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Antivirulence strategies against *Pseudomonas aeruginosa*

PhD Candidate
Valerio Baldelli

PhD Supervisor: Dr. Giordano Rampioni

PhD Coordinator: Prof. Paolo Visca

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“I grew up thinking that a research scientist was a natural thing to be”

“Be curious”

Stephen Hawking

Abstract

The long-term use of antibiotics has dramatically accelerated the emergence of multi-drug resistant (MDR) bacterial pathogens worldwide, leading to an alarming increase of difficult-to-treat infections. In parallel, only a handful of new antibiotics have been approved by the U.S. Food and Drug Administration (FDA) in the last decades, with many companies considering the R&D for new antibiotics a less attractive asset compared to more rewarding therapeutic areas. The serious health and economic problems caused by MDR pathogens, alongside the progresses in understanding the antibiotic resistance and virulence mechanisms in many bacterial pathogens, have fostered research not only into new antibiotics, but also into antibiotic adjuvants and antivirulence drugs. Antibiotic adjuvants target antibiotic resistance determinants (*e.g.,* efflux pumps, antibiotic inactivating enzymes), thus decreasing bacterial resistance to conventional antibiotics. Antivirulence drugs target bacterial virulence processes rather than growth; consequently, they are expected to reduce bacterial adaptability to the host environment with lower selective pressure for the emergence of resistance compared to antibiotics.

Pseudomonas aeruginosa is a model organism for studies focused on antibiotic adjuvants and antivirulence drugs. This Gram-negative bacterium is one of the most dreaded nosocomial pathogens and the main cause of death in cystic fibrosis patients, and has recently been included by the World Health Organization in the list of pathogens for which new therapeutic options are urgently needed (Priority 1: Critical; http://www.who.int/en/news-room/detail/27-02-2017-who-publishes-list-of-bacteria-for-which-new-antibiotics-are-urgently-needed).

The active efflux of antibiotics *via* efflux pumps contributes to the MDR phenotype of *P. aeruginosa*. Hence, inhibitors of *P. aeruginosa* efflux pumps hold promise to overcome *P. aeruginosa* antibiotic resistance. Moreover, the notion that efflux pumps could play a role in *P. aeruginosa* infection is emerging, thus implying that efflux pumps inhibitors (EPIs) could also be endowed with antivirulence properties. Nevertheless, EPIs are usually considered only for their antibiotic adjuvant activity, while their antivirulence potential is seldom taken into account. On this basis, during the first part of this PhD thesis, the effect of the model EPI Phe-Arg-β-naphthylamide (PAβN, also named MC-207,110) on *P. aeruginosa* transcriptome and virulence was evaluated.

Quorum sensing (QS) is an intercellular communication system that plays a key role in controlling the expression of key virulence determinants in a number of bacterial pathogens, including *P. aeruginosa*. *P. aeruginosa* QS defective strains show decreased virulence in animal and plant infection models, therefore QS has received attention as a potential therapeutic target for the development of antivirulence agents against *P. aeruginosa*. Over the last decade, a number of

compounds inhibiting the *P. aeruginosa* QS circuitry have been identified, most of which are active on the *las* QS system. Recently, few studies have described inhibitors also of the *pqs* QS system. This complex QS circuit is required for full virulence and is active during *P. aeruginosa* infections in humans. Unfortunately, most of the QS inhibitors identified so far are cytotoxic or display unfavourable pharmacological properties, thus limiting their transfer to the clinical practice. The discovery and development of new drugs for use in humans is a challenging task that usually requires decade-long laboratory experimentation followed by extensive clinical trials. Searching for off-target activities in drugs already approved for use in humans represents a potential shortcut to develop new therapeutic options. As compared with *de novo* drug discovery, drug-repurposing (or drug-repositioning) is expected to reduce the time and costs generally associated to standard drug discovery processes. Also *in silico* approaches have been proved as valid aids to conventional drug-discovery programmes. In particular, virtual screenings carried out through molecular docking simulations allow selecting promising drug candidates in vast libraries of molecules.

On this basis, during the second part of this PhD thesis the drug-repurposing and the *in silico* screening approaches were used to identify FDA-approved drugs able to reduce *P. aeruginosa* virulence potential by targeting different elements of the *pqs* QS system.

Riassunto

Il trattamento delle infezioni causate da batteri antibiotico-resistenti rappresenta un grande problema dal punto di vista socio-economico e sanitario. Solo in Europa è stimato che le infezioni causate da tali batteri causino circa 25˙000 decessi l'anno, con costi sanitari associati superiori a 1,5 miliardi di euro. Purtroppo questa problematica non sembra essere di facile soluzione, in quanto l'emergenza di ceppi batterici resistenti agli antibiotici è da molti considerata un fenomeno evolutivo inevitabile. Se si considerano l'elevato tasso di mutazione ed il ridotto tempo di generazione dei batteri, nonché l'elevata pressione selettiva esercitata dagli antibiotici nei confronti dei microrganismi resistenti a tali molecole, non sorprende che già pochi anni dopo l'immissione in commercio di un nuovo antibiotico vengano isolati dai pazienti batteri resistenti a tali farmaci.

Ad oggi si assiste ad un allarmante incremento di batteri patogeni multi-resistenti (MDR), specialmente per quanto concerne un gruppo di batteri patogeni identificato con l'acronimo ESKAPE (*Enterococcus faecium*, *Staphylococcus aureus*, *Klebsiella pneumoniae*, *Acinetobacter baumanii*, *Pseudomonas aeruginosa* e specie di *Enterobacter*), responsabili della maggior parte delle infezioni ospedaliere nei paesi sviluppati. La scarsa disponibilità di terapie antimicrobiche

efficaci e i ridotti investimenti delle case farmaceutiche per la ricerca di nuovi antibiotici hanno portato a quella che viene da molti definita “era post-antibiotica”.

Negli ultimi decenni diverse ricerche si sono focalizzate sullo sviluppo di strategie antibatteriche innovative. Un approccio promettente in tal senso mira a rendere nuovamente efficaci gli antibiotici per i quali i batteri hanno sviluppato resistenza. Ciò è possibile sviluppando adiuvanti degli antibiotici in grado di inibire i meccanismi di resistenza (*es*. inibendo gli enzimi che degradano gli antibiotici, inattivando le pompe d’efflusso che li estrudono al di fuori della cellula). Un secondo approccio innovativo che potrebbe limitare l’insorgenza di batteri resistenti si basa sull’utilizzo di farmaci antivirulenza. Tali sostanze non inibiscono la crescita dei batteri, ma sono sviluppate in modo tale da limitare la capacità dei patogeni di causare infezioni, ad esempio inibendo la produzione di tossine o di altri fattori di virulenza che danneggiano le cellule ed i tessuti dell’ospite. È stato ipotizzato che l’uso di molecole in grado di “disarmare” i batteri patogeni del loro arsenale di fattori di virulenza possa limitare la loro capacità di causare infezioni e la diffusione di ceppi resistenti a tali molecole.

P. *aeruginosa* é un patogeno Gram-negativo molto rilevante per la salute umana, in quanto una delle maggiori cause di infezioni nosocomiali, e la principale causa di morte in pazienti affetti da fibrosi cistica. Nel 2017 l’Organizzazione Mondiale della Sanità (OMS) ha inserito tale batterio nella lista di patogeni per i quali sono urgentemente necessari nuovi approcci terapeutici. Infatti, le infezioni causate da *P*. *aeruginosa* sono difficili da eradicare, principalmente a causa dell’elevata resistenza di tale batterio alla maggior parte degli antibiotici utilizzati in terapia, in parte dovuta alla sua capacità di formare biofilm antibiotico-resistenti e di esprimere pompe di efflusso. Inoltre questo patogeno è in grado di esprimere un vasto arsenale di fattori di virulenza necessari a stabilire l’infezione. Queste caratteristiche hanno reso *P*. *aeruginosa* uno dei più importanti organismi modello per lo studio di molecole dotate di attività antibiotico-adiuvante ed antivirulenza.

Il genoma di *P*. *aeruginosa* codifica per un elevato numero di pompe di efflusso, in grado di estrudere diversi antibiotici al di fuori della cellula contribuendo allo sviluppo di fenotipi multi-resistenti. In questo contesto, gli inibitori delle pompe di efflusso rappresentano dei prometti agenti adiuvanti degli antibiotici. Negli ultimi anni, diversi studi hanno messo in evidenza il coinvolgimento dei sistemi di efflusso di *P*. *aeruginosa* in diverse fasi dell’infezione. In questo contesto, gli inibitori delle pompe di efflusso, oltre che dotati di attività adiuvante nei confronti degli antibiotici, potrebbero anche essere dotati di proprietà antivirulenza. Uno degli inibitori delle pompe di efflusso meglio caratterizzati, la Phe-Arg-β-naphthylamide (PAβN, conosciuta anche con il nome MC-207,110), inibendo le pompe di efflusso MexAB-OprM, MexCD-OprJ e MexEF-OprN di *P*. *aeruginosa*, riduce la resistenza di questo patogeno a diversi antibiotici, inclusi

fluorochinoloni e cloramfenicolo. Su tali basi scientifiche, nella prima parte di questo progetto di dottorato è stato valutato l'impatto dell'inibitore delle pompe di efflusso PAβN sul trascrittoma e sulla virulenza di *P. aeruginosa*.

In diversi batteri, incluso *P. aeruginosa*, l'espressione di fattori di virulenza e la capacità di formare biofilm sono processi regolati da un sistema di comunicazione intercellulare noto come *quorum sensing* (QS). Il QS consente ai batteri di sincronizzare l'espressione di determinati fenotipi a livello di popolazione mediante segnalazione chimica. In questo contesto, l'inibizione del QS, o "*quorum quenching*", è una strategia molto promettente per ridurre il potenziale patogeno di microrganismi come *P. aeruginosa*. Numerosi studi hanno dimostrato come mutanti difettivi in uno o più dei tre principali sistemi di QS di *P. aeruginosa* (*las*, *rhl* o *pqs*) risultino meno virulenti in diversi modelli di infezione, sia vegetali, sia animali.

Finora, la maggior parte degli studi si sono focalizzati sull'identificazione di inibitori del sistema di QS *las* di *P. aeruginosa*, e solo negli ultimi anni diversi studi sono stati condotti per identificare inibitori di meccanismi di QS più complessi, come il sistema *pqs*. L'attivazione del sistema di QS *pqs* è necessaria per la completa virulenza di *P. aeruginosa*. Purtroppo, nella maggior parte dei casi gli inibitori del QS identificati si sono rivelati citotossici, o hanno mostrato proprietà farmacologiche sfavorevoli, limitandone il potenziale utilizzo in terapia. In questo contesto, una possibile soluzione è rappresentata dalla ricerca di inibitori del QS tra molecole già approvate per l'uso nell'uomo. Questo approccio, noto come *drug repurposing* (riposizionamento del farmaco), consente di abbreviare notevolmente i tempi e di ridurre i costi necessari per il trasferimento di un nuovo farmaco dal laboratorio alla pratica clinica. Uno degli approcci di *drug repurposing* più utilizzati si basa su *screening* effettuati su librerie di farmaci già approvati per l'uso nell'uomo. La recente disponibilità di molte strutture cristallografiche di recettori del QS ha aperto la strada all'identificazione di potenziali inibitori del QS attraverso approcci *in silico*. Tali approcci, basati su *software* che simulano processi di legame tra due molecole, permettono di selezionare possibili ligandi dei bersagli molecolari di interesse in vaste collezioni di farmaci, per poi verificare l'attività inibitoria di un numero ridotto di molecole selezionate.

Su tali basi scientifiche, nella seconda parte di questa tesi di dottorato, mediante approcci *in silico* e di *drug repurposing*, sono stati identificati farmaci già approvati per uso nell'uomo in grado di ridurre il potenziale patogeno di *P. aeruginosa* inibendo il sistema di QS *pqs*.

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Chapter 1

Introduction and aims

1.1 The post-antibiotic era

Antibiotics are natural substances produced by microorganisms in order to compete in the environment. Antibiotics can enable their producers to kill (bactericidal effect) or block the growth of (bacteriostatic effect) other competitors (Walsh, 2003; D'Costa *et al*., 2011).

For thousands of years, people have been unarmed in front of bacterial infections, which cost millions of lives. The turning point in the fight against microbial infections occurred in 1928, when the discovery of antibiotics by Alexander Fleming represented the first effective resolution to combat bacterial pathogens. This discovery ushered in the "Golden Age of Antibiotics", a period of drug innovation and implementation of human and animal health. The second half of the 20th century has been characterized by a rapid expansion in antibiotic development. Most of the antibiotic classes available today were discovered and allowed to treat many of the diseases which, in those days, reached epidemic proportions, such as cholera, syphilis, plague, tuberculosis, and others (Mohr, 2016; Luepke *et al*., 2017).

After the first period of euphoria, it was quickly realized that bacteria were able to develop, acquire, and spread numerous resistance mechanisms, and the reason is related to the nature of antibiotics themselves (Werner *et al*., 2008; D'Costa *et al*., 2011). Antibiotic resistance is a natural phenomenon that can be innate, due to the nature of microorganism (*e.g*. impermeability to the antibiotic, lack of the molecular target of the antibiotic), or emerge among antibiotic-susceptible microorganisms. Selective pressure of *de novo* mutation is a way by which bacteria spread resistance mechanisms: in a bacterial population, a small fraction of bacteria could be resistant to a given antibiotic, due to the natural mutation rates. If the antibiotic kills or inhibits the growth of sensitive clones, this will enable any resistant cell to grow in a competitor-free environment, thus posing a strong selection for antibiotic resistance phenotypes. Most of the microorganisms have also the capability to acquire genetic determinants of resistance from the environment and from other microorganisms through horizontal gene transfer (Nikaido, 2009). Common antibiotic-resistance mechanisms include the alteration or overexpression of the drug targets, the enzymatic inactivation of the drug, the expression of bypass pathways, the expression of efflux pumps and the secretion of an extracellular matrix (Lewis, 2013; Schillaci *et al*., 2017) (**Fig. 1**). The latter leads to biofilm formation and confers protection from both antibiotic treatments and host immune system (Costerton *et al*., 1995).

Since the discovery of antibiotics, their massive (and often improper) use in settings like hospitals and intensive animal farming has dramatically accelerated the emergence of antibiotic-resistant strains. Today, we are witnessing an alarming increase of multi-drug resistant (MDR), or even pan-drug resistant pathogens (World Health Organization, 2014). This problem especially

concerns a group of severe pathogens particularly relevant from a clinical point of view, known with the acronym ESKAPE (*Enterococcus faecium*, *Staphylococcus aureus*, *Klebsiella pneumoniae*, *Acinetobacter baumanii*, *Pseudomonas aeruginosa*, and *Enterobacter* species), which currently cause the majority of hospital-acquired infections and effectively "escape" the effect of almost all available antibacterials (Rice, 2008; Boucher *et al*., 2009).

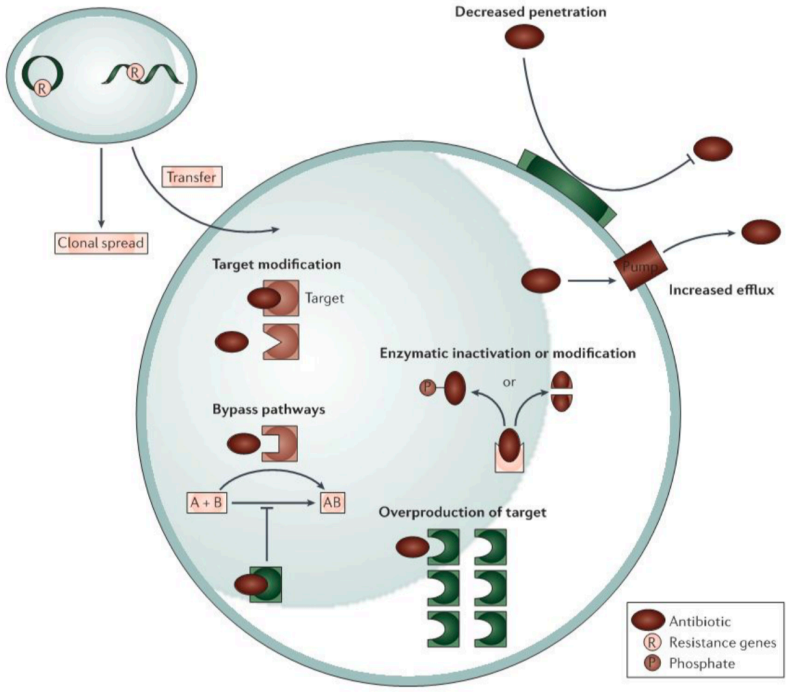


Fig. 1: Schematic representation of the main antibiotic-resistance mechanisms. Image modified from Lewis, 2013.

The steady increase in antimicrobial resistance and the decreased availability of new antibiotics are leading to the so-called "post-antibiotic era" (Mohr, 2016). Indeed, many companies are considering the development of new antibiotics a less attractive investment compared with more lucrative therapeutic areas. Even if a new antibiotic is discovered, its lifespan will probably be short due to rapid emergence of resistance. Therefore, the economic return resulting from the marketing of new antibiotics could not be sufficient to justify the large investments required for their development (Gilbert, 2010; Coates *et al*., 2011; Ventola, 2015; Fernandes and Martens, 2016; Luepke *et al*., 2017).

Progress in understanding the mechanisms that govern antibiotic-resistance and virulence processes has led researchers to consider alternative therapeutic approaches to treat bacterial pathogens. In this PhD thesis, particular attention will be given to antibiotic adjuvants and antivirulence drugs (Rasko and Sperandio, 2010; Rampioni *et al*., 2014; Brannon and Hadjifrangiskou, 2016).

1.2 Antibiotic adjuvants

In the fight against MDR bacterial pathogens, the discovery of new antibiotics is not the only viable strategy, and many studies in recent years have been focusing on the possibility of developing new therapeutic approaches for the treatment of infections caused by antibiotic-resistant microorganisms (Rasko and Sperandio, 2010; Allen *et al*., 2014; Vale *et al*., 2016; Schillaci *et al*., 2017). A promising strategy is based on the development of antibiotic adjuvants capable of inhibiting antibiotic resistance mechanisms, thus making effective again "old" antibiotics for which bacteria have developed resistance (Lomovskaya *et al*., 2001; Ejim *et al*., 2011; Blake and O'Neill, 2013).

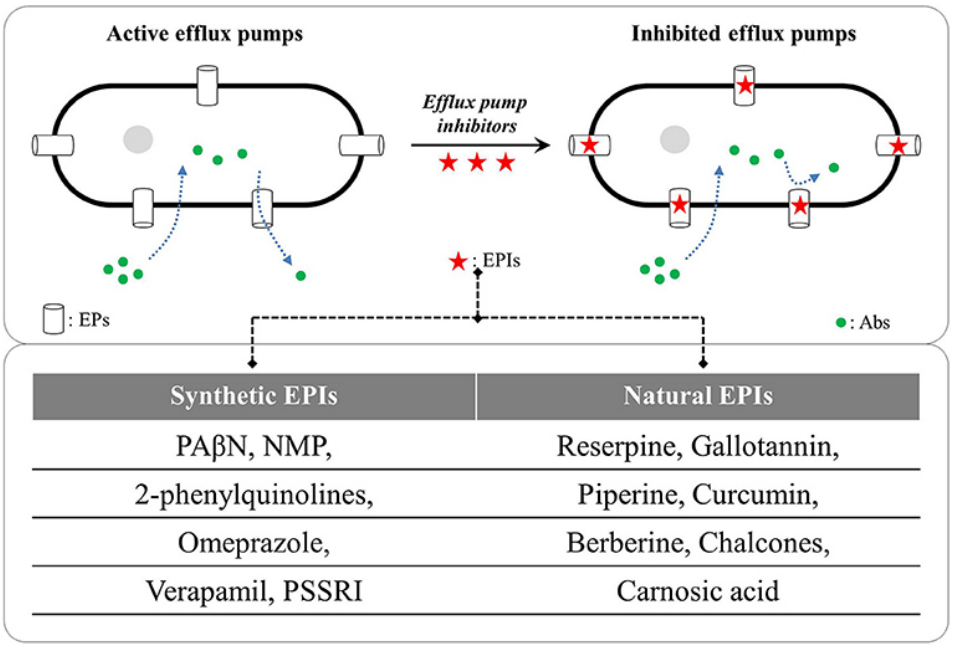
Some adjuvants inhibit enzymes that confer antibiotic resistance by degrading the antibiotic itself. A leading example of antibiotic adjuvants is clavulanic acid, a molecule that has been extensively used in combination with amoxicillin in the drug formulation Augmentin®. Clavulanic acid covalently binds to β-lactamases and irreversibly inhibits their hydrolytic activity towards β-lactams (including amoxicillin), thus making β-lactam-resistant pathogens susceptible again to the action of these antibiotics (Brogden *et al*., 1981; White *et al*., 2004).

In recent years, the research for antibiotic adjuvants is progressively shifting towards the identification of compounds capable of inhibiting the enzymatic activity of carbapenemases, which inactivate carbapenem antibiotics and represent a significant problem in ESKAPE pathogens such as *K. pneumoniae* (Carbapenem-Resistant *K. pneumoniae* CR-*Kp*; Boucher *et al*., 2009; Kalan and Wright, 2011; Oliva *et al*., 2015; Djoko *et al*., 2018; Cui *et al*., 2019).

Since in many bacterial pathogens, including the ESKAPE bacterium *P. aeruginosa*, efflux pumps contribute to the MDR phenotype by extruding antibiotics outside the cell, a promising adjuvant strategy is based on the development of efflux pump inhibitors (EPIs) (**Fig. 2**). Efflux pumps have been classified in different families on the basis of the amino acid sequence, the energy source required to drive the counter-gradient export of antibiotics, and the substrate specificity. Due to their prominent contribution to the MDR phenotype and to the absence of human homologues, the Resistance-Nodulation-cell-Division (RND) family of efflux pumps is considered a viable target for the development of drugs aimed at increasing bacterial susceptibility to antibiotics (Alvarez-Ortega *et al*., 2013; Li *et al*., 2015; Wang *et al*., 2016; Wright, 2016; Spengler *et al*., 2017).

The *P. aeruginosa* genome encodes multiple RND efflux pumps, four of which are of clinical importance for MDR and are frequently found to be up-regulated in clinical isolates, namely MexAB-OprM, MexCD-OprJ, MexEF-OprN and MexXY-OprM (Poole, 2011). The MexAB-OprM efflux pump is considered the most important RND efflux pump of *P. aeruginosa*, since it is

constitutively expressed and provides intrinsic resistance to a broad spectrum of antibiotics (Poole, 2011).



Synthetic EPIs	Natural EPIs
PAβN, NMP,	Reserpine, Gallotannin,
2-phenylquinolines,	Piperine, Curcumin,
Omeprazole,	Berberine, Chalcones,
Verapamil, PSSRI	Carnosic acid

Fig. 2: Examples of synthetic and natural efflux pump inhibitors (EPIs); PAβN: phenyl-arginine-β-naphthylamide, NMP: 1-(1-naphthylmethyl)-piperazine, PSSRIs: phenylpiperidine selective serotonin re-uptake inhibitors. Abs, antibiotics; EPs, efflux pumps. EPIs, efflux pump inhibitors. Image modified from Shiram *et al*., 2018.

The development of efflux pumps inhibitors is very attractive, since a single EPI can be active against multiple MDR pumps and could be used in adjuvant therapy. During the last decades, several compounds that hamper efflux pumps functionality have been identified *via* screening of libraries containing natural and synthetic molecules (Zechini and Versace, 2009; Salam and Quave, 2018). Efflux pumps inhibition may be obtained by dissipating the energy source required for counter-gradient transport of the antibiotic, or by competitive or non-competitive inhibition with the natural ligand (Sharma *et al*., 2019).

Since efflux pumps functionality requires energy, decoupling the energy source and efflux activity presents an interesting approach to efflux inhibition. The proton gradient or the ATPase that supplies energy to these pumps have been suggested as targets of various EPIs. This approach appears to be advantageous as many efflux pumps are dependent on the proton-motive force, making this a universal scheme for inhibiting them without direct interaction between the inhibitor and the efflux pump itself. Compounds that dissipate the proton gradient of the bacterial membrane, such as carbonylcyanide m-chlorophenylhydrazone (CCCP), valinomycin and dinitrophenol (DNP), are endowed with efflux pumps inhibition activity. As an example, alteration of the trans-membrane electrochemical potential caused by CCCP inhibits efflux pumps functionality, hence reviving the

activity of tetracycline in *Helicobacter pylori* and *Klebsiella* spp. (Anoushiravani *et al*., 2009; Fenosa *et al*., 2009).

Another mechanism of efflux pump inhibition is the direct binding of the EPIs to efflux pumps, resulting in reduced ability of the pumps to interact with their substrates. This binding could be competitive, where the EPI competes with the substrates for the same binding site, or non-competitive, where the binding of EPI to the pump decreases the affinity of the pump to its substrates (Sharma *et al*., 2019). Phe-Arg-β-naphthylamide (PAβN, also named MC-207,110) is the most active and best studied inhibitor of *P. aeruginosa* RND efflux pumps belonging to this latter class of EPIs. It was discovered in a screen for adjuvants of the fluoroquinolone levofloxacin, carried out in a *P. aeruginosa* strain over-expressing the efflux pump MexAB-OprM (Renau *et al*., 1999). Subsequent studies showed that PAβN is active against RND pumps other than MexAB-OprM, indicating that this EPI has a broad spectrum inhibitory activity. By inhibiting MexAB-OprM, MexCD-OprJ and MexEF-OprN efflux pumps in *P. aeruginosa*, PAβN reduces the resistance of this pathogen to fluoroquinolones and chloramphenicol antibiotics, normally extruded outside the cell through these export systems (Lomovskaya *et al*., 2001; Li *et al*., 2015; Wright, 2016). Unfortunately, the cellular toxicity towards mammalian cells has kept CCCP, PAβN and others EPIs limited to laboratory use only (Zechini and Versace, 2009; Sharma *et al*., 2019).

In the last decades, the notion that efflux pumps could play a role in bacterial infection is emerging. Indeed, besides extruding antibiotics outside the cell, different efflux pumps have the ability to extrude a wide variety of compounds, including antimicrobials endogenously produced by the host and bacterial virulence factors, and are required for host colonization (Piddock, 2006; Alcade-Rico *et al*., 2016). As an example, the RND efflux pump ArcAB-TolC of *Escherichia coli*, which contributes to intrinsic antibiotic resistance in this organism, confers resistance to bile salts (Thanassi *et al*., 1997), and in *Salmonella enterica* deletion of the *acrA*, *acrB*, or *tolC* genes results in decreased expression of genes involved in *E. coli* pathogenicity (Webber *et al*., 2009). RND efflux pumps contribute to both antibiotic resistance and pathogenesis also in *Vibrio cholerae*. Indeed, deletion of the genes coding for the VexAB, VexCD, VexIJK, and VexGH systems impairs the colonization of the infant mouse small intestine by *V. cholerae* (Bina *et al*., 2008). In *P. aeruginosa*, different RND efflux pumps have been shown to play a role in the export of secreted virulence factors (Evans *et al*., 1998; Aendekerk *et al*., 2005; Alcade-Rico *et al*., 2018), further supporting a crosstalk between resistance and virulence.

Overall, in different bacterial species efflux pumps contribute to the infection process, implying that EPIs, beside reducing antibiotic resistance, could also be endowed with antivirulence activity (Piddock, 2006; Martínez *et al*., 2009; Alcade-Rico *et al*., 2016).

1.3 Antivirulence drugs

Over the last decades, progress in understanding the pathogenic processes underlying bacterial infections has led researchers to focus on bacterial virulence factors as targets of new anti-infective drugs. Nowadays, inhibition of bacterial virulence rather than growth is considered a promising approach to combat bacterial infections with lower selective pressure for the emergence of resistance relative to antibiotics (Rasko and Sperandio, 2010).

Whereas conventional antibiotics target the viability of the cell, antivirulence drugs target specific functions required for the success of the infection, such as toxin function, toxin delivery, virulence gene expression, or cell adhesion (Mellbye and Schuster, 2011; Schuster *et al*., 2013). Antivirulence drugs “disarm” rather than kill pathogens and, as a consequence, they are thought to pose a reduced selective pressure for the emergence of resistance (Mellbye and Schuster, 2011). In particular, most virulence factors are considered as "public goods”, because they are secreted by the producer cells and shared among all the members of a bacterial population (Allen *et al*., 2014). *In vitro* social-evolution experiments suggest that resistant variants will not emerge if the antivirulence therapy specifically targets public goods. During infection, public goods are exploited by the entire bacterial population, as well as by the individual producer cells. In this context, mutants resistant to an inhibitor of public goods production should not experience any fitness benefit relative to sensitive clones because they would be the only cells in a sensitive population capable of producing public goods, that would be diluted among all the members of the population. Therefore, the public goods produced by few resistant mutants would not be sufficient to sustain the growth of the whole population. Additionally, mutants resistant to a drug that inhibits the production of public goods could be negatively selected with respect to the sensitive clones as a consequence of the metabolic burden associated to public goods production. Overall, mutants resistant to antivirulence drugs targeting the production of public goods are not expected to emerge in a population of sensitive bacteria (Allen *et al*., 2014; Rampioni *et al*., 2017) (**Fig. 3**).

Conversely, resistant strains are probably positively selected *in vivo* only if they gain a “private” advantage over the susceptible population. This could be the case for inhibitors of adhesion factors (Allen *et al*., 2014).

The antivirulence approach has other potential advantages relative to the use of conventional antibiotics, such as preserving the endogenous microflora and expanding the repertoire of druggable targets (Rampioni *et al*., 2017). Furthermore, antivirulence drugs could be administered in combination with conventional antibiotics to enhance their efficacy (Schuster *et al*., 2013).

Additional issues that merit consideration are the impact of reduced virulence factor production on the immune system, and the possible selection for hyper-virulent phenotypes. Indeed, virulence

factors are known to stimulate the immune response; as a consequence, decreased expression of virulence factors could reduce the clearance of pathogens by the immune system (Weigert *et al*., 2017; Rampioni *et al*., 2017).

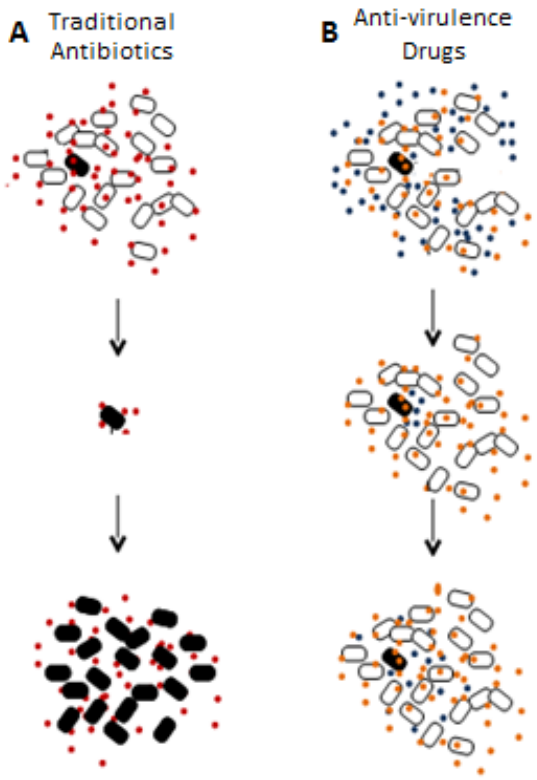


Fig. 3: Comparison between traditional antibiotics and new antivirulence strategies on the emergence of resistance. **A)** The conventional antibiotic (red dots) is added to a sensitive population (in white), which will be killed by the antibiotic, while a subpopulation of resistant cells (in black) will rapidly emerge. **B)** The antivirulence drugs (orange dots) is added to a sensitive population producing public goods, such as secreted virulence factors (blue dots). The antivirulence drug targets specifically the production of the virulence factor. In this scenario, the resistant mutant should not display any growth advantage relative to the sensitive cells, hence it should not emerge.

It has also been suggested that antivirulence drugs could select for mutants overproducing virulence factors, hence endowed with increasing virulence potential in the absence of antivirulence treatment (Ruer *et al*., 2015). However, no experimental evidence supporting this hypothesis has been provided so far. As a consequence, while additional studies are needed to assess the real benefit of antivirulence drugs *in vivo*, experimental evidence supports the antivirulence approach as a promising therapeutic option (Rampioni *et al*., 2017).

In this context, one of the most promising antivirulence strategy is based on the inhibition of quorum sensing (QS), a chemical communication system allowing many bacteria to coordinate the expression of virulence traits at the population level (Williams and Càmara, 2009; Rampioni *et al*., 2014).

1.4 Quorum sensing

QS was originally discovered in Gram-negative *Proteobacteria*, and today the diversity of bacterial taxa harboring QS circuits has grown to include hundreds of species (Moradali *et al*., 2017). QS is an intercellular communication system mediated by chemical signals that allows bacteria to synchronize certain behaviors in response to cell density on a population-wide scale (Williams and Càmara, 2009; Mukherjee and Bassler, 2019). QS is based on the production, secretion and perception of small signal molecules, which induce a physiological response in the bacterial population only if a threshold concentration of the signal molecule is reached. At low cell density, the QS signal molecule is produced at a basal level, and during bacterial growth its concentration increases. Once the signal molecule concentration reaches a threshold level, corresponding to the "*quorum*" cell density, the signal molecule binds to and activates a cognate receptor, thus triggering the expression or repression of target genes in all the members of the bacterial population (Waters and Bassler, 2005; Joint *et al*., 2007; West *et al*., 2007; von Bodman *et al*., 2008; Atkinson and Williams, 2009; Hense and Schuster, 2015; Papenfort and Bassler, 2016; Mukherjee and Bassler, 2019).

The first described QS system was observed in *Allivibrio fischeri* (formerly known as *Vibrio fischeri*) (Nealson *et al.,* 1970), a bioluminescent marine bacterium that establishes a symbiotic relation with the Hawaiian squid *Euprymna scolopes*. At sunset, *A. fischeri* colonizes the light organ of the squid wherein it grows until it reaches high cell density. At the "*quorum*" cell density, the expression of genes required for bioluminescence is induces, and consequent light emission reduces predation of the squid at night by masking its shadow on the seabed. The *A. fischeri* QS system is based on the autoinducer synthase LuxI, which produces the signal molecule 3-oxo-hexanoyl-homoserine lactone ($3OC_6$-HSL), a member of the *N*-acyl-homoserine lactones (AHLs) family of QS signal molecules, and on the cognate cytoplasmic signal receptor LuxR. As the cell density of the bacterial population increases, so does the concentration of $3OC_6$-HSL, that diffuses among the bacterial cells and accumulates until it reaches a threshold concentration. At the *quorum* cell density, $3OC_6$-HSL binds to LuxR. The LuxR/$3OC_6$-HSL complex is active as a transcriptional regulator and triggers the expression of the luciferase operon (*luxICDABE*), required for bioluminescence. Since the first gene of the *luxICDABE* operon codes for the LuxI synthase, the LuxR/$3OC_6$-HSL complex also triggers the production of $3OC_6$-HSL itself, resulting in a positive feedback loop that enhances the production of the signal molecule (Nealson *et al*., 1970; Waters and Bassler, 2005; Dunn and Stabb, 2007) (**Fig. 4**).

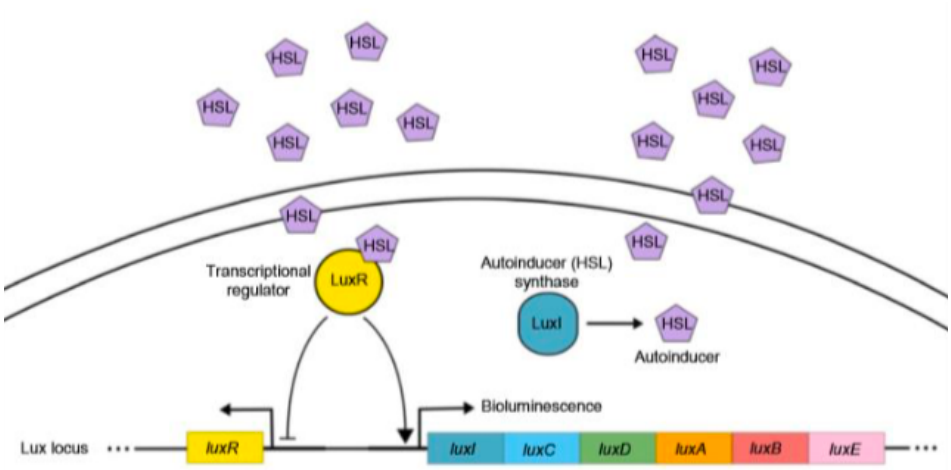


Fig. 4: Schematic representation of the QS system in *A. fischeri*. The signal molecule (HSL) produced by the LuxI synthase binds and activates the LuxR receptor, inducing transcription of *luxI* gene, thus increasing HSL biosynthesis, and of the *luxCDABE* genes, thus activating bioluminescence. Image from Reuter *et al*., 2015.

As exemplified by the QS system of *A. fischeri*, any QS circuit consists of the genes coding for the QS signal synthase (or the QS signal pro-peptide) and for the QS signal receptor (Williams, 2007; Mukherjee and Bassler, 2019). Signal molecules are small diffusible compounds or secreted modified peptides that activate or repress QS target gene(s) once a threshold concentration of the signal has been reached across the bacterial population. Some QS signal molecules interact with receptors at the cell surface, while others act following internalization (Williams and Càmara, 2009; Hense and Schuster, 2015). QS signal molecules are chemically diverse and many bacteria possess several interacting QS gene regulatory 'modules' that can employ multiple signal molecules from the same or different chemical classes, which usually constitute regulatory hierarchies, as in the case of *P. aeruginosa*, in which QS plays a pivotal role in the establishment of host infection (Atkinson and Williams, 2009; Papenfort and Bassler, 2016; Kostylev *et al*., 2019).

In general, QS systems facilitate the coordination of population behaviors to enhance access to nutrients or specific environmental niches, collective defense against other competitors or community escape where survival of the population is threatened (Williams, 2007; Papenfort and Bassler, 2016; Mukherjee and Bassler, 2019).

1.5 *Pseudomonas aeruginosa*

P. aeruginosa is a Gram-negative and ubiquitous bacterium that colonizes various environmental niches, including soil and marine habitats, plants and mammalian tissues. The ecological versatility of *P. aeruginosa* is reflected by its relatively large genome (5-7 Mbp) and genetic complexity. Indeed, compared to other bacteria, the genome of *P. aeruginosa* is particularly rich in genes coding

for metabolic functions, outer membrane transporters, and chemotaxis systems, which may contribute to its metabolic versatility. Moreover, up to 10% of the assigned ORFs are classified as transcriptional regulators or two-component systems, reflecting the remarkable capacity of this bacterium to reprogram gene expression in response to a plethora of metabolic and environmental signals (Stover *et al*., 2000; Goodman and Lory, 2004; Moradali *et al*., 2017).

P. aeruginosa is commonly known as an opportunistic human pathogen capable of causing a wide range of life-threatening acute and chronic infections, especially in immunocompromised patients. Indeed, *P. aeruginosa* is largely associated with hospital-acquired infections including pneumonia, bloodstream, surgical/transplantation and urinary catheter-related infections (Trubiano *et al*., 2015), and it is considered the leading cause of morbidity and mortality in cystic fibrosis (CF) patients (Khan *et al*., 1995; Rosenfeld *et al*., 2001; Eberl and Tümmler, 2004). *P. aeruginosa* is considered an emerging worldwide public health threat, especially because of the high mortality rates that its infections are responsible for, if compared with infections caused by other bacterial pathogens (Osmon *et al*., 2004; Boucher *et al*., 2009). The clinical relevance of *P. aeruginosa* mainly relies on the fact that its infections are hard to eradicate, mainly because this pathogen is intrinsically resistant to many antibiotics (*e.g*. macrolides, β-lactams, tetracyclines and fluoroquinolones), and it is particularly prone to acquire new resistance mechanisms by horizontal gene transfer (Latifi *et al*., 1995; Aloush *et al*., 2006). Indeed, in 2017 the World Health Organization (WHO) has top ranked *P. aeruginosa* in the list of pathogens for which new antimicrobial treatments are urgently needed (Priority 1 - Critical; www.who.int/mediacentre/news/releases/2017/bacteria-antibiotics-needed/en/).

During pathogenesis, *P. aeruginosa* can adopt two different lifestyles that reflect two different strategies of infection, the acute and the chronic infection. The acute infection is rapid, systemic and carried out by a planktonic bacterial community resulting in a severe outcome for the patient. Conversely, during chronic infections, the bacterial proliferation is restricted to a specific host tissue (*e.g*. in the CF lungs or associated with medical devices) and *P. aeruginosa* can persist in the host for extended periods of time in a slow-growing and sessile lifestyle characterized by biofilm formation (Lyczak *et al*., 2002; Folkesson *et al*., 2012; Winstanley *et al*., 2016; Moradali *et al*., 2017).

The capacity of *P. aeruginosa* to produce such diverse infections is due to a large number of virulence factors, such as LasA and LasB elastases, exotoxin A, phospholipase C, protease IV, PrpL protease, pyocyanin, siderophores, hydrogen cyanide, and rhamnolipids, and also to the production of biofilm, that allows the colonization of host tissues and the protection of bacterial cells from the immune system and antibiotics therapies. These factors are collectively capable of causing

extensive tissue damage, bloodstream invasion and dissemination in humans and animals (Smith and Iglewski, 2003; Moradali *et al*., 2017) (**Fig. 5**).

The production of virulence factors, the formation of antibiotic-tolerant biofilms and the switch from planktonic to sessile lifestyle during *P. aeruginosa* infections are finely regulated by QS (Smith and Iglewski, 2003; Lee *et al*., 2006; Williams and Càmara 2009; Papenfort and Bassler, 2016).

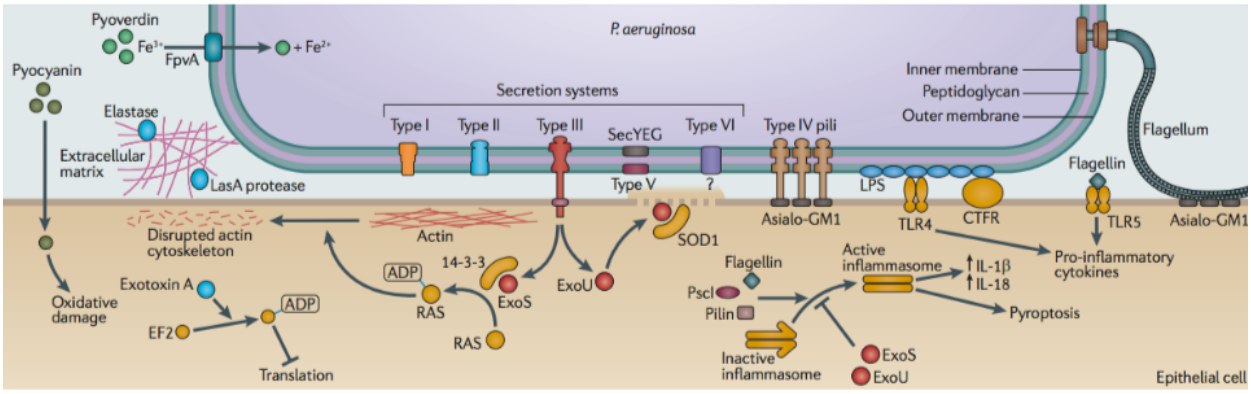


Fig. 5: Overview of some *P. aeruginosa* virulence determinants. *P. aeruginosa* has both cell-associated (*e.g*., flagellum, pili, lipopolysaccharides, alginate) and extracellular virulence factors (*e.g*., elastase, proteases, pyocyanin, exotoxin A, siderophores). Image from Hauser and Ozer, 2011.

1.5.1 Quorum sensing circuits in *Pseudomonas aeruginosa*

P. aeruginosa has three major QS systems that are interconnected and hierarchically arranged: in rich medium, the *las* QS system is at the top of this hierarchy, because it is required for full activation of the other two QS systems, namely the *rhl* and the *pqs* systems (Williams and Càmara, 2009; Papenfort and Bassler, 2016) (**Fig. 6)**. Recently, a fourth QS system based on oxylipins as QS signal molecules has been described (Martínez *et al*., 2019).

The *las* system consists of the LuxR-like transcriptional regulator LasR (encoded by the *lasR* gene), and of the LuxI-like acyl-HSL synthase LasI (encoded by the *lasI* gene), that directs the synthesis of the QS signal molecule *N*-(3-oxododecanoyl)homoserine lactone ($3OC_{12}$-HSL) (Schuster and Greenberg, 2006) (**Fig. 6**).

Similarly to the *A. fischeri lux* QS system previously described, at low-cell density the $3OC_{12}$-HSL molecule is synthesized by LasI at basal level, and it is secreted into the surrounding medium. With increasing cell density, the signal molecule accumulates until its concentration reaches the threshold level; at this critical concentration, $3OC_{12}$-HSL binds its cognate receptor LasR. The LasR/$3OC_{12}$-HSL complex triggers *lasI* transcription, generating a positive feedback loop that leads to the amplification of $3OC_{12}$-HSL production. As a consequence, the whole QS-system of *P.*

aeruginosa becomes active and its transcriptome is drastically reprogrammed. Indeed, the LasR/3OC$_{12}$-HSL complex triggers the expression of hundreds of genes, including virulence genes and genes of the *rhl* and the *pqs* QS systems (Schuster *et al*., 2003; Schuster and Greenberg, 2006; Williams and Càmara, 2009; Papenfort and Bassler, 2016). Therefore, the regulative cascade leading to the expression of virulence phenotypes in *P. aeruginosa* strongly relies on the *las* QS system (Lee and Zhang, 2015). As a whole, the QS circuit regulates about 7% of all the *P. aeruginosa* genes and has a key role in the infection process, being required for the production of many virulence factors and for biofilm formation (Kirisits and Parsek, 2006; Schuster and Greenberg, 2006).

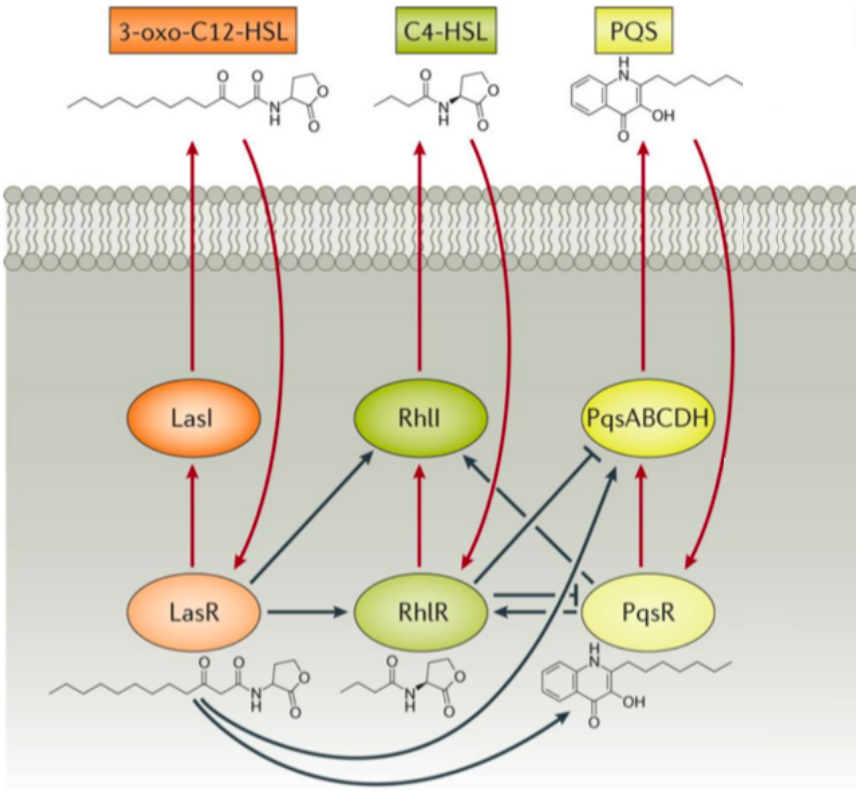


Fig. 6: The three main QS systems of *P. aeruginosa* and their interconnections. Image modified from Papenfort and Bassler, 2016.

Also the *rhl* QS system relies on the production of an acyl-homoserine lactone as signal molecule: the *N*-butanoyl-homoserine lactone (C$_4$-HSL). C$_4$-HSL is synthesised by the LuxI-like enzyme RhlI, and it is released into the extracellular environment; as the bacterial population grows, C$_4$-HSL binds to its LuxR-like cognate receptor RhlR, and the RhlR/C$_4$-HSL complex regulates the expression of target genes (**Fig. 6**).

Contrary to the *las* and *rhl* QS systems, the *pqs* QS circuit relies on the production of 2-alkyl-4(1*H*)-quinolones (AQs) as signal molecules (**Fig. 6**). The major AQs produced by *P. aeruginosa* are 2-heptyl-3-hydroxy-4(1*H*)-quinolone (PQS), its immediate precursor 2-heptyl-4-hydroxyquinoline (HHQ), and 2-heptyl-4-hydroxyquinoline *N*-oxide (HQNO) (Pesci *et al*., 1999; Déziel *et al*., 2004). Both PQS and HHQ act as QS signal molecules by binding to and activating their cognate receptor PqsR, that acts as a transcriptional regulator. Data from expression studies have revealed the extent of the *pqs* operon and its relationship with the *las* and *rhl* regulons. However, contrary to the *las* and *rhl* systems, our understanding of the molecular mechanisms governing *pqs*-dependent QS is limited, largely because of the complex and multi-component nature of this system (Heeb *et al.,* 2010; Rampioni *et al.,* 2016).

1.5.2 The *pqs* quorum sensing system

The core of the *pqs* QS system is composed of the *pqsABCDE-phnAB* operon and of the *pqsR* gene, clustering at the same genetic locus, while *pqsH* and *pqsL* genes are distally located (Winsor *et al*., 2011; Rampioni *et al*., 2016) (**Fig. 7**).

Briefly, the *pqsABCDE-phnAB* operon codes for the enzymes required for the biosynthesis of HHQ. The first step of AQs biosynthesis is performed by PqsA, an anthranilate-coenzyme A ligase (Gallagher *et al*., 2002; Déziel *et al*., 2004; Coleman *et al*., 2008), which activates anthranilate (AA) to form anthraniloyl-coenzyme A (AA-CoA). PqsD synthesizes 2-aminobenzoylacetate-CoA (2-ABA-CoA) from AA-CoA. 2-ABA-CoA is converted into 2-aminobenzoylacetate (2-ABA) by PqsE, a pathway-specific thioesterase, although loss of its function in a *pqsE* mutant can be compensated by other thioesterases (Drees and Fetzner, 2015). 2-ABA undergoes decarboxylation and reacts with octanoate *via* a reaction catalyzed by PqsC and PqsB, thus resulting in the production of HHQ, which is then oxidized to PQS by the monooxygenase PqsH (Gallagher *et al*., 2002; Déziel *et al*., 2004; Dubern and Diggle, 2008; Schertzer *et al*., 2010). Both HHQ and PQS can bind to and activate the transcriptional regulator PqsR. The PqsR/HHQ and PqsR/PQS complexes increase the activity of the *pqsABCDE-phnAB* promoter P*pqsA*, thus accelerating HHQ and PQS biosynthesis (**Fig. 7**).

Contrary to the LasR/3OC_{12}-HSL and RhlR/C_4-HSL complexes, the PqsR/AQs complex does not act as a global regulator, and its main target promoter is P*pqsA* (the promoter of the *pqsABCDE-phnAB* operon). In fact, in the *pqs* QS system the main effector protein is not the QS signal receptor PqsR, but the multifunctional thioesterase PqsE. Besides participating in the synthesis of AQs, PqsE represses P*pqsA* activity and positively controls the expression of multiple virulence factors independently of AQs and PqsR *via* a still uncharacterized mechanism (Hazan *et al*., 2010;

Rampioni *et al*., 2010; Drees and Fetzner, 2015; Rampioni *et al*., 2016). Recent studies indicate that PqsE might affect gene expression by producing an RhlR ligand alternative to C_4-HSL, thus linking the *pqs* and *rhl* QS systems (Mukherjee *et al*., 2018; McCready *et al*., 2019).

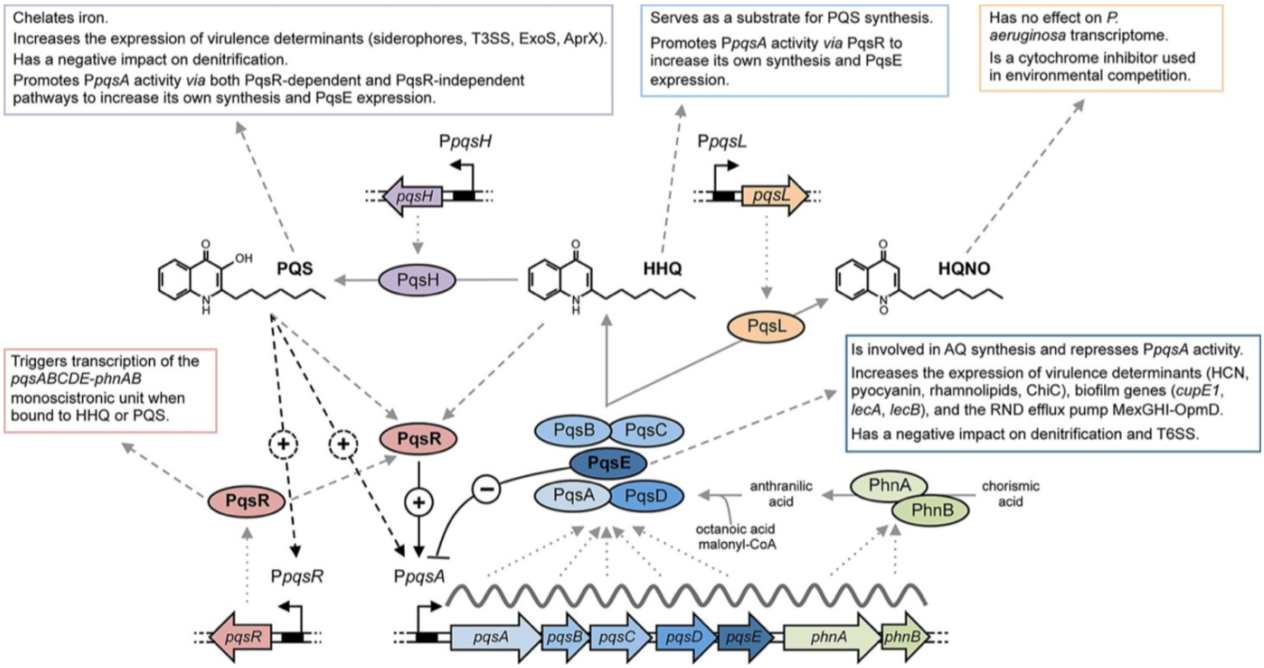


Fig. 7: Schematic representation of the *pqs* QS system in *P. aeruginosa*. The *pqsABCDE-phnAB* operon synthesizes HHQ, that binds to and activates PqsR. The PqsR-HHQ complex promotes P*pqsA* promoter activity, thus increasing HHQ and PqsE levels. Notably, the P*pqsA* promoter is the main target of the PqsR-HHQ complex. The *pqsH* and *pqsL* genes are required for PQS and HQNO biosynthesis, respectively. HQNO did not affect the *P. aeruginosa* transcriptome. PQS chelates iron triggering the iron-starvation response and increasing the transcription of virulence factor genes. PQS binds to and activates PqsR, promoting P*pqsA* activity and increasing AQs production and *pqsE* expression. Apart from its contribution to HHQ biosynthesis, PqsE influences the *P. aeruginosa* transcriptome *via* a still uncharacterized AQ-independent pathway. In this way, PqsE upregulates the expression of genes involved in virulence factor production, biofilm development, and antibiotic resistance. Conversely, PqsE down-regulates P*pqsA* activity and AQs production. Dotted grey arrows indicate gene expression; solid grey arrows represent biosynthesis; solid black arrow indicates PqsR-dependent activation (+); dashed black arrows indicate PqsR-independent activation (+); black T-line indicates negative regulation (-); dashed grey arrows represent information flow. Image modified from Rampioni *et al*., 2016.

Notably, PQS can act independently from its cognate receptor PqsR, since this QS signal molecule also acts as an iron chelator, thus inducing the iron-starvation response pathway *via* a PqsR-independent mechanism. PQS is also required for the biogenesis of outer membrane vesicles (Mashburn and Whiteley, 2005; Bredenbruch *et al*., 2006; Diggle *et al*., 2007; Rampioni *et al*., 2016; Lin *et al*., 2017).

The *pqs* QS system controls the expression of multiple virulence factors and biofilm formation, and *P. aeruginosa* mutants defective in the *pqs* QS system display attenuated pathogenicity in different plant and animal models of infection (Cao *et al*., 2001; Diggle *et al*., 2003; Déziel *et al*., 2005; Xiao *et al*., 2006; Lesic *et al*., 2007; Rampioni *et al*., 2010; Dubern *et al*., 2015).

1.6 Targeting quorum sensing: a promising antivirulence strategy

QS regulates biofilm formation and the expression of key virulence factors in plant, animal and human pathogens belonging to diverse bacterial genera. Indeed, many bacterial pathogens display markedly reduced virulence in both plant and animal infection models when their QS systems are disrupted by mutagenesis (Papenfort and Bassler, 2016). Hence, QS is considered a promising target for the development of new antivirulence drugs, and strategies aimed at inhibiting this intercellular communication system are referred to as quorum quenching (QQ) (LaSarre and Federle, 2013; Rampioni *et al*., 2014).

Since the majority of QS systems are organized in a classical scheme, which consists in the synthesis of a signal molecule that moves from a sender cell to a receiver cell, druggable QS targets are: *i*) the biosynthesis of the signal molecule in the sender cell; *ii*) the availability and functionality of the signal molecule; *iii*) the reception of the signal molecule and the consequent physiological response in the receiver cell. This recurrent scheme suggests that interference with QS can be applied at several levels by inhibiting signal molecules production, lowering signal molecule activity/availability, or inhibiting signal reception (LaSarre and Federle, 2013; Rampioni *et al*., 2014) (**Fig. 8**).

In the last decades several studies reported the identification of inhibitors of QS signal molecule biosynthesis. Christensen and co-workers, by developing a cell-free screening assay, identified two compounds in a library of over 12,000 drugs capable of reducing AHL biosynthesis by *E. coli* strains expressing recombinant LuxI-type proteins (Christensen *et al*., 2013). Nakayama and co-workers reported that the fungal metabolite ambuic acid inhibits the QS autoinducing peptide AIP biosynthesis in *S. aureus* and *Listeria* (Nakayama *et al*., 2009). In 2001, Calfee and co-workers identified methyl anthranilate as an inhibitor of the *pqs* biosynthetic pathway, by inhibiting the AQ-biosynthetic enzyme PqsA (Calfee *et al*., 2001), and more recently, compounds binding to the AQ-biosynthetic enzyme PqsD were shown to act as potent *pqs* inhibitors (Storz *et al.,* 2012; Weidel *et al.,* 2013). Although studies aimed at targeting QS signal molecule biosynthesis are rare in the QQ field, these evidences demonstrate that the inhibition of QS signal molecule production is a feasible anti-QS strategy both *in vitro* and *in vivo* for several types of autoinducers (LaSarre and Federle, 2013).

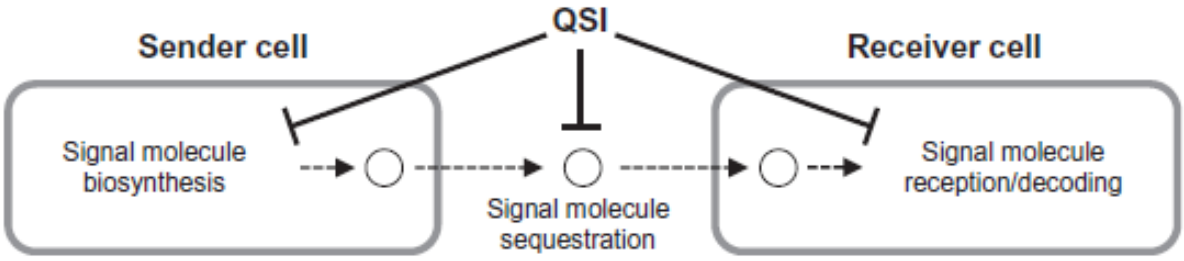


Fig. 8: Schematic representation of the potential targets of a QS inhibitor (QSI). A QSI may target the biosynthesis of the signal molecule by the sender cell, the functionality and/or availability of the signal molecule (*e.g.,* by degradation or sequestration), or the reception/decoding of the signal molecule by the receiver cell. Image modified from Rampioni *et al*., 2014.

Another QQ strategy is based on enzymes that degrade or modify QS signal molecules, in order to affect their functionality. Most enzymes identified thus far target AHL molecules, although enzymatic inactivation of other signal molecules has also been reported (Fetzner, 2015). Lactonases and acylases are two well-known examples of AHL-inactivating enzymes produced by diverse microorganisms, plants and animals (Chun *et al*., 2004; Fetzner, 2015). Acylases generally show a remarkable target specificity, as in the case of PvdQ, an acylase produced by *P. aeruginosa*, which targets the *las* signal molecule $3OC_{12}$-HSL. In a work of Koch and co-workers, a structure-based approach has been applied to PvdQ in order to find PvdQ variants that target shorter chain AHLs, such as C_8-HSL, the signal molecule produced by *Burkholderia cenocepacia*. A PvdQ variant was effective in decreasing C_8-HSL levels in *B. cenocepacia* cultures and in protecting *Galleria mellonella* larvae from *B. cenocepacia* infection (Koch *et al*., 2014). For what concerns the enzymatic inactivation of AQs signalling, Pustelny and colleagues observed that the Hod dioxygenase from *Anthrobacter nitroguajacolicus* inactivates PQS by catalysing its conversion to *N*-octanoylanthranilic acid and carbon monoxide (Pustelny *et al*., 2009). Non-enzymatic means of QS signal molecules inactivation by antibodies sequestration have also been investigated (LaSarre and Federle, 2013). Indeed, monoclonal antibodies able to sequester AHLs have been developed, limiting the bioavailability of QS signal molecules (Kaufmann *et al*., 2006).

The vast majority of QQ strategies are directed to reception inhibition. Most of these are focused on signal molecule analogues able to bind to the signal molecule cognate receptor without triggering its activation (Rampioni *et al*., 2014). Indeed, it has been hypothesized that alterations in native signal molecules can be engineered to maintain the signal molecules/receptor interaction while disrupting downstream signalling by generating non-productive signal molecules/receptor complexes that competitively block binding by the native signal molecules. Likewise, it is possible

to modify the structure of known inhibitors to enhance their activity or alter their target specificity (LaSarre and Federle, 2013).

The first QSIs targeting QS reception were identified as halogenated furanones produces by the red alga *Delisea pulchra*. These QSIs promote degradation of the LuxR proteins, and consequently hamper AHL-based QS systems in different bacterial species (Manefield *et al*., 2001 and 2002; LaSarre and Federle, 2013). As an example, the furanone-derivative compound furanone C-30 is able to inhibit the *las* QS system of *P. aeruginosa*, thus hampering the QS-dependent expression of key virulence genes required for the production of exoprotease, pyoverdin, and chitinase, and for biofilm formation and resistance to antibiotics (Hentzer *et al*., 2003). However, to conserve the steric requirements for optimal ligand/receptor interactions, antagonists have commonly been discovered through structural modification of native agonists. Moreover, high-throughput and computer-aided screens of small molecules have also expanded the list of known agonists and antagonists of various QS systems and have increased the number of structural scaffolds on which novel inhibitory compounds can be based (LaSarre and Federle, 2013; Rampioni *et al*., 2014). Recent availability of crystal structures of QS receptors like LasR has also paved the way to the identification of antivirulence drugs through *in silico* approaches. Virtual screenings and *in silico* techniques have been extensively applied for the identification of molecules hampering the *las* QS system of *P. aeruginosa* (Yang *et al*., 2009; Skovstrup *et al*., 2013; Tan *et al*., 2013; Soheili *et al*., 2015; Kalia *et al*., 2017; Xu *et al*., 2017) or QS systems in other bacteria (Ali *et al*., 2018; Ding *et al*., 2018).

1.7 Rational and aims of the PhD thesis

The post antibiotic-era is characterized by increasing emergence of MDR bacterial pathogens and by decreasing investments in the search for new antibiotics by the pharmaceutical companies. As described in the Introduction Chapter 1.1, in this context, it is mandatory to develop new antimicrobial-approaches alternative to antibiotics (Allen *et al*., 2014; Rampioni *et al*., 2017).

A promising strategy to reduce bacterial resistance to antibiotics is the development of antibiotic-adjuvants targeting resistance mechanisms (Wright, 2016). Resistance to multiple antibiotics in bacterial pathogens including the antibiotic-resistant opportunistic human pathogen *P. aeruginosa* often relies on the expression of efflux pumps extruding the antibiotic outside the cell and contributing to the MDR phenotypes. As a consequence, efflux pump inhibitors (EPIs) are of particular interest in this field (Wang *et al*., 2016).

Notably, several studies recently reported that efflux pumps are involved in *P. aeruginosa* QS and virulence other than conferring resistance to antibiotics (Aendekerk *et al*., 2005; Piddock, 2006;

Alcade-Rico *et al*., 2016). Some works have shown that the expression of different efflux pumps encoded in the *P*. *aeruginosa* genome may have an impact on the QS networks of this microorganism. The MexAB-OprM efflux pump is considered one of the most important Resistant-Nodulation cell division (RND) efflux pump of *P. aeruginosa*, since it provides intrinsic resistance to a broad spectrum of antibiotics. Recently different works support the notion that this efflux pump is directly involved in the appropriate and coordinate *las* response needed for successful host infection. Indeed, this efflux pump is highly integrated within the *las* and *rhl* QS regulons, and it is able to extrude the $3OC_{12}$-HSL QS signal molecule (Evans *et al*., 1998; Minagawa *et al*., 2012). Moreover, the overexpression of MexAB-OprM results in reduced secretion of virulence factors and $3OC_{12}$-HSL (Evans *et al*., 1998). Finally, *mexAB-OprM* defective mutants are avirulent in a mouse model of infection as well as in Madin-Darby canine kidney (MDCK) epithelial cells (Hirakata *et al*., 2002). Besides MexAB-OprM, other *P*. *aeruginosa* efflux pumps might be involved in the regulation of the expression of QS-dependent virulence factors. One of them is MexEF-OprN, an efflux pump able to extrude the QS signal HHQ (Köhler *et al*., 2001; Lamarche and Deziel, 2011; Olivares *et al*., 2012). Similar results were observed for the MexCD-OprJ efflux pump (Alcade-Rico *et al*., 2018). As it happens in the case of MexAB-OprM, mutants overexpressing MexEF-OprN or MexCD-OprJ are affected in the production of QS-regulated virulence factors and, as a consequence, are involved in the host infection process (Olivares *et al*., 2012). Another *P*. *aeruginosa* efflux pump with potential relevance in virulence is MexGHI-OprD (Aendekerk *et al*., 2005; Dietrich *et al*., 2006). It has been shown that this efflux pump is able to extrude a precursor of the phenazine pyocyanin and anthranilate, the immediate precursor of HHQ and PQS (Aendekerk *et al*., 2005). All these data suggest a role for *P*. *aeruginosa* RND efflux pumps in the regulation and export of virulence determinants, implying that certain EPIs could be endowed with antivirulence properties against *P*. *aeruginosa*. Nevertheless, EPIs are usually considered only for their antibiotic adjuvant activity, while their antivirulence potential is seldom taken into account.

On this basis, during the first part of this PhD thesis, the effect of the model EPI Phe-Arg-β-naphthylamide (PAβN, also named MC-207,110) on *P*. *aeruginosa* transcriptome and virulence has been evaluated, both *in vitro* and in an animal model of infection (Chapter 2).

Another promising strategy to treat infections caused by antibiotic-resistant pathogens is based on antivirulence drugs. These molecules specifically target virulence processes rather than bacterial viability, hence they are expected to pose a lower selective pressure for the emergence or resistance relative to antibiotics (Rasko and Sperandio, 2010). In particular, since the expression of virulence genes is controlled by QS in many bacterial pathogens, including *P*. *aeruginosa*, QS is considered

an ideal target for the development of antivirulence drugs (Rampioni *et al*., 2014; Rampioni *et al*., 2017).

As described in Chapter 1.5.2, the *pqs* QS system controls the expression of multiple virulence factors and biofilm formation in *P. aeruginosa* (Cao *et al*., 2001; Déziel *et al*., 2005; Xiao *et al*., 2006; Lesic *et al*., 2007; Rampioni *et al*., 2010; Dubern *et al*., 2015). Notably, the *pqs* QS system is active during *P*. *aeruginosa* infection (Machan *et al*., 1992; Collier *et al*., 2002; Barr *et al*., 2015), and while *P. aeruginosa* mutants impaired in the *las* QS system are frequently isolated from CF patients (Hoffman *et al*., 2009; Feltner *et al*., 2016), the highest proportion of *P. aeruginosa* strains isolated from CF lungs are proficient for AQs production (Guina *et al*., 2003; Jiricny *et al*., 2014). Moreover, AQ-based QS systems have not been described in the human microbiota so far, suggesting that drugs targeting PqsR could exert limited effects on the host microbiota. Intriguingly, recent reports indicate that the *pqs* QS system might contribute to the RhlR-dependent activation of virulence genes in the absence of functional LasR (Chen *et al*., 2019; Kostylev *et al*., 2019), and that this compensatory role might involve a yet uncharacterized signal molecule produced by PqsE and perceived by RhlR, in addition to C_4-HSL (Mukherjee *et al*., 2018). Therefore, by hampering PqsE functionality or its expression, PqsE and/or PqsR inhibitors would impact on virulence factors controlled by both the *pqs* and the *rhl* QS systems, and could be particularly active against *las*-deficient strains emerging during chronic infection.

In the last decades, many inhibitors of the *pqs* QS system have been described, proving the ability of anti-*pqs* drugs to reduce the expression of *P. aeruginosa* virulence traits both *in vitro* and in animal models of infection (Calfee *et al*., 2001; Lesic *et al*., 2007; Klein *et al*., 2012; Storz *et al*., 2012; Ilangovan *et al*., 2013; Weidel *et al*., 2013; Zender *et al*., 2013; Lu *et al*., 2014; Starkey *et al*., 2014; Maura *et al*., 2017; Maura and Rahme 2017; Soukarieh *et al*., 2018a). Despite the promise of anti-*pqs* agents for the treatment of *P. aeruginosa* infections, none of these molecules has entered clinical trials so far, likely due to poor pharmacological properties of the inhibitors (Soukarieh *et al*., 2018b).

A possible solution to this problem is searching for QS inhibitors among molecules already approved for their use in humans. This strategy is known as drug repurposing, or drug repositioning, and shows remarkable advantages compared to *de novo* drug discovery. Indeed, the drug repurposing approach is endowed with higher probability to identify safe and bioavailable molecules, that could easily and rapidly move from the laboratory to the clinical practice (Ashburn and Thor, 2004; Wermuth, 2006; Imperi *et al*., 2013; Rampioni *et al*., 2017).

Also *in silico* approaches have been proved as valid aids to conventional drug-discovery programmes. The main advantage of using computer-based approaches for drug discovery is to save

time and costs required for the wet-lab screening of broad libraries of compounds. Indeed, *in silico* approaches allow to select promising candidates *via* virtual screening, and to subsequently verify by wet-lab experiments the functionality of a reduced number of molecules (Reuter *et al*., 2015).

On this basis, the main aim of the second part of this PhD thesis was to use drug repurposing and *in silico* screening approaches to identify FDA-approved drugs able to reduce *P. aeruginosa* virulence potential by targeting different elements of the *pqs* QS system. The works reported in Chapters 3 and 4 are focused on the inhibition of the transcriptional regulator PqsR, while the manuscript in preparation presented in Chapter 5 aims at identifying new inhibitors of PqsE, the main effector protein of the *pqs* QS system.

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Chapter 2

Effect of efflux pump inhibition on *Pseudomonas aeruginosa* transcriptome and virulence

Giordano Rampioni[1], Cejoice Ramachandran Pillai[1], Francesca Longo[1], Roslen Bondì[1], **Valerio Baldelli**[1], Marco Messina[1], Francesco Imperi[2], Paolo Visca[1] and Livia Leoni[1]

[1] Department of Science, University Roma Tre, Rome, Italy; [2] Department of Biology and Biotechnology "Charles Darwin", Sapienza University of Rome.

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Preface to Chapter 2

As discussed in the Introduction section, serious economic and health problems caused by the emergence of MDR pathogens have fostered research into novel antibiotic adjuvants. Since the active efflux of antibiotics *via* efflux pumps contributes to the bacterial MDR phenotype, the development of EPIs is considered a promising adjuvant strategy. Moreover, in the last decades, the notion that RND efflux pumps could play a role in bacterial infection is emerging, implying that certain EPIs could also display antivirulence activity. Nevertheless, EPIs are usually considered only for their properties as antibiotic adjuvants, while their antivirulence potential is seldom taken into account.

In the opportunistic human pathogen *P. aeruginosa* multiple efflux pumps belonging to the RND family contribute to the MDR phenotype, and have been associated to the secretion of virulence factors and of QS signal molecules.

On this basis, in this work we investigated the impact of a well-characterized EPI, PAβN, on *P. aeruginosa* transcriptome and virulence. Briefly, data collected in this manuscript demonstrate that PAβN protects *Galleria mellonella* larvae from *P. aeruginosa* infection, and that the protective role exerted by PAβN *in vivo* well correlates with *in vitro* reduction of virulence-related phenotypes. Overall, this study highlights that EPIs should be tested not only for their ability to increase antibiotic activity, but also for their antivirulence properties.

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Effect of efflux pump inhibition on *Pseudomonas aeruginosa* transcriptome and virulence

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Giordano Rampioni[1], Cejoice Ramachandran Pillai[1,3], Francesca Longo[1], Roslen Bondi[1], Valerio Baldelli[1], Marco Messina[1], Francesco Imperi[2], Paolo Visca[1] & Livia Leoni[1]

Efflux pumps of the resistance-nodulation-cell-division (RND) family increase antibiotic resistance in many bacterial pathogens, representing candidate targets for the development of antibiotic adjuvants. RND pumps have also been proposed to contribute to bacterial infection, implying that efflux pump inhibitors (EPIs) could also act as anti-virulence drugs. Nevertheless, EPIs are usually investigated only for their properties as antibiotic adjuvants, while their potential anti-virulence activity is seldom taken into account. In this study it is shown that RND efflux pumps contribute to *Pseudomonas aeruginosa* PAO1 pathogenicity in an insect model of infection, and that the well-characterized EPI Phe-Arg-β-naphthylamide (PAβN) is able to reduce *in vivo* virulence of the *P. aeruginosa* PAO1 laboratory strain, as well as of clinical isolates. The production of quorum sensing (QS) molecules and of QS-dependent virulence phenotypes is differentially affected by PAβN, depending on the strain. Transcriptomic and phenotypic analyses showed that the protection exerted by PAβN from *P. aeruginosa* PAO1 infection *in vivo* correlates with the down-regulation of key virulence genes (*e.g.* genes involved in iron and phosphate starvation). Since PAβN impacts *P. aeruginosa* virulence, anti-virulence properties of EPIs are worthy to be explored, taking into account possible strain-specificity of their activity.

Introduction of any antibiotic in the clinical practice invariably results in ensuing resistance. The indiscriminate use of antibiotics and the increasing emergence of antibiotic resistance has drained the research in this field, resulting in a discovery rate of new antibiotics unable to compensate the escalation of antibiotic resistance in common pathogens[1,2].

The serious economic and health problems caused by multi-drug resistant (MDR) pathogens have fostered research not only into new antibiotics but also into novel adjuvants[1,2]. Different from conventional antibiotics, adjuvants share the distinctive feature of targeting bacterial factors not essential for growth, such as virulence determinants (*e.g.* toxins, adhesins and tissue-degrading enzymes) or antibiotic resistance determinants (*e.g.* efflux pumps, antibiotic inactivating enzymes). Such treatments are aimed at facilitating host immune response and/or antibiotic action in clearing the infection. As to anti-virulence drugs, they are predicted to exert a low selective pressure for the emergence of resistant strains, since they do not directly inhibit bacterial growth[2–4].

The active efflux of antibiotics *via* efflux pumps contributes to the bacterial MDR phenotype, and the development of efflux pump inhibitors (EPIs) is considered a promising adjuvant strategy[2,5–7]. Efflux pumps are categorized into different families on the basis of the amino acid sequence, the energy source required to drive antibiotic export, and the substrate specificity. The resistance-nodulation-cell-division (RND) family of efflux pumps is considered a viable target for the development of drugs aimed at increasing bacterial susceptibility to antibiotics, due to their prominent contribution to the MDR phenotype and to the absence of human homologues[2,5–7]. Notably, evidence is emerging that some RND transporters are also involved in the efflux of bacterial factors important for virulence[8,9]. These preliminary observations suggest that EPIs targeting RND efflux pumps could also affect bacterial virulence, in addition to facilitating antibiotic activity.

Pseudomonas aeruginosa is one of the most dreaded opportunistic pathogens, representing a paradigm of Gram-negative MDR "superbug" for which effective therapeutic options are limited. The ability of *P. aeruginosa* to cause a wide range of infections in humans is due to its capacity to produce a large repertoire of virulence factors

[1]Department of Science, University Roma Tre, Rome, Italy. [2]Department of Biology and Biotechnology "Charles Darwin", Sapienza University of Rome, Rome, Laboratory affiliated to Istituto Pasteur Italia – Fondazione Cenci Bolognetti, Rome, Italy. [3]Present address: Inter University Centre for Bioscience, Kannur University, Palayad, Kerala, India. Correspondence and requests for materials should be addressed to L.L. (email: livia.leoni@uniroma3.it)

and, ultimately, respond and adapt to harsh conditions as those imposed by the host immune response and antibiotic exposure[1]. The pathogenic potential of *P. aeruginosa* relies on the coordinated expression of a large array of virulence factors, the majority of which are positively controlled by quorum sensing (QS)[10]. The three main *P. aeruginosa* QS systems are based on the production of specific signal molecules, namely the *N*-acyl-homoserine lactones (AHLs) *N*-3-oxododecanoyl-homoserine lactone ($3OC_{12}$-HSL) and *N*-butanoyl-homoserine lactone (C_4-HSL), and the 2-alkyl-4-quinolones (AQs) 2-heptyl-4-hydroxyquinoline (HHQ) and 2-heptyl-3-hydroxy-4-quinolone (PQS). These systems are hierarchically organized, since $3OC_{12}$-HSL is required for optimal production of all QS signals[10]. Moreover, the *P. aeruginosa* genome is predicted to encode multiple RND efflux pumps, four of which are of clinical importance for MDR, namely MexAB-OprM, MexCD-OprJ, MexEF-OprN and MexXY-OprM, and are frequently found to be up-regulated in clinical isolates[11].

The MexAB-OprM is considered as the most important RND efflux pump for *P. aeruginosa*, since it is constitutively expressed and provides intrinsic resistance to a broad spectrum of antibiotics[11]. The emergence of *P. aeruginosa* MexAB-OprM over-expressing mutants in a rat model of acute pneumonia suggests that this efflux pump confers a selective advantage *in vivo*, also in the absence of antibiotic treatment[12]. Moreover, *P. aeruginosa* lacking the MexAB-OprM efflux pump could not invade Madin-Darby canine kidney (MDCK) epithelial cells, and invasion could be restored by supplementation with culture supernatants obtained from MDCK cells infected with wild type *P. aeruginosa*[13]. In addition, it was reported that MexAB-OprM participates in the efflux of $3OC_{12}$-HSL[14,15] and that MexEF-OprN and MexGHI-OprM could be involved in transport of some AQs[16,17]. All these data argue for a role of MexAB-OprM and other *P. aeruginosa* RND efflux pumps in the export of virulence determinants contributing to invasiveness and infection.

Phe-Arg-β-naphthylamide (PAβN, also named MC-207,110) is the most active and best studied inhibitor of *P. aeruginosa* RND efflux pumps. It was discovered in a screen for adjuvants of the fluoroquinolone levofloxacin, carried out in a *P. aeruginosa* strain that over-expressed MexAB-OprM, though this EPI was also found to be active against other RND pumps like MexCD-OprJ and MexEF-OprN[18,19], indicating that PAβN is a broad spectrum EPI[2,5]. In agreement with the results obtained with MexAB-OprM-deficient cells[13], it has been shown that PAβN reduces the invasiveness of *P. aeruginosa* in MDCK cells[20], suggesting that this compound could also inhibit some *P. aeruginosa* virulence traits. Indeed, PAβN decreases the production of the QS signals $3OC_{12}$-HSL and C_4-HSL, and of some QS-dependent virulence phenotypes in *P. aeruginosa* MDR isolates from urinary and wound infections[21]. Beside its role as EPI, it has been reported that PAβN can affect *P. aeruginosa* membrane permeability, and consequently bacterial growth, when used beyond certain concentrations (~50–200 μM)[19,22]. This side effect is particularly relevant in efflux pumps deficient genetic backgrounds[19,22], and complicates the understanding of the mechanism of action of PAβN as an EPI and as a virulence inhibitor.

This study is aimed at investigating the effect of PAβN on the general physiology and virulence of the widely studied model strain *P. aeruginosa* PAO1, by performing microarray analysis and *Galleria mellonella* infection experiments. We also provide evidence that PAβN affects to different extent virulence-related phenotypes in *P. aeruginosa* clinical isolates.

Results and Discussion

PAβN treatment extensively affects the *P. aeruginosa* transcriptome. A major requirement for anti-virulence drugs is their ability to inhibit virulence traits without affecting cell viability[3,4]. Hence, PAβN concentrations not affecting the growth rate of *P. aeruginosa* (*i.e.* ≤50 μM; Fig. S1) were used throughout this study.

The transcriptional profiles of *P. aeruginosa* PAO1 grown to an A_{600} of 2.5 in LB in the presence or in the absence of 27 μM PAβN were compared by means of high-density oligonucleotide microarrays, by using Affimetrix GeneChip® for *P. aeruginosa* PAO1. Following statistical validation of the dataset, only genes with a fold change >2 and a *p*-value <0.05 were considered for further analysis. Selected genes significantly up- or down-regulated by PAβN are listed in Tables 1 and 2, respectively (the complete gene list is given in Table S1, Supporting Information).

The transcription of 108 genes was significantly affected by PAβN (Table S1), corresponding to about 1.9% of *P. aeruginosa* PAO1 genes[23]. Of these, 39 genes were up-regulated and 69 genes were down-regulated in the presence of PAβN (Table S1). Among the 39 genes up-regulated by PAβN, the most represented categories comprise genes involved in nitrogen metabolism (*nir*, *nor* and *nos* genes; 33.3% of up-regulated genes) and in biosynthesis of phenazines (*phz* genes; 10.2% of up-regulated genes) (Tables 1 and S1). Phenazines constitute a group of nitrogen-containing heterocyclic compounds, including the virulence factor pyocyanin[24].

Among the 69 down-regulated genes, 46 genes (66.7%) were previously reported to be repressed by iron[25]. These include almost all the genes involved in the biosynthesis, uptake and regulatory response to the siderophores pyoverdine and pyochelin, including the *pvdS* sigma factor gene, which also activates the expression of *prpL* protease and *toxA* toxin genes (Tables 2 and S1). Moreover, metabolic and virulence genes previously shown to be induced in response to iron starvation were down-regulated by PAβN, including fumarate hydratase (*fumC*1), superoxide dismutase (*sodM*) and protease (*aprX*) genes (Table 2). The negative effect exerted by PAβN on the iron-starvation response pathway correlates with previous studies showing that PAβN synergizes with iron chelators in reducing the growth rate and biofilm formation of *P. aeruginosa*[26]. Moreover, PAβN treatment caused down-regulation of genes repressed by phosphate availability, including *pho*, *pst* and *pnh* genes[27] (Tables 2 and S1). Overall, the expression of many genes important for *P. aeruginosa* pathogenicity, such as *pvdS*, *phoB*, *pstS* and *vreR*[25–30], was strongly repressed by PAβN (Table 2).

The differential expression of selected genes identified as PAβN-controlled was validated by quantitative reverse transcription PCR (qRT-PCR) analysis performed on *P. aeruginosa* cultures grown under the same conditions as those used for the microarray experiment. The qRT-PCR results matched the microarray data, since the mRNA level of the *norB* and *qteE* genes increased in the presence of 27 μM PAβN, while the mRNA level of the *pvdQ*, *aprX*, *fumC*1, *pvdS* and *sodM* genes decreased in the same conditions (Fig. 1A).

PA number[a]	Gene name[a]	Fold change[b]	Product name[a]
PA0509[*]	*nirN*	2.27	NirN
PA0510[*]	*nirE*	2.33	NirE
PA0511[*]	*nirJ*	2.23	hemed_1 biosynthesis protein NirJ
PA0514[*]	*nirL*	2.3	hemed_1 biosynthesis protein NirL
PA0516[*]	*nirF*	2.3	hemed_1 biosynthesis protein NirF
PA0517[*]	*nirC*	3.53	probable *c*-type cytochrome precursor
PA0518[*]	*nirM*	3.32	cytochrome c_{551} precursor
PA0519[*]	*nirS*	4.48	nitrite reductase precursor
PA0523[*]	*norC*	2.87	nitric-oxide reductase subunit C
PA0524[*]	*norB*	5.51	nitric-oxide reductase subunit B
PA0525[*]	*norD*	2.19	probable denitrification protein NorD
PA1901[§]	*phzC1/C2*	2.24	phenazine biosynthesis protein PhzC
PA1902[§]	*phzD1/D2*	2.26	phenazine biosynthesis protein PhzD
PA1903[§]	*phzE1/E2*	2.22	phenazine biosynthesis protein PhzE
PA1904[§]	*phzF1/F2*	2.11	probable phenazine biosynthesis protein
PA2593	*qteE*	2.06	quorum threshold expression element, QteE
PA3392[*]	*nosZ*	2.16	nitrous-oxide reductase precursor
PA4810[*]	*fdnI*	2.22	nitrate-inducible formate dehydrogenase, γ subunit

Table 1. Selected genes whose transcription is up-regulated by PAßN. [a]PA number, gene name and product name are from the *Pseudomonas* Genome Database[23]. Genes previously reported as controlled by $3OC_{12}$-HSL are in bold characters[36–38]. [*]Genes involved in nitrogen metabolism; [§]Genes involved in phenazines biosynthesis. [b]Fold change in gene expression in *P. aeruginosa* PAO1 grown in LB supplemented with 27 µM PAßN with respect to the same strain grown in LB.

Despite the concentration of PAβN used in this experiment (27 µM) is not expected to destabilize the cell membrane of wild type PAO1, the possibility that this EPI controls some of the identified genes *via* membrane perturbation rather than efflux pump inhibition cannot be ruled out. However, the specificity of PAβN effect as an EPI in our settings is supported by the observation that only 2 out of the 108 PAβN-regulated genes (*i.e. phzF1* and PA4139; Table S1) were identified in a previous microarray analysis performed with sub-MIC concentration of the membrane destabilizing peptide polymyxin E (colistin)[31] (Table S1). Furthermore, none of the genes whose expression was altered upon exposure to sub-MIC concentration of polymyxin B[32] were affected by PAβN.

Additional qRT-PCR analyses were also performed to further support the primary role of PAβN as an EPI. Since previous reports showed that 1 mM Mg^{2+} completely abolished the permeabilizing effect exerted by PAβN on bacterial membranes[19,33], the effect of PAβN on the mRNA level of *qteE*, *pvdS* and *sodM* was compared in the absence and in the presence of 1 mM $MgSO_4$. The expression of the same genes was also evaluated in a *P. aeruginosa* efflux-deficient mutant (PAO1-KP Δefflux) carrying deletions in genes encoding the four major RND efflux pumps of this bacterium, namely MexAB-OprM, MexCD-OprJ, MexEF-OprN and MexXY-OprM[34] (Table S2). Since this mutant was not generated in our laboratory, and it is well known that PAO1 strains maintained in different laboratories disclose genotype variability[35], strain PAO1-KP Δefflux was compared with its isogenic wild type strain PAO1-KP[34].

This experiment revealed that 27 µM PAβN increases the mRNA level of *qteE* and decreases the mRNA level of *pvdS* and *sodM* irrespective of the presence or the absence of $MgSO_4$, both in PAO1 (Fig. 1B) and in PAO1-KP (Fig. 1C). Notably, the fold change in the mRNA level of the tested genes was similar in PAO1-KP supplemented with PAβN and in PAO1-KP Δefflux relative to untreated PAO1-KP (Fig. 1C), supporting the conclusion that the alteration in gene expression caused by PAβN relies on its ability to inhibit efflux pumps, rather than on its membrane permeabilizing effect. This is in line with previous reports suggesting that PAβN has a strong activity as an efflux pump inhibitor and a weak, concentration-dependent activity in destabilizing the cell envelope, both in *P. aeruginosa* and in *Escherichia coli*[19,33]. Unfortunately, the well-known toxic effect of PAβN to efflux pumps-deficient *P. aeruginosa* cells[2,19] does not allow to investigate the effect of PAβN on the PAO1-KP Δefflux strain.

Overall, these data indicate that the PAβN-dependent inhibition of efflux pumps has a profound impact on the *P. aeruginosa* transcriptome.

PA number[a]	Gene name[a]	Fold change[b]	Product name[a]
PA0672[∫]	*hemO*	−4.81	hemeoxygenase
PA0676[∫]	*vreR*	−4.85	sigma factor regulator, VreR
PA0707	*toxR*	−2.12	transcriptional regulator ToxR
PA1245	*aprX*	−3.44	AprX
PA1912[∫]	*femI*	−2.35	ECF sigma factor, FemI
PA2385[∫]	*pvdQ*	−2.98	$3OC_{12}$-homoserine lactone acylasePvdQ
PA2386[∫]	*pvdA*	−3.97	L-ornithine N^5-oxygenase
PA2394[∫]	*pvdN*	−2.85	PvdN
PA2395[∫]	*pvdO*	−2.32	PvdO
PA2396[∫]	*pvdF*	−3.22	pyoverdinesynthetase F
PA2397[∫]	*pvdE*	−3.16	pyoverdine biosynthesis protein PvdE
PA2398[∫]	*fpvA*	−2.03	ferripyoverdine receptor
PA2399[∫]	*pvdD*	−3.32	pyoverdinesynthetase D
PA2400[∫]	*pvdJ*	−3.36	PvdJ
PA2413[∫]	*pvdH*	−3.58	L-2,4-diaminobutyrate:2-ketoglutarate 4-aminotransferase
PA2424[∫]	*pvdL*	−3.73	PvdL
PA2425[∫]	*pvdG*	−2.58	PvdG
PA2426[∫]	*pvdS*	−10.48	sigma factor PvdS
PA2570	*lecA*	−2.42	LecA
PA3377[◊]	*phnJ*	−21.1	conserved hypothetical protein
PA3407[∫]	*hasAp*	−4.15	heme acquisition protein HasAp
PA3530[∫]	*bfd*	−2.54	bacterioferritin-associated ferredoxinBfd
PA4221[∫]	*fptA*	−2.52	Fe(III)-pyochelin outer membrane receptor precursor
PA4224[∫]	*pchG*	−2.05	pyochelin biosynthetic protein PchG
PA4225[∫]	*pchF*	−2.26	pyochelinsynthetase
PA4226[∫]	*pchE*	−2.06	dihydroaeruginoic acid synthetase
PA4228[∫]	*pchD*	−2.09	pyochelin biosynthesis protein PchD
PA4230[∫]	*pchB*	−2.69	salicylate biosynthesis protein PchB
PA4468	*sodM*	−11.65	superoxide dismutase
PA4470	*fumC1*	−8.96	fumaratehydratase
PA4708[∫]	*phuT*	−3.17	heme-transport protein, PhuT
PA4709[∫]	*phuS*	−3.37	PhuS
PA4710[∫]	*phuR*	−4.65	heme/hemoglobin uptake outer membrane receptor PhuR
PA5360[◊]	*phoB*	−15.28	two-component response regulator PhoB
PA5365[◊]	*phoU*	−9.4	phosphate uptake regulatory protein PhoU
PA5366[◊]	*pstB*	−14.02	ATP-binding component of ABC phosphate transporter
PA5367[◊]	*pstA*	−14.17	membrane protein component of ABC phosphate transporter
PA5369[◊]	*pstS*	−23.49	periplasmic phosphate-binding protein, PstS

Table 2. Selected genes whose transcription is down-regulated by PAßN. [a]PA number, gene name and product name are from the *Pseudomonas* GenomeDatabase[23]. Genes previously reported as controlled by $3OC_{12}$-HSL are in bold characters[36–38]. [∫]Genes previously reported to be controlled by iron starvation[25]; [◊]Genes previously reported to be controlled by phosphate starvation[27]. [b]Fold change in gene expression in *P. aeruginosa* PAO1 grown in LB supplemented with 27 µM PAßN with respect to the same strain grown in LB.

PAβN treatment affects *P. aeruginosa* virulence-related phenotypes. The expression of the genes involved in $3OC_{12}$-HSL and C_4-HSL synthesis and reception (*i.e. lasI-lasR*, and *rhlI-rhlR*, respectively) and of the vast majority of genes known to be controlled by these QS signal molecules[36–38] was not inhibited by PAβN in the microarray analysis (Table S1). This result and the positive effect exerted by PAβN on the expression of pyocyanin biosynthetic genes was not expected, since PAβN was previously shown to negatively affect the transcription of the *las* and *rhl* QS genes and the expression of phenotypes controlled by QS (*i.e.* pyocyanin, proteases and elastase production) in *P. aeruginosa* strains isolated from urinary tract and wound infections[21]. To clarify this issue, we measured the level of QS signals (*i.e.* $3OC_{12}$-HSL, C_4-HSL and HHQ/PQS) and of the above-mentioned QS-dependent virulence factors in supernatants collected from *P. aeruginosa* PAO1 cultures in LB supplemented with increasing concentrations of PAβN (experimental details are given in Materials and Methods). Results showed that the production of $3OC_{12}$-HSL is significantly increased in the presence of PAβN concentrations $\geq$9 µM (Fig. 2A), while C_4-HSL and HHQ/PQS production was not affected even at the maximum PAβN concentration tested (50 µM; Fig. 2A). The observation that PAβN increases $3OC_{12}$-HSL production both in PAO1 and in PAO1-KP also in the presence of 1 mM $MgSO_4$, and that $3OC_{12}$-HSL levels are higher in the supernatant of PAO1-KP Δefflux relative to

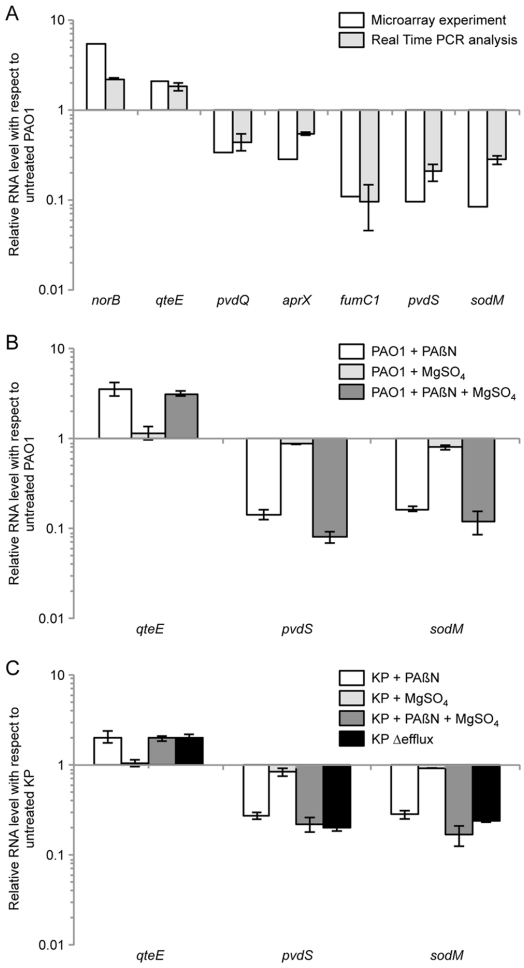

Figure 1. Validation of the microarray data by qRT-PCR. mRNA levels of the indicated genes quantified by qRT-PCR in: (**A**) The *P. aeruginosa* PAO1 strain grown to an A_{600} of 2.5 in LB supplemented with 27 µM PAßN, relative to the same strain grown in LB (grey bars), in comparison with microarray data for the same genes (white bars); (**B**) The *P. aeruginosa* PAO1 strain grown to an A_{600} of 2.5 in LB supplemented with 27 µM PAßN (white bars), with 1 mM $MgSO_4$ (light-grey bars), or with 27 µM PAßN plus 1 mM $MgSO_4$ (dark-grey bars) relative to the same strain grown in LB; (**C**) The *P. aeruginosa* PAO1-KP strain grown to an A_{600} of 2.5 in LB supplemented with 27 µM PAßN (white bars), with 1 mM $MgSO_4$ (light-grey bars), or with 27 µM PAßN plus 1 mM $MgSO_4$ (dark-grey bars), and the *P. aeruginosa* PAO1-KP Δefflux strain grown to an A_{600} of 2.5 in LB (black bars), relative to the PAO-KP strain grown in LB. The average of two independent analyses performed on three technical replicates is shown with SD.

the supernatant of PAO1-KP (Fig. 2B) indicates that the effect of PAβN on $3OC_{12}$-HSL can be ascribed to the inhibition of efflux pumps, rather than to membrane perturbation. Further experiments carried out with transcriptional fusions confirmed that PAβN did not affect *lasI* and *lasR* promoter activity in PAO1 (Fig. S2), in agreement with the microarray data. Hence, the positive effect exerted by PAβN on $3OC_{12}$-HSL production in *P. aeruginosa* does not appear to occur at the transcriptional level. Interestingly, PAβN reduced the transcription of *pvdQ* (Fig. 1 and Table 2), a gene coding for the PvdQ acylase, an enzyme responsible for $3OC_{12}$-HSL degradation[39]. Therefore, the increase in $3OC_{12}$-HSL level caused by PAβN could be due, at least in part, to a decreased degradation of this signal molecule as a consequence of *pvdQ* down-regulation. In addition, PAβN enhanced the transcription of *qteE* (Fig. 1 and Table 1), a gene coding for a protein that hampers the activity of the $3OC_{12}$-HSL-receptor protein LasR[40]. The enhanced expression of QteE in PAβN-treated cells may result in reduced levels of active LasR, thus counterbalancing the effect of increased $3OC_{12}$-HSL levels on the transcription of LasR-dependent genes.

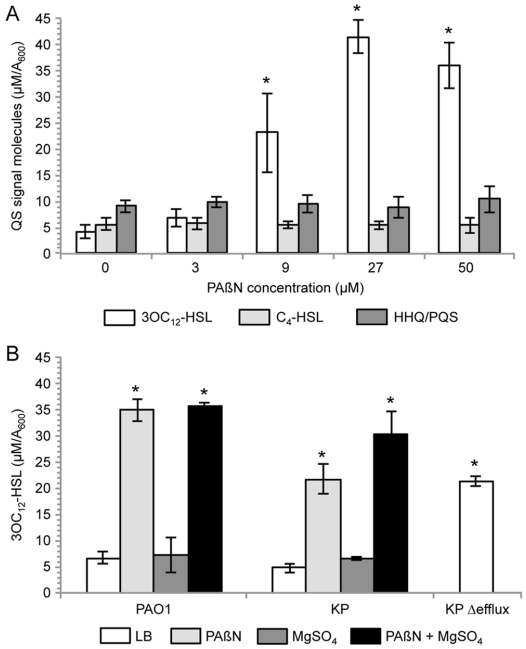

Figure 2. Effect of PAβN on QS signal molecules production. (**A**) $3OC_{12}$-HSL (white bars), C_4-HSL (light-grey bars) and HHQ/PQS (dark-grey bars) production in *P. aeruginosa* PAO1 stationary phase cultures grown in LB or in LB supplemented with PAßN at the concentrations indicated below the histogram. (**B**) $3OC_{12}$-HSL production in the indicated strains grown in LB (white bars), or in LB supplemented with 27 μM with PAßN (light-grey bars), with 1 mM $MgSO_4$ (dark-grey bars), or with 27 μM PAßN plus 1 mM $MgSO_4$ (black bars). The average of at least three independent experiments is reported with SD; statistical significance with respect to the untreated sample is indicated with one asterisk ($p < 0.05$).

As shown in Fig. 3A, pyocyanin production increased in the presence of PAβN concentrations ≥9 μM by comparison with the untreated control. Conversely, PAβN did not affect the production of proteases and elastase (Fig. 3A). These results are in agreement with the microarray data, showing that PAβN increases the transcription of pyocyanin biosynthetic genes in PAO1, without affecting the mRNA level of proteases and elastase genes (Tables 1 and S1). Therefore, it can be argued that the positive effect exerted by PAβN on pyocyanin production in *P. aeruginosa* PAO1 is likely exerted *via* a QS-independent pathways controlling phenazines biosynthesis. The increase in pyocyanin levels caused by PAβN treatment was maintained in the presence of $MgSO_4$ in both PAO1 and PAO1-KP, although the absolute pyocyanin levels were lower in PAO1-KP than in PAO1 (Fig. 3B). Moreover, pyocyanin production in PAO1-KP Δefflux was significantly increased relative to PAO1-KP (Fig. 3B). These observations suggest that pyocyanin production is affected by PAβN *via* specific EPI activity. High sequence homology of pyocyanin biosynthetic operons $phzA_1$-G_1 and $phzA_2$-G_2 in PAO1[23] does not allow discriminating their mRNAs *via* microarray or qRT-PCR analyses. Therefore, transcriptional fusions between the P$phzA_1$ or P$phzA_2$ promoters and the *luxCDABE* operon[41] were used to clarify the effect of PAβN on the pyocyanin biosynthetic operons. As shown in Fig. 3C, PAβN increased the activity of the P$phzA_1$ promoter, while it did not affect P$phzA_2$. Also in this case, the effect of PAβN was not alleviated in the presence of $MgSO_4$ (Fig. 3C).

Additional phenotypic analyses revealed that 50 μM PAβN caused a 8-fold and 2-fold reduction of twitching and swimming motility compared with the untreated control, respectively (Fig. 4A). Moreover, swarming motility was completely abrogated in the presence of 6.25 μM PAβN (Fig. 4B), in agreement with previous observations on *P. aeruginosa* clinical isolates[21]. A substantial decrease in swimming, twitching and swarming was also observed in PAO1-KP Δefflux relative to PAO1-KP (Fig. 4C), indicating that the effect of PAβN on these phenotypes is mainly dependent on efflux pumps inhibition. The negative effect exerted by PAβN on *P. aeruginosa* motility seems to be unrelated to an altered expression of pili, flagella or rhamnolipids biosynthetic genes, as suggested by the microarray results (Table S1). However, motility is a pleiotropic and energetically demanding process, strongly affected by nutrients availability. In this context, the metabolic alteration caused by PAβN (*e.g.* up-regulation of nitrogen metabolism genes and down-regulation of iron-uptake genes; Tables 1 and S1) could explain the effect of this EPI on *P. aeruginosa* motility. Moreover, it is well documented that *pvdQ* is up-regulated in swarming cells, while its deletion abrogates swarming motility in *P. aeruginosa*[42]. Thus the PAβN-mediated reduction of *pvdQ* transcription (Fig. 1 and Table 1) correlates with the strong inhibitory effect exerted by this EPI on swarming motility.

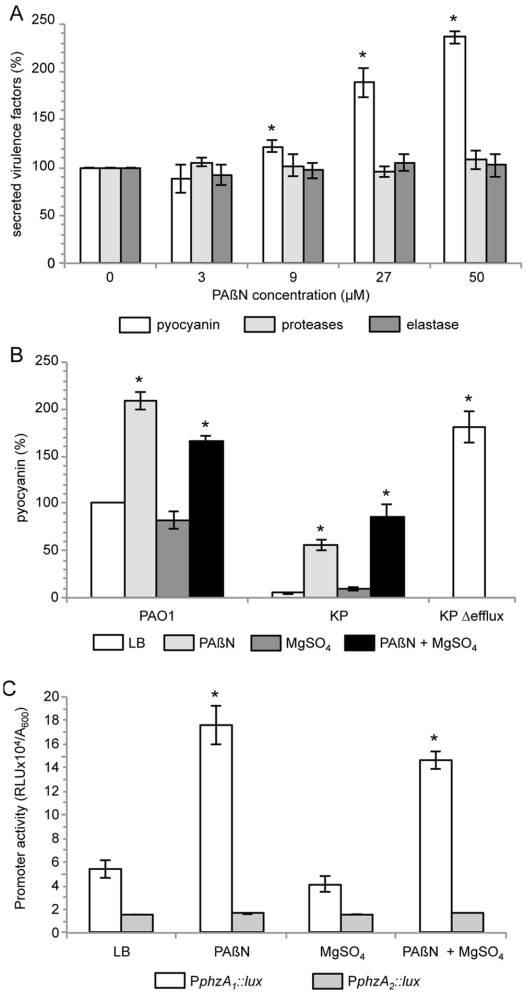

Figure 3. Effect of PAβN on pyocyanin production. (**A**) Pyocyanin (white bars), proteases (light-grey bars) and elastase (dark-grey bars) production in *P. aeruginosa* PAO1 cultures grown in LB in the absence or in the presence of PAßN at the concentrations indicated below the histogram. (**B**) Pyocyanin production in the indicated strains grown in LB (white bars), or in LB supplemented with 27 µM PAßN (light-grey bars), with 1 mM $MgSO_4$ (dark-grey bars), or with 27 µM PAßN plus 1 mM $MgSO_4$ (black bars). Pyocyanin production of strain PAO1 grown in LB is considered as 100%. (**C**) P*phzA*$_1$ (white bars) and P*phzA*$_2$ (grey bars) promoter activity measured in *P. aeruginosa* PAO1 cultures grown in LB or in LB supplemented with 27 µM PAßN, with 1 mM $MgSO_4$, or with 27 µM PAßN plus 1 mM $MgSO_4$, as indicated below the histogram. The average of at least three independent experiments is reported with SD; statistical significance with respect to the untreated sample is indicated with one asterisk ($p < 0.05$).

In summary, the effects of PAβN on *P. aeruginosa* PAO1 QS and virulence-related phenotypes are in agreement with the microarray analysis, and confirm that this molecule increases $3OC_{12}$-HSL and pyocyanin levels *via* specific EPI activity, without affecting the production of other QS signal molecules and of the QS-controlled virulence factors elastase and proteases.

***In vivo* anti-virulence activity of PAβN.** The above results show that PAβN ($\leq$50 µM) inhibits *P. aeruginosa* PAO1 processes related to motility and acquisition of micronutrients (*i.e.* phosphate and iron), relevant for pathogenesis in several models of acute infection[25,27–30]. On the other hand, in the PAO1 strain PAβN stimulates the production of both $3OC_{12}$-HSL and pyocyanin, both playing a positive role in *P. aeruginosa* virulence[10,28,43–45].

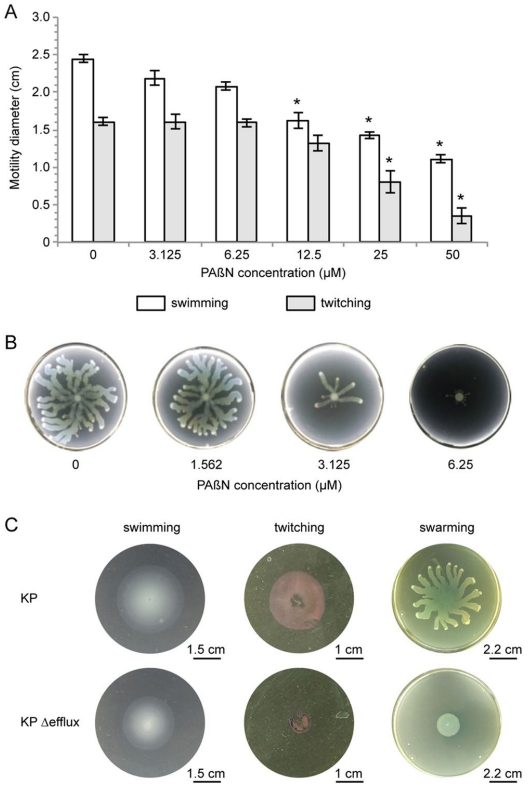

Figure 4. Effect of PAβN on *P. aeruginosa* motility. (**A**) *P. aeruginosa* swimming (white bars) and twitching (grey bars) motility in the absence or in the presence of PAßN at the concentrations indicated below the histogram. The average diameter of five independent experiments is reported with SD; statistical significance with respect to the untreated control sample is indicated with one asterisk ($p < 0.05$). (**B**) Images of *P. aeruginosa* swarming plates supplemented with the indicated concentrations of PAßN. (**C**) Images of *P. aeruginosa* PAO1-KP and PAO1-KP Δefflux swimming, twitching and swarming plates. For the swarming assay, images of the entire plates are reported, while for swimming and twitching assays, magnification of the halos are shown. Twitching halos were stained with crystal violet. One representative experiment out of three independent replicates is shown for (**B**) and (**C**).

These puzzling *in vitro* results raise the question of what kind of effect PAβN has on *P. aeruginosa* PAO1 virulence *in vivo*. To tackle this issue, the effect of PAβN on *P. aeruginosa* virulence was assessed in *Galleria mellonella*, an insect model of infection that well correlates with murine acute infection models[28]. We firstly aimed at validating the infection model by testing the virulence of the efflux-deficient mutant PAO1-KP Δefflux compared to its isogenic wild type strain PAO1-KP. The survival rate of *G. mellonella* larvae 24 h after the challenge with the tested *P. aeruginosa* strains is shown in Fig. 5A. Nearly all larvae infected with wild type *P. aeruginosa* (PAO1-KP) were killed at the maximum infective dose tested (ca. 45 colony forming units or CFU/larva), and larvae survival increased as a function of decreasing infective dose. Conversely, >50% of larvae challenged with the efflux-deficient mutant PAO1-KP Δefflux survived also at the maximum infective dose tested. The differences between the wild type and the efflux-deficient mutant survival curves were evident at all infection doses (Fig. 5A). To the best of our knowledge, this result is the first demonstration that genetic inactivation of RND efflux pumps causes a decrease in *P. aeruginosa* pathogenic potential *in vivo*. Interestingly, a PAO1 triple mutant inactivated in MexAB-OprM, MexCD-OprJ and MexEF-OprN did not show reduced virulence in the same infection model in a previous study[46]. This observation suggests that the deletion of MexXY-OprM in addition to MexAB-OprM, MexCD-OprJ and MexEF-OprN in PAO1-KP Δefflux could be critical to reduce the virulence potential of *P. aeruginosa* in the *G. mellonella* model of infection. However, this issue should be investigated by using identical experimental settings and isogenic mutants generated in the same PAO1 strain.

Since the average weight of *G. mellonella* larvae was ca. 500 mg, and arbitrarily assuming uniform dispersal of injected bacteria and PAβN in 500 µl of internal volume in each larva[43], we calculated that to reach 50 µM final concentration of PAβN, each larva should be injected with 25 µl of saline containing 1 mM PAβN. As a preliminary control experiment, we verified that the injection of 25 µl of saline containing 1 mM PAβN did not affect

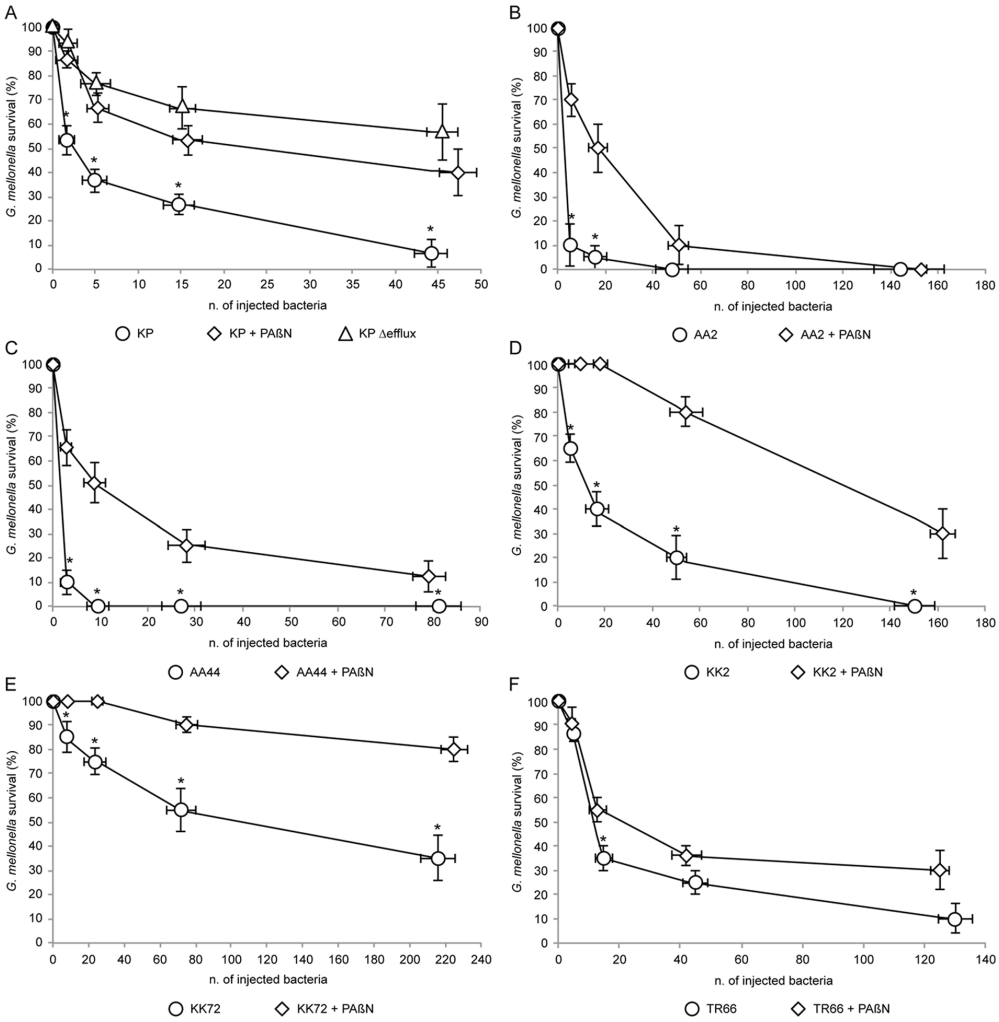

Figure 5. Effect of PAβN on *P. aeruginosa* virulence in *G. mellonella* larvae. Viability of *G. mellonella* larvae 24 h after injection with the indicated amount of bacteria. Larvae were injected with: (**A**) The *P. aeruginosa* strains PAO-KP wild type (circles), PAO-KP wild type in the presence of PAßN at ca. 50 μM final concentration (diamonds), or PAO1-KP Δefflux (triangles); (**B**–**F**) The indicated *P. aeruginosa* CF isolates in the absence (circles) or in the presence of PAßN at ca. 50 μM final concentration (diamonds). Mean values from three independent experiments, each performed on at least 30 larvae, are reported with SD; statistical significance with respect to the larvae challenged with the indicated strains in the absence of PAßN is indicated with one asterisk ($p < 0.05$).

the survival of uninfected larvae (data not shown). Then, *G. mellonella* larvae were inoculated with *P. aeruginosa* PAO1-KP in the absence or in the presence of PAβN. Results shown in Fig. 5A demonstrate that PAβN was able to protect *G. mellonella* larvae from *P. aeruginosa* PAO1-KP infection. Interestingly, the survival plot of the larvae infected with PAO1-KP and treated with PAβN was slightly lower than that of the untreated larvae infected with the efflux-deficient mutant PAO1-KP Δefflux, but was higher than the untreated control infected with wild type PAO1-KP, supporting the hypothesis that PAβN-mediated inhibition of RND efflux pumps is the cause of virulence attenuation. Also in this case, it was not possible to directly verify this hypothesis by testing the effect of PAβN on PAO1-KP Δefflux infectivity in *G. mellonella* due to the toxicity exerted by PAβN on this mutant strain[19–22]. Notably, PAβN exerted a similar protective effect when the larvae were challenged with the *P. aeruginosa* PAO1 strain routinely used in our laboratory (data not shown). Overall, these results strongly suggest that the PAβN-mediated inhibition of RND efflux pumps decreases *P. aeruginosa* PAO1 pathogenicity in *G. mellonella*, despite the increase in $3OC_{12}$-HSL and pyocyanin levels observed *in vitro* in response to PAβN.

Strain[a]	$3OC_{12}$-HSL[b]	Pyocyanin[b]	Swarming motility[b]
AA2	102%	86%	<10%
AA11	NP	125%	NS
AA12	24%	73%	NS
AA43	NP	NP	<10%
AA44	59%	142%	<10%
KK2	74%	NP	NS
KK27	81%	NP	NS
KK71	97%	NP	NS
KK72	134%	NP	NS
TR1	NP	NP	<10%
TR66	NP	NP	<10%

Table 3. Effects of PAßN treatment on virulence phenotypes in *P. aeruginosa* clinical isolates. [a]*P. aeruginosa* strains isolated from cystic fibrosis patients[47]. [b]Percentage of $3OC_{12}$-HSL levels, pyocyanin production or swarming motility in the presence of 27 µM PAßN with respect to the untreated control. The average of at least three independent experiments is reported; SD ≤ 10%. NP, non producer strain; NS, non swarmer strain.

Effect of PAβN on *P. aeruginosa* cystic fibrosis isolates. The previous observation that PAβN treatment inhibited $3OC_{12}$-HSL and pyocyanin production in *P. aeruginosa* clinical strains[21] and our results showing that this EPI has an opposite effect in the reference laboratory strains PAO1 and PAO1-KP suggest that virulence-related phenotypes could be variably affected by PAβN, depending on the test strain.

In order to verify this hypothesis, we measured the effect of 27 µM PAβN treatment on $3OC_{12}$-HSL and pyocyanin production, as well as swarming motility, in eleven *P. aeruginosa* clinical strains isolated from the lungs of cystic fibrosis (CF) patients[47]. The growth curve of all tested strains was not affected by PAβN treatment (data not shown). Interestingly, among the seven isolates producing $3OC_{12}$-HSL, PAβN increased this phenotype in one strain (*i.e.* KK72), had no effect in two strains (*i.e.* AA2 and KK71), while inhibited the production of this signal molecule in the remaining strains, though to different extents (Table 3). Out of four CF isolates that produced detectable amounts of pyocyanin, two responded to PAβN by reducing and two by increasing pyocyanin production (Table 3). Finally, only five CF isolates showed swarming motility in the absence of PAβN, and this phenotype was abrogated upon PAβN treatment in all of them (Table 3).

It appears therefore that PAβN has variable effects on QS signal and pyocyanin production, which are strain-dependent. This is in agreement with the previous study[21], showing that PAβN inhibited to a different extent $3OC_{12}$-HSL and C_4-HSL levels in two isolates from urinary tract infections, while C_4-HSL production was not inhibited in two isolates from wound infections. The extent of PAβN-mediated inhibition on all the tested virulence-related phenotypes varied significantly among the four clinical isolates previously analysed[21]. In contrast, swarming motility was invariably inhibited in all swarming-proficient CF isolates (Table 3). Moreover, for the majority of isolates, no correlation was observed between production/inhibition of the $3OC_{12}$-HSL signal molecule and the effect of PAβN on pyocyanin levels or swarming motility (Table 3), supporting our hypothesis that the effect of PAβN on pyocyanin production is exerted *via* QS-independent pathway(s).

The anti-virulence activity of PAβN against CF clinical isolates was further investigated in *G. mellonella* larvae in the presence and in the absence of 50 µM PAβN. The CF isolates AA2, AA44, KK2, KK72, and TR1 were selected based on their different pattern of virulence phenotypes and sensitivity to PAβN (Table 3). Since the CF isolates showed different pathogenicity in the *G. mellonella* larvae, the optimal range of injected bacteria to be used in the infection was preliminarily assessed (data not shown). As shown in Fig. 5C, D and E, PAβN significantly increased the survival of *G. mellonella* larvae challenged with the strains AA44, KK2 and KK72 at all the tested infective doses. Conversely, protection from AA2 infection was observed only for low doses of injected bacteria (<20 bacteria per larva; Fig. 5B), and poor protection effect was observed when *G. mellonella* larvae where challenged with the TR66 isolate (Fig. 5F).

Overall, PAβN exerted a general protective effect on *G. mellonella* larvae against *P. aeruginosa* CF isolates (Fig. 5B–F), irrespective of its positive or negative influence on $3OC_{12}$-HSL and pyocyanin production (Table S3).

Conclusions

Efflux pumps inhibition is a viable strategy to overcome the problem of antibiotic resistance. Both academic and industrial research is currently directed to the development of efflux inhibitors, and the interest in RND efflux pump inhibitors as antibiotic adjuvants is steadily increasing over the years[2,5–7]. Moreover, the notion that RND efflux pumps could play a role in bacterial infection is emerging[8,9], implying that certain EPIs could also be endowed with anti-virulence properties. Nevertheless, EPIs are usually considered only for their properties as antibiotic adjuvants, while their anti-virulence potential is seldom taken into account.

Here we demonstrate in a simple infection model that RND efflux pumps contribute to the establishment of *P. aeruginosa* PAO1 infection and, accordingly, that the EPI PAβN is able to reduce pathogenicity. In PAO1, the protective effect exerted by PAβN *in vivo* well correlates with *in vitro* suppression of some virulence-related phenotypes and repression of key virulence-related genes.

Although this study was not aimed at investigating the mechanistic link between RND efflux pumps and virulence, our findings provide relevant hints for future research. The transcriptomic analysis showed that the effect of PAβN on *P. aeruginosa* PAO1 physiology is specific, since it affects particular groups of genes, mainly related to iron and phosphate acquisition, as well as nitrogen metabolism. It is particularly relevant that PAβN inhibits the transcription of global regulators that are crucial for the establishment of a productive infection, such as the sigma factor gene *pvdS* and the response regulator gene *phoB*, controlling the regulons responding to iron and phosphate starvation, respectively[48,49].

It should be noticed that PAβN may also destabilize the outer membrane of Gram-negative bacteria, in addition to act as a nonspecific RND efflux pump inhibitor[19,22,33]. However, the majority of studies agree that the membrane-destabilizing activity of this molecule is only relevant in strains unable to extrude PAβN (*i.e.* mutants lacking RND efflux pumps), and that PAβN mainly acts as an efflux inhibitor in efflux pump-proficient isolates[19,33]. Here, PAβN had no effect on the growth rate of *P. aeruginosa* at concentrations up to 50 µM (Fig. S1), showing that in our experimental setting PAβN does not have growth-limiting effects. Most of the transcriptional and phenotypic effects observed in this study are controlled by PAβN also in the presence of the membrane stabilizing ion Mg^{2+}, and are mimicked by deletion of multiple efflux pumps in the PAO1-KP Δefflux mutant, strongly suggesting that, in our experimental setting, PAβN mainly acts as an efflux pump inhibitor.

By combining the response of clinical *P. aeruginosa* isolates to PAβN *in vitro* (Table 3) and *in vivo* (Fig. 5), no correlation could be established between the effect of this EPI on some virulence phenotypes (*i.e.* $3OC_{12}$-HSL and pyocyanin production, swarming motility) and the outcome of *G. mellonella* infection. This evidence suggests either that the protective effect of PAβN *in vivo* occurs through inhibition of virulence-related trait(s) not investigated in this study, or that the specific virulence factors affected by PAβN may be strain-specific.

Although the number of strains and virulence-related phenotypes tested here and in the previous study[21] is not sufficient to drive a definitive conclusion, the strain-dependent response to PAβN is an issue that deserves to be taken into consideration when testing the anti-virulence properties of any EPI. Unfortunately, PAβN is toxic for humans, hindering future therapeutic application and discouraging further studies aimed at characterizing the effect of this specific EPI on a wider panel of *P. aeruginosa* clinical strains. Actually, toxicity toward human cells is one of the major obstacle for microbial EPI implementation, and more efforts directed at specifically inhibiting efflux pumps operating only in prokaryotes are required. However, the search for new EPI candidates with improved pharmacological properties with respect to PAβN is in progress, as testified by the many research articles and thoughtful reviews published on this topic[2,5–7].

In conclusion, this study shows that RND efflux pump inhibition has an impact on bacterial virulence *in vivo*, and highlights that any new EPI should be tested not only for its ability to increase the inhibitory activity of antibiotics, but also for its anti-virulence effect. Given the strain-dependent response of *P. aeruginosa* to PAβN, anti-virulence properties should be tested on different virulence traits and on large panels of *P. aeruginosa* isolates from different types of infection.

Materials and Methods

Bacterial strains, growth conditions and chemicals. *P. aeruginosa* strains used in this study are listed in Table S2. All strains were routinely grown in Lysogeny Broth (LB)[50] supplemented with 50 mM 3-(*N*-morpholino)propanesulfonic acid (MOPS), pH 7.0. PAβN (Sigma-Aldrich) was suspended in dimethyl sulfoxide (DMSO) at a 10 mM final concentration.

Measurements of promoter activity and phenotypic assays. *P. aeruginosa* PAO1 strains carrying the P*phzA*$_1$::*luxCDABE* or P*phzA*$_2$::*luxCDABE* transcriptional fusions[41] were grown at 37 °C for 10 h in LB or in LB supplemented with 27 µM PAβN, 1 mM $MgSO_4$ or 27 µM PAβN plus 1 mM $MgSO_4$. Bioluminescence was determined in the resulting cultures as a function of cell density using an automated luminometer-spectrometer (Tecan Spark 10M), as previously described[41].

Levels of QS signal molecules in *P. aeruginosa* PAO1, PAO1-KP and PAO1-KP Δefflux culture supernatants were determined during bacterial growth in LB supplemented with different PAβN concentrations and/or 1 mM $MgSO_4$, by using the reporter strains specific for $3OC_{12}$-HSL, C_4-HSL and HHQ/PQS[43,51,52].

Pyocyanin was extracted with 3 ml of chloroform from 5 ml of cell-free supernatants of *P. aeruginosa* PAO1, PAO1-KP and PAO1-KP Δefflux cultures grown at 37 °C for 10 h in LB supplemented with different PAβN concentrations and/or 1 mM $MgSO_4$, and then re-extracted into 1 ml of 0.2 N HCl. The A_{520} of the resulting solution was measured to determine pyocyanin level[43,44]. Proteases and elastase activities were determined in 100 µl of the same cell-free supernatants by the azocasein and elastin-Congo red hydrolysis assays, respectively[43,44].

Swimming, swarming and twitching motilities were assessed as previously described[43,44].

Transcriptomic analysis. *P. aeruginosa* PAOI was inoculated at an A_{600} of 0.01 into 20 ml of LB with or without 27 µM PAβN. The cultures were grown at 37 °C with shaking until they reached an A_{600} of 2.5, and then 1 ml of cells was harvested by centrifugation. RNA extraction, retro-transcription and high-density oligonucleotides microarrays transcriptome analysis were performed and analysed as previously described[44,52]. RNA integrity was monitored by agarose gel electrophoresis, and the absence of contaminating chromosomal DNA was verified by PCR with primers pairs FW*pqsB*-RV*pqsB* and FW16SRT-RV16SRT (Table S3).

Processing of the *P. aeruginosa* PAO1 Affimetrix GeneChip® and statistical analysis of the dataset were performed at Lausanne Genomic Technologies Facility, Center for Integrative Genomics, University of Lausanne, Switzerland. For each condition, two different pools of RNA were compared (biological duplicate), each containing RNAs from three independent extractions (technical triplicate). Fold changes >2.0 with a p-value < 0.05 were considered as statistically significant.

qRT-PCR analyses. Novel *P. aeruginosa* PAO1 cultures were prepared specifically for qRT-PCR analysis. Growth conditions, and sampling for RNA extraction were the same used for the microarray experiments described above. When required, LB was also supplemented with 1 mM $MgSO_4$. The same setting were used also for qRT-PCR analysis performed in PAO-KP and PAO1-KP Δefflux. cDNA synthesis was performed from 1 µg of total purified RNA by using random hexamer primers and the iScript Reverse Transcription Supermix for RT-qPCR kit (BioRad).

qRT-PCR reactions were performed using the iTaq™ Universal SYBR® Green Supermix (BioRad) and primers listed in Table S3, which were designed using the Primer-Blast software (www.ncbi.nlm.nih.gov/tools/primer-blast). The reaction involved incubation at 95 °C for 1 min and 40 cycles of amplification at 95 °C for 10 s and 60 °C for 45 s. The 16 S ribosomal RNA was used as the internal control to calculate the relative fold change in gene expression by the $2^{-\Delta\Delta Ct}$ method[53]. The analysis was performed in duplicate on three technical replicates.

***Galleria mellonella* killing assay.** The *G. mellonella* killing assay was performed as previously described[43], with minor modifications. Briefly, *G. mellonella* caterpillars in the final instar larval stage (average weight, 480 ± 70 mg) were infected with 25 µl of bacterial cell suspensions in saline containing or not 1 mM PAβN. Although *P. aeruginosa* cells were incubated in the presence of PAβN for less than 15 min before injection, preliminary assays showed that 1 mM PAβN treatment *in vitro* (for up to 1 h) does not significantly affect *P. aeruginosa* cell viability (data not shown). One hundred-µl aliquots of the same suspensions were plated on LB agar to determine the number of viable cells (CFU) injected in the larvae. Larvae were incubated at 30 °C in Petri dishes (ten larvae per dish) and monitored over four days. Larvae were considered dead when they did not respond to gentle prodding. At least 30 larvae were inoculated per condition, in three independent experiments.

Statistical analysis. Statistical significance was determined by calculating the *p*-values using the two-tailed Student-t test for unpaired data sets; differences with a p-value ≤ 0.05 are considered as statistically significant.

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Author Contributions

G.R., C.R.P., F.L., R.B., V.B., M.M., and F.I. performed experiments; G.R., F.I., P.V. and L.L. conceived and designed the experiments, analyzed the data and contributed reagents/materials/analysis tools; G.R. and L.L. wrote the manuscript. All authors offered a critical review of the paper.

Additional Information

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Effect of efflux pump inhibition on *Pseudomonas aeruginosa* transcriptome and virulence

Giordano Rampioni[1], Cejoice Ramachandran Pillai[1#], Francesca Longo[1], Roslen Bondì[1], Valerio Baldelli[1], Marco Messina[1], Francesco Imperi[2], Paolo Visca[1], Livia Leoni[1*]

[1]*Department of Science, University Roma Tre, Rome, Italy;* [2]*Department of Biology and Biotechnology "Charles Darwin", Sapienza University of Rome, Rome, Laboratory affiliated to Istituto Pasteur Italia – Fondazione Cenci Bolognetti, Rome, Italy.*

Present address: [#]Inter University Centre for Bioscience, Kannur University, Palayad, Kerala, India.

[*] Corresponding author: Livia Leoni - livia.leoni@uniroma3.it

Supporting Information

Table S1. Genes whose transcription is affected by PAßN

PA number [a]	Gene name [a]	Fold change [b]	Product name [a]
PA0509*	*nirN*	2.27	NirN
PA0510*	*nirE*	2.33	NirE
PA0511*	*nirJ*	2.23	heme d_1 biosynthesis protein NirJ
PA0514*	*nirL*	2.3	heme d_1 biosynthesis protein NirL
PA0516*	*nirF*	2.3	heme d_1 biosynthesis protein NirF
PA0517*	*nirC*	3.53	probable *c*-type cytochrome precursor
PA0518*	*nirM*	3.32	cytochrome c_{551} precursor
PA0519*	*nirS*	4.48	nitrite reductase precursor
PA0523*	*norC*	2.87	nitric-oxide reductase subunit C
PA0524*	*norB*	5.51	nitric-oxide reductase subunit B
PA0525*	*norD*	2.19	probable dinitrification protein NorD
PA0526	---	2.54	hypothetical protein
PA0672∫	*hemO*	-4.81	heme oxygenase
PA0676∫	*vreR*	-4.85	sigma factor regulator, VreR
PA0688	*lapA*	-3.16	low-molecular-weight alkaline phosphatase A, LapA
PA0697	---	-3.74	hypothetical protein
PA0698	---	-3.03	hypothetical protein
PA0707	*toxR*	-2.12	transcriptional regulator ToxR
PA0842	---	-4.32	probable glycosyl transferase
PA0918	---	2.26	cytochrome b_{561}
PA1116	---	-3.13	hypothetical protein
PA1168	---	2.94	hypothetical protein
PA1169	---	2.41	probable lipoxygenase
PA1213	---	2.03	hypothetical protein
PA1215	---	2.64	hypothetical protein
PA1217	---	2.38	probable 2-isopropylmalate synthase
PA1221	---	2.01	hypothetical protein
PA1245	*aprX*	-3.44	AprX
PA1300∫	---	-4.92	ECF sigma factor
PA1301∫	---	-3.43	probable transmembrane sensor
PA1355∫	---	-2.99	hypothetical protein
PA1877	---	-2.16	probable secretion protein
PA1901§	*phzC1*	2.24	phenazine biosynthesis protein PhzC
PA1902§	*phzD1*	2.26	phenazine biosynthesis protein PhzD
PA1903§	*phzE1*	2.22	phenazine biosynthesis protein PhzE
PA1904§†	*phzF1*	2.11	probable phenazine biosynthesis protein
PA1912∫	*femI*	-2.35	ECF sigma factor, FemI
PA2033	---	-4.72	hypothetical protein
PA2034	---	-3.92	hypothetical protein
PA2143	---	-2.2	hypothetical protein
PA2384	---	-4.71	hypothetical protein
PA2385∫	*pvdQ*	-2.98	$3OC_{12}$-homoserine lactone acylase PvdQ
PA2386∫	*pvdA*	-3.97	L-ornithine N^5-oxygenase
PA2393	---	-3.09	putative dipeptidase

2

PA number [a]	Gene name [a]	Fold change [b]	Product name [a]
PA2394[∫]	*pvdN*	-2.85	PvdN
PA2395[∫]	*pvdO*	-2.32	PvdO
PA2396[∫]	*pvdF*	-3.22	pyoverdine synthetase F
PA2397[∫]	*pvdE*	-3.16	pyoverdine biosynthesis protein PvdE
PA2398[∫]	*fpvA*	-2.03	ferripyoverdine receptor
PA2399[∫]	*pvdD*	-3.32	pyoverdine synthetase D
PA2400[∫]	*pvdJ*	-3.36	PvdJ
PA2402[∫]	---	-3.43	probable non-ribosomal peptide synthetase
PA2405	---	-2.42	hypothetical protein
PA2411	---	-4.31	probable thioesterase
PA2412	---	-6.16	conserved hypothetical protein
PA2413[∫]	*pvdH*	-3.58	L-2,4-diaminobutyrate:2-ketoglutarate 4-aminotransferase
PA2424[∫]	*pvdL*	-3.73	PvdL
PA2425[∫]	*pvdG*	-2.58	PvdG
PA2426[∫]	*pvdS*	-10.48	sigma factor PvdS
PA2427	---	-3.62	hypothetical protein
PA2428	---	-18.06	hypothetical protein
PA2570	*lecA*	-2.42	LecA
PA2593	*qteE*	2.06	quorum threshold expression element, QteE
PA3195	*gapA*	2.14	glyceraldehyde 3-phosphate dehydrogenase
PA3377[◊]	*phnJ*	-21.1	conserved hypothetical protein
PA3392[*]	*nosZ*	2.16	nitrous-oxide reductase precursor
PA3407[∫]	*hasAp*	-4.15	heme acquisition protein HasAp
PA3410	*hasI*	-2.03	HasI
PA3530[∫]	*bfd*	-2.54	bacterioferritin-associated ferredoxin Bfd
PA3581	*glpF*	-2.06	glycerol uptake facilitator protein
PA3586	---	-2.2	probable hydrolase
PA3602	*yerD*	2.07	conserved hypothetical protein
PA3720	---	2.87	hypothetical protein
PA3880	---	2.63	conserved hypothetical protein
PA3899	*fecI*	-2.25	FecI
PA4139[†]	---	2.01	hypothetical protein
PA4220	*fptB*	-2.48	hypothetical protein
PA4221[∫]	*fptA*	-2.52	Fe(III)-pyochelin outer membrane receptor precursor
PA4224[∫]	*pchG*	-2.05	pyochelin biosynthetic protein PchG
PA4225[∫]	*pchF*	-2.26	pyochelin synthetase
PA4226[∫]	*pchE*	-2.06	dihydroaeruginoic acid synthetase
PA4228[∫]	*pchD*	-2.09	pyochelin biosynthesis protein PchD
PA4230[∫]	*pchB*	-2.69	salicylate biosynthesis protein PchB
PA4333	*fumA*	2.01	probable fumarase
PA4430	---	2.28	probable cytochrome *b*
PA4467	---	-3.7	hypothetical protein
PA4468	*sodM*	-11.65	superoxide dismutase
PA4469	---	-10.96	hypothetical protein
PA4470	*fumC1*	-8.96	fumarate hydratase
PA4471	*fagA*	-7.96	hypothetical protein

3

PA number [a]	Gene name [a]	Fold change [b]	Product name [a]
PA4504	*dppC*	2.18	probable permease of ABC transporter
PA4515	*piuC*	-2.48	conserved hypothetical protein
PA4570	---	-5.53	hypothetical protein
PA4587	*ccpR*	3.2	cytochrome c_{551} peroxidase precursor
PA4703	---	-2.04	hypothetical protein
PA4708[ʃ]	*phuT*	-3.17	heme-transport protein, PhuT
PA4709[ʃ]	*phuS*	-3.37	PhuS
PA4710[ʃ]	*phuR*	-4.65	meme/memoglobin uptake outer membrane receptor PhuR
PA4810[*]	*fdnI*	2.22	nitrate-inducible formate dehydrogenase, γ subunit
PA5091	*hutG*	2.11	*N*-formylglutamate amidohydrolase
PA5098	*hutH*	3.16	histidine ammonia-lyase
PA5100	*hutU*	2.95	urocanase
PA5360[◊]	*phoB*	-15.28	two-component response regulator PhoB
PA5365[◊]	*phoU*	-9.4	phosphate uptake regulatory protein PhoU
PA5366[◊]	*pstB*	-14.02	ATP-binding component of ABC phosphate transporter
PA5367[◊]	*pstA*	-14.17	membrane protein component of ABC phosphate transporter
PA5369[◊]	*pstS*	-23.49	periplasmic phosphate-binding protein, PstS
PA5410	*gbcA*	2.14	GbcA

[a] PA number, gene name and product name are from the *Pseudomonas* Genome Database[23]. Genes previously reported as controlled by $3OC_{12}$-HSL are in bold characters[36-38]. [*], genes involved in nitrogen metabolism; [§], genes involved in phenazines biosynthesis; [ʃ], genes previously reported to be controlled by iron-starvation[25]; [◊], genes previously reported to be controlled by phosphate-starvation[27]; [†], genes previously reported to be controlled by colistin[31].

[b] Fold change in gene expression in *P. aeruginosa* PAO1 grown in LB supplemented with 27 μM PAβN with respect to the same strain grown in LB.

Table S2. Bacterial strains used in this study

Strains	Relevant characteristics	Reference/Source
P. aeruginosa		
PAO1	PAO1 wild type strain ATCC15692	ATCC
PAO1-KP	PAO1 wild type strain gently provided by Prof. K. Poole, Queen's University, Kingston, Canada	[34]
PAO1-KP Δefflux	PAO1-KP mutant strain carrying mutations in the efflux pumps MexAB-OprM, MexCD-OprJ, MexEF-OprN and MexXY-OprM. Gently provided by Prof. K. Poole, Queen's University, Kingston, Canada	[34]
PA14-R3	biosensor strain for $3OC_{12}$-HSL quantification	[51]
PAO1 Δ*pqsAH PpqsA::lux*	biosensor strain for HHQ/PQS quantification	[54]
PAO-JP2 pKD-*rhlA*	biosensor strain for C_4-HSL quantification	[55]

Additional references for Table S1:

54. Fletcher, M. P. *et al*. A dual biosensor for 2-alkyl-4-quinolone quorum-sensing signal molecules. *Environ. Microbiol.* **9,** 2683-2693 (2007).

55. Duan, K. & Surette, M. G. Environmental regulation of *Pseudomonas aeruginosa* PAO1 Las and Rhl quorum-sensing systems. *J. Bacteriol.***189,** 4827-4836 (2007).

Table S3. Oligonucleotides used in this study

Name	Sequence (5'-3') [a]	Restriction site [b]
FW*pqsB*	CCGCTCGAGCGACCAGGGCTATCGCA	XhoI
RV*pqsB*	CCGGAATTCCTTATGCATGAGCTTCTCC	EcoRI
FW16SRT	AGTACGGCCGCAAGGTTAAA	-
RV16SRT	CCCAACATCTCACGACACGA	-
FW*aprX*RT	CTGCCGATCAACGTCTCCTT	-
RV*aprX*RT	TACCGTAGAACTTGGCGCTG	-
FW*sodM*RT	CGTTCAAGGATGCGTTCACC	-
RV*sodM*RT	GCGGTTCTGGTACTTCAGGT	-
FW*norB*RT	TTCCTGTTCAACGTCGGCAT	-
RV*norB*RT	GCACCCATGATCAGTTCCCA	-
FW*qteE*RT	GATGCGGTGAGCGACTACAT	-
RV*qteE*RT	GAAGATGCTGGTTGGCATCG	-
FW*pvdQ*RT	GAAGACGCTCGAGGAGATGG	-
RV*pvdQ*RT	TGAAGCGCTGGAAGTAGACG	-
FW*pvdS*RT	GGAACAACTGTCTACCCGCA	-
RV*pvdS*RT	GTAGCTGAGCTGTGCCTTGA	-
FW*fumC1*RT	GAACTGAACGTGATGCTGCC	-
RV*fumC1*RT	TTTCCGCAGCCTTCTGGTAG	-

[a] Engineered restriction sites are underlined.

[b] -, no restriction site introduced.

Figure S1. Effect of PAßN on *P. aeruginosa* growth

Growth of *P. aeruginosa* PAO1 in LB in the absence or in the presence of the indicated PAßN concentrations. The average of at least three independent experiments is reported with SD.

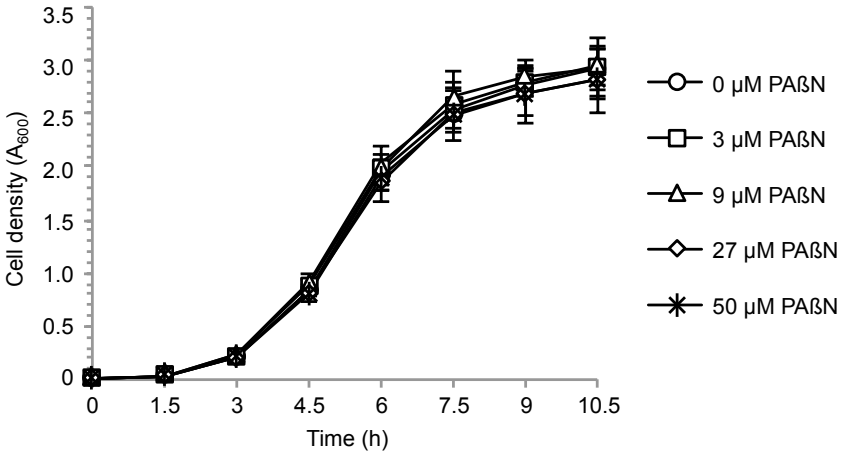
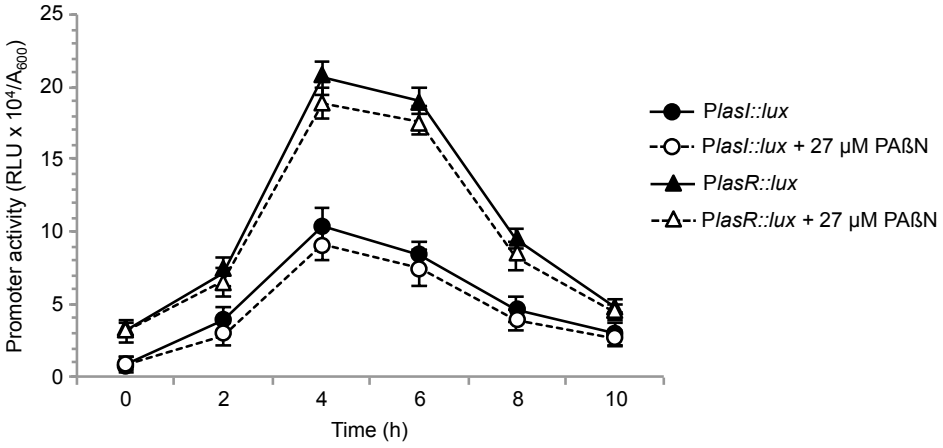


Figure S2. Effect of PAßN on the promoter activity of *lasI* and *lasR* genes

P*lasI* and P*lasR* promoter activity measured in the *P. aeruginosa* PAO1 strain carrying a chromosomally inserted cassettes P*lasI::lux* or P*lasR::lux*, respectively. The strains were grown at 37ºC with 120 rpm shaking in LB in the absence or in the presence of 27 µM PAßN. Promoter activity is reported as Relative Light Units (RLUs) normalized to bacterial cell density (A_{600}). The average of three independent experiments is reported with SD.



Chapter 3

Identification of FDA-approved drugs as antivirulence agents targeting the *pqs* quorum sensing system of *Pseudomonas aeruginosa*

Francesca D'Angelo[1], **Valerio Baldelli**[1], Nigel Halliday[2], Paolo Pantalone[2], Fabio Polticelli[1,3], Ersilia Fiscarelli[4], Paul Williams[2], Paolo Visca[1], Livia Leoni[1] and Giordano Rampioni[1]

[1] Department of Science, University Roma Tre, Rome, Italy; [2] Centre for Biomolecular Sciences and School of Life Sciences, University of Nottingham, Nottingham, UK; [3] National Institute of Nuclear Physics, Roma Tre Section, Rome, Italy; [4] Laboratory of Cystic Fibrosis Microbiology, Bambino Gesú Hospital, Rome, Italy.

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Preface to Chapter 3

As discussed in the Introduction section, a promising strategy to combat *P. aeruginosa* infections aims at identifying antivirulence agents that hamper its adaptability to the host environment without reducing its growth. In this context, the *pqs* QS system is an ideal target for the development of antivirulence drugs, since this intercellular communication system positively controls the expression of multiple virulence factors and biofilm formation, hence it is required for full pathogenicity of *P. aeruginosa*. Moreover, the *pqs* system is active during *P. aeruginosa* infection and the levels of the *pqs* QS signal molecules AQs correlate with the clinical status of cystic fibrosis (CF) patients. These evidences boosted the search for *pqs* inhibitors, leading to the identification of new antivirulence agents targeting AQs synthesis or reception. Unfortunately, none of these inhibitors has been transferred to the clinical use so far, mainly as a consequence of unfavourable pharmacological properties.

On this basis, in the work presented in Chapter 3, an *ad hoc* engineered co-cultivation-based biosensor system, in which light emission is proportional to the activation state of the *pqs* QS system, has been developed and used to screen a library of drugs already approved for their use in humans (FDA-approved). This drug repurposing approach has a high probability of yielding safe and bioavailable drug-like compounds, and it is thus expected to reduce the time and costs generally associated with standard drug discovery processes. Our screening campaign led to the identification of a new FDA-approved inhibitor of the *pqs* system, clofoctol, endowed with antivirulence activity in the model strain *P. aeruginosa* PAO1, likely by targeting the *pqs* QS receptor PqsR. Preliminary analyses indicate that clofoctol is active also against most *P. aeruginosa* clinical isolates from CF patients.

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MECHANISMS OF ACTION: PHYSIOLOGICAL EFFECTS

Identification of FDA-Approved Drugs as Antivirulence Agents Targeting the *pqs* Quorum-Sensing System of *Pseudomonas aeruginosa*

Francesca D'Angelo,[a] Valerio Baldelli,[a] Nigel Halliday,[b] Paolo Pantalone,[b] Fabio Polticelli,[a,c] Ersilia Fiscarelli,[d] Paul Williams,[b] Paolo Visca,[a] Livia Leoni,[a] Giordano Rampioni[a]

[a]Department of Science, University Roma Tre, Rome, Italy
[b]Centre for Biomolecular Sciences and School of Life Sciences, University of Nottingham, Nottingham, United Kingdom
[c]National Institute of Nuclear Physics, Roma Tre Section, Rome, Italy
[d]Laboratory of Cystic Fibrosis Microbiology, Bambino Gesú Hospital, Rome, Italy

ABSTRACT The long-term use of antibiotics has led to the emergence of multidrug-resistant bacteria. A promising strategy to combat bacterial infections aims at hampering their adaptability to the host environment without affecting growth. In this context, the intercellular communication system quorum sensing (QS), which controls virulence factor production and biofilm formation in diverse human pathogens, is considered an ideal target. Here, we describe the identification of new inhibitors of the *pqs* QS system of the human pathogen *Pseudomonas aeruginosa* by screening a library of 1,600 U.S. Food and Drug Administration-approved drugs. Phenotypic characterization of *ad hoc* engineered strains and *in silico* molecular docking demonstrated that the antifungal drugs clotrimazole and miconazole, as well as an antibacterial compound active against Gram-positive pathogens, clofoctol, inhibit the *pqs* system, probably by targeting the transcriptional regulator PqsR. The most active inhibitor, clofoctol, specifically inhibited the expression of *pqs*-controlled virulence traits in *P. aeruginosa*, such as pyocyanin production, swarming motility, biofilm formation, and expression of genes involved in siderophore production. Moreover, clofoctol protected *Galleria mellonella* larvae from *P. aeruginosa* infection and inhibited the *pqs* QS system in *P. aeruginosa* isolates from cystic fibrosis patients. Notably, clofoctol is already approved for clinical treatment of pulmonary infections caused by Gram-positive bacterial pathogens; hence, this drug has considerable clinical potential as an antivirulence agent for the treatment of *P. aeruginosa* lung infections.

KEYWORDS *Pseudomonas aeruginosa*, antivirulence, biofilm, clofoctol, clotrimazole, cystic fibrosis, drug repurposing, miconazole, *pqs*, quorum sensing

The discovery and development of new drugs for use in humans is a challenging task that usually requires decade-long laboratory experimentation followed by extensive clinical trials. This process is time-consuming and necessitates substantial economic investments with a high-risk of failure mostly due to the poor pharmacological and pharmaceutical properties of newly identified bioactive molecules. This is particularly discouraging for antibiotic discovery since the investment required cannot be adequately recovered because of the high rate at which resistance emerges (1). As a consequence, while the spread of multiresistant pathogens is accelerating at an unprecedented rate, the antibiotic discovery pipeline is running dry, with 15 big pharmaceutical companies of 18 abandoning antibacterial discovery programs in the last decade (2, 3).

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Address correspondence to Giordano Rampioni, giordano.rampioni@uniroma3.it.

The search for off-target activities in drugs already approved for human use is a promising strategy that could reduce the time and costs generally associated with conventional drug discovery processes, with a high probability of yielding bioavailable and safe compounds which can more easily and swiftly move into clinical trials (4, 5).

A number of studies have shown the promise of drug repurposing strategies for the identification of new antibacterial drugs (6, 7). Examples are gallium nitrate and 5-fluorouracil, conventionally used for the treatment of hypercalcemia and cancer, respectively, which display growth-inhibitory activities against certain Gram-negative and Gram-positive pathogens (8, 9). An alternative approach to the development of new antimicrobials is the inhibition of bacterial virulence, rather than growth (10). Recently, antivirulence activities have been identified in drugs already approved for use in humans (11). As an example, the antifungal compound 5-fluorocytosine inhibits virulence factor production in the Gram-negative human pathogen *Pseudomonas aeruginosa* both *in vitro* and in a mouse model of lung infection (12). Since antivirulence drugs attenuate rather than kill pathogens, they should in principle combat bacterial infections without exerting the strong selective pressure for resistance imposed by bactericidal antibiotics (10). The emergence of resistance is less likely to occur for drugs targeting bacterial social behaviors, such as the production of secreted virulence factors. Indeed, resistant mutants expressing extracellular factors that are shared by the members of the entire bacterial population are unlikely to experience a fitness advantage relative to susceptible clones (13). In this context, quorum sensing (QS) is considered to be a promising target for the identification and development of antivirulence drugs, since this intercellular communication system positively controls the expression of virulence factors in a number of different human pathogens, including *P. aeruginosa* (14, 15).

P. aeruginosa is one of the most problematic human pathogens in industrialized countries, since it causes a variety of severe infections, especially among hospitalized and immunocompromised patients (16, 17). These infections are difficult to treat due to the intrinsic and acquired antibiotic resistance of *P. aeruginosa* (18) that is further compounded by its ability to form antibiotic tolerant biofilms (19). *P. aeruginosa* is the predominant cause of morbidity and mortality in individuals with cystic fibrosis (CF), since it forms biofilms, thereby establishing chronic lung infections that are impossible to eradicate with antibiotic treatment (20). The necessity of new therapeutic options for the treatment of *P. aeruginosa* infections was highlighted in a recent World Health Organization report in which this pathogen is top ranked among pathogens for which new antibiotics are urgently needed (Priority 1: Critical [http://www.who.int/en/news-room/detail/27-02-2017-who-publishes-list-of-bacteria-for-which-new-antibiotics-are-urgently-needed]).

As a consequence of its importance as a human pathogen, *P. aeruginosa* has been adopted as a model organism for QS inhibition studies. This bacterium is endowed with a complex QS network consisting of four interconnected systems (i.e., *las*, *rhl*, *pqs*, and *iqs*), which collectively control social behaviors and the expression of virulence determinants, such as secreted virulence factors, swarming motility, and biofilm formation (21, 22). Over the last decade, numerous compounds interfering with the *P. aeruginosa* QS circuitry have been identified, and their effectiveness as antivirulence drugs both *in vitro* and *in vivo* has boosted the research in the field (23). Unfortunately, most of the drugs identified thus far are cytotoxic or display unfavorable pharmacological properties, thus limiting their transfer to clinical practice (15).

To combine the advantages of drug-repurposing with the antivirulence approach, we previously showed that the anthelmintic drug niclosamide has potent antivirulence activity against *P. aeruginosa* (24). Niclosamide targets the *las* QS system, thereby decreasing the expression of *las*-controlled virulence factors and protecting *Galleria mellonella* larvae from *P. aeruginosa* infection (24).

In the present study we searched for inhibitors of the *pqs* QS system of *P. aeruginosa* among drugs already approved for human use.

The *pqs* QS system of *P. aeruginosa* is based on 2-alkyl-4-quinolones (AQs) as signal

molecules, namely, 2-heptyl-3-hydroxy-4-quinolone (PQS), and its immediate precursor 2-heptyl-4-hydroxyquinoline (HHQ). Both HHQ and PQS can bind to and activate the transcriptional regulator PqsR (also known as MvfR). The PqsR/HHQ and PqsR/PQS complexes bind the P*pqsA* promoter region and trigger the transcription of the *pqsABCDE-phnAB* operon, coding for the enzymes required for the synthesis of HHQ. HHQ is in turn oxidized to PQS by the monooxygenase PqsH. Therefore, in common with other QS systems, HHQ and PQS act as autoinducers by generating an autoinductive feedback loop that accelerates their synthesis (25–28).

While HHQ only activates the expression of the *pqsABCDE-phnAB* operon, PQS has additional functionalities; it is an iron chelator, it participates in the formation of outer membrane vesicles, and it controls the expression of virulence genes via a PqsR-independent pathway (28–31).

The mechanism of action of the protein coded by the fifth gene of the *pqsABCDE-phnAB* operon, PqsE, is still poorly understood. PqsE is a pathway-specific thioesterase, which contributes to the synthesis of HHQ, although loss of its function can be compensated for by other thioesterases in a *pqsE* mutant (27). Notably, PqsE also positively controls the expression of multiple virulence factors in a *P. aeruginosa* genetic background in which it cannot participate in AQ biosynthesis, indicating that this protein has additional functions (29, 32, 33).

Overall, *P. aeruginosa* mutants defective in AQ synthesis/reception or in PqsE are severely attenuated in different plant and animal experimental models of infection (33–38). Moreover, AQs are detectable in the sputum, blood, and urine of individuals with CF, and their presence correlates with clinical status (39).

In this study, a convenient screening system has been developed and used to select for U.S. Food and Drug Administration (FDA)-approved drugs targeting the *pqs* QS system at multiple levels. This screening campaign led to the identification of the antifungal drugs clotrimazole and miconazole, as well as clofoctol, an antimicrobial compound commonly used to treat lung infections caused by Gram-positive bacteria, as inhibitors of *pqs* signaling, probably targeting the PqsR receptor protein. Phenotypic analyses performed in the laboratory strain PAO1 and in *P. aeruginosa* isolates from CF patients support the antivirulence potential of clofoctol, the most active inhibitor.

RESULTS

Development of a coculture-based system for monitoring *pqs* signaling activity. A reporter system for monitoring the activity of the *pqs* QS system has been developed. This is based on the coculture between wild-type *P. aeruginosa* PAO1 (herein referred to as PAO1) and the AQ biosensor strain *P. aeruginosa* Δ*pqsA* P*pqsA*::*luxCDABE* (here referred to as AQ-Rep; see Table S1 in the supplemental material). AQ-Rep cannot synthesize AQs due to deletion of the *pqsA* biosynthetic gene and emits light only in response to exogenously provided AQs due to PqsR-dependent activation of the P*pqsA*::*luxCDABE* transcriptional fusion integrated in a neutral chromosomal site (31). Therefore, in the PAO1/AQ-Rep coculture system the AQ signal molecules produced by PAO1 induce bioluminescence, and hence *pqs* inhibitors interfering with each step of the *pqs* signaling circuit, including AQ biosynthesis or response, should reduce bioluminescence (Fig. 1A).

Preliminary experiments directed toward setting up the screening system revealed that maximal response of AQ-Rep to exogenous PQS was obtained after 5 h of incubation in microtiter plates (Fig. S1A), when this biosensor strain was inoculated at an optical density at 600 nm (OD_{600}) wavelength of 0.1 (Fig. S1B). Cocultivation of AQ-Rep and PAO1 at different ratios and in different culture conditions showed that the highest bioluminescence signal was registered when AQ-Rep and PAO1 were inoculated in an ~3:1 ratio (AQ-Rep and PAO1 OD_{600}s of 0.1 and 0.03, respectively) (Fig. S1C), and the resulting coculture was incubated at 37°C with shaking (Fig. S1D). Therefore, the screening campaign has been set up under the above conditions to maximize the biosensor responsiveness to AQs and possibly to drugs interfering with AQ signaling.

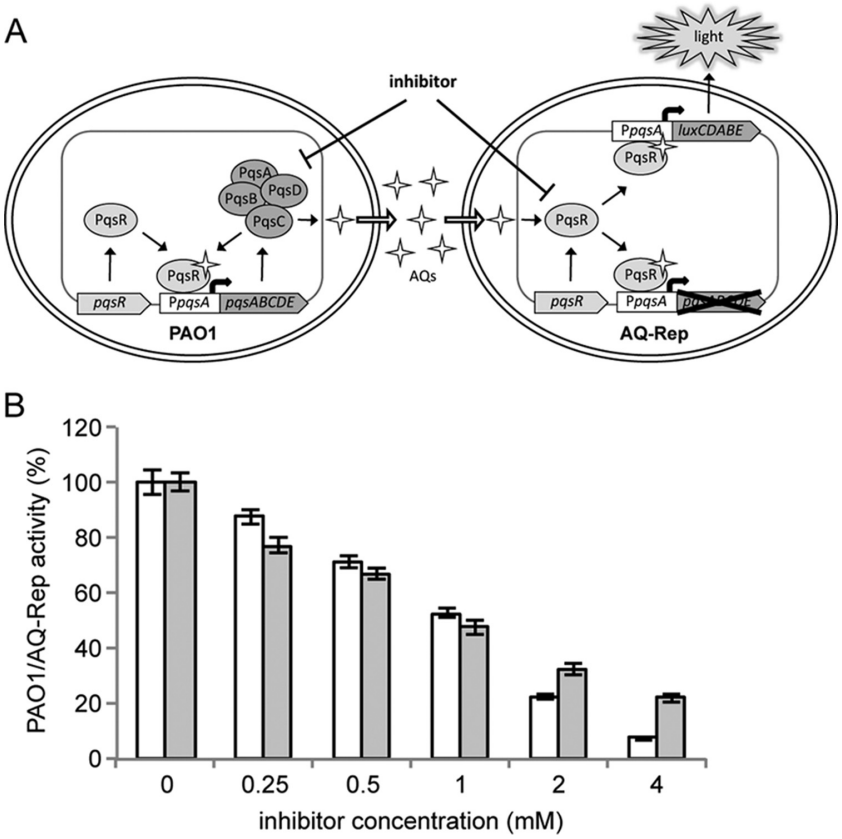
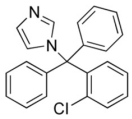
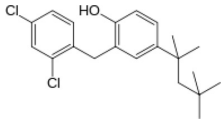
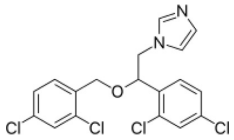


FIG 1 Validation of the screening system. (A) Schematic representation of the coculture-based reporter system. The *P. aeruginosa* PAO1 strain (PAO1) produces AQ signal molecules which activate P*pqsA*::*luxCDABE* transcription, that results in light emission in the biosensor strain AQ-Rep. Drugs interfering with AQ biosynthesis or response are expected to reduce bioluminescence in the PAO1/AQ-Rep coculture, relative to the untreated samples. (B) Activity of the PAO1/AQ-Rep coculture system treated with the indicated concentrations of the *pqs* inhibitors methyl anthranilate (white bars) or farnesol (gray bars). The bioluminescence of the untreated PAO1/AQ-Rep coculture normalized to the cell density is considered 100%.

The functionality of the PAO1/AQ-Rep coculture system for the identification of anti-*pqs* drugs was assessed using the commercially available compounds methyl anthranilate and farnesol. Methyl anthranilate inhibits AQs biosynthesis by competing with the HHQ precursor anthranilate for binding to PqsA (40), while farnesol decreases the expression of HHQ biosynthetic genes via an unknown mechanism (41). As expected, both methyl anthranilate and farnesol reduced bioluminescence from the PAO1/AQ-Rep coculture in a dose-dependent manner, with a 50% inhibitory concentration (IC_{50}) of ca. 1 mM (Fig. 1B), in accordance with literature data (40, 41).

Identification of new anti-*pqs* drugs. The PAO1/AQ-Rep coculture system was used to screen a library of 1,600 FDA-approved compounds with known biological activities selected for their high chemical and pharmacological diversity and safety in humans (PHARMAKON). In the primary screening, each drug was tested at two different concentrations, 20 and 200 μM, for the ability to reduce bioluminescence in the PAO1/AQ-Rep coculture. Since compounds from the library are dissolved in dimethyl sulfoxide (DMSO), untreated samples containing the same amount of DMSO as the treated samples were used as controls. Cell density and bioluminescence of the untreated samples were considered 100%, and the criteria for the selection of anti-*pqs* drugs were (i) inhibition of bioluminescence of $\geq$20% at 20 μM, (ii) inhibition of

TABLE 1 Anti-*pqs* compounds identified by screening the PHARMAKON library of FDA-approved drugs

Drug	Property	Structure	IC_{50} (μM)[a]	ΔG (kcal/mol)[b]
Clotrimazole	Antifungal		39	−8.4
Clofoctol	Antibacterial		20	−9.8
Miconazole	Antifungal		27	−8.5

[a]The IC_{50} values were determined using the PAO1/AQ-Rep coculture system.
[b]ΔG values for drugs binding to the PqsR CBD apo form (PDB 4JVC) (59) were predicted using molecular docking simulations.

bioluminescence of ≥60% at 200 μM, and (iii) reduction in the cell density of ≤10% at both 20 and 200 μM. This primary screening led to the selection of 17 hits meeting these criteria (Fig. S2A) and possibly endowed with *pqs* inhibitory activity.

However, reduced bioluminescence in the samples treated with the selected drugs could be due to their effects on the enzymes involved in light generation or on ATP levels (42, 43). Since inhibition of P*pqsA* promoter activity in *P. aeruginosa* should decrease the production of the AQs HHQ and PQS, a secondary screening to test the ability of the 17 hits to reduce AQ production in PAO1 was performed. In this case, AQ levels were measured by means of the AQ-Rep biosensor strain in the spent medium from PAO1 cultures grown for 16 h in Luria-Bertani broth (LB) supplemented with the selected hits at concentrations of 20 or 200 μM or with corresponding amounts of DMSO. This analysis revealed that only three drugs specifically reduced the production of AQs in PAO1: clotrimazole, clofoctol, and miconazole (I-3, I-9, and I-14 in Fig. S2B, respectively). Two of the drugs identified, clotrimazole and miconazole, are antifungal compounds (44–47), while clofoctol is an antibacterial drug with efficacy in Gram-positive human lung infections (48–50) (Table 1).

To confirm the results of the primary and secondary screening, clotrimazole, clofoctol, and miconazole were purchased from an alternative supplier (Sigma-Aldrich). These drugs did not inhibit PAO1 growth in Muller-Hinton broth or LB even at the highest concentration achievable in solution (i.e., MIC for clotrimazole, >1.6 mM; MICs for miconazole and clofoctol, >6.4 mM). Moreover, these drugs did not alter the growth profile of wild-type PAO1 and of the AQ-Rep biosensor strain up to the maximum concentration used in the primary and secondary screenings (i.e., 200 μM; Fig. S3).

The *pqs* inhibitory activity of the drug hits was retested in the PAO1/AQ-Rep coculture assay. Dose-response inhibition of P*pqsA* promoter activity was observed for the three drugs (Fig. 2A). These data generated IC_{50}s of 39, 20, and 27 μM for clotrimazole, clofoctol, and miconazole, respectively (Table 1). The three hits had no effect on bioluminescence in a *P. aeruginosa* strain in which the expression of the *luxCDABE* operon for light emission is independent on the activity of the *pqs* signaling system (Fig. S4), ruling out the possibility that the inhibitory activity on the PAO1/AQ-Rep coculture was due to nonspecific inhibition of bioluminescence. Moreover, the three drugs confirmed their ability to reduce AQ production in PAO1 in a dose-dependent manner (Fig. 2B) in accordance with the repressive effect exerted on the P*pqsA* promoter.

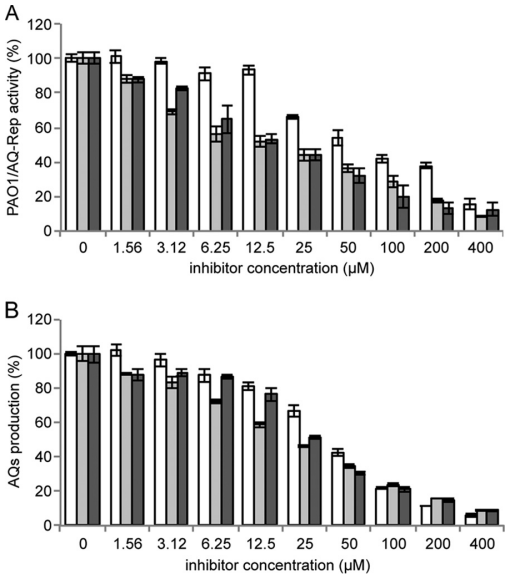


FIG 2 Clotrimazole, clofoctol, and miconazole inhibit P*pqsA* activity and AQ production. Effect of clotrimazole (white bars), clofoctol (light gray bars), and miconazole (dark gray bars) on the PAO1/AQ-Rep coculture system. Bioluminescence of the untreated PAO1/AQ-Rep coculture normalized to cell density is considered 100%. (B) Effect of clotrimazole (white bars), clofoctol (light-gray bars), and miconazole (dark gray bars) on AQ production in PAO1. The level of AQs produced by untreated PAO1 is considered 100%. For both panels A and B, the average from at least three independent experiments is reported, along with the SD.

The QS cascade in *P. aeruginosa* is a complex network of interwoven and hierarchical QS circuits (21, 22), and hence the effect of some compounds on the *pqs* QS system may be due to altered activity of the *las* and/or *rhl* QS systems. In particular, the *las* QS system is required for full activation of the *pqs* QS systems (36, 51–53), while RhlR has a negative impact on the *pqs* system by repressing PQS signal production through interference with the expression of *pqsR* and *pqsABCDE* (36, 54–56). Hence, reduced activity of the *pqs* QS system could be due to a negative or a positive effect of the hits on the *las* or the *rhl* QS systems, respectively. Therefore, possible effect of the three hits on these QS systems was investigated by using *las*- and *rhl*-specific biosensor strains. Clotrimazole, clofoctol, and miconazole did not decrease light emission in a reporter system in which the PAO1 wild type and the *las*-specific biosensor strain PA14 Δ*lasI* P*rsaL*::*luxCDABE* were cocultured (Fig. S5A) (57). Conversely, the three compounds slightly decreased (from 15 to 30% at 200 μM) light emission from a coculture system based on the PAO1 wild type and on the *rhl*-specific biosensor strain PAO1 Δ*rhlI* P*rhlA*::*luxCDABE* (Fig. S5B) (24). These data demonstrate that clotrimazole, clofoctol, and miconazole do not affect the *las* QS system, while these drugs have a slight negative effect on the *rhl* QS system. Considering that (i) the repressive effect exerted by the hits on the *pqs* QS system (Fig. 2A) occurs at lower concentration and is more pronounced than the repressive effect exerted by the same molecules on the *rhl* QS system (Fig. S5B) and that (ii) the *pqs* system exerts a positive effect on the *rhl* system (54, 58), these data support a primary activity of the hits on the *pqs* QS system that consequently reduces *rhl* activity. Overall, these data confirm that clotrimazole, clofoctol, and miconazole exert an anti-*pqs* activity without altering *P. aeruginosa* growth.

Characterization of the mechanism of action of the newly identified *pqs* inhibitors. The inhibition of P*pqsA* activity in the PAO1/AQ-Rep coculture system (Fig. 2A) may be due to inactivation of AQ biosynthesis in the PAO1 strain or of AQ reception in both PAO1 and AQ-Rep strains (Fig. 1A). Similarly, the reduced AQ levels in PAO1 (Fig. 2B)

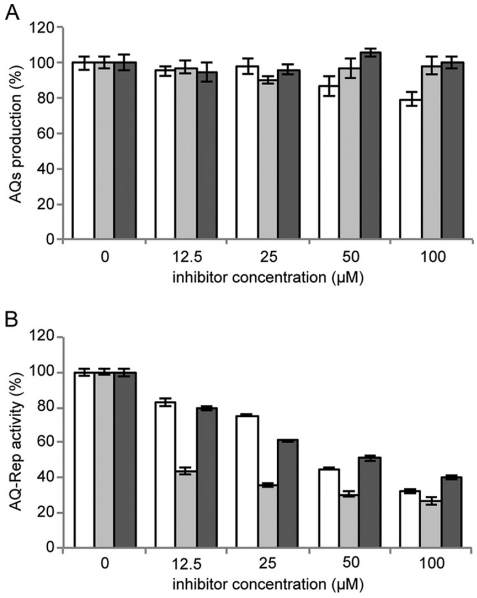


FIG 3 Clotrimazole, clofoctol, and miconazole inhibit AQ reception. (A) Production of AQs in *P. aeruginosa* PAO1 Δ*pqsAH*(pFD-*pqsABCD*) grown for 16 h in LB in the absence or in the presence of clotrimazole (white bars), clofoctol (light gray bars), and miconazole (dark gray bars). The AQ level measured in the untreated sample is considered 100%. (B) Activity of the AQ-Rep biosensor strain grown in LB supplemented with 10 μM synthetic PQS and clotrimazole (white bars), clofoctol (light gray bars), or miconazole (dark gray bars). The bioluminescence of the untreated AQ-Rep biosensor normalized to its cell density is considered 100%. For both panels A and B, the averages from at least three independent experiments are reported, along with the SD.

could also be due to inhibition of either AQ biosynthesis or response, due to the PqsR-dependent regulatory loop governing transcription of the HHQ biosynthetic enzymes (36, 55).

To discriminate between these two possibilities, the effect of the three drugs on AQ production was tested in a PAO1 Δ*pqsA* Δ*pqsH* double-mutant strain (Δ*pqsAH*; Table S1) carrying the pFD-*pqsABCD* plasmid for constitutive expression of the HHQ biosynthetic enzymes. In this genetic background, in which AQ production does not depend on the ability of AQs to activate P*pqsA* via PqsR, the inhibitors did not reduce AQ levels, demonstrating that they do not affect the functionality of the enzymes required for HHQ biosynthesis (Fig. 3A). Moreover, the inhibitors were effective in reducing bioluminescence emission by the AQ-Rep biosensor strain grown in the presence of synthetic PQS (Fig. 3B), suggesting that the inhibitors target the PqsR-dependent AQ response rather than biosynthesis.

To validate this hypothesis, we investigated the effect of the hits on the levels of *pqsR* mRNA and PqsR protein. As shown in Fig. 4A, real-time reverse transcription-PCR (RT-PCR) analysis revealed that the hits do not affect *pqsR* mRNA levels. Moreover, Western immunoblotting showed that the inhibitors do not reduce PqsR protein levels in a PAO1 Δ*pqsA* Δ*pqsH* Δ*pqsR* triple mutant strain (Δ*pqsAHR*; Table S1) carrying the pPqsR-6H plasmid for IPTG (isopropyl-β-D-thiogalactopyranoside)-inducible expression of a 6×His-tagged variant of PqsR (Fig. 4B) (59). Actually, clotrimazole increased PqsR levels, indicating that this drug has a positive effect on the translation of the *pqsR* mRNA or on PqsR stability. However, clotrimazole decreased P*pqsA* activity (Fig. 2A) and AQ production (Fig. 2B) and reduced the mRNA level of *pqs*-controlled genes, as demonstrated by real-time RT-PCR analysis performed on total mRNA extracted from PAO1 wild type grown in the absence or in the presence of 100 μM clotrimazole (Fig.

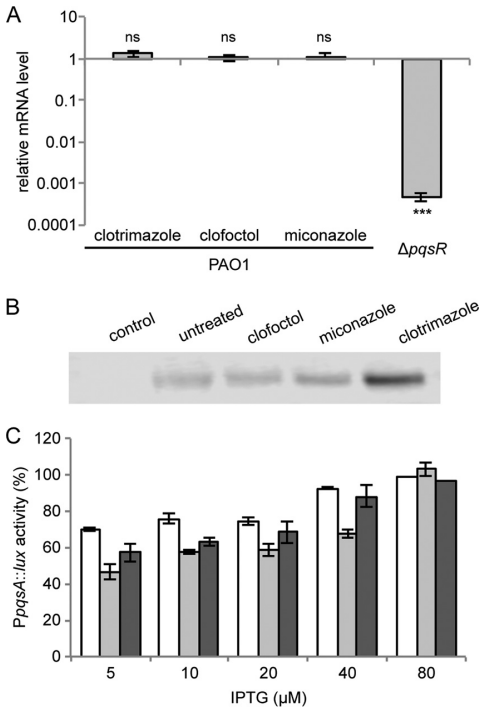


FIG 4 Clotrimazole, clofoctol, and miconazole inhibit PqsR functionality. (A) Real-time RT-PCR analysis showing the mRNA level of *pqsR* in PAO1 cultures treated with 100 μM concentrations of the indicated drugs relative to untreated PAO1 cultures. The PAO1 Δ*pqsR* strain was used as a negative control. The average from three independent experiments is reported, along with the SD. ns, nonsignificant difference; ***, $P < 0.001$ (ANOVA). (B) Western immunoblotting performed with anti-6×His antibody on crude protein extracts of PAO1 Δ*pqsAHR*(pPqsR-6H) grown in LB supplemented with 10 μM PQS and 20 μM IPTG, in the absence (untreated) or in the presence of the indicated drugs (100 μM). The PAO1 Δ*pqsAHR* strain carrying the empty vector pME6032 was used as a control. The data are representative of three independent experiments. (C) Effect of 100 μM clotrimazole (white bars), clofoctol (light gray bars), and miconazole (dark gray bars) on P*pqsA*::*lux* activity in the PAO1 Δ*pqsA* Δ*pqsH* Δ*pqsR* mutant carrying the pPqsR-6H plasmid, grown in LB supplemented with 10 μM PQS and different concentrations of IPTG as indicated in the graph. The averages from three independent experiments are reported, along with the SD.

S6). Overall, despite increasing PqsR level, clotrimazole seems to hamper the ability of this transcriptional regulator to activate gene expression.

To support PqsR as a target of the hits, we investigated their ability to reduce light emission from the P*pqsA*::*luxCDABE* transcriptional fusion in a PAO1 triple-mutant strain unable to synthesize AQs and to produce PqsR (i.e., PAO1 Δ*pqsAHR*), carrying the pPqsR-6H plasmid for IPTG-inducible expression of PqsR. Cultures of this strain were grown in LB supplemented with 10 μM PQS to induce P*pqsA* activity, with a fixed concentration of the hits (100 μM), and with increasing concentrations of IPTG. The rationale of this experiment is that increased expression of PqsR, due to increased concentration of IPTG, should decrease the repressive effect exerted by the hits on P*pqsA*, if PqsR is the target of the hits. As shown in Fig. 4C, the inhibitory effect exerted by the hits on P*pqsA* activity decreased in parallel to increasing IPTG concentration in the growth medium, thus supporting PqsR as their molecular target. Overall, these data indicate that each of the hits acts downstream of *pqsR* expression, likely hampering PqsR functionality.

To support the hypothesis that the inhibitors directly interact with PqsR, molecular docking simulations were performed based on the crystal structure of the PqsR coinducer binding domain (CBD) in the apo form (PDB 4JVC) (59). To increase the

reliability of the simulations, the docking search space encompassed the entire CBD of PqsR, i.e., a "blind" docking procedure was carried out. Amino acid residues previously reported to be involved in the binding of the natural ligand 2-nonyl-4-hydroxyquinoline (NHQ) to the PqsR CBD (59) were considered flexible (see Materials and Methods for details). This analysis indicated that the three hits bind PqsR with high affinity at the same site as the natural ligand NHQ (Fig. 5) with predicted Δ*G* values for binding of clotrimazole, clofoctol, and miconazole being −8.4, −9.8, and −8.5 kcal/mol, respectively. Interestingly, these values are lower than the predicted Δ*G* value for binding of NHQ (−7.9 kcal/mol; Table 1). Similar results were obtained when using the PqsR CBD structure bound to NHQ (PDB 4JVD) (59), from which the ligand was removed. In the latter case, the Δ*G* values for the binding of clotrimazole, clofoctol, miconazole, and NHQ were −9.4, −9.9, −8.1, and −8.1 kcal/mol, respectively. Finally, maintaining all the CBD residues in a fixed position yielded very similar results (data not shown). Interestingly, in each case the predicted affinity of the hits for PqsR parallels their efficacy as *pqs* inhibitors (Table 1).

Overall, these data suggest that the newly identified inhibitors could be endowed with a similar mechanism of action, that is to hamper PqsR functionality by competing with AQ agonists for PqsR binding. Also, the evidence that clotrimazole increases PqsR level (Fig. 4B) while hampering its ability to drive AQ production (Fig. 2B) and to activate *pqs*-controlled genes (Fig. 2A and S6) supports direct interaction of this hit to PqsR.

Notably, both activity assays and *in silico* predictions indicate that clofoctol has greater inhibitory activity relative to miconazole and clotrimazole (Table 1). To support competitive binding of PQS and clofoctol to PqsR, the ability of this drug to repress P*pqsA* activity was evaluated in the AQ-Rep biosensor grown in the presence of a range of concentrations of the native PqsR agonist PQS. This competition assay revealed the reduced ability of clofoctol to inhibit P*pqsA* activity in the presence of increasing concentrations of PQS (Fig. S7), in accordance with the activity of clofoctol as a competitive antagonist of the PQS receptor protein PqsR.

Clofoctol inhibits the expression of *pqs*-controlled virulence phenotypes. By hampering the ability of PqsR to activate the transcription of the *pqsABCDE-phnAB* operon, clofoctol is expected to reduce the expression of virulence traits controlled by both PQS and PqsE in *P. aeruginosa*. First of all, since the assays previously performed to assess the effect of clofoctol on AQ production did not discriminate between HHQ and PQS, these QS signal molecules were quantified by liquid chromatography-tandem mass spectrometry (LC-MS/MS) analysis of spent media from PAO1 cultures treated with a range of concentrations of clofoctol. As shown in Fig. 6A, this analysis confirmed that clofoctol inhibits AQ production in *P. aeruginosa*, with both HHQ and PQS concentrations being significantly reduced by the drug.

With respect to the effect of clofoctol on PQS- and PqsE-controlled virulence determinants, phenotypic analyses revealed that 100 μM clofoctol leads to >80% reduction in pyocyanin (Fig. 6B) and considerably reduced swarming motility (Fig. 6C). Moreover, 100 μM clofoctol significantly reduced biofilm formation in a PAO1 strain constitutively expressing green fluorescent protein (GFP) via the pMRP9-1 plasmid (60) (Fig. 6D). Notably, the effect of clofoctol on the tested phenotypes in PAO1 mimicked deletion of the *pqsR* gene (Δ*pqsR*; Fig. 6B to D), in accordance with the hypothesis that PqsR is the clofoctol target.

Subsequently, real-time RT-PCR analyses were performed to examine the effect of clofoctol on the expression of *pqs*-controlled virulence genes (28). The PQS-dependent *pvdS* and *pchR* genes code for the PvdS and PchR regulatory proteins required for the synthesis of the siderophores pyoverdine and pyochelin, respectively (28, 61); the PqsE-dependent *lecA* gene codes for the LecA lectin involved in the formation of antibiotic-resistant biofilms (28, 62). As a control, the mRNA level of *pqsA* was also measured. Real-time RT-PCR analyses showed that clofoctol significantly decreased the mRNA level of each of the genes tested, in agreement with the downregulation

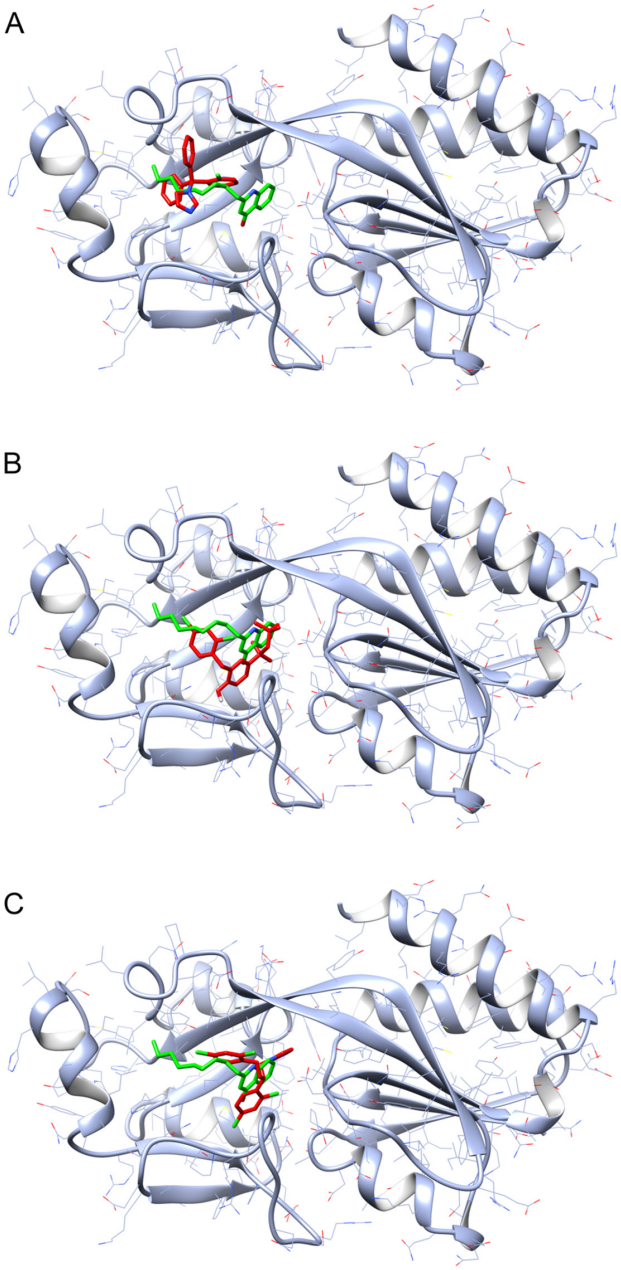


FIG 5 Putative complexes formed by clotrimazole, clofoctol, and miconazole with the PqsR CBD. Schematic representations of the complexes formed by clotrimazole (A), clofoctol (B), and miconazole (C) with the PqsR CBD, obtained by molecular docking simulations (see Materials and Methods for details), are shown. The three drugs are represented in red, while the natural ligand NHQ is represented in green.

observed in a PAO1 Δ*pqsR* mutant strain (Fig. 6E). The negative effect exerted by clofoctol on *lecA* transcription was also confirmed by promoter activity assay showing reduced activity of the P*lecA*::*luxCDABE* transcriptional fusion in PAO1 cultures treated with clofoctol (Fig. S8). Overall, these data support clofoctol as an antivirulence agent active against the *P. aeruginosa pqs* QS system.

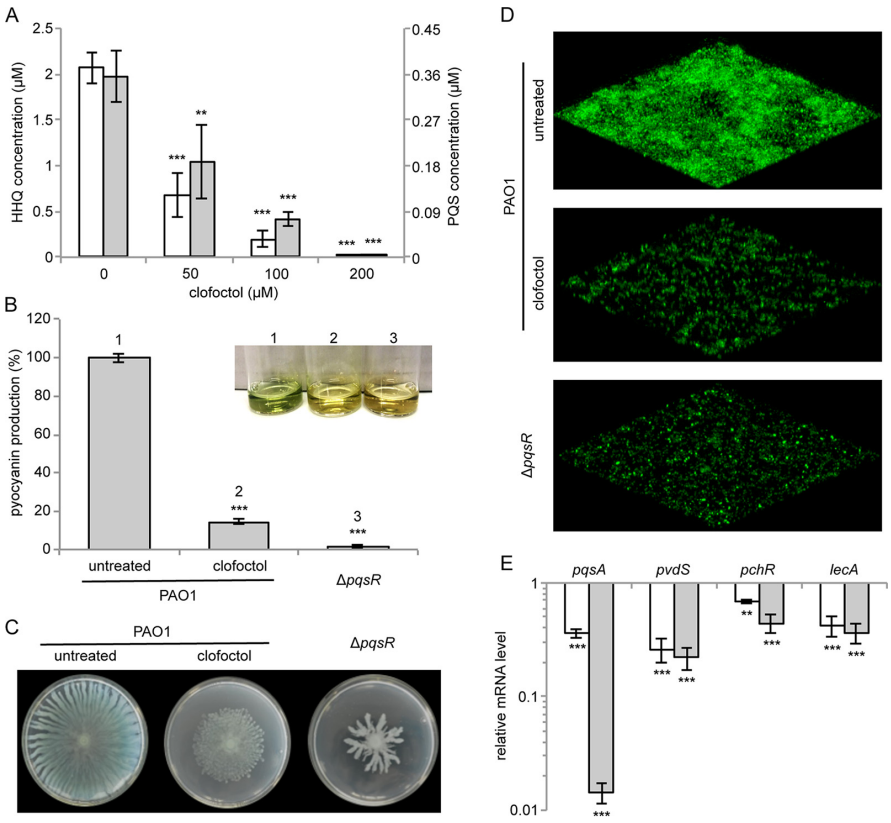


FIG 6 Clofoctol inhibits the expression of *pqs*-controlled virulence traits. (A) Concentrations of HHQ (white bars) and PQS (gray bars) measured by LC-MS/MS on supernatants of PAO1 cultures grown for 16 h in LB in the absence or in the presence of clofoctol at the indicated concentrations. The averages from three independent experiments are reported, along with the SD. **, $P = 0.0062$; ***, $P < 0.001$ (ANOVA). (B to D) Effect of 100 μM clofoctol on pyocyanin production (B), swarming motility (C), and biofilm formation (D) in PAO1. The same phenotypes were evaluated in the Δ*pqsR* mutant as a control. For pyocyanin production in panel B, the averages from three independent experiments are reported with the SD, and representative supernatants are shown in the inset picture. ***, $P < 0.001$ (ANOVA). For the swarming motility (panel C) and biofilm formation (panel D), representative pictures of three independent experiments are shown. (E) Real-time RT-PCR analysis showing the mRNA levels of the indicated genes in PAO1 treated with 100 μM clofoctol (white bars) and in the Δ*pqsR* mutant (gray bars) relative to untreated PAO1. The averages from three independent experiments are reported with the SD. **, $P = 0.0012$; ***, $P < 0.001$ (ANOVA).

Clofoctol protects *Galleria mellonella* larvae from *P. aeruginosa* infection and inhibits the *pqs* QS system in CF clinical isolates. The antivirulence activity of clofoctol was tested in *G. mellonella* larvae, an insect infection model which correlates well with *P. aeruginosa* mouse infection models (63). First, *G. mellonella* was infected with ca. 10 cells of *P. aeruginosa* PAO1 or of the isogenic Δ*pqsR* mutant and incubated at 37°C for 120 h. As shown in Fig. 7A, mutation of *pqsR* significantly reduced the ability of *P. aeruginosa* to kill the larvae, demonstrating the suitability of this insect model to investigate the antivirulence potential of drugs targeting PqsR.

Since the average weight of *G. mellonella* larvae was ca. 500 mg, and arbitrarily assuming uniform dispersal of injected bacteria and clofoctol in 500 μl of larval volume (64, 65), 10 μl of saline containing 5 mM clofoctol was injected to yield 100 μM clofoctol in each larva. Preliminarily, we verified that the injection of 10 μl of saline containing 5 mM clofoctol did not affect the survival of uninfected larvae and that 2 h of incubation of *P. aeruginosa* with 5 mM clofoctol did not affect *P. aeruginosa* growth and viability (data not shown). Then, *G. mellonella* larvae were inoculated with *P. aeruginosa*

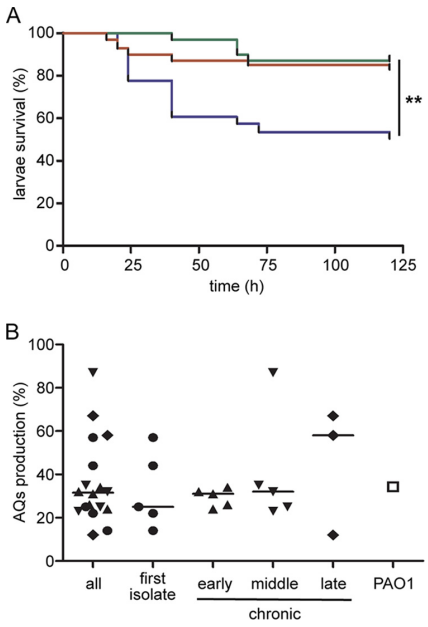


FIG 7 Clofoctol displays an antivirulence effect *in vivo* and inhibits the *pqs* QS system in *P. aeruginosa* CF clinical isolates. (A) Kaplan-Meier plot shows the percentage survival of *G. mellonella* larvae inoculated with *P. aeruginosa* PAO1 (blue line), with PAO1 and clofoctol at a final concentration of 100 μM (red line), or with Δ*pqsR* mutant (green line). The mean survival rate calculated from four independent experiments performed on at least 30 larvae per condition is reported. **, $P = 0.0033$ for PAO1 versus PAO1 plus clofoctol and $P = 0.0016$ for PAO1 versus Δ*pqsR* mutant (ANOVA). (B) Dot plot showing the inhibition of AQ production in *P. aeruginosa* CF isolates (filled symbols) and *P. aeruginosa* PAO1 (open square) treated with 100 μM clofoctol, relative to the untreated samples, which were considered 100%. Black lines represent median values: all, 31.4%; first isolate, 25.2%; early chronic, 31.1%; middle chronic, 32.1%; and late chronic, 57.8%. The AQ production in treated PAO1 cultures was 34.3% relative to untreated PAO1. Differences between the median values are not statistically significant. Mean results from three independent experiments are reported.

PAO1 in the absence or in the presence of clofoctol. The treatment with clofoctol led to a survival percentage of 87%, similar to that observed with the Δ*pqsR* mutant (83%), while only 50% of untreated *G. mellonella* larvae survived PAO1 infection (Fig. 7A). Overall, these data demonstrate that clofoctol attenuates *P. aeruginosa* PAO1 lethality in *G. mellonella*.

To verify that clofoctol is active also against clinical *P. aeruginosa* strains, its ability to reduce AQ production was evaluated in a collection of 20 *P. aeruginosa* isolates from the lungs of CF patients, grouped into four categories with respect to the stage of infection (Table S2). A preliminary analysis revealed that only two strains isolated from patients with more than 15 years of chronic infection (chronic late group) did not produce detectable levels of AQs (Table S2); hence, these strains should be considered resistant to the antivirulence effect of clofoctol. The remaining 18 clinical isolates were grown in LB for 24 h in the absence or presence of 100 μM clofoctol, and the AQ concentration was determined in the corresponding spent media by using the AQ-Rep biosensor. Residual AQ production was estimated for each treated isolate relative to the amount of AQ detected in the corresponding untreated sample, considered 100%. Notably, clofoctol decreased AQ production in each of the clinical isolates tested, with a reduction ranging from 12.7 to 88.4% (Fig. 7B). The median reduction in AQ production in the tested isolates was 68.6% and hence comparable to the reduction in AQ levels measured in PAO1 treated with 100 μM clofoctol under the same conditions (65.7%; Fig. 7B). Differences in the median reduction values among the analyzed groups were not statistically significant. Moreover, differences in the median reduction of AQ

production were not significant also when grouping the isolates according to their antibiotic resistance profiles (Table S2). Indeed, the median reductions of AQ levels were 71.6 and 67.4% in 4 antibiotic-susceptible and in 12 antibiotic-resistant strains, respectively (Fig. S9). Also the two multidrug-resistant or extensively drug-resistant strains analyzed in this study were susceptible to clofoctol, with reductions in AQ levels of 56.5 and 88.4%, respectively (Fig. S9). Although performed on a limited number of clinical isolates, this analysis indicates that clofoctol is effective in blocking the *pqs* QS system in CF strains, irrespective of their adaptation to the host environment during long-lasting chronic lung infection and of their antibiotic resistance profiles.

DISCUSSION

As a consequence of widespread antibiotic resistance, inhibition of virulence rather than growth has become a viable approach for combatting bacterial infections with lower selective pressure for emergence of resistance (10). In particular, *in vitro* evolution experiments suggest that resistant mutants will not emerge for drugs targeting public goods, such as virulence factors that are secreted and shared between individuals (66). Moreover, since antivirulence drugs target specific bacterial functions required for infection, these molecules are not expected to impact the beneficial resident microbiota relative to that of antibiotics (11, 13).

In many bacterial pathogens, QS positively controls the expression of multiple secreted virulence factors; hence, this communication system is considered a promising target for the development of antivirulence agents (23, 67). Since *P. aeruginosa* has four interconnected QS systems that positively control the production of virulence factors and biofilm formation, most of the research on QS inhibition has focused on this bacterium as a model system. Indeed, several molecules inhibiting the *las* QS system of *P. aeruginosa* have been identified (23, 67). Recently, a number of studies have described inhibitors of the *pqs* QS system. The *pqs* system positively controls the expression of multiple virulence determinants, including secreted virulence factors and biofilm formation, and *pqs* mutant strains display attenuated virulence in plant and animal models of infection (32–35, 38). Moreover, the *pqs* system is active during *P. aeruginosa* infections in humans (39, 68, 69).

Inhibitors of the *pqs* system were previously identified among analogs of anthranilate, the substrate of PqsA in the first step of the biosynthetic route leading to AQ production (37, 40). Subsequently, compounds binding to the AQ-biosynthetic enzyme PqsD were shown to act as potent *pqs* inhibitors, with IC_{50}s in the low micromolar range (from 1 to 14 μM) (70, 71). The possibility of interfering with the *pqs* system via enzymatic degradation of the AQ signals, rather than via small molecules targeting their biosynthesis, was also explored, and PQS degrading activity has been described in *Arthrobacter nitroguajacolicus* and *Achromobacter xylosoxidans* (72, 73). However, the majority of anti-*pqs* molecules identified so far are competitive inhibitors of the transcriptional regulator PqsR. Potent PqsR antagonists with IC_{50}s ranging from 0.4 to 38.5 μM have been found among analogs of the natural agonists HHQ and PQS (59, 74–76). Whole-cell high-throughput screening and structure-activity relationship analyses led to the identification of benzamide-benzimidazole PqsR inhibitors with low IC_{50}s (<1 μM), some of which also inhibited the PqsBC complex (77–79). Also, 2-sulfonylpyrimidines were identified as hampering both AQ reception and biosynthesis (80). Overall, a number of reports validated the antivirulence potential of anti-*pqs* molecules, showing their ability to reduce the expression of *pqs*-controlled virulence traits both *in vitro* and in animal models of infection. Despite the promise of anti-*pqs* agents for the treatment of *P. aeruginosa* infections, to the best of our knowledge none of these molecules has thus far entered clinical trials. This is probably due to the poor pharmacological properties of the inhibitors, including possible cytotoxicity, and to the lack of ADME-TOX studies required for their evaluation in humans. In this context, searching for off-target activities in drugs already approved for use in humans represents a potential shortcut for developing new anti-*pqs* molecules that could move straight into clinical trials.

In this study, a drug-repurposing approach led to the identification of three promising anti-*pqs* drugs already used in humans by screening a library of 1,600 FDA-approved compounds (Table 1; Fig. S2). Data on the acute and chronic toxicity are already available for these drugs, as well as information on their pharmacokinetics. Clotrimazole and miconazole are antifungal drugs used in humans to treat ringworm, pityriasis versicolor, vaginal and oral candidiasis, and skin yeast infections (44, 45, 81, 82). They both alter the permeability of the fungal cell wall by binding to phospholipids and inhibiting the biosynthesis of ergosterol and other sterols required for fungal cell membrane integrity (83, 84). Miconazole displays its activity by inhibiting fungal peroxidases, which results in peroxide-mediated cell death (83). Both of these drugs are mainly administered as creams or ointments; thus, their current formulations could be particularly suitable for the topical treatment of chronic wound infections caused by *P. aeruginosa* (85, 86). However, this opportunistic pathogen is a main cause of lung infections especially in individuals with CF, where it establishes chronic infections that can last for decades (87). The use of clotrimazole and miconazole to treat *P. aeruginosa* lung infections would require their reformulation as inhalable nanosuspensions, an approach that has recently demonstrated its value for repurposing the anthelmintic drug niclosamide as an anti-QS agent against *P. aeruginosa* (24, 88).

Of the 1,600 compounds tested in this screening campaign, the most promising anti-*pqs* drug was clofoctol, an antimicrobial used for the treatment of acute and chronic upper respiratory tract infections and for tracheobronchial infections caused by Gram-positive pathogens, especially staphylococci, pneumococci, and streptococci (48, 50). Clofoctol is also used in preventive and curative treatment of otolaryngology and stomatology (89). The mechanism of action of this drug as an antimicrobial is still poorly understood, but a detrimental effect of clofoctol on membrane and cell wall biosynthesis in Gram-positive bacteria has been reported (49, 90). Clofoctol is usually administered as suppositories since it is well absorbed through the rectal mucosa and rapidly spreads through the tissues, reaching the highest concentrations in the respiratory system (91). Since clofoctol mainly acts in the airways, it is potentially valuable as a future treatment of *P. aeruginosa* lung infections. Notably, clofoctol is used to treat infections in infants, and this is another advantageous feature considering that in CF a *P. aeruginosa* lung infection is established in early life (92).

Overall, despite their lower potency compared to other *pqs* inhibitors described thus far, the anti-*pqs* drugs identified in this study have considerable potential for human use and could be directly tested in clinical trials or serve as chemical scaffolds for future drug optimization programs.

With respect to the mechanism of action of the three FDA-approved drugs, they all affect PqsR functionality, probably by competing with the natural ligands HHQ and PQS for the PqsR ligand-binding site (Fig. 3, 4, and S7). This hypothesis is supported by docking simulations, which predict that all three compounds bind to the PqsR coinducer binding domain in the same binding site as the natural ligand NHQ (Fig. 5). This result was somehow unexpected, since the PAO1/AQ-Rep coculture used in the screening campaign should primarily identify molecules affecting both AQ biosynthesis and AQ reception (Fig. 1A). Indeed, this coculture-based reporter system was functional in identifying the PqsA-inhibitor methyl anthranilate (Fig. 1B). Intriguingly, the anti-QS activity of the anthelmintic drug niclosamide was discovered using a coculture-based reporter system similar to the one deployed in this work. In common with clofoctol, niclosamide inhibited the QS signal molecule response rather than biosynthesis (24). Therefore, the selection of drugs targeting QS receptors could be a bias intrinsic to the screening system used. In fact, in coculture-based screening systems, drugs interfering with QS signal molecule receptor would have a dual outcome since they would block both QS signal receptor and consequently signal biosynthesis in the wild type, as well as inhibiting the QS receptor in the reporter strain. Conversely, an inhibitor of QS signal molecule biosynthesis would only affect the functionality of the *P. aeruginosa* wild-type strain. Hence, the PAO1/AQ-Rep coculture system may offer a more sensitive screen for

PqsR inhibitors than for those that inhibit AQ biosynthesis, so that only drugs targeting PqsR will meet the selection criteria for the primary screen.

Since each of the hits identified in this study are likely to target PqsR, we focused our attention on the most potent inhibitor, clofoctol (Table 1).

Different elements of the *pqs* QS system have recently been shown to control distinct virulence traits. In particular, the PQS signal molecule drives the expression of genes required for the biosynthesis of siderophores and of genes coding for PrpL and AprX proteases, and exotoxin S, while PqsE is required for the production of pyocyanin, LecA and LecB lectins, hydrogen cyanide, rhamnolipids, and ChiC chitinase (28). With regard to pleiotropic virulence phenotypes such as swarming motility and biofilm formation, these appear to be regulated by both PQS and PqsE (33). Consistent with the activity of clofoctol as a PqsR inhibitor, the expression of both PQS-controlled virulence traits, such as the expression of genes required for siderophore biosynthesis (Fig. 6E), and of PqsE-dependent phenotypes, including pyocyanin production (Fig. 6B) and expression of the *lecA* gene (Fig. 6E and S8), were inhibited. Moreover, clofoctol reduced both swarming motility and biofilm formation (Fig. 6C and D). Notably, clofoctol exerted an antivirulence effect *in vivo*, since this drug-attenuated *P. aeruginosa* infection in *G. mellonella* larvae (Fig. 7A).

A major concern with respect to the use of anti-QS drugs for the treatment of CF pulmonary infection originates from evolutionary selection driving *P. aeruginosa* adaptation to the CF lung. Indeed, during chronic infections, CF isolates accumulate mutations that reduce the production of virulence factors, lead to the formation of mucoid biofilms, increase antibiotic resistance mainly as a consequence of efflux pump overexpression, and in some cases inactivate QS systems (93–96). Since *P. aeruginosa* QS-defective mutants should be considered resistant to anti-QS drugs, the suitability of QS inhibition for CF therapy is under debate. However, most studies have focused on the inactivation of the *las* QS system in chronic CF isolates, while little attention has so far been given to the *pqs* QS system (97–100). The evidence that AQs have been identified in the sputum of CF patients with both intermittent and chronic *P. aeruginosa* infections demonstrates unequivocally that the *pqs* QS system is active in the CF lung (68, 69, 99, 101). In addition, AQs can be detected in the sputum, plasma, and urine of ca. 80% of CF patients suffering with *P. aeruginosa* chronic lung infections. The levels of the AQ molecule NHQ increased at the start of a pulmonary exacerbation and positively correlated with quantitative measures of *P. aeruginosa* cells in the lung (39). This evidence is consistent with the results obtained in this study, since only 2 of the 20 clinical isolates tested did not produce detectable levels of AQs (Table S2). Notably, clofoctol reduced functionality of the *pqs* QS system in all the *pqs*-proficient CF isolates, irrespective of their antibiotic resistance profiles (Fig. 7B and S9).

Future analyses performed on a larger panel of *P. aeruginosa* clinical isolates from both CF and chronic wound patients and *in vivo* assays in murine models of infection are required to better assess the suitability of clofoctol, clotrimazole, and miconazole for the treatment of *P. aeruginosa* chronic infections. However, the results of this work should encourage further preclinical studies to aid transfer of the newly identified *pqs* inhibitors from the laboratory into clinical practice.

MATERIALS AND METHODS

Bacterial strains, media, and chemicals. The bacterial strains, clinical isolates, plasmids, and oligonucleotides used in this study are listed in Tables S1, S2, S3, and S4, respectively. Bacterial strains were routinely grown at 37°C in Luria-Bertani broth (LB) with aeration and, when necessary, antibiotics were added at the following concentrations: tetracycline, 200 μg/ml; carbenicillin, 150 μg/ml; gentamicin, 100 μg/ml; and kanamycin, 200 μg/ml. When necessary, IPTG (isopropyl-β-D-thiogalactopyranoside) was added at the concentrations indicated in the text. Muller-Hinton broth and M9 minimal medium supplemented with 20 mM glucose as a carbon source were used in the MIC assay (Clinical and Laboratory Standards Institute) and in the biofilm assay, respectively. Synthetic HHQ and PQS stock solutions were prepared in methanol. Clotrimazole, clofoctol, and miconazole were purchased from Sigma-Aldrich and dissolved in dimethyl sulfoxide (DMSO).

Primary screening for the identification of *pqs* inhibitors. *P. aeruginosa* PAO1 and the AQ-Rep biosensor strain (PAO1 Δ*pqsA* P*pqsA*::*luxCDABE*) were grown overnight at 37°C on LB agar plates. Bacteria were scraped from plate surfaces and diluted in LB to optical density at 600 nm (OD_{600}) wavelengths of

0.1 and 0.03 for the biosensor and PAO1 strains, respectively (procedure modified from [57]). Aliquots (200 μl) of the coculture were grown at 37°C in 96-well microtiter plates in LB supplemented with each compound of the PHARMAKON library (20 μM and 200 μM). The OD_{600} values and relative light units (RLU) were measured after 5 h of incubation by using a Wallac 1420 Victor³V multilabel plate reader (Perkin-Elmer). Eight samples grown in the presence of DMSO (0.2 or 2%) were used as controls in each microtiter plate. The reporter activity was determined as the RLU/OD_{600} for each sample. The residual reported activity was determined in treated samples relative to control samples grown in the presence of DMSO, for which the value was considered 100%.

A similar approach was used to investigate the effect of the hits on the *las* and *rhl* QS systems. In this case, cocultures of the *P. aeruginosa* PAO1 wild-type strain and of the PA14-R3 (PA14 Δ*lasI* P*rsaL*::*luxCDABE* [57]) or the C4-Rep (PAO1 Δ*rhlI* P*rhlA*::*luxCDABE*; 24) biosensor strains were used, respectively.

Quantification of AQs. Levels of AQ signal molecules in treated-*P. aeruginosa* PAO1 culture supernatants were determined by using the reporter strain AQ-Rep, as previously described (102). Bacterial cultures were grown in 96-well microtiter plates at 37°C with shaking. Supernatants were collected after 16 h for the experiments shown in Fig. 2B, 3A, and S2, or after 24 h for experiments shown in Fig. 7B and S9, to allow optimal AQ production in slow-growing clinical isolates. Briefly, 10 μl of culture supernatant was added to 190 μl of LB inoculated with AQ-Rep biosensor (final OD_{600} = 0.1) in 96-well microtiter plates. Microtiter plates were incubated at 37°C with gentle shaking, and the OD_{600} and RLU values were measured after 5 h of incubation. A calibration curve was generated by growing the AQ-Rep biosensor in the presence of increasing concentrations of synthetic HHQ or PQS; the resulting dose-response curve was used to calculate the concentration of the AQ signals in each culture supernatant.

AQs produced by *P. aeruginosa* PAO1 were also quantified in by LC-MS/MS analysis, as previously described (103). Briefly, PAO1 was inoculated into 5 ml of LB in the absence or in the presence of 100 μM clofoctol. After 16 h of incubation at 37°C with shaking, the cell density of the culture was recorded, and the supernatants were filter sterilized. Supernatants were solvent extracted with ethyl acetate, dried under vacuum, and redissolved in methanol prior to quantitative analysis by LC-MS/MS. For each sample, a supernatant concentration of HHQ and PQS was calculated by comparing analytic peak areas with a matched calibration line.

Pyocyanin production, swarming motility, and biofilm formation assays. Pyocyanin was extracted and quantified from *P. aeruginosa* PAO1 and Δ*pqsR* grown in LB supplemented with 100 μM clofoctol or with DMSO as a control, as previously described (104). Swarming motility assays were performed on swarming plates (0.8% [wt/vol] nutrient broth N.2, 0.5% [wt/vol] glucose, 0.5% [wt/vol] bacteriological agar). Plates were supplemented with or without clofoctol (100 μM). After 16 h of growth at 37°C, swarming motility was directly observed at the air-agar interface.

For microscopic visualization of biofilms, *P. aeruginosa* PAO1 or Δ*pqsR* strains constitutively expressing GFP via the pMRP9-1 plasmid (60) were grown in an 8-well chamber slide, as previously described (105), with minor modifications. Briefly, bacterial cells were inoculated at an OD_{600} of 0.02 in 700 μl of M9 minimal medium supplemented with 20 mM glucose as carbon source, in the absence or in the presence of 100 μM clofoctol. Cultures were incubated at 30°C for 24 h to allow the adhesion of the bacterial cells to the glass surface. To maintain bacterial viability, the medium was changed every 24 h. Biofilm formation was examined after 3 days incubation by using the Leica TCS SP5 confocal microscope.

Western immunoblotting. Crude protein extracts were collected from the *P. aeruginosa* PAO1 Δ*pqsA* Δ*pqsH* Δ*pqsR* triple-mutant strain carrying the pPqsR-6H plasmid grown in LB supplemented with 10 μM PQS and 20 μM IPTG, in the absence or in the presence of 100 μM clotrimazole, clofoctol, or miconazole. The *P. aeruginosa* PAO1 Δ*pqsA* Δ*pqsH* Δ*pqsR* strain carrying the pME6032 empty vector was used as a control. A Bradford assay (106) was used to quantify and normalize total protein content in the samples. Western immunoblotting was performed using a standard technique (107) with mouse anti-6His antibody (1:5,000; Sigma-Aldrich) and goat anti-mouse IgG horseradish peroxidase-conjugate as secondary antibody (1:6,000; Bio-Rad Laboratories). Final development was performed with Amersham ECL chemiluminescent reagents (Amersham Biosciences). A C-DiGit blot scanner (LI-COR Biosciences) was used for data acquisition.

RNA extraction and real-time RT-PCR analysis. *P. aeruginosa* PAO1 and Δ*pqsR* were inoculated at an OD_{600} of 0.02 in 5 ml of LB in the absence or in the presence of 100 μM clotrimazole, clofoctol, or miconazole. Cultures were grown at 37°C with vigorous shaking until they reached an OD_{600} of 2.0, and then 1 ml of cells was harvested by centrifugation and resuspended in 2 ml of RNAprotect bacterial reagent (Qiagen). Total RNA extraction was performed with an RNeasy Mini Columns kit (Qiagen) according to the manufacturer's instructions, including the on-column DNase I digestion step. In addition, eluted RNA was treated for 1 h at 37°C with DNase Turbo (0.2 U per μg of RNA; Ambion) and with SUPERase-In (0.4 U per μg of RNA; Ambion). DNase I was removed using the RNeasy column purification kit (Qiagen). Purified RNA was quantified using the NanoDrop 2000 spectrophotometer (Thermo-Fisher Scientific). The absence of genomic DNA in the RNA samples was verified by PCR performed with the primers FWP*pqsL* and RVP*pqsL* (Table S4). cDNA synthesis was performed with the iScript reverse transcription supermix for RT-qPCR kit (Bio-Rad Laboratories) according to the manufacturer's instructions and quantified with NanoDrop 2000. Real-time RT-PCRs were performed using an iTaq Universal SYBR Green Supermix kit (Bio-Rad Laboratories) according of the manufacturer's instructions, and the Rotor Gene 6000 thermocycler (Corbett Research). Primers employed in real-time RT-PCR analysis were designed using the Primer-BLAST software (www.ncbi.nlm.nih.gov/tools/primer-blast) and are listed in Table S4. The reaction procedure involved incubation at 95°C for 1 min and 40 cycles of amplification at 95°C for 10 s and 60°C for 45 s. Fluorescence was registered in the last 15 s of the 60°C step. 16S rRNA was chosen as an internal control (housekeeping gene) to normalize the real-time RT-PCR data in each single run and to calculate the relative fold change in gene expression by using the $2^{-\Delta\Delta CT}$

method. The average data and standard deviations (SD) were calculated from three independent experiments.

***Galleria mellonella* killing assay.** The *G. mellonella* killing assay was performed as previously described (63, 65), with minor modifications. Briefly, *G. mellonella* caterpillars in the final instar larval stage (average weight, 486 ± 67 mg) were infected with 10 μl of saline containing about 10 bacterial cells in the absence or in the presence of 5 mM clofoctol. Although PAO1 cells were incubated in the presence of clofoctol for less than 5 min before injection, preliminary assays showed that 5 mM clofoctol treatment (for up to 24 h) does not significantly affect PAO1 cell or larval viability (data not shown). *G. mellonella* larvae were incubated at 37°C in petri dishes (ten larvae *per* dish) and monitored for 120 h. Larvae were considered dead when they did not respond to gentle prodding. At least 30 larvae *per* condition were used in four independent experiments. Survival curves for the *G. mellonella* killing assay were generated using the Kaplan-Meier method.

Molecular docking simulations. Molecular docking simulations were carried out using DockingApp (108), a user friendly interface for the docking program AutoDock Vina (109). In all simulations, the search space (docking grid) included the whole PqsR coinducer binding domain (CBD) structure in order to carry out "blind" predictions of the "hit" compound binding sites.

Simulations were carried out on the apo (PDB 4JVC) and holo (PDB 4JVD) forms of the protein (59), both by keeping all protein residues rigid and by allowing flexibility only of the residues previously reported to be involved in PqsR binding to the natural ligand NHQ (i.e., ILE 149, ALA 168, VAL 170, ILE 186, LEU 189, LEU 207, LEU 208, PHE 221, ILE 236, TYR 258, ASP 264, and THR 265) (52).

Statistical analysis. Statistical analysis was performed with the software GraphPad Prism 5, using one-way analysis of variance (ANOVA), followed by Tukey-Kramer multiple comparison tests. Differences with a P value of <0.05 were considered statistically significant.

SUPPLEMENTAL MATERIAL

Supplemental material for this article may be found at https://doi.org/10.1128/AAC.01296-18.

SUPPLEMENTAL FILE 1, PDF file, 0.4 MB.

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Identification of FDA-approved drugs as antivirulence agents targeting the *pqs* quorum sensing system of *Pseudomonas aeruginosa*

Francesca D'Angelo[a], Valerio Baldelli[a], Nigel Halliday[b], Paolo Pantalone[b], Fabio Polticelli[a,c], Ersilia Fiscarelli[d], Paul Williams[b], Paolo Visca[a], Livia Leoni[a], Giordano Rampioni[a]#

[a] Department of Science, University Roma Tre, Rome, Italy; [b] Centre for Biomolecular Sciences and School of Life Sciences, University of Nottingham, Nottingham, UK; [c] National Institute of Nuclear Physics, Roma Tre Section, Rome, Italy; [d] Laboratory of Cystic Fibrosis Microbiology, Bambino Gesú Hospital, Rome, Italy.

SUPPLEMENTAL MATERIAL

Table S1. Bacterial strains used in this study.

Strains	Characteristics	References
E. coli		
S17.1λ*pir*	conjugative strain for suicide plasmids.	(1)
P. aeruginosa		
PAO1	Nottingham collection wild type strain.	
Δ*pqsR*	PAO1 mutant strain with in frame clear deletion of the *pqsR* gene.	(2)
PAO1 P*pqsA*::*lux*	PAO1 wild type strain carrying chromosomal insertion of the P*pqsA*::*lux* transcriptional fusion; Tc^R.	(3)
PAO1 mini-CTX::*lux*	PAO1 wild type strain carrying chromosomal insertion of the mini-CTX::*lux* empty vector; Tc^R.	(3)
Δ*pqsA* P*pqsA*::*lux*	PAO1 mutant strain deleted in *pqsA* gene carrying chromosomal insertion of the P*pqsA*::*lux* transcriptional fusion; Tc^R (named AQ-Rep).	(4)
Δ*lasI* P*rsaL*::*lux*	PA14 mutant strain deleted in *lasI* gene carrying chromosomal insertion of the P*rsaL*::*lux* transcriptional fusion (named PA14-R3).	(5)
Δ*rhlI* P*rhlA*::*lux*	PAO1 mutant strain deleted in *rhlI* gene carrying chromosomal insertion of the P*rhlA*::*lux* transcriptional fusion; Km^R (named C4-Rep).	(6)
Δ*pqsAH* P*pqsA*::*lux*	PAO1 double mutant strain deleted in *pqsA* and *pqsH* genes carrying chromosomal insertion of the P*pqsA*::*lux* transcriptional fusion; Tc^R.	(3)
Δ*pqsAHR* P*pqsA*::*lux*	PAO1 triple mutant strain deleted in *pqsA, pqsH* and *pqsR* genes carrying chromosomal insertion of the P*pqsA*::*lux* transcriptional fusion; Tc^R.	(7)
PAO1 P*lecA*::*lux*	PAO1 wild type strain carrying chromosomal insertion of the P*lecA*::*lux* transcriptional fusion; Tc^R.	(8)
Δ*pqsR* P*lecA*::*lux*	PAO1 mutant strain deleted in *pqsA* gene carrying chromosomal insertion of the P*lecA*::*lux* transcriptional fusion; Tc^R.	This study

Table S2. Clinical isolates used in this study.

Isolate name[a]	Colonization[b]	Years of colonization	Phenotypic characteristics[c]	Antibiotics susceptibility[d]	Residual AQs production[e]
BG 4	first isolate	-	frayed	R	22
BG 5	chronic early	2	frayed	R	31
BG 6	chronic middle	6	frayed	R	35
BG 7	first isolate	-	circular	S	25
BG 8	chronic early	2	circular	R	26
BG 10	first isolate	-	frayed	S	14
BG 11	chronic early	3	frayed	R	44
BG 12	chronic middle	5	frayed	S	32
BG 13	first isolate	-	frayed	MDR	44
BG 17[e]	chronic early	2	circular	R	24
BG 18[e]	chronic middle	6	circular	R	25
BG 36	chronic early	3	rugose	S	32
BG 56	first isolate	-	small	R	57
BG 76	chronic middle	7	mucoid	R	23
BG 80[e]	chronic middle	5	circular	R	87
BG 92[e]	chronic late	≥15	small	XDR	12
BG 93	chronic late	≥15	rugose	MDR	np
BG 96	chronic late	≥15	circular	R	58
BG 97	chronic late	≥15	frayed	XDR	np
BG 100[e]	chronic late	≥15	small	R	67

[a] CF clinical isolates from the collection of the Bambino Gesú hospital, Rome, Italy.

[b] Different categories depending on the year of infection of the clinical isolates in the lung of individuals with cystic fibrosis: first isolate; chronic early (from 2 to 3 years); chronic middle (from 5 to 7 years); chronic late (equal or more than 15 years).

[c] Characteristics observed when clinical isolates were grown as colony biofilms.

[d] Criteria to define multi-drug resistant (MDR) and extensively-drug resistant (XDR) bacteria have been taken from European Centre for Diseases Prevention and Control (ECDC) web site (http://ecdc.europa.eu/en/Pages/home.aspx): MDR, resistant to one or more antibiotics belonging to at least three different classes; XDR, resistant to one or more antibiotics belonging to all classes except two or less; S, susceptible to all classes of antibiotics; R, resistant to one or more antibiotics belonging to less than three different classes.

[e] Residual production of AQs in samples treated with 100 μM clofoctol relative to untreated samples, considered as 100%. np, strains that do not produce detectable levels of AQs.

Table S3. Plasmids used in this study.

Plasmids	Characteristics	References
pME6032	pVS1-p15A shuttle expression (IPTG-inducible) vector; Tc^R.	(9)
pPqsR-6H	pME6032 derivative for IPTG-inducible expression of the PqsR protein fused with a 6xHis tag; Tc^R.	(7)
pBBR1MCS-5	shuttle vector for constitutive expression; Gm^R.	(10)
pBBR-*pqsABCD*	pBBR1MCS-2 derivative for constitutive expression of PqsA, PqsB, PqsC and PqsD proteins in *P. aeruginosa*; Km^R.	(11)
pFD-*pqsABCD*	pBBR1MCS-5 derivative for constitutive expression of PqsA, PqsB, PqsC and PqsD proteins in *P. aeruginosa*; Gm^R. This plasmid was obtained by cloning into pBBR1MCS-5 the SalI-SacI *pqsABCD* fragment extracted from pBBR-*pqsABCD*.	This study
pMRP9-1	pMRP9 derivative for constitutive expression of GFP in *P. aeruginosa*; Ap^R/Cb^R.	(12)

Table S4. Oligonucleotides used in this study.

Name	Sequence (5'-3')
FW*pqsA*	GACCGCGAAGGACACACTAT
FW*pqsA*	TGAACAGATCGTCTTCCCGC
FW*lecA*	CAGGGCAGGTAACGTCGATT
RV*lecA*	CAACCCGGTATTGACCGGAA
FW*pchR*	CTCAGCGCACAGTTCCTTTC
RV*pchR*	CGAACACCTTGCGAAAGCC
FW*pqsR*	AACATGTTCCTCCAGGTCATCG
RV*pqsR*	TGCGCATGTAAGGGATCAGG
FW*pvdS*	GGAACAACTGTCTACCCGCA
RV*pvdS*	GTAGCTGAGCTGTGCCTTGA
FW16S	GAGAGTTTGATCCTGGCTCAG
RV16S	CTACGGCTACCTTGTTACGA
FWP*pqsL*	TCCGCTCGAGGATCGTCACCGTCAACTG
RVP*pqsL*	TAACTGCAGCGTCATGGATGAGTCTCCG

Figure S1

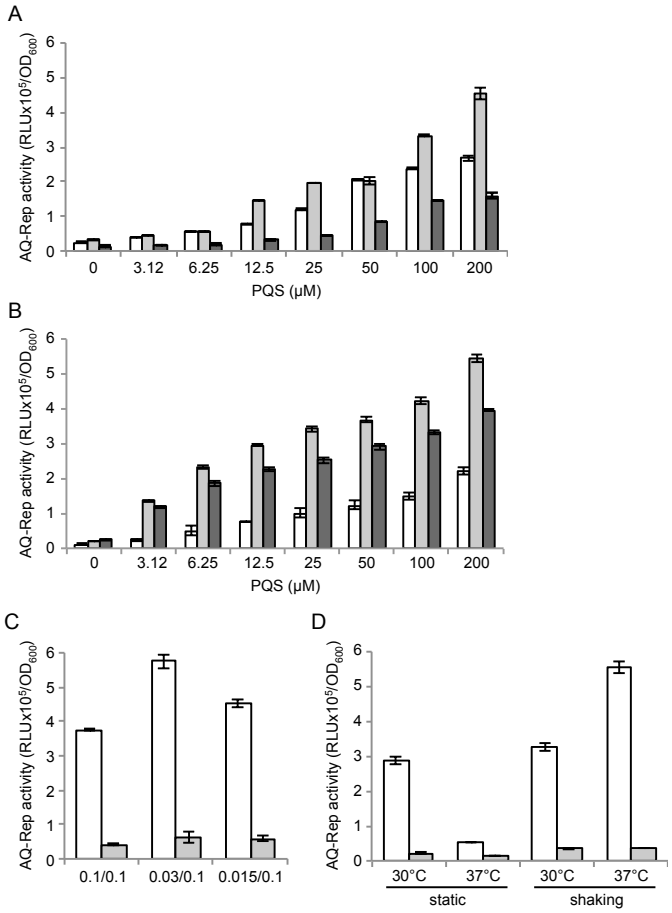

Figure S1. Set up of the PAO1/AQ-Rep coculture system.

(A) Response of the AQ-Rep biosensor after 3 h (white bars), 5 h (light-grey bars) and 7 h (dark-grey bars) incubation in LB supplemented with the indicated concentrations of PQS. **(B)** Activity of the AQ-Rep biosensor inoculated at starting optical density (OD_{600}) of 0.4 (white bars), 0.1 (light-grey bars) and 0.025 (dark-grey bars) after 5 h incubation in LB supplemented with the indicated concentrations of PQS. **(C)** Activity of the AQ-Rep biosensor after 5 h co-incubation with PAO1 (white bars) or Δ*pqsA* (grey bars) strains at the indicated starting optical density (OD_{600}). The first value refers to the PAO1 or Δ*pqsA* strains (OD_{600} from 0.1 to 0.015), the second to the AQ-Rep biosensor (OD_{600} = 0.1). **(D)** Activity of the PAO1/AQ-Rep (white bars) and Δ*pqsA*/AQ-Rep (grey bars) cocultures after 5 h incubation at 30°C or 37°C in static or shaking (200 rpm) conditions. Starting OD_{600} was 0.1 for the AQ-Rep biosensor and 0.03 for the PAO1 and Δ*pqsA* strains. For **(A)-(D)**, biosensor activity is reported as relative light units (RLU) normalized to cell density (OD_{600}); the average of three independent experiments is reported with SD.

5

Figure S2

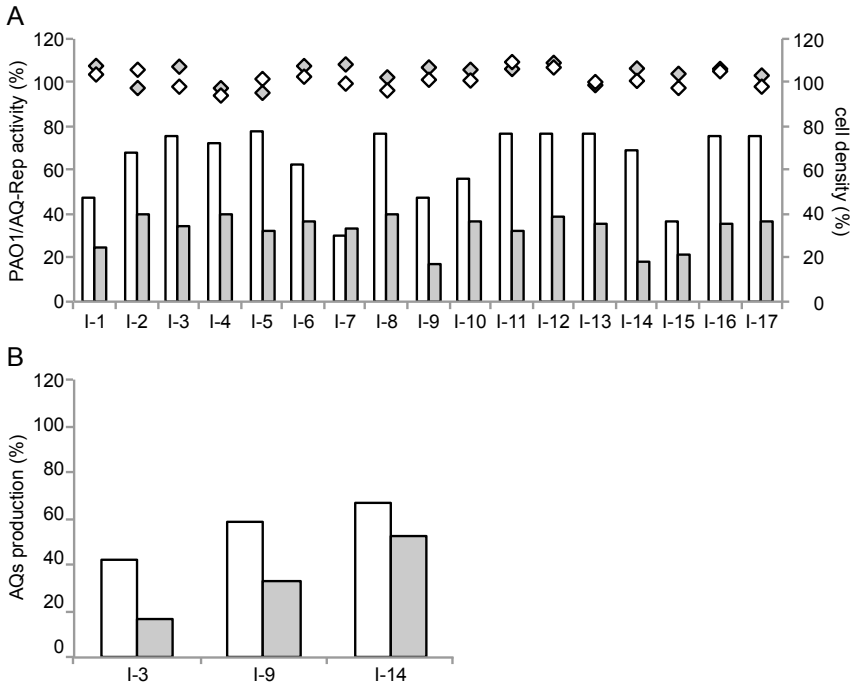

Figure S2. Primary and secondary screens of the PHARMAKON library.

(A) PAO1/AQ-Rep activity (bars) and cell density (diamonds) measured after 5 h incubation at 37°C in shaking conditions in LB supplemented with molecules of the PHARMAKON library, indicated with codes from inhibitor 1 (I-1) to inhibitor 17 (I-17), at 20 μM (white bars and diamonds) and 200 μM (grey bars and diamonds) concentration. PAO1/AQ-Rep activity and cell density measured in the presence of 0.2% and 2% DMSO were considered as 100%. **(B)** AQ production measured in supernatants of the PAO1 strain treated with PHARMAKON library compounds, clotrimazole (I-3), clofoctol (I-9) and miconazole (I-14) at 20 μM (white bars) and 200 μM (grey bars) concentration. AQs were quantified using the AQ-Rep biosensor strain.

Figure S3

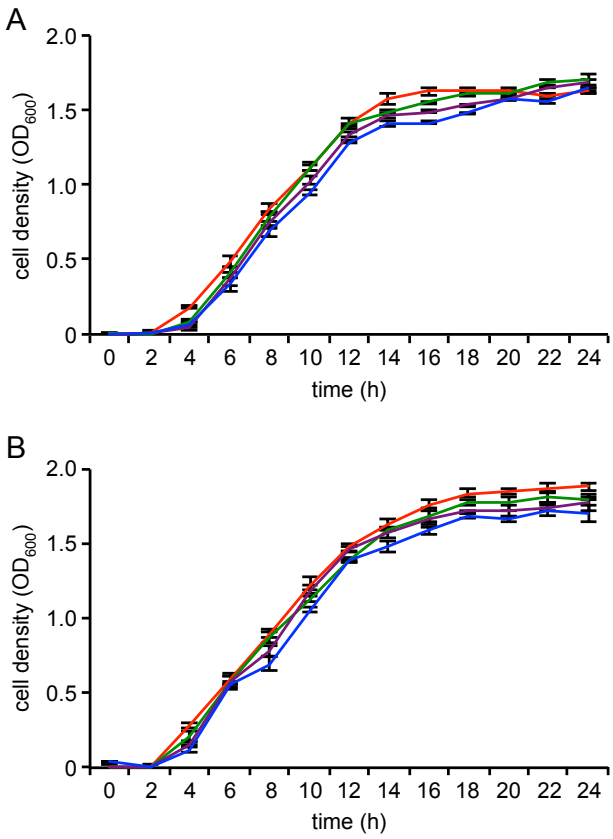

Figure S3. Effect of the *pqs* inhibitors on PAO1 growth.

Growth curves of PAO1 wild type **(A)** and of the biosensor strain AQ-Rep **(B)** incubated at 37°C in shaking conditions in LB supplemented with 200 µM clotrimazole (blue), clofoctol (green), miconazole (purple) or with the corresponding amount of DMSO (red). The average of three independent experiments is reported with SD.

Figure S4

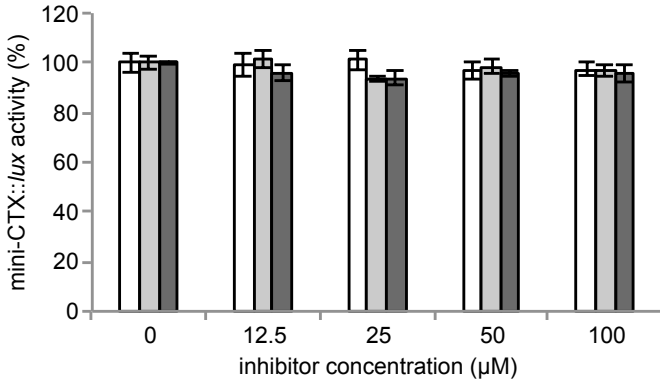

Figure S4. Effect of the *pqs* inhibitors on constitutive bioluminescence.

Percentage of light emitted by the PAO1 strain carrying the mini-CTX::*lux* empty vector grown at 37°C in shaking conditions in LB supplements with 200 μM clotrimazole (white bars), clofoctol (light-grey bars), or miconazole (dark-grey bars). Bioluminescence of the same strain grown in the presence of DMSO was considered as 100%. The average of three independent experiments is reported with SD.

Figure S5

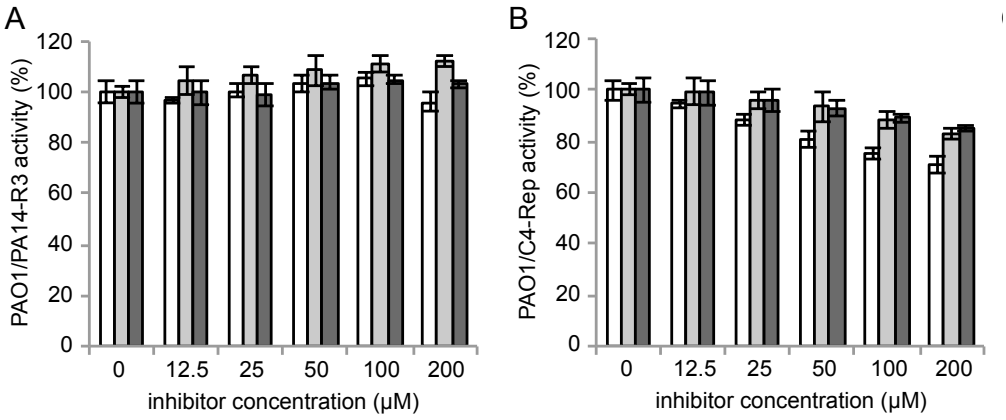

Figure S5. Effect of the *pqs* inhibitors on the *las* and *rhl* QS systems.

Effect of clotrimazole (white bars), clofoctol (light-grey bars) and miconazole (dark-grey bars) on the PAO1/PA14-R3 **(A)** and PAO1/C4-Rep **(B)** coculture systems. PA14-R3: *las*-specific biosensor strain PA14 Δ*lasI* P*rsaL*::*luxCDABE* (5); C4-Rep: *rhl*-specific biosensor strain PAO1 Δ*rhlI* P*rhlA*::*luxCDABE* (6). Bioluminescence of untreated cocultures normalized to cell density is considered as 100%.

9

Figure S6

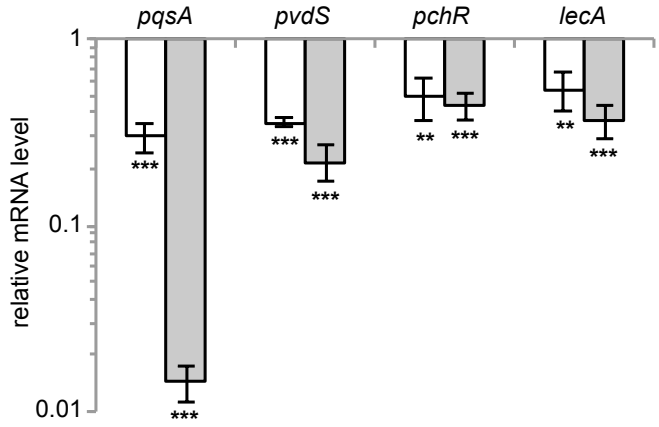

Figure S6. Effect of clotrimazole on the mRNA level of *pqs*-controlled genes.

Real Time RT-PCR analysis showing mRNA level of the indicated genes in PAO1 treated with 100 µM clotrimazole (white bars) and in Δ*pqsR* (grey bars) relative to untreated PAO1. The average of three independent experiments is reported with SD. **, $p < 0.01$; ***, $p < 0.001$ (ANOVA).

10

Figure S7

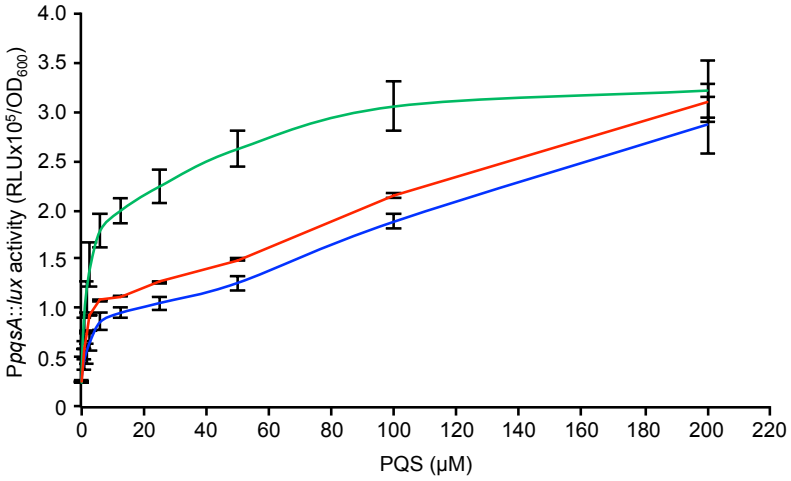

Figure S7. Competition assay between clofoctol and PQS for binding to PqsR.

P*pqsA*::*lux* activity measured in the AQ-Rep biosensor grown in LB supplemented with different concentrations of PQS in the absence (green) or in the presence of 12.5 μM (red) or 50 μM (blue) clofoctol. Promoter activity is reported as relative light units (RLU) normalized to cell density (OD_{600}).

Figure S8

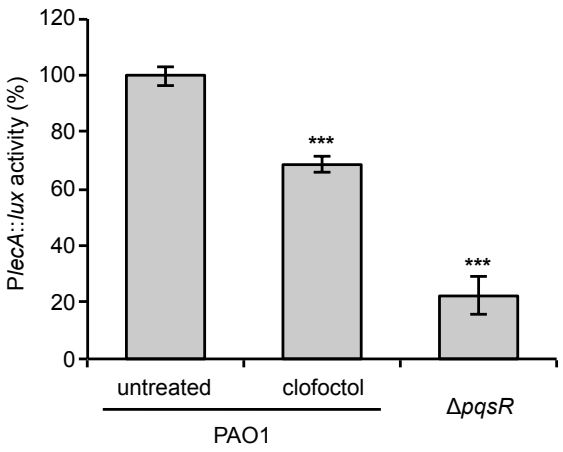

Figure S8. Effect of clofoctol on P*lecA* activity.

Activity of the P*lecA* promoter in PAO1 cultures grown in LB supplemented with DMSO (untreated) or with 100 μM clofoctol, and in the Δ*pqsR* culture grown in LB supplements with DMSO. P*lecA* activity in untreated PAO1 is considered as 100%. The average of three independent experiments is reported with SD. ***, $p < 0.001$ (ANOVA).

Figure S9

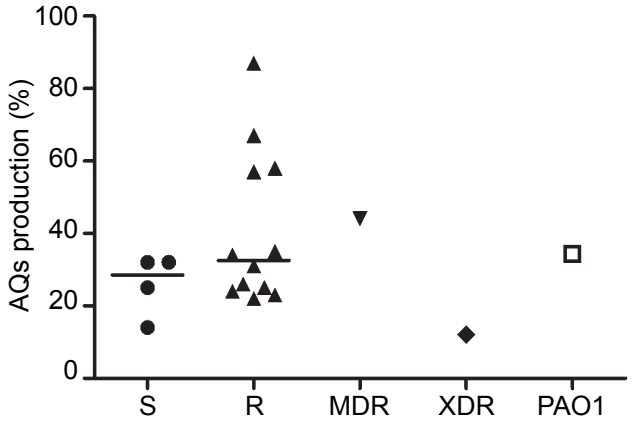

Figure S9. Effect of clofoctol on AQ production in *P. aeruginosa* CF isolates grouped according to their antibiotic resistance profile.

Dot plot showing the inhibition of AQ production in *P. aeruginosa* CF isolates (filled symbols) and *P. aeruginosa* PAO1 (open square) treated with 100 μM clofoctol, relative to the untreated samples considered as 100%. Black lines represent the median values: S, 28.4%; R, 32.6%. AQ production in the MDR, XDR and PAO1 strains were 43.5%, 11.6% and 34.3%, respectively. Differences between the median values are not statistically significant. Mean results of three independent experiments are reported.

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Chapter 4

In silico selection and experimental validation of FDA-approved drugs as anti-quorum sensing agents

Marta Mellini[1], Elena Di Muzio[1], Francesca D'Angelo[1], **Valerio Baldelli**[1], Serena Ferrillo[1], Paolo Visca[1], Livia Leoni[1], Fabio Polticelli[1,2] and Giordano Rampioni[1]

[1] Department of Science, University Roma Tre, Rome, Italy; [2] National Institute of Nuclear Physics, Roma Tre Section, Rome, Italy

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Preface to Chapter 4

The results presented in the previous Chapter 3 highlighted the potential of drug repurposing for the identification of FDA-approved compounds targeting QS in *P. aeruginosa*. The work presented in Chapter 4 aims at combining the advantages of drug repurposing and *in silico* screening to accelerate the identification of new *pqs* inhibitors among FDA-approved drugs.

In the last decades *in silico* approaches have been proved as valid aids to conventional drug-discovery programmes. In particular, virtual screenings carried out *via* molecular docking simulations allow to select promising drug candidates in vast libraries of molecules, so that validation by means of *in vitro* experiments can be performed only on a reduced number of predicted hits, thus significantly reducing time and costs associated to conventional screening campaigns.

On this basis, in this work an *in silico* library of 1,467 FDA-approved drugs has been screened by molecular docking in order to identify hits showing high predicted binding affinity for the *pqs* QS transcriptional regulator PqsR. Subsequently, *in vitro* experiments have been performed by means of *ad hoc* engineered biosensor strains to verify the ability of selected hits to hamper PqsR functionality, and hence to decrease the expression of PqsR-controlled virulence traits in *P. aeruginosa*. The identification of the antipsychotic drug pimozide as a new PqsR inhibitor endowed with antivirulence potentials highlights the promise of combining drug repurposing and virtual screening approaches to accelerate the selection of FDA-approved anti-QS drugs.

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In silico Selection and Experimental Validation of FDA-Approved Drugs as Anti-quorum Sensing Agents

Marta Mellini[1], Elena Di Muzio[1], Francesca D'Angelo[1†], Valerio Baldelli[1], Serena Ferrillo[1], Paolo Visca[1], Livia Leoni[1], Fabio Polticelli[1,2]* and Giordano Rampioni[1]*

[1] Department of Science, University Roma Tre, Rome, Italy, [2] National Institute of Nuclear Physics, Roma Tre Section, Rome, Italy

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***Correspondence:**
Fabio Polticelli
fabio.polticelli@uniroma3.it
Giordano Rampioni
giordano.rampioni@uniroma3.it

†Present address:
Francesca D'Angelo, Institut Pasteur, Paris, France

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The emergence of antibiotic resistant bacterial pathogens is increasing at an unprecedented pace, calling for the development of new therapeutic options. Small molecules interfering with virulence processes rather than growth hold promise as an alternative to conventional antibiotics. Anti-virulence agents are expected to decrease bacterial virulence and to pose reduced selective pressure for the emergence of resistance. In the opportunistic pathogen *Pseudomonas aeruginosa* the expression of key virulence traits is controlled by quorum sensing (QS), an intercellular communication process that coordinates gene expression at the population level. Hence, QS inhibitors represent promising anti-virulence agents against *P. aeruginosa*. Virtual screenings allow fast and cost-effective selection of target ligands among vast libraries of molecules, thus accelerating the time and limiting the cost of conventional drug-discovery processes, while the drug-repurposing approach is based on the identification of off-target activity of FDA-approved drugs, likely endowed with low cytotoxicity and favorable pharmacological properties. This study aims at combining the advantages of virtual screening and drug-repurposing approaches to identify new QS inhibitors targeting the *pqs* QS system of *P. aeruginosa*. An *in silico* library of 1,467 FDA-approved drugs has been screened by molecular docking, and 5 hits showing the highest predicted binding affinity for the *pqs* QS receptor PqsR (also known as MvfR) have been selected. *In vitro* experiments have been performed by engineering *ad hoc* biosensor strains, which were used to verify the ability of hit compounds to decrease PqsR activity in *P. aeruginosa*. Phenotypic analyses confirmed the impact of the most promising hit, the antipsychotic drug pimozide, on the expression of *P. aeruginosa* PqsR-controlled virulence traits. Overall, this study highlights the potential of virtual screening campaigns of FDA-approved drugs to rapidly select new inhibitors of important bacterial functions.

Keywords: *Pseudomonas aeruginosa*, anti-virulence strategy, quorum sensing inhibition, pimozide, *in silico* screening, molecular docking, new therapeutics, PqsR

INTRODUCTION

The long-term use of antibiotics has dramatically accelerated the emergence of multi-drug and even pan-drug resistant bacterial pathogens worldwide, leading to an alarming increase of difficult-to-treat infections. This worrying scenario especially concerns the ESKAPE pathogens (*Enterococcus faecium*, *Staphylococcus aureus*, *Klebsiella pneumoniae*, *Acinetobacter baumannii*, *Pseudomonas aeruginosa*, and *Enterobacter* species), a group of bacteria that "escape" the action of almost all available antibiotics (Rice, 2008; Boucher et al., 2009). The trend toward antibiotic resistance is even more alarming if considering that only a handful of new antibiotics have been approved by the U.S. Food and Drug Administration (FDA) in the last decade, with many companies considering the R&D for new antibiotics a less attractive asset compared to more rewarding therapeutic areas. Indeed, *de novo* antibiotic development requires large investments that might not grant an economic reward due to the short commercial lifespan of antibiotics, caused by the rapid emergence of resistance (Ventola, 2015; Mohr, 2016; Luepke et al., 2017).

The awareness about the risk of antibiotic resistance for human health has increased in parallel with our comprehension of bacterial pathobiology, so that virulence mechanisms are now recognized as molecular targets for the development of novel anti-virulence drugs targeting the infection process rather than bacterial growth (Rasko and Sperandio, 2010). Although resistance mechanisms to anti-virulence drugs have been described (Zhu et al., 1998; Hung et al., 2005; Maeda et al., 2012; Imperi et al., 2019), targeting virulence rather than growth is expected to pose a reduced selective pressure for the emergence of resistance (Allen et al., 2014). In particular, *in vitro* evolution experiments indicate that drug resistant clones are counterselected if "public goods" that are shared among members of a bacterial population are targeted (*e.g.,* toxins, exo-proteases, and siderophores) (Mellbye and Schuster, 2011; Vale et al., 2016). Since drug-resistant strains are likely to emerge only if they gain a "private" advantage over the susceptible population, quorum sensing (QS) is recognized as an ideal target for the development of anti-virulence agents. Indeed, QS is an intercellular communication system based on the production, secretion and reception of signal molecules that coordinate the expression of secreted virulence factors in different bacterial pathogens (Rampioni et al., 2014; Kalia et al., 2019).

The ESKAPE pathogen *P. aeruginosa* is a model organism for the development of anti-virulence drugs targeting QS (Soukarieh et al., 2018b). This Gram-negative bacterium, that is one of the most dreaded nosocomial pathogens and the main cause of death in cystic fibrosis (CF) patients, has recently been included by the World Health Organization in the list of pathogens for which new therapeutic options are urgently needed (Priority 1: Critical)[1]. The ability of *P. aeruginosa* to cause both acute and chronic infections in different districts of the human body mainly relies on its capacity to adapt to the host by fine-tuning the expression of a wide array of virulence factors, many of which are QS-controlled. As a consequence, numerous anti-virulence drugs targeting the *P. aeruginosa* QS circuitry have been identified in recent years, and their ability to reduce *P. aeruginosa* pathogenicity has been confirmed both *in vitro* and *in vivo* (Rampioni et al., 2014; Soukarieh et al., 2018b). Unfortunately, the majority of the QS inhibitors identified to date are not suitable as lead-like compounds for further drug development, mainly due to their cytotoxicity and unfavorable pharmacological properties (Maura et al., 2016; Soukarieh et al., 2018b).

With the aim to identify bioavailable and safe QS inhibitors that can faster move into clinical trials or serve as leads for drug optimization programs, our group recently undertook whole-cell biosensor-based screening campaigns of libraries of FDA-approved drugs. This drug-repurposing approach led to the identification of niclosamide, an anthelmintic drug, and clofoctol, an antibiotic active against Gram-positive bacteria, as potent and safe QS inhibitors targeting the acyl-homoserine lactones (AHL)-based and the 2-alkyl-4(1*H*)quinolone (AQ)-based QS systems of *P. aeruginosa*, respectively (Imperi et al., 2013; D'Angelo et al., 2018). These FDA-approved drugs effectively reduced *P. aeruginosa* pathogenic potential in animal models of infection, hence representing promising candidates for preclinical studies.

In the last decades *in silico* approaches have been proved as valid aids to conventional drug-discovery programs. In particular, virtual screens carried out through molecular docking simulations allow to preselect promising drug candidates in vast libraries of molecules, so that only a reduced number of predicted hits have to be validated by means of *in vitro* experiments. In this way, time and costs associated to conventional screening campaigns are reduced. In addition, docking simulations allow to predict the likely binding mode of candidate hits onto the selected target, providing a molecular basis for their optimization in terms of binding affinity (Reuter et al., 2015).

On this basis, the present study aims at combining the advantages of drug-repurposing and virtual screening approaches to identify FDA-approved drugs targeting the *pqs* QS system of *P. aeruginosa via in silico* molecular docking.

In *P. aeruginosa* the *pqs* QS system is based on the AQs 2-heptyl-4-hydroxyquinoline (HHQ) and 2-heptyl-3-hydroxy-4(1*H*)-quinolone (PQS) as signal molecules. HHQ is synthesized by the enzymes coded by the *pqsABCDE-phnAB* operon, and is converted to PQS by the monooxygenase PqsH. Both HHQ and PQS can bind to and activate the transcriptional regulator PqsR (also known as MvfR), that in the active form binds to the P*pqsA* promoter region and promotes *pqsABCDE-phnAB* transcription. Hence, HHQ and PQS act as autoinducers to accelerate their own synthesis (Bredenbruch et al., 2005; Heeb et al., 2011; Dulcey et al., 2013; Drees and Fetzner, 2015). While the main role of HHQ is to trigger this PqsR-dependent positive feedback loop, the signal molecule PQS and the protein PqsE (the latter coded by the fifth gene of the *pqsABCDE-phnAB* operon) are the main effectors of the *pqs* QS system. Besides activating PqsR, PQS acts as an iron chelator, is required for the biogenesis of outer membrane vesicles, and promotes the expression of virulence genes *via* a PqsR-independent pathway (Bredenbruch et al., 2005; Mashburn and Whiteley, 2005; Diggle et al., 2007; Rampioni et al.,

[1] http://www.who.int/en/news-room/detail/27-02-2017-who-publishes-list-of-bacteria-for-which-new-antibiotics-are-urgently-needed

2016; Lin et al., 2017). PqsE is a multifunctional protein that participates in the synthesis of HHQ and positively controls the expression of multiple virulence factors independently of AQs, likely by activating the transcriptional regulator RhlR *via* the production of an uncharacterized signal molecule that links the *pqs* and *rhl* QS systems (Drees and Fetzner, 2015; Hazan et al., 2010; Rampioni et al., 2010; Rampioni et al., 2016; Mukherjee et al., 2018). Overall, the production of PQS and the expression of PqsE require activated PqsR, and consequently PqsR-inhibitors have been shown to attenuate *P. aeruginosa* virulence both *in vitro* and in animal models of infection (Klein et al., 2012; Ilangovan et al., 2013; Zender et al., 2013; Lu et al., 2014; Starkey et al., 2014; Maura and Rahme, 2017; Maura et al., 2017; D'Angelo et al., 2018; Soukarieh et al., 2018a). Since the three-dimensional structure of the PqsR domain that interacts with HHQ and PQS (co-inducer binding domain; CBD) has recently been solved (Ilangovan et al., 2013; Kitao et al., 2018), this QS transcriptional regulator now constitutes an ideal target for the identification of new *P. aeruginosa* anti-virulence drugs *via* molecular docking simulations.

In this study, a virtual screening approach has been used to predict PqsR ligands in a library of 1,467 FDA-approved drugs. The ability of the best 5 hits to decrease P*pqsA* activity and AQs level has been tested in wild type *P. aeruginosa* and in *ad hoc* engineered strains. This process led to the identification of the antipsychotic drug pimozide as a specific PqsR inhibitor. Phenotypic assays showed that pimozide hampers the expression of PqsR-controlled virulence traits, such as the production of the virulence factor pyocyanin, swarming motility and biofilm formation, and docking simulations suggest a possible competition with native AQs for PqsR binding. These results provide a proof-of-concept that the drug-repurposing and virtual screening approaches can be combined to accelerate the selection of anti-QS molecules among FDA-approved drugs.

MATERIALS AND METHODS

Bacterial Strains, Media and Chemicals

Bacterial strains used in this study are reported in **Table 1**. Bacterial strains were routinely grown at 37°C in Luria-Bertani (LB) broth in shaking conditions, or in LB supplemented with 15 g/L agar.

When required, tetracycline (Tc; 200 μg/mL), isopropyl β-D-1-thiogalactopyranoside (IPTG), dimethyl sulfoxide (DMSO), or synthetic PQS were added to the medium. IPTG, DMSO and synthetic PQS were used at the concentrations indicated in the text. Synthetic PQS stock solution was prepared in MeOH at 20 mM concentration (synthetic PQS was kindly provided by Paul Williams and Miguel Càmara – University of Nottingham, United Kingdom). Ergotamine and pimozide were available in our laboratory as drugs of the PHARMAKON library (10 mM stock solutions in DMSO). Dutasteride, eltrombopag and conivaptan were purchased from Sigma-Aldrich, Carbosynth Ltd., and MCE Medchem Express, respectively, and dissolved in DMSO at 10 mM concentration. Pimozide was also purchased from Sigma-Aldrich for further analyses, and dissolved in DMSO at 40 mM concentration.

TABLE 1 | Bacterial strains used in this study.

***P. aeruginosa* strains**	**Characteristics**	**References**
PAO1	Nottingham collection wild type strain.	
PAO1 pMRP9-1	PAO1 wild type strain carrying a pMRP9 derivative for constitutive expression of GFP; Ap^R/Cb^R.	D'Angelo et al., 2018
Δ*pqsR*	PAO1 mutant strain with in frame clear deletion of the *pqsR* gene.	Rampioni et al., 2010
Δ*pqsR* pMRP9-1	PAO1 mutant strain with in frame clear deletion of the *pqsR* gene carrying a pMRP9 derivative for constitutive expression of GFP; Ap^R/Cb^R.	D'Angelo et al., 2018
PAO1 P*pqsA*::*lux*	PAO1 wild type strain carrying chromosomal insertion of the P*pqsA*::*lux* transcriptional fusion; Tc^R.	Fletcher et al., 2007
Δ*pqsA* P*pqsA*::*lux*	PAO1 mutant strain deleted in *pqsA* gene carrying chromosomal insertion of the P*pqsA*::*lux* transcriptional fusion; Tc^R (named AQ-Rep).	Diggle et al., 2007
Δ*pqsA* mini-CTX::*lux*	PAO1 mutant strain deleted in *pqsA* gene carrying chromosomal insertion of the mini-CTX::*lux* empty vector; Tc^R (named C-Rep).	D'Angelo et al., 2018
Δ*pqsR* (pFD-*pqsABCD*)	PAO1 mutant strain with in frame clear deletion of the *pqsR* gene, carrying the pFD-*pqsABCD* plasmid for PqsR-independent production of AQs; Km^R.	D'Angelo et al., 2018
Δ*pqsAHR* P*pqsA*::*lux* (pPqsR-6H)	PAO1 triple mutant strain deleted in *pqsA*, *pqsH* and *pqsR* genes carrying chromosomal insertion of the P*pqsA*::*lux* transcriptional fusion and the pPqsR-6H plasmid for IPTG-inducible expression of PqsR; Tc^R.	Ilangovan et al., 2013

Virtual Screening *via* Molecular Docking

Molecular docking simulations were carried out using DockingApp (Di Muzio et al., 2017), a user friendly interface to the molecular docking program AutoDock Vina (Trott and Olson, 2010), on 1,467 FDA-approved molecules extracted from the DrugBank Database and provided in ready-to-dock format as part of the DockingApp package. DockingApp is a freely available platform-independent application to perform docking simulations and virtual screening using AutoDock Vina. An intuitive graphical user interface facilitates the input phase while an embedded JMol applet allows to visualize and analyse the results. The application comes with the DrugBank set of ready-to-dock FDA-approved drugs for virtual screening and drug-repurposing purposes. In all simulations, the search space (docking grid) included the whole PqsR co-inducer binding domain (CBD) structure, in order to carry out "blind" predictions of the "hit" compounds binding sites. Simulations were first carried out on the apo form of the protein (PDB ID: 4JVC) (Ilangovan et al., 2013), by keeping all protein

residues rigid. The ten best-ranking compounds, according to the AutoDock Vina scoring function, were then selected for a refinement round in which molecular docking simulations were carried out allowing flexibility of the residues building up the PqsR binding pocket (*i.e.,* Ile149, Ala168, Val170, Ile186, Leu189, Leu207, Leu208, Phe221, Ile236, Tyr258, Asp264, and Thr265) (Ilangovan et al., 2013). The results of docking simulations were analyzed using the molecular graphics program UCSF-Chimera, version 1.12 (Pettersen et al., 2004).

Bioluminescence Assay

Analyses of PqsR activity in the presence of potential inhibitors has been performed by using *ad hoc* engineered reporter systems in which bioluminescence emission is proportional to PqsR activity.

The primary screening for potential PqsR inhibitors was performed as previously described (D'Angelo et al., 2018). Briefly, the screening was based on the co-culture of *P. aeruginosa* PAO1 wild type (PAO1) and the reporter strain PAO1 Δ*pqsA* P*pqsA*::*lux* (AQ-Rep). PAO1 and AQ-Rep were grown for 16 h at 37°C with shaking (200 rpm) in LB broth or in LB broth supplemented with 200 μg/L Tc, respectively. After growth, PAO1 and AQ-Rep were washed with sterile saline and mixed into LB broth to a final OD_{600} of 0.03 and 0.1, respectively (wild type/reporter ratio ≈ 1/3). Two-hundred μL aliquots of the diluted co-cultures were dispensed into 96-wells black clear-bottom microtiter plates. All compounds used in the primary screening were dissolved in DMSO to 10 mM concentration. The compounds were added to the microtiter plates containing the co-cultures at the final concentrations of 20 and 200 μM. As untreated controls, the same amount of DMSO alone as in the treated samples was added to the microtiter wells containing the co-culture.

For further analysis with pimozide from Sigma-Aldrich, 100 μL LB-grown aliquots of the PAO1/AQ-Rep co-culture (OD_{600} = 0.06 and 0.2, respectively) or of other reporter systems indicated in the text (OD_{600} = 0.02) were dispensed into 96-wells black clear-bottom microtiter plates, and 100 μL of pimozide diluted in LB at concentrations ranging from 50 to 400 μM were added to each well. Also in this case, DMSO alone was used as a control.

For all light emission assays, plates were incubated at 37°C with shaking (120 rpm) for 5 h, and then light emission (RLU) and cell density (OD_{600}) of the reporter system were recorded by using an automated Spark 10 M luminometer-spectrophotometer (Tecan). Reporter activity was evaluated as Relative Light Units (RLU) normalized to cell density (OD_{600}). Alteration in promoter activity induced by the tested compounds was determined by comparing the promoter activity of the specific biosensor system in untreated and treated samples.

Quantification of AQs

AQ signal molecules in *P. aeruginosa* PAO1 culture supernatants were quantified as previously described (Fletcher et al., 2007). PAO1 wild type cultures were grown at 37°C in 96-well microtiter plates with shaking (120 rpm) in LB broth supplemented with the tested compounds or solvent vehicle (*i.e.,* DMSO) as a control. After 7 h of incubation, cell-free supernatants of PAO1 wild type cultures were collected and 5 μL were added to 195 μL of the AQ-Rep biosensor (OD_{600} = 0.1) dispensed into 96-wells black clear-bottom microtiter plates. Plates were incubated for 5 h at 37°C with shaking (120 rpm), and light emission (RLU) and cell density (OD_{600}) of the cultures were recorded by using an automated Spark 10 M luminometer-spectrophotometer (Tecan). A calibration curve was generated by growing the AQ-Rep biosensor strain with synthetic PQS at concentrations ranging from ∼ 45 nM to 300 μM. The resulting dose-response curve was used as a landmark to determine the concentration of the AQs in each culture supernatant.

Pyocyanin Production, Swarming Motility and Biofilm Formation Assays

The assay for pyocyanin extraction and quantification has been performed as previously described (Essar et al., 1990) on PAO1 wild type and PAO1 Δ*pqsR* strains. Bacterial strains were grown for 16 h at 37°C with shaking (200 rpm) in LB broth in the presence of 100 μM pimozide or 0.25% (v/v) DMSO (solvent vehicle control).

Swarming motility assays were performed on PAO1 wild type and PAO1 Δ*pqsR* by using swarming plates [0.8% (w/v) nutrient broth N.2, 0.5% (w/v) glucose, 0.5% (w/v) bacteriological agar] (Rampioni et al., 2009). Plates were supplemented with 100 μM pimozide or 0.25% (v/v) DMSO (solvent vehicle control). Swarming motility was directly observed at the air-agar interface after 16 h of incubation at 37°C.

The biofilm formation assay was performed in eight-well chamber slides as previously described (Jurcisek et al., 2011; D'Angelo et al., 2018), with minor modifications. Briefly, PAO1 wild type and PAO1 Δ*pqsR* constitutively expressing GFP *via* the pMRP9-1 plasmid (Davies et al., 1998) were inoculated in an eight-well chamber slide at an OD_{600} of 0.02 in 700 μL of M9 minimal medium supplemented with 20 mM glucose as carbon source, in the presence of 0.25% (v/v) DMSO (solvent vehicle control) or 100 μM pimozide. Bacterial cultures were incubated at 30°C for 24 h. Planktonic cells were gently removed and the wells of the chamber slide were rinsed with sterile saline before confocal microscope (Leica TCS SP5) imaging of the bacterial cells adhered to the glass surface.

Statistical Analysis

For statistical analysis the software GraphPad Prism 5 was used; one-way analysis of variance (ANOVA) followed by Tukey-Kramer multiple comparison tests were performed. Differences having a p value < 0.05 were considered statistically significant.

RESULTS

Virtual Screening for the Identification of FDA-Approved PqsR Ligands

We performed a virtual screening to select possible PqsR ligands in a library of 1,467 FDA-approved compounds extracted from the DrugBank database[2] and already provided in ready-to-dock

[2]https://www.drugbank.ca/

format as part of the DockingApp software package (see section "Materials and Methods," for details).

PqsR is a multi-domain transcriptional regulator composed by a *N*-terminal helix-turn-helix DNA-binding domain and a *C*-terminal co-inducer binding domain (CBD). Since the three-dimensional structure of full-length PqsR is unavailable, molecular docking simulations were performed based on the crystal structure of PqsR-CBD. The CBD of PqsR has been crystallized in the apo form (PDB ID: 4JVC) or as a complex with the native AQ ligand 2-nonyl-4-hydroxy-quinoline (NHQ) (PDB ID: 4JVD) or with the quinazolinone (QZN) inhibitor 3-NH_2-7Cl-C9-QZN (PDB ID: 4JVI) (Ilangovan et al., 2013). It has been shown that both the native ligand and the inhibitor bind to the CBD of PqsR in a site consisting of two adjacent pockets: the quinolone ring is accommodated in pocket B, while the aliphatic chain makes hydrophobic interactions with pocket A (Ilangovan et al., 2013). More recently, the benzamide-benzimidazole inhibitor M64 was shown to bind to PqsR-CBD in a similar way as NHQ and 3-NH_2-7Cl-C9-QZN, with its benzimidazole group bound in pocket B and the phenoxy group occupying pocket A (PDB ID: 6B8A; Kitao et al., 2018). To avoid selection bias, molecular docking simulations were performed on the apo form of PqsR-CBD. In addition, to increase the reliability of the simulations, the docking search space was not restricted to the A and B binding pockets, but extended to the entire PqsR-CBD, *i.e.,* a "blind" docking procedure was carried out. To speed up the process, the initial screening of the 1,467 FDA-approved compounds was carried out by keeping all amino acid residues rigid.

Possible PqsR ligands were ranked based on the predicted binding affinity calculated with the AutoDock Vina scoring function. In the case of multiple ligands with the same binding affinity, these were prioritized based on the size-independent ligand efficiency (SILE) coefficient. SILE is a normalized parameter derived from the ligand efficiency (LE), a predictive measure of the *per*-atom binding affinity of a ligand to its binding partner (Kuntz et al., 1999).

Following the above procedure, the ten best-ranking putative PqsR ligands (predicted binding affinity ranging from -11.2 to -10.0 kcal/mol) were selected for a second round of molecular docking simulations in which residues previously reported to be involved in the binding of the natural ligand NHQ to the PqsR-CBD (Ilangovan et al., 2013) were considered flexible (see section "Materials and Methods," for details). The five molecules predicted to display the highest affinity to PqsR-CBD with fixed residues ranked in the first five positions also in the analysis with flexible residues, and are listed in **Table 2**. Conivaptan is an non-peptide inhibitor of the vasopressin receptor subtypes V1a and V2, commonly used to treat euvolemic and hypervolemic hyponatremia (Ferguson-Myrthil, 2010); ergotamine is an alkaloid acting as a serotonin agonist with vasoconstrictor and analgesic properties (Saxena and De Deyn, 1992); eltrombopag, an agonist of the thrombopoietin physiological target, is used for the treatment of thrombocytopenia (McCormack, 2015); pimozide is an antipsychotic drug used to treat schizophrenia, chronic psychosis, Tourette's syndrome, and resistant tics (Tueth and Cheong, 1993); dutasteride is a 5α-reductase inhibitor used to treat benign prostatic hyperplasia (Azzouni and Mohler, 2012).

Superimposition of the five PqsR-ligand molecular complexes obtained by docking simulations with the crystal structure of the PqsR-NHQ complex (PDB ID: 4JVD) (Ilangovan et al., 2013) predicts that all the five ligands bind in the NHQ binding site, interacting with residues building up the A and B pockets of PqsR-CDB, as observed for the natural ligand NHQ (**Figure 1**).

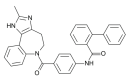
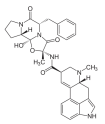
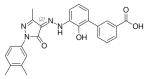
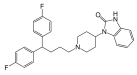
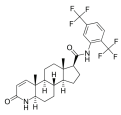
Anti-QS Activity of the Predicted PqsR Ligands

To validate the ability of the predicted PqsR ligands to inhibit PqsR activity in *P. aeruginosa*, the best 5 hits were tested for their ability to reduce bioluminescence in a co-culture system based on wild type *P. aeruginosa* PAO1 (Nottingham collection; herein referred to as PAO1) and isogenic *P. aeruginosa* PAO1 Δ*pqsA* P*pqsA*::*lux* (named AQ-Rep) (Fletcher et al., 2007; D'Angelo et al., 2018). The AQ-Rep biosensor strain is unable to synthesize HHQ and PQS signal molecules as a consequence of *pqsA* mutation, and it carries single-copy chromosomal insertion of a transcriptional fusion between the PqsR-activated promoter P*pqsA* and the *luxCDABE* operon for light emission. Therefore, in the PAO1/AQ-Rep co-culture the AQs produced by PAO1 activate PqsR in AQ-Rep, and consequently promote bioluminescence; hence, a PqsR inhibitor is expected to reduce light emission in this reporter system. The use of the PAO1/AQ-Rep co-culture recently allowed the identification of new PqsR inhibitors (D'Angelo et al., 2018), thus proving its efficacy for the selection of anti-virulence drugs targeting the *pqs* QS system. A co-culture of PAO1 and of a Δ*pqsA* derivative strain carrying the mini-CTX::*lux* empty vector for constitutive light emission (named C-Rep) was used to discriminate between molecules targeting PqsR and molecules affecting light emission in a PqsR-independent way.

The PAO1/AQ-Rep co-culture was incubated with the predicted PqsR-ligands identified *via* the preliminary virtual screening: conivaptan, ergotamine, eltrombopag, pimozide, and dutasteride. Molecules were tested at 20 μM and 200 μM concentrations. Since these drugs were dissolved in DMSO, solvent vehicle control samples in which 0.2% or 2% DMSO alone was added were also analyzed. To ensure specificity, the predicted ligands of PqsR were also analyzed by using the PAO1/C-Rep control co-culture. For each predicted ligand, the residual reporter activity (RLU/OD_{600}) was calculated by comparing the reporter activity of the culture grown in the presence of the tested molecule to the reporter activity of the same culture grown in the presence of solvent vehicle, considered as 100%.

As reported in **Table 3**, 200 μM ergotamine, eltrombopag and pimozide were able to reduce light emission in the PAO1/AQ-Rep co-culture system by 27.9, 30.4, and 46.3%, respectively, while conivaptan and dutasteride did not affect reporter activity. However, 200 μM eltrombopag reduced light emission also in the PAO1/C-Rep control co-culture system (38.5% reduction), suggesting PqsR-independent light inhibition, while ergotamine and pimozide did not significantly alter light emission in the control samples. Notably, ergotamine and

TABLE 2 | Putative FDA-approved ligands of PqsR-CBD identified *via* molecular docking.

Drug name and property	Structure	ΔDG[a]
Conivaptan: vasopressin receptors V1a and V2 inhibitor, used for the treatment of euvolemic and hypervolemic hyponatremia		−14.3 (−10.7)
Ergotamine: Alkaloid vasoconstrictor used as analgesic		−12.3 (−10.7)
Eltrombopag: Agonist of the Tpo receptor for the treatment of chronic thrombocytopenia		−12.1 (−10.6)
Pimozide: Blocker of dopaminergic receptors used as antipsychotic drug		−12.0 (−10.9)
Dutasteride: Oxo-steroid 5-α-reductase inhibitor for the treatment of benign prostatic hyperplasia		−11.3 (−11.2)

[a] *ΔGvalues (kcal/mol) for drugs binding to the PqsR-CBD apo form (PDB ID: 4JVC; Ilangovan et al., 2013) with flexible residues, predicted by the AutoDock Vina scoring function; the ΔG values (kcal/mol) in parenthesis refer to the affinity of the same molecules for the PqsR-CBD obtained in docking simulations with fixed residues.*

pimozide reduced reporter activity at 20 μM concentration (10.1 and 12.3% reduction, respectively), and none of the tested drugs significantly altered *P. aeruginosa* cell density (data not shown). Since ergotamine and pimozide were effective in specifically reducing light emission in the PAO1/AQ-Rep co-culture, without altering bacterial growth, these molecules were selected for further investigations.

A secondary screening was performed investigating the ability of ergotamine and pimozide to affect the production of the signal molecules AQs. To this end, PAO1 was grown in LB supplemented with ergotamine or pimozide (20 μM and 200 μM) or with DMSO, as a control. The amount of AQs in the corresponding cell-free supernatants was evaluated by using the AQ-Rep biosensor strain, in which light emission is proportional to the amount of AQs present in the medium. As shown in **Table 3**, ergotamine slightly decreased the level of AQs at both 20 μM and 200 μM (4.7 and 6.2% reduction, respectively), while pimozide reduced the production of these QS signal molecules of 15.1% and 49.5% at 20 μM and 200 μM, respectively.

Pimozide Inhibits the *pqs* QS System and PqsR-Controlled Virulence Traits

According to the primary and secondary screenings (**Table 3**) pimozide from the PHARMAKON library reduced P*pqsA* activity in the co-culture system PAO1/AQ-Rep and AQs production in PAO1, without altering bacterial growth. To confirm these data, experiments were replicated with pimozide purchased from a different vendor (Sigma-Aldrich).

Growth curves reported in **Figure 2A** show that up to 400 μM pimozide does not alter the growth profile of PAO1. Data reported in **Figure 2B** confirmed that pimozide significantly reduces light emission in the PAO1/AQ-Rep co-culture in a dose-dependent manner. In detail, 100 μM, 200 μM and 400 μM pimozide significantly reduced PAO1/AQ-Rep activity of 30.1, 45.2, and 64.7%, respectively (**Figure 2B**, *white bars*). Hence, the decrease in light emission from the co-culture system was comparable to what previously observed for 200 μM pimozide in the primary screening (reduction of PAO1/AQ-Rep activity = 46.3%; **Table 3**). Conversely, pimozide did not affect bioluminescence in the PAO1/C-Rep control system up to 400 μM (**Figure 2B**, *gray bars*).

Pimozide was effective in reducing the activity of the PqsR-controlled P*pqsA* promoter in a dose-dependent manner also in PAO1 wild type, with reductions in bioluminescence emission of 17.6%, 33.1 and 45.5% for pimozide concentrations of 100 μM, 200 μM, and 400 μM, respectively (**Figure 2C**, *white bars*).

To validate the results of the secondary screening, PAO1 was grown in the absence or in the presence of pimozide at different concentrations, and AQ levels were measured in cell-free supernatants by means of the AQ-Rep biosensor strain. In these conditions, pimozide significantly decreased AQ production in a dose-dependent manner only at 200 μM (22.9% reduction) and 400 μM (33.8% reduction) (**Figure 2C**, *gray bars*).

Due to its ability to hamper the *pqs* QS signaling system, pimozide is expected to reduce the expression of *pqs*-controlled virulence traits, such as pyocyanin production, swarming motility and biofilm formation. As shown in **Figure 3A**, 100 μM pimozide reduced pyocyanin production in PAO1 (24.8% reduction of pyocyanin level in pimozide-treated cultures relative to the control sample supplemented with the solvent vehicle DMSO). Moreover, exposure to 100 μM pimozide significantly altered the

swarming motility phenotype by abolishing dendrites formation (**Figure 3B**) and reduced biofilm formation in a PAO1 strain constitutively expressing GFP (**Figure 3C**). Despite exerting a milder effect, the inhibition exerted by pimozide on the tested phenotypes in wild type PAO1 mimicked *pqsR* deletion (Δ*pqsR* isogenic strain; **Figure 3**), thus supporting the hypothesis that PqsR is the likely target of pimozide.

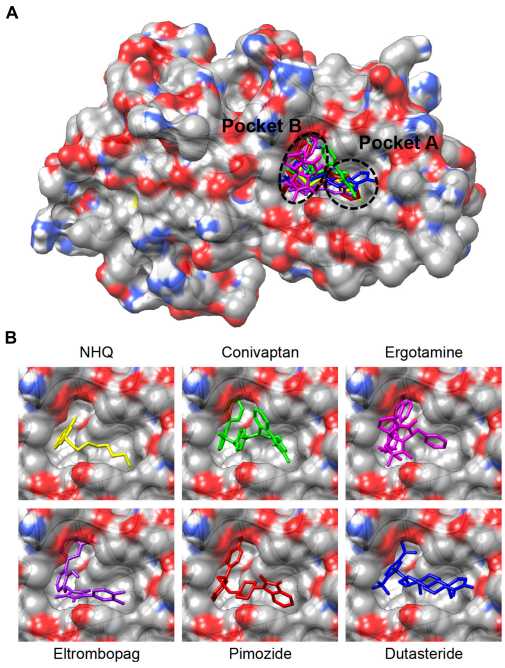


FIGURE 1 | (A) Superimposition of the putative complexes formed by conivaptan (green), ergotamine (magenta), eltrombopag (purple), pimozide (red) or dutasteride (blue) with the PqsR-CBD, obtained by molecular docking simulations. Binding of the natural ligand NHQ (yellow) is also shown (Ilangovan et al., 2013). The binding pockets A and B are indicated. **(B)** Enlargement of the ligand-binding site from **(A)**.

Validation of the Molecular Mechanism of Action of Pimozide

The inhibitory activity exerted by pimozide on P*pqsA* activity, combined with the reduction of AQs level and attenuation of *pqs*-dependent virulence traits, does not allow to rule out the possibility that pimozide affects AQs biosynthesis instead of, or in addition to, AQs reception by PqsR. To tackle this issue, the effect of pimozide on P*pqsA* promoter activity has been tested in the AQ-Rep biosensor strain grown in the presence of 5 μM synthetic PQS. As shown in **Figure 4A**, pimozide reduced P*pqsA* activity also in this experimental setting, in which the AQ molecule PQS required to activate PqsR is not endogenously produced by PAO1. Secondly, AQs production was measured in a PAO1 Δ*pqsR* mutant strain carrying the pFD-*pqsABCD* plasmid for constitutive expression of the AQs biosynthetic enzymes. In this genetic background, in which AQ synthesis is PqsR-independent, pimozide did not reduce AQ levels (**Figure 4B**), indicating that this drug does not affect the activity of the AQs biosynthetic enzymes. Taken together, these experiments indicate that pimozide targets the PqsR-dependent AQs response rather than AQs biosynthesis.

To further support target specificity, the effect of pimozide on P*pqsA* activity was evaluated in a *P. aeruginosa* recombinant strain with tunable levels of PqsR, named PAO1 Δ*pqsAHR* P*pqsA*::*lux* (pPqsR-6H). This strain carries the P*pqsA*::*lux* transcriptional fusion and deletion of the *pqsA*, *pqsH* and *pqsR* genes, therefore it does not synthesize AQs and does not produce the native PqsR regulator, which can be expressed upon IPTG induction *via* the pPqsR-6H plasmid. Therefore, in the absence of IPTG and in the presence of synthetic PQS, the PAO1 Δ*pqsAHR* P*pqsA*::*lux* (pPqsR-6H) strain should express basal level of active PqsR, and the effect of a PqsR inhibitor on P*pqsA* activity should be maximal due to target paucity. Conversely, increasing concentrations of IPTG in the presence of synthetic PQS should result in increased levels of active PqsR, thus reducing the effect of PqsR inhibitors due to increased target abundance. As shown in **Figure 4C**, in this recombinant strain the repressive effect exerted

TABLE 3 | Primary and secondary screenings.

Drug name	Residual reporter activity (%)[a]		Residual AQ production (%)[b]	
	20 μM	200 μM	20 μM	200 μM
Conivaptan	103.7 (103.1)	100.5 (95.6)	n.d.	n.d.
Ergotamine	89.9 (99.8)	72.1 (98.3)	95.3	93.8
Eltrombopag	99.4 (109.7)	69.6 (61.5)	n.d.	n.d.
Pimozide	87.7 (109.9)	53.7 (99.4)	84.9	50.5
Dutasteride	105.9 (102.4)	111.2 (98.7)	n.d.	n.d.

[a] *The first value refers to the residual reporter activity of the PAO1/AQ-Rep reporter system treated with 20 μM or 200 μM of the indicated drug relative to the untreated sample (in this case the same amount of DMSO was used as control). The second value, in brackets, refers to the residual reporter activity of the PAO1/C-Rep control coculture in the same conditions. Activity of the reporter systems in untreated samples are considered as 100%. The average of three independent experiments is reported.*
[b] *Residual production of AQ molecules in P. aeruginosa PAO1 cultures treated with 20 μM or 200 μM of the indicated drug relative to the untreated sample (also in this case DMSO was used as control). AQ levels measured in untreated samples are considered as 100%. The average of three independent experiments is reported. n.d., not determined.*

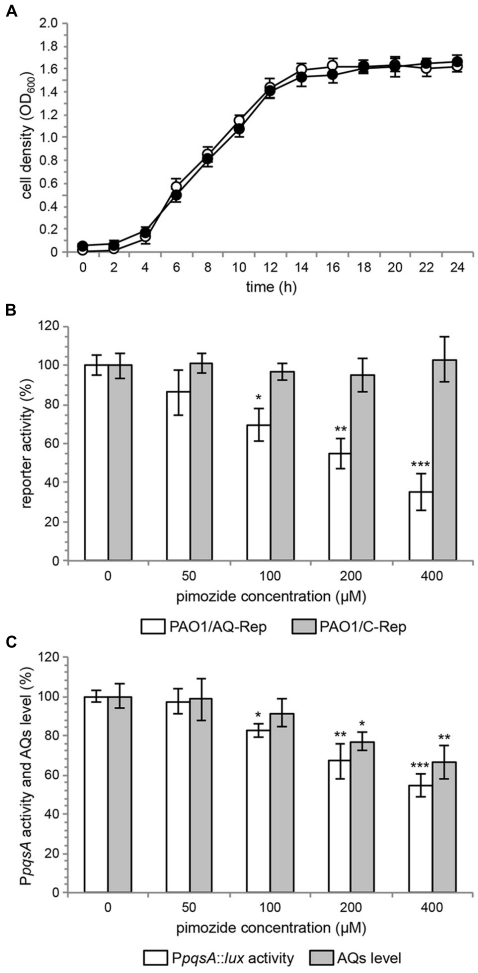


FIGURE 2 | (A) Growth curves of PAO1 wild type incubated at 37°C in shaking conditions in LB supplemented with 400 μM pimozide (black circles) or with the corresponding amount of solvent vehicle [*i.e.,* 1% (v/v) DMSO] (open circles). **(B)** Activity of the PAO1/AQ-Rep (white bars) and PAO1/C-Rep (gray bars) co-cultures treated with the indicated concentrations of pimozide. Bioluminescence of the untreated co-cultures normalized to cell density are considered as 100%. **(C)** P*pqsA*::*lux* activity (white bars) and AQ production (gray bars) in PAO1 treated with the indicated concentrations of pimozide. P*pqsA* activity and AQ level measured in untreated PAO1 normalized to cell density are considered as 100%. For **(A–C)**, the average of three independent experiments is reported with *SD*. $*p < 0.05$; $**p < 0.01$; $***p < 0.001$ (ANOVA).

by 100 μM pimozide on P*pqsA* activity was apparent only for IPTG concentrations ≤ 10 μM, while pimozide had no significant effect on the P*pqsA* promoter for IPTG concentrations ≥ 20 μM. These observations support the hypothesis that pimozide is a ligand and an inhibitor of PqsR.

As shown in **Figure 5**, pimozide is predicted to bind to the PqsR CBD establishing mainly hydrophobic interactions that closely match those of the natural ligand NHQ (Ilangovan et al., 2013), at least as far as pocket A is concerned. Slightly different interactions are instead observed in pocket B, likely due to a rearrangement of the pocket residues needed to accommodate the bulkier bis(fluorophenyl) moiety of pimozide. Interestingly, pimozide binding to pocket A is predicted to be stabilized also by a π-stacking interaction between the drug benzimidazole group and the Tyr258 aromatic ring (**Figure 5**, bottom panel), an interaction that mimics the π-stacking interaction experimentally observed between the phenoxy group of the PqsR competitive inhibitor M64 and Tyr258 (Kitao et al., 2018).

DISCUSSION

Anti-virulence drugs that do not affect bacterial growth hold promise as new therapeutic agents since they are expected to decrease bacterial adaptability to the host environment and to pose a reduced selective pressure for the emergence of resistance with respect to antibiotics. Moreover, virulence mechanisms are often pathogen-specific, thus anti-virulence drugs could avoid dysbiosis usually associated to antibiotic treatments (Rampioni et al., 2014, 2017; Monserrat-Martinez et al., 2019).

The *pqs* QS system controls the expression of multiple virulence factors and biofilm formation, so that *P. aeruginosa* mutants defective in the *pqs* QS system display attenuated pathogenicity in different plant and animal models of infection (Cao et al., 2001; Déziel et al., 2005; Xiao et al., 2006; Lesic et al., 2007; Rampioni et al., 2010; Dubern et al., 2015). Notably, the *pqs* QS system is active during the infection (Collier et al., 2002; Barr et al., 2015), and while *P. aeruginosa* mutants impaired in the *las* QS system are frequently isolated from CF patients (Hoffman et al., 2009; Feltner et al., 2016), the highest proportion of *P. aeruginosa* strains isolated from CF lung are proficient for AQs production (Guina et al., 2003; Jiricny et al., 2014). Moreover, AQ-based QS systems have not been described in the human microbiota so far, suggesting that drugs targeting PqsR should exert a limited effect on the host microbiota. Intriguingly, recent reports indicate that the *pqs* QS system might contribute to the RhlR-dependent activation of virulence genes in the absence of functional LasR (Chen et al., 2019; Kostylev et al., 2019), and that this compensatory role might involve a yet uncharacterized signal molecule produced by PqsE and perceived by RhlR in addition to C4-HSL (Mukherjee et al., 2018). Therefore, by hampering PqsE expression, PqsR inhibitors would impact on virulence factors controlled by both the *pqs* and the *rhl* QS systems, and could be particularly active against *las*-deficient strains emerging during chronic infection in CF patients. On this basis, many inhibitors of the *pqs* QS system have been described in the last decade, proving the ability of anti-*pqs* drugs to reduce the expression of *P. aeruginosa* virulence traits both *in vitro* and in animal models of infection (Calfee et al., 2001; Lesic et al., 2007; Klein et al., 2012; Storz et al., 2012; Ilangovan et al., 2013; Sahner et al., 2013; Weidel et al., 2013; Zender et al., 2013; Lu et al., 2014; Starkey et al., 2014; Sahner et al., 2015; Ji et al., 2016; Thomann et al.,

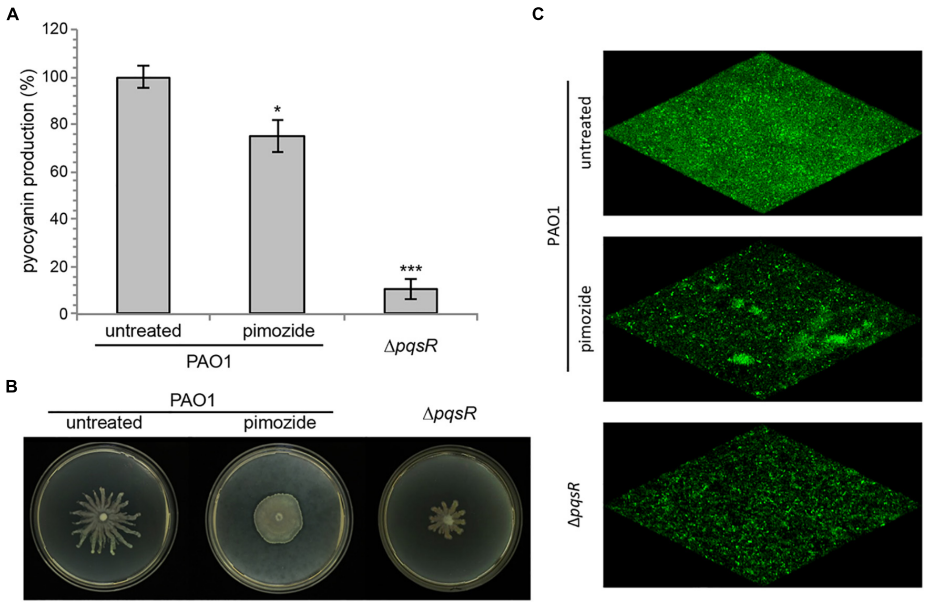


FIGURE 3 | Effect of 100 μM pimozide on pyocyanin production **(A)**, swarming motility **(B)**, and biofilm formation **(C)** in PAO1. The same phenotypes were evaluated in the Δ*pqsR* mutant as a control. For pyocyanin production, the average of three independent experiments is reported with *SD*. *$p < 0.05$; ***$p < 0.001$ (ANOVA). For swarming motility and biofilm formation, representative pictures of three independent experiments are shown.

2016; Maura and Rahme, 2017; Maura et al., 2017; D'Angelo et al., 2018; Soukarieh et al., 2018a).

Despite the promise of anti-*pqs* agents for the treatment of *P. aeruginosa* infection, none of these molecules has entered clinical trials so far, likely due to poor pharmacological properties and to the lack of ADME-TOX studies required for their evaluation in humans (Maura et al., 2016; Soukarieh et al., 2018b). To overcome this limitation, we recently exploited a drug-repurposing strategy for the identification of anti-*pqs* drugs *via* whole-cell biosensor-based screening. This strategy succeeded in identifying the FDA-approved drugs clofoctol, miconazole and clotrimazole as new inhibitors of PqsR (D'Angelo et al., 2018).

Most *pqs*-inhibitors have been identified *via* costly and time-consuming biosensor-based screenings or *via* the rational design and experimental validation of AQ analogs or precursors based on the structure of PqsR and of AQ biosynthetic enzymes. Virtual screenings could reduce the time and costs associated to conventional drug discovery programs, hence *in silico* techniques have been extensively applied for the identification of molecules hampering the *las* QS system of *P. aeruginosa* (Yang et al., 2009; Skovstrup et al., 2013; Tan et al., 2013; Soheili et al., 2015; Gökalsın et al., 2017; Kalia et al., 2017; Xu et al., 2017) or QS systems in other bacteria (Zhu et al., 2012; Ali et al., 2018; Ding et al., 2018, 2019; Medarametla et al., 2018). To the best of our knowledge, only synthetic quinoline-based molecules have so far been identified as PqsR antagonists by means of *in silico* docking analyses (Soukarieh et al., 2018a).

In this study we combined the advantages of drug-repurposing and *in silico* screening approaches by exploiting recent knowledge of PqsR-CBD structure and availability of advanced molecular docking tools to identify new FDA-approved drugs with anti-*pqs* activity. The virtual screening led to selection of five hits for which high binding affinity for PqsR was predicted, and *in vitro* experiments demonstrated the anti-*pqs* activity of two of them, namely pimozide and ergotamine. Since pimozide showed the highest inhibitory activity, this drug was experimentally characterized. Phenotypic assays showed that exposure of *P. aeruginosa* PAO1 to pimozide decreased key PqsR-controlled virulence determinants, such as AQ signal molecules, pyocyanin, swarming motility and biofilm formation, without altering bacterial growth, as one would expect for an anti-virulence drug. Additional experiments performed with *ad hoc* engineered *P. aeruginosa* strains and refined *in silico* docking simulations suggest that pimozide competes with the natural ligands HHQ and PQS for PqsR binding, hence hampering the activity of the P*pqsA* promoter. Indeed, analysis of the highest ranking pimozide-PqsR docking complex indicated that the drug interacts with the binding pocket occupying the same position of the natural ligand NHQ (**Figure 5**), and establishes interactions experimentally demonstrated for both the natural ligand and the competitive inhibitor M64 (Ilangovan et al., 2013; Kitao et al., 2018).

The inability of the predicted PqsR ligands conivaptan and dutasteride to hamper the *pqs* QS system in *P. aeruginosa* and

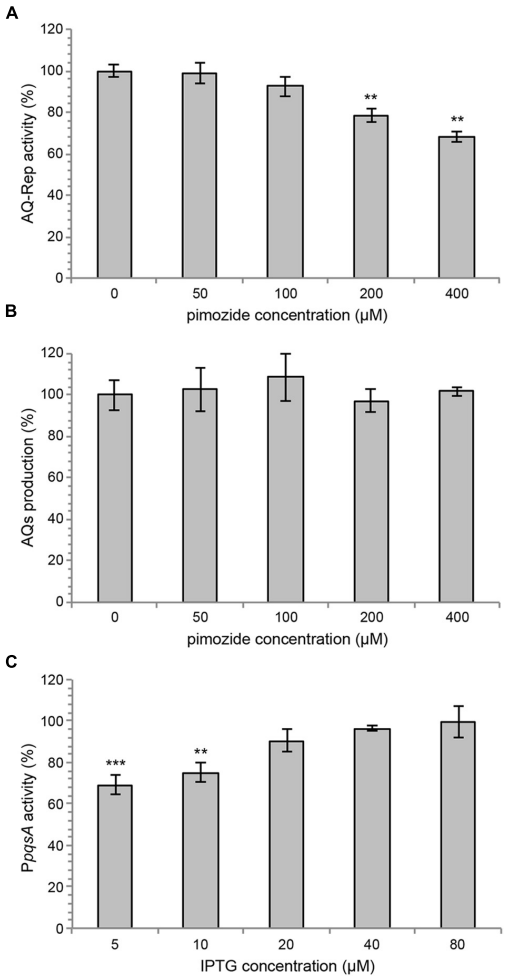


FIGURE 4 | (A) P*pqsA* activity in the AQ-Rep biosensor grown at 37°C in shaking conditions in LB supplemented with 5 μM synthetic PQS and the indicated concentrations of pimozide. Biosensor activity in the untreated sample is considered as 100%. **(B)** Production of AQs in *P. aeruginosa* PAO1 Δ*pqsR* (pFD-*pqsABCD*) grown for 16 h in LB in the absence or in the presence of pimozide. The AQ level measured in the untreated sample is considered as 100%. **(C)** Effect of 100 μM pimozide on P*pqsA*::*lux* activity in the PAO1 Δ*pqsAHR* triple mutant carrying the pPqsR-6H plasmid, grown in LB supplemented with 5 μM PQS and different concentrations of IPTG, as indicated in the graph. For **(A–C)**, the average of three independent experiments is reported with *SD*. $^{**}p < 0.01$; $^{***}p < 0.001$ (ANOVA).

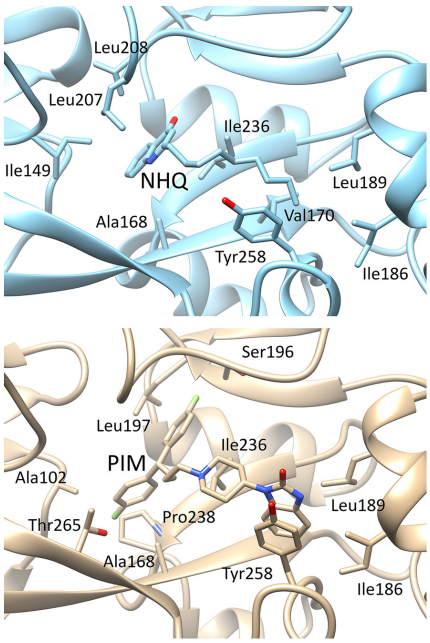


FIGURE 5 | Details of the possible interactions of NHQ (**top panel**; PDB code 4JVD) and pimozide (**bottom panel**; predicted) with the PqsR-CBD binding site. The orientation of the macromolecule is the same as in **Figure 1**; pocket B is on the left side of the macromolecule and pocket A on the right side.

to decrease bioluminescence in the control biosensor system may be related to drawbacks typically associated to virtual screening approaches, including cell impermeability to the selected compound or its modification/inactivation by cellular metabolism. This is in line with the notion that hits emerging from *in vitro* screens, as well as from screens employing heterologous organisms, may lack activity or even function as agonists when tested on the target pathogen (Galloway et al., 2011). As an example, the HHQ analog 2-heptyl-6-nitroquinolin-4*(1H)*-one acted as an antagonist in an *Escherichia coli*-based AQ-reporter strain, but as an agonist in *P. aeruginosa* as a consequence of metabolic modification (Lu et al., 2012). However, a subsequent synthetic modification of this molecule resulted in a strong PqsR antagonist also in *P. aeruginosa*, showing that agonists may still prove useful in the search for antagonists (Lu et al., 2014). More often, the inability of selected hits identified *in silico* or *in vitro* to inhibit target functionality in bacterial cell relates to a lack of internalization or to active efflux. This does not seem to be the case for eltrombopag, since the inhibitory effect exerted by this drug on both the specific and control reporter systems indicates its ability to penetrate *P. aeruginosa* cells, suggesting a QS-independent effect on bioluminescence. In a commentary on the use of whole-cell reporter systems for screens of QS inhibitors, the need for adequate control experiments to assess off-target effects of the tested compounds on reporter activity was emphasized (Defoirdt et al., 2013). For example, pyrogallol was reported to act as a potent inhibitor of AI-2 dependent QS in *V. harveyi*, but subsequent experiments revealed that the apparent inhibitory

activity of pyrogallol was a side effect of its peroxide-generating activity on the reporter system, rather than on QS itself (Defoirdt et al., 2013). In our case, unspecific effects of eltrombopag on the reporter system may mask its impact on PqsR functionality. Obviously, the possibility that the virtual screening approach could select false positive hits cannot be ruled out. When molecular assays for *in vitro* evaluation of PqsR activity will be available, it will be possible to verify the ability of the five hits identified in this study to hamper PqsR functionality in a cell-free system.

Searching for side activities in FDA-approved drugs represents a shortcut to develop new therapeutic agents, with considerable potential for shortening the time-consuming and expensive hit-to-lead and lead-optimization phases of drug-discovery programs (Rangel-Vega et al., 2015). In the last years an increasing number of studies identified some antibacterial activity in several drugs approved for different purposes, including anticancer, antifungal, cardiovascular and antipsychotic therapies (Miró-Canturri et al., 2019). However, a possible drawback of drug-repurposing approaches relies on the primary activity of the repurposed drug. As an example, the antipsychotic activity of the dopamine antagonist pimozide, clinically used for the treatement of Tourette's syndrome and schizophrenia (Tueth and Cheong, 1993), could limit its therapeutic use as anti-virulence drug against *P. aeruginosa*. In fact, it has to be considered that, although rarely, pimozide has been associated to potentially serious adverse effects, including arrhythmia, cardiac arrest, seizures, and neutropenia (Singer, 2010). Neutropenia, in particular, is a worrisome adverse effect for patients suffering a bacterial infection. In addition, the peak serum concentration of pimozide in conventional treatment as an antipsychotic drug is in the nanomolar range (Yan et al., 2010), far below the concentration required to inhibit the *pqs* QS system in *P. aeruginosa*. In spite of these limitations, the pimozide molecular scaffold could serve as the basis for chemical modifications aimed at lowering its dopamine antagonistic activity, while improving membrane permeability and affinity for the PqsR active site, in line with the selective optimization of side activity (SOSA) approach (Wermuth, 2006). However, such a hit-to-lead optimization process would partly compromise the advantage of drug reuporisng, since chemical modification of pimozide would invalidate the FDA-approval, with additional pharmacological testing being required by regulatory agencies. It must be recognized that repurposing of old drugs for new therapies can result in seamless adoption into the clinical practice only if their off-target effect overcomes their primary activity.

That said, pimozide has already been repurposed to inhibit *Listeria monocytogenes* virulence by decreasing cell invasion, vacuole escape and cell-to-cell spread in phagocytic host cells (Lieberman and Higgins, 2009), to inhibit the growth of the protozoan parasite *Toxoplasma gondii* (Dittmar et al., 2016) and for the treatment of breast cancer (Dakir et al., 2018; Elmaci and Altinoz, 2018). Notably, pimozide also inhibited Chikungunya virus (CHIKV) replication in a mouse model of Chikungunya infection when administered in combination with the fatty acid synthesis inhibitor 5-tetradecyloxy-2-furoic acid, with low toxicity *in vivo* (Karlas et al., 2016).

In conclusion, despite low potency of pimozide as a *pqs* inhibitor and predictable side-effects due to its primary antipsychotic activity, this study demonstrates for the first time the potential of virtual screening campaigns to rapidly select new FDA-approved QS inhibitors.

DATA AVAILABILITY STATEMENT

All datasets generated for this study are included in the manuscript/supplementary files.

AUTHOR CONTRIBUTIONS

FP and GR conceived the study. FP, GR, LL, and PV designed the experiments and contributed reagents, materials, and analysis tools. MM, ED, FD, VB, and SF performed the experiments. FP, GR, LL, MM, FD, ED, and VB analyzed the data. GR, FP, and MM wrote the manuscript. All authors corrected, amended the draft of the manuscript, and approved the submitted version.

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Chapter 5

Identification of FDA-approved antivirulence drugs targeting the *Pseudomonas aeruginosa* quorum sensing effector protein PqsE

Valerio Baldelli[1], Francesca D'Angelo[1], Viola Pavoncello[1], Ersilia Vita Fiscarelli[2], Paolo Visca[1], Giordano Rampioni[1] and Livia Leoni[1]

[1] *Department of Science, University Roma Tre, Rome, Italy;* [2] *Laboratory of Cystic Fibrosis Microbiology, Bambino Gesú Hospital, Rome, Italy.*

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Preface to Chapter 5

In the previous Chapters 3 and 4 a drug repurposing approached led to the identification of promising antivirulence drugs targeting the QS signal receptor PqsR. As discussed in the Introduction section, while in most AHL-based QS systems LuxR-like receptors act as global regulators that at the "*quorum*" cell density reprogram gene expression by binding to multiple promoter regions, this is not the case for the *pqs* QS system. In this case, the main role of PqsR, upon perception of the AQ signal molecules HHQ or PQS, is to promote transcription of the *pqsABCDE-phnAB* operon by binding to the P*pqsA* promoter region. This implies that activated PqsR mainly affects *P. aeruginosa* physiology by increasing AQs production and PqsE expression, being PqsE the main effector protein of the *pqs* QS system.

Intriguingly, PqsE controls the expression of key *pqs*-dependent virulence traits, including pyocyanin production and biofilm formation, *via* a still uncharacterized PqsR- and AQs-independent mechanism. While PqsE can also participate to AQs biosynthesis, the only PqsE ligand identified so far inhibits this activity, but does not affect other PqsE-controlled virulence traits, indicating that PqsE is a multifunctional protein. Recently, data have been produced indicating that one of these functions might be the production of an RhlR ligand alternative to C_4-HSL. Overall, despite the mechanism of action of PqsE is still far from being clarified, PqsE inhibitors could be endowed with significant antivirulence activity, since these drugs are expected to hamper both PqsE- and RhlR-controlled virulence determinants.

On this basis, in this work a library of 1,600 FDA-approved compounds has been screened by using a purpose-built bioluminescent *P. aeruginosa*-based biosensor strain in which light emission inversely correlates with PqsE functionality. This screening campaign led to the identification of antibiotics that hamper PqsE functionality and the expression of PqsE-controlled virulence traits at concentrations at which they do not affect *P. aeruginosa* growth. Notably, both drugs reduce the production of the PqsE-controlled virulence factor pyocyanin also in *P. aeruginosa* strains isolated from cystic fibrosis patients, and neither of the two molecules antagonizes the activity of antibiotics commonly used to treat *P. aeruginosa* infection.

In conclusion, this work led to the identification of new PqsE inhibitors with potential as antivirulence agents for *P. aeruginosa* therapeutic treatment. These drugs could also provide new insights into the molecular mechanism of action of the *pqs* effector protein PqsE.

Identification of FDA-approved antivirulence drugs targeting the *Pseudomonas aeruginosa* quorum sensing effector protein PqsE

Valerio Baldelli[1], Francesca D'Angelo[1#], Viola Pavoncello[1#], Ersilia Vita Fiscarelli[2], Paolo Visca[1], Giordano Rampioni[1*], Livia Leoni[1*]

[1] Department of Science, University Roma Tre, Rome, Italy; [2] Laboratory of Cystic Fibrosis Microbiology, Bambino Gesú Hospital, Rome, Italy.

[#] Current address: Institut Pasteur, Paris, France.

Correspondence:

[*] Giordano Rampioni: giordano.rampioni@uniroma3.it

[*] Livia Leoni: livia.leoni@uniroma3.it

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Running Head: New FDA-approved inhibitors of PqsE

Abstract

The ability of the bacterial pathogen *Pseudomonas aeruginosa* to cause both chronic and acute infections mainly relies on its capacity to finely modulate the expression of virulence factors through a complex network of regulatory circuits, including the *pqs* quorum sensing (QS) system. While in most QS systems the signal molecule/receptor complexes act as global regulators that modulate the expression QS-controlled genes, the main effector protein of the *pqs* system is PqsE. This protein is involved in the synthesis of the QS signal molecules 2-alkyl-4(1*H*)-quinolones (AQs), but it also modulates the expression of genes involved in virulence factors production and biofilm formation *via* an AQs-independent pathway. *P. aeruginosa pqsE* mutants disclose attenuated virulence in plant and animal infection models, hence PqsE is considered a good target for the development of antivirulence drugs against *P. aeruginosa*.

In this study, the negative regulation exerted by PqsE on its own transcription has been exploited to develop a screening system for the identification of PqsE inhibitors in a library of FDA-approved drugs. This led to the identification of nitrofurazone and erythromycin estolate, two antibiotic compounds that reduce the expression of PqsE-dependent virulence traits and biofilm formation in the model strain *P. aeruginosa* PAO1 at concentrations far below those affecting the bacterial growth rate. Notably, both drugs reduce the production of the PqsE-controlled virulence factor pyocyanin also in *P. aeruginosa* strains isolated from cystic fibrosis patients, and neither of the two molecules antagonizes the activity of antibiotics commonly used to treat *P. aeruginosa* infection.

1. Introduction

The spread of antibiotic resistance in bacterial pathogens is increasing at an unprecedented pace. While the mortality due to antibiotic-resistant infections rises worldwide, the antibiotic discovery pipeline is running dry, with few approvals of new antibiotics for human therapy in the last decades [1]. Consequently, new antibacterial agents active against antibiotic-resistant pathogens are urgently needed. However, traditional antibiotic research programs seem unable to cope with the rapid spread of antibiotic resistance, mainly due to the high costs and long times required for *de novo* drug-discovery [2-4].

In the last years, the repurposing of "old" drugs for new clinical applications has become a major research area in drug discovery. In principle, the identification of off-target activity in drugs already approved for their use in humans allows fast and cost-effective selection of safe drugs with high potential for seamless adoption into the clinical practice [5,6]. The search for drugs targeting the growth and/or viability of bacterial pathogens remains a primary goal, but additional innovative approaches to combat bacterial infections should be pursued in parallel. In this context, a promising antibacterial strategy aims at identifying molecules targeting bacterial virulence rather than growth/viability. This antivirulence approach has been boosted by increased knowledge on bacterial pathobiology, and it is expected to reduce bacterial adaptability to the host environment while posing a reduced selective pressure for the emergence of resistance relative to antibiotics. Moreover, by inhibiting pathogen specific targets, antivirulence drugs could be endowed with limited adverse effects on the host microbiota [7-9].

The versatile Gram-negative bacterium *Pseudomonas aeruginosa* is able to colonize a variety of harsh environments, including polluted soil and marine habitats, plants and mammalian tissues [10]. As a human pathogen, *P. aeruginosa* has evolved a number of mechanisms for adaptation and survival within the host, including intrinsic and acquired resistance to multiple classes of antibiotics [10]. In particular, antibiotic-resistant biofilms are a major cause of hard to treat *P. aeruginosa* healthcare associated infections, and the leading cause of morbidity and mortality in cystic fibrosis (CF) patients. CF is a genetic disease affecting 1/3000 newborns in the Caucasian population [11,12]. For these reasons, *P. aeruginosa* is included in the priority list of pathogens for which new antimicrobial therapies are urgently needed (Priority 1: Critical; http://www.who.int/en/news-room/detail/27-02-2017-who-publishes-list-of-bacteria-for-which-new-antibiotics-are-urgently-needed).

P. aeruginosa produces an array of toxic metabolites and enzymes, and different macromolecules contributing to the biofilm matrix [10]. Numerous efflux pumps and secretion systems complete the

dangerous armament of this tough microorganism [13,14]. Finally, multiple interwoven global regulatory systems coordinate the expression of *P. aeruginosa* virulent phenotypes in response to population structure, metabolic and environmental cues [15,16]. Indeed, *P. aeruginosa* ability to colonize different human tissues, and to resist to the immune system and to antibiotics mainly relies on its capacity to finely modulate the expression of multiple virulence factors and biofilm formation [17-19]. For these reasons, *P. aeruginosa* global regulatory systems, including the quorum sensing (QS) circuit, are considered ideal targets for the development of antivirulence drugs [9,20,21].

P. aeruginosa has three major QS systems, namely the *las*, *rhl* and *pqs* systems. The *las* and *rhl* QS systems are based on acyl-homoserine lactones (AHLs), while the *pqs* QS system is based on 2-alkyl-4(1*H*)-quinolones (AQs) as signal molecules [19,22]. *P. aeruginosa* QS-deficient mutants display attenuated virulence in different animal models of infection. Hence QS is considered a good target for the development of *P. aeruginosa* antivirulence drugs [15,17,20,21]. However, the use of QS inhibitors for CF therapy is debated, mainly as a consequence of frequent isolation of *P. aeruginosa* mutants inactivated in the *las* QS system from CF patients with years long chronic infection [23-26]. Conversely, the highest proportion of *P. aeruginosa* strains isolated from CF patients are AQ-producers [27,28], and AQ levels correlate with the clinical status of CF patients with *P. aeruginosa* lung infection [29], indicating that the *pqs* QS system could be a suitable target for innovative CF therapies.

The main AQ signal molecules of *P. aeruginosa* are 2-heptyl-3-hydroxy-4(1*H*)-quinolone (PQS) and its precursor 2-heptyl-4-hydroxyquinoline (HHQ). AQs production increases during bacterial growth, and when the level of these molecules in the environment reaches a threshold concentration, corresponding to the "*quorum*" cell density, either HHQ or PQS signal molecules bind to and activate the transcriptional regulator PqsR (also known as MvfR). The PqsR/AQ complex activates the transcription of the *pqsABCDE-phnAB* operon, coding for the enzymes required for the synthesis of HHQ, hence triggering the positive feedback loop typical of QS systems. The *pqsH* gene codes for the PqsH enzyme required to convert HHQ to PQS [30-32]. While in the majority of bacterial QS systems the signal molecule/receptor complex acts as a global regulator to modulate the expression of QS genes, the main effector protein of the *pqs* system is PqsE rather than the PqsR/AQ complex. Indeed, data produced in our laboratory indicate that the main physiological role of the PqsR/AQ complex is to trigger transcription of the *pqsABCDE-phnAB* operon, ultimately resulting in increased production of HHQ and expression of PqsE, a thioesterase coded by the fifth gene of the *pqsABCDE-phnAB* operon [33]. PqsE is involved in AQ synthesis by converting 2-aminobenzoylacetyl-CoA into 2-aminobenzoylacetate [32], that is in turn condensed with octanoyl-coenzyme A by the PqsBC

heterodimer to form HHQ [31]. However, *pqsE* inactivation does not significantly affect AQs biosynthesis [34,35], likely because PqsE thioesterase activity can be provided by alternative enzymes [32]. Intriguingly, PqsE controls the transcription of more than 140 genes, including key virulence genes (*e.g.* genes required for pyocyanin and rhamnolipids production, and genes involved in swarming motility and biofilm formation), *via* a still poorly understood pathway that is both PqsR- and AQs-independent [33-40]. Accordingly, *P. aeruginosa pqsE* mutants are impaired in biofilm formation and display reduced virulence in plant and animal models of infection [34,35,39]. Since PqsE expression requires the PqsR/AQs complex, PqsR-inhibitors have been shown to attenuate *P. aeruginosa* virulence both *in vitro* and in animal models of infection [41-51], and some of these inhibitors potentiate the effect of antibiotics used in CF therapy both *in vitro* and in murine models of infection [45,47]. To the best of our knowledge, only one PqsE inhibitor has been described so far. This molecule hampers PqsE thioesterase activity, but it does not affect PqsE ability to control the expression of virulence factors [38].

Interestingly, PqsE is also auto-regulated. Indeed, the activity of the *pqsABCDE-phnAB* promoter P*pqsA* increases in the absence of PqsE, and is abrogated in a *P. aeruginosa pqsE*-overexpressing strain [34,35]. In this study, the PqsE-dependent negative feedback loop has been exploited to develop a high-throughput screening system for the identification of molecules targeting PqsE activity. The screening system was validated by screening a library of 1,600 FDA-approved drugs, leading to the identification of anti-PqsE activity in nitrofurazone and erythromycin estolate, two antibiotics displaying antivirulence activity towards *P. aeruginosa* at concentrations that do not affect bacterial growth. The effect of both drugs on the expression of *P. aeruginosa* virulence phenotypes, on biofilm formation and on the susceptibility to antibiotics currently used to treat *P. aeruginosa* infection has been investigated, as well as their activity against *P. aeruginosa* CF isolates. Beside their possible development as antivirulence agents, the new PqsE inhibitors identified in this study might facilitate future understanding of the molecular mechanism underlying PqsE-dependent control of virulence traits in *P. aeruginosa*.

2. Materials and Methods

2.1 Bacterial strains, media and chemicals

The bacterial strains and plasmids used in this study are listed in **Table S1** and **Table S2**, respectively. *Escherichia coli* DH5αF' [52] was used for plasmid DNA amplification. Plasmids purification from *E. coli* and transformation into *P. aeruginosa* were performed with standard procedures [53].

All *E. coli* and *P. aeruginosa* strains were routinely grown at 37°C in Lysogeny Broth (LB) [53] with aeration. For some experiments, *P. aeruginosa* was grown in M9 minimal medium supplemented with 20 mM glucose as sole carbon source (M9-Glu) [53] or in BBL Mueller Hinton II Broth (Cation-Adjusted) medium (MHB, Becton Dickinson). The following antibiotics were added when required: 50 µg/mL ampicillin (Ap), for *E. coli*; 400 µg/mL carbenicillin (Cb) or 200 µg/mL tetracycline (Tc), for *P. aeruginosa*. When necessary, isopropyl β-D-1-thiogalactopyranoside (IPTG) was added at the concentrations indicated in the text. Stock solutions of 80 mM nitrofurazone (Fluka), 200 mM erythromycin estolate (Sigma-Aldrich) and 80 mM diminazene (Sigma-Aldrich) were prepared in dimethyl sulfoxide (DMSO), ethanol (EtOH) and water, respectively.

2.2 Primary screening for the identification of PqsE inhibitors

The *P. aeruginosa* PqsE-Rep biosensor strain (*i.e., P. aeruginosa* PAO1 *pqsE*^{IND} P*pqsA*::*luxCDABE* [35]) was grown overnight at 37°C on LB agar plates. Bacteria were scraped from plates surface and diluted in LB supplemented with 50 µM IPTG to an optical density at 600 nm wavelength (OD_{600}) of 0.08 (procedure modified from [54]). Two-hundred µL aliquots of the culture were grown at 37°C in 96-well black clear-bottom microtiter plates in the presence of compounds of the PHARMAKON library (20 µM and 200 µM). The OD_{600} and relative light units (RLU) were measured after 5 h incubation by using a Spark 10M multilabel plate reader (Tecan). Samples grown in the presence of the solvent vehicle DMSO [0.2% (v/v) or 2% (v/v)] were used as controls in each microtiter plate. Reporter activity was determined as RLU/OD_{600} for each sample. Residual reported activity was determined in treated samples relative to the solvent vehicle control samples grown in the presence of DMSO, considered as 100%.

2.3 Measurements of promoter activity

Bioluminescence was determined as a function of population density by using a Spark 10M multilabel plate reader (Tecan), as previously described [33]. Briefly, overnight cultures of the *P. aeruginosa* reporter strains used in this study (**Table S1**) were diluted in 200 µL of LB to an $OD_{600} \approx$ 0.01, in the presence or in the absence of PqsE inhibitors at the concentrations indicated in the text, and dispensed into 96-wells black clear-bottom microtiter plates. When required, LB was supplemented with IPTG, at the concentrations indicated in the text. Luminescence and turbidity were determined after 5 h of incubation at 37°C with gentle shacking (120 rpm). Reporter activity was determined as RLU/OD_{600} for each sample. Residual reported activity was determined in treated

174 samples relative to the solvent vehicle control samples grown in the presence of DMSO or EtOH,
175 considered as 100%.
176
177 **2.4 Analyses of virulence-related phenotypes**
178 For the *P. aeruginosa* PqsE-Rep, PAO1 wild type and PAO1 Δ*pqsE* strains, pyocyanin was
179 extracted as quantified as previously described [55]. The same method was scaled-down in order to
180 extract and quantify pyocyanin produced by *P. aeruginosa* CF isolates. Briefly, each CF strain was
181 incubated in 96-wells microtiter plates for 21 h at 37°C with shacking (120 rpm) in 200 µL of LB
182 broth, in the presence of 100 µM nitrofurazone or 50 µM erythromycin estolate. Each CF strain was
183 incubated in the presence of 0.125% (v/v) DMSO or 0.025% (v/v) EtOH as solvent vehicle control
184 (untreated samples). After 21 h incubation, two independent cultures of the same CF strain were
185 pooled, the OD_{600} was measured and cell-free supernatants were collected into 1.5 mL tubes. After
186 extraction with an isovolume of chloroform, the pyocyanin-containing chloroform phase was
187 transferred into clean 1.5 mL tubes and acidified with an isovolume of 0.2 N HCl. After
188 centrifugation, 200 µL of the aqueous-phase were transferred into 96-wells microtiter plates and A_{520}
189 was measured by using an automated Spark 10M multilabel plate reader (Tecan). Pyocyanin
190 production was evaluated by normalizing the A_{520} to the OD_{600} value measured for each CF strain.
191 Rhamnolipids in cell-free supernatants of *P. aeruginosa* cultures were quantified by the orcinol
192 method, as previously described [56] Briefly, bacterial strains were grown at 37°C for 24 h in LB
193 supplemented with 100 µM nitrofurazone, 50 µM erythromycin estolate, 0.125% (v/v) DMSO or
194 0.025% (v/v) EtOH before rhamnolipids extraction and quantification.
195 For swarming motility assay, 5 µL of *P. aeruginosa* cultures grown in LB for 8 h were spotted
196 onto swarming plates [0.8% (w/v) nutrient broth N.2, 0.5% (w/v) glucose, 0.5% (w/v) bacteriological
197 agar] supplemented with 100 µM nitrofurazone or 50 µM erythromycin estolate. Also in this case,
198 plates supplemented with the solvent vehicles 0.125% (v/v) DMSO or 0.025% (v/v) EtOH were used
199 as controls. After 16 h of growth at 37°C, swarming motility was directly observed at the air-agar
200 interface [57].
201 For microscopic visualization of biofilm, *P. aeruginosa* strains constitutively expressing GFP *via*
202 the pMRP9-1 plasmid [58] were grown in 8-well chamber slides, as previously described [59], with
203 minor modifications. Briefly, bacteria were inoculated at an OD_{600} of 0.02 in 500 µL of M9-Glu in
204 the presence and in the absence of the tested compounds (*i.e.*, 100 µM nitrofurazone, 50 µM
205 erythromycin estolate, 0.125% (v/v) DMSO or 0.025% (v/v) EtOH), and incubated at 30°C for 72 h.

To maintain bacterial viability, the medium was changed every 24 h. The biofilm structure was examined using a Leica TCS SP5 confocal microscope.

2.5 MIC and antibiotic tolerance assays

The Minimal Inhibitory Concentration (MIC) of the antibiotics ciprofloxacin, colistin, tobramycin and piperacillin was evaluated with the standard microdilution method, according to the Clinical and Laboratory Standards Institute guidelines [60]. Briefly, *P. aeruginosa* PAO1 and its isogenic Δ*pqsE* mutant were grown at 37°C with shaking in MHB or in M9-Glu [53]. After 16 h of growth, the cultures were diluted in 100 µL of MHB or M9-Glu to an $OD_{600} \approx 0.0005$ (ca. $5x10^5$ CFU/mL) in 96-well microtiter plates with increasing concentrations of the selected antibiotics. For tobramycin, possible interaction with 100 µM nitrofurazone or 50 µM erythromycin estolate was also evaluated. The MIC values were evaluated after 24 h of static incubation at 37°C.

The fraction of tolerant *P. aeruginosa* cells was determined as previously described [45]. Briefly, *P. aeruginosa* strains were grown with shaking and aeration to mid-logarithmic phase in LB, in the absence or in the presence of 100 µM nitrofurazone or 50 µM erythromycin estolate [0.125% (v/v) DMSO or 0.025% (v/v) EtOH were used as solvent vehicle controls]. Before antibiotic addition, culture aliquots were diluted in fresh LB and plated on LB agar for CFU count (pre-antibiotic). The rest of the culture was treated with 4 µg/mL tobramycin (8x MIC). At 16 h post-antibiotic addition, culture aliquots were washed twice in fresh LB to remove antibiotic carry-over, serially diluted and plated on LB agar for CFU count. This procedure was repeated at 24 h post-antibiotic addition to ensure that a killing plateau was reached. The tolerant fraction expressed as N-fold change was determined as the ratio between the CFU/mL values measured after antibiotic addition (16 h and 24 h post-antibiotic) divided by CFU/mL values measured before antibiotic addition.

2.6 Statistical analysis

Statistical analysis was performed with the software GraphPad Prism 5, using one-way analysis of variance (ANOVA) followed by Tukey-Kramer multiple comparison tests. Differences having a p value < 0.05 were considered statistically significant.

3. Results

3.1 Development and validation of a screening system for the identification of PqsE inhibitors

In this study the *P. aeruginosa* PAO1 derivative strain previously named *P. aeruginosa pqsE*^{IND} P*pqsA::lux* [35] has been re-named PqsE-Rep. This recombinant strain expresses the *pqsE* gene

239 under the control of the IPTG-inducible P*tac* promoter and carries a transcriptional fusion between
240 the promoter of the *pqsABCDE-phnAB* operon (P*pqsA*) and the *luxCDABE* operon for
241 bioluminescence emission integrated at the neutral *attB* site of the chromosome (**Fig. 1A**). A previous
242 study showed that the IPTG-dependent induction of *pqsE* expression decreases bioluminescence
243 emitted by PqsE-Rep, and that IPTG *per se* does not affect P*pqsA* activity in wild type *P. aeruginosa*
244 PAO1 up to a concentration of 1 mM [35]. These data indicate that the PqsE-Rep biosensor strain
245 could be exploited as a reporter system to identify inhibitors of PqsE activity. Preliminary
246 experiments were carried out to select culture conditions optimal for carrying out a screening
247 campaign aimed at identifying PqsE inhibitors by using the PqsE-Rep biosensor strain (**Fig. S1**). The
248 bioluminescence emitted by PqsE-Rep inversely correlated with the amount of IPTG present in the
249 medium (**Fig. 1B**). This is because the increase in IPTG concentration induces PqsE expression,
250 leading to a parallel PqsE-dependent repression of P*pqsA* activity. In the presence of IPTG, the
251 addition of a PqsE inhibitor to the PqsE-Rep biosensor strain should result in a significant increase of
252 bioluminescence with respect to the untreated control. Since 50 μM IPTG strongly decreased P*pqsA*
253 activity without saturating PqsE-Rep response (**Fig. 1B**), this IPTG concentration was used in the
254 screening campaign.
255 To identify new PqsE inhibitors, the above described reporter system was used to screen the
256 PHARMAKON library of 1,600 FDA-approved compounds with known biological activity and high
257 chemical and pharmacological diversity. Each compound was tested at both 20 μM and 200 μM
258 concentration for its ability to increase bioluminescence emission by PqsE-Rep with respect to the
259 untreated control (details in Materials and Methods). Considering bioluminescence and cell density
260 of the solvent vehicle control samples as 100%, the criteria for hits selection were: *i*)
261 bioluminescence ≥ 130% at 20 μM; *ii*) bioluminescence ≥ 200% at 200 μM; *iii*) reduction of cell
262 density ≤ 10% at both 20 μM and 200 μM. This primary screen led to the selection of 24 hits (**Fig.**
263 **S2A**).
264 Since the production of the PqsE-dependent virulence factor pyocyanin parallels IPTG-dependent
265 induction of PqsE in the PqsE-Rep biosensor strain [35], the effect of the 24 hits on pyocyanin
266 production was used as a proxy in a secondary screening aimed at deselecting hits increasing PqsE-
267 Rep bioluminescence emission *via* PqsE-independent mechanisms. Out of the 24 selected hits, only
268 nitrofurazone, erythromycin estolate and diminazene aceturate robustly reduced pyocyanin
269 production in PqsE-Rep grown in the presence of 50 μM IPTG with respect to the solvent vehicle
270 control samples, without affecting bacterial growth (**Fig. S2B**). These three compounds were
271 purchased from different providers, and their efficacy in increasing light emission and decreasing

pyocyanin production was tested again in the PqsE-Rep strain grown in the presence of 50 μM IPTG. Diminazene aceturate was still able to increase light emission (**Fig. 2A**), but it showed low activity as a pyocyanin inhibitor (**Fig. 2B**). Conversely, nitrofurazone and erythromycin estolate increased light emission (**Fig. 2A**) and strongly decreased pyocyanin production in a dose dependent manner (**Fig. 2B**), with EC_{50} and IC_{50} values of 78.58 μM and 24.65 μM for nitrofurazone, and of 6.15 μM and 5.79 μM for erythromycin estolate, respectively (**Table 1**). Hence, nitrofurazone and erythromycin estolate were selected for further analyses.

3.2 Nitrofurazone and erythromycin estolate inhibit the expression of PqsE-controlled virulence phenotypes

Nitrofurazone and erythromycin estolate are antibiotics belonging to the nitrofuran and macrolide structural classes, respectively [61,62]. However, the MIC of these drugs for *P. aeruginosa* PAO1 is ≥ 3.2 mM (see Materials and Methods), and neither nitrofurazone, nor erythromycin estolate decreased the growth rate of this strain in LB at the concentrations used in this study (**Fig. S3**), as expected for antivirulence drugs.

The PqsE-Rep biosensor used to select nitrofurazone and erythromycin estolate is an engineered strain in which *pqsE* expression is driven by the heterologous P*tac* rather than by the native P*pqsA* promoter. To validate the PqsE-dependent regulatory network as a target for nitrofurazone and erythromycin estolate, the effect of these drugs on P*pqsA* activity was evaluated by means of a P*pqsA*::*luxCDABE* transcriptional fusion in wild type *P. aeruginosa* PAO1 and in its isogenic Δ*pqsE* mutant [35,63]. As shown in **Fig. 3**, nitrofurazone and erythromycin estolate increased P*pqsA* activity in *P. aeruginosa* PAO1 of 172.1% and 280.1% relative to the solvent vehicle control samples, respectively. This effect was abolished in the Δ*pqsE* mutant and restored by *in trans* expression of the *pqsE* gene. Control experiments performed in the same genetic backgrounds carrying chromosomal integration of the empty vector mini-CTX-*lux*, in which light emission does not rely on P*pqsA* activity or PqsE functionality, revealed that nitrofurazone and erythromycin estolate do not affect constitutive bioluminescence (**Fig. S4**).

Notably, nitrofurazone and erythromycin estolate strongly reduced PqsE-dependent virulence traits in wild type *P. aeruginosa* PAO1, including pyocyanin and rhamnolipids production, and swarming motility, thus mimicking *pqsE* deletion (**Fig. 4**) [34-36,64]. Conversely, none of these drugs affected the PqsE-independent surface motilities twitching and swimming (data not shown) [35].

304 Overall, these results support the PqsE-dependent regulatory pathway as a target for nitrofurazone
305 and erythromycin estolate.
306
307 **3.3 Nitrofurazone and erythromycin estolate inhibit biofilm formation**
308 The effect of PqsE inactivation on *P. aeruginosa* biofilm formation was tested by confocal
309 microscopy analysis, using wild type and its isogenic Δ*pqsE* mutant constitutively expressing GFP.
310 In line with literature data [35], *pqsE* mutation reduced the ability of PAO1 to form biofilm (**Fig. 5**).
311 As expected for PqsE inhibitors, nitrofurazone and erythromycin estolate were able to decrease
312 biofilm formation in wild type *P. aeruginosa* PAO1 (**Fig. 5**) without affecting cell density of the
313 planktonic phase (data not shown). These results suggest that the ability of nitrofurazone and
314 erythromycin estolate to decrease biofilm formation could rely on their anti-PqsE activity, and likely
315 on the consequent reduction in pyocyanin and rhamnolipids levels.
316
317 **3.4. Effect of nitrofurazone and erythromycin estolate in combination with antibiotics**
318 The transfer of a new anti-*Pseudomonas* drug to the clinical practice requires the assessment of its
319 possible interaction with existing therapies. Moreover, antivirulence drugs targeting PqsR have been
320 shown to potentiate the effect of antibiotics commonly used to treat *P. aeruginosa* infection [45,47].
321 In a preliminary analysis, the effect of *pqsE* deletion on *P. aeruginosa* MICs for tobramycin,
322 ciprofloxacin, piperacillin and colistin was determined using both the standard rich medium Mueller
323 Hinton Broth (MHB), and the M9 glucose minimal medium (M9-Glu), that we used for the biofilm
324 assay. Results showed that *pqsE* deletion does not affect the MIC for ciprofloxacin, piperacillin and
325 colistin, irrespective of the growth medium (**Table S3**). Interestingly, the MIC of tobramycin for the
326 Δ*pqsE* mutant was 0.25 μg/mL, 2-fold lower with respect to the MIC for wild type PAO1 (*i.e.*, 0.5
327 μg/mL) in M9-Glu-grown cultures. Conversely, tobramycin MICs for both strains were identical in
328 MHB-grown cultures (**Table S3**). This supported the hypothesis that PqsE-inhibitors could increase
329 the susceptibility of *P. aeruginosa* PAO1 to tobramycin in M9-Glu medium. Intriguingly, the
330 tobramycin MIC for wild type PAO1 (*i.e.,* 0.5 μg/mL) was reduced to 0.125 μg/mL in the presence
331 of nitrofurazone, while it was unaffected by erythromycin estolate. Hence, nitrofurazone potentiates
332 the activity of tobramycin against *P. aeruginosa* planktonic cultures grown in M9-Glu medium.
333 A previous study showed that drugs targeting PqsR can restrict the formation of *P. aeruginosa*
334 antibiotic tolerant cells [45,47], hence we questioned whether PqsE inhibitors could disclose this
335 same feature. Preliminary experiments carried out with tobramycin, ciprofloxacin, piperacillin and
336 colistin showed the existence of a detectable fraction of antibiotic tolerant cells that remained

337 constant 16 h and 24 h post-treatment only in *P. aeruginosa* PAO1 cultures treated with 4 µg/mL
338 tobramycin (8 x MIC; data not shown). The fraction of tobramycin tolerant cells was not affected by
339 deletion of *pqsE* or by nitrofurazone treatment (**Fig. S5**). Surprisingly, erythromycin estolate
340 increased of about 2-logs the amount of wild type PAO1 cells tolerant to tobramycin relative to the
341 untreated control, likely *via* a PqsE-independent pathway (**Fig. S5**).
342
343 **3.4 Effect of nitrofurazone and erythromycin estolate on *P. aeruginosa* CF isolates**
344 Pyocyanin is a *P. aeruginosa* virulence factor important in CF lung infection [65], and its
345 biosynthesis strongly relies on the PqsE-dependent regulatory pathway [34-36,64]. Hence the ability
346 of the PqsE-inhibitors nitrofurazone and erythromycin estolate to reduce pyocyanin production was
347 preliminarily evaluated in a collection of 21 *P. aeruginosa* strains isolated from the lungs of CF
348 patients. The CF isolates are all AQs and pyocyanin producers and can be evenly distributed in three
349 categories with respect to the years of infection (*i.e.,* first isolation, early chronic or late chronic;
350 **Table 2**), or with respect to their antibiotic susceptibility profile (*i.e.,* sensible, resistant or multidrug
351 resistant; **Table 2**).
352 The 21 CF strains were grown in LB in the absence or in the presence of nitrofurazone or
353 erythromycin estolate, and the pyocyanin concentration was determined in the corresponding spent
354 media. Both nitrofurazone and erythromycin estolate decreased pyocyanin production of the CF
355 strains to variable extents (**Table 2 and Fig. 6A**), while the treatments did not affect bacterial growth
356 (data not shown). Overall erythromycin estolate showed a higher pyocyanin inhibitory activity
357 compared to nitrofurazone. Indeed, the residual levels of pyocyanin produced by CF isolates treated
358 with nitrofurazone were higher than those measured for erythromycin estolate-treated cultures,
359 except for 5 CF isolates out of 21 (*i.e.,* BG29, BG69, BG71, BG83 and BG94; **Table 2**). The higher
360 inhibitory activity exerted by erythromycin estolate on pyocyanin production compared to
361 nitrofurazone is also evident in the empirical distribution plot shown in **Fig. 6B**. In detail, this
362 analysis highlighted that 11 out of 21 strains (*cumulative strain fraction* = 0.524) showed a residual
363 pyocyanin production $\leq$ 13.2% and $\leq$ 61.2% when treated with erythromycin estolate and
364 nitrofurazone, respectively ($p < 0.01$; **Fig. 6B**).
365 An analysis carried out by clustering the CF isolates according to the years of infection showed
366 that no significant correlation was found between years of infection and susceptibility to
367 nitrofurazone (**Table 2 and Fig. 6C**). Conversely, CF first isolates and CF strains isolated from
368 patients with early chronic infection (from 2 to 3 years) were significantly more susceptible to
369 erythromycin estolate than the CF strains isolated from patients with late chronic infection (more

than 5 years; **Table 2 and Fig. 6C**). Indeed, 5 out of 7 strains isolated for the first time from a patient produced pyocyanin residual levels ≤ 11.1%, with the remaining 2 strains behaving as out-groups (*i.e.,* BG69 and BG71). The 6 strains isolated from patients with early chronic infection showed a similar behaviour, producing pyocyanin residual levels ≤ 13.2%. Conversely, the residual level of pyocyanin was ≤ 51.2% only in 4 out of 8 CF isolates from patients with late chronic infection (**Table 2 and Fig. 6C**).

Concerning the relationship between antibiotic resistance pattern and susceptibility to nitrofurazone or erythromycin estolate, MDR strains showed an overall lower susceptibility to both antivirulence drugs, with respect to strains susceptible to all antibiotics (S) or resistant to only one antibiotic class (R; **Table 2 and Fig. S6**). However, this observation was not supported by the statistical analysis, likely due to the limited number of samples.

4. Discussion

P. aeruginosa is considered a model organism for QS and quorum quenching studies, mainly due to the key role played by its complex QS circuitry in pathogenicity. Indeed, the ability of this bacterium to cause infection and to adapt to the host environment relies on the fine-tuning of multiple virulence genes controlled by three major QS systems, *las, rhl* and *pqs*, whose expression and activity is strictly interwoven [22,66]. Recently, an additional QS system based on oxylipins as signal molecules and possibly required for full virulence has been identified in *P. aeruginosa*, increasing the complexity of its QS circuitry [67].

The *pqs* QS system controls the expression of multiple virulence factors and biofilm formation. *P. aeruginosa* mutants defective in the *pqs* QS system display attenuated pathogenicity in different plant and animal models of infection [34,35,39,40,45,68-74]. Although the central role played by the *pqs* system in *P. aeruginosa* pathobiology has been extensively studied, a clear characterization of the molecular mechanism of action by which its individual elements control gene expression is still unclear. DNA-protein interaction studies showed that the PqsR/AQ complex can bind to different promoter and intergenic regions [75,76]. However, a transcriptomic analysis carried out in a genetic background in which PQS production or PqsE expression were independent from PqsR/AQ activity revealed that the major effectors of the *pqs* system are PQS and PqsE, rather than the PqsR/AQ complex [33]. Indeed, in the absence of PqsR, PQS and PqsE control the expression of 179 and 145 genes, respectively, with an overlap of only 30 genes among the two regulons. Hence the main role played by the PqsR/AQ complex is to trigger the transcription of the *pqsABCDE-phnAB* operon, thus increasing the synthesis of PQS and the expression PqsE [33].

The main mechanisms underlying PQS effect on gene expression could be ascribed to its ability to interact with membranes and to chelate iron [33,77-80]. Despite the crystallographic structure of PqsE was determined ten years ago [80], the mechanism of action of this protein remains elusive. Briefly, PqsE can contribute to AQs biosynthesis *via* its thioesterase activity, but this function can be replaced by other thioesterases in a *pqsE*-depleted genetic background [32]. Moreover, molecules specifically targeting the PqsE thioesterase catalytic domain do not affect the expression of PqsE-dependent virulence factors, suggesting that PqsE is a multifunctional protein [38]. This hypothesis is supported by recent studies showing that PqsE is essential for the production of a molecule able to activate the LuxR-like receptor RhlR, encoded by the *rhlR* gene, in alternative to its cognate signal molecule *N*-butyryl-homoserine lactone (C_4-HSL). Genetic data suggest that the complex between RhlR and this alternative ligand could trigger the expression of PqsE-dependent genes [39]. Notably, experiments carried out with a murine model of lung infection confirmed the central role played by PqsE in virulence. Indeed, a *P. aeruginosa* mutant unable to produce C_4-HSL was as virulent as the wild type isogenic strain, while *pqsE* and *rhlR* mutant strains showed complete loss of virulence and attenuated virulence, respectively [39]. Finally, a recent work showed that a mutated variant of RhlR active in the absence of any ligand could only partially restore pyocyanin production in a *pqsE* mutant, also depending on the environmental conditions [40]. This study, together with the previous observation that the *pqsE* mutant was less virulent than the *rhlR* mutant *in vivo*, supports the hypothesis that PqsE could have multiple mechanisms of action besides being involved in AQs synthesis and being required for the production of the RhlR alternative ligand. Finally, it remains unclear whether PqsE is directly responsible for the production of the RhlR alternative ligand, or if intermediate factor(s) are involved in the PqsE-dependent activation of virulence genes.

Overall, the current knowledge highlights the key role of PqsE as a hub in the complex regulatory QS circuitry required for full *P. aeruginosa* virulence, and supports PqsE as a target for antivirulence drugs.

Even if the actual mechanism of action of PqsE and the downstream regulative network underlying the expression of virulence genes remain largely unknown, the screening system described in this study was effective in identifying molecules specifically hampering the expression of PqsE-dependent virulence traits. Indeed, both nitrofurazone and erythromycin estolate were able to negatively affect the expression of phenotypes strictly related to PqsE activity, including pyocyanin and rhamnolipids biosynthesis, swarming motility and biofilm formation. Moreover, nitrofurazone and erythromycin estolate alleviated the PqsE-dependent repression of P*pqsA* in *P. aeruginosa* wild type. This activity was abrogated in a *pqsE*-deleted mutant and could be restored by genetic

436 complementation. Overall, these results support the PqsE regulatory pathway as a target of both
437 nitrofurazone and erythromycin estolate, thus validating our screening system.
438 Since the molecular mechanism of action of PqsE is not fully understood, it is not easy to
439 speculate about the mechanism of action of nitrofurazone and erythromycin estolate as PqsE
440 inhibitors. Nitrofuran drugs were extensively used in livestock production till 1995, when this
441 application was forbidden in Europe and other countries due to concerns about the toxicity of their
442 residues in edible tissues. However, nitrofurans are currently used worldwide for the treatment of
443 bacterial and protozoal infections in humans [82,83]. As an antibacterial, nitrofurazone is mainly
444 used for topic application on wounds and catheters infected by both Gram-positive and Gram-
445 negative pathogens. The nitrofurantoin mechanism of action is one of the best characterized among
446 nitrofurans and the main process targeted by this antibiotic is mRNA translation, through oxidative
447 damage to the ribosome [84,85]. Interestingly, a nitrofuran antibiotic named nifuroxazide was
448 previously found to have anti-QS and antivirulence properties against *P. aeruginosa* [86]. Authors
449 noticed that the effect of nifuroxazide was stronger against *rhl*- and *pqs*- than against *las*-controlled
450 virulence factors [86]. This observation, together with recent findings highlighting the strong
451 connection between the *rhl* and *pqs* QS systems [39,40], supports the hypothesis that the main
452 antivirulence activity of nitrofurans could be dependent on their ability to hamper PqsE-controlled
453 regulatory pathway(s).
454 Erythromycin is a macrolide antibiotic inhibiting bacterial growth by binding the 23S rRNA in the
455 50S subunit of the bacterial ribosome, thereby preventing the transfer of tRNA from the ribosome.
456 Erythromycin estolate is almost fallen into disuse, replaced by less toxic macrolides [62]. It is
457 important to highlight that macrolide antibiotics used at sub-MIC concentrations can inhibit *P.*
458 *aeruginosa* virulence and biofilm formation [87]. The most striking example is azithromycin, which
459 is used as adjuvant in the therapy of *P. aeruginosa* chronic lung infections, despite its lack of
460 antibiotic activity toward *P. aeruginosa* [87]. The antivirulence activity of azithromycin is still not
461 fully understood, also because it is accompanied by other biological activities, including the anti-
462 inflammatory one. However, evidences have been provided that azithromycin may selectively affect
463 the translation of distinct subsets of *P. aeruginosa* genes depending on their codon usage, including
464 the *rhlR* gene [88]. Moreover, it is worth to notice that the virulence phenotypes most strictly
465 dependent on PqsE functionality, including pyocyanin production, swarming motility and biofilm
466 formation, are strongly affected by both azithromycin [87,88] and erythromycin estolate.
467 Overall, both nitrofuran and macrolides antibiotic activity is related to ribosome inhibition,
468 leading to speculate that at low (sub-MIC) concentrations, these drugs could selectively affect the

469 expression of PqsE-dependent genes at the translational level. In this view, it is worth to cite previous
470 works inferring the presence of a ssDNA- or RNA-binding domain in PqsE [37,81], even if
471 conclusive proofs of the involvement of this domain in PqsE functionality are missing.
472 The transfer of a new antivirulence drug to the clinical use will require the assessment of its
473 possible interaction with existing treatments. Interestingly, neither nitrofurazone, nor erythromycin
474 estolate had antagonistic effects toward antibiotics commonly used to treat *P. aeruginosa* infections.
475 Moreover, *pqsE* deletion halved *P. aeruginosa* MIC values for tobramycin in M9-Glu, while this
476 effect was absent in MHB. This indicates that PqsE could positively contribute to *P. aeruginosa*
477 resistance to tobramycin depending on the growth medium, in agreement with studies showing that
478 PqsE-dependent virulence genes could be differentially regulated in diverse cultural conditions
479 [39,40]. Among the antibiotic tested, *pqsE* deletion affected only the susceptibility of *P. aeruginosa*
480 to tobramycin, indicating that PqsE could be required for the expression of factors contributing to
481 resistance to aminoglicosides. It is also interesting to highlight that, among the antibiotic tested, only
482 tobramycin targets the ribosome, supporting the existence of a link between PqsE activity and
483 ribosome activity. Surprisingly, only nitrofurazone disclosed a tobramycin potentiating activity. It is
484 not easy to explain why erythromycin estolate did not show this activity, also considering that this
485 drug showed an overall higher activity than nitrofurazone on other PqsE-dependent phenotypes. One
486 plausible explanation is that, besides PqsE, erythromycin estolate could hit multiple targets, with a
487 resulting balancing effect on tobramycin susceptibility.
488 Antibiotic tolerance is the capacity of bacterial sub-populations to tolerate exposure to lethal
489 concentrations of bactericidal antibiotics and relies upon mechanisms different from antibiotic
490 resistance [89]. Previous studies showed that PqsR inhibitors could affect *P. aeruginosa* antibiotic
491 tolerance [45]. However, this effect seems to be PqsE-independent, since in our hands antibiotic
492 tolerance was not affected by *pqsE* deletion. Nonetheless, erythromycin estolate showed a PqsE-
493 independent positive effect on tolerance to tobramycin. This result further supports the hypothesis
494 that erythromycin estolate could hit molecular targets other than PqsE.
495 To the best of our knowledge this is the first report demonstrating the contribution of PqsE-
496 dependent regulation to antibiotic resistance and providing a proof of concept that a PqsE inhibitor,
497 in addition to its antivirulence activity, can potentiate antibiotic effect. On the other hand, in line with
498 literature data [45], this study highlights that distinct anti-QS drugs targeting the same molecular
499 pathway could differentially alter antibiotic resistance and tolerance, an issue worth to be taken into
500 consideration for the development of antivirulence drugs.

As mentioned in the Introduction section, the use of QS inhibitors for the treatment of CF patients chronically infected with *P. aeruginosa* is under debate, mainly due to the high genotypic and phenotypic variability generated by within-host evolution of the infecting population. Indeed, during years of chronic infection in the lung, clonal variants of the initial population are positively selected by the peculiar CF lung environment and continuative drug administration. *P. aeruginosa* phenotypic variants with high levels of biofilm formation, high resistance to antibiotics and decreased production of secreted virulence factors, often associated to mutations in the *las* QS systems, are frequently isolated from the CF lung [23-26]. This implies that the range of activity of a novel antivirulence drug developed against *P. aeruginosa* should be determined in a broad collection of CF isolates to support its use in CF therapy. The experiments carried out in the final part of this study were not aimed at fully addressing this issue, but rather at preliminarily comparing nitrofurazone and erythromycin estolate activity in a small number of CF strains proficient in AQ and pyocyanin production, hence potentially susceptible to PqsE inhibitors. Despite further studies with a higher number of strains should be carried out before driving robust conclusions about the feasibility of anti-PqsE drugs in CF therapy, our preliminary investigation indicates that both nitrofurazone and erythromycin estolate significantly decrease pyocyanin production in the majority of the tested CF isolates, and that erythromycin estolate displays a higher range of activity compared to nitrofurazone.

In the last years, our group proved the feasibility of the drug-repurposing approach based on wet-lab or virtual screenings for the identification of FDA-approved drugs targeting *P. aeruginosa* virulence [48,51,90,91]. This additional study provides the first example of target-oriented screening aimed at identifying FDA-approved inhibitors of PqsE-dependent virulence factors. The recovery of two hits with strong anti-PqsE activity in a library of 1,600 compounds validated this system, which is suitable for future high-throughput screening of larger compound libraries. The PqsE-inhibitors here identified, nitrofurazone and erythromycin estolate, are antibiotics active against different bacterial species, though unable to inhibit *P. aeruginosa* growth. Notably, also clofoctol, an antibiotic active against Gram-positive bacteria, has strong anti-PqsR and antivirulence activity at concentrations unable to affect *P. aeruginosa* growth [48]. These observations support the hypothesis that antibiotics and other metabolites secreted at low concentration by many microorganisms in a polymicrobial natural environment might play a role as signalling molecules, rather than as inhibitors of competitors' growth [92,93].

As antibiotics, nitrofurazone and erythromycin estolate can be predicted to have a significant impact on the host microbiota. Moreover, the intrinsic toxicity of both erythromycin estolate and nitrofurazone for eukaryotic cells could be a limitation for the repurposing of these drugs to treat *P.*

534 *aeruginosa* infections. These results confirm some of the limitations of repurposing "old" drugs for
535 antivirulence therapies, as previously reviewed by our group [9]. However, it should be taken into
536 account that also a high proportion of non-antibiotic drugs has recently been associated with changes
537 in gut microbiome composition [94], and that the erythromycin analogue azithromycin is already
538 used as antivirulence drug, even if on an empirical basis [87]. In fact, the emergence of MDR and
539 extensively drug-resistant bacteria is leading to the rediscovery and/or optimization of fallen into
540 disuse drugs, in accordance to the SOSA approach [95]. As an example, nitrofuran drugs with low
541 toxicity against eukaryotic cells and high activity against *Mycobacterium tuberculosis* are under
542 development [96].
543 Finally, beyond their possible applications to the therapy, a main result of this study is the
544 identification of two compounds specifically targeting the expression of PqsE-dependent genes,
545 which could provide useful tools for future studies aimed at unravelling the mechanism of action of
546 PqsE.
547

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557 **Conflict of Interest**
558 The authors declare that the research was conducted in the absence of any commercial or financial
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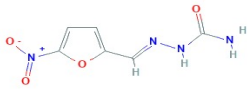
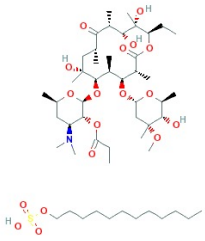
801 **Table 1.** PqsE inhibitors identified by screening the PHARMAKON library of FDA-approved drugs.

Drug name	Property	Structure	EC_{50}[a]	IC_{50}[a]
nitrofurazone	antibacterial		78.58	24.65
erythromycin estolate	antibacterial		6.15	5.79

802 [a] The EC_{50} and IC_{50} values (μM) are relative to the ability of the drugs to enhance P*pqsA::lux* activity and to inhibit
803 pyocyanin production, respectively, determined by using the PqsE-Rep reporter strain.

804 **Table 2.** Effect of PqsE inhibitors on *P. aeruginosa* CF isolates

Isolate name	Years of colonization[a]	Antibiotics susceptibility[b]	Pyocyanin residual production %	
			nitrofurazone [c]	erythromycin estolate[d]
BG6	late chronic	R	34.8	18.1
BG13	first isolation	MDR	80.5	11.1
BG14	early chronic	MDR	26.9	3.4
BG15	late chronic	MDR	61.2	39.1
BG29	late chronic	R	68.9	99.9
BG30	first isolation	S	50.7	4.9
BG34	early chronic	R	19.2	5.9
BG37	first isolation	R	48.8	1.4
BG38	early chronic	R	47.9	1.2
BG42	early chronic	R	79.2	9.3
BG45	first isolation	S	95.0	9.3
BG46	early chronic	S	70.8	1.4
BG50	first isolation	S	91.6	2.1
BG61	early chronic	S	22.9	13.2
BG69	first isolation	S	43.2	99.2
BG71	first isolation	S	73.4	99.0
BG83	late chronic	R	35.1	68.5
BG89	late chronic	MDR	95.0	51.2
BG92	late chronic	MDR	99.7	75.0
BG93	late chronic	MDR	98.4	40.6
BG94	late chronic	MDR	54.7	63.4

805
806 [a] Different categories depending on the years of infection of the clinical isolates in the lung of individuals with cystic
807 fibrosis: first isolate, early chronic (from 2 to 3 years); late chronic (more than 5 years).
808 [b] Criteria to define multi-drug resistant (MDR) bacteria according to the European Centre for Diseases Prevention and
809 Control (ECDC) web site (http://ecdc.europa.eu/en/Pages/home.aspx).
810 [c] Pyocyanin residual level in samples treated with 100 μM nitrofurazone expressed in % relative to solvent vehicle control
811 samples [0.125% (v/v) DMSO], considered as 100%.
812 [d] Pyocyanin residual level in samples treated with 50 μM erythromycin estolate expressed in % relative to solvent vehicle
813 control samples [0.025% (v/v) EtOH], considered as 100%.
814

Figure 1

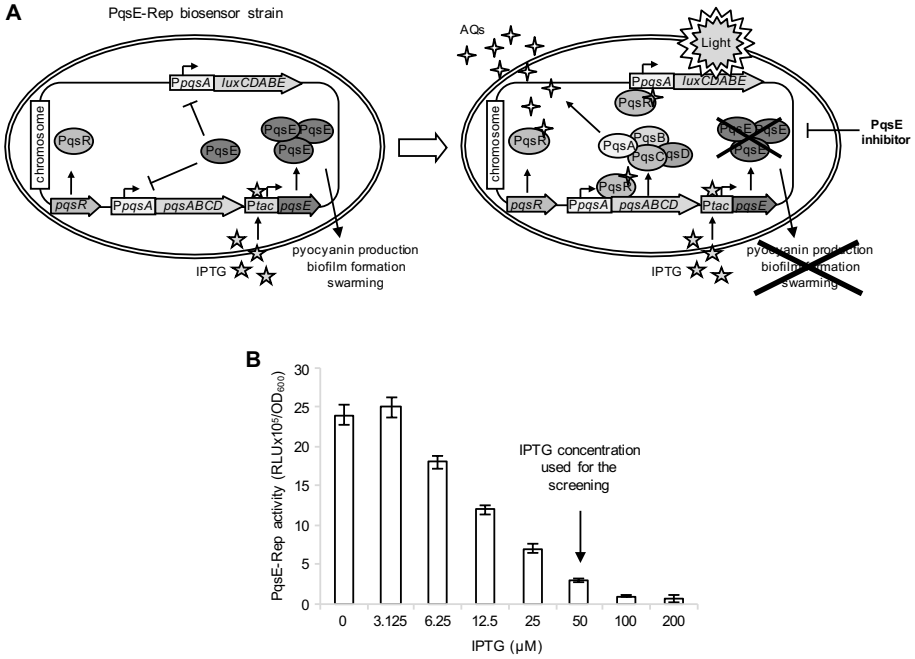


Fig. 1. Screening system developed for the identification of PqsE inhibitors

(A) Schematic representation of the PqsE-Rep-based reporter system. The PqsE-Rep strain contains the P*pqsA*::*lux* transcriptional fusion and a genetic cassette for IPTG-inducible expression of the *pqsE* gene. Since in *P. aeruginosa* PqsE represses P*pqsA* promoter activity, the PqsE-Rep biosensor emits light at basal level when grown in LB supplemented with IPTG; as a consequence, molecules affecting PqsE functionality are expected to increase light emission by P*pqsA* derepression. **(B)** Activity of the P*pqsA* promoter in the PqsE-Rep strain grown in LB supplemented with the indicated concentrations of IPTG. PqsE-Rep was inoculated at an OD_{600} of 0.08 in 0.2 mL of LB in 96-well microtiter plates and light emission was measured after 5 h of incubation at 37°C in shaking conditions. The average of three independent experiments is reported with SD.

Figure 2

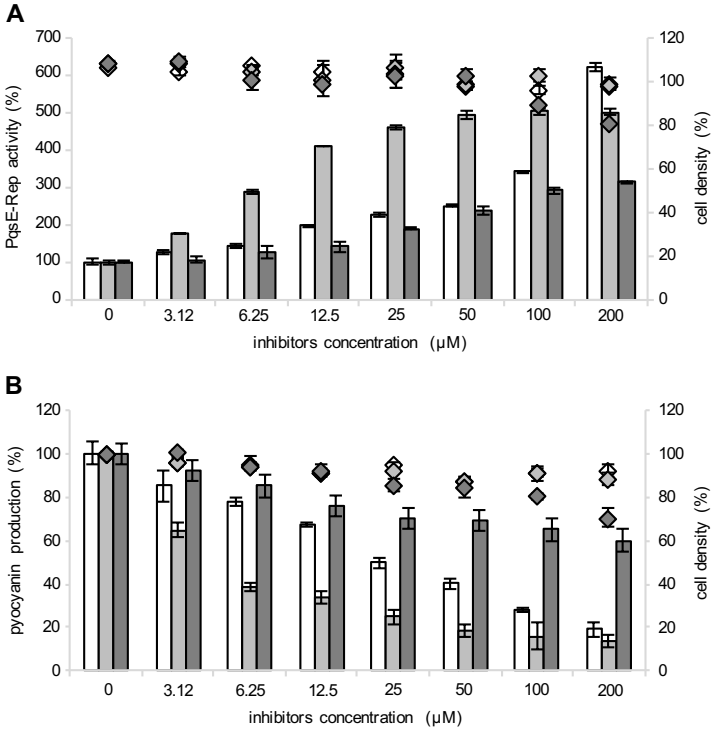
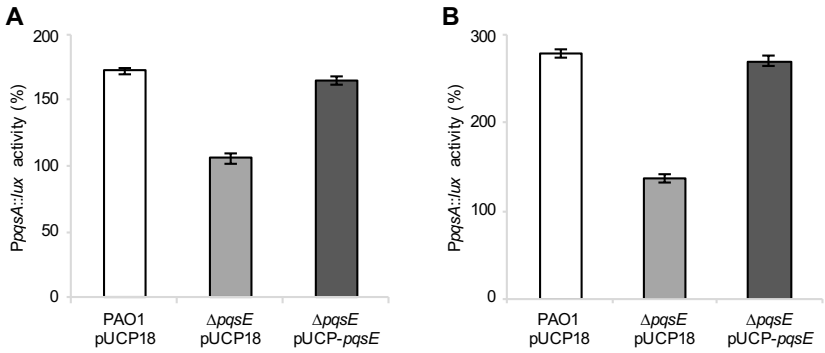


Figure 2. Selected hits increase P*pqsA* activity and reduce pyocyanin production

Effect of nitrofurazone (white bars), erythromycin estolate (light-grey bars) and diminazene aceturate (dark-grey bars) on PqsE-Rep bioluminescence emission **(A)** and pyocyanin production **(B)**. Solvent vehicle control samples were considered as 100%. The average of at least three independent experiments is reported with SD.

838 **Figure 3**

839



840

841

842 **Figure 3. Nitrofurazone and erythromycin estolate increase P*pqsA* activity only in a *pqsE*-**
843 **proficient background**

844 Effect of 100 μM nitrofurazone **(A)** or 50 μM erythromycin estolate **(B)** on P*pqsA* promoter activity
845 in the indicated strains. Promoter activity is reported as percentage with respect to the corresponding
846 solvent vehicle control sample, considered as 100%. The average of three independent experiments is
847 reported with SD.

Figure 4

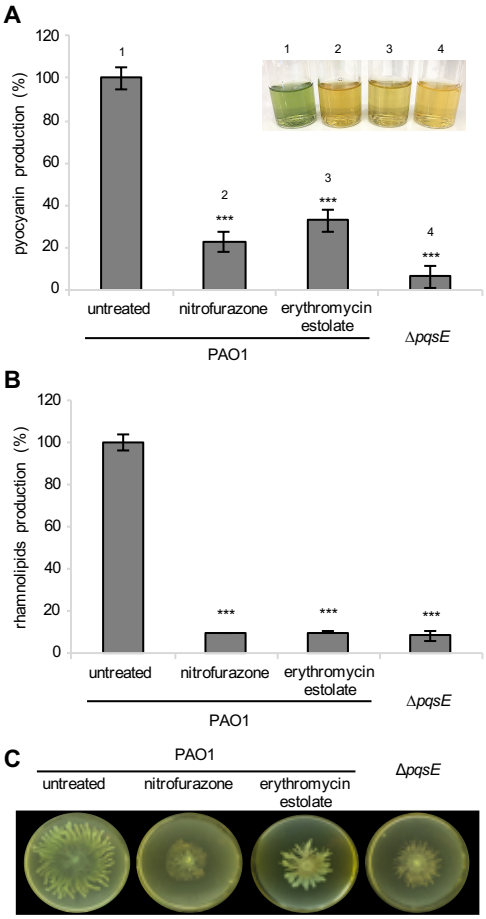


Figure 4. Nitrofurazone and erythromycin estolate inhibit the expression of PqsE-controlled virulence traits

Effect of 100 µM nitrofurazone or 50 µM erythromycin estolate on pyocyanin **(A)** and rhamnolipids **(B)** production, and on swarming motility **(C)** in *P. aeruginosa* PAO1. The isogenic Δ*pqsE* mutant was used as a control. For pyocyanin and rhamonolipids production, the average of three independent experiments is reported with SD; representative supernatants are shown in the inset picture in **(A)**. ***, $p < 0.001$ (ANOVA). For swarming motility, one representative picture of three independent experiments is shown.

Figure 5

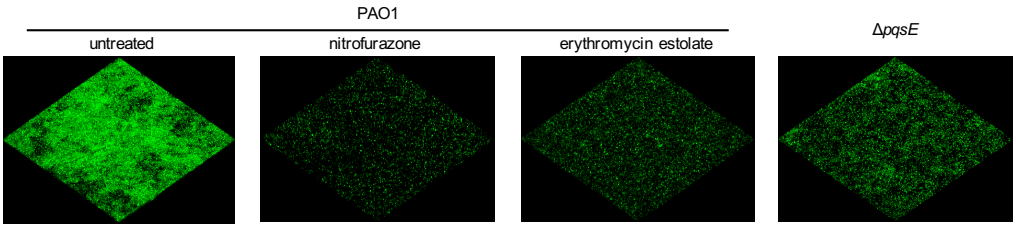
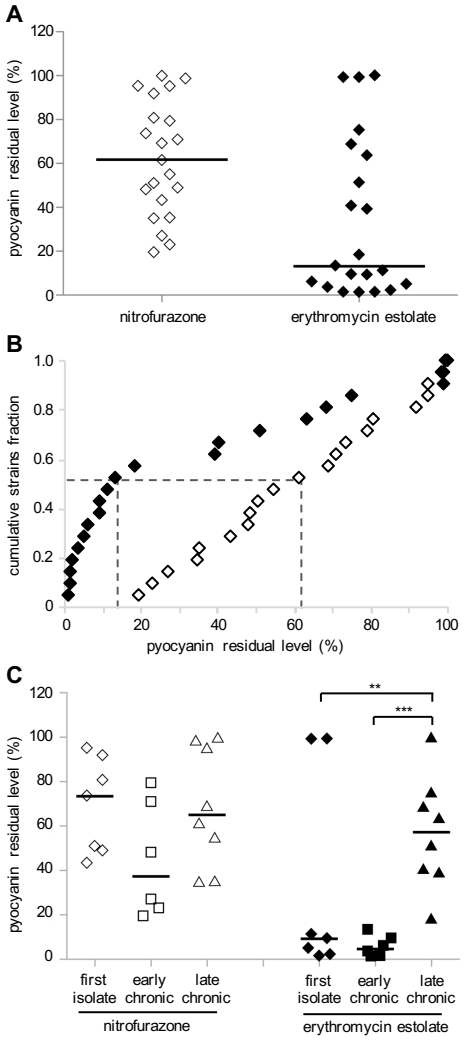


Figure 5. Nitrofurazone and erythromycin estolate decrease biofilm formation

Confocal microscope imaging of biofilms produced by the indicated *P. aeruginosa* strains constitutively expressing GFP, in M9-Glu after 72 h of incubation at 30°C. Where indicated, the PAO1 wild type strain was treated with 100 µM nitrofurazone or 50 µM erythromycin estolate. Representative pictures of three independent experiments are shown.

869 **Figure 6**

870



871

872

873 **Figure 6**. **Nitrofurazone and erythromycin estolate are active against *P. aeruginosa* CF isolates**
874 **(A)** Residual pyocyanin production in CF isolates grown in the presence of 100 μM nitrofurazone
875 (white diamonds) or 50 μM erythromycin estolate (black diamonds) relative to solvent vehicle
876 control samples, considered as 100%. Black lines represent the median values. The average of three
877 independent experiments is reported. **(B)** Empirical cumulative distribution plots based on the data in

878 **(A)**. Differences between the distribution plots of nitrofurazone (white diamonds) and erythromycin
879 estolate (black diamonds) are statistically significant ($p < 0.001$; ANOVA). Dashed lines indicate the
880 residual pyocyanin production in 11 strains out of 21 (*cumulative strains fraction* = 0.524): ≤ 13.2%
881 for erythromycin estolate and ≤ 61.2% for nitrofurazone. **(C)** Data from **(A)** clustered on the basis of
882 the years of infection: diamonds, CF strains isolated for the first time from patients; squares, CF
883 strains isolated from patients with chronic infection from 2 to 3 years; triangles, CF strains isolated
884 from patients with chronic infection for more than 5 years. ** $p < 0.05$, *** $p < 0.001$ (ANOVA).

Identification of FDA-approved antivirulence drugs targeting the *Pseudomonas aeruginosa* quorum sensing effector protein PqsE

Valerio Baldelli[1], Francesca D'Angelo[1#], Viola Pavoncello[1#], Ersilia Vita Fiscarelli[2], Paolo Visca[1], Giordano Rampioni[1*], Livia Leoni[1*]

[1] Department of Science, University Roma Tre, Rome, Italy; [2] Laboratory of Cystic Fibrosis Microbiology, Bambino Gesú Hospital, Rome, Italy.

[#] Current address: Institut Pasteur, Paris, France.

SUPPLEMENTAL MATERIAL

Table S1. Strains used in this study

Strain	Description	References
PAO1	wild type strain.	ATCC15692
PAO1 Δ*pqsE*	PAO1 derivative carrying an in-frame deletion of the *pqsE* gene.	[35]
PAO1 PqsE-Rep (*pqsE*^{IND} P*pqsA*::*lux*)	PAO1 derivative in which *pqsE* expression is IPTG inducible and containing the P*pqsA*::*luxCDABE* transcriptional fusion integrated into the chromosome at the *attB* neutral site; Tc^{R}.	[35]
PAO1 P*pqsA*::*lux*	PAO1 derivative containing the P*pqsA*::*luxCDABE* transcriptional fusion integrated into the chromosome at the *attB* neutral site; Tc^{R}.	[97]
PAO1 Δ*pqsE* P*pqsA*::*lux*	PAO1 Δ*pqsE* derivative containing the P*pqsA*::*luxCDABE* transcriptional fusion integrated into the chromosome at the *attB* neutral site; Tc^{R}.	[35]
PAO1 mini-CTX-*lux*	PAO1 derivative containing the mini-CTX-*lux* empty vector integrated into the chromosome at the *attB* neutral site; Tc^{R}.	[97]
PAO1 Δ*pqsE* mini-CTX-*lux*	PAO1 Δ*pqsE* derivative containing the mini-CTX-*lux* empty vector integrated into the chromosome at the *attB* neutral site; Tc^{R}.	[35]

Table S2. Plasmids used in this study

Plasmid	Relevant characteristics	References
pUCP18	pUC18-derivative containing a stabilising fragment for maintenance in *Pseudomonas* spp.; Ap^R, *E. coli*/Cb^R, *P. aeruginosa*.	[63]
pUCP-*pqsE*	pUCP18 derivative for *pqsE* complementation; Ap^R, *E. coli*/Cb^R, *P. aeruginosa*.	[35]
pMRP9-1	pUC18 derivative allowing constitutive expression of the *Aequorea victoria* GFP protein; Cb^R.	[58]
mini-CTX-*lux*	Promoter-probe vector containing the *luxCDABE* operon as reporter system; Tc^R.	[98]
mini-CTX-P*pqsA*::*lux*	mini-CTX-*lux* derivative used for the insertion of the P*pqsA*::*luxCDABE* transcriptional fusion into PAO1 chromosome; Tc^R.	[79]

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Table S3. MIC of selected antibiotics

	Ciprofloxacin		Colistin		Tobramycin		Piperacillin	
Strain	**MHB**	**M9**	**MHB**	**M9**	**M**	**M9**	**MH**	**M9**
P. aeruginosa PAO1	0.125	0.03125	2	4	0.5	0.5	8	2
P. aeruginosa Δ*pqsE*	0.125	0.03125	2	4	0.5	0.25	8	2

Figure S1. Set up of the PqsE-Rep biosensor system

(A) Activity of the P*pqsA* promoter in the PqsE-Rep strain grown in LB supplemented with the indicated concentrations of IPTG, after 3 h (white bars), 5 h (light-grey bars) and 7 h (dark-grey bars) of incubation at 37°C. **(B)** Activity of the P*pqsA* promoter in the PqsE-Rep strain inoculated at starting optical density (OD_{600}) of 0.08 (white bars), 0.03 (light-grey bars) and 0.01 (dark-grey bars), after 5 h of incubation at 37°C in LB supplemented with the indicated concentrations of IPTG. **(C)** Activity of the P*pqsA* promoter in the PqsE-Rep strain inoculated at a starting OD_{600} of 0.08 after 5 h of incubation in LB (white bars) or in LB supplemented with 50 µM IPTG (grey bars) at 30°C or 37°C, in static or shaking (120 rpm) conditions. For **(A)-(C)**, biosensor activity is reported as relative light units (RLU) normalized to cell density (OD_{600}); the average of three independent experiments is reported with SD.

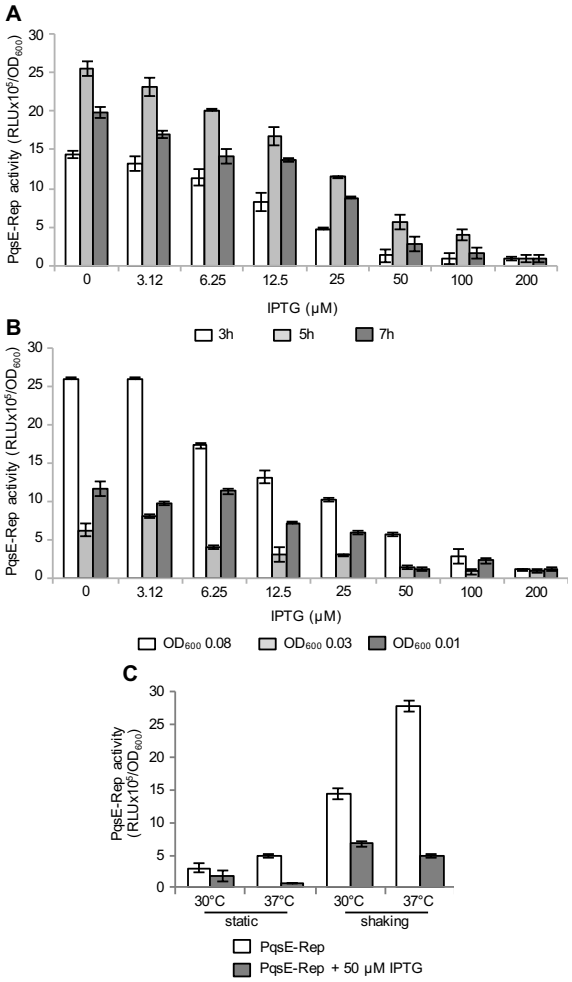

Figure S2. Primary and secondary screens of the PHARMAKON library

(A) Activity of the P*pqsA* promoter (bars) and cell density (diamonds) measured in the PqsE-Rep strain after 5 h incubation at 37°C in shaking conditions in LB supplemented with 50 µM IPTG and with the molecules of the PHARMAKON library, indicated with codes from inhibitor 1 (I-1) to inhibitor 24 (I-24), at 20 µM (white bars and diamonds) or 200 µM (grey bars and diamonds) concentration. PqsE-Rep activity and cell density measured in the presence of 0.2% (v/v) and 2% (v/v) DMSO were considered as 100%, respectively. **(B)** Pyocyanin production measured in supernatants of the PqsE-Rep biosensor strain supplemented with 50 µM IPTG and treated with the PHARMAKON library compounds nitrofurazone (I-2), erythromycin estolate (I-3) and diminazene aceturate (I-8) at 20 µM (white bars) and 200 µM (grey bars) concentration.

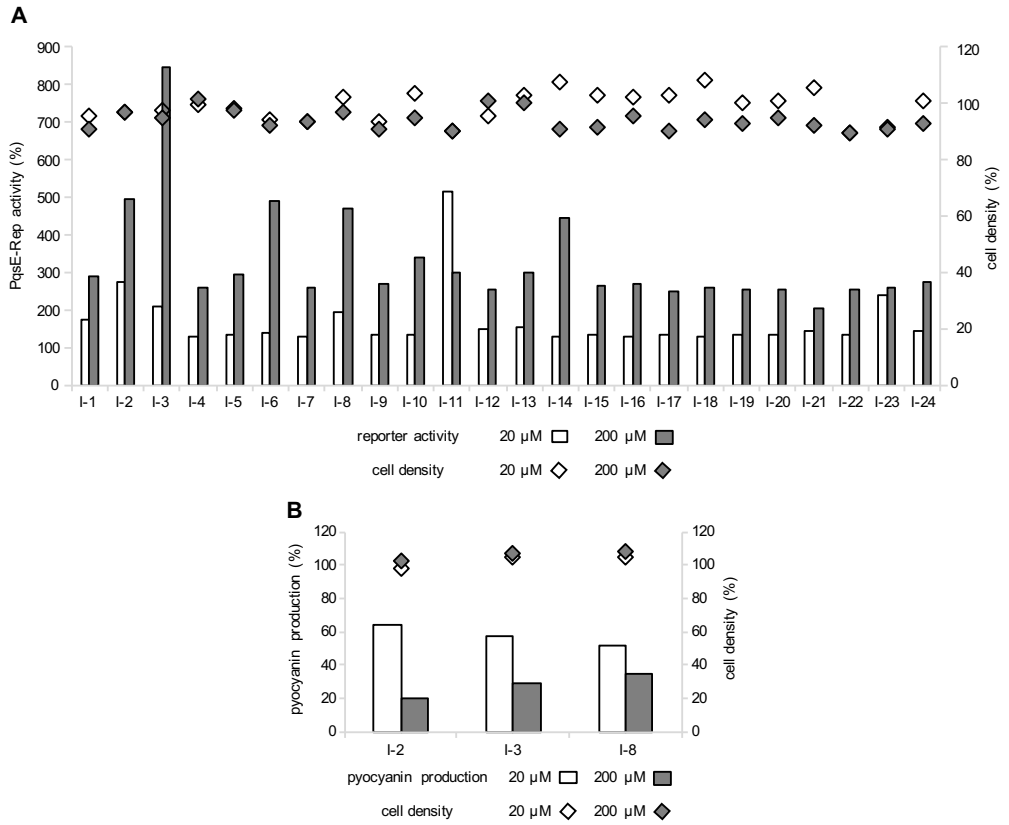

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Figure S3. Growth curves of *P. aeruginosa* in the presence of the PqsE inhibitors

Growth curves of *P. aeruginosa* PAO1 incubated at 37°C in shaking conditions in LB supplemented with: **(A)** 100 µM nitrofurazone (blue), 50 µM nitrofurazone (green), 25 µM nitrofurazone (purple), or 0.125% (v/v) DMSO (red); **(B)** 50 µM erythromycin estolate (blue), 25 µM erythromycin estolate (green), 12.5 µM erythromycin estolate (purple), or 0.025% (v/v) EtOH (red). The average of three independent experiments is reported with SD.

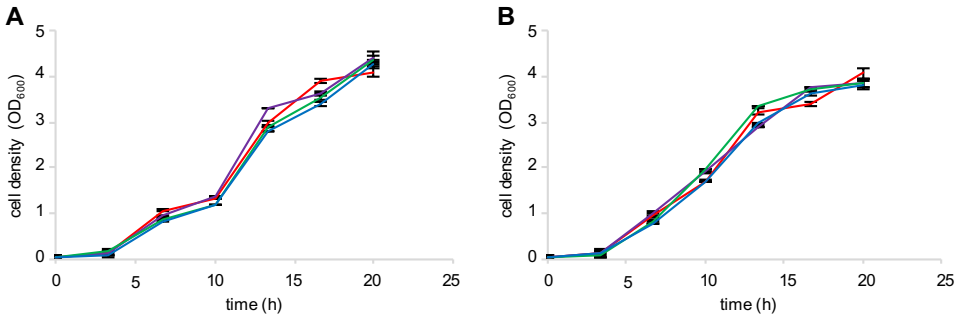


Figure S4. Effect of the PqsE inhibitors on constitutive bioluminescence

Percentage of light emitted by the indicated *P. aeruginosa* PAO1 strains carrying the mini-CTX-*lux* empty vector. The strains were grown at 37°C in shaking conditions in LB supplements with 100 µM nitrofurazone **(A)** or 50 µM erythromycin estolate **(B)**. Bioluminescence emitted by the same strains grown in the presence of 0.125% (v/v) DMSO or 0.025% (v/v) EtOH was considered as 100%. The average of three independent experiments is reported with SD.

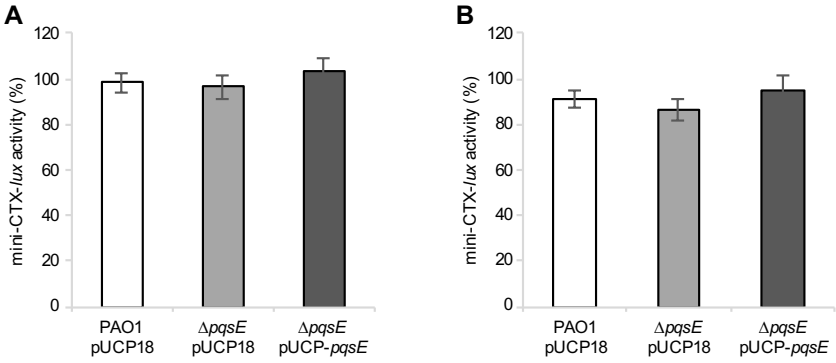


Figure S5. Effect of PqsE inhibitors on *P. aeruginosa* tolerance to tobramycin

Fraction of *P. aeruginosa* PAO1 cells tolerant to 4 µg/mL tobramycin (8x MIC) untreated (white bar) or after the treatment with 100 µM nitrofurazone (light-grey bar) or 50 µM erythromycin estolate (dark-grey bar). The untreated PAO1 Δ*pqsE* strain was used as control (black bar). The tolerant fraction expressed as N-fold change was determined as the ratio between the CFU/mL values measured after antibiotic addition (24 h post-antibiotic) divided by CFU/mL values measured before antibiotic addition. The average of three independent experiments is reported with SD. Similar results were obtained 16 h post-antibiotic treatment.

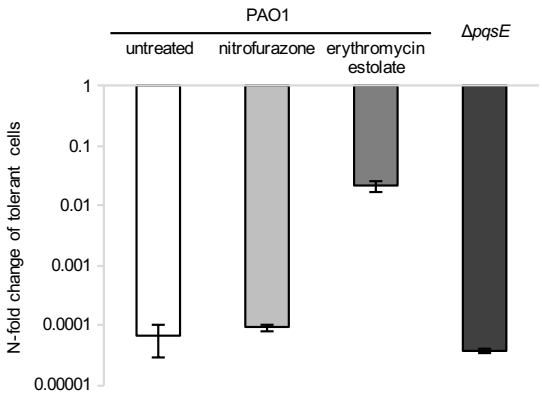
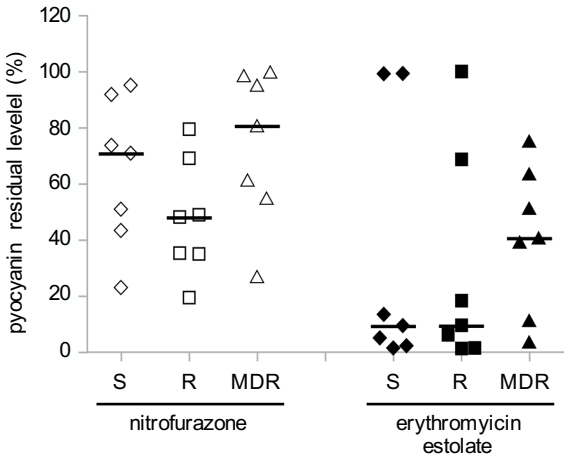


Figure S6. Correlation between susceptibility to PqsE inhibitors and to antibiotics in CF isolates

Residual pyocyanin production in CF isolates grown in the presence of 100 μM nitrofurazone (white symbols) or 50 μM erythromycin estolate (black symbols) relative to solvent vehicle control samples, considered as 100%. Black lines represent the median values. Data were clustered on the basis of the antibiotic resistance pattern: diamonds, CF strains susceptible to all antibiotics (S); squares, CF strains not susceptible to antibiotics belonging to one or two different classes (R); triangles, multi-drug resistant (MDR) CF strains. The average of three independent experiments is reported.



Chapter 6

Concluding remarks

The widespread emergence of antibiotic resistant bacterial pathogens, and the paucity of new antibiotics approved for use in humans, have boosted the research on antivirulence drugs. These molecules inhibit bacterial virulence processes rather than growth, and are expected to pose a lower selective pressure for the emergence of resistance compared to antibiotics (Rasko and Sperandio, 2010; Allen *et al*., 2014).

The Gram-negative bacterium *Pseudomonas aeruginosa* has become a model organism for studies focused on antivirulence approaches. This opportunistic human pathogen is extremely relevant for human health, since it is one of the main causative agents of nosocomial infections, and the primary cause of lung function decline and consequent premature death in cystic fibrosis (CF) patients (Eberl and Tümmler, 2004). The clinical relevance of *P*. *aeruginosa* relies on the fact that infections caused by this pathogen are particularly hard to eradicate, mainly due to its intrinsic resistant to many antibiotics and to its ability to easily acquire new resistance mechanisms (Latifi *et al*., 1995; Aloush *et al*., 2006). Moreover, *P*. *aeruginosa* is able to produce a wide array of virulence factors and to form antibiotic resistant biofilms (Smith and Iglewski, 2003; Moradali *et al*., 2017).

In this context, in this PhD thesis different molecules displaying antivirulence activity towards *P. aeruginosa* have been identified and characterized. In Chapter 2 the antivirulence property of a model efflux pump inhibitor (EPI) has been proved, while in Chapters 3, 4 and 5 new anti-quorum sensing (QS) FDA-approved drugs have been identified able to reduce the expression of *P. aeruginosa* virulence traits by targeting the *pqs* QS system. All these molecules have different clinical plausibility, but they can all serve as scaffold for future development of chemical variants with improved potency and reduced adverse effects. Moreover, these studies highlight the potential of efflux pumps and *pqs*-based QS as feasible targets for antivirulence approaches.

One of the general considerations that emerge from the studies presented in this PhD thesis concerns the strain variability in target functionality and expression when considering antivirulence drugs. As an example, in Chapter 2 we reported the antivirulence properties of the well-known EPI PAβN against the model laboratory strain *P. aeruginosa* PAO1. This EPI down-regulates the expression of virulence genes and the production of the virulence factors (*e.g*., $3OC_{12}$-HSL and pyocyanin) in PAO1. However, the results obtained by using clinical *P*. *aeruginosa* isolates indicate that no correlation could be established between the effect of this EPI on some virulence phenotypes (*i.e*., the $3OC_{12}$-HSL and pyocyanin production) and the outcome of infection in *Galleria mellonella* larvae. Despite PAβN has a general protective role against *P. aeruginosa* infection in the *G. mellonella* infection model, this evidence suggests that the specific virulence factors affected by this EPI could be strain-specific. Although PAβN is toxic for humans, hindering future therapeutic application and discouraging further studies aimed at characterizing the effect of

this EPI on a wider panel of *P. aeruginosa* clinical strains, the results obtained in this study clarify the relevance of RND efflux pumps on the pathogenic potential of this bacterium. Moreover, this molecule could serve as chemical scaffold for drug-optimization programmes in order to limit its toxicity against eukaryotic cells. The different activity exerted by antivirulence drugs against *P. aeruginosa* clinical isolates relative to the model organism PAO1 has been observed also for clofoctol (Chapter 3) and nitrofurazone and erythromycin estolate (Chapter 5). Also in this case, these QS inhibitors hamper the expression of virulence factors at different extent in the tested *P. aeruginosa* CF isolates, highlighting the importance of testing the effect of new antivirulence drugs against a broad panel of clinically relevant strains.

The discovery and development of new drugs for use in humans is a challenging task that usually requires decade-long laboratory experimentation followed by extensive clinical trials. This process is time consuming and necessitates substantial economic investments with a high-risk of failure mostly due to the poor pharmacological properties of newly identified bioactive molecules (Fernandes and Martens, 2017). Since the vast majority of the *P. aeruginosa* antivirulence drugs discovered in the last decades are endowed with unfavourable pharmacological properties, a drug repurposing approach was used in this PhD thesis leading to the identification FDA-approved drugs inhibiting the *pqs* QS system of *P. aeruginosa* (Chapters 3, 4 and 5).

Searching for off-target activities in drugs already approved for use in humans represents a potential shortcut to develop new therapeutic options, although there are still some issues that need to be addressed to make antivirulence drugs viable alternatives to current antibacterial therapies (Rampioni *et al*., 2017). Although data on the acute and chronic toxicity are already available for FDA-approved drugs, as well as information on their pharmacokinetics, some of these drugs would require reformulation to be applied as repurposed drugs. As an example, the screening campaign performed in Chapter 3 led to the identification of three promising anti-*pqs* drugs, clotrimazole miconazole, and clofoctol, able to affect PqsR functionality, probably by competing with the natural ligands HHQ and PQS for the PqsR ligand-binding site. The most promising compound was clofoctol, an antimicrobial used for the treatment of acute and chronic upper respiratory tract infections and for tracheobronchial infections caused by Gram-positive pathogens, especially staphylococci, pneumococci and streptococci (Buogo, 1981; Danesi *et al*., 1988). Phenotypic analyses performed in the laboratory strain PAO1 and in *P. aeruginosa* isolates from CF patients support the antivirulence potential of clofoctol, both *in vitro* and *in vivo* on a *G. mellonella* model of infection. Of particular interest, clofoctol is usually administered as suppositories as it is well absorbed through the rectal mucosa and rapidly spreads through the tissues, reaching the highest concentrations in the respiratory system (Del Tacca *et al*., 1987). Since clofoctol mainly acts in the

airways, it is potentially valuable as a future treatment of *P. aeruginosa* lung infections. Notably, clofoctol is used to treat infections in infants, and this is another advantageous feature if considering that in CF, *P. aeruginosa* lung infection is established in early life (Lyczak *et al*., 2002; Moradali *et al*., 2017). On the other hand, clotrimazole and miconazole are antifungal drugs used in humans to treat ringworm, pityriasis versicolor, vaginal and oral candidiasis, and skin yeast infections (Clayton and Connor, 1973; Sawyer *et al*., 1975; De Cremer *et al*., 2015; Zhang *et al*., 2016). They both alter the permeability of the fungal cell wall by binding to phospholipids and inhibiting the biosynthesis of ergosterol and other sterols required for fungal cell membrane integrity (Fothergill, 2006; Crowley and Gallagher, 2014). Miconazole displays its activity by inhibiting fungal peroxidases, which results in peroxide-mediated cell death (Fothergill, 2006). Both these drugs are mainly administered as creams or ointments; thus, their current formulations could be particularly suitable for the topical treatment of chronic wound infections caused by *P. aeruginosa* (Osmon *et al*., 2004; Driscoll *et al*., 2007). However, the use of clotrimazole and miconazole to treat *P. aeruginosa* lung infections would require their reformulation as inhalable nanosuspensions. In this context, it is important to highlight that a new formulation generally requires new absorption, distribution, metabolism, excretion and toxicity (ADMET) tests, thus partly compromising the advantage of the repurposing approach.

Several drugs with antivirulence activity have been identified by screening libraries of approved drugs, by using either experimental (wet-lab) approaches based on suitable screening systems and commercially available drug libraries, as described in Chapters 3 and 5, or *in silico* approaches based on the prediction of drug-target interaction by bioinformatics tools, as described in Chapter 4. Virtual screenings could reduce the time and costs associated to conventional drug discovery programs. However, hits emerging from *in silico* or *in vitro* screens may lack activity or even function as agonists when tested on the target pathogen (Galloway *et al*., 2011). More often, the inability of selected hits identified *in silico* or *in vitro* to inhibit target functionality in bacterial cell is due to the lack of internalization or to their active efflux outside the cell. Therefore, it is mandatory to verify the antivirulence activity of hits identified *in silico* or *in vitro* by means of whole-cell biosensor assays. In order to overcome this drawback, in Chapter 4 we combined the advantages of drug repurposing and *in silico* screening approaches by exploiting recent knowledge of PqsR-CBD structure to identify new FDA-approved drugs with anti-*pqs* activity. The virtual screening led to selection of five hits for which high binding affinity to PqsR was predicted, and *in vitro* experiments demonstrated the anti-*pqs* activity for two of them, pimozide and ergotamine. Since pimozide showed the highest inhibitory activity, this drug was experimentally characterized. Phenotypic assays showed that exposure of *P. aeruginosa* PAO1 to pimozide decreased key PqsR-

controlled virulence determinants, such as AQ signal molecules, pyocyanin, swarming motility and biofilm formation. Additional experiments performed with *ad hoc* engineered *P. aeruginosa* strains and refined *in silico* docking simulations suggest that pimozide competes with the natural ligands HHQ and PQS for PqsR binding. On the other hand, the inability of the other three predicted PqsR ligands to hamper the *pqs* QS system in *P. aeruginosa* and to decrease bioluminescence in the control biosensor system may be related to the drawbacks typically associated to virtual screening approaches, including cell impermeability to the selected compounds or their modification/inactivation by cellular metabolism. This does not seem to be the case for eltrombopag, since the inhibitory effect exerted by this drug on both the specific and control reporter systems indicates its ability to penetrate *P. aeruginosa* cells, suggesting a QS-independent effect on bioluminescence. This data highlight that the use of whole-cell reporter systems is not sufficient to unequivocally identify QS inhibitors, and that adequate control experiments are needed to assess off-target effects of the tested compounds on reporter activity in order to rule out false positive hits (Defoirdt *et al*., 2013). In our case, unspecific effects of eltrombopag on the reporter system may mask its impact on PqsR functionality.

Another possible drawback of drug repurposing approaches relies on the primary activity of the repurposed drug. As an example, the antipsychotic activity of the dopamine antagonist pimozide, clinically used for the treatment of Tourette's syndrome and schizophrenia (Tueth and Cheong, 1993), could limit its therapeutic use as antivirulence drug against *P. aeruginosa*. In fact, it has to be considered that, although rarely, pimozide has been associated to potentially serious adverse effects, including arrhythmia, cardiac arrest, seizures, and neutropenia (Singer, 2010). Neutropenia, in particular, is a worrisome adverse effect for patients suffering a bacterial infection. In addition, the peak serum concentration of pimozide in conventional treatment as an antipsychotic drug is in the nanomolar range (Yan *et al*., 2010), far below the concentration required to inhibit the *pqs* QS system in *P. aeruginosa*. In spite of these limitations, as observed in Chapter 2 for PAβN, the pimozide molecular scaffold could serve as the basis for chemical modifications aimed at lowering its dopamine antagonistic activity, while improving membrane permeability and affinity for the PqsR active site, in line with the selective optimization of side activity (SOSA) approach (Wermuth, 2006). However, as discussed for PAβN (Chapter 2), and for clotrimazole and miconazole (Chapter 3), such a hit-to-lead optimization process would partly compromise the advantage of drug repurposing, since chemical modification of pimozide would invalidate the FDA-approval, with additional pharmacological testing being required by regulatory agencies. It must be recognized that repurposing of "old" drugs for new therapies can result in seamless adoption into the clinical

practice only if their off-target effect overcomes their primary activity, as it could be the case for the PqsR inhibitor clofoctol (Chapter 3).

The last part of this PhD thesis is focused on PqsE, the main effector protein of the *pqs* QS systems required for full virulence of *P. aeruginosa* in plant and animal infection models (Gallagher *et al*., 2002; Diggle *et al*., 2003; Rampioni *et al*., 2016). Likely through multiple mechanisms, still poorly understood at the molecular level (Folch *et al*., 2013; Zender *et al*., 2016; Mukherjee *et al*., 2018; McCready *et al*., 2019), and irrespective of the presence of PqsR, PQS or HHQ, PqsE activates the transcription of more than 140 genes, including key virulence genes (*e.g*., those required for pyocyanin production, the *mexGHI-opmD* efflux pump operon, and genes involved in biofilm formation) (Rampioni *et al*., 2016). Accordingly, *P. aeruginosa pqsE* mutants have impaired biofilm formation and decreased virulence both *in vitro* and *in vivo* (Rampioni *et al*., 2010; Rampioni *et al*., 2016; Mukherjee *et al*., 2018). Since PqsE expression requires the PqsR/AQs complex, inhibition of the PqsE regulative pathway can be achieved by targeting PqsR, AQs synthesis or PqsE itself. To the best of our knowledge, none of the identified molecules active against the *pqs* QS system directly inhibit the ability of PqsE to control virulence of *P. aeruginosa*. Indeed, although Zender and co-workers identified a molecule able to bind PqsE and to inhibit its thioesterase activity, this molecule did not alter the expression of PqsE-controlled virulence traits (Zender *et al*., 2016). In Chapter 5, a drug repurposing approach on a *P. aeruginosa* whole-cell biosensor strain led to the identification of two antibiotic compounds active as anti-PqsE drugs, nitrofurazone and erythromycin estolate. These drugs were able to affect *P. aeruginosa* virulence phenotypes whose expression is PqsE-dependent.

A possible limitation of the drug repurposing approach relies on potential negative interactions of the repurposed drug with existing therapies. Notably, antivirulence drugs targeting the *pqs* systems have been shown to potentiate the effect of antibiotics against planktonic and biofilm *P. aeruginosa* cultures (Starkey *et al*., 2014; Maura and Rahme, 2017). In Chapter 5, we addressed this issue by performing different experiments with nitrofurazone and erythromycin estolate in combination with tobramycin, one of the antibiotics most commonly used in CF therapy (Ratjen *et al*., 2009; Maselli *et al*., 2017). The results obtained with our *P. aeruginosa* PAO1 laboratory strain revealed that both nitrofurazone and erythromycin estolate did not show antagonistic effects toward this antibiotic in planktonic *P. aeruginosa* cultures. Interestingly, nitrofurazone was actually able to potentiate the effect of tobramycin. This observation encourages further studies, and indicates that the clinical plausibility of an antivirulence drug is not only related to its ability to reduce the pathogenic potential of the bacterium, but also to its possible positive interaction with existing therapies.

Despite the intrinsic toxicity of both erythromycin estolate and nitrofurazone for eukaryotic cells could limit their direct repurposing for anti *P. aeruginosa* therapies, the identification of these drugs by means of an *ad hoc* engineered reporter system highlights the feasibility of identifying new *P. aeruginosa* antivirulence drugs targeting the PqsE regulatory network, and will likely boost the future identification of more potent and less toxic PqsE inhibitors. In addition, beyond their possible applications to the therapy, erythromycin estolate and nitrofurazone could provide useful tools for future studies aimed at unravelling the mechanism of action of PqsE.

A peculiar aspect of this PhD thesis relies on the primary activity of most drugs that have been here identified for their secondary quorum quenching ability. In general, antivirulence drugs should target bacterial functions specifically required for infection, hence these molecules are not expected to impact the beneficial host microbiota (Allen *et al*., 2014; Rampioni *et al*., 2017). However, the majority of the antivirulence compounds identified during this PhD thesis belong to the antibiotic class. Even if these compounds seems to act as antivirulence agents targeting QS at concentrations lower than those required to inhibit bacterial growth, the possible impact of these drugs on human microbiota has to be taken into account. However, it should be taken into account that also a high proportion of non-antibiotic drugs have recently been associated with changes in gut microbiome composition (Maier *et al*., 2018). More in general, the results of this PhD thesis support the notion proposed by the eminent scientist Julian Davies that the most part of low-molecular-weight organic compounds made and secreted by bacteria, including the molecules that are used in clinic as antibiotics, at physiological concentrations play roles as cell-signalling molecules in the environment (Yim *et al*., 2007).

Overall, the research carried out in this PhD thesis led to the identification of new drugs potentially useful for the treatment of infections caused by the human pathogen *P. aeruginosa*. Although most of them displayed some limitations for the use in therapy, clofoctol (Chapter 3) showed high clinical plausibility, despite additional work is needed to assess the potential antivirulence effect of this drug *in vivo* (*e.g.*, by using mouse models of chronic lung and wound infections, alone or in combination with existing therapies). Moreover, the results obtained in this PhD thesis could increase the comprehension of some elusive mechanisms underlying *P. aeruginosa* QS and virulence.

The increasing effort in searching for antivirulence activity in FDA-approved drugs raises the hope that the number of promising candidates for future antivirulence therapies will increase in the next future. This success could boost the interest of pharmaceutical companies for antivirulence drugs discovery, reasonably attracted by the promise of developing new antimicrobials with reduced side effects and/or extended clinical lifespan as compared with traditional antimicrobial drugs.

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2. D'Angelo F, **Baldelli V**, Halliday N, Pantalone P, Polticelli F, Fiscarelli E, Williams P, Visca P, Leoni L, Rampioni G (2018) Identification of FDA-approved drugs as antivirulence agents targeting the *pqs* quorum sensing system of *Pseudomonas aeruginosa*. *Antimicrob Agents Chemother* 62:11. doi:10.1128/AAC.01296-18.
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4. **Baldelli V**, D'Angelo F, Pavoncello V, Fiscarelli EV, Visca P, Rampioni G, Leoni L. Identification of FDA-approved drugs targeting the *Pseudomonas aeruginosa* quorum sensing effector protein PqsE. Manuscript in preparation for submission to *Virulence*.

Communications to conferences (O = oral presentation; P = poster presentation)

1. **Baldelli V**, D'Angelo F, Leoni L, Rampioni G (2015) Identification of FDA-approved compounds targeting the *pqs* quorum sensing system of *Pseudomonas aeruginosa.* 31st Conference of the Italian Society of General Microbiology and Microbial Biotechnologies (SIMGBM), September 23rd-26th 2015, Ravenna, Italy. (P)
2. D'Angelo F, **Baldelli V**, Leoni L, Rampioni G (2016) Identification of FDA-approved compounds targeting the *pqs* quorum sensing system of *Pseudomonas aeruginosa*. Conference of the Italian Federation of Life Sciences (FISV), September 20th-23rd 2016, Rome, Italy. (P)
3. **Baldelli V**, D'Angelo F, Pavoncello V, Visca P, Leoni L, Rampioni G (2017) Identification of FDA-approved anti-virulence drugs targeting PqsE. 16th International Conference on *Pseudomonas*, September 5th-9th 2017, Liverpool, UK. (P)
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5. **Baldelli V**, D'Angelo F, Pavoncello V, Visca P, Rampioni G, Leoni L (2017) Identification of FDA-approved anti-virulence drugs targeting PqsE. 32nd Conference of the Italian Society of

General Microbiology and Microbial Biotechnologies (SIMGBM), September 17th-20th 2017, Palermo, Italy. (P)

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8. **Baldelli V**, D'Angelo F, Pavoncello V, Visca P, Rampioni G, Leoni L (2019) Identification of FDA-approved anti-virulence drugs targeting PqsE. 8th Congress of European Microbiologist (FEMS 2019), July 7th-11th 2019, Glasgow, UK. (P)
9. **Baldelli V**, D'Angelo F, Pavoncello V, Visca P, Rampioni G, Leoni L (2019) Identification of FDA-approved anti-virulence drugs targeting PqsE. 33rd Conference of the Italian Society of General Microbiology and Microbial Biotechnologies (SIMGBM), June 19th-22nd 2019, Florence, Italy. (P)

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