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Homodimeric Tobramycin Adjuvant Repurposes Novobiocin as an Effective Antibacterial Agent against Gram-negative Bacteria

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ABSTRACT

Low permeability across the outer membrane is a major reason why most antibiotics are ineffective against Gram-negative bacteria. Agents that permeabilize the outer membrane are typically toxic at their effective concentrations. Here, we report the development of a broad-spectrum homodimeric tobramycin adjuvant that is non-toxic and more potent than the gold standard permeabilizing agent, polymyxin B nonapeptide. In pilot studies, the adjuvant confers potent bactericidal activity on novobiocin against Gram-negative bacteria, including carbapenem-resistant and colistin-resistant strains bearing plasmid-borne *mcr*-1 genes. Resistance development to the combination was significantly reduced, relative to novobiocin alone, and there was no induction of cross-resistance to other antibiotics, including the gyrase-acting fluoroquinolones. Tobramycin homodimer may allow the use of lower doses of novobiocin, overcoming its twin-problem of efficacy and toxicity.

INTRODUCTION

The problem of antibacterial drug resistance is complex and multifaceted, and antibiotic drug development is being massively outpaced by emerging resistance against available antibiotics.[1] Gram-negative bacteria are more difficult to eradicate because of their dual membrane topology and over expressed efflux pumps of broad substrate specificities.[2,3] The outer membrane (OM) of Gram-negative bacteria, which serves as a barrier to the permeation of potentially noxious molecules, including antibiotics, is composed of lipopolysaccharide (LPS) on the outer leaflet and phospholipid on the inner leaflet. The mechanism by which bacteria assemble this well-organized protective barrier has been well elucidated.[4] To breach the OM, agents that interact directly with LPS stability (e.g. polybasic molecules such as polymyxins, aminoglycosides (AGs), and chelating agents such as EDTA)[5] or indirectly with mechanisms that synthesize, assemble, and/or transport the LPS (e.g. LpxC inhibitors, novobiocin, etc.)[6,7] have been investigated as potential resistance breakers for Gram-negative bacteria.

Polybasic compounds destabilize the OM by displacing divalent metals that cross-bridge adjacent phosphate groups attached to LPS core sugar, thereby altering the well-ordered polyelectrolyte barrier. Truncation and/or modification of the polymyxin class of drugs has resulted into adjuvants such as polymyxin B nonapeptide (PMBN)[8] and SPR741[9], where intrinsic antimicrobial activity was decoupled from their OM-destabilizing properties. Whereas PMBN seems to potentiate several OM-impermeable antibiotics against various colistin-susceptible Gram-negative bacteria,[8] the activity of SPR741 excludes antipseudomonal effects.[9] PMBN was shown to be generally less toxic than polymyxin B, but it causes similar proximal renal tubular injury in male rats.[10] Similarly, conjugation and site-specific modification of AGs has resulted into scaffolds that lose the primary ribosomal properties of AGs but adopts an enhanced membrane effect.[11–13] Among membrane-acting AGs, tobramycin-based adjuvants seem to be more effective against *Pseudomonas aeruginosa* than other

Gram-negative bacteria.[14] A major problem associated with the use of AGs is their propensity to cause irreversible hearing loss, an effect linked to the lack of precise selectivity for prokaryotic ribosomes.[15–17] Synthetic AG analogs with lower bacterial and human mitochondrial ribosome specificities have been shown to exhibit reduced ototoxic potentials in cochlear explants, in culture and in guinea pig.[18] Hence, non-ribosomal AGs may exhibit lower idiosyncratic toxicities and drug-induced hearing loss. Consequently, we sought to expand the spectrum of activity of tobramycin-derived adjuvants beyond *P. aeruginosa* by dimerizing the core scaffold of an amphiphilic tobramycin, such that the hydrophobic domain is sandwiched between two identical polar heads (Figure 1). This was hypothesized to overcome the hemolytic problem of classic cationic amphiphiles.[19–22]

As a proof of concept, we investigated the possibility of repurposing novobiocin against clinically-relevant Gram-negative bacteria using the newly synthesized adjuvant. Novobiocin (Figure 1) is an orally active dihydroxy-glycosylated aminocoumarin antibiotic that inhibits DNA gyrase by binding the ATP-binding site in the ATPase subunit.[7] In 2011, the oral form of novobiocin (novobiocin sodium capsule, 250 mg) was withdrawn from US market for "reasons of safety or effectiveness".[23] However, recent pharmacokinetic trials in non-infected subjects (Phase I and II studies) have demonstrated novobiocin plasma concentration of 150 μM (~ 90 – 100 mg/L) for 24 h after a 5.5 g dose, with no serious toxicities.[24,25] Novobiocin displays limited activity against Gram-negative bacteria (MICs far higher than clinically achievable serum concentrations), even though their GyrB is sensitive to the antibiotic, due to the LPS-containing OM that act as a permeability barrier. PMBN had previously been investigated in combination with novobiocin to increase penetrance,[26,27] but effective concentrations and dose-limiting toxicities are of serious concern. Herein, we report the development of a non-toxic broad-spectrum antibiotic adjuvant that is more potent than PMBN and restores potent GyrB-dependent activity of novobiocin against multidrug (MDR) and extensively drug-resistant (XDR)

Gram-negative bacteria. Concentrations as low as 0.25 μg/ml (0.1 μM) of the adjuvant were enough to cause a measurable effect and the addition of ≤ 7.1 μM resulted in the attainment of MICs levels below clinically achievable plasma concentration of novobiocin in all 28 isolates studied. We also provide insights into the mechanism of action and resistance development to this combination.

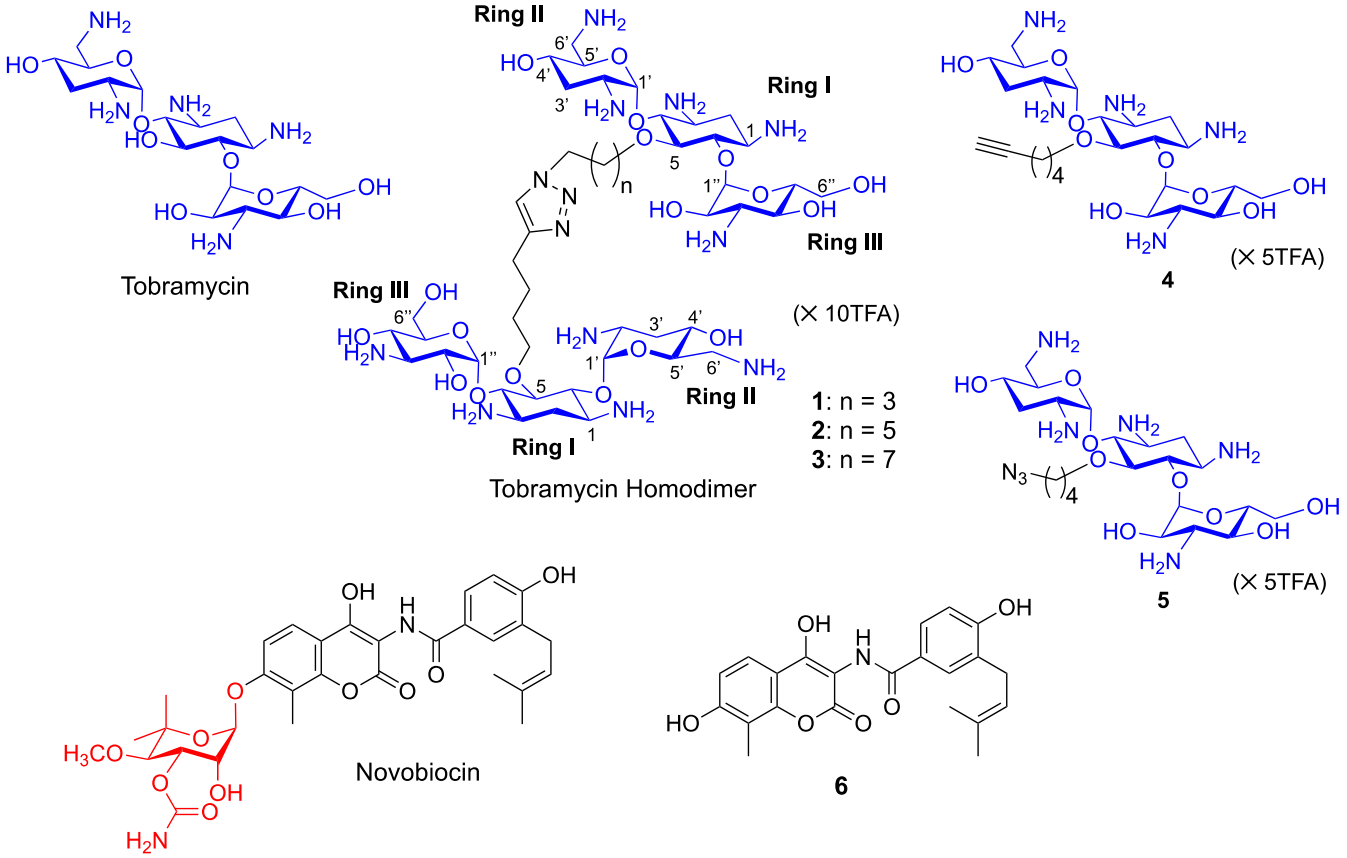


Figure 1. Structures of newly synthesized and reference compounds. Compounds **1**–**3** are tobramycin homodimers conjugated at the C-5 position of tobramycin with different tether lengths, compounds **4** and **5** are fragments of lead structure **1**, and compound **6** is an aglycone derivative of novobiocin.

RESULTS AND DISCUSSION

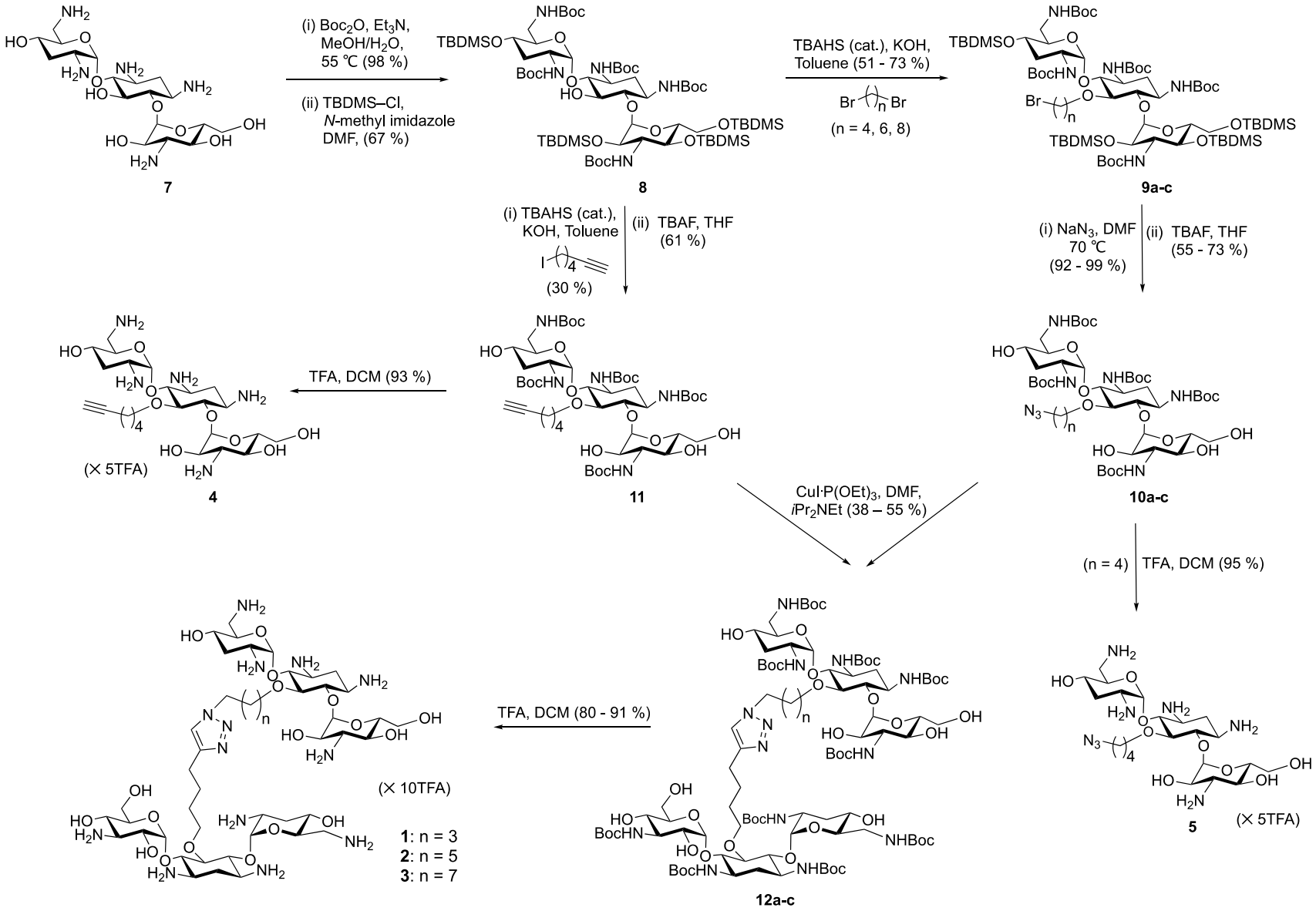
Design and Synthesis

The design of tobramycin homodimers **1–3** was guided by previous SAR.[13] Amphiphilic tobramycins with lipophilic groups at the 5-OH of deoxystreptamine (ring I; Figure 1) have been shown to lose ribosomal activities but retain the ability to permeabilize the OM.[13,14] Dimerization of ribosome-targeting antibiotics has also been shown to result in poor inhibitors of *in vitro* protein translation.[28] Hence, to prepare non-ribosomal amphiphilic-like tobramycin homodimers with potentially broad-spectrum OM permeabilizing properties, we dimerized two fragments of short-chain amphiphilic tobramycins ligated at the 4,6-disubstituted 2-deoxystreptamine via a copper(1)-catalyzed azide-alkyne cycloaddition reaction (Scheme 1). This afforded regioselective 1,4-disubstituted 1,2,3-triazole products **1–3**. Analogs with different tether length were synthesized to investigate the optimal spatial separation between the two domains while compounds **4** and **5** were prepared to study the SAR of the lead compound **1**. The full synthetic strategy for preparing compounds **1–5** is outlined in Scheme 1. Compound **6** an aglycone derivative of novobiocin, was prepared to investigate the role of the L-noviose sugar on the gyrase activity of novobiocin (Scheme 2).

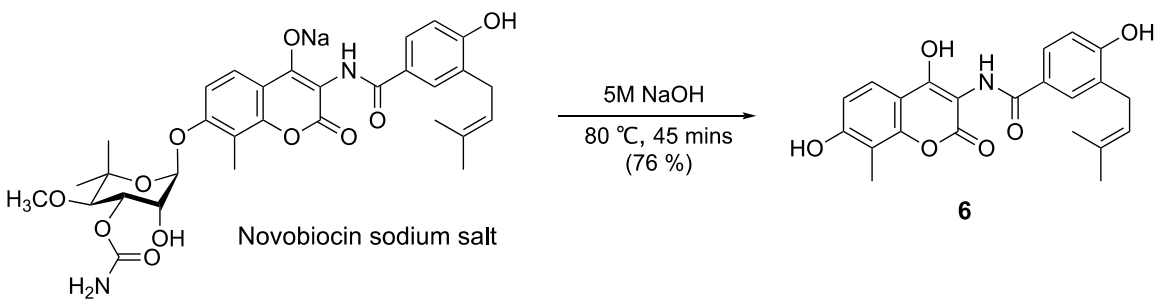
Chemical Synthesis of Tobramycin Homodimers (1–3), Fragments 4–5 and Novobiocin Aglycone (6).

The two amphiphilic tobramycin domains **4** and **5** were prepared following previously reported protocol.[49] Tobramycin **7** was purchased from a commercial source and the amino groups were first protected using di-*tert*-butyl dicarbonate (Boc anhydride), followed by silylation of the *N*-Boc-tobramycin intermediate with excess TBDMSCl to afford a partially protected derivative **8** with free OH at the *C*-5 position of the deoxystreptamine ring. In the presence of a phase-transfer catalyst (TBAHS), **8**

was alkylated with 1,*n*-dibromoalkane (*n* = 4, 6, 8) in toluene to afford alkylated TBDMS-Boc-protected tobramycin intermediates **9a-c**. Similarly, alkylation of **8** with iodohexyne under the same conditions followed by TBDMS deprotection afforded **11**. The terminal bromine of **9a-c** was then displaced by an azido nucleophile under anhydrous condition and the TBDMS protecting groups were deblocked – using TBAF – to give compounds **10a-c**, followed by Boc deprotection (using TFA) to give compound **5**. Similarly, compound **4** was prepared by deblocking compound **11** with TFA. Dimerization of tobramycin was achieved by conjoining compounds **10a-c** and **11** via "click chemistry" to afford **12a-c**. Global deprotection of Boc-protecting groups using TFA afforded the final compounds **1**–**3** (Scheme 1). Compound **6** was prepared by exposing novobiocin sodium salt to a highly basic condition (5M NaOH) at 80 °C (Scheme 2).



Scheme 1. Synthesis of tobramycin homodimers **1**–**3** and fragments **4-5**. Conjugates differ in the length of tether.



Scheme 2. Synthesis of novobiocin aglycone **6**

Susceptibility and Toxicity Screening

Susceptibilities of different Gram-positive and Gram-negative bacteria to the newly synthesized molecules **1–3** were determined and compared to the progenitor molecule tobramycin. The lack of activity of compounds **1–3** (MIC ≥ 16 μg/ml, Table S1) against a panel of organisms, relative to tobramycin, is consistent with loss of ribosomal binding. To investigate toxicity, we tested and found that compounds **1–3** were: i) non-hemolytic against porcine erythrocytes at 1024 μg/ml, ii) non-cytotoxic to human embryonic kidney (HEK293) and human liver carcinoma (HepG2) cells at 50 μM (>128 μg/ml), and iii) non-toxic *in vivo* against *Galleria mellonella* wax moths at 200 mg/kg (Figure S1). On the contrary, a single dose administration of 100 mg/kg colistin was toxic to *G. mellonella* and killed 90 % of the larvae after 96 h (Figure S1c).

Checkerboard Assay with different Classes of Antibiotics

The lack of antibacterial activity and non-toxic properties of **1–3** further encouraged us to screen their adjuvant properties. An ideal adjuvant is a bioactive helper molecule that is inactive by itself but can potentiate the activity of a primary antibiotic and/or delay resistance development when used in combination. These types of molecules are less likely to select for resistance.[29] To investigate this,

checkerboard assay was used to assess the interactions between compounds **1–3** and nineteen different antibiotics (representing all major classes) against wild-type *P. aeruginosa* PAO1. *P. aeruginosa* was selected for this initial screen because OM permeability is a major mechanism of intrinsic resistance to antibiotics,[30] and it is often regarded as a highly challenging model organism for new antibiotics.[31] Compounds **1–3**, investigated at ≤ 7.1 μM based on achievable plasma concentrations (20 – 200 μM) of aminoglycosides,[32,33] exhibit concentration-dependent synergistic relationships with all antibiotics tested against PAO1, except tobramycin, vancomycin, and colistin (Figure 2, Table S2). OM-impermeable antibiotics (such as rifampicin, linezolid, clindamycin and novobiocin), efflux-prone antibiotics (such as tetracyclines, fluoroquinolones, chloramphenicol, etc.), β-lactam antibiotics (such as monobactams, carbapenems, cephalosporins, and penicillins), and fosfomycin were all potentiated by 4- to 128-fold (Table S2). Tobramycin by itself is not synergistic with these antibiotics (Table S3). The antagonistic relationship between compounds **1–3** and tobramycin or colistin (FICI > 4) is consistent with observed antagonism between tobramycin and colistin at high concentrations (Figure S2). This is perhaps due to competition for LPS binding by both polybasic molecules. The lack of potentiation of vancomycin is consistent with other OM permeabilizing agents such as PMBN and pentamidine,[26,34] where synergy is generally more pronounced with large hydrophobic molecules (e.g. rifampicin) than with large hydrophilic molecules (e.g. vancomycin). Compound **1** is the most potent (Table S2) and least toxic of the three (Figure S1), hence, it was used for further studies.

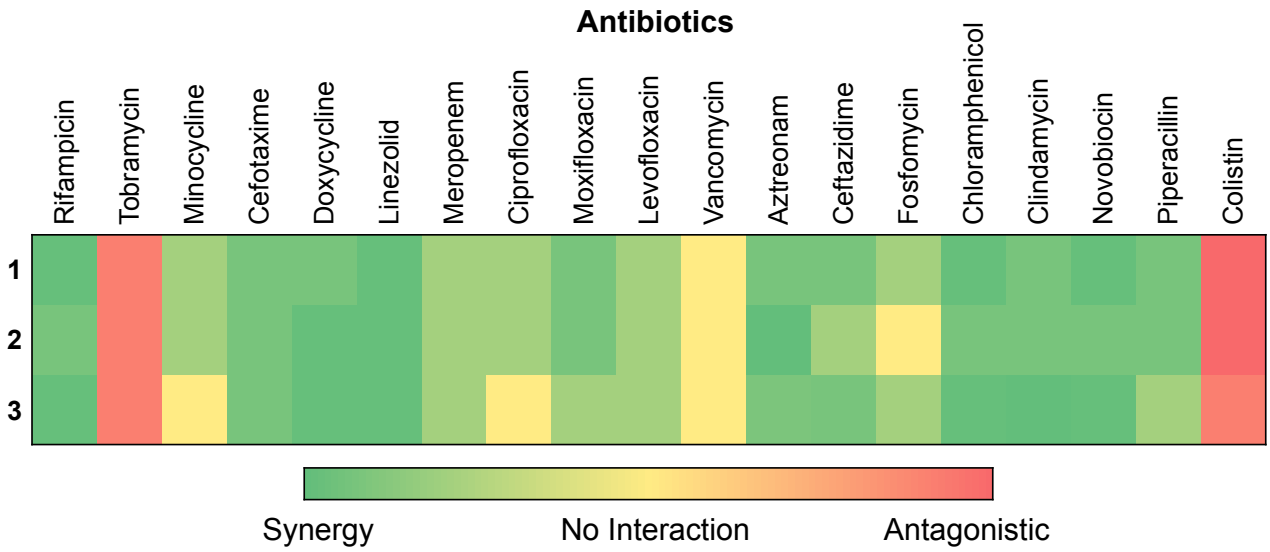


Figure 2. Interactions of compounds **1**–**3** (at ≤ 7.1 μM) with different antibiotics against *P. aeruginosa* PAO1. FICI ≤ 0.5 = Green (synergistic); FICI > 0.5 but < 1 = Yellow (no interaction); FICI > 4 = Red (antagonistic)

To investigate the spectrum of activity of the newly synthesized adjuvants, we examined a combination of compound **1** (at ≤ 7.1 μM) and novobiocin against MDR/XDR *P. aeruginosa*, *Acinetobacter baumannii*, *Escherichia coli*, *Klebsiella pneumoniae*, and *Enterobacter cloacae* exhibiting multiple resistance patterns (Table S4). Novobiocin, an ATPase inhibitor of bacterial DNA gyrase and topoisomerase IV, was selected for this study because of its unique mechanism of action but lack of meaningful activity against Gram-negative bacteria even though their GyrB is sensitive to the antibiotic.[26,27] Synergy was retained in 100% of all isolates tested (8- to 256-fold potentiation; Table 1) – including carbapenem- and colistin-resistant clinical isolates (Table S4), isolates bearing GyrA mutations (Table S5), and strains carrying the mobilized colistin resistance (*mcr*-1) gene that confer plasmid-mediated resistance to colistin.[35] We conclude that resistance to novobiocin in Gram-negative

bacteria is primarily due to OM permeability, and that resistance to fluoroquinolones (via GyrA mutations) does not necessarily confer resistance to novobiocin. To contextualize the potency of compound **1**, we compared its ability to potentiate novobiocin to the 'gold standard' potentiator molecule, PMBN, and observed that at equimolar molar concentrations of 7.1 μM, compound **1** reproducibly displayed superior activity to PMBN against colistin-resistant Gram-negative bacteria (Table 2, Figure S3a). Next, we investigated whether tobramycin by itself would potentiate novobiocin against Gram-negative bacteria. We tested a combination of tobramycin and novobiocin against tobramycin-susceptible and tobramycin-resistant Gram-negative bacteria strains and observed no synergy (FICI ≥ 0.75) (Table S6). Other antibiotics such as minocycline, ceftazidime, rifampicin, etc. also did not show any synergy with novobiocin, consistent with a previous chemical screen of 30,000 small molecules that identified just a handful of compounds (four hits) capable of synergizing with novobiocin.[36] To establish whether the synergistic relationship of compound **1** with novobiocin was specific to Gram-negative bacteria, we assessed the combination against Gram-positive bacteria. Whereas novobiocin displayed potent activity against MRSA ATCC 33592 (MIC, 0.125 μg/ml), MRSE 61589 (MIC, 0.0625 μg/ml), and *E. faecium* ATCC 27270 (MIC, 2 μg/ml), consistent with prior literature,[37] addition of compound **1** did not improve the activity of novobiocin further (Table S7). This suggests that OM-permeabilization in Gram-negative bacteria, but not in Gram-positive, is the predominant mechanism by which compound **1** (and PMBN) enhances the activity of novobiocin. Notably, a high level of susceptibility to novobiocin (MIC ≤ 1 μg/ml) was attained in wild-type and MDR *A. baumannii* clinical isolates in the presence of ≤ 7.1 μM of compound **1** (Table 1).

Table 1. **Tobramycin homodimer potentiates novobiocin against Gram-negative bacteria.** Synergistic effects of novobiocin and compound **1** (at ≤ 7.1 μM, i.e. ≤ 16 μg/ml) against wild-type and clinical isolates of Gram-negative bacteria. MIC of compound **1** is > 128 μg/ml against all strains. †Wildtype; ‡Clinical isolate; §Carbapenem-resistant; *Colistin resistant; #*mcr*-1 gene positive.

Bacteria	Strain	MIC (μg/ml) of Novobiocin		Fold Potentiation
		Alone	+ Compound **1**	
P. aeruginosa	PAO1†	512	4	128
	259-96918‡,§	512	8	64
	260-97103‡,§	256	1	256
	262-101856‡,§	512	8	64
	264-104354‡,§	512	8	64
	91433‡,§,*	16	1	16
	100036‡,§	1024	8	128
	101243‡,§,*	128	1	128
	101885‡,§	1024	8	128
	114228‡,§,*	512	16	32
A. baumannii	ATCC 17978†	16	0.5	32
	027‡	8	0.5	16
	031‡	8	0.5	16
	92247‡,*	4	0.25	16
	110193‡	256	1	256
	LAC-4‡	8	0.5	16
E. coli	ATCC 25922†	64	2	32
	94393‡,*#	128	2	64
	94474‡,*#	256	16	16
	107115‡,§	128	8	16
K. pneumoniae	113250‡,*	512	16	32
	113254‡,*	256	16	16
	116381‡	1024	8	128

	117029‡	1024	8	128
E. cloacae	118568‡,*	1024	32	32
	121187‡,*	64	8	8

Table 2. Compound **1** potentiates Novobiocin better than PMBN against colistin-resistant Gram-negative bacteria. PA = *P. aeruginosa*; AB = *A. baumannii*; KP = *K. pneumoniae*; EC = *E. cloacae*. †Wild type; ‡Clinical isolate.

Organism	MIC (μg/mL) of Novobiocin		
	Alone	+ PMBN (7.1 μM)	+ **1** (7.1 μM)
PAO1†	512	4	4
PA 91433‡	16	4	1
PA 114228‡	512	128	16
PA 101243‡	128	8	1
E. coli 94393‡	128	4	2
E. coli 94474‡	256	32	16
AB 92247‡	4	1	0.25
KP 113254‡	256	64	16
EC 118568‡	1024	512	32

To investigate SAR of tobramycin homodimers, we prepared the constituent fragments (alkyne **4** and azide **5**) of compound **1**, the most potent of the synthesized derivatives (Scheme 1). We also examined the adjuvant properties of a Boc-protected derivative **12a** to investigate the role of free amines as it relates to OM destabilization. Compounds **4** and **5** did not potentiate novobiocin against *P. aeruginosa* PAO1 (Table S8), suggesting that covalent linkage of both domains is critical for the function of the adjuvant. This observation is consistent with optimal hydrophobic-charge threshold that

must be maintained by cationic amphiphiles in order to destabilize bacterial membranes.[19–22] Similarly, compound **12a** did not potentiate novobiocin against *P. aeruginosa* PAO1 (Table S8), indicating that electrostatic interactions between the positively charged amines in compound **1** and the negatively charged phosphate residues on the OM of Gram-negative bacteria is central to the potency of the adjuvant.

Potentiation of Novobiocin is Independent of RND Efflux Pumps

To investigate whether efflux pumps play a role in the restoration of potent antibacterial activity of novobiocin by tobramycin homodimers, we assessed and compared the presence of synergy in efflux-deficient *P. aeruginosa* strains to wild type PAO1. Efflux mutants PAO200 and PAO750, lacking different clinically-relevant efflux pumps that extrude different classes of antimicrobial agents, exhibited higher level of susceptibilities to novobiocin (MIC, 32 μg/ml) than wild type PAO1 (MIC, 512 μg/ml), suggesting that novobiocin is a substrate of the RND efflux pumps. The MIC of compound **1** was unaffected by these pumps (Table 3). However, compound **1** (at 7.1 μM) further increased the susceptibilities of the mutants to novobiocin and lowered its MICs in both strains from 32 μg/ml to 0.125 μg/ml (256-fold potentiation) (Table 3). This suggests that whereas novobiocin is a substrate of the RND pumps, its potentiation by compound **1** is independent of these pumps.

Table 3. Potentiation of novobiocin in *P. aeruginosa* by compound 1 is independent of RND efflux pumps. PAO1 = wild-type, PAO200 (ΔmexAB-oprM) and PAO750 (ΔmexAB-oprM, ΔmexCD-oprJ, ΔmexEF-oprN, ΔmexJK, ΔmexXY, and ΔopmH outer membrane) are efflux-deficient strains.[38,39] MICs are reported in μg/ml.

Strain	MIC of Compound **1**	MIC of Novobiocin		Fold Potentiation
		Alone	+ 7.1 μM Compound **1**	
PAO1	>128	512	4	128
PAO200	>128	32	0.125	256
PAO750	>128	32	0.125	256

Time-Kill Assay

To scrutinize the data generated by checkerboard assay and provide more complementary evidence for the observed synergy, time-kill assays were performed on four different Gram-negative bacteria. Novobiocin is predominantly bacteriostatic versus most bacteria but could also be bactericidal against some pathogens. We investigated the kinetics of killing in LB media at fixed concentrations of 32 μg/ml (50.4 μM) for novobiocin and 16 μg/ml (7.1 μM) for compound **1** because these concentrations (in combination) inhibit visible growth of all isolates studied in MHB media (Table 1). For PAO1 and *K. pneumoniae* 116381, 32 μg/ml of novobiocin alone resulted in bacterial growth identical to their respective controls (without drug) while a combination of novobiocin (32 μg/ml) and compound **1** (7.1 μM) inhibited the growth of both pathogens in LB media (Figure 3). The combination resulted in bactericidal (PAO1) and synergistic relationships against both strains after 24 h. For *A. baumannii* ATCC 17978, 32 μg/ml novobiocin alone was bacteriostatic after 24 h, while a combination of novobiocin (32 μg/ml) and compound **1** (7.1 μM) was bactericidal (> 3-Log reduction) and

synergistic after 9 h of incubation (Figure 3). After 24 h, the bacterial culture containing novobiocin and compound **1** was completely sterilized, representing > 5-Log reduction from the starting inoculum. For *E. coli* ATCC 25299, 32 μg/ml novobiocin alone was bactericidal after 24 h, while a combination of novobiocin (32 μg/ml) and compound **1** (7.1 μM) exhibited bactericidal effects after 6 h of incubation (Figure 3). Novobiocin and compound **1** became synergistic against *E. coli* ATCC 25922 after 9 h of incubation and the culture was completely sterilized within this period. Overall, the species-dependent degree of bacterial load reduction reflects, to a large extent, the fold potentiation of novobiocin by compound **1** in checkerboard assay.

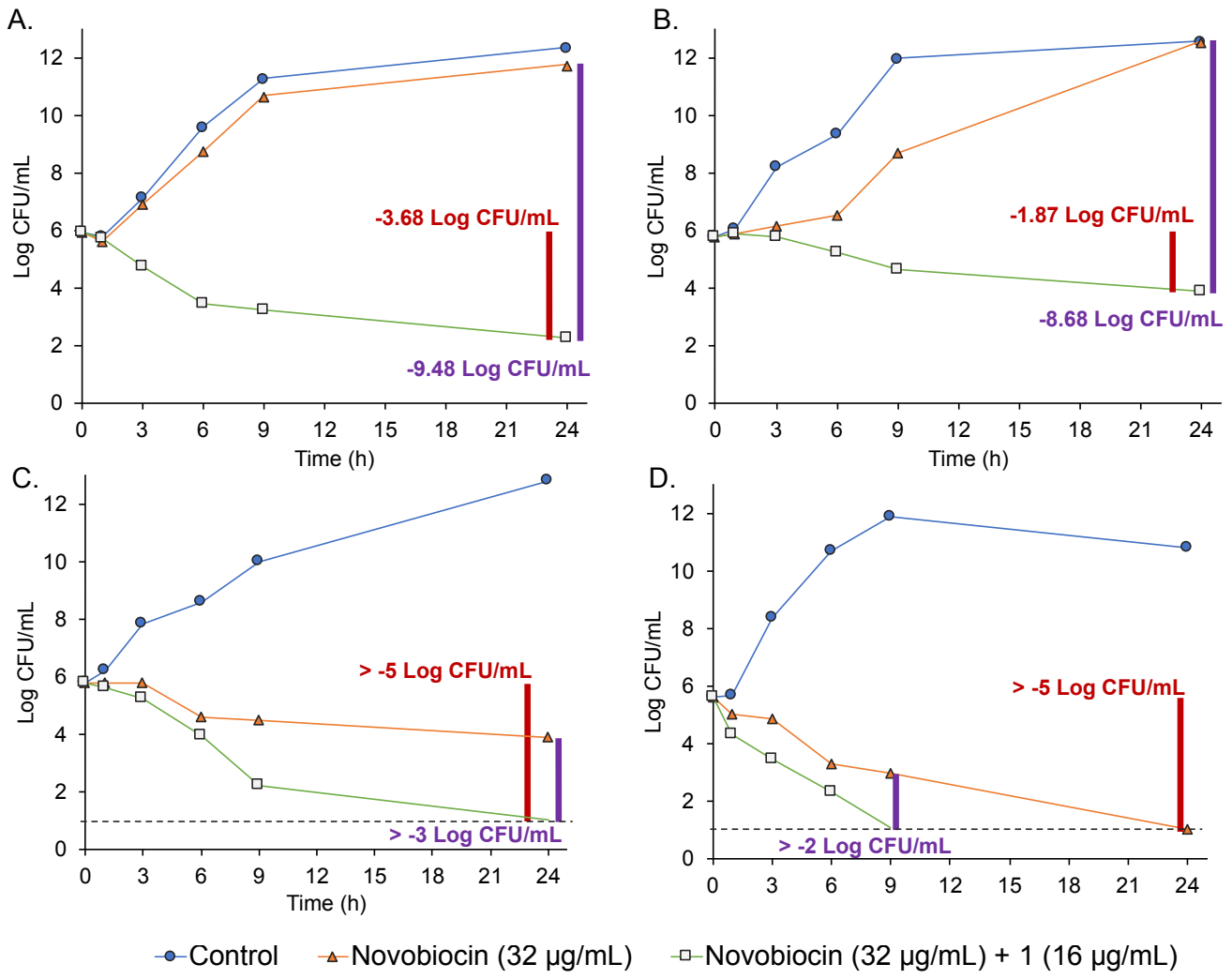


Figure 3. Time-kill synergy graphs. The activities of novobiocin (32 µg/ml) in combination with compound **1** (16 µg/ml, i.e. 7.1 µM) against (A) *P. aeruginosa* PAO1, (B) *K. pneumoniae* 116381, (C) *A. baumannii* ATCC 17978, (D) *E. coli* ATCC 25922. Red bars and numbers indicate differences in bacterial concentrations between the starting inoculum and drug combination at 24 h. Purple bars and numbers indicate differences in bacterial concentrations between the combination and the most active single agent at 24 h (9 h for *E. coli*). The dashed lines represent the lower limit of detection. Each data point represents an average of three independent determinations.

Outer Membrane Permeabilization Assay

To investigate whether compound **1** enhances the uptake of novobiocin by permeabilizing the OM, we measured the intensity of fluorescence of the nonpolar probe 1-*N*-phenylnaphthylamine (NPN) using NPN uptake assay.[40] An intact OM will ordinarily prevent the uptake of NPN dye which fluoresces strongly in phospholipid environments but only weakly in an aqueous environment. We observed that tobramycin homodimer **1** permeabilizes the OM of *P. aeruginosa* PAO1 in a dose-dependent manner, reminiscent of PMBN, while tobramycin alone showed weak fluorescence (Figure 4). PMBN is a known OM permeabilizer,[26] and our results showed that compound **1** (at 16 µg/ml) caused an increased fluorescence of NPN relative to PMBN (8 µg/ml) at similar micromolar concentrations (Figure 4). This observation is consistent with data from checkerboard assay (Table 2).

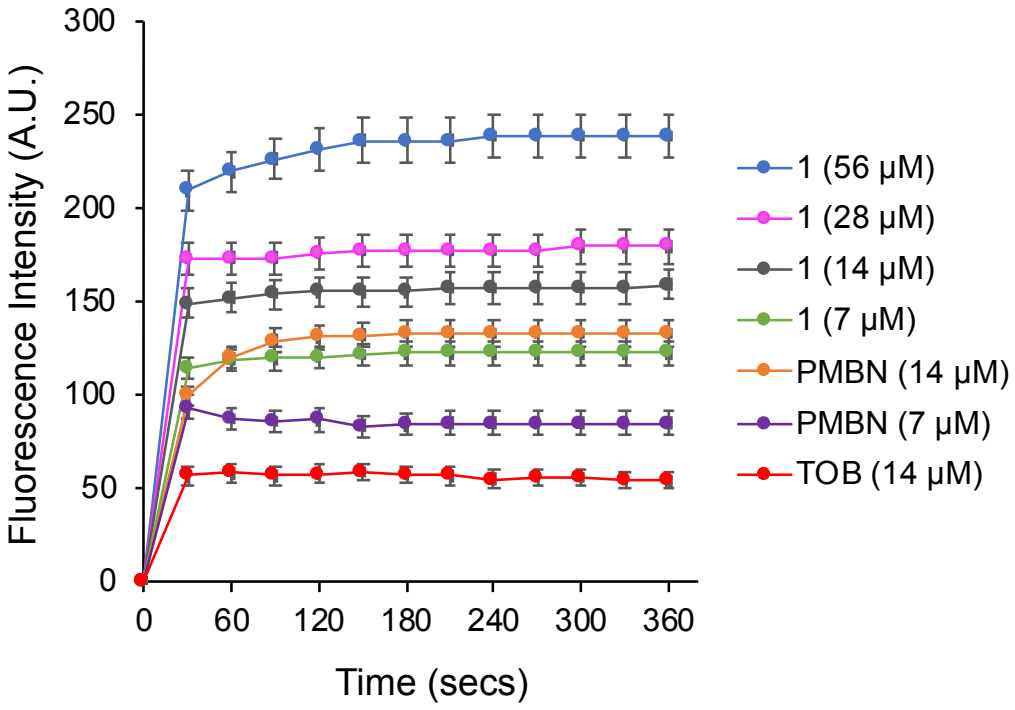


Figure 4. Outer membrane permeabilization by compound **1**, polymyxin B nonapeptide (PMBN) and tobramycin (TOB) was determined by measuring the accumulation of 1-*N*-phenylnaphthylamine (NPN) in *P. aeruginosa* PAO1 cells. Each data point is an average of four independent determinations ± SD.

Mechanism of Resistance Study

To investigate the possible mechanisms of resistance development to the combination, we generated some *A. baumannii* mutants by exposing wild-type ATCC 17978 to sub-MICs of novobiocin, alone and in combination with compound **1**. *A. baumannii* was used for this study because all strains exhibited a high level of susceptibility to novobiocin + compound **1** combination (Table 1). Tobramycin-resistant and colistin-resistant mutants were also generated to investigate the mechanism and pattern of resistance development. Emergence of resistance to novobiocin alone was fast and high level, consistent with GyrB mutation,[41] with a 64-fold change in MIC (from 8 μg/ml to 512 μg/ml) after just 3 days and a 256-fold change in MIC (from 8 μg/ml to 2048 μg/ml) after 5 days (Figure 5). On the other hand, a combination of novobiocin and compound **1** (7.1 μM) resulted in a slow and steady 2-fold increase in MIC every generation, with a 4-fold increase in MIC (from 0.5 μg/ml to 2 μg/ml) after 3 days and a 16-fold change in MIC (from 0.5 to 8.0 μg/ml) after 5 days (Figure 5). Tobramycin alone resulted in a 512-fold increase in MIC (from 1 to 512 μg/ml) after 7 days while colistin resulted in a 1024-fold change in MIC after 7 days (Figure 5).

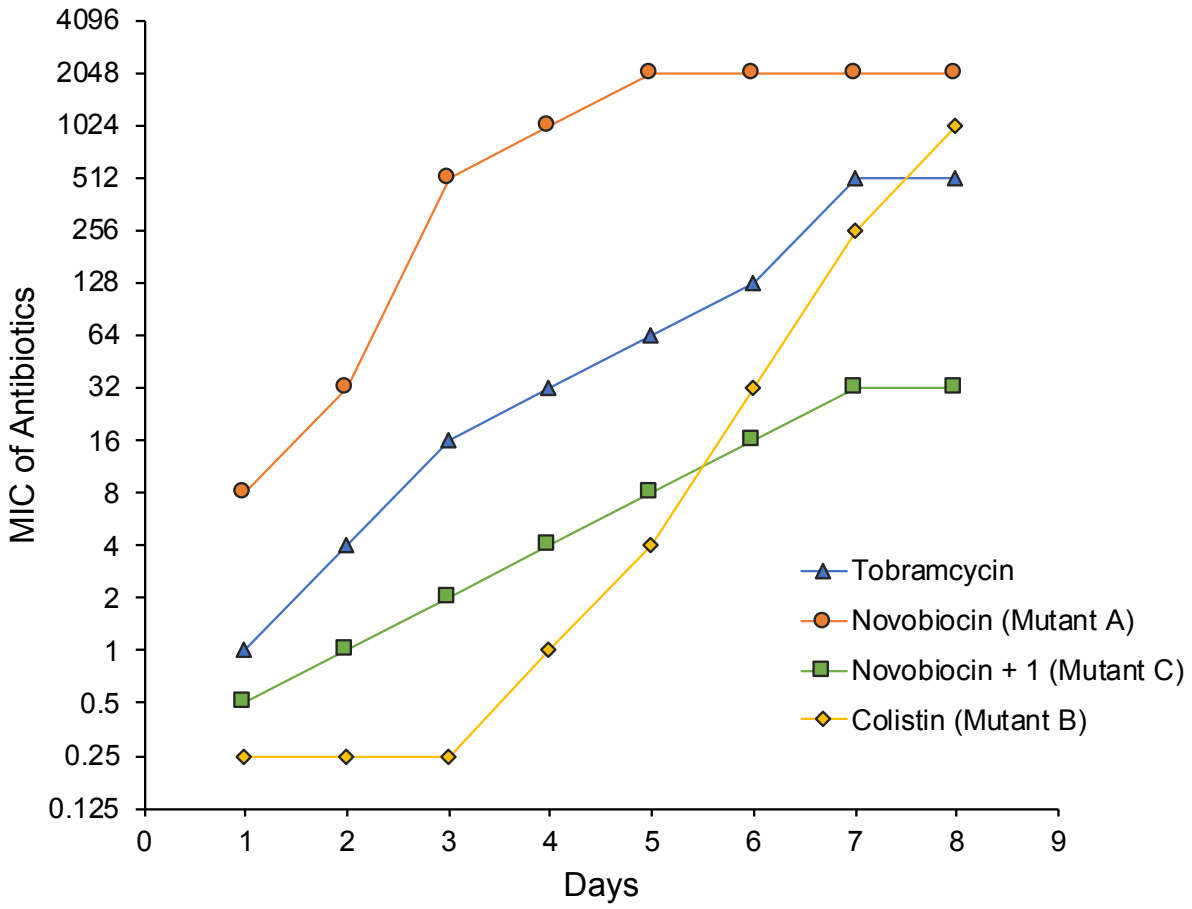


Figure 5. Emergence of resistance study. Resistance acquisition during serial passaging of *A. baumannii* ATCC 17978 in the presence of sub-MIC levels of antibiotics. For combination study, compound **1** was kept constant at a concentration of 7.1 μM throughout the experiment.

To gain insights into the emerging mechanism(s) of resistance to novobiocin/compound **1** combination, we assessed the susceptibilities of all generated mutants to different antibiotics (Table 4) and performed checkerboard assay on the novobiocin-resistant and colistin-resistant mutants A and B,

respectively. Exposure of *A. baumannii* ATCC 17978 to sub-MIC levels of tobramycin homodimer **1** for seven serial passages did not generate tobramycin-resistant phenotype, whereas tobramycin alone did, suggesting that tobramycin homodimers do not bind to any critical target in the bacteria. Novobiocin-resistant mutant A (Table 4) was found to be resistant to ciprofloxacin (MIC,16 μg/ml) and colistin (MIC, 8 μg/ml), suggesting gyrase mutation(s) as a primary mechanism of resistance but also LPS modification and efflux. Compound **1** was able to further potentiate novobiocin against this highly novobiocin-resistant mutant by 16-fold (Figure S3b). Surprisingly, the generated colistin-resistant mutant B was hyper-susceptible to all antibiotics tested, consistent with a complete loss of LPS production that resulted in collateral sensitivity to antibiotics,[42] or/and mutations in the PmrAB two-component system.[43] The lipid A component of LPS is critical for the activity of colistin,[42] and the development of such high level resistance to colistin under clinical conditions might constitute a huge fitness cost to the pathogen. PMBN and compound **1** did not potentiate novobiocin further against this strain (Figure S3c), suggesting that LPS interaction is critical for OM destabilization by both compounds. Resistance to novobiocin/compound **1** combination did not confer resistance to tobramycin, ciprofloxacin, and colistin, but conferred low level resistance to novobiocin alone (Mutant C; Table 4), suggesting that the combination did not trigger the production of aminoglycoside modifying enzymes (AMEs), overexpression of efflux pumps and/or LPS modifications. By comparing the MICs of novobiocin versus mutants A and C, it is evident that compound **1** suppressed the development of resistance to novobiocin. Overall, the mechanism of resistance development to novobiocin/compound **1** combination is consistent with gyrase mutation, not LPS modification and AMEs production, and compound **1** significantly delayed this process.

Table 4. Susceptibility profiles (MIC in μg/ml) of wild-type *A. baumannii* ATCC 17978 versus resistant mutants generated from seven serial passages (Day 8) of exposure to sub-MICs novobiocin (Mutant A), colistin (Mutant B) and novobiocin + 7.1 μM compound **1** (Mutant C).

Antibiotics	Wild-type	Mutant A	Mutant B	Mutant C
Novobiocin	16	2048	<0.031	128
Tobramycin	1	1	0.25	1
Ciprofloxacin	1	16	0.0625	1
Minocycline	0.125	0.5	<0.031	0.125
Rifampicin	2	2	<0.031	2
Ceftazidime	16	32	1	16
Chloramphenicol	64	32	8	64
Colistin	0.063	8	1024	0.031
Novobiocin + **1**	0.5	128	<0.031	16

Potentiation of Novobiocin by Compound 1 is dependent on Gyrase B Activity

A study has shown that synergistic relationship between novobiocin and polymyxin B is independent of gyrase activity of novobiocin.[44] To ascertain whether potentiation of novobiocin by compound **1** is indeed based on the binding of novobiocin to the ATP-binding site in the ATPase subunit of GyrB, we prepared novobiocin aglycone **6** (Scheme 2) lacking the L-noviose sugar of novobiocin, which is known to make important contacts with GyrB.[45] Consistent with a loss of gyrase activity, potent antibacterial activity was completely lost in compound **6**, even against Gram-positive organisms that are susceptible to novobiocin (Table S9). Novobiocin aglycone **6** has been reported to lose *in vitro* supercoiling activity against *A. baumannii* gyrase by at least 260-fold, relative to novobiocin.[44] We thereafter evaluated whether any synergistic relationship exists between compounds **1** and **6** against *P. aeruginosa* PAO1, *A. baumannii* ATCC 17978, and *E. coli* ATCC 25922. Tobramycin homodimer **1**

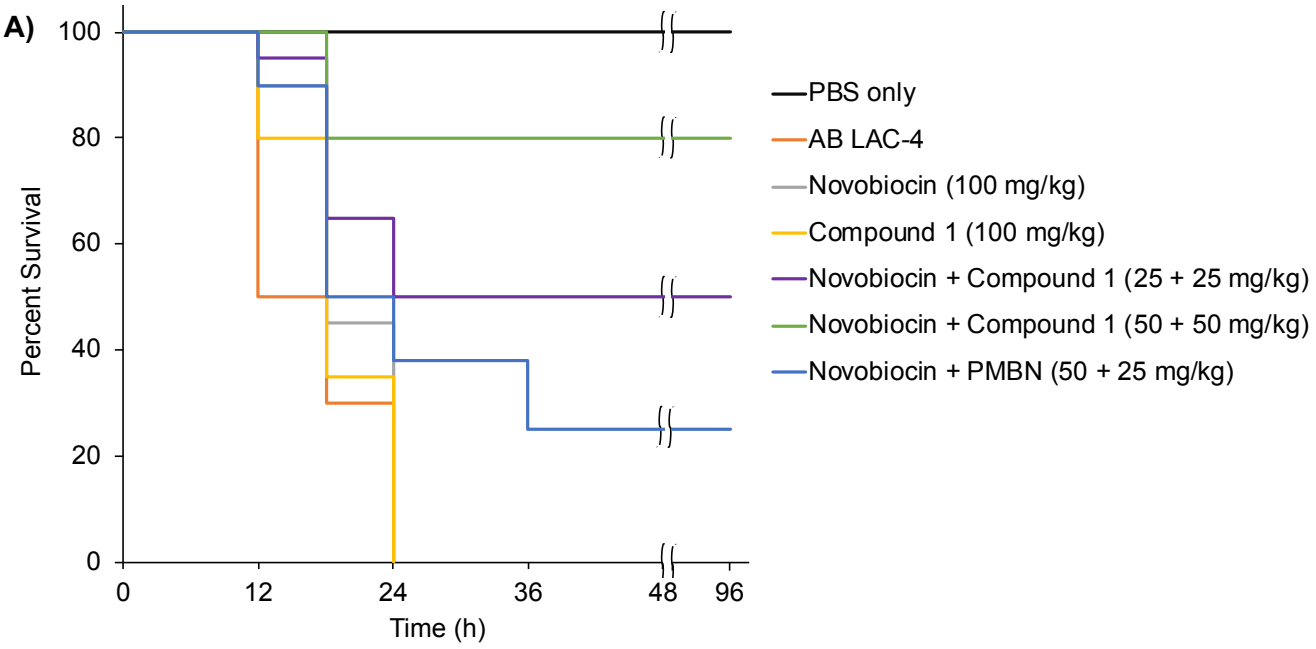
potentiates novobiocin against all of these strains (Table 1) but there was no potentiation of compound **6** against any of these pathogens (FICI > 1) (Table S10a). On the contrary, compound **6** was found to potentiate colistin against *A. baumannii* ATCC 17978 and *E. coli* ATCC 25922 (Table S10b), similar to a previous report.[44] This suggests that whereas the synergistic relationship between colistin and novobiocin (or compound **6**) might not be gyrase-dependent, the synergistic relationship between tobramycin homodimers and novobiocin is DNA-gyrase dependent.

In Vivo Efficacy in *Galleria mellonella* Larvae

To expand the robustness of the data generated using *in vitro* assays, we performed preliminary *in vivo* investigation on the ability of novobiocin and compound **1** to protect *G. mellonella* moths from two different MDR *A. baumannii* infections. Despite its well-described limitations, the use of *G. mellonella* larvae as preliminary *in vivo* model system to study host-pathogen interactions, virulence, toxicity, and efficacy of novel compounds has been demonstrated with clinically used therapeutic agents.[46–49] We found that the larvae tolerated 200 mg/kg each of compound **1** and novobiocin alone, and a 100 + 100 mg/kg combination of both, for more than 4 days, whereas exposure to 100 mg/kg of colistin (single dose administration) resulted in 90 % mortality after 4 days (Figure S1c). A multiple, but not single, dose administration of 25 mg/kg novobiocin + 50 mg/kg PMBN had previously been reported to protect mice challenged with MDR *P. aeruginosa* or *K. pneumoniae*.[26] In pilot studies, we investigated the *in vivo* therapeutic potential of novobiocin + compound **1** (25 + 25 and 50 + 50) mg/kg in the infection model. Untreated larvae challenged with MDR *A. baumannii* died within 24 h of infection. A single dose administration of 100 mg/kg each of novobiocin alone or compound **1** alone also resulted in 100 % mortality of infected larvae after 24 h. However, a single dose administration of novobiocin + compound **1** (50 + 50 mg/kg) protected ~80 % of the larvae after 24 h for more than 96 h

(Figure 6). An approximate equimolar concentration of novobiocin + PMBN (50 + 25 mg/kg) only protected ~35 % of the larvae from the same infection (Figure 6).

To ascertain that the combination therapy does not elevate toxicity against eukaryotic cells, we assessed the toxicity of a combination of compound **1** and novobiocin against HEK293 and HepG2 cell lines and found the combination to be non-cytotoxic (Figure S1d). These preliminary data provide a baseline for future studies in mice.



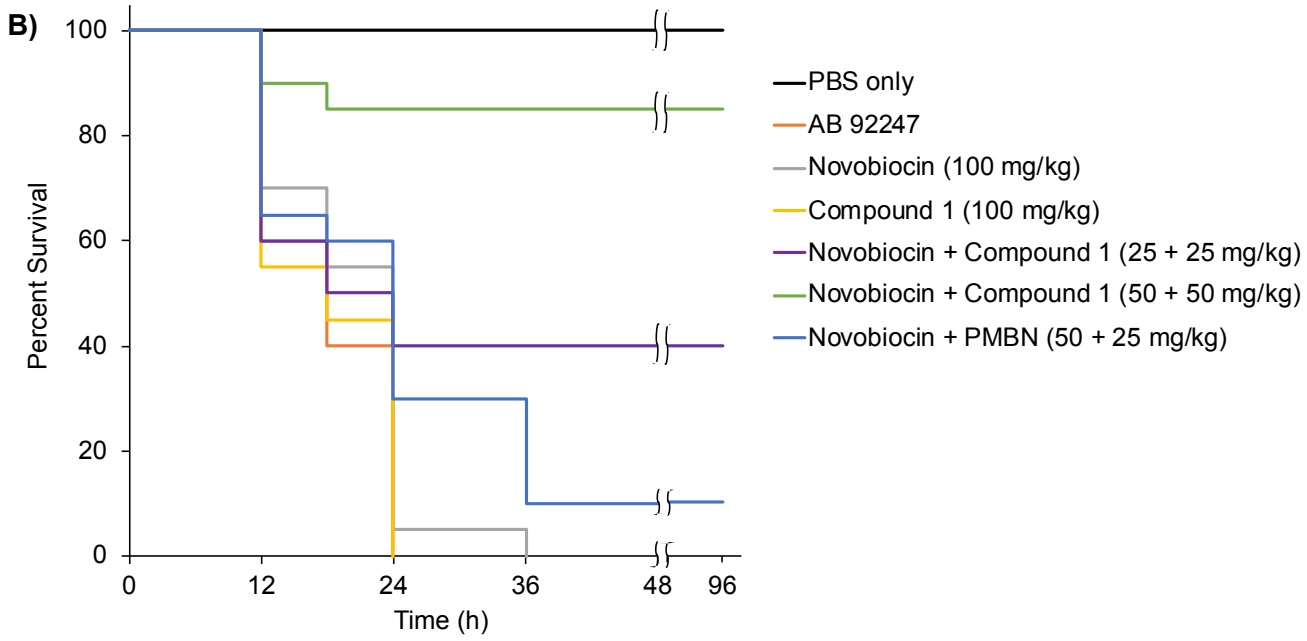


Figure 6. Preliminary efficacy studies of mono- and combination therapies in MDR *Acinetobacter baumannii* (AB)-challenged *Galleria mellonella* wax worms. A) AB LAC-4, a multidrug-resistant hypervirulent strain isolated from an hospital outbreak in LA county, California. B) AB 92247, a colistin-resistant MDR clinical isolate. Survivability of the larvae was scored every 6 h for 96 h.

CONCLUSION

The problem of antimicrobial resistance is forcing a re-think and re-evaluation of our old antibiotics, with a view to repurposing them to combat emerging threats. Antibiotic adjuvants are becoming increasingly attractive as a strategy to address the immediate needs of effective treatment options against MDR pathogens. We report the development of a broad-spectrum homodimeric tobramycin adjuvant that is more potent than the gold standard potentiator molecule, PMBN, and confers potent bactericidal activity on novobiocin against MDR/XDR Gram-negative bacteria. *A.*

baumannii isolates exhibit a high level of susceptibility to this combination, allowing the use of lower doses of novobiocin that could consequently improve tolerability and safety. Our data and others[26] suggest that intrinsic, but not acquired, resistance is the predominant mechanism of resistance to novobiocin in Gram-negative bacteria. Emergence of resistance to novobiocin alone by *A. baumannii* was spontaneous and conferred resistance to ciprofloxacin (consistent with target mutation)[41] whereas a combination of novobiocin and compound **1** suppressed this process and did not induce cross-resistance to other antibiotics. Preliminary *in vivo* evaluation in *G. mellonella* wax moths did not reveal any toxic interaction between the combination (colistin alone was toxic), but rather suggests a therapeutic potential for this combination. Future work will involve efficacy studies in animals and optimization of dosing regimens for each constituent molecule.

EXPERIMENTAL SECTION

Chemistry. All chemicals and reagents were purchased from Sigma-Aldrich (Oakville, ON, Canada) except tobramycin that was purchased from AK Scientific Inc. (CA, USA). The chemicals were all used without further purification. Air and moisture-sensitive reactions were performed with dry solvents under nitrogen atmosphere. Thin-layer chromatography (TLC) plates were visualized by staining within ninhydrin solution in n-butanol. Yields refer to chromatography-purified homogenous materials, except otherwise stated. ^{1}H and ^{13}C NMR spectra were recorded on Bruker AMX-300 and AMX-500 spectrometers (Germany) as solutions and reported in the order of chemical shifts (δ) in ppm relative to the indicated solvent, multiplicity (s, singlet; d, doublet; t, triplet and m, multiplet), number of protons, and coupling constants (*J*) in hertz (Hz). ESI-MS and MALDI-TOF MS analyses were performed on Varian 500-MS ion trap mass spectrometer (USA) and Bruker Daltonics Ultraflextreme MALDI

TOF/TOF mass spectrometer (Germany), respectively. Purity of final compounds, as determined by elemental analysis, was >95 % (Table S11).

General Procedure A: Global Amine Deprotection (Removal of Boc Protecting Groups) for Preparation of Compounds 1–3. Solution of Boc-protected compounds **12a–c** in DCM (2.0 mL) were treated with trifluoroacetic acid (2.0 mL), stirred at RT for 1 h and concentrated under low *vacuo*. 2% methanol in diethylether (2.0 mL) was then added, stirred gently for 1 min and the solvent carefully decanted to give off-white solid compounds. The crude products were subsequently purified by reverse-phase flash chromatography (eluted with 100% deionized water) to afford analytically pure compounds **1**–**3** (80 – 91 %) as off-white TFA salt solid compounds.

Tobramycin Homodimers 1–3. Final compounds **1**–**3** were prepared by global amine deprotection of compounds **12a-c**, according to general procedure A.

Tobramycin Homodimer 1. Yield (80 %). ^{1}H NMR (500 MHz, D_2O) δ 7.75 (s, 1H, C*H* of triazole), 5.19 (d, *J* = 2.5 Hz, 1H, anomeric H-1'), 5.17 (d, *J* = 2.5 Hz, 1H, anomeric H-1'), 4.97 (d, *J* = 2.7 Hz, 2H, anomeric H-1''), 4.30 (t, *J* = 7.0 Hz, 2H, N-CH_2 of linker), 4.13 – 4.06 (m, 2H, H-5'), 3.96 (t, *J* = 9.7 Hz, 2H, H-5''), 3.78 – 3.49 (m, 22H, H-5, H-6, H-4, H-2', H-4', H-2'', H-4'', H-6'', O-CH_2 of linker ×2), 3.44 – 3.31 (m, 6H, H-1, H-3, H-3''), 3.25 – 3.17 (m, 2H, H-6'), 3.14 – 3.08 (m, 2H, H-6'), 2.61 (m, 2H, CH_2 of linker), 2.36 (dt, *J* = 12.9, 4.3 Hz, 2H, H-2), 2.09 – 2.00 (m, 4H, H-3'), 1.84 – 1.71 (m, 4H, H-2, CH_2 of linker), 1.57 – 1.41 (m, 6H, CH_2 of linker ×3), 1.19 – 1.16 (m, 2H). ^{13}C NMR (125 MHz, D_2O) δ 163.28 (TFA), 163.00 (TFA), 162.72 (TFA), 162.44 (TFA), 124.16 (*C*H of triazole), 117.92 (TFA) 115.54 (TFA), 101.27 (C-1''), 101.17 (C-1''), 92.76 (C-1'), 82.09 (C-4''), 81.99 (C-4''), 81.80 (C-5), 81.77 (C-5), 77.07 (C-5''), 76.96 (C-5''), 76.14 (C-5'), 75.99 (C-5'), 73.21 (C-4), 73.18 (C-4), 73.06 (O-*C*H_2 of linker), 72.51 (O-*C*H_2 of linker), 68.55 (C-2''), 64.86 (C-6), 64.80 (C-6), 63.10 (C-

4'), 59.35 (C-6''), 59.24 (C-6''), 54.82 (C-3''), 54.44, 50.86 (*C*H_2 of linker), 49.67 (C-1), 49.61 (C-1), 48.36 (C-3), 47.27 (C-2'), 47.24 (C-2'), 38.36 (C-6'), 38.32 (C-6'), 28.89 (*C*H_2 of linker), 27.98 (C-3'), 27.70 (C-2), 26.39 (*C*H_2 of linker), 25.99 (*C*H_2 of linker), 24.97 (*C*H_2 of linker), 24.12 (*C*H_2 of linker), 17.79, 16.32. MALDI: *m/e* calcd for $C_{46}H_{89}N_{13}O_{18}H^+$, 1112.653; found 1112.650 $[M + H]^+$

Tobramycin Homodimer 2. Yield (85 %). ^{1}H NMR (500 MHz, D2O) δ 7.68 (s, 1H, C*H* of triazole), 5.21 (d, *J* = 2.6 Hz, 1H, anomeric H-1'), 5.20 (d, *J* = 2.6 Hz, 1H, anomeric H-1'), 4.99 (d, *J* = 3.5 Hz, 2H, anomeric H-1''), 4.24 (t, *J* = 7.1 Hz, 2H, N-C*H*$_2$ of linker), 4.14 – 4.07 (m, 2H, H-5'), 3.97 (td, *J* = 9.8, 4.8 Hz, 2H, H-5''), 3.79 – 3.50 (m, 22H, H-5, H-6, H-4, H-2', H-4', H-2'', H-4'', H-6'', O-C*H*$_2$ of linker ×2), 3.46 – 3.34 (m, 6H, H-1, H-3, H-3''), 3.27 – 3.19 (m, 2H, H-6'), 3.18 – 3.08 (m, 2H, H-6'), 2.65 – 2.54 (m, 2H, C*H*$_2$ of linker), 2.37 (dt, *J* = 12.8, 4.4 Hz, 2H, H-2), 2.14 – 1.97 (m, 4H, H-3'), 1.82 – 1.69 (m, 4H, H-2, C*H*$_2$ of linker), 1.60 – 1.41 (m, 6H, C*H*$_2$ of linker ×3), 1.21 – 1.11 (m, 4H, C*H*$_2$ of linker ×2). ^{13}C NMR (125 MHz, D_2O) δ 163.37 (TFA), 163.09 (TFA), 162.81 (TFA), 162.53 (TFA), 123.62 (*C*H of triazole), 119.96 (TFA), 117.67 (TFA), 115.35 (TFA), 113.04 (TFA), 101.32 (C-1''), 101.30 (C-1''), 92.76 (C-1'), 81.96 (C-4''), 81.84 (C-5), 76.93 (C-5''), 76.88 (C-5''), 75.99 (C-5'), 75.89 (C-5'), 73.49 (O-*C*H_2 of linker), 73.18 (C-4), 73.13 (O-*C*H_2 of linker), 68.55 (C-2''), 64.79 (C-6), 64.78 (C-6), 63.14 (C-4'), 63.10 (C-4'), 59.23 (C-6''), 54.82 (C-3''), 50.59 (*C*H_2 of linker), 49.69 (C-1), 48.37 (C-3), 47.29 (C-2'), 47.25 (C-2'), 38.41 (C-6'), 38.34 (C-6'), 29.41 (*C*H_2 of linker), 29.28 (*C*H_2 of linker), 28.85 (*C*H_2 of linker), 28.05 (C-3'), 27.98 (C-3'), 27.71 (C-2), 25.83 (*C*H_2 of linker), 25.19 (*C*H_2 of linker), 24.65 (*C*H_2 of linker), 24.31 (*C*H_2 of linker). MALDI: *m/e* calcd for $C_{48}H_{93}N_{13}O_{18}H^+$, 1140.684; found 1140.695 $[M + H]^+$

Tobramycin Homodimer 3. Yield (91 %). ^{1}H NMR (500 MHz, D_2O) δ 7.73 (s, 1H, C*H* of triazole), 5.21 (d, *J* = 2.6 Hz, 1H, anomeric H-1'), 5.20 (d, *J* = 2.6 Hz, 1H, anomeric H-1'), 4.98 (d, *J* = 3.4 Hz, 2H, anomeric H-1'')), 4.25 (t, *J* = 7.0 Hz, 2H, N-C*H*$_2$ of linker), 4.10 (dt, *J* = 8.4, 3.8 Hz, 2H, H-

5'), 3.97 (td, *J* = 9.8, 5.1 Hz, 2H, H-5''), 3.80 – 3.50 (m, 22H, H-5, H-6, H-4, H-2', H-4', H-2'', H-4'', H-6'', O-C*H*$_2$ of linker ×2), 3.46 – 3.33 (m, 6H, H-1, H-3, H-3''), 3.27 – 3.20 (m, 2H, H-6'), 3.16 – 3.09 (m, 2H, H-6'), 2.66 – 2.56 (m, 2H, C*H*$_2$ of linker), 2.37 (dt, *J* = 12.5, 4.3 Hz, 2H, H-2), 2.09 – 2.01 (m, 4H, C-3'), 1.84 – 1.68 (m, 4H, H-2, C*H*$_2$ of linker), 1.61 – 1.41 (m, 6H, C*H*$_2$ of linker ×3), 1.19 – 1.06 (m, 8H, C*H*$_2$ of linker ×4). ^{13}C NMR (125 MHz, D_2O) δ 163.35 (TFA), 163.07 (TFA), 162.79 (TFA), 162.50 (TFA), 123.92 (*C*H of triazole), 120.02 (TFA), 117.70 (TFA), 115.37 (TFA), 113.05 (TFA), 101.38 (C-1''), 101.27 (C-1''), 92.75 (C-1'), 81.97 (C-4''), 81.91 (C-5), 81.81 (C-4''), 76.90 (C-5''), 76.87 (C-5''), 75.96 (C-5'), 75.91 (C-5'), 73.75 (O-*C*H$_2$ of linker), 73.18 (C-4), 73.06 (O-*C*H$_2$ of linker), 68.56 (C-2''), 64.81 (C-6), 64.77 (C-6), 63.15 (C-4'), 63.12 (C-4'), 59.23 (C-6''), 59.21 (C-6''), 54.82 (C-3''), 50.98 (*C*H$_2$ of linker), 49.72 (C-1), 49.68 (C-1), 48.38 (C-3), 47.30 (C-2'), 47.25 (C-2'), 38.41 (C-6'), 38.35 (C-6'), 29.42 (*C*H$_2$ of linker), 29.30 (*C*H$_2$ of linker), 28.82 (*C*H$_2$ of linker), 28.78 (*C*H$_2$ of linker), 28.18 (*C*H$_2$ of linker), 28.06 (C-3'), 27.99 (C-3'), 27.70 (C-2), 25.57 (*C*H$_2$ of linker), 25.19 (*C*H$_2$ of linker), 25.09 (*C*H$_2$ of linker), 24.12 (*C*H$_2$ of linker). MALDI: *m/e* calcd for $C_{50}H_{97}N_{13}O_{18}H^+$, 1168.715; found = 1168.719 $[M + H]^+$

5-*O*-(Hexyne)-Tobramycin (4). Compound **4** was prepared by exposing compound 11 to TFA according to general procedure A. Yield (93 %). ^{1}H NMR (500 MHz, D_2O) δ 5.40 (d, *J* = 2.4 Hz, 1H, anomeric), 5.18 (d, *J* = 3.4 Hz, 1H, anomeric), 4.26 – 4.22 (m, 1H), 4.07 (t, *J* = 9.7 Hz, 1H), 3.97 – 3.71 (m, 11H), 3.61 – 3.51 (m, 2H), 3.48 – 3.44 (m, 1H), 3.44 – 3.29 (m, 3H), 2.49 – 2.45 (m, 1H), 2.45 – 2.40 (m, 1H), 2.33 – 2.25 (m, 3H), 2.25 – 2.16 (m, 1H, alkyne), 1.91 (m, 1H), 1.82 – 1.72 (m, 2H), 1.64 – 1.50 (m, 2H). ^{13}C NMR (126 MHz, D_2O) δ 101.3 (anomeric), 92.7 (anomeric), 81.9, 81.8, 76.6, 75.6, 73.5, 73.2, 68.6, 64.9, 63.3, 59.3, 54.8, 51.2, 49.8, 48.5, 47.4, 38.6, 29.4, 28.2, 28.0, 27.8, 26.2, 25.3, 24.8, 19.4. MALDI TOF-MS *m/e* calcd for $C_{24}H_{45}N_5O_9$, 547.3217; measured *m/e* 548.3219 $[M + H]^+$.

5-*O*-(4-Azidobutyl)-Tobramycin (5). Compound **5** was prepared by deprotecting compound **10a** according to general procedurę A. Yield (95 %). ^{1}H NMR (300 MHz, D_2O) δ 5.39 (d, J = 2.4 Hz, 1H, anomeric), 5.20 (d, J = 3.4 Hz, 1H, anomeric), 4.34 – 4.17 (m, 2H), 4.00 – 3.71 (m, 11H), 3.63 – 3.52 (m, 3H), 3.49 – 3.27 (m, 4H), 2.61 – 2.48 (m, 1H), 2.36 – 2.19 (2H), 2.11 – 1.93 (m, 1H), 1.81 – 1.56 (m, 4H). ^{13}C NMR (75 MHz, D_2O) δ 101.1 (anomeric), 92.6 (anomeric), 81.9, 81.7, 76.6, 75.7, 73.1, 72.7, 68.5, 64.8, 63.2, 59.3, 54.8, 51.0, 49.6, 48.4, 47.3, 38.5, 28.0, 27.7, 26.7, 24.6. MALDI TOF-MS *m/e* calcd for $C_{22}H_{44}N_8O_9$, 565.3231; measured *m/e* 565.35541 $[M + H]^+$.

Novobiocin Aglycone (6) was prepared as previously reported and NMR data were consistent with literature.[44]

1,3,2′,6′,3″-penta-*N*-Boc-4′,2″,4″,6″-tetra-*O*-TBDMS-tobramycin (8). Commercial tobramycin **7** (4.00 g, 8.56 mmol) was dissolved in a 2:1 mixture of methanol and water (150 mL) and treated with Boc_2O (14.25 g, 65.29 mmol) in the presence of Et_3N (8.0 mL, 57.4 mmol). The reaction mixture was stirred under reflux (at 55 °C) overnight (~ 20 h), concentrated under vacuo and thoroughly dried under high vacuum for 24 h to afford a white powdery solid (7.48 g, 90 %). The dried crude penta-*N*-boc-protected tobramycin (1.04 g, 1.07 mmol) was dissolved in anhydrous DMF (6.0 mL) and treated with *tert*-butyldimethysilyl chloride, TBDMSCl (1.13 g, 7.49 mmol) and *N*-methylimidazole (0.6 mL, 7.49 mmol). The reaction was stirred at RT for 4 days under nitrogen gas atmosphere, and the resulting mixture was poured into water (50.0 mL) and extracted with DCM (50 mL, ×3). The organic layer was dried over anhydrous Na_2SO_4, concentrated in *vacuo*, and purified by flash chromatography using gradient elution (hexanes/ ethyl acetate, 15:1 to 8:1, v/v) to afford **8** (1.05 g, 67%) as a white solid. NMR data are consistent with an earlier report.[13]

General Procedure B: 5-*O*-Alkylation of Boc and TBDMS protected Tobramycin for the Preparation of Compounds 9a–c. A solution of **8** (1 equiv.) in toluene was treated with KOH (3

equiv.), 1,*n*-dibromoalkane (3 equiv.), and a catalytic amount of tetrabutylammonium hydrogen sulphate, TBAHS (0.1 equiv.). The reaction mixture was stirred at RT overnight, dispersed in water and extracted with an equal volume of ethyl acetate (×3). The organic layers were combined, washed with brine (×1), dried over anhydrous Na_2SO_4 and concentrated *in vacuo*. The crude products were then purified by column chromatography (hexanes/ethyl acetate, 12:1 to 10:1, v/v). to afford compounds **9a-c** as white solids.

5-*O*-(n-Bromoalkyl)-1,3,2′,6′,3″-penta-*N*-Boc-4′,2″,4″,6″-tetra-*O*-TBDMS-tobramycin (9a–c). Compounds **9a**, **9b**, and **9c** were prepared by treating **8** with 1,4-dibromobutane, 1,6-dibromohexane, and 1,8-dibromooctane, respectively, according to general procedure B.

5-*O*-(4-Bromobutyl)-1,3,2′,6′,3″-penta-*N*-Boc-4′,2″,4″,6″-tetra-*O*-TBDMS-tobramycin (9a). Yield (51 %). ^{1}H NMR (300 MHz, $CDCl_3$) δ 5.24 – 5.12 (m, 2H, anomeric), 4.28 – 4.09 (m, 3H), 3.93 – 3.14 (m, 17H), 2.61 – 2.37 (m, 1H), 2.14 – 1.84 (m, 5H), 1.72 – 1.56 (m, 3H), 1.61 – 1.35 (m, 45H, *Boc*), 1.11 – 0.72 (m, 36H, TBDMS, *tert*-butyl), 0.24 – -0.09 (m, 24H, TBDMS –CH_3). ESI-MS: *m/z* calcd for $C_{71}H_{140}BrN_5O_{19}Si_4Na^+$, 1583.2; found 1583.2 $[M + Na]^+$.

5-*O*-(6-Bromohexyl)-1,3,2′,6′,3″-penta-*N*-Boc-4′,2″,4″,6″-tetra-*O*-TBDMS-tobramycin (9b). Yield (73 %). ^{1}H NMR (300 MHz, Chloroform-*d*) δ 5.24 – 5.07 (m, 3H), 5.05 – 4.95 (m, 2H), 4.17 – 4.00 (m, 3H), 3.79 – 3.60 (m, 5H), 3.60 – 3.26 (m, 11H), 3.24 – 3.15 (m, 2H), 2.47 – 2.32 (m, 1H), 1.98 – 1.88 (m, 1H), 1.82 – 1.69 (m, 3H), 1.54 – 1.24 (m, 52H), 0.96 – 0.73 (m, 36H), 0.15 – -0.10 (m, 24H). ^{13}C NMR (75 MHz, $CDCl_3$) δ 96.34, 85.70, 79.81, 79.29, 79.16, 79.11, 75.20, 73.04, 71.49, 68.03, 66.96, 63.09, 57.18, 50.50, 48.85, 48.27, 36.66, 35.67, 33.54, 33.32, 32.73, 30.32, 28.57, 28.43, 28.35, 27.19, 26.06, 25.93, 25.73, 25.23, 24.67, 18.40, 18.23, 18.02, 17.84, -3.54, -3.85, -4.26, -4.94, -4.98, -5.10, -5.21, -5.26. MALDI: Exact mass calcd for $C_{73}H_{144}BrN_5O_{19}Si_4Na^+$, 1608.861; found 1608. 886$[M + Na]^+$

5-*O*-(8-Bromooctyl)-1,3,2′,6′,3″-penta-N-Boc-4′,2″,4″,6″-tetra-*O*-TBDMS-tobramycin (9c). Yield (60 %). ^{1}H NMR (300 MHz, $CDCl_3$) δ 5.26 – 5.12 (m, 2H, anomeric), 4.39 – 3.97 (m, 3H), 3.89 – 3.07 (m, 16H), 2.47 (d, J = 12.8 Hz, 1H), 2.08 – 1.94 (m, 2H), 1.93 – 1.77 (m, 2H), 1.65 (m, 1H), 1.56 – 1.39 (m, 45H, *Boc*), 1.38 – 1.14 (m, 8H), 1.13 (m, 1H), 1.05 – 0.75 (m, 36H, TBDMS *tert*-butyl), 0.34 – -0.15 (m, 24H, TBDMS –CH_3). ^{13}C NMR (75 MHz, $CDCl_3$) δ 85.77, 79.42, 79.24, 57.27, 50.52, 34.06, 32.88, 32.83, 31.60, 30.67, 30.05, 29.67, 29.58, 29.47, 29.44, 28.82, 28.66, 28.52, 28.42, 28.22, 28.18, 26.16, 26.04, 26.01, 25.80, 18.52, 18.36, 18.12, 17.93, -3.38, -3.77, -4.17, -4.93, -5.06, -5.21. ESI-MS: *m/z* calcd for $C_{75}H_{148}BrN_5O_{19}Si_4Na^+$, 1636.89; found 1636.80 $[M + Na]^+$.

General Procedure C: Conversion of Bromoalkyl to Azide. A solution of bromoakylated compounds **9a-c** (1 equiv.) in anhydrous DMF was treated with sodium azide (10 equiv.) and stirred at 70 °C for 3 h. The resulting mixture was concentrated in *vacuo*, re-dispersed in water and extracted with ethyl acetate. The combined organic layers were subsequently washed with brine (×1), dried over anhydrous Na_2SO_4 and concentrated in *vacuo* to give yellow solids.

General Procedure D: Deprotection of Hydroxyl Groups (Removal of TBDMS Protecting Groups). A solution of TBDMS- and Boc-protected compounds in anhydrous THF (5.0 mL) were treated with tetrabutylammonium fluoride (TBAF, 6 equiv.) and stirred under nitrogen atmosphere for 2 h. The reaction mixture was concentrated under *vacuo*, dissolved in water and extracted with DCM (×3). The organic layers were combined, dried over anhydrous Na_2SO_4, concentrated *in vacuo*, and purified by column chromatography (hexanes/ethyl acetate, 1:1, v/v, then dichloromethane/methanol, 25:1 to 20:1, v/v) to afford off-white solids.

5-*O*-(*n*-Azidoalkyl)-1,3,2',6',3"-penta-*N*-Boc-tobramycin (10a-c). Compounds **10a-c** were prepared by converting **9a-c** to azido compounds, according to general procedure C, and subsequently removing the TBDMS protecting groups according to general procedure D.

5-*O*-(4-Azidobutyl)-1,3,2',6',3"-penta-*N*-Boc-tobramycin (10a). Overall yield (45 %). ^{1}H NMR (300 MHz, $CDCl_3$) δ 5.35 – 5.27 (m, 1H), 5.19 – 5.09 (m, 1H), 3.98 – 3.59 (m, 12H), 3.55 – 3.35 (m, 4H), 3.35 – 3.19 (m, 3H), 2.24 – 2.05 (m, 2H), 1.73 – 1.58 (m, 5H), 1.55 – 1.33 (m, 45H), 1.31 – 1.12 (m, 4H). ESI-MS: *m/z* calcd for $C_{47}H_{84}N_8O_{19}Na^+$, 1087.58; found 1087.61 $[M + Na]^+$

5-*O*-(6-Azidohexyl)-1,3,2',6',3"-penta-*N*-Boc-tobramycin (10b). Overall yield (65 %). ^{1}H NMR (300 MHz, $CDCl_3$) δ 5.33 – 5.12 (m, 3H), 4.31 – 4.09 (m, 1H), 3.96 – 3.53 (m, 12H), 3.48 – 3.33 (m, 3H), 3.31 – 3.05 (m, 4H), 2.22 – 2.01 (m, 2H), 1.76 – 1.46 (m, 6H), 1.46 – 1.08 (m, 52H). ^{13}C NMR (75 MHz, $CDCl_3$) δ 80.59, 80.01, 79.40, 78.06, 73.25, 72.42, 70.23, 64.99, 62.08, 56.52, 52.33, 51.31, 49.10, 33.12, 29.66, 28.67, 28.47, 28.40, 28.33, 26.68, 25.21, 20.07, 13.53. ESI-MS: *m/z* calcd for $C_{49}H_{88}N_8O_{19}Na^+$,1116.3; found 1116.9 $[M + Na]^+$

5-*O*-(8-Azidooctyl)-1,3,2',6',3"-penta-*N*-Boc-tobramycin (10c). Overall yield (57 %). ^{1}H NMR (300 MHz, $CDCl_3$) δ 5.49 – 5.35 (m, 1H), 5.25 – 5.11 (m, 1H), 3.88 – 3.30 (m, 16H), 3.23 – 3.02 (m, 4H), 2.14 – 1.90 (m, 2H), 1.73 – 1.04 (m, 62H). ^{13}C NMR (75 MHz, $CDCl_3$) δ 158.77, 157.88, 155.70, 155.30, 155.09, 96.60, 80.36, 79.83, 79.50, 79.20, 73.21, 70.31, 64.94, 61.95, 56.33, 53.42, 51.30, 48.99, 32.98, 29.76, 29.53, 29.38, 28.94, 28.68, 28.44, 28.34, 28.27, 28.03, 26.49, 25.53. MALDI: *m/e* calcd for $C_{51}H_{92}N_8O_{19}Na^+$, 1143.638; found 1143.659 $[M + Na]^+$

5-*O*-Hexyne-1,3,2',6',3"-penta-*N*-Boc-tobramycin (11). Compound **11** was prepared by reacting **8** with 6-iodohexyne, according to general procedure B, followed by TBDMS deprotection following general procedure D. Yield (30 %). ^{1}H NMR (500 MHz, $CDCl_3$) δ 5.31 – 5.23 (m, 2H), 5.15 – 4.97 (m, 2H), 3.95 – 3.50 (m, 14H), 3.48 – 3.34 (m, 4H), 3.29 – 3.20 (m, 1H), 3.18 – 3.06 (m, 1H), 2.24 – 2.06 (m, 4H), 1.97 – 1.93 (m, 1H, alkyne), 1.73 – 1.62 (m, 3H), 1.60 – 1.31 (m, 49H), 1.27 – 1.20 (m, 2H). MALDI: *m/e* calcd for $C_{49}H_{85}N_5O_{19}Na^+$, 1070.574; found 1070.596 $[M + Na]^+$

General Procedure E: Copper(1)-catalyzed azide-alkyne cycloaddition reaction ("Click Chemistry") for the Preparation of compounds 12a-c. Compounds **10a-c** (2 equiv.) and **11** (1 equiv.) were dissolved in an anhydrous DMF (4.0 mL) and treated with CuI·P(OEt)$_3$ (3 equiv.) and *i*Pr$_2$NEt (3 equiv.) The reaction was stirred under nitrogen gas for 2 h. The reaction mixture was concentrated under *vacuo*, dissolved in water and extracted with DCM (×3). The organic layers were combined, dried over anhydrous Na_2SO_4, concentrated *in vacuo*, and purified by column chromatography (dichloromethane/methanol, 40:1 to 20:1, v/v) to afford compounds **12a-c** (38 – 55 %) as white solids.

5-*O*-Alkylated-1,3,2',6',3"-penta-*N*-Boc-tobramycin homodimers (12a-c). Compounds **12a-c** were prepared via a copper(1)-catalyzed azide-alkyne cycloaddition reaction between **10a-c** and **11**, according to general procedure E.

Compound 12a. Yield (38 %). ^{1}H NMR (300 MHz, $CDCl_3$) δ 5.39 – 4.99 (m, 4H), 4.32 (s, 2H), 3.88 – 3.33 (m, 27H), 3.15 (m, 2H), 2.87 – 2.52 (m, 2H), 2.45 – 2.02 (m, 5H), 2.03 – 1.81 (m, 1H), 1.66 – 1.05 (m, 103H). MALDI: *m/e* calcd for $C_{96}H_{169}N_{13}O_{38}Na^+$, 2135.159; found 2135.169 $[M + Na]^+$

Compound 12b. Yield (55 %). ^{1}H NMR (300 MHz, $CDCl_3$) δ 5.38 – 4.98 (m, 4H), 4.42 – 4.21 (m, 1H), 4.02 – 3.24 (m, 25H), 3.23 – 2.88 (m, 2H), 2.78 – 2.56 (m, 1H), 2.32 – 2.07 (m, 2H), 1.98 – 1.82 (m, 2H), 1.69 – 0.88 (m, 106H). MALDI: *m/e* calcd for $C_{98}H_{173}N_{13}O_{38}Na^+$, 2164.514; found 2164.519 $[M + Na]^+$

Compound 12c. Yield (42 %). ^{1}H NMR (500 MHz, $CDCl_3$) δ 7.37 (s, 1H), 5.28 – 5.11 (m, 5H), 4.28 (t, J = 6.5 Hz, 2H), 4.13 (s, 2H), 3.93 – 3.25 (m, 34H), 3.20 – 3.00 (m, 2H), 2.80 – 2.63 (m, 2H), 2.48 (s, 3H), 2.29 – 1.98 (m, 4H), 1.93 – 1.77 (m, 2H), 1.71 – 1.04 (m, 108H). ^{13}C NMR (125 MHz, $CDCl_3$) δ 158.83, 157.95, 155.99, 155.24, 155.17, 121.36, 96.64, 80.49, 80.04, 79.42, 79.38, 73.16, 70.88, 70.58, 65.21, 56.47, 53.39, 50.59, 50.09, 49.26, 49.15, 40.88, 33.20, 31.89, 29.66, 29.19, 28.47,

28.44, 28.42, 28.39, 28.34, 25.16, 24.97, 22.65, 14.08. MALDI: *m/e* calcd for $C_{100}H_{177}N_{13}O_{38}Na^+$, 2191.222; found 2191.233 $[M + Na]^+$

Microbiology. Bacteria isolates were either obtained from the American Type Culture Collection (ATCC), the Canadian National Intensive Care Unit (CAN-ICU) surveillance study[50] or the Canadian Ward (CANWARD) surveillance study[51,52], and were categorized, where appropriate, as multidrug resistant, extensively drug-resistant or pan-drug resistant.[53]

Antimicrobial Susceptibility Assay. The *in vitro* antimicrobial activity of all compounds/antibiotics was evaluated in accordance with the Clinical and Laboratory Standards Institute (CLSI) guidelines (using ATCC strains as quality control strains),[54] as previously reported.[49]

Checkerboard Assay. Combination studies with different antibiotics were performed in 96-well plates as previously described.[19]

Cytotoxicity Assay. Cytotoxicity against human embryonic kidney cells (HEK293) and HepG2 cells was evaluated as previously reported,[49] and interpreted as earlier described.[21,55]

Hemolytic Assay. The hemolytic activities of the newly synthesized tobramycin homodimers were determined and quantified as the amount of hemoglobin released by lysing porcine erythrocytes following previously reported protocols.[49]

Time-kill Assay. Time-kill curve analyses were performed following established protocols.[48]

Outer Membrane Permeabilization Assay. The ability of the newly synthesized and reference compounds (compound **1**, PMBN and tobramycin) to permeabilize the outer membrane of *P. aeruginosa* PAO1 was assessed using the nonpolar membrane-impermeable fluorescent probe 1-*N*-phenylnapthylamine (NPN).[40] Briefly, an overnight grown *P. aeruginosa* PAO1 culture was subcultured (1 in 100) in fresh LB broth and grown to a mid-logarithmic phase (approximately 2 h, OD_{600} = 0.5 – 0.6). The cells were harvested by centrifuging for 10 min at 1000 *g* and room temperature, washed twice in PBS, and resuspended in half volume of PBS. This suspension was used in standard microtiter plate assay. Care was taken not to cool the suspension at any stage, since cooling the bacterial suspension below room temperature (i.e. during refrigerated centrifugation) could cause considerable increase in initial NPN uptake levels.[40] To a black 96-well plate containing the cell culture was added NPN (10 μM final concentration), alone and in combination with various concentrations of test compounds. The resulting change in NPN fluorescence was measured immediately and continuously (every 30 secs) for 10 mins, with intermittent shaking, on a FlexStation 3 (Molecular Devices, Sunnyvale, USA) microplate reader at an excitation wavelength of 350 nm and emission wavelength of 420 nm. PMBN, a known outer membrane permeabilizer, served as a positive control while cells + NPN (without test compounds) served as a negative control. Four independent replicates were conducted, and the data were corrected for any background fluorescence.

Development of Resistance Study. The ability of novobiocin + compound **1** to suppress resistance development was determined by serial passaging, as previously described.[19,56] Briefly, wild-type *A. baumannii* ATCC 17978 cells were grown in 1 mL MHB media containing novobiocin (at ¼ MIC, ½ MIC, 1× MIC, 2× MIC, and 4× MIC), alone and in combination with compound **1**. Tobramycin and

colistin were included as controls. At 24-hour intervals, the cultures were assessed for growth. Cultures from the second highest concentrations that allowed visible growth were harvested and diluted to 0.5 McFarland in sterile PBS, followed by 1:50 dilution into fresh MHB media containing ¼ MIC, ½ MIC, 1× MIC, 2× MIC, and 4× MIC of each antibiotic. This serial passaging was repeated for 8 days. For novobiocin/compound **1** combination, the concentration of compound **1** was kept constant at 7.1 μM throughout the experiment. For cultures that grew at higher than the MIC levels, cultures in highest concentrations that permit growth were passaged on drug-free LB plates and MICs were determined by microbroth dilution in MHB.

***Galleria mellonella In vivo* Larvae–Infection Model.** *In vivo* synergistic effects were determined using *Galleria mellonella* infection model, as previously described.[49] Briefly, larvae were purchased from The Worm Lady® Live Feeder (ON, Canada), stored in their natural habitat at 16 °C, and used within 10 days of delivery. The larvae (average weight of 250 mg) were used for tolerability and efficacy studies. Tolerability study was performed by injecting a 10 μL aliquot of antimicrobial agents only at concentrations equivalent to 100 mg/kg or 200 mg/kg. The larvae (ten in each group) were incubated at 37 °C and monitored for 96 h. For efficacy studies, the virulence and bacterial load required to kill 100 % of the larvae within 24 h (with no treatment) was first determined, which is approximately 10 CFU. Overnight grown culture of respective MDR *A. baumannii* isolate was standardized to 0.5 McFarland standard and diluted in PBS to a final concentration of 10^3 CFU/mL. A 10 μL aliquot of this solution (~10 CFU) was injected into each larva and incubated for 3 h at 37 °C. After the 3 h challenge, larvae in monotherapy experimental groups (fifteen per group) were treated with a 10 μL aliquot solution (containing different concentrations) of novobiocin, PMBN, compound **1**, or PBS alone. The larvae in combination therapy groups were treated with novobiocin + compound **1** (25 + 25 mg/kg or 50 + 50

mg/kg) and novobiocin + PMBN (50 + 25 mg/kg). Larvae treated with 10 μL of PBS or high concentrations of test antibiotics served as negative and positive control, respectively. The larvae were incubated at 37 °C in Petri dishes lined with filter paper and scored for survivability every 6 h for up to 36 h. This experiment was repeated to give a total of 30 larvae in each case. Survival data curves were plotted using Kaplan-Meier survival analysis. Larvae were considered dead if they do not respond to touch.

ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website.

Elemental analysis, extended biological data, supplementary figures, NMR spectra (PDF)

Molecular formula strings (CSV)

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Author Contributions

TI and FS conceived the project. TI, DA, and HR were involved in chemical synthesis. TI performed all biological studies. GGZ and FS supervised the project. TI conducted *in vivo* experiments. TI wrote the manuscript. All authors agree with the final version of the manuscript.

Notes

The authors declare that a provisional patent USSN 62/849,264, has been filed on the contents of this paper.

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ABBREVIATIONS USED

FICI, Fractional Inhibitory Concentration Index; MDR, multidrug-resistant; OM, outer membrane; RND, Resistance Nodulation Division; XDR, extensively drug-resistant.

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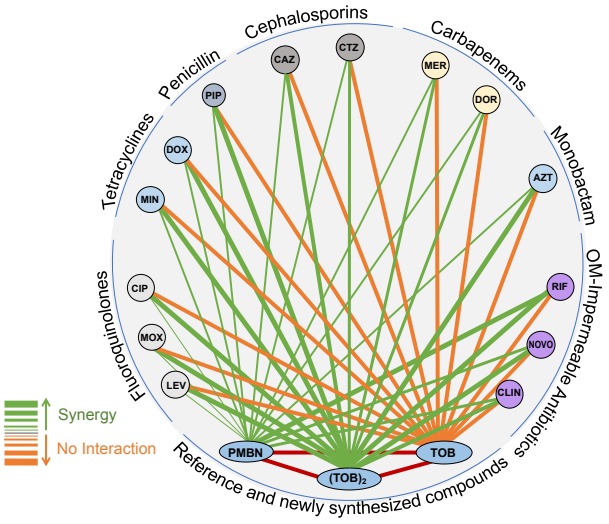
ToC Graphic


Cephalosporins
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Fluoroquinolones
Reference and newly synthesized compounds
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Synergy
No Interaction