Chapter 8
Engineered OAKs Against Antibiotic Resistance and for Bacterial Detection

Amram Mor

Abstract As bacterial resistance to antibiotics continues to threaten modern healthcare worldwide, the need for new approaches that control bacterial infections becomes evermore urgent. Membrane-active compounds (MACs) are currently gaining interest for their potential to address various antibiotic resistance challenges. Since MACs are able to target multiple vital bacterial functions simultaneously, they may have the advantage of fighting the infection while avoiding many of the known resistance mechanisms. This chapter reviews current data regarding the attempts to use oligomers of acylated cations (OACs) as a platform for optimizing the hydrophobic/cationic balance required for selective nonspecific membrane interactions of MACs, under in vitro and in vivo conditions. With the perspective gained over nearly a decade after their conception and after a few dozen investigations involving several hundreds of analogs, we describe the properties of a few representative lysyl-based OAC (OAK) sequences. These sequences reflect the OAC concept evolution from the original focus on bactericidal MACs that later shifted onto bacteriostatic derivatives and presently concentrates on seemingly inactive analogs that nonetheless improve the control of bacterial infections. Collectively, the current data appear to substantiate the potential of OAC-based MACs as a valuable resource for therapeutic antibacterial development, including for systemic applications.

8.1 Introduction

The continuous escalation of multidrug resistant (MDR) bacteria (Schaberle and Hack 2014; Eckert 2011) is inevitably leading to the dwindling supply of clinical treatment options (Haney and Hancock 2013). Thus, along with the multitude of strategies currently employed in an attempt to maintain an effective arsenal of

A. Mor (✉)
Department of Biotechnology & Food Engineering, Technion-Israel Institute of Technology, 32000 Haifa, Israel
e-mail: amor@tx.technion.ac.il

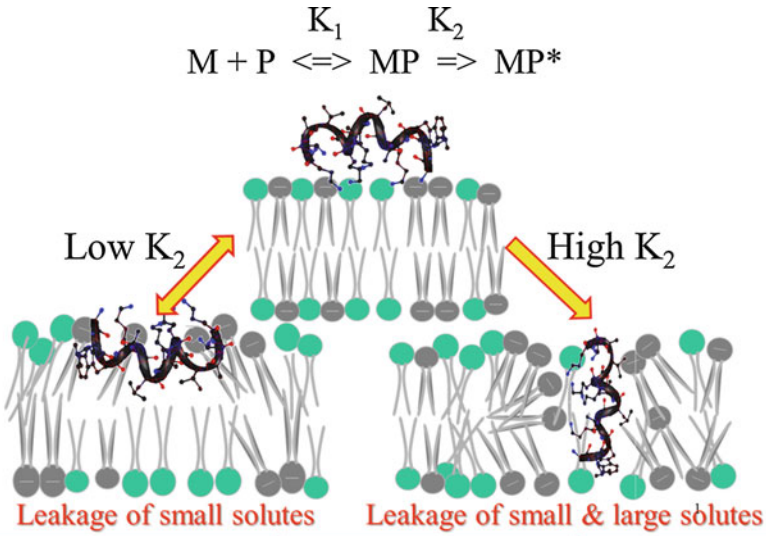
© Springer International Publishing Switzerland 2016

R.M. Epand (ed.), *Host Defense Peptides and Their Potential as Therapeutic Agents*, DOI 10.1007/978-3-319-32949-9_8

available chemotherapeutic products (Kinch et al. 2014; Silver 2011; Fischbach and Walsh 2009), there is a genuine need for new infection control approaches in order to meet the formidable capacity of bacteria to challenge new and old generations of antibiotics (Blair et al. 2015; McCallum et al. 2010; Poole 2012). In this respect, membrane active compounds (MACs) are presently gaining a renewed interest for their potential to control MDR infections (Klitgaard et al. 2008; Hurdle et al. 2011; Allen et al. 2014) by affecting critical processes that rely on common principles such as in bacterial sensing/communication (Gooderham and Hancock 2009; Daly et al. 2015), membrane proteins localization during division (Strahl and Hamoen 2010) and virulence (Daly et al. 2015; Sully et al. 2014). The molecular basis for these effects are relatively ill understood, however.

Based on their hydrophobic attributes MACs are dividable into two main classes; MACs having a pronounced hydrophobic character and borderline hydrophobic MACs. Members of the first class, tend to disrupt the bilayer structure abruptly, following deep insertion within the cytoplasmic membrane (Epand and Vogel 1999; Epand et al. 2010; Westerhoff et al. 1989) which, often culminates in a rapid bactericidal outcome at low micromolar concentrations (Hancock and Chapple 1999; Rotem and Mor 2009). Members of the second class are subject to more superficial membrane interactions and consequently believed to cause milder structural damages at the same low concentrations. As many bacteria can readily repair these damages (Hicks et al. 1994; Padan et al. 2005), such MACs might be considered altogether inactive molecules, although they can exhibit a minimal inhibitory concentration (MIC) at higher doses or display a bacteriostatic mode of action. Thus, even though transient, such superficial membrane damages appear nonetheless to inflict crippling injuries that clearly bare high cost on bacterial metabolism. For instance, the ordered packing of phospholipid can be distorted by the steric hindrance of bulky MACs, to the point that allows leakage of small ions such as protons, thereby leading to loss of the transmembrane potential (TMP). The repair process therefore, can be an exploitable window of opportunity toward controlling bacterial infections since their energy sources become depleted following membrane depolarization, thereby inhibiting vital bioenergetics and transport functions. Thus, despite maintaining near-normal proliferation rates, the penalties for bacteria can be devastating since, by inhibiting efflux pumps or export of resistance factors, such MACs might in fact sensitize bacteria to efflux substrate antibiotics (as they can now accumulate in the cytoplasm and exert their toxic effect) or restore sensitivity to formerly efficient antibiotics (for lack of resistance factors), respectively. By extension, such MACs might also significantly affect bacterial communication and virulence, as discussed in Sect. 8.2.

Host defense peptides (HDPs) can include both classes of MACs, as illustrated in Fig. 8.1. One might wonder which of these MAC classes are preferable for the developing therapeutic drugs. As the issue is out of the scope of this review, we briefly illustrate the debate with two opposing arguments: on one hand, the latter compounds might be advantageous since their milder action reduces the risk for complications associated with endotoxins released by bactericidal counterparts (Marr et al. 2006; Schuerholz et al. 2012). On the other hand, exposure of bacteria to sublethal drug



Cationic MAC	K_1 (M^{-1}) Adhesion	K_2 Insertion
$C_{12}K\text{-}7\alpha_8$	0.6×10^5	1.7
$C_{12(\omega 7)}K\text{-}\beta_{12}$	8.6×10^5	0.006

Fig. 8.1 Hypothetical interactions between a MAC and a mixed phospholipid bilayer. At the top is an equation describing the interaction between a MAC (M) and the phospholipid membrane (P). The cartoon underneath, illustrates the idea that electrostatic attraction is the initial force driving adhesion between a cationic M and anionic P to form a reversible complex (MP). This complex can reorganize to form different types of a more stable complex MP*, thereby perturbing the membrane structure in a manner that depends mainly on M's insertion within the membrane. The table at the bottom, lists parameters describing the binding of representative OAKs to a P composed of POPE:PG (3:1) as determined by SPR and analyzed by the 2-step model (Gaidukov et al. 2003). Our interpretation of these data, envisions that the apparent affinity constant describing the global interaction is the product of the individual constants for each step (K_1 and K_2), respectively, describing the kinetic ratio (k_{on}/k_{off}) for the adhesion and the insertion steps. In that case, the observed values suggest that despite the fact that roughly 10 times less $C_{12}K\text{-}7\alpha_8$ molecules adhere to the membrane (compared with $C_{12\omega 7}K\text{-}\beta_{12}$), their tendency for insertion is much stronger. High tendency for insertion, in turn, can be correlated to massive bilayer disturbances (Rotem et al. 2008a). In contrast, $C_{12\omega 7}K\text{-}\beta_{12}$ molecules tendency for insertion is very low and hence likely to remain stuck in superficial interactions that cause milder damages (e.g., membrane depolarization) (Sarig et al. 2010)

concentrations is not without potential detrimental effects, as various multicomponent sensory systems were implicated in bacterial resistance to MACs. For instance, Gram-positive bacteria (GPB) sense sub-MIC levels of antimicrobial peptides and confer resistance to these peptides in a GraRS–VraFG pathway-dependent manner

(Yang et al. 2012; Koprivnjak and Peschel 2011). The subsequent cell modifications can reduce the cytoplasmic membrane net negative charge (e.g., by addition of a lysine or alanine to phosphatidyl glycerol and lipoteichoic acid, respectively (Andra et al. 2011; Fedtke et al. 2004)), thereby reducing the electrostatic attraction between bacteria and cationic MACs. The fact that this induction occurred on exposure to polymyxin B and to RP-1 but not to daptomycin or hNP-1 (Yang et al. 2012), suggests that it might concern only certain antimicrobial peptides. In Gram-negative bacteria (GNB), several similar two-component systems for magnesium ions (Gooderham and Hancock 2009; Fernandez et al. 2010) were shown to be activated by a variety of HDPs (Fernandez et al. 2010; Shprung et al. 2012) and resulted in the modification of lipid A by addition of amino arabinose and phosphoethanolamine (Koprivnjak and Peschel 2011).

Many antibacterial HDPs increase outer membrane permeability through perturbation of the lipopolysaccharides (LPS) structure/function of Gram-negative species (Vaara et al. 2008; Zhang et al. 2000; Sawyer et al. 1988). These peptides can ultimately alter functions of the cytoplasmic membrane such as the permeability barrier (Epand et al. 2010; Ruhr and Sahl 1985; Zasloff 2002; Hancock 2005) and cell wall synthesis (Reisinger et al. 1980; Sass et al. 2010), namely as a consequence of charge clustering (Epand et al. 2010, 2008a; Epand and Epand 2009; Jean-Francois et al. 2008). Similarly, various chemical mimics of HDPs also interact with LPS (Jahnsen et al. 2013; Rotem et al. 2008a) and perturb the outer (Mensa et al. 2011) and cytoplasmic membranes, even at sub-MIC (Jammal et al. 2015; Goldberg et al. 2013; Livne et al. 2010), suggesting that certain membrane damages, such as those sustained at sub-MIC conditions, may underlie bacterial sensitization to antibiotics, as illustrated in Fig. 8.2.

Furthermore, as evident in current literature, there is an emerging interest in developing new combination therapies involving mixtures of classical antibiotics and antimicrobial HDPs (Dhand et al. 2011; Sakoulas et al. 2014; Paul et al. 2014; Li et al. 2014). However, despite their promising attributes, some HDPs can suffer from shortcomings such as protease sensitivity, systemic toxicity and/or high production costs, which hamper their systemic therapeutic potential. Therefore, at least theoretically, de novo designed chemical mimics of HDPs may be better adapted in addressing some of these challenges (Rotem and Mor 2009; Jahnsen et al. 2013; Jammal et al. 2015; Goldberg et al. 2013; Livne et al. 2010; Kaneti et al. 2013; Liu et al. 2004). HDP-mimics may better promote efficient systemic therapies owing to their improved pharmacokinetics (Jammal et al. 2015; Radzishevsky et al. 2007; Choi et al. 2009) whereas their structural simplicity should better support fine-tuning mechanistic studies. In the following sections, the review will focus on attempts to mimic natural MACs using oligomers of acylated cations (OACs). Table 8.1 lists a few representative lysyl-based OACs (OAKs) that will be emphasized throughout the review as they reflect the actual evolution of the concept, which originally concentrated on bactericidal MACs, moved on to bacteriostatic derivatives and ended up converging on seemingly inactive but promising analogs.

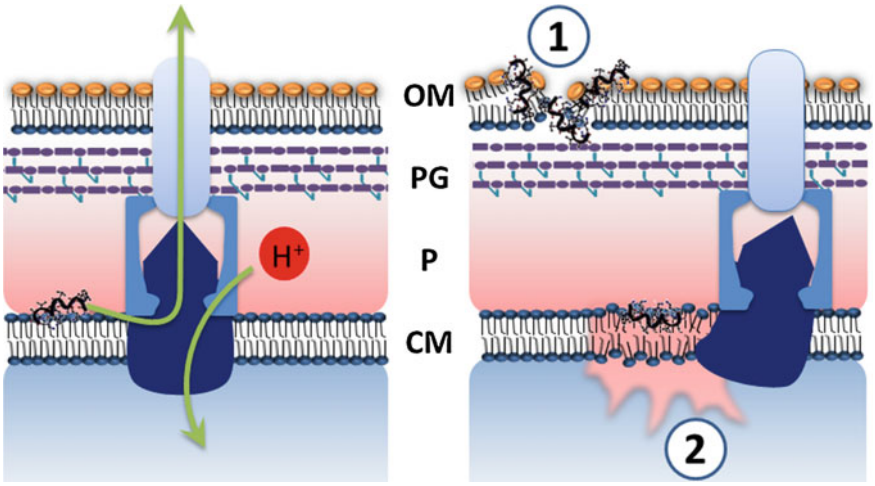


Fig. 8.2 Potential membrane damages affecting permeability and efflux functions. The *left panel* is a cartoon representation of a typical efflux pump, AcrAB-TolC, a member of the resistance nodulation division (RND) family, exclusively found in Gram-negative bacteria. It is able to extrude an antibiotic (or an HDP) from the cytoplasm or periplasm (P) in exchange for proton influx (Paulsen et al. 1996). The *right panel* illustrates two potential MAC-induced damages: (1) Cations (e.g., Ca^{++}) that normally stabilize the negative phosphate charges of the outer membrane (OM) LPS layer, are displaced by a MAC, owing to its higher affinity to LPS. However, due to its bulkier size, the MAC distorts the ordered packing of LPS molecules, thereby leading to cracks that allow entry of solutes (including of MACs), to the peptidoglycan (PG) layer, as described by the self-promoted uptake theory (Hancock and Chapple 1999). Option (2) illustrates potential fates of a moderately hydrophobic MAC (such as $C_{12\omega 7}K\text{-}\beta_{12}$ from Fig. 8.1) that adheres to anionic phospholipids of the cytoplasmic membrane (CM), a step likely facilitated by the negative inside electrochemical difference of potential existing across the CM. Such a MAC is predicted to assume only a superficial position on the CM outer leaflet (Sarig et al. 2010). Nonetheless, MAC accumulation over the CM might distort (again) the phospholipids ordered packing, to the point that allows leakage of small ions (such as protons) thereby leading to loss of the transmembrane potential and consequently loss of the energy source driving the function of efflux pumps and many other membrane proteins. Moreover, such MACs are also likely to alter the lipid environment of membrane proteins whose function relies on specific chemophysical characteristics (such as charge, fluidity, or bilayer thickness), or modify the proteins relative positions (see, for instance, the distorted alignment between AcrB and TolC in the CM and OM, respectively) which could also lead to a malfunctioning complex

8.2 Membrane-Active Antibacterial OAKs

Unlike animal cells whose cytoplasmic membrane contains a very low amount of anionic lipids, bacterial membranes are typically rich in anionic phospholipids whose relative proportion can reach 20–30 % in Gram-negative bacteria and nearly 100 % in Gram-positive bacteria (Ratledge and Wilkinson 1988; Yeaman and Yount 2003). As the OAC platform consists exclusively of fatty acyls and amide-linked

Table 8.1 Biophysical properties of representative OAKs

Designation	Sequence	Q	H	[e]MIC_{90} (μM)	Known damage in vitro	MOA at MIC	Systemic efficacy
[a]$C_{12}K\text{-}7\alpha_8$	$C_{12}\mathbf{K}\text{-}c_8\mathbf{K}c_8\mathbf{K}c_8\mathbf{K}c_8\mathbf{K}c_8\mathbf{K}c_8\mathbf{K}c_8\mathbf{K}_{NH2}$	8	47	GNB: 5 GPB: ≥50	CM charge clustering	Bactericidal	Only in combination therapy and after encapsulation
[b]$C_{12}K\text{-}3\beta_{10}$	$C_{12}\mathbf{K}\text{-}\mathbf{K}c_{10}\mathbf{K}\mathbf{K}c_{10}\mathbf{K}\mathbf{K}c_{10}\mathbf{K}_{NH2}$	7	45	GNB: 5 GPB: 5	CM charge clustering	Bactericidal	Yes
[c]$C_{12\omega7}K\text{-}\beta_{12}$	$C_{12\omega7}\mathbf{K}\text{-}\mathbf{K}c_{12}\mathbf{K}_{NH2}$	3	49	GNB: ≥50 GPB: 5	CM depolarization	Bacteriostatic	Only in combination therapy (without encapsulation)
[d]$C_{10}K\text{-}\beta_{12}$	$C_{10}\mathbf{K}\text{-}\mathbf{K}c_{12}\mathbf{K}_{NH2}$	3	46	GNB: >50 GPB: >50	CM depolarization	Inactive	Only in combination therapy (without encapsulation)

[a]Dodecanoyllysysl-[aminooctanoyllysyl]$_7$; [b]Dodecanoyllysysl-[lysylaminodecanoyllysyl]$_3$; [c]ω7-dodecenoyllysysl-lysylaminododecanoyl lysyl; [d]Decanoyl-lysysl-lysylaminododecanoyllysyl; [e]MIC_{90}, as determined on 90 % of at least 50 bacterial strains belonging to at least 10 different species; C, N-terminal acyl; c, aminoacyl. Q, Charge at physiological pH; H, Hydrophobicity as estimated by HPLC, reflecting percent acetonitrile/water required for elution from a C18 column; GNB, Gram-negative bacteria; GPB, Gram-positive bacteria; CM, Cytoplasmic membrane; MOA, Mode of action

cationic aminoacyls (Radzishevsky et al. 2007; Livne et al. 2009; Radzishevsky et al. 2008), it is particularly suitable for engineering high-affinity MACs.

One of the first sequences investigated for its membrane-active properties was dodecanoyllysysl-[aminooctanoyllysyl]$_7$ referred to as $C_{12}K\text{-}7\alpha_8$, (Radzishevsky et al. 2007) that preferentially targeted Gram-negative bacteria by exerting a rapid biocidal effect. The bactericidal outcome was proposed to stem from the peptides high binding affinity to the cytoplasmic membrane phospholipids, despite strong interactions with cell wall components as well. Various mechanistic studies support the view that $C_{12}K\text{-}7\alpha_8$ causes rapid bacterial death through disruption of the cytoplasmic membrane (Radzishevsky et al. 2007; Rotem et al. 2008b; Epand et al. 2008b). Conversely, a shorter analog ($C_{12}K\text{-}5\alpha_8$) having significantly lower aptitude for disrupting the membrane and displaying significantly slower time-kill curves, was proposed to rather inhibit the biosynthetic process. Being more hydrophobic but less cationic, $C_{12}K\text{-}5\alpha_8$ was allegedly able to reduce many electrostatic interactions on the way from the cell wall to the cytoplasmic membrane. Hence, unlike the case of $C_{12}K\text{-}7\alpha_8$, the functional transmembrane potential difference might actually promote internalization of $C_{12}K\text{-}5\alpha_8$, thereby enabling its interaction with intracellular targets, as exemplified with nucleic acids (Rotem et al. 2008b).

Two different studies have used either isothermal titration calorimetry (ITC (Epand et al. 2008b)) or surface plasmon resonance (SPR (Rotem et al. 2008b)) technologies to compare the binding properties of these analogs to model phospholipid membranes. The studies independently confirmed the higher binding affinity of $C_{12}K\text{-}7\alpha_8$ (also observed using DSC and NMR studies) while moreover indicating that only $C_{12}K\text{-}7\alpha_8$ had the ability to induce the clustering of anionic lipids. This clustering effect may lead to the lateral segregation of domains rich in anionic versus zwitterionic lipids, producing phase boundary defects that ultimately breach the permeability barrier of the cytoplasmic membrane.

Further studies of this ability to induce clustering of anionic lipids eventually led to the idea to exploit this property for co-encapsulation of synergistic drugs in lipid-based stable structures, called cochleates, whose aim would be to shield initially, and ultimately co-deliver the drugs. Inspired by reports on antimicrobial peptides that exhibited synergistic action with conventional antibiotics (Livne et al. 2010), such a potential role for $C_{12}K\text{-}7\alpha_8$ was investigated toward fighting MDR phenomena in Gram-negative bacteria. MIC determination against multiple *E. coli* MDR strains revealed combinations with sub-MIC OAK levels that acted synergistically with several antibiotics, thus lowering their MICs by several orders of magnitude. Attempts to shed light into the molecular basis for this synergism suggested that bacterial sensitization to antibiotics was derived mainly from the OAK's capacity to overcome the efflux-enhanced resistance mechanism, by promoting backdoor entry of otherwise excluded antibiotics (Fig. 8.2). Synergistic action between distinct molecular entities is, however, likely to suffer from numerous challenges during systemic therapy (namely owing to differential pharmacokinetics, body distribution, or tissue penetration), that might challenge the sensitization effect observed in vitro. Consequently, a follow-up work has aimed to

facilitate the simultaneous delivery of the synergistic drugs to the infection site while co-encapsulated within a delivery system. Out of several systems screened, OAK-based cochleates turned out to be quite remarkable in their capacity for rapid, stable, and high capacity co-encapsulation of drugs (Sarig et al. 2011; Epand et al. 2011). Such cochleates have also demonstrated advantages in systemic therapeutic efficacy in treating severe murine bacterial infection, as elaborated in Sect. 8.3.

While $C_{12}K\text{-}7\alpha_8$ is preferentially active on Gram-negative bacteria, its analog dodecanoyllysysl-[lysylaminodecanoyllysyl]$_3$ (referred to as $C_{12}K\text{-}3\beta_{10}$) presents an indiscriminate activity over many more bacterial species (MIC_{90} = 5 μM), yet displays low hemotoxicity, namely at concentrations as high as >100 μM. The main difference between these closely related OAKs (i.e., having similar HQ properties as shown in Table 8.1) pertains to their building blocks in that the acyl-lysyl (α) subunits were replaced with lysyl-acyl-lysyl (β) subunits. Consequently, subunits juxtaposition creates a structural motive (lysyl-lysyl) that is absent in the α-OAKs. This motive turned out to be critical in broadening the spectrum of activity, namely to include a wide range of Gram-positive bacteria (Livne et al. 2009) and of cancer cells (Held-Kuznetsov et al. 2009).

Interestingly, although $C_{12}K\text{-}3\beta_{10}$ exerts an essentially bactericidal effect, *E. coli* bacteria, are killed faster than *S. aureus* (i.e., within minutes versus hours), suggesting the involvement of different mechanisms of action. This contrasted with data obtained from SPR analysis that compared the OAK's binding properties using POPG:PE and POPG:CL bilayers (respective molar ratio of 20:80 and 60:40, to mimic the cytoplasmic membranes of the investigated bacteria), suggesting that the OAK presented quite similar membrane binding affinities (i.e., K_{app} = 1.1 and 0.9×10^6 M^{-1}, respectively). Mechanistic studies addressing this discrepancy suggested a peculiar mode of action involving OAK accumulation in the cell wall due to its differential affinity to GPB cell wall specific components. This blocks the advancement of OAK (and other) molecules toward the cytoplasmic membrane and leads to the observed outcome where *S. aureus* bacteria have undergone a transient rapid bactericidal stage that over time converted to a bacteriostatic effect (Livne et al. 2009).

Contrasting with the broad-spectrum activity of $C_{12}K\text{-}3\beta_{10}$, its shorter version ω7-dodecenoyl-lysyl-lysyl-aminododecanoyl-lysyl (referred to as $C_{12\omega7}K\text{-}\beta_{12}$) is principally active on Gram-positive bacteria only (Sarig et al. 2010). Additionally, while maintaining a similar potency in inhibiting bacterial growth (MIC_{90} = 5 μM), $C_{12\omega7}K\text{-}\beta_{12}$ is no longer endowed with the capability for rapid killing of bacteria, even at concentrations of several MIC multiples. These characteristics of $C_{12\omega7}K\text{-}\beta_{12}$ antibacterial activity correlate well with its membrane binding properties, exhibiting clearly lesser binding affinity to model bilayers mimicking the cytoplasmic membrane of Gram-negative compared with Gram-positive bacteria, i.e., $K_{app} = 5 \times 10^3$ versus 2×10^7 M^{-1}, respectively, as determined by SPR (Sarig et al. 2010). Here again, the tempting option to conclude for a causative relationship, is counterargued by the finding that, $C_{12\omega7}K\text{-}\beta_{12}$ was potently active on the isogenic mutant strains where efflux pump components were deleted (Goldberg et al. 2013). This suggested

that the OAK's inactivity rather resulted from the GNB capacity for rapid extrusion of this OAK through efflux pumps, unlike the previous analogs (i.e., $C_{12}K\text{-}7\alpha_8$ and $C_{12}K\text{-}3\beta_{10}$).

Otherwise, $C_{12\omega7}K\text{-}\beta_{12}$ is believed to exert a bacteriostatic effect over GPB owing to its distinct interactions with their cytoplasmic membrane. Once the OAK has adhered to the bilayer outer leaflet (e.g., in *S. aureus*), the deep insertion within the bilayer (observed for bactericidal OAKs such as $C_{12}K\text{-}7\alpha_8$ and $C_{12}K\text{-}3\beta_{10}$) is prevented by its particular chemophysical attributes, thereby limiting the extent of membrane damage that this OAK can inflict (Sarig et al. 2010). In support of this view is the fact that its hydrophobic analogs such as $C_{16\omega7}K\text{-}\beta_{12}$ are rapidly bactericidal to GPB (Sarig et al. 2008). This superficial interaction of $C_{12\omega7}K\text{-}\beta_{12}$ with the cytoplasmic membrane can nonetheless drastically alter various membrane protein functions that rely on its specific chemophysical characteristics (e.g., charge and/or fluidity) for carrying out a function, such as during signal transduction. Moreover, small solutes might leak out owing to the steric hindrance introduced by the OAK that distorts the membrane and induces its depolarization, which in turn also affects additional membrane functions, such as efflux.

Although exhibiting lesser binding affinity for GNB cytoplasmic membrane mimics, various assays assessing membrane damages provide evidence for the ability of $C_{12\omega7}K\text{-}\beta_{12}$ to induce membrane depolarization at low micromolar concentrations (Goldberg et al. 2013). As mentioned above, $C_{12\omega7}K\text{-}\beta_{12}$ seems to be a good substrate for GNB efflux pumps, unlike $C_{12}K\text{-}7\alpha_8$ or $C_{12}K\text{-}3\beta_{10}$, hence, its high MIC over these species ($MIC_{90} \geq 50$ μM). From both respects therefore, membrane depolarization of GNB by $C_{12\omega7}K\text{-}\beta_{12}$, is unexpected (Goldberg et al. 2013). Moreover, the literature often reports that depolarization is associated with bacterial death that normally occurs shortly thereafter (Silverman et al. 2003). It is therefore surprising (again), why is depolarization dissociated from bacterial death, since the number of colony-forming units remains unchanged over time, for at least several hours (Sarig et al. 2010). A possible explanation for these discrepancies maybe directly related to $C_{12\omega7}K\text{-}\beta_{12}$ binding properties to GNB versus GPB cytoplasmic membranes, as determined by SPR. The binding parameters suggest not only a lower apparent binding affinity (recall, $K_{app} = 5 \times 10^3$ versus 2×10^7 M^{-1}, respectively), they also suggested a lower propensity for insertion within the membrane (i.e., $K_{insertion} = k2_{on}/k2_{off} < 1$ versus >1, respectively, as illustrated in Fig. 8.1). Consequently, the events taking place upon adhesion to the cytoplasmic membrane of GNB are probably only slightly deviant from those described above for GPB, summarized as follows: The OAK can readily translocate across the outer membrane, like many HDPs (Hancock and Chapple 1999) as illustrated in Fig. 8.2. After reaching the periplasmic space, the OAK molecules undergo partitioning as they are simultaneously attracted to the inner membrane phospholipids and the efflux pumps. Since the OAK's tendency for insertion within the bilayer is quite low (as illustrated in Fig. 8.1), it is likely to be extruded by RND pumps, unlike $C_{12}K\text{-}7\alpha_8$ for instance, whose deeper insertion in the bilayer likely contributes to its ability to evade extrusion by the efflux pump. Therefore, the observable rapid membrane

depolarization of Gram-negative bacteria by $C_{12\omega7}K\text{-}\beta_{12}$, must be due to the action of the partitioned fraction of OAK molecules that managed to escape efflux.

Furthermore, membrane depolarization itself was associated with the sensitization of various MDR pathogenic species (e.g., Pseudomonas *aeruginosa*, *Klebsiella pneumoniae*, *Salmonella enterica,* and *Escherichia coli*) to ribosome-targeting antibiotics such as erythromycin, an excellent substrate of RND pumps. On this basis, sensitization was proposed to be TMP dependent since it correlated well with inhibition of their efflux pumps (Goldberg et al. 2013), that require a working membrane potential as energy source (Paulsen et al. 1996; Poole 2005). Thus, besides illustrating an interesting case of a highly potent synergistic antibacterial activity that emanated from combination of individually inactive compounds, this study has moreover highlighted the ability of MACs to overcome innate resistance to erythromycin and alike antibiotics.

Further investigation of this sub-MIC bacterial sensitization effect and its pertinence to the TMP revealed additional consequences in Gram-positive bacteria. As for GNB, $C_{12(\omega7)}K\text{-}\beta_{12}$ was able to simultaneously overcome multiple resistance mechanisms in multidrug resistant clinical isolates of *Staphylococcus aureus* strains, which became significantly sensitive to several antibiotics including cell wall-targeting β-lactams (e.g., oxacillin, piperacillin, and penicillin G) as well as ciprofloxacin and tetracycline, respectively, targeting DNA and ribosomes. However, while sensitization of the cytoplasm targeting drugs might be attributed to proton motive force-dependent efflux pumps (e.g., norA and tetK), this could not be the case for *S. aureus* sensitization to β-lactams, whose resistance mechanism is more likely to involve alterations in processing enzymes such as β-lactamase and/or penicillin binding protein 2a. Also noteworthy is the fact that, in addition to the OAC's ability to reduce the β-lactams MIC by up to three orders of magnitude (Kaneti et al. 2013), the study revealed that the rate at which *S. aureus* acquired resistance to β-lactams was considerably delayed in the presence of $C_{12(\omega7)}K\text{-}\beta_{12}$. Thus, the OAC interaction with *S. aureus* has achieved a double score: (a) resensitization to an antibiotic and (b) prevention of developing renewed resistance to that antibiotic. Importantly, antibiotic sensitization was shown to prevail under in vivo conditions, as well (Kaneti et al. 2013) as elaborated in Sect. 8.3.

Studies attempting to shed light into the molecular basis for this remarkable phenomenon suggest that the OAC's ability to resensitize *S. aureus* to β-lactam antibiotics is linked to inhibition of signal transduction cascades, as follows. Binding of a β-lactam antibiotic to its receptor extracellular domain induces a conformational change in the intracellular domain thereby allowing it to function as a protease that cleaves the β-lactamase gene repressor and consequently permits transcription of the Bla divergon (Wilke et al. 2004). Accordingly, qPCR was used to show that bacterial exposure to a β-lactam has indeed induced the signal transduction cascade for both *blaZ* and *mecA* (respectively encoding for β-lactamase and penicillin binding protein 2a). In contrast, addition of sub-MIC OAK has significantly reduced expression of both resistance factors, thereby providing support to the view that $C_{12(\omega7)}K\text{-}\beta_{12}$ interactions with the plasma membrane led to superficial damages (as evidenced by the depolarization assay) which in turn inhibited the signal transduction cascade.

A similar sensitization effect was obtained for bacteria exposed to sub-MIC of another MAC, the ionophore carbonylcyanide 3-chloro-phenylhydrazone (CCCP), in the presence of oxacillin, thereby further supporting the causative relationship between membrane damages (as reflected by depolarization) and the synergistic effects observed with the OAK (Kaneti et al. 2013).

This ability to interfere with expression of resistance factors has motivated additional investigations as to the effects of $C_{12(\omega 7)}K\text{-}\beta_{12}$ on MRSA, namely with respect to the occurrence of additional important signal transduction systems that are disrupted by sub-MIC OAK. The findings argue for the ability of sub-MIC OAK to inhibit quorum sensing (QS)-mediated lipolytic activity and activities of various virulence factors such as α-hemolysin and phenol-soluble modulins (PSM)-mediated cytotoxicity to erythrocytes and neutrophils (unpublished data). Similar effects were observed for the cyclodepsipeptide solonamide B, that was reported to reduce expression of RNAIII, the effector molecule of the *agr* quorum sensing system (Nielsen et al. 2014). Solonamide B too did not exhibit antimicrobial activity but displayed specific QS inhibitory traits that reduced the *S. aureus* cytotoxicity toward human neutrophils and rabbit erythrocytes in a dose-dependent manner. The authors have concluded that solonamide B interferes with *agr* activation by binding to the transmembrane (AgrC) sensor histidine kinase and thereby preventing interactions between AgrC and the auto-inducing peptides. The similarities observed between $C_{12(\omega 7)}K\text{-}\beta_{12}$ and solonamide B suggest a similar mode of action whereby $C_{12(\omega 7)}K\text{-}\beta_{12}$ like solonamide B may inhibit AgrC function through direct binding, or that both compounds indirectly interfere with the signal transduction as MACs do. Future investigation might resolve this issue.

These findings also prompted the undertaking of a structure–activity relationships (SAR) study focusing on the sequence $C_{12(\omega 7)}K\text{-}\beta_{12}$, aiming to assess the ability of OAKs to generate MACs that are devoid of antibiotic activity per se, but whose membrane perturbing properties might enhance the potency of some other antibacterial entity. Such a compound could be exploited for widening the sensitivity spectrum of GNB to include excellent antibiotics that are excluded by the outer membrane, namely due to their hydrophobicity. Also, the established inactivity of such a compound would have a mechanistic advantage in clarifying the issue of "who is doing what" during combination studies. This study revealed an analog ($C_{10}K\text{-}\beta_{12}$) that was a very good substrate of the RND family of efflux pumps and therefore inefficient on its own, in affecting the growth of Gram-negative bacteria (actually, even less efficient than $C_{12(\omega 7)}K\text{-}\beta_{12}$, being less hydrophobic). Yet, these analogs have also exhibited similar MAC properties, inducing membrane damages at sub-MIC (e.g., at 1–2 micromolar), including permeabilization of the outer membrane and depolarization of the cytoplasmic membrane (Jammal et al. 2015). In fact, $C_{10}K\text{-}\beta_{12}$ has enabled erythromycin and rifampicin to, respectively, exert their mode of action (i.e., bacteriostatic and bactericidal, respectively), likely by permeabilizing the outer membrane to rifampin and the cytoplasmic membrane to erythromycin. This study, therefore, provided strong arguments for the capacity of an OAK that is devoid of antibiotic activity to

sensitize GNB to rifampicin, reducing its MIC by up to four orders of magnitude, which was significantly higher than for the gold standard polymixin B. Possibly, this is due to the multiplicity of the types of simultaneous damages inflicted by such OAKs, including their ability to avoid interactions with cell wall components such as LPS, as well as the reciprocal drug's ability (OAK and antibiotic) to potentiate each other. Intriguingly, in the absence of exogenous antibiotics, $C_{10}K\text{-}\beta_{12}$ exhibited an improved capacity to control infection in vivo, as discussed below.

8.3 OAKs In vivo Properties

Several OAKs have shown efficacy in various mice models of infection. The sequence $C_{12}K\text{-}7\alpha_8$ was one of the first investigated for its in vivo properties using the peritonitic sepsis model (intraperitoneal (ip) treatment an hour after ip infection of neutropenic mice and the effect evaluated by monitoring survival for 6 days). In this model, the infecting bacterial inoculum ($4 \pm 1 \times 10^6$ CFU of extended spectrum beta-lactamase producing *E. coli*) corresponded to 2–3 times the LD_{50} (lethal dose at which half the animals are killed) and survival was as low as 0 % in the vehicle-treated groups. Under these conditions $C_{12}K\text{-}7\alpha_8$ prevented mortality to a similar extent as ciprofloxacin which increased the survival rates by up to 100 % after either single or multiple doses (1 or 4 mg/kg). As the lowest therapeutic dose was 2 mg/kg/day these results predict a therapeutic index (ratio of toxic to therapeutic dose >10) (Rotem et al. 2008a; Radzishevsky et al. 2007). Analysis of mice blood after single-dose ip administration revealed that $C_{12}K\text{-}7\alpha_8$ was present in the bloodstream within minutes but did not exceed the low micromolar level. *A propos*, noteworthy is the fact that a short version of this OAK (i.e., $C_{12}K\text{-}2\alpha_8$) demonstrated a maximal level of about 5 μM in mice blood upon ip administration of 5 mg/kg of body weight, whereas sustainable significantly higher concentrations in blood were also achievable (e.g., nearly 0.1 mM at 25 mg/kg). While $C_{12}K\text{-}2\alpha_8$ was devoid of antibacterial activity, it showed efficacy in experimental malaria where the blood stage of the disease revealed to be quite sensitive. Thus, in *Plasmodium vinckei*-infected mice, $C_{12}K\text{-}2\alpha_8$ presented an ED_{50} (50 % effective dose) of 22 mg/kg while toxicity emerged at the dose 4 × 50 mg/kg/day (Zaknoon et al. 2011). Interestingly, $C_{12}K\text{-}2\alpha_8$ inhibited in vitro parasite growth at submicromolar concentrations IC_{50} (50 % inhibitory concentration) was 0.3 ± 0.1 μM, but was devoid of hemolytic activity (i.e., displaying <1 % hemolysis at a concentration 1000-fold higher than IC_{50}). The fact that the early (ring) stage of the parasite developmental cycle was more sensitive (by 4- to 5-fold) than the intracellular feeding stage (trophozoite), further supports the view that the antiplasmodial mechanism was non-membranolytic to the host red blood cells.

Another antibacterial study of $C_{12}K\text{-}7\alpha_8$ used a pneumonia infection model, where mice were infected with *Pseudomonas aeruginosa* and treated by inhalation (25 μg per mouse). The OAK was similarly efficient as the antipseudomonal antibiotic tobramycin, reducing the lung bacterial population by up to 2 log units as

compared to inoculated vehicle controls (unpublished data). Collectively, these studies seem to indicate that $C_{12}K\text{-}7\alpha_8$ might be useful in the treatments of severe infections caused by Gram-negative pathogens. However, when tested against the thigh infection model (e.g., ip treatment an hour after intramuscular infection of neutropenic mice and the effect evaluated by monitoring viable bacteria extracted from the thigh 24 h post treatment), $C_{12}K\text{-}7\alpha_8$ failed to reduce bacterial load significantly, suggesting a rather poor potential for systemic efficacy probably due to poor tissue penetration.

Given its in vitro synergistic action with conventional antibiotics (Livne et al. 2010), a potential role of $C_{12}K\text{-}7\alpha_8$ in systemic therapy was investigated against Gram-negative bacteria (Epand et al. 2011). As mentioned above (Sect. 8.2) co-encapsulation of synergistic drugs in OAK-based cochleates may offer advantages toward overcoming potential problems arising during a systemic combination therapy, such as attenuating toxicity, shielding from undesired interactions and/or rectifying differential pharmacokinetic traits of the synergistic drugs. The maximal tolerated dose (MTD) of free $C_{12}K\text{-}7\alpha_8$ was compared by single IV administration to ICR mice of free or cochleated $C_{12}K\text{-}7\alpha_8$. While the MTD of free OAK was estimated at 5 mg OAK/kg of mouse weight, the MTD observed for the cochleated version was estimated at least 5-fold higher, as no detectable signs of toxicity were apparent at the highest tested dose (i.e., 20 mg/kg), indicating that encapsulation of $C_{12}K\text{-}7\alpha_8$ has significantly reduced its systemic toxicity. As mention above, previous attempts aiming to assess the therapeutic potential of $C_{12}K\text{-}7\alpha_8$ have shown encouraging outcome in topical but not in genuine systemic treatment models. While not necessarily promising an improved outcome, these acute toxicity results open the possibility for increasing the administrated doses beyond the free MTD, which in turn might achieve an improved therapeutic outcome. Regardless of this issue, the cochleates approach demonstrated moreover, that systemic treatments using single-dose administrations of co-encapsulated $C_{12}K\text{-}7\alpha_8$ and erythromycin, have significantly increased the therapeutic efficacy and protected mice from lethal bacterial infections in a dose-dependent manner (Livne et al. 2010; Sarig et al. 2011).

The use of lysyl-acyl-lysyl (β) building blocks in OAKs design also appears beneficial. We already mentioned that it enabled to broaden the spectrum of activity, namely to include a wide range of Gram-positive and Gram-negative bacteria. As it turned out, this structural motif seems also to improve the OAK's bioavailability since at least from preliminary efficacy studies using the thigh infection model, various β-OAKs demonstrated the ability to significantly affect the colony forming units (CFU) upon systemic administration. Thus, unlike α-OAKs (e.g., $C_{12}K\text{-}7\alpha_8$ and $C_{12}K\text{-}5\alpha_8$) that exhibited in vivo antibacterial efficacy only upon using topical (or semi-topical such as ip-ip) applications, $C_{12}K\text{-}3\beta_{10}$ and $C_{12(\omega7)}K\text{-}\beta_{12}$ were efficient at 2 mg/kg in reducing the viability of *Staphylococcus aureus*, an important human and animal pathogen (Zetola et al., 2005), albeit, they were assessed under somewhat different conditions (i.e., using normal ICR mice infected with *S. aureus* ATCC 29213). However, assessment of the shorter β-OAK version ($C_{12(\omega7)}K\text{-}\beta_{12}$) in the thigh infection model indicated quite comparable

efficacies on using different systemic routes for administrating the OAK (including ip, subcutanous (sc) and intreavenous (iv)), where the OAK has reduced bacterial load similarly to vancomycin. The MTD for iv and ip routes were 5 and 10 mg/kg, respectively, whereas the sc route was well tolerated at least up to 20 mg/kg. It is estimated that $C_{12(\omega7)}K\text{-}\beta_{12}$ rapidly enters circulation and remains stable for several hours.

Another antibacterial β-OAK worth mentioning is the sequence $C_{12}K\text{-}2\beta_{12}$ that demonstrated in vitro and in vivo efficacies against *Helicobacter pylori*, namely when using an experimental infection of Mongolian gerbils treated orogastrically (Makobongo et al. 2012), suggesting that the OAK concept may be a valuable resource for therapeutic treatment of *H. pylori* infection, as well. Together, these studies suggest that the potential of β-OAKs for antibacterial therapeutic development includes systemic monotherapy.

$C_{12(\omega7)}K\text{-}\beta_{12}$ was also investigated for its potential in combination therapy. As the OAK was able to overcome resistance of *S. aureus* clinical isolates to β-lactam antibiotics (e.g., oxacillin, piperacillin, and penicillin G) under in vitro conditions, it was verified whether this resensitization effect could prevail under in vivo conditions as well. Using the ip-ip version of the mouse peritonitis-sepsis model, various single doses of oxacillin and OAK combinations were able to prevent death induced by a lethal infection, in a synergistic dose-dependent manner (Kaneti et al. 2013).

Another study targeting GNB by combining $C_{12(\omega7)}K\text{-}\beta_{12}$ and erythromycin, tested their ability to affect disease course systemically, using the mouse thigh infection model in neutropenic mice that were inoculated intramuscularly with a clinically isolated MDR strain of *E. coli* and treated subcutaneously. Unlike individual treatments with OAK or erythromycin, treatments with the combined drugs have significantly enhanced growth inhibition of *E. coli* in most mice (Goldberg et al. 2013). Collectively, these findings suggest a potentially useful approach for expanding the antibiotics sensitivity spectrum of MDR Gram-negative bacteria to include efflux substrates. Another important outcome of this study is the realization that in vivo antibiotic sensitization of bacteria can prevail without the requirement for encapsulation and delivery of the synergistic drugs.

Possibly more interesting is the combination of two virtually inactive drugs on GNB such as rifampin and $C_{10}K\text{-}\beta_{12}$, whose systemic efficacy was further challenged by distinct modes of administration (oral and subcutaneous, respectively) without encapsulation (Jammal et al. 2015). Vehicle treatment of neutropenic mice inoculated with *K. pneumoniae* resulted in rapid death of most mice (20 and 10 % survival) within 1–2 days. Under these conditions, single dose treatments with rifampin, $C_{12(\omega7)}K\text{-}\beta_{12}$ or $C_{10}K\text{-}\beta_{12}$, were unable to significantly improve the survival rates, as they yielded 10, 20 and 25 % survivors at day 7, respectively. In contrast, administration of rifampin combined with $C_{12(\omega7)}K\text{-}\beta_{12}$ increased mice survival from 20 to 40 %, while the combination including $C_{10}K\text{-}\beta_{12}$ has further increased the survival rate to 60 %. The improved in vivo performance of $C_{10}K\text{-}\beta_{12}$ compared with $C_{12(\omega7)}K\text{-}\beta_{12}$ were attributed to two factors: a better bioavailability and a higher capacity to permeabilize the outer membrane of GNB.

Noteworthy is the most recent SAR study that revealed a closely related analog of $C_{10}K\text{-}\beta_{12}$ displaying quite intriguing in vivo properties (unpublished data). Namely, it is the first OAK to exhibit potent systemic efficacy against GNB using single dose monotherapy. It is intriguing not only because the systemic efficacy was the highest achieved in the OACs history thus far, but also because this outcome was achieved despite lack of antibiotic activity in vitro. Thus, using neutropenic mice infected ip with *E. coli* or *K. pneumonia*, the new analog increased the number of surviving mice in the sepsis-peritonitis model (by up to 90 %) and fully inhibited the number of viable bacteria in the thigh infection model after subcutaneous OAC administrations. The still ongoing mechanistic studies suggest that the new analog is endowed with improved bioavailability, as its free concentration in mice blood was higher than that of $C_{10}K\text{-}\beta_{12}$ (for instance, achieving 12 versus 5 micromolar, 60 min after administration of 12 mg/kg). However, while this quantitative information might justify the higher potency, the cause for the antibiotic effect remains to be determined since the MIC is consistently >50 micromolar (in culture medium, this concentration inhibited growth of some strains by about 10 %, at most). One direction taken is to verify the possibility of an OAK-mediated recruitment of the immune system.

8.4 Resin-Linked OAKs

Besides investigating OAKs potential in controlling bacterial infections, their ability to capture bacteria in the resin-linked (ROAK) form, was also investigated (Rotem et al. 2010; Marjieh et al. 2015). The first idea examined was whether OAK's binding affinity to bacteria might be exploited toward bacterial filtration from liquid media and/or eventual additional downstream applications. Having established their capacity to capture bacteria under different environmental conditions, it was next attempted to improve that capacity by investigating the SAR involved. Subsequently, the ROAKs aptitude to release the captured bacteria was examined and finally, the potential use of ROAKs in downstream applications was assessed by exploiting the capture/release capacities.

Following a preliminary SAR study, the sequence $K\text{-}7\alpha_{12}$ was initially selected for this investigation as its charge and hydrophobic characteristics were clearly implicated in bacterial capture by ROAK beads (Rotem et al. 2010). Using confocal microscopy for visualization of ROAK-bound bacteria (Fig. 8.3), and SPR technology for measuring bacterial binding to an OAK-linked chip, it was concluded that ROAKs are highly apt for rapid capture of various pathogens in different media, under incubation or continuous flow conditions. A single ROAK bead (average diameter of 50–100 μm) is estimated to capture >1,000 bacteria in contaminated culture medium, saline, or tap water. Moreover, after a brief ethanol treatment/elution, the ROAK-bound bacteria were readily identifiable by real-time PCR.

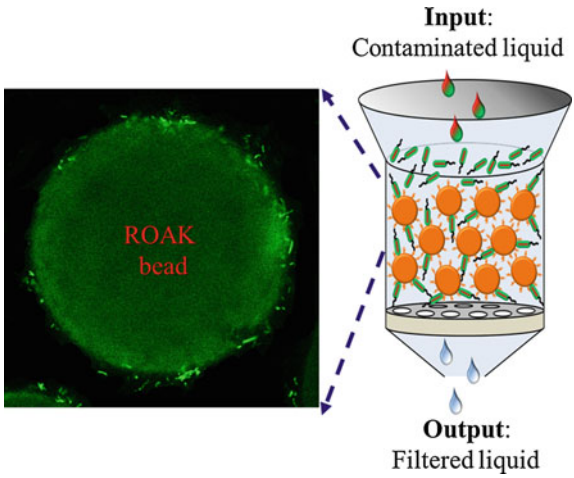


Fig. 8.3 Bacterial filtration using a ROAK column. The right panel is cartoon illustrating the principle of a ROAK-packed column used for continuous flow analysis of bacterial contaminations. Typically, 10 mg ROAK beads are packed in a glass pipette (restrained by glass fibers) and preconditioned in saline. Contaminated liquid media (e.g., tap water, saline, buffered solutions, or biological fluids) are passed through the column at a flow rate of 2.5 ml/min using a peristaltic pump. The captured bacteria can be released using a minimal elution solution (typically 2 ml of 70 % ethanol or 0.5 M $CaCl_2$ in water, to obtain dead or live free bacteria, respectively) for downstream analysis such as bacterial quantification by qPCR or determination of the numbers of colony forming units (Marjieh et al. 2015). The left panel is a "zoom" image showing GFP-expressing bacteria associated with a ROAK bead as analyzed by confocal fluorescence microscopy (Rotem et al. 2010)

Further characterization of bacterial capture by ROAKs in a recent follow-up study is summarized as follows:

(1) ROAKs maintained high-capture efficacy (80–100 %) for various representative species including medically relevant bacteria, while using inoculums differing by several orders of magnitude, starting from 1×10^4 CFU per milliliter medium;
(2) Bacterial capture in water and in the presence of salts at concentrations at least up to 100 mM was essentially similar, whereas only molar concentrations achieved significant levels of inhibition, bivalent salts being more potent inhibitors;
(3) No significant interference was detected at pH range 3–9, reflecting the hydrophobic forces at play;
(4) Partial bacterial capture (up to 23 %) occurred in contaminated whole blood, whereas 10-fold blood dilution enabled to increase the captured fraction to 50 %. These findings stand in line with previous data demonstrating efficient bacterial capture in wastewaters (Rotem et al. 2010), thereby consolidating the view of a high-affinity interaction between bacteria and ROAK beads;

(5) Free bacteria can be recovered nonetheless, as demonstrated after washing ROAK-bound *E. coli* with an eluting agent (for instance, ethanol, NaCl and $CaCl_2$, respectively yielding <1, 5 and 17 %, recovery).

Attempts to establish the minimal requirements for effective bacterial capture, point to an N-terminal lysyl residue being critical for maintaining significant capture activity, whereas OAK sequences composed of 3–4 acyl-lysyl subunits are sufficient for efficient capture. However, the data also suggest that such optimum maybe species- and/or strain dependent. For instance, K-4α_{12} was the shortest sequence to maintain similar capture of *Pseudomonas aeruginosa* as its parent sequence K-7α_{12}, whereas for *Escherichia coli* or *Klebsiella pneumonia*, it was K-3α_{12}. Thus, further studies are needed to validate this notion.

Based on these data, the capture and release capabilities were exploited for active filtration of bacteria-contaminated liquids in column chromatography. For this purpose, a glass pipette loaded with ROAK beads was utilized (as illustrated in Fig. 8.3) to filter saline inoculums spiked with a constant number of *E. coli* bacteria (6.0 ± 0.5 log CFU). Both the K-7α_{12} and K-3α_{12} ROAKs maintained a high capacity for bacterial capture under these continuous flow conditions, however, the elution yield from the K-3α_{12} column was substantially higher. The data therefore argue that the K-3α_{12} ROAK column assay represents a rapid bacterial enrichment procedure since bacterial counts were increased by a factor of about 7. Moreover, by applying a higher inoculum volume (100 ml, as often required in standard tests (Guidelines for drinking water quality. WHO 2008; (Rompre et al. 2002)), the concentration factor was increased to about 20-fold, a number that, at least theoretically, should further increase with increasing sample volumes. Collectively, these data provide evidence for the ability of ROAKs to deplete a sample of bacteria using extremely high-affinity sequences (e.g., K-7α_{12}) or, the ability to improve the sensitivity of qPCR-based bacterial detection by using moderate affinity OAKs (e.g., K-3α_{12}). Thus, in addition to its compositional simplicity and robustness, the new attributes highlight potential advantages of the OAK approach over approaches that use antibodies (Iqbal et al. 2000) or AMPs (Mannoor et al. 2010), including in terms of how environmental conditions (pH, ionic strength, and complexity) might affect their performances.

8.5 Concluding Remarks

Various studies sustain the notion that combination therapies targeting the membrane potential may represent an advantageous approach for controlling bacterial infections by disabling the devastating effects related to both antibiotic resistance and virulence factors. This notion was illustrated herein, through MAC investigations using the OAC platform. Together, the data show promise as to the concept's capacity to generate small molecules that simultaneously affect multiple membrane functions, control systemic bacterial infections in single and combination therapy

and overcome (and/or delay) innate and acquired resistance to antibiotics. In the future, therefore, side-by-side with conventional antibiotics development programs, we anticipate to witness an increased interest for exploring MACs that target signal transduction. In particular, owing to their simplicity and robustness, new OAC generations maybe also useful in elucidating the mechanisms involved in the alleged inhibition of quorum sensing and possibly for immune modulation.

Similarly, ROAKs were able to deplete liquid samples of bacterial content after incubation and during flow settings, illustrating the efficient capture of different bacterial species under a wide range of ionic strength and pH conditions. The studies also showed circumstances for the significant release of captured bacteria, live or dead, toward further analysis. The data therefore support the potential usefulness of this simple, robust, and efficient approach for rapid capture/analysis of bacteria from tap water and, possibly, from more complex media. As an effective tool for dissecting the relative roles of parameters considered most crucial for antimicrobial activities (i.e., charge and hydrophobicity), new OAC-linked surfaces might also pave the way to new potential applications (e.g., as biosensors, magnetic beads, etc.), so as to allow sensitive detection of bacteria in water and foods and/or their filtration from biological fluids such as blood.

Acknowledgments This work was supported by the Israel Science Foundation (grant 909/12).

References

Allen RC, Popat R, Diggle SP, Brown SP (2014) Targeting virulence: can we make evolution-proof drugs? Nat Rev Microbiol 12:300–308

Andra J, Goldmann T, Ernst CM, Peschel A, Gutsmann T (2011) Multiple peptide resistance factor (MprF)-mediated resistance of Staphylococcus aureus against antimicrobial peptides coincides with a modulated peptide interaction with artificial membranes comprising lysyl-phosphatidylglycerol. J Biol Chem 286:18692–18700

Blair JM, Webber MA, Baylay AJ, Ogbolu DO, Piddock LJ (2015) Molecular mechanisms of antibiotic resistance. Nat Rev Microbiol 13:42–51

Choi S, Isaacs A, Clements D, Liu D, Kim H, Scott RW, Winkler JD, DeGrado WF (2009) De novo design and in vivo activity of conformationally restrained antimicrobial arylamide foldamers. Proc Natl Acad Sci USA 106:6968–6973

Daly SM, Elmore BO, Kavanaugh JS, Triplett KD, Figueroa M, Raja HA, El-Elimat T, Crosby HA, Femling JK, Cech NB, Horswill AR, Oberlies NH, Hall PR (2015) ω-Hydroxyemodin limits staphylococcus aureus quorum sensing-mediated pathogenesis and inflammation. Antimicrob Agents Chemother 59:2223–2235

Dhand A, Bayer AS, Pogliano J, Yang SJ, Bolaris M, Nizet V, Wang G, Sakoulas G (2011) Use of antistaphylococcal beta-lactams to increase daptomycin activity in eradicating persistent bacteremia due to methicillin-resistant Staphylococcus aureus: role of enhanced daptomycin binding. Clin Infect Dis 53:158–163

Eckert R (2011) Road to clinical efficacy: challenges and novel strategies for antimicrobial peptide development. Future Microbiol 6:635–651

Epand RM, Epand RF (2009) Lipid domains in bacterial membranes and the action of antimicrobial agents. Biochim Biophys Acta 1788:289–294

Epand RM, Vogel HJ (1999) Diversity of antimicrobial peptides and their mechanisms of action. Biochim Biophys Acta 1462:11–28

Epand RM, Rotem S, Mor A, Berno B, Epand RF (2008a) Bacterial membranes as predictors of antimicrobial potency. J Am Chem Soc 130:14346–14352

Epand R, Rotem S, Mor A, Berno B, Epand R (2008b) Bacterial membranes as predictors of antimicrobial potency. J Am Chem Soc 130:14346–14352

Epand RF, Maloy WL, Ramamoorthy A, Epand RM (2010) Probing the "charge cluster mechanism" in amphipathic helical cationic antimicrobial peptides. Biochemistry 49:4076–4084

Epand RF, Sarig H, Ohana D, Papahadjopoulos-Sternberg B, Mor A, Epand RM (2011) Physical properties affecting cochleate formation and morphology using antimicrobial oligo-acyl-lysyl peptide mimetics and mixtures mimicking the composition of bacterial membranes in the absence of divalent cations. J Phys Chem B 115:2287–2293

Fedtke I, Gotz F, Peschel A (2004) Bacterial evasion of innate host defenses-the Staphylococcus aureus lesson. Int J Med Microbiol 294:189–194

Fernandez L, Gooderham WJ, Bains M, McPhee JB, Wiegand I, Hancock RE (2010) Adaptive resistance to the "last hope" antibiotics polymyxin B and colistin in Pseudomonas aeruginosa is mediated by the novel two-component regulatory system ParR-ParS. Antimicrob Agents Chemother 54:3372–3382

Fischbach MA, Walsh CT (2009) Antibiotics for emerging pathogens. Science 325:1089–1093 (New York, N.Y.)

Gaidukov L, Fish A, Mor A (2003) Analysis of membrane-binding properties of dermaseptin analogues: relationships between binding and cytotoxicity. Biochemistry 42:12866–12874

Goldberg K, Sarig H, Zaknoon F, Epand RF, Epand RM, Mor A (2013) Sensitization of gram-negative bacteria by targeting the membrane potential. FASEB J 27:3818–3826

Gooderham WJ, Hancock RE (2009) Regulation of virulence and antibiotic resistance by two-component regulatory systems in Pseudomonas aeruginosa. FEMS Microbiol Rev 33:279–294

Hancock RE (2005) Mechanisms of action of newer antibiotics for Gram-positive pathogens. Lancet Infect Dis 5:209–218

Hancock RE, Chapple DS (1999) Peptide antibiotics. Antimicrob Agents Chemother 43:1317–1323

Haney EF, Hancock RE (2013) Peptide design for antimicrobial and immunomodulatory applications. Biopolymers 100:572–583

Held-Kuznetsov V, Rotem S, Assaraf YG, Mor A (2009) Host-defense peptide mimicry for novel antitumor agents. FASEB J 23:4299–4307

Hicks DB, Cohen DM, Krulwich TA (1994) Reconstitution of energy-linked activities of the solubilized F1F0 ATP synthase from Bacillus subtilis. J Bacteriol

Hurdle JG, O'Neill AJ, Chopra I, Lee RE (2011) Targeting bacterial membrane function: an underexploited mechanism for treating persistent infections. Nat Rev Microbiol 9:62–75

Iqbal SS, Mayo MW, Bruno JG, Bronk BV, Batt CA, Chambers JP (2000) A review of molecular recognition technologies for detection of biological threat agents. Biosens Bioelectron 15:549–578

Jahnsen RD, Haney EF, Franzyk H, Hancock RE (2013) Characterization of a proteolytically stable multifunctional host defense peptidomimetic. Chem Biol 20:1286–1295

Jammal J, Zaknoon F, Kaneti G, Goldberg G, Mor A (2015) Sensitization of Gram-negative bacteria to rifampin and OAK combinations. Scientific Reports 5

Jean-Francois F, Castano S, Desbat B, Odaert B, Roux M, Metz-Boutigue MH, Dufourc EJ (2008) Aggregation of cateslytin beta-sheets on negatively charged lipids promotes rigid membrane domains. A new mode of action for antimicrobial peptides? Biochemistry 47:6394–6402

Kaneti G, Sarig H, Marjieh I, Zaknoon F, Mor A (2013) Simultaneous breakdown of multiple antibiotic resistance mechanisms in S. aureus. FASEB J

Kinch MS, Haynesworth A, Kinch SL, Hoyer D (2014) An overview of FDA-approved new molecular entities: 1827–2013. Drug Discov Today 19:1033–1039

Klitgaard JK, Skov MN, Kallipolitis BH, Kolmos HJ (2008) Reversal of methicillin resistance in Staphylococcus aureus by thioridazine. J Antimicrob Chemother 62:1215–1221
Koprivnjak T, Peschel A (2011) Bacterial resistance mechanisms against host defense peptides. Cell Mol Life Sci 68:2243–2254
Li SA, Liu J, Xiang Y, Wang YJ, Lee WH, Zhang Y (2014) Therapeutic potential of the antimicrobial peptide OH-CATH30 for antibiotic-resistant Pseudomonas aeruginosa keratitis. Antimicrob Agents Chemother 58:3144–3150
Liu D, Choi S, Chen B, Doerksen RJ, Clements DJ, Winkler JD, Klein ML, DeGrado WF (2004) Nontoxic membrane-active antimicrobial arylamide oligomers. Angew Chem Int Ed Engl 43:1158–1162
Livne L, Kovachi T, Sarig H, Epand RF, Zaknoon F, Epand RM, Mor A (2009) Design and characterization of a broad-spectrum bactericidal acyl-lysyl oligomer. Chem Biol 16:1250–1258
Livne L, Epand RF, Papahadjopoulos-Sternberg B, Epand RM, Mor A (2010) OAK-based cochleates as a novel approach to overcome multidrug resistance in bacteria. FASEB J 24:5092–5101
Makobongo MO, Gancz H, Carpenter BM, McDaniel DP, Merrell DS (2012) The oligo-acyl lysyl antimicrobial peptide C(1)(2)K-2beta(1)(2) exhibits a dual mechanism of action and demonstrates strong in vivo efficacy against Helicobacter pylori. Antimicrob Agents Chemother 56:378–390
Mannoor MS, Zhang S, Link AJ, McAlpine MC (2010) Electrical detection of pathogenic bacteria via immobilized antimicrobial peptides. Proc Natl Acad Sci USA 107:19207–19212
Marjieh I, Meir O, Zaknoon F, Mor A (2015) Improved bacterial detection using immobilized acyl-lysyl oligomers. Appl Environ Microbiol 81:74–80
Marr AK, Gooderham WJ, Hancock RE (2006) Antibacterial peptides for therapeutic use: obstacles and realistic outlook. Curr Opin Pharmacol 6:468–472
McCallum N, Berger-Bächi B, Senn MM (2010) Regulation of antibiotic resistance in Staphylococcus aureus. Int J Med Microbiol 300:118–129
Mensa B, Kim YH, Choi S, Scott R, Caputo GA, DeGrado WF (2011) Antibacterial mechanism of action of arylamide foldamers. Antimicrob Agents Chemother 55:5043–5053
Nielsen A, Mansson M, Bojer MS, Gram L, Larsen TO, Novick RP, Frees D, Frøkiær H, Ingmer H (2014) Solonamide B inhibits quorum sensing and reduces staphylococcus aureus mediated killing of human neutrophils. PLoS One 9
Padan E, Bibi E, Ito M, Krulwich TA (2005) Alkaline pH homeostasis in bacteria: new insights. Biochim Biophys Acta 1717:67–88
Paul M, Carmeli Y, Durante-Mangoni E, Mouton JW, Tacconelli E, Theuretzbacher U, Mussini C, Leibovici L (2014) Combination therapy for carbapenem-resistant Gram-negative bacteria. J Antimicrob Chemother 69:2305–2309
Paulsen IT, Brown MH, Skurray RA (1996) Proton-dependent multidrug efflux systems. Microbiol Rev 60:575–608
Poole K (2005) Efflux-mediated antimicrobial resistance. J Antimicrob Chemother 56:20–51
Poole K (2012) Bacterial stress responses as determinants of antimicrobial resistance. J Antimicrob Chemother 67:2069–2089
Radzishevsky IS, Rotem S, Bourdetsky D, Navon-Venezia S, Carmeli Y, Mor A (2007) Improved antimicrobial peptides based on acyl-lysine oligomers. Nat Biotechnol 25:657–659
Radzishevsky IS, Kovachi T, Porat Y, Ziserman L, Zaknoon F, Danino D, Mor A (2008) Structure-activity relationships of antibacterial acyl-lysine oligomers. Chem Biol 15:354–362
Ratledge C, Wilkinson SG(1988) Microbial lipids. Academic Press, London
Reisinger P, Seidel H, Tschesche H, Hammes WP (1980) The effect of nisin on murein synthesis. Arch Microbiol 127:187–193
Rompre A, Servais P, Baudart J, de-Roubin MR, Laurent P (2002) Detection and enumeration of coliforms in drinking water: current methods and emerging approaches. J Microbiol Methods 49:31–54

Rotem S, Mor A (2009) Antimicrobial peptide mimics for improved therapeutic properties. Biochim Biophys Acta 1788:1582–1592

Rotem S, Radzishevsky IS, Bourdetsky D, Navon-Venezia S, Carmeli Y, Mor A (2008a) Analogous oligo-acyl-lysines with distinct antibacterial mechanisms. FASEB J 22:2652–2661

Rotem S, Radzishevsky IS, Bourdetsky D, Navon-Venezia S, Carmeli Y, Mor A (2008b) Analogous oligo-acyl-lysines with distinct antibacterial mechanisms. FASEB J 22:2652–2661

Rotem S, Raz N, Kashi Y, Mor A (2010) Bacterial capture by peptide-mimetic oligoacyllysine surfaces. Appl Environ Microbiol 76:3301–3307

Ruhr E, Sahl HG (1985) Mode of action of the peptide antibiotic nisin and influence on the membrane potential of whole cells and on cytoplasmic and artificial membrane vesicles. Antimicrob Agents Chemother 27:841–845

Sakoulas G, Okumura CY, Thienphrapa W, Olson J, Nonejuie P, Dam Q, Dhand A, Pogliano J, Yeaman MR, Hensler ME, Bayer AS, Nizet V (2014) Nafcillin enhances innate immune-mediated killing of methicillin-resistant Staphylococcus aureus. J Mol Med 92:139–149 (Berlin, Germany)

Sarig H, Rotem S, Ziserman L, Danino D, Mor A (2008) Impact of self-assembly properties on antibacterial activity of short acyl-lysine oligomers. Antimicrob Agents Chemother 52:4308–4314

Sarig H, Livne L, Held-Kuznetsov V, Zaknoon F, Ivankin A, Gidalevitz D, Mor A (2010) A miniature mimic of host defense peptides with systemic antibacterial efficacy. FASEB J 24:1904–1913

Sarig H, Ohana D, Epand R, Mor A, Epand R (2011) Functional studies of cochleate assemblies of an oligo-acyl-lysyl with lipid mixtures for combating bacterial multidrug resistance. FASEB J 25:3336–3343

Sass V, Schneider T, Wilmes M, Korner C, Tossi A, Novikova N, Shamova O, Sahl HG (2010) Human beta-defensin 3 inhibits cell wall biosynthesis in Staphylococci. Infect Immun 78:2793–2800

Sawyer JG, Martin NL, Hancock RE (1988) Interaction of macrophage cationic proteins with the outer membrane of Pseudomonas aeruginosa. Infect Immun 56:693–698

Schaberle TF, Hack IM (2014) Overcoming the current deadlock in antibiotic research. Trends Microbiol 22:165–167

Schuerholz T, Brandenburg K, Marx G (2012) Antimicrobial peptides and their potential application in inflammation and sepsis. Crit Care 16:207

Shprung T, Peleg A, Rosenfeld Y, Trieu-Cuot P, Shai Y (2012) Effect of PhoP-PhoQ activation by broad repertoire of antimicrobial peptides on bacterial resistance. J Biol Chem 287:4544–4551

Silver LL (2011) Challenges of antibacterial discovery. Clin Microbiol Rev 24:71–109

Silverman JA, Perlmutter NG, Shapiro HM (2003) Correlation of daptomycin bactericidal activity and membrane depolarization in Staphylococcus aureus. Antimicrob Agents Chemother 47:2538–2544

Strahl H, Hamoen W (2010) Membrane potential is important for bacterial cell division. Proc Natl Acad Sci USA 107:12281–12286

Sully EK, Malachowa N, Elmore BO, Alexander SM, Femling JK, Gray BM, DeLeo FR, Otto M, Cheung AL, Edwards BS, Sklar LA, Horswill AR, Hall PR, Gresham HD (2014) Selective chemical inhibition of agr quorum sensing in staphylococcus aureus promotes host defense with minimal impact on resistance, PLoS Pathogens 10

Vaara M, Fox J, Loidl G, Siikanen O, Apajalahti J, Hansen F, Frimodt-Møller N, Nagai J, Takano M, Vaara T (2008) Novel polymyxin derivatives carrying only three positive charges are effective antibacterial agents. Antimicrob Agents Chemother 52:3229–3236

Westerhoff HV, Juretic D, Hendler RW, Zasloff M (1989) Magainins and the disruption of membrane-linked free-energy transduction. Proc Natl Acad Sci USA 86:6597–6601

Wilke MS, Hills TL, Zhang HZ, Chambers HF, Strynadka NC (2004) Crystal structures of the Apo and penicillin-acylated forms of the BlaR1 beta-lactam sensor of Staphylococcus aureus. J Biol Chem 279:47278–47287

Yang SJ, Bayer AS, Mishra NN, Meehl M, Ledala N, Yeaman MR, Xiong YQ, Cheung AL (2012) The Staphylococcus aureus two-component regulatory system, GraRS, senses and confers resistance to selected cationic antimicrobial peptides. Infect Immun 80:74–81

Yeaman M, Yount N (2003) Mechanisms of antimicrobial peptide action and resistance. Pharmacol Rev 55:27–55

Zaknoon F, Wein S, Krugliak M, Meir O, Rotem S, Ginsburg H, Vial H, Mor A (2011) Antiplasmodial properties of acyl-lysyl oligomers in culture and animal models of malaria. Antimicrob Agents Chemother 55:3803–3811

Zasloff M (2002) Antimicrobial peptides of multicellular organisms. Nature 415:389–395

Zhang L, Dhillon P, Yan H, Farmer S, Hancock RE (2000) Interactions of bacterial cationic peptide antibiotics with outer and cytoplasmic membranes of Pseudomonas aeruginosa. Antimicrob Agents Chemother 44:3317–3321