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Revision for Antimicrobial Agents and Chemotherapy

The polyaminoisoprenyl **R2.1**potentiator NV716 revives old disused antibiotics against intracellular forms of infection by *Pseudomonas aeruginosa*

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Abstract:

Active efflux confers intrinsic resistance to multiple antibiotics in *Pseudomonas aeruginosa*, including old disused molecules. Beside resistance, intracellular survival is another reason for failure to eradicate bacteria with antibiotics. We evaluated the capacity of polyaminoisoprenyl **R2.1.potentiators (designed as efflux pump inhibitors** [EPIs]) NV716 and NV731 compared to PA β N to restore the activity of disused antibiotics (doxycycline, chloramphenicol [substrates for efflux], rifampin [not substrate]) in comparison with ciprofloxacin against intracellular *P. aeruginosa* (strains with variable efflux levels) in THP-1 monocytes exposed during 24h to antibiotics alone (0.003-100x MIC) or combined with EPIs. Pharmacodynamic parameters (apparent static concentrations [C_s]; maximal relative efficacy [E_{max}]) were calculated using the Hill equation of concentration-response curves. PA β N and NV731 moderately reduced (0-4 doubling dilutions) antibiotic MICs but did not affect their intracellular activity. NV716 markedly reduced (1-16 doubling dilutions) the MIC of all antibiotics (substrates or not for efflux; strains expressing efflux or not); it improved their relative potency and maximal efficacy (lower C_s ; more negative E_{max}) intracellularly. In parallel, NV716 reduced the persister fraction in stationary cultures when combined with ciprofloxacin. In contrast to PA β N and NV731 that act as EPIs against extracellular bacteria only, NV716 can resensitize *P. aeruginosa* to antibiotics whether substrates or not for efflux, both extracellularly and intracellularly. This suggests a complex mode of action that goes beyond a simple inhibition of efflux and reduces bacterial persistence. NV716 may appear as a useful adjuvant, including to disused antibiotics with low antipseudomonal activity, to improve their activity, including against intracellular *P. aeruginosa*.

1 **INTRODUCTION**

2

3 *Pseudomonas aeruginosa* is one of the so-called ESKAPE (*Enterococcus faecium*, *Staphylococcus*
4 *aureus*, *Klebsiella pneumoniae*, *Acinetobacter baumannii*, *Pseudomonas aeruginosa*, and *Enterobacter*
5 *species*) pathogens (1) that harbor multiple mechanisms of drug resistance. It is considered by the
6 World Health Organization (WHO) as a critical priority species for the search of innovative therapies (2).
7 *P. aeruginosa* has acquired a series of resistance mechanisms to all classes of commonly used
8 antibiotics (β -lactams, fluoroquinolones, aminoglycosides, and, more recently, polymyxins). Moreover,
9 it is also innately resistant to many antibiotics, mainly due to poor outer membrane permeability
10 and/or active efflux (3). Four main efflux systems, named MexAB-OprM, MexCD-OprJ, MexEF-OprN,
11 and MexXY-OprM, have been associated with increased MICs for a broad range of antimicrobial agents
12 (4), bringing the active concentrations above the levels that can be achieved in the serum of patients.
13 This explains why drugs like tetracyclines or chloramphenicol cannot be used against *P. aeruginosa* or
14 other Gram-negative bacteria, although they are capable of binding to their respective target in these
15 bacteria when tested in acellular systems (5, 6).

16

17 Another, often neglected, reason for antibiotic failure against *P. aeruginosa* is the capacity of
18 this bacterium to adopt specific lifestyles, such as intracellular survival. Although considered as an
19 extracellular pathogen, *P. aeruginosa* has been shown by many researchers as capable of invading and
20 surviving inside epithelial (7, 8) and phagocytic cells (9-11) *in vitro*, as well as in lung epithelial cells and
21 macrophages *in vivo* (12, 13). In these intracellular niches, bacteria are protected from the host
22 humoral immune defenses but also from antibiotics, which need to have access, accumulate, and
23 express their activity in the infected subcellular compartment (14, 15). Moreover, they can adopt
24 dormant phenotypes that do not respond anymore to antibiotics, like persisters. These are
25 subpopulations of otherwise antibiotic-susceptible bacteria that switch to a transient non-dividing
26 phenotype surviving to high concentrations of bactericidal antibiotics (16). We have recently provided
27 evidence for another species, *Staphylococcus aureus*, that bacteria surviving to antibiotics
28 intracellularly are persisters, explaining why antibiotics fail to eradicate them (17). Preliminary data
29 suggest that the same may hold true for *P. aeruginosa* (18).

30

31 As *P. aeruginosa* resistance is worryingly increasing, few new antibiotics have been approved
32 over the last years, and are essentially new derivatives in existing classes with improved activity or
33 reduced susceptibility to resistance mechanisms (19). In this context, reviving old and disused
34 antibiotics may appear as an attractive strategy (20), which should be accompanied by an in-depth
35 reassessment of their efficacy according to the modern standards imposed by
36 pharmacokinetic/pharmacodynamic (PK/PD) concepts, including in models of persistent infections like
37 intracellular survival.

38 One of the approaches proposed to increase the potency of drugs that are substrates for efflux
39 is to combine them with efflux pump inhibitors (EPIs)(19). PA β N (MC 207,110; Phenylalanine-arginine
40 β -naphthylamide (21)) and CCCP (Carbonyl cyanide m-chlorophenyl hydrazone (22)) have been widely
41 used *in vitro* to increase the accumulation of antibiotics within Gram-negative bacteria, but their
42 toxicity **R1.2** and inadequate pharmacokinetic properties preclude clinical applications (23, 24). Efforts
43 have been made to synthesize new efflux pumps inhibitors with improved safety profile (25). Among
44 them, polyaminoisoprenyl compounds **NV716** and **NV731** (see chemical structures in **Figure S1**)
45 demonstrated their capacity to restore chloramphenicol activity against *Enterobacteria* spp (26) and
46 one of them (**NV716**), that of chloramphenicol and doxycycline against *Pseudomonas aeruginosa* (27),
47 or of florfenicol against *Bordetella bronchiseptica* (28).

48

49 The aim of the present study was to evaluate the effects of **NV716** in comparison with that of
50 **NV731** (less potent against *P. aeruginosa* (27)) and PA β N (reference EPI) in combination with old and
51 disused antibiotics against the intracellular forms of *P. aeruginosa*, using strains expressing or not
52 efflux pumps to infect THP-1 monocytes. We exploited a previously established *in vitro*
53 pharmacodynamic model (11) that allows comparing key pharmacological descriptors of antibiotic
54 intracellular activity, namely their relative potency and maximal efficacy (29). As antibiotics, we
55 selected doxycycline, chloramphenicol (both substrates for efflux (see **Table S1**) and for which
56 preliminary data do exist regarding the beneficial effect of **NV716** (27)), rifampin (not substrate for
57 efflux), and ciprofloxacin, selected as the most active drug against intracellular *P. aeruginosa* in this

58 pre-established *in vitro* model (11). The results were examined in the light of the effect of these EPIs on
59 antibiotic MIC and on the selection of persisters in broth culture.

60

61 We showed that **NV716**, contrarily to **NV731** and PA β N, was capable of increasing the relative
62 potency and maximal efficacy of these antibiotics against intracellular *P. aeruginosa*, whether
63 substrates of not for efflux and in strains expressing or not efflux pumps. These effects were related to
64 drastic reductions in MIC and in persister fractions. Altogether, these data suggest that **NV716** does
65 not simply act by inhibiting efflux but could be a useful adjuvant, including to disused antibiotics with
66 low antipseudomonal activity, to improve their activity also against intracellular *P. aeruginosa*.

67 RESULTS

68

69 Antimicrobial susceptibility

70 **Table 1** shows the MICs of antibiotics against PAO1, PAO509 (deleted in the genes encoding 5 efflux
71 pumps), 205-2 and BV1 (two clinical isolates expressing respectively a non-functional and a functional
72 MexAB-OprM efflux pump), in the absence of or in the presence of 20 mg/L (38 μ M) PA β N, or 4 mg/L
73 (10 μ M) **NV731** or **NV716**. These concentrations were selected because commonly used in previous
74 works (21, 27). In control conditions (no inhibitor), ciprofloxacin, doxycycline, and chloramphenicol
75 showed much lower MICs against PAO509 than against PAO1, in accordance with the fact they are
76 substrates for efflux (see **Table S1**). Conversely, the MIC of rifampin, which is not a substrate, remained
77 unchanged. BV1 was less susceptible than PAO1 to ciprofloxacin and doxycycline and more susceptible
78 to chloramphenicol, while 205-2 was less susceptible to ciprofloxacin only. PA β N and **NV731** reduced
79 antibiotic MICs in few cases, while **NV716** systematically decreased them, except for ciprofloxacin in
80 PAO509, the MIC of which was already very low. Of note, the effect of **NV716** was as (or even more)
81 important when testing strains that do not express efflux pumps or those that do, as well as when
82 using rifampin (not substrate for efflux) or other antibiotics (all substrates for efflux).

83

84 Cytotoxicity of inhibitors and antibiotics

85 In a first set of experiments, we evaluated the cytotoxicity of the EPIs and antibiotics in the conditions
86 of their use for intracellular infection by trypan blue exclusion test after 24 h incubation. Infection of
87 THP-1 cells by PAO1 caused 85% cell mortality in the absence of antibiotic. The inhibitors alone caused
88 concentration-dependent cell mortality, which was minimal at the concentration used for intracellular
89 infection studies (**Figure 1, left panel**). Doxycycline, chloramphenicol, and rifampin showed toxic effects
90 at high concentrations, with IC₅₀ (i.e. concentration causing 50% cell mortality) reaching approx. 30
91 times their respective MIC in infected cells (**Figure 1, right panel**). Ciprofloxacin was much less toxic at
92 equivalent multiples of its MIC (which corresponds to much lower mass concentrations). IC₅₀ values for
93 toxicity of inhibitors, antibiotics or their combinations in non-infected and infected cells are detailed in
94 supplementary **Figure S2** and **Table S2** but do not differ from what is described in Figure 1.

95

96 **Activity of antibiotics alone or combined with EPIs against intracellular *P. aeruginosa* in an *in vitro***
97 **pharmacodynamic model**

98 **Figure 2** shows the concentration-response curves against intracellular PAO1 in the absence of or in
99 the presence of **NV716**. The activity of antibiotics alone developed following a sigmoidal
100 concentration-response curve, as previously described (11). At low, subMIC concentrations, a $\sim 3 \log_{10}$
101 increase in CFUs was noticed over the 24 h incubation period. A static effect was observed at
102 extracellular concentrations close to the MIC of each drug. At the highest concentration tested, the
103 reduction in bacterial counts ranged from 2 \log_{10} CFU decrease for chloramphenicol, doxycycline, and
104 rifampin, to 2.6 \log_{10} CFU decrease for ciprofloxacin. In the presence of **NV716** at 10 μM , all curves
105 (except that of ciprofloxacin) were shifted to the left, meaning that the corresponding antibiotic was
106 more potent, a static effect being reached at lower extracellular concentrations. Furthermore, the
107 maximal reduction in intracellular counts was also increased, indicating that the antibiotics were more
108 effective.

109 The same type of experiment was performed with the other EPIs and the other strains. **Figure 3**
110 illustrates the pharmacodynamic parameters calculated based on the Hill equation of these
111 concentration-response curves (see methods for a definition of these parameters), namely the C_s (a
112 measure of the relative potency of the drug or the drug combination) and the E_{max} (a measure the
113 relative maximal efficacy of the drug alone or in combination). C_s of antibiotics alone were close or
114 slightly higher than the MICs and systematically reduced of 1 doubling dilution or more by **NV716**,
115 except for ciprofloxacin against PAO1 and PA0509, which were already highly susceptible to this drug.
116 **NV731** and PA β N did not show any significant effect on C_s values. Similarly, **NV716** was the only EPI
117 capable of increasing the efficacy of the 4 drugs against all strains, with E_{max} values being at least 0.5
118 \log_{10} CFU more negative than those measured for the antibiotics alone.

119

120 **Concentration-response curves of NV716 combined with fixed concentrations of antibiotics against**
121 **intracellular *P. aeruginosa***

122 **NV716** being the only adjuvant capable of improving both the relative potency and the relative
123 maximal efficacy of antibiotics, we examined the concentration-dependency of its effect when
124 combined with antibiotics at fixed concentrations corresponding to their respective C_{min} and C_{max} in

125 human serum (Table S1 for these values). The data are illustrated in Figure 4. Considering first the
126 activity of NV716 alone, a $\sim 3 \log_{10}$ increase in CFU was observed after 24 h incubation with low NV716
127 concentrations ($\leq 10 \mu\text{M}$) for all strains except PAO509, for which a slight reduction in CFU was already
128 observed at $10 \mu\text{M}$. A static effect was obtained for NV716 concentrations close to its MIC in RPMI-
129 1640 ($25 \mu\text{M}$) for all strains. At $50 \mu\text{M}$, NV716 caused a reduction in bacterial counts that ranged from
130 $\sim 1 \log_{10}$ CFU decrease for PAO1, 205-2 and BV-1, to $\sim 2 \log_{10}$ CFU decrease for PAO509. These data are
131 coherent with those obtained in broth (Figure S3), showing that NV716 did not prevent PAO1 growth
132 for concentrations $\leq 10 \mu\text{M}$ but caused a rapid decrease in bacterial counts at $50 \mu\text{M}$. In the presence
133 of antibiotics at their C_{\min} , we noticed that the intracellular concentration-response curves were
134 globally parallel to those obtained with NV716 alone, reflecting thus essentially the activity of the EPI.
135 Three exceptions need however to be highlighted. First, the activity of chloramphenicol was increased
136 for NV716 concentrations $> 3 \mu\text{M}$ against all strains. Second, an improvement in antibiotic activity was
137 noticed against PAO509, as soon as NV716 concentration was $> 3 \mu\text{M}$ for all drugs that are substrates
138 for efflux (ciprofloxacin, chloramphenicol and doxycycline). Third, at the highest concentration of
139 NV716 tested ($50 \mu\text{M}$), the reduction in CFU counts was $0.5\text{-}1.8 \log_{10}$ more important than that
140 observed with NV716 alone for all drugs, denoting some sort of synergy. Combining NV716 with
141 antibiotics at their C_{\max} caused a drastic improvement of activity, with CFU counts being reduced from
142 $0.5\text{-}2.2 \log_{10}$ at NV716 concentrations $\leq 10 \mu\text{M}$ to $1.4\text{-}5.6 \log_{10}$ at higher NV716 concentrations in
143 comparison with CFU counts measured for the antibiotic alone.

144

145 **Persister assay**

146 The fact that NV716 increases the intracellular efficacy of antibiotics (improved killing capacity)
147 suggests it may decrease the proportion of antibiotic persisters in the population, recently identified as
148 a cause of failure to eradicate intracellular *S. aureus* with antibiotics (30). We therefore examined the
149 persister fraction surviving to ciprofloxacin (selected as highly bactericidal antibiotic) alone or
150 combined with NV716 in stationary phases cultures. As shown in the left panel of Figure 5A,
151 ciprofloxacin at $50\times$ MIC caused a rapid drop in CFU counts of PAO1 over the 2 first hours of incubation,
152 after which no further change was observed, leaving a persisting fraction of 0.005% of the initial
153 inoculum. The same type of experiment was then performed with PAO1 and PAO509 exposed to

154 **NV716** at increasing concentrations and used alone or combined with 50x MIC of ciprofloxacin. The
155 right panels of Figure 5A show the proportion of persisters after 5 h of incubation, expressed in
156 percentage of the CFU counts measured in the absence of drugs. **NV716** alone had no effect on
157 persisters for concentrations $\leq 100 \mu\text{M}$ but caused a marked reduction in bacterial counts at higher
158 concentrations (3-5 \log_{10} CFU for concentrations $> 300 \mu\text{M}$ for PAO1 and $> 100 \mu\text{M}$ for PAO509).
159 Strikingly, **NV716** at lower concentrations (10-100 μM) was already capable to reduce up to 11- (PAO1)
160 to 28- (PAO509) fold the percentage of persisters selected by ciprofloxacin.

161 **R2.3** To investigate whether **NV716** needs to be administered simultaneously with ciprofloxacin to
162 obtain a maximal activity, we repeated killing experiments with stationary phase cultures exposed to
163 50 x MIC of ciprofloxacin alone or combined with **NV716** added at different time points (Figure 5B) (31).
164 CFUs were significantly reduced after the addition of **NV716** at any time point, for concentrations ≥ 10
165 μM and in both PAO1 and PAO509, indicating that **NV716** does not need to be added at the same time
166 as the drug to be able to increase its activity. In a next step, we aimed at examining whether **NV716**
167 action was related to a direct capacity to kill persisters or rather to resensitize them to the killing effect
168 of ciprofloxacin. To this end, we isolated the persisters surviving after 5 h of incubation with
169 ciprofloxacin at 50 x MIC and reexposed them to ciprofloxacin at 50 x MIC, **NV716** at different
170 concentrations, or a combination thereof (Figure 5C) (31). As expected, incubation of the isolated
171 persisters with ciprofloxacin alone caused only a marginal decrease in CFUs for both strains, confirming
172 the effective isolation of persister cells. When used alone, **NV716** caused a significant decrease in CFUs
173 only at the highest concentration tested (250 μM). In contrast, a significant reduction in CFUs was
174 already observed when it was combined at 10 μM with ciprofloxacin for both strains.

175

176 **DISCUSSION**

177

178 In this study we show that, in contrast with PA β N and **NV731** that act as efflux inhibitors against
179 planktonic bacteria only, **NV716** is capable to re-sensitize *P. aeruginosa* to antibiotics whether
180 substrates (doxycycline, chloramphenicol, ciprofloxacin) or not (rifampin) for efflux, not only in broth
181 but also intracellularly.

182

183 Considering first susceptibility data in broth, we observed a remarkable effect of **NV716** on the
184 antibiotic intrinsic activity. While PA β N and **NV731** moderately reduced the MICs of antibiotics
185 (essentially those that are substrates for efflux), **NV716** caused a marked decrease in the MICs of all
186 drugs, comprising rifampin (not substrate for efflux; see Table S1) against all strains, including PAO509
187 that does not express efflux pumps or BV1 that expresses a non-functional MexAB-OprM efflux pump.
188 This suggests that its mode of action is not related to the inhibition of efflux only, but probably also
189 relies on its capacity to permeabilize the outer membrane of the *P. aeruginosa* (27). Of note, the
190 synergy with antibiotics is obtained at sub-inhibitory concentrations of **NV716**, R1.3, indicating that, if a
191 direct effect on bacteria takes place, it is not sufficient to cause bacterial death. PA β N has also been
192 shown to permeabilize the outer membrane of Gram-negative bacteria by displacing Mg²⁺ and Ca²⁺
193 (32), but at concentrations higher than the one we used here, established as adequate to inhibit efflux
194 in princeps studies with this molecule (21). The activity of **NV716** and of the two other EPIs is observed
195 at concentrations that do not cause cytotoxicity for eukaryotic cells, suggesting no or minimal
196 interaction with eukaryotic membranes.

197

198 This contrasts with the cytotoxicity noticed for the three old and disused antibiotics, which
199 caused significant loss of viability of THP-1 monocytes at the highest concentrations tested here, which
200 are largely above those measured in the plasma of treated patients, owing to the high MIC of these
201 drugs against *P. aeruginosa*. We had thus to limit the range of antibiotic concentrations tested in our
202 concentration-response curves against intracellular bacteria in order to avoid a too massive loss of cells.

203

204 Considering then intracellular activity data, we noticed that all antibiotics displayed a
205 bacteriostatic activity at concentrations close or slightly higher than their MIC (C_s being considered as a
206 measure of the intracellular MIC), despite the fact they all accumulate to some extent in eukaryotic
207 cells (33) (Table S1). This is in line with our previous observations with other antibiotics in the same
208 model (11) or in similar models of intracellular infections by other bacterial species (34, 35), and was
209 interpreted as denoting a poor intracellular bioavailability. While PA β N and **NV731** did not increase this
210 relative potency (minimal modification in C_s), **NV716** caused a shift of the concentrations-response
211 curves to lower concentrations, with a commensurate reduction in the C_s , except for ciprofloxacin
212 against PAO1 and PAO509, the MIC of which was already rather low. Thus, **NV716** can decrease the
213 MIC of antibiotics not only extracellularly, but also intracellularly. We did not measure the cellular
214 concentration of **NV716** in the cells, but these activity data suggest that it has access to intracellular
215 bacteria in sufficient concentrations to exert its synergistic effect. The absence of gain in relative
216 potency observed with PA β N and **NV731** can be consecutive to their lower intrinsic effects on MICs (as
217 observed in broth) or possibly also to a lower penetration inside the cells, which has not been
218 investigated.

219

220 Turning then our attention towards the intracellular maximal efficacy, we observed, for
221 antibiotics used alone, reductions of 2-2.5 \log_{10} CFU with ciprofloxacin, in accordance with our previous
222 results (11), and of 1-2 \log_{10} CFU for the other antibiotics, the lowest effect being observed for
223 chloramphenicol and doxycycline against 205-2, indicating individual differences in responsiveness
224 among strains. In a broader context, the extent of intracellular killing may also differ depending on the
225 bacterial species. An intracellular E_{\max} of -1.8 \log_{10} CFU was noticed for rifampin against a small colony
226 variant of *S. aureus* (36), but the activity of chloramphenicol and doxycycline has not been investigated
227 in this model. Chloramphenicol demonstrated bacteriostatic activity against intracellular *Salmonella*
228 Typhimurium at 10x MIC (37) while doxycycline reduced of 1.5 \log_{10} the intracellular CFU of *Francisella*
229 *tularensis* after 24 h of incubation at a concentration close to its MIC (38). For fluoroquinolones, a
230 maximal reduction of 2 to 4 \log_{10} CFU was observed in concentration-response experiments similar to
231 those performed here against *S. aureus*, *Listeria monocytogenes*, *Legionella pneumophila*, *Burkholderia*
232 *thailandensis*, *Yersinia pseudotuberculosis*, or *Francisella philomiragia* (34, 35, 39).

233 While PAβN and **NV731** did not affect the efficacy of any antibiotic against none of the strains,
234 **NV716** systematically improved it (more negative E_{max}), suggesting an effect on bacterial
235 responsiveness to antibiotics. We can most likely preclude interference related to the release of
236 bacteria out of the cells before lysis or to the **R1.6** remaining amount of antibiotics accumulated in cells
237 if considering that (a) **NV716** did not increase the cytotoxicity of antibiotics, and (b) charcoal was
238 added to culture plates to adsorb residual antibiotic. Thus, the gain in intracellular efficacy observed in
239 the presence of **NV716** is rather suggestive of improved bacterial responsiveness to the drugs. We
240 previously showed an increase in the intracellular efficacy of fluoroquinolones against an insertion
241 mutant in a putative deacetylase in PAO1, which showed a lower persister character in planktonic
242 cultures (18). Here, we found that **NV716** was capable of decreasing the proportion of persisters
243 selected by ciprofloxacin in a stationary phase culture at concentrations for which it did not cause any
244 bacterial killing by itself **R2.3** and also of reducing at subMIC concentrations the number of persisters
245 when combined with ciprofloxacin but not when used alone, suggesting a mode of action unrelated to
246 a direct bactericidal effect but rather to a resensitization of persisters to the antibiotic (31). Of interest,
247 other molecules capable of disrupting membrane integrity also reduced the residual fraction of
248 persisters upon exposure to antibiotics for different bacterial species (40-42). One of them (SPI009; 1-
249 ((2,4-Dichlorophenethyl)Amino)-3-Phenoxypropan-2-ol)) was also shown to increase the efficacy of
250 ciprofloxacin against intracellular *P. aeruginosa* (43).

251

252 This work suffers from some limitations. Firstly, we used a single phagocytic cell line (THP-1
253 monocytes), which does not necessarily represent the repertoire of cells infected by *P. aeruginosa* *in*
254 *vivo*. The reason for selecting this permissive cell line was that it allows assessing the effect of
255 antibiotics without interference of cell defense mechanisms. Secondly, we did not measure the cellular
256 accumulation of antibiotics and EPIs in our experimental conditions, but setting-up the necessary
257 methodologies would represent a work by itself. Thirdly, we did not study in details the occurrence of
258 intracellular persisters, the aim of the study being rather oriented towards an in-depth
259 pharmacodynamic evaluation of the possible interest of EPIs to restore the activity of old and disused
260 antibiotics against intracellular *P. aeruginosa*.

261

262 If we thus examine our data in a more clinically-oriented perspective, we see that **NV716** is
263 capable of improving the activity of all antibiotics in the range of concentrations achieved in the serum
264 of patients as soon as its extracellular concentration exceeds 3 μM . Ten times higher concentrations
265 allow to reach a 2-3 \log_{10} CFU decrease when combined with antibiotics at their human C_{max} , pleading
266 for examining the possible interest of this compound *in vivo*.
267

268 **MATERIALS AND METHODS**

269

270 **Bacterial strains and culture media**

271 **Table 1** shows the reference and clinical strains used in the study. R2.m2BV-1 has been isolated in 2006
272 at the Queen's University Belfast, Belfast, UK, from a patient with cystic fibrosis. It shows a truncation
273 in MexB (672 amino acids instead of 1046) that drastically reduced the activity of efflux transporter
274 and its impact of the MIC of the preferential MexAB-OprM antibiotic substrate temocillin (44). 205 has
275 been isolated in 2012 at the University Hospital Münster, Münster, Germany, from a patient with cystic
276 fibrosis (45), and shows a fully active MexAB-OprM efflux pump, based on the elevated MIC of
277 temocillin.

278 Bacteria were R2.m1 incubated overnight at 37°C in Tryptic Soy Agar (TSA; VWR; Radnor, PA). A single
279 colony was then R2.m1 added in 10mL cation-adjusted Mueller-Hinton Broth (CA-MHB; BD Life Sciences,
280 Franklin Lakes, NJ) and incubated at 37°C overnight under gentle agitation (130 rpm). TSA
281 supplemented with 2g/L charcoal (Sigma-Aldrich, St Louis, MO) was used for CFU counting (colony-
282 forming unit). Cell culture media (RPMI-1640) and human and fetal bovine sera were from
283 Gibco/ThermoFisher Scientific (Waltham, MA).

284

285 **Antibiotics and Inhibitors**

286 Chloramphenicol (potency, 98%), doxycycline (potency, 98%), and rifampin (potency, 98%) were
287 obtained as microbiological standards from Sigma-Aldrich (St Louis, MO), ciprofloxacin HCl (potency,
288 89%) from Bayer (Leverkusen, Germany) and gentamicin sulfate (potency, 60.7%), from PnReac
289 AppliChem (Darmstadt, Germany). **Table S1** shows relevant properties of the antibiotics used in this
290 study. The reference efflux pump inhibitor Phe-Arg β -Naphthylamide (PA β N; potency, 98%) was
291 purchased from Sigma-Aldrich. The hydrosoluble hydrochloride polyamino-isoprenic salt derivatives
292 **NV716** and **NV731** were synthesized at Aix-Marseille University (26). Stock solutions of these inhibitors
293 were prepared in dimethyl sulfoxide (DMSO) at concentrations of 96 mM [50 mg/mL] for PA β N or in
294 water at 10 mM [4 mg/L] for **NV716** and **NV731**, respectively, and stored at -20°C until use.

295

296 Susceptibility Testing

297 MICs of antibiotics against *P. aeruginosa* were measured by serial 2-fold microdilution in MHB-CA
298 according to CLSI guidelines in control conditions or in the presence of 38 μM [20 mg/L](21) PA β N or of
299 10 μM [4 mg/L] **NV731** or **NV716** (27).

300

301 Cytotoxicity assessment (Trypan blue exclusion assay)

302 THP-1 cells (7.5×10^5 cells per mL) were incubated for 24 hours in 96-wells plates with the antibiotics
303 or inhibitors under study over a wide range of concentrations. At the end of the incubation, viability
304 was assessed using trypan blue exclusion test (vital colorant excluded from viable cells) using a
305 previously described method (29), with specific adaptations to the purpose of our study. Briefly, 50 μL
306 of trypan blue reagent was added to 50 μL of cell suspension. After 10 min of incubation at 37°C, non
307 colored (viable) cells were counted using a Fuchs-Rosenthal counting chamber (Tiefe 0.2mm). Cells
308 were counted in the same surface for all conditions (1 big square made of 16 single squares (0.0625
309 mm^2 for each single square). The percentage of cytotoxicity was evaluated based on the reduction in
310 the number of living cells according to the following equation:

$$311 \text{ cytotoxicity (\%)} = \frac{\text{Nb uncolored cells in controls} - \text{Nb uncolored treated cells}}{\text{Nb uncolored cells in controls}} \times 100.$$

312

313 Intracellular Infection

314 All experiments were performed using human THP-1 monocytes cultured in RPMI-1640 medium
315 supplemented with 10% fetal bovine serum (FBS) based on a previously established protocol (11). In
316 brief, bacteria were opsonized by incubation during 1 h in RPMI-1640 medium supplemented with 10%
317 human serum at 37°C with gentle agitation (130 rpm). Phagocytosis of opsonized bacteria was then
318 allowed for 2 h using a bacterium:cell ratio of 10:1, after which non-phagocytosed bacteria were
319 eliminated by incubation for 1 h with gentamicin at 50x MIC. After 3 washings with PBS, infected cells
320 were resuspended in the original volume of RPMI-1640 with 10% FBS. The post-phagocytosis inoculum
321 was consistently 5 to 7×10^5 CFU/mg of cell protein (defined as time zero). Infected cells were then
322 incubated for 24 h with antibiotics over a broad range of concentrations (0.003-100x their MIC) in 12-
323 well plates. After 24 h of incubation, infected cells were pelleted by centrifugation, washed once with
324 PBS at room temperature to eliminate extracellular bacteria and collected in 1 mL distilled water, to

325 achieve complete cell lysis. Lysates were used for determining CFU counts by spreading on agar
326 supplemented with 2g/L charcoal (to avoid carry-over effect, especially in samples that had been
327 incubated with high antibiotic concentrations [R1.5.no difference in colony counts was observed
328 between plates supplemented or not with charcoal for samples exposed to low antibiotic
329 concentrations, ruling out any interfering effect of charcoal in the assay]) and protein content using a
330 commercially available kit (Bio-Rad DC Protein Assay, Bio-Rad Laboratories, Hercules, CA). The activity
331 was expressed as the change in CFU (normalized by mg of cell protein) from the initial inoculum after
332 24 hours of incubation. The data were used to fit a sigmoidal function and calculate pharmacodynamic
333 parameters based on the corresponding Hill-Langmuir equation (apparent static concentrations [C_s], i.e.
334 extracellular concentration resulting in no apparent intracellular growth, and maximal relative efficacy
335 [E_{max}], i.e. maximal decrease in bacterial counts compared to the post-phagocytosis inoculum as
336 extrapolated for an infinitely large antibiotic concentration).

337

338 **Persister assay and isolation**

339 A single colony from overnight culture of PAO1 and PAO509 on TSA was R2.m1.added to in 200mL
340 MHB-CA in a 1 L flask and incubated for 24 h under agitation (130 rpm) at 37°C. 10 mL of the bacterial
341 suspension were then incubated with 50x MIC ciprofloxacin for 5 hours at 37°C under agitation (130
342 rpm), in the presence of or in the absence of **NV716**. Aliquots were serially diluted in PBS and spread
343 on TSA supplemented with 2 g/L charcoal for CFU counting. The persister fraction was defined as the
344 number of CFU growing at the end of this experiment as compared to the initial inoculum (46). R2.3For
345 experiments using isolated persisters, stationary-phase cultures were incubated with ciprofloxacin (50x
346 MIC) during 5 hours, i.e. condition for which a plateau of killing was reached. The remaining bacteria
347 were washed twice with sterile Phosphate Buffered Saline (PBS), centrifuged (5,200 g, 15 min, 4°C) and
348 used for killing assays (31).

349

350 **Curve fittings and statistical analyses.**

351 Curve fittings were performed with GraphPad Prism (version 8.4.3) software for Windows (GraphPad
352 Prism Software, San Diego, CA). Pharmacodynamic parameters were calculated based on Hill equations

353 of concentration-response curves and statistical analyses were executed with GraphPad InStat 3
354 version 3.10 (GraphPad Prism Software) or Graphpad Prism version 8.4.3.

355

356

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361

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References

1. Rice LB. 2008. Federal funding for the study of antimicrobial resistance in nosocomial pathogens: no ESKAPE. *J Infect Dis* 197:1079-1081. <https://doi.org/10.1086/533452>
2. Shrivastava S, Shrivastava P, Ramasamy J. 2018. World health organization releases global priority list of antibiotic-resistant bacteria to guide research, discovery, and development of new antibiotics. *Journal of Medical Society* 32:76-77. https://doi.org/10.4103/jms.jms_25_17
3. Mesaros N, Nordmann P, Plesiat P, Roussel-Delvallez M, Van Eldere J, Glupczynski Y, Van Laethem Y, Jacobs F, Lebecque P, Malfroot A, Tulkens PM, Van Bambeke F. 2007. *Pseudomonas aeruginosa*: resistance and therapeutic options at the turn of the new millennium. *Clin Microbiol Infect* 13:560-578. <https://doi.org/10.1111/j.1469-0691.2007.01681.x>
4. Poole K. 2001. Multidrug efflux pumps and antimicrobial resistance in *Pseudomonas aeruginosa* and related organisms. *J Mol Microbiol Biotechnol* 3:255-264.
5. Olson MW, Ruzin A, Feyfant E, Rush TS, 3rd, O'Connell J, Bradford PA. 2006. Functional, biophysical, and structural bases for antibacterial activity of tigecycline. *Antimicrob Agents Chemother* 50:2156-2166. <https://doi.org/10.1128/AAC.01499-05>
6. Vazquez D. 1964. Uptake and Binding of Chloramphenicol by Sensitive and Resistant Organisms. *Nature* 203:257-258. <https://doi.org/10.1038/203257a0>
7. Fleiszig SM, Zaidi TS, Fletcher EL, Preston MJ, Pier GB. 1994. *Pseudomonas aeruginosa* invades corneal epithelial cells during experimental infection. *Infect Immun* 62:3485-3493.
8. Emam A, Carter WG, Lingwood C. 2010. Glycolipid-Dependent, Protease Sensitive Internalization of *Pseudomonas aeruginosa* Into Cultured Human Respiratory Epithelial Cells. *Open Microbiol J* 4:106-115. <https://doi.org/10.2174/1874285801004010106>
9. Del Porto P, Cifani N, Guarnieri S, Di Domenico EG, Mariggio MA, Spadaro F, Guglietta S, Anile M, Venuta F, Quattrucci S, Ascenzioni F. 2011. Dysfunctional CFTR alters the bactericidal activity of human macrophages against *Pseudomonas aeruginosa*. *PLoS One* 6:e19970. <https://doi.org/10.1371/journal.pone.0019970>
10. Garai P, Berry L, Mousouni M, Bleves S, Blanc-Potard AB. 2019. Killing from the inside: Intracellular role of T3SS in the fate of *Pseudomonas aeruginosa* within macrophages revealed by *mgtC* and *oprF* mutants. *PLoS Pathog* 15:e1007812. <https://doi.org/10.1371/journal.ppat.1007812>
11. Buyck JM, Tulkens PM, Van Bambeke F. 2013. Pharmacodynamic evaluation of the intracellular activity of antibiotics towards *Pseudomonas aeruginosa* PAO1 in a model of THP-1 human monocytes. *Antimicrobial Agents and Chemotherapy* 57:2310-2318. <https://doi.org/10.1128/aac.02609-12>
12. Schmiedl A, Kerber-Momot T, Munder A, Pabst R, Tschernig T. 2010. Bacterial distribution in lung parenchyma early after pulmonary infection with *Pseudomonas aeruginosa*. *Cell Tissue Res* 342:67-73. <https://doi.org/10.1007/s00441-010-1036-y>
13. Zaas DW, Swan ZD, Brown BJ, Li G, Randell SH, Degan S, Sunday ME, Wright JR, Abraham SN. 2009. Counteracting signaling activities in lipid rafts associated with the invasion of lung epithelial cells by *Pseudomonas aeruginosa*. *J Biol Chem* 284:9955-9964. <https://doi.org/10.1074/jbc.M808629200>

14. Carryn S, Chanteux H, Seral C, Mingeot-Leclercq MP, Van Bambeke F, Tulkens PM. 2003. Intracellular pharmacodynamics of antibiotics. *Infect Dis Clin North Am* 17:615-634. [https://doi.org/10.1016/s0891-5520\(03\)00066-7](https://doi.org/10.1016/s0891-5520(03)00066-7)
15. Van Bambeke F, Barcia-Macay M, Lemaire S, Tulkens PM. 2006. Cellular pharmacodynamics and pharmacokinetics of antibiotics: current views and perspectives. *Curr Opin Drug Discov Devel* 9:218-230.
16. Balaban NQ, Helaine S, Lewis K, Ackermann M, Aldridge B, Andersson DI, Brynildsen MP, Bumann D, Camilli A, Collins JJ, Dehio C, Fortune S, Ghigo JM, Hardt WD, Harms A, Heinemann M, Hung DT, Jenal U, Levin BR, Michiels J, Storz G, Tan MW, Tenson T, Van Melderen L, Zinkernagel A. 2019. Definitions and guidelines for research on antibiotic persistence. *Nat Rev Microbiol* 17:441-448. <https://doi.org/10.1038/s41579-019-0196-3>
17. Peyrusson F, Varet H, Nguyen TK, Legendre R, Sismeiro O, Coppée J-Y, Wolz C, Tenson T, Van Bambeke F. 2020. Intracellular *Staphylococcus aureus* persists upon antibiotic exposure. *Nature Communications* 11:2200. <https://doi.org/10.1038/s41467-020-15966-7>
18. Khandekar S, Liebens V, Fauvart M, Tulkens PM, Michiels J, Van Bambeke F. 2018. The Putative De-N-acetylase DnpA Contributes to Intracellular and Biofilm-Associated Persistence of *Pseudomonas aeruginosa* Exposed to Fluoroquinolones. *Front Microbiol* 9:1455. <https://doi.org/10.3389/fmicb.2018.01455>
19. Burrows LL. 2018. The Therapeutic Pipeline for *Pseudomonas aeruginosa* Infections. *ACS Infect Dis* 4:1041-1047. <https://doi.org/10.1021/acsinfecdis.8b00112>
20. Theuretzbacher U, Van Bambeke F, Canton R, Giske CG, Mouton JW, Nation RL, Paul M, Turnidge JD, Kahlmeter G. 2015. Reviving old antibiotics. *J Antimicrob Chemother* 70:2177-2181. <https://doi.org/10.1093/jac/dkv157>
21. Lomovskaya O, Warren MS, Lee A, Galazzo J, Fronko R, Lee M, Blais J, Cho D, Chamberland S, Renau T, Leger R, Hecker S, Watkins W, Hoshino K, Ishida H, Lee VJ. 2001. Identification and characterization of inhibitors of multidrug resistance efflux pumps in *Pseudomonas aeruginosa*: novel agents for combination therapy. *Antimicrobial agents and chemotherapy* 45:105-116. <https://doi.org/10.1128/AAC.45.1.105-116.2001>
22. Pages JM, Masi M, Barbe J. 2005. Inhibitors of efflux pumps in Gram-negative bacteria. *Trends Mol Med* 11:382-389. <https://doi.org/10.1016/j.molmed.2005.06.006>
23. Lomovskaya O, Bostian KA. 2006. Practical applications and feasibility of efflux pump inhibitors in the clinic--a vision for applied use. *Biochem Pharmacol* 71:910-918. <https://doi.org/10.1016/j.bcp.2005.12.008>
24. Mahamoud A, Chevalier J, Alibert-Franco S, Kern WV, Pages JM. 2007. Antibiotic efflux pumps in Gram-negative bacteria: the inhibitor response strategy. *J Antimicrob Chemother* 59:1223-1229. <https://doi.org/10.1093/jac/dkl493>
25. Douafer H, Andrieu V, Phanstiel Ot, Brunel JM. 2019. Antibiotic Adjuvants: Make Antibiotics Great Again! *J Med Chem* 62:8665-8681. <https://doi.org/10.1021/acs.jmedchem.8b01781>
26. Brunel JM, Lieutaud A, Lome V, Pages JM, Bolla JM. 2013. Polyamino geranic derivatives as new chemosensitizers to combat antibiotic resistant gram-negative bacteria. *Bioorg Med Chem* 21:1174-1179. <https://doi.org/10.1016/j.bmc.2012.12.030>

27. Borselli D, Lieutaud A, Thefenne H, Garnotel E, Pages JM, Brunel JM, Bolla JM. 2016. Polyamino-Isoprenic Derivatives Block Intrinsic Resistance of *P. aeruginosa* to Doxycycline and Chloramphenicol In Vitro. PLoS One 11:e0154490. <https://doi.org/10.1371/journal.pone.0154490>
28. Borselli D, Brunel JM, Gorge O, Bolla JM. 2019. Polyamino-Isoprenyl Derivatives as Antibiotic Adjuvants and Motility Inhibitors for *Bordetella bronchiseptica* Porcine Pulmonary Infection Treatment. Front Microbiol 10:1771. <https://doi.org/10.3389/fmicb.2019.01771>
29. Buyck JM, Lemaire S, Seral C, Anantharajah A, Peyrusson F, Tulkens PM, Van Bambeke F. 2016. In Vitro Models for the Study of the Intracellular Activity of Antibiotics. Methods Mol Biol 1333:147-157. https://doi.org/10.1007/978-1-4939-2854-5_13
30. Nguyen TK, Peyrusson F, Dodémont M, Pham NH, Nguyen NH, Tulkens PM, Van Bambeke F. 2020. The persister character of clinical isolates of *Staphylococcus aureus* contributes to faster evolution to resistance and higher survival in THP-1 monocytes: a study with moxifloxacin. . Front Microbiol in minor revision.
31. Liebens V, Defraigne V, Knapen W, Swings T, Beullens S, Corbau R, Marchand A, Chaltin P, Fauvart M, Michiels J. 2017. Identification of 1-((2,4-Dichlorophenethyl)Amino)-3-Phenoxypropan-2-ol, a Novel Antibacterial Compound Active against Persisters of *Pseudomonas aeruginosa*. Antimicrob Agents Chemother 61. <https://doi.org/10.1128/AAC.00836-17>
32. Lamers RP, Cavallari JF, Burrows LL. 2013. The efflux inhibitor phenylalanine-arginine beta-naphthylamide (PAbetaN) permeabilizes the outer membrane of gram-negative bacteria. PLoS One 8:e60666. <https://doi.org/10.1371/journal.pone.0060666>
33. Tulkens PM. 1991. Intracellular distribution and activity of antibiotics. Eur J Clin Microbiol Infect Dis 10:100-106. <https://doi.org/10.1007/BF01964420>
34. Barcia-Macay M, Seral C, Mingeot-Leclercq MP, Tulkens PM, Van Bambeke F. 2006. pharmacodynamic evaluation of the intracellular activities of antibiotics against *staphylococcus aureus* in a model of thp-1 macrophages. Antimicrob Agents Chemother 50:841-851. <https://doi.org/10.1128/aac.50.3.841-851.2006>
35. Lemaire S, Van Bambeke F, Tulkens PM. 2011. Activity of finafloxacin, a novel fluoroquinolone with increased activity at acid pH, towards extracellular and intracellular *Staphylococcus aureus*, *Listeria monocytogenes* and *Legionella pneumophila*. Int J Antimicrob Agents 38:52-59. <https://doi.org/10.1016/j.ijantimicag.2011.03.002>
36. Nguyen HA, Denis O, Vergison A, Theunis A, Tulkens PM, Struelens MJ, Van Bambeke F. 2009. Intracellular activity of antibiotics in a model of human THP-1 macrophages infected by a *Staphylococcus aureus* small-colony variant strain isolated from a cystic fibrosis patient: pharmacodynamic evaluation and comparison with isogenic normal-phenotype and revertant strains. Antimicrob Agents Chemother 53:1434-1442. <https://doi.org/10.1128/AAC.01145-08>
37. Chiu CH, Lin TY, Ou JT. 1999. In vitro evaluation of intracellular activity of antibiotics against non-typhoid *Salmonella*. Int J Antimicrob Agents 12:47-52. [https://doi.org/10.1016/s0924-8579\(99\)00038-2](https://doi.org/10.1016/s0924-8579(99)00038-2)
38. Maurin M, Mersali NF, Raoult D. 2000. Bactericidal activities of antibiotics against intracellular *Francisella tularensis*. Antimicrob Agents Chemother 44:3428-3431. <https://doi.org/10.1128/aac.44.12.3428-3431.2000>

39. Chalhoub H, Harding SV, Tulkens PM, Van Bambeke F. 2019. Influence of pH on the activity of fleroxacin against extracellular and intracellular *Burkholderia thailandensis*, *Yersinia pseudotuberculosis* and *Francisella philomiragia* and on its cellular pharmacokinetics in THP-1 monocytes. *Clin Microbiol Infect* doi:10.1016/j.cmi.2019.07.028. <https://doi.org/10.1016/j.cmi.2019.07.028>
40. Defraigne V, Liebens V, Loos E, Swings T, Weytjens B, Fierro C, Marchal K, Sharkey L, O'Neill AJ, Corbau R, Marchand A, Chaltin P, Fauvart M, Michiels J. 2018. 1-((2,4-Dichlorophenethyl)Amino)-3-Phenoxypropan-2-ol Kills *Pseudomonas aeruginosa* through Extensive Membrane Damage. *Front Microbiol* 9:129. <https://doi.org/10.3389/fmicb.2018.00129>
41. Nicol M, Mlouka MAB, Berthe T, Di Martino P, Jouenne T, Brunel JM, De E. 2019. Anti-persister activity of squalamine against *Acinetobacter baumannii*. *Int J Antimicrob Agents* 53:337-342. <https://doi.org/10.1016/j.ijantimicag.2018.11.004>
42. Naclerio GA, Sintim HO. 2020. Multiple ways to kill bacteria via inhibiting novel cell wall or membrane targets. *Future Med Chem* 12:1253-1279. <https://doi.org/10.4155/fmc-2020-0046>
43. Defraigne V, Verstraete L, Van Bambeke F, Anantharajah A, Townsend EM, Ramage G, Corbau R, Marchand A, Chaltin P, Fauvart M, Michiels J. 2017. Antibacterial Activity of 1-[(2,4-Dichlorophenethyl)amino]-3-Phenoxypropan-2-ol against Antibiotic-Resistant Strains of Diverse Bacterial Pathogens, Biofilms and in Pre-clinical Infection Models. *Front Microbiol* 8:2585. <https://doi.org/10.3389/fmicb.2017.02585>
44. Chalhoub H, Pletzer D, Weingart H, Braun Y, Tunney MM, Elborn JS, Rodriguez-Villalobos H, Plésiat P, Kahl BC, Denis O, Winterhalter M, Tulkens PM, Van Bambeke F. 2017. Mechanisms of intrinsic resistance and acquired susceptibility of *Pseudomonas aeruginosa* isolated from cystic fibrosis patients to temocillin, a revived antibiotic. *Sci Rep* 7:40208. <https://doi.org/10.1038/srep40208>
45. Mustafa M-H, Chalhoub H, Denis O, Deplano A, Vergison A, Rodriguez-Villalobos H, Tunney MM, Elborn JS, Kahl BC, Traore H, Vanderbist F, Tulkens PM, Van Bambeke F. 2016. Antimicrobial Susceptibility of *Pseudomonas aeruginosa* Isolated from Cystic Fibrosis Patients in Northern Europe. *Antimicrobial agents and chemotherapy* 60:6735-6741. <https://doi.org/10.1128/AAC.01046-16>
46. De Groote VN, Verstraeten N, Fauvart M, Kint CI, Verbeeck AM, Beullens S, Cornelis P, Michiels J. 2009. Novel persistence genes in *Pseudomonas aeruginosa* identified by high-throughput screening. *FEMS Microbiol Lett* 297:73-79. <https://doi.org/10.1111/j.1574-6968.2009.01657.x>
47. Stover CK, Pham XQ, Erwin AL, Mizoguchi SD, Warrener P, Hickey MJ, Brinkman FS, Hufnagle WO, Kowalik DJ, Lagrou M, Garber RL, Goltry L, Tolentino E, Westbrook-Wadman S, Yuan Y, Brody LL, Coulter SN, Folger KR, Kas A, Larbig K, Lim R, Smith K, Spencer D, Wong GK, Wu Z, Paulsen IT, Reizer J, Saier MH, Hancock RE, Lory S, Olson MV. 2000. Complete genome sequence of *Pseudomonas aeruginosa* PAO1, an opportunistic pathogen. *Nature* 406:959-964. <https://doi.org/10.1038/35023079>
48. Mima T, Joshi S, Gomez-Escalada M, Schweizer HP. 2007. Identification and characterization of TriABC-OpmH, a triclosan efflux pump of *Pseudomonas aeruginosa* requiring two membrane fusion proteins. *J Bacteriol* 189:7600-7609. <https://doi.org/10.1128/jb.00850-07>

Table 1. MICs of antibiotics and adjuvants (EPIs) alone or combined against reference strains and clinical isolates in broth.

Strains	Phenotype	Reference	Antibiotics ^b	MIC (mg/L) ^a and (fold-dilution decrease vs antibiotic alone)						R1.6 NV716 alone (μM)
				AB alone (mg/L)	+PAβN (38μM) [20mg/L]	PAβN alone (μM)	+NV731 (10μM) [4mg/L]	NV731 alone (μM)	+NV716 (10μM) [4mg/L]	
PAO1	Wild-type	(47)	CIP	0.25	0.06 (2)		0.06 (2)		0.03 (3)	50
			DOX	16	4 (2)	>200	4 (2)	>250	1 (4)	
			CHL	32	8 (2)		16 (1)		1 (5)	
			RIF	16	8 (1)		8 (1)		0.125 (7)	
PAO509	PAO1[Δ(MexAB-OprM), Δ(MexCD-OprJ), Δ(MexJK), Δ(MexXY), Δ(MexEF-OprN)]	(48)	CIP	0.016	0.008 (1)		0.016 (0)		0.008 (1)	25
			DOX	1	1 (0)		0.125 (3)		0.03 (4)	
			CHL	2	2 (0)	>200	1 (1)	>250	0.125 (4)	
			RIF	16	4 (2)		8 (1)		0.06 (7)	
BV1	Clinical isolate (expressing truncated, non-functional MexAB-OprM)	(44)	CIP	1	1 (0)		0.5 (1)		0.125 (3)	50
			DOX	64	8 (3)		2 (5)		0.125 (9)	
			CHL	8	4 (1)	>200	0.5 (4)	>250	0.06 (7)	
			RIF	32	16 (1)		4 (3)		0.008 (12)	
205-2	Clinical isolate (overexpressing functional MexAB)	(45)	CIP	4	2 (1)		4 (0)		1 (2)	50
			DOX	16	4 (2)	>200	8 (1)	>250	0.5 (5)	
			CHL	64	32 (1)		32 (1)		1 (6)	
			RIF	16	8 (1)		16 (0)		0.125 (16)	

^a Values in bold denote a decrease of at least 2 doubling dilutions vs. antibiotics alone

^b Abbreviations: AB: antibiotic; CIP, ciprofloxacin; DOX, doxycycline; CHL, chloramphenicol; RIF, rifampin. MICs were determined in at least two independent experiments.

Figure 1. Cytotoxicity of EPIs and antibiotics as determined by trypan blue exclusion test.

Left: cytotoxicity of efflux inhibitors in non-infected THP-1 cells incubated with increasing concentrations of NV716, NV731, or PA β N during 24 h. Right: cytotoxicity of antibiotics in THP-1 cells infected by PAO1 and incubated with increasing concentrations of ciprofloxacin (CIP), doxycycline (DOX), rifampin (RIF), or chloramphenicol (CHL) during 24 h. **R1.6** Antibiotic concentrations are expressed in multiples of their MIC against PAO1 (Log_{10} scale) The tick horizontal dotted lines show the percentage of cell mortality measured in untreated infected cells. The thin dotted line shows a 50% cell mortality threshold, allowing to estimate IC_{50} values for each compound (see Figure S2 and Table S2 for these values). The vertical arrows in the left panel show the inhibitor concentrations used in intracellular experiments **R1.2** ($10 \mu\text{M}$ for NV716 and $38 \mu\text{M}$ for PA β N). All data are means \pm SEM (triplicates from 3 independent experiments).

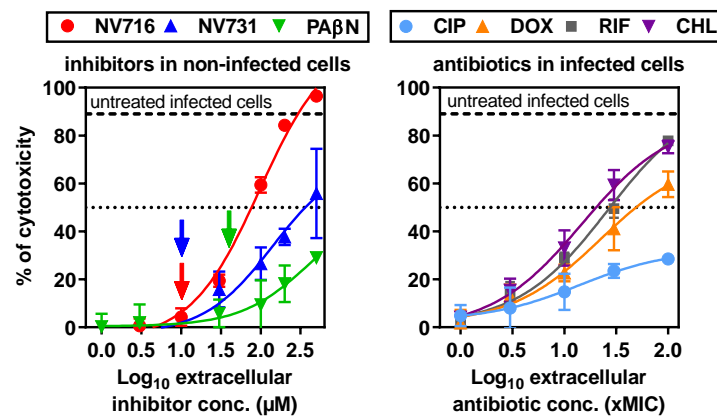


Figure 2. Concentration-response curves of antibiotics alone or combined with NV716 against intracellular PAO1 in a model of THP-1 monocytes. The graphs show the changes in CFU counts per mg of cell protein from the initial, post-phagocytosis inoculum after 24 h of incubation with increasing extracellular concentrations of antibiotics alone (ciprofloxacin [CIP], doxycycline [DOX], chloramphenicol [CHL] or rifampin [RIF]) or combined with a fixed concentration (10 μ M; 4 mg/L) of NV716. The horizontal dotted line highlights a static effect and the vertical dotted line, the MIC of each antibiotic. All data are means \pm SEM (triplicates from 3 experiments).

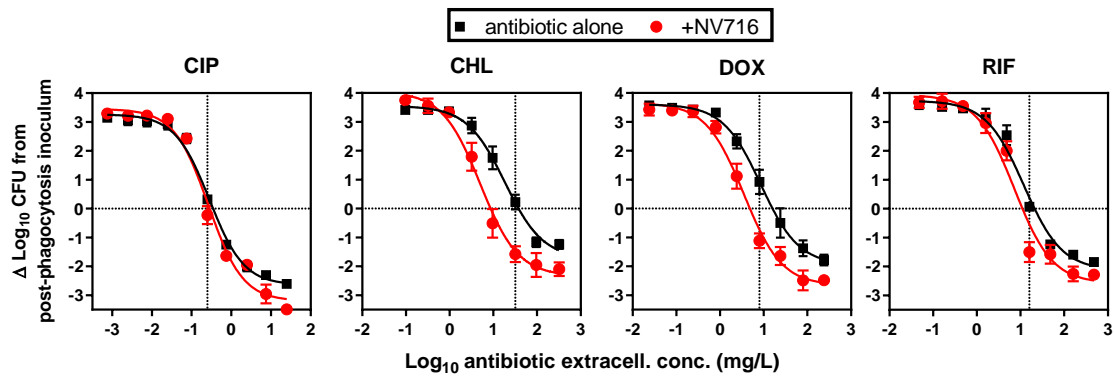


Figure 3. Intracellular pharmacodynamic parameters as calculated from the Hill equation of the concentration-response curves. The upper panel shows the C_s (in mg/L) for the 4 antibiotics (ciprofloxacin [CIP], doxycycline [DOX], chloramphenicol [CHL] or rifampin [RIF]) against the 4 strains, with horizontal dotted yellow-black lines corresponding to the MIC of the antibiotic in the absence of inhibitor. R.1.4.NV716 and NV731 were used at a concentration of 10 μ M and PA β N, at a concentration of 38 μ M. The lower panel shows the E_{max} expressed in \log_{10} CFU reduction from the post-phagocytosis inoculum. All data are expressed as means \pm SEM (triplicates from 3 experiments). Statistical analysis: One-way ANOVA with Dunnett post hoc test vs antibiotic alone (*: $p < 0.05$; **: $p < 0.01$; ***: $p < 0.001$ and ****: $p < 0.0001$).

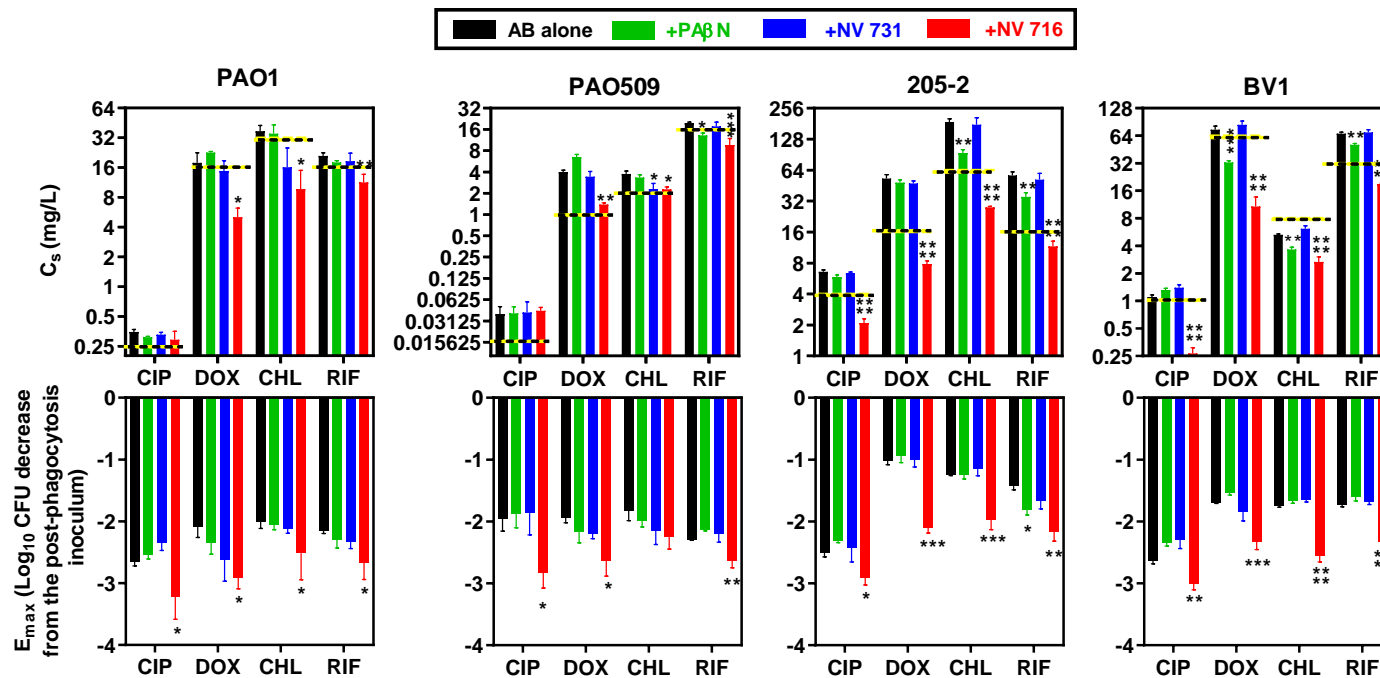
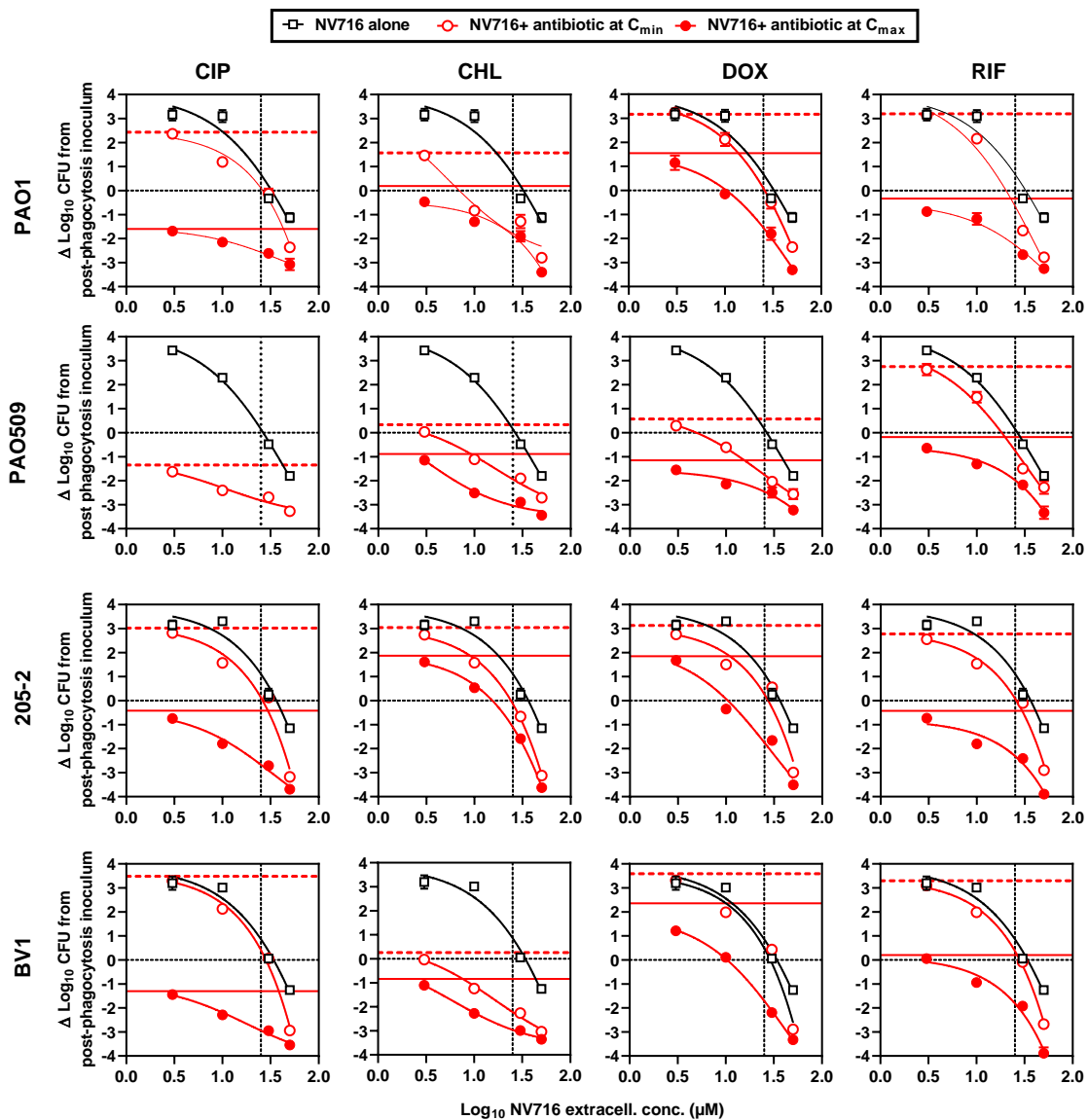


Figure 4. Concentration-response curves of NV716 alone or combined with antibiotics at their human C_{\min} or C_{\max} against intracellular *Pseudomonas aeruginosa* in a model of THP-1 monocytes. The graphs show the changes in CFU counts per mg of cell protein from the initial, post-phagocytosis inoculum after 24 h of incubation with increasing extracellular concentrations of NV716 alone or combined with ciprofloxacin [CIP], doxycycline [DOX], chloramphenicol [CHL] or rifampin [RIF] at concentrations corresponding to their human C_{\min} or C_{\max} (see values in Table S1). The combination with CIP at its C_{\max} could not be studied, CFU counts being already close to the limit of detection with the antibiotic alone. The horizontal lines highlight respectively a static effect (black dotted line), the effect of the antibiotic alone at its C_{\min} (red dotted line) or at its C_{\max} (red plain line) and the vertical dotted line, the MIC of NV716 in RPMI-1640 (25 μM). All data are means \pm SEM (triplicates from 3 experiments).



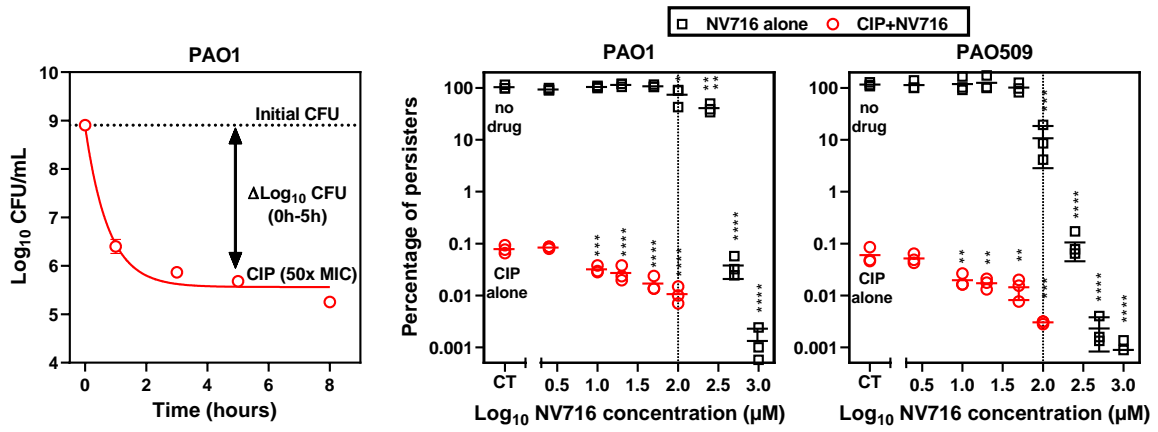
R2.3. Figure 5. Influence of NV716 on persisters selected by ciprofloxacin.

A. Persister fraction assay for ciprofloxacin (CIP) alone, NV716 alone or their combination against reference strains. Left: kill curve of a stationary phase culture of PAO1 exposed to ciprofloxacin at 50x MIC over time. The persister fraction is calculated as the difference in CFU counts between control and treated samples at 5 h, when a plateau value has been reached. Right: Percentage of persisters (as compared to the CFU counts of untreated samples) after 5 h of incubation with increasing concentrations of NV716 alone or combined with 50x MIC of ciprofloxacin. All data are expressed as means \pm SEM (triplicates from 3 experiments). Statistical analysis: One-way ANOVA with Dunnett post hoc analysis comparing samples exposed to a given concentration of NV716 with the corresponding control (no drug or ciprofloxacin alone): *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$; ****, $p < 0.0001$).

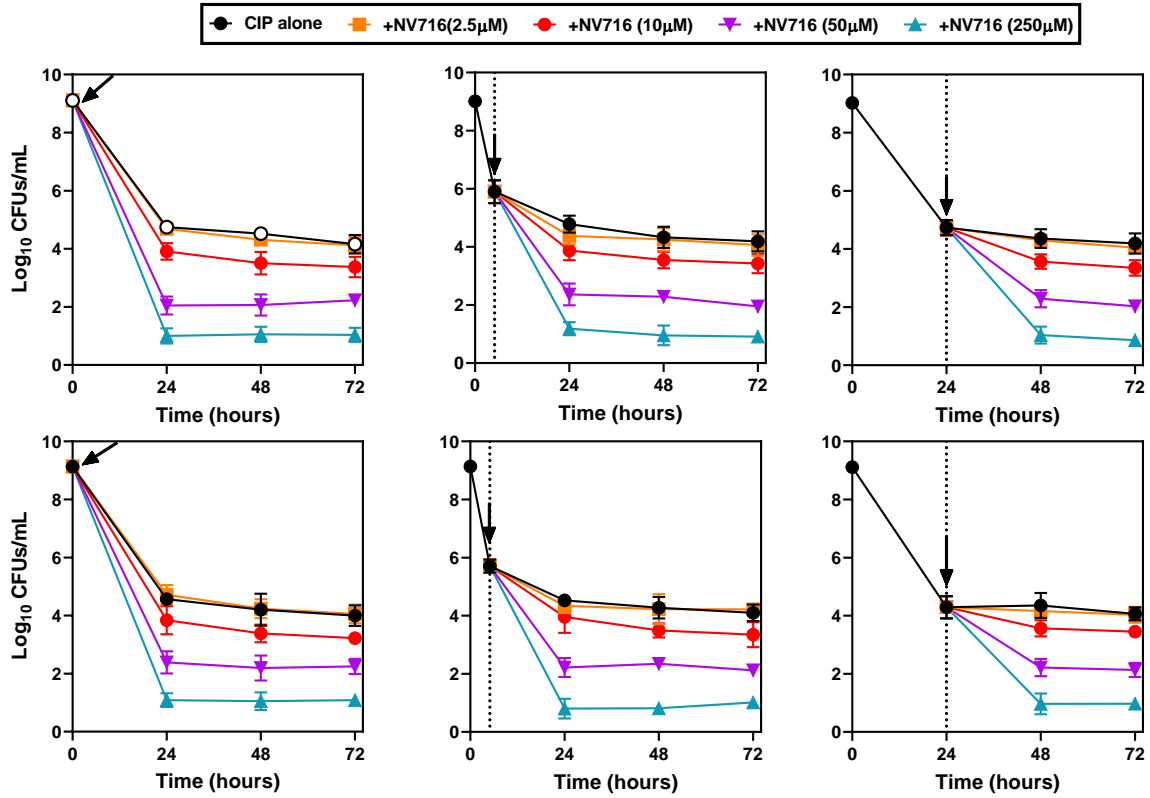
B. Kinetics of killing of stationary phase cultures of PAO1 (top) or PAO509 (bottom) by ciprofloxacin at 50x MIC alone or combined with NV716 at different concentrations. NV716 was added at different timings (0h [left], 5h [middle] or 24h [right]), highlighted by the vertical dotted line and the arrow. All data are expressed as means \pm SEM (triplicates from 3 experiments).

C. Killing of persisters cells of PAO1 (left) or PAO509 (right) by ciprofloxacin, NV716 or their combinations. Persister cells were isolated after 5 hours of incubation with ciprofloxacin at 50x MIC (persisters), then incubated with either ciprofloxacin (CIP), NV716 at different concentrations, or their combination. All data are expressed as means \pm SEM (triplicates from 3 experiments). Statistical analysis: One way ANOVA with Dunnett's post hoc test comparing samples exposed to CIP alone: *, $P \leq 0.05$; ** $p \leq 0.01$; ****, $P \leq 0.0001$.

A



B



C

