

Cyclen-Hybrid Compound Captures Copper to Protect INS-1 Cells from Islet Amyloid Polypeptide Cytotoxicity by Inhibiting and Lysing Effects

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I₁-Cu(II) inhibited hIAPP aggregation

Fibril is one of the possible folding products of protein.¹ When hIAPP fibril formation was inhibited by I₁-Cu(II), it had the possibility that the blocked hIAPP monomers might have formed other types of aggregates that could escape detection by ThT fluorescence (Figure 1a) and TEM (Figure S1a). Thus, light scattering assay was performed to investigate the total precipitation of hIAPP. This confirmed a significant difference between hIAPP alone and hIAPP co-incubated with the I₁-Cu(II) complex (Figure S1b). Taken together, these findings suggested that I₁-Cu(II) prevented hIAPP from forming fibrils and other types of aggregates. This activity was responsible for attenuating hIAPP cytotoxicity.

The I₁-Cu(II) recognition group increases the specificity and efficiency

We investigated whether recognition was necessary for the inhibition of hIAPP aggregation. ThT fluorescence was used to measure the rate of amyloid formation. hIAPP was incubated over 6 d in the presence of NYGAIL (I₁A) alone, cyclen-Cu(II) mixed with I₁A, I₁ alone, or I₁-Cu(II) (Figure S5a). We found that hIAPP aggregation was not inhibited by I₁A alone, cyclen-Cu(II) mixed with I₁A, or I₁ alone. In contrast to these ineffective building blocks, I₁-Cu(II) dramatically inhibited the formation of ThT-positive aggregates. TEM confirmed that hIAPP fibril formation was effectively blocked only by I₁-Cu(II) (Figure S5b).

Cyclen-Cu(II) complex was crucial for amide cleavage

The cyclen group of I₁ was modified by acetylation to form triacetylcyclen-NYGAIL (Ac₃I₁) (Figure S6a). Ac₃I₁ was less capable of forming a complex with Cu(II) due to the electron withdrawing property of the acetyl group. After incubation with copper, I₁ turned blue, which indicated the occurrence of chelating. In contrast, Ac₃I₁ did not change color after incubation with copper. Next, MALDI-TOF/MS was employed to monitor the occurrence of cleavage. After 13 d of incubation, hIAPP fragments were less apparent with Ac₃I₁ than with I₁ in the presence of Cu(II) (Figure S6b). These results indicated that the successful formation of the cyclen-Cu(II) was important for the inhibitor function.

Materials and Methods

Preparation of I₁, I₁A and Ac₃I₁.

I₁, I₁A and Ac₃I₁ were synthesized by routine solid phase peptide synthetic methods. The stock solutions of I₁-Cu(II) and other Cu(II) complexes were prepared according to reported methods². Briefly, an aqueous solution of CuCl₂ (0.8 equiv) was added

to the compounds (1 equiv). The CuCl_2 solution for all the experiments but cellular assay is protected by glycine (6 equiv to CuCl_2). The stock solutions were incubated at 37°C for 4 h before use.

Fresh hIAPP stock solution preparation.

hIAPP (1-37) (Beijing Boisynthesis Biotechnology Co., LTD., China) stocks were prepared by dissolving in dimethyl sulfoxide (DMSO, AMRESCO, USA) to $10\text{ mg}\cdot\text{mL}^{-1}$ for cellular assay and to $1\text{ mg}\cdot\text{mL}^{-1}$ for the other experiments, sonicating at room temperature for 15 min. Stock solutions were stored in aliquots in polypropylene Eppendorf tubes and frozen at -20°C prior to use. Prior to use all buffers and solutions were filtered through a $0.20\ \mu\text{m}$ filter to remove any particulate matter.

Preformed hIAPP aggregates (fhIAPP) preparation .

Fresh hIAPP ($10\ \mu\text{mol}\cdot\text{L}^{-1}$) was incubated in PBS pH 7.4 at 37°C for 6 h, monitored by ThT fluorescence assay.

Peptide aggregation.

hIAPP aggregation was induced by adding hIAPP stock solution to PBS, pH 7.4, to the final concentration and incubated at 37°C .

Cell culture.

INS-1 rat insulinoma cell line was a gift from Prof. Tao Xu (Institute of Biophysics, Chinese Academy of Science, China). Cells were grown in monolayer cultures in RPMI-1640 (GIBCO Invitrogen, USA) medium supplemented with 10% FBS (GIBCO Invitrogen, USA), $2\text{ mmol}\cdot\text{L}^{-1}$ L-glutamine, $100\text{ U}\cdot\text{mL}^{-1}$ penicillin, and $100\text{ U}\cdot\text{mL}^{-1}$ streptomycin, at 37°C in a humidified (5% CO_2 , 95% air) atmosphere. The cells were seeded in 96-well microplate (Corning Costar Corporation, USA) at a density of 1.2×10^4 cells per well for MTT assays. For TUNEL/DAPI assays, cells were seeded in 6-well plates (Corning Costar Corporation, USA) at a density of 4×10^4 cells per well.

MTT assay.

INS-1 cells seeded in 96-well microplate were cultured at 37°C in a humidified atmosphere for 48 h, and then the cells were exposed to different treatments for 48 h. $10\ \mu\text{mol}\cdot\text{L}^{-1}$ glycine was added to the system in order to exclude the toxic effect of free Cu(II). After incubation, medium was changed and Hank's Balancing Buffer (SIGMA, USA) was added followed by adding $20\ \mu\text{L}$ per well of MTT (AMRESCO, USA) buffer ($5\text{ mg}\cdot\text{mL}^{-1}$ in PBS) and incubated for 4 h. The medium was aspirated and replaced with $80\ \mu\text{L}$ per well of DMSO to dissolve the formazan salt. The color intensity of the formazan solution, which reflects the cell viability, was measured at 550 nm using a Synergy 4 Plate Reader (Biotek Company, USA).

TUNEL assay and DAPI staining .

DNA fragmentation is one of the hallmarks of cell apoptosis which can be detected by TUNEL assay. This assay was performed according to the manufacturer's (Promega Corporation, USA) instructions. Briefly, treated INS-1 cells on cover glass in 6-well plates (Corning Costar Corporation, USA) were fixed with 4% paraformaldehyde for 30 min at 4°C . After washed in PBS, cells were permeabilized with 0.2% Triton X-100 for 20min, and then incubated with $50\ \mu\text{L}$ TUNEL reaction mixture for 60 min at 37°C . The nuclei of the cells were double stained with $1\ \mu\text{g}/\text{mL}$ of DAPI (Promega Corporation, USA) for 30 min. Stained cells were washed with PBS and observed on a fluorescence microscopy (Olympus IX71, Japan)

ThT fluorescence assay.

ThT fluorescence assay was performed by combining $20\ \mu\text{L}$ of incubated solution with $700\ \mu\text{L}$ solution of $10\ \mu\text{mol}\cdot\text{L}^{-1}$ ThT in $12\text{ mmol}\cdot\text{L}^{-1}$ phosphate buffer, pH 7.4. Fluorescence measurements were recorded in a Hitachi FP-4500 fluorescence

spectrometer at RT using a 1cm path length quartz cell. The excitation wavelength was set to 440 nm (slit width = 5 nm), and emission was monitored at 482 nm (slit width = 10 nm).

Light scattering assay.

For determination of total aggregate growth, light scattering was measured in a Hitachi FP-4500 fluorescence spectrophotometer at RT using a 1cm path length quartz cell. Both the excitation and emission wavelengths were set to 405 nm (slit width = 1 nm).

Transmission electron microscopy.

The TEM samples were prepared by placing 8 μ l of the incubated solution in the ThT fluorescence assay on 300 mesh formvar-coated copper grids for 2 min before removing excess solution. Then the sample was stained with 1% fresh tungstophosphoric acid for another 2 min. The grid was blotted on filter paper and allowed to dry before observing the specimen in a JEOL-1200EX electron microscope (JEOL, Japan) at 100 kV.

NuPAGE analysis and silver stain.

Peptides or peptide mixtures were incubated in PBS buffer, pH 7.4, at 37°C. Samples were dissolved and heated to 70°C with NuPAGE sample buffer for 10 min and then subjected to NuPAGE electrophoresis in 4-12 % Bis-Tris gels with MES running buffer according to the manufacturer's (Invitrogen) recommendations. The gels were run under reducing conditions. Silver stain kit (Beyotime Company, China) was used to demonstrate the protein content.

Matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF/MS).

Samples were first passed through a reverse phase C18 Ziptip (Eppendorf, Germany) to remove salts, according to the manufacturer's instructions, then diluted 1:1 with matrix solution (a saturated solution of 3, 5-dimethoxy-4-hydroxycinnamic acid in 50% acetonitrile with 0.1% trifluoroacetic acid in water), loaded onto a plate, and allowed to dry. The sample was then analyzed on an Applied Biosystems 4700 Proteomics Analyzer MALDI-TOF/TOF (USA) operated in reflection mode. Myoglobin was used as an internal standard.

Additional references

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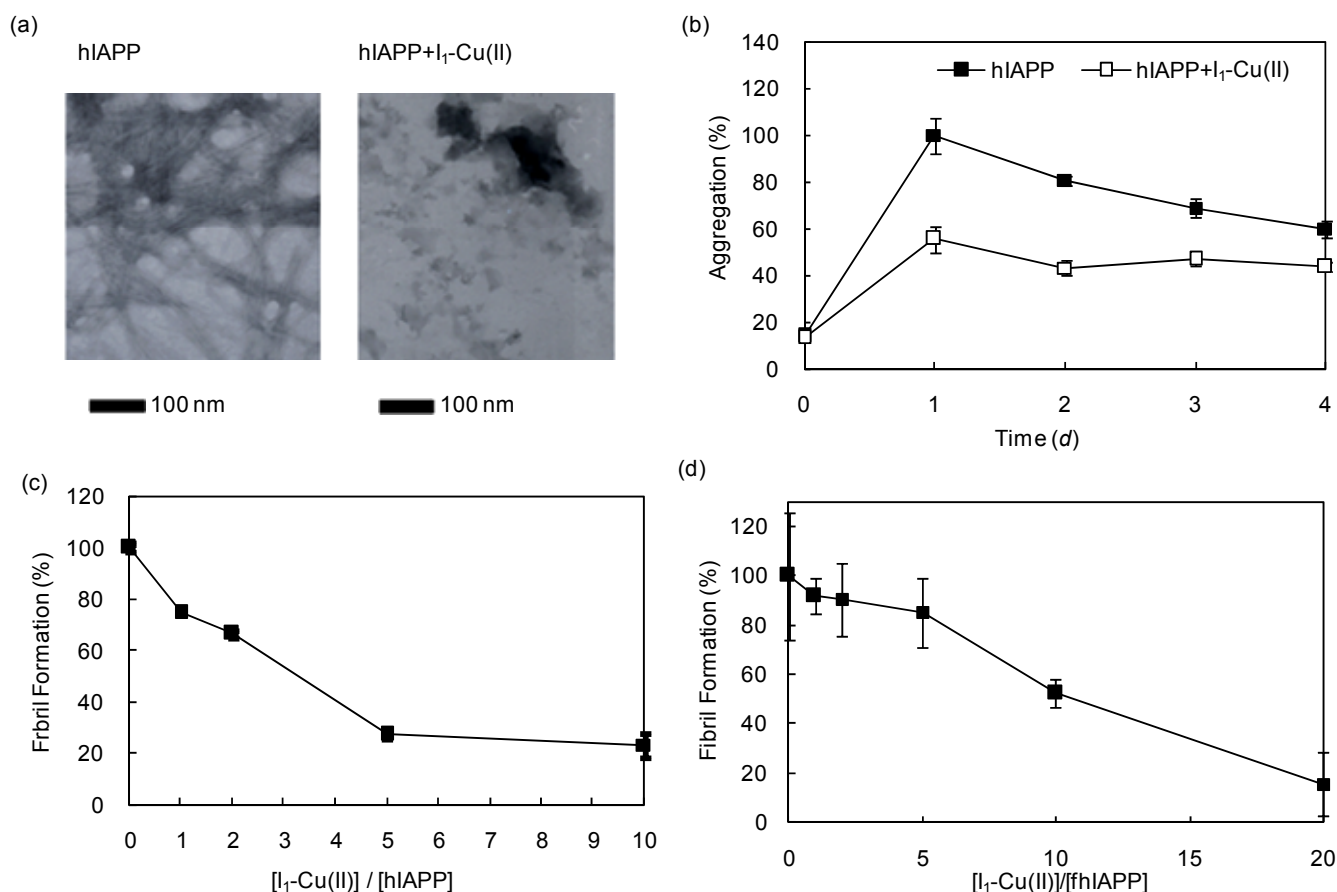


Fig. S1 I₁-Cu(II) interfered with hIAPP aggregation. (a) TEM analysis of I₁-Cu(II) effect on hIAPP fibril formation. hIAPP (10 μmol·L⁻¹) was incubated for 5 d without (left) or with (right) I₁-Cu(II) (100 μmol·L⁻¹). (b) Effects of I₁-Cu(II) (100 μmol·L⁻¹) on the aggregation of hIAPP (10 μM), monitored with a light scattering assay over 5 d. Data are means ± SD, n=3. (c) I₁-Cu(II) inhibited hIAPP aggregation in a dose-dependent manner. Effect of different concentrations of I₁-Cu(II) (10 μmol·L⁻¹, 20 μmol·L⁻¹, 50 μmol·L⁻¹, 100 μmol·L⁻¹) on hIAPP (10 μmol·L⁻¹) aggregation was monitored by ThT fluorescence assay after 2-d incubations. Data are means ± SD, n=3. (d) I₁-Cu(II) reversed hIAPP aggregation in a dose-dependent manner. Effect of different concentrations of I₁-Cu(II) (10 μmol·L⁻¹, 20 μmol·L⁻¹, 50 μmol·L⁻¹, 100 μmol·L⁻¹, 200 μmol·L⁻¹) on fhIAPP (10 μmol·L⁻¹) was monitored by ThT fluorescence after another 55-h incubation. Data are means ± SD, n=3.

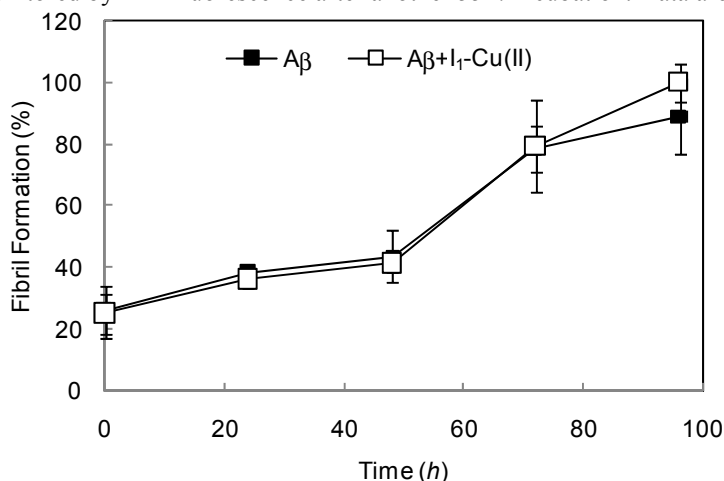


Fig. S2 Effects of I₁-Cu(II) (100 μM) on the fibril formation of Aβ (10 μmol·L⁻¹) monitored by ThT fluorescence assay over 5 d. Data are means ± SD, n=3.

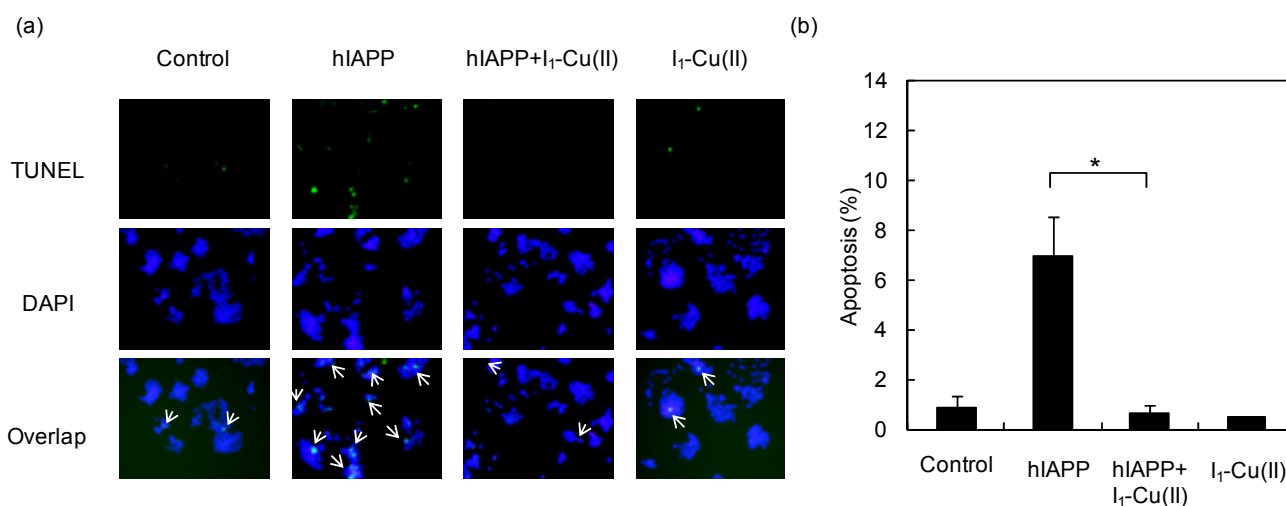


Fig. S3 I₁-Cu(II) inhibited hIAPP induced cell apoptosis. (a) Apoptosis of INS-1 cells was determined with TUNEL labeling (green) and DAPI (blue). INS-1 cells were incubated with hIAPP (10 $\mu\text{mol}\cdot\text{L}^{-1}$) with/without I₁-Cu(II) (100 $\mu\text{mol}\cdot\text{L}^{-1}$) for 48 h. (b) Apoptotic nuclei from (a) were quantified. Data are means \pm SD, n = 3. *: $p < 0.05$ vs hIAPP 10 μM ; **: $p < 0.01$ vs hIAPP 10 $\mu\text{mol}\cdot\text{L}^{-1}$ (by Student's *t*-test).

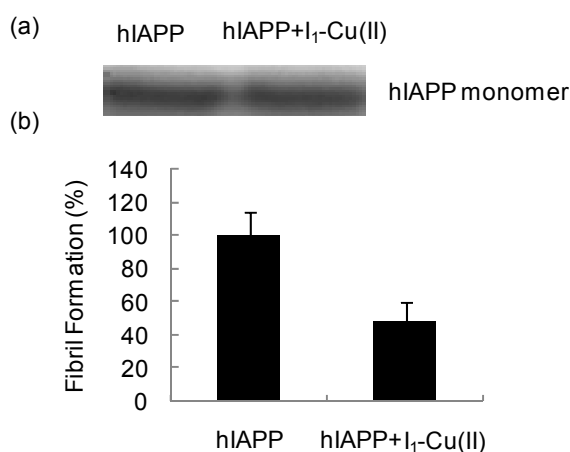
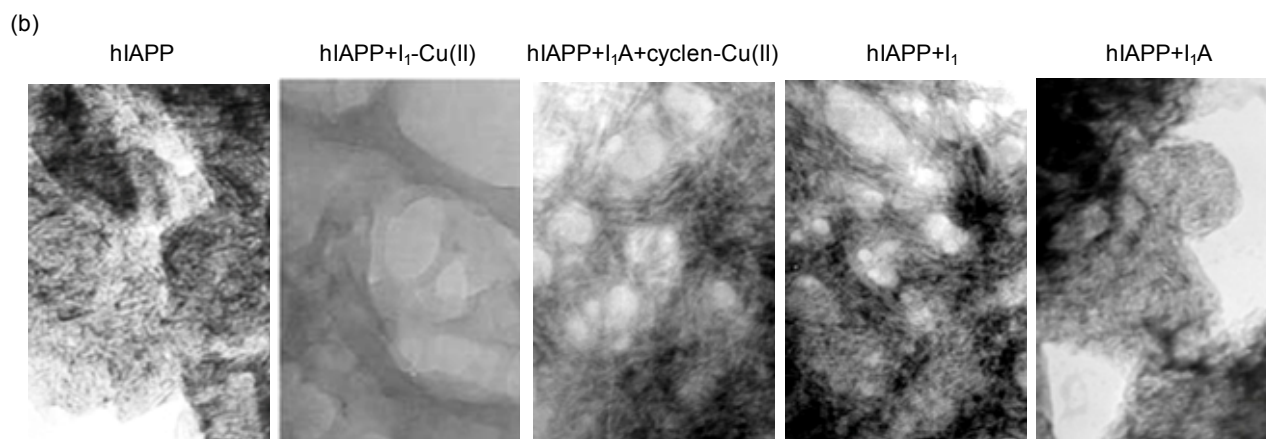
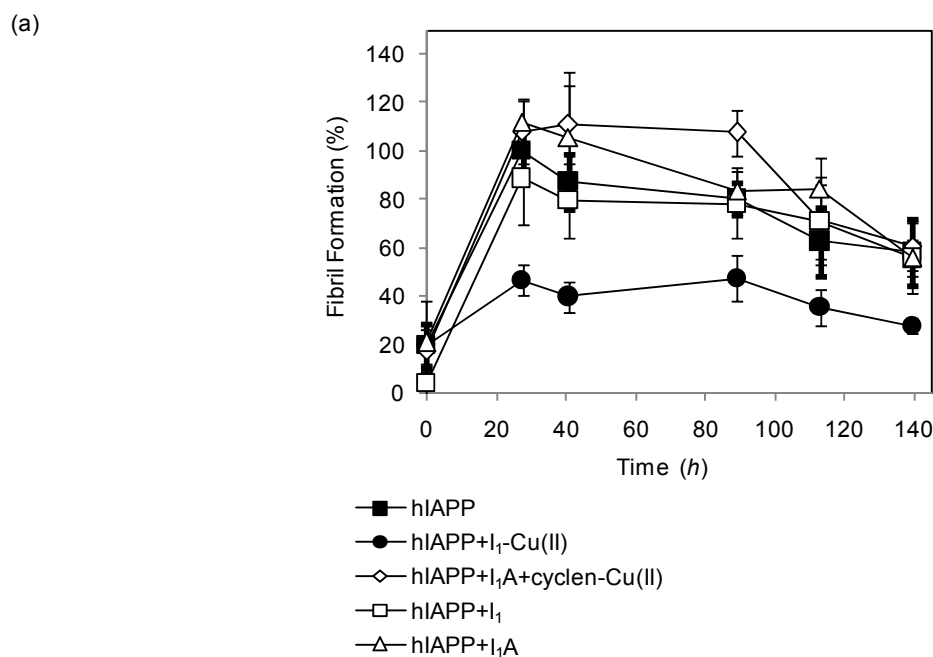


Fig. S4 Mechanism of I₁-Cu(II)'s functioning on hIAPP. (a) hIAPP (10 $\mu\text{mol}\cdot\text{L}^{-1}$) was incubated in the absence or presence of I₁-Cu(II) (100 $\mu\text{mol}\cdot\text{L}^{-1}$) for 1 h and samples were analyzed by electrophoresis. (b) The samples suspended in SDS were also monitored with a ThT fluorescence assay. Data are means \pm SD, n=3.



— 100nm

Fig. S5 I₁-Cu(II) was relatively specific for hIAPP and its function depended on the cooperation of the recognition and cleaving moieties. (A) Effects of I₁A (100 μmol·L⁻¹) alone, cyclen-Cu(II) (100 μmol·L⁻¹) mixed with I₁A (100 μmol·L⁻¹), I₁ (100 μmol·L⁻¹) alone, or I₁-Cu(II) (100 μmol·L⁻¹) on the fibril formation of hIAPP (10 μmol·L⁻¹) was monitored by ThT fluorescence assay over 6 d. Data are means ± SD, n=5. (B) Representative TEM images of hIAPP aggregation after incubations of hIAPP (10 μmol·L⁻¹) alone, hIAPP with I₁-Cu(II) (100 μmol·L⁻¹), hIAPP with cyclen-Cu(II) (100 μmol·L⁻¹) mixed with I₁A (100 μmol·L⁻¹), hIAPP with I₁ (100 μmol·L⁻¹), or hIAPP with I₁A (100 μmol·L⁻¹) for 6 d incubations.

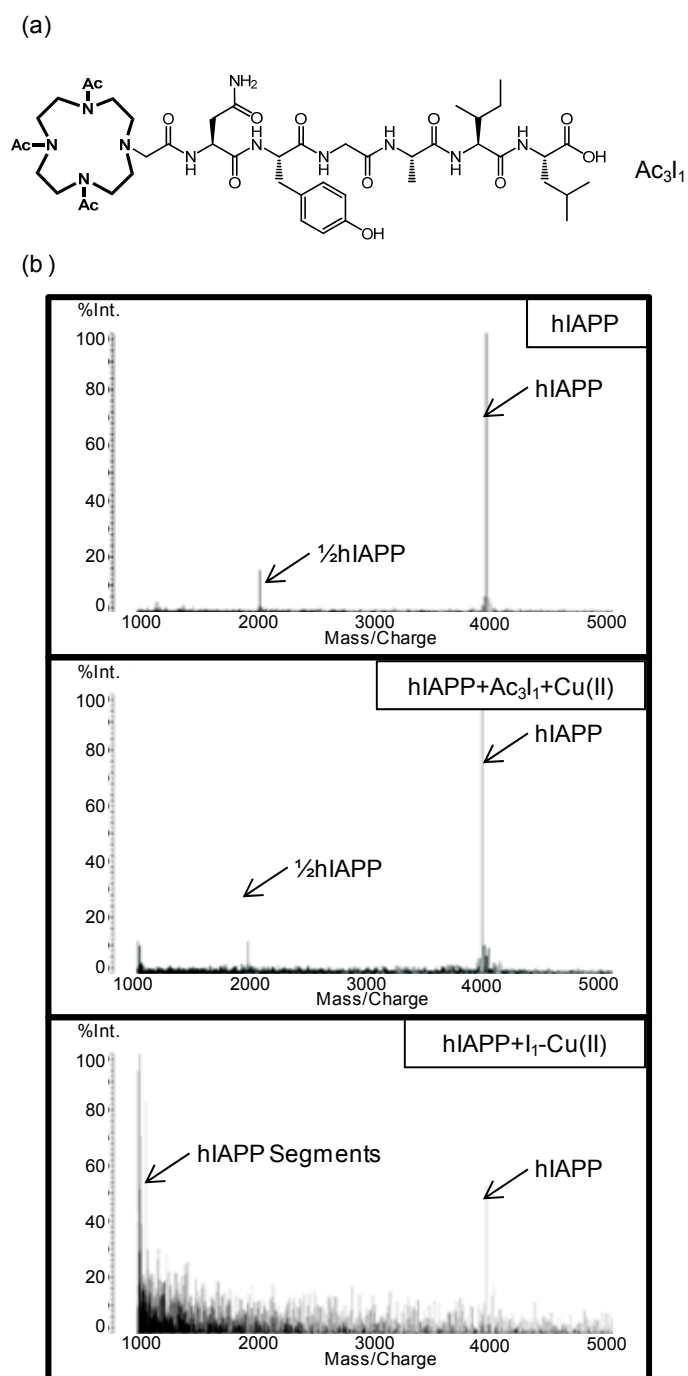


Fig. S6 The acetylated inhibitor, Ac_3I_1 showed little ability to cleave hIAPP. (A) Chemical structure of Ac_3I_1 . (B) MALDI-TOF/MS results of hIAPP ($10 \mu\text{mol}\cdot\text{L}^{-1}$) incubated without (top) or with either $\text{Ac}_3\text{I}_1+\text{Cu}(\text{II})$ ($100 \mu\text{mol}\cdot\text{L}^{-1}$) (middle) or $\text{I}_1\text{-Cu}(\text{II})$ ($100 \mu\text{mol}\cdot\text{L}^{-1}$) (bottom) for 13 d.

Table S1: Peptides Identified by MALDI-TOF/MS. Data are taken from Figure 2a. Only fragment masses above 1000 were analyzed to avoid misidentification.

Observed mass	Possible fragment	Calculated mass	Δ Mass (Da)
985.8 ^a	12-20	986.5	0.7
985.8 ^a	13-21	987.5	1.7
988.8	26-35	990.5	1.7
1006.9	23-32	1007.5	0.6
1037.7	1-10	1039.4	1.7
1048.7	5-14	1047.5	-1.2

a Two possible fragment assignments, based upon observed mass