Supporting Information

Repurposing of Intestinal Defensins as Multi-Target, Dual-Function Amyloid Inhibitors via Cross-seeding

Yijing Tang[1], Dong Zhang[1], Xiong Gong[2], and Jie Zheng[1*]

[1]Department of Chemical, Biomolecular, and Corrosion Engineering
The University of Akron, Ohio, USA

[2]School of Polymer Science and Polymer Engineering
The University of Akron, Ohio, USA

* Corresponding author: zhengj@uakron.edu

Materials and Method
Reagents
Full-length amyloid peptides including amyloid β_{1-42} (Aβ, purity $\geq$ 95.0%), human islet amyloid polypeptide$_{1-37}$ (hIAPP, purity $\geq$ 95.0%), human calcitonin$_{1-32}$ (hCT, purity $\geq$ 95.0%) peptides, human α defensin-6 (HD-6, purity $\geq$ 95.0%) and human β defensin-1 (HBD-1, purity $\geq$ 95.0%) were obtained from CPC Scientific (CA, USA). 1,1,1,3,3,3-hexafluoro-2-propanol (HFIP, 99.9%), 10 mM PBS buffer (PH=7.4), dimethyl sulfoxide (DMSO, 99.9%), and thioflavin T (ThT, 98%) were purchased from Sigma-Aldrich (MO, USA). All other chemicals used in this work were of the highest grade.

Peptide Purification and Preparation
All the lyophilized peptide powder was stored at -20 °C immediately after received. To break the preformed peptide aggregates and obtain the monomeric species, all pre-packaged peptides were redissolved in HFIP at 1 mg/ml concentration for 2 h, followed by 30 min sonication in ice bath, 30 min centrifugation at 14000 rpm and 4 °C, and stored at -80 °C before use. Unless otherwise states, all peptides were pre-solubilized in 10 mM NaOH, then further dissolve in different buffers to reach 20 µM concentration.

Thioflavin T (ThT) Fluorescence Assay
ThT spectra of amyloid aggregation was monitored by using SpectraMax M3 microplate reader (CA, USA) and measured at excitation wavelength of 450 nm and emission wavelength at the range of 470 nm to 500 nm under kinetic top-read mode. Samples were prepared on ice by dissolving amyloid peptides in 10 µM ThT- 10 mM Tris buffer solution (pH=7.4) with and without 0.1-20 µM defensins. After transferring samples to 96-well plate (200 µL/well), aggregation was initiated at 37 °C and fluorescence intensity data were recorded consistently at 30 min intervals for 24-30 h.

Circular Dichroism Spectroscopy (CD)
The secondary structure transition of amyloid peptides was examined by far-UV CD spectroscopy with a J-1500 spectropolarimeter (Jasco Inc., Japan) under the continuous scanning mode at room temperature. Samples were prepared by dissolving amyloid peptides in 10 mM sodium phosphate buffer (pH=7.4) and incubated at 37 °C in the presence or absence of defensins for 0, 12, 24, 72 h. The spectra of the solution samples were recorded over a wavelength range of 190-250 nm using a cuvette of 1 mm optical path length with a step size of 0.5 nm and 50 nm/min scan rate. All spectra were corrected by subtracting the buffer baseline and averaged by three successive scans for each sample. The secondary structural contents were determined by using the Beta Structure Selection (BeStSel) algorithm[38] (http://bestsel.elte.hu/).

Atomic Force Microscopy (AFM)
The tapping-mode AFM was employed to study the morphological changes of amyloids aggregation mediated by defensins. To consistent with the conformational changes, 20 µL sample used in CD experiment was taken for AFM measurement at different time points. i.e., 20 µL samples at 6 and 24 h were deposited onto a freshly cleaved mica substrate for 5 min, rinsed three times with Mill-Q water to totally remove additional salt and then dried with an air stream before the AFM experiment. All images were recorded at the 256 × 256 pixel resolution at a typical scan rate of 1.0-2.0 Hz and with the vertical tip oscillation frequency of 250-350 kHz. For each sample,

the representative AFM images were obtained by scanning six different locations on the mica surface. Depth data is acquired by using the Nanoscope analysis software and length data was calculated from all the fibrils shown in AFM images (up to N=40).

Surface Plasmon Resonance (SPR) Spectroscopy

A custom-built four-channel SPR instrument was used to measure the binding affinity between amyloid peptides and defensins. The detailed preparation procedure for carboxymethylated dextran modified gold surface is described in previous works[39]. Briefly, the clean gold surfaces were first immersed in 5mM 11-mercapto-1-undecanol in ethanol/water (8:2) solution for 24h to obtain the thiol-based surface. Then, these surfaces were reacted with epichlorohydrin (2% v/v) in 0.1M NaOH for 3 h and transferred to 6 kDa dextran solution in 0.1M NaOH for 24 h, followed by immersing in 1M bromoacetic acid in 2M NaOH for another 24 h to obtain the carboxymethylated dextran modified SPR chips. Finally, these modified SPR chips were dried with air stream, sealed, and stored in glass container.

For defensins immobilization, the SPR chips were first activated by an equimolar mixture of NHS (N-hydroxysuccinimide) and EDC (N-ethyl-N-(diethylaminopropyl) carbodiimide). Subsequently, defensins dissolved in PBS (10 mM, pH=7.4) were introduced to the sample surface for 10 min, and the remaining NHS-ester groups were blocked by flowing 1 M ethanolamine HCL for another 10 min. The binding affinity of defensins with amyloid peptides was performed by injecting a serial diluted amyloid peptides (2.5-20 µM) in running buffer (10 mM PBS, pH 7.4) over channels at a flow rate of 5 µL/min, followed by PBS buffer to remove any unbounded amyloid peptides. Dissociation constant (K_D) values were evaluated using Anabel software (http://anabel.skscience.org/) by fitting the data using a 1:1 Langmuir binding model and observed binding constant (kobs) linearization method.

Cell Culture

For Aβ and hCT system, human SH-SY5Y neuroblastoma cells (ATCC® CRL-2266TM, VA, USA) were used, while for hIAPP system, the rat insulinoma cells RIN-m5F (ATCC® CRL-11605TM, VA, USA) were chosen. SH-SY5Y cells were cultured in sterile-filtered Eagle's minimum essential medium (Sigma-Aldrich, MO, USA) supplemented with 10% fetal bovine serum (FBS) and 1% penicillin/streptomycin. Similarly, RIN-m5F cells were cultured in sterile-filtered RPMI-1640 medium (Sigma-Aldrich, MO, USA) supplemented with 10% fetal bovine serum (FBS) and 1% penicillin/streptomycin. All the cells were maintained in a humidified incubator with 5% CO_2 at 37 °C and replaced with fresh medium every 4-7 days until cells get to 80% confluence. Cells were then harvested by using 0.25 mg/mL Trypsin/EDTA solution (Sigma Aldrich, MO, USA) and seeded in 96-well plate (2×10^4 per well).

MTT and LDH Cytotoxicity Assay

3-(4,5-dimethylthiazole-2-yl)-2,5-diphenyltetrazolium bromide (MTT) and lactate dehydrogenase (LDH) were applied to study the cell toxicity induced by amyloid peptides and mediated by defensins. Cells were allowed to attach and grow for 24 h after seeding in 96-well plate. The media was then replaced with fresh media containing amyloid peptides (20 µM), defensins (0.1-20 µM), and amyloid peptides- defensins, which was further incubated for 24 h. 100 µL supernatant was carefully transferred to another clean 96-well plate for further LDH test. After removing the all the remaining supernatant of each well, 0.5 mg/mL MTT fresh media was added to each well. After 4

h incubation, the media was replaced by DMSO to dissolve the formazan crystals. The absorbance intensity was read at 540 nm, and the cell viability was determined as the percentage of MTT reduction as compared to untreated cells.

For the spontaneous LDH activity positive or negative control group, 10 µL sterile water or Triton-X-100 solution was added to each well, respectively. The plates were then incubated for 10 min at room temperature, followed by transferring 90 µL of medium from each well to a clean 96-well plate. 10 µL of LDH cytotoxicity assay reagent was then added to each well and incubated for 30 min. Finally, OD_{450} value was read by using SpectraMax M3 microplate reader. All LDH activity values were normalized to negative control, and cytotoxicity values were calculated in percentages of positive control. For both MTT and LDH assay, data were exhibited in mean±s.d. of six independent tests.

Bacterial Growth Assays
Escherichia coli (ATCC 8739), *Pseudomonas aeruginosa* (ATCC 27853), *Staphylococcus aureus* (ATCC 6538P), and *Staphylococcus epidermidis* (ATCC 14990) were cultured overnight and further diluted to an OD_{600} value of 0.05, which is ready to use. For each well, 100 µL bacterial solution was mixed with 1 µL amyloid peptides, defensins, amyloid peptides- defensins dissolved in DMSO at desired concentration. As a control, an equal volume of PBS was added correspondingly in control groups. The growth curves of bacteria were then recorded by measuring the OD_{600} in the following 12 h at 30 min intervals at 37 °C by SpectraMax M3 microplate reader.

LIVE/DEAD Viability/Cytotoxicity Assay
The representative images of the live and dead bacteria/cells were acquired to evaluate the antibacterial/protection effects of each defensins, amyloid peptides and their complexes. Bacteria or cell samples were stained using a LIVE/DEAD® BacLight™ Bacterial Viability Kit (L7012, Invitrogen) or a LIVE/DEAD™ Viability/Cytotoxicity Kit for mammalian cells (L3224, Invitrogen), respectively. Followed by imaged using fluorescence microscope (Olympus IX81) to visualize the live and dead bacteria or cells.
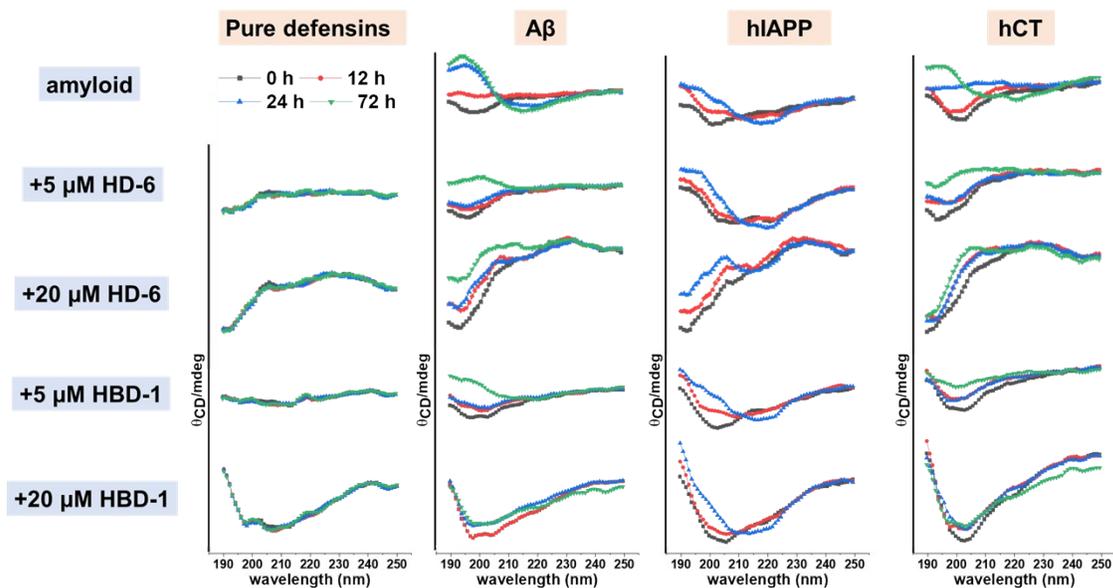
Figure S1. Defensins delay secondary structures transitions of amyloid peptides. Original time-dependent circular dichroism (CD) spectra to monitor the secondary structure changes of Aβ, hIAPP, and hCT (a) in the absence and presence of 5-20 μM (b, c) HD-6 and (d, e) HBD-1 at different molar ratios of 1:0.25 and 1:1 at 0, 12, 24, and 72 h.
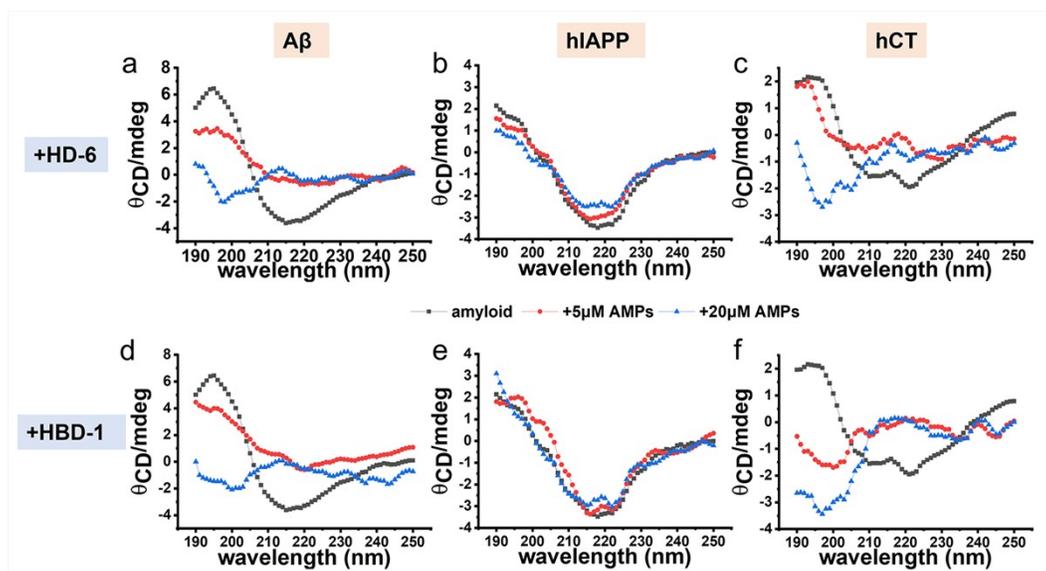
**Figure S2.** Analyzed CD spectra of (a, d) Aβ, (b, e) hIAPP, and (c, f) hCT co-culture with 5-20 µM (a-c) HD-6 and (d-f) HBD-1 to reach the final state (i.e., 72 h for Aβ and hCT, 24 h for hIAPP). Original CD spectra was further analyzed by subtracting corresponding pure defensins signals from co-aggregation signals in **Fig. S1**.
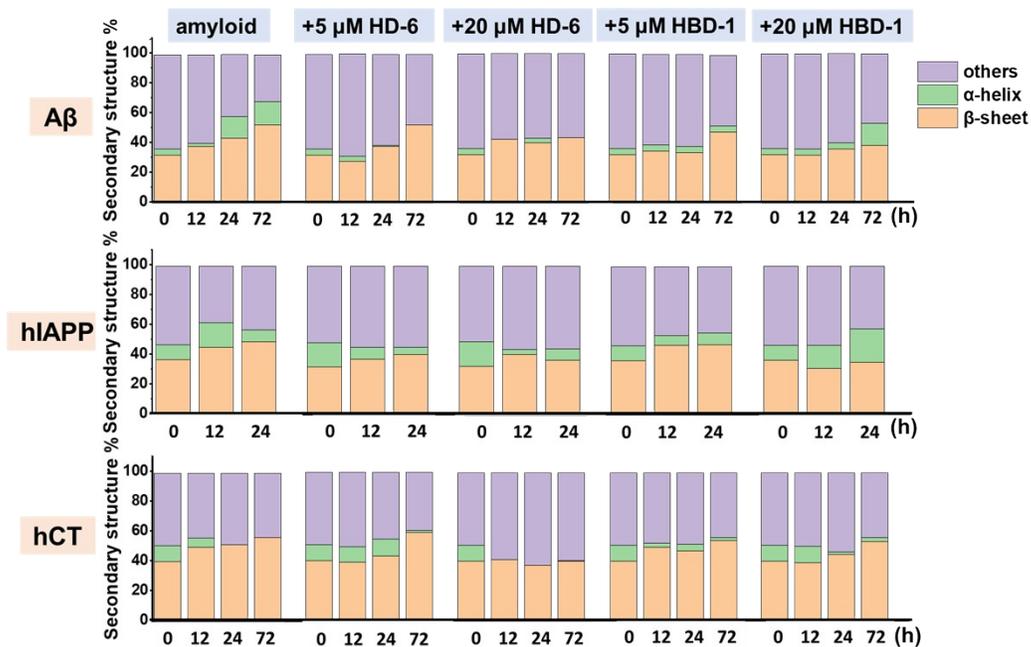
Figure S3. Secondary structure contents of 20 μM Aβ, hIAPP, hCT in the absence and presence of 5-20 μM HD-6 or HBD-1 analyzed from Fig. S2.
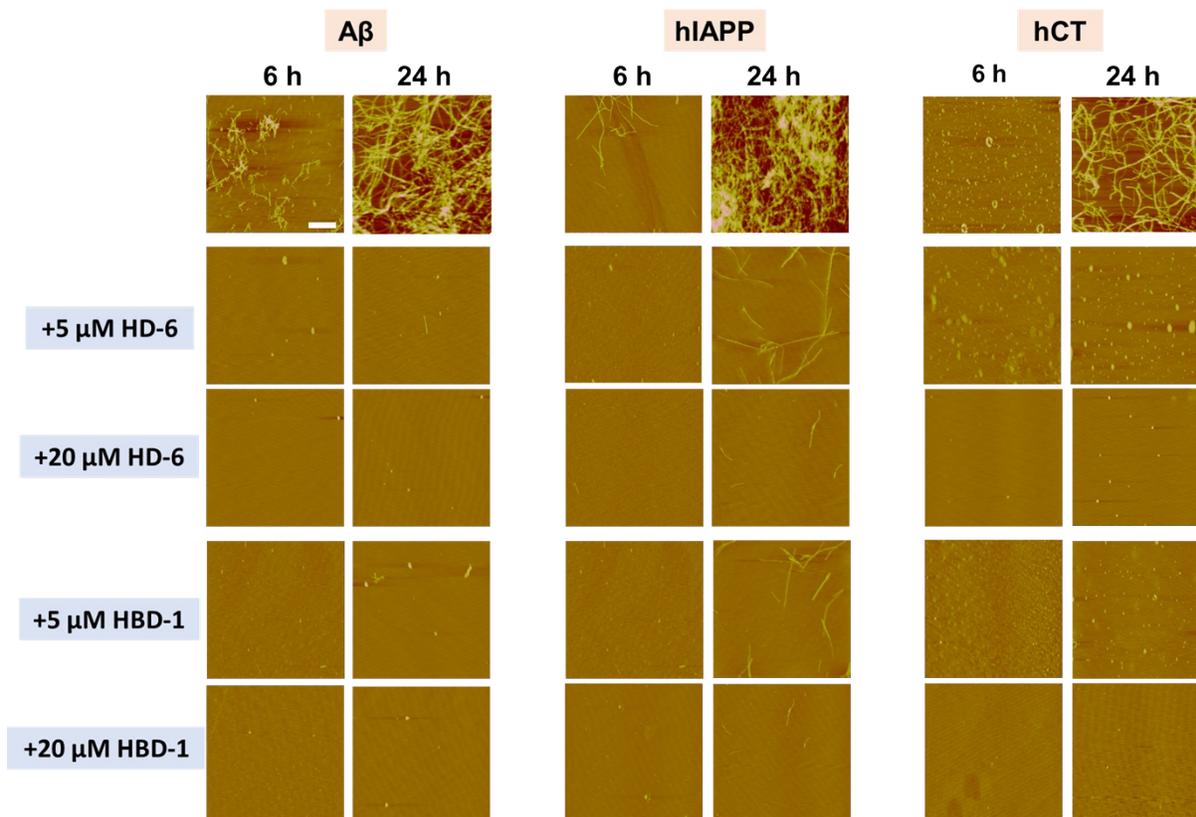
Figure S4. Defensins suppress the formation of amyloid aggregates. AFM characterization of the morphological changes of 20 µM Aβ, hIAPP and hCT, in the absence (1st row) and presence of HD-6 (2nd-3rd row) and HBD-1 (4th-5th row) at different concentrations after 6 h and 24 h incubation. Scale bars=1 µm.
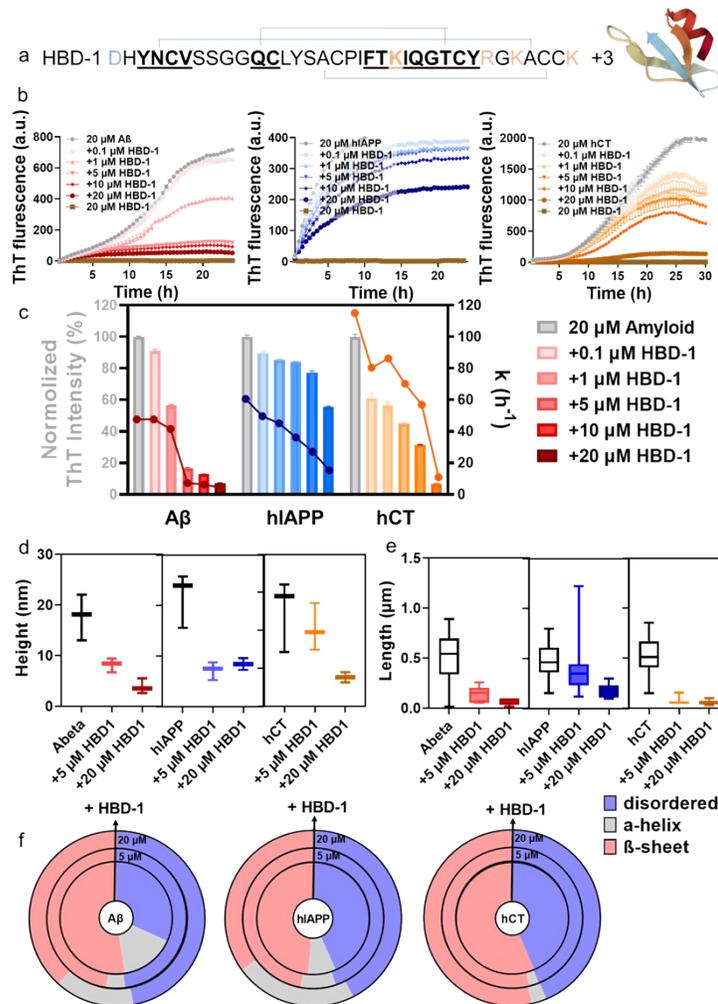
Figure S5. HBD-1 exhibits a general cross-seeding-induced inhibition capacity against different amyloid aggregation. (a) Sequence of HBD-1. Color ID: positive charged residues (orange letters), negatively charged residues (blue letters), and β-structure fragments (bold & underline). (b) ThT aggregation kinetic profiles and (c) Quantitative analysis of ThT profiles to show the dose-dependent inhibition effects of HBD-1 on Aβ, hIAPP, and hCT aggregation at different HBD-1:amyloid ratios (1:0.005-1:1). Inhibition efficiency of HBD-1 against amyloid aggregation is determined by the relative final fluorescence intensity (%) as normalized by that of pure amyloid aggregation (left axis). Error bar represents the standard deviation (s.d.) of triplicate measurements. The corresponding aggregation rate constant (k) is determined by a time point of t1/2, at which the fluorescence intensity reaches half of the maximum fluorescence intensity between the baseline and the plateau (right axis). (d) Secondary structure distributions of Aβ, hIAPP, or hCT (20 μM) in presence of HBD-1 of 0 μM (inner cycle), 5 μM (middle cycle), and 20 μM (outer cycle), as analyzed by the BESTSEL program from circular dichroism (CD) spectra. Morphological characterization of cross-seeding aggregates formed by 20 μM amyloid (Aβ, hIAPP, or hCT) and HBD-1 of 5-20 μM in (e) heights and (f) lengths, as analyzed from AFM images in **Fig. S4**. The upper, middle, and lower bars define the max, mean, and min values of aggregate heights and lengths, respectively.
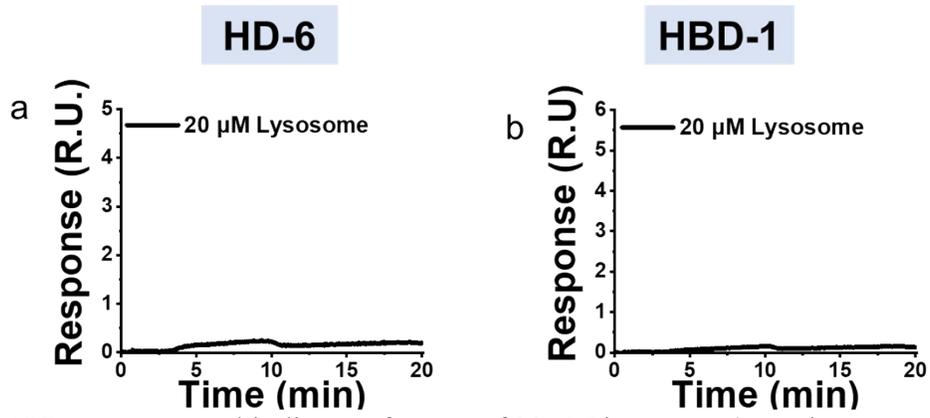
Figure S6. SPR sensorgrams binding preference of 20 µM lysosome (negative control) to (a) HD-6 and (b) HBD-1 coated SPR surface.
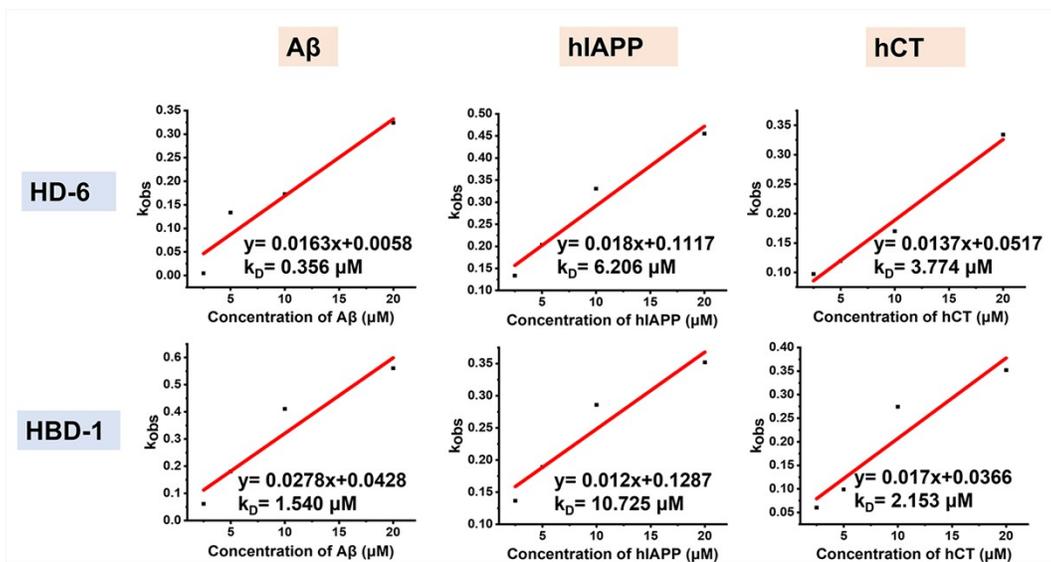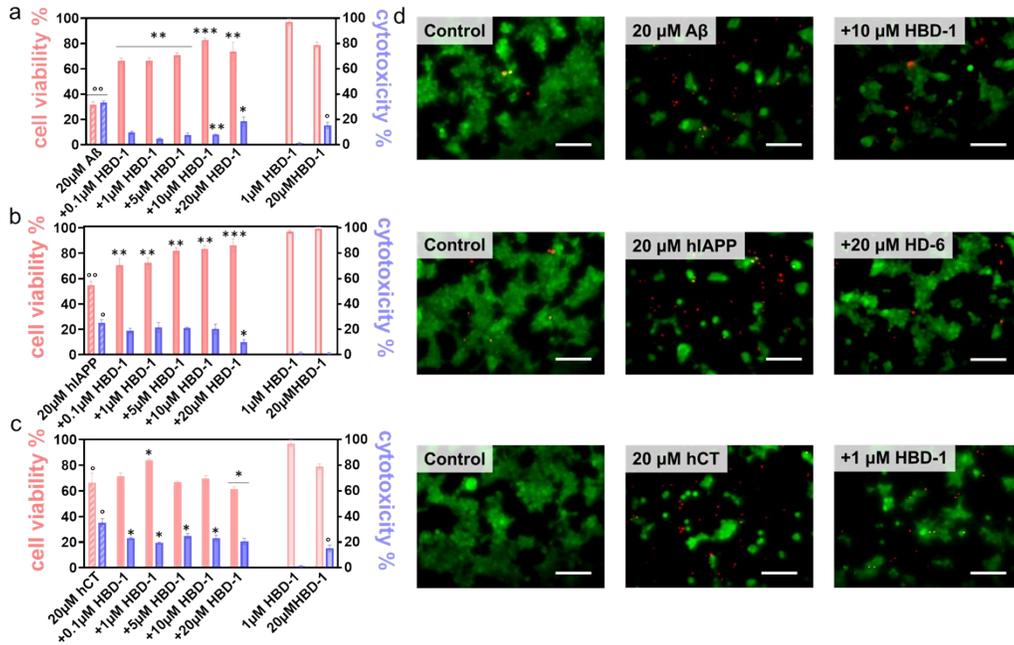
Figure S7. Binding constant (K_D) of HD-6 and HBD-1 to amyloid peptides calculated from SPR sensorgrams (**Fig. 2b-d & Fig. S8a-c**) by fitting observable binding constant k_{obs} to amyloid concentrations.
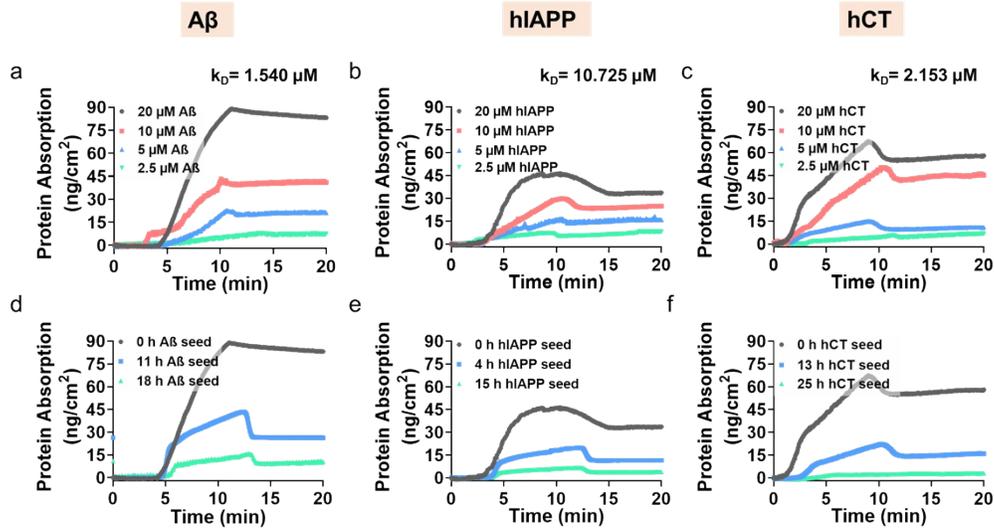
Figure S8. Binding affinity of HBD-1 to amyloid aggregates of different concentrations and sizes by SPR. (a) Schematic workflow for immobilizing defensins on the SPR chip for amyloid binding. SPR sensorgrams to show the adsorption amount (binding preference) of (b) Aβ, (c) hIAPP, and (d) hCT monomers of varied concentrations of 2.5-20 μM on HBD-1 coated SPR surface. SPR sensorgrams to show the adsorption amount (binding preference) of 20 μM (e) Aβ, (f) hIAPP, and (g) hCT monomers (black), oligomers (blue), and fibrillar species (green) on HBD-1 coated SPR surface.
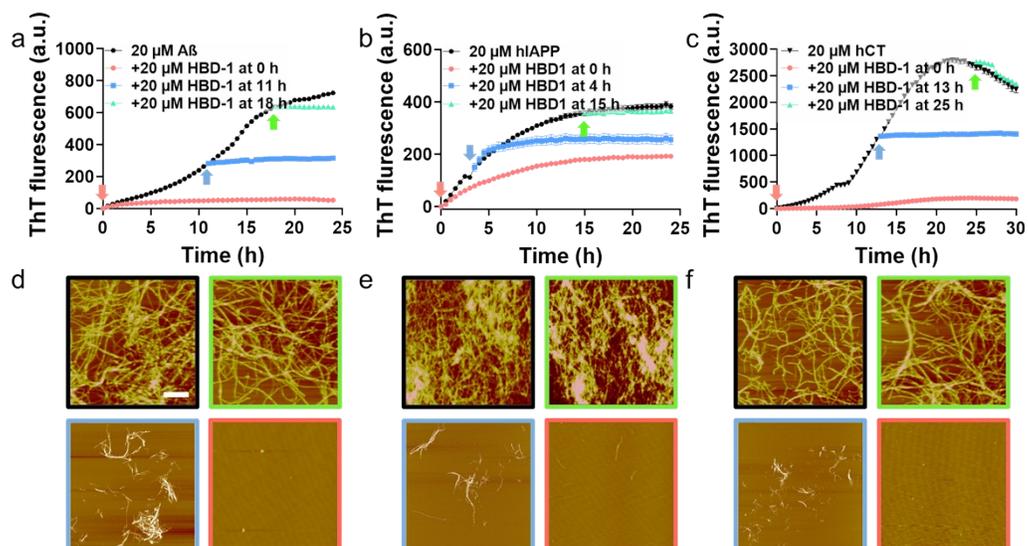
Figure S9. HBD-1 interacts with different amyloid seeds to inhibit their aggregation to different extents. Time dependent (a-c) ThT fluorescent profile and (d-f) representative AFM images for pure amyloid proteins (black) and the addition of 20 μM HBD-1 to 20 μM (a, d) Aβ, (b, e) hIAPP, and (c, f) hCT seeds preformed at the monomeric (red), oligomeric (blue), and fibrillar (green) states. Time points for HBD-1 to different amyloid seeds solution are indicated by arrows in ThT profiles, while scale bars are 1 μm in AFM images.

Figure S10. HBD-1 rescues cells from amyloid-induced cytotoxicity. Dose-dependent protection role of HBD-1 in (a) Aβ-, (b) hIAPP-, and (c) hCT-mediated cytotoxicity using cell viability from MTT assay (red bar, left axis) and cell cytotoxicity from LDH assay (blue bar, right axis), in which SH-SY5Y cells or RIN-m5F cells are treated with amyloid (20 μM) with and without 0.1-20 μM HBD-1 for 24 h. Statistical analysis (n = 3) was conducted for cells treated with HBD-1 or amyloid proteins alone relative to control (°, $p < 0.05$; °°, $p < 0.01$; °°°, $p < 0.001$), as well as cells treated with both HBD-1 and amyloid proteins relative to cells treated with amyloid proteins alone (∗, $p < 0.05$; ∗∗, $p < 0.01$; ∗∗∗, $p < 0.001$). (d) Representative fluorescence microscopy images of cells upon treatment with 20 μM HBD-1 (1st column), 20 μM amyloid proteins (2nd column), and their cross-seeds (3rd column) for 24 h. Scale bars=180 μm.

Figure S11. Antimicrobial activity of defensins, amyloid peptides, and cross-species of defensins-amyloid peptides. Growth profiles of representative Gram-negative bacteria *E. coli* and *P. aeruginosa*, Gram-positive bacteria *S. aureus* and *S. epidermidis* cultured in the absence (black) and presence of 20 µM pure defensins, 20 µM pure amyloid peptides, and defensin-amyloid heterocomplexes. All data represent mean ± standard error of triplicate measurements.
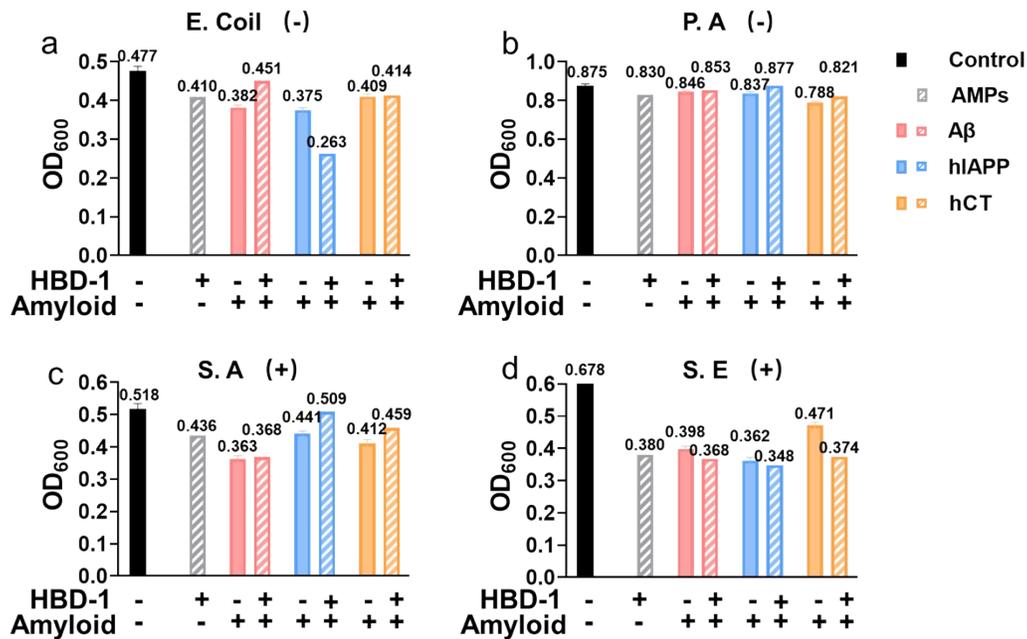
Figure S12. HBD-1-amyloid heterocomplex retains a broad-spectrum antimicrobial activity. Antimicrobial activity of 20 μM HBD-1, 20 μM amyloid peptides, and cross-species of HBD-1-amyloid peptides against Gram-negative (a) *E. Coli* and (b) *P. aeruginosa*, Gram-positive (c) *S. aureus* and (d) *S. epidermidis* quantified by final bacterial density. Bacterial density is determined by OD600. (e) Representative fluorescence microscopy images of *E.coli* treated with freshly prepared amyloid peptides (20 μM) in the absence and presence of 20 μM HBD-1. Red fluorescent propidium iodide and green fluorescent SYTO 9 are used to identify dead bacteria with damaged membrane and live bacteria with intact membranes, respectively. Scale bars=180 μm.
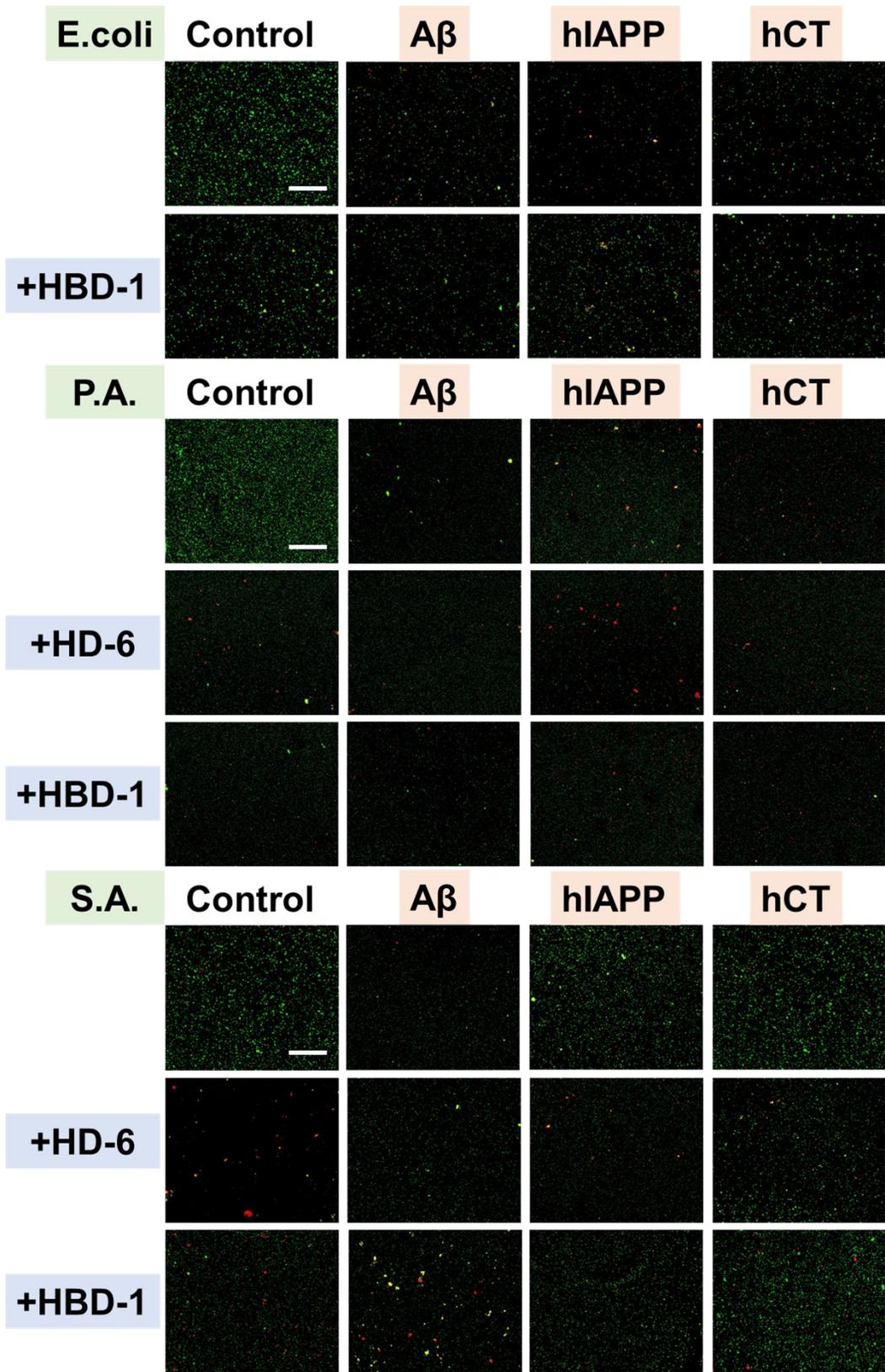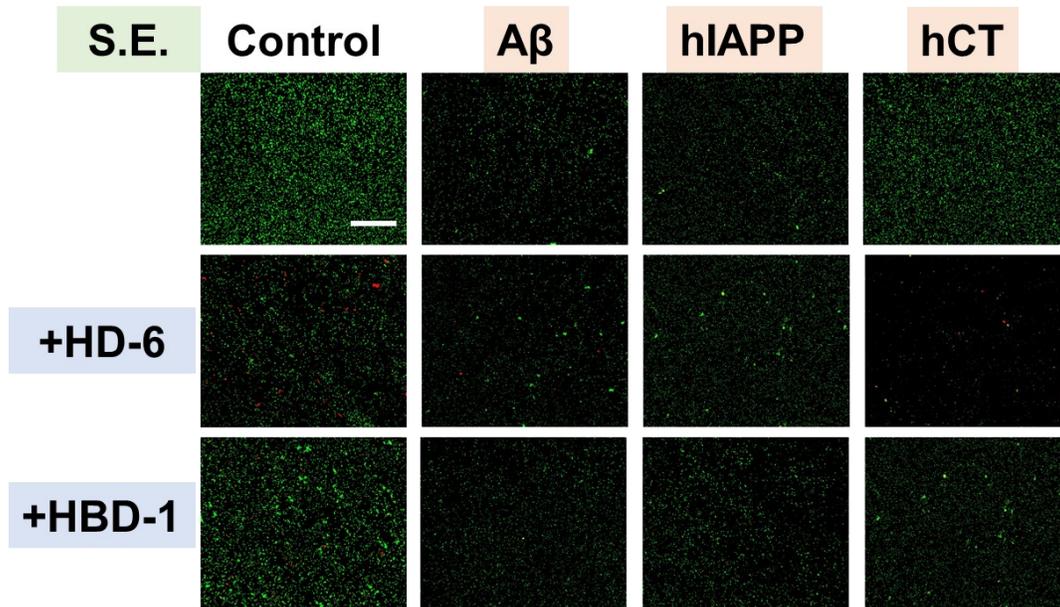
Figure S13. Representative fluorescence microscopy images of Gram-negative *E. Coli* and *P. aeruginosa*, Gram-positive *S. aureus* and *S. epidermidis* treated with or without 20 μM amyloid peptides, 20 μM defensins, and their co-assemblies for 6 h. Red fluorescent propidium iodide and green fluorescent SYTO 9 are used to identify dead bacteria with damaged membrane and live bacteria with intact membranes, respectively. Scale bars=180 μm.