



Dioctanoyl Ultrashort Tetrabasic β -Peptides Sensitize Multidrug-Resistant Gram-Negative Bacteria to Novobiocin and Rifampicin

Danyel Ramirez¹, Liam Berry¹, Ronald Domalaon¹, Yanqi Li², Gilbert Arthur³, Ayush Kumar² and Frank Schweizer^{1,4*}

¹Department of Chemistry, University of Manitoba, Winnipeg, MB, Canada, ²Department of Microbiology, University of Manitoba, Winnipeg, MB, Canada, ³Department of Biochemistry and Medical Genetics, University of Manitoba, Winnipeg, MB, Canada, ⁴Department of Medical Microbiology and Infectious Diseases, University of Manitoba, Winnipeg, MB, Canada

OPEN ACCESS

Edited by:

Wang Jiajun,
Northeast Agricultural University,
China

Reviewed by:

Henrik Franzyk,
University of Copenhagen, Denmark
Kevin Bicker,
Middle Tennessee State University,
United States

*Correspondence:

Frank Schweizer
schweize@cc.umanitoba.ca

Specialty section:

This article was submitted to
Antimicrobials, Resistance and
Chemotherapy,
a section of the journal
Frontiers in Microbiology

Received: 27 October 2021

Accepted: 29 November 2021

Published: 23 December 2021

Citation:

Ramirez D, Berry L, Domalaon R,
Li Y, Arthur G, Kumar A and
Schweizer F (2021) Dioctanoyl
Ultrashort Tetrabasic β -Peptides
Sensitize Multidrug-Resistant Gram-
Negative Bacteria to Novobiocin and
Rifampicin.
Front. Microbiol. 12:803309.
doi: 10.3389/fmicb.2021.803309

Recently reported peptidomimetics with increased resistance to trypsin were shown to sensitize priority multidrug-resistant (MDR) Gram-negative bacteria to novobiocin and rifampicin. To further optimize proteolytic stability, β -amino acid-containing derivatives of these compounds were prepared, resulting in three dioctanoyl ultrashort tetrabasic β -peptides (dUSTB β Ps). The nonhemolytic dUSTB β P 3, comprised of three β^3 -homoarginine residues and two fatty acyl tails eight carbons long, enhanced the antibacterial activity of various antibiotics from different classes. Notably, compound 3 retained the ability to potentiate novobiocin and rifampicin in wild-type Gram-negative bacteria against MDR clinical isolates of *Pseudomonas aeruginosa*, *Acinetobacter baumannii*, *Escherichia coli*, *Klebsiella pneumoniae*, and *Enterobacter cloacae*. dUSTB β P 3 reduced the minimum inhibitory concentration of novobiocin and rifampicin below their interpretative susceptibility breakpoints. Furthermore, compound 3 exhibited improved *in vitro* stability (86.8 \pm 3.7% remaining) relative to its α -amino acid-based counterpart (39.5 \pm 7.4% remaining) after a 2 h incubation in human plasma.

Keywords: antibiotic adjuvant, novobiocin, rifampicin, peptidomimetic, β -amino acid, *Pseudomonas aeruginosa*, *Acinetobacter baumannii*, *Escherichia coli*

INTRODUCTION

Antimicrobial resistance is a major threat to the global healthcare system that has caused a lack of treatment options for challenging bacterial infections (Prestinaci et al., 2015). The relative decrease in successful antibiotic development in the past decades in addition to the rising transmission of resistance genes has accelerated the problem and new therapies are urgently needed (O'Neill, 2016). Antimicrobial resistance can occur through several distinct mechanisms, including efflux, reduced antibiotic membrane penetration, modification of antibiotic targets, and the production of enzymes to degrade antibiotics (Reygaert, 2018). Of special concern are Gram-negative bacteria due to their highly restrictive outer membrane (OM), which prevents entry of many antibiotics (Zgurskaya and Rybenkov, 2019). Recent advancements have shown the successful development of a number of new antibiotics, as well as increased

interest in the use of antibiotic adjuvants to overcome antimicrobial resistance (Årdal et al., 2020).

Adjuvants are molecules that enhance the efficacy of partner antibiotics in combination therapy (Wright, 2016). Adjuvants work primarily by disabling either innate or adaptive bacterial resistance mechanisms. Examples include β -lactamase inhibitors that prevent the enzymatic degradation of β -lactam antibiotics (Drawz and Bonomo, 2010), bacterial efflux pump inhibitors (Lamers et al., 2013), and OM-permeabilizing agents derived from cationic antimicrobial peptides (AMPs). Currently, the only adjuvants approved for clinical use are β -lactamase inhibitors, including clavulanic acid (combined with amoxicillin), vaborbactam (combined with meropenem), and others (Carcione et al., 2021). Membrane-permeabilizing adjuvants derived from the polymyxin family of AMPs have also seen some pre-clinical success. One example is polymyxin B nonapeptide (PMBN), which lacks the fatty acyl tail and the Dab₁ amino acid characteristic of polymyxins (Ofek et al., 1994). Despite a reduction in antibacterial activity, PMBN is able to permeabilize the OM of Gram-negative bacteria and allow other antibiotics to enter the cell at increased rates (Vaara, 1992). Spero Therapeutics is also presently developing SPR741, an adjuvant derived from PMBN, which has reduced toxicity and improved pharmacokinetics (Eckburg et al., 2019; Vaara, 2019). SPR741 was assessed for safety, tolerability, and in combination with partner antibiotics in Phase I clinical trials (Eckburg et al., 2019; Vaara, 2019). Various short cationic lipopeptide adjuvants have also been reported that display similar membrane permeabilizing properties (Domalaon et al., 2018a,b, 2019a; Ramirez et al., 2020).

A common feature of the activity of AMPs against Gram-negative bacteria is the presence of basic sidechains, which can be protonated at physiological pH (Velkov et al., 2010). These confer an overall positive charge to the peptide, which can electrostatically interact with negatively charged phosphate groups embedded in the lipid A component of the lipopolysaccharide of the OM. This interaction results in the displacement of divalent cations such as Ca²⁺ and Mg²⁺ which normally stabilize the negatively charged lipopolysaccharide (Hancock, 1984). After OM integrity has been disrupted, the fatty acyl tail of polymyxin inserts into the phospholipid bilayer causing lipid rearrangement that consequently results in cell lysis and cell death (Moubareck, 2020). Although PMBN lacks this fatty acyl tail and thus is unable to lyse the cell, localized perturbation of the membrane still occurs (Tsubery et al., 2000). Despite their potent activity as both antibiotics and adjuvants (Melander and Melander, 2017; Hollmann et al., 2018; Marquette and Bechinger, 2018; Sheard et al., 2019; Vaara, 2019), AMPs face significant challenges toward clinical use. Notable challenges include high production cost, relatively poor metabolic stability, and overall high toxicity due to hemolysis and nephrotoxicity (Chen and Lu, 2020).

Various approaches to improve the drug-likeness of AMPs have been reported. For instance, the above-mentioned PMBN and SPR741 have reduced toxicity (Vaara, 2019). We have previously reported dilipid ultrashort cationic lipopeptides (dUSCLs) containing two shorter instead of one longer fatty

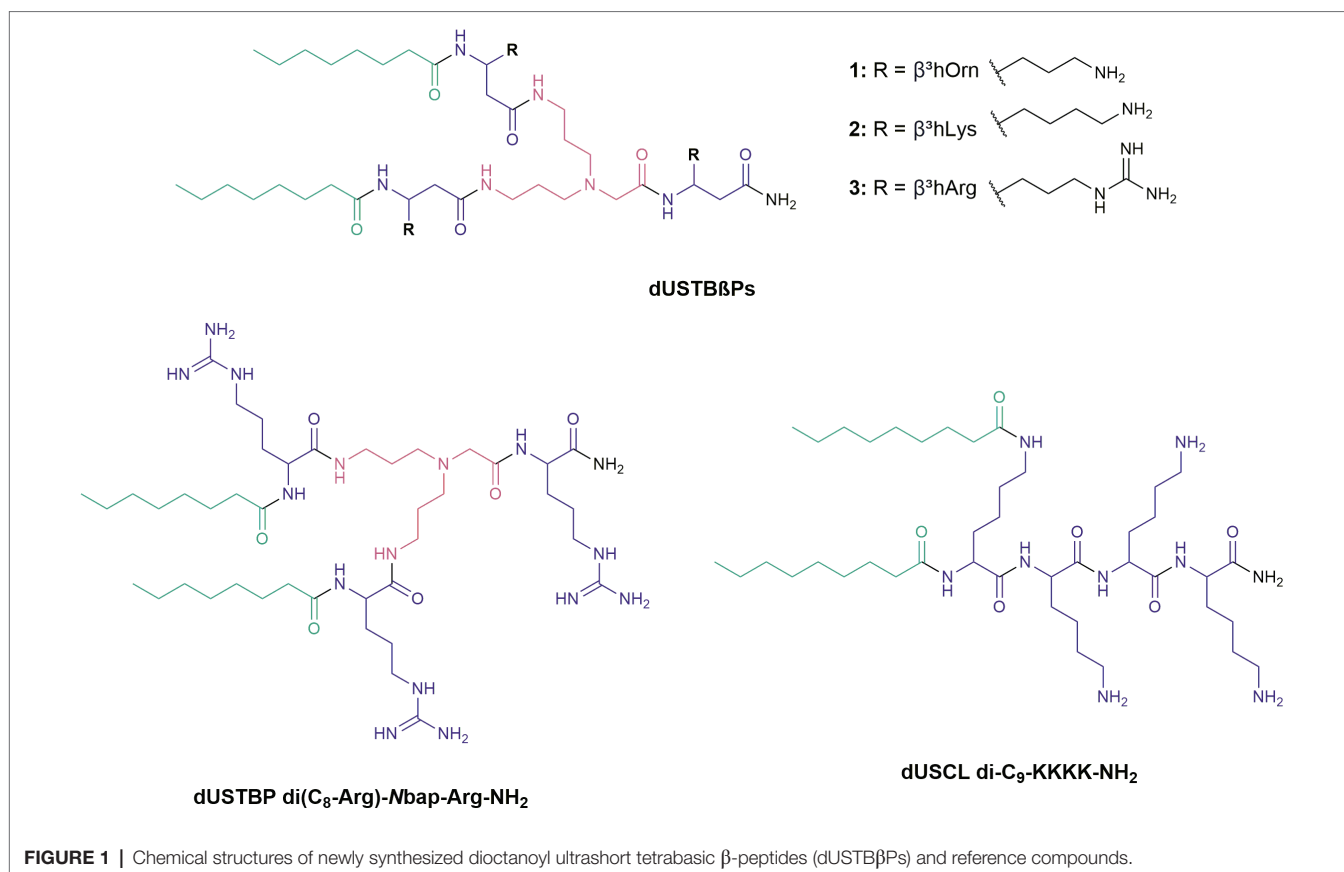
acyl tail that resulted to reduced hemolysis (Domalaon et al., 2019a). Metabolic instability due to protease activity remains a significant challenge for AMPs. Peptidomimetic approaches can mitigate proteolytic susceptibility by removing the peptide-like character of AMPs thereby resulting in the inability of proteases to recognize and degrade the resulting mimic molecule (Domalaon et al., 2016). Several peptidomimetic strategies can be enacted in lead AMP candidates including isosteric replacement of the peptide backbone, replacing naturally occurring L-amino acids with D-amino acids, and the use of β -amino acids and peptoid building blocks (Rink et al., 2010; Molchanova et al., 2017; Baker et al., 2019; Mood et al., 2021). For instance, dilipid ultrashort tetrabasic peptidomimetics (dUSTBPs) were previously developed from dUSCL lead candidates by introducing the branched molecular scaffold, *N,N*-bis(3-aminopropyl)glycine (Nbap), into the structure to interrupt the peptide backbone (Ramirez et al., 2020). The nonhemolytic dUSTBP di(C₈-Arg)-Nbap-Arg-NH₂ (**Figure 1**) enhanced the antibacterial activity of novobiocin and rifampicin, and was shown to have increased resistance to trypsin relative to dUSCL di-C₉-KKKK-NH₂ (**Figure 1**; Ramirez et al., 2020). Although improved peptide stability was observed in dUSTBPs, our continued effort in optimizing our lead candidates by incorporating further peptidomimetic structural features is reported herein.

Replacement of traditional α -amino acids in dUSTBPs with β -amino acid analogues resulted in dioctanoyl ultrashort tetrabasic β -peptides (dUSTB β Ps). Three dUSTB β Ps were prepared by incorporating fatty acids eight carbons (C₈) long and β -amino acids including β^3 -homoornithine (β^3 hOrn), β^3 -homolysine (β^3 hLys), or β^3 -homoarginine (β^3 hArg; **Figure 1**). The dUSTB β Ps were all nonhemolytic and potentiated novobiocin and rifampicin against wild-type Gram-negative bacteria. Peptidomimetic 3, consisting of three β^3 hArg residues, enhanced the antibacterial activity of various antibiotics from different classes, as well as retained novobiocin and rifampicin potentiation against multidrug-resistant (MDR) clinical isolates. Moreover, compound 3 was shown to possess enhanced plasma stability relative to its α -amino acid-based counterpart di(C₈-Arg)-Nbap-Arg-NH₂. These results indicate that multiple peptidomimetic approaches can serve to further improve proteolytic resistance of AMPs without compromising adjuvant activity.

MATERIALS AND METHODS

Preparation of dUSTB β Ps

The Rink amide 4-methylbenzylhydramine (MBHA) resin was obtained from Sigma-Aldrich (United States), Fmoc- β^3 -hOrn(Boc)-OH was obtained from A2B Chem (United States), and Fmoc- β^3 -hArg(Pbf)-OH was obtained from 1Click Chemistry (United States). Fmoc- β^3 -hLys(Boc)-OH and *N,N*-bis(*N'*-Fmoc-3-aminopropyl)glycine potassium hemisulfate were purchased from Chem-Impex (United States). All other reagents and solvents were obtained from Sigma-Aldrich (United States) and used without further purification.



All dUSTB β Ps were prepared by following a standard fluorenylmethyloxycarbonyl (Fmoc) solid-phase peptide synthesis (SPPS) protocol using Rink amide MBHA resin (Chan and White, 2000). N-terminus of the amino acids was protected with Fmoc. The ω - and ω -amine sidechains of β^3 hOrn and β^3 hLys, and the ω -amine sidechain of β^3 hArg were protected with *tert*-butyloxycarbonyl and 2,2,4,6,7-pentamethyldihydrobenzofuran-5-sulfonyl, respectively. Fmoc deprotection was carried out using 20% piperidine in dimethylformamide (DMF; v/v). Peptide and fatty acid coupling were done *via* addition of a preactivated coupling solution to the resin and subsequent constant gentle agitation with nitrogen gas for 45 min. The coupling solution which consists of 3 molar equivalents (mol. eq.) of protected amino acid or lipid, 3 mol. eq. of *O*-(benzotriazol-1-yl)-*N,N,N',N'*-tetramethyluronium tetrafluoroborate, and 8 mol. eq. of *N*-methylmorpholine in DMF was preactivated for 5 min. Following both Fmoc deprotection and coupling steps, the resin was washed with DMF (3 \times), dichloromethane (DCM; 3 \times), and DMF (3 \times) to remove traces of piperidine from the reaction vessel. The chloranil test (2% chloranil in DMF) was performed on a small amount of resin to verify the completion of the reaction. Deprotection of the amino acid sidechains and cleavage of the peptide from the resin were done using 95:5 trifluoroacetic acid (TFA)/water (v/v) for 30 min. The resin was washed with DCM (3 \times), and the solvent was removed *in vacuo* to afford the solid compounds as TFA salts. Molecular weights

of the dUSTB β Ps in TFA salt form are shown in **Supplementary Table 1**.

The dUSTB β Ps were purified *via* reverse-phase flash chromatography using C₁₈ silica gel (40–63 μ m) purchased from Silicycle (United States). The solvent system used consisted of methanol and water containing 0.1% TFA. Purity of the compounds was determined to be $\geq 95\%$ using high-performance liquid chromatography (HPLC) on a Thermo Scientific Vanquish UHPLC (United States) equipped with Phenomenex Kinetex (100 mm \times 4.6 mm) 2.6 μ m XB-C18 reverse-phase column and VF-D40 variable wavelength detector. The HPLC gradient used for purity analysis is shown in **Supplementary Table 8**. Chemical characterization of each dUSTB β P was assessed by one- and two-dimensional nuclear magnetic resonance experiments, such as ¹H, ¹³C, COSY, HSQC, and HMBC on a Bruker AMX-500 (500 MHz) instrument (Germany). Matrix-assisted laser desorption ionization-time of flight mass spectrometry experiments were carried out on a Bruker Ultraflex (Germany) in positive ion mode with 2,5-dihydroxybenzoic acid as the matrix.

Bacterial Strains and Growth Conditions

Bacterial isolates were obtained from the American Type Culture Collection (ATCC), the Canadian National Intensive Care Unit (CAN-ICU) surveillance study (Zhanel et al., 2008), and the Canadian Ward (CAN-WARD) surveillance study (Hoban and Zhanel, 2013). CAN-ICU and CAN-WARD bacterial isolates

were collected from patients diagnosed with presumed infectious diseases admitted in participating medical centers across Canada. All pharmaceutical-grade antibiotics and reagents were purchased from commercial sources.

Hemolysis Assay

The degree of hemolysis induced by dUSTB β Ps was determined by the amount of hemoglobin released from human erythrocytes upon incubation. Fresh human blood supplied by a commercial vendor was obtained from normal healthy volunteers following informed consent. The agent of interest was serially diluted in vehicle consisting of phosphate-buffered saline (PBS), saline, 5% glucose, or equivalents. The samples were incubated with gentle mixing at 37°C for 45 min and were subsequently centrifuged. The supernatant (plasma layer) was removed and centrifuged once more to completely pellet the cells. The plasma layer was diluted with Drabkin's reagent and analyzed at a wavelength of 540 nm. Experiments were conducted in triplicates, and a calibration curve prepared by diluting blood (after addition of vehicle) was used to quantitate heme release. The vehicle or Triton X-100 were used as negative or positive controls, respectively.

Antimicrobial Susceptibility Assay

The *in vitro* antibacterial activities of dUSTB β Ps were evaluated using microbroth dilution susceptibility test, in accordance with the Clinical and Laboratory Standards Institute (CLSI) guidelines (Clinical and Laboratory Standards Institute, 2019). Overnight-grown bacterial culture diluted in saline to 0.5 McFarland turbidity was successively diluted 1:50 in Mueller-Hinton broth (MHB) to achieve a final concentration of 5×10^5 colony forming units (CFU)/mL for inoculation. The agents were serially diluted 2-fold in MHB on a 96-well plate; mixed with bacterial inoculum of equal volumes, and incubated at 37°C for 18 h. Antibacterial activity was determined by the minimum inhibitory concentration (MIC), which is the lowest concentration of agent required for inhibition of visible bacterial growth in the form of turbidity. Confirmation of turbidity was done using an EMax Plus microplate reader (Molecular Devices, United States) at a wavelength of 590 nm. Wells comprising MHB alone or MHB inoculated with bacteria were used as negative or positive controls, respectively.

Checkerboard Assay

The adjuvant activities of dUSTB β Ps were evaluated using checkerboard assay as previously described (Berry et al., 2019). Overnight-grown bacterial culture diluted in saline to 0.5 McFarland turbidity was successively diluted 1:50 in MHB to achieve a final concentration of 5×10^5 CFU/mL for inoculation. The antibiotic and adjuvant were serially diluted 2-fold along the *x*-axis and *y*-axis, respectively, resulting in varying concentrations of both agents in each well. Subsequently, the 96-well plate was incubated with equal volumes of bacterial inoculum at 37°C for 18 h. Confirmation of turbidity was done using an EMax Plus microplate reader (Molecular Devices, United States) at a wavelength of 590 nm. Wells comprising MHB alone or in the presence of bacterial cells were used as negative

or positive controls, respectively. Fractional inhibitory concentration index (FICI) is determined by adding the FICs of both antibiotic and adjuvant. The FIC of the antibiotic is calculated by dividing the MIC of the antibiotic in the presence of the adjuvant by the MIC of the antibiotic alone. Similarly, the FIC of the adjuvant is calculated by dividing the MIC of the adjuvant in the presence of the antibiotic by the MIC of the adjuvant alone. $FICI \leq 0.5$, $0.5 < x \leq 4$, and > 4 were deemed synergistic, additive, and antagonistic, respectively (Meletiadiis et al., 2010).

Time-Kill Assay

The concentration-dependent killing kinetics of the combinations of dUSTB β P 3 and novobiocin or rifampicin was studied using time-kill assay as previously described (Domalaon et al., 2019b). Overnight-grown bacterial culture diluted in PBS to 0.5 McFarland turbidity was successively diluted 1:50 in lysogeny broth (LB). Cell cultures in the presence of dUSTB β P 3, novobiocin, or rifampicin, or combinations of adjuvant and antibiotic were incubated at 37°C. At designated intervals, 100 μ L aliquots acquired from each culture tube were serially diluted in PBS and plated on LB agar plates. After incubation of the plates at 37°C for 18 h, the bacterial colonies were counted.

OM Permeabilization Assay

The ability of dUSTB β P 3 to permeabilize the OM of *Acinetobacter baumannii* ATCC 17978 and *Escherichia coli* ATCC 25922 was assessed using 1-*N*-phenyl-naphthylamine (NPN) as previously described with minor modifications (Yang et al., 2017; Akhoundsadegh et al., 2019). Overnight grown culture was subcultured (1 in 100) in fresh LB broth and grown to a mid-logarithmic phase ($OD_{600} = 0.4-0.6$). The cells were pelleted by centrifugation for 10 min at $1,200 \times g$ at room temperature, washed, and resuspended in half volume of 5 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES; pH 7.2) with 5 mM glucose. NPN (10 μ M final concentration) was added to a black 96-well plate containing the cell culture and incubated in 5 mM HEPES (pH 7.2) supplemented with 5 mM glucose and 5 μ M carbonyl cyanide 3-chlorophenylhydrazone at room temperature for 30 min in darkness. Varying concentrations of compound were added onto the suspension, and the resulting change in NPN fluorescence was measured continuously (every 30 s) on a SpectraMax M2 microplate reader (Molecular Devices, United States) at an excitation wavelength of 350 nm and an emission wavelength of 420 nm. Cells with NPN and the OM permeabilizer PMBN served as a positive control, while cells with NPN alone served as a negative control. Three replicates were conducted, and the data were corrected for any background fluorescence.

Tryptic Digest Assay

Proteolytic resistance of dUSTB β P 3 and previously reported dUSCL di-C₉-KKKK-NH₂ was evaluated with tryptic digest assay as previously described (Ramirez et al., 2020). The compounds were diluted with 50 mM ammonium bicarbonate (pH 7.8) and were incubated with sequencing-grade modified trypsin from Promega (United States) at a molar ratio of

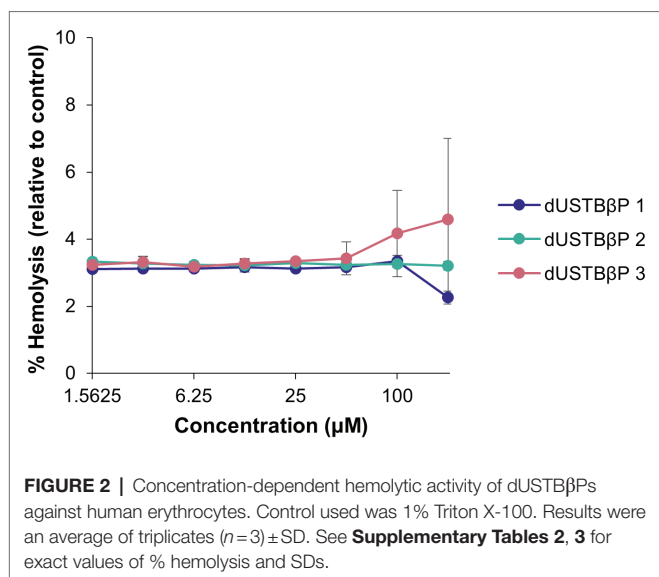


TABLE 1 | Antibacterial activity of dUSTB β Ps against wild-type Gram-negative bacteria.

Organism	MIC (μ g/mL)			<i>P. aeruginosa</i> PAO1
	1	2	3	
>128	>128	128	<i>A. baumannii</i> ATCC 17978	>128
>128	>128	<i>E. coli</i> ATCC 25922	>128	>128
128				

1:5,000 (enzyme/compound) at 37°C for 2 h. Termination of the reaction was done by overnight freezing at -18°C , and the samples were purified and concentrated by using Pierce C_{18} tips (10 μL) from Thermo Scientific (United States). Stability toward trypsin was assessed by mass fragmentation analysis in positive ion mode on a Varian 500-MS ion trap mass spectrometer (United States).

Plasma Stability Assay

Stability of dUSTB β P 3 was evaluated in human plasma. Compound 3 was incubated at 37°C in prewarmed plasma for a final compound concentration of 1 μM . At designated time points (0, 0.5, 1, 1.5, and 2 h), aliquots of the mixture were diluted with acetonitrile, and centrifuged at $1,000\times g$ for 15 min at 4°C. The supernatant was analyzed by HPLC-tandem mass spectrometry using selected reaction monitoring. The % compound remaining was determined by comparing peak areas at different time points to time zero. Assuming first-order kinetics, the half-life was extrapolated from the slope of the initial linear range of the logarithmic curve of compound remaining over time. Experiments were performed in duplicates, and the reference compounds include propantheline and propoxycaine.

Cell Viability Assay

Cell viability assay was performed essentially as previously described (Ammeter et al., 2019). Human embryonic kidney cells (HEK293) and human liver carcinoma cells (HepG2) were grown in Dulbecco's modified eagle's medium with 10% fetal bovine serum at 37°C using a humidified 5% CO_2 incubator in 75 mm tissue culture flasks. The cells were detached with trypsin and equal numbers of cells (8,000 cells in 50 μL) were dispersed into five rows of each column in 96-well plate; the remaining three rows of each column, designated as blanks, received 50 μL of the media without cells. After 24 h incubation, aliquots of 50 μL of dUSTB β P 3 at varying concentrations were added to each well and incubated for 48 h. Thereafter, cell viability was measured using PrestoBlue Cell Viability reagent from Invitrogen (United States) according to manufacturer's protocol and fluorescence (540/590 nm) was measured with a SpectraMax M2 plate reader (Molecular Devices, United States). Values from the wells without cells (blank wells) were subtracted from the corresponding sample wells and cell viability values of the treated samples relative to the vehicle controls set to 100% was determined. Thus, relative cell viability of 0% indicates that there are no viable cells. The results represent the mean \pm SD of two independent experiments with five samples per experiment. The concentration that causes 50% cytotoxicity (CC_{50}) was also estimated by using non-linear regression analysis. Colistin was used as a negative control and doxorubicin, an anticancer drug, was used as a positive control.

RESULTS

Hemolytic and Susceptibility Screening of dUSTB β Ps

The ability of the compounds to cause lysis of red blood cells was determined by measuring the amount of hemoglobin released in plasma upon treatment (Figure 2). The β -amino acid-containing derivatives elicited low levels of hemolysis at all concentrations examined. At 200 μM , compounds 1, 2, and 3 only resulted in 2.26 ± 0.19 , 3.21 ± 0.13 , and $4.59 \pm 2.42\%$ hemolysis, respectively. To examine the susceptibility of wild-type Gram-negative bacteria to dUSTB β Ps, MICs of the synthesized compounds were determined (Table 1). Limited activity of ≥ 128 $\mu\text{g/mL}$ was observed for all the compounds against all tested strains.

dUSTB β Ps Potentiated Novobiocin and Rifampicin Against Wild-Type Gram-Negative Bacteria

The capability of dUSTB β Ps to potentiate novobiocin and rifampicin was assessed against wild-type *Pseudomonas aeruginosa* (Table 2), *A. baumannii* (Table 3), and *E. coli* (Table 4) by means of a checkerboard assay. The FICI was used to evaluate interactions between the two agents. FICI of ≤ 0.5 , $0.5 < x \leq 4$, and > 4 were interpreted as synergy, additive, and antagonistic, respectively (Meletiadis et al., 2010). All interactions of dUSTB β Ps with novobiocin and rifampicin were synergistic against all tested strains. The combinations were most effective against

TABLE 2 | Synergy evaluation of combinations consisting of dUSTB β Ps and novobiocin or rifampicin against wild-type *P. aeruginosa* PAO1.

dUSTB β P	Antibiotic	MIC _{dUSTBβP} [MIC _{combo}] (μ g/mL)	MIC _{antibiotic} [MIC _{combo}] (μ g/mL)	FICI	Interpretation	Absolute MIC ^a _{antibiotic} (μ g/mL)	Potentialion ^b
1	Novobiocin	>128 [8]	1,024 [128]	0.125 < x < 0.188	Synergy	128	8-fold
	Rifampicin	>128 [16]	16 [4]	0.25 < x < 0.375	Synergy	8	2-fold
2	Novobiocin	>128 [8]	1,024 [128]	0.125 < x < 0.188	Synergy	128	8-fold
	Rifampicin	>128 [16]	16 [4]	0.25 < x < 0.375	Synergy	8	2-fold
3	Novobiocin	128 [8]	1,024 [32]	0.094	Synergy	32	32-fold
	Rifampicin	128 [8]	16 [0.25]	0.078	Synergy	0.25	64-fold

^aMIC of antibiotic in the presence of 8 μ g/mL (6 μ M) dUSTB β P.^bDegree of antibiotic potentiation in the presence 8 μ g/mL (6 μ M) dUSTB β P.**TABLE 3** | Synergy evaluation of combinations consisting of dUSTB β Ps and novobiocin or rifampicin against wild-type *A. baumannii* ATCC 17978.

dUSTB β P	Antibiotic	MIC _{dUSTBβP} [MIC _{combo}] (μ g/mL)	MIC _{antibiotic} [MIC _{combo}] (μ g/mL)	FICI	Interpretation	Absolute MIC ^a _{antibiotic} (μ g/mL)	Potentialion ^b
1	Novobiocin	>128 [8]	16 [2]	0.125 < x < 0.188	Synergy	2	8-fold
	Rifampicin	>128 [8]	2 [0.125]	0.063 < x < 0.125	Synergy	0.125	16-fold
2	Novobiocin	>128 [8]	16 [2]	0.125 < x < 0.188	Synergy	2	8-fold
	Rifampicin	>128 [8]	2 [0.125]	0.063 < x < 0.125	Synergy	0.125	16-fold
3	Novobiocin	>128 [8]	16 [0.25]	0.016 < x < 0.078	Synergy	0.25	64-fold
	Rifampicin	>128 [8]	2 [0.008]	0.004 < x < 0.066	Synergy	0.008	256-fold

^aMIC of antibiotic in the presence of 8 μ g/mL (6 μ M) dUSTB β P.^bDegree of antibiotic potentiation in the presence 8 μ g/mL (6 μ M) dUSTB β P.**TABLE 4** | Synergy evaluation of combinations consisting of dUSTB β Ps and novobiocin or rifampicin against wild-type *E. coli* ATCC 25922.

dUSTB β P	Antibiotic	MIC _{dUSTBβP} [MIC _{combo}] (μ g/mL)	MIC _{antibiotic} [MIC _{combo}] (μ g/mL)	FICI	Interpretation	Absolute MIC ^a _{antibiotic} (μ g/mL)	Potentialion ^b
1	Novobiocin	>128 [4]	64 [2]	0.031 < x < 0.063	Synergy	2	32-fold
	Rifampicin	>128 [8]	4 [0.25]	0.063 < x < 0.125	Synergy	0.25	16-fold
2	Novobiocin	>128 [8]	64 [1]	0.016 < x < 0.078	Synergy	1	64-fold
	Rifampicin	>128 [8]	4 [0.125]	0.031 < x < 0.094	Synergy	0.125	32-fold
3	Novobiocin	128 [8]	64 [0.125]	0.064	Synergy	0.125	512-fold
	Rifampicin	128 [8]	4 [0.008]	0.064	Synergy	0.008	512-fold

^aMIC of antibiotic in the presence of 8 μ g/mL (6 μ M) dUSTB β P.^bDegree of antibiotic potentiation in the presence 8 μ g/mL (6 μ M) dUSTB β P.

E. coli (FICI of 0.016–0.125) and *A. baumannii* (FICI of 0.004–0.188), and least effective against PAO1 (FICI of 0.078–0.375).

dUSTB β P 3 Retains Novobiocin and Rifampicin Potentiation Against MDR Gram-Negative Bacteria

Potentiation of novobiocin (Table 5) and rifampicin (Table 6) by dUSTB β P 3 was also examined against MDR clinical isolates of *P. aeruginosa*, *A. baumannii*, and *Enterobacteriaceae*. Supplementary Tables 4–6 contain the full data. MDR is characterized by nonsusceptibility to at least one agent in ≥ 3 different antibiotic categories (Magiorakos et al., 2012). The antibiotic susceptibility profile of the tested strains can be found in Supplementary Table 9. Testing of the α -amino acid-based

counterpart di(C₈-Arg)-Nbap-Arg-NH₂ (Figure 1) was included for comparison. Compound 3 synergized with both antibiotics against all clinical isolates tested. Similar to the trend found for the dUSTB β P, the combinations of 8 μ g/mL (6 μ M) compound 3 and novobiocin or rifampicin were most potent against *A. baumannii* (absolute MIC values of 0.002–0.125 μ g/mL) and *Enterobacteriaceae* (absolute MIC values of 0.004–32 μ g/mL). Moreover, novobiocin or rifampicin in the presence of dUSTB β P 3 was least active against *P. aeruginosa* (absolute MIC values of 0.5–1,024 μ g/mL).

Time-Kill Kinetics of dUSTB β P 3 and Novobiocin or Rifampicin in Wild-Type and MDR *A. baumannii*

The ability of the combinations of dUSTB β P 3 and novobiocin (Figure 3) or rifampicin (Figure 4) to enhance the bacterial killing of wild-type *A. baumannii* ATCC 17978 and MDR

TABLE 5 | Potentiation of novobiocin by dUSTBP di(C₈-Arg)-Nbp-Arg-NH₂ or dUSTB β P 3 at a fixed concentration of 8 μ g/mL (6 μ M) against MDR *P. aeruginosa*, *A. baumannii*, and *Enterobacteriaceae*.

Organism	MIC _{novobiocin} (μ g/mL)		
	Alone	+ di(C ₈ -Arg)-Nbp-Arg-NH ₂ ^a	+ dUSTB β P 3 ^a
<i>P. aeruginosa</i> PA259-96196	1,024	16	32
<i>P. aeruginosa</i> PA262-101856	1,024	32	1,024
<i>P. aeruginosa</i> PA264-104354	1,024	4	512
<i>P. aeruginosa</i> PA91433	1,024	32	1,024
<i>P. aeruginosa</i> PA114228	1,024	256	256
<i>A. baumannii</i> AB027	8	0.031	0.063
<i>A. baumannii</i> AB031	4	0.031	0.031
<i>A. baumannii</i> LAC-4	1	0.008	0.002
<i>A. baumannii</i> 92247	4	0.063	0.063
<i>A. baumannii</i> 110193	128	0.25	0.125
<i>E. coli</i> 94393	64	0.5	0.125
<i>E. coli</i> 94474	256	0.5	0.5
<i>E. coli</i> 107115	128	0.25	0.5
<i>K. pneumoniae</i> 113250	128	2	2
<i>K. pneumoniae</i> 113254	256	2	4
<i>K. pneumoniae</i> 116381	256	1	1
<i>E. cloacae</i> 117029	512	0.5	0.25
<i>E. cloacae</i> 118564	256	0.5	0.5
<i>E. cloacae</i> 121187	32	2	2

^aMIC of novobiocin in the presence of 8 μ g/mL (6 μ M) compound. MIC of dUSTBP di(C₈-Arg)-Nbp-Arg-NH₂ and dUSTB β P 3 is ≥ 64 μ g/mL against all strains tested, with the exception of *A. baumannii* LAC-4 (MIC of 16 μ g/mL).

A. baumannii 110193 was determined. Bactericidal and bacteriostatic activity is defined by $a \geq 3 \log_{10}$ and $a < 3 \log_{10}$ decrease in CFU/mL from the original inoculum after 24 h, respectively (Clinical and Laboratory Standards Institute, 1999). Treatment of both strains with 8 μ g/mL (6 μ M) dUSTB β P 3 alone resulted in growth curves similar to the negative controls, indicating that compound 3 does not have intrinsic antibacterial activity. Bacteriostatic activity was exhibited by 32 μ g/mL novobiocin or 0.5 μ g/mL rifampicin in both wild-type and MDR *A. baumannii* strains. Combinations of 8 μ g/mL (6 μ M) compound 3 with either 8 or 32 μ g/mL novobiocin lowered the bacterial load of both *A. baumannii* strains below the detection limit after 24 h. The addition of 8 μ g/mL (6 μ M) dUSTB β P 3 to either 0.125 or 0.5 μ g/mL rifampicin sterilized *A. baumannii* 110193 after 24 h. While the combination of 8 μ g/mL (6 μ M) compound 3 and 0.125 μ g/mL rifampicin was not able to suppress regrowth of *A. baumannii* ATCC 17978 after 4 h, increasing the rifampicin concentration to 0.5 μ g/mL resulted in bactericidal effects after 24 h.

dUSTB β P 3 Potentiated Multiple Classes of Antibiotics

In addition to novobiocin and rifampicin, synergy between dUSTB β P 3 and 19 other antibiotics were also screened against wild-type *P. aeruginosa*, *A. baumannii*, and *E. coli* (Figure 5). Synergy is defined by at least a 4-fold reduction in MIC of an antibiotic in combination with 8 μ g/mL (6 μ M) (>1/4 \times MIC of dUSTB β P 3) compound 3. dUSTB β P 3

potentiated multiple classes of antibiotics, including the aminocoumarins, ansamycins, antifolates, fluoroquinolones, lincosamides, macrolides, oxazolidinones, penicillins, pleuromutilins, and tetracyclines.

dUSTB β P 3 Permeabilizes the OM

To determine whether dUSTB β P 3 increases the intracellular concentration of novobiocin or rifampicin by permeabilizing the OM, the ability of the compound to increase the uptake of the nonpolar membrane-impermeable fluorescent probe NPN was measured in wild-type *A. baumannii* ATCC 17978 and *E. coli* ATCC 25922 (Figure 6). NPN uptake is normally prevented when the OM is intact (Idowu et al., 2019). Moreover, NPN fluoresces strongly and weakly in phospholipid and aqueous environments, respectively (Idowu et al., 2019). Since increasing concentrations of the OM permeabilizer PMBN or compound 3 resulted in increased fluorescence of NPN, this suggests that dUSTB β P 3 dose-dependently permeabilizes the OM.

dUSTB β P 3 Displayed Enhanced Resistance to Proteases

Previously reported dUSTBP di(C₈-Arg)-Nbp-Arg-NH₂ remained intact after incubation with trypsin for 2 h (Ramirez et al., 2020). Therefore, the ability of dUSTB β P 3 to resist tryptic degradation was also examined as an initial study of stability toward proteases. Compound 3 was incubated in the presence of trypsin for 2 h, and the resulting degradation mixture was subsequently assessed by electrospray ionization mass spectrometry molecular fragmentation analysis. To verify trypsin cleavage, dUSCL di-C₉-KKKK-NH₂ (Figure 1) was selected as a positive control. Solutions consisting of trypsin alone and peptide alone were chosen as negative controls. Complete degradation of the dUSCL occurred, as indicated by the loss of parent mass ions characteristic to untruncated di-C₉-KKKK-NH₂ (Supplementary Figure 1). However, resistance to trypsin was observed with the dUSTB β P, as parent mass ions corresponding to untruncated compound 3 were still present after 2 h (Supplementary Figure 2). Proteolytic stability was further evaluated by incubating dUSTB β P 3 in human plasma (Figure 7). Compound 3 (86.8 \pm 3.7% remaining) was found to be stable for 2 h in plasma, unlike the α -amino acid-based derivative di(C₈-Arg)-Nbp-Arg-NH₂ (39.5 \pm 7.4% remaining).

dUSTB β P 3 Is Noncytotoxic to Eukaryotic Cells

Toxicity of dUSTB β P 3 was evaluated against the eukaryotic HEK293 and HepG2 cell lines (Supplementary Figure 3). Testing of the anticancer drug doxorubicin was included as a positive control. Compound 3 at 125 μ M and doxorubicin at 1 μ M were noncytotoxic (86.4 \pm 8.7% cell viability) and toxic (16.6 \pm 2.3% cell viability) to HEK293 cells, respectively. Similarly, dUSTB β P 3 and doxorubicin exhibited limited activity (CC₅₀ of 100.9 μ M) and potent activity (CC₅₀ of 0.032 μ M) against HepG2 cells, respectively.

TABLE 6 | Potentiation of rifampicin by dUSTBP di(C₈-Arg)-N_{bap}-Arg-NH₂ or dUSTB β P 3 at a fixed concentration of 8 μ g/mL (6 μ M) against MDR *P. aeruginosa*, *A. baumannii*, and *Enterobacteriaceae*.

Organism	MIC _{rifampicin} (μ g/mL)		
	Alone	+ di(C ₈ -Arg)-N _{bap} -Arg-NH ₂ ^a	+ dUSTB β P 3 ^a
<i>P. aeruginosa</i> PA259-96196	16	0.5	0.5
<i>P. aeruginosa</i> PA262-101856	1,024	32	64
<i>P. aeruginosa</i> PA264-104354	16	0.063	0.5
<i>P. aeruginosa</i> PA91433	16	1	8
<i>P. aeruginosa</i> PA114228	16	2	8
<i>A. baumannii</i> AB027	1	0.031	0.008
<i>A. baumannii</i> AB031	1	0.016	0.002
<i>A. baumannii</i> LAC-4	0.5	0.016	0.004
<i>A. baumannii</i> 92,247	2	0.031	0.016
<i>A. baumannii</i> 110,193	1	0.031	0.008
<i>E. coli</i> 94393	8	0.031	0.016
<i>E. coli</i> 94474	8	0.031	0.008
<i>E. coli</i> 107115	32	0.002	0.004
<i>K. pneumoniae</i> 113250	32	0.5	1
<i>K. pneumoniae</i> 113254	16	2	0.5
<i>K. pneumoniae</i> 116381	512	32	32
<i>E. cloacae</i> 117029	8	0.016	0.004
<i>E. cloacae</i> 118564	8	0.25	0.125
<i>E. cloacae</i> 121187	4	2	0.25

^aMIC of rifampicin in the presence of 8 μ g/mL (6 μ M) compound. MIC of dUSTBP di(C₈-Arg)-N_{bap}-Arg-NH₂ and dUSTB β P 3 is \geq 64 μ g/mL against all strains tested, with the exception of *A. baumannii* LAC-4 (MIC of 16 μ g/mL).

DISCUSSION

dUSTB β Ps were designed based on our previously reported dUSTBPs (Figure 1). Basic amino acids were incorporated into the dUSTBP structure to achieve selective interaction with the anionic bacterial surface, two short hydrophobic fatty acyl tails for bacterial membrane destabilization, and the molecular scaffold N_{bap} to improve proteolytic stability. Improved resistance to trypsin was observed with dUSTBP di(C₈-Arg)-N_{bap}-Arg-NH₂ (Figure 1) in comparison to dUSCL di-C₉-KKKK-NH₂ (Figure 1; Ramirez et al., 2020). To further increase the resistance of dUSTBPs to proteases, β -amino acid-containing derivatives were produced. Particularly, β^3 -amino acids were used in which the sidechains are adjacent to the amine (Cabrele et al., 2014). Since β -amino acids contain an additional methylene in the backbone, interaction with protease active sites may be impeded, potentially resulting in decreased enzymatic degradation (Godballe et al., 2011).

All dUSTB β Ps were produced by using SPPS on a Rink amide MBHA resin following an Fmoc protection strategy. Due to the nature of the MBHA resin, all synthesized compounds have an amidated C-terminus. The basic amino acids β^3 hOrn, β^3 hLys, or β^3 hArg were attached at three points on N_{bap}. The N-terminus of both terminal amino acids was also acylated with C₈ fatty acids, yielding three dUSTB β Ps. Structural activity relationships studies revealed that C₈ fatty acyl tails were relatively nonhemolytic and exhibited promising adjuvant potency by sensitizing Gram-negative bacteria to

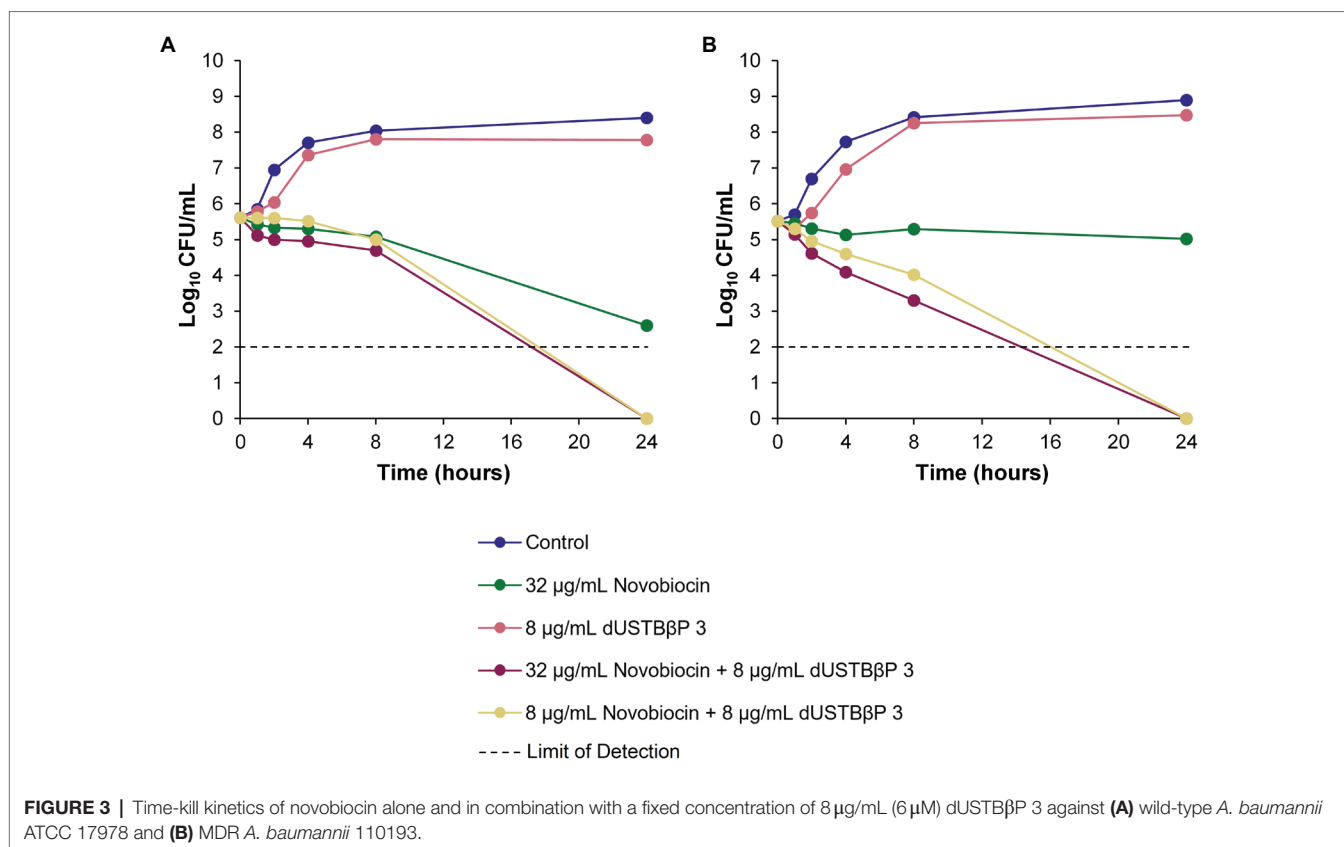
several antibiotics (Ramirez et al., 2020). However, fatty acyl tails four carbons long resulted to no potentiation possibly due to insufficient membrane interaction, and fatty acyl tails 12 carbons long exhibited hemolysis (Ramirez et al., 2020).

Selectivity of dUSTB β Ps to bacterial cells rather than eukaryotic cells is a significant aspect to consider for clinical application. We previously reported di-C₈ dUSTBPs to be nonhemolytic against porcine erythrocytes (Ramirez et al., 2020). Thus, we evaluated the propensity for hemolysis of the prepared compounds on human erythrocytes (Figure 2). Red blood cells were subjected to dUSTB β Ps at concentrations ranging from 1.5625 to 200 μ M. Even at the highest concentration tested, the compounds were found to be nonhemolytic (<5% hemolysis).

Susceptibility of wild-type *P. aeruginosa*, *A. baumannii*, and *E. coli* to dUSTB β Ps was also assessed (Table 1). Similar to the di-C₈ α -amino acid-based derivatives, the compounds did not display intrinsic antibacterial activity (Ramirez et al., 2020). While previous studies have shown that C₈ fatty acyl tails were not sufficient to confer inherent activity, they displayed potent synergy with novobiocin and rifampicin against wild-type and MDR clinical isolates of Gram-negative bacteria (Ramirez et al., 2020). Therefore, the adjuvant properties of dUSTB β Ps were also investigated.

The di-C₈ dUSTBPs acted as adjuvants that consistently enhanced the antibacterial activity of novobiocin and rifampicin against wild-type Gram-negative bacteria (Ramirez et al., 2020). Hence, the ability of dUSTB β Ps to also potentiate these two antibiotics against *P. aeruginosa* PAO1 (Table 2), *A. baumannii* ATCC 17978 (Table 3), and *E. coli* ATCC 25922 (Table 4) was studied. In general, the synthesized compounds displayed similar novobiocin and rifampicin potentiation in comparison with their α -amino acid-based counterparts. Out of the three compounds, dUSTB β P 3 (with β^3 hArg residues) proved most promising. For instance, compound 3 reduced the MIC of novobiocin and rifampicin 4- to 32-fold better than the β^3 hOrn and β^3 hLys derivatives. This matches previous structural activity relationship studies that have shown that guanidino functions may confer preferential membrane activity compared to primary amines (Nakase et al., 2012; Andreev et al., 2014; Ramirez et al., 2020). As such, the adjuvant activity of compound 3 with novobiocin and rifampicin was further explored against MDR Gram-negative bacteria.

Potentiation of novobiocin (Table 5) or rifampicin (Table 6) by dUSTB β P 3 was examined against MDR clinical isolates of *P. aeruginosa*, *A. baumannii*, *E. coli*, *Klebsiella pneumoniae*, and *Enterobacter cloacae*. Relative to the α -amino acid-based derivative di(C₈-Arg)-N_{bap}-Arg-NH₂, a slight reduction in antibiotic potentiation was observed with compound 3 against *P. aeruginosa* while an increase in rifampicin potentiation was observed against *A. baumannii* and *E. coli*. For instance, 8 μ g/mL (6 μ M) compound 3 potentiated rifampicin 8-fold less in PA264-104354 and PA91433. Moreover, 8 μ g/mL (6 μ M) dUSTB β P 3 potentiated rifampicin between 2- and 8-fold

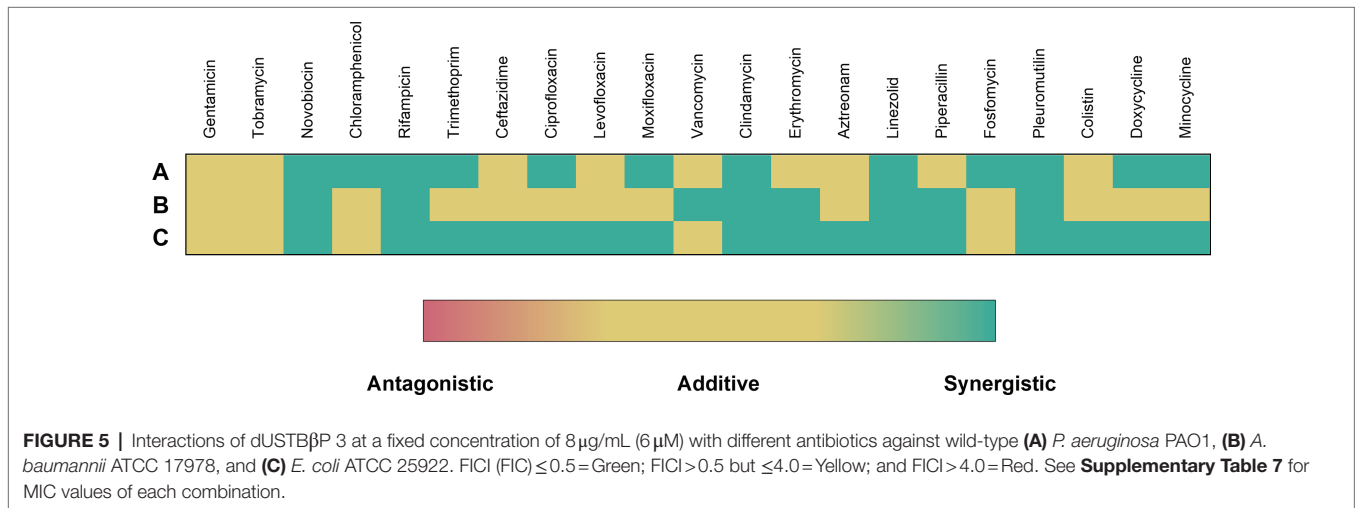
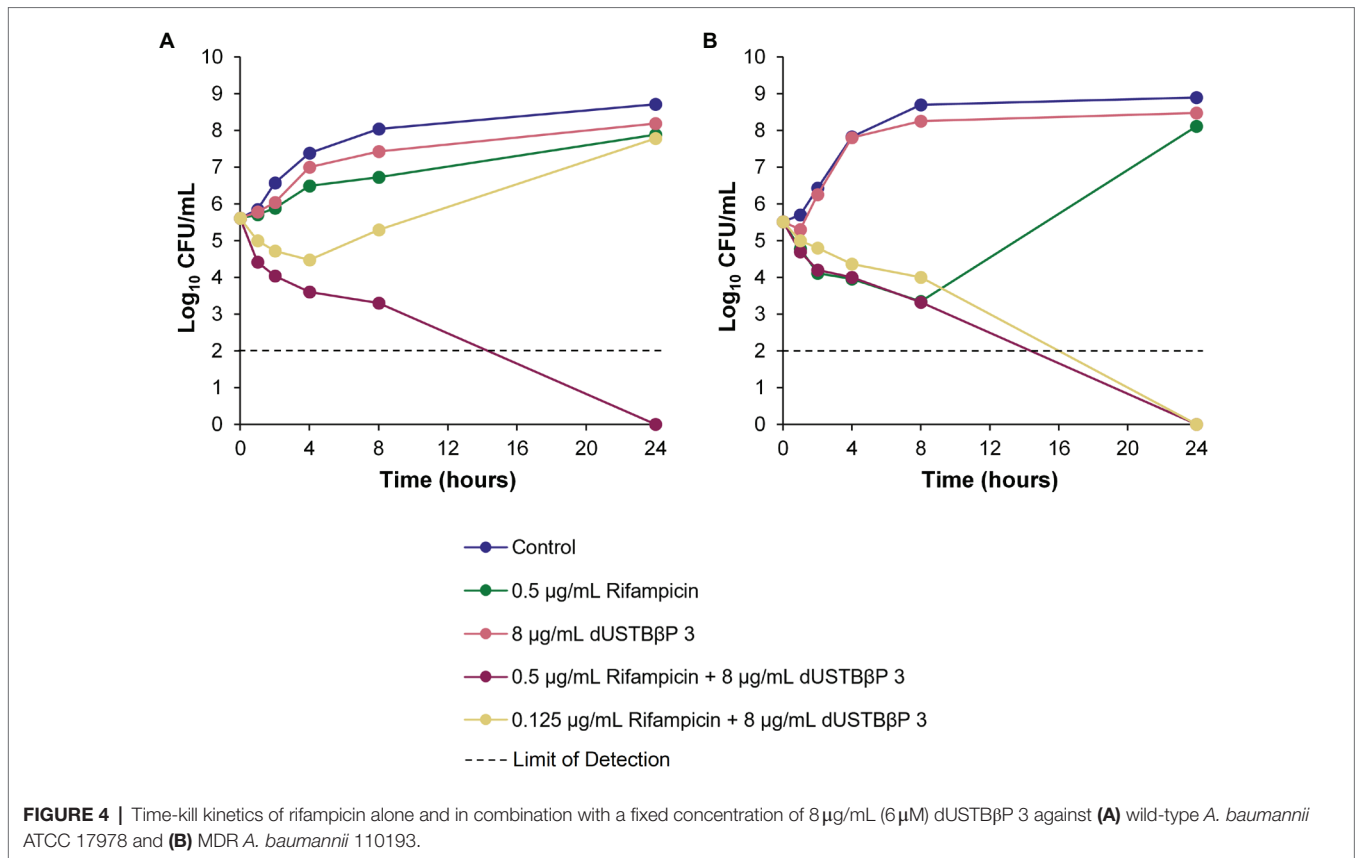


more than di(C_8 -Arg)-Nbp-Arg-NH₂ against *A. baumannii* and *E. coli*, except against *E. coli* strain 107115.

The ability of dUSTB βP 3 to reduce absolute MICs of novobiocin and rifampicin below their clinical breakpoints was evaluated. Currently, both the CLSI and the European Committee on Antimicrobial Susceptibility Testing have no breakpoint values listed for these two antibiotics against *P. aeruginosa*, *A. baumannii*, and *Enterobacteriaceae*. Thus, established breakpoints for other organisms were cautiously selected as reference. For rifampicin, a CLSI susceptibility breakpoint of $\leq 1 \mu\text{g/mL}$ for *Staphylococcus* spp. was used (Clinical and Laboratory Standards Institute, 2019). For novobiocin, an interpretative susceptibility breakpoint of $4 \mu\text{g/mL}$ based on bovine mastitis pathogens was used (Thornsberry et al., 1997). It has also been previously reported that novobiocin and colistin combination therapy which potentiates novobiocin to below the steady-state serum concentration of $5 \mu\text{g/mL}$ may have clinical potential (MacNair et al., 2018). In combination with $8 \mu\text{g/mL}$ (6 μM) compound 3, the MIC of novobiocin was reduced below the susceptibility breakpoint in all *A. baumannii*, *E. coli*, *K. pneumoniae*, and *E. cloacae* strains (Table 5). Moreover, MICs of rifampicin below the clinical breakpoint were achieved in two of the five *P. aeruginosa* strains, all *A. baumannii* strains tested, and eight of the nine *Enterobacteriaceae* strains (Table 6). Indeed, these results indicate that dUSTB βP 3 is a potent adjuvant for novobiocin and rifampicin against MDR

Gram-negative bacteria. The drastic potentiation of rifampicin in *A. baumannii* is of particular note. A concentration of $8 \mu\text{g/mL}$ SPR741 has previously been shown to reduce the MIC of rifampicin against *A. baumannii* to between 0.002 and $0.03 \mu\text{g/mL}$ against 25 clinical isolates (Corbett et al., 2017). All observed MIC values for rifampicin in combination with compound 3 fall within the same range, indicating that dUSTB βP 3 displays comparable potentiation with SPR741 (Corbett et al., 2017). To study the bacteriostatic or bactericidal activity of the combinations of compound 3 and the two antibiotics, time-kill kinetics against *A. baumannii* were conducted.

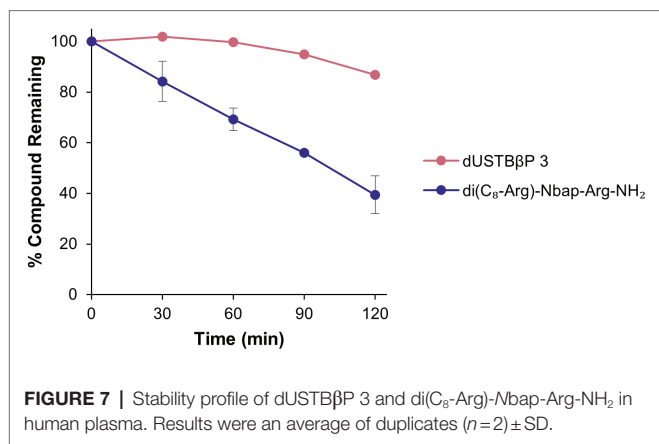
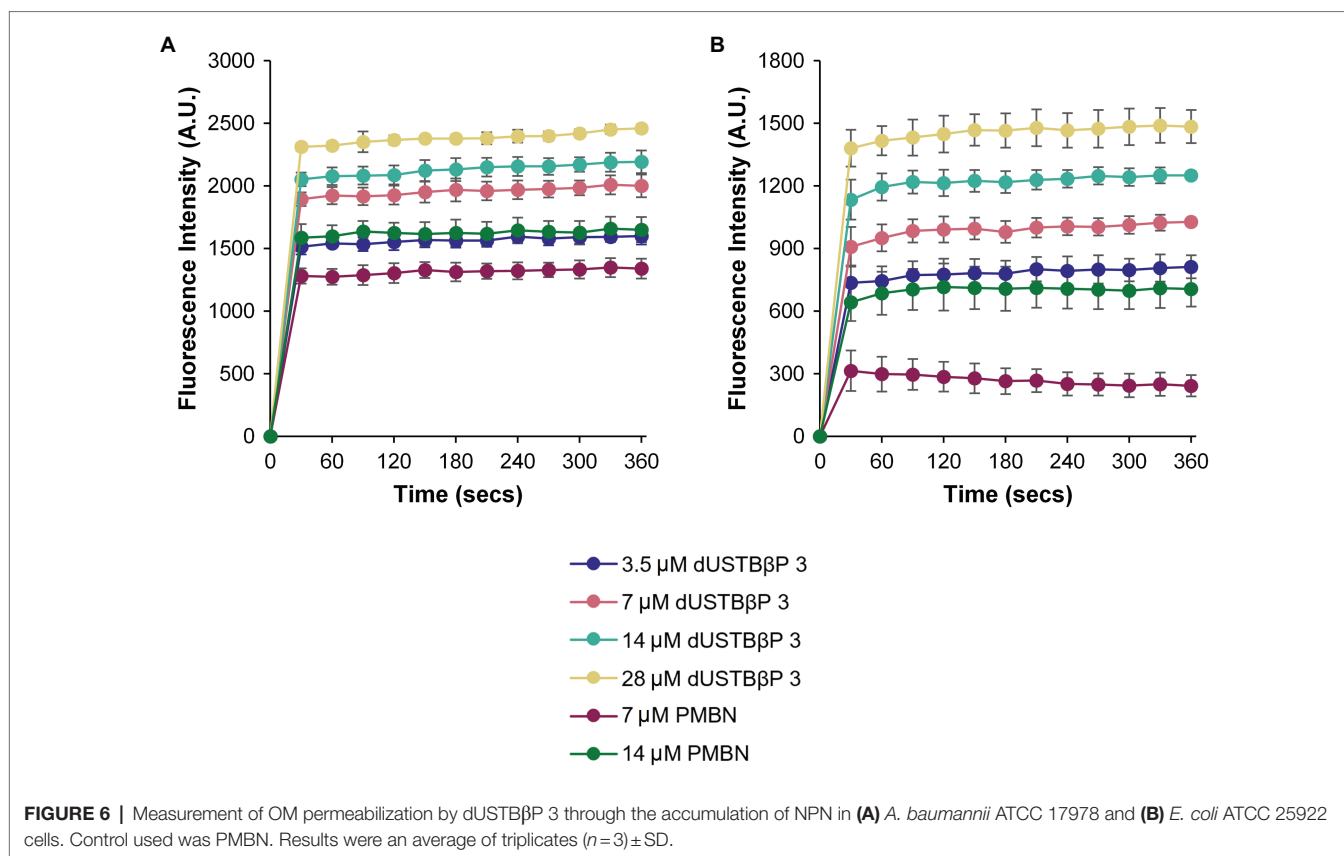
The combinations of dUSTBP di(C_8 -Arg)-Nbp-Arg-NH₂ and novobiocin or rifampicin were previously described to enhance the bacterial killing of wild-type *A. baumannii* ATCC 17978 and MDR *A. baumannii* 110193 (Ramirez et al., 2020). To determine whether this effect is conserved with dUSTB βP 3, time-kill assays were performed against the same strains (Figures 3, 4). Interestingly, the combination of $0.5 \mu\text{g/mL}$ rifampicin with $8 \mu\text{g/mL}$ (6 μM) di(C_8 -Arg)-Nbp-Arg-NH₂ resulted in complete eradication of the wild-type strain in just 8 h (Ramirez et al., 2020). Whereas the combination of $0.5 \mu\text{g/mL}$ rifampicin with $8 \mu\text{g/mL}$ (6 μM) compound 3 only reached the same effect past 8 h. This suggests that the β -amino acid-containing derivative possibly exhibits slower killing kinetics than its α -amino acid-based counterpart. Overall, our data indicate that enhanced bacterial



killing of both *A. baumannii* strains can be achieved with combination therapy consisting of dUSTB β P 3 and novobiocin or rifampicin.

Potential for synergy between dUSTB β P 3 and other clinically relevant antibiotics was also examined in wild-type Gram-negative bacteria (Figure 5). Tested antibiotics include, but are not limited to, those that have minimal activity against Gram-negative bacteria but are potent against

Gram-positive bacteria. Thus, dUSTB β P 3 synergized with antibiotics considered OM-impermeable (novobiocin, rifampicin, vancomycin, clindamycin, erythromycin, and linezolid) and efflux-susceptible (chloramphenicol, trimethoprim, ceftazidime, ciprofloxacin, levofloxacin, moxifloxacin, clindamycin, aztreonam, linezolid, piperacilin, fosfomicin, pleuromutilin, doxycycline, and minocycline). Of the 21 tested antibiotics, compound 3 potentiated 12,



eight, and 16 in PAO1, *A. baumannii* ATCC 17978, and *E. coli* ATCC 25922, respectively. Besides novobiocin and rifampicin, clindamycin and pleuromutilin were also consistently potentiated against all tested wild-type strains. These initial results suggest that dUSTB β Ps increase the uptake of antibiotics by permeabilizing the OM, as well as by potentially suppressing efflux. Future investigation will explore the precise mode of action to confirm these results.

To elucidate the mechanism of antibiotic potentiation, the ability of dUSTB β P 3 to permeabilize the OM was

initially studied in *A. baumannii* ATCC 17978 and *E. coli* ATCC 25922 (Figure 6). The two wild-type strains were selected since the combinations of compound 3 with novobiocin or rifampicin were most active against these organisms. NPN assay was performed using compound 3 at concentrations ranging from 3.5 to 28 μ M. Testing of the known OM permeabilizer PMBN was included as a positive control. The data confirm the synergy displayed in the checkerboard studies, and that compound 3 permeabilizes the OM of both strains in a concentration-dependent manner. Moreover, at equimolar concentrations (7 and 14 μ M), dUSTB β P 3 caused higher NPN fluorescence than PMBN.

The systemic usage of peptide therapeutics has been limited due to the poor bioavailability and proteolytic susceptibility associated with these molecules (Latham, 1999). To overcome these challenges, peptidomimetic strategies such as backbone modifications and the integration of unnatural amino acids can be employed (Avan et al., 2014). We have formerly shown that the incorporation of the *N*-substituted glycine Nbap to the dUSTBP design resulted in increased stability toward trypsin (Ramirez et al., 2020). β^3 -amino acids were introduced herein into the lead dUSTBP structure to improve resistance to nonspecific proteolysis. To assess the proteolytic stability of dUSTB β P 3, the ability of the compound to resist degradation by trypsin and in human plasma was assessed (Figure 7; Supplementary Figure 2). After 2 h incubation in both conditions, it was found that

compound 3 was stable. In addition, compound 3 has an extrapolated *in vitro* plasma half-life (10.43 ± 3.70 h) comparable with the *in vivo* elimination half-lives of three of the seven AMPs approved by the Food and Drug Administration: colistin (5 h), daptomycin (8–9 h), and the lipoglycopeptide telavancin (8 h; Chen and Lu, 2020). These results indicate that employing multiple peptidomimetic strategies into one molecule can result in enhanced resistance to proteases.

The effect of dUSTB β Ps on eukaryotic cells was initially screened against human erythrocytes (Figure 2). dUSTB β P 3, which was found to be nonhemolytic, showed the greatest adjuvant potency out of the three derivatives. Therefore, the probability of dUSTB β P 3 to induce cytotoxicity to HEK293 and HepG2 human cells was further examined (Supplementary Figure 3). Cell viability assay was performed at concentrations ranging from 1 to 125 μ M. At the highest concentration tested, dUSTB β P 3 was noncytotoxic to HEK293 cells ($86.4\% \pm 8.7\%$ cell viability). In contrast, dUSTB β P 3 has a CC_{50} (100.9 μ M) against HepG2 cells 17-fold higher than the effective adjuvant concentration (6 μ M) used in the synergistic studies.

To improve resistance to nonspecific proteolytic degradation, three β -amino acid-containing derivatives of dUSTBPs were prepared. In comparison to the α -amino acid-based counterpart, novobiocin and rifampicin potentiation by compound 3 were conserved against wild-type and MDR clinical isolates of *P. aeruginosa*, *A. baumannii*, and *Enterobacteriaceae*. The nonhemolytic dUSTB β P 3, consisting of β^3 hArg residues, lowered the MICs of novobiocin and rifampicin below their interpretative susceptibility breakpoints. Furthermore, compound 3 showed excellent *in vitro* plasma stability with an extrapolated half-life of 10.43 ± 3.70 h.

REFERENCES

- Akhoundsadegh, N., Belanger, C. R., and Hancock, R. E. W. (2019). Outer membrane interaction kinetics of new polymyxin B analogs in Gram-negative bacilli. *Antimicrob. Agents Chemother.* 63, e00935–e00919. doi: 10.1128/AAC.00935-19
- Ammeter, D., Idowu, T., Zhanel, G. G., and Schweizer, F. (2019). Development of a nebramine-cyclam conjugate as an antibacterial adjuvant to potentiate β -lactam antibiotics against multidrug-resistant *P. aeruginosa*. *J. Antibiot.* 72, 816–826. doi: 10.1038/s41429-019-0221-9
- Andreev, K., Bianchi, C., Laursen, J. S., Citterio, L., Hein-Kristensen, L., Gram, L., et al. (2014). Guanidino groups greatly enhance the action of antimicrobial peptidomimetics against bacterial cytoplasmic membranes. *Biochim. Biophys. Acta Biomembr.* 1838, 2492–2502. doi: 10.1016/j.bbmem.2014.05.022
- Årdal, C., Balasegaram, M., Laxminarayan, R., McAdams, D., Outtersson, K., Rex, J. H., et al. (2020). Antibiotic development—economic, regulatory and societal challenges. *Nat. Rev. Microbiol.* 18, 267–274. doi: 10.1038/s41579-019-0293-3
- Avan, I., Dennis Hall, C., and Katritzky, A. R. (2014). Peptidomimetics via modifications of amino acids and peptide bonds. *Chem. Soc. Rev.* 43, 3575–3594. doi: 10.1039/c3cs60384a
- Baker, K. R., Jana, B., Hansen, A. M., Nielsen, H. M., Franzky, H., and Guardabassi, L. (2019). Repurposing azithromycin and rifampicin against gram-negative pathogens by combination with peptidomimetics. *Front. Cell. Infect. Microbiol.* 9:236. doi: 10.3389/fcimb.2019.00236

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/Supplementary Material; further inquiries can be directed to the corresponding author.

AUTHOR CONTRIBUTIONS

DR and FS conceived the study. DR prepared the compounds and performed the antimicrobial susceptibility, checkerboard, time-kill, and tryptic digest assays. DR and LB characterized the compounds. YL and DR performed the NPN assays. GA performed the cell viability assays. FS and AK supervised the microbiological and biochemical assays. DR, LB, RD, and FS wrote the manuscript. All authors contributed to the article and approved the submitted version.

FUNDING

This work was supported by the Canadian Institutes of Health Research (CIHR) in the form of a pilot project (162159) and the Natural Sciences and Engineering Research Council of Canada (NSERC) in the form of a discovery grant (2018-06047).

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: <https://www.frontiersin.org/articles/10.3389/fmicb.2021.803309/full#supplementary-material>

- Berry, L., Domalaon, R., Brizuela, M., Zhanel, G. G., and Schweizer, F. (2019). Polybasic peptide-levofloxacin conjugates potentiate fluoroquinolones and other classes of antibiotics against multidrug-resistant Gram-negative bacteria. *Med. Chem. Commun.* 10, 517–527. doi: 10.1039/C9MD00051H
- Cabrele, C., Martinek, T. A., Reiser, O., and Berlicki, L. (2014). Peptides containing β -amino acid patterns: challenges and successes in medicinal chemistry. *J. Med. Chem.* 57, 9718–9739. doi: 10.1021/jm5010896
- Carcione, D., Siracusa, C., Sulejmani, A., Leoni, V., and Intra, J. (2021). Old and new beta-lactamase inhibitors: molecular structure, mechanism of action and clinical use. *Antibiotics* 10:995. doi: 10.3390/antibiotics10080995
- Chan, W. C., and White, P. D. (2000). *Fmoc Solid Phase Peptide Synthesis: A Practical Approach. 1st Edn.* New York: Oxford University Press Inc.
- Chen, C. H., and Lu, T. K. (2020). Development and challenges of antimicrobial peptides for therapeutic applications. *Antibiotics* 9:24. doi: 10.3390/antibiotics9010024
- Clinical and Laboratory Standards Institute (1999). *Methods for Determining Bactericidal Activity of Antimicrobial Agents; Approved Guideline, CLSI document M26-A. 29th Edn.* Wayne, PA: Clinical and Laboratory Standards Institute.
- Clinical and Laboratory Standards Institute (2019). *Performance standards for antimicrobial susceptibility testing. CLSI supplement M100. 29th Edn.* Wayne, PA: Clinical and Laboratory Standards Institute.
- Corbett, D., Wise, A., Langley, T., Skinner, K., Trimby, E., Birchall, S., et al. (2017). Potentiation of antibiotic activity by a novel cationic peptide: potency and spectrum of activity of SPR741. *Antimicrob. Agents Chemother.* 61, e00200–e00217. doi: 10.1128/AAC.00200-17
- Domalaon, R., Berry, L., Tays, Q., Zhanel, G. G., and Schweizer, F. (2018a). Development of dilipid polymyxins: investigation on the effect of hydrophobicity

- through its fatty acyl component. *Bioorg. Chem.* 80, 639–648. doi: 10.1016/j.bioorg.2018.07.018
- Domalaon, R., Brizuela, M., Eisner, B., Findlay, B., Zhanel, G. G., and Schweizer, F. (2019a). Dilipid ultrashort cationic lipopeptides as adjuvants for chloramphenicol and other conventional antibiotics against Gram-negative bacteria. *Amino Acids* 51, 383–393. doi: 10.1007/s00726-018-2673-9
- Domalaon, R., Malaka De Silva, P., Kumar, A., Zhanel, G. G., and Schweizer, F. (2019b). The anthelmintic drug niclosamide synergizes with colistin and reverses colistin resistance in Gram-negative bacilli. *Antimicrob. Agents Chemother.* 63, e02574–e02518. doi: 10.1128/AAC.02574-18
- Domalaon, R., Sanchak, Y., Cheroni Koskei, L., Lyu, Y., Zhanel, G. G., Arthur, G., et al. (2018b). Short proline-rich lipopeptide potentiates minocycline and rifampin against multidrug- and extensively drug-resistant *Pseudomonas aeruginosa*. *Antimicrob. Agents Chemother.* 62, e02374–e02317. doi: 10.1128/AAC.02374-17
- Domalaon, R., Zhanel, G. G., and Schweizer, F. (2016). Short antimicrobial peptides and peptide scaffolds as promising antibacterial agents. *Curr. Top. Med. Chem.* 16, 1217–1230. doi: 10.2174/1568026615666150915112459
- Drawz, S. M., and Bonomo, R. A. (2010). Three decades of β -lactamase inhibitors. *Clin. Microbiol. Rev.* 23, 160–201. doi: 10.1128/CMR.00037-09
- Eckburg, P. B., Lister, T., Walpole, S., Keutzer, T., Utley, L., Tomayko, J., et al. (2019). Safety, tolerability, pharmacokinetics, and drug interaction potential of SPR741, an intravenous potentiator, after single and multiple ascending doses and when combined with β -lactam antibiotics in healthy subjects. *Antimicrob. Agents Chemother.* 63, e00892–e00819. doi: 10.1128/AAC.00892-19
- Godballe, T., Nilsson, L. L., Petersen, P. D., and Jessen, H. (2011). Antimicrobial β -peptides and α -Peptoids. *Chem. Biol. Drug Des.* 77, 107–116. doi: 10.1111/j.1747-0285.2010.01067.x
- Hancock, R. E. (1984). Alterations in outer membrane permeability. *Annu. Rev. Microbiol.* 38, 237–264. doi: 10.1146/annurev.mi.38.100184.001321
- Hoban, D. J., and Zhanel, G. G. (2013). Introduction to the CANWARD study (2007–11). *J. Antimicrob. Chemother.* 68, i3–i5. doi: 10.1093/jac/dkt021
- Hollmann, A., Martinez, M., Maturana, P., Semorile, L. C., and Maffia, P. C. (2018). Antimicrobial peptides: interaction with model and biological membranes and synergism with chemical antibiotics. *Front. Chem.* 6:204. doi: 10.3389/fchem.2018.00204
- Idowu, T., Ammeter, D., Rossong, H., Zhanel, G. G., and Schweizer, F. (2019). Homodimeric tobramycin adjuvant repurposes novobiocin as an effective antibacterial agent against gram-negative bacteria. *J. Med. Chem.* 62, 9103–9115. doi: 10.1021/acs.jmedchem.9b00876
- Lamers, R. P., Cavallari, J. F., and Burrows, L. L. (2013). The efflux inhibitor phenylalanine-arginine Beta-Naphthylamide (PA β N) Permeabilizes the outer membrane of Gram-negative bacteria. *PLoS One* 8:e60666. doi: 10.1371/journal.pone.0060666
- Latham, P. W. (1999). Therapeutic peptides revisited. *Nat. Biotechnol.* 17, 755–757. doi: 10.1038/11686
- MacNair, C. R., Stokes, J. M., Carfrae, L. A., Fiebig-Comyn, A. A., Coombes, B. K., Mulvey, M. R., et al. (2018). Overcoming mcr-1 mediated colistin resistance with colistin in combination with other antibiotics. *Nat. Commun.* 9:458. doi: 10.1038/s41467-018-02875-z
- Magiorakos, A. P., Srinivasan, A., Carey, R. B., Carmeli, Y., Falagas, M. E., Giske, C. G., et al. (2012). Multidrug-resistant, extensively drug-resistant and pandrug-resistant bacteria: an international expert proposal for interim standard definitions for acquired resistance. *Clin. Microbiol. Infect.* 18, 268–281. doi: 10.1111/j.1469-0691.2011.03570.x
- Marquette, A., and Bechinger, B. (2018). Biophysical investigations elucidating the mechanisms of action of antimicrobial peptides and their synergism. *Biomol. Ther.* 8:18. doi: 10.3390/biom8020018
- Melander, R. J., and Melander, C. (2017). The challenge of overcoming antibiotic resistance: an adjuvant approach? *ACS Infect Dis.* 3, 559–563. doi: 10.1021/acscinfed.7b00071
- Meletiadis, J., Pournaras, S., Roilides, E., and Walsh, T. J. (2010). Defining fractional inhibitory concentration index cutoffs for additive interactions based on self-drug additive combinations, Monte Carlo simulation analysis, and in vitro-in vivo correlation data for antifungal drug combinations against aspergillus fumigatus. *Antimicrob. Agents Chemother.* 54, 602–609. doi: 10.1128/AAC.00999-09
- Molchanova, N., Hansen, P. R., and Franzky, H. (2017). Advances in development of antimicrobial peptidomimetics as potential drugs. *Molecules* 22:1430. doi: 10.3390/molecules22091430
- Mood, E. H., Goltermann, L., Brolin, C., Cavaco, L. M., Nejad, A. J., Yavari, N., et al. (2021). Antibiotic potentiation in multidrug-resistant Gram-negative pathogenic bacteria by a synthetic Peptidomimetic. *ACS Infect. Dis.* 7, 2152–2163. doi: 10.1021/acscinfed.1c00147
- Moubareck, C. A. (2020). Polymyxins and bacterial membranes: A review of antibacterial activity and mechanisms of resistance. *Membranes* 10:181. doi: 10.3390/membranes10080181
- Nakase, I., Okumura, S., Katayama, S., Hirose, H., Pujals, S., Yamaguchi, H., et al. (2012). Transformation of an antimicrobial peptide into a plasma membrane-permeable, mitochondria-targeted peptide via the substitution of lysine with arginine. *Chem. Commun.* 48, 11097–11099. doi: 10.1039/c2cc35872g
- O'Neill, J. (2016). Tackling Drug-Resistant Infections Globally: Final Report and Recommendations. Available at: https://amr-review.org/sites/default/files/160518_Final%20paper_with%20cover.pdf (Accessed October 25, 2021).
- Ofek, I., Cohen, S., Rahmani, R., Kabha, K., Tamarkin, D., Herzig, Y., et al. (1994). Antibacterial synergism of polymyxin B nonapeptide and hydrophobic antibiotics in experimental gram-negative infections in mice. *Antimicrob. Agents Chemother.* 38, 374–377. doi: 10.1128/AAC.38.2.374
- Prestinaci, F., Pezzotti, P., and Pantosti, A. (2015). Antimicrobial resistance: A global multifaceted phenomenon. *Pathog. Glob. Health* 109, 309–318. doi: 10.1179/2047773215Y.0000000030
- Ramirez, D., Berry, L., Domalaon, R., Brizuela, M., and Schweizer, F. (2020). Dilipid ultrashort tetrabasic Peptidomimetics potentiate Novobiocin and rifampicin against multidrug-resistant Gram-negative bacteria. *ACS Infect. Dis.* 6, 1413–1426. doi: 10.1021/acscinfed.0c00017
- Reygaert, W. C. (2018). An overview of the antimicrobial resistance mechanisms of bacteria. *AIMS Microbiol.* 4, 482–501. doi: 10.3934/microbiol.2018.3.482
- Rink, R., Arkema-Meter, A., Baudoin, I., Post, E., Kuipers, A., Nelemans, S. A., et al. (2010). To protect peptide pharmaceuticals against peptidases. *J. Pharmacol. Toxicol. Methods* 61, 210–218. doi: 10.1016/j.vascn.2010.02.010
- Sheard, D. E., O'Brien-Simpson, N. M., Wade, J. D., and Separovic, F. (2019). Combating bacterial resistance by combination of antibiotics with antimicrobial peptides. *Pure Appl. Chem.* 91, 199–209. doi: 10.1515/pac-2018-0707
- Thornsberry, C., Burton, P. J., Yee, Y. C., Watts, J. L., and Yancey, R. J. (1997). The activity of a combination of penicillin and Novobiocin Against bovine mastitis pathogens: development of a disk diffusion test. *J. Dairy Sci.* 80, 413–421. doi: 10.3168/jds.S0022-0302(97)75952-6
- Tsubery, H., Ofek, I., Cohen, S., and Fridkin, M. (2000). Structure-function studies of Polymyxin B nonapeptide: implications to sensitization of Gram-negative bacteria. *J. Med. Chem.* 43, 3085–3092. doi: 10.1021/jm0000057
- Vaara, M. (1992). Agents that increase the permeability of the outer membrane. *Microbiol. Rev.* 56, 395–411. doi: 10.1128/mr.56.3.395-411.1992
- Vaara, M. (2019). Polymyxin derivatives that sensitize Gram-negative bacteria to other antibiotics. *Molecules* 24:249. doi: 10.3390/molecules24020249
- Velkov, T., Thompson, P. E., Nation, R. L., and Li, J. (2010). Structure-activity relationships of polymyxin antibiotics. *J. Med. Chem.* 53, 1898–1916. doi: 10.1021/jm900999h
- Wright, G. D. (2016). Antibiotic adjuvants: rescuing antibiotics from resistance. *Trends Microbiol.* 24, 862–871. doi: 10.1016/j.tim.2016.06.009
- Yang, X., Goswami, S., Gorityala, B. K., Domalaon, R., Lyu, Y., Kumar, A., et al. (2017). A tobramycin vector enhances synergy and efficacy of efflux pump inhibitors against multidrug-resistant gram-negative bacteria. *J. Med. Chem.* 60, 3913–3932. doi: 10.1021/acs.jmedchem.7b00156
- Zgurskaya, H. I., and Rybenkov, V. V. (2019). Permeability barriers of Gram-negative pathogens. *Ann. N. Y. Acad. Sci.* 1459, 5–18. doi: 10.1111/nyas.14134
- Zhanel, G. G., DeCorby, M., Laing, N., Weshnowski, B., Vashisht, R., Tailor, F., et al. (2008). Antimicrobial-resistant pathogens in intensive care units in Canada: results of the Canadian National Intensive Care Unit (CAN-ICU) study, 2005–2006. *Antimicrob. Agents Chemother.* 52, 1430–1437. doi: 10.1128/AAC.01538-07

Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher's Note: All claims expressed in this article are solely those of the authors and do not necessarily represent those of their affiliated organizations, or those of the publisher, the editors and the reviewers. Any product that may

be evaluated in this article, or claim that may be made by its manufacturer, is not guaranteed or endorsed by the publisher.

Copyright © 2021 Ramirez, Berry, Domalaon, Li, Arthur, Kumar and Schweizer. This is an open-access article distributed under the terms of the Creative Commons

Attribution License (CC BY). The use, distribution or reproduction in other forums is permitted, provided the original author(s) and the copyright owner(s) are credited and that the original publication in this journal is cited, in accordance with accepted academic practice. No use, distribution or reproduction is permitted which does not comply with these terms.