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

Matthew J. A. Hein, Marc Kvansakul, Fung T. Lay, Thanh Kha Phan and Mark D. Hulett

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].
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.
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 conformation 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].

### Table 1. Examples of lipid-binding defensins

<table>
<thead>
<tr>
<th>HDP</th>
<th>Organism</th>
<th>Lipid</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>Copsin</td>
<td>Coprinopsis cinerea</td>
<td>Lipid II</td>
<td>[33]</td>
</tr>
<tr>
<td>DmAMP1</td>
<td>Dahlia merckii</td>
<td>Sphingolipids</td>
<td>[34]</td>
</tr>
<tr>
<td>Eurocin</td>
<td>Eurotium amstelodanii</td>
<td>Lipid II</td>
<td>[35]</td>
</tr>
<tr>
<td>Gallicin</td>
<td>Mytilus galloprovincialis</td>
<td>Lipid II</td>
<td>[36]</td>
</tr>
<tr>
<td>HNP-1</td>
<td>Homo sapiens</td>
<td>Palmitoyloleoylphosphatidyglycerol (POPG), Lipid II</td>
<td>[37]</td>
</tr>
<tr>
<td>HNP-2</td>
<td>Homo sapiens</td>
<td>POPG</td>
<td>[14,38]</td>
</tr>
<tr>
<td>HsAPF1</td>
<td>Heuchera sanguinea</td>
<td>PA, P(3,4,5)P₃, P(3,4)P₂</td>
<td>[39]</td>
</tr>
<tr>
<td>Lo-def</td>
<td>Lens culinaris</td>
<td>POPG</td>
<td>[40]</td>
</tr>
<tr>
<td>Lucifensin</td>
<td>Lucilla sericata</td>
<td>Lipid II</td>
<td>[36]</td>
</tr>
<tr>
<td>MtDef4</td>
<td>Medicago truncatula</td>
<td>PA</td>
<td>[16]</td>
</tr>
<tr>
<td>MtDef5</td>
<td>Medicago truncatula</td>
<td>PA; PS; P(3)P; P(4)P; P(5)P; P(3,5)P₂; P(4,5)P₂</td>
<td>[21]</td>
</tr>
<tr>
<td>NoD173</td>
<td>Nicotiana occidentalis</td>
<td>P(4,5)P₂</td>
<td>[20]</td>
</tr>
<tr>
<td>NaD7</td>
<td>Nicotiana suaveolens</td>
<td>PA</td>
<td>[19]</td>
</tr>
<tr>
<td>Oryzeacin</td>
<td>Aspergillus oryzea</td>
<td>Lipid II</td>
<td>[36]</td>
</tr>
<tr>
<td>Plectasin</td>
<td>Pseudoplectania nigrella</td>
<td>Lipid II</td>
<td>[36]</td>
</tr>
<tr>
<td>Psd1</td>
<td>Pisum sativum</td>
<td>Ergosterol, Glycosphingolip</td>
<td>[42]</td>
</tr>
<tr>
<td>PsdF2</td>
<td>Psoralius sativus</td>
<td>Glucosylceramides, Sphingolipids</td>
<td>[30]</td>
</tr>
<tr>
<td>Sd5</td>
<td>Saccharum officinarum</td>
<td>Glucosylceramides</td>
<td>[44]</td>
</tr>
<tr>
<td>TPP3</td>
<td>Solanum lycopersicum</td>
<td>P(4,5)P₂</td>
<td>[17]</td>
</tr>
</tbody>
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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)P2) [15]. Like PA, upon PI(4,5)P2 binding, NaD1 dimerises and adopts the same lipid-binding cationic grip dimer (Figure 2B), and the NaD1–PI(4,5)P2 crystal structure adopts a distinct arch-shaped higher-order complex. This is also observed during NsD7–PI(4,5)P2 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)P2 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)P2 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
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)P₂ interaction for membrane targeting has also been demonstrated for tomato pistil predominant defensin 3 (TPP3) and HBD-3, which bind exclusively or preferentially to PI(4,5)P₂ [17,27]. Effectively, TPP3 and HBD-3 deploy a similar mechanism to NaD1 that requires internalisation before binding to intracellular PI(4,5)P₂ to induce membrane blebbing and tumour cell permeabilisation [17,27]. In further support of the importance of PI(4,5)P₂-targeting by TPP3 in membrane permeabilisation, the sequestration of PI(4,5)P₂ 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)P₂ 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)P₂ binding is indispensable for its potent antifungal activity [18]. Interestingly, unlike the plant defensins, the HBD-2–PI(4,5)P₂ 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)P₂ binding between human and plant defensins is due to the fundamental differences in their tertiary structures. Namely, the orientation of disulphide bonds around a central α-helix that determines their classification into either the cis (two disulphide bonds to the α-helix; plant defensins) or trans (one disulphide bond to the α-helix; human defensins) defensin superfamily [4,18].

In addition to PI(4,5)P₂, 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].
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 RsAFP2 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 Ca²⁺, thus disturbing the homeostasis of cellular ion concentrations (Figure 3A) [51]. Additionally, both *Medicago* defensins MsDef1 and MtDef4 reportedly induce the dysregulation of homeostatic Ca²⁺ level (Figure 3C,D) [52,53]. A study comparing Ca²⁺ modulation by both MsDef1 and MtDef4 in *N. crassa* reported a significant decrease in Ca²⁺ amplitude compared with mechanical perturbation, with defensin-treated fungi also failing to restore resting Ca²⁺ levels [53]. Interestingly, MtDef4 treatment of a *N. crassa* Δgcs mutant (lacking Glucer synthase) no longer reduced Ca²⁺ amplitude when compared with mechanical perturbation indicating a role for Glucer in MtDef4 Ca²⁺ modulation.

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**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 Ca²⁺ and efflux of K⁺, thus causing disturbance of the homeostasis of cellular ion concentrations (Figure 3A) [51]. Additionally, both *Medicago* defensins MsDef1 and MtDef4 reportedly induce the dysregulation of homeostatic Ca²⁺ levels (Figure 3C,D) [52,53]. A study comparing Ca²⁺ modulation by both MsDef1 and MtDef4 in *N. crassa* reported a significant decrease in Ca²⁺ amplitude compared with mechanical perturbation, with defensin-treated fungi also failing to restore resting Ca²⁺ levels [53]. Interestingly, MtDef4 treatment of a *N. crassa* Δgcs mutant (lacking Glucer synthase) no longer reduced Ca²⁺ amplitude when compared with mechanical perturbation indicating a role for Glucer in MtDef4 Ca²⁺ modulation.
channel Kv1.3 in transformed T lymphocytes [9]. Binding of Kv1.3 in the membrane by EgK5 induces a to natural defensins such as NaD1, is able to bind lipids in the plasma membrane, also binds to the potassium oxidative and endoplasmic reticulum stress responses [56]. A designed defensin analogue EgK5, which similarly [54], which is involved in proliferation and differentiation along with cell stress responses, especially metabolic, [30]. MsDef1 can block the L-type calcium channel Ca_{1.2} 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_{1.2} 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_{1.2} 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 Mkv1p, a downstream interaction partner of Pkc1p both involved in the CWI pathway in C. albicans (Figure 3A) [32]. F. graminearum mutants lacking MGV1 (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 Mgvl 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_{v}1.3 in transformed T lymphocytes [9]. Binding of K_{v}1.3 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_{v}1.3) triggers the K_{v}1.3 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 disulphide 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
phosphoinositides (including PI(4,5)P2, phosphatidylinositol 3,4,5-trisphosphate (PI(3,4,5)P3), 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 Galliera 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 HsAFP2 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 (ACTRN1261800131257). 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...
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 biphosphate (PI(3,4)P₂) via localisation to invadopodium enhances tumour cell migration whilst PI(4,5)P₂ 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 EGRF 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.

### Table 2. Defensins under development for clinical applications

<table>
<thead>
<tr>
<th>Compound</th>
<th>Defensin</th>
<th>Trial Number</th>
<th>Application</th>
<th>Phase</th>
<th>Company</th>
<th>Outcomes</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pezadeftide</td>
<td>Plant defensin</td>
<td>ACTRN12618000131257</td>
<td>Fungal nail disease</td>
<td>Phase Ila</td>
<td>Hexima Limited</td>
<td>Excellent clinical efficacy Safe and well tolerated 2-fold higher mycological cure rate than current treatments</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>(complete); Stage Ila (ongoing)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Brilacidin</td>
<td>Synthetic defensin derivative</td>
<td>NCT02324335; NCT01211470</td>
<td>Oral mucositis in patients with head and neck cancer</td>
<td>Phase 2 complete</td>
<td>Innovation Pharmaceuticals, Inc.</td>
<td>High potential as preventative treatment</td>
</tr>
<tr>
<td></td>
<td></td>
<td>NCT02052388</td>
<td>Acute bacterial skin and skin structure infections</td>
<td>Phase 2 complete, Phase 3 planned</td>
<td>Innovation Pharmaceuticals, Inc.</td>
<td>Single dose equivalent in safety and efficacy to a 7-day antibiotic regimen</td>
</tr>
<tr>
<td></td>
<td></td>
<td>NCT04754897</td>
<td>COVID-19 hospitalised infections</td>
<td>Phase 2 complete</td>
<td>Innovation Pharmaceuticals, Inc.</td>
<td>To determine safety and efficacy for COVID-19 treatment</td>
</tr>
<tr>
<td>Plectasin (also known as NZ2114)</td>
<td>Defensin variant</td>
<td>N/A</td>
<td>Treatment of Gram-positive infections</td>
<td>Pre-clinical</td>
<td>Novozymes</td>
<td>Effective against 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.</td>
</tr>
</tbody>
</table>
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 (a 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 Defensins. 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 monocyteic 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 little 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...
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**
M.J.A.H., M.K., F.T.L., T.K.P. and M.D.H. conceived, wrote and edited the manuscript.

**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.

**References**


