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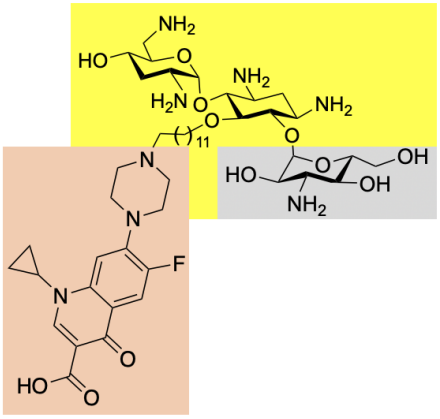

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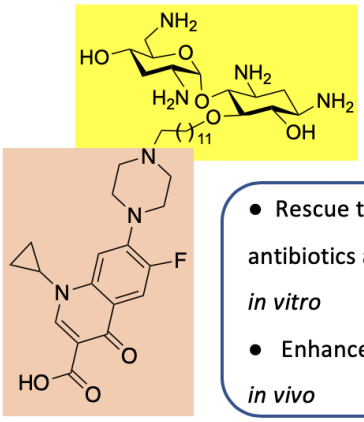

- Rescue the activity of legacy antibiotics against MDR GNB *in vitro*
- Enhance rifampicin efficacy *in vivo*

Tobramycin-ciprofloxacin hybrid adjuvant

Nebramine-ciprofloxacin hybrid adjuvant

Amphiphilic Nebramine-based Hybrids Rescue Legacy Antibiotics from Intrinsic Resistance in Multidrug-resistant *Gram-negative Bacilli*

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Abstract

The inability to discover novel class of antibacterial agents, especially against Gram-negative bacteria (GNB), compel us to consider a broader non-conventional approach to treat infections caused by multidrug-resistant (MDR) bacteria. One such approach is the use of adjuvants capable of revitalizing the activity of current existing antibiotics from resistant pathogens. Recently, our group reported a series of tobramycin (TOB)-based hybrid adjuvants that were able to potentiate multiple classes of legacy antibiotics against various MDR GNB. Herein, we report the modification of TOB-based hybrid adjuvants by replacing TOB domain by the pseudo-disaccharide nebramine (NEB) through selective cleavage of the α-D-glucopyranosyl linkage of TOB. Potent synergism was found for combinations of NEB-based hybrid adjuvants with multiple classes of legacy antibiotics including fluoroquinolones (moxifloxacin and ciprofloxacin), tetracyclines (minocycline), or rifamycin (rifampicin) against both wild-type and MDR *P. aeruginosa* clinical isolates. We also demonstrated that a combination of the optimized NEB-CIP hybrid **1b** and rifampicin protects *Galleria mellonella* larvae from the lethal effects of extensively drug-resistant (XDR) *P. aeruginosa*. Mechanistic evaluation of NEB-based hybrid adjuvants revealed that the hybrids affect the outer- and inner membranes of wild-type *P. aeruginosa* PAO1. This study describes an approach to optimize aminoglycoside-based hybrids to yield lead adjuvant candidates that are able to resuscitate the activity of partner antibiotics against MDR GNB.

Keywords: Nebramine; Hybrids; Adjuvants; Synergy; Antibacterial; Multi-drug resistant Gram-negative baccilli

1. Introduction

Starting from Fleming's discovery of penicillin in 1929 [1], a large number of antibiotics have been discovered, developed, and marketed. Antibiotics have saved countless lives and played a key role in the advancement of medical science for the past 70 years [2,3]. However, rampant and indiscriminate use of antibiotics has escalated the prevalence of multidrug-resistant (MDR) bacterial infections, especially those that are caused by Gram-negative pathogens. Worse still, there is a steady decline in the discovery of novel drug classes able to eradicate MDR Gram-negative pathogens which is largely due to the lack of understanding of the physicochemical properties necessary for antibacterial agents to efficiently traverse and accumulate inside Gram-negative bacterial cell [4,5]. There is an urgent need to find novel and perhaps unconventional approaches to address bacterial infection. Co-administration of helper molecules called adjuvants capable of enhancing the activity of currently used antibiotics and extend the life of legacy antibiotics is a viable strategy to overcome antimicrobial resistance [6,7].

Our group recently has demonstrated that amphiphilic TOB-based conjugates were able to revive the antibacterial activity of multiple classes of antibiotics against MDR Gram-negative bacilli (GNB), especially against *P. aeruginosa* [7–14]. For example, we first reported TOB-ciprofloxacin hybrids with poor intrinsic antibacterial activity were able to restore the activity of fluoroquinolone antibiotics against ciprofloxacin-resistant MDR or XDR (extensively drug-resistant) *Pseudomonas aeruginosa* in combination therapy [8]. Structure-activity studies revealed that the presence of both TOB and ciprofloxacin pharmacophores tethered by a 12-carbon-long (C_{12}) aliphatic linker is critical to the potentiation of fluoroquinolone antibiotics [8]. Encouraged by these results, an unconventional structure-activity relationship study was pursued by replacing the ciprofloxacin fragment of TOB-ciprofloxacin hybrid by other pharmacophores.

Since then, we have developed a series of TOB-moxifloxacin hybrids [9], TOB-efflux pump inhibitors conjugates [10,11], TOB-lysine peptoid conjugates [12,13], as well as TOB-polymyxin B_3 hybrids [14]. Biological evaluations revealed that these TOB-based conjugates retained the adjuvant properties of TOB-ciprofloxacin hybrids to a variable extent. These results suggest that the TOB fragment linked to C_{12} tether is the core scaffold that is responsible for the adjuvant properties. Mechanistic studies revealed that these TOB-based conjugates permeabilize the outer membrane and dissipate the proton motive force (PMF) located in the cytoplasmic membrane of *P. aeruginosa* [9,10,12].

TOB is known to eradicate Gram-negative bacteria by disruption of the outer membrane at higher concentrations (≥ 8 μg/mL) [15]. However, at lower concentrations (< 4 μg/mL), TOB, selectively interacts with the 16S rRNA, thereby causing inhibition of bacterial protein translation [15,16]. The TOB's pseudo-disaccharide segment (ring I and ring II), namely NEB (NEB), is the essential pharmacophore responsible for most of the specificity of the interactions with the ribosome [17–21]. In addition, it was recently reported that an amphiphilic NEB derivative displayed potent activity against certain TOB-resistant Gram-negative bacteria suggesting that amphiphilic NEB analogs possess a different mode of action than TOB [22]. In addition, neamine-based and neosamine-based amphiphiles have been reported to possess potent antipseudomonal properties by interacting with the outer membrane of *P. aeruginosa* [23–25].

To understand the effect of TOB on the overall adjuvant activity of previously reported TOB-based conjugates, we decided to replace TOB by NEB and evaluate its microbiological activity. We questioned whether the modification of the TOB domain would retain the adjuvant properties of our reported conjugates. To accomplish this aim, we selected TOB-moxifloxacin and TOB-ciprofloxacin as lead compounds and replaced TOB by NEB, while keeping the

moxifloxacin (MOX) and ciprofloxacin (CIP) fragments and the C_{12} hydrocarbon tether as shown in hybrid NEB-MOX (**1a**) and NEB-CIP (**1b**) (Fig. 1). In addition, we also prepared a NEB-NMP (1-(1-napthylmethyl)-piperazine) hybrid **2** containing a slightly reduced C_{10} hydrocarbon tether to potentially reduce non-specific protein binding and investigated its adjuvant properties. NMP is a well-known efflux pump inhibitor (EPI) of various efflux pumps in Gram-negative bacteria except *P. aeruginosa* [26].

2. Results and Discussion

2.1 Chemistry

The preparation of NEB-based hybrids (**1a**, **1b**, and **2)** were done by selective degradation of TOB-based hybrids as outlined in Scheme 1. Commercially available tobramycin was transformed into the *N*-Boc-, and *O*-TBDMS-protected tobramycin **3** with the exception of the sterically hindered C-5 alcohol, following previously reported procedures (Scheme 1) [9,10,12,27]. Alkylation of **3** with 1,12-dibromododecane or 1,10-dibromodecane in the presence of a phase transfer catalyst, tetrabutylammonium hydrogen sulfate (TBAHS), afforded bromoalkylated tobramycin (**4a** and **4b**). This bromide (**4a**) was then converted to primary alcohol **5** followed by oxidation reaction using pyridinium chlorochromate (PCC) to generate aldehyde **6** in good yield. Protected hybrid **7a** and **7b** were synthesized via reductive amination between moxifloxacin methyl ester or ciprofloxacin methyl ester and aldehyde **6**. Heating hybrids **7a** and **7b** in aqueous HCl solution resulted in regioselective hydrolysis of the α-D-glucopyranosyl bond [21,22] along with the simultaneous removal of Boc and TBDMS protecting groups. To avoid a laborious separation and purification of the obtained pseudo-

disaccharides, NEB-MOX and NEB-CIP hybrids, the four free amino groups of NEB were protected by $(Boc)_2O$ to afford the corresponding *N*-Boc-protected NEB-MOX hybrid **8a** and *N*-Boc-protected NEB-CIP hybrid **8b** that could easily be purified by flash chromatography. De-esterification and subsequent global deprotection of the amino groups finally resulted in desired the NEB-MOX (**1a**) and NEB-CIP (**1b**) compounds (Scheme 1). A related strategy was used to synthesize NEB-NMP (**2**) (Scheme 1).

2.2 Combination Study of Hybrids with Antibiotics

To determine whether the NEB-MOX hybrid **1a** retain the adjuvant properties of previously reported TOB-moxifloxacin hybrids, checkerboard studies were performed. Initially, we assessed the combination of hybrid **1a** with three different classes of clinically-used antibiotics including the fluoroquinolone antibiotic moxifloxacin, the tetracycline antibiotic minocycline, and the rifamycin antibiotic rifampicin against wild-type *P. aeruginosa* PAO1 (Table 1) by using the fractional inhibitory concentration index (FICI) as a measure of the interaction between two agents. FICI of ≤ 0.5, > 0.5 to ≤ 4, and > 4 indicate synergy, no interaction, and antagonism, respectively [28]. In accordance with previous findings against wild-type *P. aeruginosa* PAO1 [8,10,12], NEB-MOX hybrid **1a** displayed weak antibacterial activity (MIC = 32 μg/mL) as a stand-alone agent. However, it was found to be synergistic (FICI of 0.25) with the fluoroquinolone moxifloxacin (Table 1). Synergism was also observed with minocycline (FICI of 0.38) as well as the outer membrane-impermeable antibiotic rifampicin (FICI of 0.07) (Table 1). The absolute MICs [the MIC of antibiotics in the presence of 8 μg/mL (7.5 μM) hybrid **1a**] of three tested antibiotics, moxifloxacin, minocycline, or rifampicin, in combination therapy with hybrid **1a** were significantly reduced compared to monotherapy,

especially for rifampicin (≥ 256-fold potentiation). It should be noted that the clinically-approved β-lactamase inhibitor avibactam is typically administered at 15 μM concentration to potentiate the cephalosporin ceftazidime in *in vitro* studies [29,30].

To validate our findings in wild-type *P. aeruginosa* strain, we performed the same checkerboard study using a panel of eight MDR or XDR *P. aeruginosa* clinical isolates (Supplementary Information Table S1) as previously studied for TOB-based hybrids [8,10–12]. Notably, among this panel of clinical isolates, two strains (*P. aeruginosa* 91433 and 101243) are non-susceptible or resistant to colistin that is considered to be the antibiotic of last resort for the treatment of carbapenem-resistant Gram-negative bacterial infections [31]. We determined the FIC index of hybrid **1a** in combination with moxifloxacin, ciprofloxacin, minocycline, or rifampicin across the eight clinical isolates panel. Strong potentiation was seen with moxifloxacin, ciprofloxacin, minocycline, or rifampicin (FIC indices of 0.004 to 0.28) against these pathogens, with the exception of ciprofloxacin against *P. aeruginosa* 100036 and 101885 strains (FICI > 0.5) (Table 2, Table 3, Table 4).

Next, we evaluated the adjuvant potencies of hybrid **1a** by comparing the absolute MICs [in the presence of 8 μg/mL (7.5 μM) hybrid **1a**] of the four antibiotics to their established susceptibility breakpoints. According to the Clinical and Laboratory Standards Institute (CLSI), the susceptible breakpoint, a chosen concentration (μg/mL) of an antibiotic which defines a strain of bacteria whether it is susceptible to this antibiotic, of ciprofloxacin for *P. aeruginosa* is 1 μg/mL [32]. However, the established susceptibility breakpoints for the other three tested antibiotics against *P. aeruginosa* are not available since they are unconventional antibiotics for the treatment of *P. aeruginosa* infections. Therefore, the susceptibility breakpoints of minocycline for *Acinetobacter* spp. (≤ 4 μg/mL) and rifampicin for *Enterococcus* spp. (≤1

μg/mL) reported by CLSI were used as interpretative guidelines [32]. It is noteworthy that the French Society for Microbiology has established a rifampicin breakpoint for *Acinetobacter baumannii* based on MIC distributions (susceptible, ≤ 4 μg/mL; intermediate, 8–16 μg/mL; and resistant, ≥16 μg/mL) [33]. In addition, we conservatively considered the susceptibility breakpoint of moxifloxacin for *P. aeruginosa* to be similar to that of ciprofloxacin, as both belong to the fluoroquinolone class of antibiotics.

For the two fluoroquinolones, combinations of NEB-MOX hybrid **1a** with moxifloxacin yielded stronger potentiation than ciprofloxacin against the panel of MDR/XDR *P. aeruginosa* clinical isolates (Table 2). The adjuvant potency of hybrid **1a** in combination with moxifloxacin is comparable to previously reported TOB-ciprofloxacin hybrid [8]. In 37.5% of cases, both hybrid **1a** and TOB-ciprofloxacin hybrid **1b**, at concentrations of ≤ 8 μg/mL (6.8 – 7.5 μM), were able to bring down the MIC of moxifloxacin below its interpretative susceptibility breakpoint (≤ 1 μg/mL) against moxifloxacin-resistant MDR/XDR *P. aeruginosa* isolates (Table 2) [8]. However, the same susceptibility breakpoint was not reached for ciprofloxacin in combination with hybrid **1a** at concentration of 8 μg/mL (7.5 μM) against all the tested ciprofloxacin-resistant MDR/XDR *P. aeruginosa* isolates (Table 2). In contrast, the MICs of minocycline (8/8 minocycline-resistant MDR/XDR *P. aeruginosa* isolates) and rifampicin (7/8 rifampicin-resistant MDR/XDR *P. aeruginosa* isolates) were strongly reduced below their susceptibility breakpoints in the presence of 8 μg/mL (7.5 μM) hybrid **1a**, an effect that is consistent with previously reported TOB-efflux pump inhibitor conjugates and TOB-lysine peptoid conjugates (Table 3, Table 4) [10,12].

A summarized result of antibacterial activity of minocycline (MIN) and rifampicin (RIF) alone or in combination with a fixed concentration of 8 μg/mL (7.5 μM) hybrid **1a** against the

panel of eight MDR/XDR *P. aeruginosa* clinical isolates is shown in Table 5. The MIC_{80} of minocycline and rifampicin in combination with 8 μg/mL (7.5 μM) hybrid **1a** were significantly lower in comparison to the MIC_{80} of the antibiotic alone. Moreover, the absolute MIC_{80} of minocycline (1 μg/mL) and rifampicin (0.13 μg/mL) were found to be less than their respective CLSI susceptibility breakpoints. Similarly, we demonstrated strong synergy of NEB-CIP (**1b**) with minocycline or rifampicin against wild-type and MDR *P. aeruginosa* strains (Table 6). For instance, in presence of only 4 μg/mL (4.1 μM) of hybrid **1b**, minocycline showed a 32-fold potentiation while rifampicin resulted in a 128 – ≥256-fold potentiation in wild-type and MDR *P. aeruginosa* strains (Table 6).

The observed potentiation of rifampicin by hybrid **1a** and **1b** may be explained by our previous findings that demonstrated that amphiphilic TOB-based hybrid adjuvants perturb the outer membrane of *P. aeruginosa* in a dose-dependent manner, thus facilitating the entry of antibiotics that are unable to cross the outer membrane of Gram-negative bacteria, such as rifampicin [8–10,12]. NEB-based hybrids seem to have a similar membrane effect as that of TOB-based hybrids. Since rifampicin is a poor substrate for *P. aeruginosa* RND efflux pumps [10,12], outer membrane perturbation is most likely to be the reason to explain the observed strong synergistic effects of hybrid **1a** and **1b** in combination with rifampicin against *P. aeruginosa*.

We also investigated synergy of NEB-NMP hybrid **2** with various antibiotic classes including fluoroquinolones (moxifloxacin, ciprofloxacin), tetracyclines (minocycline), or rifamycin (rifampicin) against wild-type and MDR *P. aeruginosa* strains (Table 7). More importantly, NEB-NMP (**2**) reduced the MIC of minocycline below its CLSI susceptibility

breakpoint (≤ 4 μg/mL) against all tested *P. aeruginosa* strains. The observed adjuvant property of NEB-NMP (**2**) is consistent with that of reported TOB-NMP conjugate [10].

Besides *P. aeruginosa*, we also explored the synergistic effects of NEB-based hybrids (**1a** and **1b**) with minocycline or rifampicin against other MDR GNBs such as *Acinetobacter baumannii*, *Escherichia coli*, *Klebsiella pneumoniae*, and *Enterobacter cloacae* (Tables 8 and 9). Again, both NEB-MOX **1a** and NEB-CIP **1b** displayed poor antibacterial activity by themselves against these pathogens (MICs of ≥ 8 μg/mL). In the case of *A. baumannii*, minocycline was not potentiated against the four tested isolates while NEB-MOX **1a** was able to synergize rifampicin, leading to 32- to 64-fold reductions in MICs at a concentration of 8 μg/mL (7.5 μM) of the adjuvant. Similarly, against *A. baumannii*, we observed an additive relationship of NEB-CIP (**1b**) with minocycline while the combination of NEB-CIP (**1b**) with rifampicin remained synergistic (Table 9). With respect to *K.* pneumonia, *E. cloacae*, or *E. coli*, NEB-MOX (**1a**) and NEB-CIP (**1b**) displayed strong synergism with rifampicin against all isolates tested while synergism of NEB-MOX (**1a**) and NEB-CIP (**1b**) with minocycline was only observed in few isolates (Tables 8 and 9).

2.3 Time-kill Curve

To confirm the synergistic activity between NEB-based hybrids and minocycline or rifampicin, time-kill assays were performed. We first studied the time killing kinetics of minocycline at 4 μg/mL (½ × MIC) in combination with NEB-MOX **1a** at sub-inhibitory concentration (½ × MIC = 16 μg/mL or ¼ × MIC = 8 μg/mL) against *P. aeruginosa* wild-type PAO1 (Fig. 2A). We set a fixed concentration of 4 μg/mL minocycline for the kinetic study since the CLSI susceptibility breakpoint of minocycline is ≤ 4 μg/mL. It was demonstrated that

combination of bacteriostatic minocycline (4 μg/mL) with ½ × MIC of hybrid **1a** became bactericidal and resulted in complete eradication of *P. aeruginosa* PAO1 over a 24 h time period. This enhanced killing efficiency of minocycline in combination with hybrid **1a** is consistent with our previous findings for TOB-based hybrids [10,12] and are likely the results of the membrane effects induced by hybrid **1a**. Furthermore, we also studied the killing kinetics of NEB-CIP **1b** in combination with rifampicin against XDR *P. aeruginosa* PA259. A combination of sub-MIC of **1b** (1 μg/mL, MIC of **1b** is >128 μg/mL) and rifampicin (1/16 × MIC = 1 μg/mL) yielded a 3-order magnitude decrease in viable bacterial counts over 8 h time period (Fig. 2B). Complete eradications were observed at a higher concentration of **1b** (4 μg/mL) in combination with rifampicin at 1 μg/mL or 4 μg/mL for only 4 h of antimicrobial exposure (Fig. 2B).

2.4 Hemolytic Activity and *In Vivo* Efficacy Study

To investigate whether the adjuvant properties of the NEB-based hybrids translates into a measurable *in vivo* effect, we selected the established *in vivo Galleria mellonella* larvae infection model to study the efficacy of hybrid **1b**-rifampicin combination therapies against *P. aeruginosa* [34,35]. Initially, we demonstrated that NEB-CIP **1b** was non-hemolytic to pig erythrocytes (<10% at 512 μg/mL) (Fig. 3). We also examined the tolerability of **1b** on *G. mellonella* and found that the larvae survived beyond 96 h when administered with 100 mg/kg dosage of **1b** (Fig. 4). However, colistin resulted in 70% and 90% larvae deaths at the dosage of 75 mg/kg and 100 mg/kg, respectively, after 96 h, consistent with known toxicity of colistin to eukaryotic cells. We also established that 5 CFU of XDR *P. aeruginosa* PA264 alone resulted in 100% lethality of the larvae after 18 h. To assess the ability of combination therapy of **1b** and rifampicin to protect against XDR *P. aeruginosa* PA264-challenge larvae, single treatment doses of rifampicin +

compound **1b** (25 + 25 mg/kg, 50 + 50 mg/kg, and 75+75 mg/kg) were administered 2 h post inoculation with 5 CFU XDR *P. aeruginosa* PA264 (bacterial isolate was only susceptible to colistin). The results showed that monotherapy with a single dose of rifampicin (75 mg/kg) or **1b** (100 mg/kg) resulted in 10% and 0% survival of the larvae after 18 h, respectively (Fig. 5). In contrast, combination of rifampicin and **1b** improved the survival of the wax moth larvae in a dose-dependent manner (Fig. 5). For instance, a single dose combination of rifampicin with **1b** (75 + 75 mg/kg) resulted in 87% and 53% survival after 18 h and 24 h respectively. A 50 + 50 mg/kg single dose combination of rifampicin and **1b** resulted in 60% and 33% survival after 18 h and 24 h, respectively, while a 25 + 25 mg/kg single dose combination of rifampicin and **1b** resulted in a 33% and 13% survival after 18 h and 24 h, respectively. This clearly demonstrates a dose-dependent survivability of the infected larvae when treated with a combination of rifampicin and compound **1b**. It is interesting to note that a 25 + 25 mg/kg rifampicin + **1b** was more effective (13% survival) than 25 mg/kg of colistin (0% survival) after 24 h, although it is unclear whether the inability of colistin to protect the larvae is due to lack of effectiveness or a colistin-induced toxicity. Overall, these results demonstrate the therapeutic potential of NEB-based hybrid **1b** + rifampicin to treat MDR/XDR infections *in vivo*. We also assessed the toxicity of adjuvants **1a** or **1b** against the HepG2 and HEK293 cell lines alone and in combination with rifampicin. These results confirmed that adjuvants **1a** and **1b** do not possess elevated toxicity at their synergistic concentration alone and in combination with Rifampicin. (Figure S1).

2.5 Tetracycline Uptake Assay

To gain insight to the synergistic mechanism of NEB-MOX hybrid **1a** with minocycline, fluorescence-based tetracycline uptake assay for Gram-negative bacteria [36] was performed to investigate the effect of hybrid **1a** on the uptake of tetracycline (Fig. 6). Our results indicate that, similar to TOB-based hybrids [10], hybrid **1a** enhances the uptake of tetracycline in *P. aeruginosa* PAO1 in a concentration-dependent manner. Comparable enhancements in tetracycline uptake were also observed with membrane-targeting antibiotic colistin (Fig. 6). We previously reported that TOB-based hybrids not only permeabilize the Gram-negative bacterial outer membrane but also depolarize the cytoplasmic membrane [8–10,12]. TOB-based hybrids specifically dissipate the electrical component ($\Delta\Psi$) of the proton motive force (PMF) resulting in a compensatory increase in transmembrane chemical component (ΔpH) in order to counter this effect and maintain ATP synthesis level. The effect of TOB-based hybrids on $\Delta\Psi$ is likely retained in NEB-MOX hybrid **1** which is consistent with the observed synergy of hybrid **1a** with minocycline, as tetracycline uptake is ΔpH-dependent [36]. Disruption of $\Delta\Psi$ by hybrid **1a** is compensated by an increase in ΔpH that in turn enhances the uptake of tetracycline antibiotics. This was further corroborated by the observation that CCCP (carbonyl cyanide *m*-chlorophenyl hydrazone) inhibits tetracycline accumulation (Fig. 6). CCCP is an uncoupler of oxidative phosphorylation that disrupts the proton gradient (ΔpH) of bacterial membranes [37]. Moreover, minocycline is known to inhibit preferentially the biosynthesis of envelop proteins [38] which, perhaps, elicits further compromise of the intrinsic resistance barrier (the Gram-negative bacterial outer membrane), thereby augmenting the effects of NEB-based hybrid **1a**.

3 Conclusions

In this study, we demonstrated that NEB-based hybrids (**1a**, **1b**, and **2**) are capable of potentiating multiple classes of antibiotics including fluoroquinolones (moxifloxacin, ciprofloxacin), tetracycline (minocycline), and rifamycin (rifampicin) against wild-type and MDR/XDR GNBs including *P. aeruginosa*, *A. baumannii, K. pneumonia, and E. cloacae* strains. The adjuvant potencies of NEB-based hybrids (**1a**, **1b**, and **2**) are comparable to that of TOB-based hybrids as studied before [7–14], suggesting that cleavage of the 3-deoxy-3-amino α-D-glucosidic linkage in TOB to yield NEB did not significantly alter the adjuvant properties of this scaffold. Mechanistic study of NEB-MOX **1a** confirm that it also retains the membrane effects of TOB-based hybrid adjuvants including TOB-fluoroquinolone hybrids. Modification of the tobramycin domain of TOB-based hybrid suggests that the pseudo-disaccharide NEB linked to C_{12} tether is the essential membrane active core responsible for the adjuvant properties. This study provides further insight into structural optimization of previously investigated TOB-based hybrid adjuvants. Moreover, the reduced number of basic functions in NEB when compared to TOB may result in reduced aminoglycoside-induced cytotoxicity.

4 Experimental section

4.1 Synthetic Chemistry

4.1.1 General Comments

Reactions were monitored by thin-layer chromatography (TLC) on silica gel 60 F254 (0.25 mm, Merck) and the compounds were visualized using ultraviolet light and/or stain with

ninhydrin solution (ninhydrin and acetic acid in ethanol). 1D and 2D (^{1}H, ^{13}C, DEPT, COSY, HSQC, HMBC) nuclear magnetic resonance (NMR) characterization experiments were performed on either Bruker AMX-500 or Bruker AMX-300 spectrophotometer in the noted deuterated solvents. Chemical shifts (δ) are reported in parts per million with $CHCl_3$ (7.26 ppm), DHO (4.79 ppm), and CD_2HOH (3.31 ppm) used as internal standards. NMR spectra were analyzed using Mnova Software 8.0 version. Electrospray ionization (ESI) mass spectrometry (MS) experiments were carried out on a Varian 500 MS ion trap mass spectrometer. Matrix-assisted laser desorption ionization (MALDI) MS experiments were performed on a Bruker Daltonics Ultraflex MALDI TOF/TOF mass spectrometer. Analytical high-performance liquid chromatography (HPLC) was carried out on Breeze HPLC Waters with 2998 PDA detector (1.2 nm resolution) connected to a Synergi 4 μm Polar-RP 80 Å LC column (50 mm × 4.6 mm, Phenomenex). Yields are given following purification, unless otherwise stated. All of the tested compounds are at least 95% pure as estimated by HPLC.

4.1.2 Synthetic Procedures and Characterizations

Detailed experimental procedures of compounds **3**, **4a**, **4b**, **5**, **6**, **9**, moxifloxacin methyl ester and ciprofloxacin methyl ester were described in the supporting information.

*General synthetic procedure A: final deprotection of compounds **8a** and **8b***. Compound **8a** or **8b** (0.014 mmol) was dissolved in MeOH (2 mL). 2 N lithium hydroxide solution (1 mL) was then added to the solution while stirring. The reaction mixture was stirred at room temperature for 30 min. In an ice bath, careful acidification of the solution to pH = 6 was done by slow addition of 1 N HCl (a.q.). The solvent was removed *in vacuo*. The residue was purified by flash chromatography (elution with a gradient of DCM/MeOH from 9:1 to 1:1, *v*/*v*) to afford a

white solid which was dissolved in TFA (2 mL) and H_2O (1 mL) solution and stirred for 2 h at room temperature. The solvent was removed under reduced pressure to give a yellow residue. 20 mL of methanol and ether solution (1:20, *v*/*v*) was added to this residue in several portions and then the solvent was decanted to get a yellow solid as nebramine-based hybrid TFA salt. Stoichiometric amount of HCl aqueous solution was added into it before lyophilizing the solution to afford a yellow solid as the final product as the HCl salt.

*General synthetic procedure B: synthesis of **7a** and **7b** via reductive amination.* Moxifloxacin methyl ester (0.49 mmol) or ciprofloxacin methyl ester was mixed with aldehyde **6** (0.41 mmol), followed by the addition of dry DCE (25 mL) and AcOH (2.3 μL, 0.041 mmol) under N_2 gas. The reaction mixture was stirred at room temperature for 7 h before $NaBH(OAc)_3$ (1.64 mmol) was added at 0 ºC. The solution was gradually warmed to room temperature while stirring overnight. The reaction mixture was cooled to 0 ºC and quenched carefully by the drop-wise addition of saturated $NaHCO_3$ solution (10 mL). The solution was then extracted with DCM (3 × 15 mL). The organic layer was washed with brine, dried over anhydrous sodium sulfate and concentrated in vacuo. The crude product was purified by flash chromatography (elution with a gradient of DCM/MeOH from 50:1 to 10:1, v/v) to afford the desired product as a white solid.

*General synthetic procedure C: synthesis of Boc-proteted NEB-based hybrids **8a**, **8b** and **10*** [21,22]. 40% HCl (3 mL) and MeOH (5 mL) were added to **7a**, **7b** or **9** (0.048 mmol) slowly. The reaction was heated to 70 °C and stirred 24 h. The solution was cooled down to room temperature and neutralized with solid sodium bicarbonate before concentrated to dryness. The residue was taken up in 30 mL of MeOH, filtered and concentrated to give a crude 5-*O*-(dodecyl-moxifloxacin methyl ester)-nebramine or 5-*O*-(dodecyl-ciprofloxacin methyl ester)-nebramine HCl salt. The above crude product was dissolved in MeOH (15 mL) at room temperature.

Triethylamine (69 μL, 0.48 mmol) and $(Boc)_2O$ (0.38 mmol) were added into the solution and stirred overnight at 55 ºC. Upon completion, the reaction mixture was concentrated *in vacuo*. Purification by flash column chromatography (elution with a gradient of DCM/MeOH from 30:1 to 10:1 for **8a** and **8b**, elution with a gradient of DCM/MeOH from 100:1 to 40:1 for compound **10**) to afford a white solid as desired product.

*5-O-(dodecyl-moxifloxacin)-nebramine 5·HCl (**1a**).* Synthesized following general procedure A. Yield: 8 mg (53%). ^{1}H NMR (500 MHz, deuterium oxide) δ 8.96 (s, 1H, N-C*H* of aromatic ring), 7.58 (d, J = 69.9 Hz, 1H, F-C-C*H* of aromatic ring), 5.67 – 5.53 (m, 1H, C*H* of H-1'), 4.39 – 4.02 (m, 5H, C*H* of cyclopropyl, 2×N-C*H*H of moxifloxacin, C*H* of H-5', C*H* of H-4), 4.02 – 3.56 (m, 13H, OCH_2 of linker, C*H* of H-4', C*H* of moxifloxacin, N-C*H*H of moxifloxacin, C*H* of H-2', N-C*H*H of linker, OCH_3 of moxifloxacin, C*H* of H-5, C*H* of H-6, C*H* of H-1), 3.56 – 3.29 (m, 5H, 2×N-C*H*H of moxifloxacin, CH_2 of H-6', C*H* of H-3), 3.28 – 3.09 (m, 2H, N-C*H*H of moxifloxacin, N-C*H*H of linker), 3.09 – 2.95 (m, 1H, C*H* of moxifloxacin), 2.58 – 2.50 (m, 1H, C*H*H of H-2), 2.39 – 2.28 (m, 1H, C*H*H of H-3'), 2.23 – 2.14 (m, 1H, CH*H* of H-3'), 2.14 – 0.82 (m, 29H, CH*H* of H-2, 10×CH_2 of linker, 2×C-CH_2 of moxifloxacin, 2×CH_2 of cyclopropyl). ^{13}C NMR (125 MHz, deuterium oxide, some carbons are doubling due to fluorine atom) δ 176.37 (CO of quinoline), 169.71 (CO of carboxylic acid), 153.23 (CF of quinoline), 152.25 (CF of quinoline), 151.04 (CH, C-2 of quinoline), 141.77 (C-8 of quinoline), 135.02 (C-7 of quinoline), 134.85 (C-8a of quinoline), 117.57 (C-4a of quinoline), 106.84 (CH, C-5 of quinoline), 106.67 (C-3 of quinoline), 92.08 (C-1'), 82.73 (C-6), 75.07 (C-4), 73.48 (O-CH_2 of linker), 73.22 (C-5'), 72.69 (C-5), 63.83 (C-4'), 62.24 (O-CH_3), 56.96 (N-CH_2 of linker), 54.21 (CH_2 of pyrolidine), 52.41 (CH of pyrolidine), 50.90 (CH_2 of pyrolidine), 49.87 (C-1), 48.94 (C-3), 47.53 (C-2'), 41.36 (N-CH_2 of piperidine), 39.30 (C-6'), 36.00 (CH of cyclopropyl),

35.56 (CH of pyrolidine), 29.64 (O-CH_2-$\underline{C}H_2$ of linker), 29.27 (CH_2 of linker), 29.12 (CH_2 of linker), 28.99 (C-3'), 28.82 (CH_2 of linker), 28.70 (CH_2 of linker), 28.21 (CH_2 of linker), 28.06 (C-2), 27.92 (CH_2 of linker), 26.04 (CH_2 of linker), 25.30 (CH_2 of linker), 20.15 (CH_2 of piperidine), 17.95 (CH_2 of piperidine), 9.90 (CH_2 of cyclopropyl), 8.17 (CH_2 of cyclopropyl). MALDI-TOF-MS *m/e* calcd for $C_{45}H_{72}FN_7O_9Na$ $[M+Na]^+$: 896.5273, found: 896.5290.

*5-O-(dodecyl-ciprofloxacin)-nebramine 5·HCl (**1b**).* Synthesized following general procedure A. Yield: 13.4 mg (32%). ^{1}H NMR (500 MHz, deuterium oxide) δ 8.67 (s, 1H, N-C*H* of aromatic ring), 7.62 – 7.44 (m, 2H, C-C*H* of aromatic ring), 5.58 (d, J = 3.2 Hz, 1H, anomeric H-1'), 4.14 – 4.06 (m, 2H, H of piperazine), 4.05 – 3.94 (m, 3H, NC-H of cyclopropyl, C-H of C-O of linker), 3.89 – 3.72 (m, 7H, H-6, H-2', H-4', 2C-H of C-O of linker, 2C-H of C-N of linker), 3.69 – 3.58 (m, 2H, H-1, H-5'), 3.48 – 3.26 (m, 9H, H-3, 2H of H-6', 4H of piperazine, 2C-H of linker), 2.53 (m, 1H, H-2), 2.32 (m, 1H, H-3'), 2.16 (m, 1H, H-3'), 1.96 (m, 1H, H-2), 1.88 – 1.78 (m, 2H, C-H of linker), 1.69 – 1.58 (m, 2H. C-H of linker), 1.50 – 1.28 (m, 18H, CH_2 of cyclopropyl and CH_2 of linker), 1.23 (m, 2H, CH_2 of cyclopropyl). ^{13}C NMR (125 MHz, deuterium oxide) δ 176.11 (CO of quinoline), 169.10 (CO of carboxylic acid), 154.48 (CF of quinoline), 152.48 (CF of quinoline), 148.48 (CH, C-2 of quinoline), 144.32 (C-7 of quinoline), 144.24 (C-7 of quinoline), 139.10 (C-8a of quinoline), 118.99 (C-4a of quinoline), 110.97 (C-5 of quinoline), 110.78 (C-5 of quinoline), 106.85 (C-8 of quinoline), 105.83 (C-3 of quinoline), 92.05 (C-1'), 82.69 (C-5), 74.99 (C-4), 73.59 (*O*-CH_2 of linker), 73.19 (C-5'), 72.68 (C-6), 63.86 (C-4'), 57.17(*N*-CH_2 of linker), 51.46 (*N*-CH_2 of piperazine), 49.87 (C-1), 48.96 (C-3), 47.54 (C-2'), 46.61(*N*-CH_2 of piperazine), 39.33 (C-6'), 36.28 (CH of cyclopropyl), 29.55 (*O*-CH_2-$\underline{C}H_2$ of linker), 28.93 (CH_2 of linker), 28.89 (CH_2 of linker), 28.82 (C-3'), 28.81 (CH_2 of linker), 28.68 (CH_2 of linker), 28.34 (CH_2 of linker), 27.90 (CH_2 of linker), 25.85 (CH_2 of linker), 25.33 (CH_2

of linker), 23.43 (CH_2 of linker), 7.60 (CH_2 of cyclopropyl). MALDI-TOF-MS *m/e* calcd for $C_{41}H_{66}FN_7O_8Na$ $[M+Na]^+$: 826.4851, found: 826.4820.

*5-O-((10-(4-(naphthalen-1-ylmethyl)piperazin-1-yl)docyl)-nebramine 6·HCl (**2**).* Compound **10** (9 mg, 0.008 mmol) was dissolved in TFA (0.5 mL) and H_2O (0.25 mL) solution and then stirred for 2 h at room temperature. The solvent was removed under reduced pressure to give a white residue. Amount of 5 mL of methanol and ether solution (1:20, *v*/*v*) was added to this residue in several portions and then the solvent was decanted to get the final product **2** as TFA salt. Stoichiometric amount of HCl aqueous solution was added into it before lyophilizing the solution to afford a white solid as the final product **2** as HCl salt. Yield: 5.4 mg (72%). ^{1}H NMR (500 MHz, deuterium oxide) δ 8.27 – 8.21 (m, 1H), 8.07 – 7.97 (m, 2H), 7.72 – 7.61 (m, 2H), 7.61 – 7.54 (m, 2H), 5.56 (d, J = 3.1 Hz, 1H, anomeric C*H* of H-1'), 4.22 (s, 2H, CH_2 of naphthylmethyl), 4.08 – 3.93 (m, 3H), 3.88 – 3.48 (m, 8H), 3.43 – 3.29 (m, 4H), 3.28 – 2.78 (m, 7H), 2.56 – 2.45 (m, 1H, C*H*H of H-2), 2.34 – 2.27 (m, 1H, C*H*H of H-3'), 2.18 – 2.10 (m, 1H, C*H*H of H-3'), 1.92 – 1.82 (m, 1H, C*H*H of H-2), 1.74 – 1.66 (m, 2H, N-CH_2-CH_2 of linker), 1.66 – 1.58 (m, 2H, O-CH_2-CH_2 of linker), 1.41 – 1.24 (m, 12H, 6×CH_2 of linker).^{13}C NMR (125 MHz, deuterium oxide) δ 133.69 (C of naphthyl), 131.88 (C of naphthyl), 129.30 (CH of naphthyl), 129.13 (CH of naphthyl), 128.82 (CH of naphthyl), 126.77 (CH of naphthyl), 126.33 (CH of naphthyl), 125.53 (CH of naphthyl), 123.90 (CH of naphthyl), 92.10 (C-1'), 82.70 (C-6), 75.26 (C-4), 73.47 (O-CH_2 of linker), 73.11 (C-5'), 72.67 (C-5), 63.73 (C-4'), 58.24 (CH_2 of naphthylmethyl), 56.82 (4×CH_2 of piperazine, N-CH_2 of linker), 49.82 (C-1), 48.85 (C-3), 47.47 (C-2'), 39.18 (C-6'), 29.48 (O-CH_2-$\underline{C}H_2$ of linker), 28.80 (C-3'), 28.70 (2×CH_2 of linker), 28.55 (CH_2 of linker), 28.23 (CH_2 of linker), 28.03 (C-2), 25.77 (CH_2 of linker), 25.26 (CH_2 of linker),

23.46 (N-CH_2-$\underline{C}H_2$ of linker). MALDI-TOF-MS *m/e* calcd for $C_{37}H_{62}N_6O_5Na$ $[M+Na]^+$: 693.468, found: 693.469.

5-O-(dodecyl-moxifloxacin methyl ester)-1,3,2′,6′,3″-penta-N-(tert-butoxycarbonyl)-4′,2″,4″,6″-tetra-O-TBDMS−tobramycin (**7a**). Synthesized following general procedure B. Yield: 764 mg (93%). ^{1}H NMR (500 MHz, methanol-d_4) δ 8.71 (s, 1H), 7.68 (d, J = 14.1 Hz, 1H), 5.51 – 5.39 (m, 2H, anomeric H), 4.26 – 4.17 (m, 1H), 4.16 – 4.08 (m, 1H), 4.07 – 3.89 (m, 3H), 3.87 (s, 3H), 3.82 – 3.52 (m, 16H), 3.53 – 3.35 (m, 5H), 3.31 – 3.25 (m, 1H), 2.87 – 2.78 (m, 1H), 2.66 – 2.55 (m, 1H), 2.52 – 2.35 (m, 3H), 2.15 – 2.00 (m, 1H), 1.99 – 1.90 (m, 1H), 1.90 – 1.81 (m, 1H), 1.80 – 1.72 (m, 1H), 1.72 – 1.16 (m, 72H), 1.03 – 0.90 (m, 37H), 0.26 – 0.06 (m, 24H). ^{13}C NMR (125 MHz, methanol-d_4, some carbons are doubling due to fluorine atom) δ 174.88, 174.86, 166.74, 158.19, 158.03, 157.49, 157.44, 157.00, 155.93, 153.95, 152.25, 142.63, 142.57, 137.98, 137.89, 135.13, 121.96, 121.90, 109.81, 108.78, 108.59, 96.80 (2C, anomeric C), 86.59, 80.66, 80.57, 80.32, 80.16, 79.52, 78.28, 75.22, 74.83, 73.57, 72.62, 68.81, 65.12, 62.90, 61.65, 57.67, 56.98, 55.53, 55.48, 52.95, 52.08, 51.78, 50.17, 49.93, 42.14, 41.20, 38.54, 36.69, 31.90, 31.24, 30.84, 30.76, 30.73, 30.70, 29.29, 29.24, 29.12, 28.96, 28.91, 28.90, 28.71, 28.10, 27.69, 27.02, 26.93, 26.73, 26.68, 26.64, 26.54, 26.21, 25.18, 23.85, 19.52, 19.11, 18.97, 18.93, 10.00, 9.96, -3.26, -3.82, -3.93, -4.16, -4.21, -4.42, -4.62, -4.87. MALDI-TOF-MS m/e calcd for $C_{101}H_{181}FN_8O_{23}Si_4Na$ $[M+Na]^+$: 2028.2198, found: 2028.2174.

5-O-(dodecyl-ciprofloxacin methyl ester)-1,3,2′,6′,3″-penta-N-(tert-butoxycarbonyl)-4′,2″,4″,6″-tetra-O-TBDMS−tobramycin (**7b**). Synthesized following general procedure B. Yield: 724 mg (69%). ^{1}H NMR (300 MHz, $CDCl_3$) δ 8.52 (s, 1H), 8.00 (d, J = 13.2 Hz, 1H), 7.26 (d, J = 7.1 Hz, 1H), 5.29 – 5.00 (m, 4H), 4.88 – 4.70 (m, 1H), 4.64 – 4.50 (m, 1H), 4.34 – 4.00 (m, 3H), 3.90 (s, 3H), 3.84 – 3.11 (m, 22H), 2.75 – 2.65 (m, 3H), 2.50 – 2.39 (m, 2H), 2.06

– 1.94 (m, 1H), 1.60 – 1.08 (m, 76H), 0.97 – 0.81 (m, 36H), 0.19 – -0.01 (m, 24H). ^{13}C NMR (75 MHz, $CDCl_3$, some carbons are doubling due to fluorine atom) δ 173.11, 166.49, 155.08, 148.38, 144.56, 138.00, 123.02, 113.39, 113.08, 109.98, 104.79, 79.44, 79.25, 58.67, 52.89, 52.09, 49.83, 49.77, 48.33, 34.53, 30.08, 29.74, 29.67, 28.65, 28.52, 28.42, 27.59, 26.72, 26.16, 26.02, 25.80, 18.53, 18.36, 18.12, 17.93, 8.15, -3.77, -4.17, -4.87, -4.95, -5.05, -5.20. MALDI-TOF-MS m/e calcd for $C_{97}H_{175}FN_8O_{22}Si_4Na$ $[M+Na]^+$: 1958.178, found: 1958.125.

*5-O-(dodecyl-moxifloxacin methyl ester)-1,3,2',6'-tetra-N-(tert-butoxycarbonyl)-nebramine (**8a**)*. Synthesized following general procedure C. Yield: 40 mg (65%, two steps). ^{1}H NMR (300 MHz, methanol-d_4) δ 8.71 (s, 1H), 7.68 (d, J = 14.5 Hz, 1H), 5.31 – 5.25 (m, 1H, anomeric H-1'), 4.16 – 4.06 (m, 1H), 3.99 – 3.88 (m, 1H), 3.87 (s, 3H), 3.76 – 3.51 (m, 12H), 3.50 – 3.36 (m, 4H), 3.31 – 3.23 (m, 3H), 2.92 – 2.81 (m, 1H), 2.68 – 2.58 (m, 1H), 2.50 – 2.37 (m, 3H), 2.07 – 1.90 (m, 2H), 1.89 – 1.40 (m, 46H), 1.39 – 1.14 (m, 19H), 0.99 (dd, J = 4.7, 2.4 Hz, 1H). ^{13}C NMR (75 MHz, methanol-d_4, some carbons are doubling due to fluorine atom) δ 174.97, 166.77, 158.24, 157.95, 157.49, 156.56, 153.28, 152.30, 142.59, 142.49, 138.06, 137.91, 135.19, 121.93, 121.83, 109.81, 108.84, 108.52, 97.46 (anomeric C), 87.23, 80.81, 80.42, 80.25, 78.93, 77.23, 74.00, 73.26, 67.14, 63.05, 61.65, 56.74, 55.35, 52.29, 52.12, 49.00, 41.20, 38.59, 35.93, 34.84, 31.55, 30.80, 30.69, 30.51, 28.90, 28.88, 28.81, 28.76, 28.49, 27.85, 27.31, 24.98, 23.59, 21.01, 13.95, 10.01, 9.91. MS (ESI) *m/e* calcd for $C_{66}H_{107}FN_7O_{17}$ $[M+H]^+$: 1289.6, found: 1289.4.

*5-O-(dodecyl-ciprofloxacin methyl ester)-1,3,2',6'-tetra-N-(tert-butoxycarbonyl)-nebramine (**8b**)*. Synthesized following general procedure C. Yield: 52 mg (31%, two steps). ^{1}H NMR (300 MHz, $CDCl_3$) δ 8.54 (s, 1H), 7.99 (d, J = 13.2 Hz, 1H), 7.28 (d, J = 7.3 Hz, 1H), 5.36 – 5.12 (m, 2H), 5.09 – 4.88 (m, 3H), 3.96 – 3.21 (m, 25H), 3.18 – 3.08 (m, 2H), 2.78 – 2.68 (m,

4H), 2.52 – 2.43 (m, 2H), 2.37 – 2.25 (m, 1H), 2.21 – 2.08 (m, 1H), 1.72 – 1.03 (m, 76H). ^{13}C NMR (75 MHz, $CDCl_3$) δ 173.28, 166.28, 157.97, 155.12, 148.41, 144.56, 138.06, 122.91, 113.37, 113.07, 109.80, 104.90, 80.26, 79.40, 58.62, 52.85, 52.09, 49.65, 46.06, 34.66, 30.16, 29.60, 29.54, 29.50, 28.45, 28.42, 28.35, 28.33, 27.46, 26.52, 26.09, 8.18. MALDI-TOF-MS *m/e* calcd for $C_{62}H_{100}FN_7O_{16}Na$ $[M+Na]^+$: 1240.711, found: 1240.658.

*5-O-((10-(4-(naphthalen-1-ylmethyl)piperazin-1-yl)docyl)-1,3,2',6'-tetra-N-(tert-butoxycarbonyl)-nebramine (**10**).* Synthesized following general procedure C. Yield: 27 mg (53%, two steps). ^{1}H NMR (500 MHz, methanol-d_4) δ 8.29 – 8.25 (m, 1H), 7.87 – 7.83 (m, 1H), 7.81 – 7.77 (m, 1H), 7.52 – 7.44 (m, 2H), 7.44 – 7.37 (m, 2H), 5.25 (d, J = 3.8 Hz, 1H, anomeric C*H* of H-1'), 3.94 (s, 2H, CH_2 of naphthylmethyl), 3.93 – 3.88 (m, 1H), 3.70 – 3.47 (m, 5H), 3.47 – 3.31 (m, 5H), 3.28 – 3.19 (m, 3H), 2.83 – 2.44 (m, 8H), 2.44 – 2.37 (m, 1H), 2.01 – 1.92 (m, 1H), 1.92 –1.85 (m, 1H), 1.65 – 1.58 (m, 1H), 1.55 – 1.48 (m, 4H), 1.48 – 1.38 (m, 36H, 4 × *t*-Bu of Boc), 1.33 – 1.22 (m, 12H, 6 × CH_2 of linker). ^{13}C NMR (125 MHz, methanol-d_4) δ 159.35, 158.27, 157.98, 157.49, 135.47, 134.63, 133.92, 129.42, 129.29, 128.92, 126.80, 126.70, 126.08, 125.78, 97.43, 87.27, 80.83, 80.43, 80.29, 78.92, 77.28, 74.04, 73.25, 67.14, 67.13, 61.63, 59.62, 54.06, 53.50, 52.85, 50.84, 50.29, 42.28, 35.95, 34.82, 31.54, 30.77, 30.61, 30.59, 30.56, 28.89, 28.86, 28.80, 28.75, 28.57, 27.30, 27.14. MALDI-TOF-MS *m/e* calcd for $C_{57}H_{95}N_6O_{13}$ $[M+H]^+$: 1071.696, found: 1071.716.

4.2 Microbiology

4.2.1 Clinical Isolates

Clinically-relevant bacterial strains were collected from the Canadian National Intensive Care Unit (CAN-ICU) study [39] and Canadian Ward Surveillance (CANWARD) studies [40,41]. All pathogens obtained from CAN-ICU and CANWARD studies have received ethics approval from the University of Manitoba Ethics Committee. In addition, participating Canadian health centers have obtained appropriate ethics approval to submit clinical specimens.

4.2.2 Antimicrobial Susceptibility Testing

The antimicrobial activity of the compounds against a panel of bacteria was evaluated by broth microdilution assay in accordance with the Clinical and Laboratory Standards Institute (CLSI) guidelines [32]. was performed to assess the *in vitro* antibacterial activity. Bacterial cultures grown overnight were diluted in saline to achieve a 0.5 McFarland turbidity, followed by 1:50 dilution in Mueller-Hinton broth (MHB) for inoculation to a final concentration of 5×10^5 CFU/mL. The minimum inhibitory concentrations (MICs) of the antimicrobial agents were determined using 96-well plates containing 2-fold serial dilutions with MHB and incubated with equal volumes of inoculum for 18 h at 37 ºC. MIC was determined as the lowest concentration to inhibit visible bacterial growth in the form of turbidity, which was confirmed using an EMax Plus microplate reader (Molecular Devices, San Jose, CA, USA) at a wavelength of 590 nm. The wells containing MHB broth with or without bacterial cells were used as positive or negative controls, respectively.

4.2.3 Checkerboard Assay

The checkerboard method [42] was used to assess synergism in all tested combinations. Fractional inhibitory concentrations (FICs) were calculated as follows: $FIC_{antibiotic}$ = $MIC_{combo}/MIC_{antibiotic\ alone}$; $FIC_{adjuvant}$ = $MIC_{combo}/MIC_{adjuvant\ alone}$, where MIC_{combo} is the lowest inhibitory concentration of drug in the presence of the adjuvant. The FIC index was calculated by adding the FIC values. FIC indices (FICI) were interpreted as follows: ≤0.5, synergy, 0.5< FICI ≤4.0, no interaction, and ≥4.0, antagonism [28].

4.2.4 Time-kill Curve Assay

The kinetics of bacterial killing was measured using *P. aeruginosa* PAO1 and PA259 as previously described [12]. Overnight bacterial culture was diluted in saline to 0.5 McFarland turbidity and then 1:50 diluted in Luria-Bertani broth (LB). The cell suspension (*P. aeruginosa* PAO1) was incubated with the combination of ¼ × MIC (8 μg/mL) or ½ × MIC (16 μg/mL) of hybrid **1a** with 4 μg/mL of minocycline. Untreated cells in media and cells treated with 4 × MIC (4 μg/mL) of colistin were used as negative and positive controls respectively (Fig. 2A). Respect to the time-kill curve of **1b** (Fig. 2B), the cell suspension (*P. aeruginosa* PA259) was incubated with rifampicin (4 μg/mL) and **1b** (4 μg/mL) alone or the combination of **1b** with rifampicin at various concentrations as shown in Fig 2B. Samples were incubated at 37 °C for 24 h. At specific intervals (Fig. 2), aliquots (100 μL) were removed from the samples, serially diluted in PBS, and plated on LB agar plates. Bacterial colonies were formed and counted after 20 h of incubation at 37 °C.

4.2.5 Hemolytic Assay

The hemolytic activities of the newly synthesized compounds were determined and quantified as the amount of hemoglobin released by lysing porcine erythrocytes. Fresh blood drawn from the antecubital vein of a pig (Animal Care and Use Program, University of Manitoba) was centrifuged at 1000 g at 4 °C for 10 mins, washed with PBS thrice and resuspended in the same buffer. The final cell concentration used was 3×10^8 cells/mL. Compounds were serially diluted with PBS and added to wells in a 96-well plate at twice the desired concentrations. Equal volumes of erythrocyte solution were then added to each well and incubated at 37 °C for 1 h. Intact erythrocytes were subsequently pelleted by centrifuging at 1000 g at 4 °C for 10 mins, and the supernatants were transferred to a new 96-well plate. Hemoglobin release was determined by measuring the absorbance on EMax® Plus microplate reader (Molecular Devices, Sunnyvale, CA, USA) at 570 nm. Blood cells in PBS (0% hemolysis) and 0.1 % Triton X-100 (100% hemolysis) were used as negative and positive controls, respectively. Percent hemolysis was calculated as [% hemolysis = (X – 0%) / (100% – 0%)], where X is the optical density values of the compounds at different concentrations.

4.2.6 Galleria mellonella Model of P. aeruginosa Infection

In vivo synergistic effects were determined using *Galleria mellonella* infection model, as previously described [8]. Briefly, worms were purchased from The Worm Lady® Live Feeder (ON, Canada), stored in their natural habitat at 16 ºC, and used within 10 days of delivery. The worms (average weight of 250 mg) were used for tolerability and efficacy studies. Tolerability study was performed by injecting 10 μL of antimicrobial agents only at concentrations

equivalent to 100 mg/kg or 200 mg/kg. The worms (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 worms within 12 – 18 h was first determined, which is approximately 5 CFU. Overnight grown culture of MDR *P. aeruginosa* PA264 isolate was standardized to 0.5 McFarland standard and diluted in PBS to a final concentration of 5×10^2 CFU/mL. 10 μL of this solution (~ 5 CFU) was injected into each worm and incubated for 2 h at 37 °C. After the 2 h challenge, worms in monotherapy experimental groups (fifteen worms per group) were treated with 10 µL injection of rifampicin, compound **1b**, or PBS alone. The worms in combination therapy groups were treated with rifampicin + compound **1b** (25 + 25 mg/kg, 50 + 50 mg/kg, or 75 + 75 mg/kg). Worms treated with 10 µL PBS negative control. The worms were incubated at 37 °C in Petri dishes lined with filter paper and scored for survivability every 6 h for up to 24 h. This experiment was repeated to give a total of thirty worms (n = 30) in each case. Survival data curves were plotted using Kaplan-Meier survival analysis. Worms were considered dead if they do not respond to touch.

4.2.7 Tetracycline Uptake Assay

Fluorescence-based tetracycline uptake assay in bacterial cells was performed following previously reported method [36]. Culture of *P. aeruginosa* PAO1 was grown to OD_{600} = 0.6 followed by washing and re-suspending it in ¼ volume of 10 mM HEPES, pH 7.2, 100 μL/well cell suspension was treated with varying concentrations of test compounds in the presence of 128 μg/mL of tetracycline. Fluorescence was recorded at a continuous interval of 1 min for 30 min at room temperature on a FlexStation 3 (Molecular Devices, Sunnyvale, USA) microplate reader at

the excitation wavelength of 405 nm and emission wavelength of 535 nm. Experiments were performed in triplicates. Averages of triplicate experiments are shown in Fig. 6.

Conflicts of Interest

There are no conflicts of interest to declare.

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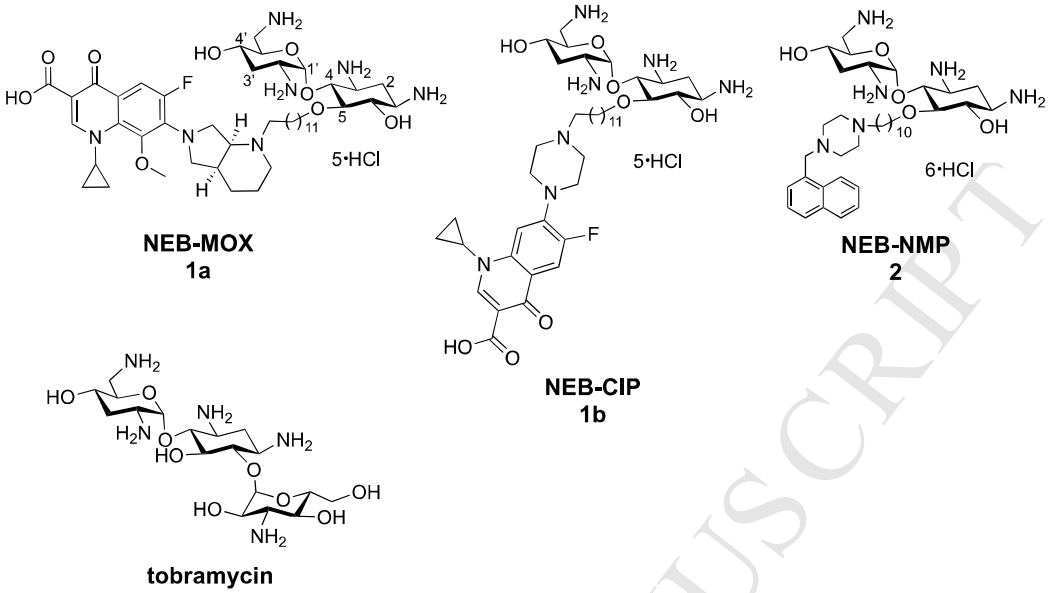


Fig. 1. Structures of the nebramine-moxifloxacin (NEB-MOX) hybrid **1a**, nebramine-ciprofloxacin (NEB-CIP) hybrid **1b**, nebramine-NMP (NEB-NMP) hybrid **2**, and tobramycin.

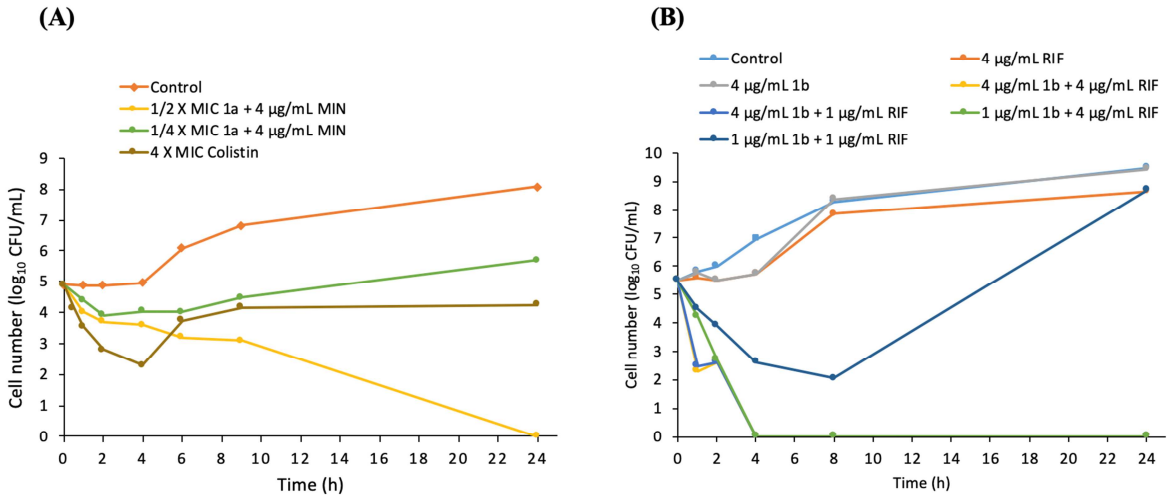


Fig. 2. (A) Time killing kinetics of minocycline (MIN) (4 μg/mL) in combination with NEB-MOX (**1a**) at ½ × MIC (16 μg/mL) or ¼ × MIC (8 μg/mL) against *P. aeruginosa* PAO1.

Untreated cells in media and cells treated with 4 × MIC (4 μg/mL) of colistin were used as negative and positive controls respectively. (B) Time killing kinetics of rifampicin in combination with NEB-CIP (**1b**) at various concentrations against XDR *P. aeruginosa* PA259. Untreated cells in media was used as a negative control. MIC of **1b** is >128 μg/mL and MIC of RIF is 16 μg/mL against *P. aeruginosa* PA259 strain.

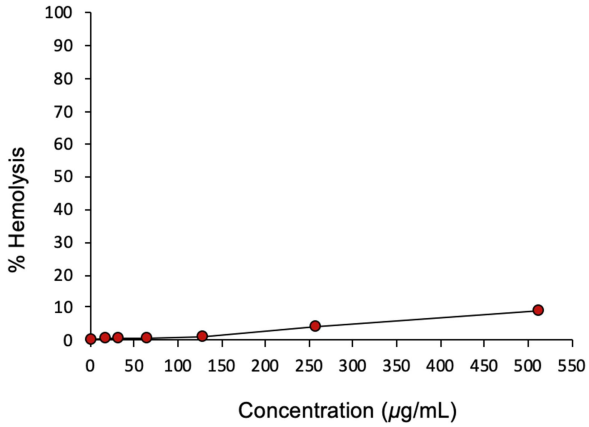


Fig. 3. Hemolytic activity of NEB-CIP (**1b**). Triton X-100 (0.1%) was employed as positive control to calculate the percentage of hemolysis.

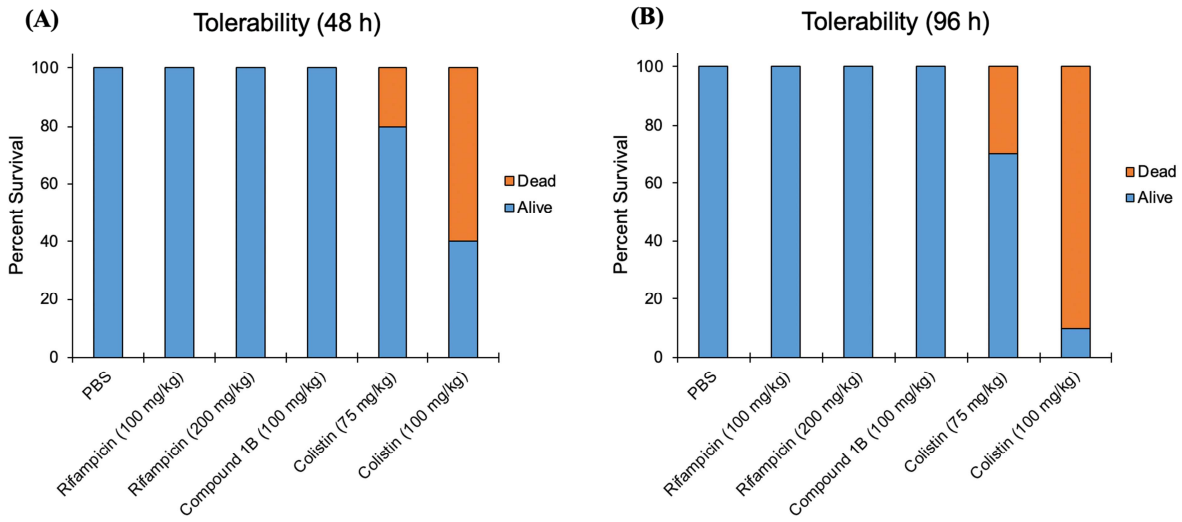


Fig. 4. Tolerability dosages of NEB-CIP (**1b**), rifampicin, and colistin on *G. mellonella* larvae (*n* = 10). Larvae survived up to 96 h when administered with 100 mg/kg dosage of **1b**.

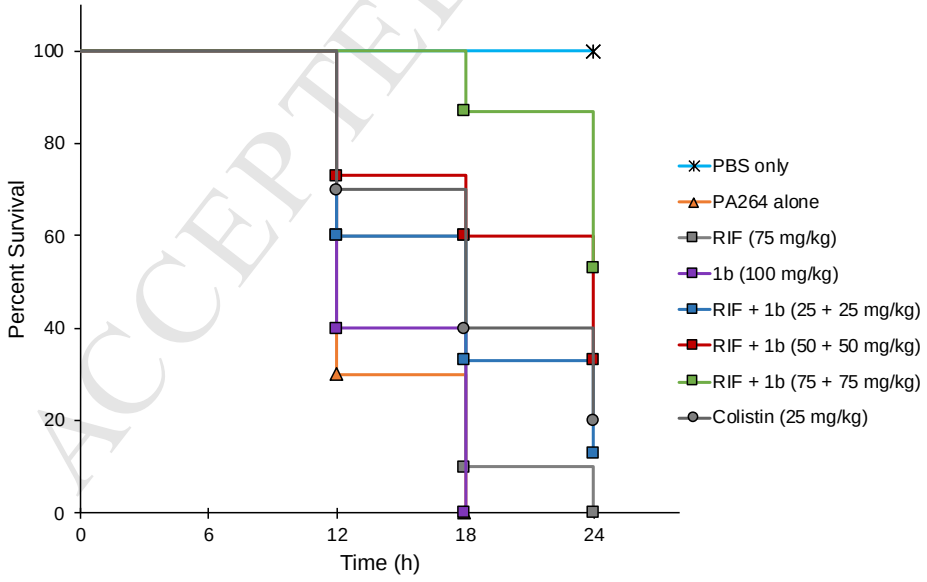


Fig. 5. Effect of treatment of *G. mellonella* larvae (inoculated with ~ 5 CFU of XDR *P. aeruginosa* PA264, n = 30 for each drug and dose combination) with rifampicin (75 mg/kg) and **1b** (100 mg/kg) alone, or rifampicin in combination with **1b** (25 + 25 mg/kg, 50 + 50 mg/kg, and 75+75 mg/kg) on survival. Single dose treatment administered at 0 h (2 h after inoculation).

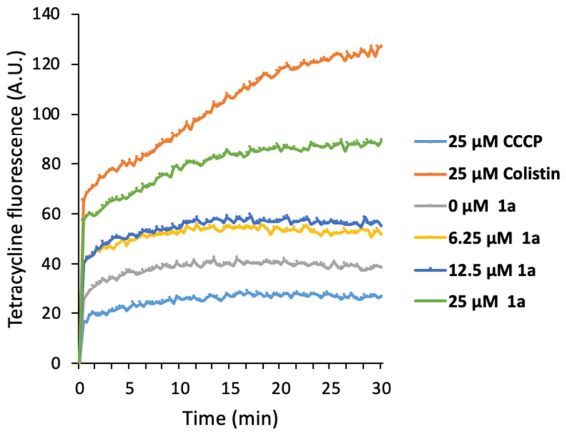
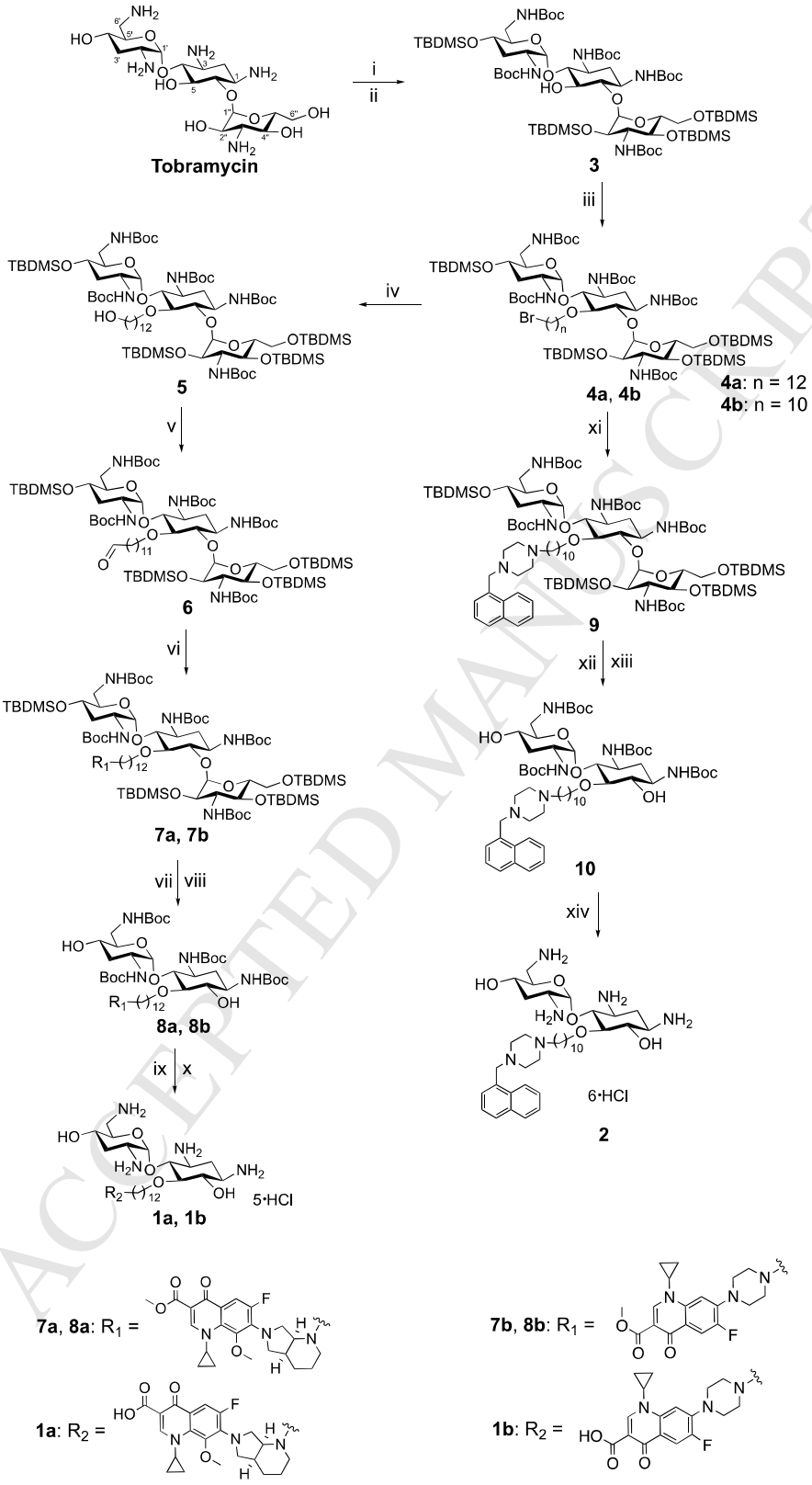


Fig. 6. Tetracycline uptake in *P. aeruginosa* PAO1 in the presence of increasing concentrations of NEB-MOX **1a**. Concentration of tetracycline was 128 μg/mL. Averages of triplicate experiments are shown.



Scheme 1. Synthesis of NEB-MOX (**1a**), NEB-CIP (**1b**), and NEB-NMP (**2**). Reagents and conditions: (i) $(Boc)_2O$, Et_3N, MeOH/H_2O (2:1), rt to 55 ºC, overnight, 97%. (ii) TBDMS-Cl, 1-methylimidazole, DMF, N_2, rt, 4 days, 90%. (iii) 1,12-dibromododecane or 1,10-dibromodecane, KOH, TBAHS, toluene, rt, overnight, 78–81%. (iv) Cs_2CO_3, H_2O, DMF, 75 ºC, 8 h, 67%. (v) PCC, NaOAc, DCM, rt, 2 h, 90%. (vi) moxifloxacin methyl ester, $NaBH(OAc)_3$, AcOH, DCE, rt, 93% for **7a**, 69% for **7b**; (vii) 40% HCl, MeOH, 70 ºC, 48 h. (viii) $(Boc)_2O$, Et_3N, MeOH, 55 ºC, overnight, 65% for **8a**, 31% for **8b** (two steps). (ix) 2 N LiOH, MeOH, rt, 30 min. (x) TFA/H_2O 2:1 (v/v), rt, 2 h, 52% for **1a**, 32% for **1b**. (xi) NMP (1-(1-naphthylmethyl)piperazine), K_2CO_3, DMF, 75 ºC, 50%. (xii) 40% HCl, MeOH, 65 ºC, 48 h. (xiii) $(Boc)_2O$, Et_3N, MeOH/H_2O (2/1, v/v), rt to 55 ºC, overnight, 53% (two steps). (xiv) TFA/H_2O (2/1, v/v), rt, 30 min, 72%.

Table 1 Combination studies of NEB-MOX **1a** with moxifloxacin (MOX), minocycline (MIN) or rifampicin (RIF) against wild-type *P. aeruginosa* PAO1 strain.

Antibiotic (MIC[a])	Hybrid (MIC[a])	FICI	Absolute MIC[b]	Potentiation (fold)[c]
MOX (1)	**1a** (32)	0.25	0.13	8
MIN (8)	**1a** (32)	0.38	1	8
RIF (8)	**1a** (32)	0.07	≤0.03	≥256

[a] All MIC data presented in μg/mL.

[b] Absolute MIC (μg/mL) of antibiotic was determined in the presence of 8 μg/mL (7.5 μM) of hybrid **1a**.

[c] Antibiotic activity potentiation at 8 μg/mL (7.5 μM) of hybrid **1a**.

Table 2 Combination studies of NEB-MOX **1a** with moxifloxacin (MOX) or ciprofloxacin (CIP) against MDR/XDR *P. aeruginosa* clinical isolates.

P. aeruginosa	Antibiotic (MIC[a])	Hybrid (MIC[a])	FICI	Absolute MIC[b]	Potentiation (fold)[c]
PA262-101856[d]	MOX (64)	**1a** (>256)	0.13<x<0.16	8	8
PA262-101856[d]	CIP (32)	**1a** (>256)	0.25<x<0.28	8	4
PA260-97103[d]	MOX (64)	**1a** (32)	0.13	1	64
PA260-97103[d]	CIP (32)	**1a** (32)	0.25	4	8
100036[d]	MOX (128)	**1a** (128)	0.08	8	16
100036[d]	CIP (32)	**1a** (128)	0.56	16	2
101885[d]	MOX (64)	**1a** (128)	0.25	16	4
101885[d]	CIP (16)	**1a** (128)	>1	NA	NA
PA259-96918[d]	MOX (256)	**1a** (>256)	0.06<x<0.07	16	16
PA259-96918[d]	CIP (128)	**1a** (>256)	0.25<x<0.27	32	4

PA264-104354[d]	MOX (128)	**1a** (256)	0.09	8	16
PA264-104354[d]	CIP (32)	**1a** (256)	0.16	4	8
91433[e]	MOX (8)	**1a** (32)	0.31	0.5	16
91433[e]	CIP (1)	**1a** (32)	0.25	0.06	16
101243[e]	MOX (8)	**1a** (64)	0.16	0.25	32
101243[e]	CIP (1)	**1a** (64)	0.25	0.13	8

[a] All MIC data presented in μg/mL.

[b] Absolute MIC (μg/mL) of antibiotic was determined in the presence of 8 μg/mL (7.5 μM) of hybrid **1a**.

[c] Antibiotic activity potentiation at 8 μg/mL (7.5 μM) of hybrid **1a**.

[d] with ^{83}Thr to ^{83}Ile mutation in gyr A [8].

[e] without ^{83}Thr to ^{83}Ile mutation in gyr A [8].

Table 3 Combination studies of NEB-MOX **1a** with minocycline (MIN) against MDR/XDR *P. aeruginosa* clinical isolates.

P. aeruginosa	Antibiotic (MIC[a])	Hybrid (MIC[a])	FICI	Absolute MIC[b]	Potentiation (fold)[c]
PA262-101856	MIN (64)	**1a** (>256)	0.03<x<0.05	2	32
PA260-97103	MIN (8)	**1a** (32)	0.09	0.25	32
100036	MIN (16)	**1a** (128)	0.06	0.5	32
101885	MIN (16)	**1a** (128)	0.07	1	16
PA259-96918	MIN (16)	**1a** (>256)	0.03<x<0.04	0.5	32
PA264-104354	MIN (32)	**1a** (256)	0.05	0.5	64
91433	MIN (16)	**1a** (32)	0.19	0.5	32
101243	MIN (4)	**1a** (64)	0.13	0.25	16

[a] All MIC data presented in μg/mL.

[b] Absolute MIC (μg/mL) of antibiotic was determined in the presence of 8 μg/mL (7.5 μM) of hybrid **1a**.

[c] Antibiotic activity potentiation at 8 μg/mL (7.5 μM) of hybrid **1a**.

Table 4 Combination studies of NEB-MOX **1a** with rifampicin (RIF) against MDR/XDR *P. aeruginosa* clinical isolates.

P. aeruginosa	Antibiotic (MIC[a])	Hybrid (MIC[a])	FICI	Absolute MIC[b]	Potentiation (fold)[c]
PA262-101856	RIF (1024)	**1a** (>256)	0.008<x<0.02	4	512
PA260-97103	RIF (4)	**1a** (32)	0.06	≤0.03	≥128
100036	RIF (8)	**1a** (128)	0.01	0.03	256
101885	RIF (8)	**1a** (128)	0.05	0.06	128
PA259-96918	RIF (8)	**1a** (>256)	0.004<x<0.01	≤0.03	≥256
PA264-104354	RIF (16)	**1a** (256)	0.02	≤0.06	≥256
91433	RIF (16)	**1a** (32)	0.16	0.13	128
101243	RIF (4)	**1a** (64)	0.13	0.13	32

[a] All MIC data presented in μg/mL.

[b] Absolute MIC (μg/mL) of antibiotic was determined in the presence of 8 μg/mL (7.5 μM) of hybrid **1a**.

[c] Antibiotic activity potentiation at 8 μg/mL (7.5 μM) of hybrid **1a**.

Table 5 *In vitro* antibacterial activity of minocycline (MIN) and rifampicin (RIF) alone or in combination with a fixed concentration of 8 μg/mL (7.5 μM) NEB-MOX **1a** against MDR/XDR *P. aeruginosa* clinical isolates (n = 8).

Antimicrobial/Hybrid	MIC_{50}[a] (μg/mL)	MIC_{80}[a] (μg/mL)	MIC Range (μg/mL)
MIN	16 ⊥	32 ⊥	4–64
MIN+**1**	0.5 †	1 †	0.25–2
RIF	8 ⊥	16 ⊥	4–1024
RIF+**1**	0.06 †	0.13 †	≤0.03–4

†, susceptible; ⊥, resistant;

[a] MIC_{50} and MIC_{80} are the MIC that inhibit the growth of 50% or 80% of all (n = 8) tested isolates.

Table 6 Combination studies of NEB-CIP **1b** with antibiotics against wild-type *P. aeruginosa* PAO1 and XDR *P. aeruginosa* strains.

P. aeruginosa	Antibiotic (MIC[a])	Hybrid (MIC[a])	FIC index	Absolute MIC[b]	Potentiation (fold)
PAO1	MIN (16)	**1b** (64)	0.047	0.5	32
PAO1	RIF (16)	**1b** (64)	0.047	≤0.06	≥256
PA259-96918	MIN (16)	**1b** (>128)	0.031<x<0.063	0.5	32
PA259-96918	RIF (16)	**1b** (>128)	0.008<x<0.016	≤0.06	≥256
PA264-104354	MIN (32)	**1b** (128)	0.039	1	32
PA264-104354	RIF (16)	**1b** (128)	0.039	0.13	128

[a] All MIC data presented in μg/mL.

[b] Absolute MIC (μg/mL) of antibiotic was determined in the presence of 4 μg/mL (4.1 μM) of hybrid **1b**.

[c] Antibiotic activity potentiation at 4 μg/mL (4.1 μM) of hybrid **1b**.

Table 7 Combination studies of NEB-NMP **2** with antibiotics against wild-type *P. aeruginosa* PAO1 and MDR/XDR *P. aeruginosa* strains.

P. aeruginosa	Antibiotic (MIC[a])	Hybrid (MIC[a])	FICI	Absolute MIC[b]	Potentiation (fold)[c]
PAO1	MOX (1)	**2** (256)	0.05	0.03	32
PAO1	MIN (8)	**2** (256)	0.09	0.5	16
PAO1	RIF (16)	**2** (256)	0.02	0.06	256
PA262-101856[d]	MOX (64)	**2** (512)	0.09	4	16
PA262-101856[d]	CIP (32)	**2** (512)	0.13	4	8
PA262-101856[d]	MIN (128)	**2** (512)	0.04	4	32
PA262-101856[d]	RIF (1024)	**2** (512)	0.02	4	256
PA260-97103[d]	MOX (64)	**2** (32)	0.08	0.5	128
PA260-97103[d]	CIP (16)	**2** (32)	0.38	2	8
PA260-97103[d]	MIN (16)	**2** (32)	0.09	0.25	64
PA260-97103[d]	RIF (4)	**2** (32)	0.05	0.06	64
100036[d]	MOX (128)	**2** (>512)	0.063<x<0.07	8	16
100036[d]	CIP (64)	**2** (>512)	0.125<x<0.133	8	8
100036[d]	MIN (64)	**2** (>512)	0.031<x<0.033	2	32
100036[d]	RIF (16)	**2** (>512)	0.004<x<0.012	0.06	256
101885[d]	MOX (64)	**2** (512)	0.07	4	16
101885[d]	CIP (32)	**2** (512)	0.13	4	8
101885[d]	MIN (32)	**2** (512)	0.04	0.5	64
101885[d]	RIF (16)	**2** (512)	0.02	0.13	128
PA259-96918[d]	MOX (512)	**2** (>512)	0.016<x<0.023	8	64
PA259-96918[d]	CIP (256)	**2** (>512)	0.063<x<0.078	16	16

PA259-96918[d]	MIN (32)	**2** (>512)	0.016<x<0.031	0.5	64
PA259-96918[d]	RIF (16)	**2** (>512)	0.004<x<0.006	≤0.06	≥256
91433[e]	MOX (8)	**2** (32)	0.25	1	8
91433[e]	CIP (2)	**2** (32)	0.38	0.25	8
91433[e]	MIN (64)	**2** (32)	0.19	2	32
91433[e]	RIF (16)	**2** (32)	0.50	4	4
101243[e]	MOX (4)	**2** (512)	0.13	0.5	8
101243[e]	CIP (2)	**2** (512)	0.16	0.5	4
101243[e]	MIN (4)	**2** (512)	0.09	0.5	8
101243[e]	RIF (8)	**2** (512)	0.05	0.25	32

[a] All MIC data presented in μg/mL.

[b] Absolute MIC (μg/mL) of antibiotic was determined in the presence of 8 μg/mL (9.0 μM) of hybrid **2**.

[c] Antibiotic activity potentiation at 8 μg/mL (9.0 μM) of hybrid **2**.

[d] with ^{83}Thr to ^{83}Ile mutation in gyr A. [8]

[e] without ^{83}Thr to ^{83}Ile mutation in gyr A. [8]

Table 8 Combination studies of NEB-MOX **1** with minocycline (MIN) or rifampicin (RIF) against MDR *Acinetobacter baumannii*, *Klebsiella pneumoniae*, and *Enterobacter cloacae*.

Organisms	Antibiotic (MIC[a])	Hybrid (MIC[a])	FICI	Absolute MIC[b]	Potentiation (fold)[c]
A. baumannii AB027	MIN (1)	**1a** (>256)	>1	NA	NA
A. baumannii AB027	RIF (1)	**1a** (>256)	0.031<x< 0.047	0.03	32
A. baumannii AB030	MIN (2)	**1a** (>16)	>1	NA	NA
A. baumannii AB030	RIF (1024)	**1a** (>16)	0.031<x<0.281	32	32
A. baumannii AB031	MIN (1)	**1a** (128)	>1	NA	NA
A. baumannii AB031	RIF (1)	**1a** (128)	0.04	0.02	64
A. baumannii 110193	MIN (1)	**1a** (>256)	>1	NA	NA
A. baumannii 110193	RIF (1)	**1a** (>256)	0.031<x<0.047	0.03	32
K. pneumoniae 116381	MIN (64)	**1a** (>256)	0.063<x<0.078	4	16
K. pneumoniae 116381	RIF (1024)	**1a** (>256)	0.002<x<0.006	≤1	≥1024
E. cloacae 117029	MIN (64)	**1a** (32)	0.19	4	16
E. cloacae 117029	RIF (4)	**1a** (32)	0.06	≤0.03	≥128

[a]All MIC data presented in μg/mL.

[b]Absolute MIC (μg/mL) of antibiotic was determined in the presence of 8 μg/mL (7.5 μM) of hybrid **1a**.

[c]Antibiotic activity potentiation at 8 μg/mL (7.5 μM) of hybrid **1a**.

NA, not available (no synergy was observed).

Table 9 Combination studies of NEB-CIP **1b** with minocycline (MIN) or rifampicin (RIF) against wield-type or MDR *Acinetobacter baumannii*, *Escherichia coli*, *Klebsiella pneumoniae*, and *Enterobacter cloacae*.

Organisms	Antibiotic (MIC[a])	Hybrid (MIC[a])	FICI	Absolute MIC	Potentiation (fold)[d]
A. baumannii ATCC 17978	MIN (0.25)	**1b** (128)	0.520	0.13[b]	2
A. baumannii ATCC 17978	RIF (2)	**1b** (128)	0.016	0.008[b]	256
A. baumannii AB92247	MIN (0.125)	**1b** (128)	0.531	0.06[b]	2
A. baumannii AB92247	RIF (2)	**1b** (128)	0.039	0.02[b]	128
A. baumannii AB110193	MIN (1)	**1b** (>128)	>1	NA	NA
A. baumannii AB110193	RIF (1)	**1b** (>128)	0.016<x<0.031	0.02[b]	64
E. coli ATCC 25922	MIN (1)	**1b** (8)	0.504	NA	NA
E. coli ATCC 25922	RIF (4)	**1b** (8)	0.133	0.03[c]	128
E. coli 94474	MIN (64)	**1b** (>128)	0.063<x<0.078	4[b]	16
E. coli 94474	RIF (8)	**1b** (>128)	0.004<x<0.035	0.03[b]	256
E. coli 107115	MIN (32)	**1b** (32)	0.133	2[b]	16
E. coli 107115	RIF (32)	**1b** (32)	0.015<x≤0.020	≤0.13[b]	≥256
K. pneumoniae 113250	MIN (2)	**1b** (128)	0.504	1[b]	2
K. pneumoniae 113250	RIF (32)	**1b** (128)	0.039	0.25[b]	128
K. pneumoniae 113254	MIN (2)	**1b** (128)	0.504	1[b]	2
K. pneumoniae 113254	RIF (16)	**1b** (128)	0.047	0.25[b]	64
K. pneumoniae 116381	MIN (64)	**1b** (>128)	0.063<x<0.070	4[b]	16
K. pneumoniae 116381	RIF (>128)	**1b** (>128)	≤0.039	1[b]	≥128
E. cloacae 117029	MIN (32)	**1b** (32)	0.125	2[b]	16
E. cloacae 117029	RIF (8)	**1b** (32)	0.023	≤0.03[b]	≥256

[a]All MIC data presented in μg/mL.

[b]Absolute MIC (μg/mL) of antibiotic was determined in the presence of 4 μg/mL (4.1 μM) of hybrid **1b**.

[c]Absolute MIC (μg/mL) of antibiotic was determined in the presence of 2 μg/mL (2.0 μM) of hybrid **1b**.

[d]Antibiotic activity potentiation at 8 μg/mL (4.1 μM) or 2 μg/mL (2.0 μM) of hybrid **1b**.

NA, not available (no synergy was observed).

Highlights:

- New amphiphilic nebramine (NEB)-based hybrids were prepared from tobramycin-based (TOB)-based hybrids.
- Potent synergism was found for combinations of NEB-based hybrid adjuvants with multiple classes of antibiotics against multi-drug resistant (MDR) Gram-negative bacilli.
- Combination of NEB-CIP hybrid **1b** with rifampicin protects *Galleria mellonella* larvae from the lethal effects of extensively drug-resistant (XDR) *P. aeruginosa*.