**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. *P. aeruginosa* 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. *P. aeruginosa* infections are diverse, because they have a multiplicity of resistance mechanisms and have developed different metabolic and nutritional pathways.

Of the resistance mechanisms to be noted is the formation of biofilms, which provide better protection against different antibiotics and body defense systems. 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). Moreover, the high expression of efflux pumps can rapidly expand antibiotic resistance as well. 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. 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. Substrates of note of the efflux pumps are oxazolidinones.

---

**Abstract**

*Pseudomonas aeruginosa* is one of the most important opportunistic human pathogen that can lead to serious nosocomial infections. The treatment of *pseudomonas* infections due to the high emergence of resistance to antimicrobial agents is challenging. One of the mechanisms of antibiotic resistance in *P. aeruginosa* is the active efflux pumps. 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. These pumps include MexAB-OprM, MexCD-OprJ, MexEF-OprM, MexGHI-OprD, MexK-OprM/OpmH, MexMN, MexPQ-OprE, MexVW-OprM, MexXY-OprM, TriABC-OpmH, and MuxABC-OpmB. Recently, Efflux Pump Inhibitors (EPIs) have been considered as novel antimicrobial agents to overcome drug efflux mechanisms of bacterial pathogens. In recent years, numerous EPIs have been identified or synthesized for multi-drug resistant (MDR) bacteria; these include existing pharmacologic drugs, naturally occurring compounds, and synthetic derivatives thereof. This review describes the current progress in the assessment and development of EPI for using against *P. aeruginosa*.

Keywords: *Pseudomonas aeruginosa*, Efflux System, Antibiotic Resistance, Antibacterial Agent, Efflux Pump Inhibitor

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.\textsuperscript{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 glycyclcycline tigecycline confuses a number of tetracycline-specific efflux pumps of gram-negative bacteria.\textsuperscript{12} Recently it has been shown that some EPIs can increase the activity of tigecycline.\textsuperscript{11} Of course, the majority of efflux systems are not drug-specific proteins and extrude a broad spectrum of compounds from bacteria.\textsuperscript{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.\textsuperscript{14} Therefore, by understanding the mechanism of action pumps and ways to dominate their pathogenicity, we can establish a promising path for novel antibiotics.\textsuperscript{15,16}

Efflux pumps were reported both in gram-negative and gram-positive bacteria and even in a few eukaryotic cells.\textsuperscript{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.\textsuperscript{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., \textit{E. coli} EmrE), the ATP-binding cassette (ABC) superfamily, and the MATE (multidrug and toxic efflux family).\textsuperscript{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).\textsuperscript{2,16,20} RND and MFS are the most frequently used systems. RND is in gram-negative bacteria only (represented by \textit{P. aeruginosa} Mex pumps and \textit{E. coli} AcrAB-ToIC), but MFS is in both gram-negative and gram-positive bacteria.\textsuperscript{21}

### Efflux Pumps in \textit{Pseudomonas aeruginosa}

\textit{P. aeruginosa} has various efflux pumps such as RND and MFS superfamilies; however, the main efflux pump belongs to the RND family.\textsuperscript{22,23} Table 1 shows the major transporters identified in \textit{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).\textsuperscript{24,25}

There are 11 types of RND efflux pumps in \textit{P. aeruginosa} that differ in their substrates and release of multi-class drugs.\textsuperscript{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.\textsuperscript{27} These 2 systems are permanently expressed with an amount specified in the wildtype strains and are responsible for the intrinsic resistance to fluoroquinolones.\textsuperscript{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.\textsuperscript{28}

The most studied tripartite efflux pumps are the MexAB-OprM and AcrAB-ToIC transporters from \textit{P. aeruginosa} and...

<table>
<thead>
<tr>
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<th>Efflux pump</th>
<th>Pump Family</th>
<th>References</th>
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<tbody>
<tr>
<td>Chloramphenicol</td>
<td>CmlA</td>
<td>MFS</td>
<td>16, 23</td>
</tr>
<tr>
<td>Chloramphenicol</td>
<td>MexEF-OprN, MexCD-OprJ, MexAB-OprM</td>
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<td>RND</td>
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<td>Macrolides, Lincosamides,</td>
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<td>RND</td>
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<td>Glycylcyclines</td>
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<td>32, 34</td>
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<td>β-lactams</td>
<td>MexAB-OprM, MexCD-OprJ</td>
<td>RND</td>
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<td>Aminoglycosides</td>
<td>EmrE homologs</td>
<td>SMR</td>
<td>23, 25, 32</td>
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<td>Oxazolidinones</td>
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<td>Fluoroquinolones</td>
<td>MexAB-OprM, MexCD-OprJ, MexEF-OprN, MexXY-OprM, MexVV-OprM</td>
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<td>Fluoroquinolones</td>
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<tr>
<td>Nalidixic acid, norfloxacine</td>
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<td>MexCD-OprJ, MexEF-OprN, MexAB-OprM</td>
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<td>Tetracyclines</td>
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<tr>
<td>Tetracyclines</td>
<td>Tet A, C, E</td>
<td>MFS</td>
<td>23</td>
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<td>Sulfamides</td>
<td>MexAB-OprM</td>
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Table 1

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<tr>
<td>2</td>
<td>DNP</td>
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<tr>
<td>3</td>
<td>Valinomycin</td>
</tr>
<tr>
<td>4</td>
<td>CCCP</td>
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</table>

**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. Among these compounds, resistance-modifying agents, more especially EPIs, can be noted. EPIs are the molecules which disorder the process of extruding antibiotics and toxic substances from the bacterial cells with different mechanisms. 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. 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. 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. 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.

**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.

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. MDR1 crosses a broad spectrum of substrates which,
in addition to chemotherapy, may be used for allergy relief, hypertension, immunosuppression, infections, inflammation, and neurology. 

Gemifloxacin is a newer fluoroquinolone and causes a wide range of activity against gram-negative bacteria. 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-571 and 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.

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 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 drug.

Most natural products extracted from medicinal plants (called secondary metabolites) exhibit anticancer properties. The main classes of secondary metabolites with anticaner 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.

Some secondary metabolites with inhibitory ABC transporters overcome multidrug resistance in cancer cells. For example, the MDR modulatory activity of Chelidonium major 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. 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. Nevertheless, for this issue to happen, the physiological mechanisms and structures of efflux pumps must be known.

### 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. As a result, very few are special for gram-negative bacteria. A lot of synthetic or natural 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.

### 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. It was also revealed that PAβN can increase the potency of other antibiotics such as macrodides and chloramphenicol; therefore, it is considered as a broad spectrum EPI. Unfortunately, it expands the penetration of the OM for other cells which lack the MexAB-OprM pump.
This character of PAßN is because of its dicationicity.\textsuperscript{57} Albeit the efficacy of PAßN on membrane integrity is lower than that of polymyxin B.

In the following, improved the activity and pharmaceutical properties of PAßN and finally discovered MC-04,124 and MC-02,595 (\textit{Figure 3A}). All these compounds increase the potency of Lev.MC-04,124 (\textit{Figure 3A}) and show lower toxicity levels, better solubility, more stability in biological fluids, and more activity against \textit{P. aeruginosa} overexpressing efflux pumps.\textsuperscript{58} MC-02,595 exhibited lower toxicity and more stability than the other 2 compounds.\textsuperscript{59}

In addition to PAßN derivatives, pyridopyrimidines have also been shown to have high efficiency in inhibiting \textit{P. aeruginosa} efflux pumps (\textit{Figure 3B}).\textsuperscript{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.\textsuperscript{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 \textit{P. aeruginosa}.\textsuperscript{15} Finally, another pyranopyridine, MBX2319 (\textit{Figure 3B}), has recently been characterized as having high activity against Enterobacteriaceae and low activity vs. \textit{P. aeruginosa}. This inhibitor is in the initial phases of optimization and has not been evaluated in in vivo models.\textsuperscript{37}

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.\textsuperscript{29,59-61} For example, the moiety of PAßN that were applied for activity in \textit{P. aeruginosa} infections caused nephrotoxicity.\textsuperscript{27}

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 \textit{P. aeruginosa}.\textsuperscript{1,62}

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.\textsuperscript{63} Among the alkylaminoquinazoline derivatives, the compounds that encompass a morpholine group along with a propyl chain are more effective than the others.\textsuperscript{64}

\textbf{Table 3} shows the compounds that act as EPIs against \textit{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.\textsuperscript{29}

\textbf{Natural Compounds Against \textit{Pseudomonas aeruginosa}}

The use of natural knowledge of traditional physicians provides helpful data for investigating new drugs.\textsuperscript{71} The potential antibacterial characteristics of diverse natural medicinal substances are being widely studied in different parts of the world.\textsuperscript{72} Among these natural substances, it has been established that many medicinal plant substances have activity against \textit{P. aeruginosa}.\textsuperscript{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.\textsuperscript{75} Plant antimicrobials have not been applied in systemic infections.\textsuperscript{39} 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.\textsuperscript{68}

\textbf{Natural 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.\textsuperscript{49} In accordance with \textbf{Table 3} and \textbf{Figure 4}, some plant extracts show EPI properties against \textit{P. aeruginosa}. In addition, the extracts of \textit{Thymus maroccanus} and \textit{Thymus broussonetii} represented synergistic properties when mixed with Cam against efflux pump-overexpressing strains of \textit{P. aeruginosa}.\textsuperscript{75} Moreover, ethanolic extracts of \textit{Vernonia adoensis} and \textit{Mangifera indica} in the accumulation of rhodamine 6G (R6G) showed the potential properties of EPIs against \textit{P. aeruginosa}.\textsuperscript{39}
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.\textsuperscript{39,76}

EPIs derived from microbial origins are somewhat rare. The study of microbial fermentation has led to the identification of 2 new EPIs,\textsuperscript{65} which were derived from Streptomyces MF-EA-371-NS1, which is a new strain with a close relationship to \textit{Streptomyces vellosus}.\textsuperscript{65} EA-371\textalpha\textsuperscript{65} and EA-371\textdelta\textsuperscript{61} both block the MDR pump of \textit{P. aeruginosa} PAM1032, which overexpresses MexAB-OprM.\textsuperscript{8}

To use natural 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.\textsuperscript{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.\textsuperscript{89}

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.\textsuperscript{25} To evaluate the phenotypic effectivity 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.\textsuperscript{77} To evaluate another phenotypic method for checking the activity potential of antibiotics by EPIs is the agar doubling dilution method.\textsuperscript{25} As clarified by Lomovskaya et al,\textsuperscript{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.\textsuperscript{77} In this modified method, after MIC determination of the natural substance and antibiotic (A) using standard broth dilution methods, 50

<table>
<thead>
<tr>
<th>Compound</th>
<th>Source</th>
<th>Protein</th>
<th>Actions</th>
<th>Ref.</th>
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<tbody>
<tr>
<td>Peptidomimetic</td>
<td>Synthetic</td>
<td>MexAB-OprM, MexCD-OprJ, MexEF-OprN</td>
<td>Synergies with Cam, Quinolones, Carb, Macrolides, and Tet</td>
<td>59</td>
</tr>
<tr>
<td>Pyridopyrimidines</td>
<td>Synthetic</td>
<td>MexAB, OprM</td>
<td>Improves the potency of levofloxacin and aztreonam</td>
<td>23</td>
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<tr>
<td>Fluoroquinolone analog</td>
<td>Synthetic</td>
<td>MexAB-OprM</td>
<td>Improves the potency of macrolides and fluoroquinolones</td>
<td>23</td>
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<td>4-(3-morpholinopropylamino)-quinazoline Derivatives</td>
<td>Non-antibiotic drugs</td>
<td>MexAB-OprM</td>
<td>Reduced MIC of Cam, Nal, Carb and Lev</td>
<td>60</td>
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<td>Natural Compounds</td>
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<tr>
<td>EA-371\textalpha\textsuperscript{65} and EA-371\textdelta\textsuperscript{61}</td>
<td>Streptomyces MF-EA-371-NS1</td>
<td>MexAB-OprM</td>
<td>Reduce MIC of Lev</td>
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<td>Geraniol</td>
<td>Helichrysum italicum</td>
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<td>Reduced MIC of β-lactams, Quinolones, and Cam</td>
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<tr>
<td>Curcumin</td>
<td>Curcuma longa</td>
<td>-</td>
<td>Reduced MIC Mem, Carb, Caz, Gen, and Cin</td>
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<td>Lanatoside C and daidzein</td>
<td>Digitalis lanata and Kwao krua, respectively</td>
<td>MexAB-OprM</td>
<td>Increased uptake of EtBr, Reduced MIC of Carb and Lev</td>
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<td>Protocatechic acid</td>
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<td>Pheophorbide a</td>
<td>Berberis aetnensis</td>
<td>MexAB-OprM</td>
<td>Synergize with Cip</td>
<td>68, 69</td>
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<tr>
<td>Theobromine</td>
<td>Theobroma cacao</td>
<td>MexAB-OprM</td>
<td>Synergize with Cip</td>
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<td>Non-antibacterial drugs</td>
<td>Non-antibiotic drugs</td>
<td>-</td>
<td>Reduced MIC of Pen, Cxm and Tob</td>
<td>70</td>
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<td>Amitryptiline, Trans-chlorprothixene</td>
<td>Selective Serotonin Re-uptake Inhibitors</td>
<td>MexAB-OprM</td>
<td>Inhibition of Nile Red efflux</td>
<td>49</td>
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</table>

Abbreviations: Cam (Chloramphenicol); Carb (Carbenicillin); Caz (Cefazidine); 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).

| Table 3. Summary of Compounds That Are Active as EPIs Against \textit{Pseudomonas aeruginosa} |
| Compound | Source | Protein | Actions | Ref. |
| 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 Derivatives | Non-antibiotic drugs | MexAB-OprM | Reduced MIC of Cam, Nal, Carb and Lev | 60 |
|  | Natural Compounds |  |  |  |
| EA-371\textalpha\textsuperscript{65} and EA-371\textdelta\textsuperscript{61} | 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 Cin | 67 |
| Lanatoside C and daidzein | Digitalis lanata and Kwao krua, respectively | MexAB-OprM | Increased uptake of EtBr, Reduced MIC of Carb and Lev | 60 |
| Protocatechic 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 | Non-antibiotic drugs | - | Reduced MIC of Pen, Cxm and Tob | 70 |
| Amitryptiline, Trans-chlorprothixene | Selective Serotonin Re-uptake Inhibitors | MexAB-OprM | Inhibition of Nile Red efflux | 49 |

Abbreviations: Cam (Chloramphenicol); Carb (Carbenicillin); Caz (Cefazidine); 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).
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. The bacterial strains were adjusted to 10⁶ 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:

\[(\text{MIC of A in mixture}/\text{MIC of A singly}) + (\text{MIC of B in mixture}/\text{MIC of B singly})\]

The FIC index was applied to interpret the potency of the combinations. Synergism was considered as FICI ≤0.5, indifferent was considered as a FICI = 0.5–4, and antagonism was considered as FICI >4.

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.

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. Reserpine, a plant alkaloid, was initially extracted from the Rauwolfia vomitoria and was found to block Nor A pumps. 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. According to the, Cip is a substrate of a lot of bacterial efflux pumps, and primary experiments applied a simple bioassay to recognize plant extracts that synergize with Cip.

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. Using phenotypic methods, Touani et al showed that Brassica oleracea var. butyris and Brassica oleracea 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.

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

<table>
<thead>
<tr>
<th>Criteria</th>
<th>Techniques</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>It should potentiate the potency of an antibiotic against generated resistance in result of the overexpression of an efflux pump.</td>
<td>Synergism is measured using checkerboard assay.</td>
<td>9</td>
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<tr>
<td>It should enhance the amount of accumulation and reduce the amount of extrusion for substrates of the efflux pump.</td>
<td>This capability can be measured by fluorescent compound accumulation assays in the presence and absence of the putative EPI (drug efflux/accumulation assay).</td>
<td>29</td>
</tr>
<tr>
<td>It should not affect the sensitive strains which do not have the intended efflux pump.</td>
<td>Use of a sensitive strain and a wild type strain (resistant) and then the checkerboard method can be executed on the resistant strain.</td>
<td>29</td>
</tr>
<tr>
<td>It should not decrease the MIC of antibiotics which are not extruded.</td>
<td>Use of a sensitive strain and a wild type strain (resistant) and then the checkerboard method can be executed on the resistant strain.</td>
<td>29</td>
</tr>
<tr>
<td>It should not cause penetrable OM.</td>
<td>Periplasmic β-lactamase property in nitrocefin (a β-lactam) hydrolysis, which alters from yellow to red when hydrolyzed, can be used.</td>
<td>29</td>
</tr>
<tr>
<td>It should not change the proton gradient available on the IM; in other words, it should not disturb the membrane integrity.</td>
<td>DNA stains with fluorescence properties such as SYTOX Green or propidium iodide, which do not pass the intact membrane of bacterial cells, can be used.</td>
<td>78</td>
</tr>
</tbody>
</table>
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 al86 reported that the combination of Cip and Piperine could reduced 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.69 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.37,39,68 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*.50,67

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 berberine enhances the fluorescence measure inside the cells, and accumulates 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.79 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.53 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).60

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 al showed 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.29

### 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 effective 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.28 One inferential point of the application RT-qPCR by *P. aeruginosa* is that a 2-fold increment in the overexpression of mexA and mexB genes leads to an increase in MIC values, while expression of the mexX 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...
Many plant extracts have been against host- 
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However, β-lactam antibiotics or various classes 
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The idea of using a compound which 
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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.

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Antimicrobial resistance is a serious global health concern, with bacteria developing resistance to multiple antibiotics. Efflux pumps play a crucial role in this resistance, exporting drugs out of the bacterial cell, thus reducing their intracellular concentration and efficacy. Efflux pump inhibitors (EPIs) are compounds that block these pumps, allowing antibiotics to remain effective. This review summarizes the progress made in the field of efflux pump inhibitors (EPIs), with an emphasis on recent developments in the characterization of inhibitors of multidrug resistance efflux pumps. It provides an overview of the mechanisms by which efflux pumps contribute to resistance and discusses the potential of EPIs as new antimicrobial agents against a wide range of pathogens, including antibiotic-resistant bacteria such as Pseudomonas aeruginosa, a major cause of hospital-acquired infections and infections in immunocompromised patients. The review highlights the importance of understanding the molecular basis of efflux pump function and the development of specific inhibitors that can be used in combination therapy to overcome multidrug resistance.


Poonsuk K, Chunachuen R. Contribution of the MexXY multidrug efflux pump and other chromosomal mechanisms on...


