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Research paper

Bis(indolyl)phenylmethane derivatives are effective small molecules for inhibition of amyloid fibril formation by hen lysozyme

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ABSTRACT

Amyloid or similar protein aggregates are the hallmarks of many disorders, including Alzheimer's, Parkinson's, Huntington's diseases and amyloidoses. The inhibition of the formation of these aberrant species by small molecules is a promising strategy for disease treatment. However, at present, all such diseases lack an appropriate therapeutic approach based on small molecules. In this work we have evaluated five bis(indolyl)phenylmethane derivatives to reduce amyloid fibril formation by hen egg white lysozyme (HEWL) and its associated cytotoxicity. HEWL is a widely used model system to study the fundamentals of amyloid fibril formation and is heterologous to human lysozyme, which forms amyloid fibrils in a familial form of systemic amyloidosis. HEWL aggregation was tested in the presence and absence of the five compounds, under conditions in which the protein is partially unfolded. To this purpose, various techniques were used, including Congo red and Thioflavin T binding assays, atomic force microscopy, Fourier-Transform Infrared spectroscopy and cell-based cytotoxicity assays, such as the MTT reduction test and the trypan blue test. It was found that all compounds inhibited the formation of amyloid fibrils and their associated toxicity, diverging the aggregation process towards the formation of large, morphologically amorphous, unstructured, nontoxic aggregates, thus resembling class I molecules defined previously. In addition, the five compounds also appeared to disaggregate pre-formed fibrils of HEWL, which categorizes them into class IA. The half maximal inhibitory concentration (IC50) was found to be ca 12.3 ± 1.0 μM for the forefather compound.

1. Introduction

Amyloid fibrils forming from specific peptides and proteins are involved in the pathophysiology of a wide range of diseases including Alzheimer's disease, Parkinson's disease, diabetes type II and systemic amyloidosis [1,2]. A number of proteins that are unrelated to protein deposition diseases may form fibrillar aggregates that possess the properties of amyloid fibrils [2,3]. It has thus been suggested that the ability to form amyloid fibrils may be considered a generic property of polypeptide chains [3]. The study of amyloid fibril formation by disease-unrelated proteins could shed light on our understanding of the mechanisms of protein aggregation and may suggest novel strategies to hinder it.

Human lysozyme is one of the proteins associated with amyloid fibril formation in human disease [2,4] In particular, mutations of human lysozyme have been known to be associated with a hereditary type of systemic amyloidosis [4]. Moreover, wild-type

Abbreviations used: HEWL, hen egg white lysozyme; ThT, Thioflavin T; MTT, 3-(4, 5- dimethylthiazol)-2, 5- diphenyltetraazolium bromide reduction test; CR, congo red; AFM, Atomic force Microscopy; FTIR, Fourier transform infrared spectroscopy; Aβ, amyloid β −protein; NMR, nuclear magnetic resonance; TLC, thin-layer chromatography; DMSO, dimethyl sulfoxide; mp, melting point; DMEM, Dulbecco's Modified Eagle's Medium; PBS, phosphate buffered saline.

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lysozymes from different organisms, including human, chicken and horse, have been shown to undergo amyloid fibril formation under well-defined conditions in vitro [5–7]. Since hen egg white lysozyme (HEWL) is known to form amyloid fibrils under denaturing conditions in organic solvents, detergents, chemical denaturants or acid pH with high temperature [6,8,9] and the properties of such fibrils and the fibrillation process have been studied in considerable detail [10,11], this disease-unrelated protein is considered a useful model in amyloid-related research.

The identification of compounds that could inhibit formation of amyloid fibrils and their precursor oligomers and suppress their related cytotoxicity would provide potential therapeutic agents. They could also be effective tools to study the mechanism of the amyloid fibril formation process, which still has many aspects to be elucidated. In order to identify such inhibitors, different compounds have been tested, including synthetic peptides [12,13], peptidomimetics [14–16], clioquinol [17], epigallocatechin-3-gallate [18] and small molecule libraries [19–22]. So far, many compounds have been proposed as potential anti-amyloidogenic agents, but to obtain clinically effective agents, there is still a need for further studies and subsequent validations.

Heterocyclic compounds possess a wide range of biological properties and are extensively employed in food, cosmetic and pharmaceutical industries. Coumarin, indole, and their derivatives are currently the subject of a great deal of attention, due to their therapeutic potential as anti-biotic, anti-inflammatory, anti-coagulant, analgesic, anti-tumor, anti-HIV, anti-apoptotic, anti-oxidant and insecticidal activities [23–25]. The synthesis of indole and their derivatives has been studied worldwide [26]. The indole ring has become an important structural requirement in many pharmaceutical drugs because of the structural diversity of biologically active indoles and their derivatives [27].

Several studies in vitro have shown the inhibitory ability of small molecules containing heterocyclic groups against amyloid fibril formation [28–31]. In particular, various studies have showed that indole and indolyl derivatives inhibit amyloid fibril formation by many systems [32–36]. They showed that the indole ring is likely to play the main role and its side chains contribute to a positive and/or negative role in this process [32,33,37].

Among the small molecules containing indole groups, bis(indolyl)methane derivatives are one of the most promising category of compounds. Medical epidemiologists believe that bis(indolyl) methane plays an important role in lowering the risk of cancer for people consuming plenty of cruciferous vegetables [38]. Bis(indolyl)methane is also able to normalize abnormal cell growth associated with cervical dysplasia [38]. Scientists reported on the potential of bis(indolyl)methanes to proliferate and induce apoptosis in human prostate and breast cancer [39,40]. Bhowmik [41] claimed that bis(indolyl)methane induces apoptosis in breast cancer cells by inhibiting epidermal growth factor receptor pathway [41]. The recent studies examined a selected series of para-phenyl–substituted bis(indolyl)methane compounds that display anti inflammatory and neuroprotective efficacy in vitro. The studies indicated that these compounds could be effective therapeutic agents to prevent neurodegeneration [42].

Following this paradigm, some *N*-heterocyclic compounds belonging to the category of bis(indolyl)arylmethanes have been synthesized as previously described [43] and tested for their possible effects on HEWL amyloid fibril formation. The effects of the compounds on amyloid fibril formation by HEWL and on the biological properties of the resulting aggregates have been investigated through the use of various techniques, including Congo red and thioflavin T assays, atomic force microscopy, Fourier transform infrared spectroscopy, and the MTT reduction and trypan blue assays.

2. Results

2.1. Chemistry

An efficient and economic route was performed for the preparation of substituted bis(indolyl)arylmethanes according to the electrophilic substitution of indole with the aromatic aldehydes and/or acetaldehyde under the solvent-free conditions by the catalytic mediation of a familiar heteropoly acid $H_5PW_{10}V_2O_{40}$ as described in Scheme 1 [43].

The heteropoly acid catalyst activates the aldehyde molecule towards the electrophilic attack of indole to generate an indolyl carbinol which further converts to azafulvenium intermediate. The produced azafulvenium salt acts as an electrophile and provides nucleophilic addition of the second molecule of indole to give the desired bis(indolyl)arylmethane derivative [43]. Using this procedure, five compounds belonging to the category of bis(indolyl) arylmethanes have been synthesized and purified as previously described [43] and have been numbered compounds 1–5 (Scheme 1). These are bis(indolyl)phenylmethane (Compound 1), bis(indolyl)-2-chlorophenylmethane (Compound 2), bis(indolyl)-3-nitrophenylmethane (compound 3), bis(indolyl)-3-methoxylphenylmethane (Compound 4), bis(indolyl)-2-methylphenylmethane (Compound 5). The chemical formulas and NMR spectra of all five compounds are reported in the supplementary information.

2.2. The effect of bis(indolyl)arylmethanes on heat-induced aggregation of HEWL

Aggregation of HEWL was induced by incubating the protein for 48 h at a concentration of 2 mg/ml in 50 mM glycine buffer, pH 2.5 and 57 °C in the absence or presence of 50 μM compounds 1–5. Since HEWL is highly stable, amyloid fibril formation does not occur at physiological values of pH and temperature [44]. Under such conditions of low pH and high temperature, by contrast, HEWL is known to be partially unfolded and prone to aggregation and such conditions are therefore ideal to study the aggregation process of HEWL in a controlled and reproducible manner [45,46]. Aliquots of the samples were withdrawn at regular time intervals to carry out the CR and ThT binding assays. The CR absorbance measured in the absence of HEWL and compounds was found to be low, with a peak at 490 nm (Fig. 1A). The CR absorbance measured in the presence of HEWL pre-incubated for 48 h under aggregating conditions in the absence of any compound exhibited a considerable increase accompanied by a red shift of the peak to 520–540 nm (Fig. 1A). The higher CR absorbance obtained in the presence of HEWL is likely to originate from the presence of protein aggregates that scatter light, whereas the red-shift of the peak from 490 nm to 520–540 nm indicates the presence of β-sheet containing aggregates.

The CR absorbance measured in the presence of HEWL pre-incubated with compounds 2 or 3 were found to be slightly lower than that measured in the absence of the compounds, with peaks at 520–540 nm (Fig. 1A). This indicates that compounds 2 and 3 were able to inhibit HEWL amyloid fibril formation only moderately. The CR absorbance measured in the presence of HEWL and compound 1 was found to be markedly lower, with a lower red shift, indicating that this compound was more effective in inhibiting HEWL amyloid formation. Finally, the CR absorbance measured in the presence of HEWL and compounds 4 or 5 was dramatically lower, with peaks at 500–510 nm, indicating their higher efficacy.

The same protein samples were also used for the ThT binding assay. The ThT fluorescence spectrum recorded in the absence of HEWL/compounds displayed a weak peak at 485 nm, amounting to 23 ± 2 a.u. (Fig. 1B). By contrast, the ThT fluorescence spectrum