UNIVERSITÀ POLITECNICA DELLE MARCHE

**DEPARTMENT OF LIFE AND ENVIRONMENTAL SCIENCES**

**PhD Course in**

## Life and Environmental Sciences

**Curriculum:** ***Biomolecular Sciences***

# Validation of novel inhibitors of MexXY-OprM and NorA efflux pumps to counteract the antibiotic resilience of *P. aeruginosa* and *S. aureus*

PhD candidate:
Nicholas Cedraro

Tutor Chiar.ma
Prof.ssa Francesca Biavasco

XXXIII Cycle

2017 - 2020

# SUMMARY

## Introduction



## Aim of the work

## Material and Methods

## Results and Discussions



## Conclusions

## Bibliography

## Sources of images

# INTRODUCTION

# Antibiotics and Antibiotic Resistance

*A short history*

Among the greatest achievements of medicine in human history, the discovery of antibiotics is considered one of the most important and maybe, the most revolutionary. Actually, the use of antimicrobial compounds is much more antique than commonly thought: although the existence of bacteria (and their role in infectious diseases) was still far from being discovered, moldy bread and medicinal soils were recommended and used from ancient populations in the treatment of open wounds [52]; moreover, a 1000-years ago Anglo-Saxon formula was recently shown able to exert antimicrobial activity and to kill methicillin-resistant *Staphylococcus aureus* [51]. In the late nineteenth century the interest of scientists over molds' inhibiting effect on bacteria increased [76] and in 1895 Vincenzo Tiberio proved the antibacterial activity of extracts of molds belonging to *Penicillum* and *Aspergillum* genera but his work, published on a local journal, went completely unnoticed [20].

Humankind had to wait the beginning of the XX century for the first synthetic preparation with an intended antimicrobial activity: the arsenic-derivate *salvarsan*, developed by Paul Ehrlich and released in 1913 was the first real chemotherapic used for the treatment of syphilis [46]. Other similar efforts have been made in subsequent years, such as the sulfonamide *prontosil*, discovered by Gerhard Domagk [88], but the real breakthrough arrived with the discovery of penicillin by Alexander Fleming in 1928 (and its purification and large-scale production ten years later) that gave rise to the "antibiotic era" [52].

The "miraculous effect" of penicillin and sulfonamides during World War II entered the collective imagination (**Fig.1**) and the search for new antibiotics accelerated considerably: from the 1940s to the 1960s almost all of today's known antibiotic classes were discovered, making this period known as "the Golden Age" of antibiotics discovery [76].

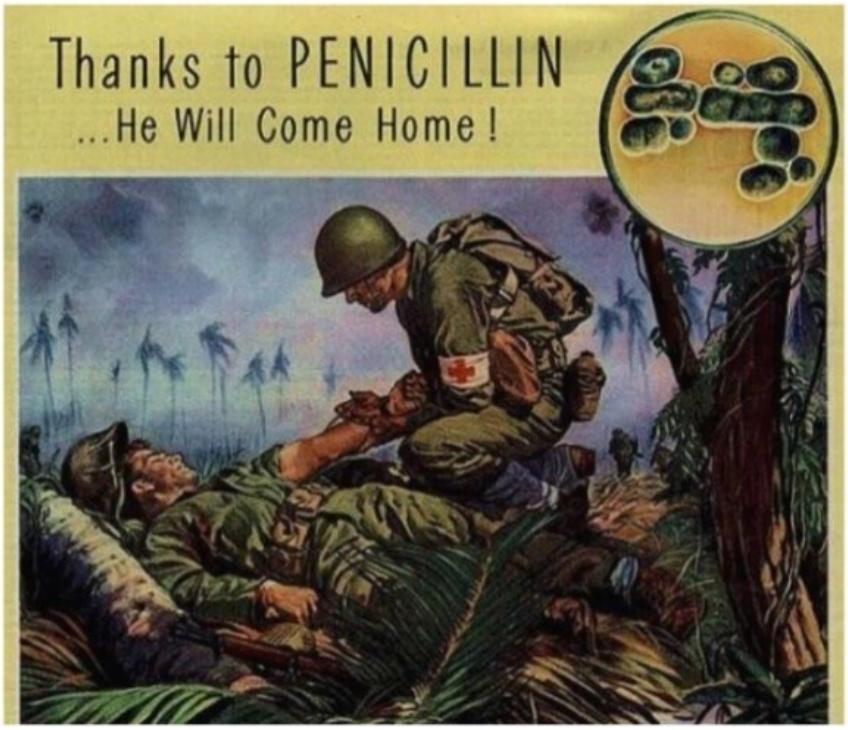


**Figure 1** The famous poster claiming the miraculous effect of penicillin, appeared in August 1944 on *Life* magazine.

To date, it's difficult to estimate the overall amount of lives saved by antibiotics, but after their introduction life expectancy grew by about 30 years in USA [2] and complex chirurgical operations like organ transplants and open-heart surgery became routinely executed [52].

Unfortunately, the illusion of an ultimate victory over bacterial infections was short-lived: the first cases of resistance to sulfonamides were reported in the late '30s, namely few years after their discovery [32]. The same happened with penicillin: a bacterial penicillase (an enzyme able to destroy the drug) was discovered in 1940, practically at the same time as the start of the "antibiotic era" [1]. This pattern repeated itself continuously in the subsequent years: the discovery or the synthesis of a new antibiotic was followed (shortly thereafter) by the report of a bacterial strain that could survive in its presence, or of a bacterial enzyme able to impair the drug (**Fig.2**).

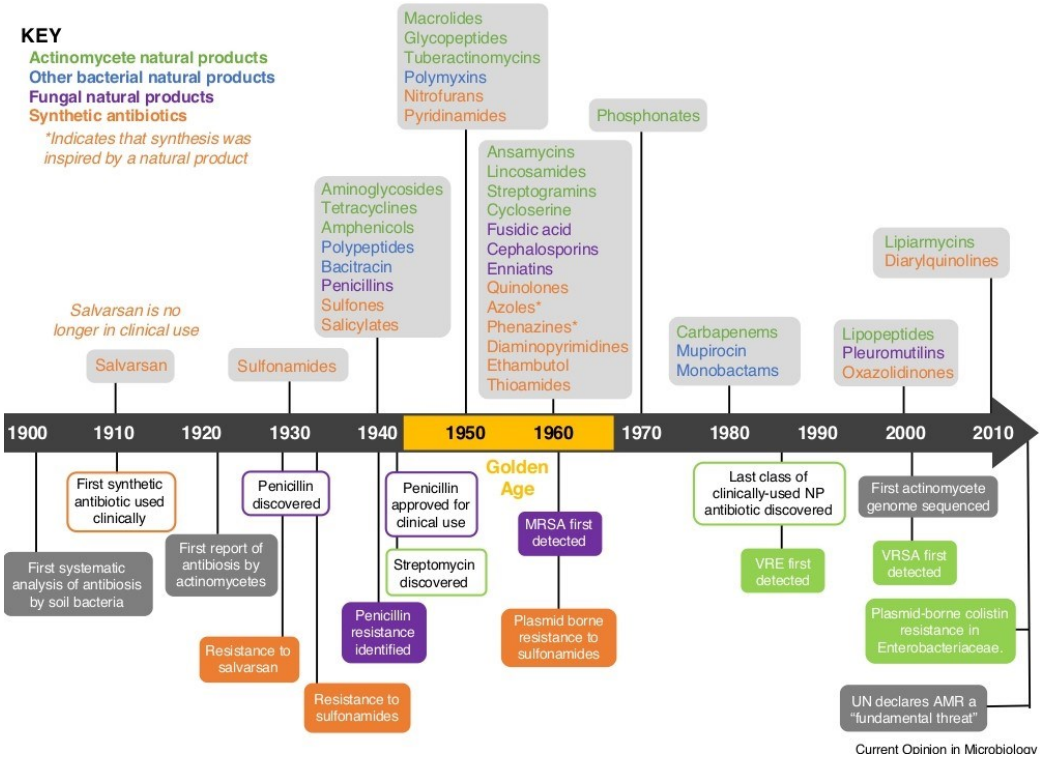


**Figure 2** Timeline showing the principal progresses in antibiotic discovery and synthesis as well as the first reporting of resistance.

The problem began to concern scientists and made clear the possible vulnerability of antimicrobials: Alexander Fleming itself wrote in 1946: "there is probably no chemotherapeutic drug to which in suitable circumstances the bacteria cannot react by in some way acquiring 'fastness' [resistance]" [42]. And this was only the beginning, as in the mid 50's Japanese scientists reported strains of *Shigella dysenteriae* simultaneously resistant to multiple classes of antibiotics, rapidly spreading all over Japan, and whose resistance genes were harbored in conjugative plasmids. Practically, resistance determinants were found to be able to transfer themselves and to accumulate in a microorganism's genome [4, 31].

Although the rising of the resistance phenomenon was therefore known and well-studied, the continuous discovery/synthesis of new antimicrobial agents has somehow hidden under the carpet the threat of the return of a pre-antibiotic era for decades. However, the race to the discovery of new antibiotics has slowed down during the years, while the resistances have been simultaneously more and more reported in practically every clinically significant microorganism. Only in the very last few years the trend seemed to be slightly changed, as 9 new molecules or combination of molecules have

been approved by the American Food and Drug Administration (FDA) in 2018 and 2019 [8], but the last completely new antibiotic was daptomycin, discovered in 1986 and approved by FDA for clinical use in 2003 [39]: therefore, antibiotic resistance is currently "running" faster than new antibiotics development.

How could antimicrobial resistance appear and spread so quickly? Actually, although antibiotics have been used only for the last 80 years, they are mostly natural compounds produced by bacterial or fungal species, or closely-derived synthetic molecules: this means that they existed in nature since, at least, thousands of years. Likewise, bacterial mechanisms have evolved simultaneously in order to survive and counteract the effect of these compounds. For example, microorganisms belonging to the order of the *Actinomycetales* (Gram-positives typical of the soil environment) are prolific producer of antimicrobial compounds so that most of the antibiotics currently used are derived or produced by them: it follows that they must also have "instruments" without whom they would die by their own antibiotics. So, most antibiotic resistance genes may originate from antibiotic-producer bacteria [93]. For a long time it has been thought that the production of antibiotics by microorganisms just helps them in the ruthless competition for resources (nutrients, oxygen, space, etc…) in the soil environment, but probably reality is much more intricate than that, considering that most of the antimicrobial compounds act as transcriptional regulators at low and sub-inhibitory concentrations, and many intermicrobial communication signal molecules can be toxic at high concentrations. Also, many resistance determinants have been shown to be involved in other important functions as detoxification of metabolic intermediates, virulence, and signal transferring [72].

Therefore, the whole "arsenal" of antibiotic-producing and antibiotic resistance determinants was already developed and present in the microbial world for a very long time: with the beginning of the antibiotic era a strong selective pressure has been enforced on bacteria, promoting in a short time the spread of those species able to survive and replicate in the presence of antimicrobial drugs; this phenomenon was then strongly enhanced by the abuse and misuse of antibiotics in the clinical practice and by their extended usage in animal farms [4].

Today, the size of the problem is massive and, according to the scientific community, it can easily get out of control in the next future. Infections are the second leading killing cause in the world; every year about 25000 people in Europe and 23000 people in USA die from infections of antibiotic resistant bacteria [71]. A recent review

commissioned by the United Kingdom government estimated about 700000 global deaths per year caused by antibiotic resistant infections today and, in the worst scenario, the toll could rise to 10 million deaths per year in 2050, with a countless economic and social cost [85]. With such alarming perspectives, there is a growing commitment from the global scientific community to defuse this threat by a deeper understanding of the antimicrobial resistance mechanisms, a better and more optimized use of antibiotics and a growing effort in finding new drugs or therapeutic options; as stated in the "Global action plan on antimicrobial resistance" endorsed by the World Health Organization in 2015.

*Resistance mobility*

Different classes of cellular mechanisms and many ways to survive the toxic effect of antibiotics have been described in bacteria. In every case, resistance allows the microorganism to grow in presence of antibiotics and is heritable; however, the resistant phenotype can be lost in the different generations if the mechanism underlying has a high metabolic and fitness cost [7]. The first important distinction that is usually made in the description of the antibiotic resistance is between intrinsic and acquired resistance. A microorganism is considered "intrinsically resistant" to an antibiotic when the bacterium's physical characteristics prevent the drug from working properly: a typical example is the outer membrane of Gram-negative bacteria, often impermeable to many antimicrobial compounds, or the possible lack of the drug target in the bacterial cell [16]. Acquired resistance mechanisms are much more worrisome, since they are not only able to nullify the antibiotic action, but also to "move" and transfer themselves in other bacterial cells in an intraspecific or interspecific way. A bacterial strain with acquired resistance has thus switched from a susceptible to a resistant phenotype, with the natural consequence of an unpredictability of its behavior, especially if involved in an infection. The acquisition of resistance determinants (and in general, the acquisition of new genetic material) is also referred to as Horizontal Gene Transfer (HGT), and can occur through three ways: transformation, transduction and conjugation [7] (**Fig.3**):

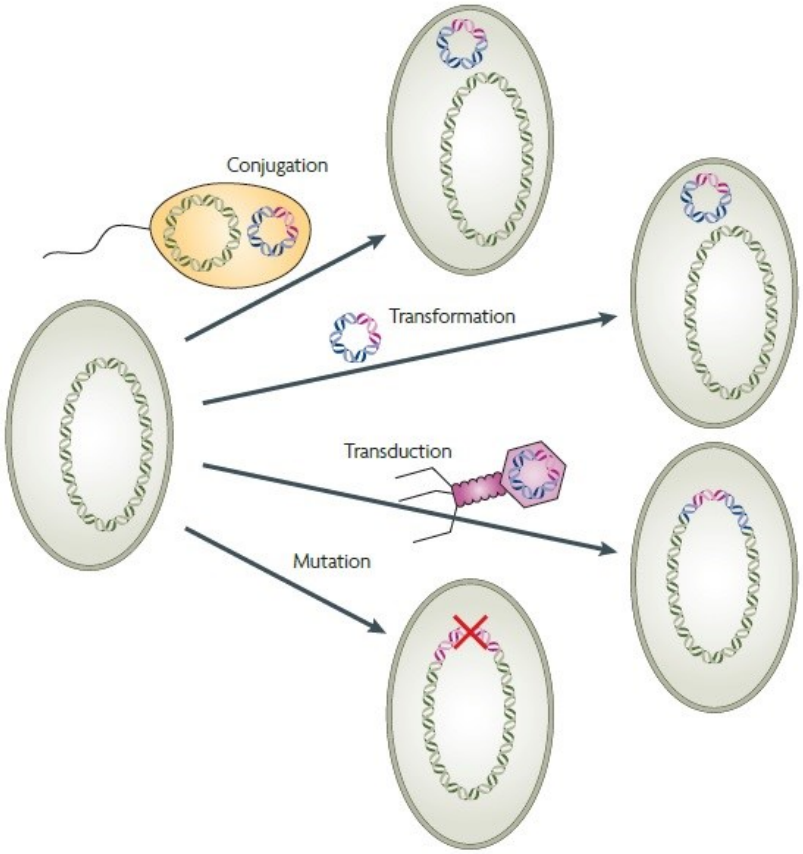


**Figure 3** Four possible ways of acquiring resistance: through a mutation or from the outside via conjugation, transformation or transduction.

Horizontal Gene Transfer (HGT) is probably the main responsible for the rapid spread of antibiotic resistance: thanks to this ability, bacteria can develop features necessary for survival much faster than through spontaneous mutation [63]. MGEs in particular have been demonstrated to play a crucial role in the sharing of resistance determinants and, *de facto*, they end up constituting a large resistance gene pool, from which each cell of the microbial community can draw in case of necessity [92].

Different types of mobile genetic elements (MGEs) have been described so far. Insertion sequences (IS), transposons (Tn) and integrons (In) are able to transfer themselves within and between DNA molecules. Usually ISs cannot carry inside them genes other than transposases. Sometimes two adjacent IS can move together, along with all the sequence between them that can possibly contain resistance genes, in a so-called "composite transposon" [92].

Transposons are not so different from the latters: they consist of a DNA section bounded by two IR sequences and they include a *tnp* gene and internal passenger genes. Transposons can move inside the cell and are divided in families; in particular, antibiotic resistance genes are associated mostly with Tn3 family and Tn7 family: one of the most famous, Tn1546, carries the *vanA* operon responsible for vancomycin resistance in enterococci and staphylococci [9].

Integrons can move through site-specific recombination and can carry one or more "gene cassettes" into it. Gene cassettes may contain genes (also resistance genes) but they usually lack a promoter, that they "find" in the integrons. Integrons can thus harbor multiple resistance genes, each in a gene cassette that can be inserted in the integron through *attI/C* recombining site. Different classes of integrons have been described, with the class 1 reported as the most common in antibiotic resistant clinical isolates [50].

Intercellular genomic mobility is instead mediated by plasmids and Integrative Conjugative Elements (ICE).

Probably, plasmids are the first contributors of the dissemination of resistance, both in Gram-negative and Gram-positive bacteria and can contain resistance genes or resistance regions constituted by IS, Tn or integrons containing resistance genes [92]. Several plasmids have been described to date, classified by different systems and usually identified by plasmid multi-locus sequence typing (pMLST) or PCR-based replicon typing (PBRT). Plasmids are spreaders of resistance so efficient that it's not rare to find completely identical plasmids, that can be called "epidemic plasmids" in unrelated or geographically distant bacterial strains, without an apparent epidemiological link [22].

Integrative conjugative elements are mobile DNA molecules able to transfer themselves by conjugation but uncapable to subsist as autonomous elements as well as of self-replication. They have been found both in Gram-positive and Gram-negative bacteria and usually consist of a "backbone", coding for maintenance/replication and integration functions, and accessory genes including resistance genes [35].

Horizontal gene transfer contributes significantly to the extreme "plasticity" of the bacterial genomes, conferring survival chances to microorganisms in unfavorable conditions. One of the most worrying potentials that HGT offers to bacteria is the possibility to accumulate resistance determinants, thus acquiring simultaneous resistance to multiple antibiotics.

Bacteria are defined Multi-Drug Resistant (MDR) when resistant to at least one antibiotic of three different classes and are of particular concern in clinical settings since

they limit or nullify the therapeutic options. The maximum expression of the antibiotic resistance threat is the formation of extensively (XDR)- or pan- (PDR)- resistant strains, which are unsusceptible to five or all the available antibiotics [69]. The isolation of these nearly invincible strains, that have gained the media attention with the name of "superbugs", is an increasingly common event, especially in hospital environment where even small surgeries can now cause severe bacterial infections and immunocompromised patients are completely defenseless towards them [18]. A particular group of bacterial pathogens, potentially problematic in nosocomial settings, is emerging all over the world for their pathogenesis, their easily transmission and their strong proneness to survive the action of antibiotics: the ESKAPE group, which refers to *Enterococcus faecium, Staphylococcus aureus, Klebsiella pneumoniae, Acinetobacter baumanii, Pseudomonas aeruginosa* and *Enterobacter* species [105]. They are all mentioned in the WHO "Priority Pathogen List" for the research and development of new antibiotics published in 2017 [117] (**Fig.4**), and therefore they represent today a true challenge in global public health.

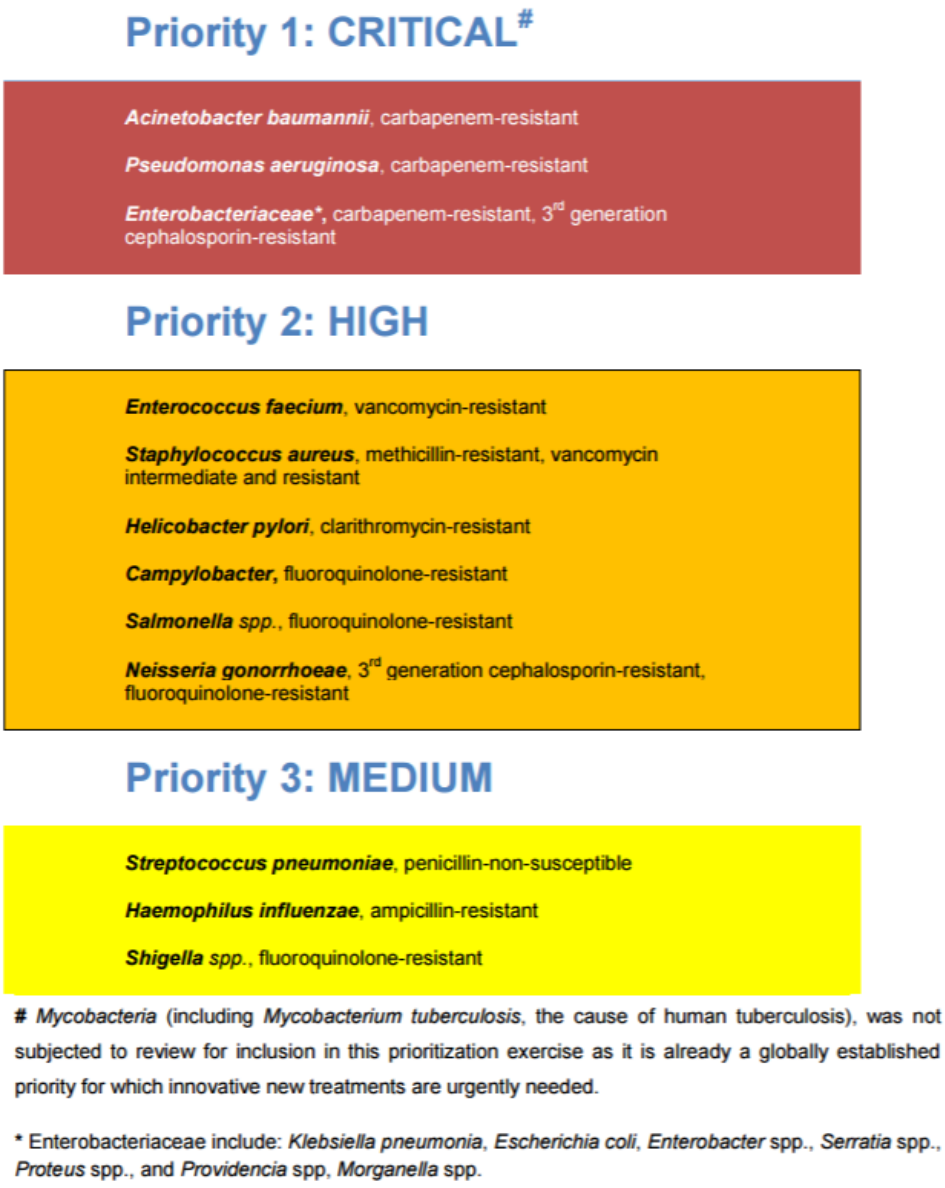


**Figure 4** The priority list for the research and development of new antibiotics, released by WHO in 2017. Microorganisms whose antibiotic resistance represents an emergency are divided in three categories based on their urgency: critical, high and medium.

*Resistance mechanisms*

Antimicrobial drugs exert their action in multiple ways: many antibiotic classes have been discovered and synthetized during the years, but all of them need to reach and bind their target, usually interrupting a bacterial metabolic pathway. Likewise, microorganisms developed several different ways to escape antibiotic's activity. Briefly, acquired resistance mechanisms can be summarized in 4 key concepts [14] (**Fig.5**):

- Lower permeability to the drug
- Modification/protection of the antibiotic target (and "metabolic bypass")
- Modification/inactivation of the antibiotic
- Expulsion of the antibiotic (efflux)

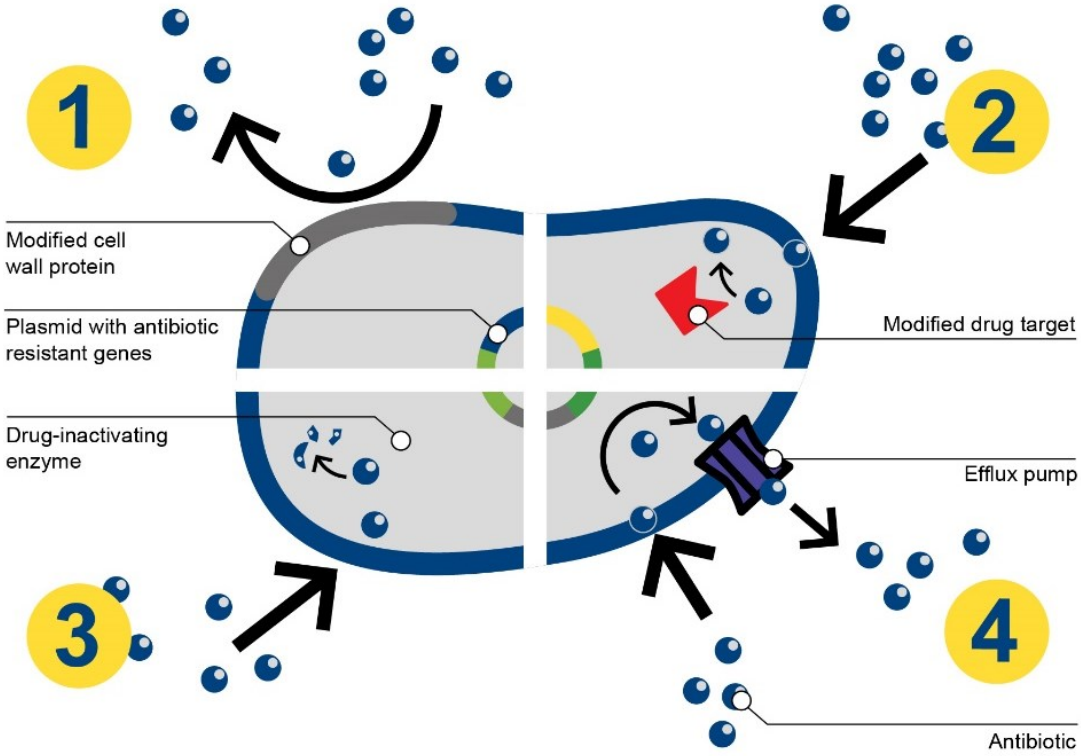


**Figure 5** Schematic representation of acquired resistance mechanisms: lower permeability (**1**), target modification/protection (**2**), antibiotic modification/inactivation (**3**) and antibiotic efflux (**4**).

**Lower permeability**

In many cases, the target of the antibiotic is inside the bacterial cell, in the cytoplasmatic space. Therefore, a lower permeability to the entrance of the drug prevents very efficiently the antibiotic-target interaction that would be lethal to the bacterial cell. Gram-negatives are able to achieve this result thanks to a very selective outer membrane: the particular composition of phospholipids and lipopolysaccharides (LPS) constitutes a very low-fluid and cross-linked membrane, through which the passage of both hydrophilic and hydrophobic molecules is extremely difficult [83]. The passage of molecules, especially hydrophilic, is thus possible only through porins (specialized transmembrane proteins with a water-filled inner channel), which may vary highly in number and dimensions, but often an upper size threshold prevents the entrance of large antibiotics thus conferring resistance to that drug [111].

Reduced expressions of porins genes or their mutation could lead to increased levels of antibiotic resistance. *Pseudomonas aeruginosa* can provide a typical example, as one of the keys of its high-level resistance relies on an extremely low-permeable outer membrane, 12-100 times less permeable than *E. coli* [19]: most of its porins are small and dedicated to nutrients; the porin oprF is highly expressed, but most of times in a closed conformation and oprD, can be less expressed or lost, conferring to *P. aeruginosa* the resistance to the beta-lactam imipenem [14].

**Modification and protection of the antibiotic target**

Microorganisms can prevent the activity of antibiotics also with the modification of their target, that becomes unrecognizable by the drug. The modification can occur as a mutation in the target coding gene or as a post-translational change in the protein [14]. In particular, mutations of the target gene is very common and able to confer high-level resistance to specific antibiotics: one of the most typical examples consists in the mutations in the *gyrA* subunit of DNA gyrase and in the *parC* subunit of topoisomerase II, that are the molecular targets of the broad-spectrum antibiotic class of fluoroquinolones [108]. Other examples are the *rmt* resistance determinants in *Pseudomonas aeruginosa*, that confer resistance to aminoglycosides by the methylation of their principal target: the 16S rRNA of the 30S ribosomal subunit [38], or *mcr-1*, able to invert the polarity of LPS in Gram-negative bacteria from negative to positive and thus impairing the action of the antibiotic colistin [96].

Also the protection of the target has been detected several times as an antibiotic resistance mechanism: one of the most famous examples is the synthesis of the Ribosome Protection Proteins (RPPs) that are able to protect bacterial ribosomes, breaking the tetracycline-ribosome binding. [14, 123].

Another interesting resistance mechanism is the overproduction of the target: it is rarely reported, probably for a too elevated fitness cost, but an example can be found in the *Escherichia coli* strains carrying mutations in the promoter of the dihydrofolate reductase (DHFR). These mutations enhance the expression of this important enzyme, involved in the DNA replication, eventually conferring to *E. coli* the resistance to trimethoprim [111].

Finally, a very efficient way to survive the action of the antibiotic is the development or acquisition of alternative metabolic pathways or enzymes, not affected by the drug action: the "metabolic bypass" mechanism, whose maybe the most famous example is the one that led to the arise of the methicillin-resistant *Staphylococcus aureus* (MRSA). β-lactam antibiotics functioning is based on the inhibition of the transpeptidase reaction catalyzed by the penicillin binding proteins (PBPs): this causes the cell lysis and death [124]. Very often resistance to β-lactams is mediated by β-lactamases (enzymes able to disrupt drugs belonging to this class), but in the case of MRSA, the resistance is possible thanks to the acquisition of a new PBP, named PBP2A and encoded by the *mecA* gene (carried on the SCC*mec* mobile genetic element), which is completely immune to the antibiotic activity [14, 45].

A further example of metabolic bypass/target modification is the one encoded by the *vanA* operon, a gene cluster composed by 5 genes and able to confer resistance to vancomycin in Gram-positive bacteria [14] through the substitution of the D-ala-D-ala at the C-terminal of the peptidoglycan precursor with the depsipeptide D-ala-D-lac unrecognized by the antibiotic. First reported in Enterococci in the late '80s, the *vanA*-mediated vancomycin resistance has been described also in *Staphylococcus aureus* leading to the emergence of resistant *S. aureus* (VRSA) [74].

**Inactivation or Modification of the antibiotic**

Antibiotic resistance can also be a consequence of the action of specific bacterial enzymes that are able to degrade the drug, modifying it or add chemical groups: in every case, the drug is inactivated and no more able to exert its effect. Many enzymes have been discovered having an inactivating effect on antibiotics, but the most studied ones are the β-lactamases, that are also one of the most common and clinically important mechanisms of antibiotic resistance overall, considering the very high and widespread use of β-lactam antibiotic in infection treatments [111].

β-lactamases can bind and break the β-lactam ring and can be sorted by several classification systems; the most used one, the Ambler classification, divides them in 4 groups: groups A, C and D having a serine in their catalytic site; and group B (named "metallo-β-lactamases", MBL) having at least one divalent zinc ion in their catalytic site [120].

Particularly worrisome are the so called Extended-Spectrum β-lactamases (ESBL), able to hydrolyze third and fourth generation cephalosporines, such as the TEM, SHV, CTX-M and OXA families. Even more recent and cause of concern is the discovery and description of carbapenemases: β-lactamases able to disrupt carbapenems, a very recent β-lactam antibiotic class often used as a last-resort drug for the treatment of ESBL-producing bacterial infections. [120]. This particular group of hydrolyzing enzymes has widely spread all over the globe and reported in several Gram-negative (particularly *Enterobacteriaceae*) bacteria: NDM-1 in particular, described in 2008 and able to inactivate all β-lactams except aztreonam; it is carried by a class 1 integron and strongly prone to be disseminated via HGT [14].

The antibiotic modification can occur through the addition of a specific chemical group to the drug. The most typical example is the resistance to aminoglycosides mediated by the aminoglycoside modifying enzymes (AMEs). AMEs catalyze structural modifications in the -OH or $-NH_2$ groups of the 2-deoxystreptamine nucleus of aminoglycosides and can be sorted on the basis of the chemical group added: acetyltransferases (AACs), nucleotidyltranferases (ANTs) or phosphotransferases (APHs). The huge number of AMEs described and their impressive transfer efficiency make them one of the most important aminoglycoside resistance determinants: due to mutations new variants are described continuously and most of them are carried in mobile genetic elements. Consequently, AMEs can be found in almost all bacterial species, both Gram-negative and Gram-positive [104].

*Antibiotic extrusion*

Resistance mediated by active antibiotic expulsion from the cell relies on specialized proteins (or protein complexes) localized in bacterial envelopes, named efflux pumps (EPs). These systems have been known for many years as one of the most important and versatile resistance mechanisms; moreover, recent findings increasingly suggest additional roles in bacterial physiology. Their importance is highlighted by the fact that about 5 – 10 % of all bacterial genes are involved in transport of molecules and most of them encode efflux pumps [125], which are able to expel a number of toxic and signaling molecules, e.g. heavy metals, quorum sensing signals, bacterial catabolites, etc. EPs are very ancient mechanisms and can be found in almost all bacterial species (as well as eukaryotic cells); they are generally chromosomal but can be also encoded by MGEs such as plasmids [95]. Some EPs can be specific for only one substrate, while others can extrude several different molecules and thus be associated with a multi-drug resistance phenotype [125]. What makes efflux pump an efficient resistance mechanism is its ability of lowering the intracellular concentration of drug, thus limiting the antibiotic-target interaction; this strategy results in a longer bacterial survival in presence of antibiotics allowing enough time to the cell to accumulate mutations or acquire external determinants leading to resistance [94].

Efflux pumps can be found in all bacterial cells, and when over-expressed result in antibiotic resistance, a condition that has been often reported in strains isolated from persistent infections. The expression of efflux systems genes is strictly controlled and can be highly influenced by environmental conditions [125]: several studies demonstrate the role of "inducers", molecules that affect the expression of efflux pumps determinants. Plant-produced compounds and biocides have been observed to trigger the expression of the pumps genes, but more interesting insights emerge from the study of the efflux pumps expression in clinical strains involved in infections: it has been demonstrated that substances commonly found in the human intestinal tract (such as bile salts), as well as chemicals that bacteria can encounter during an infection (such as cationic peptides or fatty acids), are able to induce a greater expression of acrAB in *E. coli*, one of the most studied efflux pumps [17]. a similar "activating" effect has been also observed for different stress factors, including antibiotics: a typical example is the overexpression of *Pseudomonas aeruginosa* MexXY-OprM efflux pump caused by aminoglycosides [73]. The activation of efflux pumps mediated by antibiotics is particularly worrisome, not only

because it provides a direct “feedback” defense mechanism to bacteria in case of antibiotic treatment, but also because in case of a multidrug efflux pump, a single antibiotic could possibly trigger the resistance against multiple drugs simultaneously ( a phenomenon known as “cross resistance”).

5 families of efflux pumps have been described so far (**Fig.6**):

- Major Facilitator Superfamily (**MFS**)
- ATP binding cassette (**ABC**) family
- Small Multidrug Resistance (**SMR**) family
- Multidrug And Toxic Compound Extrusion (**MATE**) family
- Resistance Nodulation Division (**RND**) family

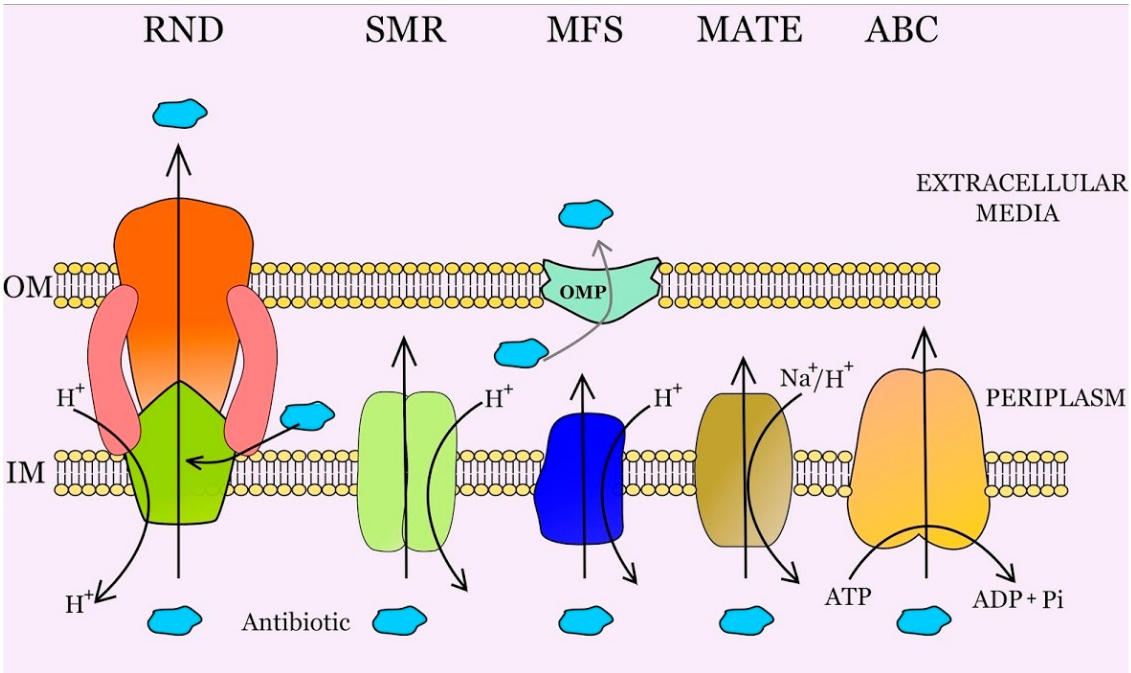


**Figure 6** Schematic representation of the five efflux pumps superfamilies described so far (RND, SMR, MFS, MATE and ABC) and their localization in the bacterial envelope. OM: outer membrane, IM: inner membrane, OMP: outer membrane protein.

In every case, the active efflux of the substrates needs a source of energy. For all these families the energy required is obtained from the proton-motive force generated by cellular metabolism, with the sole exception of the ABC family efflux pumps which catalyzes the hydrolysis of ATP [14].

The involvement of all these families in antibiotic resistance has been proved, but particularly relevant are the MFS and RND efflux pumps families.

**Major Facilitator Superfamily** efflux pumps are composed by a single aminoacidic chain (400-600 aa) with 12-14 trans-membrane helix domains and are responsible for most of efflux-mediated resistances in Gram-positive bacteria; however they can be also found in Gram-negatives (**Fig. 7**). The crystal-resolved structure of the *E. coli* EP EmrD, reveals a compact structure in which 4 helices are external, interacting with the cellular membrane, while the remaining helices design an internal channel that exposes several hydrophobic residues (thus favoring the transport of lipophilic compounds) [116]. This superfamily comprehends thousands of EPs sorted in sub-families, with a high grade of conservation from prokaryotic to eukaryotic cells. They use the proton-motive force by symport, antiport or uniport mechanisms and were firstly described in 1987 as sugar transporters [59]; nowadays they are known to recognize a broad range of substrates including ions, nucleosides, amino acids, short peptides, lipids and, most importantly, antibiotics.

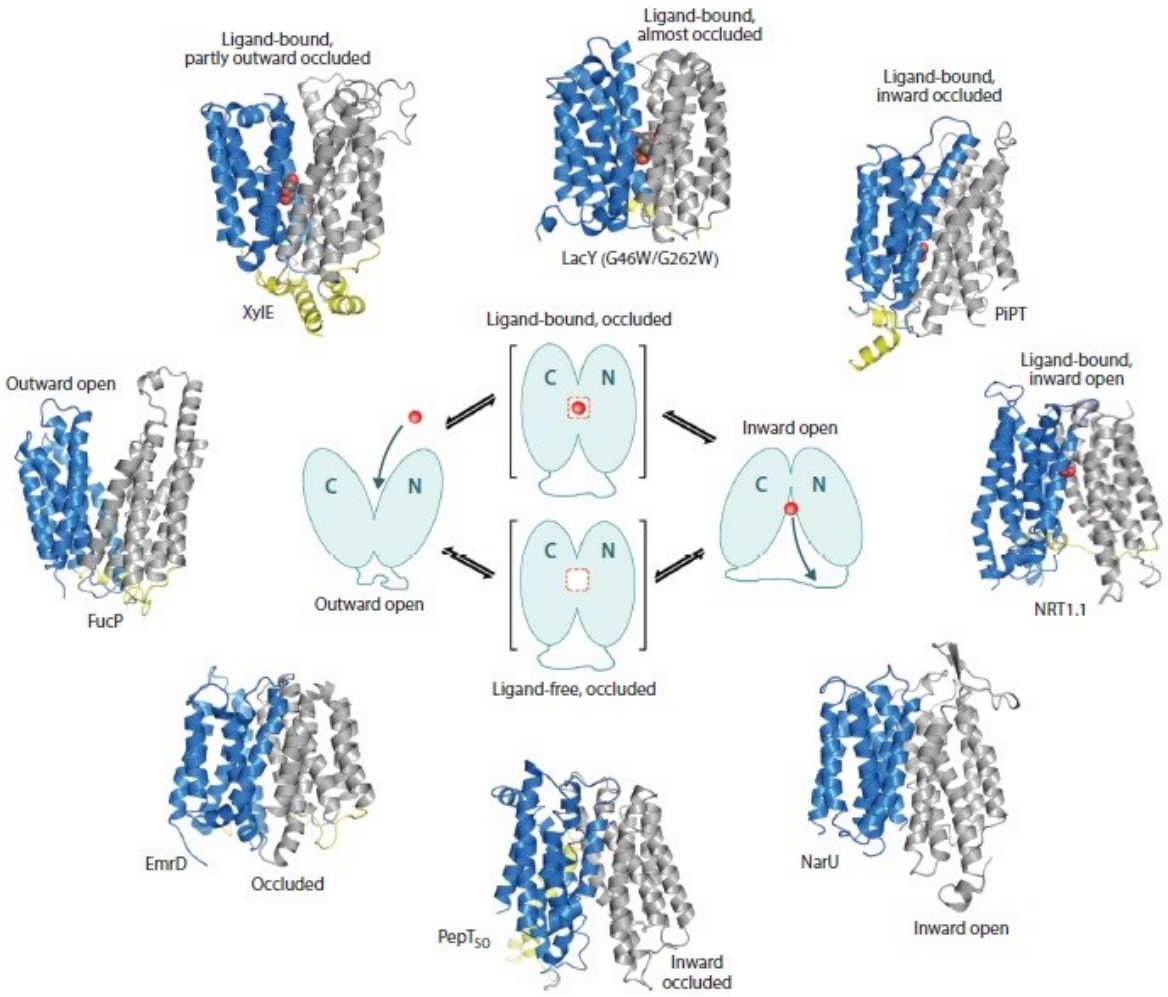


**Figure 7** Tridimensional structures of some of the most studied MFS pumps, with the N-terminal and C-terminal of the chains colored in silver and blue, respectively. In the middle, the conformational changes from inward open, to close, to outward open (and vice versa) during which the substrate is transferred from one side to the other of the membrane.

The best-known EP belonging to the MFS is NorA of *Staphylococcus aureus*. It is composed by 388 amino acids folded in 12 transmembrane segments and it has been shown to extrude quaternary ammonium compounds, dyes such as ethidium bromide and biocides. Moreover, it is one of the most important fluoroquinolones (mainly norfloxacin and ciprofloxacin) resistance systems in *S. aureus*. When expressed at a basal level NorA causes a slightly reduction of the susceptibility to the aforementioned compounds, however when overexpressed results in resistance to fluoroquinolones, biocides and dyes [30].

NorA overexpression is mostly caused by mutations in the promoter region of the *norA* gene, causing an amplified transcription or a more stable *norA* mRNA, or by the presence of "inducers", such as the transcriptional regulator MgrA, the two-component regulatory system ArlRS (involved in adhesion, autolysis and extracellular proteolytic activity of *S. aureus)*, and the ferric uptake regulator Fur (making NorA iron-responsive and probably involved in the export of siderophores in *S. aureus*). Although high-level resistance to fluoroquinolones in *S. aureus* is mostly caused by mutations in topoisomerase IV and DNA gyrase genes, the contribution of EPs shouldn't be overlooked, since it is widely spread in clinical isolates [30].

**Resistance-Nodulation-Division** family efflux pumps have been described only in Gram-negative bacteria and differ from other families by their peculiar tripartite structure, composed by an inner membrane protein, an outer membrane protein and a periplasmic membrane fusion protein that connect the other two components. The three components together form a continuous channel that connect directly the cytoplasmatic space with the external environment (**Fig.8**).

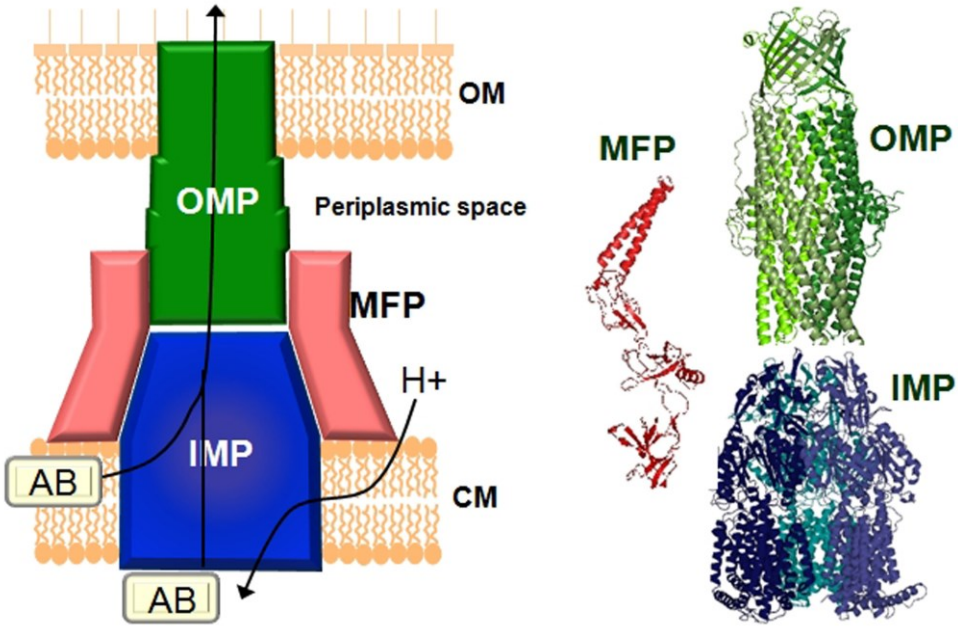


**Figure 8** Tridimensional structure of the RND superfamily efflux pumps and localization of the three components in the bacterial membranes. OM: outer membrane, OMP: outer membrane protein, MFP: membrane fusion protein, IMP: inner membrane protein, CM: cell membrane, AB: antibiotic.

The inner membrane protein in particular is able to recognize the substrate and extrude it through a substrate/$H^+$ antiport mechanism, the "caught" molecule crosses then all the channel and is released directly in the extracellular space, thus preventing a re-entry into the cell. Each of the three components is essential, as the absence or malfunction of just one of these would make the whole system inoperative [84].

The most studied RND EP is AcrAB-TolC: AcrA corresponds to the periplasmic fusion protein, AcrB to the inner membrane protein and TolC is the outer membrane channel. The AcrAB system is constitutively expressed in *E. coli* and it has been shown to confer an intrinsic resistance to several lipophilic drugs such as penicillin G, oxacillin, macrolides, linezolid, fusidic acid, etc. Moreover, the range of substrates recognized by AcrAB is extremely wide, including crystal violet, ethidium bromide, Triton X-100 and additional antibiotics such as cephalosporins, fluoroquinolones, tetracyclines, oxazolidinones, rifampicin, chloramphenicol, etc. These compounds largely differ in structure, however they share a hydrophobic domain that is believed to interact with a hydrophobic binding site of AcrB [84]. Similarly to what happen to all efflux pumps, also for RND transporters the expression level is crucial and strictly controlled: of particular importance in the *acrB* regulation in *E. coli* is the local transcriptional repressor AcrR, that in normal conditions controls and limits the expression of the efflux pump, but can be bound and inactivated by antimicrobial agents or impaired by mutations, leading in both cases to high expression levels of *acrB* and, consequently, higher efflux-mediated antibiotic resistance [94]. The mechanism of substrate binding and expulsion by AcrAB has been deeply investigated and represents the functioning model of all RND EPs. The interaction with the substrate occurs in the inner membrane protein (e.g. AcrB) with a peculiar rotating mechanism. AcrB is a homotrimer, and all of the three monomers can adopt 3 different conformations: "loose" (L), in which the binding site is free and available for binding with a substrate; "tight" (T), in which the monomer is bound to the substrate; and "open" (O), soon after the release of the molecule in the inner channel (**Fig.9**)

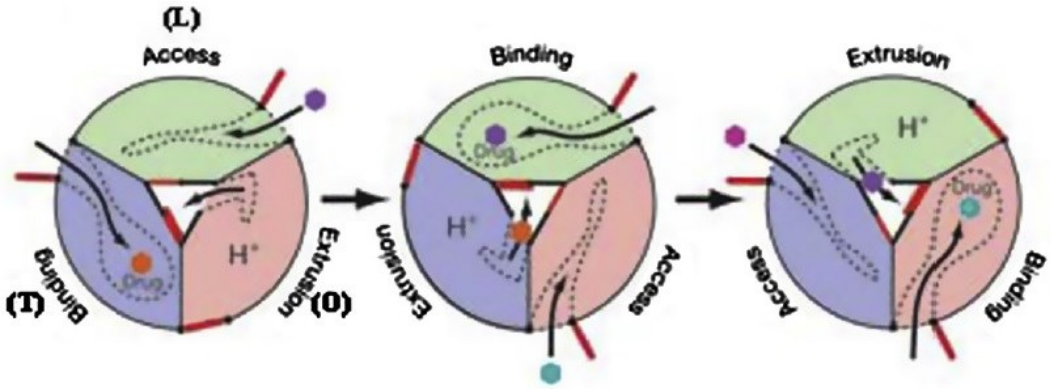


**Figure 9** Schematization of the conformation assumed by the three monomers of the inner membrane protein of RND pumps during the extrusion process: loose (L) during the access of the drug, tight (T) during the binding and open (O) during the extrusion.

Every monomer adopts cyclically a different conformation, assuring a continuous expulsion of the substrate molecules through the inner channel to the outside of the cell [116].

The most common and important efflux pumps in *Pseudomonas aeruginosa* belong to the Mex subfamily (RND superfamily), they are largely similar to AcrAB-TolC and show the same operating mechanism. Different efflux pumps belonging to the Mex family have been described in this bacterial species, MexAB-OprM and MexXY-OprM are the most widely studied.

MexAB-OprM was the first RND transporter described in *P. aeruginosa*, it is constitutively expressed and, just like the analogous AcrAB-TolC from *E. coli*, it shows a wide range of very different substrates: antibiotics such as β-lactams, fluoroquinolones, tetracyclines, aminoglycosides (but at a low level), sulfonamides, macrolides, etc., as well as different compounds such as dyes, ethidium bromide and solvents. The importance of this pump in *P. aeruginosa* is given by its clear association with the resistance to the recognized antibiotic, in particular β-lactams and fluoroquinolones [65]. The similarity between this system and AcrAB involves not only the inner membrane protein (MexB, which is identical in structure and functioning to AcrB), but also the regulation system, which is mainly controlled by the transcription repressor MexR. As expected, it has been demonstrated that any mutational event inactivating MexR leads to an overexpression of the efflux pump with a concomitant increase of resistance in the recognized substrate molecules (condition observed in many clinical strains isolated from infections). Moreover, it has been shown that oxidative stress (very often produced by antibiotic

action) causes the unbinding of MexR from the MexAB promoter, thus leading to an increased expression of the pump [24]. An interesting aspect of the activity of MexAB-OprM is its observed involvement in biofilm formation, one of the most important bacterial mechanism of virulence and defense, most likely because quorum sensing signals (pivotal for the production of biofilm) such as acyl-homoserine lactones (AHLs) are among the substrates of the pump. This feature confirms the diversified role of efflux pumps in bacteria, much more articulated than just an antibiotic resistance mechanism [3].

Another significant and well-studied RND efflux pump in *P. aeruginosa* is MexXY-OprM. The two components MexX (periplasmatic fusion protein) and MexY (inner membrane protein) are encoded in the same operon *mexXY*, while OprM is encoded by the *mexAB-oprM* operon. MexXY-OprM weight in *P. aeruginosa* resistance is due to its marked affinity to aminoglycosides, that are commonly used in *P. aeruginosa* infections treatment, especially in pulmonary infections of cystic fibrosis (CF) patients [98]. MexXY-OprM is the only RND efflux pump shown to mediate aminoglycoside resistance in *P. aeruginosa* and is considered indeed one of the most important resistance mechanisms against this antibiotic class: this resistance is particularly common in CF patients [98]. However, this pump also extrudes, to a lesser extent, β-lactams, fluoroquinolones, tetracycline, erythromycin dyes and ethidium bromide [78]. Although, as for MexAB-OprM, a crystallized model is still lacking, the high similarity with the RND archetypal pump AcrAB-TolC suggests an identical tridimensional organization and the same operating mechanism. The regulation of expression, finely controlled, is mediated mostly by the transcription repressor MexZ, which blocks the operon transcription by binding in the region between *mexX* and *mexY*. A high expression level of MexXY-OprM caused by mutations in *mexZ*, is usually observed in clinical strains (both CF and non-CF). Three types of mutants have been associated with MexXY-OprM overexpression so far [65]:

- AgrZ, in which the operon-repressor binding is prevented by mutations in MexZ or in the MexX-MexY intergenic region.
- AgrW1, characterized by impaired protein synthesis: in *P. aeruginosa* any mutation disrupting the translation process seems to activate the expression of

the protein ArmZ that, in turn, act as a repressor of *mexZ*, resulting in efflux pump over-expression.

- AgrW2, carrying mutations in the two-component system parRS, leading to a general increase of resistance mechanisms, including MexXY-OprM.

As mentioned before, antibiotic resistance is no more considered as the unique role of bacterial efflux pumps: an involvement in biofilm formation was observed not only for *P. aeruginosa* MexAB-OprM, but also in *E. coli* (in which efflux pumps are usually overexpressed in biofilm lifestyle), *Acinetobacter baumanii*, *Proteus mirabilis* and *Staphylococcus aureus*, just to mention a few. Efflux pumps probably play a role in biofilm formation in four ways: efflux of quorum sensing signals and exopolysaccharides, efflux of harmful molecules, indirect regulation of biofilm genes and promoting aggregation between cells (**Fig.10**) [3].

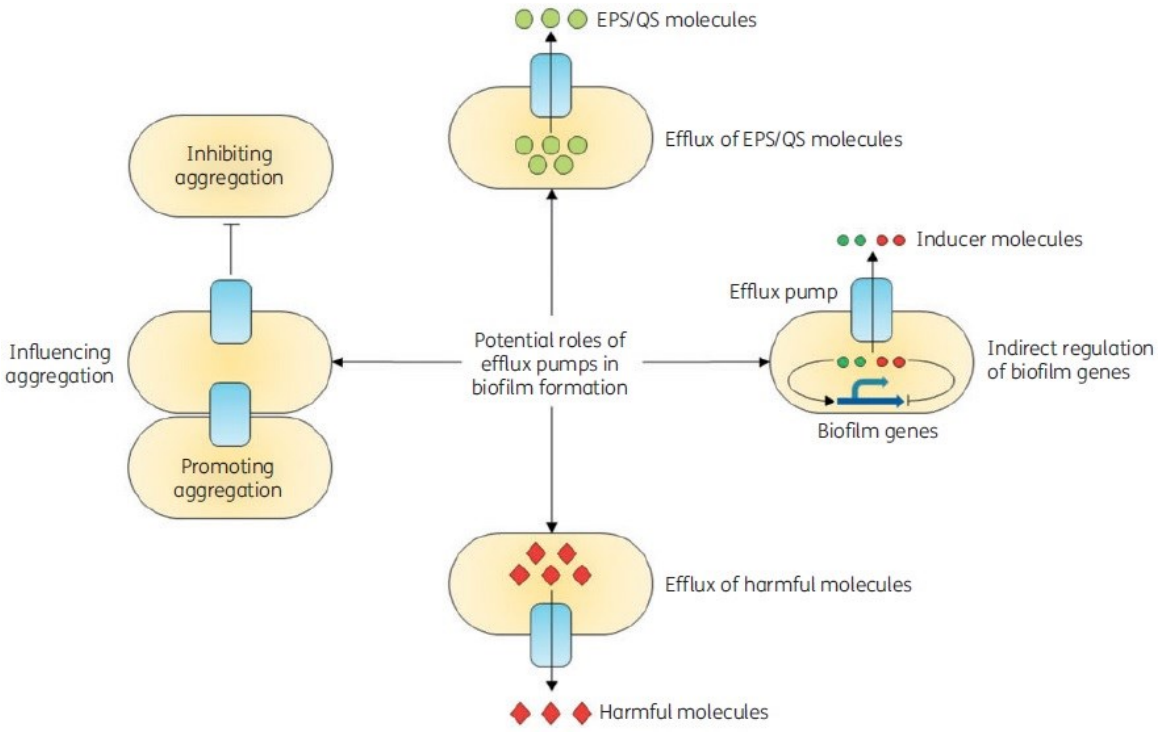


**Figure 10** The four ways efflux pumps can influence biofilm formation: efflux of exopolysaccharides (EPS) and quorum sensing (QS) molecules; indirect regulation of biofilm genes through efflux of inducers; efflux of harmful molecules and promoting aggregation between cells.

Moreover, efflux pumps also have an important task of detoxification from organic pollutants or heavy metals, but also endogenous metabolites that can be harmful if accumulated inside the cell: a typical example can be the efflux of 2,3-dyhidroxibenzoate, an intermediary metabolite of the synthesis of the siderophore enterobactin, by the AcrAB-TolC efflux pump [17]. Another important role is in virulence and pathogenicity of bacteria: *P. aeruginosa* strains lacking MexAB–OprM, MexEF–OprM and MexXY–OprM efflux pumps were observed to have a drastically reduced ability to invade epithelial cells [116]. Moreover, the efflux of host-derived molecules helping the adaptation and survival of bacteria in the human intestinal tract was first reported for *E. coli* AcrAB-TolC and then confirmed for several others similar efflux pumps in *P. aeruginosa*, *Neisseria gonorrhoeae* and *Salmonella typhimurium* [116]. Finally, a cell-to-cell communication role as been associated to bacterial efflux pumps, as in the already mentioned quorum sensing signals efflux.

All these findings underline the vital role of efflux pumps not only in bacterial survival to antibiotics, but also in their virulence and physiology.

# Biofilm

Increasing evidences during the years have set aside the idea of bacterial populations as free cultures of dispersed single cells: especially in infections, the most likely lifestyle adopted by bacteria is in biofilm. A biofilm is an articulate polymicrobial aggregate immersed in a complex matrix of hydrated extracellular substances that may include polysaccharides, lipids, proteins and nucleic acids. The matrix, produced by the microorganisms themselves, can account for over 90% of the whole biofilm and is essential for the microbial community adhesion to any type of surfaces [43]. The propensity to grow in biofilm lifestyle has been documented in practically all bacterial species, because of the clear advantages that it gives to the microorganism survival, but it is particularly marked in *P. aeruginosa* both in laboratory and clinical growth conditions. Therefore, this bacterial species has become the most studied model for the understanding of biofilm formation, structure and composition [118]. Several exopolysaccharides have been observed in biofilm matrices and, in particular, three have been described in *P. aeruginosa*: alginate, which is usually produced from mucoid strains recovered from chronic lung infections in CF patients; psl, observed in *P. aeruginosa* strains unable to produce alginate but still capable of biofilm formation; and pel, associated to biofilms in the air-liquid surfaces [126]. The development of a biofilm always passes through 4 stages (**Fig.11**):

- **Adherence**. For the formation of a mature biofilm, one or a group of cells need to adhere to a surface, that can be abiotic (plastic, glass, metal, environmental, ecc.) or biotic, thanks to the action of pili and flagella. In *P. aeruginosa*, type IV pili are believed to play a pivotal role in the first adhesion, and they result up-regulated in this phase. Once adhered, the cell(s) begins to produce extracellular matrix.

- **Microcolony formation**. Within 24 hours, the initial cells begin to divide up to form a microcolony. The continuous production of extracellular matrix strengthens its adhesion to the surface and starts protecting the inner cells from the environment. On the other hand the cells, especially the most “internal” ones, start to modify their gene expression in order to adapt to a sessile lifestyle.

- **Maturation**. A biofilm can reach maturity after 48-72 hours from adhesion. This stage shows all the distinctive characteristics; cell communication and regulation of the community is finely controlled and leads to the formation of specialized sub-colonies with different phenotypes along the biofilm layers.
-
- **Dispersal**. A mature biofilm can go through a detachment of its most external layers: cells re-acquire a planktonic lifestyle, recover motility and disperse in order to reach and colonize different districts. The disrupt of external fractions of the biofilms can be caused physically by external stimuli or triggered by community signals.

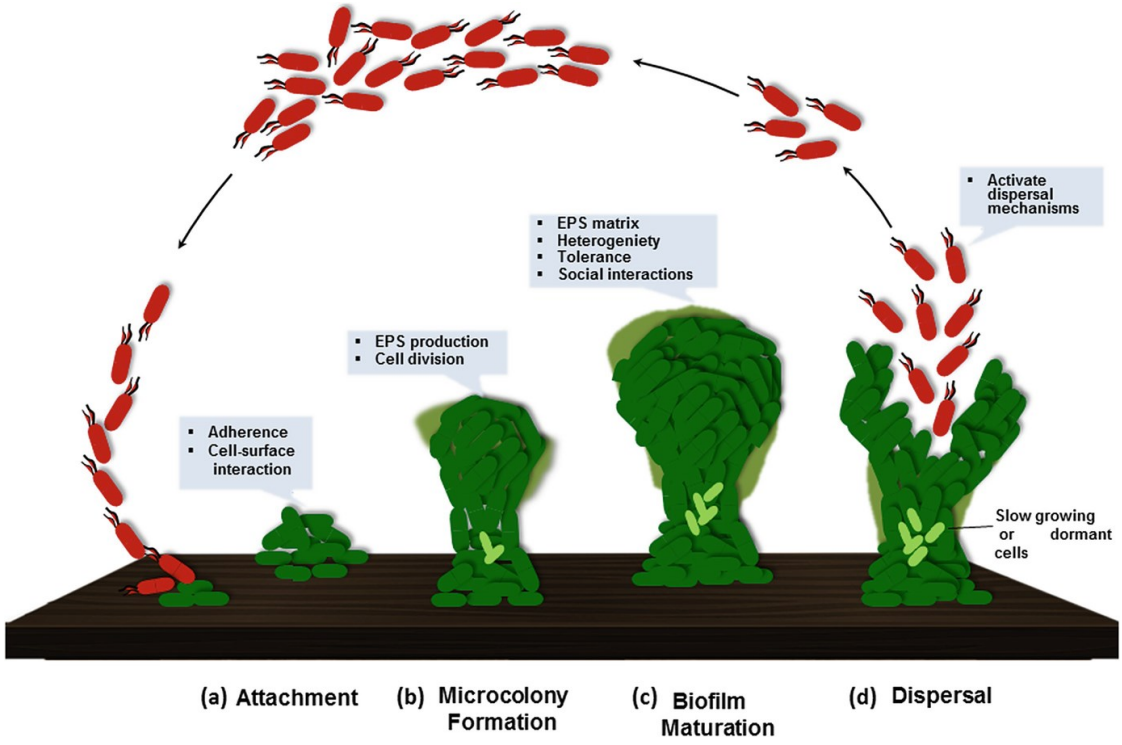


**Figure 11** The four stages of a biofilm life: attachment, microcolony formation, biofilm maturation and dispersal of the cells that can eventually colonize another district starting a new biofilm.

The biofilm maturation is a finely regulated process involving hundreds of genes; however, two molecules in particular seem to play a principal coordinator role: (p)ppGpp and c-di-GMP. The small nucleotide alarmones guanosine tetra- and penta-phosphate [(p)ppGpp] are the principal mediators of the so-called "stringent response" (a bacterial emergency response to hard environmental conditions): their synthesis is triggered by cellular stress (nutrients starvation or oxidative stress), through the enzymes RelA and SpoT, and they in turn stimulate different stress responses including biofilm formation [107]. The cyclic diguanylic acid (c-di-GMP) is a secondary messenger extremely conserved in most bacterial species, responsible for the regulation of several cell processes. In biofilm formation, it has been demonstrated to be a pivotal regulator of the matrix production and of many processes guiding the cell transition from the planktonic to the sessile lifestyle. Here too, the c-di-GMP producing enzymes (diguanylate cyclases) seem to be induced by stress conditions, including starvation, suggesting once again the protecting role of biofilm for the survival of the microbial community [47].

Another very important mechanism responsible for biofilm formation is quorum sensing. It seems to have a role in the biofilm initiation and maturation, although through still not completely clear mechanisms. In *P.aeruginosa* three quorum sensing systems have been described: the *las* and *rhl* systems synthesize and detect the signal molecule *N*-acyl-homoserine-lactone (AHL), that has probably a role during biofilm development; the PQS system acts through the signal molecule "*Pseudomonas* quinolone signal" [118].

The advantages of biofilm lifestyle for the microbial community are countless; firstly, in case of infection, the dense matrix protect with impressive efficacy the cells within from the host immune system. Various studies evidenced the difficulty (or, in some cases, the impossibility) for polymorphonuclear cells to infiltrate in the biofilm and clear the infection: they lie immobilized at the extremities of the matrix and they go into necrosis, due to the action of rhamnolipids secreted from the bacterial cells. Moreover, *P. aeruginosa* biofilm seems also to evade the complement system [109].

However, biofilms importance in clinics is mostly due to the protection it confers against antibiotics; some of the commonly used antibiotics diffuse slowly in the matrix (that become a real diffusion barrier) and can be bound and inactivated by elements of the biofilm itself. A typical example is the case of the big positively charged drugs such as aminoglycosides, that in *P. aeruginosa* biofilms are tethered by the molecules of external DNA that are present in the matrix (eDNA also increase the matrix viscosity). Biofilm lifestyle also induce a modification in cells gene expression, often activating or

upregulating antibiotic resistance determinants such as efflux pumps or the synthesis of the β-(1→3)-cyclic glucans that, released in the matrix binds and inactivate aminoglycosides [109]. The biofilm feature that probably contributes the most to antibiotic survival is the cell populations variability (**Fig.12**): if on one hand the cells at the most external layer benefit from a considerable amount of resources and nutrients, on the other hand cells from the most internal layers live in an extremely poor environment, where nutrients struggle to arrive and toxic waste compounds permeate the ambiance. The bacterial response to this tough condition is a significant change in gene expression that lead to dormancy, a lifestyle characterized by a "standby" of most cell metabolism; as a consequence if antibiotics that affect the dormant metabolic pathways enter the cell, they result totally ineffective. These dormant cells are generally called "persister" (a wider discussion will be addressed below) and represent a concrete clinical threat since while virtually unaffected by antibiotics they can re-activate, resulting in the infection recurrence or exacerbation [64, 109].

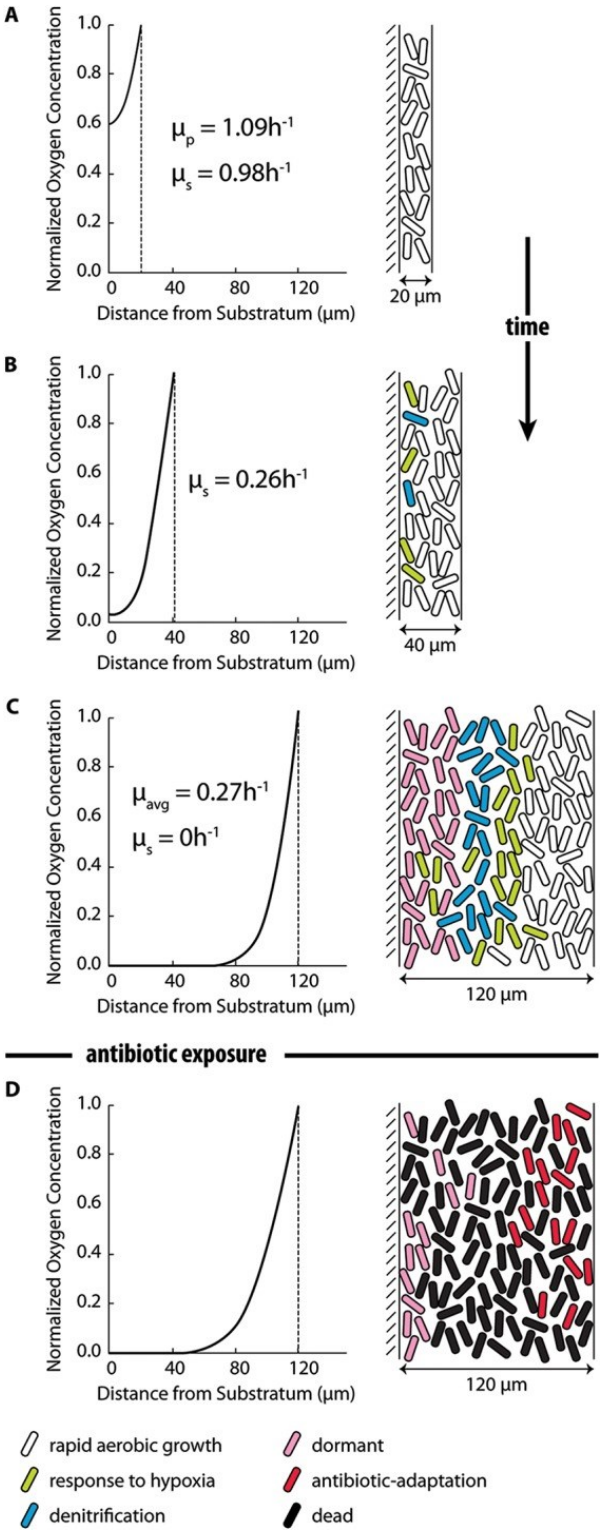


**Figure 12** The variation in oxygen concentration (oxygen decreases dramatically moving towards the inside) along the thickness of the biofilm correlates with the formation of different bacterial subpopulations. During the biofilm growth (A, B, C), the portion of the matrix oxygen-depleted increases stimulating the development of dormant cells. When challenged with antibiotic (D), most of the active cells die, while the dormant cells manage to survive.

Last but not less important, biofilm community shows a very high genetic variation and mobility; the mutation rate has been demonstrated to be much higher than planktonic bacteria, thus increasing the probability to escape the antibiotic action and thus the survival chances; moreover, the horizontal gene transfer, especially by conjugation, is favored thanks to the spatial proximity of cells within the matrix. This elevated genetic instability turns out to be a successful advantage as resistance determinants can arise easily and, most importantly, can efficiently spread among all the cells of the biofilm [49].

## Antibiotic Persistence, Tolerance and VBNC cells

For decades, when talking about bacterial strategies to survive the antibiotic activity the concept which is referred to the most is "resistance". Today, increasing evidences reveal that this notion is not completely correct: by definition, antibiotic resistance is the ability, conferred by a genetic determinant, of a bacterial strain to replicate in presence of antibiotics and to transmit the determinants of this phenotypic attribute to its progeny or to other cells through HGT. This property is usually evaluated through the measurement of the Minimal Inhibitory Concentration (MIC), i.e. the lowest concentration of a certain antibiotic causing the growth inhibition of the tested bacterial strain, which must be higher than resistance breakpoint.

The limitations of this definition have emerged already in 1940s, when Gladys L. Hobby and, shortly after, Joseph Bigger observed the incapacity of antibiotics considered bactericidal to fully sterilize bacterial cultures; a small subpopulation, that J. Bigger named "persisters", managed always to survive. When re-cultured, these cells give rise to a new population with a MIC below the susceptibility breakpoint [128]. Hence, it is not rare to find bacterial cells able to transiently survive the action of antibiotics, without undergoing genetic changes and not transferring resistance to the progeny, thus failing to fall under the definition of *resistant*.

These unclassifiable and unpredictable bacterial behaviors led us, more generally, to talk about "survival" or "resilience" of a bacterial population towards antibiotics, and evidenced the need for new definitions: a Consensus Statement published by Balaban in 2019 [13] helps us to better define the concepts underneath bacterial survival to antibiotic treatment. Firstly, it is important to distinguish "persistent infection" from "antibiotic persistence". The former refers to infections that are not cleared by the host immune system for multiple reasons involving both the infectious agent and the host's defenses, while antibiotic persistence is the ability of a sub-population to survive the exposure to bactericidal drug concentrations. It differs from resistance for some features:

- Persistence usually involves only a subset of the entire bacterial population.
- Persistence regards only for bactericidal drug.
- The insusceptibility to the drug is temporary: persistent cells re-cultured in absence of antibiotic give rise to a population as susceptible as the parental population; neither increase of the MIC nor any genotypic change occur.

- If far above the MIC, the drug concentration weakly affects the level of persistence.
- Contrary to resistant cells, persistent cells do not replicate in presence of the antibiotic any better than the non-persistent cells, but the killing rate is definitely lower.

A further definition reported in the above mentioned Consensus Statement concerns the "tolerant cells": similarly to persisters, tolerant cells show an increased survival to antibiotics without an increase in the MIC, but while persistence involves only a fraction of the bacterial population (can also be referred to as "heterotolerance"), tolerance involves the whole bacterial population. A hallmark to discriminate persistent from tolerant and susceptible cells is the survivors rate over time in the presence of antibiotic (**Fig.13**).

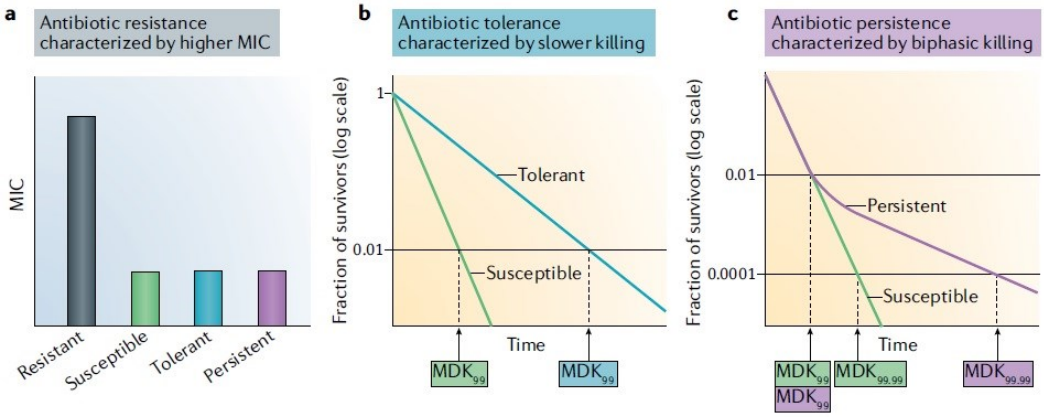


**Figure 13** Different behaviors of susceptible, tolerant and persistent cells when exposed to antibiotics. a: the MIC of resistant cells in is several times higher than the MIC of susceptible, tolerant and persistent cells b: tolerant cells are killed at a slower rate than susceptible cells. c: persistent cells exhibit biphasic killing curve, similar to the susceptible subpopulation at the beginning of the antibiotic treatment and slower afterwards.

Tolerant cells show a continuous killing curve, but at a much slower rate compared to susceptible cells; instead, persistent cells show a specific and characteristic biphasic killing curve, that reflects the presence in the population of a susceptible fraction (killed at a faster rate) and a persistent fraction whose killing rate is slowed down. There is still a debate in the scientific community about the second "phase" of the killing curve, since it has also been recently proposed that "real" persisters reach a sort of a plateau, thus flattering the second part of the curve over time [113]. However, the biphasic killing curve

is the hallmark of a persistent sub-population, that, in order not to confuse them with other mechanisms of survival, are usually recovered by treating the original bacterial population with high doses of antibiotics (several times the MIC) in laboratory experiments to exclude the involvement of resistant mutants in the survival rate [13].

Once defined what persisters are, it is interesting to understand how they can arise. Many evidences support the idea of two possible ways of persistence emerging: spontaneously or triggered (**Fig.14**).

Spontaneous persisters arise randomly in a bacterial population, without any external cue; and as long as the conditions are maintained their amount remains stable. Much more common are the so called "triggered persisters", that emerge as a consequence of environmental stimuli. Many stress conditions have been demonstrated to trigger the development of persisters such as starvation, high density population, immune factors, DNA damage, extreme pH, high toxic waste compounds and exposure to immune cells. Moreover, antibiotics too can favor their emergence: it has been observed that bactericidal drugs at very high concentrations can instead have a bacteriostatic effect on a bacterial population activating this stress response [13, 40].

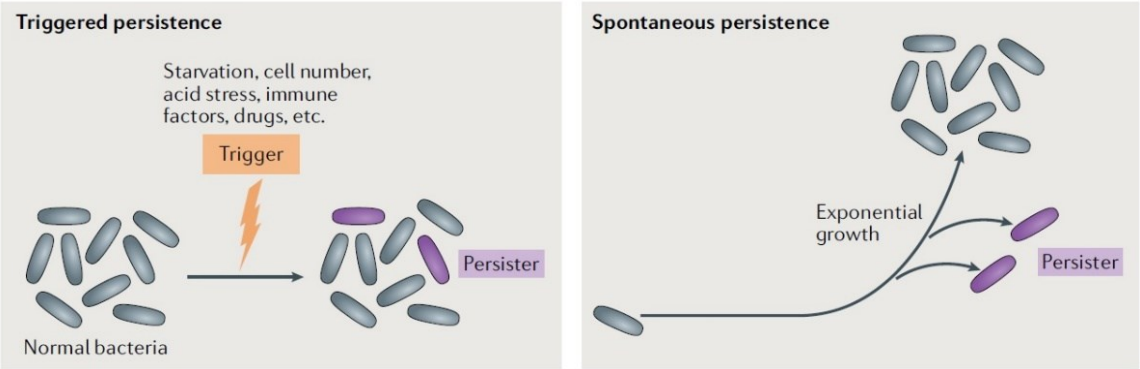


**Figure 14** persistent cells can be induced by environmental stress factors (left) or arise spontaneously, due to stochastic gene expression during exponential growth (right)

Several efforts were made during the years to find the molecular mechanisms underneath this survival phenomenon. Nowadays every attempt to find a single "master" process regulating all the mechanism has not been found, and the hypothesis of a redundant system is still the most likely. In every case, in most cases persistence is achieved through dormancy and in particular three factors have been demonstrated to play a very important role in bacterial quiescence: Toxin-Antitoxin systems, (p)ppGpp and indole [100].

Toxin-Antitoxin (TA) modules are a widespread expression regulator that controls many pathways in bacterial cells. Generally, TA modules code for a toxin able to shut down metabolic processes and trigger dormancy (usually a RNase) and an antitoxin which disengages the toxin activity. Six types of TA modules have been described so far: in type I and III the antitoxin is a noncoding RNA, while in the other types the antitoxin is a protein [89]. Actually, the first gene associated with persistence was a TA module, the *hipAB* operon of *E. coli*; a mutant carrying the allele called *hipA7* was demonstrated to cause a 1000-fold persisters increase [57]. However, confirming the redundancy hypothesis, the knock-out of any single TA module alone does not influence the number of persisters: at least in *E. coli*, a significant difference can be observed only when disactivating at least five TA modules [100].

The second messenger (p)ppGpp, as effector of the stringent response, is involved in numerous stress responses in bacterial cells: from the production of biofilm (already discussed) to a large-scale transcription repressor that leads to dormancy. The involvement of (p)ppGpp has been demonstrated to have a role in persisters formation, probably through inactivation of the ribosomes [114].

Indole and its derivates are very common bacterial signals that affects several aspects of the cell physiology. Indole molecules are synthesized in response to environmental changes and have been shown to influence the persisters formation [100].

Interestingly, in the last years the idea of dormancy as the only cause of persistence begins to be overcome: some studies suggest that some processes of the persistent cell remain active or even increase. It has been shown, for instance, that starvation-induced persisters produce more ATP per mol of carbon source consumed than do nutrient-supplied cells [102]. Moreover, another study demonstrated the unaltered ability of persisters to catabolize carbon source and an increased electron transport chain activity and membrane potential [5].

Finally, also active efflux has been suggested to have a very important role in persisters formation and maintenance by keeping low the intracellular concentration of drug and thus favoring the survival of the bacterial cell. In particular, Pu et al. demonstrated in 2016 that in *E. coli* the TolC portion of the efflux pump AcrAB-TolC was over-expressed in persistent cells, and its deletion led to a large decrease of persisters amount [101].

Another extreme response of bacterial cells has been observed in very hard environmental conditions: the development of the Viable But Non-Culturable (VBNC) cells. The VBNC condition defines a bacterial subpopulation that have transiently lost its ability to grow on culture media but is still alive [103]. These cells, first described in 1982 in *E. coli* and *Vibrio cholerae*, show a reduced metabolic activity but still an intact membrane and low but present protein synthesis. As long as stress factors persist, VBNC cells do not grow and divide but remain viable thanks to a very basal metabolism; when a significant improvement of the environment conditions occurs, they can eventually activate and re-gain culturability (a phenomenon known as "resuscitation"). The insurgence of VBNC cells has been observed in clinical and environmental settings and, so far, over 100 species have been demonstrated to enter in VBNC state, while only some of them showed to be able to resuscitate, thus indicating a real VBNC state and not just a *pre-mortem* phase [11]. When occurs, resuscitation needs the contribute of specific factors in addition to the presence of nutrients. Just to mention few examples, in *E. coli* combinations of amino acids such as methionine, glutamine, threonine, serine, and asparagine have been shown to help resuscitation, but also autoinducers and reactive oxygen scavengers. Several enteric pathogens artificially induced in the VBNC state were resuscitated by co-culturing them with various eukaryotic cell lines and the chelator diethyldithiocarbamate was demonstrated to resuscitate *P. aeruginosa* VBNC [103].

Several molecular mechanisms have been shown to have a role in VBNC formation: some of them, such as the stringent response mediated by (p)ppGpp, intracellular proteolysis (the stress-induce protease Clp may have a role both in persistence and VBNC) and TA modules are the same involved in pesisters formation. Moreover, following the hormesis hypothesis, also antibiotics at low concentrations could, acting as transcriptional regulators, trigger the stress-response in bacterial cells eventually leading them to a VBNC state [33].

Considering the common causes and, in some cases, the shared mechanisms underlying persistence and VBNC, it has been proposed that the two phenomena may be different stages of the same stress-response process (**Fig.15**): therefore a stress exposure applied to a bacterial population could induce the formation of persisters which, in case of a prolongation of the stress could fall into a deeper dormancy and become VBNC cells [10].

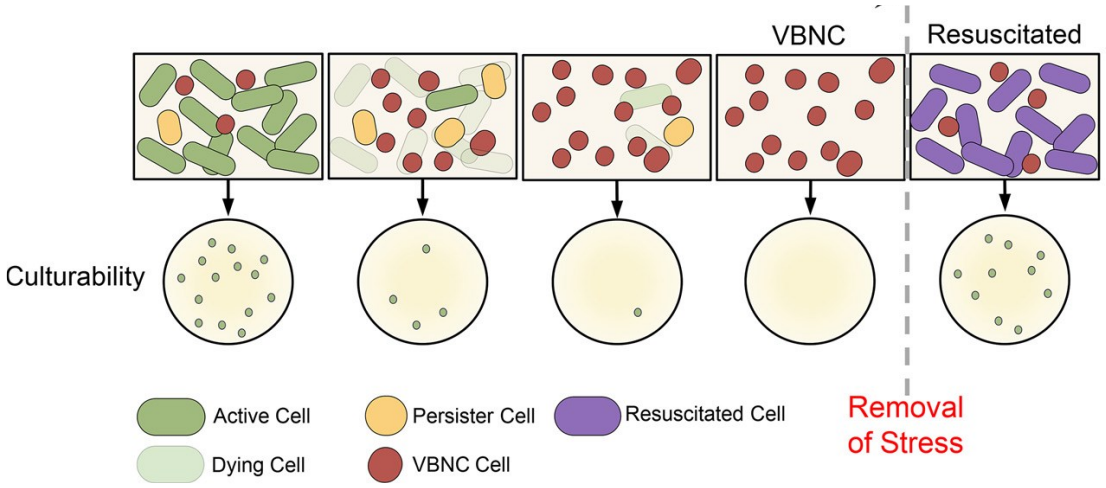


**Figure 15** increase of the persistent forms in a bacterial population exposed to stress factors: the amount of culturable cells decrease as the persisters enter the VBNC state. Once the stress is removed, some of them could "resuscitate" re-gaining culturability.

Although the importance of persistent and VBNC cells in clinical settings appeared immediately clear, surprisingly their importance in persistent/chronic infections has been overlooked for years, mostly because of the difficulty of correlating persisters to difficult-to-treat infections. Recently this situation changed and the scientific community attention over these phenomena increased significantly. Indeed, most of the antibiotic treatments are chosen on the basis of the *in vitro* susceptibility tests and, as a consequence of persistence, the antibiotic therapy often fails to achieve the clearance of the infection although the bacterial isolate strain showed a clear susceptibility. Moreover, as mentioned above, both phenotypes are commonly found in the deepest layers of bacterial biofilms and definitely have a major weight in biofilm's refractoriness to antibiotic treatment. Finally, given the inability of VBNC cells to grow on normal laboratory media, they are undetected by the standard culture-based microbiological diagnosis. Given all these features, persistent and VBNC cells are now believed to play a pivotal role in chronic and recurrent infections caused by several pathogens, explaining convincingly many events of antibiotic therapy failure especially in immunocompromised patients [122]. An example comes from the various studies that have shown in *P. aeruginosa* chronic lung infections in cystic fibrosis patients the presence and involvement of persisters and VBNC forms [36, 70, 81]. All these insights have now changed the scientific community's perception on bacterial behavior during infections and the need for new therapeutic strategies targeting persistent cells is crucial.

## *Pseudomonas aeruginosa*

*Pseudomonas aeruginosa* can be found in several different settings such as soil, coastal marine habitats, plants and animal tissues [115]. *P. aeruginosa* importance in clinics has been widely recognized since it's one of the most versatile and strong opportunistic pathogen both in plants and animals, including humans: the list of infections caused by this microorganism includes hospital-acquired pneumonia (HAP), ventilator-associated pneumonia, burn-associated infections, gastrointestinal infections, dermatitis, urinary tract infections, skin infections, respiratory tract infections and many others [12]. It is a major cause of hospital acquired infections and extremely harmful for immunocompromised patients and in particular cystic fibrosis patients, that affects with chronic and recurrent lung infections. Actually, *P. aeruginosa* is a secondary colonizer in cystic fibrosis, but during the life of the patient it tends to become the most prevalent pathogen and, ultimately, the first cause of morbidity and mortality [68].

The incredible adaptability that characterizes *P. aeruginosa* is a phenotypic reflection of its genome: the complete sequencing of the laboratory strain PAO1 performed in 2000 by Stover et al., revealed a 6.3 million bp genome (one of the largest among pathogenic bacteria) and, most importantly, it shows the highest percent of regulatory genes observed in a bacterial genome [115]. This particular feature can explain its great variety of virulence factors spanning from flagella, pili and lipopolysaccharide, involved in the adhesion to host tissues, to secreted molecules like pyocyanin, pyoverdine, proteases, elastases, lipases and additional toxins [56]. Even more importantly, the great variability of *P. aeruginosa* genotype is translated in a huge versatility and adaptability to different environmental conditions and to antibiotic treatments. This is the reason why *P. aeruginosa* has become the study model of bacterial survival strategies to antimicrobials in terms of resistance, persistence or biofilm growing.

*Pseudomonas aeruginosa* weaponry against antibiotics is vast and many of the mechanisms have been described in the previous chapters. Briefly, as many Gram-negative bacteria, this pathogen shows a considerable intrinsic resistance towards many antibiotics firstly thanks to a very low membrane permeability (due to a low amount of porins with a sufficiently large channel). A basal resistance to β-lactams is also observed thanks to the constitutive expression of a β-lactamase, encoded by *ampC*, that can eventually be induced by the presence of β-lactams themselves. Vital in *P. aeruginosa* resistance is also the intrinsic armamentarium of efflux pumps, in particular of the RND

pumps, which have been widely discussed before, that not only are able to confer resistance to a large number of antibiotics but can also be involved in the release of quorum sensing signals and in formation of biofilm.

A distinct attitude in HGT makes *P. aeruginosa* also a *hot spot* for the accumulation of acquired resistance mechanisms, carried by genetic mobile elements, such as extended spectrum β-lactamases, carbapenemases, aminoglycoside modifying enzymes, etc.

Moreover, an incredibly efficient activation of all the stress-response systems highly involved in the survival to antibiotics, such as biofilm formation, persistent and VBNC cells, is demonstrated to be the real hallmark of *Pseudomonas aeruginosa* infections, rendering this pathogen's chronic infection one of the most challenging (sometimes impossible) to treat and eradicate [12, 77, 90, 112].

## *Staphylococcus aureus*

*Staphylococcus aureus* is normally found in the environment, in the human skin and in the squamous epithelium of the human nasopharinx [119]. It has been observed that 20-30% of the human population is colonized by *S. aureus* and about 60% are occasional carriers. Although part of the human flora, *Staphylococcus aureus* can occasionally invade human tissues and start a very wide variety of infections, from minor to life-threatening. In particular, this pathogen can cause skin infections, endocarditis, pneumonia, bloodstream infections, osteomyelitis as well as necrotizing fasciitis [41].

*S. aureus* shows a wide array of virulence factors that allows this pathogen to be the causative agent of such a variety of infections; some of them are carried by mobile genetic elements or prophages. Worth mentioning are all the surface proteins that mediate the adhesion to host tissues, promote iron uptake and help preventing the immune system recognition, but also extremely powerful toxins (such as the superantigen toxin and the toxic shock syndrome toxins) and the Panton-Valentine leucocidin, as well as a series of lipases, nucleases and proteases [44].

When talking about antibiotic resistance in *Staphylococcus aureus*, our mind goes immediately to the methicillin resistant strains (MRSA), emerged after the acquisition of a *SCCmec* cassette carrying the gene *mecA* which encodes for the PBP2a. First reported in 1961, MRSA spread rapidly becoming one of the major and most dangerous causative agents of hospital acquired infections (HA-MRSA). MRSA spread also outside the hospital environment: community-acquired MRSA (CA-MRSA) have been described since 1999, causing equally (or even more) virulent infections, but usually showing fewer antibiotic resistances than the hospital counterpart [91]. In general, most of the antibiotic resistances of *S. aureus* are acquired and often carried by mobile genetic elements. A significant exception comes from the resistance to fluoroquinolones, that can be caused by both mutations in the so-called quinolone-resistance-determining-region (QRDR) of the DNA gyrase and topoisomerase IV (the fluoroquinolone targets) and drug extrusion by efflux pumps. [91].

Efflux pumps importance in *S. aureus* is gaining more and more attention, since although at a low-level, they are able to confer multi-drug resistance. Different efflux systems have been described in *S. aureus*, the most studied are those of the Nor family (NorA, NorB and NorC), belonging to the MFS superfamily, and MepA, belonging to the MATE superfamily. NorA in particular has been described to be the prevalent one; it has

been shown to play an important role in fluoroquinolone resistance, especially since it is usually overexpressed in *S. aureus* isolates and strongly upregulated in response to fluoroquinolones treatment [30]. Finally, also in *Staphylococcus aureus* the formation of biofilm has been associated to chronic infections, as well as persistent cells [28]. Especially the mechanisms underlying the latters are not completely clarified, although here too the role of the quorum sensing, *agr* (*accessory gene regulator*), seems to have a pivotal role [23, 66].

## New antimicrobial strategies: efflux pump inhibitors

The increasing failure rate of the conventional antibiotic treatments, linked to the steadfast rising of the antibiotic resistance phenomenon is currently pushing the scientific community to find innovative drugs and, in general, innovative strategies to counteract chronic and recurrent infections. Currently, the combination (and synergy) of multiple antibiotics is probably the most common strategy adopted, but still doesn't seem to put a definitive solution on this problem: the threat of the so-called "post-antibiotic era" is real, but some new approaches seem to be really promising.

A first strategy considered for the overcoming of resistance is the use of antivirulence drugs. Considering that every compound able to kill bacteria is in the same time applying a strong selective pressure, as long as we are under the evolution laws, resistant strains will always emerge and the match against pathogenic bacteria could only be tied. Hence, the idea behind antivirulence drug is render pathogens harmless, rather than kill them, thus avoiding a selective pressure. By consequence the targets of these drugs are the virulence factors of the most troublesome pathogens, such as adhesins, toxins, secretions systems, siderophores, immune evasion factors and biofilms/quorum sensing regulator factors. Already three antitoxins drugs against *Clostridium botulinum*, *Clostridium difficile* and *Bacillus anthracis* have been approved by the US Food and Drug Administration (FDA) and others promising drugs targeting toxins, adhesins, quorum sensing etc. are currently under evaluation against *S. aureus*, *P. aeruginosa*, *E. coli*, *K. pneumoniae*, *A. baumanii* [37].

Worth mentioning is also the research of antipersisters drugs that, given the importance of persistence phenomenon, could be an effective weapon against chronic and recurrent infections. So far, Antipersistence strategies can be sorted in three categories (**Fig.16**): directly kill the metabolic quiescent cells (thus not targeting active cell processes); forcing a "reactivation" of the cells, rendering them again susceptible to conventional antibiotics; preventing the persisters formation targeting the mechanisms underneath.

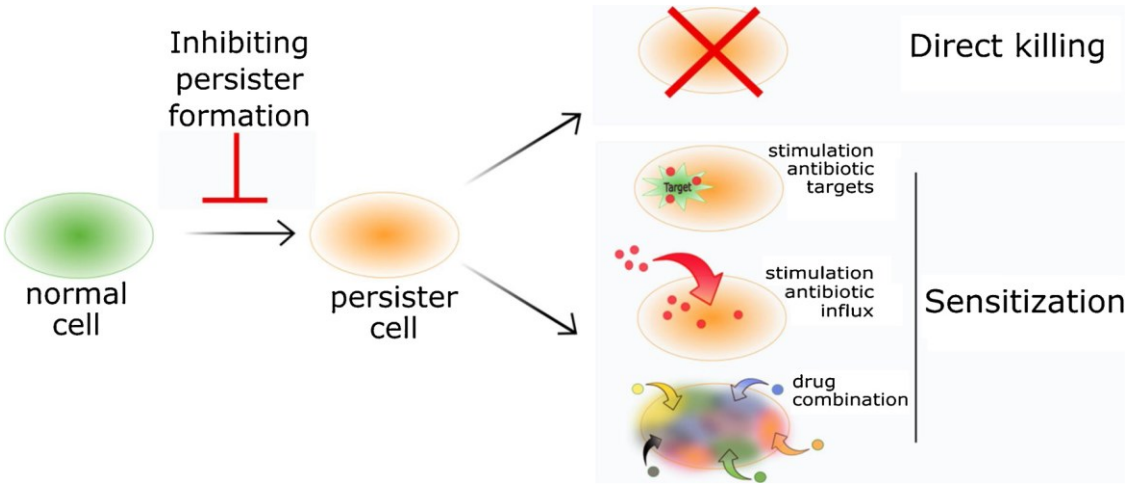


**Figure 16** Three strategies to counteract persisters: acting upstream inhibiting their formation; killing them using non-metabolic targeting drugs; re-activating them (sensitization) and then using conventional antibiotics.

Several compounds have been shown to kill bacteria although dormant, for example by acting on the membranes, the DNA and essential enzymes, or by generating reactive oxygen species. Likewise, some molecules have been taken into account because apparently able to revert the persistent phenotype, thus making conventional antibiotics effective again (e.g. the fatty acid signaling molecule cis-2-decanoic acid on *E. coli* and *P. aeruginosa* and spent medium with its protein and peptide factors on *S. aureus*). Finally, also compounds targeting quorum sensing and (p)ppGpp seem to be promising in preventing the persisters formation. All these molecules are still at a *in vitro* testing stage, but they can potentially be very effective in clinic therapies [34].

Finally, one of the most promising strategies to revert antibiotic resistance is inhibiting the efflux pumps that, as explained above, are considered main actors in antibiotic resistance and could also have a role in quorum sensing signaling and biofilm formation. Efflux pump inhibitors (EPIs) could impair the efflux pump activity in several ways: as antisense nucleic acids that bind the efflux pump mRNA thus hindering their expression, or preventing the assembly of the efflux system in case of multi-component pumps (such as the RND transporters). Moreover, since most of the pumps functioning is based on antiport systems with $H^+$ ions, proton-motive force breakers such as carbonyl cyanide m-chlorophenylhydrazine (CCCP) could be considered EPIs, although general and aspecific. More commonly, the inhibition can occur by competition with the antibiotic: an EPI with high affinity for the binding site could competitively bind the binding site, block it and thus preventing the efflux pump-antibiotic interaction [6].

Considered the major role of efflux pumps in the bacterial physiology, resistance and, probably persistence to drugs, using EPIs in association with antibiotics could potentially increase the drug action and restore the susceptibility if the resistance is efflux mediated; it thus represent one of the most promising strategies to counteract difficult to treat infections. To date, many molecules have been showed to have a considerable EPI activity, such as the aforementioned CCCP, the peptidomimetic Phenylalanine-arginine β-naphthylamide (PAβN, particularly active on RND pumps), the pyridopyrimidinone D13-9001, the pyranopyridine MBX2319 and some derivatives of quinolines and indoles, but unfortunately none of them have passed the clinical trials to date, mostly due to their toxicity [61]. However, this approach still gets the attention of the scientific community for its undoubted potential.

# AIM OF THE WORK

This study aims to provide further insights on the role of efflux pumps in antibiotic resistance and persistence in *P. aeruginosa* and *S. aureus*, and to confirm the promising efficacy of efflux pump inhibitors as antibiotic resistance breakers and inhibitors of persistence phenomenon, in order eventually to gather more tools to counteract the challenge of bacterial resilience to antibiotics.

Novel EPIs against *P. aeruginosa* MexXY-OprM and *S. aureus* NorA have been selected/developed and evaluated for their ability to inhibit EP activity resulting in i) an increased antibiotic (tobramycin or ciprofloxacin) susceptibility and ii) a decreased antibiotic-persisters development. The natural alkaloid berberine has been selected among hundreds of natural compounds on the bases of an *in silico* screening for its ability to bind the inner membrane protein MexY of MexXY-OprM, hopefully resulting in a significant decrease of survivors to tobramycin, one of the most commonly used antibiotics in the treatment of *P. aeruginosa* CF chronic infections; newly developed quinoline-based compounds have been tested against the *S. aureus* EP NorA to enhance ciprofloxacin bactericidal activity.

# MATERIALS AND METHODS

## Bacterial strains, antibiotics and compounds

A total of 175 *Pseudomonas aeruginosa* (PA) clinical isolates (157 from CF patients, 18 from non-CF patients) were collected anonymously from the microbiology laboratories of Ospedali Riuniti Torrette (Ancona, Italy) and Ospedale A. Murri (Fermo, Italy) in 3 different campaigns: from April 2014 to March 2015 (*P. aeruginosa* C1-C51, C54-C104); from October 2015 to January 2016 (P. aeruginosa AR1-AR96) and from July 2018 to March 2019 (*P. aeruginosa* NC01-NC10). PA laboratory strains PAO1 and PA14 were kindly provided by Prof. Olivier Jousson (Integrated Biology Center, University of Trento, Trento, Italy), and PA K767 (PAO1) and K1525 (K767Δ*mexY*) were kindly provided by Prof. Keith Poole (Department of Biomedical and Molecular Sciences, Queen's University, Kingston, Canada). PAO1 carrying the plasmid pHERD30T was kindly provided by Prof. Paul Williams of the Centre of Biomolecular Sciences, University of Nottingham (UK). *Staphylococcus aureus* (SA) strains SA1199 (*norA* wildtype) and its mutant SA1199B overexpressing *norA* and possessing a A116E GrlA substitution and the isogenic strain pair SAK1902 (Δ*norA*) and SAK2378 (*norA*++) were kindly provided by Prof. Stefano Sabatini (Department of Pharmaceutical Sciences, Chemistry and Technology of the Drug Section, Università degli Studi di Perugia, Perugia, Italy). PA ATCC 27853 and SA ATCC 25923 belong to the collection of the Microbiology section of the Department of Life and Environmental Sciences, Polytechnic University of Marche (Ancona, Italy).

All PA strains were cultured in Lysogenic broth (LB) or LB agar and sub-cultured in McConkey agar, while all SA strains were cultured in Triptone Soy Broth or agar and sub-cultured in Mannitol Salt agar. All strains were stored at −80 °C in LB (PA) or Triptone Soy Broth (SA) supplemented with 20% glycerol.

All bacteriological media were purchased from OXOID (Oxoid spa, Milano, Italy) and all antibiotics were purchased from Sigma-Aldritch (Sigma-Aldrich SRL, Milano, Italy).

Berberine was kindly provided from Dr. Roberta Galeazzi of the Biomolecular Modeling group of Department of Life and Environmental Sciences (Polytechnic University of Marche), while the 24 NorA putative EPIs were kindly provided by Prof. Stefano Sabatini.

## Antibiotic susceptibility tests

The 175 PA strains were firstly screened for their resistance to tobramycin (TOB), gentamycin (GEN) and amikacin (AMK) by a routine antibiotic susceptibility test (Sensititre™ Complete Automated AST System, Thermo Fisher Scientific, Waltham, MA, USA). The resistant phenotype was confirmed by determination of the Minimal Inhibitory Concentration (MIC) by broth microdilution according to clinical and laboratory standard institute (CLSI) guidelines [27] using PA ATCC 27853 as the reference strain. Strains showing intermediate susceptibility were considered resistant. The following antibiotic concentration ranges were used: GEN, 0.5–32 μg/ml; AMK, 1–64 μg/ml; TOB, 0.25–16 μg/ml initially and then 0.125-128 for the confirmation of resistance; The results were evaluated after 24 and 48 h at 37 °C to allow visible growth of all tested strains. The determination of the ciprofloxacin (CPX) MIC of the SA laboratory strains were performed identically, using SA ATCC 25923 as the reference strain; the ciprofloxacin range tested was 0.01-10 μg/ml.

The MIC of the candidate EPIs were performed by broth microdilution method, following CLSI guidelines, testing the range 10-320 μg/ml for berberine and 0.78-50 μg/ml for the NorA candidate EPIs.

The MIC of tobraycin and ciprofloxacin in association with EPIs, were performed (accordingly to CLSI guidelines) in PA and SA in presence of 80 μg/ml of berberine and 12.5 μg/ml of NorA EPI, respectively. The association was considered as synergistic when its MIC was ≥ 4fold lower than the MIC of the antibiotic alone.

## PCR assays

Two chromosomally encoded (*mexY* and *ndvB*) and four additional aminoglycoside resistance genes (*aac(3)-Ia, aph(3')-IIa, ant(2")-Ia* and *rmtA*) were sought in the aminoglycoside resistant *P. aeruginosa* isolates by PCR assays. Bacterial DNA was obtained from crude cell lysates [53] and 5 μl were used in each PCR reaction together with 1.25 U Dream-Taq Polymerase (Thermo Fisher Scientific), 1x PCR Buffer, 0.2 mM dNTPs and 0.5 μM of each primer. The target genes and the specific primer pairs used are reported in **Table 1**.

**Table 1**: genes sought by PCR and their primers.

| Target gene | | Primer pair (5'-3') | Amplicon size (bp) | Reference |
|---|---|---|---|---|
| *mexY* | mexY-F | TGGAAGTGCAGAACCGCCTG | 270 | [86] |
| | mexY-R | AGGTCAGCTTGGCCGGGTC | | |
| *ndvB* | ndvB JB-F | GGCCTGAACATCTTCTTCACC | 138 | [15] |
| | ndvB JB-R | GATCTTGCCGACCTTGAAGAC | | |
| *rmtA* | rmtA-F | CTAGCGTCCATCCTTTCCTC | 635 | [129] |
| | rmtA-R | TTTGCTTCCATGCCCTTGCC | | |
| *aac(3)-Ia* | aac3-F | GGCTCAAGTATGGGCATCAT | 389 | [75] |
| | aac3-R | TCACCGTAATCTGCTTGCAC | | |
| *aph(3')-IIa* | npt2-F | GATCTCCTGTCATCTCACCTTGCT | 129 | [127] |
| | npt2-R | TCGCTCGATGCGATGTTTC | | |
| *ant(2")-Ia* | ant2bi-F | GACACAACGCAGGTCACATT | 500 | [75] |
| | ant2bi-R | CGCAAGACCTCAACCTTTTC | | |

*P. aeruginosa* PAO1 and PA14 DNA was used as positive control in PCRs targeting *mexY* and *ndvB*, respectively; *P. aeruginosa* PAO1 carrying the plasmid pHERD30T, which harbors the *aac(3)-Ia* gene, was used as a positive control in the related PCR assays. Amplicons of *rmtA*, *aph(3')-IIa* and *ant(2")-Ia* of the right size were purified (Gene Elute PCR Cleanup kit, Sigma-Aldrich) and sequenced using BigDye Terminator v.1.1 Cycle Sequencing kit according to the manufacturer's instructions. The sequences were analyzed on an ABI Prism 310 Genetic Analyzer (Applied Biosystems, Foster City, CA, USA), and used as positive controls in PCRs targeting the corresponding gene. RNase-free water (Thermo Fisher Scientific) was used as the negative control. The PCR products were controlled on electrophoresis in 1.5% agarose gel.

## Checkerboard assays

Checkerboard assays were performed following CLSI guidelines [26]. For *P. aeruginosa*, checkerboard assays were performed against *P. aeruginosa* C25 using 2-fold increasing concentrations of both tobramycin (from 0.125 to 128 μg/mL) and berberine (from 10 to 320 μg/mL). Since berberine was resuspended in 50% DMSO and 50% methanol, the upper limit of the concentrations range tested was determined keeping in mind the recommended final concentration of 1.5% DMSO; a 4-fold decrease of tobramycin MIC was interpreted as synergy, as already reported in literature [60].

For *S. aureus*, checkerboard assays were performed against SA1199B, SAK1902 and SAK2378 using 2-fold increasing concentrations of both ciprofloxacin (from 0.001 to 20 µg/mL) and tested EPI (from 0.39 to 25 µg/mL).

## Persistence assays

*Planktonic persistence assays*

For *P. aeruginosa*, persistence assays were performed on planktonic cultures as follows: the strains were grown in LB overnight at 37°C and then exposed to tobramycin 20xMIC, alone or combined with berberine 80 µg/ml and finally incubated for 24h at 37 °C. 1 ml aliquots of culture were collected, centrifuged 5 minutes at 3300xg, resuspended in fresh LB and suitable dilutions were plated on cystine-lactose-electrolyte-deficient (CLED) agar plates at 0, 7 and 24 hours from exposure. The abundance, evaluated in colony forming units (CFU)/ml, of bacterial survivors was evaluated after 24 h of incubation at 37 °C.

For *S. aureus*, persistence assays were performed identically but exposing the SA planktonic cultures in stationary or exponential phase to ciprofloxacin 20xMIC and plating the aliquots on Triptone Soy Agar after 0, 1, 3, 5, 7 and 24 hours of exposure. All tests were run in biological duplicate.

*Biofilm persistence assays*

*P. aeruginosa* biofilms developed overnight in LB in 35 mm petri dishes at 37 °C were incubated for 24 h at 37 °C in LB supplemented with tobramycin 1000xMIC, alone or in association with berberine (80 or 320 µg/ml). Immediately before tobramycin exposure ($T_0$) or after 24-hour incubation ($T_{24}$), biofilms were mechanically detached, resuspended in phosphate-buffered saline (PBS) and serially 10-fold diluted. Suitable dilutions were plated on CLED agar plates and incubated for 24 h at 37 °C before the CFU counts. These tests were run in biological triplicate.

## Biofilm production assays

Biofilm production was evaluated as described previously (Camilli et al., 2011), with some modifications. Briefly, *P. aeruginosa* biofilms were developed in flat-bottom microtiter plates in LB without / with berberine 80 µg/ml. After overnight incubation at 37 °C, they were washed with sterile deionized water (DW) and stained with 1% crystal violet for 15 min. After removing the dye, biofilms were washed with DW and resuspended in 96% ethanol. Biofilms were quantified by measuring the optical density (OD) of the sessile phase at 570 nm. Biofilm production/bacterial cell was determined by dividing this value by the $OD_{600}$ of the planktonic phase. These tests were run in triplicate.

## *mexY* sequence analysis

The main variable regions of the *mexY* gene were identified by comparing the gene sequence of *P. aeruginosa* PAO1 (accession number, AB015853.1) and additional strain-specific sequences. Two amplicons of these regions (respectively of 270 and 588 bp) were obtained by PCR using the primer pairs mexY-F 5'-TGGAAGTGCAGAACCGCCTG-3' / mexY-R 5'-AGGTCAGCTTGGCCGGGTC-3' [86] and YF 5'-CGTGAGCATGGACGAGATCA-3' / YR 5'-ATGATGGTGATCAGGCCGAC-3' (this study). The amplicons were purified using Gene Elute PCR Cleanup kit (Sigma-Aldrich SRL) and sequenced using BigDye Terminator v.1.1 Cycle Sequencing kit according to the manufacturer's instructions. Sequences were analyzed on an ABI Prism 310 Genetic Analyzer (Applied Biosystems, Foster City, California, USA). The consensus sequences thus obtained were compared to the PAO1 sequence. The MAFFT algorithm implemented in AliView software (https://github.com/andersla/Assseq, [62] was used to align the nucleotide sequences analyzed with the *P. aeruginosa* PAO1 *mexY* corresponding regions.

## Time-kill curve assays

Time−kill curve analysis were performed as previously described [54]. For *P. aeruginosa*, tobranycin concentrations ranging from ½x to 2×MIC were used alone and in combination with the lowest active concentration of berberine resulting from checkerboard assays. *P. aeruginosa* C95 was used as the test strain. The dynamic of the bactericidal activity of the combination tobramycin-berberine was evaluated by CFU counts after plating on CLED agar at 2, 4, 6, 8, and 24 h of incubation at 37 °C. Any increase ≥ 1 log of the bactericidal power of the association compared to that of the antibiotic alone was considered as synergistic.

For *S. aureus*, the time-kill curve assays were performed identically, with some modifications: ciprofloxacin concentrations ranging from ¼x to 1xMIC were used alone and in combination with the tested compound at 3.13 and 6.25 µg/ml; aliquots at the various timepoints were plated on Triptone Soy Agar. SA1199B was used as the test strain.

## Ethidium bromide efflux assays

Ethidium bromide (EtBr) efflux assays were performed as previously described [53]. *S. aureus* cells were grown overnight in cation-supplemented Mueller Hinton broth (SMHB) at 37 °C. The culture was then diluted 25-fold into the same medium and has been grown exponentially until an $OD_{600}$ of 0.7-0.8. Cells were then pelleted and resuspended at $OD_{600}$ = 0.8 in 0.5 ml aliquots of SMHB containing EtBr plus carbonyl cyanide m-chlorophenylhydrazone (CCCP) to “load” cells with EtBr (final concentrations, 25 µM for EtBr and 100 µM for CCCP). After 20 min at room temperature, cells were pelleted then resuspended in 1 ml of fresh SMHB, and 200 µl aliquots were immediately transferred into the wells of opaque 96-well plates containing no or 50 µM test compound. Fluorescence was monitored using a BioTek Synergy HT microplate reader (BioTek Instruments Inc., Winooski, VT, USA) at excitation and emission wavelengths of 485 and 645 nm, respectively, for 5 min. Experiments were conducted in triplicate performing two technical replicates for each biological replicate. Efflux activity of SA-1199B was expressed as fluorescence decrease (%) over a 5 min time course. Efflux inhibition was determined using the equation ([efflux in the absence]

- [efflux in the presence of test [compound])/[efflux in the absence of test compound] x 100, giving the percent efflux inhibition observed.

## Toxicity assays

Hemolysis assays were performed as described previously [25]: 4 mL of freshly drawn, heparinized human blood was diluted with 25 ml of phosphate buffered saline (PBS), pH 7.4. After washing three times in 25 ml of PBS, the pellet was resuspended in PBS to ~20 vol %. A 100 µl amount of erythrocyte suspension was added to 100 µL of different concentrations of compound to be tested (1:2 serial dilutions in PBS, from 10 to 320 µg/ml for berberine and from 0.20 to 25 for NorA EPIs). The negative and the positive control were 100 µL of PBS and 100 µL of 0.2 vol % Triton X-100, respectively. After 1 h of incubation at 37 °C each well was supplemented with 150 µL of PBS and the plate centrifuged at 1.200g for 15 min. The supernatant was diluted 1:3 and transferred in a new plate, and its $OD_{540}$ was measured using the BioTek Synergy HT microplate reader spectrophotometer. The hemolysis (%) was determined as follows:

$$[(A - A_0)/(A_{\text{total}} - A_0)] \times 100$$

where A is the absorbance of the test well, $A_0$ the absorbance of the negative control, and $A_{\text{total}}$ the absorbance of the positive control; three technical replicates for every condition were performed.

The cytotoxic effect of the NorA targeting compounds was determined by MTT assays performed on THP-1 and A549 (CCL-185TM) cells after 24 h exposure, as described previously [29].

# RESULTS & DISCUSSION

# *Pseudomonas aeruginosa* and MexXY-OprM

## Preliminary study: isolation and characterization of aminoglycoside resistant *P. aeruginosa*

One hundred forty-seven *P. aeruginosa* clinical strains collected from the sputum samples of CF patients have been screened by Sensititre$^{TM}$ for their resistance to the aminoglycosides tobramycin, gentamicin and amikacin. The screening revealed that 78 of them (53%) were resistant to at least one of the tested antibiotics. The resistance was further confirmed by broth microdilution method showing that, 66 were resistant to GEN (84.62%) , 66 to AMK (84.62%) and 27 resistant to tobramycin (34.62%), thus revealing a significant higher ($p < 0.01$) abundance of gentamicin or amikacin resistance compared to tobramycin (**Fig.17A**).

The simultaneous resistance to all 3 antibiotics (26.92%) was more common than resistance to gentamicin (7.69%) or tobramycin (2.56%) alone ($p < 0.05$ and $p < 0.01$, respectively). 36 (44.87%) isolates showed both gentamicin and amikacin resistance, 4 (5.13%) showed both gentamicin and tobramycin resistance, while a simultaneous resistance both to amikacin and tobramycin was never observed (**Fig.17B**).

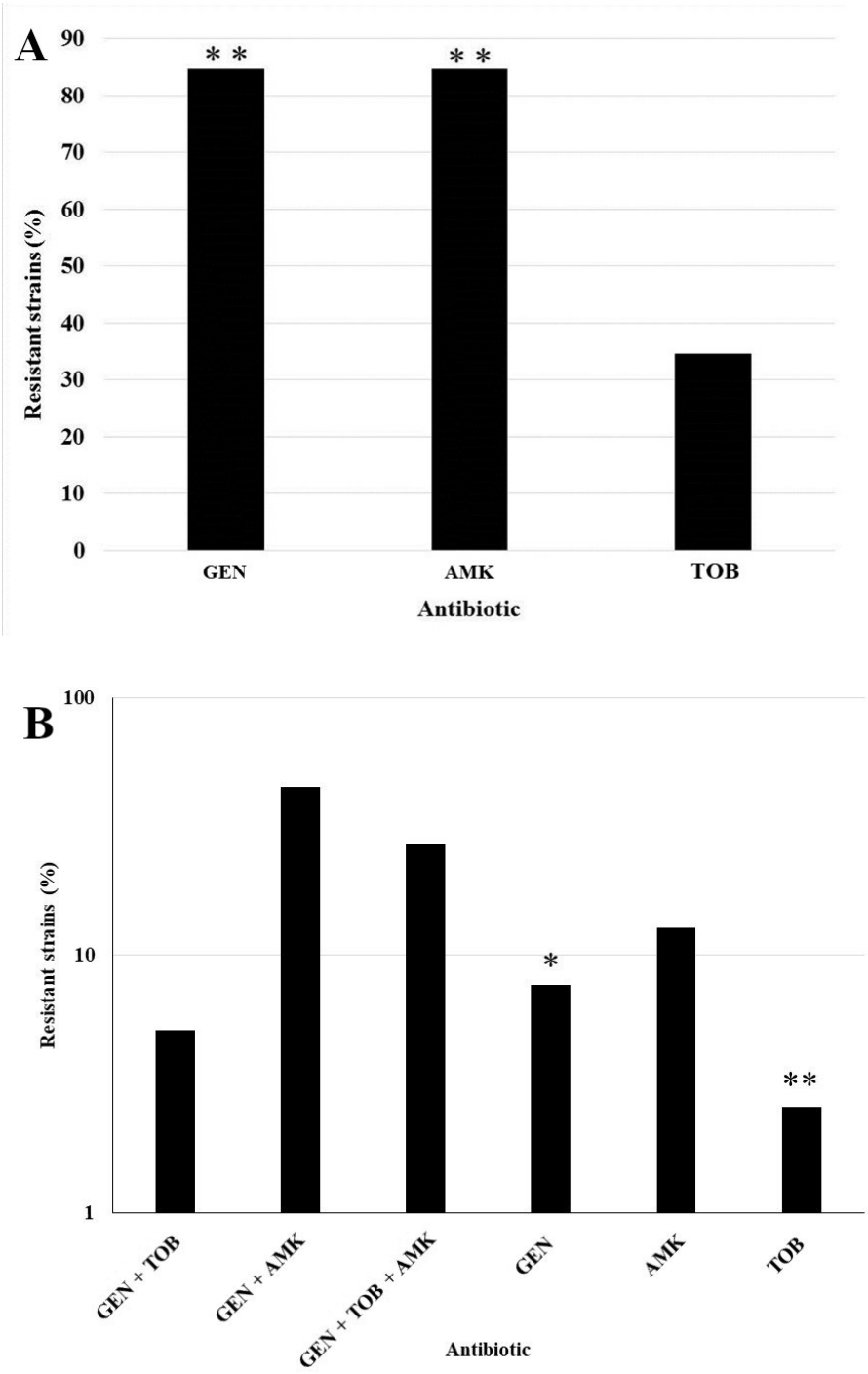


**Figure 17** Percent amount of *P. aeruginosa* isolates resistant to: (**A**) GEN, AMK or TOB; (**B)** a single (GEN, AMK or TOB) or multiple (GEN+TOB, GEN+AMK, GEN+TOB+AMK) antibiotic(s). As 100% was considered the total amount (78) of investigated aminoglycoside resistant strains. A significant difference between the resistance to a single antibiotic and its combinations was marked. GEN: gentamicin, AMK: amikacin, TOB: tobramycin. *$p < 0.05$, **$p < 0.01$.

The 78 strains were further analyzed for the presence of 6 genes (2 intrinsic, chromosome-encoded and 4 acquired) coding for different aminoglycoside resistance mechanisms, in order to estimate the role of each mechanism in the phenotypic resistance observed. The genes analyzed and their function are summarized in **Table 2**.

**Table 2**: aminoglycoside resistance determinants, their genomic location and the aminoglycoside resistance mechanism they encode.

| Gene | Location | Function |
|---|---|---|
| *mexY* | intrinsic | Inner membrane protein of the MexXY-OprM efflux pump [86] |
| *ndvB* | intrinsic | Synthesis of periplasmic cyclic glucans able to bind aminoglycosides [86] |
| *rmtA* | acquired | Ribosome modifying enzyme (methylation of 16S rRNA) [129] |
| *aac(3)-Ia* | acquired | Aminoglycoside modifying enzyme (acetyltransferase) [75] |
| *aph(3')-IIa* | acquired | Aminoglycoside modifying enzyme (phosphotransferase) [127] |
| *ant(2")-Ia* | acquired | Aminoglycoside modifying enzyme (nucleotidyltransferase) [75] |

*mexY* was detected in all strains; *ndvB* was detected in all strains except one (C36); *aac(3)-Ia* and *ant(2")-Ia* were detected in 4 (C40, AR15, AR39 and AR66) and 7 (C44, C45, AR45, AR49, AR86, AR88 and AR89 ) isolates, respectively; finally, *rmtA* and *aph(3')-IIa* were never detected. Sequencing of *ant(2")-Ia* amplicon of *P. aeruginosa* AR86 demonstrated a similarity of 99% to the sequence of *P. aeruginosa* strain PB350 (Accession no. CP025055.1) and was thus selected as positive control for the PCR analysis of this gene. Interestingly, *aac(3)-Ia* and *ant(2")-Ia* genes seem to be mutually exclusive, since they were never found together in the same strain.

When comparing bacterial phenotypes and genotypes of *P. aeruginosa* isolates carrying the acquired aminoglycoside resistance determinants, we observed that all strains with *aac(3)-Ia* were resistant to gentamicin alone or to gentamicin and amikacin. *ant (2")-Ia* was detected in 50% of strains resistant to tobramycin or tobramycin+gentamicin and in 10% of strains resistant to amikacin, in 9.52% of strains

resistant to tobramycin+amikacin+gentamicin and in 2.86% of those resistant to gentamicin +amikacin (**Table 3**).

**Table 3**: AMG resistance phenotype and genotype of the 78 *P. aeruginosa* aminoglycoside resistant isolates. R: resistant; S: susceptible. Breakpoints: GEN and TOB, S ≤ 4 R > 4; AMK, S ≤ 16, R > 16 [27].

| | PHENOTYPE | | | GENOTYPE | | |
|---|---|---|---|---|---|---|
| **Strain** | **GEN** | **AMK** | **TOB** | ***mexY*** | ***ndvB*** | **Acquired resistance gene** |
| C2 | R | R | S | + | + | / |
| C5 | R | S | S | + | + | / |
| C6 | R | R | R | + | + | / |
| C7 | R | R | S | + | + | / |
| C8 | R | R | S | + | + | / |
| C9 | R | R | R | + | + | / |
| C10 | R | S | S | + | + | / |
| C11 | R | R | R | + | + | / |
| C12 | R | R | S | + | + | / |
| C14 | S | R | S | + | + | / |
| C15 | R | R | R | + | + | / |
| C16 | R | R | S | + | + | / |
| C19 | R | R | S | + | + | / |
| C21 | S | R | S | + | + | / |
| C22 | R | R | S | + | + | / |
| C25 | R | R | R | + | + | / |
| C26 | R | R | R | + | + | / |
| C30 | S | S | R | + | + | / |
| C31 | R | S | R | + | + | / |
| C33 | R | R | S | + | + | / |
| C35 | R | S | S | + | + | / |
| C36 | R | R | R | + | - | / |
| C38 | R | R | S | + | + | / |
| C40 | R | R | S | + | + | ***aac(3)-Ia*** |
| C43 | R | R | S | + | + | / |
| C44 | R | R | R | + | + | ***ant(2")-Ia*** |
| C45 | R | R | R | + | + | ***ant(2")-Ia*** |
| C47 | R | R | S | + | + | / |
| C49 | R | R | S | + | + | / |
| C51 | R | R | R | + | + | / |
| AR1 | R | R | S | + | + | / |
| AR4 | R | R | S | + | + | / |
| AR5 | R | R | R | + | + | / |
| AR7 | R | R | S | + | + | / |
| AR8 | R | R | S | + | + | / |
| AR9 | R | R | S | + | + | / |
| AR11 | S | R | S | + | + | / |

| | | | | | | |
|---|---|---|---|---|---|---|
| AR12 | S | R | S | + | + | / |
| AR13 | R | S | S | + | + | / |
| AR14 | R | R | S | + | + | / |
| AR15 | R | S | S | + | + | ***aac(3)-Ia*** |
| AR18 | S | R | S | + | + | / |
| AR19 | R | R | S | + | + | / |
| AR25 | S | R | S | + | + | / |
| AR26 | R | R | R | + | + | / |
| AR28 | R | R | S | + | + | / |
| AR29 | R | R | S | + | + | / |
| AR35 | R | R | R | + | + | / |
| AR36 | R | R | S | + | + | / |
| AR37 | R | R | R | + | + | / |
| AR38 | R | R | S | + | + | / |
| AR39 | R | R | S | + | + | ***aac(3)-Ia*** |
| AR45 | R | S | R | + | + | ***ant(2")-Ia*** |
| AR46 | S | R | S | + | + | / |
| AR47 | R | R | R | + | + | / |
| AR48 | R | S | R | + | + | / |
| AR49 | R | S | R | + | + | ***ant(2")-Ia*** |
| AR50 | S | R | S | + | + | / |
| AR51 | R | R | R | + | + | / |
| AR52 | R | S | S | + | + | / |
| AR53 | R | R | R | + | + | / |
| AR60 | R | R | S | + | + | / |
| AR61 | R | R | R | + | + | / |
| AR62 | R | R | R | + | + | / |
| AR66 | R | R | S | + | + | ***aac(3)-Ia*** |
| AR68 | R | R | S | + | + | / |
| AR69 | R | R | S | + | + | / |
| AR70 | R | R | S | + | + | / |
| AR71 | S | R | S | + | + | / |
| AR81 | R | R | S | + | + | / |
| AR82 | R | R | S | + | + | / |
| AR83 | R | R | S | + | + | / |
| AR84 | R | R | R | + | + | / |
| AR85 | R | R | R | + | + | / |
| AR86 | S | S | R | + | + | ***ant(2")-Ia*** |
| AR87 | R | R | S | + | + | / |
| AR88 | S | R | S | + | + | ***ant(2")-Ia*** |
| AR89 | R | R | S | + | + | ***ant(2")-Ia*** |

Analyzing these data, the first outcome that strongly emerges is the high amount (53%) of aminoglycoside resistant strains isolated from CF patients' sputum, thus highlighting the fact that one of the most commonly used class of antibiotics in *P. aeruginosa* treatment seems to be not effective in half of the cases. Moreover, the higher frequency of co-resistances ( GEN+AMK, 44,87%; GEN+AMK+TOB, 26.92; GEN+tobramycin, 5.13%) compared to the resistance to gentamicin, amikacin and tobramycin alone (7.69%, 12.82% and 2.56%, respectively) suggests the important role of cross-resistance mechanisms, of which the largest contributor could be, very likely, the MexXY-OprM efflux pump, in particular when over-expressed. This hypothesis is also supported by the presence of multi-aminoglycoside resistant strains lacking additional resistance genes. The absence of isolates simultaneously resistant to amikacin and tobramycin but susceptible to gentamicin suggest that mechanisms able to counteract the effect of the former two antibiotics are always able to confer resistance also to the latter. Anyway, tobramycin seems to be the most effective aminoglycoside among the three tested, having a resistance rate (34.62%) significantly lower than the other two aminoglycosides (84.62% each), this confirms the literature data which still elect this antibiotic as the best therapeutic option in the treatment of *P. aeruginosa* CF lung infection [80, 82, 98].

Analyzing the genotypic aminoglycoside resistance profiles of the tested isolates, it's not unexpected the uniform presence of the two chromosome-encoding resistance genes *mexY* and *ndvB*, with the former detected in all strains and the latter in all but one (C36), probably due to mutations in the primer target region. Considering that *ndvB* resistance action is associated only to a biofilm mode of growth [15], the resistant phenotypes observed in strains lacking acquired aminoglycoside resistance genes is likely mediated by the MexXY-OprM efflux pump, that has been already demonstrated to be responsible of multi-aminoglycoside resistance phenotype and which is very commonly over-expressed in CF chronic lung infections [99]. Additional genes were rarely found (on 11/78 isolates) in agreement with literature data which show that aminoglycoside resistance in CF isolates, besides efflux pumps overexpression, is mostly caused by mutations [67]. As expected, the four strains carrying *aac(3)-Ia* were all resistant to gentamicin, while the isolates harboring *ant(2")-Ia* were resistant to gentamicin and tobramycin [104], except for AR88 and AR89, which resulted susceptible to tobramycin, likely due to gene silence or downregulation.

In conclusion, this preliminary study highlights and confirms that aminoglycoside resistance in *P. aeruginosa* CF lung infections is widespread, underlining once again the need for an alternative strategy for their eradication. Resistance to tobramycin, both due to EP overexpression and to the acquired gene *ant(2")-Ia*, still appears to be the less frequent among the investigated strains, supporting its common use for the treatment of *P. aeruginosa* lung infections, in agreement with literature data [82], Though the involvement of aminoglycoside modifying enzymes different from those sought in this study can't be excluded, the main responsible of aminoglycoside resistance in our strains seems to be an enhanced MexXY-OprM-mediated tobramycin efflux, possibly due to mutations in the regulatory genes.

## Berberine as MexXY-OprM inhibitor and evidence of its synergy with tobramycin

After demonstrating the key role of the efflux pump MexXY-OprM in *P. aeruginosa* aminoglycoside resistance, my study focused on the search and validation of an inhibitor able to bind and disrupt its activity. The aim was to investigate whether the combination antibiotic-EPI could decrease the resistance level to the drug in *P. aeruginosa* clinical and laboratory strains. This study was performed in collaboration with the Biomolecular Modeling group of Dr. Roberta Galeazzi (Department of Life and Environmental Sciences, Polytechnic University of Marche, Italy), which performed the *in silico* preliminary studies.

In particular, since not yet determined, the tridimensional structure of the MexY subunit was built through a homology modeling approach based on the already deposited X-ray crystal structures of the homologous EP inner membrane proteins AcrB of *E. coli* and MexB of *P. aeruginosa* (PDB accession code 1T9Y and 2V50, respectively). A monomeric model of MexY, was first built, followed by the corresponding trimeric complex, using a comparative modeling approach. Once energy-minimized and then stabilized by full-atom molecular dynamics simulation in its biological environment (i.e., bacterial membrane), the structure was analyzed comparing its structural domains with those of the reference pump AcrB (**Fig.18**).

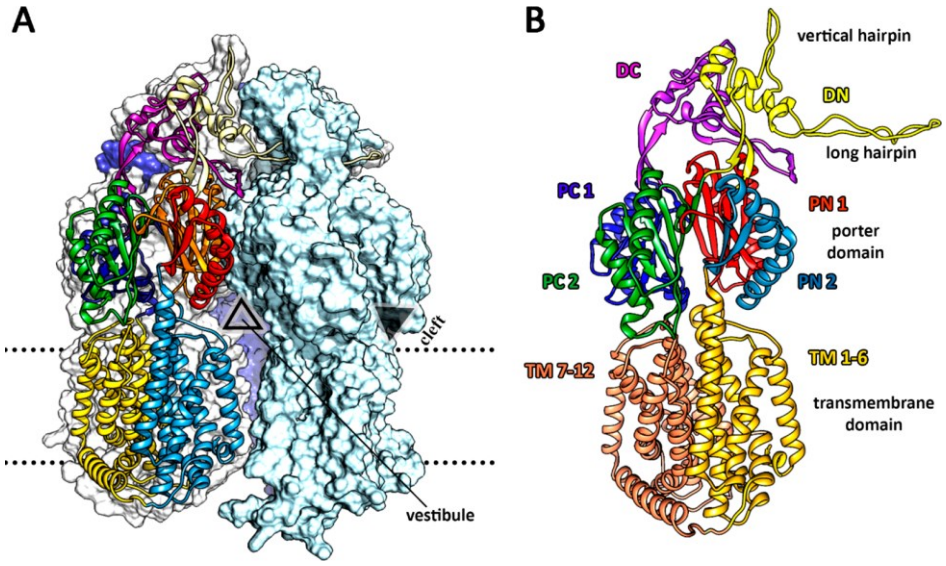


**Figure 18** Tridimensional structure of MexY in the trimeric (A) and monomeric (B) conformation. The different domains (evidenced with different colors) were determined by homology with those of AcrB.

The analysis of the tridimensional structure of MexY revealed two possible binding pockets: an external periplasmic binding site ("Site 1", or "Cleft site") and the extrusion antibiotic site ("Site 2" or "Antibiotic site").

In order to find a natural compound as MexXY-OprM inhibitor, a molecular docking approach was performed on the natural compounds database ZINC (http://zinc.docking.org) against the MexY subunit and the molecules showing the highest affinity for the two binding sites were considered for further analysis. Promising results were achieved with berberine (Be). This compound is a nonbasic, quaternary benzylisoquinoline alkaloid that can be extracted from plants such as *Hydrastis anadensis*, *Berberis aristata*, *Berberis vulgaris* and many others, and has been used in Ayurvedic and Chinese medicine since thousands of years because of its countless beneficial effects: more recent studies have demonstrated, among the others, its significant anti-inflammatory, antihypertensive, anti-hyperglycemic, antioxidant activity and antimicrobial activities (**Fig.19**) [58]. Moreover, a possible MexXY-OprM inhibitor activity has recently been suggested, although not deeply investigated in synergy with tobramycin [79].

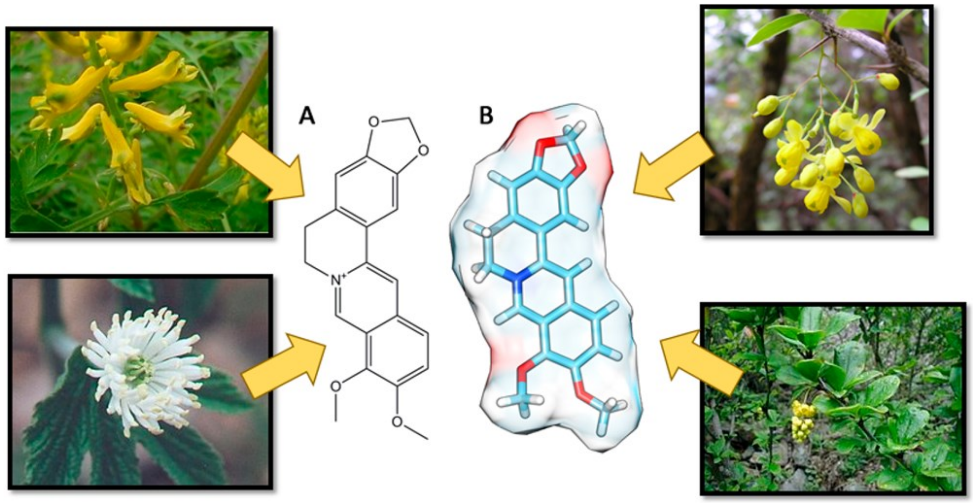


**Figure 19** 2D (A) and 3D (B) structures of berberine. On the left and on the right, some of the plants from which berberine is extracted: *Coptis chinensis* (upper left), *Hydrastis canadensis* (lower left), *Berberis aquifolium* (upper right), and *Berberis vulgaris* (lower right).

Berberine showed a high affinity for the antibiotic site (Binding Energy = -11.42 Kcal/mol (Free Gibbs Energy = -864.31 ± 80.79 KJ/mol), actually superior than tobramycin (Binding Energy = -8.21 Kcal/mol, Free Gibbs Energy = -454.02 ± 75.44 KJ/mol) (**Fig.20**), which makes this molecule, at least *in silico*, an excellent competitor for the binding of the MexY antibiotic site.

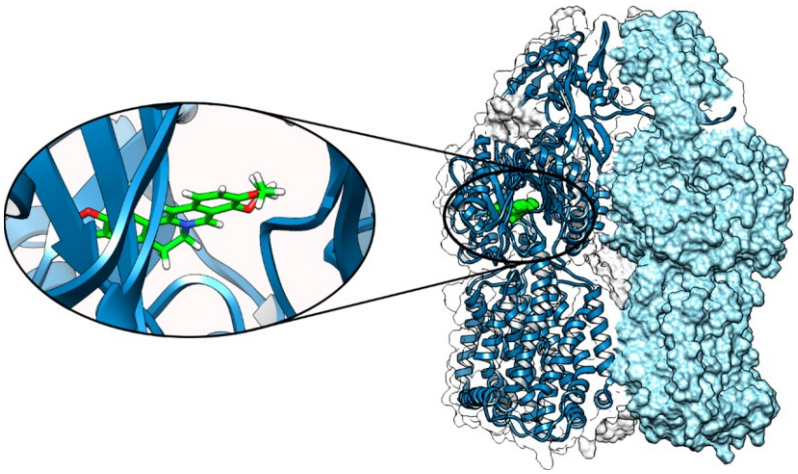

**Figure 20** Binding pose of berberine (in green) in the antibiotic binding site of the MexY trimer.

Given these promising preliminary results, the EP inhibitory activity of berberine and thus its putative synergistic effect in combination with tobramycin was assessed *in vitro*. Firstly, MIC assays on *P. aeruginosa* laboratory strain ATCC 27853 revealed no antimicrobial activity of the compound alone up to a concentration of 320 µg/ml. In order to determine its possible synergy with tobramycin, checkerboard assays were performed against the CF clinical strain *P. aeruginosa* C25, overexpressing mexY (5-fold higher than laboratory strain PAO1) and lacking acquired tobramycin resistance genes. Although a calculation of the FIC index was not possible (due to the absence of a MIC value of the compound at the tested concentrations), a significant reduction (16-fold, from 16 to 1 µg/ml) of tobramycin MIC, when used in combination with berberine concentrations starting from 80 µg/ml, was observed with values which changed from resistance to susceptibility, (**Table 4**).

**Table 4:** Checkerboard results of the combination berberine/tobramycin against *P. aeruginosa* C25. In order to evaluate a possible effect of the diluent (50% DMSO / 50% MeOH) on TOB MIC, the strain was also challenged with the diluent alone (0+diluent) at the max. concentration used (3%).

| Berberine (µg/ml) | Tobramycin MIC (µg/ml) |
|---|---|
| 0 | 16 |
| 0 + diluent | 16 |
| 20 | 16 |
| 40 | 4 |
| 80 | 1 |
| 160 | 1 |
| 320 | 1 |

Subsequently, berberine effect on tobramycin MIC was assessed in 2 further laboratory and 32 clinical PA strains: 9 clinical CF strains were chosen among the isolates of the previous study having no acquired tobramycin resistance genes (C6, C9, C15, C30, C31, C51, AR48, AR51 and AR61); further 23 clinical non-CF (C52-C104) and CF (NC01-NC10) both resistant and susceptible to tobramycin were selected after assessing the absence of acquired tobramycin resistance genes. Tobramycin MIC was evaluated in absence and presence of berberine at the fixed concentration of 80 µg/ml: the lowest active concentration determined in the checkerboard assay against *P. aeruginosa* C25. *P. aeruginosa* C6, C9, C51 and AR61 tobramycin MIC was corrected respect to the previous study after multiple repeats of the assay.

A good synergistic effect (i.e. at least 4-fold decrease) was observed on further 15 isolates (**Table 5**).

**Table 5:** Tobramycin MIC in the absence/presence of 80 µg/ml berberine against 35 isolates. L: laboratory strain, CF: cystic fibrosis isolate, C: clinical non-CF isolate. * Reduction of tobramycin MIC ≥ 4-fold.

| Strain | Source | MIC (µg/ml) | |
|---|---|---|---|
| | | tobramycin | tobramycin + berberine |
| *P. aeruginosa* PA14 | L | 0,125 | 0,125 |
| *P. aeruginosa* K767 | L | 0,25 | 0,125 |
| *P. aeruginosa* C6 | CF | 2 | 0,5* |
| *P. aeruginosa* C9 | CF | 2 | 1 |
| *P. aeruginosa* C15 | CF | 8 | 8 |
| *P. aeruginosa* C25 | CF | 16 | 1* |
| *P. aeruginosa* C30 | CF | 8 | 8 |
| *P. aeruginosa* C31 | CF | 8 | 8 |
| *P. aeruginosa* C51 | CF | 0,125 | <0,125 |
| *P. aeruginosa* C52 | C | >128 | 128 |
| *P. aeruginosa* C54 | C | 32 | 16 |
| *P. aeruginosa* C59 | C | 64 | 32 |
| *P. aeruginosa* C60 | C | >128 | 64* |
| *P. aeruginosa* C61 | C | 64 | 16* |
| *P. aeruginosa* C67 | C | 16 | 16 |
| *P. aeruginosa* C70 | C | 32 | 8* |
| *P. aeruginosa* C73 | C | 128 | 64 |
| *P. aeruginosa* C76 | C | 32 | 16 |
| *P. aeruginosa* C83 | C | 128 | 32* |
| *P. aeruginosa* C84 | C | 0,5 | 0,125* |
| *P. aeruginosa* C86 | C | 128 | 16* |
| *P. aeruginosa* C89 | C | >128 | 64* |
| *P. aeruginosa* C93 | C | >128 | 64* |
| *P. aeruginosa* C95 | C | 32 | 4* |
| *P. aeruginosa* C98 | C | 64 | 32 |
| *P. aeruginosa* C103 | C | 4 | 4 |
| *P. aeruginosa* C104 | C | 32 | 8* |
| *P. aeruginosa* AR48 | CF | 32 | 8* |
| *P. aeruginosa* AR51 | CF | 16 | 8 |
| *P. aeruginosa* AR61 | CF | 0,125 | 0,125 |
| *P. aeruginosa* NC01 | CF | 16 | 8 |
| *P. aeruginosa* NC02 | CF | 32 | 16 |
| *P. aeruginosa* NC04 | CF | 0.5 | 0* |
| *P. aeruginosa* NC06 | CF | 2 | 0.5* |
| *P. aeruginosa* NC10 | CF | 1 | 0.25* |

Time kill curve analysis were then performed to compare the dynamics of the bactericidal activity of different combinations of tobramycin (½x, 1x and 2xMIC) and berberine (80 µg/ml) against the *P. aeruginosa* clinical strain C95, which showed a tobramycin MIC reduction comparable to that of *P. aeruginosa* C25, but endowed of a faster visible growth (24h instead of 48h) and thus easier to test. When testing tobramycin alone, a bactericidal activity (i.e. at least 3 log decrease of bacterial amount compared to the condition with no antibiotic) was observed only when using a tobramycin concentration of 2xMIC (i.e. 64 µg/ml) starting from 4 hours of antibiotic exposure. All tested antibiotic concentrations showed an increase of the *P. aeruginosa* surviving cells between 8 and 24 hours, particularly evident for the 2x MIC. In combination with berberine, 1xMIC and 2xMIC tobramycin resulted bactericidal after 2 hours and ½ MIC tobramycin after 4 hours of exposure. Contrary to what observed when exposed to the antibiotic alone at 1x and 2xMIC, *P. aeruginosa* C95 did not show any increase of the surviving cells at 24 hours when treated with the same antibiotic concentrations in combination with berberine (**Fig.21**).

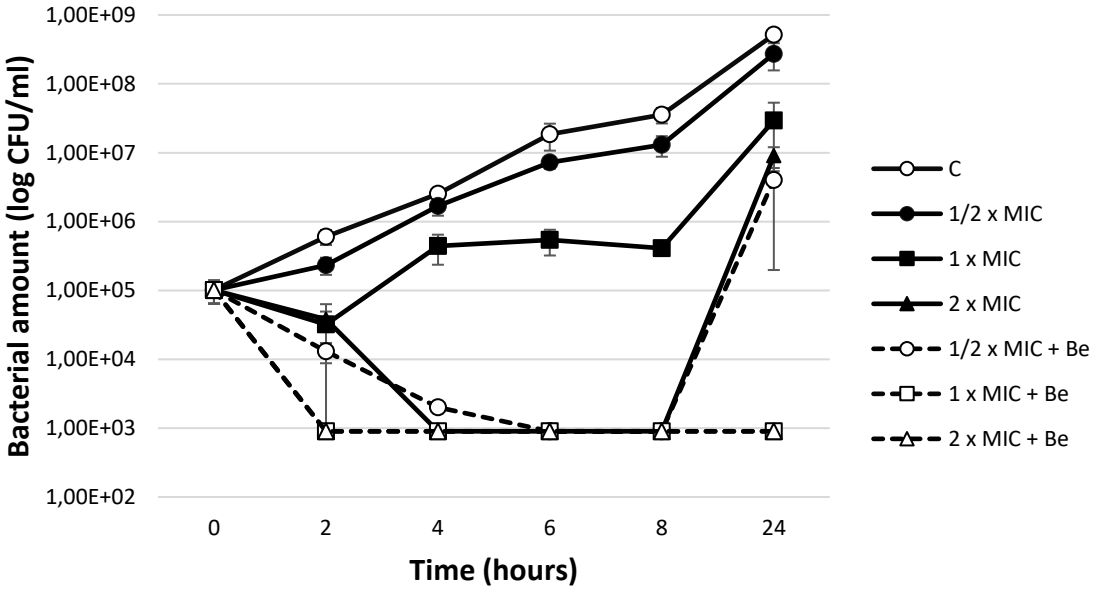


**Figure 21** Time kill curve of *P. aeruginosa* C95 performed using no antibiotic (C) or 16 (1/2×MIC), 32 (MIC), and 64 µg/mL (2×MIC) tobramycin, alone (solid lines) or in combination with 80 µg/ml berberine (Be) (dashed lines).

Finally, hemolytic assays were performed using berberine concentrations from 10 to 320 μg/ml in order to evaluate its toxic effect against human cells. Both berberine (at every concentration) and the solvent vehicle (50% DMSO / 50% MeOH) used as control didn't show any hemolytic effect.

Overall, these data support the validity of the used *in silico-in vitro* approach for the search of new effective EPIs. The natural alkaloid berberine, selected after the promising results in the *in silico* screening, was able to show a very good synergistic effect in combination with tobramycin on the *mexY*-overexpressing clinical strain C25 and confirmed its activity on further 15/34 isolates at the concentration of 80 μg/ml. Time-kill curve assays allow us to observe the effect of different tobramycin-berberine combinations and showed a strongly enhanced bactericidal activity (at least 2 log CFU decrease) of the combination respect to tobramycin alone; worthy of note, in every condition with only antibiotic, a marked increase of CFU was observed between 8 and 24 hours of exposure, probably due to a drop in the antibiotic effectiveness or to the selection of a tolerant or persistent population. However, this pattern wasn't observed when treating with 1x and 2x MIC tobramycin in combination with berberine: the lack of any increase of the bacterial population between 8 and 24 hours of treatment implies a much more incessant activity of the combination compared to tobramycin alone and suggests a possible anti-persister activity (although higher concentrations of antibiotics must be used to properly evaluate this feature).

These data strongly support the *in silico* findings which propose berberine as a solid competitor of tobramycin for the *mexY* antibiotic binding site (as evidenced by the differences in binding energes). Finally, it is important to stress that the screening of a database of natural compounds can overcome of the toxicity issues that affects most of the EPIs actually under study. On the other hand, berberine benefic effects have long been known and this study confirms its lack of toxicity. The lack of synergistic effect on 20/35 *P. aeruginosa* isolates needs to be furtherly investigated; it can be due either to the presence of additional acquired tobramycin resistance genes not sought in the preliminary screening or to mutations in the MexY binding sites, eventually hampering the affinity with berberine.

## Berberine as a *P. aeruginosa* biofilm production inhibitor

Given the documented involvement of efflux pumps in biofilm formation [3], that is recognized as one of the most important bacterial virulence factor, this study aimed to assess the influence of berberine in the biofilm production by *P. aeruginosa*, through the inhibition of MexXY-OprM.

Biofilm production assays were performed *in vitro* on the isogenic pair *P. aeruginosa* K767 (wild type) / K1525 (Δ*mexY*), in absence or presence of berberine 80 μg/ml**.**

In the absence of berberine the two strains did not exhibit significant differences in biofilm production, whereas in its presence production was significantly enhanced in both strains, being significantly greater (100%, $p<0.001$) in the WT strain than in the mutant (50%, $p<0.01$) (**Fig.22**).

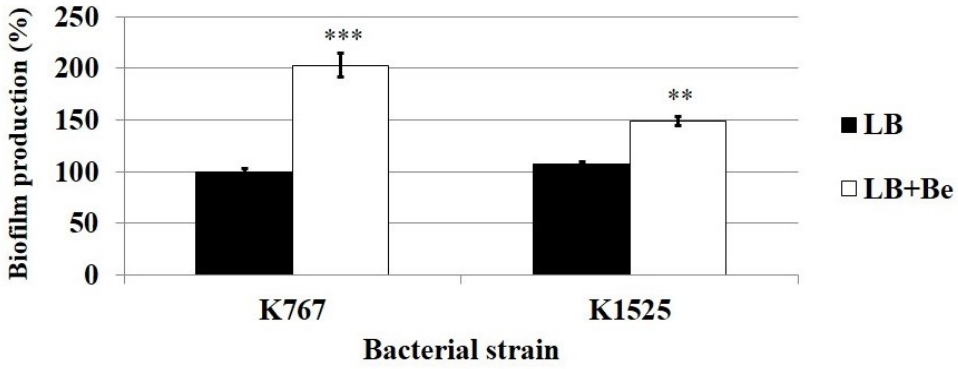


**Figure 22** Biofilm production assay on *P. aeruginosa* K767 and K1525 in absence (LB) or presence (LB + Be) of berberine at 80 μg/ml). ** $p<0.01$, *** $p<0.001$.

Without berberine no difference was observed between the wild type and the mutant strain in normal conditions, whereas, contrary to what expected, the inhibition of the pump by berberine lead to an increase of biofilm formation by both strains. This can be explained by a much greater involvement of MexAB-OprM efflux pump rather than MexXY-OprM in biofilm formation, as already reported [3]. The inhibition of MexXY-OprM could elicit a *P. aeruginosa* response leading to the over-expression of different EPs, including MexAB-OprM. Further studies are warranted to confirm this hypothesis.

## Role of MexXY-OprM in *P. aeruginosa* persistence to tobramycin

The MexXY-OprM inhibitory effect of berberine and its synergy with tobramycin confirmed the inhibition of efflux pumps as a promising strategy to counteract antibiotic resistance. Considered the recent findings about the involvement of the *E. coli* efflux pump AcrAB-TolC also in antibiotic persistence [100], we evaluated a possible anti-persisters activity of berberine through inhibition of MexXY-OprM in *P. aeruginosa*. For this purpose, a preliminary study aimed to assess of the role of MexXY-OprM in tobramycin persistence of *P. aeruginosa* was performed.

*In vitro* persistence assays of planktonic and biofilm cultures of the isogenic pair *P. aeruginosa* K767 (wt)/ K1525 (Δ*mexY*) were performed in the presence of tobramycin and the combination tobramycin/berberine.

In planktonic cultures treated with tobramycin 20x MIC (5 μg/ml) for 24 hours the wild type strain showed a 1 log CFU reduction (from $4.70x10^8$ to $4.11x10^7$ CFU/ml), while the Δ*mexY* strain exhibited a 3 log CFU decrease (from $2.2x10^8$ to $2.5x10^5$ CFU/ml): therefore, in absence of a functional MexXY-OprM pump, a significant ($p<0.01$) reduction of persisters compared to the wild type strain was observed.

When treated with tobramycin 20x MIC + Berberine 80 μg/ml, the wt strain showed a persisters amount 10 times lower compared to tobramycin alone after 24 hours of exposure, while in K1525 no difference in persisters formation was observed in presence or absence of berberine (**Fig.23**).

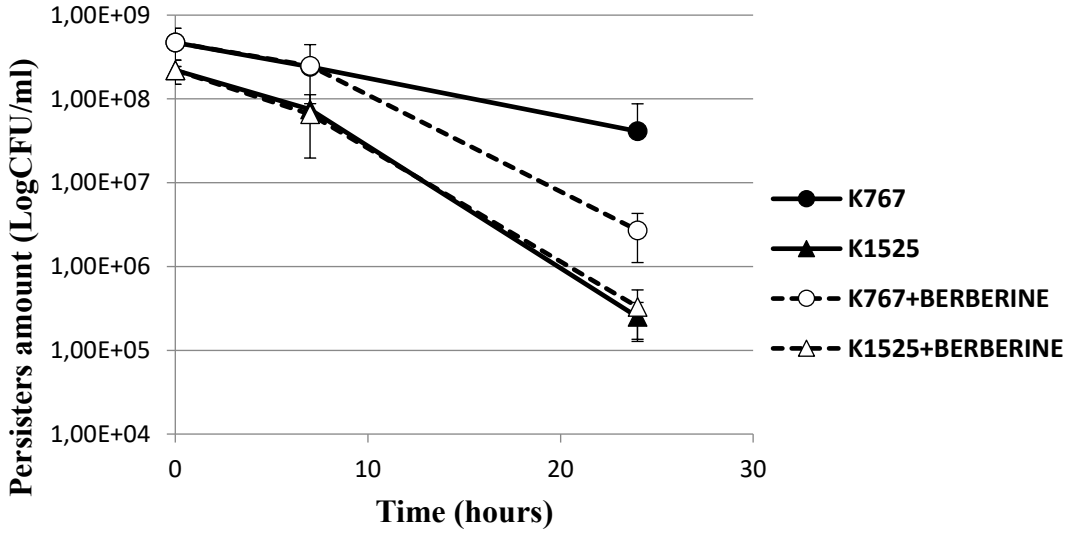


**Figure 23** Tobramycin persistence assays on K767 (wt) and K1525 (Δ*mexY*) in absence (solid lines) or presence (dashed lines) of berberine 80μg/ml.

Tobramycin persistence assays on *P. aeruginosa* K767 and K1525 have then been repeated in biofilm mode of growth: 24 hours mature biofilms were challenged with tobramycin 1000x MIC in absence/presence of berberine at 80 and 320 µg/ml. 24 hours exposure to tobramycin induced the production of a significantly lower amount (3 log, $p<0.001$) of persisters by the *mexY* mutant compared to the wild type (1x10 versus $1.33x10^4$, respectively), consistent with the results obtained with the planktonic cultures. Moreover, despite berberine at 80 µg/ml did not induce a significant reduction in K767 persisters compared to the effect of tobramycin alone, the combination tobramycin 1000x MIC + berberine 320 µg/ml was capable of a complete eradication of the culture: no persisters were recovered after 24 hours of exposure (**Fig. 24**).

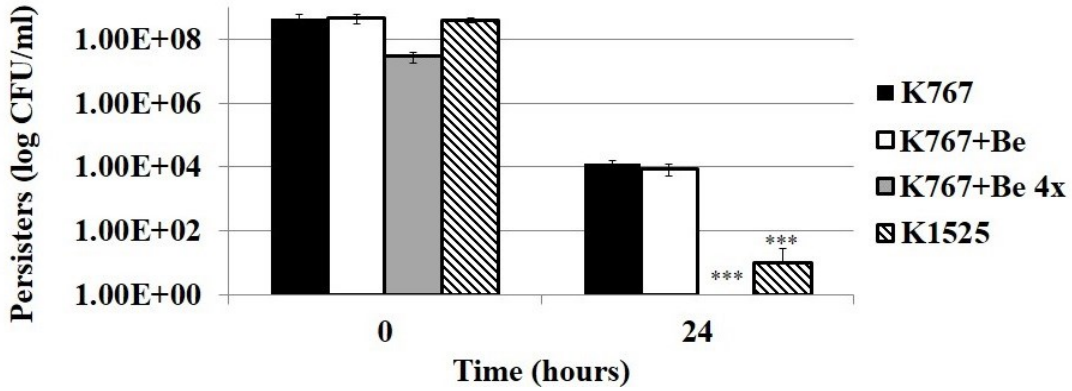


**Figure 24** Tobramycin persistence assays on K767 (wt) and K1525 (Δ*mexY*) biofilms, alone or in combination with berberine (Be) at 80 µg/ml or 320 (4x) µg/ml. *** $p< 0.001$.

These results convincingly suggest a major role of MexXY-OprM in tobramycin persistence of *P. aeruginosa*: in planktonic tobramycin persistence assays a much lower persisters amount was observed in the *mexY* deleted strain. With the addition of berberine, wild type persisters decreased furtherly while the mutated strain was not affected, suggesting: i) once again a specific interaction between berberine and MexXY-OprM and ii) the inhibition of this pump as a strategy to decrease tobramycin persisters.

These results were confirmed when treating biofilm cultures with antibiotic alone or in combination with berberine. The higher amount of berberine (320 µg/ml) needed to observe a considerable effect on persisters reduction is likely due to a poor penetration of the weakly positively charged compound into the thick matrix, as reported for several drugs including tobramycin [87, 121]. However at this concentration, previously reported as non-toxic, the association tobramycin + berberine was able to completely eliminate *P. aeruginosa* survivors, highlighting the great potential of this combination for the

treatment of *P. aeruginosa* infections, which very often occur in a biofilm mode of growth [106]. Further studies, involving the *P. aeruginosa* K1525 strain complemented with *mexY* are needed to confirm the role of MexXY-OprM in persisters formation.

## Berberine as anti-persisters in *P. aeruginosa* clinical strains

Once assessed the role of MexXY-OprM in *P. aeruginosa* tobramycin persistence, the subsequent step aimed to evaluate a possible antipersister activity of berberine in further 22 strains: 2 laboratory strains (PAO1 and PA14) and 20 clinical strains selected among the isolates (C6-C104, AR48, AR51 and NC01-NC10) showing different behaviors to the combination berberine-tobramycin. Planktonic cultures were challenged with 20x MIC tobramycin alone or in combination with berberine 80 µg/ml. The results showed a diversified and strain-specific response.

After exposure to tobramycin alone, 6/22 strains (27.3%) exhibited a 100- to 10,000-fold reduction of the starting inoculum, 9/22 strains (40.9%) showed a 10-fold reduction, 4/22 strains (18%) showed a more limited (< 10-fold) reduction, and 3/22 (13.6%), showed no CFU reduction. Remarkably, 6/7 (85.7%) isolates showing a low or no persisters reduction were from CF patients.

Combined with tobramycin, berberine 80 µg/ml induced a reduction of the starting inoculum 1-3 log greater than the one induced by tobramycin alone in 16/22 (72.7%) strains, while the remaining isolates were unaffected. Notably, in the case of *P. aeruginosa* C61 20xMIC tobramycin induced a 1 log reduction of the bacterial population, whereas the combination tobramycin-berberine left no survivors (≥ 7 log CFU reduction) (**Fig.25**).

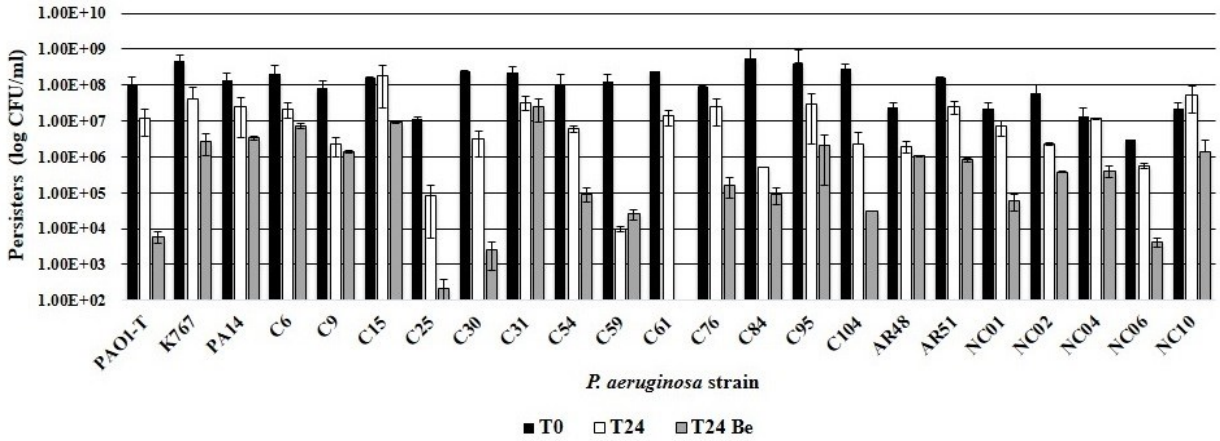


**Figure 25** Persistence assays of 23 *P. aeruginosa* laboratory and clinical (CF and non-CF) isolates to 20xMIC Tobramycin in absence / presence of berberine (Be) 80 µg/ml. Plate counts were performed immediately before (T0) and after 24-hour (T24) tobramycin exposure incubation at 37 °C.

The results of the combination berberine-tobramycin in terms of both tobramycin resistance and persistence are showed in **table 6**. Berberine reduced both tobramycin MIC and persistence in 7/23 (30.4%) strains (at least 4-fold reduction of tobramycin MIC and at least a 1 log reduction of persisters); in 10/23 strains (43.5%) it only reduced persisters, and in 3/23 (13.05%) it only reduced the MIC. Finally, 3/23 (13.04%) strains resulted uniformly unaffected.

**Table 6:** Effect of berberine on the reduction of tobramycin MIC and tobramycin-persisters amount against 23 *P. aeruginosa* strains. L: laboratory strain, CF: cystic fibrosis isolate, C: clinical non-CF isolate. * Effective berberine activity (at least 4-fold reduction of TOB MIC or 1 log reduction of persisters), ND: not detected.

| strain | source | MIC reduction | Persisters reduction |
|---|---|---|---|
| PAO1 | L | 2 folds | 3 log* |
| K767 | L | 2 folds | 1 log* |
| PA14 | L | ND | 1 log* |
| C6 | CF | 4 folds* | 0.5 log |
| C9 | CF | 2 folds | ND |
| C15 | CF | ND | 1 log* |
| C25 | CF | 16 folds* | 2.5 log* |
| C30 | CF | ND | 3 log* |
| C31 | CF | ND | ND |
| C54 | C | 2 folds | 1.5 log* |
| C59 | C | 2 folds | ND |
| C61 | C | 4 folds* | 7 log* |
| C76 | C | ND | 2 log* |
| C84 | C | 4 folds* | 0.5 log |
| C95 | C | 8 folds* | 1 log* |
| C104 | C | 4 folds* | 2 log* |
| AR48 | CF | 4 folds* | ND |
| AR51 | CF | 2 folds | 1.5 log* |
| NC01 | CF | 2 folds | 2 log* |
| NC02 | CF | 2 folds | 1 log* |
| NC04 | CF | > 4 folds* | 1.5 log* |
| NC06 | CF | 4 folds* | 2 log* |
| NC10 | CF | 4 folds* | 1.5 log* |

Overall, berberine showed a good antipersisters activity, significantly reducing their amount in most (72.7%) isolates; in one case (*P. aeruginosa* C61) the combination berberine-tobramycin seemed even able to eradicate all the persisters subpopulation with a 7 log-decrease of persisters compared to tobramycin alone. These outcomes, combined with the results of the previous studies, enhances the potential of berberine in the treatment of *P. aeruginosa* infections; through the binding and inhibition of MexXY-OprM efflux pump. The different responses to the combination tobramycin-berberine and the recovery of isolates unaffected by berberine is to be explained. A low expression level of the efflux pump's coding genes could be involved, however the tobramycin MICs in the range of resistance against strains lacking acquired resistance determinants doesn't support this hypothesis. More likely, differences in the MexY sequence and structure could hamper a correct binding of berberine in the binding sites, hampering its inhibitory action. To verify this, we decided to analyse the *mexY* sequences of isolates showing different responses to the treatment with berberine.

## MexY polymorphism analysis

The variability of the effect of berberine on the 23 *P. aeruginosa* strains prompted a study on the MexY polymorphisms, to investigate their role in the successful binding of berberine which might underly the observed lack of synergy.

For this purpose, internal sequences of *mexY* of 8 *P. aeruginosa* strains selected on the basis of their different response to berberine were analyzed. In particular, *P. aeruginosa* C25 and NC06 were selected because showing a reduction both in resistance and persistence, C30 and C54 showed only a reduction on persistence, C84 and AR48 showed only a reduced MIC (and no or very limited reduction in persister abundance), and strains C31 and C59 showed no change in either susceptibility or persistence. Two internal sequences of *mexY* of 270 bp (sequence 1) and 588 bp (sequence 2) (**Fig.26**), were analyzed on the bases of preliminary *in silico* investigations, which have indicated the amino acids probably involved in the binding sites.

ATGGCTCGTTTCTTCATTGACCGGCCGGTCTTCGCCTGGGTGATCTCCCTGCTGATCGTGCTCGCCGGGGT
CCTGGCGATCCGCTTCCTGCCGGTCGCCCAGTACCCGGACATCGCGCCGCCGGTGGTCAACGTCAGCGCC
ACGTATCCCGGCGCCTCGGCCAAGGTGGTCGAGGAAGCGGTGACCGCGATCATCGAGCGCGAGATGAAC
GGCGCGCCCGGCCTGCTCTACACCAAGGCCACCAGCAGCACCGGCCAGGCCTCGCTGACCCTGACCTTCC
GCCAGGGCGTGAACGCGAACCTCGCCGCGGTGGAAGTGCAGAACCGCCTGAAGATCGTCGAGTCGCGCC
TGCCCGAATCGGTGCGGCGCGACGGCATCTACGTGGAGAAGGCGGCGGACAGCATCCAGCTGATCGTTA
CCCTTACCTCCTCCAGCGGCCGCTACGACGCCATGGAGCTGGGCGAGATCGCCTCGTCCAACGTGTTGCA
GGCGCTGCGCCGGGTGGAGGGCGTGGGCAAGGTCGAGACCTGGGGCGCCGAGTACGCCATGCGCATCTG
GCCCGACCCGGCCAAGCTGACCTCGATGAACCTCAGCGCCAGCGACCTGGTCAACGCCGTGCGCCGGCAC
AACGCCCGCCTCACCGTGGGCGACATCGGCAACCTCGGGGTCCCCGACTCGGCGCCGATCAGCGCCACGG
TGAAGGTCGACGACACCCTGGTGACGCCCGAGCAGTTCGGCGAAATTCCGCTGGCGCATCCGCGCGACGG
CGGCGCGATCCGCCTGCGCGACGTGGCCCGCGTCGAGTTCGGCCAGAGCGAGTACGGCTTCGTCTCGCGG
GTCAACCAAATGACCGCCACCGGCCTGGCGGTGAAGATGGCGCCCGGCTCCAACGCGGTGGCCACCGCC
AAGCGCATCCGCGCCACCCTCGACGAGCTGTCGCGCTACTTCCCGGAGGGCGTGAGCTACAACATCCCCT
ATGACACCTCGGCGTTCGTCGAGATCTCGATCAGGAAGGTGGTCAGCACCCTGCTCGAGGCGATGCTGCT
GGTGTTCGCCGTGATGTACCTGTTCATGCAGAACTTCCGCGCCACCCTGATCCCGACACTGGTGGTGCCGG
TGGCCCTGCTGGGCACCTTCACGGTGATGCTCGGCCTGGGCTTCTCGATCAACGTGCTGACCATGTTCGGC
ATGGTCCTGGCGATCGGCATCCTGGTGGACGACGCGATCATCGTGGTGGAGAACGTCGAGCGGCTGATGG
CCGAGGAAGGCCTGTCGCCGCACGACGCCACGGTCAAGGCGATGCGCCAGATCAGCGGGGCCATCGTCG
GCATCACCGTAGTGCTGGTCTCGGTGTTCGTGCCGATGGCGTTCTTCAGCGGCGCGGTGGGCAACATCTAC
CGCCAGTTCGCGGTGACCCTGGCGGTCTCCATCGGCTTCTCGGCGTTCCTCGCGCTGTCGCTGACCCCGGC
CCTGTGCGCCACCCTGCTGCGCCCGATCGACGCCGACCACCACGAGAAGCGCGGCTTCTTCGGCTGGTTC
AACCGCGCCTTCCTGCGCCTGACCGGACGCTACCGCAACGCGGTGGCCGGCATCCTCGCCCGGCCGATCC
GCTGGATGCTGGTCTACACCCTGGTCATCGGCGTGGTCGCCCTGCTCTTCGTGCGCCTGCCGCAGGCGTTC
CTGCCGGAAGAGGACCAGGGCGACTTCATGATCATGGTGATGCAGCCCGAAGGCACGCCGATGGCGGAG
ACCATGGCCAACGTCGGCGACGTCGAGCGCTACCTGGCGGAGCACGAACCGGTGGCCTACGCCTATGCG
GTCGGCGGCTTCAGCCTGTACGGCGACGGCACCAGCTCGGCGATGATCTTCGCCACCCTGAAGGACTGGT
CGGAACGCCGGGAGGCCAGCCAGCACGTCGGCGCCATCGTCGAGCGCATCAACCAGCGCTTCGCCGGCC
TGCCCAACCGTACGGTGTATGCGATGAACTCGCCGCCGCTGCCGGACCTGGGTTCCACCAGCGGCTTCGA
CTTCCGCCTGCAGGACCGTGGCGGGGTTGGCTACGAGGCCCTGGTCAAGGCCCGCGACCAGTTGCTGGCG
CGCGCCGCCGAGGACCCGCGCCTGGCCAACGTGATGTTCGCCGGCCAGGGCGAGGCGCCGCAGATCCGC
CTGGACATCGACCGGCGCAAGGCGGAGACCCTTGGCGTGAGCATGGACGAGATCAACACCACCCTGGCG
GTGATGTTCGGCTCGGACTACATCGGCGACTTCATGCACGGCAGCCAGGTGCGCAAGGTGGTGGTCCAGG
CCGACCGGCGCAAGCGCCTGGGCATCGACGACATCGGCCGGCTTCACGTGCGCAACGAGCAGGGCGAGA
TGGGTGCCGCTGGCGACGTTCGCCAAGGCCGCCTGGACCCTCGGCCCGCCGCAACTGACCCGCTACAACG
GCTATCCCTCGTTCAACCTCGAGGGCCAGGCCGCGCCGGGCTACAGCAGCGCGAAGCCATGCAGGCGATG
GAGCAATTGATGCAGGGAACTGCCCGAGGCATTCGCCCACGAGTGGTCCGGCCAGTCCTTCGAAGAACGC
CTGTTGCCGGCGCCCAGGCGCCGGCGCTGTTCGCCCTCTCGGTGTTGATCGTGTTCCTCGCCCTGGCCGCC
CTCTACGAAAGCTGGTCGATCCCGCTGGCGGTGATCCTGGTGGTGCCGCTGGGCGTACTCGGCGCACTGC
TCGGGGTGAGCCTGCGCGGTCTGCCCAACGACATCTACTTCAAGGTCGGCCTGATCACCATCATCGGCCT
CTCGGCGAAGAACGCCATCCTCATCATCGAGGTGGCCAAGGACCATTACCAGGAAGGCATGAGCCTGCTG
CAGGCGACCCTGGAGGCCGCGCGCCTGCGCCTGCGACCGATCGTCATGACCTCGCTGGCGTTCGGTTTCG
GCGTGGTCCCGCTGGCGCTCTCCAGCGGCGCCGGTATCCGCGCCCAGGTCGCCATCGGCACCGGGGTGCT
CGGCGGGATCGTCACCGCCACGGTACTCGCGGTGTTCCTGGTACCGCTGTTCTTCCTGGTGGTCGGGCGCC
TGTTCCGGTTGCGCAAGGCGCCGCGCACCGGCAACTCGCCCCAGATCCCCACGGAGCAAGCCTGA

**Figure 26** Complete *mexY* sequence of *P. aeruginosa* PAO1. The region amplified and sequenced are evidenced in green (sequence 1) and blue (sequence 2).

The alignment of the two sequences to the corresponding sequences of *P. aeruginosa* PAO1 (accession number, AB015853.1) showed a small number of point mutations. Six single nucleotide polymorphisms (SNPs) were found in the 270 bp sequence and 27 SNPs in the 588 bp sequence, as showed below.

Sequence 1: c.71C>T, c.80G>A, c.98G>A, c.107T>C, c.113T>C, c.146G>A.

Sequence 2: c.110C>G, c.112G>C, c.113C>G/A, c.114G>C, c.179G>C, c.294C>G, c.295G>C, c.296A>G, c.298G>A, c.317C>G, c.331A>G, c.332A>C, c.343C>G, c.344A>C, c.345T>A, c.388T>C, c.428T>C, c.451C>G, c.511A>G, c.521C>G, c.522T>C, c.523G>T, c.524G>C, c.525T>C, c.526C>A, c.527G>C, c.531T>A (**Fig. 27**)

**Sequence 1 (270 bp)**

```
PAO1     TGGAAGTGCAGAACCGCCTGAAGATCGTCGAGTCGCGCCTGCCCGAATCGGTGCGGCGCG 60
C59      ----------------------CTACGTCGGTCGCGCCTGCCCGAATCGGTGCGGCGCG 37
C84      ---------------------TGTACGTCGGTCGCGCCTGCCCGAATCGGTGCGGCGCG 38
C25      ---------------------TGTACGTCGGTCGCGCCTGCCCGAATCGGTGCGGCGCG 38
C31      ------------------------CGTACTCGGTCGCGCCTGCCCGATCGGTGCGGCGCG 36
AR48     ----------------------CGTACGTCGATCGCGCCTGCTCCGATCGGTGCGGCGCG 38
C54      ----------------------GGTACGTCGATCGC-GCCTGCCCGATCGGTGCGGCGCG 37
C30      ----------------------CGTACGTCGATCGC-GCCTGCCCGATCGGTGCGGCGCG 37
NC06     ----------------------CGTACGTCGATCGC-GCCTGCCCGATCGGTGCGGCGCG 37
                                             *    *  **************

PAO1     ACGGCATCTACGTGGAGAAGGCGGCGGACAGCATCCAGCTGATCGTTACCCTTACCTCCT 120
C59      ACGGCATCTACGTGGAGAAGGCGGCGGACAGCATCCAGCTGATCGTTACCCTTACCTCCT 97
C84      ACGGCATCTACGTGGAGAAAGCGGCGGACAGCATCCAGCTGATCGTTACCCTTACCTCCT 98
C25      ACGGCATCTACGTGGAGAAGGCGGCGGACAGCATCCAACTGATCGTTACCCTTACCTCCT 98
C31      ACGGCATCTATGTGGAGAAGGCGGCGGACAGCATCCAACTGATCGTCACCCTTACCTCCT 96
AR48     ACGGCATCTATGTGGAGAAGGCGGCGGACAGCATCCAACTGATCGTCACCCTTACCTCCT 98
C54      ACGGCATCTACGTGGAGAAGGCGGCGGACAGCATCCAGCTGATCGTTACCCTTACCTCCT 97
C30      ACGGCATCTATGTGGAGAAGGCGGCGGACAGCATCCAACTGATCGTCACCCTTACCTCCT 97
NC06     ACGGCATCTATGTGGAGAAGGCGGCGGACAGCATCCAGCTGATCGTCACCCTCACCTCCT 97
         ********** ******** ***************** ******** ***** *******


PAO1     CCAGCGGCCGCTACGACGCCATGGAGCTGGGCGAGATCGCCTCGTCCAACGTGTTGCAGG 180
C59      CCAGCGGCCGCTACGACGCCATGGAGCTGGGCGAGATCGCCTCGTCCAACGTGTTGCAGG 157
C84      CCAGCGGCCGCTACGACGCCATGGAGCTGGGCGAGATCGCCTCGTCCAACGTGTTGCAGG 158
C25      CCAGCGGCCGCTACGACGCCATGGAGCTGGGCGAGATCGCCTCGTCCAACGTGTTGCAGG 158
C31      CCAGCGGCCGCTACGACGCCATGGAGCTGGGCGAGATCGCCTCGTCCAACGTGTTGCAGG 156
AR48     CCAGCGGCCGCTACGACGCCATGGAGCTGGGCGAGATCGCCTCGTCCAACGTGTTGCAGG 158
C54      CCAGCGGCCGCTACGACGCCATGGAGCTGGGCGAGATCGCCTCGTCCAACGTGTTGCAGG 157
C30      CCAGCGGCCGCTACGACGCCATGGAGCTGGGCGAGATCGCCTCGTCCAACGTGTTGCAGG 157
NC06     CCAGCGGCCGCTACGACGCCATGGAACTGGGCGAGATCGCCTCGTCCAACGTGTTGCAGG 157
         ************************ *********************************
```

```
PAO1     CGCTGCGCCGGGTGGAGGGCGTGGGCAAGGTCGAGACCTGGGGCGCCGAGTACGCCATGC 240
C59      CGCTGCGCCGGGTGGAGGGCGTGGGCAAGGTCGAGACCTGGGGCGCCGAGTACGCCATGC 217
C84      CGCTGCGCCGGGTGGAGGGCGTGGGCAAGGTCGAGACCTGGGGCGCCGAGTACGCCATGC 218
C25      CGCTGCGCCGGGTGGAGGGCGTGGGCAAGGTCGAGACCTGGGGCGCCGAGTACGCCATGC 218
C31      CGCTGCGCCGGGTGGAGGGCGTGGGCAAGGTCGAGACCTGGGGCGCCGAGTACGCCATGC 216
AR48     CGCTGCGCCGGGTGGAGGGCGTGGGCAAGGTCGAGACCTGGGGCGCCGAGTACGCCATGC 218
C54      CGCTGCGCCGGGTGGAGGGCGTGGGCAAGGTCGAGACCTGGGGCGCCGAGTACGCCATGC 217
C30      CGCTGCGCCGGGTGGAGGGCGTGGGCAAGGTCGAGACCTGGGGCGCCGAGTACGCCATGC 217
NC06     CGCTGCGCCGGGTGGAGGGCGTGGGCAAGGTCGAGACCTGGGGCGCCGAGTACGCCATGC 217
         ************************************************************

PAO1     GCATCTGGCCCGACCCGGCCAAGCTGACCT    270
C59      GCATCTGGCCCGACCCGGCCAGCTGACCTA    247
C84      GCATCTGGCCCGACCCGGCCAGCTGACCTA    248
C25      GCATCTGGCCCGACCCGGCCAGCTGACCTA    248
C31      GCATCTGGCCCGACCCGGCCAGCTGACCTA    246
AR48     GCATCTGGCCCGACCCGGCCAGCTGACCTA    248
C54      GCATCTGGCCCGACCCGGCCAGCTGACCTA    247
C30      GCATCTGGCCCGACCCGGCCAGCTGACCTA    247
NC06     GCATCTGGCCCGACCCGGCCAGCTGACCTA    247
         **********************     *
```

**Sequence 2 (588 bp)**

```
PAO1     CGTGAGCATGGACGAGATCAACACCACCCTGGCGGTGATGTTCGGCTCGGACTACATCGG 60
C30      ----------------------------CCTGGCGGTGATGTTCGGCTCGGACTACATCGG 33
C59      ---------------------------CCCTGGCGGTGATGTTCGGCTCGGACTACATCGG 34
C25      ------------------------CCCCCCTGGCGGTGATGTTCGGCTCGGACTACATCGG 37
C54      ---------------------------CCCTGGCGGTGATGTTCGGCTCGGACTACATCGG 34
C84      ----------------------------CCTGGCGGTGATGTTCGGCTCGGACTACATCGG 33
NC06     -------------------------------GCGGTGATGTTCGGCTCGGACTACATCGG 29
AR48     ----------------------------CCTGGCGGTGATGTTCGGCTCGGACTACATCGG 33
C31      ---------------------------CCCTGGCGGTGATGTTCGGCTCGGACTACATCGG 34
                                     ****************************

PAO1     CGACTTCATGCACGGCAGCCAGGTGCGCAAGGTGGTGGTCCAGGCCGACCGGCGCAAGCG 120
C30      CGACTTCATGCACGGCAGCCAGGTGCGCAAGGTGGTGGTCCAGGCCGACGGCGCCAAGCG 93
C59      CGACTTCATGCACGGCAGCCAGGTGCGCAAGGTGGTGGTCCAGGCCGACGGCGCCAAGCG 94
C25      CGACTTCATGCACGGCAGCCAGGTGCGCAAGGTGGTGGTCCAGGCCGACGGCGCCAAGCG 97
C54      CGACTTCATGCACGGCAGCCAGGTGCGCAAGGTGGTGGTCCAGGCCGACGGCGCCAAGCG 94
C84      CGACTTCATGCACGGCAGCCAGGTGCGCAAGGTGGTGGTCCAGGCCGACGGCACCAAGCG 93
NC06     CGACTTCATGCACGGCAGCCAGGTGCGCAAGGTGGTGGTCCAGGCCGACGGCGCCAAGCG 89
AR48     CGACTTCATGCACGGCAGCCAGGTGCGCAAGGTGGTGGTCCAGGCCGACGGCGCCAAGCG 93
C31      CGACTTCATGCACGGCAGCCAGGTGCGCAAGGTGGTGGTCCAGGCCGACGGCGCCAAGCG 94
         ************************************************ *   ******

PAO1     CCTGGGCATCGACGACATCGGCCGGCTTCACGTGCGCAACGAGCAGGGCGAGATGGGTGC 180
C30      CCTGGGCATCGACGACATCGGCCGGCTTCACGTGCGCAACGAGCAGGGCGAGATG-GTCC 152
C59      CCTGGGCATCGACGACATCGGCCGGCTTCACGTGCGCAACGAGCAGGGCGAGATG-GTCC 153
C25      CCTGGGCATCGACGACATCGGCCGGCTTCACGTGCGCAACGAGCAGGGCGAGATG-GTGC 156
C54      CCTGGGCATCGACGACATCGGCCGGCTTCACGTGCGCAACGAGCAGGGCGAGATG-GTGC 153
C84      CCTGGGCATCGACGACATCGGCCGGCTTCACGTGCGCAACGAGCAGGGCGAGATG-GTGC 152
NC06     CCTGGGCATCGACGACATCGGCCGGCTTCACGTGCGCAACGAGCAGGGCGAGATG-GTCC 148
AR48     CCTGGGCATCGACGACATCGGCCGGCTTCACGTGCGCAACGAGCAGGGCGAGATG-GTCC 152
C31      CCTGGGCATCGACGACATCGGCCGGCTTCACGTGCGCAACGAGCAGGGCGAGATG-GTCC 153
         ********************************************************* ** *

PAO1     CGCTGGCGACGTTCGCCAAGGCCGCCTGGACCCTCGGCCCGCCGCAACTGACCCGCTACA 240
C30      CGCTGGCGACGTTCGCCAAGGCCGCCTGGACCCTCGGCCCGCCGCAACTGACCCGCTACA 212
C59      CGCTGGCGACGTTCGCCAAGGCCGCCTGGACCCTCGGCCCGCCGCAACTGACCCGCTACA 213
C25      CGCTGGCGACGTTCGCCAAGGCCGCCTGGACCCTCGGCCCGCCGCAACTGACCCGCTACA 216
C54      CGCTGGCGACGTTCGCCAAGGCCGCCTGGACCCTCGGCCCGCCGCAACTGACCCGCTACA 213
C84      CGCTGGCGACGTTCGCCAAGGCCGCCTGGACCCTCGGCCCGCCGCAACTGACCCGCTACA 212
NC06     CGCTGGCGACGTTCGCCAAGGCCGCCTGGACCCTCGGCCCGCCGCAACTGACCCGCTACA 208
AR48     CGCTGGCGACGTTCGCCAAGGCCGCCTGGACCCTCGGCCCGCCGCAACTGACCCGCTACA 212
C31      CGCTGGCGACGTTCGCCAAGGCCGCCTGGACCCTCGGCCCGCCGCAACTGACCCGCTACA 213
         ************************************************************
```

```
PAO1      ACGGCTATCCCTCGTTCAACCTCGAGGGCCAGGCCGCGCCGGGCTACAGCAGCGCGAAG- 299
C30       ACGGCTATCCCTCGTTCAACCTCGAGGGCCAGGCCGCGCCGGGCTACAGCAGCGGCGAAG 272
C59       ACGGCTATCCCTCGTTCAACCTCGAGGGCCAGGCCGCGCCGGGCTACAGCAGCGGCGAAG 273
C25       ACGGCTATCCCTCGTTCAACCTCGAGGGCCAGGCCGCGCCGGGCTACAGCAGCGGCGAAG 276
C54       ACGGCTATCCCTCGTTCAACCTCGAGGGCCAGGCCGCGCCGGGCTACAGCAGCGGCGAAG 273
C84       ACGGCTATCCCTCGTTCAACCTCGAGGGCCAGGCCGCGCCGGGCTACAGCAGCGGCGAAG 272
NC06      ACGGCTATCCCTCGTTCAACCTCGAGGGCCAGGCCGCGCCGGGCTACAGCAGCGGCGAAG 268
AR48      ACGGCTATCCCTCGTTCAACCTCGAGGGCCAGGCCGCGCCGGGCTACAGCAGCGGCGAAG 272
C31       ACGGCTATCCCTCGTTCAACCTCGAGGGCCAGGCCGCGCCGGGCTACAGCAGCGGCGAAG 273
          ********************************************************   *

PAO1      CCATGCAGGCGATGGAGCAATTGATGCAGGGAACTGCCCGAGGCATTCGCCCACGAGTGG 359
C30       CCATGCAGGCGATGGAGGAATTGATGCA-GGGCCTGCCCGAGGGCATCGCCCACGAGTGG 331
C59       CCATGCAGGCGATGGAGGAATTGATGCA-GGGCCTGCCCGAGGGCATCGCCCACGAGTGG 332
C25       CCATGCAGGCGATGGAGGAATTGATGCA-GGGCCTGCCCGAGGGCATCGCCCACGAGTGG 335
C54       CCATGCAGGCGATGGAGCAATTGATGCA-GGGACTGCCCGAGGGCATCGCCCACGAGTGG 332
C84       CCATGCAGGCGATGGAGGAATTGATGCA-GGGCCTGCCCGAGGGCATCGCCCACGAGTGG 331
NC06      CCATGCAGGCGATGGAGCAATTGATGCA-GGGCCTGCCCGAGGGCATCGCCCACGAGTGG 327
AR48      CCATGCAGGCGATGGAGGAATTGATGCA-GGGCCTGCCCGAGGGCATCGCCCACGAGTGG 331
C31       CCATGCAGGCGATGGAGGAATTGATGCA-GGGCCTGCCCGAGGGCATCGCCCACGAGTGG 332
          **************** ********** **  **********   **************

PAO1      TCCGGCCAGTCCTTCGAAGAACGCCTGTTGCCGGCGCCCAGGCGCCGGCGCTGTTCGCCC 419
C30       TCCGGCCAGTCCTTCGAAGAACGCCTGTC--CGGCGCCCAGGCGCCGGCGCTGTTCGCCC 389
C59       TCCGGCCAGTCCTTCGAAGAACGCCTGTC--CGGCGCCCAGGCGCCGGCGCTGTTCGCCC 390
C25       TCCGGCCAGTCCTTCGAAGAACGCCTGTC--CGGCGCCCAGGCGCCGGCGCTGTTCGCCC 393
C54       TCCGGCCAGTCCTTCGAAGAACGCCTGTC--CGGCGCCCAGGCGCCGGCGCTGTTCGCCC 390
C84       TCCGGCCAGTCCTTCGAAGAACGCCTGTC--CGGCGCCCAGGCGCCGGCGCTGTTCGCCC 389
NC06      TCCGGCCAGTCCTTCGAAGAACGCCTGTC--CGGCGCCCAGGCGCCGGCGCTGTTCGCCC 385
AR48      TCCGGCCAGTCCTTCGAAGAACGCCTGTC--CGGCGCCCAGGCGCCGGCGCTGTTCGCCC 389
C31       TCCGGCCAGTCCTTCGAAGAACGCCTGTC--CGGCGCCCAGGCGCCGGCGCTGTTCGCCC 390
          ****************************   ****************************

PAO1      TCTCGGTGTTGATCGTGTTCCTCGCCCTGGCCGCCCTCTACGAAAGCTGGTCGATCCCGC 479
C30       TCTCGGTGCTGATCGTGTTCCTCGCCCTGGCCGCCCTCTACGAAAGCTGGTCGATCCCGC 449
C59       TCTCGGTGTTGATCGTGTTCCTCGCCCTGGCGGCCCTCTACGAAAGCTGGTCGATCCCGC 450
C25       TCTCGGTGTTGATCGTGTTCCTCGCCCTGGCCGCCCTCTACGAAAGCTGGTCGATCCCGC 453
C54       TCTCGGTGTTGATCGTGTTCCTCGCCCTGGCCGCCCTCTACGAAAGCTGGTCGATCCCGC 450
C84       TCTCGGTGTTGATCGTGTTCCTCGCCCTGGCCGCCCTCTACGAAAGCTGGTCGATCCCGC 449
NC06      TCTCGGTGCTGATCGTGTTCCTCGCCCTGGCCGCCCTCTACGAAAGCTGGTCGATCCCGC 445
AR48      TCTCGGTGCTGATCGTGTTCCTCGCCCTGGCCGCCCTCTACGAAAGCTGGTCGATCCCGC 449
C31       TCTCGGTGCTGATCGTGTTCCTCGCCCTGGCCGCCCTCTACGAAAGCTGGTCGATCCCGC 450
          ******** ********************** ****************************

PAO1      TGGCGGTGATCCTGGTGGTGCCGCTGGGCGTACTCGGCGCACTGCTCGGGGTGAGCCTGC 539
C30       TGGCGGTGATCCTGGTGGTGCCGCTGGGCGTGCTCGGCGCAGCTGCACGGGAGTGA---- 505
C59       TGGCGGTGATCCTGGTGGTGCCGCTGGGCGTACTCGGCGCACTGCTCGGGGTGAGCCTGC 510
C25       TGGCGGTGATCCTGGTGGTGCCGCTGGGCGTACTCGGCGCACTGCTCGGGGTGAGCCTGC 513
C54       TGGCGGTGATCCTGGTGGTGCCGCTGGGCGTACTCGGCGCACTGCTCGGGGTGAGCCTGC 510
C84       TGGCGGTGATCCTGGTGGTGCCGCTGGGCGTACTCGGCGCACTGCTCGGGGTGAGCCTGC 509
NC06      TGGCGGTGATCCTGGTGGTGCCGCTGGGCGTGCTCGGCGCACTGCTCGGGGTGAGCCTGC 505
AR48      TGGCGGTGATCCTGGTGGTGCCGCTGGGCGTGCTCGGCGCACTGCTCGGGGTGAGCCTGC 509
C31       TGGCGGTGATCCTGGTGGTGCCGCTGGGCGTGCTCGGCGCACTGCTCGGGGTGAGCCTGC 510
          ******************************** *********       *** * *

PAO1      GCGGTCTGCCCAACGACATCTACTTCAAGGTCGGCCTGATCACCATCATCGGCCTCTCGG 599
C30       ------------------------------------------------------------ 505
C59       GCG--------------------------------------------------------- 513
C25       GCGG-------------------------------------------------------- 517
C54       GCGGTCTG---------------------------------------------------- 518
C84       GCGGTCTGCCCAACGA-------------------------------------------- 525
NC06      GCGGCCTGCCCAACGA-------------------------------------------- 521
AR48      GCGGTCTGCCCAACGAC------------------------------------------- 526
C31       GCGGT------------------------------------------------------- 515
```

**Figure 27** Nucleotide alignment of two internal sequences of *mexY* of the 8 selected *P. aeruginosa* strains and of *P. aeruginosa* PAO1 (accession number, AB015853.1). * presence of a SNP.

The CF strains *P. aeruginosa* C30, C31, AR48 and NC06 carried the highest number of SNPs in both sequences, however, a clear correlation between the nucleotide mutations and the strain-specific behaviors in presence of berberine was not observed.

For this purpose, the polymorphisms were deeper examined in collaboration with biomolecular modeling group of Dr. Roberta Galeazzi. The nucleotide sequences were firstly translated into aminoacidic sequences, aligned, and investigated. When localizing the aminoacidic mutations in the tridimensional structure of MexY, it was observed that most of them fell in the binding sites for berberine, thus corroborating our hypothesis of MexY-berberine different affinities in the strains unsusceptible to drug combination. In particular, sequence 1 only showed one mutation (I112Y), shared by all strains, while Sequence 2 exhibited 22 substitutions; of these, 12 (R771G, E833R, A834S, M835H, Q836A, A837G, M838D, E839G, L841I, M842D, Q843A, and I849H) were shared by all strains; T845P was shared by all strains but C54; R772A was detected in 7 strains; Q840G and A794P were detected respectively in 6 and 5 strains; Q840A was found in two strains; and R772T, L908A, L909A, G910H, and V911G were found in a single strain (**Fig.28**).

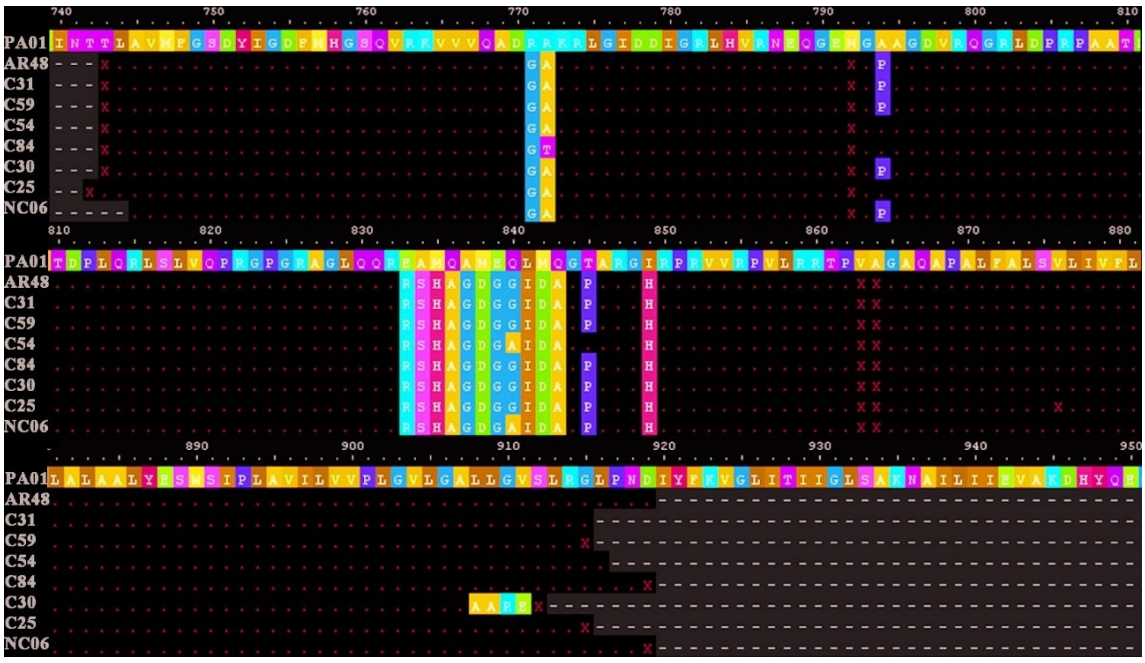


**Figure 28** Aminoacidic alignment of the translated sequence 2. Any difference with the PAO1 sequence was evidenced with different colors and the single letter of the mutated amino acid.

As observed for the nucleotide sequences, *P. aeruginosa* C30 showed the highest number of substitutions. Some of the mutations involved amino acids characterized by different chemical-physical properties (polar/apolar), probably resulting in different

numbers and types of interactions between berberine and MexY. This could likely explain the similar behavior of *P. aeruginosa* C31 and C59 (both unresponsive to berberine) which carry the same four polar/apolar amino acid substitutions; moreover, the substitutions may affect berberine binding not only directly, through changes in binding interactions, but also indirectly, through changes in the receptor conformation. However, the aminoacidic substitutions didn't clearly fit with the observed different response to berberine (**Table 7**).

**Table 7:** Correlation between specific amino acid substitutions and tobramycin susceptibility / persistence variations in the presence of berberine in the eight *P. aeruginosa* strains selected for MexY sequence analysis. CF: cystic fibrosis strain, C: clinical strain.

| Bacterial strains | Amino acid substitutions | MIC (µg/ml) reduction | Persister reduction (log) |
|---|---|---|---|
| *P. aeruginosa* C25 (CF) | R771G, R772A. E833R, A834S, M835H, Q836A, A837G, M838D, E839G, Q840G, L841I, M842D, Q843A, T845P, I849H, | 16-fold | 2.5 |
| *P. aeruginosa* C30 (CF) | R771G, R772A, A794P, E833R, A834S, M835H, Q836A, A837G, M838D, E839G, Q840G, L841I, M842D, Q843A, T845P, I849H, L908A, L909A, G910H, V911G | No | 3 |
| *P. aeruginosa* C31 (CF) | R771G, R772A, A794P, E833R, A834S, M835H, Q836A, A837G, M838D, E839G, Q840G, L841I, M842D, Q843A, T845P, I849H, | No | No |
| *P. aeruginosa* C54 (C) | R771G, R772A, E833R, A834S, M835H, Q836A, A837G, M838D, E839G, Q840A, L841I, M842D, Q843A, I849H, | 2-fold | 1.5 |
| *P. aeruginosa* C59 (C) | R771G, R772A, A794P, E833R, A834S, M835H, Q836A, A837G, M838D, E839G, Q840G, L841I, M842D, Q843A, T845P, I849H, | 2-fold | No |
| *P. aeruginosa* C84 (C) | R771G, R772T, E833R, A834S, M835H, Q836A, A837G, M838D, E839G, Q840G, L841I, M842D, Q843A, T845P, I849H, | 4-fold | 0.5 |
| *P. aeruginosa* AR48 (CF) | R771G, R772A, A794P, E833R, A834S, M835H, Q836A, A837G, M838D, E839G, Q840G, L841I, M842D, Q843A, T845P, I849H, | 4-fold | No |
| *P. aeruginosa* NC06 (CF) | R771G, R772A, A794P, E833R, A834S, M835H, Q836A, A837G, M838D, E839G, Q840A, L841I, M842D, Q843A, T845P, I849H, | 4-fold | 2 |

For this reason, the aminoacidic sequence of 5 strains (*P. aeruginosa* C25, AR48, C30, C31, C59), showing different response to berberine, was used to build the tridimensional model of their MexY protein. Molecular docking assays were then performed in order to better investigate the specific binding poses and affinities of berberine in the binding sites. From the docking assays it emerged that the binding affinity of berberine for the antibiotic and the periplasmic sites was different from strain to strain.

In particular, berberine showed a very high affinity to both sites in *P. aeruginosa* C25, creating specific hydrogen bonds (with Ile135 at the antibiotic site and with Gln831 at the cleft site); in *P. aeruginosa* C30 berberine bind to the antibiotic site of MexY with the same orientation as C25 and with a strong hydrogen bond with Ile135; in *P. aeruginosa* AR48 instead, berberine showed a greater affinity for the cleft site with the formation of two stable hydrogen bonds (with Tyr35 and Gln831). Finally, in *P. aeruginosa* C31 and C59 berberine affinity for both sites were considerably lower and no hydrogen bond formation was observed (**Table 8**).

Very interesting insights emerges from these results, as a reduction of resistance seems to be correlated with a strong binding of berberine to the cleft site of MexY, while a reduction of persisters seems to be correlated with a strong binding of berberine to the antibiotic site of MexY (**Table 8**).

**Table 8:** Comparison of the effect of berberine on reduction of tobramycin resistance and persistence and the energy of binding of berberine at the two binding sites of MexY in the different strains. H: formation of a hydrogen bond; ND, not detectable * Effective berberine activity (at least 4-fold reduction of TOB MIC or 1 log reduction of persisters)

| Strain | Resistance reduction | Persistence reduction | $E_b$ MexY Antibiotic Site (kcal/mol) | $E_b$ MexY Cleft Site (kcal/mol) |
|---|---|---|---|---|
| **C25** | (16 folds) * | (2.5 log) * | -8.05 (**H**) | -7.62 (**H**) |
| **AR48** | (4 folds) * | ND | -7.31 | -7.77 (**HH**) |
| **C30** | ND | (3 log) * | -8.02 (**H**) | -7.30 |
| **C31** | ND | ND | -6.51 | -5.98 |
| **C59** | ND | ND | -7.03 | -6.93 |

In conclusion, although the mere comparison of both nucleotide and amino acidic sequences was not enough to yield a clear correlation between MexY sequence and specific response to berberine, the reconstruction of the strain-specific tridimensional models of the protein and the *in silico* simulation of berberine binding seem to confirm the presence of MexY variants that could explain not only a lack of response to berberine in some strains, but also the tendency of berberine in other strains to reduce more specifically resistance or persistence, due to a higher affinity and a more stable binding to the MexY cleft site or antibiotic site, respectively. Although this hypothesis needs to be validated and confirmed by analyzing the whole MexY sequence of a larger number of strains, these preliminary results can provide useful information for the development of more effective berberine derivatives.

## *Staphylococcus aureus* and NorA

Although in *Staphylococcus aureus* efflux pumps have a minor role than in *P. aeruginosa*, the attention of the scientific community on these transport systems are increasing, for their role in antibiotic resistance as well as for their involvement in several aspects of bacterial physiology [30]; therefore, the use of EPIs could support antibiotic treatments against *S. aureus* infections.

A search and validation of novel inhibitors of the *S. aureus* efflux pump NorA, has been conducted in collaboration with the Medicinal Chemistry group of Prof. Stefano Sabatini (Department of Pharmaceutical Sciences, Chemistry and Technology of the Drug Section, Università degli Studi di Perugia, Perugia, Italy). The lack of a NorA tridimensional model still hinders the utilization of a structure-based drug design approach, but many molecules such as indoles, quinolines, boronic acids, chalcones, and piperine derivatives, have shown promising inhibitory activity [61].

The work of the chemistry group aimed, using a pharmacophoric model, to find, develop and synthesize derivates of the quinoline that could act as inhibitors of NorA breaking the resistance to fluoroquinolones (the main NorA substrate) in *S. aureus*.

## NorA-inhhibitory activity of 2-phenylquinoline based compounds

A first set of 16 compounds based on a 2-phenylquinoline core with different modifications at the C-2 position, (the 4'-proproxyphenyl replaced with different pyridine or thiophene moieties) was tested in order to assess their synergy in combination with ciprofloxacin. The 16 compounds were named from 30a,b to 37a,b according to the substituent in the C-2 position (**Fig. 29**).

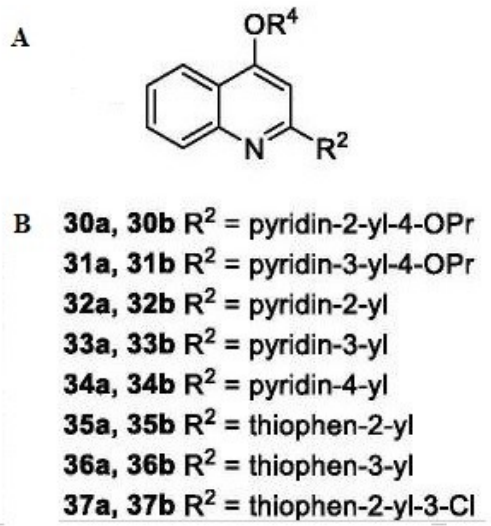


**Figure 29** Structure of the 2-phenylquinoline (**A**) and (**B**) all the tested substitutions at the C-2 position ($R^2$ site).

In order to assess their NorA inhibitory activity the 16 compounds were firstly used in ethidium bromide efflux inhibition assays on the NorA over-expressing *S. aureus* strain SA1199B. EtBr is a well-known substrate of NorA [30], so any inhibition of this efflux pump leads to a lower efflux of the dye detectable as a higher fluorescence under UV light. Three compounds (30a, 30b and 37a) at a concentration of 25 µg/ml showed a substantial (66.97%, 82.14% and 74.26% respectively) decrease of the EtBr efflux, compared to the untreated control. These results were comparable to those obtained with the starting hit "1" (74.19% efflux inhibition), i.e. the quinoline core unsubstituted in C-2, which had been already demonstrated as an effective EPI [110]. All the other compounds showed a much lower (<40%) inhibitory activity, (**Fig.30**).

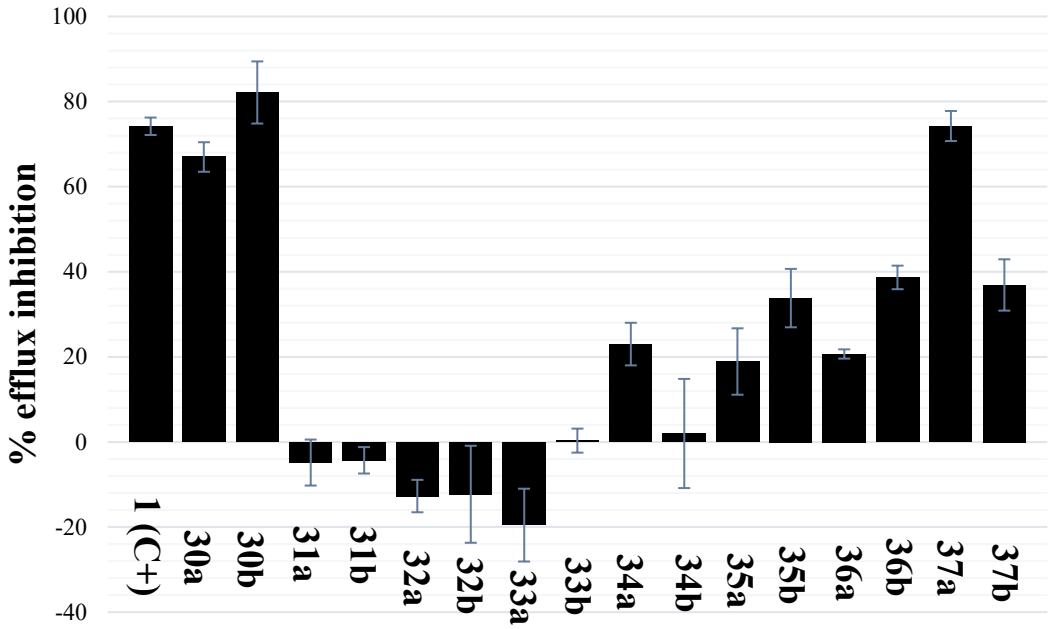


**Figure 30** Ethidium bromide efflux inhibition exerted by the 16 quinoline-derivatives against the NorA over-expressing strain *S. aeureus* SA1199B. The compound 1 (quinoline)was used as the reference compound.

Given the strong efflux inhibition, the MIC of the three compounds 30a, 30b and 37a was determined against the isogenic pair SA1199 (wt) / SA1199B (overexpressing *norA*) alone (to exclude any antimicrobial activity) and in combination with different concentration of ciprofloxacin in checkerboard assays against SA1199B. The MIC assays revealed no antimicrobial activity against both strains up to a concentration of 25 µg/ml, while, in combination with ciprofloxacin, compounds 30a, 30b and 37a were able to reduce at least 4-fold the drug MIC. In particular, at the maximum concentration tested of 25 µg/ml, 30b was able of a 4-fold reduction of ciprofloxacin MIC (from 5 to 1.25 µg/ml), 30a of a 8-fold reduction (0.63 µg/ml) and 37a of a 32-fold reduction (0.16 µg/ml) (**Table 9**).

**Table 9:.** Checkerboard assays results of the combination of ciprofloxacin and 30a, 30b or 37a.

| | | 30a | 30b | 37a |
|---|---|---|---|---|
| | | CPX MIC (µg/ml) | | |
| Compound concentration (µg/ml) | **0** | 5 | 5 | 5 |
| | **0.02** | 5 | 5 | 5 |
| | **0.05** | 2.5 | 2.5 | 2.5 |
| | **0.10** | 5 | 2.5 | 2.5/5 |
| | **0.20** | 2.5 | 2.5 | 2.5 |
| | **0.39** | 2.5 | 2.5 | 1.25 |
| | **0.78** | 5 | 2.5 | 1.25 |
| | **1.56** | 2.5 | 2.5 | 1.25 |
| | **3.13** | 5 | 5 | 2.5 |
| | **6.25** | 1.25 | 2.5 | 0.6 |
| | **12.5** | 1.25 | 1.25 | 0.3/0.6 |
| | **25** | 0.6 | 1.25 | 0.1 |

The compound 37a exhibited the best inhibitory activity, with a 4-fold reduction of ciprofloxacin MIC starting from 0.39 µg/ml. It was thus considered for further analysis. In order to exclude any effect of this compound other than the inhibition of NorA, it was tested at 25 µg/ml in combination with different ciprofloxacin concentration against *S. aureus* SA1199, assessing if it was able to reduce ciprofloxacin MIC also in the wild type strain. Only a non-significant 2-fold reduction of the ciprofloxacin MIC (from 0.07 to 0.03) was observed, thus confirming the inhibitory activity of NorA of compound 37a.

Time kill curve assays were then performed using SA1199B to evaluate the synergistic bactericidal activity over time of the combination ciprofloxacin + 37a. When used in association with ¼ MIC ciprofloxacin, compound 37a at either 3.13 µg/ml and 6.25 µg/ml showed a bactericidal effect similar to that exhibited by ciprofloxacin alone at a concentration corresponding to the MIC; moreover, all tested concentrations of 37a used in combination with ciprofloxacin caused a significant (at least 1 log) reduction of the SA1199B survivors compared to that observed using the antibiotic alone and always resulted bactericidal (> 3 log CFU reduction) compared to the untreated control (**Fig.31**).

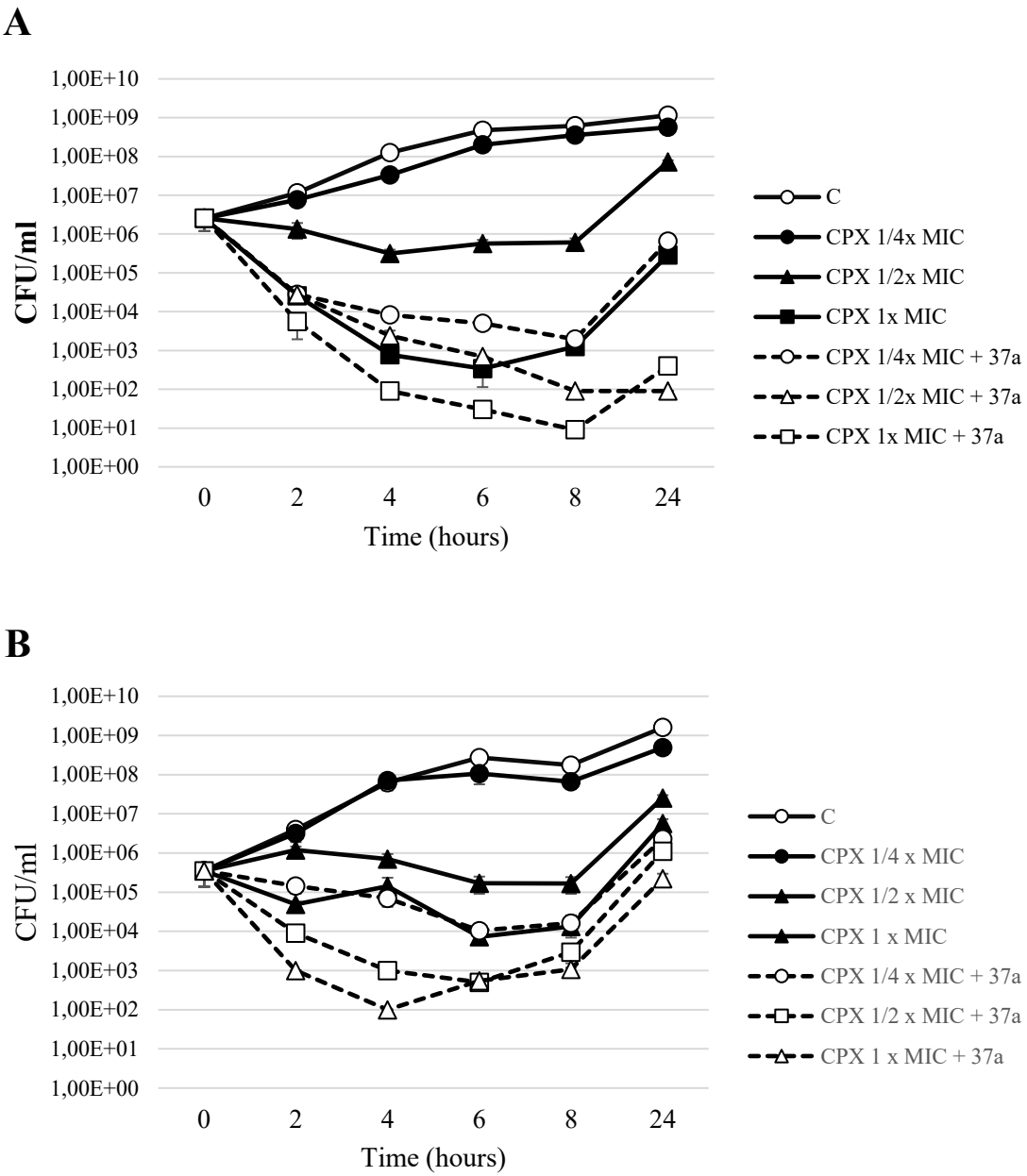


**Figure 31** Time kill curves of the combination ¼x, ½x and 1x MIC CPX with 6.25 µg/ml (A) and 3.13 µg/ml (B) of compound 37a.

Once assessed the strong inhibiting effect of 37a on NorA efflux pump and its synergy with ciprofloxacin against *S. aureus*, its possible toxic effect was evaluated through hemolysis assays, which showed a 10% hemolysis at the higher concentration used (25 µg/ml) that, however, decreased to very low levels (< 5%) at lower concentrations, encompassing also those showing synergistic activity (**Fig. 32**).

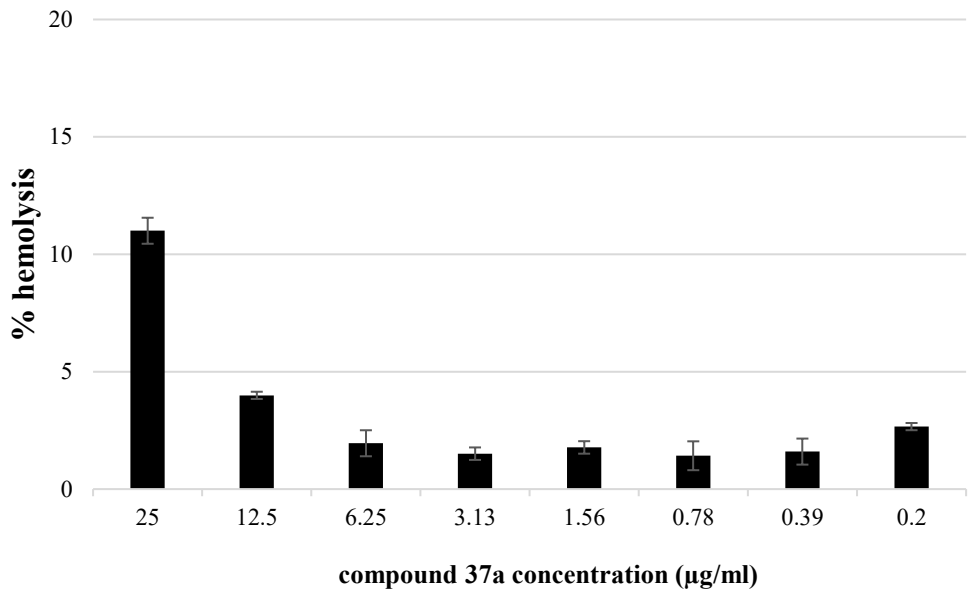


**Figure 32** Hemolysis assays of the compound 37a at different concentrations.

All together these results confirm the quinoline as a good scaffold for the development of novel NorA inhibitors. Starting from the compound 1, whose EPI activity had been already assessed [110], different substitutions at the C-2 of the quinoline core led to the development of 16 EPI candidates.

Compound 37a in particular showed a promising activity, the 74.26 % inhibition of EtBr efflux suggests its specific binding to NorA, which, although not the only efflux pump in *S. aureus*, results by far the most common [30]; moreover, the checkerboard assays and the time-kill curve exhibited a considerable potentiation of the ciprofloxacin antimicrobial activity, while the checkerboard assays showed an impressive 32-fold ciprofloxacin MIC reduction when in combination with this compound, the time kill assays showed a synergistic effect very evident also at very low concentrations (3.13 and 6.25 µg/ml). On the other hand, compound 37a showed a considerable hemolytic effect (~10%) at 25 µg/ml, but this drawback becomes insignificant considering the high efficacy of the compound at lower, and non-toxic, concentrations.

## Structural modifications of quinolin-4-yloxy based compounds as NorA EPIs

In a further study, the same workflow was followed for the analysis of the NorA EPI activity of 8 additional molecules, developed and synthesized by the Medicinal Chemistry group of Prof. Stefano Sabatini, which, using a pharmacophore model approach, started from a quinolin-4-yloxy core molecule with the 4-propoxyphenyl group at C-2 position and the alkylamino chain linked to the oxygen at C-4 and searched, in a FDA-approved drugs database, new possible EPIs by changing both the core and the functional groups.

The 8 molecules (3a, 3b, 4a, 4b, 5a, 5b, 6a and 6b) (**Fig.33**) were firstly assessed for an intrinsic antimicrobial activity through MIC assays on the isogenic pair SA1199 (wild type) / SA1199B (*norA* overexpressing mutant). The results did not show any antibacterial activity against both strains at concentrations ≤ 25 µg/ml.

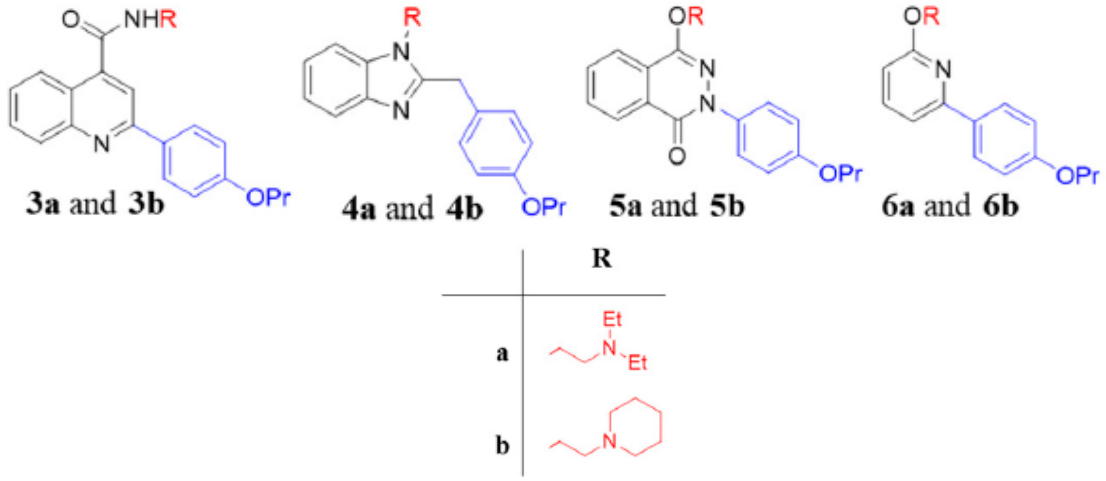


**Figure 33** Chemical structure of the 8 tested compounds.

Subsequently, ciprofloxacin MIC assays in absence / presence of the compounds at 12.5 µg/ml were performed against both SA1199 and SA1199B to rapidly screen a possible synergistic compound. Only compounds able to induce at least a 4-fold reduction of the ciprofloxacin MIC in the *norA* overexpressing strain and no significant reduction (≤ 2-fold) in SA1199 ciprofloxacin MIC were considered for further studies, since an evident activity on the wild type strain, comparable to SA1199B, could suggest a NorA-independent effect (such as the permeabilization of the bacterial membrane).

Three compounds (3a, 3b and 4a) met the required criteria for further studies as possible EPIs: they caused a 4-fold reduction of ciprofloxacin MIC in SA1199B and a negligible 2-fold reduction in the wild type strain (**Table 10**).

**Table 10:** CPX MIC assays in absence / presence of the tested compounds at 12.5 µg/ml against SA1199 (wild type) and SA1199B (*norA* overexpressing). * Good synergistic effect (at least 4-fold reduction of CPX MIC against SA1199B).

| **Compound** | **CPX MIC (µg/ml)** | |
|---|---|---|
| **(12.5 µg/ml)** | **SA1199** | **SA1199B** |
| **none** | 0.16 | 5 |
| **3a** | 0.08 | 1.25* |
| **3b** | 0.08 | 1.25* |
| **4a** | 0.08 | 1.25* |
| **4b** | 0.16 | 2.5 |
| **5a** | 0.08 | 2.5 |
| **5b** | 0.08 | 2.5 |
| **6a** | 0.08 | 2.5 |
| **6b** | 0.08 | 2.5 |

The dose-dependent synergistic effect with ciprofloxacin of the three promising compounds was then tested by checkerboard assays against *S. aureus* SA1199B. The result confirmed the synergy of the three compounds, particularly evident for compounds 3a and 3b, which showed a 4-fold reduction of a ciprofloxacin MIC already at a concentration of 3.13 µg/ml and 1.56 µg/ml, respectively, until leading to a 8-fold and 16-fold ciprofloxacin MIC reduction, respectively, at a concentration of 25 µg/ml (**Table 11**).

**Table 11:** Checkerboard assays of the compounds 3a, 3b and 4a in combination with different concentrations of CPX against *S. aureus* SA1199B.

| | | 3a | 3b | 4a |
|---|---|---|---|---|
| | | CPX MIC (µg/ml) | | |
| Compound concentration (µg/ml) | **0** | 5 | 5 | 5 |
| | **0.39** | 5 | 2.5 | 2.5 |
| | **0.78** | 2.5 | 2.5 | 2.5 |
| | **1.56** | 2.5 | 1.25 | 2.5 |
| | **3.13** | 1.25 | 1.25 | 2.5 |
| | **6.25** | 1.25 | 1.25 | 2.5 |
| | **12.5** | 1.25 | 0.6 | 1.25 |
| | **25** | 0.6 | 0.3 | 1.25 |

In order to further confirm that the synergy of these tested compounds with ciprofloxacin was due to a specific inhibition of NorA, the checkerboard assays of 3a and 3b were repeated against the additional isogenic pair *S. aureus* SAK1902 (*norA* deleted) / SAK2378 (complemented with the over-expressed *norA* of SA1199B). Once assessed the lack of a antimicrobial activity of the two compounds alone on both strains (MIC > 25 µg/ml), the checkerboard assays showed for both compounds a good synergistic activity: a 4-fold reduction of ciprofloxacin MIC already at 0.39 µg/ml and a 16-fold reduction at 6.25 µg/ml for both compounds; with compound 3a able to achieve a 32-fold ciprofloxacin MIC reduction at 12.5 and 25 µg/ml (**Table 12**). On the other hand, no visible effect on SAK1902 ciprofloxacin MIC was observed with both compounds.

**Table 12:** Checkerboard assays performed with compounds 3a and 3b in combination with different concentrations of CPX against *S. aureus* SAK1902 (Δ*norA*) and SAK2378 (*norA*++).

| | | SAK1902 | | SAK2378 | |
|---|---|---|---|---|---|
| | | 3a | 3b | 3a | 3b |
| | | CPX MIC (µg/ml) | | | |
| Compound concentration (µg/ml) | **0** | 0.08 | 0.08 | 1.25 | 1.25 |
| | **0.39** | 0.04 | 0.4 | 0.31 | 0.31 |
| | **0.78** | 0.04 | 0.4 | 0.31 | 0.31 |
| | **1.56** | 0.04 | 0.4 | 0.31 | 0.15 |
| | **3.13** | 0.04 | 0.8 | 0.15 | 0.15 |
| | **6.25** | 0.04 | 0.8 | 0.08 | 0.08 |
| | **12.5** | 0.04 | 0.8 | 0.04 | 0.08 |
| | **25** | 0.04 | 0.8 | 0.04 | 0.08 |

Ethidium bromide efflux assays revealed a very good EtBr efflux inhibition of the compounds 3a, 3b and 4a, thus confirming the NorA inhibitory power of the three tested compounds; in particular, 50 μM (~25 μg/ml) 3a and 3b reduced the EtBr efflux by 98.1% and 96.4%, respectively (**Table 13**).

**Table 13:** EtBr efflux inhibition of SA1199B by the three compounds 3a, 3b and 4a.

| Compound | EtBr Efflux Inhibition (%) | |
|---|---|---|
| | 50 μM | 10 μM |
| 3a | 98.1 | 56.6 |
| 3b | 96.4 | 66.2 |
| 4a | 74.5 | 35.8 |

Finally, cytotoxicity assays against human THP-1 and A549 cell lines were performed by MTT assays on compounds 3a and 3b in collaboration with Dr. Salvatore Vaiasicca (Department of Life and Environmental Sciences, Polytechnic University of Marche). Both compounds used at 3.13 μg/ml, reduced the vitality of THP1 cells by less than 40% (3a, 28% and 3b, 38%), while no effect on the vitality of A549 cells was observed for both compounds.

In conclusion, the structural modification approach of a quinolin-4-yloxy core molecule led to a set of 8 new EPIs candidate, all sharing a 4-propoxyphenyl group at the C-2. Two molecules in particular, 3a and 3b (quinoline-4-carboxamide derivatives), showed a very good synergistic activity with ciprofloxacin, leading to at least a 4-fold MIC reduction of the *norA* over-expressing strain SA1199B already at 3.13 and 1.56 μg/ml, without showing any effect in the wild type strain SA1199. A very good efflux inhibition was observed from both compounds in SA1199B (98.1% and 96.4% at ~25 μg/ml for 3a and 3b, respectively), and the checkerboard assays on a different isogenic strain pair (*S. aureus* SAK1902,Δ*norA* and SAK2378,*norA*++) confirmed a very good synergy with ciprofloxacin (at least 4-fold MIC reduction) already at the very low concentration of 0.39 μg/ml, without affecting the ciprofloxacin MIC of the deleted strain SAK1902, thus corroborate a specific action of the tested compounds on NorA.

Toxicity assays showed a moderate toxicity on the human cell line THP-1 at 3.13 μg/ml (72% and 62% vitality after treatment with 3a and 3b, respectively) and no toxicity

at all on the human cell line A549. These results, coupled with their quite satisfying synergistic activity at very low concentrations indicate 3a and 3b as promising EPIs to be further studied towards the development of an effective treatment, in combination with ciprofloxacin, against antibiotic resistance *S. aureus* infections.

## Studies on the role of NorA in *Staphylococcus aureus* persistence to high concentrations of ciprofloxacin

Considering what reported for AcrAB-TolC in *E. coli* [100] and MexXY-OprM in *P. aeruginosa* (present study, see above), a possible role for NorA in the *S. aureus* persistence to ciprofloxacin was investigated.

For this purpose, persistence assays were performed on the isogenic pairs *S. aureus* SA1199 (wild type) / SA1199B (*norA*++), and SAK1902 (Δ*norA*) / SAK2378 (*norA*++), by treating both stationary and exponential phase with ciprofloxacin 20x MIC.

When treating the cultures in stationary phase, no difference in persisters was observed between the *norA* defective and the *norA* overexpressing strains during the 24 hours of exposure. In both the isogenic pairs SA1199 / SA1199B and the SAK1902 /SAK2378 the persisters amount always remain stable at $10^9$ CFU/ml.

When treating the cultures in exponential phase, the two strain of the pair behaved differently.

SA1199 and SA1199B both showed a 1 log persisters decrease after 1 hour of treatment (from $10^9$ to $10^8$ CFU/ml), afterwards, SA1199 persisters remain stable at about $10^8$ CFU/ml until the end of the experiment, while SA1199B persisters remain stable at at about $10^8$ CFU/ml until 7 hours of exposure and then showed a 2 log decrease between 7 and 24 hours.

SAK1902 and SAK2378 both showed a 1 log persisters decrease after 1 hour of treatment (from $10^8$ to $10^7$ CFU/ml), after that, SAK1902 remain stable at about $10^7$ CFU/ml until the end of the experiment, while SAK2378 persisters progressively decreased until a $10^4$ CFU/ml after 7 hours of exposure and then furtherly dropped to $10^2$ CFU/ml at 24 hours (**Fig. 34**).

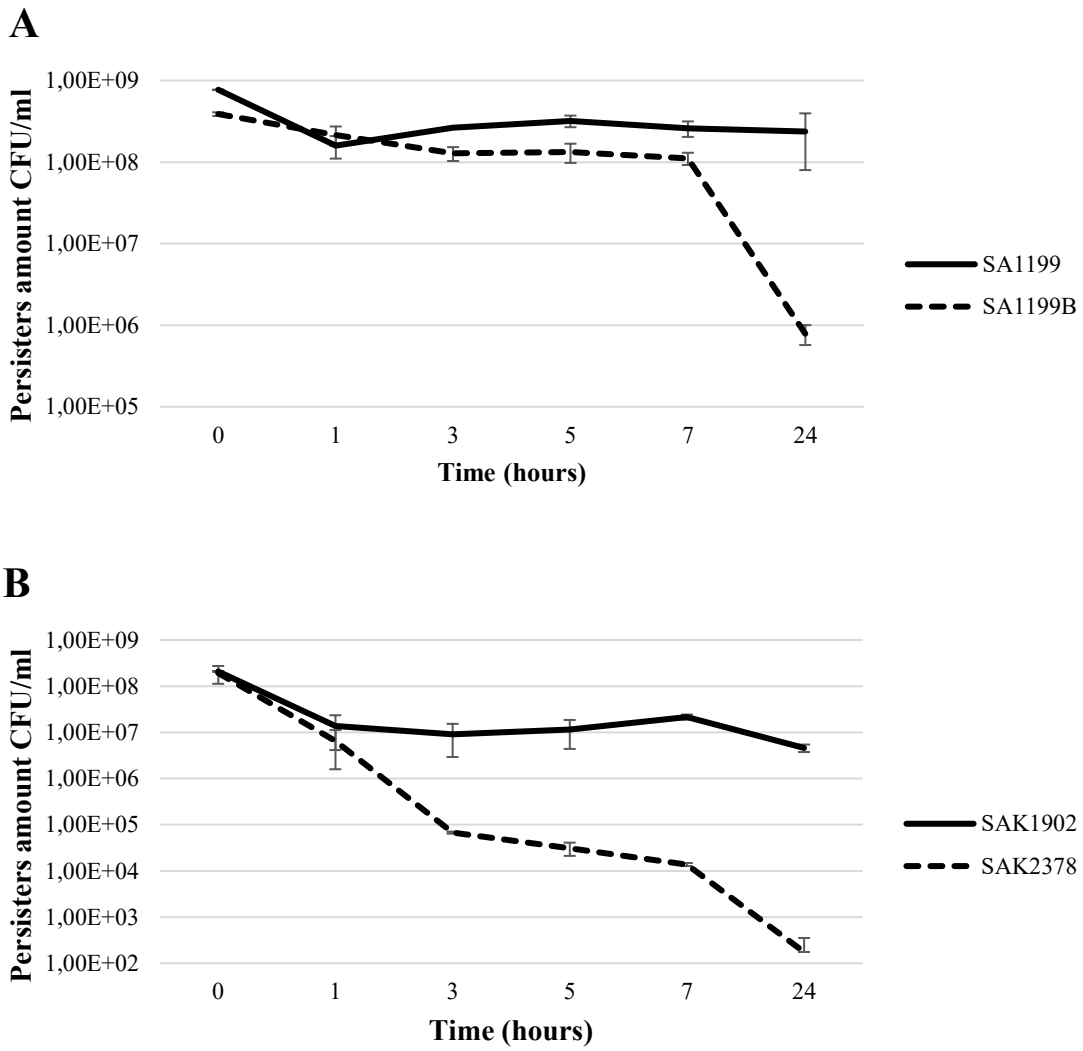


**Figure 34** persistence assays of the isogenic pairs SA1199/SA1199B (A) and SAK1902/SAK2378 (B) exposed to 20x MIC ciprofloxacin in mid-log phase. The *norA* wild type or defective strain is indicated by a solid line while the *norA* over-expressing strains are indicated by a dashed line.

Although worrisome from a clinical point of view, the insusceptibility of *S. aureus* stationary phase cultures to ciprofloxacin is not surprising. It is in agreement with previous studies reporting that, when in stationary phase, the whole *S. aureus* population exhibit persistence or tolerance to a large panel of antibiotics, including ciprofloxacin [28].

Much more surprising results come from the persistence assays in the exponential phase, where apparently a higher (and stable over time) persisters amount was observed in the *norA* wild type (SA1199) and deleted (SAK1902) strains, whereas the two *norA* over-expressing strains SA1199B and SAK2378 showed a clearly decreasing persisters

population. Therefore, in contrast to our hypothesis, the presence of a working NorA efflux pump seems to cause a reduction of persisters to ciprofloxacin in *S. aureus*. Although the same result was reported also by a recent study of a different research group [48], still a possible explanation for this phenomenon is missing. In *S. aureus* the lack of NorA and the consequent higher accumulation of antibiotic inside the bacterial cell might trigger a dormant state as a stress response, thus increasing the survival rate. However, at present no evidence to demonstrate this hypothesis is available.

# CONCLUSIONS

Antibiotic resistance is globally considered one of the biggest human health threats and could become a real emergency in the next future. The unresponsiveness of bacterial infections to the antibiotic treatment is a complex and multifaceted phenomenon that can be influenced by many underlying factors and mechanisms [32]. Bacterial tolerant and persistent cells are further complicating the picture; they exhibit antibiotic insensitivity although being virtually susceptible, through a transient modification of genes expression leading to a transient ability to overcome antibiotic killing rather than a stable and inheritable mutation [13].

Of all the mechanisms of bacterial antibiotic resistance, efflux pumps are one of the most widespread and able to confer a multidrug resistant phenotype. These transmembrane transporter systems are able to extrude toxic compounds, including antibiotics, outside of the cells thus heavily contributing to antibiotic resistance both in Gram-negative and in Gram-positive bacteria [97]. Moreover, recent findings unravel several others functions of efflux pumps in the bacterial physiology: from inter-cellular communication to biofilm formation and virulence; moreover, a recent study demonstrated the role of efflux pumps also in persisters formation in *E. coli* [100].

This study succeeded in validating new potent EPIs against the RND efflux pump MexXY-OprM, the major responsible for aminoglycoside resistance in *P. aeruginosa*, and the MFS efflux pump NorA, responsible for fluoroquinolone resistance in *S. aureus*, to be used in combination with tobramycin and ciprofloxacin to overcome antibiotic resistance. Moreover, it provided some additional information on the role of these efflux pumps in the bacterial ability to survive to high antibiotic concentrations by persisters development.

The results also provide further evidence of the high frequency of gentamycin and amikacin resistance among CF *P. aeruginosa* strains and of a less common tobramycin resistance. A central role of MexXY-OprM efflux pump in aminoglycoside resistance was also evidenced and different *in silico* docking assays and *in vitro* experimental evidences that the natural alkaloid berberine, already known for its benefic properties in traditional medicine, can act as a MexXY-OprM inhibitor were provided .

Berberine actually exhibited a good synergistic effect with tobramycin on 15/35 isolates in combination assays, and time kill curves showed a 2-3 log greater tobramycin killing activity when in combination with berberine, which on the other hand resulted devoid of toxic effect on human red blood cells at the tested concentrations. A significant role of MexXY-OprM in *P. aeruginosa* persistence to tobramycin has been also observed,

both in planktonic and biofilm cultures. Considering the observed involvement of MeXY-OprM in tobramycin persisters development in *P. aeruginosa*, blocking the pump could be a promising strategy to inhibit the formation of this worrisome phenotype. Indeed, the combination berberine-tobramycin was able to induce a 1-3 log persisters reduction, compared to tobramycin alone, in ~ 73% of the tested isolates, completely eradicating the survivals population in one case. These results strongly promote berberine as tobramycin adjuvant, to be used in drug combinations to overcome both antibiotic resistance and persistence

However, a strain-specific behavior of berberine and the non-negligible amount of strains completely unresponsive to the EPI were observed. Sequencing assays and *in silico* docking studies evidenced a correlation between the synergy of the combination berberine/tobramycin in terms of both resistance and persistence reduction and its specific affinity for a particular binding site. A resistance reduction seems to correlate with the berberine affinity for the cleft site of MexY, while a persisters reduction seems to correlate with the berberine affinity for the MexY antibiotic binding site. As expected, a poor affinity of berberine for both sites resulted with no reduction of both tobramycin resistance level and persisters amount.

As regards *S. aureus*, the search for NorA inhibitors led to the validation of two sets of molecules: the first based on a 2-phenylquinoline scaffold with different C-2 substitutions, the second based on a quinoline-4-yloxy scaffold with different changes in both in the C-2/4 and in the core structure; they were tested in association with ciprofloxacin as NorA inhibitors able to counteract the antibiotic efflux. Three compounds in particular (3a, 3b and 37a) showed promising features. They resulted synergistic with ciprofloxacin, reverting the tested strain from resistant to susceptible while not showing toxicity against different human cell lines at the active concentrations.

This whole study lays the foundations for future investigations: from a microbiological point of view, efflux pumps involvement in the bacterial life still has several unclear aspects, as well as the exact mechanisms underneath antibiotic persistence; furthermore, a deep study on the possible MexY variants could help in the setup of "universal" EPIs, able to exert a more stable and certain inhibitory activity. From a chemical point of view, on the other hand, the possibilities for searching and/or developing new and more effective EPIs are countless, as well as the development of specific delivery systems for an improved activity also in biofilms. Finally, an

advancement to *in vivo* tests would better confirm the activities of these molecules and would bring them closer to a clinical use.

Altogether the results of this study endorse the research on efflux pump inhibitors in two bacterial species among the most life-threatening due to their unresponsiveness to antibiotic treatments; furthermore, they demonstrate the successful multi-disciplinary cooperation between *in silico* chemical studies and *in vitro* microbiological studies for the identification of adjuvant molecules synergistic with antibiotics. Once overcome the toxicity issues, functioning EPIs combined with a deeper understanding of the bacterial antibiotic survival could really lead to a breakthrough in chronic and recurrent infections, in which the traditional antibiotic treatments fail to eradicate the resilient bacterial population.

# BIBLIOGRAPHY

1. Abraham, E. P., & Chain, E. (1940). An enzyme from bacteria able to destroy penicillin. *Nature*, *146*(3713), 837-837.
2. Adedeji, W. A. (2016). The treasure called antibiotics. *Annals of Ibadan postgraduate medicine*, *14*(2), 56.
3. Alav, I., Sutton, J. M., & Rahman, K. M. (2018). Role of bacterial efflux pumps in biofilm formation. *Journal of Antimicrobial Chemotherapy*, *73*(8), 2003-2020.
4. Alekshun, M. N., & Levy, S. B. (2007). Molecular mechanisms of antibacterial multidrug resistance. *Cell*, *128*(6), 1037-1050.
5. Allison, K. R., Brynildsen, M. P., & Collins, J. J. (2011). Metabolite-enabled eradication of bacterial persisters by aminoglycosides. *Nature*, *473*(7346), 216-220.
6. AlMatar, M., Albarri, O., Makky, E. A., & Köksal, F. (2020). Efflux pump inhibitors: new updates. *Pharmacological Reports*, 1-16.
7. Andersson, D. I., & Hughes, D. (2010). Antibiotic resistance and its cost: is it possible to reverse resistance?. *Nature Reviews Microbiology*, *8*(4), 260-271.
8. Andrei, S., Droc, G., & Stefan, G. (2019). FDA approved antibacterial drugs: 2018-2019. *Discoveries*, *7*(4).
9. Arthur, M. I. C. H. E. L., Molinas, C., Depardieu, F., & Courvalin, P. (1993). Characterization of Tn1546, a Tn3-related transposon conferring glycopeptide resistance by synthesis of depsipeptide peptidoglycan precursors in *Enterococcus faecium* BM4147. *Journal of bacteriology*, *175*(1), 117-127.
10. Ayrapetyan, M., Williams, T. C., & Oliver, J. D. (2015). Bridging the gap between viable but non-culturable and antibiotic persistent bacteria. *Trends in microbiology*, *23*(1), 7-13.
11. Ayrapetyan, M., Williams, T., & Oliver, J. D. (2018). Relationship between the viable but nonculturable state and antibiotic persister cells. *Journal of bacteriology*, *200*(20).
12. Azam, M. W., & Khan, A. U. (2019). Updates on the pathogenicity status of *Pseudomonas aeruginosa*. *Drug discovery today*, *24*(1), 350-359.
13. Balaban, N. Q., Helaine, S., Lewis, K., Ackermann, M., Aldridge, B., Andersson, D. I., ... & Zinkernagel, A. (2019). Definitions and guidelines for research on antibiotic persistence. *Nature Reviews Microbiology*, *17*(7), 441-448.
14. Baylay, A. J., Piddock, L. J., & Webber, M. A. (2019). Molecular Mechanisms of Antibiotic Resistance–Part I. *Bacterial Resistance to Antibiotics–From Molecules to Man*, 1-26.
15. Beaudoin, T., Zhang, L., Hinz, A. J., Parr, C. J., & Mah, T. F. (2012). The biofilm-specific antibiotic resistance gene ndvB is important for expression of ethanol oxidation genes in *Pseudomonas aeruginosa* biofilms. *Journal of bacteriology*, *194*(12), 3128-3136.
16. Blair, J. M., Webber, M. A., Baylay, A. J., Ogbolu, D. O., & Piddock, L. J. (2015). Molecular mechanisms of antibiotic resistance. *Nature reviews microbiology*, *13*(1), 42-51.
17. Blanco, P., Hernando-Amado, S., Reales-Calderon, J. A., Corona, F., Lira, F., Alcalde-Rico, M., ... & Martinez, J. L. (2016). Bacterial multidrug efflux

pumps: much more than antibiotic resistance determinants. *Microorganisms*, *4*(1), 14.
18. Bravo, A., Ruiz-Cruz, S., Alkorta, I., & Espinosa, M. (2018). When humans met superbugs: strategies to tackle bacterial resistances to antibiotics. *Biomolecular concepts*, *9*(1), 216-226.
19. Breidenstein, E. B., de la Fuente-Núñez, C., & Hancock, R. E. (2011). *Pseudomonas aeruginosa*: all roads lead to resistance. *Trends in microbiology*, *19*(8), 419-426.
20. Bucci, R., & Gallì, P. (2011). Public Health History Corner Vincenzo Tiberio: a misunderstood researcher. *Italian Journal of Public Health*, *8*(4).
21. Camilli, R., Pantosti, A., & Baldassarri, L. (2011). Contribution of serotype and genetic background to biofilm formation by *Streptococcus pneumoniae*. *European journal of clinical microbiology & infectious diseases*, *30*(1), 97-102.
22. Carattoli, A. (2013). Plasmids and the spread of resistance. *International Journal of Medical Microbiology*, *303*(6-7), 298-304.
23. Chang, J., Lee, R. E., & Lee, W. (2020). A pursuit of *Staphylococcus aureus* continues: A role of persister cells. *Archives of Pharmacal Research*, 1-9.
24. Chen, H., Hu, J., Chen, P. R., Lan, L., Li, Z., Hicks, L. M., ... & He, C. (2008). The *Pseudomonas aeruginosa* multidrug efflux regulator MexR uses an oxidation-sensing mechanism. *Proceedings of the National Academy of Sciences*, *105*(36), 13586-13591.
25. Chongsiriwatana, N. P., Patch, J. A., Czyzewski, A. M., Dohm, M. T., Ivankin, A., Gidalevitz, D., ... & Barron, A. E. (2008). Peptoids that mimic the structure, function, and mechanism of helical antimicrobial peptides. *Proceedings of the National Academy of Sciences*, *105*(8), 2794-2799.
26. Clinical and Laboratory Standards Institute (CLSI), (2017). Methods for Dilution Antimicrobial Susceptibility Tests for Bacteria That Grow Aerobically; Approved Standard-Tenth ed.; M07-A10
27. Clinical and Laboratory Standards Institute. (2017). Performance standards for antimicrobial susceptibility testing. *CLSI supplement M100*.
28. Conlon, B. P. (2014). *Staphylococcus aureus* chronic and relapsing infections: Evidence of a role for persister cells: An investigation of persister cells, their formation and their role in *S. aureus* disease. *Bioessays*, *36*(10), 991-996.
29. Corradetti, B., Vaiasicca, S., Mantovani, M., Virgili, E., Bonucci, M., & Hammarberg Ferri, I. (2019). Bioactive immunomodulatory compounds: a novel combinatorial strategy for integrated medicine in oncology? BAIC exposure in cancer cells. *Integrative cancer therapies*, *18*, 1534735419866908.
30. Costa, S. S., Viveiros, M., Amaral, L., & Couto, I. (2013). Multidrug efflux pumps in *Staphylococcus aureus*: an update. *The open microbiology journal*, *7*, 59.
31. Davies, J. (1995). Vicious circles: looking back on resistance plasmids. *Genetics*, *139*(4), 1465.
32. Davies, J., & Davies, D. (2010). Origins and evolution of antibiotic resistance. *Microbiology and molecular biology reviews*, *74*(3), 417-433.
33. Davies, J., Spiegelman, G. B., & Yim, G. (2006). The world of subinhibitory antibiotic concentrations. *Current opinion in microbiology*, *9*(5), 445-453.

34. Defraine, V., Fauvart, M., & Michiels, J. (2018). Fighting bacterial persistence: Current and emerging anti-persister strategies and therapeutics. *Drug Resistance Updates*, *38*, 12-26.
35. Delavat, F., Miyazaki, R., Carraro, N., Pradervand, N., & van der Meer, J. R. (2017). The hidden life of integrative and conjugative elements. *FEMS microbiology reviews*, *41*(4), 512-537.
36. Deschaght, P., Schelstraete, P., Van Simaey, L., Vanderkercken, M., Raman, A., Mahieu, L., ... & Vaneechoutte, M. (2013). Is the improvement of CF patients, hospitalized for pulmonary exacerbation, correlated to a decrease in bacterial load?. *PLoS One*, *8*(11), e79010.
37. Dickey, S. W., Cheung, G. Y., & Otto, M. (2017). Different drugs for bad bugs: antivirulence strategies in the age of antibiotic resistance. *Nature Reviews Drug Discovery*, *16*(7), 457.
38. Doi, Y., & Arakawa, Y. (2007). 16S ribosomal RNA methylation: emerging resistance mechanism against aminoglycosides. *Clinical Infectious Diseases*, *45*(1), 88-94.
39. Durand, G. A., Raoult, D., & Dubourg, G. (2019). Antibiotic discovery: History, methods and perspectives. *International journal of antimicrobial agents*, *53*(4), 371-382.
40. Fisher, R. A., Gollan, B., & Helaine, S. (2017). Persistent bacterial infections and persister cells. *Nature Reviews Microbiology*, *15*(8), 453.
41. Fitzgerald, J. R. (2014). Evolution of *Staphylococcus aureus* during human colonization and infection. *Infection, genetics and evolution*, *21*, 542-547.
42. Fleming, A. (1946). *Chemotherapy: yesterday, today and tomorrow*. Cambridge University Press.
43. Flemming, H. C., & Wingender, J. (2010). The biofilm matrix. *Nature reviews microbiology*, *8*(9), 623-633.
44. Foster, T. J. (2004). The *Staphylococcus aureus* "superbug". *The Journal of clinical investigation*, *114*(12), 1693-1696.
45. Fuda, C., Suvorov, M., Vakulenko, S. B., & Mobashery, S. (2004). The basis for resistance to β-lactam antibiotics by penicillin-binding protein 2a of methicillin-resistant Staphylococcus aureus. *Journal of Biological Chemistry*, *279*(39), 40802-40806.
46. Gelpi, A., Gilbertson, A., & Tucker, J. D. (2015). Magic bullet: Paul Ehrlich, Salvarsan and the birth of venereology. *Sexually transmitted infections*, *91*(1), 68-69.
47. Ha, D. G., & O'Toole, G. A. (2015). c-di-GMP and its effects on biofilm formation and dispersion: a *Pseudomonas aeruginosa* review. *Microbial Biofilms*, 301-317.
48. Habib, G., Zhu, J., & Sun, B. (2020). A novel type I toxin-antitoxin system modulates persister cell formation in *Staphylococcus aureus*. *International Journal of Medical Microbiology*, *310*(2), 151400.
49. Hall, C. W., & Mah, T. F. (2017). Molecular mechanisms of biofilm-based antibiotic resistance and tolerance in pathogenic bacteria. *FEMS microbiology reviews*, *41*(3), 276-301.

50. Hall, R. M. (2012). Integrons and gene cassettes: hotspots of diversity in bacterial genomes. *Annals of the New York Academy of Sciences*, *1267*(1), 71-78.
51. Harrison, F., Roberts, A. E., Gabrilska, R., Rumbaugh, K. P., Lee, C., & Diggle, S. P. (2015). A 1,000-year-old antimicrobial remedy with antistaphylococcal activity. *MBio*, *6*(4).
52. Hutchings, M. I., Truman, A. W., & Wilkinson, B. (2019). Antibiotics: Past, present and future. *Current Opinion in Microbiology*, 51, 72-80.
53. Hynes, W. L., Ferretti, J. J., Gilmore, M. S., & Segarra, R. A. (1992). PCR amplification of streptococcal DNA using crude cell lysates. *FEMS microbiology letters*, *94*(1-2), 139-142.
54. Isenberg, H. D. (1992). *Clinical microbiology procedures handbook*. American Society of Microbiology.
55. Kaatz, G. W., Seo, S. M., O'Brien, L., Wahiduzzaman, M., & Foster, T. J. (2000). Evidence for the existence of a multidrug efflux transporter distinct from NorA in *Staphylococcus aureus*. *Antimicrobial agents and chemotherapy*, *44*(5), 1404-1406.
56. Kipnis, E., Sawa, T., & Wiener-Kronish, J. (2006). Targeting mechanisms of Pseudomonas aeruginosa pathogenesis. *Medecine et maladies infectieuses*, *36*(2), 78-91.
57. Korch, S. B., Henderson, T. A., & Hill, T. M. (2003). Characterization of the hipA7 allele of Escherichia coli and evidence that high persistence is governed by (p) ppGpp synthesis. *Molecular microbiology*, *50*(4), 1199-1213.
58. Kumar, A., Chopra, K., Mukherjee, M., Pottabathini, R., & Dhull, D. K. (2015). Current knowledge and pharmacological profile of berberine: an update. *European journal of pharmacology*, *761*, 288-297.
59. Kumar, S., Lekshmi, M., Parvathi, A., Ojha, M., Wenzel, N., & Varela, M. F. (2020). Functional and structural roles of the major facilitator superfamily bacterial multidrug efflux pumps. *Microorganisms*, *8*(2), 266.
60. Lamers, R. P., Cavallari, J. F., & Burrows, L. L. (2013). The efflux inhibitor phenylalanine-arginine beta-naphthylamide (PAβN) permeabilizes the outer membrane of gram-negative bacteria. *PloS one*, *8*(3), e60666.
61. Lamut, A., Peterlin Mašič, L., Kikelj, D., & Tomašič, T. (2019). Efflux pump inhibitors of clinically relevant multidrug resistant bacteria. *Medicinal Research Reviews*, *39*(6), 2460-2504.
62. Larsson, A. (2014). AliView: a fast and lightweight alignment viewer and editor for large datasets. *Bioinformatics*, *30*(22), 3276-3278.
63. Lerminiaux, N. A., & Cameron, A. D. (2019). Horizontal transfer of antibiotic resistance genes in clinical environments. *Canadian journal of microbiology*, *65*(1), 34-44.
64. Lewis, K. (2010). Persister cells. *Annual review of microbiology*, *64*, 357-372.
65. Li, X. Z., Plésiat, P., & Nikaido, H. (2015). The challenge of efflux-mediated antibiotic resistance in Gram-negative bacteria. *Clinical microbiology reviews*, *28*(2), 337-418.
66. Lister, J. L., & Horswill, A. R. (2014). *Staphylococcus aureus* biofilms: recent developments in biofilm dispersal. *Frontiers in cellular and infection microbiology*, *4*, 178.

67. López-Causapé, C., Rubio, R., Cabot, G., & Oliver, A. (2018). Evolution of the *Pseudomonas aeruginosa* aminoglycoside mutational resistome *in vitro* and in the cystic fibrosis setting. *Antimicrobial agents and chemotherapy*, *62*(4).
68. Lyczak, J. B., Cannon, C. L., & Pier, G. B. (2002). Lung infections associated with cystic fibrosis. *Clinical microbiology reviews*, *15*(2), 194-222.
69. Magiorakos, A. P., Srinivasan, A., Carey, R. T., Carmeli, Y., Falagas, M. T., Giske, C. T., ... & Paterson, D. T. (2012). Multidrug-resistant, extensively drug-resistant and pandrug-resistant bacteria: an international expert proposal for interim standard definitions for acquired resistance. *Clinical microbiology and infection*, *18*(3), 268-281.
70. Mangiaterra, G., Amiri, M., Di Cesare, A., Pasquaroli, S., Manso, E., Cirilli, N., ... & Biavasco, F. (2018). Detection of viable but non-culturable *Pseudomonas aeruginosa* in cystic fibrosis by qPCR: a validation study. *BMC infectious diseases*, *18*(1), 1-7.
71. Martens, E., & Demain, A. L. (2017). The antibiotic resistance crisis, with a focus on the United States. *The Journal of antibiotics*, *70*(5), 520-526.
72. Martínez, J. L. (2008). Antibiotics and antibiotic resistance genes in natural environments. *Science*, *321*(5887), 365-367.
73. Masuda, N., Sakagawa, E., Ohya, S., Gotoh, N., Tsujimoto, H., & Nishino, T. (2000). Contribution of the MexX-MexY-OprM efflux system to intrinsic resistance in *Pseudomonas aeruginosa*. *Antimicrobial agents and chemotherapy*, *44*(9), 2242-2246.
74. McGuinness, W. A., Malachowa, N., & DeLeo, F. R. (2017). Focus: infectious diseases: vancomycin resistance in Staphylococcus aureus. *The Yale journal of biology and medicine*, *90*(2), 269.
75. Michalska, A. D., Sacha, P. T., Ojdana, D., Wieczorek, A., & Tryniszewska, E. (2014). Prevalence of resistance to aminoglycosides and fluoroquinolones among *Pseudomonas aeruginosa* strains in a University Hospital in Northeastern Poland. *Brazilian Journal of Microbiology*, *45*(4), 1455-1458.
76. Mohr, K. I. (2016). History of antibiotics research. In *How to Overcome the Antibiotic Crisis* (pp. 237-272). Springer, Cham.
77. Moradali, M. F., Ghods, S., & Rehm, B. H. (2017). *Pseudomonas aeruginosa* lifestyle: a paradigm for adaptation, survival, and persistence. *Frontiers in cellular and infection microbiology*, *7*, 39.
78. Morita, Y., Tomida, J., & Kawamura, Y. (2012). MexXY multidrug efflux system of *Pseudomonas aeruginosa*. *Frontiers in microbiology*, *3*, 408.
79. Morita, Y., Nakashima, K. I., Nishino, K., Kotani, K., Tomida, J., Inoue, M., & Kawamura, Y. (2016). Berberine is a novel type efflux inhibitor which attenuates the MexXY-mediated aminoglycoside resistance in *Pseudomonas aeruginosa*. *Frontiers in microbiology*, *7*, 1223.
80. Mostofian, F., Alkadri, J., Tang, K., Thampi, N., & Radhakrishnan, D. (2019). A real world evaluation of the long-term efficacy of strategies to prevent chronic *Pseudomonas aeruginosa* pulmonary infection in children with cystic fibrosis. *International Journal of Infectious Diseases*, *85*, 92-97.
81. Mulcahy, L. R., Burns, J. L., Lory, S., & Lewis, K. (2010). Emergence of *Pseudomonas aeruginosa* strains producing high levels of persister cells in patients with cystic fibrosis. *Journal of bacteriology*, *192*(23), 6191-6199.

82. Mustafa, M. H., Chalhoub, H., Denis, O., Deplano, A., Vergison, A., Rodriguez-Villalobos, H., ... & Van Bambeke, F. (2016). Antimicrobial susceptibility of *Pseudomonas aeruginosa* isolated from cystic fibrosis patients in northern Europe. *Antimicrobial agents and chemotherapy*, *60*(11), 6735-6741.
83. Nikaido, H. (2003). Molecular basis of bacterial outer membrane permeability revisited. *Microbiology and molecular biology reviews*, *67*(4), 593-656.
84. Nikaido, H., & Takatsuka, Y. (2009). Mechanisms of RND multidrug efflux pumps. *Biochimica et Biophysica Acta (BBA)-Proteins and Proteomics*, *1794*(5), 769-781.
85. O'Neill, J. (2014). Antimicrobial resistance: tackling a crisis for the health and wealth of nations. Review on antimicrobial resistance. *Review on Antimicrobial Resistance, London, United Kingdom: https://amr-review. org/sites/default/files/AMR% 20Review% 20Paper*.
86. Oh, H., Stenhoff, J., Jalal, S., & Wretlind, B. (2003). Role of efflux pumps and mutations in genes for topoisomerases II and IV in fluoroquinolone-resistant *Pseudomonas aeruginosa strains*. *Microbial Drug Resistance*, *9*(4), 323-328.
87. Olivares, E., Badel-Berchoux, S., Provot, C., Prévost, G., Bernardi, T., & Jehl, F. (2020). Clinical impact of antibiotics for the treatment of *Pseudomonas aeruginosa* biofilm infections. *Frontiers in microbiology*, *10*, 2894.
88. Otten, H. (1986). Domagk and the development of the sulphonamides. *Journal of Antimicrobial Chemotherapy*, *17*(6), 689-690.
89. Page, R., & Peti, W. (2016). Toxin-antitoxin systems in bacterial growth arrest and persistence. *Nature chemical biology*, *12*(4), 208-214.
90. Pang, Z., Raudonis, R., Glick, B. R., Lin, T. J., & Cheng, Z. (2019). Antibiotic resistance in *Pseudomonas aeruginosa*: mechanisms and alternative therapeutic strategies. *Biotechnology advances*, *37*(1), 177-192.
91. Pantosti, A., Sanchini, A., & Monaco, M. (2007). Mechanisms of antibiotic resistance in *Staphylococcus aureus*. *Future microbiology*, *2*(3), 323.
92. Partridge, S. R., Kwong, S. M., Firth, N., & Jensen, S. O. (2018). Mobile genetic elements associated with antimicrobial resistance. *Clinical microbiology reviews*, *31*(4).
93. Perry, J., Waglechner, N., & Wright, G. (2016). The prehistory of antibiotic resistance. *Cold Spring Harbor perspectives in medicine*, *6*(6), a025197.
94. Piddock, L. J. (2006). Clinically relevant chromosomally encoded multidrug resistance efflux pumps in bacteria. *Clinical microbiology reviews*, *19*(2), 382-402.
95. Piddock, L. J. (2006). Multidrug-resistance efflux pumps? not just for resistance. *Nature Reviews Microbiology*, *4*(8), 629-636.
96. Poirel, L., Jayol, A., & Nordmann, P. (2017). Polymyxins: antibacterial activity, susceptibility testing, and resistance mechanisms encoded by plasmids or chromosomes. *Clinical microbiology reviews*, *30*(2), 557-596.
97. Poole, K. (2007). Efflux pumps as antimicrobial resistance mechanisms. *Annals of medicine*, *39*(3), 162-176.
98. Poole, K. (2011). *Pseudomonas aeruginosa*: resistance to the max. *Frontiers in microbiology*, *2*, 65.

99. Prickett, M. H., Hauser, A. R., McColley, S. A., Cullina, J., Potter, E., Powers, C., & Jain, M. (2017). Aminoglycoside resistance of *Pseudomonas aeruginosa* in cystic fibrosis results from convergent evolution in the mexZ gene. *Thorax*, *72*(1), 40-47.
100. Pu, Y., Ke, Y., & Bai, F. (2017). Active efflux in dormant bacterial cells–new insights into antibiotic persistence. *Drug Resistance Updates*, *30*, 7-14.
101. Pu, Y., Zhao, Z., Li, Y., Zou, J., Ma, Q., Zhao, Y., ... & Bai, F. (2016). Enhanced efflux activity facilitates drug tolerance in dormant bacterial cells. *Molecular cell*, *62*(2), 284-294.
102. Radzikowski, J. L., Vedelaar, S., Siegel, D., Ortega, Á. D., Schmidt, A., & Heinemann, M. (2016). Bacterial persistence is an active σS stress response to metabolic flux limitation. *Molecular systems biology*, *12*(9), 882.
103. Ramamurthy, T., Ghosh, A., Pazhani, G. P., & Shinoda, S. (2014). Current perspectives on viable but non-culturable (VBNC) pathogenic bacteria. *Frontiers in public health*, *2*, 103.
104. Ramirez, M. S., & Tolmasky, M. E. (2010). Aminoglycoside modifying enzymes. *Drug resistance updates*, *13*(6), 151-171.
105. Rice, L. B. (2008). Federal funding for the study of antimicrobial resistance in nosocomial pathogens: no ESKAPE.
106. Römling, U., & Balsalobre, C. (2012). Biofilm infections, their resilience to therapy and innovative treatment strategies. *Journal of internal medicine*, *272*(6), 541-561.
107. Ronneau, S., & Hallez, R. (2019). Make and break the alarmone: regulation of (p) ppGpp synthetase/hydrolase enzymes in bacteria. *FEMS microbiology reviews*, *43*(4), 389-400.
108. Ruiz, J. (2003). Mechanisms of resistance to quinolones: target alterations, decreased accumulation and DNA gyrase protection. *Journal of Antimicrobial Chemotherapy*, *51*(5), 1109-1117.
109. Rybtke, M., Hultqvist, L. D., Givskov, M., & Tolker-Nielsen, T. (2015). *Pseudomonas aeruginosa* biofilm infections: community structure, antimicrobial tolerance and immune response. *Journal of molecular biology*, *427*(23), 3628-3645.
110. Sabatini, S., Gosetto, F., Manfroni, G., Tabarrini, O., Kaatz, G. W., Patel, D., & Cecchetti, V. (2011). Evolution from a natural flavones nucleus to obtain 2-(4-Propoxyphenyl) quinoline derivatives as potent inhibitors of the *S. aureus* NorA efflux pump. *Journal of medicinal chemistry*, *54*(16), 5722-5736.
111. Sharkey, L. K., & O'Neill, A. J. (2019). Molecular Mechanisms of Antibiotic Resistance–Part II. *Bacterial Resistance to Antibiotics–From Molecules to Man*, 27-50.
112. Soares, A., Alexandre, K., & Etienne, M. (2020). Tolerance and Persistence of *Pseudomonas aeruginosa* in Biofilms Exposed to Antibiotics: Molecular Mechanisms, Antibiotic Strategies and Therapeutic Perspectives. *Frontiers in microbiology*, *11*, 2057.
113. Song, S., & Wood, T. K. (2020). Are We Really Studying Persister Cells?. *Environmental Microbiology Reports*.

114. Song, S., & Wood, T. K. (2020). ppGpp ribosome dimerization model for bacterial persister formation and resuscitation. *Biochemical and Biophysical Research Communications*, *523*(2), 281-286.
115. Stover, C. K., Pham, X. Q., Erwin, A. L., Mizoguchi, S. D., Warrener, P., Hickey, M. J., ... & Olson, M. V. (2000). Complete genome sequence of *Pseudomonas aeruginosa* PAO1, an opportunistic pathogen. *Nature*, *406*(6799), 959-964.
116. Sun, J., Deng, Z., & Yan, A. (2014). Bacterial multidrug efflux pumps: mechanisms, physiology and pharmacological exploitations. *Biochemical and biophysical research communications*, *453*(2), 254-267.
117. Tacconelli, E., Magrini, N., Kahlmeter, G., & Singh, N. (2017). Global priority list of antibiotic-resistant bacteria to guide research, discovery, and development of new antibiotics. *World Health Organization*, *27*, 318-327.
118. Taylor, P. K., Yeung, A. T., & Hancock, R. E. (2014). Antibiotic resistance in *Pseudomonas aeruginosa* biofilms: towards the development of novel anti-biofilm therapies. *Journal of Biotechnology*, *191*, 121-130.
119. Taylor, T. A., & Unakal, C. G. (2019). *Staphylococcus Aureus*. In *StatPearls [Internet]*. StatPearls Publishing.
120. Tooke, C. L., Hinchliffe, P., Bragginton, E. C., Colenso, C. K., Hirvonen, V. H., Takebayashi, Y., & Spencer, J. (2019). β-Lactamases and β-lactamase inhibitors in the 21st century. *Journal of molecular biology*, *431*(18), 3472-3500.
121. Tseng, B. S., Zhang, W., Harrison, J. J., Quach, T. P., Song, J. L., Penterman, J., ... & Parsek, M. R. (2013). The extracellular matrix protects *Pseudomonas aeruginosa* biofilms by limiting the penetration of tobramycin. *Environmental microbiology*, *15*(10), 2865-2878.
122. Van den Bergh, B., Fauvart, M., & Michiels, J. (2017). Formation, physiology, ecology, evolution and clinical importance of bacterial persisters. *FEMS Microbiology Reviews*, *41*(3), 219-251.
123. Warburton, P. J., Amodeo, N., & Roberts, A. P. (2016). Mosaic tetracycline resistance genes encoding ribosomal protection proteins. *Journal of Antimicrobial Chemotherapy*, *71*(12), 3333-3339.
124. Waxman, D. J., & Strominger, J. L. (1983). Penicillin-binding proteins and the mechanism of action of beta-lactam antibiotics. *Annual review of biochemistry*, *52*(1), 825-869.
125. Webber, M. A., & Piddock, L. J. V. (2003). The importance of efflux pumps in bacterial antibiotic resistance. *Journal of Antimicrobial Chemotherapy*, *51*(1), 9-11.
126. Wiens, J. R., Vasil, A. I., Schurr, M. J., & Vasil, M. L. (2014). Iron-regulated expression of alginate production, mucoid phenotype, and biofilm formation by *Pseudomonas aeruginosa*. *MBio*, *5*(1).
127. Woegerbauer, M., Zeinzinger, J., Springer, B., Hufnagl, P., Indra, A., Korschineck, I., ... & Allerberger, F. (2014). Prevalence of the aminoglycoside phosphotransferase genes aph (3′)-IIIa and aph (3′)-IIa in *Escherichia coli*, *Enterococcus faecalis*, *Enterococcus faecium*, *Pseudomonas aeruginosa*, *Salmonella enterica* subsp. enterica and *Staphylococcus aureus* isolates in Austria. *Journal of medical microbiology*, *63*(2), 210-217.

128. Wood, T. K., Knabel, S. J., & Kwan, B. W. (2013). Bacterial persister cell formation and dormancy. *Applied and environmental microbiology*, *79*(23), 7116-7121.
129. Yamane, K., Doi, Y., Yokoyama, K., Yagi, T., Kurokawa, H., Shibata, N., ... & Arakawa, Y. (2004). Genetic environments of the rmtA gene in *Pseudomonas aeruginosa* clinical isolates. *Antimicrobial agents and chemotherapy*, *48*(6), 2069-2074.

# SOURCES OF IMAGES

**Fig.1:** Levy, S. B. (2013). *The antibiotic paradox: how miracle drugs are destroying the miracle*. Springer.

**Fig.2:** Hutchings, M. I., Truman, A. W., & Wilkinson, B. (2019). Antibiotics: Past, present and future. *Current Opinion in Microbiology*, 51, 72-80.

**Fig.3:** Andersson, D. I., & Hughes, D. (2010). Antibiotic resistance and its cost: is it possible to reverse resistance?. *Nature Reviews Microbiology*, *8*(4), 260-271.

**Fig.4:** Tacconelli, E., Magrini, N., Kahlmeter, G., & Singh, N. (2017). Global priority list of antibiotic-resistant bacteria to guide research, discovery, and development of new antibiotics. *World Health Organization*, *27*, 318-327.

**Fig.5:** https://amr.biomerieux.com/en/about-amr/what-is-antibiotic-resistance/

**Fig.6:** Blanco, P., Hernando-Amado, S., Reales-Calderon, J. A., Corona, F., Lira, F., Alcalde-Rico, M., ... & Martinez, J. L. (2016). Bacterial multidrug efflux pumps: much more than antibiotic resistance determinants. *Microorganisms*, *4*(1), 14.

**Fig.7:** Yan, N. (2015). Structural biology of the major facilitator superfamily transporters. *Annual review of biophysics*, *44*, 257-283.

**Fig.8:** Venter, H., Mowla, R., Ohene-Agyei, T., & Ma, S. (2015). RND-type drug efflux pumps from Gram-negative bacteria: molecular mechanism and inhibition. *Frontiers in microbiology*, *6*, 377.

**Fig.9:** Sun, J., Deng, Z., & Yan, A. (2014). Bacterial multidrug efflux pumps: mechanisms, physiology and pharmacological exploitations. *Biochemical and biophysical research communications*, *453*(2), 254-267.

**Fig.10:** Alav, I., Sutton, J. M., & Rahman, K. M. (2018). Role of bacterial efflux pumps in biofilm formation. *Journal of Antimicrobial Chemotherapy*, *73*(8), 2003-2020.

**Fig.11:** Bhando, T., Dubey, V., & Pathania, R. (2019). Biofilms in Antimicrobial Activity and Drug Resistance. In *Bacterial Adaptation to Co-resistance* (pp. 109-139). Springer, Singapore.

**Fig.12:** Stewart, P. S., White, B., Boegli, L., Hamerly, T., Williamson, K. S., Franklin, M. J., ... & Wallqvist, A. (2019). Conceptual model of biofilm antibiotic tolerance that integrates phenomena of diffusion, metabolism, gene expression, and physiology. *Journal of bacteriology*, *201*(22), e00307-19.

**Fig.13 and Fig 14:** Balaban, N. Q., Helaine, S., Lewis, K., Ackermann, M., Aldridge, B., Andersson, D. I., ... & Zinkernagel, A. (2019). Definitions and guidelines for research on antibiotic persistence. *Nature Reviews Microbiology*, *17*(7), 441-448.

**Fig.15:** Ayrapetyan, M., Williams, T., & Oliver, J. D. (2018). Relationship between the viable but nonculturable state and antibiotic persister cells. *Journal of bacteriology*, *200*(20).

**Fig.16:** Defraine, V., Fauvart, M., & Michiels, J. (2018). Fighting bacterial persistence: Current and emerging anti-persister strategies and therapeutics. *Drug Resistance Updates*, *38*, 12-26.