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Studies of anti-fibrillogenic activity of phthalocyanines of zirconium containing out-of-plane ligands

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ABSTRACT

Series of phthalocyanines of zirconium containing lysine, citric, nonanoic acid residues and dibenzolylmethane groups as out-of-plane ligands are firstly studied as inhibitors of fibrillogenesis using cyanine-based fluorescent inhibitory assay. It was shown that studied phthalocyanines at concentration of 20 μM inhibited aggregation reaction on 38.5–57.6% and inhibitory activity of phthalocyanines depended on the chemical nature of out-of-plane ligand. For the most active compound $PcZrLys_2$ (zirconium phthalocyanine containing lysine fragment) the efficient inhibitor concentration was estimated to be 37 μM. AFM studies have shown that in the presence of $PcZrLys_2$ the inhibition of fibrils formation and formation of spherical oligomeric aggregates took place. Due to the ability of phthalocyanines to decrease efficiently protein aggregation into the amyloid fibrils, modification of phthalocyanine molecules via out-of-plane substitutions was proposed as approach for design of anti-fibrillogenic agents with required properties.

1. Introduction

A large class of proteins can form elongated insoluble aggregates, known as amyloid fibrils, which are involved in the clinical manifestations or in the etiology of many diseases, including Creutzfeldt-Jacob's, Alzheimer's disease, Parkinson's disease (PD) and type II diabetes.[1,2] The aggregation pathway of proteins into the amyloid fibrils is suggested to be the obvious target for therapeutic intervention in such disorders. In such a way the range of small molecules from different chemical classes demonstrated the ability to inhibit the formation of fibrillar aggregates of amyloidogenic proteins.

It was shown that series of polyphenols, phenothiazines, porphyrins, polyene macrolides, Congo red and its derivatives, BSB and FSB inhibited alpha-synuclein filament assembly having IC_{50} values in the low micromolar range. Many compounds that inhibited alpha-synuclein assembly were also found to inhibit the formation of Abeta and tau filaments.[3] The polyphenol (−)-epigallocatechin gallate efficiently inhibited the fibrillogenesis of both alpha-synuclein and amyloid-beta by direct binding to the natively unfolded polypeptides and preventing their conversion into toxic, on-pathway aggregation intermediates.[4] It was shown that low micromolar concentrations of baicalein, and especially its oxidized forms, inhibited the formation of alpha-synuclein fibrils.[5] The inhibition of islet amyloid polypeptide fibril formation by small molecules based on a rhodanine scaffold at submicromolar concentrations was also reported.[6]

Among the methods of evaluation the inhibitory effects of anti-aggregation agents on proteins fibrillization reaction[7,8] successful application of fluorescence-based assay using Thioflavin T was reported. Recently we have proposed cyanines as fluorescent probes for amyloid formations detection.[9,10] Later trimethine cyanine dye 7519 (Fig. 1) was applied as probe in screening assay of potential inhibitors of insulin aggregation in vitro, demonstrating high results reproducibility.[11]

Cyclic tetrapyrroles is a class of compounds that includes biologically important heme and chlorophylls; porphyrins and relative to them phthalocyanines also belong to this class. These compounds are known to be able to bind strongly to proteins and effect changes in protein conformation. Hence porphyrines and phthalocyanines were reported to have high anti-prion activity in vitro and in vivo.[12] For metal-free phthalocyanine tetrasulfonate (PcTS) (Fig. 1) noticeable anti-amyloidogenic properties were shown, PcTS was found to destroy efficiently tau filaments[13] and inhibit ASN fibril formation.[14]

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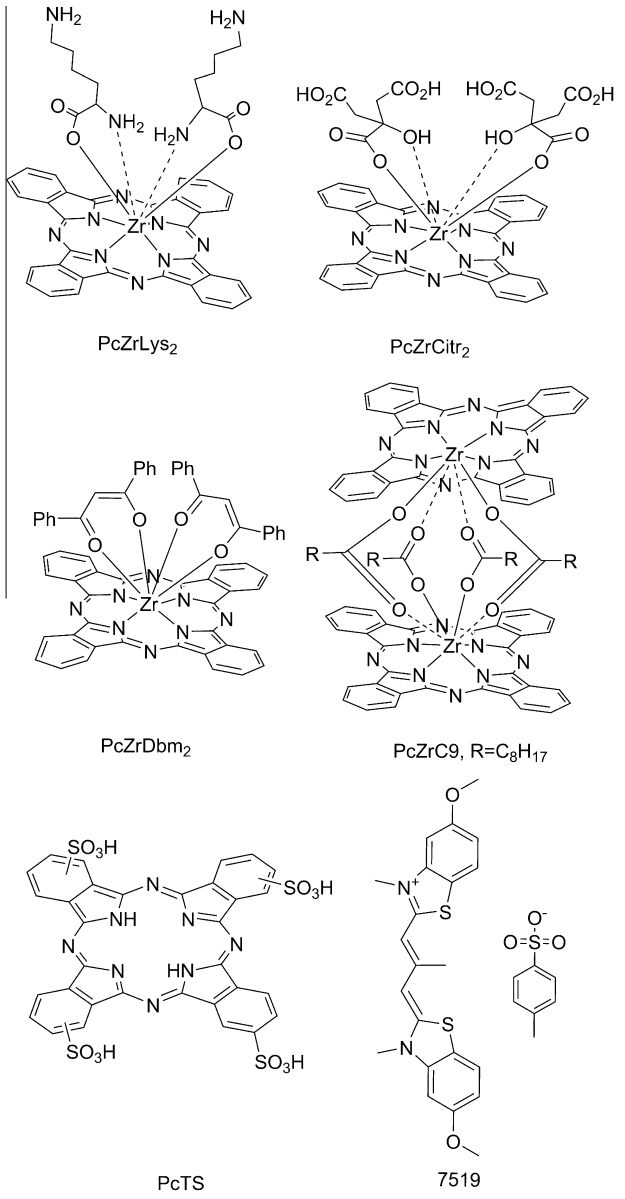

Figure 1. Chemical structures of zirconium phthalocyanines with out-of-plane ligands, metal free phthalocyanine tetrasulfonate and amyloid sensitive trimethine cyanine dye 7519.

Phthalocyanines with out-of-plane ligands are the types of metallocomplexes having substituents bound to central metal atom. This gives steric content (volume) to the molecule in opposite to usually planar porphyrine and phthalocyanines molecules. Bulky substituents when bound to central metal atom noticeably changed the size and shape of molecules, the electrical charge distribution, and the geometry of bond angles. Phthalocyanines with out-of-plane ligands were firstly reported in[15] and next studied as anti-tumor agents, demonstrating noticeable cytotoxicity.[16]

In current research zirconium phthalocyanines containing out-of-plane ligands, namely lysine ($PcZrLys_2$) and citric acid ($PcZrCitr_2$) residues, dibenzoylmethane groups ($PcZrDbm_2$) and nonanoic acid (PcZrC9)[15] (Fig. 1) were studied as inhibitors of fibrillogenesis using insulin as model protein. Cyanine-based fluorescence inhibitory assay was used to estimate inhibitory activity of phthalocyanines and to determine the efficient inhibitor concentration for the most active compound. Products of fibrillization reaction in the absence and in presence of most the efficient inhibitor were studied by atomic force microscopy (AFM).

2. Experimental

2.1. Materials

Complexes of zirconium phthalocyanines with out-of-plane ligands were synthesized as described in.[16–18] These compounds were of intensive green–blue color and had moderate solubility in DMF. Phthalocyanines were stable under the experimental conditions. Composition and structure of complexes were confirmed by ^{1}H NMR and element analysis on metal. Stock solutions of phthalocyanines in concentration of 2 mM were prepared by dissolving the compound in DMF for $PcZrDbm_2$, $PcZrCitr_2$, $PcZrLys_2$ and in THF for PcZrC9. Cyanine dye 7519[19] was kindly provided by Prof. O.I. Tolmachev and Dr. Yu.L. Slominskii (Institute of Organic Chemistry of NASU). 2 mM dye stock solution was prepared in DMF.

2.2. Insulin fibril formation

Bovine insulin (Sigma–Aldrich) was dissolved at 340 μM concentration in 100 mM water solution of HCl (pH 1.8). Fibrils were formed by incubating the protein solutions in a water bath at 65 °C for about 5 h. Aliquots of the reaction mixture were withdrawn from each tube at about 1 h interval to allow spectral measurements using dye 7519.[19] For this, 10 μl aliquots of the aggregation mixture were added to 1 ml of a 2 μM dye solution in 50 mM Tris–HCl buffer, pH 7.9, insulin concentration was thus 3.4 μM. The dye 7519 earlier was shown to specifically bind to the fibrillar form of insulin accompanied with sharp increase of the fluorescence intensity.[19] Recently we demonstrated this dye to bind to oligomeric aggregates of alpha-synuclein with about 6 times lower fluorescence intensity increase as compared to dye in complex with fibril (data to be published). As it will be shown in this manuscript, 7519 binds to insulin oligomeric aggregates with the increase of fluorescence intensity as well. Thus since the dye specifically binds to aggregated form of insulin (both fibrillar and oligomeric) demonstrating sharp increase of its fluorescence intensity, we believe the dye fluorescence intensity to be an adequate characteristic to estimate the quantity of aggregated protein (intensity of the unbound dye is much lower as compared to this of the dye bound to oligomer or fibril). The dye fluorescence was excited at 580 nm, and the emission intensity at the maximum wavelength (590 nm) was measured. All spectroscopic measurements were made immediately after mixing the protein and dye solutions. Fluorescence spectra were registered using fluorescent spectrophotometer Cary Eclipse (Varian, Australia).

2.3. Inhibitory assay

The phthalocyanines $PcZrDbm_2$, $PcZrCitr_2$, $PcZrLys_2$, and PcZrC9 were added to the corresponding insulin fibrillization mixture immediately before the beginning of aggregation. The concentrations of inhibitors in aggregation mixture were 20 μM. For the most efficient inhibitor compound $PcZrLys_2$ the efficient inhibitor concentration has been calculated. For this, inhibitor was added to the monomeric insulin solutions to make the final concentration of 0, 4, 10, 40, and 100 μM. The concentration of monomeric insulin in all reaction mixtures was of 340 μM. The procedure of insulin fibrillogenesis was performed, and the quantity of the formed aggregates was monitored using the 7519 dye fluorescence as described for inhibitor-free insulin solution (Section 2.2). The inhibitor-free sample was used as aggregation reference; corresponding

aliquot of DMF or THF was added to this sample to exclude the solvent effect. The efficient inhibitor concentration was than calculated as the inhibitor concentration at which the dye fluorescence intensity is the half of this for noninhibited solution.

2.4. Atomic force microscopy studies

AFM studies of products of fibrillization reaction of free insulin and in presence of inhibiting compounds were carried out using 'Solver Pro M' system (NT-MDT (Russia)). For the formation of sub nanolayer consisting from separate spatially resolved nanoobjects, the reaction solutions were diluted in 30 times with ultrapure water.

The sample was prepared by dropping of the solution on the freshly cleaved surface of mica. AFM measurements were carried out in semicontact (tapping) mode of AFM after full evaporation of the solvent. AFM probes of type NSG01 (NT-MDT) were used. Average diameter of nanoobjects was determined as the high of their z-profiles.

3. Results and discussion

The kinetics of insulin fibrillization in presence and in the absence of phthalocyanines was monitored by fluorescent response of 7519 dye (Fig. 2). Fluorescence of the dye 7519 was excited and emission was recorded at the wavelength of corresponding maxima—580 and 590 nm, respectively.

When monomeric insulin was aggregated both in the absence and in presence of metallocomplexes the fluorescence intensity of the reference dye increased. As it could be seen from the graph, the presence of 20 μM of studied phthalocyanines partially suppressed the fibrillogenesis of insulin. The quantity of formed aggregates was estimated by measurements of the fluorescence intensity (I) of amyloid-specific dye 7519 performed on different stages of insulin fibrillization in presence of inhibitors. Quantity of fibrils in the absence of inhibitor was assigned to be 100%, and, respectively, fluorescence intensity of the 7519 dye in unbound state was I_0. Thus the ratio I/I_0 was considered to be adequate estimation of quantity of protein aggregated in presence of certain inhibitor concentration C_{inh} as compared to noninhibited fibrillization process. For used phthalocyanines the values of inhibition efficiency (defined as $(1-I/I_0)\times 100\%$) from 38.5 to 57.6% were observed. The Fig. 2 demonstrates that the inhibitory activity of phthalocyanines increases in the row PcZrC9 (38.5%) <$PcZrDbm_2$ (43.6%) ≈ $PcZrCitr_2$ (43.8%) < $PcZrLys_2$ (57.6%). Besides, it could be seen from the Fig. 2 that fibrillization in presence of $PcZrCitr_2$ and $PcZrLys_2$ occurs with noticeable lag phase, while in the presence of PcZrC9 and $PcZrDbm_2$, as well as for noninhibited process, the lag phase is not observed.

Phthalocyanine molecules are known to form aggregates in water solutions.[16,20] Because of strong intermolecular interaction that occurs during phthalocyanines aggregation processes, the aggregated macrocyclic systems could act as single (sole) system. Recently it was shown that association is important in the anti-prion mechanism of cyclic tetrapyrroles and related phthalocyanines.[21] Also the inhibitory activity of poorly aggregating PcTS-Cu^{2+} is considerably lower comparing with that of well-aggregating PcTS.[22] Thus we could suppose that inhibitory activity of studied phthalocyanines is connected with their tendency to aggregate. This tendency was mostly pronounced for compounds $PcZrCitr_2$ and $PcZrLys_2$, which demonstrated the highest inhibitory activity in fluorescent-based assay.

Besides, the groups able to electrostatic interaction were proposed to play a role in inhibitory activity of PcTS. It was revealed that electrostatic interactions between negative charged sulfonates of PcTS and positive centers on ASN, likely provided by lysine residues, contributed to the strength of the binding.[14] In structure of out-of-plane ligands of compounds $PcZrLys_2$ and $PcZrCitr_2$ amino and carboxyl groups correspondingly are present. These groups are able to electrostatic interaction and could provide additional stabilization to the protein/phthalocyanine complex due to ability to interact with locally charged groups of aminoacids or groups able to hydrogen bonding. It should be noted that inhibitory activity of phthalocyanine $PcZrCitr_2$ with negatively charged acidic ligand (43.8%) was somewhat lower than that for phthalocyanine $PcZrLys_2$ containing ligand with basic properties (57.6%).

With the aim to further characterize the inhibition activity of the most efficient inhibitor $PcZrLys_2$, the efficient inhibitor concentration that equals to the inhibitor concentration at which the dye fluorescence intensity is the half of this for noninhibited

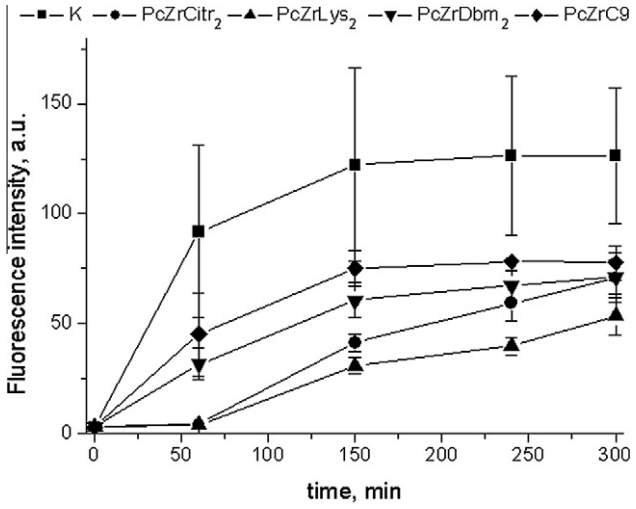

Figure 2. Effects of $PcZrLys_2$, $PcZrCitr_2$, $PcZrDbm_2$ and PcZrC9 on the kinetics of insulin fibrils formation, plotted with phthalocyanine-free insulin control (K). The reaction mixture containing 340 μM of insulin in 0.1 M water solution of HCl and 20 μM of corresponding inhibitor were incubated at 65 °C for 5 h. Fibrillogenesis efficiency was assessed with 7519 fluorescence emission using a 2 μM dye concentration. Experiment was performed three times. Standard deviation of the mean is presented as the error bars.

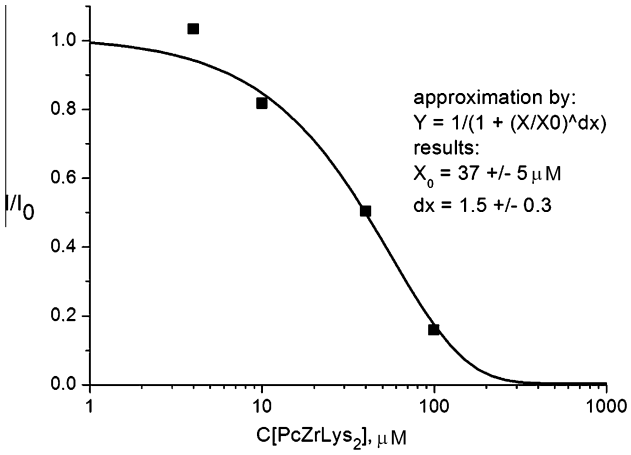

Figure 3. Dose-dependent inhibition of bovine insulin aggregates formation by $PcZrLys_2$. The reaction mixtures containing 340 μM of insulin, 100 mM HCl, and 0, 4, 10, 40, or 100 μM of $PcZrLys_2$ inhibitor in distilled water were incubated at 65 °C for 4 h. Fibrillogenesis efficiency was assessed with 7519 fluorescence emission (I) using a 2 μM dye concentration. The emission intensity for the case of absence of inhibitor (I_0) was regarded as 100%. Experimental dependence (squares) was approximated by the sigmoid dependence (line). The approximation parameter X_0 equals to the efficient inhibitor concentration.

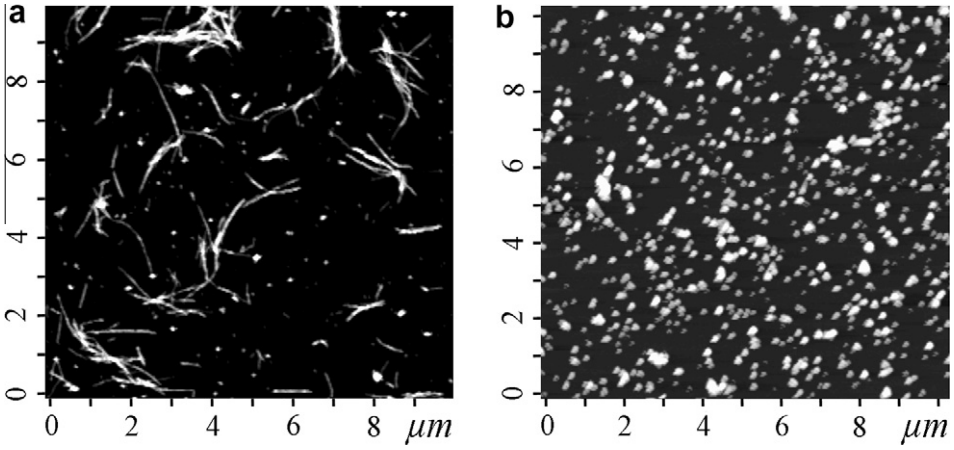

Figure 4. AFM images of products of insulin fibrillogenesis reaction (a) in absence of inhibitor, (b) in presence of $PcZrLys_2$ deposited on atomic-smooth surface of mica (tapping mode).

solution was estimated. When insulin was incubated with various concentrations of $PcZrLys_2$ metalocomplex the inhibitory effect increased dose-dependently.

Dependence of I/I_0 on C_{inh} for the inhibitor $PcZrLys_2$ is presented in the Fig. 3. Approximation of this dependence with the sigmoid curve gives the efficient inhibitor concentration which characterizes the inhibition activity of the potential inhibitor. Thus for the $PcZrLys_2$ the efficient inhibitor concentration value of insulin aggregation inhibition was found to be equal to 37 ± 5 μM.

AFM studies were carried out to estimate the effect of phthalocyanine $PcZrLys_2$ on the fibrillogenesis reaction pathway. It was shown that insulin itself forms long unbranched fibrils (as shown in Fig. 4a) with the length of up to 5 μm and average heights of about 10 nm; cord-like structures formed by twisted fibrils were also observed. In the presence of $PcZrLys_2$ full inhibition of long fibrils formation and formations of spot-like protein structures with the height about 10 nm was observed (Fig. 4b). These structures the most probably could be spherical protein aggregates with average diameter of about 10 nm. It should be noted that images, obtained for these aggregated protein formations are similar to the images and characteristics of early stage aggregates described for insulin.[23] Thus it could be concluded that phthalocyanine $PcZrLys_2$ redirected the aggregation pathway to the spherical oligomeric aggregates formation. The 'residual' fluorescence of the dye 7519 in the presence of oligomeric products of $PcZrLys_2$-inhibited reaction pointed on the dye's ability to bind with early-stage aggregates. We can propose that both in the case of mature fibrils and oligomeric aggregates the dye binds to beta-sheet formed structures. At the same time noticeable difference in dye emission intensity in the complexes with mature fibrils and oligomeric spices is an evidence of structural distinction between these types of aggregates.

It is thus concluded that phthalocyanines with out-of-plane ligands could efficiently redirect the fibrillogenesis reaction and thus could be of interest as anti-fibrillogenic compounds. Also due to out-of-plane (axial) modification a wide range of substituents could be easily inserted to the phthalocyanine molecule which is 'hard for modification' on periphery. Thus out-of-plane modification is considered to be a promising approach to obtain phthalocyanines with certain physico-chemical properties and pre-defined affinity to certain proteins.

4. Conclusions

Series of phthalocyanines containing out-of-plane ligands was firstly studied as inhibitors of proteins fibrillogenesis. According to the data of fluorescent titration inhibitory activity of compounds (at 20 μM concentration) increased in the row $PcZrC9 < PcZrDbm_2 \approx PcZrCitr_2 < PcZrLys_2$ from 38.5% for PcZrC9 to 57.6% for $PcZrLys_2$. That was point of noticeable influence of chemical nature of out-of-plane substituent on inhibitory properties of phthalocyanine. For the most active compound $PcZrLys_2$ the efficient inhibitor concentration was found to be equal to 37 μM. According to AFM data, presence of $PcZrLys_2$ inhibited the fibril formation and redirected the aggregation reaction to the formation of spherical aggregates. Using of out-of-plane modification is proposed as 'chemically easy' way to insert a wide range of substituents to the phthalocyanine molecules and thus to design anti-fibrillogenic agents on the base of these metallocomplexes.

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Supplementary data

Supplementary data (spectral data of phthalocyanines of zirconium containing out-of-plane ligands) associated with this article can be found, in the online version, at doi:10.1016/j.bmc.2011.10.083.

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