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Efflux pump inhibitors: Turning the tide against resistant bacteria

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Abstract- There are several ways by which resistance develops in bacteria for antimicrobial drugs and antibiotics. The primary reason for antimicrobial and antibiotic resistance is the efflux pump mechanism. Gram-positive and gram-negative bacteria both have these pumps. These pumps are also linked with the multiple drug resistance (MDR). Efflux pump pumps are the transport proteins and function in bacteria by expelling foreign material outside the bacterial cell and leading to decreased concentration of antibiotic in the cell. Therefore, to increase the usefulness of antibiotics, new compounds are discovered, that can inhibit the efflux pumps and increase the concentration of antibiotics inside the cells. These efflux pumps inhibitors are known as efflux pump inhibitors (EPIs). Efflux pump inhibitors can be isolated from natural sources (plants) and can be synthesized. EPIs are an important procedure to get rid of antibiotic resistance. In this article, we discuss the different EPIs and their mechanism of action.

Keywords: Efflux Pump, Antibiotics, Synthetic, Resistance, Inhibitors, Pathogens

INTRODUCTION

Antimicrobial drugs like antibiotics, antivirals, and antifungals are essential for fighting against infections. The widespread usage and exploitation of these drugs have led to genetic changes in bacteria, and this is the main reason for the development of antibiotic-resistant bacteria.[1] As the microbes are evolving and making genetic changes, the conventional medicines are becoming ineffective. Antibiotic-resistant bacteria are the major problem globally. It is spreading infections that are untreatable by the antibiotics present in the market.[2] According to the World Health Organization (WHO), the resistance developed by the efflux pump is one of the top ten threats to humanity. To overcome this problem, new drugs need to be developed, which can inhibit the efflux pumps of bacteria. The compounds which can inhibit the efflux pump of bacteria are known as efflux pump inhibitors (EPIs). These efflux pump inhibitors (EPIs) increase the concentration of antibiotics inside the bacterial cells, thereby increasing the effectiveness of existing antibiotics.[3]

Bacteria transform themselves into antibiotic-resistant bacteria for the following reasons mentioned below:

- Antibiotics are not able to enter the bacteria due to changes in cell wall permeability.
- Antibiotics are not able to act on molecular targets for a longer time.
- Enzymatically changing also renders the antibiotics inactive.
- Efflux pumps and porins do not allow the antibiotic to stay in the bacterial cells.[4]

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Bacteria aren't going down without a fight! They've developed a clever defence mechanism called efflux pumps, which are protein bouncers that physically throw antibiotics out of the cell. These pumps occur in both regular and antibiotic-resistant bacteria. Therefore, scientists are looking for advanced ways to disarm these pumps. The entry of efflux pump inhibitors (EPI) is the most recent figure in the drug discovery world. EPIs act like bouncer blockers, preventing the pumps from expelling antibiotics. This allows existing antibiotics to stay inside the bacteria and potentially reviving their effectiveness against resistant strains.[4,5]

Bacterial efflux pump

Efflux pumps are found in almost all cells including bacteria and eukaryotes. Efflux pumps present in cell walls and act like a cellular gatekeeper, clearing out a wide variety of unrelated compounds. The gatekeeper type of pump is particularly concerning because they play a major role in multidrug resistance (MDR) in bacteria.[6] They can pump out multiple antibiotics at once.[7] Energy-dependent these pumps can move substrates against a concentration gradient. In bacteria six types of efflux pump[8] families have been identified.

- **ATP-binding cassette (ABC) superfamily**: ABC transporters play a vital role in the virulence and pathogenesis of several pathogenic bacteria by transporting essential molecules, such as metal ions, amino acids, peptides and vitamins. These translocate a wide variety of substrates from, ions to proteins, across cellular membranes.[1]
- **Major facilitator superfamily (MFS):** MFS superfamily transport compounds like simple sugars, oligosaccharides, inositols, drugs, amino acids, nucleosides, organophosphate esters, Krebs cycle metabolites, and a large variety of organic and inorganic anions and cations.[9]
- **Small multidrug resistance (SMR) family: SMR** transport the small, charged metabolite guanidinium, bulky hydrophobic drugs and antiseptics, polyamines, and glycolipids across the membrane bilayer.[10,11]
- **Proteobacterial antimicrobial compound efflux (PACE) family:** PACE family of transport proteins, which was only recently described. PACE family transport proteins can confer resistance to a range of biocides.[12]
- **Multidrug and toxin extrusion (MATE) family:** MATA is a large family of secondary active transporters. It is involved in the transport of various xenobiotics and metabolites across cellular and organellar membranes.[13,14]
- **Resistance nodulation division efflux pumps (RND HAE) Super family:** These help in transport of metal ions, xenobiotics and drugs from cell. These also transport of hydrophobic and amphiphilic compounds.[15]

In gram-positive bacteria; MFS shows its dominance and RND in gram-negative bacteria.[16]

Bacterial efflux pump structure

Bacterial efflux pump (tripartite efflux pumps) of gram-negative bacteria are complex molecular assemblies that expel antibiotics and other toxic agents from the cell. The multi-drug resistance of gram-negative bacteria, growing global threat of incurable infections which are caused by the tripartite pump systems that transport a wide range antibiotics and other bactericidal substrates out of cell.[17]

The well-characterized AcrAB-TolC tripartite efflux pump in *Escherichia coli* serves as a key model system for understanding the structure and function of similar three-part efflux pumps. It is found in a wide variety of bacteria, including whose bacteria which are responsible for drug-resistant infections.[18] Tripartite efflux pumps and the related type 1 secretions systems(T1SSs) serve a variety of purposes in gram-negative organisms, including structural organization, energization. Tripartite efflux systems consist of a transmembrane inner-membrane transporter protein, a periplasmic adaptor protein (PAP) that spans the periplasm, and an outer membrane factor (OMF) protein that penetrates the outer membrane. These all present in the cell envelope of the gram-negative species. AcrB protein is the energy-transducing element of the AcrAB–TolC system. The energized inner membrane transporters (belonging to ABC, RND, and MFS families), the outer membrane factor channel-like proteins, and linking the two, the periplasmic adaptor proteins (PAPs), also known as the membrane fusion proteins (MFPs). AcrB has a peptide partner (AcrZ) that affects its transport activity for a subset of substrates. TolC is a representative of the outer membrane protein (OMP) component of a tripartite pump assembly. This entire system works together to pump out a

wide range of harmful substances, keeping the bacteria safe from antibiotics and other threats.[19-21]

Mechanism of bacterial efflux pump

Efflux pumps allow the microorganisms to regulate their internal environment by removing toxic substances, including antimicrobial agents, metabolites and quorum sensing signal molecules. In higher concentrations of antibiotic, efflux pump systems help multidrug-resistant (MDR) bacteria to survive by lowering intracellular concentrations of antibiotics.[22] The ability of efflux pumps to remove diverse compounds is a major mechanism driving bacterial resistance to multiple antibiotics. Furthermore, these pumps are involved in various other bacterial processes, including stress response, virulence, biofilm development, and modulation of the host environment.[23]

Table 1. Some examples of efflux pump in gram-positive bacteria (GPB)and Gram-negative bacteria (GNB)[24].

Gram-positive bacteria (GPB)	
Bacteria	**Efflux pumps**
Methicillin-resistant *Staphylococcus aureus* MRSA	NorA, NorB [25]
Streptococcus pneumoniae	mefE[26]
Clostridium difficile	CdeA[27]
Enterococcus spp	efrA and efrB[28]
Listeria monocytogenes	MdrL and Lde[29]
Gram-negative bacteria (GNB)	
Acinetobacter baumannii	AdeABC[30]
Escherichia coli	AcrAB-TolC[31]
Klebsiella pneumoniae	AcrB[32]
Stenotrophomonas maltophilia	SmeYZ[33]
Campylobacter jejuni	CmeABC[34]
Pseudomonas aeruginosa	MexAB-OprM, MexXY, MexCD-OprJ, and MexEF-OprN.[35]
Neisseria gonorrhoeae	MtrCDE[36]

Efflux pumps are also involved in biofilm formation by affecting factors such as physical-chemical interactions, mobility, gene regulation, quorum sensing, extracellular polymeric substances, and the removal of toxic compounds. Their specific role within the biofilm varies based on the stage of formation, gene expression levels, and substrate characteristics[37].

The mtr efflux pump region in *Neisseria gonorrhoeae* can gain mosaic-like DNA sequences from harmless *Neisseria* bacteria through DNA transfer. This can make the gonorrhea bacteria resistant to different types of antibiotics. The changes in the mtrR gene and mtrD pump protein, which controls the mtrCDE pump genes and its promoter region, have been shown to lower the bacteria's sensitivity to many antibiotics, including azithromycin (Azi). This shows how these genetic changes lead to this reduced sensitivity to drugs that are normally removed by the mtrCDE pump.[38]

Efflux pump interplays with other resistance mechanisms, such as the membrane permeability barrier[39], enzymatic inactivation/modification of drugs, and/or antibiotic target changes/protection, in significantly increasing the levels and profiles of resistance.[40]

Porins, which control antibiotic entry into bacteria, and efflux pumps, which expel antibiotics, significantly contribute to the development of antibiotic resistance through both genetic mutations and adaptive responses. These changes in porin function or increased efflux pump activity, driven by either mutation in the bacterial DNA or the bacteria's ability to adjust to their environment, directly reduce the effectiveness of antibiotic treatments by limiting the drug's ability to reach its target inside the cell.[41]

Mechanism of efflux pump inhibitors

A new weapon for the fight against antibiotic-resistant bacteria are the efflux pump inhibitors in its arsenal. These are molecules specifically designed to target the bacterial pumps that eject antibiotics. Researchers are currently testing some promising EPIs in clinical trials to see if they can effectively block these pumps. A successful, EPIs could become a game-changer in our fight against infections. EPIs could allow existing antibiotics to work more effectively against resistant strains. This would represent a significant advancement in the battle against antimicrobial resistance.[42]

The establishment of EPI's mechanism is on the trial to pin point the actual pathway. It is supposed that, when these are admistrated along with antibacterials, then they build up their concentration inside the bacterial cell to exert their action and enhance the therapeutic benefits.

To understand the strategy of EPIs following mechanisms have been proposed

1. Hampering the mechanism of gene regulation and expression decreases the expression of the of efflux pump.
2. Novel antibiotics discovery that are not familiar to the substrates
3. Obstructive the structure of efficient efflux pumps
4. Clogging antibiotics to bind the active pump site.

5. Breaking up the energy mechanism answerable for stimulating these pumps.[43]

In lab settings, EPIs have demonstrated encouraging potential as therapeutic adjuvants. Even though numerous EPIs with various mechanisms of action have been reported.

Energy dissipation

Efflux pumps depend on the cellular energy, by disrupting the power source of efflux pumps leads to inhibition efflux. The proton gradient or the ATPase that supplies energy to these pumps has been tried as targets of various EPIs. This approach is effective against specific pumps that rely on this energy source. It's a smart way to target the pump's weakness and prevent it from ejecting antibiotics.[43] Carbonyl Cyanide-m-chlorophenylhydrazone, or CCCP lab-made EPI works like a gremlin in the pump's machinery. It revives the activity of tetracycline in *Helicobacter pylori* and *Klebsiella spp*. It's a type of ionophore, a molecule that disrupts the electrical current powering the pump. CCCP specifically targets two key components of this current, the membrane potential ($\Delta\psi$) and the proton gradient (ΔpH). By messing with these components, CCCP essentially cuts off the pump's power supply, preventing it from throwing out antibiotics. It is not appropriate for use as a medicine due to its side effects. However, it leads to develop of safer and more targeted EPIs in the future.[44]

Inhibition by straight binding

EPIs directly bind to efflux pump and lead to reduce ability of the pumps to interact with their substrates. The will of two types competitive or non-competitive. If it is competitive type then EPIs compete with the substrates for the same binding site on efflux pump but if it is non-competitive then EPIs decrease the decrease in the affinity of pump towards its substrates.[45] PAβN a synthetic EPI increases the activity of levofloxacin, erythromycin and chloramphenicol for inhibiting multidrug resistance pump of *P. aeruginosa* cells.[46-48]

Sources of efflux pump inhibitors

Depending upon their ability, they are subdivided in two major categories:

Derived from Natural Sources

Similar to all living creatures, plants also produce several beneficial chemicals that offer defence against invasive bacterial species. Knowledge gathered from past research shows that, this plant based chemical agents possess the ability to reduce the activity of efflux pumps in the patients suffering with multi drug resistance bacterial infections.[49,50]

Reserpine

Reserpine, a plant alkaloid derived from Rauwolfia serpentina Figure 1 shows promise as an efflux pump inhibitor (EPI) by targeting efflux pumps of the MFS and RND superfamilies.[51] Literature shows that reserpine can enhance antibiotic activity by directly interacting with efflux transporter proteins like Bmr in *Bacillus subtilis* (mediating tetracycline efflux) and reversing NorA-mediated resistance in *Staphylococcus aureus* leading to a significant increase in norfloxacin activity[52] However, its clinical application in combination with antibiotics is currently limited due to its nephrotoxic properties.[53]

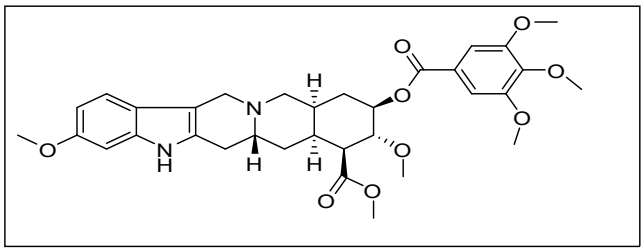

Figure. 1: Reserpine

Piperine

Piperine, an alkaloid Figure 2 obtained from *Piper nigrum* (black pepper). Piperine is an efflux pump inhibitor, combats bacterial resistance by controlling the expression of key components of the MexAB-OprM efflux system. It reduces the levels of the efflux pump proteins MexA, MexB, and OprM. It leades to increased intracellular accumulation of the antibiotic imipenem within bacteria and consequently enhancing the bacteria's susceptibility to this antibiotic.[54]

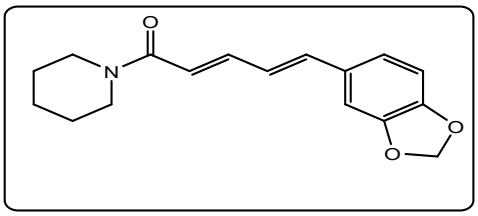

Figure 2: Piperine

Berberine

Berberine Figure 3 is benzylisoquinoline alkaloid extracted from the rhizomes of *Coptis japonia* and bark of *Phellodendron.* It reduces the reduced aminoglycoside resistance of *P. aeruginosa* by inhibiting MexXY-dependent aminoglycoside efflux pump. Phenylalanine-

arginine beta-naphthylamide (PAβN) a well-known EPI lowers the concentration of berberine to reduce amikacin resistance of *P. aeruginosa*. In the presence of PAβN., berberine enhanced the synergistic effects of amikacin and piperacillin in multidrug-resistant *P. aeruginosa* strains.[55,56]

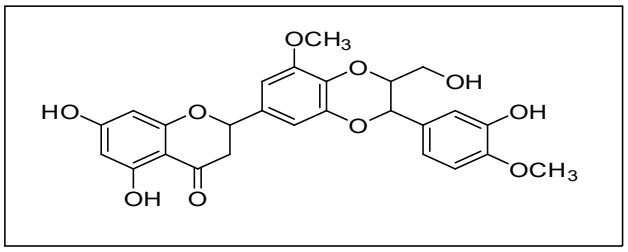

Figure 3: Berberine

Silibinin

Silybin Figure 4 is a flavonolignan component of the extract from the milk thistle seed. It inhibits the efflux system of methicillin-resistant *Staphylococcus aureus* (MRSA). The expression of the quinolone resistance protein NorA and quaternary ammonium resistance proteins A/B efflux genes in MRSA is reduced by Silibinin.[57]

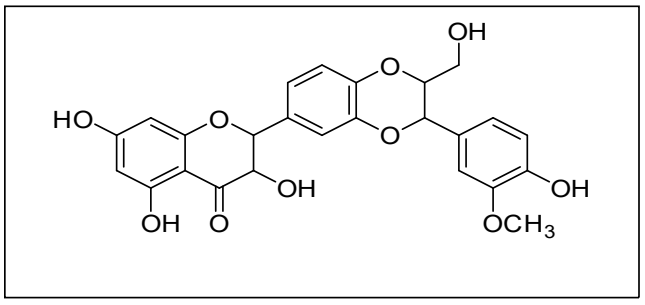

Figure 4: Silibinin

Baicalein

Baicalein, Figure 5, a bioflavonoid from *Scutellaria baicalensis*, in modulating tetracycline resistance in *S. aureus* by inhibiting efflux pumps. It was observed in the ethidium bromide accumulation assay that baicalein significantly inhibits the efflux pump with a dose dependent increase in fluoresce. The MIC of tetracycline is reduced by it by eightfold. And enhances tetracycline efficacy to combat multidrug-resistant *S. aureus* infections.[58]

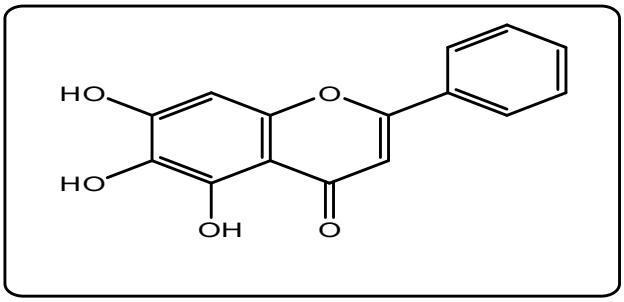

Figure 5: Baicalein

Genistein, Orobol and biochanin-A

Genistein, orobol and biochanin-A in Figure 6 are isoflavones obtained from *Lupinus argenteus*. They increase the activity of alpha-linolenic acid, berberine and norfloxacin in *Staphylococcus aureus* cells This suggest that it may be inhibiting the multidrug resistance pump [59].

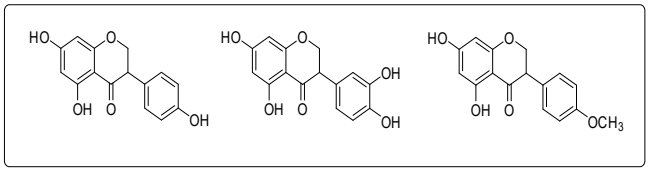

Figure 6: Genistein, Orobol, Biochanin-A

Neohesperidosides

Acylated neo hesperidosides enhance the activity of the berberine, rhein, ciprofloxacin, and norfloxacin against *S. aureus,* which is isolated from a *Geranium caes pitosum*. Against.[60]

Catechin gallates

The polyphenols isolated from green tea extracts are Epicatechin gallate and epigallocatechin gallate in Figure 7 have been proven to reverse MRSA resistance. When used against norfloxacin-resistant strain of *S. aureus* with high expression of the NorA multidrug efflux pump, both these compounds increase the activity of norfloxacin. This shows that both the compounds have efflux inhibitor activity.[60,61]

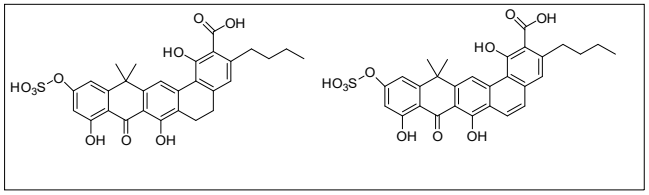

Figure 7: Epicatechin gallate and Epigallocatechin

Synthetically produces compounds

L-Phenylalanyl-L-arginine-β-naphthylamide (PaβN)

A peptidomimetic compound PaβN increases the activity of various classes of antibiotics such as 4-fluoroquinolones, macrolides, and chloramphenicol by inhibiting efflux pumps in a widevspreadede of clinical pathogens.[62]

Biricodar (VX-710), Timcodar (VX-853)

Biricodar (VX-710), Timcodar (VX-853) potentiate the activity of ethidium bromide (EtBr), efflux substrate, against gram-positive pathogens *Staphylococcus aureus, Enterococcus faecalis*, and *Streptococcus pneumoniae.* These compounds also lower the MIC of several clinically used antibiotic like fluoroquinolones.[63-65]

Phenothiazines and Thioxanthene derivatives

Phenothiazine and two geometric stereoisomers of the thioxanthene, when combined with common MDR efflux pump substrate showed synergistic interactions against the strains of *S. aureus*. Structure-activity relationship shows that the trans isomer of thioxanthene has showed MDR efflux pump inhibitory activity against wide range of pathogens.[66,67]

Quinoline derivatives

Quinoline derivatives like pyridoquinolones, has ability to combat efflux-mediated resistance. Pyridoquinolones inhibit the AcrAB-TolC efflux pump of *E. aerogenes* strains and retain the efficacy of norfloxacin in *E. aerogenes* strains. Furthermore, other synthetic quinolone analogs with substitutions at the 4-position (thioalkyl, alkylamino, and alkoxy groups) have demonstrated the capacity to enhance the activity of tetracyclines, norfloxacin, and chloramphenicol in clinical isolates of *K. pneumoniae* and *E. aerogenes*. Modification of the flavone scaffold has also yielded a series of 2-phenyl-4(1H) quinolone and 2-phenyl-4-hydroxyquinoline derivatives that act as potent inhibitors of the NorA efflux pump in *S. aureus*.[68-70]

Aryl piperazine derivatives

Several arylpiperazine derivatives reversed multidrug resistance of *E. coli* due to over-expression acrAB. Inhibition of AcrB efflux pump by interfering with its functional assembly and movement of the G-loop, 1-(1-Naphthylmethyl)-piperazine plays a significant role of substrate extrusion.[70]

Pyridopyrimidine derivatives

Pyridopyrimidine analogues D2 and D13-9001 have been reported as inhibitor of MexAB-OprM in pump *P. aeruginosa* under both in vitro and in vivo conditions. It is also propped that D13-9001 inhibits the AcrB in *E. coli* and MexB in *P. aeruginosa*.by binding with the specific sites of pump.[71-74]

Pyrazolopyridine derivatives

A synthetic pyrazolopyridine derivatives MBX2319. Inhibit the AB1157 efflux pump of *E. coli* and also the activity of antibiotics such as ciprofloxacin levofloxacin, and piperacillin *E. coli* AB1157.[75,76]

Analysis between natural and synthetic sources of EPIs.

Natural EPIs. are derived from plants as secondary metabolites like alkaloids, flavonoids, and terpenoids. They show their synergistic effects with antibiotics and has lower inherent toxicity compared to some synthetic counterparts. They are used as tractional medicine from the ancient time. The problems relate to these natural EPIs are inconsistent yields from source materials, complex extract, and the need for extensive research to validate their efficacy and safety.

Synthetic EPIs offer the possibility of rational drug design, allowing for the optimization of specific structural features to enhance potency. They can be produced with greater consistency and purity. However, synthetic EPIs may be associated with higher risks of toxicity, off-target effects, and the development of resistance mechanisms.

The increasing interest in natural EPIs reflects a desire for safer and potentially synergistic approaches to combat antibiotic resistance, although both natural and synthetic sources hold promise and require continued investigation to translate into effective clinical applications.

CONCLUSION

During the recent decades, important progresses have been made in understanding the mechanisms of action of efflux pumps, how these are making antibiotics ineffective, and their functions. Several efflux pump inhibitors have been discovered, but they are still undergoing clinical trials. These traditional inhibitors may have secondary pharmacological and toxicological effects, as well as potential issues related to their accumulation in different tissues, leading to toxicity. The current period calls for addressing the shortcomings of existing efflux pump inhibitors and exploring alternative EPI molecules, including different formulations, to minimize potential adverse effects and enhance their effectiveness.

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CONFLICT OF INTEREST

The authors declare no conflicts of interest relevant to this article.

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