Efflux pumps in *Acinetobacter baumannii*: role in antibiotic resistance and interest of efflux pump inhibitors as additional therapeutic weapons

C. Mullié¹, B. Bouharkat², R. Guiheneuf³, C. Serra³, A. Tir Touil-Meddah² and P. Sonnet¹

¹ Laboratoire de Glycochimie, des Antimicrobiens et des Agroressources UMR7378 CNRS, Université de Picardie Jules Verne, UFR de Pharmacie, 1 rue des Louvels, 80037 Amiens Cedex 1, France
² Laboratoire de Bioconversion, Génie Microbiologique et Sécurité Sanitaire N°145, Faculté des Sciences de la Nature et de la vie, Université Mustapha Stambouli, 29000 Mascara, Algérie
³ Laboratoire de Bactériologie, Centre Hospitalier Universitaire d’Amiens-Picardie, 80054 Amiens Cedex 1
⁴ Unité de Virologie Clinique et Fondamentale, EA4294, Université de Picardie Jules Verne, Amiens, France

Due to an ever increasing proportion of multi-drug resistant strains, *Acinetobacter baumannii* is a pathogen of rising clinical significance, especially in hospital-acquired infections. Among the mechanisms causing these resistances to marketed antibiotics, efflux of antibiotic molecules currently used for the treatment of *A. baumannii* has been described, especially for fosfomycin and tigecycline. Various families of efflux pumps co-exist in *A. baumannii* such as the Resistance-Nodulation-Division (RND) tripartite systems (e.g. AdeABC) or Major Facilitator Superfamily (MFS) proteins. After describing their structures, their proposed physiological roles and the regulation systems governing their expression, their relevance in biofilm formation and in antibiotic resistance will be reviewed. Finally, the potential interest of designing specific or generic efflux pump inhibitors to help restoring antibiotic activity on resistant strains will be discussed.

**Keywords**: *Acinetobacter baumannii*; antibiotic resistance; efflux pump; inhibitor; review

1. **Introduction**

The genus *Acinetobacter* includes Gram negative, short rod shaped aero-anaerobic bacteria. It belongs to the class Gamma-proteobacteria, the order Pseudomonadales, and the family Moraxellaceae. Among the many species classified in this genus, only a few have been described as pathogenic for the human being. The most significant of those pathogenic species are *Acinetobacter baumannii*, *Acinetobacter pittii* and *Acinetobacter nosocomialis*, with *A. baumannii* exhibiting the highest occurrence rates in infections [1]. Most infections caused by *A. baumannii* are hospital acquired and can range from ventilator-associated pneumonia to urinary tract infections, also encompassing catheter-associated bloodstream infections as well as soft tissue infections [2, 3]. The concern raised by this pathogen is forever increasing because (i) it has the ability to disseminate and survive extremely well in the environment and (ii) some strains have rapidly acquired an extensive drug resistance (XDR) phenotypic profile, leaving few viable alternatives for their treatment [2]. The mechanisms underlying this resistance to a wide array of antibiotic classes include the production of inactivating enzymes (e.g. carbapenemases that hydrolyze members of the β-lactam family, ArmA methylase that inactivates aminoglycosides), the modification of antibiotic targets (e.g. mutations in *parC* and/or *gyrA* genes leading to fluoroquinolone resistance), the impermeability of the outer membrane of the bacterium caused by the loss and/or modification of porins (e.g. carO and oprD for β-lactams) and finally, the overexpression of efflux pumps (EPs) extruding unwanted molecules from the cytoplasm of the bacterium (e.g. adeABC-mediated aminoglycoside expulsion) [4]. The aim of this review is to focus on the latter mechanism of resistance by describing the major EPs found in *A. baumannii* as well as their physiological functions before highlighting their clinical importance in antibiotic resistance. Finally, the helpfulness of naturally occurring or synthetic efflux pump inhibitors (EPIs) in reversing *A. baumannii* resistance to certain antibiotics will be discussed.

2. **Structure and regulation of the major efflux pumps found in *Acinetobacter baumannii***

EPs are functional entities consistently present in bacteria. Depending on their biochemical structures and action mechanisms, they can be classified in several superfamilies:

- ATP-binding Cassette (ABC) transporters;
- the Small Mutidrug Resistance (SMR) family;
- the Major Facilitator Superfamily (MFS);
- Multidrug And Toxic compound Extrusion (MATE) transporters;
- the Resistance-Nodulation-Division (RND) superfamily.

Several other families of putative EPs have been described such as MAR (Multi Antimicrobial Resistance) or PACE (Proteobacterial Antimicrobial Compound Efflux) families [5, 6] but this review will not dwell on them because of the
scarcity of information available for these EPs in *A. baumannii*. Similarly, the implication of ABC transporters in *A. baumannii* efflux is poorly documented and will not be developed.

While most EPs are constituted by a single protein enshrouded in the plasma membrane, the members of the RND family comprise 3 proteins (Fig. 1). This EP family is found in Gram-negative bacteria and the protein bearing the actual efflux activity (in other words, the real EP), is located in the inner membrane of the cell wall and picks the substrate from the periplasmic space [7]. The substrate processed by this protein is then managed by a “funnel-like” protein in the periplasmic space, called Membrane Fusion Protein (MFP), and delivered to the third protein of the system, enshrouded in the outer membrane. This latter protein is termed Outer Membrane Protein (OMP) (Fig. 1).

Therefore, efflux by all EPs but RND EPs carries substrates from the cytosol to the periplasmic space of Gram negative bacteria, while RND EPs directly extrude substrates outside the bacterial cell.

Fig. 1 Major families of bacterial efflux pumps (adapted from [6] and [8]).

As can also be seen on Figure 1, most EPs found in bacteria act as antiports, coupling the drug efflux to the downhill transport of sodium ions or protons along a concentration gradient. Only ABC transporters use ATP to enable the extrusion of molecules from the bacterial cytosol [6].

Most EPs are chromosomally encoded [8, 9] but some acquired efflux systems have also been described in *A. baumannii* such as MFS EPs Tet(A), Tet(B), CmlA and FloR or SMR EP QacE. For example, tet(A), tet(B) and other tetracyclines genes have been found on plasmids or transposons [10].

- **RND EPs in *A. baumannii***

  Eversince the first description of AdeABC in 2001 [11], these EPs have been the most widely studied in *A. baumannii*. The encoding genes of these tripartite structures are organised in operons (Fig. 2). For example, the three genes adeA, adeB and adeC have been shown to be co-transcribed but evidence of mRNA corresponding to adeAB and adeC was pinpointed, suggesting an independent transcription of adeC or a cleavage of the original co-transcript [12]. Three main RND EPs have been described: AdeABC [11], AdeIJK [13] and AdeFGH [14]. Lately, a homologue of the EmrA transporter found in the tripartite EmrAB-TolC EP of *Escherichia coli* was described but information on this pump is sparse [15]. It has to be noted that some authors classify EmrAB in the MFS [16]. RND EPs usually display a broad substrate range, making them good candidates for a role in antibiotic resistance [7].

  For each of the three major RND EPs, regulation systems exist. For AdeABC, a two-component regulation system has been uncovered: AdeRS [12]. These two genes are found upstream of the AdeABC operon but are co-transcribed in the opposite direction. Two component regulation systems in bacteria are responsible for response to environmental conditions [17] and are constituted by a sensor histidine protein kinase and its cognate response regulator, in our case adeS and adeR, respectively. Inactivation of either adeS or adeR led to the restoration of aminoglycoside susceptibility in *A. baumannii* resistant strains while point mutations in these genes drove to a rise in antibiotic resistance and/or in efflux [12, 18, 19]. Truncation of AdeS generated through an insertion (IS*Aba1*) was correlated to tigecycline resistance [20]. More recently, the binding of AdeR to a direct-repeat motif in the intercistronic spacer (Fig. 2) was demonstrated [21]. The authors postulated that mutations impacting AdeR structure might lessen its binding to the intercistronic spacer, hence favouring the transcription of adeABC operon and increasing efflux. Another two-component regulatory system, BaeSR, also impacts AdeABC expression [1, 22].
Fig. 2 Schematic representation of RND operons in *Acinetobacter baumannii*. Arrows indicate the coding sequences and the transcription direction. Their sizes are not representative of the actual sequence size (Adapted from [9] and [21]).

For AdeFGH, a sequence coding for a LysR-type transcriptional regulator, termed adeL, was found upstream from the adeFGH operon, once more with a divergent transcription. As for the AdeRS two-component system, mutations in this sequence were identified in *adeFGH*-overexpressing mutants, suggesting a part in the regulation of AdeFGH expression [14]. LysR-type transcriptional regulators are highly conserved and ubiquitous in bacteria [23]. These proteins bind DNA and can either activate or repress single genes or gene operons. However, AdeL might not be the sole regulator of AdeFGH [18].

Finally, AdeIJK expression is regulated by a TetR–type transcriptional regulator called AdeN [24]. The *adeN* gene is located 813 kbp upstream of the *adeIJK* operon and is a repressor of this operon as its inactivation leads to an increase in antibiotic resistance and *adeIJK* expression. AdeIJK is intrinsically present in all *A. baumannii* strains but appears to be tightly down-regulated as high-levels of expression for this RND EP have been shown as toxic for the bacterium [13]. As for AdeABC, BaeSR is also thought to be implied in AdeIJK expression [1].

### MFS EPs in *A. baumannii*

As mentioned above, tetracycline EPs (Tet) are not part of the intrinsic genome of *A. baumannii* but are horizontally transferred from other bacteria. Their acquisition leads to tetracycline resistance only, due to their narrow substrate range. The sole exception is Tet(B) EP that impacts minocycline as well as tetracycline [25]. Tet(A) and Tet(B) are the two major EPs of this family but Tet(C), Tet(D), Tet(E) and Tet(H) have also been described in bacteria, although they are scarcely (if ever) reported in *A. baumannii* strains [26].

AmvA is another MFS EP characterised in a MDR strain of *A. baumannii* [27]. It is constituted by a 14-transmembrane-domain system that mainly extrudes dyes, disinfectants, and detergents. As for antibiotics, only erythromycin is clearly impacted by the overexpression of this EP. The presence of *amvA* was detected in all *A. baumannii* strains tested so far.

CraA [28] and AbaF [29] are two other MFS EPs identified in *A. baumannii* that will be further discussed in paragraph 4.2 along with their antibiotic substrates. Similarly, SMR EP AbeS and MATE EP AbeM will be considered in paragraphs 4.3 and 4.4.

### 3. Physiological roles of efflux pumps in *Acinetobacter baumannii*

#### 3.1 Role in molecule transportation and virulence

Data on the original physiological function of EPs is scarce but this function most likely implies transport of molecules through the bacterial cell membrane. The importance of membrane transport systems in prokaryotes is emphasized by the fact that transporter genes constitute between 5% and 12% of the total number of genes in each sequenced genome [30]. The primary physiological role of EP systems is thought to be elimination of waste products and evasion of molecules toxic for the bacterium, hence allowing its survival in its ecological niche [16]. For example, in *E. coli*, the RND EP AcrB is able to efflux bile salts and fatty acids. Moreover, an overexpression of efflux was reported in *Vibrio cholera* and *Bacteroides fragilis* when these bacteria were grown in the presence of bile salts [31, 32]. Overall, there is mounting evidence that EPs are great contributors to bacterial stress responses, whatever the stress and whether EPs pre-exist or are activated in response to stress. In this latter case, inducers of EPs’ expression are often substrates for these pumps [33]. What has come to light in recent years is that some EPs of *A. baumannii* are involved in the resistance to osmotic and oxidative stress, possibly through the overexpression of RND EPs, AbeD and/or EmrAB [1, 34, 35]. Nevertheless, the positive impact of osmotic stress on RND EPs’ expression is not universally reported [36].

A further hypothetical role for EPs put forward by some authors is the exportation of virulence factors although direct evidence of this function is lacking for *A. baumannii* [16]. In terms of virulence, what has lately been published is that (i) the deletion of *adeB* impacts the *in vivo* virulence of *A. baumannii* in a *Galleria mellonella* model for the sole
Singapore strain 1 [37] and (ii) an A. baumannii strain in which an insertion sequence (ISAba1) was present in the adeN gene displayed a higher virulence both in vitro in terms of cell invasion (on the A549 cell line) and in vivo on the G. mellonella model [38].

3.2 Role in quorum sensing, adherence and biofilm formation

From a general point of view, biofilms are complex communities of bacteria associated with biological or artificial surfaces. Biofilms are involved in many human infectious diseases such as lung, bone or dental infections, for example.

Bacteria organised in biofilms are often less susceptible to the host immune system as well as to antibiotics than planktonic cells. Moreover, it has been shown that EPs are highly activated in biofilms [39]. For some bacterial species, the same authors described a reduction in biofilm when efflux pump inhibitors (EPIs) are present [39].

Over the last decade, several studies have focused on or reported the implication of EPs in A. baumannii’s adherence and capacity to form biofilms. Quorum-sensing signal molecules (N-decanoyl homoserine lactone and C-12 homoserine lactone) are detected in biofilm-forming clinical isolates of Acinetobacter spp. [40]. These molecules could be substrates for A. baumannii EPs and their efflux from the bacterial cell then facilitated by the overexpression of these EPs.

Some evidence support the association between EPs’ overexpression and biofilm formation in A. baumannii. For example, the pmt gene encoding a MFS transporter was identified in a clinical strain of A. baumannii. It was overexpressed in bacteria isolated from a biofilm as compared to their planktonic counterparts. The cloning of this pmt gene in E. coli DH5α enabled the strain to more readily form biofilm, adhere to polystyrene as well as to HeLa and Saccharomyces cerevisiae cells [5]. The disruption of abaF, coding for another MFS transporter, was also associated with a decrease in biofilm formation and virulence [29]. Additionally, a few studies have focused on the role of RND efflux pumps in biofilm formation. Some report a positive association between AdeABC, AdeIJK and/or AdeFGH expression and biofilm formation [41-43]. For instance, experimental levofloxacin induction of RND EPs in 3 clinical strains led to a consistent increase in adeG expression correlated to biofilm induction [41] while expressions of adeB, adeG and adeJ were positively correlated with biofilm formation [43]. However, another work concluded that overexpression of AdeABC and AdeIJK was associated with a decreased biofilm formation [44].

The importance of RND EPs’ regulation systems in biofilm formation has also been underlined. The two-component system AdeRS appears as mandatory for the formation of biofilm in an ex vivo porcine mucosal model. Moreover, deletion of adeB impacted the formation of biofilm on plastic [1, 37]. Also noteworthy, an A. baumannii strain lacking AdeRS displayed an increased expression of genes involved in adherence (pil and com), whereas the loss of adeB resulted in a decreased expression of these same genes.

Whether down- or upregulated, EPs therefore appear as contributors to bacterial adherence and biofilm formation both in artificial and biological conditions. As biofilm formation has been pointed out as a risk factor for the acquisition and/or chronic persistence of infections in humans, it would therefore be interesting to further explore A. baumannii’s EPs expression in environmental and clinical settings to pinpoint their role and possibly propose alternatives targeting EPs to enable A. baumannii’s biofilm reduction/eradication.

4. Role of Acinetobacter baumannii efflux pumps in antibiotic resistance

Overexpression of EPs has been postulated to be a first step towards the development of a Multidrug Resistance (MDR) phenotype in bacteria [5, 8]. Therefore, this section will deal with the implication of A. baumannii’s EPs in antibiotic resistance.

4.1 RND EPs

The role of RND EPs in A. baumannii antibiotic resistance has been studied through the overexpression and knock-out of the three major EPs belonging to this family (i.e. AdeABC, AdeFGH and AdeIJK) as well as through their expression in series of clinical isolates. This EP family is the most thoroughly investigated in A. baumannii antibiotic resistant strains. From the data available today, it is the most important EP family in terms of contribution to antibiotic resistance, especially for tigecycline resistance.

- AdeABC

Aminoglycosides and fluoroquinolones were the first substrates described for AdeABC [11]. The prevalence of adeA and adeB genes is high among A. baumannii strains (70% and up to 100%) [45-51]. In wild strains, AdeABC is thought to contribute to the natural resistance to molecules such as nalidixic acid [52]. However, the clinical relevance of RND EPs is mainly revealed when they are overexpressed. After overexpression induction, a broad range of antibiotic substrates can be identified as expelled from A. baumannii strains by RND EPs [7, 52].

Indeed, the artificial overexpression of AdeABC in a mutant strain leads to an increase in MICs for most major antibiotic classes: β-lactams, aminoglycosides, fluoroquinolones, trimethoprim, tetracyclins-tigecycline, macrolides-
lincosamides as well as chloramphenicol. Clinical resistance is even reached for aminoglycosides. The same set of experiments shows that colistin, rifampin and fusidic acid remain unaffected [44].

Tigecycline being a last chance treatment for *A. baumannii* multi-resistant strains, efflux-mediated tigecycline resistance has been extensively investigated in such strains. In multi-resistant isolates of *A. baumannii*, tigecycline resistance appears to be linked to AdeABC overexpression. Indeed, comparisons between tigecycline susceptible and non-susceptible strains quite consistently report higher expressions of AdeABC in non-susceptible strains [18, 49, 53-57]. Whether adeB mRNA levels correlate with tigecycline MICs remains a matter of debate. Some studies found a positive relationship between these two variables [58-60] hinting at a possible cause-effect relationship while others failed to detect the same pattern [18, 49]. Interestingly, adeC gene was only found in 20% of tigecycline resistant isolates, suggesting that the AdeABC pump can remain functional using an outer membrane protein other than AdeC [51]. Previously, the disruption of adeC did not generate the loss of antibiotic resistance, as compared to the parent strain [12]. Therefore, AdeC appears as expendable in the EP function. Additionally, a lower prevalence of adeC in genotypically diverse *A. baumannii* strains, as compared to adeA and adeB, was also reported in earlier studies [45, 48] and the use of an outer membrane protein different from AdeC pointed out [9, 12]. The genomic study of tigecycline resistance determinants also underlines the part of regulator gene adeR as its deletion triggers an 8-fold decrease in the MIC<sub>50</sub> of a panel of *A. baumannii* resistant strains [61]. Another study pointed out two mutational hotspots in conserved domains of AdeRS leading to the overexpression of AdeABC [18] and mutation in either adeR or adeS were detected in tigecycline-resistant clinical isolates with adeB overexpression [59].

As for β-lactams, a contribution of overexpressed AdeABC to carbapenem resistance is also strongly suspected. For example, a 10- to 40-fold increase in the expression of adeB was witnessed in 6 human strains resistant to imipenem as compared to their susceptible counterpart [62]. However, a high level resistance to imipenem is unlikely to be solely linked to the overexpression of an RND EP, as pointed out by recent works on 303 and 46 imipenem resistant strains, respectively [63, 64]. Similarly to what was witnessed for imipenem, the reduction in cefepime susceptibility in AmpC-linked to the overexpression of an RND EP, as pointed out by recent works on 303 and 46 imipenem resistant strains, [18, 65] that would not give norepinephrine sufficient time to significantly impact AdeFGH expression before bacterial cells are killed [65].

### AdeIJK

This EP was first described in 2008 [13] and the prevalence of adeJ has been found to oscillate between 87.5% [49] and 100% [47, 50] in *A. baumannii* clinical isolates.

An AdeIJK overexpressing mutant was shown to display major increases in MICs for various β-lactams as well as for trimethoprim, fusidic acid and chloramphenicol. The deletion of adeJ restored the susceptibility to all β-lactams to the levels of the parent strain. Additionally, MICs for trimethoprim, sulfadoxine, fusidic acid, chloramphenicol, macrolides-lincosamides, tetracyclins and quinolones were lower for the adeJ-deleted strain as compared to the parent strain, pointing to a possible role of AdeIJK in the intrinsically reduced susceptibility to these molecules [44]. Tigecycline resistance was also related to adeJ expression in a series of 21 MDR *A. baumannii* strains [57].

### AdeFGH

With reported prevalences ranging from 95% [49] to 100% [50], this is the last major RND EP described in *A. baumannii* to this day [14].

MICs for quinolones, chloramphenicol and anti-folates were increased in a mutant overexpressing AdeFGH [44]. Inaba et al. recently showed that norepinephrine induced a 4 to 6-fold increase in the expression of adeG (but not of adeB or adeJ) which correlated with a reduced bactericidal activity for tigecyclin but not for colistin. This differential impact on colistine and tigecyclin bactericidal effects could be linked with the faster action of the former (less than 8 hours) that would not give norepinephrine sufficient time to significantly impact AdeFGH expression before bacterial cells are killed [65].

### E. coli’s ErmAB-ToIC homologue

As mentioned above, a homologue of emrA was recently described in the literature for *A. baumannii* and, based on in silico data, could accept aminoglycosides (netilmicin, tobramicin) as well as imipenem as substrates [15].

Resistance to colistin could be a function of emrB-like genes as deletion of one of these genes in an *A. baumannii* strain induced an increase of susceptibility to colistin but also to spiramycin, paromomycin and nafcillin, as compared to the parent strain. Conversely, overexpression of emrB-like genes induced by colistin backs their part in colistin resistance [57].

### AbeD

An *A. baumannii* mutant strain in which abeD was invalidated proved to have lower MICs for ceftriaxone, gentamicine, tobramicine and rifampicine. Meanwhile, transformation of a susceptible *Escherichia coli* strain with this gene led to higher MICs for erythromycin, ertapenem and amikacin. Therefore, AbeD display a large substrate range and could contribute to the native resistance phenotype in *A. baumannii* [34].
4.2. MFS EPs

- Tet(A) & Tet(B)

These EPs display a rather narrow substrate specificity and thus only mainly contribute to tetracycline resistance [26, 66] and also, to a lesser extent, to minocycline resistance for the sole Tet(B) EP. In A. baumannii isolates resistant to tetracycline, studies have situated the prevalence of tet(A) between 32 and 44% [26, 67]. For tet(B), it ranges from 32.5% [26] to 70% [68]. In Argentina, a recent report points out that tet(B) is widespread in XDR A. baumannii and could be the reason for the emergence of tetracycline resistance in this country [69].

- AbaF

Lately, the cloning of a gene found in an A. baumannii isolate resistant to fosfomycin (called abaF) in an efflux-deficient E. coli strain generated an increase in fosfomycin resistance. Ethidium bromide was also less accumulated in the abaF supplemented E. coli, ascertaining its role as an EP. Additionally, disruption of abaF restored fosfomycin susceptibility to some extent while the antibiotic was shown to induce abaF expression and abaF expression to be higher in resistant clinical strains as compared to susceptible ones [29]. These results advocate for a part of AbaF in fosfomycin resistance for A. baumannii strains.

- CraA

Described with a prevalence of 100% in a series of 82 A. baumannii isolates, craA gene, when deleted, led to a drastic drop in chloramphenicol MIC while quinolones, tetracyclines, aminoglycosides, and imipenem susceptibility levels remained unaffected [28]. Complementation of the deleted strain with the gene restored its resistance to chloramphenicol. CraA might therefore contribute to A. baumannii intrinsic resistance to this molecule.

4.3 SMR EPs

- AbeS

First characterised by Srinivasan et al. in 2009, this EP was shown to increase MICs for erythromycin and novobiocin by a 5- to 6-fold ratio when its encoding gene was transfected to an E. coli susceptible strain. Resistance to detergent such as sodium dodecyl sulfate and benzalkonium chloride were also registered [70]. An increase in abeS expression was witnessed after exposure to chlorhexidine digluconate, confirming its potential part in A. baumannii biocide resistance [71]. A recent report linked abeS overexpression with amikacin resistance in MDR A. baumannii clinical isolates [57]. Further information on AbeS substrate specificity can be found in a paper by Lytvynenko et al. [72].

4.4. MATE EPs

- AbeM

In MDR A. baumannii strains, the prevalence of abeM oscillates between 63.3% [47] and 100% [73]. Its substrate specificity was studied through deletion/complementation experimentations and it was concluded that quinolones and aminoglycosides were the two main antibiotic families impacted by this EP [74].

However, most studies on clinical strains conclude that abeM expression does not correlate with phenotypic resistance to antibiotics [49, 58, 73, 75].

The main EPs that can be present in A. baumannii, often simultaneously, have been described here. Their part in the emergence of resistance to a wide array of antibiotics and biocides is clear. They therefore constitute an interesting target for the development of auxiliary therapeutic molecules (EPIs) that would help restore the activity of known antibiotics, either by co-administration or by forming bi-drugs. The search for such EPIs started over 15 years ago and is still going on, as will be described in the next paragraph.

5. Efflux pump inhibitors as a potential tool to restore antibiotic susceptibility in A. baumannii

The chemical structures of the main EPIs studied in the literature are found in Figure 3. MICs reported for these various EPIs are presented in Table 1. These EPIs do not exhibit a strong intrinsic antibiotic activity and would therefore only be of use on resistant clinical strains in combination with antibiotics for which an efflux-mediated resistance is suspected.

Omeprazole, reserpine and verapamil are well known pharmaceutical drugs. Omeprazole is used to treat gastric ulcers because of its ability to block H⁺ pumps [76]. It was therefore proposed as a potential inhibitor of EP families using the H⁺ gradient (drug/H⁺ antiporters) to eject antimicrobial drugs for the cytosol such as MFS, SMR and RND. Reserpine and verapamil are inhibitors of the P-glycoprotein, an ABC EP found in humans. As ABC EPs are not described as major contributors to antibiotic resistance, a low impact of these two molecules on clinical A. baumannii strains can be expected.
Carbonyl cyanide m-chlorophenyl-hydrazone (CCCP) acts by uncoupling oxidative phosphorylations, hence disrupting the proton gradient of membranes [77] while the precise mechanism of action of 1-(1 naphthylmethyl)-piperazine (NMP) has never been described in A. baumannii. Finally the mode of action of Phenylalanine-Arginine-β-naphthylamide (PAβN, also called MC207-110 in earlier publications) remains a matter of debate, as will be discussed below.

These EPIs have not been employed in Phase III clinical trials so far and are mostly used in vitro to assess whether an efflux-mediated resistance is present in bacteria. The cut-off value usually accepted as a signature of an efflux-driven resistance is a 4-fold reduction in the original MIC for a given antibiotic when an EPI is added [78, 79]. The main reason behind their lack of clinical use is the potential side effects that would result from their use at the concentrations that are found to be active in vitro, especially for well known drugs such as verapamil or reserpine but also for PAβN [80].

Table 1. MICs (µg/mL) of the main EPIs studied in the literature on Acinetobacter baumannii strains.

<table>
<thead>
<tr>
<th>Reference</th>
<th>CCCP</th>
<th>NMP</th>
<th>PAβN</th>
<th>Reserpine</th>
<th>Omeprazole</th>
<th>Verapamil</th>
</tr>
</thead>
<tbody>
<tr>
<td>[79]</td>
<td>40-80/80-200</td>
<td>≥250/200</td>
<td>&gt;200/1000</td>
<td>&gt;100/100</td>
<td>&gt;100/100</td>
<td>&gt;400/400</td>
</tr>
<tr>
<td>[78]</td>
<td>-</td>
<td>400</td>
<td>NR b</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>

a: Values obtained on strains susceptible/resistant to colistin
b: Not reported

- **PAβN**

PAβN was initially described as an inhibitor of P. aeruginosa EPs [81]. Shortly afterwards, the first assay of this EPI (at 20 µg/mL) on 22 clinical strains of A. baumannii strains concluded that it was only effective to reduce nalidixic acid but not ciprofloxacin MICs (45% of the strains had at least an 8-fold reduction in the nalidixic acid MIC) [82].

A direct inhibition of RND EPs through competition with their substrates was initially believed to be its mechanism of action [81]. Indeed, when the operon adeFGH was cloned in a Pseudomonas aeruginosa strain deprived of its EPs to assess the specific effect of PAβN on AdeFGH, trimethoprim, chloramphenicol and clindamycin saw their activities potentiated in the presence PAβN (10 µg/mL) as well as in 3 A. baumannii strains [83]. However, membrane permeabilisation was subsequently described as another mechanism of action for PAβN [84].

Further studies on clinical isolates showed that 756 (~10%) of tigecycline-resistant strains had their MICs lowered by 4-fold or more in the presence of PAβN (70µg/mL) [49]. PAβN (25 µM) was ineffective in an attempt to reduce colistin MICs in A. baumannii strains [85] while a 100 µg/mL concentration induced a 4-fold or higher drop in imipenem MICs for 58/60 isolates resistant to this latter molecule [86].
**NMP**

First described as an inhibitor on *E. coli* AcrAB-TolC EP [87], NMP reduced levofloxacin MICs of *E. coli* strains from 8 to 16-fold. NMP was found to be more effective in reducing MICs than PAβN on *A. baumannii* strains at high concentrations (100 µg/mL) while both EPIs had a very limited effect at lower concentrations (25 µg/mL) [78]. The effect of NMP on tetracyclines and tigecycline resistance was investigated. A paradoxical effect of NMP was witnessed on tigecycline as determined by the disc diffusion and E-test methods. A rise in the resistance to this molecule was observed for 96 clinical strains out of 104 while other tetracyclines displayed lower MICs in the presence of NMP (at 64 µg/mL). This is possibly linked to the blockage of a component required for uptake of the drug [88]. A similar work on β-lactams, gentamicin, and fluoroquinolones concluded that NMP (100 µg/mL) mainly promoted fluoroquinolones’s activities on 42 *A. baumannii* clinical strains [89].

As for tigecycline, over 55% of 81 tigecycline-resistant *A. baumannii* strains demonstrated a reduction in MIC equal to or greater than 4-fold in the presence of NMP (100 µg/mL) [55] while another study testing the same amount of NMP did not report such a reduction [49].

**CCCP**

On a series of 4 *A. baumannii* strains resistant to colistin, CCCP (10 µg/mL) was able to reduce colistin MICs by a 128 to 512-fold factor but not other EPIs such as PAβN (25 µg/mL), omeprazole (20 µg/mL), NMP (25 µg/mL) or verapamil (80 µg/mL) [77], supporting the results obtained previously for colistin and CCCP (10 µM) [85]. Tigecycline MIC were reduced by 4-fold at least by the addition of CCCP (5µg/mL) in ~12% of tigecycline-resistant isolates and 30% of tigecyclin susceptible isolates.

A decrease of at least 4-fold was witnessed in ciprofloxacin MICs for nearly 46% of ciprofloxacin resistant strains when CCCP (25µg/mL) was used in conjunction [90].

**Verapamil**

The poor inhibitory effect of verapamil on *A. baumannii* EPs was confirmed [77, 91]. The molecule was even reported to increase tigecycline MICs in a series of Chinese *A. baumannii* isolates [49].

**Other EPIs**

Some natural molecules can also be act as EPI. A recent paper demonstrated that subinhibitory concentrations (16 or 128 µg/mL) of epigallocatechin3-gallate (EGCG) were able to sensitize carbapenem-resistant isolates of *A. baumannii* and displayed a synergy with meropenem [92]. Catechins are able to bind outer membrane proteins of Gram negative bacteria [93]. Therefore, EGCG could possibly interact with the OMP of RND EPs and subsequently allow for the intercellular accumulation of associated antibiotics, resulting in an increased susceptibility of *A. baumannii* isolates.

**New perspectives in EPIs**

Recently, *N*-tert-butyl-2-(1-tert-butyltetrazol-5-yl)sulfanylacetamide and (E)-4-((4-chlorobenzylidene)amino) benzenesulfonamide were shown to induce minocycline accumulation in *A. baumannii* during growth in serum [94]. They were also active on *Pseudomonas aeruginosa* antibiotic efflux but devoid of cytotoxicity (on human HepG2 cells) and exhibited no inhibitory effect on a mammalian calcium channel, giving interesting clues on their selectivity for a potential future use in humans.

The activity of a series of pyronaridine EPIs targeted towards RND EPs of *E. coli* and *P. aeruginosa* has also been related lately but their actual efficiency on *A. baumannii* was not tested which is a major drawback as some EPIs (e.g. pyridopyrimidine derivative D13-9001) have proved to be highly specific of a given EP [7, 95]. Piperazine aryldenedimidazolones were tested on *E. coli* AcrAB-TolC EP and a *P. aeruginosa* overexpressing MexAB-OprM EP [96]. Some show promising activities but again, they were not tested on *A. baumannii* and no preclinical data is available for these products.

Lastly, Verma et al. proposed an *in silico* model for the screening of AdeABC inhibitors. This model has the advantage of including an ADMET (absorption, distribution, metabolism, excretion and Toxicity) screening of EPI candidates, which is a first step towards selecting viable leads for a potential clinical use [97].

To sum up on EPIs, inhibitors such as verapamil, omeprazole and reserpine only show little to non efficiency in blocking *A. baumannii* EPs. Through the numerous studies on clinical isolates around the world, NMP, CCCP and PAβN have displayed interesting but sometimes divergent results. This might be accounted for by the variety of EPI concentrations tested as well as by the varying panels of strains. These strains certainly harbor more than one EP at a time and that would skew the experimental results. A standardized frame for the testing of EPIs would therefore be an interesting advance along with the use of strains genetically adapted to only express one EP to ascertain the effect of putative EPI molecules [83].
6. Conclusion

EPs, especially those belonging to the RND family, are an important feature in *A. baumannii* both from an environmental and clinical point of view. They enable the bacterium to adapt to environmental conditions and are a first step towards acquisition of a MDR-phenotype in clinical strains. The use of EPs has therefore been proposed as another pathway to fight antimicrobial resistance in *A. baumannii* and other bacterial strains. However, studies published so far mainly deal with *in vitro* assays of molecules which have little to no chance to reach clinical development because of toxicity issues. Future research should therefore focus on the development of new EPs, addressing their selectivity as well as efficiency in a standardised framework to ensure their future role in clinical settings.

Acknowledgements The support of Bakhta Bouharkat by a Partenariat Hubert Curien Tassili PhD grant 16MDU974/35118Z is gratefully acknowledged.

References


