Epigallocatechin Gallate – Unravelling its Effect on Amyloid and Toxic Oligomers

Susan J. Tzotzos

Apeptico Research & Development GmbH, 136 Mariahilfer Strasse, 1150 Vienna, Austria; e-mail: s.tzotzos@apeptico.com

Many medical disorders are associated with amyloid formation, Alzheimer disease (AD) and Parkinson disease (PD) being perhaps the most well-known. All such diseases have in common the pathological aggregation of a normally soluble polypeptide chain into a series of oligomeric intermediates and ultimately into insoluble amyloid fibrils that accumulate within specific organs and tissues. The polyphenol (−)-epigallocatechin gallate (EGCG), extracted from green tea, is amongst the most promising small molecules with therapeutic potential to interfere with the pathogenic aggregation of amyloidogenic peptides and redirect their self-association into nontoxic assemblies. Investigation of the complexes formed by EGCG and amyloidogenic peptides has thus become the focus of many studies, in some cases producing apparently contradictory results. Two recent studies are briefly examined here in the light of results of other researchers.

AMYLOID FORMATION AND DISEASE

Protein misfolding and self-association of the polypeptide chain into deposits of amyloid fibrils are characteristics of several devastating diseases such as Alzheimer (AD) and Parkinson (PD) diseases. So far around 40 peptides or proteins have been found to form amyloid deposits in human pathologies, in most cases the polypeptides being secreted, with resulting deposits located in the extracellular space (Chiti & Dobson, 2017).

The extent to which the amyloid deposits *per se* are the toxic, disease-causing element in these disorders is a matter of hot debate. There is increasing evidence that intermediate prefibrillar or oligomeric species which accumulate during the course of fibril formation represent the most pathogenic species. An important determinant of the toxicity of these misfolded oligomers appears to be their small size as does the exposure of hydrophobic groups on the oligomer surface. Specifically in neuropathic diseases involving the central nervous system, it is increasingly clear that the pathogenicity arises from the oligomeric forms generated in the process of aggregation (Chiti & Dobson, 2017). Aggregates smaller than full amyloid fibrils of beta-amyloid (Abeta), termed oligomers, are now believed by some scientists to be the toxic entities in AD (Ahmed et al, 2017; Eisenberg & Sawaya, 2017). In the case of alpha-synuclein (alphaS), aggregates of which are the major constituent of Lewy bodies found intraneuronally in PD patients, the so-called "toxic oligomer hypothesis" took hold without any precise definition of what exactly constitutes a toxic oligomer and is perhaps an over-simplification (Roberts & Brown, 2015).

TOXIC OLIGOMERS – THE ELUSIVE INTERMEDIATES

Characterisation of the pathogenic species in disease defines the molecular target for drug design and is a crucial step in developing a therapy. Therefore, in order to develop an effective therapy against such devastating diseases as AD and PD, it is essential to define and characterise the disease-causing molecular target(s) to enable rational design of effective drug molecules.

Many research groups have carried out extensive searches for well-defined oligomers in protein aggregation reactions, with the aim that determination of their structure would pinpoint the disease-causing elements. However, such efforts present a formidable task given the structural heterogeneity and transient nature of any given oligomer population and the existence of multiple parallel pathways to oligomeric species, which results in the existence of many accessible polymorphs (Chiti & Dobson, 2017). On top of these considerations is the added factor of different experimental conditions used by various research groups, which makes direct comparison between results of different groups difficult.

Fusco *et al.* in their recent publication in Science (15th December, 2017) characterised two main types of alphaS oligomer: type-A* which are nontoxic and type-B* which are toxic (Fusco *et al*., 2017). The ability of type-B* oligomers to disrupt synthetic and cellular membranes strongly correlated with their ability to generate cellular toxicity as measured by their ability to cause substantial increases in intracellular reactive oxygen species (ROS) and reduce mitochondrial activity in neuronal cells. The cellular damage observed with type-B* alphaS oligomers resembled that observed in neuronal models of PD. Fusco *et al.* investigated the structure of type-A* and type-B* alphaS oligomers using solid state nuclear magnetic resonance (ssNMR) spectroscopy and found that type-B* alphaS oligomers have considerable beta-sheet content in rigid regions, whereas type-A* oligomers showed negligible secondary structure in such regions. Paramagnetic relaxation enhancement (PRE) experiments using magic angle spinning (MAS) ssNMR spectroscopy were carried out to examine the membrane insertion properties of both types of oligomer and it was found that although both types of oligomer interact strongly with membranes, only type B* oligomers interacted with the hydrophobic interior of the lipid bilayer.

Furthermore it was found that although there is no defined structural motif in type-A* oligomers which is tightly bound to the membrane, type-B* oligomers show evidence that amphipathic alpha helices in the *N*-terminal region of alphaS are involved in strong membrane interaction.

In view of the well-established fact that toxic forms of amyloid-related aggregates of many proteins bind to membranes and disrupt cellular function, the authors suggested that the ability of the accessible *N*-terminal region of alphaS to bind strongly to lipid bilayers is a vital step in enabling oligomers of this protein to disrupt cellular membranes and, consequently, to induce neuronal toxicity.

EPIGALLOCATECHIN GALLATE – A POTENTIAL THERAPY FOR AMYLOID DISEASE?

With respect to potential therapeutic molecules, the results from several groups researching into the effect of the polyphenol (−)-epigallocatechin gallate (EGCG), the most abundant catechin in green tea, give cause for optimism, although in some cases produce apparently contradictory results and differ in their interpretation of what constitutes the toxic element. Two recent studies will be briefly examined to illustrate this point.

Yang *et al*. (2017) refer to three types of alphaS oligomer; two types, "transient" and "stable" having been previously defined by others and a third so-called "active" type described for the first time in their publication. Transient oligomers, whose accumulation and subsequent depletion precede actual amyloid fibril formation, are considered on-pathway intermediates, although whether or not they are converted back to monomers for fibrillar extension remains unknown. Stable oligomers remain after completion of fibrillation and are considered off-pathway oligomers. The authors define active oligomers (AOs) as beta-sheet free, metastable oligomers which instantly turn into amyloid fibrils *via* a unit-assembly process in which AOs act as a growing unit in the presence of diverse external stimuli such as shear force, temperature change, pH, and organic solvents. Previous work from this group had shown that AOs rapidly self-assemble into radiating amyloid fibrils (RAFs) on the surface of liposomes, resulting in a drastic disruption of the membrane structure. Since AOs caused membrane disruption and represent a potential therapeutic target, the effect of EGCG on formation of these alphaS oligomers was investigated. SDS-PAGE was applied to analyse oligomers in the absence or presence of EGCG and thioflavin-T (ThT) binding fluorescence to monitor fibrillation; circular dichroism (CD) spectroscopy, dynamic light scattering (DLS), Fourier transform infrared (FTIR) spectroscopy, atomic force microsocopy (AFM) and transmission electron microscopy (TEM) were used to examine the amyloid fibrils. Notably, EGCG had previously been reported by other researchers to effectively suppress alphaS fibrillation by inhibiting fibrillogenesis and stimulating the conversion of large fibrils into smaller nontoxic, amorphous protein aggregates (Ehrnhoefer *et al.*, 2008; Bieschke *et al*., 2010). Yang *et al.* refer to these latter non-toxic aggregates as "compact oligomers" (COs).

Yang *et al*. found that after prolonged incubation, EGCG transformed alphaS into two types of oligomers: SDS-resistant, off-pathway "compact oligomers" not participating in amyloid fibril formation and "transient" oligomers suggested to be SDS-sensitive and prone to grow into amyloid fibrils. The fibrils induced by EGCG had a slightly different appearance from those formed in its absence, being of a more curly nature than the straight fibrils usually formed from alphaS alone. Thus in contrast to previous work which demonstrated that EGCG reduced cellular toxicity by converting mature fibrillar alphaS into benign aggregates (Bieschke *et al.*, 2010), Yang *et al*. reported that EGCG facilitated, rather than inhibited, alphaS fibrillation; they suggested that EGCG exhibits its protective effect against alphaS-mediated cytotoxicity by not only producing the off-pathway COs, but also facilitating the conversion of AOs into amyloid fibrils and thereby accelerating the removal of AOs which, if left undisturbed, could cause membrane disruption (through RAF formation) and subsequent cellular degeneration. The authors remarked that ThT binding fluorescence may not be an appropriate method for monitoring the inhibitory effect of EGCG on amyloid fibril formation, hence the different conclusions produced by the two research groups. Yang *et al.* concluded that EGCG interacts selectively with alphaS, inducing its fibrillar polymorphism and is a potential prophylactic or even therapeutic agent for PD by either suppressing a population of toxic AOs or promoting the non-toxic CO formation of alphaS. The authors propose to further characterise the various oligomer types they observed. Further characterisation would indeed benefit interpretation of their intriguing results, enabling comparison with recent rigorous studies, such as that described above by Fusco *et al.* (2017) which give insights into the structural basis for the toxicity of alphaS oligomers.

Zhao *et al*. (2017) investigated the effects of EGCG on fibrillation and disaggregation of alphaS at the molecular level, using ThT fluorescence spectroscopy, CD spectroscopy, NMR spectroscopy, AFM and TEM. Structural characterization using ^{1}H NMR provided evidence that EGCG binds to Ile Phe and Tyr residues of the alphaS monomer which would inhibit its transition from random coil to beta-sheet conformation. At a concentration of 200 microM EGCG completely inhibited fibrillation, resulting in the production of large amorphous aggregates instead of ordered fibrils of alphaS. Furthermore, EGCG caused disaggregation of mature alphaS fibrils by binding to Leu, His, Phe and Tyr residues. These results suggest that EGCG reverses mature alphaS fibrils from beta-sheet to soluble random coil conformation. Crucially, in parallel experiments with PC12 cells (a stable rat pheochromocytoma cell line expressing wild-type alphaS by alphaS gene knock-in) overexpressing alphaS, it was demonstrated that EGCG attenuated cell death, inhibited ROS production, and exerted a protective effect against alphaS induced-damage by inhibiting overexpression and fibrillation of alphaS (Figure 1).

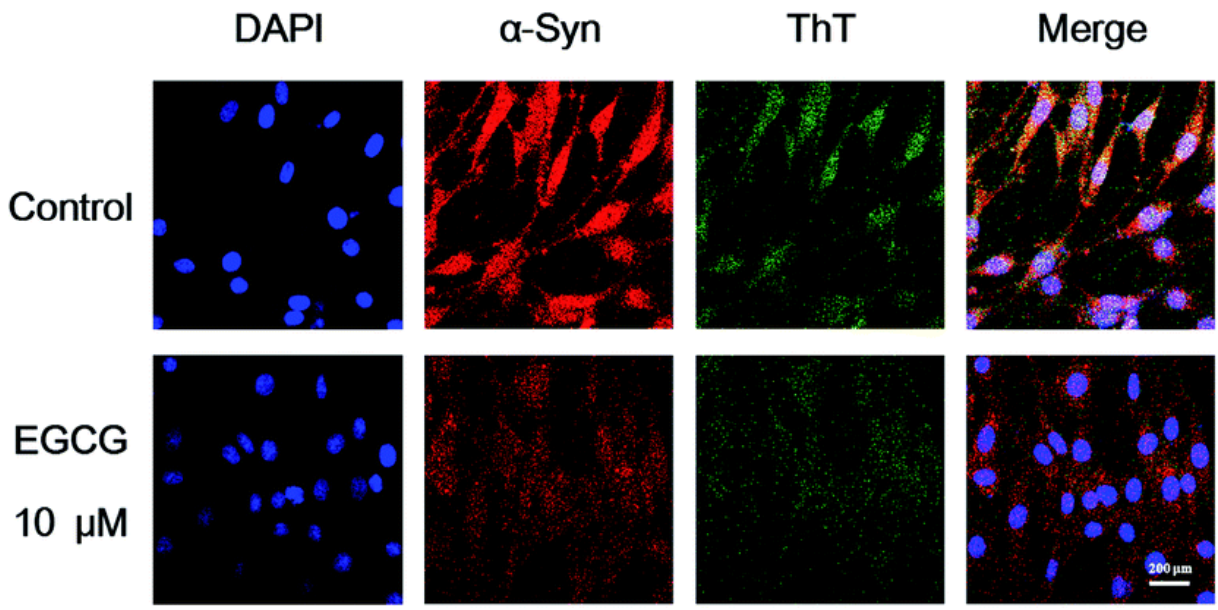

Figure 1. Laser scanning confocal microscope (LSCM) images of alphaS aggregates in the transduced PC12 treated with 0 (control) and 10 μM EGCG for 48 h. Cells were stained by DAPI (blue) for nuclei, rabbit anti-human α-Syn antibody (red) for alphaS and ThT (green) for amyloid fibrils. (Reproduced from: (−)-Epigallocatechin-3-gallate (EGCG) inhibits fibrillation, disaggregates amyloid fibrils of α-synuclein, and protects PC12 cells against α-synuclein-induced toxicity. J. Zhao, Q. Liang, Q. Sun, C. Chen, L. Xu, Y. Ding and P. Zhou, *RSC Adv.*, 2017, 7, 32508
DOI: 10.1039/C7RA03752J - Published by The Royal Society of Chemistry).

Thus the study of Zhao *et al.* implicates mature amyloid fibrils as the toxic species, responsible for cell death, whereas the study by Yang *et al.* attributes pathogenic properties to so-called active oligomers which precede mature fibrils in the aggregation process. In the disease model of Yang *et al.*, amyloid fibrils sequester the toxic active oligomers and mitigate their pathogenic effect.

CONCLUSION

Owing to the complex nature of amyloidogenic protein aggregation and the heterogeneity of intermediates as well as the particular conditions of the cell environment in disease states, it seems rather unlikely that one unique type of toxic species will be defined for any given protein folding disease. Rather as our knowledge of protein aggregation and amyloid formation advances, a variety of a therapeutic approaches will become available, each tailored to a particular pathogenic situation.

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