

UDC 577.336 + 667.287.4

Towards the anti-fibrillogenic activity of phthalocyanines with out-of-plane ligands: correlation with self-association proneness

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Aim. The activity of five hafnium phthalocyanines containing out-of-plane ligands as inhibitors of reaction of insulin fibril formation is studied and correlation between their inhibitory properties and tendency to self-association is discussed. **Methods.** Fluorescence and absorption spectroscopy. **Results.** For the complexes with weak proneness to self-association PcHfDbm_2 , PcHfPyr_2 , and PcHfBtfa_2 the values of inhibitory activity were estimated as 60–73 %. For phthalocyanines with the pronounced tendency to self-association PcHfPiromelit and PcHfCl_2 , the noticeably higher inhibitory activity values (about 95 %) were shown. In the presence of native or fibrillar insulin the destruction of self-associates of metal complex occurs in buffer pH 7.9. Besides upon the conditions of insulin fibrillization reaction (0.1 M HCl) phthalocyanines exist predominantly as monomers. **Conclusions.** The phthalocyanines with out-of-plane ligands with higher tendency to self-association have shown higher inhibitory activity in the insulin fibril formation comparing with the poorly aggregated metal complexes. At the same time low-order self-associates are not involved directly in the mechanism of inhibition of insulin fibrillization and the phthalocyanines bind with protein in monomeric form. Tendency of phthalocyanines to self-association in aqueous media seems to be an «indicator» of their proneness to stack with protein aromatic amino-acids and thus of anti-fibrillogenic properties.

Keywords: amyloid fibril, phthalocyanines, inhibitors of fibril formation, insulin, aggregation of phthalocyanines, fluorescent dye.

Introduction. Pathogenesis of many diseases, including neurodegenerative disorders (Alzheimer's, Parkinson's, Huntington's chorea) is accompanied by formation of the β -pleated aggregates of proteins, so-called amyloid fibrils [1]. In each of these pathological conditions specific proteins or protein fragments alter the natural soluble form to insoluble fibrils that accumulate in various organs and tissues [2].

Among the compounds able to suppress the amyloid fibril formation, planar macrocyclic metal complexes – phthalocyanines were reported [3]. Due to their high in-

hibitory activity, low toxicity and ability to overcome blood-brain barrier [4], the compounds of this class are considered as prospective anti-fibrillogenic agents.

Among the properties that could affect the anti-fibrillogenic and anti-prion activity of phthalocyanines is the tendency of metal complex to self-association [5, 6]. Lamberto *et al.* have shown that the differences in binding capacity and anti-amyloid activity of phthalocyanines towards alpha-synuclein are attributed to their relative ability to self-stack through π - π interactions modulated by the nature of a metal ion. The low order stacked aggregates of phthalocyanines were identified as the active amyloid inhibitory species [5].

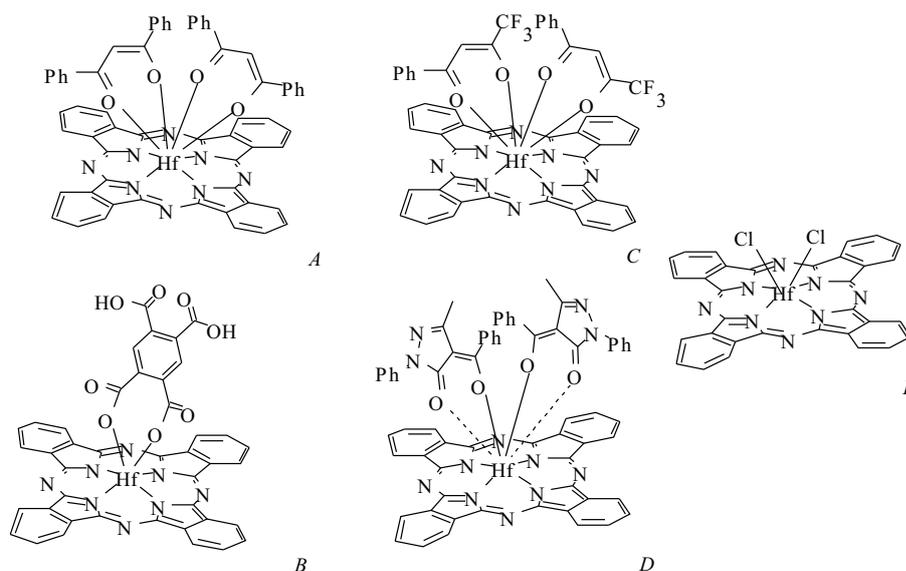


Fig. 1. Chemical structure of hafnium phthalocyanine with out-of-plane ligands: dibenzylmethane (*A* – PcHfDbm₂); pyromellitic acid (*B* – PcHfPiromelit); benzoyl-1,1,1-trifluoroacetone (*C* – PcHfBtfa₂); 1-phenyl-3-methyl-4-benzoyl-pyrazolone-5 (*D* – PcHfPyr₂); chlorine atoms (*E* – PcHfCl₂)

Recently the anti-fibrillogenic activity of phthalocyanines with out-of-plane ligands has been reported [7]. In contrast to usually planar porphyrine and phthalocyanine molecules these metal complexes have a steric content due to the presence of out-of-plane ligands bound to the central metal atom. These complexes are able to inhibit the reaction of insulin fibrillization and to redirect its pathway to the formation of oligomeric aggregates [7].

The current work is aimed at studying the inhibitory activity of series of five hafnium phthalocyanines containing out-of-plane ligands of various chemical nature (Fig. 1) in the reaction of insulin fibrillization and at inquiring into the role of phthalocyanines self-association in anti-fibrillogenic properties. The inhibitory activity of metal complexes is determined using earlier reported fluorescent cyanine based inhibitory assay [8]. For the study on phthalocyanines self-association the absorption spectra were obtained for both free metal complexes and in the presence of native and fibrillar insulin.

Materials and methods. Hafnium phthalocyanines with out-of-plane ligands were synthesized as described in [9–11]. 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 ¹H NMR and element analysis on metal. Stock solutions of phthalocyanines in concentration of 2 mM were prepared by dissolving the compound in DMF. Amyloid-specific cyanine dye 7519 [12] was kindly pro-

vided by Dr. O. I. Tolmachev and Dr. Yu. L. Slominskii (Institute of Organic Chemistry of NAS of Ukraine). 2 mM dye stock solution was prepared in DMF.

Insulin fibril formation. Human insulin (Private Joint Stock Company «On the production of insulin «Indar», Ukraine) was dissolved at 340 μM concentration in 100 mM water solution of HCl (pH 2). Fibrils were formed by incubating the protein solution in a water bath at 65 °C for about 5 h. Aliquots of the reaction mixture were withdrawn from each tube at about 90, 150, 240, and 300 min intervals to allow spectral measurements using dye 7519. 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 [7]. The dye 7519 was earlier shown to bind specifically to the fibrillar form of insulin accompanied with sharp increase in fluorescence intensity. Since the dye specifically binds predominantly to the aggregated form of insulin (both fibrillar and oligomeric) we believe the dye fluorescence intensity to be an adequate characteristic to estimate the quantity of aggregated protein (the free dye demonstrates much lower emission intensity as compared to its bound form). All spectroscopic measurements were made immediately after mixing the protein and dye solutions. Fluorescence spectra were registered using fluorescent spectrophotometer Cary Eclipse («Varian», Australia).

Inhibitory assay. The phthalocyanines PcHfPiromelit, PcHfDbm₂, PcHfPyr₂, PcHfBtfa₂ and PcHfCl₂ were added to the corresponding insulin solution right prior

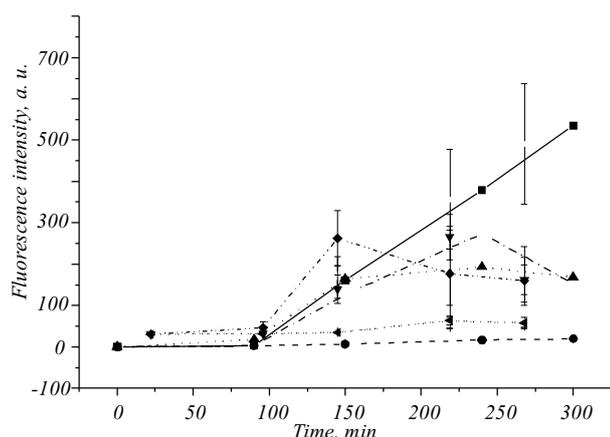


Fig. 2. Effects of presence of PcHfPiromelit (2), PcHfBtfa₂ (3), PcHfPyr₂ (4), PcHfDbm₂ (5) and PcHfCl₂ (6) on the kinetics of insulin fibrils formation, plotted with phthalocyanine-free insulin control ($K - I$). The reaction mixture containing 340 μM of insulin in 0.1 M water solution of HCl and 100 μM of corresponding inhibitor was incubated at 65 °C for 5 h. Fibril formation efficiency was assessed with fluorescence intensity of dye 7519 using 2 μM concentration. Experiment was performed three times. Standard deviation of the mean is presented as the error bars

the beginning of aggregation reaction. The used concentration of monomeric insulin was 340 μM , the concentration of inhibitors was 100 μM . The procedure of insulin fibrillization was performed, and the quantity of the formed aggregates was monitored using the 7519 dye fluorescence as described above.

Inhibitory activity was defined as $(1 - I/I_0) \cdot 100\%$, where I_0 and I are the 7519 fluorescence intensity values for inhibitor-free and inhibitor-containing insulin solutions respectively. The ratio I/I_0 was considered to be an adequate estimation of quantity of protein aggregated in the presence of certain inhibitor concentration C_{inh} as compared to noninhibited fibrillization process. The inhibitor-free sample was used as aggregation reference; corresponding aliquot of DMF was added to this sample to exclude the solvent effect.

Absorption spectroscopy. Absorption spectra were recorded using Specord M-40 spectrophotometer («Carl Zeiss», Germany). Phthalocyanines were studied in 50 mM Tris-HCl buffer, pH 7.9 and in 0.1 M HCl (concentration range 0.5–10 μM); in DMSO (concentration 5 μM); and in the presence of 34 μM native or fibrillar insulin (concentration 10 μM).

Results and discussion. Inhibitory activity of phthalocyanines in insulin fibrillization reaction. The kinetics of insulin fibrillization in the presence and in the ab-

sence of phthalocyanines was monitored by the changes in fluorescence intensity of amyloid-sensitive dye 7519 according to the method described earlier [6].

With the passing of insulin fibrillization reaction we observed the changes in dye fluorescence intensity upon addition of inhibitor-free samples and samples containing compounds PcHfDbm₂, PcHfBtfa₂, PcHfPyr₂ (Fig. 2). The presence of samples containing complexes PcHfPiromelit and PcHfCl₂ slightly affected the fluorescence intensity of dye during the reaction pathway.

Thus it could be concluded that the presence of phthalocyanines with spatial substituents containing multiple phenyl groups (PcHfDbm₂, PcHfBtfa₂, PcHfPyr₂) partially suppressed the formation of insulin fibrils. For these compounds the values of inhibition efficiency (defined as $(1 - I/I_0) \cdot 100\%$; see Experimental section) varied in the range PcHfBtfa₂ (60 %) < PcHfPyr₂ (66 %) < PcHfDbm₂ (73 %).

At the early stage ($t = 150$ min) of the insulin aggregation reaction in the presence of the sample containing phthalocyanine PcHfDbm₂ it was demonstrated a higher dye emission comparing with inhibitor-free sample. Thus, we can suppose that the presence of PcHfDbm₂ promotes the formation of beta-pleated structures at the early stage comparing with inhibitor free sample. The ability of phthalocyanines with out-of-plane ligands to redirect the insulin fibrillization reaction to the formation of spherical aggregates was shown earlier.

A higher inhibitory activity was noted for phthalocyanines PcHfPiromelit and PcHfCl₂. Their presence almost completely suppressed the formation of both beta-pleated insulin aggregates at the early stages of reaction and amyloid fibrils. The final inhibitory effects for these complexes were about 94 and 96 % correspondingly.

This observation points to the dependence of inhibitory activity of phthalocyanines on the chemical nature of out-of-plane ligand. Thus the conclusion can be made about high antifibrillogenic properties of the complexes with «small size» ligand (PcHfCl₂) or ligand containing carboxy group (PcHfPiromelit) that allows the electrostatic interaction or hydrogen bonds formation with protein aminoacids.

Determination of the efficient inhibitory concentration for PcHfPiromelit. Phthalocyanine PcHfPiromelit demonstrated a high anti-fibrillogenic activity in the insulin fibrillization reaction, thus for this complex the

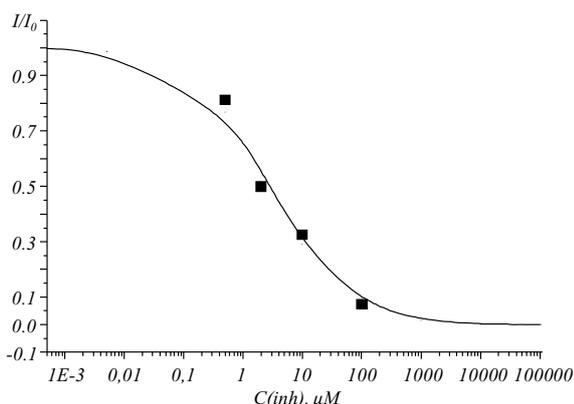


Fig. 3. Dose-dependent inhibition of insulin aggregates formation by PcHfPiromelit. The reaction mixtures containing 340 μM of insulin in 100 mM HCl, and 0, 0.5, 2, 10, or 100 μM PcHfPiromelit were incubated at 65 °C for 5 h. Fibrillogenesis efficiency at the given inhibitor concentration was assessed with 7519 fluorescence emission (I). The emission intensity in the 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 ($Y = 1/(1 + (x/x_0)^{dx})$; $x_0 = 2.8 \pm 0.6$; $dx = 0.7 \pm 0.1$) to the efficient inhibitor concentration

efficient inhibitor concentration was identified according to the method described in [6]. The efficient inhibitor concentration is estimated as a concentration of phthalocyanine at which the dye fluorescence intensity is half concentration for the inhibitor free solution. It is shown that the inhibitory effect enhances with increasing concentration of metal complex in the reaction mixture. The dependence of I/I_0 on $C(\text{inh})$ for PcHfPiromelit is presented in Fig. 3.

Approximation of experimental dependence of I/I_0 on $C(\text{inh})$ with the sigmoid curve gives the efficient inhibitor concentration IC_{50} which characterizes the inhibition of the fibrillization reaction by the studied metal complex. The efficient inhibitor concentration for PcHfPiromelit was determined as $\text{IC}_{50} = 2.8 \pm 0.6 \mu\text{M}$, that is more than one order lower than the corresponding value (obtained for inhibition of bovine insulin) for zirconium phthalocyanine containing out-of-plane lysine ligands PcZr Lys₂ ($\text{IC}_{50} = 37 \pm 0.6 \mu\text{M}$), determined by us earlier [6].

Aggregation of phthalocyanines in buffer, pH 7.9, and DMSO. To study the aggregation ability of phthalocyanines, we recorded their absorption spectra in polar solvent DMSO and in 0.05 M Tris-HCl buffer (pH 7.9) (Fig. 4).

In the spectra of complexes in DMSO two bands were observed, short-wavelength vibronic satellite band

with maximum near 615 nm and sharp intensive long-wavelength band with maxima on 679–693 nm. The profiles of spectra and band positions are typical for monomeric phthalocyanines.

In absorption spectra of complexes in the buffer the bands are much wider than in DMSO. They contain two bands, short-wavelength band or shoulder in the range 642–646 nm and long-wavelength one in the range 685–700 nm.

It is shown that for PcHfBtfa₂ (Fig. 4, B), PcHfDbm₂, and PcHfPyr₂ the long-wavelength band is the most pronounced and that an increase in the complex concentration does not lead to redistribution of intensities between the absorption bands. This behavior points to a low propensity of phthalocyanines to self-association. For these complexes the absorption spectra correspond to the monomeric phthalocyanine molecules, the short-wavelength band could be attributed to the vibronic transition. The inhibitory activity of these complexes varies in the range 60–73 %.

In spectra of phthalocyanines PcHfPiromelit (Fig. 4, A) and PcHfCl₂ in buffer, the relative intensity of short-wavelength band increases at higher complex concentration, while that of long-wavelength band decreases. Starting from about 5 μM concentration the short-wavelength band dominates in the spectra. Such behavior is an evidence of high tendency of the PcHfPiromelit and PcHfCl₂ metal complexes to self-association. In this case the short-wavelength band belongs to aggregates of phthalocyanines, while the long-wavelength one corresponds to their monomers. At the same time, these complexes demonstrated a very high inhibitory activity – 96 % for PcHfPiromelit and 94 % for PcHfCl₂.

In this way we observed a higher anti-fibrillogenic activity for phthalocyanines with pronounced tendency to self-association.

An addition of native or fibrillar insulin to the complex with high inhibitory activity PcHfPiromelit leads to the significant decrease of short-wavelength aggregation band and increase of long-wavelength monomeric one (Fig. 5, A). Thus it could be concluded that phthalocyanine interacts with both native and fibrillar proteins and this interaction leads to the destruction of metal complex self-associates. It is supposed that phthalocyanine molecules bind to the surface of protein in monomeric form.

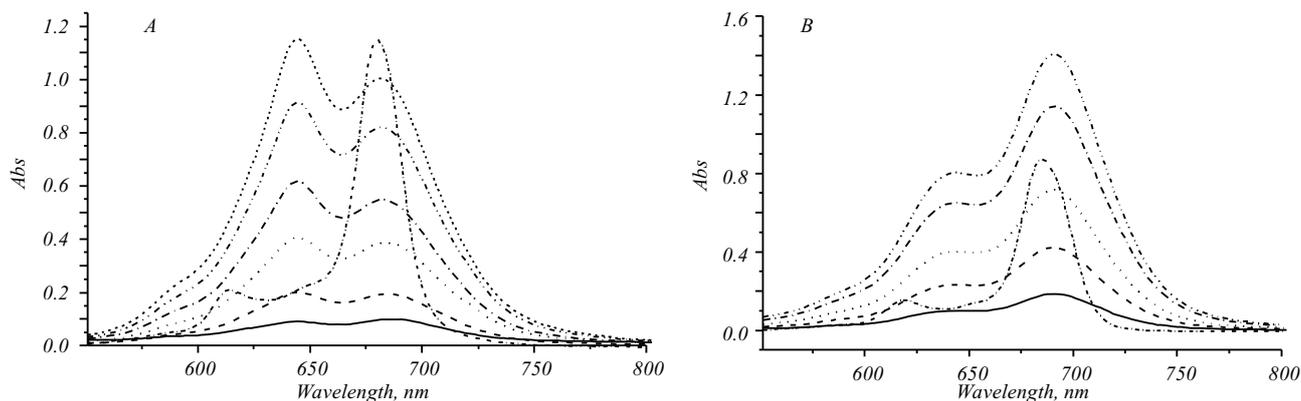


Fig. 4. Absorption spectra PcHfPiromelit (A) and PcHfBtfa, (B). Spectra are shown for concentrations of A: 1 – 0.5 μM ; 2 – 1 μM ; 3 – 2 μM ; 4 – 5 μM ; 5 – 8 μM ; 6 – 10 μM ; 7 – 5 μM , DMSO; B: 1 – 1 μM ; 2 – 2 μM ; 3 – 5 μM ; 4 – 8 μM ; 5 – 10 μM ; 6 – 5 μM , DMSO in Tris-HCl (pH 7.9) and 5 μM in DMSO

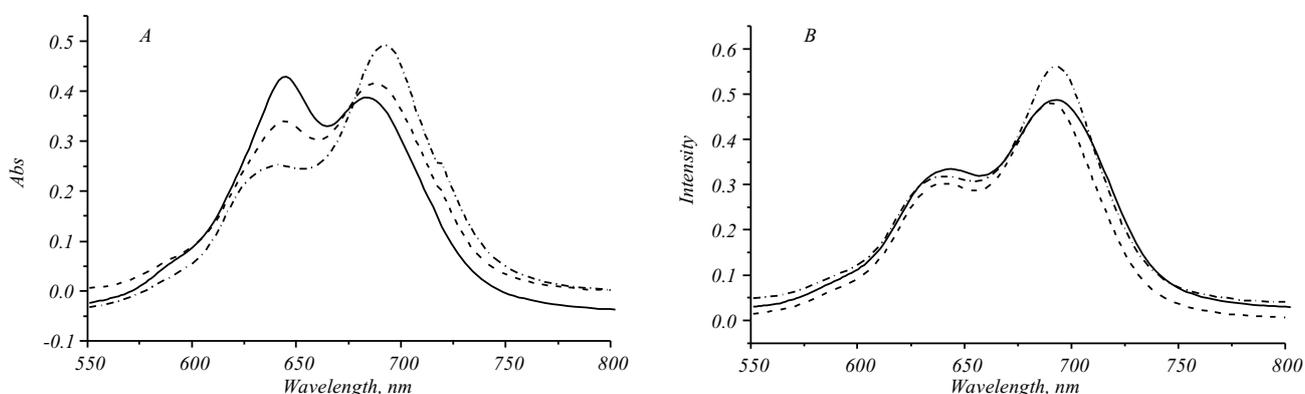


Fig. 5. Absorption spectra of PcHfPiromelit (1, concentration 10 μM) in the presence of native (2) and fibrillar insulin (3, concentration 34 μM) in buffer (A) and in 0.1 M HCl (B)

Aggregation of phthalocyanines upon conditions of fibrillization reaction. Since the reaction of insulin fibrillization is performed in acidic media – 0.1 M HCl, the aggregation tendency of phthalocyanines in these conditions was studied. In absorption spectra of all studied metal complexes in acidic media the short-wavelength band (near 650 nm) was observed as less intensive shoulder, while the long-wavelength band (maximum about 700 nm) was the most pronounced. Besides, an increase in the phthalocyanines concentration up to 10 μM did not lead to the redistribution between intensities of short- and long-wavelength bands (Fig. 6). Thus, the observed absorption spectra belong mainly to monomeric form of the phthalocyanine. Therefore, we can suppose that irrespectively of the prone of phthalocyanines to self-association in aqueous buffer at neutral or low-basic pH, these complexes did not form aggregates in acidic media at low pH.

It is shown that in acidic media for phthalocyanine with high inhibitory activity PcHfPiromelit the presence of native or fibrillar insulin slightly affects the relation between intensities of short- and long-wavelength absorption bands (Fig. 5, B) and thus – the aggregation degree of metal complex.

Hence, we can conclude that in acidic media the metal complexes exist mostly in monomeric form and their interaction with proteins does not cause the additional destruction of phthalocyanine self-associates or formation of associates on protein surface.

The axial coordination with imidazole ring of histidine was reported among the mechanisms of complex formation of Zn(II) phthalocyanine tetrasulfonate molecules and protein α -synuclein [4]. For studied phthalocyanines we can exclude the possibility of their chemical binding to aminoacids due to the nature of metal atoms and the presence of spatial out-of-plane ligands.

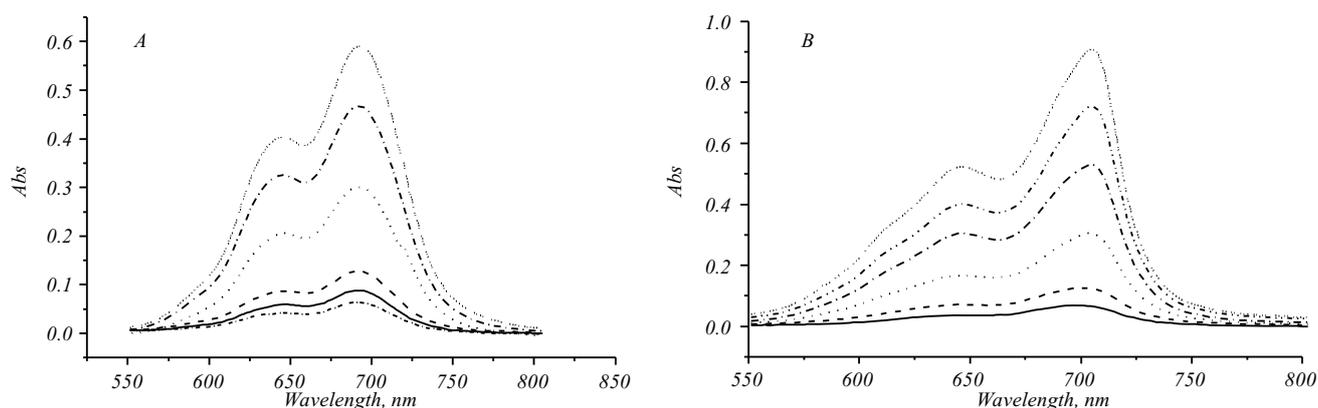


Fig. 6. Absorption spectra of PcHfPiromelit (A) and PcHfCl₂ (B) in 0.1 M HCl. Spectra are shown for concentrations of: 1 – 0.5 μM; 2 – 1 μM; 3 – 2 μM; 4 – 5 μM; 5 – 8 μM; 6 – 10 μM

We propose that high self-association ability of phthalocyanines in aqueous media points to a strong stacking interaction between molecules of the metal complex. In the presence of protein the stacking of phthalocyanine molecules with aromatic aminoacids could become more preferable than self-stacking, that results in the destruction of phthalocyanine aggregates and fixation of the metal complex molecules on the surface of protein. We could further suppose that even in acidic media where the aggregation does not occur, the stronger stacking between metal complexes and aromatic aminoacids still takes place leading to the inhibition of insulin fibrillization

At the same time, the compounds with less pronounced tendency to stacking interactions poorly form self-associates and weakly bind with aromatic aminoacids. Therefore, the tendency of phthalocyanines to self-association in aqueous media is considered as «an indicator» of their ability to stack with aromatic aminoacids of protein and thus their anti-fibrillogenic activity.

Conclusions. Series of hafnium phthalocyanines containing out-of-plane ligands were firstly studied as anti-fibrillogenic agents using the fluorescent cyanine dye inhibitory assay.

For the compounds with weak proneness to self-association in aqueous media (PcHfDbm₂, PcHfPyr₂, PcHfBtfa₂) the value of inhibitory activity varies in the range 60–73 %. For the complexes with the pronounced tendency to self-association in water solution (PcHfPiromelit and PcHfCl₂) the inhibitory activity was noticeably higher (about 95 %). For the complex PcHfPiromelit the micromolar efficient inhibitor con-

centration ($IC_{50} = 2.8 \pm 0.6 \mu M$) in the insulin fibrillization reaction was determined.

The addition of native or fibrillar insulin to the aggregation-prone phthalocyanines in aqueous buffer leads to the destruction of self-associates of metal complex. We can suppose that phthalocyanines interact with protein through the stacking with aromatic aminoacids in monomeric form. Upon the conditions of insulin fibrillization reaction (0.1 M HCl) phthalocyanines exist predominantly as monomers, the addition of native or fibrillar insulin does not affect the degree of self-association of metal complexes.

Thus, we conclude that low-order self-associates are not involved directly in the mechanism of inhibition of insulin fibrillization, phthalocyanines bind with protein in monomeric form through the stacking to it aromatic aminoacids. We suggest that the tendency of phthalocyanines to self-association in aqueous media is «an indicator» of the proneness of metal complex molecules to stack with aromatic aminoacids of protein and thus of their anti-fibrillogenic activity.

Acknowledgements. This work was supported by STCU-NASU project N 5508.

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Антифібрилогенна активність фталоціанінів з позаплосцинними лігандами: кореляція зі схильністю до самоасоціації

Резюме

Мета. Дослідити активність п'яти фталоціанінів гафнію, що містять позаплосцинні ліганди як інгібітори реакції фібрилоутворення інсуліну, та встановити кореляцію між інгібіторними властивостями фталоціанінів і їхньою схильністю до самоасоціа-

ції. **Методи.** Флуоресцентна спектроскопія і спектроскопія електронного поглинання. **Результати.** Для комплексів $PcHfDbm_2$, $PcHfPur_2$ і $PcHfVtfa_2$, які практично не утворюють агрегатів у водному буфері, інгібіторна активність становить 60–73 %. Фталоціаніни $PcHfPiromelit$ і $PcHfCl_2$ з високою схильністю до утворення агрегатів практично повністю пригнічують формування амілоїдних фібрил інсуліну (інгібіторна активність приблизно 95 %). У водному буфері за присутності мономерного або фібрилярного інсуліну відбувається руйнування агрегатів металокомплексів. Крім того, за умов проведення реакції фібрилізації інсуліну (0,1 М HCl) фталоціаніни існують переважно у вигляді мономерів. **Висновки.** Фталоціаніни з позаплоскоцинічними лігандами з тенденцією до самоасоціації демонструють вищу інгібувальну активність у реакції фібрилізації інсуліну порівняно з металокомплексами, які агрегують гірше. Фталоціанінові самоасоціати низького порядку безпосередньо не залучені до механізму інгібування агрегатоутворення інсуліну, тобто фталоціанін зв'язується з білком у мономерній формі. Тенденцію до самоасоціації фталоціанінів у водному середовищі можна розглядати як «індикатор» їхньої схильності до взаємодії з ароматичними амінокислотами і, отже, як показник їхньої антифібриллогенної активності.

Ключові слова: амілоїдні фібрили, фталоціаніни, інгібітори фібрилоутворення, інсулін, агрегація фталоціанінів, флуоресцентний барвник.

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Антифибриллогенная активность фталоцианинов с внеплоскостными лигандами: корреляция со склонностью к самоассоциации

Резюме

Цель. Исследовать активность пяти фталоцианинов гафния, со-держащих внеплоскостные лиганды в качестве ингибиторов реакции фибриллообразования инсулина, и установить корреляцию между их ингибиторными свойствами и склонностью к самоассоциации. **Методы.** Флуоресцентная спектроскопия и спектроскопия электронного поглощения. **Результаты.** Для комплексов $PcHfDbm_2$, $PcHfPur_2$ и $PcHfVtfa_2$, практически не образующих агрегатов в водном буфере, ингибиторная активность составляет 60–73 %. Фталоцианины $PcHfPiromelit$ и $PcHfCl_2$ с высокой склонностью к образованию агрегатов практически полностью подавляют формирование амилоидных фибрилл инсулина (ингибиторная активность около 95 %). В водном буфере в присутствии мономерного или фибриллярного инсулина происходит разрушение агрегатов металлокомплексов. Кроме того, в условиях проведения реакции фибриллизации инсулина (0,1 М HCl) фталоцианины существуют преимущественно в виде мономеров. **Выводы.** Фталоцианины с внеплоскостными лигандами с тенденцией к самоассоциации демонстрируют высокую ингибирующую активность в реакции фибриллизации инсулина по сравнению с металлокомплексами, агрегирующими хуже. Фталоцианиновые самоассоциаты низкого порядка непосредственно не вовлечены в механизм ингибирования агрегатообразования инсулина, т. е. фталоцианин связывается с белком в мономерной форме. Тенденцию к самоассоциации фталоцианинов в водной среде можно рассматривать как «индикатор» их склонности ко взаимодействию с ароматическими аминокислотами и, следовательно, как показатель их антифибриллогенной активности.

Ключевые слова: амилоидные фибриллы, фталоцианины, ингибиторы фибриллообразования, инсулин, агрегация фталоцианинов, флуоресцентный краситель.

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Received 18.03.13