

Cover image:

*P. aeruginosa* was treated with NV716 for 1 h and visualized with transmission electron microscopy at 6000x magnification. Adapted from a paper submitted for publication



**Reviving disused antibiotics against Gram-negative ESKAPE  
pathogens: combination with the polyaminoisoprenyl  
potentiator NV716**

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Pharmaceutical Sciences.

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Deng Ke Hou  
《登科后》

Xi Ri Wo Chuo Bu Zu Kua, Jin Zhao Fang Dang Si Wu Ya  
昔日龌龊不足夸，今朝放荡思无涯。

Chun Feng De Yi Ma Ti Ji, Yi Ri Kan Jin Chang An Hua  
春风得意马蹄疾，一日看尽长安花。

<Succeeded in Imperial Exam>

*The past has become the past and can no longer be mentioned. Today is full of high spirits and full of energy.*

*Facing the mighty spring breeze, we should gallop proudly. It is as if you can see the prosperity of Chang'an in one day.*

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## **Abstract**

Gram-negative ESKAPE pathogens (*Klebsiella pneumoniae*, *Acinetobacter baumannii*, *Pseudomonas aeruginosa* and *Enterobacter spp.*) are responsible for increases in multidrug-resistant infections worldwide. Poor permeability and active efflux are the main reasons for intrinsic resistance to many antibiotics. In addition, these pathogens can also adopt specific lifestyles like intracellular survival and biofilm formation where bacteria can switch to dormant phenotypes poorly responsive to antibiotics. The increase of antibiotic resistance together with the paucity of new drugs against Gram-negative bacteria urgently prompted the development of new antibacterial strategies.

This thesis investigated the capacity of the polyaminoisoprenyl compound NV716 to revive disused antibiotics against Gram-negative bacteria using planktonic, intracellular and biofilm models, and attempted to reveal the mechanisms of its action at molecular, genomic and transcriptional levels. We show that NV716 was the most effective potentiator of disused antibiotics against Gram-negative bacteria in our models, as compared with other adjuvants. Moreover, NV716 can not only effectively prevent the selection of resistance to antibiotics, but also significantly improve their efficacy against persisters. Mechanistic studies showed that NV716 binds to LPS of the outer membrane, disturbing its permeability and inhibiting RND efflux pumps as well as downregulating quorum-sensing regulated processes.

Therefore, our work highlights a strong potential for this compound as an adjuvant therapy against difficult-to-treat Gram-negative organisms, and suggests that NV716 deserves further investigations, notably in in-vivo models of infections.



## List of abbreviations

|                  |                                                                  |
|------------------|------------------------------------------------------------------|
| AB               | Antibiotic                                                       |
| AHLs             | Acyl homoserine lactones                                         |
| ALE              | Alexidine                                                        |
| ATP              | Adenosine triphosphate                                           |
| AZI              | Azithromycin                                                     |
| BC               | BODIPY <sup>TM</sup> -TR-cadaverine                              |
| CA               | Community-acquired                                               |
| CA-MHB           | Cation-adjusted Mueller-Hinton Broth                             |
| CAT              | Chloramphenicol acetyltransferase                                |
| Cc               | Cellular concentration                                           |
| CCCP             | Carbonyl cyanide-m-chlorophenylhydrazone                         |
| CE               | Extracellular concentration                                      |
| CF               | Cystic fibrosis                                                  |
| CFU              | Colony-forming unit                                              |
| CHL              | Chloramphenicol                                                  |
| CIP              | Ciprofloxacin                                                    |
| C <sub>max</sub> | Maximum concentration of a drug in the serum of treated patients |
| C <sub>min</sub> | Minimum concentration of a drug in the serum of treated patients |
| CRKP             | Carbapenem-resistant <i>K. pneumoniae</i>                        |
| C <sub>s</sub>   | Static concentration                                             |
| CTC              | 5-Cyano-2,3-ditolyl-tetrazolium chloride                         |
| DAEC             | Diffusely adherent <i>E. coli</i>                                |
| DDD              | Defined Daily Dose                                               |
| DHFR             | Dihydrofolate reductase                                          |
| DHPS             | Dihydropteroate synthase                                         |
| DMSO             | Dimethyl sulfoxide                                               |
| DNA              | Deoxyribonucleic acid                                            |
| DOX              | Doxycycline                                                      |
| EAEC             | Enteraggregative <i>E. coli</i>                                  |
| EARS-Net         | European Antimicrobial Resistance Surveillance Network           |
| ECM              | Extracellular matrix                                             |
| ECR              | Elastin Congo red                                                |
| eDNA             | Extracellular DNA                                                |
| EHEC             | Enterohaemorrhagic <i>E. coli</i>                                |
| EIEC             | Enteroinvasive <i>E. coli</i>                                    |
| E <sub>max</sub> | Maximal relative efficacy                                        |
| EPEC             | Enteropathogenic <i>E. coli</i>                                  |
| EPIs             | Efflux pump inhibitors                                           |

|                  |                                                                |
|------------------|----------------------------------------------------------------|
| EPS              | Extracellular polymeric substances                             |
| ESBLs            | Extended-spectrum- $\beta$ -lactamases                         |
| ESBLs            | Extended Spectrum Beta-Lactamases.                             |
| ETEC             | Enterotoxigenic <i>E. coli</i>                                 |
| EU               | European Union                                                 |
| ExPEC            | Extraintestinal pathogenic <i>E. coli</i>                      |
| FBS              | Fetal bovine serum                                             |
| FDA              | Fluorescein diacetate                                          |
| FIC              | Fractional inhibitory concentration                            |
| FUS              | Fusidic acid                                                   |
| GDP              | Gross domestic product                                         |
| HA               | Hospital-acquired                                              |
| HICs             | High-income countries                                          |
| hvKP             | Hypervirulent <i>K. pneumoniae</i>                             |
| IC <sub>50</sub> | Concentration causing 50% of the studied effect cell mortality |
| ICUs             | Intensive care units                                           |
| IM               | Inner membrane                                                 |
| LMICs            | Low- and middle-income countries                               |
| LPS              | Lipopolysaccharide                                             |
| LZD              | Linezolid                                                      |
| MBC              | Minimum bactericidal concentration                             |
| MDK              | Minimum duration for killing                                   |
| MDR              | Multidrug-resistance                                           |
| MFP              | Membrane fusion protein                                        |
| MIC              | Minimum inhibitory concentration                               |
| MOPS             | 3-morpholinopropane-1-sulfonic acid                            |
| MRSA             | Methicillin-resistant <i>S. aureus</i>                         |
| NOV              | Novobiocin                                                     |
| NMP              | 1-(1-naphthylmethyl)-piperazine                                |
| NPN              | 1-N-phenylnaphthylamine                                        |
| OM               | Outer membrane                                                 |
| OMPs             | Outer membrane permeabilizers                                  |
| PA $\beta$ N     | Phenylalanine-arginine $\beta$ -naphthylamide                  |
| PBS              | Phosphate Buffered Saline                                      |
| PDR              | Pandrug-resistant                                              |
| PI               | Propidium Iodide                                               |
| PK/PD            | Pharmacokinetic/pharmacodynamic                                |
| PL               | Phospholipid                                                   |
| PMBN             | Polymyxin B nonapeptide                                        |
| PQS              | <i>Pseudomonas</i> quinolone signal                            |
| PSA              | Polar surface area                                             |

|         |                                            |
|---------|--------------------------------------------|
| QS      | Quorum sensing                             |
| RIF     | Rifampicin                                 |
| RNA     | Ribonucleic acid.                          |
| RND     | Resistance–nodulation–cell division family |
| SCVs    | Small colony variants                      |
| SDS     | Sodium lauryl sulfate                      |
| SNPs    | Single nucleotide polymorphisms            |
| STEC    | Shiga toxin-producing <i>E. coli</i>       |
| T6SS    | The type VI secretion system               |
| TMP/SMX | Trimethoprim-sulfamethoxazole              |
| TSA     | Tryptic Soy Agar                           |
| UTI     | Urinary tract infection                    |
| WHO     | World Health Organization                  |
| XDR     | Extensively drug-resistant                 |



# Chapter I. Introduction

## 1. Gram-negative ESKAPE pathogens

ESKAPE is an acronym for a group of bacteria, including both Gram-positive (*Enterococcus faecium* and *Staphylococcus aureus*) and Gram-negative species (*Klebsiella pneumoniae*, *Acinetobacter baumannii*, *Pseudomonas aeruginosa*, and *Enterobacter species*). Recent reports presented these pathogens as “ESKAPing” current therapies [1, 2] and therefore causing life-threatening nosocomial infections in immunocompromised patients (Table 1) [3]. WHO therefore considered them as a priority for the search of new therapeutic strategies [4].

**Table 1.** Clinical characteristics of Gram-negative ESKAPE pathogens

| Species                                                                                                                                                          | Clinical manifestations [5-10]                                                                                                                       | Mortality rates [11-16]                                                                                                                | Treatments [17-22]                                                                                                                                                                                      |
|------------------------------------------------------------------------------------------------------------------------------------------------------------------|------------------------------------------------------------------------------------------------------------------------------------------------------|----------------------------------------------------------------------------------------------------------------------------------------|---------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|
| <i>K. pneumoniae</i>                                                                                                                                             | Pneumonia, pyogenic liver abscesses, necrotizing and soft tissue infection, bloodstream infection, meningitis, endophthalmitis, UTI                  | 40% to 70% for CRKP bloodstream infection; 40% for CRKP pulmonary infection; 25% to 47% for hvKP necrotizing and soft tissue infection | Aminoglycosides, polymyxin combination therapy, tigecycline, meropenem, meropenem-vaborbactam, ertapenem and meropenem, imipenem-cilastatin-relebactam, ceftazidime-avibactam, plazomicin, eravacycline |
| <i>A. baumannii</i>                                                                                                                                              | Ventilator-associated pneumonia, central line bloodstream infections, nosocomial meningitis, skin and soft tissue infection, catheter-associated UTI | 35% for ventilator-associated pneumonia and bloodstream infections                                                                     | Colistin, tigecycline, cefiderocol, eravacycline                                                                                                                                                        |
| <i>P. aeruginosa</i>                                                                                                                                             | UTI, bloodstream infection, ventilator-associated pneumonia, chronic respiratory infection, skin and soft tissue infection, endocarditis             | 67% for MDR bacteremia; 33.9% for UTI                                                                                                  | Piperacillin-tazobactam, ceftolozane-tazobactam, ceftazidime, meropenem, ciprofloxacin, ceftazidime-avibactam, cefiderocol, imipenem-cilastatin-relebactam                                              |
| <i>E. coli</i>                                                                                                                                                   | UTI, bloodstream infection, neonatal pneumonia, skin and soft tissue infection, intra-abdominal infection, endocarditis, septic arthritis            | Exceeds 40% for bloodstream infection                                                                                                  | Ciprofloxacin, levofloxacin, azithromycin, rifaximin and rifamycin, cephalosporins and penicillins                                                                                                      |
| Abbreviations: UTI, urinary tract infection; HA, hospital-acquired; CRKP, carbapenem-resistant <i>K. pneumoniae</i> ; hvKP, hypervirulent <i>K. pneumoniae</i> . |                                                                                                                                                      |                                                                                                                                        |                                                                                                                                                                                                         |

### **1.1. *Pseudomonas aeruginosa***

*P. aeruginosa* is a highly versatile, motile Gram-negative rod-shaped bacterium measuring 0.5-0.8 µm wide and 1.5-3.0 µm long. This pathogen mainly uses aerobic respiration but also anaerobic respiration on nitrate [23]. *P. aeruginosa* is generally described as ubiquitous in natural settings, including the human body, soil, water, oil-contaminated environments, hospital environments, and sinks as well as drains [24].

### **Clinical importance and epidemiology**

*P. aeruginosa* is a Gram-negative opportunistic human pathogen commonly associated with severe respiratory system infections in immunodeficient patients [25]. Different classes of antibiotics are used to treat *P. aeruginosa* infections, they are called antipseudomonal drugs and include β-lactams, aminoglycosides, quinolones, and polymyxins. Patients with chronic or inherited lung disease, such as bronchiectasis and cystic fibrosis (CF), are extremely susceptible to persistent pulmonary infection, with intermittent exacerbations requiring hospitalization and intravenous antibiotics, with a subsequent risk of multidrug resistance [26]. *P. aeruginosa* is the predominant pathogen infecting adult patients with cystic fibrosis (CF); approximately three-quarters of them are infected with this organism [27, 28]. Although eradication can be achieved following early detection, chronic infection occurs in over 60% of adults with CF. Chronic infection is associated with accelerated disease progression and increased mortality [20]. Multiple studies also showed that *P. aeruginosa* infections in CF patients enhance inflammatory response, which leads to progressive structural lung damage [29], accelerates the decline of lung function and is a leading cause of respiratory failure and mortality [30-34]. Moreover, *P. aeruginosa* infection increases the risk of other CF-related diseases such as diabetes [35], osteopenia and osteoporosis [36]. In addition, *P. aeruginosa* colonizes humid environments and therefore can be found in many healthcare environments, especially in the context of chronic wounds, respiratory support systems, or urinary tract devices.

*P. aeruginosa* accounts for 11-13.8 % of nosocomial infections when a microbiological isolate is identifiable. In intensive care units (ICUs), *P. aeruginosa* is typically responsible for an even higher percentage of nosocomial infections, with rates of 13.2-22.6% reported [37].

### **1.2. *Escherichia coli***

*E. coli* is a Gram-negative, facultatively anaerobic, rod-shaped, coliform bacterium of the genus *Escherichia* that is commonly found in the lower intestine of warm-blooded organisms. Most *E. coli* strains are harmless, but some serotypes (EPEC, ETEC) can cause serious food poisoning in their hosts and are occasionally responsible for food contamination incidents that prompt product recalls.

### **Clinical importance and epidemiology**

Despite being part of the normal intestinal microbiota (flora) in humans and many animals, *E. coli* is a frequent cause of bloodstream and urinary tract infections. It is also associated with intra-abdominal infections, neonatal meningitis, and foodborne infections. There are six well-studied intestinal pathotypes of *E. coli*, including enteropathogenic (EPEC), shiga toxin-producing (STEC), enteroaggregative (EAEC), enterotoxigenic (ETEC), enteroinvasive (EIEC), and diffusely adherent *E. coli* (DAEC). These strains are classified according to virulence characteristics and pathogenic mechanisms that cause gastrointestinal diseases such as diarrhea [38, 39]. Enterohaemorrhagic *E. coli* (EHEC) is one type of STEC that can cause serious intestinal diseases, such as haemolytic uraemic syndrome and haemorrhagic colitis [38], leading to acute renal failure and often death. Some *E. coli* strains that cause extraintestinal diseases, are called extraintestinal pathogenic *E. coli* (ExPEC). The ExPEC, defined by disease association, includes urinary tract pathogenic *E. coli*, neonatal meningitis-associated *E. coli* and sepsis-causing *E. coli* [40]. Different classes of antibiotics are used to treat *E. coli* infections, fluoroquinolones, such as ciprofloxacin, and levofloxacin, are usually the first-line therapy. Azithromycin is also commonly used

as a treatment for invasive *E. coli* infections. Rifaximin and rifamycin are closely related antibiotics that are FDA-approved to treat diarrhea caused by noninvasive strains of *E. coli*. Nitrofurantoin and fosfomycin are recommended as first-line therapy in the treatment of uncomplicated cystitis, as the resistance to these antimicrobial agents remains low between uropathogenic *E. coli* [22].

In addition, *E. coli* is most commonly found in the female reproductive tract, causing vaginal and/or endocervical colonization as well as different infections in pregnant women, such as intra-amniotic, puerperal and neonatal infections, such as early and late neonatal sepsis [41]. Such isolates show specialized virulence factors such as adhesins, toxins, iron acquisition systems and polysaccharide shells [42].

The incidence of *E. coli* bacteremia has been estimated at 48 per 100,000 person-years, but considerably increases with age [43]. Overall, *E. coli* accounts for 27% of documented bacteremia episodes, in which hospital-acquired infections accounted for 18%, community-onset healthcare-associated infections, for 32%, and community-acquired infections, for 33%. Urinary tract infection (UTI) represent more than half of all *E. coli* bacteremia episodes.

### **1.3. *Klebsiella pneumoniae***

*K. pneumoniae* is a Gram-negative, non-motile, encapsulated, lactose-fermenting, facultatively anaerobic, rod-shaped bacterium. It naturally occurs in the soil, and about 30% of strains can fix nitrogen in anaerobic conditions.

#### **Clinical importance and epidemiology**

*K. pneumoniae* is mainly found in the human gastrointestinal tract, the skin and the respiratory tract. The majority of infections caused by *K. pneumoniae* are healthcare-associated and can spread rapidly between patients and via the hands of hospital personnel, leading to nosocomial outbreaks. *K. pneumoniae* mainly causes urinary tract

infections, lower respiratory tract infections, intraabdominal infections and bloodstream infections.

This pathogen is among the most common causes of hospital-acquired infections (HAIs) in the United States and is responsible for about 10% of all infections [19]. The major sources of *K. pneumoniae* that cause HAIs remain unclear, but intestinal colonization [44], contaminated instruments [45], and healthcare workers' hands [46] have all been implicated in transmission. *K. pneumoniae* gastrointestinal colonization rates in hospitalized patients are estimated to be 20 to 38%, and a more recent study identified a 21.1% fecal carriage rate in healthy adults in Korea [47] with a high proportion of sequence type 23 (ST23) isolates that were associated with pyogenic liver abscess. A risk factor for colonization is prior treatment with antibiotics [48]. Previous work identified gastrointestinal colonization with *K. pneumoniae* as a reservoir for its infection [44], but such colonization may reflect the virulence potential of two predominant serotypes in the cohort. Regardless of the transmission route, *K. pneumoniae* appears to be transmitted efficiently, as evidenced by reported outbreaks [49]. Effective antibiotic options are lacking, and treatment usually relies on drugs with a risk of toxicity, like aminoglycosides and polymyxins, or with other safety concerns, like tigecycline [50].

#### **1.4. *Acinetobacter baumannii***

The *Acinetobacter* genus consists of a large number of species which can be divided into two complexes: the *Acinetobacter baumannii* complex (the group including *A. baumannii*, *A. pittii* and *A. nosocomialis*), and the generally less pathogenic *Acinetobacter non-baumannii* group. The correct identification of *Acinetobacter* isolates at the species level is difficult, although it is possible using mass spectrometry and genotypic methods.

#### **Clinical importance and epidemiology**

Species belonging to the *A. baumannii* group are opportunistic pathogens primarily associated with healthcare-associated infections including ventilator-associated

pneumonia, central-line-associated bloodstream infections, urinary tract infections and wound infections. Risk factors for infection include advanced age, presence of serious underlying disease, immune suppression, major trauma or burn injuries, invasive procedures, presence of indwelling catheters, mechanical ventilation, extended hospital stay and previous administration of antibiotics.

*A. baumannii* infections predominantly occur in countries located in tropical or sub-tropical climates, and the majority of cases are reported in Hong Kong [51], Singapore [52], Taiwan [53], South Korea [54] and Northern Australia [55]. *A. baumannii* infections are also more likely to occur in the humid months of the year, with between 71.4% [52] and 88% [55] of patients presenting to the hospital during these months. A small number of cases have occurred in areas with temperate climates. The majority of infections occurring during the warmer months are perhaps indicative of *A. baumannii* preferring warm and humid climates.

## 2. Antibiotics resistance in Gram-negative ESKAPE

The Gram-negative ESKAPE bacteria have developed several resistance mechanisms against many antibiotics, like  $\beta$ -lactams,  $\beta$ -lactam- $\beta$ -lactamase inhibitor combinations, aminoglycosides, fluoroquinolones. Table 2 shows the European Union/ European Economic Area population-weighted mean percentage with resistance to antibiotics according to European Antimicrobial Resistance Surveillance Network in 2018 [56].

**Table 2.** Antibiotic resistance to Gram-negative bacteria in European Union/ European Economic Area.

| Species              | Resistances [56] (values in brackets are population-weighted mean resistance percentage )                                             |
|----------------------|---------------------------------------------------------------------------------------------------------------------------------------|
| <i>P. aeruginosa</i> | fluoroquinolones (19.7%), piperacillin $\pm$ tazobactam (18.3%), carbapenems (17.2%), ceftazidime (14.1%) and aminoglycosides (11.8%) |
| <i>E. coli</i>       | aminopenicillins (57.4%), fluoroquinolones (25.3%), third-generation cephalosporins (15.1%) and aminoglycosides (11.1%)               |
| <i>K. pneumoniae</i> | third-generation cephalosporins (31.7%), fluoroquinolones (31.6%), aminoglycosides (22.7%) and carbapenems (7.5%)                     |
| <i>A. baumannii</i>  | Fluoroquinolones (36.2%), aminoglycosides (31.9%) and carbapenems (31.9%)                                                             |

In 2018, Kadri et al. proposed a new term to characterize the multidrug resistance profile of Gram-negative bacteria, namely difficult-to-treat resistance. It is defined as resistance in any Gram-negative bacteria demonstrating an intermediate or resistant phenotype to all first-line agents in the carbapenem, other beta-lactams, and fluoroquinolone categories [57]. The increased incidence of infections due to Gram-negative bacteria with difficult-to-treat resistance is a matter of serious concern, both in the community and in the hospital setting [58, 59].

In parallel, definitions have also been given for multidrug-resistant (MDR), extensively drug-resistant (XDR) and pandrug-resistant (PDR) bacteria. MDR is defined as non-susceptibility to at least one agent in three or more antimicrobial categories; XDR is defined as non-susceptibility to at least one agent in all but two or fewer antimicrobial categories (i.e. bacterial isolates remain susceptible to only one or two categories); PDR is defined as non-susceptibility to all agents in all antimicrobial categories (i.e. no agents tested as susceptible for that organism)[60].

The consequences of antibiotic resistance can be severe, and reasonable treatment with effective antibiotics is the most important intervention to reduce the risk of poor outcomes of serious infections. However, the abuse and misuse of antibiotics in animals and humans are contributing to the rising threat of antibiotic resistance. Nowadays, some types of bacteria that cause serious infections in humans have already developed resistance to most or all of the available treatments, and there are very few promising options in the research pipeline.

## **2.1. Resistance as a major public health concern**

Antibiotic resistance is considered to be one of the biggest threats to public health today, both globally [61] and in the European Union (EU) region[62]. A recent report estimates that, across the globe, approximately 700,000 individuals are expected to die prematurely because of antibiotic-resistant infections each year [63]. Other estimates

based on data from European Antimicrobial Resistance Surveillance Network (EARS-Net) show that each year, more than 670,000 infections occur in the EU due to bacterial resistance to antibiotics and that approximate 33,000 people die as a direct consequence of these types of infection [64]. If no action is taken, by 2050, antibiotic resistance will cause up to 10 million annual deaths globally (Figure 1).

### Deaths attributable to AMR every year by 2050

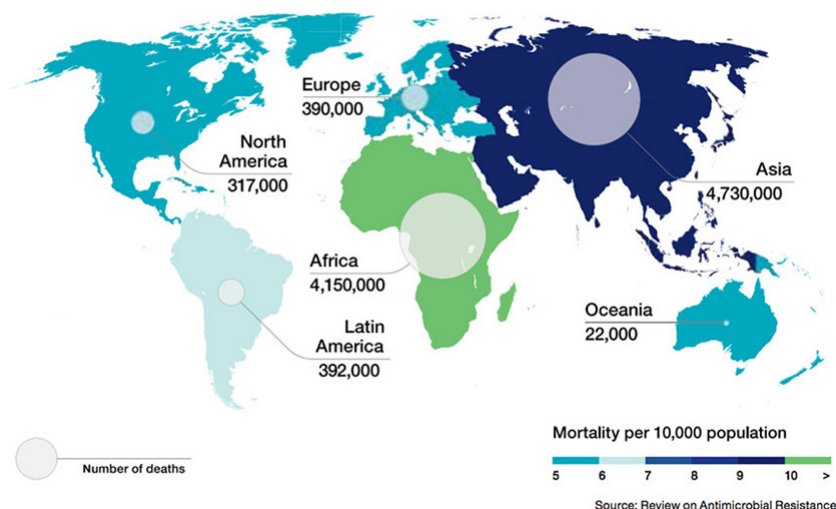


**Figure 1.** Deaths attributable to antimicrobial resistance every year by 2050 [65]

## 2.2. Main resistance mechanisms in Gram-negative bacteria

In recent years, antibiotic-resistant Gram-negative bacterial species in the clinics have caused life-threatening infections. These infections cannot be effectively treated by antibiotic monotherapy [66]. These species show intrinsic resistance to many antibiotics, albeit to various degrees, and acquire further resistance when exposed to antibiotic treatments [67]. The differences in antibiotic susceptibility among Gram-negative bacteria are attributable to various factors, including the expression of enzymes that modify antibiotics, and mutations or variations in activities or amounts of antibiotic targets

[68]. Importantly, in all Gram-negative species, the low permeability barrier of the outer membrane and multi-drug efflux pumps play important roles in preventing the access of antibiotics to their targets [69-71] (Figure 2). Due to the presence of these resistance mechanisms, Gram-negative bacteria are resistant to many different classes of antibiotics (Table 3).

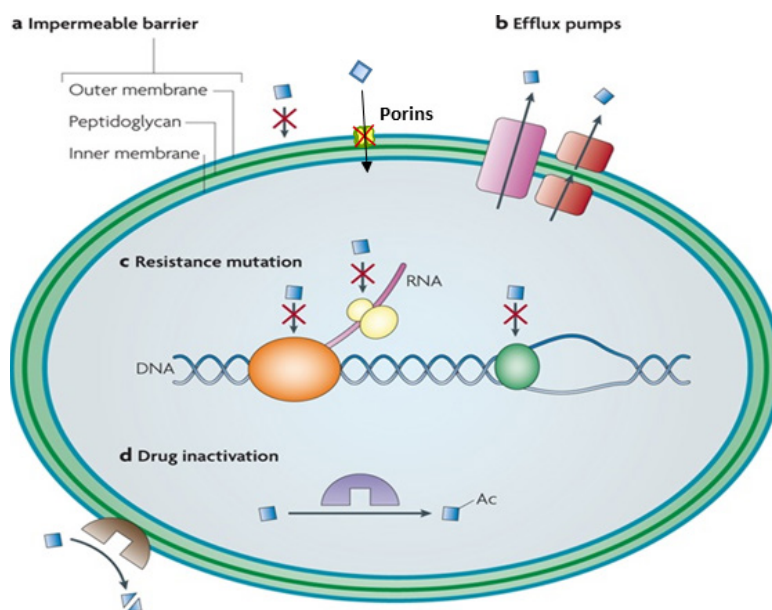


**Figure 2.** Mechanisms of antibiotic resistance in a Gram-negative bacterium. (a) Impermeable outer membrane. Some bacteria are intrinsically resistant to some antibiotics (blue squares) simply because they have an impermeable outer membrane. Loss of porins, reducing the movement of drugs through the cell membrane, also contribute to reduce drug penetration. (b) Multidrug resistance efflux pumps. These multidrug resistance pumps expel antibiotics from the cell. Some transporters, such as those of the resistance–nodulation–cell division family (RND), can pump antibiotics directly outside the cell. (c) Resistance mutations, for example, mutations in the gyrase, which cause resistance to fluoroquinolones, or in RNA polymerase subunit B, which cause resistance to rifampicin. (d) Inactivation of the antibiotic. Antibiotics inactivation can occur by covalent modification of the antibiotic, such as that catalysed by acetyltransferases (purple) acting on aminoglycoside antibiotics, or by degradation of the antibiotic, such as that catalysed by  $\beta$ -lactamases (brown) acting on  $\beta$ -lactam antibiotics. Adapted from [71]

**Table 3.** Summary of antibiotic resistance mechanisms in four Gram-negative bacterial species.[72-77]

| Antibiotic Class                                                                                                                                                                                                                                                                                                                            | <i>P. aeruginosa</i>                                                                   | <i>E. coli</i>                                                                           | <i>K. pneumoniae</i>                                                                     | <i>A. baumannii</i>                                                                                                                   |
|---------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|----------------------------------------------------------------------------------------|------------------------------------------------------------------------------------------|------------------------------------------------------------------------------------------|---------------------------------------------------------------------------------------------------------------------------------------|
| Penicillins                                                                                                                                                                                                                                                                                                                                 | AmpC, ESBLs, other $\beta$ -lactamases, efflux pumps                                   | AmpC, ESBLs, other $\beta$ -lactamases, efflux pumps                                     | AmpC, ESBLs, other $\beta$ -lactamases, efflux pumps                                     | AmpC, ESBLs, other $\beta$ -lactamases, efflux pumps                                                                                  |
| Cephalosporins 1st gen.                                                                                                                                                                                                                                                                                                                     | AmpC, ESBLs, efflux pumps                                                              | AmpC, ESBLs, efflux pumps                                                                | AmpC, ESBLs, efflux pumps                                                                | AmpC, ESBLs, efflux pumps                                                                                                             |
| Cephalosporins 2nd gen.                                                                                                                                                                                                                                                                                                                     | AmpC, ESBLs, efflux pumps                                                              | AmpC, ESBLs, efflux pumps                                                                | AmpC, ESBLs, efflux pumps                                                                | AmpC, ESBLs, efflux pumps                                                                                                             |
| Cephalosporins 3rd gen.                                                                                                                                                                                                                                                                                                                     | AmpC, ESBLs, efflux pumps                                                              | AmpC, ESBLs, efflux pumps                                                                | AmpC, ESBLs, efflux pumps                                                                | AmpC, ESBLs, efflux pumps                                                                                                             |
| Cephalosporins 4th gen.                                                                                                                                                                                                                                                                                                                     | ESBLs, efflux pumps                                                                    | ESBLs, efflux pumps                                                                      | ESBLs, efflux pumps                                                                      | ESBLs, efflux pumps                                                                                                                   |
| beta-lactamase inhibitors                                                                                                                                                                                                                                                                                                                   | AmpC, efflux pumps                                                                     | AmpC                                                                                     | AmpC                                                                                     | AmpC                                                                                                                                  |
| Aztreonam                                                                                                                                                                                                                                                                                                                                   | ESBLs, efflux pumps                                                                    | ESBLs, efflux pumps                                                                      | ESBLs, efflux pumps                                                                      | ESBLs                                                                                                                                 |
| Carbapenems                                                                                                                                                                                                                                                                                                                                 | Class B & D carbapenemases, efflux pumps, porin loss                                   | Class A, B & D carbapenemases, efflux pumps, porin loss                                  | Class A, B & D carbapenemases, efflux pumps, porin loss                                  | Class B & D carbapenemases, efflux pumps, porin loss                                                                                  |
| Tetracyclines                                                                                                                                                                                                                                                                                                                               | Efflux pumps                                                                           | Efflux pumps                                                                             | Efflux pumps                                                                             | Efflux pumps                                                                                                                          |
| Tigecycline                                                                                                                                                                                                                                                                                                                                 | Efflux pumps                                                                           | Efflux pumps, porin downregulation                                                       | Efflux pumps                                                                             | Efflux pumps, reduced membrane permeability                                                                                           |
| Macrolides                                                                                                                                                                                                                                                                                                                                  | Efflux pumps                                                                           | Efflux pumps, macrolide inactivating enzymes, target site modification                   | Efflux pumps                                                                             | Efflux pumps                                                                                                                          |
| Chloramphenicol                                                                                                                                                                                                                                                                                                                             | Efflux pumps, CAT                                                                      | Efflux pumps, CAT, rRNA methylase                                                        | Efflux pumps, CAT                                                                        | Efflux pumps, CAT                                                                                                                     |
| Fluoroquinolones                                                                                                                                                                                                                                                                                                                            | Mutations in topoisomerase IV and DNA gyrase genes, efflux pumps                       | Mutations in DNA gyrase gene, efflux pumps                                               | Mutations in DNA gyrase gene, efflux pumps, fluoroquinolone degradation enzymes          | Mutations in genes for DNA gyrase and topoisomerase IV, efflux pumps, enzyme protection proteins, fluoroquinolone degradation enzymes |
| Rifampicin                                                                                                                                                                                                                                                                                                                                  | Mutations in RNA polymerase gene                                                       | Mutations in RNA polymerase gene                                                         | Enzymatic degradation                                                                    | Mutations in RNA polymerase gene, efflux pumps, enzymatic degradation                                                                 |
| Aminoglycosides                                                                                                                                                                                                                                                                                                                             | Aminoglycoside degradation enzymes, efflux pumps                                       | Aminoglycoside degradation enzymes, efflux pumps                                         | Aminoglycoside degradation enzymes, production of 16SrRNA                                | Aminoglycoside degradation enzymes, efflux pumps                                                                                      |
| Colistin                                                                                                                                                                                                                                                                                                                                    | Reduction of membrane negative charge through addition of N4-aminoarabinose to lipid A | Reduction of membrane negative charge through addition of phosphoethanolamine to lipid A | Reduction of membrane negative charge through addition of phosphoethanolamine to lipid A | efflux pumps, loss of LPS production, alterations in the structure of lipid A                                                         |
| Abbreviations: ESBLs, extended-spectrum- $\beta$ -lactamases; TMP/SMX, trimethoprim-sulfamethoxazole; DHPS, dihydropteroate synthase; DHFR, dihydrofolate reductase; CAT, chloramphenicol acetyltransferase; RNA, ribonucleic acid; DNA, deoxyribonucleic acid; rRNA, ribosomal ribonucleic acid; ESBLs, Extended Spectrum Beta-Lactamases. |                                                                                        |                                                                                          |                                                                                          |                                                                                                                                       |

In our study, we focused on the mechanisms related to the decrease of accumulation of antibiotics, typically barrier effect of the impermeable outer membrane (OM) and efflux pumps. Only these mechanisms will be discussed in the next sections.

### **2.2.1. Resistance due to impermeability of the outer membrane in Gram-negative bacteria**

The Gram-negative bacterial cell envelope consists of two distinct membranes, namely the inner and outer membranes, along with a thin cell wall (peptidoglycan layer) in the periplasmic space [78](Figure 3). The OM is a distinguishing feature of Gram-negative bacteria; Gram-positive bacteria lack an outer membrane but have a thicker peptidoglycan layer protecting their surface.

#### **Architecture and function of outer membrane**

Like other biological membranes, the OM is a lipid bilayer, but it is not a phospholipid bilayer. The outer leaflet of the OM consists of glycolipids, principally lipopolysaccharide (LPS), and the inner leaflet is composed of phospholipids [79](Figure 3). All Gram-negative bacteria are intrinsically more resistant than Gram-positive due to the OM, which acts as a permeability barrier to reduce the uptake of antibiotics [69].

Passive diffusion of hydrophobic or larger hydrophilic compounds is relatively slow, while small and hydrophilic compounds can quickly access cells through porins that are located in the OM. For instance, the glycopeptide antibiotic vancomycin that is a relatively large antibiotic active against methicillin-resistant *S. aureus* (MRSA), is ineffective against Gram-negative bacteria due to the inability to cross the OM permeability barrier [80].

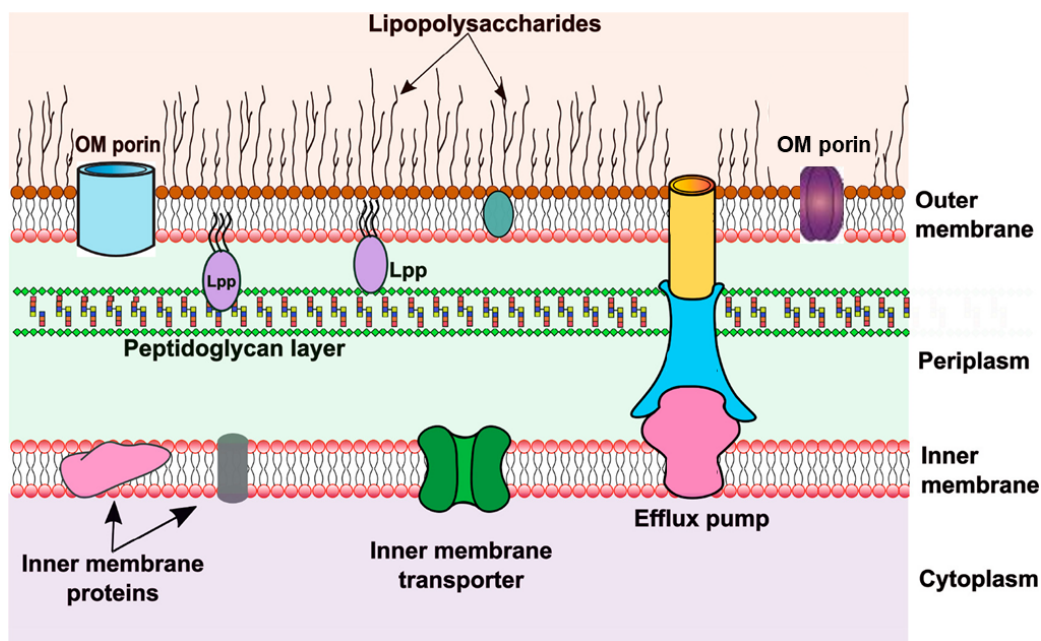


**Figure 3.** Architecture of the envelope in Gram-negative bacteria, which comprises IM, OM and cell wall (peptidoglycan). The inner membrane (IM) is made of a phospholipid (PL) bilayer and integral membrane proteins and transporters. The OM consists of LPS located in the outer leaflet and phospholipids located in the inner leaflet, and contains proteins such as porins while lipoproteins (Lpp) are anchored in this OM. Adapted from [81]

### **Architecture and function of LPS**

LPS is the main glycolipid in the outer leaflet of the OM. The classical LPS molecule has a tripartite structure, including lipid A, core oligosaccharide and O antigen polysaccharide (Figure4) [82]. Lipid A is the hydrophobic moiety of LPS that anchors the molecule in the OM. It is an acylated glucosamine disaccharide that is phosphorylated on the 1 and 4' positions. The tight packing of lipid A in the OM constitutes a gel-like permeability barrier to hydrophobic molecules [79]. Although the structure and synthesis of lipid A are generally conserved, the number of acyl chains, degree of phosphorylation, and other modifications can vary, all of which have important implications for interactions with the bacterium's environment or host [82]. The core oligosaccharide is a non-

repeating oligosaccharide that is linked to the glucosamines of lipid A [83]. The structure of the core oligosaccharide amongst Gram-negative bacteria is generally more varied than lipid A, but there are several conserved features. The core oligosaccharide is divided into two regions, the inner core and the outer core. The inner core usually contains Kdo (3-deoxy-d-manno-oct-2-ulonic acid) covalently linked to several heptose residues (either L,d-Hep, or D,d-Hep) [84], although the inner core structure of *Acinetobacter* contains Ko (D-glycero-D-threo-oct-2-ulopyranosonic acid) instead of Kdo, or no heptose [85]. The heptoses are the targets of modifications of the core oligosaccharide, such as the addition of phosphate and phosphoethanolamine [86]. The outer core region varies among bacterial species, but typically contains hexoses and hexosamines [83]. In *P. aeruginosa*, the core oligosaccharide is heavily phosphorylated and composed of Kdo, heptose, galactosamine, glucose, and rhamnose [87]. The O antigen consists of a variable number of repeating oligosaccharide units, and as such can vary in size from different molecules quite dramatically [88]. Additionally, rather than being synthesized directly on the lipid A-core complex, the O antigen is synthesized separately from it and then attached to it. Thus, not every lipid A-core complex is appended with O antigen, resulting in a heterogeneous OM surface containing LPS with and without O antigen. In the literature, bacteria containing LPS with O antigen are usually described as “smooth” whereas those devoid of O antigen are termed “rough” . The contribution of smooth LPS to virulence has been demonstrated by using animal infection models. Cryz et al. [89] with a burned-mouse infection model showed that the mutant with rough LPS has a 50% lethal dose more than 1,000-fold higher than that of its parent strain, suggesting that the parent strain of *P. aeruginosa* with smooth LPS was more virulent than the isogenic mutant with rough LPS. *P. aeruginosa* can simultaneously produce two O antigens: the common polysaccharide antigen (CPA) and the O-specific antigen (OSA) [90]. CPA has a typical, conserved structure, consisting of repeating units of  $\rightarrow 3$ D-Rha( $\alpha 1 \rightarrow 3$ )D-Rha( $\alpha 1 \rightarrow 2$ )D-Rha( $\alpha 1 \rightarrow$  whereas the OSA structure is variable, and therefore the determinant was used in serotyping to segregate this bacterial species into many groups [91].

LPS forms a layer stabilized by divalent cations like magnesium and calcium and provides a solid permeability barrier for harmful molecules such as antibiotics [79]. In addition, LPS is a major virulence factor produced by many Gram-negative bacteria. Under septic circumstances, LPS as a pathogen-associated molecular pattern can stimulate the innate immune system, mediating a local or systemic inflammatory response, and can also stimulate non-immune cells and initiate the inflammatory process. Exaggerated inflammatory responses are usually pernicious [92].

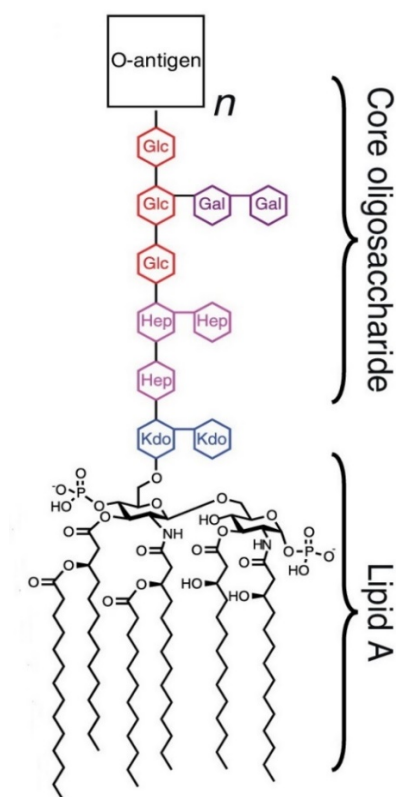


**Figure 4.** Chemical structure of Gram-negative bacteria lipopolysaccharide (LPS). LPS is a complex glycolipid that consists of three regions: lipid A, a core oligosaccharide and an O-antigen polysaccharide. adapt from [93]

## **Lipid A**

Lipid A, the hydrophobic anchor of lipopolysaccharide, is an essential component in the outer membrane of most Gram-negative bacteria. It is recognized by the TLR4/MD2 receptor of the innate immune system, which triggers an inflammatory response accompanied by massive overproduction of cytokines and leads to gram-negative septic shock. The structure [94], number and length of fatty acyl chains [95], as well as acylation patterns [96] of lipid A, are also related to the infectivity of bacteria. Some bacterial pathogens can survive in the host by modifying the structure of lipid A. In addition, the acylation pattern on Lipid A or the length of acyl chains on Lipid A perhaps could contribute to differences in antimicrobial activity in Gram-negative interspecies [97]. However, this speculation remains to be further verified via more experiments.

In *E. coli*, the fatty acyl chains of lipid A are 12 or 14 carbons long. *E. coli* K-12 and *K. pneumoniae* are described to predominantly contain hexa-acetylated lipid A that is phosphorylated at the 1- and 4' positions. *A. baumannii* shows a similar lipid A structure, but with an extra acyl chain (hepta-acetylated). However, lipid A of *P. aeruginosa* is different from *E. coli*, *K. pneumoniae*, and *A. baumannii*. In *P. aeruginosa*, lipid A is hexa-acetylated, similar to that of the other three species, but the acyl chains are distributed differently (Figure 5). Furthermore, *P. aeruginosa* lipid A generally contains shorter acyl chains than that of the other species.

Beside LPS decreasing OM permeability, porin-mediated OM permeability is also important to antibiotic resistance.

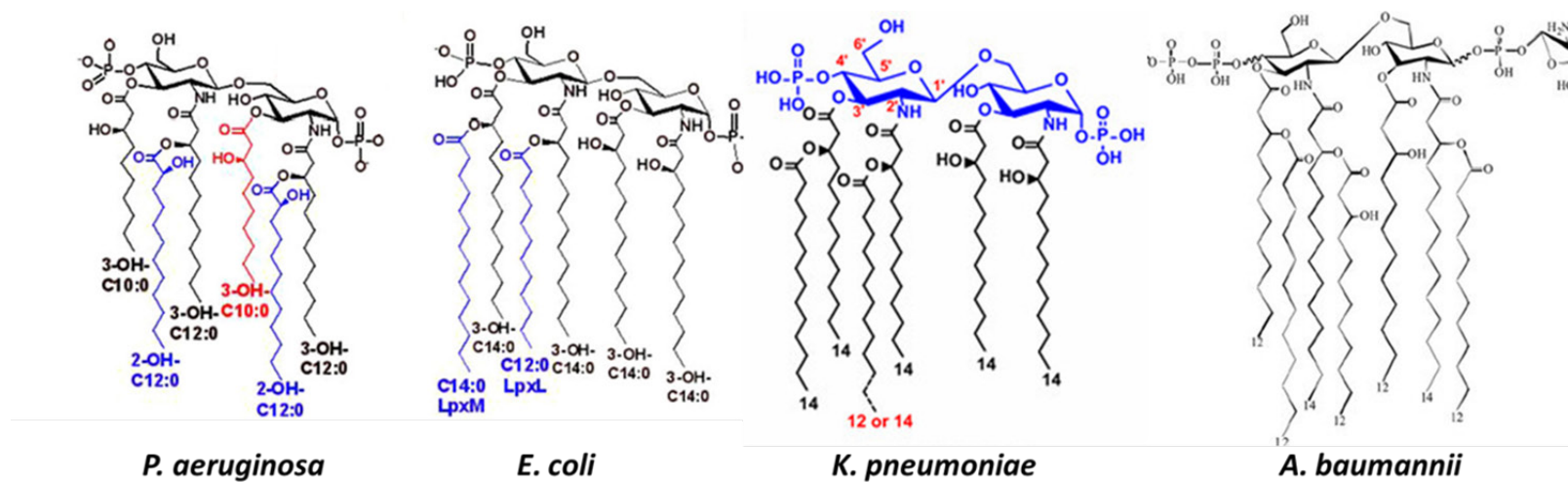


**Figure 5.** Structure of the lipid A in Gram-negative bacteria such as *P. aeruginosa*, *E. coli*, *K. pneumoniae* and *A. baumannii*. [97, 98]

### **The role of porins in antibiotic resistance**

Porins provide a path through the OM to small hydrophilic antibiotics, such as  $\beta$ -lactams, as well as tetracyclines, chloramphenicol and fluoroquinolones [79]. Any decrease in the ability or rate of entry of these compounds can lead to resistance. Many studies reported that antibiotic resistance can be acquired through loss or functional change of porins in a large number of organisms, such as *E. coli*, *P. aeruginosa*, *A. baumannii*, and *K. pneumoniae*. [79, 99-102]. Although much of the mechanistic studies described above have focused on OmpF because of its well understood structural and functional properties relative to any other major porins, many of the reports of changes in porin expression often implicated both OmpF and OmpC in *E. coli*.

#### **2.2.2. Multidrug efflux pumps**

##### **Classes of efflux pumps**

Efflux pumps are bacterial transport proteins that can extrude their substrates like antibiotics from the inside of the cell, the periplasmic space, or the inner membrane to the external environment. From the first drug-resistant efflux pump discovered in the 1990s, the development of molecular microbiology led to the characterization of many efflux pumps in Gram-negative bacteria such as *A. baumannii*, *E. coli*, *K. pneumoniae* and *P. aeruginosa* [103, 104]. Since the transport operates opposed to concentration gradients, these efflux pumps depend on energy. According to the source of deriving energy, efflux pumps are divided into two categories. The primary transporters use ATP hydrolysis as an energy source, while the secondary transporters rather use proton or ionic I gradients. Five major efflux pump families have been described in bacteria (Figure 6), namely (1) Major facilitator superfamily, (2) Multidrug and Toxin Extrusion family, (3) small multidrug resistance family, (4) Resistant nodular cell division family, which are all secondary active transporters and (5) ATP binding cassettes family, which are primary active transporters [105]. All five families are represented in the Gram-negative ESKAPE

pathogens, with individual exporters varying in terms of their substrate specificity. Of note, RND-type efflux pump-mediated resistance is of particular concern for antibiotic resistance among Gram-negative bacteria [106].

**Figure 6.** Schematic of representative structures of multidrug transporters and tripartite assemblies. [107] Abbreviation: ABC superfamily, ATP binding cassettes family; MFS, major facilitator superfamily; MATE family, multidrug and Toxin Extrusion family; SMR family, small multidrug resistance family; RND superfamily, resistant nodular cell division family.

Current models consider that intrinsic multidrug resistance in Gram-negative bacteria results from the synergy between a reduced uptake due to the impermeability barrier of the OM and active drug efflux that further contributes to reduce the intrabacterial concentrations of antibiotics [108, 109]. The transporters that are mainly involved in antibiotic resistance belong to the RND superfamily. RND is the acronym for **R**esistance/**N**odulation/**D**ivision. The RND protein family was described as a related

group of bacterial transport proteins involved in heavy metal resistance (*R. metallidurans*), nodulation (*Mesorhizobium loti*) and cell division (*E. coli*) [110]. They consist of three types of proteins: an outer membrane protein (OMP) located in the outer membrane, an efflux transporter located in the inner membrane, and a membrane fusion protein (MFP) linking the outer membrane protein and efflux transport [111]. The three components form a large multiprotein assembly that traverses both the IMs and OM of Gram-negative bacteria and therefore allows for the direct extrusion of the substrates from the cytoplasm, the periplasmic space or the periplasm to the medium (Figure 7). Worryingly also, these pumps show a broad substrate specificity and are capable of transporting several chemically-unrelated classes of drugs.

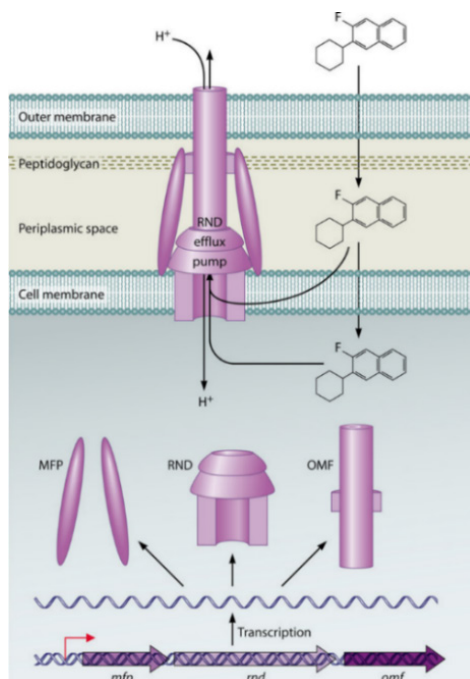


**Figure 7.** Structure and function of RND efflux pumps in Gram-negative bacteria. RND pumps typically exist in a tripartite system consisting of an efflux transporter, an MFP, and an OMF. This complex forms a channel spanning the entire membrane, allowing for the proton-driven transport of structurally different drugs from the cytoplasm of the cell to the medium. The RND efflux pumps can also extrude drugs from the periplasmic space or the inner membrane before they cross the IM. [112]

In *P. aeruginosa*, four main efflux pumps for antibiotics have been described as important for antibiotic resistance, namely, MexAB-OprM, MexCD-OprJ, MexEF-OprN, and MexXY-OprM [113-117] (Table 4). The genes encoding these pumps are operons, with the first gene encoding a membrane fusion protein like MexA, MexC, MexE, and MexX connecting the two other proteins. The second gene encodes the efflux transporter, for instance, MexB, MexD, MexF and MexY, which exports the substrate from the cytoplasm, the periplasmic space, or the inner membrane. The third gene encodes an OM protein, such as OprM, OprJ and OprN, that allows the substrate to cross the OM and be expelled in the extracellular medium. Interestingly, MexXY in the multiresistant taxonomic outlier *P. aeruginosa* PA7 can utilize either the OprA or OprM outer membrane channels [118]. The constitutive expression of MexAB-OprM contributes to the intrinsic resistance of this organism to many antibiotics, such as quinolones, tetracyclines, trimethoprim, chloramphenicol, macrolides, novobiocin,  $\beta$ -lactams and  $\beta$ -lactamase inhibitors [113, 119-122], whereas elevated expression of MexXY-OprM is the major cause of aminoglycoside resistance in the absence of modifying enzymes (acetyltransferases, nucleotidyltransferases, or phosphotransferases) [123]. In addition, efflux pumps MexCD-OprJ and MexEF-OprN are often overproduced in multiresistant clinical isolates [120]. MexAB-OprM and MexEF-OprN play important roles in *P. aeruginosa* physiology and are involved in the secretion of quorum-sensing signals and biofilm formation [124, 125]. Furthermore, the invasiveness of *P. aeruginosa* lacking MexAB-OprM is attenuated in a Madin-Darby canine kidney epithelial cell monolayer system and a murine model of endogenous septicemia [126].

**Table 4** RND family multidrug efflux systems of Gram-negative bacteria.

| Organism             | Efflux components |             |            | Regulatory genes              | Substrates [127-130]                                                                     |
|----------------------|-------------------|-------------|------------|-------------------------------|------------------------------------------------------------------------------------------|
|                      | MFP               | Efflux pump | OM channel |                               |                                                                                          |
| <i>P. aeruginosa</i> | MexA              | MexB        | OprM       | <i>mexR</i>                   | FQ, CM, NV, TP, TGt, SM, EB, AC, CV, SDS, AH, HL, CL, TL, IR, TS, TPP, RD, AZ            |
|                      | MexC              | MexD        | OprJ       | <i>nfxB</i>                   | FQ, CM, NV, TP, TG, SM, DOX, EB, AC, SDS, AH, CL, TS, TG, TPP, RD, DOX                   |
|                      | MexE              | MexF        | OprN       | <i>mexT</i>                   | FQ, CM, TP, AH, TS                                                                       |
|                      | MexX              | MexY        | OprM/OprA  | <i>mexZ (amrR)</i>            | FQ, AG, TC, ER, TG                                                                       |
| <i>E. coli</i>       | AcrA              | AcrB        | TolC       | <i>acrR, marA, robA, sdiA</i> | AH, BA, BL, NV, ER, FU, FQ, TC, CM, EB, AC, CV, SDS, TX, BS, TS, PO, FA, MX, LN, AZ, DOX |
| <i>K. pneumoniae</i> | AcrA              | AcrB        | TolC       | <i>acrR</i>                   | BL, FQ, AG, AP, AZ, DOX                                                                  |
| <i>A. baumannii</i>  | AdeA              | AdeB        | AdeC       | <i>adeSTd</i>                 | AG, CX, TC, ER, CM, TP, FQ, AZ, DOX, BL, LC                                              |

Abbreviations: AC, acriflavine; AG, aminoglycosides; AH, aromatic hydrocarbons; AP, ampicillin; AZ, azithromycin; BA, benzalkonium; BL, b-lactams; BS, bile salts; CL, cerulenin; CM, chloramphenicol; CP, ciprofloxacin; CV, crystal violet; CX, cefotaxime; DOX, doxycycline; EB, ethidium bromide; ER, erythromycin; FA, fatty acids; FU, fusaric acid; FQ, fluoroquinolones; GN, gentamicin; HL, homoserine lactones; LN, linezolid; LC, lincosamides; ML, macrolides; NV, novobiocin; PO, pine oil; RD, rhodamine; SDS, sodium dodecyl sulphate; SM, sulphonamides; TC, tetracycline; TG, tigecycline; TL, thiolactomycin; TS, triclosan; TP, trimethoprim; TPP, tetraphenyl phosphonium; TX, Triton X-100.

In *E. coli*, AcrAB-TolC is the best-characterized efflux pump, and its homologs are broadly represented in Enterobacterales like *K. pneumoniae* [131]. In this three-component complex, AcrB is an efflux transporter, TolC is an outer membrane protein, and AcrA is a periplasmic membrane fusion protein. The three proteins form a trans-envelope complex that expels multiple antibiotics from *E. coli* cells. Extensive structural and functional analyses showed that AcrB captures its substrates from the IM and the periplasm and then expels them through the TolC channel into the external medium with the help of AcrA protein [132]. At least two substrate-binding sites in AcrB, the proximal and the distal, are both located in the periplasmic domains of the protein. Thus, AcrB with the help of AcrA and TolC expels substrates across the OM from the cytoplasm and/or the periplasm to the outside medium while driven by the proton transfer across the cytoplasmic membrane.

AcrAB-TolC substrates are organic solvents, antibiotics, detergents and dyes (Table 4). Examination of chemical structures and cocrystallization and molecular dynamics studies brought further advances in our view of the molecular mechanism of multidrug recognition via AcrB [133, 134]. However, the physicochemical properties of compounds that distinguish AcrB substrates from non-substrates remain elusive. The current consensus is that AcrAB-TolC is the most effective against amphiphilic compounds that diffuse slowly across the OM. An examination of the affinity of AcrAB-TolC toward its substrates demonstrated that minimal inhibitory concentrations of antibiotics are a poor measure of whether an antibiotic is a substrate of an efflux pump [135]. In *E. coli* and *P. aeruginosa*, several efflux assays were developed to examine drug efflux. However, further characterization of the kinetic behavior of efflux pumps in the context of two-membrane envelopes of Gram-negative bacteria is needed.

The *A. baumannii* genome encodes seven RND-type efflux pumps. AdeABC and AdeIJK confer intrinsic resistance to various antibiotics including  $\beta$ -lactams, fluoroquinolones, tetracyclines, lincosamides, and chloramphenicol (Table 4). In addition, AdeABC and AdeFGH are overproduced in clinical multidrug-resistant isolates [136]. Surprisingly, overproduction of AdeABC and AdeIJK could also alter bacterial membrane composition, resulting in decreased biofilm formation but not in motility [137]. When expressed in *E. coli* at levels comparable to those of AcrAB-TolC, AdeABC was more effective to extrude lipophilic  $\beta$ -lactams, novobiocin, and ethidium bromide.

### **3. Phenotypes of Resistance, Tolerance and Persistence**

In a recent Consensus paper, Balaban and her colleagues compared these three phenotypes [138].

Resistance, tolerance and persistence are different responses to antibiotic treatment, leading to an increase in survival in comparison with susceptible bacteria [138]. Although these phenotypes have been studied for a long time, the terms or tolerance [139] and

persistence [140] were proposed to distinguish these modes of survival from resistance, but their definition and distinction from the other ones have long remained unclear [141, 142]. Now, the differences between these phenotypes have been agreed upon in terminology. These differences are illustrated in Figure 8, based on the measurement of the minimum inhibitory concentration (MIC) together with the minimum duration for killing (MDK) [138, 141], a recently defined quantitative indicator of tolerance [143]. Other differences will be described in more details in this chapter.

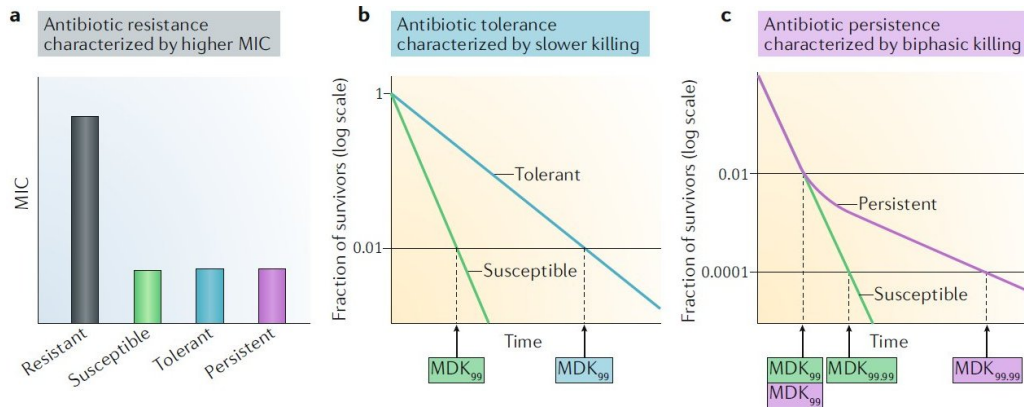


**Figure 8.** Antibiotic resistance, tolerance and persistence. (a) In comparison with susceptible bacteria, antibiotic resistance is characterized by a substantial increase in MIC, but persistence and tolerance did not lead to an increase in MIC. (b) Tolerance markedly increases the minimum duration for killing (MDK; for example, for 99% of bacterial cells in the population (MDK<sub>99</sub>)) in comparison with susceptible bacteria. (c) Persistence leads to a similar MIC and a similar initial killing of the bacterial population (MDK<sub>99</sub>) compared with susceptible bacteria, but the MDK for 99.99% of bacterial cells in the population (MDK<sub>99.99</sub>) can be substantially higher attributing to the survival of the persisters. Persisters are thus corresponding to a subpopulation of tolerant cells. Note that pure exponential killing of the susceptible strain is rarely observed because most bacterial cultures have some level of persistence. [138]

### **3.1. Resistance phenotype**

Antibiotic resistance is the ability of bacteria to grow and not just survive in the presence of a drug. The most common measure of the level of resistance is the MIC, which is the lowest concentration of the antibiotic required to prevent the bacteria replication. A higher MIC exhibits a higher level of resistance. Antibiotic resistance is defined by an increase in the MIC value above a value corresponding to the resistance breakpoint. The European Committee for Antimicrobial Susceptibility Testing considers an isolate as susceptible (S) when 'there is a high likelihood of therapeutic success using a standard dosing regimen of the agent', susceptible (I) when 'there is a high likelihood of therapeutic success using an increased dosing regimen of this agent', or resistant (R) when there is a high likelihood of therapeutic failure even when there is increased dosing regimen of this agent (<http://www.eucast.org/newsiandr/>) [144] (For more details see section 2: mechanisms of antibiotic resistance in Gram-negative bacteria).

### **3.2. Tolerance phenotype**

Antibiotic tolerance is also related to antibiotic treatment failure and recalcitrant infections. Antibiotic tolerance is a transient ability of a whole population of bacteria to survive in the presence of bactericidal antibiotics without a change in the MIC. The survival advantage of tolerant bacteria is often seen in treatments by drugs belonging to different classes, for example,  $\beta$ -lactams and fluoroquinolones [145]. However, strains highly tolerant to these antibiotics may still be killed efficiently by other drugs. Tolerance applies only to bactericidal, and not to bacteriostatic antibiotics, as all bacteria are expected to survive transient exposure to bacteriostatic antibiotics, which are not lethal but merely deter bacterial growth [146].

Importantly, longer exposure to an antibiotic, rather than a higher concentration of an antibiotic, is required to produce the same level of killing in a tolerant strain as is produced in a susceptible strain. As tolerant bacteria can have the same MIC as non-

tolerant bacteria, the MIC is not informative as a metric to evaluate tolerance [145, 147]. Recently, the MDK was described as an index for quantitative measure of tolerance, which can be calculated from time-kill curves (Figure 8). Such an index is based on the conception that a tolerant bacterial strain requires more exposure time to be effectively killed than a susceptible strain.

The MDK is defined as the duration of antibiotic treatment that is required to kill a given proportion of the bacterial population [143] at concentrations that far overstep the MIC. Such high concentration is required to avoid an effect related to drug concentration on the kill rate. For example, the minimum duration of treatment that is required to kill 99% of a bacterial population (MDK<sub>99</sub>), can be calculated from a time-kill curve. The assumption that underlies the MDK as a measure of tolerance is that the killing effect reaches saturation at high concentrations of an antibiotic so that it is almost insensitive to concentration and dependent only on the duration of exposure [148]. Similar to the MIC that can be used to compare the level of resistance between bacterial strains, the MDK can be used to compare the level of tolerance between strains. In contrast to the killing rate (that is, the rate at which survival decreases exponentially), which can only be extracted from exponential killing curves, the MDK integrates all of the different factors that together constitute a faster or slower overall killing efficacy, such as delays in killing or killing curves that are not exponential. Therefore, this quantification of tolerance is not dependent on any particular molecular mechanism. Many studies therefore suggest the MDK should be the preferred metric for the measurement of tolerance, as it enables a quantitative comparison between different bacterial strains and conditions. Furthermore, an evaluation framework that measures both the MDK and the MIC would enable a clear distinction to be made between resistance (an increase in the MIC) and tolerance (an increase in the MDK).

### **3.3. Persistence phenotype**

Antibiotic persistence corresponds to the survival of a subpopulation of tolerant bacteria in the presence of a bactericidal antibiotic. Thus, in contrast to tolerance, it concerns heterogeneous populations, with only a part of them consisting of tolerant cells. Re-culturing persisters (persistent cells) in a fresh medium will generate a susceptible subpopulation similar to the original one (tolerance being not heritable), with also the same MIC as the original strain. This contrasts with the resistance phenotype, in which bacteria can grow in the presence of antibiotics. However, the presence of the drug could prohibit the replication of persisters [138].

Persistence can be detected by the presence of a bimodal (or multimodal) time-kill curve that deviates from the simple decay expected from a uniform bacterial population [149]. In the simple case of two coexisting subpopulations, persistence is characterized by switching between two phenotypes: susceptible and persistent. Persisters constitute the less numerous subpopulation (typically less than 1%) and are killed at a slower rate than the susceptible cells [149]. Tolerance mechanisms, known as dormancy and reduced metabolism and adenosine triphosphate (ATP) levels, have also been demonstrated for persister subpopulations [150].

#### **Biphasic killing and multi-tolerance**

The hallmark of antibiotic persistence is the biphasic shape of killing curves by bactericidal antibiotics (Figure 9). The vast majority of the bacterial population is eliminated during the first phase of rapid killing, while the persister subpopulation is killed at a significantly slower rate [138]. Antibiotic persistence is not always restricted to one subpopulation, as there may have been more than one persister coexisting subpopulations, generation with a multimodal killing curve [151].

Persisters can tolerate antibiotics with different modes of action, conferring to them a phenotype of multidrug tolerance. Most antibiotics interfere with active cellular processes, such as macromolecule synthesis, thus their activity depends on the physiological status of the cell [152]. The multi-tolerance could be the consequence of a global dormant state and the shutdown of antibiotic targets [153]. This multidrug tolerance is however not always present. Different isolates could show different levels of tolerance to various antibiotics [153].

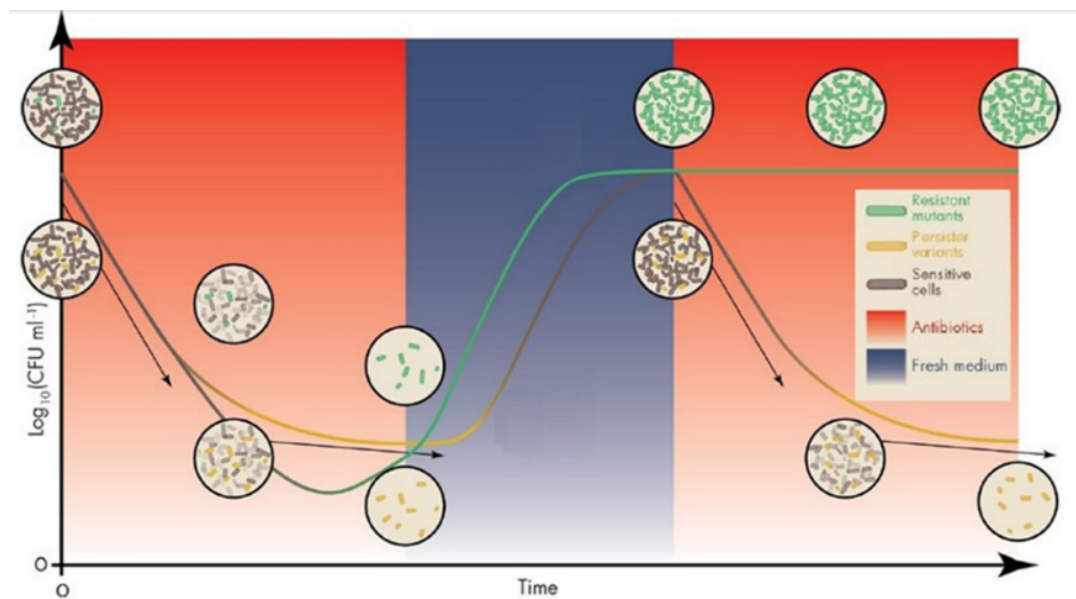


**Figure 9.** Biphasic killing curve and difference between persistence and resistance. In both cases, in the presence of antibiotics, susceptible cells are killed rapidly and killing dynamics are very similar (steep arrow). Resistant mutants are capable of dividing in the presence of antibiotics (green ascending curve) to form a population consisting completely of resistant mutants, while persisters can only survive (slowly descending arrow), leading to the typical biphasic killing pattern for persistence. Upon antibiotic removal, persisters show a lag time before dividing again at a normal rate to form a population containing the same persister subpopulation as the initial one [153].

### **Relation between persistence and therapy failure**

There are only a few reports on the relationship between persister phenotype and treatment failure, as the persister phenotype is much less characterized than resistance and not detected by routine tests of antimicrobial susceptibility (MIC) performed in clinical laboratories [154].

Persister cells are expected to reduce the efficacy of chemotherapy and prolong the duration of infection, leading to treatment failure [155]. In a study in *P. aeruginosa*, an increase in persistence levels of isolates was observed during long-term treatment with antibiotics in patients with cystic fibrosis [156]. Clinical isolates of *E. coli* usually contain multiple mutations in *hipA*, which encodes the toxin entity of the *hipBA* toxin-antitoxin mediating persistence, and thus show higher levels of persistence [157]. Persisters either cause chronic infections or are selected during long-term treatment. In addition, persisters are also considered as a reservoir of viable bacteria that can acquire resistance by random mutations or horizontal gene transfer, therefore they can accelerate the generation of antibiotic-resistant mutants [155, 158]. Interaction between persistence and antibiotic resistance has been described in many reports [158-160]. Antibiotic tolerance of persisters may promote the evolution of resistance [159] by increasing survival and mutation rates [161]. Therefore persisters are considered as an evolutive reservoir from which resistant organisms can emerge [158].

Collectively, persistence is believed to impose a significant burden on public health care, and it is responsible for the chronic and sometimes even incurable nature of many infections [162].

## 4. Persistent infection and persistent forms of infections in Gram-negative ESKAPE bacteria

### 4.1. Persistent infections

First, we would like to distinguish the concept of antibiotic persistence and of persistent infection [163]. Persistent infections are infections that are not cleared by the host immune system, in the presence of or absence of antibiotics. (Figure 10).

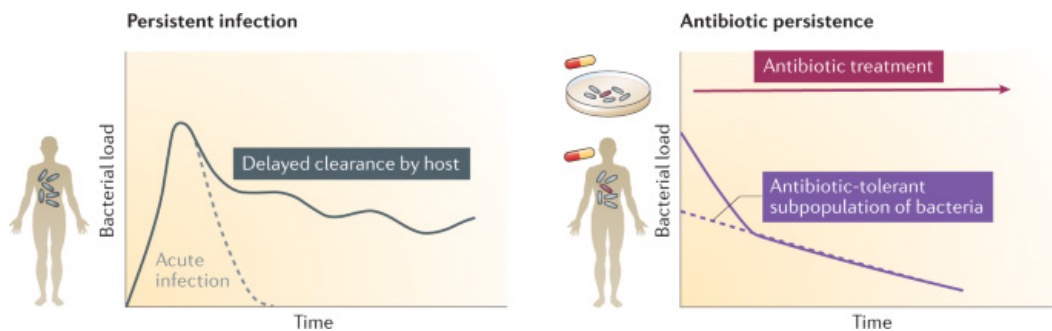


**Figure 10.** Persistent infections and antibiotic persistence. Persistent infection is defined as an infection that is not efficiently cleared by the host, in contrast with an acute infection, which resolves thanks to an appropriate host response. Antibiotic persistence specifically describes the heterogeneous response of bacterial subpopulations, in vitro or in the host, which leads to prolonging the clearance of the bacterial load by antibiotics. [138]

Conversely, and as explained earlier, antibiotic persistence describes a bacterial subpopulation that is refractory to antibiotic treatments, whether in vitro or possibly in the host. As antibiotic persistence specifically deals with the ability of bacteria to survive antibiotic treatments, it may represent an additional factor for the prolongation of persistent infections despite antibiotic treatment, for example, in recurrent urinary tract infections [164, 165]. Thus, antibiotic bacterial persistence may be an important factor in many persistent bacterial infections [165].

## **4.2. Intracellular infection**

Persistence or recurrence of Gram-negative bacteria infections has also been associated with the capacity of Gram-negative bacteria to survive intracellularly [166].

One of the important functions of immune cells is to phagocytose pathogens, which are driven in phagosomes, and then lysed in phagolysosomes after fusion of these vesicles with lysosomes [167]. However, bacteria have developed many pathways to evade the cellular mechanisms of destruction [168] and to thrive in different subcellular compartments. Intracellular bacteria are protected from the extracellular environment (including humoral immune defenses) and the distinct physicochemical conditions prevailing in the different infected compartments may affect both the bacterial responsiveness and the expression of the activity of antibiotics [163, 169].

### **4.2.1. Intracellular infection by Gram-negative bacteria**

Although they are commonly considered extracellular organisms, Gram-negative ESKAPE bacteria may behave as opportunistic intracellular organisms to invade and survive within eukaryotic cells, and eventually evade host immune defences and antibiotic killing (Figure 11). This has been documented for both epithelial [170-173] and phagocytic [174-177] cells in vitro and also observed in vivo for lung epithelial cells and alveolar macrophages of infected mice [178-181].

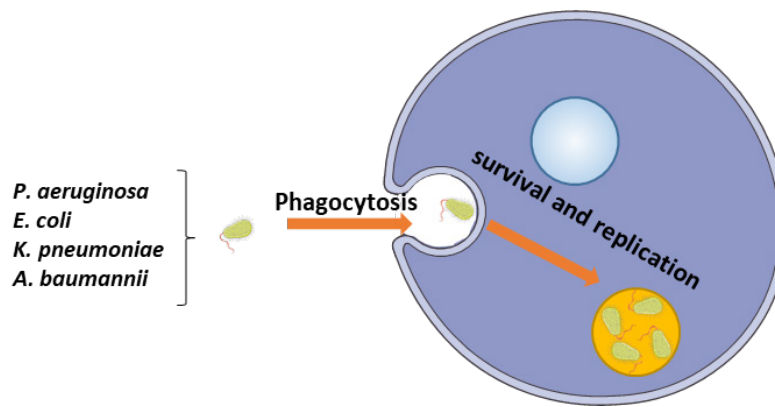


**Figure 11.** Pathways of intracellular bacteria to evade cellular mechanisms of destruction after phagocytosis. Certain bacteria (*P. aeruginosa*, *E. coli*, *K. pneumoniae*, *A. baumannii*) are capable of surviving and multiplying within phagolysosomal vacuoles. Adapted from [182]

Invasion of phagocytes by Gram-negative bacteria like *P. aeruginosa*, is mediated by many surface adhesins. *P. aeruginosa* gets endocytosed and survives within both professional and nonprofessional phagocytes, including macrophages [183]. In such instances, it is thus plausible that the microbes are able to not only evade many of the host's immune defenses but also remain insulated from the activity of cell-impermeant antibiotics, thus providing a reservoir for disseminated and/or latent infection. Intracellular bacteria can show different intracellular fates [184] (Figure 12).

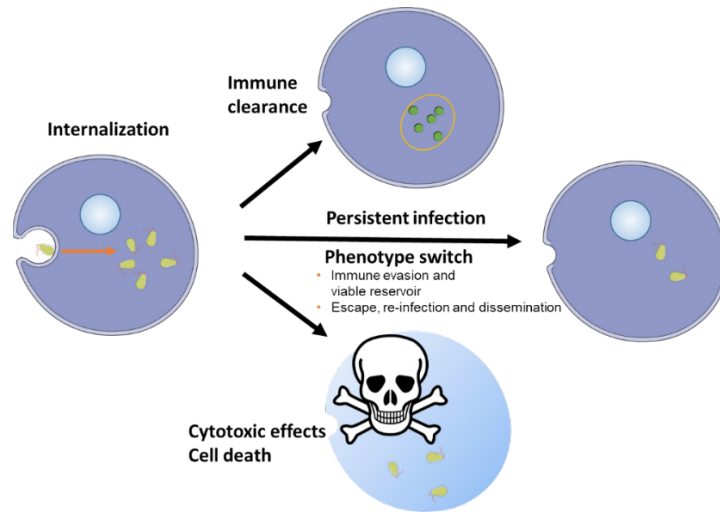


**Figure 12.** Possible outcomes after intracellular Gram-negative bacteria infection. After internalization into the macrophages, not all bacteria undergo phenotype switching. The bacteria can be cleared, it can cause cell death or it can undergo a phenotype switch and cause a persistent infection. Adapted from [185].

After phagocytosis, *P. aeruginosa* rejoins phagosomes or phagolysosomes [182]. Generally, these vacuoles are acidic and nutrient-poor compartments [168]. First, bacteria are confronted with lytic enzymes and specific antimicrobial agents of the immune cell response [167]. These defense mechanisms can succeed in clearing *P. aeruginosa*. Second, *P. aeruginosa* can also cause cell death by producing toxins. Lastly, the stressing conditions prevailing in phagolysosomes can also induce a metabolic switch and lead *P. aeruginosa* to adopt a persister phenotype [186]. This phenotype switch together with the intracellular location allows *P. aeruginosa* to evade the immune response, the antibiotic action, and leads to the formation of a viable reservoir from which the bacteria can escape and disseminate or re-infect the host [187].

#### **4.2.2. Intracellular infection: Response to antibiotics**

Intracellular forms of bacteria are less responsive to antibiotic action [188]. This results from a series of pharmacokinetic and pharmacodynamic parameters (Figure 13).

Regarding pharmacokinetics, antibiotics need to enter, accumulate, and be retained inside the infected cell and also to distribute in the infected compartment. Beta-lactams and linezolid enter eukaryotic cells but do not accumulate (cellular concentration close to the extracellular one); there are recovered in the soluble fraction after cell fractionation procedure [189]. Aminoglycosides and vancomycin are taken up by endocytosis and therefore accumulate to low levels and only very slowly; this mechanism drives them exclusively to the lysosomes [190, 191]. Macrolides accumulate to high levels by a mechanism called diffusion-segregation; this consists in the trapping of these weak basic molecules in the acidic compartments of the cells (lysosomes), from where they cannot escape, owing to the low diffusibility of their positively-charged form [192, 193]. Fluoroquinolones accumulate in the cells for still unclear reasons, and are found in the soluble fraction [194]. Most of these drugs are also substrates for efflux systems from eukaryotic cells which can reduce their accumulation [195]. This is the case for beta-lactams, macrolides, or fluoroquinolones, which are respectively substrates for MRP (Multiple Drug Resistance proteins), BCRP (Breast Cancer Resistance Protein), or P-glycoprotein [195].

Yet, high accumulation is not always synonymous with high activity. One of the reasons for this discrepancy is that some antibiotics fail to express their activity in intracellular circumstances. This is the case for example for macrolides, the activity of which is markedly defeated by the acidic pH prevailing in the lysosomes where are predominantly localized [196, 197]. This, however, does not mean that cell penetration and cell concentration are irrelevant because an absence of penetration or an insufficient local concentration can never be associated with activity [168].

Pharmacodynamic reasons also contribute to explain the absence of systematic correlation between accumulation and activity. In these intracellular niches, bacteria are protected from the host humoral immune defenses but also from antibiotics, which need to have access, accumulate, and express their activity in the infected subcellular compartment [168, 198] (Figure 13). Our team has recently provided evidence for another species, *Staphylococcus aureus*, that bacteria surviving antibiotics intracellularly are persisters, explaining why antibiotics fail to eradicate them [199].

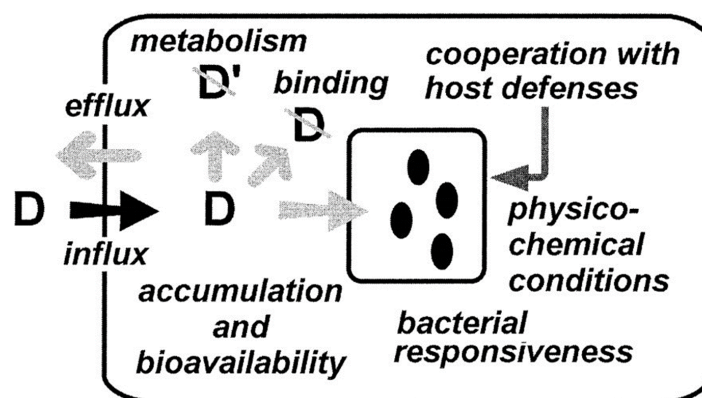


**Figure 13.** Factors affecting the intracellular activity of antibiotics. The balance between influx and efflux, metabolism, and binding determines the intracellular concentration of the free active drugs. Activity is then affected by the state of bacterial responsiveness, the physicochemical conditions prevailing at the site of infection; and the degree of cooperation (or hindrance) with the host defenses. As a result, the final outcome may bear only a very remote correlation with the actual extracellular drug concentration and even the degree of cellular concentration. This, however, does not mean that cell penetration and cell concentration are irrelevant because an absence of penetration or an insufficient local concentration can never be associated with the activity. [168]

#### 4.3. Biofilms formation

Gram-negative ESKAPE bacteria can adhere to both biological and artificial surfaces, on which they can develop strong biofilms that are a physical barrier to the penetration of antibiotics. Biofilms formation contributes to the persistence of infection by Gram-

negative bacteria. They are the most frequently encountered health-associated devices like pathogens in prosthesis or catheter infections [200].

#### 4.3.1. Definition and development overview

Biofilms are a microbially derived sessile community characterized by cells that are irreversibly attached to a substratum or interface or each other, are embedded in a matrix of extracellular polymeric substances (EPS) that they have produced and secreted, and exhibit an altered phenotype for growth rate and gene transcription [201]. The extracellular matrix is mainly composed of exopolysaccharides produced by the bacteria, proteins and extracellular DNA (eDNA). (Figure 14)

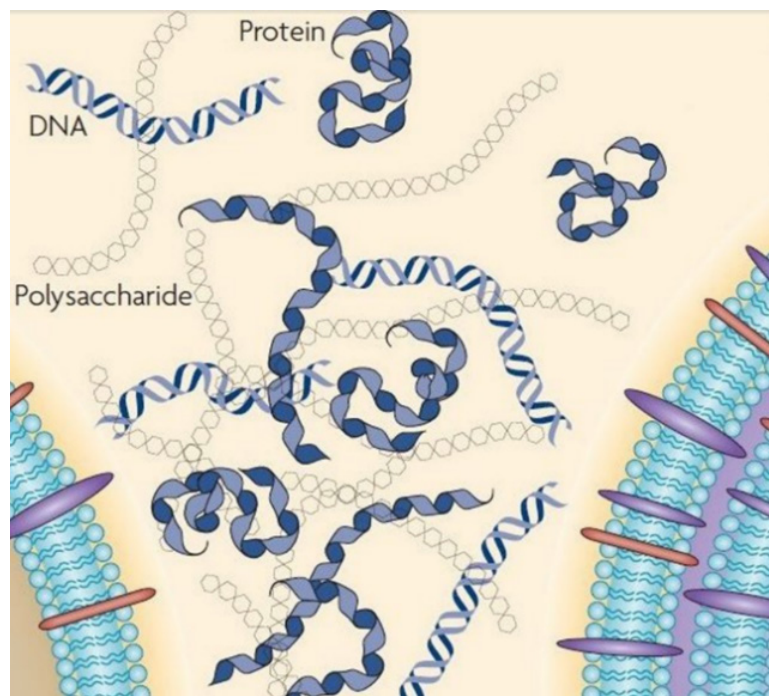


**Figure 14.** Distribution of major matrix components including polysaccharides, proteins and eDNA between the cells in a non-homogeneous pattern. [202]

Biofilm development is a complex dynamic process that follows 5 steps (i) reversible attachment, (ii) irreversible attachment and microcolonies formation, (iii) biofilm maturation, (iv) dispersion [203]. This last stage completes the circle, as dispersed bacteria will spread in the organism to uninfected regions of the host, attach and promote new biofilm formation. (Figure 15)

Microbial cells within biofilms have shown 10-1000 times more tolerance to antibiotics compared with their planktonic forms [204, 205]. Biofilms are also much more tolerant to biocides, chemical shear force and host immune defences [206], therefore allowing bacteria to persist in extremely harsh environments.

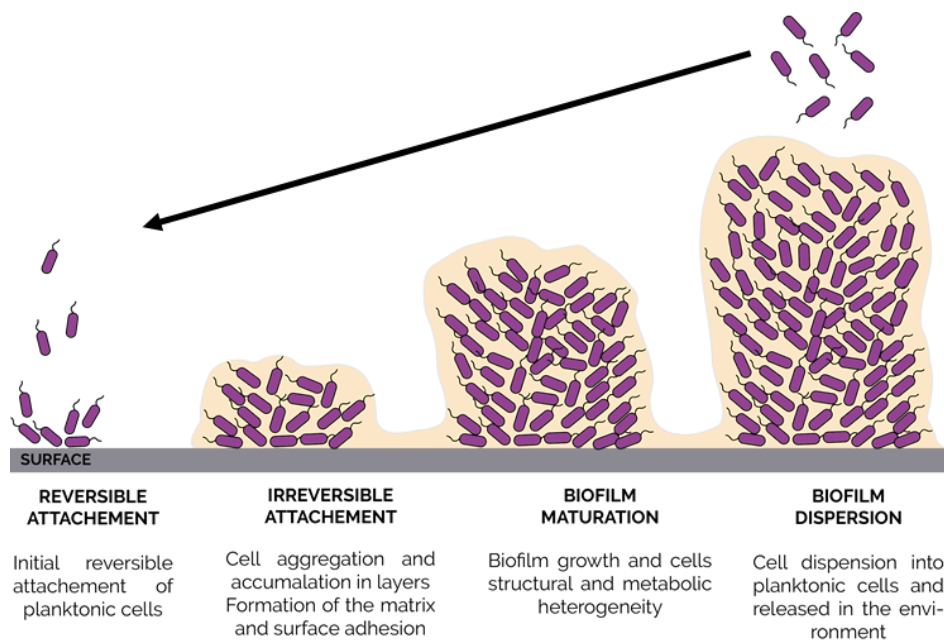


**Figure 15:** Development of biofilm in Gram-negative bacteria. (1) Biofilm formation begins with a reversible attachment of the planktonic cells followed by the adhesion to the surface. (2) The bacteria then irreversibly attach by producing an extracellular matrix, forming microcolonies (3) During later stages, the biofilm is mature (4) Finally, some cells start to detach and the biofilm will disperse. [207]

Most notably, biofilms play an important role in chronic infections, such as those involving *P. aeruginosa* in the airways of patients with cystic fibrosis and indwelling medical device infections caused by *E.coli*, *K. pneumoniae* and *A. baumannii* [208-210].

Biofilm formation and quorum sensing are two kinds of colony behaviors of bacteria. Biofilm is the aggregation of cells, and quorum sensing is the ability to detect bacterial density through the signal transmission of autoinducers between cells. Because the cells in the biofilm aggregates are very similar and connected to each other through a self-generated extracellular matrix, the biofilm thus represents an ecological environment related to quorum sensing [211].

#### **4.3.2. Quorum sensing in Gram-negative bacteria**

Quorum sensing (QS) is a density-dependent communication perception system of microbial cells. Bacteria produce and release chemical signal molecules called autoinducers that increase in concentration as a function of cell density. In general, the quorum-sensing system in Gram-negative bacteria uses LuxI-type enzymes, which produce *N*-acyl homoserine lactones (AHLs) as signal molecules that bind and activate members of the LuxR transcriptional activator protein family [212, 213]. AHL-based quorum sensing system functions through three key components: i) AHL signal molecules, ii) AHL synthase protein for synthesis of AHL signals, and iii) a regulatory protein that responds to the surrounding concentration of AHL signal [214]. This process is initiated with the synthesis and release of AHL signals into the circumambient environment. The AHL signals accumulate in a cell population-density-dependent manner. When the concentration of AHL signals reaches a higher level; the quorum sensing cells start responding and allowing them to regulate the production of secondary metabolites and to control the expression of quorum sensing genes. Most Gram-negative bacteria regulate various phenotypes through the secretion and detection of such signaling molecules. Gram-negative bacteria use quorum sensing communication circuits to regulate variously physiological behaviours including bioluminescence,

antibiotic production, extracellular polymer production, biosurfactant synthesis, sporulation, the release of virulence factors and biofilm formation [215, 216] (Figure 16).

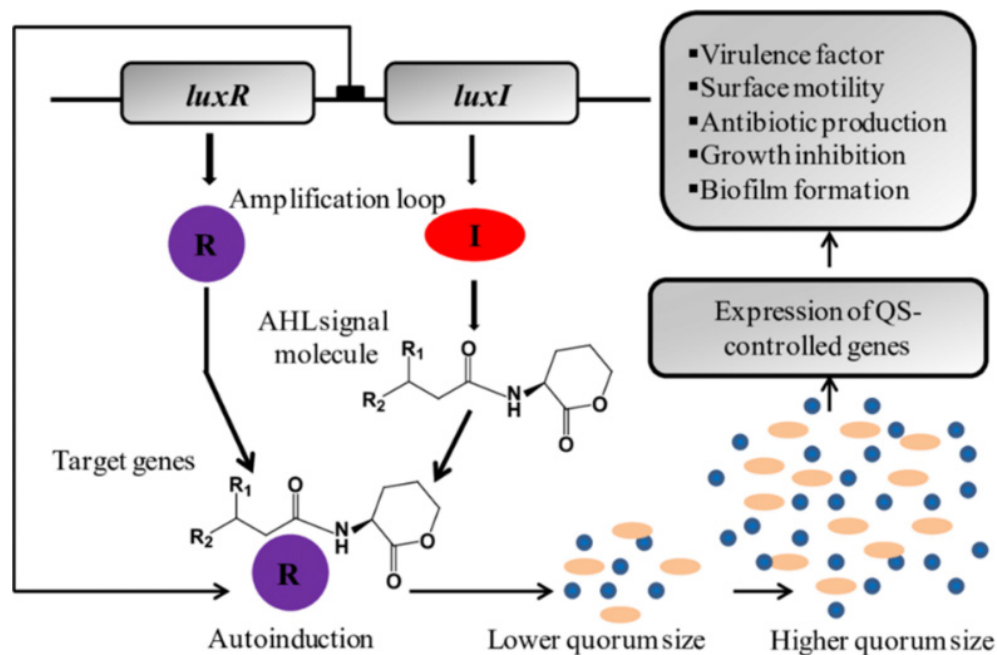


**Figure 16.** LuxR/AHL-mediated quorum-sensing regulation of multiple gene expressions in Gram-negative bacteria. The 'R' and 'I' genes refer to homologous of the LuxR and LuxI genes. The *R* protein is the AHL receptor and signal transducer while *I* protein is the AHL signal synthase. The *I* protein is responsible for the production of AHLs. After synthesis, AHLs get diffuse or pumped from the bacterial cell to the out medium prior to being taken up into nearby bacterial cells. The AHL activates *R* protein by direct binding to make AHL/*R* protein complex which rapidly increases *I* gene expression and hence AHL production. At a certain level of bacterial cells, the quorum sensing system becomes fully activated which leads to *R*-mediated expression of quorum sensing target genes. [217]

#### **4.3.3. Effects of quorum sensing on virulence factors production and biofilm formation**

Biofilm formation is largely driven by the secretion of quorum sensing molecules that allow microorganisms to communicate [218]. Quorum sensing has been documented to play a role in biofilm formation for various bacterial species, such as *P. aeruginosa*, *E. coli*, *K. pneumoniae* and *A. baumannii* [218-220].

In *P. aeruginosa*, the production of many virulence factors is regulated by two acyl-homoserine lactone (AHL) quorum-sensing (QS) systems, such as LasI-LasR (LasI/R) and RhII-RhIR (RhII/R) (Figure 17). The best-studied of these systems is the LasI/R, which includes the LasR receptor and the LasI enzyme that catalyzes the synthesis of the diffusible molecule 3OC12HSL [221]. Intracellular accumulation of 3OC12HSL is sensed by the receptor LasR, which activates the transcription of genes encoding several virulence factors, such as elastase, lipase, LasA protease, and exotoxin A. LasR also regulates the production of extracellular polymeric substances which are major constituents of biofilms matrix [222]. RhII instructs the synthesis of N-(butyryl)-L-homoserine lactone (C4-HSL). Activation of RhIR by C4-HSL regulates the production of elastase, rhamnolipids, pyocyanin, lectins A, B and lipase. These two AHL QS systems interact with a third system, which is mediated by the signal 2-heptyl-3-hydroxy-4-quinolone termed the *Pseudomonas* quinolone signal (PQS). The *pqs* operon *pqsABCDE* codes for PQS production. PQS regulates the production of *las*- and *rhl*-dependent virulence factors, including elastase, pyocyanin, rhamnolipids, lectins A, B and lipase

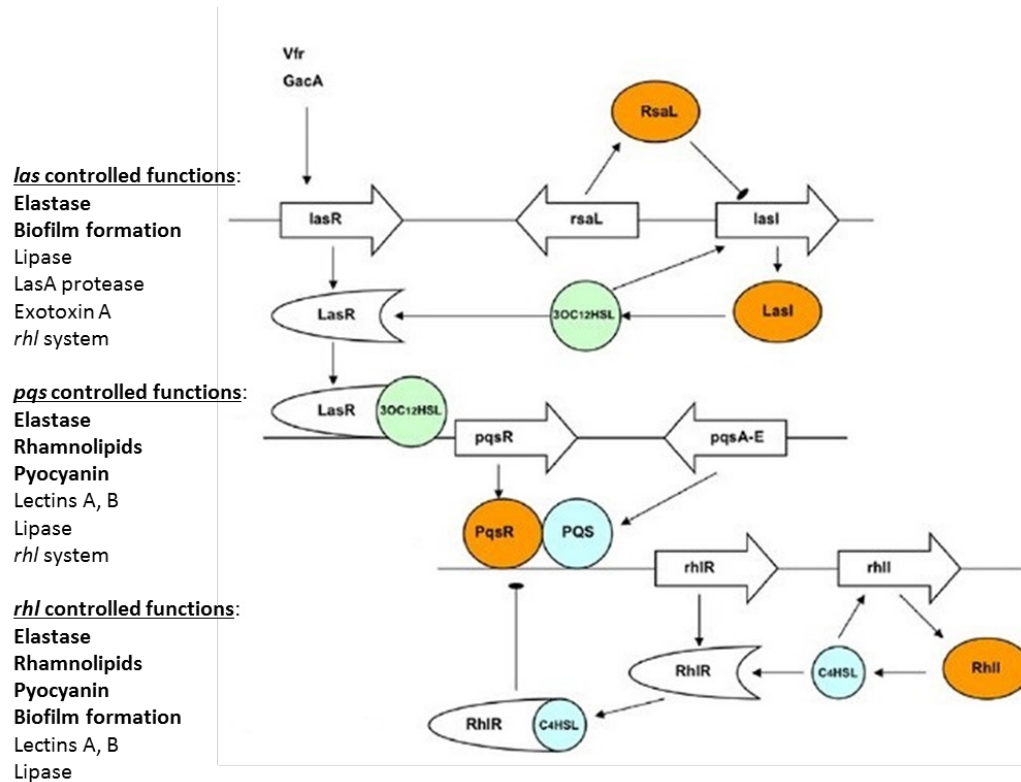


Figure 17. Schematic overview of the complex QS systems present in *P. aeruginosa*. Adapted from [223]

Among the functions regulated by the LasI-LasR and RhII-RhIR systems, several virulence factors are related to biofilm formation, such as rhamnolipids and lectins [224]. Rhamnolipids are required to maintain the biofilm aggregation, affecting a type of surface movement called clustering that is involved in the development of biofilms. LecA and LecB, two lectins-based QS carbohydrate-binding, have been shown to affect the biofilm formation, with mutant strains unable to form mature biofilms [225, 226].

In addition, quorum sensing also plays a role in the production of extracellular DNA that functions as a biofilm matrix component in biofilms [227]. Only a small amount of

extracellular DNA is present in the initial phase of biofilms, the release of a large amount of extracellular DNA occurs at a later stage of biofilm formation, regulated via the PQS system in *P. aeruginosa* [228]. The release of extracellular DNA has been shown to perhaps result from the lysis of a small subpopulation of the bacteria in biofilm [227]. In agreement with a role for the PQS system in the production of extracellular DNA, biofilms formed by *P. aeruginosa* PQS mutants are defects, developing thin biofilms that contain little extracellular DNA [229]. Although the mechanism of PQS-mediated DNA-release is elusive, PQS has been shown to act as a cell-sensitizing pro-oxidant [230], lysing a subpopulation of the *P. aeruginosa* bacteria during biofilm formation.

Lastly, quorum-sensing molecules also regulate the production of virulence factors and motility. The virulence factors include biosurfactant rhamnolipids, pyocyanin and elastase. Pyocyanin production is an intricate QS-driven process involving two identical operons termed *phz1* and *phz2* (*phzABCDEFG* genes) and two modifying enzymes, PhzM and PhzS [231]. Rhamnolipid production from the *rhlAB* operon and *rhlC* gene is under the stringent control of QS genes, particularly the transcriptional regulator RhlR [232]. Caiazza et al. [233] and Kohler et al. [234] reported that the regulation of swarming motility depends on rhamnolipid biosynthesis controlled by the *rhl* QS system in *P. aeruginosa*. Rhamnolipids are suggested to overcome surface tension and allow flagellum-based propulsion of *P. aeruginosa* over semisolid surfaces. In addition some reports also link the other types of motilities with quorum sensing. A study by Glessner et al. [235] showed that both the *las* and *rhl* quorum-sensing systems are required for 4 pilus-dependent twitching motility. Another research performed by Li et. al [236] indicates that PA5022 (*aefA*) links swimming motility and *las* and *rhl* quorum sensing. In addition, elastases are metalloproteases regulated by all the three QS systems of *P. aeruginosa* [237]. Meanwhile, the virulence factors themselves also regulate biofilm formation, such as motility for rhamnolipids as well as for T6SS, the nucleoside diphosphate kinase for elastase, or the release of extracellular DNA for pyocyanin in *P. aeruginosa* [238, 239]. Another important factor in the biofilm formation of bacteria is

swimming motility regulated by N-acyl homoserine lactones, a QS signal molecule [240]. The motility contributes to the early development of the biofilm architecture (microcolony formation) [241].

#### 4.3.4. Response to antibiotics in biofilms

Bacteria encased in biofilms are characterized by an increased capacity to survive in difficult environmental conditions (poor nutrients, high cell density, temperature, pH and osmotic stresses), or in the presence of host defense cells or antimicrobials. Focusing on the impact of this lifestyle on antibiotics, different mechanisms are proposed to explain their lack of activity, including impaired antibiotic diffusion, drug indifference, expression of biofilm-specific genetic mechanisms, and the presence of persister cells (Figure 18)

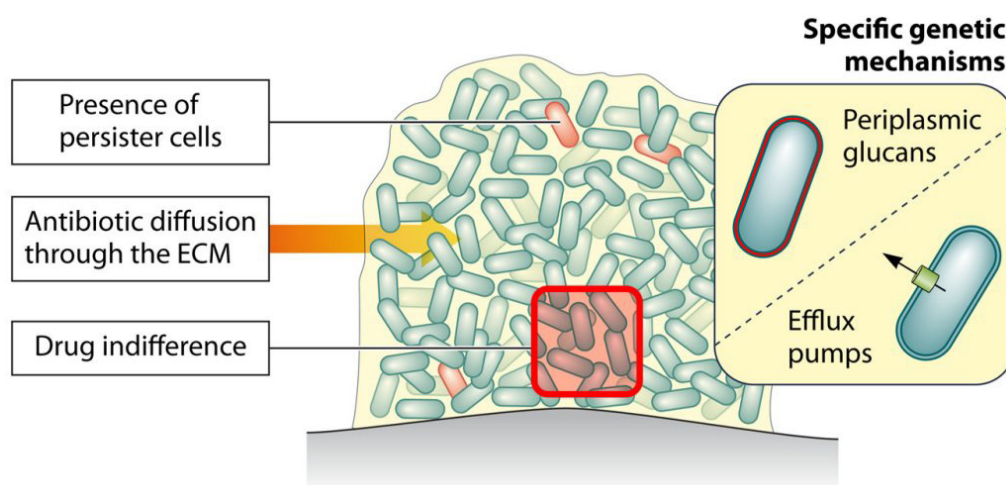


**Figure 18:** Summary of the main mechanisms involved in recalcitrance of biofilms toward antibiotics [242]

### **Antibiotic penetration**

It has been reported that the extracellular matrix (ECM) surrounding bacteria was mainly responsible for biofilm recalcitrance. Meanwhile, many reports suggest that mechanical and physicochemical properties of the biofilm matrix can reduce or delay the penetration of numerous compounds, such as antibiotics [243]. For example, recent studies suggest a slow penetration of positively charged aminoglycosides through negatively charged polymers of the biofilm matrix [244]. Thus, the chemical structure of the biofilm matrix is important, and it has been shown that even a single pathogen can involve different types of exopolysaccharides, relying on the environment of the biofilm formation [245]. Monitoring of antibiotic diffusion through cardiac vegetation in an in-vivo model of endocarditis suggested that a diffusion gradient could be observed in the presence of teicoplanin (a glycopeptide) and penicillin [246], while tobramycin was observed to be homogeneously distributed. Conversely, in *P. aeruginosa* biofilm, tobramycin was observed to exhibit delayed and reduced diffusion in-vitro [247]. Thus, the situation of diffusion of an antibiotic through the biofilm matrix cannot be simply extrapolated to another bacterial strain or biofilm matrix.

### **Drug indifference and altered microenvironment**

Deep biofilm layers correspond to a particular physicochemical microenvironment due to various gradients of nutrients, waste, pH, oxygen, and metabolic by-products through the ECM [248]. Since many antibiotics are more active against bacteria that are metabolically active and growing, these microenvironments lacking nutrients or anoxia can reduce the effects of antibiotics [249]. For example,  $\beta$ -lactam antibiotics can target the bacterial cell wall synthesis and are effective only against actively growing bacteria undergoing cell division [250]. Consistently, the effects of  $\beta$ -lactams against *P. aeruginosa* biofilms have been correlated with the metabolic activity of bacteria embedded in the biofilm [251]. Similarly, the low oxygen concentrations observed in deep layers of *P. aeruginosa* biofilms can reduce tobramycin and ciprofloxacin

bactericidal effects [252]. Other physicochemical characteristics can impair the effects of antibiotics, such as low pH and the anaerobic environment, leading to decreased activities of aminoglycosides against biofilms [253, 254].

### **Contributions of genetically determined mechanisms**

Several studies performed in *P. aeruginosa* and *E. coli* suggested that efflux pumps, induced specifically under biofilm conditions, could be related to biofilm-specific recalcitrance. In *P. aeruginosa*, MexAB-OprM is involved in the efflux of many structurally different antibiotics such as azithromycin and ofloxacin [255] and while MexXY-OprM contributes to resistance to colistin [256]. MexEF-OprN and MexXY-OprM are upregulated in response to reactive oxygen species (ROS) and could help in removing cellular elements damaged by ROS [257]. Recently, the DNA-binding transcriptional regulator BrlR was shown to contribute to *P. aeruginosa* tolerance [258]. Biofilm bacteria with inactivated BrlR, are more susceptible to hydrogen peroxide and antibiotics of different classes, including tobramycin and norfloxacin in comparison with biofilm bacteria with activated BrlR. Overexpression of *brlR* increased *P. aeruginosa* tolerance toward antibiotics. Moreover, BrlR is an activator of MexAB-OprM and MexEF-OprN [259] and the two-component hybrid protein SagS is a possible upstream regulator of BrlR [260].

In *E. coli*, many efflux pumps, typically AcrAB-TolC, are upregulated in biofilms and can remove toxic compounds, including antibiotics [261]. High-throughput screening of *E. coli* mutants suggested that *rapA* mutants displayed decreased tolerance toward many antibiotics such as penicillin, norfloxacin, chloramphenicol, and gentamicin [262]. It was demonstrated that *rapA* not only regulates *yhcQ* gene that encodes a putative multidrug resistance pump, but also *yeeZ* gene, a gene is suspected to be involved in cell wall biogenesis [262]. Thus, a dual *rapA*-mediated action was proposed, with increased antibiotics efflux and reduced penetration [262].

### **Bacterial persistence**

Although the aforementioned mechanisms play a critical role in the inability of antibiotics to eradicate biofilm bacteria, they cannot fully explain biofilm recalcitrance. For example, fluoroquinolones, which can kill non-dividing cells and diffuse easily throughout the biofilm matrix, cannot completely eradicate biofilm [263]. The presence of persisters is now considered to explain most of the biofilm recalcitrance toward antibiotics [264]. As shown in a previous study, a subpopulation of tolerant bacteria survives in the presence of antibiotics despite increased concentrations and times of exposure [265].

## **5. New strategies against antibiotic-resistant Gram-negative bacteria infection**

Drug options for the treatment of infections are currently limited. The discovery of new antibiotic classes acting on still unexploited targets is more and more complex, most of the classical targets having already been exploited. Since the early 1960s, only 4 new classes of totally new antibiotics have been approved: quinolones, lincosamides, oxazolidinones, and cyclic lipopeptides [266].

### **5.1. Old or disused antibiotics**

In the face of increasing antibiotic resistance and the paucity of new drugs, new treatment strategies are urgently needed. One of these is to revisit old or disused antibiotics to ensure that they are used correctly and to their full potential, as well as to determine whether one or several of them can help alleviate the pressure on the scarcity of new antibiotics [267]. The old or disused antibiotics may still be active against some of the existing multidrug-resistant or extensively drug-resistant pathogens. These antibiotics were developed during the 1950s–70s but some of them fell out of favour,

mainly due to the availability of newer antibiotics with better tolerability, improved activity or more convenient administration routes, or were used to treat Gram-positive bacteria only.

In this work, we selected as disused antibiotics rifampicin (not substrate for efflux), doxycycline, and chloramphenicol against *P. aeruginosa*. We then also added antibiotics the spectrum of which is limited to Gram-positive bacteria due to poor penetration in Gram-negative organisms, like azithromycin, linezolid, fusidic acid and novobiocin (all substrates for efflux) and tested them against the other Gram-negative bacteria. (Table 5)

## **5.2. Reviving old or disused antibiotics by combination with potentiators**

The combination of antibiotics has already been used in clinical therapy to cover a wider spectrum of microbes, to achieve better efficacy and to overcome treatment resistance. These combinations have represented effective and successful strategies to increase the efficacy of existing antibiotics.

In the same line, it is also feasible to combine an antibiotic with a potentiator, i.e. a molecule that increases its activity at a concentration lower than that at which it shows itself antibacterial activity. This strategy can be applied to revive antibiotics that are inactive on Gram-negative bacteria due to intrinsic mechanisms of resistance altering their capacity to rejoin their target (impermeability of the outer membrane or active efflux). These adjuvants may thus include efflux pump inhibitors (EPIs), outer membrane permeabilizers (OMPs), and LPS-disturbing agents. We will focus here on those that have been used in our experimental work.

**Table 5.** Typical examples of the antibiotics we used in the thesis with selected physicochemical or pharmacokinetic properties relevant for our experimental work. They were selected as being substrates for efflux pumps of the RND family or poorly diffusible through the outer membrane in four species Gram-negative bacteria.

| Antibiotics     | Resistance by efflux                                              |                                                                                     | Antibiotic targets<br>[268-270]       | LogD values at pH 7.4 (octanol:water partition coefficient at pH 7.4); calculated using LogD Predictor ( <a href="https://disco.chemaxon.com/calculators/demo/plugins/logd/">https://disco.chemaxon.com/calculators/demo/plugins/logd/</a> ). | Accumulation level in eukaryotic cells (at equilibrium) [168] | $C_{min} / C_{max}$ in human serum (mg/L) [271-274] |
|-----------------|-------------------------------------------------------------------|-------------------------------------------------------------------------------------|---------------------------------------|-----------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|---------------------------------------------------------------|-----------------------------------------------------|
|                 | Pumps shown to transport the drug [275]                           | Bacterial species [275, 276]                                                        |                                       |                                                                                                                                                                                                                                               |                                                               |                                                     |
| Rifampicin      | Poor substrate for efflux                                         | NA                                                                                  | bacterial RNA polymerase (RNAP)       | 2.98                                                                                                                                                                                                                                          | 1-4                                                           | 0.1-10                                              |
| Azithromycin    | MexAB-OprM; MexCD-OprJ; AcrAB-TolC, AdeABC                        | <i>P.aeruginosa</i> , <i>E. coli</i> , <i>K. pneumoniae</i> and <i>A. baumannii</i> | 50S subunit of the bacterial ribosome | -1.50                                                                                                                                                                                                                                         | 40-300                                                        | 0.04-1                                              |
| Linezolid       | MexAB-OprM; AcrAB-TolC                                            | <i>P.aeruginosa</i> , <i>E. coli</i>                                                | 50S subunit of the bacterial ribosome | 0.64                                                                                                                                                                                                                                          | 1                                                             | 2.5-15                                              |
| Fusidic acid    | MexAB-OprM; AcrAB-TolC, AdeABC                                    | <i>P.aeruginosa</i> , <i>E. coli</i> and <i>A.baumannii</i>                         | 50S subunit of the bacterial ribosome | 1.50                                                                                                                                                                                                                                          | ND                                                            | 30-200                                              |
| Novobiocin      | MexAB-OprM; MexCD-OprJ; AcrAB-TolC                                | <i>P.aeruginosa</i> , <i>E. coli</i>                                                | DNA gyrase                            | 1.76                                                                                                                                                                                                                                          | ND                                                            | 15-60                                               |
| Chloramphenicol | MexAB-OprM; MexCD-OprJ; MexEF-OprN AcrAB-TolC, AdeABC             | <i>P.aeruginosa</i> , <i>E. coli</i> , <i>K. pneumoniae</i> and <i>A. baumannii</i> | 50S subunit of the bacterial ribosome | 0.87                                                                                                                                                                                                                                          | 2-5                                                           | 5-15                                                |
| Doxycycline     | MexAB-OprM; MexCD-OprJ; MexXY-OprM AcrAB-TolC, AdeABC             | <i>P.aeruginosa</i> , <i>E. coli</i> , <i>K. pneumoniae</i> and <i>A. baumannii</i> | 30S subunit of the bacterial ribosome | -3.48                                                                                                                                                                                                                                         | 2-10                                                          | 1-10                                                |
| Ciprofloxacin   | MexAB-OprM; MexCD-OprJ; MexEF-OprN MexXY-OprM; AcrAB-TolC, AdeABC | <i>P.aeruginosa</i> , <i>E. coli</i> , <i>K. pneumoniae</i> and <i>A. baumannii</i> | DNA gyrase                            | -0.86                                                                                                                                                                                                                                         | 4-10                                                          | 0.05-5                                              |

### 5.2.1. Efflux Pumps Inhibitors

EPIs are molecules that can inhibit the activity of efflux pumps; they have been considered potential therapeutic adjuvants for rejuvenating the activity of antibiotics that have already lost their activity against Gram-negative bacteria. Efflux inhibition could be achieved in six different ways. (Figure 19).

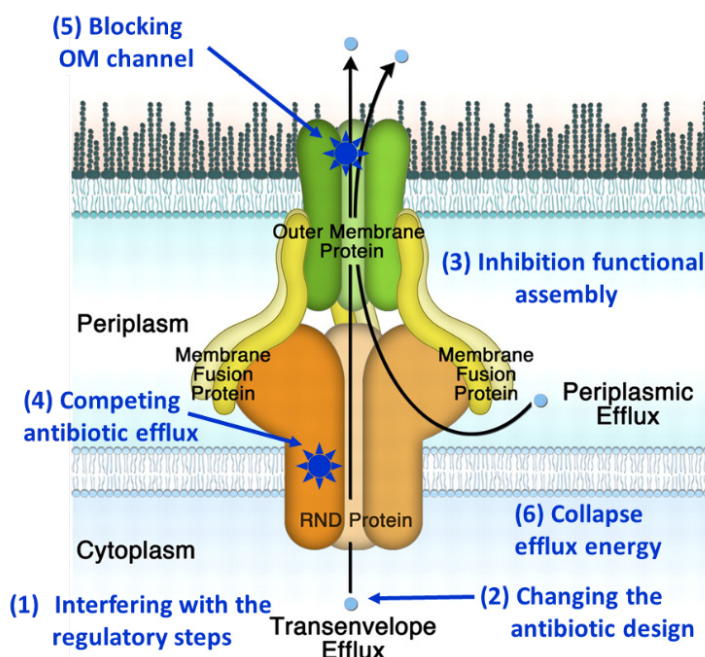


**Figure 19.** Possible mechanisms of action of EPI. Efflux inhibition could be achieved through different ways, including: interfering with the regulation of efflux pump expression, altering the chemical structures of the antibiotic substrate to prevent its recognition by the pump, interfering with the folding of efflux pump components, inhibiting substrate (antibiotic) binding by competitive or non-competitive mechanisms using other compounds, blocking the pump pores through which efflux occurs, and collapsing the energy source needed for the pump function. Adapted from [277]

Since EPI can help antibiotics to achieve adequate concentrations inside the bacterial cells, they can be used as adjuncts in combination with antibiotics to enhance their activity against bacteria expressing efflux pumps. The possibility of using EPIs to recover the activity of antibiotics has been at an experimental stage since the beginning of this century.

EPI has shown good prospects as therapeutic adjuvant in experimental models. Although many EPIs with different modes of action have been reported, they can be roughly divided into two categories: (1) energy dissipation: target the proton gradient or the ATPase that supplies energy to these pumps. For example, carbonyl cyanide-m-chlorophenylhydrazone (CCCP), has been reported to revive the activity of tetracycline in *Klebsiella spp.* by affecting disruption the proton motive force [278]. In addition, Bhattacharyya et al. [279] recently reported that a synthetic EPI, IITR08027, can effectively reverse the resistance to fluoroquinolones in both recombinant *E. coli* and clinical strains of *A. baumannii* by disturbing the proton gradient that is necessary for energizing the pump. (2) Inhibition by direct binding: by binding of the EPIs to functional efflux pumps, resulting in a reduced ability of the pumps to interact with their substrates. This binding may be competitive, when the EPI competes with the substrate for the same binding site; or non-competitive, when the combination of the EPI and the pump results in a decrease in the affinity of the pump for its substrate. For instance, 1-(1-naphthylmethyl)-piperazine (NMP) resulted in increased accumulation of levofloxacin in *E. coli* cells, which enhanced its activity [280]. Mechanistic studies showed that NMP interacts with the F610 residue and causes a conformational change in AcrB, resulting in inhibition in a non-competitive manner [281]. Another EPI is phenylalanine-arginine  $\beta$ -naphthylamide (PA $\beta$ N or MC-207,110), the first broad-spectrum inhibitor of the RND family pumps. More details of this compound are given here below.

## **PAβN**

High-throughput screening for molecule libraries using *P. aeruginosa* drug-resistant strains enabled the discovery of peptidomimetics that block antibiotic efflux [282]. PAβN (MC-207,110) and D-Orn-D-hPhe-3-aminoquinoline (MC-02,595) (both dipeptide amide products) showed high chemosensitizer properties and inhibit *P. aeruginosa* efflux pumps [283, 284]. PAβN (structure in Figure 20) was originally described in 1999 [285] and further characterized in 2001 [286] as a broad-spectrum efflux pump inhibitor, capable of significantly reducing fluoroquinolone resistance in *P. aeruginosa*. Subsequently, PAβN itself was determined to be a substrate for the MexAB–OprM pumps in *P. aeruginosa* and AcrAB–TolC pumps in *E. coli* [286, 287]; it is therefore considered as a competitive efflux inhibitor. Among the dipeptide amide products, PAβN inhibits efflux in various Gram-negative bacteria, including *P. aeruginosa*, *E. coli*, *A. baumannii* and *K. pneumoniae* [288-290].

In addition to its efflux activity, PAβN was reported to permeabilize membranes at concentrations  $\geq 16$   $\mu\text{g/mL}$ , but only in *P. aeruginosa* mutants deficient in the MexAB–OprM efflux pump. Its effect of OM permeability can be abolished by the addition of 1mM  $\text{Mg}^{2+}$  [286]. A more recent study showed that a lower concentration of 4  $\mu\text{g/mL}$  PAβN permeabilizes wild-type *E. coli* with potency similar to the antimicrobial peptide, Polymyxin B nonapeptide [291]. Both studies used increased access of small molecules to the periplasm as a measure of permeabilization.

However, the accumulation of polycationic compounds within acidic organelles such as lysosomes can lead to toxicity [292, 293]. Thus, this compound exhibits toxicity *in-vivo*, which has been related to its high level of accumulation in a variety of tissues, particularly the kidney [284].

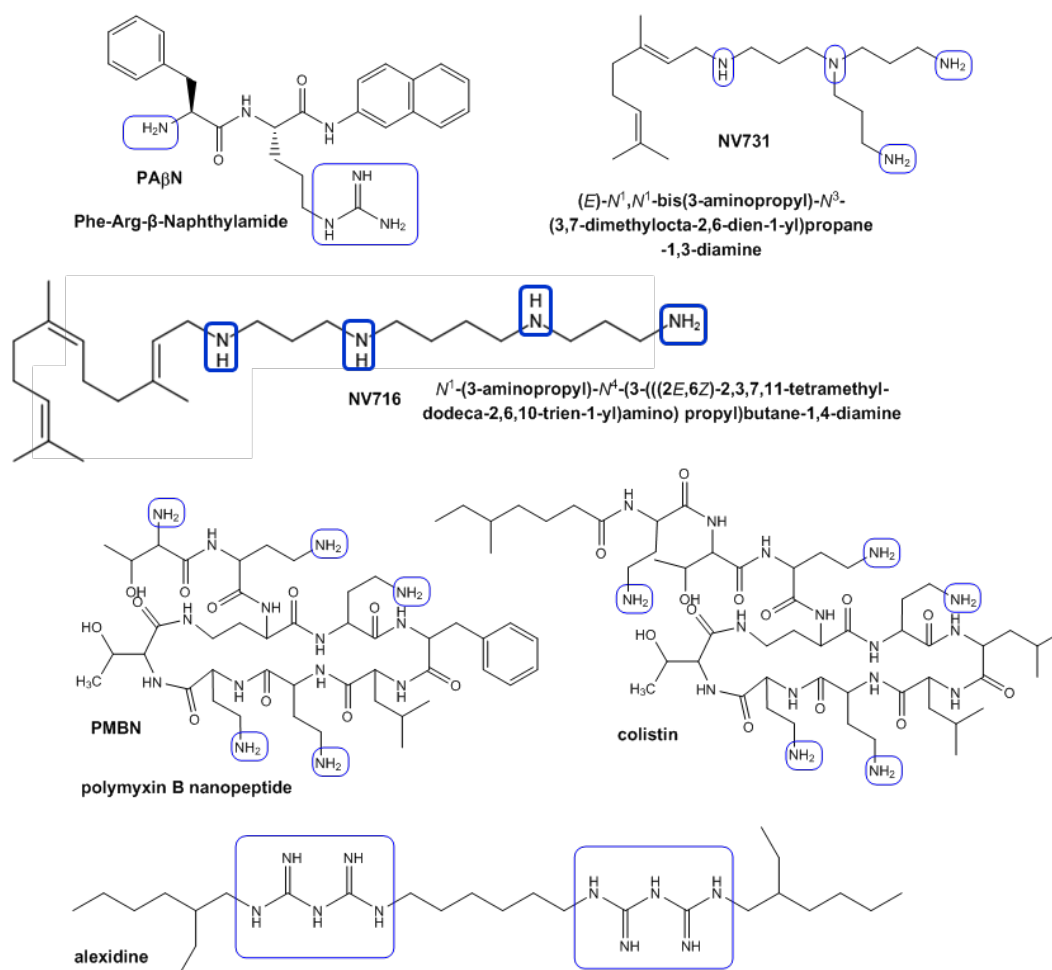


**Figure 20:** Chemical structures of potentiators that will be used in this thesis. The aminated functions of potentiators that are partially protonated at physiological pH are evidenced by blue rectangles.

### 5.2.2. Agents that act as outer membrane permeabilizers

Currently, most available antibiotics exert their action at the intracellular level, which requires their penetration inside the bacterial cell through the cell membrane. New strategies are needed to design and use potentiators that can disturb OM to facilitate the diffusion of antibiotics and increase their intracellular accumulation [294]. In this context, several compounds that alter the OM permeability have been proposed such as polymyxins (colistin), and polycationic/cationic antimicrobial peptides (polymyxin B nonapeptide, PMBN) [295, 296]. They have been used in combination with antibiotics against resistant strains [297, 298].

#### Colistin

Polymyxins (see structure in Figure 20) belong to a group of cyclic lipopeptides with potent antimicrobial characteristics. The heptapeptide ring of polymyxins is enriched with the unusual cationic diaminobutyric acid (DAB); the C-terminus of the exocyclic tripeptide is fused to the macrocyclic ring, whereas the N-terminus is linked to a fatty acid tail (prominently 6-methyl-octanoic acid). Colistin, also known as polymyxin E, was initially extracted from cultures of the Gram-positive bacterium *Bacillus colistinus*.

Some reports claim that it is effective at a few micromolar concentrations to inhibit and kill Gram-negative bacteria [299], thus colistin is used as an antibiotic itself mostly against Gram-negative organisms. It is active against the common species of *Enterobacteriaceae* and *Aeromonas*, and against the none-fermentative Gram-negative, among which *P. aeruginosa* and *Acinetobacter* species are naturally susceptible [300]. Moreover, *E. coli*, *Enterobacter*, *Salmonella*, *Shigella*, and *Klebsiella* are also sensitive to colistin [300].

On another hand, owing to its ability to permeabilize OM, it is used to restore the activity of poorly permeable drugs such as rifampicin. Many reports based on in-vitro and in-vivo

studies showed the synergy of a combination of colistin and rifampicin against Gram-negative bacteria like *P. aeruginosa*, *E. coli*, *K. pneumoniae* and *A. baumannii* [301-304].

Colistin has a high affinity for LPS of OM [305, 306]. This is exemplified by the anti-endotoxic effect of colistin in treating septic shock caused by free LPS molecules known as endotoxins [307]. The strong binding of colistin to LPS also explains the formation of rod-shaped projections from the cell wall of Gram-negative bacteria when exposed to colistin [308]. The displacement of divalent cations like  $Mg^{2+}$  and  $Ca^{2+}$  by colistin impairs the stabilization of the LPS layer. A weakened LPS layer allows more colistin (or other antibiotics) to traverse the OM [309, 310]. The mechanism was considered as the self-promoted uptake pathway. Collectively, the first step in the action of colistin against Gram-negative bacteria is to modify the structure and organization of the LPS of OM. Subsequent colistin inserts in the inner membrane and disrupts the physical integrity of the phospholipid bilayer [296](Figure 21).

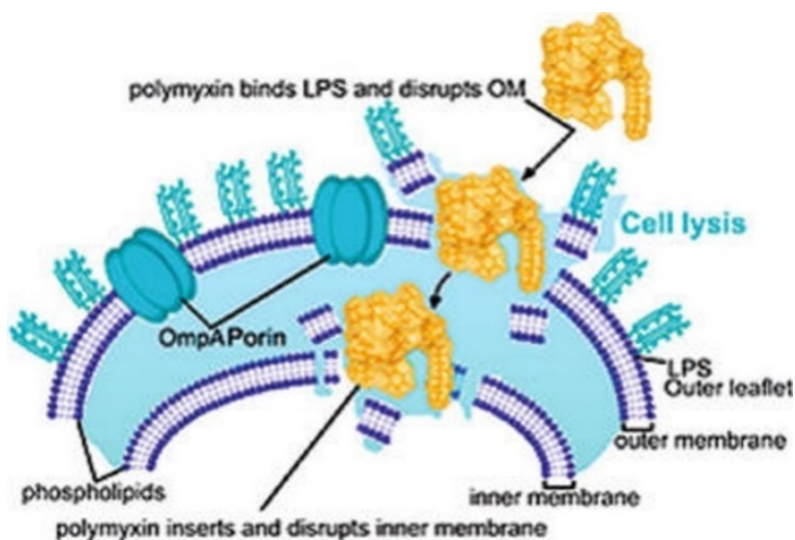


**Figure 21.** Diagram depicting putative antimicrobial action of colistin on Gram-negative bacterial membrane. [296]

Colistin was used as a broad-spectrum antibiotic for clinical application starting in the 1950s. Later, clinical experiences showed that colistin treatment was often accompanied with neurotoxic and nephrotoxic effects, although the toxicity was reversible upon discontinuation of the drug [311]. The potential toxicity rendered colistin less desirable compared to other antibiotics. The usage of colistin was gradually abandoned in the early 1980s. However, many studies have shown that colistin is generally well tolerated, with less nephrotoxicity and neurotoxicity than was once thought, which is most likely caused by lacking pharmacokinetic, pharmacodynamic, and toxicity studies earlier [312]. Also, some studies suggested that incorrect dosing, which may have resulted directly from confusing dosage forms and units, the coadministration of other nephrotoxic drugs or the presence of comorbidities may have contributed to these toxicities [313].

Recently, multidrug-resistant bacterial pathogens, mostly Gram-negative species such as *P. aeruginosa*, *A. baumannii*, and *K. pneumoniae*, have caused a huge global health threat. Because of its effective antimicrobial activities, colistin has regained interest in combating MDR infections by Gram-negative bacteria [314, 315]. Unfortunately, resistance to colistin has emerged. In Gram-negative bacteria, resistance to colistin is mostly caused by modifications of the drug target, i.e. the lipid A of LPS. Such modification results in the addition of cationic groups to the lipid A, resulting in a decrease in the negative charge on the bacterial OM. In *P. aeruginosa*, the mechanisms of resistance to colistin mostly results from the modification of lipid A by addition of 4-amino-L-arabinose (L-Ara4N) molecules to the 1 and/or 4' phosphate groups on the lipid A [316]. The synthesis and the transport of L-Ara4N molecules is encoded by the large *arnBCADTEF-UgD* operon (simplified as *arn*) [317]. Intensive research in the field has showed that at least five two-component systems (TCSs) may play a role in colistin resistance, namely PmrA/PmrB, PhoP/PhoQ, ParR/ParS, CprR/CprS, and ColR/ColS [318-321]. In *E. coli*, the addition of phosphoethanolamine (pEtN) and L-Ara4N molecules to the phosphate groups of lipid A by enzymes EptA and ArnT, respectively, strongly increases the resistance of *E. coli* to colistin [322]. Various membrane stress

conditions are also known to trigger the modification of lipid A of LPS through activation of the TCSs PhoPQ and PmrAB [323]. As in *E. coli*, colistin resistance in *K. pneumoniae* results from the addition of L-Ara4N and/or pEtN to the lipid A. The synthesis and the transport of L-Ara4N or pEtN is encoded by the *arnBCADTEF* operon (also called *pmrHFIJKLM* or *pbgPE*) and the *pmrC* gene, and are under positive control of the TCSs PhoPQ and PmrAB [324, 325]. Unlike *Enterobacteriaceae*, *A. baumannii* lacks the genes required for L-Ara4N biosynthesis [323]. Consequently, colistin resistance is caused by the addition of pETN to the 1 and/or 4' phosphate groups on the lipid A by a pETN transferase encoded by the *pmrC* gene [326]. As well, mutations in the *pmrA* and *pmrB* genes result in the activation of the PmrA/PmrB two-component system, which in turn upregulates the expression of *pmrC* gene [327, 328]. Additionally, some studies with in vitro-selected mutants demonstrated that resistance of *A. baumannii* to colistin was caused by mutations in the genes related to the lipid A biosynthesis, like *lpxA*, *lpxC*, *lpxD* [329] and to LPS core biosynthesis like *lpsB* [330].

Besides the mechanisms described above, the emergence of plasmid-mediated *mcr*-like genes such as *mcr-1* and *mcr-2* was recently described as a major cause of colistin resistance in *P. aeruginosa* [331], *E. coli* [332], *K. pneumoniae* [333], and *A. baumannii* [334]. The resistance pattern was found to be similar to that conferred by the *pmrC* gene that encodes Mcr-1 protein pEtN transferase [335].

### **Polymyxin B Nonapeptide**

The classic permeabilizer, polymyxin B nonapeptide (PMBN, see structure in Figure 20) was discovered by Vaara, published in 1983, and its properties have been extensively reviewed [336]. PMBN lacks the fatty acyl tail as well as the N-terminal diaminobutyryl (Dab) residue found in polymyxins and does not show any direct antibiotic activity against enterobacterial species [337, 338]. However, even at low concentrations (5 mg/L), it does expand the spectrum of many “anti-Gram-positive” antibiotics to cover Gram-negative bacteria. These antibiotics include rifampin (decrease in the MIC, 100-

fold) as well as erythromycin and fusidic acid (decrease in the MIC, 30-fold) [336]. Target organisms include *E. coli*, *K. pneumoniae*, several other enterobacterial species, and *P. aeruginosa*. It is reported that the combination of PMBN with erythromycin and with novobiocin administered intraperitoneally could protect mice infected with *K. pneumoniae* or *P. aeruginosa* [339].

PMBN was shown to be 15 times less toxic than polymyxin B in an acute-toxicity assay in mice, 25 times less active in releasing histamine from rat mast cells, approximately 100 times less toxic in a eukaryotic cytotoxicity assay, and approximately 150 times less active in causing neuromuscular blockade [336]. PMBN at 3.0 mg/kg did not show nephrotoxicity in a 23-day dog study and a 29-day rat study, while polymyxin B showed nephrotoxicity at the same conditions [340]. This study has confirmed the reduced nephrotoxic potential of PMBN. In vitro, PMBN was >50-fold less cytotoxic than polymyxin B toward human kidney proximal tubule cell line HK-2 [341]. Furthermore, polymyxin B and colistin preferentially accumulate in the renal cortical region, while PMBN is more uniformly distributed throughout the kidney [342].

### **5.2.3. LPS disturbing agents**

These agents can cause OM permeability via the LPS disturbance. This class molecule generally possesses one or several amino-functional groups, which can be protonated at physiological pH, then acting on negative charges of lipid A, by replacing magnesium and calcium to disturb LPS, consequently resulting in OM permeability. Alexidine is such a compound [343].

#### **Alexidine**

Alexidine (ALE, see structure in Figure 20) is a biguanide disinfectant that shows antimicrobial activity towards Gram-positive and Gram-negative bacteria. Callahan et al. [344] reported that alexidine-based multipurpose solutions demonstrate significantly

greater biocidal activity compared with other agents against Gram-negative pathogens associated with corneal infiltrative events.

Although its mechanism of bactericidal action is still unclear, recent studies have shown that, as polymyxins, alexidine establishes electrostatic interactions with the negative charges of LPS of OM, displacing  $Mg^{2+}$  from its binding to LPS and disrupting the stabilizing effect afforded by  $Mg^{2+}$  cross-bridging of adjacent LPS molecules [343]. Zorko et al [345], have reported that combining alexidine with different types of antibiotics prevented macrophage activation in the presence of Gram-negative bacteria by binding and neutralizing LPS. In addition, alexidine can also cause the leakage of the cytoplasmic content by interacting with the cell wall in Gram-negative bacteria [346] and membrane of bacteria [347].

Previous studies of alexidine have shown that it exhibits toxicity for a variety of mammalian cells [348, 349], by perturbing the cristae structure of mitochondria [350].

#### **5.2.4. Polyaminoisoprenyl derivatives**

The aforementioned potentiators have been widely used in vitro to increase the accumulation of antibiotics within Gram-negative bacteria, among which colistin and alexidine itself show antibiotics activity, but their toxicity and inadequate pharmacokinetic properties preclude clinical applications [345, 351]. Thus, efforts have been made to synthesize new efflux pump inhibitors with improved safety profiles [352].

Collaborators of our group from Aix-Marseille University synthesized a series of polyaminoisoprenyl derivatives and determined the synergistic effect combined with old antibiotics such as rifampin and doxycycline against Gram-negative bacteria [353]. These polyaminoisoprenyl derivatives originate from the observation that geraniol acts as efflux pumps inhibitor [354] and squalamine and claramine A, as outer membrane permeabilizers (resulting from the interaction of the polyamino moiety with the

membrane which can be protonated at physiological pH [355]. J.M. Brunel and his team synthesized many polyaminated compounds and selected the most active ones as efflux inhibitors. Finally, they assemble the two types of fragments to obtain polyaminoisoprenyl derivatives, among which NV716 and NV731 [356] (structure see Figure 20 and 22).

NV716 and NV731 demonstrated their capacity to restore nalidixic acid and chloramphenicol activity against *Enterobacteria spp.* [357], and NV716 also restored chloramphenicol and doxycycline activity against *Pseudomonas aeruginosa* [353] and florfenicol activity against *Bordetella bronchiseptica* [358]. Furthermore, NV716 can overcome the natural resistance of *P. aeruginosa* given its polyamino-farnesyl structure, which facilitates the diffusion of the antibiotic through the OM of *P. aeruginosa* [353]. Its positively-charged spermine moiety could interact with the negative charge of the lipid A of LPS, leading to its perturbation and increased permeability of the OM. NV716 was also shown to inhibit *P. aeruginosa* efflux pumps by impairing the proton gradient energy source [353].

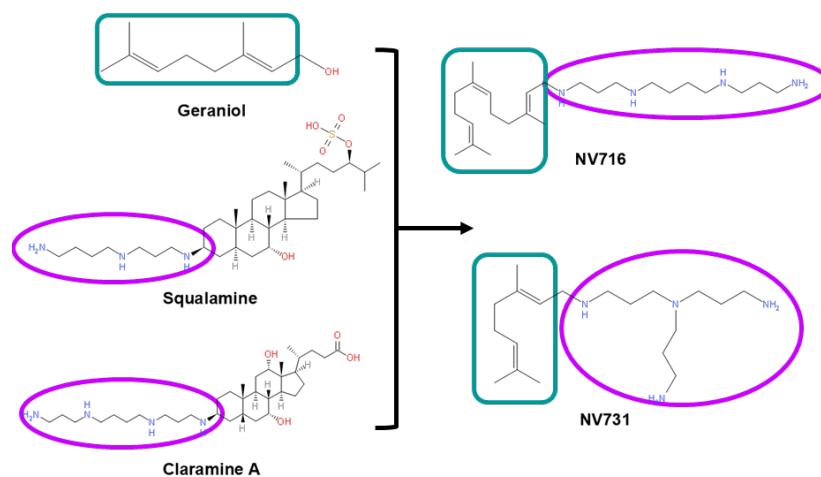


**Figure 22.** These polyaminoisoprenyl derivatives, NV716 and NV731, originate from the observation of interesting activity for their two moieties. Geraniol (green box) is the part of the carbohydrate chain of polyaminoisoprenyl derivatives, and their amino moiety originates from squalamine and claramine A (purple).

**Conclusion:** These Gram-negative ESKAPE pathogens show many resistance mechanisms and specific lifestyles (like intracellular survival and biofilm). Moreover, we are now facing the paucity of new antibiotics and an increase of multidrug resistance, consequently causing treatment failure. Therefore, we urgently need innovative strategies to tackle the worldwide crisis of antimicrobial resistance. One of them is to revisit old or disused antibiotics by combining them with potentiators (Figure 23). However, the potentiators like PA $\beta$ N, PMBN, colistin and alexidine preclude clinical applications because of their toxicity and inadequate pharmacokinetic properties. In this thesis, we have evaluated a new potentiator with improved safety profile, namely NV716.

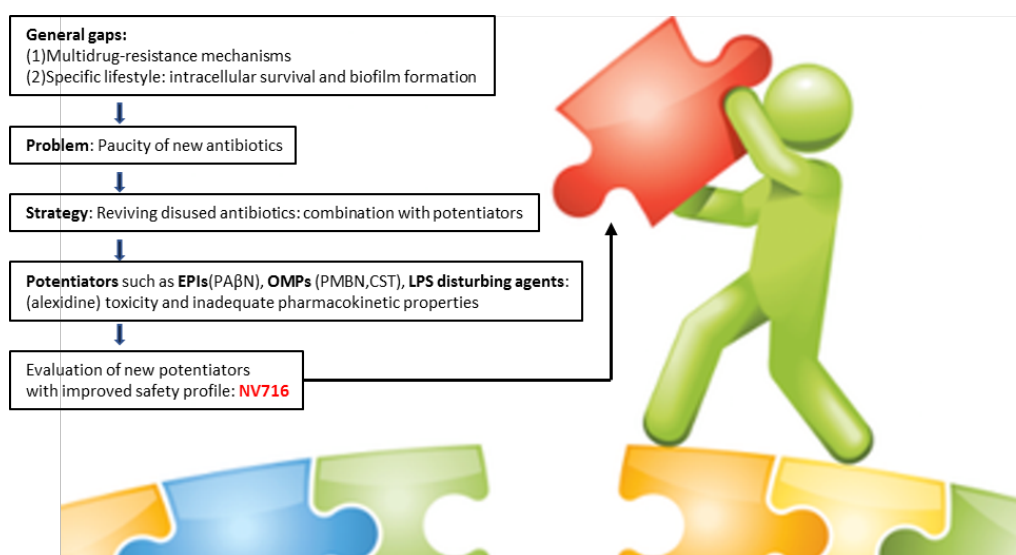


**Figure 23.** The gaps in the work are based on the current situation.

## Chapter II. Aim of the thesis

Resistance in Gram-negative bacteria causes a serious public health threat, aggregating economic burden worldwide. World Health Organization considers a group of Gram-negative ESKAPE bacteria as pathogens that urgently need new therapeutical strategies. These pathogens are *Pseudomonas aeruginosa*, *Escherichia coli*, *Klebsiella pneumoniae* and *Acinetobacter baumannii*. Indeed, these pathogens can intrinsically resist to many antibiotics due to the impermeability of the outer membrane peculiar to Gram-negative bacteria and activity of efflux pumps. Moreover, these bacteria can adopt specific lifestyles, like intracellular survival and biofilm formation, poorly responsive to antibiotics. In the face of increasing multidrug resistance and the paucity of new antibiotics, new antimicrobial strategies are urgently needed. One of these is to revisit old or disused antibiotics by combining them with potentiators.

The general objective of this thesis was therefore to determine the capacity of the polyaminoisoprenyl derivative NV716 to restore the activity of old or disused antibiotics against Gram-negative ESKAPE bacteria, including in models of intracellular and biofilm infection. As disused antibiotics, we selected doxycycline, chloramphenicol, linezolid, novobiocin, azithromycin and fusidic acid (all substrates for efflux), or rifampin (not a substrate for efflux), and ciprofloxacin, selected as the most active drug against Gram-negative bacteria. These disused antibiotics are abandoned to treat the infection by Gram-negative bacteria in the clinics due to their unfavorable pharmacokinetic properties or low antimicrobial effect.

To reach these objectives, the project involved 4 steps:

The first step (Chapter III, paper 1) was to evaluate the effect of NV716 in comparison with that of NV731 (less potent against *P. aeruginosa*) and PA $\beta$ N (reference EPI) in combination with doxycycline, chloramphenicol, or rifampin against the planktonic and

intracellular forms of infection by *P. aeruginosa*, using strains expressing or not efflux pumps to infect THP-1 monocytes.

The second step (Chapter III, paper 2) consisted in the evaluation of the effect of NV716 in comparison with that of other potentiators such as NV731, PA $\beta$ N, colistin, alexidine and polymyxin B nonapeptide in combination with doxycycline and chloramphenicol and rifampin against the planktonic forms of infection by *P. aeruginosa*, using 67 clinical isolates expressing or not efflux pumps as well as in biofilms.

The third step (Chapter III, paper 2) was to determine the mechanism of action of NV716. We examined the interaction of NV716 with LPS and its effects on OM and IM permeability, and attempted to discover its potential targets by using the genomic and transcriptomic analysis of bacteria exposed for serial passages to this molecule.

The last step (Chapter III, paper 2) was to examine the effect of NV716 on the activity of disused antibiotics against other Gram-negative bacteria (*E. coli*, *K. pneumoniae* and *A. baumannii*) in extracellular, intracellular and biofilm forms of infection.

## Chapter III.Results

### 1. The polyaminoisoprenyl 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 potentiators NV716 and NV731 compared to PA $\beta$ 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 potentiators. Pharmacodynamic parameters (apparent static concentrations [ $C_s$ ]; maximal relative efficacy [ $E_{max}$ ]) were calculated using the Hill equation of concentration-response curves. PA $\beta$ 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 $\beta$ N and NV731 that act as potentiators 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*.

## Introduction

*Pseudomonas aeruginosa* is one of the so-called ESKAPE (*Enterococcus faecium*, *Staphylococcus aureus*, *Klebsiella pneumoniae*, *Acinetobacter baumannii*, *Pseudomonas aeruginosa*, and *Enterobacter species*) pathogens [1] that harbor multiple mechanisms of drug resistance. It is considered by the World Health Organization (WHO) as a critical priority species for the search of innovative therapies [359].

*P. aeruginosa* has acquired a series of resistance mechanisms to all classes of commonly used antibiotics ( $\beta$ -lactams, fluoroquinolones, aminoglycosides, and, more recently, polymyxins). Moreover, it is also innately resistant to many antibiotics, mainly due to poor outer membrane permeability and/or active efflux [360]. Four main efflux systems, named MexAB-OprM, MexCD-OprJ, MexEF-OprN, and MexXY-OprM, have been associated with increased MICs for a broad range of antimicrobial agents [361], bringing the active concentrations above the levels that can be achieved in the serum of patients. This explains why drugs like tetracyclines or chloramphenicol cannot be used against *P. aeruginosa* or other Gram-negative bacteria, although they are capable of binding to their respective target in these bacteria when tested in acellular systems [362, 363].

Another, often neglected, reason for antibiotic failure against *P. aeruginosa* is the capacity of this bacterium to adopt specific lifestyles, such as intracellular survival. Although considered as an extracellular pathogen, *P. aeruginosa* has been shown by many researchers as capable of invading and surviving inside epithelial [364, 365] and phagocytic cells [357] *in vitro*, as well as in lung epithelial cells and macrophages *in vivo* [366, 367]. In these intracellular niches, bacteria are protected from the host humoral immune defenses but also from antibiotics, which need to have access, accumulate, and express their activity in the infected subcellular compartment [168, 198]. Moreover, they can adopt dormant phenotypes that do not respond anymore to antibiotics, like persisters. These are subpopulations of otherwise antibiotic-susceptible bacteria that

switch to a transient non-dividing phenotype surviving to high concentrations of bactericidal antibiotics [138]. We have recently provided evidence for another species, *Staphylococcus aureus*, that bacteria surviving to antibiotics intracellularly are persisters, explaining why antibiotics fail to eradicate them [199]. Preliminary data suggest that the same may hold true for *P. aeruginosa* [186].

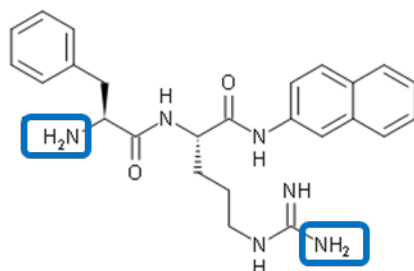
As *P. aeruginosa* resistance is worryingly increasing, few new antibiotics have been approved over the last years, and are essentially new derivatives in existing classes with improved activity or reduced susceptibility to resistance mechanisms [368]. In this context, reviving old and disused antibiotics may appear as an attractive strategy [267], which should be accompanied by an in-depth reassessment of their efficacy according to the modern standards imposed by pharmacokinetic/pharmacodynamic (PK/PD) concepts, including in models of persistent infections like intracellular survival.

One of the approaches proposed to increase the potency of drugs that are substrates for efflux is to combine them with potentiators [368]. PA $\beta$ N [286]) and CCCP [369]) have been widely used *in vitro* to increase the accumulation of antibiotics within Gram-negative bacteria, but their toxicity and inadequate pharmacokinetic properties preclude clinical applications [351, 370]. Efforts have been made to synthesize new potentiators with improved safety profile [352]. Among them, polyaminoisoprenyl compounds **NV716** and **NV731** (see chemical structures in Figure S1) demonstrated their capacity to restore chloramphenicol activity against *Enterobacteria* spp [357] and one of them (**NV716**), that of chloramphenicol and doxycycline against *Pseudomonas aeruginosa* [353], or of florfenicol against *Bordetella bronchiseptica* [358].

The aim of the present study was to evaluate the effects of **NV716** in comparison with that of **NV731** (less potent against *P. aeruginosa* [353]) and PA $\beta$ N (reference EPI) in combination with old and disused antibiotics against the intracellular forms of *P. aeruginosa*, using strains expressing or not efflux pumps to infect THP-1 monocytes. We exploited a previously established *in vitro* pharmacodynamic model [188] that allows

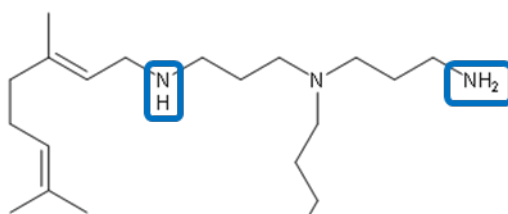
comparing key pharmacological descriptors of antibiotic intracellular activity, namely their relative potency and maximal efficacy [371]. As antibiotics, we selected doxycycline, chloramphenicol (both substrates for efflux (see Table S1) and for which preliminary data do exist regarding the beneficial effect of **NV716** [353]), rifampin (not impacted by efflux), and ciprofloxacin, selected as the most active drug against intracellular *P. aeruginosa* in this pre-established *in vitro* mode [188]. The results were examined in the light of the effect of these potentiators on antibiotic MIC and on the selection of persisters in broth culture.

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



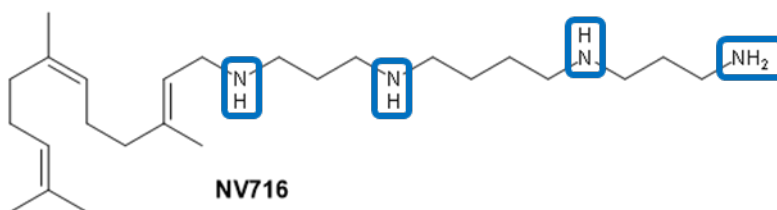
**PAβN**

**(S)-2-((S)-2-amino-3-phenylpropanamido)-5-guanidino-N-(naphthalen-2-yl)pentanamide**



**NV731**

**(E)-N1,N1-bis(3-aminopropyl)-N3-(3,7-dimethylocta-2,6-dien-1-yl)propane-1,3-diamine**



**NV716**

**N1-(3-aminopropyl)-N4-(3-(((2E,6Z)-2,3,7,11-tetramethyldodeca-2,6,10-trien-1-yl)amino)propyl)butane-1,4-diamine**

**Figure S1:** Chemical structures of the potentiators used in this study. The aminated functions that are partially protonated at physiological pH are evidenced by blue squares.

## Materials and Methods

### Bacterial strains and culture media

**Table 1** shows the reference and clinical strains used in the study. BV-1 has been isolated in 2006 at the Queen's University Belfast, Belfast, UK, from a patient with cystic fibrosis. It shows a truncation in MexB (672 amino acids instead of 1046) that drastically reduced the activity of efflux transporter and its impact of the MIC of the preferential MexAB-OprM antibiotic substrate temocillin [372]. 205 has been isolated in 2012 at the University Hospital Münster, Münster, Germany, from a patient with cystic fibrosis [373], and shows a fully active MexAB-OprM efflux pump, based on the elevated MIC of temocillin.

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

### Antibiotics and Potentiators

Chloramphenicol (potency, 98%), doxycycline (potency, 98%), and rifampin (potency, 98%) were obtained as microbiological standards from Sigma-Aldrich (St Louis, MO), ciprofloxacin HCl (potency, 89%) from Bayer (Leverkusen, Germany) and gentamicin sulfate (potency, 60.7%), from PnReac AppliChem (Darmstadt, Germany). **Table S1** shows relevant properties of the antibiotics used in this study. The reference efflux pump inhibitor PAβN (potency, 98%) was purchased from Sigma-Aldrich. The hydrosoluble hydrochloride polyamino-isoprenic salt derivatives **NV716** and **NV731** were synthesized at Aix-Marseille University [357]. Stock solutions of these potentiators were prepared in

dimethyl sulfoxide (DMSO) at concentrations of 96 mM [50 mg/mL] for PA $\beta$ N or in water at 10 mM [4 mg/L] for **NV716** and **NV731**, respectively, and stored at -20°C until use.

**Table S1.** Relevant properties of the antibiotics used in this study

| Antibiotics     | Efflux pumps shown to increase the antibiotic MIC in <i>Pseudomonas aeruginosa</i> [374-376] | Apparent cellular accumulation in eukaryotic cells (at equilibrium) [168] <sup>a</sup> | Human serum C <sub>min</sub> (mg/L) [271, 274] | Human serum C <sub>max</sub> (mg/L) [274, 377] |
|-----------------|----------------------------------------------------------------------------------------------|----------------------------------------------------------------------------------------|------------------------------------------------|------------------------------------------------|
| Ciprofloxacin   | MexAB-OprM, MexCD-OprJ, MexEF-OprN, MexXY-OprM                                               | 5                                                                                      | 0.05                                           | 5                                              |
| Doxycycline     | MexAB-OprM, MexCD-OprJ, MexXY-OprM                                                           | 2-10                                                                                   | 1                                              | 10                                             |
| Chloramphenicol | MexAB-OprM, MexCD-OprJ, MexEF-OprN                                                           | 2-5                                                                                    | 5                                              | 20                                             |
| Rifampin        | Poor substrate for these four efflux pumps                                                   | 1-4                                                                                    | 0.5                                            | 20                                             |

<sup>a</sup> apparent accumulation factor ( $C_o/C_E$ : ratio between ( $C_o$ ) cellular concentration and ( $C_E$ ) extracellular concentration).

## Susceptibility Testing

MICs of antibiotics against *P. aeruginosa* were measured by serial 2-fold microdilution in MHB-CA according to Clinical & Laboratory Standards Institute guidelines in control conditions or in the presence of 38  $\mu$ M [20 mg/L][286] PA $\beta$ N or of 10  $\mu$ M [4 mg/L] **NV731** or **NV716** [353].

## Cytotoxicity assessment (Trypan blue exclusion assay)

THP-1 cells ( $7.5 \times 10^5$  cells per mL) were incubated for 24 hours in 96-wells plates with the antibiotics or potentiators under study over a wide range of concentrations. At the end of the incubation, viability was assessed using trypan blue exclusion test (vital

colorant excluded from viable cells) using a previously described method [371], with specific adaptations to the purpose of our study. Briefly, 50  $\mu$ L of trypan blue reagent was added to 50  $\mu$ L of cell suspension. After 10 min of incubation at 37°C, non colored (viable) cells were counted using a Fuchs-Rosenthal counting chamber (Tiefe 0.2mm). Cells were counted in the same surface for all conditions (1 big square made of 16 single squares (0.0625 mm<sup>2</sup> for each single square). The percentage of cytotoxicity was evaluated based on the reduction in the number of living cells according to the following equation: cytotoxicity (%) =  $\frac{Nb \text{ uncolored cells in controls} - Nb \text{ uncolored treated cells}}{Nb \text{ uncolored cells in controls}} \times 100$ .

### **Intracellular Infection**

All experiments were performed using human THP-1 monocytes cultured in RPMI-1640 medium supplemented with 10% fetal bovine serum (FBS) based on a previously established protocol [188]. In brief, bacteria were opsonized by incubation during 1 h in RPMI-1640 medium supplemented with 10% human serum at 37°C with gentle agitation (130 rpm). Phagocytosis of opsonized bacteria was then allowed for 2 h using a bacterium: cell ratio of 10:1, after which non-phagocytosed bacteria were eliminated by incubation for 1 h with gentamicin at 50x MIC. After 3 washings with PBS, infected cells were resuspended in the original volume of RPMI-1640 with 10% FBS. The post-phagocytosis inoculum was consistently 5 to 7 x 10<sup>5</sup> CFU/mg of cell protein (defined as time zero). Infected cells were then incubated for 24 h with antibiotics over a broad range of concentrations (0.003-100x their MIC) in 12-well plates. After 24 h of incubation, infected cells were pelleted by centrifugation, washed once with PBS at room temperature to eliminate extracellular bacteria and collected in 1 mL distilled water, to achieve complete cell lysis. Lysates were used for determining CFU counts by spreading on agar supplemented with 2g/L charcoal (to avoid carry-over effect, especially in samples that had been incubated with high antibiotic concentrations [no difference in colony counts was observed between plates supplemented or not with charcoal for samples exposed to low antibiotic concentrations, ruling out any interfering effect of

charcoal in the assay]) and protein content using a commercially available kit (Bio-Rad DC Protein Assay, Bio-Rad Laboratories, Hercules, CA). The activity was expressed as the change in CFU (normalized by mg of cell protein) from the initial inoculum after 24 hours of incubation. The data were used to fit a sigmoidal function and calculate pharmacodynamic parameters based on the corresponding Hill-Langmuir equation (apparent static concentrations  $[C_s]$ , i.e. extracellular concentration resulting in no apparent intracellular growth, and maximal relative efficacy  $[E_{max}]$ , i.e. maximal decrease in bacterial counts compared to the post-phagocytosis inoculum as extrapolated for an infinitely large antibiotic concentration).

### **Persister assay and isolation**

A single colony from overnight culture of PAO1 and PAO509 on TSA was added to in 200mL MHB-CA in a 1 L flask and incubated for 24 h under agitation (130 rpm) at 37°C. 10 mL of the bacterial suspension were then incubated with 50x MIC ciprofloxacin for 5 hours at 37°C under agitation (130 rpm), in the presence of or in the absence of **NV716**. Aliquots were serially diluted in PBS and spread on TSA supplemented with 2 g/L charcoal for CFU counting. The persister fraction was defined as the number of CFU growing at the end of this experiment as compared to the initial inoculum [378]. For experiments using isolated persisters, stationary-phase cultures were incubated with ciprofloxacin (50x MIC) during 5 hours, i.e. condition for which a plateau of killing was reached. The remaining bacteria were washed twice with sterile Phosphate Buffered Saline (PBS), centrifuged (5,200 g, 15 min, 4°C) and used for killing assays [379].

### **Curve fittings and statistical analyses.**

Curve fittings were performed with GraphPad Prism (version 8.4.3) software for Windows (GraphPad Prism Software, San Diego, CA). Pharmacodynamic parameters were calculated based on Hill equations of concentration-response curves and statistical analyses were executed with GraphPad InStat 3 version 3.10 (GraphPad Prism Software) or Graphpad Prism version 8.4.3.

## Results

### Antimicrobial susceptibility

**Table 1** shows the MICs of antibiotics against PAO1, PAO509 (deleted in the genes encoding 5 efflux pumps), 205-2 and BV1 (two clinical isolates expressing respectively a non-functional and a functional MexAB-OprM efflux pump), in the absence of or in the presence of 20 mg/L (38  $\mu$ M) PA $\beta$ N, or 4 mg/L (10  $\mu$ M) **NV731** or **NV716**. These concentrations were selected because commonly used in previous works [286, 353]. In control conditions (no potentiator), ciprofloxacin, doxycycline, and chloramphenicol showed much lower MICs against PAO509 than against PAO1, in accordance with the fact they are substrates for efflux (see Table S1). Conversely, the MIC of rifampin remained unchanged in strains expressing efflux or not. BV1 was less susceptible than PAO1 to ciprofloxacin and doxycycline and more susceptible to chloramphenicol, while 205-2 was less susceptible to ciprofloxacin only. PA $\beta$ N and **NV731** reduced antibiotic MICs in few cases, while **NV716** systematically decreased them, except for ciprofloxacin in PAO509, the MIC of which was already very low. Of note, the effect of **NV716** was as (or even more) important when testing strains that do not express efflux pumps or those that do, as well as when using rifampin (not impacted by efflux) or other antibiotics (all substrates for efflux).

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

| Strains | Phenotype                                                             | Reference | Antibiotics <sup>b</sup> | MIC (mg/L) <sup>a</sup> and (fold-dilution decrease vs antibiotic alone) |                       |                 |                       |                  |                       |                  |
|---------|-----------------------------------------------------------------------|-----------|--------------------------|--------------------------------------------------------------------------|-----------------------|-----------------|-----------------------|------------------|-----------------------|------------------|
|         |                                                                       |           |                          | AB alone (mg/L)                                                          | +PAβN (38μM) [20mg/L] | PAβN alone (μM) | +NV731 (10μM) [4mg/L] | NV731 alone (μM) | +NV716 (10μM) [4mg/L] | NV716 alone (μM) |
| PAO1    | Wild-type                                                             | [380]     | CIP                      | 0.25                                                                     | <b>0.06 (2)</b>       |                 | <b>0.06 (2)</b>       |                  | <b>0.03 (3)</b>       |                  |
|         |                                                                       |           | DOX                      | 16                                                                       | <b>4 (2)</b>          | >200            | <b>4 (2)</b>          | >250             | <b>1 (4)</b>          | 50               |
|         |                                                                       |           | CHL                      | 32                                                                       | <b>8 (2)</b>          |                 | 16 (1)                |                  | <b>1 (5)</b>          |                  |
|         |                                                                       |           | RIF                      | 16                                                                       | 8 (1)                 |                 | 8 (1)                 |                  | <b>0.125 (7)</b>      |                  |
| PAO509  | PAO1[Δ(MexAB-OprM), Δ(MexCD-OprJ), Δ(MexJK), Δ(MexXY), Δ(MexEF-OprN)] | [381]     | CIP                      | 0.016                                                                    | 0.008 (1)             |                 | 0.016 (0)             |                  | 0.008 (1)             |                  |
|         |                                                                       |           | DOX                      | 1                                                                        | 1 (0)                 |                 | <b>0.125 (3)</b>      |                  | <b>0.03 (4)</b>       |                  |
|         |                                                                       |           | CHL                      | 2                                                                        | 2 (0)                 | >200            | 1 (1)                 | >250             | <b>0.125 (4)</b>      | 25               |
|         |                                                                       |           | RIF                      | 16                                                                       | <b>4 (2)</b>          |                 | 8 (1)                 |                  | <b>0.06 (7)</b>       |                  |
| BV1     | Clinical isolate (expressing truncated, non-functional MexAB-OprM)    | [372]     | CIP                      | 1                                                                        | 1 (0)                 |                 | 0.5 (1)               |                  | <b>0.125 (3)</b>      |                  |
|         |                                                                       |           | DOX                      | 64                                                                       | <b>8 (3)</b>          | >200            | <b>2 (5)</b>          | >250             | <b>0.125 (9)</b>      | 50               |
|         |                                                                       |           | CHL                      | 8                                                                        | 4 (1)                 |                 | <b>0.5 (4)</b>        |                  | <b>0.06 (7)</b>       |                  |
|         |                                                                       |           | RIF                      | 32                                                                       | 16 (1)                |                 | <b>4 (3)</b>          |                  | <b>0.008 (12)</b>     |                  |
| 205-2   | Clinical isolate (overexpressing functional MexAB)                    | [373]     | CIP                      | 4                                                                        | 2 (1)                 |                 | 4 (0)                 |                  | <b>1 (2)</b>          |                  |
|         |                                                                       |           | DOX                      | 16                                                                       | <b>4 (2)</b>          | >200            | 8 (1)                 | >250             | <b>0.5 (5)</b>        | 50               |
|         |                                                                       |           | CHL                      | 64                                                                       | 32 (1)                |                 | 32 (1)                |                  | <b>1 (6)</b>          |                  |
|         |                                                                       |           | RIF                      | 16                                                                       | 8 (1)                 |                 | 16 (0)                |                  | <b>0.125 (16)</b>     |                  |

<sup>a</sup> Values in bold denote a decrease of at least 2 doubling dilutions vs. antibiotics alone

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

## Cytotoxicity of potentiators and antibiotics

In a first set of experiments, we evaluated the cytotoxicity of the potentiators and antibiotics in the conditions of their use for intracellular infection by trypan blue exclusion test after 24 h incubation. Infection of THP-1 cells by PAO1 caused 85% cell mortality in the absence of antibiotic. The potentiators alone caused concentration-dependent cell mortality, which was minimal at the concentration used for intracellular infection studies (Figure 1, left panel). Doxycycline, chloramphenicol, and rifampin showed toxic effects at high concentrations, with  $IC_{50}$  (i.e. concentration causing 50% cell mortality) reaching approx. 30 times their respective MIC in infected cells (Figure 1, right panel). Ciprofloxacin was much less toxic at equivalent multiples of its MIC (which corresponds to much lower mass concentrations).  $IC_{50}$  values for toxicity of potentiators, antibiotics or their combinations in non-infected and infected cells are detailed in supplementary Figure S2 and Table S2 but do not differ from what is described in Figure 1. In addition, consistent results were obtained from both the trypan blue exclusion test and the LDH release assay (data not shown).

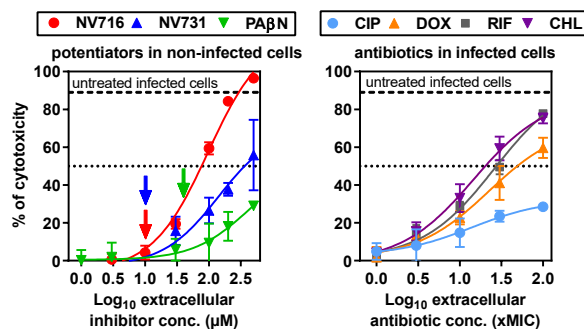


**Figure 1.** Cytotoxicity of potentiators and antibiotics as determined by trypan blue exclusion test. Left: cytotoxicity of potentiators in non-infected THP-1 cells incubated with increasing concentrations of NV716, NV731, or PAβN during 24 h. Right: cytotoxicity of antibiotics in THP-1 cells infected by PAO1 and incubated with increasing concentrations of ciprofloxacin (CIP), doxycycline (DOX), rifampin (RIF), or chloramphenicol (CHL) during 24 h. Antibiotic concentrations are expressed in multiples of their MIC against PAO1 (Log<sub>10</sub> 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 (10 μM for NV716 and 38 μM for PAβN). All data are means ± SEM (triplicates from 3 independent experiments).

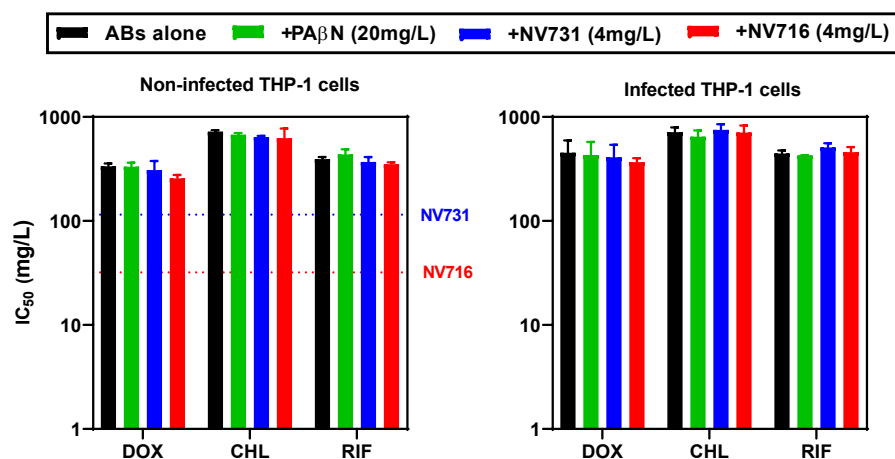


**Figure S2.** Cytotoxicity of antibiotics alone or combined with potentiators, as assessed by the trypan blue exclusion test. The graphs show the  $IC_{50}$  for three antibiotics (doxycycline [DOX], chloramphenicol [CHL] or rifampin [RIF] alone or combined potentiators at the indicated concentrations, calculated based on the Hill equation of concentration-response curves experiments similar to those illustrated in Figure 1, in non-infected THP-1 cells (left) or infected THP-1 cells (right). These  $IC_{50}$  values are higher than 400 mg/L in all cases, i.e. similar to the highest concentration tested in our model. The same type of experiment was performed with ciprofloxacin, but 50% cytotoxicity was not reached over the range of concentrations investigated (up to 25 mg/L), so that the  $IC_{50}$  could not be calculated. The dotted horizontal dotted lines in the left panel show the  $IC_{50}$  of potentiators alone (blue: NV731 [ $IC_{50}$ : 354  $\mu$ M or 115 mg/L]; red: NV716 [ $IC_{50}$ : 75  $\mu$ M or 32 mg/L]). These values are above the concentration used in most experiments (10  $\mu$ M). All data are shown as means  $\pm$  SEM (triplicates from 3 experiments). Statistical analysis (1-way ANOVA; Tukey's Multiple Comparison Test): no significant difference was noticed when comparing the different conditions for each antibiotic ( $p > 0.05$ ). See further details in Table S2.

**Table S2.** *IC<sub>50</sub> values from cytotoxicity tests, for antibiotics alone or combined with potentiators, and expressed in different units (x MIC, mg/L,  $\mu$ M)*

| Antibiotic | units   | THP-1 non-infected |                           |                    |                     | THP-1 infected by PAO1 |                           |                     |                     |
|------------|---------|--------------------|---------------------------|--------------------|---------------------|------------------------|---------------------------|---------------------|---------------------|
|            |         | AB alone           | +PA $\beta$ N<br>(20mg/L) | +731 (10 $\mu$ M)  | +716 (10 $\mu$ M)   | AB alone               | +PA $\beta$ N<br>(20mg/L) | +731 (10 $\mu$ M)   | +716 (10 $\mu$ M)   |
| RIF        | x MIC   | 24.6 $\pm$ 1.1     | 27.3 $\pm$ 3.3            | 23.1 $\pm$ 2.6     | 22.0 $\pm$ 0.9      | 27.8 $\pm$ 1.9         | 26.6 $\pm$ 0.2            | 31.9 $\pm$ 2.9      | 28.7 $\pm$ 3.4      |
|            | mg/L    | 393.0 $\pm$ 18.3   | 436.1 $\pm$ 52.6          | 369.3 $\pm$ 41.5   | 351.6 $\pm$ 14.2    | 445.5 $\pm$ 30.2       | 426.2 $\pm$ 2.8           | 510.0 $\pm$ 47.1    | 459.0 $\pm$ 55.1    |
|            | $\mu$ M | 477.5 $\pm$ 22.3   | 529.9 $\pm$ 63.9          | 448.8 $\pm$ 50.5   | 427.2 $\pm$ 17.3    | 541.4 $\pm$ 36.7       | 517.9 $\pm$ 3.4           | 619.7 $\pm$ 57.3    | 557.8 $\pm$ 66.9    |
| DOX        | x MIC   | 41.9 $\pm$ 2.8     | 41.6 $\pm$ 3.7            | 38.5 $\pm$ 8.7     | 32.1 $\pm$ 2.5      | 56.7 $\pm$ 17.7        | 53.8 $\pm$ 17.9           | 51.2 $\pm$ 16.4     | 46.0 $\pm$ 4.2      |
|            | mg/L    | 334.8 $\pm$ 22.6   | 333.0 $\pm$ 29.3          | 307.9 $\pm$ 69.3   | 256.7 $\pm$ 19.9    | 453.7 $\pm$ 141.7      | 430.5 $\pm$ 143.4         | 409.8 $\pm$ 131.0   | 368.0 $\pm$ 34.0    |
|            | $\mu$ M | 753.4 $\pm$ 50.8   | 749.3 $\pm$ 65.9          | 692.9 $\pm$ 155.9  | 577.6 $\pm$ 44.9    | 1,020.8 $\pm$ 318.8    | 968.6 $\pm$ 322.6         | 922.2 $\pm$ 294.8   | 828.0 $\pm$ 76.4    |
| CHL        | x MIC   | 22.5 $\pm$ 0.8     | 21.0 $\pm$ 0.8            | 20.1 $\pm$ 0.5     | 19.5 $\pm$ 4.7      | 22.3 $\pm$ 2.5         | 20.2 $\pm$ 3.0            | 23.5 $\pm$ 3.1      | 22.1 $\pm$ 3.7      |
|            | mg/L    | 719.1 $\pm$ 25.3   | 673.2 $\pm$ 27.0          | 642.0 $\pm$ 16.2   | 624.4 $\pm$ 150.2   | 714.1 $\pm$ 80.4       | 645.9 $\pm$ 95.0          | 752.6 $\pm$ 98.4    | 707.0 $\pm$ 118.6   |
|            | $\mu$ M | 2,225.6 $\pm$ 78.4 | 2,083.3 $\pm$ 83.7        | 1,986.9 $\pm$ 50.1 | 1,932.4 $\pm$ 464.8 | 2,210.0 $\pm$ 248.8    | 1,998.8 $\pm$ 294.1       | 2,329.2 $\pm$ 304.4 | 2,187.9 $\pm$ 366.9 |

### Activity of antibiotics alone or combined with potentiators against intracellular *P. aeruginosa* in an *in vitro* pharmacodynamic model

Figure 2 shows the concentration-response curves against intracellular PAO1 in the absence of or in the presence of **NV716**. The activity of antibiotics alone developed following a sigmoidal concentration-response curve, as previously described [188]. At low, subMIC concentrations, a  $\sim 3 \log_{10}$  increase in CFUs was noticed over the 24 h incubation period. A static effect was observed at extracellular concentrations close to the MIC of each drug. At the highest concentration tested, the reduction in bacterial counts ranged from 2  $\log_{10}$  CFU decrease for chloramphenicol, doxycycline, and rifampin, to 2.6  $\log_{10}$  CFU decrease for ciprofloxacin. In the presence of **NV716** at 10  $\mu\text{M}$ , all curves (except that of ciprofloxacin) were shifted to the left, meaning that the corresponding antibiotic was more potent, a static effect being reached at lower extracellular concentrations. Furthermore, the maximal reduction in intracellular counts was also increased, indicating that the antibiotics were more effective.

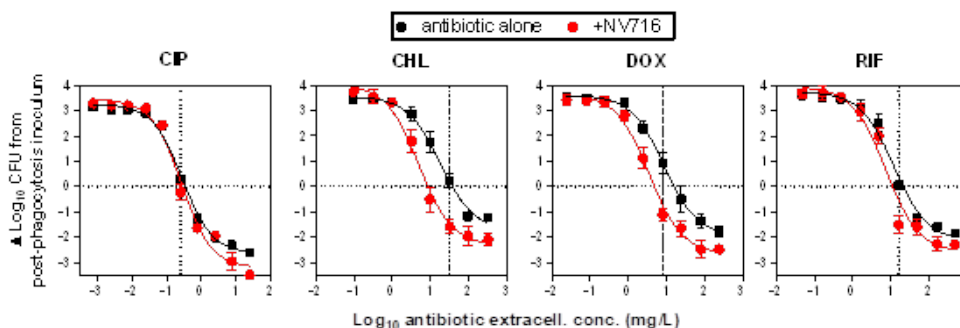


**Figure 2.** Concentration-response curves of antibiotics alone or combined with NV716 against intracellular PAO1 in a model of THP-1 monocytes. The graphs show the changes in CFU counts per mg of cell protein from the initial, post-phagocytosis inoculum after 24 h of incubation with increasing extracellular concentrations of antibiotics alone (ciprofloxacin [CIP], doxycycline [DOX], chloramphenicol [CHL] or rifampin [RIF]) or combined with a fixed concentration (10  $\mu\text{M}$ ; 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)

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

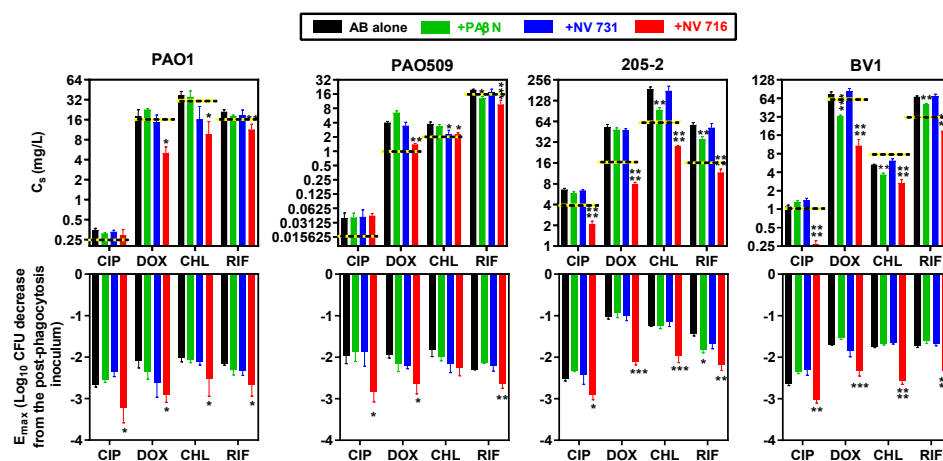


**Figure 3.** Intracellular pharmacodynamic parameters as calculated from the Hill equation of the concentration-response curves. The upper panel shows the  $C_s$  (in mg/L) for the 4 antibiotics (ciprofloxacin [CIP], doxycycline [DOX], chloramphenicol [CHL] or rifampin [RIF]) against the 4 strains, with horizontal dotted yellow-black lines corresponding to the MIC of the antibiotic in the absence of potentiator. NV716 and NV731 were used at a concentration of 10  $\mu$ M and PA $\beta$ N, at a concentration of 38  $\mu$ M. The lower panel shows the  $E_{max}$  expressed in log<sub>10</sub> 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 \*\*\*\*:  $p < 0.0001$ ).

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

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

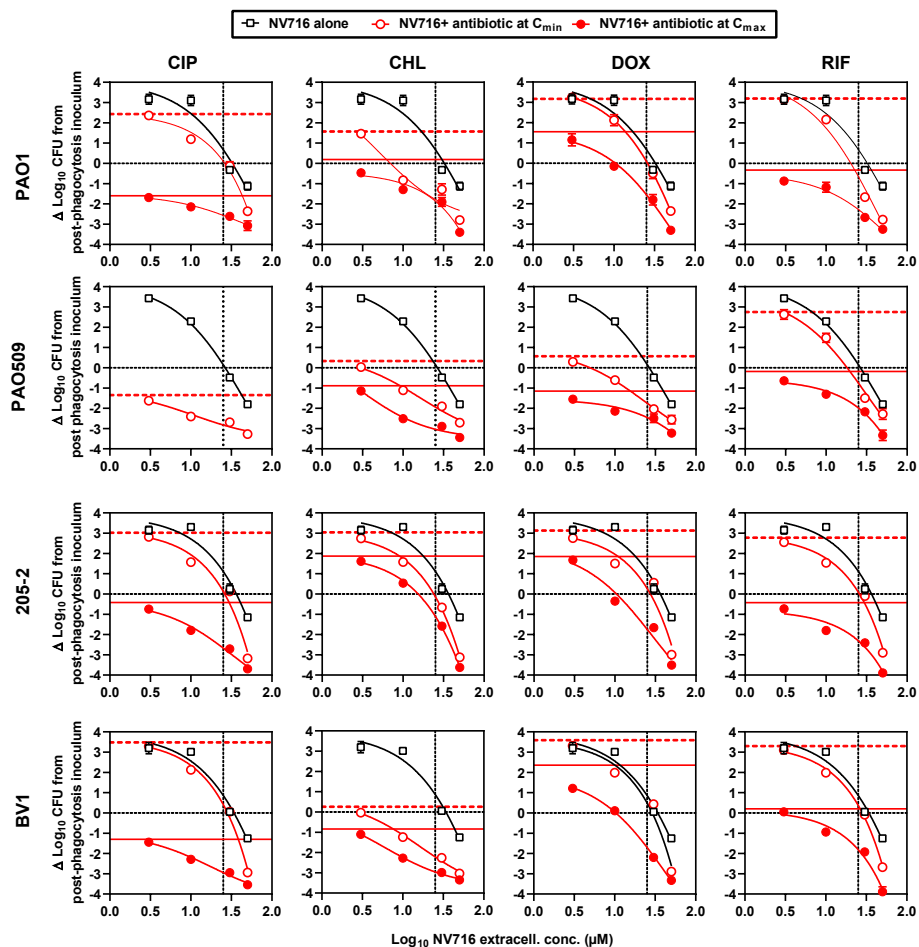


**Figure 4.** Concentration-response curves of NV716 alone or combined with antibiotics at their human  $C_{min}$  or  $C_{max}$  against intracellular *Pseudomonas aeruginosa* in a model of THP-1 monocytes. The graphs show the changes in CFU counts per mg of cell protein from the initial, post-phagocytosis 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  $\mu$ M). All data are means  $\pm$  SEM (triplicates from 3 experiments).

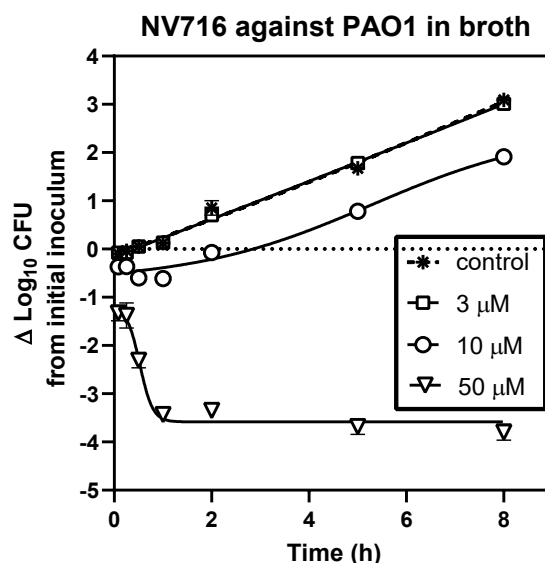


**Figure S3.** Kill curve of extracellular PAO1 by NV716 at increasing concentrations. The graph shows the changes in the Log<sub>10</sub> CFU counts from the initial inoculum per mL of broth over 8 h of incubation with NV716 at the indicated concentrations (expressed in μM). All data are means ± SEM (n=3).

Corresponding method: PAO1 was incubated on TSA overnight. A single colony was inoculated in 10 mL MHB-CA and incubated overnight at 37°C with gentle agitation (130 rpm). The density of the culture was adjusted at 0.5 McF (around 10<sup>8</sup> CFU/mL) in PBS, and diluted 100 times in MHB-CA to obtain a starting inoculum of 10<sup>6</sup> CFU/mL. Concentration-kill curves were determined over time with NV716 concentrations ranging from 3 to 50 μM. Fifty μL aliquots were diluted and plated on agar containing 2g/L charcoal to adsorb the residual antibiotic.

### Persister assay

The fact that **NV716** increases the intracellular efficacy of antibiotics (improved killing capacity) suggests it may decrease the proportion of antibiotic persisters in the population, recently identified as a cause of failure to eradicate intracellular *S. aureus* with antibiotics [382]. We therefore examined the persister fraction surviving to ciprofloxacin (selected as highly bactericidal antibiotic) alone or combined with **NV716** in stationary phases cultures. As shown in the left panel of Figure 5A, ciprofloxacin at

50x MIC caused a rapid drop in CFU counts of PAO1 over the 2 first hours of incubation, after which no further change was observed, leaving a persisting fraction of 0.005% of the initial inoculum. The same type of experiment was then performed with PAO1 and PAO509 exposed to **NV716** at increasing concentrations and used alone or combined with 50x MIC of ciprofloxacin. The right panels of Figure 5A show the proportion of persisters after 5 h of incubation, expressed in percentage of the CFU counts measured in the absence of drugs. **NV716** alone had no effect on persisters for concentrations  $\leq 100 \mu\text{M}$  but caused a marked reduction in bacterial counts at higher concentrations (3-5 log<sub>10</sub> CFU for concentrations  $> 300 \mu\text{M}$  for PAO1 and  $> 100 \mu\text{M}$  for PAO509). Strikingly, **NV716** at lower concentrations (10-100  $\mu\text{M}$ ) was already capable to reduce up to 11- (PAO1) to 28- (PAO509) fold the percentage of persisters selected by ciprofloxacin.

To investigate whether **NV716** needs to be administered simultaneously with ciprofloxacin to obtain a maximal activity, we repeated killing experiments with stationary phase cultures exposed to 50 x MIC of ciprofloxacin alone or combined with **NV716** added at different time points (Figure 5B) [379]. CFUs were significantly reduced after the addition of **NV716** at any time point, for concentrations  $\geq 10 \mu\text{M}$  and in both PAO1 and PAO509, indicating that **NV716** does not need to be added at the same time as the drug to be able to increase its activity. In a next step, we aimed at examining whether **NV716** action was related to a direct capacity to kill persisters or rather to resensitize them to the killing effect of ciprofloxacin. To this end, we isolated the persisters surviving after 5 h of incubation with ciprofloxacin at 50 x MIC and reexposed them to ciprofloxacin at 50 x MIC, **NV716** at different concentrations, or a combination thereof (Figure 5C) [379]. As expected, incubation of the isolated persisters with ciprofloxacin alone caused only a marginal decrease in CFUs for both strains, confirming the effective isolation of persister cells. When used alone, **NV716** caused a significant decrease in CFUs only at the highest concentration tested (250  $\mu\text{M}$ ). In contrast, a significant reduction in CFUs was already observed when it was combined at 10  $\mu\text{M}$  with ciprofloxacin for both strains.

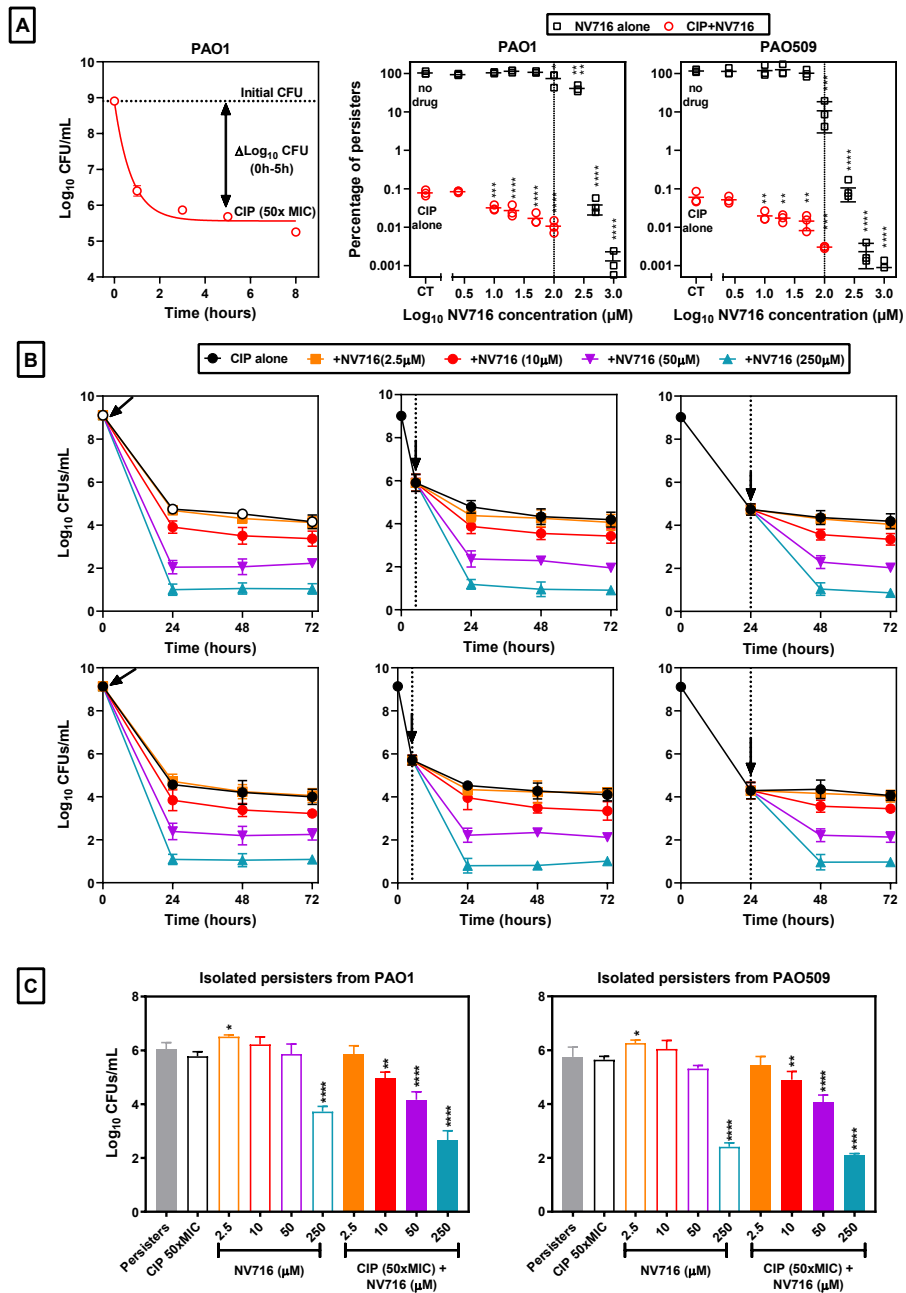


**Figure 5.** Influence of NV716 on persisters selected by ciprofloxacin. **A.** Persister fraction assay for ciprofloxacin (CIP) alone, NV716 alone or their combination against reference strains. Left: kill curve of a stationary phase culture of PAO1 exposed to ciprofloxacin at 50x MIC over time. The 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: \*,  $P \leq 0.05$ ; \*\*  $p \leq 0.01$ ; \*\*\*\*,  $P \leq 0.0001$ .

## Discussion

In this study we show that, in contrast with PA $\beta$ N and **NV731** that act as efflux inhibitors against planktonic bacteria only, **NV716** is capable to re-sensitize *P. aeruginosa* to antibiotics whether affected (doxycycline, chloramphenicol, ciprofloxacin) or not (rifampin) by efflux, not only in broth but also intracellularly.

Considering first susceptibility data in broth, we observed a remarkable effect of **NV716** on the antibiotic intrinsic activity. While PA $\beta$ N and **NV731** moderately reduced the MICs of antibiotics (essentially those that are substrates for efflux), **NV716** caused a marked decrease in the MICs of all drugs, comprising rifampin (not affected by efflux; see Table S1) against all strains, including PAO509 that does not express efflux pumps or BV1 that expresses a non-functional MexAB-OprM efflux pump. This suggests that its mode of action is not related to the inhibition of efflux only, but probably also relies on its capacity to permeabilize the outer membrane of the *P. aeruginosa* [353]. Of note, the synergy with antibiotics is obtained at sub-inhibitory concentrations of **NV716**, indicating that, if a direct effect on bacteria takes place, it is not sufficient to cause bacterial death. PA $\beta$ N has also been shown to permeabilize the outer membrane of Gram-negative bacteria by displacing Mg<sup>2+</sup> and Ca<sup>2+</sup> [383], but at concentrations higher than the one we used here, established as adequate to inhibit efflux in princeps studies with this molecule [286]. The activity of **NV716** and of the two other potentiators is observed at concentrations that do not cause cytotoxicity for eukaryotic cells, confirming previous data obtained using another cell type [384], and suggesting no or minimal interaction with eukaryotic membranes.

This contrasts with the cytotoxicity noticed for the three old and disused antibiotics, which caused significant loss of viability of THP-1 monocytes at the highest concentrations tested here, which are largely above those measured in the plasma of treated patients, owing to the high MIC of these drugs against *P. aeruginosa*. We had thus to limit the

range of antibiotic concentrations tested in our concentration-response curves against intracellular bacteria in order to avoid a too massive loss of cells.

Considering then intracellular activity data, we noticed that all antibiotics displayed a bacteriostatic activity at concentrations close or slightly higher than their MIC ( $C_s$  being considered as a measure of the intracellular MIC), despite the fact they all accumulate to some extent in eukaryotic cells [190] (Table S1). This is in line with our previous observations with other antibiotics in the same model [188] or in similar models of intracellular infections by other bacterial species [197, 385], and was interpreted as denoting a poor intracellular bioavailability. While PA $\beta$ N and **NV731** did not increase this relative potency (minimal modification in  $C_s$ ), **NV716** caused a shift of the concentrations-response curves to lower concentrations, with a commensurate reduction in the  $C_s$ , except for ciprofloxacin against PAO1 and PAO509, the MIC of which was already rather low. Thus, **NV716** can decrease the MIC of antibiotics not only extracellularly, but also intracellularly. We did not measure the cellular concentration of **NV716** in the cells, but these activity data suggest that it has access to intracellular bacteria in sufficient concentrations to exert its synergistic effect. The absence of gain in relative potency observed with PA $\beta$ N and **NV731** can be consecutive to their lower intrinsic effects on MICs (as observed in broth) or possibly also to a lower penetration inside the cells, which has not been investigated.

Turning then our attention towards the intracellular maximal efficacy, we observed, for antibiotics used alone, reductions of 2-2.5  $\log_{10}$  CFU with ciprofloxacin, in accordance with our previous results [188], and of 1-2  $\log_{10}$  CFU for the other antibiotics, the lowest effect being observed for chloramphenicol and doxycycline against 205-2, indicating individual differences in responsiveness among strains. In a broader context, the extent of intracellular killing may also differ depending on the bacterial species. An intracellular  $E_{\max}$  of -1.8  $\log_{10}$  CFU was noticed for rifampin against a small colony variant of *S. aureus* [386], but the activity of chloramphenicol and doxycycline has not been investigated in

this model. Chloramphenicol demonstrated bacteriostatic activity against intracellular *Salmonella* Typhimurium at 10x MIC [387] while doxycycline reduced of 1.5 log<sub>10</sub> the intracellular CFU of *Francisella tularensis* after 24 h of incubation at a concentration close to its MIC [388]. For fluoroquinolones, a maximal reduction of 2 to 4 log<sub>10</sub> CFU was observed in concentration-response experiments similar to those performed here against *S. aureus*, *Listeria monocytogenes*, *Legionella pneumophila*, *Burkholderia thailandensis*, *Yersinia pseudotuberculosis*, or *Francisella philomiragia* [197, 385, 389].

While PAβN and **NV731** did not affect the efficacy of any antibiotic against none of the strains, **NV716** systematically improved it (more negative E<sub>max</sub>), suggesting an effect on bacterial responsiveness to antibiotics. We can most likely preclude interference related to the release of bacteria out of the cells before lysis or to the remaining amount of antibiotics accumulated in cells if considering that (a) **NV716** did not increase the cytotoxicity of antibiotics, and (b) charcoal was added to culture plates to adsorb residual antibiotic. Thus, the gain in intracellular efficacy observed in the presence of **NV716** is rather suggestive of improved bacterial responsiveness to the drugs. We previously showed an increase in the intracellular efficacy of fluoroquinolones against an insertion mutant in a putative deacetylase in PAO1, which showed a lower persister character in planktonic cultures [186]. Here, we found that **NV716** was capable of decreasing the proportion of persisters selected by ciprofloxacin in a stationary phase culture at concentrations for which it did not cause any bacterial killing by itself and also of reducing at subMIC concentrations the number of persisters when combined with ciprofloxacin but not when used alone, suggesting a mode of action unrelated to a direct bactericidal effect but rather to a resensitization of persisters to the antibiotic [379]. Of interest, other molecules capable of disrupting membrane integrity also reduced the residual fraction of persisters upon exposure to antibiotics for different bacterial species [390-392]. One of them (SPI009; 1-((2,4-Dichlorophenethyl)Amino)-3-Phenoxypropan-2-ol)) was also shown to increase the efficacy of ciprofloxacin against intracellular *P. aeruginosa* [393].

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

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

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## **2. NV716, a membrane-active polyaminoisoprenyl compound that re-sensitizes *Pseudomonas aeruginosa* to antibiotics and reduces bacterial virulence**

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## Abstract

*Pseudomonas aeruginosa* is intrinsically resistant to many antibiotics due to the impermeability of its outer membrane and to the constitutive expression of efflux pumps. Here, we show that the polyaminoisoprenyl compound **NV716** at sub-MIC concentrations re-sensitizes *P. aeruginosa* to disused antibiotics by binding to the lipopolysaccharides (LPS) of the outer membrane, permeabilizing this membrane and increasing antibiotic accumulation inside the bacteria. It also prevents selection of resistance to antibiotics and increases their activity against biofilms. No stable resistance could be selected to **NV716**-itself after serial passages with subinhibitory concentrations, but the transcriptome of the resulting daughter cells shows an upregulation of genes involved in the synthesis of lipid A and LPS, and a downregulation of quorum sensing-related genes. Accordingly, **NV716** also reduces motility, virulence factors production, and biofilm formation. **NV716** shows a unique and highly promising profile of activity when used alone or in combination with antibiotics against *P. aeruginosa*.

## Introduction

The Gram-negative bacterium *Pseudomonas aeruginosa* is an important opportunistic pathogen that may cause life-threatening infections. These are difficult to treat because *P.aeruginosa* has developed many mechanisms of resistance to currently-used antibiotics. Beside acquired resistance mechanisms, like the production of enzymes modifying or hydrolyzing antibiotics and mutations in antibiotic targets, intrinsic resistance mediated by the poor permeability of the outer membrane to many antibiotics as well as by the expression of multidrug efflux pumps [394] contributes to restrict the number of antibiotic classes showing useful activity against this species. This is notably the case for a series of old-disused drugs otherwise characterized by a broad spectrum of activity.

The envelope of *P.aeruginosa* consists of three layers: the inner membrane (IM), made of a fluid phospholipid bilayer, the periplasmic space containing the peptidoglycan cell wall, and the outer membrane (OM), a hallmark of Gram-negative bacteria. The outer membrane is structurally asymmetric, with its inner leaflet containing phospholipids, and the outer leaflet, made of lipopolysaccharides (LPS). LPS molecules comprise a polysaccharidic antigenic part, a core oligosaccharide and a lipidic part (lipid A) insuring the anchoring in the membrane. LPS packing is enhanced by the intercalation of divalent cations such as  $Mg^{2+}$  and  $Ca^{2+}$  that neutralize negative charges within the LPS molecules [79]. This assemblage contributes to the structural integrity of the bacteria, and opposes a barrier to the diffusion of lipophilic or of high-molecular-weight compounds [79].

In addition, this envelope contains a series of transmembrane proteins, among which different efflux systems belonging to the resistance-nodulation-division (RND) superfamily. These efflux systems are made of three proteins spanning the entire envelope, including an efflux transporter embedded in the inner membrane, a periplasmic membrane fusion protein, and an outer membrane channel. Among these

transporters, MexAB-OprM, MexCD-OprJ, MexEF-OprN, and MexXY-OprM are those mainly contributing to multidrug resistance by extruding different structurally-unrelated classes of antibiotics [361]. Almost all antibiotics are substrates for active efflux in *P. aeruginosa* [361], increasing the MIC of many of them above clinically-achievable levels, although these drugs show high affinity for their target in acellular systems, as demonstrated for example for tetracyclines or chloramphenicol [363, 395].

Permeabilizing the outer membrane while at the same time impairing active efflux, therefore appears as an appealing strategy to improve the susceptibility to antibiotics of Gram-negative bacteria, including *P. aeruginosa* [396]. In this respect, a promising approach consists in combining antibiotics with potentiators interfering with these barrier effects.

The efflux pump inhibitor Phenyl-Arginine- $\beta$ -naphthylamide (PA $\beta$ N) and the outer membrane permeabilizer polymyxin nonapeptide (PMBN) have been widely used in-vitro to increase the potency of antibiotics towards Gram-negative bacteria [286, 397], but their toxicity and inadequate pharmacokinetic properties hinder clinical applications [340, 370]. Efforts have thus been made to synthesize new adjuvant molecules with improved safety profile and enhanced potency [352]. Among them, the polyaminoisoprenyl compounds **NV716** and **NV731** proved capable to restore the activity of chloramphenicol towards *Enterobacteria spp* [357] and one of them (**NV716**), that of chloramphenicol and doxycycline towards *P. aeruginosa* [353] or florfenicol towards *Bordetella bronchiseptica* [358]. Moreover, **NV716** also improved the potency and the efficacy of doxycycline, chloramphenicol, rifampicin, and ciprofloxacin against intracellular forms of *P. aeruginosa* by its capacity to inhibit efflux and to reduce the fraction of persisters in the bacterial population [398]. Previous mechanistic studies suggest a complex mode of action, including the inhibition of the activity of efflux pumps and an alteration of the outer membrane permeability barrier [353] at concentrations that do not alter eukaryotic cell viability [398].

The aim of the present study was to examine in details the effects of **NV716** on the activity of disused antibiotics against the *P. aeruginosa*, namely doxycycline, chloramphenicol (both substrates for efflux [361]), rifampicin (poor substrate for efflux [376] but showing low outer membrane permeation [399]), and of ciprofloxacin, selected as an active drug against *P. aeruginosa*. We also characterized in details its interaction with the bacterial envelope, its capacity to prevent selection of resistance, and to improve antibiotic activity against biofilms. Lastly, we performed a genomic and transcriptomic analysis of bacteria exposed for serial passages to this molecule in order to get more insight on its mechanism of action. Throughout this work, we compared **NV716** with **NV731** (less potent against *P. aeruginosa* [353]) and PA $\beta$ N (as a reference efflux pump inhibitor). We also included in our study colistin, PMBN, and alexidine as positive controls, as they all show well-characterized effects on *P. aeruginosa* envelope (see Figure S1 for the structure of all these compounds). The polymyxin colistin binds to the outer membrane via electrostatic interactions with the negatively-charged LPS molecules, more specifically with the lipid A component. By displacing Mg<sup>2+</sup> and Ca<sup>2+</sup>, polymyxins destabilize the outer membrane, cross it via a self-promoted uptake mechanism, and subsequently insert in the inner membrane and disrupt the physical integrity of the phospholipid bilayer [296]. PMBN nonapeptide (i.e. a derivative of polymyxin B lacking the lipophilic tail) also increases outer membrane permeability by binding with high affinity to LPS and lipid A via electrostatic interactions. Nevertheless it cannot establish hydrophobic interactions with lipid A, explaining why it is a poor antibacterial compound, but it is still synergistic with hydrophobic antibiotics by increasing their capacity to cross the outer membrane barrier [400]. Alexidine is a biguanide antiseptic showing a rapid bactericidal effect [345]. As polymyxins, it establishes electrostatic interactions with the negative charges of LPS, displacing Mg<sup>2+</sup> from its binding to LPS and disrupting the stabilizing effect afforded by Mg<sup>2+</sup> cross-bridging of adjacent LPS molecules [343]. In addition, it also causes the leakage of the cytoplasmic content by interacting with membrane lipids, inducing the formation of lipid domains [347].

We show that NV716, contrarily to its comparators, markedly decreased the MICs of all antibiotics against *P. aeruginosa*, whether substrates or not for efflux and in strains expressing or not efflux pumps. These effects could be attributed to a strong interaction with LPS and disturbance of outer membrane integrity that increased the intracellular accumulation of antibiotics. In addition, NV716 prevents the selection of resistance, improves antibiotic activity including in biofilms, prevents biofilm formation, reduces motility and production of virulence factors. Thus, NV716 shows a unique and promising profile of activity, combining in a single molecule the beneficial effects of alexidine and PMBN and demonstrating additional useful adjuvant properties, while proving un toxic to eukaryotic cells and avoiding the risk of resistance selection observed with colistin.

## Materials and Methods

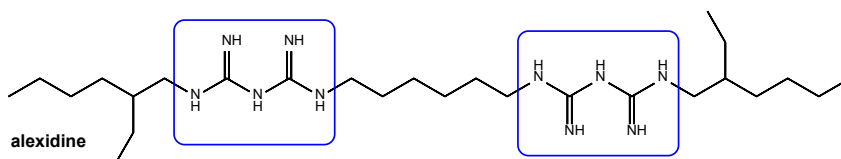
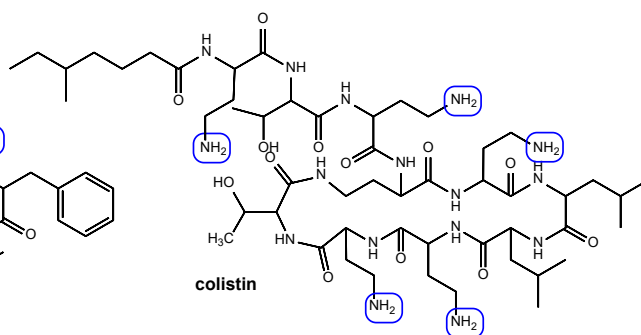
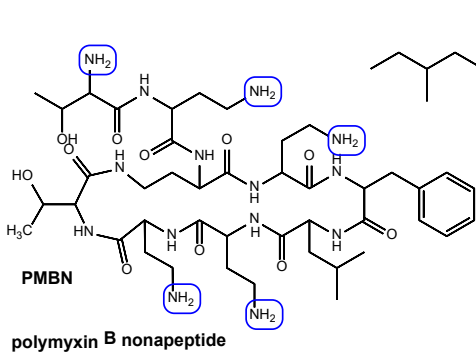
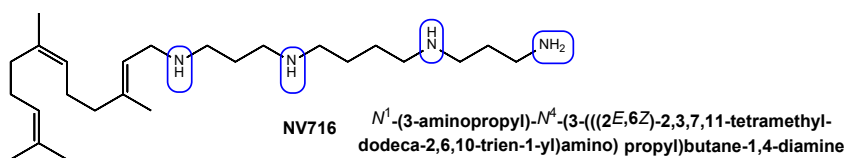
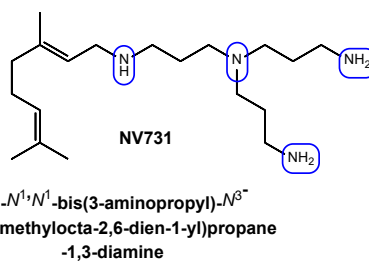
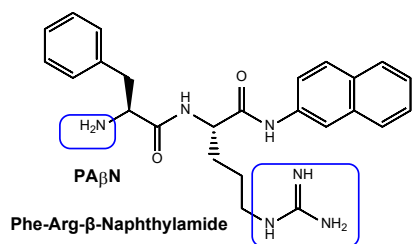
### Bacterial strains and culture media

Four reference strains and 67 clinical isolates (Table 1 and S1) obtained from patients with cystic fibrosis (n=43), urinary tract infections (n=10), or hospital-acquired pneumonia (n=14) were used in this study. Among them all isolates from CF patients and from hospital-acquired pneumonia are MDR strains. For specific experiments, additional 4 clinical isolates resistant to colistin [401] were also included (Table 1, Figures S2 and S4). Bacteria were inoculated overnight at 37°C in tryptic soy agar (VWR). A single colony was then added to 10 mL cation-adjusted Mueller-Hinton Broth (CA-MHB; Sigma-Aldrich) and incubated at 37°C overnight under gentle agitation (130 rpm). CFUs (colony-forming units) were counted on Tryptic soy agar.

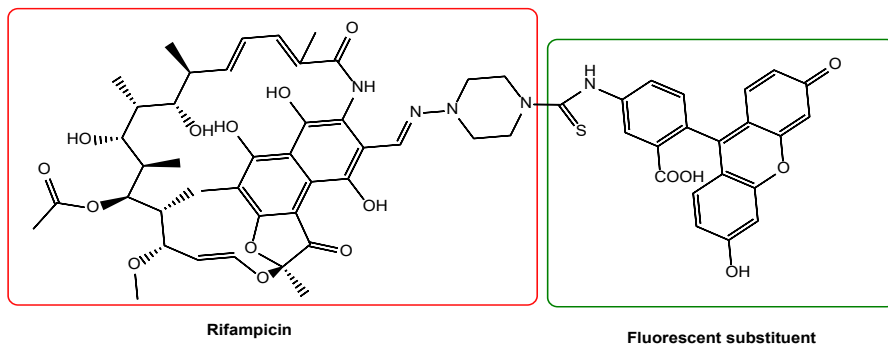
### Antibiotics and adjuvant compounds

Chloramphenicol (potency, 98%), doxycycline (potency, 98%), rifampicin (potency, 98%), colistin (potency, 92%) and alexidine (potency, 98%) were obtained as microbiological standard from Sigma-Aldrich, and ciprofloxacin HCl (potency 89%), from Bayer (Leverkusen, Germany). The reference efflux pump inhibitor Phe-Arg- $\beta$ -Naphthylamide (PA $\beta$ N; potency, 98%) and the membrane permeabilizer polymyxin B nonapeptide (PMBN) were purchased from Sigma-Aldrich. The hydrosoluble hydrochloride polyamino-isoprenic salt derivatives, **NV716** and **NV731**, were synthesized at Aix-Marseille University [384]. Figure S1 shows the structure of the potentiators. Stock solutions were prepared in DMSO at a concentration of 96 mM [50 mg/mL] for PA $\beta$ N or in water at a concentration of 10 mM for PMBN [10 mg/mL], **NV716** [4 mg/mL] and **NV731** [3.2 mg/mL], and stored at -20°C until use.

**A**

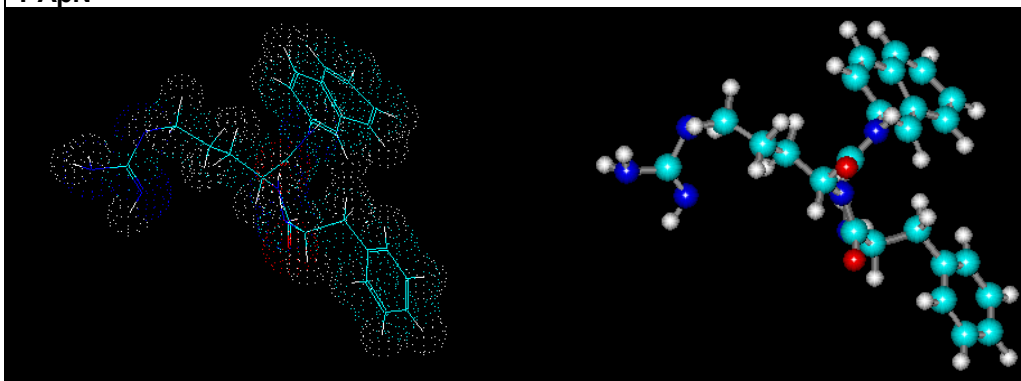


NV1532

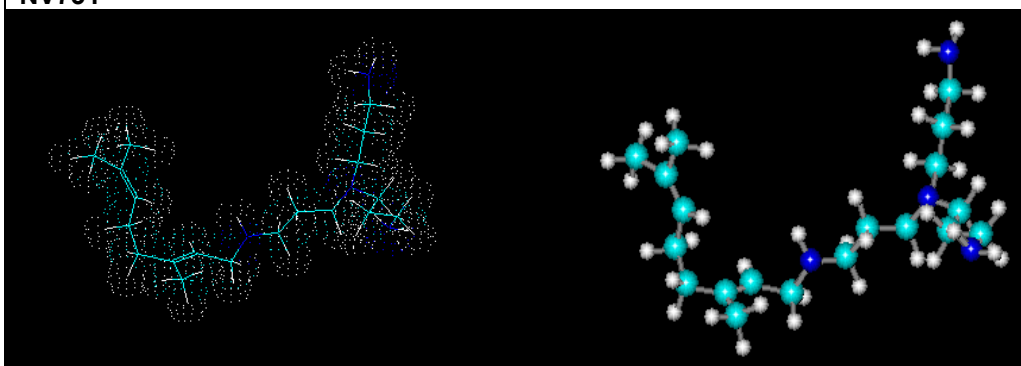


B.

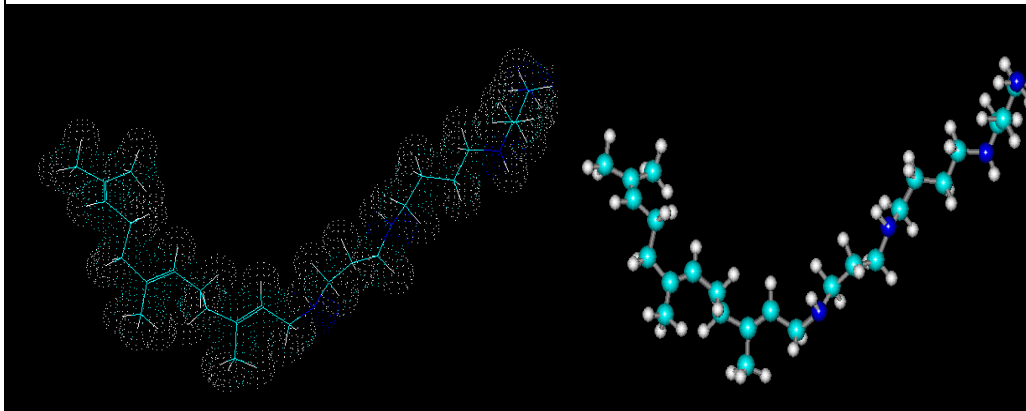
PAβN



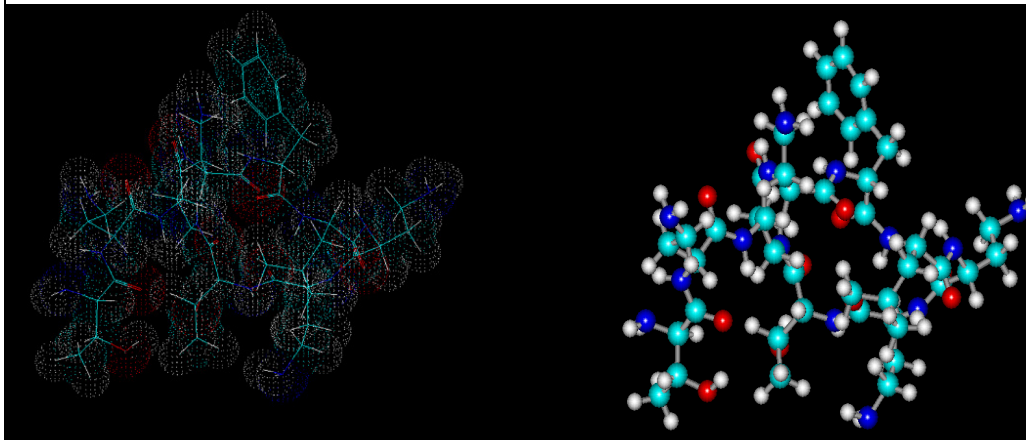
NV731



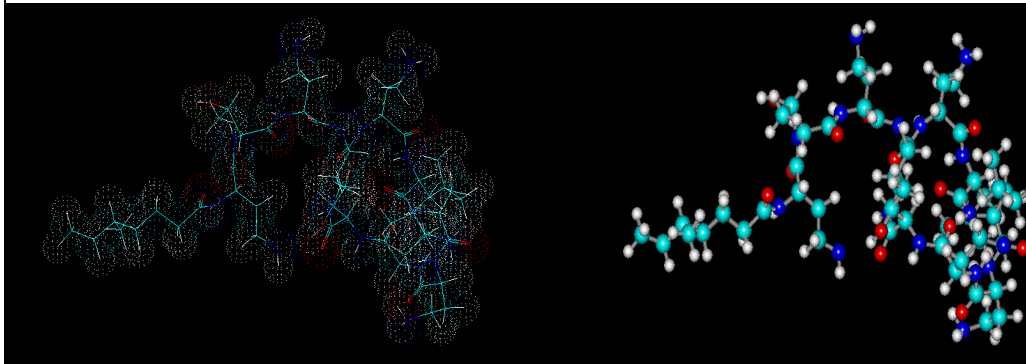
**NV716**

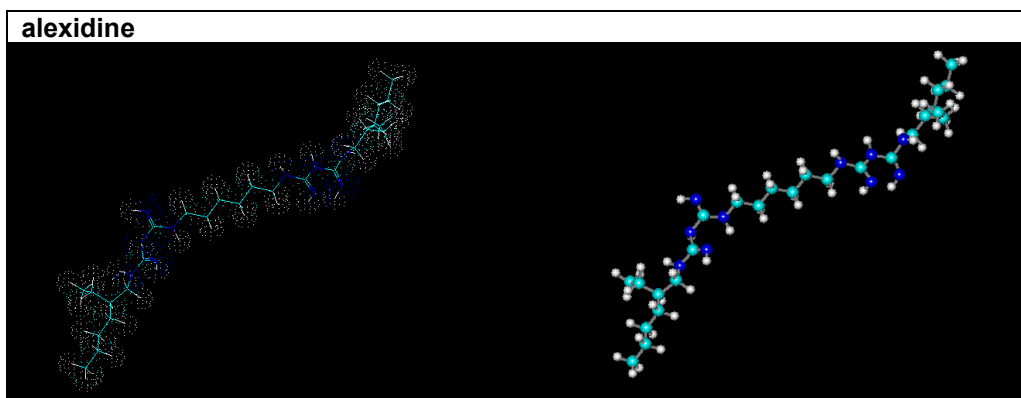


**PMBN**



**colistin**





**C**

| Parameters                                             | Alexidine | PMBN    | Colistin | NV716  | NV731  | PABN   |
|--------------------------------------------------------|-----------|---------|----------|--------|--------|--------|
| LogD (pH 7.4)                                          | -3.9      | -19.1   | -20.2    | -4.2   | -6.3   | -1.2   |
| Van der Waals molecular Surface Area (Å <sup>2</sup> ) | 932.33    | 1450.38 | 1835.51  | 794.91 | 633.42 | 655.21 |
| Polar Surface Area (Å <sup>2</sup> )                   | 167.58    | 432.46  | 490.66   | 62.11  | 67.31  | 146.12 |
| Van der Waals volume (Å <sup>3</sup> )                 | 543.41    | 901.17  | 1113.8   | 462.27 | 368.73 | 415.59 |

**Figure S1:** **A.** Chemical structures of potentiators and of the fluorescent derivative of rifampicin used in this study (**NV1532**). The aminated functions of potentiators that are partially protonated at physiological pH are evidenced by blue rectangles. **B.** Three-dimensional structure (drawn with Hyperchem Pro 6, Hypercube Inc. ) of potentiators (left: Van der Waals 2 D surface; right: ball-and-stick representation). **C.** comparison of structural properties of the potentiators used in this study (estimated with MarvinSketch 20.6, Chemaxon)

#### MIC determination for antibiotics alone or combined with adjuvants

MICs were determined by microdilution in CA-MHB, following the guidelines of the Clinical and Laboratory Standards Institute. After 18-20 hours of incubation, the MICs were defined as the minimal concentrations of antibiotics for which there was no visible bacterial growth.

### Checkerboard assay

Checkerboard assays were performed in 96-wells plates. Compound A was serially diluted from columns 1 to 11, and compound B, from rows B to H, with serial dilutions of compound A alone found in row A and of compound B alone in column 12. MICs of compounds alone and in combinations were used to calculate the fractional inhibitory concentration (FIC) index, as follows:  $FIC = \frac{C_A}{MIC_A} + \frac{C_B}{MIC_B}$ , where  $MIC_A$  and  $MIC_B$  are the MICs of compounds A and B alone, and  $C_A$  and  $C_B$  are the MIC of compounds A or B in combination. The combination was considered synergistic when the FIC index was  $< 0.5$ , and antagonistic when the FIC index was  $> 4$  [402].

### BODIPY<sup>TM</sup>-TR-Cadaverine displacement assay

Binding affinity of potentiators to lipopolysaccharides (LPS) from *P.aeruginosa* was investigated in living bacteria using the BODIPY<sup>TM</sup>-TR-cadaverine (BC) displacement assay. When bound to LPS, BC is weakly fluorescent (self-quenching) but its fluorescence is increased when it is competitively displaced by other compounds displaying affinity for LPS (dequenching). As previously described [403], BC (Thermo Fisher Scientific; final concentration, 5  $\mu$ M) and bacteria (final OD<sub>620nm</sub>, 0.1) were mixed, kept for 30 mins in the dark at room temperature, after which 50  $\mu$ L of this mixture was incubated with 50  $\mu$ L of test compounds in 96-wells black plates during 30 min before fluorescence measurements.

### Outer Membrane permeability assay

Outer membrane permeability was assessed using the 1-*N*-phenylnaphthylamine (NPN) uptake assay [401]. The fluorescence of this probe increases when incorporated in the hydrophobic core of a permeabilized outer membrane. Concisely, 20  $\mu$ L of NPN solution (final concentration, 10  $\mu$ M) containing or not the test compounds were mixed with 180  $\mu$ L of bacteria suspension (final OD<sub>620nm</sub> = 0.5, resuspended in phosphate buffer [NaCl 110 mM; KCl 7mM; NH<sub>4</sub>Cl 40mM; NA<sub>2</sub>HPO<sub>4</sub> 0.4 mM; Tris base 62 mM; Glucose 0.2%;

pH 7.5 adjusted with HCl]) in 96-wells plates. The fluorescence was rapidly measured using a Synergy H1 Hybrid Multi-Mode Microplate Reader (BioTek) with 4 seconds intervals during 5 min ( $\lambda_{exc}/\lambda_{em}$ : 340/410 nm).

#### **Inner Membrane permeability assay**

Inner membrane permeability was determined using the Propidium Iodide (PI) assay as previously described [404]. PI becomes fluorescent when intercalated in the DNA of bacteria, the outer and inner membrane of which are permeabilized. Briefly, overnight cultures were centrifuged and resuspended in phosphate buffer (same composition as above) to reach a  $OD_{620nm}$  of 0.1. Five  $\mu$ L PI (final concentration, 6  $\mu$ M) was added to 5 mL of bacterial suspension and the mixture was incubated during 30 min in the dark. Fifty  $\mu$ L of this mixture were added by 50  $\mu$ L of test compounds in a 96-wells plate. After 60 min incubation at room temperature, the fluorescence of PI was measured ( $\lambda_{exc}/\lambda_{em}$ : 535/617 nm) in a SpectraMax M3 plate reader.

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

Ciprofloxacin accumulation inside bacteria was determined by a fluorimetric method [405]. Briefly, 10 mL of bacterial suspension (approx.  $10^9$  cells/mL) were incubated with ciprofloxacin (final concentration, 10 mg/L) with or without potentiators at selected concentrations during 15 min (i.e. a duration longer than the predicted time required to reach saturation [278], but during which the antibiotic did not affect bacterial growth [no significant reduction in CFUs]). After appropriate dilution in PBS, 50  $\mu$ L aliquots were spread on TSA and colony-forming units (CFUs) were counted after overnight incubation at 37°C. The pellet was then collected by centrifugation at 3,000  $g$  for 7 min at 4°C, washed 3 times in cold PBS to remove ciprofloxacin from the medium and then resuspended in 0.1 M glycine-HCl buffer (pH 3). Samples were kept in the dark overnight to allow bacteria lysis and ciprofloxacin release. After centrifugation at 20,000  $g$  for 7 min, ciprofloxacin fluorescence was measured in the supernatant ( $\lambda_{exc}/\lambda_{em}$ : 275/450

nm) in a Spectramax M3 plate reader. Ciprofloxacin concentrations were normalized to CFUs counts.

Rifampicin accumulation was measured using a fluorescent derivative (**NV1532**, see structure in Figure S1) synthesized by one of us (JMB; see Supplementary method S1). This compound remained microbiologically active, with a MIC of 32 mg/L against PAO1 (vs. 16 mg/L for rifampicin). The fluorescence emission peak of **NV1532** in PBS was determined by an emission scan at an excitation wavelength of 470 nm. The lower limit of detection and of linearity of the fluorescence calibration curve were 15.6 µg/L to 1000 µg/L (Figure S11). In brief, 10 mL of bacterial suspension (approx.  $10^9$  cells/mL) were incubated for 15 min with **NV1532** (final concentration, 20 mg/L) combined or not with potentiators. Aliquots were processed for CFU counts and the rest of the samples were centrifuged, washed to remove **NV1532** as described for ciprofloxacin assay, resuspended in 500 µL PBS and sonicated 60 min in a bath to lyse the bacteria. After centrifugation, the supernatant was used to measure fluorescence ( $\lambda_{exc}/\lambda_{em}$ : 470/525 nm) in a spectramax M3 plate reader and data normalized to CFU counts.

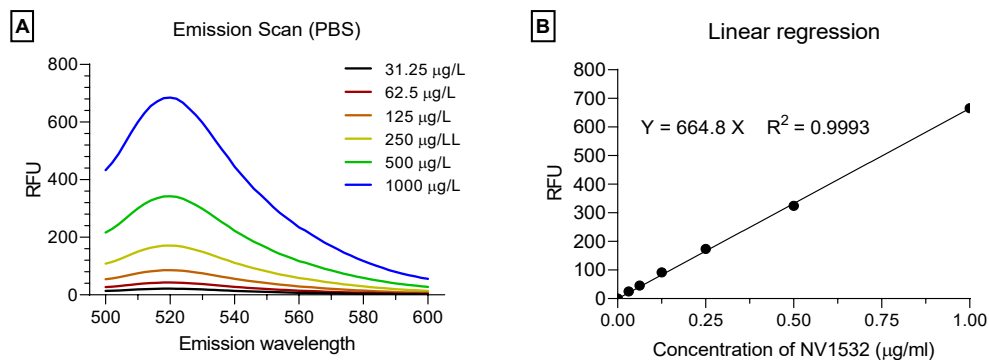


**Figure S11.** Fluorescence emission spectrum of **NV1532** at an excitation wavelength of 470nm (A) and calibration curve showing the quantitative relationship between the fluorescence signal and the concentration of NV1532 (B) in PBS. The fluorescence signal of PBS is deduced from all values (background). All values are the means from three independent experiments.

### **Frequency of selection of resistance and selection of resistance by serial passages**

PAO1 ( $10^9$  CFU/mL) was spread on TSA plates containing ciprofloxacin or rifampicin at a final concentration of 4 x MIC alone or combined with potentiators. The frequency of selection of antibiotic-resistant mutants was determined as the ratio between the number of colonies that appeared after 48 h of incubation at 37°C on the drug-containing plates and control plates [406]. MIC increase was also followed over serial passage cultures in broth exposed to (i)  $\frac{1}{4}$  MIC of the antibiotic alone or combined with potentiators or (ii) potentiators alone at  $\frac{1}{2}$  MIC, with daily 1/1000 dilution of the bacteria and readjustment of the concentration to (i)  $\frac{1}{4}$  MIC or (ii)  $\frac{1}{2}$  MIC of the previous day, over approx. (i) 30 or (ii) 50 days.

### **Genomic and transcriptomic analysis**

PAO1 and daughter bacteria collected after the 52<sup>th</sup> passage in the presence of potentiators at  $\frac{1}{2}$  MIC were spread on TSA containing or not potentiators at  $\frac{1}{2}$  MIC and incubated overnight, after which 3 colonies were independently inoculated in 2mL MHB-CA containing or not  $\frac{1}{2}$  MIC of potentiators and incubated overnight at 37°C with shaking at 130 rpm.

DNA was extracted from cell pellets using the DNeasy Blood & Tissue Kit (Qiagen) and 0.8 ng/ $\mu$ L DNA was used for the library preparation based on a modified version of the Illumina Nextera XT protocol with unique dual indices. The library pool was size selected (500 – 1500 bp) on the BluePippin™ (Sage Science) instrument. Libraries were sequenced in paired-end mode on an Illumina NovaSeq 6000 device (2 x 50 bp). The obtained reads were quality controlled by “fastqc” with default settings [407]. The tool suite “bbtools” was used for cleaning and merging reads, and for performing statistical analyses on coverage and insert sizes (BBMap – Bushnell B. – [sourceforge.net/projects/bbmap/](https://sourceforge.net/projects/bbmap/)). The trimmed reads were de-novo assembled using

“shovill” (<https://github.com/tseemann/shovill>) with the assembler “SPAdes” with the optional setting ‘only-assembler’. Single nucleotide polymorphisms (SNPs) were detected by “parsnp” [408] with default settings and with PAO1 as the reference. The raw sequencing files have been deposited in the NCBI SRA database under the accession number PRJNA785668.

RNA was extracted from cell pellets using the InviTrap® Spin Cell RNA Mini Kit (INVITE). Ribosomal RNA was removed using the Ribo-Zero Bacteria Kit (Illumina) and libraries for transcriptomics were generated according to references [409, 410]. Libraries were sequenced in paired-end mode on an Illumina NovaSeq 6000 device (2 x 50 bp). The obtained reads were quality controlled (option “--nextseq-trim=20”) and clipped using cutadapt [411]. Reads were mapped to the reference genome PAO1 (NC\_002516.2) using bowtie2 [412]. The resulting sam-files were converted to indexed binary format using SAMtools [413]. Counts of the mapped reads were extracted with featureCounts [414] using reads with a minimum mapping quality of 20. Differential gene expression analysis was performed with the R package edgeR [415]. Normalization factors to scale the raw library sizes were calculated using the weighted trimmed mean of M-values (TMM) method [416]. Dispersions were estimated and data were fitted using the edgeR functions estimateDisp and glmQLFit, respectively. Genes were considered as differentially expressed if their Log<sub>2</sub> fold change expression was significantly (FDR ≤ 0.05) greater than |1| (edgeR function glmTreat). Functional enrichment of significant gene sets was done by hypergeometric testing (R function phyper). Functions were considered as significantly enriched with a FDR adjusted p-value ≤ 0.05. The raw sequencing files have been deposited in the NCBI GEO database under the accession number GSE190194.

## Assays of virulence factors in PAO1 supernatants

### *Elastase activity assay*

Elastolytic activity in *P. aeruginosa* culture was determined by elastin Congo red (ECR) assay [417]. Briefly, bacteria from mid-log-phase ( $OD_{620nm}$ : 0.6-0.8) in MHB-CA were pelleted and re-suspended in fresh MHB-CA in the absence of or presence of potentiators so as to obtain an  $OD_{620nm}$  of 0.05, then incubated at 37°C with rotation of 130 rpm for 21 h. Bacteria were pelleted by centrifugation (20,000 g; 5 min) and the pellet was re-suspended in the original volume of water to measure the  $OD_{620nm}$ . The supernatants were passed through 0.22  $\mu m$  filters; 50  $\mu L$  of filtrates were added to 1 ml of Tris-maleate buffer (0.1 M Tris [pH 7.2], 1 mM  $CaCl_2$ ) containing 20 mg of ECR (Sigma). Mixtures were incubated for 18 h at 37°C with rotation of 130 rpm and then placed on ice after the addition of 0.1 mL of 0.12 M EDTA. Insoluble ECR was removed by centrifugation (20,000 g, 5 min), and the  $OD_{495nm}$  was measured. Elastase activity was expressed as ( $OD_{495nm}$  of the filtrate/ $OD_{620nm}$  of the resuspended pellet), in percentage of the control value (untreated bacteria).

### *Rhamnolipid assay*

Rhamnolipids were quantified in the culture medium as previously described [417]. Briefly, an aliquot from an overnight culture was added into fresh MHB-CA in the absence of or presence of potentiators to obtain an  $OD_{620nm}$  of 0.05 and incubated at 37°C with rotation of 130 rpm for 48 h. Then the bacteria were pelleted by centrifugation (20,000 g, 5 min) and the pellet was resuspended in the original volume of water to measure  $OD_{620nm}$ . Rhamnolipids (containing 3-deoxy-hexose) were assayed in the culture supernatant by the orcinol assay. Rhamnolipids from 300  $\mu L$  of supernatants were extracted twice with 600  $\mu L$  diethyl ether (Merck-Millipore). The pooled ether extracts were evaporated to dryness, then reconstituted in 100  $\mu L$  distilled water and mixed with 100  $\mu L$  1.6 % [w/v] orcinol (Sigma) and 800  $\mu L$  of 60% (v/v) sulfuric acid.

After heating to 80°C and shaking at 175 rpm for 30 min, the OD<sub>421nm</sub> was measured. The content of rhamnolipids in the samples was expressed as (OD<sub>421nm</sub> of the supernatant/OD<sub>620nm</sub> of the resuspended pellet), in percentage of the control value (untreated bacteria).

#### *Pyocyanin assay*

Cultures were prepared as for the rhamnolipids assay, but incubated during 10 h only before centrifugation and resuspension of the pellet in water. Pyocyanin was extracted from 5 mL supernatant with 3 mL of chloroform (Merck-Millipore) and then re-extracted into 1 mL of 0.2 N HCl [417]. The absorbance of 520nm (A<sub>520nm</sub>) was measured and the amount of pyocyanin in the samples was expressed as A<sub>520nm</sub>/OD<sub>620nm</sub> of the resuspended pellet), in percentage of the control value (untreated bacteria).

#### *Bacterial motility*

Swimming, swarming, and twitching motilities were assessed as previously described [417, 418]. Briefly, 2µL of PAO1 grown in MHB-CA for 14 h were either directly stabbed into swimming (0.1% [w/v] tryptone, 0.05% [w/v] yeast extract (Becton Dickinson), 0.5% [w/v] NaCl, 0.3% [w/v] agar) and twitching (1.0% [w/v] tryptone, 0.5% [w/v] yeast extract, 0.5% [w/v] NaCl, 1.0% [w/v] agar) plates or diluted in fresh LB medium to obtain an OD<sub>620 nm</sub> of 0.1 and then spotted onto swarming plates (0.8% [w/v] nutrient broth N.2, 0.5% [w/v] glucose, 0.5% [w/v] agar). Plates were supplemented or not with potentiators). After 24 h of incubation at 37°C, swimming and swarming motilities were directly observed at the air-agar interface. To better visualize the twitching motility, the plates were immersed with a small volume of cold twitching motility developer solution (TMDS; 400 mL deionized water, 100 mL glacial acetic acid [Merck-Millipore], 500 mL methanol [Merck-Millipore], stored at 4°C) for 30 min. Then the top colonies were gently scraped

with a plastic disposable loop. All images of the plates were taken using a Bio-Rad Molecular imager (Gel Doc XR system).

### *Biofilm formation*

Cultures were grown to mid-log phase in MHB-CA and diluted to  $10^6$  CFU/mL in fresh TGN (Trypticase soy broth supplemented with 1% glucose and 2% NaCl), and 200  $\mu$ L bacterial suspension containing or not potentiators was added to 96-well plate. After 24 h of incubation at 37°C without agitation, the medium was carefully removed and biofilms were washed with 200  $\mu$ L of 3-morpholinopropane-1-sulfonic acid (MOPS) buffer (20.9 g/liter of MOPS [Sigma-Aldrich], 5.6 g/liter NaCl; pH adjusted to 7 with NaOH). Then the biomass of biofilm was quantified using crystal violet, a cationic dye that non-specifically stains negatively charged constituents in biofilms. Washed biofilms were dried at 60°C for 30 min and 1 % (v/v) crystal violet (100%, Sigma) was added to the plates. After 10 min of incubation at room temperature, the excess of dye was removed under running water and the residual amount was resolubilized with 66% acetic acid and incubated 1 hour at room temperature. Absorbance was then measured at 570 nm using a SpectraMax M3 plate reader and data expressed in percentage of the untreated control.

### **Activity against preformed biofilms**

Biofilms were formed in 96-well plates [418]. Briefly, overnight cultures of bacteria were diluted in TGN to obtain an OD<sub>620</sub> of 0.05. Two hundred  $\mu$ L of this suspension were distributed in 96-well plates, which were then incubated at 37°C during 24 h to obtain mature biofilms. Mature biofilms were then incubated with 200  $\mu$ L fresh TGN containing rifampicin alone, potentiators alone, or their combination during 24 h, after which the medium was removed and the biofilm was washed once with 200  $\mu$ L MOPS buffer and biomass quantified by crystal violet staining as described above. The metabolic activity in the biofilms was determined using the fluorescein diacetate (FDA) assay. The non-fluorescent FDA is hydrolysed by living bacteria into the yellow highly fluorescent

fluorescein. Washed biofilms were incubated with 100 µg/mL fluorescein diacetate (Sigma-Aldrich) for 15 min at 37°C in the dark. Fluorescein fluorescence was measured in a SpectraMax M3 plate reader ( $\lambda_{exc}/\lambda_{em}$ : 494/518 nm).

### **Confocal laser scanning microscopy for visualization of biofilms**

Biofilms were grown on round coverslips placed at the bottom of the wells of 24-well plates. After 24 h of incubation, the culture medium was discarded. Biofilms were washed once with 1 mL PBS and reincubated with antibiotics alone or in combination with potentiators for 1 h, washed twice with 1 mL PBS and stained for 30 min in the dark with LIVE/DEAD (SYTO 9/PI) bacterial viability kit (L-7007; Thermo Fisher Scientific) or with 0.5 mM 5-cyano-2,3-ditolyl tetrazolium chloride (CTC, Sigma-Aldrich), as described previously [419]. CTC is a colorless, nonfluorescent, and membrane-permeable compound that is reduced in viable cells to a fluorescent insoluble CTC-formazan. Stained biofilms were then washed with 1 mL PBS to remove the probes in excess.

Biofilms were observed using a cell observation spinning disk microscope (Carl Zeiss) with an oil immersion 40× objective. SYTO9 and **NV1532** were detected in the green channel ( $\lambda_{exc}/\lambda_{em}$ : 488/502-538 nm), and PI and CTC, in the red channel ( $\lambda_{exc}/\lambda_{em}$ : 488/580-654 nm). Images were acquired at a resolution of 1388 x 1040 pixels, and 20 µm-deep scans were obtained using Z-stack scanning mode. 3D-images construction and quantitative analysis of the images were performed using ZEN 2.6 (blue edition) software. For quantification of fluorescent signals, an area of 37000 µm<sup>2</sup> was selected on 2D-projection of the image, and the fluorescence intensities of the fluorophores were recorded in all Z-stacks of each sample. The analyses were performed for three different zones of the coverslips, and each experiment was repeated three times.

### **Transmission Electron Microscopy**

Samples were prepared as previously described [420]. In brief, PAO1 was incubated in CA-MHB overnight at 37°C. After centrifugation, bacteria were resuspended in fresh CA-MHB containing NV716 at different concentrations during 1 h. Bacteria were fixed in 1 % glutaraldehyde in 0.1 M phosphate buffer for 4 h. After post-fixation in 1% osmium tetroxide for 1 h and dehydration in ethanol, samples were embedded in resin. 70 nm sections were cut with an Leica ultracut UCT ultramicrotome and examined (unstained) in a JEOL 1400 transmission electron microscope equipped with an 11 Mpxl EMSIS Quemesa camera.

### **Statistical analysis**

Statistical analyses and curve fitting were performed using GraphPad Prism software for Windows (version 9.0.0; GraphPad Software, Inc., San Diego, CA).

## Results

### Antimicrobial susceptibility

Table 1 shows the MIC of antibiotics alone or combined with potentiators against four reference strains and 71 clinical isolates, among which, 2 resistant to colistin (MICs of potentiators against reference strains are shown in Table S1). In reference strains, the MIC of chloramphenicol, doxycycline and ciprofloxacin against PAO1 and PT629 (MexAB-OprM overproducer) was higher than against PAO1*mexAB* ( $\Delta$ *mexAB*) and PAO509 (deleted in the genes encoding 5 efflux pumps), indicating that these three antibiotics are substrates for efflux. The MIC of rifampicin alone was the same in the four reference strains, confirming that this drug is a poor substrate for efflux pumps. Alexidine (2.5  $\mu$ M) and colistin (0.5  $\mu$ M; subMIC concentration) did not affect the MIC of all tested antibiotics. PA $\beta$ N (38  $\mu$ M) and **NV731** (2.5 and 10  $\mu$ M) reduced antibiotic MICs of up to 2 doubling dilutions, the lowest effect being observed in combination with rifampicin. Polymyxin B nonapeptide (PMBN; 30  $\mu$ M) and **NV716** (2.5 and 10  $\mu$ M) systematically decreased MICs, with reductions of 4 to 9 doubling dilutions observed for doxycycline, chloramphenicol or rifampicin, and of 1-3 doubling dilutions observed for ciprofloxacin (lowest effect against PAO509, as the MIC of ciprofloxacin was already low against this strain). FIC index calculations revealed a higher degree of synergy for **NV716** (2.5  $\mu$ M) than for PMBN (Figure S2) combined with all antibiotics against all strains, except for ciprofloxacin against PAO1*mexAB* and PAO509. PA $\beta$ N showed some degree of synergy when combined with antibiotics substrates for efflux in PAO1, PT629, and PAO1*mexAB*. No synergy was observed when antibiotics were combined with alexidine, colistin or **NV731**. In clinical isolates, PA $\beta$ N and **NV731** caused only minor changes in MIC<sub>50</sub> and MIC<sub>90</sub> (0-3 doubling dilutions), while **NV716** reduced them from 2 to 5 doubling dilutions (Table 1). Thus, in the presence of **NV716**, MICs of all antibiotics were in the range of therapeutically achievable concentrations for a large proportion (> 90%) of the collection (Figure S3).

**Table 1** MIC of antibiotics alone or combined with potentiators against *P. aeruginosa* reference strains and clinical isolates.

| Strains   | Relevant genotype or phenotype            | Reference | Experimental conditions | MIC (mg/L) <sup>a</sup> |                          |                        |                           |                              |
|-----------|-------------------------------------------|-----------|-------------------------|-------------------------|--------------------------|------------------------|---------------------------|------------------------------|
|           |                                           |           |                         | Compounds alone         | Doxycycline              | Chloramphenicol        | Rifampicin                | Ciprofloxacin                |
| PAO1      | wild type                                 | [380]     | Antibiotic (AB) alone   |                         | 16                       | 32                     | 16                        | 0.25                         |
|           |                                           |           | + PAβN (38 μM)          | >200 μM                 | <b>4 (2)</b>             | <b>8 (2)</b>           | 8 (1)                     | <b>0.0625 (2)</b>            |
|           |                                           |           | + PMBN (30 μM)          | 128 μM                  | <b>1 (4)</b>             | <b>1 (5)</b>           | <b>0.03125 (9)</b>        | <b>0.0625 (2)</b>            |
|           |                                           |           | + 731 (2.5μM)/ (10 μM)  | >250 μM                 | 8 (1)/ <b>4 (2)</b>      | 32 (0)/16 (1)          | 16 (0)/8 (1)              | <b>0.062 5(2)/0.0625 (2)</b> |
|           |                                           |           | + 716 (2.5μM)/ (10 μM)  | 50 μM                   | <b>2 (3)/1 (4)</b>       | <b>2 (4)/1 (5)</b>     | <b>0.25 (6)/0.125 (7)</b> | <b>0.0625 (2)/0.031 (3)</b>  |
|           |                                           |           | + Alexidine (2.5 μM)    | 12 μM                   | 16 (0)                   | 32 (0)                 | 16 (0)                    | 0.25 (0)                     |
|           |                                           |           | + Colistin (0.5 μM)     | 1 μM                    | 8 (1)                    | 16 (0)                 | 16 (0)                    | 0.25 (0)                     |
| PT629     | MexAB overproducer                        | [421]     | Antibiotic (AB) alone   |                         | 64                       | 128                    | 16                        | 0.5                          |
|           |                                           |           | + PAβN (38 μM)          | >200 μM                 | <b>16 (2)</b>            | <b>32 (2)</b>          | 16 (0)                    | <b>0.0625 (3)</b>            |
|           |                                           |           | + PMBN (30 μM)          | 128 μM                  | <b>2 (5)</b>             | <b>4 (5)</b>           | <b>0.0625 (8)</b>         | <b>0.0625 (2)</b>            |
|           |                                           |           | + 731 (2.5μM)/ (10 μM)  | >250 μM                 | 32 (1)/ <b>16 (2)</b>    | 128 (0)/ <b>32 (2)</b> | 16(0)/8(1)                | 0.250 (1)/ <b>0.125 (2)</b>  |
|           |                                           |           | + 716 (2.5μM)/ (10 μM)  | 50 μM                   | <b>4 (4)/2 (5)</b>       | <b>16 (3)/4 (5)</b>    | <b>0.5 (5)/0.125 (7)</b>  | <b>0.0625 (3)/0.031 (4)</b>  |
|           |                                           |           | + Alexidine (2.5 μM)    | 12 μM                   | 64 (0)                   | 128 (0)                | 16 (0)                    | 0.5 (0)                      |
|           |                                           |           | + Colistin (0.5 μM)     | 1 μM                    | 64 (0)                   | 128 (0)                | 8 (1)                     | 0.5 (0)                      |
| PAO1mexAB | PAO1 ΔmexAB                               | [381]     | Antibiotic (AB) alone   |                         | 4                        | 16                     | 16                        | 0.0625                       |
|           |                                           |           | + PAβN (38 μM)          | >200 μM                 | 2 (1)                    | <b>4 (2)</b>           | 16(0)                     | <b>0.016 (2)</b>             |
|           |                                           |           | + PMBN (30 μM)          | 128 μM                  | <b>0.0625 (6)</b>        | <b>0.5 (5)</b>         | <b>0.0625 (8)</b>         | <b>0.016 (2)</b>             |
|           |                                           |           | + 731 (2.5μM) / (10 μM) | >250 μM                 | 2 (1)/ <b>1 (2)</b>      | 16 (0)/8 (1)           | 16 (0)/8(1)               | 0.031 (1)/0.031 (1)          |
|           |                                           |           | + 716 (2.5μM)/ (10 μM)  | 50 μM                   | <b>0.5 (3)/0.125 (5)</b> | <b>2 (3)/1 (4)</b>     | <b>2 (3)/0.25 (6)</b>     | 0.031 (1)/ <b>0.016 (2)</b>  |
|           |                                           |           | + Alexidine (2.5 μM)    | 12 μM                   | 4 (0)                    | 16 (0)                 | 16 (0)                    | 0.0625 (0)                   |
|           |                                           |           | + Colistin (0.5 μM)     | 1 μM                    | 4 (0)                    | 8 (1)                  | 8 (1)                     | 0.0625 (0)                   |
| PAO509    | PAO1 Δ(mexAB-oprM) Δ(mexCD-oprJ) Δ(mexEF- | [381]     | Antibiotic (AB) alone   |                         | 1                        | 2                      | 16                        | 0.016                        |
|           |                                           |           | + PAβN (38 μM)          | >200 μM                 | 1 (0)                    | 2 (0)                  | 4 (2)                     | 0.008 (1)                    |
|           |                                           |           | + PMBN (30 μM)          | 128 μM                  | <b>0.008 (7)</b>         | <b>0.12 (4)</b>        | <b>0.03125 (9)</b>        | 0.008 (1)                    |

|                             |                                               |       |                                                                                                                  |                                  |                                                                              |                                                                   |                                                                    |                                                                      |
|-----------------------------|-----------------------------------------------|-------|------------------------------------------------------------------------------------------------------------------|----------------------------------|------------------------------------------------------------------------------|-------------------------------------------------------------------|--------------------------------------------------------------------|----------------------------------------------------------------------|
|                             | oprN) Δ(mexJK)<br>Δ(mexXY)                    |       | + 731 (2.5μM) / (10 μM)<br>+ 716 (2.5μM) / (10 μM)<br>+ Alexidine (2.5 μM)<br>+ Colistin (0.5 μM)                | >250 μM<br>25 μM<br>6 μM<br>1 μM | <b>0.25 (2)/0.125 (3)</b><br><b>0.0625 (4)/0.031 (5)</b><br>1 (0)<br>0.5 (1) | 1 (1)/1 (1)<br><b>0.5 (2)/0.125 (4)</b><br>1 (1)<br>128 (0)       | 16 (0)/8 (1)<br><b>0.25(6)/0.0625(8)</b><br>16 (0)<br><b>4 (2)</b> | 0.016 (0)/0.016 (0)<br>0.016 (0)/0.008 (1)<br>0.016 (0)<br>0.004 (1) |
| PA313                       | Colistin-resistant<br>(colistin MIC 32 mg/L)  | [401] | AB alone<br>+ PAβN (38 μM)<br>+ PMBN (30 μM)<br>+ NV716 (2.5μM) / (10 μM)                                        | >200 μM<br>>256 μM<br>50 μM      | 32<br><b>8 (2)</b><br><b>2 (4)</b><br><b>4 (3)/4 (3)</b>                     | 128<br>64 (1)<br><b>16 (3)</b><br><b>32 (2)/16 (3)</b>            | 256<br>256 (0)<br><b>2 (7)</b><br><b>32 (3)/4 (6)</b>              | 8<br>4 (1)<br><b>1 (3)</b><br><b>2 (2)/1 (3)</b>                     |
| PA2938                      | Colistin-resistant<br>(colistin MIC 64 mg/L)  | [401] | AB alone<br>+ PAβN (38 μM)<br>+ PMBN (30 μM)<br>+ NV716 (2.5μM) / (10 μM)                                        | >200 μM<br>>256 μM<br>50 μM      | 32<br>32 (0)<br><b>4 (3)</b><br>16 (1)/8 (2)                                 | 128<br>64 (1)<br><b>8 (4)</b><br><b>32 (2)/16 (3)</b>             | 64<br>32 (1)<br><b>8 (3)</b><br>32 (1)/8 (3)                       | 0.5<br>0.25 (1)<br><b>0.125 (2)</b><br>0.25 (1)/ <b>0.0625 (3)</b>   |
| PA307                       | Colistin-resistant<br>(colistin MIC 64 mg/L)  | [401] | AB alone<br>+ PAβN (38 μM)<br>+ PMBN (30 μM)<br>+ NV716 (2.5μM) / (10 μM)                                        | >200 μM<br>>256 μM<br>50 μM      | 16<br>8 (1)<br><b>2 (3)</b><br>8 (1)/4 (2)                                   | 64<br>64(0)<br><b>8(3)</b><br>32(1)/16(2)                         | 64<br>32 (1)<br><b>4 (5)</b><br>32 (1)/8 (3)                       | 4<br>4 (0)<br><b>0.5 (3)</b><br>2 (1)/ <b>0.5 (3)</b>                |
| PA272                       | Colistin-resistant<br>(colistin MIC 128 mg/L) | [401] | AB alone<br>+ PAβN (38 μM) <sup>d</sup><br>+ PMBN (30 μM)<br>+ NV716 (2.5μM) <sup>d</sup> / (10 μM) <sup>e</sup> | >200 μM<br>>256 μM<br>50 μM      | 32<br><b>8 (2)</b><br><b>4 (3)</b><br><b>4 (3)/4 (3)</b>                     | 128<br>64 (1)<br><b>8 (4)</b><br>64 (1)/ <b>16 (3)</b>            | 32<br>16 (1)<br><b>8 (2)</b><br>32 (0)/8 (2)                       | 8<br>4 (1)<br><b>1 (3)</b><br><b>2 (2)/0.5 (4)</b>                   |
|                             |                                               |       |                                                                                                                  |                                  | MIC <sub>50</sub> <sup>b</sup> /MIC <sub>90</sub> <sup>c</sup>               | MIC <sub>50</sub> <sup>b</sup> /MIC <sub>90</sub> <sup>c</sup>    | MIC <sub>50</sub> <sup>b</sup> /MIC <sub>90</sub> <sup>c</sup>     | MIC <sub>50</sub> <sup>b</sup> /MIC <sub>90</sub> <sup>c</sup>       |
| Clinical isolates<br>(n=67) |                                               |       | AB alone<br>+ PAβN (38 μM)<br>+ NV731 (2.5μM)<br>+ NV716 (2.5μM)                                                 |                                  | 16/64<br><b>4 (2)/8 (3)</b><br>8 (1)/32 (1)<br><b>0.5 (5)/2 (5)</b>          | 64/256<br>32 (1)/128 (1)<br>32 (1)/128 (1)<br><b>2 (5)/16 (4)</b> | 16/32<br>8 (1)/16 (1)<br>16 (0)/32 (0)<br><b>1 (3)/4 (3)</b>       | 0.5/16<br>0.25 (1)/8 (1)<br>0.5 (0)/8 (1)<br>0.25 (1)/ <b>4 (2)</b>  |

<sup>a</sup> values in bold highlight significant decrease in MIC (at least 2 doubling dilutions); values in brackets: number of doubling dilution decrease (log<sub>2</sub> fold change) for MIC of antibiotic combined with potentiator compared with antibiotic alone.

<sup>b</sup> MIC<sub>50</sub> and <sup>c</sup> MIC<sub>90</sub>, Minimum Inhibitory Concentration required to inhibit the growth of 50% or 90% of the isolates, respectively.

**Table S1:** MICs of potentiators against the strains used in Figure 1.

| Strain<br>identification | MIC ( $\mu\text{M}$ / [mg/L]) |             |         |              |            |           |
|--------------------------|-------------------------------|-------------|---------|--------------|------------|-----------|
|                          | ALE                           | PMBN        | CST     | PA $\beta$ N | NV731      | NV716     |
| PAO1                     | 12 / 7                        | 128 / 128   | 1 / 1   | >250 / >125  | >250 / >80 | 50 / 21   |
| PAO509                   | 6 / 3.5                       | 128 / 128   | 1 / 1   | >250 / >125  | >250 / >80 | 25 / 10.5 |
| 2938                     | 12 / 7                        | >256 / >256 | 64 / 64 | >250 / >125  | >250 / >80 | 50 / 21   |
| 307                      | 12 / 7                        | >256 / >256 | 64 / 64 | >250 / >125  | >250 / >80 | 50 / 21   |

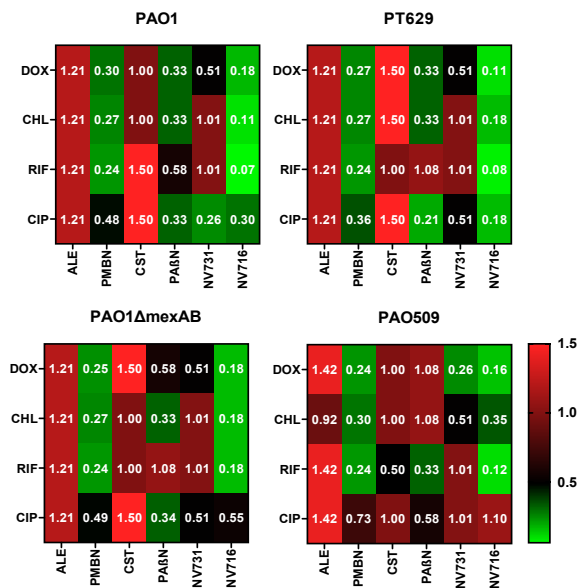
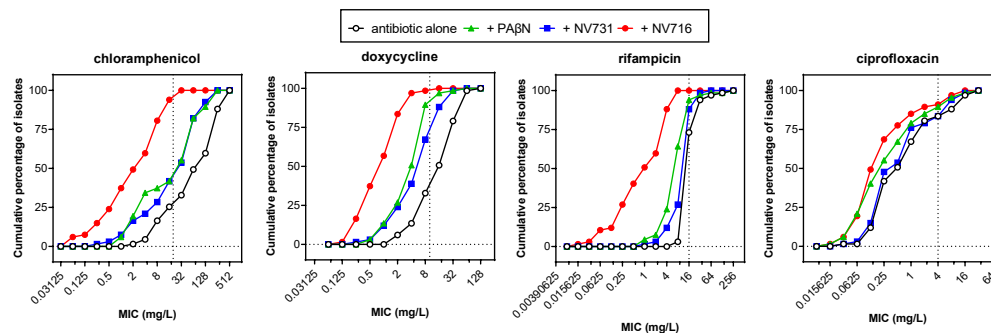


**Figure S2.** Heatmaps describing interactions between antibiotics and potentiators against reference strains. FIC index were calculated for concentrations of 2.5  $\mu\text{M}$  Alexidine, 0.5  $\mu\text{M}$  colistin (CST), 30  $\mu\text{M}$  PMBN, 38  $\mu\text{M}$  PA $\beta$ N, 2.5  $\mu\text{M}$  NV731 and 2.5  $\mu\text{M}$  NV716. Synergy is defined as FIC < 0.5 (appearing in green on the graphs). All data are mean from at least two independent experiments.



| Antibiotics     | C <sub>max</sub> (mg/L)[274, 377] | Percentage of isolates with MICs below human C <sub>max</sub> |               |                 |                 |
|-----------------|-----------------------------------|---------------------------------------------------------------|---------------|-----------------|-----------------|
|                 |                                   | ABs alone                                                     | +PAβN (38 μM) | +NV731 (2.5 μM) | +NV716 (2.5 μM) |
| Chloramphenicol | 20                                | 25.37                                                         | 41.79         | 41.79           | 94.03           |
| Doxycycline     | 10                                | 32.84                                                         | 89.55         | 67.16           | 98.51           |
| Rifampicin      | 16                                | 73.13                                                         | 94.03         | 88.06           | 100.00          |
| Ciprofloxacin   | 4                                 | 83.58                                                         | 89.55         | 83.58           | 91.04           |

**Figure S3.** Cumulative MIC distribution for antibiotics alone or combined with potentiators (38 μM PAβN, 2.5 μM NV731 and NV716) against clinical isolates. The vertical dotted line shows the human C<sub>max</sub> values for each antibiotic. The table shows the proportion of isolates for which MICs are below this C<sub>max</sub> value, and thus falls in the range of therapeutically-achievable concentrations. All MICs were determined in at least 2-3 independent experiments (if values differing of 1-2 dilutions were obtained, the most frequent one was considered).

The fact that **NV716** increases antibiotic susceptibility, including in strains that do not express efflux (PAO509) or for antibiotics that are not substrates for efflux (rifampicin), indicates that it does not act only as an inhibitor of efflux. Its amphipathic structure suggests a possible interaction with the bacterial membrane. We therefore examined its effect in combination with the same antibiotics against colistin-resistant strains (PA313, 2938, 307, 272), which are characterized by a highly ordered state of LPS [422], a reduction in the net negative charge of lipid A[423], or the presence of an additional fatty acyl chain on lipid A [98]. **NV716** at 10 μM (as well as PMBN at 30 μM) remained able

to decrease the MIC of antibiotics against these strains, though to a lower extent than against PAO1 (Table 1), and to maintain a synergistic effect (Figure S4).

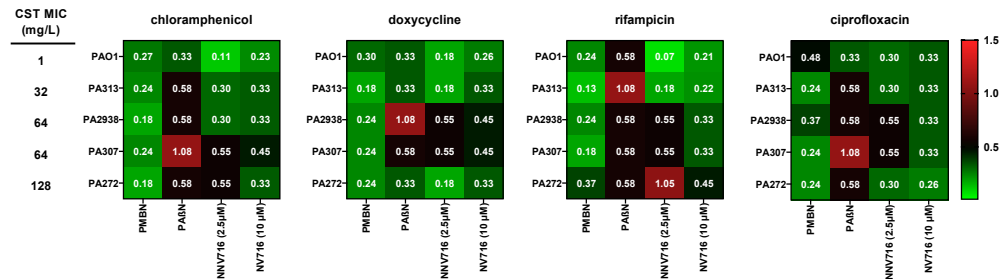


**Figure S4.** Heatmaps describing interactions between antibiotics and potentiators. Combination of chloramphenicol, doxycycline, rifampicin, and ciprofloxacin with 38  $\mu$ M PA $\beta$ N, 30  $\mu$ M PMBN, or 2.5 or 10  $\mu$ M NV716 against 4 colistin-resistant isolates [401] compared to PAO1. Colistin MICs are shown on the left.

### Interaction of NV716 with *Pseudomonas aeruginosa* membranes

Because the activity of NV716 is reduced against isolates resistant to colistin, we examined its capacity to bind to LPS, using alexidine and colistin as positive controls [345, 401], imipenem as a negative control, and the other potentiators as comparators. To this effect, we measured the capacity of each molecule to displace Bodipy-cadaverine from its binding to the LPS of PAO1, PAO509 and of 2 colistin-resistant isolates PA307 and PA2938 (Figure 1A). The effect of NV716 was similar to that of alexidine over the whole range of concentrations investigated against PAO1 and PAO509, and for concentrations > 50  $\mu$ M and 6.25  $\mu$ M (two-way ANOVA) for isolates 307 and 2938, respectively. Colistin, PMBN, PA $\beta$ N and NV731 were less effective over the whole range of concentrations and against all strains, while imipenem was ineffective. The effect of all these molecules was attenuated in a concentration-dependent fashion by the addition of Mg<sup>2+</sup> either to bacteria or to LPS in solution (Figure S5).

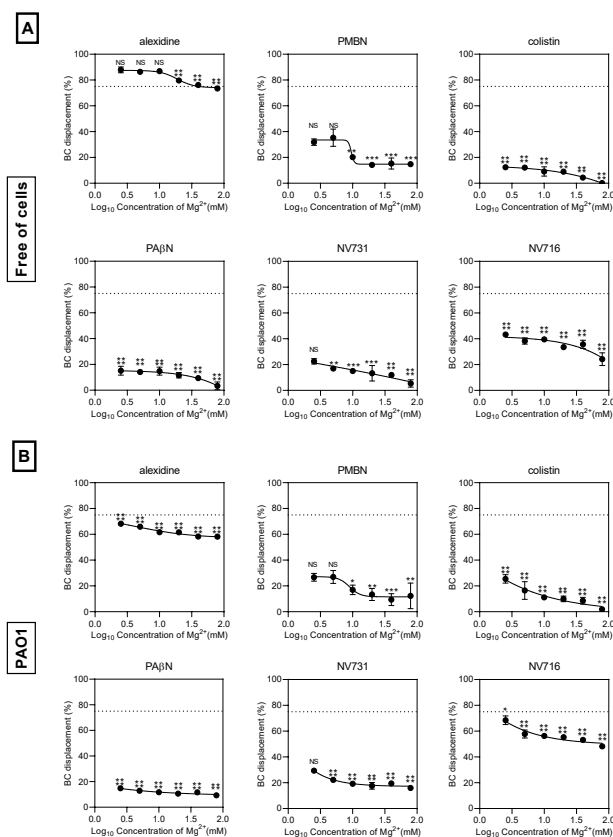


**Figure S5. (Next page)** Influence of  $Mg^{2+}$  concentration on the binding of potentiators to LPS in solution (A) or in whole bacteria (PAO1; B). Potentiators were used at a fixed concentration (10  $\mu M$  for alexidine, colistin, and NV716; 30  $\mu M$  for PMBN, 50  $\mu M$  for PA $\beta$ N and 25  $\mu M$  for NV731). The dotted line shows the value for the corresponding control (without  $Mg^{2+}$ ). All data are mean  $\pm$  SEM (triplicates from 3 independent experiments). Statistical analysis: one-way ANOVA with Dunnett's post-hoc test (NS: no significant [ $p > 0.05$ ]; \*,  $p < 0.05$ ; \*\*,  $P < 0.01$ ; \*\*\*  $p < 0.001$ ; \*\*\*\*  $p < 0.0001$ ).

Method for assay of binding to LPS in solution: stock solutions of 500  $\mu M$  BC and 5 mg/mL LPS extracted from *Pseudomonas aeruginosa* serotype 10 (Sigma-Aldrich) were prepared in Tris buffer (50 mM, pH 7.4), then mixed to reach the final desired concentrations (BC: 5  $\mu M$ ; LPS: 10  $\mu g/mL$ ) and maintained in the dark at room temperature. After 4 hours of incubation, 50  $\mu L$  of a solution of the test compounds were added to 50  $\mu L$  of this mix in a 96-wells black plate, which was kept for 30 mins in the dark at room temperature. Fluorescence was read ( $\lambda_{exc}/\lambda_{em}$ : 580/620 nm) in a SpectraMax M3 plate reader (Molecular Devices LLC, Sunnyvale, CA).

We then evaluated the capacity of potentiators to permeabilize the outer membrane by measuring the fluorescence of NPN when incorporated in the hydrophobic core of this membrane [424]. NPN uptake proceeded following a 1-phase association, with a first rapid and almost linear uptake during the first four seconds, followed by a plateau (Figure S6). As NPN is a substrate for efflux in *P. aeruginosa* [425], we calculated the rate of its influx for the early time points (0-4 seconds) to minimize a possible contribution of efflux in the reduction of its rate of uptake, assuming a linear uptake over this period of time. As expected, the rate of uptake was 70% higher in PAO509 than in PAO1. It was 55-60% lower in colistin-resistant isolates PA2938 and PA307 than in PAO1 (Figure S6). The potentiators alexidine (2.5  $\mu$ M) and PMBN (30  $\mu$ M) increased this rate of uptake to a significantly larger extent than colistin (2.5  $\mu$ M), **NV716** (2.5  $\mu$ M) and PA $\beta$ N (38  $\mu$ M) in PAO1 and PAO509 but not in the two colistin-resistant isolates. **NV731** (2.5  $\mu$ M) did not show any effect (Figure 1B). The addition of Mg<sup>2+</sup> counteracted the effect of potentiators.

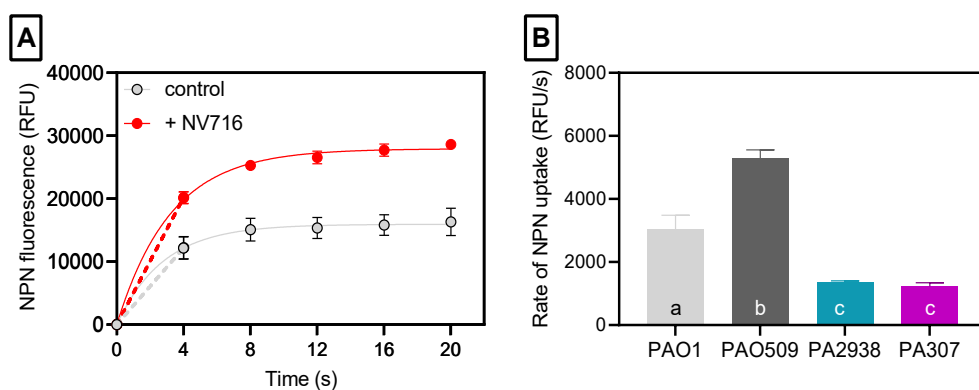


**Figure S6.** Rate of NPN uptake in *P. aeruginosa*. **(A)** Kinetics of NPN uptake in PAO1 in the absence or in the presence of **NV716**. **(B)** Rate of NPN uptake over the early time point (0-4s) in 4 *P. aeruginosa* strains (2 reference strains [PAO1 and PAO509] and two colistin-resistant isolates [PA2938 and PA307]). Statistical analysis: 1-way ANOVA with Tukey post-hoc test: data series with different letters are different from one another ( $p < 0.05$ ).

Lastly, we determined how potentiators affect the capacity of propidium iodide (PI) to get access to the DNA and generate a fluorescent signal, which implies a permeabilization of both the outer and the inner membrane. Alexidine, and to some extent colistin, increased PI fluorescence, though at higher concentrations in colistin-resistant isolates (Figure 1C). **NV716** caused a modest increase in fluorescence signal (approx. 50 %) at high concentrations (50  $\mu$ M) and only in PAO1 and PAO509. Yet, its effect was of the same order of magnitude as that of colistin but much lower than that of alexidine when these compounds were all compared at an equipotent concentration of 1 x MIC (Figure S7). PMBN, PA $\beta$ N, and **NV731** were ineffective in this assay.

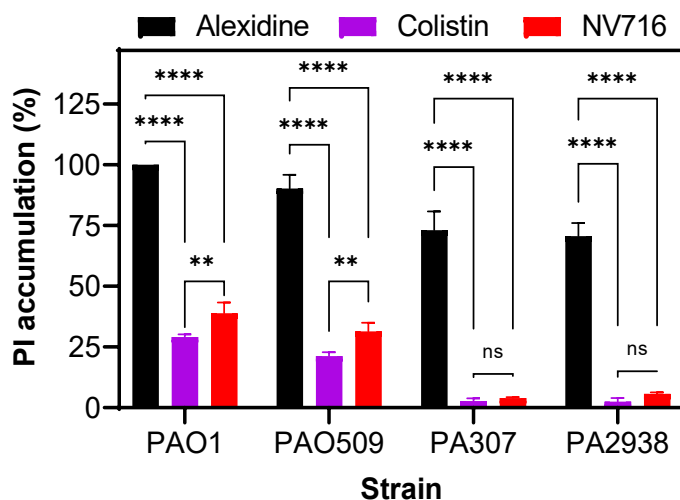


**Figure S7.** Effect of alexidine, colistin, and **NV716** each at a concentration of 1 x MIC (See Table S1) on inner membrane permeability as assessed by measuring the fluorescence of propidium iodide (PI) after 1 h of incubation with PAO1, PAO509, or two colistin-resistant clinical isolates (PA307 and PA2938). The effect measured with 0.5 % SDS (w/v) after 1 h was taken as 100% (positive control). All data are means  $\pm$  SEM (triplicates from 3 independent experiments). Statistical analysis: two-way ANOVA with Tukey post-hoc test: \*\*\*\*,  $p < 0.0001$ ; \*\*\*,  $p < 0.001$ ; \*\*,  $p < 0.01$ .

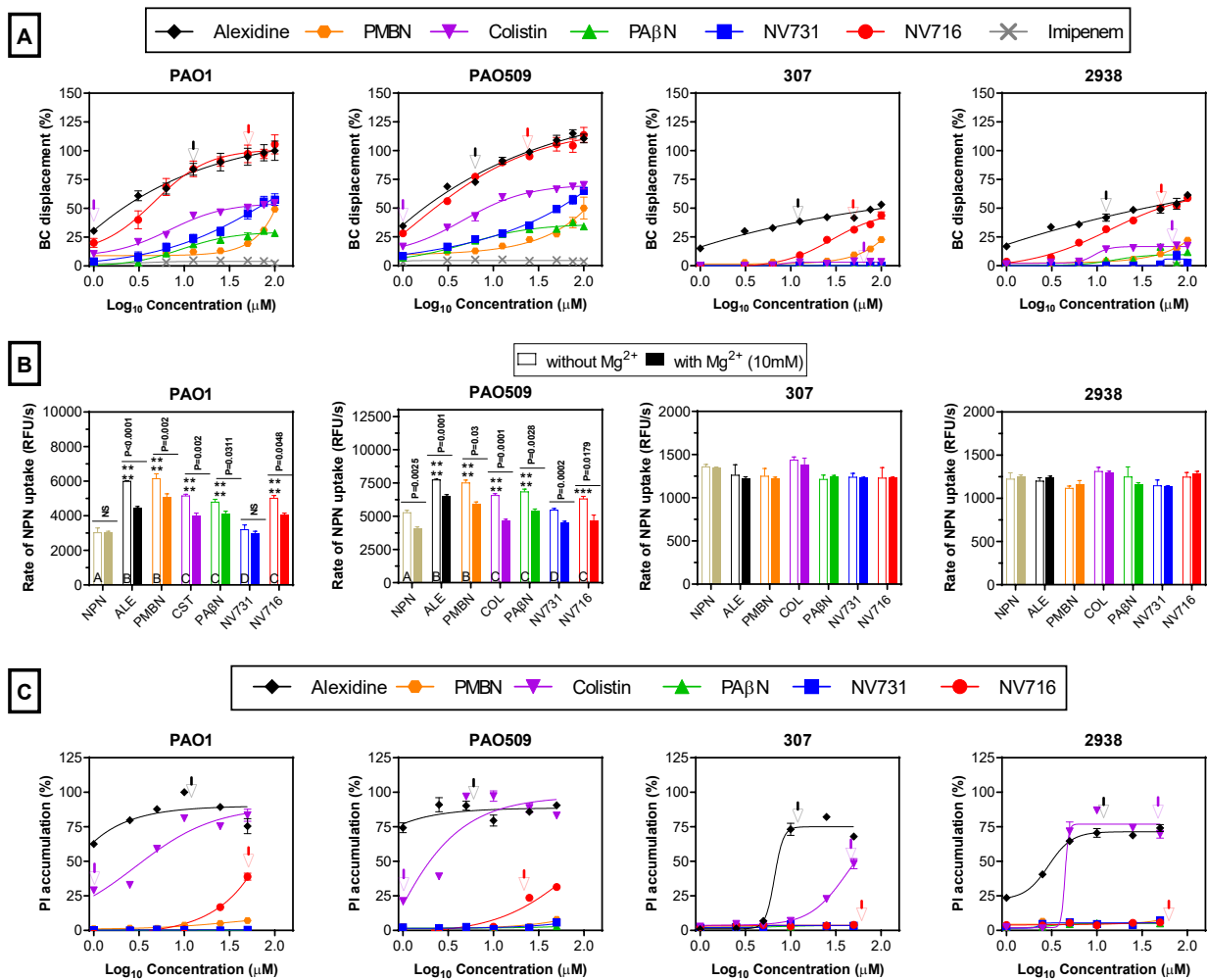


**Figure 1.** Effect of potentiators on membrane properties of the 2 reference strains PAO1 and PAO509 and 2 colistin-resistant clinical isolates PA2938 and PA307. **(A)** Binding of potentiators over a wide range of concentrations to LPS, assessed by measuring the displacement of BODIPY-cadaverine (BC) after incubation during 30 minutes. The effect of alexidine at 30 minutes was taken as 100% (positive control). Imipenem was included as a negative control. **(B)** Effect of potentiators on outer membrane permeability, assessed by measuring the rate of 1-N-phenylnaphthylamine (NPN) uptake at early-time points (0-4 seconds). Potentiators were added at fixed concentrations (PA $\beta$ N: 38  $\mu$ M; polymyxin B nonapeptide [PMBN]: 30  $\mu$ M; alexidine [ALE], colistin [CST], **NV731**, **NV716**: 2.5  $\mu$ M), in the absence (open bars) or in the presence (closed bars) of 10 mM Mg<sup>2+</sup>. Statistical analysis: one-way ANOVA with Tukey post-hoc test comparing NPN uptake in all conditions: open bars with different letters are different from one another. One-way ANOVA with Dunnett's post-hoc test for comparison between NPN uptake alone (as a control) or combined with each of the potentiator: \*\*\*,  $p < 0.001$ ; \*\*\*\*,  $p < 0.0001$ . Two-tailed Student t-test for comparison for each condition without and with Mg<sup>2+</sup>: p value indicated above each pair of columns. **(C)** Effect of potentiators over a wide range of concentrations on inner membrane permeability, assessed by measuring the fluorescence of propidium iodide (PI) after 1 h of incubation. The effect measured with 0.5 % SDS (w/v) after 1 h was taken as 100% (positive control). All data are means  $\pm$  SEM (triplicates from 3 independent experiments). In panels A and C, the arrows point to the MIC of the corresponding compound (see Table S1; when not shown, the MIC is higher than the highest concentration tested).

### Transmission electron microscopy

To examine whether **NV716** membrane effects were accompanied by visible changes in bacteria envelope, we examined in electron microscopy PAO1 cells that had been exposed to a concentration of 10  $\mu$ M during 1 h. As shown in Figure S8, bacteria maintained a morphology similar to that of control cells, and remained capable of dividing. We can exclude a massive loss of destroyed bacteria during washing procedures, because samples exposed to **NV716** did not show a reduction in their protein content (11%; SDS used as positive control). Therefore, these images suggest at least that **NV716**, at a concentration for which it proves active as a potentiator and disturbs outer membrane permeability, does not cause clear-cut alterations of bacterial integrity.

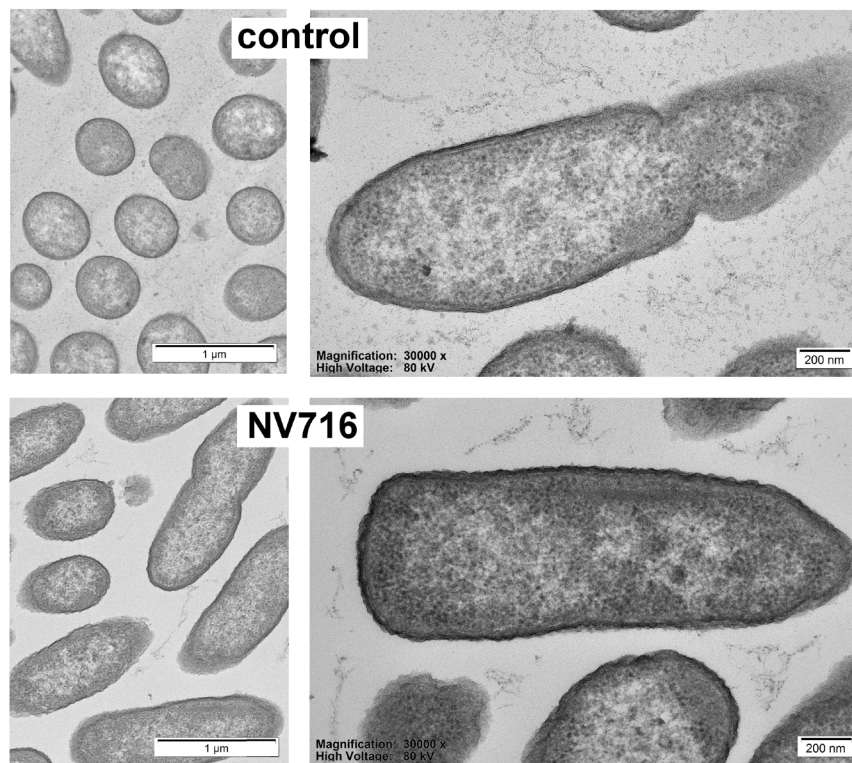


**Figure S8.** Transmission electron microscopy images of PAO1 in control conditions (top) or after 1 h of incubation with **NV716** at 10 μM (bottom). Bars: 1 μm (left) or 200 nm (right).

### Antibiotic accumulation

The outer membrane is a barrier to the diffusion of antibiotics. We therefore assessed the effect of potentiators on the accumulation of ciprofloxacin and **NV1532** (a fluorescent derivative of rifampicin), as representative of antibiotics that are, or are not, substrate for efflux, respectively. As expected, the level of accumulation of ciprofloxacin was approximately 3 times higher in PAO509 than in PAO1 (Figure 2A). In PAO1, alexidine and colistin did not increase ciprofloxacin accumulation at the concentrations tested, while PMBN and PAβN caused a significant increase at the highest concentration tested, and **NV731** and **NV716** displayed concentration-dependent effects. In PAO509, none of

the compounds caused a significant increase in ciprofloxacin accumulation, even at high concentrations. The accumulation of the rifampicin analog was similar in both strains (Figure 2B). PMBN, colistin and **NV716** were the only potentiators capable of causing a significant, concentration-dependent increase in **NV1352** accumulation in both strains, while PA $\beta$ N was only effective in PAO509 and **NV731**, in both strains but only at the highest concentration tested. A significant correlation (Figure 2C) was observed between the accumulation level of each drug inside bacteria as measured in all these conditions and the outer membrane permeability, as assessed by the fluorescence signal of NPN (data from Figure 1A).

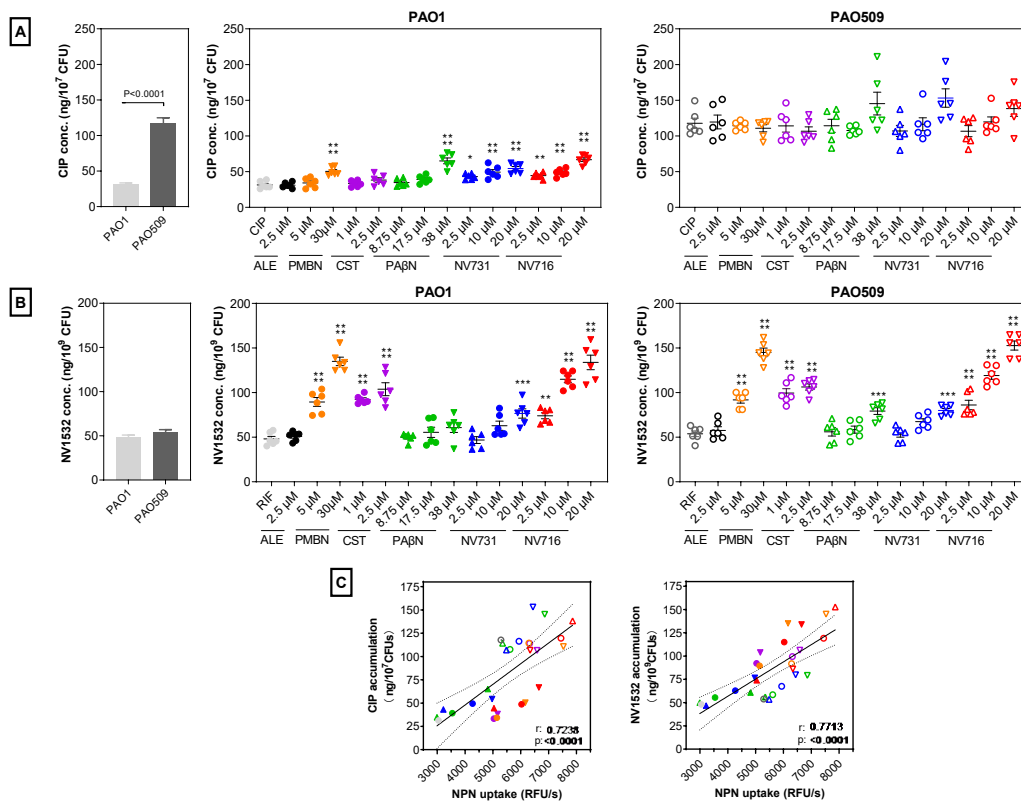


**Figure 2.** Accumulation of antibiotics (ciprofloxacin **(A)** and the rifampicin analog **NV1532 (B)**) alone or combined with potentiators in PAO1 and PAO509. Potentiators were added at the indicated concentrations (alexidine [ALE]; polymyxin B nonapeptide [PMBN]; colistin [CST]). All data are mean  $\pm$  SEM (triplicates from three independent experiments). Statistical analysis: Left-panels: Two-tail student t-test for comparison of antibiotics alone in PAO1 and PAO509; other panels: one-way ANOVA with Dunnett's post-hoc test for comparison of antibiotics alone and combined with each potentiator: \*,  $p < 0.05$ ; \*\*,  $p < 0.01$ ; \*\*\*,  $p < 0.001$ ; \*\*\*\*,  $p < 0.0001$ . **(C)** Correlation between the accumulation of ciprofloxacin or **NV1532** in bacteria and the outer membrane permeability as assessed by the fluorescence signal of NPN (see Figure 1A). 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.

## Resistance selection

We then determined whether potentiators were able to prevent selection of resistance to these two drugs. We first determined the frequency of resistance in PAO1 after 48 h of incubation with antibiotics at 4 x MIC, alone or combined with potentiators. PAO1 displayed a resistance frequency of  $2.4 \times 10^{-7}$  and  $4.5 \times 10^{-7}$  for ciprofloxacin and rifampicin, respectively. At the concentrations we used, the addition of PA $\beta$ N significantly reduced the frequency of resistance to  $6.4 \times 10^{-8}$  for ciprofloxacin (4-fold decrease) and to  $1.5 \times 10^{-7}$  (3-fold decrease) for rifampicin, while **NV716** as well as PMBN caused an almost 10-fold reduction in the frequency of resistance for ciprofloxacin ( $2.6 \times 10^{-8}$ ) and rifampicin ( $4.0 \times 10^{-8}$ ) (Figure 3A). Alexidine and colistin had no effect at the concentration tested (< MIC of the respective compound).

We then evaluated the development of resistance by serial passaging over 30 days, with daily readjustment of the antibiotic concentration to  $\frac{1}{2}$  MIC (Figure 3B). In the absence of potentiator, PAO1 rapidly developed resistance to both ciprofloxacin and rifampicin, with a 128-fold increase in MIC observed after 20 and 16 passages, respectively (up to MICs of 32 and 2048 mg/L, respectively). Alexidine and colistin were largely ineffective to prevent selection of resistance to both drugs, while PMBN was highly effective. PA $\beta$ N and **NV731** only slightly delayed this selection for rifampicin while their effect remained important for ciprofloxacin, with increases in MICs of only 3 doubling dilutions observed at the end of the experiment. **NV716** proved highly effective in the assay, the MIC having increased only of 2 and 4 doubling dilutions at the end of the 30 days incubation when combined with ciprofloxacin and rifampicin, respectively.

Lastly, we examined whether it was possible to select resistance to the potentiators themselves by serial passages over 50 days, focusing on those that show low MICs (alexidine, colistin, and **NV716**). Resistance selection was much slower than for ciprofloxacin/rifampicin, since we observed an increase of MIC of 2/3 doubling dilutions only at passage 9/23 with alexidine, 13/21 with colistin and 16/>40 with **NV716** (Figure

3C). These change in MICs were rapidly reversible, as MICs values were reduced of 1-2 dilutions after one passage culture in the absence of compound.. No cross-resistance was observed between the three potentiators in the final daughter cells (Figure 3D).

### Genomic and transcriptomic analysis

Resistance acquisition is often related to genomic modifications occurring at the target level of a drug. To get more insight in the mechanism of action of **NV716**, we passaged the PAO1 strain 52 times in control conditions or in the presence of ½ MIC of **NV716**, colistin or alexidine. We selected three colonies of each of the passaged PAO1 and subjected the DNA to whole-genome sequencing. A total of 15 SNP, which could be unambiguously assigned to the PAO1 genome, were detected in the passaged strains (Table S2). 5 SNPs were located in intergenic regions and 3 SNPs were synonymous mutations. The remaining 7 SNPs were found in overall 6 ORFs. In the strain treated with **NV716**, SNPs were found in *morA* and *roxR*. In the colistin-treated strain we found non-synonymous mutations in *phoQ*, *aer* and *pmrB*. In the strain treated with alexidine, SNPs in *rpoB* and *roxR* (however a different position than in the **NV716**-treated strain) were observed (Table S2). Signal transduction via PhoQ, Aer, RoxR and PmrB, has been described to be involved in resistance to polymyxins. However, the acquisition of these mutations in the strains exposed to each of the three tested agents was obviously not sufficient to induce a stable resistance phenotype against **NV716**, colistin or alexidine.

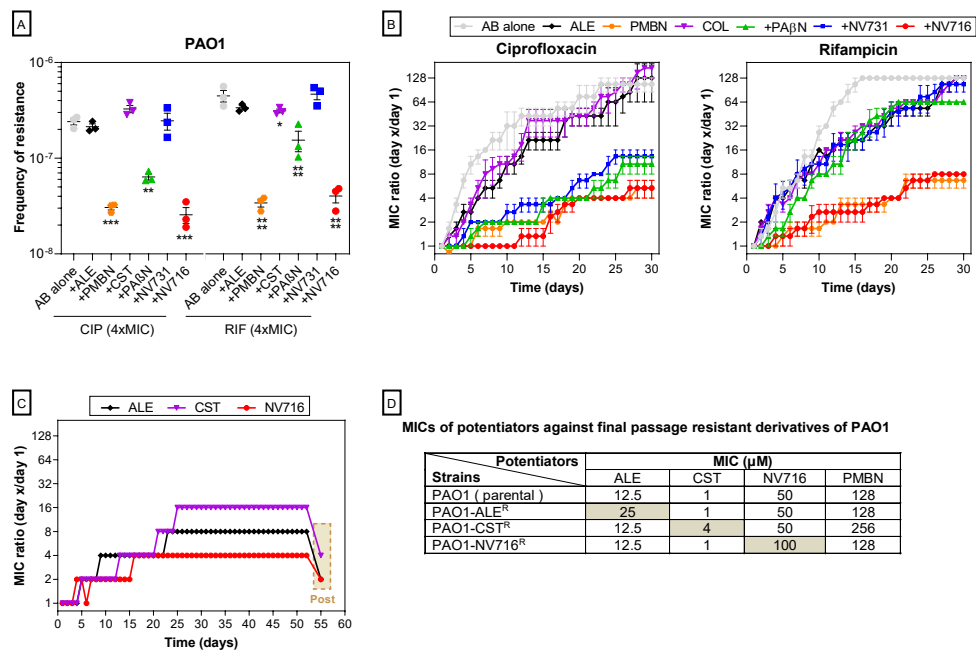


**Figure 3.** Effect of potentiators on resistance selection in PAO1. **(A)** Frequency of resistance to ciprofloxacin and rifampicin after 24 h of incubation with each antibiotic at 4 x MIC, in control conditions (antibiotic alone) or in the presence of alexidine (ALE, 2.5  $\mu$ M), polymyxin B nonapeptide (PMBN, 30  $\mu$ M), colistin (CST, 0.5  $\mu$ M), PA $\beta$ N (38  $\mu$ M), **NV731** and **NV716** (2.5  $\mu$ M). 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 with antibiotic alone (\*,  $p < 0.05$ ; \*\*,  $p < 0.01$ ; \*\*\*,  $p < 0.001$ ; \*\*\*\*,  $p < 0.0001$ ). **(B)** Evolution of the MIC of ciprofloxacin or rifampicin over passages in the presence of each antibiotic at  $\frac{1}{2}$  MIC (with daily readjustment of the concentration) alone or combined with potentiators at the same concentrations as in panel A. All data are mean  $\pm$  SEM (duplicates from three independent experiments). **(C)** Evolution of the MIC of alexidine, colistin, and **NV716** over passages in the presence of each potentiator at  $\frac{1}{2}$  MIC (with daily readjustment of the concentration). After 52 passages, MICs were measured for all compounds after 1 culture on agar in the absence of compounds (beige box in panel C and values in panel **(D)**).

**Table S2** SNPs identified in PAO1 strains after 52 passages in the presence of potentiators at ¼ MIC compared to the untreated control. The sequence is separated by a dot and the SNP occurs after the dot (red). REF, Reference, ALT, alternative nucleotide.

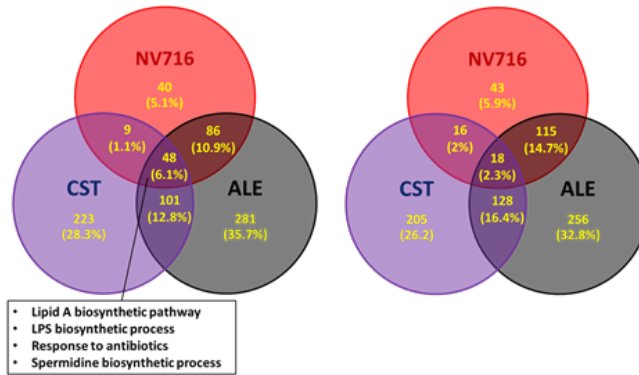
| PAO1 control | NV716 | Colistin | Alexidine | Genome location (gene, intergenic)                  | Synonymous (S) / Nonsynonymous (N) | ID                    | REF | ALT |
|--------------|-------|----------|-----------|-----------------------------------------------------|------------------------------------|-----------------------|-----|-----|
| 0            | 1     | 1        | 1         | intergenic between PA5015 and PA5016                | N/A                                | CGGCGCCGCC.CACGGGCGGC | C   | A   |
| 0            | 0     | 1        | 1         | PA5267 ( <i>hcp</i> )                               | S                                  | ACTGGATCTC.CACCTTGGTC | C   | G   |
| 0            | 0     | 1        | 1         | PA5267 ( <i>hcp</i> )                               | S                                  | CCAGCAGCAG.GGGCGAGGCC | G   | C   |
| 0            | 0     | 1        | 0         | PA1180 ( <i>phoQ</i> )                              | N (V260G)                          | CTGGTCGCCG.ACCCCCTGCA | A   | C   |
| 0            | 0     | 0        | 1         | intergenic between PA3811 and PA3810                | N/A                                | GTTAAGACAG.AGCATGGCCC | A   | G   |
| 0            | 0     | 1        | 0         | PA1561 ( <i>aer</i> )                               | N (G428D)                          | CTGCTGCAGG.CCGCTCTCCA | C   | T   |
| 0            | 0     | 0        | 1         | intergenic between PA1843 and PA1844 ( <i>tse</i> ) | N/A                                | CGAATGAGTC.TGGCTCATTC | T   | G   |
| 0            | 1     | 0        | 0         | intergenic between PA1843 and PA1844 ( <i>tse</i> ) | N/A                                | ATGAGTCTGG.CTCATTGCGA | C   | T   |
| 0            | 1     | 0        | 0         | PA4601 ( <i>morA</i> )                              | N (A1149V)                         | CAGGGCGCGA.GCGTTCATCT | G   | A   |
| 0            | 0     | 0        | 1         | PA4493 ( <i>roxR</i> )                              | N (D116E)                          | AGCCGGCGGA.TGCCGATGAC | T   | G   |
| 0            | 1     | 0        | 0         | PA4493 ( <i>roxR</i> )                              | N (A123V)                          | GTGTTGGCCG.CGCTGCTCTC | C   | T   |
| 0            | 0     | 1        | 0         | PA1511 ( <i>vgrG2a</i> )                            | S                                  | CTTCGGGCCG.GGATGGGTC  | G   | A   |
| 0            | 1     | 1        | 1         | intergenic between PA2348 and PA2327                | N/A                                | CCGGTACCCC.CTCCGGCGGA | C   | G   |
| 0            | 0     | 1        | 0         | PA4777 ( <i>pmrB</i> )                              | N (M48I)                           | CGAAGTCCAC.CATGTCGTCG | C   | T   |
| 0            | 0     | 0        | 1         | PA4270 ( <i>rpoB</i> )                              | N (Q468P)                          | GCCGAGAACC.AGTTCCGCGT | A   | C   |

We also performed a transcriptomic analysis of the same strains, since transient changes in susceptibility could be related to modifications in the expression level of genes involved in specific pathways. The number of differentially expressed genes (DEGs) between control bacteria and potentiator-exposed cells after 52 passages with **NV716**, colistin and alexidine was 375, 748, and 1034, respectively. They were ranked according to their false discovery rate and log<sub>2</sub> fold change in Figure S9. Among these genes, 48 were commonly upregulated and 18 downregulated after exposure to the three potentiators (only 3 and 2 expected by chance). The upregulated genes are involved in lipid A biosynthetic process and response to antibiotics as well as LPS synthesis (Figure 4A-C). This Closer inspection of the genes that were exclusively down-regulated in the **NV716** treated PAO1 revealed quorum sensing and pathogenesis-related functions (Figure 4B). Table S3 shows in details the DEG involved in QS, as well as in elastase, rhamnolipids and pyocyanin production, all under the control of QS [187]. The most downregulated QS-related genes encode 2-heptyl-3-hydroxy-4(1H)-quinolone synthase (*pqsH*), acyl-homoserine-lactone synthase (*lasI*), acyl-homoserine-lactone synthase (*rhII*) and the regulatory protein RsaL (*rsaL*). A selection of genes involved in elastase, rhamnolipid and pyocyanin production were also found to be down-regulated in alexidine-treated PAO1 cells. To validate these observations, we evaluated the effects of potentiators on motility (swarming, swimming, twitching), biofilm formation, and elastase, rhamnolipid, and pyocyanin production by comparing these parameters after exposure of the initial and the final (passage 52) bacteria. **NV716** was the most effective (at passage 0 and even more at passage 52) to reduce motility, biofilm formation, and virulence factor production, while alexidine reduced twitching and virulence factors secretion. Colistin rather increased swimming, biofilm biomass and production of elastase and pyocyanin (Figure 4D-H).

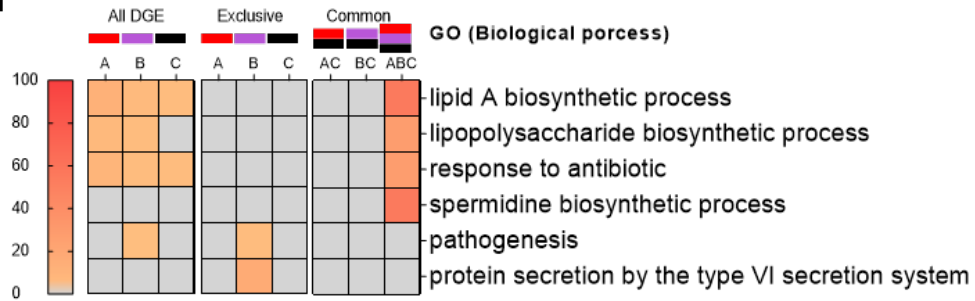
## A Venn diagrams for intersecting genes

Up-regulated

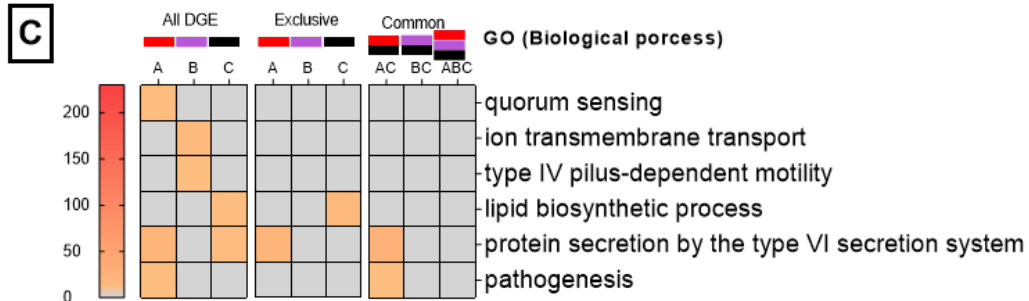
Down-regulated



## B Differentially expressed genes (DEGs) - Up regulation



## C Differentially expressed genes (DEGs) - Down regulation



# D Motility

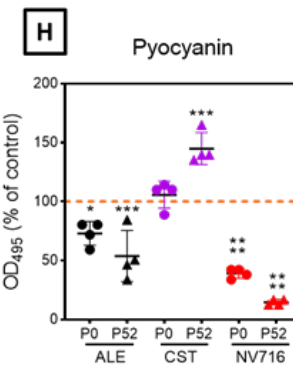
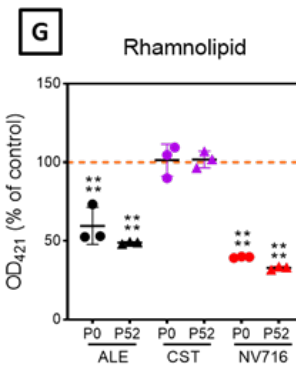
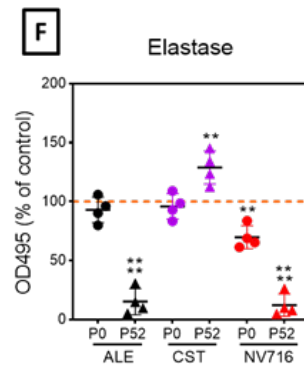
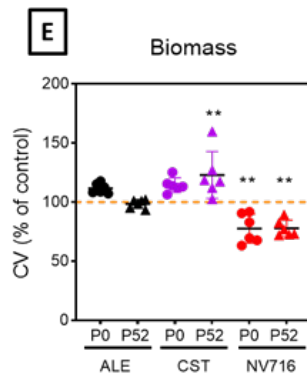
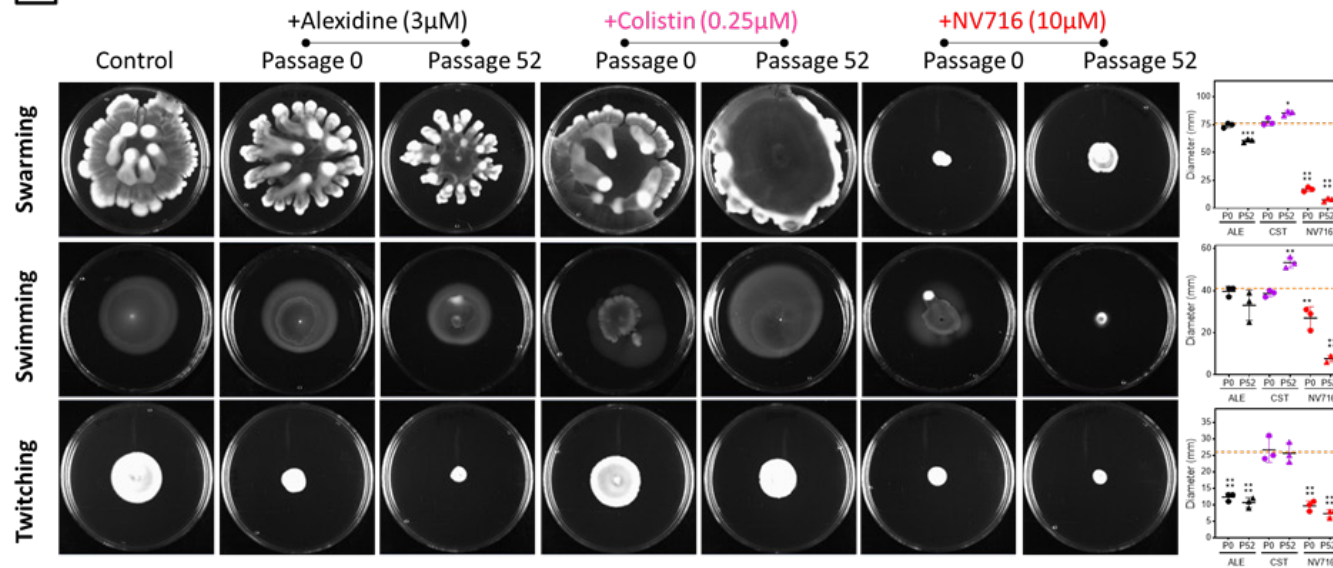


**Figure 4:** Transcriptomic analysis and phenotypic validation. **(A)** Venn diagrams showing the commonly differentially expressed genes in PAO1 after 52 passages of culture in the absence or in the presence of **NV716**, colistin (CST) or alexidine (ALE) at ½ MIC. Pathways for intersecting genes that are relevant for this study are depicted in boxes. **(B-C)** GO term enrichment analysis of up- and down-regulated genes, respectively, highlighting the biological functions that are exclusively regulated by a single potentiator or shared by two or three of them (A, red: **NV716**, B, purple: colistin, C, black: alexidine). The color gradient represents the value of the fold-change enrichment. All processes shown in these panels are significant (FDR <0.05). **(D-H)** Validation assays, performed for the parental strain or the final isolate (passage 52) exposed to each potentiator at ½ MIC: motility assays **(D)** of swarming (top), swimming (medium), and twitching (bottom); biofilm formation assessed by biomass quantification **(E)** and virulence factors secretion **(F-H)**. In these panels, all data are expressed in percentage of the untreated control. Data are means of triplicates from 3-4 experiments. Statistical analysis: A: the number of up- or down-regulated genes is higher than expected by chance (higher than 97.5% of permuted data) for those up- or down-regulated by the three potentiators or by NV716 and alexidine. D-H: one-way ANOVA with Dunnett's post-hoc test (\*,  $p < 0.05$ ; \*\*,  $p < 0.01$ ; \*\*\*,  $p < 0.001$ ; \*\*\*\*,  $p < 0.0001$ ).

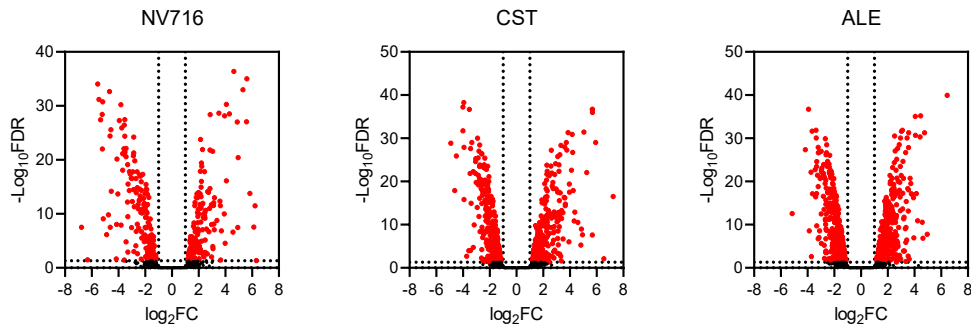


**Figure S9.** Volcano plot of the differentially expressed genes (DEGs) in PAO1 after 52 serial passages in the presence of NV716, colistin (CST) or alexidine (ALE) at ½ MIC. The x- and y-axes represent the level of change in expression ( $\log_2$  Fold Change scale) and the degree of statistical significance (negative  $\log_{10}$  transformed False Discovery Rate), respectively. Red dots represent genes which are significantly regulated ( $FDR \leq 0.05$ ; horizontal dotted line) to levels at least 2-fold (vertical dotted line) higher or lower than the untreated control (function `glmTreat` of the R package `edgeR`).

**Table S3.** DEGs involved in elastase, rhamnolipid and pyocyanin production in PAO1 after 52 passages in the presence of potentiators at ¼ MIC. Down-regulated genes are highlighted in blue; upregulated genes, in red. Genes related to QS are in bold [426].

| Regulated elements               | LOCUS TAG     | Name         | NV716                            |                  | Colistin            |          | Alexidine           |          |
|----------------------------------|---------------|--------------|----------------------------------|------------------|---------------------|----------|---------------------|----------|
|                                  |               |              | Log <sub>2</sub> FC <sup>a</sup> | FDR <sup>b</sup> | Log <sub>2</sub> FC | FDR      | Log <sub>2</sub> FC | FDR      |
| Elastase                         | <b>PA3724</b> | <b>lasB</b>  | -2.87                            | 3.40E-15         | 1.25                | 0.01656  | -2.50               | 1.11E-15 |
|                                  | <b>PA1871</b> | <b>lasA</b>  | -1.76                            | 1.10E-05         | 1.28                | 0.01215  | -1.84               | 2.38E-08 |
|                                  | <b>PA1432</b> | <b>lasI</b>  | -1.99                            | 1.80E-12         |                     |          | -1.36               | 5.83E-05 |
| Rhamnolipid                      | <b>PA3479</b> | <b>rhIA</b>  | -2.97                            | 5.30E-13         |                     |          |                     |          |
|                                  | <b>PA3478</b> | <b>rhIB</b>  | -3.69                            | 7.30E-22         |                     |          | -2.13               | 7.30E-14 |
|                                  | <b>PA1130</b> | <b>rhIC</b>  | -1.38                            | 0.03808          |                     |          |                     |          |
|                                  | <b>PA3476</b> | <b>rhII</b>  | -1.66                            | 0.00106          | 2.08                | 1.90E-11 |                     |          |
|                                  | <b>PA0996</b> | <b>pqsA</b>  | -5.46                            | 6.72E-32         |                     |          |                     |          |
|                                  | <b>PA0997</b> | <b>pqsB</b>  | -4.57                            | 2.70E-26         |                     |          | -1.43               | 2.90E-05 |
|                                  | <b>PA0998</b> | <b>pqsC</b>  | -4.11                            | 7.10E-21         |                     |          | -1.30               | 0.00871  |
|                                  | <b>PA0999</b> | <b>pqsD</b>  | -3.52                            | 4.28E-25         |                     |          | -1.45               | 2.70E-06 |
|                                  | <b>PA1000</b> | <b>pqsE</b>  | -4.68                            | 2.18E-33         |                     |          | -2.28               | 1.00E-21 |
|                                  | <b>PA1001</b> | <b>ph</b>    | -4.88                            | 6.91E-07         |                     |          | -2.09               | 0.00011  |
|                                  | <b>PA1002</b> | <b>phnB</b>  | -3.18                            | 1.54E-18         |                     |          | -2.29               | 4.11E-15 |
|                                  | <b>PA2587</b> | <b>pqsH</b>  | -1.68                            | 0.00116          |                     |          |                     |          |
|                                  | <b>PA1431</b> | <b>rsaL</b>  | -2.43                            | 2.53E-15         | 1.30                | 0.00093  | -1.80               | 6.15E-11 |
|                                  | PA2656        | bqsS         | 2.85                             | 1.58E-22         |                     |          | 1.26                | 0.00799  |
|                                  | PA2657        | bqsR         | 4.64                             | 4.07E-37         | 1.42                | 1.63E-05 | 2.33                | 3.74E-20 |
|                                  | PA5360        | phoB         | 1.97                             | 1.94E-07         |                     |          | 1.70                | 8.74E-06 |
|                                  | <b>PA2591</b> | <b>vqsR</b>  | -2.09                            | 9.84E-07         |                     |          |                     |          |
|                                  | PA1898        | qscR         |                                  |                  | -3.02               | 1.77E-20 | -2.11               | 4.40E-12 |
|                                  | PA2586        | gacA         |                                  |                  | -1.39               | 2.02E-07 |                     |          |
|                                  | PA4723        | dksA         |                                  |                  |                     |          | 1.33                | 0.00517  |
| Pyocyanin                        | PA1905        | phzG2        | -5.14                            | 8.40E-10         |                     |          | -5.15               | 2.70E-13 |
|                                  | <b>PA4210</b> | <b>phzA1</b> | -6.31                            | 0.0324           |                     |          |                     |          |
|                                  | <b>PA4211</b> | <b>phzB1</b> |                                  |                  | 4.31                | 1.37E-11 |                     |          |
|                                  | <b>PA4216</b> | <b>phzG1</b> | -4.75                            | 1.60E-10         |                     |          | -3.32               | 1.80E-10 |
|                                  | <b>PA4217</b> | <b>phzS</b>  | -5.55                            | 8.90E-35         |                     |          | -3.66               | 2.20E-32 |
| Type VI protein secretion system | <b>PA1657</b> | <b>HsiB2</b> | -3.32                            | 6.32E-13         | 2.74                | 4.6E-19  |                     |          |
|                                  | <b>PA1658</b> | <b>HsiC2</b> | -2.53                            | 1.97E-12         | 2.29                | 3.21E-17 |                     |          |
|                                  | <b>PA1659</b> | <b>HsiF2</b> | -3.30                            | 3.21E-10         | 2.27                | 1.82E-11 |                     |          |
|                                  | <b>PA1660</b> | <b>HsiG2</b> | -3.51                            | 3.93E-22         |                     |          | -2.09               | 1.50E-14 |
|                                  | <b>PA1661</b> | <b>HsiH2</b> | -3.44                            | 2.52E-21         |                     |          | -2.69               | 3.14E-20 |
|                                  | <b>PA1662</b> | <b>clpv2</b> | -2.67                            | 1.25E-14         |                     |          | -1.69               | 2.92E-08 |
|                                  | <b>PA1663</b> | <b>sfa2</b>  | -3.05                            | 2.43E-17         |                     |          | -2.16               | 1.8E-13  |
|                                  | <b>PA1665</b> | <b>HsiH2</b> | -3.32                            | 7.74E-20         |                     |          | -2.28               | 1.29E-15 |
|                                  | <b>PA1666</b> | <b>lip2</b>  | -4.05                            | 2.15E-14         |                     |          | -1.84               | 5.81E-06 |
|                                  | <b>PA1668</b> | <b>DotU2</b> | -2.33                            | 5.48E-11         |                     |          | -2.35               | 4.63E-14 |
|                                  | <b>PA1669</b> | <b>lcmF2</b> | -2.35                            | 1.01E-17         |                     |          | -1.66               | 1.13E-10 |
|                                  | <b>PA1670</b> | <b>stp1</b>  | -2.99                            | 2.89E-17         |                     |          | -2.18               | 8.46E-14 |
|                                  | PA1671        | stk1         | -3.19                            | 4.71E-15         |                     |          | -2.30               | 2.10E-12 |

<sup>a</sup> logarithm of fold change ratio

<sup>b</sup> false discovery rate

### Activity against biofilms

Biofilms are tolerant to antibiotic treatments, as they oppose a barrier to the penetration of antibiotics and they host metabolically less active bacteria. Noteworthy, the matrix of biofilms of Gram-negative bacteria are predominantly composed of extracellular polymeric substances (EPS) that are negatively-charged, allowing association with divalent cations to improve biofilm stability [201]. A similar type of bridging is described between  $Mg^{2+}$  and LPS. Based on our observation that **NV716** can displace  $Mg^{2+}$  from LPS, we speculated that it might also impact antibiotic activity against biofilms. We therefore examined the activity of antibiotics alone or combined with **NV716** or the other potentiators against biofilms made by PAO1 and PAO509 (Figure 5). Considering first the effects on metabolic activity, **NV716**, and to some extent, alexidine were the only potentiators capable of reducing the fluorescence signal emitted by fluorescein, the metabolite of fluorescein diacetate produced by metabolically active bacteria (left panels). This effect can be interpreted as denoting a reduction in the number of viable bacteria rather than in the intrinsic metabolic activity of bacteria, as **NV716** did not decrease, but rather increased, fluorescein diacetate metabolization in planktonic cultures, probably as a consequence of an increase uptake of the dye in the cells (Figure S10). The other potentiators also improved antibiotic activity at 1 x MIC with the noticeable exception of PA $\beta$ N. Changes in CFU counts were in general parallel to those in metabolic activity (middle panels). None of the potentiators was capable of reducing biofilm biomass when used alone (right panels) but **NV716**, PMBN, and colistin improved the effects of rifampicin against PAO509 biomass and of ciprofloxacin against PAO1 biomass.

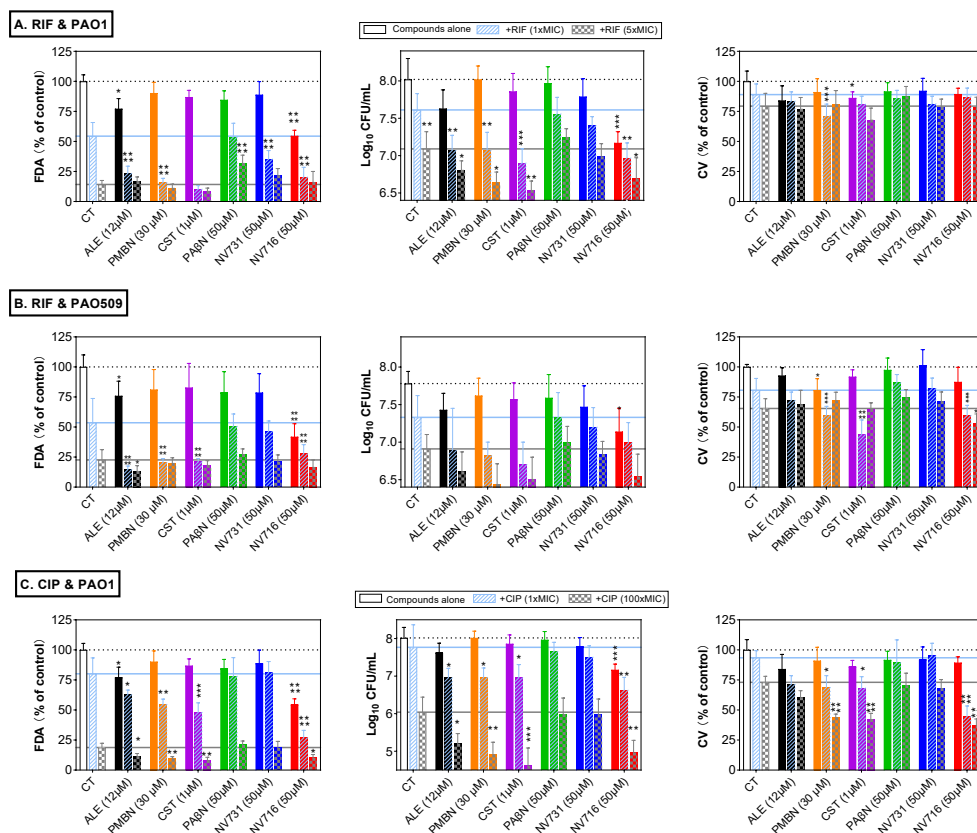


**Figure 5.** Effect of potentiators on the activity of rifampicin against biofilms of PAO1 (A) and PAO509 (B) and of ciprofloxacin against biofilms of PAO1 (C). Pre-formed biofilms were exposed during 24 h to rifampicin alone at 1 x MIC or 5 x MIC or to ciprofloxacin alone at 1 x MIC or 100 x MIC, the potentiators alone (ALE: alexidine; PMBN: polymyxin B nonapeptide; CST: colistin) at fixed concentrations, or their combination (hatched bars: + RIF or CIP 1 x MIC; checkerboard bars: + RIF 5 x MIC or CIP at 100 x MIC). Metabolic activity and biomass were quantified using fluorescein diacetate assay (FDA) and crystal violet (CV) staining, respectively. All data are mean  $\pm$  SEM (triplicates from three independent experiments). In each graph, the black horizontal dotted line is the value for the control (non treated biofilm), the blue line, the value for the biofilm exposed to the antibiotic alone at 1 x MIC, and the grey line, for the biofilm exposed to RIF at 5 x MIC or CIP at 100 x MIC, respectively. Statistical analysis: comparison of potentiators alone to control biofilm or to potentiator combined with rifampicin to rifampicin alone (at the same concentration) by one-way ANOVA with Dunnett's post-hoc test: \*,  $p < 0.05$ ; \*\*,  $p < 0.01$ ; \*\*\*,  $p < 0.001$ ; \*\*\*\*,  $p < 0.0001$ .

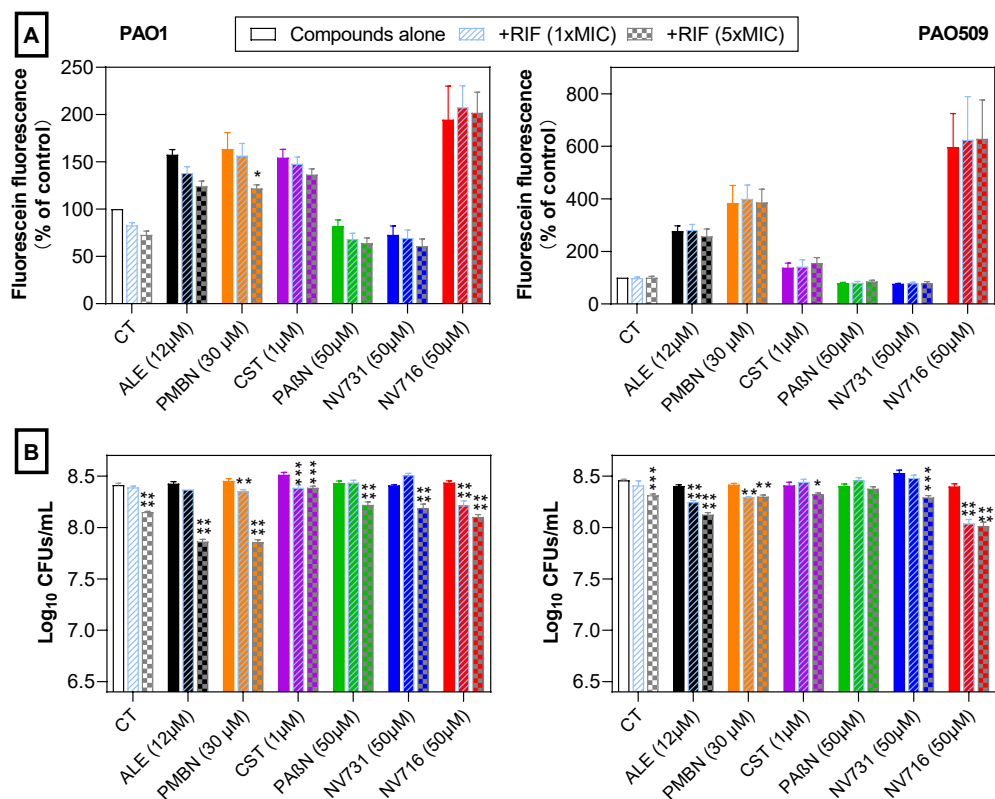
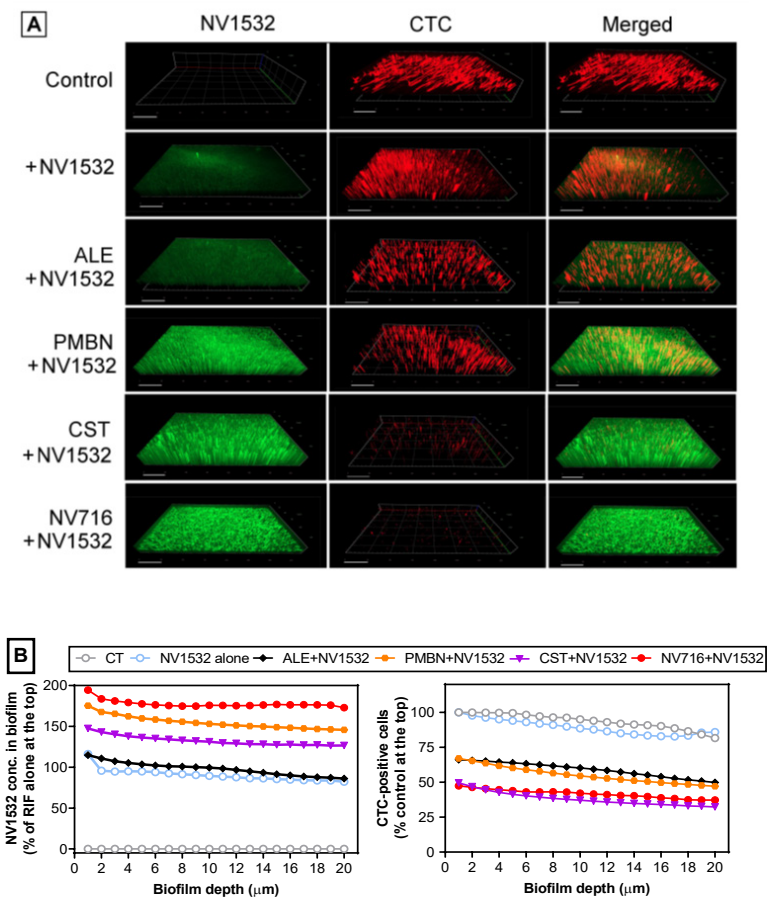


**Figure S10. (A)** Influence of potentiators on metabolic activity (fluorescein diacetate metabolization) in planktonic cultures of PAO1 and PAO509, in the same conditions as those described for the biofilm assay (Figure 5, incubation during 15 min with potentiators alone, rifampicin alone at 1 x or 5 x MIC, and their combination). **(B)** CFUs count in the same conditions. All data are mean  $\pm$  SEM (triplicates from three independent experiments). Statistical analysis: two-way ANOVA with Tukey post-hoc test: \*\*\*\*,  $p < 0.0001$ ; \*\*\*,  $p < 0.001$ ; \*\*,  $p < 0.01$ .

**Results:** An increase in fluorescence signal is observed for agents causing a permeabilization of the outer membrane, suggesting it is related to an increased uptake of the dye inside bacteria. These increase in fluorescence occurs without increase in bacterial counts (rather a decrease when bacteria are exposed to rifampin at 5 x MIC alone or in combination).

Biofilms of PAO1 were then observed in confocal microscopy to visualize the penetration of **NV1532** (fluorescent derivative of rifampicin) in parallel with that of live cells (Figure 6, panels A and B). Colistin, PMBN, and **NV716** (in that order) increased the penetration of **NV1532** and the four potentiators tested increased its effect on viability. When combined with ciprofloxacin (panels C and D), all potentiators allowed to observe a marked increase in the proportion of dead cells, **NV716** being the more effective. These data are coherent with quantitative data from Figure 5.



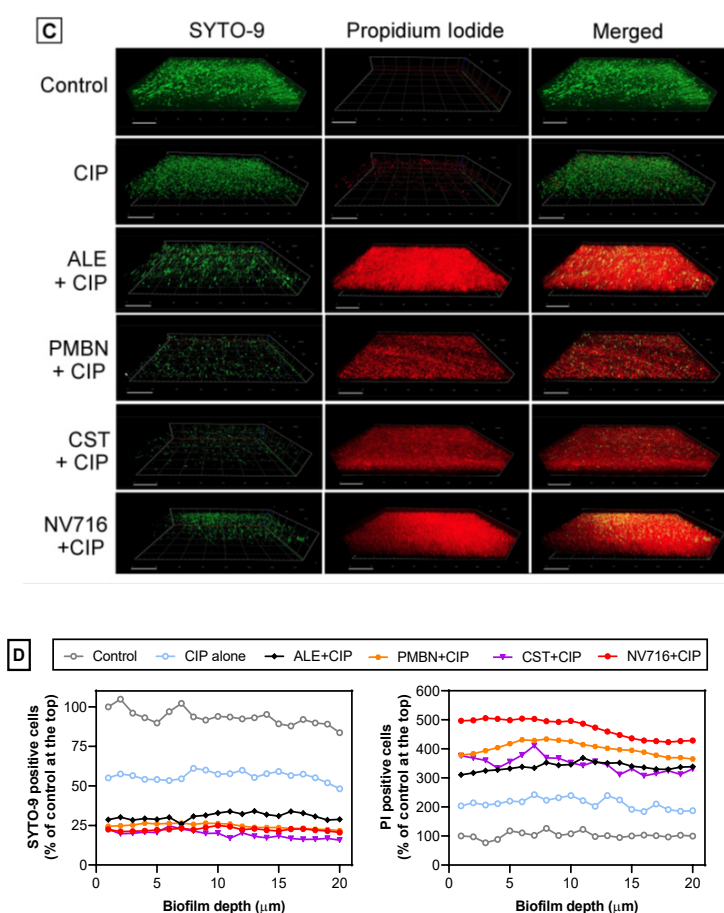


**Figure 6.** confocal images of biofilms of PAO1 exposed to antibiotics and potentiators in the conditions described in Figure 5 (ALE: alexidine 12  $\mu$ M; PMBN: polymyxin B nonapeptide 30  $\mu$ M; CST: colistin 1  $\mu$ M; **NV716** 50  $\mu$ M). **(A)** Biofilms incubated with NV1532 (fluorescent derivative of rifampicin) at 100 mg/L alone or with potentiators, with fluorescence of NV1532 recorded in the green channel (left) and fluorescence of CTC in the red channel (middle). The right panels show the merged images. **(B)** quantitative analysis of the data, showing the fluorescence of NV1532 (left) or of CTC (right) in percentage of the value measured at the top of the biofilm. **(C)** Biofilms incubated with ciprofloxacin at 50 mg/L alone or with potentiators, with fluorescence of SYTO-9 recorded in the green channel (left) and fluorescence of propidium iodide (PI) in the red channel (right). The right panel images show the merged images. **(D)** quantitative analysis of the data, showing the fluorescence of SYTO-9 (left) or of PI (right) in percentage of the value measured at the top of the biofilm.

## Discussion

The present study demonstrates that the polyaminoisoprenyl compound **NV716** is a potent potentiator of poorly permeable antibiotics in *P. aeruginosa*. At subMIC concentration (0.1-fold MIC), it proves capable of markedly decreasing the MIC of several of these antibiotics. This effect is at least partially independent of its previously demonstrated capacity to inhibit the activity of efflux transporters [353], as it is also efficient against strains that do not express efflux pumps or for antibiotics that are not substrates for efflux. Importantly, **NV716** does not easily select for resistance, essentially causing reversible transcriptomic changes after prolonged exposure; it also prevents selection of resistance to antibiotics, which are clear advantages for a molecule directed towards a multidrug-resistant pathogen like *P. aeruginosa*. Lastly, its potentiator effect is observed in models of difficult-to-treat infections like intracellular infection [398] or biofilms, which broadens the spectrum of infections where it could prove useful. Beside these potentiator effects on antibiotic activity, **NV716** also decreases the expression of quorum-sensing, with subsequent reduction in virulence, biofilm formation, and motility. Our current understanding of its mode of action is depicted in Figure 7.

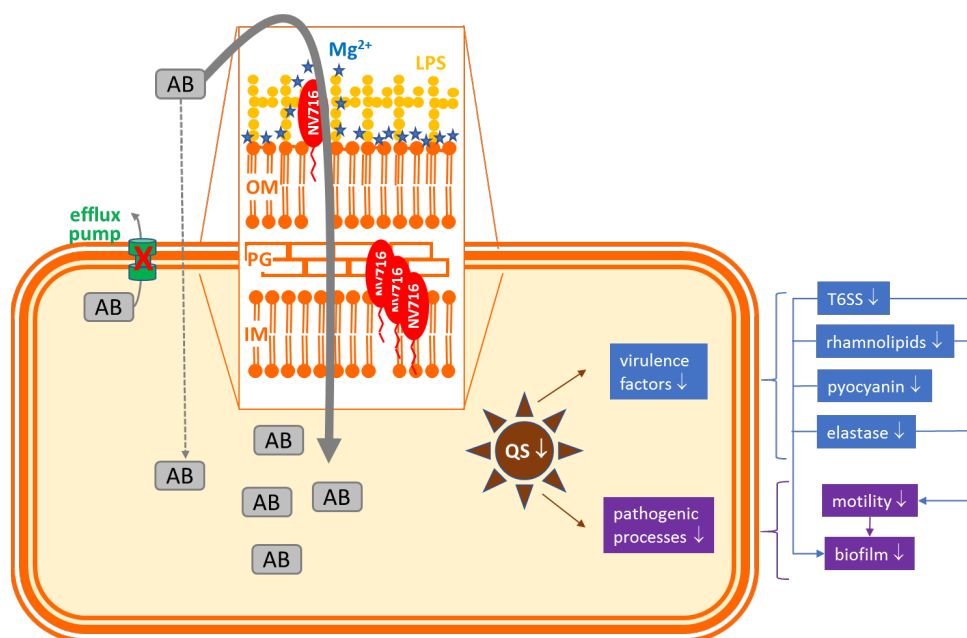


**Figure 7.** Model describing the putative mode of action of NV716. At subMIC concentration, NV716 interacts with LPS and increases the permeability of the outer membrane of *P. aeruginosa*, while at the same time inhibiting the activity of efflux transporters by an unknown mechanism [353], which facilitates the penetration of antibiotics. It also reduces the expression of quorum sensing-related genes, causing a downregulation of virulence factors like rhamnolipids, pyocyanin, elastase, and of T6SS-related genes, as well as a subsequent reduction in motility and biofilm formation. At its MIC, it also permeabilizes the inner membrane of the bacteria, leading to their death without gross alteration of the bacterial morphological integrity.

Intriguingly, its mechanism of action shares some similarities but also notable differences with that of colistin, as well as with that of alexidine and PMBN. **NV716** shares with alexidine the highest potency to bind to LPS and permeabilize the outer membrane, but, in contrast to **NV716**, alexidine has only minor effects on antibiotics MIC and uptake inside bacteria. The 3D molecular model of alexidine and **NV716** suggests a similar, extended conformation of their positively charged moiety, which may explain this privileged interaction with LPS (see Figure S1B). Conversely, PMBN shows similar effects as **NV716** on MICs but less on displacement of bodipy-cadaverine from LPS and

on the accumulation of antibiotics that are substrates for efflux. PMBN and alexidine differ in their mode of interaction with LPS. While the small lipophilic tail of PMBN is described as critical for its interaction with the outer membrane [427], one of the guanidinium groups in alexidine associates with the negatively-charged phosphate group on lipid A, while the other symmetrical end of the molecule is closely associated with the phosphate group of the core portion of LPS [343]. This therefore suggests that an interaction with the lipophilic core of the membrane, which does not occur with alexidine, is needed to allow antibiotic permeation. Conversely, colistin, with a bulky charged cyclic peptide and a short lipophilic tail, is as potent as **NV716** in terms of outer membrane and inner membrane permeation, the first being obtained at equimolar concentrations, and the latter, at their respective MIC. However, **NV716** can increase antibiotic accumulation at subMIC concentrations (i.e. when it permeabilizes only the outer membrane), which was not the case for colistin. Studies on outer membrane models, planar bilayers, or using molecular modeling suggest that colistin interacts with the headgroups of the lipids in the outer membrane, and causes their reorientation and local disorganization as well as the formation of LPS clusters, which promote outer membrane destabilization and gives access to colistin or other antibiotics into the periplasmic space [428-430]. Our electron microscopic images suggest that the permeation effects of **NV716** occur without gross disruption of the bacterial envelope, which is coherent with the fact they occur at subMIC concentrations. Our study was not powered to perform in-depth structure-activity relationships, but encourages further efforts with homogeneous series of compounds in order to better understand these interactions at the molecular level. In this context, the comparison of the physicochemical properties of the potentiators used in this study is instructive (Figure S1C). On the one hand, alexidine and **NV716** show similar calculated logD values at pH 7.4, as well as similar van der Waals surface and volume, which may therefore be determinant in their capacity to interact with LPS. On the other hand, NV716 and NV731 are the only molecules showing a calculated polar surface area (PSA; defined as the surface sum over all polar atoms or molecules, primarily oxygen and nitrogen) smaller than 140 Å<sup>2</sup>.

This parameter is a commonly-used medicinal chemistry metric to optimize the ability of a drug to cross biological barriers. A value higher than this threshold predicts a poor permeation, at least across the blood-brain barrier [431]. Whether it also applies to bacterial cells is unknown, but this exclusive property may contribute to explain the unique combination of actions shown for **NV716** as compared to other potentiators. In addition, the strong difference in the spatial arrangement of the two polyaminoisoprenyl compounds (Figure S1B), globular for **NV731** (only two nitrogen groups able to interact at the same time with the negatively-charged groups of the membrane) but linear for the spermine moiety of **NV716** (all aminogroups capable of interaction with the negative charges) could explain the much higher potency of **NV716** in all our assays.

The global similarity in the targets of **NV716**, colistin, and alexidine is confirmed by our transcriptomic analysis, which shows that a transient resistance to these molecules can be achieved by an upregulation of the metabolic pathways involved in the synthesis of lipid A and of LPS. These pathways are known to be affected in stable mutants resistant to polymyxins [432, 433]. Interestingly enough, the present work also revealed downregulation of quorum-sensing related genes and functions that were phenotypically confirmed by a reduction in the secreted amounts rhamnolipids, pyocyanin or elastase, of biofilm formation and of motility, all under the control of the three closely interconnected Rhl, Las, and PQS systems [238]. The downregulated virulence factors themselves also contribute to reduce biofilm formation, notably by impairing motility for rhamnolipids as well as for T6SS, by activating the nucleoside diphosphate kinase for elastase, or by favoring the release of extracellular DNA for pyocyanin [238, 239].

Other types of membrane-active agents have been described in the literature as potentially useful alternatives for the treatment of Gram-negative multidrug-resistant bacteria. These include notably a series of amphipathic antimicrobial peptides (see [434-436] for a few recent examples). We notice however some drawbacks for these peptides as compared to **NV716**. First, their peptidic nature makes them more expensive to

produce or to isolate from natural sources and confers to them unfavorable pharmacokinetic properties [437]. Second, many of them are hemolytic or cytotoxic, sensitive to hydrolysis by proteases, or inactivated by salt or serum [437]. Third, their proteic nature raises concern regarding a potential immunogenicity [438]. Fourth, they usually act as antibiotics rather than as potentiators, which is more likely to lead to resistance [439]. This risk has been demonstrated for the AA139 derivative of the natural arenicin-3, which selects for mutations in *mlaC*, a phospholipid transport gene [436], or for polymyxins, which induce modifications in LPS or lipid A [432]. Non-peptidic membrane-permeabilizing antibiotics have also been developed, which do not fully alleviate the risk of resistance development or of cytotoxicity, as shown for 1-((2,4-dichlorophenethyl)amino)-3-phenoxypropan-2-ol (SPI009) or amphiphilic derivatives of aminoglycosides [390, 440]. Likewise, many membrane-active compounds isolated from plants, like triterpenes, lack of selectivity and are cytotoxic [441].

Although we did not uncover the precise molecular target of **NV716** in *P. aeruginosa*, we nevertheless made significant progress in the elucidation of the physiological processes it can affect in bacteria as well as in the demonstration of their consequences for antibiotic activity and bacterial virulence. The promising profile of activity we evidenced here already at sub-inhibitory concentrations, coupled with its previously demonstrated lack of toxicity in vitro at microbiologically-active concentrations [353, 398], encourages further research in pertinent in-vivo models of infections in order to better delineate its potential as an adjuvant to current antibiotics. In a broader context, this work confirms that membrane-targeting approaches are promising strategies that can revive antibiotic activity, including against persistent forms of infections like biofilms or intracellular survival.

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### **Author contributions**

W.G. designed and performed the experiments, analyzed the data and wrote the manuscript. J.M.Br. synthesized NV716, NV731, and NV1532, calculated the structural properties of potentiators, and supervised the work. M.P. performed the RNAseq analysis; N.M., confocal microscopy experiments; S.D.W., DNaseq analysis, and P.B., electron microscopy studies. S.H. supervised RNAseq and DNaseq analyses and the writing of this part of the manuscript. J.M.Bo supervised the work. F.V.B. designed the study, supervised the work, and wrote the manuscript. All the authors contributed to the writing of the manuscript and approved the submitted version.

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### **Competing interests**

The authors have no competing interests to declare.



**3. 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 that can improve antibiotic activity against difficult-to-treat Gram-negative bacteria like *Escherichia coli*, *Klebsiella pneumoniae* or *Acinetobacter baumannii* are an appealing strategy in view of the paucity of therapeutic alternatives in case of multidrug resistance. Here, we study 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 the underlying mechanism of action. **NV716** considerably reduced the MICs of these antibiotics (2 to 9 doubling dilutions), the highest synergy being observed with the more lipophilic drugs. This potentiation was related to a strong interaction of **NV716** with LPS and ensuing permeabilization of the outer membrane, leading to an increased accumulation of the antibiotics inside bacteria. In addition, **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 is evidenced towards eukaryotic cells. Altogether, these data highlight the potential interest of **NV716** as an adjuvant against these Gram-negative pathogens put in the priority list of WHO for search of new therapies.

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

## Introduction

The rapid emergence of antibiotic resistance has become a problem of global concern [442]. The world health organization (WHO) published a list of bacteria for which new antibiotics are urgently needed, among which *Acinetobacter baumannii* and various Enterobacteriaceae, like *Klebsiella* spp. and *Escherichia coli* [443].

These species display high levels of resistance to a broad range of antimicrobials via the acquisition of a series of resistance mechanisms [444, 445]. Moreover, they also show intrinsic resistance to many antibiotics, due either to the barrier effect of their envelope or to the presence of broad-spectrum efflux pumps in their membrane. The envelope of Gram-negative bacteria comprises an inner membrane (IM) and an outer membrane (OM) separated by a periplasmic space containing the cell wall made of peptidoglycan layers [78]. The OM is an asymmetric bilayer, containing phospholipids in the inner leaflet and lipopolysaccharides (LPS) in the outer leaflet [446]. This asymmetric character confers to the OM a role of selective barrier that protects bacterial cells from cytotoxic molecules, including lipophilic and/or large size antibiotics (rifampicin, macrolides and glycopeptides) [104, 447].

Additionally, these Gram-negative bacteria also constitutively express efflux systems that expel unrelated classes of antibiotics out of the bacteria, reducing thereby the amount of drug that can reach intracellular targets [448]. These efflux pumps consist of three proteins, namely an efflux transporter located in the IM, an OM channel protein, and a fusion protein connecting the two transmembrane proteins to allow the transport of the antibiotic substrates from the cytosol or the inner membrane directly to the extracellular environment. Typical examples include AcrAB-TolC in *E. coli* and *K. pneumoniae* or AdeABC in *A. baumannii*, which can extrude structurally distinct classes of antibiotics such as quinolones, tetracyclines, chloramphenicol, macrolides, linezolid, fusidic acid, or novobiocin [275].

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

In addition to resistance, intracellular survival and biofilm formation also contribute to antibiotic treatment failure [449, 450]. Although considered essentially as extracellular pathogens, *E. coli*, *K. pneumoniae* and *A. baumannii* may also behave as opportunistic intracellular organisms. Their survival has been documented in phagocytic cells in-vitro [174, 451, 452] as well as in alveolar macrophages of infected mice in-vivo [453-455]. In intracellular niches, bacteria are both less accessible and less responsive to antibiotics. Our team recently documented for *Staphylococcus aureus* that poor responsiveness of bacteria surviving to antibiotics intracellularly is due to their switch to a persister phenotype [199]. Persisters are subpopulations of otherwise antibiotic-susceptible bacteria that show a transient non-dividing phenotype under stressful conditions and can survive to high concentrations of antibiotics [138], contributing to failure to eradicate the infection.

These three Gram-negative bacterial species can also adhere to biological and artificial surfaces to form biofilms [456, 457]. Biofilms are defined as microbial community of cells living in a self-produced matrix essentially made of polysaccharides, extracellular DNA (eDNA), and proteins [458]. These structures protect microorganisms from host defenses and antibiotics due to the barrier effect of the extracellular matrix and to the alteration of the metabolic activity of bacteria, which adopt dormant phenotypes poorly responsive to antibiotics [459].

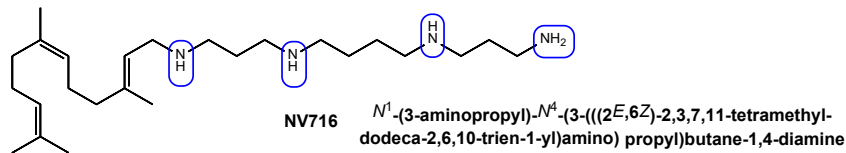
In a previous work, we showed that the polyaminoisoprenyl compound **NV716** (see chemical structure in Figure S1) was capable to restore the activity of a series of disused antibiotics (rifampicin, doxycycline and chloramphenicol) against *Pseudomonas*

*aeruginosa* both extracellularly and intracellularly [398], by increasing the permeability of the outer membrane, inhibiting efflux, and also improving the efficacy of bactericidal drugs against persisters [353, 398, 460] at concentrations that are not toxic for eukaryotic cells. The aim of the present study was (a) to extend the demonstration of the potential interest of this compound as potentiator of a series of disused antibiotics against planktonic, intracellular, or biofilm infections by other problematic Gram-negative pathogens like *A. baumannii*, *E. coli* and *K. pneumoniae*, in comparison with **NV731** (less active derivative against *P. aeruginosa*) and the well-characterized efflux inhibitor PA $\beta$ N (Phenylalanine-Arginine  $\beta$ -Naphthylamide) [286, 461, 462] and (b) to evaluate its effects on the bacterial membranes of these bacteria.

We adapted to these species an in-vitro pharmacodynamic model of THP-1 human monocytes infected by *Pseudomonas aeruginosa* [188] that allows comparing key pharmacodynamic descriptors of antibiotic intracellular activity, namely their relative potency (static concentration,  $C_s$ ) and maximal efficacy ( $E_{max}$ ), as well as an in-vitro model of biofilm growing in 96-well plates [188]. As disused antibiotics, we selected rifampicin (not substrate for efflux), doxycycline, chloramphenicol as in our previous work with *P. aeruginosa*, and added antibiotics the spectrum of which is limited to Gram-positive bacteria due to poor penetration in Gram-negative organisms, like azithromycin, linezolid, fusidic acid and novobiocin (all substrates for efflux; see Table S1). Ciprofloxacin was used as a control of active drug against intracellular *P. aeruginosa* [398]. As positive controls for mechanistic studies, we included colistin and alexidine (see Figure S1 for the structure of these compounds). Colistin binds to the lipid A component of the negatively-charged LPS molecules via electrostatic interactions, by displacing the  $Mg^{2+}$  and  $Ca^{2+}$  ions that bridge and stabilize the LPS monolayer. It perturbs thereby the OM permeability, transits through it via a self-promoted uptake mechanism, and subsequently inserts itself and disrupts the physical integrity of the phospholipid bilayer of the IM via membrane thinning [296]. Alexidine is an amphipathic biguanide antiseptic showing fast bactericidal activity thanks to its rapid ability to

permeabilize the bacterial membranes [345]. As colistin, it establishes electrostatic interactions with the negatively-charged phosphate groups present on LPS, displacing  $Mg^{2+}$  ions from their binding to LPS and perturbing the stabilizing effect afforded by  $Mg^{2+}$  cross-bridging of adjacent LPS molecules [343]. In addition, it also causes the leakage of the cytoplasmic content by inducing the formation of lipid domains in the IM [347].

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



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

| Antibiotics     | Resistance by efflux                    |                                                               | LogD values at pH 7.4<br>(octanol:water partition coefficient at pH 7.4);<br>calculated using LogD Predictor<br>( <a href="https://disco.chemaxon.com/calculators/demo/plugins/logd/">https://disco.chemaxon.com/calculators/demo/plugins/logd/</a> ) | Accumulation level in eukaryotic cells (at equilibrium) [168] | C <sub>min</sub> / C <sub>max</sub> in human serum (mg/L) [271-274] |
|-----------------|-----------------------------------------|---------------------------------------------------------------|-------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|---------------------------------------------------------------|---------------------------------------------------------------------|
|                 | Pumps shown to transport the drug [275] | Bacterial species [275, 276]                                  |                                                                                                                                                                                                                                                       |                                                               |                                                                     |
| Rifampicin      | Poor substrate for efflux               | NA                                                            | 2.98                                                                                                                                                                                                                                                  | 1-4                                                           | 0.1-10                                                              |
| Azithromycin    | AcrAB-TolC, AdeABC                      | <i>E. coli</i> , <i>K. pneumoniae</i> and <i>A. baumannii</i> | -1.50                                                                                                                                                                                                                                                 | 40-300                                                        | 0.04-1                                                              |
| Linezolid       | AcrAB-TolC                              | <i>E. coli</i>                                                | 0.64                                                                                                                                                                                                                                                  | 1                                                             | 2.5-15                                                              |
| Fusidic acid    | AcrAB-TolC, AdeABC                      | <i>E. coli</i> and <i>A. baumannii</i>                        | 1.50                                                                                                                                                                                                                                                  | ND                                                            | 30-200                                                              |
| Novobiocin      | AcrAB-TolC                              | <i>E. coli</i>                                                | 1.76                                                                                                                                                                                                                                                  | ND                                                            | 15-60                                                               |
| Chloramphenicol | AcrAB-TolC, AdeABC                      | <i>E. coli</i> , <i>K. pneumoniae</i> and <i>A. baumannii</i> | 0.87                                                                                                                                                                                                                                                  | 2-5                                                           | 5-15                                                                |
| Doxycycline     | AcrAB-TolC, AdeABC                      | <i>E. coli</i> , <i>K. pneumoniae</i> and <i>A. baumannii</i> | -3.48                                                                                                                                                                                                                                                 | 2-10                                                          | 1-10                                                                |
| Ciprofloxacin   | AcrAB-TolC, AdeABC                      | <i>E. coli</i> , <i>K. pneumoniae</i> and <i>A. baumannii</i> | -0.86                                                                                                                                                                                                                                                 | 4-10                                                          | 0.05-5                                                              |

*LogD values of the potentiators*

|               | NV716 | NV731 | PABN |
|---------------|-------|-------|------|
| LogD (pH 7.4) | -4.2  | -6.3  | -1.2 |

## Materials and Methods

### Antibiotics and potentiators

Colistin (potency, 73%), chloramphenicol (potency, 98%), doxycycline (potency, 98%), fusidic acid (potency, 98%), novobiocin (potency, 98%) and rifampicin (potency, 98%) were obtained as microbiological standards from Sigma-Aldrich (St Louis, MO), ciprofloxacin HCl (potency, 89%) from Bayer (Leverkusen, Germany), gentamicin sulfate (potency, 60.7%), from PnReac AppliChem (Darmstadt, Germany), linezolid (potency, 100%) from Rib-X Pharmaceuticals (presently Melinta Therapeutics, New Haven, CT), and azithromycin (potency, 100%) from Teva (Petach Tikva, Israel). Alexidine (potency, 98%) and the reference efflux pump inhibitor Phe-Arg- $\beta$ -Naphthylamide (PA $\beta$ N; potency, 98%) were purchased from Sigma-Aldrich. **NV716** and **NV731**, were synthesized at Aix Marseille University [384].

### Bacteria and culture media

For each species, one (*E. coli* ATCC 47076, *A. baumannii* ATCC 19606) or two (*K. pneumoniae* ATCC 700603 and ATCC 43816) reference strains were obtained from the American Type Culture Collection (Manassas, VA, USA) and two clinical isolates (*E. coli* 15 and 51, *K. pneumoniae* 58 and 74, and *A. baumannii* 112 and 113) were provided by Johan W. Mouton, Department of Medical Microbiology and Infectious Diseases, Erasmus MC, Rotterdam, The Netherlands [130, 463, 464]. All isolates harbored CTX-M-, SHV-, OXA-, or TEM-type extended-spectrum  $\beta$ -lactamases (Table S2)

**Table S2.** Beta-lactamases identified in the clinical isolates used in the study.

| Strains                 | beta-lactamases                                      | References      |
|-------------------------|------------------------------------------------------|-----------------|
| <i>E. coli</i> 15       | CTX-M 15                                             |                 |
| <i>E. coli</i> 51       | OXA-1, CTX-M 15                                      |                 |
| <i>K. pneumoniae</i> 58 | TEM-84, SHV-11                                       | [130, 463, 464] |
| <i>K. pneumoniae</i> 74 | CTX-M 1                                              |                 |
| <i>A. baumannii</i> 112 | OXA-23-24-like (plasmid) + OXA-51-like (chromosomal) |                 |
| <i>A. baumannii</i> 113 | OXA-23-24-like (plasmid) + OXA-51-like (chromosomal) |                 |

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

### **Susceptibility Testing**

MICs were determined by serial 2-fold microdilution in CA-MHB according to CLSI guidelines in control conditions or in the presence of 38 µM PAβN [20 mg/L] and **NV731** [12 mg/L] or 10 µM **NV716** [4 mg/L]. MICs of antibiotics alone and in combination were used to calculate the fractional inhibitory concentration (FIC) index, as follows:  $FIC = C_A/MIC_A + C_B/MIC_B$ , where  $MIC_A$  and  $MIC_B$  are the MICs of compounds A and B alone, and  $C_A$  and  $C_B$  are the MIC of compounds A or B in combination. The combination was considered as synergistic or antagonistic when the FIC index was  $< 0.5$  or  $> 4$ , respectively [402].

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

Binding to lipopolysaccharides (LPS) of bacteria was investigated by using BODIPY<sup>TM</sup>-TR-cadaverine displacement assay [465]. BODIPY-TR- cadaverine (BC) can bind to the lipid A region of the LPS. The ability of compounds to interact with LPS was evaluated by measuring the displacement of the BC as previously described [403]. Ten milliliters of bacterial suspension ( $OD_{620nm}$  of 0.1 in Tris buffer saline, 50 mM, pH 7.4) containing 5µM BODIPY<sup>TM</sup>-TR-cadaverine were incubated in the dark for 30 min. Then 50 µL of this suspension were mixed with 50 µL of potentiators in a 96-wells black plate and kept in the dark for 30 min. The fluorescent intensity was then measured in a Spectramax M3 plate reader (Molecular Devices LLC, Sunnyvale, CA) at  $\lambda_{ex}$  580nm and  $\lambda_{em}$  620nm.

Alexidine at 50  $\mu$ M was used as positive control and samples added by Tris buffer saline as negative control (100%).

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

Outer membrane permeability was assessed using nitrocefin, a non-permeant  $\beta$ -lactam which is converted by  $\beta$ -lactamases in a colored derivative in the periplasmic space of permeabilized bacteria [466]. Overnight cultures were incubated with 0.25  $\mu$ g/mL of imipenem for 1 h to induce  $\beta$ -lactamase expression [467]. After centrifugation at 3000 *g* for 7 min, cells were washed twice with 1x PBS and adjusted to obtain an OD<sub>620nm</sub> of 0.5. One hundred  $\mu$ L of cell suspension were mixed with 50  $\mu$ L of potentiator in 96-well plate, after which 50  $\mu$ L of nitrocefin (final concentration of 20  $\mu$ g/mL) were added. Absorbance at 490 nm was monitored over 60 min with an interval of 5 min using a Spectramax M3 plate reader.

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

The effect of potentiators on the integrity of the inner membrane was determined by using a membrane-impermeable fluorescent dye propidium iodide (PI) as previously described [404]. A stock solution of PI (10 mM DMSO) was diluted 1000 times with the bacterial suspension (OD<sub>620nm</sub> 0.1 in BET buffer). 100  $\mu$ L potentiator (in BET) were added to 100  $\mu$ L PI-containing bacterial suspension in 96-well plates. The fluorescence intensity was measured using a Spectramax M3 plate reader ( $\lambda_{ex}$ , 540nm;  $\lambda_{em}$ , 610nm) after 1 h of incubation at room temperature. Alexidine at 50  $\mu$ M was used as positive control and imipenem as negative control [404].

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

The inner membrane depolarization was evaluated by using DiSC3(5) as previously described [353]. Overnight cultures were pelleted by centrifugation (3000 *g* for 7 min), washed (1x PBS) and resuspended with 50 mM Tris buffer saline (pH 7.4) containing 2

mM EDTA. After 5 min of incubation, cells were pelleted (3000 *g* for 7 min), washed (1x PBS), resuspended with Tris buffer saline containing 50 mM glucose and adjusted to obtain an OD<sub>620nm</sub> of 0.5 after which 3, 3-dipropylthiadicarbocyanine iodide (DiSC3(5)) (final concentration of 10  $\mu$ M) was added. 100  $\mu$ L of potentiator were mixed with 100  $\mu$ L bacteria suspension in a 96-well plate. After 30 min of incubation, the fluorescent intensity was measured in a Spectramax M3 plate reader at  $\lambda_{ex}$  622nm and  $\lambda_{em}$  670nm. 0.5% SDS was used as a positive control and cells added by Tris buffer saline as negative control [384].

### **Inhibition of efflux**

The efflux activity in bacteria was assessed using the substrate 1,2'-dinaphthylamine as previously described [468]. Ten milliliters of overnight culture were pelleted by centrifugation with 3000 *g* for 7 min, washed once with 1 x PBS and then incubated with 1,2'- dinaphthylamine (TCI-Europe SA, Zwijndrecht, Belgium) at a final concentration of 32  $\mu$ M, and CCCP at a final concentration of 5  $\mu$ M, after which, cells were centrifuged at 3000 *g* for 7 min, resuspended in PBS and adjusted to an OD<sub>620nm</sub> of 0.5. 100  $\mu$ L of potentiator solution was mixed with 100  $\mu$ L bacteria suspension in a 96-well plate. The fluorescence ( $\lambda_{ex}$ , 370nm;  $\lambda_{em}$ , 420nm) was monitored each 30 sec during 620 sec using a Spectramax M3 plate reader. Active efflux was triggered by adding 5  $\mu$ L glucose (final concentration of 50 mM) at 180 sec. The maximal efflux activity (100%) was defined as the difference between the value obtained without and with glucose addition after 620 sec. Data were expressed as the ratio between the fluorescence signal recorded in control conditions or in the presence of potentiator at 620 sec. This experiment was performed only with *E. coli* and *K. pneumoniae*, preliminary experiment having shown that 1,2'-dinaphthylamine was not actively effluxed in *A. baumannii*.

### **Antibiotics accumulation in bacteria**

The concentration of ciprofloxacin and of a fluorescent analog of rifampicin (**NV1532**) inside bacteria was determined by a previously set-up fluorimetric assay [460]. In brief,

10 mL of overnight culture (approximately  $10^9$  CFU/mL) were incubated at 37°C with 10mg/L ciprofloxacin and 20mg/L of **NV1532**. After 15 min of incubation (a duration longer than the predicted time required to reach saturation [278], but during which the antibiotic did not affect bacterial growth [no significant reduction in CFUs]), 20  $\mu$ L of suspension were collected to count CFUs and the remaining cells were collected by centrifugation at 3000 *g* for 7 min at 4°C and washed three times with 1 mL cold PBS. When assaying ciprofloxacin, cells were resuspended in 0.5 mL 100 mM glycine buffer (pH 3 adjusted with HCl), and then kept in the darkroom overnight. When assaying **NV1532**, cells were resuspended in 0.5 mL PBS and then lysed by three cycles of freezing/thawing (-80°C freezer to 60°C water bath). All samples were centrifuged at 14000 *g* for 5 min, the supernatant was then collected to measure the antibiotic content at  $\lambda_{\text{ex}}$  275nm and  $\lambda_{\text{em}}$  450nm for ciprofloxacin and  $\lambda_{\text{ex}}$  470nm and  $\lambda_{\text{em}}$  525nm for **NV1532**. All data were normalized based on CFUs counts using a Spectramax M3 (Molecular devices, LLC, Sunnyvale, CA) plate reader.

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

THP-1 cells ( $7.5 \times 10^5$  cells per mL) were incubated for 24 hours in 96-well plates with the antibiotics alone or a combination of antibiotics and potentiators. The viability of cells was determined using the trypan blue exclusion test (vital colorant excluded from viable cells) as previously described [398]. Briefly, 50  $\mu$ L of trypan blue reagent was mixed with 50  $\mu$ L of cell suspension. After 10 min of incubation at 37°C, non-colored (viable) cells were counted using a Fuchs-Rosenthal counting chamber (Tiefe 0.2mm). The percentage of cytotoxicity was evaluated based on the reduction in the number of living cells according to the following formula:

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

### **Intracellular models of infection**

We adapted the protocol previously developed for *P. aeruginosa* infection in THP-1 monocytes [188](see Fig. S2 and S3 for details and model validation). All strains (opsonized 1 h with 10% human serum) were added to monocytes at a multiplicity of infection (bacteria/cell ratio) of 25 and incubated at 37°C in a 5% CO<sub>2</sub> atmosphere to allow phagocytosis. After 1 hour of incubation, cells were pelleted by centrifugation at 3000 *g* for 7 min and incubated for 1 h with 50 x MIC of gentamicin to remove non-internalized bacteria (more than 5 log<sub>10</sub> CFUs reduction, data not shown). Cells were then pelleted by centrifugation at 3000 *g* for 7 min, washed 2 times with 1 x sterile PBS and resuspended in the original volume of RPMI-1640 with 10% FBS. The cells were collected by centrifugation and washed once with PBS to eliminate extracellular bacteria and collected in 1 mL distilled water, to achieve complete lysis. Lysates were used for determining CFU counts by spreading on agar containing 2 g/L charcoal (to avoid carry-over effect, particularly in samples incubated with high antibiotic concentrations [no difference in colony counts was observed between plates supplemented or not with charcoal for samples exposed to low antibiotic concentrations, ruling out any interfering effect of charcoal in the assay]) and protein content using a commercially available kit (Bio-Rad DC Protein Assay, Bio-Rad Laboratories, Hercules, CA). The post-phagocytosis inoculum (defined as time zero) was consistently 5 to 7 x 10<sup>5</sup> CFU/mg of cell protein.

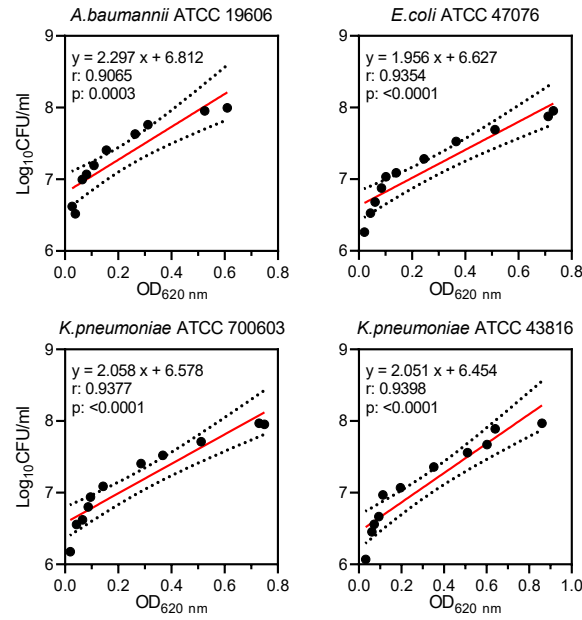


**Figure S2.** Relationship between the  $OD_{620\text{ nm}}$  and the  $\log_{10}$  CFU/mL of the 1 (*A. baumannii* and *E. coli*) or 2 (*K. pneumoniae*) reference strains suspension for the three bacterial species in CA-MHB.

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

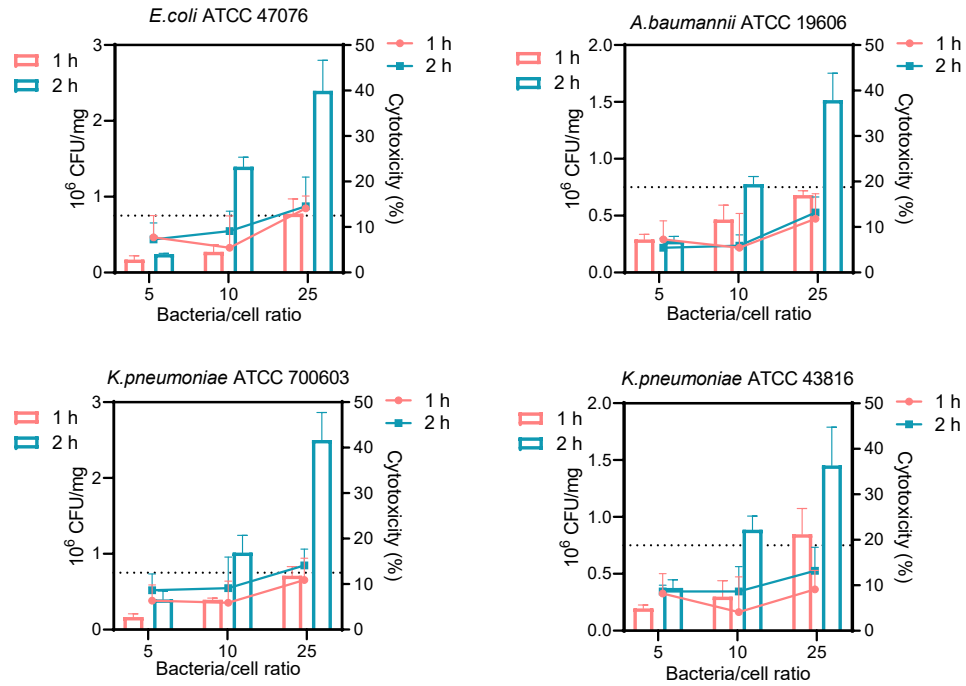


**Figure S3.** Development of the intracellular models for 1 (*A. baumannii* and *E. coli*) or 2 (*K. pneumoniae*) reference strains. The graphs show the number of CFUs normalized to the mg proteins in the samples after 1 h (open bars with red border) or 2 h (open bars with blue border) at increasing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

### **Intracellular antibiotic activity**

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

### **Persister assay**

A single colony from overnight culture of reference strains on TSA was grown in 200mL MHB-CA in a 1 L flask and incubated for 24 h with 130 rpm at 37°C. 10 mL of the bacterial suspension were then incubated with 50x MIC ciprofloxacin for 5 hours at 37°C under agitation (130 rpm), in the presence of or in the absence of NV716. To explore the effects of different treatment regimens, NV716 was added at 0, 5, or 24 h after ciprofloxacin treatment, and the cells were treated for a total of 72 h. Aliquots were serially diluted in PBS and spread on TSA supplemented with 2 g/L charcoal for CFU counting.

### **Antibiotics activity against biofilms**

Biofilms were grown in 96-well plates (VWR) as previously described [418]. In brief, one single colony was inoculated in MHB-CA overnight with 130 rpm at 37°C. A bacterial suspension was then prepared in TGN (Trypticase soy broth (VWR) supplemented with 1% glucose (Sigma) and 2% NaCl (Sigma)) using overnight cultures. 96-well plates were inoculated (200  $\mu$ L/well) at approximately  $10^7$  CFU/ml (the OD<sub>620nm</sub> of 0.05) and then

incubated at 37°C for 24 h to obtain a mature biofilm. The culture medium was removed and replaced with fresh medium (control) or medium supplemented with rifampin (1 x MIC and 5 x MIC) alone or combined with potentiators and then reincubated at 37°C for 24 h. The medium was then removed and the biofilm was washed once with 200 µl of 3-morpholinopropane-1-sulfonic acid (MOPS) buffer (20.9 g/liter of MOPS (Sigma-Aldrich), 5.6 g/liter NaCl; the pH was adjusted to 7 with NaOH) [469]. Biofilm biomass was evaluated by measuring the absorbance of crystal violet, a cationic dye that nonspecifically stains negatively charged constituents in biofilm constituents based on ionic interactions. The washed biofilms were fixed by heat at 60°C for about 24 h and incubated for 10 min at room temperature with 200 µl of 1 % (v/v) crystal violet (Sigma-Aldrich). The absorbance at 570 nm was measured using a Spectramax M3 plate reader. The metabolic activity (vitality) in the biofilms was quantified using the fluorescein diacetate (FDA) assay. It is based on the hydrolysis by living bacteria of the non-fluorescent white dye fluorescein diacetate in the yellow highly fluorescent fluorescein [418]. The washed biofilms were incubated with 100 mg/L fluorescein diacetate (Sigma-Aldrich) for 15 min at 37°C in the dark. Fluorescence was measured using a Spectramax M3 plate reader at  $\lambda_{\text{ex}}$  494 nm and  $\lambda_{\text{em}}$  518 nm.

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

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

## Results

### Antimicrobial susceptibility

Table S3 shows the MICs of antibiotics alone or combined with potentiators (20 mg/L (38  $\mu$ M) Pa $\beta$ N; 12 mg/L (38  $\mu$ M) **NV731**; 4 mg/L (10  $\mu$ M) **NV716**) against reference strains and clinical isolates of *E. coli*, *A. baumannii*, and *K. pneumoniae*. These concentrations were selected as active based on previous works, but well below the MIC of each compound [286, 398]. In control conditions, the MIC of antibiotics was generally elevated and higher than their human  $C_{max}$  (except for rifampicin against *A. baumannii*, doxycycline and ciprofloxacin against most of the strains, and chloramphenicol against most *K. pneumoniae*; see Table S1 for  $C_{max}$  values). In the presence of potentiators, MICs were globally reduced, although to a lower extent for ciprofloxacin against all strains, for azithromycin, chloramphenicol, and doxycycline against *A. baumannii*, and for fusidic acid against *K. pneumoniae*. Pa $\beta$ N and **NV731** were systematically less potent than **NV716** even though the latter was used at a lower concentration. Figure 1A illustrates these MICs data and also shows the (geometrical) mean fold decrease in MIC in the presence of potentiators. The most important reduction in this mean MIC values were noticed in combination with **NV716** for rifampicin, fusidic acid and novobiocin against *E. coli* (8-9 doubling dilutions), rifampicin and novobiocin against *K. pneumoniae* (7 doubling dilutions), and fusidic acid and novobiocin against *A. baumannii* (7-8 doubling dilution). The lowest effect (mean reduction of 1 to 2 doubling dilutions) was noticed for ciprofloxacin against all bacteria as well as for azithromycin and doxycycline against *A. baumannii*. Importantly, MIC of the combinations with **NV716** fall in the range of therapeutically-achievable concentrations in all cases (yellow zones in the graphs). FIC index calculations indicated a higher degree of synergy between antibiotics and **NV716** than the other potentiators (Figure S4), except for ciprofloxacin against *K. pneumoniae* ATCC 43816 and 74, against which ciprofloxacin alone was already very active. A highly significant correlation was observed between the reduction in MIC caused by **NV716** and the calculated logD at pH 7.4 of each antibiotic (Table S1), a

modest effect being observed for antibiotics with a negative logD value, and a progressive increase in this effect along with the lipophilicity of the drugs once their logD value becomes higher than 0 (Figure 1B).

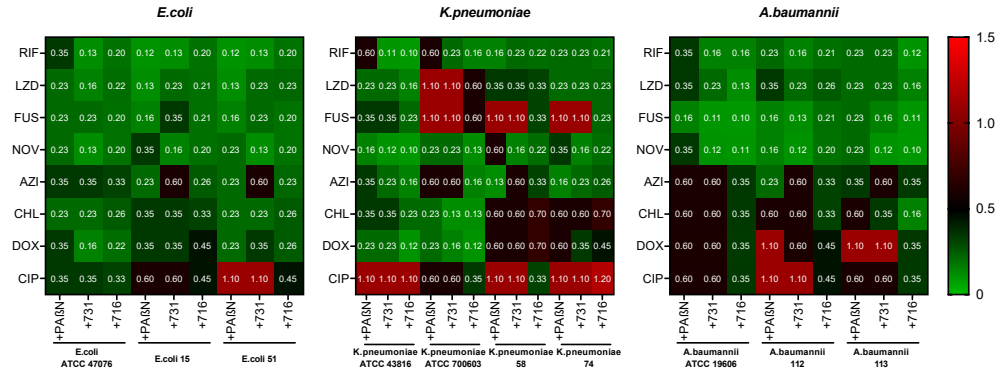


**Figure S4.** Heat maps describing interactions between antibiotics and potentiators against 1 (*E. coli* and *A. baumannii*) or 2 (*K. pneumoniae*) reference strains and 2 to clinical isolates of each species. FIC index was calculated for concentrations of 38  $\mu$ M PA $\beta$ N, 38  $\mu$ M NV731 and 10  $\mu$ M NV716. Synergy is defined as FIC < 0.5 (appearing in green on the graphs). All data are mean from at least two independent experiment

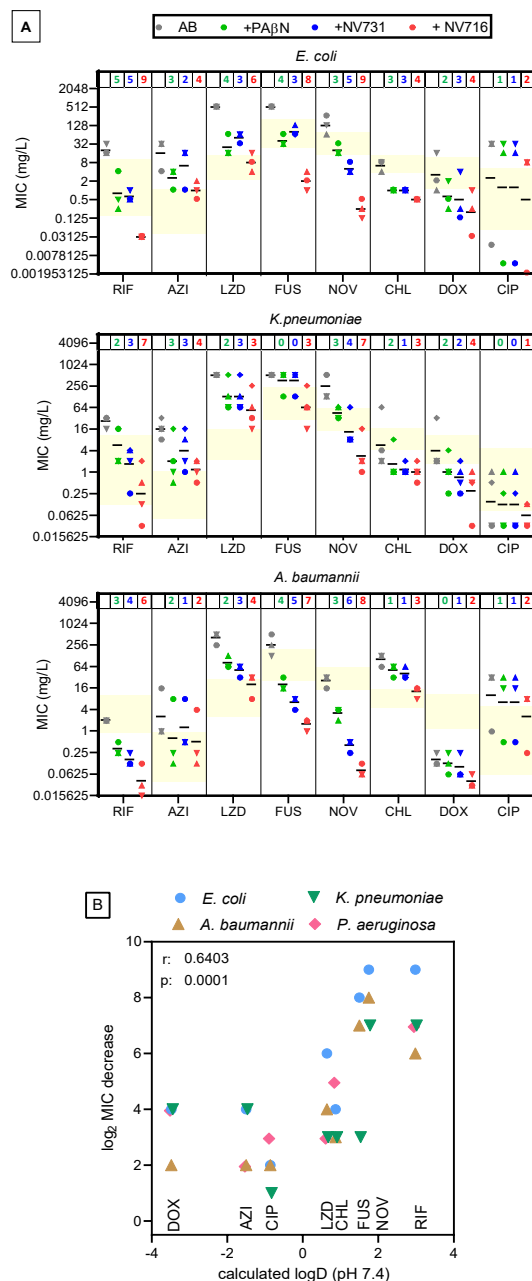


**Figure 1. A.** Activity of antibiotics alone or combined with potentiators against various Gram-negative bacteria in broth. 1 symbol/strain: 1 (*E. coli*; *A. baumannii*) or 2 (*K. pneumoniae*) reference strains and 2 to clinical isolates of each species. Grey: antibiotics alone; green: combined 38  $\mu$ M PA $\beta$ N; blue: combined 38  $\mu$ M NV731; red: combined with 10  $\mu$ M NV716. Horizontal bars: geometric mean; 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 10  $\mu$ M NV716. 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<sub>2</sub> fold decrease; geometric means) in combination with 10  $\mu$ M NV716 and the logD value of each antibiotic calculated at pH 7.4 (See Table S1). Each symbol corresponds to a bacterial species. Data previously obtained with *P. aeruginosa* PAO1 are added for completing the analysis. The Pearson's correlation coefficient *r* and the *p* value (*t*-tailed) are shown on the graph.

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

| Strains                                | Conditions    | MIC (mg/L) <sup>a</sup> |                  |               |               |               |                  |               |                 |                 |
|----------------------------------------|---------------|-------------------------|------------------|---------------|---------------|---------------|------------------|---------------|-----------------|-----------------|
|                                        |               | Potentiators            | RIF              | AZI           | LZD           | FUS           | NOV              | CHL           | DOX             | CIP             |
| <i>E. coli</i><br>ATCC<br>47076        | AB alone      |                         | 16               | 4             | 512           | 512           | 256              | 8             | 2               | 0.016           |
|                                        | +PaβN (38μM)  | >380μM                  | <b>4(2)</b>      | <b>1(2)</b>   | <b>64(3)</b>  | <b>64(3)</b>  | <b>32(3)</b>     | <b>1(3)</b>   | <b>0.5(2)</b>   | <b>0.004(2)</b> |
|                                        | +NV731 (38μM) | >380μM                  | <b>0.5(5)</b>    | <b>1(2)</b>   | <b>32(4)</b>  | <b>64(3)</b>  | <b>8(5)</b>      | <b>1(3)</b>   | <b>0.125(4)</b> | <b>0.004(2)</b> |
|                                        | +NV716 (10μM) | 50μM                    | <b>0.031(9)</b>  | <b>0.5(3)</b> | <b>8(6)</b>   | <b>2(8)</b>   | <b>0.5(9)</b>    | <b>0.5(4)</b> | <b>0.031(6)</b> | <b>0.002(3)</b> |
| <i>E. coli</i> 15                      | AB alone      |                         | 16               | 32            | 512           | 512           | 64               | 4             | 1               | 32              |
|                                        | +PaβN (38 μM) | >380 μM                 | <b>0.25(6)</b>   | <b>4(3)</b>   | <b>16(5)</b>  | <b>32(4)</b>  | <b>16(2)</b>     | <b>1(2)</b>   | <b>0.25(2)</b>  | 16(1)           |
|                                        | +NV731 (38μM) | >380 μM                 | <b>0.5(5)</b>    | 16(1)         | <b>64(3)</b>  | <b>128(2)</b> | <b>4(4)</b>      | <b>1(2)</b>   | <b>0.25(2)</b>  | 16(1)           |
|                                        | +NV716 (10μM) | 50 μM                   | <b>0.031(9)</b>  | <b>2(4)</b>   | <b>4(7)</b>   | <b>4(7)</b>   | <b>0.25(8)</b>   | <b>0.5(3)</b> | <b>0.25(2)</b>  | <b>8(2)</b>     |
| <i>E. coli</i> 51                      | AB alone      |                         | 32               | 32            | 512           | 512           | 128              | 8             | 16              | 32              |
|                                        | +PaβN (38 μM) | >380 μM                 | <b>0.5(6)</b>    | <b>4(3)</b>   | <b>16(5)</b>  | <b>32(4)</b>  | <b>16(3)</b>     | <b>1(3)</b>   | <b>2(3)</b>     | 32(0)           |
|                                        | +NV731 (38μM) | >380 μM                 | <b>1(5)</b>      | 16(1)         | <b>64(3)</b>  | <b>64(3)</b>  | <b>4(5)</b>      | <b>1(3)</b>   | <b>4(2)</b>     | 32(0)           |
|                                        | +NV716 (10μM) | 50 μM                   | <b>0.031(10)</b> | <b>1(5)</b>   | <b>16(5)</b>  | <b>1(9)</b>   | <b>0.125(10)</b> | <b>0.5(4)</b> | <b>1(4)</b>     | <b>8(2)</b>     |
| <i>K. pneumoniae</i><br>ATCC<br>43816  | AB alone      |                         | 32               | 8             | 512           | 512           | 512              | 4             | 2               | 0.031           |
|                                        | +PaβN (38μM)  | >380μM                  | 16(1)            | <b>2(2)</b>   | <b>64(3)</b>  | <b>128(2)</b> | <b>32(4)</b>     | <b>1(2)</b>   | <b>0.25(3)</b>  | 0.031(0)        |
|                                        | +NV731 (38μM) | >380μM                  | <b>0.25(7)</b>   | <b>1(3)</b>   | <b>64(3)</b>  | <b>128(2)</b> | <b>8(6)</b>      | <b>1(2)</b>   | <b>0.25(3)</b>  | 0.031(0)        |
|                                        | +NV716 (10μM) | 100μM                   | <b>0.031(10)</b> | <b>0.5(4)</b> | <b>32(4)</b>  | <b>64(3)</b>  | <b>1(9)</b>      | <b>0.5(3)</b> | <b>0.031(6)</b> | 0.031(0)        |
| <i>K. pneumoniae</i><br>ATCC<br>700603 | AB alone      |                         | 32               | 32            | 512           | 512           | 512              | 64            | 32              | 0.5             |
|                                        | +PaβN (38μM)  | >380μM                  | 16(1)            | 16(1)         | 512(0)        | 512(0)        | <b>64(3)</b>     | <b>8(3)</b>   | <b>4(3)</b>     | 0.25(1)         |
|                                        | +NV731 (38μM) | >380μM                  | <b>4(3)</b>      | 16(1)         | 512(0)        | 512(0)        | <b>64(3)</b>     | <b>2(5)</b>   | <b>2(4)</b>     | 0.25(1)         |
|                                        | +NV716 (10μM) | 100μM                   | <b>2(4)</b>      | <b>2(4)</b>   | 256(1)        | 256(1)        | <b>16(5)</b>     | <b>2(5)</b>   | <b>0.5(6)</b>   | <b>0.125(2)</b> |
|                                        | AB alone      |                         | 32               | 16            | 512           | 512           | 128              | 2             | 2               | 1               |
|                                        | +PaβN (38 μM) | > 380 μM                | <b>2(4)</b>      | <b>0.5(5)</b> | <b>128(2)</b> | 512(0)        | 64(1)            | 1(1)          | 1(1)            | 1(0)            |

|                                                                                                                                                                                                                                                                              |               |         |                 |                 |               |              |                 |              |                 |                 |
|------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|---------------|---------|-----------------|-----------------|---------------|--------------|-----------------|--------------|-----------------|-----------------|
| <i>K. pneumoniae</i><br>58                                                                                                                                                                                                                                                   | +NV731 (38µM) | >380 µM | <b>4(3)</b>     | 8(4)            | <b>128(2)</b> | 512(0)       | <b>8(4)</b>     | 1(1)         | 1(1)            | 1(0)            |
|                                                                                                                                                                                                                                                                              | +NV716 (10µM) | 50 µM   | <b>0.5(6)</b>   | <b>2(3)</b>     | <b>64(3)</b>  | <b>64(3)</b> | <b>2(6)</b>     | 1(1)         | 1(1)            | <b>0.125(3)</b> |
| <i>K. pneumoniae</i><br>74                                                                                                                                                                                                                                                   | AB alone      |         | 16              | 16              | 512           | 512          | 128             | 2            | 2               | 0.031           |
|                                                                                                                                                                                                                                                                              | +E50(38 µM)   | >380 µM | <b>2(3)</b>     | <b>1(4)</b>     | <b>64(3)</b>  | 512(0)       | <b>32(2)</b>    | 1(1)         | 1(1)            | 0.031(0)        |
|                                                                                                                                                                                                                                                                              | +NV731 (38µM) | >200 µM | <b>2(3)</b>     | <b>2(3)</b>     | <b>64(3)</b>  | 512(0)       | <b>8(4)</b>     | 1(1)         | <b>0.5(2)</b>   | 0.031(0)        |
|                                                                                                                                                                                                                                                                              | +NV716 (10µM) | 50 µM   | <b>0.125(7)</b> | <b>1(4)</b>     | <b>16(5)</b>  | <b>16(5)</b> | <b>2(6)</b>     | 1(1)         | <b>0.5(2)</b>   | 0.031(0)        |
| <i>A. baumannii</i><br>ATCC<br>19606                                                                                                                                                                                                                                         | AB alone      |         | 2               | 16              | 256           | 512          | 16              | 64           | 0.125           | 1               |
|                                                                                                                                                                                                                                                                              | +PaβN (38 µM) | >380µM  | <b>0.5(2)</b>   | 8(1)            | <b>64(2)</b>  | <b>32(4)</b> | <b>4(2)</b>     | 32(1)        | 0.063(1)        | 0.5(1)          |
|                                                                                                                                                                                                                                                                              | +NV731 (38µM) | >380µM  | <b>0.125(4)</b> | 8(1)            | <b>32(3)</b>  | <b>4(7)</b>  | <b>0.25(6)</b>  | 32(1)        | 0.063(1)        | 0.5(1)          |
|                                                                                                                                                                                                                                                                              | +NV716 (10µM) | 100µM   | <b>0.125(4)</b> | <b>4(2)</b>     | <b>8(5)</b>   | <b>2(8)</b>  | <b>0.125(7)</b> | <b>16(2)</b> | <b>0.031(2)</b> | <b>0.25(2)</b>  |
| <i>A. baumannii</i><br>112                                                                                                                                                                                                                                                   | AB alone      |         | 2               | 1               | 512           | 256          | 32              | 128          | 0.125           | 32              |
|                                                                                                                                                                                                                                                                              | +PaβN (38 µM) | >380 µM | <b>0.25(3)</b>  | <b>0.125(3)</b> | <b>128(2)</b> | <b>16(4)</b> | <b>2(4)</b>     | 64(1)        | 0.125(0)        | 32(0)           |
|                                                                                                                                                                                                                                                                              | +NV731 (38µM) | >380 µM | <b>0.125(4)</b> | 0.5(1)          | <b>64(3)</b>  | <b>8(5)</b>  | <b>0.5(6)</b>   | 64(1)        | 0.063(1)        | 32(0)           |
|                                                                                                                                                                                                                                                                              | +NV716 (10µM) | 50 µM   | <b>0.031(6)</b> | <b>0.125(3)</b> | <b>32(4)</b>  | <b>2(7)</b>  | <b>0.063(9)</b> | <b>16(3)</b> | <b>0.031(2)</b> | <b>8(2)</b>     |
| <i>A. baumannii</i><br>113                                                                                                                                                                                                                                                   | AB alone      |         | 2               | 1               | 512           | 128          | 32              | 128          | 0.25            | 32              |
|                                                                                                                                                                                                                                                                              | +PaβN (38 µM) | >380µM  | <b>0.25(3)</b>  | <b>0.25(2)</b>  | <b>64(2)</b>  | <b>16(3)</b> | <b>4(3)</b>     | 64(1)        | 0.25(0)         | 16(1)           |
|                                                                                                                                                                                                                                                                              | +NV731 (38µM) | >380µM  | <b>0.25(3)</b>  | 0.5(1)          | <b>64(2)</b>  | <b>8(4)</b>  | <b>0.5(6)</b>   | <b>32(2)</b> | 0.25(0)         | 16(1)           |
|                                                                                                                                                                                                                                                                              | +NV716 (10µM) | 100µM   | <b>0.016(7)</b> | <b>0.25(2)</b>  | <b>32(3)</b>  | <b>1(7)</b>  | <b>0.063(9)</b> | <b>8(4)</b>  | <b>0.063(2)</b> | <b>8(2)</b>     |
| <sup>a</sup> values in bold highlight a significant decrease in MIC (at least 2 doubling dilutions); values in brackets: number of doubling dilution decrease (log <sub>2</sub> fold change) for MIC of antibiotic combined with potentiator compared with antibiotic alone. |               |         |                 |                 |               |              |                 |              |                 |                 |

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

Our previous study suggested that **NV716** can bind to LPS in *P. aeruginosa*, increase the permeability of its outer membrane, and to a much lower extent, of its inner membrane [460]. We therefore examined these properties in the three bacterial species under study here. Alexidine and colistin were used as positive controls [345, 401], and **NV731** and PA $\beta$ N, as comparators. We first measured the capacity of each compound to displace Bodipy-cadaverine from its binding to LPS in the 4 reference strains (Figure 2A; IC<sub>50</sub> values in Table S4). The effect of **NV716** was similar to that of alexidine over the whole range of concentrations investigated against all strains, with similar IC<sub>50</sub> values for both molecules. The IC<sub>50</sub> was about 10-times higher **NV731** and not reached at 100  $\mu$ M for colistin and PA $\beta$ N.

We then determined the effects of these compounds on the outer membrane permeability (Figure 2B; IC<sub>50</sub> values in Table S4). To this effect, we measured the absorbance of the hydrolysis product of the non-permeant cephalosporin nitrocefin, which is degraded by periplasmic  $\beta$ -lactamases when the outer membrane has been permeabilized [466]. Alexidine was the most potent in this assay, followed by **NV716** and colistin, which also allowed to reach a same effect as alexidine but at higher concentrations (IC<sub>50</sub> 2 to 3 times higher). **NV731** and PA $\beta$ N were less potent (IC<sub>50</sub> 7 to 13 times higher than for alexidine) and less effective.

The effects of potentiators on the inner membrane permeability was also assessed by measuring the fluorescence signal of propidium iodide (PI), generated when PI intercalates in the DNA of bacteria when both their outer and inner membranes have been permeabilized [404]. Alexidine and colistin similarly increased PI fluorescence in a concentration-dependent manner, while **NV716** was 20-30 times less potent, causing only 50% of permeabilization at the highest concentrations tested (50  $\mu$ M) (Figure 2C; IC<sub>50</sub> values in Table S4). Yet, the effect of **NV716** was slightly higher than that of colistin

but much lower than that of alexidine when these compounds were all compared at an equipotent concentration of 1 x MIC (Figure S5). **NV731** and PA $\beta$ N were inactive in this assay.

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

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

Previous studies documented that **NV716** can inhibit activity of efflux pumps in *P. aeruginosa* [353]. We therefore evaluated the capacity of **NV716** and its comparators to inhibit the active efflux of the fluorescent probe 1,2'-dinaphthylamine [468]. We first examined the rate of efflux in *E. coli* ATCC 47076 after the addition of **NV716** at different concentrations (Figure 2D, left panel). A concentration-dependent reduction of the fluorescence was observed readily after the addition of **NV716**, which reached a plateau at 620 sec. The same experiments were performed with the other potentiators and also against *K. pneumoniae* reference strains. The other panels of Figure 2D show the inhibition of efflux calculated at 620 sec. **NV716** proved more potent and effective in this assay than the other potentiators (Figure 2D). While the effect of **NV716**, alexidine and PA $\beta$ N increased all over the range of concentration investigated, that of **NV731** and colistin tend to a plateau at high concentrations.

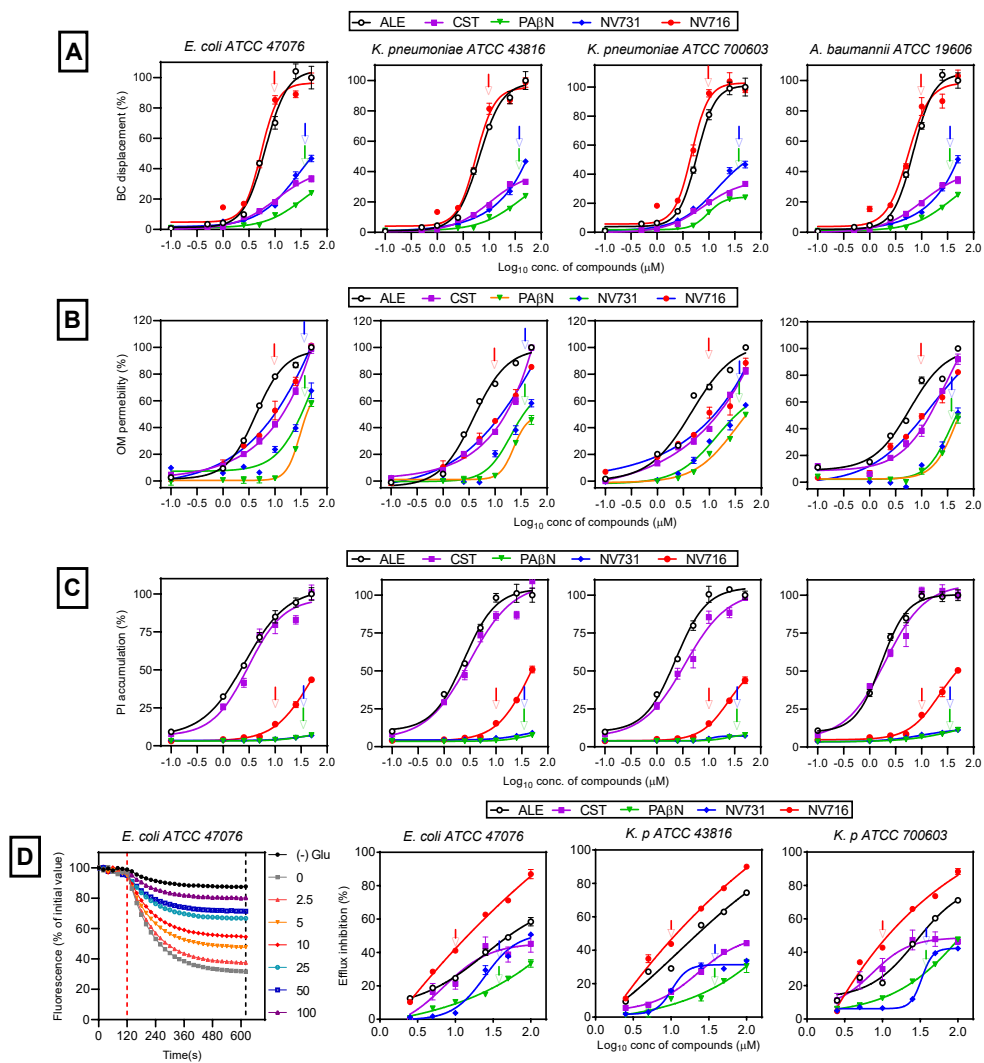


**Figure 2.** Influence of potentiators, colistin and alexidine over a wide range of concentrations on membrane properties of 1 (*E. coli*; *A. baumannii*) or 2 (*K. pneumoniae*) reference strains. (A) Binding to LPS, assessed by measuring the displacement of BODIPY-cadaverine (BC) after 30 minutes of incubation. Positive control (100%): effect of 50  $\mu$ 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  $\mu$ 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  $\mu$ M alexidine after 1 h of incubation; negative control: buffer. (D) Left: kinetics of efflux dinaphthylamine from *E. coli* ATCC47076 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* ATCC47076. 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  $\mu$ M for **NV716**; 38  $\mu$ M for **NV731** and PA $\beta$ N). All data are means  $\pm$  SEM (triplicates from 3 independent experiments). IC<sub>50</sub> values for these concentration-responses are shown in Table S4.

**Table S4.** IC<sub>50</sub> values from interaction of potentiators with Gram-negative bacterial membrane. IC<sub>50</sub> values were calculated based on the Hill equation of concentration-response curves in BC displacement, OM permeability, and PI accumulation assays as well as efflux inhibition. All data are shown as means (triplicates from 3 experiments).

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

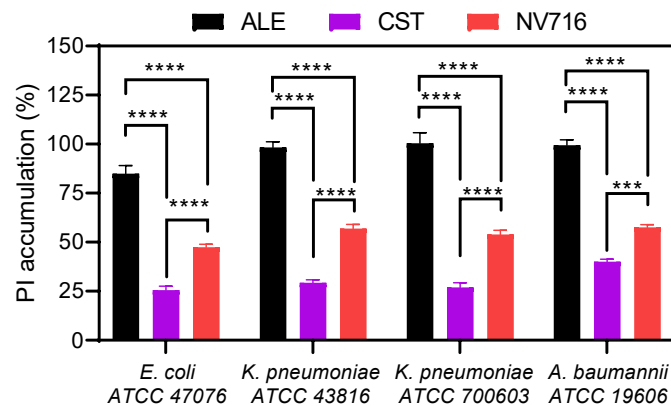


**Figure S5.** Effect of alexidine, colistin, and NV716 each at a concentration of 1 x MIC (See Table S1) on inner membrane permeability as assessed by measuring the fluorescence of propidium iodide (PI) after 1 h of incubation with 1 (*A. baumannii* and *E. coli*) or 2 (*K. pneumoniae*) reference strains.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

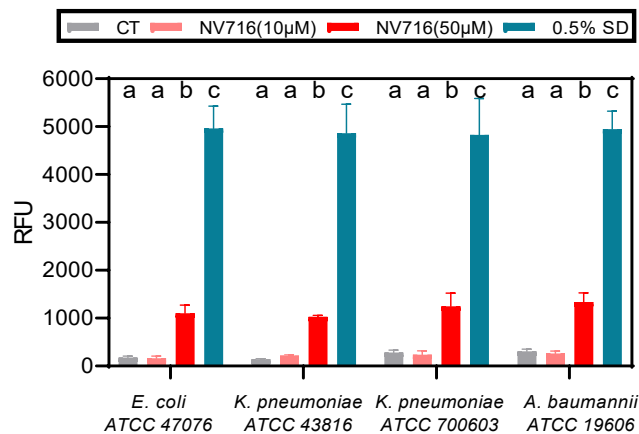


**Figure S6.** Inner membrane depolarization caused by NV716 at two concentrations, as assessed by measuring the DiSC3(5) fluorescence after 15 min incubation with 1 (*A. baumannii* and *E. coli*) or 2 (*K. pneumoniae*) reference strains. The effect measured with 0.5% (w/v) SDS after 15 min was taken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

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

As **NV716** was shown to increase outer membrane permeability and inhibit efflux, we assessed its effect compared to that of the other potentiators on the accumulation of ciprofloxacin and **NV1532** (a fluorescent derivative of rifampicin; same MIC as rifampicin against all tested strains) inside bacteria. These antibiotics were selected as representatives of antibiotics that are substrates or not for efflux, respectively. The level of accumulation of ciprofloxacin alone was higher in *E. coli* ATCC 47076 and *K. pneumoniae* ATCC 43816 than in the other two strains (Figure 3A). PA $\beta$ N and **NV731** at 38  $\mu$ M increased its accumulation in *E. coli* ATCC 47076 only, while **NV716** at 10  $\mu$ M caused a significant increase in all strains except *K. pneumoniae* ATCC 43816 in which it did not decrease ciprofloxacin MIC (Table S3). The accumulation of **NV1532** was higher in *A. baumannii* ATCC 19606 than in the other strains. PA $\beta$ N increased its accumulation in *E. coli* ATCC 47076 and *A. baumannii* ATCC 19606, while **NV731** and **NV716** caused a significant increase for all strains tested, but the effect of **NV716** was more important even though it was used at a lower concentration (Figure 3B). A significant correlation was observed between the fold change (i) in the accumulation of antibiotics and (ii) in MIC when comparing the combination with potentiators vs antibiotics alone (Figure 3C and 3D).

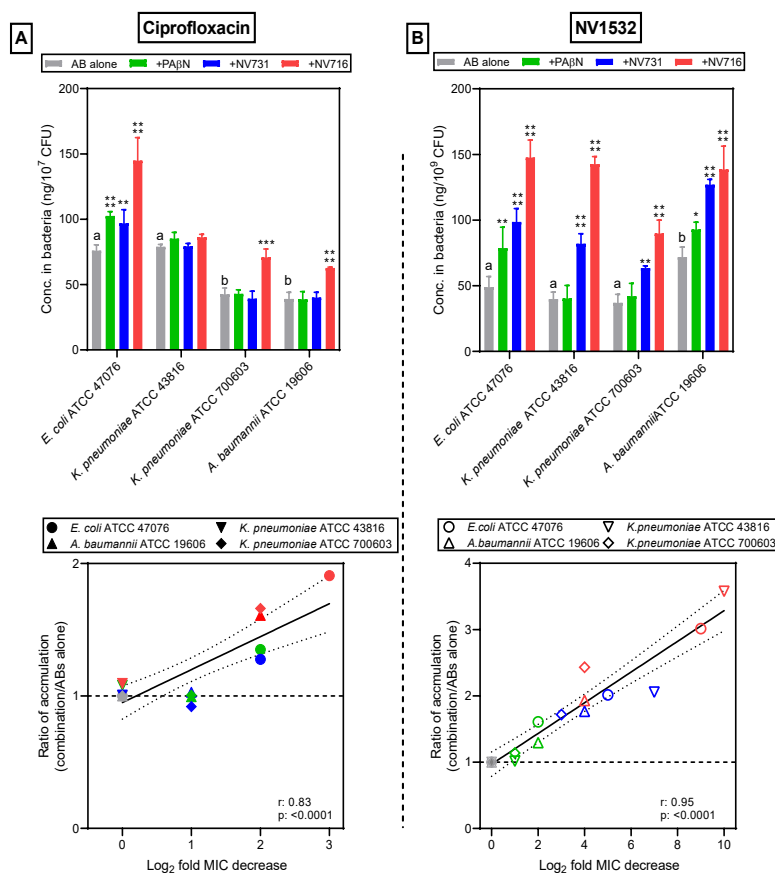
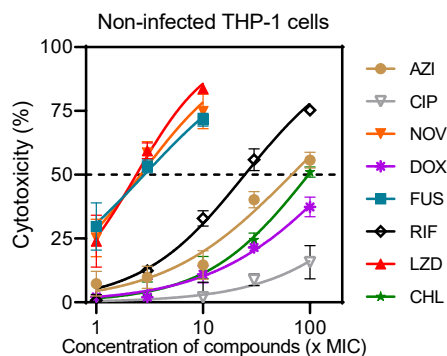


**Figure 3.** Top: Accumulation of antibiotics alone (A, ciprofloxacin; B, rifampicin analog **NV1532**) or combined with potentiators in 1 (*E. coli* and *A. baumannii*) and 2 (*K. pneumoniae*) reference strains. Grey: antibiotics alone; green: combined 38  $\mu$ M PA $\beta$ N; blue: combined 38  $\mu$ 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<sub>2</sub> 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.

## Cytotoxicity of antibiotics

As a preliminary to intracellular infection experiments, we examined whether antibiotics were cytotoxic for eukaryotic cells. This was critical as most of them had to be used at high concentrations in view of their high MIC. The cytotoxicity of potentiators had been previously examined and found to be minimal in their conditions of used for these experiments [398].  $IC_{50}$  (i.e. concentration causing 50% cell mortality) were reached at concentrations corresponding to approximately 3 x (fusidic acid, linezolid, novobiocin), 30 x (rifampicin), 60 x (azithromycin), 90 x (chloramphenicol), or above 100 x (doxycycline and ciprofloxacin) their respective MIC against *E. coli* ATCC 47076, both in infected and non-infected cells (Table S5).  $IC_{50}$  values do not differ for antibiotics alone or combined with a potentiator, both in non-infected and infected cells (Table S5).

**Table S5.**  $IC_{50}$  values from cytotoxicity tests, for antibiotics alone or combined with potentiators, and expressed in different units (x MIC, mg/L,  $\mu$ M [MIC are vs. *E. coli* ATCC 47076]). The graph above the Table show the concentration-response curves for seven antibiotics alone (rifampicin [RIF], azithromycin [AZI], linezolid [LZD], fusidic acid [FUS], novobiocin [NOV], chloramphenicol [CHL] and doxycycline [DOX]) at the indicated concentrations in non-infected THP-1 cells. The dotted horizontal line shows the 50 % cytotoxicity ( $IC_{50}$ ).  $IC_{50}$  values shown in the Table were calculated based on the Hill equation of concentration-response curves in non-infected THP-1 cells or THP-1 cells infected with *E. coli* ATCC 47076 based on experiments similar to that shown in the graph. All data are shown as means  $\pm$  SEM (triplicates from 3 experiments). Statistical analysis (1-way ANOVA; Tukey's Multiple Comparison Test): no significant difference was noticed when comparing the different conditions for each antibiotic ( $p > 0.05$ ).



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

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

Figure 4 shows the concentration-response curves against intracellular *E. coli* ATCC 47076 in the absence of or in the presence of **NV716** (the highest concentration tested for each antibiotic was that causing 50% cytotoxicity). The activity of antibiotics alone developed following a sigmoidal concentration-response curve, as previously described [188]. At low, subMIC concentrations, a ~3-4 log<sub>10</sub> increase in CFUs was noticed over the 24 h incubation period. A static effect was observed at extracellular concentrations close or slightly higher than the MIC of each antibiotic. At the highest concentration tested, the reduction in bacterial counts ranged from a static effect for linezolid, fusidic acid, or novobiocin (note that the highest concentration tested is low for these drugs because of their toxicity), 1 log<sub>10</sub> CFU decrease for azithromycin and chloramphenicol, 2 log<sub>10</sub> CFU decrease for rifampicin and doxycycline, and 2.5 log<sub>10</sub> CFU decrease for ciprofloxacin. In the presence of **NV716** at 10 µM, all curves were shifted to the left, meaning that the corresponding antibiotic was more potent, a static effect being reached at lower extracellular concentrations. The extent of the shift varied however among drugs. Furthermore, for those drugs for which the maximal effect could be reached (i.e., the plateau value of the Hill equation), the reduction in intracellular counts was also increased ( $E_{\max}$  more negative), indicating an increase in the antibiotic efficacy. For the other drugs (linezolid, fusidic acid, novobiocin), an increased effect was also observed at the highest concentration tested.

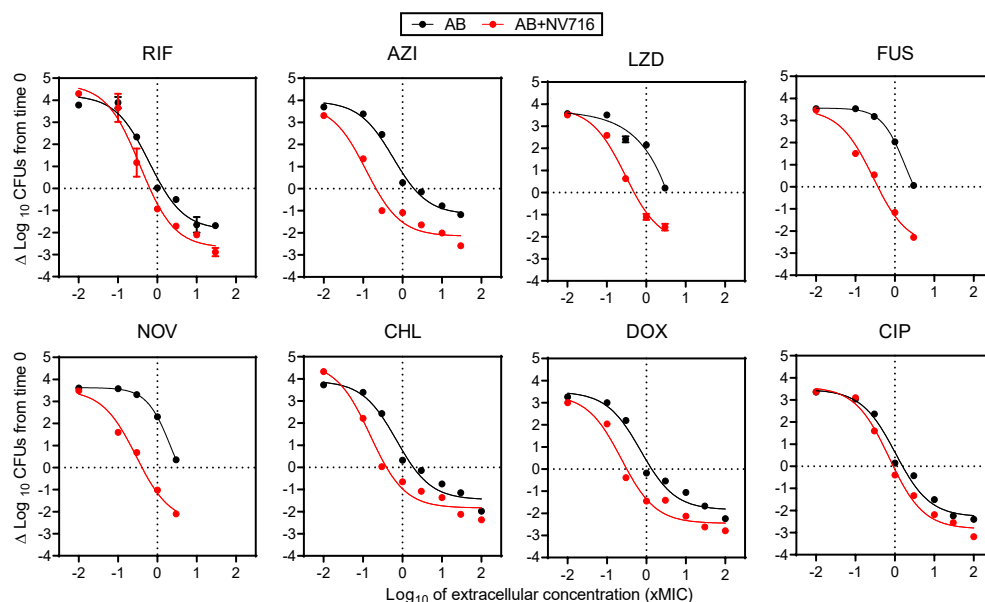


**Figure 4.** Concentration-response curves of antibiotics alone or combined with **NV716** against intracellular *E. coli* ATCC 47076 in a model of THP-1 monocytes. The graphs show the changes in CFU counts from the initial, post-phagocytosis inoculum after 24 h of incubation with increasing extracellular 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  $IC_{50}$  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).

The same type of experiment was performed with the 2 other potentiators against 1 (*E. coli*; *A. baumannii*) or 2 (*K. pneumoniae*) reference strains and 2 clinical isolates. Figure 5 and 6 illustrate the pharmacodynamic parameters calculated based on the Hill equation of these concentration-response curves (see methods for a definition of these parameters), namely the  $C_s$  (a measure of the relative potency of the drug or the drug combination) and the  $E_{max}$  (a measure the relative maximal efficacy of the antibiotics alone or in combination; when the  $E_{max}$  was not reached, the maximal reduction in

bacterial counts at the highest concentration [ $E_{\text{highest}}$ ] tested was plotted).  $C_s$  of antibiotics alone was globally slightly higher than their MIC in broth, and **NV716** markedly reduced all  $C_s$  values. These reductions ranged from approx. 1 [chloramphenicol and ciprofloxacin] to 3-4 [linezolid, fusidic acid and novobiocin] doubling dilutions against *E. coli*; 1 [chloramphenicol and ciprofloxacin] to 3 [novobiocin] doubling dilutions against *K. pneumoniae*; 1 [rifampicin and ciprofloxacin] to 3-4 [fusidic acid, novobiocin and chloramphenicol] doubling dilutions against *A. baumannii*, respectively (Figure 5 and Table S6). Ciprofloxacin was the less affected because it was the most active drug against these bacteria. **NV731** and PA $\beta$ N caused less marked effects on  $C_s$  values. Intracellular  $E_{\text{max}}$  or  $E_{\text{highest}}$  ranged between 0.6 [linezolid against *E. coli*] to 2.3 [ciprofloxacin against *E. coli*]  $\log_{10}$  CFU decrease for antibiotics alone. **NV716** showed the most important potentiation of antibiotic efficacy, with the gain in  $E_{\text{max}}$  or  $E_{\text{highest}}$  values ranging from 0.7 (doxycycline and ciprofloxacin) to 1.7 (fusidic acid)  $\log_{10}$  CFU for *E. coli*; 0.6 (chloramphenicol and doxycycline) to 1.3 (linezolid)  $\log_{10}$  CFU for *K. pneumoniae*; and 0.6 (doxycycline) to 1 (all test antibiotics except for doxycycline)  $\log_{10}$  CFU for *A. baumannii* (Figure 6 and Table S6). Pa $\beta$ N did not improve antibiotic efficacy, and **NV731** effects were more modest than those of **NV716** in all cases.

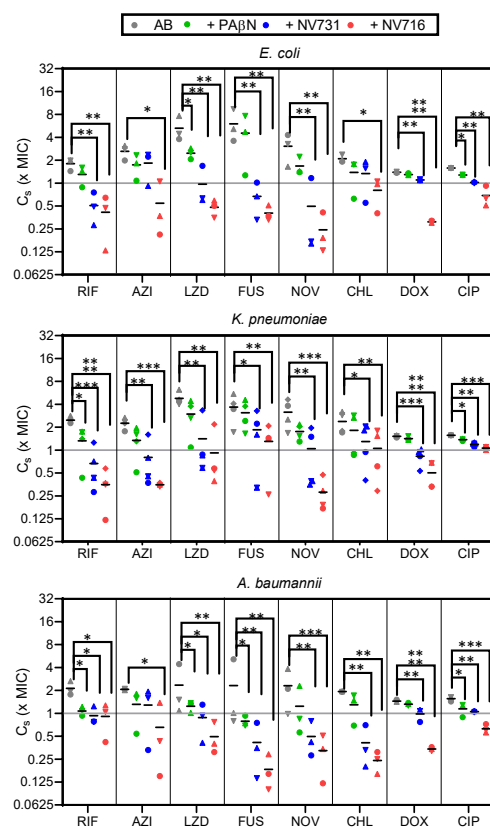


**Figure 5.** Static concentration of antibiotics alone (rifampicin [RIF], azithromycin [AZI], linezolid [LZD], fusidic acid [FUS], novobiocin [NOV], chloramphenicol [CHL], doxycycline [DOX], or ciprofloxacin [CIP]) or combined with potentiators against various multidrug resistant Gram-negative bacteria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

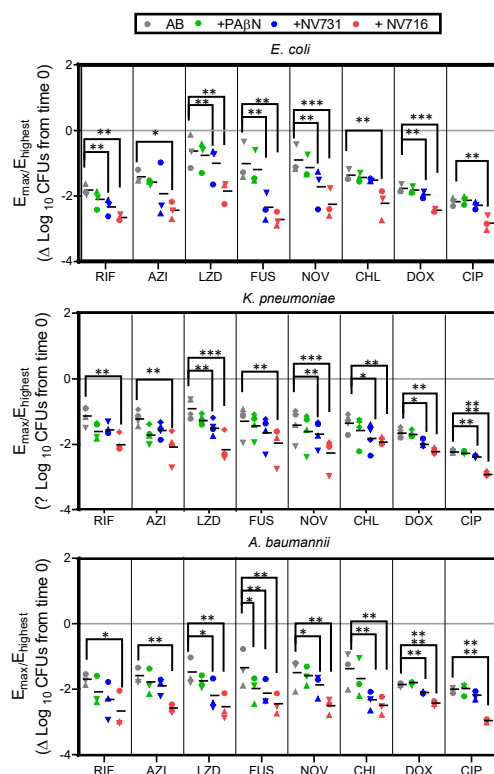


**Figure 6.**  $E_{max}$  (extrapolated for an infinitively large concentration) or  $E_{highest}$  (measured at the highest concentration tested but the plateau was not yet reached) for antibiotics alone (rifampicin [RIF], azithromycin [AZI], linezolid [LZD], fusidic acid [FUS], novobiocin [NOV], chloramphenicol [CHL], doxycycline [DOX], or ciprofloxacin [CIP]) in the intracellular infection model (THP-1). Values are calculated based on the equation of the Hill response curves from experiments similar to those described in 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. Statistical analysis: one-way ANOVA with Dunnett post-hoc test (paired) comparing each combination to the antibiotic alone: \*,  $p < 0.05$ ; \*\*,  $p < 0.01$ ; \*\*\*,  $p < 0.001$ ; \*\*\*\*,  $p < 0.0001$ .

**Table S6.** Gain in potency (expressed as the Log<sub>2</sub> fold dilution decrease in C<sub>s</sub> between antibiotics alone (rifampicin [RIF], azithromycin [AZI], linezolid [LZD], fusidic acid [FUS], novobiocin [NOV], chloramphenicol [CHL], doxycycline [DOX], or ciprofloxacin [CIP]) or combined with potentiators, and in maximal efficacy (expressed as the difference in E<sub>max</sub> between combinations and antibiotics alone) against intracellular *E. coli*, *K. pneumoniae* and *A. baumannii*.

| Strains              | ABs | Log <sub>2</sub> fold dilution decrease in C <sub>s</sub><br>(ABs alone/Combinations) |       |        | Change of E <sub>max</sub> /E <sub>highest</sub><br>(Combinations-ABs alone) |       |        |
|----------------------|-----|---------------------------------------------------------------------------------------|-------|--------|------------------------------------------------------------------------------|-------|--------|
|                      |     | +NV716                                                                                | +PAβN | +NV731 | +NV716                                                                       | +PAβN | +NV731 |
| <i>E. coli</i>       | RIF | 2                                                                                     | 0     | 2      | -0.8                                                                         | -0.3  | -0.5   |
|                      | AZI | 2                                                                                     | 1     | 0      | -1.0                                                                         | -0.2  | -0.5   |
|                      | LZD | 3                                                                                     | 1     | 2      | -1.2                                                                         | -0.1  | -0.4   |
|                      | FUS | 4                                                                                     | 0     | 3      | -1.7                                                                         | -0.2  | -1.3   |
|                      | NOV | 4                                                                                     | 1     | 3      | -1.3                                                                         | -0.2  | -0.8   |
|                      | CHL | 1                                                                                     | 1     | 1      | -1.1                                                                         | -0.1  | -0.2   |
|                      | DOX | 2                                                                                     | 0     | 0      | -0.7                                                                         | -0.1  | -0.2   |
|                      | CIP | 1                                                                                     | 0     | 1      | -0.6                                                                         | 0.1   | -0.1   |
| <i>K. pneumoniae</i> | RIF | 3                                                                                     | 1     | 2      | -0.9                                                                         | -0.5  | -0.4   |
|                      | AZI | 3                                                                                     | 1     | 1      | -0.9                                                                         | -0.5  | -0.4   |
|                      | LZD | 2                                                                                     | 1     | 2      | -1.2                                                                         | -0.4  | -0.6   |
|                      | FUS | 1                                                                                     | 0     | 1      | -0.7                                                                         | -0.1  | -0.4   |
|                      | NOV | 3                                                                                     | 1     | 2      | -0.8                                                                         | -0.2  | -0.3   |
|                      | CHL | 1                                                                                     | 0     | 1      | -0.6                                                                         | -0.2  | -0.5   |
|                      | DOX | 2                                                                                     | 0     | 1      | -0.6                                                                         | 0.0   | -0.3   |
|                      | CIP | 1                                                                                     | 0     | 0      | -0.7                                                                         | 0.0   | -0.1   |
| <i>A. baumannii</i>  | RIF | 1                                                                                     | 1     | 1      | -1.0                                                                         | -0.4  | -0.6   |
|                      | AZI | 2                                                                                     | 1     | 1      | -1.0                                                                         | -0.2  | -0.3   |
|                      | LZD | 2                                                                                     | 1     | 1      | -1.1                                                                         | -0.3  | -0.7   |
|                      | FUS | 4                                                                                     | 2     | 2      | -1.1                                                                         | -0.6  | -0.8   |
|                      | NOV | 3                                                                                     | 1     | 2      | -1.0                                                                         | -0.1  | -0.4   |
|                      | CHL | 3                                                                                     | 1     | 2      | -1.1                                                                         | -0.3  | -1.0   |
|                      | DOX | 2                                                                                     | 0     | 1      | -0.6                                                                         | 0.1   | -0.2   |
|                      | CIP | 1                                                                                     | 0     | 1      | -1.0                                                                         | 0.0   | -0.2   |

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

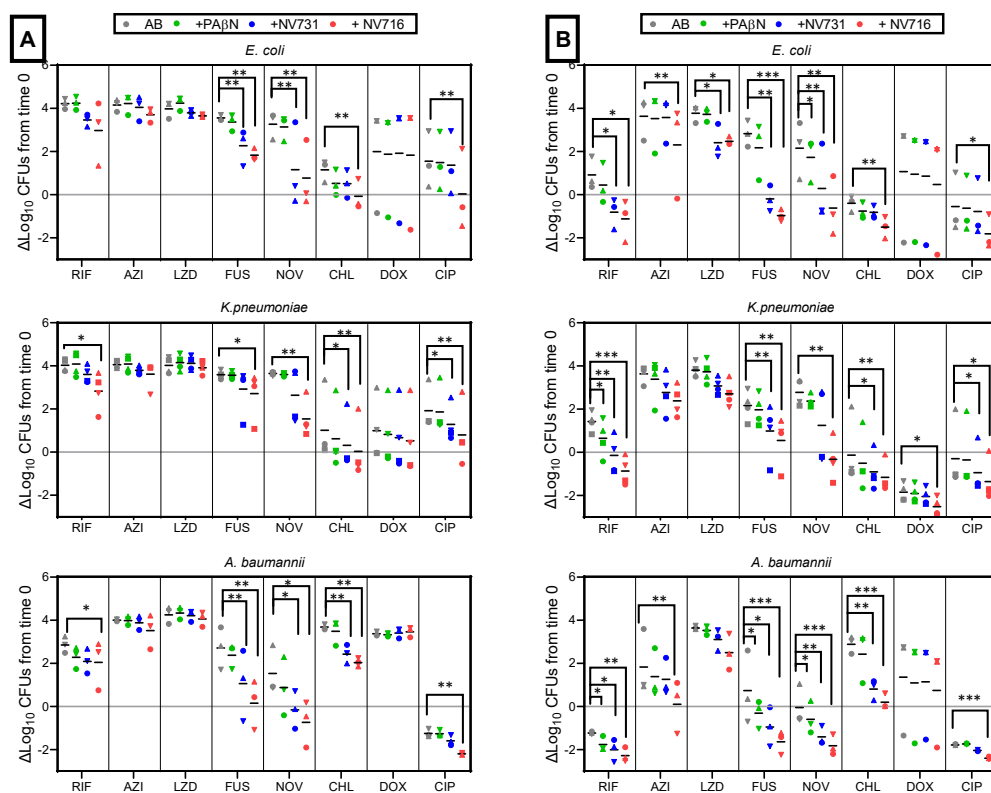


**Figure S7.** Effect of potentiators on antibiotics (rifampicin [RIF], azithromycin [AZI], linezolid [LZD], fusidic acid [FUS], novobiocin [NOV], chloramphenicol [CHL], doxycycline [DOX], or ciprofloxacin [CIP]) against 1 (*E. coli*; *A. baumannii*) or 2 (*K. pneumoniae*) reference strains and 2 clinical isolates for each species at  $C_{min}$  (A) and  $C_{max}$  (B); see Table S1 for the corresponding values. Horizontal bars: mean; horizontal line: static effect. Grey: antibiotics alone; Green: combined 38  $\mu$ M PA $\beta$ N; Blue: combined 38  $\mu$ M NV731; Red: combined 10  $\mu$ M NV716. Statistical analysis: one-way ANOVA with Dunnett post-hoc test (paired): \*,  $p < 0.05$ ; \*\*,  $p < 0.01$ ; \*\*\*,  $p < 0.001$ .

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

The fact that NV716 increases the intracellular efficacy of all antibiotics against *E. coli*, *A. baumannii* and *K. pneumoniae* suggests it may decrease the proportion of antibiotic persisters in the population, as previously demonstrated for *P. aeruginosa* [398]. We, therefore, determined the persister fraction surviving to ciprofloxacin (selected as a highly bactericidal antibiotic) at high concentration (50 x MIC) against stationary-phase cultures ( $10^9$  CFUs/mL) of reference strains. To this effect, we first examined the kinetics of killing by ciprofloxacin when used alone or combined with **NV716** added either at the same time as ciprofloxacin or later to determine whether it was able to improve activity even the maximal effect was already reached for ciprofloxacin alone [379](Figure 7A). CFUs were significantly reduced after the addition of **NV716** at any time point, suggesting that **NV716** does not need to be added at the same time as the antibiotic to be able to increase its activity. In the next step, we aimed at determining whether **NV716** action was related to a direct capacity to kill persisters or rather to resensitize them to the killing effect of ciprofloxacin. To this end, we isolated the persisters surviving after 5 h of incubation with ciprofloxacin at 50 x MIC and reexposed them to ciprofloxacin at 50 x MIC, NV716 at different concentrations, or a combination thereof (Figure 7B). As expected, incubation of the isolated persisters with ciprofloxacin alone caused only a negligible reduction in CFU for all strains, confirming the effective isolation of persister cells. When used alone, **NV716** caused a marked decrease in CFU only at the highest concentration tested (250  $\mu$ M). In contrast, a significant reduction in CFU was observed when **NV716** was combined at 10  $\mu$ M with ciprofloxacin for all strains, indicating it rather acts as a potentiator of ciprofloxacin against persisters.

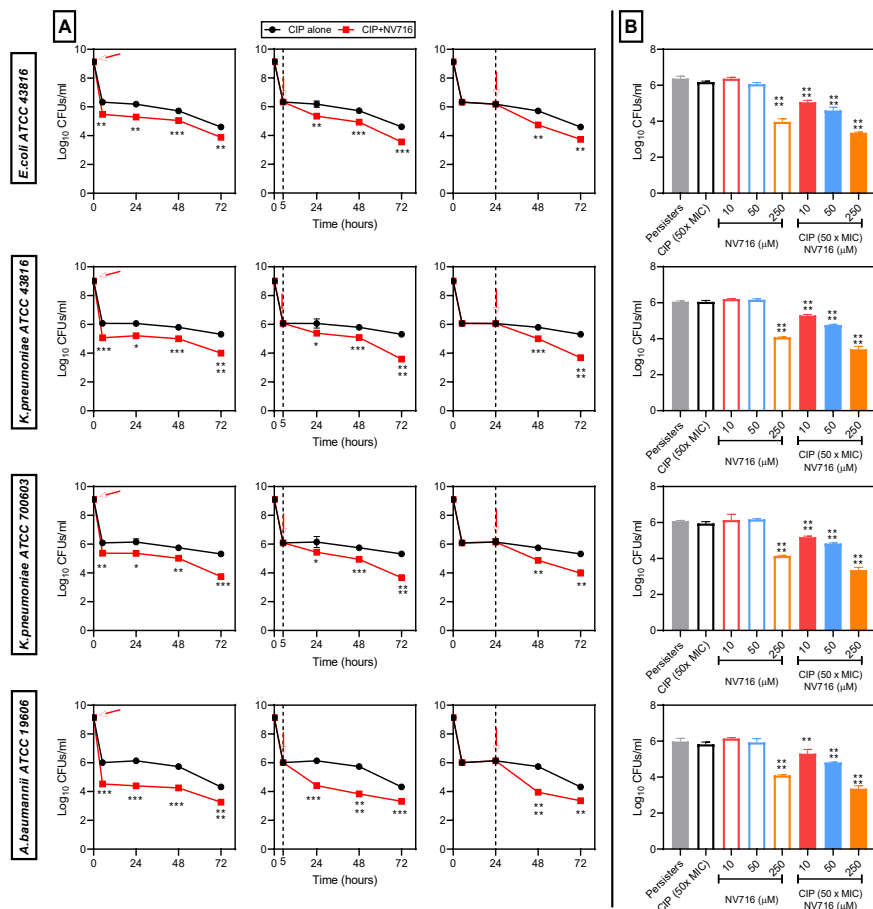


**Figure 7.** Influence of **NV716** on persisters selected by ciprofloxacin. (A) The time-killing assay for stationary-phase cultures of 1 (*E. coli*; *A. baumannii*) or 2 (*K. pneumoniae*) reference strains by ciprofloxacin at 50 x MIC alone or combined with 10 μM **NV716** added at different time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

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

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

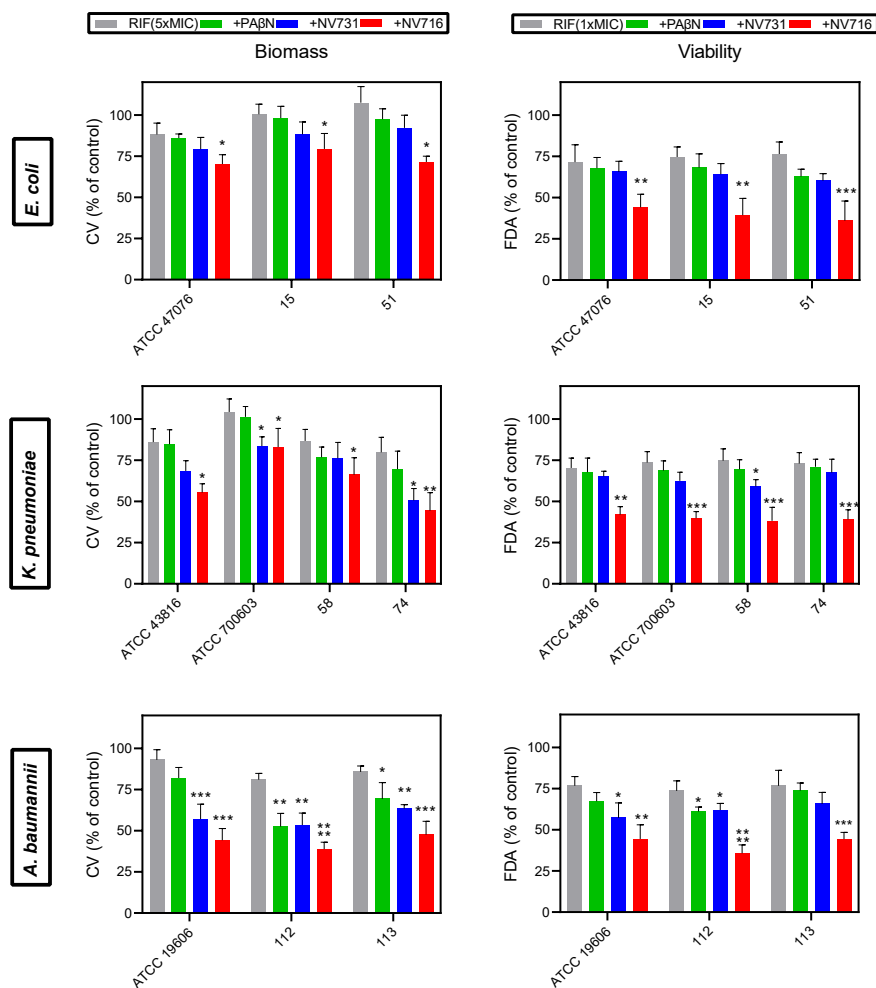


**Figure 8.** Effect of potentiators on the activity of rifampin against biofilms of 1 (*E. coli*; *A. baumannii*) or 2 (*K. pneumoniae*) reference strains and 2 to clinical isolates of each species. Mature biofilms were exposed during 24 h to rifampin alone at 1 x MIC (right panels) or 5 x MIC (left 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: \*,  $p < 0.05$ ; \*\*,  $p < 0.01$ ; \*\*\*,  $p < 0.001$ ; \*\*\*\*,  $p < 0.0001$ .

## Discussion

The emergence and rapid spread of antibiotic resistance in Gram-negative bacteria represents a critical threat to public health worldwide [470]. Identification of novel adjuvants that restore existing antibiotic efficacy and improve clinical cure has been considered as a cost-effective strategy for combating superbugs, in particular *E. coli*, *K. pneumoniae* and *A. baumannii* [471]. In this study, we show that the polyaminoisoprenyl compound **NV716** is capable of resensitizing *E. coli*, *K. pneumoniae*, and *A. baumannii* to all tested antibiotics whether affected (doxycycline, chloramphenicol, azithromycin, ciprofloxacin, fusidic acid, novobiocin and linezolid) or not (rifampicin) by active efflux [275, 376, 472], not only in broth but also intracellularly or in biofilm. In all our experiments, **NV731** and the efflux pump inhibitor PA $\beta$ N proved less potent.

Considering first the susceptibility data in broth, we observed a remarkable improvement in the intrinsic activity of antibiotics when combined by **NV716**. This synergistic effect is obtained at sub-MIC concentrations of the potentiator, demonstrating it is related to an adjuvant mode of action and not to bacterial killing. Potentiation is observed in all three species (as well as in *P. aeruginosa* [460]), and its extent (in terms of gain of activity, see Figure 1B) is largely independent of the species, even for antibiotics showing markedly different MICs against these species when used alone (see e.g. rifampicin, chloramphenicol, or doxycycline). Actually, the degree of potentiation offered by **NV716** is depending on the lipophilicity of the antibiotic, as evaluated by its logD value. This is very coherent with the fact that the external leaflet of the outer membrane is known to oppose a barrier to the diffusion of lipophilic compounds, which are also incapable to cross this obstacle via hydrophilic porin channels [79]. LPS barrier effect is mediated by the low fluidity of the layer, the quasi-crystalline, ordered arrangement of its hydrocarbon chains, and the strong intermolecular interaction between LPS heads via cation-mediated bridging [79]. In this context, **NV716**, with a calculated logD of -4.2 at physiological pH (Table S1), shows a strong capacity to interact with LPS, ensuing a subsequent permeabilisation of the outer membrane. It is as potent in this respect as the

biguanide alexidine, with which it shares an extended conformation of its polyaminated moiety, and, interestingly enough, much more potent than colistin, often used as a potentiator of other drugs against multiresistant Gram-negative bacteria, for its membrane-destabilizing effects [473, 474]. As previously discussed [460], **NV731** does not show this extended conformation, which may contribute to explain its lower potency. The capacity of **NV716** to interact with LPS and permeabilize the outer membrane is similar among the three species tested here as well as against *P. aeruginosa*. This indicates the importance of its interaction with the negative charges of the phosphate groups and subsequent displacement of stabilizing divalent cations for its action [460], as this is a common feature of all LPS. Yet, the differences in LPS between the species under study remain modest. As compared to the LPS of *E. coli*, that of *A. baumannii* shows an additional acyl chain at the 2 position containing an additional hydroxyl group [475]; that of *K. pneumoniae* is hexa-acylated with C14 chains in position 2 and 3 (instead of C12 in position 3 for *E. coli*) and possible additional modifications in the lipidic part [476], while that of *P. aeruginosa* PAO1 is hexa- or penta-acetylated (lacking in this case the primary O-linked acyl substitution at position 3) [90].

Beside its destabilizing effect on the outer membrane, **NV716** also inhibits the activity of efflux pumps, but we do not know whether this results from a direct interaction with the pump or is indirectly related to the perturbation in membrane properties it induces. **NV716** can disturb the inner membrane potential, which could reduce the energy source of the RND pumps [353], but this effect is observed at concentrations higher than those for which potentiation of antibiotic activity is reached. Moreover, the increase in antibiotic accumulation inside bacteria is also observed for rifampicin, which is not substrate for efflux, indicating that the predominant mode of action of **NV716** is related to the perturbation of the integrity of the outer membrane. Importantly, the activity of **NV716** (and the two other potentiators) is observed at concentrations that do not cause cytotoxicity for eukaryotic cells [398], suggesting no or minimal interaction with eukaryotic membranes.

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

Considering first antibiotic potency, we observed that  $C_s$  was close to the respective MIC (1.5 to 4 x the MIC) in all cases, as previously described in other models of intracellular infection [188, 197, 477], and independently of the capacity of the drug to accumulate inside monocytes (Table S1) [190, 478]. This is interpreted as denoting a poor intracellular bioavailability [477]. Although gain in potency is a pharmacokinetic-driven parameter, meaning that it essentially reflects a change in the antibiotic concentration needed to reach a specified effect (bacteriostatic effect for  $C_s$ ; [477]), it was not related to the lipophilicity of the antibiotic (Figure S8A). We do not have a simple explanation for this absence of correlation, but wish to emphasize that the system here is more complex. In particular, accumulation of antibiotics in eukaryotic cells is not directly related to the hydrophilic/lipophilic balance, with azithromycin accumulating to much larger extent than ciprofloxacin, for example, while their calculated logD value is similar. In addition, the subcellular localization of antibiotics and bacteria may also play a critical role in this context. Interestingly, however, we notice that (i) for all drugs, the  $C_s$  measured in the presence of **NV716** is reduced to a similar value (0.3 to 1.2-fold the MIC)

and (ii) as a corollary, the highest gain in relative potency is obtained for antibiotics that were the less potent when used alone (Figure S8B).

Considering then antibiotic efficacy, we observed, as in our previous models, that all antibiotics fail to eradicate the infection intracellularly. In the present study, we were limited in the range of antibiotic concentrations we could test because of their intrinsic toxicity at high multiples of their respective MICs, but it remains that the  $E_{\max}$  or  $E_{\text{highest}}$  was ranging between reductions of 0.5 (linezolid against *E. coli*) to 2.5  $\log_{10}$  CFUs (ciprofloxacin against all strains), with most of the drugs causing a reduction of 1 to 2  $\log_{10}$  CFUs. These dissimilarities are probably attributable to differences in the mode of action of the antibiotics, and most conspicuously, to their bacteriostatic/cidal character, the less efficacious being the bacteriostatic drugs linezolid and fusidic acid, and the most efficacious, the highly bactericidal drugs ciprofloxacin and rifampicin. This is very coherent with data assembled for other models of intracellular infections, with linezolid, fusidic acid, and azithromycin showing no or low reductions in CFUs counts against intracellular *Staphylococcus aureus*, *Staphylococcus epidermidis* or *Listeria monocytogenes* [479-481], and rifampicin being highly effective against intracellular *S. aureus* and its small colony variants [482]. The intracellular activity of fluoroquinolones has been largely demonstrated against a vast array of pathogens like *S. aureus*, *L. monocytogenes*, *L. pneumophila*, *Burkholderia thailandensis*, *Yersinia pseudotuberculosis*, *Francisella philomiragia*, or *Coxiella burnetii* [188, 385, 389, 483]. The relatively high efficacy of doxycycline (bacteriostatic in broth) is rather unexpected but has been already reported in models of intracellular infection by *C. burnetii* [483], *Chlamydia trachomatis* [484], or *L. pneumophila* [485], possibly suggesting that the intracellular environment may contribute to improve its killing capacity. As efficacy is a pharmacodynamic-driven parameter reflecting bacterial responsiveness to antibiotics [477], it is not surprising that the gain of efficacy triggered by **NV716** does not correlate with the lipophilicity of the antibiotics (Figure S8C). But again, it is interesting to note that the mean maximal efficacy reached in combination is very close (-2.2 to -2.4  $\log_{10}$

CFUs) for all drugs except ciprofloxacin, which reached  $-2.8 \log_{10}$  CFUs, and that the difference in  $E_{\max}$  offered by **NV716** is all the more important for poorly effective drugs (Figure S8D). This suggests that part of the intracellular inoculum is refractory to antibiotic action and that this pool is relatively constant except if using an antibiotic that is already highly efficient by itself like ciprofloxacin. For *S. aureus*, intracellular survivors to antibiotics have been identified as persisters, i.e. phenotypic variants that adopt a dormant, non-replicative, and transiently non-responsive phenotype [138, 199]. This is why we examined the effect of **NV716** of persisters selected by ciprofloxacin and found that it was capable of decreasing the proportion of persisters in *E. coli*, *K. pneumoniae* and *A. baumannii* when combined with ciprofloxacin, but not when used alone, suggesting that it can resensitize persisters to the antibiotic but not kill them [379, 398]. Of interest, other molecules capable of disrupting membrane integrity also reduced the residual fraction of persisters upon exposure to antibiotics for different bacterial species [390, 391], and one of them (SPI009; 1-[[2,4-dichlorophenethyl] amino]-3-phenoxypropan-2-ol) was also shown to increase the efficacy of ciprofloxacin against intracellular *P. aeruginosa* [393]. Recently, another membrane-damaging small molecule, JD1, was found capable of reducing the survival of the intracellular Staphylococci and of persister cells in broth, as well as of disrupting biofilms [486].

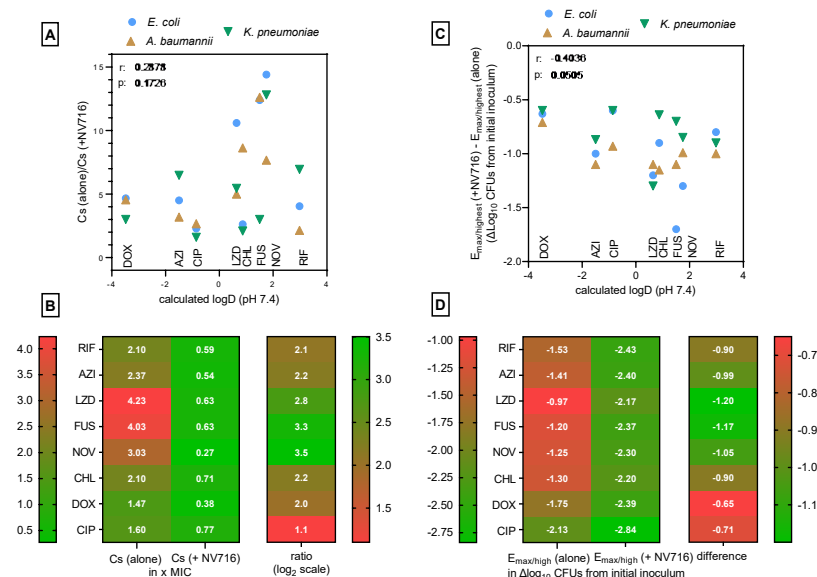


**Figure S8. Top:** correlation between the gain in potency (A) or in Efficacy (C) for antibiotics when used alone or combined with **NV716** in the intracellular model and the logD values of antibiotics (See Table S1). Data are expressed as the ration between the  $C_s$  calculated for the antibiotic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

Lastly, we also examined the effects of **NV716** combined with rifampicin on biofilms, and found that the potentiator can improve the activity of the antibiotic on the biomass and viability of the three Gram-negative bacterial biofilm models. This is consistent with our recent work in *P. aeruginosa* [460], where we also showed that **NV716** at sub-MIC concentrations enhances the penetration of the antibiotics in the deepness of the biofilm, downregulates quorum-sensing regulated processes, and therefore prevents biofilm formation. Thus, its effects partially contrast with those of JD-1, which does not prevent

biofilm formation and is effective alone against biofilm but only at concentrations higher than its MIC [486], which is also the case for **NV716** against *P. aeruginosa* [460].

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

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

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**Author contributions**

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

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**Competing interests**

The authors have no competing interests to declare.

## Chapter IV. Discussion and perspectives

### 1. Main achievements of this work

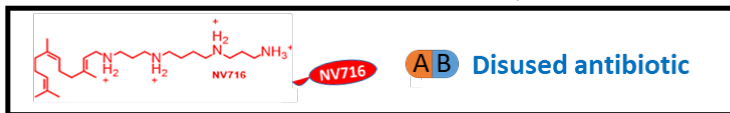
Antibiotic resistance represents an enormous global health crisis and one of the most serious threats facing humanity today. Some bacterial strains have acquired resistance to almost all antibiotics. In 2017, WHO released a list of antibiotic-resistant priority pathogens that cause a great threat to humans and are urgently needed new antibiotics. The majority of the WHO list is Gram-negative bacterial pathogens, such as *P. aeruginosa*, *E. coli*, *K. pneumoniae* and *A. baumannii*. Due to the active efflux and impermeable barrier, Gram-negative bacteria are intrinsically resistant to many antibiotics. Moreover, Gram-negative bacteria also can adopt specific lifestyles such as intracellular survival and biofilm formation, in which they can switch their phenotypes like persistence, poorly responsive to antibiotics.

Meanwhile, declining private investment and lack of innovation in the development of new antibiotics are undermining efforts to combat drug-resistant infections. Two recent reports reveal a weak pipeline for antibiotic agents. The 60 products in development (50 antibiotics and 10 biologics) bring little benefit over existing treatments and very few target Gram-negative bacteria [4]. Among them, antibiotics mainly include  $\beta$ -Lactams, tetracyclines, aminoglycosides, topoisomerase inhibitors, enoyl-[acyl-carrier-protein] reductase (FabI) inhibitor, oxazolidinones, macrolides and ketolides, and polymyxins; biologics include monoclonal antibodies, polyclonal antibodies and phage-derived endolysins.

In this context, the main aim of this thesis was to explore a novel therapeutic option, consisting in reviving disused antibiotics by combining them potentiators. We explored in details the potential interest of the polyaminoisoprenyl derivative NV716 to enhance the activity of antimicrobials against planktonic, intracellular and biofilm infection models,

### (1) Planktonic culture & persisters

The diagram illustrates the OmpC porin channel in the outer membrane of *E. coli*. The channel is a beta-barrel structure with LPS (lipopolysaccharide) on the outer surface and OMV (outer membrane vesicle) on the inner surface. A  $Mg^{2+}$  ion is shown entering the channel. A red arrow indicates the direction of transport. A green arrow labeled "efflux pump" points away from the cell. A yellow lightning bolt symbol is shown at the bottom left. A legend on the right lists various virulence factors: toxin, chemotaxis, protease, elastase, motility, and biofilm.



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### **1.1. Effect of NV716 on the activity of disused antibiotics against four Gram-negative bacteria**

Our data suggest that NV716 is the most effective compound to enhance antibiotic intrinsic activity in comparison with other potentiators such as PA $\beta$ N and NV731 against a series of Gram-negative bacteria. (section III. 1 and 3).

The MIC test is viewed as a convenient metric for resistance, and a bacterial strain with a higher MIC than another strain will be regarded as more resistant. But MIC has two major limitations: first, it is not informative for strains that are tolerant, rather than resistant; second, the MIC measured in vitro can vary according to the experimental conditions, which may affect the usefulness of this metric as a predictor of the effectiveness of the antibiotic in vivo [146]. However, the simplicity of the MIC determination justifies why it is currently the only metric that is routinely informative for treatment decisions of bacterial strains isolated in the clinics [487].

An in-depth reassessment of the efficacy of disused antibiotics needs therefore additional characterization, including using the modern standards imposed by pharmacokinetic/pharmacodynamic (PK/PD) concepts, and considering models of persistent infections like intracellular survival or biofilms.

Regarding the intracellular infection model, we compared the activity of drugs alone and combined with potentiators using two pharmacodynamic parameters:  $C_s$  (static concentration, intracellular MIC) and  $E_{max}$  (relative maximum efficacy) to compare the effectiveness of the treatment strategy. These parameters are respectively related to the active concentration of the drug ( $C_s$ ), and thus possibly to the expression of resistance, and to the killing capacity of the drug ( $E_{max}$ ), rather related to persistence. Our data show that NV716 can significantly reduce both values of  $C_s$  and  $E_{max}$ , indicating that NV716 can not only reverse four Gram-negative bacteria resistance to antibiotics, but also increase their effect on persisters.

Regarding biofilms, we assessed the effect of NV716 - antibiotics combinations towards biomass and viability (section III.2 and 3). Simplified in-vitro biofilm models have been instrumental in addressing basic questions about biofilm formation, physiology, and architecture. They generally mimic hallmarks of biofilm biology like different gradients of nutrients and metabolic products, as well as the production of extracellular matrix [488]. In vitro biofilm models can typically be classified into three distinct groups: (i) closed or static models, such as biofilm in microtiter plates [489], which allow direct rapid quantification of biofilm mass (crystal violet assay) or viability of cells (Fluorescein diacetate assay); (ii) open or dynamic systems, which allow the control the environmental parameters (shear forces) and mimic fluctuations of antibiotic concentrations over time [490], but are less adapted to high throughput analysis and often demand specialized equipment and technical skills (iii) microcosms models, which include more environmental parameters and take into consideration the complexity and heterogeneity of natural settings [491], as well as better simulate the diversity of biofilm species in the clinics as microcosm biofilms are directly developed from different niches in patients [491]. In our study, we developed the biofilms in 96-well plates, because of low cost, good repeatability, possibility of testing many conditions in parallel, and reliable results. We found that NV716 alone can prevent biofilm formation and that it increases the activity of antibiotics notably by improving their penetration in the biofilm, suggesting it has a direct destabilizing effect on the matrix. We cannot exclude an additional effect related to an improved penetration of the antibiotics inside bacteria (as shown in planktonic culture) neither to an enhanced antibiotic effect on persisters (as shown in stationary-phase cultures), but deciphering the contribution of each of these mechanisms would require specific studies.

## **1.2. Cytotoxicity of potentiators in THP-1 monocytes**

Due to unfavorable pharmacokinetic or toxicological properties, most of the current potentiators, for instance, PA $\beta$ N (MC-207,110; a typical EPI), PMBN (Outer Membrane Permeabilizers, OMPs) as well as alexidine (LPS-disturbing agents), are not used

(PA $\beta$ N, alexidine, PMBN) in the clinics. Our data show that NV716 exhibits no toxicity in vitro, with IC<sub>50</sub> (50% cytotoxic concentration) value approximately 10 times higher than the concentration at which it potentiates antibiotics (section III. 1).

Pharmaceutical safety is an essential factor in the development of every medicament [492]. The early stage of the development of the new compound must include wide toxicity screening. Traditional drug toxicity tests are a greatly important process for pharmaceutical developers, not only because of the associated loss of human life or health, but also because of immense financial loss of investment [310]. Early application of appropriate cell-based assays in drug development provides the most effective solution to the challenge of human toxicity [310]. Cell line models used to evaluate toxicity may contribute to predict human-specific toxicity. A single viability test or cell line model is unlikely sufficient as a final decision point for toxicity [493]. Thus, different test types on different cell cultures must be performed in a well-constructed toxicity screening procedure and help to select non toxic compounds for evaluation of their safety profile in vivo.

### **1.3. Possible mechanism of action at the molecular and transcriptomic level**

We found that NV716 can bind to LPS by replacing magnesium and calcium cations, causing OM permeability. Our transcriptomic analysis suggests that NV716 up-regulates genes involved in lipid A and LPS biosynthesis, while it also down-regulates bacterial virulence genes, resulting, among other effects, in impairment of biofilm formation (section III. 2).

The OM barrier plays an important role in antibiotic resistance, with LPS playing a major role in its barrier effects. OMPs are compounds that weaken the OM barrier and can non-specifically enhance the activity of many antibiotics that interact with intracellular targets. The use of OM permeabilizers, in combination with antibiotics, may provide an impressive strategy against Gram-negative bacterial infection. For example, as early as 2010, Vaara et al. [494] reported that SPR741 (also namely NAB741), a derivative of

PMBN, shows notably synergistic with rifampin and clarithromycin against *E. coli*, *K. pneumoniae*, *E. cloacae*, and *A. baumannii*. In 2017, SPR741 completed phase Ia and Ib clinical trials with a promising pharmacokinetic and tolerability profile [495]. In addition, MacNair et al. reported in 2020 that SPR741 as OMP can overcome intrinsic, acquired, and spontaneous antibiotic resistance, and enhance antibiotic activity against biofilms by OM disruption [396], which is consistent with the results we obtained with NV716.

Our results also suggest that inhibition of quorum sensing may provide additional antibacterial effects useful for treating bacterial infections. Due to the fact that QS is involved in the control of the expression of genes encoding many virulence factors and is important for biofilm formation, compounds that inhibit these processes are a class of therapeutics that may work well as antibiotic adjuvants. There are three ways in which QS can be inhibited. First, by interfering with the binding of QS signaling molecules to their receptors. Bassler and colleagues [496] showed that meta-bromo-thiolactone not only prevents virulence factor expression and biofilm formation, but also protected *C. elegans* and human A549 lung cells from killing by *P. aeruginosa*. Tan et al. conducted virtual screening on a library of natural compounds and were able to identify five molecules that bind to the LasR protein in *P. aeruginosa* and alter downstream gene expression [497]. The most promising inhibitor was also able to decrease the amount of extracellular DNA released by *P. aeruginosa* in biofilms. The natural product furanone was able to inhibit *P. aeruginosa* biofilm formation, modestly protect mice from chronic *P. aeruginosa* infections and act synergistically versus biofilms with the antibiotic tobramycin [498, 499]. Second, by destroying the quorum-sensing molecule itself. It has been shown that many enzymes can cleave acyl-homoserine lactones and quinolones produced by various species into molecules that cannot bind to QS receptors [499, 500]. Chow et al. [501] recently designed a lactonase that can significantly decrease the thickness and mass of biofilms formed by *A. baumannii*. Third, by inhibiting the production of QS molecules by impairing the activity of the enzymes synthesizing them, such as LasI and RhII. In a high-throughput screen for such inhibitors, Christensen et al.

found two inhibitors that were active against the acyl-homoserine lactone synthases of both *Burkholderia mallei* and *Yersinia pestis* [502]. Regarding NV716, our results suggest that it may affect QS systems by inhibiting the production of QS molecules, based on the fact it causes a downregulation of *lasI* and *rhII* as well as *pqsH* genes, which are involved in the synthesis of 3-oxo-C12-HSL, C4-HSL and PQS molecules.

#### **1.4. Comparison of the effect of NV716 on disused antibiotics activity against different species of Gram-negative bacteria**

Our findings indicate that NV716 shows a synergistic effect when combined with disused antibiotics against *P. aeruginosa*, *E. coli*, *K. pneumoniae* and *A. baumannii* in planktonic, intracellular and biofilm models, by disturbing OM permeability and inhibiting efflux (section III. 3).

Although all these pathogens are Gram-negative bacteria, their outer membrane proteins, permeability and efflux pumps vary. In *E. coli*, the integral membrane proteins, such as OmpA and the general porins OmpF/C, are expressed at high levels [69]. The permeability properties of *E. coli* membranes are largely defined by general porins, like OmpF, which have an exclusion limit of about 600 Da [69]. Unlike *E. coli*, the outer membrane of *P. aeruginosa* and *A. baumannii* do not contain general trimeric porins such as OmpF and OmpC. It is estimated that the permeability of *P. aeruginosa* and *A. baumannii* outer membranes is only 1 - 8% of that of *E. coli*. This low permeability is attributed to the presence of nonspecific 'slow' porins in their outer membrane, like OprF and OmpA-AB, respectively. These 'slow' porins restrict the access of molecules larger than about 200 Da, which is the case for most antibiotics [503, 504]. *K. pneumoniae* produces *E. coli*-like trimeric porins OmpK36 (an OmpC homolog) [505] and OmpK35 (an OmpF homolog) [506]. Although the permeability of these porins has not been systematically compared with that of *E. coli* porins, it is reported that the permeability to benzylpenicillin of the OM of this species higher than via *E. coli* OmpF, while the permeability to ampicillin is actually similar [507]. In our study, we find that the capacity

of NV716 to decrease the MIC of DOX and RIF is higher in *E. coli* than in *P. aeruginosa*, while the opposite is observed for chloramphenicol, in spite of the fact that NV716 proved equipotent and equi-efficient to increase OM permeability in both species. This indicates that the factors determining antibiotic penetration inside Gram-negative bacteria are complex and intermingled.

In particular, outer membrane proteins like efflux pumps also affect OM permeability and contribute to multidrug resistance in Gram-negative bacteria. A report recently elaborated that synergy between active efflux and the outer membrane barrier universally protects Gram-negative bacteria from antibiotics, that the diversity of antibiotics affected by active efflux and outer membrane barriers is wider than previously thought, and that antibiotics accumulate in cells according to specific biological determinants such as the requirement of specific porins in the OM, targeting of the OM, or specific recognition by efflux pumps. [508].

Whittle et al. [509] recently reported that efflux pumps are important to maintain a low accumulation of antibiotics only in actively growing cells, which have a more permeable envelope. However, if the cells are in a slow-growing or non-growth state, a low membrane permeability is fundamental to maintain low drug accumulation and thus efflux inhibitors may not be an effective treatment option. This offers us enlightenment that in stationary bacteria the effect of a triple combination of antibiotics, EPIs and OMPs may be more effective than that of a double combination of antibiotics plus EPIs or OMPs. Espada [510] recently found that a synergy between PMBN and EPI boosted the activity of azithromycin by 2133 times, and this triple combination resulted in a 10 million-fold decrease in the viability of biofilm-forming cells. The compound NV716 that shows effects on both efflux pumps and outer membrane can simultaneously play the role of EPI and OMP, with, as a consequence, a more important synergistic effect with antibiotics than typical EPIs (PA $\beta$ N) or OMPs (PMBN).

### **1.5. The General Relationship Between Efflux Pump and QS**

A previous study reported that lack of MexAB-OprM pump results in the accumulation of QS molecule 3OC12-HSL in the cell and limits cell-cell communication [511]. A posterior study suggested that overexpression of MexAB-OprM results in increased secretion of a quorum-sensing molecule such as 3OC12-HSL, as this molecule is a substrate for MexAB-OprM [512], which decreases the expression of the virulence factors controlled by QS. Conversely, expression of MexAB-OprM is induced by quorum-sensing molecule C4HSL, which results in more 3OC12-HSL-LasR binding specificity and additional control of the gene expression regulated by QS [513]. In our works, NV716 can downregulate the QS regulated genes, however, it did not affect the expression of *mexAB* or *oprM* genes.

## **2. Limitations of this work**

### **2.1. The study models were far from the clinical situation**

In the pharmacodynamic model, our experimental work is mainly limited to a simple in vitro model of THP-1 human monocytes and a biofilm developed in a 96-well polystyrene plate. These models do not consider the complexity of factors that interfere with the development of infections in the body.

For intracellular bacterial infection, we chose human THP-1 monocytes as the cell model, which formed a homogeneous and reproducible population [514, 515]. They have been successfully used in various studies on the interaction between *Pseudomonas aeruginosa* and phagocytes [516-518] and the intracellular activity of antibiotics [519, 520]. THP-1 cells show a low level of intrinsic bactericidal mechanisms and are therefore more susceptible to infection than macrophages. This allows us to study the effects of antibiotics without (or with limited) interference of the host cell defense mechanisms [197]. Our team has recently shown that human macrophages as non-permissive cells can kill intracellular bacteria and select an intracellular reservoir of persisters in the

absence of antibiotics [199]. Therefore, using permissive cells seems more appropriate for a study dealing with the evaluation of antibiotic activity. Moreover, such cells may better mimic the situation faced by immunodeficient patients, who are the most suffering from severe infections by the bacteria we studied here.

Regarding biofilms, 96-well polystyrene plates are commonly used carriers to develop biofilm models in vitro for *P. aeruginosa* or other Gram-negative bacteria [186, 521]. The interest of this model is that it allows to test many experimental conditions in parallel. Previous work in our laboratory with *S. aureus* biofilms has also shown that the results obtained in this model are consistent with the results produced by more complex in vitro models (such as biofilms grown on catheters) and even in vivo [522].

Despite their intrinsic limitations, in vitro experiments have maintained their advantages and can bring useful information to further study. In particular, they may contribute to a more detailed understanding of biological processes and pharmacological reactions. Because they are simpler, each factor involved in the research process can be examined. Therefore, in vitro models have been considered useful for preclinical evaluation of drug actions. At this stage, it is impossible to directly extrapolate our conclusions to the in-vivo situation, but our work may pave the way to directions for future investigations.

## **2.2. The cellular accumulation of antibiotics and potentiators were not examined**

Another limitation in our study is that we did not measure the cellular accumulation of antibiotics and potentiators in our intracellular models. As we described in the introduction, antibiotics first should enter and accumulate inside the eukaryotic cells like THP-1 monocytes before they exert killing intracellular bacteria. As in bacteria, the accumulation of drugs inside eukaryotic cells is modulated depending on the mechanisms responsible for their uptake and efflux.

Likewise, we did not measure the accumulation of potentiators inside bacteria. The pharmacological targets of the antibiotics we tested are located in the cytoplasm of

bacteria. Thus these antibiotics need to cross two membranes (OM and IM) before they can rejoin their targets to exert an antibacterial effect. This is not necessarily the case for potentiators, as we showed that they interact with the membrane, and that this may suffice to explain their ability to increase the accumulation of antibiotics inside the bacteria. Nevertheless, this does not exclude that NV716 could also enter inside the bacteria and possibly exert additional intrabacterial effects.

### **3. Perspectives**

These limitations, coupled with the innovative conclusions brought by our work, open a series of perspectives for future work.

#### **3.1. Other phagocytic cells (less permissive to infection, like human macrophages) or airway or intestinal epithelial cells**

As introduced in the previous section, a limitation of our work is that we used an in-vitro model of THP-1 human monocytes only, which cannot mimic the complexity of factors involved in the development of clinical infections. It would therefore be interesting to evaluate the effect of NV716 on disused antibiotics activity in intracellular models with other cell types like airway or intestinal epithelial cells, in which the fate of bacteria could be different [523, 524]. Human macrophages collected from fresh blood differentiated into macrophages from patients with CF and healthy individuals could be included as well. but, as explained above, this will give us a global picture of the effect of antibiotics, potentiators, and cell defense on intracellular survival. In addition, macrophages obtained from mice with CF and control mice could be included, such as pulmonary macrophages, peritoneal macrophages and alveolar macrophages.

#### **3.2. Determination of the cellular accumulation of antibiotics and potentiators**

According to our results, different antibiotics show various levels of synergistic effects with NV716 both in planktonic cultures and intracellularly. For drugs with different modes

of action, there is no reason to have a direct relationship between their concentration at the target site (whether extra- or intra-cellular) but it remains of interest to examine to which extent potentiators can modulate their concentrations in bacteria as well as in eukaryotic cells.

Regarding the accumulation of potentiators, a specific method for quantifying them with high sensitivity would be required to evaluate their concentration in cells and bacteria. Yet, we found that higher concentrations of NV716 are needed to improve antibiotic activity against intracellular than against extracellular bacteria, suggesting that it does not well penetrate the eukaryotic cells and/or that its activity is defeated intracellularly, which would need to be specifically studied. To test for this hypothesis, it would be useful to synthesize fluorescent derivative NV716 (as has been done for rifampicin) to determine the cellular accumulation of the potentiator.

### **3.3. Gram-positive bacteria**

Our entire research focused on the effect of NV716 at sub-MIC on the OM of Gram-negative bacteria. But we also found that NV716 can disturb the IM at its MIC, which could encourage to extend our studies to other species, notably among problematic Gram-positive bacteria, in planktonic cultures, as well as intracellularly or in biofilms.

### **3.4. Animal models**

Although we have produced a large amount of in-vitro experimental data, we are still far away from clinical applications. Animal models of infections should be used if wishing to bring NV716 to possible therapeutic applications in a long-term future.

This study should cover pharmacokinetic/pharmacodynamic and toxicokinetic studies. In-vivo pharmacology models generally include thigh infection, peritonitis, pneumonia (including in CF mice, although they badly reproduce the pulmonary manifestations of the disease) and urinary tract infection. For biofilms, infected catheters implanted in

mice represent a reproducible model [525]. For intracellular infection, our lab previously developed in collaboration with a Danish team a mixed model of extracellular and intracellular in vivo mice peritonitis model by *S. aureus* that could possibly be adapted to other bacterial species [526].

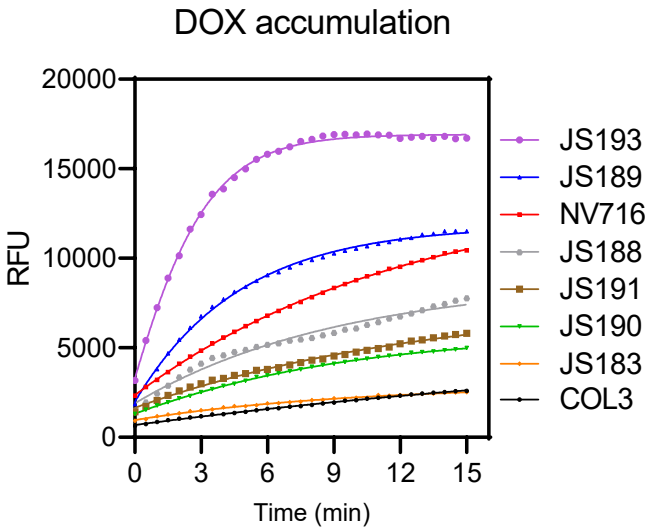
### **3.5. Screening of potentiators with a wider spectrum**

The current state of the chemical and biological sciences required for pharmaceutical development dictates that 5,000–10,000 chemical compounds must undergo laboratory screening for each new drug approved for use in humans. Of the 5,000–10,000 compounds that are screened, approximately 250 will enter preclinical testing, and 5 will enter clinical testing. In addition, less than 15% of the compounds entering clinical development are finally approved, although most of them are considered safe [527].

We tested here a very limited number of compounds. However, during a short-term study exchange with Aix-Marseille University, we identified more compounds (namely JS193, JS188 and JS189) that are based on the structure of polyaminoisoprenyl derivatives, which shows a synergy with tetracyclines against *P. aeruginosa* and *E. coli* even higher than that of NV716, while also proving less toxic (Table 6). Among them, JS193 was the most effective compound to increase doxycycline accumulation inside bacteria cells, while JS188 and JS189 were similar to NV716. These results encourage us to continue exploring these promising compounds.

**Table 6.** MIC of tetracyclines against *P. aeruginosa* PAO1 and *E. coli* AG100 (both strains are wild type) in presence of or absence of potentiators. Values in bold highlight significant decrease in MIC (at least 2 doubling dilutions); values in brackets: number of doubling dilution decrease ( $\log_2$  fold change) for MIC of antibiotic combined with potentiator compared with antibiotic alone. The figure (below) shows the doxycycline accumulation kinetics in *P. aeruginosa* PA263 (PAO1 derivative impaired in pyoverdine production) with or without these potentiators.

| Strains | Abs             | Abs alone | NV716            | JS189       | JS193           | JS188       | +JS190         | +JS183 | +JS191         |
|---------|-----------------|-----------|------------------|-------------|-----------------|-------------|----------------|--------|----------------|
| PAO1    | Doxycycline     | 16        | <b>2 (3)</b>     | <b>2(3)</b> | <b>0.125(7)</b> | <b>2(3)</b> | 16 (0)         | 16(0)  | 8(1)           |
|         | Tetracycline    | 32        | <b>8(2)</b>      | <b>8(2)</b> | <b>2(4)</b>     | <b>8(2)</b> | 16(1)          | 16(1)  | 16(1)          |
|         | Oxytetracycline | 32        | <b>8(2)</b>      | <b>8(2)</b> | <b>2(4)</b>     | <b>8(2)</b> | 16(1)          | 16(1)  | 16(1)          |
|         | Minocycline     | 16        | <b>2(3)</b>      | <b>4(2)</b> | <b>0.5(5)</b>   | <b>2(3)</b> | 16(0)          | 16(0)  | 8(1)           |
| AG100   | Doxycycline     | 1         | <b>0.125 (3)</b> | 0.25 (2)    | 0.008 (7)       | 0.25 (2)    | 0.5(1)         | 1(0)   | <b>0.25(2)</b> |
|         | Tetracycline    | 1         | 0.5 (1)          | 0.25 (2)    | 0.008 (7)       | 0.5 (1)     | 1(0)           | 1(0)   | 1(0)           |
|         | Oxytetracycline | 1         | 1 (0)            | 0.13 (3)    | 0.008 (7)       | 1 (0)       | 1(0)           | 1(0)   | 1(0)           |
|         | Minocycline     | 1         | <b>0.25 (2)</b>  | 0.13 (3)    | 0.004 (8)       | 0.13 (3)    | <b>0.25(2)</b> | 0.5(1) | <b>0.25(2)</b> |



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