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Monografia de Mestrado Integrado em Ciências Farmacêuticas apresentada à Universidade de Lisboa através da Faculdade de Farmácia

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Abstract

In the past few years, humanity has struggled with an emergent problem: multidrug bacterial infections. What was then the solution has now become part of the problem – antibiotics. Many bacterial species have become resistant to a wide range of molecules and such example is the Gram-negative bacillus *Acinetobacter baumannii* – a pathogen responsible for respiratory and skin infections in hospital environment.

Patients who are chronically ill and those who have invasive medica devices such as catheters, sutures, ventilators and those treatments such as dialysis or antimicrobial therapy in the past 90 days are at the highest risk of infection.

A. baumannii has a mechanism of resistance based in the extrusion of antibiotic molecules throughout pumps located in its membrane. These pumps are called efflux pumps and they decrease the susceptibility of *A. baumannii* to some fluoroquinolones such as ciprofloxacin, a second-generation fluoroquinolone with a wide range of action.

Portugal was one of the countries with the higher (25% to 50%) percentages of invasive isolates with resistance to fluroquinolones in 2018. The development of new drugs is time and resource consuming and so it is more profitable to develop new molecules that will restore efficacy to old already studied and safety to use antibiotics.

These new molecules are called EPIs - Efflux Pump Inhibitors and they synergize with the antibiotic molecules by inactivating the efflux pumps in *A. baumannii*.

Resistance-Nodulation-Division (RND) is one of the families of pumps in which the efflux pumps are grouped and 1-(1-naphthylmethyl)-piperazine (NMP) is one of the EPIs that have effect in inhibiting this pump in *A. baumannii*. The major efflux pump in these Gram negative species is the AdeABC and the encoding genes of these tripartite structures are *adeA*, *adeB* and *adeC* and whose expression is regulated by a two-component regulation system: AdeRS.

In this study we tested a series of analogs of NMP and their ability to restore the antibiotic efficacy of ciprofloxacin in several isolates of *A. baumannii* and the genes that regulate these pumps. It was shown that the EPIs that could restore the activity of the ciprofloxacin against *A. baumannii* were the ones in which the amine was unprotected – the EPIs 2,6,7 and 8.

Key-words: A. baumannii, Efflux Pump Inhibitors, NMP, antimicrobial resistance, RND family, AdeABC, AdeRS

Resumo

Nos últimos anos, a Humanidade tem combatido um problema cada vez mais emergente: infeções bacterianas resistentes. O que foi outrora a solução faz agora parte do problema: antibióticos. Muitas espécies bacterianas tornaram-se resistentes a um largo expectro de moléculas e um desses exemplos é o bacilo Gram-negativo *Acinetobacter baummannii* – um patogénio responsável por infeções respiratórias e da pele em ambiente hospitalar.

Os pacientes crónicos e/ou sujeitos a dispositvos médicos invasivos como catétes ou ventiladores e ainda aqueles cujos tratamentos incluem diálise ou terapêutica antimicrobana nos últimos 90 dias, são os que apresentam maior risco de infeção.

*A. baumanni*i apresenta um mecanismo de resistência baseado na extrusão de moléculas de antibiótico através das bombas localizadas na sua membrana. Estas bombas são chamadas bombas de efluxo e a sua presença diminui a susceptilibilidade de *A. baumannii* a algumas fluoroquinolonas como a ciprofloxacina, uma fluoroquinolona de segunda geração com um largo espectro de actividade antimicrobiana.

Portugal foi um dos países com as maiores taxas (entre 25% e 50%) de isolados invasivos com resistência a fluoroquinolonas em 2018. O desenvolvimento de novas moléculas requer o uso de tempo e recursos, pelo que é mais vantajoso o desenvolvimento de moléculas que permitam restabelecer o poder antimicrobiano das moléculas já conhecidas e estudadas.

No caso das bombas de efluxo, uma das vias possíveis consiste na utilização de moléculas que são denominadas de EPIs – Inibidores das Bombas de Efluxo que aorsentam actividade sinergística com as moléculas de antibiótico através da inativação das bombas de efluxo.

As bombas da família resistência, nodulação e divisão celular são uma das famílias em que estão agrupadas os vários tipos de bombas de efluxo, tendo a 1-(1-naftilmetil)-piperazina (NMP) um efeito inibidor nas bombas desta família em *A. baumannii*. A principal bomba de efluxo nesta bactéria Gram-negativa é a AdeABC e os genes que codificam para esta estrutura tripartida são os genes *adeA*, *adeB* e *adeC* cuja expressão se encontra sob o controlo do sistema de regulação de dois componentes AdeRS.

Neste estudo, testámos uma série de análogos de NMP e a sua capacidade para restaurar a atividade da ciprofloxacina em diferentes isolados de *A. Baumannii,* tendo ainda sido avaliada a expressão dos genes que codificam para estas bombas bem como os respectivos genes reguladores. Demonstrou-se que os EPIs que podiam reconstituir a atividade da ciprofloxacina contra *A. baumannii* foram aqueles cuja estrutura apresentava a amina deprotegida: . EPIs 2, 6,7 e 8.

Palavras-chave: *A. baumannii*, Efflux Pump Inhibitors, NMP, antimicrobial resistance, RND family, AdeABC, AdeRS

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First, I want to thank my supervisor, Professor João Perdigão for all the support and direction he offered throughout the realization of this work. I would also like to dedicate a special acknowledgement to my Erasmus supervisor Professor Catherine Mullié-Demailly for teaching me so much and to guide me so wisely through my research, always patient and helpful in every step. To Morgane Choquet, thank you for your time and for trusting me with your precious EPIs. To Emilie and Hugo, thank you for all the bagels and the numerous questions answered in the lab. To my Erasmus friends for letting me know what adventure was like and showing me the world that lives in Amiens. To all my friends and colleagues that were always there during this process. To my Sisters for the unconditional support and for always being by my side during the hard times. To Cata, for being my guidance and calm voice during hard times. To my family for always being proud of me and supportive. To Ana for letting me use her PC, patience, and love. To my parents for being my biggest support, my home and giving the best advices. To my little sister for always cheering me up and making me want to always do by best. To everyone who made these 5 years, *Erasmus* and this work possible, from the bottom of my heart: thank you.

Abbreviations

16SrRNA - 16S ribosomal Ribonucleic acid
A. baumannii – Acinetobacter baumannii
ABAM - Acinetobacter Bumannii Amiens
AFLP - Amplified fragment length polymorphism
AGIR laboratory
AMEs - aminoglycosides-modifying enzymes
ATP - Adenosine triphosphate
BOC - tert-butoxycarbonile
cDNA – complementary deoxyribonucleic acid
CHU Centre Hospitalier Universitarie
DNA - deoxyribonucleic acid
E. coli – Escherichia coli
EARS-Net – European Antimicrobial Resistance Surveillance Network
ECDC – European Centre for Disease Prevention and Control
EP – Efflux Pump
EPI - Efflux Pumps Inhibitors
EUCAST – European Committee on Antimicrobial Susceptibility Testing
EU/EEA – European Union/ European Economic Area
gyrB – DNA gyrase subunit B
HAI - Healthcare-associated Infections (HAIs)
IC1-3 international clonal lineages
MATE -Multidrug and toxic Compound Extrusion
MDR - Multi-Drug Resistance
MDS - Molecular Dynamic Simulation
MGEs - mobile genetic elements
MIC - minimum inhibitory concentration
MLST - Multilocus sequence typing
MSF - Major facilitator Superfamily
NCR - Normalized Calibrated Ratio
NH -amine
NMP - 1-(1-naphthylmethyl)-piperazine
Omp - outer membrane permeability
OprD outer membrane porins such as
PAβN - arginyl β-naphthylamide
PCR - Polymerase chain reactio
PDR - pandrug-resistent bacteria
PEIF – phenotypic efflux inhibition factor
P-gp - P-glycoprotein
PLP2a - Protéine de liaison à la pénicilline or PBP (penicillin binding protein)
QMD - quantum molecular dynamics
Qnr quinolone resistance gene
QRDRs quinolone resistance determining regions
RNA - Ribonucleic acid
RND Resistance-Nodulation-Division
RT-qPCR - Quantitative reverse transcription PCR
SMR - Small Multidrug Resistance
SSTIs skin and soft skin infections
TM - transmembrane helices
US – United States
XDR - Extensive Drug Resistance

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1. Chapter I – A review on efflux pump mediated resistance in Acinetobacter baumannii

1.1 Introduction

The fourth major cause of disease in industrialized countries is considered to be Healthcare-associated Infections (HAIs), increasing the costs, time of stay in hospitals and morbidity/mortality. (1) The number of HAIs has been rising over the last couple of decades, mainly due to the dissemination of multi-drug resistant strains of bacteria.

The Acinetobacter genus consists in a large number of species which can be divided into two complexes: the *Acinetobacter baumannii* complex – where we find the most pathogenic, disease-causing species like *A. baumannii*, *A. pittii* and *A. nosocomiallis*; and a group comprising less pathogenic species – the *Acinetobacter non-baumannii group*.(2)

A. *baumannii* is an aerobic, pleomorphic and non-motile Gram-negative bacillus and a bacterial pathogen associated with hospital infections of the lower respiratory tract in ventilator assisted patients, urinary tract infections; bloodstream infections associated with catheters and in patients with severe underlying diseases. (3–5) The risk factors for infection include advanced age, presence of serious underlying disease, immune suppression, major trauma or burn injuries, invasive procedures, presence of indwelling catheters, mechanical ventilation, extended hospital stay and previous administration of antibiotics. (2)

A. baumannii was first isolated in 1911 by a Dutch microbiologist, Beijerinck, and thought to only have one genus variation (6) which was changed in 1971 by the sub-committee on the Taxonomy of Moraxella and Allied Bacteria that officially acknowledged the genus Acinetobacter. (7)

1.2. Infection and disease

A. baumannii has been referred to as “Iraqibacter” since its emergence in the United States military treatment facilities causing serious problematic infections among soldiers during the Iraq and Afghanistan wars. (8) In this conflict zone, the dry and sandy desert made the wounds of injured soldiers the perfect environment for *A. baumannii* to grow. Globally 4.1% of all skin and soft skin infections (SSTIs) encountered in the injured US combat forces situated on the Persian Gulf were *A. baumannii* related.(6,9)

This opportunistic pathogen has a high incidence among immunocompromised individuals, particularly those who have stayed in the hospital for a prolonged period of time (>90 days).(6,10) If *A. baumannii* is isolated from a hospital environment, there’s a significant risk specially for the patients who are chronically ill and for those who have artificial devices such

as catheters, sutures, ventilators and those with treatments including dialysis or antimicrobial therapy in the past 90 days. (6)

As a pathogen, it specifically targets moist tissues such as mucous membranes or areas of the skin that are exposed – by accident or due to an injury. (6) The most frequently colonize sites are the toe webs, axilla and groin. The skin and soft tissues that are infected with *A. baumannii* present an appearance similar to the skin of an orange, like cellulitis and then it changes to a sandpaper-like appearance. The next stage will eventually be the presence of clear skin, where necrotic tissue and bacteremia can develop. If left untreated this infection will lead to septicemia and death. (6)

1.3. Epidemiology and distribution

The official report on Surveillance of antimicrobial resistance in Europe in 2018 shows that more than half of the Acinetobacter species isolates reported by EU/EEA countries to EARS-Net for 2018 were resistant to at least one of the groups of antibiotics under surveillance such as fluoroquinolones, carbapenems and aminoglycosides. The *Acinetobacter baumannii* species is the one that shows the biggest inter-country variation in resistance percentages for 2018, with higher numbers for the Baltic countries, southern and south-eastern Europe.(2)
In the context of Europe (EU/EEA), Portugal was one of the countries with the highest (25% to 50%) percentages of invasive isolates with resistance to fluroquinolones in 2018 (Figure 1). Concerning aminoglycosides and carbapenems, the percentage drops to a range of 10 to 25 %. (2)

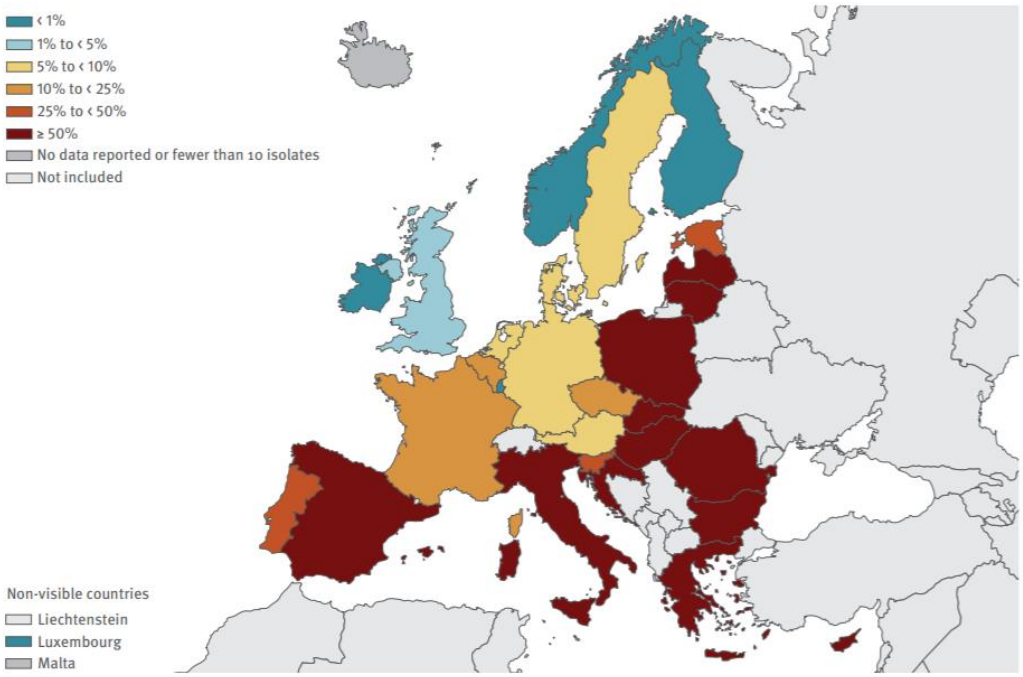

Figure 1- Acinetobacter spp. Percentage (%) of invasive isolates with resistance to fluoroquinolones, by country, EU/EEA countries, 2018 (Source: ECDC Report- Surveillance of antimicrobial resistance in Europe, 2018)

The *A*. *baumannii* population structure is clonal in nature. Three of eight described international clonal lineages (IC1-3) are dominant in Europe and have been found in nearly all European countries.(11)

A limited number of widespread clones are the ones responsible for hospital outbreaks in many countries. Strain typing by a variety of techniques has shown genotypic diversity within *A. baumannii*. (12,13) There were two major groups of epidemic strains – clone I and II delineated after comparison based on cell envelope protein profiling, ribotyping and AFLP genomic fingerprinting of epidemic and non-epidemic *A. baumannii* strains from geographically distinct European hospitals. Using the same technics, a third pan-european outbreak clone – clone III was determined. These European clones are now also called 'international clones' as they have spread all across the world because of the multidrug resistance often associated with isolates that belong to these international clones.(14,15)

Multilocus sequence typing (MLST) is the current standard for investigating the population structure of bacterial species. This method can discriminate different strains of A. *baumannii* but this technique is limited – it can only be performed in a small number of genes and does not read the majority of the genes in the bacterial genome.(16)

1.4. Antibiotics resistance and Efflux Pumps

A. baumannii has the ability to disseminate and survive in the environment and can last longer on dry surfaces than other bacteria. (17)

These bacteria have the ability to form biofilms. Biofilms are aggregates of microbial cells that are surrounded by self-produced matrices on the surfaces, either biotic or abiotic. Biofilms demonstrate greater protection against antibiotics, host immune defense and adverse environmental conditions than the planctonic cells.(8)

Some *A. baumannii* strains have developed Multi-Drug Resistance (MDR) or Extensive Drug Resistance (XDR). Such bacteria are resistant to a wide range of antibiotics like fluoroquinolones, macrolides, trimethoprim, β-lactams, tetracyclines, aminoglycosides, and chloramphenicol. (3–6)

MDR can be defined as non-susceptibility to at least one agent in three or more antimicrobial categories. XDR is defined as non-susceptibility to at least one agent in all but two or fewer antimicrobial categories. Another term is pandrug-resistent bacteria (PDR) and it is defined as non-susceptibility to all agents in all antimicrobial categories. (18)

To better combat MDR pathogens like *A. baumannii,* it is necessary to define the molecular mechanisms underlying these antibiotic resistances. In non-fermenting Gram-negative bacilli such as *A. baumannii*, the resistance can be acquired by various mechanisms:

Four main categories of resistance mechanisms are usually described : (i) production of antibiotic-inactivating enzymes such as β-lactamases, (ii) antibiotic-target modifications such as the production of PLP2a or mutated DNA-gyrase, (iii) decreased permeability through the loss of outer membrane porins such as OprD and (iv) overexpression of efflux pumps (Fig. 2).(19)

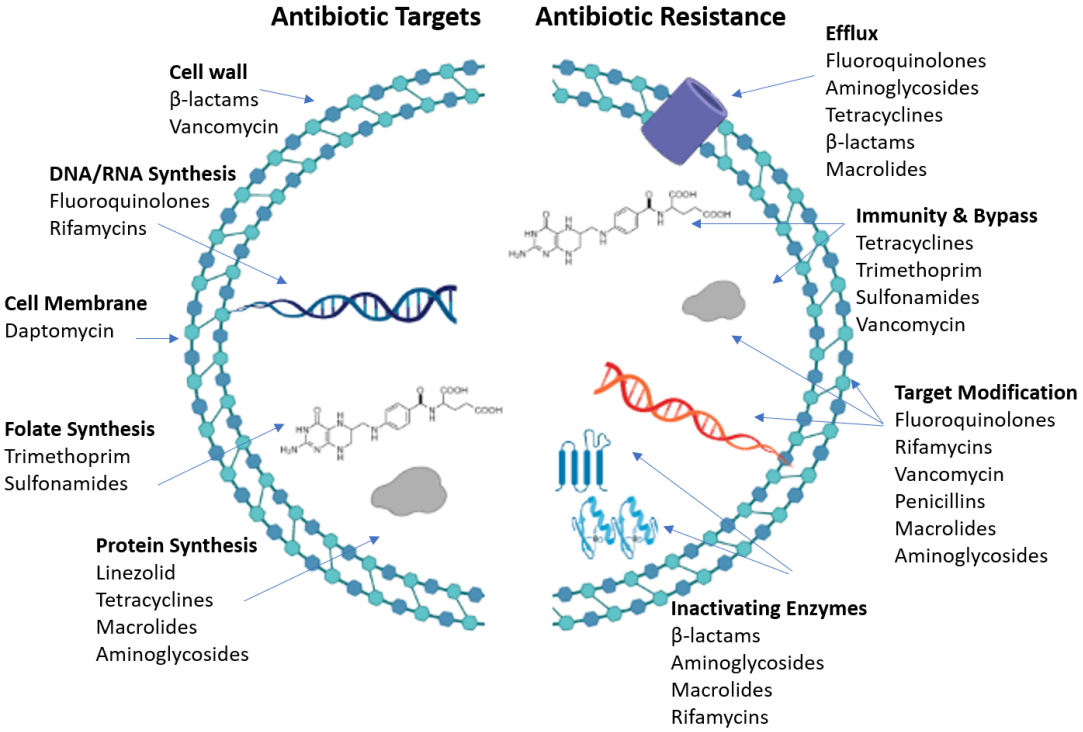

Figure 2 - Antibiotic targets and bacterial resistance mechanisms (Based on: Gerard D Wright - http://www.biomedcentral.com/content/figures/1741-7007-8-123-1-l.jpg Antibiotic targets and mechanisms of resistance. BMC Biology 2010 8:123 doi:10.1186/1741-7007-8-123)

The previous stated mechanisms are the intrinsic ones, that naturally occur. Acquired mechanisms involve mutations in genes targeted by the antibiotic and the transfer of resistance determinants borne on plasmids, bacteriophages, transposons and other mobile genetic elements (MGEs).

Horizontal transfers of MGEs carrying resistance genes, most notably plasmid-encoding beta-lactamases, aminoglycosides-modifying enzymes (AMEs), or non-enzymatic mechanisms such as Qnr for fluoroquinolone resistance in Enterobacteriaceae is a main mean of acquiring antibiotic-resistances. These plasmids can indeed be enough to confer a multidrug resistance phenotype to the recipient strain.(19,20)

In the case of resistance to fluoroquinolones, resistance normally involves chromosomal mutations in the quinolone resistance determining regions (QRDRs) of either one or both of the DNA gyrase or topoisomerase IV (parC) genes that represent the primary and secondary intracellular targets for this class of antibiotics. (21) However, posterior studies

confirmed the existence of a non-specific efflux pump (EP) mechanism leading to quinolone resistance in *A. baumannii.*(22) In Gram-negative bacteria such as *A. baumannii*, the outer membrane limits what enters the cell and the efflux pumps actively export multiple, structurally-distinct classes of molecules. These efflux transporters are expressed in cells as a way of protecting them from toxic products and organic chemicals. Over expression of these pumps can result in an increased expelling rate of the antimicrobials out of the cell. (23)

The main five families of efflux pumps in *A. baumannii* are the ATP - binding cassette (ABC) transporters; the Small Multidrug Resistance (SMR) family ; the Major facilitator Superfamily (MSF); Multidrug and toxic Compound Extrusion (MATE) family and the Resistance-Nodulation-Division (RND) one. (21) The major RND efflux pump in *A. baumannii* is called AdeABC.(24) To date, three RND system pumps, two MFS system pumps, and one member each of the MATE and SMR families of pumps have been reported to be involved in antibiotic efflux in *A. baumannii*. (25)

Members of the RND family are proton antiporters, using the proton gradient to power efflux, exchanging one H^+ ion for one drug molecule.(26) The members of the RND family comprise three proteins and their mechanism is based on the direct extrusion of substrates outside the bacterial cell. (Figure 3) The encoding genes of these tripartite structures are organized in operons located on the bacterial chromosome. The structural genes *adeA*, *adeB* and *adeC* are co-transcribed, contiguous and directly orientated. (27) (21) They are preceded by adeR and adeS regulation genes that are transcribed in the opposite direction and their products are very similar to the proteins of other species two-component regulatory system, inferring they work as such.

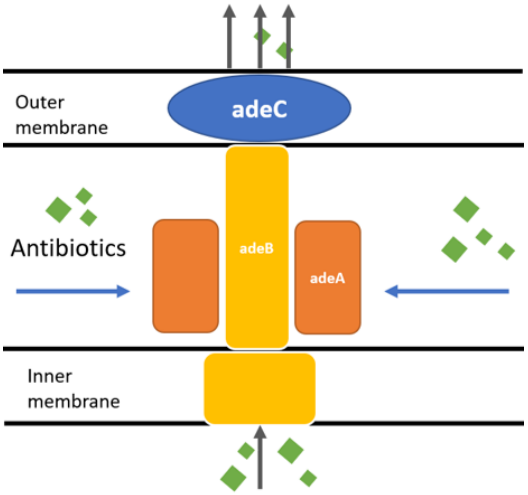

Figure 3 - AdeABC efflux pump in the cell wall of *A. baumannii*. (Based on: New Microbes New Infect., 2019, 30: 100549)

A two-component regulation system such as AdeRS exists for each of the three major RND EPs - AdeABC, AdeIJK and AdeFGH. AdeRS is responsible for responding to environmental conditions and constituted by a sensor histidine protein kinase (adeS) and by its cognate response regulator (adeR). It was shown that inactivation of *adeS* or *adeR* leads to a restoration of the susceptibility to aminoglycosides in *A. baumannii* strains and to other substrates for the pump in resulting mutants. (21,26) The AdeIJK system – another member of the RND family – appears to be present in all A. *baumannii* strains and is known to pump out a broad range of antibiotics, including β-lactams, chloramphenicol, tetracyclines, and erythromycin. A gene inactivation study showed little evidence that the third system, AdeFGH, contributes to resistance; its overexpression would be necessary to see its functions. (25)

The first described substrates for *A. baumannii* pumps were aminoglycosides and fluoroquinolones. It is now known that AdeABC plays a role in controlling resistance to a number of antibiotics including aminoglycosides, β-lactams, tetracyclines, erythromycin and chloramphenicol. (28) In previous studies, an artificial overexpression of AdeABC in modified strains led to an increase in the minimum inhibitory concentration (MIC) for the majority of antibiotics.(29) More globally, the overexpression of most EPIs in *A. baumannii* leads to an increase in the MIC of ciprofloxacin as previously stated. (5,30) Ciprofloxacin is a second generation fluoroquinolone with a wide range of action. (31)

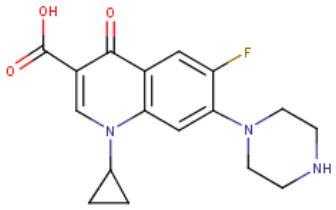

Figure 4 – Chemical structure of ciprofloxacin

The efflux pumps play a complex role in bacteria beyond their role in drug resistance – they also play a role in bile tolerance in enteric bacteria, leading to colonization, increase virulence, biofilm secretion and bacterial survival in the host.(32)

1.5. Efflux Pumps Inhibitors

The efflux pumps are one of the major causes of antibiotics resistance and there are different approaches when it comes to inhibiting their activity: i) by modification of the chemical structure of the antibiotics to decrease their binding affinity to the transporter cavities; ii) by using permeabilizers to increase the concentration of the antibiotic in the bacterial cell; iii) by downregulating efflux pump gene expression hence decreasing the number of active efflux pumps; iv) by destroying the source of energy of the drug transporter; v) by blocking the

functional assembly of the components of efflux systems; vi) by designing inhibitors that bind covalently to the cavities where the substrate binds or that block the channel of antibiotic transporter pumps; vii) by applying a decoy substrate as a competitive inhibitor for antibiotic transport inside the pump. (33)

Therefore, one path of studies about the search to decrease the resistance/ increase the susceptibility to antibiotics is Efflux Pumps Inhibitors (EPI's). Such inhibitors are capable of successfully interfering with membrane-bound efflux pumps that systematically work together to remove toxic metabolites to promote the survival of the bacterial cell. (33)

EPIs are molecules that synergize with currently used antibiotics, restore their efficacy, reduce the incidence of drug-resistant strains as well as, reduce the ability of pathogens to infect the host as the inhibition of efflux attenuates some of the bacterium virulence factors, and prevent the development of highly drug resistant biofilms. (34)

There are different classes of EPIs. Some derivate from natural sources such as plant alkaloids, phenolic metabolites or polyacylated neohesperidosides. Some are fermentation products such as heterocyclic macrocycles while other EPIs are from synthetic sources such as peptidomimetic compounds, multi-cyclic compounds, phenothiazines and thioxanthene derivatives, quinoline derivatives, arylpiperazine derivatives, pyridopyrimidine derivatives, pyranopyridine derivatives. (33)

Investigations towards efflux inhibitors were originally initiated around the same time as the discovery of the first efflux transporter, P-glycoprotein (P-gp) in 1976.(33)

However, scientists are still struggling to design and develop new EPIs because many problems with pharmacokinetics and toxicity have arisen. The lack of computational, biochemical and structural methods has also been restricting the discovery of new and more efficient EPIs, since the scientific community is still missing information about the binding sites and the details about the mechanism of drugs transportation in EPs. (35)

Inhibitors can also be designed to interact with genes that encode for efflux pumps to disrupt pump assembly, obstruct the channel through the pump or cause the collapsing of the energy-dependent processes that some bacteria rely on to pump out toxic molecules. (33)

For a molecule to be qualified as an EPI, a compound must be able to satisfy the following criteria: it must potentiate the activity of antibiotics to which a strain has developed resistance as a result of the expression/overexpression of an efflux pump; it cannot have an effect on sensitive strains lacking the efflux pump; it must not reduce the MIC of antibiotics which are not effluxed; it must increase the level of accumulation and decrease the level of extrusion of compounds which are substrates of the efflux pump; it must not permeabilize the outer membrane and it must not affect the proton gradient across the inner membrane.(34,36)

There are several tests that need to be done to assure these criteria are met, such as antibacterial assays and checkboard assays to make sure the EPI synergizes with antibiotics. Other tests can also be performed such as Substrate transport, Nitrocefine hydrolysis and $DiOC_2$(3) fluorescence to prove the effect is specific to drug efflux pump inhibition and does not interfere with membranes. Moreover, tests like RT-qPCR, Cross-linking & co-purification, substrate transport and measure of Omp (outer membrane permeability) conductance can be added to further explain the mechanism of action. (34,37,38)

Earlier experiments have highlighted the causal connection between adeB overexpression and responsiveness to EPIs in various *A. baumannii* strains. Since reductions in various antibiotic MICs after the addition of EPIs were verified in isolates with the presence of the AdeB efflux pumps, it is likely that EPIs increasing susceptibility to the antibiotics may have affinity sites similar to those for these antibiotics inside the efflux pump.(39)

For example, N-heterocyclic compounds including phenyl piperazine derivatives have shown activity against *A. baumannii* strains displaying efflux-mediated resistance to antibiotics (33) and 1-(1-naphthylmethyl)-piperazine (NMP) (Figure 5) increased the diameters zone of antibiotics (levofloxacin and ciprofloxacin) in the isolates of *A. baumannii* in previous studies. (30)

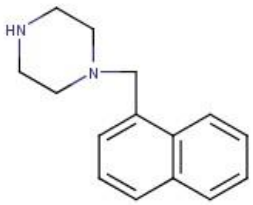

Figure 5 - Figure 5. Structure of 1-(1-naphthylmethyl)-piperazine

NMP also inhibits the function of RND-family efflux systems in a number of Gram-negative bacteria.(40) A study from 2013 concluded that responsiveness of the isolates to NMP was due to the inhibition of functional RND-type drug efflux, particularly the AdeB pump.(41)

Several studies have been conducted to identify putative EPI target residues in order to better understand mechanisms of pump inhibition. The best-known mechanism of inhibition was studied for AcrB, the pump part of the major EP of *Escherichia coli* which is homolog of those found in *A. baumannii*. (42)

AcrB is part of the tripartite complex AcrAB-TolC that bridges the periplasmic space and the inner and outer membrane. Twelve transmembrane helices (TMs) anchor the AcrB pump within the inner membrane. A periplasmic domain is built from two large periplasmic

loops comprising almost two-thirds of the protein. Topological modeling of RND proteins reveals two large periplasmic loops of approximately 300 amino acids each between TMDs 1 and 2 and TMs 7 and 8, and this accounts for their large sizes.(43) The determinants of differences in substrate specificity have been localized within the two large periplasmic loops between TM1 and TM2 and between TM7 and TM8. (42)

The promiscuity in substrate recognition is thought to be enabled by a large phenylalanine-rich deep (distal) binding pocket within the periplasmic pore domain of the protein. Compounds are thought to bind within distinct areas of this flexible and predominantly hydrophobic cavity. In addition, recent studies have discovered an access (proximal) pocket separated from the distal binding pocket by a so-called “switch-loop.” (42)

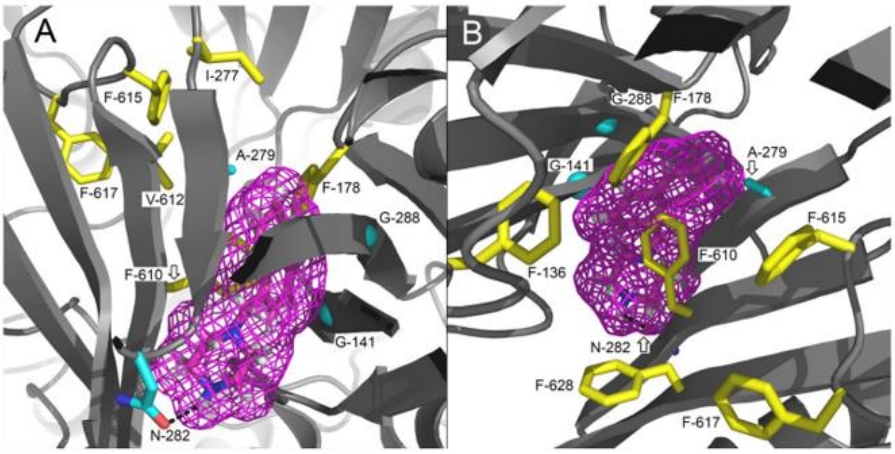

Figure 6 – Two views of suggested NMP binding within the AcrB binding state promoter. Binding pocket side chain are in yellow, residues detected from random mutagenesis are cyan sticks, and glycines are cyan spheres. The NMP molecules is in magenta. The dotted black line indicates a putative hydrogen bond. Oxyge is in red, nitrogen is blue and hydrogen is white. **Source** : Schuster et. al Random Mutagenesis of the Multidrug Transporter AcrB from Escherichia coli for Identification of Putative Target Residues of Efflux Pump Inhibitors, 2014.

1.6. Future perspectives

There is no doubt EPIs could play an important role and offer advantage as therapeutic agents, especially when the development of antibacterial therapy has almost completely dried out. Using EPIs is a time and money saving strategy since it allows the reuse of already marketed antibiotics. The importance of EPIs falls under the capacity they have on reversing antibiotic resistance, which economically translates in saving money by the large production of already optimized and stockpiled antibiotics. The other advantage of the use of EPIs is the very low frequency of generation of resistant mutants. (32)

X-ray crystallography and Molecular Dynamic Simulation (MDS) and advanced three-dimensional structure resolution along with molecular modeling can aid the identification of pharmacophores able to link with a specific binding site located on the efflux pump. (33,44)

Understanding the mechanisms of inhibition of efflux pumps by running long-term (micro seconds long) molecular dynamics (MD) simulations and quantum molecular dynamics (QMD) simulations can have a great impact in discovering new EPIs. (33)

It is urgent we learn how to develop the early diagnosis of the MDR phenotype by using techniques as real-time PCR and biochemical assays to identify clear targets to block the efflux mechanism. It is also fundamental to learn to select the molecules that present a high inhibitory potential with the minimum possible of adverse effects, which means having no toxic effect on eukaryotic pumps. Advantages and drawbacks of the characteristics of the two different types of EPIs – the ones that inactivate all pumps transporting antibiotics or the ones more specific that collapse the transport of only one drug family – must be analyzed.(45)

The above-mentioned techniques will have to suffice in the findings for a new and optimized design of high affinity and activity compounds.

2. Chapter 2 – Efflux pump inhibitory activity of novel NMP derivatives against *Acinetobacter baumannii* and ciprofloxacin resistance

2.1. Introduction

A. baumannii is an opportunistic pathogen that can lead to serious nosocomial infections. This bacteria has the ability to adhere to surfaces, form biofilms and presents a pattern of resistance to majority of the antibiotics. (3,4)

One of its resistance mechanisms is the active expulsion of antibiotic molecules, through efflux pumps present on *A. baumannii* membrane.(34) Ciprofloxacin is one of the antibiotics that is a good substrate for of these pumps. (22)

When in presence of an EPI efficient on a pump involved in CIP efflux, a reduction of the MIC of ciprofloxacin is witnessed.(34)

One well-known EPI is the 1-(1-naphthylmethyl)-piperazine (NMP) (Figure 5) It is a phenylpiperazine, a naphthyl derivative (46) that reverses the resistance to fluoroquinolones by blocking RND efflux pumps in bacteria such as *A. baumannii*. (47)

NMP is a N-heterocyclic compound that has previously shown activity in *A. baumannii* and *E. coli*.(33) An interesting fact is that NMP and phenylalanyline arginyl β-naphthylamide (PAβN) – another EPI – showed effects on different antibiotics and different species of bacteria which suggests different mechanisms of action. In *E. coli*, the NMP inhibits the AcrB-TolC efflux pump by interfering with its functional assembly and movement of the G-loop that is responsible for the extrusion of some substrates. (33)

Analysis of the relationship between the structure of phenylpiperazines and MDR reversal activity suggests that elongation of the spacer between the benzene ring and the piperazine ring would enhance potency. (48)

In our study we tested analogs of NMP to see if they could improve the inhibitory properties of ciprofloxacin in different clinical strains *A. baumannii*. In previous studies, it was determined that NMP was the most efficient inhibitor in comparison with others EPIs tested for *A. baumannii*. Therefore, we studied the effect of NMP along with 8 (eight) of its analogs EPI 1 to 8 on a series of 15 *A. baumannii* strains previously identified as possessing an efflux-mediated resistance to ciprofloxacin.

As previously stated a study from 2013 concluded a correlation between the inhibition of a RND pump, the AdeB and the responsiveness of the isolates to NMP.(41)

This study was conducted to evaluate the effects of ceftazidime, imipenem, meropenem, levofloxacin, ciprofloxacin and gentamicin on 42 clinical *A. baumannii* isolates. After testing the ciprofloxacin MICs of clinical *A. baumannii* isolates for some of the isolates, it was found that there was a significant decrease or absence on the bacterial growth in the

presence of NMP. In this study, the antibiotics most affected by the presence of NMP were levofloxacin and ciprofloxacin, which indicated the inhibition of efflux pump by NMP.(40)

A reference strain showed a susceptibility pattern after addition of NMP different from the one seen with PAβN. At a concentration of 100 mg/L, NMP reduced by a 4-fold or more the MICs of many test drugs, including ciprofloxacin, except aminoglycosides. The PaβN at the same concentration only reduced the MICs of clarithromycin, rifampicin and linezolid.(24)

As reported previously for clinical isolates, (49) PAβN was virtually ineffective in reducing the MIC of fluoroquinolones but NMP affected more agents than PAβN, in particular linezolid, chloramphenicol and tetracycline.

The active site of NMP is the amine NH. Therefore, to ascertain that the derivatives worked in a similar fashion, putative EPIs were tested with and without the terminal amine bonded to tert-butoxycarbonile (BOC).

2.2. Methods and materials

2.2.1 Study population and bacterial isolates

This study initially included 15 *A. baumannii* strains that have been isolated from patients from the Centre Hospitalier Universitarie (CHU) Amiens-Picardie. All strains were collected from patients of the hospital. They were kept frozen at 20°C on cryobeads (VWR, France) until use. The strains were named ABAM (Acinetobacter Bumannii AMiens) and a number. The list of strains is presented in Table 1.

Table 1 Isolation dates and sites of Acinetobacter baumannii clinical strains used in this study along with their NMP phenotypic efflux factor

Strain name	Isolation date	Isolation site/Clinical origin	NMP efflux factor
ABAM 7	08-2016	Rectal swab	2
ABAM 9	04-2016	Rectal swab	4
ABAM 14	10-2016	Rectal swab	4
ABAM 16	05-2016	Rectal swab	4
ABAM 26	08-2016	Rectal swab	8
ABAM 28	02-2014	Rectal swab	8
ABAM 30	01-2015	Rectal swab	2
ABAM 35	1995	Unknown	0
ABAM 48	10-1997	Bronchial aspiration	0
ABAM 57	01-1998	Cervical fluid	4
ABAM 65	03-2017	Bronchial aspiration	4
ABAM 77	08-2016	Rectal swab	8
ABAM 97	11-2017	Rectal swab	4
ABAM 118	10-2016	PTP	0
ABAM 132	06-2017	Urine	4

2.2.2. Phenotypic characterization of efflux

NMP and ciprofloxacin were purchased from SIGMA – Aldrich (Lyon, France). Ciprofloxacin was the chosen test antibiotic because, as stated above, it has previously been shown to be effluxed by numerous A. *baumannii* pumps.

New EPIs were synthesized in AGIR laboratory by Morgane CHOQUET, a PhD student working on this topic. Their structures and identities are presented in Figure 7.

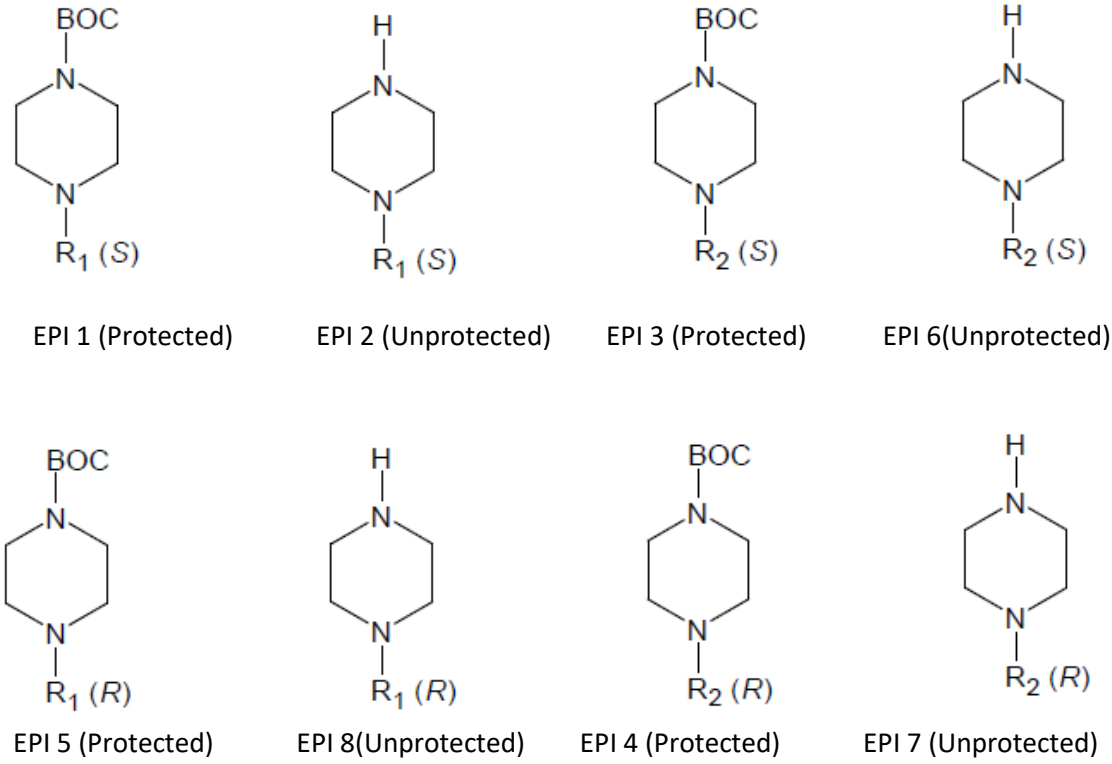

Figure 7 - Chemical structures of the tested EPIs

All bacterial isolates were incubated at 37ºC over a period of 18 to 24 hours after being spread on sheep blood agar plates (Biomérieux, Marcy l'Etoile, France) from frozen stocks.

The bacterial suspensions made from these isolates were adjusted to a turbidity equal to 0.5 McFarland in 0,9% NaCl and diluted in a proportion of 1:10 to generate the inoculums. MIC determinations were carried out in 96-well plates for ciprofloxacin, over a concentration range of 0.25 to 512 µg/mL and for NMP analogs alone over a concentration range of 0,1 to 200 µg/mL.

The effect of the combination of ciprofloxacin with the various EPIs was tested at a fixed EPI concentration, chosen below the EPI's own MIC for a given strain (Table 3).

Accordingly, all protected EPIs tested in a combination with ciprofloxacin were added at a fixed final concentration in the wells of 50 µg/mL in the inhibition tests. Unprotected EPIs were either added at a concentration of 50 µg/mL (strains ABAM7, 14, 26 and 28) or 10 µg/mL (strains ABAM 9, 16, 30, 35, 48, 57, 65, 77, 97, 118 and 132).

The culture medium used was Mueller-Hinton broth (Merck, France). The microplates were incubated at 35°C for 18 to 24 h. The MIC was defined as the lowest concentration for which no visible bacterial growth was observed. The lay-out of a typical plate is presented in Figure 6.

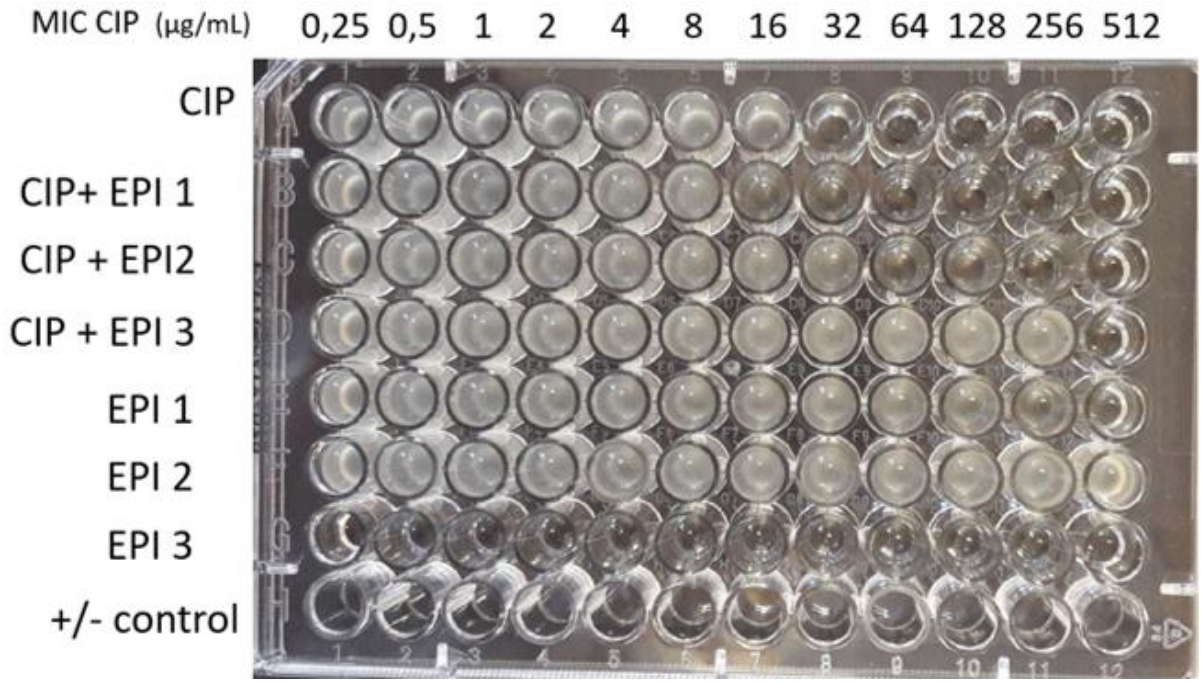

Figure 8 – Lay out of a plate test

The phenotypic inhibition efflux factor (Tables 3 and 4) was calculated by dividing the ciprofloxacin MIC without each EPI by the one with each EPI for each strain. An efflux-based resistance mechanism is suspected when this phenotypic efflux factor is of at least 4. (50)

2.2.3 Genotypic characterization of efflux

a) RNA extraction

An overnight culture of each of 15 strains studied above and of a reference strain susceptible to ciprofloxacin (calibrator) was subjected to RNA extraction according to the RNeasy extraction kit instructions (Qiagen, Courtaboeuf, France). Main steps included enzymatic cell wall lysis and precipitation/purification of nucleic acids, all of which done in the presence of an RNA-protecting agent (RNAprotect, Qiagen). RNA extracts were immediately stored at -20°C until further use. The amount of RNA extracted was quantified spectrophotometrically (Nanodrop, ThermoFisher Scientific, France). RNA extraction was carried out twice (biological replicate) on each of the 15 strains tested.

b) Reverse transcription

QuantiTect Reverse Transcription KIT (Qiagen, Courtaboeuf, France) was used according to the manufacturer's recommendations. Outside of incubations, all reactions were carried out on ice to preserve the RNA extract.

Briefly, 1µg of template RNA under an adjusted volume of 12 µL was mixed with 2 µL of gDNA Wipeout Buffer and incubated 2 min at 42°C (Verity thermocycler, Applied Biosystems, France) to remove genomic DNA from the reaction. Six µL of reverse transcription mix (comprising Quantiscript Reverse Transcriptase, Quantiscript RT buffer and RT primer mix) were added to each sample to obtain a final reaction volume of 20 µL. The cDNA was retrieved after a 30-minute incubation at 42°C. The reaction was then inactivated by incubation at 95°C for 3 minutes. The resulting cDNAs were diluted 20-fold and stored at -20°C until use.

c) Quantitative PCR

The quantitative polymerase chain reaction (qPCR) for each one of the samples obtained above was performed in duplicate (technical replicates) on a Light Cycler 480 apparatus (Roche, Boulogne-Billancourt, France).
Amplification primers used in this study are presented in Table 2.

Table 2 - Amplification primers and their characteristics.

Target gene	Function	Sequence (5'-3') FWD/REV	Amplification product size
recA	Genetic transformation	TGAAGGCACATGTACCACCAG ACCAAAAGGCCGTATTATCG	109
gyrB	DNA gyrase	TTCACAAACAACATTCCACAAAAAG GCATCATCACCAGTCACATTCA	139
rpoB	RNA polymerase β subunit	βGAGTCTAATGGCGGTGGTTC ATTGCTTCATCTGCTGGTTG	109
16S	16S rRNA	CGTAAGGGCCATGATGACTT CAGCTCGTGTCGTGAGATGG	150
adeB	RND	AACGGACGACCATCTTTGAGTATT CAGTTGTTCCATTTCACGCATT	83
adeG	RND	ATCGCGTAGTCACCAGAACC CGTAACTATGCGGTGCTCAA	90
adeJ	RND	TGCGTATCTGGCTTGATCCA CACCTAACTGACCTACGGCAACT	110
adeR	RND	TGGGTTAAAAGGCTTCACCA ACGCCAAAAAGCTCAGACTC	114
adeS	RND	GCATTTTTGACGGAAACCTC TTAGTCACGGCGACCTCTCT	120
abeM	MATE	TGCCAATTGGTTTAGCTGTG TACTTGGTGTGCGGCAATAA	100
abaQ	MFS	ATCCCAAATGGACCGACATA TTGGCTGTAGTTGCGTTCTG	148
amvA	MFS	ACGATTGATGCAACGGTAATGC TCCATAAAAGCTGATTGGCAGT	82

craA	MFS	TGTGCAACTCTTTCCTGCATT GCAATGATTGAGCTTGTACGCTAT	140

Reactions were carried out in 384–well microplates under a final volume of 10 µL comprising 2.5 µL of 1/20 diluted template cDNA, 1 µl of each primer at 0.5 µM, 5 µL of Quantitect SYBR Green PCR Master Mix (Qiagen, France) and 0.5 µL RNase/DNase free water. Each run consisted in several *A. baumannii* strains, including the wild-type strain (susceptible to ciprofloxacin), that would serve as calibrator. Four house-keeping genes were investigated to serve as reference gene for the normalization of results.

The relative expression between the target and reference genes, was calculated using the formula $\frac{(E_{Goi})^{\Delta CTGoi}}{(E_{Ref})^{(\Delta CT\ Ref)}}$, where E stands for the PCR efficiency factor, Goi for gene of interest and ref for reference. Results were subsequently expressed as Normalized Calibrated Ratios (NCRs).

2.2.4. Reverse Transcription and PCR assays

The *recA*, *gyrB*, *rpoB* and *16SrRNA* genes have previously been used as housekeeping genes for RT-qPCR analysis on *A. baumannii*. Their stability was therefore tested on our set of 16 strains using the RefFinder calculator (available at https://www.heartcure.com.au/reffinder/?type=reference) in order to choose a suitable reference gene to normalize gene expression and allow for a sound comparison. The *rpoB* gene was the most stably expressed in our panel of strains and all gene expressions were therefore normalized against it.

Target genes comprised the components of the 3 main RND EPs of *A. baumannii* as well as their regulator genes *adeR* and *adeS*. Several other EPs (belonging to MFS and MATE families) previously implicated in antibiotic resistances were also included in the study (Table 2).

2.3. Results and Discussion

2.3.1.Phenotypic characterization of efflux

The MICs for EPIs and ciprofloxacin with and without EPIs are presented in tables 3 and 4.

This step allowed us to phenotypically characterization whether our EPIs were efficient or not.

Table 3 - MICs (µg.mL^{-1}) of EPIs tested in this study.

Strain	R1 (*S*) derivatives		R2 (*S*) derivatives		R1 (*R*) derivatives		R2 (*R*) derivatives	
	Protected (EPI 1)	Unprotected (EPI 2)	Protected (EPI 3)	Unprotected (EPI 6)	Protected (EPI 5)	Unprotected (EPI 8)	Protected (EPI 4)	Unprotected (EPI 7)
ABAM 7	> 200	100	> 200	100	> 200	100	> 200	100
ABAM 9	> 200	50	> 200	50	> 200	50	> 200	25
ABAM 14	> 200	100	> 200	100	> 200	100	> 200	100
ABAM 16	> 200	25	> 200	25	> 200	> 200	> 200	25
ABAM 26	> 200	100	> 200	100	> 200	100	> 200	100
ABAM 28	> 200	100	> 200	100	> 200	100	> 200	100
ABAM 30	> 200	50	> 200	50	> 200	> 200	> 200	25
ABAM 35	> 200	50	> 200	50	> 200	100	> 200	25
ABAM 48	> 200	50	> 200	50	> 200	50	> 200	25
ABAM 57	> 200	50	> 200	25	> 200	25	> 200	25
ABAM 65	> 200	50	> 200	50	> 200	25	> 200	25
ABAM 77	> 200	50	> 200	50	> 200	50	> 200	50
ABAM 97	> 200	50	> 200	50	> 200	50	> 200	25
ABAM 118	> 200	50	> 200	50	> 200	50	> 200	50

ABAM 132	> 200	50	> 200	50	> 200	100	> 200	25

From these results, we can already see that unprotected NMP analogs had lower MIC concentrations than their protected counterparts. On some strains, some of the unprotected EPIs displayed MICs within the range of antibiotic effect (e.g. EPIs 7 & 8 on strains ABAM 48 and, to a lesser extent, ABAM 77). Therefore, the fixed concentration at which EPIs were tested in addition to ciprofloxacin was adjusted below their respective MICs.

The phenotypic efflux inhibition factor (PEIF) (Table 4) was calculated by dividing the ciprofloxacin MIC obtained without each EPI by the one with each EPI for each strain. As mentioned above, an efflux-based resistance mechanism is suspected when this phenotypic efflux inhibition factor is of at least 4.(51)

Table 4 - Ciprofloxacin MICs without/with addition of EPIs and calculated phenotypic efflux factor

Strain	**CIP MIC**	**MIC CIP+EPI (PEIF)**							
		R1 (*S*) derivatives		R2 (*S*) derivatives		R1 (*R*) derivatives		R2 (*R*) derivatives	
		Protected (EPI 1)	Unprotected (EPI 2)	Protected (EPI 3)	Unprotected (EPI 6)	Protected (EPI 5)	Unprotected (EPI 8)	Protected (EPI 4)	Unprotected (EPI 7)
ABAM 7	128[a]	512(0.25)[b]	**8(16)[c]**	512(0.25)[b]	**16(8)**	512(0.25)[b]	**0.25(512)**	512(0.25)[b]	**16(8)**
ABAM 9	256	512(0.5)	128(2)	512(0.5)	128(2)	512(0.5)	128(2)	512(0.5)	128(2)
ABAM 14	64	512(0.125)	**0,25(256)**	512(0.125)	**1(64)**	512(0.125)	**0.25(256)**	512(0.125)	**0.25(256)**
ABAM 16	256	512(0.5)	**64(4)**	512(0.5)	**64(4)**	512/(0.5)	**32(8)**	512(0.5)	**16(16)**
ABAM 26	64	512(0.125)	**0.25(256)**	512(0.125)	**2(32)**	512(0.125)	**0.25(256)**	512(0.125)	**0.25(256)**
ABAM 28	64	512(0.125)	**0.25(256)**	512(0.125)	**2/(32)**	512(0.125)	**0.25(256)**	512(0.125)	**0.25(256)**
ABAM 30	256	512(0.5)	**64(4)**	512(0.5)	**64(4)**	512(0.5)	**32(8)**	512(0.5)	**32(8)**
ABAM 35	128	512(0.25)	64(2)	512(0.25)	64(2)	512(0.25)	128(1)	512(0.25)	128(1)
ABAM 48	32	512(0.0625)	16(2)	512(0.0625)	**8(4)**	512(0.0625)	**0.25(128)**	512(0.0625)	**4(8)**
ABAM 57	128	512(0.25)	64(2)	512(0.25)	64(2)	512(0.25)	**32(4)**	512(0.25)	**32(4)**
ABAM 65	256	512(0.5)	**64(4)**	512(0.5)	**64(4)**	512(0.5)	**32(8)**	512(0.5)	**32(8)**

ABAM 77	128	512(0.25)	**16(8)**	512(0.125)	**16(8)**	512(0.125)	**2(64)**	512(0.125)	**8(32)**
ABAM 97	256	512(0.5)	**64(4)**	512(0.5)	**64(4)**	512(0.5)	**64(4)**	512(0.5)	**64(4)**
ABAM 118	256	512(0.5)	128(2)	512(0.5)	**64(4)**	512(0.5)	**64(4)**	512(0.5)	**32(8)**
ABAM 132	256	512(0.5)	**64(4)**	512(0.5)	**64(4)**	512(0.5)	**32(8)**	512(0.5)	**32(8)**

[a]: MIC results expressed as µg.mL^{-1}

[b]: MIC (µg.mL^{-1})/Phenotypic efflux inhibition factor

[c]: Efflux factor equal or superior to 4 are in bolt

These results show that all of the EPIs bearing the amine function bound to the protective group were not effective on these strains previously identified as having an efflux mechanism of resistance to ciprofloxacin, whatever their structure (R_1 or R_2) or their stereochemical configuration ((*S*) or (*R*)). On the contrary, EPIs with the free amine function displayed better efflux inhibiting results. This proves that the free amine function is indeed mandatory to block *A. baumannii* efflux pumps.

Three EPIs (EPI 2, EPI 7 and EPI) were able to reduce the CIP MIC for three strains (ABAM14, ABAM26 and ABAM28) to levels below 1.0 µg/mL, the EUCAST resistance breakpoint for *A. baumannii*. (52) Further tests using a CIP concentration spectrum spanning across lower concentrations are nevertheless needed to verify if the final MICs fall bellow the 0.01 µg/mL susceptibility breakpoint as defined for EUCAST for A. *baumannii*.

Considering all EPIs tested, a phenotypic efflux inhibition factor of 4 was not met for only 2 strains out of 15 (ABAM 9 and ABAM 35, Table 4). This might be due to the overexpression of an EP not inhibited by the EPIs tested here. It would therefore be interesting to see in the genotypic analysis of EP expression if these two strains overexpress different EPs than the other strains. .However, it also has to be mentioned that these two strains did not reach a PEIF of 4 for the parent drug NMP but were included in this experiment because the addition of PAβN in a previous experiment induced a reduction in CIP MICs of at least 4-fold, hence pointing towards an existing efflux in those strains. (data not shown)

Several other studies showed the same results and the ones where NMP and other EPIs where tested, all showed a co-relation between the 4-fold reduction of MIC in the presence of NMP for resistant strains to ciprofloxacin. (24)

Overall, EPIs with a (*R*) stereochemical configuration displayed slightly better phenotypic efflux inhibition factors than their (*S*) counterparts. As for a better activity linked to an R_1 or R_2 structure, no clear-cut pattern could be seen. R_1 sometimes gave slightly better results than R_2 (ABAM 7, ABAM 14, ABAM 26, ABAM 28 for the (*S*) series) while the reverse was also found (ABAM 48 and ABAM 118).

To try and establish a link between the phenotypic efflux inhibitions witnessed here, the work was pursued to assess which one(s) of *A. baumannii* EPs were overexpressed

2.3.2. Differential expression of efflux pumps across distinct A. baumannii strains

Next, in an attempt to establish a link between the phenotypic efflux inhibitory activity observed and the constitutive expression levels of different efflux pumps across the A. baumannii strains used, the expression level of different genes was determined by RT-qPCR. A gene was considered as overexpressed when its NCR was found as superior to 2 (Table 5) witnessed here, the work was pursued to genotypically assess which one(s) of *A. baumannii* EPs were overexpressed

.

Table 5 - Overexpressed genes in the 15 clinical A. baumannii strains used in this study

Strain	Overexpressed gene
ABAM 7	None
ABAM 9	*adeB, adeJ*
ABAM 14	*adeB, adeG, adeJ, adeR, abeM, abaQ, craA*
ABAM 16	*adeJ*
ABAM 26	*adeG, adeJ, adeR, abeM, abaQ, craA*
ABAM 28	*adeG, adeJ, abeM, abaQ, craA*
ABAM 30	None
ABAM 35	None
ABAM 48	*adeJ, abeM, abaQ, craA*
ABAM 57	*adeB*
ABAM 65	None
ABAM 77	*adeB, adeG, adeJ, abeM, abaQ, amvA, craA*
ABAM 97	None
ABAM 118	None
ABAM 132	None

None of the efflux gene tested were found to be overexpressed for ABAM 7, 30, 35, 65, 97, 118 and 132. This result was expected for strains ABAM 7, 30, 35 and 118 for which the phenotypic efflux inhibition factor was of 0 or 2. However, ABAM 48 also had a PEIF of 0 but displayed the overexpression of several efflux genes (Table 5). This conflicting result could partly be explained because of a better phenotypic inhibition obtained with PAβN than with NMP for this strain (4 vs. 0) in previous results not published yet, obtained in the early stages of this study.

No correlation was observed between the overexpression of the pumps and the MIC values obtained for CIP. Nevertheless, for which no efflux pump overexpression was detected the CIP MICs also lowered in the presence of EPIs, which suggests that test EPIs aren't just targeting these pump genes.

For example, the strains ABAM 7(no overexpressed genes detected) and the strain ABAM 57 (gene adeB overexpressed) had the same (128) CIP MIC value (128µg/mL) and the presence of EPIs 2,6,7 and 8 showed higher folds for ABAM 7 than for ABAM 57, supporting the suggestion of another mechanism involved responsible for the decreasing the MICs values in presence of these EPIs.

Also, *adeB* was not overexpressed by this strain so it could be surmised that PAβN is a better inhibitor of non-RND EPs (such as those encoded by *abeM*, *abaQ* and *craA*) in *A. baumannii* strains and that NMP is more specific of *adeB*. As for ABAM 118, it was originally included in the study because it was reported by the French national reference center as overexpressing *adeB*. This result was neither confirmed by the phenotypic efllux inhibition assay (PEIF of 0 for NMP and PAβN), nor by the RT-qPCR assay. For strains ABAM 65, 97 and 132, the discrepancy between the witnessed phenotypic inhibition of efflux by NMP and the lack of EP gene overexpression might be that other EPs, the genes of which were not investigated in the RT-qPCR study, are implicated in the efflux of ciprofloxacin.

On the 10 strains with PEIF of 4 and above, 8 overexpressed at least one member of the RND family. The most frequent overexpressed gene was *adeJ* (7 strains) followed by *adeB* and *adeG* with 4 strains each. AdeABC regulatory genes *adeR* and *adeS* were seldom seen as overexpressed (1 out of 10 strains). For three of the 10 strains, RND EPs were the only ones to be overexpressed.

As for non-RND EPs 5 strains out of 10 were found to overexpress the newly described AbaQ EP. This EP belongs to the MFS along with AmvA and CraA. The *craA* and *abaQ* genes (53) were overexpressed simultaneously in the 5 strains while *amvA* was only overexpressed in one.

Finally, the MATE AbeM EP was also found to be overexpressed in 5 strains.

3. Conclusions

NMP analogs tested in this study proved to have a moderate intrinsic antibiotic activity in their unprotected form. These forms were also found to lower CIP MICs in the majority of the strains tested. The genotypic investigation on the expression of 7 EPs belonging to *A. baumannii* showed that 80% of the strains displaying a phenotypic inhibition of efflux with NMP overexpressed at least one of these EPs. Further work is needed to better understand the link between the phenotypic inhibition of efflux witnessed with NMP and the overexpression of EP genes in those strains.

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