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Hybrid Antibiotic Overcomes Resistance in *P. aeruginosa* by Enhancing Outer Membrane Penetration and Reducing Efflux

Bala Kishan Gorityala,[†] Goutam Guchhait,[†] Sudeep Goswami,[†] Dinesh M. Fernando,[#] Ayush Kumar,[#,§] George G. Zhanel,[§] and Frank Schweizer*[,†,§]

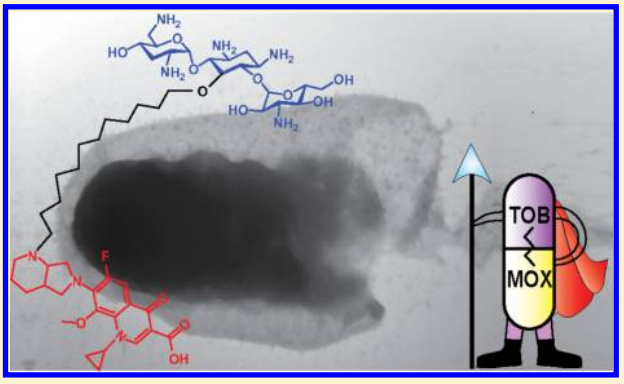
[†]Department of Chemistry, University of Manitoba, 144 Dysart Road, 450 Parker Building, Winnipeg, Manitoba R3T 2N2, Canada

[#]Department of Microbiology, University of Manitoba, Winnipeg, Manitoba R3T 2N2, Canada

[§]Department of Medical Microbiology, University of Manitoba, Winnipeg, Manitoba R3T 1R9, Canada

Supporting Information

ABSTRACT: Therapeutic interventions to treat multidrug-resistant (MDR) *Pseudomonas aeruginosa* infections are severely limited and often require the use of colistin as drug of last resort. The major challenges impeding the development of novel antipseudomonal agents are the lack of cell penetration and extensive efflux. We have discovered a tobramycin−moxifloxacin hybrid core structure which enhances outer membrane permeability and reduces efflux by dissipating the proton motive force of the cytoplasmic membrane in *P. aeruginosa*. The optimized hybrid protects *Galleria mellonella* larvae from the lethal effects of MDR *P. aeruginosa*. Attempts to select for resistance over a period of 25 days resulted in a 2-fold increase in the minimal inhibitory concentration (MIC) for the hybrid, while moxifloxacin or tobramycin resulted in a 16- and 512-fold increase in MIC. Although the hybrid possesses potent activity against MDR, *P. aeruginosa* isolates the activity that can be synergized when used in combination with other classes of antibiotics.



INTRODUCTION

The world is facing an enormous and growing threat from the emergence and dissemination of Gram-positive and Gram-negative bacteria that are resistant to almost all currently available antimicrobials,[1] but the problem is arguably most serious for Gram-negative bacilli, which are frequently multi-drug-resistant-MDR (resistant to ≥3 different antimicrobial classes) and for which no novel drug (or drug class) has been developed in over 30 years.[2] Among Gram-negative pathogens, *Pseudomonas aeruginosa* is the leading cause of nosocomial infections and chronic lung infections in cystic fibrosis patients, with mortality rates ranging from 30 to 50%.[3] In the United States, *P. aeruginosa* is the most common Gram-negative pathogen causing nosocomial pneumonia and it is frequently implicated in hospital-acquired urinary tract and bloodstream infections.[4,5] The Infectious Disease Society of America includes *P. aeruginosa* in its list of "ESKAPE" pathogens that pose the greatest public health threat due to a combination of increasing prevalence and ineffectiveness of existing antibacterial agents.[6] Infections caused by *P. aeruginosa* are particularly difficult to treat because the organism is both intrinsically resistant and capable of acquiring resistance to most antibiotics. This is in large part the result of the low permeability of its outer membrane, which is 12−100 times less permeable than that of *Escherichia coli* owing to its selective porins.[7] The intrinsic resistance mechanisms are part of the genetic makeup of *P. aeruginosa*, leading to very high baseline minimal inhibitory concentrations (MIC) and rendering many common antibiotics ineffective against *P. aeruginosa*. The reduced penetration of antibiotics across the outer membrane in *P. aeruginosa* enables secondary adaptive resistance mechanisms to function more efficiently.[8] These mechanisms include efflux as a result of intrinsic or induced expression of efflux pumps and enzymatic antibiotic modifications (e.g., β-lactamases). Besides intrinsic and adaptive resistance, *P. aeruginosa*, can further reduce susceptibility to antibacterials by the acquisition of inheritable traits such as horizontal transfer of genetic element and mutational resistance generally referred to as acquired resistance. As a result of intrinsic, adaptive, and acquired resistance the antibiotic arsenal to treat *P. aeruginosa* infections are limited to select penicillins (e.g., piperacillin/tazobactam), cephalosporins (e.g., ceftazidime), carbapenems (e.g., imipenem), fluoroquinolones (e.g., ciprofloxacin), aminoglycosides (e.g., tobramycin), and colistin, but resistance to these agents is steadily increasing and there are few novel antipseudomonal agents in clinical or preclinical development.[6,9]

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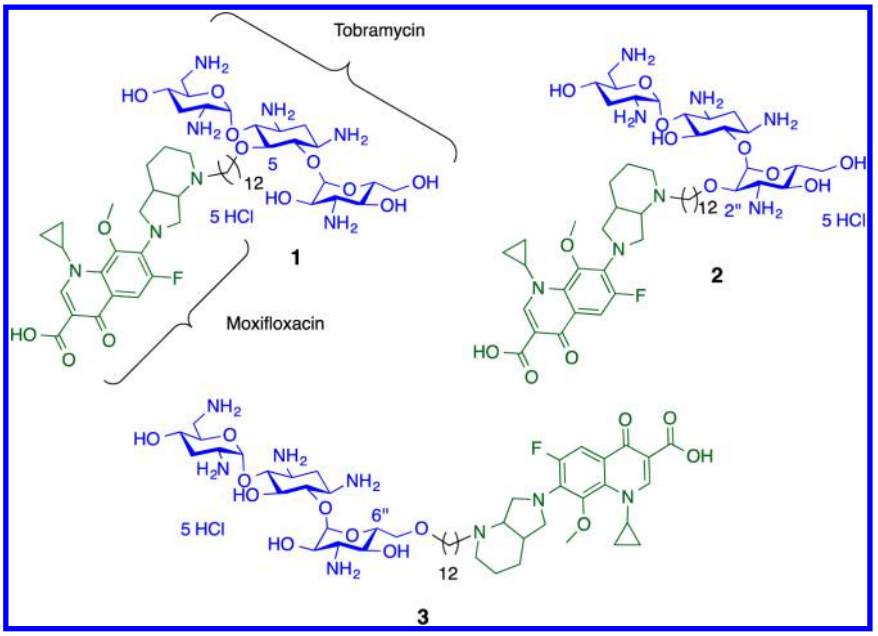

Figure 1. Overview of tobramycin−moxifloxacin hybrids used in this study. Hybrids differ in the linkage to tobramycin. In hybrid **1**, moxifloxacin is linked via a C12-tether to the C-5 position of tobramycin, while hybrids **2** and **3** are linked to the C-2″ and C-6″ positions in tobramycin.

Two strategies, multicomponent antibiotic adjuvants[10−12] and single component-based antibacterial polypharmacology,[11] are currently investigated to combat bacterial resistance. Both strategies have in common to exploit multiple modes of action. There is strong evidence that antibacterials need to interact with multiple targets and induce pleiotropic effects on the bacterial cell in order to be successful antibiotics as observed for β-lactams, fluoroquinolones, and aminoglycosides.[12,13] Multitargeting antibiotics are anticipated to limit the frequency of spontaneous resistance that can arise from mutation in the target gene.[13] For instance, it is quite likely that the rate of a spontaneous chromosomal mutation conferring reduced susceptibility or resistance to an antibiotic would be $\sim 1 \times 10^{-6}$ to 1×10^{-9} per target, depending on the concentration of the agent used. If the antibiotic is active against two independent targets in the organism, the chances of two independent spontaneous mutations occurring would be $1 \times 10^{-6\,(-9)} \times 1 \times 10^{-6(-9)}$, meaning the chances of resistance being conferred versus both targets through spontaneous mutation would be dramatically less likely occur. The expectation would be that one of the targets would still be active. Our hypothesis would be that even if an antibiotic acts on more than one target (e.g., two different unrelated targets) that an adaptive resistance mechanism (e.g., efflux) may confer reduced susceptibility or resistance to the multitarget antibiotic assuming that the efflux pump recognizes this new molecular entity and effluxes it out of the cell. Several approaches to develop multitargeting antibacterials have been utilized. These include the development of agents which target the bacterial cell membrane and the incorporation of additional binding sites into existing classes of antibiotics. Molecules which target bacterial membrane function intrinsically possess multimodal antibacterial properties.[12] This can be achieved through the interaction of a lipophilic moiety with the bacterial membrane, causing disruption of membrane architecture and functional integrity through steric or functional inhibition of membrane-embedded proteins and/or through alteration of the proton motive force which drives flagellar motility, protein excretion, export of toxic metabolites, and efflux.[12] In spite of this, traditional discovery efforts have not focused on the membrane as a target for antibiotic development, possibly owing to concern over the potential for these agents to disrupt the mammalian plasma membrane and the lack of knowledge regarding chemical optimization of such molecules to attain pathogen selectivity. However, the successful use of membrane-active antibiotics such as daptomycin, televancin, oritavancin, and dalbavancin to treat Gram-positive infections indicate that bacterial specificity is achievable.[14] Another approach to design multitargeting antibacterials involves the covalent attachment of two different pharmacophores that inhibit dissimilar targets in the bacterial cell-generating hybrid antibiotics.[15] Over the past 40 years, more than 25 hybrid antibiotics containing fluoroquinolone, aminoquinolone, vancomycin, rifamycin, oxazolidinone, β-lactam, and aminoglycoside moieties have been described. To date, four of them, a β-lactam-fluoroquinolone hybrid RO-23-9423, a fluoroquinolone−oxazolidinone hybrid MCB-3827, a rifamycin−fluoroquinolone hybrid CBR-2092, and a vancomycin−cephalosporin hybrid TD-1792, entered clinical phase studies with TD-1792 being the only one advancing to phase III.[15] Despite their composition of at least one pharmacophore entailing, excellent inherent Gram-negative activity, all clinically evaluated hybrid antibiotics are devoid of potent Gram-negative activity. The generally observed poor Gram-negative activity of hybrid antibiotics against Gram-negative bacteria and *P. aeruginosa* in particular has been attributed to reduced penetration of the outer membrane due to increased molecular weight.[13,15,16] There is mounting evidence that the Achilles' heel of synthetic compounds as new antibiotic drugs is their inability to penetrate readily the cell envelope of Gram-negative bacteria, which comprises inner and outer membrane, porins, and complex carbohydrate polymers, coupled with their susceptibility to active efflux by membrane-associated pumps.[17] Recently, we have shown that tobramycin−ciprofloxacin hybrids devoid of meaningful antibacterial activity nullify resistance (restore susceptibility) to fluoroquinolone antibiotics

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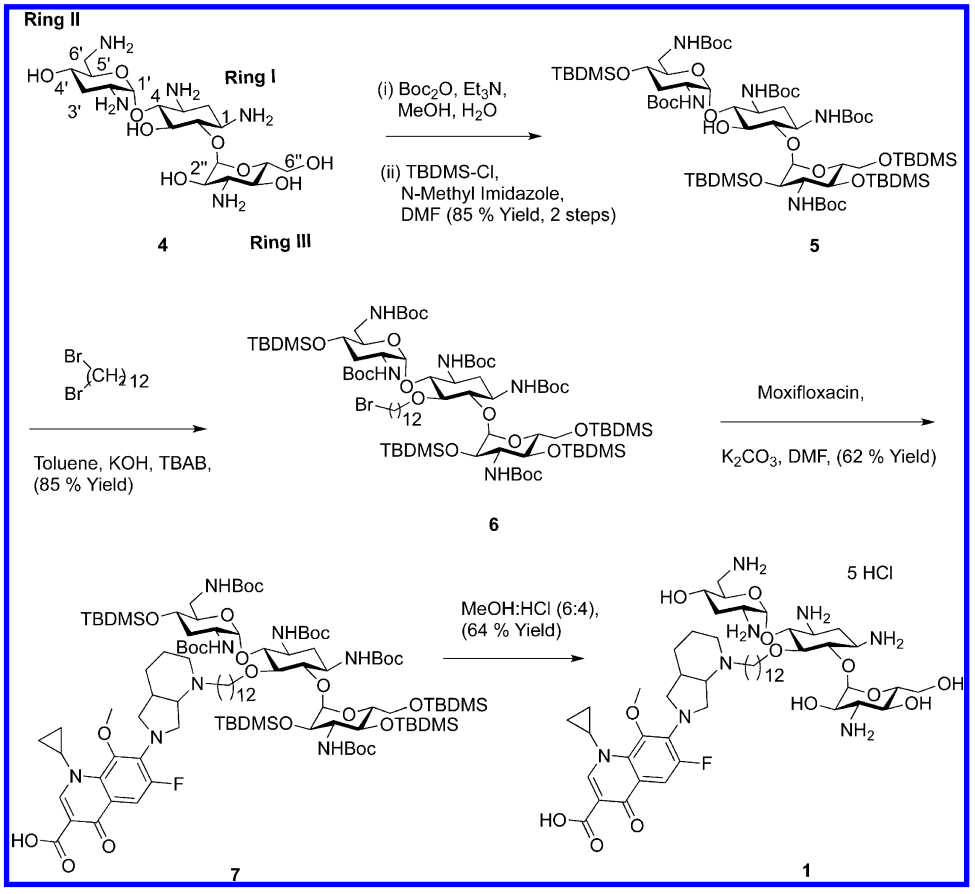
Scheme 1. Synthesis of Hybrid 1



in multidrug-resistant (MDR) *P. aeruginosa* isolates.[18] Mode of action studies indicated that the function of the hybrid is linked to outer membrane-destabilizing effects in *P. aeruginosa* while the poor antipseudomonal properties of the hybrid was caused in part by efflux. To further optimize the antipseudomonal properties of the tobramycin−ciprofloxacin hybrids, we now report on a new class of tobramycin−moxifloxacin hybrids with potent antipseudomonal activity against MDR, extremely drug-resistant (XDR) [resistant to cephalosporins (cefepime), carbapenems (meropenem and imipenem), fluoroquinolones (ciprofloxacin and moxifloxacin), and aminoglycosides (tobramycin and gentamicin) but susceptible to colistin], and pandrug-resistant (PDR) [XDR and resistant to colistin] *P. aeruginosa* isolates and low likelihood of resistance development. Moreover, we demonstrate that the potent antipseudomonal properties of the hybrid can be synergistically potentiated with other classes of antibiotics including fluoroquinolones, thereby lowering the absolute minimal inhibitory concentration (MIC) of the hybrid to <1 μg/mL against a panel of MDR, XDR, and PDR isolates from Canadian hospitals while at the same time reaching susceptible CLSI breakpoints for fluoroquinolone antibiotics.

RESULTS

Design and Synthesis. Design of the lead tobramycin−moxifloxacin hybrids (Figure 1) was established from the previous structure−activity relationships.[19] It has been reported that attachment of lipophilic groups at C-5 position of tobramycin retain antibacterial activities in Gram-negative bacteria.[19,20] The fourth-generation fluoroquinolone moxifloxacin was selected as a second pharmacophore as it possesses (a) potent antipseudomonal activity (MIC = 1 μg/mL) against wild-type *P. aeruginosa* PAO1, (b) is less prone to efflux than second- and third-generation fluoroquinolone antibiotics, and (c) interacts as a hydrophobic fluoroquinolone more strongly with the cytoplasmic membrane than ciprofloxacin. The secondary amino function in moxifloxacin was selected for point of modification as alkylation of this function retains potent antibacterial activity against bacterial pathogens.[21] A C-12 tether length was selected based on previous results obtained with the tobramycin−ciprofloxacin hybrid antibiotics that demonstrated that this tether length is optimal for outer membrane penetration and synergism with other classes of antibiotics in *P. aeruginosa*.[18] We also prepared hybrids **2** and **3** bearing a tether at the C-2″ and C-6″ positions to study how the nature of the tobramycin linkage affects the antibacterial activity. The synthetic strategy for the synthesis of hybrids **1**−**3** is outlined in Schemes 1, 2 and 3.

Chemical Synthesis of Tobramycin−Moxifloxacin Hybrid Linked to 5 Position of Tobramycin. Initially, all the amino groups in tobramycin **4** were Boc-protected by employing di-*tert*-butyl dicarbonate. Further, silylation of *N*-Boc-tobramycin with excess TBDMSCl afforded partially protected derivative **5** with free OH at 5 position in ring I.[19] Alkylation of **5** in toluene with 1,12-dibromododecane in the presence of KOH and phase transfer catalyst (TBAB) afforded bromoalkylated tobramycin **6**. Protected hybrid **7** was synthesized by directly coupling commercial moxifloxacin with bromoderivative of tobramycin **6** in anhydrous DMF.

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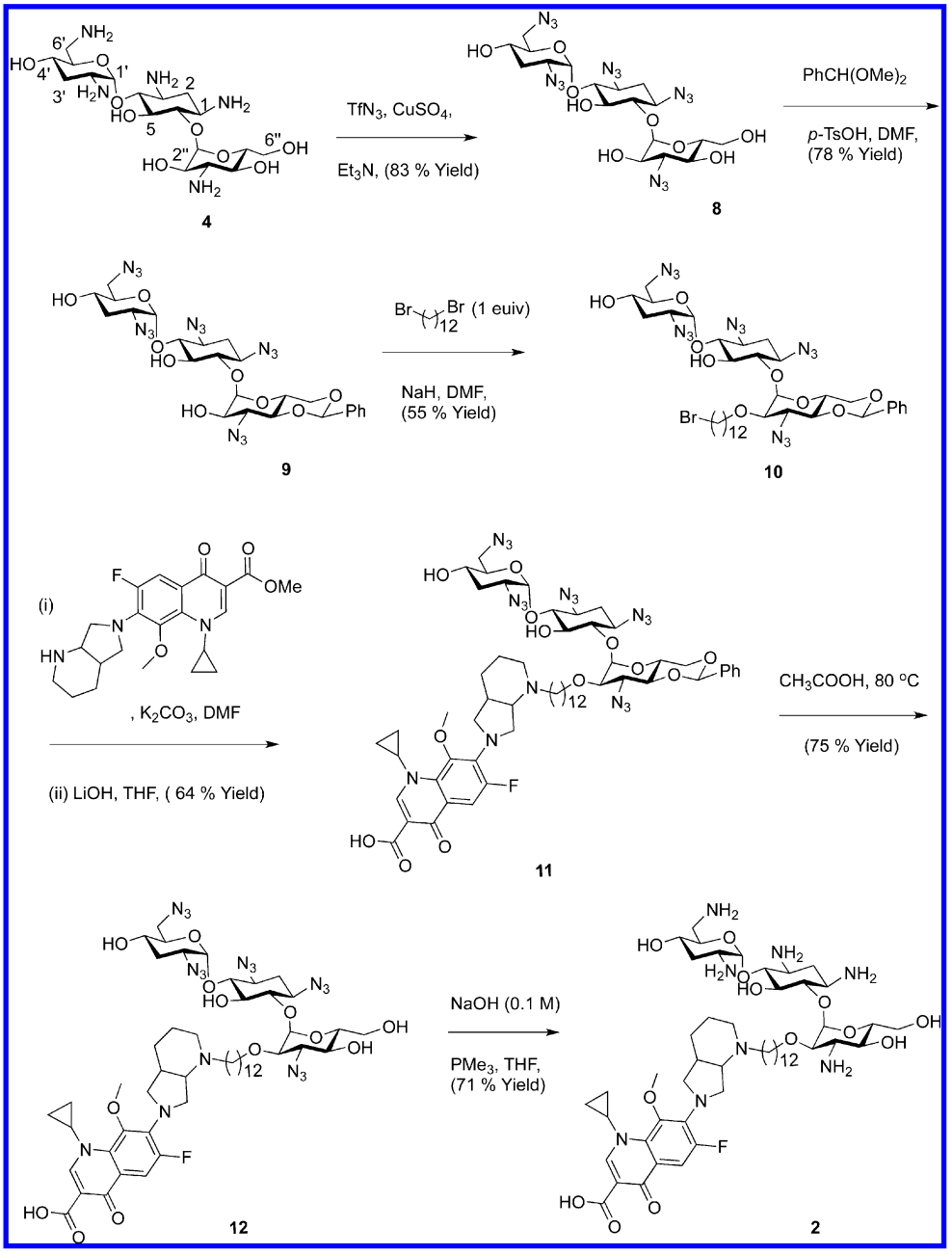
Scheme 2. Synthesis of Hybrid 2



Deprotection in methanolic HCl afforded analytically pure fused tobramycin−moxifloxacin hybrid **1** (Scheme 1).

Synthesis of Tobramycin−Moxifloxacin Hybrid Linked to 2″ Position of Tobramycin. Tobramycin upon treatment with triflylazide furnished per-azido derivative **8**.[22] Benzaldehydedimethylacetal assisted selective protection of 4″ and 6″ hydroxyl groups in ring III gave tobramycin analogue **9**.[23] Regioselective alkylation[24] at 2″ position was achieved by treating **9** with 1,12-dibromododecane in the presence of a base to afford monoalkylated derivative **10**. In the following step, tobramycin bromoderivative **10** was conjoined with moxifloxacin methyl ester to afford protected hybrid derivative **11**.[25] De-esterification followed by deprotection of benzylidene protection group resulted in derivative **12**, which was further subjected to Staudinger reaction[26] to yield final tobramycin−moxifloxacin hybrid **2** (Scheme 2).

Synthesis of Tobramycin−Moxifloxacin Hybrid Linked to 6″ Position of Tobramycin. Selective primary hydroxyl group protection of per-azido derivative **8** at 6″ position was achieved by TIPSCl to yield analogue **13**.[27] Treating this compound with *p*-methoxybenzyl (PMB)[24] ether subsequently furnished secondary hydroxyl groups protected tobramycin derivative **14**. Further selective deprotection of silylether[28] with the aid of TBAF resulted in compound **15**, which upon treatment with 1,12-dibromododecane/NaOH furnished 6″-appended bromododecyl derivative **16**. Ceric ammonium nitrate (CAN)[29] assisted deprotection of secondary hydroxyl groups provided derivative **17**. Protected hybrid analogue **18** was synthesized by reaction between moxifloxacin methyl ester and corresponding tobramycin bromo derivative **17** under basic conditions. De-esterification and subsequent Staudinger re-

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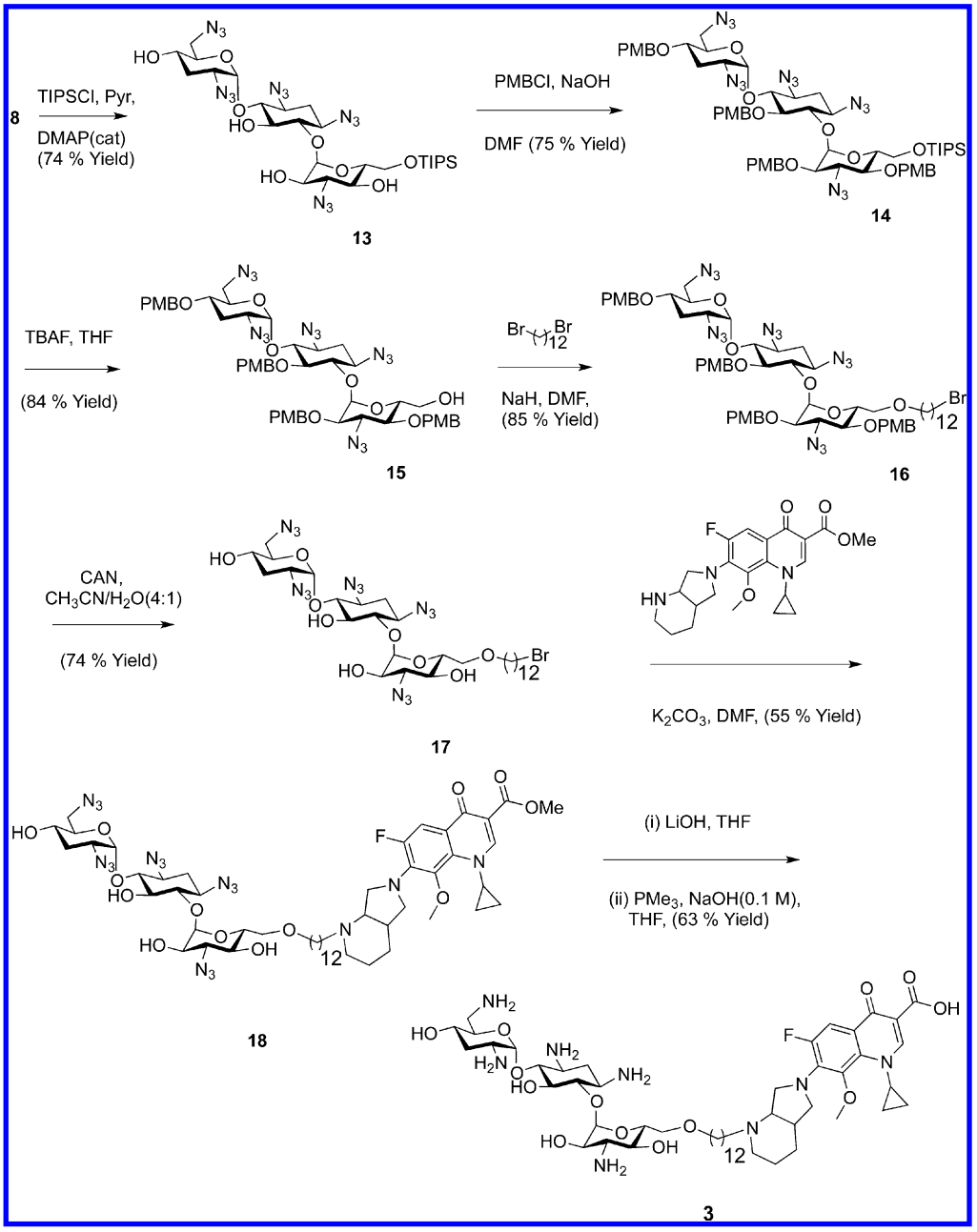
Scheme 3. Synthesis of Hybrid 3



action finally resulted in tobramycin−moxifloxacin hybrid **3** (Scheme 3).

Antibacterial Properties, Toxicity, And in Vivo Data. The hybrids **1**−**3** were tested for antibacterial activity by determining the minimal inhibitory concentration (MIC) against select Gram-negative and Gram-positive bacteria (Table 1). Among hybrids **1**−**3**, hybrid **1** showed consistently the highest antibacterial activity and displayed good activity (MIC = 1 μg/mL) against *Staphylococcus aureus* and methicillin-resistant *Staphylococcus aureus* (MRSA). Of special consideration is the potent activity of hybrid **1** against three *P. aeruginosa* strains (MIC = 4−8 μg/mL) including two gentamicin-resistant *P. aeruginosa* strains. Interestingly, hybrid **1** (MW = 1217.5) displays comparable antipseudomonal activity against these three *P. aeruginosa* strains than ciprofloxacin (MW = 367.8) when the changes in molecular weight are factored in. The promising antipseudomonal properties of hybrid **1** provided the rationale to study the antibacterial activity of hybrid **1** against a select panel of MDR and XDR *P. aeruginosa* isolates obtained from multiple Canadian hospitals which are resistant to tetracyclines (doxycycline), cephalosporins (cefepime), carbapenems (meropenem and imipenem), fluoroquinolones (ciprofloxacin and moxifloxacin), and aminoglycosides (tobramycin and gentamicin) but remain susceptible to colistin. We also included two PDR *P. aeruginosa* strains (no. 91433 and no. 101243) resistant to all known antipseudomonal agents including colistin (Table 2). Our results demonstrate that hybrid **1** possesses potent activity (MIC = 1−8 μg/mL) against a panel of eight MDR, XDR, and PDR *P. aeruginosa* isolates. In comparison, hybrids **2** and **3** in which the linker is attached to a different position in tobramycin are >8 times less active than hybrid **1**, indicating that the positioning of the tether in tobramycin is crucial for potent antibacterial activity. Similar MIC ranges (2−8 μg/mL)

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Table 1. Antibacterial Activity of Hybrids 1−3, Tobramycin (TOB), Moxifloxacin (MOX), and Ciprofloxacin (CIP) against Gram-Positive and Gram-Negative Pathogens

			MIC (μg/mL)			
test organism	**1**	**2**	**3**	TOB[c]	MOX	CIP[c]
S.aureus ATCC 29213	1	4	16	≤0.25	≤0.25	≤0.25
MRSA ATCC 33592	1	8	16	≤0.25	≤0.25	≤0.25
MSSE CANWARD-2008 81388	2	2	8	≤0.25	≤0.25	≤0.25
MRSE CAN-ICU 61589 (CAZ > 32)	16	8	8	1	64	128
E. faecalis ATCC 29212	4	32	32	8	≤0.25	1
E. faecium ATCC 27270	16	16	16	8	2	8
S. pneumoniae ATCC 49619	1	32	64	2	≤0.25	1
E. coli ATCC 25922	0.5	4	16	0.5	≤0.25	≤0.25
E. coli CAN-ICU 61714 (GEN-R)	4	64	64	8	0.5	≤0.25
E. coli CAN-ICU 63074 (AMK 32)	4	32	64	8	1	≤0.25
E. coli[a] CANWARD-2011 97615	128	128	128	128	32	256
P. aeruginosa ATCC 27853	4	16	64	0.5	4	1
P. aeruginosa CAN-ICU 62308 (GEN-R)	8	128	32	16	16	2
P. aeruginosa[b] CANWARD-2011 96846	4	>128	64	256	16	4
S. maltophilia CAN-ICU 62584	32	>128	>128	>512	4	32
A. baumannii CAN-ICU 63169	16	128	>128	32	1	2
K. pneumoniae ATCC 13883	1	4	16	≤0.25	≤0.25	≤0.25

[a](GEN-R, TOB-R, CIP-R) aac(3′)iia. [b](GEN-R, TOB-R) CIP = ciprofloxacin, TOB = tobramycin, GEN = gentamicin, AMK = amikacin. [c]TOB, CIP MIC values have been reported in ref 17.

Table 2. Antibacterial Activity MIC (μg/mL) of Hybrids 1−3 and Select Classes of Antibiotics against MDR, XDR, and PDR *P. aeruginosa* Isolates

	antibiotic (MW)											
clinical PA isolate[a]	DOX 444.4	CPM 480.6	MER 383.5	DOR 420.5	CIP 331.3	MOX 401.4	GEN 477.6	TOB 467.5	COL 1155.4	**1** 1217.5	**2** 1217.5	**3** 1217.5
100036	>32	4	4	5	64	128	>32	64	2	8	ND	ND
101885	>32	8	1	0.5	32	128	>0.5	<0.5	1	8	ND	ND
259-96918	>32	>64	>32	>32	256	512	>32	>64	1	2	>32	>32
260-97103	32	16	16	32	64	64	>32	32	1	1	>32	32
262-101856	>32	32	32	8	32	128	>32	>64	2	4	32	>32
264-104354	>32	32	>32	16	32	64	>32	>64	1	4	32	>32
91433	32	16	8	8	4	16	32	8	4	8	ND	ND
101243	8	64	16	16	2	8	>32	>64	1024	8	ND	ND
PAO1	ND	ND	ND	ND	<0.2	1	ND	0.5	1	4	>32	>32

[a]PA = *Pseudomonas aeruginosa*, DOX = doxycycline (tetracycline), CPM = cefepime (cephalosporin), MER = meropenem (penem), DOR = doripenem (penem), CIP = ciprofloxacin (fluoroquinolone), MOX = moxifloxacin (fluoroquinolone), GEN = gentamicin (aminoglycoside), TOB = tobramycin (aminoglycoside), COL = colistin, ND = not determined. All control antibiotic MIC values have earlier been reported in ref 17.

Table 3. Antibacterial Activity of Hybrids 1 against Tobramycin Susceptible *P. aeruginosa* Clinical Isolates[a]

	MIC (μg/mL)									
P. aeruginosa clinical isolate	**1**	CPM	CEFT	MER	MOX	IMI	CIP	GEN	TOB	COL
86052	2	16	>32	8	>16	32	>16	4	1	1
86053	4	64	>32	>32	16	16	>16	4	1	1
86067	4	64	>32	>32	16	>32	4	>32	>64	0.5
86079	4	32	>32	32	>16	16	>16	8	2	1
86141	4	16	>32	16	>16	16	8	8	1	1
92014	4	16	>32	8	16	8	4	4	1	1
108590	2	16	32	8	16	32	2	16	4	1
79199	8	32	32	16	>16	4	8	4	2	1
80621	8	8	>32	16	8	32	2	16	2	2
83182	8	16	>32	16	16	16	4	4	1	1
84745	8	16	>32	16	16	16	2	8	1	2
85322	8	16	>32	16	8	32	2	4	1	1

[a]**1** = hybrid **1**, CPM = cefepime, CEFT = ceftazidime, MER = meropenem, MOX = moxifloxacin, IMI = imipenem, CIP = ciprofloxacin, GEN = gentamicin, TOB = tobramycin, COL = colistin.

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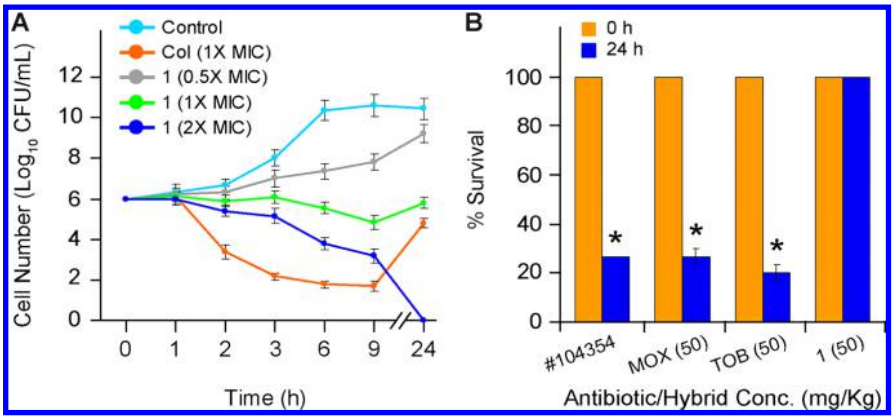

Figure 2. Effects of hybrid **1** on bacterial killing and in vivo efficacy. (A) Time-kill curves showing the effect of varying concentrations of hybrid **1** and MIC concentration of colistin (Col) on the viability of *P. aeruginosa* PAO1 cells grown in MH broth. No colony-forming units (CFU) were found with **1** at 2 × MIC (blue) after 24 h. Experiment was performed three times independently, and each data point is an average of three determinations ± SEM. (B) Enhanced dose dependent efficacy of hybrid **1** in comparison with tobramycin (TOB) and moxifloxacin (MOX) in XDR *P. aeruginosa* (no. 104354) over a period of 24 h was demonstrated in a *Galleria mellonella* in vivo infection model. Larvae infected with no. 104354 (10^6 CFU/mL) resulted in 73% killing after 24 h. In contrast, single dosage monotherapy (50 mg kg) of moxifloxacin or tobramycin or hybrid **1** resulted in 27%, 20%, or 100% survival of the larvae, respectively, after 24 h. No killing was observed due to physical trauma when larvae were injected with equal volumes of PBS. Two independent experiments were conducted for each dosage of antibiotic/hybrid, where each expermient involved 15 worms (n = 30). Significant differences between 0 and 24 h are indicated by * (p value ≤0.05).

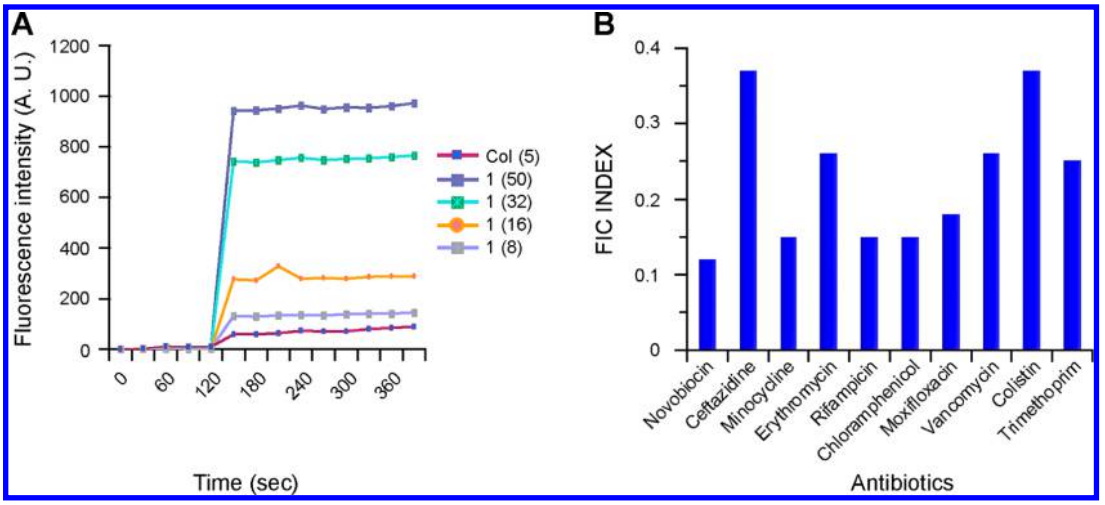

Figure 3. (A) Effects of hybrid **1** on the outer membrane. Concentration-dependent permeabilization of the outer membrane by tobramycin−moxifloxacin hybrid **1** is indicated by the accumulation of 1-*N*-phenylnaphthylamine (NPN) in *P. aeruginosa* PAO1 cells; 50 μg/mL (medium plum), 32 μg/mL (cyan), 16 μg/mL (gold), or 8 μg/mL (pale lavender) of hybrid **1** were used, along with 5 μg/mL colistin (Col) (pale-violet red) as positive control. Hybrid **1** was added at 120 s. Experiment was performed in triplicate, and each data point is an average of three determinations ± SEM. (B) Synergistic FIC index of hybrid **1** in combination with various classes of antibiotics against PAO1.

of hybrid **1** were also seen against a panel of 12 fluoroquinolone-resistant (ciprofloxacin-resistant and moxifloxacin-resistant) but tobramycin-susceptible MDR *P. aeruginosa* isolates from multiple Canadian intensive care units indicating potent activity against diverse *P. aeruginosa* phenotypes (Table 3). It is interesting to note that hybrid **1** performed poorly compared to moxifloxacin and tobramycin in wild-type *P. aeruginosa* PAO1 but at the same time was much more potent in the clinical isolates when compared to moxifloxacin and tobramycin. This suggests that the resistance mechanisms which deactivate moxifloxacin and tobramycin in *P. aeruginosa* do not apply to hybrid **1**. We also assessed whether the potent antipseudomonal properties of hybrid **1** can be extended to a panel of MDR *E. coli* and MDR *Acinetobacter baumanii* strains. These studies indicate that hybrid **1** did not display potent activity against *E. coli* (MIC ≥ 64 μg/mL) and *A. baumannii* (8−64 μg/mL) clinical isolates but in contrast retained potent activity against wild-type *E. coli* strains (MIC ≤ 2 μg/mL Table 1 and Supporting Information, Table S1). This likely reflects differences in pathogen-specific resistance mechanisms. It is likely that hybrid **1** primarily affects intrinsic resistance in *P. aeruginosa* as it relates to outer membrane permeability barrier and efflux which is less pronounced in *E. coli* and *A. baumannii*. After demonstrating that hybrid **1** possesses potent and selective antipseudomonal activity against diverse MDR and XDR *P. aeruginosa* pathogens, we then studied the killing kinetics of *P. aeruginosa* PAO1 and observed complete eradication of *P. aeruginosa* PAO1 at 2× MIC concentration over a 24 h time period (Figure 2A). Efficacy of hybrid **1** was demonstrated in a *Galleria mellonella* infection model. *Galleria mellonella* larvae is an established in vivo model to study the efficacy of antimicrobial therapy.[30,31] In pilot studies, we

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J. Med. Chem. 2016, 59, 8441−8455

determined that hybrid **1** causes <2.5% hemolysis of human erythrocytes at 1000 μg/mL (Supporting Information, Figure S1) and shows low cytotoxicity $CC_{50} \gg 30$ μM against cancer cell lines (Supporting Information, Figure S2). We assessed the tolerability of hybrid **1** in *Galleria mellonella* and did not see any toxic effects up to the maximal dose of 600 mg/kg over a period of 96 h (Supporting Information, Figure S3). Efficacy studies were performed by infecting the larvae with a dose (10^6 CFUs) of XDR *P. aeruginosa* strain no. 104354 (resistant to all classes of antipseudomonal agents except colistin) followed by injection of hybrid **1** or tobramycin or moxifloxacin at 2 h post infection. Therapy with a single dose of moxifloxacin (50 mg/kg) or tobramycin (50 mg/kg) or no drug resulted in 27%, 20%, or 27% survival of the larvae at 24 h, respectively, indicating that both antibiotics provide insufficient protection. In contrast, single dose therapy (50 mg/kg) of hybrid **1** resulted in 100% survival after 24 h (Figure 2B). We also observed enhanced long-term survival effects upon treatment with hybrid **1**. For instance, treatment of the infected larvae with a single dose (75 mg/kg) of moxifloxacin or tobramycin or hybrid **1** resulted in 7%, 0%, or 20% survival after a 96 h period, respectively. Survival rate of the larvae upon therapy with hybrid **1** was dose-dependent (Supporting Information, Figure S4).

Mode of Action Studies. To understand the protective function of hybrid **1**, we studied the membrane interactions of **1** with *P. aeruginosa* PAO1. Initially, we demonstrated that hybrid **1** permeabilizes the outer membrane of *P. aeruginosa* in a dose-dependent manner using the NPN (1-*N*-phenylnaphthylamine) assay (Figure 3A).[32] In addition, we also assessed whether the combination of hybrid **1** with other classes of antibiotics were additive or synergistic by using the fractional inhibitory concentration (FIC) index as a measure of the interaction between two antibacterial agents.[33] FIC indices of 1−2, ≤0.5, and ≥4 indicate no interaction, synergy, and antagonism, respectively.[34] We observed strong synergy of hybrid **1** with outer membrane impermeable agents interacting with intracellular targets including novobiocin (FIC index = 0.12), rifampicin (FIC index = 0.15), vancomycin (FIC index = 0.26), and erythromycin (FIC index = 0.26), indicating that hybrid **1** enhances cellular uptake of these agents into *P. aeruginosa*. Moreover, we also observed strong synergy with other classes of antibiotics including chloramphenicol (FIC index 0.15), minocycline (FIC index 0.15), moxifloxacin (FIC index = 0.18), trimethoprim (FIC index = 0.25), ceftazidime (FIC index = 0.37), and colistin (FIC index = 0.37) but not with meropenem (FIC index = 2), tobramycin (FIC index > 2), and gentamicin (FIC = 5) against *P. aeruginosa* PAO1 (Figure 3B and Supporting Information, Table S2). Importantly, hybrid **1** strongly synergizes with the fluoroquinolones ciprofloxacin and moxifloxacin against a panel of MDR and XDR *P. aeruginosa* isolates. For instance, ciprofloxacin-susceptible (MIC = 1 μg/mL) CLSI break points were reached for 6/6 ciprofloxacin-resistant, MDR, XDR, or PDR *P. aeruginosa* isolates at 0.25 × MIC of hybrid **1** (≤2 μg/mL). In comparison, the same susceptible breakpoints were reached for moxifloxacin in 5/6 moxifloxacin-resistant, MDR, XDR, or PDR isolates, indicating that hybrid **1** strongly synergizes with both clinically used fluoroquinolone antibiotics (Table 4). At the same time, the presence of ciprofloxacin or moxifloxacin at or below their respective 0.25 × MIC (≤4 μg mL) synergizes hybrid **1** to lower its MIC < 1 μg/mL for the six tested MDR, XDR, and PDR *P. aeruginosa* isolates (Table 5).

Table 4. Effect of Fixed Concentration of Hybrid 1 (≤2 μg/mL; ≤0.25 × MIC) on MIC of Ciprofloxacin (CIP) or Moxifloxacin (MOX)

	MIC (μg/mL)			
clinical PA isolate	CIP	absolute MIC[a]	MOX	absolute MIC[b]
PA259-96918	256	<1	512	<1
PA260-97103	16	1	32	1
PA262-101856	16	1	64	2
PA264-104354	16	1	32	1
91433[c]	8	0.25	8	0.12
101243[c]	2	0.06	8	0.06

[a]MIC of ciprofloxacin when <0.25 × MIC (≤2 μg/mL) of **1** was used. [b]MIC of moxifloxacin when <0.25 × MIC (≤2 μg/mL) of **1** was used. [c]PDR strain resistant to all antipseudomonal classes including colistin.

Table 5. Effect of Fixed Concentration of Ciprofloxacin (≤4 μg/mL; ≤0.25 × MIC) or Moxifloxacin (≤4 μg/mL; 0.25 × MIC) on MIC of Hybrid 1

	MIC (μg/mL)		
clinical PA isolate	**1**[a]	absolute MIC[b]	absolute MIC[c]
PA259-96918	2	<0.5	<1
PA260-97103	1	<0.25	0.25
PA262-101856	4	<0.5	<1
PA264-104354	4	<0.5	<1
91433[d]	8	<1	<1
101243[d]	8	<1	<1

[a]**1** = hybrid **1**. [b]MIC of 1 when ≤0.25 × MIC (≤4 μg/mL) of ciprofloxacin was used. [c]MIC of **1** when ≤0.25 × MIC (≤4 μg/mL) of moxifloxcin was used. [d]PDR strain resistant to all antipseudomonal classes including colistin.

Next, we assessed the ability of hybrid **1**, moxifloxacin, and tobramycin to depolarize the cytoplasmic membrane in *P. aeruginosa* PAO1 using the membrane potential-sensitive dye bis(1,3-dibutybarbituric acid) trimethine oxonol.[35] Our results demonstrate that the tobramycin−moxifloxacin hybrid **1** induces dose-dependent depolarization of the cytoplasmic membrane in a comparable manner to colistin (Figure 4A,B). In contrast, moxifloxacin was not able to induce membrane depolarization while tobramycin showed weak dose-dependent cytoplasmic membrane depolarization (Figure 4C,D).

These results indicate that the cytoplasmic membrane destabilizing effects of tobramycin can be enhanced by linking it to moxifloxacin. Moreover, flagellum-dependent swimming motility of *P. aeruginosa* PAO1 was strongly reduced in a concentration-dependent manner at sub-MIC concentration of hybrid **1** (Figure 5, and Supporting Information, Figure S5). As flagellar function requires an intact proton motive force (PMF),[36] our results suggest that hybrid **1** dissipates the PMF. Moreover, as the PMF drives the function of RND-based efflux pumps in *P. aeruginosa*,[8] the observed synergistic effects of sublethal hybrid **1** with efflux susceptible antibiotics such as fluoroquinolones likely reflect reduced efflux. The effects of hybrid **1** on the PMF are consistent with the observed antagonistic effect of hybrid **1** with the aminoglycoside gentamicin and additive effects with tobramycin in *P. aeruginosa* PAO1 as aminoglycosides require the electrical component ($\Delta\Psi$) of the intact PMF for cellular uptake (Supporting Information, Table S2).[37]

This suggests that hybrid **1** dissipates the electrical component ($\Delta\Psi$) of the proton motive force at sublethal

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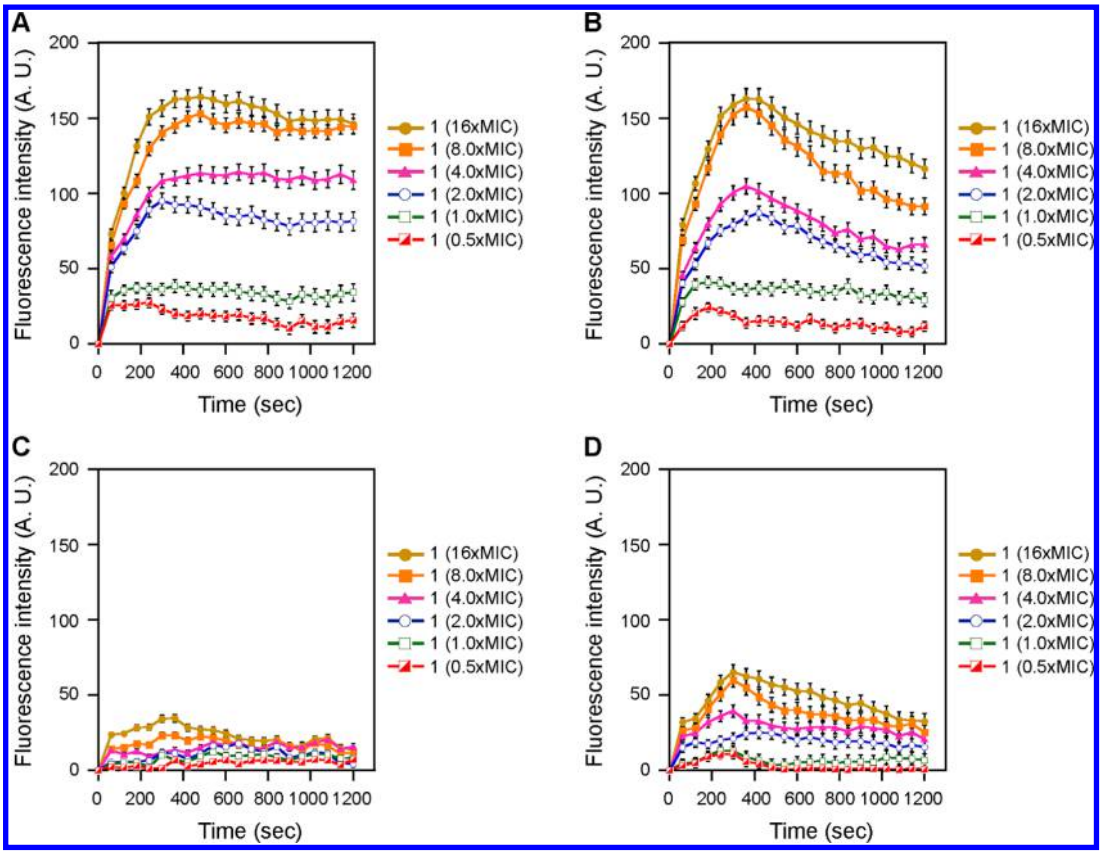

Figure 4. Cytoplasmic membrane depolarization assay ascertained by $DiSC_3$ fluorescence in *P. aeruginosa* PAO1. Comparative membrane depolarization induced by varying concentrations of (A) hybrid **1** (MIC = 4 μg/mL), (B) colistin (MIC = 1 μg/mL), (C) moxifloxacin (MIC = 1 μg/mL), and (D) tobramycin (MIC = 0.5 μg/mL). Each data point represents ± SEM from three independent samples.

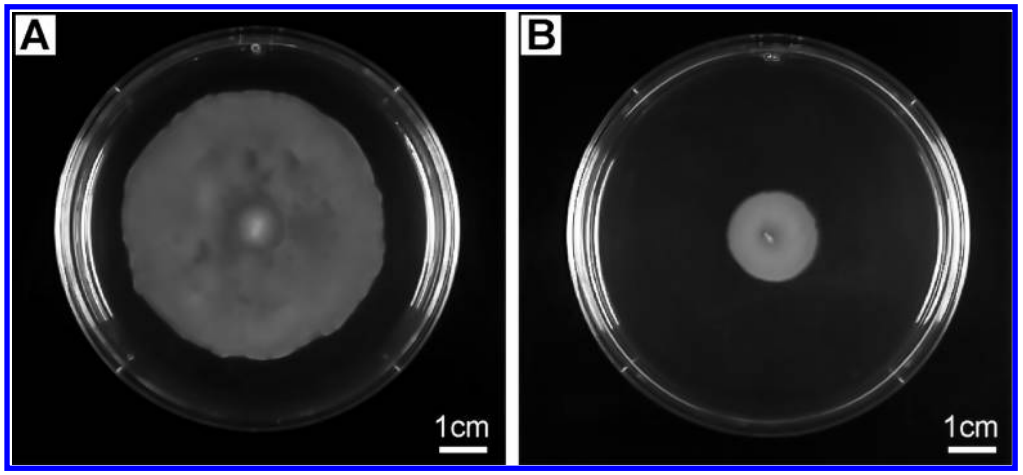

Figure 5. Motility of *P. aeruginosa* PAO1. Motile phenotype of cells in swim plates when inoculated to an optical density (600 nm) of 1.0. (A) Swim plates with no drug. (B) Plate containing 1/16 × MIC hybrid 1 (0.25 μg/mL). Swim plates were prepared using 0.3% agar, dried, and solidified for 60 min under laminar flow. Images were taken after 20 h incubation at 37 °C.

concentration. The effect of hybrid **1** on the PMF may suggest that the hybrid alters the function of RND efflux pumps. We therefore determined the MIC of hybrid **1** and moxifloxacin in two efflux-deficient strains. We tested a MexAB-OprM deletion strain (PAO200), as well as an efflux-sensitive strain (PAO750)[38,39] that lacks five different clinically relevant RND pumps (MexAB-OprM, MexCD-OprJ, MexEF- OprN, MexJK, and MexXY) and the outer membrane protein OpmH for potentiation of antibiotic activity by hybrid **1**. As expected, the antibacterial activity of moxifloxacin is greatly affected by the absence of RND pumps, leading to a 4- and 128-fold reduction of the MIC in the efflux-deficient in PAO200 and PAO750 strains (Table 6). In contrast, the MIC of hybrid **1** was not affected by the absence of the MexAB-OprM pump and showed only an 8-fold reduction in the PAO750 strain, indicating that hybrid **1** strongly resists efflux by *P. aeruginosa* RND pumps.

To assess the effects of hybrid **1** on both outer- and inner-membrane permeability barrier, we used the membrane impermeable fluorescent dye PI which stains nucleic

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Table 6. Antibacterial Activity of Moxifloxacin and Hybrid 1 in Efflux Pump Deletion Strains Derived from *P. aeruginosa* PAO1

	MIC (μg/mL)	
P. aeruginosa strains	moxifloxacin	hybrid **1**
PAO1	1	4
PAO200[a]	0.25	4
PAO750[b]	0.0078	0.5

[a]PAO1 strain deficient in MexAB-OprM. [b]PAO1 strain deficient in (MexAB-OprM, MexCD-OprJ, MexEF-OprN, MexJK and MexXY) and the outer membrane protein OpmH.

acids.[40,41] Cells grown in the presence of growth-inhibitory amounts of hybrid **1** (8 μg/mL) could be uniformly stained with intracellular dye while cells in the absence of hybrid **1** did not show fluorescence, indicating that hybrid **1** enables internalization of PI (Figure 6).

We also examined the cellular morphology of *P. aeruginosa* PAO1 treated with 8 μg/mL (2 × MIC) hybrid **1** in Mueller–Hinton (MH) broth for a period of 4 h by transmission electron microscopy.[42] The obtained images indicate significant outer membrane damage and blebbing (Figure 7) that can cause membrane rupture, cytosolic leakage, and cell death (Supporting Information, Figure S6).

Finally, we assessed whether the hybrids retain their original modes of action. In a protein translation assay, hybrids **1** and **2** showed a >750-fold and >20-fold reduction in activity, respectively, when compared to tobramycin (Figure 8A). Similarly, hybrid **1** was a 20-fold less potent inhibitor of gyrase A when compared to moxifloxacin while hybrids **2** and **3** displayed equipotency than moxifloxacin (Figure 8B). These studies indicate that the predominant, original antibacterial effects of tobramycin and moxifloxacin are greatly reduced in hybrid **1**. Next, we studied the potential of hybrid **1** to select for resistance. We used a procedure of selective pressure in which *P. aeruginosa* PAO1 was exposed to subinhibitory (0.5 × MIC) concentrations of moxifloxacin, tobramycin, and hybrid **1** during 24 successive subcultures (Figure 8C).[43] As can be seen from the data, the relative MIC values of tobramycin, moxifloxacin, and hybrid **1** increased by 512-, 16-, and 2-fold, respectively, against PAO1, indicating that hybrid **1** strongly delays resistance development when compared to moxifloxacin and tobramycin over the 25 day period of this experiment. The delayed resistance of hybrid **1** is consistent with other classes of membrane-active antibacterials which generally display reduced likelihood of resistance development.[12]

DISCUSSION

Antimicrobial resistance is a growing threat to global public health. Hospital-acquired MDR and XDR *P. aeruginosa* infections remain one of the most challenging infections to treat. Antibacterial researchers have struggled to identify new small molecules with meaningful activity against MDR *P. aeruginosa* infections. The difficulty stems from an incomplete understanding of efflux systems and compound permeation through inner and outer membranes in *P. aeruginosa*.[44] To enhance membrane permeability, we sought to exploit the outer membrane penetrating properties of tobramycin against *P. aeruginosa*. Tobramycin is one of the most potent antipseudomonal agents and exerts pleiotropic effects on the bacterial cell. At low concentrations (<4 μg/mL), tobramycin binds to the 30S ribosomal subunit, thereby leading to the disruption of protein synthesis while at higher concentrations (>8 μg/mL) destabilization of the outer membrane is observed.[45] This involves interactions at sites where divalent cations cross-bridge adjacent polyanionic lipopolysaccharide (LPS) that cause destabilization of the outer membrane and result in self-promoted uptake of the antibiotic or other extracellular molecules.[46] Using tobramycin as an outer membrane transport vehicle, we linked it at positions 5, 2″, and 6″ to the secondary amino function of the fluoroquinolone moxifloxacin which inhibits two type II DNA topoisomerase enzymes, DNA gyrase, and topoisomerase IV that play an essential role in DNA relaxation, the partitioning of replicated chromosomal DNA during cell division and decatenation reactions.[47] Except for hybrid **1**, hybrids **2** and **3** displayed weak antibacterial activity. The potent antipseudomonal properties of hybrid **1** cannot be rationalized by inhibition of protein translation or gyrase A activity as this hybrid displays the weakest inhibitory activity against these targets but rather reflect other modes of action. Instead, hybrid **1** exerts its antipseudomonal effects by affecting the outer membrane integrity as supported by the TEM-study, NPN-permeability assay, PI staining, and high synergy with outer membrane impermeable antibiotics. In addition, besides its effect on the outer membrane, hybrid **1** also affects the state of the cytoplasmic membrane by depolarizing the cytoplasmic membrane in a concentration-dependent manner and reducing swimming motility at sub-MIC concentration. Combination studies of hybrid **1** with the aminoglycoside gentamicin are antagonistic, suggesting that hybrid **1** affects the electrical

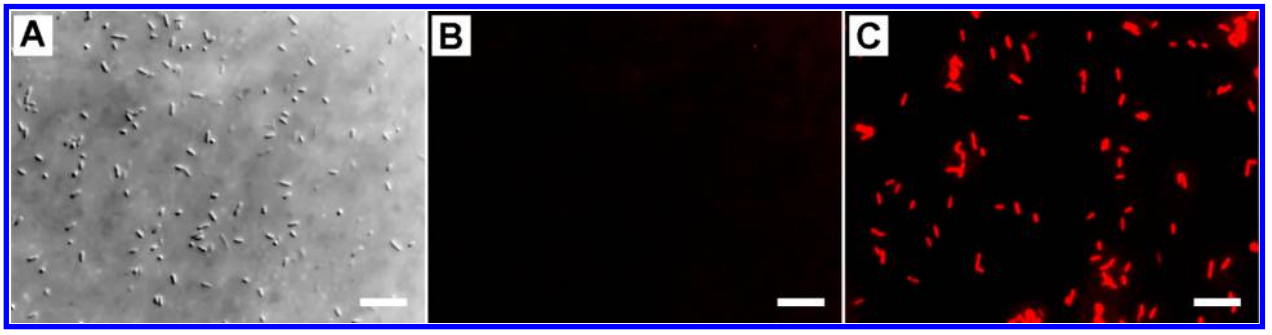

Figure 6. (A) Differential interference contrast microscopic (DIC) image of *P. aeruginosa* PAO1 cells. Fluorescence microscopic image of (B) untreated *P. aeruginosa* PAO1 cells labeled with propidium iodide (PI) with no fluorescence, indicating intact cell membrane. (C) Cells treated with 2 × MIC (8 μg/mL) of hybrid **1** for 3 h at 37 °C that gives bright-red fluorescence due to PI uptake, indicating membrane permeabilizing effect of hybrid **1**. Scale bar represents 10 μm.

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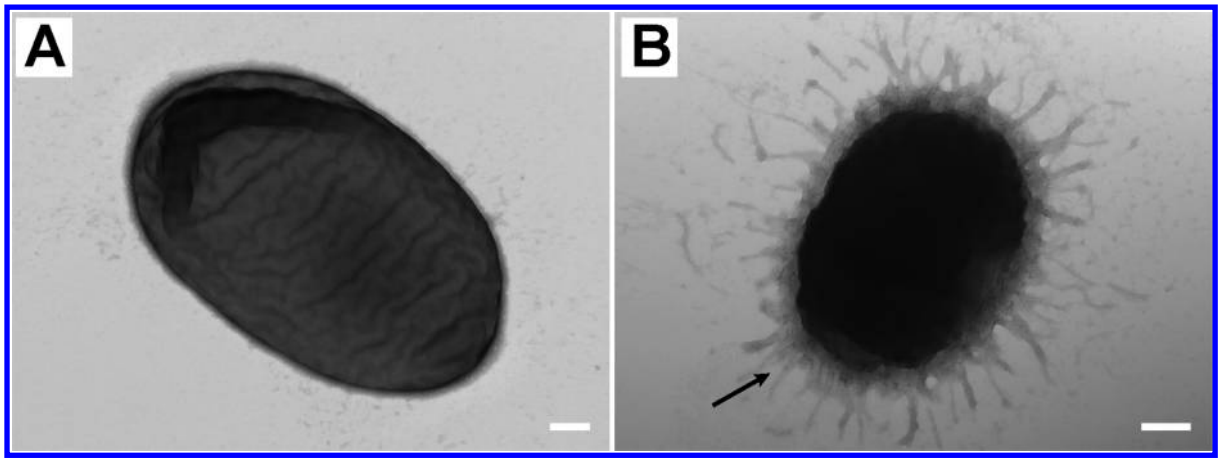

Figure 7. Transmission electron microscopic images of (A) untreated *P. aeruginosa* PAO1 cells and (B) cells treated with 2 × MIC (8 μg/mL) of hybrid **1** for 3 h at 37 °C. Arrow in B indicates loss of characteristic cell morphology, membrane blebbing, and disintegration of the cell membrane. Scales of the images are 100 nm.

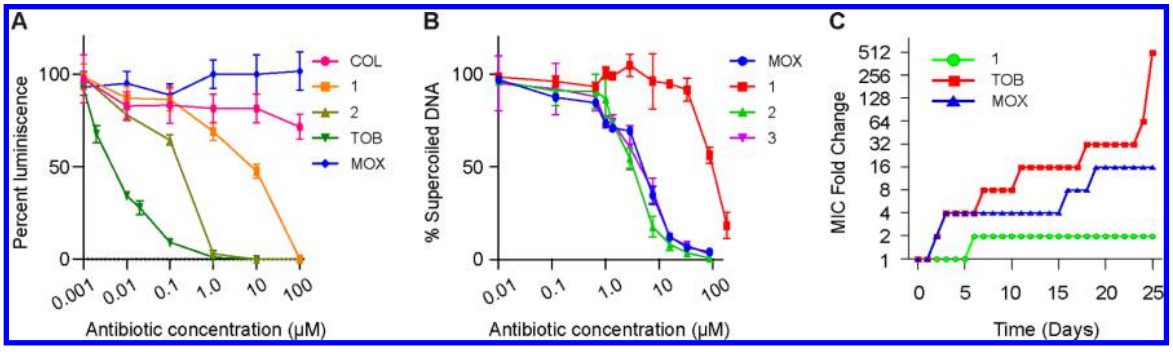

Figure 8. Protein translation activity, gyrase A activity, and emergence of resistance. (A) Inhibition of in vitro protein translation in *E. coli* S30 extract by tobramycin, moxifloxacin, hybrid **1**, and **2**. Protein translation assays were performed as described in methods section. The percentage luminescence was calculated compared to no antibiotic control (assumed to be 100%). Each data point represents an average luciferase activity of the triplicates with ± percentage error. The IC_{50} values (μM) for tobramycin (0.0062 ± 0.0002), hybrid **1** (4.7 ± 0.21), and hybrid **2** (0.13 ± 0.003) were obtained. (B) Inhibition of *P. aeruginosa* DNA gyrase A activity in the presence of moxifloxacin, hybrid **1**, **2**, or **3**. Each data point represents an average value of band (supercoiled) intensities from two independent sets of reactions (see appendix in the Supporting Information). The percentage of supercoiled DNA was measured compared to the no-antibiotic control (assumed to be 100%). IC_{50} values (μM) for moxifloxacin (2.6 ± 0.02), hybrid **1** (54.7 ± 2.9), **2** (2.1 ± 0.08), and **3** (2.6 ± 0.14) were calculated. (C) Emergence of resistance studies in *P. aeruginosa* after 25 serial passages in the presence of tobramycin, moxifloxacin, and hybrid **1**: Col = colistin, TOB = tobramycin, MOX = moxifloxacin.

component (ΔΨ) of the proton motive force in *P. aeruginosa*. Compounds which affect the PMF (uncouplers) in bacteria have recently attracted attention as a new strategy to develop novel antibiotics that can overcome resistance.[48,49] Although, uncouplers are expected to be generally cytotoxic, many FDA-approved drugs have activity as uncouplers including the antiinfectives clofazimine and bedaquiline.[49]

CONCLUSION

Our study demonstrates that hybrid **1** exerts pleiotropic effects by affecting the integrity of both outer and inner membranes in *P. aeruginosa*. The effects on the outer membrane result in enhanced cell penetration, while the effects on the cytoplasmic membrane result in membrane depolarization that perturbs the PMF. As the PMF plays a necessary role in excretion of proteins, toxic metabolites, and efflux of antibiotics multimodal antibacterial effects of hybrid **1** are expected. Besides displaying potent antipseudomonal properties against MDR, XDR, and PDR, *P. aeruginosa* isolates the activity of **1** that can be synergized with other classes of antibiotics including fluoroquinolone antibiotics, thereby reaching ciprofloxacin-susceptible (MIC = 1 μg/mL) CLSI breakpoints while at the same time reducing the MIC of **1** to (<1 μg/mL) against MDR, XDR, and PDR *P. aeruginosa* isolates. Lastly, hybrid **1** possesses a low likelihood of resistance development when compared to its individual antibiotic components tobramycin and moxifloxacin. As a result, our study opens up new therapeutic opportunities against one of the most feared bacterial pathogens.

EXPERIMENTAL SECTION

General Procedures. 1D and 2D NMR spectra (^1H, ^{13}C, DEPT, 2D COSY, 1D TOCSY, HSQC, HMBC) were recorded on a Bruker Avance 500 MHz spectrometer, and chemical shifts reported (in ppm) are relative to internal Me_4Si (δ) 0.0 with $CDCl_3$ as the solvent and to HOD (δ) 4.79 with D_2O as the solvent. ^{13}C NMR spectra were recorded on a Bruker Avance 500 spectrometer at 126 MHz, and the chemical shifts were reported (in ppm) relative to the residual solvent signal for $CDCl_3$ (δ) 77.00 or CD_3OD (δ) (49.0) as the solvent. EI-MS analyses were performed on a Varian 500 MS ion trap mass spectrometer. MALDI TOF MS were performed on a Bruker Daltonics Ultraflex MALDI TOF/TOF mass spectrometer. Chromatographic separations were performed on a silica gel column by flash chromatography (Kiesel gel 40, 0.040−0.063 mm; Merck). All reactions were carried out under an argon atmosphere with anhydrous

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solvents unless otherwise noted. Analytical HPLC was performed on Hitachi LC system equipped with autosampler by using Superspher 100 RP-18 column and a detection wavelength of 271 nm. Compounds were named following IUPAC rules as applied by Beilstein-Institute AutoNom (version 2.1) software for systematic names in organic chemistry. All chemicals, unless otherwise stated, were obtained from commercial sources. Purity of the hybrids were determined by using HPLC analysis and elemental analysis which indicated ≥95% purity of each product. TEM images were recorded by using a Hitachi scanning/transmission electron microscope (STEM) model H-7000 equipped with an Advanced Microscopy Techniques (AMT) CCD camera (model 1600 M Woburn, Massachusetts, United States of America), operating at 75 kV. Fluorescence images were recorded by using Zeiss Axio Imager Z1 eqipped with Zeiss Axio Cam MRm fluorescence microscope. Mechanistic studies were carried out on a Molecular Devices (Sunnyvale, USA) microplate reader.

5-O-(Dodecylmoxifloxacin)-1,3,2′,6′,3″-penta-N-(tert-butoxycarbonyl)-4′,2″,4″,6″-tetra-O-TBDMS−Tobramycin (7). In a round-bottom flask, compound **6** (1 mmol) was dissolved in anhydrous DMF (5.0 mL) then was mixed with moxifloxacin (1.5 mmol) and K_2CO_3 (4 mmol). After reaction, the mixture was stirred at 80 °C for 24 h, and then mixture was evaporated and dissolved in CH_2Cl_2:CH_3OH (1:1) followed by filtration through Celite. Evaporation of the filtrate and subsequent flash column chromatography (eluted with CH_2Cl_2/CH_3OH from 100/0 to 100/30) afford the desired compound 7 (62%) as a yellow solid. ^{1}H NMR (500 MHz, $CDCl_3$): δ 8.49 (s, 1H, H-2 of moxifloxacin), 7.79 (d, *J* = 14.0 Hz, 1H, C_5-H of moxifloxacin), 4, 11 (br s, 1H, NC*H*, moxifloxacin), 4.00−3.90 (m, 3H, 3 C*H* of moxifloxacin), 3.60 (s, 3H, OCH_3), 3.75−3.55 (m, 3H, OCH_2 of linker, CH_2 of moxifloxacin), 3.54−3.38 (m, 3H, CH_2 of moxifloxacin), 3.28−3.21 (m, 1H, CH_2 of moxifloxacin), 2.92−2.87 (m, 1H, CH_2 of moxifloxacin), 2.64−2.58 (m, 1H, CH_2 of moxifloxacin), 2.54−2.51 (m, 1H, CH_2 of moxifloxacin), 1.92−1.84 (m, 1H, CH_2 of moxifloxacin), 1.76−1.74 (m, 1H, CH_2 of moxifloxacin), 1.67−1.49 (m, 4H, CH_2 of linker), 1.45−1.41 (m, 45 H, C-CH_3), 1.33−1.19 (m, 20H, 9 CH_2 of linker, 2H of cyclopropyl), 1.18−0.86 (m, 4H, cyclopropyl), 0.96−0.85 (m, 36H, Si-CCH_3), 0.16−0.04 (m, 24H, Si-CH_3). Ring II: 5.21 (br s, 1H, H-1′), 5.05 (br s, 1H, H-5′), 3.80−3.75 (m, 2H, H-2′, 4′), 3.65−3.51 (m, 1H, H-6′), 3.42−3.34 (m, 2H, H-6′), 2.03−1.98 (m 1H, H-3′), 1.54−1.47 (m, 1H, H-3′). Ring I: 3.59−3.42 (m, 5H, H-1, H-3, H-4, H-6, H-6′), 3.59−3.42 (m, 4H, H-1, H-3, H-4, H-6), 2.48−2.41 (m, 1H, H-2), 1.58−1.48 (m, 1H, H-2). Ring III: 5.14 (br s, 1H, H-1″), 4.77 (br s, 1H, H-5″), 4.27−4.21 (m, 1H, H-4″), 4.16−4.13 (m, 1H, H-2″), 3.74−3.64 (m, 2H, H-6″), 3.36−3.29 (m, 2H, H-3″). ^{13}C NMR (126 MHz, $CDCl_3$): δ 172.8, 172.7, 165.9, 155.5, 154.7, 154.5, 154.3, 152.3, 150.2, 140.7, 135.9, 135.8, 133.3, 121.5, 121.4, 109.9, 108.7, 108.5,97.6, 85.7, 79.8, 79.3, 79.2 (2 C), 77.2, 68.0, 66.8, 64.9, 63.1, 60.7, 58.2, 58.1, 56.6, 52.3,52.2, 50.5, 48.3, 44.9, 39.4, 63.8, 35.6, 30.6, 30.1, 29.8, 29.7, 29.6, 29.5, 29.4, 29.3, 28.8, 28.7, 28.6, 28.5, 28.4, 28.3, 26.1, 26.0, 25.9, 25.8, 23.4, 22.1, 18.5, 18.3, 18.1, 17.9, 9.9, 8.9, −3.4, −3.8, −4.2, −4.8, −4.9, −5.1, −5.2, −5.2. EIMS: *m*/*z* calcd for $C_{100}H_{179}FN_8O_{23}Si_4Na$, 2015.9; found, 2015.7 $[M + Na]^+$.

2″-O-(Dodecylmoxifloxacin)-1,3,2′,6′,3″-tetraazido-2″-bromododecyloxy)-4″,6″-O-benzylidene−Tobramycin (11). To a mixture of compound **10** (1.00 mol) and moxifloxacin methyl ester (2 mol) in DMF (10 mL), potassium carbonate (3 mol) was added and the reaction mixture was stirred at 90 °C for overnight. When TLC (MeOH/CH_2Cl_2, 1:10) indicated completion of the reaction (14 h), the mixture was dissolved in water (100 mL) and extracted from the aqueous phase with CH_2Cl_2 (50 mL × 3). The organic layer was dried over Na_2SO_4 and evaporated under reduced pressure. The yellow solid compound was dissolved in THF:H_2O (4:1), and LiOH (4 mol) was added. The reaction progress was monitored by TLC (MeOH/CH_2Cl_2, 1:10), which indicated completion of reaction after 12 h. The mixture was diluted with CH_2Cl_2 (50 mL) and washed with brine. Subsequently, the organic layer was dried over Na_2SO_4, evaporated, and purified by flash column chromatography (MeOH/CH_2Cl_2, 1:20) and was used to yield compound **11** (64%) as a yellow solid. ^{1}H NMR (500 MHz, $CDCl_3$): δ 8.62 (s, 1H, H-2 of moxifloxacin), 7.67 (d, *J* = 14.0 Hz, 1H, C_5-H of moxifloxacin), 7.52−7.53 (m, 2H, Ar), 7.42−7.38 (m, 3H, Ar), 5.58 (Ar*H*), 4.08−3.90 (m, 4H, C*H* of moxifloxacin, OCH_3), 3.60 (s, 3H, OCH_3), 3.75−3.55 (m, 3H, OCH_2 of linker, CH_2 of moxifloxacin), 3.54−3.38 (m, 3 H, CH_2 of moxifloxacin), 3.28−3.21 (m, 1H, CH_2 of moxifloxacin), 2.92−2.87 (m, 1H, CH_2 of moxifloxacin), 2.64−2.58 (m, 1H, CH_2 of moxifloxacin), 2.54−2.51 (m, 1H, CH_2 of moxifloxacin), 1.92−1.84 (m, 1H, CH_2 of moxifloxacin), 1.76−1.74 (m, 1H, CH_2 of moxifloxacin), 1.67−1.49 (m, 4H, CH_2 of linker), 1.33−1.16 (m, 20H, 9 CH_2 of linker, 2H of cyclopropyl), 1.18−0.86 (m, 4H, cyclopropyl). Ring II: 5.50 (d, *J* = 3.0 Hz, 1H, H-1′), 4.08−4.03 (m, 1H, H-5′), 3.74−3.69 (m, 1H, H-4′), 3.38−3.32 (m, 1H, H-2′), 2.27−2.20 (m, 1H, H-3′), 2.06−1.99 (m, 1H, H-3′). Ring I: 3.75−3.62 (m, 1H, H-4), 3.58−3.49 (m, 2H, H-1, H-3), 3.40−3.35(m, 1H, H-5), 2.42 (dt, *J* = 13.1, 4.2 Hz, 1H, H-2), 1.68−1.59 (m, 1H, H-2). Ring III: 5.38 (d, *J* = 3.2 Hz, 1H, H-1″), 4.34−4.26 (m, 2H, H-6″, H-5″), 4.03−3.96 (dd, J_1 = J_2 = 10.0 Hz, 1H, H-3″), 3.76−3.69 (m, 1H, H-6″), 3.45−3.41 (m, 1H, H-4″), 3.37−3.34 (m, 1H, H-2″). ^{13}C NMR (126 MHz, $CDCl_3$): δ 173.9, 166.3, 165.4, 154.4, 152.4, 150.7, 141.1, 136.9, 134.3, 133.7, 129.6, 129.2, 128.4, 126.2, 108.4, 108.2, 101.7, 97.4, 96.9, 82.0, 80.3, 79.6, 75.0, 72.8, 72.4, 68.8, 65.2, 63.3, 61.7, 61.3, 59.9, 59.1, 57.0, 56.0, 55.8, 53.6, 52.5, 52.4, 51.4, 43.6, 40.1, 39.0, 35.8, 32.3, 31.3, 29.9, 29.8, 29.7, 29.6, 29.6, 29.5, 29.4, 29.1, 28.8, 26.1, 25.9, 24.1, 23.0, 22.4, 20.1, 14.0, 11.1, 10.0, 8.8. EIMS: *m*/*z* calcd for $C_{58}H_{77}FN_{18}O_{13}[M + H]^+$, 1254.3432; found, 1254.3424 $[M + H]^+$.

2″-O-(Dodecylmoxi)1,3,2′,6′,3″-tetraazido−Tobramycin (12). Compound **11** was dissolved in 80% acetic acid (CH_3COOH, 50 mL) and was stirred at 80 °C for 1 h. The reaction progress was monitored by TLC (MeOH/CH_2Cl_2, 1:10), which indicated completion after 1 h. The mixture was evaporated, and purified by flash chromatography (silica gel, MeOH/CH_2Cl_2) to afford **12** (75%) as a yellowish solid. ^{1}H NMR (500 MHz, CD_3OD): δ 8.74 (s, 1H, H-2 of moxifloxacin), 7.66 (d, *J* = 14.5 Hz, 1H, C_5-H of moxifloxacin), 4.14−4.07 (m, 3H, NC*H* of moxifloxacin, CH_2 of moxifloxacin), 3.94−3.88 (m, 3H, CH_2 of moxifloxacin), 3.81−3.80 (m, 1H, CH_2 of moxifloxacin), 3.66 (s, 3H, OCH_3), 3.73−3.67 (m, 3H, OCH_2 of linker, CH_2 of moxifloxacin), 3.52−3.46 (m,1H, CH_2 of moxifloxacin), 3.28−3.21 (m, 1H, CH_2 of moxifloxacin), 2.98−2.91 (m, 1H, CH_2 of moxifloxacin), 1.92−1.85 (m, 3H, CH_2 of moxifloxacin), 1.77−1.71 (m, 3H, CH_2 of moxifloxacin), 1.63−1.56 (m, 4H, 2CH_2 of linker), 1.44−1.32 (m, 18H, 9 CH_2 of linker), 1.15−1.09 (m, 1H, cyclopropyl), 1.05−0.99 (m, 2H, cyclopropyl), 0.90−0.84 (m, 1H, cyclopropyl). Ring II: 5.60 (d, *J* = 3.5 Hz, 1H, H-1′), 4.12−4.07 (m, 1H, H-5′), 3.60−3.53 (m, 1H, H-4′), 3.52−3.37 (m, 2H, H-6′), 3.19 (dt, *J* = 7.9, 4.4 Hz, 1H, H-2′), 2.14 (1H, dt, *J* = 11.5, 4.6 Hz, H-3′), 2.03−1.96 (m, 1H, H-3′). Ring I: 3.75−3.62 (m, 1H, H-4, H-6), 3.58−3.49 (m, 2H, H-1, H-3), 3.40−3.35 (m, 1H, H-5), 2.37 (dt, *J* = 13.1, 4.2 Hz, 1H, H-2), 1.68−1.63 (m, 1H, H-2). Ring III: 5.37 (d, *J* = 3.5 Hz, 1H, H-1″), 4.00−3.94 (ddd, J_1 = 7.0, 4.9, 2.2 Hz,1H, H-5″), 3.83−3.77 (m, 1H, H-6″), 3.73−3.64 (m, 1H, H-3″), 3.70−3.64 (m, 1H, H-6″), 3.31−3.28 (m, 1H, H-4″), 3.28−3.22 (m, 1H, H-2″). ^{13}C NMR (126 MHz, CD_3OD): δ 175.3, 167.1, 153.8, 152.4, 142.6, 135.5, 135.1, 130.5, 108.9, 108.7, 98.5, 97.7, 84.6, 80.5, 80.5, 76.9, 74.1, 74.0, 72.3, 69.7, 68.7, 66.9, 66.7, 62.2, 62.0, 61.2, 60.7, 57.8, 57.0, 53.3, 52.6, 45.8, 44.4, 41.4, 40.3, 36.8, 33.7, 33.2, 32.3, 31.9, 31.1, 30.7, 30.7, 30.6, 30.6, 30.6, 30.6, 30.5, 30.5, 30.4, 30.1, 29.7, 27.2, 27.1, 26.9, 25.1, 24.0, 22.9, 20.4, 14.4, 11.4, 10.6, 9.5. EIMS: *m*/*z* calcd for $C_{51}H_{73}FN_{18}O_{13}$ $[M + H]^+$, 1166.2365; found, 1166.2353 $[M + H]^+$.

6″-O-(Dodecylmoxifloxacin-OMe)-1,3,2′,6′,3″-Tetraazido-2″-bromododecyloxy)-4″,6″-O-benzylidene−Tobramycin (18). Same procedure as described for compound 7. Yield: 55%. ^{1}H NMR (500 MHz, CD_3OD): δ 8.69 (s, 1H, H-2 of moxifloxacin), 7.68 (d, *J* = 14.5 Hz, 1H, C_5-H of moxifloxacin), 4.14−4.10 (m, 1H, C*H* of moxifloxacin), 3.84 (s, 3H, OCH_3), 3.60 (s, 3H, OCH_3), 3.72−3.54 (m, 5H, OCH_2 of linker, CH_2 of moxifloxacin), 3.55−3.38 (m, 3H, CH_2 of moxifloxacin), 3.27−3.20 (m, 1H, CH_2 of moxifloxacin), 2.87−2.84 (m, 1H, CH_2 of moxifloxacin), 2.64−2.58 (m, 1H, CH_2 of moxifloxacin), 2.47−2.38 (m, 1H, CH_2 of moxifloxacin), 1.85−1.83 (m, 1H, CH_2 of moxifloxacin), 1.68−1.64 (m, 4H, 2CH_2 of linker), 1.33−1.16 (m, 20H, 9CH_2 of linker, 2H of cyclopropyl), 1.01−0.86 (m, 2H,

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J. Med. Chem. 2016, 59, 8441−8455

cyclopropyl). Ring II: 5.61 (d, J = 3.5 Hz, 1H, H-1′), 4.11−4.07 (m, 1H, H-5′), 3.59−3.56 (m, 1H, H-4′), 3.54−3.47 (m, 2H, H-6′), 3.17 (dt, J = 7.9, 4.0, 1H, H-2′), 2.14 (dt, 1H, J = 9.1, 4.5, H-3′), 2.00−1.97 (m, 1H, H-3′). Ring I: 3.69−3.65 (m, 2H, H-4, H-6), 3.56−3.41 (m, 2H, H-1, H-3), 3.39−3.35 (m, 1H, H-5), 2.38 (dt, J = 8.7, 4.4 Hz, 1H, H-2), 1.64−1.53 (m, 1H, H-2). Ring III: 5.19 (d, J = 3.8 Hz, 1H, H-1″), 4.14−4.10 (m, 1H, H-5″), 3.67−3.62 (m, 1H, H-3″), 3.58−3.56 (m, 2H, H-6″), 3.45−3.42 (m, 1H, H-2″), 3.32−3.28 (m, 1H, H-4″). ^{13}C NMR (126 MHz, CD_3OD): δ 175.1, 166.8, 153.9, 152.3, 144.4, 142.5, 138.0, 135.2, 109.8, 108.8, 108.6, 99.8 (anomeric C-1″), 98.4 (anomeric C-1″), 83.5, 80.5, 72.8, 72.7, 72.3, 70.8, 70.3, 68.4, 66.6, 61.6, 61.6, 60.8, 57.8, 56.7, 52.5, 52.1, 50.4, 49.5, 48.5, 41.2, 38.6, 33.3, 32.3, 30.7, 30.7, 30.7, 30.6, 30.6, 30.5, 28.5, 27.8, 27.2, 10.1, 9.9. EIMS: m/z calcd for $C_{52}H_{75}FN_{18}O_{13}$ $[M + Na]^+$, 1202.2631; found, 1202.2611 $[M + Na]^+$.

*5-O-(Dodecyl-moxifloxacin)−Tobramycin·5HCl (**1**).* Compound 7 was treated with 40% HCl in MeOH at room temperature for 5 h. Methanolic HCl was removed under reduced pressure. Then 2% methanol in ether was added to the residue and the solvent was decanted to get the solid tobramycin conjugate as salt. Further, the crude of final compound was purified with C-18 reverse-phase flash column chromatography (eluted with deionized water) to get analytically pure compound **1** (64%) as a yellow solid. ^{1}H NMR (500 MHz, D_2O): δ 8.97 (s, 1H, H-2 of moxifloxacin), 7.69 (d, J = 13.7 Hz, 1H, C_5-H of moxifloxacin), 4.33−4.30 (m, 2H, NC*H* of moxifloxacin), 4.25−4.06 (m, 3H, C*H*, CH_2 of moxifloxacin), 3.83−3.81 (m, 1H, CH_2 of moxifloxacin), 3.66 (s, 3H, OCH_3), 3.72−3.58 (m, 3H, OCH_2 of linker, CH_2 of moxifloxacin), 3.53−3.42 (m, 1H, CH_2 of moxifloxacin), 3.35−3.27 (m, 1H, CH_2 of moxifloxacin), 3.09−2.99 (m, 1H, CH_2 of moxifloxacin), 2.74−2.65 (m, 1H, C*H* of moxifloxacin), 1.99−1.91 (m, 3H, CH_2 of moxifloxacin), 1.63−1.56 (m, 2H, CH_2 of linker), 1.42−1.16 (m, 16H, 8CH_2 of linker), 0.99−0.93 (m, 6H, CH_2 of linker, 4H of cyclopropyl). Ring II: 5.41 (d, J = 2.4 Hz, 1H, H-1′), 4.31−4.29 (m, 1H, 5.15, H-5′), 3.97−3.94 (m, 1H, H-4′), 3.75−3.73 (m, 1H, H-2′), 3.43 (dd, J_{1-3} = 14.0 Hz, J_{1-2} = 9.0 Hz, 1H, H-6′), 3.34 (dd, J_{1-3} = 14.0 Hz, J_{1-2} = 3.8 Hz, 1H, H-6′), 2.29−2.27 (m, 2H, H-3′). Ring I: 4.21 (t, J_1 = J_2 = 9.8 Hz, 1H, H-4), 3.95−3.94 (m, 1H, H-6), 3.82−3.79 (m, 1H, H-5), 3.62−3.61 (m, 1H, H-3), 3.68−3.65 (m, 1H, H-1), 2.55−2.51 (m, 1H, H-2), 1.98 (q, J_1 = J_2 = J_3 = 12.6 Hz, 1H, H-2). Ring III: 5.18 (d, J = 3.4 Hz, 1H, H-1″), 3.97−3.94 (m, 1H, H-2″), 3.88−3.86 (m, 1H, H-6″), 3.86−3.84 (m, 1H, 3.82, H-4″), 3.84−3.82 (m, 1H, H-5″), 3.73−3.71 (m, 1H, H-6″), 3.60−3.58 (m, 1H, H-3″). ^{13}C (NMR, 126 MHz, D_2O): δ_C 175.0 (CO of moxifloxacin), 154.3 (C-6 of moxifloxacin), 152.4, 149.5 (C-2 of moxifloxacin), 143.6, 143.6 (C-6 of moxifloxacin), 138.3, 111.8, 108.5, 107.1, 101.3 (anomeric C-1″), 92.6 (anomeric C-1′), 81.8 (C-2), 76.6, 75.7, 73.7, 68.4, 65.7 (CH_2 of linker 12), 64.7, 63.1, 59.2 (C-6″), 54.7, 49.6, 48.4, 47.3, 46.7, 46.6 (piperazine), 43.3 (piperazine), 38.4 (C-6′), 35.5 (CH of cyclopropane), 29.4, 28.9, 28.8, 28.7, 28.3, 28.1, 27.8, 27.7, 25.3, 25.2, 7.5 (cyclopropane). MALDI TOFMS calcd for $C_{51}H_{83}FN_8O_{13}$.[M + Na]$^+$, 1057.5961; found, 1057.5961 $[M + Na]^+$

*2″-O-(Dodecyl-moxifloxacin)−Tobramycin·5HCl (**2**).* The tetraazidotobra−moxifloxacin conjugate **12** (1 mol) was dissolved in THF (10 mL), 0.1 M NaOH (2.0 mL) and stirred at 60 °C for 10 min, after which PMe_3 (1 M solution in THF, 1.2 mmol/azide) was added. The reaction progress was monitored by TLC (CH_2Cl_2/MeOH/NH_4OH, 2:4:1), which indicated completion after 3 h. The reaction mixture was evaporated to dryness to give an amorphous solid then dissolve in 1 M methanolic HCl to adjust the pH 4−5. The solid residue was washed with hexane−acetic acid mixture, CH_2Cl_2, 2% methanol in ether, and the solvent was decanted to get the solid tobramycin−moxifloxacin conjugate **2** as salt. Further the crude of final compound has been purified with C-18 column chromatography (eluted with deionized water) to get analytically pure compound **2** (71%). ^{1}H NMR (500 MHz, D_2O): δ8.97 (s, 1H, H-2 of moxifloxacin), 7.69 (d, J = 13.7 Hz, 1H, C_5-H of moxifloxacin), 4.33−4.30 (m, 2H, NC*H* of moxifloxacin), 4.25−4.06 (m, 3H, C*H*, $CH_2$of moxifloxacin), 3.83−3.81 (m, 1H, CH_2 of moxifloxacin), 3.66 (s, 3H, OCH_3), 3.72−3.58 (m, 3H, OCH_2 of linker, CH_2 of moxifloxacin), 3.53−3.42 (m, 1H, CH_2 of moxifloxacin), 3.35−3.27 (m, 1H, CH_2 of moxifloxacin), 3.09−2.99 (m, 1H, CH_2 of moxifloxacin), 2.74−2.65 (m, 1H, C*H* of moxifloxacin), 1.99−1.91 (m, 3H, CH_2 of moxifloxacin), 1.63−1.56 (m, 2H, CH_2 of linker), 1.42−1.16 (m, 16H, 8CH_2 of linker), 0.99−0.93 (m, 6H, CH_2 of linker, 4H of cyclopropyl). Ring II: 5.78 (d, J = 3.5 Hz, 1H, H-1′), 3.99−3.95 (m, 1H, H-5′), 3.79−3.73 (m, 1H, H-4′), 3.74−3.69 (m, 1H, H-2′), 3.48−3.42 (m, 1H, H-6′), 3.34−3.32 (m, 1H, H-6′), 2.34 (dt, J = 8.9, 4.3 Hz, 1H, H-3′), 2.11−2.03 (m, 2H, H-3′), 2.11−2.03 (m, 2H, H-3′). Ring I: 4.12−4.05 (m, 1H, H-5), 3.97−3.87 (m, 1H, H-6), 3.89−3.85 (m, 1H, H-4), 3.75−3.60 (m, 2H, H-1, H-3), 2.62 (dt, J = 8.4, 4.1 Hz 1H, H-2), 2.09−2.02 (m, 1H, H-2). Ring III: 5.38 (dd, J = 14.7, 3.1 Hz, 1H, H-1″), 3.98−3.87 (m, 2H, H-5″, H-6″), 3.83−3.76 (m, 1H, H-6″), 3.80−3.74 (m, 1H, H-2″), 3.74−3.71 (m, 1H, H-4″), 3.61−3.55 (m, 1H, H-3″). ^{13}C NMR (126 MHz, D_2O): δ 176.1, 169.8, 155.2, 151.1, 141.7, 141.5, 135.0, 118.0, 106.7, 105.9, 97.8, 94.6, 83.6, 77.8, 76.3, 74.3, 73.2, 72.3, 70.3, 65.6, 65.0, 64.5, 62.2, 61.5, 59.9, 57.9, 57.1, 55.7, 54.3, 53.7, 53.0, 50.7, 49.4, 48.5, 47.9, 41.4, 39.9, 36.0, 35.5, 29.4, 29.3, 29.2, 29.1, 29.1, 28.9, 28.9, 28.8, 28.7, 28.4, 28.3, 27.8, 27.7, 26.1, 25.5, 25.0, 24.9, 23.7, 23.3, 22.4, 21.6, 20.1, 17.9, 9.9, 8.2. MALDI TOFMS m/z calcd for $C_{51}H_{83}FN_8O_{13}$.[M + Na]$^+$, 1057.5961; found, 1057.5952 $[M + Na]^+$

*6″-O-(Dodecyl-moxifloxacin)−Tobramycin·5HCl (**3**).* Same procedure as described for compound **2** (63%). ^{1}H NMR (500 MHz, D_2O): δ8.88 (s, 1H, H-2 of moxifloxacin), 7.72 (d, J = 14.5 Hz, 1H, C_5-H of moxifloxacin), 4.29−4.24 (m, 2H, NC*H* of moxifloxacin), 4.08−3.94 (m, 3H, CH_2 of moxifloxacin), 3.81−3.80 (m, 1H, CH_2 of moxifloxacin), 3.66 (s, 3H, OCH_3), 3.72−3.54 (m, 3H, OCH_2 of linker, CH_2 of moxifloxacin), 3.50−3.46 (m, 1H, CH_2 of moxifloxacin), 3.25−3.20 (m, 1H, CH_2 of moxifloxacin), 1.99−1.87 (m, 3H, CH_2 of moxifloxacin), 1.63−1.56 (m, 2H, 2CH_2 of linker), 1.44−1.09 (m, 22H, 9 CH_2 of linker, 4H of cyclopropyl). Ring II: 5.83(d, J = 3.5 Hz, 1H, H-1′), 3.99−3.95 (m, 1H, H-5′), 3.81−3.76 (m, 1H, H-4′), 3.71 (dt, J = 7.9, 3.8 Hz 1H, H-2′), 3.49−3.44 (m, 2H, H-6′), 2.33 (dt, J = 8.7, 4.4 Hz 1H, H-2′), 2.11−2.04 (m, 1H, H-3′). Ring I: 4.08 (dd, J = 10.1, 9.1 Hz, 1H, H-5), 3.92−3.88 (m, 1H, H-6), 3.89−3.85 (m, 1H, H-4), 3.79−3.59 (m, 2H, H-1, H-3), 2.61 (dt, J = 8.4, 4.1 Hz, 1H, H-2), 2.09−2.02 (m, 1H, H-2). Ring III: 5.14 (d, J = 3.6 Hz, 1H, H-1″), 4.03−3.95 (m, 2H, H-6″, H-5″),4.00−3.94 (m, 1H, H-2″),3.81−3.78 (m, 1H, H-4″), 3.77−3.74 (m, 1H, H-6″), 3.55−3.51 (m, 1H, H-3″). ^{13}C NMR (126 MHz, D_2O) δ176.1, 175.0, 170.9, 154.4, 153.0, 150.8, 141.9, 134.8, 120.8, 118.5, 106.8, 106.2, 100.7, 94.0, 84.1, 74.3, 71.9, 71.8, 70.1, 68.1, 65.4, 64.6, 61.5, 58.7, 54.9, 49.9, 48.6, 47.9, 46.1, 44.4, 40.8, 40.0, 36.5, 35.7, 29.6, 28.9, 28.8, 28.7, 28.7, 28.5, 25.1, 20.6, 16.1, 15.6, 9.0, 8.1. MALDI TOFMS m/z calcd for $C_{51}H_{83}FN_8O_{13}$[M + Na]$^+$:1057.5961, found 1057.5968 $[M + Na]^+$

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acs.jmedchem.6b00867.

Additional figures, tables, biochemical methods, and NMR spectra (PDF)

Molecular formula strings (CSV)

■ AUTHOR INFORMATION

Corresponding Author

*Phone: (204) 474-7012. Fax: (204) 474-7608. E-mail: Frank.Schweizer@umanitoba.ca.

Author Contributions

B.K.G. and G.G. carried out organic synthesis; B.K.G., S.G., and D.F. performed mechanistic studies; B.K.G carried out in vivo experiments; F.S. supervised the project; F.S. wrote the manuscript with contributions from all authors.

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Notes

The authors declare that provisional patent USSN 62/329274 on the design, synthesis, and biological activities of all the hybrid molecules has been filed.

The authors declare the following competing financial interest(s): A provisional patent has been filed.

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■ ABBREVIATIONS USED

CCR2, CC chemokine

■ REFERENCES

(1) Rice, L. B. Federal funding for the study of antimicrobial resistance in nosocomial pathogens: NO ESKAPE. *J. Infect. Dis.* **2008**, *197*, 1079−1081.

(2) Peleg, A. Y.; Hooper, D. C. Hospital-acquired infections due to gram-negative bacteria. *N. Engl. J. Med.* **2010**, *362*, 1804−1813.

(3) Wisplinghoff, H.; Bischoff, S. M.; Tallent, S. M.; Seifert, H.; Wenzel, R. P.; Edmond, M. B. Nosocomial bloodstream infections in US hospitals: analysis of 24, 179 cases from a prospective nationwide surveillance study. *Clin. Infect. Dis.* **2004**, *39*, 309−317.

(4) Weinstein, R. A.; Gaynes, R.; Edwards, J. R. Overview of nosocomial infections caused by Gram-negative bacilli. *Clin. Infect. Dis.* **2005**, *41*, 848−854.

(5) Hidron, A. L.; Edwards, J. R.; Patel, J.; Horan, T. C.; Sievert, D. M.; Pollock, D. A.; Fridkin, S. K. NHSN annual update: antimicrobial-resistant pathogens associated with healthcare-associated infections: annual summary of data reported to the National Healthcare Safety Network at the Centers for Disease Control and Prevention, 2006−2007. *Infect. Control Hosp. Epidemol.* **2008**, *29*, 996−1011.

(6) Boucher, H. W.; Talbot, G. H.; Bradley, J. S.; Edwards, J. E.; Gilbert, D.; Rice, L. B.; Scheld, M.; Spellberg, B.; Bartlett, J. Bad bugs, no drugs; no ESKAPE! An update from the Infectious Diseases Society of America. *Clin. Infect. Dis.* **2009**, *48*, 1−12.

(7) Breidenstein, E. B. M.; de laFuente-Núñez, C.; Hancock, R. E. W. Pseudomonas aeruginosa: all roads lead to resistance. *Trends Microbiol.* **2011**, *19*, 419−426.

(8) Nikaido, H. Prevention of drug access to bacterial targets: permeability barriers and active efflux. *Science* **1994**, *264*, 382−388.

(9) Wagner, S.; Sommer, R.; Hinsberger, S.; Lu, C.; Hartmann, R. W.; Empting, M.; Titz, A. Novel strategies for the treatment of *Pseudomonas aeruginosa* infections. *J. Med. Chem.* **2016**, *59*, 5929−5969.

(10) Kalan, L.; Wright, G. D. Antibiotic adjuvants: multicomponent anti-infective strategies. *Expert Rev. Mol. Med.* **2011**, *13*, e5.

(11) Gill, E. E.; Franco, L.; Hancock, R. E. Antibiotic adjuvants: diverse strategies for controlling drug-resistance pathogens. *Chem. Biol. Drug Des.* **2015**, *85*, 56−78.

(12) Hurdle, J. G.; O'Neill, A. J.; Chopra, I.; Lee, R. E. Targeting bacterial membrane function: an underexploited mechanism for treating persistent infections. *Nat. Rev. Microbiol.* **2011**, *9*, 62−75.

(13) Brötz-Osterhelt, H.; Brunner, N. A. How many modes of action should an antibiotic have? *Curr. Opin. Pharmacol.* **2008**, *8*, 564−573.

(14) Zhanel, G. G.; Calic, D.; Schweizer, F.; Zelenitsky, S.; Adam, H.; Lagacé-Wiens, P. R.; Rubinstein, E.; Gin, A. S.; Hoban, D. J.; Karlowsky, J. A. New lipoglycopeptides: a comparative review of dalbavancin, oritavancin and telavancin. *Drugs* **2010**, *70*, 859−886.

(15) Pokrovskaya, V.; Baasov, T. Dual-acting hybrid antibiotics: a promising strategy to combat bacterial resistance. *Expert Opin. Drug Discovery* **2010**, *5*, 883−902.

(16) Pokrovskaya, V.; Belakhov, V.; Hainrichson, M.; Yaron, S.; Baasov, T. Design, synthesis, and evaluation of novel fluoroquinolone-aminoglycoside hybrid antibiotics. *J. Med. Chem.* **2009**, *52*, 2243−2254.

(17) Brown, E. D.; Wright, G. D. Antibacterial drug discovery on the resistance era. *Nature* **2016**, *529*, 336−343.

(18) Gorityala, B. K.; Guchhait, G.; Fernando, D. M.; Deo, S.; McKenna, S. A.; Zhanel, G. G.; Kumar, A.; Schweizer, F. Adjuvants based on hybrid antibiotics overcome resistance in Pseudomonas aeruginosa and enhance fluoroquinolone efficacy. *Angew. Chem., Int. Ed.* **2016**, *55*, 555−559.

(19) Hanessian, S.; Tremblay, M.; Swayze, E. E. Tobramycin analogues with C-5 aminoalkyl ether chains intended to mimic rings III and IV of paromomycin. *Tetrahedron* **2003**, *59*, 983−993.

(20) Guchhait, G.; Altieri, A.; Gorityala, B. K.; Yang, X.; Findlay, B.; Zhanel, G. G.; Mookherjee, N.; Schweizer, F. Amphiphilic tobramycins with immunomodulatory properties. *Angew. Chem., Int. Ed.* **2015**, *54*, 6278−6282.

(21) Li, Q.; Xing, J.; Cheng, H.; Wang, H.; Wang, J.; Wang, S.; Zhou, J.; Zhang, H. Design, synthesis, antibacterial evaluation and docking study of novel 2-hydroxy-3-(nitroimidazolyl)-propyl-derived quinolone. *Chem. Biol. Drug Des.* **2015**, *85*, 79−90.

(22) Alper, P. B.; Hendrix, M.; Sears, P.; Wong, C.-H. Probing the specificity of aminoglycoside-Ribosomal RNA interactions with designed synthetic analogs. *J. Am. Chem. Soc.* **1998**, *120*, 1965−1978.

(23) Evans, M. E. Methyl 4,6-*O*-benzylidene-α- and -β-D-glucosides. *Carbohydr. Res.* **1972**, *21*, 473−475.

(24) Takaku, T.; Kamaike, K. Synthesis of oligoribonucleotides using 4-methoxybenzyl group as a new protecting group of the 2′-hydroxyl group of adenosine. *Chem. Lett.* **1982**, *11*, 189−192.

(25) Sachin, K.; Kim, E.-M.; Cheong, S.-J.; Jeong, H.-J.; Lim, S. T.; Sohn, M.-H.; Kim, D. W. Synthesis of N_4'-[^{18}F]Fluoroalkylated Ciprofloxacin as a Potential Bacterial Infection Imaging Agent for PET Study. *Bioconjugate Chem.* **2010**, *21*, 2282−2288.

(26) Staudinger, H.; Meyer, J. Über neue organische Phosphorverbindungen III. Phosphinmethylenderivate und Phosphinimine. *Helv. Chim. Acta* **1919**, *2*, 635−646.

(27) Cunico, R. F.; Bedell, L. The triisopropylsilyl group as a hydroxyl-protecting function. *J. Org. Chem.* **1980**, *45*, 4797−4798.

(28) Aizpurua, J. M.; Cossio, F. P.; Palomo, C. Reaction of hindered trialkylsilyl esters and trialkylsilyl ethers with triphenylphosphine dibromide: preparation of carboxylic acid bromides and alkyl bromides under mild neutral conditions. *J. Org. Chem.* **1986**, *51*, 4941−4943.

(29) Werz, D. B.; Seeberger, P. H. Total Synthesis of Antigen *Bacillus Anthracis* Tetrasaccharide-Creation of an Anthrax Vaccine Candidate. *Angew. Chem., Int. Ed.* **2005**, *44*, 6315−6318.

(30) Hill, L.; Veli, N.; Coote, P. J. Evaluation of *Galleria mellonella* larvae for measuring the efficacy and pharmacokinetics of antibiotic therapies against *Pseudomonas aeruginosa* infection. *Int. J. Antimicrob. Agents* **2014**, *43*, 254−261.

(31) Krezdorn, J.; Adams, S.; Coote, P. J. A *Galleria mellonella* infection model reveals double and triple antibiotic combination therapies with enhanced efficacy versus a multidrug-resistant strain of *Pseudomonas aeruginosa*. *J. Med. Microbiol.* **2014**, *63*, 945−955.

(32) Gooderham, W. J.; Bains, M.; McPhee, J. B.; Wiegand, I.; Hancock, R. E. Induction by cationic antimicrobial peptides and involvement in intrinsic polymyxin and antimicrobial peptide resistance, biofilm formation, and swarming motility of PsrA in *Pseudomonas aeruginosa*. *J. Bacteriol.* **2008**, *190*, 5624−5634.

(33) Pillai, S. K.; Moellering, R. C.; Eliopoulos, G. M. In *Antimicrobial combinations; Antibiotics in Laboratory Medicine* Lorian, V., Ed.; Williams and Wilkins: Baltimore, MD, 2005; pp 365−440.

(34) Odds, F. C. Synergy, antagonism and what the checkerboard puts between them. *J. Antimicrob. Chemother.* **2003**, *52*, 1.

(35) Ouberai, M.; El Garch, F.; Bussiere, A.; Riou, M.; Alsteens, D.; Lins, L.; Baussanne, I.; Dufrêne, Y. F.; Brasseur, R.; Decout, J. L.; Mingeot-Leclercq, M. P. The *Pseudomonas aeruginosa* membranes: A target for a new amphiphilic aminoglycoside derivative? *Biochim. Biophys. Acta, Biomembr.* **2011**, *1808*, 1716−1727.

(36) Paul, K.; Erhardt, M.; Hirano, T.; Blair, D. F.; Hughes, K. T. Energy source of flagellar type III secretion. *Nature* **2008**, *451*, 489−492.

DOI: 10.1021/acs.jmedchem.6b00867
J. Med. Chem. 2016, 59, 8441−8455

(37) Taber, H. W.; Mueller, J. P.; Miller, P. F.; Arrow, A. S. Bacterial uptake of aminoglycoside antibiotics. *Microbiol Rev.* **1987**, *51*, 439−457.
(38) Kumar, A.; Chua, K. − L.; Schweizer, H. P. Method for regulated expression of single-copy efflux pump genes in a surrogate *Pseudomonas aeruginosa* strain: Identification of the BpeEF-OprC chloramphenicol and trimethoprim efflux pump of *Burkholderia pseudomallei* 1026b. *Antimicrob. Agents Chemother.* **2006**, *50*, 3460−3463.
(39) Lomovskaya, O.; Warren, M. S.; Lee, A.; Galazzo, J.; Fronko, R.; Lee, M.; Blais, J.; Cho, D.; Chamberland, S.; Renau, T.; Leger, R.; Hecker, S.; Watkins, W.; Hoshino, K.; Ishida, H.; Lee, V. J. Identification and characterization of inhibitors of multidrug resistance efflux pumps in Pseudomonas aeruginosa: Novel agents for combination therapy. *Antimicrob. Agents Chemother.* **2001**, *45*, 105−116.
(40) Sträuber, H.; Müller, S. Viability states of bacteria − specific mechanisms of selected probes. *Cytometry, Part A* **2010**, *77A*, 623−634.
(41) Horobin, R. W.; Stockert, J. C.; Rashid-Doubell, F. Fluorescent cationic probes for nuclei of living cells: why are they selective? A quantitative structure-activity relations analysis. *Histochem. Cell Biol.* **2006**, *126*, 165−175.
(42) Hartmann, M.; Berditsch, M.; Hawecker, J.; Ardakani, M. F.; Gerthsen, D.; Ulrich, A. S. Damage of the bacterial cell envelope by antimicrobial peptides gramicidin S and PGLa as revealed by transmission and scanning electron microscopy. *Antimicrob. Agents Chemother.* **2010**, *54*, 3132−3142.
(43) Pollard, J. E.; Snarr, J.; Chaudhary, V.; Jennings, J. D.; Shaw, H.; Christiansen, B.; Wright, J.; Jia, W.; Bishop, R. E.; Savage, P. B. *In vitro* evaluation of the potential for resistance development to ceragenin CSA-13. *J. Antimicrob. Chemother.* **2012**, *67*, 2665−2672.
(44) Tommasi, R.; Brown, D. G.; Walkup, G. K.; Manchester, J. I.; Miller, A. A. Eskaping the labyrinth of antibacterial discovery. *Nat. Rev. Drug Discovery* **2015**, *14*, 529−542.
(45) Bulitta, J. B.; Ly, N. S.; Landersdorfer, C. B.; Wanigaratne, N. A.; Velkov, T.; Yadav, R.; Oliver, A.; Martin, L.; Shin, B. S.; Forrest, A.; Tsuji, B. T. Two mechanisms of killing *Pseudomonas aeruginosa* by tobramycin assessed at multiple inocula via mechanism based modeling. *Antimicrob. Agents Chemother.* **2015**, *59*, 2315−2327.
(46) Hancock, R. E. W.; Sahl, H. G. Antimicrobial and host-defense peptides as new anti-infective therapeutic strategies. *Nat. Biotechnol.* **2006**, *24*, 1551−1557.
(47) Zhanel, G. G.; Ennis, K.; Vercaigne, L.; Walkty, A.; Gin, A. S.; Embil, J.; Smith, H.; Hoban, D. J. A critical review of the fluoroquinoloes: focus on respiratory infections. *Drugs* **2002**, *62*, 13−59.
(48) Farha, M. A.; Verschoor, C. P.; Bowdish, D.; Brown, E. D. Collapsing the proton motive force to identify synergistic combinations against *Staphyloccoccus aureus*. *Chem. Biol.* **2013**, *20*, 1168−1178.
(49) Feng, X.; Zhu, W.; Schurig-Briccio, L. A.; Lindert, S.; Shoen, C.; Hitchings, R.; Li, J.; Wang, Y.; Baig, N.; Zhou, T.; Kim, B. K.; Crick, D. C.; Cynamon, M.; McCammon, J. A.; Gennis, R. B.; Oldfield, E. Antiinfective targeting enzymes and the proton motive force. *Proc. Natl. Acad. Sci. U. S. A.* **2015**, *112*, E7073−E7082.

DOI: 10.1021/acs.jmedchem.6b00867
J. Med. Chem. 2016, 59, 8441−8455