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Review

New alternative therapeutic strategies against *Pseudomonas aeruginosa*, an opportunistic multi-resistant pathogen with a myriad of virulence factors

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Abstract

Pseudomonas aeruginosa (PA) has emerged as a significant cause of Gram-negative infections, particularly in patients with impaired host defenses. It is one of the six ESKAPE pathogens that majorly cause severe nosocomial infections. In addition to biofilm formation, PA possesses various virulence factors. It can be life-threatening due to his remarkable capacity to resist antibiotics, either intrinsically, developing adaptative resistance, or following the acquisition of resistance genes. The situation worsens when these mechanisms co-exist, conferring worrying multi-resistant phenotypes. Therapeutic options are becoming limited, which has led to the development of new antibiotics and novel alternative therapeutic strategies that require the exploration of other therapeutic avenues. Although mostly at the preclinical stages, many recent studies have reported several innovative therapeutic technologies that have demonstrated pronounced effectiveness in fighting against drug-resistant Pa strains. This literature review aims to discuss the mechanism of pyocyanic bacillus resistance to antibiotics, highlight the current state of some novel antibiotics and combination therapies, and the new alternative therapeutic approaches for treating PA infections.

Key words: *Pseudomonas aeruginosa*; genetic diversity; antibiotic resistance; resistance mechanisms; virulence factors; alternative therapeutics.

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Introduction

The name of *Pseudomonas aeruginosa* (PA) species derives from the Latin word *aeruginosus* (rust-covered), formerly known as “pyocyanic bacillus”. A French military pharmacist, A. Gessard discovered it in 1882. PA belongs to the *Pseudomonas* genus in the *Pseudomonadaceae* family. PA is a ubiquitous Gram-negative bacillus, saprophytic, strictly aerobic, with oxidative metabolism. It is adaptable to many environments; it can be isolated from various biotopes, including plants, animals, humans, and natural humid sites [2,3].

PA has emerged as a significant clinical opportunistic pathogen due to the number and severity of severe nosocomial infections caused [1]. This germ, associated with high morbidity and mortality levels, especially in immunodeficient patients, poses serious therapeutic difficulties worldwide. Infections of PA became difficult to eradicate due to its multi-drug resistance, and even the emergence of "pan-resistant" strains [2,3], and to its ability to produce a vast arsenal of virulence factors that allow its survival in several ecological niches and its colonization of a broad spectrum of hosts [4].

The World Health Organization published the first-ever list of antibiotic-resistant "priority pathogens" that have emerged as a major therapeutic challenge by developing resistance to numerous antibiotics, including carbapenems and third-generation cephalosporins (the most effective drugs currently available for treating MDR bacteria) [1,4]. PA is designated as one of these six ESKAPE (*Enterococcus faecium, Staphylococcus aureus, Klebsiella pneumoniae, Acinetobacter baumannii, Pseudomonas aeruginosa*, and *Enterobacter* spp) in the most critical group according to the urgency of the need for new antibiotics [1]. The development of novel therapeutics to treat drug-resistant infections, especially those caused by ESKAPE pathogens, is essential at this time [1].

Special attention has recently focused on the emergence and dissemination of PA virulent and multi-drug resistant (MDR) strains [2]. The increasing incidence of infections associated with acquired resistance and the limited antibiotic-therapeutic treatments have warned against developing new antibiotics and novel antibacterials [3]. Additionally, compared to non-MDR-PA infections, cases with MDR-PA infections have more extended hospital stays, higher readmission rates, and expenses per infection [5]. In this context, our review focuses on elucidating the current state of resistance mechanism to antibiotics of pyocyanic bacillus and highlighting some novel therapeutic approaches for treating PA infections.

Genetic diversity of PA

The capacity of PA to thrive in diverse environments (that relies on its metabolic versatility), to infect a large range of its hosts, and to resist (intrinsically or after the acquisition of the resistance genes) to a large number of antimicrobial agents are the consequence of the large genome of the species. PA has a large circular genome of 6.3 million bp. The PA pan-genome (the totality of genes described in the species) varies between 10,000 and 40,000 genes [6,7]. The core genome's size and content are open for debate as their estimation depends on the set of genomes considered and the pipeline of gene detection and clustering [7].

Valot *et al.* (2015) used a clustering approach to find that the average PA genome contained 5,972 genes and the pan-genome 9,344 genes. These authors redefined the size and the content of the core genome of PA from fully re-analyzed genomes of 17 reference strains. After the optimization of gene detection and clustering parameters, the core genome was defined at 5,233 orthologs, which represented ~ 88% of the average genome, that are common to the majority of the PA strains [6,7]. The core genome is accompanied by genes called “mobile genetic elements” that are present in a smaller number of strains, and it varies from one strain to another which is described as an “accessory genome”; these differences have been associated with virulence and antibiotic resistance [7,8].

The PA accessory genome has an essential role in disseminating resistance; it is an important driver of its ability to persist in a particular environment [9]. The mobile genetic elements comprise integrons, transposons, insertion sequences, genomic islands, prophages, plasmid, and integrative and conjugative elements (ICEs) species. Most of these elements can mobilize and frequently possess a mosaic structure comprising modules from different mobile genetic elements [8,11]. The dissemination of these mobile genetic elements may then contribute to the spread of MDR phenotypes [10]. Even though plasmids and ICEs are essential drivers of antimicrobial resistance, the underlying evolutionary traits that promote this dissemination are poorly understood. Understanding the gene networks shared by most PA isolates is essential for developing effective drugs to treat many human infections [11].

Regulation of virulence factors by quorum sensing (QS) system

This mechanism plays an essential role in bacterial motility, antibacterial resistance, expression of virulence factors, and biofilm development [12]. A common feature of all QS networks is the transcriptional modulation of a large regulon of QS-controlled genes after the threshold level of specific auto-inducers is reached [13]. Two acyl homoserine lactone (AHL) regulate the QS system in PA, LasI-LasR (LasIR) and RhlI-RhlR (RhlIRLasI) catalyze the production of QS signal N-3-oxo-dodecanoyl homoserine lactone (3OC12- HSL) [14,12]. Signal-bound RhlR activates the expression of RhlI and other genes, including LasR regulon. Together, these pathways regulate the expression of ~10% of the PA transcriptome, including different virulence factors (pyocyanin, rhamnolipids, hydrogen cyanide, and exotoxin A) [14]. The previous two AHL QS systems interact with a third system called *Pseudomonas* quinolone signal (PQS) and its biosynthetic precursor 2-heptyl-4-2quinolone (HHQ). PQS and HHQ bind to the transcriptional regulator pqsR (also called MvfR) to activate the expression of the PQS synthesis genes. Multiple genes, including LasR and RhlR are activated by the PQS system [13]. The Las system is at the top of the hierarchy as it exerts regulatory control over the Rhl and the PQS signaling system [14].

In PA, ecological adaptation and expression of pathogenicity factors are controlled by QS. This system is based on the ability of bacteria to communicate with each other, which accentuate its pathogenicity, adaptability, and flexibility under environmental stress and makes this pathogen a successful infectious agent [14].

PA, a challenged pathogen with a myriad of virulence factors

PA is one of the most severe opportunistic bacteria that can cause chronic infections, especially urinary tract infections, burn infections, chronic otitis, and corneal infections [15]. It is a significant cause of

healthcare-associated infections (10–20%), particularly problematic in intensive care units [14]. PA infections are associated with high morbidity and mortality in many groups, including individuals with healthcare-associated pneumonia, chronic obstructive pulmonary disease, or cystic fibrosis (CF) [16]. The severity and persistence of these infections depend on the bacteria's metabolic adaptability, which is supported by several virulence factors and enzymes that allow it to adapt to different environments and colonize various types of hosts [3].

The membrane-associated virulence factors such as flagellum, biofilm, lipopolysaccharide (LPS), OprF protein, alginate, and pili are mainly involved in adhesion and motility [13] are generally involved in the colonization phase and chronic infection, whereas the extracellular factors, which are highly toxic, are associated with intensive infections [3].

PA produces three extracellular polysaccharides (or exopolysaccharides) alginate, Psl, and Pel [17]. They provide many protective properties and confer surface and self-adherence. They are constituents of the biofilm matrix, are involved in surface colonization, and promote host immune evasion and bacterial persistence through several mechanisms. Mucoid PA overproduces the exopolysaccharide alginate. These strains are frequently linked to other chronic lung disorders, including chronic CF lung infections. It interferes with opsonophagocytosis, complements activation, scavenges reactive oxygen species, and inhibits phagocytic killing [18]. PA also secretes factors such as exotoxin A, phospholipases, metalloproteases (elastin Las B or pseudolysin, elastase Las A or staphylolysin, alkaline protease or aeruginolysin), serine-proteases (protease IV or arginyl peptidase, Las D), leucocidin, phenazines, pyoverdines, rhamnolipids, and lectins. PA produces two small soluble proteins lectins, Lec A and LecB (also named PAI-L and PAII-L, respectively), binding specific sugars; galactose and its derivatives, fucose, mannose, and mannose-containing oligosaccharides (Lec B). The primary functional role attributed to these lectins is to mediate the attachment of the pathogen to human cells during infection. LecA is involved in host cell invasion and cytotoxicity, while LecB reduces the ciliary beating of airway epithelium. Both lectins also play a crucial role in forming PA biofilm [19]. In addition, various secretion systems are present in Gram-negative bacteria allowing toxins injection directly inside the cell. PcrV is an important protein secretion system on the tip of the PA T3SS injectisome complex responsible for host cell toxicity by the ExoU, ExoT, and ExoS cytotoxins [19].

Types and resistance mechanisms of PA

PA is increasingly resistant to many antimicrobial agents, highlighting the ability of this species to acquire and disseminate enzymatic and nonenzymatic resistance mechanisms [20]. The principal mechanisms of PA used to counter antibiotic attacks can be classified into intrinsic, acquired, and adaptive resistance.

Intrinsic resistance

The intrinsic resistance of PA to many antibiotics, including β-lactams, results from the combined action of several mechanisms that tend either to inactivate the antibiotics, inhibit their ability to penetrate the cell, or to reach their intracellular target through **i)** the production of the chromosomal inducible cephalosporinase of a broad-spectrum called AmpC [21]. Wild-type strains of PA produce the cephalosporinase AmpC at a relatively low level, whose expression is induced by certain beta-lactams. This cephalosporinase hydrolyzes aminopenicillins (amoxicillin and ampicillin), 1st generation and 2nd generation cephalosporins, ceftriaxone, cefotaxime, and ertapenem [21]. **ii)** Insufficient porin size that results in low membrane permeability to ß-lactams. Antibiotics with cytoplasmic targets must traverse all three layers of the cell envelope. Among Gram-negative bacteria, PA is particularly adept at reducing antibiotic entry, and **iii)** Export of antibiotics out of the cell by the membrane efflux system, it can be rapidly extruded by RND (resistance-nodulation-division) family efflux pumps. These pumps consist of an inner membrane-spanning motor (named "Mex" for multi-drug efflux) connected by a periplasmic adaptor to an outer membrane porin, and together, the pump complexes traverse the entire cell envelope (MexAB–OprM). In some cases, the outer membrane porin interacts with inner membrane components from other systems; OprM can pair with MexAB and MexXY. Furthermore, each efflux pump has different specificities. MexAB–OprM extrudes β-lactams and quinolones, MexCD–OprJ is specific for β-lactams, MexEF–OprN pumps out quinolones, and MexXY–OprM extrudes aminoglycosides [22].

Acquired resistance

Resistance can be acquired either from mutation of a chromosomally resident gene or from the transfer of genetic material encoding resistance genes *via* extra-chromosomal mobile genetic carriers against several classes of antibiotics [23], including ß-lactams, fluoroquinolones, aminoglycosides, and polycationic antimicrobials [23]. One of the most frequent

mechanisms of acquired resistance to FQs is chromosomal mutations in the Quinolone Resistance Determining Regions (QRDRs) encoding subunits of the enzymes DNA gyrase (gyrA-gyrB) and topoisomerase IV (parC-parE) [2,23]. Additional mechanisms to this type of resistance include the alteration of porins due to the loss of D2 porin in this species. The preferential pathway for carbapenem penetration is the main mechanism by which PA acquires resistance to carbapenems. This porin deletion is effectively responsible for an increased MIC, making the strain intermediate or resistant to carbapenems when it's accompanied by hyperproduction of the cephalosporinase AmpC. Furthermore, it includes mutational upregulation of efflux pumps; decreased permeability to antibiotics; target site alterations through mutations such alterations are phosphorylation chloramphenicol and aminoglycosides), acetylation (chloramphenicol, streptogramins, and aminoglycosides), and adenylation (aminoglycosides and lincosamides) [23,3].

This resistance can also be due to an enzymatic mechanism by the constitutive hyperproduction of the cephalosporin AmpC, which affects the activity of ticarcillin, piperacillin/tazobactam combination, ceftazidime, aztreonam, and to a lesser extent cefepime [23]. Usually, the enzyme is produced in low quantities. Its action escapes the action of β-lactamase inhibitors such as clavulanic acid or tazobactam [23]. Another enzymatic mechanism is the production of ß-lactamases that inactivates enzymes of the serine-type (classes A, C, and D) or metallo-enzymes (class B), whose substrates are β-lactams which affects its cycle and consequently provokes losing the activity of the antibiotic [23].

The classification of β-lactamases A is made between penicillinases and extended-spectrum β-lactamases (ESBL). Five types of ESBL class A (TEM, SHV, PER, VEB, and GES) have been detected in PA. These enzymes are usually detected by a synergy between a C3G (notably Ceftazidime) or Aztreonam and clavulanic acid appearing as a "champagne cork" on an antibiogram. The diffusion of ESBL class A genes plays an essential role in disseminating antibiotic resistance and may therefore limit the possibilities of treating infections caused by PA [24]. The β-lactamases class D, including oxacillinases, are penicillinases whose spectrum has been extended in some studies to C3Gs and others to carbapenems. In PA, ESBLs derived from OXA- 10 and OXA-2 have been isolated (OXA10, 11, 14, 15, 16, 19), as well as the β-lactamase OXA-18. These enzymes are localized on plasmids (except OXA-18). They hydrolyze most β-lactams, including cephalosporins, imipenem, and meropenem. Aztreonam and piperacillin are less affected, but their activity is not inhibited by clavulanic acid or tazobactam. The OXA-type carbapenemases show a wide diversity of protein sequences but have a fairly similar spectrum of activity. In the absence of other resistance mechanisms (other than ESBL or plasmid AmpC β-lactamases, loss of porins, or efflux pumps), they only decrease carbapenem sensitivity [25].

Some PA clones are known to be high-risk clones according to their prevalence, global spread, and association with MDR/XDR profiles, and regarding ESBLs and carbapenemases, the worldwide top 10 PA high-risk clones include ST235, ST111, ST233, ST244, ST175, ST277, ST654, ST357, ST308, and ST298. These last three are also potentially associated with higher virulence [20]. To develop strategies against high-risk clones, it is necessary to have a better understanding of the underlying causes of their success.

Adaptive resistance

Adaptive resistance is an inducible resistance that occurs in response to antimicrobial agents or other environmental stresses (chemical or physical), such as a change in media, pH, temperature, oxygen, and other growth conditions. The exposure of PA to aminoglycosides frequently selects for recalcitrant sub-populations exhibiting an unstable adaptive résistance to these antibiotics [26]. The best-characterized mechanisms of adaptative resistance in PA are the formation of biofilm and the generation of persister cells.

Biofilm formation

PA is a well-known biofilm former which makes it an excellent model to study biofilm formation. The important length, complexity, and diversity of its genome provide it with a panoply of genes that expresses during the formation of biofilm (e.g., pilB, pilA, algD, rsmZ, LasA, phzI, phzII, PslA, LecA, LecB) [12].

It has been estimated that biofilms have a substantial bearing on over 90% of chronic wound infections by PA, resulting in poor wound healing [80]. Bacteria living in biofilms are more resistant to antimicrobial agents than planktonic bacteria, which indicates that the mechanisms involved in biofilm resistance to antimicrobials may differ from those of planktonic bacteria [3]. Biofilm formation concerns bacterial populations structured and enclosed within a matrix containing polysaccharides, proteins, and

extracellular DNA and fixed on a natural or artificial surface [27]. It depends on environmental signals and the metabolic state of bacteria in the biofilm, relying on the availability of oxygen and nutrients that will consequently influence antibiotic resistance. The extracellular matrix will also modulate the activity of the antibiotic by reducing its diffusion within the biofilm [26].

PA also effectively colonizes a variety of surfaces including medical materials (urinary catheters, implants, contact lenses, etc.), and food industry equipment (mixing tanks, vats and tubing) [4,80]. A resilient biofilm is a critical weapon for PA to compete, survive and dominate in the CF lung polymicrobial environment. It's often recognized as a co-colonizer along with other microbes such as *Staphylococcus aureus* (*S. aureus*), *Burkholderia cenocepacia* (*B. cenocepacia*), and *Streptococcus parasanguinis* (*S. parasanguinis*). For example, colonization of the biofilm-forming bacteria PA and S. aureus coinfects the lungs of CF patients and in diabetic and chronic wounds [80]. During co-infection, PA could sequester iron and nutrients through the lysis of Gram-positive bacteria, including *S. aureus*, *Streptococcus pneumoniae,* and *Bacillus anthracis* [80], as well as other Gram-negative bacteria *Burkholderia cepacia* [80].

It is important therefore to diagnose PA infections at an early stage before biofilm development which could enhance the susceptibility of PA to antimicrobial treatments. However, the increasing incidence of acute and persisting infections worldwide also highlights the need to develop therapeutic strategies as an alternative to traditional antibiotics, expectedly to disarm and eradicate this Gram-negative bacterium [80].

Persister cells

"Persister" cells (also called small-colony variants SCVs) correspond to a transient phenotypic variant of bacteria that are not genetically resistant to antibiotics. However, under the conditions mentioned above, they can withstand very high concentrations of these drugs (pharmacodynamic barrier). Persister cells are responsible for recalcitrance and relapse of infections [27]. This resistance increases the ability of a bacterium to survive antibiotic attack due to transient alterations in genes and proteins expression in response to an environmental stimulus. It is reversible when the stimulus is removed. PA is a highly adaptable species that can change its response according to its environment [28].

Persisters (corresponding to 1–2% of the bacterial population) opt not to proliferate during antibiotic exposure, but they resume replication if the stressors are removed from the environment. Persisters may also be important in the recurrence and chronicity of PA infections [28]. Meanwhile, "swarming" or rapid proliferation is a propagation due to a fast movement of hyper-flagellated cells, allowing faster colonization of surfaces during infection. The differentiation of these specialized cells involves QS. They are resistant to antibiotics, mainly due to the overexpression of efflux pumps during their differentiation. However, this transient phenotype's mechanism of antibiotic resistance is poorly understood [29].

Exposure to low concentrations of antibiotics leads to an "immediate" development of resistance that disappears in the absence of antibiotics [3]. This reversible response is thought to result from DNA methylation or a high level of point mutations; it is a "fast and transient" response. The slow and stable response corresponds to prolonged contact with the antibiotic, which leads to the progressive and cumulative appearance of mutations, as well as the duplication and amplification of genes that confer permanent resistance. Adaptive resistance is, therefore, a bridge between natural resistance and acquired resistance, allowing bacteria to develop defense systems in accordance with their environment [26, 27].

Conventional therapy

Conventional therapy is a treatment that is widely accepted and used by most healthcare professionals. It differs from alternative or complementary therapies, which are not as widely used. Antibiotic therapy is one of the conventional treatments against bacterial infections.

Recent discovered antibiotics and their mode of action

The resistance to current antimicrobial agents has continued to grow among various pathogens, including PA, indicating a need for new antimicrobials to fight against MDR organisms [30]. Newer antibiotics seem more effective against PA; they have new routes of administration and a lower frequency of resistance development compared to existing antibiotics. This is due to their new mechanisms of action, the efficacy of administration (e.g., inhaled antibiotics), and resistance to modification by bacterial enzymes [30]. Some of these newer antibiotics show excellent *in vitro* antibacterial activity against PA and lower minimum inhibitory concentration than conventional antibiotics [28]. While there have been advances in the marketing authorization of novel antibiotics and combination therapy in the recent decade, their price and availability

may hinder their widespread use in the near future. Additionally, it is questionable how long the new agents can manage the worsening resistance situation [8].

Plazomicin

Plazomicin is a next-generation semi-synthetic aminoglycoside synthetically derived from a natural product, sisomicin [32]. It can resist a broad spectrum of aminoglycosides modifying enzymes but not 16S rRNA ribosomal methyl transferases [32]. Plazomicin demonstrates potent *in vitro* activity against Gram-negative and Gram-positive bacterial pathogens, and it has similar activity to amikacin against multidrug-resistant PA strains. Furthermore, Pankuch *et al.* (2011) reported an *in vitro* synergistic activity of plazomicin against PA clinical isolates when combined with cefepime, doripenem, imipenem, or piperacillin-tazobactam, suggesting that plazomicin is a potential candidate for combination therapies in the treatment of MDR PA infections. Nevertheless, Plazomicin can still cause mild to moderate nephrotoxic and ototoxic effects [32].

POL7001

POL7001 is a macrocycle antibiotic belonging to the novel class of Protein Epitope Mimetic molecules, with selective and potent activity against PA. Some of these molecules inhibit the transport of LPS to the bacterial outer membrane [31]. Cigana *et al.* (2016) evaluated the efficacy of POL7001 both *in vitro* and in murine models with PA acute and chronic pneumonia. They found that the multidrug-resistant PA isolates from CF patients were sensitive to POL 7001 and that the POL7001-treated mice had a significantly reduced bacterial burden and decreased lung inflammation levels during PA acute and chronic infection. The new mode of action, the efficient pulmonary delivery, and the potent *in vitro* and *in vivo* activity suggest POL7001 as a novel therapeutic agent for future clinical trials. The side effects of POL7001 have not been reported yet [3].

Murepavadin (POL7080)

Murepavadin (formerly POL7080), a 14-amino-acid cyclic peptide that represents the first member of a novel class of outer-membrane-protein-targeting antibiotics, is being developed for the treatment of serious infections caused by PA. Its mechanism of action is not precise, but it was known in 2018 by Sader's study that murepavadin targets the LPS transport protein D (LptD). Through binding to LptD in the bacterium's outer membrane, murepavadin causes LPS alterations and ultimately kills the bacterium. Murepavadin is under development for hospital-acquired pneumonia and ventilator-associated pneumonia caused by PA [34].

Murepavadin has minimal or no effect on other bacterial species, which may reduce the risk of cross-resistance with other antibiotics. Its development as an intravenous formulation was recently halted due to unexpected findings of kidney damage. However, the development of a formulation for inhalation therapy is underway. It may be a valuable addition to therapeutic options for treating PA pulmonary infection in CF patients or other patients with chronic bronchial colonization by this organism, such as those with bronchiectasis [35].

Cefiderocol

Cefiderocol is a cephalosporin currently in phase III of development. It targets Gram-negative bacteria with an extremely broad spectrum [36]. More than 95% of PA isolates are sensitive to this antibiotic, even in cases of resistance to the ceftolozane-tazobactam combination [37]. This excellent activity is explained by its original structure, close to that of ceftazidime but with a side chain replaced by a siderophore, i.e., an iron-binding group [38]. This siderophore is actively transported intracellularly by dedicated bacterial systems. In addition to this "Trojan horse" strategy, the siderophore protects the molecule from the hydrolytic activity of most β-lactamases, including those belonging to Ambler's class B [36]. These structural properties make it necessary to test the sensitivity of the molecule in a particular medium with low iron concentration. Cefiderocol has significant potential in treating documented infections due to multidrug-resistant Gram-negative bacilli [37]. It is essential to emphasize the progress represented by this antibiotic, which will probably be one of the only active molecules in infections due to PA producing β-lactamases. It is more potent than meropenem and ceftazidime-avibactam and targets four Penicillin-binding Protein (PBPs) (PBP1, 1a, 2, and 3), although its greatest affinity is for PBP3 [39].

Combination therapies: New ß-lactam/ß-lactamases inhibitor combinations

Using multiple antibiotics in combination or combining antibiotics with potentiators or adjuvants is an appealing and frequently used strategy to treat PA infections.

Ceftazidime-avibactam

Ceftazidime-avibactam is a semi-synthetic cephalosporin coupled to a non-β-lactam β-lactamase inhibitor [40]. The broader activity of ceftazidime-avibactam is attributed to the addition of avibactam. This non-β-lactam β-lactamase inhibitor inhibits class A, class C, and most class D β-lactamases [41].

Avibactam is shown to be effective against the class A and C β-lactamases expressed in PA [38]. However, the activity of avibactam toward class D β-lactamases (i.e., OXA-2, OXA-5/10, and OXA- 50 [poxB] families from PA is limited. Winkler and Papp-Wallace (2016) have demonstrated that ceftazidime-avibactam possesses potent activity against MDR PA, raising optimism regarding its potential use in the treatment of infections [42,38].

Ceftolozane-tazobactam

Ceftolozane-tazobactam is an expanded-spectrum cephalosporin of the fifth generation, combined with a well-known β-lactamase inhibitor. This combination is characterized by enhanced activity [43] due to ceftolozane affinities for various PBPs that are at least 2-fold higher than that of ceftazidime (inhibiting a broader set of PBPs (including PBP1b, PBP1c, and PBP3 present in PA) [43].

According to the recent assessment of the European Society of Clinical Microbiology and Infectious Diseases guidelines [44], ceftolozane-tazobactam represents an effective treatment option for susceptible MDR/XDR PA infections, essentially in the case of carbapenem-resistant PA as well as when it comes to serious infections in intensive care facilities [45,46]. In addition, its new 3D structure gives superior stability to the hydrolysis caused by AmpC –lactamase compared to other cephalosporins, although AmpC-mediated pathways have recently been shown to be involved in the emergence of ceftolozane-tazobactam-resistant PA strains [47]. PA resistant isolates *in vivo* to ceftolozane-tazobactam have emerged by acquisition of class D β-lactamases and oxacillinases (OXA), which hydrolyze ceftolozane and are not efficaciously tazobactam inhibited [47,43]. Ceftolozane is minimally affected by other resistance mechanisms in *Pseudomonas*, such as porin loss and efflux pumps, even if, already in 2017, data from BSAC bacteremia surveillance revealed resistance due to increased efflux [49], and more recently, the emergence of PA resistance, following ceftolozane-tazobactam treatment, mediated, among others, also by OprD porin mutation and upregulation of efflux pumps was reported [49].

Imipenem-relebactam/imipenem-cilastatin-relebactam

Imipenem/cilastatin/relebactam (Recarbrio™), approved in the USA and EU, is an intravenously administered combination of the carbapenem imipenem, the renal dehydropeptidase-I inhibitor cilastatin, and the novel β-lactamase inhibitor relebactam [51]. Relebactam has a similar chemical structure to avibactam that effectively inhibits ESBL, *Klebsiella Pneumoniae* Carbapenemase KPC, and AmpC β-lactamases [52]. In contrast to avibactam, relebactam has minimal activity against any oxacillinaseOXA-type β-lactamases, including OXA-48. Relebactam also has no activity against metallo-β-lactamasesMBLs such as New Delhi MedicalNDM, Verona Integron–encoded Metallo-β-Lactamase VIM, and imipénèmase IPM [53]. It is currently in phase III development in combination with imipenem-cilastatin. The main benefit of adding relebactam is to restore the activity of imipenem against class A carbapenemases (KPC-2) [54]. The combination of imipenem-cilastatin and relebactam was more tolerable and effective than the combination of imipenem-cilastatin and colistin in various infections caused by Gram-negative bacteria [52,53,54]. Imipenem/cilastatin/relebactam has been shown to be an effective and well-tolerated treatment in adults with severe infections due to Gram-negative bacteria, including carbapenem-resistant pathogens [51].

Ceftazidime-avibactam-fosfomycin

Importantly, by combining ceftazidime-avibactam with fosfomycin and targeting penicillin-binding protein 3 (PBP-3), β-lactamases (e.g., PA-derived cephalosporinase [PDC] also known as PA-AmpC), and MurA (a UDP- N-acetylglucosamine-1-carboxy vinyl transferase), the susceptibility was restored *in vitro* to most ceftazidime-avibactam–resistant PA isolates [50]. Several recent studies revealed synergism between fosfomycin and β-lactams (i.e., carbapenems and ceftolozane-tazobactam) [55]. The decision to combine ceftazidime-avibactam with fosfomycin was based on the rationale that even though fosfomycin can down-regulate expression of PBP-3 and induce PDC expression, avibactam is so potent that it can significantly hinder the hydrolytic activity of PDC, as well as other class A and C β-lactamases present in PA [55].

New alternative therapeutic strategies

PA is challenging to treat with currently available antibiotics. The discovery of novel antibacterial agents with a distinct mechanism of action is not enough.

Therefore, new therapeutic strategies against MDR PA are essential. With the daunting increase in antimicrobial resistance rates in all types of bacteria, one of the main aims of antimicrobial research is the exploration of new approaches past conventional antibiotics [8], namely bacteriophages, antimicrobial peptides with diverse structures and mechanisms of action, virulence inhibitors, siderophores, compounds from natural origins (like essential oils), and adjuvants (e.g., efflux pump inhibitors, monoclonal antibodies). It is possible that these agents will play a significant role in managing severe bacterial infections caused by PA and other pathogens of critical importance [3].

QS inhibition

Recent attempts to interrupt the QS-dependent signaling pathway have attenuated PA virulence and biofilm formation in clinical infections [13]. Small-molecule inhibitors were identified for different QS systems of this bacterium [56]. Up to now, many QS and biofilm inhibitory compounds like curcumin, halogenated furanone, meta-bromo-thiolactone, triazole-containing 2-phenylindole, salicylic acid, 6-gingerol, ellagic acid, trans-anethole, ajoene, cinnamic acid, 7-fluoroindole, equisetin, 2-substituted 3-hydroxy-6-methyl-4H-pyran-4-one derivatives and also natural products such as two structurally related flavonoids have been identified [8,56]. The compounds that do not interfere with viability or growth possibly make less resistance than traditional antibiotics. Ideally, these reagents should not disturb bacterial and host metabolism without any harmful side effects [8].

Lectin Inhibition

Lectin inhibitors may block lectin binding to host cell surfaces, acting as key virulence factors. These inhibitors, such as glycoclusters, glycopolymers and glycodendrimers, have high binding affinity for lectins and inhibit their functioning [28,57]. The β-phenylgalactosyl peptide dendrimer (GalAG2), a glycopeptide dendrimer, showed a strong binding affinity to PA LecA and inhibited biofilm formation *in vitro*. Dendrimer FD2 binds to PA Lec B and facilitates the inhibition of biofilm formation and dispersion of preformed biofilms on a steel surface. In addition to being chemically synthesized, the PA lectin inhibitors also occur naturally. Royal jelly is a secretion from young worker honeybees and contains nutrients for larvae development. It has been shown to inhibit PA biofilm formation by interacting with lectins and disrupting the initial attachment of PA to human lung epithelial cells *in vitro* [65]. Inhibition of lectin binding may be helpful in preventing and treating PA infections for its high stability and low risk of developing bacterial resistance. These *in vitro* studies showed that lectin inhibitors effectively inhibited PA biofilm formation, but they require further assessment *in vivo* models and clinical trials. However, it is noteworthy that PA expresses a variety of adhesins that can reduce the efficacy of lectin inhibitors [3].

Iron Chelation

PA utilizes the siderophores pyoverdine and pyochelin to acquire iron from the extracellular environment [3]. Accordingly, limiting extracellular iron concentration or disrupting PA uptake by PA is a strategy to counter PA infections [3]. Free iron in humans is naturally limited as the proteins lactoferrin and transferrin sequester it. Lactoferrin prevents biofilm formation by promoting type IV pilus–mediated twitching motility. On the other hand, PA secretes proteases that can break down lactoferrin to obtain iron. Iron chelators can be used alongside conventional antibiotics. In this regard, combining tobramycin with the iron chelators deferoxamine and deferasirox significantly reduced the biomass of PA biofilm on C Fairway epithelial cells *in vitro* and enhanced the tobramycin-mediated killing of PA in biofilm [3].

Bacteriophage therapy

Phage therapy is based on using bacteriophages, viruses specific to bacteria, in human therapeutics [3]. They offer several advantages, as they are very specific, replicate at the site of infection, and no serious adverse effects of their administration have been described [58]. Their specificity toward a bacterial species makes bacteriophages the ideal candidates for treating multi-resistant bacteria. This specificity is such that many phages are only active on a limited number of strains within a species. They can also not infect eukaryotic cells, thus limiting their potential toxicity. Several studies have shown bacteriophages efficacy in treating experimental infections caused by PA in animals. These studies indicate that bacteriophages might also be helpful in the therapy of infections caused by MDR bacterial strains in humans [59]. Bacteriophages may be administered alone or in combination with antibiotics and can be given prophylactically or as a therapy for infections [3]. Although, further studies are needed in order to assess their therapeutic use in humans [3].Phage therapy is one of the new therapeutic solutions for the destruction of PA biofilm due to the non-applicability of antibiotics. Bacteriophages can be used to eradicate PA biofilm by destroying the

extracellular matrix, increasing the permeability of antibiotics into the inner layer of the biofilm, and inhibiting its formation by stopping the QS activity. Furthermore, the combined use of bacteriophages and other compounds with anti-biofilm properties, such as nanoparticles, enzymes, and natural products, can be of more interest because they invade the biofilm by various mechanisms and can be more effective than the one used alone. On the other hand, using bacteriophages for biofilm destruction has some limitations, such as limited host range, high-density biofilm, sub-populate phage resistance in biofilm, and inhibition of phage infection via QS in biofilm [60].

An endolysin, LysPA26, containing a lysozyme-like domain, was screened against PA in a study by Guo *et al.* (2017) [61]. It had activity against MDR PA without pretreatment with an outer-membrane permeabilizer. LysPA26 could kill up to 4 log units PA in 30 min. In addition, temperature and pH effect assays revealed that LysPA26 had good stability over a broad range of pH and temperatures. Moreover, LysPA26 could kill other Gram-negative bacteria, such as *Klebsiella pneumoniae, Acinetobacter baumannii* and *Escherichia coli*, but not Gram-positive bacteria. Furthermore, LysPA26 could eliminate *PA* in biofilm formation [61].

Vaccine strategy

The easiest way to mitigate the consequences of PA infection is to prevent it by using a vaccine. Different types of vaccines are being designed to improve the immune response against many substances involved in this process. The most common targets are components of the bacterial surface, such as outer membrane proteins (Opr) and different polysaccharides (LPS, mucoid exopolysaccharide, and O-polysaccharides), structures involved in PA adhesion and movement, such as flagella, pili, and several virulence factors, such as TTSS, exotoxin A, or proteases [3]. Developing an effective vaccine is difficult due to the high variability between *Pseudomonas* species, the complexity of its infection process, and its interaction with the host immune response. In many cases during phase I, II, and III studies, some molecules failed to provide adequate coverage against different PA strains or showed a low immunogenicity capacity or an insecure profile [62]. Passively administered therapeutic antibodies are an alternative immune-based treatment for this bacterium. However, the ability of this pathogen to cause both acute and chronic infections means that it is necessary to consider whether the targets of those antibodies are highly expressed under relevant conditions [63]. Development of novel PA vaccines is currently ongoing; the protective efficacy of a novel PA vaccine PcrV28-294-OprI25-83-Hcp11-162 (POH), containing PcrV, OprI, and a vital component of the type VI secretion system Hcp1, was evaluated in murine pneumonia and burn models. It was found that the POH vaccination significantly triggered Tcell responses and proliferation and protected mice against clinical PA strains [80]. The development of multivalent vaccines may provide a means of protecting against PA infections in the future.

Liposomal antimicrobial drug delivery

Nanoparticles such as chitosan nanoparticles, quantum dots, dendrimers, and liposomes are under study as antimicrobial agents. Polymyxin B-loaded liposomes represent a successful example of liposomal antimicrobial drug delivery. It has been reported that liposomal encapsulation of polymyxin B dramatically diminishes side effects and improves its antimicrobial activity against resistant strains of PA. The action mechanism of liposomal polymyxin B against bacteria has been identified as membrane fusion. Membrane fusion between liposomes and bacteria is a rapid and spontaneous process driven by non-covalent forces such as van der Waals force and hydrophobic interactions that minimize the free energy within the system. When liposomes fuse with cell membranes, a high dosage of drug contents is immediately delivered to the bacteria, potentially suppressing the antimicrobial resistance of the bacteria by overwhelming the efflux pumps, thereby improving drugs' antimicrobial activity [3].

According to Ibarakia *et al.* (2020), the liposomes whose surface properties can be easily controlled will become an effective tool for treating infections associated with bacterial biofilms delivering antimicrobial agents to bacteria located inside bacterial biofilm [3].

Electrochemical scaffolds

The use of electrochemical scaffolds to generate a low and constant concentration of H_2O_2, is enough to destroy bacterial biofilms and allow better antibiotic penetration. In this regard, a study by Istanbullu *et al.* (2012) indicated that the H_2O_2 produced by a steel surface significantly reduced PA biofilm formation. A more recent study by Sultana *et al.* (2016) demonstrated that an electrochemical scaffold could enhance tobramycin susceptibility of PA PAO1 and effectively eradicate persister cells in biofilms. To date, electrochemical scaffolds have not been implanted into

patients; thus, the clinical efficacy of this approach remains to be demonstrated [3].

Monoclonal antibodies

Current research in managing PA infections has been directed toward preventing infection in high-risk patients with vaccines and modulation of virulence with monoclonal antibodies instead of focusing on bacterial clearance attainment [62]. The bispecific antibody MEDI3902 is under study (phase II) to prevent pyocyanic pneumonia [65]. It targets a protein secretion system (PcrV) and the exopolysaccharide PsI [65]. An engineered human antibody Fab fragment that binds to the PAPcrV protein with high affinity has been identified and has potent *in vitro* neutralization activity against the TTSS. Instilling a single dose of Fab into the lungs of mice protected against the lethal pulmonary challenge of PA and led to a substantial reduction of viable bacterial counts in the lungs. These results demonstrate that blocking the TTSS by a Fab lacking antibody Fc-mediated effector functions can be sufficient for the adequate clearance of pulmonary PA infection [65]. AR-101 and AR-105 are being developed (phase II) to treat ventilator-associated pneumonia caused by PA [18]. One of the most successful monoclonal anti-type three secretion system antibodies; is the KB001, a high-affinity, PEGylated Fab antibody, which, in a phase II study, has been well tolerated and showed a safety profile in mechanically ventilated patients colonized by PA. Different monoclonal antibodies against alginate have been developed, showing an increase in PA phagocytosis [66]. In some cases, as with the monoclonal antibody F429, his improvement in the immune response against PA infection was also reported in different models of infection such as pneumonia, sepsis, and keratitis in animal models, being promising as an adjunctive strategy in PA infection management. Panobacumab (AR-101) is an IgM-type human monoclonal antibody that is directed against IATS 011 serotype PA, one of the most prevalent serotypes associated with nosocomial pneumonia. A multicenter Phase II study using panobacumab in combination with different antipseudomonal antibiotics in critical patients with nosocomial pneumonia due to PA serotype O11, almost all with VAP, showed a good safety profile with good PKs. More recently, Hebert *et al.* (2020) [65] showed that MEDI3902, a multifunctional antibody that targets the *PA* persistence factor, Psl exopolysaccharide, and the type 3 secretion protein PcrV may be clinically effective as an antimicrobial agent in treating *P. aeruginosa* keratitis [66].

Cysteamine

Cysteamine is an endogenous stable aminothiol (a reduced form of cystamine). It's an FDA-approved drug, compound licensed to treat cystinosis, tested as an anti-pseudomonal therapy, mucolytic (presumably through reduction of disulphide bonds), and directly antimicrobial. It has been shown to prevent biofilm formation and enhance antibiotic activity against PA in a mouse model [67]. There is also the unexpected possibility that cysteamine may improve CF transmembrane conductance regulator function when used in combination with the polyphenol compound epigallocatechin gallate, as assessed by many physiological endpoints [67,68,69]. Cysteamine also shows additional powerful anti-virulence effects that target PA, further bolstering its potential for treating CF and other infections [67].

Alginate oligosaccharides (AOS)

The AOS is, a low molecular weight oligomer containing 2 to 25 monomers, which can be obtained *via* organic synthesis by hydrolysis of glycosidic bonds, or through biosynthesis (produced by the brown seaweed *Laminaria hyperborea*). These oligosaccharides have attracted interest from both basic and applied researchers due to their immunomodulatory, antimicrobial, antioxidant, prebiotic, antihypertensive, antidiabetic, antitumor, and anticoagulant activities [70].

The ability of AOS to potentiate the effect of antibiotics against Gram-negative MDR bacteria and to inhibit biofilm formation *In vitro* has been described [68]. In addition, binding of AOS to PA cell surfaces (and flagella) induces a greater negative charge on the cell surface, thereby reducing bacterial adherence (to epithelial mucin and/or cells, for example) and biofilm formation [71]. AOS has also been shown to inhibit the motility of bacteria, including PA, by binding to the bacterial flagella, which are important virulence factors [71].

Gallium

Metal-based antimicrobials have been gaining attention as potential alternatives to traditional antimicrobials in the fight against antibiotic resistance [72]. They can disrupt multiple bacterial physiological processes and boost and restore antibiotic efficacy, improving antibiotic-resistant infections' cure rates [73]. Gallium, are active metal ion that disrupts bacterial biofilm formation and protects against PA infection in mouse models and *in vitro* in human serum [72]. While the therapeutic capacities of gallium have

always been attributed to its chemical similarity to Fe (III), a recent study Yuchuan *et al*., (2019) shows that Ga (III) acts at the transcriptional level by targeting the transcription enzyme; RNA polymerase by binding to RpoB and RpoC, two subunits of this enzyme identified for the first time by Yuchuan *et al.* (2019). This results in the suppression of RNA synthesis, which in turn affects energy metabolism.

Several studies focus on the development of novel formulations to deliver gallium, including polymeric or solid materials that offer a slow release of gallium [75]; co-encapsulated to deliver gallium along with a conventional antibiotic; as well as gallium complexed with biological and synthetic chelating agents [74,76,77]. Banin *et al.* (2008) combines a strong siderophore, desferrioxamine (DFO), with gallium (DFO-Ga) as a Trojan horse delivery system. This complex kills free-living bacteria and blocks biofilm formation. Furthermore, a combination of DFO-Ga and the anti-*Pseudomonas* antibiotic gentamicin caused a massive killing of PA cells in mature biofilms [74,76,77].

Peptidomimetic antibiotics

Given its essential function in many Gram-negative bacteria, the LPS transport pathway is an interesting target for developing new antibiotics [78]. Peptidomimetic antibiotics are part of a family of novel synthetic cyclic peptides derived from an existing (biological) antimicrobial peptide. They bind to the LptD protein involved in LPS transport from their assembly site on the inner membrane to the outer membrane and hence impact PA by interfering with the transport of LPS [78,79]. The related molecule, Murepavadin, is under clinical development to treat life-threatening infections caused by PA [78].

Peptidomimetic molecules have also been studied on PA strains producing the MexAB efflux pump. Some of them can block the efflux of levofloxacin (used as a marker for fluoroquinolone transport). A strategy based on the inhibition of these molecules' efflux mechanisms has been developed to restore the activity of pump-substrate antibiotics without having any intrinsic effect on the bacteria [78].

Issues and hypothesis

Over the years, PA has served as a paradigm for studying gene expression, metabolism, and pathogenesis. The large genome, which approaches the complexity and size of lower eukaryotes, and an abundance of regulators, facilitates adaptation to almost any environment. During evolution, the competition with other prokaryotes and the acquisition of defensive mechanisms to fend off eukaryotic predators have allowed the maintenance of antibiotic-resistance markers, degradative enzymes, and secretion systems, which impact the human infection. The surveillance of nosocomial infections caused by PA requires the identification of reliable epidemiological markers. In addition to traditional typing techniques such as antibiotyping and serotyping, the contribution of molecular biology techniques can make the determination of the epidemic nature of these infections possible and, consequently, improve their management and limit the diffusion of multi-resistant strains.

Authors' contributions

Benzaarate Ihssane: Conceptualization, Methodology, Writing-Review & Editing; Elotmani Fatima: Validation, Supervision; Khazaz Aboubakr: Methodology; Timinouni Mohammed: Supervision, Project Administration; Nayme Kaotar: Supervision, Project Administration

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