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Benzofuranone Derivatives as Effective Small Molecules Related to Insulin Amyloid Fibrillation: A Structure–Function Study

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Amyloids are protein fibrils of nanometer size resulting from protein self-assembly. They have been shown to be associated with a wide variety of diseases such as Alzheimer’s and Parkinson’s and may contribute to various other pathological conditions, known as amyloidoses. Insulin is prone to form amyloid fibrils under slightly destabilizing conditions in vitro and may form amyloid structures when subcutaneously injected into patients with diabetes. There is a great deal of interest in developing novel small molecule inhibitors of amyloidogenic processes, as potential therapeutic compounds. In this study, the effects of five new synthetic benzofuranone derivatives were investigated on the insulin amyloid formation process. Protein fibrillation was analyzed by thioflavin-T fluorescence, Congo red binding, circular dichroism, and electron microscopy. Despite high structural similarity, one of the five tested compounds was observed to enhance amyloid fibrillation, while the others inhibited the process when used at micromolar concentrations, which could make them interesting potential lead compounds for the design of therapeutic antiamyloidogenic compounds.

Key words: aggregation, amyloid, benzofuranone, bovine insulin, inhibition

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Amyloids are now believed to be a generic property of proteins that is in principle reachable by all polypeptides (1). A glance at the recent literature reveals that the amyloid state of proteins that are involved in the so-called amyloidosis diseases (i.e., alpha peptide, alpha-synuclein, and tau-protein) has been more focused on. On the other hand, even proteins that are commonly thought as only ‘models’ of amyloid structure have also been shown to be involved in disease states [e.g., lysozyme (2) and insulin (3)].

Amyloid structures, characterized by cross-beta-sheets structural elements, are suggested to possess a quasi-crystalline composition that makes them different from amorphous aggregates which could also be rich in beta-sheets (4). In the course of amyloid fibril formation, various stages exist, which could be typically stated as: monomers, small soluble oligomers, protofibrils formed from the assembly of smaller units, and finally mature fibrils, which, for example in the case of the abeta peptide, could contribute to the formation of plaques (5). While fibrils were once considered to be toxic species, further studies have shown that in fact oligomeric states of the protein are responsible for the cellular toxicity observed in amyloidosis (6), and it has even been suggested that besides an obvious therapeutic benefit derived from the inhibition of oligomers formation, prompting the formation of mature fibrils could also have such a potential (7).

Inhibiting the formation of amyloid structures has gained more and more attention from multiple research groups in the recent years. These inhibitors could be roughly divided into two groups of peptide-based and small molecules, with the latter encompassing a wide range of different chemical structures [such a variety could be for example seen in compounds tested in ref. (7)]. As protein–protein interaction is stated to possess a ‘hot spot’ component, i.e., while occurring at large surfaces, having a smaller number of critical residues that are important for these
interactions, the search for small molecules that would be able to target these areas has regained strength in recent years (8). Interestingly, these 'hot spots' have been found to be rich in aromatic and charged residues (9), and a large number of compounds tested for anti-amyloidogenic activities possess also aromatic components, which are potentially able to interact with these residues (7).

Different compounds have been proposed as potential anti-amyloidogenic agents, but further investigation is needed to find new candidates that would eventually make clinically validated agents (10).

In this study, five novel compounds belonging to the benzofuranone class (Scheme 1) have been tested for their effects on insulin amyloid formation. Insulin is a small polypeptide of 51 amino acids and has been suggested to form oligomers composed predominantly of alpha-helical structures (11), reaching a flat beta-sheet-rich state upon fibrillation (12). It is involved in the formation of fibrils at the site of frequent insulin injection in patients with diabetes (3) and could relatively easily be driven to form fibrils under in vitro conditions. The effect of benzofuranone compounds on this process has been investigated through the use of fluorometry, circular dichroism (CD), and electron microscopy. Four of these compounds have been shown to slow down amyloid formation of insulin.

Methods and Materials

Bovine insulin, Thioflavin-T (ThT), and Congo red (CR) were purchased from Sigma (St Louis, MO, USA), and all salts and organic solvents were from Merck (Darmstadt, Germany). The five synthetic benzofuranone derivatives have been synthesized in the Medicinal Chemistry Department, Tehran University of Medical Sciences.

Synthesis of benzofuranone derivatives

3(2H)-Benzofuranones, the key intermediates for the production of the desired compounds, were prepared according to previously described procedures (13,14). Title compounds (1–4) were prepared by condensation of appropriate 3(2H)-benzofuranone with 5-nitrofuran-2-carbaldehyde or 5-nitrothiophene-2-carbaldehyde in acetic acid, in the presence of catalytic amount of sulfuric acid (15) (Scheme 2), while compound 5 was prepared by a different method, in ethanol in the presence of catalytic amount of HCl (16).

Chemistry

Melting points were determined with a Reichert-Jung hot-stage microscope and are uncorrected. Infrared spectra were recorded on a Nicolet Magna 550-FT spectrometer. $^1$H NMR (400 MHz) spectra were measured on a Varian Unity plus 400 spectrometer in CDCl₃ with tetramethylsilane (TMS) as the internal standard, where $J$ (coupling constant) values are estimated in Hertz. Elemental microanalyses were carried out with a Perkin-Elmer 240-C apparatus and were within ±0.4% of the theoretical values for C, H, and N.

General procedure for the preparation of substituted (Z)-2-(5-nitrofuran (or thiophene)-2-ylmethylene)-3(2H)-benzofuranones (1–4)

Equimolar amounts of the appropriate substituted 3(2H)-benzofuranone (1 mmol) and 5-nitrofuran-2-carbaldehyde (or 5-nitrothiophene-2-carbaldehyde) in acetic acid (5 mL) and sulfuric acid (98% w/w, 0.1 mL) were stirred overnight at room temperature. Then, 10 mL of

Scheme 1: Structures of the five new benzofuranone compounds.
methanol was added and the insoluble product was filtered off and recrystallized from methanol.

(Z)-6,7-Dimethoxy-2-(5-nitrothiophene-2-ylmethylene)-3(2H)-benzofuranone (1)
Yield, 59%; mp 248–249 °C.

\[ ^1\text{H-NMR (CDCl}_3\text{): } \delta 7.91 (d, J = 4.4 \text{ Hz}, 1\text{H}), 7.53 (d, J = 8.4 \text{ Hz}, 1\text{H}), 7.33 (d, J = 4.4 \text{ Hz}, 1\text{H}), 6.98 (s, 1\text{H}), 6.84 (d, J = 8.4 \text{ Hz}, 1\text{H}), 4.26 (s, 3\text{H}), 4.02 (s, 3\text{H}). \]

IR (KBr): \( \nu (\text{cm}^{-1}) 1697 (\text{CO}), 1519, 1337 (\text{NO}_2) \).

Anal. Calcd for C\(_{17}\)H\(_{16}\)N\(_2\)O\(_6\)S: C, 54.05; H, 3.33; N, 4.20. Found: C, 53.88; H, 3.49; N, 4.06.

(Z)-6,7-Dimethoxy-2-(5-nitrofuran-2-ylmethylene)-3(2H)-benzofuranone (2)
Yield, 50%; mp 215–216 °C.

\[ ^1\text{H-NMR (CDCl}_3\text{): } \delta 7.52 (d, J = 8.4 \text{ Hz}, 1\text{H}), 7.43 (d, J = 3.6 \text{ Hz}, 1\text{H}), 7.13 (d, J = 3.6 \text{ Hz}, 1\text{H}), 6.82 (d, J = 8.4 \text{ Hz}, 1\text{H}), 6.74 (s, 1\text{H}), 4.24 (s, 3\text{H}), 4.01 (s, 3\text{H}). \]

IR (KBr): \( \nu (\text{cm}^{-1}) 1709 (\text{CO}), 1519, 1358 (\text{NO}_2) \).

Anal. Calcd for C\(_{15}\)H\(_{11}\)NO\(_7\): C, 56.79; H, 3.49; N, 4.42. Found: C, 56.96; H, 3.62; N, 4.33.

(Z)-6-Methoxy-2-(5-nitrothiophene-2-ylmethylene)-3(2H)-benzofuranone (3)
Yield, 57%; mp 254–255 °C.

\[ ^1\text{H-NMR (CDCl}_3\text{): } \delta 7.91 (d, J = 4.4 \text{ Hz}, 1\text{H}), 7.71 (d, J = 8.4 \text{ Hz}, 1\text{H}), 7.31 (d, J = 4.4 \text{ Hz}, 1\text{H}), 6.96 (s, 1\text{H}), 6.85 (d, J = 2.0 \text{ Hz}, 1\text{H}), 6.81 (dd, J = 8.4, 2.0 \text{ Hz}, 1\text{H}), 3.97 (s, 3\text{H}). \]

IR (KBr): \( \nu (\text{cm}^{-1}) 1701 (\text{CO}), 1484, 1334 (\text{NO}_2) \).

Anal. Calcd for C\(_{14}\)H\(_{9}\)NO\(_5\)S: C, 55.44; H, 2.99; N, 4.62. Found: C, 55.60; H, 3.17; N, 4.49.

(Z)-6-Methoxy-2-(5-nitrofuran-2-ylmethylene)-3(2H)-benzofuranone (4)
Yield, 45%; mp 251–252 °C.

\[ ^1\text{H-NMR (CDCl}_3\text{): } \delta 7.71 (d, J = 8.4 \text{ Hz}, 1\text{H}), 7.43 (d, J = 4.0 \text{ Hz}, 1\text{H}), 7.18 (d, J = 4.0 \text{ Hz}, 1\text{H}), 6.81 (dd, J = 8.4, 2 \text{ Hz}, 1\text{H}), 6.79 (s, 1\text{H}), 6.73 (d, J = 2 \text{ Hz}, 1\text{H}), 3.95 (s, 3\text{H}). \]

IR (KBr): \( \nu (\text{cm}^{-1}) 1700 (\text{CO}), 1463, 1350 (\text{NO}_2) \).

Anal. Calcd for C\(_{14}\)H\(_{9}\)NO\(_6\): C, 58.54; H, 3.16; N, 4.88. Found: C, 58.67; H, 3.02; N, 5.03.

Protein concentration
Protein concentration was determined by absorbance measurement at 280 nm, using an extinction coefficient of 1.0 (17).

Amyloid preparation
Bovine insulin was dissolved at 1 mg/mL in 0.1 M phosphate buffer of (pH 8) containing 1 mM EDTA, followed by dialysis against the same buffer (devoid of EDTA) for 24 h. The protein solution was then incubated at 37 °C for the specified duration while being stirred gently by Teflon magnetic bars. In another series of test, acidic medium was used to induce amyloid formation in insulin. Bovine insulin was dissolved at 1 mg/mL in 50 mM glycine (pH 2.5) and incubated at 37 °C for 24 h, in agitation conditions similar with the previous experiment. The main work is related to the first set of conditions, and each time an experiment has been performed in acidic conditions, this fact is explicitly mentioned in the manuscript.

Scheme 2: Reagents and conditions: (a) acetic acid/sulfuric acid, RT, 24 h.


Benzofuranone Derivatives as Antiamyloid Compounds
**Thioflavin T binding assays**

All fluorescence experiments were carried out on a Cary Ellipse Varian fluorescence spectrophotometer (Mulgrave, Australia) at room temperature. To investigate the formation of amyloid fibrils, 10 μL of insulin samples (1 mg/mL) was added to 590 μL of 13 μM ThT solution (from 2.5 mM ThT stock solution in 10 mM sodium phosphate, 150 mM NaCl, pH7, passed through a 0.45 μM filter paper), mixed completely, and incubated for 5 min. Fluorescence emission spectra were then taken using excitation at 440 nm. The excitation and emission slit widths were set a 5 nm (18).

**Congo red binding assay**

A stock of 7 mg/mL CR was prepared in 5 mM potassium phosphate buffer containing 150 mM NaCl. Insoluble particles were removed by filtering twice through a center-glass N4 filter and the filtrate was diluted 2000 times with the same buffer. Ten microliters of a 1 mg/mL protein solution was taken at 24 h after incubation for amyloid formation and was diluted into CR solution with a final volume of 235 μL followed by incubation at 25°C without further stirring for at least 5 min in the dark. The absorbance spectrum was acquired on a Shimadzu UV-visible spectrophotometer (Kyoto, Japan) in the region of 400–600 nm (18).

**Circular dichroism**

Circular dichroism spectra in the far-UV region (190–260 nm) were obtained on an AVIV 215 spectropolarimeter (Aviv Associates, Lakewood, NJ, USA), using a 1-mm path cell at room temperature. Protein concentration was 0.2 mg/mL.

**Transmission electron microscopy (TEM)**

Ten microliters of insulin samples was put on copper 400-mesh grids, which had been covered with carbon coated formvar films. After 2 min, excess fluid was drawn out using a paper filter and 1% uranyl acetate added. After another 2 min, excess dye was removed. Finally, the grids were viewed with a CEM 902A Zeiss microscope (Oberkochen, Germany).

**Monitoring the effect of benzofuranone derivatives**

The five different synthetic compounds were diluted into DMSO while keeping the final concentration of the solvent at 12%. Concentrations of insulin and the five synthetic compounds were kept at 1 mg/mL and 4 μM, respectively. To drive insulin toward amyloid fibril formation, the protein was incubated at pH 8.0 and 37°C. Formation of amyloid fibrils in the presence and absence of these compounds was then verified through the above-mentioned specific methods of amyloid detection; DMSO alone had no effect on insulin fibril formation.

**Docking experiment**

Docking was performed using the graphical interface MGL Tools 1.5.1 (The Molecular Graphics Laboratory, Scripps Research Institute) for Autodock. The executable file vina was used for the actual
Blind docking method was used with grid boxes encompassing bovine insulin structure from the 1EV3.pdb file. pKa prediction module of moe 2009.10 (Chemical Computing Group Inc., Montreal, QC, Canada) was used to achieve a probable protonation state of the protein at pH 8 and 2.5. Both protonation states of the protein were used in the docking experiment. Visualization of best-obtained docking poses and preparation of image were carried out using moe 2009.10.

Results and Discussion

Bovine insulin could form hexamers that are connected to each other via two zinc ions complexed with histidine residues. Insulin monomers are composed of two chains (A and B), which are linked together by two disulfide bonds. Dissociation of hexamers increases insulin propensity to form amyloid structures. Insulin amyloid units are thus formed from monomeric insulin that is thought to remain intact with regard to its disulfide bonds (20). In this study, insulin samples were dissolved in phosphate buffer (pH 8) containing EDTA, to facilitate monomer formation. Dialysis against the same buffer was subsequently carried out to remove EDTA and zinc.

Formation of amyloid fibrils was verified through specific methods of amyloid detection. Spectrophotometric methods included ThT binding assay which showed increased fluorescence in the presence of the protein incubated in the amyloidogenic conditions (37 °C and pH 8) in the absence of any additives, as well as CR test, in which increase in absorbance was observed in the wavelength range of 400–600 nm, with a red shift from 490 to 520 nm (Figures S1 and S2). Absorbance at 520 nm was used as an indicator of amyloid structures formation (Figure 1). Addition of four of five synthetic compounds (compounds 1–5) at 4 μM concentration inhibited amyloid structure formation, as monitored by a decrease in ThT fluorescence intensity and CR absorbance at 520 nm. On the other hand,
one of the synthetic compounds (compound 5) appeared to enhance the process (Figure 1). It should be mentioned that the process of amyloid formation was first studied in the absence of additives, where ThT and CR spectra were taken at intervals during a 24-h period, and mature fibrils were detected at this time.

Insulin being an all-alpha-protein, a far-UV spectrum is obtained from its native structure with typical minima at 208 and 222 nm, characteristics of α-helical structures (Figure 2, inset). Utilizing the amyloidogenic condition and in the absence of any additives, this spectrum is changed to one with a deep minimum peak at 215 nm that is expected for β-sheet-containing structures (Figure S3). Incubation of insulin with compounds no. 1–4 (at 4 μM concentration) resulted in an increase in the molar ellipticity (Figure 2). On the other hand, compound 5 was again shown to have an enhancing effect on this process: its presence resulted in the appearance of a deeper peak at 215 nm (Figure 2); β-structures were clearly detectable after 4–5 h of incubation of the protein (results not shown).

As a final proof, TEM was performed. Samples of the protein were drawn after 24 h incubation for which the corresponding TEM images are shown in Figure 3. In the absence of any additives, clearly observable mature fibrils were detected. Upon addition of four of five synthetic compounds at 4 μM concentration, amyloid fibrillation was considerably inhibited by compound no. 3, to a lesser degree by compounds no. 1, 2, and 4, and noticeably enhanced by compound no. 5. To get more information on the antiamyloidogenic capacity of the compounds, acidic amyloidogenic conditions were used. Here too, all four effective compounds were able to prevent fibril formation to some extent, with compounds no. 4 and 3 acting more effectively as compared to no. 1 and 2. These results were obtained by inspecting TEM images of samples, after a first assessment by CR spectra (results not shown).

Preserving the native structure of a protein would be an ideal way to prevent formation of amyloid structures. In this case, however, it seems that all the effective compounds are capable of interfering with beta-sheets assembly as suggested by CD spectra. As seen in TEM images, the amount of fibrils varies depending on the compound used.

Introducing a compound in the amyloidogenic medium that would prevent the formation of beta-sheets by the target protein is one of the early proposed therapeutic approaches for the prevention of abeta amyloid neurotoxicity. This idea resulted in the design of a number of synthetic peptides that were shown to be effective in this regard (21), from which iAβ5Sp is probably the most known. Low stability under in vivo conditions and the need for peptides to be injected have always been a major problem in the use of this class of molecules as drugs. This is why a search toward smaller molecules possessing beta-sheet breaking properties may have great practical potential.

To our knowledge, there has not been any report on antiamyloidogenic property of compounds containing benzofuranone; however, different benzofuranone derivatives have been studied in this regard. One of the first studies of this kind relates to some bulky benzofuranone derivatives as inhibitors of abeta peptide fibril formation.
thought to have specific interaction with this protein. Smaller benzofuranones which could be comparable to the present compounds related to their size were found to have much higher IC50 toward abeta fibril formation (more than 1 μM) than bulkier derivatives which possessed extra aromatic branching in their structure (22). It could be suggested that these compounds were also acting by a beta-sheet-breaking mechanism. One of the smaller benzofuranone containing structure (SKF-63058) has also been recently used as the antifibrillation component of a hybrid ligand designed to act simultaneously at multiple targets including the cholinesterase enzyme (23). Dibenzo-furan-4,6-dicarboxylic acid derivatives have also been shown to strongly inhibit transthyretin amyloid formation. These structures are bulkier and contain more aromatic components than the benzofuranones tested here, besides the fact that in the case of transthyretin, these compounds, as well as many other small molecules, have been found to be able to bind to a specific cavity of the protein, namely the thyroxine binding site (24). Another series of compounds that could be more closely compared with the ligands are aminostyrylbenzofuran derivatives, which were recently studied on abeta peptides fibrillation, with the use of a ThT test. In this study, the most effective compounds were found to bear one methoxy group on their benzofuran moiety (25).

A preliminary structure–function relationship could be formulated based on the results of the current study, where it was observed that compounds no. 3 and 4, possessing only one methoxy group on the benzofuranone core, acted more effectively than their counterparts which contain two methoxy groups on the same moiety. As mentioned above, this was observed to be the case for two of the potent aminostyrylbenzofuran derivatives studied on the abeta peptide (25), a point that brings again the possibility of a generic effect of these compounds, i.e., an inhibitory action toward beta-sheets. Compounds no. 3 and 4 are also effective in a similar manner toward insulin fibrils formed under acidic conditions, i.e., under a different set of amyloidogenic conditions. It could be suggested that the common point of these two experiment would be the beta-component of these amyloid structures. On the other hand, thiophene derivatives work better than furane containing derivatives, because compounds no. 1 and 3 are both more effective than their counterparts no. 2 and 4. It is interesting to note that compound no. 5 lacks any substitution on the benzofuranone core, but a rational explanation for the reverse effect of this ligand on fibrillation is not straightforward. There has been a report about the unawaited effect of small aromatic compounds that enhance amyloid formation in insulin (in contrast with compounds containing multiple rings). This effect is especially remarkable for benzene and phenol which result in the formation of more compact fibrils (26). Thus, the unsubstituted benzene component of ligand no. 5 could be suggested to be the reason for its different effect.

As a matter of fact, the use of compounds that increase fibrillation has been recently proposed as a possible alternative to compounds that would prevent the starting metamorphosis of the proteins toward fibril formation (7). However, this concept undoubtedly needs more evidence before being considered as a novel line for therapeutics research. Compound no. 5 and similar compounds could be of use in this regard, for example, for testing their cytotoxicity with the use of in vitro systems.

A docking experiment was performed to provide an insight into a possible binding mode of the compounds with insulin. A native structure of human insulin crystallized with cresol was used in this experiment. Interestingly, the compounds were found to bind insulin in an extended conformation (Figure 4A), which could suggest them to be suitable for binding to the subsequently formed beta-sheets. The whole protein structure was given to the docking program, to find putative binding sites, and interestingly, only two sites were found, in a total of 100 poses for all the ligands with the best one being the B-chain of insulin, which has the potential to form amyloid structure independently (Figure 4B). The second location is the phenol binding pocket, where phenol molecules interact, a process that is known to afford stabilization of the protein structure (27). In both protonation states of the protein (pH 8 and 2.5), a similar binding position was obtained for the ligands. In Figure 4C, best poses of all five ligands have been superimposed in the B-chain location and in the acidic protonation state. As shown there, the main interacting residues are His5 and Tyr26. Putative hydrogen bonds could be formed with the side chain of Tyr26 by all the ligands. However, the possible hydrogen bonds with His5 would be only made by compounds bearing a 7-methoxy group (i.e., compounds no. 1 and 2). The other potential interaction would be an aren–cation interaction between the aryl groups of the ligands and His5. The potential hydrogen binding with His5 would be thus the most prominent difference between the potential interactions of the effective ligands and should have some deleterious effect on the inhibition process. It should be pointed out that this is only a first theoretical attempt to explain a very complex process which involves many intermediate structures of insulin and a wide range of potential interactions between the ligands and these structures.

Results presented in the present communication may be considered as a preliminary report on the beneficial effect of benzofuranone derivatives as inhibitors of amyloid fibrillation. The present class of compounds possess the characteristics of being relatively simple to derivatize and small enough to fit the drug-like requirements. As these could have a beta-sheet breaker property, they also present an interesting new series to be explored in that direction.

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Conflicts of Interests

The authors declare to have no conflict of interest.

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