Front Biosci (Landmark Ed). **2017**;22:732-756.

Cellular efflux transporters and the potential role of natural products in combating efflux mediated drug resistance

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TABLE OF CONTENTS

1. ABSTRACT

Efflux mediated multidrug resistance (MDR) is a major problem in the treatment of bacterial, fungal and protozoal infections in addition to cancer chemotherapy. Among other well known mechanisms, efflux pumps are significant contributors to chemo-resistance. Efflux mediated resistance generally occurs through up-regulation of genes responsible for the expression of transporter proteins extruding drugs from the cell to create intracellular sub-therapeutic concentrations leading to resistance. The rapid expansion of MDR pathogens necessitates the discovery of resistance modifying drugs, which in combination with antimicrobial or chemotherapeutic agents would tend to reinstate the action of these drugs and avert the emergence of acquired resistance. This review describes the existence of efflux pumps in prokaryotes and eukaryotes as well as their role in chemo-resistance with a special focus on natural product-derived efflux pump inhibitors.

2. INTRODUCTION

Multidrug Resistance (MDR), mediated by efflux pumps present in prokaryotes and eukaryotes is a major problem in the management not only of infectious diseases but also cancers (1-4). The role of efflux mediated drug resistance is now well established in bacteria, fungi, protozoa and cancer cells. In this context, efflux pumps are transport proteins which facilitate the efflux of intracellular toxic substances, including drugs out of the cell, thus inhibiting cellular attainment of therapeutic concentrations subsequently leading to resistance (5, 6). Efflux pumps mediate their function through energy dependent mechanisms, specified as transporters. Among these transporters, ATP is employed as the energy source for the transport process (i.e. ATP-binding cassette transporters or ABC transporters) and these are termed primary transporters. In another group of transporter proteins, drug efflux is coupled to an electrochemical ionic disparity across the cell membrane (Na^+/H^+ driven active anti-porters) and they are commonly known as secondary transporters. In eukaryotes, the efflux process is frequently carried out

by primary transporters, whereas prokaryotes mainly employ secondary active anti-porters. Some of these transport proteins are drug specific while others are non-specific drug transporters and they are largely responsible for MDR (7, 8).

Phylogenetically, bacterial efflux pumps have been grouped into five super-families (9, 10), based on their differences in amino acid sequence and the energy source utilized for the efflux of substrates. They are namely: the ATP binding cassette (ABC) super-family, the major facilitator super-family (MFS), the small multidrug resistance (SMR) super-family, the multidrug and toxic compound extrusion (MATE) super-family and the resistance-nodulation-cell division (RND) super-family. Mechanistically, these super-families have been grouped into primary and secondary transporters, the ATP-binding cassette (ABC) super-family consisting of primary transporters while the remaining super-families all comprise secondary transporters.

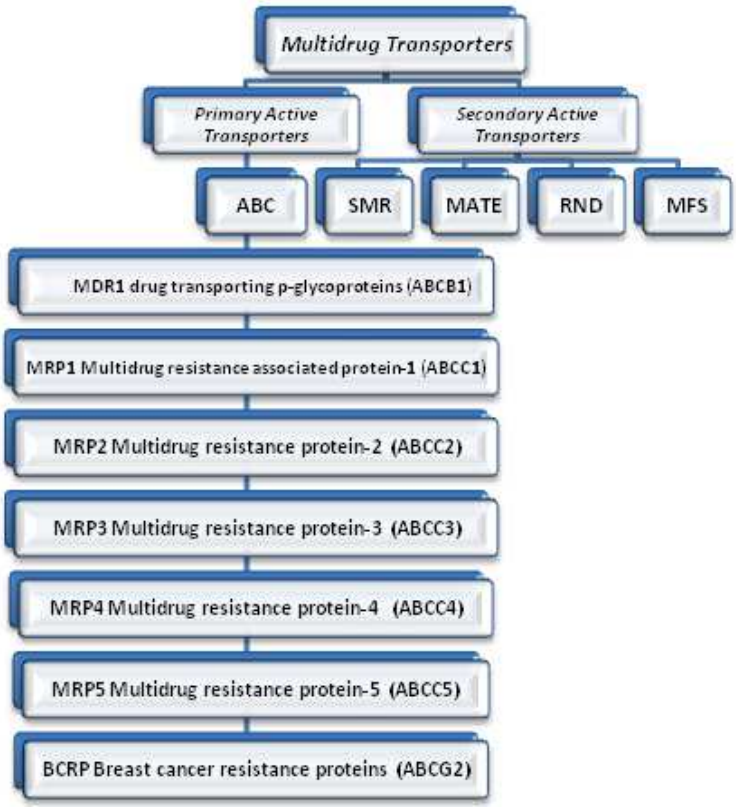


Figure 1. Schematic representation of multidrug transporters.

3. ABC TRANSPORTER PROTEINS

Transporter proteins that couple substrate transport with ATP utilization belong to an important group of primary transporters called the ABC super-family. Members of this family are ubiquitous membrane systems reported in prokaryotic as well as eukaryotic cells (11). Bacterial ABC efflux pumps typically possess higher specificity for several drugs including antibiotics, amino acids, iron complexes, vitamins, sugars and metallic cations. Typical examples of ABC transporters in humans include P-glycoproteins (P-gps) and multi-drug resistance proteins 1 and 2 (MRP1 and MRP2) which confer resistance to anticancer drugs (12, 13). ABC transporters contain two hydrophobic transmembrane domains and a cytoplasmic domain that carries signature motifs implicated in ATP binding (11). Several ABC transporter proteins are present in antibiotic fabricating microbes such as *Streptomyces* species and they impart resistance to the antimicrobial agents that they generate (14). ABC transporters also exist in Gram-positive bacteria, including *Staphylococci* and *Enterococci* conferring resistance to macrolides and bacitracin (15). Amongst the ABC efflux proteins, a frequently studied MDR ABC efflux pump is *Lmr*A found in *Lactococcus lactis*, which is structurally and functionally allied to human P-gp (16). In addition, other MDR ABC pumps have also been described in Gram-positive and Gram-negative microorganisms such as *Enterococcus faecalis*, *Vibrio cholerae* and *Mycoplasma* species (17-19).

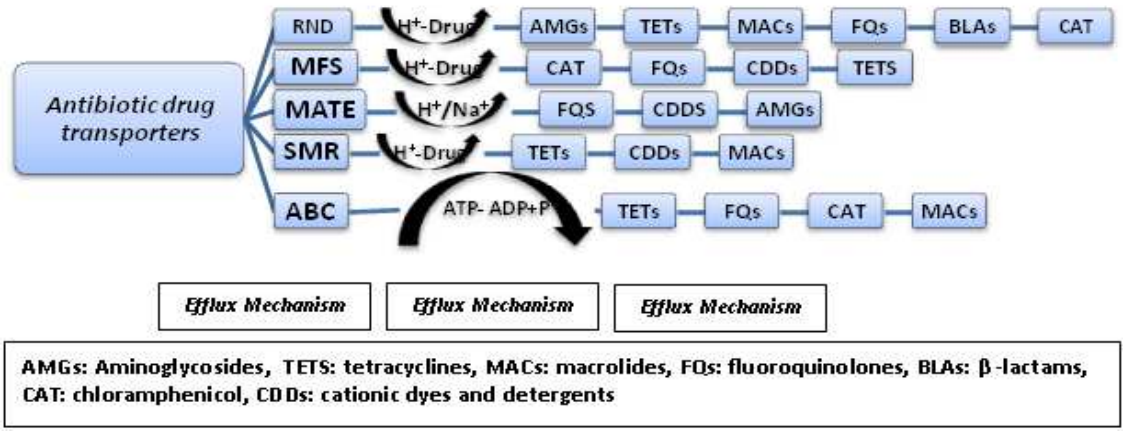


Figure 2. Antibiotic efflux pumps, mechanism of transport and drugs substrates.

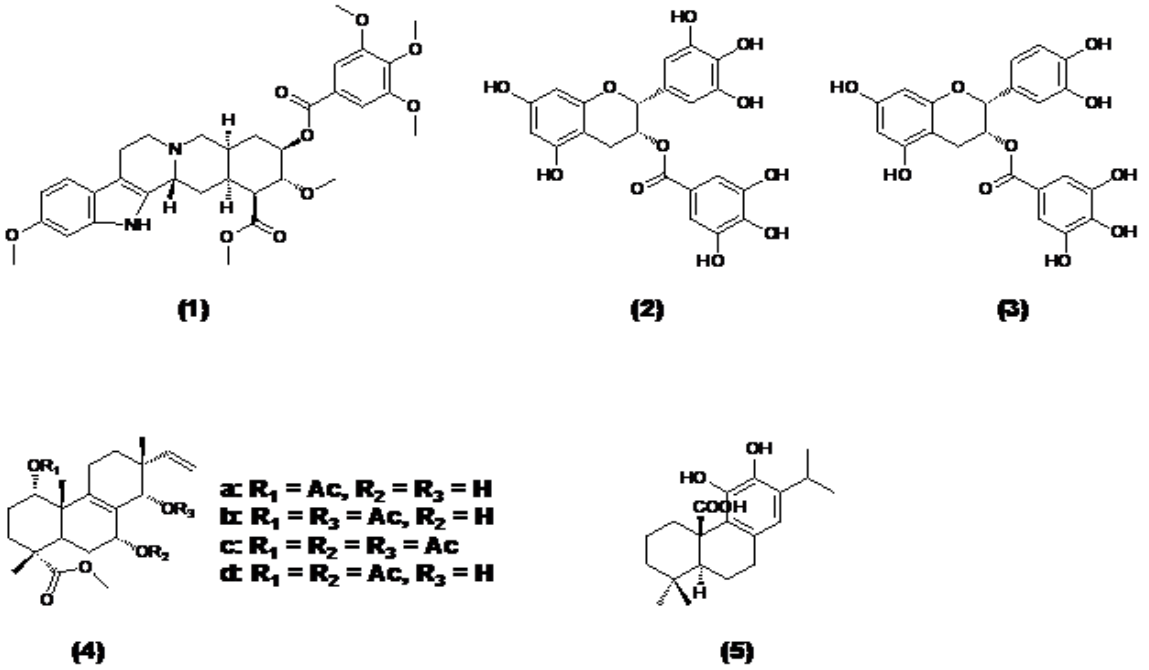


Figure 3. Structures of Compounds 1-5. Compound 1 (Reserpine), 2 (Epigallaocatechin gallate), 3 (Epicatechin gallate), 4a, b, c, d (Isopimarine diterpenes) and 5 (Carnosic acid).

3.1. Mammalian ABC transporters and drug resistance

The mammalian ABC efflux transporter system is a highly diverse group of transport proteins. These transporters play a vital role in the extrusion of clinically important drugs and the family comprises five types of MDR proteins known as (P-gp 1-5 plus breast cancer resistance proteins (BCRP's) (20). P-gp is a membrane bound ATP dependent transporter protein system implicated in MDR during cancer chemotherapy (21). These five types of human ABC transporters and BCRP are discussed below.

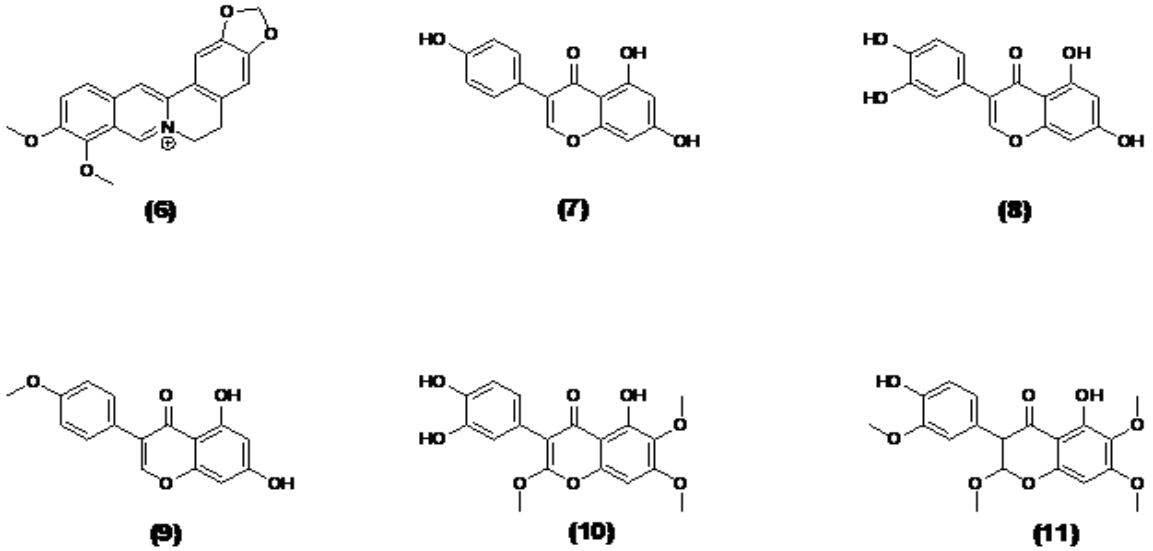


Figure 4. Structures of Compounds 6-11. Compound 6 (Berberine), 7 (Genistein), 8 (Orobol), 9 (Biochanin A), 10 (chrysosplenol-D) and 11 (chrysoplenetin).

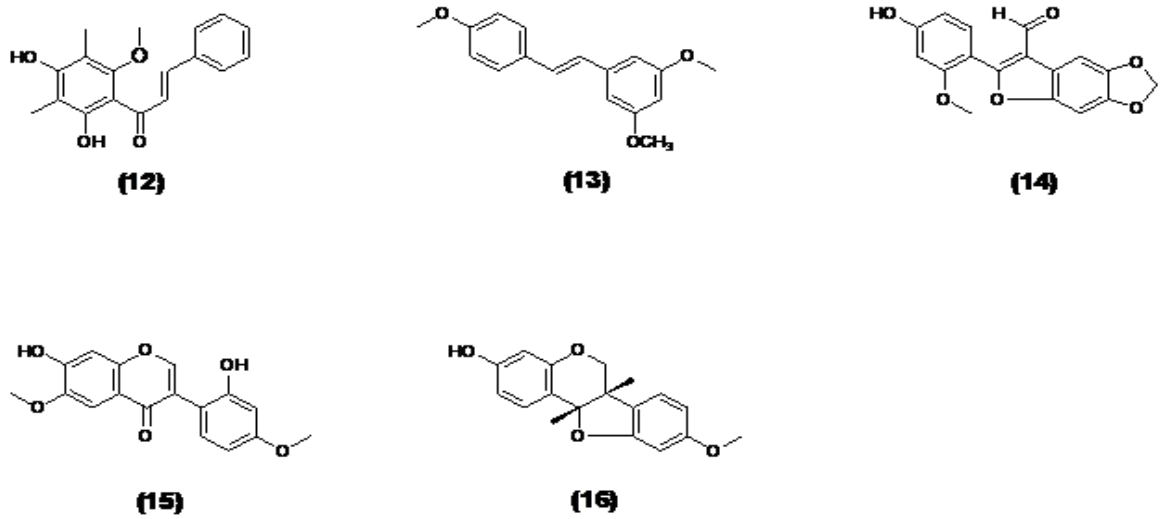


Figure 5. Structures of Compounds 12-16. Compound 12 (Chalcone), 13 (Stilbene), 14 (Spinosan A), 15 (Isoflavone) and 16 (Pterocarpan).

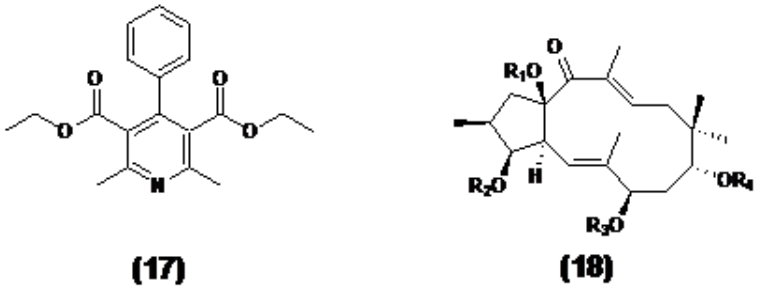


Figure 6. Structures of Compounds 17,18. Compound 17 (2,6-dimethyl-4-phenyl-pyridine-3,5-dicarboxylic acid diethyl ester) and 18 (Jatrophane diterpene).

3.1.1. Multidrug resistant (MDR1) drug-transporting P-glycoprotein (ABCB1)

This type of transport protein was first discovered by Juliano and Ling (21) and it is one of the most studied of the ABC proteins (22, 23). The distinctiveness of MDR1 originates from the sheer variety of substrates it is capable of transporting out of the cell. Many of these substrates are clinically employed chemotherapy drugs for example methotrexate, vinblastine, vincristine, paclitaxel, doxetaxel, doxorubicin, epirubicin, daunorubicin, bisantrene, mitoxantrone, etoposide, teniposide and topotecan in addition to HIV protease inhibitors like nelfinavir, lopinavir, ritonavir, saquinavir, indinavir and amprenavir. Moreover, other drug substrates include cimetidine, erythromycin, valinomycin, dexamethasone, hydrocortisone, corticosterone, triamcinolone, loperamide, cyclosporine, digoxin, verapamil, colchicine, emetine and morphine (20).

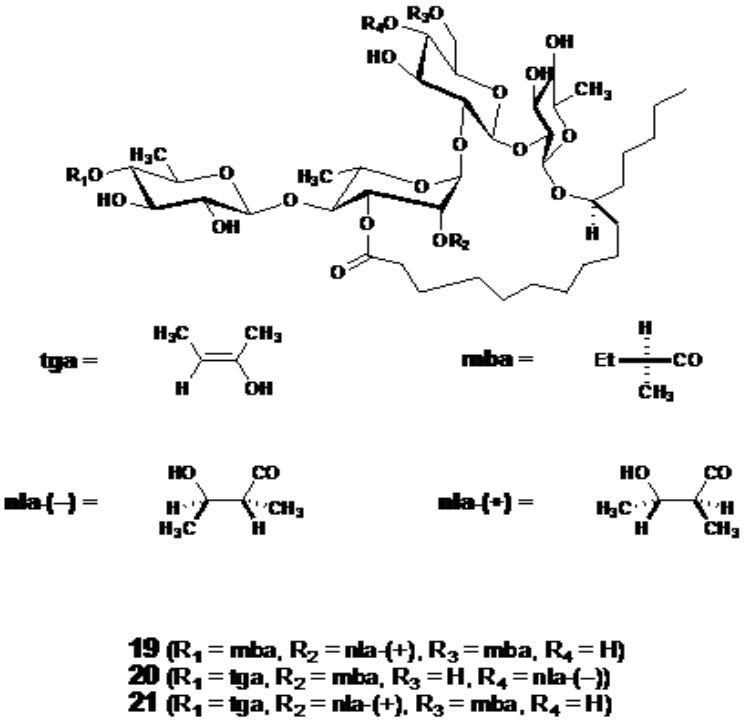


Figure 7. Structures of Compounds 19-21. Compound 19 (Orizabin XIX), 20 (Orizabin IX) and 21 (Orizabin XV).

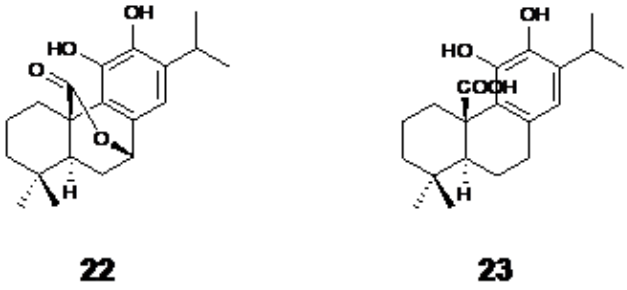


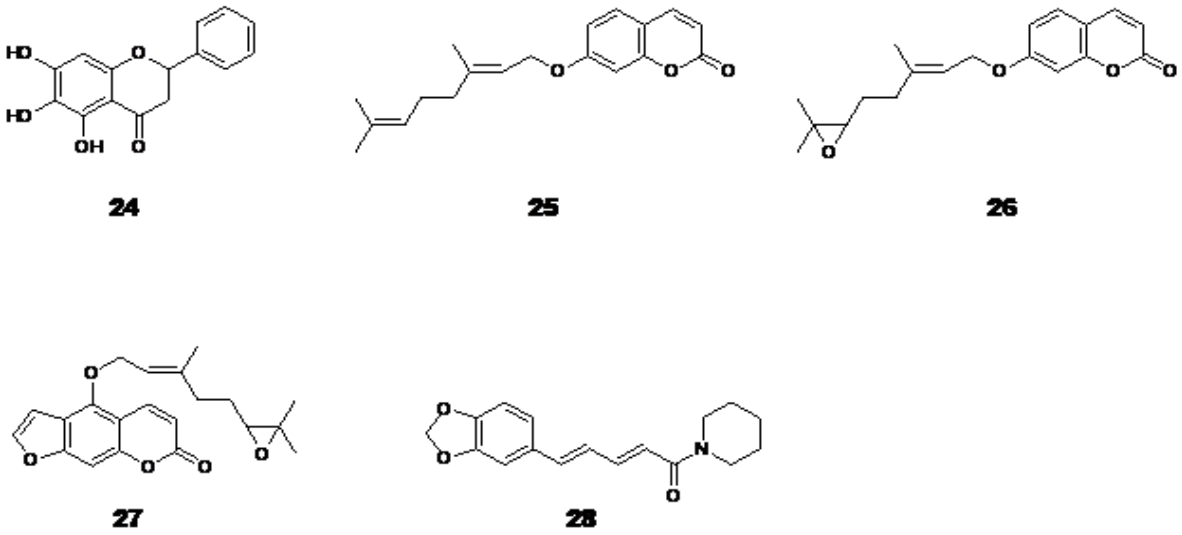
Figure 8. Structures of Compounds 22-23. Compound 22 (Carnosol) and 23 (Carnosic acid).

3.1.2. Multidrug resistance associated protein 1 (MRP1 or ABCC1)

MRP1/ABCC1 was originally recognized in cell lines which were rendered particularly resistant to the anticancer drug doxorubicin and subsequent studies revealed that this protein was responsible for resistance against a diversity of cytotoxic agents (24, 25). This range of anticancer drugs consisted of the vinca alkaloids, epipodophyllotoxins, mitoxantrone, anthracyclines and methotrexate which were apparent substrates for this transporter protein (26, 27). However, MRP1 did not confer high level resistance against either bisantrene or paclitaxel (25, 28).

3.1.3. Multidrug resistance protein 2 (MRP2 or ABCC2)

This transport system is well characterized and individuals lacking the MRP2 gene experience Dubin–Johnson syndrome which is typically associated with symptoms of hyperbilirubinemia and mild jaundice (29, 30). Accordingly, it is the hepatocyte canalicular membranes of affected individuals that are MRP2 deficient and which give rise to hepatobiliary elimination of glucuronidated bilirubin (31). The transport of numerous compounds by MRP2 is validated on recognition of the drugs that are carried into the bile of healthy rats but missing in the bile of MRP2 lacking mutants. Furthermore, the difference in uptake of compounds in canalicular membrane vesicles within MRP2 deficient and normal healthy rats, transduction of human/rat MRP2 into cell lines with drug resistance analysis and transepithelial movement of drugs, has established the role of MRP2 in drug efflux and MDR acquisition (29, 32). The anticancer drugs transported by MRP2, include methotrexate, anthracyclines, vincristine, vinblastine, doxorubicin, epirubicin, etoposide and cisplatin (27, 32).



24 25 26

27 28

Figure 9. Structures of Compounds 24-28. Compound 24 (Baicalein), 25 (Coumarin derivative) and 26 (coumarin epoxide derivative), 27 (Bergamottin epoxide derivative) and 28 (Piperine).

3.1.4. Multidrug resistance protein 3 (MRP3 or ABCC3)

Unlike MRP1 and MRP2, to date MRP3 has not been studied in depth although this group of transport proteins is known to exist in the basolateral membrane of polarized cells (29). The human MRP3 transporter system is reported to exhibit resistance against anticancer agents like methotrexate, tenoposide and etoposide, whereas rat MRP3 mediates the transport of methotrexate and bile salts (33, 34). It has also been reported that organic anion transport inhibitors including indomethacin, benzbromarone, sulphinpyrazone and probenecid can reverse an MRP3 dependent decline in etoposide accumulation (35).

3.1.5. Multidrug resistance protein 4 (MRP4 or ABCC4)

MRP4 has been studied in detail by Lee *et al*., (36). This transporter protein group is commonly expressed in skeletal muscles, bladder, prostate, testes, small intestine, lungs, kidneys, gall bladder, tonsils and ovaries. It is capable of extruding a number of drugs including anticancer agents like azidothymidine (AZT) and in a cell line made highly resistant to these drugs, while other analogues are transported even more rapidly out of the cell (37). Additionally, MRP4 based resistance has been described to additional anticancer drugs like thioguanine (TG) and 6-mercaptopurine (6-MP) (38).

3.1.6. Multidrug resistance protein 5 (MRP5 or ABCC5)

MRP5 has been studied but details about this important group of proteins remain essentially in the early stages (39, 40). Thus MRP5 is reported to offer low level resistance against potassium antimonyl tartrate and cadmium chloride transport but no resistance has been documented against anticancer agents (39, 40).

3.1.7. Breast cancer resistance protein (BCRP or ABCG2)

Cancer is the next major cause of mortality after cardiac diseases in developed countries (41). The incidence of MDR cancer cells and concomitant cross resistance of tumor cells to a variety of anticancer drugs has been acknowledged since the early application of chemotherapy for human malignancies (42). Correspondingly, BCRP transporter protein has been recognized for its involvement in resistance against anticancer drugs such as doxorubicin, daunorubicin and mitoxantrone. Due to the fact that the gene for this particular protein was first identified in a breast cancer cell line, it was designated the breast cancer resistance protein (BCRP) gene and transporters of this nature are predictably involved in resistance to breast cancer chemotherapy (41).

3.2. ABC transporter proteins and antibacterial drug resistance

The majority of prokaryotic drug efflux systems entail active transporters coupled to an electrochemical differential on either side of the cell membrane (proton exchange systems). A number of drug transporting proteins, including ABC drug transporters, also utilize energy for drug transport generated through hydrolysis of ATP (43). Most of these transporter proteins mediate single drug resistance (SDR) in Gram-positive microbes and typically transport a single group or very similar groups of compounds. An example is expressed from the *ard*1 gene cloned from *Streptomyces capreolus* which encodes resistance against an aminonucleoside antibiotic (44). Furthermore, a genomic library of *Streptomyces longisporoflavus* DNA cloned in *S. lividans* revealed a determinant (tnrB) which conferred resistance to the polyether-ionophore antibiotic tetronasin, the mechanism of which is most likely via an ATP-dependent efflux system (45). Due to the fact that antibiotic resistance among human pathogens such as *S. epidermidis* and *S. aureus* is accomplished by ABC super-family members, strategies to inhibit these pumps represent a clinical need. Another ATP-dependent prokaryotic MDR transporter protein *Lmr*A, was first identified in *Lactococcus lactis* (46) and possesed comparable pharmacological features to P-gp with an extensive range of substrates as shown in Table 1. Interestingly, no biologically active SDR or MDR efflux pumps belonging to the ABC drug transporter family have been recognized in Gram-negative bacteria (47).

Table 1. Microbial, Parasitic and Fungal ABC transporters related with chemo-resistance

Microorganism	ABC transporter	Substrates / Drugs	References
Streptomyces capreolus	*Ard1*	A201A	(72)
Streptomyces logisporoflavus	*TnrB*	Tetronasin	(73)
Streptomyces peuceticus	*DrrAB*	Daunorubicin, Doxorubicin	(74)
Staphylococcus epidermidis	*MsrA*	Erythromycin	(75)
Lactococcus lactis	*LmrA*	Anthracyclines, Quinolones, Vinca alkaloids, Macrolides, Tetracycline's Aminoglycosides, Chloramphenicol	(16. 46, 76)
Lactobacillus brevis	*HorA*	Novobiocin	(77)
Aloferax volcanii	*Not identified*	Anthracyclines, Vinca alkaloids	(78, 79)
Saccharomyce scerevisiae	*Pdr12p,Pdr5p, Snq2p, Ycf1p*	Antimycotics, Antibiotics, Anticancer drugs, Fungicides	(80, 81)
Candida albicans	*Cdr1p, Cdr2*	Antimycotics	(82-86)
Plasmodium falciparum	*Phg1*	Chloroquine,mefloquine,quinine, Halofantrine, artemisinin	(57, 58)
Leishmania tarentolae	*PGPA*	Antimony drugs, Arsenic drugs	(66, 87)
Entamoeba histolytica	*EhPgp1, EhPgp2*	Amoebicides	(70, 71)

3.3. ABC transporter proteins and antifungal drug resistance

Two major groups of xenobiotic transporters systems, ABC and MFS, which offer resistance against several drugs have been recognized. In contrast to prokaryotic ABC transporters, fungal ABC transport proteins consist of numerous membrane spanning pumps (48). The first reported non-mammalian ABC transporter protein was discovered in *Saccharomyces cerevisiae* (49). These transporters expel a diversity of chemically dissimilar compounds/drugs including several antibiotics, anti-mycotic agents, anticancer agents, herbicides, mycotoxins and agricultural fungicides (50). Among yeast species, within the genome sequence of *Schizosaccharomyces pombe*, there are determinants with prospective implications in MDR against clinically important drugs (51). The most frequent substrates of these efflux pumps include anticancer agents, antifungal drugs, leptomycin B, actinomycin, cerulenin and cytochalasin B (52, 53). *Candida albicans* is reported as the major causative agent (> 50%) in many fungal infections. It is a major clinical problem particularly in immunocompromised, HIV infected, organ transplant and cancer patients owing to the emergence of MDR Candida strains (54). The Cdr1p efflux system of *Candida albicans* is reported to bestow resistance against a variety of antifungal drugs namely miconazole, fluconazole as well as morpholines, allylamines and others. On the other hand, the Cdr2p transporter of the same fungus confers resistance to terbinafine, amorolfine, azole antifungals and a range of metabolic inhibitors (54, 55).

3.4. ABC transporter proteins and parasitic drug resistance

MDR has turned out to be a major barrier in the treatment of protozoal parasitic diseases. Malaria is the most widespread of the tropical parasitic diseases affecting about 300-500 million individuals with almost 2-3 million deaths globally. Amongst the four species of malarial parasites, *Plasmodium falciparum* generates high mortality rates in untreated cases. It has been reported that more than 41% of the world population is at risk due to the emergence of MDR protozoans (56). At least three different ABC transporter protein encoding genes and two P-glycoprotein homologues, Phg1 and Phg2, have been identified in *P. falciparum* (57, 58). The Phg1 transporter system is located on the vacuole of the parasite which is the site of action for chloroquine and this may present resistance against clinically useful drugs not only to chloroquine but also quinine, halofantrine and mefloquine (59, 60).

Leishmanial infections have proliferated drastically and become the second most common cause of global deaths from parasitic infections (61). Among the treatment strategies for intracellular Leishmanial infections, the use of pentavalent

antimonials including glucantime and pentostam are the only effective options (62). However, these metalloid antimony based compounds are not very useful due to their toxicity and the emergence of multiple drug resistant pathogens (63). In light of this, numerous ABC transporter proteins associated with MDR have been identified in *Leishmania* species (64). The *pgpA* gene encoding an ABC protein pgpA has been identified in *Leishmania tarentolae* confering resistance to antiprotozoal drugs (65, 66). Some species of *Leishmania* have multidrug resistant ABC transporter proteins (MDR1) which are similar to human P-gp and impart resistance to cytotoxic drugs (67). *Entamoeba histolytica* is another clinically important parasite which infects about 500 million people with an annual death rate of 100,000 worldwide (68). The basic therapeutic options for amoebiasis include the use of nitroimdazole drugs such as metronidazole. However, due to divergence in the susceptibility of clinical isolates and standard strains of *E. histolytica*, it appears that the parasite is capable of acquiring MDR (68, 69). Studies suggest that verapamil sensitive ABC transporter proteins are implicated in the development of MDR against amoebicidal drugs. What is more, six P-gp-like genes (EhPgp1-6) have been recognized among which the expression of EhPgp1 and EhPgp2 strongly correlate with resistance against the trophozoites (70, 71).

Table 2. Families with in the MFS, their substrates and transport mechanisms

Family Name	Mechanisms	Substrates	Examples
Sugar porter (SP)	Sugar : uniport Sugar: proton Symport Sugar: sugar Antiport	Monosaccharide's, Disaccharides, Inisitols , Organo-cations,	XylE transporter of Escherichia coli
Drug: H1 antiporter (DHA14)	Drug: H1 Antiport	Multiple or single drugs	QacA of S. aureus
Drug:H1 antiporter (DHA14)	Drug:H1 Antiport	Multiple or single drugs	Bmr transporter of Bacillus subtilis
Organophosphate: antiporter (OPA)	Organophosphate: Pi Antiport	Sugar-phosphates, glycerol phosphate, phosphoglycer-ates, phosphoenol pyruvate	UhpT transporter of Escherichia coli
Oligosaccharide: H1 Symporter (OHS)	Sugar: H1 Symport Sugar: sugar Antiport	Di-and trisaccharides	LacY transporter of Escherichia coli
Metabolite: H1 symporter (MHS)	Solute: H1 Symport	Citrate, a-ketoglutarate, proline, betaine,methylphtha-late, dicarboxylates	Kgt transporter Escherichia coli
Fructose-galactose-glucose: H1 symporter	Hexoses uniport Hexoses: H1 Symport	Fructose, glucose, galactose	FucP transporter of Escherichia coli
Nitrate/nitrite porter (NNP)	Nitrite uniport Nitrate: H1 Symport	Nitrite, nitrate	NarK transporter of Escherichia coli
Phosphate: H1 Symporter (PHS)	Pi :H1 Symport	Inorganic phosphate	Pho-5 transporter of Neurospora crassa
Nucleoside: H1 Symporter (NHS)	Nucleoside: H1 Symport	Nucleosides	NupG transporter of Escherichia coli
Oxalate: formate Antiporter (OFA)	Anion: anion Antiport	Oxalate, formate	OxlT Oxalobacter formigenes
Sialate:H^+ symporter (SHS)	Substrate: H1 Symport	Sialate	NanT transporter of Escherichia coli
Anion: Cation Symporter (ACS)	Substrate: H^+ Na^+ Symport	Glucarate, tartrate, 4-hydroxy phenyl acetate, inorganic phosphate, allantoate	ExtU transporter of Escherichia coli
Aromatic acid: H^+ Symporter (AAHS)	Substrate: H1 Symport	Muconate, benzoate	PcaKo transporter of P. putida

4. MAJOR FACILITATOR SUPERFAMILY (MFS)

The major facilitator super-family (MFS) is a transporter system of ubiquitous proteins, which extrude sugars, drugs, metabolic end products, and other toxic substances (90, 91). In 1998, Pao *et al.,* explored this transmembrane transporter system in detail and seventeen distinct sub-families were primarily classified based on phylogenetic data (92, 93). However, with the passage of time and research endeavor, this has been revised to a broad total of 74 families (94).

MFS efflux pumps are single polypeptide secondary carriers capable of transporting small molecules by means of chemi-osmotic ion gradients (95). These proteins transport a variety of drugs, Krebs cycle metabolites, organophosphates and oligosaccharides (96). MFS proteins are distributed in all major groups of living organisms including eukaryotic protists, bacteria, yeasts, plants, fungi and animals. These transporter proteins consist of twelve transmembrane segments (TMS) and operate as symporters, uniporters and antiporters. The first type of the originally classified sub-families of MFS employs a sugar porter (SP) which transports hexoses, pentoses, inisitols, disaccharides and organic cations. The most widespread example of the SP family is the arabinose-proton symport present in *E. coli* (97). Sub-families 2 and 3 transfer proteins are composed of fourteen and twelve transmembrane segments respectively. These efflux systems are well recognized in bacteria as well as eukaryotes and actuate drug efflux (H^+ Antiport) (43). Sub-families 4-6 were recognized as members of MFS in 1993 and utilize organophosphate antiporters (OPA), oligosaccharide symporters H^+ (OHS) and metabolite symporters H^+ (MHS) correspondingly (96). Sub-Family 7 is specific for sugars and functions through a fructose-glucose-galactose H^+ symporter (FGHS) as a proton symport mechanism. Sub-family 8 of MFS is distributed in plants, yeasts and bacteria, and acts through a nitrate-nitrate porter (NNP). These transporter proteins have a larger size (395-547 residues) and initiate nitrate efflux or uptake. Sub-Family 9 operates via a phosphate H^+ symporter (PHS) and is limited to yeast and plants. Proteins of this family are bigger in size with 518-587 residues. Sub-family 10 which relies on a nucleoside H^+ symporter (NHS) has been reported in bacterial

species and consists of 418 residues. Both of these transporter proteins have been recognized in *E. coli* and have no substrate specificity (92). The oxalate/formate antiporter (OFA-sub-family 11) is a small and diverse group which is distributed in bacterial, eukaryotic and archaeal kingdoms. Sub-family 12 exploits the sialate H^+ symporter and is mainly present in Gram-negative bacteria such as *E. coli* and *Haemophilus influenza* (98). Sub-family 13 functions through a monocarboxylate porter (MCP) consisting of thirteen sub-proteins. These proteins are reported in bacteria and mammalian cells, however, only the mammalian members of this family have been functionally characterized (99) but they all operate by a proton symport to transport pyruvate, lactate and mevalonate (100). Sub-family 14 implements an anion-cation symporter (ACS) and commonly transports organic and inorganic anions. These efflux proteins are extensively distributed in animals, fungi and bacteria being recognized not only in *Rattus novegicus* but also *B. subtilis, E. coli* and other microbes. Sub-family 15 of MFS performs through an aromatic acid H^+ symporter (AAHS) which is limited to Gram-negative bacteria transporting aromatic acids. Sub-family 16 is recognized as "unknown major facilitator super-family" (UMFS) since none of these protein members have been functionally characterized (94, 101). Family 17 of MFS relies on cyanate permease (CP) transport protein and is commonly present in *B. subtilis* and *E.coli.* Another reported sub-family of the MFS series is the Proton reliant Oligopeptide Transporter (POT) family which comprises 34 proteins. Members of this family are widely distributed in bacteria, fungi, yeast, animals and plants (102).

5. SMALL MULTIDRUG RESISTANCE (SMR) SUPERFAMILY

The Small Multidrug Resistance (SMR) superfamily is the smallest known group of transporter proteins the members of which are comprised of four transmembrane segments (103). Efflux pumps within this family are reported to extrude a variety of lipophilic cationic drugs in microorganisms via a proton drug antiport pathway. Structural studies of these pumps in *E. coli* (*Emr*E) have demonstrated that they are closely correlated to bacterial MFS transporter proteins (104).

6. MULTIDRUG AND TOXIC COMPOUNDS EXTRUSION (MATE) SUPERFAMILY

Tasuchiya *et al.,* described groups of multidrug transporter proteins in *E. coli* and *Vibrio parahaemolyticus* in 1998 and named them YdhE and NorM respectively (105). Since these proteins were composed of twelve transmembrane segments, like true MFS proteins, they were initially included in the MFS superfamily (105). Subsequently Skurray and his co-workers reported that these proteins belonged to a dissimilar group of transporter proteins because they possessed no sequence resemblance to other known multidrug transporters. Thus, they were assigned to a novel family of proteins named the Multidrug and Toxic compound Extrusion (MATE) superfamily (103). MATE proteins are extensively distributed in almost all living kingdoms ranging from protozoa and bacteria to eukaryotes and fungi. This super-family has been grouped into three subfamilies. Family 1 consists of bacterial MATE transporter proteins like NorM in *V. parahaemolyticus*, a prototype transporter. Family 2 has been allocated to eukaryotic MATE proteins and has been further divided into four subfamilies. Subfamily 2A represents fungal and yeast MATES, 2B represents plant MATES, 2C comprises animal MATES and 2D consists of protozoal MATES. Family 3 has been allocated to bacterial and archaeal MATES (103, 105).

Table 3. Distribution of MATE efflux proteins and their substrates

Microorganism	MATE Efflux Pump	Multidrug Resistance	Reference
Vibrio parahaemolyticus	*NorM*	Ciprofloxacin, Berberine, Acriflavine Streptomycin, Doxorubicin, Trimethoprim	(105, 109)
P. aeroginosa	*PmpM*	Norfloxacin, Ciprofloxacin, Ofloxacin, Fradiomycin, Chlorhexidine gluconate,	(111)
Haemophilus influenzae	*HmrM*	Norfloxacin, Streptomycin, Doxorubicin, Daunomycin, Trimethoprim, Acriflavine, Berberine	(112)
Escherichia coli	*YdhE*	Ciprofloxacin, Acriflavine, Streptomycin, Berberine, Kanamycin, Doxorubicin,Daunomycin, Trimethoprim	(105)
Brucella melitensis	*NorM*	Ciprofloxacin, Gentamycin, Acriflavine, Berberine	(113)
Staphylococcus Aureus	*MepA*	Norfloxacin, Ciprofloxacin, Acriflavine, Cimitidine, Rhodamine, Chlorhexidine	(114, 115)
Acinetobacter baumannii	*AbeM*	Kanamycin, Gentamycin, Ofloxacin, Ciprofloxacin, Erythromycin, Doxorubicin, Chloramphenicol, Daunomycin, Trimethoprim, Rhodamine	(116)
Clostridium difficile	*CdeA*	Acriflavine	(117)
Vibrio cholerae	*VcmA, VcrM, etc*	Ciprofloxacin, Ofloxacin, Acriflavine, Streptomycin, Kanamycin, Chloramphenicol	(118)
Bacteroides thetaiotaomicron	*BexA*	Ciprofloxacin, EtBr, Reserpine, Verapamil,	(119)
Saccharomyces cerevisiae	*Erc1*	Ethionine	(120)
Erwinia amylovora	*NorM*	Norfloxacin, Ciprofloxacin, Ampicillin, Phloretin Kanamycin, Methylene blue, Berberine,	(121)

6.1. Bacterial MATEs

Seventeen MATE transporter proteins have been recognized in bacteria which are implicated in the development of MDR against clinically important drugs (105-107). MATE transporter proteins confer resistance to numerous cationic drugs including norfloxacin, berberine, acriflavine and ethidium bromide. Thus it has been concluded that bacterial MATE multidrug transporters express elevated selectivity for the transport of organic cations. It is clear from the literature that overexpression of MepA, a MATE transporter protein in *S. aureus*, elicits resistance to tigecycline, which is a clinically vital antibiotic against vancomycin resistant *S. aureus* (VRSA) as well as methicillin resistant *S. aureus* (MRSA) (108). The efflux of organic cations across the plasma membrane in eight MATE transporters, including NorM in *V. parahaemolyticus*, are understood to be coupled to a Na^+ motive force mediated either by a primary Na^+ pump or a Na^+-H^+ antiporter coupled with respiration. On the other hand, the activity of AbeH and PmpM pumps in the plasma membrane are coupled to a H^+ motive force. Thus it can be concluded that the currently known bacterial MATE proteins are either Na^+ coupled or H^+ coupled secondary antiporters of organic cations although the mechanism involved in the transport of organic cations has yet to be fully elucidated (109).

6.2. Fungal MATEs

Erc1 is the only representative MATE efflux pump reported in the yeast strain *Saccharomyces cerevisiae* (110). This efflux pump confers resistance against numerous antimycotic drugs and is implicated in the vesicular storage of S-adenosylmethionine.

6.3. Mammalian MATEs

Two types of genes encoding MATE 1 and MATE 2, which are closely located on chromosome 17, have been identified in humans (109). MATE 1 is distributed throughout the entire body, but mainly in the brush border membranes of the kidneys and bile canaliculi of the liver. It is positioned on the plasma membrane and mediates H^+ coupled transport of various substrates including organic cations. The second transporter, MATE 2, is exclusively expressed in the kidneys localized in the brush border membranes (109) and it transports a variety of organic cations including cimetidine, N-methylnicotinamide (NMN) and metformin by means of H^+ exchange (105).

In bacteria, fungi and protozoa, inhibitors of MATE transporters may well reduce the emergence of MDR strains against antimicrobial/antifungal/antiprotozoal drugs in addition to toxic organic cations. Furthermore, in mammalian cells, either inhibitors or substrates of MATE efflux pumps are likely to diminish the extrusion of xenobiotics and metabolic products thus raising their intracellular concentrations. Hence, cimetidine, a substrate of the MATE efflux pump, restrains the excretion of several cationic drugs like procainamide causing significant clinical toxicity. Consequently further comprehensive studies regarding the structure and characteristics of MATE transporters are needed for a deeper understanding of their pharmacological, toxicological and physiological significance (105).

7. RESISTANCE NODULATION DIVISION (RND) SUPERFAMILY

Gram-negative bacterial strains tend to be inherently resistant to antibacterial drugs. This feature has been attributed to the existence of an additional membrane which acts as an efficient permeability barrier. Consequently, the influx of hydrophobic drugs is impaired via porin channels due to a larger molecular size than common nutrient molecules (122). Furthermore, lipophilic drug entry through these channels is countered by the extremely ordered nature of the water molecules inside these channels. Thus, these molecules diffuse very slowly across the lipid bilayer of the external membrane lipopolysaccharide outer leaflet which has very little fluidity (123). The discovery of RND multidrug efflux pumps (i.e. AcrAB of *E. coli*) confounded the simple and fixed idea which was based completely on the outer membrane permeability barrier (124). Subsequently, gene sequencing and cloning established that AcrAB is coded for an active efflux pump of the RND super-family and is responsible for the efflux of a variety of drugs (10). RND transporter proteins are typically present in Gram-negative bacteria. However, their presence has also been noted in Gram-positive bacteria, eukaryotes and archaea (125). RND transporter proteins are tripartite efflux systems coupled to a drug-proton antiporter (126). These multidrug transporter proteins are also associated with two other proteins viz. another periplasmic membrane fusion protein (MFP) and an outer membrane channel forming protein in such a way that drug efflux is facilitated (127). The proteins forming these transporters are composed of 12 transmembrane segments (TMS) having two bulky periplasmic loops that exhibit substrate specificity (128).

The MDR transporters MexB and AcrB, belonging to the RND super-family have been documented in the Gram-negative bacteria *P. aeroginosa* and *E. coli* respectively (129). RND type efflux pumps have extremely wide substrate specificities and afford safety to bacterial cells from the injurious effects of numerous drugs. Major groups of antibiotics that are effluxed by RND transporter proteins include cephalosporins, β-lactam antibiotics, β-lactamase inhibitors, fluoroquinolones and macrolides (130). Genomic sequencing of *P. aeroginosa* has revealed the presence of 12 RND transporter proteins (127) although only 6 efflux pumps have been structurally and functionally characterized. Among these, the first one (MexAB-OprM), is implicated in the efflux of β-lactam antimicrobials, fluoroquinolones, macrolides, tetracyclines, chloramphenicol, triclosan and

trimethoprim (127). Likewise, a third RND transporter system (MexEF-OprN) is associated with the efflux of fluoroquinolones, aromatic hydrocarbons, trimethoprim and chloramphenicol (127, 131). Additionally, MexXY has been shown to extrude aminoglycosides, fluoroquinolones, erythromycin and tetracyclines, whilst MexJK and MexEF-OpmD transport less commonplace substrates (131). RND efflux pumps are also acknowledged in other bacteria whose cell wall characteristics are similar to *P. aeruginosa*. An example is *Burkholderia cepacia,* a plant and human pathogen which is extremely resistant to antibiotics like fluoroquinolones and chloramphenicol primarily due to the presence of RND efflux pumps (132). *Burkholderia pseudomallei* the primary cause of meliodosis, is also highly resistant to macrolides and aminoglycosides owing to the presence of RND transporters (133). Furthermore, *Stenotrophomonas maltophilia*, a widespread cause of nosocomial infections particularly in immunocompromised individuals, is extremely antibiotic resistant due to the presence of RND efflux proteins (134).

Apart from the less permeable outer membrane present in Gram-negative bacteria, RND efflux pumps are major contributors to the development of MDR. Detailed studies of these transporters in pathogenic microbes is of major clinical significance because they are expressed in clinical isolates and involved in the extrusion of valuable drugs. Although RND transporters have been examined to a high degree, a more profound understanding of their physiological functioning, control of their expression and outer membrane channel protein features are warranted. The study and development of broad spectrum RND pump inhibitors will inevitably decrease the emergence of intrinsic resistance to clinically important drugs (135). Therefore, inhibition of efflux pumps is a notable stratagem to enhance the efficacy not only of available antimicrobials, but also anticancer agents and this will certainly involve continuing research time and investment.

8. BACTERIAL EFFLUX PUMP INHIBITORS (EPIs)

In order to curtail MDR, an imperative strategy is to employ efflux pump inhibitors (EPIs) with the capability of counteracting resistance mechanisms in order to potentiate the efficacy of clinically available drugs. In applying this idea, a drug would be co-administered with an inhibitor agent capable of offsetting the resistance thus rendering the drug effective even against previously resistant microbes (136, 137) or cells. Another approach already in use, is the combination of drug with an inhibitor of its inactivating enzyme(s) a classic example being amoxicillin co-administered with the β-lactamase inhibitor clavulanic acid.

Efflux pumps are potentially important antimicrobial targets and the discovery of safe and effective EPIs for bacterial, fungal or cancerous cells is without doubt, a worthwhile priority. Such inhibitors are likely to reduce the activity of MDR strains, decrease the intrinsic resistance of microbes and restrict/limit acquired resistance allied to target mutations. EPIs may perform their function mechanistically for example, by chemical alteration of substrates and/or disruption of binding with their respective transporters thereby hindering efflux. It may not be unreasonable to speculate that these binding processes might either be competitive or non-competitive in nature and EPI affinity for substrates leading to a drug-EPI complex may conceivably restrict extrusion through expansion of molecular size. Putative EPIs might also give rise to exhaustion of the energy necessary for efflux transport by inhibiting either the proton gradient or through inhibition of ATP binding.

9. EFFLUX PUMP INHIBITORS FROM NATURAL SOURCES

Plants are a potential source of new medicinal compounds including EPIs (138, 139). Reserpine **(1),** an antihypertensive plant alkaloid is known to inhibit BMR efflux pump-mediated multidrug-resistance and restrain the function of P-gps (140, 141). As an inhibitor agent, reserpine has been shown to potentiate the antibacterial effect of fluoroquinolones against MDR bacterial strains and reduce the emergence of resistant variants of *S. aureus* and *S. pneumonia* (142, 144). Reserpine has also been reported to inhibit the MDR efflux pump (LmrA) in *Lactococcus lactis* and augment the antimicrobial efficiency of tetracycline against methicillin resistant strains of *S. aureus* (MRSA) (145). However, as an EPI, the effective concentration of the drug required for laboratory experiments is too high to be clinically applicable. Two phenolic compounds, epigallocatechin gallate **(2)** and epicatechin gallate **(3)** obtained from green tea, have not only been shown to be antimicrobial but also to offset the resistance of MRSA strains and inhibit the activity of P-gps (146). As EPI's, these compounds correspondingly reversed TetB and TetK mediated *S. aureus* resistance against tetracycline as well (147). They additionally potentiated the antibacterial effect of norfloxacin against *S. aureus* via NorA pump inhibition although their inhibitory action on ethidium bromide efflux was moderate (148). Likewise, some diterpenes, namely isopimarine **(4a,b,c,d)** and carnosic acid **(5)**, obtained respectively from *Rosmarins officinalis* and *Lycopus europaeus*, have been evaluated as EPIs. These compounds inhibited the ABC type efflux pump (MrsA) in macrolide resistant *S. aureus* strains and potentiated the antibacterial effect of erythromycin (149, 150).

Another alkaloid, berberine **(6)**, isolated from *Berberis fremontii* possesses inhibitory activity on the MDR efflux pump present in *S. aureus* (Stermitz, *et al.* (151). In fact, it transpired that berberine by itself exhibited only weak antibacterial activity against *S. aureus* (MIC = 256 mg/L). However, a flavonolignan compound (5-methoxy hydnocarpin-D) also present in *B. fremontii*, acted synergistically with berberine resulting in a sixteen fold reduction in the berberine MIC and this combination also reversed NorA mediated resistance to norfloxacin (151). A number of methoxylated flavones and isoflavones isolated from Berberis species have also been shown to inhibit the MDR NorA efflux pump in the presence of sub-therapeutic concentrations of berberine and norfloxacin (152, 153). Moreover, genistein **(7)**, orobol **(8)** and biochanin A **(9),** which are isoflavones found in

Lupinus argenteus also reduce the MICs of berberine and norfloxacin by 16- and 4-fold factors respectively against *S. aureus* (154). Similarly, chrysosplenol-D **(10)** and chrysoplenetin **(11)**, two flavones acquired from *Artemisia annua,* have been shown to potentiate the anti-malarial effect of *Artemisia annua* against the parasite *P. falciparum* (155)

Phenolic compounds isolated from organic extracts of *Dalea versicolor* (Fabaceae) have been found to enhance the antimicrobial activity of erythromycin, tetracycline and even berberine against *S. aureus.* One of the isolated compounds in particular, chalcone **(12)**, induced a reduction in MICs to levels comparable to those observed in NorA knock out mutants, signifying that it is an inhibitor of the NorA efflux pump (156). In the same study, both chalcone **(12)** and stilbene **(13)** also enhanced the antimicrobial effect of erythromycin and tetracycline against *Bacillus cereus*. In another investigation by the same research group, arylbenzofuran aldehyde (spinosan A) **(14)**, isoflavone **(15)** and pterocarpan **(16)** obtained from *Dalea spinosa* (smoke tree), potentiated the inhibitory activity of berberine against *S. aureus* lowering MICs by factors of between 4-8 times (157). These agents enhanced the antimicrobial efficacy of berberine against knockout isogenic efflux mutants for the NorA pump and caused a 2-15 fold reduction in MICs. However, these compounds were not effective against NorA over-expressing mutants indicating that the activity of these compounds is not exclusively related to the NorA efflux pump of *S. aureus*.

In 2005, Marquez *et al.,* isolated and characterized a penta-substituted pyridine, 2,6-dimethyl-4-phenyl-pyridine-3,5-dicarboxylic acid diethyl ester **(17)** from *Jatropha elliptica* (158). This compound was tested against MsrA and NorA efflux pumps in *S. aureus* and it was confirmed as an EPI against NorA with the capability to restore intracellular antibiotic concentrations. Others have examined jatrophane diterpene polyesters **(18)** isolated from the Euphorbiaceae family as potential inhibitors of MDR. It was revealed that overproduction of permeability P-gp, acting as an energy-dependent efflux transporter in mouse lymphoma cells, was concentration-dependently inhibited by these compounds and this was likely to reduce the intracellular accumulation of anticancer drugs (159, 160).

In another study on Mexican Morning Glory species, three oligosaccharides in particular, orizabin XIX **(19)**, orizabin IX **(20)** and orizabin XV **(21)** were isolated along with a range of 19 others (161). These compounds were notable in that they potentiated the activity of norfloxacin against the NorA over-expressing *S. aureus* strain, SA-1199B. Out of the three compounds, amphipathic orizabin XIX augmented the activity of norfloxacin instigating a 4-fold decrease in MIC, whereas, orizabin IX enhanced norfloxacin activity 16-fold. In an ethidium efflux inhibition assay conducted on SA-1199B, it was further disclosed that orizabin IX and orizabin XV were of equivalent potency. In all instances, these compounds were more potent than reserpine and as such, they were considered to possess a prospective capacity as EPIs.

Two abietane diterpenes, namely carnosol **(22)** and carnosic acid **(23)** isolated from *Rosmarinus officinalis*, have been found to boost the activity of erythromycin and tetracycline versus Tet(K) and Msr(A) transporters in *S. aureus* (162). Interestingly, carnosic acid markedly augmented the antimicrobial activity of erythromycin against an erythromycin effluxing staphylococcal strain and both compounds enhanced the activity of tetracycline against *S. aureus*. Furthermore, carnosic acid had an inhibitory effect in the ethidium efflux assay but this was thought in all probability, to derive from a NorA-independent pump in *S. aureus*.

A trihydroxy flavone, baicalein **(24),** isolated from *Thymus vulgaris* has been reported to synergize the activity of tetracycline and three β-lactam antibiotics against MRSA (163). Baicalein also induced a 4-fold decline in the MIC of tetracycline against another MRSA isolate, OM584. Expression of an *S. aureus*-derived tet(K) gene into *E. coli* resulted in up to a 16-fold MIC increase, but baicalein reduced this by a factor of four times. In contrast, there was also a decrease in MIC detected in an *E. coli* strain not expressing the Tet(K) efflux transporter protein. Thus, the MRSA isolate OM481, does not possess the Tet(K) protein suggesting that baicalein may inhibit another MDR pump in this MRSA isolate or at least exhibit more than one mechanism of action, such as interference with cell wall integrity.

A biological investigation of grape fruit oils isolated from *Citrus paradisi*, led to the isolation and characterization of three compounds: a coumarin derivative **(25)**, coumarin epoxide derivative **(26)** and a bergamottin epoxide **(27)** derivative 164). These compounds were all found to be modulators of efflux pumps in MRSA strains and enhanced the activities of norfloxacin and ethidium bromide causing a 2-6 fold reduction in MICs. *Piper longum* and *Piper nigrum* which belong to the Piperaceae family contain the plant alkaloid piperine **(29)**. In effect, it has been revealed that piperine enhances ciprofloxacin accumulation in *S. aureus* leading to a 2-4 fold reduction in MIC. Moreover, this alkaloid was also able to decrease the MIC of ciprofloxacin against the MRSA strain 15187, by a factor of 2 and the outright similarity of piperine's action profile with known EPIs, indicates that it is yet another P-gp inhibitor like reserpine for example (165). It is noteworthy overall, that the antibacterial and efflux pump inhibitory portfolios of many of these natural products outlined so far, make them interesting potential targets for synthesis and may represent promise as therapeutic agents.

10. CONCLUSIONS

Efflux mediated MDR is now an established clinical dilemma, rendering various antibiotics superfluous and some clinicians speculate justifiably that we are facing distressing infections reflective of the pre-antibiotic era. Even though very effective antimicrobial drugs have been developed, microbes have evolved new protective and acquired resistance coping

mechanisms and the emergence of MDR pathogens is a global health problem. As a consequence, several infectious diseases have become intractable, a phenomenon commonly attributed to inappropriate and indiscriminate use of antimicrobial agents. Undoubtedly, modern antimicrobial drugs target specific mechanisms and as result, antibiotic resistant microbes and resistant genes appear. Generally, microorganisms, including bacteria, adapt to environmental pressures via different mechanisms and their response towards antibiotic effects is no exemption. Unfortunately, the discovery of novel drugs and molecular targets has been limited and is inadequate in fulfilling the current need for effective antimicrobial drugs. Recognition of novel and susceptible drug targets is a demanding process and it appears that there will inevitably be a reliance on existing agents at least for the foreseeable future. Furthermore, owing to the emergence of MDR pathogens, maintenance of the currently available drugs in an efficacious state is a major challenge to the scientific community. Plant extracts are a natural source of lead drugs against numerous diseases and have been extensively studied by researchers (166-169). Development of new antimicrobial and anticancer drugs with novel modes of action is a prerequisite in the control of MDR pathogens and neoplasias and this includes natural products (170, 174). One alternative approach is to identify molecules capable of interfering with the process of drug efflux. Despite ongoing research, both in academic institutions and the pharmaceutical industry to discover new EPIs, there is no EPI-antibiotic combination available on the market even though this need is well recognized these days. Some success has been accomplished in the development of resistance modifying agents through P-gp MDR efflux pumps in mammalian cancer cells, but much more needs to be done (175).

Several groups of researchers are working on natural products to identify EPIs but their findings are at an emergent stage and are limited to preliminary potentiation and efflux inhibition assays. Further, biochemical studies regarding decisive confirmation of EPI-efflux pump interactions, toxicity and pharmacokinetic studies are required for the development of potential EPIs. Limiting factors in the development of EPIs involve high costs, a considerable time outlay and conceivable toxicity associated with their use at effective concentrations. Like reserpine, a plant alkaloid, development as an EPI was stopped due to its neurotoxic effects at concentrations required to inhibit the *S. aureus* NorA efflux pump (176) Nevertheless, the chemical diversity of compounds in medicinal plants makes them an attractive option for researchers.

This review has highlighted the diversity of efflux pumps in prokaryotic and eukaryotic cells along with their role in MDR development and a particular focus has been placed on plant-derived EPIs. The findings with a number of such molecules are extensive, but they require further investigation for lead optimization. In order to select a compound that could act as an EPI or synergistically interact with a drug, it is important to recognize the range of molecular mechanism(s) of the drug in the presence and absence of an EPI. Benefit may be maximized when the pharmacokinetics of a natural compound/EPI plus an antibiotic combination occurs in the absence of any major side effects. The optimum drug-EPI ratio and dosing regimen must be studied for optimum efficacy and minimum toxic effects. Genetically modified animal models and knock out microbial strains can be utilized to define both kinetic as well as dynamic targets and this should be followed up by carefully designed clinical trials. Recent advances in proteomics, metabolomics and genomics provide a platform for researchers to determine the mode of action and synergistic interactions of natural products with other drugs. An important issue when identifying a potential EPI is to ensure that the activity stems from the efflux pump rather than any other antimicrobial activity although complementary effects would be an undoubted bonus.

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Abbreviations: AAHS (Aromatic acid H^+ symporter), ABC (ATP binding cassette), ACS (Anion-cation symporter), ATP (Adenosine tri-phosphate), BCRP (Breast cancer resistance proteins), EPIs (Efflux pump inhibitors), FGHS (Fructose-glucose-galactose H^+ symporters), MATE (Multidrug and toxic compound extrusion), MCP (Monocarboxylate porter), MDR (Multiple drug resistance/resistance), MFS (Major facilitator superfamily), MHS (Metabolites H^+ symporters), MRP (Multidrug resistance protein), MRSA (Methicillin resistant *Staphylococcus aureus*), NHS (Nucleoside H^+ symporter), NNP (Nitrate-nitrate porter), OFA (Oxalate/formate antiporter), OHS (Oligosaccharide H^+ symporters), OPA (Organophosphate antiporters), P-gp (p-glycoprotein), PHS (Phosphate H^+ symporter), POT (Proton dependent oligopeptide transporter), RND (Resistance nodulation

division), SDR (Single drug resistance/resistant), SHS (Sialate / H^+ symporter), SMR (Small multidrug resistance), SP (Sugar porter), UMFS (Unknown major facilitator superfamily).

Key Words: Efflux pumps, EPIs, Natural products, Chemo-resistance, MDR, Infectious Diseases, Review

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