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MULTIDRUG EFFLUX PUMPS IN BACTERIA AND EFFLUX PUMP INHIBITORS

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Abstract: Antimicrobial resistance is becoming a paramount health concern nowadays. The increasing drug resistance in microbes is due to improper medications or over usage of drugs. Bacteria develop many mechanisms to extrude the antibiotics entering the cell. The most prominent are the efflux pumps (EPs). EPs play a significant role in intrinsic and acquired bacterial resistance, mainly in Gram-negative bacteria. EPs may be unique to one substrate or transport several structurally different compounds (including multi-class antibiotics). These pumps are generally associated with multiple drug resistance (MDR). EPs are energized by a proton motive force and can pump a vast range of detergents, drugs, antibiotics and also β-lactams, which are impermeable to the cytoplasmic membrane. There are five leading efflux transporter families in the prokaryotic kingdom: MF (Major Facilitator), MATE (Multidrug And Toxic Efflux), RND (Resistance-Nodulation-Division), SMR (Small Multidrug Resistance) and ABC (ATP Binding Cassette). Apart from the ABC family, which utilizes ATP hydrolysis to drive the export of substrates, all other systems use the proton motive force as an energy source. Some molecules known as Efflux Pump Inhibitors (EPI) can inhibit EPs in Gram-positive and Gram-negative bacteria. EPIs can interfere with the efflux of antimicrobial agents, leading to an increase in the concentration of antibiotics inside the bacterium, thus killing it. Therefore, identifying new EPIs appears to be a promising strategy for countering antimicrobial drug resistance (AMR). This mini-review focuses on the major efflux transporters of the bacteria and the progress in identifying Efflux Pump Inhibitors.

1. Introduction. 2. Major classes of efflux pumps. 2.1. ATP-Binding Cassette Superfamily. 2.2. Major Facilitator Superfamily. 2.3. Multidrug And Toxic Compound Extrusion Family. 2.4. Small Multi-drug Resistance Family. 2.5. Resistance-Nodulation-Division Superfamily. 3. Efflux pumps and their role in virulence and biofilm formation. 4. Efflux Pump Inhibitors

Keywords: Antimicrobial resistance, MDR, Multidrug efflux pumps, Biofilm, Efflux Pump Inhibtor (EPI)

1. Introduction

Antimicrobial resistance (AMR) arises when microorganisms develop strategies to evade antimicrobial agents, making them ineffective. AMR is a global threat to the public health system across the globe. According to a recent report from the WHO, drug-resistant diseases claim the lives of at least 700,000 individuals each year [1]. Due to the inappropriate dosage and use of current antimicrobials, many pathogens become multidrug-resistant (MDR) [2]. It is generally assumed that resistance to antibiotics and other antimicrobials has developed due to selective pressures resulting from indiscriminate and inappropriate use. Increased antibiotic resistance has resulted in fewer treatment options for patients and increased morbidity and death. Due to this, we are now confronted with more acute diseases that require more intense treatment, relatively extended hospital stays and expensive hospitalization [3]. Bacteria can gain resistance through diverse mechanisms such as restricting drug uptake into the cell, altering a drug target, enzymatic degradation of a drug, and active efflux of a drug. The efflux mechanism involves the extrusion of drugs from the interior to the external environment by protein transporters called multidrug efflux pumps (EPs). An EP reduces the efficacy of antibiotics by preventing their intracellular accumulation. The efflux-mediated resistance is widespread in the bacteria [4]. Numerous studies have shown that EPs, such as AcrAB-TolC of *Escherichia coli*, MexAB-OprM of *Pseudomonas aeruginosa*, and AdeFGH of *Acinetobacter baumanii* help in biofilm formation, pathogenicity, stress tolerance, and quorum sensing (QS) [5–8]. The link between EPs and QS has been investigated in *P. aeruginosa.* Mutation in RND EP leads to the downregulation of QS-dependent LecA-Lux pathways, thereby increasing the expulsion of QS molecules and biofilm formation [9]. EPs can interact with host-derived antimicrobials such as bile salts, contributing to the virulence of enteric bacteria; the RND EP, AcrAB-TolC from *E. coli* is involved in the extrusion of bile salt, is a good example [10]. Other RND EPs, VexAB, VexCD, VexIJK, and VexGH, increase the pathogenicity of *Vibrio cholerae* by encoding the two major virulence factors: cholera

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toxin (CT) and toxin co-regulated pilus (TCP) [11]. This review will focus on the major classes of EPs identified in bacteria and discuss the newly identified EPIs.

2. Major classes of efflux pumps

The EPs are predominantly found in Gram-positive bacteria (GPB) such as *Streptococcus pneumoniae,* methicillin-resistant *Staphylococcus aureus* (MRSA), *Listeria monocytogenes* and Gram-negative bacteria (GNB) including *E. coli, A. baumanii, Klebsiella pneumoniae*, *Camphylobacter jejuni*, *P. aeruginosa*, *Neisseria gonorrheae* and *V. cholerae.* EPs are energy-dependent as they expel toxic substrates against a concentration gradient. The EPs can be classified into two types based on their energy source. The primary EPs directly utilize energy from ATP hydrolysis, while the secondary EPs derive energy from the chemical gradients from protons or sodium ions. The GNB EPs are more complex than GPB EPs and possess tripartite assembly. They can expel a broad spectrum of antibiotics such as quinolones, β-lactams, and tetracycline AMR in GPB is chiefly mediated by MFS transporter, NorA, and MATE transporter, MepA protein in *S. aureus*, ABC transporter LmrA of *Lactococcus lactis* [12–15]. Major efflux in GNBs are AcrAB-TolC efflux (*E. coli*), AcrAD-TolC and AcrEF-TolC (*Salmonella enterica*). KdeA, KmrA, KpnEP and EefABC in (*K. pneumoniae*), MexAB-OprM, MexJK-OprM, MexEF-OprN, MexXY--OprM, MexCD-OprJ and MexVW-OprM (*P. aeruginosa*), SsmE, SdeAB, SdeCDE, SdeXY, SmdAB and SmfY (*Serratia marcescens*) MarA and AcrAB (*Yersinia pestis*) CmeABC (*C. jejuni*) and AdeIJK, AdeABC and AdeFGH (*A. baumanii*) [16].

The EPs are classified into five families based on the number of membrane-spanning regions, sequence similarity, substrate specificity and energy source used by the pump and the types of molecules exported (Fig. 1).

2.1. ATP-binding cassette superfamily

ATP-binding cassette (ABC) transporters are a large superfamily that uses the energy released upon ATP hydrolysis to pump chemicals [22]. They function as influx and efflux proteins, transporting nutrients into cells and removing toxins and drugs from the cell. However, in Eukaryotes, ABC transporters behave as efflux proteins that protect the cell against toxins [23]. A distinguishing feature of ABC transporters is the presence of two transmembrane domains, which help in substrate translocation, and two cytoplasmic ATP--binding domains that generate energy by ATP hydrolysis to move the substrates across the membrane [24]. ABC transporters contain highly conserved motifs such as Walker A and Walker B motifs (binds to ATP) and LSGGQ/KQR (C-motif) [25]. LmrA (involved in the efflux of ethidium, rhodamine G, daunorubicin) was the first bacterial MDR ABC transporter reported and was identified in *Lactococcus lactis* [26]. A homolog of LmrA, named BmrA, was identified from *Bacillus subtilis* which expelled drugs such as Hoechst 33342, doxorubicin and 7-amino-actinomycin-D [27]. MacAB-TolC, initially identified as a tripartite pump, is involved in the efflux of macrolide antibiotics, protoporphyrin and heat--stable enterotoxins [28]. Similarly, DrrA (ATP binding) and DrrB (integral membrane protein) from *Mycobacterium tuberculosis* imparted resistance to doxorubicin and daunorubicin [29]. PatAB in *S. pneumoniae* is implicated in the efflux of several drugs, including fluoro-

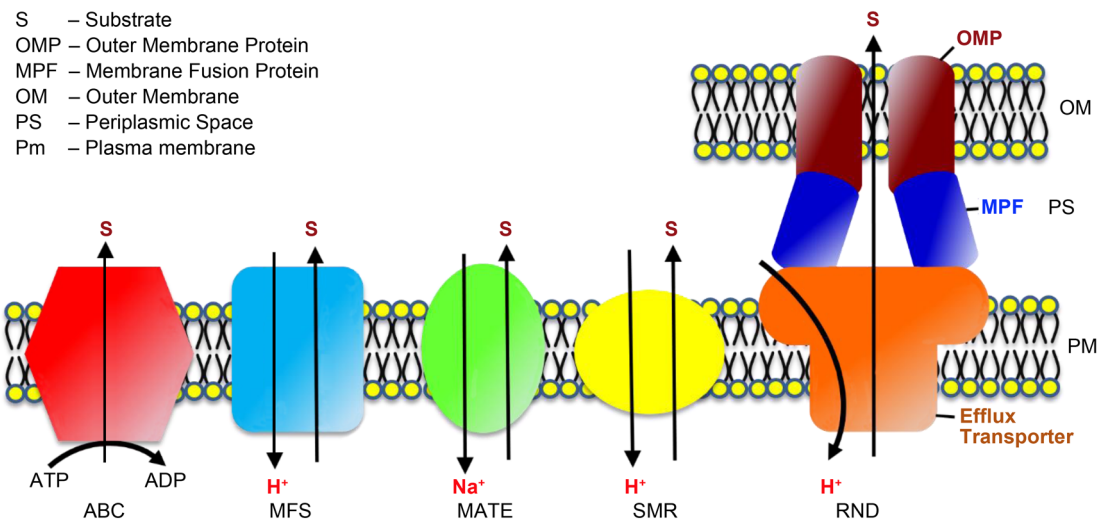


Fig. 1. Schematic representation of five superfamilies of EPs found in bacteria

(ABC) ATP-Binding Cassette Superfamily [17]; (MFS) Major Facilitator Superfamily [18]; (MATE) Multidrug And Toxic Compound Extrusion Family [19]; (SMR) Small Multi-Drug Resistance Family [20]; (RND) Resistance-Nodulation-Division Superfamily [21].

quinolones [30]. EfrCD, an ABC transporter characterized in *Enterococcus faecalis* demonstrated enhanced sensitivity to several drugs, such as daunorubicin and doxorubicin [31]. SmrA is an ABC transporter identified in the nosocomial pathogen, *Stenotrophomonas maltophilia,* which conferred increased resistance to fluoroquinolones and tetracycline [32]. SmdAB, a multidrug efflux transporter, was identified in *S. marcescens* involved in the transport of antibiotics, norfloxacin and tetracycline [33]. A multidrug EP, VcaM, was identified in *V. cholerae,* conferring resistance to fluoroquinolones and tetracycline [34]. Recently, YddA was identified as an ABC-type multidrug transporter associated with exporting several substrates, including norfloxacin [35].

2.2. Major Facilitator Superfamily

Major Facilitator Superfamily (MFS) transporters are found in most living forms, including humans, and they transport many small compounds across the cell membranes [36]. The gene encoding the MFS transporters is present in high copy numbers. For example, *E. coli* K-12 likely has more than 70 transporters [37]. Unlike ABC transporters which are primary active transporters depending on ATP hydrolysis, MFS transporters are secondary active transporters moving smaller solute particles depending on the ion gradient created by active transporters [18]. The MFS transporters function as symport, antiport, or uniport and transport a wide range of compounds, including glucose, oligosaccharides, inositols, drugs, amino acids, and nucleosides. Structurally, MFS transporters are composed of 400–600 amino acids that fold into 12 or 14 transmembrane helices [38].

MdfA, an MDR EP identified in *E. coli* is involved in the transport of lipophilic compounds such as ethidium bromide, rhodamine, daunomycin, rifampin, tetracycline, and puromycin [39]. LmrP, a proton/drug antiport pump from *L. lactis* is involved in the extrusion of lincosamide, streptogramin, and tetracycline [40]. Fluoroquinolones, biocides, dyes, quaternary ammonium compounds and antiseptics are substrates of NorA EP from *S. aureus* and *Staphylococcus epidermis* [41]. Bmr and Blt of *B. subtilis*, and QacA of *S. aureus* are other examples of MFS transporters in GPB [42]. MFS transporters are monomeric in GPB, whereas they possess tripartite assembly in GNB. Tripartite EmrAB-TolC and EmrKY--TolC of *E. coli* enable the transportof the substrates ie. thiolactomycin, cerulenin, nalidixic acid and nitroxolone across the outer and inner membranes of GNB [43, 44].

2.3. Multidrug and toxic compound extrusion family

Multidrug and toxic compound extrusion (MATE) family comprises active secondary transporters and contributes to MDR in *V. cholerae* and *N. gonorrhoeae*. MATE family of transporters pump a wide range of toxic compounds from mammalian and bacterial cells harnessing the proton motive force and cation gradient. Many toxic metabolites and antimicrobial drugs are transported across the membrane by the MATE family, contributing to multidrug tolerance [45]. The NorM transporters from *V. cholerae* and *N. gonorrhoeae* and DinF transporters from *Pyrococcus furiosus* and *Bacillus halodurans* are well characterized [46]. NorM from *Vibrio parahaemolyticus* can extrude antibiotics, norfloxacin and ciprofloxacin outside the cells energy--dependent [47]. All MATE EPs are frequently made up of 12 transmembrane helices except mammalian MATE transporters, containing one additional helix [48]. MDR EP of the MATE family, MepA, is responsible for the extrusion of norfloxacin, ciprofloxacin and tigecycline [49, 50]. Interestingly, human MATE transporters (hMATE1-K and hMATE2-K) contribute to the transport of drugs, such as cimetidine, metformin, procainamide, cephalexin, and acyclovir [51]. PmpM, a proton-drug anti-transporter belonging to MATE family, associated with extrusion of fluoroquinolones, was identified in *P. aeruginosa* [52].

2.4. Small multidrug resistance family

As their name suggests, SMR transporters are small (~12 kDa) proteins consisting of 100 to 140 amino acids and involved in transporting a variety of lipophilic compounds and antibiotics [53, 54]. A proton gradient or ATP-dependent mechanism drives the transport of the substrates across the membrane. All SMR transporter consists of 4 transmembrane helix with primarily α-helical structure [55]. EmrE is an SMR type transporter in *E. coli* exchanging H^+ with ethidium and tetraphenylphosphonium compounds [56].The SMR transporters are further classified into three subclasses: the small multidrug pumps (SMP), suppressors of *groEL* mutation proteins (SUG), and paired small multidrug resistance proteins (PSMR) [54]. SMR proteins are encoded by bacterial chromosomes or plasmids and may be present in integrons. SMR transporters confera high level of resistance to several classes of antibiotics, such as β-lactams, cephalosporins co-trimoxazole, and a few aminoglycosides [39].

2.5. Resistance-Nodulation-Division Superfamily

The Resistance-Nodulation-Division (RND) efflux protein superfamily was initially identified as proteins related to Heavy Metal Resistance (*Ralstonia metalliduransans*), Nodulation (*Mesorhizobium loti*) and Cell division (*E. coli*) [57]. AdeABC is the first characterized RND EP in *A. baumannii,* conferring multidrug resistance [58]. The components of AdeABC EP are adeA, adeB and adeC encoding membrane fusion proteins, multidrug transporter and outer membrane channel

protein, respectively [59]. The adeABC is associated with the active extrusion of fluoroquinolones, tetracycline, macrolides and aminoglycosides [58]. The members of the RND family play a key role in conferring antibiotic resistance in GNB, whereas the MATE family is mainly concerned with resistance in GPB [60]. RND pumps are proton gradient dependent and possess a tripartite assembly with three subunits, an inner membrane protein (IMP), an outer-membrane protein (OMP), and a periplasmic membrane fusion protein (MFP) which connect the other two components. The AcrAB-TolC EP, a well-characterized RND pump, encompasses the outer-membrane channel TolC, the transporter AcrB in the inner membrane, and AcrA, a periplasmic component interacting with the TolC and AcrB. The crystal structure of AcrB confirmed that it is a homotrimer [61]. The AcrAB-TolC EP transports several compounds and imparts resistance to antibiotics [62]. Another well-studied RND transporter is MexA--MexB-OprM from *P. aeruginosa,* actively extruding tetracycline, norfloxacin, and chloramphenicol [63]. *E. coli* RND transporter, SecDF, is a proton-dependent protein translocation factor that functions as a protein exporter [64]. RND efflux system, VexB, VexD, VexK and VexH, identified in *V. cholerae*, exhibited resistance to bile salts and several antimicrobial agents [11].

3. Efflux pumps and their role in virulence and biofilm formation

It has been demonstrated that the efflux of several host-derived antimicrobials agents, such as bile salts, facilitates colonization and increases bacterial adaptation to the host digestive tract [65]. In *E. coli*, the RND EP, AcrAB-TolC, primarily involved in drug efflux, can also impart bile salt resistance [10]. Biofilms are complex microbial communities attached to several surfaces, including implanted devices such as urinary catheters. It is well-known that bacteria encased in biofilm show a greater degree of antibiotic resistance than planktonic cells. The relationship between antimicrobial tolerance of biofilm and EPs has been reported in several bacterial species [66]. For example, the antimicrobial tolerance of biofilms in *P. aeruginosa* increases due to the expression of the multidrug EPs MexAB--OprM and MexEF-OprN [67]. The upregulation of EPs affects the flagellar motility, which plays a crucial role in biofilm formation [68]. The deletion of genes encoding RND EP diminished the ability of biofilm formation in *S. maltophilia* and the retraction of flagellar formation [69]. Intriguingly, the upregulation of RND efflux causes inhibition of the type III secretion system in *P. aeruginosa*, which deliver bacterial toxins into the host cell, thus reducing the virulence [70].

AcrAB-TolC, MexAB-OprM, AdeFGH and AcrD are crucial in biofilm formation. Numerous studies have examined the relationship between EPs and biofilm formation [71–73]. Gene expression studies using microarrays have shown that efflux encoding genes, *mdtF* and *lsrA* are upregulated during biofilm formation and QS in *E. coli* [8]. *Klebsiella* sp. isolates exhibiting efflux activity formed strong biofilm [74]. A strong correlation exists between the overexpression of the AdeFGH EP and biofilm formation by clinical isolates of *A. baumannii* [6]. Further, efflux genes *yihN* and *mdtO* are overexpressed in *E. coli* biofilms and are involved in the efflux of glucose, a major constituent of the extracellular polymer matrix [75]. An MDR EP, YhcQ confer drug resistance in the *E. coli* biofilm, whereas TolC plays an important role in the adhesion and biofilm formation in enteroaggregative *E. coli* [66]. MexAB-OprM EP extruded tetracycline, chloramphenicol, quinolones and β-lactams in *P. aeruginosa* biofilms [76]. Correlation between biofilm formation, drug resistance, and efflux mechanism has been reported in *P. aeruginosa* recently. In addition, the occurance of such cases may be a major public health concern in the treatment of infections caused by the pathogen [77]. Several EP genes, ie. *acrA*, *emrB*, *oqxA* are overexpressed in *K. pneumoniae* biofilms [71]. Deletion of the *bcr* gene decreased the biofilm formation of *P. mirabilis* and reduced catheter blockage [78]. Similarly, deletion of EP encoding genes (acrB, acrD, acrEF, emrAB, macAB, mdfA, mdsABC, mdtABC, mdtK, and tolC) impaired biofilm formation in *Salmonella enterica* [79]. The EPs play an important role in *Helicobacter pylori* biofilm drug resistance. Studies have shown upregulation of EP encoding genes (kefB, hefA, yckJ, tetA, gln, crdB/hefG and ybhS) in biofilm compared to planktonic cells [80]. These data, taken together, strongly show a relationship between efflux activity and biofilm development.

4. Efflux Pump Inhibitors

Efflux abolition could be accomplished by various means: (i) controlling the expression of EPs (ii) discovering new antibacterial agents that do not act as substrates, (iii) identifying small molecules inhibiting the EPs or mimicking the substrates and subsequently blocking EP [15]. The Efflux Pump Inhibitors (EPIs) are molecules capable of inhibiting EPs and preventing the extrusion of foreign compounds. EPIs, inhibit EPs by one or more mechanisms mentioned above. The synergistic activity of EPI and the antibiotics can strengthen their efficacy against bacteria expressing EPs, as this might lead to an adequate accumulation of an antibiotic inside the cell. Eventhough several EPIs have been identified at the experimental level in recent years, none have been approved by the FDA and used therapeutically.

The relationship between EPs and biofilm formation is well understood, therefore, EPIs can also reduce biofilm formation. Several EPIs act as biofilm disruptors, e.g., the combinations of EPIs, thioridazine with Phenylalanine-arginine β-naphthylamide (PaβN) and thioridazine with 1-naphthylmethyl-piperazine (NMP) reduced 80–99% of biofilm formation in *E. coli* [81]. Biofilm inhibitors such as reserpine, linoleic acid, berberine and curcumin exhibited efflux inhibitory activity in *K. pneumoniae* [82]. EPIs can also act as adjuvants, e.g., PAβN and NMP can compete with levofloxacin for the binding site of RND pumps (MexAB, MexCD and MexEF) in *P. aeruginosa* and *E. coli* (AcrAB and AcrEF), thereby increasing the accumulation of levofloxacin [83, 84]. A competitive interaction between PAβN and polyamine potentiates the tetracycline concentration and abolishes biofilm formation in *P. aeruginosa* [85]. Other clinically approved drugs such as nilotinib, dihydroergotamine, ergoloid, azelastine, doxazosin and telmisartan are competitive inhibitors of ciprofloxacin [86]. Mahey et al., identified azoles as putative TetK EPI that reduced the *S. aureus* associated biofilm [87]. Fluoxetine and thioridazine drugs can strongly inhibit the biofilm-associated Bcr/CflA efflux system and swarming motility of *Proteus mirabilis* [88]. Quinazoline derivatives enhanced the inhibitory activity of chloramphenicol and nalidixic acid in EP over-expressing strains of *Enterobacter aerogenes*, *P. aeruginosa* and *K. pneumonia* [89]. Similarly, peptidomimetic EPI, PaβN, increases the antibacterial activity of levofloxacin and erythromycin in MexAB-OprM overexpressing clinical isolates of *P. aeruginosa* [90]. A novel EPI, conessine reduced the MIC of all antibiotics by 8-fold in MexAB-OprM overexpressed *P. aeruginosa* through competitive inhibition [91]. Carbonyl-cyanide 3-chlorophenylhydrazone (CCCP) is an important EPI that can disrupt the energy or ATP levels of bacteria (oxidative phosphorylation) and abolishes the efflux of various molecules. It could reverse the colistin resistance of GNB without affective tigecycline and carbapenem resistance [92]. In another study, CCCP showed synergism with ciprofloxacin, imipenem, gentamicin and cefepime in *P. aeruginosa* [93]. CCCP is a known proton motive force inhibitor of MexAB-OprM overexpressing *P. aeruginosa* biofilm [94]. Xanthone derivatives effectively inhibit specific EPs such as AcrAB-TolC in *S. typhimurium* and NorA in *S. aureus* [95]. Oliveira-Tintino et al., reported that 1,8 naphthyridines reduced the MIC of norfloxacin and ethidium bromide in NorA overexpressing *S. aureus* strains [96]. The calcium channel blocker verapamil, clinically used to treat cardiac disorders, can inhibit ATP-dependent multidrug resistant EPs and reverse the resistance of rifampicin, ofloxacin, streptomycin, and ethidium bromide in *M. tuberculosis*. Valinomycin is a potassium-specific EPI extracted from *Streptomyces* that targets the MFS and ABC EPs. They have been shown to inhibit the P55, an MFS EP that relies on the electrochemical gradient for the active efflux of substrates in *M. tuberculosis* [97]. The list of EPIs, their mechanism of action, origin and their corresponding target EPs are shown in Table I.

Table I.
List of Efflux Pump Inhibitors based on mechanism of action, origin, targets and substrates

Mechanism of action/Compounds	EPIs	Target EPs	Bacteria	Substrates	References
1. Mechanism of Action					
Energy Disruption	CCCP-Carbonyl cyanide chlorophenyl hydrazone	MFS- *tetA*, *tetB*	*H. pylori*, *Klebsiella* spp.	Tetracycline	[98, 99]
	Synthetic EPI- IITR08027	MATE- *abeM*	*E. coli, A. baumanii*	Fluoroquinolones	[100]
	PAβN	RND- *mexAB-oprM*, *mexCD-oprJ*, *mexEF-oprN*	*P. aeruginosa*	Levofloxacin Erythromycin Streptomycin	[90, 101]
Competitive Inhibition	Verapamil	MATE- *dinF* and *norM*	*M. tuberculosis*	Bedaquiline Ofloxacin	[102]
	1-(1-napthylmethyl)-piperazine (NMP)	RND- *acrAB, acrEF*	*E. coli*	Levofloxacin Rifampin Chloramphenicol	[103]
2. Plant origin					
Alkaloids	Reserpine (*Rawfolia serpentia*)	MFS- *norA, tetK, Bmr* MATE- *mepA*	*S. aureus* *Bacillus subtilis* *Streptococcus pneumoniae*	Norfloxacin Tetracycline Ciprofloxacin	[104]
	Piperine (*Piper nigram*)	ABC transporters MFS- *norA*	*S. aureus, Mycobacterium tuberculosis*	Ciprofloxacin Rifampicin	[105]
	Conessine (*Holarrheaa antidysenterica*)	RND- *adeIJK*, *mexAB-oprM*	*Acinetobacter baumannii*	Novobiocin Rifampicin	[91]

Table I Continued

	EPIs	Target EPs	Bacteria	Substrates	References
Flavanoids	Baicalein (*Thymus vulgaris*)	MFS- *tetK, norA*	*Staphylococci*	Tetracycline Ciprofloxacin	[106, 107]
	5'-methoxy-hydnocarpin (*Berberis fremontii*)	MFS- *norA*	*S. aureus*	Tetracycline Norfloxacin Berberine	[108]
	Genistein (Isoflavone)	MFS- *norA*	*S. aureus*	Berberine	[109]
	Epigallocatechin gallate	MFS- *tetK*	*Staphylococci Camphylobacter*	Tetracycline Erythromycin Ciprofloxacin	[110]
Polyphenols	Curcumin (*Curcuma longa*)	MFS- *norA* RND	*S. aureus P. aeruginosa*	Norfloxacin Ciprofloxacin Gentamicin	[111, 112]
	Coumarin (*Mesua ferrea*)	MFS- *norA*	*S. aureus*	Norfloxacin Ciprofloxacin	[113]
Phenolic diterpenes	Carnosic acid (*Rosmarinus officinalis)*	ABC transporter *msrA*	*S. aureus*	Erythromycin	[114]
Monoterpenoid	Carnosol (*Rosmarinus officinalis)*	ABC transporter *msrA* MFS- *tetK*	*S. aureus*	Tetracycline	[114]
	Geraniol (*Helichrysum italicum*)	RND- *acrAB-tolC*	*Enterobacter aerogenes*	Chloramphenicol	[115]
	Catharanthine (*Catharanthus roseus*)	RND- *mexAB-oprM*	*P. aeruginosa*	Tetracycline Streptomycin	[116]
3. Synthetic origin					
Quinolone derivatives	Pyridoquinolones	RND- *acrAB-tolC*	*Enterobacter aerogenes*	Norfloxacin	[117]
Arylpiperidines and aryl piperazine derivatives	Phenylpiperadines	RND- *acrAB-tolC*	*E. coli*	Linezolid	[118]
Pyridopyrimidine and pyranopyridine derivatives	D2 and D13-9001	RND- *mexAB-oprM*	*P. aeruginosa*	Fluoroquinolones	[119, 120]
	MBX2319	RND- *acrAB-tolC*	*E. coli*	Fluoroquinolones	[121]
Naphthyridine derivatives	1,8-naphthyridines sulfonamide	MFS- *norA*	*S. aureus*	Norfloxacin Ethidium bromide	[97]
Boronic acid derivatives	6-(3-Phenylpropoxy) pyridine-3-boronic acid	MFS- *norA*	*S. aureus*	Ciprofloxacin Ethidium bromide	[122]
Indole derivatives	3-amino-6-carboxyl-indole 3-nitro-6-amino-indole	RND- *acrAB-tolC*	*E. coli*	Chloramphenicol Tetracycline Erythromycin Ciprofloxacin	[123, 124]
4. Clinically approved drug					
Hypoglycemic biguanide drug	Metformin	RND- *acrAB-tolC* MATE- *mdtK*	*K. pneumoniae*	Ampicillin-sulbactam Meropenem Amikacin	[125]
Tyrosine kinase Inhibitor	Nilotinib	MFS- *EmrD*	*S. aureus*	Ciprofloxacin	[86]
Ergot alkaloid--vasoconstrictor	Dihydroergotamine	MFS- *norA*	*S. aureus*	Ciprofloxacin	[86]
5. Microbial origin					
	EA-371α and EA-371d (fermentation extract of *Streptomyces* spp.	RND- *mexAB-oprM*	*P. aeruginosa*	Levofloxacin	[126]

The chemical compound must follow specific criteria to make it an ideal EPI. The first and foremost rule is that the molecule must not be antibacterial. Secondly, it should be selective and target only bacterial EPs. Thirdly, it should be non-toxic with high therapeutic and safety indices and good ADMET

(Absorption, Distribution, Metabolism, Excretion and Toxicity) [127]. The toxicity of EPI can be lowered by co-administering them with membrane permeabilizing antimicrobial peptides (AMP) such as Polymyxin B nonapeptide (PMBN), which has five times lower toxicity than the parent compound polymyxin B [128]. The nephrotoxicity of PMBN was low when compared to polymyxin B (PMB) and polymyxin E (Colistin) in mice [129]. The cytotoxicity of polyamines towards eukaryotic cells are relatively low, and it would strongly enhance the antibacterial activity [85].

Computational approaches have led to the discovery of novel EPIs such as PAβN, novel pyranopyridine (D13-9001), and novel pyranopyridine (MBX2319) [130]. Molecular dynamics simulation (MDS), advanced three-dimensional structural resolution and molecular modelling can help identify possible inhibitors with pharmacophores that can detect a specific binding site on the EP [131]. Several studies have been made on the correlation of molecular interactions between EPIs and bacterial pumps via molecular docking [132].

In summary, EPs significantly contribute to drug resistance and survival of bacteria in the biofilm by extruding clinically relevant antibiotics. Therefore, the present investigation highlights that EPs could be an attractive target for antimicrobial drug development.

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