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Modulators of Amyloid β -Protein ($A\beta$) Self-Assembly

F. Rahimi, H. Li, S. Sinha, G. Bitan

OUTLINE

Introduction	97	Vitamins	138
Peptidic or Peptidomimetic		Proteins or Peptides	140
Modulators of $A\beta$ Assembly	100	Other Compounds	142
$A\beta$ Fragments or $A\beta$ Derivatives	100	$A\beta$ Assembly Modulators	
Peptides and Peptidomimetics		From Nonnatural Sources	144
From Other Sources	115	Small-Molecule Modulators of $A\beta$	
Conclusions	119	Assembly and Toxicity	144
$A\beta$ Assembly Modulators Derived		Large Molecules That Modulate $A\beta$	
From Natural Sources	120	Assembly and Toxicity	160
Polyphenols	120	Conclusions	170
Inositol Derivatives	136	References	172

INTRODUCTION

Amyloid β -protein ($A\beta$) is produced mainly as 40- or 42-amino acid polypeptides from the amyloid β -protein precursor (APP) through sequential cleavage by enzymatic activities called β - and γ -secretases (Esler and Wolfe, 2001; Martins et al., 1991). It is produced in a monomeric form as part of normal physiology by different cells in the body throughout life (Haass et al., 1993); yet, although the peptide was discovered in the 1980s (Glennner and Wong, 1984; Masters et al., 1985), more than 30 years later its physiological role is poorly understood. Most studies of $A\beta$ have focused on its pathogenic role in disease. The original discovery of $A\beta$ was in brain extracts from patients with Alzheimer's disease (AD). Today, AD still is the main

disease associated with $A\beta$, though deleterious effects of $A\beta$ aggregates also have been found in other diseases, including Down syndrome (Wilcock and Griffin, 2013), inclusion-body myositis (Askanas et al., 2012), and age-related macular degeneration (Ratnayaka et al., 2015).

Following the discovery that $A\beta$ was the main proteinaceous component of amyloid plaques, the amyloid cascade hypothesis posited that formation of $A\beta$ fibrils along with their deposition in amyloid plaques was the key event initiating the disease process, and that all the other pathologic processes, including abnormal tau hyperphosphorylation and aggregation, oxidative stress, and inflammation, were consequences of $A\beta$ fibrillation (Hardy and Higgins, 1992). Later, this original hypothesis was revised when it became clear that elusive, metastable oligomers were substantially more neurotoxic than amyloid fibrils (Dahlgren et al., 2002; Lambert et al., 1998), and oligomer concentration showed high correlation with the progression of AD, whereas fibril abundance and location did not (Hardy, 2002; Selkoe, 2004).

Over the past three decades, major progress has been made in elucidating the central role of $A\beta$ in AD and uncovering the contribution of specific forms of $A\beta$. In addition to the increasing understanding that oligomers are the proximal neurotoxins acting in AD, research has shown that the 42-amino-acid-long form of $A\beta$, $A\beta_{42}$, is substantially more toxic than the shorter form, $A\beta_{40}$. $A\beta_{40}$ is ~ 10 times more abundant than $A\beta_{42}$, yet because of its lower toxicity, it is believed to be a lesser contributor to AD pathology (Rahimi and Bitan, 2012). Alongside these shifts in conception and understanding of the rank order of importance of different $A\beta$ species, the search for therapeutic intervention has been shifting slowly from fibrils to oligomers and from $A\beta$ in general to $A\beta_{42}$.

Therapeutic approaches targeting $A\beta$ generally have focused on three main strategies—inhibition of $A\beta$ production, enhancement of $A\beta$ clearance, and inhibition/modulation of $A\beta$ assembly. Theoretically, inhibition of $A\beta$ formation would be the most straightforward approach. A major strength of the pharmaceutical industry has been discovering and optimizing enzyme inhibitors. Thus, searching for inhibitors of β - and γ -secretases appeared to be a promising strategy after the major enzymes responsible for these enzymatic activities were identified as presenilin 1 (Wolfe et al., 1999) and β -site APP-cleaving enzyme 1 (BACE1) (Vassar et al., 1999). However, in both cases, efforts of more than a decade have not led to effective drugs, primarily due to the fact that each enzyme is responsible for processing multiple substrates. Thus, discovering inhibitors with sufficient specificity and safety has been challenging (Pollack and Lewis, 2005; Wolfe, 2012; Yan and Vassar, 2014). γ -Secretase therapy suffered a major recent setback with the failure of semagacestat, which not only did not improve symptoms in patients with AD, but actually worsened symptoms (Doody et al., 2013). β -Secretase inhibitors are currently being tested in several clinical trials (Vassar, 2014), yet recent research showing that these inhibitors may lead to accumulation of N-terminally extended APP fragments with substantial neurotoxicity are worrisome (Kaneko et al., 2014; Willem et al., 2015).

Clearance of $A\beta$ from the brain has been the most advanced therapeutic strategy explored to date following initial discoveries showing that antibodies targeting $A\beta$ could reduce $A\beta$ burden dramatically in animal models (Frenkel et al., 2000; Schenk, 2002). Early attempts to immunize patients with AD with fibrillar $A\beta$ were halted abruptly after some patients developed subacute meningoencephalitis (Orgogozo et al., 2003). Subsequently, several clinical trials used passive immunization with antibodies targeting different $A\beta$ epitopes. Unfortunately, these trials have not been successful due to lack of efficacy and/or because of

safety issues, decreasing hope for a breakthrough therapy (Cummings et al., 2014; Doody et al., 2014; Salloway et al., 2014). Very recently, results reported in the 2015 Alzheimer's Association International Conference provided cautious hope with news of possible delayed cognitive decline in patients with mild AD treated by Eli Lilly's monoclonal antibody, solanezumab, and positive target engagement by Roche's gantenerumab. These reports followed positive data from a small phase 1 trial showing efficacy of Biogen's aducanumab earlier in the year, yet safety issues with this antibody are a concern (https://www.alz.org/aaic/releases_2015/wed-7amET.asp). Current trepidation exists regarding the general approach using antibody clearance of A β due to fear that finding the right balance between efficacy and safety may be more difficult than previously perceived. Another general worry is that virtually all the major trials conducted to date were done too late in the disease process to have an effect. New clinical trials in asymptomatic patients (A4 NCT02008357) or patients with familial AD (DIAN-TU NCT01760005, PSEN1 E280A NCT01998841) are expected to address this concern and potentially provide hope for effective disease-modifying therapy in the next few years. Other strategies for enhancement of A β clearance, such as increased autophagy (Maiese, 2014) or induction of efflux from the brain (Paganetti et al., 2014; Roberts et al., 2014) have been explored but are not as advanced as immunological approaches.

A general strategic caveat in inhibition of A β production and enhancement of A β clearance is that they affect normal physiological processes. The limited understanding of the physiological role of A β itself increases this concern even further (Rajasekhar et al., 2015). In contrast, because A β self-assembly is a purely pathologic process, targeting this process, at least conceptually, is a highly attractive strategy. The problem here is that the therapeutic target—the metastable toxic oligomers—is an unusual and highly challenging one. The oligomers exist in mixtures of constantly changing structures, and the conformational stability of each monomer in these oligomers is low, making targeting the oligomers using typical strategies, for example, screening of small molecules, a frustrating endeavor.

Monomeric A β is nontoxic. Upon A β self-assembly, the toxicity increases substantially as oligomers form and then decreases with formation of fibrils. Because assembly state has such a strong impact on toxicity, researchers have asked whether particular oligomers are the most toxic or most important pathologic determinants in AD (Reed et al., 2011). However, despite intense research, no consensus has emerged, and it appears that most oligomers ranging from dimers to protofibrils are indeed toxic, increasing the complexity of the problem and the difficulty of developing effective inhibitors of A β oligomer toxicity. An even more confusing aspect of the problem is that because the oligomers are metastable structures, even molecules that interact with them with moderate affinity can affect their structure and assembly state. Many examples of such molecules are discussed in this chapter. However, to date, there are no clear criteria allowing the prediction of whether modulation in a particular direction would be therapeutically viable.

Despite these difficulties, but also perhaps because obtaining an initial effect is relatively easy and low-potency hits are easy to find, multiple groups have examined natural products, peptides, peptidomimetics, and various synthetic compounds as potential modulators of A β assembly and inhibitors of its toxicity (Liu and Bitan, 2012). In this chapter we review prominent examples of each category, emphasizing molecules that have “made it” to clinical trials or to advanced preclinical studies, yet also highlighting other compounds and interesting strategies that are still in their early stages.

PEPTIDIC OR PEPTIDOMIMETIC MODULATORS OF $A\beta$ ASSEMBLY

Several strategies have been used to design and discover peptides and peptidomimetics that modulate $A\beta$ assembly and inhibit its toxicity (summarized in [Table 6.1](#)). These strategies include using fragments of $A\beta$ itself based on the principle of self-recognition, generation of sequences mimicking the binding domains of $A\beta$ -binding proteins/peptides, de novo peptide design, peptide–small-molecule conjugates, and screening of random peptide libraries. Many comprehensive reviews have discussed peptide inhibitors previously, and readers are encouraged to refer to these reviews ([Doig and Derreumaux, 2015](#); [Kumar and Sim, 2014](#); [Luo and Abrahams, 2014](#); [Sciarretta et al., 2006](#); [Soto and Estrada, 2005](#); [Takahashi and Mihara, 2008](#)) in addition to this chapter.

$A\beta$ Fragments or $A\beta$ Derivatives

Self-aggregation is a predominant characteristic of amyloid proteins. An attractive strategy for developing amyloid aggregation inhibitors is to use fragments of the wild-type peptide, particularly those derived from sequences known to mediate aggregation because of their known tendency to self-assemble. Thus, the $A\beta$ fragments 17–21 (central hydrophobic cluster, CHC) and 29–42 (C-terminus) have been used frequently as starting points for developing inhibitors.

CHC-Derived Sequences

[Tjernberg et al. \(1996\)](#) were the first to show that $A\beta$ (16–21), KLVFFA, bound full-length $A\beta$, thereby preventing its fibrillization. Subsequently, the β -sheet breaker LPFFD (termed i $A\beta$ 5), an analog of $A\beta$ (17–21), was designed with Val17→Pro and Ala21→Asp substitutions to reduce the aggregation propensity of the peptide ([Soto et al., 1998](#)). This peptide inhibited $A\beta$ fibrillization and prevented the neurotoxic effect of $A\beta$ fibrils in cultured cells ([Poduslo et al., 1999](#); [Soto et al., 1998](#)). The C- and N-terminal-protected version of i $A\beta$ 5 (Ac-LPFFD-NH₂), referred to as i $A\beta$ 5p, and one of its N-methylated derivatives, i $A\beta$ 5p-A1 (Ac-LP-(NMe)F-FD-NH₂), also inhibited $A\beta$ fibrillization and $A\beta$ -induced neuron death. These peptides also significantly reduced $A\beta$ deposition in a transgenic mouse model. In addition, most of the N-methylated derivatives were highly stable against enzymatic degradation in human plasma and in 10% rat brain homogenates ([Adessi et al., 2003](#); [Permanne et al., 2002](#)).

Building on the pioneering studies of Tjernberg and Soto, various peptides and peptide derivatives subsequently were synthesized based on the CHC sequence. For example, three trehalose-conjugated peptide derivatives of i $A\beta$ 5p were prepared, in which the disaccharide was covalently attached to different sites of the LPFFD peptide chain, including the N-terminus (Th-NT), C-terminus (Th-CT), or the Asp side chain (Th-SC) ([De Bona et al., 2009](#)). All the sugar–peptide conjugates were more stable in 10% rat brain homogenates than i $A\beta$ 5p. Th-NT or Th-CT at 500 μ M effectively inhibited β -sheet formation of 100 μ M $A\beta$ 42 in N-ethylmaleimide buffer, but not in phosphate-buffered saline, in thioflavin T (ThT) fluorescence experiments ([De Bona et al., 2009](#)). The reason for the difference in activity between the two buffer systems was not discussed. About 50% of $A\beta$ 42 formed fibrils longer than 100 nm, whereas in the presence of fivefold excess Th-NT or Th-CT, only 27 and 30% of $A\beta$ 42, respectively, formed fibrils as seen by scanning-force microscopy. All three peptides protected ~80–95% of primary cortical

TABLE 6.1 Peptidic or Peptidomimetic Modulators of A β Assembly

Compound	Type	In vitro studies		Animal studies		Human studies/ clinical trials
		Inhibition of A β -induced cytotoxicity	Modulation of A β assembly	Decrease of pathological A β	Effect on behavioral deficits	
LPFFD (iA β 5) and derivatives	CHC-derived sequence, β -sheet breaker	Yes	Inhibit A β fibrillization	Reduce A β deposition in a transgenic mouse model	Unknown	Unknown
Th-NT, Th-CT, and Th-SC	CHC-derived sequence, β -sheet breaker	Yes	Th-NT and Th-CT inhibit β -sheet formation and fibril formation	Unknown	Unknown	Unknown
iA β 5-PEG5000	CHC-derived sequence, β -sheet breaker	Unknown	Inhibits A β 42 fibrillization more strongly than iA β 5- PEG3200 and similarly to iA β 5	Unknown	Unknown	Unknown
OR1 and OR2	CHC-derived sequence, β -sheet breaker	Yes (OR2)	Both inhibit fibrillization; OR2 inhibits oligomerization	Unknown	Unknown	Unknown
RI-OR2	CHC-derived sequence	Yes	Prevents formation of A β fibrils and oligomers	Unknown	Unknown	Unknown
RI-OR2-TAT	CHC-derived sequence conjugated to TAT	Yes	Inhibits A β 42 oligomer and fibril formation	Reduces cortical A β oligomer levels, amyloid plaque count, activated microglia, and oxidative damage	Unknown	Unknown
Fc-KLVFF	CHC-derived sequence, β -sheet breaker	Unknown	Inhibits A β aggregation similar to KLVFF	Unknown	Unknown	Unknown
Ac-L-D(OBzl)-F-F- D-NH ₂	CHC-derived sequence, β -sheet breaker	Unknown	Inhibits A β 40 fibrillization	Unknown	Unknown	Unknown
H102	CHC-derived sequence, β -sheet breaker	Yes	Inhibits A β 42 fibrillization	Unknown	Improves memory in transgenic mice	Unknown

(Continued)

TABLE 6.1 Peptidic or Peptidomimetic Modulators of A β Assembly (*cont.*)

Compound	Type	In vitro studies		Animal studies		Human studies/ clinical trials
		Inhibition of A β -induced cytotoxicity	Modulation of A β assembly	Decrease of pathological A β	Effect on behavioral deficits	
Ac-L-Ant-FFD-NH ₂	CHC-derived sequence	Unknown	Inhibits A β 40 fibrillization	Unknown	Unknown	Unknown
A β (16–22)m and A β (16–22)mR	CHC-derived sequence	Unknown	Inhibit A β 40 fibril formation and disassemble preformed A β 40 fibrils	Unknown	Unknown	Unknown
SEN304	CHC-derived sequence	Yes, and protects LTP in rat hippocampal slices	Delays β -sheet formation, and remodels toxic oligomers into nontoxic aggregates	Unknown	Unknown	Unknown
inD and inrD	CHC-derived sequence	Yes	Inhibit A β fibrillization	Unknown	Unknown	Unknown
ABSM-1a and ABSM-1b	CHC-derived sequence	Yes	Inhibit A β 40 fibrillization	Unknown	Unknown	Unknown
Compound 17	Small molecule based on CHC- derived cyclic peptide	Unknown	Inhibits A β 42 oligomerization and fibrillization	Unknown	Unknown	Unknown
A β (31–42) and A β (39–42)	C-terminal fragments	Yes, and prevent disruptions of synaptic activity	Inhibits A β 42 oligomerization	Unknown	Unknown	Unknown
N-methylated A β (32–37)	C-terminal fragment	Yes	Penta-N-methylated peptide inhibits A β aggregation	Unknown	Penta-N- methylated peptide increases <i>Drosophila</i> longevity and improves locomotion	Unknown

RIIGLa	C-terminal fragment	Yes and prevents disruptions of synaptic activity	Inhibits A β fibril formation	Unknown	Unknown	Unknown
G16	Mimicking the binding domain of A β -binding proteins	Yes	Moderately inhibits A β 40 fibrillization; promotes formation of large, nonfibrillar aggregates	Unknown	Unknown	Unknown
PP-Leu	Shares common structural features with A β fragments	Yes	Inhibits A β 42 oligomerization and fibrillization	Unknown	Unknown	Unknown
D-Trp-Aib	Rationally designed small peptides	Yes	Inhibits A β oligomerization	Reduces cerebral amyloid deposits in transgenic mice	Improves cognitive performance of transgenic mice	Unknown
RR (RYYA AFFARR)	Rationally designed peptide	Yes	Inhibits A β 40 fibrillization	Unknown	Unknown	Unknown
CILFWG, CTLWWG, CTIYWG	De novo design	Yes	Inhibit A β 42 fibrillization	Unknown	Unknown	Unknown

“Yes” means protection to a statistically significant extent from A β -induced reduction of cell viability.

FIGURE 6.1 Structures of KLVFFA or curcumin with cyclopeptide scaffold conjugates. Source: Modified from Ouberai et al. (2009).

neurons against $A\beta_{42}$ toxicity in the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay, but only when added at fivefold molar excess to 25 μ M $A\beta_{42}$, first upon $A\beta_{42}$ addition to cultured cells and then again 48 h later (De Bona et al., 2009).

In another study, two copies of an $A\beta$ -binding motifs [either curcumin (see section: Curcumin and Curcumin Derivatives) or KLVFFA] were conjugated to a constrained cyclopeptide scaffold designed to interfere with $A\beta$ aggregation (Fig. 6.1) (Ouberai et al., 2009). The conjugates strongly inhibited $A\beta_{40}$ fibrillization at low molar ratios relative to $A\beta$ (eg, 1:50 in the case of the KLVFFA conjugate), whereas the nonconjugated compounds, curcumin or KLVFFA, were inactive in ThT and atomic-force microscopy (AFM) experiments under the same conditions.

iA β 5 also was linked to polyethylene glycol (PEG) with the goal to reduce its immunogenicity and increase its molecular weight, and, as a result, to prolong its half-life in vivo (Rocha et al., 2009). In a binding competition assay, the pegylated iA β 5 had similar affinity to iA β 5 for $A\beta_{42}$. Using transmission electron microscopy (TEM), iA β 5-PEG5000 inhibited $A\beta_{42}$ fibrillization more strongly than iA β 5-PEG3200 and similarly to iA β 5. Caspase-3 activation by $A\beta_{42}$ in SH-SY5Y neuroblastoma cells was inhibited by treatment with both pegylated iA β 5 derivatives. This work showed that pegylation of the β -sheet-breaker peptides effectively maintained anti-amyloidogenic properties of iA β 5 (Rocha et al., 2009). However, the half-lives of the pegylated compounds were not determined.

Other potential peptide-based aggregation inhibitors were designed to contain the KLVFF sequence with Arg-Gly and Gly-Arg residues added at N- and C-terminal ends, respectively, to aid solubility (Austen et al., 2008). Two peptides, RGKLVFFGR, named OR1, and RGKLVFFGR-NH₂, named OR2, inhibited $A\beta_{40}$ fibrillization effectively in ThT and TEM assays, but only OR2 inhibited $A\beta$ oligomer formation in an enzyme-linked immunosorbent assay (ELISA) using monoclonal antibody 6E10 for capture and a biotinylated form of the same antibody for detection. This antibody was produced against a peptide comprising the first 16 amino acid residues of $A\beta$ (Kim et al., 1988, 1990). OR2 also inhibited $A\beta_{40}$ toxicity in human SH-SY5Y cells using the MTT assay.

FIGURE 6.2 Chemical transformation of Asp(OP)–Axx to aspartimide-containing peptide. *Source: Adapted from Nadimpally et al. (2014).*

A ferrocenoyl (Fc) group was conjugated to the N-terminus of KLVFF (Fc-KLVFF) (Wei et al., 2011) to increase the hydrophobicity and stability toward proteolysis of the conjugated compound. Ferrocene was chosen because of its high prevalence in different types of drugs, including antimalarial, antitumor, and antibacterial drugs. Using the ThT assay, Fc-KLVFF inhibited A β 42 aggregation with a similar potency to KLVFF. AFM and TEM confirmed the inhibitory effect on A β 42 fibrillization. Even after 5 months of incubation at room temperature, there was no sign of fibril formation. As expected, Fc-KLVFF showed improved lipophilicity and significant resistance toward proteolytic degradation compared to its parent peptide.

A series of β -sheet-breaker dipeptides (BBDPs) was designed with the general formula Asp(OP)–Axx as the β -sheet-breaker unit, where P designates a suitable side-chain-protecting group of the Asp residue, and Axx can be any amino acid (Nadimpally et al., 2014). Conceptually, Asp could be replaced by Glu or artificial amino acids bearing a carboxyl group at a suitable position of their side chains. The BBDPs incorporated into β -sheet-breaker sequences, for example, A β (17–21), and then chemically transformed into aspartimide-containing peptides at physiological pH (Fig. 6.2), generating a kink in the peptide backbone in situ and thereby breaking the aggregation pattern. One designed model peptide, Ac–L–D(Obzl)–F–F–D–NH₂, was found to increase fibril formation at the initial reaction stages (until ~24 h). As the reaction progressed, a gradual decrease in ThT fluorescence was observed, and TEM showed amorphous aggregates after 4 days of incubation, suggesting that the peptide might have incorporated into the fibrils, but then gradually disrupted them as the aspartimide moiety formed.

A 10-residue β -sheet-breaker called H102, HKQLPFFED, was reported to inhibit A β 42 aggregation in the ThT fluorescence assay and A β 42-induced toxicity effectively in SH-SY5Y cells (Lin et al., 2014; Xu et al., 2008). In the Morris water-maze test, 3 μ L of 80 mM of H102 in a saline solution injected into the lateral ventricle of APP695 mice daily improved memory performance compared to vehicle-treated mice. This transgenic model expresses human APP695 containing the Swedish (KM670/671/NL) and Indiana (V717F) mutations under the control of the hamster prion gene promoter (Chishti et al., 2001). No significant difference was found between wild-type animals and H102-treated mice, indicating that H102 improved learning and memory in the transgenic mice to the level of wild-type mice (Lin et al., 2014). Expression of inflammatory factors, including inducible nitric oxide synthase, interleukin-1 β , and tumor necrosis factor- α in the CA1 hippocampal region, was lower, and apoptosis was reduced in the H102-treated APP transgenic mice as assessed by immunohistochemistry for BCL2-associated X protein, a regulator of apoptosis (Lin et al., 2014).

FIGURE 6.3 Examples of structure-restricting strategies as tools for peptide-based drug design.

CONFORMATIONALLY RESTRICTED PEPTIDES

Conformationally restricted peptides have emerged as an important peptide-based drug-design strategy by stabilizing peptides of interest in an active conformation, which simultaneously protects them against proteases. Most reported conformation-restricting strategies include modified α -amino acids or β -amino acids, *N*-methylation, and/or cyclization by linking together two compatible groups in the peptide, such as two cysteines in a disulfide bond, or a carboxyl and an amine in a lactam ring (Luo and Abrahams, 2014). See Fig. 6.3 for general

FIGURE 6.4 Structures of α -aminoisobutyric acid (Aib) and *o*-aminobenzoic acid (Ant).

examples of chemical modifications that confer conformational stability on peptides or otherwise change their backbones to increase metabolic stability.

MODIFIED AMINO ACIDS Substituted α -amino acids or β -amino acids may limit a peptide's conformational space, for example, favoring an α -helical conformation to a β -strand. These structures can be effective β -sheet-breaker elements. For example, α -aminoisobutyric acid (Aib) (Gilead and Gazit, 2004) and anthranilic acid (Ant, Fig. 6.4) (Paul et al., 2015) have been introduced into short peptides for this purpose. The known propensity of Ant to induce either a helix or a turn conformation and its stability toward proteolytic degradation prompted Paul et al. to use it as a novel β -breaker element in peptides. Ac-L-Ant-FFD-NH₂ was found to resist aggregation itself and at 10-fold excess reduced A β 40 fibril formation by ~72% at physiological pH and temperature using the ThT assay. This inhibitory effect was higher than that of iA β 5p, which was used as a positive control and inhibited A β 40 fibril formation by 48% under the same conditions. In a disaggregation assay, Ac-L-Ant-FFD-NH₂ also performed substantially better than iA β 5p (Paul et al., 2015).

N-METHYLATED PEPTIDES *N*-methylated peptides are a common class of aggregation inhibitors. Because the *N*-methyl group replaces a backbone N—H group, the hydrogen bonds that stabilize the cross- β structure of amyloid fibrils cannot form at the *N*-methylated site. Thus, the *N*-methylated peptide can join a growing fibril and cap it, preventing further growth.

Gordon et al. (2001) described an *N*-methylated derivative of A β (16–22), termed A β (16–22)m, which incorporated *N*-methyl residues in alternating positions: K-(NMe)L-V-(NMe)F-F-(NMe)A-E-NH₂, and its reverse sequence, A β (16–22)mR, E-(NMe)L-V-(NMe)F-F-(NMe)A-K-NH₂, both of which inhibited A β 40 fibril formation and disassembled preformed A β 40 fibrils as observed by ThT fluorescence assay and TEM. In contrast, nonmethylated A β (16–22) and an analog methylated in consecutive positions (ie, K-L-(NMe)V-(NMe)F-(NMe)F-(NMe)A-E-NH₂), showed weak or no inhibition of A β fibrillization. A β (16–22)m and A β (16–22)mR were highly soluble in aqueous media and resistant to chymotrypsin digestion (Gordon et al., 2001), and A β (16–20)m passed spontaneously through natural and artificial phospholipid bilayers, suggesting good potential for drug development (Gordon et al., 2002).

A β (16–20) was used in later studies as a template for five libraries with variations in peptide length, *N*-methylation sites, *N*-terminal acetylation and/or C-terminal amidation, side-chain identity, and chirality (Kokkoni et al., 2006). Multiple assays, including ThT fluorescence, A β -affinity chromatography, TEM, and MTT, were used to test the inhibitory activity. Favorable structures for inhibiting A β aggregation included all-D chirality, a free N-terminus, an

FIGURE 6.5 Structure of SEN 304.

amidated C-terminus, large branched hydrophobic side chains at positions 1–4, and a single *N*-methyl group. An analog termed SEN304, D-[chG–Y–chG–chG–(NMe)L]–NH₂ (chG stands for cyclohexylglycine) (Fig. 6.5) was the most active inhibitor of A β 40 and A β 42 aggregation by the ThT assay and an effective inhibitor of A β 42-induced toxicity by the MTT assay in rat pheochromocytoma (PC-12) cells (Kokkoni et al., 2006). Circular dichroism (CD) spectroscopy measurements suggested that SEN304 caused only a subtle change in β structure, yet size-exclusion chromatography (SEC) experiments using equimolar concentrations (10 μ M) of A β 42 and SEN304 showed that SEN304 slowed A β 42 aggregation and prevented formation of A β 42 oligomers. Using surface-plasmon resonance (SPR), SEN304 was found to bind directly to monomeric A β 40 and A β 42 and to oligomeric A β 42, with $K_d = 14$, 2.5, and 11 μ M, respectively. TEM data showed that SEN304 did not stop fibril formation, but changed the morphology of the resulting fibrils, which were noticeably wider than those formed by A β 42 alone. In addition, SEN304 was found to inhibit A β 42-induced cytotoxicity in human SH-SH5Y cells, A β 42-induced decrease of synaptophysin in cortical neurons, and both A β 40-induced and A β 42-induced inhibition of long-term potentiation (LTP) in rat hippocampal slices (Amijee et al., 2012).

Giralt and coworkers applied the retro-inverso (RI) concept (Goodman and Chorev, 1979) to design an *N*-methylated peptide inhibitor of A β 42 aggregation (Grillo-Bosch et al., 2009). The RI strategy flips both the chirality of the residue from L to D and the direction of the peptide bond, thus maintaining the overall topology of the native peptides. Previously, this group identified a peptide they termed inL (KKLVF–(NMe)F–A–NH₂), which decreased A β 42-induced cytotoxicity in PC-12 cells in the MTT assay (Cruz et al., 2004). Considering developing a proteolysis-resistant, active version of the peptide, they designed and synthesized two analogs of inL composed of all-D amino acids, inD (kklvf–(NMe)f–a–NH₂) (lowercase letters represent D-amino acids) and an RI analog, inrD (a–(NMe)f–fvllk–NH₂) (Grillo-Bosch et al., 2009). ThT and TEM experiments showed that inD and inrD inhibited A β 42 fibrillization more efficiently than inL. InD and inrD strongly lowered the ThT plateau value of A β 42 aggregation, whereas inL did not lower the plateau. Using photo-induced cross-linking of unmodified proteins (PICUP) experiments (Bitan, 2006; Fancy and Kodadek, 1999), monomers, dimers, and pentamers were found to be more abundant in A β 42 solutions incubated with inD or inrD than in A β 42 solution treated with inL. Both inD and inrD reduced A β 42 toxicity in SH-SY5Y cells using the [3-(4,5-dimethylthiazol-2-yl)-5-(3

FIGURE 6.6 General structure of an amyloid β -sheet mimic (ABSM) peptide. Source: Reproduced from [Cheng et al. \(2012\)](#).

carboxymethoxyphenyl)-2(4-sulfophenyl)-2H-tetrazolium salt] (MTS) assay. Both inD and inrD also were found to be stable in cerebrospinal fluid (CSF), indicating their therapeutic potential in the central nervous system (CNS) ([Grillo-Bosch et al., 2009](#)).

CYCLIC PEPTIDES Another way to achieve conformational restriction is by cyclization. Cyclization may stabilize peptide structures into bioactive conformations, and cyclic peptides often are more biostable than their noncyclic analogs because of their resistance to enzymatic proteolysis. Therefore, cyclization is an attractive method for developing peptides into drugs.

Cheng and coworkers investigated an “amyloid β -sheet mimic” (ABSM), which was composed of a heptapeptide β -strand (the upper strand) and one “Hao” unit [an unnatural amino acid containing hydrazine, 5-amino-2-methoxybenzoic acid, and oxalic acid groups, designated as Hao ([Nowick et al., 2000](#)), a tripeptide β -strand mimic] flanked by dipeptides (the lower strand) and two δ -linked ornithine turns ([Fig. 6.6](#)) ([Cheng et al., 2012](#)). The upper β -strand of ABSM-1 incorporated different heptapeptide fragments from A β or other amyloid proteins. For A β , fragments derived from the CHC and C-terminal regions were selected. The lower strand was expected to serve as a template for intramolecular hydrogen bonding, assuming the designed cyclic peptide would fold well. The structural design of “Hao” helped prevent the cyclic peptides from aggregating in solution ([Nowick et al., 2000](#)). Fulfilling the expectation, ABSM-1a, incorporating A β (16–22), and ABSM-1b, incorporating A β (17–23), inhibited A β 40 aggregation in the ThT assay. In contrast, ABSMs incorporating C-terminal fragments (CTFs) showed little or no inhibition. ABSM-1a reduced A β 40- and A β 42-induced toxicity in PC-12 cells using the MTT assay.

Starting from cyclic A β (16–20), Arai et al. synthesized cyclo-D-peptides, RI peptides, Ala-substituted peptides, and single D-amino-acid-substituted peptides, and evaluated their inhibitory effects on A β fibrillization using the ThT assay. Based on their structure–activity relationship studies, they then simplified the structure into a small molecule, designated compound 17, containing isopentyl carboxamide (Leu side-chain mimic), benzyl (Phe side-chain

FIGURE 6.7 Structures of compounds 17 and 18. Source: Reproduced from [Arai et al. \(2014\)](#).

mimic), and phenoxy (Phe side-chain mimic) groups arranged at positions 2, 4, and 6 of a pyridine ring core, respectively (Fig. 6.7) ([Arai et al., 2014](#)). Using ThT and AFM experiments, the compound was found to inhibit $A\beta_{42}$ aggregation. In ThT experiments, fivefold excess of compound 17 coincubated with $A\beta_{42}$ at 10 μ M decreased the fluorescence intensity to $\sim 71\%$ relative to $A\beta_{42}$ alone. A follow-up analog, termed compound 18 (Fig. 6.7), decreased the fluorescence to $\sim 47\%$. In comparison, the parent peptide cyclo-[klvff] and its analog cyclo-[klaff] reduced ThT fluorescence to 39 and 58%, respectively.

D-PEPTIDES

D-Peptides are less susceptible to enzymatic degradation than native L-peptides, making them attractive candidates for drug discovery; for a recent review in the context of AD, see [Kumar and Sim \(2014\)](#). In that review, the authors categorized the D-peptides into two types ([Kumar and Sim, 2014](#)): (1) those preventing fibril formation, such as an 11-residue peptide called D-iA β 1 (rdlpffvpid) prepared by [Soto et al. \(1998\)](#), D versions of $A\beta(16-20)$ or $A\beta(16-22)$, and derivatives thereof, and (2) those promoting the formation of stable, nontoxic, off-pathway assemblies, including 12-residue D-peptides, qshyrhispaqv and rprtrlhthnr, discovered through screening of a mirror-image phage display (see section: Peptide Library Screening), and the short pentapeptide, SEN304, discussed in section: N-Methylated Peptides. However, this division likely is misleading and simply based on incomplete information with regard to the first class. Inhibition of fibril formation typically yields nonfibrillogenic assemblies, such as those defined in the second class, but this may have not been characterized in early studies that simply reported inhibition of fibril formation without asking what the resulting structures were.

Austen et al. designed an RI peptide (RI-OR2, Ac-[rGffvlkGr]-NH₂) ([Taylor et al., 2010](#)) based on a previously described inhibitor of $A\beta$ oligomer formation (OR2, RGKLVFFGR-NH₂) ([Austen et al., 2008](#)). RI-OR2 was highly stable to proteolysis and completely resisted degradation in human plasma and brain extracts, in contrast to the parent peptide, OR2,

which was almost completely degraded under the same conditions. RI-OR2 blocked the formation of oligomers and fibrils of A β 40 or A β 42, as assessed by ThT fluorescence, an immunoassay for A β oligomers, sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) fractionation of “stable oligomers,” and AFM, and was more effective against A β 42 than against A β 40. In SPR experiments, RI-OR2 bound to immobilized A β 42 monomers and fibrils, with $K_d = 9\text{--}12\ \mu\text{M}$, and also inhibited A β 42 fibril extension. In two different cytotoxicity assays, lactate dehydrogenase (LDH) release and MTS, RI-OR2 reduced A β 42 toxicity significantly in SH-SY5Y cells.

In subsequent experiments, an RI version of the HIV protein transduction domain TAT was attached to RI-OR2, with the goal to target this new inhibitor, RI-OR2–TAT (Ac-[rGffvlkGrrrrqrkkkGy]–NH₂), into the brain (Parthasarathy et al., 2013). The fluorescein-tagged peptide crossed the blood–brain barrier, and bound to amyloid plaques and to activated microglial cells present in the cerebral cortex of 17-month-old APP_{SWE}/PS1 Δ E9 transgenic mice—PSAPP mice bearing exon-9-deleted presenilin 1 and the Swedish APP_{K595N/M596L} mutations (Jankowsky et al., 2001, 2004). Daily intraperitoneal injection of 266 mg/kg RI-OR2–TAT for 21 days into 10-month-old APP_{SWE}/PS1 Δ E9 mice resulted in a 25% reduction ($p < 0.01$) of A β oligomer levels in the cerebral cortex detected by a sandwich ELISA after ultracentrifugation at 100,000g at 4°C for 1 h to remove fibrils. The treatment also led to significant 32% reduction in amyloid plaque count detected by A β immunostaining, 44% reduction in the number of activated microglial cells shown by Iba-1 immunostaining, and 25% reduction in oxidative damage shown in 8-oxo-guanine immunostaining, whereas the number of young neurons in the dentate gyrus was significantly increased by 210%.

Jagota and Rajadas (2013) examined the effects of D-peptides, pgklvya, kklvffarrrra, and kklvffa, derived from the A β CHC, on A β aggregation in vitro and on toxicity in a transgenic *Caenorhabditis elegans* model expressing human A β 42. ThT, CD, and 4,4'-bis(1-anilino-naphthalene 8-sulfonate) (bis-ANS) binding assays suggested that kklvffarrrra and kklvffa effectively inhibited A β fibrillization. Of the three peptides, pgklvya and kklvffa, but not kklvffarrrra, prolonged survival of the transgenic *C. elegans* by ~20%.

C-Terminal Fragments

The C-terminus of A β 42 is a key region controlling oligomerization (Bitan et al., 2003). A β 42 differs from A β 40 by having two extra C-terminal amino acids, Ile41 and Ala42. A β 42 is more toxic (Dahlgren et al., 2002) and its C-terminus more rigid than that of A β 40 (Yan and Wang, 2006). Discrete molecular dynamics studies suggested that oligomerization of A β 42, but not A β 40, was driven primarily by intermolecular interactions involving the C-terminal region (Ile31–Ala42) (Urbanc et al., 2004, 2010). Based on these data, our group hypothesized that peptides derived from the C-terminus of A β 42 may coassemble with the full-length peptide and thereby disrupt oligomer formation and inhibit A β 42-induced neurotoxicity. We prepared a series of CTFs with the general formula A β ($x\text{--}42$), $x = 28\text{--}39$ (Condron et al., 2008), and evaluated their capability to disrupt A β 42 assembly and neurotoxicity (Fradinger et al., 2008). A cell-viability screen identified A β (31–42) (IIGLMVGGVVIA) as the most potent inhibitor. In addition, the shortest peptide, A β (39–42) (VVIA), also had surprisingly high activity. Both A β (31–42) and A β (39–42), at micromolar concentrations, inhibited A β -induced cell death by the MTT and LDH assays in differentiated PC-12 cells and prevented disruption

of synaptic activity by $A\beta$ 42 oligomers in experiments measuring miniature excitatory post-synaptic currents in primary hippocampal mouse neurons.

Investigation of the biophysical properties of this peptide series revealed that CTFs up to eight residues long were soluble at concentrations $>100\ \mu\text{M}$ and had a low propensity to aggregate (Li et al., 2010a). Longer CTFs were soluble at $\sim 1\text{--}80\ \mu\text{M}$, and most but not all readily formed β -sheet-rich fibrils. Dynamic light scattering (DLS) experiments showed that inhibition of $A\beta$ 42-induced toxicity correlated with stabilization of small oligomers with hydrodynamic radius (R_H) $\approx 8\text{--}12\ \text{nm}$ and attenuation of formation of larger oligomers with $R_H \approx 20\text{--}60\ \text{nm}$ (Li et al., 2010b). In contrast, inhibition of $A\beta$ 42 hexamer formation, analyzed by PICUP, correlated with CTF solubility and the degree to which CTFs formed amyloid fibrils themselves, but not with inhibition of $A\beta$ 42-induced toxicity (Li et al., 2010b).

The amphipathic nature and small size of $A\beta(39\text{--}42)$ suggested that it could be used as a drug lead. Therefore, we explored the effects of structural modifications on inhibitory activity to determine the key structural features, including D-configuration, Ala-substitution, N-terminal acetylation, and C-terminal amidation (Li et al., 2011). We found that an L-configuration, bulky side chains, and a free N-terminus were important for inhibition of neurotoxicity. Thus, despite the small size of $A\beta(39\text{--}42)$ and the hydrophobic, aliphatic nature of all four side chains, the interaction of $A\beta(39\text{--}42)$ with $A\beta$ 42 was controlled by specific intermolecular contacts requiring a combination of hydrophobic and electrostatic interactions and a particular stereochemistry.

To elucidate the binding site(s) of $A\beta(39\text{--}42)$ on $A\beta$ 42, intrinsic Tyr fluorescence of $A\beta$ 42 was measured in analogs containing Tyr at positions 1, 10, 20, 30, or 40, in the presence or absence of $A\beta(39\text{--}42)$. In addition, solution-state nuclear magnetic resonance (NMR), as well as $^{15}\text{N}\text{--}^1\text{H}$ and $^{13}\text{C}\text{--}^1\text{H}$ heteronuclear single-quantum coherence (HSQC), were used to study the interaction between $A\beta(39\text{--}42)$ and $A\beta$ 42. Both Tyr fluorescence and NMR data suggested that $A\beta(39\text{--}42)$ bound at several sites, of which the predominant one was located at the $A\beta$ 42 N-terminus. These data agreed with modeling studies (Urbanc et al., 2011) predicting that $A\beta(39\text{--}42)$ bound to several regions of $A\beta$ 42, including the Ala2–Phe4 region. The findings of predominant binding sites at the $A\beta$ 42 N-terminus were surprising and did not agree with our original rationale for preparing CTFs, which postulated that they would interrupt the intermolecular association of $A\beta$ C-termini into a hydrophobic oligomer core.

Interestingly, ion-mobility spectroscopy–mass-spectrometry (IM–MS), ThT fluorescence, and TEM experiments indicated that $A\beta(39\text{--}42)$ disrupted the early assembly of full-length $A\beta$ 42, but did not prevent fibril formation (Gessel et al., 2012). Specifically, the IM–MS results showed that $A\beta(39\text{--}42)$ prevented the formation of large decamer/dodecamer $A\beta$ 42 species. At the same time, ThT fluorescence and TEM results showed that the CTF did not inhibit fibril formation, lending strong support to the hypothesis that oligomers and not amyloid fibrils are responsible for toxicity.

These data recently were corroborated by a study of $A\beta(39\text{--}42)$ analogs in which either the N-terminus was acetylated or the C-terminus was amidated (Zheng et al., 2015b). Previously, acetylation of the N-terminus was found to abrogate the inhibitory activity of the peptide against $A\beta$ 42-induced toxicity, whereas amidation of the C-terminus yielded an active analog (Li et al., 2011). The new study used IM–MS and TEM, and showed that the difference between these two analogs was subtle. Both disrupted formation of $A\beta$ 42 dodecamers, but they bound to different regions and different assembly sizes of $A\beta$ 42. Similarly to the parent peptide, neither analog affected fibril formation (Zheng et al., 2015b).

FIGURE 6.8 A two-step strategy for identifying positions sensitive to *N*-methylation. In the first step, each residue is replaced by *N*-Me-Ala. In the second step, in positions found sensitive to the substitutions, the original side chain is restored, leaving the *N*-Me group intact.

As mentioned earlier, *N*-methylation helps inhibit aggregation of peptides and often yields highly soluble analogs due to disruption of hydrogen bonding and β -sheet formation. We used this approach to increase the solubility of A β (31–42), which was the strongest inhibitor of A β 42-induced toxicity in our original study (Fradinger et al., 2008), using a two-step substitution strategy (Li et al., 2012). In the first step, to achieve a systematic structural study and to improve aqueous solubility, each residue was replaced by NMe-Ala. In the second step, to distinguish between the effects of side-chain minimization and *N*-methylation, side chains with substantial effects on activity were reintroduced, while keeping the *N*-Me moiety in that position (Fig. 6.8). We found that introduction of *N*-Me amino acid substitution at position 9 or 11 improved the aqueous solubility and increased the inhibitory activity relative to the parent peptide. The data suggested that inhibition of A β 42 toxicity by short peptides was highly structure specific, providing a basis for the design of new peptidomimetic inhibitors with improved activity, physicochemical properties, and metabolic stability.

The effect of peptides derived from A β (32–37), with none, two, three, or five *N*-methylated amino acids (Fig. 6.9) on A β 40 and A β 42 aggregation was assessed using CD (Pratim Bose et al., 2009). Deconvoluted A β 42 CD spectra in the absence or presence of inhibitors showed that peptide 4 (containing five *N*-methylated amino acids) increased unordered structures significantly. Peptide 4 also reduced the formation of Congo red (CR)-positive assemblies of A β 40 or A β 42 in agreement with the CD results. Peptides 1–3 (Fig. 6.9) were less effective than peptide 4 in this assay. The authors also found that increasing the extent of *N*-methylation improved the efficiency of the peptides in inhibiting A β -mediated toxicity in PC-12 cells in the MTT assay (Pratim Bose et al., 2009). The tri-*N*-methylated and penta-*N*-methylated peptides were the strongest inhibitors, and the latter protected the cells to the same level as the untreated cells (Pratim Bose et al., 2009). In a *Drosophila melanogaster* model expressing A β 42 in the CNS, 300 μ M peptide 4 increased the longevity by 13% relative to untreated flies ($n \approx 100$, $p = 0.004$) and improved locomotion. This effect was specific to the action of the peptide on A β 42 because no effect on life span was observed in peptide-treated wild-type *Drosophila* (Pratim Bose et al., 2009).

Peptides derived from the region A β (31–34), including Pr-IIGLa (propionyl-IIGL-NH₂) and RIIGLa (RIIGL-NH₂), were studied by Penke and coworkers (Fülöp et al., 2004; Harkany et al., 1999; Laskay et al., 1997; Szegedi et al., 2005). The two peptides were tested in CR-binding assay to study whether they could influence the process of A β 42 aggregation. RIIGLa strongly inhibited CR binding to A β 42 fibrils, and was more effective than Pr-IIGLa. Fivefold

FIGURE 6.9 Structure of N-methylated $A\beta(32-37)$ peptides. Source: Reproduced from Pratim Bose et al. (2009).

molar excess RIIGLa completely inhibited $A\beta_{42}$ fibril formation over a 6-day incubation with $A\beta_{42}$ as observed by TEM (Fülöp et al., 2004). RIIGLa inhibited $A\beta_{42}$ -induced cytotoxicity significantly using the MTT assay in SH-SY5Y cells. In contrast, Pr-IIIGLa itself was found to be toxic and thus did not show any protective effect (Fülöp et al., 2004). Electrophysiological experiments in rat brain slices demonstrated that RIIGLa prevented the attenuation of field excitatory postsynaptic potential by $A\beta_{42}$ (Szegedi et al., 2005).

Peptides and Peptidomimetics From Other Sources

Mimicking the Binding Domain of A β -Binding Proteins

Due to A β 's propensity to self-assemble, A β -derived peptides are highly likely to interact with A β and interrupt its aggregation, as discussed in the preceding section. By the same token, peptides mimicking, or derived from, the A β -binding regions of A β ligands would be expected to have a high potential to interact with A β and inhibit its aggregation and toxicity.

Transthyretin, a stably folded homotetrameric protein abundant in the plasma and CSF, has been shown to inhibit A β aggregation and reduce its toxicity (Buxbaum et al., 2008). Strand G of the inner β -sheet of transthyretin was identified and characterized as a specific A β -binding domain (Cho et al., 2014). A 12-mer peptide, TIAALLSPYSYS, corresponding to transthyretin residues 106–117, was identified as the strongest A β binder of all the contiguous sequences tested (Cho et al., 2014). The authors further studied the minimal sequence length required for A β 40 binding and single-amino-acid substitution using this peptide as a starting point to identify individual amino acids required for binding. These experiments eventually led to a modified and somewhat longer peptide, G16 (PRRYTLAALLSPYSWS), and control peptides, which were synthesized and studied in A β 40-binding and cytotoxicity assays. G16 was found to interact with A β 40 in PICUP experiments, in which a smear of oligomers (~30 to ~100 kDa) was observed in G16–A β 40 mixtures but not in A β alone, indicating that G16 bound A β 40 and promoted its self-assembly. These observations were corroborated by DLS data showing that G16 dramatically increased the average size and growth rate of A β 40 aggregates. At the same time, G16 moderately inhibited A β 40 fibrillization observed by TEM and reduced cross- β -sheet formation in ThT experiments, suggesting that it promoted formation of large nonfibrillar aggregates. G16 inhibited A β 40 toxicity dose dependently in primary cortical neurons examined by the MTS assay. Inhibition of cytotoxicity by G16 was less potent than by the recombinant human transthyretin mutant F87M/L110M, which inhibited A β 40 oligomerization, rather than enhancing A β 40 aggregation. The study introduced a general strategy for designing inhibitors of A β toxicity. Other proteins, such as cystatin C or clusterin, also are present in the CSF and bind A β oligomers. Thus, peptides derived from these proteins may also protect against A β -induced cytotoxicity.

Peptides Sharing Common Structural Features With A β Fragments

A peptide or a protein that shares with A β fragments such characteristics as hydrophobicity and propensity to incorporate into β -sheet structures may interact with, and inhibit, A β 42 aggregation. Based on this rationale, Teplow and coworkers described a θ -defensin analog, a 13-residue peptide hairpin, called PP-Leu, as a potential inhibitor of A β 42 oligomerization and fibrillization. The PP-Leu structure was stabilized by a C₂–C₁₃ disulfide bond and a type II β -turn formed by a D-P₇–L-P₈ moiety (Fig. 6.10) (Yamin et al., 2009). PP-Leu lowered the rate of A β 42 assembly in a concentration-dependent manner in ThT experiments. In a

FIGURE 6.10 Structure of PP-Leu. Source: Reproduced from Yamin et al. (2009).

mixture containing respective 5:1 or 10:1 molar ratios of PP-Leu and $A\beta$ 42 ThT, fluorescence did not increase significantly up to 300 h, whereas under the same conditions, $A\beta$ 42 alone displayed a rapid increase in ThT fluorescence which plateaued after $\sim$ 24 h. Addition of fivefold PP-Leu excess to preformed $A\beta$ 42 fibrils resulted in an immediate and significant drop in ThT fluorescence. In this case, a mesh-like assembly instead of fibrils was observed by TEM. PICUP experiments showed that fivefold molar excess PP-Leu blocked $A\beta$ 42 paranucleus formation completely and produced an oligomer size distribution similar to that of noncrosslinked $A\beta$ 42.

Rationally Designed Small Peptides

Gazit and coworkers demonstrated that a hybrid molecule composed of an aromatic moiety and α -amino-isobutyric acid (Aib) inhibited $A\beta$ fibrillization (Frydman-Marom et al., 2009). The dipeptide D-Trp-Aib interacted with early and intermediate $A\beta$ assemblies and inhibited their further growth into toxic oligomers. Intraperitoneally administered D-Trp-Aib reduced the extent of cerebral amyloid deposits and improved cognitive performance in the Morris water maze in male APP_{SweLon} mice. These mice overexpress human APP containing the Swedish double mutation (K670M/N671L) and the London mutation (V717I) (Lamb et al., 1997). D-Trp-Aib was completely stable in both mouse and human sera for 24 h as determined by LC-MS/MS, and showed excellent bioavailability upon oral (39%) and intranasal (55%) administration to mice (Frydman-Marom et al., 2009).

Liu et al. (2014) identified a decapeptide inhibitor of $A\beta$ 40 aggregation, nicknamed RR (RYYA AFFARR). They reasoned that the relatively weak inhibitory activity of some CHC β -sheet breakers, which merely prolong the lag time of $A\beta$ aggregation but do not completely inhibit the aggregation, may be due to low affinity of these agents for $A\beta$. RR was designed by using molecular simulations targeting the $A\beta$ (11–23) sequence, which was expected to bind $A\beta$ 40 by hydrophobic and electrostatic interactions and hydrogen bonding. RR had substantially stronger affinity for $A\beta$ 40 ($K_d = 1.1 \mu\text{M}$) than i $A\beta$ 5 ($K_d = 156 \mu\text{M}$) by SPR. TEM showed that RR inhibited $A\beta$ 40 fibrillization completely at fourfold molar excess, and by $\sim$ 75% at equimolar concentration. In the MTT assay, when used at fourfold molar excess to $A\beta$ 40, RR reduced $A\beta$ 40-induced cytotoxicity and rescued the viability of differentiated PC-12 cells to $\sim$ 95% of untreated cells.

De Novo Design

Computer-aided modeling and characterization of the binding sites of a target protein could be used to construct an “ideal drug” by docking experiments using the target binding sites and evaluating their interactions, provided that a stable structure exists and is known. The de novo design method using this strategy has been applied broadly in peptide-based drug discovery campaigns of anticancer, antiangiogenic, and antiinflammatory agents (Kliger, 2010). In the case of $A\beta$, applying this strategy is challenging because the most toxic species, $A\beta$ oligomers, do not have a stable structure. Nonetheless, the popularity of the approach has motivated some researchers to explore ways of modeling the interaction between potential inhibitors and $A\beta$, mostly based on the common structural motifs of $A\beta$ fibrils.

Wang et al. (2014a) developed a hybrid, high-throughput computational method to screen and design hexapeptides against $A\beta$ aggregation and toxicity. Considering that all amyloid peptides have a strong self-aggregating propensity and share common β -sheet structural

characteristics, they proposed a “like-interacts-like” hypothesis to design peptide-based amyloid inhibitors. They combined bioinformatics, molecular dynamics simulations, and in vitro experiments to test the principles they used for selecting new peptide-based aggregation inhibitors from a large pool of sequences, unrelated to the A β sequence. Of the 8000 sequences with self-assembling propensity predicted by quantitative structure–activity relationship, they randomly selected 35 hexapeptides with predicted high, moderate, or low self-assembly propensity determined by molecular dynamics simulations. The simulations showed that 11 out of 35 peptide dimers had relatively high structural stability. Of the 11 selected hexapeptides, three different effects (inhibition, promotion, or no effect) were observed on A β 42 fibrillization using the ThT fluorescence assay and AFM, but their effects on A β 42 fibrillization did not correlate with their binding affinity for A β 42 measured by SPR. In cell-culture experiments, three moderate-to-high-affinity peptides (CILFWG, CTLWWG, CTIYWG) strongly suppressed A β -induced cytotoxicity in SH-SY5Y cells in the MTT assay.

Peptide Library Screening

Using phage display, combinatorial chemistry, or existing/virtual libraries, a large number of peptides could be produced for drug screening. If an efficient high-throughput screening method were established, the library screening method would be a powerful tool for finding potential drug candidates with structural variation (Bratkovic, 2010; Lazo and Wipf, 2000; Smith, 1985). With that in mind, a variation of a novel protein-fragment-complementation assay was used to generate and screen peptide libraries in bacterial cells (Acerra et al., 2013). A β (25–35) was used in the design scaffold as the target, and the selection peptide library was based on A β (29–35), a peptide known to self-associate into toxic fibrils (Hughes et al., 2000; Hung et al., 2008; Pike et al., 1995). The A β (25–35) sequence was fused to one half of an essential murine enzyme, dihydrofolate reductase, and the peptide library fused to the other half of the same enzyme. Following expression in *Escherichia coli*, binding of a library component to A β (25–35) could reconstitute murine dihydrofolate reductase, which was used as an indicator of expression, and result in bacterial growth and colony formation under the selection conditions. Using this protein-fragment-complementation assay and a library containing 8000 peptides led to selection of an initial pool of sequences interacting with A β (25–35). A second library of 160,000 peptides based on the first hit (GAKATLM) as a design scaffold yielded two additional novel interacting sequences (L2P1, FSKATSN, and L2P2, PVKATTA). These peptides shared no homology with the starting A β (29–35) design template. All selected peptides bound A β 42, inhibited its fibril formation, and disaggregated preformed fibrils. In follow-up work, Acerra et al. (2014) generated RI analogs of these peptides to increase their metabolic stability. The RI peptides KAR-R1 (mltakag–NH₂), L2P1a-RI (nstaksf–NH₂), L2P1b-RI (pagnstaksfsa–NH₂), L2P2a-RI (attakvp–NH₂), and L2P2b-RI (ragattakvp-sa–NH₂) blocked fibrillization and disaggregated A β fibrils efficiently, similarly to the parent peptides, as shown by ThT fluorescence, CD spectroscopy, and oblique angle fluorescence microscopy. They also modestly reduced A β 42-induced cytotoxicity in PC-12 cells in the MTT assay and restored locomotion in a *Drosophila* model of A β toxicity (Acerra et al., 2014).

Biyani et al. (2014) applied a progressive library method to obtain A β 42-binding peptide aptamers. In the primary and secondary library selections, peptides with a moderate binding affinity to A β 42 (K_d in the micromolar range) were found (Tsuji-Ueno et al., 2011). Then, the peptides were further evolved by the paired-peptide method (Ghimire Gautam et al., 2014),

in which the library was constructed by random shuffling of peptide blocks selected from the primary and secondary libraries. The evolved peptide aptamers contained two peptide moieties, which were combined arbitrarily through a linker sequence. The selected two peptides (P84, CGILDPIPWGGSGGSCGILDPIPW, and P131, GCPCIGIIGGSGGSDCSSDLTPS, where GGS GGS is a linker) had higher binding affinity for $A\beta$ 42 ($K_d = 20$ nM and 12 nM, respectively), as determined by SPR experiments, than the primary and secondary $A\beta$ 42-binding peptides. Both peptides at 10 μ M inhibited $A\beta$ 42 (100 μ M) fibrillization as shown by ThT fluorescence and AFM experiments. P84 inhibited $A\beta$ 42 oligomerization evidenced by AFM experiments. Both P84 and P131 inhibited the toxic effects of $A\beta$ 42 fibrils and $A\beta$ 42 oligomers, yet P84 was significantly more effective than P131, in PC-12 cells using the cell-counting kit-8, CCK-8, and water-soluble tetrazolium salt-8 assays.

Kawasaki et al. (2010) constructed a random heptapeptide library (XX-P-XXXX) using phage display to search inhibitors specific against formation of globulomers, which are 37–48 kDa oligomers of $A\beta$ 42 formed in the presence of SDS. Pro was introduced into the sequences as a β -sheet-breaker element. An antibody raised against globulomers showed increased reactivity in the brains of patients with AD versus healthy individuals, suggesting that globulomers or similar structures exist in the AD brain (Barghorn et al., 2005). Eight peptides selected after the fifth round of panning revealed that a common feature was the presence of Arg residues. The strongest binder (RGPRGRV) was used in an inhibition test against formation of globulomers. Using SDS-PAGE and SEC, the peptide strongly inhibited formation of globulomers, but did not affect formation of low-molecular-weight oligomers. To reduce the peptide length, 3-residue and 4-residue random libraries prepared by phage display were tested. The 3-residue peptides did not significantly inhibit globulomer formation, likely because they were too small. In contrast, the tetrapeptide RFRK inhibited globulomer formation similarly to the best heptapeptide. Interestingly, RFRK had little effect on $A\beta$ fibrillization (Kawasaki et al., 2011), similarly to the activity of SEN304 (Kokkoni et al., 2006) or $A\beta$ (39–42) and its analogs (Li et al., 2011; Zheng et al., 2015b). To discover a small molecule that might inhibit formation of both $A\beta$ 42-derived globulomers and fibrils, Kawasaki et al. then searched for Arg-containing small molecules and tested their inhibitory activity against $A\beta$ 42 aggregation by SDS-PAGE and ThT fluorescence assay. The search yielded Arg-Arg-7-amino-4-trifluoromethylcoumarin (RR-AFC) (Fig. 6.11), which was found to inhibit $A\beta$ 42-derived globulomer formation (37- and 48-kDa bands on SDS-PAGE) at fivefold excess and was a weak inhibitor of $A\beta$ 42 aggregation at the same concentrations in the ThT fluorescence assay (Kawasaki and Kamijo, 2012).

FIGURE 6.11 Structure of Arg-Arg-7-amino-4-trifluoromethylcoumarin (RR-AFC).

Luo et al. (2013) synthesized an on-bead library consisting of 38,416 unique peptoids, which are *N*-substituted glycine oligomers (Fig. 6.3), to develop selective and high-affinity A β 42 ligands. The generated peptoid library was screened and arrays of A β 42-selective peptoid ligands were identified. One of those ligands, IAM1, was evaluated further by different biochemical assays. ELISA experiments determined that IAM1 was approximately 10-fold more selective for A β 42 than for A β 40. IAM1 inhibited A β 42 aggregation in a concentration-dependent manner in the ThT assay. To increase the binding affinity, the authors prepared a dimeric form of IAM1, designated (IAM1)₂. Using the same ELISA, a K_d value = 0.06 ± 0.04 μ M for A β 42 was determined, indicating a 7.4-fold increase in affinity relative to the monomeric IAM1. However, affinity for A β 40 was also increased, resulting in some loss of specificity. (IAM1)₂, but not IAM1, protected primary hippocampal neurons against A β 42-induced toxicity. In this experiment, mouse hippocampal neurons were incubated for 3 days with A β -containing conditioned media (Luo et al., 2013) and then fixed, permeabilized, and stained for the neuronal marker MAP2. A 73% decrease in neuronal viability was found in neurons incubated with A β compared to control cells incubated with media alone.

Rahimipour and coworkers hypothesized that due to the strong similarities between self-assembled cyclic D,L- α -peptides and amyloids, such peptides likely would bind to, and stabilize, nontoxic forms of A β (Richman et al., 2013). By screening a focused library of 6-residue head-to-tail cyclic D,L- α -peptides containing Lys, Glu, Ser, Leu, Trp, and His, and by optimizing the activity of the lead peptide, the authors discovered the peptide CP-2, cyclo-[I-I-w-H-s-K] (I denotes L-norleucine), which interacted strongly with A β 40 or A β 42 and inhibited their aggregation. By TEM, ThT fluorescence, and cytotoxicity assays, CP-2 inhibited both A β 40 and A β 42 assembly, entirely disassembled preformed fibrillar A β 40, and protected PC-12 cells from A β 40-induced or A β 42-induced toxicity, without showing toxicity itself. NMR spectroscopy indicated that CP-2, likely in a self-assembled form, interacted with monomeric and low-oligomeric A β 40 and induced weak secondary structures (α -helix) in A β 40 during the initial stage of aggregation, followed by a shift to a parallel β -sheet structure. The NMR data suggested that CP-2 might promote the conformational transition of A β from a more toxic antiparallel β -sheet to the less toxic parallel β -sheet.

Conclusions

During the past 20 years, peptide-based A β protein aggregation inhibitors were discovered using many sources and strategies, including A β fragments, sequences sharing common structural features with A β , sequences mimicking the binding domain of A β -binding proteins, de novo designed peptides, and library screening. Sequences based on A β itself have been the most popular method, especially those derived from the central hydrophobic region. Regardless of the chosen strategy, peptides made of natural amino acids serve as leads but require subsequent optimization due to poor bioavailability and metabolic stability. Introduction of unnatural amino acid, backbone modification, cyclization, and unnatural secondary structural mimics are popular directions to pursue. Currently, there are no guidelines or algorithms that can predict what modification is likely to succeed. Rather, modifications typically are screened and sometimes combined with other strategies. Structural modification directions are all geared to fulfill biological and biophysical criteria. Therefore, correct evaluation of the structure–activity relationship is important for optimization of the compounds so

they can best fulfill these criteria. Reviewing the methods used by different labs reveals large variations in sample preparation and testing methods, which often makes comparison of results difficult. We therefore would like to emphasize the need for standardized protocols for $A\beta$ preparation for biophysical and biological tests, and for standard positive controls, which may allow comparing the activities of inhibitors from different laboratories.

$A\beta$ ASSEMBLY MODULATORS DERIVED FROM NATURAL SOURCES

Molecules derived from natural foods likely are the largest group of nonpeptidic compounds explored as modulators of $A\beta$ assembly. These “nutraceutical” compounds are appealing because they have been consumed for thousands of years and therefore are expected to be safe and bioavailable. Compounds from sources including tea, coffee, wine, or spices such as turmeric or cinnamon are attractive due to their abundance and the perception that consuming them may begin immediately, without the need for approval by regulatory agencies, and be beneficial and protective in one’s lifetime. It is important to note, however, that the safety and bioavailability of *purified* compounds likely differ from their safety and bioavailability in the context of a consumed beverage or spice. Not only can the dose of a pure compound taken, for example, as a dietary supplement be substantially different from the dose consumed through food intake, but also the combination or interaction of the compound with other alimentary ingredients, which is what we have become accustomed to through thousands or millions of years of evolution, may be required to elicit a desirable outcome.

In this chapter, we have classified the nutraceutical compounds into five groups: polyphenols, inositol derivatives, vitamins, natural proteins or peptides (as opposed to the synthetic peptides discussed in section: Peptidic or Peptidomimetic Modulators of $A\beta$ Assembly), and other compounds. The examples we discuss are summarized in [Table 6.2](#).

Polyphenols

Polyphenols are organic compounds with multiple phenol groups in their structure. They are a major component of the naturally occurring, plant-derived phytochemicals. Polyphenols have received much attention in health research because they are free-radical scavengers and antioxidants. More than 8000 naturally occurring polyphenols have been categorized as flavonoids, phenolic acids, lignans, and stilbenes. Some polyphenols that inhibit $A\beta$ assembly and/or toxicity are discussed next.

Curcumin and Curcumin Derivatives

Curcumin (diferuloylmethane, [Fig. 6.12A](#)) is a natural compound that has been studied extensively among potential therapeutics for AD. It is the principal antioxidant component of the spice turmeric (*Curcuma longa*) and is also responsible for its yellow color. It was first demonstrated by [Lim et al. \(2001\)](#) that curcumin substantially reduced $A\beta$ plaque burden in the transgenic Tg2576 mouse model of AD ([Hsiao et al., 1996](#)). This mouse model bears the isoform 695 of the human APP gene containing the double mutation K670N/M671L (Swedish mutation) under the control of the hamster prion protein ([Hsiao et al., 1996](#)). [Lim et al. \(2001\)](#) used 24 mg or 744 mg of curcumin per kilogram of body weight and monitored inflammation,

TABLE 6.2 Modulatory Effects of Naturally Sourced Compounds on A β Assembly and Toxicity

Compound	Type	In vitro, cell culture, and ex vivo studies		Animal studies		
		Inhibition of A β -induced cytotoxicity	Modulation of A β assembly	Decrease of pathological A β	Effect on behavioral deficits	Human studies/ clinical trials
Curcumin and derivatives	Polyphenol	Yes	Prevent oligomerization	Reduce plaques in model mice; stain plaques; FMeC1 binds plaques in model mice	Improve life span and locomotion in flies; improve spatial memory and learning in mice; nanoparticle-conjugated preparations reverse learning and memory deficits in a rat model of AD	Curumin C3 Complex® used in trials with minimal effect due to poor bioavailability
Inositol derivatives	Sugars	Yes and rescue LTP in mouse hippocampal slices	Some isoforms cause micelle formation whereas others cause β -sheet formation	Reduce plaque burden in mice	Improve learning in rats; improve cognitive deficits in mice; fluorinated derivatives improve spatial memory in mice	Clinical trials inconclusive because of toxicity at high doses and lack of effectiveness at low doses
EGCG	Polyphenol	Yes	Causes formation of nontoxic assemblies; reduces A β levels in cultured cells	Reduces A β oligomerization and aggregation in a <i>C. elegans</i> model; reduces A β 42 levels in the hippocampus of a mouse model measured by ELISA	Rescues cognitive deficits in senescence-accelerated mice by intragastric administration (5 and 15 mg/kg) for 60 days; improves learning and memory in APP/PS1 mice	A phase 2/phase 3 trial is ongoing with EGCG formulation Sunphenon; results expected in 2015
Brazilin	Polyphenol	Yes	Inhibits fibrillization by redirecting the misfolding pathway toward nontoxic assemblies	Unknown	Unknown	Unknown

(Continued)

TABLE 6.2 Modulatory Effects of Naturally Sourced Compounds on A β Assembly and Toxicity (*cont.*)

Compound	Type	In vitro, cell culture, and ex vivo studies		Animal studies		
		Inhibition of A β -induced cytotoxicity	Modulation of A β assembly	Decrease of pathological A β	Effect on behavioral deficits	Human studies/ clinical trials
Myricetin	Flavonoid	Yes and inhibits synaptotoxicity in hippocampal slices	Interferes with oligomerization	No effect on plaque burden but reduces levels of soluble A β and A11-positive oligomers	Unknown	Unknown
Quercetin and derivatives	Flavonoid	Inhibit cytotoxicity and synaptotoxicity	Inhibit aggregation	Reverse AD pathology	Restore spatial memory in an AD model	Unknown
Taxifolin	Flavonoid	Yes	Inhibits aggregation	Unknown	Unknown	Unknown
Rosmarinic acid	Similar to polyphenols	Yes	Inhibits A β fibrillization and oligomerization; disintegrates preformed fibrils	Decreases A β deposition in brain of a mouse model	Unknown	Unknown
Tannic acid	Similar to polyphenols	Yes	Inhibits A β fibrillization; disintegrates preformed fibrils	Reduces brain parenchymal and cerebral vascular A β deposits; reduces A β oligomers in a mouse model	Prevents behavioral impairment, including hyperactivity, decreased object recognition, and defective spatial reference memory in a mouse model of AD	Unknown
Theaflavins	Polyphenol	Yes	Remodel preformed toxic A β fibrils into nontoxic spherical assemblies	Unknown	Unknown	Unknown

<i>Ginkgo biloba</i>	Mixture of flavonoids and terpenoids	Inhibits A β -induced cytotoxicity in cultured cells, primary hippocampal neurons, and in <i>C. elegans</i>	Inhibits formation of A β -derived diffusible ligands	Did not affect plaque burden	Recovers spatial memory deficits in mice	Rates of decline of memory, attention, visuospatial abilities, language, and executive functions did not differ between treated and placebo groups
Vitamin A	Vitamins	Yes	Inhibits oligomerization, fibrillization, and fibril extension; disintegrates preformed fibrils	Reduces A β deposition	Rescues memory deficits in two mouse models of AD	Dietary supplementation has shown improvement of clinical symptoms of AD
Vitamin C	Vitamins	Yes	Suppresses A11-positive A β oligomers in cultured cells	Supplementation reduces A β plaque deposition in a rodent model deficient of vitamin C	Improves spatial memory in a mouse model of AD	Inconclusive
Vitamin E	Vitamins	Yes	α -Tocopherolquinone, a derivative of vitamin E, inhibits A β 42 fibrillization, disaggregates preformed fibrils, and interferes with intracellular A β oligomerization	Vitamin E administered at a younger age caused a significant reduction in A β levels and amyloid deposition in mouse model of AD, but mice supplemented with vitamin E at a later age did not show any significant difference from placebo	Combination with vitamin C supplementation rescued behavioral deficits in a mouse model of AD	Inconclusive

(Continued)

TABLE 6.2 Modulatory Effects of Naturally Sourced Compounds on $A\beta$ Assembly and Toxicity (*cont.*)

Compound	Type	In vitro, cell culture, and ex vivo studies		Animal studies		
		Inhibition of $A\beta$ -induced cytotoxicity	Modulation of $A\beta$ assembly	Decrease of pathological $A\beta$	Effect on behavioral deficits	Human studies/ clinical trials
Colostrinin	Polypeptide	Yes	Changes fibril morphology	Unknown	Improves life span and motor and sensory parameters in a mouse model	Improves outcomes in patients with mild or moderate dementia
Laminin	Polypeptide	Yes	Inhibits aggregation; disaggregates preformed fibrils	Unknown	Unknown	Unknown
Docosahexaenoic acid (DHA)	Miscellaneous	No. Forms toxic oligomers. Some studies suggest DHA inhibits and reverses formation of toxic $A\beta$ oligomers	Prevents fibrillization by stabilizing $A\beta$ protofibrils	Reduces $A\beta$ plaque density	Improves cognitive function in a mouse model	Unknown
Melatonin	Miscellaneous	Yes	Inhibits $A\beta$ aggregation	Decreases $A\beta$ in organotypic cultures; improves neuroplasticity in a rat model of sporadic AD; attenuates $A\beta$ pathology	Improves memory in mouse models of AD	A prolonged-release formulation was shown to improve cognitive function and sleep maintenance in AD patients compared with placebo

FIGURE 6.12 Chemical structures of the enol (A) and keto (B) forms of curcumin, and of the curcumin derivative FMeC1 (C).

oxidative damage, and plaque pathology in the treated model mice. Curcumin reduced insoluble A β by 43–50% without affecting APP levels. In the same year, curcumin derivatives (curcumin, demethoxycurcumin, and bisdemethoxycurcumin) also were reported to prevent A β -induced toxicity in cultured cells (Kim et al., 2001).

These initial results inspired many follow-up studies, which investigated the effects and mechanisms of action of curcumin in relation to AD (Balasubramanian, 2006; Banerjee, 2014; Garcia-Alloza et al., 2007; Kumaraswamy et al., 2013; Masuda et al., 2011; Mithu et al., 2014; Ngo and Li, 2012; Thapa et al., 2013; Yang et al., 2005a; Zhao et al., 2012). Curcumin was found to have antioxidant, antiinflammatory, neurogenic, neuroprotective, and immunomodulatory effects along with modulatory effects on A β oligomerization and aggregation. During in vitro aggregation, curcumin inhibited A β 40 aggregation (IC_{50} = 0.8 μ M) and disaggregated fibrillar A β 40 (IC_{50} = 1 μ M). It also prevented A β 42 oligomerization and cytotoxicity between 0.1 and 1 μ M (Yang et al., 2005a; Ye and Zhang, 2012).

Curcumin crossed the blood–brain barrier and stained A β plaques, further demonstrating direct binding to A β (Yang et al., 2005a). Ab initio density functional theory studies showed that penetration of curcumin through the blood–brain barrier and curcumin binding to A β plaques were facilitated by critical hydrophobic–hydrophilic balance involving the polar enolic and two phenolic moieties separated by an aliphatic bridge (Balasubramanian, 2006). The polar enol form of curcumin is favored structurally over the keto form (Fig. 6.12A) when crossing the blood–brain barrier and when binding to A β .

In a transgenic *Drosophila* model of A β , toxicity, life span, and locomotion were improved by 75% in flies treated with curcumin compared to control flies. Interestingly, this effect did

not correlate with a significant decrease in $A\beta$ deposition. In fact, flies treated with curcumin had accelerated $A\beta_{42}$ deposition, suggesting that faster kinetics of fibrillization and lower steady-state concentrations of toxic low-molecular-weight assemblies were responsible for the improved life span and phenotype (Caesar et al., 2012).

An accelerating effect on $A\beta$ aggregation has been observed in many additional studies. However, the therapeutic potential of this effect in AD is questionable because, unlike the human disease, most animal models do not develop cerebral amyloid angiopathy (CAA), which would be exacerbated by accelerated $A\beta$ aggregation, potentially leading to microhemorrhage, increasing the risk of vascular dementia (Thal et al., 2008).

Curcumin interacts with $A\beta$ at several sites. In monomeric $A\beta_{40}$, two-dimensional, 1H - ^{15}N HSQC NMR spectroscopy showed that curcumin interacts with Arg5, Ser8, Gly9, His13, Lys16, Leu17, Asp23, and Ile31 (Ono et al., 2012). Solid-state NMR studies of curcumin-induced $A\beta_{42}$ aggregates indicated major structural changes in the Asp23–Lys28 salt bridge and near the C-terminus, similar to structural changes in the presence of Zn^{2+} (Mithu et al., 2014). Solid-state NMR using dipolar-assisted rotational resonance of curcumin-complexed $A\beta_{42}$ fibrils showed that curcumin interacted with Val12 and the CHC region of $A\beta$, which makes up part of the β -sheet structure in the $A\beta_{42}$ fibrils (Masuda et al., 2011). The methoxy and hydroxyl groups attached to the aromatic rings of curcumin were found to be important for the binding to $A\beta_{42}$ fibrils (Masuda et al., 2011).

Although curcumin is a potent inhibitor of $A\beta$ cytotoxicity in vivo, its poor bioavailability and low solubility in water have restricted its use as a drug for AD. In a short pilot study of AD patients, curcumin did not significantly improve the Mini Mental State Examination (MMSE) score (Baum et al., 2008). MMSE is a test used extensively in clinical or research settings to measure cognitive impairment. In a different 24-week phase 2 trial, no significant score improvement ensued in the MMSE, Alzheimer's Disease Assessment Scale Cognitive portion (ADAS-Cog), neuropsychiatric inventory, and Alzheimer's Disease Cooperative Study–Activities of Daily Living (ADCS-ADL). No appreciable differences in plasma or CSF biomarkers of $A\beta_{40}$, $A\beta_{42}$, total tau, or hyperphosphorylated tau were observed in this trial (Ringman et al., 2008). In another 24-month-long clinical study of 30 patients with AD, curcumin was well tolerated except in three patients who suffered gastrointestinal symptoms. Unfortunately, in this trial, again the efficacy of the curcumin formulation (curcumin C3 Complex®) was minimal (Ringman et al., 2012), possibly due to its poor bioavailability. These findings inspired attempts to improve the water solubility of curcumin derivatives (Endo et al., 2014). To improve the bioavailability and efficacy of curcumin, use was made of formulations containing carriers, such as liposomes, poly(lactic-co-glycolic acid), or gold nanoparticles (AuNPs) (Hoppe et al., 2013; Lazar et al., 2013; Palmal et al., 2014; Tsai et al., 2011); curcumin nanoparticles (80 nm, prepared by flash precipitation of curcumin, polyethylene glycol-poly(lactic acid) coblock polymer, and polyvinylpyrrolidone, followed by freeze-drying with β -cyclodextrin) (Cheng et al., 2013); chemically modified curcumin derivatives (eg, FMeC1; Fig. 6.12B) (Yanagisawa et al., 2015); or curcumin conjugates with other compounds, such as melatonin, cyclodextrin, sugars, or metals (Al(III), Cu(II), or Zn(II)) (Banerjee, 2014; Chojnacki et al., 2014; Dolai et al., 2011; Gautam et al., 2014; Picciano and Vaden, 2013). An inhalable aerosolized formulation of FMeC1 led to binding of the compound to hippocampal and cortical plaques in $5 \times$ FAD mice (McClure et al., 2015). The $5 \times$ FAD mice bear two transgenes: (1) mutant human APP with

the Swedish (K670N/M671L), Florida (I716V), and London (V717I) mutations, and (2) mutant human *PSEN1* including M146L and L286V mutations. Both transgenes are driven by the mouse Thy1 promoter (Oakley et al., 2006).

In APP_{SWE}/PS1 Δ E9 transgenic mice (Jankowsky et al., 2001, 2004) treated with 200 mg/kg or 400 mg/kg body weight per day of a curcumin preparation, spatial learning and memory were improved compared with untreated mice tested by the Morris water maze (Wang et al., 2014d). In the same study, SDS-PAGE and Western blotting of hippocampal tissue extracts and immunohistochemistry of hippocampal tissue sections of mice treated with curcumin showed lower A β 40 or A β 42 levels, and lower A11-reactive species than those measured in samples of vehicle-treated control mice (Wang et al., 2014d). A11 recognizes a common secondary structure shared by toxic assemblies of a number of amyloid proteins (Kayed et al., 2003). Besides changes in A β levels, Western blotting and immunohistochemistry showed that treated transgenic mice expressed low levels of the γ -secretase component, presenilin 2, and higher levels of A β -degrading enzymes—insulin-degrading enzyme and neprilysin (Wang et al., 2014d). Overall, Wang et al. (2014d) described curcumin's action mechanisms that are distinct from its potential modulatory effects on A β self-assembly, but are likely clearing A β through presenilin 2 inhibition and expression of A β -degrading enzymes.

In rats treated intraperitoneally with curcumin-encapsulated poly(lactic-co-glycolic acid) nanoparticles (Cur-PLGA-NPs) at 5, 10, or 20 mg/kg body weight, nanoparticles could cross the blood-brain barrier, act as prolonged-release curcumin carriers, and induce hippocampal stem cell proliferation and neurogenesis (Tiwari et al., 2014). Mechanistically, the Wnt/ β -catenin pathway was activated while the glycogen kinase-3 β pathway was inhibited in the treated rats. The Wnt/ β -catenin pathway is involved in survival, proliferation, and differentiation of neural stem cells during embryonic and adult neurogenesis (Machon et al., 2003; Zhang et al., 2011). To assess whether curcumin nanoparticles could reverse A β effects in the brain, anesthetized rats were injected stereotactically with 2 μ L of 0.2 μ g/ μ L A β 42 in bilateral hippocampi and then treated with 20 mg/kg body weight curcumin nanoparticles. As a result of this treatment, A β -induced inhibition of neurogenesis and the ensuing learning and memory deficits were prevented by Cur-PLGA-NP treatment (Tiwari et al., 2014). This study did not investigate the modulation of A β self-assembly but described other mechanisms of curcumin action.

In addition to using curcumin as a drug, owing to its affinity for A β , curcumin derivatives have been used for diagnostic applications in AD (Asti et al., 2014; Lee et al., 2011; Liu et al., 2012; Zhang et al., 2014). For example, radiolabeled fluorinated and pegylated curcumin derivatives could cross the blood-brain barrier better than the fluorinated derivatives (Lee et al., 2011; Rokka et al., 2014). In addition, intravenously administered curcumin was shown to detect retinal A β plaques by noninvasive optical imaging applied in live, anesthetized, APP_{SWE}/PS1 Δ E9 transgenic mice (Koronyo-Hamaoui et al., 2011).

Several factors may limit the use of curcumin as an AD therapeutic. Curcumin interferes with certain physiological functions, including actin and tubulin polymerization, and corneal neovascularization and angiogenesis (Bhandarkar and Arbiser, 2007; Dhar et al., 2015). Curcumin interaction with actin causes loosening of the actin structure, leading to destabilization of filaments and reduced cell proliferation and angiogenesis (Arbiser et al., 1998). Hence, testing curcumin as an anti-AD drug must be explored with caution.

FIGURE 6.13 Structure of (–)-epigallocatechin-3-gallate.

(–)-Epigallocatechin-3-Gallate

(–)-Epigallocatechin-3-gallate (EGCG; Fig. 6.13) is a natural polyphenol and a key bioactive ingredient of green tea (*Camellia sinensis*). EGCG was found to inhibit $A\beta$ assembly and toxicity by directing its aggregation pathway toward formation of nontoxic assemblies (Ehrnhoefer et al., 2008). Electron micrographs of $A\beta$ aged in the presence of EGCG showed amorphous aggregates in contrast to long fibrils that formed in the absence of the polyphenol. The amorphous $A\beta$ aggregates formed in the presence of EGCG did not react with the oligomer-specific antibody A11 (Sinha et al., 2012a), which recognizes a common secondary structure shared by toxic assemblies of various amyloid proteins (Kayed et al., 2003). In cytotoxicity experiments using PC-12 cells, fivefold excess EGCG to $A\beta$ completely inhibited $A\beta$ oligomer toxicity (Bieschke et al., 2010; Ehrnhoefer et al., 2008). Similar results were observed in primary rat hippocampal neurons and mixed glial and neuronal cultures (Sinha et al., 2012a).

Several experimental and theoretical studies have attempted to clarify the mechanisms underlying $A\beta$ –EGCG interaction. Isothermal calorimetry and molecular dynamics simulations showed that $A\beta$ 42–EGCG interactions depend on their concentration ratio ($m = [EGCG]/[A\beta$ 42]) (Wang et al., 2010). The binding stoichiometry N is linearly related to m . At $m < 16$, the process is always enthalpy driven, suggesting that it is driven predominantly by hydrogen bonding. At $m > 46$, binding is entropy driven, suggesting increased hydrophobic interactions. At m values between 16 and 46, both hydrogen bonding and hydrophobic interactions are involved. The study also showed that at pH values close to the isoelectric point of $A\beta$ 42 ($pI = 5.5$), $A\beta$ 42 self-association became dominant due to low repulsive electrostatic forces, and the aforementioned trend for $A\beta$ 42–EGCG interaction did not hold (Wang et al., 2010).

Solution-state two-dimensional NMR showed that EGCG interacted with the entire peptide backbone (Sinha et al., 2012a), in agreement with previous studies (Ehrnhoefer et al., 2008). Magic angle spinning solid-state NMR showed that EGCG interacted with the aromatic hydrophobic core of $A\beta$. In the EGCG-induced oligomeric $A\beta$ structure, the C-terminal residues (22–39) assumed a β -sheet conformation, the N-terminal $A\beta$ residues 1–20 were unstructured, and the salt bridge between Asp23 and Lys28 was retained (Lopez del Amo et al., 2012).

Another report showed that EGCG autooxidation was critical for remodeling $A\beta$ 40 fibril structures (Palhano et al., 2013). Here, a centrifugation–wash protocol was used, whereby the soluble unbound EGCG was removed from fibrillar $A\beta$ by centrifugation before taking measurements. Using this protocol, when ThT was added to $A\beta$ fibrils incubated with EGCG, no change in ThT fluorescence was observed immediately after ThT addition, yet the ThT fluorescence

signal decreased substantially after 24 h. In the unwashed samples, there was an initial rapid decrease in ThT fluorescence followed by a gradual decrease. This suggested that EGCG interfered with ThT fluorescence either by competitively binding to ThT binding sites on the fibrils or by quenching the ThT fluorescence. The color of the mixture of A β aggregates with EGCG became brown, and A β aggregates inspected by AFM were amorphous. The authors hypothesized that upon oxidation, EGCG-derived quinones covalently interacted with free amines and formed Schiff bases. Presumably, such covalent binding prevents formation of small toxic oligomeric species and drives the equilibrium toward nontoxic assemblies (Palhano et al., 2013).

EGCG also reduced A β aggregation, oligomerization, and toxicity in a transgenic *C. elegans* model of AD (Abbas and Wink, 2010), and significantly reduced cell-secreted A β levels detected by immunoprecipitation and Western blotting using the 6E10 antibody in Chinese hamster ovarian (CHO) cells (Reznichenko et al., 2006). EGCG treatment in N2a cells overexpressing the Swedish mutant of human APP, or in primary neurons from transgenic mice bearing the Swedish APP mutation, demonstrated reduction in A β levels assessed by immunohistochemistry using monoclonal antibody 4G8 (Rezai-Zadeh et al., 2005), which recognizes residues 18–22 of A β (Kim et al., 1988).

In a different report, 16 or 32 μ M EGCG treatment caused proliferation of an A β 42-overproducing cell model (M146L) which was established by overexpressing APP and mutant PS1 genes into CHO cells; EGCG did not affect proliferation of the CHO cells themselves, and untreated M146L cells normally undergo slow growth rates compared to CHO cells (Chang et al., 2015). In the same report, EGCG significantly prevented A β 42 accumulation in the hippocampi of SAM-P/8 mice (Yagi et al., 1988) and in M146L model cells measured by Western blotting and ELISA (Chang et al., 2015). It was also found that EGCG treatment upregulated neprilysin expression in M146L cells and in the hippocampi of SAM-P/8 mice, suggesting that EGCG-induced neprilysin could potentially prevent A β accumulation in this cell and animal model. SAM-P/8 is a senescence-accelerated mouse line that occurs naturally (Yagi et al., 1988). SAM-P/8 mice suffer from marked spatial learning and memory deficits in the Morris water maze test. EGCG treatment improved spatial learning and memory in these mice (Chang et al., 2015).

When APP/PS1 mice (Borchelt et al., 1996, 1997) were treated with EGCG at 6 mg/kg body weight per day by gavage, and spatial memory was assessed by the Morris water maze, treated mice recovered their spatial learning and memory (Jia et al., 2013). When levels of soluble and insoluble A β 42 were measured by ELISA in the hippocampal extracts of the same mice, EGCG at 2 mg/kg per day or at 6 mg/kg per day reduced soluble A β 42 levels in the hippocampi of transgenic mice by 25 or 38% in comparison with vehicle-treated transgenic mice, respectively (Jia et al., 2013). Insoluble A β 42 levels were reduced by 18 or 31%, respectively (Jia et al., 2013). Mechanistically, these observations were ascribed to inactivation of hippocampal TNF α /JNK signaling (antiinflammatory), restoration of dysregulated Akt/GSK3 β signaling (gluconeogenic), improved insulin signaling, and reduction of hippocampal A β 42 levels in this AD mouse model (Jia et al., 2013). GSK3 β has been associated with tau phosphorylation, APP–A β cascades, and neurodegeneration in AD (Kaytor and Orr, 2002; Phiel et al., 2003; Planel et al., 2002).

A phase 2/3 clinical trial (NCT00951834) with an EGCG formulation called Sunphenon in early-stage AD cases was completed in Aug. 2015, but the results have not yet reported at the completion of writing this chapter.

FIGURE 6.14 Structures of common flavonoids.

Flavonoids—Myricetin, Quercetin, Taxifolin, and Fisetin

Flavonoids are described as secondary metabolites in plants. They perform important functions, including antioxidation, chelation of redox-active metals, regulation of cell growth and differentiation, and imparting colors to plant petals or other components. Several flavonoids have been tested for their efficacy against AD symptoms based on their ability to interfere with $A\beta$ assembly.

Myricetin (Fig. 6.14), which is derived from vegetables, fruits, nuts, berries, and tea (Ross and Kasum, 2002) and is also present in red wine (Basli et al., 2012), was shown to alter $A\beta$ assembly and interfere with its oligomerization patterns as examined by CD spectroscopy, ThT fluorescence, PICUP, SEC, and AFM experiments (Ono et al., 2012). At a peptide:myricetin concentration ratio of 5:2, myricetin completely inhibited the self-assembly of $A\beta$ 40 and moderately that of $A\beta$ 42. At a 1:4 ratio, respectively, $A\beta$ 42 oligomerization, examined by PICUP and AFM, also was completely inhibited (Ono et al., 2012). At the same concentration ratio, curcumin (section: Curcumin and Curcumin Derivatives) and rosmarinic acid (section: Rosmarinic Acid and Tannic Acid) failed to inhibit $A\beta$ oligomerization. Solution-state NMR studies showed that myricetin stabilized the statistical-coil structures in $A\beta$ 42 by binding to the monomeric peptide. The chemical shifts of monomeric ^{15}N -labeled $A\beta$ 42 peptide in the presence of myricetin (1:10, $A\beta$ 42:myricetin concentration ratio) showed preferential binding to $A\beta$ (16–24) and $A\beta$ (31–33) (Ono et al., 2012). Myricetin prevented the toxicity of crosslinked or noncrosslinked $A\beta$ 40 and $A\beta$ 42 oligomers in HEK293 cells in the MTT assay. In electrophysiological experiments, myricetin reversed $A\beta$ 42-induced inhibition of LTP ($97.5 \pm 7.6\%$) to baseline values ($157 \pm 5.1\%$) in mouse CA1 hippocampal slices (Ono et al., 2012). Myricetin was

found to be more potent against Cu²⁺-associated or Zn²⁺-associated A β 40 than metal-free A β 40 species. The compound inhibited metal-induced A β 40 assembly in vitro ([A β 40] = 25 μ M, [CuCl₂] or [ZnCl₂] = 25 μ M, [myricetin] = 50 μ M) and neurotoxicity in human neuroblastoma SK-N-BE(2)-M17 cells at twofold excess to A β 40 ([A β 40] = 10 μ M, [CuCl₂ or ZnCl₂] = 10 μ M, [myricetin] = 20 μ M) (DeToma et al., 2011). These observations suggest that myricetin may inhibit A β 40 assembly by metal chelation similar to clioquinol or PBT2 (Crouch et al., 2011); however, there is no experimental evidence to support this hypothesis.

Tg2576 mice fed a diet containing 0.5% myricetin showed high levels of soluble A β (detected by antibodies against human A β 40, A β 42, or aggregated A β) and low levels of A11-reactive oligomers (using immunohistochemistry), with no substantial reduction in A β plaque deposition (Hamaguchi et al., 2009). These results suggest that myricetin can induce formation of nontoxic A β structures and supports its future therapeutic development for AD.

Another flavonoid, (+)-taxifolin (Fig. 6.14), also was reported to inhibit A β assembly and toxicity (Sato et al., 2013a,b). ThT experiments showed that taxifolin (50 μ M) oxidized with sodium periodate (100 μ M) inhibited A β 42 (25 μ M) aggregation more effectively than unoxidized taxifolin (Sato et al., 2013b). The combination of taxifolin and periodate did not suppress A β 40 fibrillization under anaerobic or reducing conditions (Sato et al., 2013b). The exact mechanism of (+)-taxifolin action on A β is unclear; however, it was suggested that oxidation of the catechol moiety of taxifolin to an *o*-quinone may be responsible. This change subsequently causes formation of covalent conjugates with susceptible side chains in A β and leads to inhibition of A β assembly (Sato et al., 2013b). This inhibition mechanism is similar to the data reported for EGCG (section: (–)-Epigallocatechin-3-Gallate) and is likely to be the same for all the catechol-containing categories of flavonoids.

The 3'- and 4'-hydroxyl groups of taxifolin (Fig. 6.14) are critical for inhibition of A β aggregation, whereas the 7-hydroxyl group and the stereochemistry at positions 2 and 3 are not, as was shown by methylation of these hydroxyl groups. (+)-Taxifolin–A β adducts were analyzed by an ion-trap-type LC–MS equipped with a time-of-flight mass analyzer. These experiments showed oxidized A β 42–taxifolin adducts that resulted from the Michael addition of the *o*-quinone groups to Lys or Arg residues (Sato et al., 2013b).

To identify residues involved in adduct formation, A β 42 isoforms with Arg5, Lys16, or Lys28 mutated to norleucine (Nle) were examined. Five different A β 42 mutants, Arg5Nle–A β 42, Lys16Nle–A β 42, Lys28Nle–A β 42, Lys16Nle/K28Nle–A β 42, and Arg5Nle/Lys16Nle/Lys28Nle–A β 42, were generated. The intensity of ThT-positive aggregates of the singly mutated Lys16Nle–A β 42 was similar in the presence or absence of (+)-taxifolin, whereas that of the singly mutated Lys28Nle–A β 42 in the presence of (+)-taxifolin was slightly lower than that in its absence. (+)-Taxifolin did not alter the aggregation pattern of the doubly mutated Lys16Nle/Lys28Nle–A β 42 and the triply mutated Arg5Nle/Lys16Nle/Lys28Nle–A β 42. However, (+)-taxifolin predominantly blocked the aggregation of the singly mutated Arg5Nle–A β 42. Taken together, these results showed that Lys residues at positions 16 and 28 likely are the targets of oxidized taxifolin. Lys16, which is believed to be exposed to the solvent (Zhang et al., 1998, 2000), appears to be the primary target, as (+)-taxifolin could not alter the aggregation profiles of Lys16Nle–A β 42 or Lys16Nle/Lys28Nle–A β 42. Moreover, ThT assays done in the presence of catechol-type flavonoids (dihydromyricetin, (+)-taxifolin, myricetin, or quercetin) or noncatechol-type flavonols (morin, kaempferol, or datiscetin) using Lys16Nle–A β 42, Lys16Nle/Lys28Nle–A β 42, and A β 42 showed that catechol-type flavonoids suppressed

$A\beta$ 42 aggregation by forming covalent adducts on Lys residues. The study suggested that Lys residues are important determinants of $A\beta$ 42 assembly and toxicity, and may be targets for therapeutic approaches developed against abnormal self-assembly of amyloidogenic proteins in AD and other amyloidoses (Sato et al., 2013b). These findings dovetail with studies in our group showing that substitution of the Lys residues by Ala dramatically decreased the neurotoxicity of both $A\beta$ 40 and $A\beta$ 42 (Sinha et al., 2012b), and that labile complexation of Lys residues in multiple amyloidogenic proteins by molecular tweezers (section: Molecular Tweezers) inhibited the aggregation and cytotoxicity of these proteins (Sinha et al., 2011).

Fisetin (Fig. 6.14) has been found to be neurotrophic (Maher, 2006), induce neuron differentiation (Sagara et al., 2004), enhance memory (Maher et al., 2006), and inhibit $A\beta$ aggregation (Kim et al., 2005). Fisetin also inhibited $A\beta$ 42 aggregation and toxicity in cultured hippocampal neurons (Akaishi et al., 2008). Similarly to the findings with taxifolin, a comparative study of several related flavonoids suggested that the 3',4'-dihydroxyl group, and not the 3-hydroxyl or 7-hydroxyl group, were essential for fisetin's inhibitory activity (Akaishi et al., 2008). Although no direct studies of the mechanism of interaction between fisetin and $A\beta$ have been published to our knowledge, the compound likely targets Lys residues on $A\beta$ covalently as in the case of most catechol-type flavonoids.

Quercetin and its derivatives reportedly have many beneficial biochemical and pharmacological properties, including free-radical scavenging (Fiorani et al., 2010) and effects on immune and inflammatory functions (Garcia-Mediavilla et al., 2007). They could also have beneficial effects in AD by reducing age-associated cellular damage that causes metabolic by-production of reactive oxygen species (Weinreb et al., 2004). Quercetin and its derivatives were tested as modifiers of $A\beta$ assembly to find out whether they could reverse the primary pathological hallmarks of AD as an additive outcome to their other beneficial properties (Sabogal-Guaqueta et al., 2015). Quercetin (Fig. 6.14) and its derivatives, such as quercetin-3'-O-glucoside and quercetin-3-O-rutinoside, were found to inhibit $A\beta$ 42 aggregation as seen in ThT and TEM assays, and attenuated $A\beta$ 42-induced cytotoxicity in SH-SY5Y neuroblastoma cells (Jimenez-Aliaga et al., 2011; Wang et al., 2012). In a triple-transgenic ($3 \times$ Tg) AD mouse model, which expresses human APP (KM670/671NL), PSEN1 (M146V), and tau (P301L) (Oddo et al., 2003), quercetin reversed AD pathology and restored spatial memory tested by the elevated plus maze (Sabogal-Guaqueta et al., 2015). In this study, quercetin administered via intraperitoneal injection every 48 h at 25 mg/kg for 3 months decreased significantly hippocampal and amygdala $A\beta$ 40 and $A\beta$ 42 deposition in $3 \times$ Tg AD mice.

Quercetin-3-O-glucuronide significantly decreased $A\beta$ in primary corticohippocampal neurons derived from the Tg2576 AD mouse model (Ho et al., 2013). PICUP experiments showed that quercetin-3-O-glucuronide interacted with $A\beta$ monomers and inhibited $A\beta$ self-assembly and oligomer formation. In electrophysiologic assays, quercetin-3-O-glucuronide significantly rescued deficits in basal neuronal transmission in the CA1 region of hippocampal slices compared to vehicle-treated hippocampal slices from Tg2576 mice (Ho et al., 2013).

Rosmarinic Acid and Tannic Acid

Several natural antioxidants such as rosmarinic acid and tannic acid have neuroprotective properties. These compounds structurally resemble the polyphenols and flavonoids discussed earlier, suggesting that they may have similar activity, modifying $A\beta$ assembly and reducing its toxicity.

FIGURE 6.15 Structures of (A) rosmarinic acid and (B) tannic acid.

Rosmarinic acid (Fig. 6.15A) inhibited the formation of β -sheet-rich fibrils of A β 40 and A β 42 in ThT fluorescence and TEM experiments. The compound also disintegrated pre-formed A β fibrils with $EC_{50} = 0.1\text{--}1\text{ }\mu\text{M}$ (Ishigaki et al., 2013; Ono et al., 2004b), prevented the formation of A11-positive oligomers, and inhibited A β -induced neurotoxicity in PC-12 cells with significant inhibition at $0.1\text{ }\mu\text{M}$ (Camilleri et al., 2013; Hamaguchi et al., 2009; Iuvone et al., 2006). Rosmarinic acid included at 0.5% in the diet of a Tg2576 mouse model of AD (Hsiao et al., 1996) significantly reduced the total A β plaque burden in the brain as assessed by immunohistochemistry using the antibody 4G8 (Hamaguchi et al., 2009). This monoclonal antibody recognizes residues 18–22 of A β (Kim et al., 1988). Solution state 2D HSQC-NMR studies with monomeric ^{15}N -labeled A β 42 in the presence of 20-fold excess rosmarinic acid showed no substantial movement of crosspeaks compared to the A β 42 peptide alone, suggesting that rosmarinic acid does not bind to specific regions in monomeric A β 42 (Ono et al., 2012), similarly to EGCG (section: (–)-Epigallocatechin-3-Gallate). In contrast, there was substantial binding of myricetin to A β 42 under similar conditions (see section: Flavonoids—Myricetin, Quercetin, Taxifolin, and Fisetin).

Tannic acid (Fig. 6.15B) dose dependently inhibited A β 40 and A β 42 fibrillization and disintegrated preformed fibrils (Ono et al., 2004a). Tannic acid did not extend the length of the lag phase, but decreased the rate of aggregation in A β 40 and A β 42. In samples seeded with sonicated A β 42 fibrils, tannic acid completely inhibited ThT-fluorescence increase, suggesting that it bound to fibril ends. Electron micrographs of tannic acid-treated A β 40 or A β 42 fibrils showed smaller disintegrated fibrils compared to the untreated preparations (Ono et al., 2004a). When administered by gavage at 30 mg/kg body weight for 6 months (Mori et al., 2012), tannic acid reduced brain parenchymal and cerebral vascular

$A\beta$ deposits (visualized by immunohistochemistry) and reduced $A\beta$ oligomers (measured by ELISA in SDS-containing extracts) in the PSAPP mouse model of cerebral amyloidosis [bearing exon-9-deleted presenilin 1 and the Swedish $APP_{K595N/M596L}$ mutations (Jankowsky et al., 2001, 2004)]. Behaviorally, hyperactivity, perturbed object recognition, and defective spatial reference memory were also prevented by tannic acid treatment in the mice. Mechanistically, these findings were ascribed to inhibition of β -secretase activity and dampening of neuroinflammation resulting in reduced $A\beta$ burden (Mori et al., 2012).

Theaflavins

Theaflavins, the major polyphenolic components of fermented black tea, include theaflavin 1, theaflavin 2a, theaflavin 2b, and theaflavin 3 (Fig. 6.16). These compounds were reported to remodel preformed toxic $A\beta$ fibrils into nontoxic, ThT-negative assemblies. $A\beta$ toxicity was measured in PC-12 cells (Grelle et al., 2011). AFM experiments showed formation of spherical $A\beta$ aggregates in the presence of theaflavins, as opposed to fibrils forming by $A\beta$ alone. Theaflavins, especially theaflavin 3, were found to be less susceptible to oxidation than EGCG and flavonoids (sections: (–)-Epigallocatechin-3-Gallate; Flavonoids—Myricetin, Quercetin, Taxifolin, and Fisetin) (Grelle et al., 2011). To determine the potential theaflavin-binding sites of $A\beta$, an array of 33 $A\beta$ -derived overlapping peptides covering the sequence of $A\beta$ 42 was used, and binding was determined using dot blots followed by densitometric analysis. Theaflavin 3 preferentially was found to interact with the regions $A\beta$ (12–23) and $A\beta$ (24–36) with different affinities, yet the binding mainly was based on nonspecific hydrophobic interactions rather than sequence specificity (Grelle et al., 2011).

Ginkgo Biloba Extract

Ginkgo biloba extract from the medicinal maidenhair tree is a mixture of flavonoids and terpenoids (isoprenoids). EGb 761® is a standard, proprietary extract of *G. biloba* (DeFeudis, 2003; Adis R&D Profile, 2003). The extract was tested in a number of small-to-medium clinical trials for dementia or specifically for AD, and has shown mild positive effects (Itil and Martorano, 1995; Kanowski et al., 1996; Le Bars et al., 1997; Maurer et al., 1997). Based on these results, several mechanistic studies tested the direct effect of EGb 761 on $A\beta$ assembly and toxicity. The extract was shown to prevent $A\beta$ -mediated toxicity in PC-12 cells, human neuroblastoma cells, rat hippocampal cell lines, primary hippocampal neurons, and in *C. elegans* (Bastianetto et al., 2000; Smith and Luo, 2003; Wu et al., 2006; Yao et al., 2001). Interestingly, in the study by Smith and Luo (2003), pretreatment with EGb 761 did not prevent $A\beta$ -induced toxicity but prevented generation of $A\beta$ -induced reactive oxygen species. However, in a different study using PC-12 cells, EGb 761 was reported to interact directly with $A\beta$ and inhibit formation of $A\beta$ -derived diffusible ligands (ADDLs) dose dependently (Yao et al., 2001). ADDLs are particular $A\beta$ 42 oligomers originally reported by Klein and coworkers (Lambert et al., 1998; Oda et al., 1995). They are highly toxic in cell culture and in vivo (Gong et al., 2003; Wang et al., 2002). EGb 761 (10–100 μ g/mL) also protected hippocampal neurons against toxicity induced by $A\beta$ 40 or $A\beta$ 42 dose dependently with complete protection achieved at 100 μ g/mL.

Treatment of Tg2576 mice with *G. biloba* extract in water led to spatial memory recovery to comparable levels measured in wild-type mice, without affecting soluble $A\beta$ levels or plaque burden (Stackman et al., 2003). The reported dose was 70 mg/kg per day, yet this is an

FIGURE 6.16 Structures of different theaflavins, major polyphenols derived from fermented black tea.

estimate because the *G. biloba* extract in the water was administered ad libitum. Surprisingly, the brain levels of protein carbonyls were elevated in the treated mice (Stackman et al., 2003). If certain compounds in the extract undergo self-oxidation, similar to the process described earlier for EGCG (section: (–)-Epigallocatechin-3-Gallate) and certain flavonoids (section: Flavonoids—Myricetin, Quercetin, Taxifolin, and Fisetin) and form Schiff-base adducts with lysine residues, they may contribute to oxidative stress and explain the observation of increased carbonyls in the mice.

A 2009 clinical trial showed that compared with placebo ($n = 1524$), the use of *G. biloba* at 120 mg twice daily ($n = 1545$), did not change the rate of cognitive decline in older adults

FIGURE 6.17 Structure of brazilin.

(72–96 years of age) with normal cognition or with mild cognitive impairment (Snitz et al., 2009). The annual rates of decline did not differ between the *G. biloba* and placebo groups in all the measured parameters, including memory, attention, visuospatial abilities, language, and executive functions (Snitz et al., 2009). Thus, overall, in multiple trials the effect of *G. biloba* on patients with AD has been underwhelming. The extract is available as an over-the-counter supplement but is not expected to have a significant therapeutic effect on patients with AD.

Brazilin

Brazilin (Fig. 6.17) is a red pigment obtained from the wood of *Caesalpinia spinosa*. It was part of a >600 compound library screened virtually by Du et al. (2015) for docking onto a fibrillar $A\beta(17-42)$ pentamer. Out of the hits discovered, bicuculline and brazilin were chosen for further characterization based on their hydrophathy characteristics. ThT fluorescence experiments suggested that brazilin was a stronger inhibitor than bicuculline, and therefore subsequent experiments focused on brazilin only.

ThT, TEM, and CD suggested that, similarly to EGCG, brazilin inhibited $A\beta_{42}$ fibrillization by redirecting the misfolding pathway toward nontoxic assemblies. Brazilin reduced $A\beta_{42}$ -induced toxicity in the LDH release assay. Molecular dynamics simulations suggested that brazilin inhibited $A\beta_{42}$ fibrillization by directly binding to $A\beta_{42}$ species using hydrophobic interactions and hydrogen bonding, and altered the structure of mature $A\beta_{42}$ fibrils by disrupting the Asp23–Lys28 salt bridge (Du et al., 2015).

Inositol Derivatives

Inositol exists as nine potential stereoisomers, which are involved in diverse physiological functions, including gene expression, fat metabolism, maintenance of membrane potential, regulation of intracellular calcium levels, cytoskeletal assembly, control of telomere length, and insulin secretion (Michell, 2008). They are synthesized physiologically by the enzymatic conversion of glucose-6-phosphate—a glycolysis metabolite (Parthasarathy et al., 2006).

Four physiologically active stereoisomers of inositol (cyclohexane-1,2,3,4,5,6-hexol) (*epi*-, *scyllo*-, *myo*-, and *chiro*-inositol, Fig. 6.18) had been tested for their ability to modulate $A\beta$ assembly and toxicity (McLaurin et al., 2000). *Myo*-inositol bound to $A\beta_{42}$ in vitro forming small, stable micelles, whereas *epi*- and *scyllo*-inositol induced β -sheet formation in $A\beta_{42}$. *Chiro*-inositol did not induce structural changes in $A\beta_{42}$ (Fenili et al., 2007). *Myo*-, *epi*-, *chiro*-, and *scyllo*-inositol reduced $A\beta_{42}$ oligomers' toxicity to different degrees when tested in differentiated PC-12 cells (McLaurin et al., 2000). *Scyllo*-inositol was the most potent inhibitor, and therefore subsequent studies focused mainly on this stereoisomer.

FIGURE 6.18 Structures of four physiologically active isoforms of inositol.

Scyllo-inositol dose dependently rescued LTP in mouse hippocampal slices treated with soluble assemblies of cell-derived human A β (Townsend et al., 2006). In vivo, *scyllo*-inositol given in drinking water completely rescued the learning deficits caused by A β oligomers injected intracerebroventricularly in rats (Townsend et al., 2006). Orally administered *scyllo*- or *myo*-inositol reversed the cognitive deficits and substantially reduced A β plaque burden in the TgCRND8 mouse model of AD (Fenili et al., 2007). This transgenic model expresses human APP695 containing the Swedish (KM670/671/NL) and Indiana (V717F) mutations under the control of the hamster prion gene promoter (Chishti et al., 2001). Administration of *scyllo*-inositol at 10-fold excess over endogenous concentration levels (1 mM) did not interfere with general physiological functions, such as phospholipid synthesis, suggesting that *scyllo*-inositol could be used therapeutically at higher concentrations than the endogenous levels (Fenili et al., 2007; Townsend et al., 2006). However, administration of high doses of *scyllo*-inositol was found to be lethal in a clinical trial (see following discussion).

To understand the nature of interactions between *scyllo*-inositol and A β , a series of deoxy-inositol derivatives was used (Sun et al., 2008). A β 42 aggregated in the presence of 1-deoxy-*scyllo*-inositol or 1,4-dideoxy-*scyllo*-inositol, suggesting that both the equatorial and axial hydroxyl groups were important for inhibiting aggregation. Incubation of A β 42 with 1-deoxy-1-fluoro-*scyllo*-inositol resulted in ThT-positive, amorphous aggregates, similar to those in the presence of *scyllo*-inositol, whereas A β 42 incubation with 1,4-dideoxy-1,4-difluoro-*scyllo*-inositol showed both amorphous aggregates and fibrils. Later in vivo studies showed that 1-deoxy-1-fluoro-*scyllo*-inositol dose-dependently improved spatial memory in TgCRND8 mice (Hawkes et al., 2012). The compound did not change the overall cerebral A β 40 or A β 42 concentration, but decreased A β plaque burden (Hawkes et al., 2012).

The derivative 1,4-di-*O*-methyl-*scyllo*-inositol inhibited A β 42 fibrillization in vitro by stabilizing A β 42 protofibril-like structures (Hawkes et al., 2010; Shaw et al., 2012). However, unlike protofibrils of A β itself, which are highly neurotoxic (Nilsberth et al., 1999; O'Nuallain et al., 2010; Walsh et al., 1999), the structures formed in the presence of 1,4-di-*O*-methyl-*scyllo*-inositol apparently were nontoxic. Thus, in the TgCRND8 AD mouse model, 1,4-di-*O*-methyl-*scyllo*-inositol improved spatial memory measured by the Morris water-maze test and significantly decreased cerebral amyloid pathology (Hawkes et al., 2010). In agreement with the in vitro data, the total A β plaque burden decreased by 30%, whereas soluble A β levels increased (Hawkes et al., 2010; Shaw et al., 2012). As oligomeric A β forms are more toxic than the mature fibrils, and those found in the mice were not well characterized, the data suggested caution in testing 1,4-di-*O*-methyl-*scyllo*-inositol in humans.

A randomized, 78-week, phase 2 trial using *scyllo*-inositol was conducted in 353 patients with mild or moderate AD who received 250, 1000, or 2000 mg/day. Clinical scores of the “neuropsychological test battery” and ADCS-ADL tests were used as primary outcome measures. The high doses of 1000 and 2000 mg/day, however, were discontinued due to significant incidence of adverse reactions in patients. These included nine deaths, in addition to less severe side effects, such as falls, depression, and confusion. The trial continued with only the low dose, 250 mg/day, which did not lead to significant cognitive or functional improvement (Salloway et al., 2011). In this trial, *scyllo*-inositol concentrations increased in the CSF, and cerebral and CSF $A\beta$ concentrations decreased significantly compared to the placebo group ($p = 0.009$), suggesting that these parameters may not serve as meaningful pharmacodynamics biomarkers for the treatment’s efficacy since the clinical outcome measures were insignificant. Alternatively, the reason for the lack of efficacy might be that the trial was conducted too late in the disease.

The insignificant effect of *scyllo*-inositol in the clinical trial correlated with an in vitro/cell-culture study by our group, in which the effects of *scyllo*-inositol on $A\beta_{42}$ oligomerization, aggregation, and toxicity were substantially weaker than those of EGCG (section: (–)-Epigallocatechin-3-Gallate) or CLR01 (section: Molecular Tweezers) (Sinha et al., 2012a).

Vitamins

Vitamins are essential nutrients our body cannot synthesize in sufficient quantities and thus are acquired through food. Vitamins play diverse functions in growth, development, immune regulation (vitamin A), metabolism (vitamin B), mineral absorption (vitamin D), and antioxidant defense (vitamins C and E). In AD patients, levels of several vitamins are lower than normal. These include particularly vitamins A, C, and E. Supplementation of some vitamins in AD patients could improve AD symptoms clinically and therefore has been researched as a potential treatment for AD. Perhaps as a fashionable addition, in some cases the effect of certain vitamins on $A\beta$ assembly also has been explored.

Vitamin A

Vitamin A is a mixture of retinol, retinal, retinoic acid, and β -carotene (Fig. 6.19). It is important for human growth, development, and visual sensation. In AD patients, plasma and CSF levels of vitamin A reportedly are lower than those in age-matched normal individuals

FIGURE 6.19 Components of vitamin A, which is a mixture of retinol (A), retinal (B), retinoic acid (C), and β -carotene (D).

(Lopes da Silva et al., 2014; Ono and Yamada, 2012). Dietary supplementation of vitamin A has been shown to improve clinical AD symptoms (Obulesu et al., 2011). To test if there is a correlation between decrease in levels of vitamin A and A β aggregation, inhibition of A β assembly by vitamin A was examined. Vitamin A inhibited oligomerization, fibrillization, and fibril extension of A β 40 or A β 42 dose dependently in ThT fluorescence and TEM experiments. Vitamin A also disintegrated fibrillar A β dose dependently (Ono et al., 2004c; Takasaki et al., 2011). All-*trans* retinoic acid, an active metabolite of vitamin A, was found to decrease cerebral A β deposition and rescue memory deficits in APP/PS1 transgenic mice (Ding et al., 2008) and in a streptozotocin-induced model of dementia of Alzheimer type in Swiss albino mice (Sodhi and Singh, 2013). Immunodetection using 6E10 and CR staining were used to assess amyloid plaque burden in APP/PS1 and in albino mice, respectively (Sodhi and Singh, 2013).

Vitamin C

Vitamin C (L-ascorbic acid, Fig. 6.20A), is an important cofactor in several different physiological processes, including collagen synthesis, wound healing, and scavenging of reactive oxygen species. It is a powerful antioxidant and plays an important role in prevention of cancer and cardiovascular diseases, which involve oxidative damage. In AD, plasma vitamin C levels are significantly reduced (0.56 mg/dL vs 0.8 mg/dL in control individuals) (Charlton et al., 2004). However, it is unclear whether vitamin C deficiency is a cause or an effect of AD pathogenesis. In several in vivo and cell-culture assays, vitamin C acted as a potent inhibitor of A β -induced insults. Vitamin C completely prevented A β -induced increase of cellular calcium levels, suppressed A11-positive A β oligomers in Tg2576 fibroblasts, and prevented A β -induced death of PC-12 cells (Cheng et al., 2011; Yallampalli et al., 1998). Studying vitamin C deficiency in rodents has been difficult because, unlike humans, rodents produce sufficient endogenous amounts of vitamin C. To overcome this practical challenge, 5 $\times$ FAD mice (Oakley et al., 2006) were cross-bred with mice lacking the enzyme l-gulonon- γ -lactone oxidase (EC 1.1.3.8), which produces vitamin C in rodents, but is nonfunctional in haplorhines,

FIGURE 6.20 Structures of vitamins C and E. (A) L-ascorbic acid, (B) α -tocopherol, (C) α -tocopherolquinone.

including humans (Nishikimi et al., 1988). This animal model was tested for the effect of vitamin C in AD. The crossbred mice, which could not synthesize vitamin C, showed heavy amyloid plaque deposition in the brain (Kook et al., 2014). The mice were supplemented with low (0.66 g/L) or high (3.3 g/L) doses of vitamin C. The higher dose reduced the amyloid plaque burden in the cortical and hippocampal regions of these mice, suggesting a prospective potential for using vitamin C for AD prevention (Kook et al., 2014). Vitamin C treatment was shown to improve memory performance in the middle-aged APP/PS1 mouse model of AD and in wild-type mice (Kennard and Harrison, 2014). Human data, however, calls for caution regarding vitamin C treatment (Arlt et al., 2012) (and see section: Vitamin E).

Vitamin E

Vitamin E (α -tocopherol, Fig. 6.20B) is an antioxidant, which scavenges reactive oxygen species upon oxidation of lipids. It plays other important physiological roles in gene expression, cell signaling, and inhibition of platelet aggregation (Azzi, 2007; Bell, 1987; Zingg and Azzi, 2004). Although endogenous vitamin E levels are not altered in patients with AD, exogenous vitamin E reportedly reversed $A\beta$ -induced damage in several experimental paradigms. α -Tocopherolquinone (Fig. 6.20C), an important derivative of α -tocopherol, inhibited $A\beta$ 42 fibrillization and $A\beta$ 42-induced neurotoxicity in SH-SY5Y neuroblastoma cells, disaggregated preformed fibrils, and interfered with intracellular $A\beta$ oligomerization (Yang et al., 2010). In the Tg2576 transgenic mouse model of AD, vitamin E treatment as 2 IU/g diet, which was started at 5 months of age and continued until 13 months, significantly reduced lipid peroxidation, $A\beta$ levels, and $A\beta$ deposition (Sung et al., 2004). By contrast, mice taking diet-supplemented vitamin E at 14 months of age until month 20 did not show any significant difference when compared with placebo (Sung et al., 2004). In a different study, combined vitamin E (400 IU/kg diet) and vitamin C (1 g/kg diet) treatment prevented spatial memory deficits in APP/PS1 mice tested in the water maze. However, $A\beta$ deposition was not reduced by this treatment (Harrison et al., 2009). A clinical trial of vitamin E for AD was carried out in 341 patients with moderate or severe AD (Sano et al., 1997). Patients received 2000 IU of vitamin E a day for 2 years. However, no effect was observed in the MMSE test. In another study, 769 patients with mild cognitive impairment received the same dose of vitamin E. Unfortunately, there were no significant differences in the rate of AD progression between vitamin E and placebo groups (Petersen et al., 2005). Negative results were reported in AD patients taking a cholinesterase inhibitor supplemented with 1000 mg/day vitamin C and 400 IU/day vitamin E (Arlt et al., 2012).

In a clinical trial that included 78 patients with mild-to-moderate AD, the patients were given a combination of antioxidants, including vitamin E, vitamin C, α -lipoic acid, and co-enzyme Q3. The treatment was well tolerated by the subjects but showed no effect on the CSF biomarkers related to amyloid or tau pathology. Moreover, in certain groups, there was accelerated decline in MMSE scores, suggesting against this strategy for AD treatment (Galasko et al., 2012).

Proteins or Peptides

Though we dedicated section: Peptidic or Peptidomimetic Modulators of $A\beta$ Assembly to peptides and peptidomimetics, those are *synthetic* compounds synthesized and tested based

on specific rationales. Here, we discuss two examples of *natural* peptides and proteins tested as inhibitors of A β assembly and toxicity, and therefore we include them in the section dealing with molecules of natural sources.

Colostrinin

Colostrinin is a proline-rich polypeptide mixture derived from mammalian colostrum. Reportedly, colostrinin is made up of 32 polypeptides (molecular weight 500–3000 Da) resulting from the proteolytic cleavage of colostrum proteins, for example, β -casein. These peptides contain 25% proline and 40% hydrophobic residues (Janusz and Zablocka, 2013). In primary rat hippocampal cells, colostrinin alleviated A β 42-induced toxicity (Froud et al., 2010). Upon treatment with 0.25 μ M colostrinin, 25 μ M preparations of A β 40 formed shorter and less dense fibrils than untreated A β 40 as documented by TEM experiments (Schuster et al., 2005). When given orally to mice, colostrinin increased the life span and improved various motor and sensory activities (Boldogh and Kruzel, 2008). However, the exact mechanism of colostrinin action is still unclear (Boldogh and Kruzel, 2008; Rocha et al., 2010). In a double-blind, placebo-controlled study to test the effect of colostrinin on daily activities of AD patients, orally administered colostrinin improved cognitive AD outcomes in patients with mild or moderate dementia. This trial consisted of 105 patients and comprised a 15-week double-blind phase comparing colostrinin with placebo, followed by a second 15-week period in which all patients received colostrinin. The patients received 100 μ g drug on alternate days for 3 weeks followed by no drug for 2 weeks. At week 15 and week 30, parameters were evaluated for ADAS-Cog, Clinical Global Impression of Change (CGIC), instrumental activities of daily living (IADL), MMSE, ADAS Noncognitive test (ADAS-Noncog), and overall patient response. Colostrinin showed an alleviating effect on cognitive function in ADAS-Cog and on daily function in IADL. The overall patient response was also in favor of the drug (Bilikiewicz and Gaus, 2004). Colostrinin is used as a nutraceutical for treating the early stages of cognitive decline in North America, Australia, and Europe (Stewart, 2008).

Laminin

Extracellular matrix (ECM) proteins can serve as important traps for extracellular A β . Laminin, which is a major component of the basement membrane, is a high-molecular-weight extracellular membrane protein, which was found to bind A β 40 with nanomolar affinity (Castillo et al., 2000) and colocalize with A β in senile plaques. Among several other ECM components, laminin inhibited A β 40 aggregation significantly better than perlecan, type IV collagen, or fibronectin (Castillo et al., 2000). Inhibition of A β 40 aggregation by other ECM proteins was not statistically significant. Laminin caused a ninefold inhibition by day 3, and a 21-fold inhibition 1 week after initiation of A β 40 assembly, measured by ThT fluorescence. Incubation of A β 40 with laminin resulted in formation of amorphous aggregates and pre-formed A β 40 fibrils were disassembled upon incubation with laminin as detected by TEM.

The potential A β -interacting domains of laminin were investigated using proteolysis with a combination of V8 protease and elastase, which identified 55-kDa globular repeats in laminin that bound A β 40 (Castillo et al., 2000). Interestingly, laminin receptor precursors/laminin receptors (LPR/LR) were shown to bind and internalize A β 42. The LPR-/LR-A β complexes facilitated endocytosis of the peptide, thus enhancing neurotoxicity and accumulation. In view of a recent report suggesting that cellular internalization and accumulation of A β are

FIGURE 6.21 Structure of docosahexaenoic acid (DHA).

critical for induction of synaptotoxicity (Ripoli et al., 2014), anti-LPR/LR antibodies and LPR/LR-based gene therapy could serve as potential strategies for treating AD (Da Costa Dias et al., 2014).

Other Compounds

Docosahexaenoic Acid

Docosahexaenoic acid (DHA, Fig. 6.21) is a polyunsaturated ω -3 fatty acid present in high amounts in fish oil and breast milk. DHA is a major component of the human eye, brain, skin, and sperm. Elevated levels of DHA have been linked with decreased risk of dementia (Cole and Frautschy, 2010; Laurin et al., 2003). This instigated further investigations on the use of DHA as a therapeutic agent for AD. ThT fluorescence and TEM showed that DHA inhibited $A\beta$ 42 fibril formation (Hossain et al., 2009). In viability assays using SH-S5Y5 cells or brain endothelial cells, DHA reduced $A\beta$ 42-induced toxicity (Hossain et al., 2009; Veszelka et al., 2013). In Tg2576 mice, DHA supplementation noticeably reduced $A\beta$ 42 accumulation and oxidative damage, corrected synaptic deficits, and improved cognitive function (Cole and Frautschy, 2006). In the APP/PS1 rat model of AD, rats fed a DHA-supplemented diet showed lower hippocampal $A\beta$ plaque density and modest behavioral improvements relative to those animals fed a DHA-lacking diet. But DHA supplementation also increased overall fibrillar $A\beta$ in the hippocampus (Teng et al., 2015). On the other hand, previous in vitro assays had shown that DHA micelles stabilized protofibrillar $A\beta$ 42, preventing further fibrillization (Johansson et al., 2007). Unfortunately, these protofibrils induced toxicity in PC-12 cells, suggesting that DHA may act as a double-edged sword and, depending on the context, may actually exacerbate AD pathology (Cole and Frautschy, 2006; Hashimoto et al., 2008; Johansson et al., 2007).

Melatonin

Melatonin (Fig. 6.22) is a hormone produced by the pineal gland. It is involved in many physiological processes, including circadian rhythm, blood pressure regulation, oncogenesis, retinal physiology, reproduction, ovarian physiology, immune regulation, and osteoblast differentiation (Altun and Ugur-Altun, 2007). Melatonin also acts as a potent free-radical scavenger and an antioxidant by direct and indirect mechanisms (Hardeland, 2005). Melatonin

FIGURE 6.22 Structure of melatonin.

levels tend to decline with aging. Although no direct correlation between melatonin levels and AD pathogenesis has been established, the antioxidant activity of the hormone and its declining concentration levels with aging prompted its investigation in the context of AD.

Melatonin inhibited A β aggregation in CD, TEM, and NMR experiments. Electrospray ionization–mass spectrometry and proteolysis experiments showed that melatonin bound to the 29–40 region of A β 40, mainly by hydrophobic interactions (Pappolla et al., 1998; Skribanek et al., 2001). Pretreatment of PC-12 cells with melatonin rescued them from A β 42-induced apoptotic insults as evident from MTT cell viability assay, Hoechst 33342 nuclear staining, DNA fragmentation analysis, and flow cytometry (Feng and Zhang, 2004). However, the neuroprotective effect observed for melatonin was reduced considerably when picrotoxin, an antagonist of the γ -amino butyric acid (GABA) receptor, was added to the system. Therefore, the neuroprotective effect of melatonin actually might be mediated by its action on the GABA receptor rather than directly on A β (Paula-Lima et al., 2003). Melatonin was reportedly neuroprotective in organotypic hippocampal cultures maximally at 1 μ M in combination with 1 μ M galantamine, an acetylcholinesterase inhibitor. Mechanistically, this treatment caused antiapoptotic effects and reduced oxidative stress, neuroinflammation, tau phosphorylation, and aggregates positively stained with thioflavin S (Buendia et al., 2015). In a recent study, melatonin rescued A β -induced memory deficits in mice. Wild-type male C57BL/6N mice (25–30 g, 8 weeks old) were stereotactically administered human A β 42 (aggregated for 4 days at 37°C at a concentration of 1 mg/mL) intracerebroventricularly (Ali and Kim, 2015). Using Y-maze, Morris water maze, and immunohistochemistry, the authors showed that melatonin had protective effects against A β 42-induced neurotoxicity by decreasing memory impairment, synaptic disorder, and tau hyperphosphorylation. In another study using Tg2576 mice, melatonin treatment improved pathology and memory performance in 4- to 12-month-old mice by targeting activated GSK3 β (Peng et al., 2013). Melatonin supplementation decreased A β 42 and A β 40 levels in the hippocampus and A β 42 levels in the frontal cortex of OXYS rats, and improved treated rats' reference memory function (Rudnitskaya et al., 2015). OXYS rat is a model of senescence-accelerated sporadic AD that suffers from deficient melatonin secretion (Kolosova et al., 2014; Stefanova et al., 2014a,b).

Excess melatonin is associated with several physiological disorders, including seasonal affective disorder, mood changes, abnormalities in thyroid and adrenal function, cancer, cardiovascular diseases, and hypertension. Thus because melatonin affects multiple physiological systems, its therapeutic use probably will be difficult and is not likely to be a viable option for AD. However, a prolonged-release formulation containing 2 mg melatonin was recently tested in 80 patients who suffered from mild-to-moderate AD, with or without insomnia comorbidity, but receiving standard therapy (acetylcholinesterase inhibitors with or without memantine) (Wade et al., 2014). In this randomized, double-blind, parallel-group study, patients were treated for 2 weeks with placebo and subsequently received 2 mg of prolonged-release melatonin or placebo nightly for 24 weeks, followed by 2 weeks placebo treatment. In the comorbid AD–insomnia subgroup, prolonged-release melatonin resulted in significant and better effects than the placebo when patients' performances were scored by IADL, MMSE, ADAS-Cog, and sleep efficiency. Prolonged-release melatonin was well tolerated by the patients. The authors concluded that melatonin positively affected cognitive functioning and sleep regulation in AD patients, particularly in those suffering insomnia (Wade et al., 2014).

$A\beta$ ASSEMBLY MODULATORS FROM NONNATURAL SOURCES

In this section, we discuss typical and atypical small molecules, as well as large molecules or molecular complexes used to modulate $A\beta$ assembly and toxicity (summarized in Table 6.3). Although most typical small molecules are ≤ 500 Da according to Lipinski's guidelines (Lipinski et al., 2001) because $A\beta$ self-assembly is an atypical target, we define small molecules as those ranging from ~ 300 to 1000 Da in molecular mass, and large molecules as those whose molecular mass is ≥ 1000 Da.

Small-Molecule Modulators of $A\beta$ Assembly and Toxicity

Methylene Blue

Methylene blue or methylthioninium chloride (3,7-bis(dimethylamino)-phenothiazin-5-ium chloride, trade name: RemberTM, Fig. 6.23A) belongs to the tricyclic phenothiazine family (reviewed by Guerrero-Muñoz et al., 2014; Schirmer et al., 2011). It is used in a wide range of clinical indications, including methemoglobinemia, ifosfamide-induced neurotoxicity in cancer patients, urinary tract infections in the elderly, vasoplegic adrenalin-resistant shock, and pediatric malaria (Guerrero-Muñoz et al., 2014; Schirmer et al., 2011). It is also used for intraoperative visualization of nerves, nerve tissues, endocrine glands, parathyroid glands, or pathologic fistulae (Guerrero-Muñoz et al., 2014; Schirmer et al., 2011).

Methylene blue or its derivatives currently are in phase 2 clinical trials for AD (Wischik et al., 2015), and patient recruitment is under way for phase 3 trials. These clinical trials are based on the evidence that methylene blue functions as a tau aggregation modulator/inhibitor (Taniguchi et al., 2005; Wischik et al., 1996, 2008, 2015). Because our chapter does not focus on modulators of tau aggregation (see Chapter 15), here we summarize studies related to the effects of methylene blue on $A\beta$ aggregation and discuss experimental animal studies that showed reduction in $A\beta$ levels following methylene blue treatment.

In 2007, azure C (3-amino-7-methylaminophenothiazin-5-ium chloride, Fig. 6.23B), an oxidation product of methylene blue (Marshall, 1976), was shown to modulate $A\beta_{42}$ oligomerization and promote fibrillization in vitro when tested by dot-blotting using the oligomer-targeting antibody, A11 (Necula et al., 2007b). Rather than completely inhibiting oligomerization, azure C modulated oligomerization such that A11 reactivity was lost but fibrillization still continued; $A\beta$ formed 6E10-positive, high-molecular-weight species which could not enter the SDS-PAGE gels (Necula et al., 2007b). These findings were further confirmed by TEM experiments (Necula et al., 2007a).

A different study showed modulation of $A\beta_{42}$ oligomerization, enhanced disappearance of nonaggregated $A\beta_{42}$, and formation of larger, potentially fibrillar aggregates using capillary electrophoresis (Brinet et al., 2014). However, this study did not specify the exact chemical name or source of methylene blue, or whether its derivatives or oxidation products were used.

In 2014, effects of methylene blue on AD-like pathology and behavioral deficits were tested (Mori et al., 2014) in PSAPP mice bearing exon-9-deleted presenilin 1 and Swedish APP_{K595N/M596L} (APP_{SWE}) (Jankowsky et al., 2001, 2004), modeling cerebral amyloidosis. The authors administered methylene blue at 3 mg/kg once daily for 3 months to 15-month-old transgenic mice by gavage and examined the effect of the treatment on cognitive function, behavior,

TABLE 6.3 A β Assembly and Toxicity Modulators from Nonnatural Sources

Compound	Type	In vitro studies		Animal studies		Human studies/ clinical trials
		Inhibition of A β -induced cytotoxicity	Modulation of A β assembly	Decrease of pathological A β	Effect on behavioral deficits	
Methylene blue	Small molecule	Yes, and also reduces APP processing in cultured cells	Promotes fibrillization (azure C)	Reduces brain parenchymal and vascular A β deposits; reduces levels of extracted A β in treated mice compared to vehicle-treated mice	Prevents behavioral deficits in mouse models of AD	Currently in clinical trials based on its inhibition of tau aggregation, not its effects on A β
Mitoxantrone, bithionol, and hexachlorophene	Small molecule	Bithionol alone is nontoxic; mitoxantrone and hexachlorophene reduce cell viability, mitoxantrone or bithionol reduce cytotoxicity significantly	Inhibit seeded A β fibrillization and fibrillization of nonaggregated A β preparations	Transgenic mice treated with bithionol or mitoxantrone showed diffuse A β plaques and significantly lower A β 42 in the frontal cortex and in the hippocampus (only biothionol) than untreated APPtg mice. Both compounds protected neurons and synapses; hexachlorophene was ineffective	Unknown	Unknown
D737	Small molecule	Inhibits cytotoxicity of high-molecular-weight A β 42 oligomers but not that caused by low-molecular-weight oligomers	Inhibits fibril formation	Unknown	Improves life span and locomotion in a fly model of AD	Unknown
D737 analogs D744 and D830	Small molecule	Expected similar to parent compound	Expected similar to parent compound	Unknown	More effective than D737	Unknown
Cromolyn sodium	Small molecule	Increases A β clearance by microglial activation	Inhibits A β fibrillization and oligomerization but does not dissociate preexisting assemblies	Lowers soluble A β levels but not insoluble A β burden in a mouse model; enhances microglial activation and microglia-mediated A β clearance in vivo	Unknown	Unknown

(Continued)

TABLE 6.3 A β Assembly and Toxicity Modulators From Nonnatural Sources (*cont.*)

Compound	Type	In vitro studies		Animal studies		Human studies/ clinical trials
		Inhibition of A β -induced cytotoxicity	Modulation of A β assembly	Decrease of pathological A β	Effect on behavioral deficits	
Aminopyrazole derivatives	Small molecule	Yes	Some derivatives suppress fibril formation more efficiently than others	Unknown	Unknown	Unknown
Ruthenium complexes, for example, PRMU20	Small molecule	Yes	Inhibits A β fibrillization	Unknown	Unknown	Unknown
Molecular tweezers	Small molecule	Inhibit A β -induced cell death, retraction of dendritic spines, impairment of basal synaptic activity, and suppression of LTP	Inhibit A β fibril formation and oligomerization; dissociate preformed fibrils	Reduce A β plaques in transgenic AD mice and rats	Unknown in AD models Rescue disease phenotype and improve behavioral deficits in models of Parkinson's disease	Unknown
Cucurbit[7]uril	Large molecule	Yes	Inhibits fibrillization	Unknown	Unknown	Unknown
Metallohelical complexes	Large molecule	Yes	Inhibit fibrillization	Unknown	Unknown	Unknown
Polyoxometalates (POMs)	Large molecule	Yes but after a long, 7-day incubation with A β ; some derivatives dissociate preformed fibrils	Wells–Dawson POMs were more effective than other POMs	Unknown	Unknown	Unknown
Artificial molecular chaperones	Large molecule	Yes	Attenuates nucleation and elongation of A β fibrillization; does not affect preformed aggregates/fibrils	Unknown	Unknown	Unknown

FIGURE 6.23 Structures of (A) methylene blue and (B) azure C, a methylene blue derivative.

CAA, and APP processing. Transgene-associated behavioral deficits, including hyperactivity and impaired spatial, working, and reference memory, were prevented upon treatment (Mori et al., 2014). Moreover, brain parenchymal and vascular A β deposits and levels of extracted A β were reduced in methylene blue-treated mice compared to vehicle-treated mice (Mori et al., 2014). Mechanistically, the reported beneficial effects in this animal model were attributed to inhibition of amyloidogenic APP proteolysis, whereby protein expression of β -carboxyl-terminal APP fragment and expression and activity of BACE1 were found to be reduced (Mori et al., 2014). Although these mechanisms are not directly relevant to our chapter, which deals with inhibition and/or modulation of A β self-assembly rather than A β production, we mention them here briefly for completeness of the discussion. Methylene blue treatment of CHO cells overexpressing wild-type human APP also significantly decreased A β production and proteolytic APP processing (Mori et al., 2014).

The therapeutic or preventive uses of oral or intraperitoneal methylene blue treatment against A β pathology also was tested (Paban et al., 2014) in the transgenic mouse model APP/PS1, bearing the chimeric human–mouse APP gene with the Swedish mutation (K549N/M595L) and the human presenilin 1 A246E variant, APP_{SWE}/PS1 (A246E) (Borchelt et al., 1996, 1997). Cognitive impairment assessed by social, learning, or exploratory tasks was prevented in treated transgenic mice in this study (Paban et al., 2014). Correlating with the behavioral findings, immunoreactive hippocampal and cortical A β deposition was significantly reduced in treated mice, again likely reflecting effects on A β production rather than assembly.

Besides methylene blue's effects on A β and tau aggregation, it partially improves mitochondrial function (Paban et al., 2014) and cellular metabolism, and influences cholinergic, serotonergic, and glutamatergic neurotransmitters, all of which are involved in AD pathogenesis (Oz et al., 2009). Collective evidence suggests that the combination of methylene blue's diverse roles likely mediates its potentially beneficial effects in animal models and disease settings.

The aforementioned studies used a multifaceted rationale for employing methylene blue as a modulator of A β and/or tau aggregation:

- Methylene blue is a small molecule with many properties suitable for a drug that can enter and act in the brain.
- It has high aqueous solubility.

- It has low toxicity in rats and humans (Kupfer et al., 1994; Riha et al., 2005).
- It can cross the blood–brain barrier in rats, and it yields high cerebral levels irrespective of its administration route (O’Leary et al., 1968; Peter et al., 2000; Walter-Sack et al., 2009).
- It has been approved for use in humans (DiSanto and Wagner, 1972; Guerrero-Muñoz et al., 2014; Kupfer et al., 1994; Schirmer et al., 2011). However, it should never be given intrathecally in humans (Schirmer et al., 2011); when administered through this route, it can reportedly cause reversible paraplegia with residual perineal anesthesia and bladder dysfunction (Evans, 1959; Sivak, 1959).

The major caveat in using methylene blue for treating AD is that the compound accelerates $A\beta$ fibrillization. This may provide initial relief due to reduction in the steady-state concentration of oligomers, which are the most toxic $A\beta$ species and are more toxic than fibrils. However, fibrils form amyloid plaques and accumulate therein; plaques impinge on functional neurites, take up parenchymal space, and resist proteolysis and clearance as AD progresses. In addition, fibrillar $A\beta$ deposits in the brain vasculature lead to CAA (Ellis et al., 1996), a major comorbidity in AD that may lead to microhemorrhage and stroke, and complicates use of $A\beta$ -targeting strategies (Sperling et al., 2011). Also, $A\beta$ normally regulates brain function and memory at picomolar physiological levels (Morley and Farr, 2014), and lowering free $A\beta$ by hastening fibrillization may compromise $A\beta$ ’s potential physiological role. Thus, a therapeutic strategy based on increasing fibril load is questionable.

Mitoxantrone, Bithionol, and Hexachlorophene

Addition of preformed $A\beta$ fibril “seeds” to a fibrillization reaction shortens the nucleation-dependent lag phase of $A\beta$ self-assembly, and accelerates fibrillization in vitro and in vivo (Harper and Lansbury, 1997; Stöhr et al., 2012, 2014). Seeded $A\beta$ fibrillization in vivo likely is crucial for $A\beta$ plaque formation (Walker et al., 2013) and $A\beta$ dissemination by a potential prion-like mechanism of propagation (Frost and Diamond, 2010).

Seeded fibrillization of $A\beta_{42}$ was targeted to identify molecules that could disrupt this process, prevent $A\beta_{42}$ toxicity, and potentially prevent the prion-like spread of $A\beta$ plaques (Eleuteri et al., 2015). A library of 1040 compounds (80% of which were approved by the US Food and Drug Administration) was screened in a high-throughput seeded fibrillization assay in vitro. Fibril fragments of 100–300 nm length were added to monomeric $A\beta_{42}$ solutions at molar ratio 4:10, respectively, in 384-well plates. The test compounds were added to the reaction mixture at 10 μ M final concentration per well and incubated at 37°C for 3 h under agitation. Fibrillization kinetics was assayed by ThT fluorescence. Three compounds were found to inhibit seeded $A\beta$ fibrillization at low micromolar concentrations: mitoxantrone (IC_{50} = 1.7 μ M, Fig. 6.24A), bithionol (IC_{50} = 5.33 μ M, Fig. 6.24B), and hexachlorophene (IC_{50} = 5.9 μ M, Fig. 6.24C). They also strongly inhibited fibrillization of freshly prepared monomeric $A\beta_{42}$ by SEC (Di Giovanni et al., 2010) with similar IC_{50} values.

The potential protective effect of the three compounds against $A\beta_{42}$ -induced toxicity was tested in primary murine cortical neurons. Although bithionol alone was nontoxic, mitoxantrone and hexachlorophene reduced cell viability at 20 and 50 μ M, respectively. Primary neurons were ~50–60% less viable when treated for 24 h with crude $A\beta_{42}$ preparations (40 μ M), which contained predominantly monomeric and protofibrillar species. In the presence of 5 or 10 μ M mitoxantrone or bithionol, $A\beta_{42}$ cytotoxicity was significantly reduced. According to the authors, this was consistent with the proposed inhibitory action of the compounds

FIGURE 6.24 Structures of mitoxantrone (A), bithionol (B), and hexachlorophene (C).

that target fibril growth rather than stabilizing monomeric A β 42. However, stabilization of A β 42 monomers also would be consistent with inhibition of toxicity when oligomerization is prevented. Hexachlorophene only modestly protected against A β 42-induced cytotoxicity despite inhibiting conversion of protofibrils to fibrils and inhibiting seeded fibrillization.

In view of the strong antiamyloidogenic and neuroprotective properties of bithionol and mitoxantrone and the modest effects of hexachlorophene observed *in vitro* and in tissue culture, the authors examined the compounds next in an APP transgenic mouse model of AD (mThy1-APPtg; [Rockenstein et al., 2001, 2002](#)). Each compound was administered intravenously at 10 mg/kg to 8-month-old mice, and their pharmacokinetic properties were studied in plasma and brain extracts by liquid chromatography–mass spectrometry (LC–MS/MS). Bithionol showed 20% brain penetration with plasma and brain half-lives of 7.62 and 1.77 h, respectively. Mitoxantrone surprisingly showed 100% brain penetration. Hexachlorophene showed 50% brain permeability with plasma and brain half-lives of 4.4 and 5.64 h, respectively ([Eleuteri et al., 2015](#); [DiMeco et al., 2002](#); [Green et al., 1988](#)).

The authors then examined the effects of the three compounds on A β 42 deposition, A β 42 oligomer levels, synaptic loss, neuronal damage, and astrogliosis, as representative neuropathological hallmarks of AD, and compared the corresponding data among the treated animals, vehicle-treated APP transgenic mice, and the nontransgenic vehicle-treated control mice. Because bithionol and mitoxantrone cross the blood–brain barrier, they were administered daily by intraperitoneal injection for 1 month at 10 and 1 mg/kg, respectively. A confusing point in the report is that despite finding 50% brain permeability for hexachlorophene, it was treated as if it could not cross the blood–brain barrier and thus was administered intracerebroventricularly ([Eleuteri et al., 2015](#)).

$A\beta$ 42 immunohistochemistry using antibody 6E10 and thioflavin S staining in vehicle-treated tissue sections from the APPtg mice showed dense amyloid plaques in the frontal cortex and the hippocampus. Treated mice showed diffuse $A\beta$ plaques in the frontal and hippocampal regions and significantly lower $A\beta$ 42 immunoreactivity in the frontal cortex when treated with bithionol or mitoxantrone and in the hippocampus when treated with bithionol, relative to untreated APPtg mice. In contrast, hexachlorophene-treated APPtg mice showed dense plaques and high $A\beta$ 42 immunoreactivity.

Bithionol- or mitoxantrone-treated APPtg mouse brains also showed significantly reduced $A\beta$ monomers and intermediate species. Total $A\beta$ levels in membrane fractions and in guanidine-HCl-extracted samples were dramatically reduced following administration of bithionol or mitoxantrone compared with that in vehicle-treated APPtg mice as measured by ELISA. Hexachlorophene did not significantly reduce $A\beta$ 42 levels. Together, immunoblotting and ELISA confirmed that bithionol and mitoxantrone strongly reduced $A\beta$ 42 levels in brain tissue extracts from treated mice. In addition, these compounds protected neuronal and synaptic integrity in the APPtg mice, as shown by immunohistochemistry and/or Western blotting of several markers, including synaptophysin, a major membrane constituent of the presynaptic vesicles; postsynaptic density-95 protein; the neuronal marker microtubule-associated protein 2; and NeuN, a vertebrate neuron-specific nuclear protein.

D737

The discovery of D737 ensued from screening of 65,000 small molecules in a high-throughput fluorescence assay to isolate those that modulate $A\beta$ 42 assembly; the fluorescence assay used a recombinant fusion system of $A\beta$ and green fluorescent protein (GFP) (McKoy et al., 2012). In this system, aggregation and/or insolubility of the upstream $A\beta$ 42 in the fusion sequence prevents correct folding of the downstream GFP, whereby $A\beta$ 42-GFP fusion expressed in *E. coli* does not fold properly or fluoresce. Thus, compounds that inhibit $A\beta$ 42 assembly and allow proper GFP folding can be found by fluorescence screening (Kim et al., 2006; Waldo et al., 1999). Of all the screened compounds, 269 constituted positive hits. From these, eight commercially available compounds were chosen for further testing, and D737 (Fig. 6.25A) was identified by ThT fluorescence to interfere with $A\beta$ fibrillization more significantly than the others. This effect depended on D737 concentration; 10 μ M D737 inhibited ThT fluorescence of 20 μ M $A\beta$ 42 by 50%. In cytotoxicity assays using the MTT reduction assay, D737 inhibited cytotoxicity of high-molecular-weight $A\beta$ 42 oligomers but did not affect that caused by low-molecular-weight oligomers formed after 1 h of incubation. D737 also inhibited the cytotoxicity of insoluble high-molecular-weight oligomers formed after 2–24 h of incubation and of fibrils formed after 8 h of incubation.

D737 significantly improved both the life span and locomotive ability of transgenic *Drosophila* expressing either wild-type $A\beta$ 42 or $A\beta$ 42(E22G) in their CNS, thus confirming D737's effect in vivo. D737 at 20 μ M increased the median life span of $A\beta$ 42-expressing transgenic flies to 43 days, compared with control flies with a median life span of 37 days. The median life span for untreated transgenic flies expressing $A\beta$ 42(E22G) was 30 days whereas treatment with 20 μ M D737 extended the median life span to 34 days. The flies' locomotive activity was assessed using a climbing assay (Crowther et al., 2006). Transgenic flies treated with 20 μ M D737 showed dramatically improved climbing ability relative to untreated flies (McKoy et al., 2012).

FIGURE 6.25 Structures of D737 (A), D744 (B), and D830 (C), with fluorine substitutions at the *para*- and *meta*-positions in the R1 phenyl group of D737, respectively. A methyl group in the R2 position was essential for the function of all three compounds.

In a follow-up study, structure–activity relationships of 11 commercially available D737 analogs were reported (McKoy et al., 2014). D744 (Fig. 6.25B) and D830 (Fig. 6.25C), containing fluorine substitutions at the *para*- and *meta*-positions, respectively, in the R1 phenyl group of D737, were found to be more effective than the parent compound in extending the life span and in improving the locomotive ability of A β 42-expressing flies (McKoy et al., 2014).

Cromolyn Sodium (Disodium Cromoglycate)

Cromolyn sodium (Fig. 6.26) was tested for modulation of A β self-assembly based on the rationale that its structure is similar to that of fisetin (Fig. 6.14) (Hori et al., 2015). Furthermore, pharmacological use of cromolyn sodium in asthma and other diseases (Ammit et al., 2009; Castells et al., 2011; Daniels et al., 2013; Ng and Ohlsson, 2012) and its ability to cross the blood–brain barrier after injection in rodent addiction models (Leza et al., 1995; San-Martin-Clark et al., 1993, 1995) provided additional incentives to study the compound in relation to AD.

FIGURE 6.26 Structure of cromolyn sodium.

ThT assays were used first to assess the role of cromolyn sodium in modulating $A\beta$ 40 and $A\beta$ 42 self-assembly. Increasing concentrations of cromolyn sodium (5, 50, 500 nM) inhibited ThT fluorescence over 1 h, and TEM confirmed that fibrillization of $A\beta$ 42 at 0.2 mg/mL ($\sim 44 \mu\text{M}$) was inhibited by 500 nM of the compound (Hori et al., 2015). Next, a split luciferase complementation assay (Hashimoto et al., 2011) was used for indirectly monitoring $A\beta$ oligomer formation. HEK293 cells overexpressing both N-terminally or C-terminally luciferase-conjugated $A\beta$ 42 showed a significantly lower, dose-dependent luminescence signal when treated with cromolyn sodium compared to untreated cells (Hori et al., 2015). However, cromolyn sodium inhibited intracellular $A\beta$ oligomerization only at concentrations $>10 \mu\text{M}$ in cells expressing the split luciferase system. Addition of cromolyn sodium to pre-formed luciferase-conjugated $A\beta$ oligomers did not abrogate the luminescence signal. Altogether, the authors concluded that cromolyn sodium prevented $A\beta$ self-assembly into high-order oligomers or fibrils, but could not dissociate preexisting assemblies (Hori et al., 2015).

To extend these findings in vivo, APP/PS1 mice were treated with increasing doses of cromolyn sodium (1.05, 2.1, 3.15 mg/kg), injected daily intraperitoneally (Hori et al., 2015). A 7-day treatment of mice with 2.1 or 3.15 mg/kg cromolyn sodium significantly reduced buffer-soluble $A\beta$ x-40 and $A\beta$ x-42 species by $>50\%$. Treating mice with the highest dose of cromolyn sodium, 3.15 mg/kg, also led to significantly lower concentrations of “detergent-resistant” $A\beta$ ($A\beta$ x-40 and $A\beta$ x-42) species, extracted sequentially in buffers containing 2% Triton or 2% SDS, than in control animals treated with phosphate-buffered saline. In contrast, in mice treated with cromolyn sodium, levels of insoluble $A\beta$ species extracted in formic acid, and the overall $A\beta$ plaque burden assessed by immunohistochemistry, were unaffected by the treatment. Because cromolyn sodium treatment only lowered soluble $A\beta$ levels and did not affect insoluble $A\beta$ burden in APP/PS1 mouse brain treated for 1 week, the authors concluded that cromolyn sodium predominantly modulated $A\beta$ aggregation rather than dissociating preformed $A\beta$ assemblies, similar to their in vitro findings (Hori et al., 2015). Reduction of the concentration of soluble $A\beta$ would be expected to induce disaggregation of plaques, but such disaggregation was not observed, leaving the in vivo mechanism of action of cromolyn sodium unclear.

Because treatment with cromolyn sodium decreased the amount of soluble $A\beta$ rather than aggregated species in mice, the authors measured soluble $A\beta$ species in the hippocampal interstitial fluid (ISF) using an in vivo microdialysis system (Hori et al., 2015). Treating the APP/PS1 mice with cromolyn sodium at 3.15 mg/kg daily for 1 week decreased ISF $A\beta$ x-40 by 30% (control, 387 pM; cromolyn-treated mice, 283 pM). ISF $A\beta$ x-42 and $A\beta$ oligomers followed a similar trend, but the changes were not statistically significant.

Besides modulating $A\beta$ assembly, cromolyn sodium reportedly enhanced microglial activation and microglia-mediated $A\beta$ clearance in vivo. These postulates were based on a 50% reduction in $A\beta$ half-life in ISF of mice treated with cromolyn sodium compared with that in control mice, and a higher percentage of overlapping immunoreactivity of microglia and $A\beta$ in brain sections of mice treated with 3.15 mg/kg cromolyn sodium compared with untreated animals. These findings were supported by tissue-culture experiments in which cromolyn-sodium-treated microglia cleared $A\beta$ 40 or $A\beta$ 42 levels dose dependently after 16 h. Microglia treated with 1 mM cromolyn sodium decreased $A\beta$ 40 or $A\beta$ 42 levels 1.5-fold or 2-fold, respectively, more efficiently than untreated cells. Thus, the combination of in vivo and in vitro results suggests that cromolyn sodium may modulate $A\beta$ self-assembly and promote microglial activation and resultant $A\beta$ clearance (Hori et al., 2015).

FIGURE 6.27 Interaction of aminopyrazole trimers with an extended β -strand. (A) Hydrogen-bonding interactions of an aminopyrazole trimer with A β (17–21). (B) A β 42 fibrils stacked as a pentameric unit. A β 42 fibril structure was determined by NMR (Lührs et al., 2005). Four regions likely to interact with C-terminally extended aminopyrazole trimers are indicated in dark gray. The aminopyrazole moiety and the interacting phenylalanine residues are shown in light gray stick models, respectively. Adapted with permission from Hochdörffer K, März-Berberich J, Nagel-Steger L, Eppele M, Meyer-Zaika W, Horn AH, Sticht H, Sinha S, Bitan G, Schrader T: Rational design of β -sheet ligands against A β 42-induced toxicity, *J Am Chem Soc* 133:4348–4358, 2011; Copyright 2011 American Chemical Society.

Aminopyrazole Derivatives

Aminopyrazole derivatives are a class of rationally designed, β -sheet-binding, small molecules with specific hydrogen bonding donor–acceptor–donor configurations that interact complementarily with hydrogen bonding groups of a peptide in a β -sheet configuration (Kirsten and Schrader, 1997; Schrader and Kirsten, 1996). As such, aminopyrazole derivatives were shown initially to stabilize β -sheet structures by forming cooperative hydrogen bonds (Schrader and Kirsten, 1996). An example of such interactions is presented in Fig. 6.27A. Since then, several different aminopyrazole-containing derivatives have been generated and tested with the aim of increasing their length for providing more hydrogen bonding or π -stacking groups for interactions with β -sheet peptide (Hellmert et al., 2015; Hochdörffer et al., 2011; Kroth et al., 2012; Rzepecki et al., 2003, 2004; Rzepecki and Schrader, 2005). For example, with consideration of complementary binding sites on the A β 42 protofilament structure determined previously by NMR (Lührs et al., 2005), also shown in Fig. 6.27B, “trimeric” aminopyrazoles were designed to feature various C-terminal extensions (Hochdörffer et al., 2011). These extended “trimers” (Fig. 6.28) were designed to bestow additional favorable interacting groups besides hydrogen bonding and π -stacking by targeting specific A β regions (Fig. 6.27B):

- For binding to the polar A β 42 region spanning residues Glu22–Lys28 (Fig. 6.27B), carboxylate and ammonium groups were placed close to the aminopyrazoles, for example, trimer-diamine, trimer-GABA-OH, and trimer TEG-OH (Fig. 6.28).

FIGURE 6.28 Structures of unprotected aminopyrazole trimer derivatives. Small neutral, anionic, and cationic moieties, and nonpolar and TEG-spacer groups are shown on the left. Peptidic attachments are shown on the right. R = aminopyrazole trimer moiety.

- For binding to the Glu22 ladder (Fig. 6.27B) formed by the Glu22 side chains on adjacent cross- β -sheet layers, pentacationic groups (eg, five Lys residues) were added, for example, trimer-KKKKKG-OH and trimer-TEG-KKKKKG-OH (Fig. 6.28).
- For potential binding to the central U-shaped turn of A β joining its polar and nonpolar regions (Fig. 6.27B), a triethyleneglycol unit was added for extended van der Waals interactions, for example, trimer-TEG-OEt, trimer-TEG-OH, trimer-TEG-Che, trimer-TEG-Dd, and trimer-TEG-Lys-OMe (Fig. 6.28).
- For binding to the nonpolar residues Ile31–Val36 (Fig. 6.27B), flexible, branched hydrocarbons such as cyclohexylglycine were included (for hydrophobic and dispersive interactions), for example, trimer-Che, trimer-Lys-Che, trimer-TEG-Che, and trimer-Chg-Che (Fig. 6.28).
- Small peptides from the CHC also were attached to aminopyrazole trimers for promoting self-recognition, for example, trimer-LPFFD-OH, trimer-KLVFF-OH, and trimer-TEG-LPFFD-OH (Fig. 6.28).

Overall, the derivatives were divided into type A (polar), type B (very polar), and type C (nonpolar). Molecular dynamics simulations suggested that extended aminopyrazole trimers interacted with the A β 42 fibril structure as expected (Hochdörffer et al., 2011). To confirm the simulations experimentally, some of the designed aminopyrazole derivatives were synthesized and tested in ThT, CD, TEM, and cytotoxicity assays. Of all the tested aminopyrazole trimers, the trimer TEG-K-OMe (Fig. 6.28) suppressed A β fibril formation most efficiently. However, of all tested trimer aminopyrazoles, this ligand was found to form colloidal aggregates. Colloidal aggregates have been reported to inhibit amyloid formation in a nonspecific manner (Feng et al., 2008) and therefore are not considered viable therapeutics candidates.

In ThT assays assessing disaggregation, the percentage of remaining ThT fluorescence for most trimer derivatives was identical to the final ThT fluorescence in aggregation assays (Table 6.4). This observation led the authors to conclude that aminopyrazoles functioned in a reversible fashion, ultimately reaching an open equilibrium where the most thermodynamically stable complexes with A β were favored (Hochdörffer et al., 2011).

Generally, the various trimer aminopyrazoles did not prevent fibrillization but caused formation of compact and thin fibrils of 5–10 nm diameter and 800 nm length, in some cases with

TABLE 6.4 Reported Experimental Results of Aminopyrazole Derivatives

	Glu22 ladder (polylysines)	Polar region (single charged groups)	Nonpolar region (extended alkyl residues)	Central pore (TEG derivatives)
ThT (inhibition) ^a	24%	25%	27–50%	48–54%
ThT (disaggregation) ^a	28%	21%	35–62%	42–68%
TEM	Amorphous material	Unstructured material and thin bent fibrils	Thick twisted fibrils (70 $\times$ 600 nm)	Thin, delicate structures
MTT ^b	IC ₅₀ > 80 μ M	IC ₅₀ $\geq$ 22 μ M	IC ₅₀ $\geq$ 3 μ M	IC ₅₀ $\geq$ 35 μ M

^aPercent ThT fluorescence of a mixture containing 10 μ M A β 42 and 60 μ M of an aminopyrazole derivative.

^bPC-12 cells treated with 10 μ M A β 42 and aminopyrazoles at 0.3–100 μ M.

From Hochdörffer K, März-Berberich J, Nagel-Steger L, Eppele M, Meyer-Zaika W, Horn AH, Sticht H, Sinha S, Bitan G, Schrader T: Rational design of β -Sheet ligands against A β ₄₂-induced toxicity, J Am Chem Soc 133:4348–4358, 2011.

no typical helical twist (Hochdörffer et al., 2011). For example, presence of trimer-TEG-OH or trimer-OMe caused formation of delicate thin fibrillar structures with no helical twist (Table 6.4). The authors postulated that the compact nature of these structures prevented ThT binding.

In cytotoxicity assays, all compounds were screened at 100 μ M using the MTT assay in differentiated PC-12 cells. The compounds by themselves did not reduce cell viability at this concentration. When treated for 24 h with 10 μ M $A\beta$ 42 in the presence of 100 μ M of each compound, the cells were completely protected against $A\beta$ 42-induced toxicity by most of the derivatives except for trimer-OMe and trimer-OH, which only increased viability by <10% (Hochdörffer et al., 2011).

Two important binding motifs greatly enhanced the β -sheet-breaker ability of the aminopyrazoles. Lipophilic moieties interacted with nonpolar amino acid clusters between Ile31 and Val36, and the oligo-Lys residues interacted with the Glu22 ladder.

Ruthenium Complexes

Messori et al. (2013) used the rationale that metal-containing complexes, such as platinum-phenanthrolines, bind $A\beta$ 42, interfere with its self-assembly, and protect against $A\beta$ 42-induced cytotoxicity (Barnham et al., 2008; Kenche and Barnham, 2011; Valensin et al., 2011). However, they decided to test various ruthenium complexes because they posited that ruthenium-containing compounds are less cytotoxic than platinum complexes (Messori et al., 2013). Ruthenium compounds have affinity toward His residues and may modulate $A\beta$ 42 assembly by interacting with His6, His13, and His14 residues (Messori et al., 2013). Three representative ruthenium compounds were tested, namely NAMI-A (Alessio et al., 2004), KP1019 (Hartinger et al., 2008), and PMRU20 (Mura et al., 2005). NAMI-A ([ImH][*trans*-RuCl₄(DMSO-S)(Im)]), where DMSO is dimethyl sulfoxide and Im is imidazole; Fig. 6.29A) and KP1019 (indazolium *trans*-[tetrachlorobis(¹H-indazole)ruthenate(III)]; Fig. 6.29B) are two experimental anticancer compounds. PMRU20 (2-aminothiazolium [*trans*-tetrachlorobis(2-aminothiazole)ruthenate(III)]; Fig. 6.29C), which is a relatively new compound, was prepared and characterized by the same group (Mura et al., 2005).

The activity of the three compounds was tested against $A\beta$ 42-induced cytotoxicity using the MTT assay in rat primary cortical neurons. PMRU20 was active whereas NAMI-A was poorly active and KP1019 was nearly inactive in the MTT assay (Messori et al., 2013). To

FIGURE 6.29 Structures of ruthenium complexes, including NAMI-A (A), KP1019 (B), and PMRU20 (C).

test whether the protective effect of PMRU20 was due to the metal-containing center of the compound and not due to the antioxidant properties that were shown for aminothiazole derivatives (Uchikawa et al., 1996), its counter-ion, 2-aminothiazole, was tested in the presence of A β 42. Because 2-aminothiazole alone was ineffective, the authors concluded that the protective effects of the compound were primarily due to the metal-containing center. However, using only the aminothiazole counter-ion in these experiments does not necessarily demonstrate that the protective activity is specific to the metal-containing center of the molecule.

The authors used the ThT assays to compare the effects of the ruthenium compounds on A β 42 aggregation. PMRU20 addition at twofold molar excess to A β 42 caused a large decrease in ThT fluorescence after 10 h of incubation, whereas NAMI-A was less effective and KP1019 reacted with ThT and precipitated. In contrast to the aforementioned report, a different study found that KP1019 at ≥ 0.5 equivalents to A β 42 (5 μ M in the assay) limited ThT fluorescence and caused formation of amorphous A β 42 assemblies observed by TEM (Jones et al., 2015).

To explore the binding site of KP1019, reactivity of antibodies 6E10 and 4G8 with A β 42 in the presence of KP1019 was assessed (Jones et al., 2015). At a 1:1 KP1019-to-A β 42 ratio over 24, 48, or 72 h incubation, 6E10 still bound A β 42. However, at 2:1, 4:1, or 10:1 ratios, 6E10 reactivity was reduced, possibly due to saturated binding of KP1019 at the A β 42 N-terminus. In contrast, 4G8 reactivity to the peptide was unaffected on dot blots over time, suggesting that the binding site of KP1019 was not near the CHC of A β 42. Native PAGE and Western blotting showed that increasing concentrations of KP1019 (0.25–2 molar equivalents to A β) caused disappearance of monomeric A β and small oligomeric A β 42 bands but increased the intensity of high-molecular-weight species between 37 and 250 kDa, and higher aggregates that did not enter the gel. These results combined with TEM experiments showed that KP1019 drove formation of large A β aggregates but prevented formation of small oligomeric A β 42 assemblies. Induction of formation of high-molecular-weight assemblies might have been responsible for the change in 6E10 reactivity. When preincubated with A β 42 from 5 min to 24 h, KP1019 rescued SH-SY5Y cells from A β 42 toxicity when used at ≥ 0.1 molar equivalents to A β 42 (Jones et al., 2015).

Molecular Tweezers

Molecular tweezers are supramolecular compounds prepared originally by Klärner et al. (1996). They have a rigid hydrocarbon skeleton comprising alternating aromatic and aliphatic rings. The aliphatic rings are constrained in norbornene-like structures forcing the molecules into a horseshoe-shape structure (Fig. 6.30A). This structure creates a high electron density in the cavity of the tweezers (Fig. 6.30B), making them welcoming “hosts” for electron-deficient “guests” (Klärner et al., 1996, 2000). Attachment of negatively charged groups, for example, phosphonates and phosphates by Schrader and coworkers to the central aromatic ring of the tweezers has rendered them water soluble, allowing examination of potential biological applications and leading to the discovery that these compounds bound with low micromolar affinity and high selectivity to Lys residues (Fokkens et al., 2005; Talbiersky et al., 2008). The molecular tweezers also bound Arg, yet with 5–10 times lower affinity, and did not bind to other amino acid residues or small, positively charged biological cofactors (Fokkens et al., 2005).

The selectivity of the molecular tweezers for Lys (and Arg) is due to inclusion of the alkyl chain of these residues inside the cavity of the tweezer, facilitating hydrophobic interaction

FIGURE 6.30 Molecular tweezers. (A) A schematic structure of water-soluble molecular tweezers. At least one of the X and Y groups must be negatively charged. (B) Electrostatic potential surface showing the high electron density inside the tweezer cavity. Color code: -25 kcal/mol: dark gray; $+25$ kcal/mol: black. (C) Schematic structure of the complex between CLR01 and Lys. Source: Part B, adapted from Fokkens *et al.* (2005).

between the tweezer's side arms and the butylene chain of Lys, and simultaneous strong electrostatic attraction between the ϵ -NH₃⁺ group of Lys and the negatively charged groups of the molecular tweezer (Fig. 6.30C).

Importantly, binding of the molecular tweezers to their target amino acids is labile and has a high on-off rate (Bier *et al.*, 2013). Our group discovered that these characteristics allow the molecular tweezers to disrupt selectively the abnormal self-assembly process of amyloid-forming proteins without affecting normal proteins, even though their binding is not selective to a particular protein (Attar and Bitan, 2014). This somewhat counterintuitive selectivity profile is due to the difference between normal protein and abnormal proteins assemblies. Stable proteins, whose structure was optimized by millions of years of evolution, are unaffected by the gentle binding of the molecular tweezers, whereas the weak forces mediating formation of metastable oligomers and nuclei, which (mis)fold considerably more slowly and through a rougher energy landscape (Yu *et al.*, 2015), are disrupted by the molecular tweezers' binding. Moreover, there is a profound difference between freely accessible Lys on unstructured proteins, which can all be complexed by CLR01—the derivative in which both polar groups are phosphates (depicted in Fig. 6.30C), and folded proteins whose surface often does not

allow the sterically demanding tweezer to approach a Lys residue close enough for binding. This was evidenced by X-ray crystallography, isothermal titration calorimetry, and molecular dynamics simulations of the complex between the molecular tweezer CLR01, which has two phosphate groups attached to the central aromatic ring (Fig. 6.30C), and the small adapter protein, 14-3-3, in which only 5 of 17 surface Lys residues were complexed by CLR01 (Bier et al., 2013). In contrast, in unfolded proteins, virtually all lysine residues are expected to be accessible to the molecular tweezers.

ThT fluorescence and TEM showed that CLR01 prevented fibril formation by A β 40 or A β 42, tau, and six other disease-associated proteins (Sinha et al., 2011). Additional experiments showed that CLR01 inhibited formation of A β 42 oligomers in dot blots using the oligomer-specific antibody, A11 (Kayed et al., 2003), attenuated the transition from an unstructured conformation to β -sheet measured (using CD spectroscopy), and prevented formation of large aggregates observed by DLS. ThT and TEM studies also showed that CLR01 dissociated preformed fibrils of A β 40 or A β 42 (Sinha et al., 2011). Recently, using IM-MS, CLR01 was shown to directly decrease the formation of A β 42 hexamers and dodecamers (Zheng et al., 2015a). Previously, these oligomers have been shown to form selectively by A β 42 (Bernstein et al., 2009; Bitan et al., 2003), and dodecamers were found to correlate with AD pathology in human brain and transgenic mouse brain (Gong et al., 2003; Lesné et al., 2006).

The predicted binding sites for CLR01 in A β are Lys16 and Lys28, and to a lower extent Arg5. Binding to these three sites was confirmed by two independent methods—2D heteronuclear solution-state NMR, and top-down, electron-capture dissociation–mass spectrometry (Sinha et al., 2011). Lys16 and Lys28 were found to be the primary and secondary binding sites, respectively, because the former is exposed to the solvent, whereas the latter is involved in a salt bridge with Glu22 and/or Asp23 (Lazo et al., 2005).

Experiments using the MTT-reduction assay in differentiated PC-12 cells showed that CLR01 prevented the toxicity induced by 20 μ M A β 40 or 10 μ M A β 42 oligomers with $IC_{50} = 14 \pm 11$ and 52 ± 18 μ M, respectively (Sinha et al., 2011). In the same cells, inhibition of 5 μ M A β 42 toxicity was achieved with $IC_{50} = 9 \pm 4$ μ M in the LDH assay (unpublished data), likely reflecting the fact that decreasing the protein's concentration reduced the concentration of toxic oligomers nonlinearly.

Subsequent experiments in rat or mouse primary neurons showed that CLR01 inhibited synaptotoxic effects of A β 42 (Attar et al., 2012), including hippocampal dendritic-spine retraction, reduction in frequency and amplitude of basal synaptic activity, and suppression of LTP, a cellular correlate of learning and memory.

To test the hypothesis that CLR01 would disrupt aberrant protein self-assembly but not normal assembly, we compared the protein–CLR01 concentration ratio needed for inhibiting aggregation of amyloid proteins to that required for interfering with a normal assembly process—tubulin polymerization. In experiments examining what ratio would be necessary for inhibition of amyloidogenic proteins, the protein–CLR01 concentration ratios needed for complete inhibition of different amyloid proteins were mostly in the range 1:0.1 to 1:3 (Lopes et al., 2015; Prabhudesai et al., 2012; Sinha et al., 2011). These concentration ratios had no effect on tubulin polymerization. Only at a tubulin:CLR01 concentration ratio of 1:55 was some disruption observed, but not complete inhibition of the process (Attar et al., 2014). Schrader and coworkers also have tested CLR01 as an inhibitor of enzymes containing key Lys residues

at or near the active site. The enzyme:CLR01 ratios needed for inhibiting the activity of alcohol dehydrogenase and PARP-1 were 1:865 (Talbiersky et al., 2008) and 1:1435 (T. Schrader, personal communication), respectively.

The selectivity of CLR01 also was demonstrated in cultured cells, in which CLR01 itself reduced cell viability only at concentrations 1–3 orders of magnitude higher than those needed for inhibition of toxicity by $A\beta$ or other amyloidogenic proteins. CLR01 toxicity depended on the cell type tested. For example, in differentiated PC-12 cells, no toxicity was observed at $\leq 200 \mu\text{M}$ CLR01, and $\sim 15\%$ viability reduction (MTT assay) occurred at $400 \mu\text{M}$ (Sinha et al., 2011). In recent experiments, no reduction in viability (XTT assay) was found at $\leq 500 \mu\text{M}$ in the HIV reporter cell line TZM-bl (a HeLa cell line that enables quantitative analysis of HIV using β -gal or luciferase as reporter genes) (Lump et al., 2015).

CLR01 was evaluated in the $3 \times \text{Tg}$ mouse model of AD (Oddo et al., 2003). Mixed-gender, 15-month-old mice were treated for 28 days with 0.04 mg/kg per day CLR01 in saline (vehicle), or with vehicle alone, applied subcutaneously, continuously using osmotic minipumps (Attar et al., 2012). Immunohistochemistry of brain sections of vehicle-treated mice using antibody 6E10 showed extracellular amyloid plaques deposited predominantly in the subiculum and CA1 regions of the hippocampus, as reported previously (Oddo et al., 2003). In addition, the mice showed neurofibrillary tangles detected by the anti-p-tau mAb AT8, predominantly in the CA1 and CA3 regions.

Mice treated with CLR01 showed a significant decrease in $A\beta$ in multiple brain regions. Similarly, reduction in AT8-positive p-tau was observed in the CA1 (33%) and CA3 (46%) regions (Attar et al., 2012). Therapeutic effects of CLR01 were also observed in animal models of other diseases (Ferreira et al., 2014; Prabhudesai et al., 2012), and CLR01 was shown to have a high safety margin in mice (Attar et al., 2014). In the context of AD, the ability of the compound to affect both $A\beta$ and tau is particularly attractive.

Large Molecules That Modulate $A\beta$ Assembly and Toxicity

In this section, cyclic molecules such as cucurbit[7]uril, metal-containing complexes such as POMs and helical metallocomplexes, and artificial molecular chaperones are discussed. These compounds are mainly experimental, with most data available only in vitro and in cell culture.

Three important potential concerns must be considered, especially in relation to the compounds described later:

1. Are their molecular weights prohibitive of blood–brain barrier penetration?
2. Do these compounds form colloids?
3. If they form colloids, does the colloidal mechanism of inhibition apply?

As discussed previously in section: Aminopyrazole Derivatives, the colloidal mechanism of inhibition (Feng et al., 2008) is relevant when assessing whether certain modulators or inhibitors of $A\beta$ assembly have the potential to become drug candidates. This is important because excess of other proteins, such as albumin, can prevent the colloidal inhibition of amyloid assembly by sequestering the colloid aggregates (Feng et al., 2008). This mechanism can be tested by addition of a nonionic detergent, such as Triton X-100, in the system. Detergents disrupt the colloidal aggregates and thus prevent their nonspecific, promiscuous inhibitory

activity toward an enzyme, for example, β -lactamase. Assaying for the detergent-sensitive inhibition of β -lactamase activity thus can be used to determine which molecules likely act through a colloidal mechanism of inhibition (Coan and Shoichet, 2007; Feng et al., 2005, 2008; McGovern et al., 2003). Recently, electrospray ionization–ion-mobility spectrometry–mass spectrometry (ESI–IMS–MS) was used successfully to identify the colloidal mechanism of inhibition in the context of A β and amylin (Young et al., 2014, 2015a,b).

Cucurbit[7]uril

Cucurbituril designates a family of macrocyclic supramolecular compounds that derives its name from its physical resemblance to a gourd or pumpkin (botanical family Cucurbitaceae) and to a cucurbit, a vessel connected to a distillation alembic used by Persian alchemists (Mandadapu et al., 2014). The rigid and symmetrical cucurbituril structure is made of two identical hydrophilic portals surrounded by partially negative carbonyl groups, and a hydrophobic cavity (Fig. 6.31) (Gürbüz et al., 2015; Mandadapu et al., 2014). Nanomaterials, including nanoparticles, nanocomposites, vesicles, and rods, can be prepared by altering the cavity size of cucurbituril homologs (Gürbüz et al., 2015). Cucurbituril homologs take the cucurbit[*n*]uril (*n* = 5–8, 10, 14) composition (Mandadapu et al., 2014). Of the different homologs, cucurbit[7]uril binds specifically and avidly ($K_a = 10^4$ – 10^7 M $^{-1}$) to phenylalanine residues in peptides and proteins (Chinai et al., 2011; Mandadapu et al., 2014). It was hypothesized that capturing the phenylalanine residues by cucurbit[7]uril would block phenylalanine-mediated hydrophobic interactions in amyloid proteins (Lee et al., 2014). Effectively, this interaction could prevent amyloid fibrillization because phenylalanine residues may initiate and enhance hydrophobic clustering in amyloid proteins (Vivekanandan et al., 2011).

Based on the aforementioned rationale, cucurbit[7]uril (1163 Da) was used to target A β 40 or A β 42 self-assembly (Lee et al., 2014). Cucurbit[7]uril was found to inhibit A β self-assembly effectively by increasing the kinetic energy barrier to fibrillization. The authors tested

FIGURE 6.31 Cucurbit[*n*]uril has a rigid and symmetrical structure which is made of two identical hydrophilic portals surrounded by partially negative carbonyl groups and a hydrophobic cavity.

cucurbit[7]uril in a ThT fluorescence assay first. Without cucurbit[7]uril, $A\beta$ 40 aggregation led to an increase in the ThT fluorescence signal to a plateau in 4 h. When 50-fold or 100-fold excess cucurbit[7]uril was added to $A\beta$ 40, $A\beta$ 40 fibrils were not observed (ThT and TEM). In experiments with $A\beta$ 42, the time-dependent ThT fluorescence intensity decreased with increasing concentration ratios of added cucurbit[7]uril, thus showing slowing down of $A\beta$ 42 fibrillization. In accordance with the ThT experiments, the morphology of $A\beta$ 40 and $A\beta$ 42, assessed by TEM, changed from fibrillar to amorphous aggregates with increasing concentrations of cucurbit[7]uril. However, cucurbit[7]uril was effective only when added before initiation of fibrillization.

Using fluorescence titration, the K_a of interactions between cucurbit[7]uril and $A\beta$ 40 was calculated to be $7.1 \times 10^4 \text{ M}^{-1}$ (Lee et al., 2014). Structure–activity relationship analysis using $A\beta$ 40 analogs, in which the Phe, Tyr, or His residues were substituted by Ala, showed that the three Phe residues (at positions 4, 19, and 20) contributed equally to the interaction with cucurbit[7]uril. Next, cucurbit[7]uril was examined for its ability to protect SH-SY5Y cells from $A\beta$ -mediated cytotoxicity using the MTT reduction assay. $A\beta$ 40 or $A\beta$ 42 fibrils reduced SH-SY5Y cell viability to $\sim 75\%$ of control. Applying cucurbit[7]uril at 50-fold or 100-fold molar excess to $A\beta$ 40 and at 500-fold molar excess to $A\beta$ 42 restored the viability of treated SH-SY5Y cells to values comparable to untreated cells, 90 and 100%, respectively.

Helical Metallocomplexes

Stereochemistry and enantioselectivity importantly determine the therapeutic efficacy of a drug. Generally, one of any two hypothetical enantiomers of a compound is therapeutically active whereas the other is inactive or likely bears unwanted side effects (Nakanishi et al., 2004; Schapira et al., 2009). Considering stereochemistry and enantioselectivity, two triple helical, dinuclear, chiral metallosupramolecular complexes were designed and synthesized (Li et al., 2014). The aim was to modulate $A\beta$ aggregation (Li et al., 2014). The study rationale was that these metallohelicates could bind to α -helical $A\beta$ regions and prevent or modulate $A\beta$ oligomerization and self-assembly. The authors postulated that the α -helical structure of these complexes could target early stages of aggregation at α/β discordant $A\beta$ stretches—segments predicted to form a β -strand but preferentially form an α -helix (Kallberg et al., 2001). An α/β discordant segment in $A\beta$ spans amino acids 13–23 (Kallberg et al., 2001).

The designed helicate complexes were thermodynamically stable and composed of single enantiomers of monometallic units connected by organic linkers: $\Lambda 1$, $\Delta 1$ and $\Lambda 2$, $\Delta 2$ (Fig. 6.32). Fluorescence screening and other biophysical and biochemical techniques were used to test these compounds. In the fluorescence assay, $A\beta$ 42 fused to enhanced cyan fluorescent protein (ECFP) through a short linker sequence ($A\beta$ –ECFP) was used to measure fluorescence, which depends on the fused $A\beta$ 42's folding and solubility. Similar to the $A\beta$ –GFP system (see section: D737), ECFP fluoresces when it folds normally but fails to fluoresce if the fused $A\beta$ 42 aggregates or precipitates. Thus, blocking or preventing $A\beta$ 42 aggregation would allow normal ECFP folding and resultant ECFP fluorescence. The design of this probe system follows the original work of Hecht and coworkers, who used $A\beta$ fused to GFP for the same purpose (Kim et al., 2006; Wurth et al., 2002).

When testing the compounds, complex 1 ($[\text{Fe}_2\text{L}_3^1]\text{Cl}_4$) was more potent than complex 2 ($[\text{Fe}_2\text{L}_3^2]\text{Cl}_4$) and enantiomers $\Lambda 1$ ($\Lambda\text{Fe}_2\text{S}_C\text{--}[\text{Fe}_2\text{L}_3^1]\text{Cl}_4$) and $\Lambda 2$ ($\Lambda\text{Fe}_2\text{R}_C\text{--}[\text{Fe}_2\text{L}_3^2]\text{Cl}_4$) were more effective than their corresponding mirror images, $\Delta 1$ ($\Delta\text{Fe}_2\text{R}_C\text{--}[\text{Fe}_2\text{L}_3^1]\text{Cl}_4$) and $\Delta 2$

FIGURE 6.32 Structures of helical metallocomplexes, $\Delta 1$ ($\Delta\text{Fe}_2\text{SC}[\text{Fe}_2\text{L}_{13}]\text{Cl}_4$), $\Delta 1$ ($\Delta\text{Fe}_2\text{RC}[\text{Fe}_2\text{L}_{13}]\text{Cl}_4$), $\Delta 2$ ($\Delta\text{Fe}_2\text{RC}[\text{Fe}_2\text{L}_{23}]\text{Cl}_4$), and $\Delta 2$ ($\Delta\text{Fe}_2\text{SC}[\text{Fe}_2\text{L}_{23}]\text{Cl}_4$).

($\Delta\text{Fe}_2\text{SC}[\text{Fe}_2\text{L}_3^2]\text{Cl}_4$). Thus, less polar complexes (complex 1) were more effective than polar complexes, ΔFe enantiomers were more effective than the ΔFe enantiomers, and ThT fluorescence plunged to the lowest values with equimolar concentration of metal complexes. The latter finding was consistent with the postulate that A β 40 and metal complexes interacted at an equimolar ratio. Thus, the authors speculated that the metal complexes could interact with monomeric A β before early oligomers formed. They used ESI-MS experiments to show masses corresponding to A β –metal complexes of a 1:1 ratio. AFM and CD data also showed that $\Delta 1$ and $\Delta 2$ prevented A β 40 fibrillization more effectively than the corresponding mirror images, $\Delta 1$ and $\Delta 2$. These findings were supported by dialysis experiments, which showed enrichment of $\Delta 1$ and $\Delta 2$ in dialysis tubes containing A β 40, suggesting preferential binding of the Δ compounds to A β 40. NMR experiments showed that complex $\Delta 2$ interacted with Phe19 and Phe20. Similarly, less polar complexes (complex 1) were more effective than polar complexes in destabilizing preformed A β 40 fibrils. The most effective complex, $\Delta 1$, dissociated A β 40 fibrils with $\text{IC}_{50} = 1.97 \mu\text{M}$. Again in these experiments, ΔFe enantiomers were more effective than ΔFe enantiomers (Li et al., 2014).

Because the complexes modulated A β assembly, the authors postulated that they could also inhibit A β -induced cytotoxicity, and measured their effect using the MTT assay. Complex 1 or 2 (1–5 μM) inhibited A β 40-induced cytotoxicity dose dependently and did not affect cell viability on their own; aged A β 40 was used at 5 μM in these experiments. ΔFe enantiomers were more effective than the ΔFe enantiomers, in agreement with their stronger ability to modulate A β 40 aggregation. In addition, ΔFe enantiomers inhibited A β 40-mediated intracellular calcium changes more effectively than ΔFe enantiomers.

To test whether the complexes could cross the blood–brain barrier, mice were injected with the compounds and levels of CSF iron were measured, as an indirect indicator for complexes, using inductively coupled plasma–mass spectrometry (ICP–MS) analysis. Iron accumulation was more than that in control mice by 1.5 and 0.7% for complexes 1 and 2, respectively. To draw solid conclusions, however, experiments should measure the complexes directly in the CSF.

In contrast to the aforementioned study, stereospecific interactions cannot be generalized to other enantiomeric molecules potentially interacting with $A\beta$. As an example, a previous study (Sood et al., 2009), which assessed the effects of enantiomeric trifluoromethyl-indolyl-acetic acid ethyl esters on $A\beta$ self-assembly, found that both enantiomers inhibited $A\beta$ self-assembly with apparently similar potencies, suggesting lack of stereospecific interactions between these small molecules and $A\beta$ (Sood et al., 2009).

Polyoxometalates

POMs are synthetic, nonbiological clusters of oxygen anions and certain transition metals (especially Mo, W, and V). POMs can be used to construct versatile nanoscale molecular structures with important catalytic, electronic, magnetic, interactive, or structural properties (Hasenknopf, 2005; Miras et al., 2012). POMs are effective antiviral, antibiotic, and anticancer agents (Leon et al., 2014; Wang et al., 2014b,c). Most of the transition metals within the composition of POMs are generally toxic and tolerated only at low concentrations (Barceloux, 1999; Imtiaz et al., 2015; Okamoto et al., 2015; Vyskocil and Viau, 1999; Witten et al., 2012). Despite the toxicity of transition metals within POMs, Qu and coworkers recently assessed POMs for modulating $A\beta$ aggregation and fibrillization in a number of articles (Gao et al., 2014, 2015; Geng et al., 2011; Li et al., 2013a,b). Their rationale for testing POMs for this purpose included POMs' catalytic, electronic, and structural versatility and researchers' knowledge of how to control POMs' physicochemical properties (Rhule et al., 1998); previous work showing their interaction with proteins (Judd et al., 2001; Sarafianos et al., 1996; Seko et al., 2009) and their structural similarity to fullerene derivatives that have anti-amyloid activity (Geng et al., 2011; Kim and Lee, 2003).

Initially, a screening method based on bacterial expression of $A\beta$ fused to ECFP (Kim et al., 2006) was used to test different POMs with Wells–Dawson ($K_8[P_2CoW_{17}O_{61}]$), Keggin ($\alpha Na_9H[SiW_9O_{34}]$), or Anderson ($Na_5[IMo_6O_{24}]$) structures (Geng et al., 2011). The Wells–Dawson phosphotungstate ($K_8[P_2CoW_{17}O_{61}]$; POMD–Co) inhibited $A\beta$ aggregation more effectively than the others in the $A\beta$ –ECFP system; POMs with Keggin structure caused moderate to high inhibition of $A\beta$ aggregation; POMs with Anderson structure, for example, $Na_5[IMo_6O_{24}]$, were inactive. The inhibitory effect related to the size and surface charge of the POMs. Thus, POMs with the largest structure and strongest binding affinity to $A\beta$, commensurate with their electrostatic interactions, were the most active (Geng et al., 2011). Complementary methods such as ThT fluorescence assay, native gel electrophoresis, and TEM also confirmed the initial findings by the $A\beta$ –ECFP aggregation screening (Geng et al., 2011).

The authors next used trypsin digestion, competition experiments using *bis*-ANS, and a shorter $A\beta$ fragment, $A\beta_{12-28}$ (VHHQKLFFFAEDVGSNK), to investigate how POMs interacted with $A\beta$ (Geng et al., 2011). POMD–Co prevented trypsin digestion of $A\beta$ dose dependently and completely inhibited trypsin digestion at twofold molar excess to $A\beta$. The authors tested whether POMD–Co could inhibit trypsin digestion of lysozyme under similar conditions. They showed that lysozyme could be digested by trypsin in the presence of

POMD-Co. They concluded that the positively charged Lys or Arg residues of A β likely were important in POMD-Co-A β interactions (Geng et al., 2011). Further, POMD-Co competed off *bis*-ANS binding to A β concentration dose dependently. Thus, the study suggested that POMD-Co could bind close to the *bis*-ANS binding site on A β . Reportedly, *bis*-ANS binds to the A β CHC near the HHQK segment (Ferrão-Gonzales et al., 2005; LeVine, 2002); thus, the authors postulated the same region as the POMD-Co binding site. POMD-Co also rescued A β 12–28 from trypsin digestion and competed off *bis*-ANS binding, suggesting that POMD-Co likely binds to His13–Lys16 of A β . However, the authors did not test whether POMD-Co could form colloidal aggregates and cause colloidal sequestration (Feng et al., 2008) of A β rather than specifically interacting with it, as was discussed also in section: Aminopyrazole Derivatives.

The authors suggested two possible mechanisms whereby POMDs could inhibit A β fibrillization. First, POMDs likely bind A β monomers, shifting the equilibrium away from oligomerization and fibrillization. Second, POMDs likely block the self-contact points among monomers or among other prefibrillar A β assemblies, rendering nucleation and fibrillization less favorable (Geng et al., 2011). The authors postulated that this could be because of the large size and electrostatic interactions of POMD-Co (Geng et al., 2011). Such interactions likely also could sequester A β molecules by the colloidal mechanism of inhibition, as described previously (Feng et al., 2008).

After determining the effect of POMs on A β self-assembly, the authors tested whether POMs could block A β -mediated cytotoxicity in PC-12 cells by the MTT reduction assay. POMs by themselves were nontoxic, and they rescued viability of PC-12 cells to 90%, the level of untreated cells after a 7-day preincubation with A β (Geng et al., 2011). This requirement for long preincubation suggests that POMs are not strong inhibitors of A β -mediated toxicity.

Because POM-A β interactions depended mainly on POM size and nonspecific electrostatic properties, Qu and coworkers aimed to improve POM specificity for A β in their following studies (Gao et al., 2015; Li et al., 2013b). One of their approaches was to modify POMs by introducing specific A β self-recognition motifs. Thus, the peptide Ac-QKLVEFF-NH₂ of A β was conjugated to POMD-Co, and the conjugate used to target A β 40 self-assembly (Li et al., 2013b). This POMD-Co-A β (15–20) complex was compared with POMD-Co or the peptide alone using the ThT fluorescence assay. The conjugate, which self-assembles when aqueous solutions of A β (15–20) and POMD-Co are mixed and matured at room temperature for 6 h, was approximately twice as effective as either component alone in inhibiting β -sheet formation by A β 40 (Li et al., 2013b).

In cytotoxicity assays, the POMD-Co-peptide conjugate was nontoxic. A β fibrils (10 μ M) reduced cell viability to 46%. Addition of POMD-Co-A β (15–20) (6 μ M), POMD-Co (10 μ M), or A β 15–20 (6 μ M) increased the cell viability to 82, 72, or 69%, respectively. Next, the authors conjugated the CR dye to the POMD-Co-A β (15–20) complex, and tested the ability of POMD-Co-A β (15–20)-CR to affect A β fibrillization in murine CSF. A β 40 incubated with CR in the CSF for 7 days showed birefringent particles by polarized microscopy. A β 40 samples incubated with POMD-Co-A β (15–20)-CR in the CSF under the same conditions showed less CR-positive deposits (Li et al., 2013b). However, the CR component of POMD-Co-A β (15–20)-CR could compete with CR binding at the same sites, thus reducing CR fluorescence without affecting fibrils. Moreover, CR was one of the first molecules to show colloidal inhibition (McGovern et al., 2002).

Because POMDs presumably engage the cationic HHQK cluster of $A\beta$ (Geng et al., 2011), it was proposed that the two neighboring His residues of this cluster likely act as specific chelators of POMDs' transition metal ions (such as Ni(II) or Co(II)) to achieve specific $A\beta$ recognition (Gao et al., 2014). They hypothesized that POMDs with a defined His-binding site would greatly inhibit $A\beta$ fibrillization and toxicity (Gao et al., 2014). Thus the authors used a semirational approach to test a series of POMDs, functionalized with different known His-chelating metals (Cu, Fe, Ni, Co, or Mn), by using the same high-throughput $A\beta$ -ECFP screening method (Geng et al., 2011). The aim was to verify binding affinity of the tested POMDs to $A\beta$.

Among the tested derivatives of POMDs (POMD-Mn, POMD-Cu, and POMD-Fe) and unmodified Wells-Dawson POM, only those with Ni or Co inhibited $A\beta$ 42 aggregation; thus, only $K_8P_2NiW_{17}O_{61}$ (POMD-Ni) and $K_8P_2CoW_{17}O_{61}$ (POMD-Co) were used in the following tests. The compounds suppressed ThT fluorescence with the following potencies: POMD-Ni > POMD-Co > POMD derivatives. Native-gel electrophoresis of $A\beta$ 40, AFM experiments, and IC_{50} calculations confirmed these findings; IC_{50} values of POMD-Ni and POMD-Co were approximately 4.5-fold and 1.8-fold lower than that of POMD, respectively. The authors also tested whether POMDs could inhibit $A\beta$ oligomerization following a 24 h Cu^{2+} treatment at 37°C. Native PAGE showed that in $A\beta$ treated with Cu^{2+} the monomer band was absent, whereas when POMD-Co or POMD-Ni were incubated with $A\beta$ samples, a strong monomer band and weaker oligomers appeared (Gao et al., 2014).

The enhanced binding affinity of POMDs to $A\beta$ was attributed to two features: First, there were high electrostatic interactions between POMDs and $A\beta$ because POMs have six negative charges and POMDs have eight; the two additional negative charges would increase electrostatic interactions with the cationic HHQK cluster. Second, His binding was improved by the metal-chelated POMDs to recognize the HHQK cluster. This postulate was confirmed by the higher apparent K_a value of POMD-Ni than that of POMD-Co. The authors suggested two possible mechanisms whereby POMDs could interfere with $A\beta$ aggregation and fibrillization: binding between POMDs and $A\beta$ assemblies could substantially lower the concentration of the monomeric $A\beta$ in solution, thus tilting the equilibrium away from aggregation. In addition, interactions between POMDs and oligomeric $A\beta$ species could block the contact points between various $A\beta$ species, effecting unfavorable conditions for further nucleation or fibril growth.

In cytotoxicity assays using the MTT assay in PC-12 cells, $A\beta$ was incubated in the presence or absence of POMD, POMD-Ni, or POMD-Co again for 7 days, and subsequently the cells were exposed to different $A\beta$ -POMD mixtures. POMD-Ni was more efficacious than POMD or POMD-Co, while they were all nontoxic by themselves (Gao et al., 2014). The long, 7-day duration of preincubation with $A\beta$ was used by the authors presumably because ThT experiments and $A\beta$ fibril formation were monitored that long. However, this long incubation suggests an unfavorable therapeutic potential for these compounds, which would likely require continual administration or a durable pharmacokinetic availability. This therapeutic postulate is notwithstanding their potential systemic toxicity (yet to be determined), which would more likely emerge with prolonged pharmacokinetic availability.

Because the blood-brain barrier is a major impediment for experimental compounds for AD treatment (100% of large-molecule drugs and 98% of small-molecule drugs fail to cross the blood-brain barrier; see Pardridge, 2009), POMDs were tested to determine whether they

cross the blood–brain barrier, by treating wild-type mice with POMD–Ni, which was the most effective *in vitro*. In the test, 25 mg/kg of POMD–Ni was administered to mice intravenously (Gao et al., 2014). Plasma and brain tissue were collected 5, 10, 30, and 60 min after POMD–Ni treatment. Tungsten levels were then measured as an indirect indicator of POMD–Ni in brain homogenates and in plasma by ICP–MS. Tungsten levels in the plasma rapidly increased and plateaued in POMD–Ni-treated animals after 5 min (~ 8.9 mg/kg). However, peak tungsten levels in the brain did not coincide at the same time as in the plasma but rather 10 min after POMD–Ni administration; the brain tungsten level (~ 0.0231 mg/kg) was extremely low, 0.26% of that in the blood at 5 min. Thereafter, brain and plasma tungsten concentrations markedly decreased and returned to initial levels after 48 h.

In similar experiments, wild-type Wistar rats were treated with POMD–Ni intravenously at 25 mg/kg of body weight. Rats' CSF was collected 60 min after dosing and analyzed by high-performance liquid chromatography. The samples contained POMD–Ni at the same retention time as the pure POMD–Ni. Thus the authors concluded that POMD–Ni entered the brain, distributed extracellularly in the brain, and appeared in the CSF where A β could exist in different aggregation states.

In a recent study, the Wells–Dawson POMs (POMDs) were conjugated to AuNPs (Gao et al., 2015). The AuNPs already were conjugated using sulfur–gold chemistry to a β -sheet-breaker peptide derived from the A β sequence, LPFFD, and containing a Cys residue at its N-terminus (*N*-acetyl–Cys–LPFFD) for Au–S conjugation to AuNPs. The authors postulated that the POMD–AuNP–peptide conjugate could inhibit A β fibrillization, dissociate preformed fibrils, and easily cross the blood–brain barrier because the AuNPs could efficiently deliver the drug through the blood–brain barrier (Yang et al., 2005b). After confirming the POMD–AuNP–peptide synthesis success, the substances were tested by ThT fluorescence assays. The 40 nM POMD–AuNP–peptide with 40 μ M A β 40 reduced ThT fluorescence by $\sim 47\%$, and POMD–AuNP–peptide dissociated preformed A β fibrils; these results were confirmed using TEM experiments, DLS, and native PAGE. Trypsin digestion experiments using A β 12–28 showed that the POMD–AuNP–peptide bound this A β fragment and inhibited its degradation by trypsin.

Similar to studies conducted by Qu and coworkers, three different multimolybdenum POM anionic nanoclusters were used against A β 40 assembly (Chen et al., 2014). By using ThT fluorescence, native PAGE, turbidity, CD, and TEM, it was shown that the three tested POM complexes significantly inhibited A β 40 self-assembly and metal-induced A β 40 assembly (Chen et al., 2014). They also reported that PC-12 cell viability, which was reduced by 20 μ M A β 40 in the absence or presence of 40 μ M Cu²⁺ or 40 μ M Zn²⁺, was rescued by 10 μ g/mL POM complexes to up to 80% of control levels (Chen et al., 2014). Morphological studies assessing cell shape and dendritic processes also confirmed that POM complexes protected cells from the toxic effects of Zn²⁺–A β 40 or Cu²⁺–A β 40 complexes. Flow cytometry and annexin V staining confirmed that POM complexes protected cells against metal-induced apoptosis, and fluorescence staining indicated that cells were protected against insults by reactive oxygen species induced by A β 40 assemblies or metal–A β 40 complexes (Chen et al., 2014).

Artificial Molecular Chaperones

Molecular chaperones control protein misfolding and maintain protein homeostasis *in vivo* (Kim et al., 2013; Smith et al., 2015). Chaperones, including heat-shock proteins, chaperonins,

FIGURE 6.33 Depiction of a possible mechanism underlying interactions between mixed-shell polymeric micelles and $A\beta$ peptide.

apolipoproteins, DNAJB6, heparan sulfate proteoglycans, and αB crystallin, to name a few, can modulate pathological neurodegenerative processes by targeting misfolded proteins for refolding or degradation (Holtzman, 2004; Kim et al., 2013; Mansson et al., 2014; Niforou et al., 2014; Shammass et al., 2011; Smith et al., 2015; Wilhelmus et al., 2007).

In an attempt to mimic endogenous chaperones, artificial molecular chaperones were developed to modulate $A\beta$ self-assembly (Huang et al., 2014). These chaperones were synthesized by self-assembly of two amphiphilic di-block copolymers, poly(ϵ -caprolactone)-*block*-poly(ethylene oxide) (PCL-*b*-PEG) and poly(ϵ -caprolactone)-*block*-poly(*N*-isopropylacrylamide) (PCL-*b*-PNIPAM) in aqueous solution. The self-assembly process resulted in complex micelles composing a PCL core and a mixed shell comprising PEG and PNIPAM (Fig. 6.33) (Huang et al., 2014). At 37°C, the PNIPAM chains, which form the micellar shell, transition from hydrophilic to hydrophobic character spontaneously and fold to form hydrophobic domains on the PCL core. These hydrophobic domains were thought to bind to $A\beta$ monomers or oligomers through hydrophobic interactions, whereas the extended hydrophilic PEG chains likely repel excessive $A\beta$ to prevent aggregation of the micelles themselves. Importantly, the PNIPAM surfaces could be altered by changing the proportion of the two hydrophilic-hydrophobic copolymers on the surface of the mixed-shell polymeric micelles (MSPM) to enhance their antiaggregative effects. Adjusting this ratio modulated MSPMs' surface properties and controlled their effect on $A\beta$ fibrillization. Moreover, MSPMs with an optimal surface hydrophilicity-hydrophobicity balance had advantageous circulatory pharmacokinetics (Gao et al., 2013), a critical characteristic for potential in vivo applications as amyloid modulators. The same authors had shown previously that such MSPMs did not interact or affect functions of other proteins (Gao et al., 2013; Liu et al., 2013).

When MSPMs were added to A β , the lag phase of ThT fluorescence was prolonged and the final intensity at plateau was reduced, suggesting that the artificial chaperones attenuated both the nucleation and the elongation of A β fibrillization (Huang et al., 2014). Among the different MSPMs with varying PEG–PNIPAM ratios, MSPM–50PNIPAM was the best inhibitor of A β fibrillization, suggesting that controlling the balance between PEG chains and PNIPAM domains was essential for preventing A β self-assembly. MSPMs abrogated A β aggregation in a concentration-dependent manner. For example, MSPM–70PNIPAM was most effective at 0.4 mg/mL and least effective at 0.05 mg/mL. In the presence of MSPMs, A β showed an α -helical structure for 48 h by CD, and eventually converted to the spectral characteristic of the A β transition state from α -helical to β -sheet structure (decreased intensity at 222 nm and disappearance of the dip at 208 nm), suggesting that MSPMs fixed A β structures in the conformational transition state, and typical β -sheet spectra were not observed for the duration of the experiment (144 h) (Huang et al., 2014).

Using DLS, the authors showed that immediately after dissolution, A β particles of ~ 10 nm R_H predominated, consistent with A β oligomers, which changed over time to larger aggregates as A β fibrillized. When MSPMs were mixed with A β , the hydrodynamic radius, R_H , was similar to that of MSPMs initially, but over time increased from ~ 65 to ~ 98 nm, indicating formation of MSPM–A β complexes. These complexes, examined by TEM, showed dark spherical structures, whereas MSPMs alone formed distinctly white structures (Huang et al., 2014). Furthermore, the authors used a quartz crystal microbalance with dissipation monitoring (QCM-D) as a mass sensor to determine the adsorption/desorption and binding kinetics of MSPMs for A β . A QCM comprises a thin quartz disk sandwiched between two electrodes. When an alternative electrical current is applied across the electrodes, the disk oscillates at its acoustic resonance frequency. When the voltage is cut off, the disk oscillation decays exponentially. This decay is recorded, and the resonance frequency (f) and the energy dissipation factor (D) are calculated.

When surface-adsorbed MSPMs at different hydrophilic–hydrophobic ratios were exposed to A β preparations at 37°C, resonance frequency rapidly decreased. The largest decrease of frequency was for MSPM–50PNIPAM, entailing that MSPM–50PNIPAM interacted with A β most avidly, in agreement with ThT results. All types of MSPMs bound unaggregated A β preparations more preferably than A β oligomers and showed negligible binding to fibrils. It was thus hypothesized that the PEG chains triggered adsorption of A β by MSPMs and the PNIPAM domains then strongly captured the peptides, signifying the synergistic roles of hydrophilic PEG chains and hydrophobic PNIPAM domains in modulating A β assembly (Huang et al., 2014).

MSPMs at concentrations ranging from 6.25 to 400 μ g/mL were negligibly toxic to PC-12 or SH-SY5Y cells ($\sim 90\%$ cell viability) and rescued cell survival to $\sim 80\%$ of control when used at fourfold excess to A β (40 μ M) in the MTT assay. Although MSPMs modulated A β fibrillization, they could not decrease A β -induced cytotoxicity completely. This effect can be attributed to the inherent cytotoxic effect of the MSPMs, averaging 10% cell death at almost all the different concentrations used.

The authors also performed proteinase-K proteolysis experiments to compare the stability of A β fibrils and MSPM–A β complexes. ThT fluorescence intensity of MSPM–A β complexes treated with proteinase K decreased with time, whereas mature amyloid fibrils showed a slight reduction in the first 16 h and then the relative fluorescence intensity climbed again. Thus, MSPM–A β complexes were more susceptible to proteinase K than amyloid fibrils *in vitro*. Moreover, the authors demonstrated that MSPMs did not have any effect on 7-day-matured amyloid fibrils

in a disaggregation ThT assay (Huang et al., 2014). Thus, the two aforementioned observations suggest that MSPMs would not affect preexisting amyloid plaque if they were to successfully pass through the blood–brain barrier. This makes their potential as an AD therapeutic unlikely.

CONCLUSIONS

In this chapter, we surveyed more than 50 peptides and peptidomimetics, about 30 natural compounds (of which two-thirds were polyphenols), and approximately the same number of nonnatural molecules tested for their ability to modulate $A\beta$ assembly and inhibit $A\beta$ toxicity. Yet we are far from covering this vast field. A PubMed search for the terms “amyloid*” and “inhibit*” in the Title field yielded 1718 hits in Sep. 2015. Even if we assume arbitrarily that 50% of these hits do not discuss unique inhibitors of $A\beta$ assembly or toxicity, the number of remaining articles is much larger than could be covered in a typical review.

Despite the major effort dedicated to discovery, design, optimization, and characterization of “ $A\beta$ inhibitors” (a term we use hereafter to convey modulators and/or inhibitors of $A\beta$ assembly and inhibitors of $A\beta$ -induced toxicity), none of the compounds studied thus far has yielded a drug for AD. So are we, as a field, barking up the wrong tree as some have questioned in recent years? In agreement with others in the field (Karran and Hardy, 2014; Lo et al., 2014), we believe that $A\beta$ self-assembly and toxicity play a central role in AD and most likely are the causative pathologic event, yet the efforts targeting the self-assembly process have not been effective to date.

Reading through this chapter, several trends can be pointed out. First, although peptides and peptidomimetics are the largest group of molecules explored as $A\beta$ inhibitors, none of them has progressed to clinical trials, and the frequency of publication of peptide-based $A\beta$ inhibitors studies seems to have declined in recent years compared to the previous decade. This trend may reflect the boundaries posed by the known limitations of peptides as drug leads—low bioavailability and metabolic stability compared to nonpeptidic small molecules. Have we exhausted the attempts to turn peptides into effective $A\beta$ inhibitors? Some of the peptidomimetics reviewed in this chapter actually did show relatively high metabolic stability and bioavailability (eg, sections: D-Peptides; Rationally Designed Small Peptides), yet to the best of our knowledge, they have not been pursued further, possibly due to lack of continued research funding and/or lack of interest by the pharmaceutical industry.

We suggest that there are important lessons to be learned here. The pharmaceutical industry may be too conservative and miss important opportunities offered by peptidomimetics that fulfill druggability criteria. On the other side of the equation, academic scientists considering using peptide-based strategies for development of $A\beta$ inhibitors should consider pharmacokinetic and toxicity factors early, rather than the traditional approach of focusing on efficacy with the assumption that pharmacokinetic and safety characteristics can be improved later. The same considerations should be taken into account when designing nonpeptidic compounds, such as those discussed in section: $A\beta$ Assembly Modulators From Nonnatural Sources. The common side to most of the compounds discussed in sections: Peptidic or Peptidomimetic Modulators of $A\beta$ Assembly; $A\beta$ Assembly Modulators From Nonnatural Sources is the existence of a scientific rationale for making and testing them. At the same time, similarly to peptides, some of the nonnatural, nonpeptidic compounds explored, especially

the ones discussed toward the end of section: A β Assembly Modulators From Nonnatural Sources, have obvious liabilities, such as large size and incorporation of metal ions, which would be expected to limit their translation potential into drugs.

In section: A β Assembly Modulators Derived From Natural Sources, the broad use of polyphenolic compounds is an obvious trend. Of the different compounds we reviewed, compounds in this group most often have reached clinical trials. Yet the reason for this higher rate of success likely is simply the relative ease of reaching the point of human studies due to low toxicity, rather than compelling rationales or preclinical data. As the public health need is increasingly overwhelming, testing compounds with a potential to provide even moderate improvement is understood, though we must remember that the resources are limited, and dedicating much effort and funding to compounds expected to have only a moderate effect may come at the expense of developing a disease-modifying therapy for AD. Funding agencies must keep the need for balance between these approaches in mind.

As section: Polyphenols illustrates, the popularity of polyphenols may be related to the ease of obtaining them and the hope that due to their abundance in natural sources they would be easy to develop into drugs. However, to date, these premises have not proven successful (we note that this statement will change dramatically if the anticipated Sun-phenon results show significant disease-modifying effects). The realization that flavonoids tend to form covalent adducts with susceptible amino groups in A β (and likely those in off-targets) is a concern that should be taken into account in every study considering these compounds.

Despite many doubts in the validity of A β assembly and toxicity as a therapeutic target for AD in recent years, the genetic evidence for the causative role of A β in AD, including both familial-AD-causing mutations ([Attar et al., 2013](#)) and the recently discovered rare, protective mutation in an Icelandic kindred ([Jonsson et al., 2012](#)), is very strong. We maintain that targeting A β assembly as a purely pathogenic process is an attractive therapeutic approach. That said, we emphasize two important considerations: (1) Because the most relevant species, the highly toxic yet structurally elusive oligomers, are held together by weak forces, many compounds will interact with them nonspecifically and will affect their structure and aggregation kinetics. But this does not mean that the compounds can become useful drugs for AD. In particular, as discussed in the Introduction, compounds that accelerate aggregation are poor candidates for drug development. (2) AD is a complex disease involving multiple pathologic mechanisms. Therefore, a single therapeutic approach focused only on A β is unlikely to be successful in isolation. Developing effective therapy for AD, and ultimately a prevention strategy or a cure, most likely will require a multipronged approach targeting A β , tau, inflammation, and possibly other mechanisms.

Abbreviations

ABSM Amyloid β -sheet mimic

A β Amyloid β -protein

AD Alzheimer's disease

ADAS-Cog Alzheimer's Disease Assessment Scale–Cognitive portion

ADCS-ADL Alzheimer's Disease Cooperative Study–Activities of Daily Living

AFM Atomic-force microscopy

ANS 1-Anilinonaphthalene 8-sulfonate

APP Amyloid β -protein precursor

AuNPs Gold nanoparticles
BBDPs β -Sheet-breaker dipeptides
CAA Cerebral amyloid angiopathy
CD Circular dichroism
CHC Central hydrophobic cluster
CHO Chinese hamster ovarian cells
CR Congo red
CSF Cerebrospinal fluid
CTFs C-terminal fragments
Cur-PLGA-NPs Curcumin-encapsulated poly(lactic-co-glycolic acid) nanoparticles
DHA Docosahexaenoic acid
DLS Dynamic light scattering
ECFP Enhanced cyan fluorescent protein
ECM Extracellular matrix
EGCG (–)-Epigallocatechin-3-gallate
ELISA Enzyme-linked immunosorbent assay
ESI Electrospray ionization
GABA γ -Amino butyric acid
GFP Green fluorescent protein
HSQC Heteronuclear single-quantum coherence
IADLs Instrumental activities of daily living
ICP-MS Inductively coupled plasma–mass spectrometry
IM-MS Ion-mobility–mass spectrometry
ISF Interstitial fluid
LDH Lactate dehydrogenase
LPR/LR Laminin receptor precursors/laminin receptors
LTP Long-term potentiation
MMSE Mini Mental State Examination
MSPM Mixed-shell polymeric micelles
MTS [3-(4,5-Dimethylthiazol-2-yl)-5-(3 carboxymethoxyphenyl)-2(4-sulfophenyl)-2H-tetrazolium salt]
MTT 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
NMR Nuclear magnetic resonance
PAGE Polyacrylamide gel electrophoresis
PCL Poly(ϵ -caprolactone)
PEG Polyethylene glycol
PICUP Photo-induced crosslinking of unmodified proteins
PNIPAM Poly(*N*-isopropylacrylamide)
POM Polyoxometalate
POMD The Wells–Dawson polyoxometalate
QCM-D Quartz crystal microbalance with dissipation monitoring
 R_H Hydrodynamic radius
RI Retro-inverso
SDS Sodium dodecyl sulfate
SEC Size-exclusion chromatography
SPR Surface-plasmon resonance
TEM Transmission electron microscopy
ThT Thioflavin T

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