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Microbial Efflux Pump Inhibition: Tactics and Strategies

George P. Tegos^{1,2,3,4,*}, Mark Haynes¹, J. Jacob Strouse¹, Mohiuddin Md. T. Khan¹, Cristian G. Bologa^{1,5}, Tudor I. Oprea^{1,5}, and Larry A. Sklar^{1,2}

¹Center for Molecular Discovery, University of New Mexico, Albuquerque, NM 87131

²Department of Pathology, University of New Mexico, Albuquerque, NM 87131

³Wellman Center for Photomedicine, Massachusetts General Hospital

⁴Department of Dermatology, Harvard Medical School

⁵Division of Biocomputing, University of New Mexico Health Sciences Center, Albuquerque, NM 87131

Abstract

Traditional antimicrobials are increasingly suffering from the emergence of multidrug resistance among pathogenic microorganisms. To overcome these deficiencies, a range of novel approaches to control microbial infections are under investigation as potential alternative treatments. Multidrug efflux is a key target of these efforts. Efflux mechanisms are broadly recognized as major components of resistance to many classes of chemotherapeutic agents as well as antimicrobials. Efflux occurs due to the activity of membrane transporter proteins widely known as Multidrug Efflux Systems (MES). They are implicated in a variety of physiological roles other than efflux and identifying natural substrates and inhibitors is an active and expanding research discipline. One plausible alternative is the combination of conventional antimicrobial agents/antibiotics with small molecules that block MES known as multidrug efflux pump inhibitors (EPIs). An array of approaches in academic and industrial research settings, varying from high-throughput screening (HTS) ventures to bioassay guided purification and determination, have yielded a number of promising EPIs in a series of pathogenic systems. This synergistic discovery platform has been exploited in translational directions beyond the potentiation of conventional antimicrobial treatments. This venture attempts to highlight different tactical elements of this platform, identifying the need for highly informative and comprehensive EPI-discovery strategies. Advances in assay development genomics, proteomics as well as the accumulation of bioactivity and structural information regarding MES facilitates the basis for a new discovery era. This platform is expanding drastically. A combination of chemogenomics and chemoinformatics approaches will integrate data mining with virtual and physical HTS ventures and populate the chemical-biological interface with a plethora of novel chemotypes. This comprehensive step will expedite the preclinical development of lead EPIs.

Keywords

Multidrug resistance; efflux pump substrates and inhibitors; natural antimicrobials; high-throughput screening

MICROBIAL MULTIDRUG EFFLUX SYSTEMS; OVERVIEW

Multidrug resistance (MDR) has expanded dramatically in a broad range of organisms from bacteria to humans resulting in a global increase in life threatening infections and deaths [1]. There is a high medical need to systematically explore the etiology and principles as well as to devise strategies leading to implementation of effective countermeasures. Efflux mechanisms are broadly recognized as major components of resistance to many classes of chemotherapeutic agents as well as antimicrobials. Efflux occurs due to the activity of membrane transporter proteins widely known as multidrug efflux systems (MES). They are implicated in a variety of physiological processes other than efflux and identifying natural substrates and inhibitors is an active and expanding research discipline [2–4].

MES perform essential roles in cellular metabolism and activity. They differ in membrane topology, energy coupling mechanisms, and most importantly in substrate specificities [5]. Based on their sequence similarity, they are classified into six super-families including; ATP-binding cassette super family (ABC), major facilitator super family (MFS), resistance-nodulation cell division super family (RND), small multidrug resistance family (SMR), multi-antimicrobial extrusion protein family (MATE), and multidrug endosomal transporter (MET) (Fig. 1) The first five families are found in microorganisms (the MET family appears to be restricted to higher eukaryotes), but representatives of all groups are also expressed in mammalian cells [6]. ABC transporters comprise the largest super family, with seven subfamilies that are designated A to G based on sequence and structural homology [4]. The best-studied families of fungal MES are those from *Sacharomyces cerevisiae*. The group of *S. cerevisiae* ABC transporters most closely associated with drug resistance is the pleiotropic drug resistance (PDR) subfamily. There are 28 ABC transporter genes in *S. cerevisiae*, and 9 of these encode PDR transporters (between them PDR5, PDR10, PDR15, SNQ2, and YOR1 as well as the hexose transporter genes HXT9 and HXT11) that are highly conserved among pathogenic fungal species [7–9]. A challenging clinical scenario involves MES in *Pseudomonas aeruginosa* [10]. Although sequence analysis of the *P. aeruginosa* genome has revealed the presence of MES from all five super families, the largest number of predicted pumps, with a total of 12, belong to the RND family [11].

Crystal structures of MFS and ABC transporters have been resolved (Fig. 2) in a range of organisms (e.g. Multidrug Resistance Protein D (EmrD) [12], Lactose Permease [13] and the Glycerol-3-Phosphate Transporter [14–17]). Recently, advances have been made for the RNDs crystal structures of the components of i) Ac-rAB-TolC from *Escherichia coli* [18–20] ii) MexAB-OprM from *P. aeruginosa* - MexB [21], OprM [22], and MexA [23] iii) VceC from the VceCAB (VCE) in *Vibrio cholerae*, [24] and iv) CusA from CusCBA a member from the heavy-metal efflux (HME) subfamily [25]. Furthermore, the assembled structure of a complete tripartite bacterial RND multidrug efflux pump has been published [26]. There is a special emphasis in the structure of transcriptional regulation systems such as the MexR [27] and MexZ [28], TTgR, which regulates ttgABC, a key system in *P. putida* DOT-T1E [29, 30], BldR, a member of the multiple antibiotic resistance regulator (MarR) protein family from *Sulfolobus solfataricus* [31] and SmeT, the repressor of the *Stenotrophomonas maltophilia* system SmeDEF [32]. Significant progress has also been made in MATE structural insights [33, 34].

For the last two decades the pieces of the efflux puzzle have been gradually coming together. A wide variety of MES mutants and MES-associated functional efflux assays have been used to identify both substrates and inhibitors, and efforts at translational studies as well as clinical implementation of lead molecules and therapies based on efflux inhibition have been described. In this report, we assemble a portion of these innovative pieces and we highlight the principals for future discovery strategies aimed at MES.

THE QUEST FOR NATURAL SUBSTRATES - RATIONALE

It has been suggested that amphipathic cations represent the natural MES substrate pool [35]. Specifically, SMR family members expressed by Gram-positive bacteria efflux amphipathic cations exclusively [36, 37, 38] as do most MFS members. For example, the NorA pump of the human pathogen *Staphylococcus aureus* extrudes cations, quinolones, biocides and dyes [39–41]. The BMR pump of *Bacillus subtilis* primarily extrudes cations and neutral chloramphenicol [42, 43]. Furthermore, the RND super-family has a broad substrate spectrum that includes, apart from antibiotics, amphipathic cations, biocides, dyes, and steroid hormones [44–49]. This substrate pattern is similarly observed in ABC-transporters expressed by a wide range of organisms [50–51]. Thus, the need to protect a cell from amphipathic cations has evolved in different families of MES across different organisms despite a lack of overall molecular homology or similarity in their mechanism of action. Quite surprisingly, one does not find amphipathic cations in a general list of natural antimicrobials. The known cationic antibiotics of the aminoglycoside group such as streptomycin and kanamycin are hydrophilic substances that get trapped in the cell via specific translocases [52] and are not general MES substrates.

A series of studies were directed at finding the basis of Gram-negative bacterial resistance to plant antimicrobials. In *Rhizobium etli*, there is an operon activated by a number of plant phytoalexins. This operon appears to code for an RmrAB MES [53]. Expression of a mutant *rmrAB* resulted in diminished root colonization, and a 30% increase in susceptibility to the phytoalexins, naringenin and coumaric acid. The soybean antifungal phytoalexin coumestrol induces LfeAB MES expression in *Agrobacterium tumefaciens* [54]. Mutation in this MES increases the accumulation of coumestrol in the pathogen, and the mutant was out-competed by wild type in colonizing the plant. Interestingly, neither wild type nor mutant was sensitive to coumestrol. In a slightly different approach, a panel of plant antimicrobials was tested using a combination of efflux mutants and EPIs on a set of bacteria that represent the main groups of plant and human pathogens [55]. One of the main observations was the strong potentiation of antimicrobial action in strains with disabled MES. The activity of the majority of plant antimicrobials was considerably greater against the Gram-positive bacteria *S. aureus* and *B. megaterium*. Disabling MES in Gram-negative species led to a striking increase in antimicrobial activity. For instance, the activity of the anthraquinone rhein, the principal antimicrobial from rhubarb [56], was potentiated 100–2000-fold depending on the bacterial species. A similar effect was observed with plumbagin [57], resveratrol, gossypol, coumestrol, and berberine. Direct measurement of the uptake of berberine confirmed that MES inactivation significantly increased berberine accumulation into Gram-negative bacteria.

The outer-membrane protein-encoding gene *tolC* expressed by the bacterial plant pathogen *Erwinia chrysanthemi* EC16 has been identified and characterized [58]. *E. chrysanthemi* *tolC* plays an important role in the survival and colonization of the pathogen in plant tissue conferring resistance to plant antimicrobials. It was established that combination of the signaling molecule in local and systemic plant resistance to salicylic acid and its precursors, *t*-cinnamic acid and benzoic acid, can activate expression of specific MES-encoding genes in *E. chrysanthemi* and enhance survival of the bacterium in the presence of model as well as plant-derived antimicrobials [59]. The *E. chrysanthemi* gene functionally complements the *E. coli* *tolC* gene in MES. A *tolC* mutant of *E. chrysanthemi* is extremely sensitive to an array of plant-derived chemicals including berberine, rhein, plumbagin, pyrithione, genistein (4,5,7-trihydroxyisoflavone), *p*-coumaric acid and *t*-cinnamic acid (phenolic acids), (2-mercaptopyridine-1-oxide) and esculetin (6,7-dihydroxycoumarin). This mutant is unable to grow *in planta* and its ability to cause plant tissue maceration is severely compromised. Similarly, two host-induced *Ralstonia solanacearum* genes, *acrA* and *dinF* encode for MES

that contribute to the overall aggressiveness of this phytopathogen by protecting the bacterium from the toxic effects of host antimicrobial compounds [60].

EPI-DISCOVERY, PROOF OF PRINCIPAL

Discovery of EPIs is a promising approach to deal with MES that may improve clinical performance of antibiotics and chemotherapeutic agents [61, 62]. The potential for developing broad acting EPIs is exemplified by reserpine that effectively inhibits both bacterial and mammalian ABC-system P-glycoprotein (P-gp) [63–65], as well as by biricodar (VX-710), timcodar (VX-853) [66, 67] and verapamil [68, 69] (Fig. 3). EPI activity can be verified *ab initio* by testing the combined action of an EPI compound with a MES substrate added at a sub-inhibitory concentration. The NorA inhibitors 5-nitro-2-phenylindole (INF55) and diphenyl urea INF271 [70] (Fig. 3) were identified by screening a synthetic chemical library using ethidium bromide as a substrate.

Other approaches involve the discovery of EPIs from natural sources through bioassay-driven purification and structural determination. Several *Berberis* medicinal plants (*Berberis repens*, *B. aquifolia*, and *B. fremontii*) that produce the plant antimicrobial berberine also synthesize 5'-methoxyhydrnocarpin (5'-MHC), an inhibitor of *S. aureus* NorA [71]. This discovery intensified the search for plant-derived EPIs [72]. Using the berberine efflux assay, investigators discovered a number of EPIs in various plant sources that exhibit activities similar to 5-MHC. Bioassay purification from various plant sources yielded a number of EPIs acting against Gram-positive bacteria with activities similar to 5-MHC, as detected in the berberine efflux assay. The list includes pheophorbide a from *Berberis* sp., and *Mahonia* [73, 74], crysopenol and crysopenetin from *Artemisia annua* [75], polyacylated neohesperidosides from *Geranium caespitosum* [76], chalcones and a stilbene from *Dalea versicolor* [77, 78]. Additionally, isoflavones such as genistein, orobol, and biochanin also exhibit EPI activity against the NorA pump of Gram-positive bacteria [79].

Similar natural product screening and synthesis campaigns have led to the identification of additional NorA EPIs. The list includes catechin gallates [80], the resin glycosides and tetrasaccharide agents of *Ipomoea murucoides* [81], polyacylated oligosaccharides from the medicinal Mexican morning glory species [82], N-caffeoylphenalkylamide derivatives [83], citral derived amides [84] kaempferol glycoside from *Herissantia tiubae* (Malvaceae) [85] and a set of plant-based alkaloids against methicillin-resistant *S. aureus* [86]. A series of plant phenolic compounds have been functioning as ethidium bromide EPIs in *Mycobacterium smegmatis*. [87]. A series of synthetic efforts have been concentrated in paroxetine and femoxetine [88], piperine and fluoroquinolone as the basis for designing structural analogues [89, 90], phenothiazines [91] with emphasis on thioridazine and chlorpromazine (Fig. 3) [92]. In conclusion an array of lead chemotypes both natural products and synthetic compounds are available for further analysis.

NORA AS A MODEL FOR SYNERGISTIC DISCOVERY STRATEGIES

This efflux system has been explored beyond the identification of EPIs with emphasis in combinatorial antimicrobial strategies with high probability of preclinical implementation. A hybrid compound (SS14, Fig. 3) created by combining the plant antimicrobial berberine with the synthetic EPI INF55 is an effective antimicrobial against *S. aureus* including mutant strains that over express NorA [93]. MIC's for SS14 against *S. aureus* are 2–16 times lower than berberine and INF55. The hybrid rapidly accumulates in bacterial cells and shows higher efficacy than vancomycin in a *Caenorhabditis elegans* model of enterococcal infection [93]. SS14 analogues exhibit similar antimicrobial activities [94, 95] suggesting that significant structural changes can be made to these hybrids without adversely affecting their ability to block MES or their antibacterial activity. Such hybrids should have an

advantage over separate compound administration in terms of synchronous or near synchronous delivery to the appropriate bacterial target sites.

Photodynamic therapy (PDT) has recently gained regulatory approval in USA for treating various cancers and age-related macular degeneration [96, 97]. Certain non-toxic photosensitizers (PS) accumulate preferentially in malignant tissues and can be selectively activated by light. A relatively novel application of PDT is in the treatment of localized infections [98]. Clinically used PS for photoinactivation (PDI)/PDT includes phenothiazinium salts, such as toluidine blue O [99] and methylene blue [100]. Phenothiazinium salts, structurally characterized as amphipathic cations, are substrates of microbial MES [101] and specific EPIs have been shown to potentiate antimicrobial photoinactivation both in Gram-positive and Gram-negative bacteria overexpressing MFS and RND efflux systems [102]. NorA specific EPIs dramatically enhanced the phenothiazinium mediated phototoxicity in *S. aureus*. Recently, a similar theme emerged when a set of porphyrin-based PSs were identified as substrates of the ABCG2-breast cancer related transporter [103, 104] even though they have an inconclusive recognition pattern in microbial MFS systems [105]. All the reported studies have found that PDT can kill drug-resistant microbes as easily as their native counterparts [106, 107]. European and US-based companies that currently employ PDT for localized infections include Ondine Biopharma, who gained regulatory approval in Canada (and is in process in U.S.) for endodontics, nasal decolonization and gingivitis and Nomir Medical Technologies (Waltham MA) who have developed a near-infrared light inactivation platform providing evidence for its involvement in microbial efflux mechanisms [108].

RND-BASED EPIs

It has been a challenging task to identify RND-based EPIs basically due to the complexity of MES in Gram-negative bacteria. Phenyl-arginine beta naphthylamide (PAβN, Fig. 3), was identified by assaying an array of synthetic compounds and natural product extracts using *P. aeruginosa* strains over-expressing each of the three MES (MexAB-OprM, MexCD-OprJ, MexEF-OprN) in the presence of levofloxacin. It has been a valuable tool for drug discovery [109, 110]. This venture has explored i) the development of preclinical candidates including strategies for lead optimization [111], ii) activity *in vivo* through the use of alternative scaffolds [112], iii) optimization of potency in the pyridopyrimidine series through the application of a pharmacophore model [113] and iv) extensive structural activity relationships testing toxicity, stability, and solubility [114–117]. Mechanistically, PAβN itself is a MES substrate that acts as a competitive inhibitor [117–120]. It seems that PAβN may recognize and bind to the substrate pocket specific for the potentiated antibiotics. Alternatively, due to a close location of binding site, EPI binding may also generate steric hindrance, impairing antibiotic binding at its affinity site. PAβN has been validated against the AcrAB-TolC in *K. pneumoniae*, *E. coli*, *S. typhimurium* and *E. aerogenes* [121–124], and in multiple homologous systems including *Acinetobacter baumannii* [125], *Campylobacter jejuni* and *Campylobacter coli* [126, 127].

Naphthylpiperazines, most notably 1-naphthylmethyl-piperazine (NMP, Fig. 3), are among the most potent unsubstituted arylpiperazines [128], with a dose-dependent ability to increase the intracellular concentration of several antibiotics [129]. NMP seems to be effective in *A. baumannii* and several *Enterobacteriaceae*, but not in *P. aeruginosa* [129, 130]. The list also includes trimethoprim and epinephrine [131], indole derivatives [132] for the AcrAB-TolC, phenothiazines for the BpeAB-OprB MES in *Burkholderia pseudomallei*. [133], quinoline derivatives in *Enterobacter aerogenes* isolates [134] and *K. pneumoniae* [135], and carbonyl cyanide m-chlorophenyl hydrazone (CCCP) and pantoprazole in *Helicobacter pylori* [136].

FUNGAL EPIs

Yeast EPIs that have been so far identified and characterized belong to five groups: (i) Tacrolimus (FK506) potentiates azoles in strains over expressing the CDR1 and CaMDR genes [137]. (ii) Phenothiazines potentiate ketoconazole in strains overexpressing Pdr5p, Snq2p and Yor1p while also exhibiting intrinsic antimicrobial activity [138]. (iii) Propafenones potentiate azoles and terbinafine in strains lacking Cdr1p and Cdr2p [139]. (iv) Isonitrile and enniatins against *Fusarium* sp Y-53 potentiate cycloheximide or cerulenin in Pdr5p-overexpressing cells [140]. The mode of Pdr5p inhibition by enniatin is competitive against FK506, and its inhibitory activity is more potent with less toxicity than that of FK506. Enniatins exhibit a similar profile as FK506 against mutants of Pdr5p. They were identified after screening a 1.8-million-member designer D-octapeptide combinatorial library for surface-active Pdr5p antagonists. However, enniatins (Fig. 3) did not inhibit Snq2p, a homologue of Pdr5p and they show only modest toxicity against yeast cells [141]. A similar screening process yielded isonitrile 3-(3'-isocyano-cyclopent-2'-enylidene)-propionic acid, a compound whose carboxyl residue is essential for its EPI-activity [140]. (v) *N*-methylpiperazine (quinazolinone derivative) and milbemycin derivatives (Fig. 3) have been characterized as fungal EPIs that enhance fluconazole efficacy in *C. albicans* [142, 143]. Attempts to improve the aqueous solubility of screening hits led to the discovery of an analog with improved physical properties and activity against clinically-relevant *Candida* spp. [144]. The list of fungal EPIs is further expanded with the cyclic peptides unnarmicin A and unnarmicin C that were identified by testing a library of marine microorganisms [145] sulfated sterols from the marine sponge *Topsentia* sp [146], acridone derivatives [147] and ibuprofen [148].

EPIs & BIOFILMS

Since MES are a key resistance factor in microbial cells, it has been suggested that their expression contributes to biofilm persistence [149–153]. The exact role of MES in biofilm growth and their importance in biofilm-mediated antibiotic resistance are, however, elusive, and studies of Gram-negative bacteria have produced somewhat incongruous results. In *E. coli*, the putative multidrug resistance pump YhcQ was reported to be involved in antibiotic resistance of biofilms [154], and the AcrAB-TolC pump has been found to be upregulated under stress conditions, such as stationary-phase growth or, as in the natural habitat of this species, exposure to bile acids [155]. Interestingly, exposure to these agents renders *E. coli* resistant to lipophilic antibiotics, suggesting an upregulation of AcrAB-TolC [155].

In *P. aeruginosa*, the MexAB-OprM and MexCD-OprJ efflux pumps have been shown to be involved in biofilm-specific mechanisms of resistance [156], especially with regards to the macrolide azithromycin [156]. In contrast, it was found that neither pump was up regulated in a developing biofilm [157]. MES expression may be influenced by factors such as growth rate and the accumulation of metabolites. Most probably, biofilm-mediated resistance is achieved through multiple factors, such as slow growth due to deficient nutrition, reduced penetration due to production of protective extracellular polysaccharides, and efficient efflux [158]. Additionally, it was found that the *ndvB* gene, coding for a glucosyltransferase, is a genetic determinant of antibiotic resistance in *P. aeruginosa* [159].

A number of EPIs including thioridazine, NMP, and PAβN reduced biofilm formation, and in combination could abolish biofilm formation completely in MFS (norA) and RND (AcrAB, F, MexAB CD, EF) expressing strains. [160] This synergy hypothesis was successfully applied for the photodynamic inactivation in an *Enterococcus faecalis* biofilm model using TBO and verapamil [161].

FROM TACTICS TO STRATEGIES

The natural role of MES is very complex as they are important for bacterial metabolism, physiology, and pathogenicity [2, 3]. It has been shown that mutant strains of *Salmonella enteric*, serovar typhimurium, *E. coli*, and *C. jejuni* over expressing MES are resistant to high concentrations of bile salts. In addition, over expression of the MtrCDE efflux system in *N. gonorrhoeae* enhances bacterial survival [3, 84]. It has been established that RND MES have a role in invasion, adherence and colonization of the host cell. Therefore, the EPI-approach in some cases might also reduce bacterial virulence *in vivo*. A major obstacle, however, may arise from the fact that MES manipulation may cause unexpected toxicities due to the variety of their physiological roles. In this context, efforts directed at specifically inhibiting efflux pumps operating only in prokaryotes may offer a significantly greater chance of effective therapeutic success. Interestingly, it has been shown that target bacteria respond to clinical challenge with EPIs by developing resistance mutations that decrease the efficacy of the EPI [162, 163]. Recently, it has been demonstrated that reserpine can select multidrug resistant *S. pneumoniae* strains [164]. The threat of cross-resistance to antibiotics elevates the complexity of discovery ventures. To date, the efficacy of EPIs has been demonstrated in the therapy of malignancies *in vitro* and *in vivo*; with clinical trials (Table 1). The only documented microbial EPI is currently MP-601,205 (Fig. 3), used for respiratory infections in patients with cystic fibrosis (CF) and ventilator-associated pneumonia. The compound is an aerosol formulation of an approved drug that functions as an EPI.

Natural sources such as specific plants have a distinct role in the effort to identify lead EPIs as well as potential antimicrobial agents. The natural antimicrobial discovery approach, a process ranging from identifying a hit to isolating a pure compound, has increased over the last decade and is thought of as more than promising [165–169]. Still, there are significant technical bottlenecks. There are a limited number of natural product extract libraries and their analysis typically involves exacting isolation of different components of the extract and subsequent time-consuming spectroscopic identification of the separate compounds. HTS campaigns for EPIs using natural or synthetic libraries also suffers from distinct bottlenecks (variety of representative chemotypes in addition to limited number of compounds tested) as well as considerable disadvantages, such as the lack of defined secondary and tertiary evaluation assays.

An innovative HTS strategy based on a functional EPI assay coupled with a comprehensive secondary validation flowchart is capable of overcoming barriers while being highly informative. The University of New Mexico's Center for Molecular Discovery (UNMCMD) is pioneering the development of cell suspension HTS for EPI discovery utilizing a sensitive multiplex flow cytometry platform. The approach incorporates the Prestwick Chemical Library (PCL, consisting of 1120 FDA approved drugs) along with the Molecular Libraries Small Molecules Repository (MLSMR, http://mlsmr.glp.com/MLSMR_HomePage/). It paved the way for a series of innovations in chemical genetics including novel flow cytometry efflux assays (Fig. 4 and 5) in a set of yeast transporters including ABC (CDR1, CDR2), MFS (MDR1) and vacuolar V-ATPases [170–173]. Moreover this initiative has generated a number of projects targeting cancer MES including dye-substrate profiling and EPI discovery [170]. UNMCMD has initiated a quest for ABC and MFS substrates and inhibitors both in microbial and mammalian systems. We have proposed a hybrid chemogenomics-chemoinformatics discovery platform employing MES from specific organisms, the MLSMR library and HTS flow cytometry in the hope of developing a transporter-ligand interactome. This approach integrates data from genomic, proteomic and medicinal chemistry databases in concert with physical screening campaigns. It provides the rationale to chemically characterize MES substrates and subsequently accelerate the

discovery of potent functional inducers or repressors for transporters as well as lead EPIs. Novel chemical probes will undergo lead optimization and detailed biological characterization. This project attempts to provide “MES signature specific” chemical probes from the MLSMR library to elucidate potential evolutionary relationships and facilitate collaborative ventures in the framework of a broad consortium including European Union Cost Action ATENS, (COST BM0701) and The International Transporter Consortium [174].

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ABBREVIATIONS

MES	Multidrug efflux systems
EPIs	Efflux pump inhibitors
HTS	high-throughput screening
ABC	ATP-binding cassette
MFS	Major facilitator super
RND	Resistance-nodulation
SMR	Small multidrug resistance
MATE	Multi-antimicrobial extrusion
MET	Multidrug endosomal transporter
PDR	Pleiotropic drug resistance
HXT	Hexose transporter
HME	Heavy-metal efflux

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Fig. 1.

Representative members of the five characterized families of MES [3]. The ABC family including ATP-driven multidrug pumps such as P-glycoprotein and LmrA from *Lactococcus lactis*. The MFS consists of secondary transporters driven by chemiosmotic energy and includes proton/drug antiporters such as QacA from *S. aureus*. Both the resistance/nodulation/cell division (RND) and the small multidrug resistance (SMR) families include proton-driven drug efflux pumps such as *E. coli* AcrB and EmrE, respectively. AcrB functions as a multisubunit complex in association with the outer membrane channel TolC and the membrane fusion protein AcrA. The multidrug and toxic compounds efflux (MATE) family consists of sodium ion-driven drug efflux pumps such as NorM from *Vibrio parahaemolyticus*.

Fig. 2.

(a,b) Lateral and axial view of the overlap of the crystal structures of Multidrug Resistance Protein D (red) Lactose Permease (green) and the Glycerol-3-Phosphate Transporter (blue) from MFS family; (c,d) Lateral and axial view of the crystal structure of EmrE multidrug transporter from SMR family (C α atoms only);(e,f) Lateral and axial view of the crystal structure of the MATE transporter NorM from *Vibrio cholerae*. Crystal structures of (g) MexA, (h) MexB, (i) OprM, and (j) a model of the assembly of AcrAB-TolC from RND family; (k,l) Perpendicular views of the crystal structure of the multidrug ABC transporter Sav1866 from *Staphylococcus aureus*.

Fig. 3.
Structures of representative microbial EPIs

Fig. 4.
Duplex format flow cytometric assay for identification of ABCB1, ABCC1 and ABCG2 EPIs

Fig. 5. R6G (**A**) and Nile red (**B**) content of *S. cerevisiae* strain AD/pABC3 (vector only) and strains expressing *C. albicans* Cdr1p (AD/CDR1), Cdr2p (AD/CDR2), or Mdr1p(AD/MDR1) MES genes. Yeast cells were incubated with 15 μ M R6G (**A**) or 7 μ M Nile red (**B**). Enniatin (50 μ M) was added to strains preloaded with substrate and incubated for 20 min. Each bar represents the median $\pm$ standard deviation (n = 3).

Table 1

Panel of Representative EPIs and their Biological Features

Inhibitor	Limitations	Toxicity	MES	Clinical Trials	Reference
reserpine	NR	neurotoxicity	MFS, ABC	No	[175]
INF55	NR	NR	MFS	No	[70]
INF271	NR	NR	MFS	No	[70]
Biricodar (VX-710)	Pharmacokinetic interaction	NR	MFS, ABC	Yes (Pgp)	[176]
Timcodar (VX-853)	NR	NR	MFS, ABC	Site effects	[177]
Phenothiazines	Low potency	NR	MFS, ABC	No	[91, 92, 178–180]
PaβN	Pharmacokinetic interaction	Potential Toxicity	RND	No	[118]
NMP	Pharmacokinetic interaction	NR	RND	No	[129]
MP-601,205	NR	NR	RND	Yes	[118]
Milbemycin	NR	NR	ABC	No	[181]
Verapamil	Low potency	Hypotension	MFS, ABC	Yes	[182]
Enniatins	NR	mitochondrial dysf	ABC	No	[183, 184]

NR, not reported, INF555-nitro-2-phenylindole, INF271, diphenyl urea

Pgp, P-glycoprotein, PaβN, Phenyl-arginine beta naphthylamide, NMP, 1-naphthylmethyl-piperazine