



Cite this: *Chem. Sci.*, 2025, **16**, 2105

## Multi-target-directed therapeutic strategies for Alzheimer's disease: controlling amyloid- $\beta$ aggregation, metal ion homeostasis, and enzyme inhibition

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Alzheimer's disease (AD) is the most prevalent neurodegenerative dementia, marked by progressive cognitive decline and memory impairment. Despite advances in therapeutic research, single-target-directed treatments often fall short in addressing the complex, multifactorial nature of AD. This arises from various pathological features, including amyloid- $\beta$  (A $\beta$ ) aggregate deposition, metal ion dysregulation, oxidative stress, impaired neurotransmission, neuroinflammation, mitochondrial dysfunction, and neuronal cell death. This review illustrates their interrelationships, with a particular emphasis on the interplay among A $\beta$ , metal ions, and AD-related enzymes, such as  $\beta$ -site amyloid precursor protein cleaving enzyme 1 (BACE1), matrix metalloproteinase 9 (MMP9), lysyl oxidase-like 2 (LOXL2), acetylcholinesterase (AChE), and monoamine oxidase B (MAOB). We further underscore the potential of therapeutic strategies that simultaneously inhibit A $\beta$  aggregation and address other pathogenic mechanisms. These approaches offer a more comprehensive and effective method for combating AD, overcoming the limitations of conventional therapies.

Received 6th October 2024  
Accepted 2nd January 2025

DOI: 10.1039/d4sc06762b  
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## 1. Introduction

Among neurodegenerative diseases, Alzheimer's disease (AD) is the most prevalent form of dementia, characterized by a progressive decline in cognitive function and memory.<sup>1</sup> Despite extensive research since its discovery in 1906, effective treatments for this fatal disorder continue to be elusive.<sup>2</sup> The pathophysiology of AD remains incompletely understood, highlighting the challenges in identifying its underlying causes and their interconnections.<sup>3</sup> Pathological features found in AD-affected brains include amyloid- $\beta$  (A $\beta$ ) aggregate deposition, metal ion dysregulation, oxidative stress, impaired neurotransmission, neuroinflammation, mitochondrial dysfunction, and neuronal cell death, as illustrated in Fig. 1a.<sup>4–15</sup> While no single hypothesis can fully explain the complex pathology of AD, it is evident that multiple factors simultaneously contribute to its progression.<sup>16</sup>

Several U.S. Food and Drug Administration (FDA)-approved or experimental single-target-directed therapies, ranging from small molecules and monoclonal antibodies aimed at disrupting A $\beta$  aggregation<sup>17–21</sup> to metal chelators<sup>22–27</sup> and neurotransmission

enhancers,<sup>28–33</sup> have not demonstrated significant disease-modifying effects.<sup>3,16,34</sup> Although these treatments effectively modulate specific pathological mechanisms, the multifactorial characteristic of AD likely limits their overall efficacy and can lead to severe side effects. Considering the complexity of AD, it is crucial to prioritize the development of multi-target-directed strategies that engage the multifaceted nature of the disease in drug design and discovery.<sup>3,35,36</sup> Given recent advancements in targeting A $\beta$  as a clinical approach and its direct interactions with factors like metal ions and enzymes under pathological conditions, this review primarily illustrates A $\beta$ , metal ions, and AD-related enzymes as pathogenic elements (Fig. 1a). The objective is to provide an overview of integrated therapeutic designs while evaluating the limitations of traditional single-target-directed treatments.<sup>17–21</sup> Additionally, this review explores frameworks that simultaneously address these features, focusing on multi-target-directed ligands (MTDLs) and discussing their potential advantages in treating AD, which highlights promising directions for future therapeutic development (Fig. 1b).

## 2. Single-target-directed drugs and their limitations

### 2.1 A $\beta$ peptides

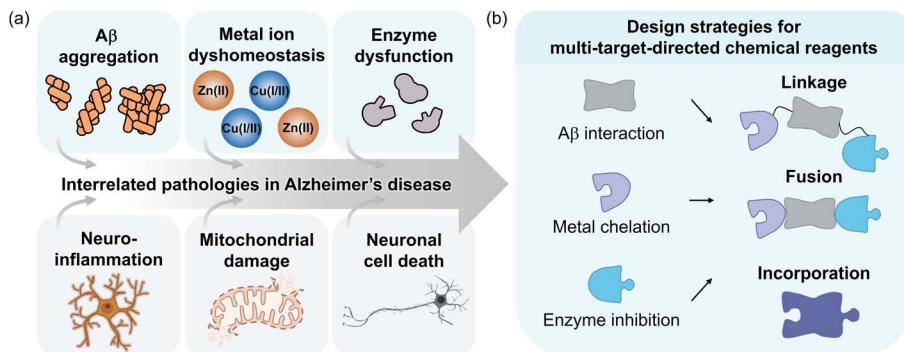
In 1984, A $\beta$  peptides were identified as the central component of extracellular amyloid plaques found in the brains of

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**Fig. 1** Pathological features in AD and design strategies for multi-target-directed chemical reagents. (a) Multifaceted pathology associated with AD. (b) Schematic illustration of design strategies (linkage, fusion, and incorporation) to develop small molecules against A $\beta$  aggregation, metal ion dyshomeostasis, and enzyme dysfunction simultaneously.

patients.<sup>1,37</sup> Since then, they have been recognized as a critical driver of AD pathology, leading to the development of the “amyloid cascade hypothesis,” which remains a prominent theory in understanding AD pathogenesis.<sup>1,38</sup> As illustrated in Fig. 2a, A $\beta$  peptides are derived from a transmembrane protein known as amyloid precursor protein (APP).<sup>2</sup> APP undergoes processing *via* non-amyloidogenic and amyloidogenic pathways.<sup>44,45</sup> In the amyloidogenic pathway, sequential proteolytic cleavages by  $\beta$ - and  $\gamma$ -secretases generate intrinsically disordered, aggregation-prone A $\beta$  peptides.<sup>2,46</sup> Due to the imprecise nature of these cleavages, A $\beta$  peptides are yielded in various forms, such as A $\beta_{1-x}$ , A $\beta_{4-x}$ , A $\beta_{8-x}$ , and A $\beta_{9-x}$ , with A $\beta_{40}$  and A $\beta_{42}$  being the most prevalent species found in amyloid plaques (Fig. 2b).<sup>46</sup>

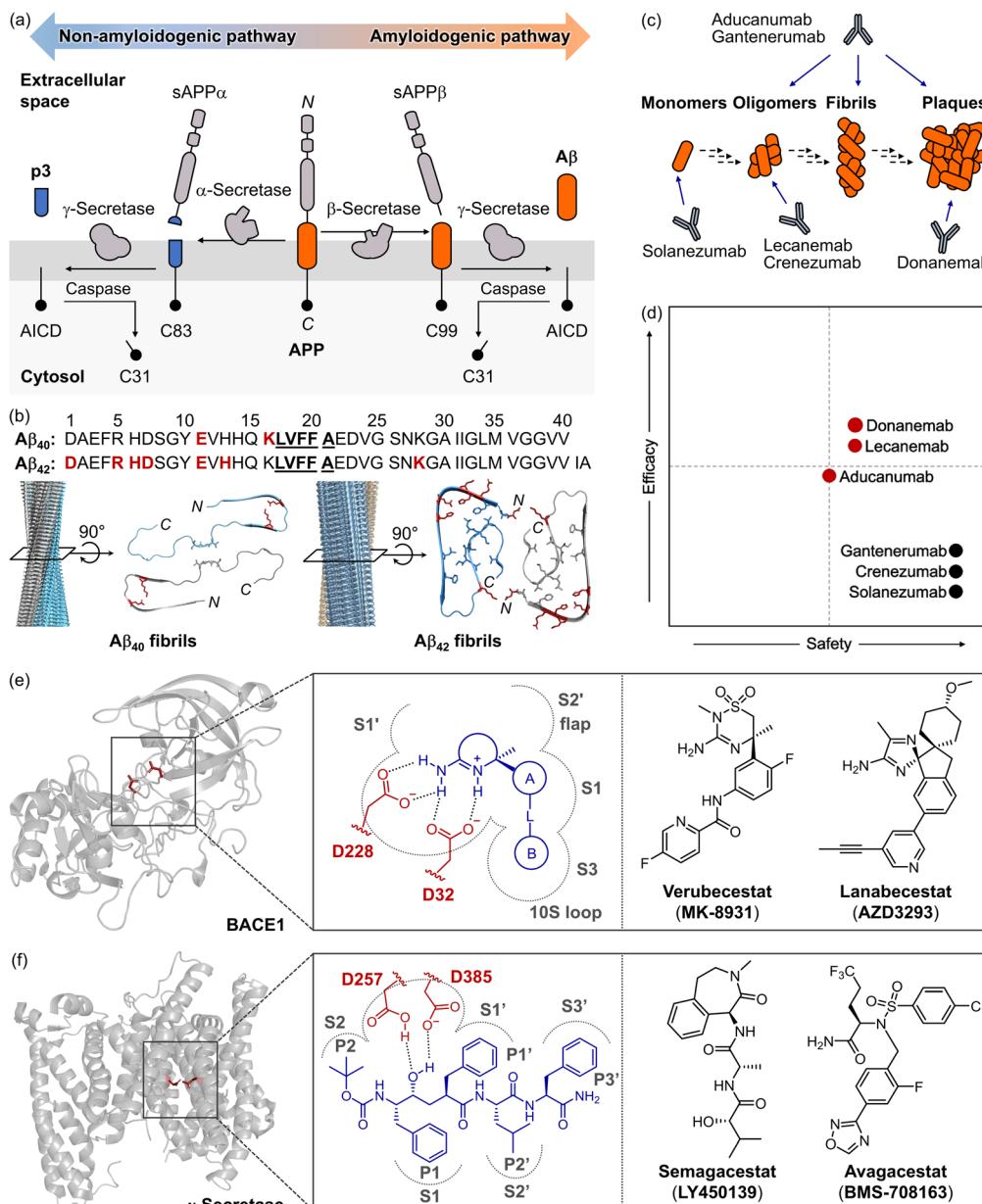
Under physiological conditions, the levels of A $\beta$  peptides are tightly regulated.<sup>47</sup> In the brains of AD patients, however, an imbalance between the production and clearance of A $\beta$  leads to its accumulation.<sup>47</sup> Fibrils and plaques are believed to form through intermolecular interactions at the self-recognition and hydrophobic C-terminal regions.<sup>39,40</sup> Recent advances in cryogenic electron microscopy (cryo-EM) have elucidated the structures of A $\beta$  fibrils found in the brains of AD patients (Fig. 2b).<sup>39,40</sup> Both A $\beta_{40}$  and A $\beta_{42}$  fibrils consist of two stacks of peptides, each constructing a right-handed twist along a central axis. In A $\beta_{40}$  fibrils, the peptide stack typically contains four sets of cross- $\beta$  sheets spanning residues A2–S8, Y10–H13, Q15–F19, and I32–L34.<sup>39</sup> In A $\beta_{42}$  fibrils, additional interactions, such as salt bridges between D1 and K28, D7 and R5, and E11 with H6 and H13, are observed, with those involving E11 stabilizing a kink in the N-terminal part of the  $\beta$ -sheet around Y10.<sup>40</sup> These interactions promote the assembly of monomers into oligomers, protofibrils, and fibrils. Notably, A $\beta$  oligomers are neurotoxic and contribute to cellular dysfunction, inflammation, and synaptic impairment, all of which drive neurodegeneration in AD.<sup>48</sup>

Given that the accumulation of both soluble and insoluble A $\beta$  aggregates can trigger pathological processes in AD,<sup>17</sup> previous research has focused on reducing cerebral A $\beta$ ,<sup>1</sup> or diminishing its production to alleviate the A $\beta$  burden.<sup>49,50</sup> These approaches have been pursued either through the use of anti-amyloid monoclonal antibodies<sup>20,41,51</sup> or by inhibiting  $\beta$ - or  $\gamma$ -

secretases,<sup>49</sup> as illustrated in Fig. 2c–f. Recently, the U.S. FDA has approved three human monoclonal antibodies directed against A $\beta$  (Fig. 2c and d): **aducanumab** (approved in 2021), **lecanemab** (approved in 2023), and **donanemab** (approved in 2024). Each of these therapies has shown potential to slow the progression of AD by targeting specific forms of A $\beta$ : **aducanumab** addresses soluble oligomers and fibrils;<sup>21</sup> **lecanemab** engages with soluble protofibrils;<sup>17,18</sup> **donanemab** is designed to target amyloid plaques.<sup>19,20</sup> These therapeutics have demonstrated promise in decreasing amyloid plaque burden and potentially delaying cognitive decline.<sup>17–21</sup> Despite these encouraging results, the outcomes of clinical trials have often failed to meet expectations.<sup>18,20,21,52</sup> Confronting A $\beta$  aggregates with anti-amyloid monoclonal antibodies remains controversial due to mixed treatment outcomes and concerns about adverse effects, such as brain swelling and microhemorrhages, collectively known as amyloid-related imaging abnormalities (ARIA).<sup>18,20,21</sup>

In recent decades, the inhibition of  $\beta$ -secretases has garnered attention as a therapeutic strategy for AD, with a particular focus on targeting  $\beta$ -site APP cleaving enzyme 1 (BACE1) to reduce the production of A $\beta$ .<sup>53</sup> The structure of BACE1, as illustrated in Fig. 2e, includes a protease domain with two catalytic aspartates (D32 and D228) and several sub-pockets within the catalytic clefts (e.g., S1, S1', S2', and S3 pockets).<sup>42,54</sup> Amidine-based BACE1 inhibitors have been extensively studied, with some advancing to phase II or III clinical trials. These inhibitors interact with the aspartate dyad through salt bridges and specifically bind to the enzyme's active site.<sup>54</sup> Notably, **verubecestat** (MK-8931) and **lanabecestat** (AZD3293) (shown in Fig. 2e) significantly reduced the levels of A $\beta_{40}$ , A $\beta_{42}$ , and soluble APP $\beta$  (sAPP $\beta$ ) in cerebrospinal fluid (CSF) by up to 80%, accompanied by a modest decrease in plaque load as confirmed by amyloid positron emission tomography (PET) imaging.<sup>55,56</sup> Over fourteen  $\beta$ -secretase inhibitors have been developed, showing promising results in lowering A $\beta$  burden in advanced clinical trials. They were discontinued, however, due to adverse effects, including liver toxicity, cognitive worsening, weight loss, sleep disturbances, and skin rashes.<sup>52</sup>





**Fig. 2** Recent drug development against A $\beta$  species or BACE1. (a) Illustration of the proteolytic cleavage of APP. Non-amyloidogenic and amyloidogenic pathways occur depending on the combinational action of  $\alpha$ -,  $\beta$ -, and  $\gamma$ -secretases. (b) Amino acid sequences of A $\beta$ <sub>40</sub> and A $\beta$ <sub>42</sub>, with side views of 3D reconstructed A $\beta$  fibrils, and top views of their cross-sections from cryo-EM (for A $\beta$ <sub>40</sub>, PDB 6SHS<sup>39</sup>; for A $\beta$ <sub>42</sub>, PDB 5OQV<sup>40</sup>). Amino acid residues responsible for the self-recognition sites are highlighted in bold and underlined, while those forming salt bridges in fibrillar structures are indicated in red and depicted as sticks. Core structures of A $\beta$  fibrils are also shown in stick representation. Reproduced with permission from ref. 39 and 40. Copyright© 2019 Springer Nature and 2017 The American Association for the Advancement of Science. (c) Schematic description of A $\beta$  aggregation and A $\beta$ -lowering monoclonal antibodies. (d) Relative safety and efficacy of A $\beta$ -lowering monoclonal antibodies in patients with sporadic early-symptomatic AD. Monoclonal antibodies approved by the U.S. FDA for AD are shown in red dots, while other monoclonal antibodies are presented in black. Reproduced with permission from ref. 41. Copyright© 2023 Springer Nature. (e) Structure of BACE1 (PDB 1W50<sup>42</sup>), zoom-in view of the target site, and BACE1 inhibitors that have reached phase III clinical trials. The catalytic dyad is presented in red and depicted as sticks. (f) Structure of  $\gamma$ -secretase (PDB 5A63<sup>43</sup>), illustration of the binding pocket, and  $\gamma$ -secretase inhibitors that have progressed to clinical trials. The catalytic dyad is indicated in red with sticks.

The inactivation of  $\gamma$ -secretase has also been investigated to control A $\beta$  production and attenuate the progression of AD.<sup>43,57,58</sup> As depicted in Fig. 2f,  $\gamma$ -secretase shares a similar structure to that of BACE1, containing two catalytic aspartates (D257 and D385) and five cavities (S1, S1', S2, S2', and S3' pockets) within the active site.<sup>43,57,58</sup> Several inhibitors, such as

**semagacestat (LY450139)** and **avagacestat (BMS-708163)** (Fig. 2f), have been developed to interact with these sites as potential AD treatments.<sup>59,60</sup> **Semagacestat**, was the first  $\gamma$ -secretase inhibitor to enter phase III clinical trials, but it was withdrawn due to the lack of cognitive improvement and the emergence of side effects, including further cognitive

decline.<sup>55,56,59</sup> Additionally, some  $\gamma$ -secretase inhibitors exhibited toxic effects, such as an increased risk of skin cancer, likely resulted from off-target interactions with proteins like the Notch receptor, which is involved in cell proliferation *via* nuclear signaling.<sup>59</sup> Although recent drug approvals targeting A $\beta$  aggregates highlight their relevance as a biomarker, these challenges underscore the limitations of focusing solely on a single pathological factor, such as A $\beta$  species or its associated secretases, to reverse cognitive impairment. The complexity of AD pathogenesis and the unintended effects of these inhibitors emphasize the need for therapeutic strategies that address multiple pathological factors simultaneously.<sup>41</sup>

## 2.2 Metal ion dyshomeostasis

Metal ions, such as Fe(II/III), Cu(I/II), and Zn(II), play vital roles in physiological processes, including signal transmission and catalytic reactions, through their coordination to various biological Lewis bases.<sup>61–65</sup> These metal ions are distributed between two distinct pools: the static and labile pools. The static pool consists of tightly bound metal ions embedded within proteins, often stabilizing protein structures or occupying catalytic sites of enzymes.<sup>61–66</sup> In contrast, the labile pool contains loosely-bound or surface-exposed metal ions that participate in more dynamic processes, such as signal transmission and regulation.<sup>61–66</sup>

The delicate balance between static and labile metal ion pools can be disrupted under pathological conditions, leading to metal ion dyshomeostasis in the brain. In AD-affected regions, such as the hippocampus and amygdala, an increase in unregulated labile copper ions in serum and plasma has been observed.<sup>67,68</sup> Additionally, the concentration of Zn(II) in these areas nearly doubles compared to healthy subjects.<sup>67</sup> The miscompartmentation of metal ions not only impairs their physiological functions but also induces toxicity, supporting the “metal ion hypothesis” in AD.<sup>69</sup> Specifically, the mislocalization of metal ions undermines antioxidant systems against reactive oxygen species (ROS), leading to elevated oxidative stress and severe cytotoxicity.<sup>66</sup> Excess ROS can damage biological components, such as lipids and proteins, and induce neuronal death, which further leads to neurodegeneration.<sup>69</sup>

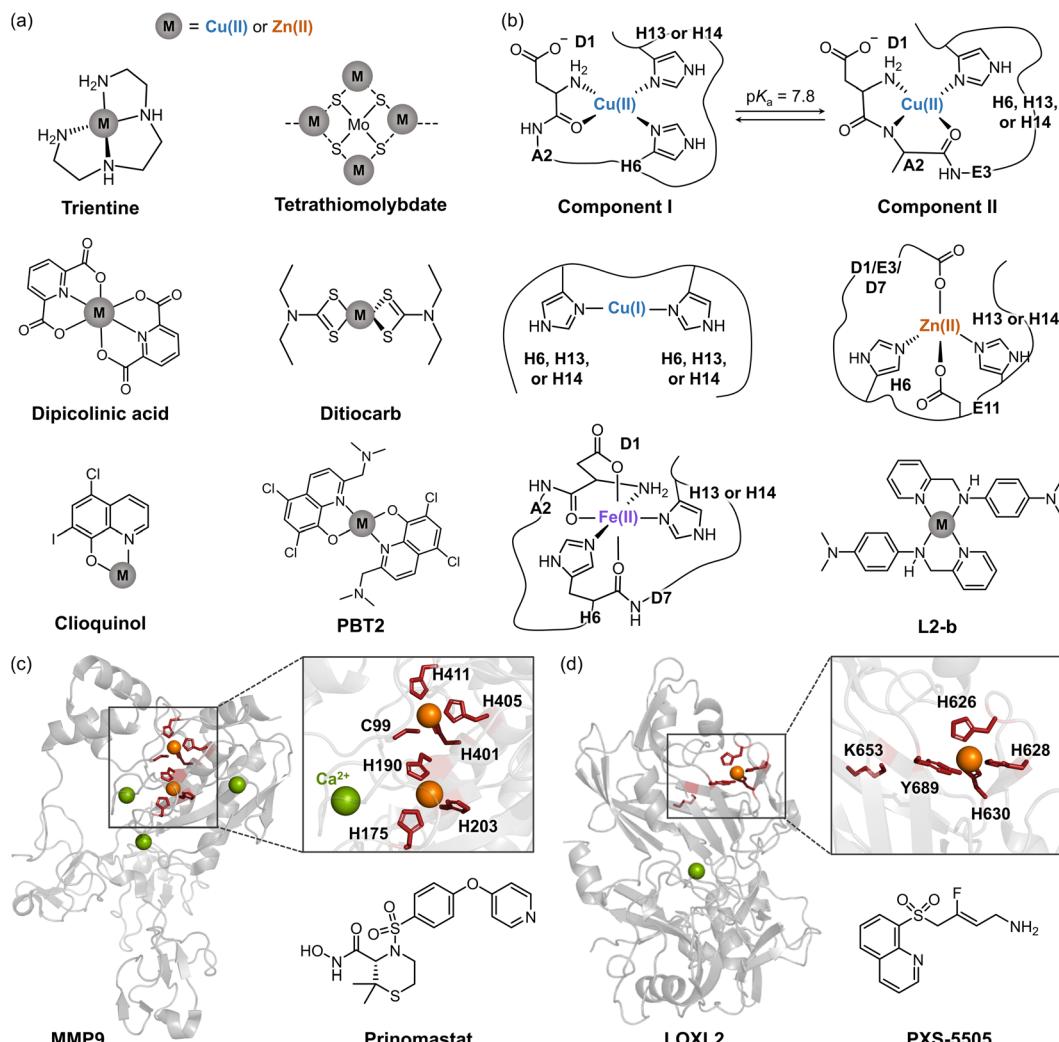
Extensive research on the regulation of Cu(I/II) and Zn(II) dyshomeostasis has led to the development of several chelators, including **trientine**, **tetrathiomolybdate**, **dipicolinic acid**, **ditio-carb**, **clioquinol**, and **PBT2**, as illustrated in Fig. 3a.<sup>22–27</sup> These chelators are designed to remove excess metal ions concentrated in plaques, prevent redox reactions, and potentially offer therapeutic benefits for AD.<sup>22–25,72–76</sup> In addition, **clioquinol** and an 8-hydroxyquinoline derivative, **PBT2**, were considered ionophores to restore metal ion homeostasis by coordinating metal ions through their nitrogen and oxygen donor atoms, reintroducing them into physiological systems and preventing their pathological interactions with A $\beta$ .<sup>26,27</sup> Despite their initial promise, chelation-based therapies have not been successful in clinical trials, with no significant improvement and severe side effects.<sup>25,77–79</sup> The failure of these therapies may be attributed to several factors, including (i) non-specific binding to target metal

ions, (ii) retinal and ocular toxicity with unclear mechanisms, and (iii) an inaccurate understanding of metal concentrations in AD.<sup>25,77–79</sup>

To address these challenges, recent strategies have focused on regulating various pathological features associated with metal ion dyshomeostasis. Excess metal ions can coordinate to amyloidogenic peptides (*e.g.*, A $\beta$ , illustrated in Fig. 3b), accelerating their aggregation and exhibiting cytotoxic effects.<sup>69,80,81</sup> The formation of metal–A $\beta$  complexes at the synaptic cleft is well-documented, with metal concentrations in A $\beta$  plaques reaching up to 0.4 mM for copper and 1.0 mM for zinc.<sup>82</sup> Specifically, two major Cu(II)-binding modes to A $\beta$  have been identified depending on pH, as shown in Fig. 3b. At pH  $\leq$  6.5 (Component I), Cu(II) primarily adopts a 3N1O coordination mode involving the N-terminal primary amine, the backbone carbonyl group between D1 and A2, and two imidazole rings from H6 and H13 or H14.<sup>83–85</sup> Additional oxygen donor atoms from water molecules or carboxylate groups from D1, E3, D7, or E11 are also proposed.<sup>83–85</sup> In contrast, at pH  $\geq$  8 (Component II), Cu(II) maintains a 3N1O coordination but with different donor atoms: the N-terminal primary amine, a deprotonated amide backbone between D1 and A2, an imidazole ring from H6, H13, or H14, and an oxygen donor atom from the backbone carbonyl group between A2 and E3.<sup>69,83,84,86</sup> Cu(I) binds to A $\beta$  in a linear mode, primarily entailing the imidazole rings from H6, H13, or H14, with a preference for engaging H13 and H14.<sup>87,88</sup> Moreover, Zn(II) coordinates to A $\beta$  through a 2N2O coordination comprising two oxygen donor atoms from the carboxylate groups of D1, E3, or D7 and E11 and two nitrogen donor atoms from the imidazole rings of H6 and H13 or H14 (Fig. 3b).<sup>69,89</sup> Furthermore, although relatively less established, Fe(II) can associate with A $\beta$  through a 3N3O coordination mode. This incorporates three nitrogen donor atoms from the N-terminal primary amine and two imidazoles from H6 and H13 or H14 and three oxygen donor atoms from the backbone carbonyl groups between D1 and A2, between H6 and D7, and the carboxylate group of D1 (Fig. 3b).<sup>90,91</sup>

These interactions facilitate A $\beta$  aggregation and elevate oxidative stress by either disrupting ROS scavenging systems or overproducing ROS through Fenton chemistry or Fenton-like reactions in the case of redox-active metal ions. For example, the varying coordination modes of Cu(I) and Cu(II), coupled with the conformational flexibility of native peptides, enable redox reactions at the metal center ( $E^\circ = 0.28$  V *vs.* NHE<sup>92</sup>), generating ROS.<sup>90,93</sup> Note that Fe(II/III) unbound and bound to A $\beta$  is also involved in ROS generation; however, the  $E^\circ$  value could not be accurately determined in aqueous media because of Fe(OH)<sub>3</sub> sedimentation.<sup>90,91</sup> Considering the impact of metal ions on A $\beta$  aggregation and ROS formation, targeting metal–A $\beta$  complexes, therefore, appears to be a promising therapeutic strategy compared to controlling individual factors.<sup>94–96</sup> Lim and coworkers rationally designed a small molecule, **L2-b** (shown in Fig. 3b), to specifically regulate the reactivities of metal–A $\beta$  complexes.<sup>94,97</sup> **L2-b** inhibited metal-induced A $\beta$  aggregation and reduced ROS produced by Cu(I/II)–A $\beta$  complexes.<sup>94</sup> *In vivo* studies further demonstrated that **L2-b** treatment mitigated amyloid pathology and improved cognitive deficits in the 5xFAD





**Fig. 3** Metal-related pathological factors associated with AD and therapeutic interventions against these components. (a) Examples of metal chelators. (b) Representative coordination modes of Cu(II), Zn(II), and Fe(II) to Aβ with a chemical structure of metal-bound L2-b. L2-b,  $N^1,N^1$ -dimethyl- $N^4$ -(pyridin-2-ylmethyl)benzene-1,4-diamine. (c) Structures of MMP9 (PDB 1L6J<sup>70</sup>) and its inhibitor (prinomastat). The Zn(II)-coordination residues are highlighted in red and depicted as sticks. (d) Structures of LOXL2 (PDB 5ZE3<sup>71</sup>) and its inhibitor (PXS-5505). The Cu(II)-coordination residues and catalytic lysine are indicated in red with sticks. Note that Cu(II) occupies the Zn(II)-binding site in human LOXL2, contrary to the crystallographic illustration.

mouse model, underscoring the importance of confronting and regulating metal–Aβ interactions.<sup>94</sup> Despite the significance of these findings, no clinical trials addressing metal–Aβ species have been reported to date, to the best of our knowledge.

Dysregulated and miscompartmentalized metal ions can significantly impact the structures and functions of metalloenzymes.<sup>98–100</sup> Among various metalloenzymes, matrix metalloproteinase 9 (MMP9) and lysyl oxidase-like 2 (LOXL2) have emerged as major pathological factors (depicted in Fig. 3c and d), as they are activated by excess labile zinc or copper ions under pathological conditions.<sup>98–105</sup> MMP9, which plays a crucial role in physiological processes, such as embryonic development, angiogenesis, and cell migration, was initially studied for its proteolytic activity against Aβ peptides.<sup>103,104</sup>

Research in AD mouse models has shown that MMP9 is overexpressed, contributing to neurotoxicity in hippocampal neurons, cognitive deficits, and the degradation of nerve growth

factors, thereby implicating MMP9 activation in the progression of AD.<sup>104,106,107</sup> *In vivo* studies further suggested that suppressing MMP9 can alleviate neurobehavioral deficits, making it a potential therapeutic target.<sup>104,106,107</sup> Current MMP9 inhibitors are designed to bind to its binuclear Zn(II) centers (Fig. 3c). The two Zn(II) ions are coordinated through two main modes: (i) 3N coordination with the imidazole rings of H175, H190, and H205 and (ii) 3N1S coordination with the free thiol of C99 and the imidazole rings from H401, H405, and H411.<sup>70</sup> The 3N1S coordination serves as the catalytic center, which becomes activated when the free thiol of C99 dissociates, creating a vacant site. Chelator-based therapeutics, such as **prinomastat** (shown in Fig. 3c), are being explored for their ability to address this vacant Zn(II)-coordination site and competitively inhibit the active site of MMP9.<sup>104,108</sup>

In parallel, LOXL2 is another metalloenzyme that contains a catalytic copper center (Fig. 3d).<sup>71,109</sup> The LOX family is

responsible for the oxidative deamination of  $\epsilon$ -amino groups in lysine residues, converting them to hydroxyllysine within collagen and elastin chains. This chemical transformation initiates covalent cross-linking in the extracellular matrix, which is crucial for maintaining the tensile strength and elasticity of connective tissues.<sup>105,109</sup> The redox-active Cu(II) in the LOXL2 active site facilitates this process through the 3N1O coordination with a distorted tetrahedral geometry involving three imidazole rings from H626, H628, and H630 and an additional oxygen donor atom from Y689 (Fig. 3d).<sup>71</sup> The catalytic copper then oxidizes the lysine tyrosylquinone (LTQ) cofactor, enabling further deamination of lysines.<sup>110</sup>

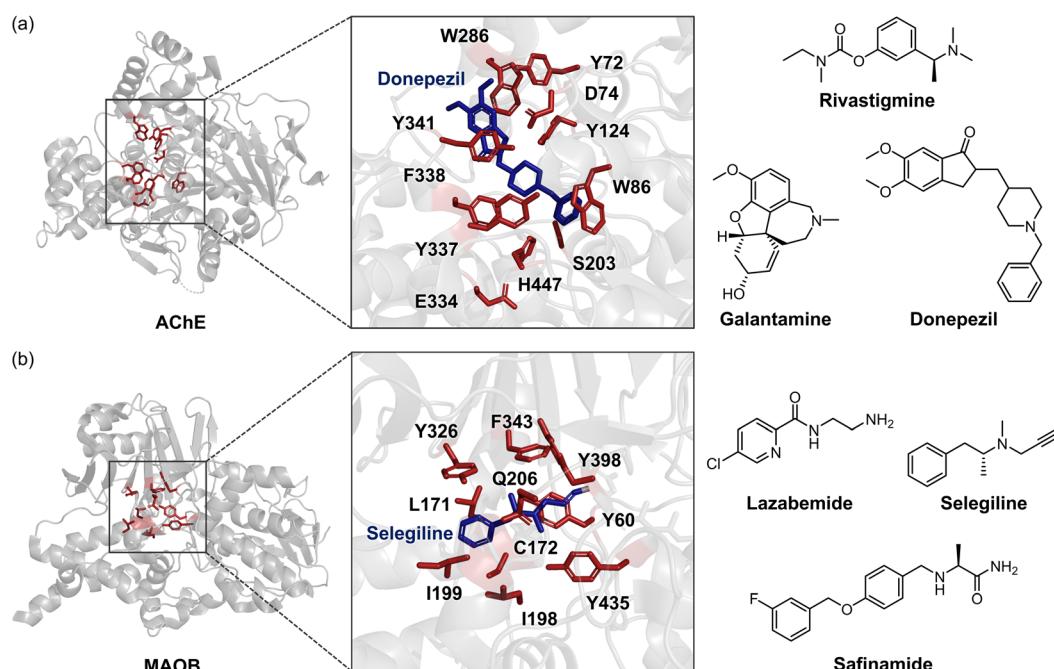
Given the diverse physiological roles of LOXL2, its dysregulation can impact various pathophysiological conditions, such as AD and other vascular diseases.<sup>105,114</sup> For example, LOXL2 activation can induce arterial stiffness, leading to brain microvascular damage and A $\beta$  deposition.<sup>105</sup> Additionally, suppression of LOXL2 has been shown to enhance the clearance of A $\beta$  aggregates, making it an intriguing therapeutic target.<sup>105</sup> LOXL2 inhibitors typically modify the chemical structure of its substrate, the LTQ cofactor, or disrupt the Cu(II)-binding site to halt enzymatic activity.<sup>109,110</sup> Fluoroallylamine-based inhibitors, such as **PXS-5505** (shown in Fig. 3d), form a covalent bond with the LTQ cofactor, resulting in irreversible termination of its function.<sup>110</sup> Other compounds, such as guaiacol derivatives, have also demonstrated modulatory effects against LOXL2 by binding to the catalytic Cu(II) center and showed potential as AD therapeutics.<sup>115</sup> Collectively, metal ion dyshomeostasis drives to inappropriate interactions with A $\beta$  and the activation of

metalloenzymes, thereby increasing cytotoxicity. These findings suggest that developing multipotent chemical reagents capable of confronting and controlling pathogenic features associated with metal ion dyshomeostasis is a promising therapeutic strategy for AD.<sup>116</sup>

### 2.3 Neurotransmitters and related enzymatic systems

Imbalances in neurotransmission caused by the dysregulation of enzymatic systems represent another pathological factor for AD.<sup>28,44,103,117-121</sup> Patients with AD often experience diminished signal transmission mediated by neurotransmitters, such as acetylcholine (ACh), dopamine, and serotonin. These neurotransmitters are regulated and degraded by enzymes, including acetylcholinesterase (AChE), monoamine oxidase (MAO), and catechol-O-methyltransferase (COMT).<sup>28,117,118</sup> The abnormality in these enzymatic systems has been observed in AD patients, suggesting that targeting neurotransmitter-related enzymes would be a valuable therapeutic strategy.<sup>28,117,118</sup>

AChE is a well-known and extensively studied factor in the pathology of AD (Fig. 4a). According to the "cholinergic hypothesis," AD is characterized by a significant loss of cholinergic neurons and a decline in ACh levels in the brain, leading to memory loss and cognitive deficits.<sup>28,122</sup> As a result, AChE becomes a major target for developing therapeutic inhibitors. The structures of AChE and commonly prescribed AChE inhibitors, such as **rivastigmine**, **galantamine**, and **donepezil**, are presented in Fig. 4a.<sup>28-32,123</sup> Recombinant human AChE (rhAChE) contains several essential binding domains. The catalytically active site (CAS) is located at the bottom of a 20 Å



**Fig. 4** AChE, MAOB, and examples of their inhibitors. (a) Structure of AChE (PDB 4EY4<sup>111</sup>), binding mode of **donepezil** at its active site (PDB 7E3H<sup>112</sup>), and three U.S. FDA-approved AChE inhibitors (**donepezil**, **rivastigmine**, and **galantamine**). The amino acid residues involved in **donepezil** binding are highlighted in red and depicted as sticks. (b) Structure of MAOB (PDB 1GOS<sup>113</sup>), interaction with **selegiline** at its catalytic domain (PDB 2BYB<sup>33</sup>), and three representative inhibitors (**lazabemide**, **selegiline**, and **safinamide**). The amino acid residues involved in **selegiline** binding are indicated in red with sticks.



deep, narrow binding gorge, and its anionic domain includes amino acid residues W86, Y130, Y337, and F338. The peripheral anionic site (PAS), positioned at the entrance of the binding gorge, comprises amino acid residues Y72, D74, Y124, W286, and Y341.<sup>111,112</sup> Current AChE inhibitors work by competitively impeding the enzyme's activity, either by blocking the CAS to prevent substrate interaction or by hindering the access to the active site through the PAS.<sup>28–32</sup> For example, **donepezil** interacts with both the CAS and the PAS of AChE, forming hydrogen bonds with water molecules that connect to the side chains of Y337 and Y341, and engaging in  $\pi$ – $\pi$  interactions with the side chains of W86 and W286, as illustrated in Fig. 4a.<sup>112</sup> These interactions interfere the breakdown of ACh into acetate and choline, thereby increasing the availability of ACh and enhancing cholinergic neurotransmission.<sup>28</sup>

Additionally, the distinctive selectivity of AChE inhibitors against butyrylcholinesterase (BuChE) plays a significant role in their efficacy and suitability for different stages of AD.<sup>124–126</sup> Unlike AChE, BuChE shows weaker substrate preference due to the absence of several aromatic residues, retaining only one aspartate and tyrosine residue in its PAS. Interestingly, BuChE activity increases during the late stage of AD, particularly in neurodegenerative regions, which compensates for the decline in AChE activity.<sup>124–126</sup> Consequently, non-selective inhibitors may offer advantages in later stages of AD by targeting both enzymes, though they also carry a higher risk of gastrointestinal and cardiovascular side effects.<sup>124–126</sup> Currently developed AChE inhibitors exhibit varying selectivity for AChE and BuChE. For example, **rivastigmine** addresses both AChE and BuChE, while **galantamine** and **donepezil** demonstrate moderate and strong selectivity for AChE, respectively.<sup>124–126</sup>

Along with the cholinergic hypothesis, the loss of dopaminergic and serotonergic neurotransmission represents another pathological factor in AD.<sup>127–129</sup> The degeneration of dopaminergic and serotonergic neurons leads to significant local inflammation and exacerbates age-related cell death, as observed in the AD-transgenic Tg2576 mice model.<sup>127</sup> This neuronal loss is associated with pathological symptoms, including impairments in hippocampal neuronal excitability, synaptic plasticity, memory, and reward performance.<sup>127–129</sup> Furthermore, the activation of enzymes that degrade dopamine and serotonin, such as MAO and COMT, has also been implicated in the pathology of AD.<sup>103,117,118,130</sup> Consequently, numerous efforts have focused on targeting these enzymes. Recent studies have highlighted MAO inhibitors, such as **lazabemide**, **selegiline**, and **safinamide**, which are either U.S. FDA-approved or in clinical trials, as displayed in Fig. 4b.<sup>131</sup> These inhibitors block the active site of MAO (primarily MAOB), which consists of two cavities: the substrate cavity near the flavin adenine dinucleotide (FAD) and the entrance cavity.<sup>113</sup> For instance, **selegiline** interacts with the substrate/FAD binding site, including amino acid residues L171, C172, I199, Q206, Y326, F343, and Y398, through van der Waals, CH– $\pi$ , and  $\pi$ – $\pi$  interactions that are essential for binding.<sup>33</sup> **Selegiline** then forms covalent bonds with FAD, irreversibly prohibiting substrate coordination and subsequent reactions, thereby alleviating the loss of dopamine and serotonin under pathological

conditions (Fig. 4b).<sup>33</sup> Despite their potential to restore neurotransmission, these drugs cannot halt disease progression and provide only modest, short-term cognitive benefits, often accompanied by significant side effects.<sup>28,132</sup> While AChE and MAO inhibitors have been the cornerstone of AD treatment, these drawbacks underscore the urgent need for novel therapies. The inadequate efficacy and adverse effects of current treatments emphasize the necessity for disease-modifying therapies that can concurrently address multiple pathophysiological mechanisms of AD.

## 2.4 Other pathological features

In addition to the abovementioned pathological factors, neuroinflammation, mitochondrial dysfunction, and neuronal cell death are closely associated with the pathogenesis of AD (Fig. 1a).<sup>4–7</sup> These features are intricately connected to other pathological elements, such as A $\beta$  and ROS, underscoring the multifaceted nature of the disease.<sup>6–15</sup> Under normal conditions, astrocytes and microglia play distinct yet complementary roles in maintaining homeostasis and managing neuroinflammation in the central nervous system (CNS). Astrocytes preserve the integrity of the blood–brain barrier (BBB), regulate cerebral blood flow, and release anti-inflammatory cytokines, contributing to a stable neural environment.<sup>133–137</sup> Meanwhile, microglia, the primary immune cells of the CNS, constantly monitor their microenvironment and secrete pro-inflammatory mediators, such as tumor necrosis factor- $\alpha$  (TNF- $\alpha$ ) and interleukin-1 $\beta$  (IL-1 $\beta$ ), in response to injury or infection.<sup>138,139</sup> Together, these cells balance neuroprotection and tissue repair to support neural homeostasis. In AD, however, this balance is disrupted, leading to chronic neuroinflammation and neuronal death.

A $\beta$  aggregates activate microglial receptors, such as Toll-like receptors (TLRs) and nucleotide-binding oligomerization domain-like receptors (NLRs), which stimulate the NLR family pyrin domain-containing 3 (NLRP3) inflammasome.<sup>140–144</sup> Once induced, the NLRP3 inflammasome promotes A $\beta$  aggregation by serving as a nucleation platform for fibrillization. It also cleaves pro-caspase-1 into active caspase-1, which processes pro-interleukin-1 $\beta$  (pro-IL-1 $\beta$ ) and pro-interleukin-18 (pro-IL-18) into their secretory forms.<sup>140–144</sup> The release of cytokines, such as IL-1 $\beta$  and TNF- $\alpha$ , amplifies inflammation and prompts astrocytes to secrete neurotoxic molecules, including ROS and complement components (e.g., complement component 3).<sup>145,146</sup> Simultaneously, sustained excitation of nuclear factor- $\kappa$ B (NF- $\kappa$ B) in astrocytes and microglia, triggered by A $\beta$  and cytokine feedback loops, further elevates the production of pro-inflammatory mediators, including interleukin-6 (IL-6) and TNF- $\alpha$ , perpetuating inflammation and oxidative stress.<sup>147</sup> Chronic glial activation also impairs microcirculation and disrupts the BBB, exacerbating A $\beta$  accumulation.<sup>148,149</sup> Through these interconnected mechanisms, astrocytes and microglia transition from protective roles to active contributors to neuronal damage, driving the progression of AD.

Therapeutic strategies to address astrocyte- or microglia-mediated neuroinflammation in AD primarily focus on



modulating the NF- $\kappa$ B pathway, a central regulator of inflammatory responses. These strategies include developing antagonists for membrane receptors that activate NF- $\kappa$ B<sup>150–153</sup> and inhibitors of key intracellular signaling molecules within this pathway.<sup>154–157</sup> Complementary approaches aim to mitigate A $\beta$  aggregation driven by neuroinflammation.<sup>158–164</sup> One promising method involves targeting the active NLRP3 inflammasome, which serves as a nucleation site for A $\beta$  aggregation, thereby attenuating harmful effects of pro-inflammatory responses.<sup>158–164</sup> Another strategy focuses on enhancing microglial phagocytosis and autophagy to promote the clearance of A $\beta$  aggregates, offering the dual benefit of reducing inflammation and preventing further neuronal damage.<sup>165–169</sup>

Mitochondrial dysfunction is another critical pathological feature of AD, closely linked to disease progression through several key mechanisms. One major manifestation is the over-production of ROS, which induces oxidative stress and damages cellular components, such as lipids, proteins, and DNA.<sup>170</sup> Bioenergetic deficits, such as reduced ATP production by disruptions in the electron transport chain (ETC), further compromise neuronal energy supply, ultimately leading to synaptic failure and cell death.<sup>171,172</sup> Mitochondrial DNA (mtDNA) degradation exacerbates these effects by impairing metabolic functions and repair mechanisms, intensifying cellular dysfunction.<sup>173,174</sup> A key contributor to mitochondrial dysfunction is the diminished clearance of damaged mitochondria, primarily regulated by the phosphatase and tensin homolog-induced kinase 1 (PINK1)-Parkin pathway.<sup>175,176</sup> This pathway is altered under pathogenic conditions due to the accumulation of hyperphosphorylated tau and A $\beta$ , resulting in mitochondrial defection and increased cellular stress.<sup>177,178</sup> Additionally, the balance between mitochondrial fusion and fission is disturbed. A $\beta$  and ROS overactivate dynamin-related protein 1 (Drp1), causing excessive mitochondrial fragmentation.<sup>179–182</sup> This unregulated cleavage compromises energy distribution, weakens synaptic function, and exacerbates neuronal damage.<sup>179–182</sup> Mitochondrial biogenesis is also hindered by oxidative stress and neuroinflammation, which suppress peroxisome proliferator-activated receptor- $\gamma$  co-activator-1 $\alpha$  (PGC-1 $\alpha$ ), thereby reducing the generation of functional mitochondria and worsening bioenergetic deficits.<sup>183–185</sup> Abnormal mitochondrial distribution, coupled with endoplasmic reticulum damage, further amplifies mitochondrial dysfunction, positioning it as a central driver of AD progression.<sup>186,187</sup>

Strategies to address mitochondrial dysfunction in AD focus on reducing excess ROS, restoring mitochondrial function, and improving bioenergetics. To mitigate ROS overproduction, various therapeutic agents with antioxidant functional groups have been explored.<sup>188–190</sup> Efforts are also underway to restore mitochondrial biogenesis, dynamics, and mitophagy by targeting essential proteins involved in these processes, such as nicotinamide adenine dinucleotide-dependent deacetylase sirtuin-1 (SIRT1) and mammalian target of rapamycin (mTOR), which inhibits PGC-1 $\alpha$  and the PINK1-Parkin signaling, respectively.<sup>191–195</sup> Furthermore, modulating mitochondrial bioenergetics by enhancing components critical to energy

production in mitochondria, such as the ETC and Krebs cycle enzymes, may improve energy production in AD.<sup>187,196–201</sup>

The interconnected pathological systems and signaling cascades drive apoptosis-mediated neuronal cell death through both intrinsic and extrinsic pathways.<sup>202–204</sup> Oxidative stress, mitochondrial dysfunction, and A $\beta$  accumulation disrupt intracellular membranes, leading to the release of proteins, such as cytochrome *c*, into the cytosol.<sup>202–206</sup> This triggers the formation of the apoptosome complex and activates caspase-9, further exacerbating cell death and promoting mitochondrial permeabilization.<sup>205</sup> Dysregulated survival pathways, including phosphoinositide 3-kinase (PI3K)/protein kinase B (Akt)/mTOR signaling network, further contribute to apoptosis by impairing protective mechanisms and initiating stress-induced responses.<sup>15,207,208</sup> Extracellular A $\beta$  aggregates also activate cell surface death receptors (e.g., Fas or TNF), forming a death-inducing signaling complex that induces the extrinsic apoptotic pathway *via* caspase-8.<sup>209,210</sup> Caspase-8 either directly stimulates caspase-3 or amplifies the intrinsic pathway by cleaving the Bcl-2 homology 3 interaction domain death agonist (Bid) into truncated Bid (tBid), which further promotes apoptosis.<sup>211,212</sup> Neuroinflammation intensifies these effects *via* pathways, such as Janus kinase/signal transducer and activator of transcription (JAK-STAT), upregulating the expression of pro-inflammatory and pro-apoptotic genes. Concurrently, alterations in Ras/extracellular signal-regulated kinase (Ras/ERK) signaling compromise neuronal survival, which accelerates apoptosis-directed neurodegeneration.<sup>213,214</sup>

Non-apoptotic cell death pathways also play a significant role in neurodegeneration associated with AD. Dysregulated autophagy, initiated by A $\beta$  aggregates and impaired lysosomal function, leads to the accumulation of dysfunctional organelles and proteins, worsening neuronal damage.<sup>215,216</sup> Pyroptosis, an inflammatory form of cell death, is driven by the activation of the NLRP3 inflammasome, releasing pro-inflammatory cytokines and causing membrane damage *via* gasdermin-D-mediated pathway.<sup>144,217</sup> Cuprotosis, arising from imbalances in copper metabolism, disrupts the mitochondrial function, elevates the oxidative stress, and results in the misprocessing of APP, ultimately contributing to neuronal death and exacerbating A $\beta$  pathology.<sup>218</sup> Ferroptosis, an iron-dependent cell death mechanism, is fueled by perturbed iron metabolism, oxidative stress, and lipid peroxidation, thereby promoting neuronal loss.<sup>219,220</sup> Collectively, these apoptotic and non-apoptotic pathways form a feedback loop of inflammation, mitochondrial dysfunction, and neuronal damage, which fosters the progression of AD. To regulate apoptosis, inhibitors confronting caspases-3, -8, and -9 are being actively investigated, with caspase-1 inhibitors to mitigate pyroptosis.<sup>221–232</sup> Additionally, metal chelators are being explored as a potential therapy to control metal ion dyshomeostasis-mediated cell death, such as cuprotosis and ferroptosis.<sup>2,218,232</sup> Taken together, neuroinflammation, mitochondrial damage, and neuronal cell death are increasingly recognized as key pathological targets in AD. In addition to the aggregation of A $\beta$ , the dyshomeostasis of metal ions, and the dysfunction of enzymatic systems, these pathological features highlight the multifaceted nature of AD.<sup>4–7</sup>



Ongoing research efforts are focused on developing therapeutic interventions to address these interconnected processes, as emphasized in recent literature.<sup>6–15</sup>

### 3. Strategies to overcome the complex pathology of AD

Numerous single-target-directed drugs have been developed to address specific pathological factors associated with AD. These treatments have been evaluated at various clinical trial stages, highlighting their potential therapeutic roles within the AD landscape.<sup>17,32,55,56</sup> Despite extensive efforts, no single treatment has been able to effectively alter the progression of the disease, achieving limited success.<sup>3,18,20,21,25,41,59,77–79,233</sup> This outcome underscores the complexity of AD, where multiple factors contribute to disease onset.<sup>16</sup> Therefore, there is a critical need for therapeutics capable of targeting several pathological factors simultaneously.

Rational design strategies for multi-target-directed molecules involve combining two or more molecular frameworks, each with established properties, into a single molecular entity, as illustrated in Fig. 1b.<sup>35,126,234,235</sup> This integration can be accomplished through three approaches: (i) linkage approach, where the original structures are connected *via* a linker; (ii) fusion approach, which directly joins two scaffolds without a linker; (iii) incorporation approach, where functional components are merged while preserving structural features essential for their activity.<sup>35,126,234,235</sup> Achieving this multifunctionality requires a careful balance of scaffold properties while maintaining their functional integrity.<sup>35,126,234,235</sup> These resulting compounds can then undergo structure–activity relationship (SAR) optimization to refine their physicochemical properties, target specificity, and BBB permeability to maximize their therapeutic potential.<sup>35,126,234,235</sup>

Multifunctional molecules developed to address A $\beta$  aggregation often use frameworks inspired by known imaging agents, *e.g.*, thioflavin-T (ThT), polyphenols, and peptide-based inhibitors.<sup>35,126,234,235</sup> These scaffolds are strategically modified to strengthen interactions with the regions of A $\beta$ , including the self-recognition and C-terminal regions, both of which play a crucial role in A $\beta$  aggregation.<sup>39</sup> By targeting these regions, the molecules can disrupt aggregation pathways and prevent the formation of toxic oligomers and fibrils.<sup>39</sup> These compounds typically incorporate hydrophobic features to enhance binding to A $\beta$ 's hydrophobic domains, as well as  $\pi$ – $\pi$  stacking or hydrogen bonding capabilities to interact with multiple amino acid residues within the peptide.<sup>39</sup>

MTDLs designed for confronting A $\beta$  aggregation and metal ion dyshomeostasis simultaneously require the incorporation of nitrogen, oxygen, or sulfur donor atoms for metal chelation into A $\beta$ -binding frameworks to achieve dual function.<sup>35,94–96</sup> By precisely tuning dissociation constant ( $K_d$ ) values and metal-binding geometries, these chelators can prohibit interactions between metal ions and A $\beta$  without disrupting systemic metal ion homeostasis or interfering with the function of metalloenzymes. Additionally, MTDLs can be optimized to block

vacant coordination sites within metal-binding domains, thereby preventing redox reactions that produce ROS. Moreover, these molecules can modulate the redox potential of the metal center to mitigate oxidative stress.

To regulate enzyme activity, rational strategies focus on targeting the active sites of enzymes.<sup>54,104,108,111,112</sup> A common approach is competitive inhibition, where molecules are designed to mimic natural substrates, enabling them to occupy the active sites of enzymes and block their substrate binding. By integrating computational modeling and crystallographic studies, the development of highly selective and effective MTDLs capable of adjusting enzyme activity is facilitated. Rational design often utilizes backbones from natural products, polyphenols, or established inhibitors, employing interactions, such as hydrogen bonding,  $\pi$ – $\pi$  stacking, and hydrophobic contacts, to acquire high specificity.<sup>236,237</sup>

Multifunctionality can be achieved by linking or incorporating key moieties addressing multiple pathways into a single molecular framework. While research on A $\beta$  aggregation remains a central focus, extensive efforts are also being directed toward combining two or more distinct structural motifs within a single molecule to simultaneously target alternative pathological pathways.<sup>35,126,234,235</sup> These efforts include inhibiting enzymes, such as BACE1, MMP9, LOXL2, AChE, and MAOB, as well as modulating their interactions with metal ions.<sup>35,126,234,235</sup> Such approaches, which have been comprehensively reviewed in several studies, highlight the versatility and therapeutic promise of MTDLs.<sup>35,126,234,235</sup> The following sections underscore the critical role of SAR studies, with an emphasis on the development of multi-target-directed compounds that focus on A $\beta$  aggregation with other interconnected pathological factors of AD.

#### 3.1 Chemical reagents capable of controlling A $\beta$ and BACE1

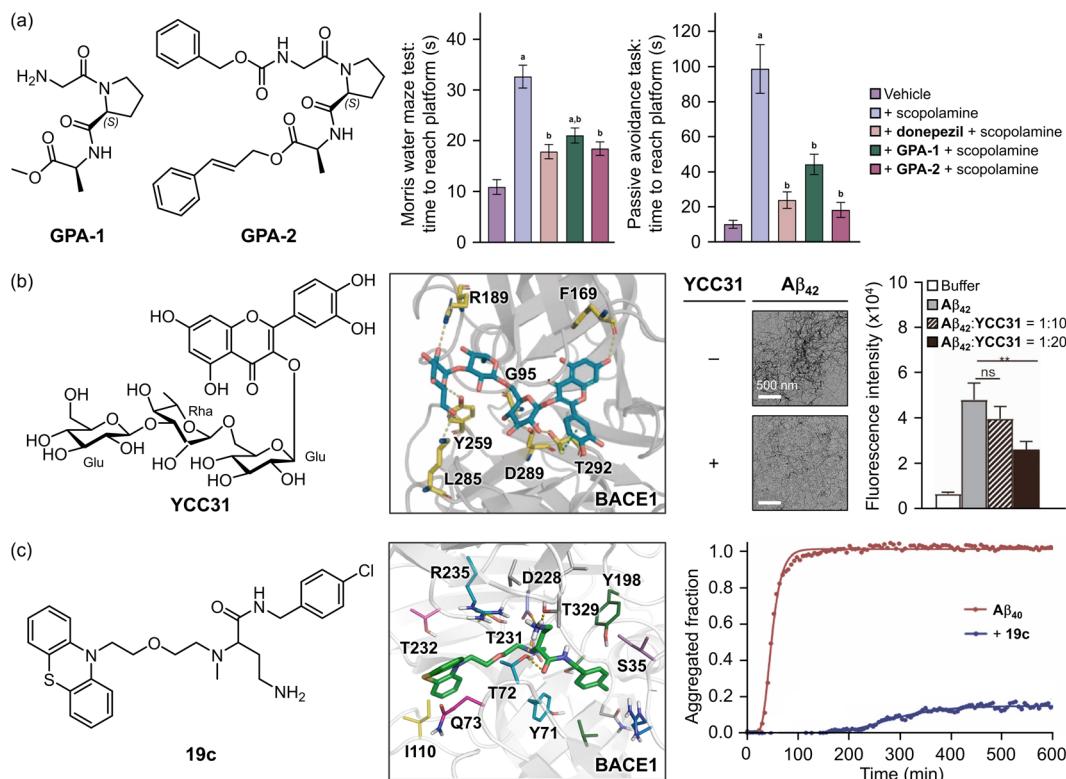
A $\beta$  remains a critical biomarker in AD therapy due to its association with the disease's pathological features and its involvement in neuroinflammation and cognitive decline.<sup>1</sup> Consequently, regulating BACE1 activity, the primary enzyme responsible for A $\beta$  production, has become a major focus in the development of AD therapeutics.<sup>50,238</sup> Single-target-directed therapies, however, have proven insufficient in slowing disease progression. This has led to a shift in research towards strategies that regulate both the accumulation and production of A $\beta$  to achieve a synergistic clinical effect.<sup>233,238</sup> Therapeutic interventions that integrate multiple characteristics can effectively form hydrophobic interactions or hydrogen bonds within the catalytic cleft and aspartate dyad to target BACE1 efficiently. Additionally, these structures can be designed to establish hydrophilic and hydrophobic contacts with A $\beta$ , thereby inhibiting its aggregation. Current approaches with the above factors are widely being explored to address both A $\beta$  and BACE1 using peptides,<sup>239</sup> natural products,<sup>240,241</sup> and modifications of existing therapeutic frameworks.<sup>242,243</sup>

Peptides naturally contain polar groups that can be modified to improve their ability to cross the BBB and interact efficiently with target enzymes. To optimize these properties, Singh and coworkers designed peptides with hydrophobic groups added at



both the N- and C-termini.<sup>239</sup> Proline was used as the template to regulate surface area and structural flexibility, with a glycine–proline–alanine (GPA) tripeptide as the model backbone.<sup>239</sup> Various derivatives, each featuring different functional groups at the N- and C-termini, were synthesized to achieve an optimal balance between hydrophobicity and hydrophilicity. Among these derivatives, **GPA-1** and **GPA-2** (shown in Fig. 5a) exhibited significant *in vivo* efficacy, effectively inhibiting both the activity of BACE1 and the aggregation of A $\beta$ <sub>42</sub>.<sup>239</sup> Both peptides displayed potent ID<sub>50</sub> values of 20 nM against BACE1.<sup>239</sup> Additionally, **GPA-1** and **GPA-2** suppressed the formation of  $\beta$ -sheet-rich A $\beta$ <sub>42</sub> aggregates by 54% and 34%, respectively.<sup>239</sup> Pretreatment with these peptides also alleviated memory deficits in a mouse model with scopolamine-induced memory impairment, as presented in Fig. 5a.<sup>239</sup> Behavioral assessments, including the Morris water maze, revealed a significant reduction in latency time to reach the platform, while the passive avoidance task demonstrated decreased latency in reaching the shock-free area.<sup>239</sup> Furthermore, peptide-based strategies have been applied to block the  $\beta$ -cleavage site of APP in *in vivo* systems.<sup>244</sup>

The use of natural products as therapeutic candidates has garnered attention for decades due to their proven efficacy, safety, and increased molecular rigidity compared to synthetic compound libraries, particularly for addressing protein–protein interactions.<sup>245</sup> Among these natural compounds, flavonoids are notable for their ability to interact with multiple biological targets through hydrogen bonding and  $\pi$ – $\pi$  stacking interactions.<sup>240,246,247</sup> Building on these properties, Yang and coworkers discovered a natural flavonoid–carbohydrate conjugate, **YCC31**, which exhibited significant potential for dual inhibition (Fig. 5b). **YCC31** reduced A $\beta$  production in 7PA2 cells by modulating the activity of BACE1.<sup>240</sup> The compound forms hydrogen bonds with key amino acid residues G95, F169, R189, Y259, K285, and D289 within BACE1 through its hydroxyl groups and ether group oxygen atom. Additionally, its phenyl group participates in CH– $\pi$  interactions with T292, effectively preventing substrate access and reducing A $\beta$  production, as depicted in Fig. 5b.<sup>240</sup> In addition to BACE1 inhibition, **YCC31** demonstrated dose-dependent regulation of A $\beta$ <sub>42</sub> aggregation, as analyzed by transmission electron microscopy (TEM), ThT assay, and molecular dynamics (MD) simulations.<sup>240</sup> The



**Fig. 5** Examples of chemical reagents controlling A $\beta$  and BACE1. (a) Small peptide-based compounds (**GPA-1** and **GPA-2**) and their effects on the cognition of mice models after inducing impaired memory with scopolamine on days 12 to 14. **GPA-1**, NH<sub>2</sub>-Gly-Pro-Ala-OMe; **GPA-2**, Cbz-Gly-Pro-Ala-O-cinnamyl. Reproduced with permission from ref. 239. Copyright© 2024 American Chemical Society. (b) A flavonoid-based natural product (**YCC31**) and its inhibitory impact on the activity of BACE1 and the aggregation of A $\beta$ <sub>42</sub>. The interactions between **YCC31** and BACE1 at the active site, the morphology of the resultant A $\beta$ <sub>42</sub> species, and its inhibitory effect on A $\beta$ <sub>42</sub> aggregation were analyzed by MD simulations, TEM, and the ThT assay, respectively. **YCC31**, quercentin-3-O-[ $\beta$ -D-glucopyranosyl-(1 $\rightarrow$ 3)-O- $\alpha$ -L-rhamnopyranosyl-(1 $\rightarrow$ 6)-O- $\beta$ -D-glucopyranoside]. Reproduced with permission from ref. 240. Copyright© 2024 Elsevier. (c) A phenothiazine-derived compound (**19c**) and its effect against the activity of BACE1 and the aggregation of A $\beta$ <sub>40</sub>. The interactions between **19c** and BACE1 at the active site were obtained from docking studies and its inhibitory effect on the formation of  $\beta$ -sheet-rich A $\beta$ <sub>40</sub> aggregates was quantitatively measured by the ThT assay. **19c**, 4-amino-N-[(4-chlorobenzyl)-2-[methyl({2-[2-(10H-phenothiazin-10-yl)ethoxyethyl]amino}butanamide]. Reproduced with permission from ref. 243. Copyright© 2023 Elsevier.



supramolecular equivalent of **YCC31** suppressed the fibrillization of  $\text{A}\beta_{42}$ , generating short fibrils by interacting with amino acid residues D22, S26, N27, and K28, which are crucial for the salt bridging that stabilizes fibrillar structures.<sup>240</sup> Moreover, **YCC31** decreased the production of ROS and nitric oxide (NO) induced by  $\text{A}\beta$ -mediated activation of microglia, thereby mitigating oxidative stress and neuroinflammation.<sup>240</sup>

Modifying existing scaffolds has emerged as a promising approach to accelerate the drug discovery process.<sup>248</sup> One notable example is *N*-benzylbutanamide.<sup>243</sup> Bajda and coworkers developed a phenothiazine derivative, **19c**, which exhibited the highest *h*BACE1 inhibitory activity ( $\text{IC}_{50} = 1.6 \mu\text{M}$ ) and anti-aggregation effects against  $\text{A}\beta_{40}$  (99% inhibition at 10  $\mu\text{M}$ ), as described in Fig. 5c.<sup>243</sup> The inhibition of *h*BACE1 by **19c** is attributed to its ability to block both substrate entry and catalytic proteolysis. The compound interacts with the S2 and S2' pockets *via*  $\text{CH}-\pi$  interactions with Y71 and a  $\pi$ -oxygen bond with Y198, while forming a cation– $\pi$  interaction with R128 in the S3' pocket. Additionally, its amide group establishes a hydrogen bond with the main chain of T72, and its  $\gamma$ -amino group engages in a network of bonds, creating two hydrogen bonds with the side chains of T72 and T329 and an ionic interaction with D328.<sup>243</sup> The phenothiazine moiety of **19c** fits into the S3 and S4 pockets of BACE1, accompanying in hydrophobic contacts with Q73 and I110.<sup>243</sup> Moreover, **19c** prevented  $\text{A}\beta_{40}$  aggregation by 86%, significantly decreasing both elongation and nucleation rates, as measured by the ThT assay (Fig. 5c). These results indicate that **19c** directly associates with fibrillar species to suppress elongation and with soluble nucleic, oligomeric, and prefibrillar forms of  $\text{A}\beta_{40}$  during the early stages of polymerization. Beyond its *in vitro* effects, **19c** demonstrated statistically significant anti-amnesic properties and improved non-spatial (contextual and recognition) memory in a scopolamine-induced amnesia mouse model.<sup>243</sup> Overall, these findings underscore the potential of multi-targeting strategies in developing effective AD therapeutics, particularly those involving precise molecular interactions with BACE1 and  $\text{A}\beta$ .

### 3.2 Chemical reagents capable of regulating $\text{A}\beta$ , metal ions, and ROS

Metal ions, such as  $\text{Cu}(\text{I/II})$ ,  $\text{Zn}(\text{II})$ , and  $\text{Fe}(\text{II/III})$  can coordinate to  $\text{A}\beta$  to form metal– $\text{A}\beta$  complexes.<sup>82,249</sup> The non-specific removal of these metal ions, however, has been unsuccessful and presents several challenges.<sup>25,77–79</sup> As a result, strategies promising alternatives to non-selective metal ion chelation. To achieve this, numerous aspects of each component must be carefully considered. Chemical reagents should incorporate either a redox-active backbone, a metal-chelation moiety, or both to effectively mitigate oxidative stress.<sup>25,77</sup> In addition, the structure should include functional groups capable of forming hydrogen bonds and hydrophobic interactions to regulate  $\text{A}\beta$  aggregation.<sup>35,234,235</sup> For these aspects, peptides have been developed.<sup>250–256</sup> Sun and coworkers discovered that integrating the  $\text{A}\beta$  self-recognition site (LVFFA) and a metal-chelating tripeptide (RTH) efficiently modulates metal-mediated  $\text{A}\beta$

aggregation and ROS generation (Fig. 6a).<sup>250–252</sup> Building on this concept, a *D*-enantiomeric peptide, **RTHLVFFARK-NH<sub>2</sub>** (**rk10**), was rationally designed and compared to its previously reported *L*-enantiomer, **RK10**. The **rk10** peptide demonstrated the ability to form stable metal coordination with the amino terminal  $\text{Cu}(\text{II})$ - and  $\text{Ni}(\text{II})$ -binding (ATCUN) motif, exhibiting a  $K_d$  value of 28 nM, comparable to that of  $\text{A}\beta$  ( $\log K_{\text{Cu}(\text{II})} = 10$ ). This  $\text{Cu}(\text{II})$  chelation prevented redox reactions and excessive ROS production by  $\text{Cu}(\text{II})$ – $\text{A}\beta$  complexes.<sup>250</sup> As shown in Fig. 6a, **rk10** treatment decreased ROS generation to half the level observed in the  $\text{Cu}(\text{II})$ – $\text{A}\beta$  control without peptide treatment.<sup>250</sup> Furthermore, **rk10** disrupted the twisted and elongated fibrillar network, suppressing fibril growth and redirecting the aggregation pathway to yield amorphous aggregates, as visualized by atomic force microscopy (AFM).<sup>250</sup> A similar trend was observed in the presence of  $\text{Cu}(\text{II})$ , where **rk10** addition noticeably decreased the formation of visible structures, compared to **RK10**.<sup>250</sup> These findings suggest that while **rk10** possesses metal-chelation properties similar to **RK10** ( $K_d = 31 \text{ nM}$ ), its enhanced interactions with  $\text{A}\beta$  further improve the inhibitory effects on  $\text{A}\beta$  fibrillization and  $\text{Cu}(\text{II})$ – $\text{A}\beta$ -induced ROS generation.<sup>250,252</sup>

An alternative strategy in AD research involves using small molecules that chemically modify  $\text{A}\beta$ , altering its aggregation pathways. A series of redox-active aromatic compounds has demonstrated reactivities towards metal-free  $\text{A}\beta$ , metal-bound  $\text{A}\beta$ , and free organic radicals.<sup>257</sup> Among these, *p*-phenylenediamine (**PPD**; Fig. 6b) and its derivatives have emerged as promising therapeutic candidates due to their structural simplicity, redox capability, and amphiphilicity.<sup>259,260</sup> **PPD**, with two electron-donating groups at the *para* position, lowered the redox potential and enhanced its ROS scavenging capacity.<sup>261,262</sup> Furthermore, **PPD** was shown to modulate  $\text{A}\beta$  aggregation, resulting in smaller fibrils and amorphous aggregates, which appeared as smearing bands in the 4–270 kDa range in gel electrophoresis with western blotting (gel/western blot) analyses (Fig. 6b).<sup>257</sup> A similar trend was observed in the presence of  $\text{Cu}(\text{II})$  or  $\text{Zn}(\text{II})$ , indicating that **PPD** further perturbed metal– $\text{A}\beta$  aggregation pathways.<sup>257</sup> Mechanistic details revealed that **PPD** undergoes oxidation to yield benzoquinone-diimine (**BQDI**) or benzoquinone (**BQ**)<sup>257</sup> during which the M35 residue of  $\text{A}\beta$  is oxidized.<sup>257</sup> In addition, **BQ** formed covalent adducts with  $\text{A}\beta$  through primary amino groups, such as K16 (Fig. 6b).<sup>257,263,264</sup> This complexation captured  $\text{A}\beta$  in a relatively compact conformation, likely preventing its involvement in  $\text{A}\beta$  fibrillization.<sup>265</sup> Supported by these *in vitro* results, **PPD** significantly reduced cerebral and hippocampal  $\text{A}\beta$  deposits and improved cognitive function in 5xFAD transgenic mice.<sup>257</sup>

The structural similarity of *N,N*-dimethylaniline (**DMA**) to **PPD** imparts comparable properties, enabling **DMA** to interact with both metal-free  $\text{A}\beta$  and metal– $\text{A}\beta$ , and participate in one- or two-electron oxidation processes, thereby contributing to ROS scavenging.<sup>266,267</sup> Building on this concept, a derivative named **L1** (presented in Fig. 6c), featuring a bidentate ligand attached to the **DMA** backbone, was developed to directly modify the  $\text{Cu}(\text{II})$ -coordination sphere in  $\text{A}\beta$  through copper–O<sub>2</sub> chemistry.<sup>258</sup> Notably, the H14 residue was modulated when  $\text{Cu}(\text{II})$ – $\text{A}\beta$  was incubated with **L1** under aerobic conditions,



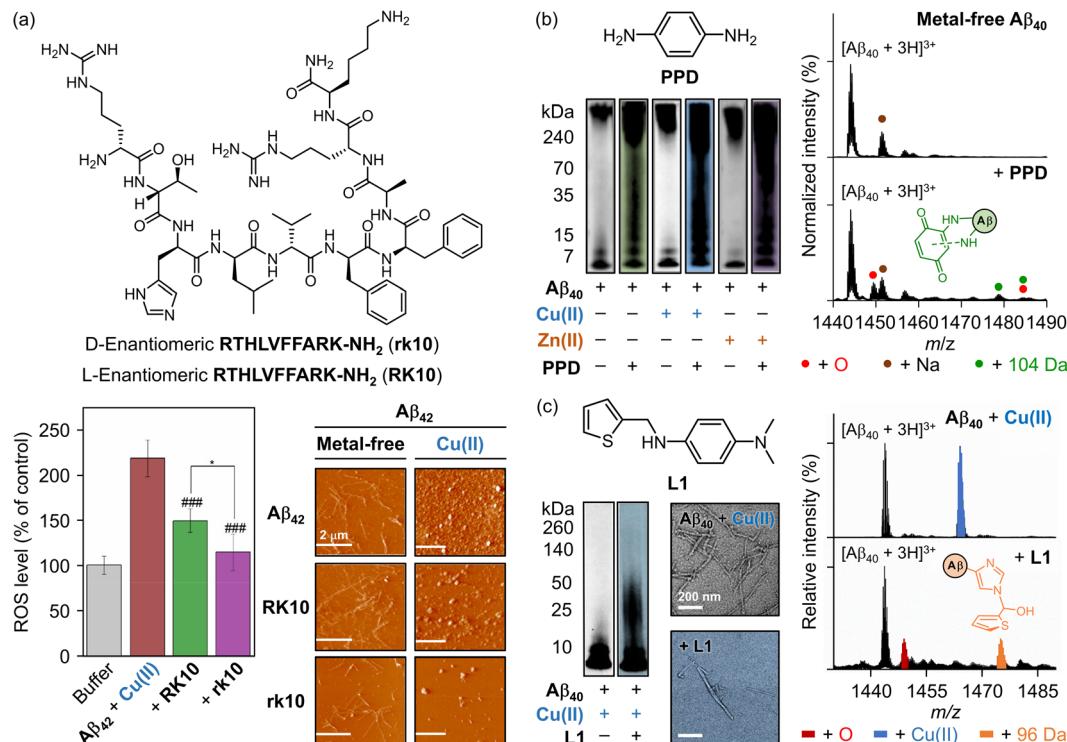


Fig. 6 Examples of chemical reagents regulating A $\beta$  peptides and metal ions. (a) Structure of the D-enantiomeric decapeptide RTHLVFFARK-NH<sub>2</sub> (rk10) and inhibitory effects of rk10 and its L-enantiomer (RK10) on the generation of ROS and metal-free and Cu(II)-A $\beta$ <sub>42</sub> aggregation. The ROS level produced by Cu(II)-A $\beta$ <sub>42</sub> in the absence and presence of rk10 or RK10 in SH-SY5Y cell was measured by a fluorescence assay with 2',7'-dichlorofluorescein diacetate (DCFH-DA). The statistical significance level is expressed by the pound (control as A $\beta$ <sub>42</sub> + Cu(II), #P < 0.001) and asterisk (control as A $\beta$ <sub>42</sub> + Cu(II) + RK10, \*P < 0.05). The morphology of A $\beta$ <sub>42</sub> aggregates generated with and without Cu(II) and rk10 or RK10 was analyzed by AFM. Reproduced with permission from ref. 250. Copyright© 2019 American Chemical Society. (b) Structure of PPD and its impact on the aggregation of both metal-free and metal-added A $\beta$ <sub>40</sub>. The size distribution of A $\beta$ <sub>40</sub> aggregates produced with and without PPD or metal ions was investigated by gel/western blot. The formation of the covalent adduct between A $\beta$ <sub>40</sub> and benzoquinone was detected by ESI-MS. PPD, *p*-phenylenediamine. Reproduced with permission from ref. 257. Copyright© 2020 American Chemical Society. (c) Structure of L1 and its influence on Cu(II)-added A $\beta$ <sub>40</sub> aggregation. The size distribution and morphology of the resultant A $\beta$ <sub>40</sub> aggregates produced with and without L1 or Cu(II) were investigated by gel/western blot and TEM, respectively. Chemical modifications onto the Cu(II)-coordination sphere of A $\beta$ <sub>40</sub> by L1 were monitored by ESI-MS. L1, *N*<sup>1</sup>,*N*<sup>1</sup>-dimethyl-*N*<sup>4</sup>-(thiophen-2-ylmethyl)benzene-1,4-diamine. Reproduced with permission from ref. 258. Copyright© 2020 United States National Academy of Sciences.

resulting in mass increments of 16 Da and 96 Da in A $\beta$ , corresponding to oxidation and covalent adduct formation, respectively.<sup>258</sup> Since H14 is associated with coordinating both Cu(I) and Cu(II) in A $\beta$ , this chemical transformation effectively disrupted the aggregation of metal-A $\beta$  complexes.<sup>258</sup> Moreover, L1 inhibited the aggregation of Cu(II)-A $\beta$ <sub>40</sub> by altering the morphology of A $\beta$  aggregates into shorter fibrils, suppressed H<sub>2</sub>O<sub>2</sub> production from the redox cycling of Cu(I/II)-A $\beta$ , and reduced cytotoxicity (Fig. 6c).<sup>258</sup>

Overall, several small molecules have been developed to address metal-induced A $\beta$  aggregation through three primary mechanisms: (i) directly interacting with both metal ions and A $\beta$ , (ii) chemically modifying A $\beta$ , and (iii) disrupting the coordination sphere of metal-A $\beta$  complexes. These strategies can precisely redirect the binding pattern of redox-active metal ions to A $\beta$ , thereby preventing ROS production. They further suggest that shifting beyond traditional approaches, which solely focus on chelating metal ions, to simultaneously targeting both metal-A $\beta$  interactions and ROS may present a more effective strategy for developing novel AD treatments.

### 3.3 Chemical reagents capable of modulating A $\beta$ and metalloenzymes

Metal ion dyshomeostasis and the resultant dysregulation of metalloenzymes are emerging as promising therapeutic targets for AD.<sup>98,104-107,120,268-272</sup> Among these, MMP9 and LOXL2 have been implicated in the pathogenesis of neurodegenerative diseases due to their involvement in A $\beta$  aggregation.<sup>104-107</sup> Consequently, there is growing interest in developing therapies that simultaneously address MMP9 or LOXL2 while inhibiting A $\beta$  aggregation as a combined approach to enhance therapeutic efficacy.

As part of metalloenzymes, regulating the activity of MMP9 and LOXL2 requires the inclusion of a metal-coordination motif, with functional groups capable of disrupting A $\beta$  self-assembly. Building on this concept, several polyphenol-based natural products have been investigated for their regulatory properties against MMP9.<sup>273,274</sup> Among them, **doxycycline (DOX)** (Fig. 7a), a tetracycline derivative, is a well-known antibiotic that also inhibits MMP9 and reduces neuronal damage in cases of focal brain ischemia.<sup>278</sup> This prospective dual-modulatory effect has advanced DOX to phase II clinical trials.<sup>279</sup> Kaur and



coworkers elucidated the binding mode and antagonistic mechanism of **DOX** against MMP9 through docking analysis.<sup>280</sup> **DOX** primarily interacts with the catalytic domain of MMP9 by forming hydrogen bonds with amino acid residues E402, E416, and L418, and through hydrophobic interactions, such as CH- $\pi$  and  $\pi$ - $\pi$  interactions with amino acid residues H401, P421, M422, Y423, and R424 (highlighted green in Fig. 7b).<sup>280</sup> Additionally, the coordination of **DOX** to Zn(II) through the hydroxyl group on its D ring further stabilizes its complexation at the active site, thereby preventing ligand association and catalytic hydrolysis.<sup>280</sup>

Recent studies have also elucidated that **DOX** directly associates with amyloidogenic fibrils, including those produced by A $\beta$ , and disassembles pre-formed aggregates.<sup>275,276,279,281,282</sup> As depicted in Fig. 7c, **DOX** engages in hydrophobic interactions with key amino acid residues of A $\beta$ <sub>42</sub> fibrils, such as L17, F19, I32, and L34, which are critical for self-recognition and fibril stabilization (Fig. 2b).<sup>275</sup> Morphological analysis revealed that **DOX** treatment significantly reduced fibril generation and promoted degradation.<sup>276</sup> In addition to its anti-aggregation properties, **DOX** has been shown to scavenge ROS and other oxidants, such as superoxide anion radicals (O<sub>2</sub><sup>-</sup>) and lipid peroxides, diminishing the production of cytotoxic malondialdehyde-acetaldehyde-protein adducts mediated by redox reactions.<sup>283</sup> These antioxidant properties of **DOX** contribute to its anti-inflammatory effects and its ability to alleviate oxidative stress in AD.<sup>283</sup>

Recent studies have shown that polyphenols can also regulate the activity of LOXL2, highlighting their potential as therapeutic agents.<sup>275,277,284</sup> Based on these findings, there is growing interest in identifying noble polyphenols that can simultaneously inhibit the activity of LOXL2 and the aggregation of A $\beta$ . **Curcumin (CUR)** (Fig. 7d), a natural polyphenol found in *Curcuma longa* plants, has gained recognition as a promising candidate for AD treatment due to its ability to target multiple pathological factors, including LOXL2 and A $\beta$ .<sup>275,277,284</sup> **CUR** modulated LOXL2 through mechanisms similar to those of **DOX**, binding directly to the catalytic domain and preventing ligand complexation and catalytic activity.<sup>284</sup> This interaction involves hydrogen bonding between the amide backbone of P716 and a water molecule, which forms a hydrogen bonding network with the side chain of S512, along with hydrophobic interactions with the side chains of V713 and F718, as displayed in Fig. 7e.<sup>284</sup> This binding mode resulted in a stable interaction, as confirmed by root mean square deviation (RMSD) analysis.<sup>284</sup>

Similar to **DOX**, **CUR** maintained continuous contact with A $\beta$  fibrils during MD simulations, interacting with the central hydrophobic core of A $\beta$  fibrils, including amino acid residues L17, F19, I32, and L34. **CUR** also disrupted the salt bridge formed by the side chain of D23. These interactions prohibited the aggregation of A $\beta$  and destabilized fibril structures, leading to shorter, fragmented fibrils, as illustrated in Fig. 7f.<sup>275</sup> In addition, **CUR** has been shown to inhibit the activity and expression of MMP9.<sup>285</sup> Furthermore, **CUR** can chelate several metal ions, such as Cu(II), Fe(II), and Zn(II), through its  $\alpha$ , $\beta$ -unsaturated  $\beta$ -diketo moiety, thereby contributing to the reduction of ROS.<sup>286,287</sup> Collectively, addressing

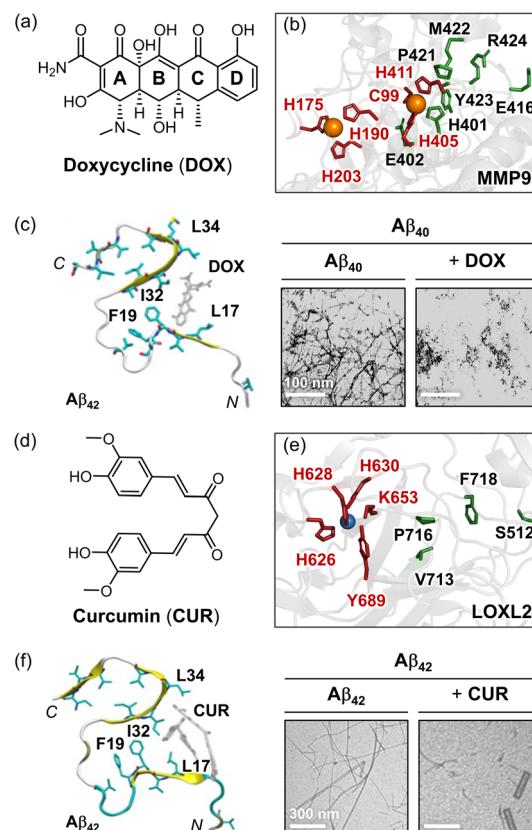


Fig. 7 Examples of chemical reagents controlling A $\beta$  and MMP9 or LOXL2. (a) Structure of doxycycline (DOX); (4S,4aR,5S,5aR,6R,12aS)-4-(dimethylamino)-3,5,10,12,12a-pentahydroxy-6-methyl-1,11-dioxo-1,4,4a,5,5a,6,11,12a-octahydrotriacene-2-carboxamide). (b) Intermolecular interactions between MMP9 (PDB 1L6J<sup>70</sup>) and DOX. The Zn(II)-coordination and DOX-interacting residues are indicated as sticks, and highlighted in red and green, respectively. (c) Interactions of DOX with A $\beta$ <sub>42</sub> fibrils (PDB 2MXU). The morphology of the resultant A $\beta$ <sub>42</sub> species was analyzed with TEM. Reproduced with permission from ref. 275 and 276. Copyright© 2019 Multidisciplinary Digital Publishing Institute and 2001 John Wiley and Sons. (d) Structure of curcumin (CUR). CUR, (1E,6E)-1,7-bis(4-hydroxy-3-methoxyphenyl)hepta-1,6-diene-3,5-dione. (e) Intermolecular interactions between LOXL2 (PDB 5ZE3<sup>71</sup>) and CUR. The Cu(II)-coordination and CUR-interacting residues are shown as sticks, and depicted in red and green, respectively. (f) Interactions of CUR with A $\beta$ <sub>42</sub> fibrils (PDB 2MXU). The formation of the smaller A $\beta$ <sub>42</sub> species was detected by TEM. Reproduced with permission from ref. 275 and 277. Copyright© 2019 Multidisciplinary Digital Publishing Institute and 2017 Elsevier.

metalloenzymes, *e.g.*, MMP9 and LOXL2, using multi-target-directed strategies offers a promising approach to enhancing specificity and therapeutic efficiency in AD treatment. Further research focusing on optimizing polyphenol backbones or modifications could significantly advance the development of AD therapeutics.

### 3.4 Chemical reagents capable of regulating metal-free A $\beta$ , metal-bound A $\beta$ , ROS, and AChE

The dysregulation of ACh, driven by the upregulation of AChE in senile plaques, is well-known to disrupt cholinergic neurotransmission and contributes to cognitive deficits in AD.<sup>35,288</sup>



Despite the development of numerous AChE inhibitors, their efficacy has not met clinical expectations.<sup>28–32</sup> As a result, recent research has shifted towards multivalent therapeutic approaches that target multiple pathological factors to maximize efficacy while minimizing side effects.<sup>101,289–291</sup>

For AChE inhibition, it is essential to prevent substrate binding to the active site by blocking the CAS, PAS, or both, thereby interrupting enzymatic activity mediated by the catalytic triad. Simultaneously, the molecule should incorporate features that enable interactions with A $\beta$ , such as hydrogen bonding and hydrophobic properties. Flavonoid derivatives have shown promise in modulating several pathological factors, including ROS, metal-free and metal-bound A $\beta$ , and AChE.<sup>289,290,292</sup> To enhance multi-targeting capabilities, structural modifications on the **isoflavone** or **flavone** core have been explored, as depicted in Fig. 8a.<sup>289,290,292</sup> These modifications increased the reactivity of flavonoid by incorporating multiple hydroxyl groups that disrupt substrate complexation at the AChE active site through hydrogen bonding. Additionally, the hydrophobic A and C rings of flavonoids interacted with aromatic side chains, such as W86, F295, and Y341, within the hydrophobic binding pocket (Fig. 8b).<sup>292</sup>

Among these derivatives, a rationally designed and optimized flavonoid, **isoflavone-3** (shown in Fig. 8a), which contains five hydroxyl groups on the **isoflavone** backbone, exhibited the highest reactivity.<sup>289,290</sup> As illustrated in Fig. 8b, the 5-OH group on the A ring formed hydrogen bonds with the backbone carbonyl group of G120–G121 and the hydroxyl group of S203. Additionally, the 3-OH group on the B ring generated a hydrogen bond with the backbone amide group between I294 and F295, while the O1 donor atom on the C ring constructed a hydrogen bond with the hydroxyl group of Y337.<sup>289,294</sup> These interactions effectively blocked the cavity located at the AChE active site, inhibiting the enzyme's function with an IC<sub>50</sub> value of 0.19  $\mu$ M.<sup>289</sup>

**Isoflavone-3** significantly modulated the oligomerization and fibrillation of both metal-free A $\beta$  and metal-A $\beta$ . As shown resulted in the formation of shorter and thinner fibrils, whereas in the presence of Cu(II) or Zn(II), it produced fragmented fibrils in Fig. 8c, the addition of **isoflavone-3** to metal-free A $\beta$ <sub>42</sub> with a mixture of amorphous and fibrillary aggregates.<sup>289</sup> These alterations in aggregation were mediated by the interaction of **isoflavone-3** with key regions of A $\beta$ <sub>42</sub>, including the  $\beta$ -turn motif (V12–Q15), self-recognition site (L17–A21), and the C-terminal region (I32–A42), which are critical for A $\beta$ <sub>42</sub> fibrillization. Additionally, **isoflavone-3** promoted the oxidation of H13, H14, and M35 in the presence of Cu(II), thereby disrupting metal coordination and the hydrophobic interactions between A $\beta$  peptides, which influenced their aggregation behavior.<sup>289</sup>

The rational design of **isoflavone-3** incorporated three hydroxyl groups at the C5–C7 positions on the A ring, a catechol moiety on the B ring, and translocation of the B ring from C2 to C3 on the C ring. This structural modification lowered its redox potential to 1.0 V (vs. SHE), enhancing its scavenging capability against free organic radicals.<sup>289</sup> Overall, flavonoids like **isoflavone-3** represent a promising platform for developing multifunctional therapeutics for AD by confronting multiple pathological features, including A $\beta$  aggregation, metal ion dyshomeostasis, cholinergic dysfunction, and oxidative stress.

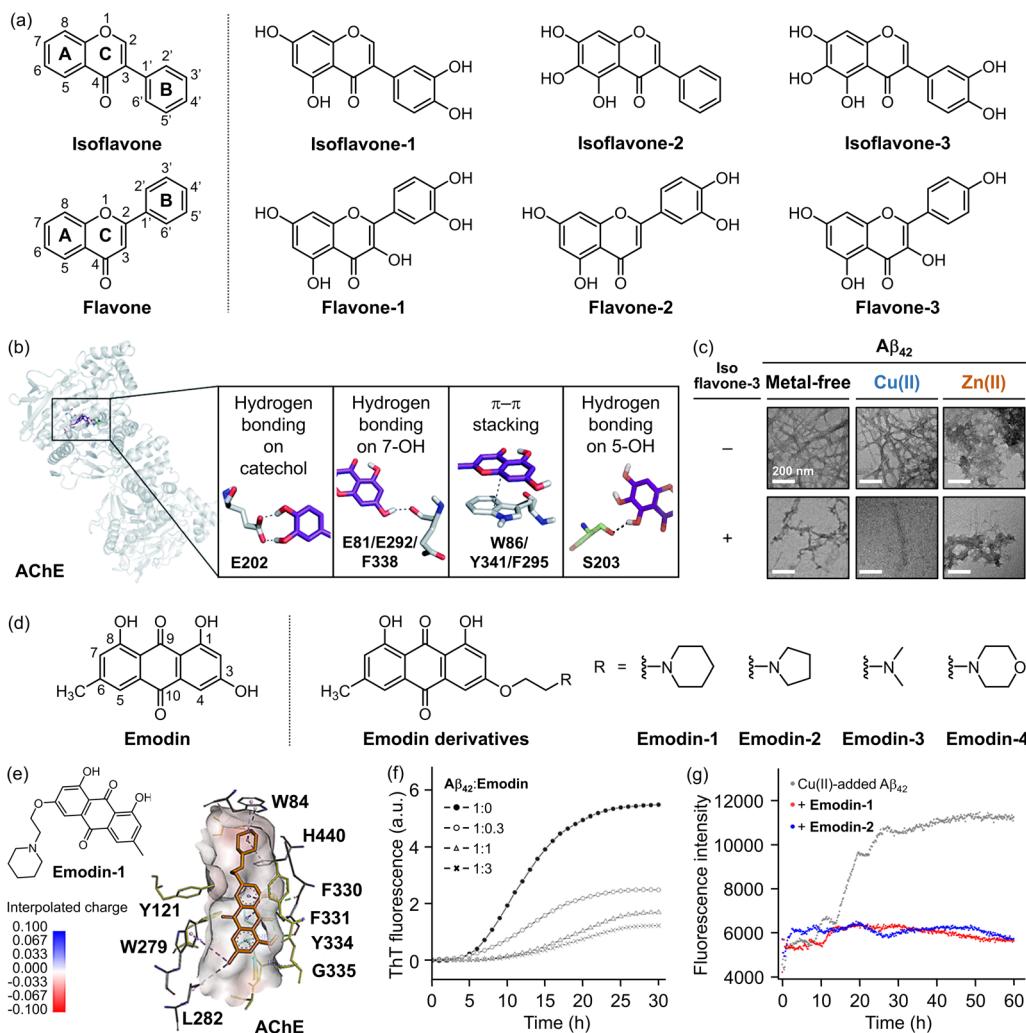
Several studies have suggested that anthraquinone compounds can alleviate AD symptoms by deactivating AChE through simultaneous targeting of the CAS and PAS, while also exhibiting antioxidant and anti-A $\beta$  aggregation properties.<sup>101,291</sup> **Emodin** (Fig. 8d), a natural anthraquinone-based polyphenol used in traditional Chinese medicine, is a well-researched chemical reagent that addresses multiple pathological factors in AD.<sup>101,291,295–297</sup> Numerous structural modifications have been made to the **emodin** backbone to improve its dual activity against AChE and A $\beta$ .<sup>291</sup> Among these derivatives, **emodin-1** (illustrated in Fig. 8d) has demonstrated the most potent AChE inhibitory activity, with an IC<sub>50</sub> value of 67 nM.<sup>291</sup> The anthraquinone backbone of **emodin-1** bound to PAS through CH– $\pi$  interactions with W279, while additional hydrogen bonds, hydrophobic interactions, and  $\pi$ – $\pi$  interactions occurred with F331.<sup>291</sup> Furthermore, functional groups, such as piperidine and pyrrolidine rings, formed CH– $\pi$  interactions with W84 and H440, effectively blocking the CAS, as displayed in Fig. 8e.<sup>291</sup> These interactions at both the CAS and the PAS contributed to the efficient downregulation of AChE, reducing the degradation of ACh and thereby preserving cholinergic function.

**Emodin** has also been reported to modulate and inhibit A $\beta$ <sub>42</sub> aggregation, both in the absence and presence of Cu(II). As presented in Fig. 8f, **emodin** reduced the  $\beta$ -sheet-rich aggregation of A $\beta$ <sub>42</sub> by up to 80% in a dose-dependent manner,<sup>101</sup> which is attributed to interactions between **emodin**'s anthraquinone backbone and the V18 and F19 residues of A $\beta$ , which form the self-recognition site.<sup>101</sup> **Emodin** derivatives with various functional groups have demonstrated similar modulatory effects on A $\beta$ <sub>42</sub> aggregation, with **emodin-1** achieving an inhibition ratio of *ca.* 76%.<sup>293</sup> A comparable trend was observed in the presence of Cu(II), where significant reductions in  $\beta$ -sheet-rich aggregates were noted in samples treated with **emodin-1** and **emodin-2** (Fig. 8g).<sup>293</sup> This effect is likely due to the interaction of **emodin-1–4** (Fig. 8d) with the hydrophobic surface of the A $\beta$ <sub>42</sub> helix in the N-terminal region, potentially stabilizing the  $\alpha$ -helix and reducing the formation of oligomers and fibrils.<sup>293</sup> Additionally, **emodin** possessed chelation properties that can regulate metal ion dyshomeostasis and oxidative stress. Treatment with **emodin-1–4** dramatically decreased the formation of hydroxyl radicals (·OH) and O<sub>2</sub><sup>·-</sup>, compared to controls with Cu(II) alone.<sup>291</sup> These findings suggest that **emodin** derivatives display multiple reactivities towards pathological factors of AD, including metal chelation, oxidative stress modulation, A $\beta$  aggregation control, and AChE inhibition, indicating their potential as promising MTDs for AD treatment.<sup>291,293</sup>

### 3.5 Chemical reagents capable of controlling A $\beta$ and MAOB

The loss of dopaminergic and serotonergic neurotransmission in AD can be triggered by the increased activity of MAOB.<sup>298,299</sup> Inhibitors of MAOB, such as **lazabemide**, **selegiline**, and **safinamide** (Fig. 4b), have been shown to improve cognitive deficits and slow the progression of AD, making them therapeutically relevant candidates for anti-Alzheimer therapies.<sup>300,301</sup> Given the limitations in efficacy and potential side effects of MAOB inhibitors, there has been an increasing focus on targeting





**Fig. 8** Examples of chemical reagents regulating metal-free or metal-bound A $\beta$  and AChE. (a) Structures of flavonoids. Isoflavone, 3-phenyl-4H-chromen-4-one; isoflavone-1, 3-(3,4-dihydroxyphenyl)-5,7-dihydroxy-4H-chromen-4-one; isoflavone-2, 5,6,7-trihydroxy-3-phenyl-4H-chromen-4-one; isoflavone-3, 3-(3,4-dihydroxyphenyl)-5,6,7-trihydroxy-4H-chromen-4-one; flavone, 2-phenyl-4H-chromen-4-one; flavone-1, 2-(3,4-dihydroxyphenyl)-3,5,7-trihydroxy-4H-chromen-4-one; flavone-2, 2-(3,4-dihydroxyphenyl)-5,7-dihydroxy-4H-chromen-4-one; flavone-3, 2-(4-hydroxyphenyl)-3,5,7-trihydroxy-4H-chromen-4-one. (b) Intermolecular interactions between the flavonoids and AChE. (c) Impact of isoflavone-3 on the formation of metal-free A $\beta$ <sub>42</sub> and metal-A $\beta$ <sub>42</sub> aggregates. The morphology of the resultant A $\beta$ <sub>42</sub> species was analyzed by TEM. (d) Structures of emodin and its derivatives with different functional groups at the R position. Emodin, 1,3,8-trihydroxy-6-methylanthracene-9,10-dione; emodin-1, 1,8-dihydroxy-3-methyl-6-(2-(piperidin-1-yl)ethoxy)anthracene-9,10-dione; emodin-2, 1,8-dihydroxy-3-methyl-6-(2-(pyrrolidin-1-yl)ethoxy)anthracene-9,10-dione; emodin-3, 1,8-dihydroxy-3-methyl-6-(2-(dimethylamino)ethoxy)anthracene-9,10-dione; emodin-4, 1,8-dihydroxy-3-methyl-6-(2-morpholinoethoxy)anthracene-9,10-dione. (e) Binding of emodin-1 against AChE. (f) Effect of emodin on A $\beta$ <sub>42</sub> aggregation. The inhibitory activity of emodin on the production of  $\beta$ -sheet-rich A $\beta$ <sub>42</sub> aggregates was quantitatively measured by the ThT assay. (g) Impact of emodin-1 and emodin-2 on Cu(II)-induced A $\beta$ <sub>42</sub> aggregation. The influence of emodin-1 and emodin-2 on the generation of  $\beta$ -sheet-rich Cu(II)-A $\beta$ <sub>42</sub> aggregates was analyzed by the ThT assay. Reproduced with permission from ref. 289 and 292. Copyright© 2023 Royal Society of Chemistry and 2020 Royal Society of Chemistry. (c) Impact of isoflavone-3 on the formation of metal-free A $\beta$ <sub>42</sub> and metal-A $\beta$ <sub>42</sub> aggregates. The morphology of the resultant A $\beta$ <sub>42</sub> species was analyzed by TEM. Reproduced with permission from ref. 289. Copyright© 2023 Royal Society of Chemistry. (d) Structures of emodin and its derivatives with different functional groups at the R position. Emodin, 1,3,8-trihydroxy-6-methylanthracene-9,10-dione; emodin-1, 1,8-dihydroxy-3-methyl-6-(2-(piperidin-1-yl)ethoxy)anthracene-9,10-dione; emodin-2, 1,8-dihydroxy-3-methyl-6-(2-(pyrrolidin-1-yl)ethoxy)anthracene-9,10-dione; emodin-3, 1,8-dihydroxy-3-methyl-6-(2-(dimethylamino)ethoxy)anthracene-9,10-dione; emodin-4, 1,8-dihydroxy-3-methyl-6-(2-morpholinoethoxy)anthracene-9,10-dione. (e) Binding of emodin-1 against AChE. Reproduced with permission from ref. 291. Copyright© 2024 Elsevier. (f) Effect of emodin on A $\beta$ <sub>42</sub> aggregation. The inhibitory activity of emodin on the production of  $\beta$ -sheet-rich A $\beta$ <sub>42</sub> aggregates was quantitatively measured by the ThT assay. Reproduced with permission from ref. 101. Copyright© 2021 John Wiley and Sons. (g) Impact of emodin-1 and emodin-2 on Cu(II)-induced A $\beta$ <sub>42</sub> aggregation. The influence of emodin-1 and emodin-2 on the generation of  $\beta$ -sheet-rich Cu(II)-A $\beta$ <sub>42</sub> aggregates was analyzed by the ThT assay. Reproduced with permission from ref. 293. Copyright© 2023 Springer Nature.

additional AD pathological features, such as A $\beta$  aggregation and elevated oxidative stress, simultaneously. Several developmental strategies have been employed to achieve this dual functionality, enabling interactions with the active site of MAOB while possessing structural moieties to address A $\beta$  aggregation. For example, modifications of existing therapeutics are emerging as viable candidates for this approach.

**Rasagiline** (Fig. 9a), a prominent MAOB inhibitor, is currently in phase II clinical trials for AD due to its dual ability

to control A $\beta$  aggregation through its 2,3-dihydro-1H-indene moieties.<sup>304,305</sup> Building on this framework, Xie and coworkers developed a selective and efficient MAOB inhibitor with the added capacity to target A $\beta$  aggregation by combining the structural advantages of another MAO inhibitor, **clorgyline**, resulting in **rasagiline-clorgyline** conjugates.<sup>302,306</sup> Among the synthesized conjugates, as described in Fig. 9a, **RC-6j**, which features a five-carbon linker, exhibited the highest modulatory potency and selectivity, with an IC<sub>50</sub> of 4.0 nM and a selectivity

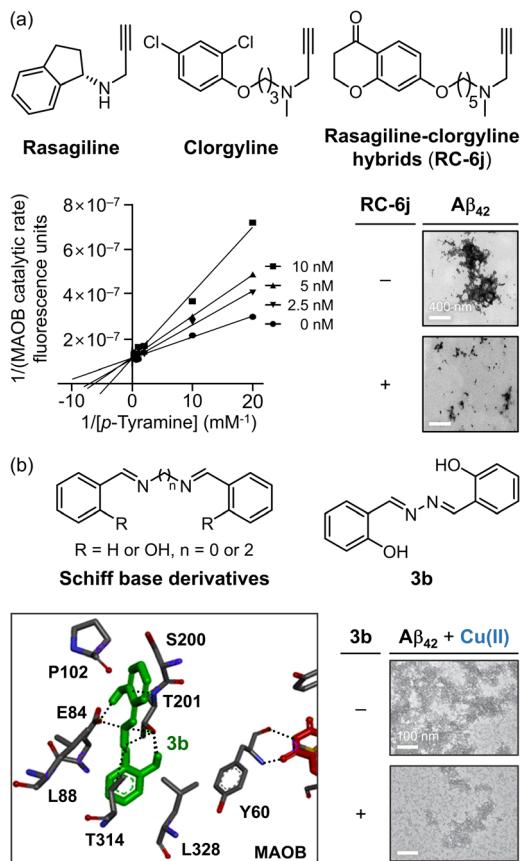


Fig. 9 Examples of chemical reagents controlling A<sub>β</sub> and MAOB. (a) Structures of **rasagiline**, **clorgyline**, and **RC-6j**. The inhibitory effect of **RC-6j** against the activity of MAOB was represented in Lineweaver–Burk reciprocal plots, with its impact on the formation A<sub>β</sub><sub>42</sub> aggregates. The catalytic rate of MAOB was measured through the amount of H<sub>2</sub>O<sub>2</sub> generated by catalytic oxidation of *p*-tyramine, observed by the Amplex Red H<sub>2</sub>O<sub>2</sub>/peroxidase assay. The morphology of the resultant A<sub>β</sub><sub>42</sub> species was monitored by TEM. **RC-6j**, 7-((5-(methyl-prop-2-yn-1-yl)amino)pentyl)oxy)chroman-4-one. Reproduced with permission from ref. 302. Copyright© 2020 Elsevier. (b) Structures of Schiff base derivatives and **3b**, binding mode of **3b** towards MAOB, and its effects on Cu(II)-added A<sub>β</sub><sub>42</sub> aggregation. The morphology of the Cu(II)-induced A<sub>β</sub><sub>42</sub> aggregates produced with and without **3b** was investigated by TEM. **3b**, 2,2'-(1*E*,1'*E*)-hydrazine-1,2-diylidene-bis(methanylidene)diphenol. Reproduced with permission from ref. 303. Copyright© 2020 Springer Nature.

index greater than 25 000.<sup>302</sup> **RC-6j** inhibited MAOB by competitive binding at the active site, interacting with the FAD cofactor and establishing a CH-π interaction with Y435 in the substrate cavity. Compared to **rasagiline**, the chromanone moiety of **RC-6j** occupied the entrance cavity, which is composed of amino acid residues W119, F168, L171, C172, I198, I199, I316, and Y326, through van der Waals and hydrophobic interactions, thereby enhancing the binding affinity.<sup>302</sup> In addition to MAOB inhibition, **RC-6j** also prevented A<sub>β</sub><sub>42</sub> aggregation, significantly reducing β-sheet-rich structures by 40% and decreasing overall aggregation (Fig. 9a).<sup>302,307</sup> Furthermore, **RC-6j** mitigated neuronal damage caused by oxidative stress induced by 6-hydroxydopamine, a ROS generator that disrupts mitochondrial function through auto-oxidation.<sup>302</sup> These

findings suggest that **RC-6j** is a promising MTDL for simultaneously controlling MAOB activity, A<sub>β</sub> aggregation, and oxidative stress in AD treatment.

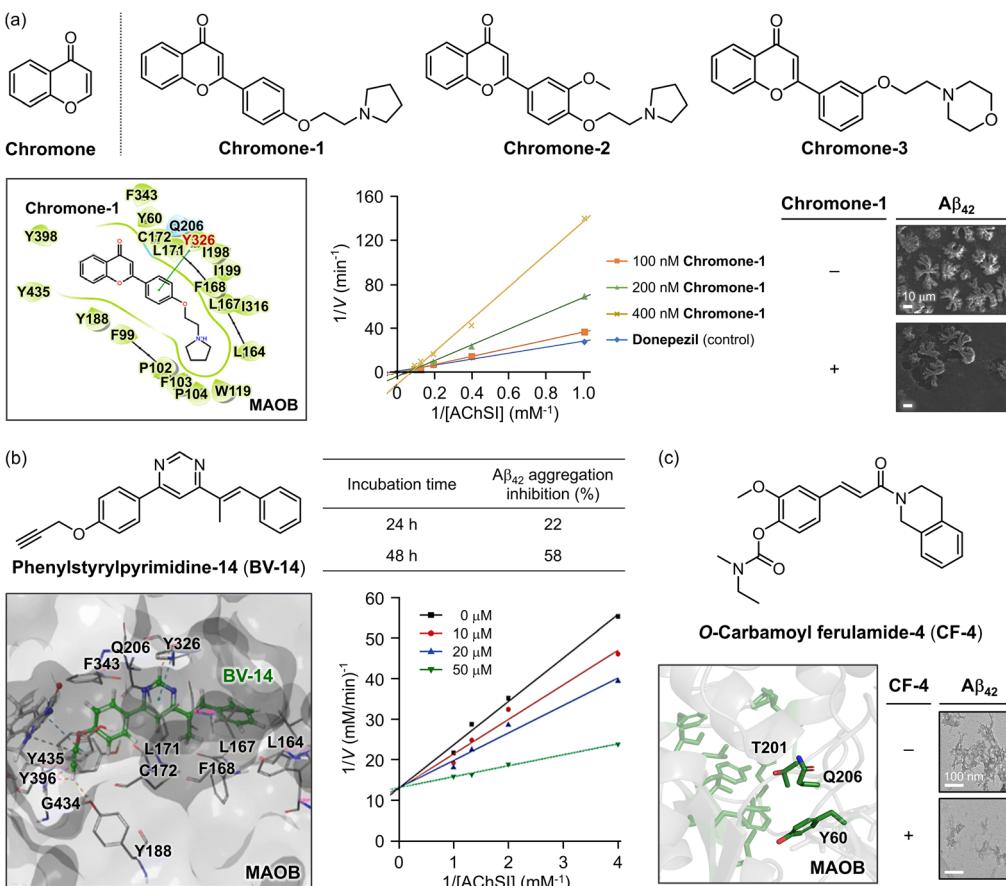
**Clioquinol** (Fig. 3a), which contains nitrogen and oxygen donor atoms for metal chelation, is also a widely utilized therapeutic framework to target metal ions and A<sub>β</sub> aggregation, as discussed in the previous section.<sup>26,27</sup> Sang and coworkers designed a combinatorial strategy to address A<sub>β</sub> aggregation, metal ion dyshomeostasis, and MAOB activity by incorporating metal-binding donor atoms like **clioquinol** and Schiff base moieties into a backbone, as depicted in Fig. 9b.<sup>303</sup> Specifically, **3b** with 2-hydroxy moiety obtained the lowest IC<sub>50</sub> value of 8.4 nM.<sup>303</sup> SAR analysis revealed that an increase in the length of the linker domain or elimination of the hydroxyl group from the aromatic ring dramatically decreased their inhibitory activity.<sup>303</sup> As illustrated in Fig. 9b, **3b** prohibited MAOB activity through binding at its active site; notably, the hydroxyl groups of **3b** generated hydrogen bonding with E84 and T201, which are essential for the catalytic activity of MAOB. In the absence of hydroxyl groups or additional methyl linkers, the compound no longer maintains its hydrogen bonding network, dramatically weakening binding affinity.<sup>303</sup> Additionally, **3b** altered A<sub>β</sub><sub>42</sub> aggregation, reducing fibril growth by 32% and 62% in the presence and absence of Cu(II), respectively. Furthermore, thinner and shorter fibrils were produced in the presence of **3b**, supporting the modulatory role of **3b** in A<sub>β</sub><sub>42</sub> aggregation.<sup>303</sup> These findings suggest that **3b** has potential as a multipotent therapeutic agent confronting MAOB, intracellular ROS, and metal-free and Cu(II)-bound A<sub>β</sub><sub>42</sub>.

### 3.6 Chemical reagents capable of controlling A<sub>β</sub>, AChE, and MAOB

While targeting A<sub>β</sub>, AChE, and MAOB individually has shown promise in addressing specific aspects of AD, the complexity of its pathology necessitates a more integrated approach.<sup>35,288,298,299</sup> The combinatorial interaction of A<sub>β</sub>, AChE, and MAOB has been shown to induce synaptic dysfunction through synergistic effects, resulting in impaired cholinergic, dopaminergic, and serotonergic neurotransmission.<sup>35,288,298,299</sup> Therefore, simultaneously tackling all three mechanisms could improve therapeutic outcomes by alleviating symptoms and slowing disease progression. As discussed earlier, compounds should incorporate structural moieties capable of interacting with the CAS and PAS of AChE, the active site of MAOB, and functional groups that effectively disrupt intermolecular hydrogen bonding and hydrophobic interactions of A<sub>β</sub>.

**Chromone** (Fig. 10a), a heterocyclic compound with a benzopyranone backbone, is a versatile scaffold in drug discovery for various diseases, including AD, due to its antioxidant activity, anti-amyloidogenic properties, and ability to modulate cholinergic and glutaminergic systems.<sup>308,310–313</sup> **Chromone** inhibits MAOB activity by forming intermolecular hydrogen bonds between C172 and Y326 through its sp<sup>2</sup> oxygen atom, a feature unique to MAOB.<sup>314</sup> Several SAR studies have further explored the **chromone** backbone to enhance its multi-targeting capabilities, as shown in Fig. 10a.<sup>308,312,313</sup> Kumar and coworkers





**Fig. 10** Examples of chemical reagents regulating A $\beta$ , AChE, and MAOB. (a) Structures of **chromone** and its derivatives, binding of **chromone-1** to MAOB, and its impact on AChE activity and A $\beta$ <sub>42</sub> aggregation. The inhibitory effect of **chromone-1** against the activity of AChE was performed using Ellman's method and represented in a double reciprocal Lineweaver–Burk plot. The morphology of A $\beta$ <sub>42</sub> aggregates generated with or without **chromone-1** was obtained by FE-SEM. Reproduced with permission from ref. 308. Copyright© 2024 American Chemical Society. **Chromone-1**, 2-(4-(2-pyrrolidin-1-yl)ethoxy)phenyl)-4H-chromen-4-one; **chromone-2**, 2-(3-methoxy-4-(2-(pyrrolidin-1-yl)ethoxy)phenyl)-4H-chromen-4-one; **chromone-3**, 2-(3-(2-morpholinoethoxy)phenyl)-4H-chromen-4-one. (b) Structure of **BV-14**, its binding mode towards MAOB, and effects on the A $\beta$ <sub>42</sub> aggregation and AChE activity. The extent of the inhibition of A $\beta$ <sub>42</sub> aggregation was assessed using the ThT assay. The AChE inhibition by **BV-14** was measured using Ellman's method and shown in an overlaid Lineweaver–Burk reciprocal plot. **BV-14**, (Z)-4-(1-phenylprop-1-en-2-yl)-6-(4-(prop-2-yn-1-yloxy)phenyl)pyrimidine. Reproduced with permission from ref. 309. Copyright© 2024 Royal Society of Chemistry. (c) Structure of **CF-4**, its binding to MAOB (PDB 1GOS<sup>313</sup>), and impact on the production of A $\beta$ <sub>42</sub> aggregates. The morphology of the resultant A $\beta$ <sub>42</sub> species was analyzed by TEM. **CF-4**, (E)-4-(3-(3,4-dihydroisoquinolin-2(1H)-yl)-3-oxoprop-1-en-1-yl)-2-methoxyphenyl ethyl(methyl)carbamate. Reproduced with permission from ref. 310. Copyright© 2020 Elsevier.

found that attaching pyrimidine derivatives to the **chromone** backbone introduces a tertiary nitrogen moiety as a pharmacophore, enabling additional cation– $\pi$  interaction with W84 in AChE.<sup>308</sup> **Chromone** derivatives (Fig. 10a) demonstrated strong inhibitory activity against MAOB and AChE, while **chromone-1**, featuring a pyrrolidine group at the C4 position, showed the highest potency ( $IC_{50} = 2.1 \mu\text{M}$  for MAOB and  $80 \text{ nM}$  for AChE).<sup>308</sup> In MAOB, the phenyl ring of **chromone-1** engages in  $\pi$ – $\pi$  stacking with Y236, while pyrrolidine and morpholine rings at C4 form electrostatic interactions with P102, F103, and W119, surrounding the FAD cofactor. These interactions are absent in **chromone-3**, which has a morpholine ring at the C3 position, emphasizing the importance of functional group positioning in designing multi-target-directed molecules.<sup>308</sup> All **chromone** derivatives exhibited similar interactions with AChE, including  $\pi$ – $\pi$  stacking with F331 and W279 at the PAS and cation– $\pi$  interactions with W84 at the CAS, underscoring the

critical role of the tertiary nitrogen group in AChE inhibition.<sup>308</sup> In addition, treatment with **chromone** compounds restricted the formation of A $\beta$  fibrils and plaques, as evidenced by the loss of aggregate structures in the images obtained by field emission scanning electron microscopy (FE-SEM) (Fig. 10a).<sup>308</sup> These findings emphasize the potential of the **chromone** scaffold in developing multi-target-directed molecules against MAOB, AChE, and A $\beta$ .

In a separate study, Kumar and coworkers designed phenylstyrylpyrimidine derivatives containing *O*-propargyl and amidine moieties, a combination of previously known pharmacophores, to evaluate their multi-target potential.<sup>309,315,316</sup> The study demonstrated the inhibition of MAOB activity, and a SAR analysis revealed that **BV-14** (Fig. 10b), featuring a formamidine group, outperformed other functional derivatives with guanidine, acetamidine, or benzamidine groups, attaining an  $IC_{50}$  of  $7.3 \mu\text{M}$ .<sup>309</sup> **BV-14** suppressed MAOB activity by binding



to the active site, with its pyrimidine ring and propargyl group engaging with the Y326 residue and the FAD cofactor of MAOB through hydrophobic interactions, including  $\pi$ - $\pi$  stacking, and additional hydrogen bonds (not shown in the figure).<sup>309</sup> In addition, **BV-14** showed the inhibitory effect on AChE, possessing  $IC_{50}$  of 7.3  $\mu$ M (Fig. 10b), which was achieved by the hydrogen bonding with F288, a residue from the PAS.<sup>309</sup> To assess the broader impact of **BV-14** on AD-related factors, researchers also evaluated its influence on prohibiting ROS generation and A $\beta$  aggregation. Although detailed insights were not provided, **BV-14** lowered ROS production by 48%, as depicted in Fig. 10b. Additionally, **BV-14** prevented A $\beta_{42}$  aggregation, reducing fibril growth by 22%. These findings suggest that **BV-14** has potential as a multipotent therapeutic agent targeting MAOB, intracellular ROS, and A $\beta_{42}$ .<sup>309</sup>

Natural products have also been explored for their potential to modulate A $\beta$  aggregation, along with AChE and MAOB activity. Ferulic acid, a widely distributed plant constituent first isolated from *Ferula foetida*, has been investigated as a therapeutic agent for AD in this manner.<sup>317,318</sup> Chen and coworkers designed *O*-carbamoyl ferulamide derivatives by combining ferulic acid with a carbamate fragment, a pharmacophore found in **rivastigmine** (Fig. 4a), to create compounds with multi-targeting potency against MAOB, AChE, and A $\beta$ .<sup>310,319</sup> Among these derivatives, **CF-4** (Fig. 10c) emerged as a promising and selective MAOB inhibitor with an  $IC_{50}$  of 5.3  $\mu$ M. **CF-4**'s carbamoyl group interacted with the FAD cofactor, forming intermolecular hydrogen bonds and  $\pi$ - $\pi$  interactions with the Y60 residue. The carbonyl group of the ferulic acid backbone generated hydrogen bonds with T201 and Q206, emphasizing the importance of the ferulic acid moiety in binding to the active site of MAOB.<sup>310</sup> Furthermore, **CF-4** not only interacted with the active site of MAOB but also exhibited inhibitory effects on AChE. The benzene ring of the 1,2,3,4-tetrahydroisoquinoline and the ferulic acid created  $\pi$ - $\pi$  interactions with key amino acid residues in AChE, enabling **CF-4** to simultaneously associate with both the CAS and the PAS, leading to effective suppression of AChE.<sup>310</sup> Additionally, **CF-4** prohibited A $\beta_{42}$  aggregation by up to 58%,<sup>310</sup> with a significant reduction in bulk A $\beta_{42}$  aggregates (Fig. 10c). Notably, **CF-4** also showed self-mediated disaggregation of A $\beta_{42}$  by 43%, demonstrating its dual capacity to alter and disaggregate A $\beta_{42}$  aggregates.<sup>310</sup> **CF-4**'s ability to cross the BBB permeability and its efficacy in improving cognitive defects induced by scopolamine further validated its potential for clinical use.<sup>310</sup> These findings on **CF-4**, with those of **chromone** derivatives and **BV-14**, underscore the importance of targeting AChE, MAOB, and A $\beta$  in AD therapy. This comprehensive approach, which combines existing therapeutics to address the complex pathology of AD, offers a more efficient strategy for disease treatment.

## 4. Conclusions

With the rising prevalence of AD, there is an increasing need for effective therapeutic strategies. The limitations of current single-target-directed treatments highlight the necessity for more comprehensive approaches that address the

multifactorial nature of AD. This *review* illustrates several pathological features in AD, particularly A $\beta$  aggregation, metal ion dysregulation, and enzymes' dysfunction. While A $\beta$  has long been considered a primary therapeutic target due to its role in disrupting cellular functions, inducing inflammation, and impairing synaptic activity, treatments focused solely on A $\beta$  have often demonstrated limited success. Although recent drug approvals aiming at A $\beta$  underscore its continued relevance as a therapeutic biomarker, it is essential to concurrently engage additional neurotoxic factors contributing to AD progression to effectively combat the disease. The complex interplay of metal ion dyshomeostasis, metalloenzyme dysregulation, impaired neurotransmission, and elevated oxidative stress further complicates the pathology of AD and constrains the success of isolated interventions.

As such, the development of multi-target-directed molecules capable of modulating multiple pathogenic pathways offers a promising strategy to enhance therapeutic efficacy. Future research should prioritize the refinement and optimization of MTDLs to address the interconnected mechanisms associated with AD. A more systematic therapeutic approach that simultaneously tackles A $\beta$  aggregation, metal ion dyshomeostasis, enzyme dysregulation, and neurotransmitter imbalance holds the potential to overcome the limitations of current single-target-directed treatments. Alongside the multi-target-directed molecules primarily focusing on A $\beta$ , extensive research has also been conducted on therapeutic combinations not covered in this *review*, which underscores their significance and potential in advancing AD treatment strategies. Furthermore, beyond the pathogenic targets discussed in this *review*, the development of MTDLs aimed at neuroinflammation, mitochondrial dysfunction, and neuronal cell death could provide new insights into their interconnected roles in AD pathogenesis and therapeutic intervention. A deeper understanding of these processes and their interplay with previously discussed factors could guide the design of MTDLs capable of effectively confronting the complex, multifaceted nature of AD. Expanding the scope of therapeutic targets, as discussed in this *review*, can serve as a foundation for future research, driving the development of disease-modifying treatments that efficiently address the complexity of AD.

## Data availability

This manuscript is a review article; therefore, no new data were generated or analyzed in this study, and a data availability statement is not applicable.

## Author contributions

All authors contributed to the conceptualization of this *review*. Jeasang Yoo, Jimin Lee, Byeongha Ahn prepared the draft, which was revised by Professors Mi Hee Lim and Jiyeon Han.

## Conflicts of interest

There are no conflicts to declare.



## Acknowledgements

This work was supported by grants of the National Research Foundation of Korea funded by the Korean Government {Creative Research Initiative [RS-2022-NR070709 (M. H. L.)]; Global Science Research Center Program [RS-2024-00411134 (M. H. L.)]; RS-2024-00353015 (J. H.)].

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