Potential role of efflux pump inhibitors as a death traps in multidrug resistant pathogens and biofilm infections

Çoklu ilaca dirençli patojenler ve biyofilm enfeksiyonlarında eflux pompa inhibitörlerinin ölüm tuzakları olarak potansiyel rolü

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ABSTRACT

Efflux systems are transport proteins that are crucial for bacteria in maintaining their internal environment and ensuring survival. Efflux pumps extrude a lots of harmful substrate that endogenous and exogenous from bacteria to the environment. These systems have gained considerable attention due to their role in drug resistance mechanisms, which has significant implications of infection disease. This review provides the main characteristics of efflux pumps in bacteria, including the structure of pumps and provides also a perspective to efflux pump inhibitors that nacavt antibiotic resistance. Efflux pump strongly related to biofilm infection and necessary to deliver quorum sensing signal molecules. Understanding the structure and function of efflux systems may provide valuable insights into the development of novel therapeutics for infectious diseases and combating drug resistance in bacteria. The rise of multidrug-resistant bacteria poses a serious threat to global public health. International authorities, such as the World Health Organization (WHO), recognize the urgent need for effective treatments against these resistant strains. With the

ÖZET

Eflux sistemleri, bakteriler için iç ortamlarını korumada ve hayatta kalmalarını sağlamada çok önemli olan taşıma proteinleridir. Eflux pompaları, bakterilerden çevreye endojen ve eksojen birçok zararlı substratı atar. Bu sistemler, enfeksiyon hastalığında önemli etkileri olan ilaç direnci mekanizmalarındaki rolleri nedeniyle büyük ilgi görmüştür. Bu derlemede, eflux pompalarının bakterilerdeki temel özelliklerinin, pompaların yapısını ve antibiyotik direncini ortadan kaldıran eflux pompası inhibitörlerine bir bakış açısı sunması amaçlanmıştır. Eflux pompası, biyofilm enfeksiyonu ile güçlü bir şekilde ilişkilidir ve quorum sensing (çevreyi algılama sistemi) moleküllerini iletmek için gereklidir. Eflux sistemlerinin yapısını ve işlevini anlamak, bulaşıcı hastalıklar için yeni terapötiklerin geliştirilmesi ve bakterilerde ilaç direnciyle mücadele konusunda değerli bilgiler sağlayabilir. Yakın zamanda hızla artan çoklu ilaca dirençli bakterilere karşı etkili mevcut antibiyotiklerin yeterli olmayacağı ve bu etkenlerle enfekte hastalara etkili bir tedavi sunulamayabileceği konusu Dünya Sağlık Örgütü (DSÖ) gibi uluslararası otoritelerin de gündeminde bulunmaktadır. Dünya Sağlık Örgütü beklenmekte olan bu felaketi önlemek adına bir taraftan yeni antibiyotiklerin keşfi

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potential exhaustion of current antibiotics, the WHO is leading multidisciplinary efforts to discover new antibiotics and increase the efficacy of existing ones. Efflux pump inhibitors have emerged as a promising approach to combat resistant bacteria by enhancing the effectiveness of existing antibiotics. While toxicity issues have hindered the clinical use of many efflux pump inhibitors, new strategies such as the discovery of novel agents or the use of bacteriophages show potential to overcome these obstacles. The main is to provide an overview of current approaches in efflux pump inhibitors to provide a solution to the treatment of multidrug resistant bacterial infections.

Anahtar Kelimeler: Efflux-mediated resistance, efflux pump inhibitors, antibiotic resistance, biofilm

konusunda diğer taraftan eldeki antibiyotiklerin etkinliğini arttırmak konusunda çok disiplinli olarak çalışmalar yürütmektedir. Bu bağlamda eflux pompa inhibitörleri mevcut antimikrobiyallerin etkinliğini arttırarak çoklu ilaca dirençli bakterilerin tedavisinde yeni yaklaşımlar sunmaktadır. Günümüze kadar tanımlanmış çok sayıda eflux pompa inhibitor adayı toksisite problemleri nedeniyle henüz klinik kullanıma giremese de yeni eflux pompa inhibitörü ajanlarının keşfi veya bakteriyofajların eflux pompa inhibitörü ajanı olarak kullanımı gibi yaklaşımlar gelecekte belki de seçici toksik etkinin sağlanması yoluyla engel teşkil eden toksisite probleminin aşılmasını sağlayabilecektir. Bu derleme çoklu ilaca dirençli bakterileri enfeksiyonlarının tedavisine bir çözüm sunabilmek adına eflux pompa inhibitörlerinde güncel yaklaşımlara bir bakış sunulmaktadır.

Key Words: Eflux pompası aracılı direnç, eflux pompa inhibitörleri, antibiyotik direnci, biyofilm

INTRODUCTION

Multidrug resistant (MDR) bacteria pose a significant challenge to the current healthcare system as they are one of the most pressing threats to public health (1). Typically, MDR bacteria are commonly associated with nosocomial infections, but the emergence of some MDR strains as prevalent causes of community-acquired infections is a crucial development that has significant implications (2). The expansion of MDR bacteria into the community is linked to elevated levels of morbidity, mortality, healthcare costs and antibiotic use, underscoring the urgency of addressing this issue. The rise of antibiotic resistance poses a serious threat to public health globally. There are growing concerns that the efficacy of current antibiotics will diminish rapidly making it challenging to prevent, treat infections and rendering classical infections one of the leading causes of mortality. Bacteria have evolved a multitude of strategies to evade the effects of antibiotics, which encompass diverse mechanisms such as chemical alterations, enzymatic inactivation of antibiotics, modification of the antibiotic's target site, modulation of intracellular antibiotic concentrations via changes in membrane permeability or upregulation of efflux pumps (EP) (3). EP, which are pump proteins located in the all eukaryotic and prokaryotic cell membrane, are responsible for the excretion of various substances that include exogenous and endogenous out of the cell. Siderephores, quorum sensing (QS) related compounds and toxic metabolites are among the endogenous substances excreted by EP; exogenous substances include antibiotics and pollutants such

as heavy metals and solvents. Efflux pumps can be found in different types of Gram positive (GP) and Gram negative (GN) bacteria. EP, can use a single substance or several different substances as their substrate. This means that bacteria can be protected from the effects of many different chemicals such as disinfectants, dyes, biocides, antiseptic agents, as well as antibiotics, by means of efflux pumps (4). These pumps are mainly chromosomally encoded, exhibiting a highly conserved arrangement (5). To solve the antibiotic resistance problem researchers have explore various approaches, such as the development of antibiotic analogues, the use of adjuvants in combination with antibiotics, the use of multidrug EP inhibitors as alternative therapeutic agents which hold the potential to re-sensitize bacteria to antibiotics. The emergence of antibiotic-resistant bacteria is a complex issue resulting from various factors such as the inappropriate use of antibiotics in humans and animals, the lack of new antibiotics and the failure to implement effective infection control measures. Given the limited options for the development of new antibiotics, it is critical to address this problem urgently by promoting rational use of existing antibiotics and implementing measures to prevent the spread of antibiotic-resistant bacteria (6).

EP Family: Their Structure and Regulation

EP systems are a major contributor to multidrug resistance in microorganisms and cancer cells. These pump systems are broadly categorized into two types: prokaryotic and eukaryotic efflux pumps. Prokaryotic efflux pumps function primarily in bacteria, while eukaryotic efflux pumps are responsible for drug resistance in fungi, protozoa and cancer cells. However, the division between these two categories is not clear-cut, as some pumps mediate resistance in both prokaryotic and eukaryotic cells. It is imperative to understand the mechanisms of EPs to develop effective strategies for combating drug resistance in various organisms (7). Prokaryotic efflux pump proteins play a critical role in the bacterial cell membrane by regulating the transport of toxic substances from the internal to the external cell environment. These non-specific systems are capable of identifying and expelling diverse compounds, including antibacterial agents and structurally unrelated chemicals, without modifying or degrading the drug. By extruding antimicrobial agents from the cell, efflux pumps reduce the intracellular concentration of antibiotics, which can lead to a decrease in the bactericidal effect of antibiotics and prolonged bacterial survival. This extended survival period can facilitate the accumulation of spontaneous mutations on target proteins, eventually leading to drug resistance. The six superfamilies of efflux pumps are classified based on their structural features and energy requirements, including ATP binding cassette (ABC); small multidrug resistance (SMR); major facilitator superfamily (MFC); multidrug and toxin extrusion (MATE); resistance-nodulation division (RND) and proteobacterial antimicrobial compound efflux (PACE) family (3, 8). The ABC superfamily harnesses the energy from ATP hydrolysis to drive the extrusion of substrates across cellular membranes. Its signature ATP-binding domains and transmembrane regions contribute to a well-defined structure essential for its pump function. In contrast, the SMR superfamily relies on proton motive force for substrate efflux, featuring four transmembrane helices that facilitate substrate transport. The MFS employs a diverse array of substrate-specific transporters, utilizing the electrochemical gradient to facilitate efflux. Another superfamily MATE utilizes ion gradients, particularly protons, for the extrusion of diverse substrates. The RND superfamily, predominantly found in GN bacteria, plays a pivotal role in antibiotic resistance through a tripartite structure involving inner and outer membranes and a periplasmic linker protein. Lastly, the PACE family is recognized for its involvement in resistance to antimicrobial compounds, employing diverse energy sources for efflux. Some antibioticsexcreted by efflux pumps are mentioned in Table 1.

Table 1. Families of EPs in bacteria and their associated drug substrates (9)

The Family of EP		Extruded Drug
Gram positive bacteria	ABC superfamily	Numerous drugs
	MFS family	Acriflavine, benzalkonium, cetrimide, chlorhexidine, pentamidine
	MATE family	Aminoglycosides, fluoroquinolones, cationic drugs
	SMR family	Acriflavine, benzalkonium, cetrimide
Gram negative bacteria	RND family	Numerous drugs
	ABC superfamily	Numerous drugs
	MFS family	Nalidixic acid, novobiocin

The EPs' substrate specificity is a variable characteristic that ranges from narrow to wide, depending on the pump type. For instance, tetracycline pumps exhibit a limited substrate range, while multidrug resistance efflux pumps can transport a broad range of substrates (9). The regulation of multidrug efflux pumps involves the participation of numerous transcriptional regulators or modulators, which are essential for controlling the expression of these drug EPs through complex pathways (10). It is well-established that mutational changes, exposure to antibiotics, diverse compounds or modulators can significantly alter the expression of efflux pumps, thereby impacting the efficacy of antimicrobial agents (5).

Efflux pumps require energy to transport drugs against the concentration gradient. The transport of substrates against their concentration gradient through efflux pumps necessitates energy expenditure, thus rendering efflux pumps reliant on energy sources. Two broad categories of efflux pumps are recognized based on the energy source utilized in their mechanism. Primary efflux pumps obtain their energy through the active hydrolysis of ATP, whereas secondary efflux pumps utilize the chemical gradient established by protons or ions such as sodium to derive energy (11). Efflux pump proteins are arranged in a way that provides a detailed understanding of the structure and mechanism of substrate transport. Mutations in the promoter site or regulatory proteins can lead to the overexpression of these pumps, resulting in antibiotic resistance (12). The emergence of drug resistance in GN bacteria primarily has been linked to the cytoplasmic membrane located efflux transporters, which function to expel drugs out of the bacterial cell. The efflux pumps found in GN bacteria exhibit a greater level of complexity due to their multi-layered cell envelop. Specifically, GN bacteria possess both inner and outer membranes that are separated by the periplasmic space, thus forming a tripartite protein channel that facilitates drug efflux. Among the various efflux pumps in GN bacteria, the RND family efflux pumps have a tripartite organization and are recognized as the primary contributors to intrinsic antibiotic resistance (11). Monopart efflux proteins have the ability to transport the antibiotic from the cytoplasm into the periplasmic space. These efflux proteins are characterized by their limited range of substrates they can recognize, making them narrow-spectrum in nature. Otherwise, three-part efflux systems are responsible for transferring the antibiotic from the cytoplasm to the external environment. This type of efflux system is capable of recognizing a broad spectrum of substrates, which in turn, leads to the development of multidrug resistance. Efflux pumps for GN and GP bacteria are shown in Figure 1. In summary, monopart efflux proteins and three-part efflux systems have distinct

modes of action in expelling antibiotics from bacterial cells, with the latter being associated with a higher potential for multidrug resistance.

EPIs can be categorized based on their origin. The first category is plant-derived EPIs, which are further divided into subclasses: plant alkaloids, phenolic diterpenes, flavonoids, and polyphenols. The second category is EPIs of synthetic origin, which are further divided into subclasses: peptidomimetic compounds, arylpiperidines and arylpiperazine derivatives, pyridopyrimidine and pyranopyridine derivatives, and quinoline derivatives. Finally, the third category is EPIs derived from microorganism. As an example of this category *Streptomyces* spp., with compounds like EA-371α and EA-371d being recognized as definitive inhibitors of the MexAB-OprM pump in *Pseudomonas aeruginosa* (10).

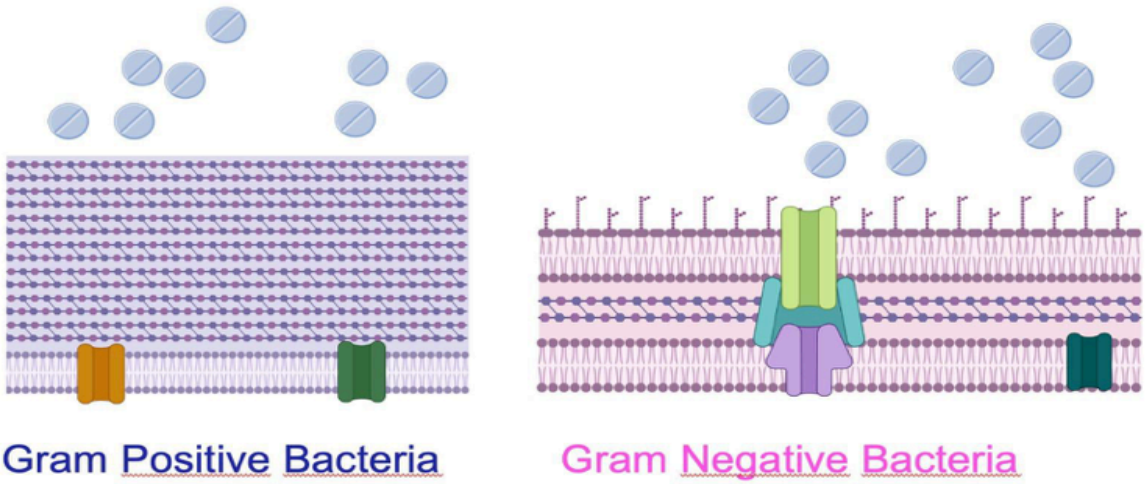

Figure 1. Efflux pump Gram positive and Gram negative bacteria

Antibiotic Resistance and Its Mechanisms

Efflux pumps are an essential component of bacterial physiology, as they facilitate the efflux of various compounds from bacterial cells. These pumps play a crucial role in the development of antibiotic resistance, as they can extrude antibiotics from bacterial cells, making the bacteria resistant to these antibiotics. Overexpressed EP cause acquired resistance in bacteria. This is due to the ability of efflux pumps to expel antibiotics from the bacterial cell, thereby reducing the efficacy of these antimicrobial agents. Efflux pumps play a significant role in both resistance and virulence. Efflux pumps have been shown to enhance the virulence properties of bacteria directly or indirectly. For example, efflux pumps can facilitate the secretion of toxins and other virulence factors and necessary to form biofilm formation. Efflux pumps present in bacterial pathogens play a crucial role in the development of antibiotic resistance, as they demonstrate a range of functions in intrinsic, acquired, and phenotypic resistance mechanisms (5). The development of potential pump inhibitors has become an interesting research area in the fight against resistance. The use of pump inhibitors in combination with traditional antibiotics has the potential to prevent efflux-induced resistance or to re-sensitize resistant bacteria to the antimicrobial agents (13).

EPIs are a class of compounds that can inhibit the function of efflux pumps, thereby reducing the ability of bacteria to resist antibiotics. EPIs work by binding to efflux pumps and blocking their ability to pump out antibiotics. EPIs allowing antibiotics to remain within bacterial cells and effectively kill the bacteria. Studies have shown that the use of EPIs can enhance the effectiveness of antibiotics against drug-resistant bacterial infections. However, the development of effective EPIs that are safe for human use remains a significant challenge in the field. Figure 2 presents the re-sensitization of drug-resistant bacteria. After the administration of drug-EPI therapy antimicrobial resistance change from resistant to sensitive. However, this observation is valid if there is only efflux pump resistance except all resistance mechanism. Presence of additional resistance mechanisms may prevent re-sensitization of resistant bacteria.

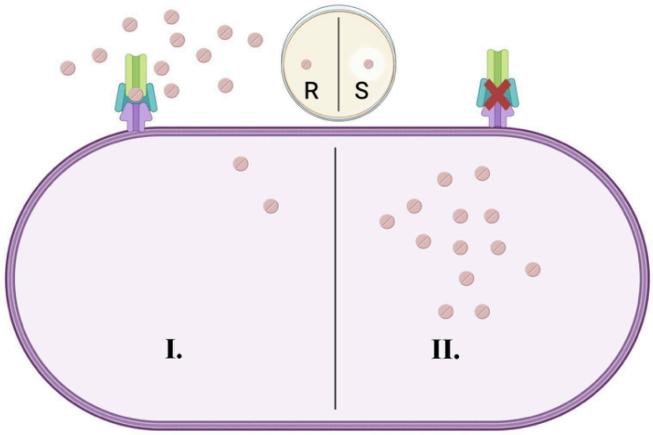

Figure 2. EP and drug resistant bacteria (I), EPI and to re-sensitized bacteria from drug resistant bacteria (II)

Efflux pump-inhibitors (EPI) are a type of antibiotic adjuvant that do not exhibit direct antimicrobial effects on their own, but rather enhance the activity of antibiotics when used in combination. By blocking the function of efflux pumps, EPIs can increase the intracellular concentration of antibiotics, which leads to improved efficacy against multidrug-resistant bacteria. One of the key advantages of using EPIs is their ability to restore the sensitivity of antibiotics that have become ineffective due to the overexpression of efflux pumps (14). Secondly, these pumps are thought to maintain low intracellular antibiotic concentrations, thereby promoting the accumulation of mutations (15). In addition to their ability to enhance antibiotic activity, EPIs can also extend the lifespan of antibiotics by reducing the development of resistance in bacterial populations. The use of EPIs represents a promising strategy for combating antibiotic resistance, as it provides a means of improving the efficacy of existing antibiotics without requiring the development of new drugs. Thirdly EPI supports the formation of ant-biofilm activity.

Ideal EPIs should be capable of restoring the activity of an antimicrobial against both intrinsic and acquired resistance. Furthermore, they have a broad range of activity against GP and GN bacterial pumps to ensure its effectiveness against a wide range of pathogens. Additionally, to minimize adverse effects, they should not interfere with the physiological efflux pumps, which are essential for the normal functioning of the cell. Thus, efficient

EPIs should meet these criteria to be a promising candidate for combating multidrug-resistant pathogens (7).

Multiple studies have demonstrated that the use of EPI results in a reduction of the minimum inhibitory concentration (MIC) values of antibiotics. The study found that the MIC values of various anti-tuberculosis agents, namely ciprofloxacin; levofloxacin; ofloxacin; moxifloxacin, were decreased when combined with certain antibiotics and EPI such as CCCP (carbonyl cyanide m-chlorophenyl hydrazone), reserpine and verapamil (16). The addition of EPI inhibitors to tuberculosis treatment is hypothesized to induce an increase in killing, which is attributed to the inhibition of macrophage calcium transporters. This inhibition results in phagolysosome acidification and activates the hydrolases necessary for the subsequent killing of intracellular *Mycobacterium tuberculosis* (17). Verapamil, an EPI, when administered concurrently with tuberculosis treatment, has been observed to enhance both the bactericidal and sterilization effects of standard tuberculosis treatment in an animal model (18).

Antibiotic persistence, a phenomenon in which a small subpopulation of bacterial cells survive lethal antibiotic treatment, has been a topic of interest in the field of microbiology. Recent studies have shown that persisters accumulate fewer antibiotics as a result of increased efflux rates, which is attributed to higher expression of efflux-associated genes. Specifically, high expression of the AcrAB-trimeric outer membrane channel (TolC) gene has been found to be critical in promoting persister formation (19, 20). Persisters are able to survive antibiotic attack by combining active efflux with passive dormancy, allowing them to evade the effects of the antibiotic and subsequently resume growth once the antibiotic is removed. These findings shed light on the mechanisms behind antibiotic persistence and may lead to the development of novel strategies for combating persistent infections (20).

EPIs have demonstrated potential as adjuvants to conventional antimicrobial agents, effectively reversing multi-drug resistance in pathogenic bacteria. Such a combination therapy has been shown to increase the efficacy of antibiotics against resistant strains, making them susceptible to multiple classes of antibiotic agents. Despite the availability of numerous potential EPIs described in the literature, significant challenges related to their using as therapeutic compounds, such as toxicity and limited efficacy (21).

The EPI as a candidate, must fulfill the subsequent requirements:

- Ability to effectively inhibit efflux pumps: The primary function of an EPI is to inhibit the function of efflux pumps, so a candidate should demonstrate a high level of potency in this regard.
- Synergy with antibiotics: An effective EPI candidate should be able to enhance the activity of one or more antibiotics when used in combination, resulting in a significant improvement in antimicrobial efficacy.
- Low toxicity and side effects: Since EPIs are administered in combination with antibiotics, they should have a low risk of toxicity and side effects when used in humans. The neurotoxicity of certain EPIs, such as reserpine, has been noted at the concentrations employed for bacterial infection management, as will be elucidated subsequently. The toxicity of certain EPIs can be attributed to their serotonin-agonist properties.
- Broad-spectrum activity: The ideal EPI candidate should be effective against a wide range of GP and GN bacteria, as well as against different classes of antibiotics.
- Low potential for resistance development: An EPI should not promote the development of resistance in bacterial populations, as this would undermine its ability to restore antibiotic sensitivity.
- Ability to penetrate bacterial cells: An EPI must be able to effectively penetrate bacterial cells to inhibit the function of efflux pumps and enhance antibiotic activity.

- Cost-effectiveness: Ideal EPI should be cost-effective, particularly in resource-limited settings where the burden of antibiotic resistance is high (22).

There exist limitations in the use of multi-drug flow pumps for the administration of antimicrobial agents in combination therapies. The incorporation of EPIs for the management of antibiotic resistance introduces an additional layer of complexity, given their potential for adverse interactions with co-administered antimicrobial agents. A notable example of such limitations is the fatal outcome observed upon co-administration of verapamil and clarithromycin, which resulted in renal dysfunction, hypotension (23).

Over the past two decades, a substantial number of inhibitors have been uncovered and patented. Despite this, the discovery, testing, and commercialization process remains notably sluggish. Among the notable inhibitors are energy decouplers, phenothiazines, analogs of widely used antibiotics, and inhibitors of serotonin re-uptake (24).

The Significance of EPI in Combating Drug-Resistant Pathogens

The emergence of multidrug-resistant bacterial infections has become a major public health concern worldwide. The escalation of antibiotic-resistant bacterial infections has emerged as a critical and pressing issue within contemporary global health contexts. Among these infectious agents, the ESKAPE (*Enterococcus faecium*, *Staphylococcus aureus*, *Klebsiella pneumoniae*, *Acinetobacter baumannii*, *Pseudomonas aeruginosa*, *Enterobacter* spp. and *Escherichia coli*) pathogen group has been identified as a primary contributor to this growing concern. Among the various multidrug-resistant pathogens, the ESKAPE group has been identified as the leading cause of these infections. Efflux pump have been found to be one of the most critical mechanisms for the evolution of multidrug resistance in ESKAPE pathogens. In this regard, upregulation of efflux pump expression represents a major mechanism employed by these pathogens to attain and sustain antibiotic resistance (21).

The WHO classifies pathogens into three priority tiers based on their degree of resistance to drugs. Among these, GN bacteria are of significant concern, given their high incidence of infections that exhibit resistance to multiple antibiotics. Table 2 presented that WHO priority pathogens list. In some cases, these strains demonstrate pan-resistance facilitated by the expression of resistance-nodulation-division (RND) superfamily efflux pumps, which are commonly found in GN bacteria. To counteract the problem of antibiotic resistance, the use of EPIs as adjuvants has emerged as a crucial strategy for restoring the effectiveness of antibiotics against drug-resistant GN bacteria (26).

EPIs assume paramount importance in addressing the antibiotic resistance exhibited by ESKAPE pathogens, as these inhibitors have the potential to enhance the efficacy of existing antibiotics. By selectively targeting and inhibiting efflux pumps, EPIs offer a strategic means to counteract the multidrug resistance mechanisms employed by ESKAPE pathogens, thereby revitalizing the therapeutic potential of antibiotics and mitigating the challenges posed by these elusive and clinically significant pathogens.

Types of Resistance

In the absence of efflux pump expression, susceptibility to antimicrobial agents is generally observed. However, other additional resistance mechanisms may also contribute to resistance in the organism. Therefore, it is important to consider the presence of such mechanisms when evaluating susceptibility to antimicrobial agents in microorganisms. The absence of efflux pump expression alone may not be sufficient to confirm susceptibility if other additional resistance mechanisms are present in the organism. Efflux-mediated antibiotic resistance can be two types:

Tablo 2. WHO priority pathogens list (24)

WHO Priority Pathogens List for Research - Development of New Antibiotics	
Priority 1: Critical	*Acinetobacter baumannii*, carbapenem-resistant *Pseudomonas aeruginosa*, carbapenem-resistant *Enterobacteriaceae* spp., carbapenem-resistant, ESBL-producing
Priority 2: High	*Enterococcus faecium*, vancomycin-resistant *Staphylococcus aureus*, methicillin-resistant, vancomycin-intermediate and resistant *Helicobacter pylori*, clarithromycin-resistant *Campylobacter* spp., fluoroquinolone-resistant *Salmonellae* spp., fluoroquinolone-resistant *Neisseria gonorrhoeae*, cephalosporin-resistant, fluoroquinolone-resistant
Priority 3: Medium	*Streptococcus pneumoniae*, penicillin-non-susceptible *Haemophilus influenzae*, ampicillin-resistant *Shigella* spp., fluoroquinolone-resistant

intrinsic and acquired (5). Intrinsic resistance is the inherent ability of an organism to resist the effect of an antibiotic, while acquired resistance is defined as the alteration in the genetic material of an organism. Intrinsic resistance is characterized by the presence of efflux pumps that are expressed at basal levels, such as MexAB-OprN in *Pseudomonas aeruginosa* or TolC in *E. coli*. Acquired resistance is related to increased expression of efflux pump genes, which can be caused by mutations of the regulatory proteins. Phenotypic resistance is another type of acquired resistance where the expression of efflux pumps can be activated by specific inducers. It is possible to downregulate the expression of efflux pump genes by using transcriptional repressors. Understanding the different types of antibiotic resistance is crucial in developing effective strategies to combat the growing problem of antibiotic resistance (10).

The findings presented in Figure 3 reveal a positive correlation between the level of antibiotic resistance and the degree of efflux pump expression. Efflux pumps play a crucial role in antibiotic resistance through three distinct mechanisms. Firstly, basal expression of efflux pumps such as *P. aeruginosa* MexAB-OprN or *E. coli* AcrAB-TolC, contribute to the inherent antimicrobial resistance of these bacteria. Secondly, acquired resistance that is stable may arise due to mutations in regulatory proteins resulting in the de-repression of efflux pump expression. Lastly, the presence of specific inducers can trigger the expression of efflux pumps, leading to transient phenotypic resistance (5).

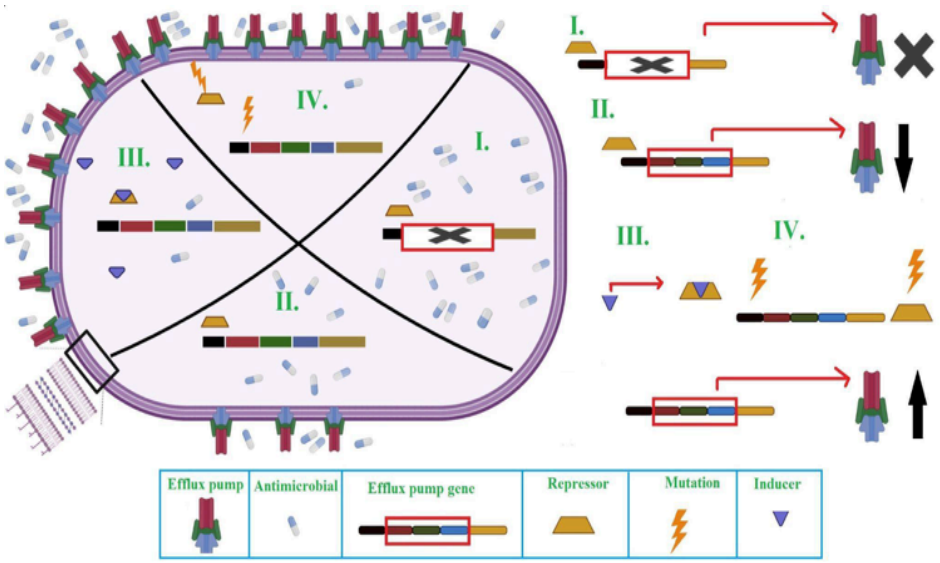

Figure 3. Efflux mediated antibiotic resistance profiles are presented in the figure.
Sensitivity of the bacteria: lack of efflux pump expression (I); intrinsic resistance: efflux pump expression at basal level (II); phenotypic resistance: triggered expression of efflux pump by inducer molecules (III); acquired resistance: de-repression of efflux pump expression as a result of mutations in regulatory proteins (IV).

The Mechanism of Efflux Pump Inhibitors as Novel Therapeutic Agents: Current Developments and Future Prospects

Efflux pump inhibition is considered to be a potentially highly efficacious strategy in combating resistance, which consequently ensures the sustained provision of the therapeutic benefits associated with existing antibiotics in the context of treatment (4). Targeting efflux pumps is a promising strategy for combating antibiotic resistance (28). There are several distinct approaches to achieve the abolishment of efflux pumps, including:

1. Downregulating the expression of efflux pump genes: This can be done by interfering with genetic regulation, for example, by using small interfering RNA (siRNA) or antisense oligonucleotides (ASOs) to target the efflux pump genes and reduce their expression (29). SiRNAs, which are composed of 21-23 base pairs (bp), have demonstrated their effectiveness as exogenous agents in manipulating gene expression in cultured cell- and animal-based systems experimentally. Additionally, siRNAs exhibit high stability and low toxicity, further enhancing their potential as therapeutic agents. The efficacy of siRNAs was determined in a murine model of chronic *P. aeruginosa* lung infection. Specifically, the research elucidates that MexB-siRNAs effectively downregulated both mRNA expression and activity of *P. aeruginosa* in vitro. In vivo, the siRNA intervention demonstrated significant efficacy in reducing the bacterial load and mitigating the *P. aeruginosa*-induced pathological changes in the chronic lung infection model. These findings suggest that siRNAs targeting MexB could serve as a promising therapeutic approach for chronic *P. aeruginosa* lung infection (30).

2. Redesigning antibiotics that are no longer recognized as substrates: This can be achieved by modifying the chemical structure of antibiotics to make them less susceptible to efflux pumps, which can increase their effectiveness against resistant bacteria. The current understanding regarding efflux pumps is that they exhibit variable sensitivity to agents

within the same antibiotic group and the underlying mechanism remains incompletely elucidated. Notably, the efflux of new generation agents is recognized at lower rates by efflux pumps relative to their older counterparts, thereby facilitating their evasion. For instance, 3rd and 4th generation quinolones are less prone to efflux compared to 1st and 2nd generation quinolones, ketolides relative to macrolides, and glycylcyclines with respect to tetracyclines. These findings suggest that an appreciation of resistance mechanisms should be integrated into the design of novel antibiotics targeting existing groups of antibiotics (31).

3. Inhibiting the assembly of functional efflux pumps: This can be done by targeting the proteins involved in the assembly of efflux pumps, such as chaperones, to prevent the formation of functional pumps.

4. Blocking the Pump: A Strategy for Inhibiting Substrate Binding to the Active Site: This can be achieved by designing inhibitors that target the efflux pump's active site, preventing the pump from binding to antibiotics and pumping them out of the cell.

5. Collapsing the energy mechanism responsible for energizing these pumps: This can be achieved by targeting the proton motive force that drives the efflux pump, for example, by using inhibitors of the ATP synthase, which can lead to the collapse of the energy mechanism and the inhibition of efflux pump activity (24).

6. Competitive or non-competitive pump inhibitors can effectively hide the antibiotic that possesses the efficacy from the pump. This approach has been proposed as a potential solution to combat antibiotic resistance, which has become a major global health concern. By using pump inhibitors, it may be possible to prevent the efflux of antibiotics from bacterial cells, thereby increasing their concentration and effectiveness (32). Figure 4 presented that some inhibition strategies for efflux pump.

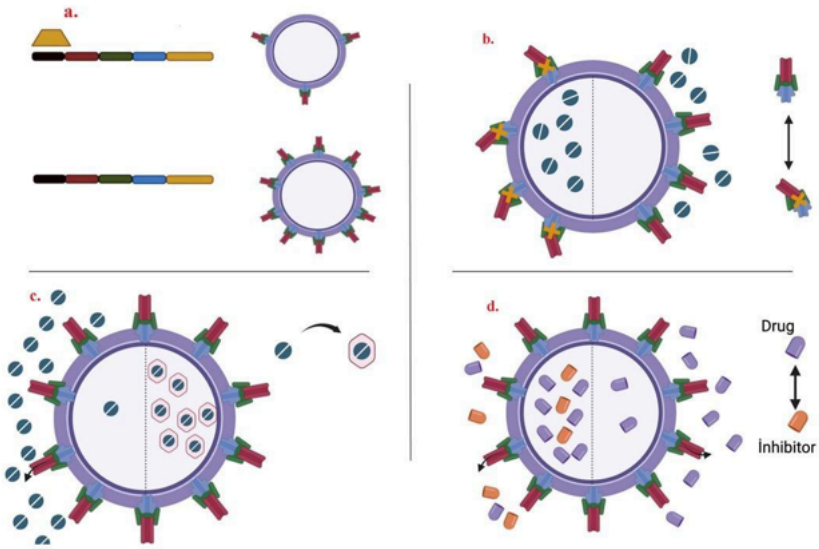

Figure 4. Inhibition strategies for EPs in bacterial cells to combat antimicrobial resistance: (Suppressed EP Gene Expression a), Disruption of Pump Assembly b), Altered Drug Structure c) and the application of competitive and non-competitive inhibitors with drugs.)

Ethidium bromide (EtBr) is a well-established substrate for assessing the activity of efflux pumps. EPIs have been developed as a potential strategy to restore the activity of antimicrobial agents against drug-resistant pathogens. EtBr is a fluorescent dye that is commonly used to estimate the potency of EPIs *in vitro*. EtBr is a substrate of efflux pumps and its accumulation within bacterial cells is inversely proportional to the activity of efflux pumps. In the presence of an EPI, the accumulation of EtBr is expected to increase, resulting in higher fluorescence intensity compared to the control without EPI (33). The potency of an EPI can be estimated by measuring the concentration at which it achieves 50% inhibition of efflux pump activity, known as the half-maximal inhibitory concentration (IC50). The use of EtBr as a marker for efflux pump activity is a well-established method for evaluating the potency of EPIs and is widely used in the field of antimicrobial drug discovery. However, it is important to note that in vitro results may not always translate to in vivo efficacy and additional studies are needed to validate the activity of EPIs in animal models and clinical trials (34).

The Role of Efflux Systems in Modulating Bacterial Virulence

It is widely believed that a symbiotic connection exists between the expression of efflux pumps and the formation of biofilms (35). Multiple investigations conducted on pathogenic organisms, have revealed a marked rise in the biofilm-producing ability of strains exhibiting specific pump protein genes. Conversely, a decrease in biofilm formation has been noted in mutants that lack the genes responsible for encoding such proteins (36).

According to several investigations, efflux pumps can perform four distinct functions in biofilm formation. First, they can transport extracellular polymeric substances (EPSs) and/or QS molecules to aid in biofilm matrix formation and regulate QS, respectively. Second, they can indirectly regulate genes involved in biofilm formation. Third, they can expel harmful molecules, such as antibiotics and metabolic intermediates that could potentially damage or inhibit biofilm growth. Finally, efflux pumps can influence aggregation by promoting or preventing adhesion to surfaces and other cells.

The growth of microorganisms in the deep layers of biofilms is markedly inhibited due to their exposure to limited oxygen and nutrient availability, enabling them to evade the effects of antimicrobial agents. Inter-bacterial signaling pathways modulate the biofilm's physiology, facilitating the expression of efflux pumps by bacteria. Persister cells exhibit a dormancy-like state in the presence of antibiotics, whereby they evade cell death, but upon the cessation of drug exposure or stress response, they revert to a proliferative state, forming normal bacterial colonies (37). Due to the recurrence of biofilm infections and the difficulties in their treatment, new treatment approaches for biofilm removal are being investigated. QS signal is a critical step in the process of biofilm formation. The QS signal can upregulate the efflux pumps of the cell. Upregulate efflux pumps are responsible for the effective effect of the QS signal. There is a mutual relationship between efflux pumps and QS that feeds each other. Considering efflux pumps as a door, QS is one of the signaling molecule that can upregulate or downregulate these doors. In this case, EPI acts as a key that locks these open doors. EPIs provide bilateral therapeutic benefit by preventing both antibiotic resistance development and biofilm formation.

Recent studies have demonstrated that certain EPIs have the ability to inhibit biofilm formation due to their anti-biofilm activity. This finding highlights the potential of EPIs as agents that can be used in combination with antibiotics to overcome antibiotic resistance. However, despite the potential of EPIs, only MP-601205, has been tested in clinical trials. The phase 1b trial of MP-601205 in cystic fibrosis patients was eventually discontinued due to toxicity-related issues. The high doses required for EPIs to

be effective are the main reason for their toxicity, thus restricting their systemic use. Nonetheless, EPIs could be locally applied to medical devices such as catheters to prevent biofilm formation and the emergence of antibiotic-resistant pathogens (38).

Future Perspectives

Bacteriophages are viruses that infect bacteria by recognizing specific receptor binding proteins (RBPs) on the bacterial cell surface. These RBPs can include efflux pump proteins, which are capable of expelling a wide range of molecules from the bacterial cell. The recognition of these efflux pump proteins by multiple phages suggests that they may play an important role in bacterial defense against viral infection (39). The process of phage infection is initiated by the binding of viral tail fibers to particular receptors on the bacterial cell wall, such as lipopolysaccharide, teichoic acid, pili, outer membrane proteins, efflux pumps, and polysaccharide, which leads to irreversible attachment. This strict receptor specificity results in the specificity of phages against their bacterial hosts (40). When the phage binds to the EP irreversibly, the EP undergoes modification and loses its residual drug efflux function. The investigation of bacteriophages that are specifically designed to target bacterial efflux systems represents a critical area of research, as it holds considerable potential for the discovery of novel EPIs. To develop new phage therapies and manage bacterial infections, it is necessary to understand how phages recognize their bacterial hosts, including the role of efflux pump protein.

CONCLUSION

Efflux-mediated resistance has become prevalent in clinical settings and is of significant concern, especially for antibiotics that exhibit resistance to specific resistance mechanisms. Efflux-mediated MDR is a major challenge in the development of effective antibacterial agents. One potential strategy to address the challenge of antimicrobial resistance is to employ EPIs in combination with traditional approaches such as the discovery of novel antimicrobial agents or the modification of existing ones to minimize efflux susceptibility. By targeting EPs, EPIs have the potential to increase the efficacy of existing antibiotics and restore susceptibility to previously resistant bacterial strains. This approach may enhance the efficacy of existing antimicrobials by reducing their expulsion from bacterial cells and increase the likelihood of successful treatment of resistant infections. Therefore, the use of EPIs in conjunction with conventional methods represents a promising avenue for combating antibiotic resistance and improving the efficacy of antibacterial therapies.

Preliminary findings have shown that the EPI approach can bring about multifaceted benefits in combating drug resistance and biofilm infection that related efflux pump. Specifically, the use of EPI is anticipated to reduce intrinsic resistance, broaden the spectrum of activity of antibiotics to previously non-susceptible bacterial species, reverse acquired resistance, and importantly, decrease the incidence of resistant strains emergence.

Most important characteristic of an ideal EPI would be the ability to provide selective toxic effect, targeting bacterial efflux pumps while sparing mammalian efflux pumps. It has been demonstrated that EPIs are capable of inhibiting a broad range of bacterial efflux pumps from different superfamilies, however, it is crucial that their mechanism of action does not lead to any adverse effects on mammalian cells by avoiding targeting mammalian efflux pumps. The utilization of efflux inhibitors is intrinsically linked to a pre-existing medication, given that such inhibitors typically function by augmenting the intracellular concentration of antibiotics rather than directly inhibiting bacterial growth. As a consequence, the combined use of these inhibitors and antibiotics presents a considerable challenge with regards to the compatibility of their pharmacokinetic properties (41).

The development of EPIs requires consideration of multiple factors, such as the physiochemical

structure of efflux pumps, their overexpression in bacteria, their poly-specificity properties, bacterial adaptation to toxic substrates, the mechanisms of drug transport across bacterial membranes and the energy utilization of efflux pumps.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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