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Narrative Review

Efflux Pump Inhibitors Derived From Natural Sources as Novel Antibacterial Agents Against *Pseudomonas aeruginosa*: A Review

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Abstract

Infections resulting from *Pseudomonas aeruginosa* are important due to their highest resistance against all clinically used antibiotics. To date, 11 different efflux pumps of the RND family in *P. aeruginosa* that enable the efflux of antibiotics/anti-microbial production have been detected. Carvacrol of *Satureja khuzestanica* is one of the most effective compounds with the ability to affect bacteria. This study aimed to evaluate herbal compounds with inhibitory activities. These pumps include MexAB-OprM, MexCD-OprJ, MexEF-OprN, MexGHI-OpmD, MexJK-OprM/OpmH, MexMN, MexPQ-OpmE, MexVW-OprM, MexXY-OprM, TriABC-OpmH, and MuxABC-OpmB. Unfortunately, among bacteria, *P. aeruginosa* are highly resistant to drug compounds. Because of this high resistance, its importance in nosocomial infections and burns, and that it often causes diseases in immunocompromised patients, finding a therapeutic supplement is essential. In this study, drug compounds against efflux pump genes were sought.

Keywords: Efflux Pump, *Pseudomonas aeruginosa*, Antibacterial, Mex, Natural

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Introduction

Pseudomonas aeruginosa is a gram-negative bacterium that can survive in different environments, including soil, plants, and animals. Moreover, it is the most opportunistic human pathogen, particularly in immunocompromised patients, and has been recognized as one of the five main pathogens in nosocomial infections.[1] *Pseudomonas* diseases are mainly seen in urinary tract infections (UTIs), burn wounds, and pulmonary infections such as cystic fibrosis (CF). Mortality rates in *P. aeruginosa* infections are up to 50% and 70% in patients with bacteremia and nosocomial pneumonia, respectively.[2] *P. aeruginosa* infections are diverse, because they have a multiplicity of resistance mechanisms and have developed different metabolic and nutritional pathways.[3]

Of the resistance mechanisms to be noted is the formation of biofilms, which provide better protection against different antibiotics and body defense systems.[3] Additionally, low outer cell membrane permeability causes intrinsic resistance to antibiotics and increases the number of multi-drug resistance (MDR) strains in *P. aeruginosa* (resistance ≥3 antibiotic classes).[4-7] Moreover, the high expression of efflux pumps can rapidly expand antibiotic resistance as well.[8] This review discusses types of efflux pumps, the efflux pumps role in pathogenicity, the importance of efflux pump inhibitors (EPIs), diagnostic methods for their detection, and compounds derived from natural sources that reduce infections caused by MDR *P. aeruginosa*.

Efflux Pumps and Their Importance in Antibiotic Resistance

Since the discovery of bacterial efflux pumps in the 1980s, many of them have been characterized.[9] Efflux pumps are membrane proteins and participate in the extrusion of antibiotics and chemicals such as organic solvents, dyes, detergents, intermediate molecules in cellular communications, metabolic products, and biocides in prokaryote and eukaryote cells.[10]

Substrates of note of the efflux pumps are oxazolidinones and tetracyclines. In the case of oxazolidinones, since the potency against gram-positive pathogens fails by intrinsic

efflux, they are only used in infections generated by gram-positive bacteria. Great efforts are currently being made toward producing oxazolidinone derivatives that confuse the relevant gram-negative efflux pump.[11]

In the tetracycline class, there are newer compounds that differ from their progenitors by having lower affinity for efflux pumps and the so-called glycylcycline subclass. For example, the new glycylcycline tigecycline confuses a number of tetracycline-specific efflux pumps of gram-negative bacteria.[12] Recently it has been shown that some EPIs can increase the activity of tigecycline.[11] Of course, the majority of efflux systems are not drug-specific proteins and extrude a broad spectrum of compounds from bacteria.[13]

Although efflux pumps are very effective in the growth of multidrug-resistant bacteria, it is obvious that the efflux of drugs is not their main function. These pumps contribute to various processes of bacterial pathogenesis, such as participation in the escape from host defense mechanisms, colonization, biofilm production, and toxin production.[14] Therefore, by understanding the mechanism of action pumps and ways to dominate their pathogenicity, we can establish a promising path for novel antibiotics.[15,16]

Efflux pumps were reported both in gram-negative and gram-positive bacteria and even in a few eukaryotic cells.[17] In gram-negative bacteria, the majority of the efflux pumps have a three-part structure that overpasses both inner and outer membranes. This assembly directly extrudes substrates from the intracellular to the extracellular and causes the ineffectiveness of drugs.[18]

The bacterial multidrug efflux pumps are categorized into the RND (resistant nodulation division superfamily), the MFS (major facilitator superfamily), the SMR (small multidrug resistance family of drug metabolite transporters [DMT] superfamily e.g., *E. coli* EmrE), the ATP-binding cassette (ABC) superfamily, and the MATE (multidrug and toxic efflux family).[16,19] For the active transport of substrates, those gain energy from proton motive force (RND, MFS, and SMR), Na+ dependent (MATE), or by ATP hydrolysis (ABC).[2,16,20] RND and MFS are the most frequently used systems. RND is in gram-negative bacteria only (represented by *P. aeruginosa* Mex pumps and *E. coli* AcrAB-TolC), but MFS is in both gram-negative and gram-positive bacteria.[21]

Efflux Pumps in *Pseudomonas aeruginosa*

P. aeruginosa has various efflux pumps such as RND and MFS superfamilies; however, the main efflux pump belongs to the RND family.[22,23] Table 1 shows the major transporters identified in *P. aeruginosa* plus the major antibiotic classes they pass. The RND pumps, which exchange antibiotic/proton, are located in the inner membrane (IM). They must interact with the outer membrane (OM) channel through periplasmic linker protein (also known as membrane fusion protein) and, therefore, produces a tripartite complex. OM ensures that the removed substrate does not stay in the periplasm (Figure 1).[24,25]

There are 10 types of RND efflux pumps in *P. aeruginosa* that differ in their substrates and release of multi-class drugs.[26] Of these, MexXY-OprM and MexAB-OprM are more important due to their high prevalence in clinical strains and their potency in expelling various classes of antibiotics.[27] These 2 systems are permanently expressed with an amount specified in the wildtype strains and are responsible for the intrinsic resistance to fluoroquinolones.[1,26] Of course, the expression of MexAB-OprM is greater than that of MexXY-OprM. Both pumps are inducible when expose bacteria with antibiotics. The other pumps (MexEF-OprN, MexJK, MexCD-OprJ, MexGHI-OpmD, MexPQ-OpmE, MexVW-OprM, MexMN-OprM, and TriABC) are not in the wild type strains, but are expressed in resistant isolates and may participate in biocide or antibiotic resistance.[28]

The most studied tripartite efflux pumps are the MexAB-OprM and AcrAB-TolC transporters from *P. aeruginosa* and

Table 1. Main Efflux Systems in *Pseudomonas aeruginosa* and Their Antibiotic Substrates

Antibiotics	Efflux pump	Pump Family	References
Chloramphenicol	CmlA	MFS	16, 23
Chloramphenicol	MexEF-OprN, MexCD-OprJ, MexAB-OprM	RND	16, 31-33
Erythromycin, Roxythromycin	MexCD-OprJ	RND	31, 33
Macrolides, Lincosamides, Ketolides	MexCD-OprJ, MexAB-OprM, MexXY-OprM	RND	16, 31, 32
Glycylcyclines	MexXY-OprM, MexAB-OprM, MexCD-OprJ	RND	32, 34
β-lactams	MexAB-OprM, MexCD-OprJ	RND	32
Aminoglycosides	EmrE homologs	SMR	23, 25, 32
Aminoglycosides	MexXY-OprM, MexAB-OprM	RND	23, 32
Oxazolidinones	MexAB-OprM, MexXY-OprM	RND	16, 31, 32
Fluoroquinolones	MexAB-OprM, MexCD-OprJ, MexEF-OprN, MexXY-OprM, MexVW-OprM	RND	16, 31-33
Fluoroquinolones	PmpM	MATE	35
Nalidixic acid, norfloxacin	Orf12-Orf11-Orf10 (plasmid)	ABC	31
Trimethoprim	MexCD-OprJ, MexEF-OprN, MexAB-OprM	RND	36
Tetracyclines	MexCD-OprJ, MexAB-OprM, MexXY-OprM	RND	20, 36
Tetracyclines	Tet A, C, E	MFS	23
Sulfamides	MexAB-OprM	RND	27

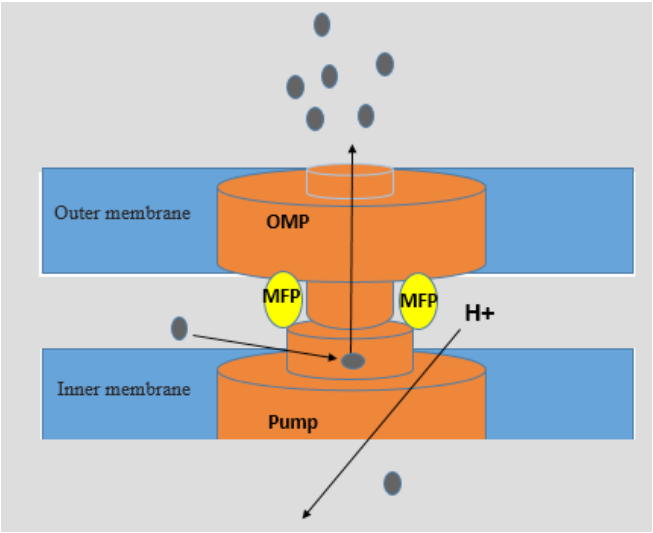

Figure 1. Schematic Representation of Drug Efflux Pumps and Pathway of Drug Efflux Across the IM and OM in *Pseudomonas aeruginosa*. Efflux transporters in *P. aeruginosa* are three components (a drug-proton transporter in the inner membrane, a membrane fusion protein (MFP) in the periplasmic space, and an outer membrane protein (OMP). For example, in the MexAB-OprM pump, the IMP is MexB, which identifies specifically with substrates and catalyzes proton dependent drug transport. MFP is MexA and links the OMP and the IMP. These efflux pumps capture the antibiotic from the IM and the periplasm and directly extrude it out of the cells. Finally, OprM plays the role of OMP.

Escherichia coli, respectively.[29] The inner membrane proteins (IMPs), MexB, and AcrB are similar too, and in research, MexB can be used as a substitute for AcrB.[30] MexEF-OprN confers resistance to doripenem and meropenem, but this resistance may be caused by the low expression of OrpD porin.[27]

Aminoglycosides have been introduced as poor substrates for efflux pumps due to their very hydrophilic trait. Not long ago, they were proven to be transported by a large number of efflux pumps from the RND superfamily, such as the MexXY-OprM pump of *P.* aeruginosa (Table 1).

Colistin, the last weapon in the fight against MDR *P. aeruginosa*, is not effluxed by these pumps. Ciprofloxacin (Cip) and levofloxacin (Lev) are antibiotics that can be substrates for all main RND pumps (Table 1).[27]

EPIs as Novel Therapeutic Instruments

The continuous prevalence of MDR strains of *P. aeruginosa* have made treatment difficult and necessitated the detection of new antibiotics. According to the decrease in antibiotic expansion, however, it is necessary to search and characterize the compounds that return the activity of older antibiotics against bacteria.[37] Among these compounds, resistance-modifying agents, more especially EPIs, can be noted.[9,38] EPIs are the molecules which disorder the process of extruding antibiotics and toxic substances from the bacterial cells with different mechanisms.[39] Applying EPIs is the most promising approach to inhibiting the multidrug efflux pumps. Indeed, based on their properties and by diverse mechanisms, EPIs can disrupt the function, assembly, and expression of efflux pumps as shown in Figure 2 and Table 2. Moreover, pump inhibitors are applied as diagnostic tools. MC- 207,110 is usually applied for the detection of active efflux pumps in gram-negative pathogen profiling and reserpine for gram-positive bacteria.[23] They can be used as diagnostic tools for the detection of active efflux in pathogens as a mechanism of resistance. For this application, narrow-spectrum inhibitors which allow the gross identification of the transporters that are expressed are preferred.

Compounds such as dinitrophenol (DNP), valinomycin, and carbonylcyanide *m*-chlorophenylhydrazone (CCCP) impacts the energy measure of the cell membrane and suppresses entirely the efflux of various molecules.[11] Valinomycin collapses the electrochemical gradient generated by potassium ions, whereas DNP and CCCP cause a waste of the proton gradient and collapse the proton-motive force of the cell membrane.[40] Changing the chemical structure of the drug might attenuate potency against its cellular target. For this reason, no novel antibiotic has been designed to date for gram-negative bacteria.[40]

Multidrug Resistance in Human Cells

MDR is a critical problem in the treatment of human diseases. One main mechanism that can cause the improvement of MDR in human cells is drug extrusion by ABC transporters. ABC transporters have 2 transmembrane domains and 2 ATP-binding cassettes. ABC transporters present a significant mechanism for supporting CNS tasks. They have diverse substrates, such as drugs and poisons. The cytotoxic drugs applied for MDR in cancer cells are as follows: Vinca alkaloids, microtubule-stabilizing taxanes (docetaxel and paclitaxel), anthracyclines, epipodophyllotoxins, antimetabolites, topotecan, and actinomycin-D.[41]

The family of ABC pumps has been classified into seven subfamilies. Most investigations into members of this family have been conducted with permeability glycoprotein 1 (abbreviated as P-gp), breast cancer resistance protein (BCRP), and multidrug resistance-associated proteins-2 (MRP2). P-glycoprotein 1, also known as multidrug resistance protein 1 (MDR1), creates resistance to antibiotics and cytotoxic drugs.[11] MDR1 crosses a broad spectrum of substrates which,

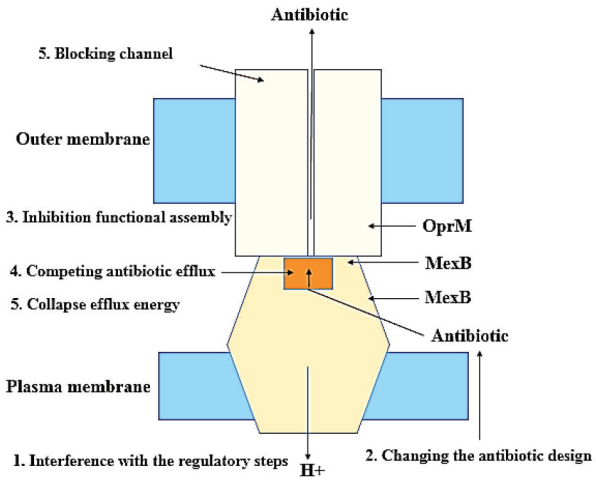

Figure 2. Schematic Showing of an Efflux Pump (MexAB-OprM as an example). The general strategies for efflux pump suppression and the targets that may be influential are displayed.

Table 2. Mechanisms to Inhibit Drug Efflux, Effective Compounds, and Their Study Methods

Mechanism of Action	Example of Inhibitors	Analysis Method	References
Interference in the regulatory steps and suppression of pump expression	*Satureja khuzestanica*	Real time PCR, RT-PCR	49-51
Alterations in the structure antibiotic so the drug easily enters the cell, but is not identified by a pump	A chemical modification, e.g., adds succinate group at taxol	Rhodamine 123 accumulations Assay	40, 46
Disruption of pump assembly	DARPin inhibitors (Ankyrin repeat proteins)	The interaction between the components of the efflux pump can be evaluated with the surface plasmon resonance (SPR) method.	30, 52
Competing inhibition of IMP directly with a high affinity antibiotic	DARPin inhibitors (Ankyrin repeat proteins)	Direct interaction between the EPI and the IMP could be measured by SPR or isothermal calorimetry (ITC)	52
Connect unmediated to the outer pores and block them (block exit duct)	A number of indole derivatives	Blocking of the OMP through the tripartite pump could be determined by suppressing antibiotic efflux.	29
Disrupt PMF and activity of the efflux pump by disorder in the proton gradient	Compounds that disrupt the proton gradient	The measure of the pmf and changes caused by the effect of EPIs on these could be evaluated by the use of fluorescent specific compounds for the ΔpH or Δψ components of the pmf.	53

in addition to chemotherapy, may be used for allergy relief, hypertension, immunosuppression, infections, inflammation, and neurology.[42]

Gemifloxacin is a newer fluoroquinolone and causes a wide range of activity against gram-negative bacteria.[43] The intracellular accumulation of (14C) erythromycin was performed singly and in the presence of gemifloxacin plus the specific inhibitors of MRP2 and P-gp, the MK-571and quinidine, respectively. In addition, gemifloxacin can stimulate the expression of both pumps by activating nuclear hormone receptors such as PXR. Nuclear factors, a superfamily of transcription factors, are stimulated by an excess of endogenous activators such as retinoids, steroids, oxysterols, and bile acids. These activated receptors are then linked to the promoter of the intended gene.[44,45]

Most inhibitor agents with reversal effects can block MDR1 competitively or noncompetitively. Competitive modulators such as verapamil act as a substrate with the cytotoxic drug for cross by the transporter. A noncompetitive inhibitor such as cyclosporin A is not a substrate but binds to the transporter. This causes a structural change in the pump and, as a result, prevents ATP hydrolysis and the extracellular transfer of the drug. Other noncompetitive inhibitors can be connected to drugs such as paclitaxel (taxol) and can cause chemical change in them. Therefore, P-glycoprotein cannot identify taxol and easily transit the blood brain barrier and approach its target without being extruded by P-glycoprotein.[29,46]

Most natural products extracted from medicinal plants (called secondary metabolites) exhibit anticancer properties. The main classes of secondary metabolites with anticancer activities are phenolics (e.g., flavonoids), terpenoids, and alkaloids. The first natural compound applied as an anticancer substrate was Podophyllotoxin that was extracted from *Podophyllum peltatum*. Afterward, taxol, vinca alkaloids (vinblastine and vincristine), and chemical derivatives (teniposide and etoposide) were identified as active parts of *Taxus brevifolia*.[47]

Some secondary metabolites with inhibitory ABC transporters overcome multidrug resistance in cancer cells. For example, the MDR modulatory activity of *Chelidonium majus* can be helpful in cancer treatment. Furthermore, some flavonoids have been proven to block MDR1-mediated transport mechanisms by directly connecting to the adjacent steroid- and ATP -binding sites.[48]

Therefore, the use of EPIs is effective and can (*a*) increase the intracellular antibiotic measure, (*b*) decrease the antibiotic minimal inhibitory concentration (MIC) for the antibiotic, (*c*) improve the activity of an antibiotic against resistant strains and eradicate the resistant bacteria, and (*d*) repress the appearance of MDR strains.[1,9] Nevertheless, for this issue to happen, the physiological mechanisms and structures of efflux pumps must be known.[54]

Types of Efflux Pump Inhibitors

Entrance of amphipathic compounds is difficult in gram-negative bacteria because of the presence of lipophilicity and an additional outer membrane.[55] As a result, very few are special for gram-negative bacteria.[9] A lot of synthetic or native compounds with different natures, such as analogs to an antibiotic, peptidomimetic inhibitors, and other chemical substrates, were examined for their efflux pump inhibition properties against *P. aeruginosa*.[40]

Synthetic Compounds against *Pseudomonas aeruginosa*

Synthetic compounds remain one of the major EPIs. Most compounds used as EPI for *P. aeruginosa* overexpressing MexAB-OprM pumps are groups of peptidomimetic molecules with phenylalanine arginine beta naphthylamide (PAβN) as a leading compound. These compounds are known as C-capped dipeptides. PaβN, or MC-207,110, was the first EPI to be recognized in resistant *P. aeruginosa* and is effective against RND pumps. Therefore, it has been developed for clinical use as an adjuvant (Figure 3a). These inhibitors act with a competitive inhibition approach and are recognized instead of the target antibiotics (quinolones, mainly Lev and Cip) by MexAB-OprM. Until the pumps extrude these inhibitors outside the cell, the antibiotic stays and increases intracellular concentration.[54] It was also revealed that PAβN can increase the potency of other antibiotics such as macrolides and chloramphenicol; therefore, it is considered as a broad spectrum EPI.[56] Unfortunately, it expands the penetrance of the OM for other cells which lack the MexAB-OprM pump.

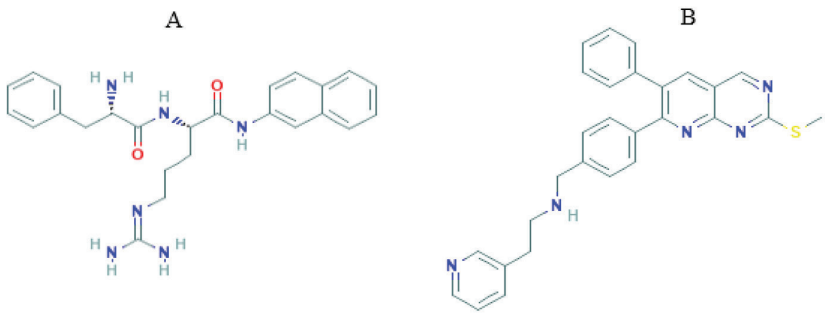

Figure 3. Synthetic EPIs Acting Against *P. aeruginosa* (a) PAβN and its derivatives (MC-02,595, MC-04,124) and (b) pyridopyrimidine derivatives (D13-9001, MBX2319)

This character of PAβN is because of its dicationicity.[57] Albeit the efficacy of PAβN on membrane integrity is lower than that of polymyxin B.

In the following, improved the activity and pharmaceutical properties of PAβN and finally discovered MC-04,124 and MC-02,595 (Figure 3A). All these compounds increase the potency of Lev.MC-04,124 (Figure 3A) and show lower toxicity levels, better solubility, more stability in biological fluids, and more activity against *P. aeruginosa* overexpressing efflux pumps.[54] MC-02,595 exhibited lower toxicity and more stability than the other 2 compounds.[58]

In addition to PAβN derivatives, pyridopyrimidines have also been shown to have high efficiency in inhibiting *P. aeruginosa* efflux pumps (Figure 3B).[40] These compounds showed higher solubility and improved activity through the import of chloramphenicol (Cam) and fluoroquinolone antibiotics as well as inhibition of the β-lactam efflux.[1,54] D13-9001, one of these synthesized compounds, is an analog of ammonium acetic acid; it improves the potency of the Lev and aztreonam and achieved a good safety level in the acute toxicity assay. Hence, it is used as MexAB-OprM EPIs in *P. aeruginosa*.[11] Finally, another pyranopyridine, MBX2319 (Figure 3B), has recently been characterized as having high activity against Enterobacteriaceae and low activity vs. *P. aeruginosa*. This inhibitor is in the initial phases of optimization and has not been evaluated in in vivo models.[57]

Unfortunately, other than EPI properties, synthetic compounds also permeate up the outer membrane. A number of these inhibitors were confirmed using in vivo models; however, they were prohibited because of toxicity.[29,59-61] For example, the moieties of PAβN that were applied for activity in *P. aeruginosa* infections caused nephrotoxicity.[57]

Other EPIs that have been studied in detail are quinolines. These compounds inhibit the AcrAB pump and elevate the activity of multiple antibiotics such as cycline, quinolone, and phenicol against gram-negative bacteria. Due to the link of the alkyl side-chain and chlorine to the heterocyclic moiety of quinoline, are synthesized quinolone analogs such as alkoxy-, thioalkoxy-, alkylamino-, and chloro-quinolines that have a slight efficiency against *P. aeruginosa*.[11,62]

On the other hand, quinazolines have also been applied in different areas as EPIs. By adding different active groups to the quinazoline, a variety of quinazoline derivatives with various biological properties have been synthesized.[63] Among the alkylaminoquinazoline derivatives, the compounds that encompass a morpholine group along with a propyl chain are more effective than the others.[64]

Table 3 shows the compounds that act as EPIs against *P. aeruginosa*. The purpose of the term EPI here is that some of the compounds were identified according to their synergism with antibiotics, whereas no analysis was performed to study non-specific impacts (e.g., membrane permeabilization) or survey the mechanism of inhibition.[29]

Native Compounds Against *Pseudomonas aeruginosa*

The use of native knowledge of traditional physicians provides helpful data for investigating new drugs.[71] The potential antibacterial characteristics of diverse natural medicinal substances are being widely studied in different parts of the world.[72] Among these natural substances, it has been established that many medicinal plant substances have activity against *P. aeruginosa*.[55,73,74] Plants are extracted with water, methanol, and chloroform. The important issue is that none of the solvents should have antimicrobial activity at the applied concentration.[37] Plant antimicrobials have not been applied in systemic infections.[55] Additionally, some plant antimicrobial peptides demonstrate side effects against mammalian cells. Therefore, before being prescribed in new clinical treatments, their toxicity to humans must be evaluated in vivo. Bioassays have shown the toxicity of plant extracts. Meanwhile, supplementary clinical trial research is necessary to obtain EPIs which are nontoxic at higher concentrations.[68]

Native Compounds With EPI Property

Due to the high chemical diversity in plant extracts, it is expected that they are a potential origin of drug resistance modifying substances. The ability of plant extracts to inhibit efflux pumps was discovered by Stavri et al.[9] In accordance with Table 3 and Figure 4, some plant extracts show EPI properties against *P. aeruginosa*. In addition, the extracts of *Thymus maroccanus* and *Thymus broussonetii* represented synergistic properties when mixed with Cam against efflux pump-overexpressing strains of *P. aeruginosa*.[75] Moreover, ethanolic extracts of *Vernonia adoensis* and *Mangifera indica* in the accumulation of rhodamine 6G (R6G) showed the potential properties of EPIs against *P. aeruginosa*.[39]

Table 3. Summary of Compounds That Are Active as EPIs Against *Pseudomonas aeruginosa*

Compound	Source	Protein	Actions	Ref.
Synthetic Compounds				
Peptidomimetic	Synthetic	MexAB-OprM, MexCD-OprJ, MexEF-OprN	Synergies with Cam, Quinolones, *Carb*, Macrolides, and *Tet*	59
Pyridopyrimidines	Synthetic	MexAB, OprM	Improves the potency of levofloxacin and aztreonam	23
Fluoroquinolone analog	Synthetic	MexAB-OprM	Improves the potency of macrolides and fluoroquinolones	23
4-(3-morpholinopropylamino)-quinazoline	4-alkylaminoquinazoline Derivatives	MexAB-OprM	Reduced MIC of Cam, Nal, Nor, and Spfx, Increased Cam uptake	64
Natural Compounds				
EA-371α and EA-371δ	*Streptomyces* MF-EA-371-NS1	MexAB-OprM	Reduce MIC of Lev	65
Geraniol	*Helichrysum italicum*	-	Reduced MIC of β-lactams, Quinolones, and Cam	66
Curcumin	*Curcuma longa*	-	Reduced MIC Mem, Carb, Caz, Gen, and Cip	67
Lanatoside C and daidzein	*Digitalis lanata* and *Kwao krua*, respectively	MexAB-OprM	Increased uptake of EtBr, Reduced MIC of Carb and Lev	60
Protocatechuic acid	*Camellia sinensis* (green tea)	MexAB-OprM	Reduced MIC of Lev	60
Pheophorbide a	*Berberis aetnensis*	MexAB-OprM	Synergize with Cip	68, 69
Theobromine	*Theobroma cacao*	MexAB-OprM	Synergize with Cip	68, 69
Non-antibacterial drugs				
Amitryptiline, Trans-chlorprothixene	Non-antibiotic drugs	-	Reduced MIC of Pen, Cxm and Tob	70
Sertraline, chlorpromazine	Selective Serotonin Re-uptake Inhibitors	MexAB-OprM	Inhibition of Nile Red efflux	49

Abbreviations: Cam (Chloramphenicol); Carb (Carbanecillin); Caz (Ceftazidime); Cip (Ciprofloxacin); Cxm (Cefuroxime); EtBr (Ethidium Bromide); Gen (Gentamicin); Lev (Levofloxacin); Mem (Meropenem); Nal (Nalidixic acid); Nor (Norfloxacin); Pen (Penicillin); Spfx (Sparfloxacin); Tet (Tetracycline); and Tob (Tobramycin).

Although plant extracts may have no antimicrobial properties singly, when they are applied simultaneously with standard antibiotics, they can act as EPIs and enhance the efficacy of drugs.[39,76]

EPIs derived from microbial origins are somewhat rare. The study of microbial fermentation has led to the identification of 2 new EPIs[65] which were derived from Streptomyces MF-EA-371-NS1, which is a new strain with a close relationship to *Streptomyces vellosus*.[65] EA-371α[40] and EA-371δ[41] both block the MDR pump *of P. aeruginosa* PAM1032, which overexpresses MexAB-OprM.[9]

To use native compounds as EPIs in clinical treatments, their spectrum of activity, potency, toxicity, and pharmacokinetics should be determined. Accurate information regarding the binding site of an EPI and the mechanism of inhibition could assist the scientific design of analogs that could overcome these importance issues.[57]

Manners for Studying EPIs

The most important problem in screening studies for EPIs is that in numerous cases the synergism could be caused by non-specific destruction in the bacterial membrane. As a result, most EPIs, especially PAβN, would act similarly against human cells, and would therefore be cytotoxic. Consequently, comprehensive research with the purpose of confirming true EPI action needs to be done.[29]

The rapid expansion of knowledge in molecular biology and biochemistry as well the accessibility of a great amount of bacterial genome data has simplified investigations for understanding the importance of antibiotic transporter inhibitors in drug resistance. To date, diverse biochemical and molecular methods have been employed to reveal EPIs and distinguish their portion in resistance.[25] To evaluate the phenotypic effectiveness of natural substrates on efflux pumps, both checkerboard and accumulation assays can be applied. These are significant screening tools that allow many EPI potential compounds to be examined easily and quickly.[9] Another phenotypic method for checking the activity potential of antibiotics by EPIs is the agar doubling dilution method.[77] As clarified by Lomovskaya et al,[56] for a substance to be certified as an EPI, it should encompass the criteria shown in Table 3. All these criteria can be researched with advanced techniques (Table 4).

Measuring Synergism (MIC of Antibiotics ± Plant Extracts)

It has been known for a long time that a number of antibiotics show synergism when used simultaneously. The checkerboard microtiter test has been utilized to recognize such agents. This method with variations and modifications has been used to recognize possible inhibitors of efflux pumps.[9] In this modified method, after MIC determination of the natural substance and antibiotic (A) using standard broth dilution methods, 50

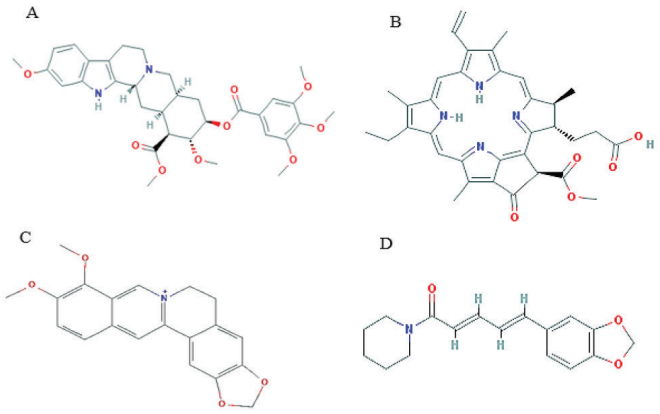

Figure 4. Chemical Structures of 4 Plant Extracts as EPIs, Including Reserpine, Berberine, Piperine, and Pheophorbide.

µL of subinhibitory concentrations for serial dilution for an antibiotic and also 50 µL of subinhibitory concentrations of serial dilution for a natural substance (B) (usually 4×less than the MIC) were combined in a 96-well microtiter plate.[79] The bacterial strains were adjusted to 10^6 CFU/mL cell density and 50 µl was used in each well. The fractional inhibitory concentration (FIC) index was determined according to the following formula[60]:

(MIC of A in mixture/MIC of A singly) + (MIC of B in mixture/MIC of B singly)

The FIC index was applied to interpret the potency of the combinations.[39,60] Synergism was considered as FICI ≤0.5, indifferent was considered as a FICI = 0.5–4, and antagonism was considered as FICI >4.[80] The varying amounts of sensitivity of the bacteria to the EPI and drugs may be explained by the varied efflux pump accessibility rates in different isolates and the combination of ingredients present in the extract.[81]

For gram-positive bacteria, one famous inhibitor, e.g., piperine, CCCP, or reserpine, can be used easily as the positive control together with a natural substrate and target antibiotics.[9,82] Reserpine, a plant alkaloid, was initially extracted from the *Rauwolfia vomitoria* and was found to block Nor A pumps.[9] Piperine is also a plant alkaloid extracted from the family Piperaceae such as *Piper longum* (long pepper) and *Piper nigrum* (black pepper) and can enhance the accumulation of Cip by *Staphylococcus aureus*.[83] According to the, Cip is a substrate of a lot of bacterial efflux pumps,[16] and primary experiments applied a simple bioassay to recognize plant extracts that synergize with Cip.[69]

On the contrary, because gram-negative bacteria utilize numerous efflux pumps, it is harder to recognize inhibitors that specifically influence one or all of these pumps. Therefore, genotypic methods are preferably utilized to study the role of EPIs in these organisms.[84] Using phenotypic methods, Touani et al showed that *Brassica oleacera* var. *butyris* and *Brassica oleacera* var. *italica* extracts containing flavonoids, alkaloids, phenols, sterols, and triterpenes manifested synergistic effects with manifold reductions in the MICs of certain antibiotics examined (antagonistic effects were also detected with some combinations) against MDR *P. aeruginosa*.[85]

In accordance with the report of Garvey et al, all extracts showed higher activity in liquid mediums than in agar. Meanwhile, specific extracts from *Melissa officinalis* and *Levisticum officinale* had the greatest activity in connection with antibiotic potentiation with either tetracycline (Te), Cip, or the dye EtBr against gram-negative bacteria. This information proposes that these extracts are inhibitors of efflux. Fractionation of the chloroform extract of *L. officinale*

Table 4. Required Principles of Compounds to be Certified as EPIs and Their Analyzing Methods

Criteria	Techniques	Reference
It should potentiate the potency of an antibiotic against generated resistance in result of the overexpression of an efflux pump.	Synergism is measured using checkerboard assay.	9
It should enhance the amount of accumulation and reduce the amount of extrusion for substrates of the efflux pump.	This capability can be measured by fluorescent compound accumulation assays in the presence and absence of the putative EPI (drug efflux/accumulation assay).	29
It should not affect the sensitive strains which do not have the intended efflux pump.	Use of a sensitive strain and a wild type strain (resistant) and then the checkerboard method can be executed on the resistant strain.	29
It should not decrease the MIC of antibiotics which are not extruded.	Use of a sensitive strain and a wild type strain (resistant) and then the checkerboard method can be executed on the resistant strain.	29
It should not cause penetrable OM.	Periplasmic β-lactamase property in nitrocefin (a β-lactam) hydrolysis, which alters from yellow to red when hydrolyzed, can be used.	29
It should not change the proton gradient available on the IM; in other words, it should not disturb the membrane integrity	DNA stains with fluorescence properties such as SYTOX Green or propdium iodide, which do not pass the intact membrane of bacterial cells, can be used.	78

revealed that of these, falcarindiol had the maximum antibacterial activity and a strong synergistic effect on the activity of Cip.[37]

In a study by Aparna et al,[60] bioinformatic calculations and checkerboard results proposed that daidzein and lanatoside C are probable inhibitors of the MexAB-OprM efflux system in *P. aeruginosa*. Lanatoside C in principle is a cardiac glycoside blocker and can increase intracellular Ca2+ concentration.[74] The blocking effect of Lanatoside C on efflux systems could probably be a result of its inhibitory effect on sodium pumps.[86]

Seasotiya et al[81] reported that the combination of Cip and Piperine could reduce the amount of MICC against *P. aeruginosa* (MTCC 7453 and MTCC 424) by half, but none of the 35 Indian medicinal herbal extracts could be effective in reducing MIC. Of course, only plants which show no direct antibacterial activity were entered in this study.[60] This issue proves that the EPI of plant extracts potentially have a limited extent. When was being fractioned a plant extract with the synergism capability, but do not have EPI-like activity none of the its components only, it can be assumed that the efflux inhibitor was either inactivated or lost during fractionation or that it is active in combination with another fraction in the extract from which it was isolated.[37]

Fractionation of Extracts

When it is realized that an extract can be synergistic with an antibiotic, the extract is fractionated and the effector molecules are revealed. This requires the use of a significant screening method which enables a lot of fractions to be analyzed easily and quickly. Fractionation was performed on silica gel by vacuum liquid chromatography (VLC). Then, the fractions were purified by thin-layer chromatography (TLC).[37] Eventually, the MIC of antibiotics in the absence and presence of fractions and purified compounds was measured.

Measuring Fluorescent Dye Accumulation ± Plant Extracts

The general methodology for assessing increases in accumulation is easily done by measuring accumulation in the intracellular concentration marker (fluorescent dyes) in the presence or absence of plant extracts with a spectrophotometer.[37,39,60] If the natural substance increases the measure of the marker after confronting, it is considered an important efflux inhibitor and vice versa. For example, lanatoside C and daidzein have shown a notably increased rate in the aggregation of EtBr for *P. aeruginosa*.[60,87]

In an accumulation assay an ionophore (e.g., CCCP or valinomycin) is first used to de-energize bacterial cells. De-energizing caused no compound to be effluxed until the energize phase. In the next stage, ionophore was eliminated by washing, and then cells were loaded with fluorescent compound. Fluorescent efflux was begun by adding glucose (energize) and EPI.[29,56,88]

The accumulation of fluorescent dyes such as ethidium bromide (EtBr), TMA-DPH, Hoechst 33342 (Bisbenzimide), *N*-phenylnaphthylamine (NPN), and berberine enhances the fluorescence measure inside the cells, but an accumulation of R6G and doxorubicin leads to a reduction in the fluorescence signal.[79,89]

Berberine, isolated from the Berberis genus, is a plant alkaloid and has properties such as EPI against the *S. aureus*, weak antibacterial activity, and the ability to intercalate with DNA similar to EtBr.[39] This fluorescent dye makes a powerful yellow when bound to DNA.[9] Therefore, its accumulation inside cells can be easily monitored by measuring the emitted fluorescence.[55] Berberine-producing plants synthesize 2 other substances, the flavonolignan 5-MHC-D and the porphyrin Pheophorbide-α, which have no antibacterial property, but have EPI activity (Table 3).[90]

The main disadvantage of applying fluorescent compounds with this method is that the potential EPI of the compounds could be strongly colored and therefore reduce the accuracy of the measurements.[29] Recently, Bohnert et al investigated using Nile Red a technique that is dominant on this problem. Nile Red has a high fluorescence yield when it is connected with cell membrane phospholipids; however, it is approximately nonfluorescent in external mediums.[91]

R6G is accumulated mostly in the cytosol by joining to proteins or nucleic acids. Results from an R6G uptake study by Chitemerere et alshowed that *Callistemon citrinus* extract inhibited antibiotic efflux pumps. Despite the fact that *P. aeruginosa* is a gram-negative bacterium and due to it having a double membrane, it is less sensitive to drugs or extracts. However, Chitemerere et al reported that *P. aeruginosa* after *S. aureus* is the second most sensitive strain to plant essences.[39]

Use of Molecular Methods in Evaluating EPI

Since approximately all efflux pumps contribute to other systems of resistance in *P. aeruginosa* and a high level of resistance mechanisms hide the influence of the expression of efflux pumps on MICs, obtaining a differential diagnosis using phenotypic antimicrobial methods is difficult. Furthermore, efflux systems can be overexpressed during therapy, which may clarify treatment failures with drugs that are considered useful according to the primary susceptibility profile.[23] Thus, it is better to use the genotypic test along with phenotypic tests to study the expression of efflux pump genes in *P. aeruginosa*.

It has been proven that molecular methods are the only ways to survey the expression of efflux systems in clinical strains. The western blotting technique was introduced first, but subsequently, the reverse transcriptase quantitative PCR method (RT-qPCR) quickly became famous because of its greater rapidity and specificity. Thus, RT-qPCRs were expanded to detect and measure the expression of the genes coding for the diverse proteins of an RND pump. These methods stays can be used in clinical laboratories.[92] One inferential point of the application RT-qPCR by *P. aeruginosa* is that a 2-fold increment in the overexpression of *mex*A and *mex*B genes leads to an increase in MIC values, while expression of the *mex*X should be greater (≥5-fold) in order to increase antibiotic resistance.[93]

In contrast, reverse transcriptase PCR (RT-PCR) is less costly, but laborious. RT-PCR allows the amplification products to quickly be observed and is easily applicable in clinical laboratories where a real-time PCR device is not available.[92] Since the quantity of the expressed efflux pumps is not measured, the efficiency of RT-PCR may be lower in

comparison with real time RT-qPCR. In a study by Jalalvandi et al, the results of RT-PCR revealed that the expression of *mexA* and *mexR* genes, related to MexAB-OprM in *P. aeruginosa*, were significantly reduced after confronting *Satureja khuzestanica* essence.[51] Furthermore, there are commercial kits for use in checking the expression of *P. aeruginosa* efflux pumps, such as the mexQ-TesT kit that facilitates the analysis of *mex*A and *mex*X genes and the expression of clinical strains versus PAO1 (wild strain).[27]

Conclusion

As regards the increasing prevalence of MDR *P. aeruginosa,* particularly in hospitalized patients, and the restrictions in the utilization of broad spectrum antibiotics in immunocompromised patients, pseudomonas infections are considered to be a developing threat to the community.[4] There are 3 ways to combat MDR *P. aeruginosa*:

1. Development of novel antibiotics. Although new antibiotics may be helpful in the short term, the organisms rapidly adapt to the changes.[94]
2. Alternative therapy and attention to novel antimicrobial factors, including phages, antibodies, and selective peptides or the use of medicinal natural products.[95]
3. Combination therapy in order to reach bactericidal synergism.

With the lack of progress in novel antimicrobial development, an interest has emerged in the detection of compounds that restore the activity of licensed antibacterial agents that until recently had extraordinary affections against *P. aeruginosa*.[37] The idea of using a compound which contains a conventional antibiotic and an inhibitor resistance is well verified; co-amoxiclav is a good example.[96] Another example of a combinational command is the prescription of novel β-lactamase inhibitors with cephalosporins or penicillins.[97] However, β-lactam antibiotics or various classes of aminoglycosides are, unfortunately, ineffective in the treatment of MDR Pseudomonas infections.

Efflux pumps have an important role in developing resistance to antimicrobial agents, particularly in *P. aeruginosa*. For this reason, they can be targets for natural antimicrobial compounds.[56] Many plant extracts have been recognized as EPIs when applied as adjuvants in combination with the special antibiotics. To fight the MDR *P. aeruginosa,* a combination of plant extracts with EPI properties and antibiotics would be a better way.[51]

This review has emphasized a number of bacterial EPIs obtained from natural sources, mainly from plants. Some of these substances have remarkable activities and can be optimized in the future. It is suggested that plant extracts must be further studied for their potential to block efflux pumps and these compounds be consumed together with antibiotics as chemotherapeutic agents.

Conflict of Interest Disclosures

The authors declare they have no conflicts of interest.

Ethical Approval

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