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EFFLUX PUMPS: AN OVERVIEW

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ABSTRACT: Antibiotic efflux pumps appear a major component of microbial resistance to many classes of major antibiotics namely the tetracycline, the macrolides, and the fluoroquinolones. These are totally synthetic, amphiphilic compounds with no known 'natural' counterpart, antibiotic efflux appears sufficient per se to confer a medium or high level of resistance, defeating medically applicable treatments of the corresponding infections with these antibiotics. Antibiotic efflux may be found in association with other mechanisms, such as antibiotic inactivation, to confer high-level resistance on bacteria. In some respects, this phenomenon bears similarities with the cooperation of drug-extruding pumps and the cytochrome P450-based degradation pathways in enterocytes. An increasing knowledge about a variety of efflux pumps that are involved in MDR of pathogens, is an important challenge for current medicinal chemistry. The existence of antibiotic efflux pumps, and their impact on therapy, must now be taken fully into account for the selection of novel antimicrobials. The design of specific, potent inhibitors appears to be an important goal for the improved control of infectious diseases in the near future. Clinical statistics reveal significant prevalence of antibiotic efflux pumps on resistance with that of the other resistance mechanisms. Recent surveys point to alarming figures of 40 - 90% of some bacterial pathogens (*S.pneumonia, S. pyogenes,* and *P. aeruginosa*) bearing efflux mechanisms for the major classes of clinically available antibiotics. An increasing number of patents indicates the intense role of efflux pumps in field of microbiology. Patent searches carried out on various online databases concluded the latest perspectives of efflux pump inhibitors.

INTRODUCTION: Antibiotic: The history of mankind indicated from time to time that infectious diseases are a major cause of death and disability accounting for major global disease burden. The discovery of penicillin in the 1940s and several other antibiotics in subsequent years led to great improvements in the management of infectious diseases.

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Antibiotics are defined as substances produced by microorganisms (bacteria, fungi and actinomycetes), which suppress the proliferation of other (pathogenic) microorganisms and can eventually destroy these. The term "antibiotics" first used in 1942 by Selman Waksman, also includes synthetic antimicrobials such as the sulfonamides and quinolones, which are not actually synthesized by microbes.

The discovery of antibiotics was a turning point in human history, revolutionizing medicines in many respects, and countless lives have been saved by their use. Antibiotics like aminoglycoside, chloramphenicol, macrolide, and tetracycline can inhibit protein synthesis while quinolone and

rifampin interact with the synthesis of DNA and RNA. some groups such as β-lactam and glycopeptides inhibit the synthesis of bacterial cell wall and some others like sulfonamide and trimethoprim modify the energy metabolism of a microbial cell. Thus, several different classes of antimicrobial agents are known having Different modes of action explained in **Table 1**.

These compounds target vital microbial biochemistry at vulnerable metabolic and physiologic hubs such as translation, DNA replication, and cell wall biosynthesis. Thus, one classification system of antibiotics is based on their mode of action while another classification system is based on biological activity [1].

TABLE 1: MODES OF ACTION AND RESISTANCE MECHANISMS OF COMMONLY USED ANTIBIOTICS

Antibiotic class	Examples	Target	Mode of resistance
β-Lactams	Penicillins (ampicillin), cephalosporins (cephamycin), penems (meropenem), monobactams (aztreonam)	Peptidoglycan biosynthesis	Hydrolysis, efflux, altered target
Aminoglycosides	Gentamicin, streptomycin, spectinomycin, Amikacin	Translation	Phosphorylation, acetylation, nucleotidylation, efflux, altered target
Glycopeptides	Vancomycin, teicoplanin	Peptidoglycan biosynthesis	Reprogramming peptidoglycan biosynthesis
Tetracyclines	Minocycline, tigecycline, Tetracyclin	Translation	Monooxygenation, efflux, altered target
Macrolides	Erythromycin, azithromycin	Translation	Hydrolysis, glycosylation, phosphorylation, efflux, altered target
Lincosamides	Clindamycin	Translation	Nucleotidylation, efflux, altered target
Streptogramins	Synercid	Translation	C-O lyase (type B streptogramins), acetylation (type A streptogramins), efflux, altered target
Oxazolidinones	Linezolid	Translation	Efflux, altered target
Phenicols	Chloramphenicol	Translation	Acetylation, efflux, altered target
Quinolones	Ciprofloxacin, Norfloxacin	DNA replication	Acetylation, efflux, altered target
Pyrimidines	Trimethoprim	C1 metabolism	Efflux, altered target
Sulfonamides	Sulfamethoxazole	C1 metabolism	Efflux, altered target
Rifamycins	Rifampin	Transcription	ADP-ribosylation, efflux, altered target
Lipopeptides	Daptomycin	Cell membrane	Altered target
Cationic peptides	Colistin	Cell membrane	Altered target, efflux

However, despite this success, the increased use of antibiotics led to the inevitable development of resistance, with the effect that diseases that were hitherto thought to have been controlled by antibiotics later re-emerged as resistant infections [2]. The successful use of any therapeutic agent is compromised by the potential development of tolerance or resistance to that compound from the time it is first employed. Mutation and selection together with the mechanism of genetic exchange enable many bacterial species to adapt quickly to the introduction of antimicrobial agents into their environment.

Antibiotic resistance genes database lists the existence of more than 20,000 potential resistance genes (r genes) of nearly 400 different types, predicted from available bacterial genome sequences [3]. Bacteria carrying resistance genes have the ability to spread these genes to other species *via* horizontal gene transfer.

Therefore, even if the specific antibiotic is no longer introduced into the environment, antibiotic-resistance genes will persist through the bacteria that have since replicated without continuous exposure.

Summarily, resistance to antimicrobials is as a result of three main strategies namely enzymatic inactivation of the drug [4], modification of target sites [5] and extrusion by efflux [6]. Several studies have shown that active efflux can be a mechanism of resistance for almost all antibiotics [7, 8, 9]. The majority of the efflux systems in bacteria are non-drug-specific proteins that can recognize and pump out a broad range of chemically and structurally unrelated compounds (including antibiotics) from bacteria in an energy-dependent manner, without drug alteration or degradation [10]. The consequence of this drug extrusion is that, it leads to a reduced intracellular concentration of the antimicrobial/ antibiotics such that the bacterium can survive

under conditions of elevated anti-microbial/ antibiotics concentration [11]. The MIC of the drug against such organisms will be higher than predicted.

Role of Efflux Pumps in Antibiotic Resistance: These efflux mechanisms are broadly recognized as major components of resistance to many classes of chemotherapeutic agents as well as anti-microbials. Efflux occurs due to the activity of membrane transporter proteins widely known as multidrug efflux systems (MES). Efflux pumps contribute to multidrug resistance as they expel different types of antibiotics and chemicals such as dyes, organic solvents, detergents, molecules needed for the cell–cell communication, biocides, and metabolic products.

Hence understanding the mechanisms by which these pumps act and how to overcome its activity opens the door for restoring the antibiotic activity and constitute a promising target for novel antibacterial agents. All bacterial genomics contain efflux pump genes *e.g. E. coli* housekeeping efflux system AcrAB-TolC include chloramphenicol, fluoroquinolone, tetracycline, novobiocin, rifampin, fusidic acid, nalidixic acid and β-lactam antibiotics [12].

Similarly, the AcrAB-TolC efflux system in *S. typhimurium* also found to be able to expel different classes of antimicrobial agents such as quinolones, chloramphenicol, tetracycline and nalidixic acid [13]. In *P. aeruginosa*, MexAB–OprM and MexXY-OprM, are constitutively expressed and both of the systems can actively export fluoroquinolones, tetracycline, chloramphenicol. In addition to these common substrates, MexAB–OprM system can also export novobiocin and b-lactams, such as carbenicilline, and MexXY system can also export aminoglycosides [14]. The expression is subject to tight regulation by various local and global transcriptional regulators indicates that drug efflux pumps have physiological functions, especially during the stress adaptation, development and pathogenesis and virulence of bacteria. The bacterial efflux pumps have the capacity to extrude various host-derived anti-microbial compounds and facilitate the adaptation and survival of bacteria in their ecological and physiological niches [15].

Multidrug transporters in bacteria are generally classified into five families on the basis of sequence similarity [16].

- The Major Facilitator super family (MFS)
- The Resistance-Nodulation - Cell Division (RND) family
- The Small Multidrug Resistance (SMR) family
- The Multidrug and Toxic compound Extrusion (MATE) family
- The ATP-Binding Cassette (ABC) family

Those five classes obtain energy required for the active transporting either from H^+ protons (RND, SMR, and MFS), Na^+ dependent (MATE), or by hydrolysis ATP (ABC).

Transporters has been classified on the basis of 4-digit nomenclature, constructed in analogy with enzyme nomenclature and in which the first group of digits refers to the mode of transport and energy source, the second and the third to the phylogeny (super families and families), and the fourth to the substrate [51]. The primary active transporters use various forms of energy and constitute the bulk of the drug efflux pumps in eukaryotic cells; drug efflux transporters are classically energized by ATP. The secondary active transporters, acting as symports and antiports (*i.e.* coupling the drug efflux to the downhill transport of an ion along a concentration gradient), are predominant in bacteria. Phylogenic studies classify each further into super families, families, and clusters, in correlation with their substrate specificity.

Although most drug efflux pumps confer a multidrug resistance phenotype, corresponding to the large variety of substrates they may recognize, including several classes of antibiotics as well as non-antibiotic drugs. Another significant characteristic of the antibiotic efflux pumps is the variety of molecules they may transport, which actually can be related directly to their well-known poor substrate specificity. Considering the pharmacochemical aspects first, it is clear that only very minimal common structural determinants are necessary to obtain detectable transport. Nevertheless, for each class of transporters, investigators are trying to determine which substrate features are the most specific. Although these substrate specificities may appear difficult to establish, a unifying hypothesis is that most,

transporters recognize molecules with a polar, often slightly charged head associated with a hydrophobic domain, the importance of lipophilia is to ascertain in present scenario [17]. Interestingly antibiotic-specific efflux pumps appear to be restricted to organisms producing antibiotics and are often an integral part of the corresponding biosynthetic pathway.

The eukaryotic cells systems, achieve efficient protection against 'chemical invaders' *via* active drug efflux pumps as complementing the cytochrome P450 - like any other enzyme-based detoxification systems. Antibiotic efflux transport has now been observed for many classes of drug efflux pumps. It seems antibiotic efflux transport mechanisms play important roles in eukaryotic cells by modulating the pharmacological and toxicological profile of antibiotics. So among eukaryotes, six families belonging to the ATP-binding cassette super family, and including the P-glycoprotein in the MDR (Multi Drug Resistance) group and the MRP (Multidrug Resistance Protein), have been recognized as primary active transporters.

Role of Efflux Pumps to Antibiotic Resistance in Biofilms: It is well known that biofilms can play an important role in resistance to antibiotics due to the extracellular polysaccharide matrix, higher bacterial cell density, and lower bacterial growth that provide a good protective means for the bacterial cells against antimicrobial agents. In addition, a novel efflux pump has been identified as important to increase the bacterial antibiotic resistance in biofilms rather than the planktonic cells where the deletion of the genes encoding for this pump render the cells in biofilms more sensitive to antibiotics, such as to bramycin, indicating that this efflux pump is essential to pseudomonas ant defect in efflux activity impairs biofilm formation, which linked the physiological function of efflux pump to biofilm formation [18] reported that inhibition of efflux activities by efflux pump inhibitors (EPIs) reduced biofilm formation in both *E. coli* and *Klebsiella strains*, while simultaneous treatment with different EPIs abolished biofilm formation completely.

The efflux pump inhibitors as new therapeutic agents. The continuous increase in the development of multidrug resistance by many pathogens has resulted in difficulties fighting many infectious diseases. In view of the fact that the majority of those multidrug resistant pathogens expresses and overproduces efflux pumps that are responsible for the expelling and extruding of the antibiotics from inside the cells, the new direction for other chemotherapeutics is the use of efflux pump inhibitors (EPIs). Using the (EPIs) together with antibiotics can reduce the invasiveness of *P. aeruginosa* besides its role in lowering the antibiotic minimal inhibitory concentration [19]. For example, the sensitivity to ciprofloxacin by *P. aeruginosa* is largely increased upon using this inhibitor proving that efflux pumps play a role in the resistance of this organism to this antibiotic [20].

Thus the inhibition of the efflux pumps is promising in order to (1) increase the intracellular drug concentration, (2) restore the drug activity against the resistant strains, and (3) minimizes further development of resistant strains. However, this requires the understanding of the structural and physiological mechanisms of the responsible efflux pumps (**Fig. 1**) [21, 22].

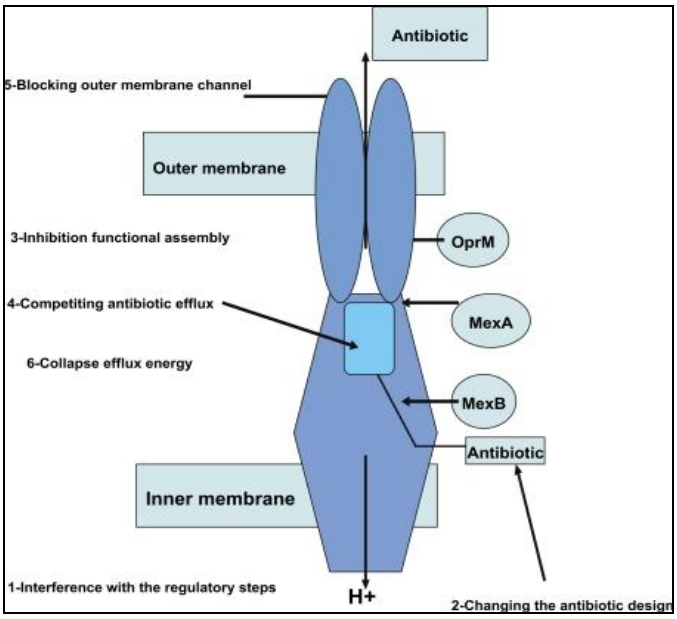

FIG. 1: THE INHIBITION OF EFFLUX PUMPS CAN BE ACHIEVED BY DIFFERENT MECHANISMS

(1) interference with the regulatory steps needed for the expression of the efflux pump, (2) chemical changes in the antibiotic structure hence hindering its attachment as the specific substrate, (3) disruption of the assembly of the efflux pump-components, (4) inhibition of the substrate

(antibiotic) binding by either competitive or non-competitive binding using other compounds, (5) blocking the outer most pores responsible for the efflux of antibiotic compound, (6) interference with the energy required for the pump activity.

By passing efflux pump activity may be achieved through a variety of different approaches: (1) by modifying the chemical design of previous antibiotics to reduce their respective affinity for binding sites and cavities located inside the pump transporter; (2) by increasing the influx of antibiotics, using membrane permeabilizers that subsequently increase the intracellular concentration of drugs; (3) by down-regulating the expression of efflux pump genes and/or decreasing the level of active efflux complexes in the bacterial envelope; (4) by collapsing the energy required to support the drug transport; (5) by inhibiting the functional assembly of efflux pump components; (6) by inserting a carefully-designed molecular plug inside the membrane channels responsible of antibiotic transport (inside the pump cavities or inside the exit channel component) or (7) by generating a dynamic competition, between a decoy- substrate and the antibiotic, during transport flux inside the pump [23].

To date, at least two classes of broad-spectrum EPI, such as peptidomimetics and pyridopyrimidines, have been extensively characterized. Phenyl-arginine beta-naphthylamide (PAbN) [MC-207,110] was the first identified EPI that successfully inhibited clinically relevant *P. aeruginosa* efflux systems, MexAB–OprM, MexCD-OprJ, MexEF–OprN, and MexXY–OprM [24]. It successfully reduced the emergence of levofloxacin-resistance in *P. aeruginosa* strain PAO1 treated with levofloxacin [25]. PAbN also has an activity against the AcrAB-TolC in a variety of Gram-negative pathogens, such as *E. coli*, *S. typhimurium* and *K. pneumonia*. PAbN derivatives have also been explored in order to improve the efficacy. Further modify of this led to the development of pyridopyrimidine compounds.

The scaffolds redesign approach was explored by Nakayama *et al*., 2003 [26], by addition of hydrophilic chains, as well as introduction of quaternary ammonium salt side chains [27, 28], the compound [[2-({[((3R)-1-{8-{[(4-tert-butyl - 1, 3-thiazol-2-yl) amino]carbonyl}-4-oxo-3-[(E)-2-(1H-tetrazol-5-yl) vinyl]-4H-pyrido [1,2-a]pyrimidin-2-yl}piperidin-3-yl)oxy]carbonyl}amino) ethyl] (di-methyl) ammonio] acetate [D13-9001]. This was referred to as AcrAB/MexAB-specific inhibitor of pyridopyrimidine derivative (ABI-PP) by Nakashima *et al*., 2013 hinders the rotation of the pump [29].

Another group of EPIs called the quinoline derivatives [22] include chloroquinolone, alkoxy-quinolone, alkylaminoquinolone, pyrridoquinolone, and thioalkoxyquinolone [30.] Some of them have been shown to be able to reduce the MICs of quinolones and cyclines. Moreover, these derivatives can increase intracellular concentration of radiolabelled norfloxacin or chloramphenicol [31, 32] and increase antibiotic susceptibility for various strains including the clinical isolates of *Enterobacter aerogenes* and *K. pneumoniae*. They do not destroy the membrane integrity of bacteria as measured by the potassium leakage and periplasmic activity tests. High-throughput assays have also screened compounds that might be putative EPIs.

Thorarensen *et al*., 2001 revealed for putative inhibitor of *E. coli* in the presence of novobiocin, a 3-arylpiperidine derivative [33]. Another screening of an N-heterocyclic organic compound library was conducted to identify putative EPIs reversing multidrug resistance in *E. coli* over-expressed AcrAB and AcrEF efflux pumps [34]. Among the compounds tested, naphthylpiperazines (NMP) was the most potent arylpiperazines that has been shown to increase the intracellular accumulation of several antibiotics, such as fluoroquinolones, chloramphenicol, and linezolid.

Despite the abundant literature about the antimicrobial properties of plant extracts, none of the plant derived chemicals have successfully been used for clinical use as antibiotics antibasis. Different phytochemicals present in the plant extracts might inhibit bacteria by different mechanisms. In addition to plants being potential sources of direct antibacterial drugs, research has also shown that some secondary metabolites of plants with no intrinsic anti-microbial activity are useful in sensitizing bacterial cells to anti-microbial agents [35, 36]. These compounds are believed to play

a role in the plant's defense against infection by working in synergy with intrinsic antimicrobials. It has therefore been suggested recently, that such compounds can potentially be used to improve the efficacy of antibiotics against bacterial pathogens.

Various findings of Indian medicinal plants have confirmed that indeed plants can be sources of compounds that can potentiate the activity of antibiotics against resistant bacterial pathogens [37, 38, 39, 40, 41]. These compounds have variably been termed resistance modifying, modulating or reversal agents. The search for resistance modifying compounds that can improve the efficacy of antibiotics when used in combination, appears attractive as it allows for the recycling of old and relatively cheaper antibiotics that have been rendered ineffective due to resistance. It is therefore imperative that instead of new antibiotics, resistance-modifying agents and, more specifically, efflux pump inhibitors (EPIs) needs to be characterized. The use of bacterial resistance modifiers such as EPIs could facilitate the re-introduction of therapeutically ineffective antibiotics back into clinical use.

Natural products (NP) are often less toxic than synthetic compounds and have been implicated in efflux inhibition have also attracted intensive attention [42]. Two compounds, EA-371a and EA-371d, in microbial fermentation extracts have been specific inhibitors of the MexAB–OprM pump in *P. aeruginosa*. In addition, *Berberis aetnensis* extracted compound pheophorbidea was shown to lower the MIC of ciprofloxacin against *E. coli* and *P. aeruginosa* [43, 44]. Genistein isolated from *Lupinus argenteus*, spinosan A isolated from *Dalea spinosa* and Tiliroside isolated from *Herissantia tiubae* have been found to be EPIs against *S. aureus* NorA[45 - 47].

Though considerable efforts have been made to the development of EPIs, none of these EPIs is used in clinics currently. One main reason is that the mechanisms of most EPIs remain unknown, except the extensively investigated PAbN.

Patents: The purpose of the Patent Search/ Literature review is to inform, provide appropriate research, and demonstrate continued progress towards the updated information available related to the efflux pump inhibitor. Patent searchers are carried out on various online databases available and concluded with the latest technology available in efflux pump inhibitor. Wu Junwei, Zhang has filed Patent (CN102988400) in 2012 at China in which he briefed about the Application of liquiritin in preparing *E. coli* fluoroquinolone efflux pump inhibitor [48].

In this invention, he observed that, this invention broadens application fields of an efficient and safe bacteria efflux pump inhibitor for anti-infective therapy along with the liquiritin and improves market value of the liquiritin. The liquiritin can certain decreases the lowest inhibitory concentration of fluoroquinolone of drug resistant *E. coli* (fluoroquinolone active efflux phenotype and acrA gene high expression strain), apparently increase drug accumulation concentration in a thallus, and evidently decreases the expression quantity of an efflux pump gene acrA.

Hence, it is proved that susceptibility of the drug-resistant *Escherichia coli* to the fluoroquinolone is raised and usage amounts of drugs are reduced. The treatment cost is reduced and food safety problems such as drug residues are decreased. However, the liquiritin is a natural compound existing in plants and it has no side effects such as teratogenesis and carcinogenesis. In 2005, Tomasz Glinka has filed the Patent Application no. WO2005113579A1 in USA [49]. He worked on Bacterial efflux pump inhibitors and methods of treating bacterial infections. In this invention, he focused on antimicrobial agents and more specifically it relates to EPI compounds to be co-administered with antimicrobial agents for the treatment of infections caused by drug resistant pathogens. The EPI compounds are soft drugs which exhibit a reduced propensity for tissue accumulation. In the experiment, he includes novel compounds useful as efflux pump inhibitors, compositions and devices comprising such efflux pump inhibitors, and therapeutic use of such compounds.

This invention β-lactam compounds and analogous compositions as efflux pump inhibitors and/or porin modulators are used. Tomasz Glinka and his team focused on administered the mixture with antimicrobial agents for the treatment of infections caused by various microorganisms, in particular

drug resistant microorganisms. Essential Therapeutics, Inc. filed a Patent Application no. US 6495591 B1 in 1998 on the fungal efflux pump inhibitors [50]. In this Invention, the use of compounds of the milbemycin class as inhibitors of efflux pumps in microbes or other cells is described, along with pharmaceutical compositions incorporating a milbemycin. In this experiment, it is also developed a method of screening for compounds which inhibit a CDR1, CDR2, BEN, or FLU1 efflux pump or a pump with components having a high level of protein level sequence similarity with the components of those efflux pumps. Many patents and experiments are filed in the field of efflux pump inhibitor.

CONCLUSION: An increasing knowledge about a variety of efflux pumps that are involved in MDR of pathogens, is an important challenge for current medicinal chemistry. The search for new efflux pump inhibitors performed for few decades indicates that chemical structures of the NorA has been thoroughly studied and target to generate EPIs. It can also be observed that the most active agents belongs to the family of compounds possessing conjugated double bonds, *e.g*. chalcones, piperine-like compounds, N-cinnamoyl phenalkylamides or citral amide derivatives. Indole-, dihydronaphthyl-, 2-chloro-5-bromo-phenyl- or piperidine moieties seem to be profitable for the EPI properties as well. Human proteins expelling toxic substances out of tissues display significant similarities to those involved in bacterial MDR, bacterial EPIs probably inhibit human detoxification simultaneously with the inhibition of bacterial MDR. The active compounds give a new hope for their future therapeutic usage as antibiotics "adjuvants" and should be a subject of wider investigations, including their pharmacokinetic properties and toxic effects. The search for new EPIs should pay more attention to developing MDR efflux protein targets, including SMR, MATE, ABC or other members of the MFS family.

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