LL37 and hBD-3 elevate the β-1,3-exoglucanase activity of *C. albicans* Xog1p, resulting in reduced fungal adhesion to plastic

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SYNOPSIS

The opportunistic fungus *Candida albicans* causes oral thrush and vaginal candidiasis, as well as candidemia in immunocompromised patients including those undergoing cancer chemotherapy, organ transplant and those with AIDS. We previously found that the antimicrobial peptides (AMPs) LL37 and hBD-3 inhibited *C. albicans* viability and its adhesion to plastic. For this study, the mechanism by which LL37 and hBD-3 reduced *C. albicans* adhesion was investigated. After AMP treatment, *C. albicans* adhesion to plastic was reduced by up to ~60% and was dose-dependent. Our previous study indicated that LL37 might interact with the cell-wall β-1,3-exoglucanase Xog1p, which is involved in cell-wall β-glucan metabolism, and consequently the binding of LL37 or hBD-3 to Xog1p might cause the decrease in adhesion. For our current study, Xog1p(41–438)-6H, an *N*-terminus truncated, active, recombinant construct of Xog1p, and Xog1p fragments were produced and used in pull-down assays and ELISA *in vitro*, which demonstrated that all constructs interacted with both AMPs. Enzymatic analyses showed that LL37 and hBD-3 enhanced the β-1,3-exoglucanase activity of Xog1p(41–438)-6H by about two-fold. Therefore, elevated Xog1p activity might compromise cell-wall integrity and decrease *C. albicans* adhesion. To investigate this hypothesis, *C. albicans* was treated with 1.3 μM Xog1p(41–438)-6H, and *C. albicans* adhesion to plastic decreased 47.7%. Taken together, the evidence suggests that Xog1p is one of LL37/hBD-3 targets, and elevated β-1,3-exoglucanase activity reduces *C. albicans* adhesion to plastic.

INTRODUCTION

Candida albicans infections occur with an incidence of 1.1 to 24 cases per 100,000 humans [1, 2]. Over the past three decades, *C. albicans* infections have emerged as a significant cause of human morbidity and mortality [3, 4]. In the USA, ~3,000 to 11,000 individuals die annually from nosocomial candidaemia [5]. Those at high risk for *C. albicans* infection include cancer patients undergoing immunosuppressive chemotherapy and patients that have undergone major surgery, are on supportive ventilation, and/or have inserted central venous and/or urinary catheters [4, 6]. Anti-fungal therapies are of limited effectiveness against systemic infection by *C. albicans*, as drug resistance and extreme toxicity results in treatment failure and, consequently, a mortality rate >40% [7].

The pathogenesis of *C. albicans* infection requires several steps, i.e., adhesion to the host mucosal surface, cell-surface colonization, cell invasion, and tissue disruption [8]. Therefore, if adhesion can be prevented, pathogens cannot colonise mucosal surfaces. *C. albicans* cell-wall macromolecules are required for adhesion to host mucosal cells. Certain heat shock proteins function as adhesins on the cell surfaces of pathogens such as *Helicobacter pylori*, *Haemophilus influenzae*, *Mycobacterium avium*, *Histoplasma capsulatum* [9-12]. Cell-surface HSP70 and HSP100 family proteins, which protect certain pathogens against host-induced stress [13, 14], also interact with antimicrobial peptides (AMPs) produced by the host cells [15, 16]. Moreover, the agglutinin-like sequence gene family of *C. albicans* encodes eight glycosylphosphatidylinositol-anchored cell-wall proteins that are adhesion molecules and bind to host cell surfaces [17-20]. Heterologous gene-expression and gene-deletion experiments demonstrated that the different agglutinin-like sequence proteins have different binding affinities towards different host cells, e.g., oral mucosa and buccal epithelial cells [19, 21-23]. Antibody neutralization experiments

demonstrated that when the *C. albicans* surface protein, complement receptor 3-related protein, was blocked, *C. albicans* adhesion on plastic and cell surfaces was reduced [24]. Therefore, *C. albicans* cell-wall macromolecules are crucially involved in the first step of infection.

During adhesion of *C. albicans* to cell surfaces, the cell-wall components of pathogens is remodelled to attach the various surfaces [25]. *C. albicans* cell-wall β-glucan and chitin, which are associated with mannoproteins, form the main structural microfibrillar polymer and provide the cell wall with structural rigidity [26]. Two metabolic enzymes, β-1,3-exo-glucanase and chitinase, are responsible for cell-wall morphogenetic events as they hydrolyse β-glucan and chitin, respectively [27]. In *C. albicans*, the major β-1,3-exoglucanase is Xog1p, a homolog of *Saccharomyces cerevisiae* Exg1p [28]. Knockout experiments demonstrated that in the null *C. albicans* strain 5314 *xog1*Δ/*xog1*Δ, exoglucanase activity decreased by 60% compared with that in wild-type *C. albicans* [28], which indicated that Xog1p functions in β-glucan metabolism. Moreover, an Xog1p-deficient mutant was more susceptible to antifungal agents that inhibit β-1,3-glucan biosynthesis, e.g., Papulacandin B and cilofungin, and had a reduced capacity to colonise the brain during systemic infection [28]. Therefore, Xog1p may participate in *C. albicans* adhesion and colonization. The Xog1p-deficient strain was equally viable in minimal or rich medium at 30 °C, 37 °C, and 42 °C, and no morphological differences observed by scanning and transmission electron microscopes [28].

Mammalian AMPs are secreted mainly by epithelial cells and neutrophils, and AMPs are the first line of defence against infectious microorganisms [29, 30]. AMPs have distinct functions in response to different pathogens. For example, human β-defensin-1 (hBD-1) and hBD-2 have substantial microbicidal activity against Gram-negative bacteria but not against Gram-positive bacteria. Conversely, hBD-3 is a broad-spectrum AMP that kills many pathogenic bacteria and opportunistic pathogenic yeast, including *C. albicans* [31]. Recently, Schroeder *et al.* reported that after reduction of disulphide bridges hBD-1 becomes a potent AMP against a wide range of pathogens including *C. albicans*, anaerobic, Gram-positive commensals of Bifidobacterium and Lactobacillus species [32]. The AMP LL37 is also a broad-spectrum antimicrobial that is active against Gram-positive and Gram-negative bacteria and pathogenic fungi [33]. In addition to its antimicrobial activity, LL37 neutralises the effect of bacterial lipopolysaccharide and consequently reduced endotoxic shock in a murine model [34]. We previously showed that LL37 interacts with *C. albicans* cell-wall carbohydrates and reduces *C. albicans* adhesion to plastic and mouse bladders [35]. We also found that *C. albicans* cell-wall Xog1p is an LL37 receptor [36], which suggested that LL37 may prevent *C. albicans*/host cell interactions. The study herein demonstrates that LL37 and hBD-3 elevate Xog1p activity by interacting with the enzyme and that elevated Xog1p activity is key to reduced *C. albicans* adherence.

MATERIALS AND METHODS

Candidacidal activity of LL37, hBD-3 and Xog1p(41–438)-6H

A colony-forming unit (CFU) assay was used to assess the antifungal activities of LL37 and hBD-3. *C. albicans* SC5314 was grown in liquid YPD medium (10 g yeast extract, 20 g peptone, 20g glucose in 1 L water) at 30 °C with shaking. After 14 hr,

the cells were diluted into fresh YPD medium at an initial OD_{600} of 0.1 and cultured for 3 to 4 hr at 30 °C until the OD_{600} reached 1.0. Cells were harvested by centrifugation at 2,000 × *g* at room temperature, washed twice using PBS, and suspended in PBS at a concentration of 4,000 cells/ml. To determine the lethal doses of the AMPs and Xog1p(41–438)-6H, samples of 400 cells were incubated with 0, 0.1, 0.3, 1, 3, or 10 μM of an AMP at 30 °C for 30 min, or with 0, 0.1, 0.3, 1, 3, 10, 30, 79 μM of Xog1p(41–438)-6H at 30 °C for 24 hr. Then, the sample supernatants that contained the AMPs and Xog1p(41–438)-6H were removed by centrifugation at 2,000 × *g* at room temperature, the cells were plated onto YPD agar, and incubated at 30 °C overnight. Finally, colonies were counted, and for each sample, the relative survival value was calculated as mean of [CFUs for AMP samples/CFUs for the control samples]. The assays were performed in triplicate and then the standard error of the mean (SEM) was calculated. To determine the growth inhibition effects of Xog1p(41–438)-6H, 400 cells were treated with 1.3 μM of Xog1p(41–438)-6H at 30 °C at time indicated. Similarly with previous procedures, the CFUs were counted and the relative survival value was calculated.

C. albicans adhesion assay

C. albicans cells were prepared as described in the previous section. Then, the samples were centrifuged at 2,000 × *g* for 10 min, and the cells were washed with PBS three times. Cells were then diluted with PBS to a final density of 3,000 cells/ml and mixed with LL37 (LLGDFFRKSKEKIGKEFKRIVQRIKDFLRNLVPRTES) or hBD-3 (GIINTLQKYYCRVRGGRCAVLSCLPKEEQIGKCSTRGRKCCRRKK) or one of the control peptides, CYC3-3 (GWFWADKPS), TAT (YGRKKKRQRRR), or 3× FLAG (MDYKDHDGDYLDHDIDYLDDDDL) at a 1:1 (v/v) ratio to obtain the desired peptide concentration. The peptides were synthesised chemically by MDBio, Inc. in Taiwan. The *C. albicans*/peptide mixtures (250 μl each) were each added into a well of a 24-well plate and incubated at 37 °C for 30 min. Wells were then washed three times with PBS. The cells were scraped from each well, each cell sample was plated onto YPD agar, incubated at 30 °C for 24 hr, and then the colonies were counted. The relative adhesion was normalized by CFUs without peptide treatment. Assays were performed in triplicate.

To determine the effect of Xog1p(41–438)-6H on *C. albicans* adhesion, samples containing 300 cells were each treated with 0.33, 0.65 and 1.3 μM Xog1p(41–438)-6H at 30 °C for 1.5 hr. Then, 250 μl of each sample was added into a well of a 24-well plate and incubated at 37 °C for 30 min. Wells were washed three times with PBS, and the cells were then scraped from the wells, spread onto YPD agar, and incubated at 30 °C overnight. The CFUs were counted and the relative adhesion was normalised by the CFUs without Xog1p(41–438)-6H treatment. Assays were performed in triplicate.

To characterise cell-wall morphology, *C. albicans* cells that had been treated with or without 1.3 μM Xog1p(41–438)-6H at 30 °C for 2 hr were visualised by scanning electronic microscopy. Briefly, *C. albicans* cell suspension was prepared and pipetted drop-wise onto the shiny side of a polycarbonate membrane with a 1 μm pore size (Nucleopore, Pleasanton, CA), allowed to settle for 5 min without drying and then immersed in 2% (w/v) aqueous osmium tetroxide (OsO_4) for 12 hr at 4 °C in the dark. Fixed material was washed in distilled water for 15 min to remove excess osmium tetroxide, and dehydrated in a 10 % graded ethanol series, 15-min in each

step from 10 % to 90 % ethanol. The membrane was washed in 95% ethanol followed by rising three times in absolute ethanol for 15 min each. The membrane was then immerse in ethanol:acetone (2:1), ethanol: acetone (1:2) and finally immerse in absolute acetone for three times (15 min each). The dehydrated sample was further dried in a Hitachi HCP-2 Critical Point Dryer, and coated with Pt (10 nm thickness) in a Hitachi E-1045 ion sputter. The morphology of *C. albicans* was examined in a Hitachi S-4700 Scanning Electron Microscope (SEM) at 3.0 kV.

Expression, purification and refolding of recombinant Xog1p-6H truncated fragments

XOG1(41–438) DNA fragments were PCR amplified using *C. albicans* genomic DNA as the template and the primers 5'-ATATCATATGGGACATAATGTTGCTTGG-3' and 5'-ATATCTCGAGGTGAAAGCCACATTGGTTTG-3' (*Nde*I and *Xho*I sites are singly and doubly underlined, respectively). By using the forward primer, the first 120 nucleotides of *XOG1*, which encoded a highly hydrophobic *N*-terminus, were deleted so that the gene for *N*-terminus truncated Xog1p was synthesised. Four fragments of *XOG1* were amplified using the primers: 5'-ATATCATATGGGACATAATGTTGCTTGG-3' and 5'-ATATCTCGAGTTGACCTTGGACGTATGGAT-3' for *XOG1*(41–150), 5'-GCATCATATGGTTCAGTATTTGGAAAAGGC-3' and ATATCTCGAGTTGGAAAGCATCGTGAATGA for *XOG1*(151–268), 5'-GCGCCATATGGTCTTTGGCTATTGGAATAA-3' and 5'-ATATCTCGAGCTCATAACGTGCTCCTCTGT-3' for *XOG1*(269–352), and 5'-GCATCATATGGGTGCTTACGATAATGCTCC-3' and 5'-ATATCTCGAGGTGAAAGCCACATTGGTTTG-3' for *XOG1*(352–438) (*Nde*I and *Xho*I sites are singly and doubly underlined, respectively). □□□□□□□□ *XOG1*(41–438), *XOG1*(41–150), *XOG1*(151–268), *XOG1*(269–352), and *XOG1*(352–438) were isolated by digestion with *Nde*I *and Xho*I, ligated into pGEM-T Easy vectors, and sequenced. The genes were then each cloned into a pET23a(+) vector to generate the plasmids pET23-*XOG1*(41–438), pET23-*XOG1*(41–150), pET23-*XOG1*(151–268), pET23-*XOG1*(269–352), and pET23-*XOG1*(353–438). All the constructs contain a *C*-terminal hexahistidine sequence (6H) derived from the pET23a(+) vectors.

For protein expression, the plasmids were each transformed into *E. coli* BL21(DE3)pLysS and the transformants were plated onto LB agar plates. Single colonies, each of which contained one of the plasmids, were individually added into 15 ml LB broth containing 100 μg/ml carbenicillin and 50 μg/ml chloramphenicol at 37 °C, and the cultures were shaken at 200 rpm overnight. The cultures were then subcultured in 500 ml of fresh LB broth that contained the same antibiotics at 37 °C until the OD_{600} of each culture was between 0.5 and 0.8. Protein expression was induced by addition of 0.5 mM isopropyl β-D-thiogalactoside at 37 °C for 5 hr. Cell pellets were harvested by centrifugation, suspended in 15 ml PBS, sonicated, and centrifuged at 10,000 × *g* at 4 °C for 10 min. Inclusion bodies in the insoluble fractions were dissolved in 10 ml of 20 mM Tris-HCl (pH 7.9) containing 6 M urea and 0.5 M NaCl (binding buffer) and incubated at 4 °C overnight. After centrifugation at 10,000 × *g* and 4 °C for 30 min, the supernatants were each chromatographed through HisLink resin (Promega), and unbound proteins were removed first by elution

with binding buffer and then by elution with 10 mM imidazole in binding buffer. Hexahistidine-tagged proteins were then eluted in a 50–300 mM imidazole gradient in binding buffer. The purities of the recombinant proteins were assessed by SDS-PAGE through a 12% polyacrylamide gel that was subsequently stained with Coomassie Blue.

As preparation for experimentation, the purified proteins were each incubated with 80 mM glutathione (reduced form) at 25 °C for 30 min for reducing the disulphide bonds, rapidly diluted 100-fold in 0.1 M Tris-HCl (pH 7.5) containing 10% glycerol, 1 mM EDTA, 0.5 M L-arginine, 1 mM phenylmethylsulphonyl fluoride, 40 μM benzamidine, 40 μg/ml aprotinin, 20 μg/ml leupeptin, 20 μM 4-(2-aminoethyl) benzenesulphonyl fluoride hydrochloride, and 1:4 reduced/oxidised glutathione at 4 °C, and then slowly stirred for 24 hr. Then proteins were individually concentrated through Vivaflow 200 (Sartorius, Germany) and Centricon (MWCO: 10 K, Millipore, Billerica, MA) modules at 4 °C, and protein concentrations were determined using the BCA assay (Thermo Scientific, MA).

Enzyme-linked immunosorbent assay (ELISA)

The relative binding affinities of LL37, hBD-3, CYC3-3, TAT, and 3× FLAG for Xog1p(41–438)-6H were measured by ELISA. Each peptide (10 μg) was dissolved in 50 μl of 50 mM sodium carbonate (pH 9.6), added into a well of a 96-well ELISA plate (GeneDireX, NV), and incubated at 4 °C overnight. The wells were then blocked with 0.5% BSA (Gibco, New Zealand) in 100 μl of PBS at room temperature for 1 hr. Each well was then washed three times with PBS. Xog1p(41–438)-6H (50 μl, 200 μg/ml) was added to each well, and the mixtures were incubated at room temperature for 2 hr. After washing with PBS, 100 μl of His-tag antibody (Santa Cruz Biotechnology, Inc., CA; diluted 1:1,000 in 0.5% BSA/PBS) was added into each well, and the samples were incubated at room temperature for 4 hr. Subsequently, each well was washed three times with PBS, and the samples were incubated with 100 μl of horseradish peroxidase (HRP)-conjugated goat anti-mouse antibody (Jackson ImmunoResearch, PA; diluted 1:5,000 in 0.5% BSA/PBS) at room temperature for 2 hr. After a final wash with PBS, 100 μl of 1,1,3,3-tetramethoxypropane (KPL, MD) was added into each well, and the samples were incubated for 2 min in the dark at room temperature. Reactions were stopped by addition of 100 μl of 1 N HCl (Sigma, LA), and the absorbance of each sample at 450 nm was immediately measured using an ELISA microtiter reader (Thermo Scientific). The assays were performed in triplicate. Each value is reported as the mean ± SEM (Prism 5.0, GraphPad Software, Inc., CA).

***In vitro* pull-down binding assay**

Streptavidin beads (20 μl, GE Healthcare, Sweden) in PBS were mixed with 10 μg of *N*-terminal biotinylated (BA)-LL37 or BA-hBD-3. Xog1p(41–438)-6H, Xog1p(41–150)-6H, Xog1p(151–268)-6H, and Xog1p(269–352)-6H (10 μg each), were individually added into a PBS solution containing BA-LL37 or BA-hBD-3 (final volume, 200 μl), and the solutions were incubated at 4 °C for 3 hr. Each solution was centrifuged at 10,000 × *g* for 1 min, and the pelleted beads were each washed eight times with PBS. Finally, the beads were mixed with 20 μl of 1× SDS-loading buffer and boiled. The proteins in the loading buffers were electrophoresed through an SDS-

15% (w/v) polyacrylamide gel. Because Xog1p(151–268)-6H migrated at the same position as streptavidin derived from the streptavidin beads, western blotting was employed to detect Xog1p(151–268)-6H. After SDS-PAGE of the Xog1p(151–268)-6H sample, proteins were transferred onto a PVDF membrane (Pall, Mexico). The membrane was then blocked with 3% BSA/PBST (PBS containing 0.1% Tween-20) and incubated with His-tag antibody (Santa Cruz Biotechnology, Inc., 1:1,000 dilution) at 25 °C for 2 hr. After washing with PBST, HRP-conjugated goat anti-mouse antibody (Jackson ImmunoResearch, 1:10,000) was incubated with the membrane for 1 hr at 25 °C. Immunoreactive bands were visualised using enhanced chemiluminescence (Millipore, MA).

Dissociation constants (K_d values) for Xogp1(41–438)-6H and the AMPs

The Affinity Detection System (ADS, Affinity-sensor New Technology Co. Ltd., Taiwan) employing quartz crystal microbalance was used for measuring the dissociation constants [37]. The molecular interaction could be observed by the decrease of vibration frequency of the quartz chip, ΔF. Briefly, AT-cut quartz chips were first activated with 2.5% glutaraldehyde for 30 min, then washed with doubly distilled water, and coated with Xog1p(41–438)-6H (100 µl, 100 µg/ml), all at room temperature. After 1 hr, Xog1p(41–438)-6H solution was discarded, and the chip was washed twice with PBS. Free aldehydes were then blocked using 1 M ethanolamine at room temperature for 30 min. After an additional wash with doubly distilled water, the chip was bathed in 100 mM sodium cyanoborohydride for 10 min and then washed with 50 mM sodium acetate (pH 5.5) (Amresco, OH) at 37 °C for 30 min. The Xog1p(41–438)-6H chip was installed in the flow cell of the affinity detection system, and 50 mM sodium acetate (pH 5.5) was pumped through the cell at 50 µl/min. After the frequency had stabilised, 300 µl of an AMP (0.5, 1, 2, 5, 10, 15 or 20 µM), which had been passed through a 0.22-mm filter, was injected into the cell and the frequency decrease recorded. The K_d values were calculated using the specific binding model of Prism 5.0 (GraphPad Software, Inc.).

Xog1p(41–438)-6H exoglucanase activity

Each of the peptides, LL37, hBD-3, CYC3-3, TAT, and 3× FLAG (final concentrations 0, 0.1, 1, 3, or 10 µM), was incubated with 0.5 nM Xog1p(41–438)-6H at 4 °C immediately before use. After a 30-min incubation period, each Xog1p(41–438)-6H/peptide mixture was incubated with 8 mg/ml of laminarin (Sigma) in 50 mM sodium acetate (pH 5.5). Glucose oxidase/peroxidase reagent (100 µl; *o*-dianisidine dihydrochloride, 50:1 dilution; Sigma) was added to each mixture, and all samples were then incubated at 37 °C for 15 min. The reactions were stopped by adding 100 µl of 12 N sulphuric acid (J.T. Baker), and the absorbance of each mixture was immediately measured at 540 nm to determine the concentration of hydrogen peroxide, which is a side product of glucose oxidation and is produced in the same molar amount as glucose. The experiments were performed in triplicate. The glucose concentration was calculated as the mean ± SEM using Prism 5.0.

RESULTS

LL37 and hBD-3 kill *C. albicans* in a dose-dependent manner

Although LL37 and hBD-3 are both cationic peptides, they have different secondary structures — α-helical [38] and β-sheet [39], respectively. To assess the functional effect(s) of secondary structure on a cationic AMP, both LL37 and hBD-3 were used in this study. Both AMPs were chemically synthesised and their candidacidal activities assessed. After each AMP, at various concentrations, had been incubated with *C. albicans* at 30 °C for 30 min, the cells were plated and the number of colonies counted the next day (Figure 1). At 10 μM, hBD-3 reduced the number of colonies by 67.9% in comparison with the number of control colonies. Conversely, 3 μM hBD-3 had no apparent candidacidal activity. Only above a concentration of 10 μM did LL37 display candidacidal activity, and even then it was less effective than was hBD-3. Both AMPs at a concentration of 100 μM killed all *C. albicans* cells.

LL37 and hBD-3 inhibit the adhesion of *C. albicans* to plastic

To study the inhibition of *C. albicans* adhesion to plastic, yeast cells were treated with each of the AMPs at a concentration of 0, 3, or 10 μM at 37 °C for 30 min. Then cells that had adhered to the wells were scraped and plated onto agar that contained YPD medium. After overnight culture, the colony numbers were counted. At 10 μM, LL37 decreased *C. albicans* adhesion by 59%, and at 3 μM, hBD-3 decreased adhesion by 35% (both concentrations are non-lethal doses). At the concentration tested (10 μM), the control peptides, CYC3-3, TAT and 3× Flag, chosen because their net charges are neutral, positive, and negative, respectively, did not affect adhesion. Therefore, the interaction of both LL37 and hBD-3 with the cell-wall components reduced the ability of *C. albicans* to adhere to plastic.

Xog1p-6H fragments interact with LL37 and hBD-3

Xog1p(41–438)-6H, which did not contain the *N*-terminal hydrophobic region of the full-length protein (residues 1–40), Xog1p(41–150)-6H, Xog1p(151–268)-6H, Xog1p(269–352)-6H, and Xog1p(232–438)-6H were constructed, expressed, purified, and prepared as described above (Figure 2A). However, Xog1p(232–438)-6H was not expressed in *E. coli*. After purification and SDS-PAGE analyses, the positions of the other fragments in an 12% polyacrylamide gel corresponded to 46 kDa (Xog1p(41–438)-6H), 14 kDa (Xog1p(41–150)-6H), 13 kDa (Xog1p(151–268)-6H), and 10 kDa (Xog1p(269–352)-6H), which are the expected molecular weights of the fragments (Figure 2B).

ELISA was used to assess the interactions between Xog1p(41–438)-6H and LL37 or hBD-3. Both AMPs were coated onto ELISA plates with Xog1p(41–438)-6H serving as the probe. The interactions of Xog1p(41–438)-6H with LL37 and hBD-3 were 2.5- and 3-fold stronger, respectively, than were the interactions measured with the control sample (no peptide) or samples containing CYC3-3, TAT, or 3× FLAG (Figure 3A). Furthermore, to identify the portions of Xog1p(41–438)-6H that interacted with the AMPs, the Xog1p-6H fragments were pulled down with the biotinylated AMPs. Figure 3B shows Xog1p(41–438)-6H, Xog1p(41–150)-6H, Xog1p(269–352)-6H, and Xog1p(232–438)-6H interacted with BA-LL37 and BA-hBD-3. Because Xog1p(151–268)-6H has the same molecular weight as monomeric streptavidin, we assessed the ability of Xog1p(151–268)-6H to interact with the AMPs using western blotting (Figure 3C) and found that Xog1p(151–268)-6H also bound LL37 and hBD-3. Therefore, Xog1p possesses multiple regions that interact with LL37 and hBD-3.

The binding affinities of Xog1p(41–438)-6H for the AMPs were next quantified using affinity detection, which relied on a reduction in the acoustical frequency (ΔF) after mixing immobilised Xog1p(41–438)-6H with a solution that contained an AMP at a specified concentration. The observed ΔFs indicated that the AMPs interacted with immobilised Xog1p(41–438)-6H. Using the values of ΔF/[peptide] and ΔF, and the specific binding function in Prism 5.0, binding isotherm and Scatchard plots were obtained and K_d values determined. The K_d for Xog1p(41–438)-6H and LL37 was 1.41 ± 0.10 μM, and for Xog1p(41–438)-6H and hBD-3 was 7.52 ± 0.64 μM (Figure 4).

Xog1p(41–438)-6H β-1,3-exoglucanase activity is elevated by LL37 and hBD-3

Because LL37 and hBD-3 bound Xog1p(41–438)-6H *in vitro*, the question of their possible biological function(s) in relation to Xog1p needed to be addressed. We hypothesised that the hydrolytic activity of Xog1p might be affected when the enzyme bound LL37 or hBD-3. Xog1p(41–438)-6H was incubated with each peptide at a specified concentration for 30 min at 4 °C. Then, the exoglucanase activity in each mixture was measured using laminarin as the substrate (Figure 5). The concentration of hydrogen peroxide, the side product of glucose oxidation, was then measured. LL37 and hBD-3 both increased the concentration of hydrogen peroxide (reported as glucose concentration in Figure 5) and, at a concentration of 1 μM or greater, LL37 and hBD-3 enhanced Xog1p(41–438)-6H activity more than 1.8- and 1.9-fold, respectively, compared with the control. Conversely, the control peptides did not enhance Xog1p(41–438)-6H activity.

***C. albicans* adhesion is reduced by treatment with exogenous Xog1p(41–438)-6H**

Exoglucanase activity is crucial for maintaining and remodelling the *C. albicans* cell wall. Therefore, an abnormal concentration or activity of Xog1p may damage the cell wall, thereby reducing the potential infectivity of *C. albicans*. We hypothesised that an elevated level of Xog1p activity would affect the normal metabolism of cell-wall glucan, and that the observed decreased adhesion of *C. albicans* to plastic was a consequence of abnormal remodelling of the cell wall. To investigate if Xog1p(41–438)-6H was cytotoxic to *C. albicans*, cells were treated with various concentrations of Xog1p(41–438)-6H at 30 °C for 24 hr, and surviving colonies were then counted (Figure 6A). Xog1p(41–438)-6H killed cells in a dose-dependent and somewhat sigmoidal manner with an IC_{50} of 1.3 μM. To determine the effect of Xog1p(41–438)-6H on *C. albicans* adhesion, the candidacidal activity of the enzyme had to be avoided. To determine non-candidacidal conditions for Xog1p(41–438)-6H, a time-course experiment was conducted (Figure 6B). After 2 hr at 30 °C, *C. albicans* suspended in PBS and not treated with Xog1p(41–438)-6H began replicating, and replication of *C. albicans* treated with Xog1p(41–438)-6H was still inhibited after 2 hr (Figure 6B). Therefore, 1.3 μM Xog1p(41–438)-6H and an incubation time of 2 hr comprised the non-candidacidal condition, which was then used to investigate reduction in adhesion to plastic by *C. albicans* caused by an increase in Xog1p activity. Figure 6C shows that 0.65 and 1.3 μM Xog1p(41–438)-6H reduced *C. albicans* adhesion to plastic by 24.1% and 47.7%, respectively. Therefore, upregulation of Xog1p activity *in vivo* may interfere with adhesion. Notably, the cell-wall morphology of *C. albicans* that had been exposed to these conditions was apparently not destroyed (Figure 7). These findings suggested that the AMPs may elevate the β-1,3-exoglucanase activity, which subsequently results in abnormal cell-

wall glucan metabolism that leads to the inhibition of *C. albicans* adhesion, even though cell-wall morphology appears to be unaltered.

DISCUSSION

LL37 and hBD-3 are highly cationic, low molecular weight AMPs. Cell-membrane and cell-wall carbohydrates are often receptors or co-receptors for positively charged AMPs. For example, the AMP eosinophil cationic protein (pI 10.8) kills mammalian cells via its interaction with cell-membrane heparan sulphate [40, 41] and kills bacteria via its association with lipopolysaccharides [42, 43]. The electrostatic affinity between positively charged AMPs and negatively charged membrane carbohydrates is probably a consequence of the fact that carbohydrates are often modified with negatively charged sulphate, phosphate, and carboxylate moieties [43]. In addition to membrane carbohydrates, membrane (or cell-wall) proteins serve as receptors [44]. We reported herein that the recombinant *C. albicans* cell-wall β-1,3-exoglucanase Xog1p bound LL37 and hBD-3, and as a consequence its exoglucanase activity increased. This upregulated activity might abnormally enhance the hydrolysis of cell-wall β-glucan, thereby damaging the cell wall integrity, and consequently reducing the ability of *C. albicans* to adhere to epithelial cells in vivo. However, our previous study showed that the activity of β-1,3-exoglucanase in *C. albicans* cell wall was decreased with the treatment of LL37 [36]. The cell wall components are composed of lipids, glycans, and proteins to form molecular complexes for regulating biological functions. In addition to Xog1p, our previous study also indicated that LL37 preferentially binds mannan, the main component of the *C. albicans* cell wall, and partially binds chitin or glucan, which underlie the mannan layer [35]. Therefore, some unidentified cell-wall components might form large complexes with LL37, carbohydrates and Xog1p resulting in the reduction of Xog1p activity. In this study, to clarify the direct effect, the pure recombinant Xog1p(41-438)-6H was incubated with synthetic LL37, and we found that Xog1p activity was upregulated. The opposite result suggests that other cell-wall components such as glucans may also affect the activity of Xog1p.

Carbohydrates account for 80% to 90% of *C. albicans* cell-wall mass, with β-glucan, chitin, and mannoproteins as the major components. β-glucan and chitin maintain the structural skeleton of the cell wall, and β-glucan accounts for 47% to 60% of the cell-wall mass [45, 46]. It has been thought that β-glucan is buried in the cell wall under a layer of mannoprotein [26]. However, a recent study found that the anti-β-glucan monoclonal antibody IgG2b (mAb 2G8) specifically binds to β-1,3-glucan epitopes found on the outer surface of the *C. albicans* cell wall and, by doing so, inhibits fungal replication and adhesion to human epithelial cells [47]. Therefore, it is possible that at least some of the *C. albicans* cell-wall β-1,3-glucan is on the outer surface of the cell wall. In our current study, we treated Xog1p(41–438)-6H with 0.33, 0.65 and 1.3 μM for 2 hr and observed reduced adhesion of *C. albicans* to plastic; therefore, the enhanced activity of Xog1p(41–438)-6H may have damaged the cell wall.

Taken together, our results indicate that, at high concentrations, LL37 and hBD-3 kill *C. albicans*, but at smaller, non-cytotoxic concentrations, the AMPs prevent *C. albicans* adhesion to plastic by elevating the β-1,3-glucanase activity of Xog1p. Furthermore, the AMPs may be developed as peptide drugs for preventing the infection via the inhibition of the *Candida albicans* adhesion.

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FIGURE LEGENDS

Figure 1 The antifungal activities of LL37 and hBD-3 and their abilities to reduce *C. albicans* adhesion to plastic. (A) LL37 and hBD-3 were incubated with *C. albicans* at 30 °C for 30 min. The surviving cells were grown on YPD agar, and the relative number of CFUs that were present 24 hr later in comparison with cells incubated in the absence of an AMP was determined. (B) *C. albicans* incubated with or without a peptide and that had adhered to the wells of a 24-well plate were scrapped after 30 min and were plated onto YPD agar. The number of cells that had adhered to the wells in the presence of an AMP or a control peptide was normalised to the number of cells found when peptides were not present. CYC3-3, TAT, and 3× FLAG served as control peptides. ***, $p < 0.001$; **, $p < 0.01$; *, $p < 0.05$.

Figure 2 Recombinant Xog1p-6H fragments. (A) Schematic of the Xog1p(41–438)-6H, Xog1p(41–150)-6H, Xog1p(151–268)-6H, Xog1p(269–352)-6H and Xog1p(353–438)-6H. (B) The purified Xog1p-6H fragments were analysed using a 12% SDS-PAGE.

Figure 3 Binding assays for Xog1p(41–438)-6H and the peptides used in this study. (A) Relative affinities of the peptides for Xog1p(41–438)-6H normalised to the control sample (no peptide). The "Xog1p" sample is that for an ELISA plate coated with Xog1p(41–438)-6H . (B) Pull-down assays that used a biotinylated (BA) AMP in conjunction with the various Xog1p-6H fragments were shown in protein gels. (C) Due to the same molecular weight of Xog1p(151–268)-6H and streptavidin, Western blotting was used to detect the recombinant Xog1p(151–268)-6H. **, $p < 0.01$; *, $p < 0.05$.

Figure 4 Binding isotherm and Scatchard plots for the measurement of the Xog1p(41–438)-6H/LL37 and Xog1p(41–438)-6H/hBD-3 dissociation constants obtained by affinity detection. The measured values for the dissociation constants between Xog1p(41–438)-6H and LL37, and Xog1p(41–438)-6H and hBD-3 are 1.41

± 0.10 μM and 7.52 ± 0.64 μM, respectively. ΔF is the difference in the response of quartz crystal microbalance at equilibrium on the chip surface.

Figure 5 Xog1p(41–438)-6H activity assay. Xog1p(41–438)-6H was incubated with each of the peptides at the indicated concentrations, and its activity was assessed using laminarin as the substrate and measuring hydrogen peroxide, the side-product of glucose oxidation, which is released upon hydrolysis of laminarin. *, $p < 0.05$.

Figure 6 Cytotoxic effect on and adhesion inhibition of *C. albicans* by Xog1p(41–438)-6H. (A) Xog1p(41–438)-6H at various concentrations was incubated with *C. albicans* at 30 °C for 24 hr. The surviving cells are counted. (B) Xog1p(41–438)-6H, at a concentration of 1.3 μM, inhibited *C. albicans* replication for the initial 2 hr of culture. (C) *C. albicans* was treated with Xog1p(41–438)-6H for 2 hr, and cells that had adhered to plastic were then counted. *$p < 0.05$; **$p < 0.01$.

Figure 7 *C. albicans* cell-wall morphology. *C. albicans* was not treated (A) or treated (B) with 1.3 μM Xog1p(41–438)-6H at 30 °C for 2 hr. Cell-wall morphology was visualised by scanning electron microscopy.

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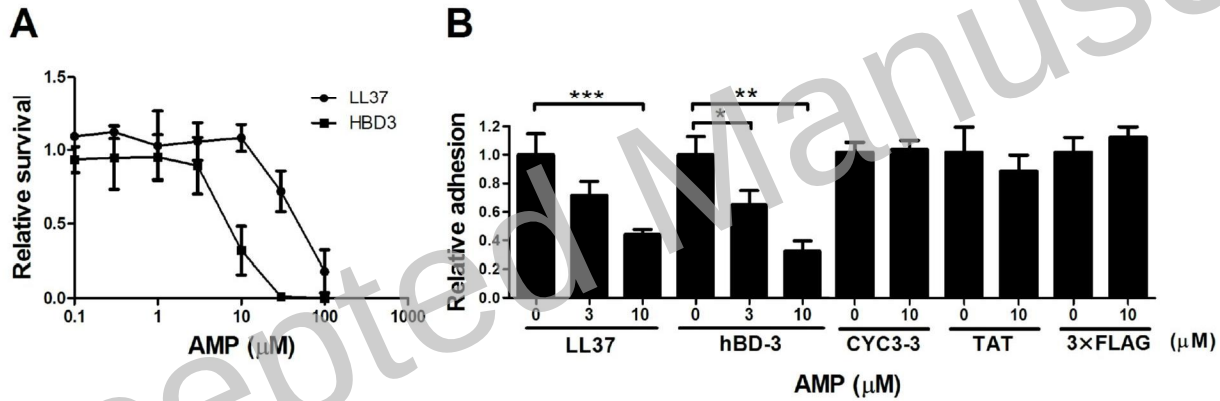

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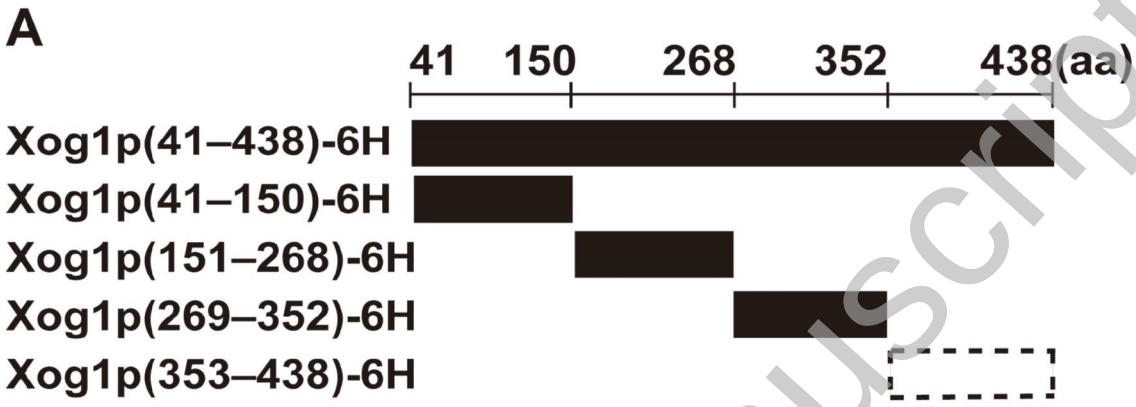

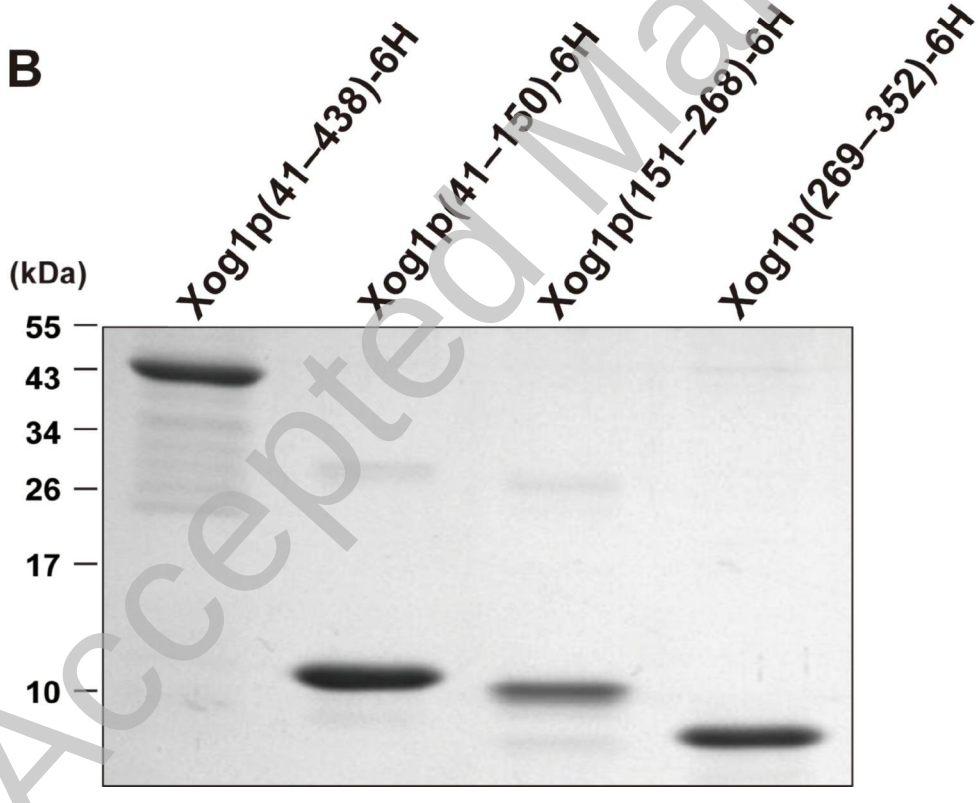

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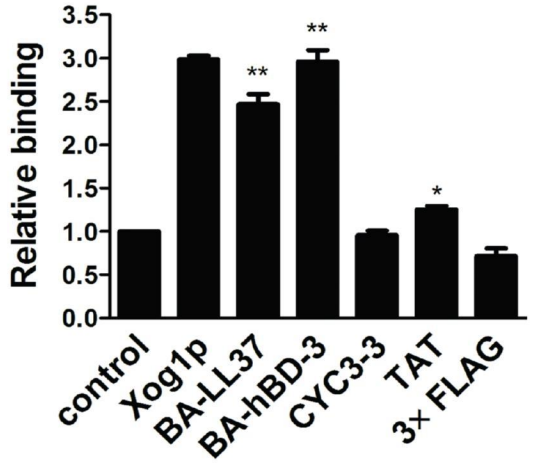

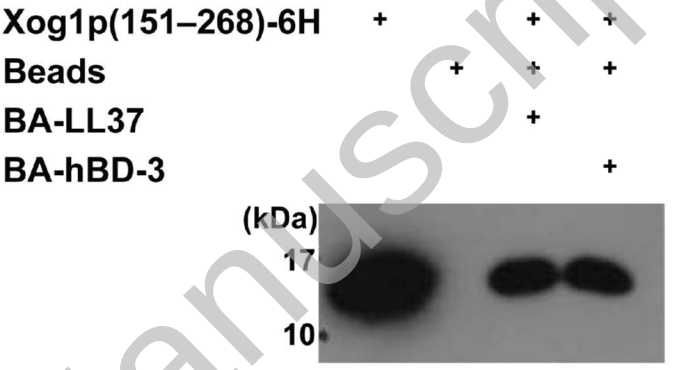

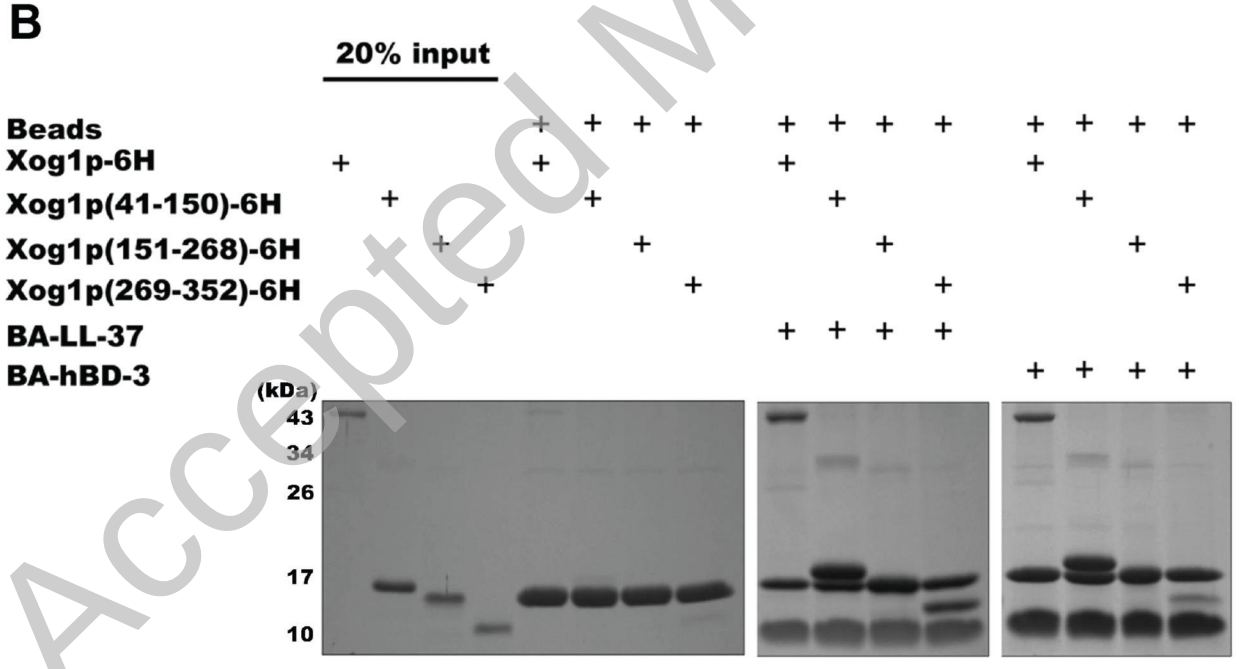

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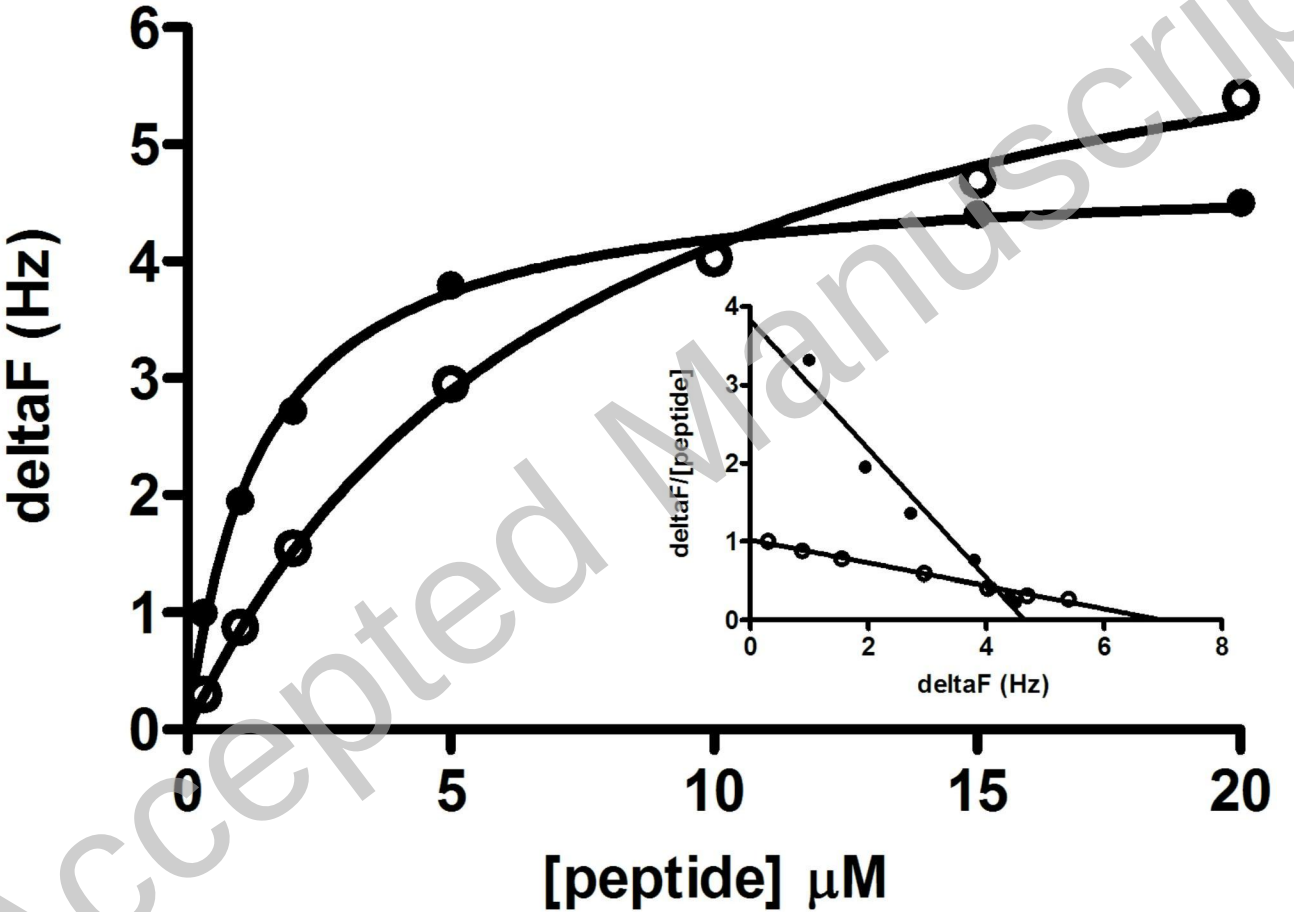

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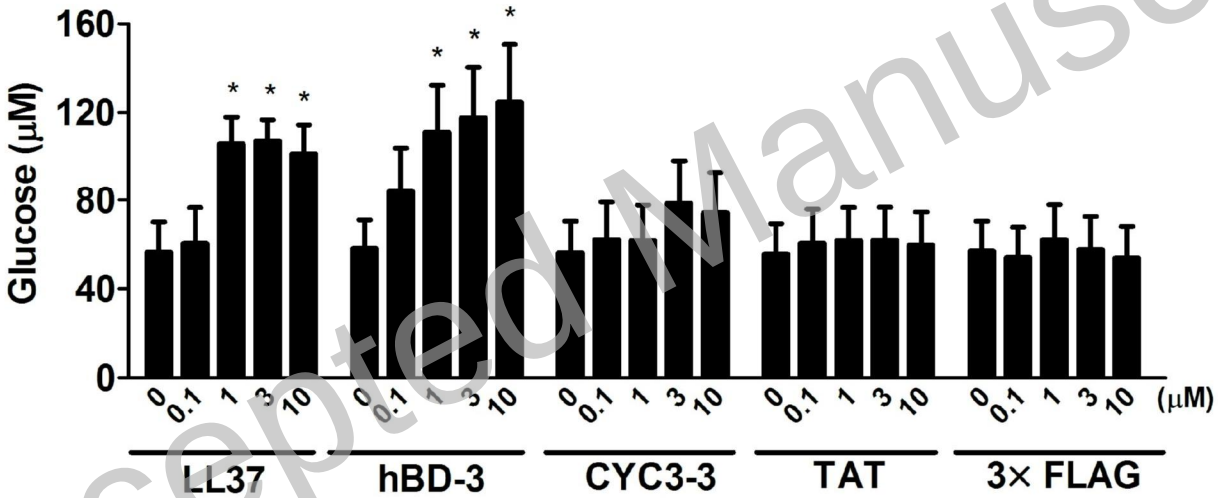

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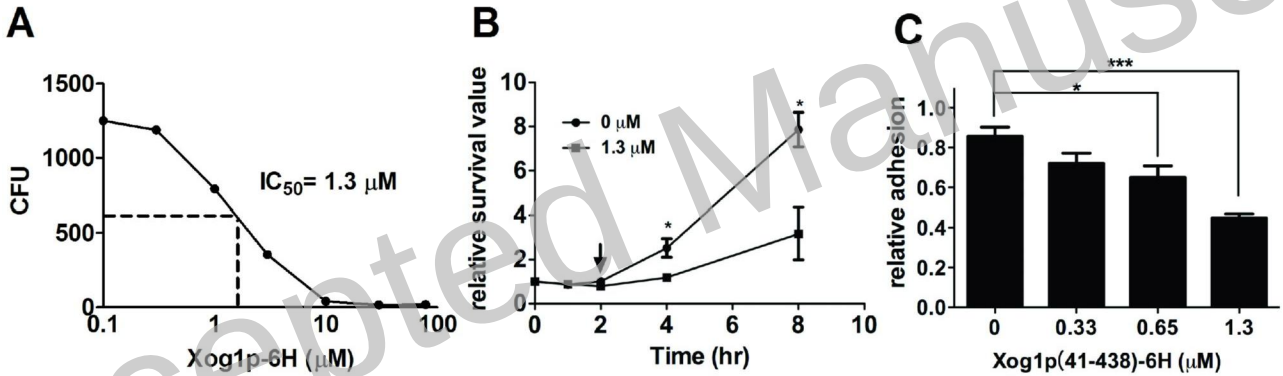

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