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Review Article

Defensin–lipid interactions in membrane targeting: mechanisms of action and opportunities for the development of antimicrobial and anticancer therapeutics

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Defensins are a class of host defence peptides (HDPs) that often harbour antimicrobial and anticancer activities, making them attractive candidates as novel therapeutics. In comparison with current antimicrobial and cancer treatments, defensins uniquely target specific membrane lipids via mechanisms distinct from other HDPs. Therefore, defensins could be potentially developed as therapeutics with increased selectivity and reduced susceptibility to the resistance mechanisms of tumour cells and infectious pathogens. In this review, we highlight recent advances in defensin research with a particular focus on membrane lipid-targeting in cancer and infection settings. In doing so, we discuss strategies to harness lipid-binding defensins for anticancer and anti-infective therapies.

Introduction

Host defence peptides (HDPs; also referred to as cationic antimicrobial peptides) are key components of the innate immune system across all kingdoms of life [1,2]. Defensins, a prominent HDP class, are typically cationic, β-sheet and cysteine-rich and maintain conserved disulfide-stabilised structures [3,4]. The arrangement of two specific disulfide bonds in defensins define their classification into either the *cis*- or *trans*-defensin superfamilies, which are evolutionally convergent (see [4,5] for more details). For *cis*-defensins (dominated by plant defensins), the two disulfide bonds are parallel and tether the final β-strand to an α-helix. Conversely, in *trans*-defensins (including animal and human defensins), the two analogous disulfide bonds are orientated in opposite directions from the final β-strand to different secondary structure elements [4,5]. The disulfide bond framework and the functionally important β2–β3 loop between two antiparallel β-strands are highly conserved amongst defensin family members [4,6].

Like other HDPs, many defensins exhibit potent antimicrobial and anticancer activity, with additional roles including but not limited to ion channel blocking and immune modulation [7–9]. These antimicrobial and anticancer effects have largely been attributed to their membrane-permeabilising property (Figure 1A), for which three mechanistic models have been proposed: the barrel stave (Figure 1B), toroidal pore (Figure 1C) and carpet models (Figure 1D) [10–12]. The ornamental tobacco (*Nicotiana alata*) defensin NaD1 in complex with phosphatidic acid (PA) was the first direct structural evidence for the carpet model (Figure 1E) [13]. Intriguingly, emerging evidence suggests a novel membrane targeting and membrane disrupting mechanism, in which defensins including NaD1, human β-defensin 2 (HBD-2) and human β-defensin 3 (HBD-3) can preferentially bind specific phosphoinositides, ultimately leading to membrane permeabilisation in tumour, fungal and bacterial cells (Figure 1F) (elaborated below) [6,14–16].

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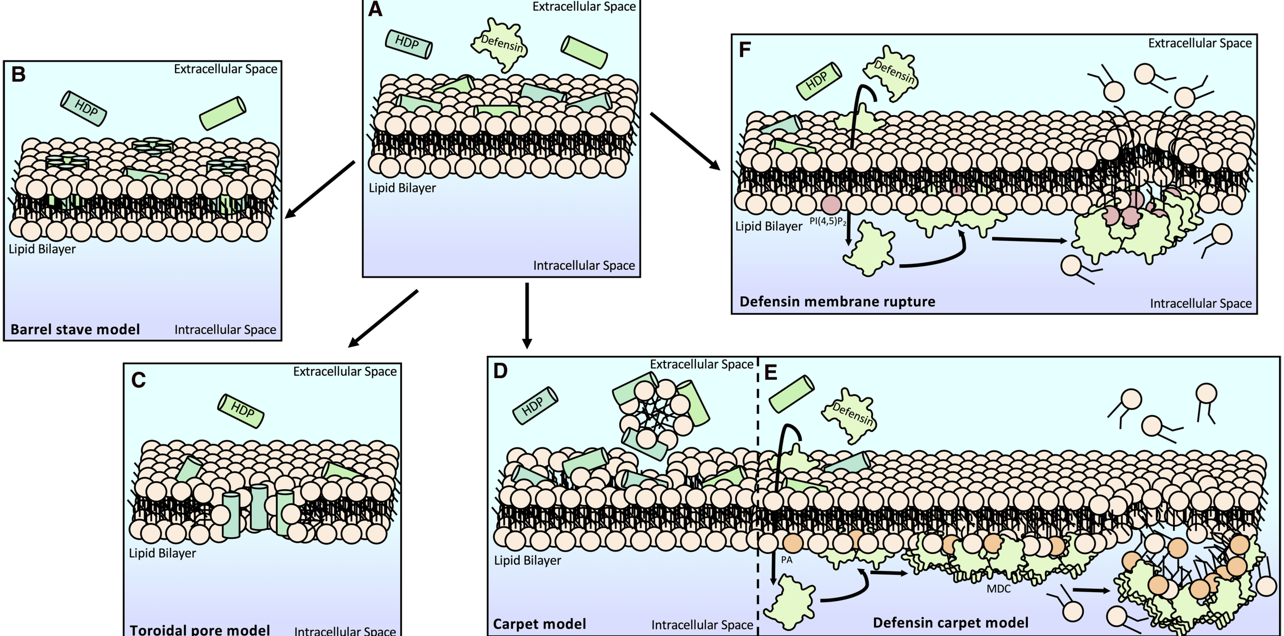

Figure 1. Models of membrane permeabilisation induced by HDPs and defensins.
(**A**) HDPs and defensins first associate with the cell membrane via electrostatic charge interactions. (**B**) In the barrel stave model, the HDPs embed themselves through the membrane, forming a pore completely lined with peptide. (**C**) In the toroidal pore model, the HDPs force the lipid membrane to curve and form a continuous lipid layer. The pore is lined with lipid head groups and peptide. (**D**) In the carpet model, the HDPs spread over the surface of the membrane like a surfactant until a critical concentration is reached at which point, micelles are formed that as the membrane breaks apart. (**E**) In the NaD1–PA carpet model, NaD1 monomers cross the membrane before engaging PA and forming dimers that assemble into the MDC. The MDC induces membrane curvature stress and subsequent membrane rupture. (**F**) In the defensin membrane disruption model (as exemplified by NaD1 and NsD7), defensin monomers cross the membrane before engaging PI(4,5)P_2 and forming dimers that oligomerise into arch-shaped assemblies to induce membrane rupture. The defensins may also act from the extracellular membrane surface in some cases as abnormal cells such as in cancer often demonstrate disrupted membrane asymmetry.

The potent antimicrobial and anticancer activities of defensins make them attractive candidates for development as novel therapeutics. Indeed, the specific lipid-targeting and membrane-permeabilising activities of defensins have the potential to address some of the key concerns for current antimicrobial and anticancer therapeutics, such as drug/antibiotic resistance and severe off-target effects [2]. In this review, we discuss the current understanding of the molecular interactions of defensins with cell membranes as well as highlight evidence supporting that this process differs from those previously proposed for other HDPs. We also outline the potential utility of these lipid-binding peptides as novel antimicrobial and anticancer agents.

Lipid binding-mediated membrane permeabilisation by defensins

Recent studies have shown that defensins interact with microbial pathogens and/or tumour cell membranes by binding specific phospholipids to cause membrane permeabilisation [6,15,17–21]. Biochemical, structural and functional evidence for various defensin–lipid interactions have been reported (Table 1), highlighting the conservation of key lipid binding regions in defensins and the overall mechanism that ultimately result in membrane permeabilisation. The first defensin–lipid interactions were demonstrated for bacterial membrane components such as 1-palmitoyl-2-oleoyl-sn-glycero-3-phosphatidylglycerol (POPG), date back to 1994 [14]. However, it was not until recently that the structure–function relationship and detailed mechanisms of these lipid interactions were reported for several plant and human defensins.

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Table 1. Examples of lipid-binding defensins

HDP	Organism	Lipid	References
Copsin	*Coprinopsis cinerea*	Lipid II	[33]
DmAMP1	*Dahlia merckii*	Sphingolipids	[34]
Eurocin	*Eurotium amstelodami*	Lipid II	[35]
Gallicin	*Mytilus galloprovincialai*	Lipid II	[36]
HBD-2	*Homo sapiens*	PS, PI(3)P; PI(4)P, PI(5)P, PI(3,4)P_2, PI(3,5)P_2, PI(4,5)P_2, PI(3,4,5)P_3, Cardiolipin, Sulfatide	[18]
HBD-3	*Homo sapiens*	PA, PS, PE, PI(3)P, PI(4)P, PI(5)P, PI(3,4)P_2, PI(3,5)P_2, PI(4,5)P_2, PI(3,4,5)P_3, Cardiolipin, Sulfatide	[27]
HNP-1	*Homo sapiens*	Palmitoyloleoylphosphatidylglycerol (POPG), Lipid II	[37]
HNP-2	*Homo sapiens*	POPG	[14,38]
HsAPF1	*Heuchera sanguinea*	PA, PI(3,4,5)P_3, PI(3,4)P_2	[39]
Lc-def	*Lens culinaris*	POPG	[40]
Lucifensin	*Lucilia sericata*	Lipid II	[36]
MsDef1	*Medcago sativa*	PA, PI(3)P, PI(4)P, PI(5)P, PI(3,4)P_2, PI(3,5)P_2, PI(4,5)P_2, PI(3,4,5)$P_{3,}$ Glucosylceramide	[16]
MtDef4	*Medicago truncatula*	PA	[16]
MtDef5	*Medicago truncatula*	PA; PS; PI(3)P; PI(4)P; PI(5)P;PI(3,5)P_2; PI(4,5)P_2	[21]
NaD1	*Nicotiana alata*	PA, PS; PI(3)P, PI(4)P, PI(5)P, PI(3,4)P_2, PI(3,5)P_2, PI(4,5)P_2, PI(3,4,5)P_3, Cardiolipin, Sulfatide	[15]
NoD173	*Nicotiana occidentalis*	PI(4,5)P_2	[20]
NsD7	*Nicotiana suaveolens*	PA	[19]
Oryzeacin	*Aspergillus oryzea*	Lipid II	[36]
OsAFP1	*Oryza sativa*	PI(3)P, PI(4)P, PI(5)P, PI(3,5)P_2, PI(4,5)P_2	[41]
Plectasin	*Pseudoplectania nigrella*	Lipid II	[36]
Psd1	*Pisum sativum*	Ergosterol, Glycosphingolipid	[42]
Psd2	*Pisum sativum*	Ergosterol, Glucosceramides, Phosphatidylcholine, PI(3)P, PI(5)P, PS	[43]
RsAFP2	*Raphanus sativus*	Glucosylceramides, Sphingolipids	[30]
Sd5	*Saccharum officinarum*	Glucosylceramides	[44]
TPP3	*Solanum lycopersicum*	PI(4,5)P_2	[17]

Medicago truncatula defensin 4 (MtDef4) has been shown to bind PA and interact with cells via its positively charged β2–β3 loop region. Notably, the substitution of cationic loop residues to alanine residues perturbed both the lipid binding and antifungal activities of MtDef4 [16]. NaD1 also binds to PA as a part of its fungal killing mechanism. X-ray crystallographic analysis showed that the β2–β3 loop was important for lipid binding, and mutagenesis studies confirmed this finding with mutants of proposed key lipid-binding residues showing reduced efficacy against fungal cells [13]. Lipid binding also plays a role in defensin oligomerisation and complex formation, which has been proposed as a key event in the membrane permeabilising activity of some defensins [14,16,20,23]. Intriguingly, the NaD1–PA crystal structure reveals an oligomeric structure, termed the membrane disruption complex (MDC). The MDC is formed via the assembly of groups of defensin dimers (containing either three or four dimer pairs; Figure 2A) with each dimer in a conserved cationic grip configuration engaged with the head group of a single PA molecule (Figure 2B) [13]. The formation of the MDC at the membrane is postulated to generate curvature stress on the membrane, which aids in membrane destabilisation and rupture [13]. Furthermore, this NaD1 MDC appears to engage PA from one side, resembling the carpet model of membrane binding and disruption that has been previously proposed for HDPs (Figure 1E) [13].

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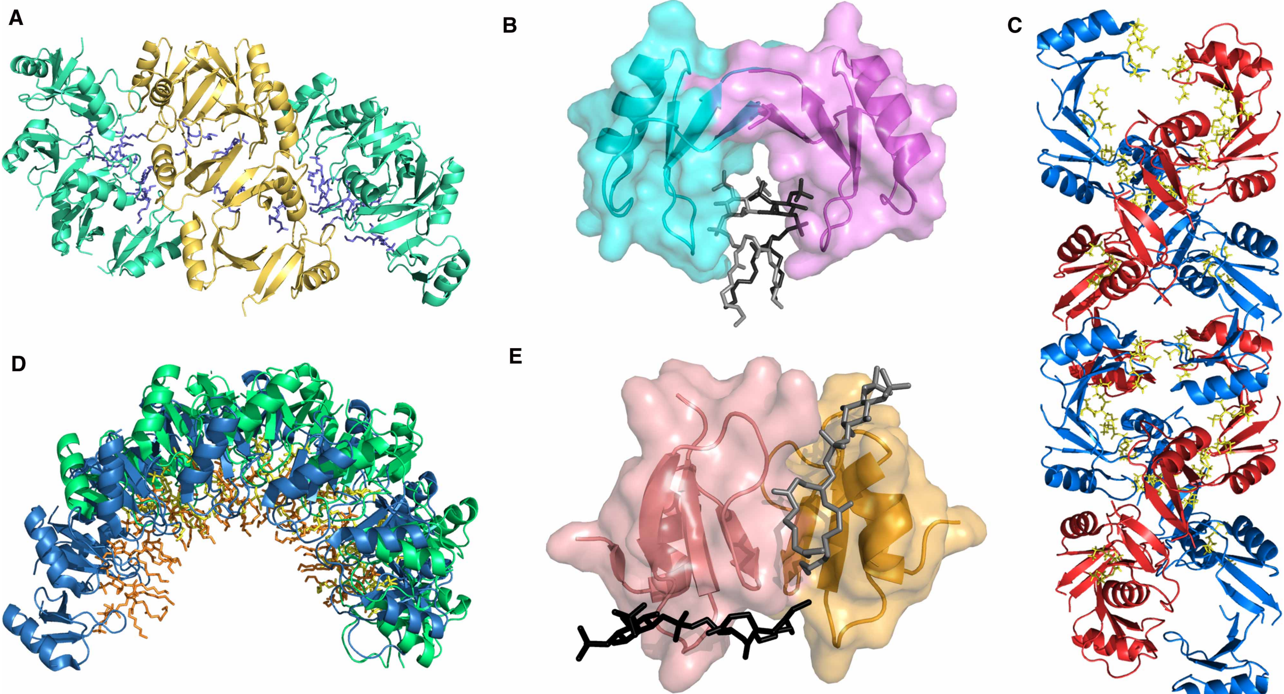

Figure 2. X-ray crystal structures of defensin–lipid complexes.
(**A**) The membrane disruption complex formed in a carpet-like model of membrane disruption by NaD1 (PDB: 6B55) engaging PA (three dimer pairs shown in aqua, four dimer pairs shown in gold). (**B**) The conserved cationic grip of an NaD1 dimer (PDB: 4CQK) (monomer 1 in cyan, monomer 2 in pink) binding two PI(4,5)P_2 molecules (grey and black). (**C**) The oligomer of NsD7–PA (PDB: 5KK4) showing the NsD7 double helix (helix 1 in blue, helix 2 in red). PA (yellow) is bound in between the dimers and in the cationic grip. (**D**) Comparison of NsD7 (PDB: 5VYP) (green) and NaD1 (PDB: 4CQK) (blue) engaging PI(4,5)P_2 (orange for NaD1, yellow for NsD7), both of which form multimeric arch-shaped oligomers that are proposed to exert torsional strain on the membrane. (**E**) The asymmetric dimer of HBD-2 (PDB: 6CS9) (monomer 1 in salmon, monomer 2 in orange) showing the two PI(4,5)P_2 binding sites (lipids shown in grey and black). Images generated using PyMOL.

Additionally, *Nicotiana suaveolens* defensin 7 (NsD7) adopts a double-helical oligomer upon interaction with PA that was demonstrated by X-ray crystallography (Figure 2C) [19]. Site-directed mutagenesis of key lipid-binding residues, which impaired NsD7 oligomerisation, had a subsequent effect on membrane permeabilisation. This underscores the importance of lipid binding for oligomerisation that is required for defensin membrane targeting [15,19,22].

In addition to PA, NaD1 binds phosphorylated phosphatidylinositols, particularly phosphatidylinositol 4,5 bisphosphate (PI(4,5)P_2) [15]. Like PA, upon PI(4,5)P_2 binding, NaD1 dimerises and adopts the same lipid-binding cationic grip dimer (Figure 2B), and the NaD1–PI(4,5)P_2 crystal structure adopts a distinct arch-shaped higher-order complex. This is also observed during NsD7–PI(4,5)P_2 binding (Figure 2D) [13,15,22]. These structural studies of NaD1 and NsD7 indicate that defensins can form structurally different oligomeric complexes driven by different phospholipids [22]. Furthermore, these studies indicate that PI(4,5)P_2 binding-induced tumour cell membrane permeabilisation by NaD1 and NsD7 may be executed through a novel mechanism that differs from either carpet-like or other proposed models. While it is tempting to speculate that defensin arch-shaped complexes may come together to form 'carpet-like' configurations on a biological membrane, which of these models is more physiologically relevant remains to be experimentally determined. In any case, the mechanism first necessitates the entry of NaD1 into the tumour cell and binding to PI(4,5)P_2 at the inner membrane leaflet prior to inducing membrane blebbing and ultimately resulting in cell lysis (Figure 1F) [15]. It should be noted that membranes feature a complex mix of lipids. As such, defensins are likely to interact with multiple lipid targets during membrane rupture.

Defensin uptake into some fungal cells is energy-dependant. When *Candida albicans* was subjected to cold or latrunculin A treatment (to inhibit ATP production and endocytosis, respectively, inhibiting NaD1

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internalisation), NaD1-induced cell death was significantly reduced [23]. Additionally, MtDef4 uptake into *Neurospora crassa* and *Fusarium graminearum* was significantly reduced following cold or sodium azide treatment both of which block ATP synthesis required for energy dependant internalisation [24]. These studies suggest that defensins require both active uptake into the cytoplasm and intracellular targeting for their membrane rupturing effect [23,24]. It is worth noting that in some cases such as in tumour cells where membrane asymmetry is often disrupted, defensins may also be able to act on the outer leaflet of the membrane in addition to the requirement for internalisation [25]. Morphologically, tumour cells undergoing NaD1-induced cell death show large necrotic-like membrane blebs, and become permeable to the nucleic acid dye propidium iodide, indicating damage to membrane integrity in a process distinct from apoptosis [26].

The importance of $PI(4,5)P_2$ interaction for membrane targeting has also been demonstrated for tomato pistal predominant defensin 3 (TPP3) and HBD-3, which bind exclusively or preferentially to $PI(4,5)P_2$ [17,27]. Effectively, TPP3 and HBD-3 deploy a similar mechanism to NaD1 that requires internalisation before binding to intracellular $PI(4,5)P_2$ to induce membrane blebbing and tumour cell permeabilisation [17,27]. In further support of the importance of $PI(4,5)P_2$-targeting by TPP3 in membrane permeabilisation, the sequestration of $PI(4,5)P_2$ by neomycin has been shown to cause dose-dependent inhibition of TPP3-induced cell death. In contrast, neomycin had no effect on the membrane-lysing ability of LL-37, a toroidal pore-forming peptide [18]. These data not only suggest the conserved critical role of $PI(4,5)P_2$ in mediating defensin-induced membrane permeabilisation, but also further emphasise a distinct mechanism of specific lipid-targeting by defensins compared with the aforementioned general membrane-binding models of most HDPs. This notion is further supported in a more recent report on HBD-2 that describes how $PI(4,5)P_2$ binding is indispensable for its potent antifungal activity [18]. Interestingly, unlike the plant defensins, the HBD-2–$PI(4,5)P_2$ crystal structure reveals a uniquely asymmetric conformation with two different lipid-binding sites (Figure 2E), one which is positively charged whilst the other is more hydrophobic and engages the acyl chain of the lipid molecule [18]. Mutations of key lipid-binding residues in either site substantially impede fungal cell killing by HBD-2 [18]. It remains unknown whether the conformational disparity in $PI(4,5)P_2$ binding between human and plant defensins is due to the fundamental differences in their tertiary structures. Namely, the orientation of disulfide bonds around a central α-helix that determines their classification into either the *cis* (two disulfide bonds to the α-helix; plant defensins) or *trans* (one disulfide bond to the α-helix; human defensins) defensin superfamily [4,18].

In addition to $PI(4,5)P_2$, the binding of other membrane phospholipids by defensins has been shown to mediate the antifungal activity. *M. truncatula* defensin MtDef5, a novel bi-domain defensin, reportedly binds strongly to monophosphorylated phosphoinositol such as phosphatidylinositol 3-phosphate (PI(3)P) and phosphatidylinositol 4-phosphate (PI(4)P) as a part of its mechanism of action against plant bacterial pathogens [6,21]. It is, therefore, implied that MtDef5 has likely evolved multifaceted anti-infective mechanisms involving both membrane targeting and interaction with intracellular targets [6]. Of great interest will be the determination of MtDef5–lipid structures as this will provide valuable insights into how the bi-domains associate and whether they form the lipid-binding cationic grip that is observed for other plant defensins [22].

The bacterial cell wall precursor lipid II is another target of defensins. Lipid II is utilised in the final step of peptidoglycan synthesis and is a target of current antibiotic treatments such as vancomycin [28]. Oyster defensins Cg-Defh1, Cg-Defh2 and Cg-Defm all bind essentially irreversibly to lipid II [28]. Interestingly, the binding of oyster defensins to lipid II-containing liposomes varied among the three defensins tested, and the strength of binding as measured via surface plasmon resonance correlated with the ability to inhibit the growth of *Staphylococcus aureus* [28]. As defensins Cg-Defh1, Cg-Defh2 and Cg-Defm were able to inhibit the growth of Gram-positive but not Gram-negative bacteria, Cg-Defh1, Cg-Defh2 and Cg-Defm are thought to interact with lipid II at the extracellular interface [28].

Glucosylceramide (GluCer), a membrane sphingolipid regulating fungal growth, hyphal formation and fungal virulence, is a key binding partner for many antifungal defensins including RsAFP2 (from *Raphanus sativus*) and *Medicago sativa* defensin 1 (MsDef1) [29–31]. RsAFP2 is selective for fungal GluCer and is unable to bind to the structurally related human GluCer. Fungal strains which lack GluCer or its synthesising enzyme glucosylceramide synthase are resistant to RsAFP2 treatment [29,32]. Unlike many of the defensins listed above, the GluCer-binding RsAFP2 does not appear to form pores to permeabilise membranes, but instead activates downstream pathways that ultimately lead to fungal cell death (details below) [32]. Similarly, GluCer binding also contributes to MsDef1-induced antifungal activity against *F. graminearum*, which also become resistant upon GluCer deficiency [30].

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Mechanisms downstream of lipid binding: more than just membrane disruption

In addition to membrane permeabilisation, other downstream effects of membrane binding by defensins have been suggested, further highlighting their multifaceted mechanisms in combating microbial pathogens and tumour cells. Generally, defensins can trigger different cellular effects including, but not limited to, reactive oxygen species (ROS) and/or nitric oxide (NO) production, activation of cell wall integrity (CWI) pathway and dysregulation of ionic homeostasis, ultimately contributing to cell death (Figure 3) [45].

Defensins NaD1 and RsAPF2 have both been shown to induce the formation of ROS (and NO in the case of NaD1) in fungal cells, hence significantly damaging key cellular components and processes [45,46] (Figure 3A,B). RsAFP2 is believed to cause induction of ROS as a downstream signal from its binding to GluCer in the membrane. This increase in intracellular ROS is believed to induce apoptosis in yeast which is also observed upon RsAFP2 treatment (Figure 3A) [47]. NaD1 induces ROS and NO as the final step in a three-step mechanism of action against fungal cells. Initiation of the process involves interactions with cell wall components such as glycosylated proteins or 1,3-β-glucan, which drives energy-dependant import (step 2), allowing lipid binding and ROS/NO production (step 3) (Figure 3B) [15,23,48,49]. The induction of ROS/NO by NaD1 occurs via interaction with yeast mitochondria, as *Saccharomyces cerevisiae* with an inactive mitochondria respiratory chain are more resistant to NaD1 than wild-type fungi [48]. NaD1 binds cardiolipin (an abundant mitochondrial inner membrane lipid) which, along with the mitochondrial respiratory chain components in yeast, may provide explanation of the mechanism of ROS/NO generation by NaD1 [15,48,50].

In addition to its ability to induce cellular ROS and apoptosis, RsAFP2 interaction with GluCer in the membrane is able to induce the efflux of K^+ and influx of Ca^{2+}, thus disturbing the homeostasis of cellular ion concentrations (Figure 3A) [51]. Additionally, both *Medicago* defensins MsDef1 and MtDef4 reportedly induce the dysregulation of homeostatic Ca^{2+} level (Figure 3C,D) [52,53]. A study comparing Ca^{2+} modulation by both MsDef1 and MtDef4 in *N. crassa* reported a significant decrease in Ca^{2+} amplitude compared with mechanical perturbation, with defensin-treated fungi also failing to restore resting Ca^{2+} levels [53]. Interestingly, MtDef4 treatment of a *N. crassa* Δ*gcs* mutant (lacking GluCer synthase) no longer reduced Ca^{2+} amplitude when compared with mechanical perturbation indicating a role for GluCer in MtDef4 Ca^{2+} modulation

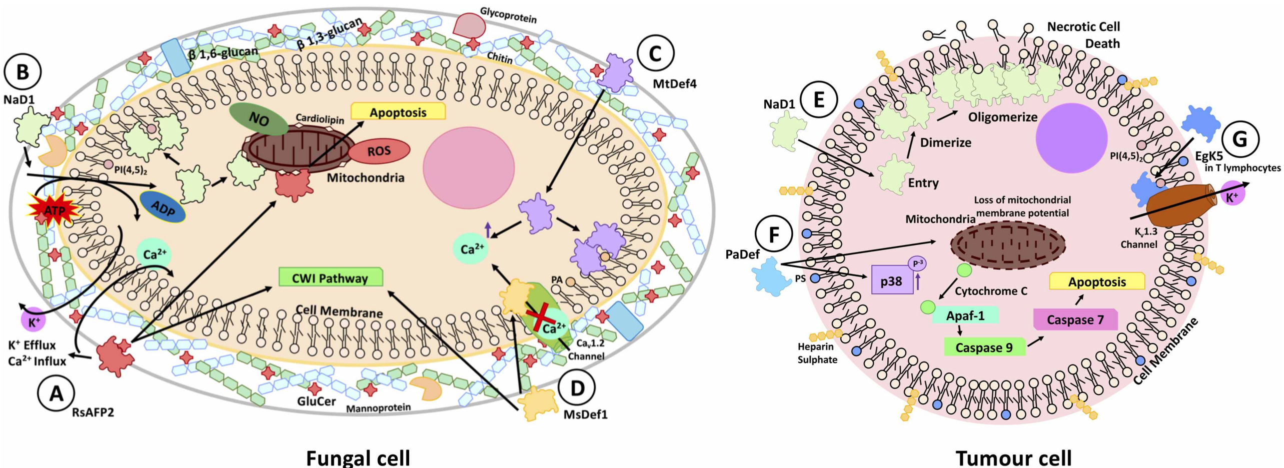

Figure 3. Mechanisms of defensin action downstream of lipid binding.
(**A**) Binding of RsAFP2 to GluCer in the membrane of fungi induces the influx of Ca^{2+} and efflux of K^+ along with activation of the CWI pathway and ROS formation, leading to apoptosis. (**B**) NaD1 kills fungi by a three-step mechanism involving cell wall interaction, energy-dependent import followed by ROS and NO production along with lipid binding and membrane permeabilisation. (**C**) MtDef4 induces dysregulation of Ca^{2+} levels by mechanisms involving GluCer, resulting in dysregulated Ca^{2+} levels. (**D**) MsDef1 blocks Ca^{2+} by interaction with $Ca_v1.2$ channels in the membrane. Additionally, MsDef1 is able to induce the activation of the CWI repair pathway. (**E**) NaD1 induces membrane damage and necrotic cell death in tumour cell settings, via first dimerisation and lipid engagement before oligomerisation and membrane rupture. (**F**) PaDef induces the loss of mitochondrial membrane potential and induces apoptosis via caspases 7/9 activation downstream of Apaf-1. PaDef additionally up-regulates the levels of phosphorylated p38. (**G**) Defensin analogue EgK5 is able to bind to $PI(4,5)P_2$ in the membrane and cause the rundown of $K_v1.3$ channels.

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(Figure 3C) [53]. MsDef1 can block the L-type calcium channel $Ca_v1.2$ with MsDef1 treatment blocking up to 90% of Ca^{2+} current (Figure 3D). An Arg-38 residue at the base of the β2–β3 loop was shown to be important for the $Ca_v1.2$ channel blocking ability by MsDef1 [52]. The β2–β3 loop is also important for defensin lipid binding, as MsDef1 in addition to GluCer binding can bind phospholipids such as PA and PI(4,5)P_2 [16]. Studies investigating cofactors of ion channels reveal an important role for PI(4,5)P_2 in channel stabilisation and activation [9]. Whilst not shown experimentally it is tempting to speculate MsDef1 may block the $Ca_v1.2$ channel via a lipid-dependant mechanism, which has been shown for the defensin EgK5 (discussed later) [9].

Various cell signalling pathways have been shown to be activated in response to defensin exposure. MsDef1 and RsAFP2 both induce increased MAPK signalling and activation of the CWI pathway in response to damage caused by defensins binding to GluCer in the membrane (Figure 3A,D) [45]. RsAFP2 induces increased phosphorylation of Mkc1p, a downstream interaction partner of Pkc1p both involved in the CWI pathway in *C. albicans* (Figure 3A) [32]. *F. graminearum* mutants lacking *MGV*1 (a gene involved in the CWI pathway in *F. graminearum*) are significantly more sensitive to MsDef1 than wild type *F. graminearum*. The increased sensitivity is believed to be caused due to decreased signalling through the Mgv1 MAPK signalling cascade (Figure 3D) [30].

The downstream mechanisms of defensins against tumour cells include activation of classical cell death pathways such as apoptosis and necrosis (Figure 3E–G) [26,54]. NaD1 induces necrotic cell death as a result of membrane rupture (Figure 3G) [26]. It is likely that under subacute NaD1 treatment (<10 μM) tumour cells would induce activation of membrane repair mechanisms such as micro-particle shedding (in an effort to shed the defensin damaged areas), patch-mediated repair and blebbing [55] (see [55] for a comprehensive review of plasma membrane repair mechanisms). However, prolonged exposure to NaD1 is likely to overwhelm such mechanisms and thus render the cell non-viable. Interestingly, in contrast to NaD1, breast cancer cells (MCF-7) treated with PaDef (from avocado fruit) showed activation of the intrinsic apoptotic pathway with up-regulation of caspase 7/9 genes, along with cytochrome c and Apaf-1 (Figure 3F) [54]. Additionally, PaDef also induces loss of mitochondrial membrane potential and increases the phosphorylation of p38 (Figure 3F) [54], which is involved in proliferation and differentiation along with cell stress responses, especially metabolic, oxidative and endoplasmic reticulum stress responses [56]. A designed defensin analogue EgK5, which similarly to natural defensins such as NaD1, is able to bind lipids in the plasma membrane, also binds to the potassium channel $K_v1.3$ in transformed T lymphocytes [9]. Binding of $K_v1.3$ in the membrane by EgK5 induces a current rundown of the channel, via a PI(4,5)P_2 dependent mechanism in which EgK5 depletion of PI(4,5)P_2 (a cofactor for $K_v1.3$) triggers the $K_v1.3$ channel to release K^+ (Figure 3G) [9].

Developing lipid-targeting defensins as novel anticancer and anti-infective therapeutics

Infectious diseases and cancer remain urgent public health and medical issues. The continued emergence of new infectious agents and multidrug/antibiotic resistance is of particular concern [57,58]. Many drugs currently under development exhibit similar mode(s) of action to traditional drugs, which could make them vulnerable to the same resistance mechanisms [59,60]. Therefore, the specific lipid-targeting and potent membrane-permeabilising properties of defensins provide an exciting avenue for anticancer and anti-infective therapeutic design. Advantages of such treatments could include reduced susceptibility to resistance due to the targeting of very conserved cellular features, increased specificity for infectious pathogens and tumour cells, and the ability to target metabolically active and dormant tumour cells [2,61–63]. Furthermore, some defensins are potent at low micromolar concentration ranges against a broad spectrum of tumour cells and pathogens *in vitro* and *in vivo*, including multidrug-resistant bacteria [2,8,16]. Additionally, due to their small compact size and high disulfide content, defensins are stable to protease degradation [15,23,48].

As detailed above, defensins bind a wide range of lipids from both prokaryotic and eukaryotic organisms, speaking to the diversity of structures and functions within this family. Not surprisingly, defensins from the same species can have different lipid binding profiles and downstream mechanisms of action, likely to have arisen as a result of selective pressure to protect hosts from various pathogens [64]. In the context of human health and disease, the role of lipids is extensive but is often poorly understood. For diseases including cancer, Alzheimer's disease and liver disease, lipids represent important biomolecules for disease progression and resolution [65–67]. Additionally, various phosphoinositides are implicated in the establishment and progression of pathogenic infections [65]. Microbial pathogens are able to modulate the regulation of various

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phosphoinositides (including $PI(4,5)P_2$, phosphatidylinositol 3,4,5-trisphosphate ($PI(3,4,5)P_3$), PI(3)P and PI(4)P) in order to modulate host cell functions. These include roles in phagocytosis, membrane ruffling and cup formation, phagosomal lysis and fusion, and the modulation of endoplasmic reticulum machinery, respectively [65]. Thus, defensin-based therapeutics may offer opportunities to directly target pathogens as well as control disease progression through their lipid mediators.

In the next section, defensins and their potential applications for targeting microbial pathogenesis and cancer through lipid interactions are discussed along with limitations currently restricting defensins for therapeutic applications.

Defensins as therapeutics against antibiotic-resistant microbes

Antibiotics revolutionised disease treatment by targeting key bacterial cell processes and allowing selectivity from host tissue [62], however, their misuse has led to the rapid emergence of multidrug-resistant microbes [8]. As discussed above, defensins are unique in that they are able to specifically target specific membrane phospholipids, enabling them to kill microorganisms that are otherwise resistant to other forms of antimicrobials [68]. Furthermore, defensin-based therapeutics are less susceptible to resistance mechanisms than traditional therapeutics. In a study testing the development of *S. cerevisiae* resistance to NaD1 treatment, the authors showed that resistance was developed more slowly compared with the antifungal compound, caspofungin [63]. Furthermore, multiple genome regions in *S. cerevisiae* were identified to contribute to resistance, with resistant strains requiring multiple mutations for resistance. However, the formation of resistance also resulted in decreased cellular fitness as indicated by growth speed and size when compared with wild-type cells [63].

In vitro antimicrobial activity has been shown for several defensins including mouse α-defensins Crp-4, rhesus monkey defensin RMAD-4 and θ-defensin RTD-1 from macaques [56]. As little as 3 μM of defensins Crp-4, RMAD-4 or RTD-1 was sufficient for potent antibacterial activity against methicillin-resistant *S. aureus* (MRSA), vancomycin-resistant *S. aureus* strains and ciprofloxacin-resistant *Pseudomonas aeruginosa* [56]. Mechanistically, hydrophobic residues are important for the antibacterial activity of Crp-4 and RMAD-4 as mutagenesis of key hydrophobic residues resulted in decreased antibacterial activity compared with wild-type controls [68,69].

The defensin rRpdef1α from manila clam shows activity against both *Escherichia coli* and its biofilms, and is believed to act via mechanisms involving both targeting of extracellular ligands (such as lipopolysaccharide (LPS) and glucan) as well as membrane disruption caused by pore formation [70]. Furthermore, PaDef is active against human bacterial pathogens *E. coli* and *S. aureus* [71]. In addition to antibacterial activity, many plant and human defensins have been characterised to have activity against human fungal pathogens such as *C. albicans*, including NaD1 and HBD-2 via the mechanisms discussed above [18,48]. The common bean defensin PvD1 is active against pathogenic fungi *C. albicans*, *Candida buinensis*, *Candida tropicalis* and *Candida parapsilosis* at low micromolar concentrations [72]. PvD1 was also tested *in vivo* in a *C. albicans* infection model of *Gallieria mellonella* (greater wax moth), revealing that treatment with PvD1 significantly increased survival of *G. mellonella* upon infection with various *Candida* strains [72].

Defensins including rRpDef1α, HBD-3, PsD1, HsAFP1 and HsAPF2 are active against bacterial and fungal biofilms [70,73–75]. This defensin-mediated anti-biofilm activity may have applications in the field of medical device implants and prosthetics where infection caused by the formation of biofilms on prosthetic surfaces is of increasing concern [76,77]. HBD-3 reduces the adhesion and formation of MRSA as well as *Staphylococcus epidermidis* and methicillin-resistant *S. epidermidis* (MRSE) biofilms on a titanium surface. Additionally, HBD-3 showed the potential to clear pre-existing MRSA and MRSE biofilms from orthopaedic implants [75].

A few defensins are currently undergoing development and trial for clinical use as treatments for various fungal, bacterial, and viral infections (Table 2). A derivative of a plant defensin, Pezadeftide (previously HXP124) developed by Hexima Limited (Melbourne, Australia) is showing promise as a topical treatment for fungal nail disease following phase IIa clinical trials (ACTRN12618000131257). Brilacidin (formally PMX-30063), a synthetic defensin derivative, is currently undergoing an FDA-fast tracked clinical trials (NCT02324335; NCT01211470) for the treatment of oral mycosis (in patients with head and neck cancer). Brilacidin is also being investigated as a topical treatment for ulcerative proctitis, ulcerative proctosigmoiditis, and an intravenous treatment for acute bacterial skin infections (NCT02052388). Additionally, following successful preclinical trial demonstrating its ability to inhibit SARS-CoV-2 in cell culture [78], Brilacidin has been

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Table 2. Defensins under development for clinical applications

Compound	Defensin	Trial Number	Application	Phase	Company	Outcomes
Pezadeftide (formerly HXP124)	Plant defensin	ACTRN12618000131257	Fungal nail disease	Phase IIa (complete); Stage IIb (ongoing)	Hexima Limited	Excellent clinical efficacy Safe and well tolerated 2-fold higher mycological cure rate than current treatments
Brilacidin (formerly PMX-30063)	Synthetic defensin derivative	NCT02324335; NCT01211470	Oral mucositis in patients with head and neck cancer	Phase 2 complete	Innovation Pharmaceuticals, Inc.	High potential as preventative treatment
		NCT02052388	Acute bacterial skin and skin structure infections	Phase 2 complete, Phase 3 planned	Innovation Pharmaceuticals, Inc.	Single dose equivalent in safety and efficacy to a 7-day antibiotic regimen
		NCT04784897	COVID-19 hospitalised infections	Phase 2 complete	Innovation Pharmaceuticals, Inc.	To determine safety and efficacy for COVID-19 treatment
Plectasin (also known as NZ2114)	Defensin variant	N/A	Treatment of Gram-positive infections	Pre-clinical	Novozymes	Effective against *Streptococcus pneumococcus* Effective in reducing CSF bacterial concentration

FDA fast tracked to phase II clinical trials as an intervention for hospitalised COVID-19 patients (NCT04784897). Currently, although there are a limited number of defensins undergoing clinical trial, a number are in preclinical stages of development. Some challenges currently restrict the application of defensins as therapeutics including a limited therapeutic window, high production costs and issues with delivery and formulation [77]. Additionally, further considerations such as peptide stability, bioavailability and target specificity in biological systems are all challenges to be overcome to aid the progression of defensins into clinical trials [77]. However, more defensin-based therapeutics are likely to soon enter clinical trials on the basis of promising preclinical efficacy. Once such example is a defensin variant Plectasin, also known as NZ2114. Plectasin has undergone preclinical trials for the treatment of *Streptococcus pneumoniae* and *S. aureus* in a murine infection model, and has shown significant promise as a therapeutic [79]. Optimistically, defensin-based therapeutics, such as Plectasin, will join a host of other HDPs currently under clinical trial (extensively reviewed by Mookherjee et al. [77]) fulfilling their therapeutic potential and gaining real-life application for a range of clinical pathologies.

Defensin-based treatment against tumours

Cancer is a disease with high morbidity and mortality and many current treatment options have side effects due to toxicity towards healthy cells [2,80–83]. Additionally, the emergence of secondary treatment-related cancers is of growing concern [80,84]. Tumour cells undergo various changes to their lipid expression profiles that make them susceptible to treatment with defensins [20]. For example, increased levels of PI and its phosphoinositide derivatives are well-reported during tumourigenesis, associated with tumour growth, proliferation and metastasis. Phosphatidylinositol 5-phosphate (PI(5)P) and its metabolising enzyme PIKfyve have been shown to increase the rate of tumour cell migration, via an increase in cellular PI(5)P, which results in increased activation of Rac1 via recruitment of effectors to PI(5)P [85]. Additionally, phosphatidylinositol 3,4 bisphosphate ($PI(3,4)P_2$) via localisation to invadopodium enhances tumour cell migration whilst $PI(4,5)P_2$ influences the invasiveness, migration, cell polarity and metastasis of tumour cells via its many effectors [65,85]. Furthermore, the dysregulation of PI(3)K via activating mutations increases PI3K-Akt-mTOR pathway flux, thus promoting cancer growth and survival as well as cell polarity driven epithelial-to-mesenchymal transition [65,86]. Tumour cell plasma membrane levels of PA are also elevated due to changes in cell metabolism including increased flux via EGFR receptors and G protein-dependant activation of phospholipase D, which is involved in PA biosynthesis. Increased PA levels, in turn, activate kinases such as MAPK and ABL tyrosine kinase 1 which are implicated in cancer progression [87]. As a result of tumourigenesis-induced dysregulation

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of lipid-transporting enzymes (e.g. flippases, floppases, scramblases, aminolipid translocase), phosphatidylserine (PS) and phosphatidylethanolamine (PE) are shuffled to the outer leaflet of the tumour cell plasma membrane [87,88]. In cancers such as colorectal and metastatic liver cancer, phospholipid scramblase 1 (causes bidirectional membrane lipid scrambling), is up-regulated and thought to be responsible for a breakdown of membrane asymmetry [89,90]. The dysregulation tumour cell membrane composition has been shown in a tumour implantation model of Hodgkins lymphoma in SCID mice which Annexin V and a monoclonal antibody 9D2 (which specifically recognises anionic lipids) localised to the vascular endothelium in tumours but not normal endothelium, indicating increased exposure of anionic lipids in the membranes of tumour endothelium [91]. This may aid in sensitising the cells to defensin treatment whilst in a dormant or actively dividing state [25,90]. Furthermore, a study of tumourigenesis in *Drosophila* showed that tumour necrosis factor (TNF) caused the exposure of PS in tumour cells which made them selectively permeable to *Drosophila* Defensin. This study reported that the defensin bound to PS-rich regions in the tumours which results in cell death and tumour regression [92]. These data indicate that increased negative charge on tumour cell membranes may cause them to be more susceptible to defensin attack and permeabilisation.

Many defensins have been shown to be active against a wide variety of tumour cell lines *in vitro*. Examples include NaD1 against human colon cancer HCT-116, breast cancer MCF-7, melanoma MM170 and cervical HeLa cancer cells [15]; TPP3 against human monocytic lymphoma U937 [17]; PaDef against MCF-7 cells and myeloid leukaemia K562 cells [54,93]; HBD-3 against U937, HeLa, prostate PC3, leukaemia HL-60 and T-cell leukaemia Jurkat cells [27]; and PvD1 against brain cancer HBMEC and breast cancer MDA-MB-231 cells [94]. However, to date, there is very limited *in vivo* evidence accompanying these *in vitro* studies with the focus of the field tending towards the discovery of new defensins instead of further developing currently known defensins. The plant defensin NoD173 from *Nicotiana occidentalis* (Australian tobacco) has demonstrated *in vivo* activity, dramatically inhibiting the growth of established solid B16-F1 melanoma tumours in a C57BL/6 mouse model. When NoD173 was administered to mice intratumorally at 5 mg/kg body weight (three times per week over 2 weeks), tumour growth was significantly perturbed when compared with both the vehicle control and a chemically inactive form (by reduction and alkylation) of NoD173 [20].

Clinically, there are many opportunities for the use of defensins as anti-infective and cancer therapeutics but much work is still required in this area, including studies on bioavailability, pharmacokinetics, dosing and stability [20]. There are currently some concerns regarding the systemic administration of defensins, which are likely to require yet to be developed delivery systems to target cancer effectively [77]. Nevertheless, nanotechnology-based delivery systems are showing promise in addressing current defensin delivery concerns [95]. In addition to using defensins to directly treat cancer, defensins could be used in conjunction with current chemotherapeutic options to aid in tumour targeting and killing. An example of this approach was reported for the defensin MsDef1 and doxorubicin against triple-negative breast cancer cells (MDA-MB-231R) and oestrogen receptor-positive cells (MCF-7R). In this study, defensin treatment synergistically improved doxorubicin effectiveness [96]. Furthermore, defensins could be used to aid in protection against opportunistic infections by 'supplementing' components of the innate immune system during chemotherapeutic treatment [97]. As more research is published on defensin immune-modulatory functions, novel therapeutic opportunities may become apparent for cancer and other immune-related diseases, such as the treatment of inflammatory bowel disease by HBD-2 [98,99].

Outstanding limitations to be addressed

Despite the promise of using defensins as anticancer and antimicrobial drugs, none are currently approved for clinical use, although several are in clinical trial [77]. One key challenge that has been identified with the use of defensins as treatments for a range of human conditions is their reduced (and in many cases abolished) activity at physiological salt concentration [100,101]. A recent study on a highly charged corn defensin (ZmD32) showed that it was able to retain activity in the presence of salt concentrations as high as 100 mM, compared with other plant defensins such as NaD1 and NaD2 that lose their activity, although the kinetics of ZmD32 killing was reduced in high salt conditions [100].

Defensins often suffer from non-superior efficacy when compared with traditional treatments, potentially due to the inability to deliver defensins therapeutically [77]. Currently, *in vivo* studies typically utilise a subcutaneous injection to deliver defensins [20,98] which is less desirable clinically when compared with oral delivery mechanisms [102]. A novel delivery mechanism is desirable for defensins to address systemic delivery concerns as well as reduce potential toxicities, allowing the defensins to only be exposed to the host cellular

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environment upon arrival at the target site [103]. Such a delivery system may aid in increasing the bioavailability of defensins, thus making them an incredibly attractive area of research for the defensin field [103].

Future perspectives and concluding remarks

Defensins represent an armoury of potential anticancer and anti-infective therapeutics, with their unique ability to bind to specific lipids within the target cell membrane, resulting in the permeabilisation of the target membrane and activation of the downstream process which eventuate in cell death. As discussed above, recent studies show the preclinical efficacy of defensins in killing a wide range of human tumour cells, fungal pathogens and antimicrobial-resistant bacteria, along with reduced susceptibility to resistance. However, there are still several challenges to be addressed including delivery mechanisms, potential toxicity and bioavailability. These areas of research provide an opportunity to further advance the field. Future studies investigating both the action of defensins *in vivo*, including pharmacodynamic, bioavailability and efficacy of defensins in treating both microbial and cancer disease models in animals (or other model organisms) would prove beneficial. Together with careful evaluation of the outcomes of current clinical trials, this will provide hope that defensins can one day be used as a new arsenal against pathogens and cancer in clinical settings.

Perspectives

- The mechanism(s) of membrane interaction by defensins has been of significant interest in the HDP field. Recent research shows that the mechanism of action of defensins may differ to the models traditionally proposed for HDPs.

- Defensins bind membrane lipids via novel mechanisms, which may pave the way to a novel class of antimicrobial and anticancer peptides.

- Defensins present an untapped natural reservoir of novel antimicrobial and cancer therapeutics. Whilst there are currently limitations to their clinical use, research overcoming these limitations may provide a new class of lipid-targeting therapeutics for clinical application.

Competing Interests

The authors declare that there are no competing interests associated with the manuscript.

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Author Contributions

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Abbreviations

CWI, cell wall integrity; HBD-2, human beta-defensin 2; MDC, membrane disruption complex; MRSA, methicillin-resistant *S. aureus*; MRSE, methicillin-resistant *S. epidermidis*; PA, phosphatidic acid; PE, phosphatidylethanolamine; PS, phosphatidylserine; ROS, reactive oxygen species; TPP3, tomato pistil predominant defensin 3.

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