Research Article

Journal of PeptideScience

Received: 9 June 2011 Revised: 11 August 2011 Accepted: 18 August 2011 Published online in Wiley Online Library: 14 November 2011

(wileyonlinelibrary.com) DOI 10.1002/psc.1416

Interference of low-molecular substances with the thioflavin-T fluorescence assay of amyloid fibrils

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Abnormal fibrillization of amyloidogenic peptides/proteins has been linked to various neurodegenerative diseases such as Alzheimer's and Parkinson's disease as well as with type-II diabetes mellitus. The kinetics of protein fibrillization is commonly studied by using a fluorescent dye Thioflavin T (ThT) that binds to protein fibrils and exerts increased fluorescence intensity in bound state. Recently, it has been demonstrated that several low-molecular weight compounds like Basic Blue 41, Basic Blue 12, Azure C, and Tannic acid interfere with the fluorescence of ThT bound to Alzheimers' amyloid-β fibrils and cause false positive results during the screening of fibrillization inhibitors. In the current study, we demonstrated that the same selected substances also decrease the fluorescence signal of ThT bound to insulin fibrils already at submicromolar or micromolar concentrations. Kinetic experiments show that unlike to true inhibitors, these compounds did neither decrease the fibrillization rate nor increase the lag-period. Absence of soluble insulin in the end of the experiment confirmed that these compounds do not disaggregate the insulin fibrils and, thus, are not fibrillization inhibitors at concentrations studied. Our results show that interference with ThT test is a general phenomenon and more attention has to be paid to interpretation of kinetic results of protein fibrillization obtained by using fluorescent dyes. Copyright © 2011 European Peptide Society and John Wiley & Sons, Ltd.

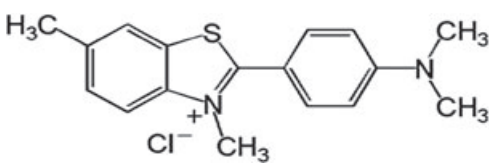
Supporting information can be found in the online version of this article.

Keywords: insulin; aggregation; fibrillization; fluorescence; amyloid

Introduction

Abnormal accumulation of amyloidogenic peptides/proteins plays an important role in more than 40 human diseases, including Alzheimers and Parkinsons disease, and type-II diabetes mellitus [1,2]. Formation of protein fibrils is a physical process of the formation of insoluble highly ordered structures. Although the amino acid sequences of amyloidogenic peptides and proteins are diverse, they all adopt a similar structure in aggregates called cross-beta-spine, where the partially unfolded β-structure-rich polypeptides interact with each other via intermolecular hydrogen bonding. Recent studies indicate that the phenomenon of protein fibrillization seems to be a generic property of polypeptide as under appropriate conditions even otherwise stable peptides and proteins may form amyloid fibrils [1,3].

The fluorescence dye thioflavin T (ThT) (1), which binds to protein fibrils and exerts increased fluorescence intesity in bound state, is a defining probe for the identification and kinetic study of amyloid fiber formation [4].



(1)

In a typical ThT fluorescence assay, ThT is added to samples containing fibril structures in micromolar concentration, and the ThT fluorescence intensity is monitored at 490 nm (excitation at 440 nm). Free ThT in an aqueous environment shows only weak fluorescence with lower (blue-shifted) excitation and emission maxima at 350 and 440 nm. As a rule, ThT does not affect the peptide fibrillation kinetics and has also been used for *in situ* monitoring of fibril formation [5,6].

The mechanism of interaction between ThT and amyloid fibrils involves the intercalation of ThT molecules within grooves between solvent-exposed side chains of the amyloid fibrils that run parallel to the fibril axis [4,7–9]. Binding within the channels is thought to provide rigidity of the molecule and the planar orientation of benzothiazole and benzene ring, which prevents the formation of a less-radiative twisted rotamers of the ThT molecule with the torsion angle between the benzothiazole and benzene ring from 37° to 90° [4,10]. The aromatic interaction with the protein is also contributing to the high quantum yield of fibril-bound ThT emission [4]. Because the interaction between ThT and amyloid is stoichiometric and reaches saturation at reasonably low ThT concentrations, the fluorescence signal from the amyloid–ThT complex provides a simple tool for the quantification of amyloid fibrils [11].

ThT test has also been used for the identification of fibrillization inhibitors applicable as tools for therapeutic intervention in

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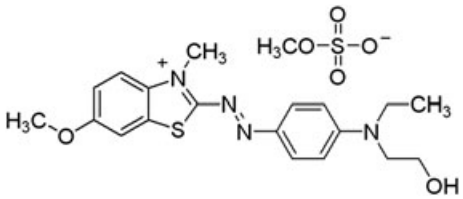
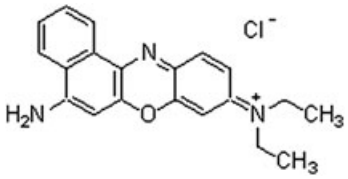
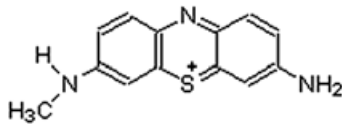
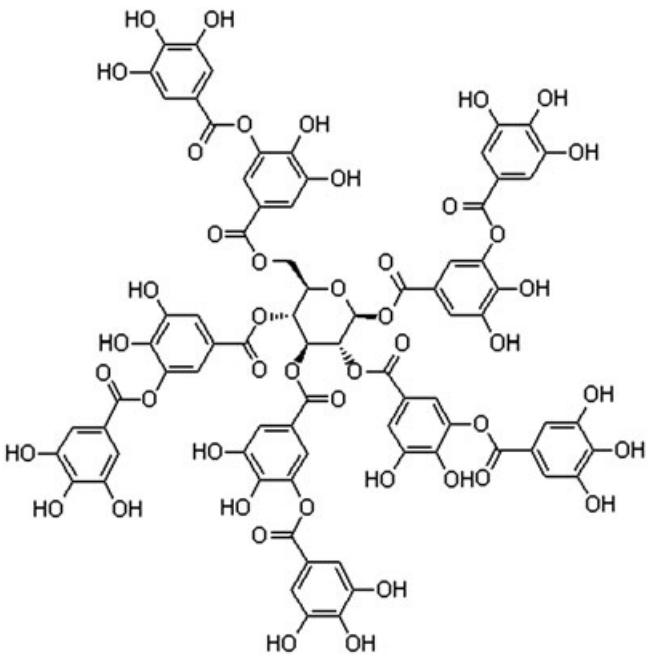
Abbreviations: *ThT, thioflavine T.*

Table 1. Effect of tested substances on maximum fluorescence observed in fibrillization of insulin and on fluorescence intensity of ThT bound to fresh and old insulin fibrils (IC_{50}^{FI})

Substance	Fibrillization IC_{50}^{FI} value (μM)	Fresh fibrils IC_{50}^{FI} value (μM)	Old fibrils IC_{50}^{FI} value (μM)
Basic Blue 41	0.025 ± 0.006	0.041 ± 0.006	0.030 ± 0.016
Basic Blue 12	0.026 ± 0.003	0.048 ± 0.004	0.039 ± 0.008
Azure C	0.96 ± 0.21	1.64 ± 0.04	0.92 ± 0.14
Tannic acid	9.02 ± 3.85	5.82 ± 0.97	0.74 ± 0.35
$ZnCl_2$	3.49 ± 0.90 (ICk value [5])$_{50}$	n.a.	n.a.

Substance	Chemical structure
Basic Blue 41	
Nile blue Chloride (Basic Blue 12)	
Azure C	
Tannic acid	

case of a variety of amyloidogenic diseases. However, in order to provide reliable data, the method for monitoring the fibril formation in the presence of a variety of substances *in vitro* should be reliable and free from artifacts. Recently, it has been demonstrated that low molecular weight substances may interfere with the ThT test and decrease ThT fluorescence in the case of Alzheimer's amyloid beta peptide (Aβ42), which may lead to false positive results in the screening of fibrillization inhibitors

Journal of PeptideScience

[12,13]. Interference with ThT test might be a rather general phenomenon that may disturb the fibrillization studies of all amyloidogenic peptides/proteins and lead to misinterpretation of experimental results obtained with application of ThT and its analogs.

Insulin is a 51-residue peptide hormone involved in glucose metabolism and universally used in diabetes treatment. Under specific conditions, that is, high temperature and low pH, it is very prone to fibrillization. Insulin fibrils exhibit common properties of amyloid fibrils, which have made insulin fibrillization a good model system for the study of protein fibrillization. There are also studies where inhibitors of insulin fibrillation at low pH have been studied using ThT fluorescence [14,15]. At the elevated temperatures and moderate agitation, zinc-free insulin aggregates rapidly at physiological pH values [5]. Considering that inhaled insulin forms toxic pulmonary amyloid aggregates [15] search for inhibitors of insulin fibrillization may also have a practical outcome.

The aim of this work was to test whether substances that have been studied as inhibitors of Aβ42 fibrillization [Tannic acid, Basic Blue 41, Basic Blue 12, Azure C (see Table 1), riboflavin, imidazole, ascorbic acid, phenolphtalein, and Zn(II)] compete with ThT for binding sites within insulin fibrils. We demonstrated that four substances (Tannic acid, Basic Blue 41, Basic Blue 12, Azure C) interfere with ThT test, which shows that interference with ThT test is a general phenomenon and more attention has to be paid to the interpretation of kinetic results of protein fibrillization obtained by using fluorescent dyes.

Materials and Methods

Lyophilized insulin, ThT, Basic Blue 41, Tannic acid, Azure C, Nile blue chloride (Basic Blue 12), riboflavin, imidazole, ascorbic acid, and phenolphtalein were from Sigma–Aldrich (St. Louis, USA). HEPES Ultrapure, MB Grade was from USB Corporation (Cleveland, USA). NaCl and $ZnCl_2$ were extra pure from Scharlau (Barcelona, Spain). All solutions were prepared in fresh MilliQ water. Stock solution of insulin was prepared as follows: Insulin was dissolved in 20 mM HEPES and 100 mM NaCl, pH 7.3 at a concentration of 50 μM. After 30 min incubation, the insulin stock solution was diluted with buffer and used for experiments. All test substances were dissolved in 20% ethanol.

Monitoring Insulin Fibrillization by ThT Fluorescence

Insulin fibrillation was monitored as described earlier [5]: in a standard experiment, the stock solution of insulin was diluted to a final concentration of 2.5 μM in 20 mM HEPES and 100 mM NaCl, pH 7.3 containing 2.5 μM of ThT and an appropriate amount of low-molecular weight substances [Basic Blue 41, Basic Blue 12, Azure C, Tannic acid, riboflavin, imidazole, phenolphtalein, ascorbic acid and ZnCl(II)]. A total of 450 μl of each sample was incubated in a 0.5 cm path length quartz cell, equilibrated to 50 °C and agitated with a magnetic stirrer at 250 rpm. The increase in the ThT fluorescence intensity at 480 nm (excitation at 440 nm) was monitored on a PerkinElmer LS-45 fluorescence

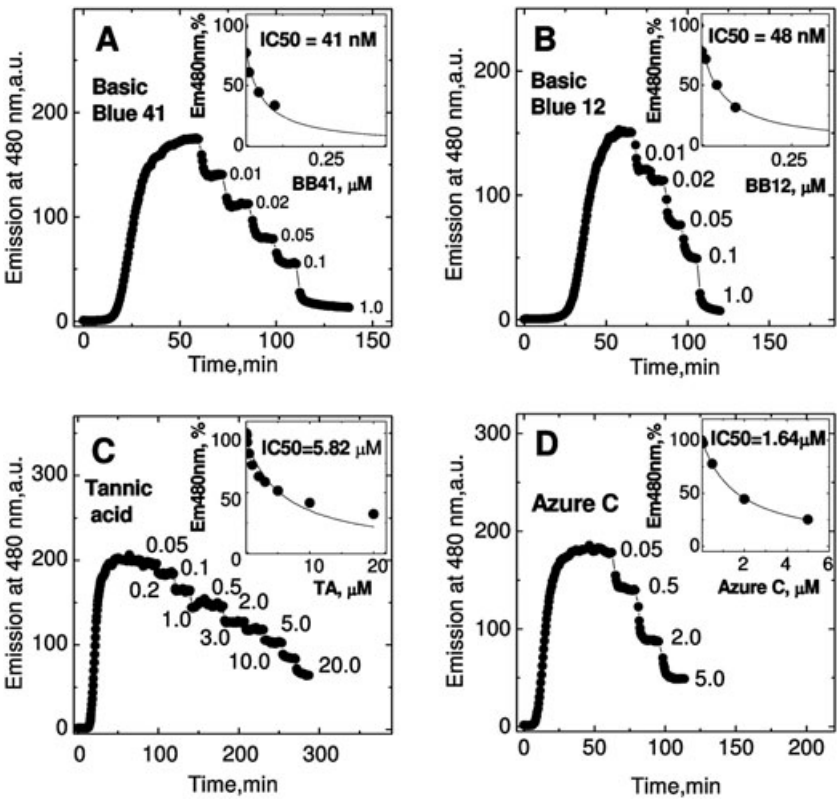


Figure 1. Effect of selected compounds on the ThT fluorescence emission in the presence of insulin fibrils. A total of 0.5 μmol/l of insulin in 20 mmol/l HEPES, 100 mmol/l NaCl at pH 7.3 was incubated at 50 °C in a quartz cell with continuous agitation at 250 rpm in the presence of 2.5 μM ThT; solid lines correspond to the fit of the data to Boltzmann equation; after completion of the fibrillization process, increasing amounts of selected compound was added. Inset – dependence of fibrillization rate constant from the concentration of selected compounds A – Basic Blue 41, B – Basic Blue 12, C – Tannic acid, D – Azure C.

spectrophotometer (PerkinElmer, Waltham, MA, USA) equipped with a custom magnetic stirrer.

The concentration of the low-molecular effectors was varied in the range 0.01–15.0 μM depending on the observed effects.

Determination of Soluble Insulin by SEC

Content of monomeric insulin in samples was determined by SEC on Superdex Peptide 10/300 column (GE Healthcare, Giles, United Kingdom) connected to an Äkta Purifier system (GE Healthcare, Giles, United Kingdom) by using 100 mM HEPES, pH 7.5 as elution buffer, flow rate 1 ml/min, sample volume 200 μl.

Calculation of Kinetic Parameters

The aggregation parameters were determined by fitting the fluorescence intensity *versus* time to Boltzmann sigmoid curve as described earlier [5]:

$$y = \frac{P_1 - P_0}{1 + e^{(t-t_0)\times k}} + P_0, \quad (2)$$

Where P_0 is the initial fluorescence level, P_1 is the maximum fluorescence, t_0 is the time t when fluorescence has reached half maximum, and k is the rate constant of the fibril elongation.

The half maximal inhibitory concentration (IC_{50}) values were calculated from the effects of substances on the final level of ThT fluorescence (IC_{50}^{FI}) and also from the effects on apparent rate constant of fibril formation (IC_{50}^{k}) according to hyperbolic dose-response curves:

$$y = P_1 - \frac{P_1 \cdot c}{IC_{50}^{FI} + c}, \quad (3)$$

where y is the fluorescence intensity of ThT, P_1 – the maximum value of ThT fluorescence, c – concentration of test substance, and IC_{50}^{FI}–concentration, which reduces ThT fluorescence by 50%.

Nonlinear regression analysis was carried out using a program Origin 6.1.

Results and Discussion

In the first set of experiments, the effect of Basic Blue 41, Basic Blue 12, Azure C, and Tannic acid on the ThT fluorescence intensity in the presence of pre-formed fibrils was estimated. Figure 1 shows typical fibrillization curves of insulin together with the decrease of the ThT fluorescence intensity upon addition the potential fibrillization inhibitors to the fibrils. The insets show the estimation of corresponding IC_{50}^{FI} values, presented also in Table 1. In principal, the abrupt decrease of fluorescence after addition of tested substances may result in (i) very fast disruption of the fibrils; (ii) competitive replacement of fibril bound ThT with the tested substances, or (iii) suppression of the ThT fluorescence. Quantitatively similar results were also obtained with matured fibrils that were incubated for 24 h before titration (Supplementary Figure 1).

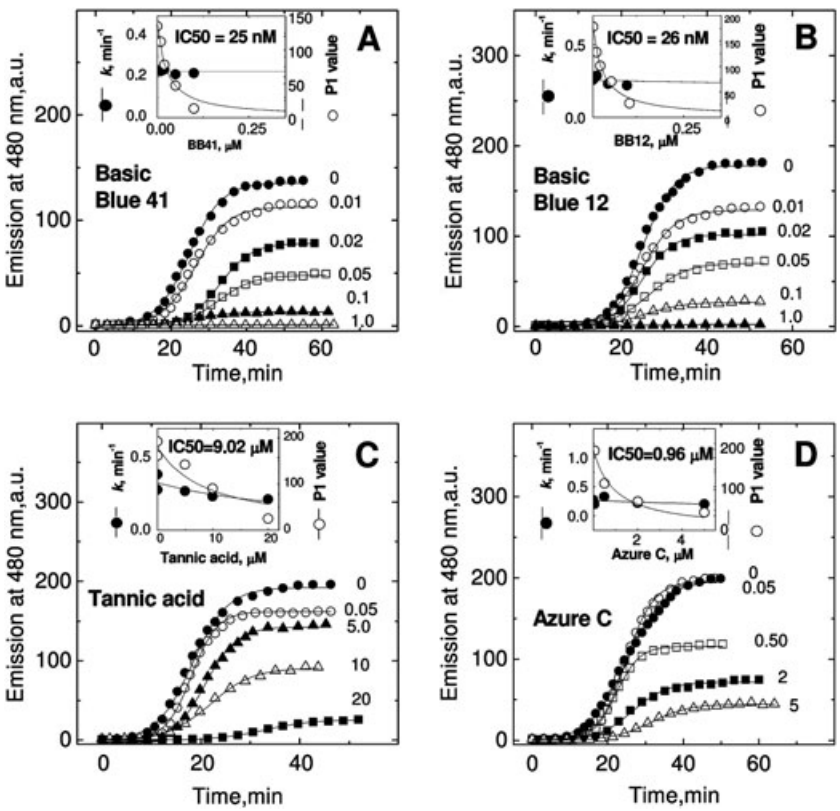


Figure 2. Effect of selected compounds on the fibrillization of insulin. A total of 2.5 μmol/l of insulin in 20 mmol/l HEPES, 100 mmol/l NaCl at pH 7.3 was incubated at 50 °C in a quartz cell with continuous agitation at 250 rpm in the presence of 2.5 μM ThT. Compounds added A: Basic Blue 41 concentrations: ● – 0 μM, ○ – 0.01 μM, ■ – 0.02 μM, □ – 0.05 μM, ▲ – 0.1 μM, – 1.0 μM. Basic Blue 12 concentrations: ● – 0 μM, ○ – 0.01 μM, ■ – 0.02 μM, □ – 0.05 μM, – 0.1 μM, ▲ – 1.0 μM. Tannic acid concentrations: ● – 0 μM, ○ – 0.05 μM, ▲ – 5.0 μM, – 10.0 μM, ■ – 20.0 μM. Azure C concentrations: ○ – 0 μM, ● – 0.05 μM, □ – 0.5 μM, ■ – 2.0 μM, – 5.0 μM. Solid lines correspond to the fit of the data to Boltzmann equation. Insets show the dependence of fibrillization rate constant k and maximal level of ThT fluorescence P1 on the concentration of effectors.

Journal of PeptideScience

Further, the kinetic curves of insulin fibrillization in the presence of Basic Blue 41, Basic Blue 12, Azure C, and Tannic acid were studied. Figure 2 shows that the maximal levels of ThT fluorescence is dropped with increasing concentration of the effector (IC_{50}^{k} values are presented in Table1), but neither the duration of the lag period nor the rate constant of fibril growth changed at the concentrations studied (Figure 2). This means that these compounds do not inhibit the insulin fibrillization process at low micromolar concentrations, but most probably compete with ThT for the same binding site in fibrils. After the addition of 1 μM Basic Blue 41 to insulin fibrils, peak of monomeric insulin was not detected by SEC in the supernatant (Figure 3), which confirms that the fibrils remain intact after addition of the compounds suppressing the ThT fluorescence. A weak inhibition of fibrillization rate was observed at elevated concentrations of Basic Blue, indicating that inhibition of fibrillization by the compounds studied occurs at higher micromolar concentration, but this cannot be determined using ThT fluorescence based methods.

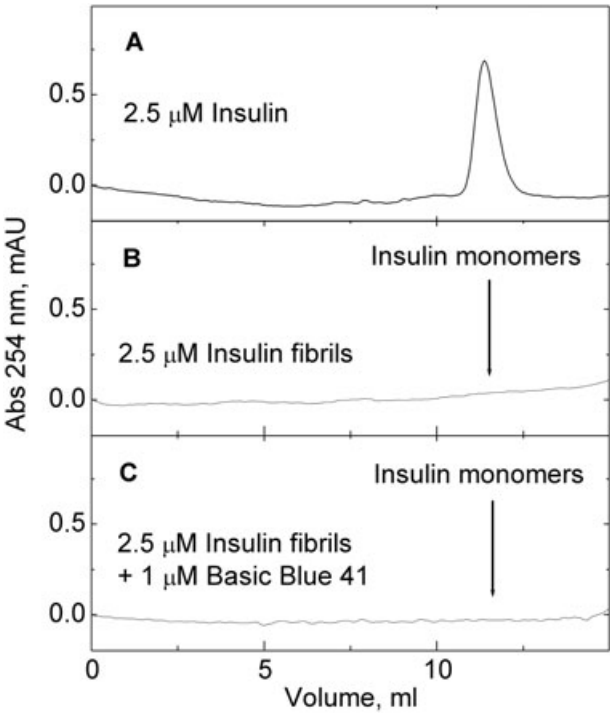


Figure 3. SEC analysis of the insulin solution before (A) and after (B) fibrillization and after addition of Basic Blue 41 to the fibrils (C).

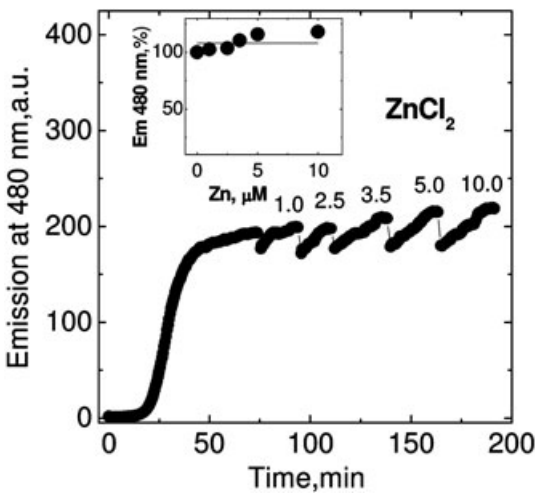


Figure 4. Effect of selected Zn(II) ions on the fibrils of insulin. A total of 2.5 μmol/l of insulin in 20 mmol/l HEPES, 100 mmol/l NaCl at pH 7.3 was incubated at 50 °C in a quartz cell with continuous agitation at 250 rpm in the presence of 2.5 μM ThT. Increasing amounts of ZnCl(II) (1.0 μM, 2.5 μM, 3.5 μM, 5.0 μM, 10.0 μM) were added. Inset shows the dependence of ThT fluorescence on the Zn(II) concentration.

It has been earlier shown in our laboratory that under similar conditions Zn(II) ions inhibit the fibrillization of monomeric insulin by decreasing the rate constant of fibril formation with $IC_{50}^{k} = 3.5$ μM [5]. Contrary to the compounds tested in this paper, Zn(II) did not decrease the fluorescence of preformed insulin fibrils (Figure 4). Riboflavin, imidazole, ascorbic acid, and phenolphthalein had also no effect on the fibrillization and ThT fluorescence.

It has been demonstrated that ThT binds to the grooves of the cross-beta structure of fibrils [4,7,9]. The binding stoichiometry of bound ThT inducing the characteristic fluorescence is about 0.09 moles of ThT per mole of insulin in fibril form [16]. Our results show that the apparent fluorescence quenching starts at very low submicromolar concentration of selected compounds and is completed at concentrations equal to 0.1 stoichiometry, which is in agreement with the binding stoichiometry for ThT determined earlier [16]. Compounds that interfere with ThT are similar to the structure of ThT (see Table 1), and, therefore, it is realistic that they compete with ThT for common binding sites on amyloid fibrils. Basic Blue 41 and Basic Blue 12 expose high, nanomolar affinity to the insulin fibrils, which is higher than affinity of ThT. This fact could be further exploited for design of novel amyloid probes or potential starting points for discovery of substances inhibiting protein fibrillization process, which are applicable as drug candidates for amyloidogenic diseases. We have to mention that Methylen Blue, which has similar structure to Basic Blue 41 and Basic Blue 12 is currently in clinical trials as disease modifying drug for Alzheimers disease [17,18].

Acknowledgements

This work was supported by the Estonian Ministry of Education, Research [SF0140055s08]; Estonian Science Foundation Grant 8811 both to Peep Palumaa, by the Enterprise Estonia Grant 30200 and by the World Federation of Scientists scholarship to Andra Noormägi.

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