A NEW STRATEGY FOR BACTERIAL RESISTANCE-EFFLUX PUMP INHIBITORS

[1]Nagma begum*,[2]Dr.M.Vijaya bhargavi, [3]Zaber unissa,[4]Dr.M.Sumakanth
Department of Pharmaceutical Chemistry
RBVRR Women's College of Pharmacy, Osmania University, Hyderabad, India.

ABSTRACT: With the advent of antibiotics, bacterial infections were supposed to be a thing of past. However, this event instead led to the selection and evolution of bacteria with mechanisms to counter the action of antibiotics. Antibiotic efflux is one of the major mechanisms, whereby bacteria pump out the antibiotics from their cellular interior to the external environment using special transporter proteins called efflux pumps. Inhibiting these pumps seems to be an attractive strategy at a time when novel antibiotic supplies are dwindling. Molecules capable of inhibiting these pumps, known as efflux pump inhibitors (EPIs), have been viewed as potential therapeutic agents that can rejuvenate the activity of antibiotics that are no longer effective against bacterial pathogens. EPIs follow some general mechanisms of efflux inhibition and are derived from various natural as well as synthetic sources. This review focuses on EPIs and identifies the challenges that have kept these futuristic therapeutics away from the commercial realm so far.

KEYWORDS: Antibiotics, Efflux pumps, Multiple drug resistance, Pathogens, Therapeutics.

INTRODUCTION

Bacterial infections are becoming more challenging to treat, as a result of the emergence of multi-drug resistant pathogenic bacteria. According to worldwide surveillance studies, multidrug resistance (MDR) is increasingly prevalent, especially in Gram-positive pathogens such as *Staphylococcus aureus*, *Streptococcus pneumoniae*and *Enterococcus* spp.[1]For instance, the emergence of vancomycin resistance in the last decade, first in *Enterococci*[2]and recently in *S. aureus*,[3]has caused considerable concern because vancomycin is the drug of last-resort for treatment of methicillin-resistant *S. aureus* (MRSA) infections. Gram-negative bacteria such as *Pseudomonas aeruginosa*, *Acinetobacter* spp. And the β-lactamase-producing *Enterobacteriaceae* are also more difficult to treat with standard therapies.

Bacteria develop resistance to antibiotics through four major mechanisms (Fig.1): (*i*) altering the cellular permeability to avoid the entry of antibiotics into the cells, (*ii*) modifying the molecular targets of the antibiotics so that they can no longer act on them, (*iii*) enzymatic modification of antibiotics to make them inactive, and (*iv*) expression of efflux pumps to pump out antibiotics from the cellular milieu.[4]

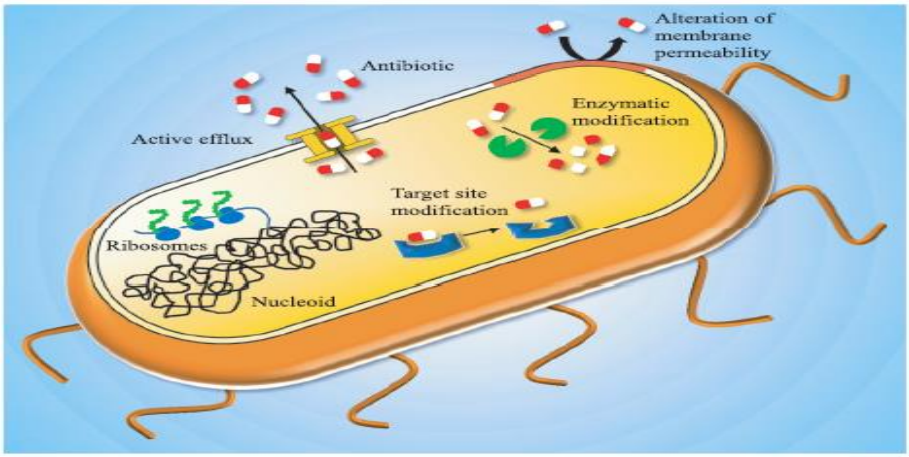

Fig. 1: Mechanisms of drug resistance

Efflux pump inhibitors (EPIs) are the substances that give the most promising approach in blocking the efflux pumps. They are the molecules that interfere with the process of removing toxic substances and antibiotics from the bacterial cell. Efflux pump inhibitors act as adjuvant to potentiate the activities of conventional antibiotics by inhibiting them either competitively or non-competitively.[5] Therefore, an attempt has been made in this review to enlist the synthetic and plant derived EPIs discovered till date against Gram negative and Gram positive bacteria of human pathogenesis to the best of our knowledge.

BACTERIAL EFFLUX SYSTEMS AS DETERMINANTS OF MULTIDRUG RESITANCE

Bacterial efflux systems are responsible for the secretion of toxins or antibiotics produced by the cell itself, efflux of toxic compounds run in to bacterial environment such as antibiotics.

From the first drug-resistant efflux pump discovered in the 1990s, the development in molecular microbiology has led to the characterization of many efflux pumps in Gram-positive bacteria (GPB) including methicillin-resistant *Staphylococcus aureus* (MRSA), *Streptococcus pneumoniae*, *Clostridium difficile*, *Enterococcus* spp. and *Listeria monocytogenes* and Gram-negative bacteria (GNB) such as *Acinetobacter baumannii*, *Escherichia coli*, *Klebsiella pneumoniae*, *Stenotrophomonas maltophilia*, *Campylobacter jejuni*, *Pseudomonas aeruginosa*, *Neisseria gonorrhoeae*, *Vibrio cholera* and *Salmonella* spp.[6,7]

Since these transport substrates against a concentration gradient, these efflux pumps are energy dependent. Based on the mechanism by which these derive this energy, the efflux pumps are broadly classified into two categories.The primary efflux pumps draw energy from active hydrolysis of ATP, whereas the secondary efflux pumps draw energy from chemical gradients formed by either protons or ions such as sodium. Five major families of efflux pumps have been described in the prokaryotes (Fig. 2), namely (*i*) ATP binding cassette (ABC), which are primary active transporters, (*ii*) small multidrug resistance family, (*iii*) multidrug and toxin extrusion(MATE) family, (*iv*) major facilitator superfamily (MFS) and (*v*) resistance nodulation cell division (RND) family, which are all secondary active transporters.[8]

ABC transporters are ubiquitous membrane systems, involved in different transport functions such as the efflux of toxins, metabolites and drugs.[9] One of the most studied ABC transporter is the mammalian P-glycoprotein (P-gp, MDR1) whose overexpression confers resistance to cytotoxic compounds used in chemotherapy.[10]

Membrane proteins of the SMR family are involved in the efflux of lipophilic cationic drugs in bacteria.[11] They are the smallest drug efflux proteins known, with only 4 predicted TMS. They may function either as homo- or hetero-oligomeric complexes.

The MFS proteins are ubiquitous systems, ensuring transport of sugars, intermediate metabolites and drugs.[12] These proteins form two separate clusters, with either 12- or 14-transmembrane segments (TMS).

RND family efflux pumps have tripartite organization and are the major contributors to intrinsic antibiotic resistance in GNB, which expel a broad spectrum of antibiotics and biocides, including fluoroquinolones, β-lactams, tetracycline and linezolid. However, in GPB, MFS transporters are predominant including NorA of *S. aureus*, PmrA of *S. pneumonia* and EmeA of *E.faecalis* that extrude a large number of antibiotics belonging to different classes.[13,7]

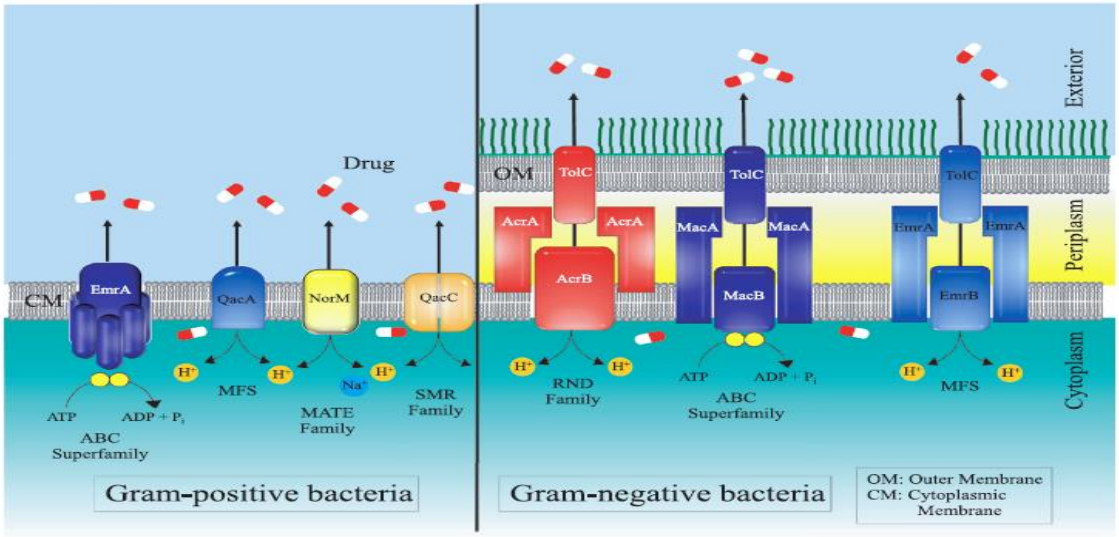

Fig. 2: The five classes of efflux pumps in bacteria, (*i*) ATP-binding cassette superfamily, (*ii*) major facilitator superfamily, (*iii*) multidrug and toxic compound extrusion family, (*iv*) small multidrug resistance family, and (*v*) resistance nodulation division family. The organization of these efflux pumps is different in Gram-positive and Gram-negative bacteria.

Efflux pumps, unlike most other determinants of resistance, are more often intrinsic. The genes coding for these transporters are found in susceptible as well as resistant bacteria[14] and are often parts of an operon whose expression is regulated at the transcriptional level. The mutations in the regulatory proteins or the mutations at the promoters result in the overexpression of these efflux pumps, resulting in drug resistance.[14]

Bacterial efflux system can be either specific, release only one or a single class of antibiotics (such as TetA and AbaF that selectively eliminate specific antibiotics such as tetracycline and fosfomycin, respectively)[15] or capable of pumping out several classes of antibiotics (such as MexAB-OprM, NorA and BmrA that extrude distinct class of antibiotics, disinfectant, dyes, and detergents) being designated as MDR efflux pumps. Most of the MDR efflux pumps are chromosomally encoded including NorA, NorB, MepA and MdeA of *S. aureus* that are responsible for intrinsic resistance in bacteria to several antibiotics, while some of the pumps are encoded on plasmids (QacA/B of *S. aureus*) or transposons (MefA and MefB of *Streptococcus* spp.)that provide the transferable mode of resistance.[16,17]

PHYSIOLOGICAL ROLES OF EFFLUX PUMPS

There has been some speculation about the physiological role of efflux pumps in bacteria. The in vivo role of efflux pumps is believed to be very complex, since they play an important role in bacterial physiology, metabolism, and pathogenicity.[18] Bacteria may use efflux pumps to eliminate toxic endogenous metabolites, for the secretion of virulence determinants, to participate in the cell stress response and in biofilm formation. Moreover, they can be responsible for removing toxins that are encountered in the environment (heavy metals, biocides) and can serve as protective tools that enable bacteria to survive the colonization process during infection in a host, when it is being attacked by noxious agents.[19,20] It has been reported that RND type MES play a role in adherence, invasion, and colonization of the host cell.[21,22]

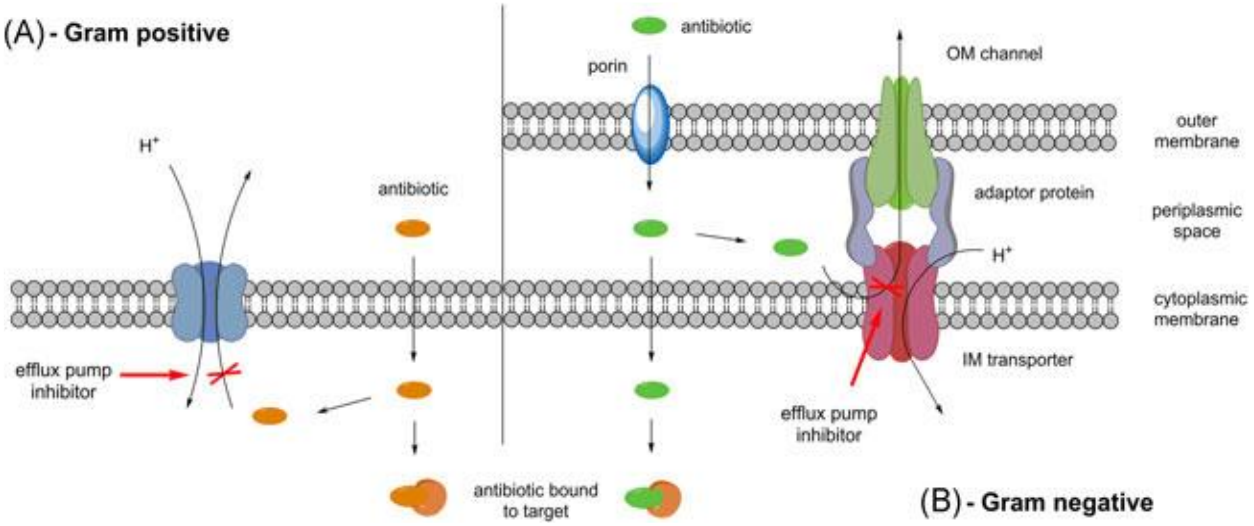

Fig.3: A schematic representation of efflux pump inhibitory activity of EPIs. MFS transporters in Gram-positive bacteria (A) and RND transporters in Gram-negative bacteria (B) use the proton gradient to drive the extrusion of an antibiotic by a proton/drug antiport mechanism. Blocking of antibiotic efflux by the action of an EPI increases antibiotic concentration in the cytosol of resistant bacteria, thus increasing its binding to the target and restoring its effectiveness.

Hence, EPIs may also reduce bacterial virulence in vivo, as was proven lately in an insect model of infection,[23] and act as anti virulence determinants that lower the pathogenic potential of microorganisms.[24,25] An interesting example of how efflux pumps serve to extrude physiological compounds made by the cells themselves is that of the N-acyl homoserine lactones, which have been shown, in *P. aeruginosa*, to diffuse through media to other cells of the population and activate many processes, thereby serving as quorum-sensing signals.[26,27]

CLASSES OF EFFLUX PUMP INHIBITORS

1) EPIs based on their mechanism of action

The EPIs in laboratories have shown good promise as therapeutic adjuvants. Although a multitude of EPIs have been reported with different modes of action, these can be broadly characterized into two categories:

a) Energy dissipation:

Since efflux pumps are dependent on cellular energy, the decoupling of the energy and efflux activity presents an interesting approach to efflux inhibition. The proton gradient or the ATPase that supplies energy to these pumps has been tried as targets of various EPIs. Such an inhibition scheme does not require any direct interaction of the inhibitor with the efflux pump itself. This approach appears to be advantageous as many efflux pumps are dependent on the proton gradient, making this a universal scheme for inhibiting them.

Carbonyl cyanide-m-chlorophenylhydrazone (CCCP) is perhaps the most well-known laboratory EPI. It is an ionophore that disrupts the proton motive force (PMF) by affecting both its components, $\Delta\psi$ and ΔpH.[28] The CCCP has been reported to revive the activity of tetracycline in *Helicobacter pylori* and *Klebsiella* spp.[29,30] Synergy between carbapenems and CCCP was also described, which was independent of the efflux inhibition activity of CCCP, supporting the previous hypothesis that CCCP leads to metabolically inactive cells giving rise to synergistic effect with antibiotics.[31]

b) Inhibition by direct binding:

Another mechanism of efflux pump inhibition is the binding of the EPIs to functional efflux pumps, resulting in reduced ability of the pumps to interact with their substrates. This binding could be competitive, where the EPI competes with the substrates for the same binding site; or non-competitive, where the binding of EPI to the pump causes decrease in the affinity of pump towards its substrates.

However, bacteria can always mutate their efflux pumps to modify the target sites of these inhibitors, rendering them useless.

Verapamil is a small molecule that acts as anion channel blocker and is used in the treatment of hypertension. Studies in *Mycobacterium tuberculosis* have shown that verapamil enhance the activity of bedaquiline and ofloxacin.[32,33] Further studies have identified that verapamil inhibits the activity of MATE pumps. It has a low amount of toxicity towards bacterial cells not expressing MATE efflux pumps, suggesting specificity towards bacteria expressing these pumps and a competitive mode of inhibition.

EPIs based on their origin

The EPIs can be categorized based on their source. This leads to three broad categories that include EPIs derived from plant products, synthetic chemistry and microorganisms.

a) Plant-derived EPIs

Plant-derived phytochemicals include a wide variety of chemical adjuvants that synergistically enhance the efficacy of antibiotics up to several folds.[34] Major subclasses of plant-derived EPIs are enumerated as follows:

i. Plant alkaloids

- **Reserpine:** an antipsychotic drug extracted from the roots of *Rauwolfia serpentina*, is a promising EPI that targets efflux pumps of the MFS and RND superfamily.[34] Reserpine is reported to potentiate antimicrobial activity of antibiotics by interacting directly with amino acid residues in the efflux transporter protein Bmr, which mediates tetracycline efflux in *B. subtilis.* In addition, reserpine has also been shown to reverse NorA-mediated resistance in *S. aureus* by enhancing the activity of norfloxacin upto four-fold.[35] The clinical application of reserpine with clinically used antibiotics, however, has not yet been achieved due to its nephrotoxic nature.[36]

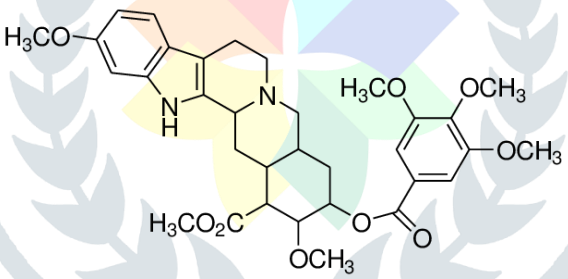

Fig.4: Reserpine

- **Piperine:** isolated from *Piper nigrum* is another alkaloid known to inhibit the human P-glycoprotein of ABC transporters via cytochrome P450-mediated pathways. The efflux pump inhibitory activity of both piperine and its derivative, piperidine, has also been reported against pathogenic bacteria including *S. aureus* and *Mycobacteria* spp.[37] A study conducted in *S. aureus* showed that piperine enhances the accumulation of ciprofloxacin by inhibiting NorA efflux pumps. In *M. tuberculosis H37Rv* and several clinical isolates, piperine has been reported to potentiate the activity of rifampicin by inhibiting an uncharacterized efflux pump-Rv1258c.

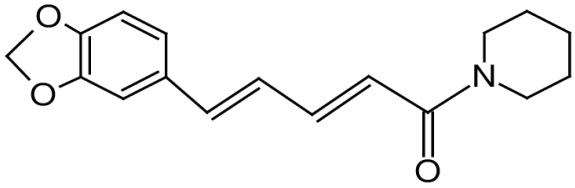

Fig.5: Piperine

ii. Flavonoids

- **Baicalein:** a 5,6,7-trihydroxyflavone, is a weak antimicrobial flavone isolated from thyme leaves (*Thymus vulgaris*). It improves the susceptibility of clinical MRSA strain towards ciprofloxacin and β-lactam antibiotics including oxacillin, cefmetazole and ampicillin.[38,39] Baicalein is also reported to increase the potency of tetracycline in TetK-overexpressing *Staphylococci* by inhibiting the uptake of [3H] tetracycline.[39]

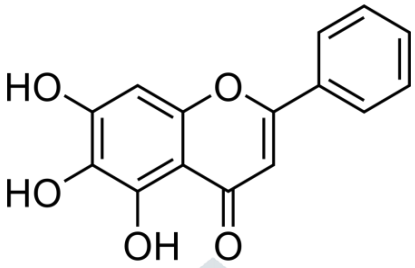

Fig.6: Baicalein

- **5'-methoxyhydnocarpin:** a flavolignan isolated from *Berberis fremontii*, has been reported to enhance the efficacy of several NorA substrates, including norfloxacin and berberine by inhibiting this proton pump. However, due to its toxic nature, its clinical success is doubtful.[40] Some of the other plant derived isoflavones (isolated from *Lupinus argenteus*) including genistein, orobol and biochanin A, have been reported to reduce the MIC of berberine and norfloxacin in clinical *S. aureus* and *M. smegmatis* by blocking the MDR efflux pumps.[41]

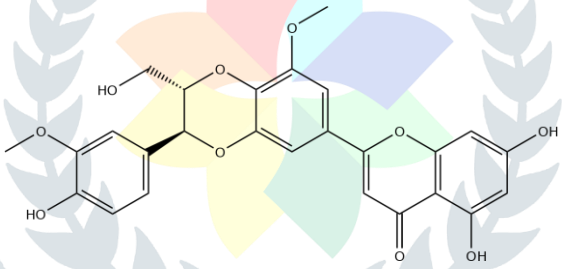

Fig.7: 5'-methoxyhydnocarpin

iii. Polyphenols:

Catechin gallates: a group of phenolic metabolites, have been reported to reverse the MRSA resistance. Catechin gallates such as epicatechin gallate and epigallocatechin gallate are weak inhibitors of NorA efflux pump, with epicatechin gallate being slightly more potent. It has been proposed that these molecules have two different binding sites on the NorA efflux transporter with different affinities. At low concentrations, catechins occupy high-affinity binding sites leading to increased efflux of NorA substrate. Their effect as EPI is observed only at a higher concentration.

- **Epigallocatechin gallate:** has also been reported to enhance the potency of tetracycline, erythromycin and ciprofloxacin in TetK-overexpressing Gram-positive *Staphylococci* and in Gram-negative *Campylobacter* spp. However, due to toxicity concerns associated with it, further in vivo and preclinical studies were not undertaken.[42]

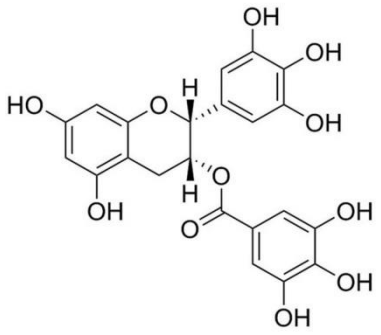

Fig.8: Epigallocatechin gallate

iv. Phenolic diterpenes:

- **Carnosic acid and Carnosol:** isolated from herb Rosemary (*Rosmarinus officinalis*), have been reported as EPIs. These enhance the potency of antibiotics such as tetracycline and erythromycin against macrolide resistant strain of *S. aureus* expressing the ABC transporter MsrA and TetK efflux pumps.[43]

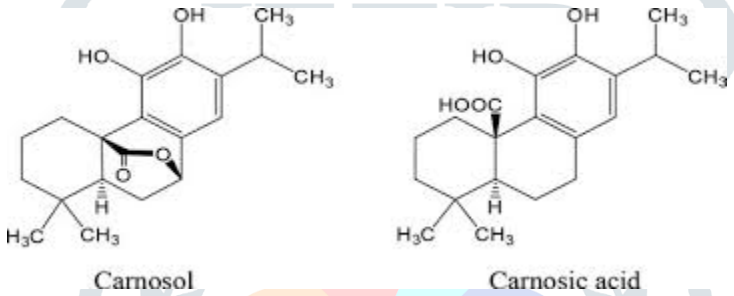

Fig.9: Carnosol and Carnosic acid

b) EPIs of synthetic origin

i. Peptidomimetic compounds: The dipeptide amide compound PAβN was one of the first EPIs discovered through chemical genetics approach. It has been reported to potentiate the activity of many antibiotics including fluoroquinolones, macrolides and chloramphenicol in GNB by inhibiting RND efflux pumps.[44,45] However, it had limited clinical potential due to toxicity towards mammalian cells. Although some synthetic derivatives with different basic properties such as reduced toxicity, enhanced stability, and better solubility were evaluated, none of the active analogues could significantly reduce the drawback of the parent molecule. This, PAβN and its novel derivatives are limited to use in laboratory as standards to determine the level of inhibitor-sensitive efflux for specific antibiotics in various bacterial pathogens.[46]

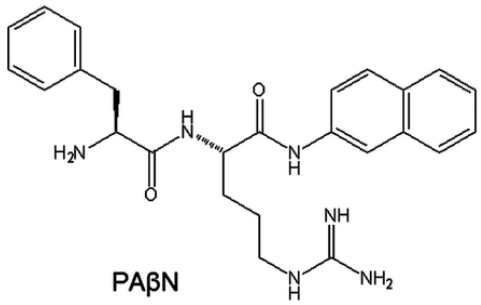

Fig.10: Phenylalanine-Arginine β-Naphthylamide

ii. **Quinoline derivatives:** This novel class of compounds was discovered by using several screening approaches against clinical MDR bacterial strains. Quinoline derivatives such as pyridoquinolones can restore the activity of norfloxacin in *E. aerogenes* overexpressing the AcrAB-TolC efflux pump, by acting as competitive inhibitor of this RND pump.[47] Some other synthetic analogues such as 4-substituted thioalkyl, alkylamino and alkoxy quinolone have also been reported to enhance the activity of tetracyclines, norfloxacin and chloramphenicol in clinical isolates of *K. pneumonia* and *E. aerogenes*.[48] A series of 2-phenyl-4(1H)-quinolone and 2-phenyl-4-hydroxyquinoline derivatives have been synthesized by modifying the flavone scaffold and these have been reported as potent inhibitors of NorA efflux pump in *S. aureus*.[49]

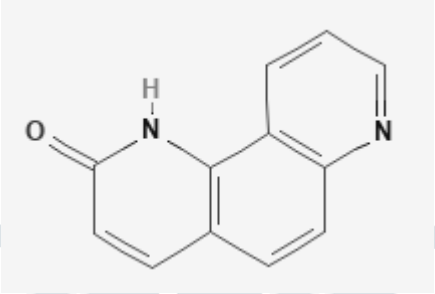

Fig.11: Pyridoquinolone

iii. **Arylpiperidines and aryl piperazine derivatives:** Arylpiperidine and its derivatives such as 3-arylpiperidine have been reported to restore susceptibility to linezolid and enhance its accumulation in *E. coli*.[50] Another series of analogues, phenylpiperidines, which are selective serotonin reuptake inhibitors, are known to inhibit the function of *S. aureus* MDR efflux pumps. These compounds also affect the activity of the AcrAB-TolC pump in *E. coli* partially but have no effect on the efflux activity of the *P. aeruginosa* RND efflux pumps such as MexAB-OprM or MexCD-OprJ.[51]

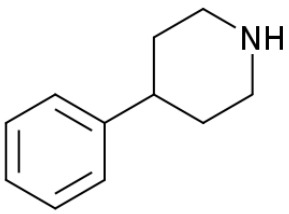

Fig.12: Phenylpiperidine

One of the leading arylpiperazine compounds, NMP(1-(1 naphthylmethyl)-piperazine), has been shown to restore the activity of RND pump substrates including levofloxacin and EtBr in *E. coli*-overexpressing AcrAB and AcrEF. However, due to serotonin reuptake inhibitor property of arylpiperazines, these compounds are likely to be toxic to mammalian cells.[52]

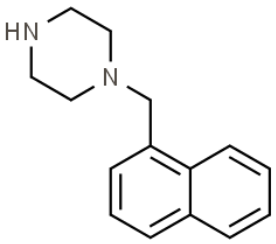

Fig.13: NMP (1-(1naphthylmethyl)-piperazine)

c) EPIs derived from microbes

Although most of the EPIs have their origin in natural products or semi-synthetic/synthetic chemical libraries, a small fraction of EPIs has been reported to originate from microbes. EA-371α and EA-371d, first extracted from fermentation extract of *Streptomyces* spp., have been recognized as specific inhibitors of the MexAB-OprM pump in *P. Aeruginosa*.[53] The novel structure of these compounds offers an opportunity to the researchers to synthesize novel derivatives with increased potency, bioavailability and reduced toxicity.With the three-dimensional crystal structure of efflux pumps available, further computational studies could also be useful to identify the molecular interaction of these compounds with such MDR pumps.

Efflux pump inhibitors from various sources[54] are in table 1.

Tab.1: List of efflux pump inhibitors (EPIs) from various sources

EPIs	Target efflux pumps	Bacterial strain	Substrate
Natural EPIs from plant sources			
Reserpine	NorA, TetK, MepA, Bmr	*S.aureus, Bacillus subtilis,Streptococcus pneumoniae*	Norfloxacin, ciprofloxacin, tetracycline
Piperine	NorA,MdeA,Rv1258c	*S. aureus, Mycobacterium* spp.	Norfloxacin, ciprofloxacin
Baicalein	NorA, TetK	*S. aureus, E. coli*	Ciprofloxacin, Tetracycline
5′-MHC	NorA	*S. aureus*	Berberine
Epigallocatechin gallate, Epicatechingallate	TetK	*S. aureus*	Tetracycline
Carnosic acid	MsrA	*S. aureus*	Erythromycin
Carnosol	MsrA, TetK	*S. aureus*	Tetracycline
Curcumin	NorA	*S. aureus*	Norfloxacin, Ciprofloxacin
Orobol	NorA	*S. aureus*	Berberine
Synthetic EPIs (chemically synthesized)			

PAβN	AdeFGH	*A. baumannii*	Trimethoprim, chloramphenicol and clindamycin
Pyridoquinolines	AcrAB-TolC	*E. aerogenes*	Norfloxacin
4-(2-piperidin-1-ylethoxy)-2-(4 propoxyphenyl) quinoline (PPQ)	NorA	*S. aureus*	Norfloxacin, ciprofloxacin
NMP(1(1naphthylmethyl)-piperazine)	AdeABC, AcrAB, AcrEF	*A.baumannii, E.coli, Enterobacter aerogenes, K. pneumonia*	Levofloxacin
Timcodar	–	*S. aureus, Mycobacterium* spp.	Norfloxacin, isoniazid, rifampicin
EPIs from microbial sources			
EA-371α and EA-371δ	MexAB-OprM	*P. aeruginosa*	Levofloxacin

CURRENT CHALLENGES FOR EPIs AS THERAPEUTIC AGENTS

Even though EPIs have been in laboratory experimentation since the 1990s, these are one of the futuristic prospects in our struggle against antibiotic resistant bacteria. However, the path leading to a successful commercial EPI has a lot of road blocks. These challenges are diverse in nature ranging from scientific and academic to administrative and economic. A major hurdle in developing and marketing an EPI is its economic worth. Major players in the pharmaceutical sector tend to stay away from this field as EPI is ultimately a new chemical entity (NCE).

The drug experts are well versed with the problems associated with NCE that is trumped by the idea of modifying the currently known antibiotics that, in turn, have a well-documented pharmacological profile and clinical data from numerous patient records.[55]

Academicians have looked for EPIs from both natural and synthetic compounds, however, their commercial production has not been taken under consideration at the laboratory level. The naturally derived EPIs have a complex and bulky structure making it difficult to synthesize. While synthetic molecules are easier to synthesize, these often suffer from poor solubility, toxicity and problems with cell permeability.

A major challenge for EPIs as therapeutic agents itself lies with their targets. Efflux pumps are one of the mechanisms but not always the only mechanismof antibiotic resistance. In bacteria such as *A.baumannii* and *P. aeruginosa*, the fluoroquinolone resistance is often mediated by the efflux pumps as well as point mutations in the gyrase-coding genes.[56] The problem is compounded by co-expression of multiple pumps and substrate redundancy. This event makes the EPI-antibiotic combinatorial therapy case-specific and casts doubts over the success at the community level.

While EPIs usually show promise with an antibiotic against the efflux pump, it is often seen that the same EPI does not potentiate the activity of other substrates of the same efflux pump. PAβN is effective at potentiating only a certain set of antibiotics while it does not really potentiate other substrate antibiotics of the pump MexAB.[44] Like PAβN, many EPIs are substrates of the pumps and act at a substrate-binding site. An indirect implication of this observation is that a high concentration of EPI would be required to ensure that these competitively prohibit the interaction of substrate antibiotics with the pump.

Unfortunately, the farewell with antibiotics that are also the substrate of the pump but have a different substrate-binding site. This greatly narrows the spectrum of an EPI, making it highly specific for only a limited number of substrates. Although it is difficult to discover an NCE that inhibits the efflux of antibiotic from a pump, it is extremely hard to find an EPI that would inhibit multiple pumps across multiple bacterial species. Although some molecules have a common mechanism of inhibition, these have been found to inhibit animal efflux pumps as well, resulting in toxicity and unfavourable pharmacological profile.[57]

FUTURE PERSPECTIVES

Efflux pump inhibitors can play a major role in tackling antimicrobial resistance by reviving antibiotics to which many clinically relevant pathogenic microorganisms have become resistant. They can be also used as a chemical tool to understand the molecular mechanisms of antimicrobial resistance that are mediated by efflux, particularly in Gram-negative bacteria. Efflux pump activities can be blocked by interfering with the functional assemblies of efflux pumps components.

Efflux pump activity may be bypassed or inhibited by employing a range of diverse approaches[44] including i) modifying the chemical structure of antibiotics to decrease their binding affinity to the transporter cavities; an approach which has been used for tetracycline antibiotics,[58] ii) using permeabilizers of the bacterial membrane to artificially increase intracellular antibiotic concentration; this approach has been applied for MexAB-OprM andMexXY-OprM efflux pump of *P. aeruginosa*,[59,60] iii) decreasing the number of active efflux complexes in the envelope of bacteria, by down regulating efflux pump gene expression or destabilising the protein component; like what has been applied for OmpF and OmpC,[61] iv) the destruction of the drug transporter energy source, using potassium cyanide and carbonyl cyanide m-chlorophenylhy- drazone(CCCP) affect the energy level of the bacterial membrane and reduce the efflux of various agents, v) blocking the functional assembly of the components of efflux systems,[62] vi) designing inhibitors that bind covalently to the substrate-binding cavities or block the channel of antibiotic transporter pumps; various natural products[63] and nanoparticles like zinc oxide[64]have been shown to inhibit bacterial efflux pumps by blocking them (a “molecular plug”) and vii) applying a decoy substrate as a competitive inhibitor for antibiotic transport inside the pump.

The use of EPIs obviates the discovery of new antibiotics, a strategy that saves a lot of time, effort and capital associated with discovery of a novel antibiotic. It allows the clinicians to exploit the already well-established pharmacological properties of known antibiotics. A very important implication of EPIs as therapeutic agents is the ability to reverse antibiotic resistance. It assumes great importance when we consider the fact that the current economic conditions also favour the large scale production of already optimized and stockpiled antibiotics. Another striking advantage of using EPIs is the extremely low frequency of generation of resistant mutants. The combination of antibiotic and EPI is, therefore, effective in not only tackling the already resistant bacteria but also providing respite from the future problems of development of resistance.

CONCLUSION

Bacterial resistance to antibiotics is a crucial issue and efflux systems strongly contribute to this phenomenon. They are responsible for intrinsic resistance of some pathogens and, as they can be located on plasmids, such systems can be acquired by many others. A number of bacterial efflux systems have been characterized but MDR transporters of the MFS and RND families, the first being mainly found in

Gram-positive pathogens and the second in Gram-negative bacteria, are the most implied in clinical resistance. Several EPIs of such systems have been identified, by rational design, testing of already known inhibitors of other transporters, or screening. Although many problems still remain (stability, selectivity, bioavailibility...), these results are encouraging to prospect on this field. The increasing number of reports on this domain indicates that searching for EPIs is actually worthwhile. It would be interesting to improve these molecules to widen their spectrum, even if a universal prokaryotic EPI might not be achievable.

REFERENCES

1. Karchmer, A.W., 2000. Nosocomial bloodstream infections: organisms, risk factors, and implications. *Clinical infectious diseases*, *31*(Supplement_4), pp.S139-S143.
2. Gholizadeh, Y. and Courvalin, P., 2000. Acquired and intrinsic glycopeptide resistance in enterococci. *International journal of antimicrobial agents*, *16*, pp.11-17.
3. Ruef, C., 2004. Epidemiology and clinical impact of glycopeptide resistance in Staphylococcus aureus. *Infection*, *32*(6), pp.315-327.
4. Blair, J.M., Webber, M.A., Baylay, A.J., Ogbolu, D.O. and Piddock, L.J., 2015. Molecular mechanisms of antibiotic resistance. *Nature reviews microbiology*, *13*(1), p.42.
5. Lamers, R.P., Cavallari, J.F. and Burrows, L.L., 2013. The efflux inhibitor phenylalanine-arginine beta-naphthylamide (PAβN) permeabilizes the outer membrane of gram-negative bacteria. *PLoS One*, *8*(3), p.e60666.
6. Schindler, B.D. and Kaatz, G.W., 2016. Multidrug efflux pumps of Gram-positive bacteria. *Drug Resistance Updates*, *27*, pp.1-13.
7. Li, X.Z., Plésiat, P. and Nikaido, H., 2015. The challenge of efflux-mediated antibiotic resistance in Gram-negative bacteria. *Clinical microbiology reviews*, *28*(2), pp.337-418.
8. Blair, J.M., Richmond, G.E. and Piddock, L.J., 2014. Multidrug efflux pumps in Gram-negative bacteria and their role in antibiotic resistance. *Future microbiology*, *9*(10), pp.1165-1177.
9. Poellarends, G., Vigano, C., Ruysschaert, J.M. and Konings, W.N., 2001. Bacterial multidrug resistance mediated by ABC transporters. *ABC proteins from bacteria to man*.
10. Lage, H., 2003. ABC-transporters: implications on drug resistance from microorganisms to human cancers. *International journal of antimicrobial agents*, *22*(3), pp.188-199.
11. Chung, Y.J. and Saier, J.M., 2001. SMR-type multidrug resistance pumps. *Current opinion in drug discovery & development*, *4*(2), pp.237-245.
12. Saier Jr, M.H., Beatty, J.T., Goffeau, A., Harley, K.T., Heijne, W.H., Huang, S.C., Jack, D.L., Jahn, P.S., Lew, K., Liu, J. and Pao, S.S., 1999. The major facilitator superfamily. *J MolMicrobiolBiotechnol*, *1*(2), pp.257-279.
13. Schindler, B.D. and Kaatz, G.W., 2016. Multidrug efflux pumps of Gram-positive bacteria. *Drug Resistance Updates*, *27*, pp.1-13.
14. Webber, M.A. and Piddock, L.J.V., 2003. The importance of efflux pumps in bacterial antibiotic resistance. *Journal of Antimicrobial Chemotherapy*, *51*(1), pp.9-11.
15. Sharma, A., Sharma, R., Bhattacharyya, T., Bhando, T. and Pathania, R., 2016. Fosfomycin resistance in Acinetobacter baumannii is mediated by efflux through a major facilitator superfamily (MFS) transporter—AbaF. *Journal of Antimicrobial Chemotherapy*, *72*(1), pp.68-74.
16. Costa, S.S., Ntokou, E., Martins, A., Viveiros, M., Pournaras, S., Couto, I. and Amaral, L., 2010. Identification of the plasmid-encoded efflux pump gene in methicillin-resistant (MRSA) strain HPV107; a representative of the MRSA Iberian clone.
17. Santagati, M., Iannelli, F., Cascone, C., Campanile, F., Oggioni, M.R., Stefani, S. and Pozzi, G., 2003. The novel conjugative transposon Tn 1207.3 carries the macrolide efflux gene mef (A) in Streptococcus pyogenes. *Microbial Drug Resistance*, *9*(3), pp.243-247.
18. Piddock, L.J., 2006. Multidrug-resistance efflux pumps? not just for resistance. *Nature Reviews Microbiology*, *4*(8), p.629.
19. Pagès, J.M. and Amaral, L., 2009. Mechanisms of drug efflux and strategies to combat them: challenging the efflux pump of Gram-negative bacteria. *BiochimicaetBiophysicaActa (BBA)-Proteins and Proteomics*, *1794*(5), pp.826-833.

20. Köhler, T., Pechère, J.C. and Plesiat, P., 1999. Bacterial antibiotic efflux systems of medical importance. *Cellular and Molecular Life Sciences CMLS*, *56*(9-10), pp.771-778.
21. Anes, J., McCusker, M.P., Fanning, S. and Martins, M., 2015. The ins and outs of RND efflux pumps in Escherichia coli. *Frontiers in microbiology*, *6*, p.587.
22. Alibert S, N'GompazaDiarra J, Hernandez J, et al. Multidrug efflux pumps and their role in antibiotic and antiseptic resistance: a pharmacodynamic perspective. Expert Opin Drug MetabToxicol. 2017;13:301-309.
23. Rampioni, G., Pillai, C.R., Longo, F., Bondì, R., Baldelli, V., Messina, M., Imperi, F., Visca, P. and Leoni, L., 2017. Effect of efflux pump inhibition on Pseudomonas aeruginosa transcriptome and virulence. *Scientific reports*, *7*(1), p.11392.
24. Blanco, P., Sanz-García, F., Hernando-Amado, S., Martínez, J.L. and Alcalde-Rico, M., 2018. The development of efflux pump inhibitors to treat Gram-negative infections. *Expert opinion on drug discovery*, *13*(10), pp.919-931.
25. P Tegos, G., Haynes, M., Jacob Strouse, J., Md T Khan, M., G Bologa, C., I Oprea, T. and A Sklar, L., 2011. Microbial efflux pump inhibition: tactics and strategies. *Current pharmaceutical design*, *17*(13), pp.1291-1302.
26. Passador, L., Cook, J.M., Gambello, M.J., Rust, L. and Iglewski, B.H., 1993. Expression of Pseudomonas aeruginosa virulence genes requires cell-to-cell communication. *Science*, *260*(5111), pp.1127-1130.
27. Hastings, J.W. and Greenberg, E.P., 1999. Quorum sensing: the explanation of a curious phenomenon reveals a common characteristic of bacteria. *Journal of Bacteriology*, *181*(9), pp.2667-2668.
28. Bhattacharyya, T., Sharma, A., Akhter, J. and Pathania, R., 2017. The small molecule IITR08027 restores the antibacterial activity of fluoroquinolones against multidrug-resistant Acinetobacter baumannii by efflux inhibition. *International journal of antimicrobial agents*, *50*(2), pp.219-226.
29. Anoushiravani, M., Falsafi, T. and Niknam, V., 2009. Proton motive force-dependent efflux of tetracycline in clinical isolates of Helicobacter pylori. *Journal of medical microbiology*, *58*(10), pp.1309-1313.
30. Fenosa, A., Fusté, E., Ruiz, L., Veiga-Crespo, P., Vinuesa, T., Guallar, V., Villa, T.G. and Viñas, M., 2009. Role of TolC in Klebsiellaoxytoca resistance to antibiotics. *Journal of antimicrobial chemotherapy*, *63*(4), pp.668-674.
31. OseiSekyere, J. and Amoako, D.G., 2017. Carbonyl cyanide m-chlorophenylhydrazine (CCCP) reverses resistance to colistin, but not to carbapenems and tigecycline in multidrug-resistant Enterobacteriaceae. *Frontiers in microbiology*, *8*, p.228.
32. Gupta, S., Cohen, K.A., Winglee, K., Maiga, M., Diarra, B. and Bishai, W.R., 2014. Efflux inhibition with verapamil potentiates bedaquiline in Mycobacterium tuberculosis. *Antimicrobial agents and chemotherapy*, *58*(1), pp.574-576.
33. Singh, M., Jadaun, G.P.S., Ramdas, K.S., Chauhan, V., Mishra, R., Gupta, K., Nair, S., Chauhan, D.S., Sharma, V.D., Venkatesan, K. and Katoch, V.M., 2011. Effect of efflux pump inhibitors on drug susceptibility of ofloxacin resistant Mycobacterium tuberculosis isolates. *The Indian journal of medical research*, *133*(5), p.535.
34. Stavri M, Piddock LJ, Gibbons S. Bacterial efflux pump inhibitors from natural sources. *J AntimicrobChemother* 2007; *59* : 1247-60.
35. Gibbons, S., Oluwatuyi, M. and Kaatz, G.W., 2003. A novel inhibitor of multidrug efflux pumps in Staphylococcus aureus. *Journal of Antimicrobial Chemotherapy*, *51*(1), pp.13-17.
36. Pfeifer, H.J., Greenblatt, D.K. and Koch-Wester, J., 1976. Clinical toxicity of reserpine in hospitalized patients: a report from the Boston Collaborative Drug Surveillance Program. *The American journal of the medical sciences*, *271*(3), pp.269-276.
37. Kumar, A., Khan, I.A., Koul, S., Koul, J.L., Taneja, S.C., Ali, I., Ali, F., Sharma, S., Mirza, Z.M., Kumar, M. and Sangwan, P.L., 2008. Novel structural analogues of piperine as inhibitors of the NorA efflux pump of Staphylococcus aureus. *Journal of Antimicrobial Chemotherapy*, *61*(6), pp.1270-1276.
38. Chan, B.C., Ip, M., Lau, C.B., Lui, S.L., Jolivalt, C., Ganem-Elbaz, C., Litaudon, M., Reiner, N.E., Gong, H., See, R.H. and Fung, K.P., 2011. Synergistic effects of baicalein with ciprofloxacin against

NorA over-expressed methicillin-resistant Staphylococcus aureus (MRSA) and inhibition of MRSA pyruvate kinase. *Journal of ethnopharmacology*, *137*(1), pp.767-773.
39. Fujita, M., Shiota, S., Kuroda, T., Hatano, T., Yoshida, T., Mizushima, T. and Tsuchiya, T., 2005. Remarkable Synergies between Baicalein and Tetracycline, and Baicalein and β-Lactams against Methicillin-Resistant Staphylococcus aureus. *Microbiology and immunology*, *49*(4), pp.391-396.
40. Stermitz, F.R., Lorenz, P., Tawara, J.N., Zenewicz, L.A. and Lewis, K., 2000. Synergy in a medicinal plant: antimicrobial action of berberine potentiated by 5′-methoxyhydnocarpin, a multidrug pump inhibitor. *Proceedings of the National Academy of Sciences*, *97*(4), pp.1433-1437.
41. Morel, C., Stermitz, F.R., Tegos, G. and Lewis, K., 2003. Isoflavones as potentiators of antibacterial activity. *Journal of agricultural and food chemistry*, *51*(19), pp.5677-5679.
42. Roccaro, A.S., Blanco, A.R., Giuliano, F., Rusciano, D. and Enea, V., 2004. Epigallocatechin-gallate enhances the activity of tetracycline in staphylococci by inhibiting its efflux from bacterial cells. *Antimicrobial Agents and Chemotherapy*, *48*(6), pp.1968-1973.
43. Oluwatuyi, M., Kaatz, G.W. and Gibbons, S., 2004. Antibacterial and resistance modifying activity of Rosmarinus officinalis. *Phytochemistry*, *65*(24), pp.3249-3254.
44. Lomovskaya, O., Warren, M.S., Lee, A., Galazzo, J., Fronko, R., Lee, M.A.Y., Blais, J., Cho, D., Chamberland, S., Renau, T. and Leger, R., 2001. Identification and characterization of inhibitors of multidrug resistance efflux pumps in Pseudomonas aeruginosa: novel agents for combination therapy. *Antimicrobial agents and chemotherapy*, *45*(1), pp.105-116.
45. Vargiu AV, Nikaido H. Multidrug binding properties of the AcrB efflux pump characterized by molecular dynamics simulations. *Proc Natl AcadSci*2012; *109* : 20637-42.
46. Opperman, T.J. and Nguyen, S.T., 2015. Recent advances toward a molecular mechanism of efflux pump inhibition. *Frontiers in microbiology*, *6*, p.421.
47. Chevalier, J., Atifi, S., Eyraud, A., Mahamoud, A., Barbe, J. and Pagès, J.M., 2001. New Pyridoquinoline Derivatives as Potential Inhibitors of the Fluoroquinolone Efflux Pump in Resistant Enterobacter aerogenes Strains. *Journal of medicinal chemistry*, *44*(23), pp.4023-4026.
48. Pradel, E. and Pagès, J.M., 2002. The AcrAB-TolC efflux pump contributes to multidrug resistance in the nosocomial pathogen Enterobacter aerogenes. *Antimicrobial agents and chemotherapy*, *46*(8), pp.2640-2643.
49. Sabatini, S., Gosetto, F., Manfroni, G., Tabarrini, O., Kaatz, G.W., Patel, D. and Cecchetti, V., 2011. Evolution from a natural flavones nucleus to obtain 2-(4-Propoxyphenyl) quinoline derivatives as potent inhibitors of the S. aureus NorA efflux pump. *Journal of medicinal chemistry*, *54*(16), pp.5722-5736.
50. Thorarensen, A., Presley-Bodnar, A.L., Marotti, K.R., Boyle, T.P., Heckaman, C.L., Bohanon, M.J., Tomich, P.K., Zurenko, G.E., Sweeney, M.T. and Yagi, B.H., 2001. 3-Arylpiperidines as potentiators of existing antibacterial agents. *Bioorganic & medicinal chemistry letters*, *11*(14), pp.1903-1906.
51. Kaatz, G.W., Moudgal, V.V., Seo, S.M., Hansen, J.B. and Kristiansen, J.E., 2003. Phenylpiperidine selective serotonin reuptake inhibitors interfere with multidrug efflux pump activity in Staphylococcus aureus. *International journal of antimicrobial agents*, *22*(3), pp.254-261.
52. Bohnert, J.A. and Kern, W.V., 2005. Selected arylpiperazines are capable of reversing multidrug resistance in Escherichia coli overexpressing RND efflux pumps. *Antimicrobial agents and chemotherapy*, *49*(2), pp.849-852.
53. Lee, M.D., Galazzo, J.L., Staley, A.L., Lee, J.C., Warren, M.S., Fuernkranz, H., Chamberland, S., Lomovskaya, O. and Miller, G.H., 2001. Microbial fermentation-derived inhibitors of efflux-pump-mediated drug resistance. *IL farmaco*, *56*(1-2), pp.81-85.
54. Sharma, A., Gupta, V.K. and Pathania, R., 2019. Efflux pump inhibitors for bacterial pathogens: From bench to bedside. *The Indian Journal of Medical Research*, *149*(2), p.129.
55. Lomovskaya, O. and Bostian, K.A., 2006. Practical applications and feasibility of efflux pump inhibitors in the clinic—a vision for applied use. *Biochemical pharmacology*, *71*(7), pp.910-918.
56. Nakajima A, Sugimoto Y, Yoneyama H, Nakae T. High-level fluoroquinolone resistance in *Pseudomonas aeruginosa* due to interplay of the MexAB-oprM efflux pump and the DNA gyrase mutation. *MicrobiolImmunol*2002; *46* : 391-5.

57. Vargiu, A.V., Collu, F., Schulz, R., Pos, K.M., Zacharias, M., Kleinekathöfer, U. and Ruggerone, P., 2011. Effect of the F610A mutation on substrate extrusion in the AcrB transporter: explanation and rationale by molecular dynamics simulations. *Journal of the American Chemical Society*, *133*(28), pp.10704-10707.
58. Chopra, I. and Roberts, M., 2001. Tetracycline antibiotics: mode of action, applications, molecular biology, and epidemiology of bacterial resistance. *Microbiol. Mol. Biol. Rev.*, *65*(2), pp.232-260.
59. Li, X.Z.; Zhang, L.; Poole, K. Interplay between the MexAMexB- OprM multidrug efflux system and the outer membrane barrier in the multiple antibiotic resistance of *Pseudomonasaeruginosa*. *J. Antimicrob. Chemother.*,2000, *45*(4), 433-436.
60. Iino, R., Nishino, K., Noji, H., Yamaguchi, A. and Matsumoto, Y., 2012. A microfluidic device for simple and rapid evaluation of multidrug efflux pump inhibitors. *Frontiers in microbiology*, *3*, p.40.
61. Dupont, M., Dé, E., Chollet, R., Chevalier, J. and Pagès, J.M., 2004. Enterobacter aerogenesOmpX, a cation-selective channel mar-and osmo-regulated. *FEBS letters*, *569*(1-3), pp.27-30.
62. Pagès, J.M., Masi, M. and Barbe, J., 2005. Inhibitors of efflux pumps in Gram-negative bacteria. *Trends in molecular medicine*, *11*(8), pp.382-389.
63. Molnár, J., Engi, H., Hohmann, J., Molnár, P., Deli, J., Wesolowska, O., Michalak, K. and Wang, Q., 2010. Reversal of multidrug resistance by natural substances from plants. *Current topics in medicinal chemistry*, *10*(17), pp.1757-1768.
64. Banoee, M., Seif, S., Nazari, Z.E., Jafari-Fesharaki, P., Shahverdi, H.R., Moballegh, A., Moghaddam, K.M. and Shahverdi, A.R., 2010. ZnO nanoparticles enhanced antibacterial activity of ciprofloxacin against Staphylococcus aureus and Escherichia coli. *Journal of Biomedical Materials Research Part B: Applied Biomaterials*, *93*(2), pp.557-561.