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ARTICLE *in* ACS CHEMICAL BIOLOGY · SEPTEMBER 2006

Impact Factor: 5.33 · DOI: 10.1021/cb600328c · Source: PubMed

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From Green Bacteria to Human Dementia: A Novel Model for Discovering Amyloid Assembly Inhibitors

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ABSTRACT The formation of amyloid assemblies is associated with major human disorders. Yet no therapeutic agents presently exist to control this process. In a recent paper, a new bacterial system is described that uses a fusion of the Alzheimer's disease β-amyloid polypeptide to the GFP. The assay detects the formation of small, soluble amyloid intermediates associated with degenerative diseases. This assay allows the researchers to use high-throughput screening methods to find inhibitors of the formation of amyloid assemblies.

The formation of amyloid assemblies, which are large protein aggregates that share biophysical, biochemical, and ultrastructural properties, is associated with >20 human disorders (*1*, *2*). Despite the amyloid-forming proteins' different origins and the lack of any simple homology among them, in all cases fine 7–10 nm fibrils are observed that have a predominant β-sheet structure and a strong birefringence upon staining with Congo red. One of the most notable properties of amyloid fibrils is their effect on the fluorescence of small aromatic dyes such as thioflavin. When the dye binds to mature amyloid fibrils, its fluorescence markedly increases. Therefore, the ability to inhibit amyloid fibril formation, as reflected by the thioflavin fluorescence, is based on the use of a major assay for the high-throughput screening of inhibitors.

Amyloid fibril formation has been associated with some of the most common and grave degenerative disorders, including Alzheimer's disease (AD), Parkinson's disease (PD), and type 2 diabetes. However, despite continuous efforts by academic groups and the pharmaceutical industry, currently no approved therapeutic agents can control the process of amyloid formation and reverse the degenerative symptoms observed in these disorders. Despite the screening of large chemical libraries that may contain hundreds or thousands of compounds that use the thioflavin assay, as mentioned above, no direct amyloid assembly inhibitors have been developed into clinical therapeutic agents. The drugs available merely treat the symptoms rather than halt or reverse the course of the disease.

One of the reasons for the lack of approved efficacious agents that can inhibit amyloid formation and improve the prognoses of patients suffering from amyloid diseases may be because of the exact molecular character of the pathological species. In recent years, accumulating evidence has indicated that soluble amyloid assemblies rather than the mature amyloid plaques may actually be the pathological culprits that result in the degenerative phenomena (Figure 1) (*3*–*13*).

This rather novel notion is most established in the case of AD. Researchers demonstrated that soluble amyloid oligomers specifically affect memory-related functions, manifested by impairment of the long-term potentiation (LTP), a cerebral synaptic activity that is associated with learning and memorizing processes (*4*–*6*). The shift in the paradigm allows us to better understand some of the apparent inconsistencies in the "amyloid hypothesis". This includes the lack of signs of dementia in some people who possess large levels of amyloid deposits. In one famous case, a nun, Sister Mary, had very good cognitive abilities before her death at 101, despite having a very significant amount of amyloid deposits (*14*). The soluble oligomer notion also provides an explanation for the observation of LTP and

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Published online August 18, 2006

10.1021/cb600328c CCC: $33.50

Fluorescence could be detected only if an inhibitor allows the correct folding and if the fusion protein exists in a nonaggregative state.

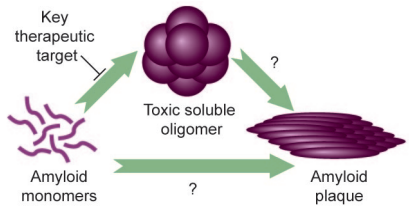

Figure 1. Soluble oligomers appear to be a major pathological element that facilitates the cognitive impairment associated with AD. It is not clear whether the formation of the oligomers is on-pathway or off-pathway in terms of the formation of the mature plaques. The sensitivity of a bacterial system to the formation of small aggregates will allow the identification of novel inhibitors to elucidate this key biochemical step.

cognitive deficiencies in AD model mice well before any amyloid deposits could be detected (*13*).

Specific, stable, soluble, dodecamer amyloid species of ~56 kDa were independently isolated and characterized by two different groups (*15*, *16*). These species are extremely stable in solution and do not disintegrate in denaturating SDS polyacrylamide gel electrophoresis (*15*). These stable species affect the LTP activity in rat brain slices and live rodents and cause memory impairment upon intracranial injection into rats (*15*, *16*). Most intriguingly, the very same dodecamer soluble species, which were termed Aβ*56, were found in the brains of AD patients and therefore can serve as novel biomarkers for the onset of AD long before the large fibrils can be observed by any imaging technique. The importance of the early soluble oligomers may be even greater given the many hints emerging that these assemblies may actually play a role in a condition known as mild cognitive impairment (MCI). The MCI disorder involves minor memory loss but not total dementia, is much more common than AD, and may affect >20 million Americans. Yet most likely, MCI may actually lead to the dreadful AD dementia.

The article by Kim *et al.* (*17*) on page 461 of this issue of *ACS Chemical Biology* offers a new way to select for amyloid formation inhibitors by using a bacterial screen, with special emphasis on early soluble assemblies (Figure 2). The assay is based on the expression of the AD β-amyloid (Aβ) polypeptide fused to a GFP in *Escherichia coli* bacteria. The authors chose to fuse the Aβ42 polypeptide, an Aβ variant that contains 42 amino acids and is considered to be the most important pathological form (compared with the shorter Aβ40 peptide). The assay is based on a reduction in the level of fluorescence that is observed upon the aggregation of the fused Aβ42 protein in the bacterium, as was previously reported by the same group (*18*). This occurs because of the slow formation of the fluorophore *in vivo*. Therefore, the fluorescence of the fusion protein depends on its correct folding and solubility. Fluorescence could be detected only if an inhibitor allows the correct folding and if the fusion protein exists in a nonaggregative state. The assay eliminates false-positive hits, which can prevent aggregation, but it allows proteins to fold correctly because of their effect on β-sheet structures in general. This is because inhibition of aggregation is only one essential factor needed to obtain a fluorescence signal. The other crucial element is the correct folding of the GFP protein. Therefore, a positive fluorescence signal can be observed only if the two events occur (*17*).

A major advantage of the assay is the fact that a decrease in fluorescence occurs already upon the formation of small oligomers, which are so important for the pathology of AD and other amyloid diseases, as previously mentioned. The bacterial assay also has other inherent advantages, because the bacteria represent a physiological environment under molecular crowding conditions and other effects that are difficult to replicate in a test tube. Furthermore, the bacterial assay allows researchers to study protein stability in a proteolytic environment and their ability to cross biological membranes. The latter property is more relevant to intracellular protein-aggregation phenomena, as described below, and less so to extracellular AD assemblies. In order not to miss agents that are effective inhibitors of Aβ aggregation but do not traverse well across biological membranes, the authors are also developing a cell-free assay that will be based on *in vitro* transcription and translation of the fusion protein.

The *E. coli* system appears to be very cost-effective. The authors used a simple, 96-well-plate rapid test to screen a library of ~1000 compounds. Members of the triazine chemical scaffold were added to the bacteria that expressed the fusion protein, and a conventional plate reader was used to determine the level of fluorescence in each well. The authors reported that the assay allowed them to distinguish between similar chemical entities and thus allows the pharmacophore definition of the identified molecules. An interesting observation that was reported is the similarity between the identified active compounds and one developed by Selkoe *et al.* (*19*) to control the formation of soluble oligomers.

The novel bacterial assay, as described in the new article (*18*), also represents a major

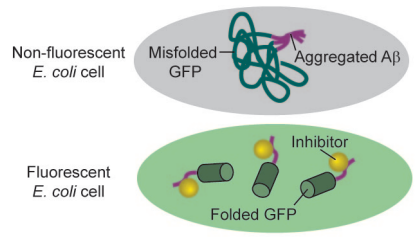

Figure 2. The bacterial systems used to select aggregation inhibitors. Fluorescence could be observed only if the GFP–Aβ fusion protein maintains its soluble structure and if correct folding of the GFP moiety occurs. The assay is sensitive enough for the aggregation of a small number of monomers. Thus, inhibitors of the early nucleation step may be identified.

advantage given some of the limitations of synthetic Aβ polypeptides. The synthetic Aβ polypeptide is relatively difficult to synthesize and thus very costly, a significant limitation to how much screening can be done. Furthermore, because of its exceptional tendency to aggregate, it is almost impossible to attain a synthetic preparation of Aβ that does not include any prenucleated seeds. The number may vary between batches, but some seed contamination always occurs. This not only results in large variability between different experiments but also may impair the ability to select for the most important inhibitors, those of the early oligomers that may represent the nucleation seeds. Biologically expressed Aβ can also be easily used to study mutated forms of Aβ, such as altered polypeptides associated with the early onset of the disease. Actually, some of the mutations in the Aβ polypeptide, most notably the Arctic mutation that was identified in families in northern Sweden, are known to result in a greater tendency to form prefibrillar amyloid assemblies as well as associated LTP impairment (*20*).

The current work paves the way for new developments in the field that will involve other amyloid-associated diseases as well as protein-aggregation disorders in general. The most challenging ones are those associated with intracellular protein-aggregation events. The author discusses Huntington's disease, which involves the aggregation of polyglutamine-rich huntingtin protein and the prion disorders, which may be hereditary, spontaneous, or infective encephalopathies. The latter form is the well-known bovine spongiform encephalopathy or "mad cow" disease. Two other key diseases are PD, in which the formation of intracellular amyloid Lewy bodies is linked to motor dysfunction, and amyotrophic lateral sclerosis (commonly known as Lou Gehrig's disease), which is associated with the intracellular aggregation of the superoxide dismutase enzyme.

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