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Review

Antibiotics: Pharmacokinetics, toxicity, resistance and multidrug efflux pumps

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ABSTRACT

The discovery of penicillin followed by streptomycin, tetracycline, cephalosporins and other natural, semi-synthetic and synthetic antimicrobials completely revolutionized medicine by reducing human morbidity and mortality from most of the common infections. However, shortly after they were introduced to clinical practice, the development of resistance was emerged. The decreasing interest from antibiotic industry in spite of rapid global emergence of antibiotic resistance is a tough dilemma from the pointview of public health. The efficiency of antimicrobial treatment is determined by both pharmacokinetics and pharmacodynamics. In spite of their selective toxicity, antibiotics still cause severe, life-threatening adverse reactions in host body mostly due to defective drug metabolism or excessive dosing regimen. The present article aims at updating current knowledge on pharmacokinetics/pharmacodynamics concepts and models, toxicity of antibiotics as well as antibiotic resistance mechanisms, resistome analyses and search for novel antibiotic resistance determinants with special emphasis given to the-state-of-the-art regarding multidrug efflux pumps and their additional physiological functions in stress adaptation and virulence of bacteria. All these issues are highly linked to each other and not only important for most efficient and prolonged use of current antibiotics, but also for discovery and development of new antibiotics and novel inhibitors of antibiotic resistance determinants of pathogens.

Contents

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1. Introduction

The identification of penicillin by Alexander Fleming in 1928 [1] is the cornerstone discovery in the history of the 'antibiotic era'. Still, the efforts of Paul Ehrlich and his co-workers to establish a systematic screening approach for discovery of antimicrobials took place much earlier and introduced the 'magic bullet' Salvarsan [2,3]. Salvarsan remained the most populously prescribed drug against syphilis till the first use of penicillin in 1941 [3,4]. Prontosil as the first sulfa drug was next discovered by the use of same approach [5] and followed by discovery of streptomycin which was the first antibiotic used for the treatment of tuberculosis [6]. In 1945, the fungus *Cephalosporium acremonium* was shown to produce an "antibiotic principle" effective against staphylococcal, streptococcal infections, typhoid fever and brucellosis [7]. Later on, the principle was demonstrated to represent a group of natural compounds called cephalosporins, and N-phenylacetyl derivative of cephalosporin C being the most effective against *Staphylococcus aureus* [8,9]. These investigations led to the production of new generation cephalosporin compounds and saved many lives as the former ones. However, resistance has eventually appeared for nearly all antibiotics, shortly after they were introduced to clinical practice [10].

According to the estimates of Bérdy (2012) as based on Bioactive MICrobial Metabolite Database of his own, of 60–80 thousand natural metabolites produced by microbes, 47% exhibit bioactivity [11]. On the other hand, when it comes to the total number of drugs in market for use in human therapy, there are ca. 3500 such compounds 200–220 of which include antibiotics made up of direct natural products, more than 250 being semisynthetic/modified derivatives of them, and synthetic antimicrobials (especially quinolones and oxazolidinones) do also have a role in the antimicrobial market. As headed by the problem of increasing resistance to antibiotics creating clinical and economic burden, the search for novel antibiotics (from nature, combinational biosynthesis, hybrid antibiotics, discovery of new molecular targets, screening of unculturable microorganisms, combinatorial chemistry as well as computerized drug design) should constitute a very hot research area for finding new antibacterial drugs. Indeed, in between late 1960s and mid 1980s, the pharmaceutical industry introduced many new antibiotics to solve resistance problem, but since then there appears only a very limited number of new antibiotics reported. To exemplify, some new antibiotics including synthetic (e.g. besifloxacin, doripenem, radezolid), and semi-synthetic (e.g. cethromycin; derived from erythromycin A) compounds were recently approved by FDA or in clinical trials [12]. In a recent study, Ling and his co-workers (2015) announced the discovery of a new class of natural antibiotic namely teixobactin which inhibits cell wall synthesis by a novel mechanism after the screening of a previously uncultured bacterium namely *Eleftheria terrae* [13]. Another research group suggested a new antimicrobial agent called lugdunin produced by *Staphylococcus lugdunensis* which is an inhabitant of human nares. They demonstrated the potential killing effect of lugdunin against many Gram-positive bacteria including methicillin-resistant *S. aureus* (MRSA) and vancomycin-resistant *Enterococcus* (VRE) isolates [14]. Also, a team at Harvard University proposed a new platform for the production of new macrolides which is based on the synthesis of different compounds that cannot be produced by traditional semi-synthetic methods [15]. The novel targets in different bacterial processes such as quorum sensing (QS) and biofilm formation are being investigated for development of new antibacterial agents mainly by academia [16]. The Infectious Diseases Society of America (IDSA) provided a platform called 'the 10×20 initiative' for development of ten new antibacterial drugs including new chemical classes and modified versions of current classes till 2020 [17]. 'Return On Investment' considerations and challenging regulatory requirements unfortunately restricted the attempts of antibiotic industry to discover and develop novel classes of agents against pathogenic bacteria which instead preferred to focus on altering existing ones or development of antiviral agents [18,19]. Thus, the decreasing interest from antibiotic industry in spite of worldwide rapid emergence of antibiotic resistance is a tough dilemma from the pointview of public health. Maintaining and prolonging the useful life span of existing antibiotics must have a high priority under these circumstances [20]. In this respect, computer-aided screening to identify potential inhibitors of the antibiotic resistance and also preferentially quorum sensing and virulence has received great attention in recent years [21].

Pharmacokinetic properties of antibiotics are mainly based on their chemical structure which absolutely affects their bioavailability, half-life, tissue penetration, distribution, degradation and elimination [22]. For each class of antibiotics, dosage application and duration of exposure have been critical issues to obtain optimum outcomes in patients while minimizing the risk of resistance development and toxicity. Expanding knowledge on the interaction between antibiotic pharmacokinetics, toxicity and resistance provided better understanding of individualized therapy [22,23]. Pharmacodynamic factors include antimicrobial activity against the pathogen, drug stability in the case of resistance and absence of organ toxicity [24]. The present article aims at overviewing pharmacokinetics/pharmacodynamics concepts and models, toxicity of antibiotics as well as antibiotic resistance mechanisms with special emphasis to multi-drug transporters, all of which are highly linked to most efficient and prolonged use of antibiotics.

2. Pharmacokinetics/pharmacodynamics and toxicity of antibiotics

2.1. Pharmacokinetics/pharmacodynamics concepts and models

Since the emergence of antibiotic resistance is mostly attributed to drug overuse, inappropriate prescribing and suboptimal dosing, certain measures must be taken for dose optimization of current antibiotics [25]. Optimization of antibiotic usage requires well-understood criteria that can be simplified as the relationships between concentration, dose and both desirable and side effects. This requirement has emerged a well established and authorities-recognized field called pharmacokinetics/pharmacodynamics (PK/PD) that basically studies the interactions between host, pathogen and drug in that infection/immune response, pharmacodynamics/drug susceptibility, pharmacokinetics/toxicity couples forming the edges of an equilateral triangle [25,26]. PK/PD concepts were originally described by Eagle et al. [27] who revealed time-dependent, concentration-dependent and mixed patterns of these for different antibiotics including penicillin and streptomycin and re-emerged by the effort of Craig (1998) [27,28]. "The optimal dosage regimen" is to be determined before the drug receives regulatory approval and should be a function of the correct dose and dosing interval rather than the duration of treatment [26]. After administration of an antibiotic to a patient, it goes through some processes in body known as ADME (Absorption, Distribution, Metabolism and Excretion). PK is after 'what the body does to the drug' with certain parameters like total body clearance, volume of distribution, bioavailability and protein binding [26]. When drug goes to the action site (i.e. pathogenic bacterium), it develops desirable effects as well as undesirable ones, the topics studied by PD which can be defined as 'what the drug does to the body' [29]. In other words, PK deals with the time course of serum level of antibiotics in body, thus its parameters

have been traditionally used for deciding antibiotic dosing regimens. It is PD, on the other hand, that establishes the relationship between antibiotic concentrations and the magnitude of killing activity, thereby very important for deciding dosing regimens which prevent antibiotic resistance. The studies of PD/PK provide integrated information, greatly affecting the selection of antibiotics and dosage adjustment by taking into account patient-specific factors such as kidney function and risk for toxicity [25,30,31].

It is basically possible to measure the effect of antibiotics through Minimum Inhibitory Concentration (MIC). In spite of its inherent drawbacks such as measurement errors or neglecting dynamic changes in growth and sensitivity (in this respect, time–kill curves provide more detailed information) as well as its limitation to extracellular pathogens, MIC is generally used as a major indicator for PK/PD analysis [25,30,32]. For intracellular infections, cellular pharmacokinetics and pharmacodynamics must focus on different parameters, as discussed in Section 2.2.

In MIC-based PK/PD analysis, mathematical and statistical methods are integrated with microbiological parameters to create models and simulations for characterization of drug behavior. The three main indices used as the common standards in this analysis are (i) Cmax/MIC, (ii) AUC/MIC, and (iii) T > MIC as schematically explained in Fig. 1 [32,33].

According to the PK/PD parameters shown in Fig. 1, killing activity of antibiotics can be described with respect to its time-dependence, concentration-dependence and persistence. Persistent effects include the Post-Antibiotic Effect (PAE) which is another PD concept referring to persistent suppression of bacterial growth following exposure to antibiotic [29,33].

For PK/PD analysis of antibiotics, both *in vitro* and *in vivo* models have been used. *In vitro* models are easier and more flexible than animal- and patient-based *in vivo* ones and provide advantages regarding ethical and cost issues. To exemplify, Ahmed and Noreddin (2012) performed simulations with STELLA® for PK/PD analysis of five different antibiotics; ampicillin and tetracycline as time-dependent antibiotics and ciprofloxacin, rifampin and streptomycin as concentration-dependent antibiotics [20]. Multiple intravenous administrations of antibiotics were applied at 8-h intervals in 24 h. The antibiotics displayed different antimicrobial activities based on Hill coefficient (a measure used for the relationship between growth rate and concentration of antibiotic) even when they have the same MIC. As based on different PK/PD nature of the antibiotics, increase in both AUC/MIC and T > MIC provided efficient therapeutic results for tetracycline while ciprofloxacin was successful only when AUC > MIC was increased by the applied dose. In another study, the simulations based on a PK/PD model that characterizes the full time course of *in vitro* time-kill curve experiments of six antibacterial drugs (benzylpenicillin, cefuroxime, erythromycin, gentamicin, moxifloxacin, and vancomycin) proved to be rather powerful [34].

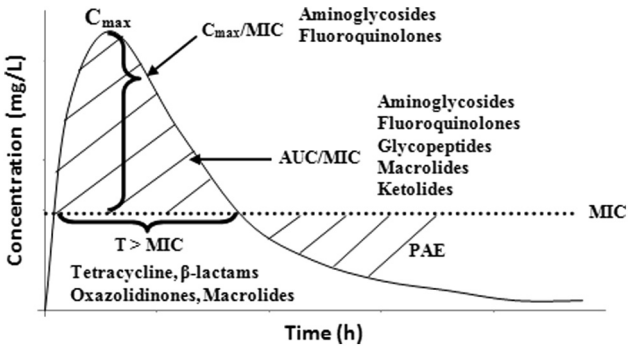

Fig. 1. The main PK/PD parameters using to predict drug efficacy. C_{max}/MIC: the ratio between the peak serum concentration (C_{max}) of the antibiotic reached in the serum and the MIC, AUC/MIC: the ratio between the 24 h Area Under the Serum concentration-time Curve (AUC) and the MIC, T > MIC: time (dosage interval) during which the serum concentration of antibiotic remains above the MIC. PAE: Post-Antibiotic Effect. Antibiotics that correlate well with each parameter are also shown in respective areas [Adapted from 32 and 33].

Of different experimental setups defined in the literature and the most commonly used models are static and dynamic systems [30]. Unlike dynamic systems, there is no exchange of media in culture in static systems where the interaction between drug administration and efficacy can be easily studied. In dynamic systems, the concentration of drug changes during process and it allows mimicking human PK *in vitro* and thus allowing time-dependent studies. Dilution and diffusion methods are two common methods used in dynamic systems. Dilution method is achieved by addition of new medium into the system during the experiment and this can be performed in open or closed systems. While the dilution of bacteria and drug takes place in open systems due to the addition of new medium, membranes and filters are used in closed systems to limiting bacterial losses during withdraw and replacement of the broth [30]. In the latter, an exchange of nutrients, drug and waste but not bacteria occurs between two compartments.

In spite of its advantages, *in vitro* models have an important drawback that host immune system is ignored during evaluation of antibacterial effect. In animal models, action and efficacy of the drug can be evaluated at the place of infection and the reaction of immune system can be monitored which provides more precise results as soon as proper modifications of the system are made to simulate the human PK profile. Besides these, new approaches are being developed including modeling and simulations to merge knowledge gained by *in vitro* and *in vivo* models [30].

2.2. Cellular pharmacokinetics and pharmacodynamics of antibiotics

Capability of invading host cells and surviving within them provide protection for intracellular pathogens against specific and nonspecific host defenses as well as antibiotics. Therefore, selection of antibiotics with intracellular action or both intracellular and extracellular effects (particularly for facultative/opportunistic intracellular ones) is critical. Cellular pharmacokinetics involves the evaluation of antibiotic's behavior in individual cells such as diffusion, degradation and efflux [32].

Drugs can enter inside of a cell via different nonspecific routes such as endocytosis or diffusion while some can access to their intracellular targets through specific transporters [35]. Molecular weight, environmental pH and ionization properties are important for diffusion across the cellular membrane. While β-lactam antibiotics are known to enter cell by passive diffusion, some antibiotics such as aminoglycosides can cross the cellular membrane via endocytosis involving surface receptors. Moreover, active inward transport may have a role in some cases where a drug is structurally similar to natural targets of transporters [32]. Efflux transporters found on cell surfaces are another concern of cellular pharmacokinetics since they can be responsible for decreased absorption of drugs, reduced intracellular accumulation and thus suboptimal drug concentration in cell [36]. In their review article focused on cellular PK/PD of antibiotics, Van Bambeke et al. (2006) provided a detailed list on the relevant properties of main antibiotic classes, including accumulation level at equilibrium, cellular concentration at equilibrium, time to equilibrium, accelerated efflux due to active transport and predominant subcellular localization, respectively [32]. Accordingly, while the accumulation of macrolides and glycopeptides is higher in cells, quinolones accumulate moderately and are concentrated in cytosol. However, β-lactams do not accumulate in cells, representing improper choice for intracellular pathogens. Due to their slow accumulation, aminoglycosides can be used to treat chronic intracellular infec-

tions. Macrolides, quinolones and rifamycins are generally considered as suitable antibiotics for the effective treatment of intracellular infections. Although cellular pharmacokinetic studies provide valuable information about the accumulation level of antibiotics in cells, this should not be taken as the sole criterion [32]. It is because only free (non-protein bound) fractions of antibiotics are capable of entering cells, plus a uniform distribution throughout cells rather than a high local concentration in specific cellular compartments is required. Insufficient bioavailability of the accumulated antibiotic as well as a shift of MICs toward higher values in the intracellular milieu generally accounts for the lack of correlation between the cellular concentrations of antibiotics and actual intracellular activity, and makes pharmacokinetic predictions incorrect, but pointing to the importance of pharmacodynamic considerations [32,37].

Most of the *in vitro* and *in vivo* models are mainly based on the use of facultative intracellular or opportunistic pathogens generally with static parameters instead of dynamic ones [38–40]. New approaches considering intracellular pathogens, the nature of infections and dynamic conditions are in demand for a better understanding of PK/PD behaviors of antibiotics. Recently, Buyck et al. (2016) presented a highly flexible *in vitro* PD model for studying the pharmacodynamics of antibiotics against intracellular bacteria [41].

2.3. Selected PK/PD studies for different antibiotic classes with reference to particular pathogens

β-lactams which act in a time-dependent manner with minimal persistent effects are thought to be passively diffused into cells and the absorption of some β-lactams takes place in gastrointestinal (GI) tract. Most β-lactams have low plasma protein binding and are mostly eliminated by the kidneys [32,42]. Their bactericidal activity is mainly related with the exposure duration (T > MIC) instead of their concentration above the MIC [22]. However, there are studies revealing the correlation between efficacy of β-lactam agents and AUC/MIC under various conditions [43,44]. Owing to its promising potency against resistant pathogens, one of the old antibiotics which is being re-evaluated for its PK/PD parameters is the monobactam antibiotic aztreonam [45]. Recent studies mostly focus on its administration with β-lactamase inhibitors such as avibactam to prevent the infections of metallo-β-lactamase producing Gram-negative pathogens [45,46]. For instance, Singh et al. (2015) determined bacterial killing capacity of aztreonam and avibactam combination by both *in vitro* and *in vivo* studies [46]. They used hollow-fiberfiber infection model (HFIM) and neutropenic mouse thigh infection model to determine PK/PD parameters and their magnitudes against six MDR Enterobacteriaceae isolates. The results showed that the activity of aztreonam was restored by avibactam against all isolates and aztreonam MIC levels were reduced by 512- and 1024-fold for *Klebsiella pneumoniae* and up to 128-fold for *Escherichia coli* isolates. It was concluded that % T > MIC was the best parameter to evaluate the efficacy of aztreonam/avibactam. In addition to monobactams, cephalosporins such as ceftolozane can be used together with β-lactamase inhibitors. Ceftolozane is used to treat urinary tract and abdominal infections in combination with tazobactam and the best PK/PD parameter associated with ceftolozane activity is known as % T > MIC [47,48]. In a population PK study in healthy subjects having different renal function levels and patients with infections, the clearance of ceftolozane/tazobactam was mainly affected by renal function [49]. In a recent study, the activity of ceftolozane/tazobactam was characterized with time-kill studies using *E. coli* strains expressing β-lactamase in different levels [48]. They reported that tazobactam had improved the activity of ceftolozane and expanded the susceptibility of *E. coli* strains producing β-lactamase. Recently, the use of ceftolozane/tazobactam and ceftazidime/avibactam were approved by FDA to treat complicated urinary tract and intra-abdominal infections [50].

Despite increasing resistance, carbapenems like doripenem, biapenem, imipenem, meropenem either in monotherapy or combination therapy maintain their essential use to treat *P. aeruginosa* and *K. pneumoniae* infections especially in critically ill and immunocompromised patients since they distribute widely into various body fluids. Thus these antibiotics do have widely documented PK/PD profiles [31,51,52] and except for imipenem it is generally accepted that high doses and long infusions improve bacterial clearance [52]. Such antibiotics are known to have time-dependent bactericidal activity against Gram-negatives when free drug concentrations remain above the MIC of the pathogen for 40–50% of the dosing interval [29]. Van Wart et al. (2009) described the results of an animal PK/PD, human PK, and *in silico* modeling work for doripenem in phase 3 clinical studies [53]. Some of the dosing regimens validated as effective included 500 mg infused over 1 h every 8 h for complicated intra-abdominal infections and 1000 mg infused over 4 h every 8 h for hospital-acquired pneumonia. Monte Carlo simulations were utilized to determine dosing regimens of meropenem [54] and doripenem [55] in critically ill patients suffering from Gram-negative infections. As meropenem remains stable up to 8 h <23 °C in infusion bags, its administration by continuous infusion (2000 mg every 8 h administered over 8 h) is possible, allowing higher concentrations in subcutaneous tissue and plasma than by intermittent bolus dosing. To note, in cases of bloodstream infections (BSI) caused by carbapenemase-producing *K. pneumoniae* (KPC), much better survival rates were obtained when meropenem was included in tigecycline–colistin combination [56]. Mainly when the meropenem MICs are ⩽16 μg/l, the 30-day mortality was significantly lower among patients treated with triple drug combination. An independent work supported these findings in that the use of a combination therapy with either colistin-polymyxin B or tigecycline and a carbapenem significantly reduced the mortality associated with bacteremia (13.3% versus 57.8%) [57]. As to doripenem which has a PK profile similar to that of imipenem and meropenem but shows high relative potency particularly against *P. aeruginosa*, Samtani et al. (2010) defined probability of PK/PD target attainment by renal function, duration of doripenem infusion, and MIC of pathogen [55]. According to their model, a 500-mg dose of doripenem infused over 4 h every 8 h could be recommended for MICs up to 4 μg/ml as based on a T > MIC 35% target and even 4 h infusions of 1 g doses might provide a better response. In a more recent work, a simulation model using adult PK/PD data was applied with 1, 5, 10, 20 and 30 mg/kg twice and three times administrations per day to investigate doripenem dosage regimen in pediatric patients [58]. These simulations revealed that 20 mg/kg three times per day could be clinically efficient in infections such as pneumonia and septicemia in children. Like monobactams and cephalosporins, carbapenem therapy with β-lactamase inhibitor can be used against resistant pathogens producing β-lactamase. A novel inhibitor (CB-618) combined with meropenem was recently tested in an *in vitro* infection model [59]. They reported AUC/MIC as PK/PD parameter related to the efficacy of CB-618 given in combination with meropenem.

Aminoglycosides which have clear concentration-dependent killing activity are absorbed from GI tract poorly and they enter cell via receptor-mediated endocytosis. The accumulation of aminoglycosides in cells occurs slowly and thus long exposure is required for active concentration [22,32]. They have low plasma protein binding, prolonged persistent effects and their primary excretion route is glomerular filtration [42]. Clinically optimized strategy for aminoglycosides usually includes the application of high-

dose, once-daily dose or high-dose, extended interval dosing with their maximal effect at C_{max}/MIC ratio of 10–12 [60]. Matthaiou et al. (2014) extensively reviewed aminoglycoside PK/PD properties in critically ill and intensive care unit (ICU) patients, emphasizing the importance of daily therapeutic drug monitoring as aminoglycoside PK changes over time [61]. Among aminoglycosides, amikacin and tobramycin are the antibiotics which are the most commonly re-evaluated in terms of PK/PD properties for different patient profiles. In a recent study conducted to optimize adult initial amikacin dose, the validated dosage regimen of amikacin (15 mg/kg/day) seemed to be suboptimal and 2500 mg single initial dose was recommended as individualized dose for adults between 40 kg and 200 kg [62]. Optimal dosage regimen of tobramycin which is a common antibiotic for the treatment of *P. aeruginosa* infections in children with cystic fibrosis (CF) was studied by comparing one, two, or three times administration of tobramycin for a dosing regimen of 10 mg/kg/day [63]. While all administrations equally achieved optimal bactericidal activity for all patients with MICs $\leqslant$1 μg/mL, none reliably achieved the PD target for MICs $\geqslant$2 μg/mL, a single administration per day reached the target in all subjects with MICs <2 μg/mL and recommended by the authors for clinical environments with continuous MIC values.

Fluoroquinolones, ciprofloxacin and ofloxacin being the most widely used ones, accumulate at higher concentration inside cells, thus can be used also against intracellular pathogens like *Listeria monocytogenes*, *Salmonella* spp., *Legionella pneumophila* and *Mycobacterium* spp. and opportunistic intracellular pathogens such as *S. aureus* [32,64]. Ciprofloxacin is particularly the most active against *P. aeruginosa.* Their primary route of excretion is renal mechanisms but some of them such as moxifloxacin are eliminated by hepatic metabolism. The PK/PD features of fluoroquinolones are similar to those of aminoglycosides, i.e. they show rapid, concentration-dependent killing profile and prolonged persistent effects [42]. For both aminoglycosides and fluoroquinolones, a C_{max}/MIC ratio of at least 10 within the first 24 h of treatment results in ca. 90% clearance [65]. AUC/MIC level is mostly associated with the efficacy of the fluoroquinolones, especially for the newer ones having a long half-life and broad-spectrum activity against both susceptible and resistant organisms, yet the C_{max}/MIC can be critical to limit the selection of resistant bacteria [28,66,67]. Chigutsa et al. (2012) studied PK/PD of ofloxacin which is routinely used against multidrug-resistant tuberculosis (MDR-TB) in South Africa [68]. An AUC/MIC ratio of 100 is known as an ideal minimal value based on *in vitro*, animal and human studies with *Mycobacterium tuberculosis*. In spite of limited sample size, it was concluded that currently recommended dose (800–1600 mg/day) of ofloxacin was inadequate so that the use of higher doses of ofloxacin or different fluoroquinolones was required for better efficacy. In another study conducted with fluoroquinolones levofloxacin and moxifloxacin for severe lower respiratory tract infections, it was demonstrated that both antibiotics display favorable action in patients, but higher levofloxacin dose was recommended against less sensitive pathogens [69].

Glycopeptides show time-dependent killing activity with poor tissue diffusion and low dosages have been recommended due to nephrotoxicity [23,60,70]. They have variable distribution profile in body and are mainly excreted by kidneys [42]. The area under AUC/MIC highly predicts the efficacy of glycopeptides, especially vancomycin and teicoplanin [70,71]. Vancomycin was used as a first-line antibiotic against MRSA and its dosage regimen was recently reconsidered due to the appearance of vancomycin intermediate-susceptible *S. aureus* and VRE [72,73]. Clinical studies and meta-analysis showed that continuous infusion (CI) of vancomycin achieved target concentration efficiently with lower nephrotoxicity risk [74,75]. In another study, CI of vancomycin was evaluated on non-intensive care unit patients and similar results were obtained [76]. In the study conducted by Lenhard et al. (2016), clinically applicable dosage of vancomycin against two MRSA strains in a HFIM model was inefficient and even an AUC/MIC of 400 could be insufficient to defeat *S. aureus* resistance [77]. Indeed, because of the increased resistance to this antibiotic along with its low penetration to infection sites and nephrotoxicity risks, it became wiser to look for newer antistaphylococcal agents like linezolid [78]. In a recent study, it was demonstrated that a target AUC/MIC of $\geqslant$ 900 μg.h/mL teicoplanin is necessary to obtain an efficient bacteriological response in patients with MRSA infections [79].

Linezolid is the only oxazolidinone antibiotic which was approved by FDA and provides an effective alternative against MRSA and VRE infections [23]. It acts in a time-dependent manner with minimal persistent effects, and shows a wide interpatient variability for which therapeutic drug monitoring and long-term administration are advised for critically-ill patients [23,42,80]. In a study aimed to compare the PK/PD of linezolid against *Enterococcus faecium* and *S. aureus*, a modified sigmoidal maximum effect (E_{max}) PK/PD model was defined by which concentration- and time-dependent effects of linezolid are quantitatively measured and interpreted. The drug was more effective against *S. aureus* than against *E. faecium* (E_{max} 1.8-fold higher) at a comparable potency [81]. In a recent study conducted in critically ill patients receiving renal replacement treatment, the PK/PD parameters of 600 mg/12 h linezolid were compared in two different conditions; continuous venovenous haemofiltration (CVVHF) and continuous venovenous haemodiafiltration (CVVHDF) [82]. In addition, they used Monte Carlo simulations to define best dosage regimens reaching PD targets in the patients. There were no significant differences between CVVHF and CVVHDF in terms of PK of linezolid and a high variability in C_{max} and AUC was observed which resulted in suboptimal achievement of PD targets at MIC = 2 mg/L. It was concluded that the patient features and the MIC values of bacteria have more influence on achieving optimal serum concentrations for linezolid than the methods of renal replacement therapy.

Macrolides have the highest accumulation capacity in eukaryotic cells among the antibiotic classes, and their wide distribution throughout the body makes them an ideal drug for fight against intracellular pathogens [42]. In terms of their PK activity, the members of this group show differences in that while the administration of erythromycin is based on the parameter T > MIC, both T > MIC and AUC/MIC are the crucial parameters for the efficacy of azithromycin and clarithromycin [23,83]. In a recent study, it was shown that current azithromycin doses (500–600 mg in combination therapy) are not sufficient for optimum bacterial killing in pulmonary *M. avium* infections and only a much higher dose (8 g/day) achieves the target point [84]. Yet, these findings were based on bacterial response and further analyses with clinical studies are expected. Ikawa et al. (2014) reported site-specific bronchopulmonary PKs of two macrolides, clarithromycin and telithromycin [85]. Population mean parameters were distribution volumes of central, peripheral and epithelial lining fluid (ELF) compartments, absorption rate constant, clearance and transfer rate constants connecting compartments. The breakpoint MIC values indicated that twice-daily doses of 250 and 500 mg of clarithromycin were effective against *S. pneumoniae*, *S. aureus*, *M. catarrhalis* and *Haemophilus influenzae* isolates for which MICs were $\leqslant$0.5 and $\leqslant$1 mg/L, respectively. For telithromycin, on the other hand, once-daily doses of 600 and 800 mg achieved a $\geqslant$90% probability in ELF in all isolates except for those of *H. influenzae*.

In polymyxin classes of antibiotics, there are two clinically used agents called colistin and polymyxin B [23]. Despite decrease in their clinical use due to nephrotoxicity and neurotoxicity issues in the 1970s, they continued to be the subjects of PK/PD analysis

against the pathogens resistant to all other antimicrobials [86]. Colistin in two forms, namely colistin sulphate and sodium colistin methanesulphonate is widely used for the treatment of *P. aeruginosa* and *K. pneumoniae* infections [23,87]. It has concentration-dependent activity and is excreted by nonrenal way [23]. Recent studies show that AUC/MIC is an important parameter for effective action of colistin and about 2 mg/L plasma concentration can be sufficient for the isolates of *P. aeruginosa* and *Acinetobacter baumannii* with MICs $\leqslant$1 mg/L [88,89]. In neutropenic mouse thigh and lung infections with *P. aeruginosa* and *A. baumannii*, exposure-response relationships between unbound colistin in plasma and antibacterial activity were studied and PK/PD target values were determined [90]. Using a static *in vitro* time-kill data, an *in silico* PK/PD model for *P. aeruginosa* exposed to colistin was developed and applied to an *in vivo* study in which the effect of colistin on *P. aeruginosa* was studied in the thigh infection model. The PK/PD model successfully predicted *in vivo* results from mice, showing an effective utility in the area of drug development [91]. Polymyxin B (PB) is used as a last-line defense against MDR Gram-negative pathogens and the study conducted with *P. aeruginosa* strains revealed that the activity of PB is concentration-dependent and AUC/MIC is the main PK/PD parameter [92]. As it seems that traditional administration of PB is not efficient against Gram-negative ESKAPE (*E. faecium*, *S. aureus*, *K. pneumoniae*, *A. baumannii*, *P. aeruginosa*, and *Enterobacter* species) pathogens, Tsuji et al. (2016) investigated the effect of increasing dosage regimen of PB in the HFIM against *A. baumannii* strains [93]. Although the authors observed a quick decrease in the total population within the first 6 h of PB administration, it was unexpectedly revealed that increasing PB exposures caused increase in resistant *A. baumannii* subpopulations over 336 h. Due to increment in resistance with high dose of PB, the use of polymyxins as a part of combination therapy was recommended. Rigatto et al., (2015) compared PB combination therapy and PB in monotherapy in critically ill patients having extensively drug resistant *P. aeruginosa* or *A. baumannii* infections [94]. The results indicated that combination therapy with a β-lactam or carbapenem provided significant decrease in the mortality risk of the patients when compared to monotherapy.

Daptomycin is a lipopeptide antibiotic which is generally used against Gram-positive pathogens including MDR *Staphylococci* and *Enterococci* species and its bacterial activity is dependent on concentration with AUC/MIC parameter of PK/PD [95]. A population-based study revealed that renal function was an important factor for daptomycin clearance and adjusted dosage regimen was recommended for patients especially on dialysis [96]. For dose optimization, Garonzik et al., (2016) suggested an active fraction approach to evaluate PD of daptomycin, i.e. quantification of active daptomycin concentration in human serum *in vitro* against MRSA [97]. In addition to daptomycin monotherapy, studies were conducted to investigate the effect of combination therapy with different antimicrobial agents. Garrigos et al., (2010), for example, examined the efficacy of daptomycin in combination with rifampin against rifampin resistant or susceptible MRSA in experimental foreign-body infection [98]. While high dose daptomycin was quite efficient in monotherapy, it enhanced the activity of the rifampin combination against MRSA in such infections. Another study which employed daptomycin in combination with β-lactams suggested a promising alternative treatment for MRSA infections [99]. Recently, synergistic effect of daptomycin and ceftolozane/tazobactam combination was evaluated in a hollow fiber (HF) PK/PD model against MRSA strains. Although there was no significant synergistic activity between ceftolozane and daptomycin, the addition of tazobactam enhanced the daptomycin efficacy against daptomycin-susceptible MRSA strains [100].

2.4. Toxicity of antibiotics

Antibiotic-related toxicity mainly results from administration of high dose, long term administration of high-dose of antibiotics or low tolerability of antibiotics in special patients with different conditions such as renal dysfunction. While the development of antibiotics can be halted due to inefficient antimicrobial activities and unusual PK behaviors, life-threatening adverse effects have been the primary factors for withdrawal of antibiotics from the market [101]. Although β-lactams are generally considered to have a wide safety range, they can lead to dose-related adverse effects [102]. Penicillins may exhibit side effects such as hepatotoxicity, neutropenia and encephalopathy [103,104]. They may cause a transient increment in liver enzymes and neuronal excitation due to inhibition of gamma amino butyric acid (GABA) system [105,106]. In a study reporting 391 patients with antibiotic-related encephalopathy (ARE), penicillins were most commonly associated with such cases accompanied by myoclonus or seizures [107]. Cephalosporins can cause neutropenia, nephrotoxicity and neurotoxicity in large doses [108]. High intratubular concentration of cephalosporins can result in lipid peroxidation and membrane protein acylation in addition to mitochondrial toxicity [109] while neurotoxicity is induced due to a reduction in GABA release from nerve terminals [110]. In a case report, high dose cefazolin induced generalized tonic clonic seizures in a patient with chronic kidney disease [111]. For another cephalosporin, cefepime, a high-dose therapy could lead to neurological toxicity such as ARE, seizures and mycoclonia in febrile neutropenic patients having mild renal dysfunction [112]. Another subclass of β-lactams called carbapenems is more linked to neurotoxicity than penicillins and cephalosporins when administered at excessive doses due to the interaction with GABA receptors [113]. High dosage regimen of imipenem/-cilastatin increases seizure risk in moderately to severely ill patients [114]. Even clinical doses could have risk in patients with different conditions. Lee and his co-workers (2015) reported a case study regarding four regular hemodialysis (HD) patients who were administrated with the recommended dose of 500 mg ertapenem, but could not tolerate this and developed ertapenem-associated central nervous system (CNS) toxicity [115].

For aminoglycosides, there is a small difference between therapeutic and toxic dose levels and these antibiotics are mostly associated with ototoxicity and nephrotoxicity [116,117]. In hair cells of ear, aminoglycosides can induce disarray of stereocilia and increase in reactive oxygen species (ROS) formation [118]. High concentration of aminoglycosides accumulates in the lysosomes of renal tubular cells where they can interfere with important biological pathways such as mitochondrial respiration, protein synthesis and sodium-potassium pump [119]. In one study with Buruli Ulcer (a tropical infectious skin disease) patients, toxicity of prolonged streptomycin administration was evaluated and a persistent hearing loss was observed in adult patients. In addition, transient nephrotoxicity occurred in both children and adult patients [120]. When high concentration of another aminoglycoside tobramycin was parenterally administered to CF patients, it was also associated with nephrotoxicity and ototoxicity [121]. Moreover, retinal toxicity can be seen after aminoglycoside administration even though it is quite rare. In a case report including a patient with suturless vitrectomy for the repair of retinal detachment, it was reported that high amount of gentamicin could accumulate in the macular area of eye and lead to a permanent effect to the patient's vision [122]. Amikacin-related macular toxicity in retina was also recorded [123].

Fluoroquinolone antibiotics are mostly associated with cardiovascular disorders, tendinopathy and phototoxicity in addition to the other rare adverse effects such as neuropathy and hepatotoxicity. Fluoroquinolones can block the cardiac voltage-gated potas-

sium channels [124] and it is suggested that fluoroquinolone-induced tendinopathy can be due to direct toxic effect on collagen, oxidative stress or allergic mechanisms [125]. Recently, FDA advised limited use of fluoroquinolones due to disabling adverse effects, especially for patients with acute bronchitis, acute sinusitis and uncomplicated urinary tract infections (UTI) [126]. According to FDA's Adverse Event Reporting System, fluoroquinolone use seems to be linked to increase in tendon rupture risk, with the greatest risk associated with levofloxacin [127]. In addition to tendinopathy, levofloxacin-associated CNS toxicity such as orofacial dyskinesia was reported in a patient with mild renal dysfunction [128]. Etminan et al. (2014) managed a case-control study within a cohort of men aged 45–80 years to evaluate the risk of oral fluoroquinolones use, demonstrating an increased risk of peripheral neuropathy [129]. In another study, ocular phototoxicity of four fluoroquinolones; ciprofloxacin, lomefloxacin, norfloxacin and ofloxacin was recorded and the authors recommended precautions such as UV blocking sunglasses when using these antibiotics [130]. Both norfloxacin and moxifloxacin induced concentration-dependent loss in melanocytes viability and suppression of melanin biosynthesis and especially norfloxacin inhibited cellular tyrosinase activity [131].

Vancomycin and teicoplanin are the most commonly used among glycopeptide antibiotics although new generation glycopeptides (especially lipoglycopeptides) such as telavancin, and dalbavancin have been developed [132]. Vancomycin is generally used in the treatment of MRSA infections and dosage regimen as well as interval of administration is an important issue in terms of side effects such as nephrotoxicity and phototoxicity [133]. Vancomycin may damage auditory nerve resulting in hearing loss and production of ROS is known as the most possible mechanism for vancomycin-induced nephrotoxicity [134,135]. A study with 60 patients having chronic MR staphylococcal prosthetic hip infections was conducted to evaluate the safety issues of continuous intravenous infusion of high dose vancomycin therapy [136]. Although the therapy was effective, 32% of the patients had mild and reversible nephrotoxicity. In another study of nephrotoxicity risk evaluation in patients with high-dose, prolonged vancomycin treatment, 24 out of 176 patients suffered from nephrotoxicity and a linear relationship was proposed between nephrotoxicity risk and duration of treatment [137]. Teicoplanin, being used in the treatment of Gram-positive infections including MRSA, is known to have lower toxicity than vancomycin. Although a previous study examining 549 patients treated with a standard (400 mg/day) or high dose (600 mg/day) of teicoplanin suggested no difference in terms of the incidence of drug toxicity [138], but a more recent study revealed high dose teicoplanin (400 mg/12 h)-induced pancytopenia in a patient diagnosed with pneumonia and UTI [139]. Neutropenic sepsis induced by teicoplanin was reported in an old patient with cardiac surgery [140]. Despite the rareness of cross-reactivity between vancomycin and teicoplanin, Yang et al. (2014) reported a case of Stevens–Johnson syndrome (SJS) induced by sequential therapy with teicoplanin and vancomycin in a patient with chronic obstructive pulmonary disease [141].

Macrolides are generally associated with cardiac toxicity, gastrointestinal disturbances, hepatotoxicity and ototoxicity [142]. A study tested cardiotoxic effects of three macrolides; azithromycin, erythromycin and clarithromycin on isolated rat heart mitochondria [143]. ROS formation was induced by these antibiotics and swelling of mitochondria following membrane permeabilization took place. Liver injury caused by azithromycin was the subject of another case report [144]. Macrolides can transform into nitrosoalkanes which bind to SH groups of proteins leading to hepatocellular necrosis [145]. Clarithromycin-induced neurotoxicity and hearing loss were also documented in the literature [146,147].

Polymyxins including colistin (polymyxin E) are mostly linked to neurotoxicity and nephrotoxicity [148]. Accumulation of colistin and elevated level of amino acid neurotransmitters in mouse brain were reported that the effects of colistin on mitochondrial activities could have a role in neurotoxicity [149,150]. Since colistin is considered as 'last-line' defense against MDR Gram-negative bacterial infections, its administration and dosage regimen should be adjusted carefully [87]. Neurotoxicity and renal toxicity were reported in a 51-year-old man after therapy with colistin [151]. Discontinuation of colistin resulted in recovery of neural functions in the patient. To minimize the side effects of colistin, Dewan and Shoukat (2014) proposed the application of high doses at prolonged intervals [152].

Although drug toxicity is mostly dose-related, some antibiotics can cause toxicity which is not related to dosage regimen. β-lactams, especially penicillin are very well-known to be associated with hypersensitivity reactions due to its major and minor determinants formed when it is metabolized. When fluoroquinolones are metabolized, reactive intermediates are formed via some mechanisms such as oxidation [153]. These products can bind to proteins and form hapten-protein complexes which are recognized by immune system and may cause hypersensitivity reactions [154]. In addition to these reactions, the structures of some fluoroquinolones such as ciprofloxacin and tosufloxacin containing C7-piperazine or C7-pyrrolidine on the quinolone nucleus have an important role in GABA-binding to its receptors which may lead to CNS toxicity [155]. It is known that oxazolidinones can inhibit mammalian mitochondrial protein synthesis (MPS) by interacting with mitochondrial ribosome which may account for clinical adverse effects of these antibiotics [156] which could be prevented by some structural modifications of the C-ring of the antibiotic [157].

To avoid time-consuming experiments during drug discovery and development, structural toxicity assessment databases such as MultiCASE Expert Systems and DEREK have been developed [158]. More recently, a new open Web-based platform called Tox-Alerts (http://ochem.eu/alerts) was introduced. This platform currently includes more than 600 structural alerts including compounds undergoing metabolic activation and those forming reactive metabolite, thus, leading to adverse reactions [159].

3. Antibiotic resistance and multidrug efflux pumps

3.1. General outlook to antibiotic-resistant pathogens, mechanisms of resistance and transfer

As declared and warned by many authorities, we are now in the "post-antibiotic era," exposed to a global "antibiotic resistance (AR) crisis" [10,160]. With respect to their clinical impact, economic impact, incidence, 10-year projection of incidence, transmissibility, availability of effective antibiotics, and barriers to prevention, the Centers for Disease Control and Prevention classified the pathogens concerning the degree of threat (Table 1) [161].

Nosocomial infections caused by AR "ESKAPE" pathogens which represent the vast majority as well as many other AR ones continue to spread and account for a significant worldwide morbidity and mortality [162–164]. Among Gram-positive representatives of ESKAPE, MRSA and VRE stand as the biggest threats. MRSA, as the first major player in the AR crisis, was recently estimated to be responsible for 60–89% of nosocomial infections. It is resistant to numerous penicillin-like β-lactam antibiotics primarily due to expression of the *mecA* gene which encodes the low affinity penicillin binding protein PBP 2a [165]. MRSA can still be treated with other antibiotics like glycopeptides, linezolid, tigecycline, daptomycin, and some newer β-lactams in spite of a couple of reports

Table 1
Bacterial pathogens of three different threat levels [161].

Threat level: urgent	*Clostridium difficile*
	Carbapenem-resistant Enterobacteriaceae (CRE)
	Drug-resistant *Neisseria gonorrhoeae*
Threat level: serious	Multidrug-resistant *Acinetobacter*
	Drug-resistant *Campylobacter*
	Extended spectrum β-lactamase producing Enterobacteriaceae (ESBLs)
	Vancomycin-resistant *Enterococci* (VRE)
	Multidrug-resistant *Pseudomonas aeruginosa*
	Drug-resistant non-typhoidal *Salmonella*
	Drug-resistant *Salmonella typhimurium*
	Drug-resistant *Shigella*
	Methicillin-resistant *Staphylococcus aureus* (MRSA)
	Drug-resistant *Streptococcus pneumoniae*
	Drug-resistant tuberculosis (MDR-TB, XDR-TB)
Threat level: concerning	Vancomycin-resistant *Staphylococcus aureus* (VRSA)
	Erythromycin-resistant Group A *Streptococcus*
	Clindamycin-resistant Group B *Streptococcus*

on emergence of resistance to some of them [166]. VRE retains a major role among hospital patients particularly by colonizing on indwelling medical devices, at the same time exerts major therapeutic challenge because of the availability of only few antimicrobial options. Some of them express enterococcal surface protein for drug resistant biofilm formation. Though VRE is known to produce several resistance genes, the most common, as with VRSA, is *vanA* rendering pathogen resistant to both vancomycin and teicoplanin via alteration of the peptidoglycan synthesis pathway [165]. Being both community- and hospital-acquired with its highly resistant spores, *C. difficile* is "urgent" in that it can lead to life threatening complications after its overgrowth in gastrointestinal tract following antibiotic usage. The first report on emergence of a hypervirulent strain of fluoroquinolone resistant *C. difficile* in North America [167] was followed by the other alerts within last decade [168–171].

Of MDR-TB resistant to the first line combination therapy of rifamycin, isoniazid, and pyrazinamide, 9.6% are estimated to be extensively drug resistant (XDR-TB), i.e. further resistant to a second-line fluoroquinolone and aminoglycoside [10,165]. Increasingly pan-resistant Gram-negative pathogens, on the other hand, are currently even more serious than the Gram-positive ones especially in health care settings since they are naturally resistant to many antibiotics owing to higher prevalence of efflux pumps (EPs). CRE are becoming increasingly challenging as they exhibit XDR phenotypes, and their infections are associated with high mortality rates (up to 70%) [166]. Colistin, fosfomycin, tigecycline and doripenem are the only therapeutic options left [172]. The toughest infections of Gram-negative pathogens are most commonly caused by *K. pneumoniae* followed by *P. aeruginosa* and *Acinetobacter*. Gram-negative MDR pathogens have become increasingly prevalent in community as well, including ESBL-producing *E. coli* and *N. gonorrhoeae* resistant to fluoroquinolones, tetracycline, penicillin and azithromycin or expanded-spectrum cephalosporins [173]. The carbapenemases in CRE can belong to Class A (*K. pneumoniae* carbapenemases, KPC, most common in the U.S.), Class B (metallo-β-lactamases, most common in the Indian Subcontinent as well as in specific European countries) and Class D (OXA-48-like carbapenemases, the epicenter in Turkey and surrounding countries) [174]. Colistin is one of the last-resort antibiotics for MDR Gram-negative pathogens. The first colistin-resistance gene (*mcr-1*) which is plasmid-borne that is carried in a plasmid and can be transferred between bacterial strains was described very recently [175–177].

The molecular mechanisms of AR have been extensively reviewed to date [178–184]. Increased efflux of antibiotics, decreased influx, target modification, target amplification, repair of damaged target, enzymatic inactivation of antibiotics, sequestration of antibiotic, target bypass (acquisition of alternative metabolic pathways), protection of target, intracellular localization, and biofilm formation, the latter as an indirect mechanism are considered among the mechanisms (Fig. 2) that can be acquired by horizontal gene transfer besides mutations (spontaneous mutations, hypermutators and adaptive mutagenesis) [185–187,189]. Physical (wind, water) and biological forces (human activities, animals, insects and birds) cause widespread dissemination of resistance genes throughout many environments [179].

Von Hoek (2011) provided very detailed lists of well-known AR genes, their specific function and distribution in bacterial genera for each chemical class of antibiotics [180]. Global regulators modulating AR were recently reviewed by Corona et al. (2016) [183]. Conjugative plasmid-mediated transfer is the most common mechanism for AR gene pick-up and transfer. Even when a conjugative plasmid cannot replicate in the new host but contains a resistance gene on a transposon, it can translocate to the bacterial chromo-

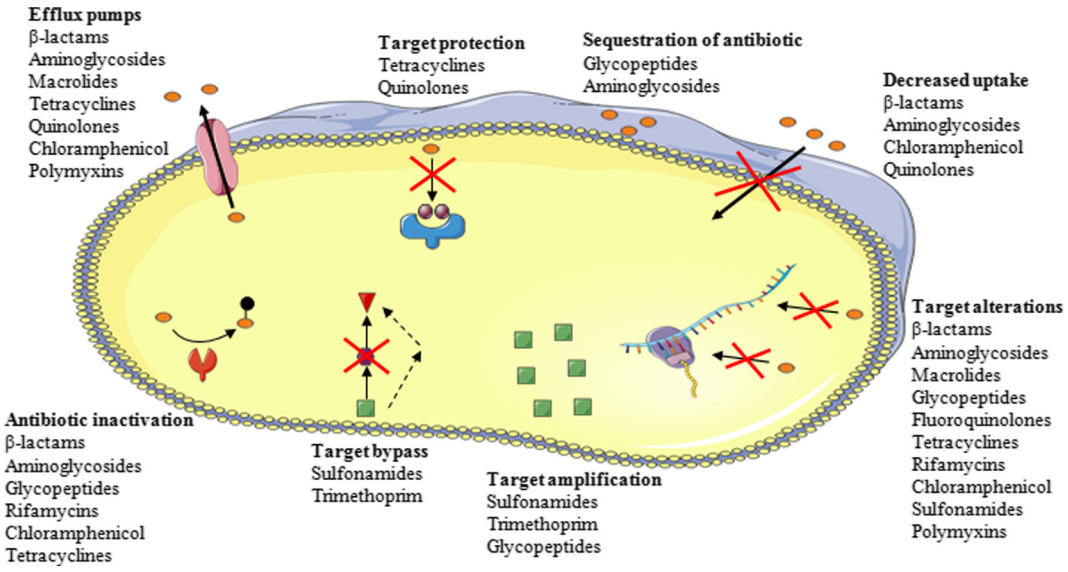

Fig. 2. Major mechanisms of bacterial resistance to different classes of antibiotics [Adapted from 180,183,185,187,188 and 189].

some. As known well, a simple transposon does contain an accessory gene often encoding AR together with the transposase, but cannot make conjugal transfer to other bacteria. Integrons, on the other hand, are genetic structures that are able to acquire, express and exchange novel 'AR gene cassettes' often associated with transposons [178,190]. Integrons' promoter element, P_{ant}, for AR gene cassette expression may also contribute to the expression of previously silent AR genes. Mobile integrons which are located on plasmids and transposons usually encode AR genes, capture and spread of these genes occurring by site-specific recombination, *intI* gene coding for integrase and *attI* target site for integration to an AR gene cassette at the *attC* of the latter [191]. These integrative conjugative elements (ICE; conjugative transposons) are mosaics, do generally have a modular organization (conjugation, recombination, regulation, and accessory modules coding for AR). Since they do not contain an origin of replication, they have to integrate into either a plasmid or chromosome, conferring them a wider host range than plasmids [192]. Of course, environmental bacteria and pathogens must inhabit the same niche for close proximity needed for above-mentioned horizontal gene transfers (HGT) to occur, as in the case of gut systems [190]. In many ecosystems, the viral communities persisting in the environment mediate transduction which is not considered to be the most frequent mechanism of AR gene transfer [193]. Since antibiotic usage is known to induce phage-mediated transfer [194], the relevant phage populations' mobility might have become as a frequent vehicle. The association between phages and other mobile genetic elements has recently been evaluated with respect to their roles in spread of AR genes [195].

3.2. Resistomes and resistome analysis for finding novel antibiotic resistance determinants

The term "resistome" refers to entire gene sets that contribute directly or indirectly to AR in pathogens, antibiotic producers and benign environmental bacteria in the world [196–198]. Intrinsic resistome encompasses the genes encoding proteins that confer antibiotic insensitivity (rather than resistance!) via inactivation of drugs, altering their targets or altering cellular permeability as well as those encoding metabolic and regulatory proteins. The latter group sharing common ancestry with housekeeping genes is termed as "proto-resistance genes" by having a potential to evolve into resistance elements through mutations and natural selection [199,200]. In other words, highly efficient resistance elements are derived from existing biochemical mechanisms, the proto-resistome. To exemplify, the housekeeping proteins like GCN5 protein acetyltransferases and D-Ala-D-Ala ligase are similar in structure and function to acetyltransferases and VanA conferring resistance to aminoglycosides and vancomycin, respectively [196]. Like proto-resistance genes, silent resistance genes do not contribute to phenotypic resistance (i.e. no growth in presence of antibiotic), but unlike proto-resistance genes they can be identified based on sequence homology to known resistance determinants [190]. The elements of intrinsic resistome are independent of previous antibiotic exposure and are not due to horizontal gene transfer [183]. In intrinsic resistance, bacteria are phenotypically resistant due to a trait common to all taxonomically related bacteria (e.g. LPS, EPs), resistance genes are located only on chromosome and only vertically transmitted, however acquired resistance genes are not natively present in other taxonomically related bacteria, present on a plasmid or on chromosome and can also be horizontally transferred [190]. Olivares et al. (2013), on the other hand, prefer to define phenotypic resistance as a situation in which a bacterial population, usually susceptible to antibiotics becomes transiently resistant when persistence, biofilm formation and swarming become a part of the intrinsic resistome only under specific growing conditions [198]. Since the emergence of resistance in pathogens generally results from natural selection in the clinical care settings, clinical resistome differs from intrinsic resistance in that the mutations or acquisition of genes and their stable integration into the bacterial chromosome causing AR may lead to clonal dissemination of the resulting resistance only within geographic limits [196]. Therefore, restriction of resistome studies to such clinically important pathogens is vital on the basis of specific outbreaks.

As to the origin and evolution of AR genes, the evidence points to conservation of function from the cave to the clinic, demonstrating that resistance genes are present in the microbial pan-genome and resistome must have evolved long before the use of antibiotics became clinically common [190]. A screen of a sample of the culturable microbiome in a region of a cave in New Mexico that has been isolated for over 4 million years revealed bacteria highly resistant to structurally different antibiotics including daptomycin, aminoglycosides, macrolides; some strains were even resistant to 14 different commercially available antibiotics [201]. Metagenomic analyses of ancient DNA from 30,000-year-old Beringian permafrost sediments identified genes conferring resistance to β-lactams, tetracycline and glycopeptides [202]. HGT from suicide-avoiding antibiotic producers [199,203] is a realistic view knowing that actinomycetes are more ancestral than pathogenic bacteria [204,205]. However, Aminov and Mackie (2007) oppose this in that dissemination and penetration of AR genes from antibiotic producers were less significant and essentially limited to other high G + C bacteria [206]. On the other hand, none of these views would exclude the fact that mutations in chromosomal genes may be positively selected by antibiotic pressure which can accelerate mobilization through transposons (Tn) or insertion sequences (IS), i.e. transported to other bacteria via HGT [207–209]. Interestingly, besides DNA mutations, epigenetic inheritance of variant gene expression patterns was shown to drive evolution of AR in bacteria [210]. Schenk and de Visser (2013) emphasized the roles of pleiotropy and epistasis to predict the evolution of AR determinants [211]. According to Baquero et al. (2009), after origination in environmental bacteria and evolution in them for millions of years to play different functions like detoxification, signal trafficking or metabolic ones, the clinical and agricultural use of antimicrobials started to exert a strong selective pressure in the last few decades [212]. Thus just only a few genes should have disseminated among pathogens to form clinical resistome while the natural ones contain a large number of potential AR genes.

Analyzing resistome at different levels (PCR-based, biochemical or -omics-based) in both environmental and clinical microbiota does not only serve for finding existing and new resistance genes, but also for defining novel targets of inactivation to make bacteria more susceptible to antibiotics, thus improving the activity of drugs currently in clinical use [183,198,213,214]. In a recent study involving single genes, fecal samples from 120 infants collected at the ages of 5–31 weeks were subjected to qPCR for the detection of β-lactamase encoding gene *cfxA*, tetracycline resistance encoding genes *tetM* and *tetQ*, macrolide resistance encoding gene *ermB*, aminoglycoside resistance encoding gene *aac(6′)-aph(2″)* and quinolone resistance encoding gene *qnrS* in gut resistome [215]. The changes in resistome over the course of several months were evaluated. To date, functional metagenomics, by identifying genes that are not closely related to known resistance genes has been the approach of choice. At the same time, knowing of the reservoirs of AR determinants, advances in next-generation sequencing enabled extensive metagenomic research in soil, human, food animal, plant, freshwater and marine biomes which led to an explosion of sequences that are presumptively associated with AR. Bioinformatics methodology to identify AR from sequence-based

metagenomes were explained by Schmieder and Edwards (2012) [188].

Currently, short-read sequencing technologies fail to assemble complex resistance gene loci, making difficult to reliably characterize mobile genetic elements [216]. In addition, the studies inferring the function solely *in silico* by similarity analyses make databases increasingly comprehensive with a large amount of 'noise' [217,218]. Elbehery et al. (2016) recently illustrated how to optimize and improve existing AR detection methodologies from metagenomic datasets [219]. According to Fitzpatrick and Walsh (2016), in order to identify potential hotspots of resistance, i.e. AR gene risk using metagenomic data, the limits of detection of this technique as well as the ways to normalize datasets containing vastly different diversities of microbes and genome sizes must be identified at the very first place [220]. The bottlenecks that affect the transfer of antibiotic resistance genes to human pathogens (ecological connectivity, the founder effect and fitness costs) were discussed and the rules for estimating the AR risks by evaluating the likelihood of their introduction into human pathogens were proposed by Martínez et al. [217,218]. The alert levels (resistance readiness condition (RESCon); from RESCon 1, the highest risk, to RESCon 7, the lowest risk) were outlined.

Soil has been the source of the majority of AR genes discovered. In one of the earlier studies on soil metagenomics with particular reference to AR determinants, nine clones expressing resistance to aminoglycosides and one expressing tetracycline resistance were identified. The resistance mechanisms included enzymatic inactivation of aminoglycosides and efflux of tetracycline as based on the predicted amino acid sequences, and almost all the sequences were considerably different from previously reported ones [221]. This study was followed by two functional metagenomics reports on bifunctional β-lactamases in a remote Alaskan soil [222] and apple orchard soil [223]. When three different soils were subjected to functional metagenomics, 11 new AR genes conferring resistance to ampicillin, gentamicin, chloramphenicol and trimethoprim were identified [224]. Of particular interest was a new trimethoprim resistance gene with dihydrofolate reductase activity. A comprehensive large-scale environmental (non-clinical) resistome analysis was conducted to compare samples of different environmental origin to include Antarctic lakes, Arctic snow, chicken gut, cow gut, deep oceans, human feces, microbial fuel cell, mouse gut, ocean, halophile sediment, activated sludge and soil. Efflux pumps and genes conferring resistance to vancomycin, tetracycline or β-lactam antibiotics were the most common types of resistance found in these metagenomes [225]. Profiles of AR genes and mobile genetic elements in relatively pristine Tibetan environment were completely distinct from modern antibiotic resistome with low potential of AR genes to be transferred among bacteria [226]. As a last note for soil environments, functional metagenomics was also employed to investigate the capacity of soil microbiota to transfer AR to human pathogens, providing evidence for the transfer of AR cassettes from environmental bacteria to human pathogens for several different antibiotics [227].

As to gut metagenomics, expression libraries from healthy adult gut microbiota were used to functionally characterize AR genes, resulting in a total of 210 genes (95 unique resistance to include 27 unique β-lactamases) and were thought to act as a reservoir for AR genes in human pathogens [228]. Metagenome-wide analysis of gut microbiota from 162 individuals identified a total of 1093 antibiotic resistance genes with a high abundance of tetracycline resistance genes and showed that Chinese individuals harbor a higher abundance of antibiotic resistance genes than Danish and Spanish individuals. SNP analysis indicated that antibiotic resistance genes from the two European populations are more closely related while the Chinese ones are clustered separately [229]. The functional metagenomic survey of gut-associated resistomes pooled from 22 healthy infants and children mostly without recent antibiotic exposure identified clinically relevant resistance genes, a probable new resistance mechanism to trimethoprim-sulfamethoxazole being particularly interesting [230]. In roughly 3% of the total resistance-conferring contigs, AR genes were in synteny with mobile elements like transposons or integrons. Analysis of an antibiotically-naive, six-month-old infant gut functional metagenomic library revealed a diverse and abundant reservoir of aminoglycoside and β-lactam resistance genes [231]. Shotgun metagenomic sequencing of the gut microbiome of 35 Swedish students returning from exchange programs in Central Africa or the Indian peninsula showed increased abundance of genes encoding resistance to sulfonamide, trimethoprim and β-lactams with 2.6, 7.7 and 2.6 fold increase, respectively **[232]**. Very relevant to the transductional transfer mechanism (section 3.1), the ecological network of the phage metagenome was explored by sequencing murine fecal phage populations following antibiotic perturbation [194]. It was shown that antibiotic treatment enriches the phage metagenome for stress-specific and niche-specific functions and leads to a more highly connected phage-bacterial network for gene exchange.

To test the hypothesis that agricultural environments have different antibiotic resistance profiles than nonagricultural ones, the numbers and kinds of AR genes were quantified in publicly available metagenomic datasets from 26 environments with between 0.7 and 4.4% of all classified genes coding for AR and toxic compounds [233]. AR genes, including those conferring resistance to antibiotics important in human medicine like macrolides (*mphA* and *erm*), cephalosporins (*bla-TEM* and *blaCTX-M*), aminoglycosides (*aph* and aad) and tetracycline (*tet*) were abundant in Chinese swine farms where swine feed contains antibiotics and metals. In correlation particularly with tetracycline resistance, transposases were enriched up to 90,000-fold in manure samples and 1000-fold in the soil samples [234]. Hypothesizing that AR genes can transfer from animal feces to the environment through manure, the resistomes (collections of AR genes) of cow feces, manure, and soil samples collected from five dairy farms were characterized using a metagenomics approach [235]. The identified AR genes were associated with 18 antibiotic resistance classes across all samples, the most abundant genes were classified under multidrug transporters, followed by resistance to vancomycin, tetracycline, bacitracin, β-lactams and macrolide-lincosamides-streptogramin (MLS) efflux pump antimicrobials. Another study of the resistome in manure, soil and wastewater from North American dairy and beef production systems also employed shotgun metagenomics and identified 34 mechanisms of antimicrobial drug resistance, the majority belonging to tetracycline resistance [236].

Comparative metagenomics revealed that two types of effluents that are entering a river catchment contributed an array of genes, the most abundant being tetracycline resistance genes *tetC* and *tetW* from farm effluents and the sulfonamide resistance gene *sul2* from wastewater treatment plant effluents [237]. In another study, metagenomic detection of a wide spectrum of 323 AR genes and 83 human bacterial pathogens were combined with a correlation-based statistical approach to construct a network of their co-occurrence relationships in municipal sewage sludge digesters [238]. Unlike β-lactam resistance genes, multidrug and MLS tended to co-occur more with pathogens. Hatosy et al. (2015) uncovered the diversity of AR genes in diverse marine environments using functional metagenomics [239]. Antibiotic-resistant clones were found at all sites, with 28% of the genes identified as known AR genes (encoding β-lactamases, bicyclomycin resistance pumps, etc.). However, the majority of AR genes were not previously classified as such but had products similar to proteins such as transport pumps, oxidoreductases, and hydrolases.

Furthermore, 44% of the genes conferring AR were found in abundant marine taxa, making the ocean a global reservoir of potentially novel AR genes. As to the clinical applications, the advantages of metagenomics are the identification of unknown pathogens, especially unculturable ones and identification of AR of the community, even ones that are not present in the disease causing pathogen, but having potential of transfer due to the high mobility of many AR genes [240].

Gel-based or gel-free proteome analyses (each with inherent advantages over the other) are generally performed before and after subjecting the target resistant strain mostly along with the susceptible one to sublethal doses of the selected antibiotic [241]. Proteomics have the power of directly showing the actual players in cell physiology, its analytical capacity in a single experiment, identifying protein localization, post-translational modifications and protein turnover rates, all should be as important as the presence of the AR gene(s) though proteomics findings usually await confirmation by transcript or transcriptome analysis and/or inactivation of relevant genes. Likewise, transcriptomics is not sufficient alone to identify AR genes. For instance, under tetracycline stress, transcript levels of *A. baumannii* outer membrane proteins (OMP) were not significantly changed while the proteomic analysis revealed that they were significantly decreased in OM fraction, but increased in secretome [242]. OM proteomics of kanamycin-resistant *E. coli* identified MipA as a novel AR-related protein [243]. Since it is not sufficient to rely on identification of putative resistance genes, coupling functional metagenomics with proteomics to study AR in diverse environments allows the identification and analysis of potential resistance genes and their resultant proteins [244]. Very recent reviews of proteomics research that contributed to the mechanisms of AR have already been published [244–247]. The study of Forsberg et al. (2015), on the other hand, constituted an example to the power of combination of structural metagenomics and biological chemistry [248]. Analysis of certain genes' function that confer high-level tetracycline resistance by enzymatic inactivation as obtained from their soil functional metagenomic selections revealed catalysis of the oxidation of tetracyclines *in vitro* both by known mechanisms and a previously undescribed activity, the latter remains to be investigated for clinical isolates. When compared to the other systems of biology techniques, metabolomics has not yet found much use in understanding AR mechanisms. In one study with methicillin-resistant strains of *S. aureus*, up to 210 metabolites representing a substantial 50% improvement over previously published data could be identified by using hydrophilic interaction LC coupled to high-resolution MS [249].

3.3. Multidrug efflux pumps (MDEPs)

Five main multidrug efflux pump (MDEP) superfamilies are associated with AR in prokaryotes, as extensively reviewed earlier [250–254] and shown in Fig. 3.

These pumps can be plasmid- or chromosome-borne to contribute to both acquired and intrinsic AR [253]. While the SMR family, the MFS, the MATE family and the ABC superfamily are widespread in both Gram-negative and -positive microorganisms, the RND family is found only in Gram-negative bacteria. Whereas the ABC family transporters are the primary transporters utilizing ATP hydrolysis, the others are considered as the secondary transporters obtaining energy from proton or sodium gradient [254].

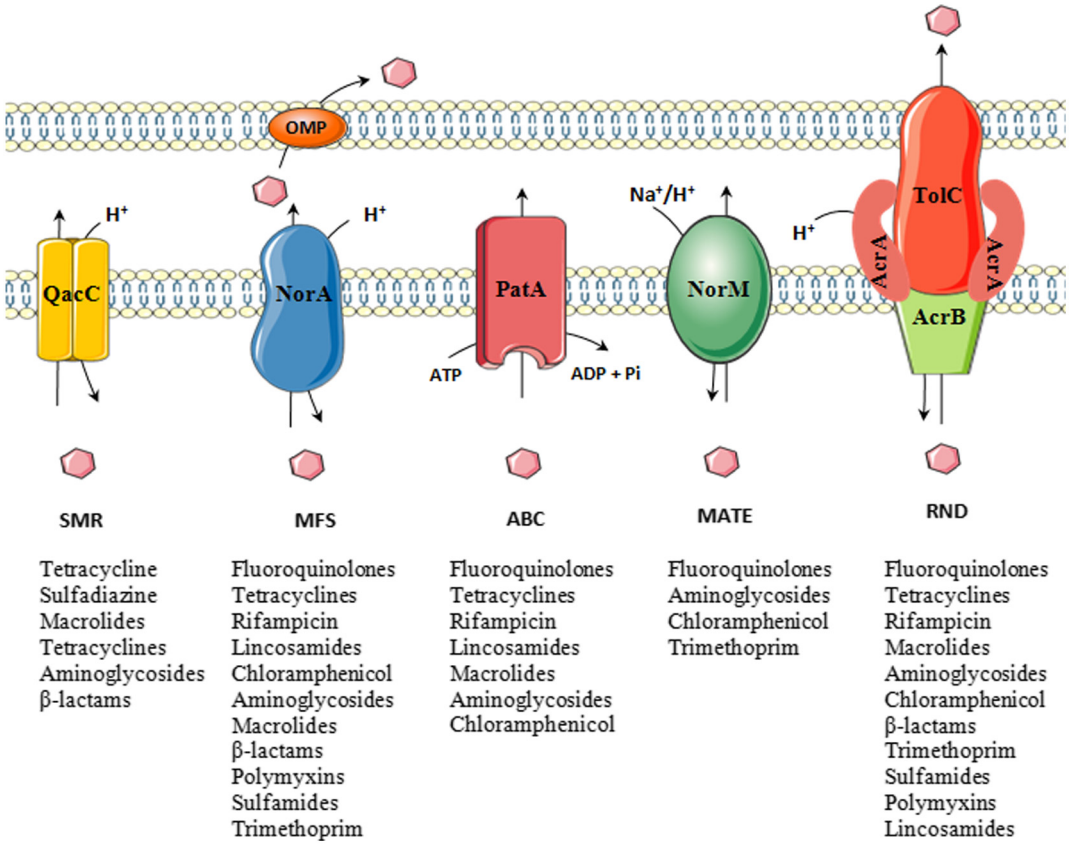

Fig. 3. Five main MDEPs with their well-known examples and antibiotic substrates. OMP: outer membrane protein; SMR: the small multidrug resistance family; MFS: the major facilitator superfamily; ABC: ATP-binding cassette superfamily; MATE: the multidrug and toxic compound extrusion family and RND: the resistance-nodulation-division family [Adapted from 250,251,253 and 254].

Some MFS pumps have single component to transport compounds from cytoplasm to periplasm while the others can function with OMPs to efficiently export compounds across the inner and outer membrane of Gram-negative bacteria [252]. RND family ones locate in the inner membrane and function as a system with a periplasmic adaptor protein and an OMP [182] and are associated with clinically important Gram-negative pathogens including *E. coli*, *A. baumannii*, *S. enteric*, *K. pneumoniae*, *P. aeruginosa* and *N. gonorrhoeae* [251,253]. Of RND-type EPs, AcrAB-TolC in *E. coli* and Mex efflux system in *P. aeruginosa* are the most studied EP systems which confer resistance to many antibiotics.

Despite the specificity of some EPs (e.g. tetracycline transporters), many of them can recognize a broad range of drugs contributing to MDR phenotype and understanding the molecular basis of multidrug binding properties, i.e. how structurally dissimilar compounds are recognized is widely achieved by molecular dynamics computer simulations [255,256]. In addition to their role in AR, MDEPs seem to be evolutionarily ancient detoxification tools providing extrusion of heavy metals, solvents, detergents and many other toxic materials, especially in soil- and plant-associated microbes have the highest numbers of EPs [257–260]. Since the late 1980s, the research on MDEPs increasingly continues to discover new transporters with novel molecular mechanisms [261,262]. The most-studied and well-known efflux pumps of clinically relevant pathogens with their substrates are summarized in Table 2.

RND-type MDEPs are the most prominent among Gram-negative pathogens. A structural research performed with *in vitro* random mutagenesis revealed that drug susceptibility of *E. coli* became altered when I38 and/or I671 isoleucine residues of AcrB pump was replaced by aromatic or more polar amino acids which affected low-molecular-weight drug efflux negatively while it increased resistance to selected high-molecular-weight drugs [264]. It appears that structural characterization of multidrug EPs can pave way for understanding their antibiotic selectivity. As in *E. coli*, AcrAB-TolC system is the main EP in *K. pneumoniae*, *Enterobacter* and *Salmonella* [253]. In a clinical isolate of *K. pneumoniae* displaying cross-resistance to quinolones, chloramphenicol and cefoxitin, a point mutation in the *oqxR* repressor gene was responsible for overexpression of OqxAB pump [265]. Ogawa et al. (2012) identified a new RND-type pump called KexD in *K. pneumoniae* which could cause increase in MICs of erythromycin, tetracycline, novobiocin and cloxacillin [261]. AR in *P. aeruginosa* is mostly associated with four RND-type EPs MexAB-OprM, MexCD-OprJ, MexEF-OprN and MexXY-OprmM. It has been known that antibiotic exposure in *P. aeruginosa* can lead to overexpression of EPs [266]. Riou et al. (2016) recently demonstrated that the overexpression of mexX and mexA in *P. aeruginosa* isolates from patients with nosocomial infections were significantly related to exposure of patients to cefepime and meropenem or ciprofloxacin and meropenem, respectively [267]. MexR (primary repressor), NalC and NalD (secondary repressor) are known as the transcriptional regulators of MexAB, but molecular mechanism of NalD action was unknown [268–270] till a recent study shed light on how EP expression is changed upon antibiotic exposure in *P. aeruginosa* [271]. Direct binding of novobiocin to NalD is responsible for dissociation of NalD from the promoter region on DNA and thus de-repression of MExAB expression. In *A. baumannii*, AdeABC, AdeFGH and AdeIJK are the clinically most important RND EPs [272]. The regulation of AdeABC and AdeFGH expression is mediated by AdeRS and LysR-type transcriptional regulator, respectively [272,273]. A new regulatory protein, AdeN, was identified for AdeIJK expression through whole-genome sequencing and finding that its inactivation in a susceptible *A. baumannii* strain led to an increase in resistance to erythromycin, tetracycline, β-lactams, quinolones and sulfonamides [274]. In a very recent study, Li et al. (2016) combined qPCR and 'Multiplexed Biology Phenotype Microarrays (PMs)' as a novel high-throughput phenotype screening strategy to screen phenotypes of putative drug transporters of *A. baumannii* heterologously expressed in *E. coli* [262]. Of 15 ORFs encoding putative efflux proteins, three were characterized as novel drug resistance genes one which is the first ABC drug resistance gene identified in *A. baumannii*, conferring resistance to 1,10-phenanthroline, gentamicin, kanamycin, chloramphenicol, oxytetracycline and chloroxylenol. The other two proteins were the members of the MFS-type transporters.

MFS transporters represent the largest EP group found in Gram-positive pathogens while this group is secondarily abundant in Gram-negative ones [275]. In addition to well-known NorA, B, C and D in *S. aureus* (Table 2), SdrM and MdeA are also found [276,277]. Overexpression of *norB* was significantly higher in ciprofloxacin-resistant MRSA than the susceptible ones [278]. An MFS-type EP called LmrS in *S. aureus* confers resistance to lincomycin, linezolid and kanamycin [279]. Another known MFS-type EP is PmrA in *S. pneumoniae* which confers fluoroquinolones resistance [252]. MDR (especially macrolide, chloramphenicol and tetracycline) of *S. pneumoniae* is usually associated with mobile genetic elements and two variants of *mef*, *mef*(A) and *mef*(E) are located on the defective transposons Tn1207.3 or Tn1207.1 and macrolide efflux genetic assembly (MEGA) element, respectively [280–282]. Horizontal transfer of these transposons may have role in the high prevalence of macrolide and tetracycline resistance among *S. pneumoniae*.

Sav1866 in *S. aureus* is the first characterized ABC-type MDEP and has a significant structural homology with human ABC transporter Mdr1 [283]. AbcA is another ABC-type pump in *S. aureus* conferring resistance to penicillin G, methicillin, cefotaxime and nafcillin [284]. Moreover, Yoshikai et al. (2015) demonstrated dual function of AbcA which secretes *S. aureus* cytolytic toxins such as phenol soluble modulins considered as high virulence determinants of community-acquired MRSA, indicating a relationship between virulence and drug resistance [285]. An important ABC-type EP is PatAB in *S. pneumoniae* and the reason behind constitutive overexpression of *patAB* in fluoroquinolone-resistant clinical *S. pneumoniae* isolates was investigated [286]. The authors reported three novel mutations in a Rho-independent transcriptional terminator structure located upstream of *patA*, leading to *patAB* overexpression and thus possibly high fluoroquinolone resistance in *S. pneumoniae*. MacAB plays a role in macrolide resistance in *E. coli* and it has a homolog in *N. gonorrhoeae* conferring the same function [287].

A well-known MATE-type EP NorM exporting fluoroquinolones is found in different pathogens including *N. gonorrhoeae*, *N. meningitidis* and *V. cholerae* [288,289]. Although it is known that MATE MDEPs export substrates with influx of H^+ or Na^+ as in the case of a new pump PdrM in *S. pneumoniae* which exports norfloxacin in exchange of Na^+ [290], *V. cholerae* NorM can simultaneously couple drug efflux to the proton- and sodium-motive forces [291]. In *S. aureus*, MepA belongs to MATE-type MDEPs and its expression is regulated by the transcription factor MepR whose defective function results in *mepA* overexpression [292,293].

Besides roles of MDEPs in AR, they have different physiological roles in microorganisms such as cell-cell communication, stress adaptation and survival of microbes in their niches and biofilm formation, all facilitating their pathogenesis [251,254,294]. KpnEF, a SMR-type MDEP found in *K. pneumoniae* is also directly involved in capsule synthesis indicating its contribution to virulence [295]. Padilla and co-workers (2010) demonstrated in murine models that *acrB* knockout resulted in increased antibiotic susceptibility and a decreased virulence of *K. pneumoniae* [296]. AcrAB can export bile salts and promote *E. coli* growth in the animal intestinal tract and the homologs of this protein display a similar function in var-

Table 2
The best-known MDEPs of some clinically important pathogens [Adapted from 252,253 and 263].

Pathogen	(Super) Family	Efflux Pump*	β-lactams	Aminoglycosides	Macrolides	Tetracyclines	Tigecycline	Fluoroquinolones	Trimethoprim	Lincosamides	Rifampicin	Chloramphenicol	Polymyxins	Novobiocin	Sulfamides
		MdtABC-TolC						*						*	
		AcrAB-TolC	*		*	*	*	*	*	*	*	*		*	*
	RND	AcrAD-TolC	*	*				*						*	
		AcrEF-TolC	*		*	*	*	*	*	*		*			
		OqxAB						*	*			*			
E. coli		EmrAB-TolC				*		*							
	MFS	MdfA						*	*			*			
		Bcr				*									*
	SMR	EmrE			*	*									
	ABC	MacAB-TolC			*										
	MATE	MdtK						*	*			*			
E. cloacae	RND	AcrAB	*	*	*	*	*	*		*	*	*		*	
		AdeABC		*	*	*	*	*	*			*			
A. baumannii	RND	AdeFGH				*	*	*	*	*		*			*
		AdeIJK	*		*	*	*	*	*	*	*	*		*	
	MATE	AbeM		*				*							
		NorA						*							
		NorB				*		*							
	MFS	NorC						*							
S. aureus		NorD				*		*	*				*		
	MATE	MepA					*	*							
	RND	AcrAB			*	*	*	*	*			*		*	
K. pneumoniae		OqxAB						*				*			
	SMR	KpnEF	*	*	*	*					*		*		
	MFS	KpnGH	*	*	*			*					*		
		MexAB-OprM	*		*	*	*	*	*		*		*		*
P. aeruginosa	RND	MexCD-OprJ	*		*	*	*	*	*		*		*		
		MexEF-OprM				*		*	*			*			
		MexXY	*	*	*	*	*	*							
		MexJK			*	*									
S. pneumoniae	ABC	PatA						*							
		PatB						*							
	MFS	Mef			*										

*EPs not shown in this table are mentioned in the text.

ious enteric species [297]. Δ*acrB* derivative of a clinical *K. pneumoniae* strain was significantly less virulent than its parental strain [265]. The AcrAB and TolC inactivation interfered with AR, fitness and virulence of clinical strains of *Enterobacter cloacae* in an experimental mouse model [298]. When *Salmonella enterica* serovar Typhimurium-susceptible BALB/c mice were infected with either wild-type or Δ*macAB* mutant strain, the mutant one was defective in colonization in liver. Although the mutant could invade macrophages, intracellular multiplication was highly reduced which could be ascribed to the importance of MacAB for survival under oxidative stress [299]. The OMP TolC is the central component of a number of bacterial efflux pumps, responsible for exporting cellular β-lactam antibiotics [300]. Pu et al., (2016) reported high level expression of multidrug efflux-related genes including *acrA*, *acrB*, *acrD acrF*, *macA*, *macB*, *emrA* and *emrB* and particularly TolC in persister cells of *E. coli* under β-lactam antibiotic treatment [301]. RND-type EPs AcrB and MdtABC were shown to play role in biofilm maintenance in *E. coli* [302]. A genome-wide transcriptional profiling in *P. aeruginosa* and chromatin immunoprecipitation analysis revealed that a transcriptional regulator BrlR had an important role in high-level biofilm tolerance to antibiotic through the activation of *mexAB-oprM* and *mexEF-oprN* genes [303]. He et al. (2015) reported a positive relationship between biofilm induction and AdeG upregulation in *A. baumannii* [304]. Knowing that biofilm formation is associated with QS, the authors proposed a potential role of overexpressed AdeFGH in acceleration of transport of autoinducer molecules of QS during biofilm formation. Subsequently, using porcine mucosal model, deletion of AdeRS regulator of AdeABC in *A. baumannii* was shown to cause decrease in AR and biofilm formation [305]. The evolutionary relationships and biological relevance between the regulatory systems of QS and MDR were well explained by Xu (2016) [306]. Briefly, the QS signals of cell-cell communication which are acylhomoserine lactones (AHLs) in Gram-negative bacteria and gamma-butyrolactones (GBLs) in Gram-positive bacteria may have antimicrobial activity at high concentrations while many antibiotics, at subinhibitory concentrations, act as signal molecules. Orphan LuxR receptors, pseudo GBL receptors, and MDR regulators of the TetR family are shared by QS and MDR systems. QS interference using specific inhibitors stands a new strategy for antimicrobial therapy. Guo et al. (2016) screened transposon insertion mutants of *Pseudomonas aeruginosa* with increased antibiotic susceptibility for defective cytotoxicity and biofilm formation [307]. After identifying a *pyrD* (encoding dihydroorotate dehydrogenase) mutant displayed defects in cytotoxicity, biofilm formation, quorum sensing and virulence in an acute mouse pneumonia model, they employed a computer-aided screening to identify potential inhibitors of this protein. A candidate small molecule simultaneously suppressed the expression of T3SS genes and bacterial cytotoxicity while significantly enhanced the killing efficacy of ciprofloxacin on biofilm.

AR problem needs new strategies to keep bacteria susceptible to existing antibiotics since new antibiotic discoveries are limited and MDR phenotypes continue to increase. Thus, efflux pump inhibitors (EPIs) can counteract with AR and enhance clinical performance of antibiotics [308]. Design and development of effective EPIs require a deep insight into the mechanistics and regulation of MDEPs, thus such efforts also greatly aid in detection of the presence of MDEPs in clinical isolates besides restoring the activity of current antibiotics [263]. Moreover, as shown by Baugh et al. (2014) with *S. enterica* serovar Typhimurium mutants lacking components of the AcrAB-TolC system in as well as by using carbonyl-cyanide *m*-chlorophenylhydrazone, chlorpromazine and phenyl-arginine-β-naphthylamide as three EPIs, MDR inhibition can also be used as a strategy to prevent biofilm formation [309].

In principle, EPIs can act in 6 different ways: (a) competing with antibiotic binding site of EP, (b) energy source depletion, (c) interruption of pump assembly, (d) disruption of regulatory pathways for EP expression, (e) blockage of efflux pore and (f) complex formation with antibiotic [310,311]. EPIs can be natural products isolated from biological sources such as plants. Choudhury et al., (2016) screened 328 secondary plant metabolites for their inhibitory action on MexB pump in *P. aeruginosa* via molecular docking method and reported that p-coumaric acid as well as its derivatives may have potential as EPI of RND-efflux pump [312]. Another study was conducted to reveal EPI potency of carvacrol and thymol from the aromatic plants *Satureja montana* L. and *Thymus vulgaris* L. alone and in combination with benzalkonium chloride (BC) and tetracycline [313]. Because substrates that are more susceptible for MDEPs are predicted to have a common pharmacophore feature map, a strategy of excluding compounds with efflux substrate-like features was used by Aparna et al. (2014) when screening an in-house database of phytochemicals using high-throughput virtual screening against MexAB-OprM and AcrAB-TolC systems [314]. Lanatoside C and daidzein were shown as possible inhibitors of these EPs in *P. aeruginosa* and *E. coli*. Besides natural products, EPIs can also be synthetic compounds found through screening of chemical libraries. One of the best known examples is phenylalanyl-arginine β-naphthylamide belonging to the family of peptidomimetic EPIs, namely aminoacyl β-naphthylamides (PAβN). PAβNs are competitive inhibitors of the EPs and act by binding to the same pocket or at a site close to the antibiotic substrate binding site [315]. Molecular dynamic simulations were recently employed to show the PAβN blocking action on AcrB and its homologs [316]. Although the potential use of peptidomimetic EPIs was confirmed in *in vivo* infection models, toxicity such as nephrotoxicity stands as a critical problem [315]. Pyridopyrimidinone analogs, e.g. D13-9001 and the pyranopyridine MBX2319 are also promising synthetic EPIs, the former being specific for MexAB pump of *P. aeruginosa*, but not active against MexXY, and the latter acts as an inhibitor of AcrAB-TolC EP in *E. coli* and Enterobacteriaceae [317,318]. Despite these candidates chemotherapeutic EPIs were optimized in a pre-clinical development program as adjuncts to the antibiotics, there are still no approved EPIs due to safety and clinical potency issues. The technical obstacles related with their design and development were discussed with particular reference to RND-type transporters which are the major EP components of MDR Gram-negative pathogens [318]. Further investigations are required to re-design such molecules to improve OM penetration via porins while maintaining RND pumps preferences for binding. As to the inhibition of MPS-type EPs of Gram-positive pathogens, in a recent study a series of dithiazole thione derivatives were synthesized and checked as competitive NorA inhibitors in MRSA using zebrafish infection model [319]. A putative EPI, namely DTT10, was the least toxic one, plus potentiated effect of ciprofloxacin against clinical strain of MRSA and reduced bacterial burden in muscle and skin tissue of infected zebrafish. The results of Tintino et al (2016) indicated that tannic acid has the same properties of the standard EPIs against NorA EP responsible for moderate fluoroquinolone resistance of *S. aureus*, carrying potential as a possible adjuvant on new formulations [320].

4. Conclusion

Antibiotics have been savior of humankind since their first discovery but increasing AR which is a natural ecological phenomenon of billions of years evolution and a result of escape of resistance genes from environmental 'resistome' to human pathogens created a fearful and alarming situation in recent years. Moreover, individualized therapy became the forefront of providing most proper use of antibiotics. Knowledge about evolution and

mechanisms of resistance can pave way to reduce resistance emergence. Further investigations focused on PK/PD analyses, potential synergies with combinations of antibiotics, antibiotic toxicity, AR mechanisms and their evolution, discovery of novel molecules to counteract the effects of AR determinants as well as more precise implementation of urgent measures are expected to minimize problems originating from misuse of antibiotics and to cope with AR.

Disclosures

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