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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 to 12 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.

Keywords: ESKAPE pathogen, potentiator, outer membrane, efflux, intracellular infection, biofilm

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## 1 Introduction

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

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

16 Additionally, these Gram-negative bacteria also constitutively express efflux systems that expel  
17 unrelated classes of antibiotics out of the bacteria, reducing thereby the amount of drug that can  
18 reach intracellular targets (9). These efflux pumps consist of three proteins, namely an efflux  
19 transporter located in the IM, an OM channel protein, and a fusion protein connecting the two  
20 transmembrane proteins to allow the transport of the antibiotic substrates directly to the  
21 extracellular environment. Typical examples include AcrAB-TolC in *E. coli* and *K. pneumoniae* or  
22 AdeABC in *A. baumannii*, which can extrude structurally distinct classes of antibiotics such as  
23 quinolones, tetracyclines, chloramphenicol, macrolides, linezolid, fusidic acid, or novobiocin (10).

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

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

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

45 In a previous work, we showed that the polyaminoisoprenyl compound **NV716** (see chemical  
46 structure in [Figure S1](#)) was capable to restore the activity of a series of disused antibiotics  
47 (rifampicin, doxycycline and chloramphenicol) against *Pseudomonas aeruginosa* both  
48 extracellularly and intracellularly (25), by increasing the permeability of the outer membrane,  
49 inhibiting efflux, and also improving the efficacy of bactericidal drugs against persisters (25-27)  
50 at concentrations that are not toxic for eukaryotic cells. The aim of the present study was (a) to  
51 extend the demonstration of the potential interest of this compound as potentiator of a series of  
52 disused antibiotics against planktonic, intracellular, or biofilm infections by other problematic  
53 Gram-negative pathogens like *A. baumannii*, *E. coli* and *K. pneumoniae*, in comparison with  
54 **NV731** (less active derivative against *P. aeruginosa*) and the well-characterized efflux inhibitor

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55 PA $\beta$ N (Phenylalanine-Arginine  $\beta$ -Naphthylamide) (28-30) and (b) to evaluate its effects on the  
56 membranes of these bacteria.

57 We adapted to these species an in-vitro pharmacodynamic model of THP-1 human monocytes  
58 infected by *Pseudomonas aeruginosa* (31) that allows comparing key pharmacodynamic  
59 descriptors of antibiotic intracellular activity, namely their relative potency (static concentration,  
60  $C_s$ ) and maximal efficacy ( $E_{max}$ ), as well as an in-vitro model of biofilm growing in 96-well plates  
61 (32). As disused antibiotics, we selected rifampicin (not substrate for efflux), doxycycline,  
62 chloramphenicol as in our previous work with *P. aeruginosa*, and added antibiotics the spectrum  
63 of which is limited to Gram-positive bacteria due to poor penetration in Gram-negative organisms,  
64 like azithromycin, linezolid, fusidic acid and novobiocin (all substrates for efflux; see [Table S1](#)).  
65 Ciprofloxacin was used as a control of active drug against intracellular *P. aeruginosa* (25). As  
66 positive controls for mechanistic studies, we included colistin and alexidine (see [Figure S1](#) for the  
67 structure of these compounds). Colistin binds to the lipid A component of the negatively-charged  
68 LPS molecules via electrostatic interactions, by displacing the  $Mg^{2+}$  and  $Ca^{2+}$  ions that bridge and  
69 stabilize the LPS monolayer. It perturbs thereby the OM permeability, transits through it via a  
70 self-promoted uptake mechanism, and subsequently inserts itself and disrupts the physical  
71 integrity of the IM via membrane thinning (33). Alexidine is an amphipathic biguanide antiseptic  
72 showing fast bactericidal activity thanks to its rapid ability to permeabilize the bacterial  
73 membranes (34). As colistin, it establishes electrostatic interactions with the negatively-charged  
74 phosphate groups present on LPS, displacing  $Mg^{2+}$  ions from their binding to LPS and perturbing  
75 the stabilizing effect afforded by  $Mg^{2+}$  cross-bridging of adjacent LPS molecules (35). In addition,  
76 it also causes the leakage of the cytoplasmic content by inducing the formation of lipid domains  
77 in the IM (36).

78 Our data show that **NV716**, contrarily to **NV731** and PA $\beta$ N, was capable of increasing the relative  
79 potency and maximal efficacy of all tested antibiotics against the three species of intracellular  
80 Gram-negative bacteria. It also improved the activity of rifampicin against biofilms of these three  
81 species. These effects were related to drastic reductions in MIC and persister fractions.  
82 Mechanistic studies showed that **NV716** inhibits the activity of efflux pumps and disturbs the

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83 permeability of the OM, allowing for an increased accumulation of antibiotics inside bacteria.  
84 Collectively, these data suggest that **NV716** could be a useful adjuvant to revive disused  
85 antibiotics against extracellular and intracellular infections as well as against biofilms caused by  
86 difficult-to-treat Gram-negative bacteria.

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## 87 **Materials and Methods**

### 88 **Antibiotics and potentiators**

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

### 98 **Bacteria and culture media**

99 For each species, one (*E. coli* ATCC 47076, *A. baumannii* ATCC 19606) or two (*K. pneumoniae*  
100 ATCC 700603 and ATCC 43816) reference strains were obtained from the American Type Culture  
101 Collection (Manassas, VA, USA). **Twenty-three clinical Gram-negative isolates from the same**  
102 **three species were also included for specific experiments. Twelve** clinical isolates (*E. coli* 15 and  
103 51; *K. pneumoniae* 58, 74, **99, 100, and 101**; and *A. baumannii* **109, 110, 111, 112** and 113) were  
104 provided by Johan W. Mouton, Department of Medical Microbiology and Infectious Diseases,  
105 Erasmus MC, Rotterdam, The Netherlands (38); **eleven clinical isolates (*E. coli* CPE541, 493, 472,**  
106 **144, and BISC15813; *K. pneumoniae* CPE73, 497, 370 and 532; *A. baumannii* NF2147 and 2137)**  
107 **were provided by one of us (HRV).** All isolates harbored CTX-M-, SHV-, OXA-, **KPC-, VIM-** or TEM-  
108 type extended-spectrum  $\beta$ -lactamases **and also showed resistance to many other drugs used in**  
109 **the clinics to treat infection by Gram-negative pathogens (Table S2).**

110 All bacteria were grown on trypticase soy agar (BD Life Sciences, Franklin Lakes, NJ, USA) and  
111 incubated at 37°C overnight. A single colony was then inoculated in 10 mL cation-adjusted  
112 Mueller-Hinton Broth (CA-MHB; BD Life Sciences, Franklin Lakes, NJ) and incubated at 37°C  
113 overnight under gentle agitation (130 rpm). TSA supplemented with **2 g/L** charcoal (Sigma-Aldrich,



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114 St Louis, MO) was used for colony-forming unit (CFU) counting. Cell culture media (RPMI-1640),  
115 human and fetal bovine sera were from Gibco/ThermoFisher Scientific (Waltham, MA, USA).

### 116 **Susceptibility Testing**

117 MICs were determined by serial 2-fold microdilution in CA-MHB according to CLSI guidelines in  
118 control conditions or in the presence of 38  $\mu\text{M}$  PA $\beta\text{N}$  [20 mg/L] and **NV731** [12 mg/L] or 10  $\mu\text{M}$   
119 **NV716** [4 mg/L]. MICs of antibiotics alone and in combination were used to calculate the  
120 fractional inhibitory concentration (FIC) index, as follows:  $\text{FIC} = C_A/\text{MIC}_A + C_B/\text{MIC}_B$ , where  $\text{MIC}_A$   
121 and  $\text{MIC}_B$  are the MICs of compounds A and B alone, and  $C_A$  and  $C_B$  are the MIC of compounds A  
122 or B in combination. The combination was considered as synergistic or antagonistic when the FIC  
123 index was  $< 0.5$  or  $> 4$ , respectively (39).

### 124 **BODIPY<sup>TM</sup> TR cadaverine displacement assay**

125 Binding to lipopolysaccharides (LPS) of bacteria was investigated by using BODIPY<sup>TM</sup>-TR-  
126 cadaverine displacement assay (40). BODIPY<sup>TM</sup> TR cadaverine (BC) can bind to the lipid A region  
127 of the LPS. The ability of compounds to interact with LPS was evaluated by measuring the  
128 displacement of the BC as previously described (41). Ten milliliters of bacterial suspension  
129 ( $\text{OD}_{620\text{ nm}}$  of 0.1 in Tris buffer saline, 50 mM, pH 7.4) containing 5  $\mu\text{M}$  BODIPY<sup>TM</sup> TR cadaverine  
130 were incubated in the dark for 30 min. Then 50  $\mu\text{L}$  of this suspension were mixed with 50  $\mu\text{L}$  of  
131 potentiators in a 96-well black plate and kept in the dark for 30 min. The fluorescent intensity  
132 was then measured in a Spectramax M3 plate reader (Molecular Devices LLC, Sunnyvale, CA) at  
133  $\lambda_{\text{ex}}$  580 nm and  $\lambda_{\text{em}}$  620 nm. Alexidine at 50  $\mu\text{M}$  was used as positive control and samples added  
134 by Tris buffer saline as negative control (100%).

### 135 **Outer membrane permeability assay**

136 Outer membrane permeability was assessed using nitrocefin, a non-permeant  $\beta$ -lactam which is  
137 converted by  $\beta$ -lactamases in a colored derivative in the periplasmic space of permeabilized  
138 bacteria (42). Overnight cultures were incubated with 0.25  $\mu\text{g}/\text{mL}$  of imipenem for 1 h to induce  
139  $\beta$ -lactamase expression (43). After centrifugation at 3000  $g$  for 7 min, cells were washed twice

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140 with 1x PBS and adjusted to obtain an OD<sub>620 nm</sub> of 0.5. One hundred  $\mu$ L of cell suspension were  
141 mixed with 50  $\mu$ L of potentiator in 96-well plate, after which 50  $\mu$ L of nitrocefin (final  
142 concentration of 20  $\mu$ g/mL) were added. Absorbance at 490 nm was monitored over 60 min with  
143 an interval of 5 min using a Spectramax M3 plate reader.

#### 144 **Inner membrane permeability assay**

145 The effect of potentiators on the integrity of the inner membrane was determined by using a  
146 membrane-impermeable fluorescent dye propidium iodide (PI) as previously described (44). A  
147 stock solution of PI (10 mM DMSO) was diluted 1000 times with the bacterial suspension  
148 (OD<sub>620 nm</sub> 0.1 in BET buffer). 100  $\mu$ L potentiator (in BET) were added to 100  $\mu$ L PI-containing  
149 bacterial suspension in 96-well plates. The fluorescence intensity was measured using a  
150 Spectramax M3 plate reader ( $\lambda_{\text{ex}}$ , 540 nm;  $\lambda_{\text{em}}$ , 610 nm) after 1 h of incubation at room  
151 temperature. Alexidine at 50  $\mu$ M was used as positive control and imipenem as negative control  
152 (44).

#### 153 **Inner Membrane Depolarization Assay**

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

#### 164 **Inhibition of efflux**

165 The efflux activity in bacteria was assessed using the substrate 1,2'-dinaphthylamine as  
166 previously described (45). Ten milliliters of overnight culture were pelleted by centrifugation with  
167 3000 *g* for 7 min, washed once with 1 x PBS and then incubated with 1,2'- dinaphthylamine (TCI-  
168 Europe SA, Zwijndrecht, Belgium) at a final concentration of 32  $\mu$ M, and CCCP at a final  
169 concentration of 5  $\mu$ M, after which, cells were centrifuged at 3000 *g* for 7 min, resuspended in  
170 PBS and adjusted to an OD<sub>620 nm</sub> of 0.5. **One hundred microliters** of potentiator solution was mixed  
171 with 100  $\mu$ L bacteria suspension in a 96-well plate. The fluorescence ( $\lambda_{\text{ex}}$ , **370 nm**;  $\lambda_{\text{em}}$ , **420 nm**)  
172 was monitored each 30 sec during 620 sec using a Spectramax M3 plate reader. Active efflux was  
173 triggered by adding 5  $\mu$ L glucose (final concentration of 50 mM) at 180 sec. The maximal efflux  
174 activity (100%) was defined as the difference between the value obtained without and with  
175 glucose addition after 620 sec. Data were expressed as the ratio between the fluorescence signal  
176 recorded in control conditions or in the presence of potentiator at 620 sec. This experiment was  
177 performed only with *E. coli* and *K. pneumoniae*, preliminary experiment having shown that 1,2'-  
178 dinaphthylamine was not actively **exported** in *A. baumannii*.

#### 179 **Antibiotic accumulation in bacteria**

180 The concentration of ciprofloxacin and of a fluorescent analog of rifampicin (**NV1532**) inside  
181 bacteria was determined by a previously set-up fluorimetric assay (27). In brief, 10 mL of  
182 overnight culture (approximately 10<sup>9</sup> CFU/mL) were incubated at 37°C with **10 mg/L** ciprofloxacin  
183 and **20 mg/L** of **NV1532**. After 15 min of incubation (a duration longer than the predicted time  
184 required to reach saturation (46), but during which the antibiotic did not affect bacterial growth  
185 [no significant reduction in CFUs]), 20  $\mu$ L of suspension were collected to count CFUs and the  
186 remaining cells were collected by centrifugation at 3000 *g* for 7 min at **4 °C** and washed three  
187 times with 1 mL cold PBS. When assaying ciprofloxacin, cells were resuspended in 0.5 mL 100  
188 mM glycine buffer (pH **3.0** adjusted with HCl), and then kept in the darkroom overnight. When  
189 assaying **NV1532**, cells were resuspended in 0.5 mL PBS and then lysed by three cycles of  
190 freezing/thawing (**-80 °C** freezer to **60 °C** water bath). All samples were centrifuged at 14000 *g*  
191 for 5 min, the supernatant was then collected to measure the antibiotic content at  $\lambda_{\text{ex}}$  **275 nm**  
192 and  $\lambda_{\text{em}}$  **450 nm** for ciprofloxacin and  $\lambda_{\text{ex}}$  **470 nm** and  $\lambda_{\text{em}}$  **525 nm** for **NV1532**. All data were

193 normalized based on CFUs counts using a Spectramax M3 (Molecular devices, LLC, Sunnyvale, CA)  
194 plate reader.

### 195 **Cytotoxicity assessment (Trypan blue exclusion assay)**

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

$$203 \text{ cytotoxicity (\%)} = \frac{\text{Nb uncolored cells in controls} - \text{Nb uncolored treated cells}}{\text{Nb uncolored cells in controls}} \times 100 \%$$

### 204 **Intracellular models of infection**

205 We adapted the protocol previously developed for *P. aeruginosa* infection in THP-1 monocytes  
206 (31) (see Fig. S2 and S3 for details and model validation). All strains (opsonized 1 h with 10%  
207 human serum) were added to monocytes at a multiplicity of infection (bacteria/cell ratio) or 25  
208 and incubated at 37 °C in a 5% CO<sub>2</sub> atmosphere to allow phagocytosis. After 1 hour of incubation,  
209 cells were pelleted by centrifugation at 3000 *g* for 7 min and incubated for 1 h with 50 x MIC of  
210 gentamicin to remove non-internalized bacteria (more than 5 log<sub>10</sub> CFUs reduction, data not  
211 shown). Cells were then pelleted by centrifugation at 3000 *g* for 7 min, washed 2 times with 1 x  
212 sterile PBS and resuspended in the original volume of RPMI-1640 with 10% FBS. Cells were  
213 collected by centrifugation and washed once with PBS to eliminate extracellular bacteria and  
214 collected in 1 mL distilled water, to achieve complete lysis. Lysates were used for determining  
215 CFU counts by spreading on agar containing 2 g/L charcoal (to avoid carry-over effect, particularly  
216 in samples incubated with high antibiotic concentrations [no difference in colony counts was  
217 observed between plates supplemented or not with charcoal for samples exposed to low  
218 antibiotic concentrations, ruling out any interfering effect of charcoal in the assay]) and protein

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219 content using a commercially available kit (Bio-Rad DC Protein Assay, Bio-Rad Laboratories,  
220 Hercules, CA). The post-phagocytosis inoculum (defined as time zero) was consistently 5 to 7 x  
221  $10^5$  CFU/mg of cell protein.

### 222 **Intracellular antibiotic activity**

223 Infected cells were incubated for 24 h with antibiotics over a range of concentrations (0.01 to  
224 100x MIC) alone or in combined with potentiators, after which the cells were collected and the  
225 protein content and CFU counts were measured as described above. The data were expressed as  
226 the change in CFU (normalized by mg of cell protein) from the initial inoculum after 24 hours of  
227 incubation then used to fit a sigmoidal function and calculate pharmacodynamic parameters  
228 based on the corresponding Hill-Langmuir equation (apparent static concentrations [ $C_s$ ], i.e.  
229 extracellular concentration resulting in no apparent intracellular growth, and maximal relative  
230 efficacy [ $E_{max}$ ], i.e. maximal decrease in bacterial counts compared to the post-phagocytosis  
231 inoculum as extrapolated for an infinitely large antibiotic concentration).

### 232 **Persister assay**

233 A single colony from overnight culture of reference strains on TSA was grown in 200 mL MHB-CA  
234 in a 1-L flask and incubated for 24 h with 130 rpm at 37 °C. Ten milliliters of the bacterial  
235 suspension were then incubated with 50x MIC ciprofloxacin for 5 hours at 37 °C under agitation  
236 (130 rpm), in the presence of or in the absence of NV716. To explore the effects of different  
237 treatment regimens, NV716 was added at 0, 5, or 24 h after ciprofloxacin treatment, and the cells  
238 were treated for a total of 72 h. Aliquots were serially diluted in PBS and spread on TSA  
239 supplemented with 2 g/L charcoal for CFU counting.

### 240 **Antibiotic activity against biofilms**

241 Biofilms were grown in 96-well plates (VWR) as previously described (32). In brief, one single  
242 colony was inoculated in MHB-CA overnight with 130 rpm at 37°C. A bacterial suspension was  
243 then prepared in TGN (Trypticase soy broth (VWR) supplemented with 1% glucose (Sigma) and  
244 2% NaCl (Sigma)) using overnight cultures. Ninety-six-well plates were inoculated (200  $\mu$ L/well)

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245 at approximately  $10^7$  CFU/ml (the  $OD_{620\text{ nm}}$  of 0.05) and then incubated at 37 °C for 24 h to obtain  
246 a mature biofilm. The culture medium was removed and replaced with fresh medium (control)  
247 or medium supplemented with rifampicin (1x MIC and 5x MIC) alone or combined with  
248 potentiators and then reincubated at 37°C for 24 h. The medium was then removed and the  
249 biofilm was washed once with 200  $\mu\text{L}$  of 3-morpholinopropane-1-sulfonic acid (MOPS) buffer  
250 (20.9 g/L of MOPS (Sigma-Aldrich), 5.6 g/L NaCl; the pH was adjusted to 7.0 with NaOH) (47).  
251 Biofilm biomass was evaluated by measuring the absorbance of crystal violet, a cationic dye that  
252 nonspecifically stains negatively charged constituents in biofilm constituents based on ionic  
253 interactions. The washed biofilms were fixed by heat at 60 °C for about 24 h and incubated for  
254 10 min at room temperature with 200  $\mu\text{L}$  of 1 % (v/v) crystal violet (Sigma-Aldrich). The  
255 absorbance at 570 nm was measured using a Spectramax M3 plate reader. The metabolic activity  
256 (vitality) in the biofilms was quantified using the fluorescein diacetate (FDA) assay. It is based on  
257 the hydrolysis by living bacteria of the non-fluorescent white dye fluorescein diacetate in the  
258 yellow highly fluorescent fluorescein (32). The washed biofilms were incubated with 100 mg/L  
259 fluorescein diacetate (Sigma-Aldrich) for 15 min at 37 °C in the dark. Fluorescence was measured  
260 using a Spectramax M3 plate reader at  $\lambda_{\text{ex}}$  494 nm and  $\lambda_{\text{em}}$  518 nm.

### 261 **Curve fitting and statistical analysis**

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

## 265 Results

### 266 Antimicrobial susceptibility

267 **Table S3** shows the MICs of antibiotics alone or combined with potentiators (20 mg/L [38  $\mu$ M]  
268 PA $\beta$ N; 15 mg/L [38  $\mu$ M] **NV731**; 4 mg/L [10  $\mu$ M] **NV716**) against reference strains and clinical  
269 isolates of *E. coli*, *A. baumannii* and *K. pneumoniae*. These concentrations were selected as  
270 active based on previous works, but well below the MIC of each compound (25, 28). In control  
271 conditions, the MIC of antibiotics was generally elevated and higher than their human  $C_{max}$   
272 (except for rifampicin against *A. baumannii*, doxycycline and ciprofloxacin against most of the  
273 strains, and chloramphenicol against most *E. coli* and *K. pneumoniae*; see **Table S1** for  $C_{max}$   
274 values). In the presence of potentiators, MICs were globally reduced, although to a lower  
275 extent for ciprofloxacin against all strains, for azithromycin, chloramphenicol, and doxycycline  
276 against *A. baumannii*, and for fusidic acid against *K. pneumoniae*. PA $\beta$ N and **NV731** were  
277 systematically less potent than **NV716** even though the latter was used at a lower  
278 concentration. **Figure 1A** illustrates these MICs data and also shows the (geometrical) mean  
279 fold decrease in MIC in the presence of potentiators. **The most important reduction in this**  
280 **mean MIC values was noticed in combination with NV716 for rifampicin, linezolid, fusidic acid**  
281 **and novobiocin against *E. coli* (9-12 doubling dilutions), rifampicin and novobiocin against**  
282 ***K. pneumoniae* (9 doubling dilutions), and rifampicin, novobiocin and fusidic acid against**  
283 ***A. baumannii* (8-9 doubling dilutions). The lowest effect (mean reduction of 1 to 2 doubling**  
284 **dilutions) was noticed for ciprofloxacin against all bacteria as well as for azithromycin against**  
285 ***A. baumannii* (3 doubling dilutions). Importantly, MIC of the combinations with NV716 fall in**  
286 **the range of therapeutically-achievable concentrations in all cases (yellow zones in the**  
287 **graphs). FIC index calculations indicated a higher degree of synergy between antibiotics and**  
288 **NV716 than the other potentiators (Figure S4), except for ciprofloxacin against *K. pneumoniae***  
289 **ATCC 43816 and 74, against which ciprofloxacin alone was already very active. A highly**  
290 **significant correlation was observed between the reduction in MIC caused by NV716 and the**  
291 **calculated logD at pH 7.4 of each antibiotic (Table S1), a modest effect being observed for**  
292 **antibiotics with a negative logD value, and a progressive increase in this effect along with the**  
293 **lipophilicity of the drugs once their logD value becomes higher than 0 (Figure 1B).**

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## 294 Interaction of potentiators with *E. coli*, *K. pneumoniae* and *A. baumannii* membranes

295 Our previous study suggested that **NV716** can bind to LPS in *P. aeruginosa*, increases the  
296 permeability of its outer membrane, and to a much lower extent, of its inner membrane (27).  
297 We therefore examined these properties in the reference strains of the three bacterial species  
298 under study here. Alexidine and colistin were used as positive controls (34, 48), and **NV731**  
299 and PA $\beta$ N, as comparators. We first measured the capacity of each compound to displace  
300 Bodipy-cadaverine from its binding to LPS in the 4 reference strains (Figure 2A; IC<sub>50</sub> values in  
301 Table S4). The effect of **NV716** was similar to that of alexidine over the whole range of  
302 concentrations investigated against all strains, with comparable IC<sub>50</sub> values for both  
303 molecules. The IC<sub>50</sub> were about 10-times higher **NV731** and not reached at 100  $\mu$ M for colistin  
304 and PA $\beta$ N.

305 We then determined the effects of these compounds on the outer membrane permeability  
306 (Figure 2B; IC<sub>50</sub> values in Table S4). To this effect, we measured the absorbance of the  
307 hydrolysis product of the non-permeant cephalosporin nitrocefin, which is degraded by  
308 periplasmic  $\beta$ -lactamases when the outer membrane has been permeabilized (42). Alexidine  
309 was the most potent in this assay, followed by **NV716** and colistin, which also allowed to reach  
310 a same effect as alexidine but at higher concentrations (IC<sub>50</sub> 2 to 3 times higher). **NV731** and  
311 PA $\beta$ N were less potent (IC<sub>50</sub> 7 to 13 times higher than for alexidine) and less effective.

312 The effect of potentiators on the inner membrane permeability was also assessed by  
313 measuring the fluorescence signal of propidium iodide (PI), generated when PI intercalates in  
314 the DNA of bacteria when both their outer and inner membranes have been permeabilized  
315 (44). Alexidine and colistin similarly increased PI fluorescence in a concentration-dependent  
316 manner, while **NV716** was 20-30 times less potent, causing only 50% of permeabilization at  
317 the highest concentrations tested (50  $\mu$ M) (Figure 2C; IC<sub>50</sub> values in Table S4). Yet, the effect  
318 of **NV716** was slightly higher than that of colistin but much lower than that of alexidine when  
319 these compounds were all compared at an equipotent concentration of 1 x MIC (Figure S5).  
320 **NV731** and PA $\beta$ N were inactive in this assay.

321 In addition, we investigated the effect of **NV716** on inner membrane depolarization using the  
322 DiSC3(5) fluorescence assay. We observe a depolarization at 50  $\mu$ M but not at 10  $\mu$ M (Figure



323 **S6**), indicating that **NV716** can dissipate the proton motive force (PMF) at its MIC, but not at  
324 sub-inhibitory concentrations.

### 325 **Inhibition of active efflux by potentiators**

326 Previous studies documented that **NV716** can inhibit the activity of efflux pumps in  
327 *P. aeruginosa* (26). We therefore evaluated the capacity of **NV716** and its comparators to  
328 inhibit the active efflux of the fluorescent probe 1,2'-dinaphthylamine (45). We first examined  
329 the rate of efflux in *E. coli* ATCC 47076 after the addition of **NV716** at different concentrations  
330 (Figure 2D, left panel). A concentration-dependent reduction of the fluorescence was  
331 observed readily after the addition of **NV716**, which reached a plateau at 620 sec. The same  
332 experiments were performed with the other potentiators and also against *K. pneumoniae*  
333 reference strains. The other panels of Figure 2D show the inhibition of efflux calculated at  
334 620 sec. **NV716** proved more potent and effective in this assay than the other potentiators  
335 (Figure 2D). While the effect of **NV716**, alexidine and PA $\beta$ N increased all over the range of  
336 concentration investigated, that of **NV731** and colistin tend to a plateau at high  
337 concentrations.

### 338 **Influence of potentiators on antibiotic accumulation inside bacteria**

339 As **NV716** was shown to increase outer membrane permeability and inhibit efflux, we  
340 assessed its effect compared to that of the other potentiators on the accumulation of  
341 ciprofloxacin and **NV1532** (a fluorescent derivative of rifampicin; same MIC as rifampicin  
342 against all tested strains) inside bacteria. These antibiotics were selected as representatives  
343 of antibiotics that are substrates or not for efflux, respectively. The level of accumulation of  
344 ciprofloxacin alone was higher in *E. coli* ATCC 47076 and *K. pneumoniae* ATCC 43816 than in  
345 the other two strains (Figure 3A), possibly related to differences in intrinsic outer membrane  
346 permeability or expression of efflux transporter. PA $\beta$ N and **NV731** at 38  $\mu$ M increased its  
347 accumulation in *E. coli* ATCC 47076 only, while **NV716** at 10  $\mu$ M caused a significant increase  
348 in all strains except *K. pneumoniae* ATCC 43816 in which it did not decrease ciprofloxacin MIC  
349 (Table S3). The accumulation of **NV1532** was more elevated in *A. baumannii* ATCC 19606 than  
350 in the other strains, also probably related to a higher intrinsic membrane permeability to this  
351 more lipophilic drug. PA $\beta$ N increased its accumulation in *E. coli* ATCC 47076 and *A. baumannii*

352 ATCC 19606, while **NV731** and **NV716** caused a significant increase for all strains tested, but  
353 the effect of **NV716** was more important even though it was used at a lower concentration  
354 (Figure 3B). A significant correlation was observed between the fold change (i) in the  
355 accumulation of antibiotics and (ii) in MIC when comparing the combination with potentiators  
356 vs antibiotics alone (Figure 3C and 3D).

### 357 **Cytotoxicity of antibiotics**

358 As a preliminary to intracellular infection experiments, we examined whether antibiotics were  
359 cytotoxic for eukaryotic cells. This was critical as most of them had to be used at high  
360 concentrations in view of their high MIC. The cytotoxicity of potentiators had been previously  
361 examined and found to be minimal in their conditions of used for these experiments (25). IC<sub>50</sub>  
362 (i.e. concentration causing 50% cell mortality) were reached at concentrations corresponding  
363 to approximately 3 x (fusidic acid, linezolid, novobiocin), 30 x (rifampicin), 60 x (azithromycin),  
364 90 x (chloramphenicol), or above 100 x (doxycycline and ciprofloxacin) their respective MIC  
365 against *E. coli* ATCC 47076, both in infected and non-infected cells (Table S5). IC<sub>50</sub> values did  
366 not differ for antibiotics alone or combined with a potentiator, both in non-infected and  
367 infected cells (Table S5).

### 368 **Activity of antibiotics alone or combined with potentiators against intracellular Gram-** 369 **negative bacteria in THP-1 cells**

370 Figure 4 shows the concentration-response curves against intracellular *E. coli* ATCC 47076 in  
371 the absence of or in the presence of **NV716** (the highest concentration tested for each  
372 antibiotic was that causing 50% cytotoxicity). The activity of antibiotics alone developed  
373 following a sigmoidal concentration-response curve, as previously described (31). At low,  
374 subMIC concentrations, a ~3-4 log<sub>10</sub> increase in CFUs was noticed over the 24 h incubation  
375 period. A static effect was observed at extracellular concentrations close or slightly higher  
376 than the MIC of each antibiotic. At the highest concentration tested, the reduction in bacterial  
377 counts ranged from a static effect for linezolid, fusidic acid, or novobiocin (note that the  
378 highest concentration tested is low for these drugs because of their toxicity), 1 log<sub>10</sub> CFU  
379 decrease for azithromycin and chloramphenicol, 2 log<sub>10</sub> CFU decrease for rifampicin and  
380 doxycycline, and 2.5 log<sub>10</sub> CFU decrease for ciprofloxacin. In the presence of **NV716** at 10 μM,

381 all curves were shifted to the left, meaning that the corresponding antibiotic was more potent,  
382 a static effect being reached at lower extracellular concentrations. The extent of the shift  
383 varied however among drugs. Furthermore, for those drugs for which the maximal effect  
384 could be reached (i.e., the plateau value of the Hill equation), the reduction in intracellular  
385 counts was also increased ( $E_{\max}$  more negative), indicating an increase in the antibiotic  
386 efficacy. For the other drugs (linezolid, fusidic acid, novobiocin), an increased effect was also  
387 observed at the highest concentration tested.

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

411 In order to examine our data in a clinically-oriented perspective, we estimated, based on the  
412 Hill equation of the concentration-response curves, the reduction in bacterial counts reached  
413 when antibiotics were used alone or in combination at fixed concentrations corresponding to  
414 their respective minimum and maximum concentrations ( $C_{\min}$  and  $C_{\max}$ , see **Table S1**) in  
415 human serum (**Figure S7**). Antibiotics alone at their  $C_{\min}$  were inactive, except ciprofloxacin,  
416 chloramphenicol and doxycycline against *E. coli* and *K. pneumoniae* (which reduced  
417 intracellular counts but did not reach a static effect) and ciprofloxacin, against *A. baumannii*  
418 (1  $\log_{10}$  CFU decrease). When antibiotics at their  $C_{\min}$  were combined with **NV716**, we noticed  
419 a reduction in bacterial counts, the extent of which was highly variable among drugs and  
420 bacteria, with reduction of 90% (1  $\log_{10}$  CFU) being observed for rifampicin, fusidic acid,  
421 chloramphenicol and doxycycline against all species (except *A. baumannii* for doxycycline).  
422 Yet, in most of the cases, this effect was not sufficient to decrease bacterial counts below the  
423 post-phagocytosis inoculum. When used at the human  $C_{\max}$ , most antibiotics were capable of  
424 reducing the inoculum, except linezolid (against all species) and azithromycin (against *E. coli*  
425 and *K. pneumoniae*). Chloramphenicol and doxycycline were at least bacteriostatic against *E.*  
426 *coli* and *K. pneumoniae*, and ciprofloxacin, against all species. In combination with **NV716** (10  
427  $\mu\text{M}$ ), a drastic improvement of activity was observed, with increment of activity reaching 1  
428 to almost 4  $\log_{10}$  CFU decrease as compared to the antibiotic alone. For rifampicin, novobiocin,  
429 doxycycline, and in some cases, for fusidic acid, chloramphenicol and ciprofloxacin, a 99% (2  
430  $\log_{10}$  CFUs) decrease as compared to the post-phagocytosis inoculum could be achieved by  
431 the combinations. In all these conditions, the effect of PA $\beta$ N and **NV731** was lower than that  
432 of **NV716**.

### 433 **Activity of ciprofloxacin alone or combined with potentiators against persisters**

434 The fact that **NV716** increases the intracellular efficacy of all antibiotics against *E. coli*,  
435 *A. baumannii* and *K. pneumoniae* suggests it may decrease the proportion of antibiotic  
436 persisters in the population, as previously demonstrated for *P. aeruginosa* (25). We, therefore,  
437 determined the persister fraction surviving to ciprofloxacin (selected as a highly bactericidal  
438 antibiotic) at high concentration (50 x MIC) against stationary-phase cultures ( $10^9$  CFUs/mL)  
439 of reference strains. To this effect, we first examined the kinetics of killing by ciprofloxacin  
440 when used alone or combined with **NV716** added either at the same time as ciprofloxacin or

441 later to determine whether it was able to improve activity even the maximal effect was  
442 already reached for ciprofloxacin alone (49) (Figure 7A). CFUs were significantly reduced after  
443 the addition of **NV716** at any time point, suggesting that **NV716** does not need to be added  
444 at the same time as the antibiotic to be able to increase its activity. In the next step, we aimed  
445 at determining whether **NV716** action was related to a direct capacity to kill persisters or  
446 rather to resensitize them to the killing effect of ciprofloxacin. To this end, we isolated the  
447 persisters surviving after 5 h of incubation with ciprofloxacin at 50 x MIC and reexposed them  
448 to ciprofloxacin at 50 x MIC, NV716 at different concentrations, or a combination thereof  
449 (Figure 7B). As expected, incubation of the isolated persisters with ciprofloxacin alone caused  
450 only a negligible reduction in CFU for all strains, confirming the effective isolation of persister  
451 cells. When used alone, **NV716** caused a marked decrease in CFU only at the highest  
452 concentration tested (250  $\mu$ M). In contrast, a significant reduction in CFU was observed when  
453 **NV716** was combined at 10  $\mu$ M with ciprofloxacin for all strains, indicating it rather acts as a  
454 potentiator of ciprofloxacin against persisters.

#### 455 **Activity of rifampicin alone or combined with potentiators against biofilms.**

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

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## 467 Discussion

468 The emergence and rapid spread of antibiotic resistance in Gram-negative bacteria represents a  
469 critical threat to public health worldwide (50). Identification of novel adjuvants that restore  
470 existing antibiotic efficacy and improve clinical cure has been considered as a cost-effective  
471 strategy for combating superbugs, in particular *E. coli*, *K. pneumoniae* and *A. baumannii* (51). In  
472 this study, we show that the polyaminoisoprenyl compound **NV716** is capable of resensitizing  
473 *E. coli*, *K. pneumoniae*, and *A. baumannii* to all tested antibiotics whether affected (doxycycline,  
474 chloramphenicol, azithromycin, ciprofloxacin, fusidic acid, novobiocin and linezolid) or not  
475 (rifampicin) by active efflux (10, 52, 53), not only in broth, but also intracellularly or in biofilm. In  
476 all our experiments, **NV731** and the efflux pump inhibitor PA $\beta$ N proved less potent.

477 Considering first the susceptibility data in broth, we observed a remarkable improvement in the  
478 intrinsic activity of antibiotics when combined by **NV716**. This synergistic effect is obtained at  
479 sub-MIC concentrations of the potentiator, demonstrating it is related to an adjuvant mode of  
480 action and not to bacterial killing. Potentiation is observed in all three species (as well as in  
481 *P. aeruginosa* (27)), and its extent (in terms of gain of activity, see **Figure 1B**) is largely  
482 independent of the species, even for antibiotics showing markedly different MICs against these  
483 species when used alone (see e.g. rifampicin, chloramphenicol, or doxycycline). Actually, the  
484 degree of potentiation offered by **NV716** is depending on the lipophilicity of the antibiotic, as  
485 evaluated by its logD value. This is very coherent with the fact that the external leaflet of the  
486 outer membrane is known to oppose a barrier to the diffusion of lipophilic compounds, which  
487 are also incapable to cross this obstacle via hydrophilic porin channels (54). LPS barrier effect is  
488 mediated by the low fluidity of the layer, the quasi-crystalline, ordered arrangement of its  
489 hydrocarbon chains, and the strong intermolecular interaction between LPS heads via cation-  
490 mediated bridging (54). In this context, **NV716**, with a calculated logD of -4.2 at physiological pH  
491 (**Table S1**), shows a strong capacity to interact with LPS, ensuing a subsequent permeabilisation  
492 of the outer membrane. It is as potent in this respect as the biguanide alexidine, with which it  
493 shares an extended conformation of its polyaminated moiety, and, interestingly enough, much  
494 more potent than colistin, often used as a potentiator of other drugs against multiresistant Gram-

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495 negative bacteria, for its membrane-destabilizing effects (55, 56). As previously discussed (27),  
496 **NV731** does not show this extended conformation, which may contribute to explain its lower  
497 potency. The capacity of **NV716** to interact with LPS and permeabilize the outer membrane is  
498 similar among the three species tested here as well as against *P. aeruginosa*. This indicates the  
499 importance of its interaction with the negative charges of the phosphate groups and subsequent  
500 displacement of stabilizing divalent cations for its action (27), as this is a common feature of all  
501 LPS. Yet, the differences in LPS between the species under study remain modest. As compared  
502 to the LPS of *E. coli*, that of *A. baumannii* shows an additional acyl chain at the 2 position  
503 containing an additional hydroxyl group (57); that of *K. pneumoniae* is hexa-acylated with C14  
504 chains in position 2 and 3 (instead of C12 in position 3 for *E. coli*) and possible additional  
505 modifications in the lipidic part (58), while that of *P. aeruginosa* PAO1 is hexa- or penta-  
506 acetylated (lacking in this case the primary O-linked acyl substitution at position 3) (59).

507 Beside its destabilizing effect on the outer membrane, **NV716** also inhibits the activity of efflux  
508 pumps, but we do not know whether this results from a direct interaction with the pump or is  
509 indirectly related to the perturbation in membrane properties it induces. **NV716** can disturb the  
510 inner membrane potential, which could reduce the energy source of the RND pumps (26), but  
511 this effect is observed at concentrations higher than those for which potentiation of antibiotic  
512 activity is obtained. Moreover, the increase in antibiotic accumulation inside bacteria is also  
513 observed for rifampicin, which is a poor substrate for efflux, indicating that the predominant  
514 mode of action of **NV716** is related to the perturbation of the integrity of the outer membrane.  
515 Importantly, the activity of **NV716** (and the two other potentiators) is observed at concentrations  
516 that do not cause cytotoxicity for eukaryotic cells (25), suggesting no or minimal interaction with  
517 eukaryotic membranes.

518 Turning then our attention to intracellular models of infection, we noticed that **NV716** was the  
519 most active among the potentiators tested to enhance both the relative potency (lower  $C_s$ ) and  
520 the maximal efficacy (more negative  $E_{max}$ ) of all antibiotics against all species. Although we did  
521 not examine the cellular concentration of **NV716** in human monocytes, these results suggest that  
522 it has access to intracellular bacteria in sufficient concentrations to exert its synergistic effect.

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523 The lower benefit of PA $\beta$ N and **NV731** can be attributed to their lower intrinsic effects on MICs  
524 (as observed in broth) or possibly to a lower accumulation inside the cells, which has neither been  
525 investigated. Also, we can most likely exclude that the improved efficacy is related to interference  
526 in the count of the residual CFUs, related either to the release of bacteria out of the cells before  
527 lysis or to the remaining amount of antibiotics accumulated in cells if considering that (i) **NV716**  
528 did not increase the cytotoxicity of antibiotics, and (ii) charcoal was added to culture plates to  
529 adsorb residual antibiotic.

530 Considering first antibiotic potency, we observed that  $C_s$  was close to the respective MIC (1.5 to  
531 4 x the MIC) in all cases, as previously described in other models of intracellular infection (31, 60,  
532 61), and independently of the capacity of the drug to accumulate inside monocytes (Table S1)  
533 (62, 63). This is interpreted as denoting a poor intracellular bioavailability (61). Although gain in  
534 potency is a pharmacokinetic-driven parameter, meaning that it essentially reflects a change in  
535 the antibiotic concentration needed to reach a specified effect (bacteriostatic effect for  $C_s$  (61)),  
536 it was not related to the lipophilicity of the antibiotic (Figure S8A). We do not have a simple  
537 explanation for this absence of correlation, but wish to emphasize that the system here is more  
538 complex. In particular, accumulation of antibiotics in eukaryotic cells is not directly related to the  
539 hydrophilic/lipophilic balance, with azithromycin accumulating to much larger extent than  
540 ciprofloxacin, for example, while their calculated logD value is similar. In addition, the subcellular  
541 localization of antibiotics and bacteria may also play a critical role in this context. Interestingly,  
542 however, we notice that (i) for all drugs, the  $C_s$  measured in the presence of **NV716** is reduced to  
543 a similar value (0.3 to 1.2-fold the MIC) and (ii) as a corollary, the highest gain in relative potency  
544 is obtained for antibiotics that were the less potent when used alone (Figure S8B).

545 Considering then antibiotic efficacy, we observed, as in our previous models, that all antibiotics  
546 fail to eradicate the infection intracellularly. In the present study, we were limited in the range  
547 of antibiotic concentrations we could test because of their intrinsic toxicity at high multiples of  
548 their respective MICs, but it remains that the  $E_{\max}$  or  $E_{\text{highest}}$  was ranging between reductions of  
549 0.5 (linezolid against *E. coli*) to 2.5 log<sub>10</sub> CFUs (ciprofloxacin against all strains), with most of the  
550 drugs causing a reduction of 1 to 2 log<sub>10</sub> CFUs. These dissimilarities are probably attributable to  
551 differences in the mode of action of the antibiotics, and most conspicuously, to their



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552 bacteriostatic/cidal character, the less efficacious being the bacteriostatic drugs linezolid and  
553 fusidic acid, and the most efficacious, the highly bactericidal drugs ciprofloxacin and rifampicin.  
554 This is very coherent with data assembled for other models of intracellular infections, with  
555 linezolid, fusidic acid, and azithromycin showing no or low reductions in CFUs counts against  
556 intracellular *Staphylococcus aureus*, *Staphylococcus epidermidis* or *Listeria monocytogenes* (64-  
557 66), and rifampicin being highly effective against intracellular *S. aureus* and its small colony  
558 variants (67). The intracellular activity of fluoroquinolones has been largely demonstrated against  
559 a vast array of pathogens like *S. aureus*, *L. monocytogenes*, *L. pneumophila*, *Burkholderia*  
560 *thailandensis*, *Yersinia pseudotuberculosis*, *Francisella philomiragia*, or *Coxiella burnetii* (31, 68-  
561 70). The relatively high efficacy of doxycycline (bacteriostatic in broth) is rather unexpected but  
562 has been already reported in models of intracellular infection by *C. burnettii* (70), *Chlamydia*  
563 *trachomatis* (71), or *L. pneumophila* (72), possibly suggesting that the intracellular environment  
564 may contribute to improve its killing capacity. As efficacy is a pharmacodynamic-driven  
565 parameter reflecting bacterial responsiveness to antibiotics (61), it is not surprising that the gain  
566 of efficacy triggered by **NV716** does not correlate with the lipophilicity of the antibiotics (Figure  
567 S8C). But again, it is interesting to note that the mean maximal efficacy reached in combination  
568 is very close (-2.2 to -2.4 log<sub>10</sub> CFUs) for all drugs except ciprofloxacin, which reached -2.8 log<sub>10</sub>  
569 CFUs, and that the difference in E<sub>max</sub> offered by **NV716** is all the more important for poorly  
570 effective drugs (Figure S8D). This suggests that part of the intracellular inoculum is refractory to  
571 antibiotic action and that this pool is relatively constant except if using an antibiotic that is  
572 already highly efficient by itself like ciprofloxacin. For *S. aureus*, intracellular survivors to  
573 antibiotics have been identified as persisters, i.e. phenotypic variants that adopt a dormant, non-  
574 replicative, and transiently non-responsive phenotype (19, 20). This is why we examined the  
575 effect of **NV716** of persisters selected by ciprofloxacin and found that it was capable of  
576 decreasing the proportion of persisters in *E. coli*, *K. pneumoniae* and *A. baumannii* when  
577 combined with ciprofloxacin, but not when used alone, suggesting that it can resensitize  
578 persisters to the antibiotic but not kill them (25, 49). Of interest, other molecules capable of  
579 disrupting membrane integrity also reduced the residual fraction of persisters upon exposure to  
580 antibiotics for different bacterial species (73, 74), and one of them (SPI009; 1-[[2,4-

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581 dichlorophenethyl] amino]-3-phenoxypropan-2-ol) was also shown to increase the efficacy of  
582 ciprofloxacin against intracellular *P. aeruginosa* (75). Recently, another membrane-damaging  
583 small molecule, JD1, was found capable of reducing the survival of the intracellular Staphylococci  
584 and of persister cells in broth, as well as of disrupting biofilms (76).

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

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

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

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**612 Author contributions**

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

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**621 Competing interests**

622 The authors have no competing interests to declare.

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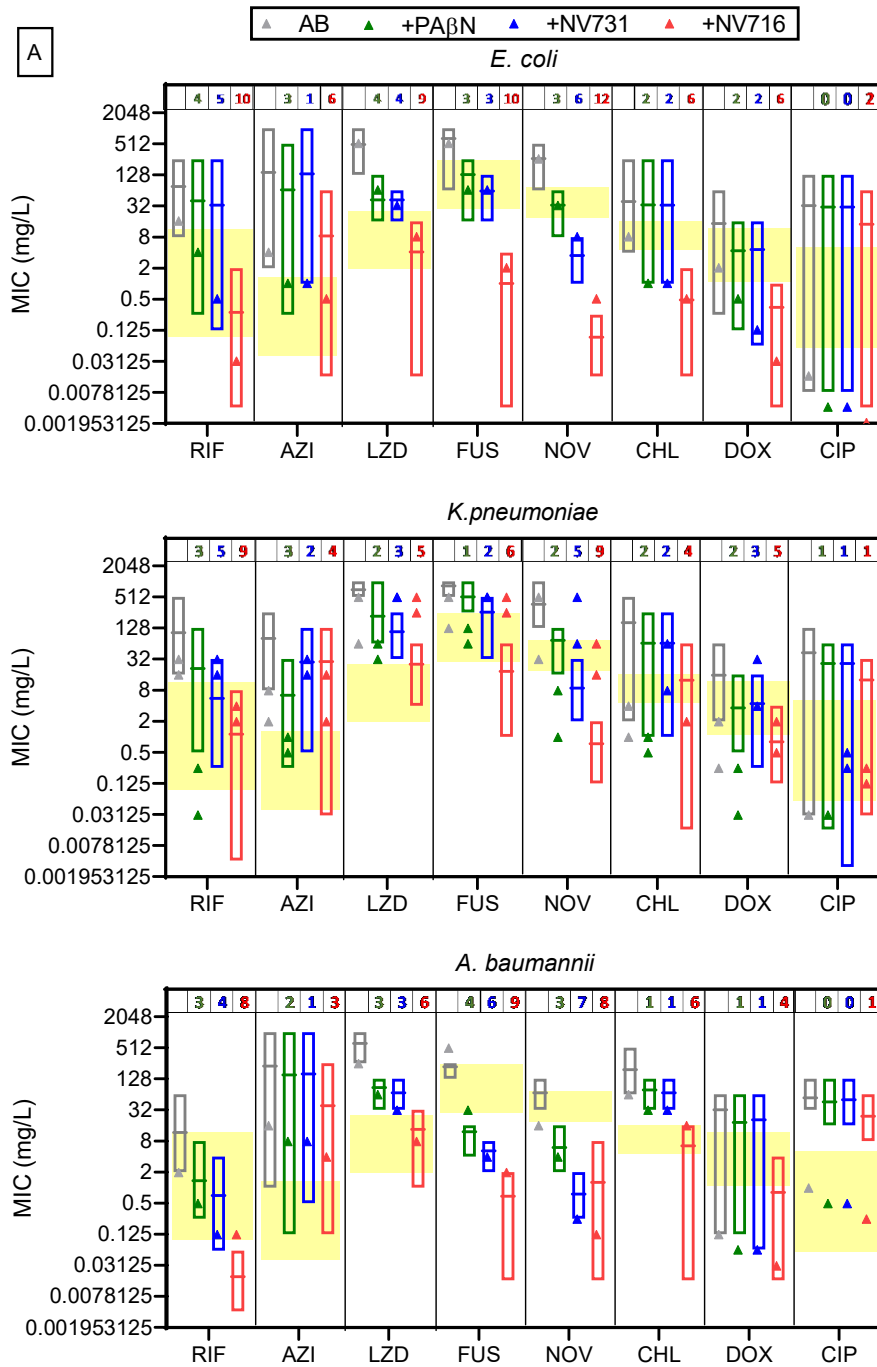
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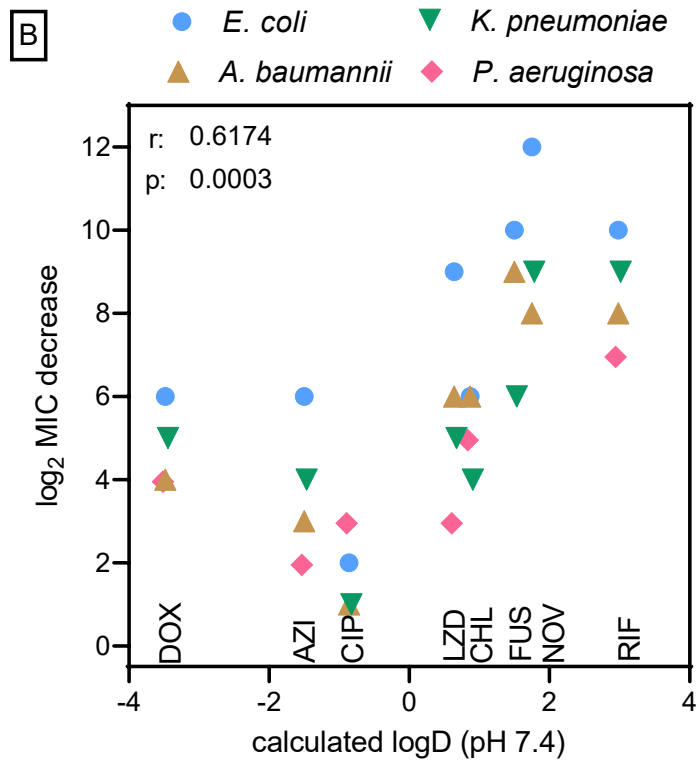
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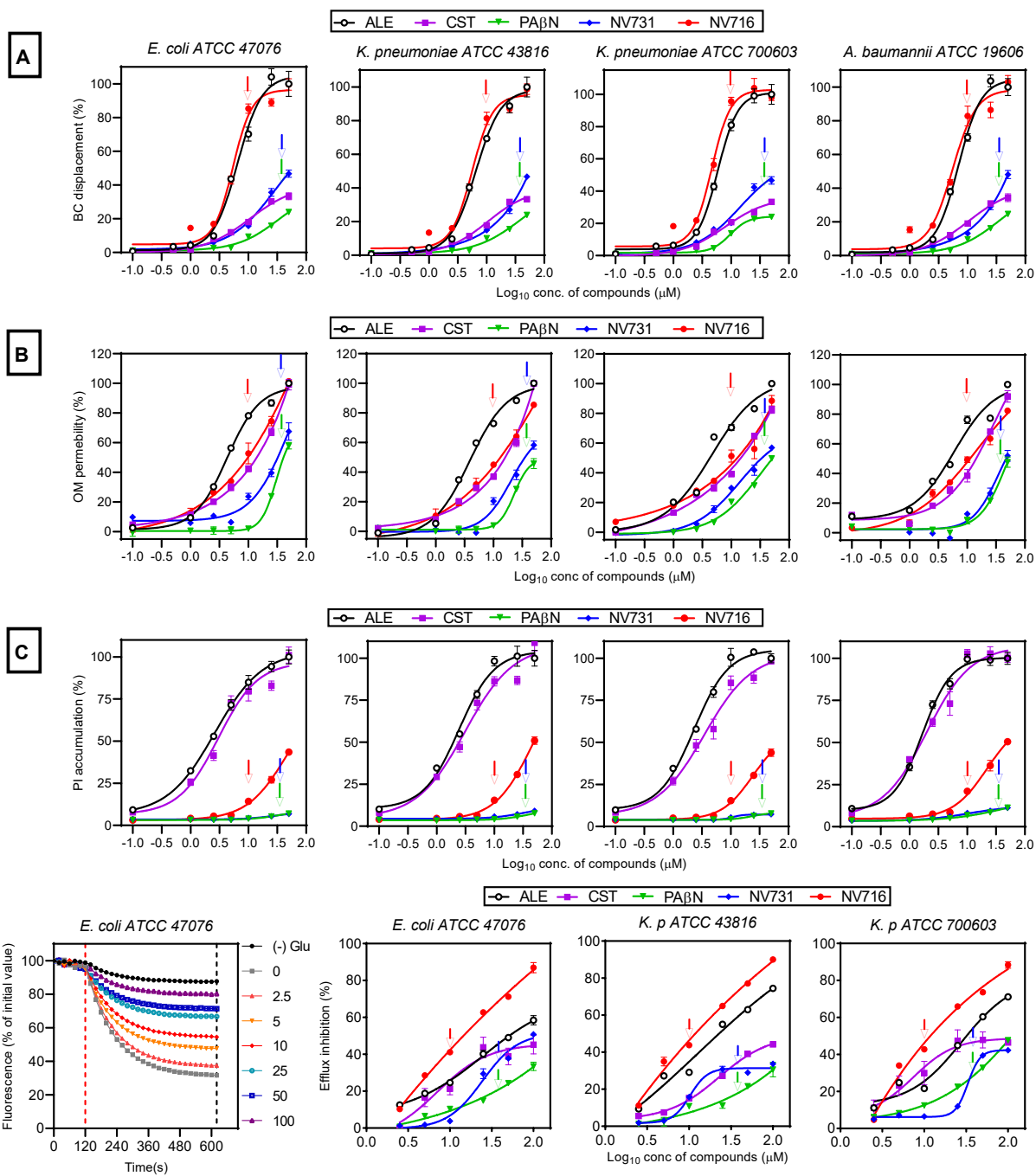
**Figure 1. A.** Activity of antibiotics alone or combined with potentiators against various Gram-negative bacteria in broth. The MICs of reference strains (One [*E. coli*; *A. baumannii*] or two [*K. pneumoniae*] strains) are represented by triangles. The boxes show the interval between the minimal 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  $\mu\text{M}$  PA $\beta$ N; blue: combined with 38  $\mu\text{M}$  NV731; red: combined with 10  $\mu\text{M}$  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  $\mu\text{M}$  NV716 and the  $\log D$  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.



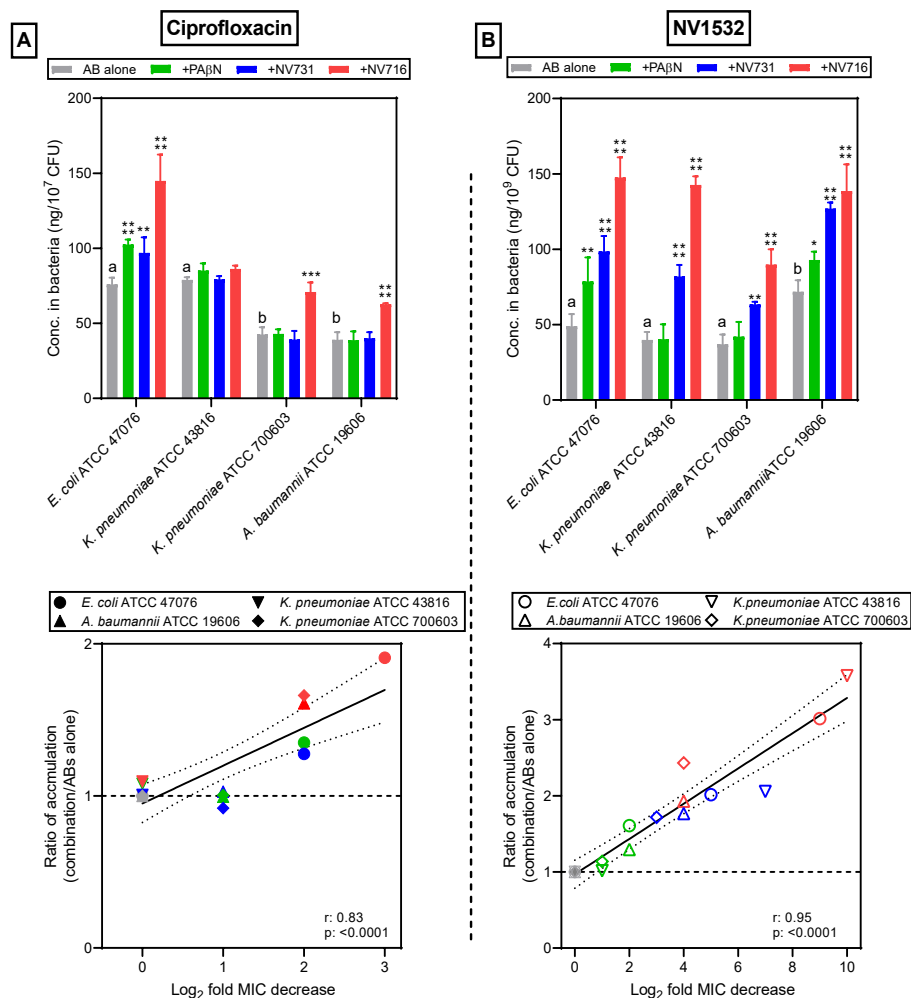


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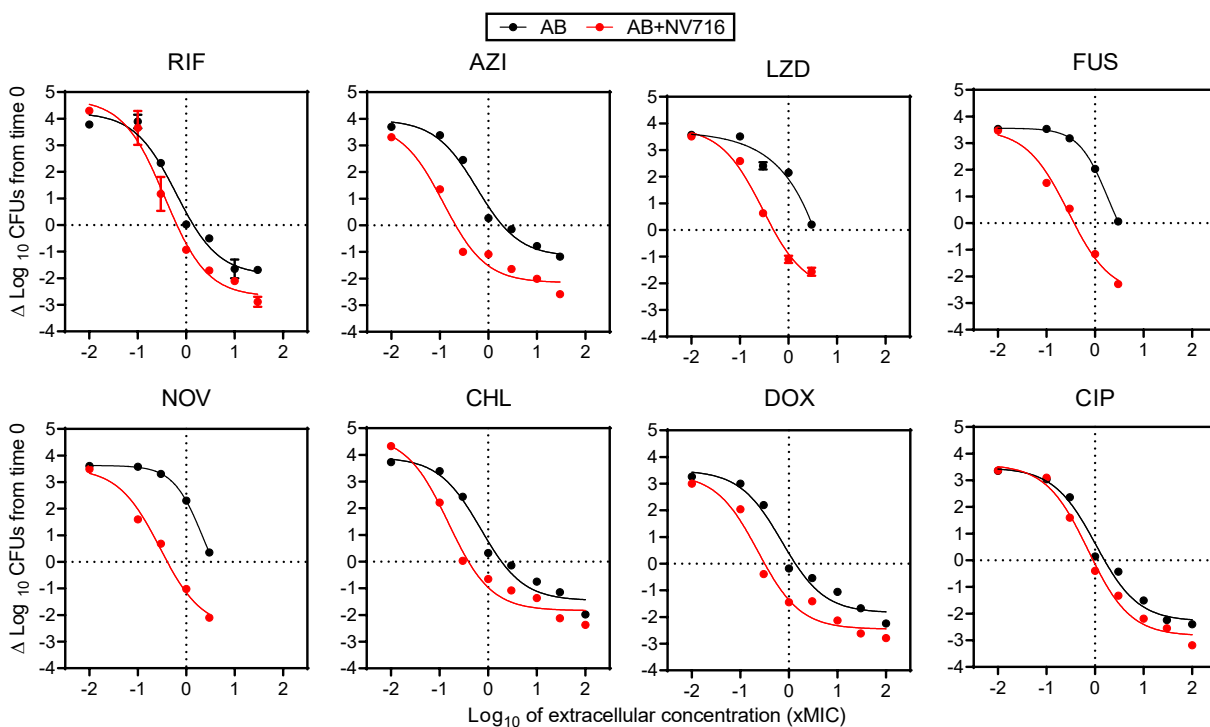
**Figure 2.** Influence of potentiators, colistin and alexidine over a wide range of concentrations on membrane properties of 1 (*E. coli*; *A. baumannii*) or 2 (*K. pneumoniae*) reference strains. (A) Binding to LPS, assessed by measuring the displacement of BODIPY-cadaverine (BC) after 30 minutes of incubation. Positive control (100%): effect of 50  $\mu\text{M}$  alexidine at 30 minutes. (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  $\mu\text{M}$  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  $\mu\text{M}$  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 sec (red vertical dotted line). Data are expressed in percentage of the value recorded in the absence of glucose. Another panel: Inhibition of efflux of 1,2-dinaphthylamine by potentiators in *E. coli* ATCC 47076. The graphs show the percentage of efflux inhibition after 620 seconds of incubation (black vertical dotted line on the left graph) (500 seconds after the addition of glucose). The arrows on each graph point to the concentration of potentiators used in most other experiments (10  $\mu\text{M}$  for **NV716**; 38  $\mu\text{M}$  for **NV731** and PA $\beta$ N). All data are means  $\pm$  SEM (triplicates from 3 independent experiments).  $\text{IC}_{50}$  values for these concentration-responses are shown in [Table S4](#).



**Figure 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  $\mu$ M PA $\beta$ N; blue: combined 38  $\mu$ M **NV731**; red: combined with 10  $\mu$ M **NV716**. All data are mean  $\pm$  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.



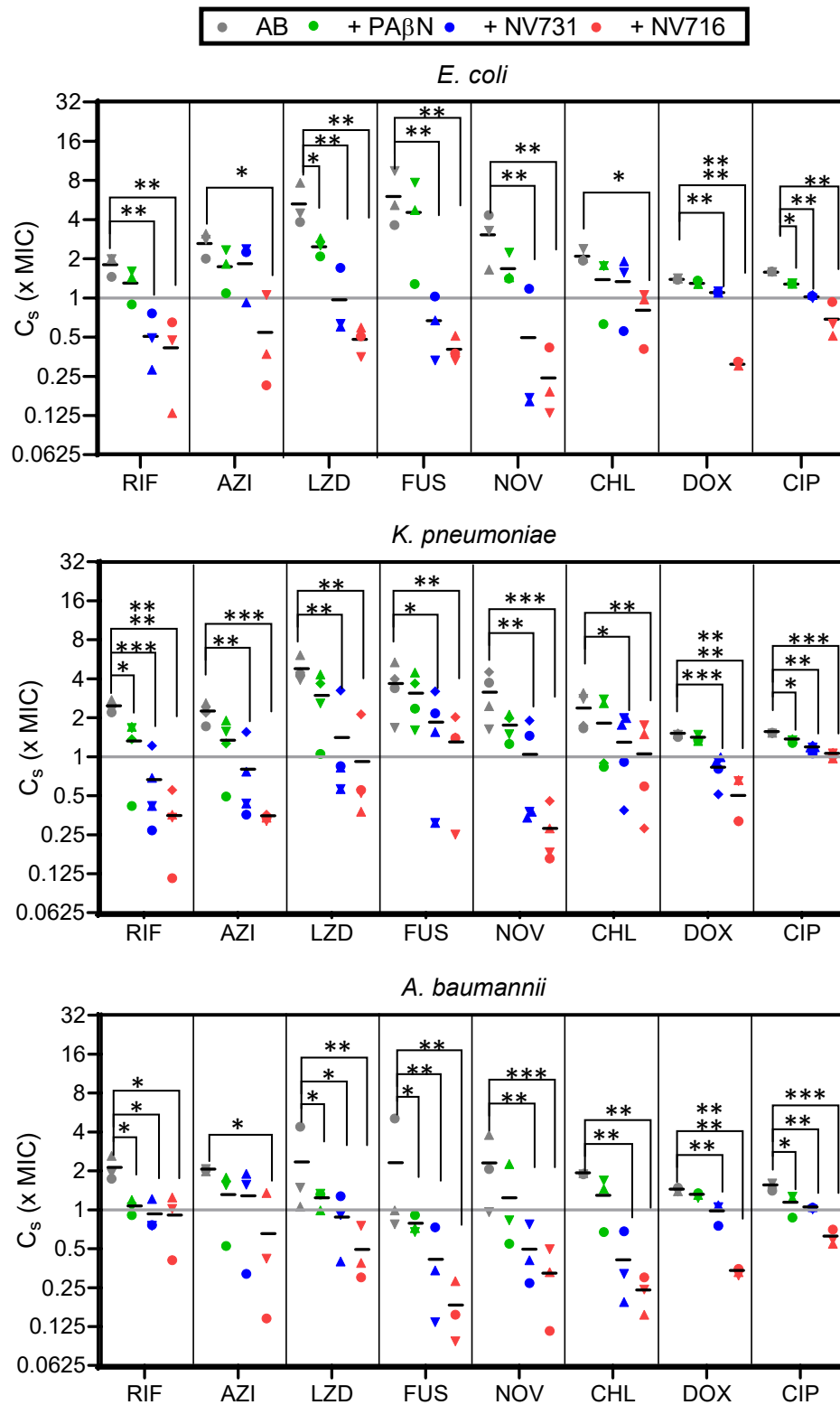
**Figure 4.** Concentration-response curves of antibiotics alone or combined with **NV716** against intracellular *E. coli* ATCC 47076 in a model of THP-1 monocytes. The graphs show the changes in CFU counts from the initial, post-phagocytosis inoculum after 24 h of incubation with increasing extracellular 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  $\mu$ M; 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<sub>50</sub> of toxicity for THP-1 cells (not applicable to CIP, not toxic). All data are means  $\pm$  SEM (triplicates from three experiments; when non-visible, error bars are smaller than the symbols).



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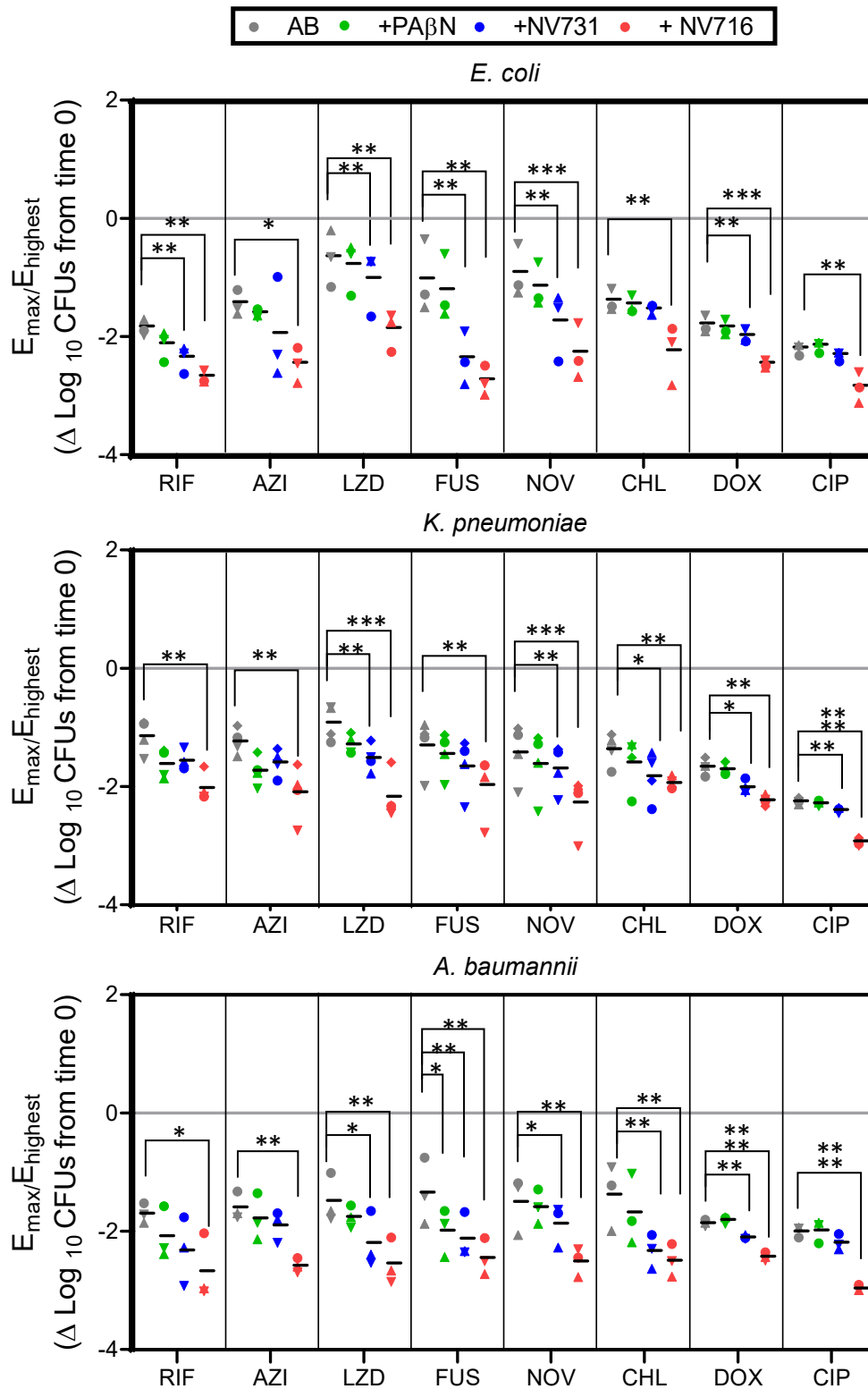
**Figure 5.** Static concentration of antibiotics alone (rifampicin [RIF], azithromycin [AZI], linezolid [LZD], fusidic acid [FUS], novobiocin [NOV], chloramphenicol [CHL], doxycycline [DOX], or ciprofloxacin [CIP]) or combined with potentiators against various multidrug resistant Gram-negative bacteria 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 **Figure 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  $\mu$ M PA $\beta$ N; blue: combined 38  $\mu$ M **NV731**; red: combined with 10  $\mu$ M **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: \*,  $p < 0.05$ ; \*\*,  $p < 0.01$ ; \*\*\*,  $p < 0.001$ ; \*\*\*\*,  $p < 0.0001$ .



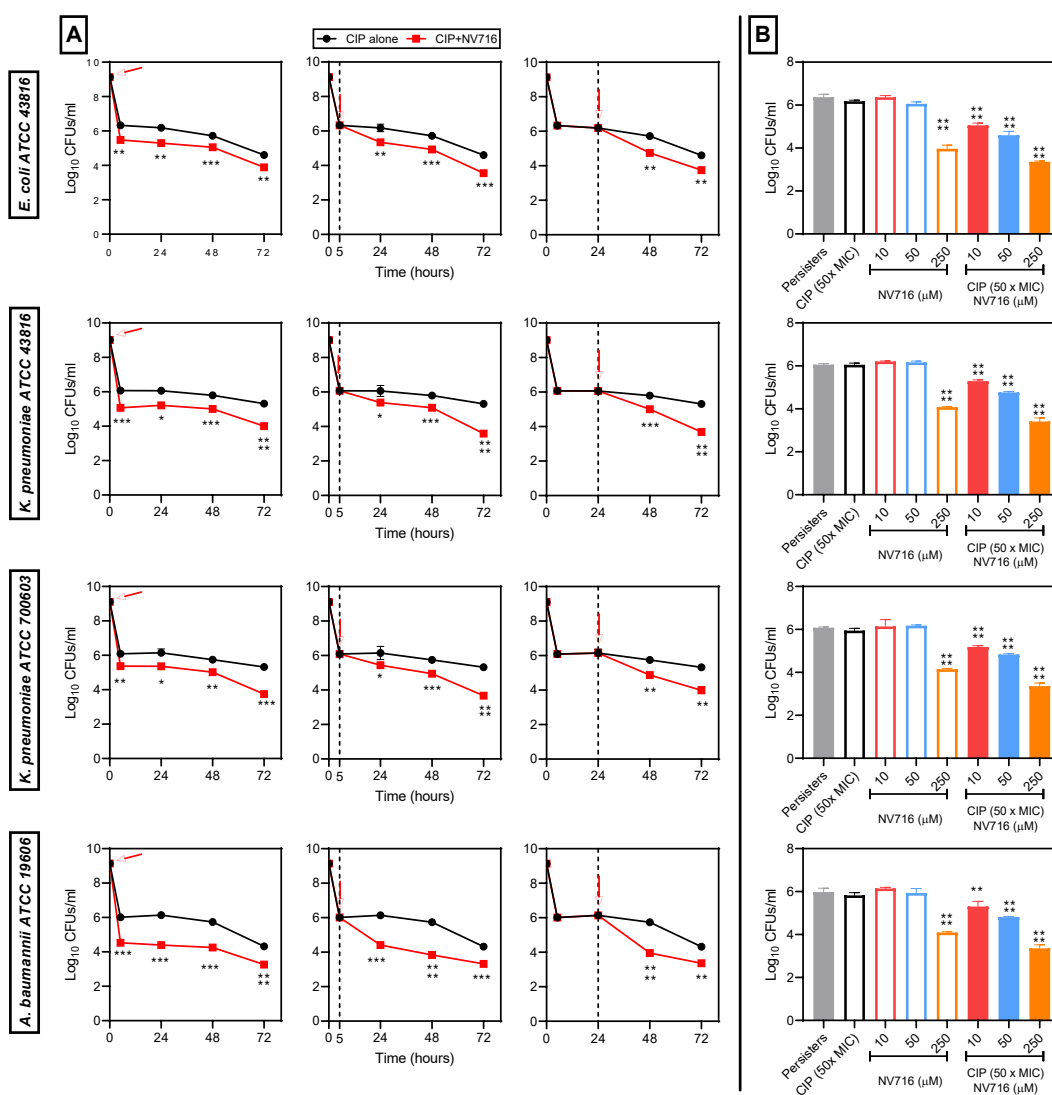


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**Figure 6.**  $E_{\max}$  (extrapolated for an infinitively large concentration) or  $E_{\text{highest}}$  (measured at the highest concentration tested but the plateau was not yet reached) for antibiotics alone (rifampicin [RIF], azithromycin [AZI], 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 figure 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  $\mu\text{M}$  PA $\beta$ N; blue: combined 38  $\mu\text{M}$  **NV731**; red: combined with 10  $\mu\text{M}$  **NV716**. Horizontal bars: mean. Statistical analysis: one-way ANOVA with Dunnett post-hoc test (paired) comparing each combination to the antibiotic alone: \*,  $p < 0.05$ ; \*\*,  $p < 0.01$ ; \*\*\*,  $p < 0.001$ ; \*\*\*\*,  $p < 0.0001$ .



**Figure 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  $\mu$ M **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  $\pm$  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  $\pm$  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: \*,  $P < 0.05$ ; \*\*,  $P < 0.01$ ; \*\*\*,  $P < 0.001$ ; \*\*\*\*,  $P < 0.0001$ .



**Figure 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  $\mu$ M PA $\beta$ N and **NV731** and 10  $\mu$ M **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  $\pm$  SEM (triplicates from three independent experiments). Statistical analysis: one-way ANOVA with Dunnett's post-hoc test comparing each combination to RIF alone: \*,  $p < 0.05$ ; \*\*,  $p < 0.01$ ; \*\*\*,  $p < 0.001$ ; \*\*\*\*,  $p < 0.0001$ .

