Structural and functional analysis of EntV reveals a 12 amino acid fragment protective against fungal infections

Fungal pathogens are a continuing challenge due to few effective antifungals and a rise in resistance. In previous work, we described the inhibition of Candida albicans virulence following exposure to the 68 amino acid bacteriocin, EntV, secreted by Enterococcus faecalis. Here, to optimize EntV as a potential therapeutic and better understand its antifungal features, an X-ray structure is obtained. The structure consists of six alpha helices enclosing a seventh 16 amino acid helix (α7). The individual helices are tested for antifungal activity using in vitro and nematode infection assays. Interestingly, α7 retains antifungal, but not antibacterial activity and is also effective against Candida auris and Cryptococcus neoformans. Further reduction of α7 to 12 amino acids retains full antifungal activity, and excellent efficacy is observed in rodent models of C. albicans oropharyngeal, systemic, and venous catheter infections. Together, these results showcase EntV-derived peptides as promising candidates for antifungal therapeutic development.

Management of disseminated fungal infections continues to be a major clinical problem. The limited spectrum of available antifungal agents contributes to both the development of acquired resistance and the rise in incidence of previously rare but intrinsically resistant pathogens. Consequently, there is an unacceptably high mortality rate in patients with fungal infections. Antifungal discovery is complicated by the similar cell biology of fungi and mammals; as a result, there are only three classes of drugs for systemic infections, the newest of which was discovered nearly 40 years ago and approved for clinical use in 2001. This illustrates the challenges of developing new antifungals, despite the dire need for them.

Life-threatening fungal infections are seen nearly exclusively in immunodeficient patients, with healthy individuals routinely exposed to these pathogens. Candida albicans is archetypical: it is a ubiquitous mammalian commensal of the gastrointestinal and urogenital tracts as well as the skin and is rarely isolated from environmental sources. The normally benign association of most C. albicans with the human host raises the prospect that successful antifungal therapy need not involve...
eradication, but rather a restoration of microbial balance, so as to confine it to a non-pathogenic association. Consequently, significant effort has been expended to understand the normal and pathological interactions of C. albicans with the host. Less attention has been paid to how these interactions may be impacted by other components of the microbiome, but C. albicans has been observed to have both synergistic and antagonistic relationships with various bacterial species, including human pathogens such as Staphylococcus aureus, Pseudomonas aeruginosa, Streptococcus mutans and Enterococcus faecalis14–21. It stands to reason that C. albicans, often considered to be an obligate mammalian commensal, would have evolved specific responses to its bacterial neighbors in the microbiome and is thus a platform for uncovering molecular signals that may have therapeutic potential.

We previously reported that the Gram-positive bacterium E. faecalis antagonizes the virulence of C. albicans22,23. These two opportunistic species share common host niches and are perhaps the most common bacterial–fungal pairing in polymicrobial infections, suggesting that they may cooperate to promote either commensal or pathogenic behaviors24,25. The effect on C. albicans is in part mediated by EntV, a bacteriocin produced by E. faecalis as a pre-pro-peptide14,18. Processing includes removal of a signal peptide, formation of a disulfide bond, and cleavage by an extracellular protease27,28. The processed, active form of EntV is 68 amino acids and is both necessary and sufficient to inhibit C. albicans biofilm formation in vitro and pathogenesis in both a nematode (Caenorhabditis elegans) infection model and a mouse model of oropharyngeal candidiasis (OPC)29. Notably, EntV appears to have no fungicidal or fungistatic action, but works to inhibit adhesion and hyphal morphogenesis, both of which are required for virulence in this species30. EntV is thus a candidate anti-virulence agent.

There are several significant obstacles to further development of EntV as an antifungal agent in its native form. Chemical synthesis of a 68 amino acid protein that contains a required disulfide bond is non-trivial and attempts to produce this recombinantly in bacteria have not been successful due to toxicity14. This is likely due to its bacteriocin activity, which poses the potential for off-target effects on the bacterial microbiome. Thus, we sought to further investigate the structural determinants of the antifungal activity of EntV and whether we could reduce the size and complexity of the active moiety and separate the bacteriocin and antifungal functions.

Here, the three-dimensional structure of the secreted form of EntV prior to extracellular cleavage is reported. By functional analysis one alpha helix with significant antifungal but no bacteriocin activity is identified. Moreover, shorter fragments of the helix, down to 12 amino acids, exhibited full antifungal activity at nanomolar concentrations in vitro assays and animal models of candidiasis, with a 10-mer variant retaining some function. These peptides have promise as broad-spectrum antifungal agents as we showed they are active against at least some strains of Cryptococcus neoformans and the multi-drug resistant Candida auris. Thus, EntV-derived peptides are candidates for development of anti-virulence agents for the treatment or prevention of fungal infections.

**Results**

*Clasping palms* structure of EntV

EntV was previously characterized as a secreted protein that undergoes multiple processing events. The 170 amino acid translated protein is directed to the secretion system by a signal sequence at its N-terminus. The signal peptide is cleaved during secretion releasing a 169 amino acid C-terminus14,17,18. A disulfide bond formed between two cysteines in the C-terminus (C106 and C167 in Fig. 1a) was also shown to be necessary for activity18. To better understand the basis for the antimicrobial properties of EntV, we sought to visualize its structure by X-ray crystallography. Unprocessed EntV16 was recombinantly purified from an E. coli strain as a selenomethionine-derivated protein and crystallized. The structure was determined using single anomalous dispersion (SAD) phasing to 1.8 Å resolution, allowing for high resolution analysis of the structure. A disulfide bond, visible in the electron density except for the first two amino acids19,20, the last two (169–170) and an internal region corresponding to residues 90–104; this latter region notably corresponds to the GelE cleavage site, and we postulate that there is natural flexibility in this region. The predicted disulfide bond between C106 and C167 was not observed in the structure due to the necessity of producing the protein under reducing conditions in the E. coli cytosol. However, the two cysteine residues are co-localized within 3.4 Å, consistent with the ability to form an intramolecular disulfide bond. Interestingly, the structure resembled two “clasping palms” comprised of three α helices each (Fig. 1b). The first three helices are in the N-terminus of EntV whereas the second three are part of the C-terminus. The two helms enclose a final C-terminal helix, α7 (Fig. 1a, b). We noticed a strong charge asymmetry between the external faces of the two helms (Supplementary Fig. 1a). The NTD (N-terminal domain) external face is negatively charged, while the CTD (C-terminal domain) external face is positively charged. The positive charge of the external face of the CTD is due to the presence of many lysine and arginine residues, while in contrast, the interior face that interacts with α7 is hydrophobic/neutral (Supplementary Fig. 1b). α7 itself is strongly hydrophobic, as 11 of its 16 residues are hydrophobic sidechains (Fig. 1c). These observations are consistent with the sheltering of the hydrophobic α7 in the interior of the EntV** structure.

**The α7 helix is sufficient for antifungal activity**

Based on the solved structure, we tested fragments of EntV for structural moieties that retained antifungal activity. We generated synthetic peptides of each of the four alpha helices (plus two flanking amino acids) from the active C-terminus of EntV—α4, α5, α6 and α7 (Fig. 1a) as well as an α4-α6 construct. The peptides were tested for their ability to protect C. elegans against C. albicans infection as established in previous work14,21. As shown in Fig. 2a, the α7 fragment at a concentration of 1 nM protected C. elegans from C. albicans infection as well or better than full-length, active EntV16 (Fig. 2a and Supplementary Data 1). The other peptide fragments also showed modest protection.

EntV was originally characterized for its antibacterial activity against some Gram-positive species6,24. Therefore, the peptides were additionally tested for their ability to inhibit the growth of Lactobacillus sakei in liquid culture. As shown in Supplementary Fig. 2, EntV abrogated the growth of L. sakei at concentrations as low as 10 nM. However, none of the EntV fragments had any activity in this assay at any of the concentrations tested. These data suggest that the antibacterial and antifungal effects of EntV have different mechanisms and may be separable.

We previously demonstrated that the full-length EntV reduces fungal burden and invasion in a mouse model of oropharyngeal candidiasis (OPC)14. This, like many manifestations of Candida infections, requires adhesion of the fungal cell to surfaces, both biotic and abiotic, whereupon biofilm structures are commonly formed. We used a model in which binding to plastic surfaces is a measure of the initial stage of biofilm differentiation. In this assay, cells are incubated in polystyrene 96 well plates with the peptides in PBS for one hour and then overlaid with artificial saliva media for 90 min before washing and quantitation of adhesion using crystal violet. Full-length EntV significantly reduced adhesion to polystyrene (Fig. 2b). The core 16aa α7 helix by itself had further increased efficacy, with significant activity remaining at 1 nM.
The magnitude of the reduction was equivalent to that of the negative control (‘neg’ in Fig. 2b), a well-characterized non-adherent and non-hyphal mutant lacking the CPH1 and EFG1 transcriptional regulators.

We next designed variants of the α7 peptide to probe the elements that were required for activity. To test if the specificity of sequence and/or the helical structure were important for enabling activity, we randomized the sequence of α7 in two ways. First, using the peptide folding prediction program PEP-FOLD, we generated peptides in which the sequence was randomized in a manner predicted to either retain or disrupt the helical structure. Neither peptide provided any significant protection in the worm assay (Fig. 2c), suggesting sequence specificity to the observed activity. Consistent with this, the scrambled peptides also largely lost the ability to inhibit adhesion (Fig. 2d). Thus, the antifungal activity results from the specific sequence and not a generic helical motif.

We observed that while most of the residues that comprise the 16 amino acid core of α7 are hydrophobic, the three polar residues (two glutamines and one cysteine) are on the same face of the helix as shown in a helical wheel projection (Fig. 1c). This cysteine forms a disulfide bond that is required for the activity of the 68 amino acid peptide. We speculated that the polar nature of the glutamines might be important and synthesized peptides in which these were both changed either to non-polar isoleucine or to charged glutamic acid. Surprisingly, the isoleucine substitution had no effect on activity in the worm or adhesion assays. In contrast, the glutamic acid variant was notably less effective. Further reducing the peptide size to 11 or 10 amino acids attenuated activity somewhat in both assays, while a 9-mer peptide was inactive. The tested peptides are illustrated in Fig. 3f. These data suggest that a 12mer of the α7 helix, retaining a full three turns of the α-helix and ending with cysteine-167, is as effective in protecting against C. albicans infection in the worm model and inhibiting surface adhesion as full-length EntV.

EntV peptides have activity against other fungal species and strains

We previously reported that EntV68 inhibited biofilm formation of other Candida species including C. tropicalis, C. parapsilosis and C. glabrata, suggesting its activity may not be limited to C. albicans. Based on these observations, whether EntV and derivative peptides protected C. elegans from other fungal species and drug resistant strains of C. albicans was tested. Candida auris has been defined by the...
Fig. 2 | The α7 helix of EntV has antifungal activity. a, c show survival over time of C. elegans infected with C. albicans and exposed to 1 mM of the indicated fragments of EntV based on the structure in Fig. 1a or versions of the α7 helix, respectively. Random sequence = TNVGLWICGLAVIQAAQLS and disrupted sequence = TNAAAQGGGICLLVVWLS. Statistical differences in survival were compared to animals in the exposure condition on the left of each legend by Mantel–Cox log rank analysis. An n of 60 animals was used and one representative trial is shown. Exact p values from top to bottom: a = <0.0001, 0.1900, 0.0361, 0.0583, 0.0059, 0.0019. b <0.0001, 0.1666, <0.0001, <0.0001. Median survival and p values of all trials are shown in Supplementary Data 1. b, d show adhesion of C. albicans to tissue-culture treated plates following incubation with different concentrations of the indicated peptides. “Neg” is a control non-adherent strain (Δegf1 Δacphd). Lines with error bars indicate the mean and the SD following normalization against the mean of the vehicle control group. Statistical significance in comparison to the DMSO control group was determined using one-way ANOVA followed by Dunnett’s multiple comparison test. An n of three biological replicates, each with six technical replicates, was used. Exact p values from left to right are as follows: b 0.1065, <0.0001, 0.0071, 0.0001, 0.9998, 0.0026, 0.9997, 0.8961, 0.9998, 0.9875, 0.0002, 0.0101, 0.1223, 0.9994, 0.8044, 0.8456, 0.9996, 0.9994, 0.993, 0.8957, <0.0001.

CDC as an emerging pathogen of concern, causing infection in immunocompromised patients that are hard to treat due to high-levels of intrinsic drug-resistance. As shown in Fig. 4a, treatment of a C. auris strain with α7 protected animals from killing. The 12aa version of the α7 fragment also protected C. neoformans infected worms from killing (Fig. 4b), a pathogen that is a leading cause of death in HIV/AIDS patients due to fungal meningitis. C. neoformans is in the phylum basidiomycota and is thus evolutionarily very distant from Candida species (phylum ascomycota), suggesting that the unknown target of EntV is widely conserved amongst fungi. We also tested paired clinical isolates of C. albicans in which fluconazole-resistance had been gained following in-patient evolution. Animals infected with both the original strain as well as the fluconazole-resistant descendents were protected by EntV (Fig. 4c). These results warrant future testing of many more species and strains of fungal pathogens to understand the extent of EntV efficacy.

The 12mer is protective in a mouse model of oropharyngeal candidiasis (OPC)

The 12mer of the α7 helix was tested for efficacy in protecting against C. albicans oral infection in mice, a model in which EntV was previously shown to be effective. Briefly, corticosteroid-suppressed mice were orally inoculated with C. albicans and provided with water containing vehicle (0.01% DMSO), EntV, or the 12mer peptide (100 nM). After five days, fungal burden was assessed in the tongue using qPCR and epithelial invasion by histological examination. As shown in Fig. 5a, treatment with EntV significantly reduced the fungal burden, as observed in our previous study, and treatment with the 12mer was equally effective. We assessed epithelial invasion of the tongues via histology and determined that the 12 amino acid peptide was at least as effective as the full length EntV (Fig. 5b, c). In contrast, to the extensive hyphal morphology and invasion in control animals, C. albicans is found mostly in the yeast morphological state when associated with tissue from the animals treated with EntV or the 12mer (Fig. 5c). Overall, these data show that the 12mer is as effective as EntV in abrogating an oropharyngeal infection of C. albicans. We also tested the shorter peptides and, consistent with the C. elegans and adhesion assays, the peptides of 11 and 10 amino acids reduced fungal burden in the OPC model, but not to the same degree as the 12mer. The 9mer exhibited no significant protection (Fig. 5d). Hyphal invasion was also most reduced following treatment with the 12mer, with some reduction observed in animals exposed to the 10mer and 11mer. The 9mer had no effect (Supplementary Fig. 5a). The results support the conclusion that the 12mer is the optimal fragment length, comparable to or better than EntV in all assays.

In these experiments, as well as in the previous study, ad libitum treatment with EntV or its truncated variants commenced as soon as the animals recovered from the oral inoculation with C. albicans by adding peptide to drinking water. To determine if the 12mer has efficacy as a treatment after the establishment of an infection, we...
inoculated mice, but did not administer peptide until day three (Fig. 5e). A group of untreated infected animals was also sacrificed at day three to check for successful establishment of the infection. Animals treated with the 12mer starting at day three and sacrificed on day five had significantly less fungal burden compared to both groups of untreated animals. In fact, the level of reduction was not significantly different from those animals who had been treated from day zero (Fig. 5e). The same patterns were observed when hyphal invasion was used as the measurement of infection severity (Supplementary Fig. 5b). These results suggest that the 12mer has efficacy in treating established OPC.

We assessed the efficacy of the 12mer relative to fluconazole, an antifungal agent recommended to treat OPC. At our standard dose of 100 nM, the 12mer was even more effective at reducing fungal burden than 4 µg/mL fluconazole, 8-times the MIC for this strain, though this was only statistically significant when we increased the concentration of EntV to 500 nM (Fig. 5f). Hyphal invasion was slightly less prevalent after treatment with 100 nM and 500 nM relative to fluconazole (Supplementary Fig. 5c). We also tested a lower dose (50 nM), which resulted in loss of efficacy in terms of fungal burden, though there was still a significant lowering of hyphal invasion (Fig. 5f and Supplementary Fig. 5c). Overall, these data show that oral treatment with 100 nM 12mer is comparable or better to treatment with fluconazole, a standard and accepted chemotherapeutic.

Given the sustained efficacy of the 12mer to reduce epithelial invasion at 50 nM, we tested yet lower concentrations. At 10 nM and 1 nM there was only a slight reduction in fungal burden on the tongue, but the reduction in invasion remained dramatic (Supplementary

**Fig. 3** Shorter variants of α7 retain antifungal activity. **a, c, e** show survival over time of C. elegans infected with C. albicans and exposed to 1 nM of the indicated α7 fragment as shown in **f**. Statistical differences in survival were compared to animals in the exposure condition on the left of each legend by Mantel–Cox log rank analysis. An n of 60 was used and one representative trial is shown. Exact p values from top to bottom: **a** < 0.0001, 0.7271, 0.4951, 0.5702, 0.3797, **c** < 0.0001, 0.7958, 0.2951, 0.5476, 0.0002. **e** < 0.0001, 0.0489, 0.5844, 0.0127, < 0.0001. Median survival and p values of all trials are shown in Supplementary Data 1. **b, d** show adhesion of C. albicans to tissue-culture treated plates following incubation with 10 nM of the α7 fragment size variants. "Neg" is the background observed with a non-adherent strain (Δefg1Δcph1). Lines with error bars indicate the mean and the SD following normalization against the mean of the vehicle control group. Statistical significance in comparison to the DMSO control group was determined using one-way ANOVA followed by Dunnett’s multiple comparison test. An n of four biological replicates, each with six technical replicates, was used. Exact p values from left to right: **b** 0.0003, 0.0210, 0.0001, **d** < 0.0001, 0.6890, < 0.0001.
Fig. 5d, e). The EntV 12mer appears to promote a commensal, non-invasive, association with the host epithelium at concentrations well below those needed to see a reduction in fungal numbers. Finally, we note that there are no differences in fungal burden, invasion, or EntV/12mer efficacy between male and female mice (Supplementary Fig. 5f, g).

The 12mer is protective in a rat venous catheter model

*C. albicans* can form prodigious biofilms on implanted medical devices and the presence of an intravenous catheter is, by itself, a significant risk factor for developing disseminated candidiasis. These infections are notoriously difficult to treat and are associated with the drug resistance characteristic of biofilms. We initially tested the full-length EntV in a model in which an intravenous catheter is implanted into the jugular vein of rats and inoculated with *C. albicans* with or without the peptide. After 24 h, the catheter is removed and sectioned to assess fungal burden by plating for CFUs and by scanning electron microscopy (SEM). Catheters treated with 100 nM EntV were essentially sterile, with fungal burden below the limit of detection (Fig. 6a). As we defined the minimal peptide length, we again utilized this model using the 12mer peptide, this time at a lower concentration to probe the extent of protection. Even as low as 20 nM, the 12mer reduced fungal burdens by 1.5 logs (Fig. 6b) and SEMs showed the catheter surfaces to be largely clear of fungal biofilm (Fig. 6a, b). The data suggest that the EntV peptides are very effective in preventing *C. albicans* biofilm formation on abiotic surfaces and has potential as a treatment and/or a preventative of *C. albicans* medical device-associated infections.

**The 12mer is protective in a systemic mouse model**

Systemic, bloodstream infections by *C. albicans* are the most life-threatening to patients. Mortality rates from disseminated candidiasis exceed 40% and new treatments are in dire need. Since EntV impairs hyphal morphogenesis and biofilm formation rather than killing *C. albicans*, it was unclear if EntV and EntV derivatives would be protective in a systemic infection model. Before injection of the 12mer, toxicity and stability of the 12mer was examined. Incubation of EntV and the 12mer caused no lysis of red blood cells (Supplementary Fig. 6a). However, they were not particularly stable in serum; about 10% of the 12mer was detectable following a 24-hour incubation (Supplementary Fig. 6b). To increase the chances of observing an effect, we pre-incubated *C. albicans* with 100 nM of EntV or the 12mer for two hours prior to intravenous (IV) injection of 1 × 10⁵ CFU. CFUs were measured both before and after the two-hour incubation and there was no significant difference, indicating that the EntV versions were not toxic to *C. albicans*. The median survival of animals injected with *C. albicans* alone was 7.5 days and all animals had expired by 11 days. In striking contrast, animals injected with *C. albicans* that had been pre-incubated with EntV or the 12mer survived much longer and about half the animals were still alive when the experiment was ended in accordance with the protocol at day 22 (Fig. 6c).

**Discussion**

Structural studies demonstrated that the pro-peptide of EntV (EntV⁽¹⁾) forms a clapping palm motif that cages the α7 helix in a manner that portends its importance. Indeed, informed by this data, the antifungal activity was isolated to this single α-helix and just 10-12 amino acids have full to partial activity in multiple in vitro, ex vivo, and animal...
**Fig. 5** | The 12mer is protective in a mouse model of oropharyngeal candidiasis.

Amount of *C. albicans* DNA detected by qPCR, **a**, and percentage of the tongue surface showing hyphal invasion, **b**, in untreated animals compared to those given 100 nM EntV and 12aa α7 in their drinking water for 5 days following inoculation. **c** Representative pictures used to score hyphal invasion. Pink staining cells in the areas that are magnified, indicated by the black arrow heads, are *C. albicans*. The cells in the control sample show an elongated, hyphal morphotype more prevalently invading the tissue compared to the treated samples. **d** The amount of *C. albicans* DNA detected by qPCR in animals treated with different fragment lengths of the α7 peptide at day 5. **e** The amount of *C. albicans* DNA detected by qPCR in animals at day 3 or 5 following treatment with 100 nM of the 12mer starting at day 0 or 3. **f** The amount of *C. albicans* DNA detected by qPCR in animals treated with different concentrations of the 12mer in comparison to fluconazole (Flu) at day 5. An n of 6-10 biologically independent animals was used with the exact number indicated by the number of data points. Averages were calculated and the error bars represent the SEM. Horizontal lines mark compared conditions. Significance was determined using one-way ANOVA followed by Tukey's multiple comparison test. Exact p values from top to bottom are as follows: **a** <0.0001, <0.0001, >0.9999, **b** <0.0001, <0.0001, 0.8811, **d** <0.0001, 0.0014, 0.0206, 0.0515, **e** <0.0001, 0.9688, 0.0030, 0.0009, **f** 0.8588, <0.0001, <0.0001, 0.0043, 0.0001, 0.1672, 0.0444, 0.9087, 0.0282.

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Virulence models. Excitingly, these peptides not only prevented the onset of infection but, in the oral candidiasis model, effectively treated an established infection. They also reduced adhesion in vitro and in intravenous catheters implanted into rats, which models a key route of infection for human patients. Finally, EntV and the 12 mer displayed efficacy in a systematic mouse model that mimics deadly human bloodstream infections. No apparent toxicity was seen in the mouse model, and our previous study observed no toxicity when EntV was incubated with human and mouse cells. Interestingly, these shorter peptides no longer have antibacterial activity as measured in the L. sakei assay (Supplementary Fig 2). Furthermore, despite its robust protection against infection, EntV is not toxic to C. albicans. Growth of C. albicans was unaffected when EntV was added to shaking cultures in concentrations as high as 30 μM. Rather than being fungicidal or fungistatic, EntV acts as an “anti-virulence” factor, interfering with key fungal virulence traits, including adhesion, morphogenesis, and biofilm formation. These have been linked to pathogenesis in a variety of fungal species, as well as in bacterial and protozoal pathogens.

**Fig. 6 | The 12mer is protective in models of venous catheter and systemic C. albicans infection.** In a, b, intravenous catheters were implanted into the jugular veins of rats and inoculated with C. albicans with or without EntV peptides using an n of three biologically independent animals. After 24 h the catheters were removed and CFUs were determined from one-half (left panels) and scanning electron micrographs were taken at 100x (left) or 1000x (right) of the other half of the catheters (right panels) with a showing treatment with EntV at 100 nM and b showing treatment with the 12mer at 20 nM. The dashed lines are the detection limit of this assay (100 CFU). The mean of the CFUs were calculated and the error bars represent the standard deviations (SD). An unpaired, two-tailed t test was used to compare the treatments to the controls and the exact p values were <0.0001 for both. C. albicans was incubated for 2 h in PBS with DMSO, EntV, or the 12mer prior to inoculation via the lateral tail vein. An n of 10 biologically independent animals was used for each group. When moribund, animals were humanely sacrificed. Survival was compared to the DMSO control by Mantel-Cox log rank analysis and the exact p values were <0.0001 and <0.0001.
The bacteriocin activity of EntV is non-lytic and therefore unlikely to form pores\(^{36}\). All four \(\alpha\)-helices of EntV were required for this activity against \(L. \text{sakei}\), although other bacterial species may respond differently and further in vitro, and microbiome study are necessary to determine the spectrum of activity. In contrast, \(\alpha_7\) is all that is required for antifungal activity. Further mutational analysis and combinatorial library screening of this peptide is underway to better understand the features necessary for activity. Additionally, how EntV and derivative peptides exert antifungal activity is actively under study and preliminary evidence suggests binding to the fungal cell surface, which would be consistent with its ability to inhibit adhesion and invasion. Because adhesion is important for many fungal pathogens, this gives some optimism that it might be active against a broad array of species beyond what was demonstrated here. Further studies will be needed to better define the mechanism, species range, and target of this intriguing peptide.

The potential of peptides to broaden the pool of antimicrobial agents has been increasingly recognized. Two of the three classes of current antifungals are modified peptides (the polyenes and echinocandins) and a variety of other peptides have been investigated for their antifungal activity\(^{34}\). Included are several mammalian antimicrobial peptides active against fungi, such as Histatin-5 and CRAMP/L1-37. In this vein, peptides derived from EntV are promising leads for further optimization and development.

**Methods**

**Ethics statement**

Vertebrate animal experiments were conducted under protocols approved by the Animal Welfare Committee of the University of Wisconsin (rat catheter, protocol number DA0031) or The University of Texas Health Science Center at Houston (mouse OPC and mouse IV, protocol numbers AWC-18-0078 and AWC-21-0047) according to the guidelines of the Animal Welfare Act, The Institute of Laboratory Animals Resources Guide for the Care and Use of Laboratory Animals, and Public Health Service Policy. Animals were housed at 22 °C, ambient humidity, and with a 12 h light–dark cycle.

**Strains and media**

Fungal strains were routinely propagated in YPD medium (1% yeast extract, 2% peptone, 2% dextrose). Most experiments used \(C. \text{albicans}\) wild-type strain SC5314\(^{35}\). For the adhesion assays the non-adherent strain HLC54 (\(\Delta \text{hisG/EFG1/URA3/hisG/ura3}\))\(^{21}\) was used as a negative control. \(C. \text{aureus}\) strain AR-382 (CDC Antimicrobial Resistance Bank), \(C. \text{neoforans}\) strain 40-71 (a gift of L. Ostrosky) and \(C. \text{albicans}\) strains TFPY412 and TFPY1002 (gifts of T. Patterson and J. Lopez-Ribot\(^{27}\)) were used in the assays shown in Fig. 4.

**Protein expression and purification**

Strain CE61, a BL21 (DE3) derivative containing pET28a encoding EntV\(^{13,14}\), was used for overexpression. 10 mL overnight culture was diluted into 1 L M9 SeMET (selenomethionine) media containing 0.5 mM TCEP and 5 mM imidazol. To remove the His-tag, this was applied to a second Ni-NTA column and the flow-through was collected, then the tag-free protein was dialysed against crystallization buffer (10 mM HEPES pH 7.5, 300 mM NaCl), and the purity of the protein was analyzed by SDS-polyacrylamide gel electrophoresis.

**Crystallization and structure determination**

The EntV\(^{136}\) crystal was grown at room temperature using the vapor diffusion sitting drop method using 2 \(\mu\)L of a 25 mg/mL protein solution, 0.1 M Tris pH 8 and 28% (v/v) PEG4K. The crystal was cryoprotected with paratone-N oil prior to flash freezing in liquid nitrogen. Diffraction data at 100 K at beamline 19-ID of the Structural Biology Center of the Advanced Photon Source, Argonne National Laboratory, HKL3000\(^{16}\) was used to process two Se-Met diffraction data sets. Computational corrections for absorption in a crystal and Lorentz factor were applied\(^{18,20}\). Anisotropic diffraction correction was necessary for structure solution\(^{19,37,38}\). Indexing, integration, and scaling indicated \(C2\) symmetry. The best Se-Met crystal, used in the refinement, diffracted to a nominal resolution of 1.8 Å but diffraction was highly anisotropic, with (200) and (110) Patterson extending to 1.9 Å resolution in the \(a\) direction (<I>/<\(\sigma\)) ~2 and to a resolution much higher than 1.8 Å in the \(b\) and \(c\) directions (<I>/<\(\sigma\)) ~19 and ~13, respectively. Initial phases were obtained from a single Se-Met crystal in a single-wavelength anomalous diffraction (SAD) experiment by performing heavy atom search to resolution 2.2 Å using SHELXD\(^{19}\). The search identified 2 Se positions with correlations coefficients: \(C_{\text{calc}} = 31.3\%\), \(C_{\text{calc}} = 25.9\%\), and \(C_{\text{calc}} = 57.2\%\) with relative occupancies of 1.000 and 0.660 with the handedness of the best solution was determined with SHELXE. The heavy atom positions were refined to 1.8 Å with MLPHARE\(^{29}\), with the final FOM reaching 0.180 for all observations. Density modification was performed with DM\(^{30,31}\). The resulting electron density map was used as the entry model for the model building with BUCCANER\(^{32}\) and refinement with REFMAC\(^{33}\), run within HKL3000. The resulting main chain was ~82% complete (116 aa), with ~79% of side chains docked into electron density maps (112 aa), and R factor = 23.3% and \(R_{\text{free}} = 24.3\%\). The resulting assembly was used to perform isomorphous replacement with another data set using MOLREP\(^{34}\) run within HKL3000, and rebuilt and refined again with BUCCANER and REFMAC run within HKL3000. The resulting main chain was ~90% complete (127 aa) with ~80% of side chains docked into electron density maps (113 aa), and R factor = 23.4% and \(R_{\text{free}} = 27.7\%\). Refinement was completed using Phenix.refine\(^{35}\) and Coot\(^{36}\). B-factors were refined as anisotropic for protein atoms and isotropic for non-protein atoms and TLS parameterization was included in the refinement. Average B-factor and bond angle/length RMSD values were calculated using Phenix. All geometry was verified using the Phenix, Coot and wwPDB validation tools. The X-ray crystallographic statistics are in Supplementary Table 1 and the structure was deposited to the Protein Data Bank under the accession code 7ROA.

**Peptide synthesis and alkylation**

Peptides were synthesized and verified by Bio-Synthesis Inc (Lewisville, TX) or prepared in-house using standard Solid Phase Peptide Synthesis (SPPS) protocols. Starting from the C-terminal residue, the peptides were synthesized on Tentagel S-Ram 0.2–0.8 mg/g beads (from Chem Impex). FMOC protected peptide monomers (from Advanced ChemTech) were dissolved in dimethylformamide (DMF) at roughly a 3x molar excess (0.528 mM) relative to the manufacturer’s stated loading capacity (0.22 mM). The reactions were catalyzed by the addition of Hexafluorophosphosphate Benzotriazolyl Tetramethyl Uronium (HBTU) (200.11 mg per residue addition), Hydroxybenzotriazole (HOBt) (71.28 mg per residue addition), and N,N-Diisopropylethylamine (DIEA) (100.34 mg per residue addition).
(DIP EA) (68 µL per residue addition). Reaction completion for each residue was confirmed by conducting a ninhydrin test on a small sample of beads taken from the reaction vessel after washing the beads with DMF three times to clean any residual or unreacted components. Following completion of amino acid addition, the beads underwent a final deprotection to removable the Fmoc group and then were washed with diethyl ether (DCM). Acid cleavage from the beads was accomplished by using Reagent B (88% v/v trifluoroacetic acid, 5% v/v phenol, 5% v/v dithio H2O, 2% v/v triisopropyl silane). The volume of cleavage solution used was roughly 6 ml per reaction vessel. Synthesis quality was verified by performing HPLC and mass spectrometry. Prior to analysis, the peptides were ether precipitated for purification, dissolving in DMSO, and lyophilized. Yields of purified peptides, as a result of synthesis, ranged from 15 to 30 mg total. Peptide alkylation was conducted by reacting at least a 5x molar excess of iodoacetamide (0.4 M) to the wild type 12mer variant of EntV (1000 µM) for 48 h. The reaction was confirmed by monitoring the retention times of the peptides via HPLC. The product was then pooled by HPLC fraction collection and then dried down for use once the product weight was confirmed by mass spectrometry. All in-house and Bio-Synthesis verification of the peptides by HPLC and mass spectrometry is presented in Supplementary Data 2. The biophysical characteristics of the peptides are presented in Supplementary Table 2.

C. elegans killing assay

C. elegans infection assays were performed with the glp-4(2m); sek-1(km-4) nematodes and maintained on E. coli OP50 that was seeded onto nematode growth medium (NGM) agar using standard techniques (Hope, 1999). To synchronize the animals, L1 stage worms on non-starved plates were washed off, filtered through a 10 µm filter (pluriSelect, pluriStrainer 10 µm), harvested by centrifugation at 750 xg for 30 seconds, transferred to OP50 seeded plates, and grown to the L4 stage. To prepare the infection plates, Fungal strains were grown in YPD broth for 24 h at 30°C without agitation. Following adjustment to a concentration of 1 x 10^6 cells/ml, cells were diluted 1:10 into fresh MRS medium containing the indicated concentrations of EntV fragments. After 24 h of growth at 30°C without agitation, OD_{595} readings were taken using a BioTek Cytation5 plate reader. Readings of blanks (containing fresh MRS medium) were subtracted from sample wells.

Lactobacillus MIC assay

For this assay, Lactobacillus sakei ATCC 15521 was used following the methodology as described with a few modifications. Briefly L. sakei was grown in Lactobacilli MRS broth (Difco, Detroit, MI, USA) for 18 h at 30°C without agitation. Following adjustment to a concentration of 1 x 10^6 cells/ml, cells were diluted 1:10 into fresh MRS medium. Peptide serum stability assay

One mL of RPMI supplemented with 10% (v/v) of human serum (type AB) were aliquoted and temperature-equilibrated at 37°C for 15 min before adding a concentrated stock peptide solution resulting in a final peptide concentration of 100 µM. For each time point, 100 µL of the reaction solution was removed and added to 200 µL 96% ethanol for precipitation of serum proteins. The samples were cooled (4°C) for 15 min and then spun at 18,000 g for 2 min to pellet the precipitated serum proteins. The reaction supernatant was analyzed using HPLC with absorbance detected at 280 nm. The peptide peak was detected first without serum treatment to assess the area under the curve for the peptide of interest. At each time point the area under the curve was calculated by HPLC and then divided by the untreated peptide area under the curve to determine the percent remaining.

Mouse OPC model

The efficacy of EntV fragments were tested in the OPC model BALB/c mice, 10 wks, 18-20 g, were immunosuppressed by injecting 225 mg/kg cortisone acetate subcutaneously 1 d before inoculation, and subsequently on days 1 and 3 of the infection. To prepare the inoculum, 1 mL of C. albicans (SCS314) overnight culture grown at 30°C in YPD broth was washed twice in PBS before resuspension in sterile Hanks’ balanced salt solution (HBSS) at a concentration of 1 x 10^6 cells/mL. Calcium alginate swabs were soaked in this inoculum for 5 min prior to inoculation. Mice were anesthetized using ketamine and xylazine and placed on pre-warmed isothermal pads and the swabs were placed sublingually for 75 min. Mice were given additional doses of ketamine (50 mg/kg), as necessary. After inoculation, mice were given drinking water with EntV fragments or DMSO (0.01%) in the drinking water ad libitum. Mice were euthanized at 5 d after inoculation, unless noted. The tongues were excised and cut in half laterally for tissue histology and assessment of fungal burden.

For tissue histology the tongue sample was placed in 10% zinc-buffered formalin overnight and stored in 80% ethanol, before...
embedding in paraffin. For each tongue, 5 μm sections were prepared using a Leica microtome and stained using Periodic Acid-Schiff (PAS) stain. Epithelial invasion was measured from 40x images taken of the entire tissue section of each tongue half with the total area of epithelium and infected epithelium were measured using ImageJ (version 1.6). Measurements were totaled and expressed as a percentage of total infected epithelium relative to the entire epithelial area.23,27

For examination of fungal burden, qPCR was used by amplifying a 269-bp fragment of the internal transcribed spacer 2 (ITS2) between the 5.8 S and 28 S ribosomal RNA genes of C. albicans using oligonucleotides GTGATACGTGARTTTGAAC and TATGTTAATTTAGCAGCGGTGA, as the forward and reverse primers, respectively.24,25 DNA was extracted from homogenized tongue tissue using the Yeast DNA Extraction Kit (Thermo Scientific) according to the protocol with minor modifications. The ITS2 fragment was amplified by qPCR with a CFX96 Real-Time System with a C1000 Touch thermal cycler (BioRad). C. albicans gDNA was quantified using FastStart Universal SYBR Green master mix with ROX (Roche). To screen for contamination and C. albicans DNA extraction from homogenized tongue tissue using the Yeast DNA Extraction Kit (Thermo Scientific) according to the protocol with minor modifications. The ITS2 fragment was amplified by qPCR with a CFX96 Real-Time System with a C1000 Touch thermal cycler (BioRad). C. albicans gDNA was quantified using FastStart Universal SYBR Green master mix with ROX (Roche). To screen for contamination and C. albicans

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Author contributions
Conceptualization: DAG, MCL. Methodology: DAG, MCL, DRA, PJS, PF, MRC, SC, SG, GBC, EE, HS, DB, PM, JY. Investigation: MRC, SC, SG, GBC, EE, HS, DB, PM, JY. Funding acquisition: DAG, MCL, AS. Project administration: DAG, MCL, AS. Supervision: DAG, MCL, DRA, AS. Visualization: DAG, MCL, MRC, SC, PJS Writing – original draft: DAG, MCL, MRC, SC, SG, PJS Writing – review & editing: DAG, MCL, DRA, PJS, PF, MRC, SC, SG, GBC

Competing interests
DAG, MCL and SG are named on patent application UTSH.P0383US.P1 related to the peptides described in this work. The other authors declare no competing interests.

Additional information
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Correspondence and requests for materials should be addressed to Michael C. Lorenz or Danielle A. Garsin.

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