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The polyamino-isoprenyl potentiator NV716 revives disused antibiotics against Gram-negative bacteria in broth, infected monocytes, or biofilms, by disturbing the barrier effect of their outer membrane

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ABSTRACT

Potentiators can improve antibiotic activity against difficult-to-treat Gram-negative bacteria like *Escherichia coli, Klebsiella pneumoniae* or *Acinetobacter baumannii.* They represent an appealing strategy in view of the paucity of therapeutic alternatives in case of multidrug resistance. Here, we examine the ability of the polyamino-isoprenyl compound **NV716** to restore the activity of a series of disused antibiotics (rifampicin, azithromycin, linezolid, fusidic acid, novobiocin, chloramphenicol, and doxycycline, plus ciprofloxacin as an active drug) against these three species in planktonic cultures, but also in infected human monocytes and biofilms and we study its underlying mechanism of action. **NV716** considerably reduced the MICs of these antibiotics (2–11 doubling dilutions), the highest synergy being observed with the more lipophilic drugs. This potentiation was related to a strong interaction of **NV716** with LPS, ensuing permeabilization of the outer membrane, and leading to an increased accumulation of the antibiotics inside bacteria. Moreover, **NV716** increased the relative potency of all drugs against intracellular infection by the same bacteria as well as their maximal efficacy, probably related to an improvement of antibiotic activity against persisters. Lastly, **NV716** also enhanced rifampicin activity against biofilms from these three species. All these effects were observed at sub-MIC concentrations of **NV716** (and thus unrelated to a bactericidal effect), and in conditions for which no toxicity was evidenced towards eukaryotic cells. Altogether, these data highlight for the first time the potential interest of **NV716** as an adjuvant against these Gram-negative pathogens placed in the priority list of WHO for search of new therapies.

1. Introduction

The rapid emergence of antibiotic resistance has become a problem of global concern [1]. The world health organization (WHO) published a list of bacteria for which new antibiotics are urgently needed, among which *Acinetobacter baumannii* and various Enterobacteriaceae, like *Klebsiella* spp. and *Escherichia coli* [2].

These species display high levels of resistance to a broad range of antimicrobials via the acquisition of a series of resistance mechanisms [3,4]. Moreover, they also show intrinsic resistance to many antibiotics, due to the barrier effect of their envelope and to the presence of broad-spectrum efflux pumps in their membrane. The envelope of Gram-negative bacteria comprises an inner membrane (IM) and an outer membrane (OM) separated by a periplasmic space containing the cell wall made of peptidoglycan layers [5]. The OM is an asymmetric bilayer, containing phospholipids in the inner leaflet and lipopolysaccharides (LPS) in the outer leaflet [6]. This asymmetric character confers to the OM a role of selective barrier that protects bacterial cells from cytotoxic molecules, including lipophilic and/or large size antibiotics (rifampicin, macrolides and glycopeptides) [7,8].

Additionally, these Gram-negative bacteria also constitutively express efflux systems that expel unrelated classes of antibiotics out of the bacteria, reducing thereby the amount of drug that can reach intracellular targets [9]. These efflux pumps consist of three proteins, namely an efflux transporter located in the IM, an OM channel protein, and a fusion protein connecting the two transmembrane proteins to allow the

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transport of the antibiotic substrates directly to the extracellular environment. Typical examples include AcrAB-TolC in *E. coli* and *K. pneumoniae* or AdeABC in *A. baumannii*, which can extrude structurally distinct classes of antibiotics such as quinolones, tetracyclines, chloramphenicol, macrolides, linezolid, fusidic acid, or novobiocin [10].

Barrier effects and efflux pumps can cooperate and reduce antibiotic activity to such a level that their active concentrations become higher than those achieved in the serum of patients. Antibiotics affected by these mechanisms are therefore unusable against Gram-negative bacteria in clinical practice, and therefore often referred to as disused antibiotics.

In addition to resistance, intracellular survival and biofilm formation also contribute to antibiotic treatment failure [11,12]. Although considered essentially as extracellular pathogens, *E. coli*, *K. pneumoniae* and *A. baumannii* may also behave as opportunistic intracellular organisms. Their survival has been documented in phagocytic cells in-vitro [13–15] as well as in alveolar macrophages of infected mice in-vivo [16–18]. In intracellular niches, bacteria are both less accessible and less responsive to antibiotics. Our team recently documented for *Staphylococcus aureus* that poor responsiveness of bacteria surviving to antibiotics intracellularly is due to their switch to a persister phenotype [19]. Persisters are subpopulations of otherwise antibiotic-susceptible bacteria that show a transient non-dividing phenotype under stressful conditions and can survive to high concentrations of antibiotics [20], contributing to failure to eradicate the infection.

These three Gram-negative bacterial species can also adhere to biological and artificial surfaces to form biofilms [21,22]. Biofilms are defined as microbial community of cells living in a self-produced matrix essentially made of polysaccharides, extracellular DNA (eDNA), and proteins [23]. These structures protect microorganisms from host defenses and antibiotics due to the barrier effect of the extracellular matrix and to the alteration of the metabolic activity of bacteria, which adopt dormant phenotypes poorly responsive to antibiotics [24].

In a previous work, we showed that the polyaminoisoprenyl compound **NV716** (see chemical structure in Fig. S1) was capable to restore the activity of a series of disused antibiotics (rifampicin, doxycycline and chloramphenicol) against *Pseudomonas aeruginosa* both extracellularly and intracellularly [25], by increasing the permeability of the outer membrane, inhibiting efflux, and also improving the efficacy of bactericidal drugs against persisters [25–27] at concentrations that are not toxic for eukaryotic cells. The aim of the present study was (a) to extend the demonstration of the potential interest of this compound as potentiator of a series of disused antibiotics against planktonic, intracellular, or biofilm infections by other problematic Gram-negative pathogens like *A. baumannii, E. coli and K. pneumoniae,* in comparison with **NV731** (less active derivative against *P. aeruginosa*) and the well-characterized efflux inhibitor PAβN (Phenylalanine-Arginine β-Naphthylamide) [28–30] and (b) to evaluate its effects on the membranes of these bacteria.

We adapted to these species an in-vitro pharmacodynamic model of THP-1 human monocytes infected by *Pseudomonas aeruginosa* [31] that allows comparing key pharmacodynamic descriptors of antibiotic intracellular activity, namely their relative potency (static concentration, C_s) and maximal efficacy (E_{max}), as well as an in-vitro model of biofilm growing in 96-well plates [32]. As disused antibiotics, we selected rifampicin (poor substrate for efflux), doxycycline, chloramphenicol as in our previous work with *P. aeruginosa,* and added antibiotics the spectrum of which is limited to Gram-positive bacteria due to poor penetration in Gram-negative organisms, like azithromycin, linezolid, fusidic acid and novobiocin (all substrates for efflux; see Table S1). Ciprofloxacin was used as a control of active drug against intracellular *P. aeruginosa* [25]. As positive controls for mechanistic studies, we included colistin and alexidine (see Fig. S1 for the structure of these compounds). Colistin binds to the lipid A component of the negatively-charged LPS molecules via electrostatic interactions, by displacing the Mg^{2+} and Ca^{2+} ions that bridge and stabilize the LPS monolayer. It perturbs thereby the OM permeability, transits through it via a self-promoted uptake mechanism, and subsequently inserts itself and disrupts the physical integrity of the IM via membrane thinning [33]. Alexidine is an amphipathic biguanide antiseptic showing fast bactericidal activity thanks to its rapid ability to permeabilize the bacterial membranes [34]. As colistin, it establishes electrostatic interactions with the negatively-charged phosphate groups present on LPS, displacing Mg^{2+} ions from their binding to LPS and perturbing the stabilizing effect afforded by Mg^{2+} cross-bridging of adjacent LPS molecules [35]. In addition, it also causes the leakage of the cytoplasmic content by inducing the formation of lipid domains in the IM [36].

Our data show that **NV716**, contrarily to **NV731** and PAβN, was capable of increasing the relative potency and maximal efficacy of all tested antibiotics against the three species of intracellular Gram-negative bacteria. It also improved the activity of rifampicin against biofilms of these three species. These effects were related to drastic reductions in MIC and persister fractions. Mechanistic studies showed that **NV716** inhibits the activity of efflux pumps and disturbs the permeability of the OM, allowing for an increased accumulation of antibiotics inside bacteria. Collectively, these data suggest that **NV716** could be a useful adjuvant to revive disused antibiotics against extracellular and intracellular infections as well as against biofilms caused by difficult-to-treat Gram-negative bacteria.

2. Materials and methods

2.1. Antibiotics and potentiators

Colistin (potency, 73%), chloramphenicol (potency, 98%), doxycycline (potency, 98%), fusidic acid (potency, 98%), novobiocin (potency, 98%) and rifampicin (potency, 98%) were obtained as microbiological standards from Sigma-Aldrich (St Louis, MO), ciprofloxacin HCl (potency, 89%) from Bayer (Leverkusen, Germany), gentamicin sulfate (potency, 60.7%), from PnReac AppliChem (Darmstadt, Germany), linezolid (potency, 100%) from Rib-X Pharmaceuticals (presently Melinta Therapeutics, New Haven, CT), and azithromycin (potency, 100%) from Teva (Petach Tikva, Israel). Alexidine (potency, 98%) and the reference efflux pump inhibitor Phe-Arg-β-Naphthylamide (PAβN; potency, 98%) were purchased from Sigma-Aldrich. **NV716** and **NV731**, were synthesized at Aix Marseille University [37].

2.2. Bacteria and culture media

For each species, one (*E. coli* ATCC 47076, *A. baumannii* ATCC 19606) or two (*K. pneumoniae* ATCC 700603 and ATCC 43816) reference strains were obtained from the American Type Culture Collection (Manassas, VA, USA). Twenty-three clinical Gram-negative isolates from the same three species were also included for specific experiements. Twelve clinical isolates (*E coli* 15 and 51; *K. pneumoniae* 58, 74, 99, 100, and 101; and *A. baumannii* 109, 110, 111, 112 and 113) were provided by Johan W. Mouton, Department of Medical Microbiology and Infectious Diseases, Erasmus MC, Rotterdam, The Netherlands [38]; eleven clinical isolates (*E. coli* CPE541, CPE73, CPE493, CPE472, CPE144, and BISC15813; *K. pneumoniae* CPE73, CPE497, CPE370 and CPE532; *A. baumannii* NF2147 and NF2137) were provided by one of us (HRV). All isolates harbored CTX-*M*-, SHV-, OXA-, KPC-, VIM- or TEM-type extended-spectrum β-lactamases and also showed resistance to many other drugs used in the clinics to treat infection by Gram-negative pathogens (Table S2).

All bacteria were grown on trypticase soy agar (BD Life Sciences, Franklin Lakes, NJ, USA) and incubated at 37 °C overnight. A single colony was then inoculated in 10 mL cation-adjusted Mueller-Hinton Broth (CA-MHB; BD Life Sciences, Franklin Lakes, NJ) and incubated at 37 °C overnight under gentle agitation (130 rpm). TSA supplemented with 2 g/L charcoal (Sigma-Aldrich, St Louis, MO) was used for colony-forming unit (CFU) counting. Cell culture media (RPMI-1640), human and fetal bovine sera were from Gibco/ThermoFisher Scientific (Waltham, MA, USA).

2.3. Susceptibility testing

MICs were determined by serial 2-fold microdilution in CA-MHB according to CLSI guidelines in control conditions or in the presence of 38 μM PAβN [20 mg/L] and **NV731** [12 mg/L] or 10 μM **NV716** [4 mg/L]. MICs of antibiotics alone and in combination were used to calculate the fractional inhibitory concentration (FIC) index, as follows: FIC= $C_A/MIC_A + C_B/MIC_B$, where MIC_A and MIC_B are the MICs of compounds A and B alone, and C_A and C_B are the MIC of compounds A or B in combination. The combination was considered as synergistic or antagonistic when the FIC index was <0.5 or >4, respectively [39].

2.4. BODIPY™ TR cadaverine displacement assay

Binding to lipopolysaccharides (LPS) of bacteria was investigated by using BODIPY™-TR-cadaverine displacement assay [40]. BODIPY™ TR cadaverine (BC) can bind to the lipid A region of the LPS. The ability of compounds to interact with LPS was evaluated by measuring the displacement of the BC as previously described [41]. Ten milliliters of bacterial suspension ($OD_{620\ nm}$ of 0.1 in Tris buffer saline, 50 mM, pH 7.4) containing 5 μM BODIPY™ TR cadaverine were incubated in the dark for 30 min. Then 50 μL of this suspension were mixed with 50 μL of potentiators in a 96-well black plate and kept in the dark for 30 min. The fluorescent intensity was then measured in a Spectramax M3 plate reader (Molecular Devices LLC, Sunnyvale, CA) at λ_{ex} 580 nm and λ_{em} 620 nm. Alexidine at 50 μM was used as positive control and samples added by Tris buffer saline as negative control (100%).

2.5. Outer membrane permeability assay

Outer membrane permeability was assessed using nitrocefin, a non-permeant β-lactam which is converted by β-lactamases in a colored derivative in the periplasmic space of permeabilized bacteria [42]. Overnight cultures were incubated with 0.25 μg/mL of imipenem for 1 h to induce β-lactamase expression [43]. After centrifugation at 3000*g* for 7 min, cells were washed twice with 1x PBS and adjusted to obtain an $OD_{620\ nm}$ of 0.5. One hundred μL of cell suspension were mixed with 50 μL of potentiator in 96-well plate, after which 50 μL of nitrocefin (final concentration of 20 μg/mL) were added. Absorbance at 490 nm was monitored over 60 min with an interval of 5 min using a Spectramax M3 plate reader.

2.6. Inner membrane permeability assay

The effect of potentiators on the integrity of the inner membrane was determined by using a membrane-impermeable fluorescent dye propidium iodide (PI) as previously described [44]. A stock solution of PI (10 mM DMSO) was diluted 1000 times with the bacterial suspension ($OD_{620\ nm}$ 0.1 in BET buffer). 100 μL potentiator (in BET) were added to 100 μL PI-containing bacterial suspension in 96-well plates. The fluorescence intensity was measured using a Spectramax M3 plate reader (λ_{ex}, 540 nm; λ_{em}, 610 nm) after 1 h of incubation at room temperature. Alexidine at 50 μM was used as positive control and imipenem as negative control [44].

2.7. Inner membrane depolarization assay

The inner membrane depolarization was evaluated by using DiSC3 (5) as previously described [26]. Overnight cultures were pelleted by centrifugation (3000 *g* for 7 min), washed (1x PBS) and resuspended with 50 mM Tris buffer saline (pH 7.4) containing 2 mM EDTA. After 5 min of incubation, cells were pelleted (3000 *g* for 7 min), washed (1x PBS), resuspended with Tris buffer saline containing 50 mM glucose and adjusted to obtain an $OD_{620\ nm}$ of 0.5 after which 3, 3-dipropylthiadicarbocyanine iodide (DiSC3(5)) (final concentration of 10 μM) was added. One hundred microliters of potentiator were mixed with 100 μL bacteria suspension in a 96-well plate. After 30 min of incubation, the fluorescence intensity was measured in a Spectramax M3 plate reader at λ_{ex} 622 nm and λ_{em} 670 nm. 0.5% SDS was used as a positive control and cells added by Tris buffer saline as negative control [37].

2.8. Inhibition of efflux

The efflux activity in bacteria was assessed using the substrate 1,2′-dinaphthylamine as previously described [45]. Ten milliliters of overnight culture were pelleted by centrifugation with 3000 *g* for 7 min, washed once with 1 x PBS and then incubated with 1,2′- dinaphthylamine (TCI-Europe SA, Zwijndrecht, Belgium) at a final concentration of 32 μM, and CCCP at a final concentration of 5 μM, after which, cells were centrifuged at 3000 *g* for 7 min, resuspended in PBS and adjusted to an $OD_{620\ nm}$ of 0.5. One hundred microliters of potentiator solution was mixed with 100 μL bacteria suspension in a 96-well plate. The fluorescence (λex, 370 nm; λem, 420 nm) was monitored each 30 s during 620 s using a Spectramax M3 plate reader. Active efflux was triggered by adding 5 μL glucose (final concentration of 50 mM) at 180 s. The maximal efflux activity (100%) was defined as the difference between the value obtained without and with glucose addition after 620 s. Data were expressed as the ratio between the fluorescence signal recorded in control conditions or in the presence of potentiator at 620 s. This experiment was performed only with *E. coli* and *K. pneumoniae,* preliminary experiment having shown that 1,2′-dinaphthylamine was not actively exported in *A. baumannii*.

2.9. Antibiotic accumulation in bacteria

The concentration of ciprofloxacin and of a fluorescent analog of rifampicin **(NV1532)** inside bacteria was determined by a previously setup fluorimetric assay [27]. In brief, 10 mL of overnight culture (approximately 10^9 CFU/mL) were incubated at 37 °C with 10 mg/L ciprofloxacin and 20 mg/L of **NV1532.** After 15 min of incubation (a duration longer than the predicted time required to reach saturation [46], but during which the antibiotic did not affect bacterial growth [no significant reduction in CFUs]), 20 μL of suspension were collected to count CFUs and the remaining cells were collected by centrifugation at 3000*g* for 7 min at 4 °C and washed three times with 1 mL cold PBS. When assaying ciprofloxacin, cells were resuspended in 0.5 mL 100 mM glycine buffer (pH 3.0 adjusted with HCl), and then kept in the darkroom overnight. When assaying **NV1532**, cells were resuspended in 0.5 mL PBS and then lysed by three cycles of freezing/thawing (−80 °C freezer to 60 °C water bath). All samples were centrifuged at 14000 *g* for 5 min, the supernatant was then collected to measure the antibiotic content at λ_{ex} 275 nm and λ_{em} 450 nm for ciprofloxacin and λ_{ex} 470 nm and λ_{em} 525 nm for **NV1532**. All data were normalized based on CFUs counts using a Spectramax M3 (Molecular devices, LLC, Sunnyvale, CA) plate reader.

2.10. Cytotoxicity assessment (trypan blue exclusion assay)

THP-1 cells (7.5×10^5 cells per mL) were incubated for 24 h in 96-well plates with the antibiotics alone or a combination of antibiotics and potentiators. The viability of cells was determined using the trypan blue exclusion test (vital colorant excluded from viable cells) as previously described [25]. Briefly, 50 μL of trypan blue reagent was mixed with 50 μL of cell suspension. After 10 min of incubation at 37 °C, non-colored (viable) cells were counted using a Fuchs-Rosenthal counting chamber (Tiefe 0.2 mm). The percentage of cytotoxicity was evaluated based on the reduction in the number of living cells according to the following formula:

$$\text{cytotoxicity}\ (\%) = \frac{Nb\ uncolored\ cells\ in\ controls - Nb\ uncolored\ treated\ cells}{Nb\ uncolored\ cells\ in\ controls} \times 100\%$$

2.11. Intracellular models of infection

We adapted the protocol previously developed for *P. aeruginosa* infection in THP-1 monocytes [31] (see Figs. S2 and S3 for details and model validation). All strains (opsonized 1 h with 10% human serum) were added to monocytes at a multiplicity of infection (bacteria/cell ratio) of 25 and incubated at 37 °C in a 5% CO_2 atmosphere to allow phagocytosis. After 1 h of incubation, cells were pelleted by centrifugation at 300*g* for 7 min and incubated for 1 h with 50 x MIC of gentamicin to remove non-internalized bacteria (more than 5 $\log_{10}$ CFUs reduction, data not shown). Cells were then pelleted by centrifugation at 300*g* for 7 min, washed 2 times with 1 x sterile PBS and resuspended in the original volume of RPMI-1640 with 10% FBS. Cells were collected by centrifugation and washed once with PBS to eliminate extracellular bacteria and collected in 1 mL distilled water, to achieve complete lysis. Lysates were used for determining CFU counts by spreading on agar containing 2 g/L charcoal (to avoid carry-over effect, particularly in samples incubated with high antibiotic concentrations [no difference in colony counts was observed between plates supplemented or not with charcoal for samples exposed to low antibiotic concentrations, ruling out any interfering effect of charcoal in the assay]) and protein content using a commercially available kit (Bio-Rad DC Protein Assay, Bio-Rad Laboratories, Hercules, CA). The post-phagocytosis inoculum (defined as time zero) was consistently 5 to 7 $\times$ 10^5 CFU/mg of cell protein.

2.12. Intracellular antibiotic activity

Infected cells were incubated for 24 h with antibiotics over a range of concentrations (0.01–100x MIC) alone or in combined with potentiators, after which the cells were collected and the protein content and CFU counts were measured as described above. The data were expressed as the change in CFU (normalized by mg of cell protein) from the initial inoculum after 24 h of incubation then used to fit a sigmoidal function and calculate pharmacodynamic parameters based on the corresponding Hill-Langmuir equation (apparent static concentrations [C_s], i.e. extracellular concentration resulting in no apparent intracellular growth, and maximal relative efficacy [E_{max}], i.e. maximal decrease in bacterial counts compared to the post-phagocytosis inoculum as extrapolated for an infinitely large antibiotic concentration).

2.13. Persister assay

A single colony from overnight culture of reference strains on TSA was grown in 200 mL MHB-CA in a 1-L flask and incubated for 24 h with 130 rpm at 37 °C. Ten milliliters of the bacterial suspension were then incubated with 50x MIC ciprofloxacin for 5 h at 37 °C under agitation (130 rpm), in the presence of or in the absence of **NV716**. To explore the effects of different treatment regimens, **NV716** was added at 0, 5, or 24 h after ciprofloxacin treatment, and the cells were treated for a total of 72 h. Aliquots were serially diluted in PBS and spread on TSA supplemented with 2 g/L charcoal for CFU counting.

2.14. Antibiotic activity against biofilms

Biofilms were grown in 96-well plates (VWR) as previously described [32]. In brief, one single colony was inoculated in MHB-CA overnight with 130 rpm at 37 °C. A bacterial suspension was then prepared in TGN (Trypticase soy broth (VWR) supplemented with 1% glucose (Sigma) and 2% NaCl (Sigma)) using overnight cultures. Ninety-six-well plates were inoculated (200 μL/well) at approximately 10^7 CFU/ml (the $OD_{620\ nm}$ of 0.05) and then incubated at 37 °C for 24 h to obtain a mature biofilm. The culture medium was removed and replaced with fresh medium (control) or medium supplemented with rifampicin (1x MIC and 5x MIC) alone or combined with potentiators and then reincubated at 37 °C for 24 h. The medium was then removed and the biofilm was washed once with 200 μL of 3-morpholinopropane-1-sulfonic acid (MOPS) buffer (20.9 g/L of MOPS (Sigma-Aldrich), 5.6 g/L NaCl; the pH was adjusted to 7.0 with NaOH) [47]. Biofilm biomass was evaluated by measuring the absorbance of crystal violet, a cationic dye that nonspecifically stains negatively charged constituents in biofilm constituents based on ionic interactions. The washed biofilms were fixed by heat at 60 °C for about 24 h and incubated for 10 min at room temperature with 200 μL of 1% (v/v) crystal violet (Sigma-Aldrich). The absorbance at 570 nm was measured using a Spectramax M3 plate reader. The metabolic activity (vitality) in the biofilms was quantified using the fluorescein diacetate (FDA) assay. It is based on the hydrolysis by living bacteria of the non-fluorescent white dye fluorescein diacetate in the yellow highly fluorescent fluorescein [32]. The washed biofilms were incubated with 100 mg/L fluorescein diacetate (Sigma-Aldrich) for 15 min at 37 °C in the dark. Fluorescence was measured using a Spectramax M3 plate reader at λ_{ex} 494 nm and λ_{em} 518 nm.

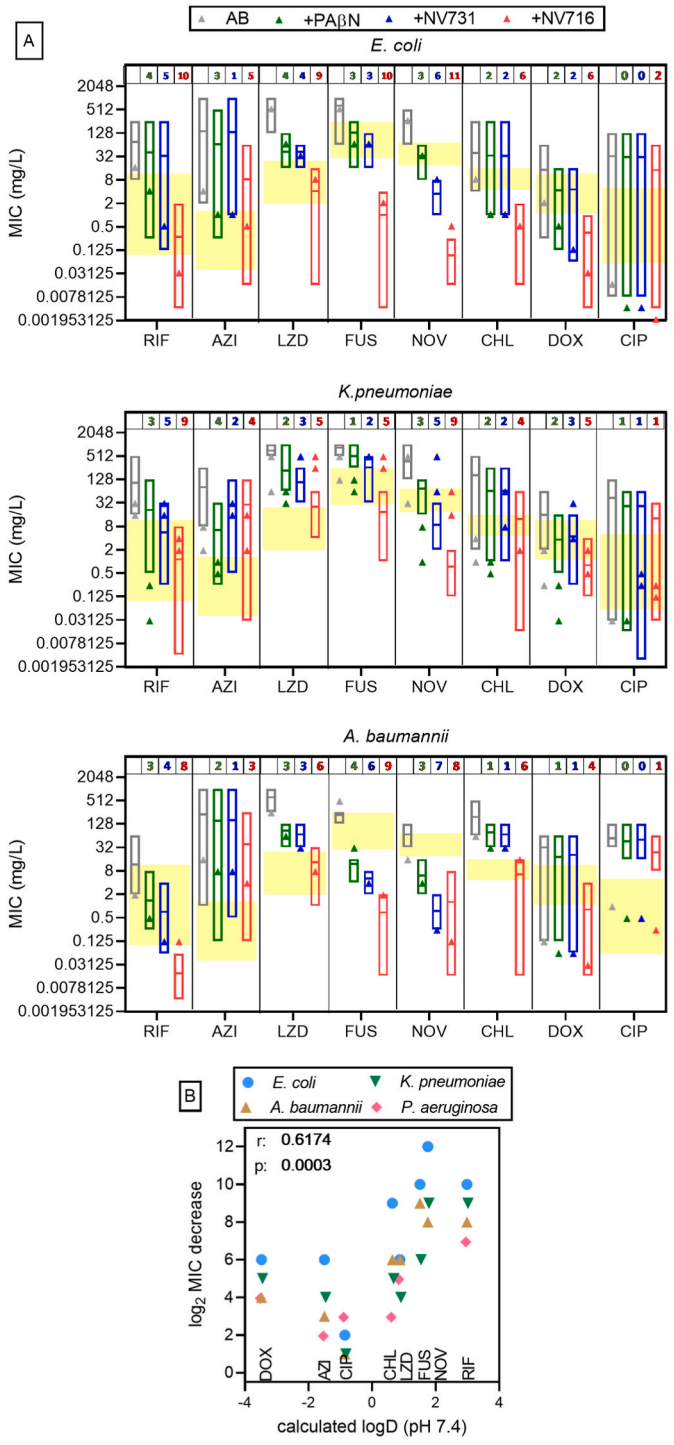
2.15. Curve fitting and statistical analysis

Statistical analyses and curves fitting were performed using GraphPad Prism version 9.1.2 (GraphPad Software, San Diego, CA, USA). Pharmacodynamic parameters were calculated based on Hill equations of concentration-response curves.

3. Results

3.1. Antimicrobial susceptibility

Table S3 shows the MICs of antibiotics alone or combined with potentiators (20 mg/L [38 μM] PAβN; 15 mg/L [38 μM] **NV731**; 4 mg/L [10 μM] **NV716**) against reference strains and clinical isolates of *E. coli, A. baumannii and K. pneumoniae*. These concentrations were selected as active based on previous works, but well below the MIC of each compound [25,28]. In control conditions, the MIC of antibiotics was generally elevated and higher than their human C_{max} (except for rifampicin against *A. baumannii*, doxycycline and ciprofloxacin against most of the strains, and chloramphenicol against most *E. coli* and *K. pneumoniae*; see Table S1 for C_{max} values). In the presence of potentiators, MICs were globally reduced, although to a lower extent for ciprofloxacin against all strains, for azithromycin, chloramphenicol, and doxycycline against *A. baumannii,* and for fusidic acid against *K. pneumoniae*. PAβN and **NV731** were systematically less potent than **NV716** even though the latter was used at a lower concentration. Fig. 1A illustrates these MICs data and also shows the (geometrical) mean fold decrease in MIC in the presence of potentiators. The most important reduction in this mean MIC values was noticed in combination with **NV716** for rifampicin, linezolid, fusidic acid and novobiocin against *E. coli* (9–11 doubling dilutions), rifampicin and novobiocin against *K. pneumoniae* (9 doubling dilutions), and rifampicin, novobiocin and fusidic acid against *A. baumannii* (8–9 doubling dilutions). The lowest effect (mean reduction of 1–2 doubling dilutions) was noticed for ciprofloxacin against all bacteria as well as for azithromycin against *A. baumannii* (3 doubling dilutions). Importantly, MIC of the combinations with **NV716** fall in the range of therapeutically-achievable concentrations in all cases (yellow zones in the graphs). FIC index calculations indicated a higher degree of synergy between antibiotics and **NV716** than the other potentiators (Fig. S4), except for ciprofloxacin against *K. pneumoniae* ATCC 43816 and 74, against which ciprofloxacin alone was already very active. A highly significant correlation was observed between the reduction in MIC caused by **NV716** and the calculated logD at pH 7.4 of each antibiotic (Table S1), a modest effect being observed for antibiotics with a negative logD value, and a progressive increase in this effect along with the lipophilicity of the drugs once their logD value becomes higher than 0 (Fig. 1B).



(caption on next column)

Fig. 1. A. Activity of antibiotics alone or combined with potentiators against various Gram-negative bacteria in broth. The MICs of reference strains (One [*E. coli*; *A. baumannii*] or two [*K. pneumoniae*] strains) are represented by triangles. The boxes show the interval between the minimal and maximal (line at mean value) MICs values for eight (*E. coli; K. pneumoniae*) or seven (*A. baumannii*) multidrug-resistant clinical isolates. Grey: antibiotics alone; green: combined with 38 μM PAβN; blue: combined with 38 μM **NV731**; red: combined with 10 μM **NV716**. Squared value: fold decrease (geometrical mean; number of doubling dilutions) in MIC (mg/L) between antibiotic alone (rifampicin [RIF], azithromycin [AZI], linezolid [LZD], fusidic acid [FUS], novobiocin [NOV], chloramphenicol [CHL], doxycycline [DOX], or ciprofloxacin [CIP]) and antibiotic combined with potentiators. Yellow zone: range of clinically-achievable concentrations in human plasma (see Table S1 for values). **B.** correlation between the reduction in antibiotic MIC (expressed as $\log_2$ fold decrease; geometric means) in combination with 10 μM **NV716** and the logD value of each antibiotic calculated at pH 7.4 (See Table S1). Each symbol corresponds to a bacterial species. Data previously obtained with *P. aeruginosa* PAO1 are added for completing the analysis. The Pearson's correlation coefficient r and the p value (t-tailed) are shown on the graph.

3.2. Interaction of potentiators with E. coli, K. pneumoniae and A. baumannii membranes

Our previous study suggested that **NV716** can bind to LPS in *P. aeruginosa,* increases the permeability of its outer membrane, and to a much lower extent, of its inner membrane [27]. We therefore examined these properties in the reference strains of the three bacterial species under study here. Alexidine and colistin were used as positive controls [34,48], and **NV731** and PAβN, as comparators. We first measured the capacity of each compound to displace Bodipy-cadaverine from its binding to LPS in the 4 reference strains (Fig. 2A; IC_{50} values in Table S4). The effect of **NV716** was similar to that of alexidine over the whole range of concentrations investigated against all strains, with comparable IC_{50} values for both molecules. The IC_{50} were about 10-times higher for **NV731** and not reached at 100 μM for colistin and PAβN.

We then determined the effects of these compounds on the outer membrane permeability (Fig. 2B; IC_{50} values in Table S4). To this effect, we measured the absorbance of the hydrolysis product of the non-permeant cephalosporin nitrocefin, which is degraded by periplasmic β-lactamases when the outer membrane has been permeabilized [42]. Alexidine was the most potent in this assay, followed by **NV716** and colistin, which also allowed to reach a same effect as alexidine but at higher concentrations (IC_{50} 2 to 3 times higher). **NV731** and PAβN were less potent (IC_{50} 7 to 13 times higher than for alexidine) and less effective.

The effect of potentiators on the inner membrane permeability was also assessed by measuring the fluorescence signal of propidium iodide (PI), generated when PI intercalates in the DNA of bacteria when both their outer and inner membranes have been permeabilized [44]. Alexidine and colistin similarly increased PI fluorescence in a concentration-dependent manner, while **NV716** was 20–30 times less potent, causing only 50% of permeabilization at the highest concentrations tested (50 μM) (Fig. 2C; IC_{50} values in Table S4). Yet, the effect of **NV716** was slightly higher than that of colistin but much lower than that of alexidine when these compounds were all compared at an equipotent concentration of 1 x MIC (Fig. S5). **NV731** and PAβN were inactive in this assay.

In addition, we investigated the effect of **NV716** on inner membrane depolarization using the DiSC3(5) fluorescence assay. We observe a depolarization at 50 μM but not at 10 μM (Fig. S6), indicating that **NV716** can dissipate the proton motive force (PMF) at its MIC, but not at sub-inhibitory concentrations.

3.3. Inhibition of active efflux by potentiators

Previous studies documented that **NV716** can inhibit the activity of efflux pumps in *P. aeruginosa* [26]. We therefore evaluated the capacity of **NV716** and its comparators to inhibit the active efflux of the fluorescent probe 1,2′-dinaphthylamine [45]. We first examined the rate of efflux in *E. coli* ATCC 47076 after the addition of **NV716** at different concentrations (Fig. 2D, left panel). A concentration-dependent reduction of the fluorescence was observed readily after the addition of **NV716**, which reached a plateau at 620 s. The same experiments were performed with the other potentiators and also against *K. pneumoniae* reference strains. The other panels of Fig. 2D show the inhibition of efflux calculated at 620 s. **NV716** proved more potent and effective in

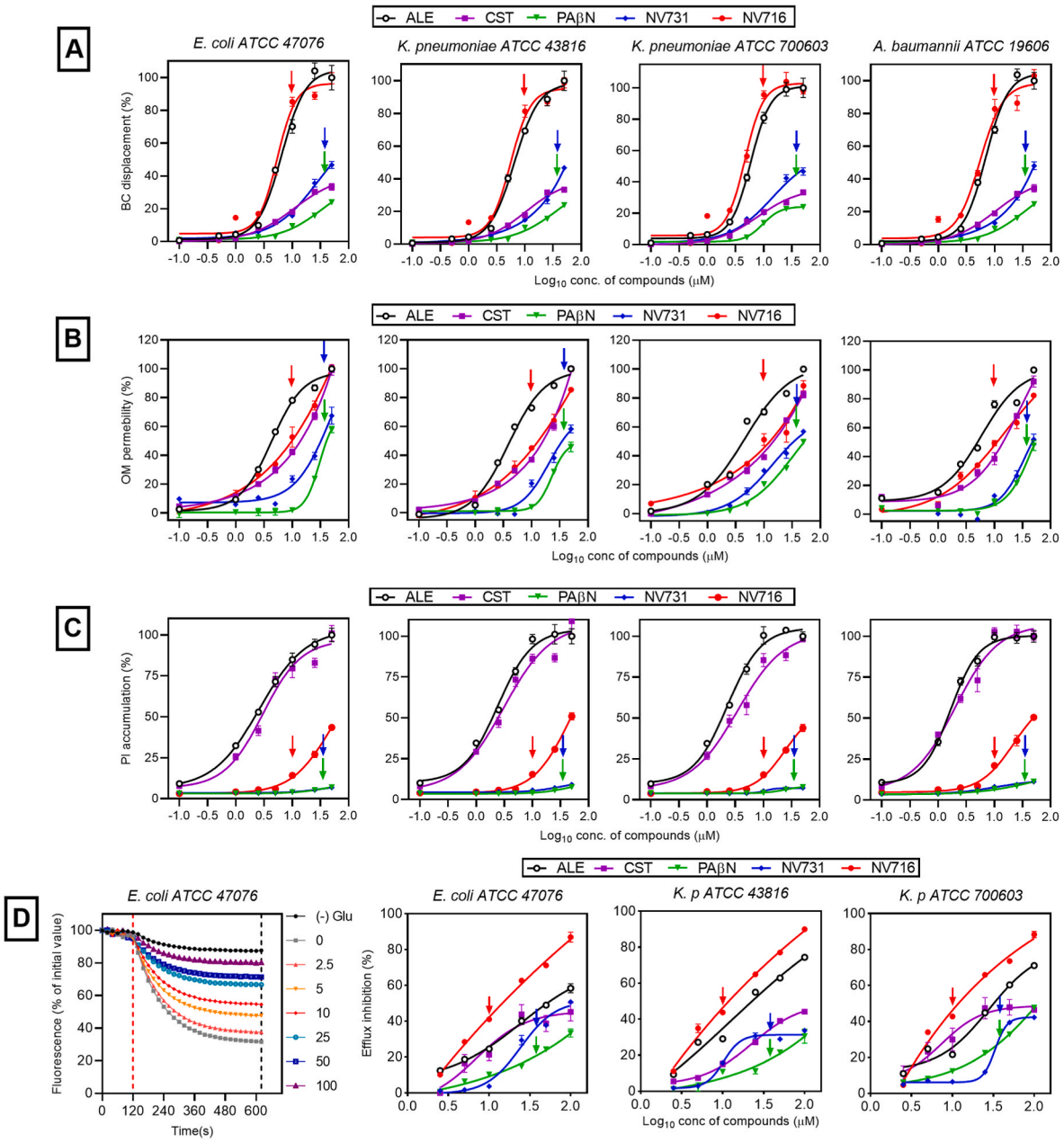


Fig. 2. Influence of potentiators, colistin and alexidine over a wide range of concentrations on membrane properties of 1 (*E. coli; A. baumannii*) or 2 (*K. pneumoniae*) reference strains. (A) Binding to LPS, assessed by measuring the displacement of BODIPY-cadaverine (BC) after 30 min of incubation. Positive control (100%): effect of 50 μM alexidine at 30 min. (B) Outer membrane permeability, evaluated by measuring the absorbance of the hydrolysis product of nitrocefin after 1 h of incubation. Positive control (100%): effect of 50 μM alexidine at 1 h; negative control: buffer. (C) Inner membrane permeability, assessed by measuring the fluorescence of propidium iodide (PI) after 1 h of incubation. Positive control (100%): effect of 50 μM alexidine after 1 h of incubation; negative control: buffer. (D) Left: kinetics of efflux dinaphthylamine from *E. coli* ATCC 47076 in the presence of increasing concentrations of **NV716** (indicated on the right of the graph). Active efflux was triggered by adding 50 mM glucose at 120 s (red vertical dotted line). Data are expressed in percentage of the value recorded in the absence of glucose. Other panels: Inhibition of efflux of 1,2-dinaphthylamine by potentiators in *E. coli* ATCC 47076. The graphs show the percentage of efflux inhibition after 620 s of incubation (black vertical dotted line on the left graph) (500 s after the addition of glucose). The arrows on each graph point to the concentration of potentiators used in most other experiments (10 μM for **NV716**; 38 μM for **NV731** and PAβN). All data are means ± SEM (triplicates from 3 independent experiments). IC_{50} values for these concentration-responses are shown in Table S4.

this assay than the other potentiators (Fig. 2D). While the effect of **NV716**, alexidine and PAβN increased all over the range of concentration investigated, that of **NV731** and colistin tend to a plateau at high concentrations.

3.4. Influence of potentiators on antibiotic accumulation inside bacteria

As **NV716** was shown to increase outer membrane permeability and inhibit efflux, we assessed its effect compared to that of the other potentiators on the accumulation of ciprofloxacin and **NV1532** (a fluorescent derivative of rifampicin; same MIC as rifampicin against all tested strains) inside bacteria. These antibiotics were selected as representatives of antibiotics that are preferential substrates for efflux or not, respectively. The level of accumulation of ciprofloxacin alone was higher in *E. coli* ATCC 47076 and *K. pneumoniae* ATCC 43816 than in the other two strains (Fig. 3A), possibly related to differences in intrinsic

outer membrane permeability or expression of efflux transporter. PAβN and **NV731** at 38 μM increased its accumulation in *E. coli* ATCC 47076 only, while **NV716** at 10 μM caused a significant increase in all strains except *K. pneumoniae* ATCC 43816 in which it did not decrease ciprofloxacin MIC (Table S3). The accumulation of **NV1532** was more elevated in *A. baumannii* ATCC 19606 than in the other strains, also probably related to a higher intrinsic membrane permeability to this more lipophilic drug. PAβN increased its accumulation in *E. coli* ATCC 47076 and *A. baumannii* ATCC 19606, while **NV731** and **NV716** caused a significant increase for all strains tested, but the effect of **NV716** was more important even though it was used at a lower concentration (Fig. 3B). A significant correlation was observed between the fold change (i) in the accumulation of antibiotics and (ii) in MIC when comparing the combination with potentiators vs antibiotics alone (Fig. 3C and D).

3.5. Cytotoxicity of antibiotics

As a preliminary to intracellular infection experiments, we examined whether antibiotics were cytotoxic for eukaryotic cells. This was critical as most of them had to be used at high concentrations in view of their high MIC. The cytotoxicity of potentiators had been previously examined and found to be minimal in their conditions of used for these experiments [25]. IC_{50} (i.e. concentration causing 50% cell mortality) were reached at concentrations corresponding to approximately 3 x (fusidic acid, linezolid, novobiocin), 30 x (rifampicin), 60 x (azithromycin), 90 x (chloramphenicol), or above 100 x (doxycycline and ciprofloxacin) their respective MIC against *E. coli* ATCC 47076, both in infected and non-infected cells (Table S5). IC_{50} values did not differ for antibiotics alone or combined with a potentiator, both in non-infected and infected cells (Table S5).

3.6. Activity of antibiotics alone or combined with potentiators against intracellular Gram-negative bacteria in THP-1 cells

Fig. 4 shows the concentration-response curves against intracellular *E. coli* ATCC 47076 in the absence of or in the presence of **NV716** (the highest concentration tested for each antibiotic was that causing 50% cytotoxicity). The activity of antibiotics alone developed following a sigmoidal concentration-response curve, as previously described [31]. At low, subMIC concentrations, a ~3–4 $\log_{10}$ increase in CFUs was noticed over the 24 h incubation period. A static effect was observed at extracellular concentrations close or slightly higher than the MIC of each antibiotic. At the highest concentration tested, the reduction in bacterial counts ranged from a static effect for linezolid, fusidic acid, or novobiocin (note that the highest concentration tested is low for these drugs because of their toxicity), 1 $\log_{10}$ CFU decrease for azithromycin and chloramphenicol, 2 $\log_{10}$ CFU decrease for rifampicin and doxycycline, and 2.5 $\log_{10}$ CFU decrease for ciprofloxacin. In the presence of **NV716** at 10 μM, all curves were shifted to the left, meaning that the corresponding antibiotic was more potent, a static effect being reached at lower extracellular concentrations. The extent of the shift varied however among drugs. Furthermore, for those drugs for which the maximal effect could be reached (i.e., the plateau value of the Hill equation), the reduction in intracellular counts was also increased (E_{max} more negative), indicating an increase in the antibiotic efficacy. For the other

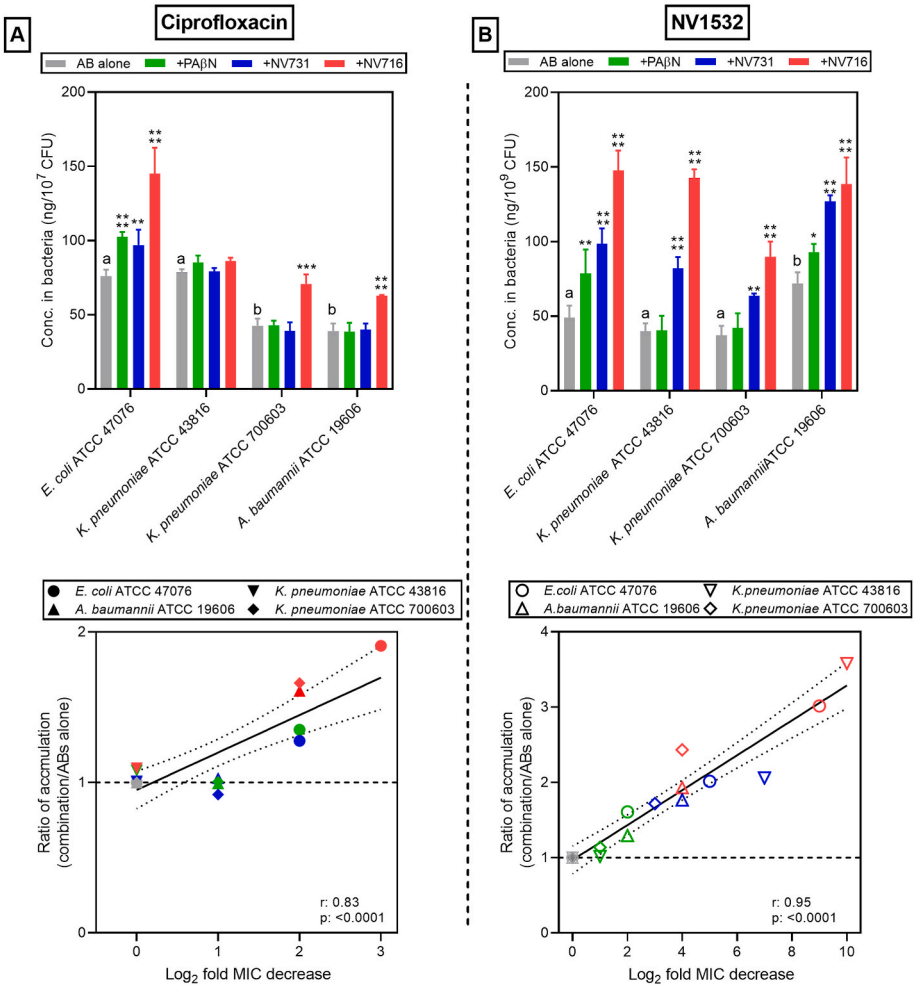


Fig. 3. Top: Accumulation of antibiotics alone (A, ciprofloxacin; B, rifampicin analog **NV1532**) or combined with potentiators in 1 (*E. coli* and *A. baumannii*) and 2 (*K. pneumoniae*) reference strains. Grey: antibiotics alone; green: combined 38 μM PAβN; blue: combined 38 μM **NV731**; red: combined with 10 μM **NV716**. All data are mean ± SEM (triplicates from three independent experiments). Statistical analysis: two-way ANOVA with Dunnett post-hoc test for comparison of antibiotics alone and combined with each potentiator for each strain: *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$; ****, $p < 0.0001$; with Tukey post-hoc test for comparison of antibiotics alone among strains: data series with different letters are different from one another ($p < 0.05$). Bottom: Correlation between the change in antibiotic accumulation in bacteria (expressed as the ratio between the concentration measured with potentiators and in control conditions) and the change in MIC (expressed as the $\log_2$ fold decrease in MIC) in the same experimental conditions. The Pearson correlation coefficient r and the p values are shown on each graph. The plain and dotted lines correspond to the linear regression with its 95% confidence interval.

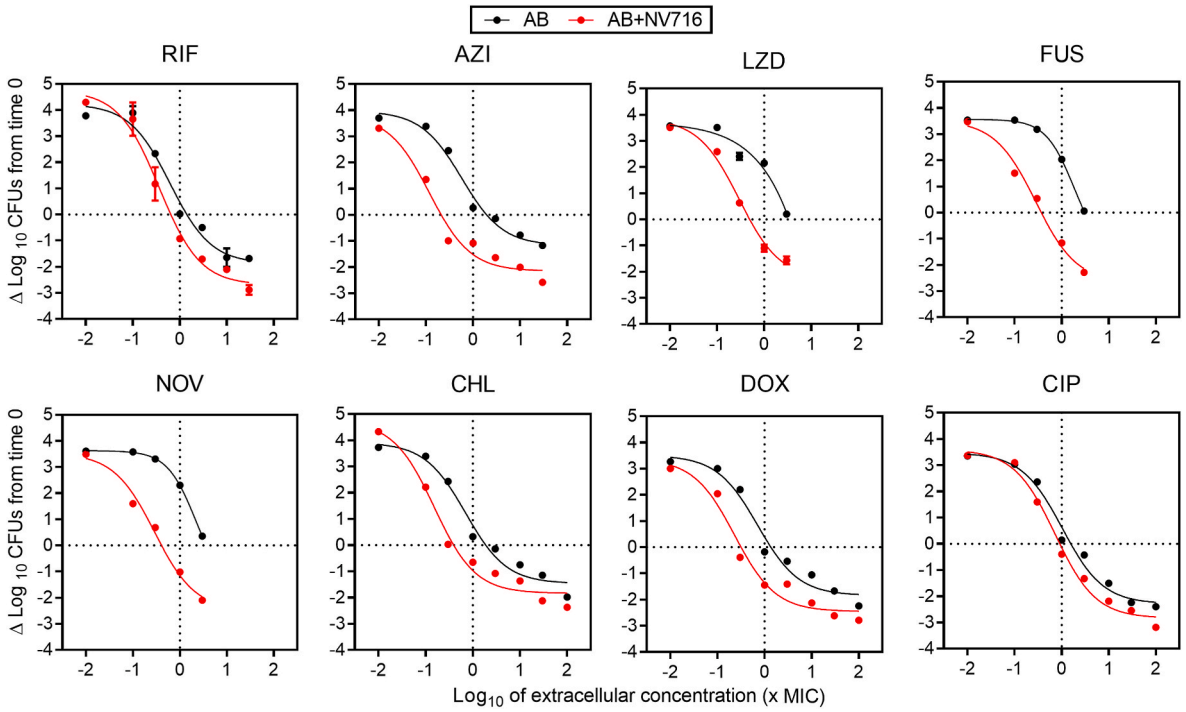


Fig. 4. Concentration-response curves of antibiotics alone or combined with **NV716** against intracellular *E. coli* ATCC 47076 in a model of THP-1 monocytes. The graphs show the changes in CFU counts from the initial, post-phagocytosis inoculum after 24 h of incubation with increasing extracellular concentrations of antibiotics (AB) alone (rifampicin [RIF], azithromycin [AZI], linezolid [LZD], fusidic acid [FUS], novobiocin [NOV], chloramphenicol [CHL], doxycycline [DOX], or ciprofloxacin [CIP]) or combined with a fixed concentration (10 μM; 4 mg/L) of **NV716**. In each graph, the horizontal dotted line highlights a static effect and the vertical dotted line indicates the MIC of each antibiotic. The highest concentration of the antibiotic tested is close to the IC_{50} of toxicity for THP-1 cells (not applicable to CIP, not toxic). All data are means ± SEM (triplicates from three experiments; when non-visible, error bars are smaller than the symbols).

drugs (linezolid, fusidic acid, novobiocin), an increased effect was also observed at the highest concentration tested.

The same type of experiment was performed with the 2 other potentiators against 1 (*E. coli*; *A. baumannii*) or 2 (*K. pneumoniae*) reference strains and 2 clinical isolates. From each species Figs. 5 and 6 illustrate the pharmacodynamic parameters calculated based on the Hill equation of these concentration-response curves (see methods for a definition of these parameters), namely the C_s (a measure of the relative potency of the drug or the drug combination) and the E_{max} (a measure the relative maximal efficacy of the antibiotics alone or in combination; when the E_{max} was not reached, the maximal reduction in bacterial counts at the highest concentration [$E_{highest}$] tested was plotted). C_s of antibiotics alone was globally slightly higher than their MIC in broth, and **NV716** markedly reduced all Cs values. These reductions ranged from approx. 1 [chloramphenicol and ciprofloxacin] to 3–4 [linezolid, fusidic acid and novobiocin] doubling dilutions against *E. coli*; 1 [chloramphenicol and ciprofloxacin] to 3 [novobiocin] doubling dilutions against *K. pneumoniae*; 1 [rifampicin and ciprofloxacin] to 3–4 [fusidic acid, novobiocin and chloramphenicol] doubling dilutions against *A. baumannii*, respectively (Fig. 5 and Table S6). Ciprofloxacin was the less affected because it was the most active drug against these bacteria. **NV731** and PAβN caused less marked effects on C_s values. Intracellular E_{max} or $E_{highest}$ ranged between 0.6 [linezolid against *E. coli*] to 2.3 [ciprofloxacin against *E. coli*] $\log_{10}$ CFU decrease for antibiotics alone. **NV716** showed the most important potentisation of antibiotic efficacy, with the gain in E_{max} or $E_{highest}$ values ranging from 0.7 (doxycycline and ciprofloxacin) to 1.7 (fusidic acid) $\log_{10}$ CFU for *E. coli;* 0.6 (chloramphenicol and doxycycline) to 1.3 (linezolid) $\log_{10}$ CFU for *K. pneumoniae;* and 0.6 (doxycycline) to 1 (all test antibiotics except for doxycycline) $\log_{10}$ CFU for *A. baumannii* (Fig. 6 and Table S6). PAβN did not improve antibiotic efficacy, and **NV731** effects were more modest than those of **NV716** in all cases.

In order to examine our data in a clinically-oriented perspective, we estimated, based on the Hill equation of the concentration-response curves, the reduction in bacterial counts reached when antibiotics were used alone or in combination at fixed concentrations corresponding to their respective minimum and maximum concentrations (C_{min} and C_{max}, see Table S1) in human serum (Fig. S7). Antibiotics alone at their C_{min} were inactive, except ciprofloxacin, chloramphenicol and doxycycline against *E. coli* and *K. pneumoniae* (which reduced intracellular counts but did not reach a static effect) and ciprofloxacin, against *A. baumannii* (1 $\log_{10}$ CFU decrease). When antibiotics at their C_{min} were combined with **NV716,** we noticed a reduction in bacterial counts, the extent of which was highly variable among drugs and bacteria, with reduction of 90% (1 $\log_{10}$ CFU) being observed for rifampicin, fusidic acid, chloramphenicol and doxycycline against all species (except *A. baumannii* for doxycycline). Yet, in most of the cases, this effect was not sufficient to decrease bacterial counts below the post-phagocytosis inoculum. When used at the human C_{max}, most antibiotics were capable of reducing the inoculum, except linezolid (against all species) and azithromycin (against *E. coli* and *K. pneumoniae*). Chloramphenicol and doxycycline were at least bacteriostatic against *E. coli* and *K. pneumoniae*, and ciprofloxacin, against all species. In combination with **NV716** (10 μM), a drastic improvement of activity was observed, with increment of activity reaching 1 to almost 4 $\log_{10}$ CFU decrease as compared to the antibiotic alone. For rifampicin, novobiocin, doxycycline, and in some cases, for fusidic acid, chloramphenicol and ciprofloxacin, a 99% (2 $\log_{10}$ CFUs) decrease as compared to the post-phagocytosis inoculum could be achieved by the combinations. In all these conditions, the effect of PAβN and **NV731** was lower than that of **NV716.**

3.7. Activity of ciprofloxacin alone or combined with potentiators against persisters

The fact that **NV716** increases the intracellular efficacy of all

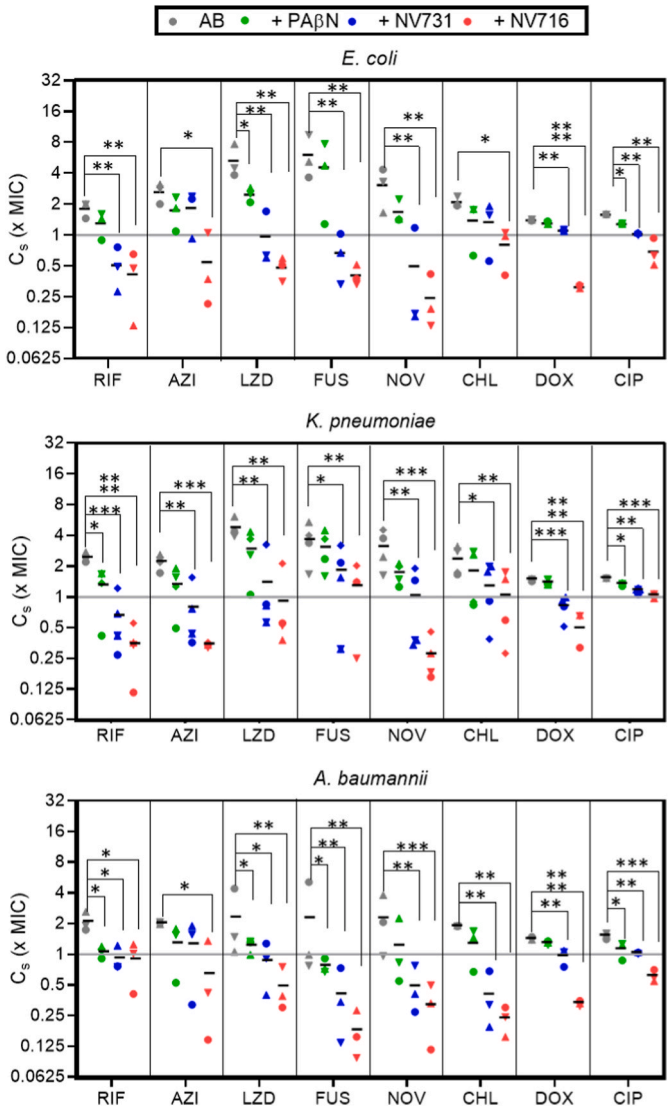


Fig. 5. Static concentration of antibiotics alone (rifampicin [RIF], azithromycin [AZI], linezolid [LZD], fusidic acid [FUS], novobiocin [NOV], chloramphenicol [CHL], doxycycline [DOX], or ciprofloxacin [CIP]) or combined with potentiators against various multidrug resistant Gram-negative bacteria in the intracellular infection model (THP-1). Values are calculated based on the equation of the Hill response curves from experiments similar to those described in Fig. 4. 1 symbol/strain: 1 (*E. coli; A. baumannii*) or 2 (*K. pneumoniae*) reference strains and 2 to clinical isolates of each species. Grey: antibiotics alone; green: combined 38 μM PAβN; blue: combined 38 μM **NV731**; red: combined with 10 μM **NV716**. Horizontal bars: mean; horizontal line: MIC. Statistical analysis: one-way ANOVA with Dunnett post-hoc test (paired) comparing each combination to the antibiotic alone: *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$; ****, $p < 0.0001$.

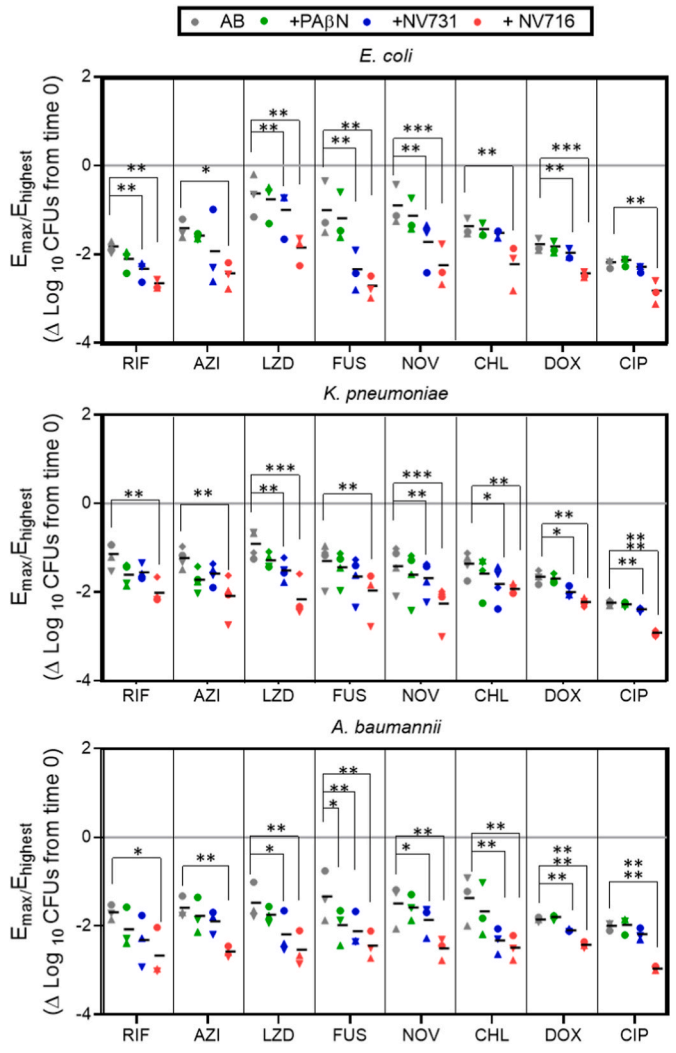


Fig. 6. E_{max} (extrapolated for an infinitively large concentration) or $E_{highest}$ (measured at the highest concentration tested but the plateau was not yet reached) for antibiotics alone (rifampicin [RIF], azithromycin [AZI], linezolid [LZD], fusidic acid [FUS], novobiocin [NOV], chloramphenicol [CHL], doxycycline [DOX], or ciprofloxacin [CIP]) in the intracellular infection model (THP-1). Values are calculated based on the equation of the Hill response curves from experiments similar to those described in Fig. 4. 1 symbol/strain: 1 (*E. coli; A. baumannii*) or 2 (*K. pneumoniae*) reference strains and 2 to clinical isolates of each species. Grey: antibiotics alone; green: combined 38 μM PAβN; blue: combined 38 μM **NV731**; red: combined with 10 μM **NV716**. Horizontal bars: mean. Statistical analysis: one-way ANOVA with Dunnett post-hoc test (paired) comparing each combination to the antibiotic alone: *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$; ****, $p < 0.0001$.

antibiotics against *E. coli*, *A. baumannii* and *K. pneumoniae* suggests it may decrease the proportion of antibiotic persisters in the population, as previously demonstrated for *P. aeruginosa* [25]. We, therefore, determined the persister fraction surviving to ciprofloxacin (selected as a highly bactericidal antibiotic) at high concentration (50 x MIC) against stationary-phase cultures (10^9 CFU/mL) of reference strains. To this effect, we first examined the kinetics of killing by ciprofloxacin when used alone or combined with **NV716** added either at the same time as ciprofloxacin or later to determine whether it was able to improve activity even the maximal effect was already reached for ciprofloxacin alone [49] (Fig. 7A). CFUs were significantly reduced after the addition of **NV716** at any time point, suggesting that **NV716** does not need to be added at the same time as the antibiotic to be able to increase its activity. In the next step, we aimed at determining whether **NV716** action was related to a direct capacity to kill persisters or rather to resensitize them to the killing effect of ciprofloxacin. To this end, we isolated the persisters surviving after 5 h of incubation with ciprofloxacin at 50 x MIC and reexposed them to ciprofloxacin at 50 x MIC, NV716 at different concentrations, or a combination thereof (Fig. 7B). As expected, incubation of the isolated persisters with ciprofloxacin alone caused only a negligible reduction in CFU for all strains, confirming the effective isolation of persister cells. When used alone, **NV716** caused a marked decrease in CFU only at the highest concentration tested (250 μM). In contrast, a significant reduction in CFU was observed when **NV716** was combined at 10 μM with ciprofloxacin for all strains, indicating it rather acts as a potentiator of ciprofloxacin against persisters.

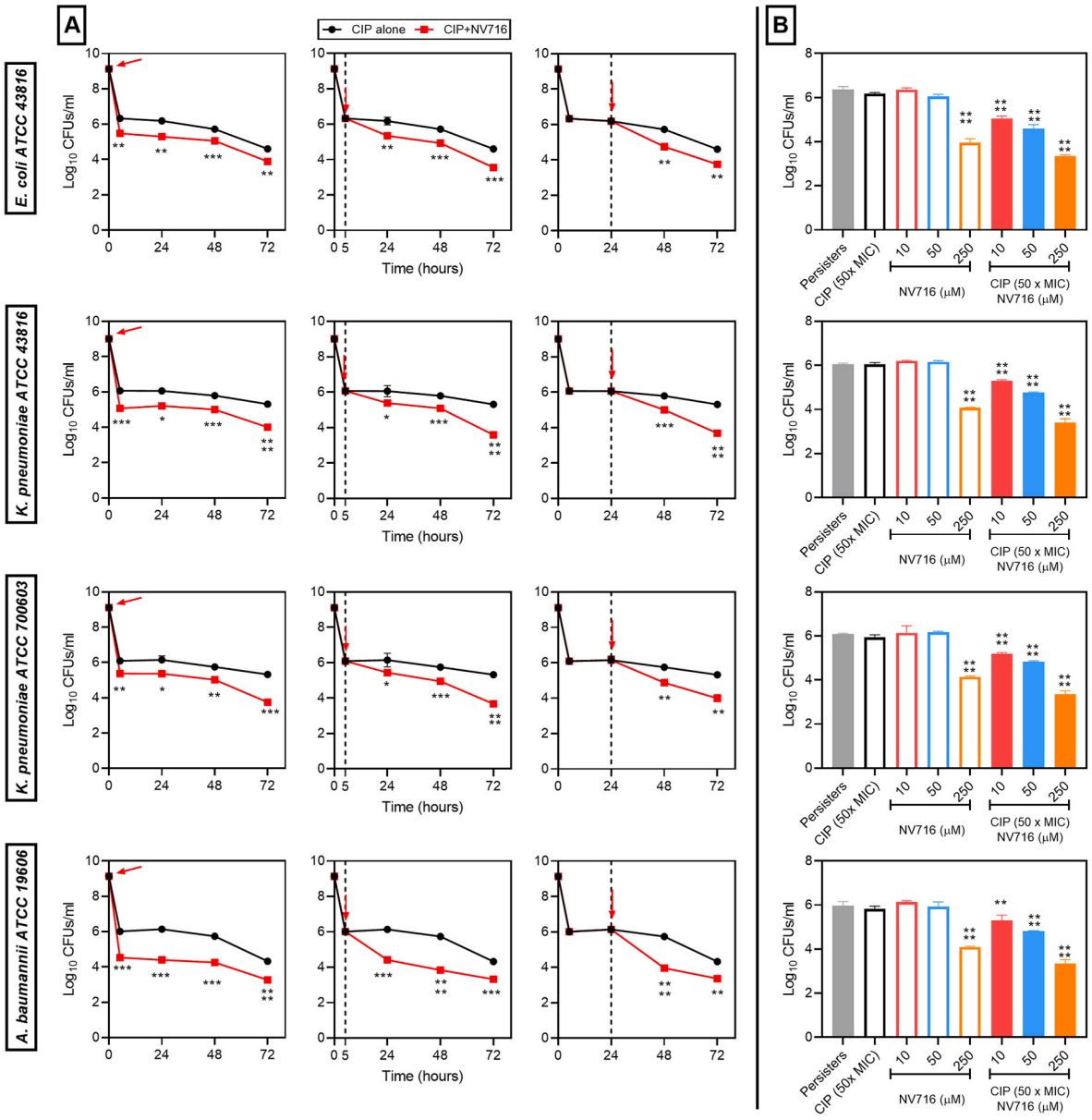


Fig. 7. Influence of **NV716** on persisters selected by ciprofloxacin. (A) The time-killing assay for stationary-phase cultures of 1 (*E. coli; A. baumannii*) or 2 (*K. pneumoniae*) reference strains by ciprofloxacin at 50 x MIC alone or combined with 10 μM **NV716** added at different time points (0 h [left], 5 h [middle] or 24 h [right]; the time of **NV716** addition is highlighted by the vertical dotted line and the red arrow). All data are expressed as means ± SEM (triplicates from three experiments). (B) Killing of persister cells of the same strains by ciprofloxacin, **NV716**, or their combinations. Persister cells were isolated after 5 h of incubation with ciprofloxacin at 50 x MIC (persisters) and then incubated with either 50 x MIC ciprofloxacin (CIP), **NV716** at different concentrations, or their combination. All data are expressed as means ± SEM (triplicates from three experiments). Statistical analysis: A: Student's t-test comparing the combination with CIP alone at each concentration; B: one-way ANOVA with Dunnett's post hoc test comparing each type of treatment to CIP alone: *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$; ****, $P < 0.0001$.

3.8. Activity of rifampicin alone or combined with potentiators against biofilms

Our previous work shows the combination of antibiotics and **NV716** can reduce the biomass and viability of *P. aeruginosa* in biofilms [27]. We therefore examined the activity of rifampicin alone or combined with potentiators against biofilms produced by reference and clinical isolates of the three species investigated here (Fig. 8). When used alone, rifampicin had no or little effect on biomass at 5x MIC (assessed by crystal violet staining; left panels) and bacterial viability at 1x MIC (assessed by fluorescein diacetate metabolization; right panels) in biofilms. Its combination with **NV716** (10 μM) significantly reduced the biomass as well as the viability (right panels). PAβN did not improve rifampicin activity, and **NV731** increased rifampicin activity against *A. baumannii* biofilms, but to a lower extent than **NV716**. None of the potentiators was capable of reducing biofilm biomass and viability when used alone at these concentrations (data not shown).

4. Discussion

The emergence and rapid spread of antibiotic resistance in Gram-negative bacteria represents a critical threat to public health worldwide [50]. Identification of novel adjuvants that restore existing antibiotic efficacy and improve clinical cure has been considered as a cost-effective strategy for combating superbugs, in particular *E. coli*, *K. pneumoniae* and *A. baumannii* [51]. In this study, we show that the

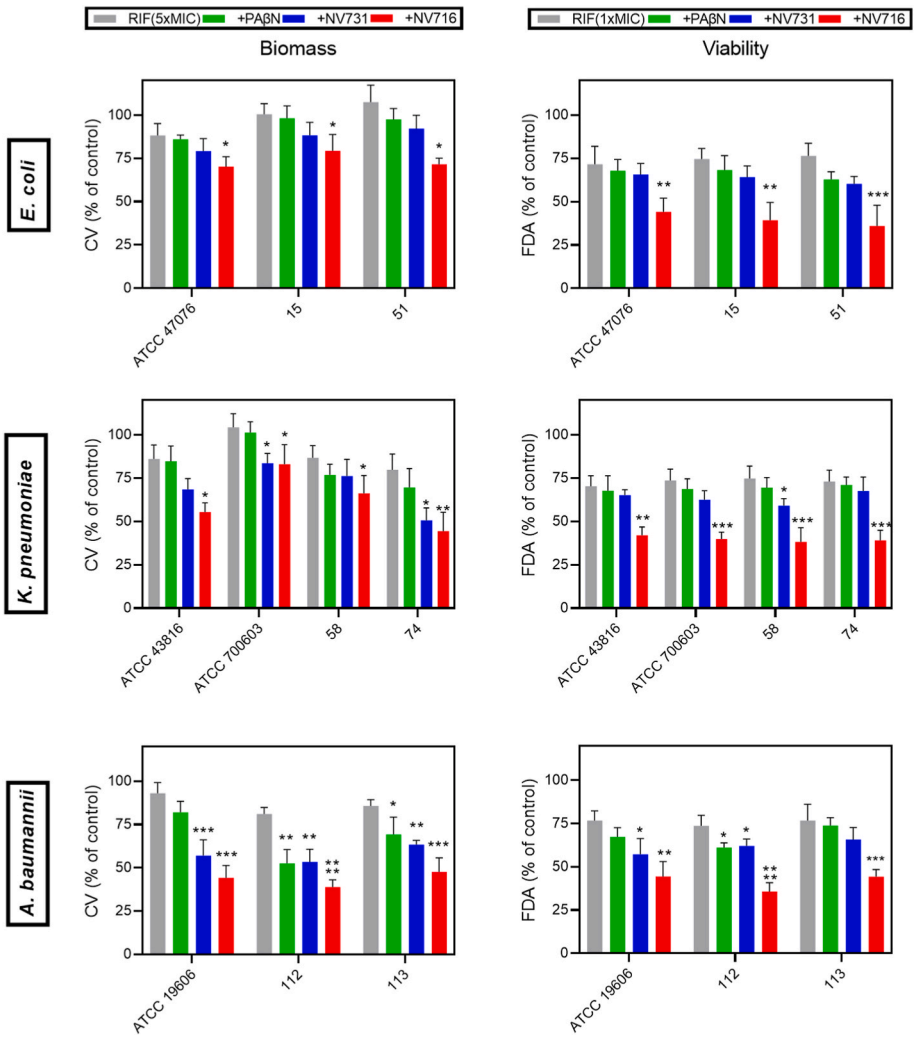


Fig. 8. Effect of potentiators on the activity of rifampicin against biofilms of 1 (*E. coli; A. baumannii*) or 2 (*K. pneumoniae*) reference strains and 2 to clinical isolates of each species. Mature biofilms were exposed during 24 h to rifampicin alone at 1 x MIC (right panels) or 5 x MIC (left panels) in order to achieve suboptimal effects, the potentiators alone (38 μM PAβN and **NV731** and 10 μM **NV716**), or their combination. Biomass (left panel) and viability (right panel) were quantified using crystal violet (CV) staining and fluorescein diacetate assay (FDA), respectively. All data are mean ± SEM (triplicates from three independent experiments). Statistical analysis: one-way ANOVA with Dunnett's post-hoc test comparing each combination to RIF alone: *, $p < 0.05$; **, $p < 0.01$; ***$p < 0.001$; ****, $p < 0.0001$.

polyaminoisoprenyl compound **NV716** is capable of resensitizing *E. coli*, *K. pneumoniae,* and *A. baumannii* to all tested antibiotics whether affected (doxycycline, chloramphenicol, azithromycin, ciprofloxacin, fusidic acid, novobiocin and linezolid) or not (rifampicin) by active efflux [10,52,53], not only in broth, but also intracellularly or in biofilm. In all our experiments, **NV731** and the efflux pump inhibitor PAβN proved less potent.

Considering first the susceptibility data in broth, we observed a remarkable improvement in the intrinsic activity of antibiotics when combined by **NV716**. This synergistic effect is obtained at sub-MIC concentrations of the potentiator, demonstrating it is related to an adjuvant mode of action and not to bacterial killing. Potentiation is observed in all three species (as well as in *P. aeruginosa* [27]), and its extent (in terms of gain of activity, see Fig. 1B) is largely independent of the species, even for antibiotics showing markedly different MICs against these species when used alone (see e.g. rifampicin, chloramphenicol, or doxycycline). Actually, the degree of potentiation offered by **NV716** is depending on the lipophilicity of the antibiotic, as evaluated by its logD value. This is very coherent with the fact that the external leaflet of the outer membrane is known to oppose a barrier to the diffusion of lipophilic compounds, which are also incapable to cross this obstacle via hydrophilic porin channels [54]. LPS barrier effect is mediated by the low fluidity of the layer, the quasi-crystalline, ordered arrangement of its hydrocarbon chains, and the strong intermolecular interaction between LPS heads via cation-mediated bridging [54]. In this context, **NV716**, with a calculated logD of −4.2 at physiological pH (Table S1), shows a strong capacity to interact with LPS, ensuing a subsequent permeabilization of the outer membrane. It is as potent in this respect as the biguanide alexidine, with which it shares an extended conformation of its polyaminated moiety, and, interestingly enough, much more potent than colistin, often used as a potentiator of other drugs against multiresistant Gram-negative bacteria, for its membrane-destabilizing effects [55,56]. As previously discussed [27], **NV731** does not show this extended conformation, which may contribute to explain its lower potency. The capacity of **NV716** to interact with LPS and permeabilize the outer membrane is similar among the three species tested here as well as against *P. aeruginosa.* This indicates the importance of its interaction with the negative charges of the phosphate groups and subsequent displacement of stabilizing divalent cations for its action [27], as this is a common feature of all LPS. Yet, the differences in LPS between the species under study remain modest. As compared to the LPS of *E. coli*, that of *A. baumannii* shows an additional acyl chain at the 2 position containing an additional hydroxyl group [57]; that of *K. pneumoniae* is hexa-acylated with C14 chains in position 2 and 3 (instead of C12 in position 3 for *E. coli*) and possible additional modifications in the lipidic part [58], while that of *P. aeruginosa* PAO1 is hexa- or penta-acetylated (lacking in this case the primary O-linked acyl substitution at position 3) [59].

Beside its destabilizing effect on the outer membrane, **NV716** also inhibits the activity of efflux pumps, but we do not know whether this results from a direct interaction with the pump or is indirectly related to the perturbation in membrane properties it induces. **NV716** can disturb

the inner membrane potential, which could reduce the energy source of the RND pumps [26], but this effect is observed at concentrations higher than those for which potentiation of antibiotic activity is obtained. Moreover, the increase in antibiotic accumulation inside bacteria is also observed for rifampicin, which is a poor substrate for efflux, indicating that the predominant mode of action of **NV716** is related to the perturbation of the integrity of the outer membrane. Importantly, the activity of **NV716** (and the two other potentiators) is observed at concentrations that do not cause cytotoxicity for eukaryotic cells [25], suggesting no or minimal interaction with eukaryotic membranes.

Turning then our attention to intracellular models of infection, we noticed that **NV716** was the most active among the potentiators tested to enhance both the relative potency (lower C_s) and the maximal efficacy (more negative E_{max}) of all antibiotics against all species. Although we did not examine the cellular concentration of **NV716** in human monocytes, these results suggest that it has access to intracellular bacteria in sufficient concentrations to exert its synergistic effect. The lower benefit of PAβN and **NV731** can be attributed to their lower intrinsic effects on MICs (as observed in broth) or possibly to a lower accumulation inside the cells, which has neither been investigated. Also, we can most likely exclude that the improved efficacy is related to interference in the count of the residual CFUs, related either to the release of bacteria out of the cells before lysis or to the remaining amount of antibiotics accumulated in cells if considering that (i) **NV716** did not increase the cytotoxicity of antibiotics, and (ii) charcoal was added to culture plates to adsorb residual antibiotic.

Considering first antibiotic potency, we observed that C_s was close to the respective MIC (1.5–4 x the MIC) in all cases, as previously described in other models of intracellular infection [31,60,61], and independently of the capacity of the drug to accumulate inside monocytes (Table S1) [62,63]. This is interpreted as denoting a poor intracellular bioavailability [61]. Although gain in potency is a pharmacokinetic-driven parameter, meaning that it essentially reflects a change in the antibiotic concentration needed to reach a specified effect (bacteriostatic effect for C_s [61]), it was not related to the lipophilicity of the antibiotic (Fig. S8A). We do not have a simple explanation for this absence of correlation, but wish to emphasize that the system here is more complex. In particular, accumulation of antibiotics in eukaryotic cells is not directly related to the hydrophilic/lipophilic balance, with azithromycin accumulating to much larger extent than ciprofloxacin, for example, while their calculated logD value is similar. In addition, the subcellular localization of antibiotics and bacteria may also play a critical role in this context. Interestingly, however, we notice that (i) for all drugs, the C_s measured in the presence of **NV716** is reduced to a similar value (0.3–1.2-fold the MIC) and (ii) as a corollary, the highest gain in relative potency is obtained for antibiotics that were the less potent when used alone (Fig. S8B).

Considering then antibiotic efficacy, we observed, as in our previous models, that all antibiotics fail to eradicate the infection intracellularly. In the present study, we were limited in the range of antibiotic concentrations we could test because of their intrinsic toxicity at high multiples of their respective MICs, but it remains that the E_{max} or $E_{highest}$ was ranging between reductions of 0.5 (linezolid against *E. coli*) to 2.5 $\log_{10}$ CFUs (ciprofloxacin against all strains), with most of the drugs causing a reduction of 1–2 $\log_{10}$ CFUs. These dissimilarities are probably attributable to differences in the mode of action of the antibiotics, and most conspicuously, to their bacteriostatic/cidal character, the less efficacious being the bacteriostatic drugs linezolid and fusidic acid, and the most efficacious, the highly bactericidal drugs ciprofloxacin and rifampicin. This is very coherent with data assembled for other models of intracellular infections, with linezolid, fusidic acid, and azithromycin showing no or low reductions in CFUs counts against intracellular *Staphylococcus aureus, Staphylococcus epidermidis* or *Listeria monocytogenes* [64–66], and rifampicin being highly effective against intracellular *S. aureus* and its small colony variants [67]. The intracellular activity of fluoroquinolones has been largely demonstrated against a vast array of pathogens like *S. aureus*, *L. monocytogenes*, *L. pneumophila*, *Burkholderia thailandensis*, *Yersinia pseudotuberculosis*, *Francisella philomiragia, or Coxiella burnetii* [31,68–70]. The relatively high efficacy of doxycycline (bacteriostatic in broth) is rather unexpected but has been already reported in models of intracellular infection by *C. burnettii* [70], *Chlamydia trachomatis* [71], or *L. pneumophila* [72], possibly suggesting that the intracellular environment may contribute to improve its killing capacity. As efficacy is a pharmacodynamic-driven parameter reflecting bacterial responsiveness to antibiotics [61], it is not surprising that the gain of efficacy triggered by **NV716** does not correlate with the lipophilicity of the antibiotics (Fig. S8C). But again, it is interesting to note that the mean maximal efficacy reached in combination is very close (−2.2 to −2.4 $\log_{10}$ CFUs) for all drugs except ciprofloxacin, which reached −2.8 $\log_{10}$ CFUs, and that the difference in E_{max} offered by **NV716** is all the more important for poorly effective drugs (Fig. S8D). This suggests that part of the intracellular inoculum is refractory to antibiotic action and that this pool is relatively constant except if using an antibiotic that is already highly efficient by itself like ciprofloxacin. For *S. aureus*, intracellular survivors to antibiotics have been identified as persisters, i.e. phenotypic variants that adopt a dormant, non-replicative, and transiently non-responsive phenotype [19,20]. This is why we examined the effect of **NV716** of persisters selected by ciprofloxacin and found that it was capable of decreasing the proportion of persisters in *E. coli, K. pneumoniae* and *A. baumannii* when combined with ciprofloxacin, but not when used alone, suggesting that it can resensitize persisters to the antibiotic but not kill them [25,49]. Of interest, other molecules capable of disrupting membrane integrity also reduce the residual fraction of persisters upon exposure to antibiotics for different bacterial species [73,74], and one of them (SPI009; 1-[[2, 4-dichlorophenethyl] amino]-3-phenoxypropan-2-ol) was also shown to increase the efficacy of ciprofloxacin against intracellular *P. aeruginosa* [75]. Recently, another membrane-damaging small molecule, JD1, was found capable of reducing the survival of the intracellular Staphylococci and of persister cells in broth, as well as of disrupting biofilms [76].

Lastly, we also examined the effects of **NV716** combined with rifampicin on biofilms, and found that the potentiator can improve the activity of the antibiotic on the biomass and viability of the three Gram-negative bacterial biofilm models. This is consistent with our recent work in *P. aeruginosa* [27], where we also showed that **NV716** at sub-MIC concentrations enhances the penetration of the antibiotics in the deepness of the biofilm, downregulates quorum-sensing regulated processes, and therefore prevents biofilm formation. Thus, its effects partially contrast with those of JD1, which does not prevent biofilm formation and is effective alone against biofilm but only at concentrations higher than its MIC [76], which is also the case for **NV716** against *P. aeruginosa* [27].

We acknowledge some limitations for this work. First, we do not know yet whether the interaction of **NV716** with LPS by itself suffices to explain both the increase in intrinsic activity of antibiotics and their improved activity on persisters, neither how it contributes to the synergy seen intracellularly and in biofilms. Second, we did not measure the concentration of **NV716** in bacteria, monocytic cells or biofilms, as a highly sensitive method should be developed for this purpose.

Nevertheless, it remains that this study highlights a strong potential for this molecule as an adjuvant therapy against difficult-to-treat Gram-negative organisms, demonstrating that it covers the most critical Gram-negative ESKAPE pathogens, including in models of persisting infections. In particular, it allows to bring MICs back to clinically–achievable concentrations for all the disused antibiotics, to confer to most of them (except linezolid and azithromycin) some killing activity against intracellular bacteria at their human C_{max}, and to improve rifampicin activity against biofilms at low multiples of its MICs. Our work therefore position **NV716** as a promising adjuvant that deserves further investigations, notably in in-vivo models of infections.

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Author contributions

W.G. designed and performed the experiments, analyzed the data and wrote the manuscript. J.M.Br. synthesized **NV716**, **NV731**, and **NV1532**, calculated the logD values of antibiotics, and supervised the work. H.R.V. collected and characterized clinical isolates. J.M.Bo supervised the work. F.V.B. designed the study, supervised the work, and wrote the manuscript. All the authors contributed to the writing of the manuscript and approved the submitted version.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.ejmech.2022.114496.

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The polyamino-isoprenyl potentiator NV716 revives disused antibiotics against Gram-negative bacteria in broth, infected monocytes, or biofilms, by disturbing the barrier effect of their outer membrane

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Supplementary material

Table S1. Characteristics of the antibiotics used in the present study.

Antibiotics	**Resistance by efflux**		**LogD values at pH 7.4** (octanol:water partition coefficient at pH 7.4); calculated using LogD Predictor (https://disco.chemaxon.com/calculators/demo/plugins/logd/).	**Accumulation level in eukaryotic cells (at equilibrium)** (1)	**C_{min} / C_{max} in human serum (mg/L)** (2-5)
	Pumps shown to transport the drug (6)	**Bacterial species** (6, 7)			
Rifampicin	Poor substrate for efflux	NA	2.98	1-4	0.1-10
Azithromycin	AcrAB-TolC, AdeABC	*E. coli*, *K. pneumoniae* and *A. baumannii*	-1.50	40-300	0.04-1
Linezolid	AcrAB-TolC	*E. coli*	0.64	1	2.5-15
Fusidic acid	AcrAB-TolC, AdeABC	*E. coli* and *A.baumanni*	1.50	ND	30-200
Novobiocin	AcrAB-TolC	*E. coli*	1.76	ND	15-60
Chloramphenicol	AcrAB-TolC, AdeABC	*E. coli*, *K. pneumoniae* and *A. baumannii*	0.87	2-5	5-15
Doxycycline	AcrAB-TolC, AdeABC	*E. coli*, *K. pneumoniae* and *A. baumannii*	-3.48	2-10	1-10
Ciprofloxacin	AcrAB-TolC, AdeABC	*E. coli*, *K. pneumoniae* and *A. baumannii*	-0.86	4-10	0.05-5

LogD values of the potentiators

	NV716	**NV731**	**PAβN**
LogD (pH 7.4)	-4.2	-6.3	-1.2

Table S2. Characteristics of the clinical isolates isolates used in this study.

Strain	Species	Beta-lactamases identified	Resistance to antibiotics[a]	References
15	*E. coli*	CTX-M 15	CAZ, PIP, CIP	(8)
51	*E. coli*	OXA-1, CTX-M 15	CAZ, PIP, TZP CIP, DOX	
CPE541	*E. coli*	OXA-48	PIP, TZP, CIP	This study
CPE73	*E. coli*	ESBL	CAZ, PIP, TZP, MER, CIP, DOX, GEN	
CPE472	*E. coli*	ESBL	CAZ, PIP, TZP, MER, CIP, DOX, GEN	
CPE144	*E. coli*	ESBL	CAZ, PIP, TZP, MER, CIP, GEN	
CPE493	*E. coli*	ESBL	CAZ, PIP, TZP, MER	
BISC15813	*E. coli*	ESBL	CAZ, PIP	
58	*K. pneumoniae*	TEM-84, SHV-11	CAZ, PIP, TZP, CIP, CST	(8)
74	*K. pneumoniae*	CTX-M 1	CAZ, PIP	
CPE532	*K. pneumoniae*	ESBL	CAZ, PIP, TZP, MER, CIP, GEN	This study
CPE497	*K. pneumoniae*	KPC	CAZ, PIP, TZP, CIP, GEN	
CPE370	*K. pneumoniae*	VIM	CAZ, PIP, TZP, MER, DOX	
101	*K. pneumoniae*	KPC-2	CAZ, PIP, TZP, MER, CIP, GEN	
99	*K. pneumoniae*	KPC-2, OmpK35-, OmpK35red	CAZ, PIP, TZP, MER, CIP, DOX, GEN	
100	*K. pneumoniae*	KPC-2	CAZ, PIP, TZP, MER, CIP, DOX, GEN	
112	*A. baumannii*	OXA-23-24-like (plasmid)+OXA-51-like (chromosomal)	CFPM, TIC, CIP	(8)
113	*A. baumannii*	OXA-23-24-like (plasmid)+OXA-51-like (chromosomal)	CFPM, TIC, MER, CIP	
111	*A. baumannii*	OXA-23-24-like (plasmid)+OXA-51-like (chromosomal)	CFPM, TIC, MER, CIP, DOX, GEN	This study
109	*A. baumannii*	OXA-23-like (plasmid)+OXA-51-like (chromosomal)	CFPM, TIC, MER, CIP, DOX, MIN, GEN	
110	*A. baumannii*	OXA-23-like (plasmid)+OXA-51-like (chromosomal)	CFPM, TIC, CIP, GEN	
NF2147	*A. baumannii*	OXA-23	CFPM, TIC, MER, CIP, DOX, GEN	
NF2137	*A. baumannii*	OXA-23	CFPM, TIC, MER, CIP, DOX, GEN	

Abbreviations: CAZ: ceftazidime; CFPM: cefepime; PIP: piperacillin; TZP: piperacillin+tazobactam (4 mg/L); TIC: ticarcillin; MER: meropenem; CIP: ciprofloxacin; CST: colistin; DOX: doxycycline; MIN: minocycline; GEN: gentamicin.

[a] breakpoints for resistance: EUCAST 2022 for all drugs (except tetracyclines) against *E. coli* and *K. pneumoniae*; CLSI 2020 for all drugs against *A. baumannii* and tetracyclines against all species.

Table S3: MIC of antibiotics alone or combined with potentiators against 1 (*E. coli* and *A. baumannii*) or 2 (*K. pneumoniae*) reference strains and 23 resistant clinical isolates (see Table S2)

Strains	Conditions	MIC (mg/L)[a]								
		Potentiators	**RIF**	**AZI**	**LZD**	**FUS**	**NOV**	**CHL**	**DOX**	**CIP**
E. coli ATCC 47076	AB alone		16	4	512	512	256	8	2	0.016
	+PAβN (38 µM)	>380 µM	**4 (2)**	**1 (2)**	**64 (3)**	**64 (3)**	**32 (3)**	**1 (3)**	**0.5 (2)**	**0.004 (2)**
	+NV731 (38 µM)	>380 µM	**0.5 (5)**	**1 (2)**	**32 (4)**	**64 (3)**	**8 (5)**	**1 (3)**	**0.125 (4)**	**0.004 (2)**
	+NV716 (10 µM)	50 µM	**0.031 (9)**	**0.5 (3)**	**8 (6)**	**2 (8)**	**0.5 (9)**	**0.5 (4)**	**0.031 (6)**	**0.002 (3)**
E. coli 15[b]	AB alone		16	32	512	512	64	4	1	32
	+PAβN (38 µM)	>380 µM	**0.25(6)**	**4 (3)**	**16 (5)**	**32 (4)**	**16 (2)**	**1 (2)**	**0.25 (2)**	16 (1)
	+NV731 (38 µM)	>380 µM	**0.5 (5)**	16 (1)	**64 (3)**	**128 (2)**	**4 (4)**	**1 (2)**	**0.25 (2)**	16 (1)
	+NV716 (10 µM)	50 µM	**0.031 (9)**	**2 (4)**	**4 (7)**	**4 (7)**	**0.25 (8)**	**0.5 (3)**	**0.25 (2)**	**8 (2)**
E. coli 51[b]	AB alone		32	32	512	512	128	8	16	32
	+PAβN (38 µM)	>380 µM	**0.5 (6)**	**4 (3)**	**16 (5)**	**32 (4)**	**16 (3)**	**1 (3)**	**2 (3)**	32 (0)
	+NV731 (38 µM)	>380 µM	**1 (5)**	16 (1)	**64 (3)**	**64 (3)**	**4 (5)**	**1 (3)**	**4 (2)**	32 (0)
	+NV716 (10 µM)	50 µM	**0.031 (10)**	**1 (5)**	**16 (5)**	**1 (9)**	**0.125 (10)**	**0.5 (4)**	**1 (4)**	**8 (2)**
E. coli CPE 541	AB alone		8	2	128	512	128	4	0.25	2
	+PAβN (38 µM)	>380 µM	**0.25 (5)**	**0.5 (2)**	**32 (2)**	256 (1)	**8 (4)**	**1 (2)**	0.125 (1)	2 (0)
	+NV731 (38 µM)	>380 µM	**0.25 (5)**	1 (1)	**16 (3)**	**32 (4)**	**1 (7)**	**1 (2)**	**0.063 (2)**	2 (0)
	+NV716 (10 µM)	50 µM	**0.004 (11)**	**0.125 (4)**	**0.016 (13)**	**0.032 (14)**	**0.016 (13)**	**0.25 (4)**	**0.004 (6)**	1 (1)
E.coli CPE 73	AB alone		16	1024	512	1024	128	256	32	64
	+PAβN (38µM)	>380µM	**1 (4)**	512 (1)	**128 (2)**	**256 (2)**	64 (1)	256 (0)	16 (1)	64 (0)
	+NV731 (38µM)	>380µM	**0.5 (5)**	1024 (0)	**64 (3)**	**64 (4)**	**4 (5)**	256 (0)	16 (1)	64 (0)
	+NV716 (10µM)	50µM	0.008 (11)	**64 (4)**	**8 (6)**	**1 (10)**	**0.125 (10)**	**2 (7)**	**1 (5)**	32 (1)
E. coli CPE 472	AB alone		256	4	512	1024	512	8	64	1
	+PAβN (38 µM)	>380 µM	**64 (2)**	**1 (2)**	**32 (4)**	**256 (2)**	**64 (3)**	**2 (2)**	**16 (2)**	1 (0)
	+NV731 (38 µM)	>380 µM	**8 (5)**	2 (1)	**32 (4)**	**128 (3)**	**4 (7)**	**2 (2)**	**16 (2)**	1 (0)
	+NV716 (10 µM)	50 µM	**0.125 (11)**	**0.125 (5)**	**4 (7)**	**0.5 (11)**	**0.125 (12)**	**0.5 (4)**	**0.5 (7)**	**0.25 (2)**
E. coli CPE 144	AB alone		16	8	1024	64	512	16	1	128
	+PAβN (38 µM)	>380 µM	**0.5 (5)**	**1 (3)**	**64 (4)**	64 (0)	**16 (5)**	**2 (3)**	**0.25 (2)**	128 (0)
	+NV731 (38 µM)	>380 µM	**0.25 (6)**	**2 (2)**	**32 (5)**	32 (1)	**1 (9)**	**2 (3)**	**0.125 (3)**	128 (0)
	+NV716 (10 µM)	50 µM	**0.008 (11)**	**0.016 (9)**	**0.5 (11)**	**0.5 (7)**	**0.016 (15)**	**0.016 (10)**	**0.016 (6)**	64 (1)
E. coli CPE 493	AB alone		8	2	256	1024	128	8	0.5	0.008
	+PAβN (38 µM)	>380 µM	**0.5 (4)**	**0.25 (3)**	**32 (3)**	**128 (3)**	**16 (3)**	**2 (2)**	**0.125 (2)**	0.008 (0)
	+NV731 (38 µM)	>380 µM	**0.125 (6)**	1 (1)	**32 (3)**	**32 (5)**	**2 (6)**	**1 (3)**	**0.125 (2)**	0.008 (0)
	+NV716 (10 µM)	50 µM	**0.008 (10)**	**0.063 (5)**	**0.063 (12)**	**1 (10)**	**0.063 (11)**	**0.031 (8)**	**0.008 (6)**	0.008 (0)
E. coli BISC 15813	AB alone		256	32	512	512	512	8	2	0.016
	+PAβN (38 µM)	>380 µM	256 (0)	**2 (4)**	**16 (5)**	**16 (5)**	**64 (3)**	**2 (2)**	**0.125 (4)**	0.016 (0)
	+NV731 (38 µM)	>380 µM	256 (0)	**8 (2)**	**32 (4)**	**16 (5)**	**8 (6)**	**2 (2)**	**0.125 (4)**	0.008 (1)
	+NV716 (10 µM)	100 µM	**2 (7)**	**0.06 (9)**	**0.25 (11)**	**0.004 (17)**	**0.016 (15)**	**0.06 (7)**	**0.008 (8)**	**0.004 (2)**

K. pneumoniae ATCC 43816	AB alone		32	8	512	512	512	4	2	0.031
	+PAβN (38 μM)	>380 μM	16 (1)	**2 (2)**	**64 (3)**	**128 (2)**	**32 (4)**	**1 (2)**	**0.25 (3)**	0.031 (0)
	+NV731 (38 μM)	>380 μM	**0.25 (7)**	**1 (3)**	**64 (3)**	**128 (2)**	**8 (6)**	**1 (2)**	**0.25 (3)**	0.031 (0)
	+NV716 (10 μM)	100 μM	**0.031 (10)**	**0.5 (4)**	**32 (4)**	**64 (3)**	**1 (9)**	**0.5 (3)**	**0.031 (6)**	0.031 (0)
K. pneumoniae ATCC 700603	AB alone		32	32	512	512	512	64	32	0.5
	+PAβN (38 μM)	>380 μM	16 (1)	16 (1)	512 (0)	512 (0)	**64 (3)**	**8 (3)**	**4 (3)**	0.25 (1)
	+NV731 (38 μM)	>380 μM	**4 (3)**	16 (1)	512 (0)	512 (0)	**64 (3)**	**2 (5)**	**2 (4)**	0.25 (1)
	+NV716 (10 μM)	100 μM	**2 (4)**	**2 (4)**	256 (1)	256 (1)	**16 (5)**	**2 (5)**	**0.5 (6)**	**0.125 (2)**
K. pneumoniae 58[b]	AB alone		32	16	512	512	128	2	2	1
	+PAβN (38 μM)	> 380 μM	**2 (4)**	**0.5 (5)**	**128 (2)**	512 (0)	64 (1)	1 (1)	1 (1)	1 (0)
	+NV731 (38 μM)	>380 μM	**4 (3)**	8 **(4)**	**128 (2)**	512 (0)	**8 (4)**	1 (1)	1 (1)	1 (0)
	+NV716 (10 μM)	50 μM	**0.5 (6)**	**2 (3)**	**64 (3)**	**64 (3)**	**2 (6)**	1 (1)	1 (1)	**0.125 (3)**
K. pneumoniae 74[b]	AB alone		16	16	512	512	128	2	2	0.031
	+E50(38 μM)	>380 μM	**2 (3)**	**1 (4)**	**64 (3)**	512 (0)	**32 (2)**	1 (1)	1 (1)	0.031 (0)
	+NV731 (38 μM)	>380 μM	**2 (3)**	**2 (3)**	**64 (3)**	512 (0)	**8 (4)**	1 (1)	**0.5 (2)**	0.031 (0)
	+NV716 (10 μM)	50 μM	**0.125 (7)**	**1 (4)**	**16 (5)**	**16 (5)**	**2 (6)**	1 (1)	**0.5 (2)**	0.031 (0)
K. pneumoniae CPE 532	AB alone		512	256	512	1024	512	4	2	64
	+PAβN (38 μM)	>380 μM	**128 (2)**	**32 (3)**	**64 (3)**	512 (1)	**64 (3)**	**1 (2)**	**0.5 (2)**	64 (0)
	+NV731 (38 μM)	>380 μM	**32 (4)**	128 (1)	**32 (4)**	**128 (3)**	**2 (8)**	**1 (2)**	**0.25 (3)**	64 (0)
	+NV716 (10 μM)	100 μM	**8 (6)**	128 (1)	**8 (6)**	**8 (7)**	**0.25 (11)**	**0.5 (3)**	**0.125 (4)**	32 (1)
K. pneumoniae CPE 497	AB alone		16	8	512	1024	512	8	2	16
	+PAβN (38 μM)	>380 μM	**1 (4)**	**0.25 (5)**	**128 (2)**	**256 (2)**	**128 (2)**	**2 (2)**	**0.5 (2)**	16 (0)
	+NV731 (38 μM)	>380 μM	**0.25 (6)**	**0.5 (4)**	**64 (3)**	**32 (5)**	**32 (4)**	**2 (2)**	**0.25 (3)**	16 (0)
	+NV716 (10 μM)	50 μM	**0.008 (11)**	**0.03 (8)**	**4 (7)**	**1 (10)**	**0.5 (10)**	**0.5 (4)**	**0.125 (4)**	**4 (2)**
K. pneumoniae CPE 370	AB alone		32	8	512	1024	256	4	32	0.03
	+PAβN (38 μM)	>380 μM	**0.5 (6)**	**0.25 (5)**	**64 (3)**	**256 (2)**	**32 (3)**	**1 (2)**	16 (1)	0.016 (1)
	+NV731 (38 μM)	>380 μM	**1 (5)**	4 (1)	**64 (3)**	**128 (3)**	**2 (7)**	**1 (2)**	16 (1)	0.03 (0)
	+NV716 (10 μM)	50 μM	**0.06 (9)**	**1 (3)**	**32 (4)**	**8 (7)**	**0.25 (10)**	**0.5 (3)**	**4 (3)**	0.03 (0)
K. pneumoniae 101	AB alone		32	64	1025	512	128	512	4	128
	+PAβN (38 μM)	>380 μM	**1 (5)**	**8 (3)**	**128 (3)**	512 (0)	128 (0)	**128 (2)**	**1 (2)**	64 (1)
	+NV731 (38 μM)	>380 μM	**1 (5)**	**16 (2)**	**128 (3)**	256 (1)	**8 (4)**	**128 (2)**	**0.5 (3)**	64 (1)
	+NV716 (10 μM)	100 μM	**0.125 (8)**	32 (1)	**32 (5)**	**16 (5)**	**0.5 (8)**	**32 (4)**	**0.25 (4)**	**32 (2)**
K. pneumoniae 99	AB alone		64	16	1024	1024	1024	512	16	4
	+PAβN (38 μM)	>380 μM	**1 (6)**	**1 (4)**	1024 (0)	1024 (0)	**128 (3)**	256 (1)	**1 (4)**	**1 (2)**
	+NV731 (38 μM)	>380 μM	**0.25 (8)**	**2 (3)**	**256 (2)**	**256 (2)**	**8 (7)**	256 (1)	**0.5 (5)**	**1 (2)**
	+NV716 (10 μM)	50 μM	**0.004 (14)**	**0.125 (7)**	**16 (6)**	**2 (9)**	**0.125 (13)**	**64 (3)**	**0.125 (7)**	**1 (2)**
K. pneumoniae 100	AB alone		128	256	1024	1024	256	256	64	128
	+PAβN (38 μM)	>380 μM	**32 (2)**	**8 (5)**	**128 (3)**	512 (1)	**16 (4)**	128 (1)	**8 (3)**	64 (1)
	+NV731 (38 μM)	>380 μM	**4 (5)**	**64 (2)**	**128 (3)**	**256 (2)**	**2 (7)**	128 (1)	**16 (2)**	64 (1)
	+NV716 (10 μM)	50 μM	**0.125 (10)**	**64 (2)**	**32 (5)**	**32 (5)**	**0.25 (10)**	**0.016 (14)**	**0.25 (8)**	**32 (2)**

A. baumannii ATCC 19606	AB alone		2	16	256	512	16	64	0.125	1
	+PAβN (38 µM)	>380 µM	**0.5 (2)**	8 (1)	**64 (2)**	**32 (4)**	**4 (2)**	32 **(1)**	0.063 (1)	0.5 (1)
	+NV731 (38 µM)	>380 µM	**0.125 (4)**	8 (1)	**32 (3)**	**4 (7)**	**0.25 (6)**	32 **(1)**	0.063 (1)	0.5 (1)
	+NV716 (10 µM)	100 µM	**0.125 (4)**	**4 (2)**	**8 (5)**	**2 (8)**	**0.125 (7)**	**16 (2)**	**0.031 (2)**	**0.25 (2)**
A. baumannii 112[b]	AB alone		2	1	512	256	32	128	0.125	32
	+PAβN (38 µM)	>380 µM	**0.25 (3)**	**0.125 (3)**	**128 (2)**	**16 (4)**	**2 (4)**	64 (1)	0.125 (0)	32 (0)
	+NV731 (38 µM)	>380 µM	**0.125 (4)**	0.5 (1)	**64 (3)**	**8 (5)**	**0.5 (6)**	64 (1)	0.063 (1)	32 (0)
	+NV716 (10 µM)	50 µM	**0.031 (6)**	**0.125 (3)**	**32 (4)**	**2 (7)**	**0.063 (9)**	**16 (3)**	**0.031 (2)**	**8 (2)**
A. baumannii 113[b]	AB alone		2	1	512	128	32	128	0.25	32
	+PAβN (38 µM)	>380 µM	**0.25 (3)**	**0.25 (2)**	**64 (2)**	**16 (3)**	**4 (3)**	64 (1)	0.25 (0)	16(1)
	+NV731 (38 µM)	>380 µM	**0.25 (3)**	0.5 (1)	**64 (2)**	**8 (4)**	**0.5 (6)**	**32 (2)**	0.25 (0)	16(1)
	+NV716 (10 µM)	100 µM	**0.016 (7)**	**0.25 (2)**	**32 (3)**	**1 (7)**	**0.063 (9)**	**8 (4)**	**0.063 (2)**	**8 (2)**
A. baumannii 111	AB alone		4	32	512	256	128	128	64	64
	+PAβN (38 µM)	>380 µM	**0.25 (4)**	**8 (2)**	**64 (3)**	**8 (5)**	**4 (5)**	64 (1)	64 (0)	64 (0)
	+NV731 (38 µM)	>380 µM	**0.25 (4)**	**8 (2)**	**64 (3)**	**4 (6)**	**0.25 (9)**	64 (1)	32 (1)	64 (0)
	+NV716 (10 µM)	50 µM	**0.008 (9)**	**8 (2)**	**16 (5)**	**0.125 (11)**	**0.032 (12)**	**16 (3)**	**4 (4)**	**32 (1)**
A. baumannii 109	AB alone		4	512	512	256	32	128	64	32
	+PAβN (38 µM)	>380 µM	**0.25 (4)**	**32 (4)**	**32 (4)**	**4 (6)**	**4 (3)**	**32 (2)**	**16 (2)**	16 (1)
	+NV731 (38 µM)	>380 µM	**0.125 (5)**	**64 (3)**	**64 (3)**	**2 (7)**	**0.5 (6)**	**32 (2)**	**16 (2)**	16 (1)
	+NV716 (10 µM)	50 µM	**0.004 (10)**	**4 (7)**	**4 (7)**	**0.016 (14)**	**0.016 (11)**	**0.25 (9)**	**0.5 (7)**	16 (1)
A. baumannii 110	AB alone		4	2	1024	256	64	512	0.125	64
	+PAβN (38 µM)	>380 µM	**0.25 (4)**	**0.125 (4)**	**128 (3)**	**16 (4)**	**4 (4)**	**128 (2)**	0.125 (0)	32 (1)
	+NV731 (38 µM)	>380 µM	**0.06 (6)**	**0.5 (2)**	**64 (4)**	**4 (6)**	**0.5 (7)**	**128 (2)**	0.063 (1)	64 (0)
	+NV716 (10 µM)	50 µM	**0.004 (10)**	**0.125 (4)**	**8 (7)**	**0.125 (11)**	**8 (3)**	**1 (9)**	**0.032 (2)**	64 (0)
A. baumannii NF 2147	AB alone		64	1024	1024	256	128	256	64	128
	+PAβN (38 µM)	>380 µM	**8 (3)**	1024 (0)	**128 (3)**	**16 (4)**	**16 (3)**	128 (1)	**16 (2)**	128 (0)
	+NV731 (38 µM)	>380 µM	**4 (4)**	1024 (0)	**128 (3)**	**8 (5)**	**2 (6)**	128 (1)	64 (0)	128 (0)
	+NV716 (10 µM)	100 µM	**0.06 (10)**	**256 (2)**	**1 (10)**	**1 (8)**	**0.5 (8)**	**4 (6)**	**1 (6)**	**32 (2)**
A. baumannii NF 2137	AB alone		2	8	256	128	64	64	32	32
	+PAβN (38 µM)	>380 µM	**0.25 (3)**	**2 (2)**	**64 (2)**	**8 (4)**	**8 (3)**	64 (0)	32 (0)	32 (0)
	+NV731 (38 µM)	>380 µM	**0.125 (4)**	8 (0)	**32 (3)**	**2 (6)**	**1 (6)**	32 (1)	32 (0)	32 (0)
	+NV716 (10 µM)	50 µM	**0.008 (8)**	**0.5 (4)**	**1 (8)**	**0.5 (8)**	**0.125 (9)**	**0.016 (12)**	**0.016 (11)**	**8 (2)**

[a] values in bold highlight a significant decrease in MIC (at least 2 doubling dilutions); values in brackets: number of doubling dilution decrease (log2 fold change) for MIC of antibiotic combined with potentiator compared with antibiotic alone.

[b] clinical isolates used to study activity in infected cells and biofilms

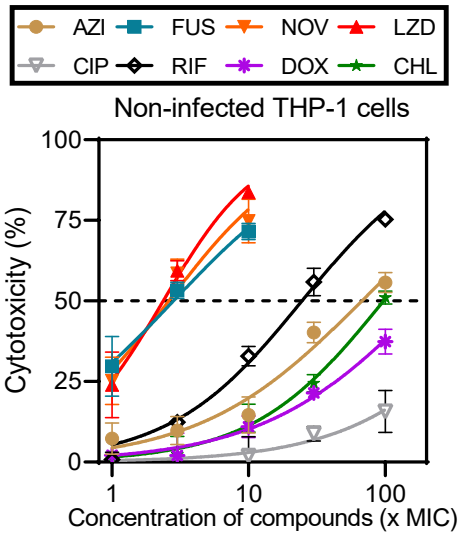
Table S4. IC_{50} values from interaction of potentiators with Gram-negative bacterial membrane. IC50 values were calculated based on the Hill equation of concentration-response curves in BC displacement, OM permeability, PI accumulation and NPN efflux assays. All data are shown as means (triplicates from 3 experiments).

Experimental conditions	Strains	IC_{50} (μM)				
		ALE	CST	PAβN	NV731	NV716
BC displacement	*E. coli ATCC 47076*	5.95	>100	>100	55.12	5.35
	K. pneumoniae ATCC 43816	6.63	>100	>100	64.05	5.55
	K. pneumoniae ATCC 700603	5.50	>100	>100	47.99	4.03
	A.baumannii ATCC 19606	6.39	>100	>100	58.52	5.23
OM permeability	*E. coli ATCC 47076*	4.18	10.14	42.13	29.57	7.59
	K. pneumoniae ATCC 43816	4.19	12.06	53.27	36.66	11.36
	K. pneumoniae ATCC 700603	3.03	7.86	36.25	23.07	5.97
	A.baumannii ATCC 19606	3.90	9.90	48.52	42.47	8.31
IM permeability	*E. coli ATCC 47076*	2.08	2.75	>100	>100	66.72
	K. pneumoniae ATCC 43816	1.78	2.32	>100	>100	51.30
	K. pneumoniae ATCC 700603	1.70	2.84	>100	>100	63.01
	A.baumannii ATCC 19606	1.43	1.48	>100	>100	46.97
Efflux inhibition	*E. coli ATCC 47076*	73.71	98.50	204.63	83.69	18.90
	K. pneumoniae ATCC 43816	54.68	102.05	262.60	85.79	15.45
	K. pneumoniae ATCC 700603	24.13	116.40	390.00	233.30	12.71
	A.baumannii ATCC 19606	32.30	68.86	118.90	120.10	13.91

Table S5. IC_{50} values from cytotoxicity tests, for antibiotics alone or combined with potentiators, and expressed in different units (x MIC, mg/L, µM [MIC are vs. *E. coli* ATCC 47076]). The graph above the Table show the concentration-response curves for seven antibiotics alone (rifampicin [RIF], azithromycin [AZI], linezolid [LZD], fusidic acid [FUS], novobiocin [NOV], chloramphenicol [CHL] and doxycycline [DOX]) at the indicated concentrations in non-infected THP-1 cells. The dotted horizontal line shows the 50 % cytotoxicity (IC_{50}).

IC_{50} values shown in the Table were calculated based on the Hill equation of concentration-response curves in non-infected THP-1 cells or THP-1 cells infected with *E. coli* ATCC 47076 based on experiments similar to that shown in the graph.

All data are shown as means ± SEM (triplicates from 3 experiments). Statistical analysis (1-way ANOVA; Tukey's Multiple Comparison Test): no significant difference was noticed when comparing the different conditions for each antibiotic ($p>0.05$).



Antibiotic	MIC (mg/L)	units	IC_{50}							
			non-infected THP-1				infected THP-1			
			AB alone	+PAβN (38 µM)	+NV731 (10 µM)	+NV716 (10 µM)	AB alone	+PAβN (38 µM)	+NV731 (10 µM)	+NV716 (10 µM)
Azithromycin	4	xMIC	65.3±4.1	68.1±11.4	64.3±8.8	64.7±3.5	66.5±3.9	64.7±2.4	65.3±1.8	73.1±3.6
		mg/L	261.4±16.3	272.3±45.7	257.4±35.4	258.7±13.9	266.1±15.6	258.9±9.5	261.0±7.0	292.6±14.4
		µM	349.0±21.7	363.6±61.0	343.6±47.2	345.4±18.6	355.3±20.8	345.7±12.7	348.5±9.4	390.6±19.3
Fusidic acid	256	xMIC	2.8±0.3	2.9±0.1	2.8±0.1	2.8±0.3	2.8±0.5	2.7±0.2	2.7±0.1	2.8±0.0
		mg/L	712.5±65.6	746.8±34.0	726.3±29.2	717.6±76.4	725.2±116.1	685.1±53.7	680.1±37.2	710.7±10.3
		µM	1378.2±126.8	1444.6±65.8	1404.8±56.4	1387.9±147.8	1402.8±224.5	1325.1±103.8	1315.5±72.0	1374.6±19.9
Linezolid	256	xMIC	2.3±0.1	2.4±0.1	2.3±0.1	2.5±0.0	2.4±0.1	2.4±0.1	2.5±0.1	2.4±0.1
		mg/L	596.0±37.4	611.0±16.7	579.1±26.5	633.9±8.1	625.5±21.6	610.6±18.4	634.4±17.4	614.6±21.7
		µM	1768.5±111.1	1813.0±49.7	1718.3±78.7	1880.9±24.1	1856.1±64.1	1812.0±54.5	1882.4±51.7	1823.7±64.5
Novobiocin	256	xMIC	2.6±0.3	2.6±0.1	2.5±0.2	2.5±0.0	3.9±0.6	3.5±0.1	3.7±0.2	3.6±0.2
		mg/L	670.8±75.7	676.9±28.0	632.9±47.3	635.7±1.2	993.1±141.8	907.9±19.6	938.0±50.6	924.2±50.5
		µM	1094.3±123.6	1104.2±45.7	1032.5±77.2	1037.1±2.0	1620.1±231.4	1481.0±32.0	1530.2±82.5	1507.6±82.3
Rifampicin	16	x MIC	24.6±1.1	27.3±3.3	23.1±2.6	22.0±0.9	27.8±1.9	26.6±0.2	31.9±2.9	28.7±3.4
		mg/L	393.0±18.3	436.1±52.6	369.3±41.5	351.6±14.2	445.5±30.2	426.2±2.8	510.0±47.1	459.0±55.1
		µM	477.5±22.3	529.9±63.9	448.8±50.5	427.2±17.3	541.4±36.7	517.9±3.4	619.7±57.3	557.8±66.9
Chloramphenicol	8	x MIC	89.9±3.2	84.1±3.4	80.3±2.0	78.1±18.8	89.3±10.0	80.7±11.9	94.1±12.3	88.4±14.8
		mg/L	719.1±25.3	673.2±27.0	642.0±16.2	624.4±150.2	714.1±80.4	645.9±95.0	752.6±98.4	707.0±118.6
		µM	2225.6±78.4	2083.3±83.7	1986.9±50.1	1932.4±464.8	2210.0±248.8	1998.8±294.1	2329.2±304.4	2187.9±366.9

Table S6. Gain in potency (expressed as the Log_2 fold dilution decrease in C_s between antibiotics alone (rifampicin [RIF], azithromycin [AZI], linezolid [LZD], fusidic acid [FUS], novobiocin [NOV], chloramphenicol [CHL], doxycycline [DOX], or ciprofloxacin [CIP]) or combined with potentiators, and in maximal efficacy (expressed as the difference in E_{max} between combinations and antibiotics alone) against intracellular *E.coli, K. pneumoniae* and *A. baumannii.*

Strains	ABs	Log_2 fold dilution decrease in C_s (ABs alone/Combinations)			Change of E_{max} /$E_{highest}$ (Combinations-ABs alone)		
		+NV716	+PAβN	+NV731	+NV716	+PAβN	+NV731
E. coli	RIF	2	0	2	-0.8	-0.3	-0.5
	AZI	2	1	0	-1.0	-0.2	-0.5
	LZD	3	1	2	-1.2	-0.1	-0.4
	FUS	4	0	3	-1.7	-0.2	-1.3
	NOV	4	1	3	-1.3	-0.2	-0.8
	CHL	1	1	1	-1.1	-0.1	-0.2
	DOX	2	0	0	-0.7	-0.1	-0.2
	CIP	1	0	1	-0.6	0.1	-0.1
K. pneumoniae	RIF	3	1	2	-0.9	-0.5	-0.4
	AZI	3	1	1	-0.9	-0.5	-0.4
	LZD	2	1	2	-1.2	-0.4	-0.6
	FUS	1	0	1	-0.7	-0.1	-0.4
	NOV	3	1	2	-0.8	-0.2	-0.3
	CHL	1	0	1	-0.6	-0.2	-0.5
	DOX	2	0	1	-0.6	0.0	-0.3
	CIP	1	0	0	-0.7	0.0	-0.1
A. baumannii	RIF	1	1	1	-1.0	-0.4	-0.6
	AZI	2	1	1	-1.0	-0.2	-0.3
	LZD	2	1	1	-1.1	-0.3	-0.7
	FUS	4	2	2	-1.1	-0.6	-0.8
	NOV	3	1	2	-1.0	-0.1	-0.4
	CHL	3	1	2	-1.1	-0.3	-1.0
	DOX	2	0	1	-0.6	0.1	-0.2
	CIP	1	0	1	-1.0	0.0	-0.2

Figure S1. structure of the potentiators tested. Aminated functions protonable at physiological pH are highlighted in blue squares.

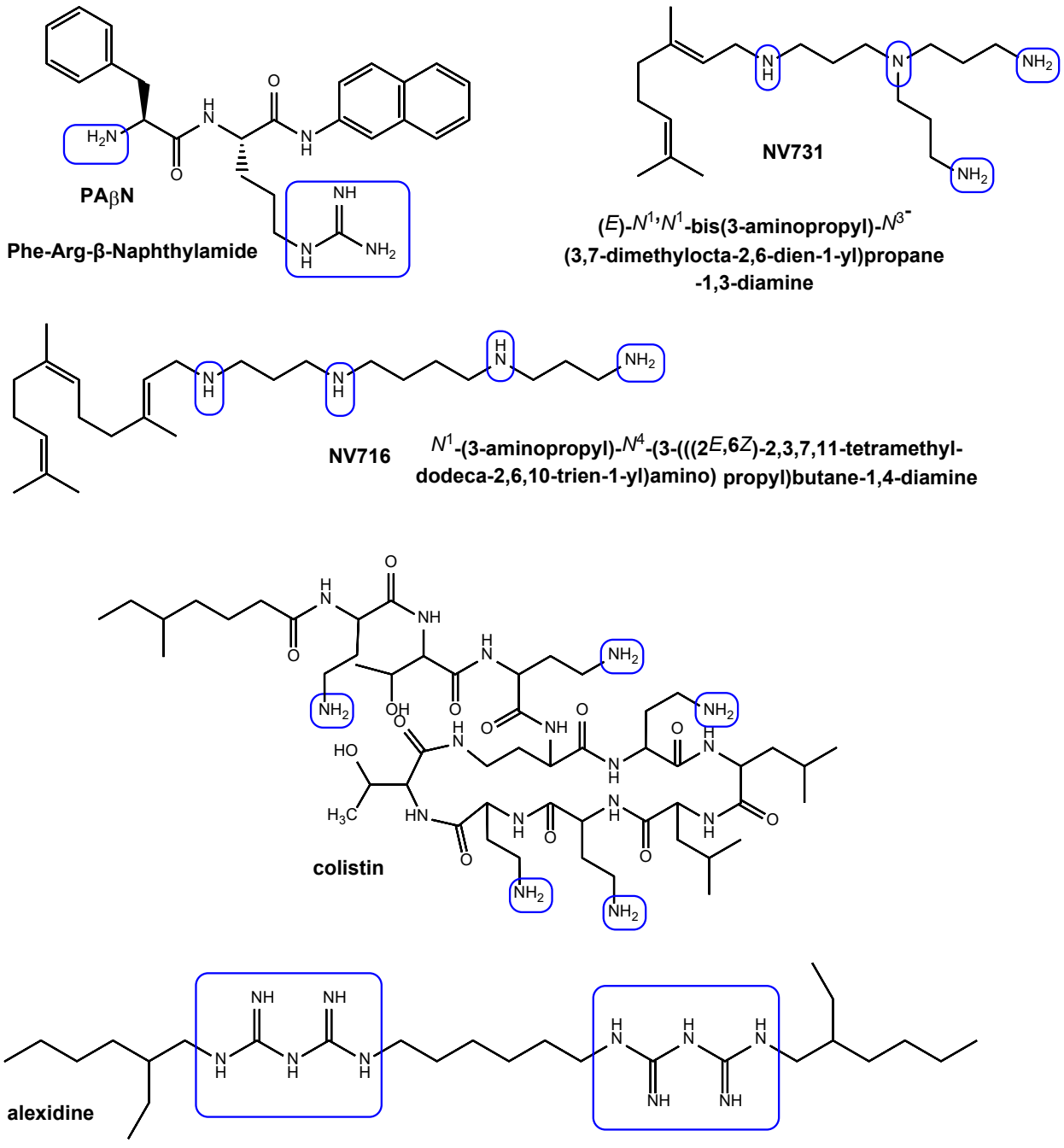


Figure S2. Relationship between the OD_{620nm} and the Log_{10} CFU/mL of the 1 (*A. baumannii* and *E. coli*) or 2 (*K. pneumoniae*) reference strains suspension for the three bacterial species in CA-MHB.

Bacteria (at an OD_{620nm} of 0.05) were grown in CA-MHB and incubated overnight with 130 rpm. Aliquots were diluted in PBS and then used to determine optical density and CFU counts. Correlation curves plotting $OD_{620\ nm}$ and Log_{10} CFU/mL were used to adjust the inocula in subsequently intracellular-infection experiments. All data are mean (triplicate from two independent experiments).

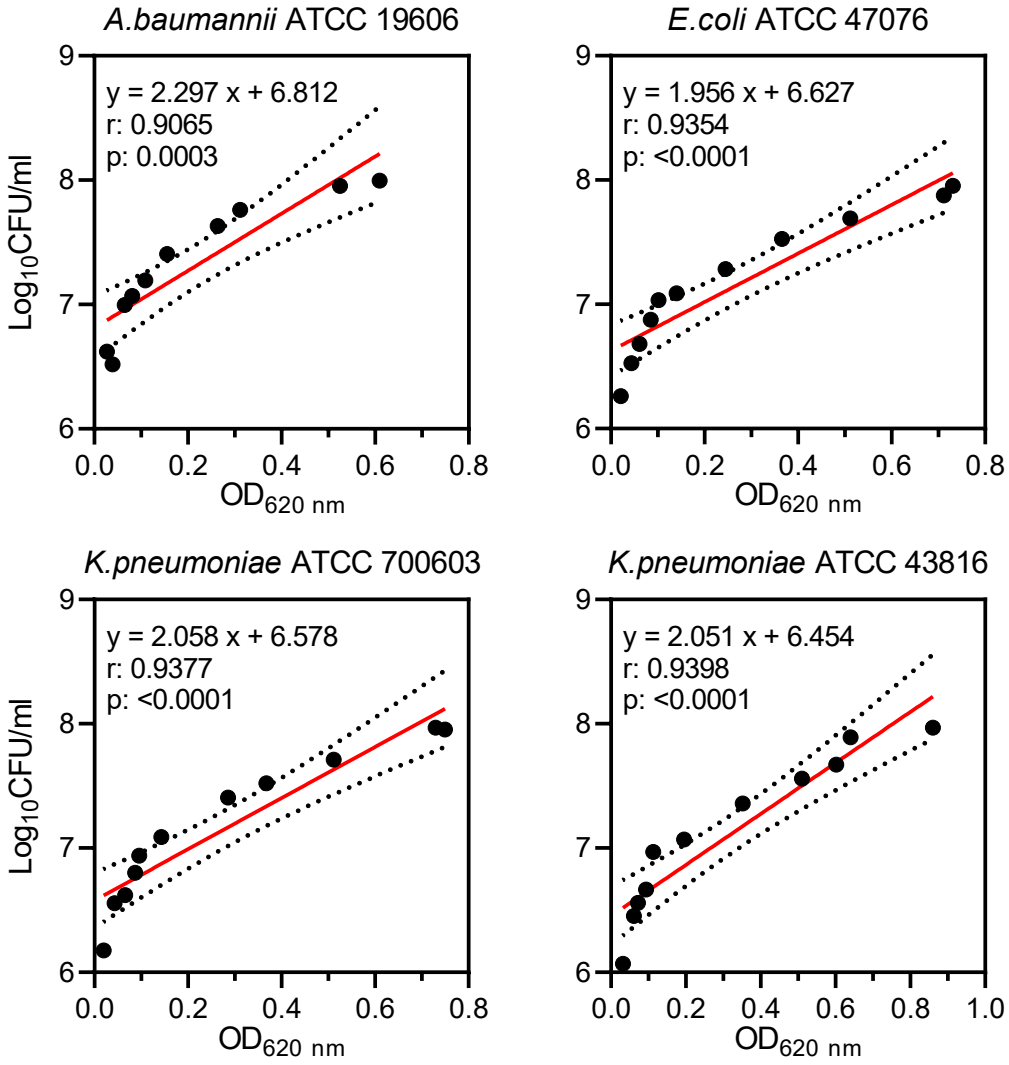


Figure S3. Development of the intracellular models for 1 (*A. baumannii* and *E. coli*) or 2 (*K. pneumoniae*) reference strains. The graphs show the number of CFUs normalized to the mg proteins in the samples after 1 h (open bars with red border) or 2 h (open bars with blue border) at increasing bacteria-to-cell ratios (left axis) and the percentage of mortality of THP-1 cells as determined at the end of the phagocytosis period (right axis; red line and symbols: 1h phagocytosis; blue line and symbols: 2h phagocytosis). The horizontal dotted line shows the inoculum targeted for intracellular experiments (7.5 x 10^5 CFUs/mg). All data are means ± SEM (triplicate from three independent experiments). Based on these experiments, we selected a bacteria/cell ratio of 25 and 1 h of phagocytosis to reach the target inoculum while maintaining adequate cell viability.

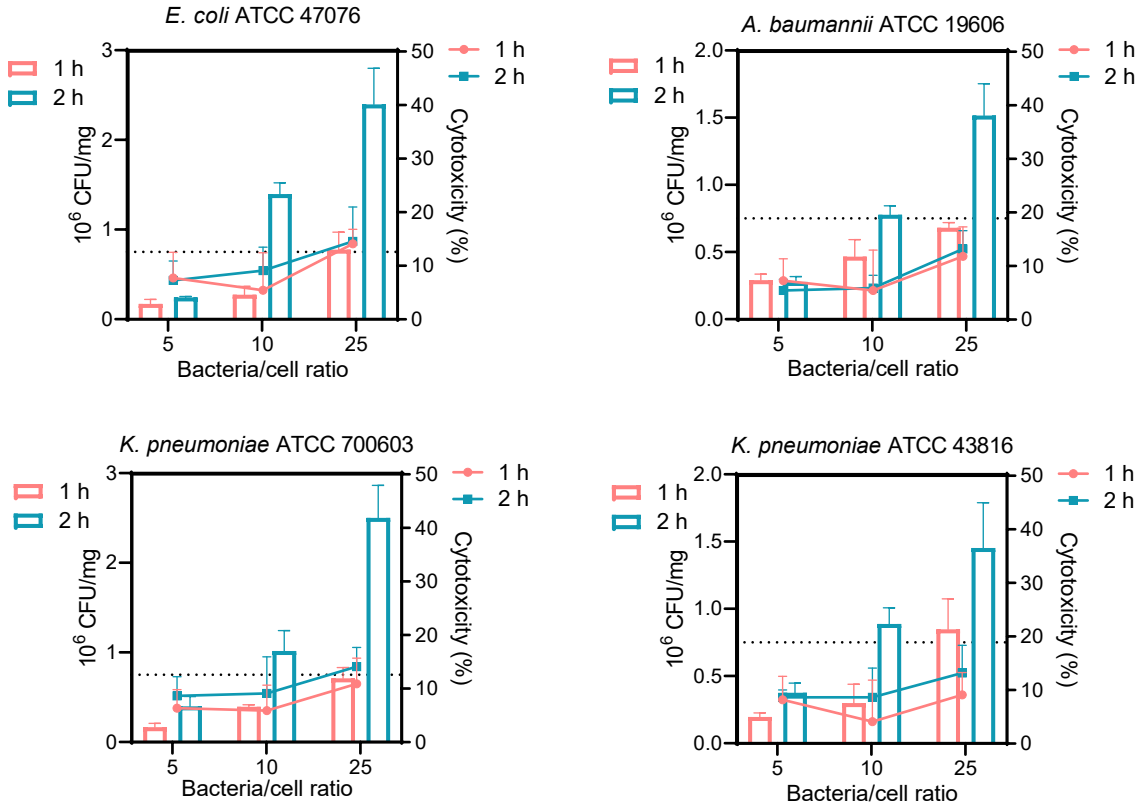


Figure S4. Heat maps describing interactions between antibiotics and potentiators against 1 (*E. coli* and *A. baumannii*) or 2 (*K. pneumoniae*) reference strains and 2 selected to clinical isolates of each species (i.e. those tested in the intracellular and biofilm models). FIC index was calculated for concentrations of 38 µM PAβN, 38 µM **NV731** and 10 µM **NV716**. Synergy is defined as FIC < 0.5 (appearing in green on the graphs). All data are mean from at least two independent experiments.

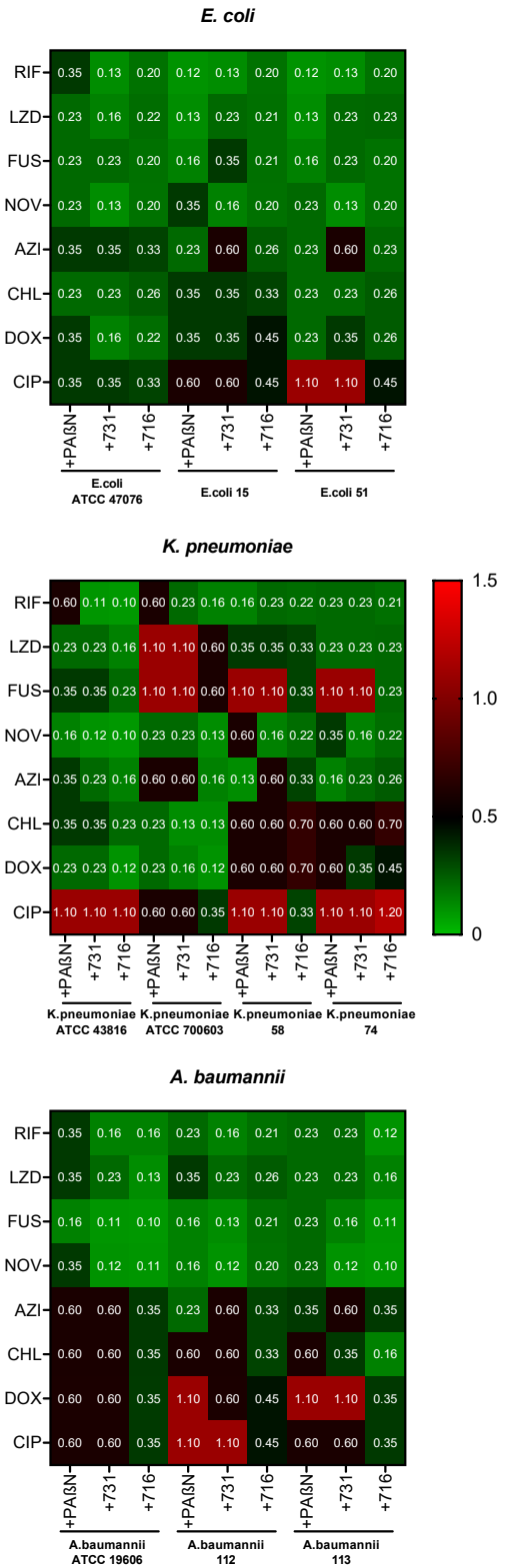


Figure S5. Effect of alexidine, colistin, and **NV716** each at a concentration of 1 x MIC (See Table S1) on inner membrane permeability as assessed by measuring the fluorescence of propidium iodide (PI) after 1 h of incubation with 1 (*A. baumannii* and *E. coli*) or 2 (*K. pneumoniae*) reference strains. The effect measured in the presence of 50 µM alexidine after 1 h was taken as 100% (positive control). All data are means ± SEM (triplicates from 3 independent experiments). Statistical analysis: two-way ANOVA with Tukey post-hoc test: ****, p<0.0001; ***, p< 0.001.

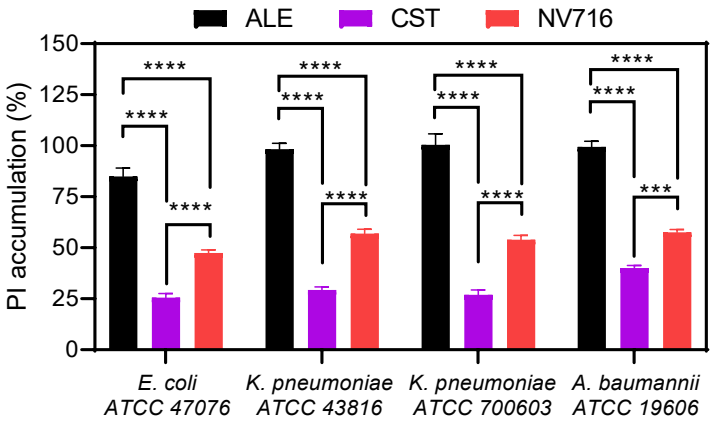


Figure S6. Inner membrane depolarization caused by **NV716** at two concentrations, as assessed by measuring the DiSC3(5) fluorescence after 15 min incubation with 1 (*A. baumannii and E. coli*) or 2 (*K. pneumoniae*) reference strains. The effect measured with 0.5% (w/v) SDS after 15 min was taken as 100% (positive control). All data are means ± SEM (triplicates from 3 independent experiments). Statistical analysis: two-way ANOVA with Tukey post-hoc test: data series with different letters are different from one another ($p < 0.05$).

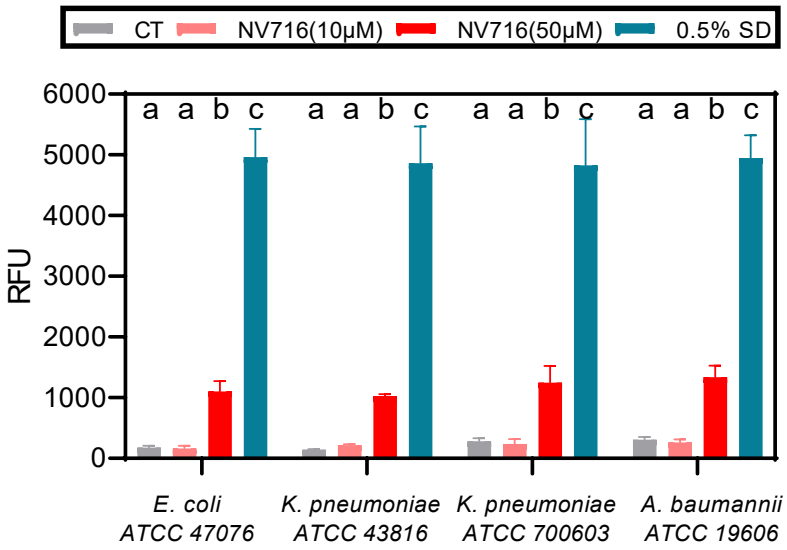


Figure S7.

Effect of potentiators on the intracellular activity of antibiotics (rifampicin [RIF], azithromycin [AZI], linezolid [LZD], fusidic acid [FUS], novobiocin [NOV], chloramphenicol [CHL], doxycycline [DOX], or ciprofloxacin [CIP]) against 1 (*E. coli; A. baumannii*) or 2 (*K. pneumoniae*) reference strains and 2 clinical isolates for each species at C_{min} (A) and C_{max} (B); see Table S1 for the corresponding values.

Horizontal bars: mean; horizontal line: static effect. Grey: antibiotics alone; Green: combined 38 µM PAβN; Blue: combined 38 µM **NV731**; Red: combined 10 µM **NV716**.

Statistical analysis: one-way ANOVA with Dunnett post-hoc test (paired): *, p<0.05; **, p < 0.01; ***, p<0.001.

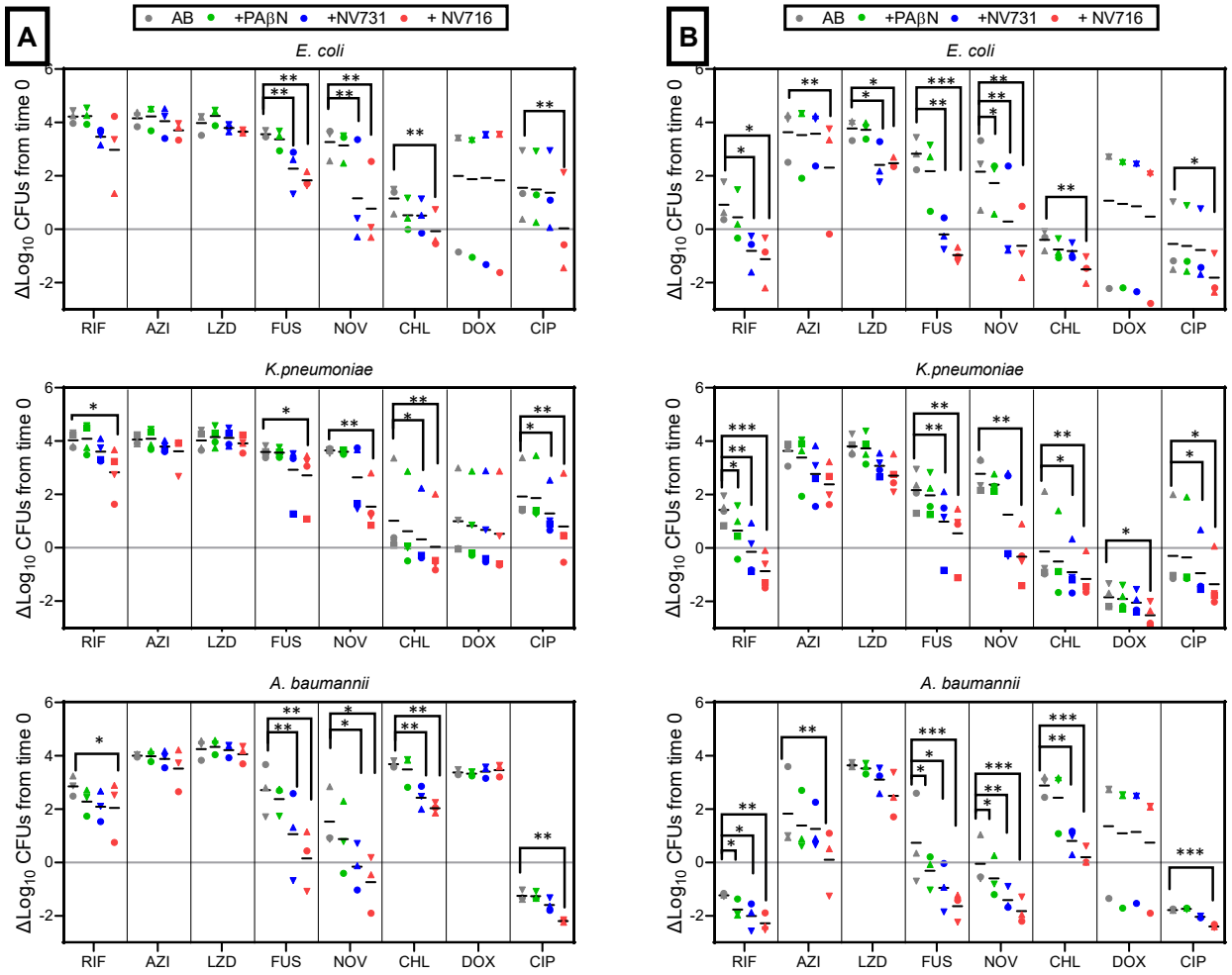
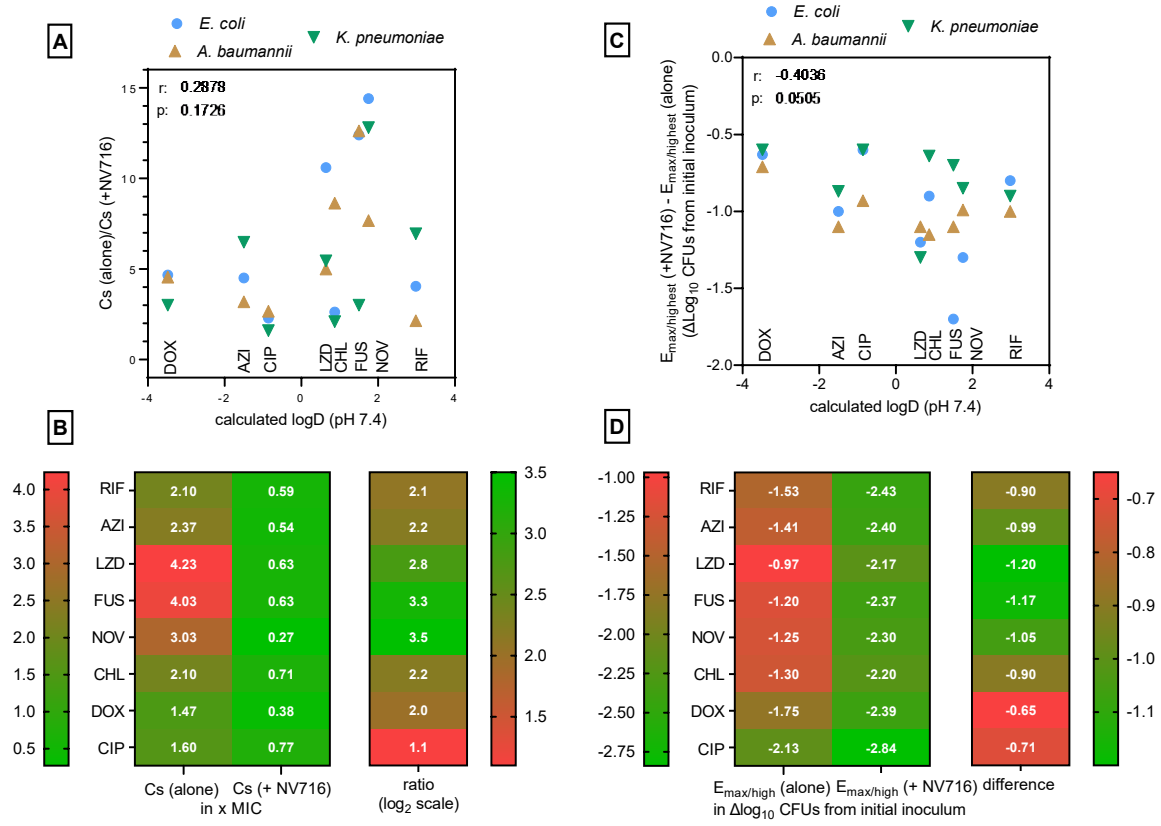


Figure S8. Top: correlation between the gain in potency (A) or in efficacy (C) for antibiotics when used alone or combined with **NV716** in the intracellular model and the logD values of antibiotics (See Table S1). Data are expressed as the ration between the C_s calculated for the antibiotic alone or combined with **NV716** (A) and as the difference between the E_{max} (or $E_{highest}$) for the combination and the antibiotic alone (C). The Pearson's correlation coefficient r and the p value (t-tailed) are shown on the graphs. **Bottom:** heat maps showing the mean C_s (B) or $E_{max/highest}$ (D) for antibiotics alone or combined with NV716 for the three bacterial species together as well as the gain in potency observed in combination (ratio of C_s or difference in $E_{max/highest}$). For drugs alone or combined with **NV716** (+ NV716), the values shown are the mean of the Cs or $E_{max/highest}$ values calculated for the three species; the C_s ratio is the mean of the C_s ratio calculated for each species expressed in a $\log_2$ scale; the difference in $E_{max/highest}$ is the mean of the difference calculated for each species. Color code: red, lowest potency/efficacy or gain for this parameter; green, potency/efficacy or gain for this parameter.



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