Bioorganic & Medicinal Chemistry 18 (2010) 2598–2601

Contents lists available at ScienceDirect

Bioorganic & Medicinal Chemistry

journal homepage: www.elsevier.com/locate/bmc

Cu(II) cyclen cleavage agent for human islet amyloid peptide

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ARTICLE INFO

Article history:
Received 8 February 2010
Revised 18 February 2010
Accepted 18 February 2010
Available online 1 March 2010

Keywords:
Type 2 diabetes mellitus
Human islet amyloid peptide
Cu(II) cyclen

ABSTRACT

Type 2 diabetes mellitus (T2DM) is characterized by a substantial reduction in β-cell mass and the amyloid fibrils which are formed by the aggregation of the human islet amyloid polypeptide (h-IAPP) in the islet of Langerhans. Cleavage agents with Co(III) cyclen as the catalytic group have been studied as a novel therapeutic option for T2DM patients. However, recent research has suggested that the cytotoxicity of h-IAPP might be mediated by interactions with Cu(II); furthermore, it has been shown in vitro that Cu(II) prevents h-IAPP from forming the β-sheet conformers. Therefore, we synthesized a cleavage agent using Cu(II) cyclen. The resulting cleaved fragments and estimated cleavage yield (8.3 mol %) were evaluated after incubation with h-IAPP.

1. Introduction

Protein misfolding and aggregation have been linked with numerous fatal diseases including Alzheimer's disease (AD), Parkinson's disease, Huntington's disease, senile systemic amyloidosis (SSA), spongiform encephalopathies (mad cow disease), and type 2 (non-insulin-dependent) diabetes mellitus (T2DM). These fatal diseases have created much interest in developing compounds that can alter the aggregation properties for the purpose of novel drug therapies.

T2DM is characterized by a substantial reduction in β-cell mass and the intra-islet formation of amyloid fibrils.[1,2] The amyloid is formed by the aggregation of the human islet amyloid polypeptide (h-IAPP: also called amylin), an oligopeptide with 37 amino acid residues, which is co-secreted along with insulin from pancreatic β-cells.[3,4]

Extensive research has been dedicated to finding drugs which can act directly with h-IAPP. Among those studies, metal ions have been widely investigated as aggregation inhibitors for h-IAPP. Metal ions have been implicated in the precipitation of amyloidogenic peptides such as Aβ,[5] ABri,[6] Amyloid Protein Non-Aβ Component (NAC),[7] and α-synuclein.[8] Recent papers showed that the cytotoxicity of h-IAPP might be mediated by interaction with Cu(II) and, in addition, that Cu(II) prevents h-IAPP from forming the β-sheet conformers in vitro.[9,10]

The development of conventional drugs against pathogenic proteins is mainly concentrated on finding small molecules that can block the active site of the target proteins such as enzymes, receptors, ion channel, etc. For these conventional drug approaches, excessive amounts of the drug molecule are needed to block the activity of the target protein. In addition, increasing dosage can result in many side effects. However, synthesized artificial enzymes, which can selectively cleave disease-related peptides, can address these two obstacles. The catalytic amount may be enough to act as a catalytic drug, potentially leading to remarkably reduced dosage and side effects. Additionally, catalytic drugs do not need extremely strong affinity toward the target proteins.

Metal complexes bearing macrocyclic structure have been proposed as therapeutic options to alleviate neurotoxicity in many previously reported studies.[11–14] When metal complexes are used to hydrolyze peptide bonds, metal binding hydroxide ion, metal binding water molecules, and the metal ion itself play key roles.[15] Among them, target-selective artificial enzyme synthesis attempts have discovered several efficient catalysts on h-IAPP.[16] The cleavage agents, which have demonstrated activity on h-IAPP, are attached with Co(III) aqua complex of 1,4,7,10-tetraazacyclododecane (Co(III) cyclen) as the catalytic group, which is oxidized from Co(II). The derivative of the imaging agent, which has aromatic moieties and an affinity for amyloid, is attached to the Co(III) cyclen cleavage agent (Fig. 1).[16]

In the present study, we prepared the cleavage agent with Cu(II) cyclen as the catalytic group against the h-IAPP, which could be potentially developed as a more optimal therapeutic option for T2DM. We subsequently determined the difference in cleavage yields between the Co(III) cyclen cleavage agent and the Cu(II) cyclen cleavage agent, and additionally investigated the reasons for this difference.

2. Materials and methods

2.1. Synthesis of cleavage agents

Catalyst moieties, not combined with metal ions, were synthesized as described previously.[17] Cleavage agents with Co(III) aqua

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doi:10.1016/j.bmc.2010.02.045

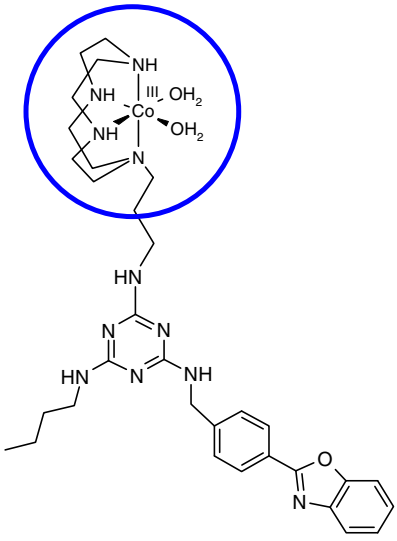

Figure 1. Cleavage agent attaching Co(III) cyclen (circle) as the catalytic group is shown.

complex of 1,4,7,10-tetraazacyclododecane (Co(III) cyclen) were also synthesized as reported previously.[16] Cu(II) aqua complex of 1,4,7,10-tetraazacyclododecane (Cu(II) cyclen) was identified by crystallographic structures.[18] Complexation with Cu(II), in order to obtain the Cu(II) cyclen aqua complex on the catalyst, was carried out as described in the literature.[19] Synthesized catalyst moiety was dissolved in methanol. The stock solution of Cu(II) aqua complex was prepared by adding 0.95 equiv of $CuCl_2$ (in methanol) to the catalyst moiety to avoid Cu^{2+} ion interaction with h-IAPP. After solvent evaporation, Cu(II) cyclen cleavage agent stock solution was obtained by dissolving in purified water. The stock solution was incubated at 37 °C for 4 h before use.

2.2. Substrate (h-IAPP) preparation

Synthetic h-IAPP (1 mg) (purchased from Ana-Spec, San Jose, CA, USA) was solubilized to 1 mg/ml in 1, 1, 1, 3, 3, 3-hexafluoroisopropanol (HFIP, Acros, USA) and was separated into 32 aliquots. The solution was sonicated at room temperature for 5 min, lyophilized, and stored at 4 °C. The lyophilized sample was re-dissolved in 1 mL HEPES buffer (50 mM, pH 7.5, Sigma, USA) to make 8 μM of h-IAPP solution. Prior to use, the solution was filtered (0.20 μm) to remove any particulates and autoclaved at 120 °C for 2 h.

2.3. Incubation

After the catalysts were activated at 37 °C for 4 h, cleavage agents with Co(III) cyclen and Cu(II) cyclen were added to 8 μM h-IAPP solution and adjusted to final concentrations of 1 μM for the catalyst and 8 μM for h-IAPP, respectively. Incubation was carried out at 37 °C for 36 h.

2.4. Measurement

After incubation, identification of the cleaved fragments of h-IAPP was carried out with MALDI-TOF MS measurement (Bruker Daltonics Autoflex II MALDI-TOF/TOF mass spectrometer). Cleavage yields were estimated by fluorescamine assay. After incubation at 37 °C for 36 h, the solution was fully filtered through a 10,000 Da MW-cutoff filter (Millipore Microcon centrifugal filter device YM-10) at 13,000*g* for 30 min at room temperature with a microcentrifuge (Hanil Model Micro 12) to obtain h-IAPP monomer, dimer, and cleaved fragments. The 100 μL of collected filtrate was transferred to a 500 μL microfuge tube to perform amino acid analysis. After addition of 10 μL of 13.5 M NaOH, the sample was placed in an autoclave at 120 °C for 2 h to hydrolyze amino acids. After cooling to room temperature, the mixture was neutralized with methanesulfonic acid (15.4 M, 7.5 μL), and boric acid solution (0.7 M, 48 μL) was added to adjust the pH to 9.0. After brief vortexing and centrifugation, samples were loaded onto a 96 well plate (F96 Cert. Maxisorp, Nunc-Immuno Plate). Fluorescamine in acetonitrile (3.0 mg/mL, 10 μL) was also loaded onto the 96 well plate and subsequently mixed. The relative value of fluorescence of the resulting solution was measured with alpha-imager (Model 1220 INT) and compared with a standard curve. As controls, after the h-IAPP buffer solution without catalyst was incubated, same molar catalysts (1 μM) were added to the solution, respectively.

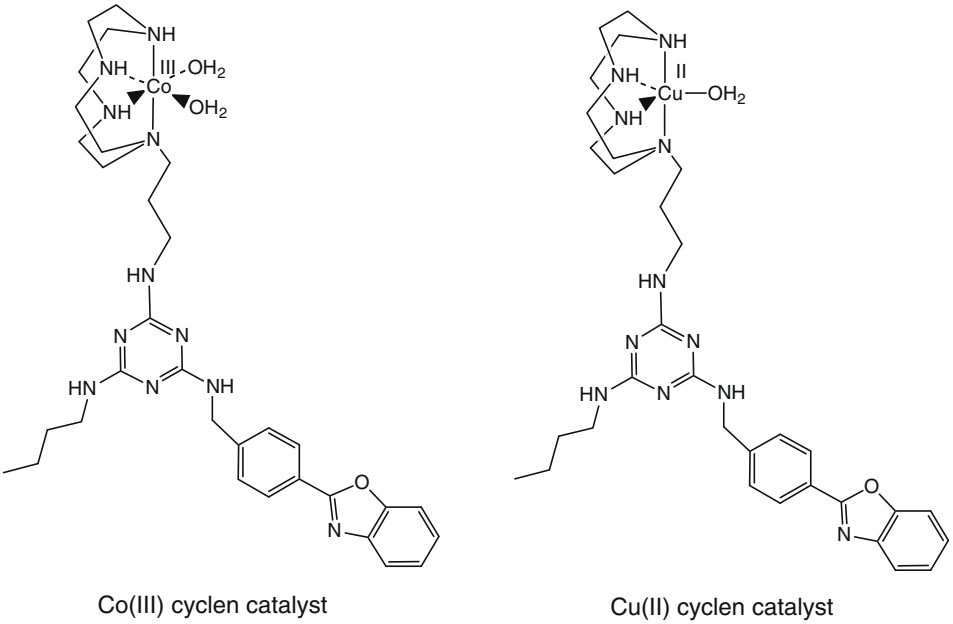

Figure 2. Synthesized cleavage agents with Co(III) cyclen and Cu(II) cyclen.

The mean fluorescence intensity of three experiments was used to determine the peptide quantity.

3. Results

The use of Co(III) cyclen as the cleavage center for h-IAPP has been reported previously.[16] Based on the previous results, we synthesized a Cu(II) cyclen cleavage agent which showed activity towards h-IAPP. In order to compare activities, the cleavage agent containing Co(III) cyclen as the catalytic group was also synthesized (Fig. 2).

The activities of the cleavage agents were judged by the appearance of peptide fragments of h-IAPP. MALDI-TOF mass spectra were obtained after incubation of h-IAPP with cleavage agents. Depending on the cleavage agents, fragments were identified as one of the following: h-IAPP$_{12-37}$, h-IAPP$_{19-37}$, and h-IAPP$_{20-37}$. The h-IAPP fragments are named by the amino acid sequence of h-IAPP. The major fragments of h-IAPP resulting from each cleavage agent were not identical (Fig. 3).

The amount of h-IAPP peptide fragments was determined by fluorescamine assay. The product solution obtained by the reaction of h-IAPP (8 μM) with the cleavage agent (1 μM) was filtered through 10 kDa MW-cutoff membrane to remove h-IAPP fibrils. The filtrate contains h-IAPP monomer, dimer, and cleaved h-IAPP fragments. The solution was hydrolyzed under alkaline conditions and amino acids were quantified with fluorescamine. Cleavage yields were estimated as mol% of the initial amount of h-IAPP (8 μM). Fluorescence value obtained by the control sample was subtracted from the result. The cleavage yield was estimated by comparison with a standard calibration curve. Therefore, the cleavage yields obtained by Co(III) cyclen cleavage agent and Cu(II) cyclen cleavage agent were estimated as 11.6 mol % and 8.3 mol %, respectively.

4. Discussion

According to a recent publication,[16] almost all (>90%) of h-IAPP cannot pass through a 10,000 MW membrane filter after 36 h of incubation. During filtration, only small amounts of h-IAPP exist as monomer and dimer in the filtrate. This concept is supported by the MALDI-MS data presented in Figure 3.

The mechanism of hydrolysis on soluble h-IAPP oligomers by cleavage agents was previously proposed; aromatic side chains of h-IAPP may play a key role in the self assembly process.[16] The ami-

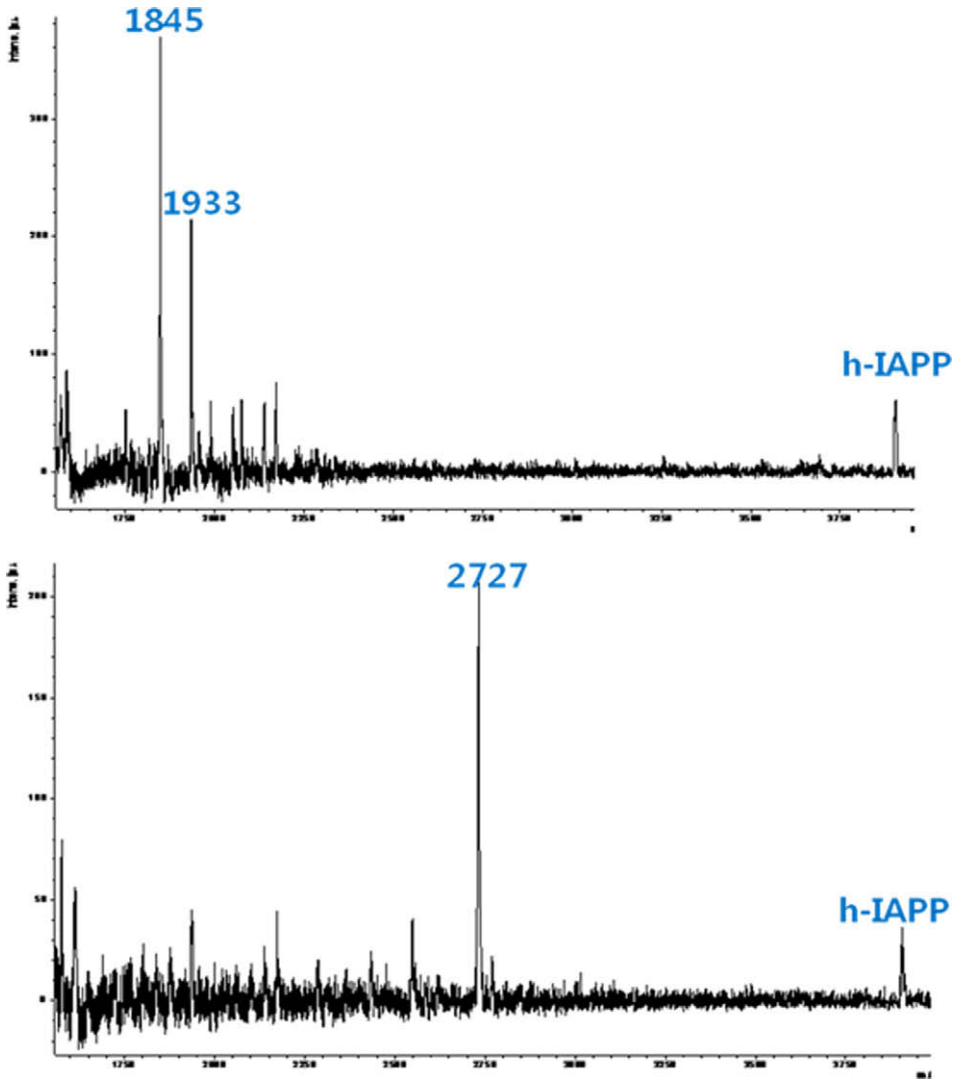

Figure 3. MALDI-TOF mass spectra of the product obtained by incubation of h-IAPP (8 μM) with Co(III) cyclen cleavage agent (1 μM, top spectrum), Cu(II) cyclen cleavage agent (1 μM, bottom spectrum) for 36 h at pH 7.50 (50 mM HEPES) in 37 °C. The peaks with m/z of 1845, 1933, and 2727 correspond to h-IAPP$_{20-37}$, h-IAPP$_{19-37}$, and h-IAPP$_{12-37}$, respectively

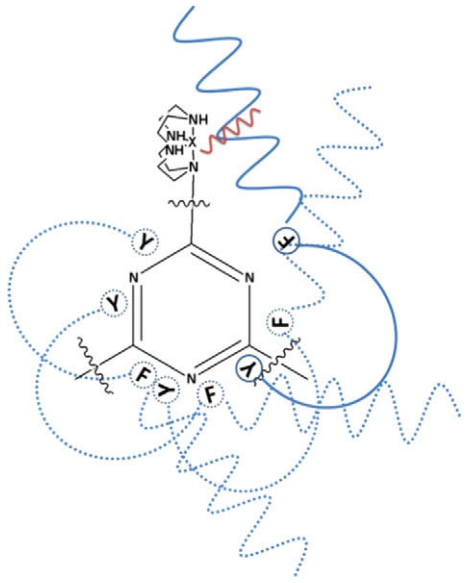

Figure 4. Schematic of proposed soluble oligomer cleavage of h-IAPP (X = Co(III) or Cu(II); Y = tyrosine, h-$IAPP_{37}$; F = phenylalanine, h-$IAPP_{23}$). The C-terminal portion of h-IAPP interacts with the catalyst moiety, leading to hydrolysis of the N-terminal portion of h-IAPP.

no acids of h-IAPP, which contain aromatic side chains, are h-$IAPP_{23}$ and h-$IAPP_{37}$ amino acids corresponding to phenylalanine and tyrosine. Based on analysis of the cleaved fragments (Fig. 3), the center, which interacts with the triazine scaffold and imaging agent, may be C-terminal portion of h-IAPP (Fig. 4).

The cleavage agents interact with the C-terminal portion of h-IAPP, thus forming a complex. In the complex, the metal center hydrolyzes the peptide bond and fragments are produced. However, as shown in Figure 3, the Cu(II) cyclen and Co(III) cyclen cleavage agents yielded fragments which were different from one another. The cleavage yield produced by the Cu(II) cyclen cleavage agent (8.3 mol %) was lower than Co(III) cyclen cleavage agent (11.6 mol %).

Metal exchange inertness may be a contributing factor in the different catalytic activities seen between Cu(II) and Co(III) cyclen cleavage agents. Metal transfer between metal-abstracting material and solvent is slower for a Co(III) complex than a Cu(II) complex due to the exchange-inertness of Co(III) ions. Followed by the study on a liability of metal-organic ligand complex with freshwater, Cu(II) ion exchange rates are approximately 1000 times higher than that for Co(III) ions.[20] By the result, water or peptides can lower the Cu(II) cyclen cleavage agent concentration in the limited hydrolysis reaction time.

We have proposed to cleave h-IAPP with Cu(II) ion, which can inhibit h-IAPP to form the β-sheet conformers. Although the liability of Cu(II) ions on the solvent would lower the cleavage agent's activity, the secreted Cu(II) ion from cleavage agent might lower toxicity towards β-cells, resulting in a more appropriate artificial protease to be used in drug design. To investigate this relationship between β-cell toxicity and these two types of cleavage agents, future in vivo studies are needed.

Acknowledgments

This study was funded by the Korea Military Academy Hwarang-dae Research Institute research program. We acknowledge Prof. Suh, Junghun of Seoul National University for allowing us to use their research facilities during the course of this study.

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