# Human Antimicrobial Peptide LL-37 Inhibits Adherence of *Candida albicans* via Interaction with Cell Wall Carbohydrates

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**Abstract**

*Candida albicans* is the major fungal pathogen of humans. Fungal adherence to host cells is the critical first step during mucosal infection. Antimicrobial peptides play important roles in the first line of mucosal immunity against *C. albicans* infection. LL-37 is the only member of human cathelicidin family and is commonly expressed in various tissues and cells, including epithelial cells of oral cavity and urogenital tract. Here we found that except candidacidal activity, LL-37 significantly reduced adherence of *C. albicans* to plastic surfaces, oral epidermoid OECM-1 cells and urinary bladders of female BALB/c mice. Moreover, the non-adherent floating *C. albicans* cells aggregated to form clumps and associated with LL-37. According to competition assay, the inhibitory effect of LL-37 on cell adhesion and induction of cell aggregation were mediated through its preferential binding to mannan, a main component of *C. albicans* cell wall and partially through its binding to chitin or glucan. These results suggest that targeting on cell wall carbohydrates provides new strategy to prevent *C. albicans* attachment and LL-37 is useful as a new tool to screen for other important components involved in *C. albicans* adherence.

## Introduction

*Candida albicans* is a major fungal pathogen in humans. *C. albicans* is commensal on cutaneous and mucosal surfaces of oral, gastrointestinal, urinary and vaginal tracts of healthy individuals [1,2]. However, *C. albicans* is an opportunistic pathogen and can cause infections ranging from superficial mucosal infections to hematogenously disseminated candidiasis. In immunocompromised patients, *C. albicans* is responsible for a number of life-threatening infections [1,2]. Moreover, with rapidly expanding use of medical devices such as indwelling catheters and increases in the number of patients receiving antibiotic and immunosuppressive therapy, there is increased risk of pathogen penetration of mucosal barriers and entry into the blood stream, which often lead to multi-organ infections. As a consequence, *C. albicans* has become a leading cause of nosocomial bloodstream infections with a mortality rate of 40% [3,4].

Studies of *C. albicans* pathogenesis suggest several phases of mucosal infection, including early colonization, invasion, and late tissue disruption phases [5,6]. *C. albicans* initiates an infection by interacting with host epithelial cells to colonize and proliferate on mucosal surfaces, followed by invasion and tissue damage [7,8]. Therefore, *C. albicans* adhesion is the critical first step in this process and is essential for the pathogen to persist on mucosal surfaces. As the outermost layer of the cell, *C. albicans* cell wall participates in the interaction between pathogen and host cells [9]. *C. albicans* expresses multiple surface components (e.g. carbohydrates and proteins) and structures that come into contact with epithelial cells and facilitate cell-cell interconnections [10,11].

Host cells defend against candidal infection by producing cytokines [12], chemokines [13] and antimicrobial peptides (AMPs) [14,15]. Human AMPs play important roles in the first line of mucosal immunity [16,17]. AMPs are generally short (10 to 50 amino acid residues), positively charged (generally +2 to +9), and contain ≥30% hydrophobic residues [18]. Because of these properties, the folded peptides exhibit an amphiphilic structure and

embed into microbial membranes to form pores, causing membrane rupture and eventual microbial death [19]. Recent studies indicate that AMPs can also inhibit biosynthesis of cell walls, nucleic acids, and proteins, and can inhibit the activity of microbial enzymes [19,20,21,22]. In humans, different types of AMPs are synthesized and secreted by various cells and tissues, including skin, mucosal surfaces, neutrophils, and epithelia [23].

LL-37 is the only member of the human cathelicidin family of AMPs [24]. LL-37 is stored in specific granules of neutrophils as an inactive proform that can be cleaved extracellularly to yield the mature active peptide [25]. In addition to exhibiting a broad spectrum of antimicrobial activity against bacteria and fungi, LL-37 has other activities related to host defense, including chemotactic migration, endotoxin neutralization, angiogenesis, and wound healing [26,27]. Up to now, study of the effects of LL-37 on *C. albicans* is relatively scarce. In a study of the candidacidal activity of AMPs, LL-37 is found to remain associate with the cell surface of *C. albicans*, while other AMPs (as exampled by histatin 5) can translocate over the membrane and accumulates intracellularly [28].

In this study, we further investigated the effects of LL-37 on *C. albicans*. We show that LL-37 inhibited the adherence activity of *C. albicans*. LL-37 significantly reduced *C. albicans* attachment not only to an abiotic surface but also onto oral epidermics and urinary bladders of mice. In addition, LL-37 associated with carbohydrates of *C. albicans* cell wall, followed by causing aggregation of *Candida* cells to protect the hosts from adherence of the pathogen. To our knowledge this is the first report of the human AMP LL-37 can interfere with adhesion of a human fungal pathogen. These results suggest new potential therapeutic strategies targeting cell wall carbohydrates or using AMPs to prevent *C. albicans* colonization and infection. Moreover, LL-37 may provide a new tool to screen for other adhesion molecules on the cell surface of *C. albicans* and other fungal pathogens.

## Results

### *Candida albicans* was killed by LL-37

The activity of LL-37 to kill *C. albicans* was measured by spot assay and FUN-1 analysis using flow cytometry (21). In the spot assay, the growth of cells with LL-37 treatment was compared to the control cells (without LL-37 treatment). It indicated that the cells were more sensitive to the treatments with higher concentrations of LL-37 (20 to 40 μg/ml) vs. the cells without or with lower concentrations (5 and 10 μg/ml) of LL-37 treatment (Fig. 1A). To have more quantitative measurement in cell mortality, FUN-1 was used to distinguish dead and live cells. In Fig. 1B, the cell death in LL-37 treatments was observed starting at the dosage of 20 μg/ml and was reached to approximately 60% for cells treated with 40 μg/ml LL-37. These results indicate that LL-37 has candidacidal effects against *C. albicans* and functions at a dose higher than 20 μg/ml.

### LL-37 affects adhesion of *C. albicans* to polystyrene dishes

During our study of cell exposure to LL-37, we found that *C. albicans* with the treatment of 5 μg/ml LL-37 was much easily to be spun down by centrifugation and did not adhere to centrifuge tubes as the control cells did (Fig. S1). Cell adherence is an important step for *C. albicans* to colonize on host tissues and to establish infection. Adherence is conferred by *C. albicans* adhesins that bind to amino acid or sugar residues on the surface of target cells, and may also promote cell binding to the surface of abiotic substrates such as plastic prostheses and catheters [29]. We thus suspected that LL-37 might affect interactions between *Candida* cells and substratum. To test this hypothesis, *C. albicans* were treated with sub-lethal doses of LL-37 (0.1-10 μg/ml) to exclude cell killing by LL-37, and the cell attachment to polystyrene dishes was determined by measuring XTT reduction and whole-cell enzyme-linked immunosorbent assay (ELISA). *C. albicans* attachment to polystyrene dishes was significantly reduced by LL-37 in a dose-dependent manner (Fig. 2A). The floating cells

were also collected and spotted onto YPD agar plate to monitor cell viability. The numbers of colonies derived from these non-adherent cells raised was dependent upon the increase of LL-37 concentration from 0.1 to 10 μg/ml (Fig. 2B), suggesting that LL-37 concentrations used in the *Candida* adhesion assays do not have a significant candidacidal activity. Together, based on Fig. 1 and 2A, very low doses of LL-37 had the potential to inhibit *Candida* cells adhering to polystyrene dishes and this inhibitory effect was not directly caused by the killing activity of LL-37 (Fig. 2B). This supposition further proposed that other mechanisms might contribute to this event. One of the mechanisms may be direct association between LL-37 and cells; this notion is evidenced by a strong aggregation observed between the non-adherent and floating cells treated with 5 μg/ml of LL-37 (Fig. 2C).

**LL-37 induces cell aggregation and reduces cell attachment to polystyrene dishes by direct binding to *C. albicans***

To determine whether LL-37 caused *Candida* cells aggregation and led to reduce cell attachment to plastic dishes, the floating non-adherent cells were collected after LL-37 treatment and observed under an optical microscope. The cells showed a higher degree of aggregation at higher concentrations of LL-37 (5-20 μg/ml), whereas cells were less aggregated at lower LL-37 concentrations (Fig. 3A). With LL-37 treatment, the cells that were non-adherent and floating from the supporting surface aggregated (Fig. 3A). Noting these dose-dependent effects of LL-37 on cell-cell interactions, we hypothesized that LL-37 may directly bind to *C. albicans* cell surface and alter adherence of the pathogen to substratum surfaces.

To test this possibility, LL-37 binding assays were performed. *C. albicans* cells were treated with BA-LL37, and the non-adherent and floating cells were collected. BA-LL37 was bound to these cells, as assessed by western blot and detected using SA-HRP. As shown in Fig. 3B, direct binding of LL-37 to non-adherent cells was obviously observed at 10 and 20 μg/ml. The low numbers of floating cells might be the cause of non-detectable LL37-binding

at lower doses. To overcome the unfavorable detection sensitivity of immunoblot, we further measured LL-37 binding by flow cytometry using SA-4,6-dichlorotriazinyl aminofluorescein. Figure 3C showed that BA-LL37 binding to *C. albicans* cells could be observed at the dose as low as 1 μg/ml of LL-37. These results strongly indicated that LL-37 directly binds to *C. albicans* cells and induces cell aggregation, resulting in cell de-adherence to supporting surfaces.

To investigate if the inhibitory effect on *C. albicans* adhesion was specific for LL-37, two different types of AMPs, human β-defensin 3 (hBD-3) and histatin 5 (Hst 5), were also included in the *C. albicans* adhesion assay. Hst 5 has a linear non-helical structure and hBD-3 folds into β-sheet, whereas LL-37 has a helical conformation [30]. All the three AMPs are reported to have candidacidal activity. Because the length of each AMP is different, corresponding molar concentration for each AMP was employed. After *C. albicans* cells were independently incubated with 2.12 μM of BA-hBD3, BA-Hst 5 or BA-LL37 (2.12 μM of BA-LL37 is approximately equal to 10 μg/ml of LL-37), and the XTT reduction assay and whole-cell ELISA were performed. As shown in Fig. 4A, BA-Hst 5 and BA-hBD3 reduced *C. albicans* cell adhesion to polystyrene dishes by 10-30%, whereas BA-LL37 largely reduced *C. albicans* adhesion by 60-80% compared to the control cells. In addition, AMP-induced aggregation of the un-adherent floating cells was examined by microscopy and aggregation clumps were counted. BA-LL37 caused the strongest cell aggregation and three big aggregation clumps were observed, while BA-hBD3 only led to two small cell aggregation clumps (Fig. 4B). Besides, BA-Hst 5 induced very small and few cell aggregations, no obvious aggregation clump was noted (Fig. 4B). Flow cytometry was also used to assess the binding of different AMPs to *Candida* cells. Base on the intensity of fluorescence, cells bound by BA-hBD3 and BA-Hst 5 were only 10-15% of that by BA-LL37 (Fig. 4C). To exclude the possibility that the inhibitory effect of AMPs on *Candida* adhesion were caused by microbial killing, cells were incubated with the same molar concentration of AMPs for

adhesion assay and the viability of cells were further determined by spot assay. The killing activity was not observed in the BA-LL37 or BA-Hst 5-treated cells, although a slight killing effect was seen in *Candida* cells treated with BA-hBD3 (Fig. 4D). In combination with our results of cell adhesion inhibition, cell aggregation induction and peptide-cell association (Fig. 4), these results indicated that AMP binding to *Candida* cells, leading to cell aggregation and resulting in the inhibition of *C. albicans* adhesion to plastic were unique for LL-37.

**LL-37 binds to *C. albicans* cell wall polysaccharides and reduces *C. albicans* adherence**

Cell wall is the outmost layer of *C. albicans* that interacts with the environment and host cells [31]. The cell wall of *C. albicans* contains approximately 80 to 90% different types of carbohydrates [2]. In a previous study, LL-37 was shown to contain an XBBXBX-motif (where X represents hydrophobic or uncharged amino acids, and B represents basic amino acids), which allowed LL-37 to bind to glycosaminoglycans, e.g., heparin and dermatan sulfate [32]. In order to evaluate the role of carbohydrates in LL37-mediated inhibition of *C. albicans* adherence, removal of carbohydrates from cell wall was performed as described in Materials and Methods. Concanavalin A (Con A) is a protein specially reacting with α-D-mannose and α-D-glucose and BA-Con A was used to monitor the efficiency of deglycosylation. In Fig. 5A, the association of BA-Con A with *C. albicans* cells was decreased about 50%, representing a removal of half carbohydrates from cell wall. In this deglycosylation condition, the binding of BA-LL37 to the *C. albicans* cells was reduced about 30% compared to the non-deglycosylated cells. Moreover, co-pull down assays was carried out to verify the interaction between LL-37 and polysaccharides. The same linkages of glucan and chitin as that in *C. albicans* cell wall were used. Mannan-agarose, glucan and chitin were incubated with BA-LL37 and the unbound peptide was separated from carbohydrates by centrifugation. As shown in Fig. 5B, LL-37 bound directly to mannan-coupled agarose beads, chitin and glucan. The interacting intensity of the polysaccharides with LL-37 was dose-dependent.

To further determine the types of carbohydrates as the target of LL-37 binding, three monosaccharides consist of *C. albicans* cell wall, D-glucose, *N*-acetyl-D-glucosamine, and mannose [33] were used for competition with LL-37 in the cell adhesion assay. Our results indicated that these three monosaccharides (400 μM each) could compete with LL-37 in inhibiting *C. albicans* adhesion (Fig. 5C). However, polysaccharides in reality account for >90% of the *C. albicans* cell wall [34], and therefore polysaccharides were also tested in the adhesion assay. As previously study described [35] that mannan of *C. albicans* itself enhanced adherence to plastic, thus the cell adhesion blocked by LL-37 was almost completely rescued in the presence of *C. albicans* mannan (Fig. 5D). On the other hand, chitin, glucan and *S. cerevisiae* mannan also abolished the adhesion inhibition of LL-37 (Fig. 5D) but exhibited minor ability on aggregation elimination (Fig. 5E). The aggregation of non-adherent and floating cells induced by LL-37 was largely reduced in the presence of *C. albicans* mannan (Fig. 5E). Nevertheless, the cell aggregations induced by LL-37 were slightly reduced in the medium containing chitin, glucan or *S. cerevisiae* mannan (Fig. 5E). In addition, the binding affinities between mannans and LL-37 were determined, and the dissociation constants ($K_d$) were measured by a high sensitive biosensor system. The $K_d$ values for mannan-LL37 complex were averaged from at least two independent experiments. LL-37 bound to *C. albicans* mannan with a $K_d$ = 0.434 ± 0.146 μM. However, *S. cerevisiae* mannan reduced the binding affinity by 11-fold ($K_d$ = 4.935 ± 0.44 μM), indicating the favor binding of LL-37 to *C. albicans* mannan than to *S. cerevisiae* mannan. Together, these results demonstrated that LL-37 has the ability to bind to polysaccharides on the *C. albicans* cell wall and results in *Candida* cells aggregation and reducing of cell adherence.

**LL-37 effects on adhesion of *C. albicans* to oral epidermal cells and urinary bladders of mice**

To *in vivo* correlate the effect of LL-37 on *C. albicans* adhesion, we examined *C. albicans* adherence onto an oral epidermal cell and the urinary tract of a mouse model in the presence

of LL-37 [36]. *Candida albicans* is related to human oral infections [37] and LL-37 is reported to play a role in preventing microbial infection in oral cavity [14]. To determine the capacity of *C. albicans* to adhere to the oral epidermal OECM-1 cells, cell ELISA was carried out. After pre-incubation with LL-37, LL37-bound *Candida* cells were added to the host cell for further incubation. The adherence of the LL37-treated *Candida* cells was significantly reduced (Fig. 6A). In *C. albicans* treated with 20 μg/ml of LL-37, cell adherence was reduced more than 70% compared to that of the control without LL-37 (Fig. 6A). In addition, *Candida* species is often isolated from urine of the patients who are suffering from microbial colonization in the bladder or urinary tract infection [38]. To determine the effects of LL-37 on *C. albicans* adherence within the host, we therefore used a mice model of urinary tract infection. LL-37 and *C. albicans* were mixed and then injected into the urinary tract of mice. After injection, *C. albicans* cells were allowed to attach to bladder mucosa for 1 h, and the numbers of *C. albicans* cells that adhered to bladders were quantified as colony-forming units on agar plates. As shown in Fig. 6B, *C. albicans* cells treated with LL-37 showed a significant reduction in their attachment to bladders of mice than control cells ($p = 0.0011$). These results indicated that LL-37 inhibited *C. albicans* adherence both *in vitro* and *in vivo*.

## Discussion

Human LL-37 is a multifunctional AMP and is commonly found at mucosal surfaces with a concentration around 2-5 μg/ml [39,40]. However, its expression can reach 30-1,500 μg/ml at some local sites during the process of infection or inflammation in a variety of tissues [41,42]. Recently, the effects of LL-37 on infection have been the focus of much research on bacterial pathogens. One study showed that, in the presence of pathogens, human lung epithelia respond by rapidly increasing LL-37 secretion onto the airway surfaces, which subsequently kills the nearby microbes [16]. Bacterial contact with epithelial cells of the urinary tract results in rapid production and secretion of human LL-37 into urine [36]. Although this epithelium-derived LL-37 substantially contributes to the protection of urinary tract against infection [36], the molecular basis of this host defense mechanism is still mostly unknown.

In this study, in addition to the microbial killing activity, we demonstrated a new role for human AMP LL-37 against fungal pathogens, in preventing *C. albicans* adherence. Similar effects of LL-37 have been shown to prevent the urinary tract from *E. coli* adherence [36] and to inhibit attachment and biofilm formation of *Pseudomonas aeruginosa* and *Staphylococcus epidermidis* [41,43] at the initial process of bacteria-host interaction. However, to our knowledge, this work is the first report that AMPs can block adherence of a fungal pathogen. We showed that physiological concentrations of LL-37 prevent the adherence of *C. albicans* to abiotic and epithelial surfaces *in vitro* and *in vivo*. The effect appeared to be specific to LL-37 because Hst 5 and hBD-3, two other candidacidal AMPs, did not have the same degree of inhibitory effect on cell adherence and did not bind *C. albicans* to the same extent (Fig. 4A and 4C). LL-37, hBD-3, and Hst 5 carry positive charges of +6, +11 and +12, respectively, at physiological pH [30]. In addition, recombinant LL-37 has a propensity to aggregate during purification [44], it is feasible that aggregation among un-adherent cells involved direct binding of LL-37 to the cell surfaces, leading to cellular aggregation (Fig. 4B). Figure 4D

shows a weaker *Candida*-killing potency of BA-hBD3 than that of BA-LL37 and BA-Hst 5 (comparison using the same dose of these AMPs). Together, these data suggest that LL-37-mediated inhibition of *C. albicans* adhesion was possibly not related to its positive charge or killing activity, but rather due to its interaction with the cell surface of *C. albicans*. After subsequent, these cells aggregation and lost their ability to adhere to substratum.

In the effort to identify components of *C. albicans* surface that may interact with LL-37, we found that certain cell wall carbohydrates are involved. Associations of AMPs with carbohydrates and importance of these associations in microbial infection have been reported. Foschiatti *et al.* showed that bacteria use exopolysaccharides to bind and segregate AMPs, thus reducing the efficiency of the primary innate host defenses [45]. Bergsson *et al.* showed that LL-37 complexes with glycosaminoglycans in cystic fibrosis lungs and inhibit its *Pseudomonas* killing activity [46]. In our study, the association of LL-37 and *C. albicans* cell wall carbohydrates led to the inhibition of *Candida* adherence both *in vitro* and *in vivo*. The abiliity of adhesion is crucial for *C. albicans* to form biofilm on an abiotic surface and to colonize on a host tissue during the process of infection [10]. Interestingly, LL-37 could associate with chitin, glucan and mannan from *S. cerevisiae*, while only *C. albicans* mannan could completely rescue the LL37-mediated cell aggregation and inhibition of cell adherence (Fig. 5B, 5D and 5E). A major difference between these two mannans is that *C. albicans* mannan contains β-1,2 linkages but *S. cerevisiae* mannan does not [47]. The β-1,2-linked mannans have been shown to act as adhesins for *Candida* interaction with buccal epithelium [35] and can be recognized by galectin-3, inducing death of *Candida* species [48]. In an *ex vivo* assay, a monoclonal antibody that recognizes β-1,2-mannans inhibits *C. albicans* attachment to murine splenic marginal-zone macrophages [49]. Dromer *et al.* demonstrated that synthetic analogs of β-1,2-oligomannosides prevent intestinal colonization by *C. albicans* [50]. Our results indicate that the reduced cell adhesion of LL-37 specifically competed by *C. albicans* mannan, suggesting that mannan bound by LL-37 may be related to its β-1,2-

linkages. This hypothesis will be interesting to be further studied. Since higher eukaryotic cells do not have a cell wall and LL-37 exerts its effects through the association with components of the cell wall, these components may be possible drug targets against *Candida* infection.

Although carbohydrates play some roles in the interactions with LL-37, as shown in Fig. 5A, BA-LL37 only reduced the adherence of 30% of the cells that were estimated to be approximately 50% being deglycosylated. Therefore, we cannot completely exclude that LL-37 may also interact with other components of the cell wall, such as proteins. Proteins are estimated to represent approximately 20-30% of the fungal cell wall by mass [11]. Particularly, mannan covalently associates with proteins (mannoproteins) and represents about 40% of the total polysaccharides on the surface of *C. albicans* [51]. Mannoproteins are considered to be involved in cell-cell recognition and trigger immune responses [52]. Several studies have showed that the functions of AMPs are via their interactions with microbial proteins. In the anaerobic bacteria *Finegoldia magna*, a protein called FAF (*F. magna* adhesion factor) blocks the killing activity of LL-37 and promotes colonization and survival of *F. magna* in the human host [53]. However, hBD-3 binds to immobilized recombinant hemagglutinin B, a non-fimbrial adhesin from *Porphyromonas gingivalis* [54,55]. Binding of hBD-3 to adhesin may prevent bacterial adherence to host tissues [55]. The possible *C. albicans* protein targets for LL-37 and the significance of their associations are currently under investigation.

Study of *C. albicans* cell wall indicate that glucan and chitin layers are buried underneath a thin but dense mannan coat [11]. In our study, in addition to interact with mannan, LL-37 also associates with chitin and glucan (Fig. 5B). Moreover, compared to mannan, chitin and glucan showed much less impact on the rescue of LL37-mediated cell aggregation and inhibition of cell adherence (Fig. 5D and 5E). Although this result supports the previous findings that mannan is the most important adhering glycan of *C. albicans* [56]

and the loss of phosphomannan is correlated with reduced levels of AMP-binding to the cells [57], it also raises a possible mechanism of LL-37 to interfere cell wall remodeling of *C. albicans*. It is considered that any carbohydrates expressed on cell surface of *C. albicans* was recognized and attacked by LL-37, leading carbohydrates degradation, impair of cell wall construction and then preventing the adhesion. Besides, the exposure of inner glucan during infection and drug (e.g. caspofungin) treatment has been reported in *C. albicans* [58,59]. To determine the possible effects of LL-37 on cell wall remodeling will be investigated in future studies.

Finally, AMP-mediated cellular aggregation has been previously reported. An AMP L8 causes cellular aggregation of *Escherichia coli* and *Listeria monocytogenes*, two important food-borne pathogens [60]. In addition, human neutrophil defensins induce aggregation of *E. coli* and *Staphylococcus aureus* and may, in part, increase the neutrophil uptake of bacteria [61]. Similarly, human β-defensins bind to bacterial components and enhance internalization of microbial antigens to dendritic cells [62]. In this work, except that LL-37 binding to *C. albicans* cells and reducing the cell adherence to a plastic surface, strong aggregation among non-adherent and floating cells was recognized. This cellular aggregation was mediated by LL-37 binding to Candida cells (Fig.2 and 3). 刪除以下劃線We thus hypothesize that LL-37 may also play a role to recruit phagocytes and cellular aggregation may help the phagocytic cells to recognize the pathogen. On the other hand, we thus hypothesize that LL37-mediated aggregation might also lead *C. albicans* to escape from LL-37 attack. These effects need to be further proved.

In summary, our results revealed that LL-37 prevented *C. albicans* adherence, in part via its interaction with *Candida* cell wall carbohydrates. Our study highlights a great potential for LL-37 to be an efficient therapeutic or preventive strategy against *Candida* infection. *Candida* polysaccharides could also serve as a target to develop monoclonal antibodies or short peptides to block the critical first step of microbial adhesion in the infection process.

## Materials and Methods

### Peptides and reagents

LL-37 (LLGDFFRKSKEKIGKEFKRIVQRIKDFLRNLVPRTES), biotinylated LL-37 (BA-LL37), biotinylated human β-defensin-3 (BA-hBD3, QKYYCRVRGGRCAVLSCLPKEEQIGKCSTRGRKCCRRKK), and biotinylated histatin 5 (BA-Hst 5, DSHAKRHHGYKRKFHEKHHSHRGY) were synthesized by MDBio, Inc. (Taipei, Taiwan). Analysis by HPLC and mass spectrometry revealed purities of 96-98% for the peptides. All reagents used in this study were obtained from Sigma-Aldrich unless otherwise mentioned.

### *C. albicans* strains, media, and growth conditions

The *C. albicans* SC5314 [63] cells were routinely maintained at –80ºC and plated on YPD agar (1% yeast extract, 2% Bacto-peptone, 2% glucose and 1.5% agar) before each experiment. A single colony was inoculated in YPD broth and incubated at 30°C overnight (12 to 14 h). This culture was then sub-cultured in YPD broth for approximately 2.5 h to reach logarithmic-phase growth. For LL-37 treatment, cells were washed twice with phosphate-buffered saline (PBS), collected by centrifugation, and resuspended in Gibco RPMI-1640 medium (Invitrogen, Carshad, CA), except in PBS for LL37-binding experiments. The carbohydrates of *C. albicans* cell wall were removed by using GlycoPro™ Enzymatic Deglycosylation Kit (Prozyme, San Leandro, CA) according to the manufacturer's instructions.

### Assay for candidacidal 刪除of activity

For the assay of LL-37 candidacidal activity, spot assay and the FUN-1 staining method were used. After 30 min incubation, treated or un-adherent cells were collected and prepared in 10-

fold serial dilutions and ten microliters of cells were spotted onto the YPD agar plates. The cell growth was observed after incubation at 30°C for 24 h.

FUN-1 staining was performed using the LIVE/DEAD® Yeast Viability Kit (Molecular Probes, Eugene, OR) according to the manufacturer's instructions and Kohatsu *et al* (21). Briefly, after LL-37 treatment for 30 min, *C. albicans* cells were harvested by centrifugation and resuspended in 500 μl of 10 mM HEPES supplemented with 2% glucose plus 5 μM FUN-1 for 30 min at 30°C in dark. Samples were then analyzed using a FACSCalibur™ analyzer (BD Bioscience, San Jose, CA) equipped with a diode laser (excitation wavelength 488 nm). The yellow-green fluorescence emission from cells with or without LL-37 treatment was determined at filter FL1 (515–545 nm). A total of 10,000 events were acquired per analysis. The mean fluorescence index (MFI) from LL37-treated cells was compared to the MFI of the control cells without LL-37 treatment. Percentages of dead cells from these comparisons were presented.

**_C. albicans_ adherence to polystyrene dishes**

Adherence of *C. albicans* was assayed in uncoated 24-well plates (Orange Scientific, Braine-I'Alleud, Belgium) or 96-well plates (Nunc™, Rochester, NY) as described [64,65]. For the XTT (2,3-bis-(2-methoxy-4-nitro-5-sulfophenyl)-2H-tetrazolium-5-carboxanilide) reduction assay*, C. albicans* cells were harvested, washed, and resuspended in RPMI-1640 medium to approximately $6 \times 10^7$ cells/ml. Next, 250 μl of cell suspension was transferred to each well of the 24-well flat-bottom plates. Different concentrations of LL-37 or other AMPs were independently added to the cell suspensions and incubated at 37°C for 30 min with shaking (100 rpm). The metabolic activity of sessile cells was then measured by detecting the reductive adduct of XTT [66]. Briefly, cells were washed three times with PBS to remove non-adherent cells. Adherent cells were incubated with 300 μl XTT (1 mg/ml) and 0.6 μl menadione (1 μM) in PBS per well at 37°C for 20 min. Absorbance at 490 nm was determined using a VICTOR3 Multilabel plate reader (PerkinElmer). For competition assays,

LL-37 was premixed with 400 μM monosaccharides or 1 mg/ml polysaccharides in RPMI for 30 min at 4°C before incubation with cells. All stock solutions of carbonhydrates were prepared in PBS. *Candida albicans* cell wall mannan was obtained from Takara Bio Inc. (Otsu, Japan). Mannan from *Saccharomyces cerevisiae* (M7054), glucan from *S. cerevisiae* (G5011) and chitin from crab shells (C9752) were purchased from Sigma-Aldrich.

Cell enzyme-linked immunosorbent assays (ELISA) were performed using a similar procedure, except that 100 μl of $5 \times 10^7$ cells/ml were used in each well of 96-well plates. The adherent cells were fixed with 3% paraformaldehyde and labeled with FITC-conjugated rabbit polyclonal antibody against *C. albicans* (Biodesign International, Saco, ME). Fluorescence was detected using a VICTOR3 Multilabel plate reader. The relative percentage of adherent cells was calculated as follows: [mean absorbance or fluorescence of (each LL-37 treatment)/(non-treatment) × 100%]. All assays were performed in triplicate and repeated three or four times.

**Microscopic observation of non-adherent *C. albicans* cells**

*Candida albicans* cells after incubated with different treatments were directly observed in 24-well tissue culture plates using an invertible microscope (Zoomkop, Taipei, Taiwan). Besides, non-adherent cells were collected and injected into μ-Slides VI flat (Ibidi, Martinsried, Germany). Samples were analyzed by using a light microscope (Zeiss).

**Assays of LL-37 binding to non-adherent and floating *C. albicans* cells**

*C. albicans* cells were suspended in PBS, mixed with BA-LL37 in 24-well tissue culture plates, and incubated at 4ºC for 30 min. Non-adherent cells were collected by centrifugation, suspended with reducing sample buffer, and denatured by heating at 100°C for 10 min. The samples were analyzed using 15% Tricine SDS-PAGE (prepared with 40% Acrylamide/Bis solution; MDBio, Inc., Taipei, Taiwan) and transferred onto polyvinylidene difluoride (PVDF) membranes (Pall Corporation, Port Washington, NY). BA-LL37 binding to *C.*

*albicans* was measured using horseradish peroxidase–conjugated streptavidin (SA-HRP) following the manufacturer's instructions (Zymed Laboratories, San Francisco, CA). The blots were processed using an ECL kit (PerkinElmer Life Sciences).

**Binding of antimicrobial peptides to *C. albicans* cells**

Detection of AMPs binding to *C. albicans* was carried out as described by Kohatsu *et al.* [48]. A total of $6 \times 10^6$ cells were mixed with different concentrations of BA-LL37 or 2.12 μM BA-LL37, BA-hBD3 or BA-Hst 5 in 750 μl PBS and incubated at 4ºC overnight. Cell-bound peptide was assessed by flow cytometry to detect SA-4,6-dichlorotriazinyl aminofluorescein (3 μg/reaction; Jackson ImmunoResearch, West Grove, PA). Reactions were analyzed using a FACSCalibur™ flow cytometer. Fluorescence data for 10,000 cells were acquired per analysis. The relative levels of AMP-binding to cells (in percentages) were obtained by comparing MFI from BA-hBD3 and BA-Hst 5-treated cells to that of the cells treated with BA-LL37. Besides, the relative levels of AMP-binding from BA-LL37 and BA-Con A-treated cells were compared to that of the cells without deglycosylation.

**Binding of LL-37 to polysaccharides**

Polysaccharide binding assays were performed as described previously [67] with modifications. Mannan-agarose, glucan and chitin were incubated with 10 μg BA-LL37 in 750 μl PBS by gentle rotation at 4ºC overnight. The samples were pelleted and washed twice with 1 ml PBS. Then, the pellets were suspended with reducing sample buffer and denatured by heating at 100ºC for 10 min. The polysaccharides-bound BA-LL37 were analyzed by 15% Tricine SDS-PAGE, transferred to PVDF membranes, and were detected by SA-HRP. The blots were processed using the ECL kit according to the manufacturer's recommendations.

**Measurement of dissociation constant of LL-37 binding to yeast mannans**

Dissociation constant ($K_d$) was measured using a quartz crystal microbalance (QCM) methodology. The amine surface of gold electrodes on a quartz chip was purchased from ANT Tech (Taipei, Taiwan) and was activated employing 2.5% glutaraldehyde. Mannan of *S. cerevisiae* or *C. albicans* (100 mg/ml each) was then cross-linked with glutaraldehyde and coated onto the chip. The binding assay was performed using an Affinity Detection System (ADS, ANT Tech). Before each QCM experiments, the system was equilibrated with PBS (pH 7.4) at the flow rate of 50 ml/min. For $K_d$ measurement, LL-37 ranging from 0.5 to 10 μM was injected onto the chip surface containing yeast cell wall mannans. All the experiments were performed in duplicate or triplicate. The calculation of $K_d$ was performed using GraphPad Prism 5.0 (GraphPad Inc.).

**Adherence of *C. albicans* to oral epidermal cells**

The oral epidermoid carcinoma cell line (OECM-1) was kindly provided by Dr. Tzong-Ming Shieh (China Medical University, Taiwan). The cells were grown in RPMI-1640 medium containing 10% heat-inactivated fetal bovine serum (BSR BIO) and 2 mM L-glutamine with penicillin and streptomycin (Biosera East Sussex, UK), at 37°C in 5% $CO_2$.

To determine the adherent ability of *C. albicans* to oral epithelial cells, a protocol similar to that of *Candida* cell ELISA was used, except that $5 \times 10^4$ OECM-1 cells were pre-grown to 95% confluency in 96-well tissue culture plates (Nunc™, Rochester, NY). Meanwhile, 100 μl of *C. albicans* cells ($1 \times 10^7$ cells/ml) were prepared in RPMI-1640 containing different concentrations of LL-37 at 4°C for 30 min. *Candida albicans* cells were incubated with OECM-1 cells at 4°C for another 30 min. The relative percentage of adhered cells was calculated as follows: [mean fluorescence of (each LL-37 treatment)/(non-treatment) × 100%]. All assays were performed in tetraplicate and repeated four times.

**Mouse model of *C. albicans* adhesion**

All animal studies were approved by the Institutional Animal Care and Use Committees of National Tsing Hua University (approval number 09730) and Animal Technology Institute Taiwan (approval number 97010). Adhesion of *C. albicans* to mouse bladder epithelium was assayed as described with modifications [36]. Briefly, 20- to 24-week-old female BALB/c mice (BioLasco Taiwan Co., Ltd.) were water-deprived overnight. Mice under anesthesia were transurethrally catheterized with 100 μl of *C. albicans* suspension ($2 \times 10^7$ cells) using soft sterile polyethylene tubing (PE 10, outside diameter 0.61 mm, inside diameter 0.28 mm, Clay Adams, Becton Dickinson) with lubricant. After 1 h of infection, mice were sacrificed and urinary bladders were removed. The bladders were washed four times with 1 ml PBS and homogenized using a tissue grinder. The number of colony-forming units (cfu) of *C. albicans* cells adhered on bladders was determined by plating serial dilutions of the sample (in PBS) on YPD agar plates containing 50 μg/ml ampicillin and incubated at 30°C overnight. Relative cell adherence was calculated as follows: [cfu of non-treatment or LL-37 treatment/average cfu of non-treatment in each set of experiment] × 100%.

## Statistical analysis

Data were assessed for statistical significance by two-tailed Student's *t* test. Difference in adherent rates in *C. albicans*-injected mice bladders were analyzed by the GraphPad Prism 5 software.

## Supporting Information

Figure S1

*Candida albicans* was treated 30 min with 5 μg/ml LL-37, pelleted by centrifugation and then compared to untreated cells by visual observation. Upper panel: 50 ml centrifuge tubes; Lower panel: 1.5 ml microcentrifuge tubes; Left panel: after centrifugation; Right panel: supernatant removal; Left tube: control; Right tube: 5 μg/ml LL-37.

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## Author contributions

Conceived and designed the experiments: PWT and HTC. Performed the experiments: PWT, HTC and CYY. Analyzed the data: PWT. Contributed reagents/materials/analysis tools: CYL, HTC and CYY. Wrote the paper: PWT and CYL.

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## Figure Legends

**Figure 1. Candidacidal activity of LL-37.** (A) Killing activity of LL-37 was determined by spot assay. *C. albicans* SC5314 strain was treated with different concentrations of LL-37 and ten-fold serial dilutions of the cells were spotted onto YPD agar plates. Each experiment was performed in triplicate. (B) Killing activity of LL-37 was monitored by using FUN-1 staining. LL-37 treated cells were stained with FUN-1 for 30 min at 30°C. The relative levels of dead cells of LL-37 treated were compared to that of the control (without LL-37 treatment). Data are obtained from average of three independent experiments, and statistical significance was determined using Student's t-test (* indicates $p < 0.05$; ** indicates $p < 0.01$ compared to control and LL-37-treated cells).

**Figure 2. Inhibition of *C. albicans* adherence by LL-37.** (A) Cell adherence to an abiotic surface. *Candida albicans* cells were adhered to polystyrene dishes for 30 min and were washed three times to remove unattached cells. The attached cells were detected by measuring XTT reduction and by whole-cell ELISA using an antibody against *C. albicans*. All assays were done in triplicate and repeated in three (XTT reduction) or four (cell ELISA) separate experiments, and statistical significance was determined using Student's t-test (* indicates $p < 0.05$; ** indicates $p < 0.01$; *** indicates $p < 0.001$ compared to control and LL-37-treated cells). (B) Mortality of LL-37 triggered un-adherent cells was determined by spot assay. Different concentrations of LL-37 were incubated with cells for 30 min and then floating cells were collected and diluted immediately to spot onto YPD agar plated for growing overnight. All assays were repeated in three separate experiments. (C) Morphology of LL-37-induced non-adherent aggregation cells was observed under microscopy. Magnification: 400 × times.

**Figure 3. LL-37 induced *Candida* cells aggregation and associated with *Candida* cells.** (A) Microscopy observation of aggregation of *C. albicans* cells causing by LL-37. *Candida albicans* were incubated with LL-37, and the non-adherent and floating cells were injected into μ-Slides. The aggregations were found in LL37-treated cells and indicated by

arrowheads. (B) Western blot analysis of LL-37 binding to floating *C. albicans* cells. *Candida albicans* were incubated with BA-LL37, and the non-adherent and floating cells were subjected to SDS-PAGE and detected with SA-HRP. BA-LL37 (10 μg) alone was used as positive control. (C) Flow cytometric analysis of LL-37 binding to *C. albicans* cells. Different doses of BA-LL37 were added to *C. albicans,* the cell-bound peptides were measured. Data from this experiment are representative of two independent experiments with similar results.

**Figure 4. Specificity of adhesion inhibition by LL-37 is due to peptide-cell binding and cell-cell aggregation.** (A) Effects of different AMPs on *C. albicans* adherence. To ensure the molecules of AMPs are the same in the reaction, a concentration of 2.12 μM (about 10 μg/ml of BA-LL37) was used for each of BA-LL37, BA-Hst 5, and BA-hBD3. All assays were done in triplicate and repeated in three separate experiments. The Student's t-test was used to determine the statistical significance (§ indicated $p < 0.05$; §§ indicated $p < 0.01$ compared to control and BA-LL37-treated cells; * indicates $p < 0.05$; ** indicates $p < 0.01$; *** indicates $p < 0.001$ compared to cells treated with BA-LL37 and that with BA-Hst 5 or BA-hBD3). (B) Microscopy observation of aggregation of *C. albicans* cells causing by LL-37. *Candida albicans* were incubated with biotinylated AMPs, and the non-adherent and floating cells were collected. The aggregations were found in BA-LL37-treated cells (as indicated by arrowheads). (C) Flow cytometric analysis of binding of different AMPs to *C. albicans*. BA-LL37, BA-Hst 5, or BA-hBD3 (10 μg of each) were independently added to *C. albicans*. The Student's t-test was used to determine the statistical significance (* indicates $p < 0.05$ compared to cells with BA-LL37 and with BA-Hst 5 or BA-hBD3 treatment). Results are the mean ± SD of two independent assays. (D) Candidacidal activity of AMPs was determined by spot assay. A concentration of 2.12 μM of each BA-LL37, BA-Hst 5, and BA-hBD3 was incubated with cells for 30 min and then cell mixtures were diluted immediately and spotted

onto YPD agar plated for growing overnight. All assays were repeated in three separate experiments.

**Figure 5. Binding of LL-37 to *Candida* cell wall polysaccharides reduced *Candida* adherence.** (A) Flow cytometric analysis of cell-bound LL-37 from *C. albicans* cells with deglycosylation. Cells were mixed with BA-LL37 or BA-Con A in PBS at 4ºC overnight, and binding of BA-LL37 or BA-Con A to *C. albicans* was measured by using streptavidin-conjugated 4,6-dichlorotriazinyl aminofluorescein. Results are the mean ± SD of two independent assays. (B) BA-LL37 (10 μg) was incubated with mannan-agarose beads or chitin or glucan. After washing, the samples were subjected to Tricine SDS-PAGE and detected by western blotting with SA-HRP. BA-LL37 alone was used as positive control. (C) Monosaccharides reverse the LL37-mediated inhibition of adhesion. LL-37 (5 μg/ml) was added to *C. albicans* in the absence or presence of 400 μM monosaccharides for 30 min, and XTT reduction assays were performed. All assays were done in triplicate and repeated in three independent experiments. The Student's t-test was used for statistical analysis (* indicates $p < 0.05$ comparing LL-37-treated cells in the absence or presence of D-glucose or LL37-treated cells with control cells). (D) *C. albicans* cell wall mannan reverses the LL37-mediated inhibition of adhesion. LL-37 (5 μg/ml) was added to *C. albicans* in the absence or presence of 1 mg/ml polysaccharide competitors, and XTT reduction assays were performed. All assays were done in triplicate and repeated in three independent experiments. The Student's t-test was used for statistical analysis (§§ indicates $p < 0.01$ compared to control and LL-37-treated cells; # indicated $p < 0.05$ comparing cells in the presence of *S. c.* glucan with control cells; ** indicates $p < 0.01$ comparing LL-37-treated cells in the absence or presence of *C. albicans* mannan or chitin from crab shells). *C. a.*: *C. albicans*; *S. c.*: *Saccharomyces cerevisiae*. (E) Microscopy observation of aggregation of *C. albicans* cells blocked by LL-37. *Candida albicans* were incubated with LL-37 in the absence or presence

of polysaccharides and the non-adherent and floating cells were collected. The aggregations were disappeared in *C. a.* mannan-treated cells.

**Figure 6.** LL-37 inhibits *C. albicans* attachment to oral epidermal cells and mice bladder mucosa. (A) *C. albicans* were pre-incubated at 4°C for 30 min with LL-37, after which the LL37-interacted organisms were added to the OECM-1 oral epidermal cell line for another 30 min. The magnitude of adherent *C. albicans* was determined by *Candida* cell ELISA. The data were expressed as a percentage of the results with LL-37 un-treated *Candida*, and are the mean ± SD of four experiments, each performed in tetraplicate. *, $p < 0.05$; **, $p < 0.01$ as compared to control and LL-37-treated cells. (B) Attachment of *C. albicans* cells to urinary bladders of mice. Percentages of adhered cells were calculated by comparing 5 μg/ml LL37-treated cells with untreated controls as described in Materials and Methods. The dot plot shows the relative adherence of *C. albicans* to bladders of mice from three independent experiments, with six to eight mice per study group in each experiment. The median for each group is shown as a horizontal line. The difference between LL-37-treated cells and controls was significant ($p = 0.0011$; Student's t-test).