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Efflux Pump Inhibitors as a Broad-Spectrum Strategy to Combat Multidrug Resistance *Acenitobacter Baumanii*

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Abstract:

The World Health Organization (WHO) identifies A. baumannii as one of the most resistant pathogens. Over the past few years, there has been a significant rise in its resistance to a wide array of antibiotics. Multidrug resistance is attributed to several mechanisms, with efflux pumps being the most notable. Recently, the development of new treatment strategies to tackle A. baumannii infections has shown great promise. This review focuses on the various families of efflux pumps in A. baumannii and explores innovative therapeutic options that have emerged over the past four years. Specifically, we emphasize synthetic and natural efflux pump inhibitors (EPIs), nanoparticles, bacteriophage therapy, and antimicrobial peptides (AMPs) as part of these novel strategies.

Key word: Efflux pump inhibitors [EPIs], Multidrug resistance, *Acenitobacter baumanii,* synthetic EPIs, natural EPIs, nanoparticles, bacteriophage, antimicrobial peptides (AMPs)

1. Introduction:

The rise of multidrug-resistant (MDR) bacteria can be attributed to the improper use and excessive consumption of antibiotics, resulting in the development of MDR strains globally, which poses a significant health threat. In 2019 alone, over 1.2 million individuals lost their lives due to MDR bacteria [1]. Drug-resistant bacteria are categorized into three levels: multi-drug resistant (MDR), extensively drug resistant (XDR), and pan-drug resistant (PDR), each indicating increasing resistance to antibiotics. [2] Bacteria can be hazardous even if resistant to only one antibiotic, leading to serious health issues. Resistance can result in harmful side effects from alternative treatments and prolong recovery. Ineffective antibiotics and antifungals would hinder our ability to treat infections and manage public health challenges. [3]. In the last twenty years, mechanisms of resistance in commensal bacteria that often cause infections have developed and become widely distributed. A particularly alarming aspect of some of these resistance mechanisms is their ability to be transferred, as they are associated with mobile

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genetic elements like integrons, transposons, and plasmids. [4]. MDR results from various mechanisms, with the (over)expression of multidrug efflux pumps being a key factor. These pumps are transmembrane transporters found naturally in Gram-negative bacteria, capable of expelling multiple types of antibiotics and imparting resistance. [5,7]. Thus, inhibiting these efflux pumps could be a promising approach to enhance the effectiveness of antibiotics, especially given that the search for new antibiotics is becoming increasingly challenging. [6]. Efflux pumps play a role beyond what has already been mentioned; they are also crucial to bacterial pathogenicity in several respects. This includes the release of bacterial exotoxins, quorum-sensing molecules, and other virulence factors. As a result, they have garnered significant medical attention and interest from numerous physicians and researchers seeking treatments for multidrug-resistant (MDR) infections. [9]. This implies that inhibition of drug efflux pumps leads to the elimination of many other health problems. In 2017, the World Health Organization published a report identifying organisms that should be prioritized for enhanced research and development of new treatment approaches. The report highlighted carbapenem-resistant Acinetobacter baumannii as the most urgent pathogen to address. [15].

A. baumannii is a non-motile, Gram-negative coccobacillus that is catalase-positive, oxidase-negative, and strictly aerobic. This bacterium is not particularly demanding in terms of growth conditions and has emerged as a significant challenge in hospitals, particularly in intensive care and burn units, due to its prevalence as a multidrug-resistant (MDR) organism [21]. A rise in the occurrence of carbapenem-resistant *A. baumannii* isolates has been noted in Northern and Eastern Europe, as well as in the Levant countries within the Arab League, including Iraq, Jordan, Lebanon, the Palestinian territories, and Syria. [22].
A. baumannii has shown various mechanisms of antimicrobial resistance to antibiotics, including aminoglycosides, carbapenems, fluoroquinolones, cephalosporins, tetracyclines, sulbactams, rifampicins, as well as to colistin and tigecycline, which are considered last-resort medications for treating its infections. Furthermore, research has documented resistance to newer antibiotics like eravacycline and cefiderocol. [16]. The search for new antibiotics is time-consuming and requires tremendous economic and labor investment. Additionally, administering antibiotics in high doses can lead to toxicity, which poses another limitation [32]. Consequently, researchers are seeking alternative approaches that might avoid producing toxic and harmful effects.

This review primarily highlights the different types of efflux pumps typically present in *A. baumannii* that contribute to the bacteria's drug resistance. Additionally, it addresses important strategies that utilize efflux pump inhibitors to effectively target these pumps in *A. baumannii*.

2. Mechanism of efflux pump-mediated resistance

The overexpression of efflux pumps is a key factor in gaining antimicrobial resistance, including resistance to multiple drugs. [14]. Efflux pumps are divided into primary and secondary types based on the energy source they use for substrate transport. Primary efflux pumps utilize energy from ATP hydrolysis to move substrates across the membrane, while secondary efflux pumps rely on the electrochemical gradients created by protons or ions (proton motive force). To date, researchers have identified six main families of efflux pumps in bacteria: the ATP-binding cassette (ABC) superfamily, which uses ATP hydrolysis for

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energy, the major facilitator superfamily (MFS), the multidrug and toxic compound extrusion (MATE), the resistance nodulation cell division (RND) family, the small multidrug resistance (SMR) family, and the proteobacterial antimicrobial compound efflux (PACE) family. [8]. *A. baumannii* has the ability to harbor efflux pump genes from all these families. [23]. These families are categorized according to particular characteristics, including membrane topology, whether they traverse single or double membranes, substrate specificity, the similarity of their primary sequences, and the stoichiometry of the multi-subunit complexes. [10].

2.1 ABC Superfamily

The ATP-binding cassette (ABC) transporter family facilitates the passage of its substrates over a lipid membrane, either into or out of the cell, by utilizing the energy provided during the hydrolysis of ATP to ADP. The four protein domains that make up an ABC transporter are two that traverse the membrane and two that are involved in the hydrolysis of ATP [15]. In Acinetobacter baumannii, the removal of macrolides, peptide toxins, virulence factors, siderophores, lipopolysaccharides, and protoporphyrins is facilitated by the tripartite MacA-MacB-TolC transporter. [24]. The inner membrane protein MacB is a homodimer complex with a C-terminal cytoplasmic tail and an N-terminal nucleotide binding domain that binds to ATP [25]. ATPase activates the membrane fusion protein MacA, which particularly binds with the lipopolysaccharide core.

2.2 Major facilitator superfamily (MFS)

MFS efflux transporters are found in all living organisms, making them highly widespread proteins. Their prevalence is a characteristic feature of one of the largest and oldest families of secondary active transporters, dating back over three billion years. [26]. Its biological mechanism relies on the transport of various substrates by taking advantage of ion gradients through secondary active transport or substrate gradients through uniport. [26]. The main MFS pumps in *A. baumannii* are TetA and TetB. The overexpression of TetA leads to resistance against tetracycline [24] and tigecycline [27], while TetB is linked to resistance against minocycline and tetracycline. Additionally, AbaF, another MFS pump, provides resistance to fosfomycin. [24,45]. Furthermore, CraA in *A. baumannii* contributed to resistance against various antibiotics, including chloramphenicol, norfloxacin, tetracycline, ceftazidime, cefepime, and streptomycin. [49].

2.3 Multidrug & toxic compound extrusion (MATE)

MATE transporters consist of roughly 450 amino acids and have a structure made up of 12 segments that span the membrane [49]. They utilize the energy from electrochemical gradients of H+ and/or Na+ across bacterial membranes to eliminate cationic compounds like norfloxacin and ethidium bromide [31]. In A. baumannii, the most prevalent gene in the MATE family is the adeM gene, which accounts for 63% to 100% of multidrug resistance (MDR) cases [29]. Some studies indicate that adeM is not linked to resistance against β-lactams or cephalosporins, but it may play a role in resistance to aminoglycosides, trimethoprim, fluoroquinolones, erythromycin, and chloramphenicol [29].

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2.4 Resistance–Nodulation–Division (RND) Superfamily Efflux Pump

The multiprotein complexes that comprise the RND superfamily span the periplasmic space, inner membrane, and outer membrane of Gram-negative bacteria. Because they typically span more than one membrane, the majority of other bacterial transmembrane proteins are smaller than these protein complexes. [12]. The amazing ability of RND pumps to eliminate a wide variety of compounds includes antibiotics of different kinds, such as fusidic acid, tetracyclines, novobiocin, chloramphenicol, and certain β-lactams. Apart from antibiotics, these pumps are also capable of handling biocides, detergents, bile salts, metals, and bacterial products like virulence factors and siderophores that bind iron. [11]
Clinical isolates of A. baumannii have been found to produce an excessive amount of one of the three major RND pumps, which has been associated with antibiotic resistance: The AdeFGH, AdeIJK, and AdeABC [17].
AdeABC is acknowledged as the most important efflux pump in the RND family in clinical settings. Studies reveal that it plays a critical role in the emergence of multidrug resistance in A. baumannii. AdeABC is overexpressed in a significant proportion of A. baumannii isolates when compared to other efflux pumps; around 80% of these isolates express the adeABC operon. [23]. AdeABC was found to be overexpressed in just 44% (28/64) of the isolates of CRAB (Carbapenem-resistant A. baumannii) in a different investigation [30]. The majority of this class of multidrug transporters interact with both an outer membrane protein (OMP) and a membrane fusion protein (MFP). This contact makes it easier for the antimicrobial chemical to pass through the bacteria's outer and inner membranes. without building up within the periplasmic gap. AdeA acts as the MFP, AdeB as the multidrug transporter, and AdeC as the OMP, making AdeABC a three-component efflux pump [18, 19]. The AdeRS two-component system controls the efflux pump's activity. Increased production of the pump due to mutations in the adeRS operon can result in an increase in antibiotic resistance [22].

2.5 Small Multidrug Resistance (SMR)

The efflux pumps belonging to the SMR family consist of small, integral proteins located in the inner membrane. These proteins feature four α-helix transmembrane domains and likely operate as either hetero- or homo-oligomers. Within this family, the efflux pumps utilize a proton gradient to expel their substrates from the cell [23]. A defining trait of SMR family proteins in both bacteria and archaea is their resistance to lipophilic cations and quaternary compounds. The AbeS efflux pump from *A. baumannii* exhibits lower susceptibility to various dyes, antimicrobials, and detergents, allowing these bacteria to withstand numerous detergents and antiseptics [23,24]. Research by Lin et al. indicated a strong correlation between increased expression of AbeS and resistance to amikacin [28]. Additionally, a study that deleted the AbeS gene in *A. baumannii*, which is responsible for the SMR efflux pump, affirmed its role in providing antibiotic resistance [20]. In *A. baumannii*, AbeS contributes to reduced susceptibility to chloramphenicol, fluoroquinolones, erythromycin, and novobiocin [31]. Comprising approximately 108 amino acids, AbeS continuously requires energy (H+), and inhibiting this energy source could enhance drug susceptibility in multidrug-resistant *A. baumannii* [29].

2.6 PACE efflux pump

Despite their structural resemblance to the SMR family, which features four transmembrane helices, PACE transporters are compact proteins made up of roughly 150 amino acids that

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provide resistance to various biocides and pesticides [29,31]. In *A. baumannii*, the gene aceI (Acinetobacter chlorhexidine efflux protein I) also plays a role in conferring resistance to chlorhexidine and oxidizing agents [29]. AceI and other proteins from the PACE family utilize the electrochemical proton gradient across the membrane as their energy source to facilitate active export [62]. Close homologues of AceI are particularly common in Proteobacteria, leading to the classification of this novel type of bacterial multidrug efflux protein as the Proteobacterial Antimicrobial Compound Efflux (PACE) family [62].

3. Inhibitors:

The identification of efflux pump inhibitors is not a recent development. It has been over 20 years since we started our research to find potential compounds that can inhibit efflux. [37]. Innovative treatments for *A. baumannii* infections that involve Chemical inhibitors [45], new natural compounds [48], nanoparticles [35], bacteriophages and antimicrobial peptides are quite intriguing. [48].

3.1 Chemical inhibitors

An appealing substitute approach to controlling this bacterium is to employ efflux pump inhibitors (EPIs) to increase the potency of currently available antibiotics. [43]. The mechanisms behind the inhibition of efflux pump systems in A. baumannii have been proposed to include changing the regulatory steps of the efflux pump expression, putting a plug in place to block the outer membrane pathways (adeC, adeK), breaking down the efflux's energy source, or attaching a non-antibiotic molecule to the affinity sites of the efflux pump for competitive or no inhibition [29]. Thioridazine is a promising antibacterial drug against MDR A. baumannii because, according to Ahmadi et al., it can inhibit bacterial isolates and the adeB efflux pump gene [52]. Several efflux pumps, such as AdeFGH, AdeIJK, and AdeABC, have been shown to be blocked by substances such carbonyl cyanide 3 chlorophenylhydrazone (CCCP) and phenylalanine–arginine β naphthylamide (PAβN), however due to their toxicity, they are not approved for clinical use [46].
Verapamil is an EPI and calcium channel blocker made artificially that is used to treat hypertension. Because verapamil can extend the Q-T interval, which raises the risk of hypotension and shock, the US FDA views it as deadly when administered with macrolide antibiotics. On the other hand, it has some inhibitory effects on A. baumannii, which result in competitive inhibition of MATE family efflux pumps and increased susceptibility to tigecycline. [23].
It has been found by Rashikesh et al. that 4,6-diaminoquoniline analogs have the potential to improve the antibacterial qualities of erythromycin, tetracycline, and novobiocin, not just in the strain of A that is susceptible to antibiotics. baumannii ATCC17978 in the laboratory, as well as in the clinical isolates AB5075 that are resistant to drugs [44]. Another harmless substance, IITR08367 [bis(4-methylbenzyl) disufide], enhances the effectiveness of fosfomycin against clinical strains of A. baumannii and inhibits the efflux pump (AbaF) to prevent biofilm formation [45].

3.2 Natural inhibitors

It has been proven that a number of natural compounds block bacterial efflux pumps. [13]. Furthermore, certain substances—such as curcumin and epigallocatechin gallate (EGCG), which are flavonoids derived from plants—may have an impact on the development of biofilms and decrease the pathogenicity of A. baumannii, a multidrug-resistant bacterium [48]. Edible

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plants are gaining popularity due to the population's widespread use of them as food. Investigations on several regularly used in traditional diet fruits (grapefruits, grapes, pomegranates), seeds (coffee and cocoa seeds), plants (lemongrass, tea leaves, and condiments), and spices (pepper) have in fact shown powerful EPIs [49].
According to a study, resveratrol (3,5,4'-trihydroxy-trans-stilbene, RV) and piperine (1-piperoyliperidine, PIP) are non-toxic substances that can prevent *A. baumannii* from expressing the EPs gene and from developing a tolerance to BZK or CHX [47]. Resveratrol (RV), a naturally occurring substance derived from grape skin and seeds, inhibited the expression of adeB in *A. baumannii*. RV and Chlorhexidine showed strong bactericidal and synergistic actions on carbapenem-resistant *A. baumannii* when combined [48]. Compounds derived from plants can supplement antibiotics to improve their therapeutic efficacy in the treatment of infectious illnesses. According to Alammery *et al*., chamomile watery extract can be considered a kind of rapid efflux pump inhibitor in tetracycline resistance isolates of *A. baumannii* [50].
Usnic acid (UA), a secondary metabolite generated from lichens and found in Usnea, Ramalina, and Cladonia, was discovered by Nagaraju et al. to be more effective than carbonyl cyanide phenylhydrazone, a typical efflux pump inhibitor, in lowering the minimum inhibitory concentration [46]. The results of one investigation show Imipenem and cinnamon oil together were the most potent combinations against all tested strains of *A. baumannii* [51].

3.3 Nanoparticles

Nanotechnology has enabled the exploration of new possibilities, and nanoparticles (NPs) have been utilized either individually or in conjunction to combat multidrug-resistant (MDR) bacteria and overcome antibacterial resistance [33]. A study has demonstrated that nanoparticles possess considerable potential in suppressing biofilm formation and efflux pumps in A. *baumannii* isolates. This finding is particularly relevant given the escalating prevalence of antibiotic resistance. [35].
Mechanical, biological, electrical conductivity, and catalytic activity are just a few of the properties that nanoparticles can dramatically change due to their minuscule size (diameter range: 1–100 nm) and high surface area to volume ratio [40], which in turn increase their ability to bind a wide variety of high affinity ligands. They are potential candidates for applications in medical imaging, drug delivery, and disease diagnostics [41]. Based on the physicochemical characteristics of each metal nanomaterial, a number of NPs, including silver (Ag) and gold (Au) NPs as well as copper (Cu) NPs, zinc (Zn) NPs, nickel (Ni) NPs, platinum (Pt) NPs, and palladium (Pd) NPs, have revealed various antibacterial capabilities. [42].

Twelve of the fifty strains of *A. baumannii* were found to have efflux pump genes, and following treatment with AgNPs, there was a significant downregulation in the expression levels of the efflux pump genes AdeA, AdeC, AdeS, AdeR, AdeI, AdeJ, and AdeK [34]. An additional study revealed that chitosan, chitosan nanoparticles, and silver nanoparticles reduced the expression of efflux pump adeB in A. baumannii and mexB in P. aeruginosa; as a result, they would be ideal candidates for efflux pump inhibitors [33]. When NDC-capped silver nanoparticles (NDC-AgNPs) were employed as efflux pump inhibitors, Privita et al. demonstrated that they not only produced reactive oxygen and nitrogen species but also altered the electrochemical gradient in CRAB in addition to their efflux inhibitory activities [36]. Because AdeABC depends on the proton gradient for proper operation, changing the electrochemical gradient also interferes with the efflux activity [36].

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CuO nanoparticles can also be utilized against *A. baumannii* as an anti-capsular agent, and when combined with gentamicin, they may boost their antibacterial activity [38]. ZnO NPs appear to have potential for application as a medication candidate in the pharmaceutical sector, given their desired inhibitory effects on the expression of AdeB and AdeRS, which are crucial in the pharmacological resistance of Acinetobacter species. [39]. Another study revealed that the new silica nanoparticles and lower doses of amoxicillin could prevent the formation of biofilm and lessen harmful side effects, making them a good substitute for high-concentration dosages [63]. Jawad et el. found that, with a higher minimum inhibitory concentration (MIC) of bismuth nanoparticles than amikacin, Bi2O3NPs exhibited more effective antibacterial activity against *A. baumannii* and *Staphylococcus aureus* [64]. In summary, the results of the experiments indicate that nanoparticles may be a promising substitute for fighting *A. baumannii*.

3.4 Bacteriophage

The application of lytic phages to inactivate infections is known as phage treatment. On the other hand, reports of bacterial mutants resistant to a therapeutic phage, or phage-resistance, are common [57].
In a study, the biofilm of multidrug-resistant A. baumannii may be reduced by up to 87% by isolated lytic phage (IsfAB78). [53]. Additionally, Wintachai et al. showed that sacha inchi oil and MDR A. baumannii, which is specific to phage vWUPSU, combined to inhibit and remove biofilms more successfully than either treatment did alone. According to the study's findings, MDR A. baumannii may be controlled using phage vWUPSU [55]. It is possible for bacteriophages to obstruct efflux pumps. Efflux pumps possess both inner and outer membrane proteins (OMPs), as was previously mentioned. OMPs are made up of β-barrel structures with extracellular loops and membrane bridges. These loops are receptors that phages can cling to and utilize. Bacteria may respond by changing or removing the extracellular OMP loops and changing the production of other OMP genes, which would suppress or remove efflux pumps. [23]. Phage resistance frequently results in basic changes in bacteria, as Wang et al. confirmed. These alterations might lead to decreased pathogenicity or antibiotic resensitization. [57]. In a mouse animal model, the clinical strain PlyF307 proved to be the first very effective therapeutic-specific A. baumannii phage against a Gram-negative bacterium, saving half of the animals [54]. Phage cocktail treatment proved effective in treating a patient infected with multi-drug resistance (MDR) A. baumannii Phage cocktail treatment of the infected patient resulted in the successful eradication of the MDR A. baumannii infection and the patient's recovery [56]. According to a different study, giving immunocompetent mice a single phage treatment improved the outcome of pneumonia after an A. baumannii lung infection. Most importantly, this study's lack of inflammatory or other adverse effects supports future efforts to develop phages for use in treating MDR A. baumannii lung infections in clinical settings [65].

3.5 Aantimicrobial peptides (AMPs)

Innate immunity depends on antimicrobial peptides (AMPs), which are naturally occurring substances made by many species, including humans and archaea. They are crucial in protecting hosts from infections caused by viruses, bacteria, fungi, and protozoa [58]. The majority of antimicrobial peptides (AMPs) contain fewer than 50 amino acids. Defensins, cathepsins, frog and insect antimicrobial peptides, modified peptides derived from tilapia antimicrobial 2, and other naturally occurring or artificially created small molecular proteins with α-helical structures are among the current AMPs used to combat drug-resistant A.

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baumannii [59].
Additionally, a number of groups have investigated the impact of AMPs and antibiotics together on MDR A. baumannii in recent years. For instance, Sacco and colleagues' research [60] amply demonstrated the synergistic effects of colistin and AMP Esc(1-21) in preventing the development and eliminating colistin-resistant A. baumannii clinical isolates, most likely by enhancing the chemicals' ability to disrupt membranes. [60].
Research has indicated that the methods by which these peptides directly target bacteria can be broadly classified into two groups: intracellular targeting and bacterial membrane attack, however certain peptides may have both processes. [59].
Six peptides—Melittin, Histatin-8, Omega76, AM-CATH36, Hymenochirin, and Mastoparan—had the strongest anti-A. baumannii activity against both susceptible and antibiotic-resistant isolates, according Neshani et al.'s Minimum Inhibitory Concentration (MIC) investigations. [61].

Octopus minor's prohibitin-2 gene is the source of octoproborantin, a synthetic antimicrobial peptide (AMP). Since an A. baumannii-infected zebrafish model demonstrated octoprohibitin's potent bactericidal action in vivo, with a higher cumulative survival percentage (46.6%) and fewer pathological signs [58]. The antimicrobial peptide Cec4 has also been approved by Peng et al. to have good and sustained action against A. baumannii both in vivo and in vitro, suggesting that it has good potential for treating infections caused by A. baumannii that are resistant to many medications [59]. Finally, but just as importantly, octominin employs a number of tactics to prevent A. baumannii from growing bacteria and changing its morphology characteristics structural alterations, surface damage, elevated membrane permeability (influencing the efflux pumps), elevated ROS generation, LPS neutralization, inhibition of biofilm formation, and biofilm eradication. In addition, higher concentrations of octominin (>100 μg/mL) in mammalian cells showed remarkably little cytotoxicity [66].

4. Conclusion

According to current research, A. baumannii's export of antibiotics is facilitated by efflux pumps belonging to the RND, MATE, SMR, MFS, ABC, and PACE families. To further understand the distinct physiological roles and contributions of each family's efflux pumps to multidrug resistance, more research is required to characterize them in greater detail. This review could provide critical insights that will aid in the development of novel efflux pump inhibitors (EPIs), improve knowledge of their mechanisms of action, and help to reduce the spread of antibiotic resistance. Furthermore, it is critical to investigate these EPIs' clinical efficacy. The development of clinically usable EPIs could extend the efficacy of existing antibiotics, possibly allowing for their use at lower dosages, thereby decreasing toxicity.

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