frontiers in Cell and Developmental Biology

REVIEW
published: 12 April 2021
doi: 10.3389/fcell.2021.649875

Membrane-Interacting Antifungal Peptides

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The incidence of invasive fungal infections is increasing worldwide, resulting in more than 1.6 million deaths every year. Due to growing antifungal drug resistance and the limited number of currently used antimycotics, there is a clear need for novel antifungal strategies. In this context, great potential is attributed to antimicrobial peptides (AMPs) that are part of the innate immune system of organisms. These peptides are known for their broad-spectrum activity that can be directed toward bacteria, fungi, viruses, and/or even cancer cells. Some AMPs act via rapid physical disruption of microbial cell membranes at high concentrations causing cell leakage and cell death. However, more complex mechanisms are also observed, such as interaction with specific lipids, production of reactive oxygen species, programmed cell death, and autophagy. This review summarizes the structure and mode of action of antifungal AMPs, thereby focusing on their interaction with fungal membranes.

Keywords: antimicrobial peptides, mode of action, antifungal activity, membrane-interaction, review

OPEN ACCESS

Edited by:
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Specialty section:
This article was submitted to Membrane Traffic, a section of the journal Frontiers in Cell and Developmental Biology

Received: *05 January 2021*
Accepted: *09 March 2021*
Published: *12 April 2021*

Citation:
Struyfs C, Cammue BPA and Thevissen K (2021) Membrane-Interacting Antifungal Peptides. Front. Cell Dev. Biol. 9:649875. doi: 10.3389/fcell.2021.649875

INTRODUCTION

In the past two decades, fungal infections have caused severe die-offs and extinctions in wild species, as they comprise the biggest threat for both plant (64%) and animal (72%) species in terms of infection-related species extinctions (Fisher et al., 2012). Moreover, fungal infections are also jeopardizing food security, as they are causing both yield and post-harvest losses (Fisher et al., 2012; Gauthier and Keller, 2013).

In human health, invasive fungal infections (IFIs) are yearly causing 1.6 million deaths worldwide (Fisher et al., 2020). This number is still increasing (i) due to a rise in the number of immunocompromised patients, e.g., patients receiving immunosuppressive therapies in context of cancer treatment or transplantation (Kim, 2016; Bongomin et al., 2017), and (ii) due to an increased use of modern medical devices, such as catheters and implants (Kojic and Darouiche, 2004; Vandecandelaere and Coenye, 2015). Notably, the number of IFI deaths is most likely an underestimation due to poor epidemiological data and misdiagnosis (Bongomin et al., 2017; Spitzer et al., 2017; Benedict et al., 2019). Currently, only three main classes of antifungal drugs are available, namely azoles, polyenes and echinocandins, while antifungal drug resistance is increasing (Fisher et al., 2018; Revie et al., 2018). *Candida* spp. and *Aspergillus* spp. are mainly responsible for IFI with mortality rates up to 50% (Brown et al., 2012; Bongomin et al., 2017). Moreover, the recently identified pathogen *Candida auris* is often multidrug resistant, with some strains resistant to all three available antifungal classes (Lockhart et al., 2017). In addition, both the Centers for Disease Control and Prevention and the World Health Organization recently placed fungal pathogens on their concern list (Centers for Disease Control and Prevention [CDC], 2019; World Health Organization [WHO], 2020). Hence, there is a clear need for novel antifungal strategies.

Antimicrobial peptides (AMPs) are host defense peptides of the nonspecific innate immune system that are part of the first line of immune defense (Hancock and Patrzykat, 2002; Teixeira et al., 2012; Shah et al., 2016, 2019; Contreras et al., 2019). Due to their broad-spectrum activity, selective targeting, multiple modes of action and often limited toxicity to human cells, great potential is attributed to AMPs. AMPs were first described in 1939 by Dubos, who isolated gramicidin from *Bacillus brevis* (Dubos, 1939). Later in the 1980s, cecropins, defensins and magainins were isolated from moths, human neutrophils and amphibians, respectively (Steiner et al., 1981; Ganz et al., 1985; Zasloff, 1987). Currently, over 3000 AMPs are described in the Antimicrobial Peptide Database (Wang et al., 2016), nevertheless only a limited number of AMPs have been studied regarding their mechanism of action. AMPs are present in bacteria, fungi, plants, invertebrates and vertebrates (van der Weerden et al., 2013; Wang et al., 2016; Kumar et al., 2018), and are known for their broad-spectrum that can include activity against bacteria, fungi, viruses, protozoa and/or even cancer cells (Wachinger et al., 1998; do Nascimento et al., 2015; El-Mounadi et al., 2016; Cools et al., 2017a; Parvy et al., 2019; Struyfs et al., 2020). Note that individual AMPs may not act against all these organisms. Apart from their direct action, AMPs can also function indirectly via immunomodulation in higher organisms. Firstly, AMPs can affect the innate immune system, e.g., enhanced wound healing by the salivary peptides histatin 1 and 2 (Oudhoff et al., 2008). Secondly, AMPs can modulate and bridge the adaptive immune response elements, e.g., human β-defensins 1, 2, and 3 (hBD-1,-2, and -3) induce the upregulation of specific cytokines, such as IL-8 and MCP-1, whereas only hBD-1,-2 induce IL-6 and IL-10 (Boniotto et al., 2006). In animals, AMPs are mostly produced by epithelial cells of skin, airways and gastrointestinal tract (Zasloff, 2002), while in plants they preferentially accumulate in the peripheral cell layer (Broekaert et al., 1997; Lay and Anderson, 2005; van der Weerden et al., 2013; Parisi et al., 2019b). AMPs can be constitutively expressed e.g., hBD-1 in epithelial cells (O'Neil et al., 1999); termicin and spinigerin in termites (Lamberty et al., 2001b) and the plant defensin Psd1 in the epidermal tissues and vascular bundles of pea pods (Almeida et al., 2000, 2002). Additionally, AMPs can be expressed upon induction e.g., hBD-2,-3, and -4 in epithelial cells upon infection and inflammation (García et al., 2001; Liu et al., 2003; Sørensen et al., 2003; Gácser et al., 2014); CRAMP in mouse skin upon *Candida albicans* infection (López-García et al., 2005) and plant peptides e.g., AtPDF1.2, AtPDF2.3 in leaves upon microbial invasion (Penninckx et al., 1996; Broekaert et al., 1997; Manners et al., 1998; Thomma and Broekaert, 1998; Lay and Anderson, 2005; van der Weerden et al., 2013; Parisi et al., 2019b).

The focus of this review will be on the structure and mode of action of membrane-interacting antifungal AMPs and on a potential link between their mode of action and membrane contact sites (MCSs), thereby focusing on AMPs of which the mode of action is already described in more detail. Additional information on AMPs can be found in Faruck et al. (2016); Parisi et al. (2019b), Buda De Cesare et al. (2020), and Mookherjee et al. (2020). Gaining insight in their mechanism of action allows to exploit this information for novel therapeutics.

STRUCTURE

Antimicrobial peptides are typically composed of 12 to 54 amino acids (AA) with an overall net positive charge at physiological pH (Teixeira et al., 2012; Mookherjee et al., 2020). However, also negatively charged peptides exist, such as dermcidin (*Homo sapiens*) and maximin H5 (*Bombina maxima*) (Schittek et al., 2001; Lai et al., 2002). In general, up to 50% of the residues are hydrophobic, thereby contributing to an amphipathic conformation that enhances their interaction with the target membrane (Gomes et al., 2018). AMPs are known for their sequence diversity and wide range of secondary structures. Based on their conformation four main groups can be distinguished, i.e., (i) α-helical peptides, (ii) β-sheet peptides with disulfide bridges, (iii) cyclic peptides, and (iv) residue-rich peptides. Firstly, α-helical peptides are usually unstructured in aqueous solvent, whereas in the presence of membranes, they fold into amphiphilic α-helices in which polar and non-polar AA are segregated along the helical axis (Almeida and Pokorny, 2012; Hollmann et al., 2018). The size of α-helical peptides ranges between 12 and 37 AA; they typically contain a kink or central hinge region (Tossi et al., 2000). Examples of α-helical peptides are cathelicidins [e.g., LL-37 (human) and CRAMP (mouse)], cecropins (moths), magainins (amphibians), and melittin (honeybee) (Zasloff, 1987; Gallo et al., 1997; Silvestro et al., 2000; Andrä et al., 2001; Den Hertog et al., 2005; Park and Lee, 2010; Ordonez et al., 2014). Also α-helical peptides with a disulfide bridge have been occasionally described. Their N-terminal region consists of an amphipathic α-helix, whereas the C-terminus contains a disulfide bridge (Almeida and Pokorny, 2012), such as brevinin 2 (*Rana pirica*) and gaegurin 1 (*Rana rugosa*). Secondly, β-sheet peptides with disulfide bridges are characterized by a well-defined number of β-strands that are stabilized by disulfide bonds (Hollmann et al., 2018). These peptides have no or only a few helical domains. Cysteine-containing β-sheet peptides represent a highly diverse group of peptides, mainly represented by defensins. These peptides have been found in fungi, plants, insects and vertebrates (van der Weerden et al., 2013). Defensins have a conserved motif, namely the γ-core, which is an essential structural element that is composed of two antiparallel β-sheets (Brogden, 2005; Sathoff et al., 2019). Although early work described the isolated γ-core as an active antifungal peptide, this is not reproducible for all peptides, as synthetic γ-core peptides have been tested that were not active (Sagaram et al., 2011; Sathoff et al., 2019). For plant defensins, the γ-core comprises surface loop 5 which is essential for antifungal activity (Parisi et al., 2019b). β-sheet peptides can contain two (e.g., tachyplesins and protegrins), three (e.g., mammalian defensins and insect defensins) or four disulfide bridges (e.g., drosomycin and plant defensins) (Broekaert et al., 1997; Parisi et al., 2019b). *Petunia hybrida* defensins 1 and 2 (PhD1,2) are, so far, the only known plant defensins containing five disulfide bridges (Janssen et al., 2003). The presence of disulfide bridges in defensins assures their stability under extreme conditions, such as exposure to high temperatures or in serum, thereby, retaining their biological activity (Osborn et al., 1995; Tavares et al., 2008). Thirdly, cyclic peptides are characterized by a cyclization of the peptide backbone, such as gramicidin

S (*Bacillus brevis*), daptomycin (*Streptomyces roseosporus*) and cyclotides, such as kalata B1 and cycloviolacins (Tam et al., 1999; Brogden, 2005; Daly et al., 2009; Almeida and Pokorny, 2012; Slazak et al., 2018). Cyclotides contain three cysteine bridges and a structural motif known as the cyclic cysteine knot, resulting in exceptional stability. Fourthly, residue-rich peptides are characterized by the predominant presence of a particular amino acid, which imposes particular constraints to their structure, such as indolicidin (tryptophan-rich), histatin (histidine-rich) and arasin 1 (proline-rich) (Helmerhorst et al., 1999; Brogden, 2005; Almeida and Pokorny, 2012; Paulsen et al., 2013).

MODE OF ANTIFUNGAL ACTION OF AMPs

Previously, it was believed that the mode of action of AMPs solely consisted of rapid physical disruption of microbial membranes causing cell death. However, numerous antifungal AMPs have a more complex mechanism of action in which they interact with the surface of fungal cells and affect intracellular targets, leading to e.g., endogenous reactive oxygen species (ROS) production, mitochondrial and vacuolar dysfunction, programmed cell death, autophagy, and cell cycle impairment as described in more detail and summarized in **Supplementary Table 1**.

Interaction of AMPs With the Fungal Cell Surface

In the past, the mechanism of action of AMPs was believed to be nonspecific, resulting in pore formation by barrel-stave, carpet or toroidal pore mechanisms. In these mechanisms, (i) peptides form a bundle with a central lumen in the membrane, (ii) accumulate on the bilayer surface, or (iii) insert into the membrane and induce the lipids to bend through the pore resulting in a central lumen consisting of both peptides and lipid head groups (Brogden, 2005; Lazzaro et al., 2020). For certain AMPs, this is indeed the case. For example, using synthetic lipid vesicles, the insect peptide cecropin A (*Hyalophora cecropia*) was demonstrated to form ion channels at low concentrations and pores at high concentrations (Silvestro et al., 2000). It remains, however, unclear whether pore formation of cecropin A is responsible for its antifungal activity. In bacteria, the vertebrate peptide magainin 2 (*Xenopus laevis*) forms pores by the toroidal pore mechanism, whereas in mammalian cells, it forms pores via the carpet mechanism (Imura et al., 2008). Also here, the mode of action on fungal cells remains unclear. Note that membrane disrupting AMPs should not be called pore formers unless there is biophysical evidence that a pore has indeed been formed, as confirmed by e.g., ion conductance and the atomic size of the pore. Nonetheless, there is currently little evidence of antifungal AMPs that form pores *in vivo* or in artificial membranes that closely resemble biological membranes. Nonspecific interaction of antifungal AMPs with antimicrobial membranes is thus not the sole mechanism of action (as discussed in Wilmes et al., 2011). Thevissen et al. (1997, 2000b) were the first to report the existence of specific, high-affinity binding sites for the plant defensins HsAFP1 and DmAMP1 on *Neurospora crassa* hyphae, and demonstrated that binding to these sites is required for their antifungal activity.

Interaction of AMPs With the Fungal Cell Membrane

The first step in the antifungal mechanism of action is thus the interaction of AMPs with their fungal target, which can be the fungal cell membrane and/or the fungal cell wall (see section "Interaction of AMPs With the Fungal Cell Wall"). The former mainly consists of sterols, phospholipids and sphingolipids. Phospholipids are composed of a glycerol backbone coupled to two fatty acids and one polar head group. Numerous phospholipids can be distinguished based on their polar head group, such as phosphatidic acid (PA), phosphatidylcholine, phosphatidylethanolamine (PE), phosphatidylglycerol, phosphatidylserine (PS), and phosphoinositides. Note that binding affinities between AMPs and lipids are rarely measured, and can thus not be compared. Examples of AMPs that interact with PA are the plant defensins HsAFP1, MtDef4, NaD1, NaD2, and NsD7 (**Figures 1**, **2C,D**); some of which interact also with other phospholipids like phosphatidylinositol phosphates (PIPs) and phosphatidylinositol bisphosphates (PIP_2s) (**Figure 1**). The RGFRRR-motif of MtDef4 was found to be of importance for the interaction with PA, as mutants devoid of this motif are characterized by reduced binding (Sagaram et al., 2013). Similarly, this motif is present within the sequence of NaD2 and AtPDF2.3 (Cools et al., 2017a). NaD2 is demonstrated to interact with PA as well as with PIPs, as evaluated using lipid overlays and lipid vesicles (Bleackley et al., 2016; Payne et al., 2016). Although lipid overlays can be useful for initial assessment of a potential peptide – lipid interaction, this technique is often unreliable due to the high lipid concentration and the lipid orientation on the strip that are not representative of *in vivo* membranes (Narayan and Lemmon, 2006; Payne et al., 2016). Therefore, potential interactions observed using lipid overlays must be further validated using different techniques, such as ELISA assays, lipid vesicles, structure analysis or deletion mutants of genes that are of importance for the biosynthesis of membrane lipids. It is important to note that by using specific deletion mutants for validating a peptide-membrane component interaction, one cannot distinguish between the membrane component being the genuine peptide interaction partner or only a secondary effector in modulating peptide susceptibility. Therefore, we have listed the specific validation methods that were used in this section. For AtPDF2.3 the interaction with PA has hitherto not been validated. Nevertheless, the presence of an RGFRRR-motif seems not essential for the interaction of AMPs with PA. Recently, using lipid overlays, reverse ELISA assays and lipid vesicles, Cools et al. (2017b) demonstrated the interaction of HsAFP1 with PA, in which histidine at position 32 and arginine at position 52 are important features. Additionally, HsAFP1 also binds, to a lesser extent, to various phosphatidylinositol moieties. AMPs are also able to bind different types of phospholipids to form oligomeric fibrils with different topologies. Note that these fibrils have been produced *in vitro* and there is currently little evidence that they are also produced *in vivo*. In this respect, the plant

defensin NsD7 forms a coiled defensin–PA double helix upon interaction with PA, in which the critical residues that enable assembly of the dimers into an oligomer are lysine at position 36 and arginine at position 39 (Kvansakul et al., 2016). NaD1 also interacts with PA, as demonstrated using lipid overlays, lipid vesicles and structure analysis. In contrast, NaD1 and PA form a near-flat, carpet-like oligomeric complex *in vitro* during the initial stages of membrane encounter. Also here, arginine at position 39 is critical for PA binding, oligomerization and fungal cell killing (Järvå et al., 2018a). Additionally, NaD1 also targets $PI(4,5)P_2$ (**Figure 1**), thereby seven dimers of NaD1 bind anionic headgroups of 14 PIP_2 molecules through a cationic grip configuration. Lysine at position 36 and arginine at position 40 are of importance for the NaD1 – PIP_2 interaction (Poon et al., 2014; Bleackley et al., 2016). This interaction seems to be essential for NaD1's activity on tumor cells, whereas it does not seem to be essential for its antifungal activity. Nevertheless, it can be challenging to identify relevant interaction partners, since AMPs, in particular plant defensins, have multiple targets so eliminating one can weaken but does not necessarily abolish antifungal activity. Likewise, TPP3 adopts a cationic grip dimer conformation that mediates binding of PIP_2 *in vitro* (Baxter et al., 2015). Also NsD7 interacts with $PI(4,5)P_2$. In this case the oligomeric topology formed by NsD7- PIP_2 is nearly identical to the previously determined NaD1–PIP_2 complex (Järvå et al., 2017). Both hBD-2 and hBD-3 are also able to interact with

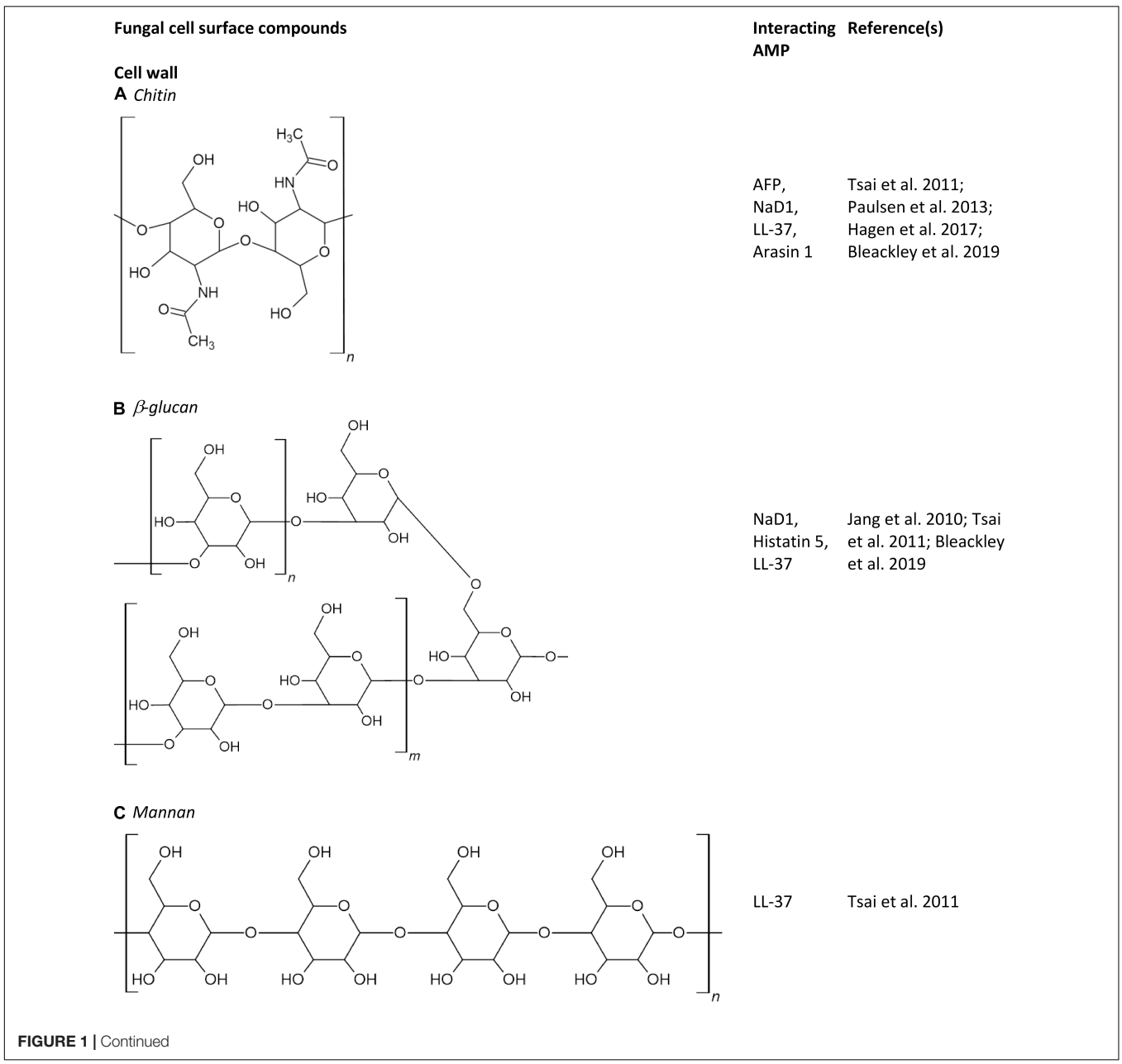


FIGURE 1 | Continued

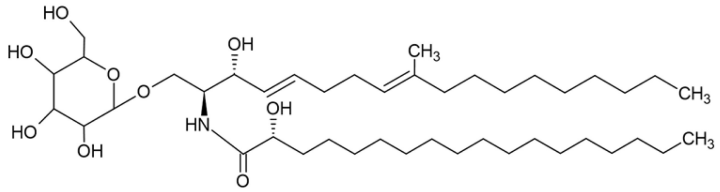
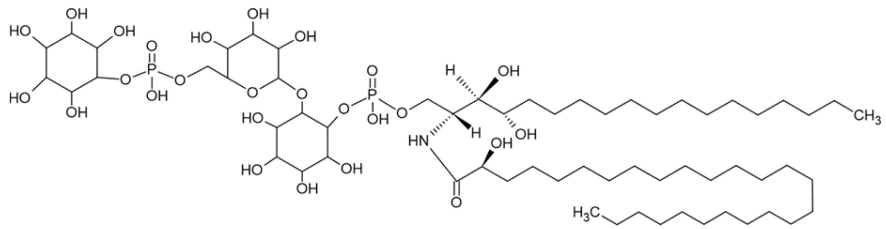
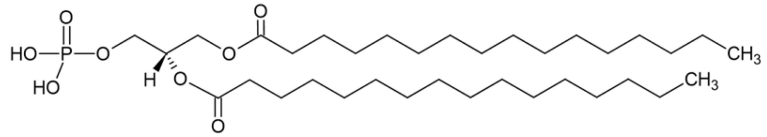
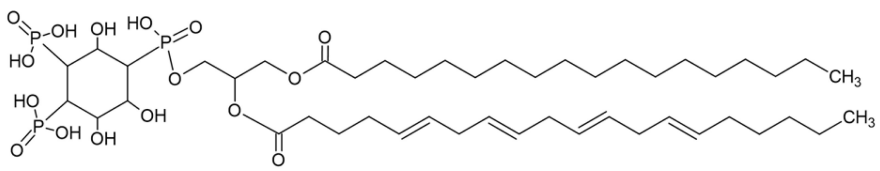
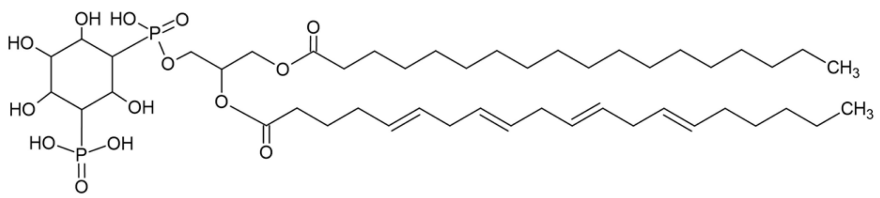
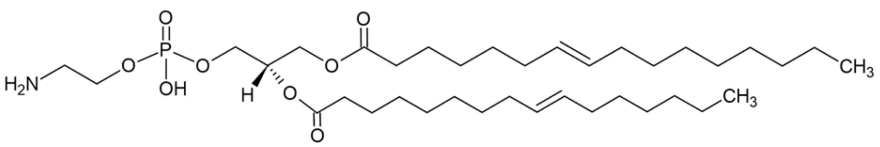
Fungal cell surface compounds	Interacting AMP	Reference(s)
Cell membrane		
A *Glucosylceramide (GlcCer)* 	MsDef1, Psd1, Psd2, PvD1, Sd5, RsAFP2, Heliomicin, PAF	Thevissen et al. 2000b; Thevissen et al. 2004; Ramamoorthy et al. 2007; De Medeiros et al. 2010; De Paula et al. 2008, 2011; De Medeiros et al. 2014; Mello et al. 2014; Muñoz et al. 2014; Amaral et al. 2019; Huber et al. 2019a
B *Mannosyldiinositolphosphorylceramide ($M(IP)_2C$)* 	DmAMP1, AtPDF2.3	Thevissen et al. 2000b; Vriens 2015
C *Phosphatidic acid (PA)* 	HsAFP1, MtDef4, NaD1, NaD2, NsD7	Sagaram et al. 2013; Kvansakul et al. 2016; Payne et al. 2016; Cools et al. 2017b; Järvå et al. 2018a
D *Phosphatidylinositol 4,5 bisphosphate ($PI(4,5)P_2$)* 	NaD1, TPP3, hBD-2, hBD-3	Baxter et al. 2015; Phan et al. 2016; Järvå et al. 2017; Järvå et al. 2018b
E *Phosphatidylinositol 3 phosphate (PI3P)* 	OefDef1.1, OsAFP1	Li et al. 2019; Ochiai et al. 2020
F *Phosphatidylethanolamine (PE)* 	cyO2	Burman et al. 2011

FIGURE 1 | (A–F) Fungal cell surface compounds known to play an important role in the antifungal mode of action of AMPs.

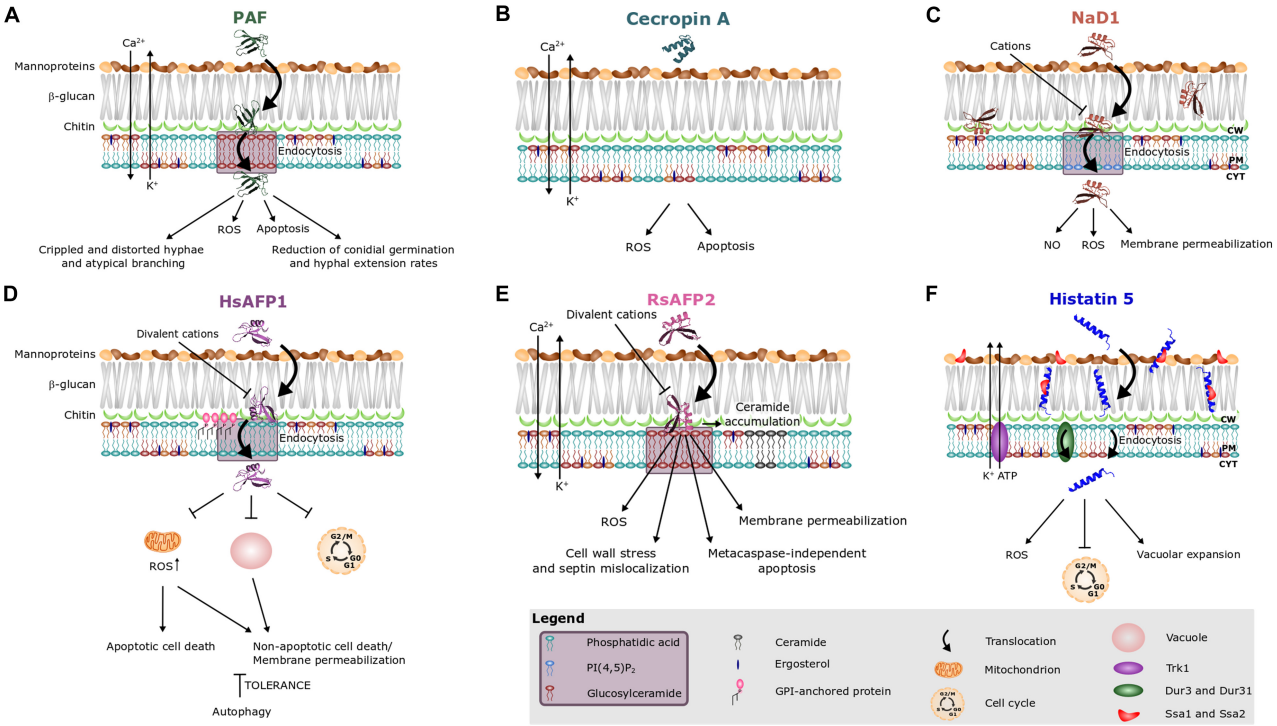


FIGURE 2 | Antifungal mode of action of **(A)** the *Penicillium chrysogenum* antifungal protein PAF and various peptides, i.e., **(B)** cecropin A, **(C)** NaD1, **(D)** HsAFP1, **(E)** RsAFP2, and **(F)** histatin 5. Different peptides are known to interact with different sphingo- and phospholipids, as indicated on the images and in the purple box in the legend. Note that treatment of fungal cells with rather high peptide concentrations can result in aspecific membrane permeabilization (Thevissen et al., 1997, 1999). This phenomenon is not taken into account in this figure.

$PI(4,5)P_2$ as evaluated using lipid overlays, liposome pulldown assays and structure determination in the case of hBD-2, and using lipid overlays and lipid vesicles in case of hBD-3 (Phan et al., 2016; Järvå et al., 2018b). HBD-2 dimers specifically interact with two distinct PIP_2-binding sites to permeabilize fungal cell membranes (Järvå et al., 2018b). Note that, $PI(4,5)P_2$ is localized in the inner leaflet of the cell membrane (Borges-Araújo and Fernandes, 2020), therefore, AMPs will first target other cell wall/cell membrane components. In contrast, glucose-containing glucosylceramide (GlcCer) is also found at the fungal cell wall (see section "Interaction of AMPs With the Fungal Cell Wall") (Rodrigues et al., 2000). The olive tree defensin OefDef1.1 and the rice defensin OsAFP1 are known to interact with both PI3P and PI5P, and PI3P, respectively, as validated using lipid overlays (**Figure 1**; Li et al., 2019; Ochiai et al., 2020). The cycloviolacin O2 (cyO2) is known to selectively target and disrupt membranes containing PE, as evaluated using lipid vesicles (**Figure 1**; Burman et al., 2011).

Apart from AMPs that interact with phospholipids, several AMPs have been identified that interact with sphingolipids. Sphingolipids, together with sterols, typically form membrane rafts, so-called lipid rafts, that are enriched in specific domains in fungal plasma membranes (Hurst and Fratti, 2020). Sphingolipids consist of a ceramide backbone linked to a polar head group. Based on this polar head group, they are classified in two groups, being phosphosphingolipids and glycosphingolipids. The phosphosphingolipid mannosyldiinositolphosphorylceramide $[M(IP)_2C]$ is the major sphingolipid in *Saccharomyces cerevisiae* (Dickson et al., 1997), and plays an important role in the antifungal mechanism of action of DmAMP1 and AtPDF2.3 (documented via an ELISA assay and/or specific yeast deletion mutants) (**Figure 1**; Thevissen et al., 2000a; Vriens, 2015). GlcCer is the most common glycosphingolipid in fungi, except for *S. cerevisiae* and *Candida glabrata*, since they do not produce GlcCer (Saito et al., 2006; Tavares et al., 2008). So far, GlcCer was identified to be of importance in the antifungal mode of action of numerous AMPs, such as the plant defensins MsDef1, Psd1, Psd2, PvD1, Sd5, and RsAFP2, the insect defensin-like peptide heliomicin, the *Aspergillus giganteus* peptide AFP and the *Penicillium chrysogenum* antifungal protein PAF (**Figures 1**, **2A,E**; Thevissen et al., 2004; De Paula et al., 2008, 2011; De Medeiros et al., 2014; Mello et al., 2014; Muñoz et al., 2014; Amaral et al., 2019; Huber et al., 2019). The involvement of GlcCer in the mode of action of these AMPs was established via lipid vesicles (e.g., Psd1, Psd2, Sd5, RsAFP2, and PAF), ELISA assays (RsAFP2 and heliomicin) and deletion mutants (e.g., AFP, MsDef1, Psd1, PvD1, RsAFP2, heliomicin, and PAF) (Thevissen et al., 2000a, 2004; Ramamoorthy et al., 2007; De Paula et al., 2008, 2011; De Medeiros et al., 2010, 2014; Mello et al., 2014; Muñoz et al.,

2014; Vriens, 2015; Amaral et al., 2019; Huber et al., 2019). Often multiple biochemical tests are used to confirm the relevance of these potential interactions. Note that RsAFP2 interacts with GlcCers isolated from *Pichia pastoris* but not with those of soybean nor human GlcCers, whereas heliomicin interacts with GlcCers isolated from both *P. pastoris* and soybean but not with human GlcCers. Moreover, RsAFP2 and heliomicin were suggested to interact with different motifs of fungal GlcCer, as the interaction of RsAFP2 with fungal GlcCer could not be competed for by heliomicin and vice versa (Thevissen et al., 2004). Interestingly, although most sphingolipids are non-essential for cell survival, they are key regulators of pathogenicity in a variety of fungi, such as *C. albicans*, which makes them essential for *in vivo* infections (Noble et al., 2010). Therefore, the incidence of *in vivo* resistance development against sphingolipid-targeting AMPs is expected to be minimal as resistant mutants with defective sphingolipid biosynthesis are not virulent (Del Poeta et al., 2014).

Antimicrobial proteins can also interact with plasma membrane proteins. Human lactoferrin targets the plasma membrane Pma1p H^+-ATPase, thereby perturbating cation homeostasis (cfr. supra) (Andrés et al., 2016, 2019).

Interaction of AMPs With the Fungal Cell Wall

Apart from the interaction of AMPs with the fungal cell membrane, AMPs can also interact with the fungal cell wall which is mainly composed of glucans, chitin and glycosylated proteins (Garcia-Rubio et al., 2020). Note that there are differences in cell wall composition between fungal species, e.g., galactomannan makes up a major part of the *Aspergillus* cell wall. Consequently, its presence is currently used as a diagnostic test for invasive aspergillosis (Mercier et al., 2018). GlcCer is also found at the fungal cell wall (Rodrigues et al., 2000), explaining the interaction of RsAFP2 with the fungal cell wall (Thevissen et al., 2012). Moreover, AMPs can also interact with other cell wall specific compounds. Recently, the interaction of both chitin and β-glucan with the tobacco plant defensin NaD1 was described (**Figures 1**, **2C**). Herein, the yeast cell wall protects target cells against the antifungal activity of NaD1, as the killing of spheroplasts occurred at lower concentrations as compared to cells with intact walls (Bleackley et al., 2019). Note that AMPs can have multiple fungal targets, as NaD1 also interacts with PA and PIP_2 (**Figure 2C**) (cfr. infra). The salivary peptide histatin 5 interacts with β-glucan, as well as with the heat shock proteins Ssa1p and Ssa2p that are present in the cell wall and cell membrane of *C. albicans* cells (**Figures 1**, **2F**; Li et al., 2003; Jang et al., 2010). Binding with the latter is required for the fungicidal activity of histatin 5 (Li et al., 2003). Similarly, the human β-defensins hBD-2 and hBD-3 bind Ssa1p and Ssa2p as part of their antifungal mode of action (Vylkova et al., 2006). The human neutrophil defensin 1 (HNP-1) competes with histatin 5 for Ssa1p and Ssa2p binding sites, however, using deletion mutants, it could be demonstrated that HNP-1 does not require Ssa1p or Ssa2p for its fungicidal activity (Edgerton et al., 2000; Vylkova et al., 2006). The human cathelicidin LL-37 preferentially interacts with mannan, but also with chitin or glucan (**Figure 1**; Tsai et al., 2011). Likewise, the proline-rich antimicrobial peptide arasin 1 from spider crab also binds chitin (**Figure 1**). However, this chitin-binding property may also arise from its involvement in host wound healing (Paulsen et al., 2013). Also AFP binds chitin as validated *in vitro* (**Figure 1**; Hagen et al., 2007).

Influence of Cations on the Initial Association of AMPs With the Fungal Cell Surface

The initial association of cationic AMPs with anionic bilayers is mostly directed by electrostatic interactions due to their net charge being opposed to that of the target membrane (von Deuster and Knecht, 2011; Alvares et al., 2017). Therefore, the presence of cations can significantly reduce the antifungal activity of AMPs, as demonstrated in case of e.g., AFP, coprisin (dung beetle), DmAMP1, HNP-1, HsAFP1, indolicidin (cattle), NaD1, OefDef1.1, P113 (a 12-amino-acid fragment of histatin 5), RsAFP2, and hBD-1,-2 (**Figures 2C–E**; Osborn et al., 1995; Goldman et al., 1997; Bals et al., 1998; Edgerton et al., 2000; Lee et al., 2003, 2012, 2014; Theis et al., 2003; Bleackley et al., 2019; Li et al., 2019; Cheng et al., 2020). Similarly, in yeast mutants lacking Agp2p, a plasma membrane regulator of polyamine and carnitine transport, the antifungal activity of NaD1 is significantly reduced. Deletion of AGP2 was suggested to result in an accumulation of cationic molecules at the cell surface, thereby repelling cationic AMPs (Bleackley et al., 2014). Since the hydrolysis of the cell wall by zymolyase treatment restored the activity of NaD1 in high salt conditions (100 mM NaCl), the loss of antifungal activity at elevated salt concentrations was recently attributed to the sequestration of cations by fungal cell wall polysaccharides (Bleackley et al., 2019). Note that Bleackley et al. (2019) only evaluated the effect of monovalent cations, being Na^+, thereby reporting the plant defensin DmAMP1 to be salt tolerant. In contrast, Osborn et al. (1995) earlier reported that the antifungal activity of DmAMP1 against both *N. crassa* and *Fusarium culmorum* is significantly reduced in the presence of both 1 and 5 mM Ca^{2+} or Mg^{2+}. Hence, it remains unclear if divalent cations are also sequestrated by fungal cell wall polysaccharides. In case of the plant defensin HsAFP1, the presence of divalent cations specifically reduced the uptake of the peptide, thereby inhibiting its antifungal activity (**Figure 2D**; Struyfs et al., 2020). The salt-sensitivity of most peptides (at least *in vitro*) is probably one of the main factors why only few AMPs have been further developed into a commercial antifungal or antibacterial product. However, there are few reports on the efficacy of AMPs upon systemic administration, thus acting in biological cation-containing fluids like blood, in preclinical models (see conclusion section of this review). For example, the plant defensin RsAFP2 demonstrated *in vivo* efficacy in a prophylactic murine model of candidiasis upon intravenous administration (Tavares et al., 2008). Only a limited number of AMPs are salt tolerant of which most originate from marine organisms (Lee et al., 1997; Fedders et al., 2008). Also the antifungal activity of dermcidin, expressed in sweat glands, is maintained at high salt concentrations that resemble human sweat conditions. Since dermcidin is negatively charged at physiological pH, electrostatic interactions with membrane lipids might not be of importance for its antifungal activity

(Schittek et al., 2001). Exceptionally, the presence of divalent cations can also enhance the antifungal activity of AMPs. Histatin 5 binds multiple cations, including Zn^{2+} which induces dimerization of the peptide. Zn^{2+} that is abundantly present in saliva, significantly increases killing activity of histatin 5 in both *C. albicans* and *C. glabrata* (Norris et al., 2020).

Downstream Pathways Involved in the Antifungal Mode of Action of AMPs

Potential AMP Uptake

Previously, it was believed that the mode of action of AMPs solely consisted of simple pore formation that resulted in rapid killing. However, antifungal AMPs can have a more complex mechanism of action that includes signaling pathways and intracellular targets. Hitherto, the mechanism of action of only a limited number of AMPs has been described. Amongst them are AMPs that (i) are internalized upon their initial interaction with the fungal cell, without causing fungal membrane disruption (e.g., histatin 5, HsAFP1, MtDef4, NaD1, *Neosartorya fischeri* antifungal protein NFAP, OefDef1.1, Psd1 and PAF) (**Figures 2A,D,F**) or (ii) remain outside the cell (e.g., RsAFP2) (**Figure 2E**; Oberparleiter et al., 2003; Kumar et al., 2011; Thevissen et al., 2012; El-Mounadi et al., 2016; Cools et al., 2017b; Hajdu et al., 2019; Li et al., 2019). Note that internalization is not essential for inducing intracellular mechanisms, as RsAFP2 is not internalized, but does induce e.g., production of ROS and programmed cell death (Aerts et al., 2007; Thevissen et al., 2012). The internalization process of AMPs can be species-specific. In *Fusarium oxysporum* and *Fusarium virguliforme*, the translocation of OefDef1.1 to the cytoplasm occurs in both germlings and conidia. However, in *Botrytis cinerea*, uptake only occurs in germlings (Li et al., 2019). In *N. crassa*, the internalization of MtDef4 depends on the presence of phospholipase D, whereas this is not the case in *Fusarium graminearum* (Sagaram et al., 2011; El-Mounadi et al., 2016). The uptake of AMPs is often an active, energy requiring process, pointing to endocytosis as in case of HsAFP1, histatin 5, MtDef4, NaD1, NFAP, and PAF (**Figures 2A,C,D,F**; Oberparleiter et al., 2003; Jang et al., 2010; Kumar et al., 2011; Puri and Edgerton, 2014; El-Mounadi et al., 2016; Cools et al., 2017b; Hayes et al., 2018; Hajdu et al., 2019). Indeed, in *N. crassa*, internalization of MtDef4 is energy dependent and requires endocytosis, while in *F. graminearum*, uptake is only partially energy dependent (El-Mounadi et al., 2016). Internalization of histatin 5 can occur via three different pathways (**Figure 2F**) being (i) endocytosis, (ii) transportation into the fungal cell via the polyamine transporters Dur3p and Dur31p in an energy-dependent process, and (iii) membrane lytic effects if the histatin concentration, estimated 15–30 μM in saliva, exceeds 30 μM (Kumar et al., 2011; Puri and Edgerton, 2014). Deletion of the GPI-anchor remodeling enzyme *BST1* results in increased resistance of *S. cerevisiae* cells to HsAFP1 treatment due to a compromised HsAFP1 internalization pathway, pointing to an important role for GPI-anchor remodeling enzymes in HsAFP1's internalization (**Figure 2D**; Struyfs et al., 2020).

Production of Endogenous ROS and the Induction of Programmed Cell Death

Similar to certain antimycotics (e.g., amphotericin B), numerous AMPs, such as the plant defensin ApDef1, cecropin A, coprisin, histatin 5, HsAFP1, human lactoferrin, the plant defensin LpDef1, NaD1, OefDef1.1, the honeybee venom peptide melittin, PAF, the *Penicillium chrysogenum* antifungal protein C (PAFC), PvD1, RsAFP2 and the centipede peptide scolopendin induce the production of ROS (**Figure 2**; Leiter et al., 2005; Andrés et al., 2008; Park and Lee, 2010; Aerts et al., 2011; Mello et al., 2011; Lee et al., 2012, 2016; Galgóczy et al., 2013; Choi et al., 2014; Yun and Lee, 2016; Soares et al., 2017; Vriens et al., 2017; Holzknecht et al., 2020). Excessive cellular levels of ROS can cause oxidative damage to proteins, nucleic acids, lipids, membranes and organelles, which in turn can lead to activation of cell death processes such as programmed cell death (Redza-Dutordoir and Averill-Bates, 2016). Programmed cell death is a tightly regulated and highly conserved process of cell death in which plasma membrane integrity is initially preserved and intracellular content is degraded in a programmed manner. Numerous AMPs are known to induce programmed cell death, such as ApDef1, cecropin A, coprisin, hBD-2, HsAFP1, human lactoferrin, melittin, NPAF, OsAFP1, PAF, RsAFP2, and scolopendin (**Figures 2A,B,D,E**; Leiter et al., 2005; Andrés et al., 2008; Yount et al., 2009; Park and Lee, 2010; Aerts et al., 2011; Lee et al., 2012, 2016; Thevissen et al., 2012; Galgóczy et al., 2013; Yun and Lee, 2016; Soares et al., 2017; Ochiai et al., 2018). In *C. albicans*, RsAFP2 treatment results in cell wall stress, septin mislocalization and increased ceramide levels, the latter having been previously demonstrated to result in programmed cell death (Thevissen et al., 2012; Rego et al., 2014). Indeed, RsAFP2 induces programmed cell death in a metacaspase-independent (Mca1) manner (Aerts et al., 2007). Nevertheless, the exact mechanism of programmed cell death induction is hitherto unclear. In contrast, ApDef1 induces chromatin condensation, resulting in cell death via metacaspase-dependent programmed cell death (Soares et al., 2017). Similarly, metacaspase-dependent programmed cell death is induced upon treatment with coprisin, preceded by the exposure of PS on the outer leaflet of the plasma membrane, dysfunctional mitochondrial transmembrane potential ($\Delta\psi m$) and cytochrome C release (Lee et al., 2012). Human lactoferrin induces metacaspase-dependent programmed cell death in *C. albicans*, as lactoferrin-treated cells exhibited PS exposure, chromatin condensation and DNA degradation (Andrés et al., 2008; Acosta-Zaldívar et al., 2016). Also scolopendin treatment induces programmed cell death via metacaspase activation, as evidenced by PS externalization, cytochrome C release, de-energization, chromatin condensation and DNA fragmentation (Lee et al., 2016). Cecropin A triggers programmed cell death characterized by PS externalization, cytochrome C release, dissipation of $\Delta\psi m$ and DNA fragmentation (Yun and Lee, 2016). Upon treatment with hBD-2, apoptotic cell death follows PS accessibility in

C. albicans (Yount et al., 2009). Likewise, melittin induces programmed cell death in *C. albicans*, as evidenced by PS externalization, and DNA and nuclear fragmentation (Park and Lee, 2010). Note that, spinigerin induces programmed cell death in a caspase independent manner in *Leishmania donovan*. So far, it remains unclear if this also occurs in fungal cells (Sardar et al., 2013).

Mitochondrial Dysfunction

Moreover, AMP treatment can also result in mitochondrial dysfunction, as demonstrated for coprisin, ETD151 (analog of the antifungal insect defensin heliomicin), HsAFP1, human lactoferrin, LpDef1 and scolopendin (**Figure 2D**; Aerts et al., 2011; Lee et al., 2012, 2016; Puri and Edgerton, 2014; Vieira et al., 2015; Acosta-Zaldívar et al., 2016; Aumer et al., 2020). ETD151 does not interact directly with complexes I and III of the mitochondrial respiratory chain. Therefore, it was suggested that mitochondrial dysfunction results from mitochondrial membrane perturbation (Aumer et al., 2020). Co-incubation of HsAFP1 and sodium azide, which blocks the respiratory electron transport chain at complex IV, antagonized the antifungal activity of HsAFP1. This points to the indispensability of a functional respiratory chain for HsAFP1 antifungal action (Aerts et al., 2011). Likewise, treatment of *C. albicans* cells with sodium azide also results in a decreased susceptibility to coprisin, hBD-2, hBD-3, and histatin 5 (Helmerhorst et al., 1999; Vylkova et al., 2007; Lee et al., 2012). *S. cerevisiae* rho^0 deletion mutants, lacking the mitochondrial genome, are more resistant to NaD1. Hence, mitochondrial respiratory activity is required for the antifungal activity of NaD1 (Hayes et al., 2013).

ATP Efflux

Another feature of the mechanism of action of AMPs is the release of ATP from target cells, as in the case of histatin 5, LL-37, hBD-2, and hBD-3 (**Figure 2F**; Koshlukova et al., 1999; Den Hertog et al., 2005; Vylkova et al., 2007). In *C. albicans*, histatin 5 causes a drastic reduction of intracellular ATP content, which results from the efflux of ATP (Koshlukova et al., 1999). Similarly, killing by both hBD-2 and hBD-3 involves ATP release. The magnitude and profile of ATP efflux are most similar between histatin 5 and hBD-2, as opposed to hBD-3 in which the maximal ATP release at 60 min peptide treatment only reached 50% of the ATP efflux level caused by histatin 5 (Vylkova et al., 2007). Notably, no gross membrane disruption occurs upon ATP efflux when tested with these AMPs, except in case of LL-37.

Cell Cycle Impairment

Antimicrobial peptides can also affect cell cycle progression, which is the case for ApDef1, histatin 5, HsAFP1 and Psd1 (**Figures 2D,F**; Baev et al., 2002; Lobo et al., 2007; Soares et al., 2017; Struyfs et al., 2020). Upon HsAFP1 treatment of *S. cerevisiae* cells, cell cycle impairment was observed. Nonetheless, this impairment was also present upon treatment with the inactive HsAFP1 mutant, HsAFP1[H32A][R52A] (Cools et al., 2017b), when tested at equimolar concentrations. Hence, these findings indicate that HsAFP1-induced cell cycle impairment is not linked to its antifungal activity (Struyfs et al., 2020). Therefore, cell cycle impairment was considered as a non-specific secondary effect rather than the primary cause of HsAFP1's killing activity.

Disruption of Cation Homeostasis

Furthermore, AMPs can also abolish cation homeostasis in target cells, herein mostly intracellular levels of Ca^{2+} and K^+ ions are disturbed. Ca^{2+} plays an important role in signal transduction pathways, where it acts as a second messenger e.g., in programmed cell death, and is mainly stored in the endoplasmic reticulum (ER) as well as in mitochondria (Kinjo and Schnetkamp, 2005). In contrast, K^+ is the major cytoplasmic cation, thereby mainly controlling the ionic strength of the cytoplasm, and its depletion is critical for progress on the apoptotic pathway (Baev et al., 2004). DmAMP1, RsAFP2, PAF, and cecropin A induce both K^+ efflux and Ca^{2+} influx upon interaction with fungal target cells (**Figures 2A,B,E**; Thevissen et al., 1996, 1999; Kaiserer et al., 2003; Binder et al., 2010, 2015; Yun and Lee, 2016), whereas scolopendin, MsDef1 and MtDef4 induce Ca^{2+} influx and human lactoferrin and histatin 5 induce K^+ efflux (**Figure 2F**; Baev et al., 2004; Andrés et al., 2008; Muñoz et al., 2014; Lee et al., 2017). Interestingly, the potassium transporter Trk1 is required for the antifungal mode of action of histatin 5, as it was suggested to provide the essential pathway for ATP loss (Baev et al., 2004).

Induction of Autophagy and Vacuolar Dysfunction

Recently, AMPs were also found to induce autophagy, which is a cytoprotective process in which intracellular material is digested in the vacuole (Carmona-Gutierrez et al., 2018). Notably, excessive levels of autophagy have been linked with cell death (Liu and Levine, 2015). Moderate HsAFP1 doses induce autophagy in *S. cerevisiae* cells, probably as a fungal tolerance/pro-survival mechanism against the HsAFP1 action (**Figure 2D**; Struyfs et al., 2020). Since functional vacuoles are indispensable for the induction of autophagy (Martínez-Muñoz and Kane, 2008; Sampaio-Marques et al., 2019), they might play a role in governing tolerance to HsAFP1. Indeed, at high HsAFP1 doses vacuoles are affected, as demonstrated by the increased vacuolar pH, possibly resulting in impaired autophagy, and therefore in effective killing of *S. cerevisiae* cells. After NbD6 treatment the vacuoles of both *S. cerevisiae* and *F. graminearum* cells fused into one large vacuole, while the peptide remained on the cell surface. Though such large vacuole resembles those that form in an autophagic response, a possible link between both must be validated (Parisi et al., 2019a). In contrast, after SBI6 treatment, cells contained multiple fragmented vacuoles, pointing to vacuolar disruption (Parisi et al., 2019a). While functional vacuoles reportedly seem to govern tolerance to HsAFP1, deletions in vacuolar genes result in increased resistance to NbD6 and SBI6 (Aerts et al., 2011; Parisi et al., 2019a). These findings indicate that these peptides do not impair vacuolar function, but that their antifungal effect rather depends on a properly functioning vacuole (Parisi et al., 2019a).

Potential Link Between the Antifungal Mode of Action of AMPs and MCSs

In eukaryotic cells, incompatible biochemical processes are spatially separated by intracellular compartmentalization in organelles. Most organelles are physically connected via MCSs (Kohler et al., 2020). Notably, at MCSs, organelle membranes are closely apposed and tethered, however, not fused (Scorrano et al., 2019). MCSs play a critical role in inter-organelle communication that is essential in maintaining cellular homeostasis, including lipid synthesis and trafficking, and intracellular signaling (e.g., Ca^{2+}) (Scorrano et al., 2019). Although there is currently no evidence of MCS involvement in the mode of action of AMPs, it could be linked with their antifungal activity. In particular, MCSs play an important role in the regulation of autophagy, as the MCSs between the phagophore and the ER allow the direct transfer of lipids that are required for membrane extension, and thus the progression of autophagy (Molino et al., 2017; Kohler et al., 2020). Moreover, the endosomal protein Vps13p transiently localizes to nucleus-vacuole junctions in yeast (Lang et al., 2015). Interestingly, the *S. cerevisiae* Δ*vps13* deletion mutant is 4-fold more resistant to HsAFP1 (Aerts et al., 2011), pointing to a potential link between MCSs and HsAFP1 functioning. Furthermore, MCSs are involved in Ca^{2+} homeostasis at the ER-mitochondria interface (Phillips and Voeltz, 2016), and disruption of cation homeostasis is part of the antifungal activity of numerous AMPs. Hence, MCSs could be involved in the antifungal mode of action of AMPs. This hypothetical link should be further investigated by different techniques, such as super-resolution microscopy, transmission electron microscopy, FRET-based reporters or proximity ligation assays, in the presence and absence of AMPs to evaluate their potential effect on MCSs (Nascimbeni et al., 2017; Scorrano et al., 2019).

CONCLUSION

Antimicrobial peptides are part of the innate immune system of numerous organisms and are known for their broad-spectrum activity. Their structure is highly diverse and grouped according to their conserved 3D structure. In the past, the mode of action of AMPs was generally considered as nonspecific, resulting in rapid physical disruption of microbial cell membranes causing cell leakage and death. However, AMPs can also exert a more complex mechanism of action. Many AMPs specifically interact with the fungal cell wall and/or cell membrane, with various sphingolipids and phospholipids identified as AMP targets. Upon target interaction, peptides can be internalized or can remain at the outside of the fungal cell. Regardless of potential uptake, antifungal peptides can affect intracellular targets, thereby resulting in a multitude of actions such as ROS production, programmed cell death, mitochondrial dysfunction, disruption of cation homeostasis, ATP efflux, cell cycle impairment, autophagy, and vacuolar dysfunction. Moreover, MCSs might be linked with the mode of action of antifungal AMPs.

Elucidating the mode of action of AMPs is a crucial step to exploit their application potential. Hitherto, the activity of certain AMPs is demonstrated *in vivo* as exemplified by RsAFP2's activity in a prophylactic murine model of candidiasis and by the histatin 5 derivative P113's activity against oral candidiasis that completed clinical phase IIb (Tavares et al., 2008; Brunetti et al., 2016; Håkansson et al., 2019; Mookherjee et al., 2020). Inactivation of AMPs by cations upon systemic administration could be limiting for commercial applications, however, alternative administration routes, such as topical administration, in which the inactivation of AMPs by cations is of less importance, could be of interest. For example, ropocamptide, developed by ProMore Pharma, Sweden, is based on LL-37 [known to be inactivated by 100 mM NaCl (Turner et al., 1998)] and has recently passed clinical phase IIb study in patients with venous leg ulcers to improve chronic would healing (Thapa et al., 2020; Promore Pharma, 2021). As compared to small molecules, peptides mostly have a greater efficacy, selectivity and specificity (Hummel et al., 2006; McGregor, 2008, Henninot et al., 2018). Nonetheless, they are also more prone to degradation as compared to small molecule-based drugs. To overcome this limitation, non-natural amino acids and/or cyclization can be introduced to enhance peptide stability (Ding et al., 2020). Numerous AMP-based drugs are currently evaluated in clinical trials [as reviewed in Mookherjee et al. (2020)] for a variety of potential applications, such as antimicrobial therapies and immunomodulatory therapies. Apart from directly exploiting antifungal peptides and their derivatives, insight in the mechanism of action of AMPs can also be used to facilitate the efficient design and development of novel AMP-like antifungal compounds with high selectivity. This can be exemplified by fungal GlcCer-specific camelid single domain antibodies, which were designed to mimic RsAFP2's interaction with GlcCer and hence, are active against a broad spectrum of plant pathogenic fungi (De Coninck et al., 2017). This points to the potential of AMPs in a myriad of novel applications.

AUTHOR CONTRIBUTIONS

KT coordinated the review of the manuscript. BC revised the review of the manuscript. CS contributed to writing of the manuscript, redaction, and revisions. All authors contributed to the article and approved the submitted version.

FUNDING

This work was received funding from Research Foundation Flanders (FWO) (SB fellowship 1S38618N, Research projects G080016N and G0C6418N). KT acknowledges receipt of a mandate of IOF, KU Leuven (IOFm/05/022).

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fcell.2021.649875/full#supplementary-material

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Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.