Efflux pumps and antimicrobial resistance: Paradoxical components in systems genomics

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ABSTRACT

Efflux pumps play a major role in the increasing antimicrobial resistance rendering a large number of drugs of no use. Large numbers of pathogens are becoming multidrug resistant due to inadequate dosage and use of the existing antimicrobials. This leads to the need for identifying new efflux pump inhibitors. Design of novel targeted therapies using inherent complexity involved in the biological network modeling has gained increasing importance in recent times. The predictive approaches should be used to determine antimicrobial activities with high pathogen specificity and microbicidal potency. Antimicrobial peptides, which are part of our innate immune system, have the ability to respond to infections and have gained much attention in making resistant strain sensitive to existing drugs. In this review paper, we outline evidences linking host-directed therapy with the efflux pump activity to infectious disease.

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Contents

1. Introduction

Antimicrobial resistance is a condition when microorganisms like bacteria, fungi, viruses and parasites change themselves when exposed to various antimicrobial drugs in such a way that the drug is rendered ineffective. These drugs are being used since 1940s and they have been successful in treating various infections. Long-term usage, inadequate dosage, frequency and evolvability of microbes have resulted in the development of resistance against these drugs. These resistant organisms are the major concern of present world

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Abbreviations

γECS	γ-glutamylcysteine synthetase
ABC	ATP Binding Cassette
ABCB1	ATP Binding Cassette-subfamily B, member 1
AMPs	Antimicrobial peptides
ATP	Adenosine triphosphate
BCRP	Breast Cancer Resistance Protein
CDC	Centers for Disease Control and Prevention
CMV	Cytomegalovirus
CS	Cysteine synthase
EPIs	Efflux Pump Inhibitors
FBA	Flux-balance analysis
GEMs	Genome-scale Metabolic Models
GSH	Glutathione
Gsp	Glutathionylspermidine
H_2O_2	Hydrogen peroxide
HBV	*Hepatitis B virus*
HIV	*Human immunodeficiency virus*
HSV	*Herpes simplex virus*
Li-TryR	*Leishmania infantum* trypanothione reductase
MATE	Multidrug and Toxic Compound Extrusion
MFS	Major Facilitator Superfamily
MRP1	Multidrug Resistance associated Protein 1
MRSA	Methicillin resistant *Staphylococcus aureus*
NADH	Nicotinamide adenine dinucleotide (reduced)
NADPH	Nicotinamide adenine dinucleotide phosphate (reduced)
NBDs	Nucleotide binding domains
PA β N	phenylalanine arginyl β-naphthylamide
PACE	Proteobacterial Antimicrobial Compound Efflux
PMF	Proton motive force
PDR	pleiotropic drug resistance
Pgp	P-glycoprotein
RND	Resistance Nodulation Division
RNS	Reactive nitrogen species
ROS	Reactive oxygen species
SAT	Serine acetyltransferase
SMR	Small Multidrug Resistance
SpdT	Spermidine transport
T(SH)$_2$	Trypanothione
TMDs	Transmembrane Domains
TMH	Transmembrane Helix
TryR	Trypanothione reductase
TryS	Trypanothione synthase
VRE	vancomycin resistant enterococci
WGS	Whole Genome Sequencing
WHO	World Health Organization

as they are difficult to treat due to longer duration of treatment and quite expensive drugs. Infections caused by resistant organisms may also result in relapse of the disease, disability or death since new resistance mechanisms are evolving at a very high rate ("CDC-drug resistance," n.d., "WHO-antimicrobial resistance," n.d.). The mechanism of resistance development may be broadly classified in three categories as in intrinsic resistance, acquired resistance and adaptive resistance. Intrinsic resistance involves such mechanisms, which are intrinsic to microbes, and they act by lowering the concentration of drug inside the cell by either decreasing the uptake of drug or their extrusion by efflux pumps. Here, we will be focusing on various efflux pumps in brief. A resistance resulting from mutation or horizontal gene transfer of resistance genes is referred to as acquired resistance. It may be achieved either by degrading the antimicrobial drug or by modifying and hence protecting the drug target. In some cases, an antimicrobial drug may interact with the other drug or proteins and may result in an induction of a gene thereby causing resistance. Such type of resistance is named as adaptive resistance (Hughes and Andersson, 2017). Of these above mechanisms, resistance mediated by efflux pumps is clinically referred to as the most significant mode of resistance.

Efflux pumps are transmembrane proteins present ubiquitously in plasma membranes of all forms of life. They have been classified in six major families (Fig. 1) namely: the Small Multidrug Resistance (SMR) superfamily, the Proteobacterial Antimicrobial Compound Efflux (PACE) superfamily, the Major Facilitator Superfamily (MFS), the Multidrug and Toxic Compound Extrusion (MATE) superfamily, the Resistance Nodulation Division (RND) family and the ATP (adenosine triphosphate)-Binding Cassette (ABC) superfamily. ABC efflux pumps are primary active transporters utilizing ATP hydrolysis as the energy source for extrusion. SMR, PACE, MATE, MFS and RND pumps acts as secondary active transporters since they use proton motive force (PMF) or sodium ion gradient as their energy source (Munita and Arias, 2016; Spengler and Amaral, 2017).

SMR transporters are the smallest efflux pumps having 100—120 amino acid length and four transmembrane helices (TMH). They generally function as homodimers and are found in bacteria and archaeabacteria. SMR family proteins transport lipophilic compounds and a variety of antibiotics (Bay et al., 2008; Spengler and Amaral, 2017). PACE family proteins are most commonly found in proteobacteria's effluxing various synthetic biocides like chlorhexidine, dequalinium, benzalkonium, proflavine, acriflavine, etc. (Hassan et al., 2015). MFS efflux pumps constitute a large family of transporters, which are ubiquitously expressed in all kingdoms of life. They transport a diverse variety of substrates like ions, oligosaccharides, amino acids, small metabolites, and various antimicrobial agents. These proteins consist of 12 TMH grouped in two six helices thereby forming a dimer like structure with a length of 400—600 amino acid residues (Blanco et al., 2016; Spengler and Amaral, 2017). MATE family or multi antimicrobial extrusion proteins function as drug/sodium or proton antiporters found in bacteria, archaea as well as eukaryotes. The length of the members of this protein family ranges from 400 to 700 amino acid residues with 12 TMH. MATE transporters can extrude various antimicrobial agents like norfloxacin, chloramphenicol, ciprofloxacin, kanamycin, ampicillin, metformin, cimetidine, ethidium and triethylammonium (Kuroda and Tsuchiya, 2009). RND efflux pumps acts as proton/drug antiporters and exists primarily in gram negative bacteria, although they are also found in gram positive bacteria, archaea and eukaryotes. One of the largest superfamily of efflux proteins is that of the primary transporters, ATP binding cassette (ABC) transport proteins, utilizing ATP hydrolysis as their energy source. ABC proteins are also known as orphan proteins as they efflux a large range of molecules like ions, phospholipids, small molecules, antibiotics etc. (Spengler and Amaral, 2017). In this review, we will be discussing about various strategies used to fight with antimicrobial resistant superbugs. We have focused on (Efflux Pump Inhibitors) EPIs and AMPs (Anti-microbial Peptides) and how they can be used for targeting efflux pumps.

2. Antimicrobial resistance

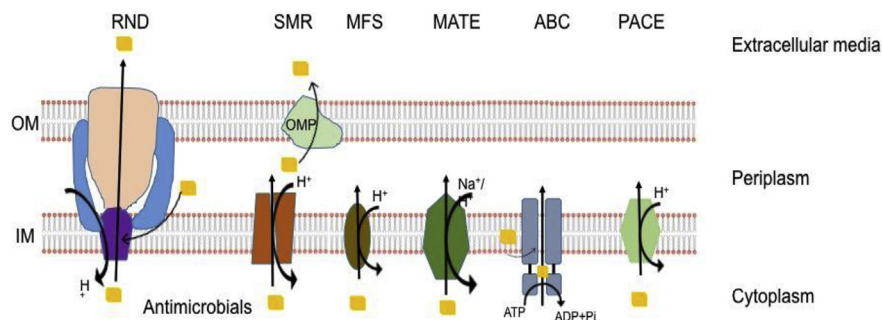Increasing resistance for various existing antimicrobial

Fig. 1. Schematic representation of six major superfamilies of efflux pumps along with their energy source. RND: Resistance Nodulation Division family, SMR: Small Multidrug Resistance superfamily, MFS: Major Facilitator Superfamily, MATE: Multidrug and Toxic Compound Extrusion superfamily, ABC: ATP (adenosine triphosphate)-Binding Cassette superfamily, PACE: Proteobacterial Antimicrobial Compound Efflux superfamily, OMP: outer membrane protein, OM: outer membrane, IM: inner membrane.

compounds has raised the urgency to develop newer strategies to fight these superbugs. Amongst various strategies, the one with the most promising future is to target efflux pumps. These pumps can be targeted either to increase the intracellular concentration of the antimicrobial compounds which are being effluxed out or making the superbugs sensitive for existing drugs. It can be achieved by modifying and improving the structure of antimicrobials to decrease their efflux, decreasing the efficacy of membrane barrier so that more amount of antimicrobials penetrate inside cell and last but not the least, by allosterically altering/blocking efflux pump function. The mechanism of action of various antimicrobials is shown in Fig. 2. The details of currently used antimicrobials against resistant strains are given in Table 1. A vast research has been done and is still going on to generate resistance reversal compounds, which are discussed in detail below.

2.1. Antibacterial

The most abundant efflux pumps in bacteria's are MFS and RND pumps. They are found in both gram-positive and gram-negative bacteria (Spengler and Amaral, 2017). Comparatively, gram-negative bacteria are more prone to resistance because of the presence of large periplasmic space along with an outer membrane. The drug is sequestered by efflux pump directly from the periplasm and extruded out even before they reach the cytoplasm (Mahamoud et al., 2007).

A nonapeptide derived from Polymyxin B was found to be effective when tested with erythromycin-resistant *Klebsiella pneumoniae*. This peptide conjugate acts by increasing the permeability of bacterial outer membrane for hydrophobic antibiotics (Tsubery et al., 2005). Compounds targeting proton motive force required for the functioning of efflux pumps like verapamil and reserpine can also be used to increase the efficacy of antibiotics. The major disadvantage of using these compounds is that the concentration required for their effectiveness can be neurotoxic. Even, the chemically synthesized derivatives of these compounds were ineffective due to their stability and solubility issues along with high cost of purification and synthesis (Li et al., 2004). Many
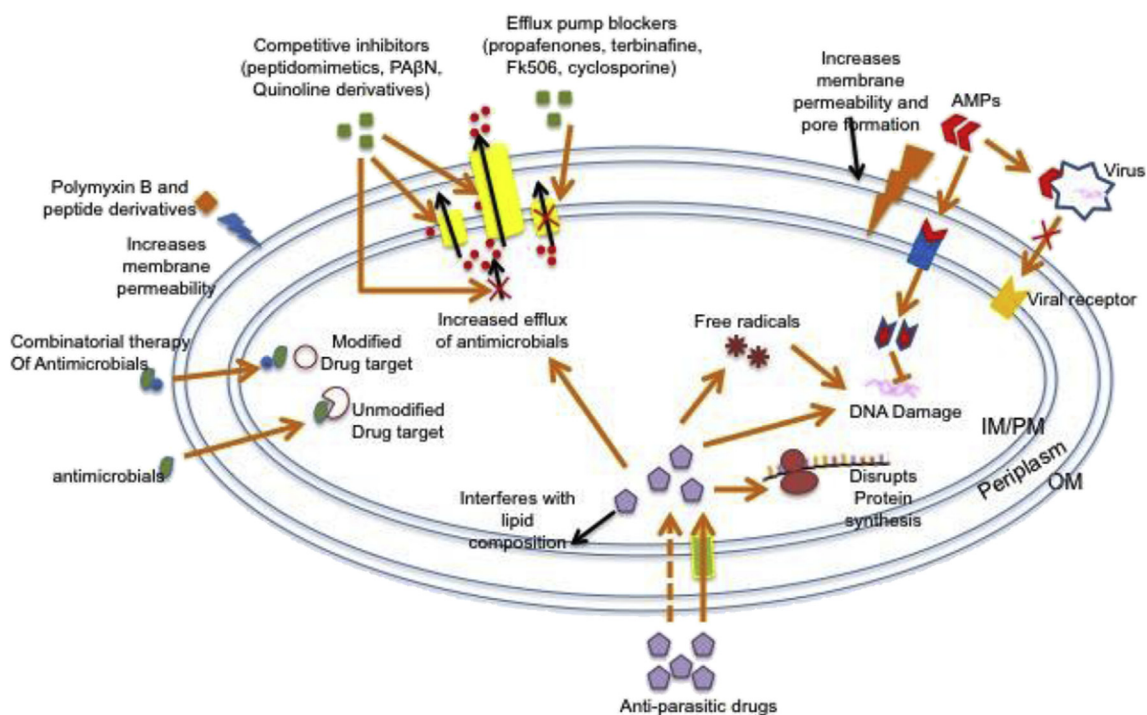

Fig. 2. Schematic representation of mechanism of action of antimicrobials. IM/PM: Inner Membrane in case of bacteria and PM: Plasma Membrane in case of fungi and parasites. OM: Outer Membrane in case of bacteria.

Table 1
Details of currently used antimicrobial agents against resistant strains and their probable mechanism of action.

S. No.	Antimicrobial agent	Strain	Probable mechanism	Reference
Antibacterial				
1.	Polymyxin B	*K. pneumoniae*	Increases permeability of bacterial outer membrane	(Tsubery et al., 2005)
2.	PAβN	*P. aeruginosa*	Inhibits RND type efflux pump	(Bohnert and Kern, 2005)
3.	Pyridopyrimidines	*P. aeruginosa*	Inhibits efflux pumps	(Mahamoud et al., 2007)
4.	Quinoline derivatives	*E. aerogenes, K. pneumoniae*	Inhibits efflux pumps	(Chevalier et al., 2004)
5.	Arylpiperidines, Arylpiperazines	*E. coli, S. aureus, A. baumannii*	Inhibits efflux pumps	(Bohnert and Kern, 2005; Kern et al., 2006)
6.	Nisin (AMP)	MRSA, VRE	Cell lysis	(Brumfitt, 2002)
Antifungal				
7.	FK506	Azole resistant baker yeast, *C. albicans*	Inhibits calcineurin pathway	(Schuetzer-Muehlbauer et al., 2003)
8.	Propafenones		Inhibits Cdr1p transporter	
9.	Terbinafine		Blocks ergosterol biosynthesis	
10.	GP382		Inhibits Cdr1p transporter	
11.	AMPs (D-V13K, P18, indolicin, defensins)	*C. albicans, T. beigelii, A. flavus* and *F. oxysporum*	Mostly target fungal cell membrane	(Bahar and Ren, 2013)
Antiviral				
12.	AMPs (defensin, lactoferrin)	HSV	Blocks virus – receptor interactions	(Jenssen et al., 2004)
Antiparasitic				
13.	Artemisinin based combinatorial therapies	Plasmodium	Alkylation of biomolecules, oxidative stress, cellular damage	(Blasco et al., 2017)
14.	AMPs (dermaseptins, cercopins, melettin, cathelicidin, magainin)	*C. elegans, P. caudatum, Leishmania*	Disrupting membrane integrity, interrupting protein, DNA, RNA synthesis, etc.	(Bahar and Ren, 2013; Hopkins and Groom, 2002; Mangoni et al., 2005)

research groups have used peptidomimetics approach either for altering or inhibiting the function of efflux pumps. Based on this, Microcide and Daiichi Pharmaceuticals have developed a large family of peptidomimetics exhibiting properties of efflux pump inhibitors. The first member of this family was MC-207 110 alternatively known as phenylalanine arginyl β-naphthylamide (PA β N). In *P. aeruginosa* resistant clinical strains, it was able to restore the levofloxacin susceptibility. Various structural derivatives of MC-207 110 as well as pyridopyrimidines have shown promising results in inhibiting efflux pumps of *P. aeruginosa* in *invitro* conditions. These EPIs (efflux pump inhibitors) failed in clinical trials owing to their toxicity (Mahamoud et al., 2007). Screening procedures with *E. aerogenes* strains and *K. pneumoniae* have led to the discovery of new class of EPIs namely, Quinoline derivatives. These compounds were successful in making resistant strains susceptible to chloramphenicol, tetracycline and norfloxacin. The derivatives with wide variety of side chains like alkylamino-, alkoxy-, thioalkoxy-, chloro-quinoline etc. showed promising results in structure-activity relationship analysis. They also have an advantage with an efficient pharmacokinetic profile as well as least side effect on permeabilization and alteration of membrane function. However, pharmacodynamics and cytotoxicity studies along with *invivo* studies are needed to check the actual therapeutic application of these compounds (Chevalier et al., 2004; Gallo et al., 2003). Aryl-piperidines and arylpiperazines and their derivatives like N-methylpyrrolidone have shown resistance reversal effect in *Escherichia coli*, *Staphylococcus aureus*, *Acinetobacter baumannii* and some species of Enterobacteriaceae (Bohnert and Kern, 2005; Kern et al., 2006; Pannek et al., 2006). Apart from these compounds, a new emerging class is of antimicrobial peptides or AMPs. They include natural occurring peptides as well as their synthetic derivatives with a broad spectrum of targets. Antibacterial AMPs are amongst the most studied ones. These can be cationic or amphipathic in nature, thereby, easy to interact with lipid bilayer hence targeting bacterial cell membrane. Nisin (an AMP) when administered with ramoplanin or chloramphenicol was able to restore sensitivity against methicillin resistant *Staphylococcus aureus* (MRSA) and vancomycin resistant enterococci (VRE). However, significant investigation is still required before reaching for clinical trials (Bahar and Ren, 2013; Brumfitt, 2002).

2.2. Antifungal

The foremost fungal pathogens affecting humans includes species of *Aspergillus*, *Candida* and *Cryptococcus* (Pfaller and Diekema, 2007). Compared to antibacterial resistance, antifungal resistance is a major problem in immuno-compromised individuals, which can be lethal. The efficacy of commonly used antifungals like fluconazole, ketoconazole, itraconazole, voriconazole, benomyl, methotrexate, etc. is decreasing mostly due to overexpression of efflux pumps. The efflux pumps responsible for multidrug resistance are ABC and MFS transporters. In *Saccharomyces cerevisiae*, multidrug resistance is known as pleiotropic drug resistance (PDR) and is the best understood mechanism. Most of the fungal drug resistance is studied with ABC and MFS transporter homologues in *S. cerevisiae* (Monk and Goffeau, 2008). FK506, propafenones, terbinafine, GP382 were found to be effective on azole resistant baker yeast as well as strains of *C. albicans* (Schuetzer-Muehlbauer et al., 2003). The antifungal AMPs belonging to all structural classes like alpha-helical, extended as well as beta-sheet are found. These AMPs (like D-V13K, P18, indolicin, defensins, etc.) mostly target fungal cell membrane and are effective even against resistant fungal cells (Bahar and Ren, 2013).

2.3. Antiviral

The common drug resistant viruses are *Cytomegalovirus* (CMV), *Herpes simplex virus* (HSV), *Human immunodeficiency virus* (HIV), *Influenza virus*, *Varicella zoster virus*, *Hepatitis B virus* (HBV) (Tanwar et al., 2014). Most of these viral strains are a result of either acquired or adaptive resistance. There is no room for intrinsic resistance. Antiviral AMPs acts either by causing membrane instability or reduction in host-virus interaction. Such a mechanism is utilized by defensin (an AMP) to render HSV unable to bind host cells (Yasin et al., 2004). Some antiviral AMPs like lactoferrin, prevent HSV infection by blocking virus-receptor interactions (Jenssen et al., 2004). In a recent study, a research group has discovered non-peptidic small molecule cyclophilin inhibitors with potent antiviral activity against hepatitis C virus, HIV and coronaviruses (Ahmed-Belkacem et al., 2016).

2.4. Antiparasitic

As per WHOs recent report, malaria stands 1st in the race of largest parasitic killer closely followed by leishmaniasis. The first report of resistance for an antimalarial drug (quinines and chloroquines) reports back to early 90s. After these, combinatorial therapies came into existence involving proguanil - atovaquone, sulfadoxine − pyrimethamine, napthoquinones, atremisinins, etc. Sooner or later *Plasmodium* showed resistance to all of them. The current first line of defense being used is artemisinin based combinatorial therapies. The instances of resistance for artemisinin combinatorial therapies shows that the partner drugs are the responsible candidates (Blasco et al., 2017; Taylor and Juliano, 2014).

Leishmania is one of the most neglected microbes causing leishmaniasis, endemic in over 98 countries across the globe. Many strains of parasite have been found which are resistant against the most recommended drugs like antimonials, miltefosine, amphotericin B, pentamidine, paromomycin, sitamaquine, etc. One of the major causes of development of resistance against these drugs is their irregular and inappropriate use in the endemic regions (Singh et al., 2012). Various *invitro* experiments have suggested that the drug resistant strains of *L. donovani* show decreased accumulation of miltefosine. This effect may be due to involvement of either an ABC transporter protein P-glycoprotein (Pgp or ABCB1) which may increase the drug efflux or a decrement in the drug uptake by the organisms which is contributed by two proteins, miltefosine transporter LdMT, a P4ATPase and its beta subunit LdROS3 (Perez-Victoria et al., 2006). A large number of ABC transporters have been identified so far, of which proteins belonging to three subfamilies; Multi Drug Resistance P-glycoprotein (P-gp, ABCB1), Multidrug Resistance associated Protein 1 (MRP1, ABCC1) and Breast Cancer Resistance Protein (BRCP, ABCG2); are believed to play an important role in drug efflux and hence multidrug resistance phenomenon (Ferreira et al., 2015). The ABC transporter, Pgp, consists of four domains: two transmembrane domains (TMDs) and two nucleotide-binding domains (NBDs) arranged in a head to tail fashion. Each TMD consist of 6 helices and are capable of recognizing a wide variety of substrates. NBDs consist of a larger (catalytic sub domain) and a smaller domain. The Walker A motif (GXXGXGKS/T) and Walker B motif (ΦΦΦΦD) are part of this domain. The smaller helical domain has conserved ABC signature motif or C-motif (LSGGQ) (Dawson and Locher, 2006).

In order to combat the resistant strains, various combinatorial therapies have been used. Due to increased drug selectivity, reduced toxicity and compatibility with the target protein structures AMPs, for example, dermaseptins, cecropins, melettin, etc., have been described as a first line of defense against pathogen invasion, including protozoa (Hopkins and Groom, 2002; Lynn et al., 2011; Mangoni et al., 2005; Marr et al., 2016; Toro et al., 2013; Ruiz-Santaquiteria et al., 2017) and are under intense investigation to improve their druggability. Their mode of action involves the perturbation of protozoan homeostasis by directly disrupting the cellular membranes, interaction and interference with key processes in the parasite metabolism (Afacan et al., 2012; Jenssen et al., 2006; Torrent et al., 2012b). Various experimental and computational platforms have been proposed to develop more efficient AMPs (Fjell et al., 2012; Hilpert et al., 2005; Lata et al., 2007; Torrent et al., 2012a, 2009; Wang, 2004). Synthetic AMPs have also been designed and were found to be effective for treating canine leishmaniasis (Alberola et al., 2004). But their success has been restricted to *invitro* conditions only. None of these have succeeded in *invivo* or in clinical trials. Besides, their antimicrobial role, AMPs are also involved in chemotaxis and redox homeostasis with rapid radical scavenging ability (Cole et al., 2003; Com et al., 2003; Ganz, 2002; Liu et al., 2010; Zhao et al., 2011). Recently, the metagenomic approaches are extensively reviewed to explore the role of AMPs in redox homeostasis (Champion and Xu, 2017).

Nifurtimox and benznidazole being used against *Trypanosoma cruzi* are showing many side effects in patients. Melarsoprol used against *Trypanosoma brucei* may be lethal in chronic cases. In the past decade, there has been a sudden increase in the cases of resistance of Schistosoma to praziquantel (Acevedo et al., 2017). Peptides like magainin for *Paramecium caudatum* and cathelicidin for *Caenorhabditis elegans* have been designed (Bahar and Ren, 2013). The efficacy for above drugs is decreasing resulting in the emergence of resistance and also having toxic effects making them unsuitable for use. These have raised an urgency to find out alternatives to decrease or reverse the resistance condition making microbes sensitive to existing drugs.

3. System genomics and antimicrobial resistance

Due to exponential increase in the size of biological data, development and advancement in systems biology, integration of mathematical methods and computational approaches has recently been of much interest. Systems biology approaches have been utilized to model various pathway systems to study their dynamics by combining metabolic network (Beiting and Roos, 2011; Pillay et al., 2013) and -omics data (Go et al., 2014). These strategies can be implied to find novel therapeutic targets.

3.1. Metabolomics approach

The systems-based application of metabolomics (a large scale analysis of small molecules) has provided important insights into the metabolic processes (Barrett et al., 2010; Creek et al., 2012; Kafsack and Llinas, 2010; Lakshmanan et al., 2011; Saunders et al., 2011; Scheltema et al., 2010; Swann et al., 2015). Another strategy to combat resistance is targeting redox metabolism pathway of microbes by the identification of novel targets using systems biology approaches (Fig. 3). Although, the host provides survival advantages to the parasites by offering unlimited nutritional supply, the environmental change also entails potential risks from host defense in which the parasite has to undergo tremendous oxidative stress that has to be neutralized. For their survival, maintenance of redox homeostasis is of principal importance. Parasitic redox metabolism has gained greater interests due to their distinctive redox systems in comparison to their hosts (Salinas, 2013).

Application of metabolomic analysis to understand the response to oxidative stresses in *E. histolytica* has been studied by exposing the trophozoites to various reactive oxygen or nitrogen species (ROS and RNS) (Jeelani and Nozaki, 2014; Vicente et al., 2009;
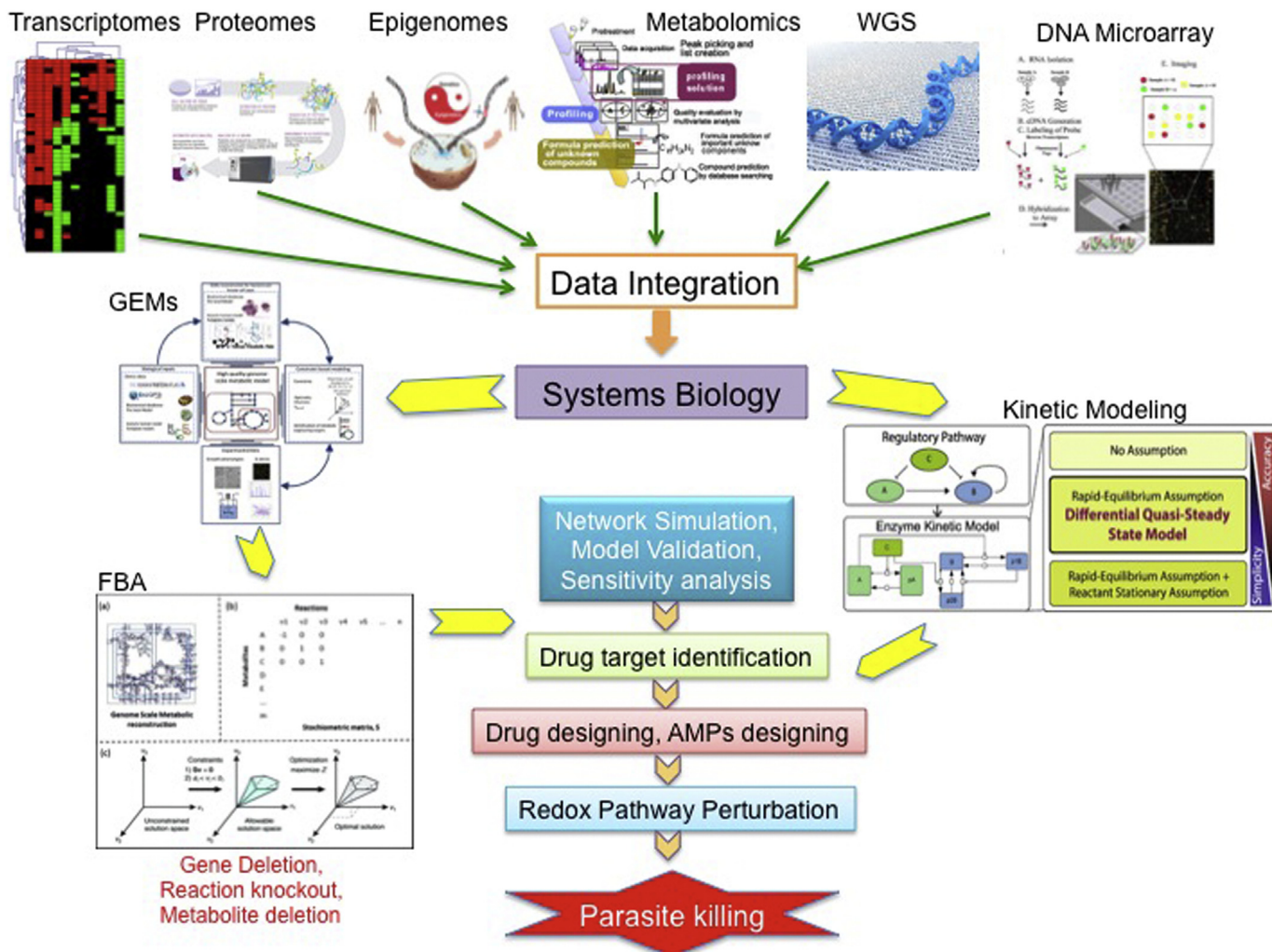
Fig. 3. Applications of Systems Genomics in identifying new drug targets and drug design.

Husain et al., 2012). These studies have revealed that oxidative stress affected the level of metabolites synthesis and inactivation of several key enzymes involved in glycolysis and other associated pathways. H_2O_2-mediated oxidative stress has helped in the identification of indispensable role of cysteine synthase (CS) and serine acetyltransferase (SAT), enzymes involved in cysteine synthesis pathway, in *E. histolytica* trophozoites in their survival, growth, attachment, elongation, motility, gene regulation, and oxidative stress defense (Fahey et al., 1984; Gillin and Diamond, 1981; Husain et al., 2011; Jeelani et al., 2010; Jeelani and Nozaki, 2016; Jeelani et al., 2017).

Importance of cysteine has also been studied in *Trypanosoma cruzi* epimastigotes during the transition from exponential to stationary growth phase (Barisón et al., 2017). It has been observed that the *de novo* cysteine synthesis pathway was increased in stationary phase revealing a crucial metabolic switching mechanism to cope up with the phase transition and environment change between exponential and stationary growth phase. Deep understanding of these metabolic switches and their mechanism would help in exploring the key metabolic checkpoints as novel targets for future therapeutic intervention and epidemiological control of Chagas disease.

Using -omics approaches, *in silico* metabolic network can be constructed which, further, are used to identify metabolic gaps, blocked reactions, and to differentiate between host and parasite

metabolism that may be exploited for drug therapy (Brauer et al., 2006; Breitling et al., 2008; Kafsack and Llinas, 2010; Kalisiak et al., 2009; May et al., 2008). Metabolic network analysis has been used to identify promising targets of therapeutic importance in *Leishmania infantum* (Chavali et al., 2008), T. *cruzi* epimastigote (Roberts et al., 2009), and *Plasmodium falciparum* (Fatumo et al., 2009; Yeh et al., 2004).

Currently, due to the lack of the kinetic parameters, genome scale metabolic models (GEMs) using the concept of flux balance analysis (O'Brien et al., 2015) is also becoming very popular among the researchers. Application of GEM has already been applied to map the effect of chloroquine in *Plasmodium falciparum* (Tewari et al., 2017) for predicting dose-dependent inhibition of DNA replication for both drug-sensitive and drug-resistant *P. falciparum* strains. *In silico* gene knockout in GEMs of *Leishmania infantum* and *Leishmania donovani* (Sharma et al., 2017; Subramanian and Sarkar, 2017) has predicted Trypanothione reductase (TryR) and 28 other genes to be essential with negligible sequence identity to the human proteins. Further, comparison of stage specific flux distribution between the promastigote and amastigote stages has illustrated the differences in metabolism and environmental conditions. Flux-balance analysis (FBA) of *P. falciparum* metabolic network was able to reproduce the experimental gene-knockout phenotypes and drug inhibition assay with up to 90% accuracy (Plata et al., 2010) and identified Thioredoxin reductase to be lethal.

Redox systems biology of artemisinin resistant and sensitive blood stage *P. falciparum* have shown the importance of mitochondrial function and glutathione redox system in surviving artemisinin stress (Chen et al., 2014; Peatey et al., 2015; Carey et al., 2017). Likewise, kinetic models of Trypanothione synthase (TryS) and TryR in *Trypanosoma brucei* were formulated. These models were able to conclude that TryS activity is tightly regulated (Leroux et al., 2013; (Olin-Sandoval et al., 2012).

Due to increasing appearance of resistant clinical isolates of many parasites, decreasing efficacy of current chemotherapy has been an issue of concern and urgent need of development of alternative agents with new mode of action is required. Traditionally, small organic molecules target the small cavities of the target protein and inhibit specific catalytic centers or the binding sites of natural substrate analogs (Drews, 2000). Protein-protein interaction networks has provided insights of drug targeting towards disruption of protein-protein interactions; where classical small molecules are not always ideally suited (Fry, 2006).

GEM has also been explored in finding new drug targets and to combat antimicrobial resistance in many bacteria. A GEM developed by integrating gene expression data with metabolic pathways to study the effect of ribosome targeting antibiotics on the metabolism of *Pseudomonas aeruginosa* was able to spot important reactions with fluxes correlating with gene activation levels (Xu et al., 2016). Metabolic simulation of another high-quality model *i*PAO1 of *Pseudomonas aeruginosa* showed mechanism of polymyxin resistance in which polymixin exerted the remarkable change in the physiochemical properties of the outer membrane (Zhu et al., 2018). Similar models to study antibiotic treatment and response were developed in multi-drug resistant bacteria, including *Acinetobacter baumannii* (Presta et al., 2017), *Mycobacterium tuberculosis* (Colijn et al., 2009), and *Yersinia pestis* (Navid and Almaas, 2012).

Mathematical modeling has been applied to model the kinetics of bacterial growth, gene transfer and antibiotic resistance (Knopoff and Sánchez Sansó, 2017). The kinetic model of gene transfer in motile and non-motile *Azotobacter vinelandii* has effectively depicted the experimentally determined rates of tetracycline resistance gene transformation and was able to explain the relationship between transformation frequency and varied DNA or cell concentrations (Lu et al., 2015).

Systems biology also offers possible methods to screen novel targets and methods to combat antibacterial resistance. Synthetic biology, one of these methods, that is becoming a method of choice among the researchers. Gene circuits in synthetic biology have provided new insights into natural gene-network dynamics leading to the identification of new drug targets (Herrera, 2005; Huang et al., 2011; Walsh, 2003). Due to the increasing popularity of systems biology in fighting antibiotic resistant bacteria, it is enabling the construction of biological systems for antibiotic production by involving complex multiple genetic circuits for efficient overexpression of all metabolites and enzymes in the same cell (Wang et al., 2012; Weber et al., 2008).

3.2. Whole Genome Sequencing (WGS)

The past decade has seen a bloom in the technology for genome sequencing. Time as well as cost for sequencing and data assembly has reduced with a great pace from weeks to hours and millions to thousands dollars respectively. The accuracy of the reads has also increased with time. The technology has evolved from 454-sequencing (http://www.my454.com/), Illumina (http://www.illumina.com), SOLiD (http://www.appliedbiosystems.com), to Helicos Heliscope (http://www.helicosbio.com), Pacific Biosciences SMRT (http://www.pacificbiosciences.com) and Oxford Nanopore (http://www.nanoporetech.com) (Gullapalli et al., 2012). These

advancements have made WGS accessible for routine use.

From vast and diverse applications of WGS, one of the important and of our focus is its utility in case of antimicrobial resistance as the main criteria of classifying a microbe as resistant to a particular drug are presence/absence of a specific gene or certain mutations. Apart from identification of resistant strain, WGS can be used in the identification of novel drug targets. A web server, ResFinder, has been developed to identify acquired antimicrobial resistance genes in bacteria using WGS data (Zankari et al., 2012). This server is still limited on detection of resistant gene obtained from horizontal transfer and not from mutations.

As it provides complete information, WGS can be used to keep a check on the spread of pathogens and hence control of infection by diagnosing the resistant strain in the early stage of infection. It will help in deciding the drug regimen for infection by specific strains, thus, minimizing the unnecessary drug exposure to resistant strains. It will be of great use in clinics giving an early warning of the emergence of drug resistance. For the researches, WGS provides a great scope in understanding the mechanisms responsible for resistance. They can look for various genomic aspects playing key role in resistance development. The only limitation for it is to analyze and interpret the vast data generated from sequencing (Köser et al., 2014; Zankari et al., 2012).

4. Conclusion

In decade's long battle between microbes and drugs, it seems like microbes have evolved into superbugs to defeat humans. They have developed various mechanisms to keep antimicrobials far away from them. Amongst vast mechanisms, the most studied and targeted are those involving efflux pumps. Microbes exhibit resistance for drugs either by overexpression of efflux pumps hence causing an increase in the efflux; or in some cases they may enhance the activity of these efflux pumps either by mutating some genes or causing some allosteric changes. Whatever be the mechanism, the end result is less accumulation of drug inside the microbes. The need of the hour is to find new strategies to make these super bugs again susceptible for the drugs. A doable method is to target efflux pumps by minimizing pump expression or decrease in affinity for drug binding or directly inhibiting pump activity. Many research groups have contributed in developing EPIs and AMPs so that the existing antimicrobial agents can be reused based on combinatorial therapy. The reason for their failure in *invivo* or in clinical trials is that mostly potential antimicrobial agents are being tested on laboratory generated resistant strains. We should focus more on testing on resistant isolates from patient samples. This will decrease the failure rate in clinical trials. Further additional efforts must be made to design EPIs or rather small peptides that would specifically target efflux pump of a particular type of microbe. These specific small peptides may be designed either to inhibit a pump or to allosterically modulate and thereby decreasing its activity. Peptides acting allosterically on a specific pump will minimize the possibility of resistance. Instead, they will contribute mostly in making microbes susceptible again for the existing antimicrobial agents. Targeting redox metabolism pathway of microbes using systems genomics approaches serves as another strategy, discussed above. These approaches may help in avoiding drug tolerance as well as efflux pump mediated resistance.

5. Future perspective

The current research scenario opens a vast scope for identifying new antimicrobials or repurposing the existing ones such that their efficacy is enhanced with combinatorial therapies. The authors think that it's of utmost importance to focus on eradication and

reversal of drug resistance lest in the coming future every existing antimicrobial drug should just not be a waste. Targeting specific efflux pumps as well as finding critically important proteins necessary for microbe survival through systems genomics approaches can surely provide us a way to minimize the occurrence of resistance.

Conflicts of interest

The authors declare no conflict of interest.

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