Nicotiana alata defensin chimeras reveal differences in the mechanism of fungal and tumour cell killing and an enhanced antifungal variant

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Abstract

The plant defensin NaD1 is a potent antifungal molecule that also targets tumour cells with high efficiency. We examined the features of NaD1 that contribute to these two activities by producing a series of chimeras with NaD2, a defensin that has relatively poor activity on fungiand no activity on tumour cells. All plant defensins have a common tertiary structure known as a cysteine stabilized α-β motif which consists of an α helix and a triple-stranded β-sheet stabilized by four disulphide bonds. The chimeras were produced by replacing loops 1-7, the sequences between each of the conserved cysteine residues on NaD1, with the corresponding loops from NaD2. The loop 5 swap replaced the sequence motif (SKILRR) that mediates tight binding with phosphatidylinositol 4,5 bisphosphosphate ($PI(4,5)P_2$) and is essential for the potent cytotoxic effect of NaD1 on tumour cells. Consistent with previous reports, there was a strong correlation between $PI(4,5)P_2$ binding and the tumour cell killing activity of all of the chimeras. However, this correlation did not extend to antifungal activity. Some of the loop swap chimeras were efficient antifungal molecules even though they bound poorly to $PI(4,5)P_2$ suggesting additional mechanisms operate against fungal cells. Unexpectedly the L1B loop swap chimera was ten times more active than NaD1 on filamentous fungi. This led to the conclusion that defensin loops have evolved as modular components that combine to make antifungal molecules with variable mechanisms of action and that artificial combinations of loops can increase antifungal activity compared to natural variants.

Introduction

Innate immunity peptides have evolved in plants to protect against the damaging effects of microbial pathogens, particularly fungi [1]. Fungi cause both persistent annual crop losses as well as

devastating epidemics [2] that are a serious threat to global food security [3]. Plants lack the adaptive immune system of mammals and instead rely on a suite of antifungal peptides to ward off infection [1]. Defensins are a major family of plant antifungal peptides [4, 5] and some members have been studied extensively because of their potential use in transgenic plants for protection against fungal disease [6-8].

Plant defensins are small peptides (45-54 amino acids) that are basic and cysteine rich. They have a conserved three dimensional structure composed of three β-strands linked to an α helix by three disulfide bonds together with a fourth disulfide bond that links the N- and C-terminal regions and creates a pseudo cyclic structure [9]. Beyond the eight cysteine residues and a conserved glycine that is required for the structural fold, there is a great deal of sequence variability across the plant defensin family. This variability is displayed in seven loops (L1-L7) that correspond to the regions between cysteine residues (Figure 1) [5]. The sequence diversity explains the wide range of functions that have been reported for plant defensins including antibacterial and antifungal activities as well as roles in plant development, sexual reproduction and metal tolerance (reviewed in [5]). Some antifungal defensins display activity against tumour cells but not healthy mammalian cells [10, 11]. However, it remains to be established if the mechanism of action on fungal and mammalian tumour cells is the same.

Fungal cell killing by the defensin NaD1 from the ornamental tobacco *Nicotiana alata* occurs through a mechanism that involves specific interaction with the fungal cell wall before it passes through the plasma membrane and enters the fungal cytoplasm. Once inside the cell, NaD1 rapidly initiates the production of reactive oxygen species, permeabilization of the fungal plasma membrane and cell death [12-14]. The activity against tumour cell lines is mediated by a specific and high affinity interaction with phosphatidylinositol 4,5 bisphosphate ($PI(4,5)P_2$). This lipid binding specificity has only been described for the class II defensins from the Solanaceae among plant defensins and is largely mediated through the sequence in loop 5 [10, 11, 15]. Class II defensins have a C-terminal pro-peptide that directs them to the vacuole whereas class I defensins lack this sequence and are secreted by default from the plant cell [9]. Interestingly even though human and plant defensins have evolved separately [16] a related sequence in loop 5 of human β-defensin 3 [17] is required for $PI(4,5)P_2$ binding and tumour cell killing. The defensins NaD1 and TPP3 from tomatoes both dimerize and form the “cationic grip” which binds two $PI(4,5)P_2$ molecules. The defensin:$PI(4,5)P_2$ dimer then forms oligomers via a different interface [10]. The interaction of NaD1 and TPP3 with $PI(4,5)P_2$ is proposed to lead to the characteristic blebbing and membrane permeabilization that occurs when tumour cells are treated with these defensins [10, 11]. Indeed, masking of $PI(4,5)P_2$ by intracellular expression of a pleckstrin homology domain, or treatment with neomycin, delayed tumour cell killing by NaD1 supporting the role for $PI(4,5)P_2$ binding in the activity against tumour cells [10, 11].

Mutations in residues that participate in PI(4,5)P_2 binding reduced both tumour and fungal cell killing by these defensins [10, 11].

NaD2 is another defensin produced by *N. alata* that has weaker antifungal activity than NaD1 against filamentous fungi and rusts [18]. NaD2 is not a member of the Solanaceous Class II defensin family but is a member of the much larger family of Class I defensins which are produced by all plant families. Here we report that unlike NaD1, NaD2 has no activity against tumour cells. We investigated the role of various sequence components in the lipid binding, tumour cell killing and antifungal activities of NaD1 by swapping the loop regions of NaD1 with corresponding loops from NaD2. These studies revealed a strong correlation between PI(4,5)P_2 binding and tumour cell killing across all of the chimeric defensins. This correlation did not extend to the antifungal activities of the loop swap chimeras indicating that the role of PI(4,5)P_2 binding is not as crucial in fungal cell killing by these defensins.

Methods

Strains and vectors

Fusarium oxysporum vas infectum used in this study was Australian isolate from cotton; Farming Systems Institute, DPI, Queensland, Australia, a gift from Wayne O'Neill. *Aspergillus niger* (5181), *Aspergillus flavus* (5310) and *Aspergillus paraciticus* (4467) spores (gift from Associate Professor Dee Carter, University of Sydney) were grown on half-strength potato dextrose agar plates (PDA).

Candida albicans (DAY185) and *Cryptococcus neoformans* (H99) (gift from Associate Professor Dee Carter, University of Sydney) were grown on 1% yeast extract, 2% peptone, 2% dextrose (YPD) agar plates and cultured at 30˚C in YPD. *S. cerevisiae* experiments were all performed in a BY4741 (MATa *his3Δ0 leu2Δ0 met15Δ0 ura3Δ0*) or BY4743 (*MATa/α his3Δ1/his3Δ1 leu2Δ0/leu2Δ0 LYS2/lys2Δ0 met15Δ0/MET15 ura3Δ0/ura3Δ0*) background. Deletion strains were retrieved from the nonessential (*inp51Δ, inp52Δ, lsb6Δ)* or heterozygous essential (*STT4/stt4Δ, PIK1/pik1Δ, MSS4/mss4Δ)* knockout collection (ThermoScientific). Native NaD1 and NaD2 were purified from *Nicotiana alata* flowers as described in [12]. LL37 was purchased from Genscript (Hong Kong).

Cloning and expression of loop swap proteins

Loops in the NaD1 defensin were substituted with corresponding loops from NaD2 using Phusion mutagenesis (New England Biolabs) of an NaD1 construct in the pHUE expression vector [19]. Loops 1 and 4 were both split into two shorter regions yielding an A and B chimera for each loop. Loops 6 and 7 are both short and were thus combined when making the chimeric proteins. Proteins were

expressed in T7 *E. coli* (New England Biolabs) according to manufacturer's instructions and were purified using nickel affinity chromatography. The ubiquitin/his tag was removed using the ubiquitin specific protease 2 at a final concentration of 70 µg/mL followed by reverse nickel affinity chromatography. Proteins were purified further by reverse phase HPLC using a Zorbax C8 analytical column (Agilent). Correct folding of loop swap variants was confirmed by circular dichroism.

Circular dichroism

Stock solutions of each of the proteins were prepared in H_2O (~0.5–1.0 mg/mL) and diluted 1:4 in 10 mM sodium phosphate buffer, pH 6.8 prior to measurements. CD spectra were recorded on a Jasco J-810 spectropolarimeter from 185–260 nm at room temperature using quartz cuvettes with a path length of 1 cm (151 data points/scan, band width: 1.7 nm, response: 1 s, scan speed 100 nm/min). The buffer blank was subtracted and spectra were smoothed using the algorithm included in the Jasco data analysis software and plotted using Graphpad Prism. Fractional helicity (f_H) was calculated as described in [20].

Antifungal assays

Antifungal assays on *F. oxysporum, F. graminearum* and *C. graminicola* were performed as described in [12]. *F. oxysporum and F. graminearum* growth was assayed after 24 h, *C. graminicola* growth was assayed after 48 h. Assays on *C. albicans, C. neoformans* and *S. cerevisiae* were performed as described in [21]. IC_{50} values for *F. oxysporum* were identified from the growth inhibition curves. Confidence intervals (95%) were calculated and overlapping CIs were assigned the same letter and values with different letters are significantly different.

Tumour cell permeabilization assays

Permeabilization of cells from a human monocytic lymphoma cell line, U937, was monitored using a propidium iodide (PI) uptake assay as described in [10] with some modifications. Protein at 0, 2.5, 5, 10 or 20 µM was incubated with 4×10^4 cells in RPMI-1640 medium (Thermo) containing 0.1% BSA (Sigma-Aldrich) for 30 min at 37 °C, before the addition of PI (Sigma-Aldrich) to a final concentration of 1 µg/mL. Samples were placed on ice and analysed by flow cytometry using a FACSCanto II with FACSDiva software v6.1.1 (BD Biosciences). Data was analysed by FlowJo software (Tree star) by gating cells based on forward and side scatter. Cell permeabilization was defined by PI fluorescence. Statistical analysis was performed as described for antifungal assays.

Permeabilization of liposomes

Liposomes were prepared as outlined in [10]. Permeabilization of liposomes was assessed by monitoring release of entrapped calcein as described in [22] and [13]. Fluorescence of released

calcein was used as a measure of permeabilization and was calculated relative to the Triton X-100 (0.1%) positive control. Statistical analysis was performed as described for antifungal assays.

Fungal membrane permeabilization assays

Membrane permeabilization was monitored using SYTOX green as outlined in [13]. Assays on the permeabilization activity of loop swap variants on *F. oxysporum* hyphae were performed using test proteins at 10 µM.

Lipid binding

Binding of protein to a variety of membrane lipids was tested using PIPstrips™ (Batch numbers KE071312 and KE020212, Echelon biosciences, Salt Lake city, UT) as described in the manufacturer's protocol and [10]. Antibodies to NaD1 and NaD2 were polyclonals developed in rabbits.

Yeast survival assays

S. cerevisiae BY4741 were grown overnight at 30˚C in YPD. Aliquots of overnight culture (500µL) were treated with neomycin (10, 1, 0.1, 0 mM) for 3 hours. Cells were washed three times with half strength potato dextrose broth (½ PDB) (BD Difco) and then diluted in ½ PDB to an OD_{600}=0.2. Cells were treated with 20 and 10 µM NaD1, 30 and 15 µM LL37, 50 and 25 µM L5 and water for 1 h at 30˚C with shaking. To determine the survival of yeast cells in each treatment a five-fold dilution series of each treatment combination was plated on YPD Agar plates and incubated at 30˚C for 2 days. Images of plates were taken using a ChemiDoc Imager (BioRad) using the epi-White settings. Data was consistent across three independent biological experiments.

Results

Protein expression and folding

Eight NaD1-NaD2 loop swap proteins were produced recombinantly in *E. coli* using the pHUE expression system. These proteins consisted of an NaD1 backbone in which each of the loops of NaD1 was replaced with the corresponding loop from NaD2 (Figure 1). Purified recombinant protein was analysed by SDS-PAGE and MALDI-MS to ensure that the expressed proteins were of the expected size (data not shown). Two of the recombinant proteins, L2 and L3, did not express well and were not included in subsequent experiments. Loops 1 and 4 are the longer loops in plant defensins and as such were each split across two chimeric defensins denoted A and B. Loops 6 and 7 are the shortest loops in plant defensins (one and three amino acids respectively) and were thus combined into a single chimeric defensin. L1A expressed at very low levels and was omitted from some experiments. The CD spectra of recombinantly expressed NaD1 and NaD2 were similar to

NaD1 and NaD2 purified from plants, demonstrating that the recombinant expression system produced defensins with the correct fold (Figure 2A). Based on the mean residue ellipticity at 222 nm (Θ_{222}), the helical content (represented as the fractional helicity, f_H) for NaD1 and NaD2 is ~16% and ~6%, respectively (Figure 2C). The CD-estimated value for NaD1 is slightly lower than the actual helical content (~23%) in the NMR and X-ray structures, but consistent with it considering that the CD data provide broad estimates only. The CD spectra of the loop swap proteins were all similar to the NaD1 spectrum, with f_H values generally in the range of 16 - 18% (Figure 2B). The largest differences were for the CD spectra of L4A, with a helicity of 24%, and L6&7 (f_H = 12%). L4A locates to the C-terminal cap region of the single α-helix in the cysteine-stabilized αβ motif structure of NaD1, and hence changes in this region can be expected to directly affect helix propensity. Loop swaps in L6&7 are expected to affect the C-terminal β-strand. Despite the relatively conservative substitutions (CLCTKPC in NaD1 vs. CFCTRPC in L6&7), the helix propensity may have been impacted indirectly because the α-helix and C-terminal β-strand are tethered via two disulfide bonds (Figure 1). L4B had a deeper trough at 200nm indicating an increase in random coil secondary structure. Loop 4 B forms one of the β strands and modification of this sequence may have affected formation of the secondary structure.

Antifungal activity of NaD1-NaD2 loop swap chimeras against F. oxysporum

The antifungal activity of the native floral defensins and the recombinantly expressed defensins was assessed against *F. oxysporum*. NaD1 (IC_{50}: 1.5 ± 0.25 μM) was more active than NaD2 (IC_{50}: 8.3 ± 2.1 μM) (Figure 3A,D). The IC_{50}s of recombinant NaD1 and NaD2 did not differ significantly from the proteins isolated from plants demonstrating that recombinant expression had no effect on activity of these defensins (data not shown). Swapping the loop 4A or loop 4B sequences of NaD2 into the NaD1 backbone (L4A and L4B) had no effect on the antifungal activity of the defensin. However, replacement of loop 1A (L1A) and loop 5 (L5) of NaD1 with the corresponding NaD2 sequence decreased the activity of the chimeric defensins. Increased activity was observed when any of loop 1B (L1B) or loops 6 and 7 (L6&7) of NaD2 were inserted into the NaD1 framework in place of the native sequence. The loop 1B swap produced the most active variant with an IC_{50} of 0.2 ± 0.04 μM compared with 1.5 ± 0.3 μM for NaD1 and 8.3 ± 2.1 μM for NaD2. The least active variant was L1A with an IC_{50} of 3.2 ± 0.8 μM (Figure 3A,D).

Activity against a model tumour cell line

Tumour cell killing by native and recombinant defensins was determined by monitoring propidium iodide (PI) uptake into the model monocytic lymphoma cell line, U937. Recombinant NaD1 was much more efficient at permeabilizing and killing U937 cells than recombinant NaD2 (Figure 3B). Replacement of either loop 4A or loops 6 and 7 of NaD1 with the corresponding loops from NaD2

had no effect on the tumour cell killing activity compared to NaD1. However, substitution of loops 1A, 1B, 4B or 5 generated chimeric defensins with less antitumour cell activity than NaD1 (Figure 3B,D). None of the loop swap variants had increased activity relative to NaD1 on U937 cells.

Permeabilization of PI(4,5)P2 liposomes

A calcein release assay was used to determine the permeabilizing activity of the native defensins and the loop swap variants on bilayers of defined composition. PC:PE:PS:PI liposomes (molar ratio 52:30:10:8) were prepared with and without PI(4,5)P_2 (4%) and were loaded with calcein prior to incubation with each of the defensins (1.25 µM) for 10 min. Liposomes without PI(4,5)P_2 were not permeabilized at a significant level by any of the defensins tested (data not shown). When PI(4,5)P_2 was present, NaD1 treatment released the most calcein whereas NaD2 had no activity(Figure 3C,D). There was no significant difference in permeabilization of the PI(4,5)P_2 liposomes by the L1B, L4A and L6&7 chimeras compared to NaD1. In contrast, the L4B chimera had significantly less permeabilization activity than NaD1 and the L5 chimera had none.

Permeabilization of the F. oxysporum plasma membrane by loop swap chimeras

Permeabilization of the *F. oxysporum* plasma membrane was assayed by monitoring the uptake and fluorescence of the non-membrane permeable dye SYTOX green over a period of 180 min (Figure 4). After a 20 min delay there was a steady increase in fluorescence of the NaD1 (10 µM) treated hyphae for the following 80 min. Hyphae incubated with 10 µM NaD2 showed no increase in fluorescence over the 180 min time period indicating this defensin did not permeabilize the plasma membrane. The loop 5 chimera displayed similar permeabilization kinetics to NaD1. All the other loop swap chimeras had permeabilization profiles intermediate between those of NaD1 and NaD2.

L1A permeabilized more slowly than NaD1 or L5 but reached a similar plateau of fluorescence after 130 min. Initial permeabilization by L4A was at a similar rate to NaD1 and L5, but the fluorescence reached a plateau at a lower level. L1B and L4B displayed the slowest permeabilization kinetics but L4B fluorescence plateaued at the same lower level as L4A, whereas L1B continued to permeabilize and achieved the same plateau as NaD1, L5 and L1A.

Lipid binding

Specificity of lipid binding of the defensin chimeras was examined using lipid strips (Figure 5). Consistent with previous studies, NaD1 interacted strongly with PI(4,5)P_2 and NaD2 interacted preferentially with PA [10, 15] The chimeras L1B and L6&7 had a similar pattern of lipid binding to NaD1 whereas L5 resembled NaD2 with a preference for PA. The chimeras L1A, L4A and L4B bound to several lipids with no preference for PA or PI(4,5)P_2.

Activity of the L1B chimera against other pathogenic fungi

As mentioned above, replacement of the loop 1B sequence of NaD1 with the corresponding sequence from NaD2 led to a substantial increase in activity against *F. oxysporum* compared to the other chimeras and NaD1. The L1B chimera was thus tested against a range of other fungi (Table 1). L1B was about 10 times more effective than NaD1 on *F. oxysporum* and was also more active on the other agriculturally-relevant pathogens; *F. graminearum* and *C. graminicola*. L1B also inhibited the growth of the human pathogen *A. flavus* with an IC_{50} of 3.5 µM whereas NaD1 had no effect at concentrations up to 10 µM. This enhanced activity relative to NaD1 did not extend to the two other *Aspergillus* species tested. Similarly, sensitivity of the yeast pathogens *C. albicans, C. neoformans* and *C. gattii* to L1B was unchangedcompared to NaD1.

Effect of the PI(4,5)P2 binding molecule neomycin on the antifungal activity of NaD1

Neomycin is an aminoglycoside antibiotic that binds to PI(4,5)P_2 [23] and blocks the antitumour activity of NaD1 and other defensins that bind to PI(4,5)P_2 [11, 17]. To determine whether neomycin could block the antifungal activity of NaD1, we employed the model yeast *S. cerevisiae* as the antifungal mechanism of action of NaD1 is conserved between filamentous fungi and yeast [21, 24].

Yeast were treated with neomycin (0.1-10 mM), washed and then treated with antifungal peptides NaD1, L5 and LL37. LL37 was included as a non-PI(4,5)P_2 binding control. Survival was determined by plating serial dilutions of the treated yeast on YPD plates and assessing colony formation (Figure 6A).

Neomycin did not protect the yeast cells against the activity of any of the antifungal peptides.

Activity of NaD1 against S. cerevisiae strains with deletions in genes that function in PI(4,5)P2 biosynthesis

The relationship between PI(4,5)P_2 binding in the antifungal activity of NaD1 was examined further using a series of *S. cerevisiae* strains that had deletions in the PI(4,5)P_2 biosynthetic machinery (Figure 7C). Three of the deleted genes, *stt4, mss4,* and *pik1,* are essential to yeast viability, thus the heterozygous diploid of each gene was assayed for changes in NaD1 sensitivity compared with the wildtype diploid BY4743. All other strains were compared with the wildtype haploid BY4741. None of the deletion strains differed significantly in their sensitivity to NaD1 (Figure 7 A&B).

Discussion

The plant defensin NaD1 is produced in the flowers of the ornamental tobacco and functions to protect the reproductive tissues from damage by fungal pathogens [25]. It also efficiently kills mammalian tumour cells at concentrations that have little effect on normal cells [10]. We prepared chimeras of NaD1 and a second defensin NaD2 that is produced in the same tissues as NaD1. NaD2 is not as potent an antifungal molecule as NaD1 and has no activity on tumour cells (Figure 3). The overall aim of the work was to identify the features of NaD1 that are responsible for its potent activity on fungal and tumour cells with the objective of using this knowledge to generate more active and specific defensins for use in agriculture and medicine. These chimeras were prepared by replacing, in turn, each of the seven surface loops on NaD1 with the equivalent loops from NaD2. Apart from the chimeras with the loop 2 and 3 substitutions all the loop swaps were tolerated and the proteins were expressed and correctly folded, allowing us to examine the relative importance of loops 1, 4, 5 ,6 and 7 to the antifungal and tumour cell activity of NaD1. The longer loops, *i.e.* loops 1 and 4 were each divided into two sections, loops 1A and 1B and loops 4A and 4B whereas the shorter loops 6 and 7 were combined. Each of the loop swap chimeras was tested for activity against fungi (*F. oxysporum*) and tumour cells (U937). The established role for $PI(4,5)P_2$ binding in tumour cell killing by NaD1 led to the investigation of the interaction between the loop swap variants and phospholipids, particularly $PI(4,5)P_2$.

Substitution of loop 1A, loop 1B, loop 4B or loop 5 of NaD1 with the corresponding sequence of NaD2 decreased activity on tumour cells compared to NaD1. Apart from L1B each of these substitutions also had a major effect on $PI(4,5)P_2$ binding and permeabilization of $PI(4,5)P_2$ liposomes, consistent with previous reports [10, 11] that $PI(4,5)P_2$ binding and oligomerisation of the defensin are crucial for the tumour cell killing activity of NaD1. The structure of NaD1 in complex with $PI(4,5)P_2$ revealed that four of the six residues from loop 5 (K36, L37, L38 and R40) form the primary lipid binding domain of NaD1 [10]. Apart from R40 these residues are very different in NaD2 explaining why NaD2 and the NaD1 chimera with loop 5 from NaD2 do not bind to $PI(4,5)P_2$ on lipid strips and do not permeabilize $PI(4,5)P_2$ containing liposomes. The crystal structure also revealed that three of the seven residues in loop 1A participate in formation of the NaD1 dimer that is needed to produce the cationic grip structure that is essential for $PI(4,5)P_2$ binding. Of these three residues, R1, K4 and E6, the lysine at position 4 is most crucial [10]. Lay and colleagues reported that substitution of K4 with an alanine prevented dimer formation and increased the IC_{50} five-fold against fungal cells [26]. The lysine at position 4 also participates in $PI(4,5)P_2$ binding. Consequently, both K4 (in loop 1A) and R40 (in loop 5) have been described as the key residues involved in the formation of the oligomeric NaD1- $PIP(4,5)P_2$ complex [10].

Another residue that interacts with PIP(4,5)P_2 is H33 [10], which is one of the four residues in loop 4B. The chimera with the SGGD sequence from NaD2 in place of the TDGH loop 4B sequence of NaD1 had decreased activity compared with NaD1 on tumour cells, was less effective at permeabilizing PI(4,5)P_2 liposomes and bound less well to PI(4,5)P_2 on lipid strips. These data support the role of H33 in the PI(4,5)P_2 interaction and the previously described role for D31 in the interaction between dimers required to form the oligomer [10]. Substitution of the five residues in loop 4A of NaD1 (ISEKF) with the corresponding residues from NaD2 (LTEGF) had no significant effect on the activity of the chimera on tumour cells, fungal cells or PI(4,5)P_2 liposomes probably because the sequences are similar. Residue E27, which is required for dimer formation, is present in both.

The loop 6&7 substitution encompassed 5 residues, F42 which was changed to a leucine and residues 44 to 46 (TKP) which were changed to TRP. K45 participates in dimer formation, but the conservative change to an arginine explains why the 6&7 substitution had no effect on activity against tumour cells, PI(4,5)P_2 binding or liposome permeabilization.

There was a strong correlation between tumour cell killing activity and the level of permeabilization of PI(4,5)P_2 liposomes across the set of loop swap variants, further highlighting the importance of the NaD1: PI(4,5)P_2 interaction for the antitumour activity of NaD1 (Figure 8). However, there was no correlation between antifungal activity and permeabilization of PI(4,5)P_2 liposomes indicating that PI(4,5)P_2 binding was not essential for the antifungal activity of all of the defensin chimeras. To further assess the role of PI(4,5)P_2 binding in the antifungal activity of NaD1 we determined whether neomycin has a protective effect in fungi similar to that observed with NaD1 and the tomato defensin TPP3 in tumour cells [10, 11] . Neomycin did not protect yeast cells against NaD1, LL37 or the L5 swap variant confirming that PI(4,5)P_2 binding is not essential for the antifungal activity of NaD1 as it is for the antitumour activity. The lack of any significant difference in the sensitivity of yeast gene deletions that cause changes in PI(4,5)P_2 levels further supports that the antifungal mechanism of NaD1 involves more than binding to PI(4,5)P_2. That is not to say that PI(4,5)P_2 binding does not have a role in antifungal activity but that other mechanisms must also be involved.

Many researchers have reported the importance of the loop 5 sequence for their antifungal defensins (reviewed in [27]) and the high sequence variability in this region led to the hypothesis that they interact with different targets on fungal hyphae and hence have different mechanisms of action. Substitution of loop 5 and loss of PI(4,5)P_2-binding in the L5 chimera only decreased the IC_{50} against *F. oxysporum* by two fold but it abolished activity on tumour cells. Indeed, even though the L5 chimera failed to release calcein from the PI(4,5)P_2 liposomes it was as effective as NaD1 in the permeabilisation assays with *F. oxysporum* hyphae. That is, it was able to enter fungal cells and disrupt the plasma membrane in a PI(4,5)P_2 independent way. Considering the increased IC_{50} for L5

it was surprising that L5 displayed similar permeabilization kinetics to NaD1. The permeabilization assay is conducted using protein concentrations in excess of the MIC and measures the rate of cell permeabilization by the peptide as opposed to the effect on fungal growth. Thus the difference in observed IC_{50}s between NaD1 and L5 must be related to differences in the antifungal mechanism of these proteins that are not related to the rate of membrane permeabilization, such as a difference in affinity for a cell surface binding partner or ability to enter the cytoplasm. NaD2, the defensin from which the loop 5 sequence in the L5 chimera was taken, binds to PA and does not permeabilize the fungal membrane. Similarly the IC_{50} of L5 was more than two fold lower than that for NaD2 even though the two defensins have very similar lipid binding profiles on PIP strips. Hence lipid binding is not the only determinant of antifungal activity and sequences other than loop 5 must be contributing to the improved activity of L5 compared to NaD2.

We hypothesize that NaD1, and by extension the loop swap chimeras, have a three step mechanism of action on fungal cells. The first interaction is with components of the fungal cell wall [13], which it traverses before coming into contact with the plasma membrane of fungal cells. It passes through the membrane by a mechanism that has not been defined and once it reaches the cytoplasm it induces oxidative stress and permeabilizes the plasma membrane [13, 21]. The reactive oxygen species (ROS) alone may be sufficient to initiate cell death. However, both the ROS production and an interaction of NaD1 with $PI(4,5)P_2$ on the inner leaflet of the membrane would compromise the membrane and ensure the cell cannot survive.

Knowing that $PI(4,5)P_2$ binding, dimerization and oligomerisation are all essential for the activity of NaD1 on tumour cells we asked why NaD2 had no effect on tumour cells. NaD2 did not bind to $PI(4,5)P_2$ on the lipid strips or the $PI(4,5)P_2$ containing liposomes. This was expected because NaD2 lacks most of the amino acids that are crucial for $PI(4,5)P_2$ binding and we have reported previously that it binds preferentially to PA [15].

As described for NaD1, loop5 contains the residues that line the cationic grip and define lipid-binding specificity for the plant defensins. The loop 5 sequence of NaD2 is RGFRRR which is present in several other antifungal plant defensins [7]. One of these, MtDef4 from *Medicago truncatula,* has potent antifungal activity and has been well studied [28]. It shares 85% sequence similarity with

NaD2 and the loop5 sequence is identical. Sagaram and colleagues [29] reported that like NaD2, MtDef4 binds to PA. Furthermore, amino acid substitutions in loop 5 impair PA binding and abolish the ability of MtDef4 to enter and kill fungal cells. Thus $PI(4,5)P_2$ binding is not required for the antifungal activity of all plant defensins. Indeed, $PI(4,5)P_2$ binding specificity has only been reported for type II defensins from the Solanaceae [15]. Conversely, lipid binding alone is not sufficient for

the antifungal activity of plant defensins. A single amino acid variant (Y38G) of RsAFP2 from radish bound to the cognate lipid glucosylceramide at similar levels to the wildtype defensin but had significantly impaired antifungal activity [30].

A common feature of antimicrobial peptides is that they carry a positive net charge to facilitate interactions with negatively charged microbial cell surfaces and the negatively charged lipid head groups in the plasma membrane [1]. Tumour cells, like microbial cells have an anionic plasma membrane outer leaflet [31] as well as greater surface area and more fluid plasma membranes than normal cells [32]. The activity of plant antimicrobial peptides against tumour cells could be the result of increased susceptibility of tumour cells to cationic membrane disrupting peptides as they lack the protective capacity of a cell wall.

One of the most intriguing results from the initial loop swap variants was the increased activity observed for L1B, L4A and L6&7 against *F. oxysporum*. NaD1 is more active as an antifungal molecule than NaD2. Thus it was expected that replacement of the loop regions of NaD1 with those of NaD2 would be detrimental to antifungal activity, as was observed for antitumour activity. L1B was the most active of the loop swap variants and, although it retained $PI(4,5)P_2$ binding activity in the PIP strip assays, it displayed decreased permeabilization of $PI(4,5)P_2$ liposomes. Electrostatic interactions are a key component of the activity of cationic peptides, including defensins, against fungi [24] and L1B has an overall positive charge of +8 compared to +6 for NaD1 (Figure 1) and +5 for NaD2. Modelling the structure of the L1B chimera based on the structure of NaD1 reveals that the two extra positively charged residues in loop 1B would not prevent formation of the dimers that are required for antifungal activity [26], would not affect $PI(4,5)P_2$ binding and are presented on the surface of the dimer (Figure 9). Enhanced activity on fungal cells and decreased activity on tumour cells suggest that L1B is interacting with a fungal-specific target and is not merely better at nonspecific disruption of negatively charged phospholipids in the plasma membrane. This is supported by the observation that L1B exhibited delayed permeabilization kinetics on fungal hyphae relative to NaD1 and was less active than NaD1 in the calcein release assay. Thus it is likely that enhanced activity of L1B is due an increased affinity for a target within the fungal cell wall or with an intracellular target that results in ROS production. L1B had enhanced activity against the filamentous fungi *F. graminearum*, *C. graminicola*, *A. flavus* and to a lesser extent *A. paraciticus*. However, the activity of L1B against yeasts and *A. niger* was similar to that of NaD1. The observation that the enhancement in antifungal activity varied widely between fungal strains points to potential involvement of the fungal cell wall which varies between fungal species [33] and/or to differences in the ability of the fungi to respond to cell wall, osmotic or oxidative stress [14, 21]. Variations in cell wall composition have been proposed as an explanation for the different effects that the defensin MtDef4 displays against *Neurospora crassa* and *F. graminearum* [34].

Similar loop swap experiments have been conducted with human β-defensins to identify regions that produce the best antibacterial activity. Some chimeras had increased activity whereas others were less active [35] leading to the proposal that sequence elements from different defensins make different contributions to antimicrobial activity. Analogously, the different loop regions in a plant defensin are likely to target different processes in the fungal cell explaining why different defensins have different mechanisms of action. Swapping loops between defensins could create new combinations of these functions in a single molecule. This has been proposed for dimers of short proline rich antimicrobial peptides that are active against bacteria. Fusion of the DNA-K binding domain of pyrrhocoricin to the cell-penetrating region of drosocin increased activity on *E. coli* and allowed for broader target specificity [30]. In the context of NaD1 it remains to be determined whether loop 1B from NaD2 conferred new antifungal activity to the NaD1 backbone or enhanced the activity of the other loops of NaD1.

It remains to be elucidated whether the combination of the loop 1B sequence of NaD2 with other defensins would lead to an increase in antifungal activity and conversely whether loop 1B sequences from other class I defensins and/or other Solanaceous Class II defensin scaffolds could also be used to enhance or broaden the activity of antifungal plant defensins.

The constant threat from fungal pathogens has led plants to evolve an arsenal of innate immunity molecules for protection against devastating diseases. Plant defensins have a high degree of sequence variability outside of the invariant cysteine residues that give them their characteristic structure. We propose that the loop regions between the cysteine crosslinks have evolved as modular components that combine to form potent antifungal molecules. Artificial combinations of these loops can both enhance and diminish antifungal activity. The increase in antifungal activity obtained with some of these combinations suggests that nature has not yet developed the most potent antifungal plant defensin. Further shuffling of defensin sequences could produce antifungal peptides that are more active than those presented here and could lead to more desirable properties of this family of peptides for use in treatment of fungal disease.

Acknowledgements

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Tables

Table 1: IC50s of the L1B loop swap chimera and NaD1 against other pathogenic fungi.

Fungal species	NaD1 (μM)	L1B (μM)
Fusarium oxysporum	1.5 ± 0.25	0.16 ± 0.04
Fusarium graminearum	0.4 ± 0.3	0.28 ± 0.03
Colletotrichum graminicola	4.4 ± 0.1	2.0 ± 0.4
***Aspergillus flavus* 5310**	>10	3.5 ± 2.28
***Aspergillus paraciticus* 4467**	4.5 ± 0.27	3.7 ± 1.17
***Aspergillus niger* 5181**	2.1 ± 0.76	2.2 ± 1.22
***Candida albicans* DAY185**	2.0 ± 0.07	2.0 ± 0.16
***Cryptococcus neoformans* H99**	2.0 ± 0.15	1.6 ± 0.56
***Cryptococcus gattii* WM276**	1.5 ± 0.59	1.6 ± 0.37

Table included in figure 7C

Strain	Gene function	Effect on PI(4,5)P_2
STT4/stt4Δ	Phosphatidylinositol-4-kinase [36]	Decreased [37]
PIK1/pik1Δ	Phosphatidylinositol 4-kinase [38]	Decreased [39]
MSS4/mss4Δ	Phosphatidylinositol-4-phosphate 5-kinase [40]	Decreased [40]
Inp51Δ	Phosphatidylinositol 4,5-bisphosphate 5phosphatase [41]	Increased [41]
Inp52Δ	Polyphosphatidylinositol phosphatase [41]	Normal [41]
Isb6Δ	Type II phosphatidylinositol 4-kinase [42]	Normal [42]

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Figures

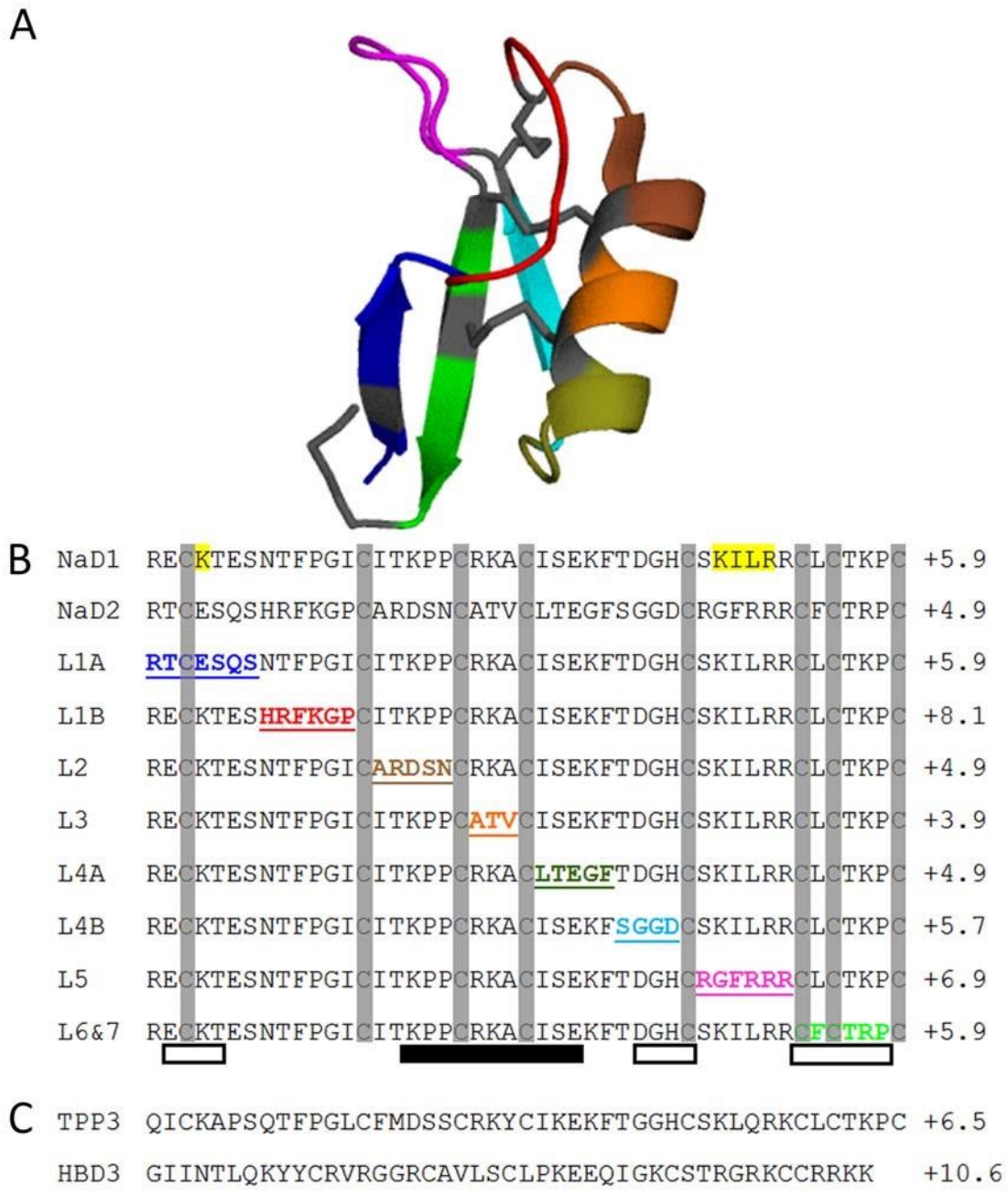
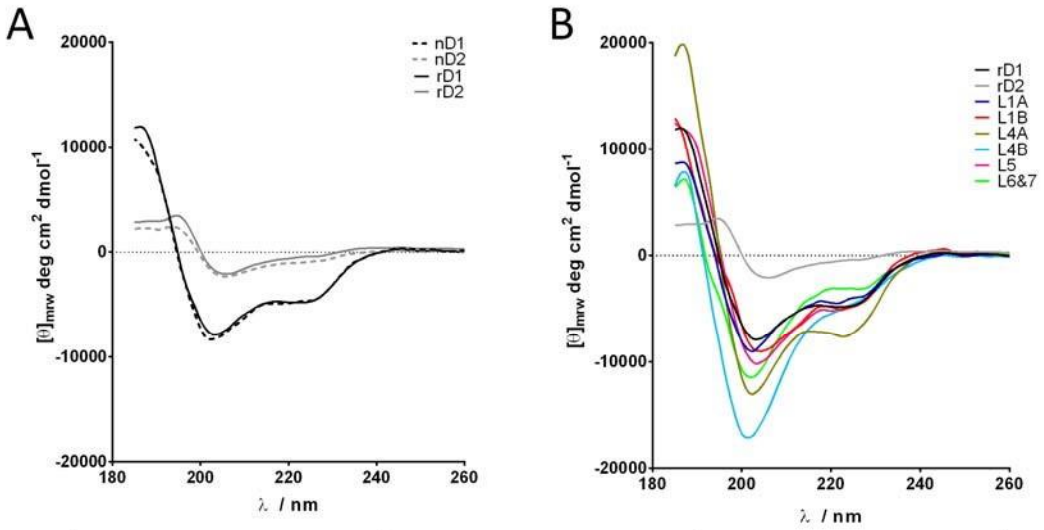


Figure 1. **Loop swap variants of NaD1 and NaD2.** (A) Loops are defined as inter-cysteine sequences (shown here using NaD1 as a model with cysteine residues and disulphides in grey). The residues that participate in the interaction between NaD1 and PI(4,5)P_2 are highlighted in yellow in the NaD1 sequence. Loop 1A (L1A, blue) consists of the first β-strand, loop 1B (L1B,

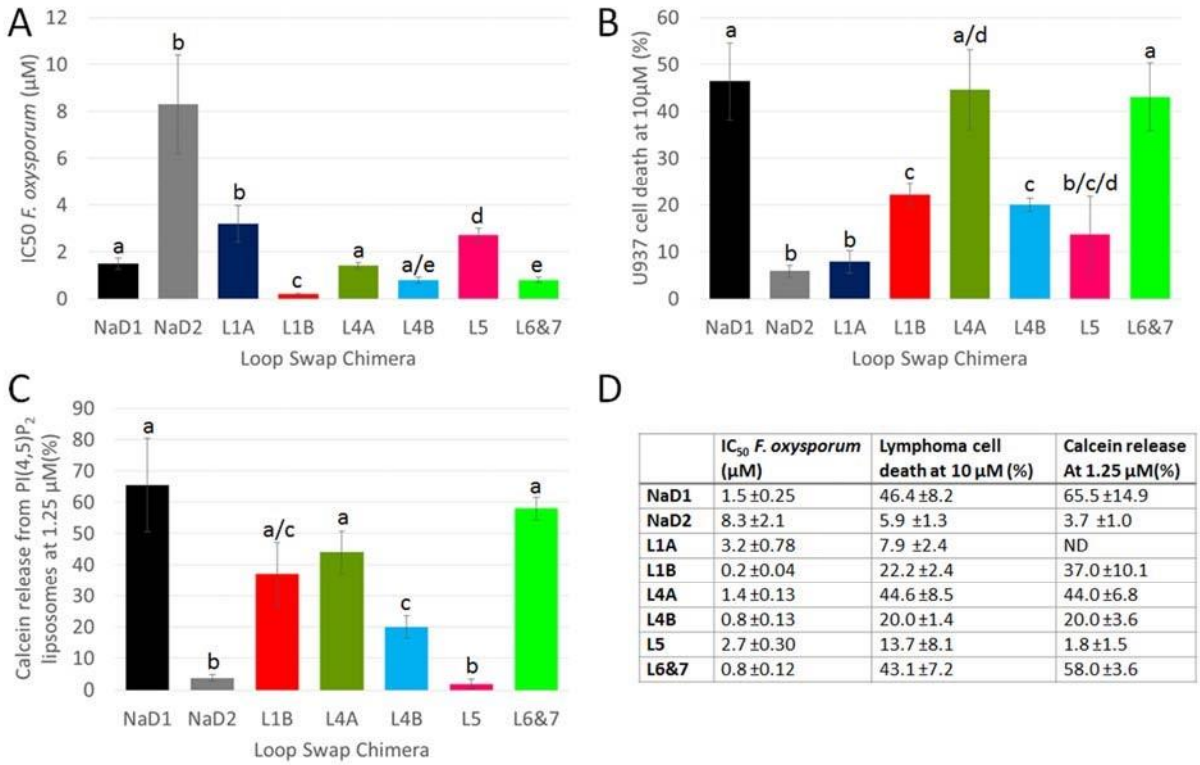
red) is the random-coil/turn region joining the strand to the α-helix. Loops 2 (L2, brown), 3 (L3, yellow) and 4A (L4A, olive) make up the α-helix. Loop 4B (L4B, cyan) forms the second β-strand. Loop 5 (L5, pink) is a surface loop connecting two β strands and loops 6 and 7 (L6&7, green) form the final β strand (PDB accession code: 4AAZ [26]). (B) Sequence alignment of the loop swapped proteins, which contain a loop of NaD2 in the NaD1 framework. The NaD2 loop sequences are colour-coded as shown in A. The column on the right has the charge of each defensin at pH 7. The white boxes below the sequences indicate regions that form β strands and the black box the helical region of NaD1.(C) Sequence and charge at pH7 of two other proteins reported to bind PI(4,5)P_2 TPP3 and HBD3.



C

Peptide	$[\theta]_{222}$ (MRW)	F_{helix}/%
rNaD1	-4813	16.5
nNaD1	-4742	13.2
rNaD2	-502	4.8
nNaD2	-1025	6.2
L1A	-4460	15.6
L1B	-5059	17.2
L4A	-7580	24.0
L4B	-5144	17.4
L5	-5174	17.5
L6&7	-3123	11.9

Figure 2. Circular dichroism of purified loop swap variants. (A) Recombinantly expressed and purified defensins (rD1, rD2) have the same CD spectra as those purified from plants (nD1, nD2) indicating that the secondary structure elements have formed correctly. (B) Loop swap variants have similar CD spectra to the native sequences demonstrating correct folding of the proteins.(C) Percent helicity of native and recombinant NaD1 and NaD2 and the loop swap variants.



	IC_{50} *F. oxysporum* (µM)	Lymphoma cell death at 10 µM (%)	Calcein release At 1.25 µM(%)
NaD1	1.5 ±0.25	46.4 ±8.2	65.5 ±14.9
NaD2	8.3 ±2.1	5.9 ±1.3	3.7 ±1.0
L1A	3.2 ±0.78	7.9 ±2.4	ND
L1B	0.2 ±0.04	22.2 ±2.4	37.0 ±10.1
L4A	1.4 ±0.13	44.6 ±8.5	44.0 ±6.8
L4B	0.8 ±0.13	20.0 ±1.4	20.0 ±3.6
L5	2.7 ±0.30	13.7 ±8.1	1.8 ±1.5
L6&7	0.8 ±0.12	43.1 ±7.2	58.0 ±3.6

Figure 3. The antifungal, antitumour and liposome permeabilizing activities of the NaD1-NaD2 loop swap proteins. (A) The concentration of the NaD1 NaD2 loop swap proteins required to inhibit 50% growth (IC50) of the filamentous fungus F. oxysporum. The loop swap proteins L4B and L6&7 and particulary L1B had enhanced antifungal activity relative to NaD1, whereas L1A and L5 had decreased activity. (B) Activity of the NaD1-NaD2 loop swap proteins against the U937 lymphoma cell line. Cell death was assessed by PI uptake into cells by flow cytometry after treatment with test protein at 10 µM. All of the loop swap proteins, apart from L4A and L6&7, had less activity than rNaD1. (C) Release of calcien from PC:PE:PS:PI:PI(4,5)P2 liposomes by loop swap chimeras (1.25 µM) after 10min relative to Triton X-100 control. NaD1 readily permeabilized liposomes while NaD2 had minimal permeabilization activity. All of the loop swap chimeras permeabilized liposomes with activities intermediate to NaD1 and NaD2 except L5 which did not permeabilize liposomes at all. Error bars are SEM, n=3. Letters above bars indicate overlapping 95% confidence intervals. Values with the same letter are not significantly different.

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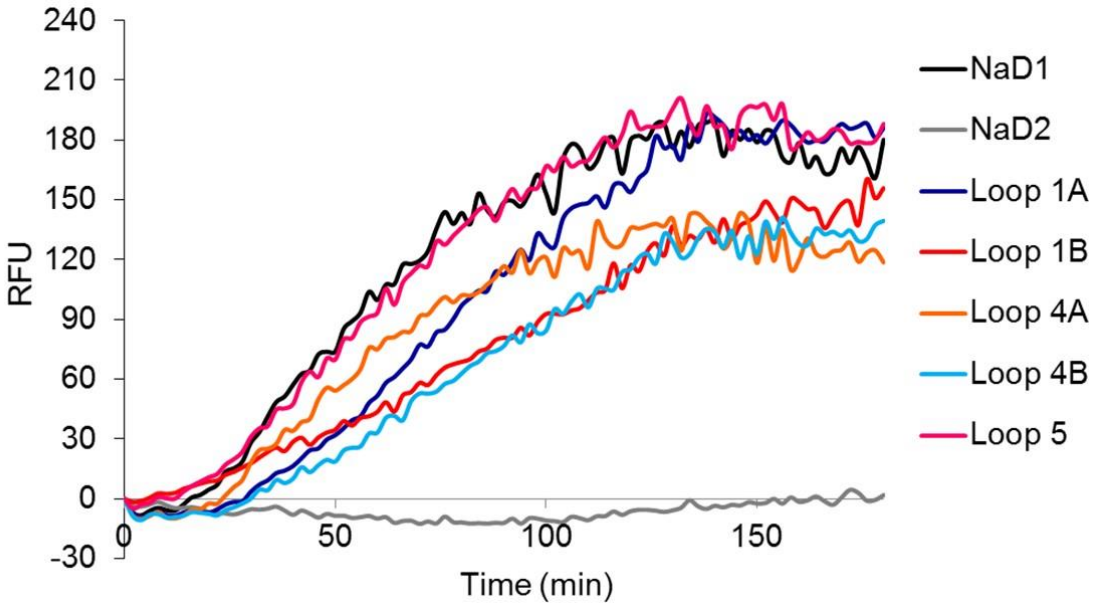

Figure 4. Permeabilization of *F. oxysporum* hyphae by NaD1- NaD2 loop swap variants. Permeabilization of *F. oxysporum* was assessed by monitoring the uptake and fluorescence of the non-cell permeable dye SYTOX green. All of the loop swap variants permeabilized hyphae but to variable extents. NaD2 did not permeabilize hyphae at all. L5 displayed similar permeabilization kinetics to NaD1 while the remaining loop swap variants all had slower rates of permeabilization than NaD1. Data is representative of 3 independent assays.

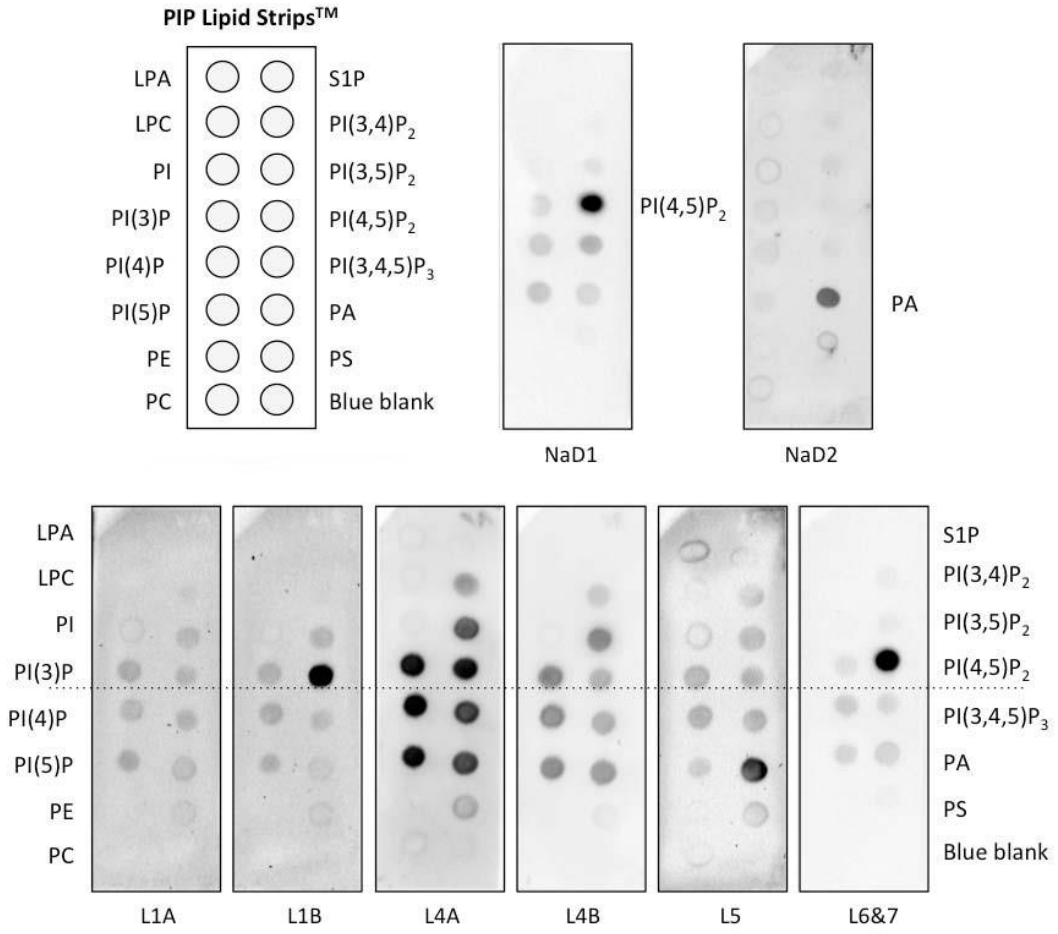

Figure 5. Lipid strip binding profiles of NaD1, NaD2 and the loop-swap chimeras.

The introduction of the different loop regions from NaD2 into NaD1 changed the lipid binding profiles of the protein. Binding of NaD1 and the loop swapped proteins to PIPstrips™ was detected by Western blot analysis with polyclonal rabbit α-NaD1 IgG, while NaD2 binding was detected with polyclonal rabbit α-NaD2 IgG. Images are representative of two separate experiments with different batches of strips. Lysophosphatidic acid (LPA), lysophosphocholine (LPC), phosphatidylinositol (PI), phosphatidylinositol 3-phosphate (PI(3)P), phosphatidylinositol 4-phosphate (PI(4)P),

phosphatidylinositol 5-phosphate (PI(5)P), phosphatidylethanolamine (PE), phosphatidylcholine (PC), sphingosine 1-phosphate (S1P), phosphatidylinositol 3,4-bisphosphate (PI(3,4)P2),

phosphatidylinositol 3,5-bisphosphate (PI(3,5)P2), phosphatidylinositol 4,5-bisphosphate (PI(4,5)P2), phosphatidylinositol 3,4,5-trisphosphate (PI(3,4,5)P3), phosphatidic acid (PA), phosphatidylserine (PS) and lipid negative control (blue blank).

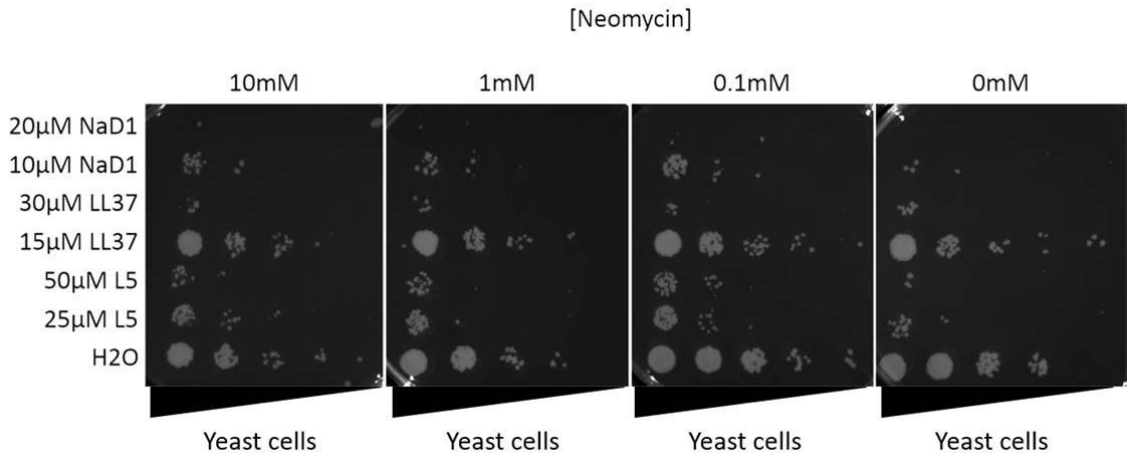

Figure 6. Neomycin does not protect against the antifungal activity of NaD1, LL37 or L5. *S. cerevisiae* (BY4741) treated with neomycin (0-10mM) for three hours, washed and then treated with NaD1 (10 and 20µM), LL37 (15 and 30µM) or L5 (25 and 50µM) for 1 hour at 30˚C. A 5 fold dilution series of each treatment was plated on YPD agar and grown at 30˚C for two days. Neomycin did not protect against any of the antifungal peptides tested. Data is representative of three independent biological experiments.

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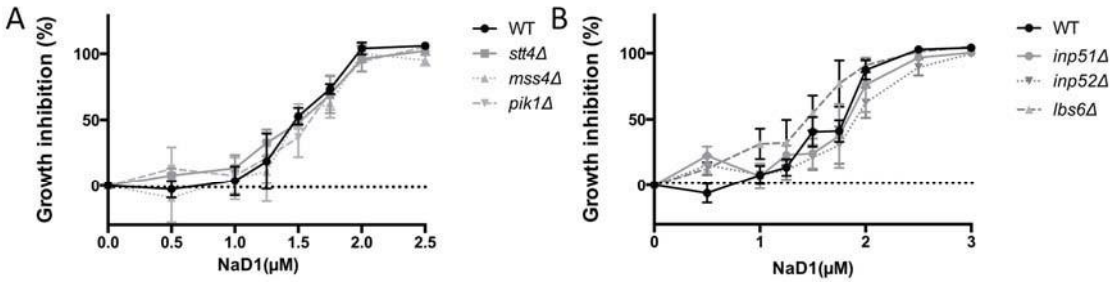

C

Strain	Gene function	Effect on PI(4,5)P_2
STT4/stt4Δ	Phosphatidylinositol-4-kinase [36]	Decreased [37]
PIK1/pik1Δ	Phosphatidylinositol 4-kinase [38]	Decreased [39]
MSS4/mss4Δ	Phosphatidylinositol-4-phosphate 5-kinase [40]	Decreased [40]
Inp51Δ	Phosphatidylinositol 4,5-bisphosphate 5-phosphatase [41]	Increased [41]
Inp52Δ	Polyphosphatidylinositol phosphatase [41]	Normal [41]
Isb6Δ	Type II phosphatidylinositol 4-kinase [42]	Normal [42]

Figure 7. Effect of NaD1 on the growth of *S. cerevisiae* mutants with gene knockouts in the PI(4,5)P_2 biosynthesis pathway. (**A**) Growth of *S. cerevisiae* cells with the heterozygous knockouts, *pik1*Δ, *mss4*Δ and *stt4*Δ was similar to the growth of the parent cell line BY4743 (WT) in the presence of NaD1 (0–2.5 μM). (**B**) Growth inhibition of the *S. cerevisiae* non-essential PI(4,5)P_2 biosynthesis knockouts, *lsb6*Δ, *inp51*Δ and *inp52*Δ was similar to the growth inhibition of the parent cell line BY4741 (WT) in the presence of NaD1 (0–3 μM). Growth was monitored by optical density at 595 nm over 24 h and percentage growth inhibition was plotted, n=4±SEM. (C) Functions of deleted genes and the effects on PI(4,5)P_2 levels.

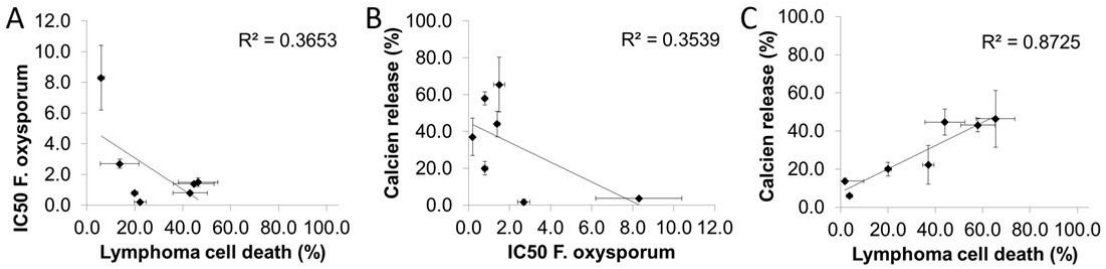

Figure 8. Correlation of lymphoma cell death, calcien release from PI(4,5)P_2 liposomes and IC_{50} against *F. oxysporum*. Linear regression analysis was performed with the values for

lymphoma death, calcien release and IC_{50} agianst *F. oxysporum* from figure 3. The IC_{50} against *F. oxysporum* had R^2 values of 0.3653 and 0.3539 for lymphoma cell death and calcien release respectively indicating a poor correlation. Calcien release and lymphoma cell death had an R^2 value of 0.8725 indicating a strong correlation between the two values across the defensin chimeras.

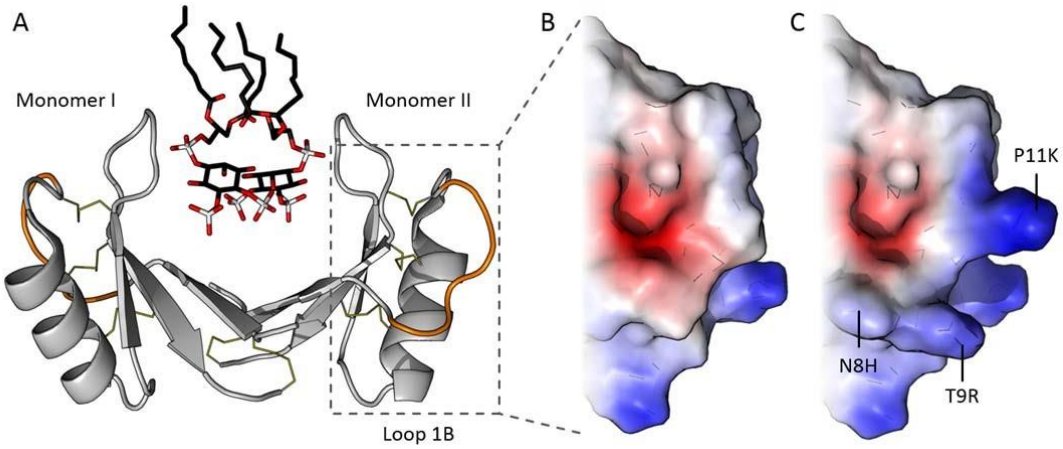

Figure 9. Location of positively charged residues of L1B modelled onto the NaD1 structure. (A) Dimer of NaD1 with loop 1B highlighted in orange. $PI(4,5)P_2$ ligands are shown in black. (B) NaD1 surface potential with positive charge in blue and negative charge in red. (C) L1B surface charge with mutations indicated that increased exposed positive charge. The increase in positive charge is on the outer faces of the defensin dimer away from the $PI(4,5)P_2$ binding pocket. Structure was modified from PDB:4CQK.