Current Trends in Microbiology
Vol. 12, 2018

Review

Bioactive agents as modulators of multidrug efflux pumps of the major facilitator superfamily in key bacterial pathogens

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ABSTRACT

Infectious disease-causing bacterial pathogens that harbor multidrug resistance may be difficult to treat effectively. Such bacterial pathogens may possess an assortment of cellular mechanisms, such as antimicrobial resistance, for conferring virulence and infectious disease. One major antimicrobial resistance mechanism involves the extrusion of growth inhibitory substances from the internal milieu of the pathogenic bacterium. Several transporter superfamilies have emerged as being chiefly responsible for the efflux of multiple antimicrobial agents from bacteria. Of these protein superfamilies, the so-called major facilitator superfamily (MFS) of solute transporters encompasses several key and well-studied multidrug efflux pump systems. These multidrug transporters of the superfamily are, thus, excellent targets for modulation. Of the various drug efflux pump modulators which have been discovered so far, those that are relatively non-toxic to humans are obviously the most promising modulatory candidates. This review article briefly summarizes several key bacterial pathogens and modulation targets exemplified by multidrug efflux systems of the major facilitator superfamily.

KEYWORDS: bioactive products, bacteria, pathogenesis, antimicrobial resistance, major facilitator superfamily, multidrug efflux pumps, modulation.

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1. Introduction

Outbreaks of bacterial infections have emerged both historically and recently, constituting a serious public health concern that is alarming on a worldwide scale. Confounding the recent problem of bacterial infectious disease is the recalcitrance that is inherent in the antimicrobial resistance mechanisms [1-4]. Of the various major antimicrobial resistance mechanisms developed by pathogenic bacteria, one clinically important mechanism includes the utilization of integral membrane transporters, which function as multidrug efflux pumps by extruding inhibitory molecules from the bacterial cytoplasm where antimicrobial targets reside [5-8]. Presently, several well-established groups of transporters have been categorized into major protein superfamilies [9].

One of these transporter superfamilies is frequently referred to as the major facilitator superfamily, and the group represents a considerable collection of related solute transporters with diverse substrates, but similar amino acid sequences, modes of energy and overall three-dimensional protein structures [7, 10-12]. Since little or no new clinically useful antimicrobial agents are in the pharmaceutical pipeline, new strategies are currently the focus of recent investigations [13, 14]. As such, the multidrug efflux pumps of the major facilitator superfamily are appropriate cellular and molecular targets for modulation, such as antimicrobial efflux inhibition [15-17].

While some natural and synthetic modulators are toxic to humans, non-toxic natural modulators are

promising avenues for multidrug efflux pump inhibition [15, 16]. This review briefly summarizes several key bacterial pathogens and current advances in antimicrobial efflux pump modulation for transporters of the major facilitator superfamily from a physiological and molecular perspective [18]. Particular attention will be paid here to the physiological effectiveness of naturally occurring and potentially non-toxic bioactive agents upon antimicrobial efflux pumps of the major facilitator superfamily.

2. *Salmonella enterica*

Salmonella enterica, a member of the Enterobacteriaceae family, is a pathogen of significance due to its inherent virulence and its increasing ability to resist multiple antimicrobial substances. Morphologically, *S. enterica* is a Gram-negative, rod-shaped microbe that is approximately 0.7 to 1.5 μm in girth and 2.0 to 5.0 μm in length. Physiologically, this bacterium is a motile facultative-anaerobe and is non-endospore forming [19]. Virulence factors of *S. enterica* include an initial acid tolerance response (ATR) system which occurs after ingestion allowing for survival of the pathogen in what would be an otherwise extremely hostile environment by maintaining the internal pH [20]. The underlying mechanisms are complex, but a number of acid shock proteins unique to *S. enterica* have been identified [21]. The ability to travel through gastric fluid allows the bacteria to reach the microfold cells (M cells) of the gut-associated lymphoid tissue where they adhere and undergo intracellular changes at the cytoskeletal level to facilitate entry into the M cells. Gene products of the fimbriae *lpfABCDE* operon mediate adherence [22, 23].

Membranes of infected M cells form vacuoles that enclose the *Salmonella* bacteria and allow for growth while avoiding internal hydrolytic lysosomes. The bacterial cells are released and travel to nearby tissues and encounter macrophages. The virulence factor SopD [24] allows *Salmonella* to replicate within macrophages. Chemotherapy has not been found to be overly beneficial unless administered within two days of the onset of symptoms and is often not prescribed in most cases [25, 26]. The treatment of salmonellosis focuses instead on replacement of fluids and electrolytes; however, when severe infections do occur the prescription of antimicrobials is used in conjunction with fluid/electrolyte replacement therapies. Among the antimicrobial agents utilized in these infections are the fluoroquinolones, trimethoprim-sulfamethoxazole, ampicillin, or wide-spectrum cephalosporins such as ceftriaxone and cefixime [27-29]. The presence of multidrug resistant strains has been widely documented with the *Salmonella* serotype Typhimurium, complicating the treatment of severe cases in affected patients [30, 31].

Antibiotic resistance mechanisms of *S. enterica* including (a) modification of drug-binding sites, (b) enzyme-mediated inactivation of antimicrobials, (c) ribosomal (or target) protection, (d) changes in membrane permeability, and (e) the formation of biofilm mirror those of other pathogens. Each of these resistance systems play a significant role in enabling *S. enterica* to become clinically resistant to a multitude of structurally distinct antimicrobials, each with distinct modes of antimicrobial action. Perhaps, the most important resistance mechanism of *S. enterica* is the presence of active drug efflux pumps of which *S. enterica* [2, 32-34]. *S. enterica* has been found to contain multiple efflux pumps from several different major families including the ATP binding cassette (ABC) transporter superfamily [35], multidrug and toxic compound extrusion (MATE) superfamily [36, 37], major facilitator superfamily (MFS) [11, 18, 38], resistance nodulation division (RND) superfamily [39], and small multidrug resistance (SMR) superfamily [40, 41]. Table 1 lists the drug efflux pumps of the MFS that confer resistance to multiple antimicrobials. Of these various antimicrobial transporter systems, ErmB, FloR and MdfA have been the most intensively studied [42-44]. Interestingly, one of these major facilitator superfamily transporter systems, EmrB, involves the formation of a so-called tripartite complex in which EmrB resides in the cytoplasmic membrane while EmrA resides as a soluble periplasmic protein to bring antimicrobials from EmrB to TolC, an outer membrane protein, in order to accommodate drug efflux to the extra-cellular milieu of the bacterium [45].

3. *Vibrio cholerae*

Vibrio cholerae, responsible for the well-known disease cholera, is a natural inhabitant of freshwater

Table 1. Antimicrobial efflux pumps of the major facilitator superfamily in *Salmonella enterica*.

Drug efflux pump	No. of amino acids	No. of TMS	Substrates	References
EmrB	513	12	Fluoroquinolones	[44, 45]
SmvA	126	14	Acriflavine, ethidium bromide, malachite green, pyronin B	[46]
FloR	404	12	Chloramphenicol, florfenicol, thiamphenicol	[42, 47]
MdfA	302	12	Tetracycline, chloramphenicol, norfloxacin, dixorubicin	[43, 48]
STY0901	403	12	Benzalkonium chloride	[49]
STY2458	396	12	Tetracycline, doxycycline, kanamycin	[49]

and coastal environments [50]. Globally, cholera is responsible for an estimated 100,000 deaths every year in countries deprived of potable water and proper sanitation [51]. Ingestion of contaminated food and water results in clinical intoxication caused by this bacterium. In countries where cholera is endemic, consumption of contaminated water is the major source of disease [52]. The symptoms of cholera start within 8-72 hours of infection, characterized by acute watery diarrhea resulting in severe dehydration, vomiting and cramps [53]. The production of the cholera toxin (CT) is central to the virulence of *V. cholerae*, although several additional virulence factors also contribute to its survival, persistence and disease-causing abilities [54]. Two serogroups, *V. cholerae* O1 and O139 (a non-O1 serovar), are responsible for cholera intoxications of epidemic scales. *V. cholerae* capable of producing CT have been responsible for several global epidemics of cholera resulting in the deaths of millions of people during each of the historical pandemics.

The remaining non-O1 serovars, roughly 200 in number, are not known to produce cholera toxin and are either non-pathogenic or cause varying degrees of intestinal and extraintestinal disease [55, 56]. The O1 serogroup consists of two biotypes, classical and El Tor, and 3 serotypes Ogawa, Inaba and Hikojima based on the distribution of their associated antigens, A, B and C [53]. The disease cholera is characterized by profuse diarrhea and if left untreated, can lead to death due to acute severe dehydration. Replacement of body fluids by rehydration therapy is frequently followed as the main course of treatment for cholera, although antimicrobials such as doxycycline are advocated to ameliorate the symptoms and shorten the duration of diarrhea, and to reduce fecal shedding of the bacterium into the environment [53]. Erythromycin in liquid form is advocated for children along with fluid replacement therapy [57].

Unfortunately, the incidence of antibiotic-resistant *V. cholerae* is steadily increasing with the emergence of strains resistant to tetracycline, fluoroquinolones, chloramphenicol, ampicillin, furazolidone, and trimethoprim-cotrimoxazole, which are drugs commonly used to treat severe cholera [58-60]. Multidrug-resistant *V. cholerae* have been reported from different parts of the world [52, 61, 62]. Infections by multidrug-resistant *V. cholerae* strains result in longer duration of illness, excessive dehydration and possible treatment failures [53]. Outbreaks of cholera involving drug-resistant *V. cholerae* have been reported from Asia and Africa [58]. Considering this, the WHO recommends that affected individuals refrain from antibiotic treatment of cholera except in cases of severe dehydration [63]. Along similar lines, antibiotic prophylaxis is also not advocated for cholera since it does not offer any additional protection against *V. cholerae* and on the contrary, can potentially lead to the emergence of antibiotic resistance by natural selection [64].

3.1. Mechanisms of antibiotic resistance in *V. cholerae*

The mechanisms of antibiotic resistance commonly found in eubacteria including enzyme hydrolysis, target modification, changes in membrane permeability and active efflux of antibiotic can also be found in *V. cholerae* [2, 61, 65]. The genetic elements responsible for these resistances are located on the chromosome or associated with mobile genetic elements such as plasmids, transposons, and integrons [66-68]. Multidrug resistance associated

with a self-transmissible, conjugative transposon-like element SXT has been widely reported in *V. cholerae* [69, 70]. The SXT element characteristically confers resistance to sulfamethoxazole, trimethoprim, chloramphenicol and streptomycin [70]. The class 1 integron-associated cluster of genes in *V. cholerae* has been shown to confer resistance to multiple antibiotics including streptomycin, cotrimoxazole, nalidixic acid, nitrofurantoin, ampicillin, chloramphenicol, amoxicillin-clavulanate and aztreonam [66, 67, 71, 72].

Outbreak strains of *V. cholerae* O1 harboring SXT, an integrated chromosomal element and a class 2 integron carrying *dfrA1*, *sat* and *aadA1* cassettes, were resistant to sulfamethoxazole-trimethoprim, along with several other antibiotics including ampicillin, nalidixic acid, tetracycline, chloramphenicol, gentamicin and cephalosporins [71]. In addition, *V. cholerae* toxigenic and non-toxigenic strains resistant to antibiotics harbor their genetic determinants of resistance on conjugative plasmids [73, 74]. Resistance to multiple antibiotics, including those clinically used to treat cholera, is carried on transmissible plasmids which could disseminate rapidly in the environment and pose severe therapeutic crisis when such strains are involved during an outbreak [75, 76].

3.2. Antimicrobial efflux pumps from *V. cholerae*

The role of efflux pumps in drug resistance and virulence in *V. cholerae* is increasingly being recognized. On average, *V. cholerae* genomes harbor at least 28 putative efflux pumps [77-79]. One of the earliest characterized efflux pumps in *V. cholerae* is VceAB, a homologue of EmrAB of *Escherichia coli*, which belongs to the major facilitator superfamily of proteins [80]. The *vceAB* genetic element is part of an operon *vceCAB* which is negatively regulated by a transcriptional regulator *vceR* [81]. Diverse hydrophobic agents such as deoxycholate (DOC), chloramphenicol, nalidixic acid and cyanide carbonyl m-chlorophenylhydrazone (CCCP) form substrates for this efflux pump [81].

The *V. cholerae* genome encodes at least six RND efflux pumps VexB, VexD, VexK, VexF, VexH, and VexM, which may also share a single TolC outer membrane protein encoded elsewhere in the genome [82]. While VexB, VexD, and VexK transporters efflux antibiotics such as erythromycin, polymyxin B, and penicillin, VexD and VexK have narrow substrate spectrums involving bile acids and detergents [82]. Studies have demonstrated that the RND efflux pumps regulate the expression of virulence factors such as CT and the toxin co-regulated pilus (TCP) [82, 83]. The role of antimicrobial efflux pumps towards the virulence of *V. cholerae* assumes significance apart from their contribution to the antibiotic resistance from the perspective of modulating these pumps for the clinical control of this human pathogen [78].

The efflux pumps of the MATE superfamily reported in *V. cholerae* include VcmB, VcmD, VcmH, VcmN, VcmA and VcrM [77, 84]. The drug extrusion by these efflux pumps is coupled to a Na^+ antiporter activity, and the substrates include 4',6-diamidino-2-phenylindole, acriflavine, rhodamine 6G, ethidium bromide, and tetraphenylphosphonium chloride (TPCL).

Table 2 shows physiologically characterized drug and multidrug efflux pumps of the MFS from *V. cholerae*. EmrD-3 is a multidrug efflux pump of the major facilitator superfamily (MFS) [85]. EmrD-3 has 379 amino acids that fold into 12-transmembrane helices. EmrD-3 actively effluxes linezolid, rifampin, minocycline, erythromycin, rifampin and chloramphenicol [85]. Considering its wide range of substrates, which includes both hydrophilic and hydrophobic compounds, EmrD-3 may be considered as an important drug efflux pump of H^+/drug antiporter in *V. cholerae* and a potential candidate pump to study the efflux pump-substrate interaction and to identify potential efflux inhibitors.

4. *Staphylococcus aureus*

S. aureus is a Gram-positive opportunistic bacterial pathogen that causes severe disease conditions in humans and non-human animals. Generally, this bacterium colonizes the skin and mucosa, and routes of entry to the body such as eyes, nasal passages, ears etc., followed by invasion of multiple organs within the body. It causes different types of infections, ranging from mild to life-threatening diseases, including abscesses of various organs, pneumonia, osteomyelitis, endocarditis, arthritis, and sepsis [90]. In livestock, *S. aureus* mainly causes mastitis, skin and soft tissue infections [91]. *S. aureus* causes disease through two possible mechanisms involving production of toxins and colonization that causes tissue invasion and

Table 2. MFS antimicrobial efflux pumps of *Vibrio cholerae*.

Drug efflux pumps	No. of amino acids	No. of TMS	Substrates	References
VceB	511	14	Nalidixic acid, deoxycholate, phenylmercuric acetate, carbonyl m-chlorophenylhydrazone	[80, 81]
VcaM	619	6	4',6-diamidino-2-phenylindole, acriflavine, rhodamine 6G, ethidium bromide, tetraphenylphosphonium chloride (TPCL), norfloxacin, ciprofloxacin tetracycline, doxorubicin	[86]
VcmA	457	12	norfloxacin, ciprofloxacin and ofloxacin, kanamycin, acriflavin	[77, 87]
VcrM	445	12	Acriflavin, 4′, 6-diamidino-2-phenylindole, Hoechst 33342, rhodamine 6G, TPCL, ethidium bromide	[84]
VcmB VcmD VcmH VcmN	460 (VcmB) 451 (VcmD) 458 (VcmH) 442 (VcmN)	12	Fluoroquinolones, aminoglycosides, ethidium bromide and Hoechst 33342, kanamycin, streptomycin	[77]
VexB	1026	12	Benzylpenicillin, erythromycin, polymyxin B, cholate, SDS, Triton X-100	[82, 88]
VexD VexK	1,016 1,037	12	Bile acids and detergents	[82, 88]
VexH			Triton X-100, ampicillin, novobiocin	[83]
EmrD-3	379	12	Linezolid, rifampicin, erythromycin, chloramphenicol	[85]
NorM	461	12	Norfloxacin, ciprofloxacin, and ethidium bromide	[89]

destruction during their pathology. The microorganism produces toxins such as exotoxins and enterotoxins, which are associated with intoxications, such as food poisoning [92]. Coupled with the actions of toxins, the production of coagulases, the proteins that help in evading phagocytosis and other defense mechanisms of the host, these virulence factors collectively play a significant functional role in mediating the pathogenesis of *S. aureus*. The expression of proteins called agglutinins that bind polymerized fibrin on the surface of bacteria also plays a key role in the virulence strategies of *S. aureus* [92].

S. aureus has acquired resistance to common anti-bacterial agents and consequently, has significantly restricted the use of chemotherapeutic alternatives against it [93, 94]. The efficacy of antibiotic therapy against *S. aureus* has been severely compromised due to the emergence of MRSA (methicillin-resistant *S. aureus*) and CA-MRSA (community-acquired MRSA) [95]. MRSA have been found in livestock workers, meat handlers, farms, food animals, and educational facilities [94, 96-98]. Certain clonal types of MRSA, such as the sequence type (ST), 398 and the *spa* type, t108, are capable of human-to-human or animal-to-human transmission [98].

The mechanisms by which *S. aureus* gains resistance to antibiotics include enzymatic inactivation of the antibiotic by penicillinase and aminoglycoside-modification enzymes, target modification with reduced affinity for the antibiotic, such as in the case of penicillin-binding protein 2a of MRSA, and the transport action of efflux pumps against fluoroquinolones and tetracycline [99]. These mechanisms may be intrinsic to the bacteria or acquired from another bacterium *via* plasmid, bacteriophage or simple uptake of DNA which carries an antibiotic resistance-conferring gene [100]. The bacteria may use either or both of these types of mechanisms based on the drug it is acting against. *S. aureus* has acquired complex genetic arrays such as the so-called staphylococcal chromosomal cassette *mec* elements or the *vanA* operon through horizontal gene transfer, whereas spontaneous mutations and positive selection have provided

resistance to other antibiotics such as the fluoroquinolones and linezolid [101, 102].

4.1. Efflux pumps of *Staphylococcus aureus*

S. aureus has an array of efflux pumps responsible for its resistance to disinfectants, dyes and antibiotics [5]. The genome sequence of *S. aureus* reportedly contains 20 putative efflux pumps [103]. Table 3 shows some of the important efflux pumps of *S. aureus* and their substrate profiles. NorA, the first efflux pump identified in *S. aureus*, belongs to the MFS family of proteins and is chromosomally encoded [104]. The other MFS efflux pumps include NorB, NorC, SdrM, LmrS and MdeA which are chromosomally encoded, and QacA and QacB which are encoded on potentially transferable plasmids [5, 105]. Several SMR transporters (QacC, QacD, QacG and QacH) have been identified in *S. aureus*, and all these pumps are plasmid-encoded. *S. aureus* has a MATE transporter, MePA, which is chromosomally encoded [106]. In addition, two chromosomally encoded efflux proteins (Sav1866 and AbcA), and five plasmid-borne efflux pumps (MsrA, VgaA, Vga(A)LC and VgaB) belong to ABC family of solute transporters [105]. The substrates of these efflux pumps include diverse compounds

Table 3. MFS antimicrobial efflux pumps of *Staphylococcus aureus*.

Drug efflux pump	No. of amino acids	No. of TMS	Substrates	References
QacA, QacB	514	14	Quaternary ammonium compounds, damidines, dyes	[108-110]
NorA	388	12	Hydrophilic quinolones, tetracyclines, chloramphenicol, quaternary ammonium compounds, rhodamine, puromycin, ethidium bromide	[104, 111]
NorB	463	12	Hydrophilic fluoroquinolones norfloxacin and ciprofloxacin), biocides (quaternary ammonium compounds) and dye (ethidium bromide). Also confers resistance to hydrophobic fluoroquinolones (moxifloxacin and sparfloxacin) which are not the substrates of NorA	[111]
NorC	462	14	Ciprofloxacin, norfloxacin, sparfloxacin, moxifloxacin, garenoxacin and to the dye rhodamine 6G	[112]
NorD		12	Unknown	[113]
LmrS	480	14	Lincomycin, kanamycin, linezolid, and fusidic acid	[114]
MdeA	479	14	Tetraphenylphosphonium chloride (TPCL), Hoechst 33342, fluoroquinolones	[115]
QacG, QacH, and QacJ	107	4	Quaternary ammonium compounds, dyes	[116, 117]
Tet(K)	459	14	Tetracycline, oxytetracycline and chlortetracycline	[118, 119]
Tet38	450	14	Tetracycline	[120, 121]
SdrM	447	14	Acriflavine, ethidium bromide, fluoroquinolone and norfloxacin	[122]
MefA	405	12	Macrolides	[123]

consisting of antibiotics, quaternary ammonium compounds, and dyes [5].

Efflux pumps play a crucial role in the survival, persistence and antimicrobial resistance of *S. aureus* [105]. Therefore, efflux pumps of *S. aureus* have been ideal targets for the discovery of novel inhibitors which can restore the susceptibility of *S. aureus* to antimicrobials [16, 107]. The availability of whole genome sequences and bioinformatics tools has made the prediction of 3-dimensional structures of membrane proteins relatively easy, and using these structures, potential modulators of efflux pumps can be discovered by virtual screening.

5. *Escherichia coli*

E. coli bacteria are Gram-negative, commensal intestinal microorganisms. With metabolism types of both fermentative and respiratory in nature, *E. coli* are facultative anaerobic bacteria. *E. coli* is a member of the Enterobacteriaceae family of bacteria. Pathogenic variants of *E. coli* are a leading cause of clinically important infections. They are moderately sized bacteria with motile peritrichous flagella [124]. *E. coli* pathogens can cause infections of the urinary tract, biliary tract, kidney and abdominal cavity in immunocompromised hosts [19]. *E. coli* are also opportunistic pathogens, and infections may be caused by strains harboring genetic determinants encoding a variety of virulence factors located on plasmids, transposons, bacteriophages and pathogenicity islands [125, 126]. Antibiotic resistance occurs mainly due to the transferability of plasmids encoding resistance genes [127]. Various previous studies have found that pathogenic strains of *E. coli* might have originated from relatively harmless commensal strains by acquiring and harboring chromosomal or extra-chromosomal virulence-encoding genetic elements from other microbial species, especially while residing in the gut [128]. Many pathogenic *E. coli* strains could have resulted from random DNA sequence variations that occur during the course of their evolution and in which adaptation has occurred to confer pathogenicity when residing within their micro-environments [129] while others may have been due to pathogenically derived adaptive mutations in which genomic deletions enhanced pathogenicity [130]. Isolates of *E. coli* with varying types of antimicrobial resistance profiles have been found in agricultural environments such as soil [131, 132], milk [133], and other food animal production locations [4, 134].

There are different categories of *E. coli* pathogens, each with distinctive pathogenic mechanisms for enteric infections [135, 136]. Enterotoxigenic *E. coli* (ETEC) is a major cause of travelers' diarrhea in adults. Enteropathogenic *E. coli* (EPEC) is a cause of infant diarrhea. Enterohemorrhagic *E. coli* (EHEC) is a food-borne pathogen of worldwide importance. Enteroaggregative *E. coli* (EAEC) was originally recognized as a predominant etiologic agent of persistent diarrhea. Enteroinvasive *E. coli* (EIEC) and diffusely adhering *E. coli* (DAEC) strains cause watery diarrhea and dysentery in humans [135, 136].

Key solute transporter systems of the major facilitator superfamily and their distinctive protein sub-families from *E. coli* are included in Table 4 [12, 137, 138]. Of these transporters, the xylose permease XylE and the LacY lactose permease represent some of the best-understood and widely studied solute transporters of bacterial origin [139-142]. These bacterial sugar transporters and other transporters of the major facilitator superfamily are homologous to the human GLUT transport systems [143]. Table 5 shows various drug and multidrug efflux pumps of the major facilitator superfamily from *E. coli*. Of these antimicrobial efflux systems TetA(B), ErmD, YajR and MdfA have been intensively studied at the functional and molecular structural levels [7, 144].

6. *Enterobacter* spp.

The *Enterobacter* genus represents a group of Gram-negative, non-endosporing bacteria of the Enterobacteriaceae family. *E. aerogenes* and *E. cloacae* continue to be recognized among the Enterobacteriaceae as important causative agents of health care-associated (nosocomial) infections [155]. Despite being widely regarded as potentially pathogenic bacteria, the molecular mechanisms of their virulence factors during infectious disease are relatively poorly understood. On the other hand, numerous reports pertaining to the *Enterobacter* bacteria have largely focused on their modes of resistance to multiple antimicrobial agents [37, 41, 155, 156]. Among the clinically relevant antimicrobial agents known, the β-lactams such as the extended-

Table 4. *Escherichia coli* solute transporters.

Transporter family	No. of family members	No. of TMS	Substrates	Transport mechanisms	Example
Anion:cation symporter (ACS)	40	12	Allantoate, acetate, glucarate, hexuronate, tartate, 4-hydroxyphenyl acetate	Substrate:H^+ or Na^+ symport	ExtU
Cyanate permease (CP)	3	12	Cyanate	Substrate:H^+ symport	CynX
Fucose-galactose-glucose: H^+ symporter (FGHS)	4	12	L-Fucose, glucose, galactose	Hexose uniport Hexose:H^+ symport	FucP
Metabolite:H^+ symporter (MHS)	16	12	Citrate, α-ketoglutarate, proline, betaine, methylphthalate	Solute:H^+ symport	KgtP
Nucleoside:H^+ symporter (NHS)	2	12	Nucleosides	Nucleoside:H^+ symport	NupG
Nitrate/nitrite porter (NNP)	13	12	Nitrite, nitrate	Nitrite uniport Nitrate:H^+ symport	NarK
Oligosaccharide:H^+ symporter (OHS)	6	12	Di- and trisaccharides	Sugar:H^+ symport	LacY
Organophosphate:P_i antiporter (OPA)	12	12	Sugar-phosphates, glycerol phosphate, phosphoglycerates	Organo phosphate:P_i antiport	UhpT
Sialate:H^+ symporter (SHS)	3	14	Sialate	Substrate:H^+ symport	NanT
Sugar porter (SP)	133	12	Monosaccharides, disaccharides, quinate, inositols	Sugar uniport Sugar:proton symport	XylE

Table 5. Antimicrobial efflux pumps of the MFS in *Escherichia coli*.

Drug efflux pump	Substrates	References
EmrB	CCCP, fluoroquinolones	[145]
EmrD	Benzalkonium, CCCP, SDS	[146, 147]
FloR	Chloramphenicol, florfenicol, thiamphenicol	[47]
MdfA (Cmr, CmlA)	Benzalkonium, chloramphenicol, fluoroquinolones	[48, 148]
MdtM	Macrolides, quaternary ammonium compounds	[149, 150]
QepA, QepA2	Fluoroquinolones	[151, 152]
TetA	Tetracyclines	[153]
YajR	Multiple drugs	[154]

spectrum cephalosporins and carbapenems are primarily used in the treatment of *Enterobacter* infections. However, the frequent usage of these antimicrobial agents has increased the prevalence of *Enterobacter* spp. infections that are recalcitrant to chemotherapy [157].

According to the National Nosocomial Infection Surveillance System, the extended-spectrum cephalosporins are no longer effective against most of the *Enterobacter* spp. [158]. Carbapenems, however, have been effective in the treatment of *Enterobacter* spp. infections due to the presence

of the extended-spectrum β-lactamase (ESBL) enzymes and AmpC in *Enterobacter* spp. [159]. The frequent use of broad-spectrum antimicrobials including β-lactams has resulted in the emergence of *Enterobacter*-resistant strains [160-162].

Resistance mechanisms to β-lactams are sustained by a number of factors, including alteration of porins, antimicrobial efflux pumps, modifications of the target sites and the production of β-lactamases [162]. Table 6 shows well-characterized multidrug efflux pumps of the MFS. Carbapenem resistance is mediated by i) the reduced binding to penicillin binding proteins which form the molecular targets for carbapenems, ii) the combination of reduced permeability of drug through the outer membrane and the excessive production of β-lactamases having weak carbapenem-hydrolyzing activities, and iii) carbapenem-hydrolyzing β-lactamase production [160-162].

7. *Neisseria* spp.

Neisseria are aerobic, Gram-negative, non-motile diplococci bacteria [172]. There are many commensal species, including *N. perflava*, *N. sicca*, *N. mucosa*, *N. flava*, *N. cinerea* and *N. meningitidis*; only two of these species cause infectious disease in human hosts: the opportunistic *N. meningitidis*, which causes meningitis and septicemia, and the primary pathogen *N. gonorrhoeae*, which causes the sexually transmitted infection gonorrhea [172, 173]. Structurally, *N. meningitidis* and *N. gonorrhoeae* are quite similar in that they have outer membrane proteins (OMPs) and pili with slight antigenic variation; however, one major difference is that *N. meningitidis* possesses a capsule, as well [172, 174, 175]. The capsule is the main cause of the pathogenic difference in the two species – *N. meningitidis* evades phagocytosis because of its capsule, allowing it to enter into the bloodstream of the host; and the lack of an *N. gonorrhea* capsule means it is phagocytosed, causing the infection to remain localized [172].

The endotoxins lipooligosaccharide (LOS) and lipopolysaccharide (LPS) cause pro-inflammatory responses during infections with both *Neisseria* species, triggering septic shock in *N. meningitidis* infections and localized inflammation/damage in *N. gonorrhoeae* infections [172, 176]. In both species, LOS is sialylated with host sialic acid to prevent complement activity of the immune system; the *N. meningitidis* capsule also serves this purpose [172].

Furthermore, antigenic shift in *N. gonorrhoeae* pili and Opa proteins (specific OMPs) hinders proper phagocytosis as the infection spreads to the submucosa of the host tissue [172]. This antigenic shift, also called phase variation, affects over 60 genes (about half of which are important for encoding surface proteins, toxin production, restriction enzymes, and LPS/sugar metabolism systems), and allows *Neisseria* to express many phenotypes throughout its life-cycle [176, 177]. If phagocytosis does occur, Laz proteins and catalase minimize oxidative stress [172, 178]. Other virulence factors include iron-scavenging proteins (bind transferrin, lactoferrin, and hemoglobin), and IgA protease, which in *N. meningitidis* infections, allows for the cleavage of the main immunoglobulin in secretions [172, 179, 180].

For meningococcal infections, penicillin is the first-line antibiotic of choice, with third-generation cephalosporin (ceftriaxone and cefotaxime) utilization in cases of β-lactam resistance [172, 176, 181]. Rifampin and ciprofloxacin are occasionally provided as prophylactics to an infected individual's family members [172]. The MCV4 vaccine can also be used to prevent meningococcal infections in any individual aged nine months or older [182]. For gonococcal infections, penicillin cannot be used

Table 6. MFS antimicrobial efflux pumps of *Enterobacter* spp.

Drug efflux pump	No. of amino acids	No. TMS	Substrates	References
QepA	511	14	Fluoroquinolones	[163, 164]
Mef(A)	419	12	Macrolides	[165-168]
CmlB	409	12	Chloramphenicols	[169-171]

due to the observed high rates of resistance; instead, third-generation cephalosporins are used, with an intramuscular injection of ceftriaxone being preferred over oral treatment [183].

Neisseria antimicrobial resistance mechanisms are vast and varied. Erythromycin resistance is conferred *via* two conjugative transposons, one that contains the genes for RNA methylase, and one that contains *mefA*, a gene that encodes an antimicrobial efflux pump [184-186]. In the same vein, horizontal gene transfer is responsible for the presence of TetM- and β-lactamase-encoding plasmids, which cause tetracycline and penicillin resistances, respectively [180, 184, 187, 188]. Target alteration causes rifampicin/spectinomycin, penicillin, cefixime/ penicillin, and ciprofloxacin resistance by changing the structure of RNA polymerase, penicillin binding protein 1 (PBP1), PBP2, and DNA gyrase/ topoisomerase IV, respectively [187, 189, 190].

Finally, *Neisseria* uses numerous efflux systems, including MacA-MacB, MtrC-MtrD-MtrE, FarA-FarB-MtrE, and NorM (Table 7) [191]. Though the *mtrCDE*-encoded efflux pump system is used by both *Neisseria* species, it is under different regulatory constraints; *N. gonorrhoeae* contains both an activator (MtrA) and repressor (MtrR), whereas *N. meningitidis* utilizes the so-called Correia element containing an integration host factor site [192, 193]. Interestingly, the expression of the *mtrR* gene has a secondary effect, causing a mutation in the PorB1b OMP in *N. gonorrhoeae*, resulting in decreased influx of penicillin, tetracycline, and ceftriaxone, as well [185, 193].

8. *Pseudomonas aeruginosa*

Pseudomonas aeruginosa can cause opportunistic pulmonary infections and acute pneumonia in immunocompromised individuals [201]. Originating from the Proteobacteria phylum, *P. aeruginosa* is a Gram-negative bacillus, and many of its strains possess numerous virulence and antimicrobial resistance factors [202]. Most of the strains are motile as well as planktonic, meaning that an aggregation of *P. aeruginosa* cells can coalesce together to produce a biofilm structure [203, 204]. The ability to partake in adherence behavior enables *P. aeruginosa* to better attach to and colonize host tissues, which is often a key bacterial property during pathogenesis and infection [205]. In addition to these characteristics, *P. aeruginosa* has quorum-sensing capabilities which allow the bacterial cells to selectively express desirable genes depending upon their environmental conditions [206, 207].

P. aeruginosa can produce enzymes that break down antimicrobial agents, and an important example is

Table 7. *Neisseria* species multidrug efflux pumps.

Drug efflux pump	Family	Structure	Substrates	References
MacA-MacB	ATP-binding cassette (ABC)	MacA: Membrane fusion protein MacB: integral membrane protein with one ATP-binding domain	Macrolides	[191, 194]
MtrC-MtrD-MtrE	Resistance Nodulation Cell Division (RND)	MtrC: Periplasmic protein MrtD: inner membrane transporter (homotrimer, each with 12 transmembrane helices) MtrE: outer membrane channel	Hydrophobic antimicrobial agents	[192, 193, 195, 196]
FarA-FarB-MtrE	Major Facilitator Superfamily (MFS)	FarA: Membrane fusion protein FarB: cytoplasmic membrane transporter protein MtrE: outer membrane channel	Antibacterial long-chain fatty acids	[197, 198]
NorM	Multi-drug and Toxic Compound Extrusion (MATE)	12 transmembrane domains	Cationic toxic compounds; fluoroquinolones	[199, 200]

β-lactamase [208]. This microorganism can also produce siderophores, which are extracellular compounds that have iron-chelating properties [209]. These siderophores allow the bacteria to grow in iron-deficient environments [210]. *P. aeruginosa* also possesses numerous multidrug efflux pumps, including several from the MFS, ABC, and RND solute transporter superfamilies [211-214]. Regarding antimicrobial efflux pumps of the MFS, the tetracycline efflux pump TetA(C) and the multidrug efflux pump CmlA from *P. aeruginosa* have been characterized physiologically [215]. Treatment of *P. aeruginosa* includes the use of antibacterial agents that target the bacterium specifically, as in the case with certain β-lactams, carbapenems, and cephalosporins [216, 217].

9. Modulators of antimicrobial transporters from the major facilitator superfamily

9.1. Early modulators

One of the earliest known modulators of solute transport activity in members of this transporter superfamily includes the protonophore and disruptor of the membrane potential, an agent called carbonyl cyanide *m*-chlorophenylhydrazone (CCCP) [218, 219]. While toxic to many eukaryotes and prokaryotes alike, the physiological uses of CCCP were instrumental in establishing the secondary active transport modes for many solute transporters of the major facilitator superfamily, distinguishing them from their passive facilitated diffuser transporters [139, 220, 221]. The notion that the TetA tetracycline efflux pump is an active transporter system was demonstrated by using dissipaters of the membrane potential such as CCCP and others like cyanide and 2,4, dinitrophenol [222].

Compounds derived from plants are considered to be very good candidates for efflux pump modulation and for controlling bacterial pathogens that are resistant to multiple antimicrobial agents [223]. Another early efflux pump inhibitor is the naturally occurring plant alkaloid compound called reserpine [224]. Along these lines the related plant compound piperine was effective in inhibiting the NorA multidrug efflux pump from methicillin resistant *S. aureus* (MRSA) clinical isolates [225, 226]. Likewise, these types of agents were good inhibitors of the Bmr multidrug transporter from *B. subtilis* [227]. These modulators were shown to inhibit a variety of multidrug efflux pumps of the major facilitator superfamily, such as CmlR1 and CmlR2 from *Streptomyces coelicolor* [228], P55 from *Mycobacterium tuberculosis* [229], Lde from *Listeria monocytogenes* [230], LmrS from MRSA [114], MdeA from *S. aureus* [231] and EmrD-3 from *V. cholerae* [85].

Frequently, as a matter of standard protocol, newly discovered secondary active solute transporters employ dissipaters of the membrane potential in order to establish their mode of energization. Two problems inherent in these and other early efflux inhibitory agents, however, included their non-specific nature of action at high concentrations and their putative host cell toxicities, thus compromising their clinical utility in treating bacterial infectious disease in humans and in veterinary medicine [4, 134].

Additional studies were conducted on the LmrP efflux pump from *Lactococcus lactis* in which modulation of ethidium transport by verapamil and quinine in a competitive manner, by nicardpin and vinblastine in a non-competitive manner and by tetraphenylphosphonium in an un-competitive manner was observed, indicating that LmrP possesses multiple sites of action for transport and modulation [232, 233]. Oligosaccharide sugars that were polyacylated and derived from medicinal plant species were shown to have successful antibacterial activity in cells with NorA and the TetA(K) tetracycline efflux pump and inhibition of ethidium bromide efflux activities in cells containing both pumps [234].

The naturally occurring plant compound curcumin from *Curcuma longa* was shown to inhibit the efflux of rhodamine 6G which is a substrate of CaMdr1p from host cells of *Saccharomyces cerevisiae* [235]. Unfortunately, the use of curcumin has become controversial, since in the case of efflux measurements for ethidium, the curcumin molecule is known to fluoresce, as well [236, 237]. Thus, care should be taken to ensure that investigators subtract any fluorescence emitted by properly using appropriate control groups consisting of "curcumin-alone" from host cells with and without the antimicrobial efflux pump being examined during transport studies in which fluorescence is used; the remaining fluorescence may be due to

effects on ethidium transport. Another set of plant-based compounds, flavonolignan and alkylated flavones showed good antibacterial activities against cells harboring NorA, although efflux activities were not measured [238, 239].

9.2. Recent modulators

The food agent in spicy peppers, capsaicin, effectively inhibited the transport of ethidium bromide in cells harboring the NorA multidrug efflux pump from *S. aureus* [240]. Another naturally occurring modulator of NorA consists of derivatives of *N*-caffeoylphenalkylamide from plant origin, shown to be effective in inhibiting ethidium bromide efflux [241]. In 2012, a series of hydantoin-derived agents inhibited drug efflux from cells containing the QacA multidrug efflux pump from *S. aureus* [242]. Natural compounds have been of immense interest as inhibitors of efflux pumps in view of their availability and the ease of preparation of extracts for testing. Several compounds of plant origin such as the plant-derived alkaloid reserpine, kaempferol rhamnoside and capsaicin which inhibit the NorA efflux pump [240, 243], and piperine which inhibits the MdeA efflux pump have been shown to be inhibitors of *S. aureus* efflux pumps [225]. The plant alkaloid reserpine effectively inhibited the efflux activities of NorA and reduced the MIC of fluoroquinolones such as norfloxacin, sparfloxacin and moxifloxacin in clinical isolates harboring NorM [106]. Along similar lines, piperine has also been shown to significantly reduce the MIC of mupirocin to *S. aureus* [225]. Structural analogues of piperine, namely SK-20, SK-56 and 5-(3,4-methylenedioxyphenyl)-2E,4E pentadienoic acid 3-cyanophenyl amide were shown to be effective inhibitors of NorA [226, 244].

The plant-derived phenolic amide *N*-trans-feruloyl 4'-*O*-methyldopamine showed inhibitory activities against *S. aureus* with the NorA efflux pump and reduced the MIC of ciprofloxacin [241, 243]. Several other natural compounds have been shown to be effective in partially or completely inhibiting the efflux activities of NorA [234, 241, 245-247]. Compounds derived from berberine plants 5'methoxyhydnocarpin and pheophorbide were shown to be potent NorA inhibitors [248, 249]. Although the exact mechanism of inhibition by these compounds is not fully understood, sequestration of H^+ critical for the energization of secondary efflux pumps could be a plausible explanation for their transport inhibitory activities [245, 250]. Synthetic compounds, on the other hand, offer structurally diverse options to screen against a specific efflux pump, and these compounds can be further modified to achieve higher efficiency of inhibition [251]. Some of the effective synthetic inhibitors of NorA include chlorpromazine, thioridazine [252], tariquidar [246], a synthetic analog of ofloxacin [253], quinolone-derivatives [251], COX-2 inhibitor celecoxib and a new class of pyrazolo[4,3-c][1,2]benzothiazine 5,5-dioxide analogues [254].

Efflux pump inhibitors work in synergy with the antibiotic and increase their potency by inhibiting the efflux-mediated reduction of the intracellular antibiotic concentrations. Several recent studies have shown that the efflux pump inhibitor-antibiotic synergy is potentially useful in restoring the efficacy of some antibiotics, as in the case of citral amide derivatives [255] and *N*-cinnamoylphenalkylamide derivatives [241] which caused significant reduction in ciprofloxacin and norfloxacin MICs. Similarly, Nargotra *et al*. [256] showed that piperine analogues in which a piperidine fragment was replaced with other amines reduced the MIC of ciprofloxacin. Several indole-derived compounds were effective as efflux pump inhibitors resulting in the increased susceptibility of *S. aureus* to ciprofloxacin [257].

The search for new EPIs among natural, natural-derived synthetic or purely synthetic compounds has yielded promising results [258]. These EPIs are capable of inhibiting efflux pumps in *S. aureus* resulting in the increased efficacy of anti-staphylococcal antibiotics. Since NorA is one of the best-characterized efflux pumps of *S. aureus*, the majority of the studies have been performed in strains harboring NorA. Although most of the studies have described the anti-NorA activities of EPIs used, the inhibition of other efflux pumps cannot be ruled out, as well, although these latter instances depend upon the mode of action of the putative EPI in question, and remain to be definitively demonstrated. Nevertheless, EPIs are promising alternatives to the search for newer antibiotics and can help to restore the efficacy of antibiotics against drug-resistant *S. aureus.*

In 2015, a group of derivatives that was synthesized from a cyclobutene-dione structural core was shown

to inhibit the efflux of substrate Nile Red from the CaMdr1p transporter of the fungal microorganism *Candida albicans* [259]; in the same study, two of these synthetic derivatives, denoted as compounds A and B, showed synergistic effects with the anti-fungal agent fluconazole [259]. Additionally, the compound B agent may also be a substrate for the CaMdr1p efflux pump [259].

In a separate study of the eukaryotic vesicular monoamine neurotransmitter transporter VMAT2, inhibition of transport was observed in the presence of the established efflux pump inhibitor tetrabenazine [260] in a non-competitive manner in which residues Val-41, Gly-308 and Pro-314 of VMAT2 are required for tetrabenzine binding and are thought to mediate conformational changes to accommodate inhibitor binding [261]. In another study, the nitrate transporter NrtA from the fungus *Aspergillus nidulans* was inhibited by chlorate while its paralog NrtB was inhibited by cesium, a cation [262].

Efflux pumps extrude clinically relevant antimicrobials thus reducing their intracellular concentrations to sub-lethal levels. This activity not only allows bacteria to survive antibiotic pressure but also to develop other mechanisms of resistance such as mutation, alteration of target or of membrane permeability. Considering their overwhelming presence in the genome of *V. cholerae*, modulation of efflux pumps is a viable approach towards restoring the potency of antibiotics [263]. Several studies substantiate this hypothesis and with the availability of bioinformatics tools, it is possible to screen vast databases of lead molecules and to identify potential novel inhibitors of efflux pumps. A recent study showed that *A. sativum* extract and allyl sulfide inhibited ethidium bromide efflux in cells harboring EmrD-3 and that *A. sativum* lowered the MICs of multiple antibacterials [264].

The efflux activity of VcaM was experimentally observed to be inhibited by reserpine and sodium *o*-vanadate [86]. Along similar lines, the compounds 1-(1-naphthylmethyl)-piperazine (NMP) and phenyl-arginine-β-naphthylamide (PAβN) could inhibit RND efflux pumps in *V. cholerae* and potentiate the activities of efflux pump substrates [265]. NMP and PAβN also inhibited the production of CT and the TCP. The fact that RND efflux pumps regulate the expression of virulence factors such as cholera toxin (CT) and the toxin co-regulated pilus (TCP) along with their antimicrobial efflux activities [82, 83], makes them ideal candidates for control of antimicrobial resistance and the virulence in *V. cholerae* [2, 15].

A great deal of attention has been focused on modulation of antimicrobial efflux transporters from the serious pathogen *S. aureus* [266, 267]. In recent work, derivatives of boronic compounds have been shown to have good anti-bacterial activities towards cells of *S. aureus* containing NorA, although antimicrobial transport was not measured directly in these studies [268, 269].

In 2016, tannic acid was indirectly shown to affect efflux from NorA in cells using the MIC of substrate ethidium while also showing good synergy with substrates ethidium and norfloxacin [270]. During this same period, the crystal structures of MdfA from *E. coli* were determined in which each structure was found to be complexed with either substrate acetylcholine or naturally occurring efflux pump inhibitor reserpine [271]. In this study, the acetylcholine and reserpine were shown to share some MdfA amino acid residues in common while differing in key contacts, residues of which are found in highly conserved sequence motifs and play important roles in mediating conformational changes that transpire during transport catalysis [271-274]. We predict that the structures formed by highly conserved sequence motifs and the transport catalysis conferred by these structures will also be important sites of action for putative modulators, especially for efflux pumps of the major facilitator superfamily [15, 272, 275].

More recently, cumin extract and one of its bioactive components, cuminaldehyde, from the plant spice *Cuminum cyminum* showed good efflux pump inhibitory activity against the LmrS multidrug efflux pump cloned from a MRSA clinical isolate [276]. In another study, garlic extract and its bioactive agent, allyl sulfide, from *Allium sativum* were antibacterial and effective inhibitors of ethidium bromide transport by EmrD-3 from *V. cholerae* [264]. Furthermore, it was established that *A. sativum* extract showed good synergistic activity with multiple antimicrobial agents, including vancomycin [264].

10. Future directions

With the ever-increasing incidence and prevalence of multidrug resistant bacterial pathogens, it is

becoming quite clear that new antimicrobial agents and naturally occurring bioactive modulators are needed in order to circumvent bacterial resistance. Towards this, future work is needed in discovering and developing new natural bioactive modulators as potential antibacterial agents and as putative inhibitors of antimicrobial efflux systems.

Another important avenue includes evaluation of the synergistic relationships between combinations of modulators and antimicrobial agents and between combinations of bioactive agents themselves. Additionally, it could be quite advantageous to evaluate the relationships at the molecular level between natural bioactive modulators and their efflux pump targets; that is, it will be important to know the binding sites on drug efflux pumps to which modulators bind.

Knowledge of such molecular interactions will greatly facilitate our efforts to produce efficient and safe efflux pump inhibition. It is predicted that such efforts will no doubt circumvent antimicrobial resistance and will aid in the eventual reduction of both morbidity and mortality rates that are due to resistant bacterial pathogens. Thus, these efforts, and others, may eventually restore the therapeutic efficacy of antimicrobials against infection.

ACKNOWLEDGMENTS

This publication was funded in part by NIH grant from the National Institute of General Medical Sciences (P20GM103451), an Internal Research Grant (IRG) from Eastern New Mexico University, and by a grant from the US Department of Education, HSI STEM program (P031C110114).

CONFLICT OF INTEREST STATEMENT

The authors declare that they have no conflicts of interest.

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