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Trimeric Tobramycin/Nebramine Synergizes β-Lactam Antibiotics against *Pseudomonas aeruginosa*

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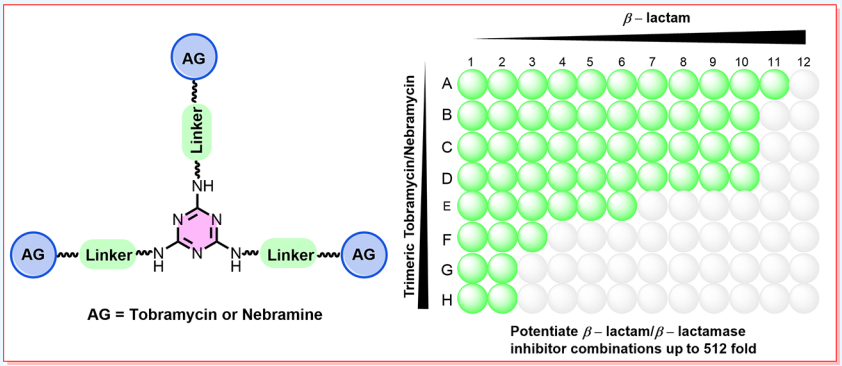

ABSTRACT: β-Lactam antibiotics remain one of the most effective therapeutics to treat infections caused by Gram-negative bacteria (GNB). However, since ancient times, bacteria have developed multiple resistance mechanisms toward this class of antibiotics including overexpression of β-lactamases, suppression of porins, outer membrane impermeability, overexpression of efflux pumps, and target modifications. To cope with these challenges and to extend the lifetime of existing β-lactam antibiotics, β-lactamase inhibitors are combined with β-lactam antibiotics to prevent antibiotic inactivation by β-lactamases. The combination therapy of an outer membrane permeabilizer with β-lactam antibiotics is an alternative approach to overcoming bacterial resistance of β-lactams in GNB. This approach is of particular interest for pathogens with highly impermeable outer membranes like *Pseudomonas aeruginosa*. Previous studies have shown that outer membrane permeabilizers can be designed by linking tobramycin and nebramine units together in the form of dimers or chimeras. In this study, we developed trimeric tobramycin and nebramine-based outer membrane permeabilizers presented on a central 1,3,5-triazine framework. The resultant trimers are capable of potentiating outer membrane-impermeable antibiotics but also β-lactams and β-lactam/β-lactamase inhibitor combinations against resistant *P. aeruginosa* isolates. Furthermore, the microbiological susceptibility breakpoints of ceftazidime, aztreonam, and imipenem were reached by a triple combination consisting of an outer-membrane permeabilizer/β-lactam/β-lactamase inhibitor in β-lactam-resistant *P. aeruginosa* isolates. Overall, our results indicate that trimeric tobramycins/nebramines can rescue clinically approved β-lactams and β-lactam/β-lactamase inhibitor combinations from resistance.

1. INTRODUCTION

Numerous antibiotics have been discovered, manufactured, and marketed since Fleming discovered penicillin in 1929. This advent of antibiotic discovery has facilitated the ability to treat bacterial infections that were once fatal, resulting in longer lifespans.[1] The global spread of multidrug-resistant (MDR) superbugs has seriously compromised our antibiotic arsenal to combat bacterial infections.[2−4] The majority of our existing antibiotics are more active against Gram-positive bacteria than Gram-negative bacteria (GNB). This is in part caused by the dual-membrane topology of GNB characterized by orthogonal uptake properties of the inner membrane and outer membrane (OM), as well as the existence of resistance−nodulation−division (RND)-type efflux pumps, which severely restrict the permeation of antibacterial agents into GNB.[5−7] Currently, β-lactams (BLs) are the most widely prescribed class of antibiotics to treat MDR priority pathogens such as *Pseudomonas aeruginosa*, *Acinetobacter baumannii*, and Enterobacterales.[8,9] BLs interfere with the bacterial cell wall synthesis by binding covalently to essential penicillin-binding proteins (PBPs) involved in the last steps of peptidoglycan

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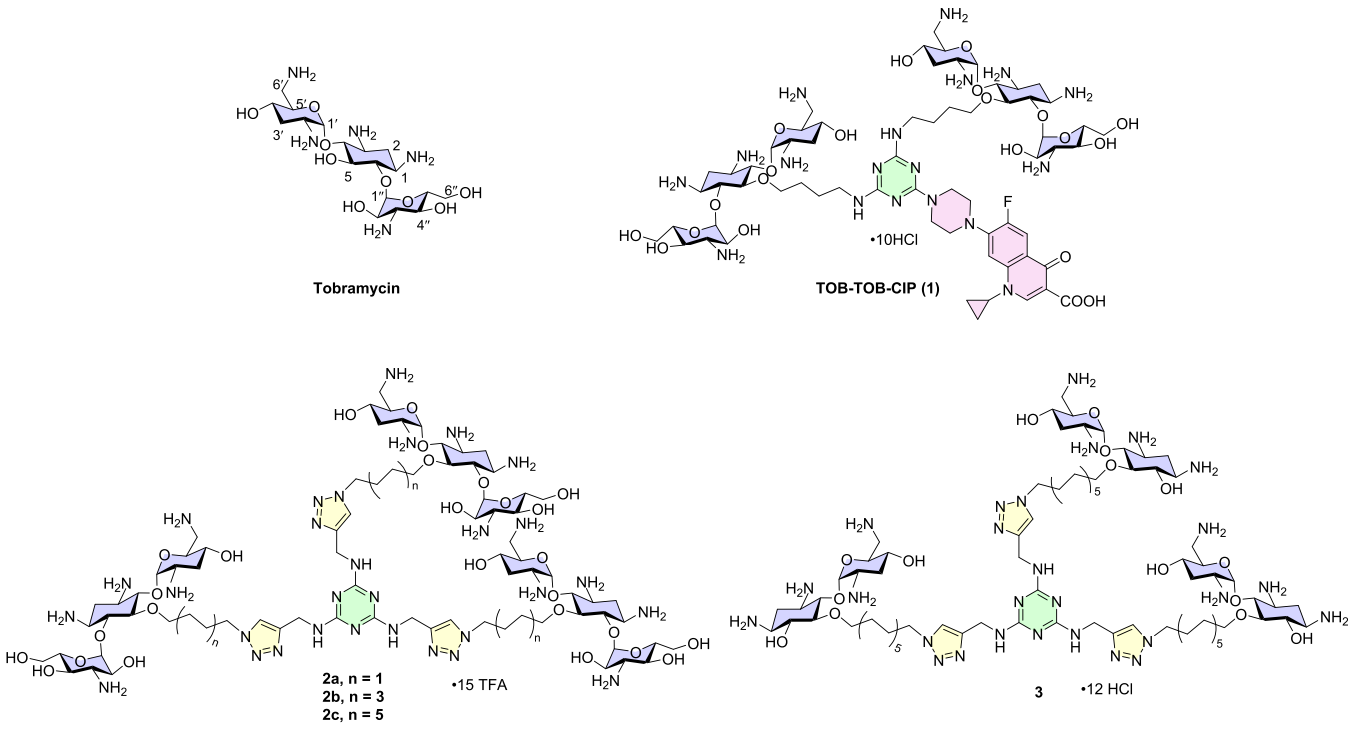

Figure 1. Structures of TOB, previously studied chimeric TOB-TOB-CIP,[32] trimeric TOB (**2a−c**) and trimeric NEB (**3**) used in this study.

cross-linking in bacteria.[10] Depending on the organism, bacteria express up to eight PBPs.[11] One of the major resistance mechanisms of BLs is their inactivation by β-lactamase enzymes. β-Lactamases hydrolyze the β-lactam ring of BLs, and the hydrolyzed adducts are unable to bind to PBPs.[7] β-Lactamases are divided into four Ambler classes A, B, C, and D.[12] According to this classification, Ambler classes A, C, and D are serine β-lactamases (Ser-BLs), which utilize the catalytic serine residue for nucleophilic attack on the carbonyl group of the BL ring in the hydrolysis step.[11] In contrast, Ambler class B is categorized as metallo-β-lactamases (MBLs) that require a zinc metal in the hydrolysis step.[12] Furthermore, acquired mutations in porin channels and PBPs, as well as suppression of porin expression, also contribute to BL resistance.[13]

In recent years, the pharmaceutical industry has developed a therapeutic approach of combining an antibiotic with an anti-resistance agent (adjuvant) to combat antimicrobial resistance (AMR).[14,15] This promising strategy provides a short-term solution to AMR and in some cases can rescue the activity of already FDA-approved antibiotics. Currently, there are at least three known types of anti-resistance agents that enhance and/or synergize the antibacterial effect of antibiotics in MDR GNB. These include outer-membrane permeabilizers (OMPs), efflux pump inhibitors (EPIs), and β-lactamase inhibitors (BLIs).[6,16] Among the anti-resistance agents, only BLIs are in clinical use. At present, six BLIs have been approved by the FDA including clavulanic acid, sulbactam, tazobactam (TAZ), avibactam (AVI), vaborbactam (VAB), and relebactam (REL), which rescue BLs by inhibiting β-lactamases.[11,17−19] Although BLIs can restore the activity of BLs in combination therapy against Ser-BLs,[20] none of the developed BLIs are effective against MBLs.[21,22] Besides β-lactamases, other factors including poor membrane permeability and efflux can further compromise the activity of BLs in GNB by reducing the intracellular concentration of antibiotics in the bacterial cell.[23]

Previous investigations have suggested that membrane impermeability may be more prevalent in *P. aeruginosa* than in other GNB.[24] As a result, the combination of OMPs with antibiotics is therefore an alternative approach to increase the intracellular concentration of antibiotics in *P. aeruginosa* and restore susceptibility to antibiotics.[25,26] OMPs destabilize the lipopolysaccharides (LPS) of OM by electrostatically interacting with the negatively charged phosphate groups of lipid A and displacing the divalent Ca^{2+} and Mg^{2+} cations, which serve as bridges between neighboring LPS molecules.[5,27] In this context, we previously reported a series of amphiphilic tobramycin-based OMPs in which tobramycin (TOB) was conjugated to antibiotics,[28,29] EPIs,[30] and ion chelators.[31] These conjugates were devoid of potent antibacterial activity but potentiated multiple classes of antibiotic classes against *P. aeruginosa*.[5] Structure−activity relationship studies indicated that the TOB moiety and the length of the tether were critical for the adjuvant properties of these conjugates. More recently, we demonstrated that homodimeric TOBs are effective OMPs capable of potentiating GPB-selective antibiotics including rifampicin (RIF) and novobiocin (NOV) but also BLs and BL/BLI combinations against *P. aeruginosa* strains.[34] We also studied TOB-based chimeras in which up to three membrane-active warheads were united into a single molecule.[32] For instance, chimera TOB-TOB-CIP (**1**) (Figure 1) was able to potentiate BLs and BL/BLI combinations in *P. aeruginosa*.[32] Collectively, these results suggested that the presence of multiple TOB moieties in a single molecular entity enhances the uptake properties of multiple antibiotic classes including BLs and BL/BLI combinations in *P. aeruginosa*. Thus, we sought to develop trimeric TOB-based OMPs (**2**) where three amphiphilic TOB moieties are presented in a single scaffold (Figure 1). Moreover, we also prepared the corresponding pseudo-disaccharide segment of TOB known as nebramine (NEB) (**3**) to study how structural changes in TOB affect antibiotic potentiation and cytotoxicity,[33,34] as previously done

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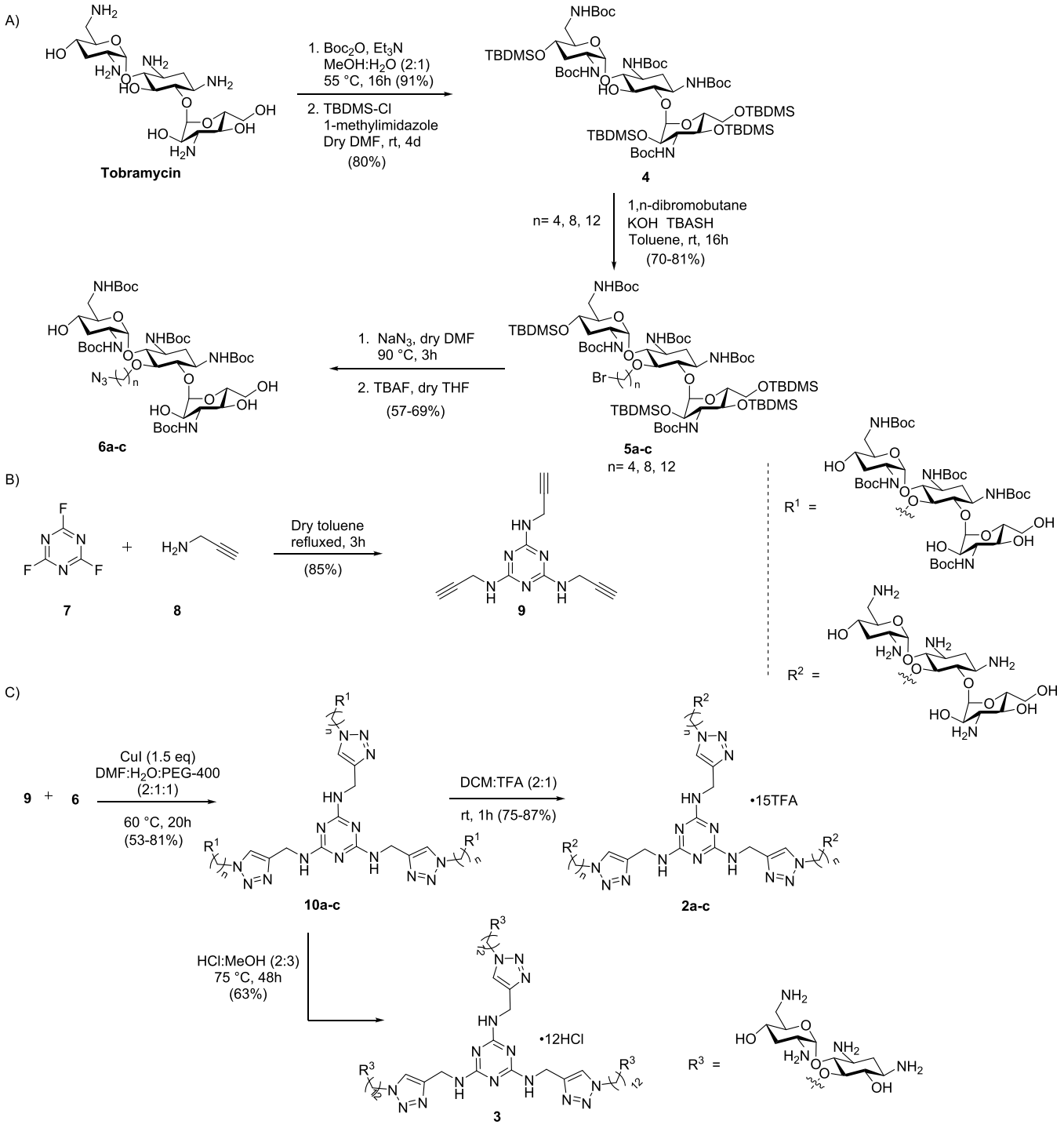
Scheme 1. Synthesis of Trimeric TOB 2a−c and Trimeric NEB 3



on related OMPs including polymyxin B, polymyxin B nonapeptide (PMBN) and SPR741 (Figure 1).[35−37]

2. RESULTS AND DISCUSSION

2.1. Chemistry. *2.1.1. Synthesis of Trimeric TOB* ***2a−c*** *and Nebramine* ***3***. The overall strategy for synthesizing trimeric TOB (**2a−c**) and NEB (**3**) is summarized in Scheme 1. The copper-catalyzed alkyne-azide cycloaddition (CuAAC)[38] approach was used for the synthesis of trimeric TOB. Both coupling partners for the CuAAC reaction were synthesized using previously reported methodologies.[39,40] For the preparation of the azido coupling partner, the amino groups and hydroxyl groups of TOB were initially protected with di-*tert*-butyl dicarbonate (Boc-anhydride) and *tert*-butyldimethylsilyl chloride (TBDMS-Cl), respectively, to obtain compound **4** with a free hydroxyl group at the C-5 position of the deoxystreptamine ring. Subsequently, the free OH group was alkylated with different 1,*n*-dibromoalkanes using potassium hydroxide (KOH) in the presence of phase transfer catalyst tetrabutylammonium sulfate (TBASH) in toluene at room temperature for 20 h to obtain compounds **5a−c** in 70−81% yields. The terminal bromide in compounds

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5a−c was then subjected to azidation using sodium azide (NaN_3) in anhydrous *N*,*N*-dimethylformamide (DMF) at 90 °C for 3 h, followed by deblocking of the TBDMS protecting groups using tetrabutylammonium fluoride (TBAF) in dry tetrahydrofuran (THF) at room temperature for 5 h to afford protected azide **6a−c** in 57−69% yields.

The CuAAC coupling partner, tris-propargylamino triazine (**9**), was synthesized by a previously reported protocol using cyanuric fluoride (**7**) and propargyl amine (**8**).[40] Coupling of azido compounds **6a−c** to alkyne **9** produced protected TOB trimers **10a−c** in 53−81% yields. Global deprotection of Boc groups using trifluoroacetic acid (TFA) in dichloromethane (DCM) (1:2) produced trimeric TOB analogues **2a−c** in 75−87% yields. Trimeric NEB analogue **3** was synthesized by treating compound **2c** with a solution of concentrated HCl in methanol (MeOH) (2:3) at 75 °C for 48 h to afford desired trimer **3** in 63% yield (Scheme 1).[33]

2.2. Microbiological Evaluation. *2.2.1. Antibacterial Susceptibility Assay.* The intrinsic antibacterial activity of trimeric TOB analogues **2a−c** and trimeric NEB **3** was determined against a panel of reference GNB organisms including *P. aeruginosa* PAO1, *A. baumannii* ATCC 17978, and *E. coli* ATCC 25922 (Table 1). Trimers **2a−c** displayed weak antibacterial activity when compared to parent TOB against all three GNB. However, trimers **2c** and **3** exhibited better antibacterial activity (32−64 μg/mL) against two GNB (*P. aeruginosa* PAO1 and *A. baumannii* ATCC 17978), respectively, in comparison to trimers **2a**,**b**.

Table 1. Antibacterial Activity of Trimeric TOB 2a−c and Trimeric NEB 3 against Selected GNB

	minimum inhibitory concentration (MIC) (μg/mL)				
strains	TOB	2a	2b	2c	3
P. aeruginosa PAO1	0.5	>128	128	32	64
A. baumannii ATCC 17978	0.5	>128	>128	64	32
E. coli ATCC 25922	2	>128	128	128	64

*2.2.2. Assessment of Antibiotic Potentiation by Trimeric TOB **2a−c**.* The weak intrinsic antibacterial activity of trimeric TOB **2a−c** and trimeric NEB **3** further stimulated our interest to investigate their use as antibiotic potentiators against GNB. Ideal potentiators are molecules with weak or no standalone antibacterial activity; however, when used in combination with an antibiotic, potentiators enhance the antibiotic's activity either by increasing OM permeability and/or by reducing efflux.[5,16,41] A fourfold reduction in the MIC of a selected antibiotic at a concentration of ≤MIC/4 of the potentiator constitutes a synergistic relationship.[42] The activity of compounds **2a−c** to potentiate five different classes of antibiotics against *P. aeruginosa* PAO1 was studied using the checkerboard assay (Figure 2). Trimeric TOB **2a−c** at a concentration of 8 μg/mL (2 μM) were able to potentiate hydrophobic and GPB-selective antibiotics such as NOV and RIF by 256-fold and 4- to 128-fold, respectively. Minocycline (MIN, tetracycline) was potentiated by 4- to 32-fold, and levofloxacin (LEV, fluoroquinolone) was potentiated 4- to 8-fold by trimers **2a−c**. Compounds **2a** and **2c** also potentiated BLs ceftazidime (CAZ) by fourfold and eightfold, respectively, while compound **2b** did not potentate CAZ in PAO1 (Figure 2). The absolute MIC values of these antibiotics in the combination with trimeric compounds **2a−c** are summarized in Table S1. Moreover, we observed that an increase in the length of the carbon chain linker (C_4−C_{12}) elevated the fold potentiation of RIF, LEV, and CAZ in PAO1. Interestingly, MIN potentiation increased when the carbon chain linker length was increased from C_4 to C_8 but then decreased with a C_{12} carbon chain linker. In contrast, our previous studies demonstrated that TOB did not synergize any of these antibiotics in PAO1.[31,39] The potentiating effects of trimer **2c**

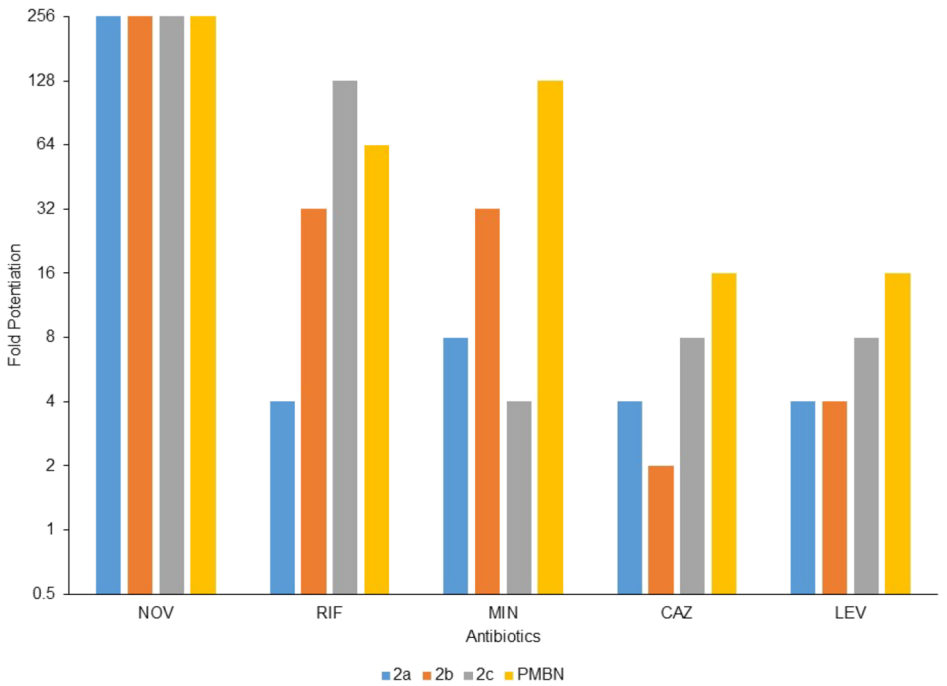

Figure 2. Potentiation of novobiocin (NOV), rifampicin (RIF), minocycline (MIN), ceftazidime (CAZ), and levofloxacin (LEV) by 8 μg/mL trimeric TOB **2a−c** (2 μM) or PMBN (7 μM) in *P. aeruginosa* PAO1.

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Table 2. Combination Studies of Trimer 2c with BLs against β-Lactamase Harboring *P. aeruginosa* Isolates[a]

strain	BL[d]	$MIC_{compd2c}$ [MIC_{combo}] (μg/mL)	MIC_{BL} [MIC_{combo}] (μg/mL)	FICI	interpretation	absolute MIC (μg/mL)[b]	fold potentiation[c]
PA86052	CAZ	128 [4]	128 [16]	0.156	synergy	16	8-fold
	ATM	128 [16]	64 [0.25]	0.128	synergy	1	64-fold
	IMI	128 [2]	64 [16]	0.265	synergy	16	4-fold
	MER	128 [16]	16 [4]	0.375	synergy	8	2-fold
	CTZ	128 [8]	8 [0.5]	0.125	synergy	0.5	16-fold
PA88949	CAZ	>128 [16]	64 [32]	$0.5 < x < 0.625$	additive	64	1-fold
	ATM	>128 [16]	32 [2]	$0.125 < x < 0.1875$	synergy	8	4-fold
	IMI	>128 [16]	64 [1]	$0.016 < x < 0.141$	synergy	64	1-fold
	MER	>128 [16]	16 [4]	$0.25 < x < 0.375$	synergy	8	2-fold
	CTZ	>128 [16]	4 [0.5]	$0.125 < x < 0.250$	synergy	2	2-fold
PA107092	CAZ	32 [4]	128 [32]	0.375	synergy	32	4-fold
	ATM	32 [8]	256 [8]	0.281	synergy	8	32-fold
	IMI	32 [8]	32 [8]	0.50	synergy	8	4-fold
	MER	32 [8]	32 [8]	0.50	synergy	8	4-fold
	CTZ	32 [8]	4 [1]	0.50	synergy	1	4-fold
PA108590	CAZ	32 [8]	64 [0.125]	0.251	synergy	0.125	512-fold
	ATM	32 [8]	32 [0.125]	0.253	synergy	0.125	256-fold
	IMI	32 [2]	32 [8]	0.312	synergy	8	4-fold
	MER	32 [8]	4 [0.5]	0.375	synergy	0.5	8-fold
	CTZ	32 [8]	8 [0.25]	0.312	synergy	0.25	32-fold
PA109084	CAZ	32 [4]	128 [32]	0.375	synergy	32	4-fold
	ATM	32 [2]	64 [16]	0.312	synergy	16	4-fold
	IMI	32 [2]	16 [8]	0.562	additive	8	2-fold
	MER	32 [2]	16 [8]	0.562	additive	8	2-fold
	CTZ	32 [4]	8 [2]	0.375	synergy	2	4-fold

[a]All the β-lactamase harboring *P. aeruginosa* isolates are colistin-susceptible.[37] [b]MIC of antibiotic in the presence of 8 μg/mL (2 μM) compound **2c**. [c]Degree of antibiotic potentiation in the presence of 8 μg/mL (2 μM) compound **2c**. [d]CAZ = ceftazidime; ATM = aztreonam; IMI = imipenem; MER = meropenem; CTZ = ceftolozane.

was also compared with the gold standard OMP PMBN against PAO1. Trimer **2c** at 8 μg/mL (2 μM) displayed similar or better potentiation of NOV, RIF, LEV, and CAZ in comparison to PMBN (7 μM). On the other hand, PMBN potentiated MIN 4-fold higher than trimer **2c** in PAO1 (Figure 2, Table S1).

The results obtained in wild-type *P. aeruginosa* encouraged us to extend the combination screening against other GNB as well, including wild-type *A. baumannii* ATCC 17978 and *E. coli* ATCC 25922. Against *E. coli* ATCC 25922, trimers **2a−c** consistently potentiated NOV (64- to 256-fold) and RIF (4- to 1024-fold). However, only trimer **2c** synergized with CAZ (fourfold) (Table S2). Against *A. baumannii* ATCC 17978, trimers **2a−c** potentiated NOV (8- to 32-fold) and trimers **2b**,**c** potentiated RIF (8- to 64-fold) (Table S3). Furthermore, all the trimeric TOB did not synergize MIN and LEV against both wild-type *A. baumannii* and *E. coli*. Collectively, these results indicate that trimer **2c** is the most potent potentiator against all three reference GNB (Tables S1−S3).

*2.2.3. BL-Potentiating Activity of Compounds **2c** and **3** against β-Lactamase Harboring P. aeruginosa Isolates.* The previous results showed remarkable synergy with CAZ against PAO1 by hit compound **2c**. To examine the spectrum of BL potentiation, we studied the combination of hit compound **2c** with different classes of BLs such as the monobactams (aztreonam (ATM)), carbapenems (imipenem (IMI) and meropenem (MER)), and cephalosporins (CAZ and ceftolozane (CTZ)) against five β-lactamase harboring *P. aeruginosa* isolates, as shown in Table 2. Compound **2c** at 8 μg/mL (2 μM) significantly potentiated CAZ (4- to 512-fold, except PA88949) and ATM (4- to 256-fold) in all tested strains. A 4- to 16-fold reduction in BL MIC was also observed for CTZ in four of the five isolates. In contrast, trimer **2c** potentiated IMI (4-fold), and MER (4- to 8-fold) in three and two of the five isolates, respectively. It is worth noting that in the presence of compound **2c** at 8 μg/mL (2 μM), the microbiological susceptibility breakpoints of ATM (MIC of 8 μg/mL),[43] CAZ (MIC of 8 μg/mL),[43] MER (MIC of 2 μg/mL),[43] and CTZ (MIC of 4 μg/mL)[43] were reached in four strains (except PA109084), in PA86052 and PA108590, in PA108590, and in all five strains, respectively. While CAZ and ATM were not synergized by compounds **2a**,**b** in four of the five strains, compound **2a** potentiated CAZ (4-fold) and ATM (8-fold) in PA108590 whereas compound **2b** potentiated ATM (4-fold) in PA107092 and PA108590 (Tables S4 and S5). Collectively, these studies suggest that compound **2c** bearing a C_{12} carbon chain linker is the most effective BL potentiator among the synthesized trimeric TOB analogues.

To further explore how the nature of the TOB moiety affects potentiating properties, we decided to replace the TOB moiety in trimer **2c** with NEB to obtain trimeric NEB analogue **3**. Combination therapy of compound **3** with BLs was then investigated to examine whether compound **3** will retain the synergistic effect with BLs in β-lactamase harboring *P. aeruginosa* isolates. A similar trend was observed with compound **3** for the potentiation of IMI, MER, and CTZ against the tested strains (Table 3). Compound **3** was also found to be more potent for the potentiation of BLs in PA86052 and PA108590 in comparison to compound **2c**, except in the case of ATM and CAZ in PA10890 (Table 3).

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Table 3. Combination Studies of Trimer 3 with BLs against β-Lactamase Harboring *P. aeruginosa* isolates[a]

strain	BL[d]	MIC_{compd3} [MIC_{combo}] (μg/mL)	MIC_{BL} [MIC_{combo}] (μg/mL)	FICI	interpretation	absolute MIC (μg/mL)[b]	fold potentiation[c]
PA86052	CAZ	>128 [4]	64 [0.5]	0.0078 < x < 0.039	synergy	0.5	128-fold
	ATM	>128 [4]	32 [0.062]	0.002 < x < 0.033	synergy	0.062	512-fold
	IMI	>128 [2]	32 [4]	0.125 < x < 0.141	synergy	4	8-fold
	MER	>128 [4]	16 [0.5]	0.031 < x < 0.062	synergy	0.5	32-fold
	CTZ	>128 [8]	4 [0.25]	0.062 < x < 0.125	synergy	0.25	16-fold
PA88949	CAZ	>128 [16]	64 [8]	0.125 < x < 0.250	synergy	64	1-fold
	ATM	>128 [16]	32 [8]	0.25 < x < 0.375	synergy	32	1-fold
	IMI	>128 [8]	64 [32]	0.50 < x < 0.562	additive	32	1-fold
	MER	>128 [16]	32 [16]	0.50 < x < 0.625	additive	32	1-fold
	CTZ	>128 [16]	4 [1]	0.25 < x < 0.375	synergy	4	1-fold
PA107092	CAZ	>128 [8]	128 [32]	0.250 < x < 0.312	synergy	32	4-fold
	ATM	>128 [4]	64 [16]	0.25 < x < 0.281	synergy	16	4-fold
	IMI	>128 [4]	32 [8]	0.25 < x < 0.281	synergy	8	4-fold
	MER	>128 [16]	64 [8]	0.125 < x < 0.250	synergy	16	4-fold
	CTZ	>128 [4]	4 [1]	0.25 < x < 0.281	synergy	1	4-fold
PA108590	CAZ	>128 [16]	64 [4]	0.062 < x < 0.187	synergy	8	8-fold
	ATM	>128 [16]	32 [0.25]	0.0078 < x < 0.132	synergy	0.5	64-fold
	IMI	>128 [8]	32 [2]	0.062 < x < 0.125	synergy	2	16-fold
	MER	>128 [8]	8 [0.125]	0.015 < x < 0.078	synergy	0.125	64-fold
	CTZ	>128 [8]	4 [0.031]	0.0078 < x < 0.070	synergy	0.0312	128-fold
PA109084	CAZ	>128 [16]	128 [32]	0.25 < x < 0.375	synergy	64	2-fold
	ATM	>128 [16]	64 [16]	0.25 < x < 0.375	synergy	32	2-fold
	IMI	>128 [2]	16 [8]	0.50 < x < 0.515	additive	8	2-fold
	MER	>128 [16]	16 [8]	0.5 < x < 0.625	additive	16	1-fold
	CTZ	>128 [16]	4 [0.5]	0.125 < x < 0.25	synergy	1	4-fold

[a]All the β-lactamase harboring *P. aeruginosa* isolates are colistin-susceptible.[37] [b]MIC of antibiotic in the presence of 8 μg/mL (3.5 μM) compound **3**. [c]Degree of antibiotic potentiation in the presence of 8 μg/mL (3.5 μM) compound **3**. [d]CAZ = ceftazidime; ATM = aztreonam; IMI = imipenem; MER = meropenem; CTZ = ceftolozane.

*2.2.4. Triple-Combination Study of Compounds **2c** or **3** with BL/BLI against β-Lactamase Harboring P. aeruginosa.* To investigate whether trimers **2c** and **3** are able to further potentiate clinically approved BL/BLI combinations, we studied the triple combination of trimer **2c** or **3** with CAZ/AVI, ATM/AVI, IMI/REL, MER/VAB, and CTZ/TAZ against five β-lactamase harboring *P. aeruginosa* isolates. Our studies indicate that adding trimers **2c** or **3** to BL/BLI combinations elevated the activity of CAZ, ATM, IMI, and CTZ (Figure 3 and Figure S1). Strikingly, the microbiological susceptibility breakpoints of CAZ, ATM, IMI (except in PA88949), and CTZ were reached against the tested strains (Figure 3 and Figure S1). Moreover, compound **2c** or **3** potentiated MER/VAB and attained the microbiological susceptibility breakpoint of MER in two isolates, PA86052 and PA108590 (Figure S2). Similar to the dual combination of compound **3**/BL, we also observed that trimer **3** was found to be more potent compared to compound **2c** with regard to BL/BLI potentiation against PA86052 and PA108590 (Figure 3).

The potentiation of BL/BLI by 8 μg/mL of compound **3** (3.5 μM) was compared with PMBN (7 μM) in PA86052 and PA108590, as well. PMBN was better in potentiating IMI/REL, MER/VAB, and CTZ/TAZ compared to compound **3** in PA86052 (Table 4). However, trimer **3** was superior to PMBN for the potentiation of BL/BLI in PA108590, while comparable potentiation of CAZ/AVI and ATM/AVI was observed in PA86052.

2.2.5. Time-Kill Kinetics in P. aeruginosa PA86052 and PA108590. Time-kill experiments were performed to verify the synergistic effect of trimeric NEB **3** with ATM or ATM/AVI against β-lactamase harboring *P. aeruginosa* PA86052 and PA108590. The CLSI susceptibility breakpoint for ATM is 8 μg/mL (18 μM), and visible growth against PA86052 was inhibited at 8 μg/mL (3.5 μM) of compound **3** in combination with ATM or ATM/AVI (8 μM). Thus, these concentrations were selected for the time-kill studies.

After 8 h, regrowth of PA86052 was observed when treated with ATM alone. However, ATM in combination with AVI or compound **3** resulted in a 2 log_{10}-fold bacterial load reduction after 24 and 12 h, respectively, whereas the combination of ATM/compound **3** entirely eradicated PA86052 after 24 h. Interestingly, the triple combination of ATM/AVI/compound **3** reduced the bacterial load by 2 log_{10}-fold after 8 h and complete sterilization was observed after 24 h (Figure 4A).

Similar to PA86052, regrowth of PA108590 occurs after 8 h. However, at 24 h, the dual combinations of either compound **3**/ATM or AVI/ATM, as well as the triple combination of ATM/AVI/compound **3** showed a bactericidal effect (Figure 4B). Our studies on both PA86052 and PA108590 revealed that addition of compound **3** boosted bacterial mortality when combined with ATM and ATM/AVI. This supports the synergism observed in the checkerboard assays.

*2.2.6. OM Permeabilization of P. aeruginosa PAO1 by Compounds **2c** and **3**.* To study the mechanism of action of the compounds, the 1-*N*-phenylnaphthylamine (NPN) uptake assay was performed using trimeric TOB **2c** and NEB **3** in wild-type *P. aeruginosa*. Since increased fluorescence of membrane impermeable NPN was observed upon addition of either compounds or gold-standard OMP PMBN, this suggests that OM permeabilization occurs, thus allowing NPN

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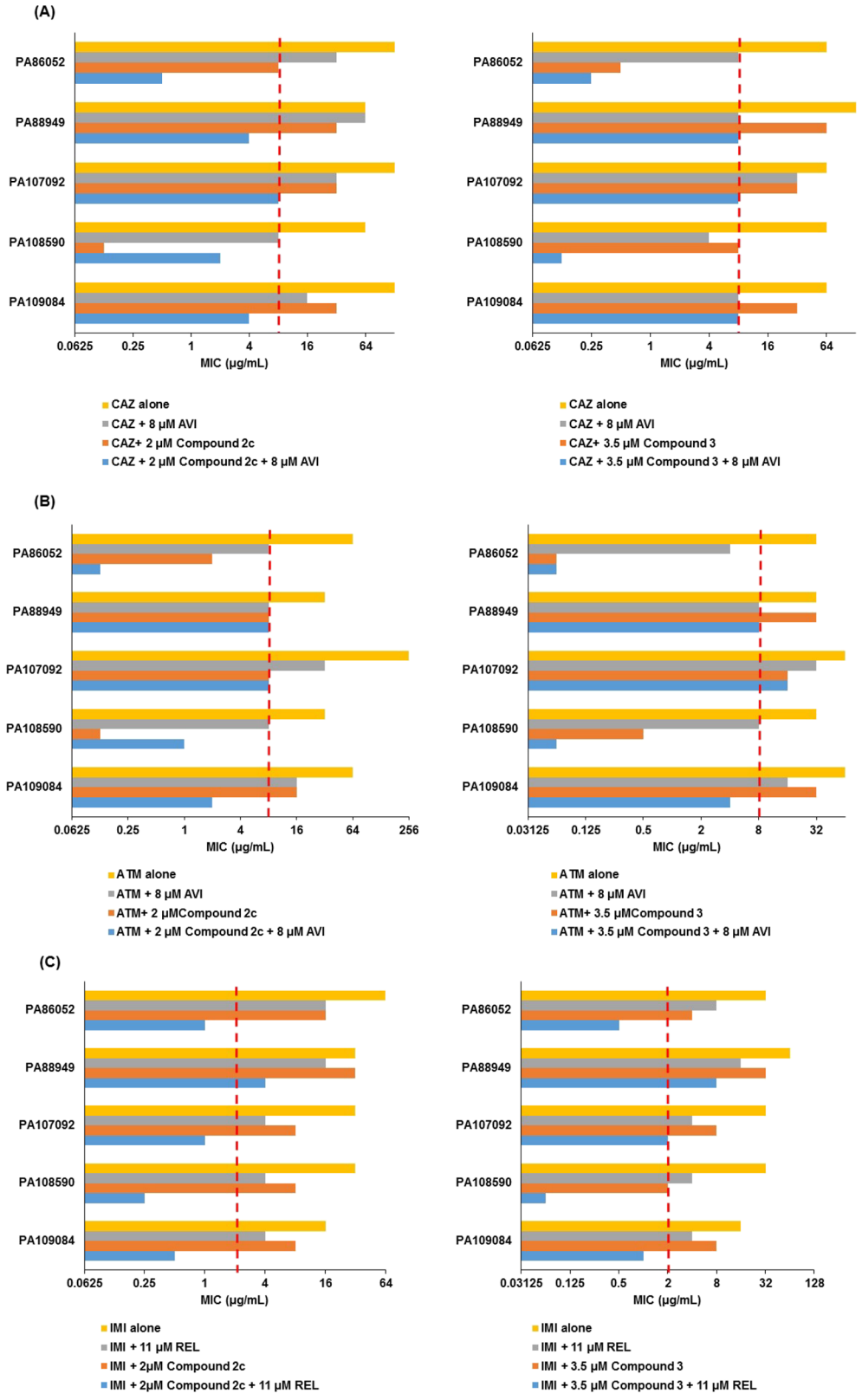

Figure 3. Triple combination studies consisting of (A) ceftazidime (CAZ) and avibactam (AVI), (B) aztreonam (ATM) and avibactam (AVI), and (C) imipenem (IMI) and relebactam (REL), with hit compounds **2c** or **3** against β-lactam-resistant *P. aeruginosa* isolates. The microbiological susceptibility breakpoints of CAZ and ATM (MIC of 8 μg/mL) and IMI (MIC of 2 μg/mL) are represented by red dashed lines.

entry into the phospholipid bilayer. Moreover, since the fluorescence intensity of NPN increases as the concentration of compound **3** increases, this indicates that permeabilization of the OM happens in a dose-dependent manner. The NPN data were also consistent with the previously discussed potentiation of outer membrane-impermeable RIF and NOV in *P. aeruginosa* PAO1.

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Table 4. Comparative Potentiation of BL/BLI by Compound 3 or PMBN in PA86052 and PA108590

strain	BL/BLI[b]	$MIC_{BL/BLI}$ [MIC_{with3}] (μg/mL)[a]	fold potentiation[a]	$MIC_{BL/BLI}$ [$MIC_{with\ PMBN}$] (μg/mL)[a]	fold potentiation[a]
PA86052	CAZ/AVI	64 [0.25]	256	128 [0.25]	512
	ATM/AVI	32 [0.0625]	512	64 [0.0625]	1024
	IMI/REL	32 [0.5]	64	64 [0.125]	512
	MER/VAB	16 [1]	16	16 [0.0312]	512
	CTZ/TAZ	4 [0.25]	16	8 [0.0625]	128
PA108590	CAZ/AVI	64 [0.125]	512	128 [4]	32
	ATM/AVI	32 [0.0625]	512	32 [2]	16
	IMI/REL	32 [0.0625]	512	32 [1]	32
	MER/VAB	8 [0.125]	64	4 [4]	1
	CTZ/TAZ	4 [0.0312]	128	4 [2]	2

[a]MIC of antibiotic in the presence of 8 μg/mL compound **3** (3.5 μM) or PMBN (7 μM). [b]CAZ/AVI = ceftazidime/avibactam; ATM/AVI = aztreonam/avibactam; IMI/REL = imipenem/relebactam; MER/VAB = meropenem/vaborbactam; CTZ/TAZ = ceftolozane/tazobactam.

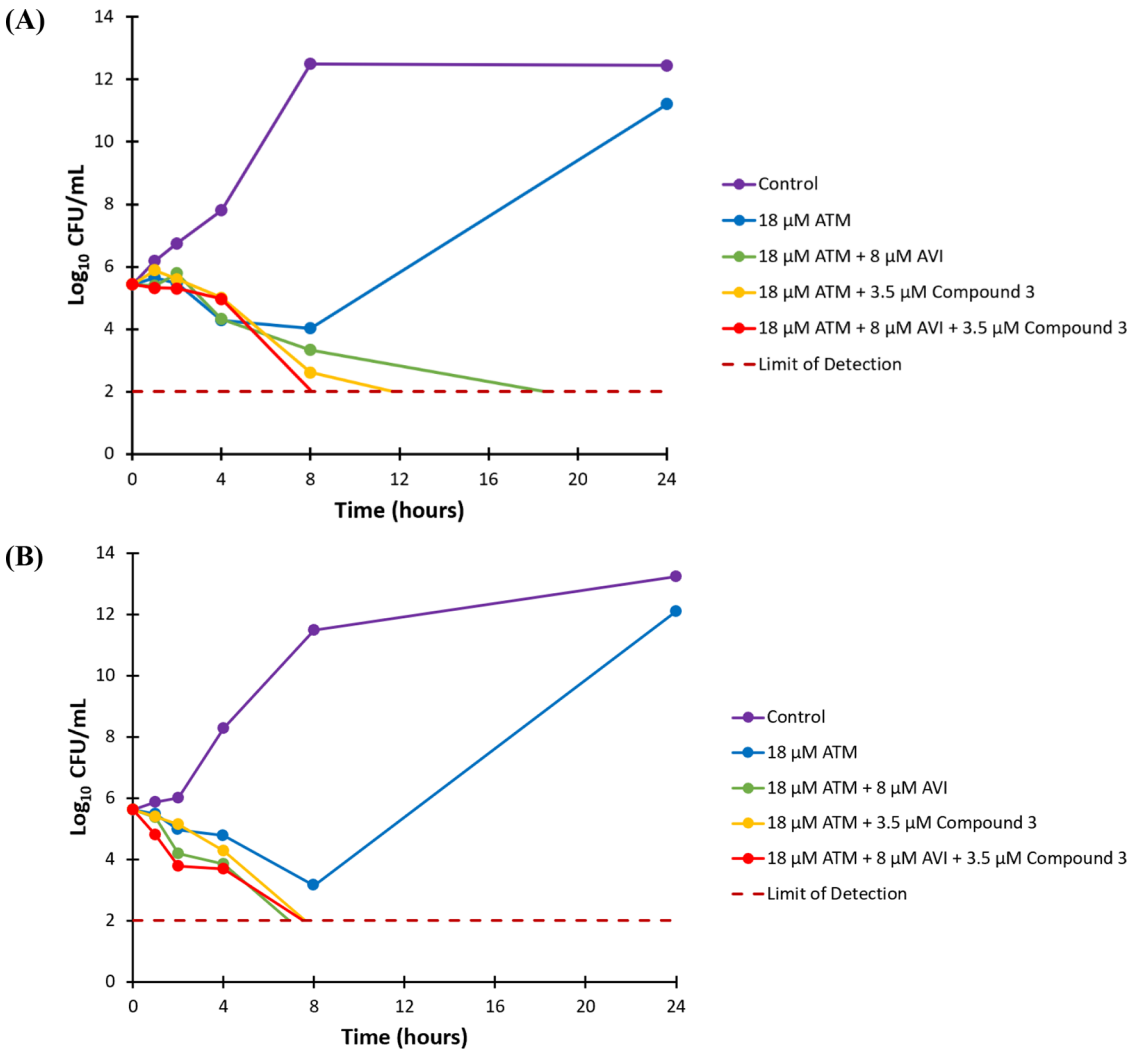

Figure 4. Time-kill kinetics of aztreonam (ATM) alone, with avibactam (AVI) at 8 μM, with compound **3** at 3.5 μM, and in combination with both AVI and compound **3** against *P. aeruginosa* (A) PA86052 and (B) PA108590.

Compounds **2c** and **3** interact with or disrupt the OM of GNB confirmed by the NPN assay. To further ascertain that trimeric tobramycin **2c** disrupts the LPS, we examined how the addition of divalent cations such as Mg^{2+} influenced the action of trimeric tobramycin **2c** for the potentiation of hydrophobic antibiotics such as RIF, as divalent cations are considered to stabilize the linking of neighboring LPS molecules in the OM by reducing the negative charge.[44,45] The results indicate that the MIC of **2c** and the potentiation effect are reduced but not abolished at elevated Mg^{2+} concentrations. For instance, 8 μg/mL (2 μM) of **2c** reduces the MIC of RIF from 32 to 0.25 μg/mL (128-fold potentiation) at CAMHB conditions while only a 16-fold potentiation is observed at Mg^{2+}-enriched CAMHB conditions. These results are consistent with an OM-targeting agent.[45] Collectively, the NPN assay, RIF-potentiating effect,

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Table 5. Comparative Potentiation of Rifampicin (RIF) by Compound 2c against PAO1 in CAMHB and Mg^{2+}-Supplemented CAMHB[a]

Mg^{2+}-enriched media	$MIC_{compd2c}$ [MIC_{combo}] (μg/mL)	MIC_{RIF} [MIC_{combo}] (μg/mL)	FICI	interpretation	absolute MIC (μg/mL)	fold potentiation
CAMHB	32 [8]	32 [0.25]	0.257	synergy	0.25	128
CAMHB with 20 mM Mg^{2+}	256 [8]	32 [2]	0.093	synergy	2	16
	256 [32]	32 [0.25]	0.132	synergy	0.25	128
	256 [64]	32 [0.125]	0.253	synergy	0.125	256

[a]CAMHB = cation-adjusted Mueller Hinton broth.

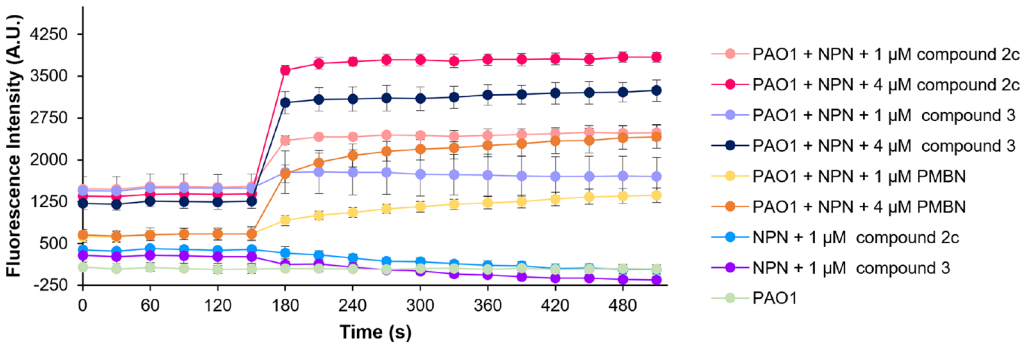

Figure 5. Measurement of NPN accumulation through OM permeabilization by compounds **2c**, **3**, or PMBN in *P. aeruginosa* PAO1.

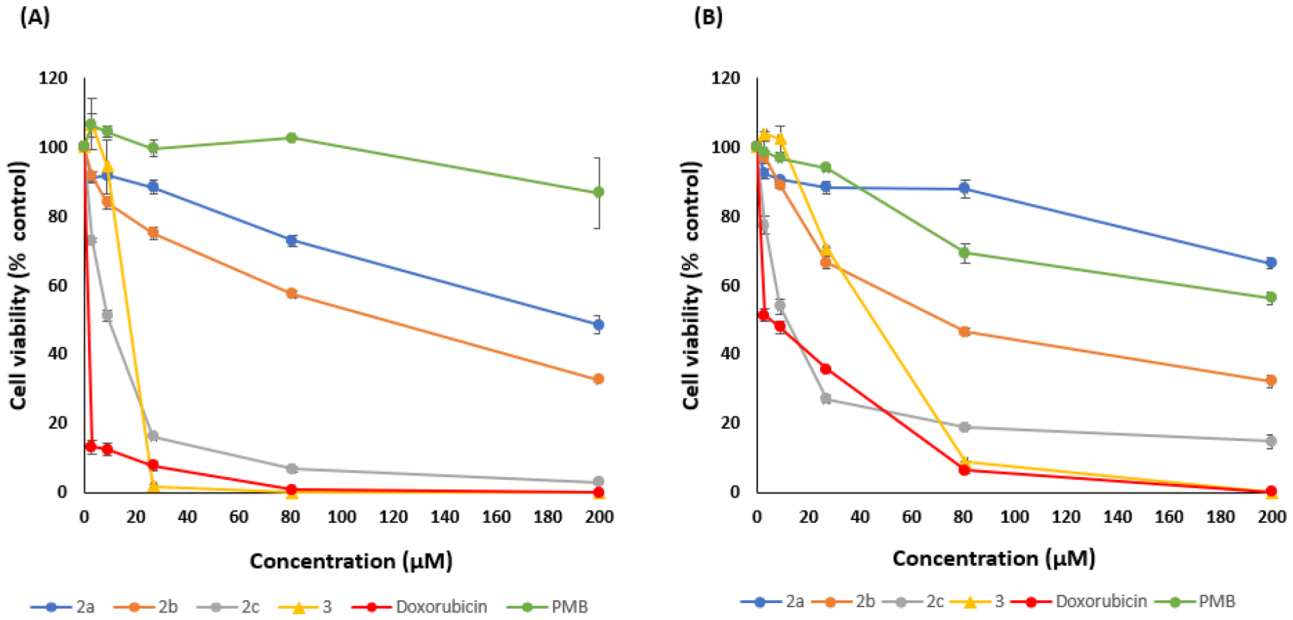

Figure 6. Cytotoxicity studies of compounds **2a−c** and **3** relative to control (vehicle) against (A) HEK293 and (B) Hep G2 cells. Doxorubicin was used as a positive control, and polymyxin B (PMB) was used as a negative control. Results represent the mean ± standard deviation of two independent experiments with five wells for each concentration.

and Mg^{2+} dependency on RIF potentiation indicate involvement of the OM as a target (Table 5 and Figure 5).

*2.2.7. Cytotoxicity Studies of Compounds **2a−c** and **3**.* Trimeric TOB **2a−c** and NEB **3** were examined for in vitro cytotoxicity against eukaryotic cells using human embryonic kidney (HEK) 293 and liver Hep G2 cell lines. Clinically used antibiotic polymyxin B (PMB) and anticancer drug doxorubicin were employed as negative and positive controls, respectively. The results, which are displayed in Figure 6, show that compounds **2a−c** and **3** were relatively noncytotoxic at their effective doses (2 and 3.5 μM, respectively). Thus, cell viabilities in HEK293 and Hep G2 were found to be 90 and 100% in the case of compounds **2a**,**b** and **3**, respectively, while in the case of compound **2c**, the cell viability was observed to be 70%. For compounds **2a−c** and **3** at a concentration of 27 μM (8−14 times their effective dose, respectively), cell viabilities in HEK293 were reduced to 88%, 75%, 16%, and 1.77%, respectively. In Hep G2 cells incubated with 27 μM of **2a−c** and **3**, the cell viabilities were found to be 88%, 66%, 27%, and 70%, respectively. Incubations with higher doses of each compound resulted in increased cytotoxicity (Figure 6). The cytotoxicity studies indicated that the degree of toxicity directly correlated with the length of the carbon chain linker of trimeric TOB **2a−c** since the order of increasing toxicity was

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Table 6. CC_{50} and S Values of Compounds 2a−c and 3 on HEK293 and Hep G2 Cell Lines[a]

compounds	$CC_{50,HEK293}$ (μM)	$S_{HEK293} = CC_{50}/ED_{OMP}$	$CC_{50,HepG2}$ (μM)	$S_{HepG2} = CC_{50}/ED_{OMP}$
2a	190.5 ± 4.2	95.25	ND	ND
2b	116.3 ± 0.4	58.15	72.3 ± 1.8	36.15
2c	9.5 ± 0.7	4.75	10.5 ± 0	5.25
3	17.8 ± 1.1	5.01	44.8 ± 0.4	12.8

[a]S = relative in vitro safety; ED_{OMP} = effective dose of the outer membrane permeabilizer to produce optimal antibiotic potentiation and retain synergy, which was achieved at $^1/_4$ MIC; CC_{50} = concentration of the compound to reduce cell viability to 50%; ND = not determined.

2a < **2b** < **2c**. It is worth noting that at lower concentrations (<20 μM for HEK293 and <30 μM for Hep G2 cells), compound **3** was less toxic than compound **2c**, but this was no longer the case at higher concentrations. These results suggested that a reduction of positive charges could reduce cytotoxicity in the compounds.

To examine the relative in vitro safety (S) of trimeric compounds **2a−c** and **3**, we calculated the CC_{50} values of these compounds from the cytotoxicity assay and the effective dose of the outer membrane permeabilizer (ED_{OMP}) by the checkerboard assay as the concentration to produce optimal antibiotic potentiation and retain synergy (typically $^1/_4$ MIC). S was determined by taking the ratio of CC_{50} to ED_{OMP} (CC_{50}/ED_{OMP}). Among hit compounds **2c** and **3**, compound **3** was found to show a higher in vitro safety than compound **2c** on Hep G2 cell lines, while against the HEK293 cell line, both the compounds have a comparable S (Table 6).

3. CONCLUSIONS

GNB like *P. aeruginosa* are resistant to many classes of antibiotics in part by the high impermeability of the OM combined with the overexpression of efflux pumps, which synergistically reduce the intracellular concentration of antibiotics. Enhancing the uptake of antibiotics into GNB by using OMPs in combination is a promising strategy to potentiate antibiotics. This approach is of particular interest when combined with other resistance mechanisms like antibiotic deactivation and efflux. Previous studies have demonstrated that conjugation of TOB to OM-active components (cationic amphiphiles, EPI, antimicrobial peptides, and metal chelators) abolishes the antibacterial activity but retains the OMP activity of TOB. In this context, we recently discovered that engineering multiple TOB moieties into OMPs can improve synergy with antibiotics. Therefore, we became interested in extending this idea to trimeric TOB analogues. Three trimeric TOB analogues and one trimeric NEB analogue were prepared, and their potentiating effects with OM-impermeable antibiotics (RIF, NOV) but also OM-permeable antibiotics (tetracyclines, fluoroquinolones, and BLs) were studied in *P. aeruginosa* and other GNB. The results demonstrate that tether length modulates the antibiotic potentiating effect in the trimers with optimal potentiation requiring a hydrophobic C_{12} tether as in hit compounds **2c** and **3**. Of special interest is the potentiation of BLs and BL/BLI combinations, which are clinically used or in clinical development to treat *P. aeruginosa* infections. Compounds **2c** or **3** strongly reduced the MIC of BLs and BL/BLI combinations to a point that microbiological susceptibility breakpoints of CAZ, AZT, and IMI were achieved by triple combination of compounds **2c** or **3** with CAZ/AVI, ATM/AVI, and IMI/REL in all tested BL-resistant *P. aeruginosa* isolates. Time-kill studies confirmed the enhanced bactericidal effect of the triple combination consisting of **3**/ATM/AVI when compared to the dual-combination ATM/AVI against two MDR *P. aeruginosa* isolates. Unfortunately, extending the tether length results in increased cytotoxicity against the two selected cell lines. Interestingly, the cytotoxicity profile and therapeutic index of NEB-based trimer **3** was better than those of compound **2c** at the effective dose, indicating that lowering the number of positive charges in the trimer reduces cytotoxicity. Collectively, our results indicate that addition of OMPs **2c** or **3** to BL/BLI combinations in clinical use or clinical development can rescue antibacterial activity against BL-resistant *P. aeruginosa* isolates. However, our study has certain limitations that need to be addressed before moving into *in vivo* studies. These include that triple-combination approaches require careful matching of the PK properties of the individual components. As ATM/AVI combinations have been optimized for clinical trials,[46] we have not investigated the PK properties of compounds **2c** and **3**. Moreover, the potential nephrotoxicity of compounds **2c** and **3** should be studied on primary kidney cells and compared to TOB and NEB controls.[47,48]

4. EXPERIMENTAL SECTION

4.1. Chemistry. Reagents and solvents were bought from commercially accessible vendors such as Sigma-Aldrich, AK Scientific, and Fisher Scientific and used without further purification. Thin layer chromatography (TLC) was used to monitor the reactions' progress on 0.25 mm silica gel 60 F254 plates from Merck and visualized by staining in ninhydrin solution. Normal and reverse-phase column chromatography was performed to purify the compounds using SiliaFlash P60 (40−63 μm) 60 Å silica gel and SiliaBond C18 (40−63 μm) 60 Å silica gel from SiliCycle, respectively. The yields of the compounds were determined after purification. The chemical structures of all intermediates and final products were characterized by nuclear magnetic resonance (^{1}H NMR and ^{13}C NMR) on a Bruker AMX-300 MHz NMR and 500 MHz spectrometer. ^{13}C NMR spectra were fully decoupled. Chemical shifts were reported in parts per million (ppm) using deuterated solvents chloroform-*d* (7.26 ppm), MeOD (3.31 ppm), and HOD (4.79 ppm) as internal standards. Matrix-assisted laser desorption ionization mass spectrometry (MALDI-MS) spectra were recorded on Bruker Daltonics ultraflex MALDI-time-of-flight (TOF)/TOF and MS-electrospray ionization mass spectrometers. Purity of the final compounds was determined by high-performance liquid chromatography (HPLC) from Thermo Scientific HPLC using a reversed-phase Synergi 100 Å C18 (50 × 2 mm) column from Phenomenex.

*4.1.1. General Procedure A for Copper-Catalyzed Azide−Alkyne Cycloaddition Reaction (CuAAC) for the Synthesis of Compounds **10a−c**.* An oven-dried round-bottom (RB) flask was charged with a Boc-protected TOB tethered azido compound (**6a−c**) (5 equiv), tris(propagylamino)-triazine (**9**) (1.0 equiv), and CuI (1.5 equiv). The solvent system

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DMF:H_2O:polyethylene glycol (PEG)-400 (2:1:1) was then added, and the reaction mixture was stirred for 24 h at 55 °C. Reaction mass was diluted with ethyl acetate (100 mL) and washed with ice-cold water (50 mL × 3) followed by brine solution. The organic layer was separated out, dried over anhydrous Na_2SO_4, and filtered, and the filtrate was concentrated to obtain the crude compound. The crude compounds were then subjected to column chromatography to afford pure compounds **10a−c** as white solids in 53−81% yields.

*4.1.2. Synthesis of Compound **10a**.* This compound was synthesized by following general procedure A from compound **9** (0.010 g, 0.042 mmol), compound **6a** (0.221 g, 0.208 mmol, 5 equiv), and CuI (1.5 equiv) in DMF:H_2O:PEG-400 (2:1:1) at 55 °C for 24 h to afford compound **10a** (0.116 g, 81%) as an off-white sold. ^{1}H NMR (500 MHz, MeOD + D_2O) δ 7.86 (s, 3H, CH of triazole), 5.32 (s, 3H, anomeric H-1′), 5.274 (s, 3H, anomeric H-1″), 4.36 (t, J = 7.0 Hz, 6H, N-CH_2 of triazole), 4.04−4.02 (m, 3H), 3.90−3.87 (m, 3H), 3.77−3.54 (m, 29H), 3.52−3.49 (m, 4H), 3.45−3.31 (m, 14H), 1.99−1.89 (m, 12H), 1.69−1.57 (m, 9H), 1.46−1.33 (m, 145H); ^{13}C NMR (125 MHz, MeOD + D_2O) δ 165.64, 158.04, 157.93, 156.65, 156.37, 156.13, 145.90, 122.86, 97.31, 95.58, 85.00, 79.67, 79.22, 79.14, 78.91, 77.38, 76.90, 72.81, 72.36, 71.89, 70.62, 68.95, 65.95, 61.22, 55.75, 51.04, 50.16, 48.97, 48.72, 41.05, 35.49, 34.73, 33.55, 27.54, 27.53, 27.50, 26.82, 26.35. MALDI-TOF: m/e [M + Na]$^+$ calcd for $C_{153}H_{264}N_{30}O_{57}Na^+$, 3456.8; measured 3456.8.

*4.1.3. Synthesis of Compound **10b**.* This compound was synthesized by following general procedure A from compound **9** (0.010 g, 0.042 mmol), compound **6b** (0.233 g, 0.208 mmol, 5 equiv), and CuI (1.5 equiv) in DMF:H_2O:PEG-400 (2:1:1) at 55 °C for 24 h to afford compound **10b** (0.107 g, 71%) as an off-white sold. ^{1}H NMR (500 MHz, MeOD + D_2O) δ 7.86 (d, J = 12.5 Hz, 3H, CH of triazole), 5.31 (s, 3H, anomeric H-1′), 5.27 (s, 3H, anomeric H-1″), 4.34 (t, J = 7.2 Hz, 6H, N-CH_2 of triazole), 3.94−3.89 (m, 6H), 3.78−3.50 (m, 32H), 3.45−3.34 (m, 16H), 3.20 (q, J = 7.3 Hz, 2H), 2.00−1.91 (m, 6H), 1.88−1.82 (m, 6H), 1.66−1.53 (m, 12H), 1.49−1.37 (m, 135H), 1.33−1.25 (m, 28H); ^{13}C NMR (125 MHz, MeOD + D_2O) δ 165.61, 158.17, 157.90, 156.65, 156.40, 156.10, 146.16, 122.84, 97.55, 95.66, 84.61, 79.64, 79.21, 79.07, 78.99, 78.02, 77.65, 77.11, 73.30, 72.67, 71.97, 70.58, 68.79, 65.90, 61.05, 55.79, 51.01, 50.01, 49.07, 48.85, 46.53, 41.05, 35.48, 34.64, 33.58, 29.88, 29.28, 28.46, 27.56, 27.53, 27.50, 27.48, 25.95, 25.33, 7.86. MALDI-TOF: m/e [M + Na]$^+$ calcd for $C_{165}H_{288}N_{30}O_{57}Na^+$, 3625.0; measured 3625.9.

*4.1.4. Synthesis of Compound **10c**.* This compound was synthesized by following general procedure A from compound **9** (0.010 g, 0.042 mmol), compound **6c** (0.245 g, 0.208 mmol, 5 equiv), and CuI (1.5 equiv) in DMF:H_2O:PEG-400 (2:1:1) at 55 °C for 24 h to afford compound **10c** (0.083 g, 53%) as an off-white sold. ^{1}H NMR (500 MHz, MeOD) δ 7.85 (d, J = 5.6 Hz, 3H, CH of triazole), 5.31 (s, 3H, anomeric H-1′), 5.26 (s, 3H, anomeric H-1″), 4.33 (t, J = 7.1 Hz, 6H, N-CH_2 of triazole), 3.95−3.89 (m, 6H), 3.77−3.50 (m, 32H), 3.45−3.34 (m, 16H), 1.99−1.91 (m, 6H), 1.87−1.82 (m, 6H), 1.65−1.55 (m, 12H), 1.49−1.37 (m, 139H), 1.31−1.23 (m, 50H); ^{13}C NMR (125 MHz, MeOD + D_2O) δ 165.58, 158.24, 157.87, 156.62, 156.37, 156.08, 146.16, 122.87, 97.53, 95.72, 84.55, 79.63, 79.21, 79.05, 78.07, 77.62, 77.18, 73.37, 72.66, 72.02, 70.55, 68.94, 65.91, 62.86, 61.06, 55.79, 51.00, 50.00, 49.09, 48.89, 41.06, 35.44, 34.64, 33.59, 29.98, 29.90, 29.66, 29.38, 29.27, 29.24, 29.21, 29.09, 28.66, 27.61, 27.59, 27.56, 27.53, 26.07, 25.55. MALDI-TOF: m/e [M + Na]$^+$ calcd for $C_{177}H_{312}N_{30}O_{57}Na^+$, 3793.2; measured 3793.2.

*4.1.5. General Procedure B for the Synthesis of Compounds **2a−c** (Deprotection of Boc Groups).* In a solution of Boc-protected compounds **10a−c** and DCM (2 mL) was added TFA (2 mL), and the reaction mixture was continuously stirred at ambient temperature for 1 h. After completion, the reaction mass was concentrated and the crude compound was stirred in 2% MeOH in diethyl ether (v/v, 3 mL) for 2 min. The solvent was then carefully decanted to obtain crude compounds as off-white crystalline solids. The crude compounds were purified by reversed-phase flash chromatography and were eluted in 100% deionized water to give desired products **2a−c** as colorless crystal TFA salt in 90−98% yields.

*4.1.5.1. Synthesis of Trimeric TOB **2a**.* Compound **2a** was prepared by following general procedure B using compound **10a** (0.100 g, 0.03 mmol) to give trimeric TOB **2a** (0.080 g, 75%) as a colorless crystalline solid. ^{1}H NMR (500 MHz, D_2O) δ 7.97 (s, 1H, CH of triazole), 7.86 (s, 2H, CH of triazole), 5.36 (d, J = 2.5 Hz, 3H, anomeric H-1′), 5.15 (d, J = 3.5 Hz, 3H, anomeric, H-1″), 4.69−4.67 (m, 6H, N-CH_2 of triazole), 4.45−4.39 (m, 6H, N-CH_2 of linker), 4.30−4.27 (m, 3H, H-5′), 4.14 (t, J = 9.8 Hz, 3H, H-5″), 3.97−3.92 (m, 5H, H-4′, H-4), 3.91−3.86 (m, 6H, H-4, H-5), 3.86−3.77 (m, 10H, H-6, H-3″ H-4″), 3.75−3.66 (m, 12H, H-2′, H-2″, H-6″, O-CH_2 of linker), 3.61−3.50 (m, 9H, H-1, H-3, H-6″), 3.44−3.39 (m, 3H, H-6′), 3.32−3.28 (m, 3H, H-6′), 2.56−2.52 (m, 3H, H-2), 2.23 (s, 6H, H-3′), 1.93 (q, J = 10.0 Hz, 9H, H-2, CH_2 of linker), 1.66−1.60 (m, 6H, CH_2 of linker); ^{13}C NMR (125 MHz, D_2O) δ 162.93 (q, J = 35.4 Hz, CO of TFA) 124.32 (CH of triazole), 123.78 (CH of triazole), 116.43 (q, J = 290 Hz, CF_3 of TFA), 101.21 (anomeric, C-1″), 92.78 (anomeric, C-1′), 82.08 (C-4″), 81.81 (C-5), 77.07 (C-5″), 76.14 (C-5′), 73.20 (O-CH_2 of linker), 72.58, 68.55 (C-2″), 64.86 (C-6), 63.10 (C-4′), 59.34 (C-6″), 54.82 (C-3″), 50.25 (N-CH_2 of linker), 49.61 (C-1), 48.35 (C-3), 47.24 (C-2′), 38.33 (C-6′), 35.47 (N-CH_2 of triazole), 27.97 (C-3′), 27.72 (C-2), 26.34 (CH_2 of linker), 26.18 (CH_2 of linker). MALDI-TOF: m/e [M + Na]$^+$ calcd for $C_{78}H_{144}N_{30}O_{27}Na^+$, 1956.07; measured 1956.16.

*4.1.5.2. Synthesis of Trimeric TOB **2b**.* Compound **2b** was synthesized by following general procedure B using compound **10b** (0.080 g, 0.022 mmol) to give trimeric TOB **2b** (0.073 g, 87%) as a colorless crystalline solid. ^{1}H NMR (500 MHz, D_2O) δ 7.96 (s, 1H, CH of triazole), 7.78 (s, 2H, CH of triazole), 5.38 (d, J = 2.6 Hz, 3H, anomeric, H-1′), 5.16 (d, J = 3.5 Hz, 3H, anomeric, H-1″), 4.66 (s, 6H, N-CH_2 of triazole), 4.44−4.32 (m, 6H, N-CH_2 of linker), 4.28−4.26 (m, 3H, H-5′), 4.13 (t, J = 9.8 Hz, 3H, H-5″), 3.96−3.83 (m, 21H, H-4, H-5, H-6, H-2′, H-4′, H-3″ H-4″), 3.78−3.70 (m, 12H, H-2″, H-6″, O-CH_2 of linker), 3.60−3.53 (m, 9H, H-1, H-3, H-6″), 3.43−3.38 (m, 3H, H-6′), 3.32−3.29 (m, 3H, H-6′), 2.55−2.53 (m, 3H, H-2), 2.26−2.24 (m, 6H, H-3′), 1.90 (q, J = 10.0 Hz, 3H, H-2), 1.85−1.82 (m, 6H, CH_2 of linker), 1.62 (s, 6H, CH_2 of linker), 1.30−1.20 (m, 24H, CH_2 of linker); ^{13}C NMR (125 MHz, D_2O) δ 162.92 (q, J = 35 Hz, CO of TFA), 146.25 (C═N of triazine), 123.53 (CH of triazole), 116.42 (q, J = 291.25 Hz, CF_3 of TFA), 101.20 (anomeric, C-1″), 92.68 (anomeric, C-1′), 82.05 (C-4″), 82.02 (C-5), 76.99 (C-5″), 74.49 (C-5′), 73.14 (O-CH_2 of linker), 72.79, 68.62 (C-2″), 64.85 (C-6), 63.61 (C-4′), 59.24 (C-6″), 54.86 (C-3″), 50.42

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(*N*-CH_2 of linker), 49.95 (C-1), 48.51 (C-3), 47.52 (C-2′), 38.86 (C-6′), 35.61 (*N*-CH_2 of triazole), 29.33 (C-3′), 29.29 (C-3′), 28.89, 28.68 (C-2), 28.02, 25.47 (CH_2 of linker), 25.01 (CH_2 of linker). MALDI-TOF: *m*/*e* $[M + Na]^+$ calcd for $C_{90}H_{168}N_{30}O_{27}Na^+$, 2124.25; measured 2124.35.

4.1.5.3. Synthesis of Trimeric TOB ***2c****.* Compound **2c** was synthesized by following general procedure B using compound **10c** (0.100 g, 0.026 mmol) to give trimeric TOB **2c** (0.088 g, 84%) as a colorless crystalline solid. ^{1}H NMR (500 MHz, D_2O) δ 7.67 (s, 3H, CH of triazole), 5.24 (s, 3H, anomeric, H-1′), 5.00 (s, 3H, anomeric, H-1″), 4.44−4.32 (m, 6H, *N*-CH_2 of triazole), 4.17 (t *J* = 7.0 Hz, 6H, *N*-CH_2 of linker), 4.10−4.07 (m, 3H, H-5′), 3.93 (t, *J* = 9.7 Hz, 3H, H-5″), 3.79−3.66 (m, 21H, H-4, H-5, H-6, H-2′, H-4′, H-3″ H-4″), 3.61−3.50 (m, 12H, H-2″, H-6″, *O*-CH_2 of linker), 3.48−3.31 (m, 9H, H-1, H-3, H-6″), 3.23−3.14 (m, 6H, H-6′), 2.37−2.33 (m, 3H, H-2), 2.10−2.04 (m, 6H, H-3′), 1.74 (q, *J* = 12.6 Hz, 3H, H-2), 1.63−1.60 (m, 6H, CH_2 of linker), 1.47−1.41 (m, 6H, CH_2 of linker), 1.15−0.91 (m, 48H, CH_2 of linker); ^{13}C NMR (125 MHz, D_2O) δ 162.86 (q, *J* = 35.2 Hz, CO of TFA), 145.86 (C═N of triazine), 123.61 (CH of triazole), 116.47 (q, *J* = 292.15 Hz, CF_3 of TFA), 101.31 (anomeric, C-1″), 92.69 (anomeric, C-1′), 82.01(C-4″), 81.95 (C-5), 76.98 (C-5″), 75.30 (C-5), 73.32 (*O*-CH_2 of linker), 73.22, 68.59 (C-2″), 64.86 (C-6), 63.40 (C-4′), 59.32 (C-6″), 54.81 (C-3″), 50.41(*N*-CH_2 of linker), 49.81 (C-1), 48.42 (C-3), 47.40 (C-2′), 38.61 (C-6′), 35.43 (*N*-CH_2 of triazole), 29.47 (C-3′), 29.29, 29.05, 28.94, 28.79, 28.63, 28.36, 28.28, 28.10, 25.61, 25.31 (CH_2 of linker). MALDI-TOF: *m*/*e* $[M + Na]^+$ calcd for $C_{102}H_{192}N_{30}O_{27}Na^+$, 2292.44; measured 2292.50.

4.1.6. Synthesis of Trimeric NEB ***3****.* An oven-dried RB flask charged with Boc-protected compound **10c** (0.050 g, 0.0132 mmol) and 2:3 conc. HCl:MeOH (10 mL). The resulting solution was stirred and heated at 75 °C for 48 h. After completion, the reaction mass was concentrated and the crude compound was stirred in 2% MeOH in diethyl ether (v/v, 3 mL) for 2 min. The solvent was then carefully decanted to obtain crude compounds as off-white crystalline solids. The crude compounds were purified by reversed-phase flash chromatography and were eluted in 10% MeOH:deionized water to give desired product **3** (0.015 g, 63%) as a colorless crystal in HCl salt. ^{1}H NMR (500 MHz, D_2O) δ 8.06 (s, 1H, CH of triazole), 7.98−7.93 (m, 2H, CH of triazole), 5.58 (s, 3H, anomeric, H-1′), 4.82 (s, 2H, *N*-CH_2 of triazole), 4.71 (s, 2H, *N*-CH_2 of triazole), 4.62 (s, 2H, *N*-CH_2 of triazole), 4.42−4.40 (m, 6H, *N*-CH_2 of linker), 4.14−4.08 (m, 6H, H-5′), 4.01−3.93 (m, 3H, *O*-CH_2 of linker), 3.87−3.84 (m, 3H, *O*-CH_2 of linker), 3.82−3.72 (m, 9H), 3.70−3.58 (m, 6H), 3.45−3.39 (m, 6H), 3.36−3.32 (m, 3H, H-6′), 2.55−2.51 (m, 3H, H-2), 2.35−2.30 (m, 3H, H-3′), 2.19−2.13 (m, 3H, H-3′), 2.00−1.93 (m, 3H, H-2), 1.85 (s, 6H, CH_2 of linker), 1.63−1.57 (m, 6H, CH_2 of linker), 1.30−0.96 (m, 48H, CH_2 of linker); ^{13}C NMR (125 MHz, D_2O) δ 154.56 (C═N of triazine), 143.87 (C═N of triazine), 125.21 (CH of triazole), 123.96 (CH of triazole), 92.08 (anomeric, C-1′), 82.70, 75.05, 73.62, 73.23, 72.69, 63.87, 50.66, 49.89, 48.96, 47.55, 39.34, 36.48, 35.33, 29.59, 29.55, 29.28, 29.04, 28.87, 28.83, 28.74, 28.62, 28.53, 28.49, 28.34, 28.26, 27.93, 27.91, 27.68, 25.52, 25.46, 25.30 (CH_2 of linker). MALDI-TOF: *m*/*e* $[M + Na]^+$ calcd for $C_{84}H_{159}N_{27}O_{15}Na^+$, 1809.24; measured 1809.24.

4.2. Microbiology Analyses. Bacterial isolates were obtained from the American Type Culture Collection (ATCC), the Canadian National Intensive Care Unit (CAN-ICU) surveillance study,[49] and the Canadian Ward (CAN-WARD) surveillance study.[50,51] Bacterial isolates from both CAN-ICU and CAN-WARD were recovered from patients diagnosed with presumed infectious diseases admitted in participating medical centers across Canada. All the pharmaceutical-grade antibiotics and reagents were purchased from Sigma-Aldrich or AK Scientific.

4.2.1. Antimicrobial Susceptibility Assay. Microbroth dilution susceptibility testing was performed to evaluate the in vitro antibacterial activity of compounds **2a−c** and **3** and antibiotics in accordance with Clinical and Laboratory Standards Institute (CLSI) guidelines.[43] Overnight grown bacterial culture was diluted with saline to achieve 0.5 McFarland turbidity and further diluted 1:50 in cation-adjusted Mueller Hinton broth (CAMHB) for inoculation to a final concentration of approximately 5×10^5 colony-forming units (cfu)/mL. 96-well plates were used for testing, and tested agents were serially diluted twofold in CAMHB and incubated with equal volumes of bacterial inoculum at 37 °C overnight. The lowest concentration of the tested agents to inhibit visible bacterial growth in the form of turbidity was considered as the minimum inhibitory concentration (MIC). Turbidity was measured with an EMax Plus Microplate Reader (Molecular Devices, USA) at a wavelength of 595 nm. The wells containing CAMHB with and without bacterial cells were used as positive and negative controls, respectively.

4.2.2. Checkerboard Assay. Using 96-well plates, the checkerboard assay was carried out using previously reported procedures.[39] The antibiotic is 2-fold serially diluted along the *x*-axis, while the adjuvant is diluted along the *y*-axis. Different concentrations of antibiotic and adjuvant were combined in the resulting wells. Overnight grown bacterial culture was diluted with saline to obtain 0.5 McFarland turbidity and further diluted 1:50 in CAMHB for inoculation to a final concentration of approximately 5×10^5 cfu/mL. The plates were incubated with equal volumes of bacterial inoculum at 37 °C overnight. The EMax Plus microplate reader (Molecular Devices, USA) operating at a 595 nm wavelength was used to verify the presence turbidity. The FIC of the tested antibiotic was determined by dividing the MIC of the antibiotic in the presence of adjuvant by the MIC of the antibiotic alone. Similarly, the FIC of the adjuvant was determined by dividing the MIC of the adjuvant in the presence of the antibiotic by the MIC of the adjuvant alone. The FIC index was calculated by summation of both the FIC values. FIC indices $\leq$0.5, 0.5 < $x \leq 4$, and > 4 were interpreted as synergistic, additive, and antagonistic, respectively.[42] The MICs of the tested compounds and antibiotics were ascertained using antimicrobial susceptible and checkerboard assays, which were performed at least twice. No repetition was carried out, if the values of all the plates were within the twofold range of agreement. If values were outside the acceptable range, the tests were repeated.

4.2.3. Time-Kill Assay. The time-kill kinetic studies were carried out following previously reported protocols.[31,32,52] Briefly, compounds and antibiotics at varying concentrations were incubated with the bacterial culture (1:50 dilution of 0.5 McFarland turbidity) in lysogeny broth (LB) at 37 °C and shaking at 250 rpm. At specific time points, an aliquot was taken from each tube, diluted in phosphate-buffered saline, and plated on LB agar plates. After incubation of the agar plates at 37 °C overnight, the bacterial colonies were counted.

4.2.4. Outer Membrane Permeabilization Assay. The outer membrane permeabilization assay was performed

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following a previously reported procedure using the nonpolar membrane-impermeable fluorescent probe NPN.[32,39,53] Briefly, NPN (10 μM final concentration) was incubated with the cell suspension (OD_{600} = 0.4−0.6) in buffer (5 mM 4-(2-hydroxyethyl)-1-piperazine-ethanesulfonic acid, 5 mM glucose, and 5 μM carbonyl cyanide 3-chlorophenylhydrazone) at room temperature for 30 min in the dark. Subsequently, varying concentrations of compound were added to the plate and the fluorescence was monitored every 30 s on a FlexStation 3 microplate reader (Molecular Devices, USA) at excitation and emission wavelengths of 350 and 420 nm, respectively.

4.2.5. Cytotoxicity Assay. The cytotoxicity assay was performed against HEK293 and Hep G2 cells, as previously described.[28,39,54] Briefly, compounds at varying concentrations were incubated with cells in media (Dulbecco's modified Eagle's medium) or in media alone (blank). After incubation of the plates at 37 °C and 5% CO_2 for 48 h, PrestoBlue reagent (Invitrogen, USA) (10% v/v final concentration) was added to the plate and incubation was proceeded for another hour. Fluorescence was measured on a SpectraMax M2 microplate reader (Molecular Devices, USA) at excitation and emission wavelengths of 560 and 590 nm, respectively. The cell viability was interpreted as previously stated. After subtracting blank values from each value of the corresponding well, the viability values of tested samples were calculated in comparison to the vehicle only controls. The mean and standard deviation of two independent experiments with five samples were used as the values for the analysis.

Supporting Information. Combination studies of compounds **2a−c** and PMBN with different antibiotics against *P. aeruginosa* PAO1; combination studies of compounds **2a−c** with different antibiotics against *E. coli* ATCC 25922; combination studies of compounds **2a−c** with different antibiotics against *A. baumannii* ATCC 17978; combination studies of compound **2a** with ceftazidime and aztreonam against β-lactamases harboring *P. aeruginosa* isolates; combination studies of compound **2b** with ceftazidime and aztreonam against β-lactamases harboring *P. aeruginosa* isolates; triple-combination studies of compounds **2c** and **3** with ceftolozane/tazobactam against β-lactam-resistant *P. aeruginosa* isolates; triple-combination studies of compounds **2c** and **3** with meropenem/vaborbactam against β-lactam-resistant *P. aeruginosa* isolates; synthetic procedures and characterizations of compounds **2−9**; NMR spectra of compounds **2−10**; and HPLC methodology and chromatograms of compounds **2a−c** and **3** (PDF)

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acsomega.3c02810.

(PDF)

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Author Contributions

F.S. and S.D. designed the experiments. S.D. conducted the synthetic work and characterized the compounds. K.G. provided the bromoalkylated tobramycin derivatives. S.D. and D.R. performed the microbiological assays. F.S., S.D., and D.R. wrote the manuscript and the supporting information. R.A. and G.A. performed the cytotoxicity assays. D.R. and R.W. performed the NPN assay. F.S. and A.K. supervised the microbiological assays.

Notes

The authors declare no competing financial interest.

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■ ABBREVIATIONS

AMR	antimicrobial resistance
ATCC	American Type Culture Collection
ATM	aztreonam
AVI	avibactam
BL	β-lactam
BLI	β-lactamase inhibitor
Boc	*tert*-butyloxycarbonyl
CAMHB	cation-adjusted Mueller Hinton Broth
CAN-ICU	Canadian National Intensive Care Unit
CANWARD	Canadian Ward
CAZ	ceftazidime
CC_{50}	cytotoxic concentration to reduce cell viability to 50%
CFU	colony-forming units
CLSI	Clinical Laboratory Standards Institute
CTZ	ceftolozane
CuAAC	copper-catalyzed alkyne-azide cycloaddition

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DCM	dichloromethane
DMF	*N*,*N*-dimethylformamide
ED	effective dose
FDA	Food and Drug Administration
GNB	Gram-negative bacteria
HEK	human embryonic kidney
HPLC	high-performance liquid chromatography
IMI	imipenem
LB	lysogeny broth
LEV	levofloxacin
LPS	lipopolysaccharides
MALDI-MS	matrix-assisted laser desorption ionization mass spectrometry
MBL	metallo-β-lactamase
MDR	multidrug resistance
MER	meropenem
MIC	minimum inhibitory concentration
MIN	minocycline
MS	mass spectrometry
NEB	nebramine
NMR	nuclear magnetic resonance
NOV	novobiocin
NPN	1-*N*-phenylnapthylamine
OD	optical density
OM	outer membrane
OMP	outer membrane permeabilizer
PBP	penicillin-binding protein
PEG	polyethylene glycol
PMB	polymyxin B
PMBN	polymyxin B nonapeptide
RB	round bottom
REL	relebactam
RIF	rifampicin
RND	resistance-nodulation-division
S	relative in vitro safety
TAZ	tazobactam
TBAF	tetrabutylammonium fluoride
TBAHS	tetrabutylammonium hydrogen sulfate
TBDMS	*tert*-butyldimethylsilyl
TBDMS-Cl	*tert*-butyldimethylsilyl chloride
THF	tetrahydrofuran
TLC	thin layer chromatography
TOF	time-of-flight
TOB	tobramycin
VAB	vaborbactam

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