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Small-Molecule Inhibitors

Small-Molecule Inhibitors of Islet Amyloid Polypeptide Fibril Formation**

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Newly synthesized proteins in the cell adopt a functional folded state resulting from a highly regulated process. Failure to form this functional state leads to the degradation of proteins inside the cell. However, under certain conditions, some proteins can also adopt an alternative state by the assembly of unfolded or partially folded monomers or protein fragments into a β-sheet structure called amyloid fibril.[1] In spite of arising from diverse amino acid sequences, they have a similar fibrillar structure that binds the dye Congo red.[2] These amyloids are involved in a number of devastating diseases including Alzheimer's disease, prion diseases, and type-II diabetes mellitus.[3]

In the type-II diabetes, deposition of extracellular amyloid plaques in pancreatic beta cells has been observed in humans. Biochemical analysis of the plaques revealed the presence of a 37-residue peptide called islet amyloid polypeptide (IAPP) or amylin, which is co-secreted with insulin. It has a disulfide bond between residues 2–7 and the C-terminus is amidated.[4–6] IAPP is also known to interact with lipid membranes which are able to induce and foster the fibril formation.[7] Recently, a possible mechanism of IAPP fibril formation at anionic lipid interfaces has been proposed in which it has been shown that IAPP forms β-sheet-rich amyloid fibrils via an intermediate α-helical state.[8] The presence of IAPP amyloid finally leads to the apoptosis of pancreatic beta cells.[9] However, it is still not clear whether the fibrils themselves or their intermediate states are responsible for the cell death.[10] In nature, IAPP amyloid fibril formation can be prevented by altering the primary amino acid sequence, such as in rat IAPP where three proline residues, which are absent in the human IAPP, are thought to prevent the amyloid fibril formation.[11] Recently, Kapurniotu's group succeeded in the synthesis of conformationally constrained analogues of IAPP, which are methylated at amide bonds and do not fibrillize.[12,13]

Inhibition of amyloid fibril formation is considered to be a potentially key therapeutic approach towards diabetes and other amyloid-related diseases.[14] Surprisingly, very little attempt has been made to inhibit IAPP fibril formation by small-molecule inhibitors.[15] Small-molecule inhibitors have advantages over peptide inhibitors because they could more easily cross the blood brain barrier, avoid immunological response, and are more stable in biological fluids and tissues.[16] In addition, the high flexibility of peptide inhibitors may, for entropic reasons, prevent efficient binding. This problem may be overcome by synthesis of conformationally restricted peptides.[12,13] The bottleneck in the discovery of small-molecule inhibitors of amyloid fibril formation is the lack of structural information about amyloids. However, this did not prevent the discovery of small-molecule inhibitors for other amyloid fibrils such as, Aβ[17,18] and tau,[19,20] which are involved in Alzheimer's disease.

In a recent study on a cellular model of tau aggregate inhibition, two rhodanine-scaffold (2-thioxothiazolidin-4-one) based inhibitors have been identified which have very low cell toxicity.[21] These compounds were chosen because of the presence of a rhodanine heterocyclic core, which is biocompatible, non-mutagenic,[22] and has a drug-like profile. Inspired by these results, we wanted to explore whether these compounds will also inhibit amyloid fibril formation of IAPP which has a completely different amino acid sequence but shares a similar fibrillar morphology with the tau aggregate. To our knowledge, this is the first study on such small-molecule inhibitors of IAPP amyloid formation.

The compounds **1** and **2** (Figure 1) were synthesized as described earlier.[21] Amyloid fibril formation was carried out in 10 mM sodium phosphate buffer at pH 7.5 for 96 h. To reveal the effect of the two potential inhibitors, different concentrations of the compounds were added to the buffer solution. Fibril formation was quantified by measuring the fluorescence intensity of the amyloid-specific dye thioflavin T (ThT) at a wavelength of 480 nm. The fluorescence intensity of amyloid fibrils increases upon binding to ThT. The efficiency of inhibition was monitored by measuring the ThT fluorescence intensity with respect to that of pure IAPP aggregate without inhibitor (100%). It is evident from Figure 1b that both compounds have a marked inhibitory effect. The concentration at which half of the fibril formation is inhibited (IC50) is 1.23 μM for compound **1**, and 0.45 μM for compound **2**. A similar trend has been observed for the aggregation of tau, with IC50 values of 0.67 and 0.26 μM for compounds **1** and **2**, respectively.[21] From the results on tau and IAPP aggregation it is clear that compound **2** is a more

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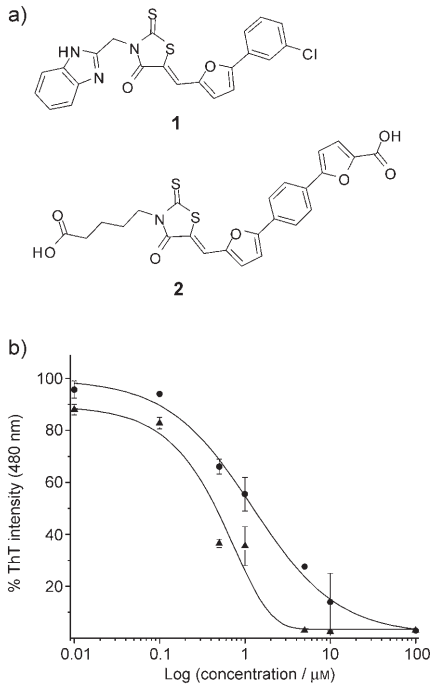

Figure 1. a) Structures of compounds **1** and **2**. b) Effect of different concentrations of compound **1** (●) and **2** (▲) on IAPP amyloid fibril formation with a constant IAPP concentration of 10 μM. After 96 h, samples were incubated for 1 h with 10 μM of ThT at room temperature. The intensity of the ThT fluorescence at 480 nm is plotted against a control IAPP sample (100%) with no inhibitor.

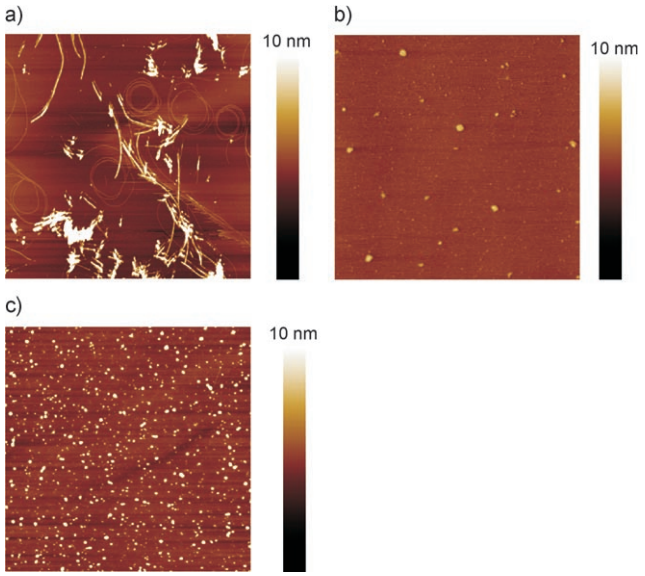

Figure 2. AFM images of the amyloid fibrils of a) pure IAPP and of IAPP in the presence of a molar ratio 1:1 of b) compound **1** and c) compound **2** (width of image: 4 μm; height scale on the right-hand side). The solution was kept for 96 h at room temperature (25 °C) before samples were taken, diluted, and measured. The IAPP concentration was 50 μM.

than two-fold more potent inhibitor than compound **1** (*P*-value = 0.0085). The results obtained also indicate that it is very likely that IAPP interacts with inhibitor **1** largely in the ratio 1:1, which suggests that the inhibition occurs at a very initial stage of the aggregation process. The non-equimolar ratio inhibition observed for compound **2** indicates that this compound is also active against small oligomers and prevents them from forming mature fibrils. The interaction is probably initialized by interaction of the heterocyclic ring system of the inhibitors with hydrophobic patches on the surface of the monomeric or oligomeric IAPP. The differences in the side chains of the scaffold of compounds **1** and **2** may explain the different extents of inhibition as was previously discussed in the case of the tau protein.[21] Notably, the striking common features shared between reported inhibitors of the aggregation of other amyloids[23,24] are the rigid planar hydrophobic central core decorated with highly polar functionalities, such as carboxylic acid, sulfonates, or phenols.

Owing to the fact that the fluorometric ThT assay may be prone to errors arising from the inhibitor–ThT interaction, additional techniques were employed to provide support of these findings. Further evidence of inhibition of IAPP fibril formation came from morphological studies using atomic force microscopy (AFM). Fibrils grown for 96 h at room temperature were diluted, applied on cleaved mica, and dried before scanning at room temperature. IAPP forms long unbranched fibrils as shown in Figure 2a, with heights of 3–4 nm which is typical for amyloid fibrils.[25] To visualize the effect of the two compounds on IAPP fibril formation, IAPP was incubated with compounds **1** and **2** in a 1:1 molar ratio. No fibril formation and appearance of a few oligomeric species only, was observed in the case of compound **1** (Figure 2b). For compound **2**, abundance of small oligomeric structures is visible (Figure 2c). Hence, the AFM data clearly support the results obtained from the ThT fluorescence assay. Morphological differences induced by the inhibition with compounds **1** and **2** indicate a differential mechanism of inhibition of IAPP fibril formation. What is clear from Figure 2c is that inactive monomers and/or oligomers are present as inhibition product, only, and compound **2** seems to preferentially interact with the rate-limiting intermediate oligomeric species.

Negatively charged lipid membranes are known to accelerate IAPP fibril formation.[7,8,26,27] It is believed that the membrane interface provides sites for initiating the aggregation process. Therefore, we tested whether these inhibitors are also able to inhibit fibril formation of IAPP in the presence of lipid membranes. Lipid bilayer membranes were prepared from large unilamellar vesicles (LUV) made up from dioleoyl phosphatidylcholine (DOPC) and dioleoyl phosphatidylglycerine (DOPG; which is negatively charged) in the molar ratio 7:3. We applied—to our knowledge for the first time—the surface-sensitive attenuated total reflection Fourier-transform infrared (ATR-FTIR) spectroscopy technique[28] to be able to follow the fibril formation at the lipid interface (top of Figure 3). The buffer solution containing the lipid vesicles was injected into the ATR cell leading to spontaneous formation of supported lipid bilayers. ATR-FTIR absorbance spectra are dominated by the signal from the supported membrane, including the membrane-bound protein, whereas—because of the low penetration depth (< 1 μm) of the evanescent IR wave—the molecules far from the membrane do not contrib-

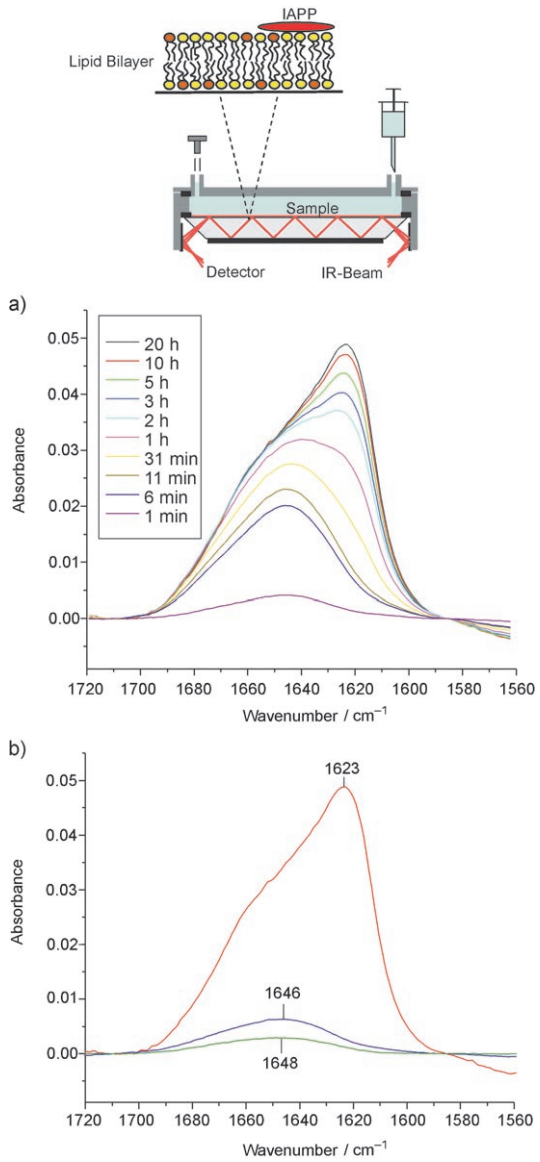

Figure 3. a) Time-evolution of the ATR-FTIR spectra of 10 μM IAPP. b) ATR-FTIR spectra of 10 μM IAPP (red), 10 μM IAPP + 1 μM compound **1** (green) and 10 μM IAPP + 1 μM compound **2** (blue) after 20 h incubation. All the spectra were collected in the presence of a lipid bilayer made up from a 7:3 molar ratio of DOPC:DOPG at 25 °C and deposited on the ATR crystal in buffer solution. Top: Scheme of the ATR sample cell with an internal Ge reflection plate covered with the DOPC–DOPG lipid bilayer. IAPP molecules adsorbing to the membrane are detected while those distant from the membrane are barely visible owing to the low penetration depth of the evanescent IR wave (< 1 μm).

ute significantly to the ATR-FTIR spectra. This situation makes ATR-FTIR spectroscopy a surface-sensitive technique that allows the study of protein binding to supported membranes, and of the structural changes in both membrane lipids and the protein that result from protein–membrane interactions. The IAPP solution was then injected into the ATR cell and allowed to adsorb at the membrane for up to 20 h. The ATR-FTIR spectra shown in Figure 3a show the development of the aggregation band of IAPP with time. Formation of β-sheet-rich amyloid fibrils is characterized by their specific amide I subband which appears at approximately 1623 cm^{-1}.[29] As not all of the amino acids are involved in the intermolecular β-sheet formation, a broad shoulder remains around 1646 cm^{-1}. The high absorbance value indicates that IAPP molecules are in close contact to or even inserted into the membrane. In agreement with other recent spectroscopic data,[7,8] no such behavior is observed for neutral lipids or in the absence of the anionic lipid bilayer in the time-range covered in these experiments. When compounds **1** and **2** were included in the buffer, no such aggregation subband is visible (Figure 3b). Rather, broad IR bands at about 1646–1648 cm^{-1} with low intensity appear, which are characteristic of a large contribution of disordered conformations arising from the presence of monomeric and small oligomeric IAPP particles in solution close to the lipid interface (in the evanescent wave region), only. Comparison of Figures 2b and 2c reveals that in the presence of compound **2**, IAPP forms small oligomeric structures of the size of 3–4 nm, which are largely absent in the case of compound **1**. Such a scenario is in accord with the infrared data which suggest that the IAPP-compound **2** complex corresponds to the existence of a significant population of structurally unordered oligomers. A slightly different picture emerges for the scenario with compound **1**, which exhibits a very low IR absorbance only, typical for the presence of unfolded monomeric IAPP in the solution. Again, this indicates that the two inhibitors effectively interfere with the strong native ability of IAPP to self-assemble, with differential specific interactions, however, which lead to different populations of the final inactive peptide species. This result also demonstrates that even minor changes in the structure of these small-molecule inhibitors may lead to several different behaviors for modulating protein aggregation.

Generally, the lack of atomic resolution structures of amyloid fibrils hampers the search for small-molecular inhibitors. By screening of guiding structures, such inhibitors may be found. Herein, we have reported the inhibition of IAPP amyloid fibril formation by non-peptidic, small molecules with a rhodanine scaffold at submicromolar concentrations. By using ThT fluorescence and ATR-FTIR spectroscopy as well as atomic force microscopy we were able to show that the compounds **1** and **2** are effective inhibitors of IAPP amyloid fibril formation. These inhibitors may be of particular interest because of their biocompatibility and negligible cytotoxicity at the concentrations investigated.[21] As the cellular membrane is considered to be the primary target for amyloid toxicity,[30] these compounds promise to be also active against IAPP fibril formation in the cell, since they already show inhibition in the presence of aggregation-fostering lipid interfaces. Inhibition of fibrillation is an important aspect in preventing (proto)fibril-induced toxicity. However, an ideal inhibitor should be able to inhibit other potential cytotoxic species, such as the oligomers as well. Hence, compounds which act in the very early stage of the fibrillation reaction, before cytotoxic species are formed, would be ideal candidates for drug development. Finally, we presume that the time-lapse ATR-FTIR spectroscopy technique introduced for studying aggregation processes of

amyloidogenic proteins at lipid interfaces may prove very valuable in forthcoming studies on related systems.

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