

MODULATORS OF AMYLOID PROTEIN AGGREGATION AND TOXICITY: EGCG AND CLR01

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Abstract

Abnormal protein folding and self-assembly causes over 30 cureless human diseases for which no disease-modifying therapies are available. The common side to all these diseases is formation of aberrant toxic protein oligomers and amyloid fibrils. Both types of assemblies are drug targets, yet each presents major challenges to drug design, discovery, and development. In this review, we focus on two small molecules that inhibit formation of toxic amyloid protein assemblies – the green-tea derivative (–)-epigallocatechin-3-gallate (EGCG), which was identified through a combination of epidemiologic data and a compound library screen, and the molecular tweezer CLR01, whose inhibitory activity was discovered in our group based on rational reasoning, and subsequently confirmed experimentally. Both compounds act in a manner that is not specific to one particular protein and thus are useful against a multitude of amyloidogenic proteins, yet they act via distinct putative mechanisms. CLR01 disrupts protein aggregation through specific binding to lysine residues, whereas the mechanisms underlying the activity of EGCG are only recently beginning to unveil. We discuss current *in vitro* and, where available, *in vivo* literature related to EGCG and CLR01's effects on amyloid β -protein, α -synuclein, transthyretin, islet amyloid polypeptide, and calcitonin. We also describe the toxicity, pharmacokinetics, and mechanism of action of each compound.

Keywords

• Amyloid • Amyloidosis • Alzheimer's disease • Parkinson's disease • Inhibitor • Molecular tweezers • Polyphenol

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Abbreviations

ADAD	– Alzheimer's disease
AFM	– Atomic force microscopy
A β	– Amyloid beta
APP	– Amyloid β -protein precursor
COMT	– Catechol-O-methyltransferase
CYP 450	– Cytochrome P450
EC	– Epicatechin
EGC	– Epicatechin-3-gallate
EGCG	– Epigallocatechin
EGCG	– Epigallocatechin-3-gallate
EM	– Electron microscopy
FAP	– Familial amyloidotic polyneuropathy
IAPP	– Islet amyloid polypeptide
IC50	– Half maximal inhibitory concentration
IP	– Intraperitoneal
MPTP	– 1-Methyl-4-phenyl-1,2,3,6-tetrahydropyridine
MT	– Molecular tweezer
MTT	– 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
NBT	– Nitroblue-tetrazolium
NMR	– Nuclear magnetic resonance

PD	– Parkinson's disease
SDS-PAGE	– Sodium dodecyl sulfate polyacrylamide gel electrophoresis
SPRI	– Surface plasmon resonance imaging
ThT	– Thioflavin T
TTR	– Transthyretin
UPS	– Ubiquitin-proteasome system

Introduction

What is common to Alzheimer's disease (AD), type-2 diabetes, medullary carcinoma of the thyroid, and human immunodeficiency virus infection? If you answered 'protein aggregation,' you answered correctly. In each of these diseases, particular proteins, which in all cases are part of normal physiology, change their behavior due to genetic, environmental, or unknown causes and begin to self-associate. The result is formation of abnormal oligomers of these proteins, which are not part of normal physiology, and in most of the cases studied to date, are cytotoxic [1]. They interfere with multiple cellular mechanisms, disrupt cell

membranes, and eventually may cause cell death, all of which are mediated by mechanisms that are not well understood and are actively investigated [2]. The oligomers are metastable structures that are difficult to detect, isolate, and study even on their own, let alone in the complex context of a cell or a tissue.

The self-assembly process does not stop at the oligomers but continues on to formation of amyloid fibrils, which are polymers characterized by a cross- β structure, in which protein monomers are arranged in β -sheets aligned perpendicular to the fibril axis [3]. The amyloid fibrils are substantially more stable structurally than the oligomers, and therefore histological analysis of tissue from disease sufferers or animal models reveals the presence of deposited amyloid fibrils in, for example, amyloid plaques and neurofibrillary tangles in AD or Lewy bodies and Lewy neurites in Parkinson's disease (PD) [4]. Intracellular deposits typically are found in dysfunctional cells, whereas extracellular amyloid often is surrounded by dead or dying cells and by an active inflammatory process. Because of their higher stability relative to oligomers, the

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amyloid deposits and fibrils were discovered earlier than the oligomers and originally were thought to be the cause of diseases, such as AD [5]. However, the discovery of the toxic oligomers, the subsequent demonstration that in many cases oligomers were substantially more toxic than the corresponding fibrils, and better correlation of disease progression with soluble oligomers than with fibrillar deposits, led most researchers to adopt the view that oligomers of amyloidogenic proteins, not amyloid fibrils, are the predominant cause of the related diseases.

Efforts to obtain assembly inhibitors for treatment of amyloid-related diseases focused initially on inhibition of fibril formation or dissociation of fibrils [6]. With the realization that oligomers, rather than fibrils, likely were the primary culprits, the focus shifted towards generation of inhibitors and modulators of the oligomers aimed at preventing their toxicity. Because in many cases oligomers are thought to be precursors of amyloid fibrils, successful prevention of oligomer formation often also precludes formation of the fibrils, thereby inhibiting the toxicity of both types of assemblies. Recently, multiple examples have been published of compounds that may decrease the toxicity of amyloidogenic proteins by actually accelerating fibril formation, thereby reducing the steady-state concentration of the toxic oligomers [7-9]. However, the therapeutic applicability of this approach is questionable because amyloid deposits may be toxic themselves, the very process of their growth may contribute to cytotoxicity and tissue damage [10], and accelerating fibril formation may induce a harmful pro-inflammatory response [11].

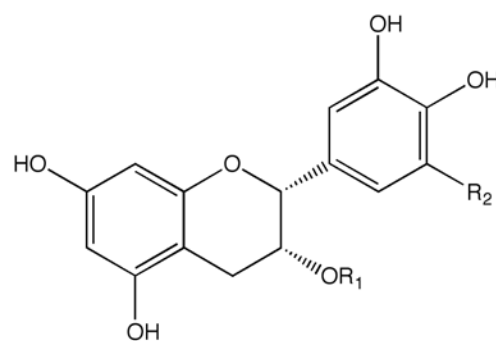
An ideal assembly inhibitor would interact with different conformations of the offending protein, which would enable the inhibitor both to block formation of toxic oligomers and to dissociate already-formed aggregates into non-toxic forms. Importantly, these forms should be soluble and amenable to degradation by the natural clearance mechanisms; otherwise, even if they are benign, they may accumulate and disrupt cellular function by sheer mass action. Examples of such inhibitors include predominantly two types of compounds –

peptides derived from the amyloid proteins themselves [12,13], or found through screening *in vitro* or *in silico* [14-16], and small molecules found empirically [17,18].

Though small-molecule drug candidates typically possess superior pharmacological characteristics relative to peptides, for example higher biological stability and bioavailability, a rational basis for prediction of small-molecule efficacy is difficult to define and studies often are based on screening of large libraries or empirical findings. A particular difficulty in the amyloid field is the lack of specific and stable structures of the targets [19] that would allow efficient screening of potential pharmacophores *in silico* or *in vitro*. The absence of well-defined structures is partially the cause of the current reality in which neither the protein self-assembly process nor the interaction of the target proteins with inhibitors or cells is sufficiently understood [20]. Due to these obstacles, most efforts geared at discovering and testing new protein oligomerization inhibitors/modulators for different amyloid-related diseases have relied on empirical observations [21]. A particularly prominent trend has been the use of nutraceuticals – compounds isolated from natural food sources, for which empirical or epidemiological data suggested therapeutic potential [22]. The advantage here is clear –

even if we do not know how or why these compounds work, at least we can reasonably expect them to be safe for human use [23].

If the main criterion for selection of aggregation modulator molecules is known safety based on many years of use, one might expect that the compounds available would have diverse structures. Surprisingly, however, the majority of the compounds reported in the literature based on this strategy are polyphenols or flavonoids [24,25]. The mode of action of these compounds, i.e., how they interact with amyloidogenic proteins or why the polyphenolic/flavonoid structures confer affinity for amyloid sequences, is unclear. Nevertheless, it has become evident that the binding of these compounds to amyloid sequences was not specific to one particular protein. Rather, compounds such as the green-tea (*Camellia sinensis*)-derived (–)-epigallocatechin-3-gallate [(2R,3R)-5,7-dihydroxy-2-(3,4,5-trihydroxyphenyl)-3,4-dihydro-2H-1-benzopyran-3-yl 3,4,5-trihydroxybenzoate (EGCG; Figure 1)] [26], the red-wine component resveratrol [27], or the coffee-bean extracted caffeic acid [28,29] can modulate the self-assembly process and inhibit the toxicity of multiple proteins [9]. Many of these compounds have additional activities, including anti-oxidant, anti-inflammatory, or chelatory actions that often are considered beneficial for treatment of amyloid-related



Major Tea Catechins		R1	R2
Epicatechin	EC	H	H
Epicatechin gallate	ECG	Gallate	H
Epigallocatechin	EGC	H	OH
Epigallocatechin gallate	EGCG	Gallate	OH

Figure 1. Chemical structures of major tea catechins. Catechins make up 10–30% of green tea weight. EGCG is the most abundant. Figure adapted from [41].

diseases and have spurred a recent surge in reports of similar, multifunctional compounds [30-37]. However, the lack of understanding of the anti-amyloidogenic action of these compounds and their multiple other activities also have raised concerns about side-effects and potential toxicity ([38,39], also see comments in [9]).

EGCG and CLR01

The most studied compound among these examples is EGCG. Habitual green tea consumption has long been associated with health benefits, including neuroprotective, cardioprotective, anti-oxidant, anti-inflammatory, and anti-carcinogenic effects [40]. These effects have been attributed to the high levels of polyphenols found in tea, especially green tea, due to the way tea leaves are processed. Polyphenols can be subdivided, with one of the larger categories being flavonoids, which can be further divided into subgroups, including catechins. These group names often are used interchangeably. The major catechins are epicatechin (EC), epigallocatechin (EGC), epicatechin-3-gallate (ECG), and (-)-epigallocatechin-3-gallate (EGCG) [41] (Figure 1). In green tea, polyphenols account for 20–40% of the weight, of which 60–80% are catechins, and EGCG is the most abundant among them – 50–80% [42]. Thus in 1 g of tea, EGCG constitutes ~130 mg. Green-tea catechins, and EGCG in particular, have been studied in many different fields of medicine, from human epidemiological studies to cellular and *in vitro* mechanistic studies. A PubMed literature search for “EGCG” results in over 2,900 hits, the majority of which are studies of the anti-oxidant and anti-carcinogenic activities of EGCG.

EGCG also has been tested against multiple amyloidogenic proteins both *in vitro* and *in vivo* and has been found to have beneficial effects in various disease models, establishing it as a broad-spectrum drug candidate. Based on screening a library of ~5,000 natural substances for inhibitors of aggregation of mutant huntingtin (Htt) exon 1 containing a polyglutamine expansion, EGCG was identified as the only purified compound (as opposed to plant extracts) with substantial inhibitory

activity [43]. Epidemiologic studies of PD and cognitive impairment [44-46] showed a moderate risk reduction in habitual tea drinkers compared to non-drinkers, supporting a role for EGCG as a protective compound in PD, though the protective activity could not be ascribed directly to inhibition of protein aggregation, because of EGCG's other various reported beneficial therapeutic effects [47-55]. Further investigation of the anti-aggregation activity showed that EGCG modulated or inhibited amyloid assembly of a number of amyloidogenic polypeptides, including amyloid β -protein ($A\beta$, related to AD), α -synuclein (related to PD), islet amyloid polypeptide (IAPP, also known as amylin, related to type-2 diabetes), Htt (related to Huntington's disease), the human immunodeficiency virus enhancer factor – semen-derived enhancer of virus

infection (SEVI), and the *Plasmodium falciparum* merozoite surface protein 2 (related to malaria) [43, 56-63]. EGCG also causes conversion of the cellular form of the prion protein, PrP^C, into a form distinct from the pathological scrapie prion protein, PrP^{Sc} [64].

Here, we compare EGCG with a drug candidate developed in our laboratory, the molecular tweezer CLR01, which in contrast to EGCG and the other small molecules mentioned above, was tested for its ability to inhibit protein self-assembly and toxicity based on a mechanistic rationale [65]. Torus-shaped molecular tweezers (MTs) were developed originally by Klärner and coworkers as host molecules for a variety of guests in organic solvents [66]. Introduction of negatively charged groups attached to the central hydroquinone in the hydrocarbon backbone

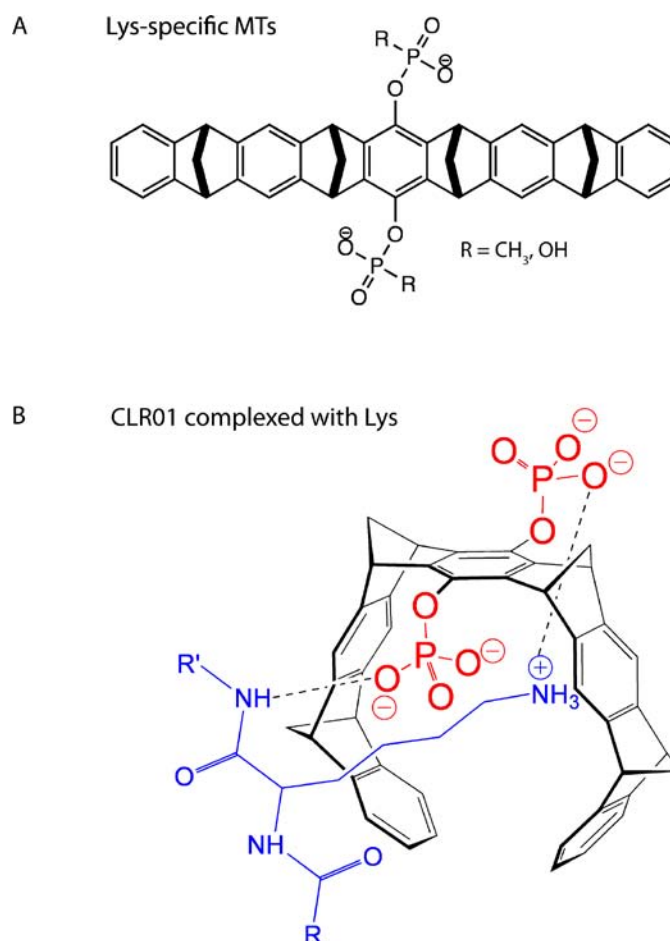


Figure 2. Chemical structures of molecular tweezers (MTs). A) Lysine-specific MTs bearing phosphate or phosphonate groups. B) Schematic representation of the interaction between CLR01 and Lys.

led to water-soluble derivatives, suitable for biological applications, and to the discovery of the specificity of diphosphonate and diphosphate MTs (Figure 2A) for Lys residues [67,68]. The specificity for Lys is achieved by inclusion of the butylene moiety in the Lys side-chain within the MT cavity enabling hydrophobic interactions, together with electrostatic attraction between the negatively charged groups of the MT and the positively charged ϵ -NH₂ group of the Lys side-chain (Figure 2B).

In amyloid fibrils, the major forces holding together the cross- β structure are hydrogen bonds and hydrophobic interactions. In contrast, in the early stages of the aberrant self-assembly of amyloidogenic proteins, when oligomers and nuclei form, the contribution of hydrogen bonding and β -sheet structure is lower. In these early steps, the self-assembly is mediated by a combination of hydrophobic and electrostatic interactions.

As Lys is the only proteinogenic amino acid that effectively forms both hydrophobic and electrostatic interactions in peptides and proteins and contributes substantially to these early self-assembly processes, the ability of MTs to bind specifically to the amino acid Lys was hypothesized to interfere with both these types of interactions within and among polypeptides [9,65]. This hypothesis was tested and concluded to be true by our group and our many collaborators over the last several years.

Lys is a ubiquitous residue and therefore the strategy held promise to produce broad-spectrum inhibitors. At the same time, binding of MTs to exposed Lys residues in virtually any protein raised obvious concerns about potential toxicity. However, this was found not to present a major problem because of the difference in forces stabilizing amyloidogenic versus normal proteins. The abnormal structures of oligomers of amyloidogenic proteins are held together by relatively weak forces, as opposed to normal proteins, whose structures have been shaped and optimized by millions of years of evolution. MTs bind to Lys residues with micromolar affinity, which is relatively weak but has been found to be sufficient for modulating the assembly and inhibit the toxicity of multiple amyloidogenic

proteins without causing toxicity in cell culture unless substantially higher concentrations were used [69]. These data are consistent with *in vitro* enzyme inhibition studies, which required MT concentrations at least an order of magnitude higher than those needed for inhibition of amyloidogenic proteins [68,70] and with data showing beneficial effects with no associated toxicity in animal models [71,72]. Below we compare and contrast in detail the *in vitro* and *in vivo* activities, modes of action, and drug-like characteristics of the broad-spectrum inhibitors EGCG and CLR01.

Effects on A β toxicity and assembly *in vitro* and in cell culture

A β was described originally as a component of vascular amyloid deposits associated with cerebrovascular amyloidosis [73] and the major component of amyloid plaques, both associated with AD [74]. The discovery of A β in these amyloid deposits led to the fibril-centered "amyloid cascade hypothesis" [5] as the driving tenet in AD pathogenesis. However, cumulative research in the last two decades has established that soluble, non-fibrillar A β assemblies, rather than fibrils, are the neurotoxic and synaptotoxic culprits underlying the protein-misfolding aspects of AD pathogenesis, which was emphasized by an updated version of the "amyloid cascade hypothesis" [75]. Therefore, efforts to find inhibitors of A β assembly and hence A β -mediated toxicity have intensified in parallel.

EGCG: EGCG's capability to inhibit A β -induced toxicity and A β assembly has been studied *in vitro* by multiple researchers. Bastianetto *et al.* found that micromolar concentrations of EGCG prevented 15- μ M A β 42-induced neurotoxicity in mixed rat primary hippocampal cells [76]. Pre-incubation of cells with EGCG for 30 min before addition of A β 42 did not promote cell survival, whereas co-incubation of EGCG with A β did. Therefore, the authors postulated that direct EGCG-A β interaction mediated the protective effect of EGCG. Thioflavin T (ThT) fluorescence [77] experiments supported this hypothesis by showing that EGCG (1–10 μ M) inhibited A β 42

(15 μ M) β -sheet formation [76]. However, it was later shown, as discussed below, that EGCG competes with ThT for hydrophobic binding sites on amyloid fibrils, which decreases the ThT fluorescence in the initial time points of the assay and causes remodeling of amyloid fibrils in later time points thus interfering with ThT binding and fluorescence [78].

A β 42 assemblies produced in the presence of EGCG were found not to be cytotoxic in rat pheochromocytoma cells (PC-12) [57] and EGCG rescued Chinese hamster ovary cells, overexpressing amyloid β -protein precursor (APP, 7PA2 cells) from the toxic effects of A β oligomers [58]. These cells previously had been shown to secrete highly neurotoxic dimers and trimers into the culture medium [79,80], though the toxic effect actually may require formation of protofibrils [81]. Pre-formed intracellular A β 42 aggregates in these cells were found by immunofluorescence microscopy to disappear after three days of EGCG treatment [58]. This finding and the cytoprotective effects of EGCG treatment against A β 42 oligomers generated in tissue-culture medium [58], led to the hypothesis that EGCG-mediated remodeling of amyloid assemblies likely protects cells from A β 42-induced toxicity [58]. Various concentrations of EGCG incubated with 15 μ M soluble A β 42 caused prolongation of the fibrillization lag phase, even when 10-fold molar excess A β 42 to EGCG were mixed together [57]. A 72-h incubation of A β 42 with EGCG caused formation of mostly spherical or amorphous structures, in contrast to typical A β 42 protofibrils and fibrils, which were the predominant morphology without EGCG treatment. These findings suggested that EGCG bound to A β oligomers and prevented their conversion into fibrils [58].

To examine the effect of EGCG on A β oligomerization, 50 μ M A β 42 were incubated in the absence or presence of EGCG and probed using the oligomer-specific antibody A11 [82] by dot-blotting [57]. A11 recognized untreated A β 42 following incubation for 6–24 h, but not A β 42 incubated with EGCG, whereas 6E10 (a monoclonal antibody against A β and APP) reacted with both treated and untreated A β 42 [57]. The authors concluded that EGCG-stabilized A β 42 oligomers, which likely differed

structurally from the A11-reactive amyloid oligomers described previously.

Patients with AD likely will be treated with any assembly inhibitor after substantial amyloid deposition has occurred in their brains. Therefore, it is valuable to test whether inhibitors not only prevent formation of new aggregates, but also can disassemble pre-formed amyloid fibrils. When equimolar concentrations of EGCG were added to A β 42 fibrils, the morphology of the fibrils was altered within 1–4 h. Besides large assemblies with typical fibrillar morphology, smaller amorphous assemblies were found in EGCG-treated samples, but not in controls [58]. These findings were supported by time-dependent reduction of ThT fluorescence suggesting that EGCG treatment disrupted the β -sheet structure of the amyloid fibrils. Interestingly, the gallate ester was found to be critical for efficient amyloid remodeling [58].

To determine whether EGCG bound directly to A β 42, Ehrnhoefer *et al.* used a nitroblue-tetrazolium (NBT) assay [57], which detects peptide-bound EGCG molecules by a color reaction [83]. NBT experiments showed that EGCG associated directly with soluble, unfolded A β 42. When natively folded proteins, including ovalbumin, carbonic anhydrase, chymotrypsinogen, or lysozyme were subjected to the same assay, NBT staining was not observed, suggesting that EGCG binding was selective for unfolded proteins [57]. However, this conclusion was not supported by later studies, which suggested that EGCG bound predominantly to β -sheet containing A β oligomers [78].

Lopez Del Amo *et al.* [84] studied the interaction between EGCG and A β 40 oligomers (which are distinct from A β 42 oligomers [85–87]) using nuclear magnetic resonance (NMR) and atomic-force microscopy (AFM). In their experiments, addition of EGCG caused rapid A β 40 precipitation. Nonetheless, well-defined chemical-shift changes in solution-state NMR spectra suggested specific EGCG–A β 40 interactions. AFM examination of the mixture revealed spherical particles of ~2.1 nm height and no fibrils. Pre-formed amyloid fibril fragments and certain oligomers can seed further polymerization and efficiently convert

unpolymerized amyloidogenic polypeptides from the soluble to the aggregated state [88]. In agreement with previous studies [57,58], A β oligomers produced in the presence of EGCG did not seed A β fibril formation, suggesting that in the presence of EGCG, A β oligomers adopt distinct structures than those formed in the absence of EGCG, and these structures cannot transform into protofibrils or fibrils [84]. Solid-state NMR experiments showed that A β 40 assemblies adopted a well-defined structure, in which residues 22–39 formed β -sheets, whereas the *N*-terminus (residues 1–20) was unstructured. The characteristic Asp23–Lys28 turn, which previously had been identified as a key structural feature in A β fibrils [89,90], monomers [91], and oligomers [92], was present in these A β assemblies [84]. Overall, the NMR data suggested a model in which EGCG binds to unfolded A β 40 by aromatic interactions. According to the model, during aggregation, A β 40 folds into a hairpin structure, in which the *C*-terminus adopts a β -sheet conformation whereas residues 10–20 cannot do so due to steric interference by EGCG [84].

Wang *et al.* used isothermal titration calorimetry to study interactions between A β 42 and EGCG and to analyze the thermodynamic parameters involved in these interactions [93]. They found that the binding stoichiometry increased linearly when EGCG:A β 42 ratio increased. Both hydrophobic interactions and hydrogen bonding were important in A β –EGCG interactions, but their relative contribution changed from predominantly hydrogen-bonding to hydrophobic interactions with increasing EGCG:A β 42 ratios. EGCG binding to A β 42 was promoted by increasing temperature and salt concentration, and by changing the pH away from the isoelectric point of A β 42 [93]. The same group extended their initial findings by probing intermolecular EGCG–A β interactions occurring at different regions of A β 42 by using three A β 42 fragments – A β (1–16), A β (1–30), and A β (31–42) [94]. Their results indicated that different interactions predominated at different A β 42 regions [94]. Hydrogen bonding occurred mainly in A β (1–16), a region rich in hydrophilic residues, whereas hydrophobic interactions predominated in A β (17–42), which is rich in hydrophobic residues [94].

Certain transition-metal ions accelerate the A β aggregation rate and alter its morphology and toxicity substantially [95–99]. Based on reports that EGCG has metal-chelating properties [100–104], Cheng *et al.* tested how the compound might affect A β fibrillization in the presence of metals [105,106]. Surface plasmon resonance imaging (SPRI), which allows monitoring intermolecular interactions, was used to study A β fibrillization in the presence of EGCG and various metal ions, including Fe³⁺, Cu²⁺, or Zn²⁺ [105]. A β 40 fibril seeds were immobilized covalently on SPRI plates and soluble “monomeric” A β 40 was introduced by continuous flow over the SPRI surface array. Consequently, fibril elongation was recorded and calculated. Incubation of Fe³⁺, Zn²⁺, or Cu²⁺ with A β 40 caused fibril elongation more efficiently than control A β 40, whereas EGCG addition resulted in slower elongation rates than A β control or A β –metal mixtures, suggesting that EGCG might have masked the immobilized fibril seeds and decreased their availability for elongation by addition of A β 40 monomers. EGCG inhibited A β fibril elongation of A β 40 alone less efficiently than in the presence of metal ions. Therefore, the authors suggested that EGCG-mediated metal chelation might have reduced the concentration of free EGCG available to interact with A β 40 [105]. If this mechanism were correct, it would suggest that direct interaction of EGCG with A β is more efficient at inhibiting A β fibrillogenesis than the indirect sequestration of metal ions by EGCG.

The ability of EGCG to modulate metal-bound A β assembly and toxicity also has been studied by Hyung *et al.* [106], who found that EGCG interacted with A β in the presence of Cu²⁺ or Zn²⁺ and caused production of low-molecular-mass, amorphous A β species whereas metal-free or metal-containing A β preparations formed fibrils in the absence of EGCG [106]. Similarly, EGCG disaggregated A β fibrils formed in the presence of metals after short incubation periods of 2–4 h, leading to formation of low-mass A β species [106]. In the presence of EGCG, regardless of the presence of metal ions, A β preparations were not toxic to murine Neuro-2a neuroblastoma cells [106]. Ion-mobility spectroscopy–mass-spectrometry and NMR experiments suggested that EGCG

interaction with metal- $A\beta$ species caused formation of more compact configurations than those formed by metal-free $A\beta$ [106].

CLR01: CLR01 was tested for inhibition of $A\beta$ fibrillogenesis and dissociation of existing fibrils using ThT fluorescence and electron microscopy (EM), and inhibition of $A\beta$ oligomerization by dot-blotting with antibody A11 [69]. Dose-response experiments showed that CLR01 inhibited formation of β -sheet-rich $A\beta$ fibrils by 10 μ M $A\beta$ 40 or $A\beta$ 42 completely at $A\beta$:CLR01 concentration ratio 1:3 and partially at 1:1 ratio [69]. In the absence of CLR01, both $A\beta$ alloforms formed typical amyloid fibrils, whereas in the presence of CLR01, only amorphous structures were observed by EM. Incubation of pre-formed fibrils of $A\beta$ 40 or $A\beta$ 42 with 10-fold excess CLR01 led to gradual dissociation of the fibrils, decrease in ThT fluorescence, and formation of amorphous aggregates [69]. In contrast to the fast dissociation of fibrils by EGCG, which was accomplished within hours [58], dissociation by CLR01 was substantially slower and required several weeks for completion [69]. A potential explanation for the major difference in reaction rate is covalent modification of $A\beta$ by EGCG [78] (see below in the Mechanisms of Action section), which shifts the equilibrium between fibrillar and soluble $A\beta$ towards formation of soluble structures and prevents dissociated monomers from re-association with the fibrils. In contrast, CLR01 binds non-covalently and the binding is highly labile [70], requiring substantially longer time for shifting the equilibrium towards fibril dissociation.

Similarly to EGCG, the presence of 10-fold excess CLR01 prevented completely formation of A11-positive $A\beta$ oligomers, whereas in the absence of CLR01, oligomers were detected readily already at $t = 0$ h, and their abundance increased gradually up to 120 h [69]. Interestingly, examination of the particle size distributions of $A\beta$ 40 or $A\beta$ 42 by dynamic light scattering showed that although CLR01 prevented fibril formation, it had little effect on the size distribution of soluble oligomers [69]. Thus, similarly to EGCG, binding of CLR01 to $A\beta$ appears to stabilize assemblies of similar sizes to those formed in the absence of inhibitor, but subtle changes in the structure prevent further aggregation and fibril formation by these assemblies.

The interaction of CLR01 with $A\beta$ strongly inhibited the toxicity of both $A\beta$ 40 and $A\beta$ 42. Addition of 20 μ M $A\beta$ 40 or 10 μ M $A\beta$ 42 to differentiated PC-12 cells caused 30–40% decrease in cell viability, which was reversed in the presence of CLR01 with half-maximal inhibitory concentration (IC_{50}) values 14 ± 11 and 52 ± 18 μ M, respectively, measured using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT)-reduction assay [69]. In primary rat or mouse neurons, CLR01 inhibited significantly $A\beta$ 42-induced retraction of dendritic spines and reversed $A\beta$ -mediated inhibition of basal synaptic activity [71]. CLR01 also inhibited significantly the typical decrease in long-term potentiation caused by treatment of hippocampal slices with $A\beta$ 42 [71].

Side-by-side comparison of EGCG and CLR01 showed that both compounds inhibited $A\beta$ fibril growth and A11-positive $A\beta$ 42 oligomerization with similar efficiency [107]. In cell-death assays using lactate dehydrogenase release in differentiated PC-12 cells, primary rat neurons, or mixed cultures of primary neurons and glial cells, EGCG was 10–20% more effective than CLR01 at inhibiting cell death following 48 h of incubation. Interestingly, examination of the interaction between the two inhibitors and $A\beta$ 40 using solution-state NMR showed that unlike CLR01, which bound readily to the two Lys and one Arg residues in $A\beta$ monomers already at 10:1 $A\beta$ 40:CLR01 concentration ratio, EGCG showed very little binding to $A\beta$ 40 monomers even when used at 4-fold excess. These observations suggested that EGCG binds to $A\beta$ oligomers but not monomers, in agreement with the study by Palhano *et al.* [78].

Effects on tau toxicity and assembly *in vitro*

Tau is a microtubule-associated protein that is most abundantly found in neurons. Hyperphosphorylation of tau occurs in AD and several other neurodegenerative diseases called tauopathies. Tau hyperphosphorylation disrupts the normal function of the protein and results in aggregation and accumulation of neurofibrillary tangles. In AD, tau hyperphosphorylation and toxicity occur

downstream of the effects of $A\beta$ and its degradation by the proteasome is inhibited by $A\beta$ signaling. Six different isoforms of tau exist in humans, which differ by the number of exons translated and the number of sequence repeats in the C-terminal region of the protein.

EGCG: EGCG has been reported to stimulate glycogen synthase kinase-3 β [108], one of the kinases known to phosphorylate tau, and therefore treatment with EGCG may exacerbate tau hyperphosphorylation under pathologic conditions. We are not aware of studies examining the direct impact of EGCG on tau aggregation or toxicity *in vitro*, except for a conference report about potential induction of toxic tau oligomers upon treatment of tau with EGCG [109].

CLR01: The effect of CLR01 on tau aggregation was examined using 4 μ M recombinant embryonic form of the protein (ON3R), which was induced to aggregate by addition of arachidonic acid [110] and monitored by ThT fluorescence. An increase in the ThT fluorescence signal began immediately following induction and reached a plateau by ~15 h [69]. At 1:1 tau:CLR01 concentration ratio, little or no change in ThT fluorescence intensity was observed, suggesting inhibition of β -sheet formation. Examination of the protein morphology by EM showed that tau samples incubated in the absence of CLR01 formed abundant fibrils, whereas in the presence of CLR01, no fibrils formed [69].

Effects on $A\beta$ and tau toxicity and assembly *in vivo*

EGCG: The discussion in this section is on both $A\beta$ and tau, the two proteins whose aggregation is implicated in AD because we find it helpful to consider the effect on the pathological lesions formed by both proteins whenever possible. Tan and colleagues reported that Tg2576 mice, a model expressing the APP_{Swe} mutation [111], treated either intraperitoneally (IP) with 20 mg/kg EGCG for two months, or orally with 50 mg/kg in water for six months, showed an increase in levels of C- and N-terminal fragments produced after cleavage of APP by α -secretase as compared to mice treated with vehicle [112].

The EGCG-treated mice also showed ~50% reduction in both total brain A β levels and in deposited A β burden [112,113]. Mice treated with EGCG through either administration route also showed improved performance in the radial arm water maze [113], a task of working memory. In a different study, one week of oral 3 mg/kg EGCG treatment of mice expressing a presenilin 2 mutation (Asn141→Ile) [114] showed decreased levels of A β 42, measured by ELISA, compared to vehicle-treated mice [115].

Intracerebroventricular injections of A β 42 in mice resulted in memory dysfunction, as measured by passive avoidance and Morris water maze tests, neuronal apoptosis, an increase in β - and γ -secretase activities, and a decrease in α -secretase activity [115]. In addition, an increase in the activation of extracellular-signal-regulated kinase, the mitogen-activated protein kinase system, and the nuclear factor κ -light-chain-enhancer of activated B cells protein complex was observed in these mice. These systems have been implicated in A β -induced neuronal cell death *in vitro* [116]. Mice pre-treated orally for three weeks with 1.5 mg/kg or 3 mg/kg EGCG showed dose-dependent improvement of all these pathological effects [115]. A rat model in which A β 40 was injected directly into the hippocampus also showed improved psychomotor coordination and working memory when the animals were pre-treated with daily IP injections of 10 mg/kg EGCG for three weeks [117].

Three weeks of oral EGCG pre-treatment of a mouse model utilizing intracerebroventricular [118] or IP [119] administration of lipopolysaccharide, a pro-inflammatory agent, to induce A β deposition by increasing levels of APP and activities of β - and γ -secretases [118–120], showed a dose-dependent decrease in APP and A β levels and the activities of the secretases [118,119]. An improvement in memory impairment measured by the passive avoidance [118] and Morris water maze tests also was observed [118,119]. Additionally, EGCG pre-treatment resulted in a decrease of lipopolysaccharide-induced apoptosis, cytokine release, inducible nitric oxide synthase, and cyclooxygenase-2 [118,119].

Both oral and IP EGCG treatment of Tg2576 mice also showed near-complete

reversal of formation of sarkosyl-soluble hyperphosphorylated tau compared to vehicle-treated mice [113]. Conversely, as mentioned above, *in vitro* studies suggest that EGCG actually stimulated dose-dependently glycogen synthase kinase-3 β -mediated phosphorylation of human recombinant tau and tau autophosphorylation [108]. The phosphorylation was thought to be stimulated by the direct binding of EGCG to human recombinant tau [108]. Additional studies are required to reconcile these contradicting results of a decrease in hyperphosphorylated tau [113] and glycogen synthase kinase-3 β -mediated phosphorylation of tau [108].

Oral EGCG treatment reduced A β levels in a *Caenorhabditis elegans* strain that expresses wild-type human A β 42, which deposits in the cytoplasm of muscle cells in this model [121,122]. Treatment with 220 μ M EGCG for four days reduced the mean number of thioflavin-S-stained deposits by ~30% compared to untreated worms. Western blot analysis using monoclonal antibody 6E10 showed a decrease in intensity of the bands identified as A β oligomers [122]. However, we and others have repeatedly warned that interpreting bands observed by Western blots or sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) as *bona fide* oligomers is misleading because upon contact with SDS and other detergents, A β forms artifactual oligomers that are not observed in the absence of detergents [1,123–126].

In addition to the A β -related effects of EGCG, the polyphenol also has mitochondrial restorative properties [47]. Mitochondria from the APP_{swE}/PS1 Δ E9 [127] double-transgenic mouse model of AD treated orally with ~37 mg/kg/day EGCG revealed a rescuing effect of EGCG on mitochondrial respiratory rates, mitochondrial membrane potential, reactive oxygen species production, and ATP levels by at least 50% in the hippocampus, cortex, and striatum. Hippocampal mitochondrial A β levels and total brain insoluble A β levels were also decreased by 50% and 13%, respectively, following EGCG treatment [47]. Whether the restorative mitochondrial effects of EGCG treatment were direct or indirect is yet to be determined.

CLR01: The effect of CLR01 on AD-related brain pathology was determined in a triple-transgenic mouse model of AD [128]. Fifteen-month-old mice were treated for a month with 40 μ g/kg/day of CLR01 using subcutaneously implanted osmotic minipumps. Immunohistochemical analysis of brain sections of CLR01-treated and vehicle-treated mice showed a significant decrease in A β burden of ~33% in AD-affected brain regions of CLR01-treated mice (Figure 3) [71]. Hyperphosphorylated tau, but not total tau, also decreased substantially following CLR01 treatment (Figure 3). Previously, CLR01 was found to inhibit tau aggregation *in vitro* [69], suggesting that the decrease in neurofibrillary tangle-like, hyperphosphorylated tau-rich structures in the brain of the CLR01-treated mice might have resulted from the direct action of CLR01. Alternatively, this effect could have been downstream of the effect of CLR01 on A β . Presumably downstream of CLR01's effect on A β burden, levels of microgliosis also decreased with CLR01 treatment, with no effect found in levels of microglia in CLR01- or vehicle-treated wild-type animals (Figure 3) [71].

Effects on α -synuclein toxicity and assembly *in vitro*

α -Synuclein, a 140-amino-acid-residue protein was characterized first in the zebra finch (*Taeniopygia guttata*) [129] brain regions that control singing (under the UniProt accession number Q91448, the organism described is *Serinus canaria* (Island canary) or *Fringilla canaria*). It was also identified using an antibody to purified synaptic vesicles in the electric organ of *Torpedo californica* [130]. It was designated as “synuclein” because of its co-localization with the nuclear envelope of presynaptic nerve terminals [131]. Although its functions are still unclear, α -synuclein point mutations have been associated causally with autosomal dominant forms of PD. Lewy bodies and dystrophic neuritis, the pathological hallmarks of idiopathic and genetic forms of PD, react strongly with antibodies against α -synuclein (reviewed in [132]). Therefore, genetic evidence and fibrillar α -synuclein accumulation in patients' neuropathological lesions point to a key role for α -synuclein in

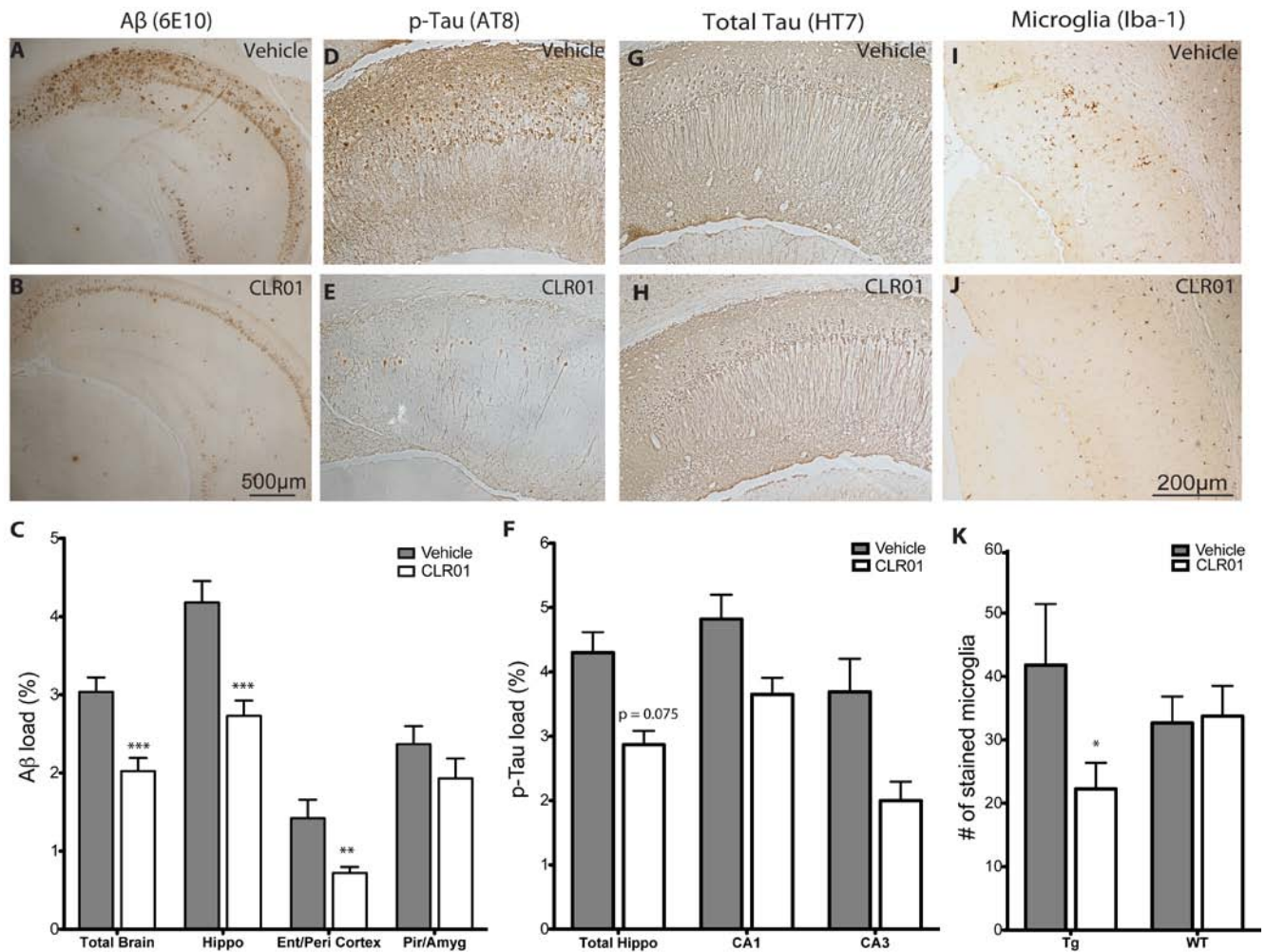


Figure 3. CLR01 decreases amyloid β -protein and p-tau deposition and ameliorates microgliosis in transgenic mouse brain. Triple-transgenic mice were treated with 40 $\mu\text{g}/\text{kg}/\text{d}$ CLR01 or vehicle. (A, D, G and I) Vehicle-treated transgenic mouse hippocampus. (B, E, H and J) CLR01-treated transgenic mouse hippocampus. (A and B) Transgenic mouse brain stained with monoclonal antibody 6E10 showing amyloid plaque deposition. (C) Percent amyloid β ($\text{A}\beta$) burden was quantified by calculating the total 6E10-stained area divided by the total area measured. (D and E) Transgenic mouse brain showing AT8-positive neurofibrillary tangles in the CA1 region. (F) Percent aggregated p-tau load was quantified by calculating the total AT8-stained area divided by the total area. (G and H) Transgenic mouse brain stained with monoclonal antibody HT7 for total tau. (I and J) Transgenic mouse brain showing Iba1-positive microglia in the subiculum and CA1 region. (K) Number of stained microglia in a 1.14 mm^2 area of hippocampus per treatment condition. Scale bars: bar in B applies to both A and B; bar in J applies to D–J. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ compared with vehicle-treated mice. Amyg = amygdala; Ent = entorhinal; Hippo = hippocampus; Peri = perirhinal; Pir = piriform cortices; WT = wild-type. Figure adapted from [71].

PD pathogenesis. Similar to $\text{A}\beta$, α -synuclein undergoes fibrillization and self-assembly and causes cytotoxicity.

EGCG: To investigate whether EGCG-generated oligomeric α -synuclein assemblies were cytotoxic, Ehrnhoefer *et al.* used the MTT-reduction assay in differentiated PC-12 cells. Concordant with previous observations [133], a mixture of α -synuclein protofibrils and fibrils (~ 75 nM) reduced cell viability by $\sim 40\%$. In contrast, when identical amounts of EGCG-generated oligomeric α -synuclein

assemblies were added to cells, no cytotoxicity was observed [57]. Similarly, EGCG-treated α -synuclein fibrils were significantly less toxic than untreated fibrils [58]. The lactate-dehydrogenase release assay also was used to investigate the effect of EGCG on the cytotoxicity of α -synuclein amyloid aggregates. Lactate-dehydrogenase activity in α -synuclein-treated cells was ~ 2.5 -fold higher than in untreated cells and this effect was rescued by 20 μM EGCG, suggesting that EGCG-mediated remodeling of α -synuclein aggregates

correlated with reduced cytotoxicity [58]. To assess whether EGCG was active intracellularly, pre-formed, insoluble α -synuclein fibrils were introduced to HEK-293 cells overexpressing α -synuclein by a tetracycline-inducible, stable transfection system [58]. Six hours later, EGCG (20 μM) was added, and cultures were incubated for additional 1–3 d. EGCG treatment strongly reduced the amount of insoluble α -synuclein aggregates in these cells time-dependently [58], suggesting that EGCG was active intracellularly, though an EGCG effect

mediated through cell-surface interactions could not be excluded based on these findings.

The hypothesis that EGCG-mediated remodeling of α -synuclein assemblies rescued cells from cytotoxicity was tested using several biochemical, biophysical, and structural techniques. First, NBT staining [57] showed a purple α -synuclein (100 μ M) band detected at ~17 kDa upon SDS-PAGE in the presence of 1 mM EGCG, whereas no colored bands were observed without EGCG treatment. This result was interpreted as indicating that EGCG bound unfolded α -synuclein directly [57]. Similar to experiments with A β , no EGCG binding was observed when control folded proteins were analyzed by NBT staining, suggesting that unfolding of α -synuclein specifically, and polypeptide chains generally, is a prerequisite for EGCG binding [57]. Furthermore, comparative studies using denatured, unfolded bovine albumin, which is natively folded, showed EGCG binding when albumin chains were denatured [57], suggesting that EGCG bound unfolded polypeptide chains irrespective of their primary sequence.

In two-dimensional NMR experiments, resonances corresponding to the C-terminus of α -synuclein disappeared at equimolar EGCG concentrations, indicating potential interactions with the flexible region of the C-terminus of α -synuclein at Asp119, Ser129, Glu130, and Asp135 [57]. At five-fold molar excess, EGCG interacted with the backbone groups of the protein along its sequence, rather randomly and non-specifically [57].

After determining the location of interactions between EGCG and α -synuclein, Ehrnhoefer *et al.* assessed the effect of EGCG on α -synuclein aggregation using 100 μ M His-tagged α -synuclein. In the absence of EGCG, ThT-positive, β -sheet-rich α -synuclein assemblies were observed following a lag phase of ~10 h. In contrast, 10 μ M EGCG suppressed formation of α -synuclein aggregates at EGCG: α -synuclein concentration ratio 1:10. Complementary EM experiments showed that EGCG markedly reduced fibril formation. In size-exclusion chromatography experiments, 100 μ M untreated α -synuclein eluted with an apparent molecular mass of ~51 kDa, representing the natively unfolded structure of the protein. In

contrast, samples treated with 200 μ M EGCG contained large soluble α -synuclein oligomers of ~500–1,500 kDa in addition to monomeric α -synuclein [57]. Apparently, these structures were conformationally disordered as circular dichroism spectroscopy showed that EGCG prevented β -sheet formation in α -synuclein in a dose-dependent manner [57].

To examine whether EGCG also could disassemble pre-formed, β -sheet-rich structures or earlier non-fibrillar assemblies, Bieschke *et al.* treated α -synuclein fibrils (100 μ M monomer concentration) with equimolar EGCG and monitored the mixture using time-resolved EM and AFM [58]. After 1–4 h, mixtures of amorphous and fibrillar structures were observed, and by 24–72 h, small amorphous assemblies predominated [58]. Quantitative analysis of EGCG binding to α -synuclein fibrils yielded an apparent dissociation constant of 100 ± 20 nM, suggesting high affinity of EGCG for the fibrils [58]. Complementary circular dichroism spectroscopy and ThT fluorescence data showed time-dependent disappearance of β -sheet conformation, in agreement with the morphological analyses. To find out whether EGCG disassembled α -synuclein fibrils into smaller species, which then could reassemble into amorphous structures or whether the remodeling occurred directly, Bieschke *et al.* used red (Cy5) and green (Alexa 488) fluorescently labeled α -synuclein fibrils (5% wt/wt). Labeled fibrils were mixed and incubated with equimolar EGCG for 1 or 24 h. Immunofluorescence microscopy showed that α -synuclein aggregates remained uniformly red or green after addition of equimolar EGCG (50 μ M), indicating that EGCG mediated direct fibril restructuring and did not cause release of monomers or small oligomeric assemblies that would subsequently reassemble into larger protein aggregates [58]. Altogether, these findings suggested that EGCG bound directly to pre-formed α -synuclein fibrils and transformed them into smaller, non-fibrillar structures.

To test whether α -synuclein assemblies generated by EGCG addition might promote fibril formation, *in vitro* seeding experiments using pre-formed α -synuclein fibril fragments and EGCG-stabilized assemblies were performed [57]. When equal amounts of either

EGCG-stabilized α -synuclein assemblies or α -synuclein fibrils were added to an excess of unpolymerized α -synuclein, fibrillization was efficiently seeded by sonicated fibrils, whereas EGCG-stabilized α -synuclein oligomers had no effect [57]. Ehrnhoefer *et al.* also characterized EGCG-stabilized α -synuclein assemblies for A11 immunoreactivity. Using time-resolved dot blotting, formation of A11-reactive amyloid assemblies was suppressed efficiently by addition of EGCG, similar to findings described for A β in the same study [57].

CLR01: The effect of CLR01 on α -synuclein aggregation was investigated using ThT fluorescence, EM, and native PAGE [72]. ThT fluorescence measurements showed that CLR01 inhibited formation of β -sheet completely at α -synuclein:CLR01 concentration ratio 1:1, respectively, and partially at 10:1 ratio. In disaggregation experiments, α -synuclein was allowed to aggregate for 60 days and monitored by ThT fluorescence. Ten-fold excess CLR01 was added on day 8, in the middle of the fibril growth phase, or on day 24, when the ThT signal had been stable for more than a week. In both disaggregation reactions, following addition of CLR01, a gradual decrease in the ThT fluorescence signal was observed, suggesting the arrest of fibril growth and dissociation of existing fibrils. EM examination confirmed the disappearance of fibrils and appearance of non-fibrillar aggregates [72].

Interestingly, the inhibition of α -synuclein aggregation by CLR01 seemed to involve stabilization of intermediate-to-high-order oligomers. Thus, when subjected to native PAGE, α -synuclein migrated as a predominant band of 127 kDa, and additional bands migrated with apparent mobility corresponding to 238 kDa and 700–900 kDa, presumably corresponding to oligomers. In the presence of CLR01, a similar pattern to that of α -synuclein alone was observed, yet the 700–900 kDa bands were substantially more abundant. The data suggested that CLR01 promoted the formation and/or stabilization of intermediate- to high-molecular-weight oligomers [72]. In contrast, experiments using Photo-Induced Cross-linking of Unmodified Proteins showed no effect of CLR01 on the distribution of small α -synuclein oligomers,

although electron-capture dissociation–mass-spectrometry data showed binding of CLR01 to monomeric α -synuclein [134].

Cell-culture experiments showed that CLR01 inhibited the toxicity of 20 μ M α -synuclein oligomers in differentiated PC-12 cells with $IC_{50} = 4 \pm 1 \mu$ M in the MTT-reduction assay [72]. In addition, CLR01 inhibited the toxicity of endogenously expressed α -synuclein in HEK 293 cells. Induction of α -synuclein expression for two days in these cells resulted in a decrease of ~40%, in cell viability. When CLR01 was simultaneously added with induction of α -synuclein expression, cell numbers did not decrease at 1 μ M CLR01 and even increased slightly at 10 μ M CLR01.

Effects on α -synuclein toxicity and assembly *in vivo*

EGCG: *In vivo* studies of EGCG in PD models have shown neuroprotection, increased lifespan, and improvement of behavioral deficits. Mutant LRRK2 (Gly2019→Ser) [135] and parkin-null [136] *drosophila melanogaster* treated with EGCG showed improvements in both dopaminergic and mitochondrial dysfunction, which was abolished upon genetic inactivation of adenosine monophosphate-activated protein kinase, suggesting that the effect of EGCG was mediated by increased adenosine-monophosphate-activated protein kinase activity [137]. In *drosophila* in which the *park2* gene was knocked-down, treatment with low doses of paraquat alone, a toxin that eliminates dopaminergic neurons at high levels through oxidative stress, or paraquat and EGCG, or propyl gallate resulted in improved survival and increased climbing ability [138]. Interestingly, the addition of EGCG, an antioxidant, did not improve the effect conferred by low doses of paraquat, a pro-oxidant, alone, but paraquat in combination with epicatechin or gallic acid actually reduced the effect of paraquat alone (Figure 4) [138]. Hormesis, also known as the upside-down U-shaped dose-response curve and an anti-oxidant supplemented pro-oxidant stimuli regimen, was suggested as the mechanism for these unexpected effects.

In studies utilizing the 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP)-mouse

model of PD, oral administration of either tea (containing ~25 mg/kg/day EGCG) or pure EGCG (25 mg/kg/day) prevented the loss of tyrosine-hydroxylase-positive cells in the substantia nigra (i.e., dopamine-producing cells) and of tyrosine hydroxylase activity and dopamine levels in the striatum [139]. In a separate study, in which EGCG was administered at 10 or 50 mg/kg/day, dopaminergic cell death rate was reduced to less than 50% of control and no toxicity was observed in either group [140]. MPTP-treated mice showed a 20% increase in levels of nitric oxide synthase, which causes toxicity by increasing nitric oxide [140]. Treatment with EGCG or oral tea administration not only reduced levels of neuronal nitric oxide synthase [139], but also lowered them down to control levels [140]. MPTP-administered mice treated with a combination of EGCG and rasagiline, a monoamine oxidase B inhibitor, showed a synergistic neuroprotective effect in the substantia nigra at doses below the therapeutic level of each compound [141]. A suggested mechanism for this response was a potential convergence of the individual effects of the compounds to increase levels of the Ser/Thr kinase Akt/protein kinase B [141]. A cDNA microarray analysis of the early and late genes involved in the MPTP model suggested a domino effect resulting from the early gene changes that led to cell death in the *substantia nigra* [142]. EGCG treatment reversed some of the gene expression changes and reduced cell death, supporting the role of those changes in neurodegeneration [142].

Rats pre-treated orally for 14 days with up to 2 mg/kg of EGCG followed by lesion surgery with 6-hydroxydopamine – a synthetic neurotoxin that selectively destroys dopaminergic and noradrenergic cells – showed subtle improvement in postural abnormalities and ability to cross a narrow beam [143]. However, this study did not demonstrate a rescue in dopaminergic cell loss [143]. In addition to the neuroprotective and nitric oxide synthase inhibitory properties of EGCG, Kang *et al.* showed that EGCG also acted as a catechol-O-methyltransferase (COMT) inhibitor [144]. COMT inhibitors typically are administered together with L-dopa or carbidopa as approved treatments

for PD to improve the brain penetration and bioavailability of the dopamine derivatives by preventing their methylation and inactivation. Thus, oral administration of EGCG to rats treated with L-dopa or carbidopa decreased the accumulation of 3-O-methyldopa in plasma and striatum [144]. The EGCG treatment also exerted a strong neuroprotective effect against kainic-acid-induced oxidative neuronal death in the rat hippocampus [144].

CLR01: CLR01's *in vivo* effects on α -synuclein aggregation and toxicity were first tested in a zebra fish (*Brachydanio rerio*) model, which expresses human wild-type α -synuclein under a neuronal promoter that causes a deformed body phenotype due to neuronal apoptosis, and early death [72]. Addition of CLR01 to the water environment of the fish at 1 or 10 μ M caused dramatic, dose-dependent improvements in both the body phenotype and the survival of the fish [72]. Immunohistochemical analysis of the zebra fish neurons showed abundant aggregated α -synuclein in untreated fish, whereas only soluble α -synuclein was observed in fish treated with CLR01. Western blot analysis in the absence or presence of the 26S ubiquitin-proteasome system (UPS) inhibitor, lactacystin, showed that by blocking α -synuclein aggregation, CLR01 prevented the known inhibition of the UPS by α -synuclein oligomers [145,146], leading to increased clearance of α -synuclein [72]. This latter finding provided important support for the “process-specific” mechanism of action of CLR01 by demonstrating that the labile binding of CLR01 to Lys residues in α -synuclein was sufficient for inhibition of aggregation, yet at the same time did not interfere with ubiquitination, which is required for UPS degradation [72].

A new zebra fish study [147] used the fungicide ziram (zinc bis(dithiocarbamate)), an inhibitor of ubiquitin E1 ligase of the UPS [148-150], which is linked to increased risk of PD [151]. Inhibition of E1 ligase has been shown to damage dopaminergic neurons preferentially and to increase α -synuclein levels and synaptic currents [150,152]. Exposure of zebra fish embryos to 10–1,000 nM ziram led to dose-dependent altered swimming, selective dopamine cell loss, and early death. UPS inhibition was measured using a degran-

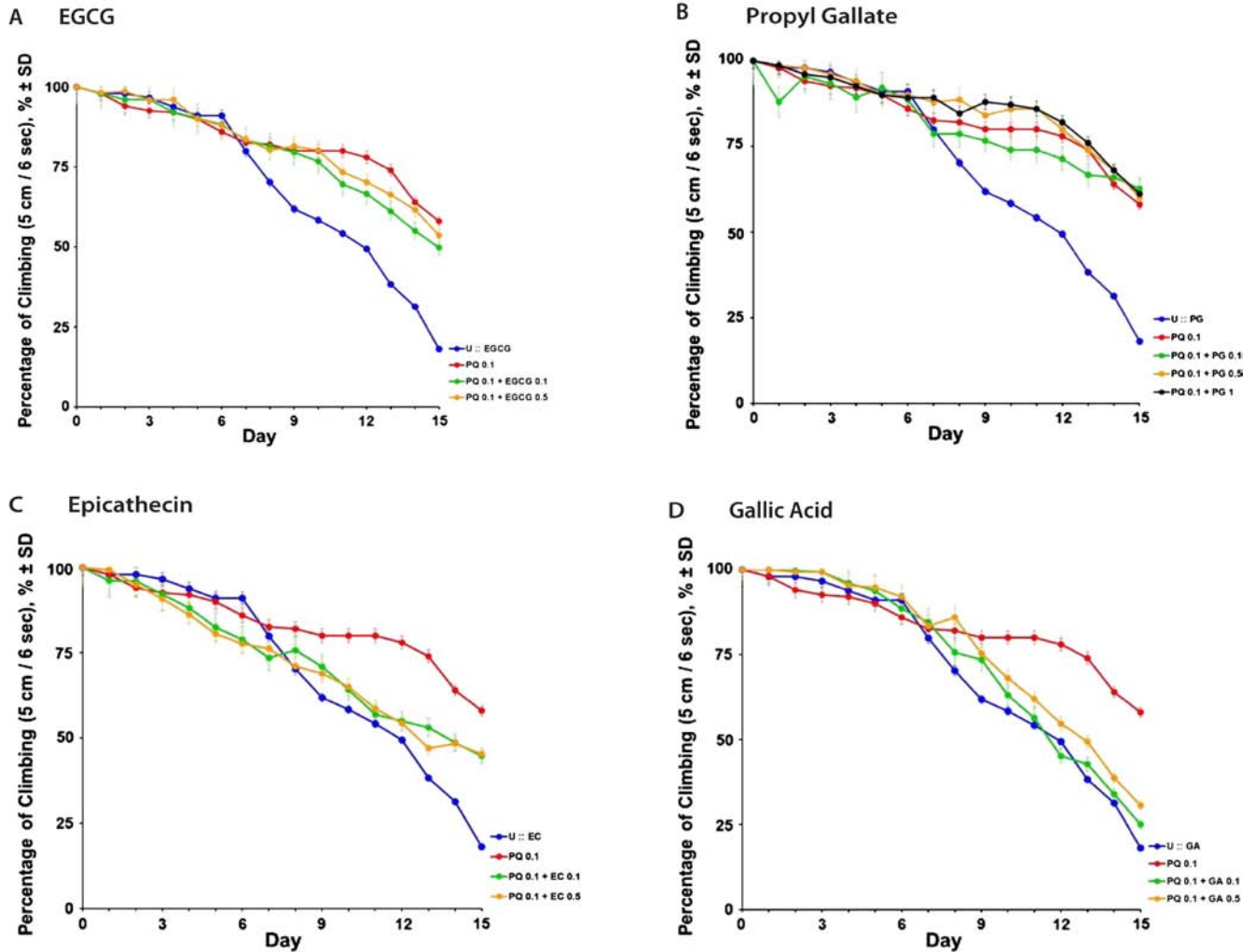


Figure 4. Effect of paraquat and/or catechins on *Drosophila melanogaster* locomotor activity. Female ($n = 50$ per treatment) parkin knock-down *Drosophila melanogaster* were treated with paraquat in the absence (0, blue bar) or presence of different concentrations of A) EGCG, B) Propyl gallate, C) Epicatechin, and D) Gallic acid. Statistical comparisons between untreated and treated flies showed $p < 0.05$ by χ^2 test for all compounds. Figure adapted from [138].

green fluorescent protein system [72] and detected at ziram concentrations as low as 1 nM. Unexpectedly, treatment of the zebra fish embryos with CLR01 attenuated ziram toxicity and improved survival, suggesting that ziram might have caused aggregation of endogenous zebra fish synuclein, a heretofore-unknown mechanism of ziram toxicity. Examination of this potential mechanism in the zebra fish confirmed that ziram promoted synuclein aggregation, suggesting that CLR01 protected the fish by mitigating ziram-induced synuclein aggregation.

Effects on TTR aggregation and toxicity *in vitro*

Transthyretin (TTR) is a tetrameric protein in which each subunit comprises 127 amino acid residues. TTR is produced by the liver, cerebral choroid plexus, and pancreas (reviewed in [153]). It is normally circulated in the plasma and cerebrospinal fluid where it transports thyroxine and retinol. Over 100 TTR mutations have been identified, some of which cause familial amyloidotic polyneuropathy (FAP), whereas others cause variable phenotypes, including neuropathy, cardiomyopathy, carpal-tunnel syndrome, vitreous TTR

deposition, and/or leptomeningeal pathology. Aggregation of wild-type TTR in the elderly causes senile systemic amyloidosis [154]. Immunohistochemical evidence suggests deposition of TTR in non-fibrillar forms in early stages of FAP before maturation into amyloid fibrils in the proximity of Schwann cells. Tissue-culture studies have provided evidence that low-molecular-mass, oligomeric assemblies of TTR (<100 kDa), but not fibrillar or large soluble TTR aggregates (>100 kDa), are cytotoxic (reviewed in [153]).

EGCG: EGCG interaction with TTR was assessed by binding assays using control plasma

or plasma from heterozygous TTR Val30→Met carriers, and recombinant wild-type TTR or recombinant TTR Val30→Met preparations incubated with ¹²⁵I-thyroxin in the absence or presence of EGCG [155]. Upon electrophoresis and phosphorimaging, no significant change in the TTR band intensity was observed regardless of EGCG presence, indicating that EGCG did not interfere with thyroxin binding to the tested TTR variants [155]. However, TTR showed a different apparent molecular weight when incubated with EGCG, suggesting that EGCG altered the electrophoretic mobility of the TTR preparations by binding, likely covalently, to a region distinct from its thyroxin-binding site [155,156]. X-ray crystallography of TTR Val30→Met complexed with EGCG showed three EGCG binding sites. EGCG interacted with these sites by hydrogen bonding or hydrophobic interactions with hydrophilic or hydrophobic side-chains of various amino acid residues between two monomers in the TTR dimer interface. Binding of EGCG was interpreted as stabilizing the TTR dimer interface and the overall tetramer structure [156].

EGCG-bound TTR was detectable by electrophoresis, NBT staining, and mass spectrometry [155]. Isothermal calorimetry allowed determination of the binding constant, $K = 2.5 \times 10^6 \pm 9 \times 10^5$ and the enthalpy change associated with the binding process, $\Delta H = -30 \pm 1 \text{ kJ mol}^{-1}$ [155]. Isoelectric focusing showed that EGCG increased TTR tetramer stability in plasma samples from heterozygote carriers of TTR Val30→Met and control individuals [155]. The effect of EGCG on TTR aggregation also was assessed by transmission electron microscopy of TTR Tyr78→Phe treated with 10- or 100-fold molar excess. Following two days of incubation at 37°C, TTR alone showed a heterogeneous mixture containing mainly oligomers, amorphous aggregates, and short twisted fibrils. In contrast, in a mixture of TTR Tyr78→Phe and 10-fold excess EGCG, small oligomeric assemblies were detected and no fibrils were visible. Incubation with 100-fold molar excess EGCG caused formation of quasi-spherical structures resembling those formed by the native, soluble protein. By day 6, TTR alone was mainly fibrillar whereas the

EGCG-containing preparations did not change [155]. Similarly, EGCG was found to inhibit TTR aggregation in media of a rat Schwannoma cell line, which secretes TTR Leu55→Pro, as determined by a filter-binding assay [155].

Because structural [156] and electrophoretic evidence [155] showed that EGCG–TTR interaction sites are distinct from the thyroxin-binding site of TTR, it was hypothesized that EGCG could stabilize TTR tetramers and suppress TTR fibril formation in variants, such as TTR Glu54→Lys, which is resistant to the effect of tetramer stabilizers (e.g., thyroxin) [156]. To test this hypothesis, recombinant wild-type TTR, TTR Val30→Met, or TTR Glu54→Lys preparations were incubated with different amounts of EGCG and analyzed by ThT fluorescence [156]. EGCG decreased TTR amyloid formation in the tested preparations in a dose-dependent manner [156]. However, whether this was caused by stabilization of the tetramer, disruption of aggregation, or a combination of both mechanisms, is yet to be determined.

To assess whether EGCG could dissociate pre-formed TTR fibrils, wild-type TTR, TTR Tyr78→Phe, and TTR Val30→Met fibril preparations were incubated in the absence or presence of 100-fold molar excess EGCG for 18 days at 37°C. In the absence of EGCG, on day 0, Tyr78→Phe preparations comprised mainly oligomers, small aggregates, and short twisted fibrils. Upon incubation with EGCG, a time-dependent decrease in assembly size was observed, demonstrating that EGCG disrupted TTR Tyr78→Phe fibrils [155]. Wild-type TTR and Val30→Met TTR fibrils, which were formed by acidification, were disrupted similarly to the Tyr78→Phe variant.

Because dissociation of TTR tetramers into monomers is necessary for TTR fibrillization, inhibition of TTR monomer formation by EGCG was investigated by Miyata *et al.* using 11 naturally occurring and clinically relevant TTR variants: Asp18→Glu, Asp18→Gly, Ala25→Thr, Val30→Met, Glu54→Gly, Glu54→Lys, Leu55→Pro, Tyr78→Phe, Ile84→Ser, Arg104→His, and Thr119→Met, and two other clinically relevant variants: Ser112→Ile and Tyr114→Cys [156]. CHO-K1 cells were transfected with the TTR variants

and incubated in the absence or presence of 20 μM EGCG for 36 h. Cell-culture media were analyzed by SDS-PAGE and Western blotting. Except for TTR variants Ala25→Thr, Ser112→Ile, and Tyr114→Cys, levels of TTR oligomers were increased while the level of corresponding monomers decreased depending on EGCG dose [156]. However, results obtained using SDS-PAGE and Western blotting should be interpreted with caution because of artifactual effects of SDS interactions with amyloidogenic proteins, as discussed above.

CLR01: Inhibition of TTR aggregation by CLR01 was examined using wild-type TTR, which was incubated at a concentration of 7.2 μM under aggregation-inducing conditions – 10 mM sodium acetate, pH 4.4, at 37 °C in the absence or presence of CLR01 [69]. The aggregation was assessed using turbidity at $\lambda = 340 \text{ nm}$ and EM. CLR01 inhibited the aggregation of TTR completely at a TTR:CLR01 concentration ratio 1:1 and partially at a 10:1 ratio. TTR alone formed abundant worm-like fibrils, whereas in the presence of CLR01, only amorphous structures were observed [69]. Surprisingly, though inhibition of TTR fibrillogenesis was achieved by equimolar concentration of CLR01, measurement of cytotoxicity in differentiated PC-12 cells showed that high concentration of CLR01 was required for inhibition. One μM oligomerized TTR caused ~20% reduction of cell death, measured using the MTT release assay. CLR01 inhibited this toxic effect with $IC_{50} = 54 \pm 19 \mu\text{M}$, suggesting that inhibition of toxicity occurred through a different mechanism from inhibition of fibrillogenesis [69]. Deciphering these mechanistic differences will require further studies.

Effects on TTR toxicity *in vivo*

EGCG: A single-center observational study conducted in patients with familial amyloidotic TTR cardiomyopathy with an intake of 500–700 mg EGCG per day for 12 months, reported either no increase or a decrease in the left ventricular myocardial mass [157]. Left ventricular function is positively correlated with better prognosis in cardiac amyloidosis [158]. Though this study was very preliminary, without a control cohort, and the increase in left ventricular mass varied

[159], the authors also found an increase in the mean mitral annular systolic velocity, a restorative effect, and a decrease in the total and low-density lipoprotein-associated cholesterol levels with no serious adverse events, suggesting that treatment with EGCG might be beneficial in these patients [157].

Six weeks of 100-mg/kg/day oral treatment with EGCG was examined in a mouse model of FAP [160]. These mice express human TTR Val30→Met on a TTR-null background and are heterozygous for heat-shock transcription factor 1 [161]. Mice were treated at a pre-amyloid deposition age resulting in a decrease of TTR deposition by ~50%, compared to untreated control mice, along the gastrointestinal tract and the peripheral nervous system [160]. Disease-associated biomarkers of endoplasmic reticulum stress, protein oxidation, and apoptosis also were reduced. Treatment of older mice at a post-amyloid deposition stage produced a decrease in amyloid deposits and the associated biomarkers [160].

CLR01: Treatment of the same FAP mouse model [161] at the pre-amyloid deposition stage with CLR01 administered subcutaneously via osmotic mini-pumps resulted in a decrease in TTR deposition and the associated endoplasmic reticulum stress, protein oxidation, and apoptosis biomarkers, compared to vehicle-treated mice, similarly to the effects found with EGCG [162]. Taking into account the different routes of administration and different doses used in the two studies, the compounds appear to have similar activity in this model [162].

Effects on IAPP aggregation and toxicity *in vitro*

IAPP (also called amylin) is a 37-residue polypeptide associated with islet amyloid deposits in type-2 diabetes mellitus [163-165]. IAPP readily self-assembles to form amyloid fibrils. IAPP fibrils, intermediate assemblies in the fibrillization process, or both, are toxic to β -cells, suggesting that IAPP fibrillization and islet amyloid formation contribute to type-2 diabetes pathogenesis [166-170].

EGCG: In efforts to interfere with IAPP fibrillization, multiple groups have reported

IAPP amyloid formation inhibitors, including EGCG [171-175]. Meng *et al.* measured the IAPP fibrillization rate in the absence or presence of EGCG using ThT-binding assays [56], where a typical sigmoidal curve, with an unusually long lag phase for IAPP – ~20 h, was observed *in vitro* in the absence of EGCG. When equimolar EGCG concentration was added, ThT fluorescence did not increase. The final ThT fluorescence intensity was reduced by 94% and 80% with 2- and 5-fold excess IAPP, respectively [56]. EM images of samples of IAPP alone showed abundant fibrils, whereas few fibrils were observed in the presence of equimolar EGCG [56]. Similarly to the experiments examining the seeding potential of EGCG-treated A β and α -synuclein assemblies (discussed above), Meng *et al.* investigated the fibril-seeding ability of EGCG-treated IAPP assemblies and found that they did not seed IAPP fibril formation [56]. EGCG also disaggregated and converted IAPP fibrils to smaller aggregates with a lower tendency to self-assemble [56].

In cytotoxicity experiments, the effects of equimolar EGCG and IAPP (30 μ M each) mixtures on rat INS-1 cells were compared [56]. Cells incubated with 30 μ M human IAPP alone for 5 h showed reduced viability relative to untreated control cells determined by alamar-blue assay. EGCG addition reduced cytotoxicity and increased the proportion of viable cells.

Engel *et al.* used a uniquely sensitive and spatially selective method called vibrational sum-frequency generation spectroscopy to probe the effects of EGCG on IAPP fibrillization at water-phospholipid interfaces [176]. Previously, Yan and colleagues used this approach to study changes in the secondary structure of IAPP at air-water or water-lipid interfaces [177, 178]. Vibrational sum-frequency generation is an optical technique that uses one visible laser beam and one infrared laser beam, which interact with molecules of analytes at interfaces, generating a third beam at the sum-frequency of the visible and infrared beams [177, 178]. In addition to vibrational sum-frequency generation, AFM combined with the Langmuir-Blodgett technique was used to investigate the morphology of aggregating species at the phospholipid interfaces [176]. The study found that EGCG

inhibited IAPP fibrillization at phospholipid interfaces considerably less effectively than in bulk solution [176]. Under the conditions used, EGCG did not disaggregate existing fibril β -sheets, in strong contrast with the efficient inhibition of fibril formation and disruption of pre-existing fibrils in bulk solution.

¹⁹F NMR and transmission electron microscopy experiments for measuring fibrillization have been used recently to examine IAPP amyloid assembly pathways and EGCG interactions by measuring monomer consumption [179]. In these experiments, a ¹⁹F-labeled IAPP (IAPP-tfmF23), in which Phe23 was substituted with 4-trifluoromethyl-Phe, was used. The rationale behind this study was that Phe23 is solvent-exposed in the unstructured monomeric peptide, but becomes buried when IAPP undergoes aggregation [179]. Therefore, the ¹⁹F chemical shifts and line widths corresponding to trifluoromethyl groups would change significantly as IAPP's secondary structure and oligomerization state change [179]. The data suggested that monomeric IAPP was converted into large, mostly β -sheet-containing species without significant accumulation of non-fibrillar intermediates during the aggregation process [179]. The aggregation kinetics of IAPP-tfmF23 then was monitored in the absence or presence of 0.2, 0.5, 1.0, or 5.0 molar equivalents of EGCG [179]. ¹⁹F measurements showed that EGCG inhibited IAPP fibrillization dose-dependently.

CLR01: Based on its mechanism of action, CLR01 would not be expected to inhibit IAPP aggregation or toxicity efficiently. CLR01 inhibits the electrostatic and hydrophobic interactions mediating amyloidogenic protein assembly by binding predominantly to Lys and to a lesser extent, to Arg residues. In IAPP there are one Lys and one Arg residues, in positions 1 and 11, respectively. Because the predominant putative binding site at Lys1 is far from the amyloidogenic sequences in IAPP, the expectation was that CLR01 would be a weak inhibitor of the peptide's aggregation and toxicity. Surprisingly, CLR01 was found to inhibit IAPP aggregation at 1:10 concentration ratio, respectively, using ThT fluorescence and EM [69]. Investigation of the role of the binding of CLR01 to the Lys and Arg residues

in IAPP suggests that binding of CLR01 to Lys1 is not crucial for the observed inhibitory effect [180]. Interestingly, inhibition of IAPP-induced toxicity in rat insulinoma (RIN5fm) cells showed that CLR01 inhibited IAPP-induced toxicity yet a large excess, ~600-fold, was required for the inhibition [69], suggesting that the non-fibrillar structures observed by EM in the presence of CLR01 were toxic at low CLR01:IAPP concentration ratio, whereas similar structures formed in the presence of large excess of CLR01 were non-toxic, although the morphology of the latter, as observed by EM was indistinguishable from that of the former.

Effects on calcitonin aggregation and toxicity *in vitro*

Calcitonin is a 32-residue polypeptide hormone secreted from the thyroid gland in response to high serum $[Ca^{2+}]$ to exert hypocalcemic effects and reduce bone resorption by osteoclasts [181–185]. Calcitonin's tendency to form amyloid fibrils in aqueous solutions limits its therapeutic applications in bone disorders such as osteoporosis.

EGCG: Studies assessing the effects of EGCG on calcitonin aggregation and fibrillization have been rather limited. Huang *et al.* [186] investigated high-resolution structures of human calcitonin at 0.3 mM and 1 mM and documented the EGCG–calcitonin interactions using one- and two-dimensional NMR spectroscopy. Based on the NMR data, the authors suggested that EGCG efficiently inhibited calcitonin fibril formation by preventing the initial peptide assembly. In addition, the authors performed EM experiments, in which 100 μ M calcitonin was incubated in the absence or presence of 500 μ M EGCG at pH 7.4 for 36 h. In the absence of EGCG, calcitonin aggregated into dense networks of amyloid fibrils, which did not form in the presence of EGCG. Hydrogen–deuterium exchange and NMR experiments indicated a much faster exchange of amide protons in the presence of EGCG than in its absence, supporting the interpretation that calcitonin was almost entirely unstructured in the presence of EGCG and formation of calcitonin oligomers was prevented. Heteronuclear

$^1H/^{15}N$ NMR experiments using calcitonin in the absence or presence of two molar equivalents of EGCG showed that EGCG bound to monomeric calcitonin in a non-specific manner. The data suggested that intermolecular aromatic interactions were important in inhibiting calcitonin fibrillogenesis [186].

CLR01: Aggregation of calcitonin in the absence or presence of CLR01 was examined using ThT fluorescence and EM. Similarly to the experiments described above with EGCG, in the presence of CLR01, calcitonin formed non-fibrillar amorphous structures, whereas abundant fibrils were observed in the absence of CLR01 [69]. ThT fluorescence experiments showed that β -sheet formation was inhibited completely by 1 molar equivalent of CLR01 and partially by 0.1 molar equivalent. The inhibitory effect was on the final ThT fluorescence level, which was ~50% of the level observed in the absence of CLR01, but not on the reaction rate [69]. The fact that CLR01 inhibited formation of β -sheet-rich calcitonin fibrils at substoichiometric concentrations was somewhat surprising because calcitonin has only one Lys and no Arg residues. Thus, apparently binding of CLR01 to Lys18 in one out of ten molecules of calcitonin recruited other molecules into assemblies with reduced β -sheet content relative to those formed by calcitonin alone. A detailed understanding of this phenomenon will require additional investigation.

The capability of CLR01 to inhibit calcitonin-induced cytotoxicity was assessed in differentiated PC-12 cells. Calcitonin was allowed to oligomerize by incubation of 1.5 mM at 37 °C for 24 h and then added to the cells in the absence or presence of increasing concentrations of CLR01 at a final concentration of 15 μ M. In the absence of CLR01, ~30% reduction of cell viability was measured using the MTT reduction assay. CLR01 inhibited this toxic effect with $IC_{50} = 28 \pm 4 \mu$ M [69].

Toxicity

EGCG: Green tea has a long history as one of the world's most popular beverages and is recognized widely as beneficial for health. However, as extracts and dietary supplements of green tea and purified EGCG have become more

popular, some case studies and some formal studies of their toxicity have shown that at high levels they often lead to hepatotoxicity [187].

Cell assays in bacteria and mouse lymphoma L5178Y tk⁺ have shown that up to 450 μ M EGCG were not genotoxic [188]. In animal experiments, a daily dose of up to ~1,000 mg/kg EGCG did not show teratogenicity in rats [189]. A topical dose of 1,860 mg/kg of EGCG resulted in minor dermal irritation in rats and guinea pigs but not in rabbits [190]. Administration of 100 mg EGCG to rabbit eye resulted in moderate-to-severe irritation and slight-to-moderate corneal opacity [190]. A single dose of 2,000 mg/kg was >80% lethal in rats [190] and single doses of 1,500 mg/kg or 1,200 mg/kg were 85% or 40% lethal in mice, respectively [191]. Two days of oral 750 mg/kg/day or 7 days of intraperitoneal 50 mg/kg/day were 50% and 67% toxic in mice, respectively [191,192], whereas 13 weeks of oral 500 mg/kg/day showed no toxicity in rats [190]. Toxicity in mice often was observed in the form of increased alanine-aminotransferase levels suggesting hepatotoxicity [192], and/or by oxidative stress [191]. Plasma EGCG concentrations in mice tend to be closer to levels reported in human studies compared to levels in rats, in which administration of similar doses results in higher plasma concentrations [188]. Chronic toxicity studies in beagle dogs, showed 67% lethality within 26 weeks of daily doses of 200–800 mg/kg Polyphenol E, a pure and standardized green tea extract, when the dogs fasted for 20 h prior to EGCG administration [193]. However, replication of the 200-mg/kg dose for 13 weeks did not result in any deaths compared to the previous study, in which 3/8 deaths were recorded at that dose [193]. Fasting dogs receiving a single dose of 120 mg/kg/day or 400 mg/kg/day died or were sacrificed due to their moribund condition starting at 7 weeks of a 13-week study [190]. Prior to death, dogs presented with occasional vomiting, frequent diarrhea, weight loss, and anorexia [190]. Autopsy of dogs from the 120-mg/kg and 400-mg/kg groups consistently showed thymus lymphoid atrophy and some kidney necrosis, liver necrosis, erosion of the stomach, and limited cases of hemolytic anemia and myocardial necrosis [190]. In

contrast, no adverse events were observed when non-fasting dogs were treated with 400 mg/kg in two doses [190].

The cytochrome P450 (CYP) superfamily of enzymes is involved in phase-1 metabolism and accounts for ~75% of metabolic reactions in the body. Thus, inhibition or activation of these enzymes may cause metabolic toxicity or drug–drug interactions [194]. EGCG showed inhibition of CYP19 (aromatase enzyme for estrogen biosynthesis) and activation of CYP3A subfamily enzymes and CYP2E1 when administered by intraperitoneal injection at 25 mg/kg/day for 7 days to female mice [192]. *In vitro*, 250 μ M EGCG inhibited CYP2B1 [195].

Acute human studies showed that single doses of 1,200 mg or 1,600 mg EGCG were safe and well tolerated, though nausea was reported [196,197]. Doses of 800 mg/day for 10 days also were safe and well tolerated, though an excretion saturation limit was reached [198]. Doses of ~800 mg/day for 4 weeks or 4 months in 1–3 doses per day showed mild adverse events, in the form of upset or achy stomach, constipation, nausea, headache and dizziness, and muscle pain. Abdominal discomfort and mild constipation also was recorded in the placebo group [199,200]. A case study reported a man drinking 6 cups of green tea (600–1200 mg EGCG) per day for 4 months who presented with jaundice and increased plasma alanine aminotransferase [201]. A 6-month chronic study of 800–1,600 mg/day administered in two doses determined the maximum tolerated dose, defined as the dose that causes 25% dose-limiting toxicity, to be 1,200 mg per day, with liver function abnormalities observed at the highest dose [202]. In addition, at least 25 reports have been made of hepatotoxicity following use of herbal supplements containing 10–29 mg/kg/day green tea extracts (Table 1 in Mazzanti et al. 2009) [203,204].

CLR01: In all the *in vitro* and *in vivo* experiments using CLR01, no signs of toxicity have been observed near the efficacious doses used. Mild toxicity was observed at 400 μ M, which is 1–3 orders of magnitude higher than concentrations needed for inhibition of the toxicity of different proteins in culture in differentiated PC-12 cells or primary neurons [69,107]. In a zebra fish model of α -synuclein

toxicity treated with up to 10 μ M CLR01 dissolved in the water environment, only improvement in pathology and no signs of toxicity were seen [72]. Mouse brain slices treated with up to 2 μ M CLR01 did not show changes in levels of long-term potentiation [71]. Importantly, mice treated with up to 1.2 mg/kg/day subcutaneously or with 10 μ M CLR01 intracerebroventricularly did not show any signs of toxicity, measured by weight loss, changes in activity, or morbidity ([71] and unpublished results). In a recent study, the toxicity of CLR01 was evaluated 24 hours following a single dose of 10 or 100 mg/kg, or after administration of 3 or 10 mg/kg for 30 days (intraperitoneal administration in both cases) [205]. Behavioral monitoring revealed signs of distress in the single, 100-mg/kg dose group, but not in the 10-mg/kg group, ~30 min following administration, which disappeared by 2 h following administration. No mortality was recorded. In the 30-day study, no signs of discomfort were observed in either group [205]. Serum chemical analysis of mice from both studies showed no differences in the low-dose groups compared to controls. In contrast, mice in the single 100-mg/kg dose group showed signs of acute hepatotoxicity [205]. Histological findings in the heart, lung, kidney, and brain of all the treated mice were indistinguishable from vehicle-treated mice, whereas liver injury was observed in the 100-mg/kg dose group [205].

In vitro studies of CLR01 inhibition of five major CYP enzymes showed IC_{50} values of 1.5 μ M for 2C19, 1.7 μ M for 3A4, 2.2 μ M for 2C9, 3.6 μ M for 2D6, and >20 μ M for 1A2 [71]. Inhibitory potencies are thought to have an increased risk for drug–drug interaction complications if the IC_{50} is <1 μ M [206], which was not found for CLR01 with any of the 5 isoforms studied. *In vitro* studies of CLR01 activation of the CYP system found minimal activation up to 50 μ M [71].

Pharmacokinetics

EGCG: In a meta-analysis of seven studies of EGCG pharmacokinetics, following consumption of an average cup of green tea (containing 112 mg EGCG, 51 mg EGC,

and 15 mg of EC), the maximum plasma concentration of total free and sulfated or glucuronidated conjugates were 125 nM EGCG, 181 nM EGC and 76 nM EC [207], indicating a higher bioavailability of EGC relative to EGCG even at lower administered doses [207–209]. Oral bioavailability of EGCG can be increased by administration to fasting people [197], albeit, with an increased risk of toxicity as suggested by the dog studies discussed above. Catechins appear in plasma after ~30 min and reach maximum levels between 1–3 h post-ingestion in a monophasic manner suggesting that the key site of absorption is the small intestine [207,208,210]. EGCG is found at very low levels, if at all, in urine [207,211,212] and interconversion or de-gallation from EGCG to EGC is very limited [211].

Conflicting results have been published regarding whether EGCG is mainly in an unconjugated or conjugated, i.e., sulfated or glucuronidated, form in plasma. Thus, one study reported 92% unconjugated EGCG [194], whereas another found 72% of EGCG to be conjugated, mostly in a sulfated form [212].

Methylated catechins, most likely by COMT [213] also have been detected in the plasma [214]. Interestingly, as mentioned above, EGCG is a COMT inhibitor [144]. The beneficial or detrimental significance of EGCG methylation has been debated. Methyl capping of other flavones has been shown to increase metabolic stability, and by preventing glucuronidation and sulfation, to improve bioavailability [215]. In addition, an increased anti-allergic effect has been reported for methylated EGCG and other catechins relative to the unmethylated forms [216]. On the other hand, other studies have reported a decrease in the antioxidant activity of methylated catechins [217].

In combination-therapy experiments, 8 mg/kg/day of fish oil added to 12.5 mg/kg/day EGCG given to a transgenic mouse model of Alzheimer's disease increased the oral bioavailability and brain penetration of EGCG up to levels similar to those obtained upon administration of 62.5 mg/kg/day of EGCG alone [218]. Nanolipid formulations of EGCG, in which EGCG is co-solubilized with a lipid carrier to form 30–80 nm diameter, non-micellar nanoparticle complexes resulted in a 2.4-fold

increase in bioavailability of free EGCG in rats administered 100 mg/kg EGCG by oral gavage [52].

CLR01: Initial studies of CLR01 have shown little, if any biotransformation. *In vitro* incubation of CLR01 with mouse or human, liver microsomes or plasma preparations for one hour showed 100% stability [71]. The initial plasma concentration measured following an intravenous injection of 1 mg/kg CLR01 to mice was 10–11 μM and the half-life was ~ 2.5 h in mice [205]. In blood–brain-barrier studies, one hour following intravenous injection, brain levels of ^3H -CLR01, measured by scintillation counting, were $\sim 2\%$ of the levels found in the blood [71].

Mechanisms of action

EGCG: Metals and oxygen promote EGCG auto-oxidation, generating superoxide and quinones [219]. Superoxide formation propagates EGCG oxidation and the resultant quinones cross-link and form polymeric species, which potentially react with sulfhydryl (SH) and/or free amino (NH_2) groups of proteins, forming covalent conjugates [220]. An important recent study by Palhano *et al.* examined the mechanism(s) whereby EGCG remodels mature amyloid fibrils using $\text{A}\beta(1-40)$, IAPP(8–24), or the yeast prion fragment Sup35NM(7–16) [78]. They observed that $\text{A}\beta(1-40)$ solutions or fibrils after centrifugation changed from clear to brown color when incubated with EGCG for 24 h, suggesting EGCG auto-oxidation. Under the experimental conditions used – 30 μM EGCG in phosphate buffer, pH 7.4, at 25°C – EGCG was oxidized fully after 6 h with a half-life of 2.7 h, which was increased to 5.5 h in the presence of superoxide dismutase 1. Addition of superoxide dismutase 1 was found to delay the decrease in ThT fluorescence caused by EGCG remodeling of $\text{A}\beta(1-40)$ fibrils following a centrifugation–wash protocol and reduced the amount of NBT-stained protein–EGCG conjugates, indicating that EGCG auto-oxidation was important for amyloid remodeling [78]. The centrifugation–wash protocol was used to remove unbound, soluble EGCG. In this protocol, aliquots of fibrillar amyloid proteins incubated in the absence or presence of EGCG were centrifuged

to obtain a pellet. The protein pellet was washed and centrifuged again before suspension and further testing by ThT or other assays. Based on these observations, the authors concluded that covalent modifications of amyloid fibrils by EGCG promoted their cross-linking and likely prevented fibril fragmentation or dissociation into toxic oligomeric assemblies, but reduction in toxicity was independent of formation of covalent conjugates between EGCG and the protein [78]. They also established that EGCG oxidation products reacted with free amines in the Lys side-chains of $\text{A}\beta(1-40)$ through Schiff base formation; however, EGCG could still bind $\text{A}\beta(1-40)$ fibrils likely near the hydrophobic ThT-binding sites when free NH_2 groups were acetylated [78]. The role of auto-oxidation of catechol-containing flavonoids in inhibiting $\text{A}\beta$ fibrillization was confirmed recently by Sato *et al.*, who showed that *o*-quinone forms of catechol-containing flavonoids (e.g., (+)-taxifolin) prevented $\text{A}\beta_{42}$ fibril formation by binding covalently to Lys16 and/or Lys28 [221].

Palhano *et al.* reported also that addition of EGCG to pre-formed $\text{A}\beta(1-40)$ fibrils caused an initial sudden decrease followed by gradual decrease in ThT fluorescence measurements with time [78]. This observation led them to hypothesize that EGCG might compete with ThT for amyloid-binding sites or might quench the ThT fluorescence signal rather than remodel fibrillar structures. To test this hypothesis, the authors used the centrifugation–wash protocol mentioned above [78], ensuring removal of excess unbound EGCG for subsequent ThT binding assays. EGCG or oxidized EGCG were found to bind amyloid fibrils and interfere with ThT-binding-associated fluorescence early during the assay. In contrast, the reduced ThT fluorescence after 24-h incubation of $\text{A}\beta(1-40)$ fibrils with EGCG was due to EGCG-mediated altered amyloid fibril structures rather than disaggregation of amyloid fibrils or interference with ThT fluorescence [78]. To assess the effect of non-covalent EGCG remodeling, acetylated IAPP(8–24), which does not contain Lys or Cys, was used. Fibrils of this IAPP analogue treated with EGCG and washed with phosphate-buffered saline alone in the centrifugation–wash protocol showed higher

ThT fluorescence than fibril preparations washed with phosphate-buffered saline and Tween-20. This experiment indicated that some of the non-covalent EGCG-mediated IAPP remodeling was reversed by Tween-20 treatment, demonstrating that the detergent could displace a fraction of non-covalently bound, oxidized EGCG from the fibrils [78]. Finally, when the hydrophilic amyloid fibrils derived from acetylated Sup35NM were treated with EGCG, a negligible effect on ThT binding was observed, suggesting no EGCG-mediated amyloid remodeling, likely because hydrophobic EGCG interactions were impaired. When the hydrophobicity of acetylated Sup35NM was increased by a Tyr \rightarrow Phe substitution, a decrease in ThT fluorescence was observed after 24 h similar to $\text{A}\beta$ and IAPP peptides. The authors concluded that oxidized EGCG could remodel amyloid fibrils sufficiently only using hydrophobic interactions [78].

CLR01: CLR01 binds selectively to Lys residues and with lower affinity to Arg residues. The selectivity for these residues is achieved by the combination of electrostatic and hydrophobic interactions, as explained in the introduction. Strong evidence for this mode of binding to different amyloidogenic proteins comes from observation of a large shift of proton NMR resonances in the side chains of Lys and Arg when they are enclosed in CLR01's cavity (Figure 2) [69,180]. This binding mode also has been observed crystallographically [70]. The combination of hydrophobic and electrostatic interactions, which in the absence of the inhibitor mediate early assembly of amyloidogenic proteins, are blocked by CLR01 binding sufficiently to modulate the self-assembly process into non-toxic and non-fibrillogenic structures, which are amenable to clearance by the 26S UPS [72] and plausibly other clearance mechanisms.

Importantly, the binding of CLR01 is highly labile and the affinity of the compound for Lys is in the micromolar range [68,69], although higher affinity may be achieved in certain cases, depending on the number of Lys residues in the target protein and the specific sequence of the protein [134]. The high lability and moderate affinity prevent

CLR01 binding to structured proteins from interfering with normal physiological function. Thus, despite its ability to bind exposed Lys residues on virtually any protein, as discussed above, the compound was found to have weak iatrogenic toxicity in cell culture and animal models. Direct demonstration of the specificity of CLR01's mechanism for modulation of aberrant assembly of amyloid proteins was provided by examining its effect on a regulated physiologic assembly process – that of tubulin polymerization. The CLR01 concentration required for modulation of tubulin polymerization was ~50-times higher than that needed for inhibition of amyloidogenic proteins [205].

Conclusion

EGCG and CLR01 are not specific to one target, but rather bind multiple amyloidogenic proteins and remodel the aberrant assembly process of amyloidogenic proteins. Although CLR01 binds directly to monomers, whereas EGCG binds to oligomers and larger assemblies, neither compound prevents monomer self-association. Rather, both compounds modulate the assembly process into formation of non-toxic structures that do not go on to form amyloid fibrils and do not seed fibril formation. Deciphering the details that distinguish these structures from transient toxic oligomers of amyloid proteins will require future investigation.

Although both CLR01 and EGCG bind to Lys residues, it is important to note the distinct nature of the binding. In contrast to the labile binding of CLR01, EGCG binds covalently to

the amino groups of Lys side chains due to its self-oxidation into quinone derivatives that subsequently form a Schiff base with the Lys side chain. Modification of the Lys amino group by reactive carbonyls has been reported in certain cases to cause toxicity (e.g., by 4-hydroxy-2-nonenal [222-224]) and may contribute to the toxicity of EGCG. At the same time, the covalent binding of EGCG makes it substantially more efficient at dissociating existing fibrils of various amyloidogenic proteins compared to CLR01, as discussed above.

Therapeutically, both CLR01 and EGCG treatment result in reduction of pathological protein deposition, such as A β and α -synuclein, in animal models of disease. Additionally, the effect of both extends to secondary disease features such as levels of microglia, proteasome and mitochondrial activity, and protein oxidation. As the study of EGCG has been ongoing for far longer and by more investigators, much more data exist about its functional *in vivo* effects. EGCG has been shown to improve memory in a mouse model of AD, climbing ability in a drosophila model of PD, and posture and movement in a mouse model of PD. CLR01's functional effects have thus far only been tested in a zebrafish model of PD with results of improved survival and postural phenotype.

EGCG has a better oral bioavailability profile than CLR01. Both compounds have relatively low toxicity, though the auto-oxidation and covalent cross-linking of EGCG to proteins and potentially other biomolecules may be a reason for concern. Nonetheless, the readily available nature of EGCG and its general protective anti-oxidant and anti-carcinogen effects increase EGCG's

short-term therapeutic use potential. CLR01 is the first example of a process-specific modulator of amyloid protein aggregation selected based on rational considerations. The high efficacy and low toxicity of this compound provide proof of concept for the idea that modulators binding with moderate affinity may be the drugs of the future for amyloidoses. Development of process-specific drugs with effects on multiple aspects of disease in many amyloidoses is an important goal of future research.

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Conflict of interest

G.B. is an inventor of a patent application entitled Molecular Tweezers for the Treatment of Amyloid-Related Diseases. International Patent Application No. PCT/US2010/026419, USA Patent Application No. 13/203,962, European Patent Application 10 708 075.6. G.B. is a founder and a shareholder of Clear Therapeutics, Inc.

References

- [1] Rahimi F, Shanmugam A, Bitan G, Structure–function relationships of pre-fibrillar protein assemblies in Alzheimer's disease and related disorders, *Curr. Alzheimer Res.*, 2008, 5, 319-341
- [2] Fändrich M., Oligomeric intermediates in amyloid formation: structure determination and mechanisms of toxicity, *J. Mol. Biol.*, 2012, 421, 427-440
- [3] Serpell L.C., Alzheimer's amyloid fibrils: structure and assembly, *Biochim. Biophys. Acta*, 2000, 1502, 16-30
- [4] Vinters H.V., Tung S., Solis O.E., Pathologic Lesions in Alzheimer disease and Other Neurodegenerative Diseases—Cellular and Molecular Components, In: Rahimi F, Bitan G. (Eds.), *Non-fibrillar Amyloidogenic Protein Assemblies—Common Cytotoxins Underlying Degenerative Diseases*, Springer, 2012
- [5] Hardy J.A., Higgins G.A., Alzheimer's disease: the amyloid cascade hypothesis, *Science*, 1992, 256, 184-185
- [6] Soto C., Estrada L., Amyloid inhibitors and β -sheet breakers, *Subcell. Biochem.*, 2005, 38, 351-364
- [7] Necula M., Kaye R., Milton S., Glabe C.G., Small molecule inhibitors of aggregation indicate that amyloid β oligomerization and fibrillization pathways are independent and distinct, *J. Biol. Chem.*, 2007, 282, 10311-10324

- [8] Ladiwala A.R., Dordick J.S., Tessier P.M., Aromatic small molecules remodel toxic soluble oligomers of amyloid β through three independent pathways, *J. Biol. Chem.*, 2011, 286, 3209-3218
- [9] Liu T., Bitan G., Modulating self-assembly of amyloidogenic proteins as a therapeutic approach for neurodegenerative diseases: strategies and mechanisms, *ChemMedChem*, 2012, 7, 359-374
- [10] Jan A., Adolphsson O., Allaman I., Buccarello A.L., Magistretti P.J., Pfeifer A., et al., A β 42 neurotoxicity is mediated by ongoing nucleated polymerization process rather than by discrete A β 42 species, *J. Biol. Chem.*, 2011, 286, 8585-8596
- [11] Eikelenboom P., Veerhuis R., Familian A., Hoozemans J.J., van Gool W.A., Rozemuller A.J., Neuroinflammation in plaque and vascular β -amyloid disorders: clinical and therapeutic implications, *Neurodegener. Dis.*, 2008, 5, 190-193
- [12] Esteras-Chopo A., Pastor M.T., Serrano L., Lopez de la Paz M., New strategy for the generation of specific D-peptide amyloid inhibitors, *J. Mol. Biol.*, 2008, 377, 1372-1381
- [13] Fradinger E.A., Monien B.H., Urbanc B., Lomakin A., Tan M., Li H., et al., C-terminal peptides coassemble into A β 42 oligomers and protect neurons against A β 42-induced neurotoxicity, *Proc. Natl. Acad. Sci. USA*, 2008, 105, 14175-14180
- [14] Doig A.J., Peptide inhibitors of β -amyloid aggregation, *Curr. Opin. Drug Discov. Devel.*, 2007, 10, 533-539
- [15] Cheng P.N., Liu C., Zhao M., Eisenberg D., Nowick J.S., Amyloid β -sheet mimics that antagonize protein aggregation and reduce amyloid toxicity, *Nat. Chem.*, 2012, 4, 927-933
- [16] van Groen T., Wiesehan K., Funke S.A., Kadish I., Nagel-Steger L., Willbold D., Reduction of Alzheimer's disease amyloid plaque load in transgenic mice by D3, A D-enantiomeric peptide identified by mirror image phage display, *ChemMedChem*, 2008, 3, 1848-1852
- [17] Belluti F., Rampa A., Gobbi S., Bisi A., Small-molecule inhibitors/modulators of amyloid- β peptide aggregation and toxicity for the treatment of Alzheimer's disease—A patent review (2010–2012), *Expert Opin. Ther. Pat.*, 2013,
- [18] Re F., Airoidi C., Zona C., Masserini M., La Ferla B., Quattrocchi N., et al., β amyloid aggregation inhibitors: small molecules as candidate drugs for therapy of Alzheimer's disease, *Curr. Med. Chem.*, 2010, 17, 2990-3006
- [19] Roberts B.E., Shorter J., Escaping amyloid fate, *Nat. Struct. Mol. Biol.*, 2008, 15, 544-546
- [20] Wang W., Protein aggregation and its inhibition in biopharmaceutics, *Int. J. Pharm.*, 2005, 289, 1-30
- [21] Bartolini M., Andrisano V., Strategies for the inhibition of protein aggregation in human diseases, *ChemBioChem.*, 2010, 11, 1018-1035
- [22] Bose M., Gestwicki J.E., Devasthali V., Crabtree G.R., Graef I.A., 'Nature-inspired' drug-protein complexes as inhibitors of A β aggregation, *Biochem. Soc. Trans.*, 2005, 33, 543-547
- [23] Cole G.M., Teter B., Frautschy S.A., Neuroprotective effects of curcumin, *Adv. Exp. Med. Biol.*, 2007, 595, 197-212
- [24] Bastianetto S., Krantic S., Quirion R., Polyphenols as potential inhibitors of amyloid aggregation and toxicity: possible significance to Alzheimer's disease, *Mini Rev. Med. Chem.*, 2008, 8, 429-435
- [25] Porat Y., Abramowitz A., Gazit E., Inhibition of amyloid fibril formation by polyphenols: structural similarity and aromatic interactions as a common inhibition mechanism, *Chem. Biol. Drug Des.*, 2006, 67, 27-37
- [26] Mandel S.A., Amit T., Weinreb O., Reznichenko L., Youdim M.B., Simultaneous manipulation of multiple brain targets by green tea catechins: a potential neuroprotective strategy for Alzheimer and Parkinson diseases, *CNS Neurosci. Ther.*, 2008, 14, 352-365
- [27] Albani D., Polito L., Signorini A., Forloni G., Neuroprotective properties of resveratrol in different neurodegenerative disorders, *BioFactors*, 2010, 36, 370-376
- [28] Cheng B., Liu X., Gong H., Huang L., Chen H., Zhang X., et al., Coffee components inhibit amyloid formation of human islet amyloid polypeptide in vitro: possible link between coffee consumption and diabetes mellitus, *J. Agric. Food Chem.*, 2011, 59, 13147-13155
- [29] Huang Y., Jin M., Pi R., Zhang J., Chen M., Ouyang Y., et al., Protective effects of caffeic acid and caffeic acid phenethyl ester against acrolein-induced neurotoxicity in HT22 mouse hippocampal cells, *Neurosci. Lett.*, 2013, 535, 146-151
- [30] Mohamed T., Yeung J.C., Vasefi M.S., Beazely M.A., Rao P.P., Development and evaluation of multifunctional agents for potential treatment of Alzheimer's disease: application to a pyrimidine-2,4-diamine template, *Bioorg. Med. Chem. Lett.*, 2012, 22, 4707-4712
- [31] Mao F., Huang L., Luo Z., Liu A., Lu C., Xie Z., et al., O-Hydroxyl- or o-amino benzylamine-tacrine hybrids: multifunctional biometals chelators, antioxidants, and inhibitors of cholinesterase activity and amyloid- β aggregation, *Bioorg. Med. Chem.*, 2012, 20, 5884-5892
- [32] Pi R., Mao X., Chao X., Cheng Z., Liu M., Duan X., et al., Tacrine-6-ferulic acid, a novel multifunctional dimer, inhibits amyloid- β -mediated Alzheimer's disease-associated pathogenesis in vitro and in vivo, *PLoS One*, 2012, 7, e31921
- [33] Bag S., Ghosh S., Tulsan R., Sood A., Zhou W., Schifone C., et al., Design, synthesis and biological activity of multifunctional α,β -unsaturated carbonyl scaffolds for Alzheimer's disease, *Bioorg. Med. Chem. Lett.*, 2013,
- [34] Nunes A., Marques S.M., Quintanova C., Silva D.F., Cardoso S.M., Chaves S., et al., Multifunctional iron-chelators with protective roles against neurodegenerative diseases, *Dalton Trans.*, 2013,
- [35] Telpoukhovskaia M.A., Patrick B.O., Rodriguez-Rodriguez C., Orvig C., Exploring the multifunctionality of thioflavin- and deferiprone-based molecules as acetylcholinesterase inhibitors for potential application in Alzheimer's disease, *Mol. Biosyst.*, 2013, 9, 792-805
- [36] Török B., Sood A., Bag S., Tulsan R., Ghosh S., Borkin D., et al., Diaryl hydrazones as multifunctional inhibitors of amyloid self-assembly, *Biochemistry*, 2013, 52, 1137-1148
- [37] Granzotto A., Zatta P., Resveratrol acts not through anti-aggregative pathways but mainly via its scavenging properties against A β and A β -metal complexes toxicity, *PLoS One*, 2011, 6, e21565
- [38] Stratton S.P., Bangert J.L., Alberts D.S., Dorr R.T., Dermal toxicity of topical (-)epigallocatechin-3-gallate in BALB/c and SKH1 mice, *Cancer Lett.*, 2000, 158, 47-52

- [39] Miyamoto Y., Haylor J.L., El Nahas A.M., Cellular toxicity of catechin analogues containing gallate in opossum kidney proximal tubular (OK) cells, *J. Toxicol. Sci.*, 2004, 29, 47-52
- [40] Mak J.C., Potential role of green tea catechins in various disease therapies: progress and promise, *Clin. Exp. Pharmacol. Physiol.*, 2012, 39, 265-273
- [41] Balentine D.A., Wiseman S.A., Bouwens L.C., The chemistry of tea flavonoids, *Crit. Rev. Food Sci. Nutr.*, 1997, 37, 693-704
- [42] Khan N., Afaq F., Saleem M., Ahmad N., Mukhtar H., Targeting multiple signaling pathways by green tea polyphenol (-)-epigallocatechin-3-gallate, *Cancer Res.*, 2006, 66, 2500-2505
- [43] Ehrnhoefer D.E., Duennwald M., Markovic P., Wacker J.L., Engemann S., Roark M., et al., Green tea (-)-epigallocatechin-gallate modulates early events in huntingtin misfolding and reduces toxicity in Huntington's disease models, *Hum. Mol. Genet.*, 2006, 15, 2743-2751
- [44] Barranco Quintana J.L., Allam M.F., Del Castillo A.S., Navajas R.F., Parkinson's disease and tea: a quantitative review, *J. Am. Coll. Nutr.*, 2009, 28, 1-6
- [45] Hellenbrand W., Seidler A., Boeing H., Robra B.P., Vieregge P., Nischan P., et al., Diet and Parkinson's disease. I: A possible role for the past intake of specific foods and food groups. Results from a self-administered food-frequency questionnaire in a case-control study, *Neurology*, 1996, 47, 636-643
- [46] Ng T.P., Feng L., Niti M., Kua E.H., Yap K.B., Tea consumption and cognitive impairment and decline in older Chinese adults, *Am. J. Clin. Nutr.*, 2008, 88, 224-231
- [47] Dragicvic N., Smith A., Lin X., Yuan F., Copes N., Delic V., et al., Green tea epigallocatechin-3-gallate (EGCG) and other flavonoids reduce Alzheimer's amyloid-induced mitochondrial dysfunction, *J. Alzheimers Dis.*, 2011, 26, 507-521
- [48] Fernandez J.W., Rezaei-Zadeh K., Obregon D., Tan J., EGCG functions through estrogen receptor-mediated activation of ADAM10 in the promotion of non-amyloidogenic processing of APP, *FEBS Lett.*, 2010, 584, 4259-4267
- [49] Lin C.L., Chen T.F., Chiu M.J., Way T.D., Lin J.K., Epigallocatechin gallate (EGCG) suppresses β -amyloid-induced neurotoxicity through inhibiting c-Abl/FE65 nuclear translocation and GSK3 β activation, *Neurobiol. Aging*, 2009, 30, 81-92
- [50] Mandel S.A., Amit T., Kalfon L., Reznichenko L., Weinreb O., Youdim M.B., Cell signaling pathways and iron chelation in the neurorestorative activity of green tea polyphenols: special reference to epigallocatechin gallate (EGCG), *J. Alzheimers Dis.*, 2008, 15, 211-222
- [51] Singh B.N., Shankar S., Srivastava R.K., Green tea catechin, epigallocatechin-3-gallate (EGCG): mechanisms, perspectives and clinical applications, *Biochem. Pharmacol.*, 2011, 82, 1807-1821
- [52] Smith A., Giunta B., Bickford P.C., Fountain M., Tan J., Shytle R.D., Nanolipidic particles improve the bioavailability and α -secretase inducing ability of epigallocatechin-3-gallate (EGCG) for the treatment of Alzheimer's disease, *Int. J. Pharm.*, 2010, 389, 207-212
- [53] Ruidavets J., Teissedre P., Ferrieres J., Carando S., Bougard G., Cabanis J., Catechin in the Mediterranean diet: vegetable, fruit or wine?, *Atherosclerosis*, 2000, 153, 107-117
- [54] Chyu K.Y., Babbidge S.M., Zhao X., Dandillaya R., Rietveld A.G., Yano J., et al., Differential effects of green tea-derived catechin on developing versus established atherosclerosis in apolipoprotein E-null mice, *Circulation*, 2004, 109, 2448-2453
- [55] Katiyar S., Elmets C.A., Katiyar S.K., Green tea and skin cancer: photoimmunology, angiogenesis and DNA repair, *J. Nutr. Biochem.*, 2007, 18, 287-296
- [56] Meng F., Abedini A., Plesner A., Verchere C.B., Raleigh D.P., The flavanol (-)-epigallocatechin 3-gallate inhibits amyloid formation by islet amyloid polypeptide, disaggregates amyloid fibrils, and protects cultured cells against IAPP-induced toxicity, *Biochemistry*, 2010, 49, 8127-8133
- [57] Ehrnhoefer D.E., Bieschke J., Boeddrich A., Herbst M., Masino L., Lurz R., et al., EGCG redirects amyloidogenic polypeptides into unstructured, off-pathway oligomers, *Nat. Struct. Mol. Biol.*, 2008, 15, 558-566
- [58] Bieschke J., Russ J., Friedrich R.P., Ehrnhoefer D.E., Wobst H., Neugebauer K., et al., EGCG remodels mature α -synuclein and amyloid- β fibrils and reduces cellular toxicity, *Proc. Natl. Acad. Sci. USA*, 2010, 107, 7710-7715
- [59] Masuda M., Suzuki N., Taniguchi S., Oikawa T., Nonaka T., Iwatsubo T., et al., Small molecule inhibitors of α -synuclein filament assembly, *Biochemistry*, 2006, 45, 6085-6094
- [60] Hauber I., Hohenberg H., Holstermann B., Hunstein W., Hauber J., The main green tea polyphenol epigallocatechin-3-gallate counteracts semen-mediated enhancement of HIV infection, *Proc. Natl. Acad. Sci. USA*, 2009, 106, 9033-9038
- [61] Popovych N., Brender J.R., Soong R., Vivekanandan S., Hartman K., Basur V., et al., Site specific interaction of the polyphenol EGCG with the SEVI amyloid precursor peptide PAP(248-286), *J. Phys. Chem. B*, 2012, 116, 3650-3658
- [62] Chandrashekar I.R., Adda C.G., MacRaild C.A., Anders R.F., Norton R.S., Inhibition by flavonoids of amyloid-like fibril formation by *Plasmodium falciparum* merozoite surface protein 2, *Biochemistry*, 2010, 49, 5899-5908
- [63] Chandrashekar I.R., Adda C.G., Macraild C.A., Anders R.F., Norton R.S., EGCG disaggregates amyloid-like fibrils formed by *Plasmodium falciparum* merozoite surface protein 2, *Arch. Biochem. Biophys.*, 2011, 513, 153-157
- [64] Rambold A.S., Miesbauer M., Olschewski D., Seidel R., Riemer C., Smale L., et al., Green tea extracts interfere with the stress-protective activity of PrP and the formation of PrP, *J. Neurochem.*, 2008, 107, 218-229
- [65] Attar A., Bitan G., Disrupting Self-Assembly and Toxicity of Amyloidogenic Protein Oligomers by "Molecular Tweezers"- from the Test Tube to Animal Models, *Curr. Pharm. Des.*, 2013, In press
- [66] Klärner F.G., Schrader T., Aromatic interactions by molecular tweezers and clips in chemical and biological systems, *Acc. Chem. Res.*, 2013, 46, 967-978

- [67] Fokkens M., Schrader T., Klärner F.G., A molecular tweezer for lysine and arginine, *J. Am. Chem. Soc.*, 2005, 127, 14415-14421
- [68] Talbiersky P., Bastkowski F., Klärner F.G., Schrader T., Molecular clip and tweezer introduce new mechanisms of enzyme inhibition, *J. Am. Chem. Soc.*, 2008, 130, 9824-9828
- [69] Sinha S., Lopes D.H., Du Z., Pang E.S., Shanmugam A., Lomakin A., et al., Lysine-specific molecular tweezers are broad-spectrum inhibitors of assembly and toxicity of amyloid proteins, *J. Am. Chem. Soc.*, 2011, 133, 16958-16969
- [70] Bier D., Rose R., Bravo-Rodriguez K., Bartel M., Ramirez-Anguita J.M., Dutt S., et al., Molecular tweezers modulate 14-3-3 protein-protein interactions, *Nat. Chem.*, 2013, 5, 234-239
- [71] Attar A., Ripoli C., Riccardi E., Maiti P., Li Puma D.D., Liu T., et al., Protection of primary neurons and mouse brain from Alzheimer's pathology by molecular tweezers, *Brain*, 2012, 135, 3735-3748
- [72] Prabhudesai S., Sinha S., Attar A., Kotagiri A., Fitzmaurice A.G., Lakshmanan R., et al., A novel "molecular tweezer" inhibitor of α -synuclein neurotoxicity in vitro and in vivo, *Neurotherapeutics*, 2012, 9, 464-476
- [73] Glenner G.G., Wong C.W., Alzheimer's disease: Initial report of the purification and characterization of a novel cerebrovascular amyloid protein, *Biochem. Biophys. Res. Commun.*, 1984, 120, 885-890
- [74] Masters C.L., Simms G., Weinman N.A., Multhaup G., McDonald B.L., Beyreuther K., Amyloid plaque core protein in Alzheimer disease and Down syndrome, *Proc. Natl. Acad. Sci. U.S.A.*, 1985, 82, 4245-4249
- [75] Hardy J., Selkoe D.J., The amyloid hypothesis of Alzheimer's disease: progress and problems on the road to therapeutics, *Science*, 2002, 297, 353-356.
- [76] Bastianetto S., Yao Z.X., Papadopoulos V., Quirion R., Neuroprotective effects of green and black teas and their catechin gallate esters against β -amyloid-induced toxicity, *Eur. J. Neurosci.*, 2006, 23, 55-64
- [77] LeVine H., 3rd, Quantification of β -sheet amyloid fibril structures with thioflavin T, *Methods Enzymol.*, 1999, 309, 274-284
- [78] Palhano F.L., Lee J., Grimster N.P., Kelly J.W., Toward the molecular mechanism(s) by which EGCG treatment remodels mature amyloid fibrils, *J. Am. Chem. Soc.*, 2013, 135, 7503-7510
- [79] Walsh D.M., Klyubin I., Fadeeva J.V., Cullen W.K., Anwyl R., Wolfe M.S., et al., Naturally secreted oligomers of amyloid β protein potently inhibit hippocampal long-term potentiation in vivo, *Nature*, 2002, 416, 535-539
- [80] Reed M.N., Hofmeister J.J., Jungbauer L., Welzel A.T., Yu C., Sherman M.A., et al., Cognitive effects of cell-derived and synthetically derived A β oligomers, *Neurobiol. Aging*, 2011, 32, 1784-1794
- [81] O'Nuallain B., Freir D.B., Nicoll A.J., Risse E., Ferguson N., Herron C.E., et al., Amyloid β -protein dimers rapidly form stable synaptotoxic protofibrils, *J. Neurosci.*, 2010, 30, 14411-14419
- [82] Kaye R., Head E., Thompson J.L., McIntire T.M., Milton S.C., Cotman C.W., et al., Common structure of soluble amyloid oligomers implies common mechanism of pathogenesis, *Science*, 2003, 300, 486-489
- [83] Paz M.A., Flückiger R., Boak A., Kagan H.M., Gallop P.M., Specific detection of quinoproteins by redox-cycling staining, *J. Biol. Chem.*, 1991, 266, 689-692
- [84] Lopez del Amo J.M., Fink U., Dasari M., Grelle G., Wanker E.E., Bieschke J., et al., Structural properties of EGCG-induced, nontoxic Alzheimer's disease A β oligomers, *J. Mol. Biol.*, 2012, 421, 517-524
- [85] Bitan G., Kirkitadze M.D., Lomakin A., Vollers S.S., Benedek G.B., Teplow D.B., Amyloid β -protein (A β) assembly: A β 40 and A β 42 oligomerize through distinct pathways, *Proc. Natl. Acad. Sci. USA*, 2003, 100, 330-335
- [86] Hoshi M., Sato M., Matsumoto S., Noguchi A., Yasutake K., Yoshida N., et al., Spherical aggregates of β -amyloid (amylospheroid) show high neurotoxicity and activate tau protein kinase I/glycogen synthase kinase-3 β , *Proc. Natl. Acad. Sci. USA*, 2003, 100, 6370-6375
- [87] Dahlgren K.N., Manelli A.M., Stine W.B., Jr., Baker L.K., Krafft G.A., LaDu M.J., Oligomeric and fibrillar species of amyloid- β peptides differentially affect neuronal viability, *J. Biol. Chem.*, 2002, 277, 32046-32053
- [88] Harper J.D., Lansbury P.T., Jr., Models of amyloid seeding in Alzheimer's disease and scrapie: mechanistic truths and physiological consequences of the time-dependent solubility of amyloid proteins, *Annu. Rev. Biochem.*, 1997, 66, 385-407
- [89] Petkova A.T., Ishii Y., Balbach J.J., Antzutkin O.N., Leapman R.D., Delaglio F., et al., A structural model for Alzheimer's β -amyloid fibrils based on experimental constraints from solid state NMR, *Proc. Natl. Acad. Sci. USA*, 2002, 99, 16742-16747
- [90] Petkova A.T., Yau W.M., Tycko R., Experimental constraints on quaternary structure in Alzheimer's β -amyloid fibrils, *Biochemistry*, 2006, 45, 498-512
- [91] Lazo N.D., Grant M.A., Condron M.C., Rigby A.C., Teplow D.B., On the nucleation of amyloid β -protein monomer folding, *Protein Sci.*, 2005, 14, 1581-1596
- [92] Yu L., Edalji R., Harlan J.E., Holzman T.F., Lopez A.P., Labkovsky B., et al., Structural characterization of a soluble amyloid β -peptide oligomer, *Biochemistry*, 2009, 48, 1870-1877
- [93] Wang S.H., Liu F.F., Dong X.Y., Sun Y., Thermodynamic analysis of the molecular interactions between amyloid β -peptide 42 and (-)-epigallocatechin-3-gallate, *J. Phys. Chem. B*, 2010, 114, 11576-11583
- [94] Wang S.H., Dong X.Y., Sun Y., Thermodynamic analysis of the molecular interactions between amyloid β -protein fragments and (-)-epigallocatechin-3-gallate, *J. Phys. Chem. B*, 2012, 116, 5803-5809
- [95] Hane F., Tran G., Attwood S.J., Leonenko Z., Cu²⁺ affects amyloid- β (1-42) aggregation by increasing peptide-peptide binding forces, *PLoS One*, 2013, 8, e59005
- [96] Solomonov I., Korkotian E., Born B., Feldman Y., Bitler A., Rahimi F., et al., Zn²⁺-A β 40 complexes form metastable quasi-spherical oligomers that are cytotoxic to cultured hippocampal neurons, *J. Biol. Chem.*, 2012, 287, 20555-20564
- [97] Mancino A.M., Hinds S.S., Kochi A., Lim M.H., Effects of cloquinol on metal-triggered amyloid- β aggregation revisited, *Inorg. Chem.*, 2009, 48, 9596-9598
- [98] Bush A.I., Masters C.L., Tanzi R.E., Copper, β -amyloid, and Alzheimer's disease: Tapping a sensitive connection, *Proc. Natl. Acad. Sci. USA*, 2003, 100, 11193-11194

- [99] Huang X., Moir R.D., Tanzi R.E., Bush A.I., Rogers J.T., Redox-active metals, oxidative stress, and Alzheimer's disease pathology, *Ann. N. Y. Acad. Sci.*, 2004, 1012, 153-163
- [100] Pirker K.F., Baratto M.C., Basosi R., Goodman B.A., Influence of pH on the speciation of copper(II) in reactions with the green tea polyphenols, epigallocatechin gallate and gallic acid, *J. Inorg. Biochem.*, 2012, 112, 10-16
- [101] Sun S.L., He G.Q., Yu H.N., Yang J.G., Borthakur D., Zhang L.C., et al., Free Zn²⁺ enhances inhibitory effects of EGCG on the growth of PC-3 cells, *Mol. Nutr. Food Res.*, 2008, 52, 465-471
- [102] Weinreb O., Amit T., Mandel S., Youdim M.B., Neuroprotective molecular mechanisms of (-)-epigallocatechin-3-gallate: a reflective outcome of its antioxidant, iron chelating and neurotogenic properties, *Genes Nutr.* 2009, 4, 283-296
- [103] Seeram N.P., Henning S.M., Niu Y., Lee R., Scheuller H.S., Heber D., Catechin and caffeine content of green tea dietary supplements and correlation with antioxidant capacity, *J. Agric. Food Chem.*, 2006, 54, 1599-1603
- [104] Zhang Y., Jiang T., Zheng Y., Zhou P., Interference of EGCG on the Zn(II)-induced conformational transition of silk fibroin as a model protein related to neurodegenerative diseases, *Soft Matter*, 2012, 8, 5543-5549
- [105] Cheng X.R., Hau B.Y., Veloso A.J., Martic S., Kraatz H.B., Kerman K., Surface plasmon resonance imaging of amyloid- β aggregation kinetics in the presence of epigallocatechin gallate and metals, *Anal. Chem.*, 2013, 85, 2049-2055
- [106] Hyung S.J., DeToma A.S., Brender J.R., Lee S., Vivekanandan S., Kochi A., et al., Insights into anti-amyloidogenic properties of the green tea extract (-)-epigallocatechin-3-gallate toward metal-associated amyloid- β species, *Proc. Natl. Acad. Sci. USA*, 2013, 110, 3743-3748
- [107] Sinha S., Du Z., Maiti P., Klärner F.G., Schrader T., Wang C., et al., Comparison of three amyloid assembly inhibitors: the sugar scyllo-inositol, the polyphenol epigallocatechin gallate, and the molecular tweezer CLR01, *ACS Chem. Neurosci.*, 2012, 3, 451-458
- [108] Miyai S., Yamaguchi A., Iwasaki T., Shamsa F., Ohtsuki K., Biochemical characterization of epigallocatechin-3-gallate as an effective stimulator for the phosphorylation of its binding proteins by glycogen synthase kinase-3 β in vitro, *Biol. Pharm. Bull.*, 2010, 33, 1932-1937
- [109] Takashima A., The Mechanism of tau aggregation and its relation to neuronal dysfunction, *Alzheimer's Association International Conference on Alzheimer's disease*, 2010, S144, Abstract No. PL-104-103.
- [110] Frost B., Ollesch J., Wille H., Diamond M.I., Conformational diversity of wild-type Tau fibrils specified by templated conformation change, *J. Biol. Chem.*, 2009, 284, 3546-3551
- [111] Hsiao K., Chapman P., Nilsen S., Eckman C., Harigaya Y., Younkin S., et al., Correlative memory deficits, A β elevation, and amyloid plaques in transgenic mice, *Science*, 1996, 274, 99-102
- [112] Rezaei-Zadeh K., Shytle D., Sun N., Mori T., Hou H., Jeannot D., et al., Green tea epigallocatechin-3-gallate (EGCG) modulates amyloid precursor protein cleavage and reduces cerebral amyloidosis in Alzheimer transgenic mice, *J. Neurosci.*, 2005, 25, 8807-8814
- [113] Rezaei-Zadeh K., Arendash G.W., Hou H., Fernandez F., Jensen M., Runfeldt M., et al., Green tea epigallocatechin-3-gallate (EGCG) reduces β -amyloid mediated cognitive impairment and modulates tau pathology in Alzheimer transgenic mice, *Brain Res.*, 2008, 1214, 177-187
- [114] Hwang D.Y., Chae K.R., Kang T.S., Hwang J.H., Lim C.H., Kang H.K., et al., Alterations in behavior, amyloid β -42, caspase-3, and Cox-2 in mutant PS2 transgenic mouse model of Alzheimer's disease, *FASEB J.*, 2002, 16, 805-813
- [115] Lee J.W., Lee Y.K., Ban J.O., Ha T.Y., Yun Y.P., Han S.B., et al., Green tea (-)-epigallocatechin-3-gallate inhibits β -amyloid-induced cognitive dysfunction through modification of secretase activity via inhibition of ERK and NF- κ B pathways in mice, *J. Nutr.*, 2009, 139, 1987-1993
- [116] Lee S.Y., Lee J.W., Lee H., Yoo H.S., Yun Y.P., Oh K.W., et al., Inhibitory effect of green tea extract on β -amyloid-induced PC12 cell death by inhibition of the activation of NF- κ B and ERK/p38 MAP kinase pathway through antioxidant mechanisms, *Brain Res. Mol. Brain Res.*, 2005, 140, 45-54
- [117] Rasoolijazi H., Joghataie M.T., Roghani M., Nobakht M., The beneficial effect of (-)-epigallocatechin-3-gallate in an experimental model of Alzheimer's disease in rat: a behavioral analysis, *Iran Biomed. J.*, 2007, 11, 237-243
- [118] Lee Y.K., Yuk D.Y., Lee J.W., Lee S.Y., Ha T.Y., Oh K.W., et al., (-)-Epigallocatechin-3-gallate prevents lipopolysaccharide-induced elevation of β -amyloid generation and memory deficiency, *Brain Res.*, 2009, 1250, 164-174
- [119] Lee Y.J., Choi D.Y., Yun Y.P., Han S.B., Oh K.W., Hong J.T., Epigallocatechin-3-gallate prevents systemic inflammation-induced memory deficiency and amyloidogenesis via its anti-neuroinflammatory properties, *J. Nutr. Biochem.*, 2013, 24, 298-310
- [120] Miklosy J., Kis A., Radenovic A., Miller L., Forro L., Martins R., et al., β -amyloid deposition and Alzheimer's type changes induced by *Borrelia spirochetes*, *Neurobiol. Aging*, 2006, 27, 228-236
- [121] Link C.D., Expression of human β -amyloid peptide in transgenic *Caenorhabditis elegans*, *Proc. Natl. Acad. Sci. USA*, 1995, 92, 9368-9372
- [122] Abbas S., Wink M., Epigallocatechin gallate inhibits β amyloid oligomerization in *Caenorhabditis elegans* and affects the daf-2/insulin-like signaling pathway, *Phytomedicine*, 2010, 17, 902-909
- [123] Bitan G., Fradinger E.A., Spring S.M., Teplow D.B., Neurotoxic protein oligomers—what you see is not always what you get, *Amyloid*, 2005, 12, 88-95
- [124] Hepler R.W., Grimm K.M., Nahas D.D., Breese R., Dodson E.C., Acton P., et al., Solution state characterization of amyloid β -derived diffusible ligands, *Biochemistry*, 2006, 45, 15157-15167
- [125] Khan J.M., Qadeer A., Chaturvedi S.K., Ahmad E., Rehman S.A., Gourinath S., et al., SDS can be utilized as an amyloid inducer: a case study on diverse proteins, *PLoS One*, 2012, 7, e29694

- [126] Watt A.D., Perez K.A., Rembach A., Sherrat N.A., Hung L.W., Johanssen T., et al., Oligomers, fact or artefact? SDS-PAGE induces dimerization of β -amyloid in human brain samples, *Acta Neuropathol. (Berl)*. 2013,
- [127] Jankowsky J.L., Fadale D.J., Anderson J., Xu G.M., Gonzales V., Jenkins N.A., et al., Mutant presenilins specifically elevate the levels of the 42 residue β -amyloid peptide in vivo: evidence for augmentation of a 42-specific γ secretase, *Hum. Mol. Genet.*, 2004, 13, 159-170
- [128] Oddo S., Caccamo A., Shepherd J.D., Murphy M.P., Golde T.E., Kaye R., et al., Triple-transgenic model of Alzheimer's disease with plaques and tangles: intracellular $A\beta$ and synaptic dysfunction, *Neuron*, 2003, 39, 409-421
- [129] George J.M., Jin H., Woods W.S., Clayton D.F., Characterization of a novel protein regulated during the critical period for song learning in the zebra finch, *Neuron*, 1995, 15, 361-372
- [130] Maroteaux L., Scheller R.H., The rat brain synucleins; family of proteins transiently associated with neuronal membrane, *Brain Res. Mol. Brain Res.*, 1991, 11, 335-343
- [131] Maroteaux L., Campanelli J.T., Scheller R.H., Synuclein: a neuron-specific protein localized to the nucleus and presynaptic nerve terminal, *J. Neurosci.*, 1988, 8, 2804-2815
- [132] Bendor J.T., Logan T.P., Edwards R.H., The function of α -synuclein, *Neuron*, 2013, 79, 1044-1066
- [133] El-Agnaf O.M.A., Jakes R., Curran M.D., Middleton D., Ingénito R., Bianchi E., et al., Aggregates from mutant and wild-type α -synuclein proteins and NAC peptide induce apoptotic cell death in human neuroblastoma cells by formation of β -sheet and amyloid-like filaments, *FEBS Lett.*, 1998, 440, 71-75
- [134] Acharya S., Safaie B., Wongkongkathep P., Ivanova M.I., Attar A., Klärner F.-G., et al., Molecular basis for preventing α -synuclein aggregation by a molecular tweezer, 2013, Submitted for publication
- [135] Ng C.H., Mok S.Z., Koh C., Ouyang X., Fivaz M.L., Tan E.K., et al., Parkin protects against LRRK2 G2019S mutant-induced dopaminergic neurodegeneration in *Drosophila*, *J. Neurosci.*, 2009, 29, 11257-11262
- [136] Wang C., Lu R., Ouyang X., Ho M.W., Chia W., Yu F., et al., *Drosophila* overexpressing parkin R275W mutant exhibits dopaminergic neuron degeneration and mitochondrial abnormalities, *J. Neurosci.*, 2007, 27, 8563-8570
- [137] Ng C.H., Guan M.S., Koh C., Ouyang X., Yu F., Tan E.K., et al., AMP kinase activation mitigates dopaminergic dysfunction and mitochondrial abnormalities in *Drosophila* models of Parkinson's disease, *J. Neurosci.*, 2012, 32, 14311-14317
- [138] Bonilla-Ramirez L., Jimenez-Del-Rio M., Velez-Pardo C., Low doses of paraquat and polyphenols prolong life span and locomotor activity in knock-down parkin *Drosophila melanogaster* exposed to oxidative stress stimuli: implication in autosomal recessive juvenile Parkinsonism, *Gene*, 2013, 512, 355-363
- [139] Choi J.Y., Park C.S., Kim D.J., Cho M.H., Jin B.K., Pie J.E., et al., Prevention of nitric oxide-mediated 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine-induced Parkinson's disease in mice by tea phenolic epigallocatechin 3-gallate, *Neurotoxicology*, 2002, 23, 367-374
- [140] Kim J.S., Kim J.M., O J.J., Jeon B.S., Inhibition of inducible nitric oxide synthase expression and cell death by (-)-epigallocatechin-3-gallate, a green tea catechin, in the 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine mouse model of Parkinson's disease, *J. Clin. Neurosci.*, 2010, 17, 1165-1168
- [141] Reznichenko L., Kalfon L., Amit T., Youdim M.B., Mandel S.A., Low dosage of rasagiline and epigallocatechin gallate synergistically restored the nigrostriatal axis in MPTP-induced parkinsonism, *Neurodegener. Dis.*, 2010, 7, 219-231
- [142] Youdim M.B., Grunblatt E., Levites Y., Maor G., Mandel S., Early and late molecular events in neurodegeneration and neuroprotection in Parkinson's disease MPTP model as assessed by cDNA microarray; the role of iron, *Neurotox. Res.*, 2002, 4, 679-689
- [143] Leaver K.R., Allbutt H.N., Creber N.J., Kassiou M., Henderson J.M., Oral pre-treatment with epigallocatechin gallate in 6-OHDA lesioned rats produces subtle symptomatic relief but not neuroprotection, *Brain Res. Bull.*, 2009, 80, 397-402
- [144] Kang K.S., Wen Y., Yamabe N., Fukui M., Bishop S.C., Zhu B.T., Dual beneficial effects of (-)-epigallocatechin-3-gallate on levodopa methylation and hippocampal neurodegeneration: in vitro and in vivo studies, *PLoS One*, 2010, 5, e11951
- [145] Emmanouilidou E., Stefanis L., Vekrellis K., Cell-produced α -synuclein oligomers are targeted to, and impair, the 26S proteasome, *Neurobiol. Aging*, 2010, 31, 953-968
- [146] Zhang N.Y., Tang Z., Liu C.W., α -Synuclein protofibrils inhibit 26 S proteasome-mediated protein degradation: understanding the cytotoxicity of protein protofibrils in neurodegenerative disease pathogenesis, *J. Biol. Chem.*, 2008, 283, 20288-20298
- [147] Lulla A., Barnhill L., Stahl M., Fitzmaurice A.G., Li S., Bronstein J.M., Neurotoxicity of the dithiocarbamate fungicide ziram is dependent on synuclein in zebrafish: Implications for Parkinson's disease, *Society of Toxicology Annual Meeting*, 2013, Abstract #1407.
- [148] Wang X.F., Li S., Chou A.P., Bronstein J.M., Inhibitory effects of pesticides on proteasome activity: implication in Parkinson's disease, *Neurobiol. Dis.*, 2006, 23, 198-205
- [149] Zhou Y., Shie F.S., Piccardo P., Montine T.J., Zhang J., Proteasomal inhibition induced by manganese ethylene-bis-dithiocarbamate: relevance to Parkinson's disease, *Neuroscience*, 2004, 128, 281-291
- [150] Chou A.P., Maidment N., Klintonberg R., Casida J.E., Li S., Fitzmaurice A.G., et al., Ziram causes dopaminergic cell damage by inhibiting E1 ligase of the proteasome, *J. Biol. Chem.*, 2008, 283, 34696-34703
- [151] Wang A., Costello S., Cockburn M., Zhang X., Bronstein J., Ritz B., Parkinson's disease risk from ambient exposure to pesticides, *Eur. J. Epidemiol.*, 2011, 26, 547-555
- [152] Rinetti G.V., Schweizer F.E., Ubiquitination acutely regulates presynaptic neurotransmitter release in mammalian neurons, *J. Neurosci.*, 2010, 30, 3157-3166
- [153] Saraiva M., Cardoso I., Transthyretin Aggregation and Toxicity, In: Rahimi F., Bitan G. (Eds.), *Non-fibrillar Amyloidogenic Protein Assemblies—Common Cytotoxins Underlying Degenerative Diseases*, Springer Netherlands, 2012

- [154] Westermark P, Senile systemic amyloidosis – An overview, *Amyloid*, 2001, 8, 121
- [155] Ferreira N, Cardoso I, Domingues M.R., Vitorino R., Bastos M., Bai G., et al., Binding of epigallocatechin-3-gallate to transthyretin modulates its amyloidogenicity, *FEBS Lett.*, 2009, 583, 3569-3576
- [156] Miyata M., Sato T., Kugimiya M., Sho M., Nakamura T., Ikemizu S., et al., The crystal structure of the green tea polyphenol (–)-epigallocatechin gallate-transthyretin complex reveals a novel binding site distinct from the thyroxine binding site, *Biochemistry*, 2010, 49, 6104-6114
- [157] Kristen A.V., Lehrke S., Buss S., Mereles D., Steen H., Ehlermann P, et al., Green tea halts progression of cardiac transthyretin amyloidosis: an observational report, *Clin. Res. Cardiol.*, 2012, 101, 805-813
- [158] Kristen A.V., Perz J.B., Schonland S.O., Hegenbart U., Schnabel P.A., Kristen J.H., et al., Non-invasive predictors of survival in cardiac amyloidosis, *Eur. J. Heart Fail.*, 2007, 9, 617-624
- [159] Mörner S., Hellman U., Suhr O.B., Kazzam E., Waldenstrom A., Amyloid heart disease mimicking hypertrophic cardiomyopathy, *J. Intern. Med.*, 2005, 258, 225-230
- [160] Ferreira N., Saraiva M.J., Almeida M.R., Epigallocatechin-3-gallate as a potential therapeutic drug for TTR-related amyloidosis: “in vivo” evidence from FAP mice models, *PLoS One*, 2012, 7, e29933
- [161] Santos S.D., Fernandes R., Saraiva M.J., The heat shock response modulates transthyretin deposition in the peripheral and autonomic nervous systems, *Neurobiol. Aging*, 2010, 31, 280-289
- [162] Ferreira N., Pereira-Henriques A., Attar A., Klärner F.-G., Schrader T., Bitan G., et al., Molecular Tweezers Targeting Transthyretin Amyloidosis, 2013, Submitted for publication
- [163] Westermark P, Wernstedt C., Wilander E., Hayden D.W., O'Brien T.D., Johnson K.H., Amyloid fibrils in human insulinoma and islets of Langerhans of the diabetic cat are derived from a neuropeptide-like protein also present in normal islet cells, *Proc. Natl. Acad. Sci. USA*, 1987, 84, 3881-3885
- [164] Cooper G.J., Willis A.C., Clark A., Turner R.C., Sim R.B., Reid K.B., Purification and characterization of a peptide from amyloid-rich pancreases of type 2 diabetic patients, *Proc. Natl. Acad. Sci. USA*, 1987, 84, 8628-8632
- [165] Kahn S.E., Andrikopoulos S., Verchere C.B., Islet amyloid: a long-recognized but underappreciated pathological feature of type 2 diabetes, *Diabetes*, 1999, 48, 241-253
- [166] Clark A., Cooper G.J., Lewis C.E., Morris J.F., Willis A.C., Reid K.B., et al., Islet amyloid formed from diabetes-associated peptide may be pathogenic in type-2 diabetes, *Lancet*, 1987, 2, 231-234
- [167] Hull R.L., Westermark G.T., Westermark P., Kahn S.E., Islet amyloid: a critical entity in the pathogenesis of type 2 diabetes, *J. Clin. Endocrinol. Metab.*, 2004, 89, 3629-3643
- [168] Lorenzo A., Razzaboni B., Weir G.C., Yankner B.A., Pancreatic islet cell toxicity of amylin associated with type-2 diabetes mellitus, *Nature*, 1994, 368, 756-760
- [169] Clark A., Wells C.A., Buley I.D., Cruickshank J.K., Vanhegan R.I., Matthews D.R., et al., Islet amyloid, increased a-cells, reduced b-cells and exocrine fibrosis: quantitative changes in the pancreas in type 2 diabetes, *Diabetes Res.*, 1988, 9, 151-159
- [170] Butler A.E., Janson J., Bonner-Weir S., Ritzel R., Rizza R.A., Butler P.C., β -Cell deficit and increased β -cell apoptosis in humans with type 2 diabetes, *Diabetes*, 2003, 52, 102-110
- [171] Bahramikia S., Yazdanparast R., Inhibition of human islet amyloid polypeptide or amylin aggregation by two manganese-salen derivatives, *Eur. J. Pharmacol.*, 2013, 707, 17-25
- [172] Cheng B., Gong H., Li X., Sun Y., Chen H., Zhang X., et al., Salvianolic acid B inhibits the amyloid formation of human islet amyloid polypeptide and protects pancreatic β -cells against cytotoxicity, *Proteins*, 2013, 81, 613-621
- [173] Cheng B., Gong H., Li X., Sun Y., Zhang X., Chen H., et al., Silibinin inhibits the toxic aggregation of human islet amyloid polypeptide, *Biochem. Biophys. Res. Commun.*, 2012, 419, 495-499
- [174] Hagihara M., Takei A., Ishii T., Hayashi F., Kubota K., Wakamatsu K., et al., Inhibitory effects of choline-O-sulfate on amyloid formation of human islet amyloid polypeptide, *FEBS open bio*, 2012, 2, 20-25
- [175] Seeliger J., Winter R., Islet amyloid polypeptide: Aggregation and fibrillogenesis in vitro and its inhibition, *Subcell. Biochem.*, 2012, 65, 185-209
- [176] Engel M.F., vandenAkker C.C., Schleegeer M., Velikov K.P., Koenderink G.H., Bonn M., The polyphenol EGCG inhibits amyloid formation less efficiently at phospholipid interfaces than in bulk solution, *J. Am. Chem. Soc.*, 2012, 134, 14781-14788
- [177] Fu L., Ma G., Yan E.C., In situ misfolding of human islet amyloid polypeptide at interfaces probed by vibrational sum frequency generation, *J. Am. Chem. Soc.*, 2010, 132, 5405-5412
- [178] Fu L., Liu J., Yan E.C., Chiral sum frequency generation spectroscopy for characterizing protein secondary structures at interfaces, *J. Am. Chem. Soc.*, 2011, 133, 8094-8097
- [179] Suzuki Y., Brender J.R., Hartman K., Ramamoorthy A., Marsh E.N., Alternative pathways of human islet amyloid polypeptide aggregation distinguished by 19F nuclear magnetic resonance-detected kinetics of monomer consumption, *Biochemistry*, 2012, 51, 8154-8162
- [180] Lopes D.H.J., Attar A., Du Z., McDaniel K., Dutt S., Bravo-Rodriguez K., et al., The molecular tweezer CLR01 inhibits islet amyloid polypeptide assembly and toxicity via an unexpected mechanism, 2013, Submitted for publication
- [181] Sexton P.M., Findlay D.M., Martin T.J., Calcitonin, *Curr. Med. Chem.*, 1999, 6, 1067-1093
- [182] Copp D.H., Calcitonin: discovery, development, and clinical application, *Clin. Invest. Med.*, 1994, 17, 268-277
- [183] Huang C.L., Sun L., Moonga B.S., Zaidi M., Molecular physiology and pharmacology of calcitonin, *Cellular and molecular biology (Noisy-le-Grand, France)*, 2006, 52, 33-43
- [184] Foster G.V., Calcitonin (thyrocalcitonin), *N. Engl. J. Med.*, 1968, 279, 349-360
- [185] Haymovits A., Rosen J.F., Calcitonin in metabolic disorders, *Adv. Metab. Disord.*, 1972, 60, 177-212

- [186] Huang R., Vivekanandan S., Brender J.R., Abe Y., Naito A., Ramamoorthy A., NMR characterization of monomeric and oligomeric conformations of human calcitonin and its interaction with EGCG, *J. Mol. Biol.*, 2012, 416, 108-120
- [187] Molinari M., Watt K.D., Kruszyna T., Nelson R., Walsh M., Huang W.Y., et al., Acute liver failure induced by green tea extracts: case report and review of the literature, *Liver Transpl.*, 2006, 12, 1892-1895
- [188] Isbrucker R.A., Bausch J., Edwards J.A., Wolz E., Safety studies on epigallocatechin gallate (EGCG) preparations. Part 1: genotoxicity, *Food Chem. Toxicol.*, 2006, 44, 626-635
- [189] Isbrucker R.A., Edwards J.A., Wolz E., Davidovich A., Bausch J., Safety studies on epigallocatechin gallate (EGCG) preparations. Part 3: teratogenicity and reproductive toxicity studies in rats, *Food Chem. Toxicol.*, 2006, 44, 651-661
- [190] Isbrucker R.A., Edwards J.A., Wolz E., Davidovich A., Bausch J., Safety studies on epigallocatechin gallate (EGCG) preparations. Part 2: dermal, acute and short-term toxicity studies, *Food Chem. Toxicol.*, 2006, 44, 636-650
- [191] Lambert J.D., Kennett M.J., Sang S., Reuhl K.R., Ju J., Yang C.S., Hepatotoxicity of high oral dose (-)-epigallocatechin-3-gallate in mice, *Food Chem. Toxicol.*, 2010, 48, 409-416
- [192] Goodin M.G., Rosengren R.J., Epigallocatechin gallate modulates CYP450 isoforms in the female Swiss-Webster mouse, *Toxicol. Sci.*, 2003, 76, 262-270
- [193] Kapetanovic I.M., Crowell J.A., Krishnaraj R., Zakharov A., Lindeblad M., Lyubimov A., Exposure and toxicity of green tea polyphenols in fasted and non-fasted dogs, *Toxicology*, 2009, 260, 28-36
- [194] Guengerich F.P., Cytochrome p450 and chemical toxicology, *Chem. Res. Toxicol.*, 2008, 21, 70-83
- [195] Huynh H.T., Teel R.W., Effects of plant-derived phenols on rat liver cytochrome P450 2B1 activity, *Anticancer Res.*, 2002, 22, 1699-1703
- [196] Ullmann U., Haller J., Decourt J.P., Girault N., Girault J., Richard-Caudron A.S., et al., A single ascending dose study of epigallocatechin gallate in healthy volunteers, *J. Int. Med. Res.*, 2003, 31, 88-101
- [197] Chow H.H., Hakim I.A., Vining D.R., Crowell J.A., Ranger-Moore J., Chew W.M., et al., Effects of dosing condition on the oral bioavailability of green tea catechins after single-dose administration of Polyphenon E in healthy individuals, *Clin. Cancer Res.*, 2005, 11, 4627-4633
- [198] Ullmann U., Haller J., Decourt J.D., Girault J., Spitzer V., Weber P., Plasma-kinetic characteristics of purified and isolated green tea catechin epigallocatechin gallate (EGCG) after 10 days repeated dosing in healthy volunteers, *International journal for vitamin and nutrition research. Internationale Zeitschrift für Vitamin- und Ernährungsforschung. Int. J. Vitam. Nutr. Res.*, 2004, 74, 269-278
- [199] Chow H.H., Cai Y., Hakim I.A., Crowell J.A., Shahi F., Brooks C.A., et al., Pharmacokinetics and safety of green tea polyphenols after multiple-dose administration of epigallocatechin gallate and polyphenon E in healthy individuals, *Clin. Cancer Res.*, 2003, 9, 3312-3319
- [200] Hsu C.H., Liao Y.L., Lin S.C., Tsai T.H., Huang C.J., Chou P., Does supplementation with green tea extract improve insulin resistance in obese type 2 diabetics? A randomized, double-blind, and placebo-controlled clinical trial, *Altern. Med. Rev.*, 2011, 16, 157-163
- [201] Jimenez-Saenz M., Martinez-Sanchez Mdel C., Acute hepatitis associated with the use of green tea infusions, *J. Hepatol.*, 2006, 44, 616-617
- [202] Crew K.D., Brown P., Greenlee H., Bevers T.B., Arun B., Hudis C., et al., Phase IB randomized, double-blinded, placebo-controlled, dose escalation study of polyphenon E in women with hormone receptor-negative breast cancer, *Cancer Prev. Res.*, 2012, 5, 1144-1154
- [203] Bonkovsky H.L., Hepatotoxicity associated with supplements containing Chinese green tea (*Camellia sinensis*), *Ann. Intern. Med.*, 2006, 144, 68-71
- [204] Mazzanti G., Menniti-Ippolito F., Moro P.A., Cassetti F., Raschetti R., Santuccio C., et al., Hepatotoxicity from green tea: a review of the literature and two unpublished cases, *Eur. J. Clin. Pharmacol.*, 2009, 65, 331-341
- [205] Attar A., Chan W.-T.C., Klärner F.-G., Schrader T., Bitan G., Safety and pharmacokinetic characterization of the molecular tweezer CLR01 in vivo, 2013, Manuscript in preparation
- [206] Obach R.S., Walsky R.L., Venkatakrishnan K., Gaman E.A., Houston J.B., Tremaine L.M., The utility of in vitro cytochrome P450 inhibition data in the prediction of drug-drug interactions, *J. Pharmacol. Exp. Ther.*, 2006, 316, 336-348
- [207] Williamson G., Dionisi F., Renouf M., Flavanols from green tea and phenolic acids from coffee: critical quantitative evaluation of the pharmacokinetic data in humans after consumption of single doses of beverages, *Mol. Nutr. Food Res.*, 2011, 55, 864-873
- [208] Yang C.S., Chen L., Lee M.J., Balentine D., Kuo M.C., Schantz S.P., Blood and urine levels of tea catechins after ingestion of different amounts of green tea by human volunteers, *Cancer Epidemiol. Biomarkers Prev.*, 1998, 7, 351-354
- [209] Chow H.H., Cai Y., Alberts D.S., Hakim I., Dorr R., Shahi F., et al., Phase I pharmacokinetic study of tea polyphenols following single-dose administration of epigallocatechin gallate and polyphenon E, *Cancer Epidemiol. Biomarkers Prev.*, 2001, 10, 53-58
- [210] Renouf M., Guy P., Marmet C., Longet K., Fraering A.L., Moulin J., et al., Plasma appearance and correlation between coffee and green tea metabolites in human subjects, *Br. J. Nutr.*, 2010, 104, 1635-1640
- [211] Van Amelsvoort J.M., Van Hof K.H., Mathot J.N., Mulder T.P., Wiersma A., Tijburg L.B., Plasma concentrations of individual tea catechins after a single oral dose in humans, *Xenobiotica*, 2001, 31, 891-901
- [212] Lee M.J., Wang Z.Y., Li H., Chen L., Sun Y., Gobbo S., et al., Analysis of plasma and urinary tea polyphenols in human subjects, *Cancer Epidemiol. Biomarkers Prev.*, 1995, 4, 393-399
- [213] Mateos R., Goya L., Bravo L., Uptake and metabolism of hydroxycinnamic acids (chlorogenic, caffeic, and ferulic acids) by HepG2 cells as a model of the human liver, *J. Agric. Food Chem.*, 2006, 54, 8724-8732
- [214] Meng X., Sang S., Zhu N., Lu H., Sheng S., Lee M.J., et al., Identification and characterization of methylated and ring-fission metabolites of

- tea catechins formed in humans, mice, and rats, *Chem. Res. Toxicol.*, 2002, 15, 1042-1050
- [215] Walle T., Methylation of dietary flavones greatly improves their hepatic metabolic stability and intestinal absorption, *Mol. Pharm.*, 2007, 4, 826-832
- [216] Maeda-Yamamoto M., Ema K., Monobe M., Tokuda Y., Tachibana H., Epicatechin-3-O-(3'-O-methyl)-gallate content in various tea cultivars (*Camellia sinensis* L.) and its in vitro inhibitory effect on histamine release, *J. Agric. Food Chem.*, 2012, 60, 2165-2170
- [217] Harada M., Kan Y., Naoki H., Fukui Y., Kageyama N., Nakai M., et al., Identification of the major antioxidative metabolites in biological fluids of the rat with ingested (+)-catechin and (-)-epicatechin, *Biosci. Biotechnol. Biochem.*, 1999, 63, 973-977
- [218] Giunta B., Hou H., Zhu Y., Salemi J., Ruscin A., Shytle R.D., et al., Fish oil enhances anti-amyloidogenic properties of green tea EGCG in Tg2576 mice, *Neurosci. Lett.*, 2010, 471, 134-138
- [219] Sang S., Lee M.J., Hou Z., Ho C.T., Yang C.S., Stability of tea polyphenol (-)-epigallocatechin-3-gallate and formation of dimers and epimers under common experimental conditions, *J. Agric. Food Chem.*, 2005, 53, 9478-9484
- [220] Ishii T., Mori T., Tanaka T., Mizuno D., Yamaji R., Kumazawa S., et al., Covalent modification of proteins by green tea polyphenol (-)-epigallocatechin-3-gallate through autoxidation, *Free Radic. Biol. Med.*, 2008, 45, 1384-1394
- [221] Sato M., Murakami K., Uno M., Nakagawa Y., Katayama S., Akagi K.I., et al., Site-specific inhibitory mechanism for amyloid- β 42 aggregation by catechol-type flavonoids targeting the Lys residues, *J. Biol. Chem.*, 2013,
- [222] Okada K., Wangpoengtrakul C., Osawa T., Toyokuni S., Tanaka K., Uchida K., 4-hydroxy-2-nonenal-mediated impairment of intracellular proteolysis during oxidative stress - Identification of proteasomes as target molecules, *J. Biol. Chem.*, 1999, 274, 23787-23793
- [223] Qin Z., Hu D., Han S., Reaney S.H., Di Monte D.A., Fink A.L., Effect of 4-hydroxy-2-nonenal modification on α -synuclein aggregation, *J. Biol. Chem.*, 2007, 282, 5862-5870
- [224] Perez M., Cuadros R., Smith M.A., Perry G., Avila J., Phosphorylated, but not native, tau protein assembles following reaction with the lipid peroxidation product, 4-hydroxy-2-nonenal, *FEBS Lett.*, 2000, 486, 270-274