REVIEW ARTICLE

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Efflux pump inhibitors (EPIs) as new antimicrobial agents against *Pseudomonas aeruginosa*

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Pseudomonas aeruginosa is an opportunistic human pathogen and one of the leading causes of nosocomial infections worldwide. The difficulty in treatment of pseudomonas infections arises from being multidrug resistant (MDR) and exhibits resistance to most antimicrobial agents due to the expression of different mechanisms overcoming their effects. Of these resistance mechanisms, the active efflux pumps in *Pseudomonas aeruginosa* that belong to the resistance nodulation division (RND) plays a very important role in extruding the antibiotics outside the bacterial cells providing a protective means against their antibacterial activity. Beside its role against the antimicrobial agents, these pumps can extrude biocides, detergents, and other metabolic inhibitors. It is clear that efflux pumps can be targets for new antimicrobial agents. Peptidomimetic compounds such as phenylalanine arginyl β-naphthylamide (PAβN) have been introduced as efflux pump inhibitors (EPIs); their mechanism of action is through competitive inhibition with antibiotics on the efflux pump resulting in increased intracellular concentration of antibiotic, hence, restoring its antibacterial activity. The advantage of EPIs is the difficulty to develop bacterial resistance against them, but the disadvantage is their toxic property hindering their clinical application. The structure activity relationship of these compounds showed other derivatives from PAβN that are higher in their activity with higher solubility in biological fluids and decreased toxicity level. This raises further questions on how can we compact Pseudomonas infections. Of particular importance, the recent resurgence in the use of older antibiotics such as polymyxins and probably applying stricter control measures in order to prevent their spread in clinical sittings.

Keywords: *pseudomonas aeruginosa*; *efflux pumps*; *antibiotic resistance*

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Pseudomonas aeruginosa is one of the most prevalent nosocomial pathogens associated with higher mortality rates and antibiotic costs. It can survive in different environments including soil, plants, and animals. It is also considered the most opportunistic human pathogen especially in immunocompromised patients and one of the top five pathogens of nosocomial diseases worldwide (1).

Pseudomonas infections are commonly reported in burns, urinary tract infections (UTI), and pulmonary diseases such as cystic fibrosis (CF). This diversity of pseudomonas infections is due to the development of various adaptive mechanisms such as the nutritional and metabolic pathways besides the regulation of gene expression. In addition, its ability to form biofilms provides greater protection against host immune defense systems and the susceptibility to various antimicrobial agents (2).

Pseudomonas aeruginosa is a multidrug resistant organism (MDR) and considered a phenomenon of bacterial resistance. This is demonstrated by different types of antibiotic resistance that are presented by this organism such as derepression of chromosomal AmpC cephalosporinase, production of plasmid or integron mediated β-lactamases from different molecular classes, lower outer membrane permeability (loss of OprD proteins), overexpression of active efflux systems, synthesis of aminoglycoside modifying enzymes (phosphoryl transferases, acetyl transferases, and adenyl transferases), and structural alterations of topoisomerases II and IV determining quinolone resistance. Taking into consideration that all

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these mechanisms are often present simultaneously thereby conferring multiresistant phenotypes (3–6).

Quinolones (fluoroquinolones) and aminoglycosides are the major classes of antibiotics used in the treatment of infections caused by *Pseudomonas aeruginosa.* Fluoroquinolones act by inhibition of DNA replication and transcription via inhibition of DNA gyrase and topoisomerase IV. The mode of action of aminoglycosides depends on the inhibition of protein synthesis by binding to the 30s ribosomal subunit resulting in misreading of mRNA by inhibition of the transfer of peptidyl-tRNA across the ribosome (7). Even the use of antibiotic combinations between β-lactams antibiotics or β-lactams with β-lactamase inhibitors or different classes of aminoglycosides is useless in the management of pseudomonas infections (8). This has directed the attention to novel antimicrobial agents such as antibodies, phages, selective peptides, novel β-lactams, or other combinations of novel β-lactamase inhibitors with known penicillins or cephalosporins (8).

Role of efflux pumps in antibiotic resistance

Efflux pumps contribute to multidrug resistance as they expel different types of antibiotics and chemicals such as dyes, organic solvents, detergents, molecules needed for the cell–cell communication, biocides, and metabolic products. Hence understanding the mechanisms by which these pumps act and how to overcome its activity opens the door for restoring the antibiotic activity and constitute a promising target for novel antibacterial agents (9–13).

The bacterial multidrug efflux transporters can be divided into five classes: (1) small multidrug resistance (SMR), (2) major facilitator superfamily (MFS), (3) resistance nodulation cell division (RND), (4) multidrug and toxic compound extrusion (MATE), (5) ATP-binding cassette (ABC). Those five classes obtain energy required for the active transporting either from H+ protons (RND, SMR, and MFS), Na+ dependant (MATE), or by hydrolysis ATP (ABC) (12, 14).

The efflux pump transporter in *Pseudomonas aeruginosa* belongs to the (RND) family. It is composed of three parts, the transporter, the linker, and the outer membrane pore that ensures that the extruded compound does not remain in the periplasm, hence, avoiding its return to the cytosol (15). There are 12 types of RND efflux systems including for example MexAB-OprM, MexCD-OprJ, MexEF-OprN, MexXY-OprM, MexPQ-OpmE, MexMN-OprM, and MexVW-OprM that differ in their substrates as shown in Table 1. Of these different types of efflux pumps, MexAB-OprM is the one constitutionally expressed in *Pseudomonas aeruginosa* accounting for the intrinsic resistance to flouroquinolones and pathogenicity of this organism (13, 16–19). As shown in Fig. 1 MexAB-OprM consists of three subunits, MexA and OprM acting by substrate recognizing energy transfer and connecting the MexB and OprM, and the antibiotic discharge duct protein (20). As a result, antibiotics are entrapped by MexB and transferred to OprM and finally extruded by MexA (21–23). Higher resistance profiles of *Pseudomonas aeruginosa* to quinolones can result from mutations in the genes encoding for efflux pump MexAB-oprM that regulate the resistance for quinolones, β-lactams, and β-lactamase inhibitors (24). Beside its well-defined activity against the known antimicrobial agents, MexAB-OprM pump confers resistance to the other non-antibiotic compounds such as tea tree oil and its monoterpene components α-terpineol and the related alcohols (25).

In spite of being important to provide resistance to many antibiotics, MexAB-OprM pump cannot confer resistance to the human antimicrobial polypeptides cathelicidins, α and β-defensins by testing the action of these antimicrobial agents versus the wild-type pseudomonas strains and strains mutant in the genes encoding for the pump expression (26). On the other hand, it was shown that MexXY-OprM has a role in the resistance to

Table 1. Substrates and the general regulators of major efflux pumps in *Pseudomonas aeruginosa*

Efflux pump	Substrates	Regulators and function	Reference
MexAB-OprM	β-lactam, β-lactam inhibitors, SDS, fluoroquinolones, tetracycline, novobiocin, chloramphenicol, macrolides, trimethoprim, triclosan, ethidium bromide, aromatic hydrocarbons, thiolactomycin, cerulenin, acylated homoserine lactones.	MexR (repressor)	(54, 58)
MexCD-OprJ	β-lactam, fluoroquinolones, tetracycline, chloramphenicol, novobiocin, macrolides, trimethoprim, triclosan, ethidium bromide, SDS, aromatic hydrocarbons, crystal violet, acriflavin.	NfxB (repressor)	(54)
MexEF-OprN	Fluoroquinolones, chloramphenicol, trimethoprim, triclosan, aromatic hydrocarbons, pseudomonas quinolone signal (precursors)?	MexS (repressor) MexT (activator)	(21, 54)
MexGHI-OprD	Vanadium, acylated homoserine lactones?	LasR? RhlR? (unknown)	(19)
MexXY	Tetracycline, erythromycin, aminoglycosides, fluoroquinolones	MexZ (repressor)	(14)

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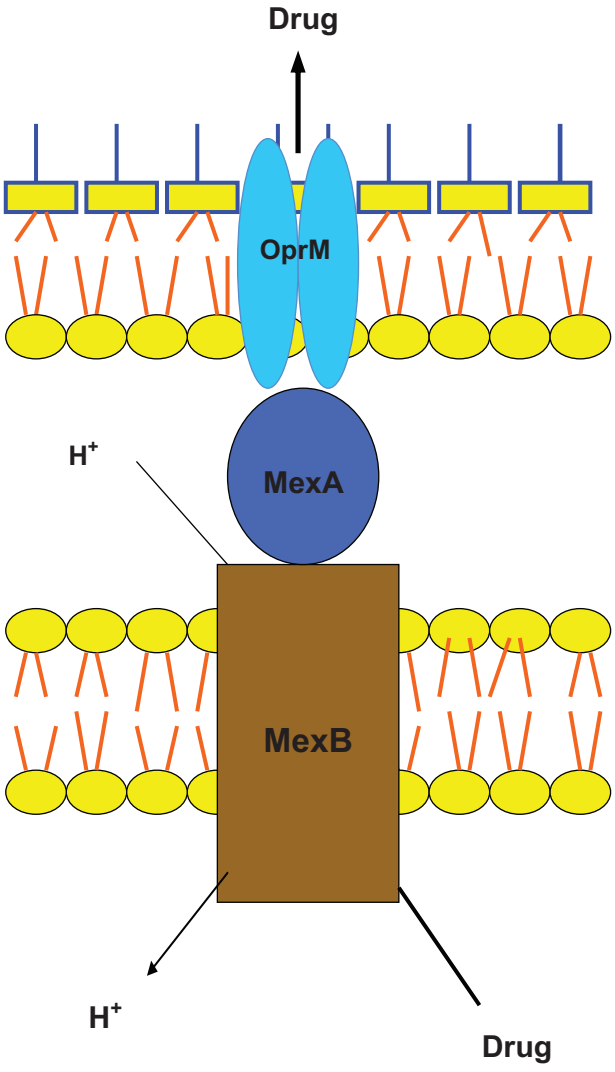

Fig. 1. Schematic illustration of the main efflux pump MexAB-OprM in *Pesudomonas aeuroginosa* as examples for the Resistance-Nodulation-Division (RND) family showing that it is energy dependant on hydrogen protons.

aminoglycoside antibiotics that was approved by the deletion of the genes responsible for this efflux pump expression resulting in the increased sensitivity of the mutant strains to aminoglycosides than the wild-type strains (27).

Role of efflux pumps to antibiotic resistance in biofilms

It is well known that biofilms can play an important role in resistance to antibiotics due to the extracellular polysaccharide matrix, higher bacterial cell density, and lower bacterial growth that provide a good protective means for the bacterial cells against antimicrobial agents (28). In addition, a novel efflux pump has been identified as important to increase the bacterial antibiotic resistance in biofilms rather than the planktonic cells where the deletion of the genes encoding for this pump render the cells in biofilms more sensitive to antibiotics, such as tobramycin, indicating that this efflux pump is essential to pseudomonas antibiotic resistance in biofilms rather than the planktonic state (29).

Genetic organization of *Pseudomonas aeruginosa* RND efflux operons

The genetic organization of RND efflux pump operons comprises of three steps. First, the genes regulating the RND transporter and membrane fusion protein are always present. Second, the genes encoding the operon regulators and the outer membrane channel proteins are not always present taking into account that some operons do not have regulatory genes or outer membrane regulatory gene linked to the efflux operon. Third, some additional regulatory genes can be present beside the regulatory genes for the efflux transporter and membrane fusion protein such as in case of *mexGHI-opmD* that contains *mexG,* which encodes a membrane protein required for pump function (30).

Regulation of expression of *Pseudomonas aeruginosa* RND efflux pumps

Efflux systems other than MexAB-OprM are tightly regulated that recently was verified by transcriptional profiling using Affymetrix Gene Chips. This expression differs from one system to the other, where the expression of MexGHI-OprM is dependent on the cell density indicating that its regulator is the quorum signaling (26), while in the case of MexXY its expression is inducible and enhanced by the presence of certain antibiotics (31). The CzrAB-OpmN divalent cation RND pump is regulated via two compartment regulatory systems in the presence of divalent cations and some isolates that resist antibiotic treatment show susceptibility to the same antibiotic when propagated in the laboratory suggesting inducible components of resistance (13). Other efflux systems are overexpressed as a result of mutations in their encoding genes for regulatory proteins due to the exposure antimicrobial agents either *in vivo* or *in vitro* taking into consideration that these mutations are persistent, which is indicated by the expression that remains long even after removal of the selective pressure (32). For MexAB-OprM, which is always expressed in low but detectable amounts, Hao et al. (33) have shown that MexR, which is a MarR family protein, can negatively regulate multidrug efflux systems in *Pseudomonas aeruginosa*. The mechanism of MexR-regulated antibiotic resistance is due to the formation of intermonomer disulfide bonds in MexR dimer that leads to its dissociation from promoter DNA, derepression of the *mexAB-oprM* drug efflux operon, and increased antibiotic resistance of *Pseudomonas aeruginosa*. It

was also shown that macrolide antibiotics can down-regulate the gene expression of MexAB-OprM, where azithromycin lowers the expression of this efflux pump by 70% resulting in the increased sensitivity of *Pseudomonas aeuroginosa* to different antibiotics such as chloramphenicol and tetracycline. Besides the previously known regulators, there are other unidentified regulators since many efflux systems are expressed in the absence of their regulatory factors (34, 35). Eisaku et al. (36) have shown that the OprM subunit of efflux pump MexAB-OprM can be replaced by the OprN subunit in MexEF-OprN without affecting its activity but the reverse is not.

The efflux pump inhibitors as new therapeutic agents

The continuous increase in the development of multidrug resistance by many pathogens has resulted in difficulties fighting many infectious diseases. In view of the fact that the majority of those multidrug resistant pathogens expresses and overproduces efflux pumps that are responsible for the expelling and extruding of the antibiotics from inside the cells, the new direction for other chemotherapeutics is the use of efflux pump inhibitors (EPIs) (37). Using the (EPIs) together with antibiotics can reduce the invasiveness of *Pseudomonas aeruginosa* besides its role in lowering the antibiotic minimal inhibitory concentration (18). For example, the sensitivity to ciprofloxacin by *Pseudomonas aeruginosa* is largely increased upon using this inhibitor proving that efflux pumps play a role in the resistance of this organism to this antibiotic (38). Thus the inhibition of the efflux pumps is promising in order to (1) increase the intracellular drug concentration, (2) restore the drug activity against the resistant strains, and (3) minimizes further development of resistant strains. However, this requires the understanding of the structural and physiological mechanisms of the responsible efflux pumps (29, 39).

The inhibition of efflux pumps can be achieved by different mechanisms as shown in (Fig. 2): (1) interference with the regulatory steps needed for the expression of the efflux pump, (2) chemical changes in the antibiotic structure hence hindering its attachment as the specific substrate, (3) disruption of the assembly of the efflux pump-components, (4) inhibition of the substrate (antibiotic) binding by either competitive or non-competitive binding using other compounds, (5) blocking the outer

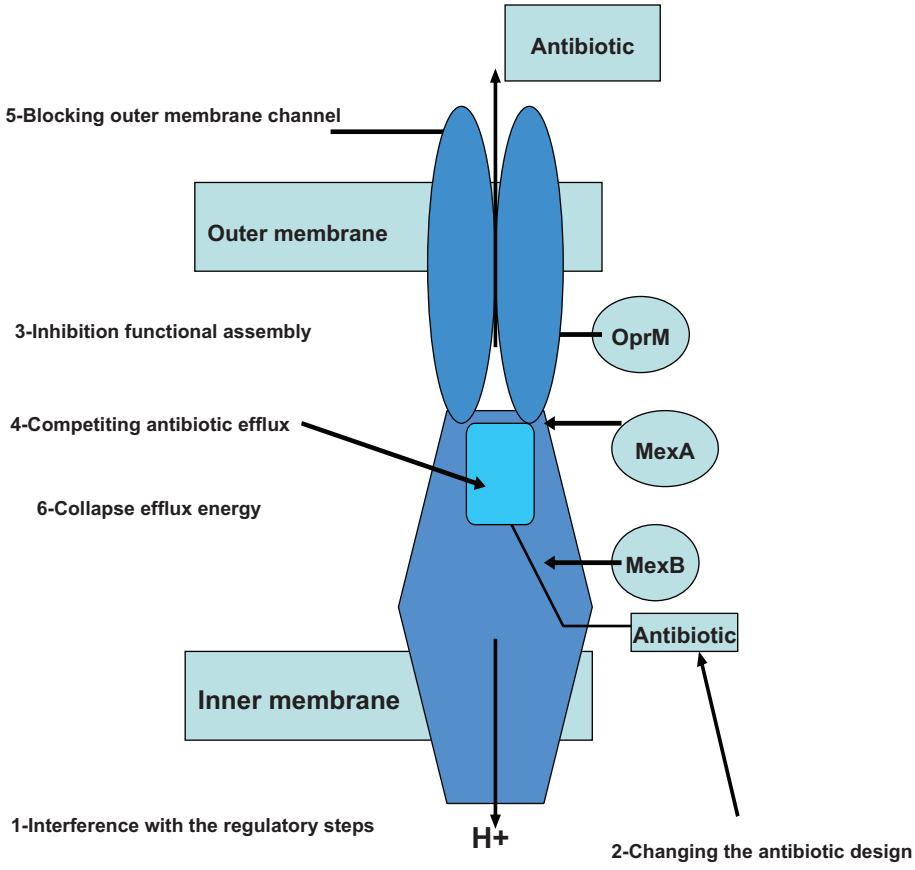

Fig. 2. Schematic illustration showing the general mechanisms of efflux pump inhibition and the targets that can be affected using efflux pump MexAB-OprM efflux pump as an example.

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most pores responsible for the efflux of antibiotic compound, (6) interference with the energy required for the pump activity (37, 40).

Many compounds were tested for their efflux pump inhibition ability including some analogues for antibiotic substrates and other chemical compounds, but few are used that take into consideration the structure-activity relationship and the spectrum of the activity (39). The general methodology used for testing the efficacy of these efflux inhibitors is simply performed by comparing the intracellular concentration of the added antibiotic to the bacterial cell culture before and after the addition of the (EPIs) such as phenylalanine arginyl β-naphthylamide (PAβN). If the compound under testing showed higher intracellular concentration of the antibiotic, it is considered a good efflux inhibitor and vice versa (41, 42).

Peptidomimetic compounds (PAβN) as efflux inhibitors against pseudomonas infections

The most widely used compounds as (EPIs) for pseudomonas overexpressing MexAB pump are the group of peptidomimetic molecules with phenylalanine arginyl β-naphthylamide (PAβN) as a leading compound (MC-207,110) (Fig. 3a) and other many derivatives being studied using the structure activity relationship. The mechanism of action of these inhibitors is through competitive inhibition mechanism, where the efflux pumps recognize them as a substrate instead of the target antibiotics (quinolones mainly ciprofloxacin and levofloxacin) and as long as the pumps expel these inhibitors outside the cells, the antibiotic remains intracellular and increasing in concentration. Taking into consideration that PAβN has a differential behavior, meaning that it can compete with certain antibiotics and not the other depending on the nature of the efflux pump and the large substrate-binding site (43–45).

It was also shown that PAβN can restore the activity of other unrelated antibiotics such as chloramphenicol and macrolides; hence, it can be considered a broad spectrum efflux pump inhibitor (43, 46). Other derivatives were designed from PAβN such as MC-04,124 (Fig. 3a), which is more stable in biological fluids, shows less toxicity levels and more activity against *Pseudomonas aeruginosa* overexpressing efflux pumps (47).

Structure-activity relationship of peptidomimetic compounds

The structural activity relationship of these compounds revealed that the modification of these compounds by the addition of various hydrophilic chains had yielded a pyridopyrimidine compound (Fig. 3b) with improved solubility (e.g. morpholine derivative) that showed higher activity through the potentiation of flouroquinolones and β-lactam antibiotics in addition to inhibition of the β-lactam efflux (17, 48, 49).

The PAβN-derived (EPIs) still remain the most studied and developed family against *Pseudomonas aeruginosa*, though more studies concerning the structure activity relationship, pharmacokinetics, and stability in biological fluids are required. The main advantage of using the PAβN-derived (EPIs) is the difficulty to develop resistance to them, where any pump mutation leading to

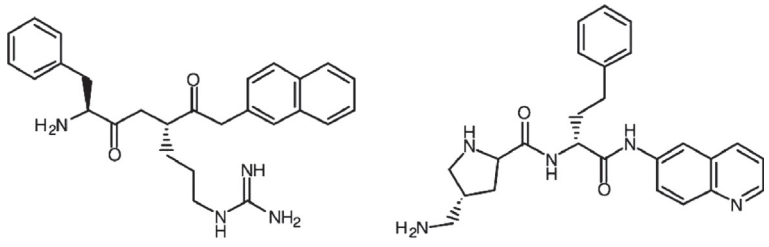

Fig. 3. Efflux pump inhibitors (EPIs) acting against *Pseudomonas aeruginosa* (a) phenylalanine arginyl β-naphthylamide, PAβN (MC-207,110) and its derivative (MC-04,124) and (b) pyridopyrimidine derivative.

inhibitor resistance will lead to resistance to the antibiotic substrate. The disadvantage of those compounds is their low affinity to the target that necessitates the use of higher doses and for a longer time in addition to only substrates that share the inhibitor binding site will be affected (40).

The main drawbacks associated with these EPI compounds is their toxic properties hindering their clinical applications; however, they are used in order to evaluate the different efflux mechanisms expressed by different pathogenic bacteria besides the measurement of the affinity of efflux pumps to them in relation to the antibiotics (46, 50). It has been demonstrated that PAβN is more potent as an efflux inhibitor against MexAB-OprM pump of *Pseudomonas aeruginosa* when compared to quinoline derivatives (another class of EPIs) that could be attributed to the difference in the screening protocols for the antibiotic used as a substrate (levofloxacin versus chloramphenicol) for the two EPI classes (peptidomimetics or quinoline derivatives) (41).

The Mpex Company has already developed a compound that is a combination of an antibiotic and EPI for the systemic treatment of serious hospital acquired infections caused by multidrug resistant pathogens such as *Pseudomonas aeruginosa* (37).

Is there a role for older antimicrobials and infection control measures?

The discovery of polymyxins goes back to 1947 as products being synthesized from *Bacillus polymyxa* (51). There use has declined in the late 1970–1980s because of their neuro- and nephrotoxicity and emergence of newer less toxic antimicrobials (52). However, as many multidrug-resistant bacteria including pseudomonas are still only susceptible to polymyxins (polymyxin B and colistin [polymyxin E]), this has leading clinicians reconsidering their application for the management of multidrug-resistant gram-negative bacterial infections in clinical practice. In a number of recent studies, it has been demonstrated that polymyxins remain active against most gram-negative bacteria in vitro, and that intravenous or inhaled polymyxins have been found to be effective and considerably less toxic than reported in older studies (52, 53). For instance, the global resistance rate against polymyxin B in different centers in Europe, Asia, and America were 1.3% for *P. aeruginosa* and 2.1% for *A. baumannii* isolates (54). Colistin is available commercially as colistin sulfate (i.e. colistin) and sodium colistin methanesulfonate (CMS), which is administered parenterally. The CMS is an inactive prodrug of colistin and, after parenteral administration, colistin is formed *in vivo* (55, 56).

On the other hand, as cross-contamination appears to be a common route for the spread of infection by MDR resistant pseudomonas spp. among patients, there is a need to underscore the importance of stricter control measures with recommended infection control practices to limit the spread of MDR pseudomonas clones inside the hospital environment (57, 58).

Conclusions

Pseudomonas aeruginosa is responsible for a high percentage of nosocomial infections. The difficulty in its treatment by antibiotics arises from its capability of nearly expressing all mechanisms of antibiotic resistance; hence, it is considered a multidrug-resistant (MDR) organism (15).

Many compounds have been identified as (EPIs) when used as adjuvants or in combination with the effective antibiotics (37). It is also important to take into account both the pharmacokinetics and pharmacodynamics when choosing the efflux inhibitors to be adapted with those combined antibiotics (59). In addition, more assay techniques are required in order to design and quantify the (EPIs) and measuring their kinetic parameters in relation to the efflux pump components. These parameters are essential for choosing between the general EPI that can inhibit the action of one transporter that expels various antibiotics in one bacterial species or a specific EPI that inhibits the pumping of one antibiotic family in various bacteria (39).

In this review it was demonstrated that efflux pumps are very important to provide resistance to antimicrobial agents especially in *Pseudomonas aeruginosa*, hence they can be targets for new promising antimicrobial compounds. The benefits of these (EPIs) will be the ability to reuse the traditional antibiotics that became no longer effective due to the development of bacterial resistance through the efflux pumps; hence, this will save a lot of cost and effort that is required to develop new antimicrobial agents, which will face the same problem of developing microbial resistance in the case that these pumps are not inhibited. Many studies showed that the use of peptidomimetic molecules as phenylalanine arginyl β-naphthylamide (PAβN) or other derivatives of PAβN by structural modifications results in a significant decrease of *Pseudomonas aeruginosa* resistant to antibiotics – mainly flouroquinolones. In the future more derivatives are needed using the structure activity relationship in order to minimize the disadvantages of the present compounds associated with higher toxic properties and less biological stability. Also more advanced techniques for assaying the (EPIs) and determination of the exact efflux system for each antibiotic are required in order to design appropriate inhibitors for antibiotics.

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