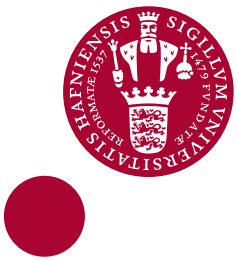

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Repurposing azithromycin and rifampicin against Gram-negative pathogens by combination with peptide potentiators

Kristin R. Baker [a, b] #, Bimal Jana [a, b], Anna Mette Hansen[d], Karina Juul Vissing [c], Hanne Mørck Nielsen [c], Henrik Franzyk [d] #, Luca Guardabassi [a, b].

[a] Department of Veterinary and Animal Sciences, Faculty of Health and Medical Sciences, University of Copenhagen, Frederiksberg, Denmark

[b] Department of Biomedical Sciences, Ross University School of Veterinary Medicine, Basseterre, St Kitts, West Indies

[c] Department of Pharmacy, University of Copenhagen, Copenhagen, Denmark

[d] Department of Drug Design and Pharmacology, University of Copenhagen, Copenhagen, Denmark

Running title: Peptide-induced antibiotic susceptibility

Corresponding author mailing address: Kristin R. Baker, Ross University School of Veterinary Medicine, Main Road, Basseterre, St. Kitts, Tel: +1 869-465-4161 Ext. 401-1211, kribake@sund.ku.dk and Henrik Franzyk, University of Copenhagen, Jagtvej 162, DK-2100, Copenhagen, Tel: +45 3533 6255, henrik.franzyk@sund.ku.dk.

Abstract

Gram-negative pathogens are intrinsically resistant to several antibiotics that are not able to penetrate the envelope barrier. The objective of this study was to identify peptides that at low concentrations induce susceptibility to these antibiotics in multidrug-resistant (MDR) Gram-negative strains of clinical relevance. A pairwise screening of 34 diverse peptides and four antibiotics (erythromycin, linezolid, rifampicin and vancomycin) with primary activity against Gram-positive bacteria identified four peptides that at sub-micromolar concentrations conferred susceptibility to rifampicin or erythromycin in *Escherichia coli* ATCC 25922. The identified peptides exhibited synergy with azithromycin and potentiated clindamycin in MDR *E. coli* ST131 and *Klebsiella pneumoniae* ST258. The low cytotoxicity toward eukaryotic cells (IC_{50} >50 μM) observed for two peptides (KLWKKWKKWLK-NH_2 and GKWKKILGKLIR-NH_2) prompted synthesis and evaluation of the corresponding all-D analogs (**D1** and **D2**), which retained similar synergistic antibacterial profiles. Low concentrations of **D1** and **D2** in combination with azithromycin and rifampicin inhibited growth of most clinical *E. coli*, *K. pneumoniae* and *Acinetobacter baumannii* strains tested. Our data demonstrate that combinatorial screening at low concentrations constitutes an efficient approach to identify clinically relevant peptide-antibiotic combinations. *In vivo* PK/PD and toxicity studies are needed to further validate the use of the peptides identified by this study for repurposing azithromycin and rifampicin against Gram-negative pathogens.

Keywords

Antimicrobial peptides, Gram-negative bacteria, multidrug resistance, antibiotic adjuvant, combination therapy, antibiotic potentiation

1. Introduction

As a consequence of the worldwide spread of multidrug-resistant (MDR) Gram-negative clones, the World Health Organization has ranked the development of new therapeutics to treat infections caused by *Enterobacteriaceae, Acinetobacter baumannii* and *Pseudomonas aeruginosa* as a critical priority [1]. Intrinsic antibiotic resistance considerably limits the therapeutic options against these pathogens, since several classes of the available antibiotics cannot effectively penetrate the envelope barrier [2]. Combination therapy represents an attractive approach for treating MDR infections as it typically reduces the required dose of the individual components and limits the risk for emergence of resistance [4,5]. Antimicrobial peptides that increase therapeutic potency and expand the spectrum of antibiotics to include Gram-negative pathogens have potential use in combination therapy [4,5,6]. Although many reports have demonstrated synergistic peptide-antibiotic interactions, the clinical potential of such findings have rarely been studied systematically.

The objective of the present study was to identify peptides that at low non-toxic concentrations render MDR Gram-negative pathogens susceptible to antibiotics to which they are intrinsically resistant. Following a systematic approach, we designed a pairwise screen based on antibacterial activity of low concentrations of a diverse set of peptides in combination with four antibiotics with primary activity against Gram-positive bacteria. Subsequently, peptide-induced antibiotic susceptibility was confirmed, and cytotoxicity was then assessed for the top four antibiotic-potentiating peptides. This resulted in identification of two lead peptides that displayed low cytotoxicity to different eukaryotic cell types and potentiated azithromycin and rifampicin against several Gram-negative species of clinical relevance.

2. Materials and methods

2.1 Media, antibiotics, bacterial strains and peptide synthesis

Bacteria were cultured on Luria-Bertani broth, cation-adjusted Mueller-Hinton agar (MHA) and broth (MHBII). All media and antibiotics were purchased from Sigma-Aldrich. ATCC reference strains

included *Escherichia coli*, *Klebsiella pneumoniae*, *Pseudomonas aeruginosa*, and *Acinetobacter baumannii*. MDR strains *E. coli* ST131 and *K. pneumoniae* ST258 are clinical isolates from urinary tract [7] and wound infections [8], respectively. A panel of β-lactamase resistant clinical isolates of were provided by Laurent Poirel. Starting materials and solvents for peptide synthesis were purchased from commercial suppliers (Iris Biotech, Sigma-Aldrich and VWR). All peptides and all-D analogs were synthesized and analyzed as previously reported [9]. The peptide stock solutions were made in deionized water, followed by dilution in MHBII.

2.2 Antimicrobial susceptibility testing

Bacterial susceptibility to compounds was determined by microbroth dilution according to CLSI guidelines [10]. In the screen, antibiotics and peptides were combined at fixed concentrations corresponding to the antibiotics' CLSI susceptibility breakpoint for *Staphylococcus* species [11]. Antibiotic minimum inhibitory concentrations (MICs) in presence of peptide concentrations (0.5 µM or 1 µM) were determined as above. For growth curve assays, the MIC plates were prepared as above, and then plates were incubated for 24 h at 37 °C with continuous shaking. Optical density (OD) at 600 nm was recorded in 10 min intervals.

2.3 Checkerboard assay

Synergy of peptide-antibiotic combinations was measured by using a two-dimensional checkerboard assay [12] and CLSI guidelines [10]. The fractional inhibitory concentration index (FICI) was calculated and interpreted as previously reported [13].

2.4 Cellular viability and IC_{50}

Cell viability was determined in ATCC NIH 3T3 fibroblasts and HepG2 hepatocytes by using the MTS/PMS assay as previously reported [14]. Peptide concentrations ranged from 0.1 to 500 µM. The relative cell viability was calculated according to eq. 1 with 100% (Abs_{pos}) and 0% cell death (Abs_{neg})

defined as the absorbance values obtained after incubation of cells with SDS (0.2%, w/v in medium) and with medium, respectively.

$$Relative\ viability\ (\%) = \frac{(Abs_{sample} - Abs_{pos})}{(Abs_{neg} - Abs_{pos})} \times 100\% \quad (1)$$

IC_{50} values were calculated using GraphPad Prism 7 (GraphPad Software, La Jolla, CA, USA) by fitting the relative viability of the cells to the concentration of the test compound using equation 2:

$$Relative\ viability\ (\%) = \frac{Top - Bottom}{1 + 10^{(LogIC_{50} - Log[peptide]) \times Hill\ slope}} (2)$$

With top and bottom values constrained to 100% and 0%, representing the mean of the highest and of the lowest observed values, respectively. Data were collected from technical triplicates.

2.5 Time-kill assay

Time-kill kinetics assays were performed in *K. pneumoniae* ATCC 13883 and *A. baumannii* ATCC 19606. Briefly, ~10^6 CFU/mL logarithmic-phase cells were transferred to 15-mL round-bottom tubes and incubated for 24 h at 37 °C with aeration in the presence or absence of antibiotic, peptide or their combination. At each time point, 100 µL cells were serially diluted in sterile 0.9% NaCl and 10 µL aliquots were plated on MHA in triplicate. The CFU/mL from each condition was calculated following 18-24 h incubation at 37 °C. The detection limit was 10^2 CFU/mL. All time-kill curves represent the average and standard deviation from biological duplicates. Synergy was defined as a ≥2-log_{10} CFU/mL decrease for the antibiotic-peptide combination relative to the individual compounds.

3. Results and Discussion

To identify peptide-induced antibiotic susceptibility, a pairwise combinatorial screening of 34 peptides and four antibiotics with poor activity against Gram-negative bacteria (rifampicin, erythromycin, vancomycin, and linezolid) was performed by assaying growth inhibition of *E. coli* ATCC 25922. Since potentiation of antibiotics is a frequent characteristic of cationic peptides, screening at low peptide

concentrations (1 or 0.5 μM) and at clinically relevant antibiotic concentrations [11] would identify the most potent antibiotic potentiators, thus expediting the discovery of peptides with potential clinical utility. Three peptides (**1**, **2**, and **3**) exhibited growth inhibition in combination with rifampicin or erythromycin (Figure S1). A fourth peptide (**4**) was selected for further analysis due to its ability to enhance susceptibility to both rifampicin and erythromycin at 0.5 μM (Figure S1). All four peptides had a low MIC of 2 μM against *E. coli* ATCC 25922. These peptides were all short (9 to 13 residues), highly cationic, and possessed similar hydrophobicity as estimated from their retention in reversed-phase analytical HPLC (Table 1) [15–17]. None of the 34 peptides induced susceptibility to linezolid or vancomycin.

The ability of the four identified peptides to induce antibiotic susceptibility in two epidemic MDR clones with high clinical relevance (i.e., *E. coli* ST131 and *K. pneumoniae* ST258) was evaluated by determining the MICs of rifampicin, erythromycin, clindamycin and azithromycin in combination with low concentrations (≤ 1 μM) of peptide. In the presence of sub-inhibitory concentrations of peptide (Table 1), the MICs of the antibiotics were reduced considerably, resulting in synergistic peptide-antibiotic combinations with estimated FICIs ranging from 0.02 for rifampicin to 0.38 for azithromycin (Tables 2 and S1). The reductions in antibiotic MICs ranged from 8-fold, for azithromycin in combination with peptides **1** and **4**, to ≥250-fold, for rifampicin in the presence of peptides **2** and **3**. All four peptides reduced the MICs of rifampicin and azithromycin to below susceptibility breakpoints [11] in both strains. For clindamycin, the most favorable interactions were observed for peptides **2** and **3** in *E. coli* ST131 with a reduction of the MICs below the resistance breakpoint [11]. In *K. pneumoniae* ST258, the clindamycin MICs remained above the resistance breakpoint despite of ≥32-fold reduction of the MICs. Susceptibility to erythromycin was not achieved, most likely due to the high MICs of this macrolide in the two strains (256 and 512 μg/mL, respectively). Consequently, azithromycin was chosen as the representative macrolide for further analyses. For the above combinations that reduced the antibiotic MICs below the resistance breakpoints, synergy was confirmed by checkerboard assays (Table S2).

As a preliminary evaluation of the toxicity, and thus potential for clinical application, we determined the cytotoxicity for peptides **1**-**4** in two relevant eukaryotic cell lines (Table 1). Peptides **1** and **2** exhibited a low cytotoxicity with IC_{50} values above 50 μM in mouse fibroblasts (NIH 3T3) and ≥100 μM in human hepatocytes (Hep G2), while peptides **3** and **4** reduced cell viability with IC_{50} values of 19-43 μM (Table 1). Regardless, the peptide-antibiotic combinations were non-toxic at synergistic concentrations (Figure S2).

All-D analogs of the four selected peptides (denoted as D-peptides **D1**-**D4** hereafter), were synthesized and tested for their ability to induce susceptibility of MDR Gram-negative pathogens to azithromycin, rifampicin and clindamycin. The all-D analogs retained the MICs of the corresponding L-forms (Table 1), and exhibited synergy with the antibiotics in MDR *E. coli* ST131 and *K. pneumoniae* ST258 (Table S2). Based on their activity profiles, both forms would be expected to retain similar toxicity profiles while the D-peptides are expected to have greater proteolytic stability [18]. As peptides **1** and **2** alone had significantly lower cytotoxicity compared to peptides **3** and **4**, it is likely that **D1** and **D2** will retain better safety profiles as compared to **D3** or **D4**; hence the first two D-peptides were studied further.

We further tested the activity of **D1** and **D2** in combination with the same three antibiotics (azithromycin, rifampicin and clindamycin) by using a collection of reference and clinical isolates of *E. coli*, *K. pneumoniae*, *A. baumannii* and *P. aeruginosa*. Overall, the MICs of rifampicin and azithromycin were reduced to below their respective susceptibility breakpoints in three reference strains when co-exposed to sub-MIC concentrations of peptides **D1** and **D2**, while the MIC of azithromycin was reduced to 2-fold above the susceptibility breakpoint in *P. aeruginosa* (Table S3). Similarly, the MICs of clindamycin were below the resistance breakpoint for *K. pneumoniae* and *A. baumannii*. In *P. aeruginosa*, the peptide-antibiotic combinations were overall not synergistic, and only borderline synergy was observed for combinations with rifampicin (Table S3). Overall, the antibacterial activity of the D-peptide-antibiotic combinations against the reference strains reflected the activity observed against the clinical isolates (Table

S4). Most (88%) and ≥50% of the isolates, except for *P. aeruginosa*, were inhibited by the D-peptides in combination with rifampicin and azithromycin, respectively (Table S4).

The above synergistic combinations were further investigated in growth curve assays, which showed that neither peptide nor antibiotic individually inhibited growth of *E. coli* or *K. pneumoniae* at the concentration present in the synergistic combination (Figure S3 A-J). However, *A. baumannii* growth was retarded in the presence of each antibiotic or peptide **D2** alone (Figure S3, K-P).

Peptide **D2** was studied further to understand the bactericidal kinetics of **D2**-antibiotic combinations. This peptide was chosen based on its high potency in synergistic combinations. Time-kill experiments with **D2** in combination with antibiotics and alone were performed with reference strains of *A. baumannii* and *K. pneumoniae* which served as the representative of Enterobacteriaceae. All **D2**-antibiotic combinations exerted synergistic bactericidal effects in the time-kill assay (Figure 1, A-F). Moreover, at sub-MIC concentrations of **D2** (i.e., ≤2 μM), all antibiotic concentrations were below their respective susceptibility breakpoints, except for clindamycin in *K. pneumoniae*.

Time-kill kinetics of the antibiotics, **D2**, and their combinations were compared to examine whether the **D2**-antibiotic combinations were able to enhance the rate and efficiency of killing relative to either component individually. In both species, faster killing kinetics were achieved for the **D2** combinations with clindamycin and rifampicin than for either antibiotic alone (Figure S4B-C and E-F). The **D2**-azithromycin combination also exhibited faster killing kinetics than azithromycin alone in *A. baumannii* (Figure S4D), while the combination displayed similar kinetics in *K. pneumoniae* (Figure S4A). However, **D2** did not exhibit efficient killing in *K. pneumoniae* (Figure S5) even at concentrations 8-fold above the MIC (Table S5).

The approach developed in this study, which combines combinatorial screening at low compound concentrations with cytotoxicity testing, can be used to expedite discovery of clinically relevant peptide-antibiotic combinations. This approach enabled rapid identification of two peptides (**1** and **2**) that at low sub-MIC non-toxic concentrations were able to circumvent intrinsic resistance to azithromycin and

rifampicin in multiple Gram-negative species of clinical relevance, including epidemic MDR clones. Furthermore, the all-D peptide analogs induced susceptibility to rifampicin and azithromycin and reduced the MICs of clindamycin by more than 500-fold. These findings may help mitigate the lack of novel antibiotics effective against Gram-negative species by opening new avenues to repurpose these antibiotics for treatment of infections caused by Gram-negative MDR pathogens.

Peptides **1** and **2** as well as their all-D analogs (**D1** and **D2**) exhibited substantial synergy with rifampicin, azithromycin, and clindamycin in *K. pneumoniae* and *A. baumannii* (Tables S2 and S3) at low (≤1 μM) non-toxic peptide concentrations. The present study constitutes the first report on antibiotic synergy of these peptides, while their antimicrobial activity, cytotoxic and haemolytic properties were reported previously [19,20]. Notably, according to these studies peptides **1** and **2** do not exhibit haemolytic activity at concentrations ≥200 μM. The use of the analogue **D2** appears to be particularly promising for antibiotic potentiation since **D2**-antibiotic combinations displayed synergistic bactericidal activity (Figure 2), and faster killing kinetics than each individual component (Figure 3). Importantly, *in vivo* PK/PD and toxicity studies are needed to fully assess the clinical potential of these findings.

4. **Conclusions**

Intrinsic resistance to azithromycin and rifampicin in Gram-negative bacteria can be overcome by very low peptide concentrations that are not toxic to eukaryotic cells. The two peptide leads identified in this study merit further investigation as antibiotic potentiators for repurposing azithromycin and rifampicin against MDR Gram-negative pathogens.

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Tables

Table 1: Peptide sequences, physicochemical characteristics, cytotoxicity and MIC in *E. coli and K. pneumoniae*

Peptide	Sequence	Length (residues)	MW (g/mol)	Molecular mass (Da)		Net charge[a]	Retention time (min)	Cytotoxicity (μM) IC_{50} (± 95% CI)		MIC (μM)		
				Calc.	Obs.			NIH3T3	HepG2	*E. coli* ATCC 25922	*E. coli* ST131	*K. pneumoniae* ST258
1	KLWKKWKKWLK-NH_2	11	2369.17	1571.01	1571.02	+7	6.73	51 ± 35	105 ± 21	2	2	64
2	GKWKKILGKLIR-NH_2	12	2121.92	1438.97	1438.97	+6	7.14	143 ± 21	175 ± 38	2	2	32
3	KKWRKWLKWLAKK-NH_2	13	2710.5	1798.15	1798.16	+8	6.93	20 ± 13	19 ± 3	2	2	4
4	KWRRWIRWL-NH_2	9	1968.85	1398.84	1398.84	+5	7.42	43 ± 16	34 ± 8	2	2	4
D1	klwkkwkkwlk-NH_2	11	2369.17	1571.01	1571.00	+7	6.66	ND[b]	ND	2	2	64
D2	gkwkkilgklir-NH_2	12	2121.92	1438.97	1438.97	+6	7.16	ND	ND	2	2	32
D3	kkwrkwlkwlakk-NH_2	13	2710.5	1798.15	1798.14	+8	6.89	ND	ND	2	2	4
D4	kwrrwirwl-NH_2	9	1968.85	1398.84	1398.84	+5	7.38	ND	ND	2	2	4

[a] Charge at pH 7.4.

[b] ND, Not determined

Table 2: MICs of azithromycin (AZM), erythromycin (ERY), rifampicin (RIF) and clindamycin (CLI) in *E. coli* ST131 and *K. pneumoniae* ST258 exposed to low concentrations of peptides **1**-**4**. Antibiotic MICs below the susceptibility breakpoint are in bold and MICs below the resistance breakpoint are underlined.

[a] CLSI clinical breakpoints for *Staphylococcus* species. S= susceptible; R= resistant.

	***E. coli* ST131**					***K. pneumoniae* ST258**						
Antibiotic (μg/mL)	**0.5 μM peptide**					**1 μM peptide**					**Clinical breakpoint[a]**	
	1	**2**	**3**	**4**	**None**	**1**	**2**	**3**	**4**	**None**	**≤S**	**≥R**
AZM	**1**	**≤0.25**	**0.25**	**1**	8	**2**	**1**	**1**	**2**	32	2	8
ERY	4	4	1	4	256	8	8	4	8	512	0.5	8
RIF	**0.25**	**≤0.03**	**≤0.03**	**0.25**	4	**0.125**	**0.06**	**≤0.03**	**0.6**	16	1	4
CLI	≥8	1	2	4	>64	>8	4	4	4	>64	0.5	4

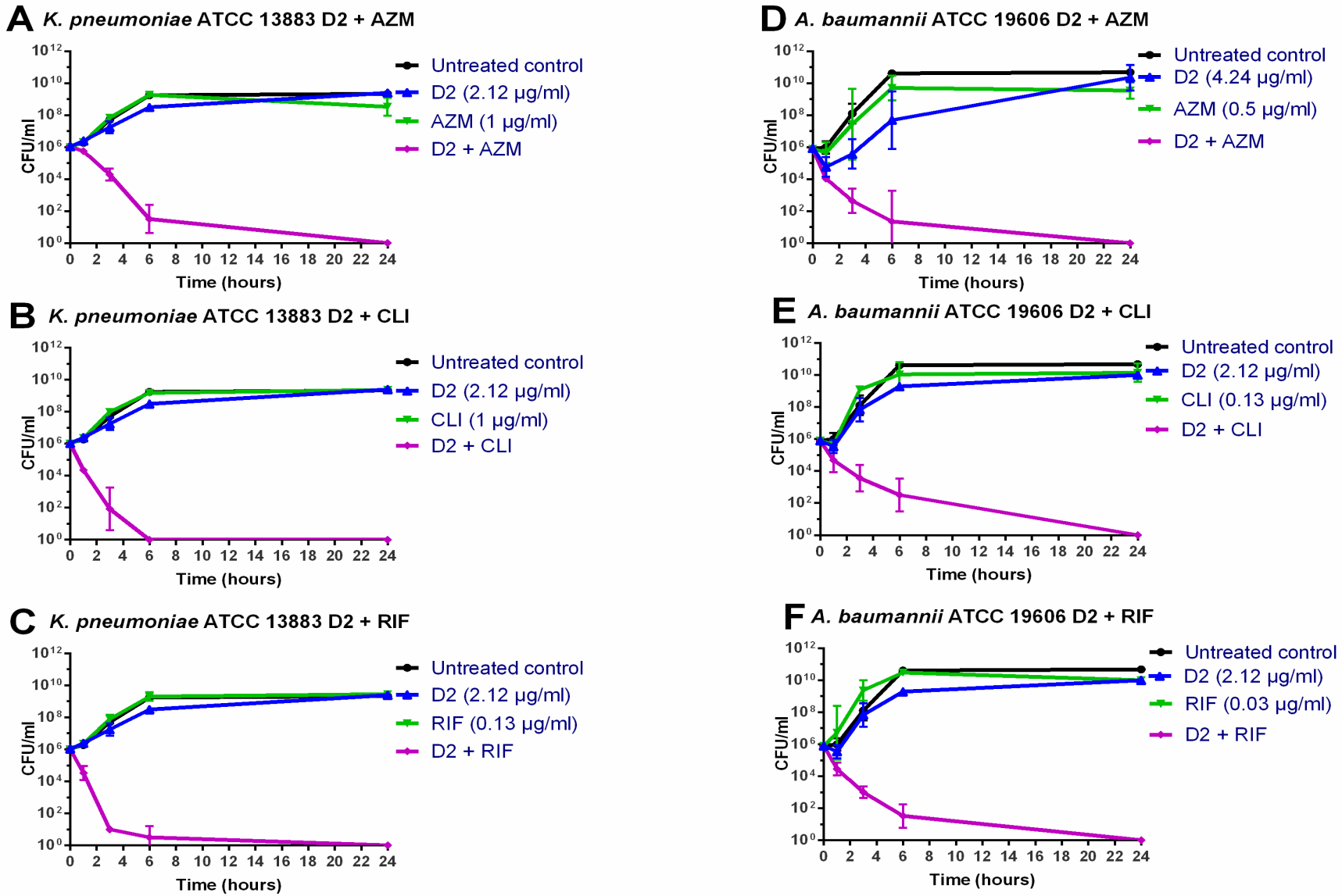

Figure 1: *Peptide **D2**-antimicrobial combination kills bacteria synergistically*. Time-kill kinetics for azithromycin (AZM), rifampicin (RIF) and clindamycin (CLI) as individual compounds and in combination with **D2** are presented for both *K. pneumoniae* ATCC 13883 (A-C) and *A. baumannii* ATCC 19606 (D-F), including only **D2** and untreated control. The curves of the synergistic combination and the untreated control are also depicted in Figure S4.