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PII: S0223-5234(19)30349-6

DOI: https://doi.org/10.1016/j.ejmech.2019.04.034

Reference: EJMECH 11269

To appear in: *European Journal of Medicinal Chemistry*

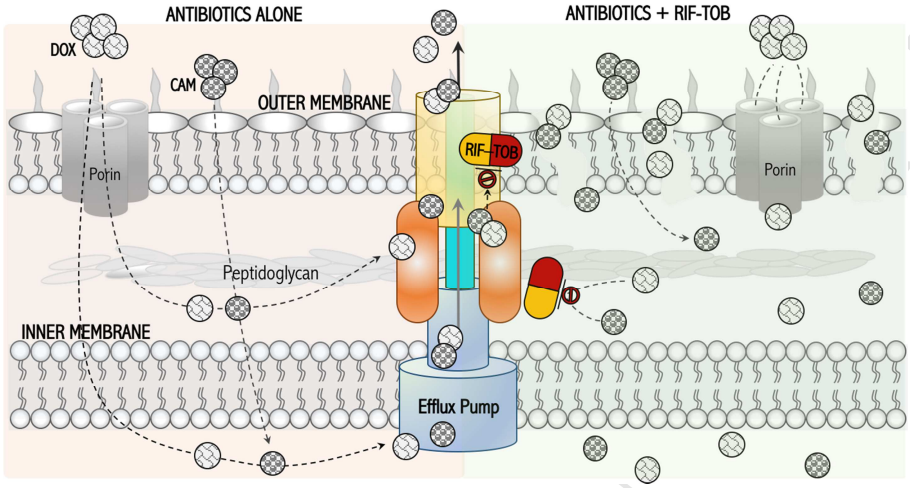
Received Date: 15 January 2019

Revised Date: 1 April 2019

Accepted Date: 13 April 2019

Please cite this article as: T. Idowu, G. Arthur, G.G. Zhanel, F. Schweizer, Heterodimeric Rifampicin–Tobramycin conjugates break intrinsic resistance of *Pseudomonas aeruginosa* to doxycycline and chloramphenicol *in vitro* and in a *Galleria mellonella in vivo* model, *European Journal of Medicinal Chemistry* (2019), doi: https://doi.org/10.1016/j.ejmech.2019.04.034.

Graphical Abstract



Heterodimeric Rifampicin–Tobramycin conjugates break intrinsic resistance of *Pseudomonas aeruginosa* to Doxycycline and Chloramphenicol *in Vitro* and in a *Galleria mellonella in Vivo* model.

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Abbreviations used

CAM, chloramphenicol; CFU, Colony-forming Unit; CLSI, Clinical and Laboratory Standard Institute; DOX, doxycycline; FICI, Fractional Inhibitory Concentration Index; MDR, multidrug-resistant; OM, outer membrane; RND, Resistance Nodulation Division; XDR, extensively drug-resistant.

Abstract

Intrinsic resistance in *Pseudomonas aeruginosa*, defined by chromosomally encoded low outer membrane permeability and constitutively over-expressed efflux pumps, is a major reason why the pathogen is refractory to many antibiotics. Herein, we report that heterodimeric rifampicin-tobramycin conjugates break this intrinsic resistance and sensitize multidrug and extensively drug-resistant *P. aeruginosa* to doxycycline and chloramphenicol *in vitro* and *in vivo*. Tetracyclines and chloramphenicol are model compounds for bacteriostatic effects, but when combined with rifampicin-tobramycin adjuvants, their effects became bactericidal at sub MIC levels. Potentiation of tetracyclines correlates with the SAR of this class of drugs and is consistent with outer membrane permeabilization and efflux pump inhibition. Overall, this strategy finds new uses for old drugs and presents an avenue to expand the therapeutic utility of legacy antibiotics to recalcitrant pathogens such as *P. aeruginosa*.

Keywords: adjuvant, amphiphilic aminoglycoside, antibacterial, antimicrobial, antipseudomonal, *Galleria mellonella*, hybrid, rifampicin, synergy.

1. Introduction

The problem of antimicrobial resistance is a global phenomenon that is threatening to reverse the gains and advances in modern medicine.[1–4] Microorganisms are developing resistance to our antibiotic pipeline at a rate that is practically impossible to keep up with and there are some phenotypes that are completely resistant to all available treatment options.[5] Regrettably, no new class of antibiotics with a novel mode of action has been successfully developed in the last few decades.[6,7] It is not surprising that for the first time ever, the WHO in 2017 published a list of pathogens for which effective treatment options are becoming elusive.[8] With a projection that antimicrobial resistance will be a leading cause of death worldwide by 2050,[9] it is expedient to, in the meantime, develop strategies that can expand and/or preserve the therapeutic usefulness of our current armamentarium.

Gram-negative bacteria are more difficult to inhibit or kill because of their outer membrane (OM) that serves as an additional layer of protection.[10] The OM is an asymmetric bilayer that limits or prevents the uptake of potentially noxious compounds to the bacterial cell, especially molecules that are hydrophobic and/or have high molecular weight.[11] Thus, most antibiotics that are active against Gram-positive bacteria are inactive against Gram-negative bacteria because of their inability to traverse the OM. Furthermore, the physicochemical requirements for a molecule to traverse the OM and inner membrane of Gram-negative bacteria are orthogonal to each another.[12] To cross the OM, a molecule needs to be small and polar (for porin-mediated uptake) or polybasic (for a self-promoted uptake), whereas it needs to be hydrophobic to traverse the inner membrane.[11] Medicinal chemists often struggle with developing agents that can tightrope this dual-membrane topology, accumulate in

the cell, and still be able to bind to a distinct target in the cytosol.[12,13] The frustrations expressed by the WHO is therefore apparent and it is clear that the priority one pathogens, which are all carbapenem-resistant (CR) Gram-negative ESKAPE[14] pathogens (i.e. CR *Acinetobacter baumannii*, CR *Pseudomonas aeruginosa*, and various CR *Enterobacteriaceae*), are major threats to public health. Of these pathogens, *P. aeruginosa* is even more difficult to inhibit or kill because of its sophisticated and high level of intrinsic resistance, such as its extremely low OM permeability and constitutively over-expressed efflux pumps of broad substrate specificities that actively extrude drug molecules from its periplasm/cytoplasm.[15] These, coupled with acquired and adaptive resistance mechanisms,[15] make *P. aeruginosa* resistant to many antibiotics that are active against other Gram-negative bacteria. As an opportunistic pathogen, *P. aeruginosa* is the leading cause of nosocomial infections such as respiratory infections in debilitated patients and patients with cystic fibrosis, and it is well known for its ability to evade antibiotic actions.[11,15]

Current thinking in the development of clinically relevant antipseudomonal agents include, but not limited to, developing agents that could potentially weaken or destabilize the OM, such that molecules that are otherwise unable to traverse this barrier can now gain access into the periplasm and/or cytoplasm.[16–19] Even for drugs with porin-mediated uptake mechanisms, OM destabilization ensures that the rate of drug influx overwhelms the rate of efflux thereby enhancing antibiotic accumulation. For instance, SPR741, a polymyxin-based adjuvant is being developed by Spero Therapeutics as an OM permeabilizer that can facilitate the entry of OM-impermeable antibiotics into Gram-negative bacteria such as *A. baumannii* and *Enterobactericeae*.[20] Unfortunately, SPR741 is not effective against *P. aeruginosa*.[20] On the other hand, tobramycin-based heterodimeric scaffolds have been reported to potentiate

several legacy antibiotics against *P. aeruginosa* than in other Gram-negative bacteria.[11,21] These two scenarios present intriguing dynamics about the physicochemical requirements for activity and antibiotic potentiation in *P. aeruginosa*. Hybridization of a second bioactive molecule to tobramycin via a tether resulted in conjugates that: i) preserve the original mode of action of the bioactive molecule, ii) abolish the ribosomal effect of tobramycin by itself, and iii) confer adjuvant properties on the resulting conjugate.[11,21] Tobramycin, an aminoglycoside antibiotic that interferes with the fidelity of ribosomal protein translation, propagates its own uptake into *P. aeruginosa* by displacing the stabilizing divalent cations that cross-bridge adjacent lipopolysaccharides on the outer leaflet of the OM.[22] This process is known as the 'self-promoted uptake mechanism' and the cationic nature of aminoglycosides is critical to their ability to induce this process.

To put all of these into perspective, we sought to expand the therapeutic usefulness of some OM-impermeable antibiotics by leveraging the self-promoted uptake mechanism of tobramycin to deliver them into Gram-negative bacteria, in a "Trojan-horse" fashion. For example, rifampicin, a large (M_w = 822.9 g/mol) hydrophobic molecule that binds to RNA polymerase and inhibit RNA synthesis, is active against Gram-positive bacteria and mycobacteria but inactive against most Gram-negative bacteria because of its inability to cross the OM. Unlike most classes of antibiotics, rifamycins are unique in that they are active against pathogens in slow growing, stationary, and non-replicating metabolic states.[23] Unfortunately, the greatest limitations of this class of drug is the rapid development of a single mutation in the β-subunit of bacterial RNA polymerase (*rpoB*),[24] and the lack of activity against Gram-negative pathogens. Surprisingly, some tobramycin-based conjugates that strongly potentiate the activity of rifampicin against *P. aeruginosa* were also shown to prevent the development of

resistance after 25 serial passages at sub MIC levels.[25] Hence, by directly conjugating rifampicin to a tobramycin scaffold using various tether lengths, we wanted to investigate the possibility of: 1) using tobramycin as a vector to shuttle rifampicin into *P. aeruginosa*, 2) preserving the original mode of action of rifampicin, i.e. RNA polymerase inhibition, in *P. aeruginosa*, 3) modulating the overall physicochemical property of the resulting conjugate such that it preserves or amplifies the known adjuvant properties of tobramycin-derived conjugates. The different tether lengths between the rifampicin and tobramycin domains were meant to investigate the optimal spatial separation between these two drug moieties. Herein, we report the synthesis and evaluation of a series of covalently-attached rifampicin–tobramycin molecules **1** – **3** (Figure 1). When hybridized as a single non-cleavable entity, the potency of rifampicin was preserved in some Gram-positive bacteria but was attenuated in *P. aeruginosa* (Table 1). However, the resulting conjugates **1** – **3** break the chromosomally-encoded intrinsic resistance of *P. aeruginosa* to doxycycline and chloramphenicol and significantly potentiate their activities against wild-type, multidrug- (MDR) and extensively drug-resistant (XDR) phenotypes *in vitro* and *in vivo*. An isolate is MDR if it is non-susceptible to at least one agent in at least three antimicrobial categories, while an XDR isolate is non-susceptible to at least one agent in all but two or fewer antimicrobial categories.[26] Typically, *P. aeruginosa* is clinically-resistant to doxycycline and chloramphenicol, but in the presence of < 10 μM of compounds **1** – **3**, the Clinical Laboratory Standard Institute (CLSI) susceptibility breakpoints (extrapolated: doxycycline ≤ 4 μg/ml for *Acinetobacter spp*, chloramphenicol ≤ 8 μg/ml for *Enterobacteriaceae*) were reached in 9 out of 10 phenotypes for doxycycline, and 7 out of 10 for chloramphenicol. This strategy expands the chemotherapeutic utility of doxycycline and

chloramphenicol to *P. aeruginosa* and makes the pathogen susceptible to and treatable by these agents.

2. Results

2.1 Design and Synthesis

To design a covalently hybridized conjugate of two bioactive molecules, the point of attachment on both molecules must be carefully identified in order not to interfere with their biological activities. Tobramycin was conceptualized as a vector to deliver rifampicin into the periplasm, due to its self-promoted uptake mechanism, hence, its cationicity must be preserved. Rifampicin was intended to mediate its antibiotic activity in the cell, hence, the point of attachment must not be directly involved with its target. Therefore, the design of rifampicin-tobramycin conjugates was established from the known structure-activity relationships of the parent molecules. Amphiphilic tobramycins with lipophilic groups at the *C*-5 position have been shown to retain its self-promoted uptake mechanism.[27,28] Solved crystal structure of RNA polymerase complexed with rifampicin revealed that the terminal piperazine ring of rifampicin is not involved in the binding of the drug to its target.[29] The drugs were therefore linked at these identified positions under a chemically benign reaction condition using aliphatic hydrocarbon of different lengths, i.e. *C*-4, *C*-6, and *C*-12. Rifampicin is hydrolyzed in both acidic and basic conditions, due to its imine and ester groups, respectively, thus, a copper(1)-catalyzed azide-alkyne cycloaddition reaction ("Click Chemistry")[30] was employed to join both compounds together under neutral conditions. This afforded regioselective 1,4-disubstituted 1,2,3-triazole products **1** – **3** (Figure 1). To achieve this, an azide moiety was incorporated on the tether length of an amphiphilic tobramycin, while an alkyne group was installed on the piperazine ring of rifampicin. The tether length was meant to investigate the

optimal spatial separation between the two domains. The synthetic strategy for preparing compounds **1** – **3** is outlined in Schemes 1, 2, and 3.

2.2 Chemical Synthesis of Rifampicin–Tobramycin Conjugates 1 – 3.

To install a propargyl group on rifampicin, a 1-amino-4-propagylpiperazine **7** precursor was synthesized (Scheme 1) and coupled to commercially available 3-formyl rifamycin SV (Scheme 3) following established procedure.[31] Briefly, **7** was prepared by converting one of the secondary amines in piperazine **4** to a nitroso- group under acidic conditions to give compound **5**, followed by a nucleophilic conjugation of the second secondary amine to propargyl bromide under basic conditions to give 1-nitroso-4-propargylpiperazine **6**. The nitroso group was then reduced to a primary amine in the presence of a strong reducing agent, $LiAlH_4$, to afford precursor **7** (Scheme 1). The amphiphilic tobramycin domain was prepared following previously reported protocol.[27] Tobramycin **8** was purchased from a commercial source and the amino groups were first protected using di-*tert*-butyl dicarbonate (Boc anhydride), followed by silylation of the *N*-Boc-tobramycin intermediate with excess TBDMSCl to afford a partially protected derivative **9** with free OH at the *C*-5 position of the cyclitol ring. Alkylation of **9** in toluene with 1,*n*-dibromoalkane (*n* = 4, 6, 12) in the presence of a phase-transfer catalyst (TBAHS) afforded bromoalkylated TBDMS-Boc-protected tobramycin intermediates. The terminal bromo-groups of these intermediates were then displaced by an azido nucleophile under anhydrous condition to give compounds **10a-c**. The protecting groups were finally removed in a stepwise manner, first by removing TBDMS groups using TBAF, and then *Boc*-protecting groups using TFA, to afford compounds **11a-c** (Scheme 2). Rifampicin-tobramycin conjugates **1** – **3** were ligated by coupling **7** to 3-formyl rifamycin SV to give **12**,

followed by a “click chemistry” conjugation to **11a-c** under neutral conditions to afford the final compounds (Scheme 3). The final compounds were retained on C18 reverse-phase silica in a column and washed with copious amount of deionized water to remove residual copper ions.

2.3 Antimicrobial Susceptibility Screening

The antibacterial activities of the newly synthesized conjugates **1** – **3** and reference compounds, rifampicin and tobramycin, were assessed against a panel of Gram-positive and Gram-negative bacteria following the CLSI guidelines. These results are presented as the minimum inhibitory concentrations (MICs) in table 1. As expected, rifampicin by itself was potent against several Gram-positive bacteria (MIC = <0.25 – 1 µg/ml) but not against Gram-negative bacteria (MIC ≥ 8 µg/ml) while tobramycin exhibits potency against most Gram-positive and Gram-negative bacteria. By design, the rifampicin-tobramycin conjugates **1** – **3** are expected to lose the protein translation inhibitory effect of tobramycin but retain the RNA polymerase inhibitory properties of rifampicin, as previously reported.[23] Tobramycin conjugated in this fashion are generally non-ribosomal,[21,25,28] hence, it was designed to only shuttle rifampicin into Gram-negative bacteria. Table 1 shows that compounds **1** – **3** exhibit slightly potent activity against some Gram-positive bacteria (MIC of 1 – 8 µg/ml) but were generally not potent against Gram-negative bacteria (MIC > 32 µg/ml). This suggests that whereas the conjugates might have retained the RNA polymerase effect of rifampicin against Gram-positive bacteria, they are unable to mediate the same effect in Gram-negative bacteria. The antipseudomonal activities of these conjugates were further assessed against a panel of MDR/XDR phenotypes and the results were consistent with the poor activity against Gram-negative bacteria (Table S1). Against Gram-positive bacteria, compound **1** with the shortest

tether length (C-4 aliphatic hydrocarbon chain) appears to be more potent than compound **3** with the longest tether length (C12 chain).

2.4 Combination Studies of Rifampicin–Tobramycin conjugates with different classes of antibiotics against wild-type *P. aeruginosa*

To investigate the impact of hybridizing a highly hydrophobic rifampicin moiety to a richly hydrophilic and polybasic tobramycin domain, the adjuvant properties of the resulting compounds were evaluated against *P. aeruginosa*. Physicochemical modulation of tobramycin-based conjugates is believed to be crucial to the nature, type, and degree of potentiation of different antibiotics.[11] Thus, we assessed the interactions between compounds **1** – **3** and twenty-one different antibiotics (representing all major classes) against wild-type *P. aeruginosa* PAO1 using checkerboard assay (Table S2). Data from this study were interpreted as a function of the fractional inhibitory concentration index (FICI), a numerical quantification of the interactions between antimicrobial agents. FICI of < 0.5, 0.5 – 4, and > 4 indicate synergy, additive or no interaction, and antagonism, respectively.[11,21] Surprisingly, rifampicin-tobramycin conjugates **1** – **3** were able to potentiate the effects of several antibiotics against WT PAO1, including rifampicin, but not tobramycin or β-lactams (Figure 2a, Table S2). Unlike in susceptibility screening against Gram-positive bacteria, conjugates **2** and **3** with longer tether lengths were better potentiators than compound **1** with four carbon chain length (Figure 2b). Compounds **2** and **3**, at ≤ 10 μM, potentiated rifampicin (FICI = 0.15 – 0.28), tetracyclines (FICI = 0.06 – 0.50), chloramphenicol (FICI = 0.06 – 0.09), fosfomycin (FICI = 0.15 – 0.28), linezolid (FICI = 0.15 – 0.28), novobiocin (FICI = 0.28), erythromycin (FICI = 0.06 – 0.15), etc. but not tobramycin (FICI = 1.03), β-lactams (FICI = 1.03), moxifloxacin (FICI = 0.53 – 1.03),

and nitrofurantoin (FICI = 0.53) (Figure 2a, Table S2). Doxycycline (FICI = 0.06 – 0.09) and chloramphenicol (FICI = 0.06 – 0.09) were exceptionally potentiated by compounds **2** and **3** against PAO1, hence, these combinations were further investigated against highly resistant phenotypes of *P. aeruginosa*. It should be noted that doxycycline and chloramphenicol are typically not used to treat *P. aeruginosa* infections because of poor susceptibility due to intrinsic resistance.

2.5 Rifampicin-Tobramycin conjugates strongly potentiate doxycycline and chloramphenicol against multidrug and extensively drug resistant *P. aeruginosa*

A combination of doxycycline or chloramphenicol with compounds **2** or **3** were further assessed against a panel of highly drug-resistant *P. aeruginosa* phenotypes. Most of these isolates are neither susceptible to doxycycline nor chloramphenicol, and are mostly resistant to carbapenems, the drugs of last resort (Table S3). Remarkably, compounds **2** and **3** significantly potentiated the effects of doxycycline and chloramphenicol against these clinical isolates (Table 2). For instance, in the presence of ≤ 10 μM of compounds **2** and **3**, susceptibility of *P. aeruginosa* clinical isolates to doxycycline was increased by 4- to 512-fold while susceptibility to chloramphenicol was increased by 4- to 256-fold (Figure S1). These effects were generally dose-dependent against WT and clinical isolates (Figure 3), suggesting an involvement of a common mechanism in both phenotypes. On the contrary, the degree of potentiation of doxycycline and chloramphenicol were not as pronounced (nil to 8-fold) in other Gram-negative bacteria (Figure S2), suggesting a strong antipseudomonal potentiating effect for **2** and **3**.

To put these findings into context, extrapolated CLSI susceptibility breakpoints were used as interpretive standards for doxycycline and chloramphenicol since they are not conventional antipseudomonal agents. Breakpoints are discriminatory antimicrobial concentrations used in the interpretation of susceptibility testing to define isolates as susceptible, intermediately-resistant, or resistant. If the MIC of an antibiotic against an organism is less than or equal to its susceptibility breakpoint, the bacterial strain is considered to be susceptible to the antibiotic. The CLSI clinical breakpoint of doxycycline for *Acinetobacter* spp. is 4 μg/ml while that of chloramphenicol for *Enterobacteriaceae* is 8 μg/ml.[32] Susceptibility equal or below these breakpoints were reached for doxycycline in wild-type and eight out of nine clinical isolates, and for chloramphenicol in wild-type and six out of nine clinical isolates (Table 2). CLSI clinical breakpoints could not be reached for both antibiotics in other resistant Gram-negative pathogens such as *Acinetobacter baumannii*, *Klebsiella pneumoniae* and *Enterobacter cloacae* in the presence of 10 μM of compounds **2** and **3** (Table S4). It is noteworthy that tobramycin alone, rifampicin alone, or a combination of both do not potentiate doxycycline or chloramphenicol against any of the *P. aeruginosa* phenotype used in this study (Table S5).

2.6 Potentiation of doxycycline and chloramphenicol in *P. aeruginosa* is efflux pump-dependent

A major contributor to the intrinsic resistance of *P. aeruginosa* to doxycycline and chloramphenicol is their highly efficient efflux mechanisms, as does the low permeability of the OM. To accumulate in cells, compounds must traverse the OM (or porins) faster than they are pumped out. The MexAB/MexXY efflux systems in *P. aeruginosa* contribute significantly to the

extrusion of tetracyclines out of the cell while resistance to chloramphenicol is in part due to the MexAB-OprM efflux system.[33] To investigate the role of these pumps in the ability of compound **2** to increase the susceptibility of *P. aeruginosa* to doxycycline and chloramphenicol in such magnitude, we assessed the synergistic relationships of these combinations in two mutant strains, PAO200 and PAO750. PAO200 is a *mexA-mexB-oprM* deletion strain while PAO750 lacks five clinically important RND pumps (MexAB–OprM, MexCD–OprJ, MexEF–OprN, Mex JK, and MexXY) and the OM protein OpmH.[34,35] As expected, the efflux-mutant strains were hypersusceptible to both doxycycline and chloramphenicol (Table 3), consistent with known contributions of RND efflux pumps to the intrinsic resistance to these agents.[36] However, at a fixed concentration of compound **2**, its ability to potentiate doxycycline and chloramphenicol was diminished in efflux-deficient mutants compared to the wild-type strain (Table 3). For instance, compound **2** (at 9.8 μM) increased the susceptibility of WT PAO1 to doxycycline by 32-fold while an increase in susceptibility of 8- and 4-fold was observed in PAO200 and PAO750, respectively. The same phenomenon was observed for the potentiation of chloramphenicol (Table 3 and Figure S3). Even between the two mutants, the test antibiotics were more potentiated in PAO200 which is deficient in only one of its pumps, compared to PAO750 which lacks five different pumps. This indicates that interaction with and/or inhibition of RND efflux pumps play an important role in the degree of potentiation seen in PAO1 but, expectedly, not in efflux-deficient mutants. However, inhibition of efflux pumps does not explain the partial increase in susceptibilities of the efflux mutants, suggesting that other mechanisms such as OM permeabilization might concomitantly be at play. OM permeabilization is indeed consistent with the ability of these conjugates to potentiate some OM-impermeable antibiotics such as rifampicin, erythromycin, etc. Overall, interaction with

RND efflux pumps seems to play a role in the ability of compound **2** to potentiate doxycycline and chloramphenicol, and strains with higher levels of expression will likely be more susceptible to these combinations.

2.7 Rifampicin–Tobramycin conjugate potentiates rifampicin and other members of tetracycline against MDR/XDR *P. aeruginosa*

To ascertain whether the ability of rifampicin-tobramycin conjugates to potentiate doxycycline is conserved across the entire class of tetracyclines, synergistic relationships between compound **2** and minocycline or tigecycline were evaluated against wild-type and MDR/XDR *P. aeruginosa*. Results of this study show that minocycline and tigecycline were similarly potentiated as doxycycline but to a lesser degree (Figure 4, Table S6). The degree of potentiation of tetracyclines is: doxycycline > minocycline > tigecycline (Figure 4), an observation that seems to correlate with the structure-activity relationships of tetracyclines. This variation is perhaps due to the fact that tigecycline evades acquired efflux and target-mediated resistance to classical tetracyclines[37] but remains vulnerable to the chromosomally-encoded multidrug efflux pumps of *P. aeruginosa*.[38] Thus, inhibition of acquired efflux pumps by compound **2** will have lesser consequential effects on tigecycline than it will on doxycycline. Rifampicin, an OM-impermeable antibiotic, was also potentiated against wild-type and MDR/XDR *P. aeruginosa* isolates (Figure 4, Table S6), suggesting a role for OM permeabilization as part of the mechanism of potentiation by compound **2**. Rifampicin is not a substrate for RND efflux pumps in *P. aeruginosa* (Table S1).

2.8 Time-kill Assay

Time-kill curves can be used to monitor bacterial growth and death over a wide range of antimicrobial concentrations to evaluate the effect of antimicrobials over time. An antibiotic could either be bactericidal or bacteriostatic under specific growth conditions. Bactericidal activity is defined as a ≥ 3-log reduction in the total CFU/mL from the original inoculum over 24 hours while bacteriostatic activity is defined as maintenance of < 3-log reduction in the total CFU/mL from the original inoculum.[32] Thus, the time-kill kinetics of wild-type *P. aeruginosa* PAO1 when incubated with doxycycline or chloramphenicol alone, or in combination with compound **2**, was assessed. Preliminary growth curve was performed to ensure that PAO1 could be grown from a starting inoculum of about 10^6 CFU/ml under our assay conditions, and that pre-incubation in antimicrobial-free LB medium will reach a stable early- to mid-log phase after 4 h. The time-kill curves of doxycycline (at 32 μg/ml, i.e. 2× MIC) and chloramphenicol (at 32 μg/ml, i.e. 1× MIC) looked similar, with almost no killing of bacteria within the assay period (Figure 5). Bacterial growth was completely inhibited by both drugs at these concentrations, an effect that is consistent with known bacteriostatic properties of tetracyclines (doxycycline) and chloramphenicol.[39] When PAO1 was incubated with sub MICs of 4 μg/ml doxycycline alone or 8 μg/ml chloramphenicol alone, i.e. their respective extrapolated CLSI breakpoints, there was bacterial growth as early as 3 h, similar to the control without antibiotics (Figure 5). However, in the presence of 4.9 μM or 9.8 μM of compound **2**, doxycycline became strongly bactericidal and chloramphenicol became weakly bactericidal at their respective CLSI breakpoints. Indeed, a combination of 4 μg/ml of doxycycline and 9.8 μM of compound **2** reduced the viability of *P. aeruginosa* PAO1 below the limits of detection (< 10 CFU/mL) after 9 h of incubation (Figure 5). Synergistic effect is defined as ≥ 2-log decrease in the number of CFU/mL between the combination and the most active component of the combination after 24

h (at least one of the drugs must be present at a concentration that does not affect the growth curve of the test organism).[40] It is clear that compound **2** exhibited synergistic relationships with both doxycycline and chloramphenicol against *P. aeruginosa* PAO1 in a dose-dependent manner and there was no re-growth after 24 h of incubation.

2.9 *In Vivo* Efficacy Studies using *Galleria mellonella* Infection Model

In vitro synergy of many antibiotic/antibiotic or antibiotic/adjuvant combinations against Gram-negative bacteria have been shown to be often incongruent with *in vivo* synergy.[41,42] To investigate whether the *in vitro* synergy observed with compound **2** and doxycycline or chloramphenicol is translated *in vivo*, we examined the ability of different combination concentrations to offer therapeutic protection against MDR *P. aeruginosa*-infected *Galleria mellonella* wax moths. The capability of this infection model to determine virulence of *P. aeruginosa* strains as well as efficacy and pharmacokinetics of antipseudomonal agents have been widely demonstrated.[11,21,25,42,43] The maximum tolerable dose was first determined by injecting high concentrations of doxycycline, chloramphenicol, and compound **2** (100 mg/kg and 200 mg/kg each) into the larvae and survivability was scored for 96 h (4 days). 100 % survival was recorded in groups injected with 100 mg/kg doxycycline, 100 and 200 mg/kg chloramphenicol, and 100 mg/kg of compound **2**, while groups injected with 200 mg/kg each of doxycycline and compound **2** recorded 85 % and 97 % survival, respectively, after 4 days (Figure 6a). This indicates that the compounds were relatively non-toxic to the larvae by themselves, an effect that is consistent with other *in vitro* toxicity studies (*vide infra*). Also, an inoculum size of 5 CFU of PA260, an extensively drug-resistant *P. aeruginosa* clinical isolate, achieved 100% lethality in the larvae after 18 h. Next, the ability of the drugs, alone and in

combination, to protect the larvae from XDR PA260 3 h post infection was determined using single doses of 100 mg/kg each as monotherapy, or 75 + 75 mg/kg or 100 + 100 mg/kg as combination therapy. PA260 is expected to have reached a stable early- to mid-log phase in the larvae after 3 h of infection. Like the untreated control, 100 % mortality was observed in the monotherapy treatments with all drugs (Figure 6b). However, a single dose combination of doxycycline and compound **2** using 75 + 75 mg/kg and 100 + 100 mg/kg resulted in 75 % and 87 % survival, respectively after 18 h, and 55 % and 75 % survival, respectively after 24 h (Figure 6b). Similarly, a single dose combination of chloramphenicol and compound **2** using 75 + 75 mg/kg and 100 + 100 mg/kg resulted in 55 % and 72 % survival, respectively after 18 h, and 10 % and 45 %, respectively after 24 h (Figure 6b). This demonstrates the ability of compound **2** to synergize with doxycycline and chloramphenicol *in vivo* and offer therapeutic protection to PA260-infected larvae in a dose-dependent manner at tolerable concentrations. The higher level of survival seen with doxycycline + compound **2** relative to chloramphenicol + compound **2** is perhaps attributable to the susceptibility of PA260 to these agents. The absolute MIC (in the presence of compound **2**) of doxycycline against PA260 is 0.125 μg/ml while that of chloramphenicol is 2 μg/ml (Table 2). Colistin, which is the only antibiotic that PA260 is susceptible to besides amikacin (Table S3), served as the positive control for this experiment while treatment with PBS only served as negative control.

2.10 Toxicity Studies

To evaluate *in vitro* toxicity and margin of therapeutic safety of compounds **1** – **3** against eukaryotic cells, they were screened against human liver (HepG2) and kidney (HEK293) cells, as well as porcine erythrocytes. The results of these studies were compared to the approved

parent antibiotics, rifampicin and tobramycin. For cytotoxicity against human cells, doxorubicin, a very potent anticancer drug, was used as a positive control for this experiment. The results of the study showed that none of the rifampicin-tobramycin conjugates **1** – **3**, rifampicin, or tobramycin was toxic to HepG2 and HEK293 (Figure 7a). At the effective adjuvant concentration of < 10 μM (< 16 μg/mL) used for this study, cell viability was greater than 90 % for both cells, and greater than 70 % at the highest concentration tested (50 μM, i.e. > 100 μg/mL). Expectedly, doxorubicin reduced the viability of HepG2 and HEK293 cells to less than 10% at about 12 μM (6.5 μg/mL), consistent with its cytotoxic properties (Figure 7a). We also investigated the toxicity of the combination therapies against HepG2 and HEK293 cells (i.e. compound **2** + doxycycline and compound **2** + chloramphenicol) and found that the combination regimen did not elevate toxicological profiles of the primary antibiotics (Figure 7b). For toxicity against freshly collected porcine erythrocytes, 0.1% Triton X-100 served as the positive control and was used to calculate percent hemolysis. All the newly synthesized conjugates **1** – **3** exhibited insignificant hemolytic effects (< 2%) at very high concentrations of 1024 μg/mL (Figure 7c), a 64-fold higher dose than the maximum synergistic concentration used in the study.

3. Discussion

The sustained and emerging resistance of recalcitrant pathogens to our antibiotic armamentarium constitutes an acute threat to public health and a cause for alarm. The growing gap between clinical needs and drug innovation is a direct consequence of the difficulty in identifying and bringing new drugs to market, especially against Gram-negative pathogenic bacteria. Intrinsic resistance in Gram-negative bacteria, such as their protective outer membrane and constitutively overexpressed efflux pumps, is a major survival weapon that renders them untreatable by most antibiotics.[11] To fill the current void in antibacterial drug discovery against Gram-negative bacteria, a plausible strategy that has gained wide attention is the development of adjuvants that could either rescue/preserve the efficacy of available treatment options,[17] or expand the therapeutic usefulness of antibiotics that are not clinically indicated for such infections.[11,20,25] For example, tazobactam, avibactam, and varborbactam are adjuvants that have all been approved by FDA to rescue/preserve the efficacies of ceftolozane, ceftazidime, and meropenem, respectively, while membrane permeabilizing agents (e.g. SPR741) have been shown to be capable of expanding the therapeutic usefulness of OM-impermeable drugs (e.g. rifampicin) to Gram-negative bacteria.[11,25,44]

To cross the OM, a molecule must either pass through the porin (usually very small and polar, e.g. fluoroquinolones, β-lactams),[45] have an active transport mechanism, or induce a self-promoted uptake mechanism (could be big, e.g. colistin, aminoglycosides). Cationicity is critical for a self-promoted uptake mechanism across the OM and hydrophobicity facilitates penetration of membrane-active compounds across bacterial membrane. Consequently,

amphiphilicity have often been employed to achieve the dual physicochemical requirement of electrostatic interactions and transmembrane navigation in cells.[13,46,47] Rifampicin–Tobramycin conjugates **1** – **3** were designed such that rifampicin could be shuttled into the periplasm of Gram-negative bacteria, especially *P. aeruginosa*, using tobramycin as a vector due its self-promoted uptake mechanism. The hydrophobic rifampicin domain may also facilitate uptake across the inner membrane. Based on earlier studies, tobramycin was expected to lose its ability to inhibit protein translation,[21,47] but rifampicin was expected to retain its activity.[29] A different group had previously used similar 'Trojan-horse' approach to deliver OM-impermeable erythromycin as an effective treatment of *Klebsiella pneumonia in vivo*.[31] The resulting conjugates **1** – **3** retained activity as standalone agents against some Gram-positive bacteria but not against Gram-negatives (Table 1). This observation is similar to that of a dual-acting rifampicin-ciprofloxacin hybrid, TNP-2092, a former drug candidate that was only active against Gram-positive bacteria.[23] Since conjugated tobramycins modified at the *C*-5 position are completely inactive against Gram-positive and Gram-negative bacteria,[25] the activity of compounds **1** – **3** against some Gram-positive pathogens is most likely driven by the rifampicin domain, suggesting that ligation to its piperazine moiety do not significantly affect its biological activities. Previous SAR study of tobramycin-based compounds have shown that the second domain of this scaffold is amenable, and that the spectrum of activity, degree of potentiation, and type of antibiotics it potentiates can be altered by changing the second domain.[11] Thus, we investigated the physicochemical impact of hybridizing a hydrophobic rifampicin with a cationic hydrophilic tobramycin domain, as it relates to the ability to permeate bacterial membrane and potentiate legacy antibiotics. The conjugates (Figure 1) were found to potentiate different classes of Gram-positive-only antibiotics against *P.*

aeruginosa, most especially doxycycline and chloramphenicol (Figure 2), while the individual parent molecules alone or in combination could not (Table S5). Compound **1** with the shortest tether was more active than **2** and **3** as standalone antibacterial agents, while compounds **2** and **3** were more potent than **1** as adjuvants. Indeed, compounds **1** – **3** potentiated antibiotics that are clinically not indicated for the treatment of *Pseudomonas* infections but did not potentiate typical antipseudomonal agents (Figure 2). This suggests a mechanism that involves the reversal of intrinsic resistance of *P. aeruginosa* to these agents, especially resistance to doxycycline and chloramphenicol.

P. aeruginosa is clinically resistant to tetracyclines and chloramphenicol due to its intrinsic and adaptive resistance mechanisms. Tetracyclines enter Gram-negative bacteria via a porin-mediated process and they inhibit the elongation phase of protein synthesis by blocking the association of aminoacyl tRNA with the bacterial ribosome.[48] On the other hand, chloramphenicol, which also prevents protein chain elongation by inhibiting the peptidyl transferase activity of bacteria ribosome,[49] is believed to be taken up into the cell via an energy-dependent process.[50] A major mechanism of resistance to these agents by *P. aeruginosa* is by actively extruding them out of the cell using its abundantly expressed efflux pumps.[48] The ability of compounds **2** and **3** to potentiate the effects of doxycycline and chloramphenicol were found to be dependent on efflux pumps (Table 3), suggesting that mitigating the effects of these pumps, in addition to outer membrane permeabilization, might be responsible for the sensitization of *P. aeruginosa* to these agents. Outer membrane permeabilization is a known property of all tobramycin conjugates synthesized to date.[11,21,25] Agents that alter transmembrane protein environment (such as membrane charge, fluidity, and thickness) and/or steric hindrance of membrane-embedded proteins can

prevent the relay of signaling cascades required to elicit conformational changes necessary to extrude substrate molecules by efflux pumps.[11] The perturbation of transmembrane efflux protein domains by compounds **2** and **3** via alteration of lipid composition surrounding the protein may, therefore, contribute to their ability to potentiate doxycycline and chloramphenicol in *P. aeruginosa*. Indeed, rapid influx and reduced efflux are simultaneous complementary processes necessary for enhanced drug bioaccumulation in bacteria cell.

Tetracyclines and chloramphenicol often act as model compounds for bacteriostatic effects,[50] and the association of tetracycline with the ribosome is reversible,[48] providing an explanation for the bacteriostatic nature of this class of drugs. The concentration-independent bacteriostatic effects of doxycycline and chloramphenicol against wild-type PAO1 were confirmed by growth curves that do not indicate any decrease in the number of CFU/mL from the initial inoculum at MIC and supra MIC levels (Figure 5). However, in the presence of compound **2**, *P. aeruginosa* was sensitized to doxycycline and chloramphenicol (Table 2) and their effects became bactericidal (Figure 5). The ability to switch the bacteriostatic effects of these drugs to bactericidal effects (in combination with compound **2**) in the same organism, under the same growth conditions, is quite remarkable. Given the lack of antimicrobial activity of compounds **2** and **3** against Gram-negative bacteria (Tables 1 and S1), the mechanism of synergistic relationships with doxycycline and chloramphenicol is not immediately obvious. It is possible that compounds **2** and **3** disrupt the OM of *P. aeruginosa*, thus, enhancing bioaccumulation of doxycycline and chloramphenicol in the cytosol. However, this does not fully explain the bactericidal effects of these combinations, given that very high concentrations of tetracyclines and chloramphenicol are often bacteriostatic against *P. aeruginosa*.[25,50] It is therefore conceivable that the bactericidal effect of these combinations emanates from the

antimicrobial/biochemical potentiation of both participating molecules. For example, ribosome-targeting antibiotics (tetracyclines, chloramphenicol, aminoglycosides) are known to preferentially inhibit the biosynthesis of envelop proteins,[48–50] thus, impacting the OM and facilitating the entry of some chemical entities.[51] We speculate that interference with the fidelity of ribosomal proteins by doxycycline and chloramphenicol enhances the uptake of compounds **2** and **3** across a structurally-weakened OM, thereby promoting the antibiotic efficacy of the rifampicin domain. Rifampicin is bactericidal against WT *P. aeruginosa*.[25] This scenario is consistent with the activity of compounds **2** and **3** against some Gram-positive bacteria that lack an OM (Table 1), an effect that is believed to be mediated by the rifampicin domain. The membrane-weakening effects of doxycycline and chloramphenicol possibly augment the OM-permeabilizing properties of the amphiphilic tobramycin domain in compounds **2** and **3**, thus facilitating their uptake across the OM, while the rifampicin domain possibly anchors the conjugate into the periplasm to mediate it antibacterial effect. The lack of meaningful synergistic relationship between a double and triple combination of doxycycline or chloramphenicol and tobramycin or rifampicin (Table S5) underscores the importance of linking the two domains in compounds **2** and **3**. Aminoglycosides (tobramycin) are taken up across the cytoplasmic membrane via an energy-dependent process[22] while rifampicin lacks the requisite physicochemical properties to navigate the OM.

It is gratifying to note that, consistent with *in vitro* potentiation, synergy was also observed *in vivo* between doxycycline or chloramphenicol and compound **2** in *G. mellonella in vivo* infection model (Figure 6). A 100 mg/kg monotherapy each of doxycycline, chloramphenicol, or compound **2** resulted in 100 % mortality of PA260-challenged *G. mellonella* larvae after 18 h, while a single dose administration of 100 + 100 mg/kg

combination therapy each of doxycycline + compound **2** or chloramphenicol + compound **2** resulted in 87 % and 72 % survival, respectively, after 18 h in a dose-dependent manner (Figure 6). This indicates the therapeutic potential of rifampicin-tobramycin conjugates in combination with doxycycline or chloramphenicol to treat infections caused by MDR/XDR *P. aeruginosa*. A 100 mg/kg of meropenem alone resulted in 100% mortality of the larvae after 24 h while colistin, the only antibiotic (besides amikacin) that the strain is susceptible to, require up to 75 mg/kg to confer ≥ 50% survivability on the larvae after 24 h. Despite the obvious limitation of this model such as the simple body plan and lack of many defined organs/tissues in the larvae, the similarities between the innate immune responses in *G. mellonella* larvae and humans, the demonstrable virulence of pathogenic bacteria and efficacy of therapies in both *G. mellonella* larvae and humans, and the logistical and ethical relief of using *G. mellonella* make it a suitable preliminary model to determine efficacy of therapeutic agents.[11,21,25,42,43] Moreover, unlike other invertebrate models such as *Caenorhabditis elegans* and *Drosophilia melanogaster*, *G. mellonella* larvae can survive at 37 °C and therefore allow the investigation of temperature-dependent microbial virulence and antibiotic action.[52] However, further studies of this combination is needed in mice infection model.

The newly synthesized compounds **1** – **3**, alone and in combination with antibiotics, were found to be non-cytotoxic to human kidney (HEK293) and liver (HepG2) cells *in vitro* (Figures 7a and b), non-hemolytic to porcine erythrocytes *in vitro* (Figure 7c), and non-toxic to *G. mellonella* larvae *in vivo* (Figure 6a) at relatively high concentrations. As a result, it rules out the suspicion of a non-specific mode of action and demonstrates the relative safety of these compounds against eukaryotic cells. Amphiphilic aminoglycosides have previously been shown to target bacterial membranes more selectively than eukaryotic membranes.[53]

4. CONCLUSIONS

The lack of a robust pipeline of new agents, particularly against resistant Gram-negative bacteria, emphasizes the importance of optimizing our current antimicrobials. Rifampicin–Tobramycin conjugates have been shown to break the intrinsic resistance of *P. aeruginosa* to doxycycline and chloramphenicol *in vitro* and *in vivo*, thus expanding the therapeutic usefulness of these agents. In the presence of <10 μM of compounds **2** or **3**, extrapolated CLSI susceptibility breakpoints were reached in nine out of ten resistant *P. aeruginosa* phenotypes for doxycycline (≤ 4 μg/ml), and seven out of ten for chloramphenicol (≤ 8 μg/ml). A single dose combination of compound **2** with doxycycline or chloramphenicol also confer survivability on MDR *P. aeruginosa*-challenged *G. mellonella* larvae while a monotherapy of compound **2**, doxycycline, or chloramphenicol does not. Whereas doxycycline alone exhibits bacteriostatic effects against *P. aeruginosa* at supra MIC levels, when combined with compound **2**, its effect became bactericidal at sub MIC levels. In developed countries, usage of chloramphenicol as broad-spectrum antibiotics has diminished over the years due to increasing resistance and its well-described adverse effects, but the ability to achieve potent bactericidal effects at lower concentrations could rejuvenate interest in this drug, especially in this era of unabating resistance development. The current study has also demonstrated that chemical entities with no intrinsic activity as standalone agents against Gram-negative bacteria does not necessarily mean they are biologically irrelevant. Indeed, the attainment of specific physicochemical threshold in a molecule is central to permeating the outer membrane of *P. aeruginosa*. Further studies with tobramycin-based conjugates in general are warranted to carefully elucidate and situate their role in clinical practice.

5. Experimental Section

5.1 Chemistry. All chemicals and reagents were purchased from Sigma-Aldrich (Oakville, ON, Canada) except 3-formyl rifamycin SV and tobramycin that were purchased from AK Scientific Inc. (CA, USA). The chemicals were all used without further purification. Air and moisture-sensitive reactions were performed with dry solvents under nitrogen atmosphere. Thin-layer chromatography (TLC) was carried out on aluminum-backed silica gel 60 F_{254} GF plates (0.25 mm) and/or aluminum-backed reverse phase silica gel 60 RP-18 $F_{254}S$ plates (Merck KGaA, Germany) with the indicated solvents, and visualized under ultraviolet light and/or by staining within ninhydrin solution in *n*-butanol. Compounds were purified by flash chromatography on silica gel 60 (230-400 ASTM mesh) and/or reverse-phase C18 silica gel (Silicyle, USA). Yields refer to chromatography-purified homogenous materials, except otherwise stated. ^{1}H and ^{13}C NMR spectra were recorded on Bruker AMX-300 and AMX-500 spectrometers (Germany) as solutions and reported in the order of chemical shifts (δ) in ppm relative to the indicated solvent, multiplicity (s, singlet; d, doublet; t, triplet and m, multiplet), number of protons, and coupling constants (*J*) in hertz (Hz). ^{1}H and ^{13}C of compounds were assigned using 1D and 2D NMR experiments such as Proton, COSY, Carbon-13, DEPT-135, HSQC, and HMBC. ESI-MS and MALDI-TOF MS analyses were performed on Varian 500-MS ion trap mass spectrometer (USA) and Bruker Daltonics Ultraflextreme MALDI TOF/TOF mass spectrometer (Germany), respectively. Analytical HPLC was performed on Breeze HPLC Waters equipped with W2998 PDA detector (1.2 nm resolution) coupled to Phenomenex Synergi Polar (50 × 4.6 mm) 4 μm reverse-phase column with phenyl ether-linked stationary

phase and a detection wavelength of 332 nm. The purity of final compounds as determined by HPLC analysis was > 95 %.

5.1.1 General Procedure A: Copper(1)-catalyzed azide-alkyne cycloaddition reaction ("Click Chemistry") for the Preparation of compounds 1 – 3. Compounds **11a-c** (2 equiv.) and **12** (1 equiv.) were dissolved in a 1:1 mixture of H_2O/1-butanol (2.0 mL). A 100 mM solution of $CuSO_4.5H_2O$ (1 equiv.) and freshly prepared 500 mM solution of ascorbic acid (4 equiv.) were added, and the mixtures were stirred at 50 °C for 4 h. The reaction mixtures were concentrated and purified by reverse-phase column chromatography (100% water to water/acetonitrile, 85/15, v/v, spiked with 0.1% v/v formic acid) to give the final compounds **1 – 3** (45 – 55%) isolated as reddish-brown solids. Excess **11a–c**, $CuSO_4$, and ascorbic acid were eluted on RP-18 column using a copious amount of 100% water while the desired compounds **1 – 3** were retained in the column until 15% v/v acetonitrile in water. The potential retention of some residual copper ions did not impact cytotoxicity of the final compounds *in vitro* and *in vivo*, consistent with an earlier report.[31] The purification process is laborious and the final compounds are stabile at neutral conditions but are labile in acidic or basic pH. CD_3CN was used as co-solvent for acquisition of NMR spectra for compounds **2** and **3**.

5.1.2 General Procedure B: 5-*O*-alkylation of *Boc* and TBDMS protected tobramycin for the Preparation of compounds 10a–c*.* A solution of **9** (1 equiv.) in toluene was treated with KOH (3 equiv.), 1,*n*-dibromoalkane (3 equiv.), and a catalytic amount of tetrabutylammonium hydrogen sulphate, TBAHS (0.1 equiv.). The reaction mixture was stirred at RT overnight, dispersed in water and extracted with an equal volume of ethyl acetate (×3). The organic

layers were combined, washed with brine (×1), dried over anhydrous Na_2SO_4 and concentrated *in vacuo*. The crude products were then purified by column chromatography (hexanes/ethyl acetate, 100/0 to 100/10, v/v). The resulting compounds were dried and subsequently dissolved in anhydrous DMF, treated with NaN_3 (20 equiv.) and stirred at 70 °C for 3 h under nitrogen atmosphere. The crude mixtures were concentrated under *vacuo* and re-dissolved in ethyl acetate. The organic layers were then washed with water (×2) and brine (×1), dried over anhydrous Na_2SO_4, and concentrated under *vacuo* to afford compounds **10a–c** as white solids.

5.1.3 General Procedure C: Deprotection of hydroxyls and amines (Removal of TBDMS and *Boc* protecting groups) for Preparation of compounds 11a–c. A solution of TBDMS- and Boc-protected compounds **10a–c** in anhydrous THF (5.0 mL) were treated with tetrabutylammonium fluoride (TBAF, 6 equiv.) and stirred under nitrogen atmosphere for 2 h. The reaction mixture was concentrated under *vacuo*, dissolved in water and extracted with DCM (×3). The organic layers were combined, dried over anhydrous Na_2SO_4, concentrated *in vacuo*, and purified by column chromatography (dichloromethane/methanol, 40/1, v/v). A solution of the resulting compounds in DCM (2.0 mL) were further treated with trifluoroacetic acid (2.0 mL), stirred at RT for 1 h and concentrated under low *vacuo*. 2% methanol in ether (2.0 mL) was then added, stirred gently for 1 min and the solvent carefully decanted to give off-white solid compounds. The crude products were subsequently purified by reverse-phase flash chromatography (eluted with 100% deionized water) to afford analytically pure compounds **11a–c** as off-white TFA salt solid compounds. Compound **11b** was converted to HCl salt before use by treating with stoichiometric amount of aqueous HCl solution.

Rifampicin-C_4-Tobramycin conjugate.5TFA (1). Compounds **11a** (0.100 g, 0.088 mmol) and **12** (0.037 g, 0.044 mmol) were conjoined via click chemistry as described in general procedure A to afford a final compound **1** (0.027 g, 45%) as a reddish brown solid. ^{1}H NMR (500 MHz, D_2O) δ 8.27 (s, 1H, triazole), 7.66 (s, 1H, imine), 6.69 – 6.42 (br. m, 2H), 6.25 – 6.04 (br. m, 2H), 5.40 (s, 1H), 5.30 – 5.04 (m, 3H), 4.52 – 4.45 (m, 3H), 4.32 – 4.21 (m, 3H), 3.94 – 3.54 (m, 30H), 3.41 – 3.30 (m, 6H), 3.20 – 3.14 (m. 3H), 3.10 – 3.05 (m, 2H), 2.58 – 2.49 (m, 2H), 2.28 – 2.22 (m, 5H), 2.11 – 2.03 (m, 5H), 1.97 – 1.91 (m, 3H), 1.84 – 1.77 (m, 3H), 1.66 – 1.57 (m, 5H), 1.52 – 1.48 (m, 2H), 1.40 – 1.27 (m, 10H), 0.97 (br. s, 3H), 0.80 (br. s, 3H), 0.58 (br. s, 3H), -0.04 (br. s, 3H). ^{13}C NMR (126 MHz, D_2O) δ 199.3, 176.2, 173.6, 147.9, 147.5, 144.9, 143.7, 142.9, 141.4, 139.8, 138.2, 137.2, 136.3, 134.8, 130.5, 127.5, 125.0, 108.3, 106.2, 101.3, 92.5, 82.1, 81.8, 77.3, 76.4, 75.6, 73.5, 73.2, 73.0, 72.1, 71.7, 69.7, 68.6, 64.8, 63.7, 63.6, 62.6, 60.4, 59.3, 56.7, 54.7, 50.6, 49.8, 48.5, 47.4, 39.2, 38.6, 37.4, 35.6, 32.6, 29.9, 29.3, 28.3, 27.6, 25.8, 25.7, 25.1, 24.6, 20.5, 20.3, 17.4, 9.9, 9.7, 9.6, 9.0. MALDI TOF-MS *m/e* calcd for $C_{67}H_{103}N_{12}O_{21}$, 1411.7282; measured *m/e,* 1411.7285 $[M + H]^+$.

Rifampicin-C_6-Tobramycin conjugate.5HCl (2). Compounds **11b** (0.100 g, 0.086 mmol) and **12** (0.036 g, 0.043 mmol) were conjoined via click chemistry as described in general procedure A to afford a final compound **2** (0.032 g, 52%) as a reddish brown solid. ^{1}H NMR (500 MHz, D_2O + CD_3CN) δ 8.03 (s, 1H, triazole), 7.66 (s, 1H, imine), 6.63 (br., 1H), 6.49 (d, *J* = 10.7 Hz, 1H), 6.26 (d, *J* = 12.6 Hz, 1H), 6.12 (br., 1H), 5.44 – 5.39 (m, 1H), 5.28 (d, *J* = 10.0 Hz, 1H), 5.25 – 5.22 (m, 1H), 5.18 – 5.13 (m, 1H), 4.32 – 4.28 (m, 2H), 4.23 (t, *J* = 9.6 Hz, 2H), 4.02 – 3.84 (m, 15H), 3.80 – 3.72 (m, 11H), 3.69 – 3.66 (m, 2H), 3.62 – 3.43 (m, 12H), 3.37 – 3.34 (m,

3H), 3.25 – 3.21 (m, 2H), 3.20 – 3.17 (m, 1H), 3.13 (s, 2H), 3.10 – 3.08 (m, 1H), 2.83 – 2.73 (m, 3H), 2.59 – 2.50 (m, 2H), 2.35 – 2.33 (m, 1H), 2.31 – 2.25 (m, 5H), 2.02 – 1.95 (m, 4H), 1.83 (s, 3H), 1.71 – 1.63 (m, 6H), 1.58 – 1.54 (m, 2H), 1.52 – 1.50 (m, 1H), 1.43 – 1.34 (m, 14H), 1.05 (d, *J* = 7.0 Hz, 3H), 0.99 (t, *J* = 6.7 Hz, 3H), 0.88 (d, *J* = 6.9 Hz, 3H), 0.65 (d, *J* = 6.8 Hz, 3H), 0.15 (br. s, 3H) . ^{13}C NMR (126 MHz, D_2O + CD_3CN) δ 196.6, 185.3, 178.1, 175.5, 171.1, 169.2, 163.3, 151.1, 144.8, 137.6, 133.3, 132.8, 131.2, 130.1, 128.7, 127.7, 118.8, 115.2, 112.9, 110.6, 104.1, 95.0, 85.0, 84.7, 79.2, 79.0, 78.2, 76.7, 76.3, 76.2, 75.2, 75.0, 72.5, 71.4, 67.6, 66.6, 65.4, 62.3, 59.3, 57.5, 53.2, 52.6, 51.3, 51.2, 50.2, 41.8, 41.5, 40.3, 38.4, 35.5, 34.3, 32.5, 32.4, 32.2, 32.1, 32.0, 31.9, 31.3, 31.0, 30.3, 28.7, 28.2, 27.9, 25.1, 23.4, 23.1, 22.5, 20.2, 16.3, 12.7, 9.9. MALDI TOF-MS *m/e* calcd for $C_{75}H_{119}N_{12}O_{21}$, 1523.8534; measured *m/e* 1523.8541 $[M + H]^+$. MALDI TOF-MS *m/e* calcd for $C_{69}H_{106}N_{12}O_{21}Na$, 1461.7493; measured *m/e,* 1461.7498 $[M + Na]^+$.

Rifampicin-C_{12}-Tobramycin conjugate.5TFA (3). Compounds **11c** (0.100 g, 0.081 mmol) and **12** (0.036 g, 0.041 mmol) were conjoined via click chemistry as described in general procedure A to afford a final compound **3** (0.034 g, 55%) as a reddish brown solid. ^{1}H NMR (500 MHz, D_2O + CD_3CN) δ 8.02 (s, 1H, triazole), 7.69 (s, 1H, imine), 6.58 (br. d, *J* = 10.5 Hz, 1H), 6.42 (d, *J* = 11.0 Hz, 1H), 6.18 (d, *J* = 12.5 Hz, 1H), 6.06 (br. d, *J* = 10.5, 1H), 5.34 (d, *J* = 2.7 Hz, 1H), 5.20 (d, *J* = 10.1 Hz, 1H), 5.17 (d, *J* = 3.5 Hz, 1H), 5.09 (dd, *J* = 12.5, 6.1 Hz, 1H), 4.24 – 4.16 (m, 2H), 4.12 (br. s, 1H), 3.95 (t, *J* = 9.6 Hz, 1H), 3.91 (dd, *J* = 11.0, 3.4 Hz, 2H), 3.87 – 3.75 (m, 9H), 3.74 – 3.32 (m, 25H), 3.27 (d, *J* = 6.6 Hz, 3H), 3.17 – 3,13 (m, 1H), 3.10 (dd, *J* = 10.3, 2.3 Hz, 1H), 3.06 (s, 3H), 2.99 – 2.91 (m, 3H), 2.53 – 2.49 (m, 1H), 2.29 (s, 3H), 2.25 – 2.15 (m, 7H), 1.92 – 1.87 (m, 3H), 1.76 (s, 3H), 1.74 – 1.71 (m, 1H), 1.66 – 1.55 (m, 8H),

1.50 – 1.43 (m, 3H), 1.38 – 1.22 (m, 32H), 0.97 (d, *J* = 6.9 Hz, 3H), 0.91 (t, *J* = 6.7 Hz, 3H), 0.77 (d, *J* = 6.9 Hz, 3H), 0.57 (d, *J* = 6.8 Hz, 3H), 0.06 (br. s, 3H). ^{13}C NMR (126 MHz, D_2O + CD_3CN) δ 196.6, 185.3, 178.1, 175.5, 171.1, 169.2, 163.3, 151.1, 144.8, 137.6, 133.3, 132.8, 131.2, 130.1, 128.7, 127.7, 118.8, 115.2, 112.9, 110.6, 104.1, 95.0, 85.0, 84.7, 79.2, 79.0, 78.2, 76.7, 76.3, 76.2, 75.2, 75.0, 72.5, 71.4, 67.6, 66.6, 65.4, 62.3, 59.3, 57.5, 53.2, 52.6, 51.3, 51.2, 50.2, 41.8, 41.5, 40.3, 38.4, 35.5, 34.3, 32.5, 32.4, 32.2, 32.1, 32.0, 31.9, 31.3, 31.0, 30.3, 28.7, 28.2, 27.9, 25.1, 23.4, 23.1, 22.5, 20.2, 16.3, 12.7, 9.9. MALDI TOF-MS *m/e* calcd for $C_{75}H_{119}N_{12}O_{21}$, 1523.8534; measured *m/e* 1523.8541 $[M + H]^+$.

1-Nitrosopiperazine (7). Piperazine **4** (5.16 g, 60.0 mmol) was dissolved in 6 M HCl (36.0 mL) and cooled to -10 °C in ice bath. A solution of $NaNO_2$ (4.14 g, 60.0 mmol) in water (72.0 mL) was slowly added (using a separatory funnel) to the reaction mixture in ice bath over 1 h. The reaction mixture was adjusted to pH 10 with 3 M NaOH and extracted with $CHCl_3$ (100 mL, ×3). The combined organic extracts were dried over anhydrous Na_2SO_4, concentrated *in vacuo* and purified by flash column chromatography (hexanes/ ethyl acetate, 4:1 to 2:1, v/v) to give **5** as a yellow oil (3.56 g, 51%). ^{1}H NMR (300 MHz, $CDCl_3$) δ 3.72 (m, 2H,), 3.29 (m, 2H), 2.57 (m, 2H), 2.32 (m, 2H). ^{13}C NMR (75 MHz, $CDCl_3$) δ 50.8, 46.2, 44.6, 40.7. ESI-MS: *m/z* calcd for $C_4H_{10}N_3O$, 116.08; found 116.2 $[M + H]^+$.

1-Nitroso-4-propargylpiperazine (6). A solution of 1-nitrosopiperazine **5** (2.61 g, 22.48 mmol) in anhydrous acetonitrile (20.0 mL) was treated with propargyl bromide (3.37 g of 80% solution in toluene, 22.48 mmol) and Et_3N (6.3 mL, 44.96 mmol). The reaction mixture was refluxed for 3 h and concentrated *in vacuo*. The crude product was subsequently dissolved in 10 % NaOH

(50.0 mL) and extracted with CH_2Cl_2 (50.0 mL, ×3). The combined organic phase was dried over anhydrous Na_2SO_4, concentrated *in vacuo* and purified by column chromatography (hexanes/ ethyl acetate, 4:1, v/v) to yield **6** as an orange oil (1.39 g, 40%). ^{1}H NMR (300 MHz, $CDCl_3$) δ 3.98 – 3.92 (m, 2H), 3.50 (dd, *J* = 6.0, 4.9 Hz, 2H), 3.09 (d, *J* = 2.6 Hz, 2H, C*H*$_2$CCH), 2.48 – 2.42 (m, 2H), 2.23–2.17 (m, 3H). ^{13}C NMR (75 MHz, $CDCl_3$) δ 77.3, 74.2, 51.6, 50.1, 49.1, 46.3, 39.0. ESI-MS: *m/z* calcd for $C_7H_{12}N_3O$, 154.09; found 154.1 $[M + H]^+$.

1-Amino-4-propargylpiperazine (7). $LiAlH_4$ (0.59 g, 15.58 mmol) was added to a flame-dried RBF, suspended in anhydrous Et_2O (15.0 mL) and stirred vigorously for 5 mins. A solution of **6** (1.20 g, 7.79 mmol) in anhydrous Et_2O (5.0 mL) was slowly added in ice bath, stirred for 2 mins at RT, and then refluxed for 6 h. The reaction mixture was then cooled in ice bath, quenched with 2 M HCl and filtered through celite. The celite was subsequently washed with 2 M HCl (100 mL) and the filtrate adjusted to pH 10 with 10% NaOH. The aqueous phase was extracted with CH_2Cl_2 (100 mL, ×3), dried over anhydrous Na_2SO_4 and concentrated *in vacuo*. The crude product was purified by column chromatography (dichloromethane/ methanol, 20:1 to 10:1, v/v) to yield compound **7** (0.33 g, 30%). ^{1}H NMR (300 MHz, $CDCl_3$) δ 3.28 (br. s, 2H), 3.24 (d, *J* = 1.5 Hz, 2H, C*H*$_2$CCH), 2.80 – 2.74 (m, 2H), 2.61 – 2.43 (br. m, 4H), 2.42 – 2.36 (m, 2H), 2.16 (t, *J* = 2.5, 1H, CH_2CCH). ^{13}C NMR (75 MHz, $CDCl_3$) δ 73.4, 73.3, 58.9, 52.5, 51.4, 46.2, 45.46. ESI-MS: *m/z* calcd for $C_7H_{14}N_3$, 140.11; found 140.2 $[M + H]^+$.

1,3,2′,6′,3″-penta-*N*-*Boc*-4′,2″,4″,6″-tetra-*O*-TBDMS-Tobramycin (9). Commercial tobramycin (4.0 g, 8.56 mmol) was dissolved in a 2:1 mixture of methanol and water (90.0 mL) and treated with Boc_2O (18.7 g, 85.56 mmol) in the presence of Et_3N (5.0 mL). The reaction

mixture was stirred under reflux (at 55 °C) overnight, concentrated under vacuo and thoroughly dried under high vacuum for 24 h to afford a white powdery solid (7.04 g, 85%). The dried crude penta-*N*-boc-protected tobramycin (7.0 g, 7.27 mmol) was dissolved in anhydrous DMF (6.0 mL) and treated with *tert*-butyldimethysilyl chloride, TBDMSCl (11.0 g, 72.98 mmol) and *N*-methylimidazole (4.0 mL). The reaction was stirred at RT for 5 days under nitrogen gas atmosphere, and the resulting mixture was poured into water (100.0 mL) and extracted with DCM (×3). The organic layer was dried over anhydrous Na_2SO_4, concentrated in *vacuo*, and purified by flash chromatography using gradient elution (hexanes/ ethyl acetate, 15/1 to 8/1, v/v) to afford **9** (7.87 g, 76%) as a white solid. NMR data are consistent with an earlier report.[21]

5-*O*-(*n*-Azidoalkyl)-1,3,2′,6′,3″-penta-*N*-Boc-4′,2″,4″,6″-tetra-*O*-TBDMS-Tobramycin (10a–c). Compounds **10a**, **10b**, and **10c** were prepared by treating **9** with 1,4-dibromobutane, 1,6-dibromohexane, and 1,12-dibromododecane, respectively, according to general procedure B.

5-*O*-(4-Azidobutyl)-1,3,2′,6′,3″-penta-*N*-*Boc*-4′,2″,4″,6″-tetra-*O*-TBDMS-Tobramycin (10a). Yield (55%). ^{1}H NMR (500 MHz, $CDCl_3$) δ 5.27 – 4.98 (m, 2H, anomeric), 4.32 – 4.00 (m, 2H), 3.87 – 3.06 (m, 17H), 2.43 (d, *J* = 7.5 Hz, 1H), 2.06 – 1.95 (m, 2H), 1.91 – 1.72 (m, 2H), 1.57 – 1.35 (m, 49), 0.98 – 0.79 (m, 36H), 0.19 – 0.04 (m, 24H, Si(CH_3)$_2$). ^{13}C NMR (126 MHz, $CDCl_3$) δ 156.9, 156.8, 156.5, 156.2, 156.0, 97.9 (anomeric), 96.6 (anomeric), 85.7, 79.7, 79.0, 75.3, 73.3, 72.9, 71.5, 68.0, 66.8, 63.1, 57.3, 50.5, 49.0, 48.5, 41.6, 35.9, 35.7, 34.1, 32.8, 30.6, 29.4, 28.4, 26.1, 26.0, 22.0, 18.0, 17.9, 17.8, 17.6, -4.7, -5.1, 5.5, -5.9, -6.3, -6.4. ESI-MS: *m/z* calcd for $C_{71}H_{140}N_8O_{19}Si_4Na$, 1543.9; found 1544.2 $[M + Na]^+$.

5-*O*-(6-Azidohexyl)-1,3,2′,6′,3″-penta-*N*-*Boc*-4′,2″,4″,6″-tetra-*O*-TBDMS-Tobramycin (10b). Yield (59%). ^{1}H NMR (500 MHz, $CDCl_3$) δ 5.28 – 4.99 (m, 2H, anomeric), 4.31 – 3.97 (m, 2H), 3.87 – 3.05 (m, 17H), 2.42 (d, *J* = 7.5 Hz, 1H), 2.06 – 1.96 (m, 2H), 1.92 – 1.72 (m, 2H), 1.57 – 1.35 (m, 49), 0.98 – 0.80 (m, 40H), 0.19 – 0.04 (m, 24H, Si(CH_3)$_2$). ^{13}C NMR (126 MHz, $CDCl_3$) δ 157.0, 156.7, 156.5, 156.1, 156.0, 97.8 (anomeric), 96.6 (anomeric), 85.7, 79.8, 78.9, 75.4, 73.3, 72.9, 71.5, 68.1, 66.7, 63.1, 57.3, 50.3, 49.1, 48.5, 41.5, 35.9, 35.5, 34.1, 32.8, 30.6, 29.4, 28.4, 26.1, 26.0, 22.0, 18.0, 17.9, 17.8, 17.6, -4.7, -5.1, 5.5, -5.9, -6.3, -6.4. ESI-MS: *m/z* calcd for $C_{73}H_{144}N_8O_{19}Si_4Na$, 1571.95; found 1571.8 $[M + Na]^+$.

5-*O*-(12-Azidododecyl)-1,3,2′,6′,3″-penta-*N*-*Boc*-4′,2″,4″,6″-tetra-*O*-TBDMS-Tobramycin (10c). Yield (66%). ^{1}H NMR (500 MHz, $CDCl_3$) δ 5.26 – 4.97 (m, 2H, anomeric), 4.32 – 4.00 (m, 2H), 3.87 – 3.06 (m, 17H), 2.43 (d, *J* = 7.5 Hz, 1H), 2.06 – 1.95 (m, 2H), 1.91 – 1.72 (m, 2H), 1.57 – 1.35 (m, 49), 0.98 – 0.79 (m, 42H), 0.19 – 0.04 (m, 24H, Si(CH_3)$_2$). ^{13}C NMR (126 MHz, $CDCl_3$) δ 156.9, 156.8, 156.5, 156.2, 156.0, 97.9 (anomeric), 96.6 (anomeric), 85.7, 79.7, 79.0, 75.3, 73.3, 72.9, 71.5, 68.0, 66.8, 63.1, 57.3, 50.5, 49.0, 48.5, 41.6, 35.9, 35.7, 34.1, 32.8, 30.6, 29.4, 28.4, 26.1, 26.0, 22.0, 18.0, 17.9, 17.8, 17.6, -4.7, -5.1, 5.5, -5.9, -6.3, -6.4. ESI-MS: *m/z* calcd for $C_{79}H_{156}N_8O_{19}Si_4Na$, 1656.04; found 1656.2 $[M + Na]^+$.

5-*O*-(n-Azidoalkyl)-Tobramycin.5TFA (11a–c). Compounds **11a**, **11b**, and **11c** were prepared by treating compounds **10a–c** with tetrabutylammonium fluoride and trifluoroacetic acid successively, according to general procedure C.

5-*O*-(4-Azidobutyl)-Tobramycin.5TFA (11a). ^{1}H NMR (300 MHz, D_2O) δ 5.39 (d, *J* = 2.4 Hz, 1H, anomeric), 5.20 (d, *J* = 3.4 Hz, 1H, anomeric), 4.34 – 4.17 (m, 2H), 4.00 – 3.71 (m, 11H), 3.63 – 3.52 (m, 3H), 3.49 – 3.27 (m, 4H), 2.61 – 2.48 (m, 1H), 2.36 – 2.19 (2H), 2.11 –

1.93 (m, 1H), 1.81 – 1.56 (m, 4H). ^{13}C NMR (75 MHz, D_2O) δ 101.1 (anomeric), 92.6 (anomeric), 81.9, 81.7, 76.6, 75.7, 73.1, 72.7, 68.5, 64.8, 63.2, 59.3, 54.8, 51.0, 49.6, 48.4, 47.3, 38.5, 28.0, 27.7, 26.7, 24.6. MALDI TOF-MS *m/e* calcd for $C_{22}H_{44}N_8O_9$, 565.3231; measured *m/e* 565.35541 $[M + H]^+$.

5-*O*-(6-Azidohexyl)-Tobramycin.5HCl (11b). ^{1}H NMR (500 MHz, D_2O) δ 5.31 (d, *J* = 2.6 Hz, 1H, anomeric), 5.10 (d, *J* = 3.6 Hz, 1H, anomeric), 4.23 – 4.17 (m, 1H), 4.14 (dd, *J* = 11.1, 8.5 Hz, 1H), 3.88 – 3.61 (m, 11H), 3.59 – 3.47 (m, 3H), 3.37 – 3.30 (m, 1H), 3.27 – 3.21 (m, 3H), 2.49 – 2.42 (m, 1H), 2.23 – 2.10 (m, 2H), 1.99 – 1.89 (m, 1H), 1.63 – 1.49 (m, 4H), 1.36 – 1.19 (m, 5H). ^{13}C NMR (126 MHz, D_2O) δ 101.3 (anomeric), 92.7 (anomeric), 81.9, 81.8, 76.6, 75.6, 73.5, 73.2, 68.6, 64.9, 63.3, 59.3, 54.8, 51.2, 49.8, 48.5, 47.4, 38.6, 29.4, 28.2, 28.0, 27.8, 26.2, 25.3, 24.8, 19.4. MALDI TOF-MS *m/e* calcd for $C_{24}H_{48}N_8O_9Na$, 615.3442; measured *m/e* 615.3555 $[M + Na]^+$.

5-*O*-(12-Azidododecyl)-Tobramycin.5TFA (11c). ^{1}H NMR (300 MHz, D_2O) δ 5.38 (d, *J* = 2.4 Hz, 1H, anomeric), 5.15 (d, *J* = 3.4 Hz, 1H, anomeric), 4.28 (dd, *J* = 8.8, 4.1 Hz, 1H), 4.20 – 4.07 (m, 1H), 3.99 – 3.48 (m, 16H), 3.45 – 3.24 (m, 4H), 2.61 – 2.47 (m, 1H), 2.30 – 2.20 (m, 2H), 2.02 – 1.85 (m, 1H), 1.73 – 1.52 (m, 4H), 1.46 – 1.20 (m, 18H). ^{13}C NMR (75 MHz, D_2O) δ 101.4 (anomeric), 92.7 (anomeric), 81.9, 81.8, 76.9, 76.0, 73.8, 73.2, 68.5, 64.8, 63.1, 59.3, 54.8, 51.3, 49.7, 48.3, 47.2, 38.3, 29.4, 28.9, 28.8, 28.7, 28.6, 28.4, 28.2, 28.1, 28.0, 27.7, 26.0, 25.3. MALDI TOF-MS *m/e* calcd for $C_{30}H_{60}N_8O_9$, 676.4483; measured *m/e* 676.4492.

***N*-propargyl-Rifampicin (12)**. A solution of Rifaldehyde (3-formyl rifamycin SV) (0.62 g, 0.854 mmol) suspended in dry THF (5.0 mL) was treated with 1-amino-4-propargylpiperazine (**7**)

(0.12 g, 0.854 mmol) and stirred at RT for 20 mins. The reaction mixture was diluted with DCM (10.0 mL) and washed with a solution of ascorbic acid (2.0 g) in 3:1 H_2O/brine (40.0 mL). The aqueous layer was then extracted with DCM (30.0 mL, ×3), and the combined organic layers were dried over anhydrous Na_2SO_4 and concentrated *in vacuo*. The crude product was purified by reverse-phase (C18) column chromatography using a gradient elution of acetonitrile /water, 1:1 to 4:1, v/v, to yield rifampicin alkyne (**12**) as a reddish orange solid (0.70 g, 97%). ^{1}H NMR (300 MHz, CD_3OD) δ 8.00 (s, 1H, imine), 6.64 – 6.51 (m, 1H), 6.45 (dd, *J* = 11.3, 1.6 Hz, 1H), 6.18 (dd, *J* = 12.8, 0.9 Hz, 1H), 5.91 (dd, *J* = 15.3, 4.7 Hz, 1H), 5.05 – 4.88 (m, 2H), 3.72 (dd, *J* = 9.2, 1.7 Hz, 1H), 3.43 – 3.33 (m, 2H), 3.32 – 3.26 (m, 1H), 3.22 – 3.03 (m, 4H, piperazine), 2.99 (dd, *J* = 10.3, 2.4 Hz, 1H), 2.91 (s, 3H), 2.86 – 2.64 (m, 4H, piperazine), 2.33 – 2.20 (m, 1H), 2.08 (s, 3H), 2.04 (s, 3H), 1.96 (s, 1H, alkyne), 1.95 (s, 3H), 1.77 (s, 3H), 1.65 – 1.53 (m, 1H), 1.33 – 1.16 (m, 2H), 1.02 – 0.80 (m, 6H), 0.46 (d, *J* = 6.8 Hz, 3H), -0.55 (d, *J* = 6.7 Hz, 3H). ^{13}C NMR (75 MHz, CD_3OD) δ 194.6, 173.8, 171.0, 169.5, 147.2, 143.3, 141.8, 138.4, 134.4, 133.7 (imine), 130.0, 123.5, 119.4, 117.4, 112.3, 111.0, 109.1, 105.4, 77.1, 76.6, 74.3, 74.0, 71.4, 55.5, 50.2, 49.6, 45.7, 40.1, 38.6, 38.1, 33.0, 29.3, 20.9, 19.4, 17.3, 9.6, 8.0, 7.8, 6.7, -0.6. MALDI TOF-MS *m/e* calcd for $C_{45}H_{58}N_4O_{12}$, 846.4051; measured *m/e* 846.4068.

5.2 Microbiology. Bacterial isolates were either obtained from the American Type Culture Collection (ATCC), the Canadian National Intensive Care Unit (CAN-ICU) surveillance study[54], or the Canadian Ward (CANWARD) surveillance study[55,56]. Clinical isolates obtained as part of the CAN-ICU and CANWARD studies from participating medical centers across Canada were cultured from body fluids and tissues of patients suffering from presumed "clinically significant" infectious diseases. Antimicrobial susceptibilities of clinical isolates were evaluated (using ATCC strains as quality control strains) and categorized, where appropriate, as either multidrug resistant (MDR), extensively drug-resistant (XDR), or pan drug-resistant (PDR). MDR is defined as acquired non-susceptibility to at least one agent in three or more antimicrobial categories, XDR as non-susceptibility to at least one agent in all but two or fewer antimicrobial categories (i.e. bacterial isolates remain susceptible to only one or two categories), and PDR as non-susceptibility to all agents in all antimicrobial categories.[26]

5.2.1 Antimicrobial Susceptibility Assay. The *in vitro* antimicrobial activity of all compounds/antibiotics against a panel of bacteria was evaluated by microbroth dilution method in accordance with the Clinical and Laboratory Standards Institute (CLSI) guidelines. Overnight grown bacterial cultures 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 approximately 5×10^5 CFU/mL. The antimicrobial agents were 2-fold serially diluted in MHB in a 96-well plate and incubated at 37 °C with equal volumes of inoculum for 18 h. The lowest concentration that prevented the visible growth of bacteria was defined as the MIC for each antimicrobial agent. The broth with or without bacterial cells was used as positive or negative control, respectively.

5.2.2 Checkerboard Assay. Combination studies with different antibiotics were performed in 96-well plates as previously described.[25] Briefly, the antibiotic of interest was serially diluted in MHB along the abiscissa while the adjuvant (newly synthesized conjugates) was serially diluted in MHB along the ordinate. This creates a 10×7 matrix wherein each well consists of a combination of different antibiotic and adjuvant concentrations. Overnight grown bacterial cultures 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 approximately 5×10^5 CFU/mL. Equal volume of this bacterial culture was then added to each well and incubated at 37 °C for 18 h. After incubation, the plates were read on EMax® Plus microplate reader (Molecular Devices, Sunnyvale, CA, USA) at 590 nm. MIC was recorded as wells with the lowest concentration of drugs with no bacterial growth. The fractional inhibitory concentration (FIC) for each antibiotic was calculated by dividing the MIC of the antibiotic in the presence of adjuvant by the MIC of the antibiotic alone. Similarly, the FIC of adjuvant was calculated by dividing the MIC of the adjuvant in the presence of antibiotic by the MIC of the adjuvant alone. FIC index is the sum of both FICs. FIC indices of < 0.5 were deemed synergistic; 0.5 - 4, no interaction; and > 4, antagonistic.

5.2.3 Time-kill assay. Time-kill curve analyses were performed by culturing *P. aeruginosa* in LB medium, in the presence of antibiotics alone and in combination with test adjuvants. MICs of antibiotics and adjuvants were determined before the experiment following CLSI microbroth dilution guidelines. Growth curves were initially performed to confirm that all strains will reach a stable early- to mid-log phase after 4 h of pre-incubation in antimicrobial-free LB medium. A

0.5 McFarland inoculum of each strain was prepared in sterile 0.85 % saline solution from an overnight grown culture. For each strain, 30 µl of the prepared inoculum was diluted to 3 ml of LB broth (containing different combinations of antimicrobial agents and adjuvants) and incubated at 37 °C shaking at 250 rpm. At specific time intervals (0, 1, 3, 6, 9, and 24 h), 100 µl was taken from each sample, serially diluted in sterile PBS, plated on LB agar plates, and incubated at 37 °C in a humid 5 % CO_2-enriched atmosphere. Bacterial colonies were counted after 20 h of incubation.

5.2.4 *In vivo* larvae–infection model. *In vivo* synergistic effects were determined using *Galleria mellonella* infection model, as previously described.[21] Briefly, worms were purchased from The Worm Lady Live Feeder (ON, Canada), stored in their natural habitat at 16 ^{o}C, 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 extensively-resistant *P. aeruginosa* PA260 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 3 h at 37 °C. After the 3 h challenge, worms in monotherapy experimental groups (fifteen worms per group) were treated with 10 µL injection of doxycycline (100 mg/kg), chloramphenicol (100 mg/kg), compound **2** (100 mg/kg), or PBS alone. The worms in combination therapy groups were treated with doxycycline + compound **2** (75 + 75 mg/kg or

100 + 100 mg/kg) or chloramphenicol + compound **2** (75 + 75 mg/kg or 100 + 100 mg/kg). Worms treated with 10 μL PBS or colistin (50 mg/kg, 75 mg/kg or 100 mg/kg) served as negative and positive control, respectively. The worms were incubated in Petri dishes lined with filter paper at 37 °C and scored for survivability every 6 h for 36 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.

5.2.5 Cytotoxicity Assay. Human embryonic kidney cells (HEK293) and HepG2 cells were grown in Dulbecco's modified eagle's medium supplemented with 10% fetal bovine serum in a humidified 5% atmospheric incubator at 37 °C. Equal number of cells (100 μl of media containing ~8000 cells) were dispersed into 96-well plates and wells with medium but no cells were used as blanks. After incubating for 24 h, 100 μl of varying concentrations of test compounds (at twice the desired concentrations) were added to each well, including the blanks. The treated cells were then incubated further for 48 h, after which PrestoBlue reagent was added to each well. The plates were then incubated for an additional hour on a nutator mixer in a 5% CO_2 incubator. The fluorescence was read at 490 nm on a SpectraMax M2 plate reader (Molecular Devices, USA). Cell viability were interpreted as previously described.[46,57] The values of blank were subtracted from each value, and the viability values of the treated samples relative to the controls with vehicle were calculated. The values for the plots are the means ± standard deviation.

5.2.6 Hemolytic Assay. The hemolytic activities of the newly synthesized compounds were determined and quantified as the amount of hemoglobin released by lysing ovine 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.

Conflicts of Interest

The authors declare no conflicts of interest

Acknowledgments

The authors thank the Natural Sciences and Engineering Research Council (NSERC) of Canada for financial support in the form of a Discovery Grant (2018-06047) and Prof. A. Kumar

(University of Manitoba) for providing us with efflux pump deficient *P. aeruginosa* mutants, PAO200 and PAO750.

Appendix A. Supporting Information

Supplementary data to this article can be found online at

Author Contributions

TI performed all chemical synthesis, biological studies, and *in vivo* experiments. GA conducted cytotoxicity testing. GGZ and FS supervised the project. TI analyzed the data and wrote the manuscript, with contributions from all authors.

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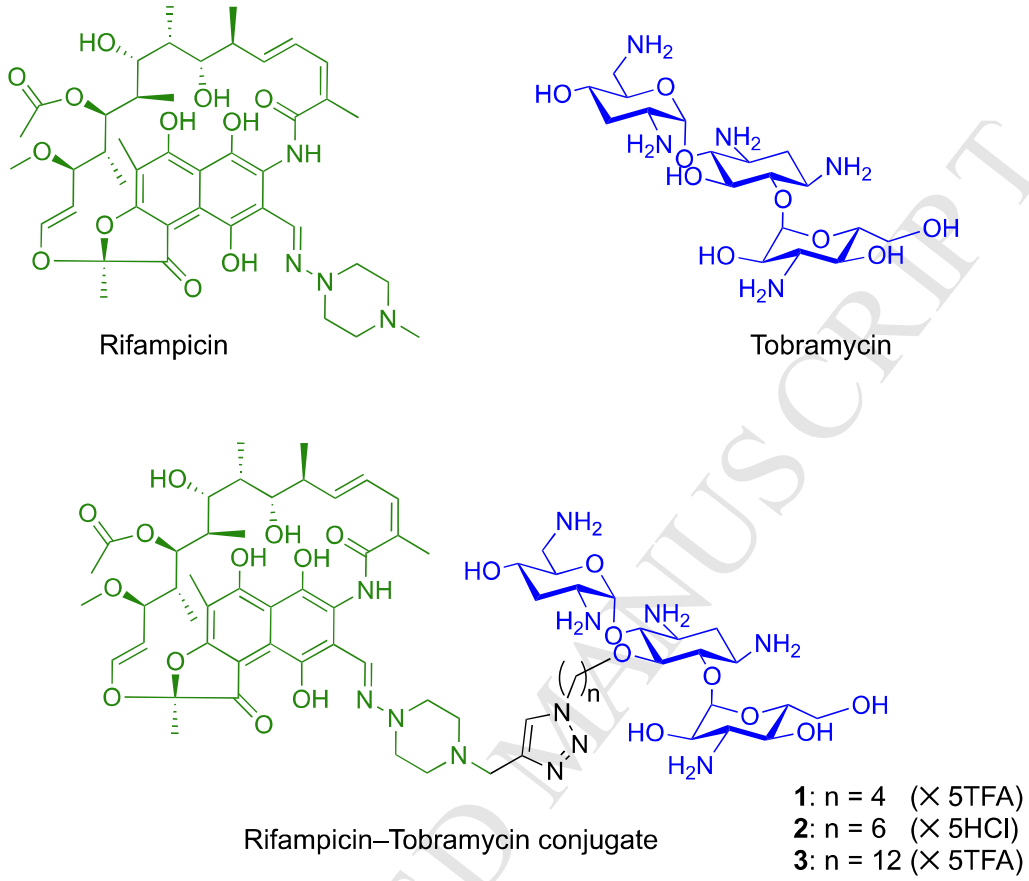
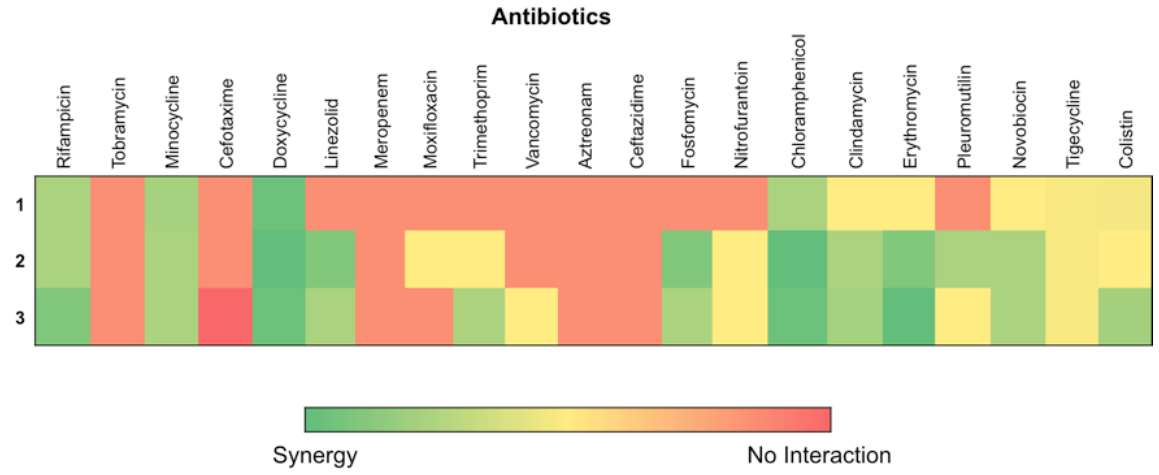


Figure 1: Structures of Rifampicin, Tobramycin, and newly synthesized Rifampicin-Tobramycin conjugates **1** – **3**. Conjugates differ in the length of carbon chains.

a)



b)

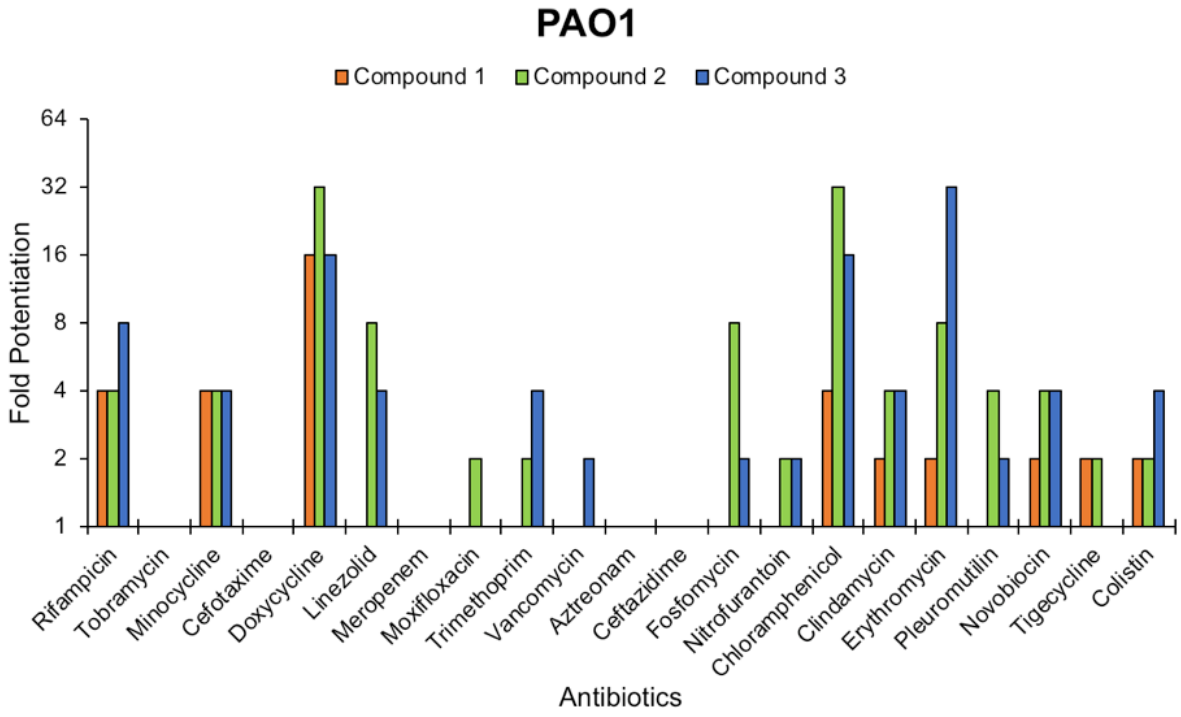


Figure 2. (a) Interactions of compounds **1 – 3** (at ≤ 10 μM, i.e. 2 -16 μg/ml) with different antibiotics against WT *P. aeruginosa* PAO1. FIC < 0.4 = Green; FIC ≥ 0.5 but < 0.6 = Yellow;

FIC > 0.6 but < 3 = Red, (b) Fold potentiation of several classes of antibiotics by rifampicin-tobramycin conjugates **1** – **3** (at ≤ 10 μM) against WT *P. aeruginosa* PAO1

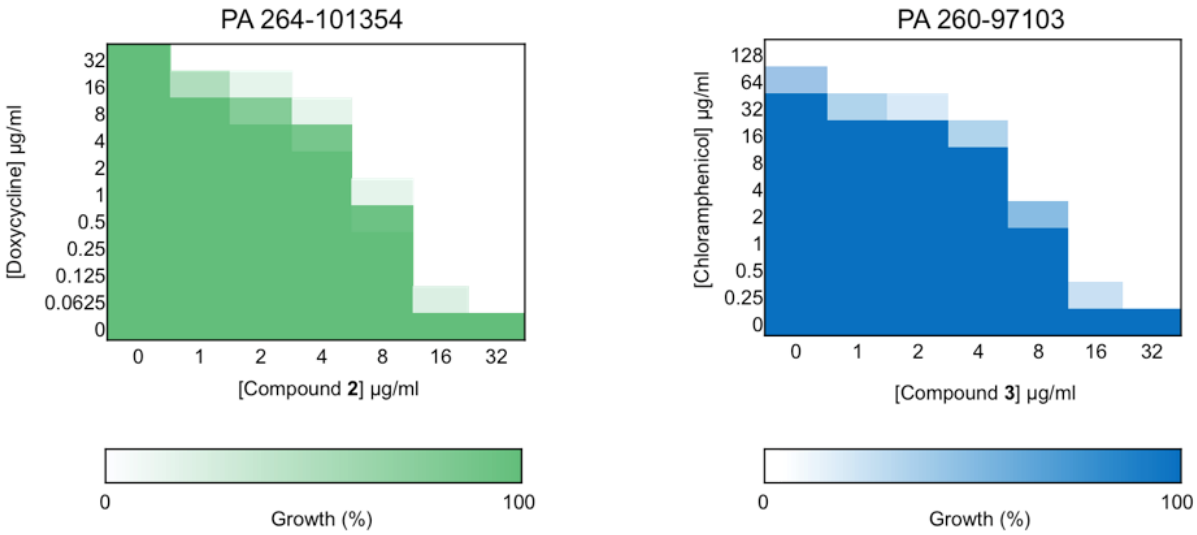


Figure 3. Representative checkerboard broth microdilution assays showing dose-dependent potentiation of doxycycline and chloramphenicol in two MDR/XDR *P. aeruginosa* clinical isolates by compounds **2** and **3**. Dark colours represent higher cell density (OD measured at 590 nm); 16 µg/ml of compounds **2** and **3** = 9.8 µM and 7.6 µM, respectively.

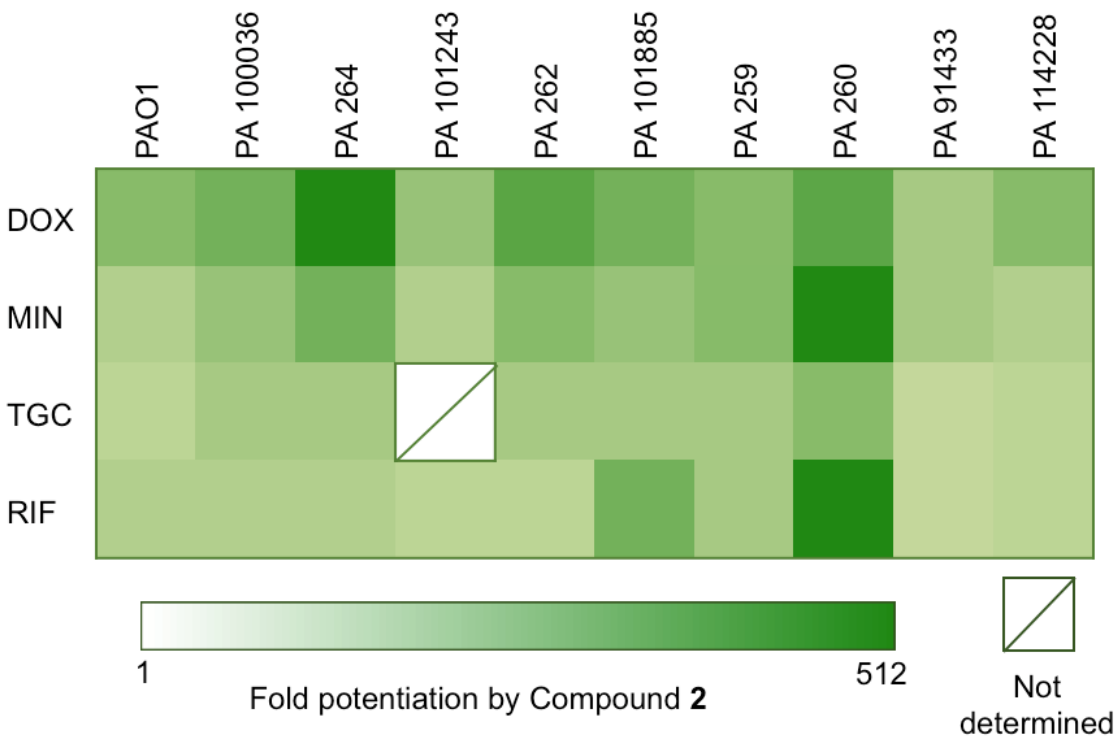


Figure 4. Fold change in susceptibilities of wild-type and MDR/XDR *P. aeruginosa* to doxycycline (DOX), minocycline (MIN), tigecycline (TGC), and rifampicin (RIF) in the presence of ≤ 9.8 μM of compound **2**.

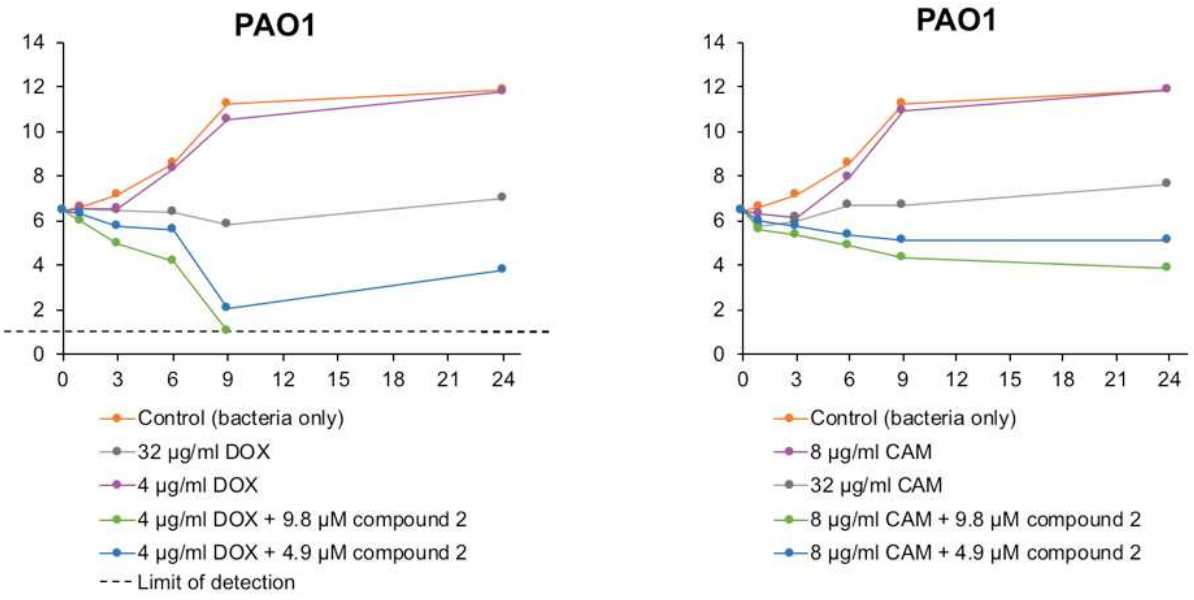
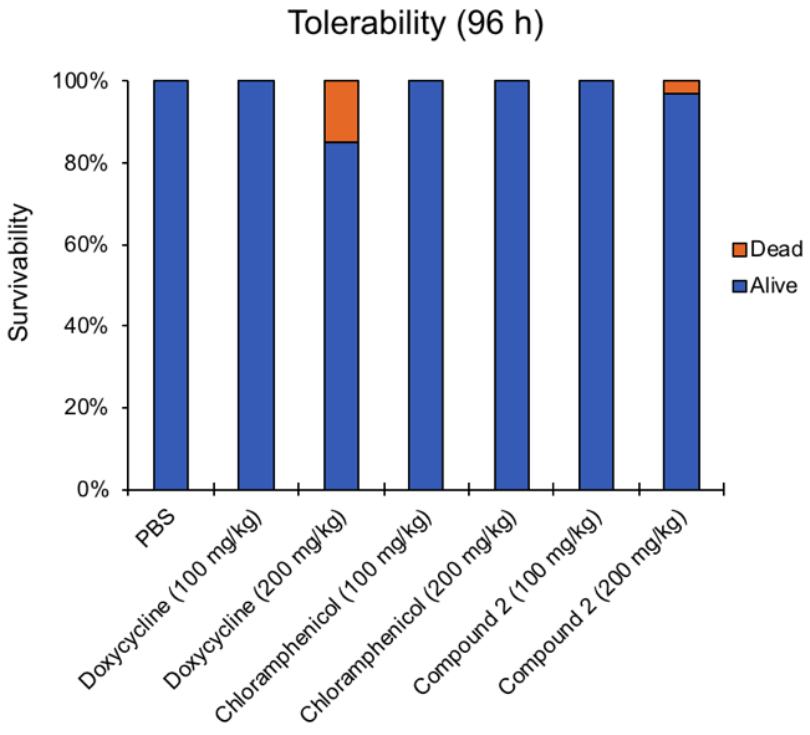
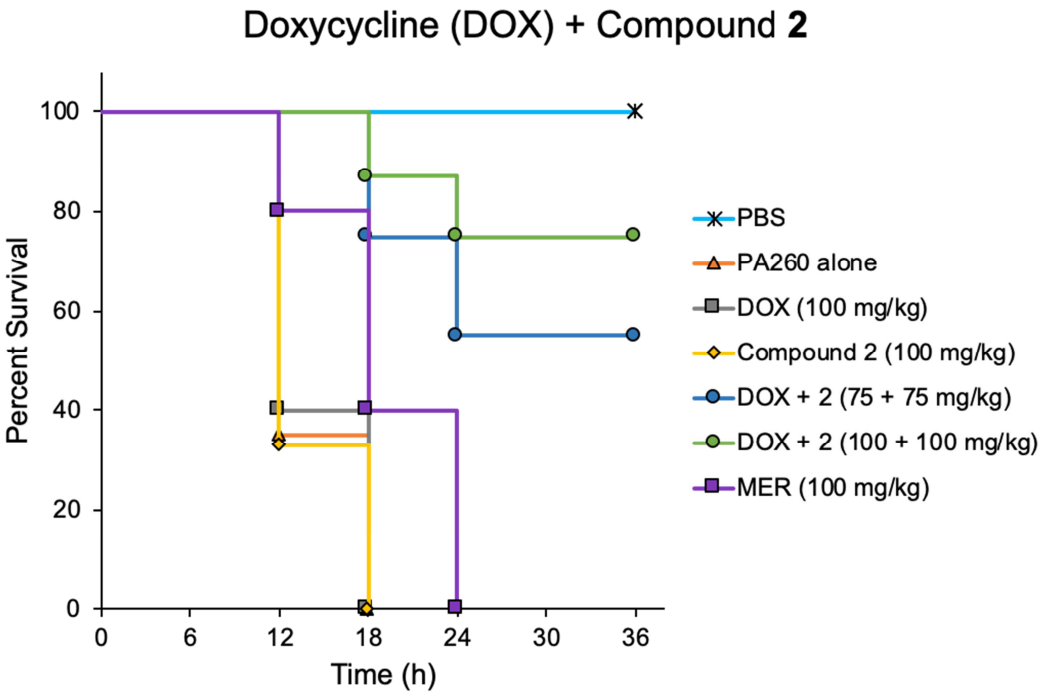


Figure 5. Time-kill kinetics of doxycycline (DOX) and chloramphenicol (CAM), alone and in combination with different concentrations of compound **2**, on the viability of wild type *P. aeruginosa* PAO1 grown in LB media. MICs of DOX and CAM are 16 µg/ml and 32 µg/ml, respectively. CLSI susceptibility breakpoint of DOX (against *Acinetobacter spp*) and CAM (against *Enterobacteriaceae*) are 4 µg/ml and 8 µg/ml, respectively. Colony-forming units (CFU) of PAO1 were reduced below the limits of detection when incubated with 4 µg/ml DOX and 9.8 4 µM of compound **2**. Each data point is an average of three independent determinations.

a)


Tolerability (96 h)
Survivability
100%
80%
60%
40%
20%
0%
Dead
Alive
PBS
Doxycycline (100 mg/kg)
Doxycycline (200 mg/kg)
Chloramphenicol (100 mg/kg)
Chloramphenicol (200 mg/kg)
Compound 2 (100 mg/kg)
Compound 2 (200 mg/kg)

b)


Doxycycline (DOX) + Compound 2
Percent Survival
100
80
60
40
20
0
0
6
12
18
24
30
36
Time (h)
PBS
PA260 alone
DOX (100 mg/kg)
Compound 2 (100 mg/kg)
DOX + 2 (75 + 75 mg/kg)
DOX + 2 (100 + 100 mg/kg)
MER (100 mg/kg)

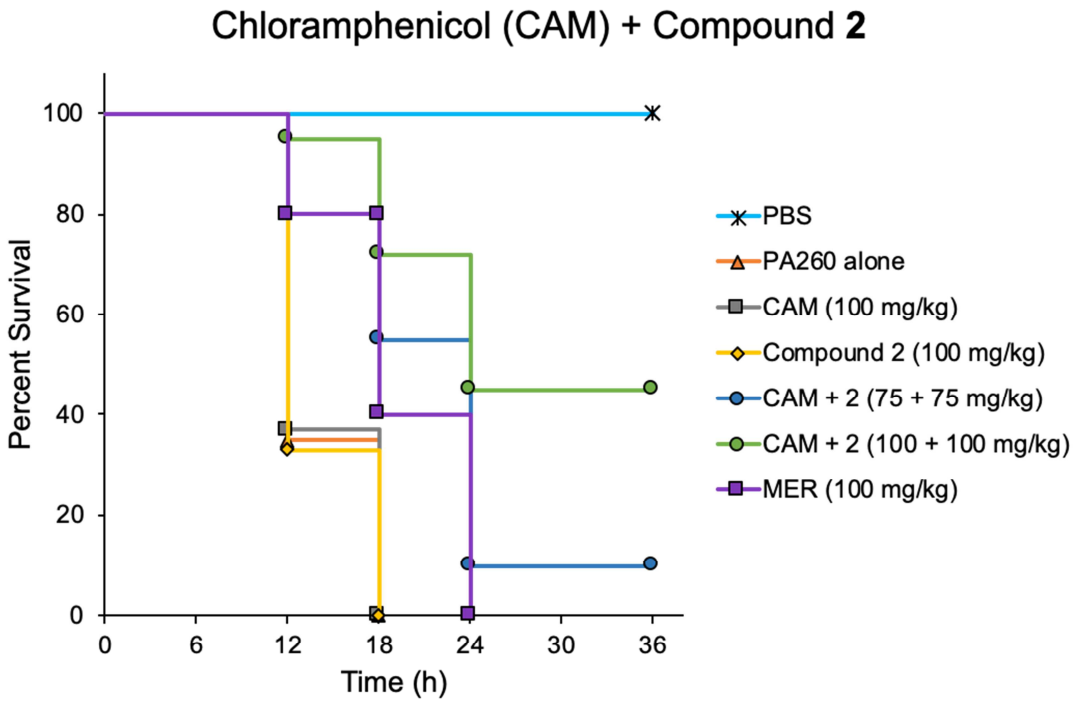
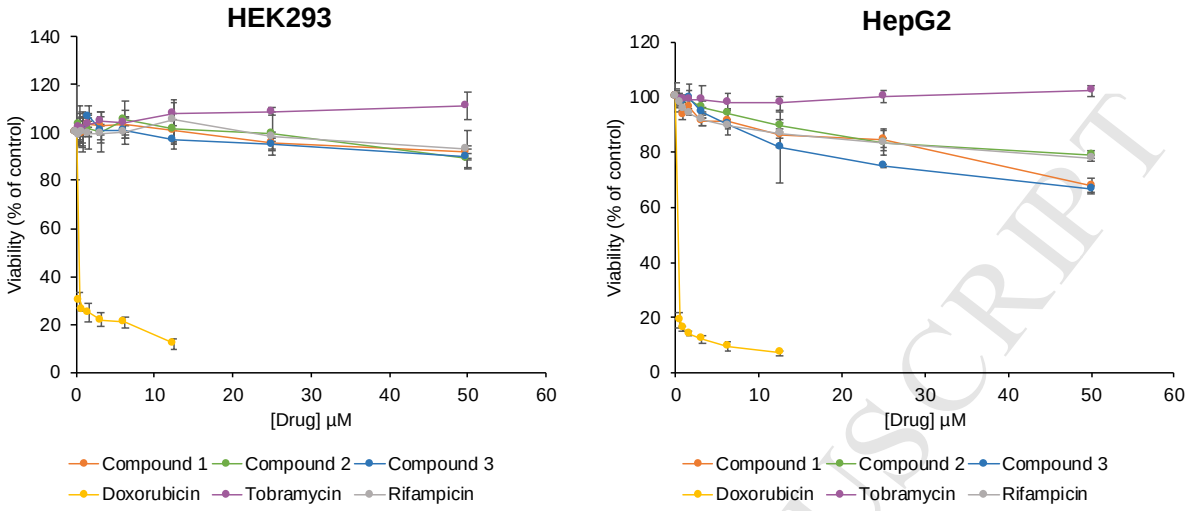
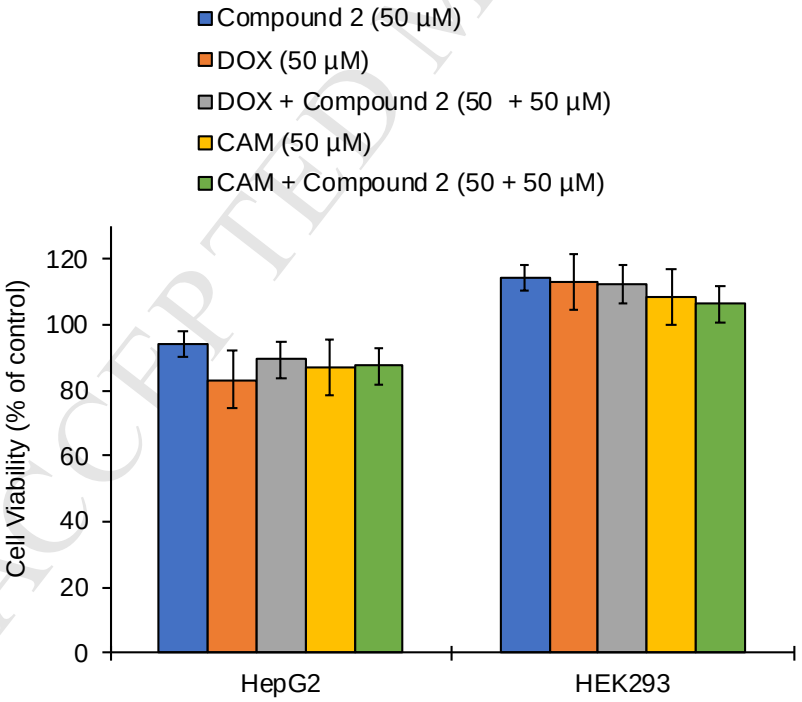


Figure 6. *In vivo* dose-dependent efficacy of a combination therapy of compound **2** and doxycycline (DOX) or chloramphenicol (CAM) demonstrated in *Galleria mellonella* infection model. a) Tolerable doses were determined by injecting 100 and 200 mg/kg of test compounds alone into the larvae and scored for survivability for 96 h (4 days). b) Efficacy studies using a single dose administration of different concentrations of mono- and combination therapies to treat PA260-challenged larvae 3 h post infection. Survivability of the larvae was scored every 6 h for 36 h.

a)



b)



c)

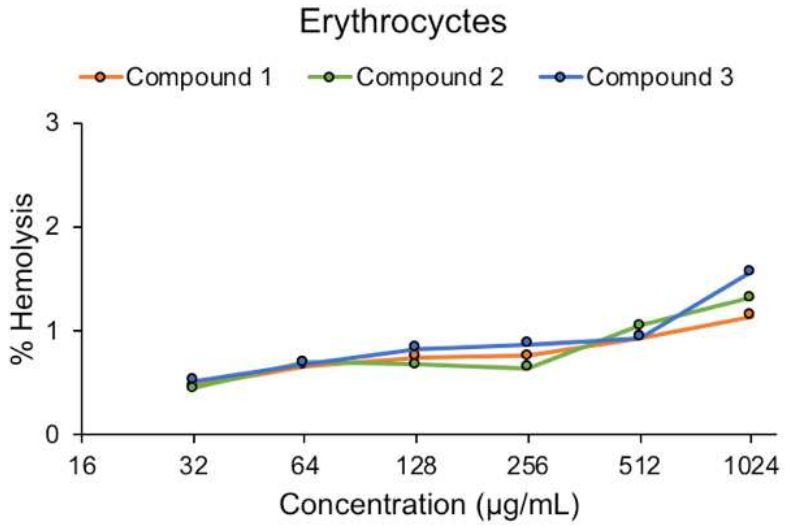


Figure 7. a) Cytotoxicity of compounds **1** – **3**, doxorubicin, tobramycin, and rifampicin against human liver carcinoma (HepG2) cells and human embryo kidney (HEK293) cells using PrestoBlue cell viability assay. Doxorubicin was used as positive control. Error bars denote standard deviation of at least four replicates. b) Cytotoxic evaluation of compound **2** + (doxycycline, DOX or chloramphenicol, CAM) combinations (at 50 µM) against HepG2 and HEK293 cell lines. c) Hemolytic activity of compounds **1** – **3** evaluated against porcine erythrocytes at different concentrations. 0.1% Triton X-100 (100 % hemolysis) was used as positive control to calculate percent hemolysis. The result represents the mean of three independent determinations.

Table 1. Minimum inhibitory concentrations (MICs, μg/ml) of Rifampicin, Tobramycin, and compounds **1 – 3** against a panel of Gram-positive and Gram-negative bacteria

Test organism	Rifampicin	Tobramycin	Rifampicin–Tobramycin conjugates		
			1	**2**	**3**
S. aureus ATCC 29213	≤0.25	0.5	4	16	16
MRSA ATCC 33592	>128	0.5	>32	>32	>32
MSSE CANWARD-2008 81388	≤0.25	≤0.25	2	4	4
MRSE CAN-ICU 61589 (CAZ >32)	≤0.25	2	2	4	8
E. faecalis ATCC 29212	1	8	>32	>32	>32
E. faecium ATCC 27270	≤0.25	16	32	>32	>32
S. pneumoniae ATCC 49619	≤0.25	2	1	2	8
E. coli ATCC 25922	8	0.5	>32	>32	16
E. coli CAN-ICU 61714 (GEN-R)	8	8	>32	>32	>32
E. coli CAN-ICU 63074 (AMK 32)	16	8	>32	>32	32
E. coli CANWARD-2011 97615 (GEN-R, TOB-R, CIP-R) aac(3')iia	16	128	>32	>32	32
P. aeruginosa ATCC 27853	32	1	>32	>32	>32
P. aeruginosa CAN-ICU 62308 (GEN-R)	32	16	>32	>32	>32
P. aeruginosa CANWARD-2011 96846 (GEN-R, TOB-R)	32	256	>32	>32	>32
S. maltophilia CAN-ICU 62584	32	>512	>32	>32	>32
A. baumannii CAN-ICU 63169	8	32	>32	>32	>32
K. pneumoniae ATCC 13883	16	≤0.25	>32	>32	>32

Table 2. Synergistic effects of 16 μg/ml each of compounds **2** (9.8 μM) and **3** (7.6 μM) with doxycycline (DOX) and chloramphenicol (CAM) against MDR/XDR *P. aeruginosa* clinical isolates. MICs are reported in μg/ml. In the presence of < 10 μM of compounds **2** and **3**, CLSI breakpoints (DOX = ≤ 4 μM; CAM = ≤ 8 μM) were reached in eight out of nine clinical isolates for doxycycline and six out of nine for chloramphenicol.

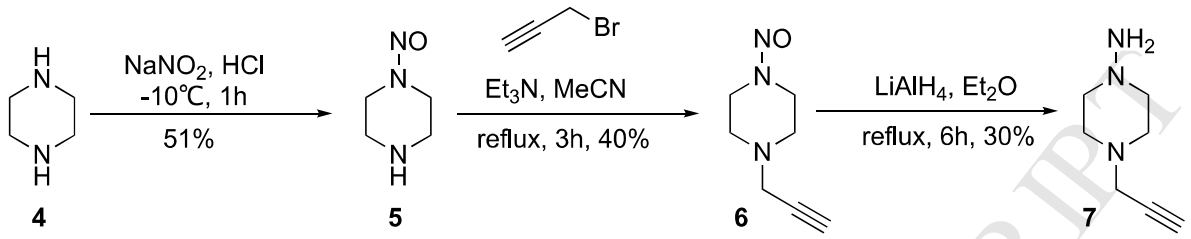
Strain	Antibiotic (MIC)	Conjugate (MIC)	$MIC_{Antibiotic}$ in the presence of < 10 μM of conjugate	FICI
PA 100036	DOX (64)	**2** (256)	1	0.078
		3 (256)	0.5	0.070
	CAM (1024)	**2** (256)	4	0.066
		3 (256)	4	0.067
PA 264	DOX (64)	**2** (256)	0.125	0.064
		3 (256)	1	0.078
	CAM (4096)	**2** (256)	128	0.063
		3 (256)	128	0.063
PA 101243	DOX (4)	**2** (256)	0.25	0.125
		3 (256)	0.125	0.094
	CAM (1)	**2** (256)	0.125	0.187
		3 (256)	0.0625	0.125
PA 262	DOX (1024)	**2** (>256)	8	<0.070
		3 (>256)	8	<0.070
	CAM (2048)	**2** (>256)	128	<0.125
		3 (>256)	128	<0.125
PA 101885	DOX (64)	**2** (256)	1	0.078
		3 (256)	2	0.094

	CAM (512)	**2** (256)	8	0.078
		3 (256)	4	0.070
PA 259	DOX (32)	**2** (>128)	1	<0.156
		3 (>128)	0.25	<0.133
	CAM (1024)	**2** (>128)	256	<0.375
		3 (>128)	512	<0.625
PA 260	DOX (16)	**2** (>128)	0.125	<0.133
		3 (>128)	0.125	<0.133
	CAM (128)	**2** (>128)	2	<0.141
		3 (>128)	0.5	<0.129
PA 91433	DOX (32)	**2** (128)	4	0.250
		3 (128)	0.25	0.133
	CAM (8)	**2** (128)	2	0.375
		3 (128)	1	0.250
PA 114228	DOX (32)	**2** (>128)	1	<0.156
		3 (>128)	2	<0.187
	CAM (64)	**2** (>128)	16	<0.281
		3 (>128)	8	<0.156

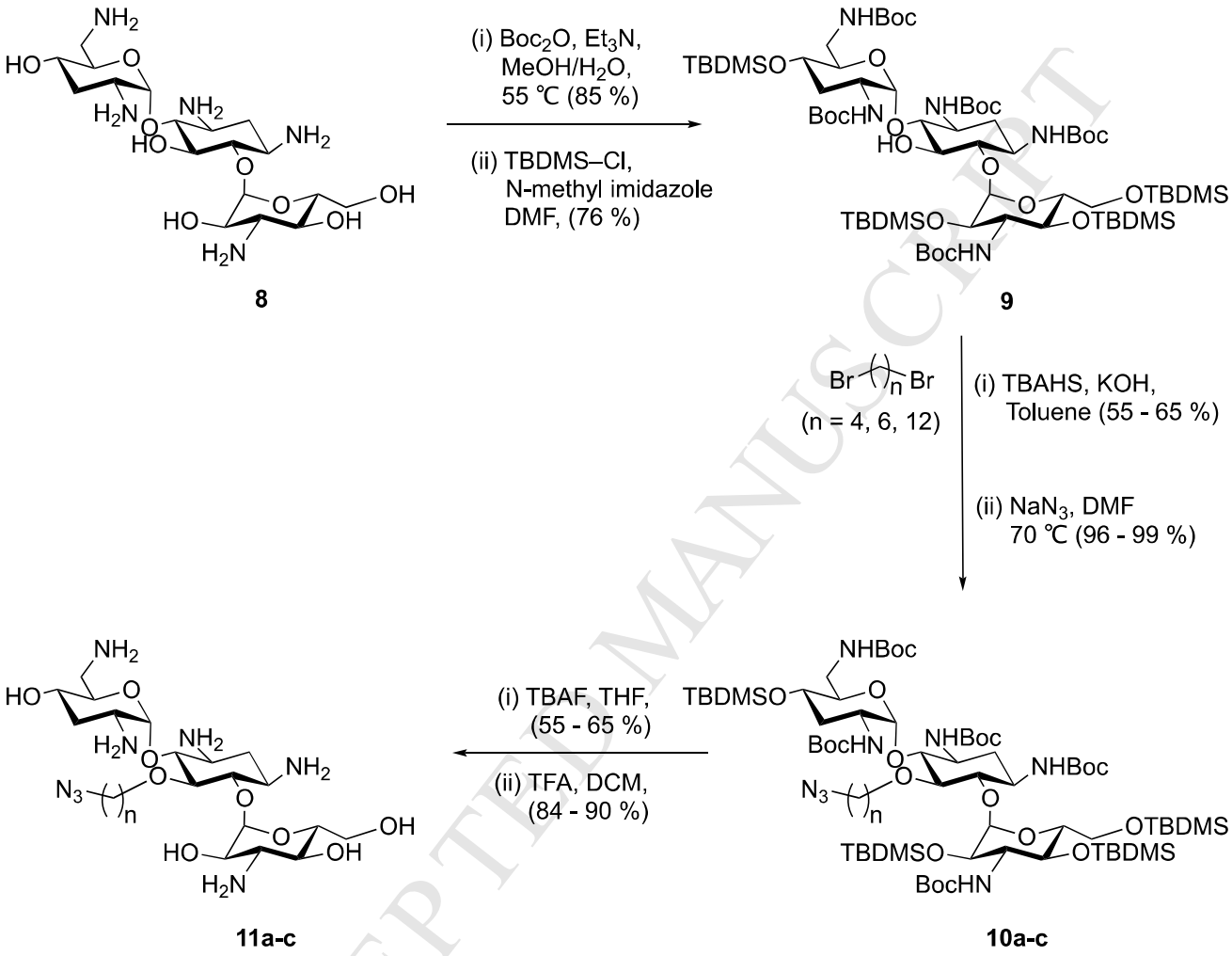
Table 3. Potentiation of doxycycline and chloramphenicol in *P. aeruginosa* by compound **2** (≤ 9.8 μM) is dependent on RND efflux pumps. PAO1 = wild-type, PAO200 and PAO750 are efflux-deficient mutants. MICs are reported in μg/ml.

Antibiotics	MIC of antibiotic alone in			MIC of antibiotic (fold change) + Compound **2** in		
	PAO1	PAO200	PAO750	PAO1	PAO200	PAO750
Doxycycline	16	0.5	0.125	0.5 (32)	0.063 (8)	0.031 (4)
Chloramphenicol	32	1	0.5	1 (32)	0.063 (16)	0.125 (4)

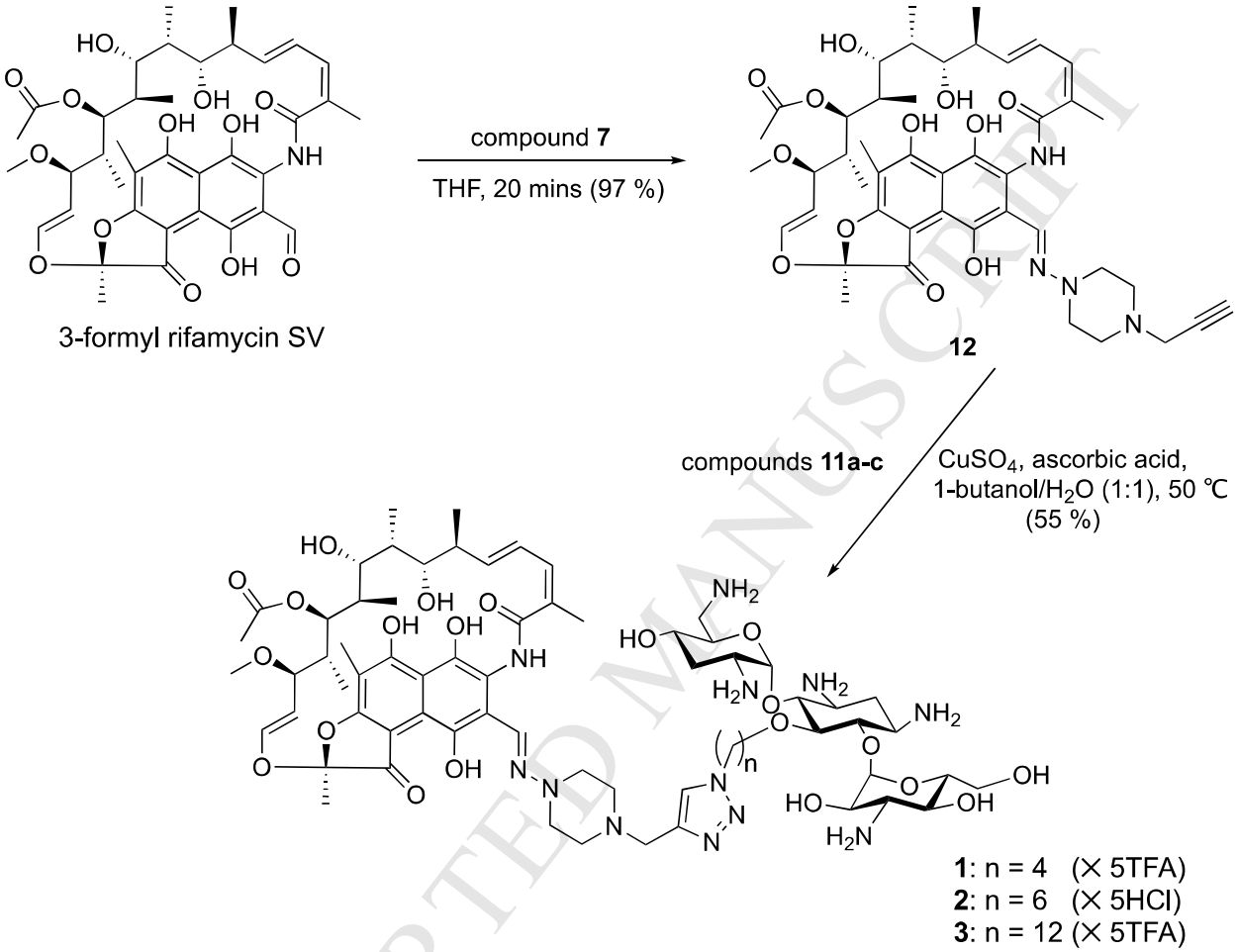
Scheme 1. Synthesis of 1-amino-4-propargylpiperazine.



Scheme 2. Synthesis of *n*-Azido Amphiphilic Tobramycins **11a–c.**



Scheme 3. Synthesis of Rifampicin–Tobramycin conjugates **1** – **3**.



Highlights:

- Rifampicin–Tobramycin (RIF-TOB) conjugates break intrinsic resistance of *P. aeruginosa*
- CLSI breakpoints were attained for doxycycline and chloramphenicol when combined with RIF-TOB
- Potency of the new conjugates was demonstrated in a *Galleria mellonella* infection model