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Review article

Small molecule therapeutics for tauopathy in Alzheimer's disease: Walking on the path of most resistance

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ABSTRACT

Alzheimer's disease (AD) is the most common form of dementia characterized by presence of extracellular amyloid plaques and intracellular neurofibrillary tangles composed of tau protein. Currently there are close to 50 million people living with dementia and this figure is expected to increase to 75 million by 2030 putting a huge burden on the economy due to the health care cost. Considering the effects on quality of life of patients and the increasing burden on the economy, there is an enormous need of new disease modifying therapies to tackle this disease. The current therapies are dominated by only symptomatic treatments including cholinesterase inhibitors and *N*-methyl-D-aspartate receptor blockers but no disease modifying treatments exist so far. After several failed attempts to develop drugs against amyloidopathy, tau targeting approaches have been in the main focus of drug development against AD. After an overview of the tauopathy in AD, this review summarizes recent findings on the development of small molecules as therapeutics targeting tau modification, aggregation, and degradation, and tau-oriented multi-target directed ligands. Overall, this work aims to provide a comprehensive and critical overview of small molecules which are being explored as a lead candidate for discovering drugs against tauopathy in AD.

Contents

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1. Introduction

Alzheimer's disease (AD) is the most common form of dementia characterized by presence of extracellular amyloid plaques (amyloid beta; Aβ) and intracellular neurofibrillary tangles (NFTs) composed of tau protein. The amyloid hypothesis being the earliest one has been explored thoroughly but several of the clinical trials targeting Aβ with small molecules and antibodies failed. Tauopathy is the abnormal and pathological intracellular accumulation of tau protein in the form of NFTs. After several failures of the amyloid targeted drugs, the academic and pharmaceutical industry researchers have increased their efforts to explore treating tauopathy for the AD therapy. Several studies suggest that neuroinflammation also contributes to the pathology of AD [1]. But again, the clinical trials with the non-steroidal anti-inflammatory drugs failed to show any evidence that treatment of inflammation as a therapeutic approach to treat AD [2].

In this review, we have begun with an overview of the tauopathy in AD. Then we summarized the development of small molecules as therapeutics targeting tau modification, aggregation, and degradation. Later, we introduced recent research examples of tau-oriented multi-target directed ligands (MTDLs). In the end, we have tried to provide a future perspective of small molecule therapeutics against tauopathy in AD.

2. Tau and tauopathy in AD

2.1. Tau protein

In humans, tau is encoded by a single gene on chromosome 17q21 containing 16 exons [3]. Alternative splicing of exons 2, 3 and 10 allows for six tau isoforms with 352−441 amino acids, differing from each other by the presence of zero, one or two inserts (0 N, 1 N and 2 N) in its amino-terminal part and three or four microtubule-binding domains (3R or 4R) in its carboxy-terminal part (Fig. 1) [4,5]. Tau is subject to a host of post-translational modifications, including phosphorylation, acetylation, ubiquitination, SUMOylation, glycosylation, nitration, methylation, prolyl-isomerization, glycation and truncation [6]. Tau is abundant in axons, where it binds to microtubules, although it is also found in the neuron's soma and dendrites at lower levels [7,8]. Under normal conditions, tau is mainly involved in microtubule assembly and stabilization and contributes to the regulation of intracellular trafficking [9].

2.2. Pathological processes in AD

2.2.1. Post-translational modification

The intra-neuronal accumulation of paired helical filaments (PHFs) in the form of NFTs is a hallmark of the brain pathology in AD. Abnormal tau is the major component of NFTs [10,11]. Even before the formation of tangles, tau undergoes a series of abnormal post-translational modifications in AD. The most important modification is hyperphosphorylation. In AD brain, tau can contain seven to eight moles of phosphate which is 3-4-fold more phosphorylated than that in healthy brain [12]. Phosphorylation reduces the association of tau with microtubules and affects the ability of tau to involve in microtubule assembly and stabilization [13,14]. This disassembly promotes relocation of tau to somatodendritic compartment where it disrupts synaptic function by inhibiting glutamate receptor trafficking or synaptic anchoring [15]. In addition, tau acetylation is elevated in patients at early and moderate Braak stages of tauopathy [16]. Tau acetylation prevents degradation of phosphorylated tau, impairs tau-microtubule interactions and promotes pathological tau aggregation [16,17]. Another modification such as proteolytic cleavage at the C-terminus of tau (truncation) has been linked to the pathogenesis of AD as well [18,19]. Abnormal proteolytic events, including caspase activation during apoptosis [20], generate truncated tau which act as "seeds" similar to filaments isolated from AD brain [21], enhance tau polymerization kinetics [22] and lead to synaptic deficits [23]. In AD brains, but not in healthy brains, tau was reported to be *N*-glycosylated which appears to be responsible for the maintenance of the PHFs' structure [24]. Conversely, AD patient cortical brain tissues reportedly have significantly reduced levels of *O*-GlcNAcylation of tau [25]. This downregulation leads to increased tau phosphorylation and onset of AD pathology [26] and inhibits the toxic tau self-assembly [27]. Other abnormal post-translational modifications of tau in AD were also identified, including ubiquitination [28−30], SUMOylation [31,32], nitration [33], glycation [34], methylation [35], and prolyl-isomerization [36,37]. These events also contribute to pathological tau aggregation and accumulation in AD.

2.2.2. Aggregation

Abnormal post-translational modifications and increasing cytoplasmic tau levels caused by loss of affinity to microtubules enhances tau to aggregate. In AD, tau aggregates are a mixture of three- and four-repeat tau isoforms (3R and 4R) [38]. Their assembly states contain PHFs [39], straight filaments [40] and oligomers [41]. NFTs are composed of PHFs which are twisted, fibrous, β-sheet-containing assemblies of tau [42]. NFTs first appear in the

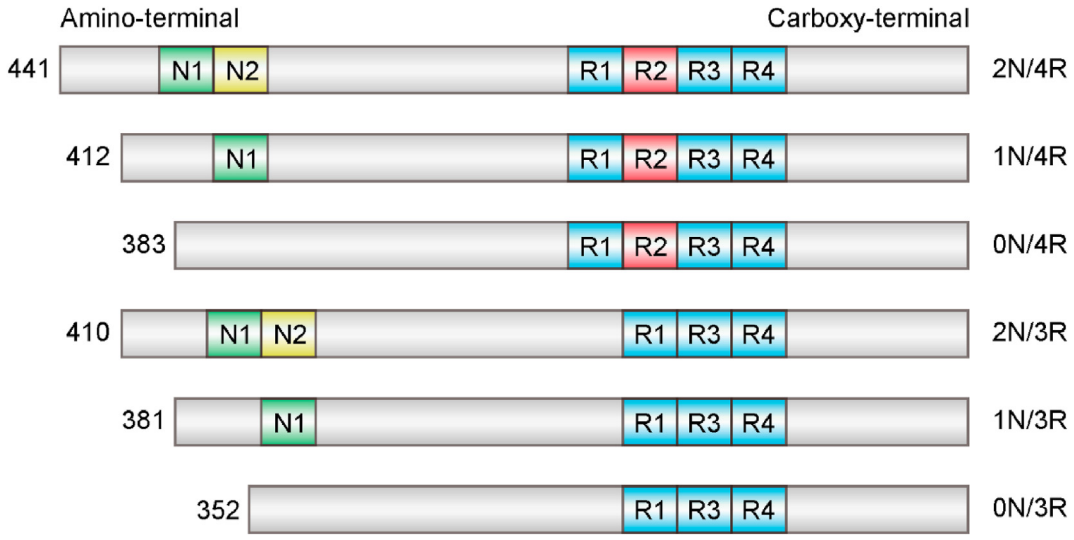

Fig. 1. Tau isoforms in the human brain.

entorhinal cortex and hippocampus before spreading to limbic system and association cortices as the disease progresses [43,44]. The number of NFTs has been positively related with the severity of dementia in AD [45–47]. Neurons containing NFTs have fewer expression of synaptic messages compared with neighbor NFTs-free neurons [48]. In addition, PHF-tau isolated from AD brains interacts with the 20S-subunit of the proteasome and inhibits its activity suggesting that PHF-tau is able to directly induce neuronal damage in AD [49]. Oligomeric tau is also an important element of the neurotoxic event [50]. The levels of tau oligomers are increased in Braak I stage, a stage which NFTs are believed to be absent, suggesting that tau oligomers may be present before NFTs form [51]. Tau oligomers cause degeneration of human neurons [52], impair cognition and induce synaptic and mitochondrial dysfunction in mice [53,54]. Furthermore, pathological aggregation of tau causes indirect detrimental effects with reduced levels of native soluble tau decreasing its physiological function [55].

3. Small molecules targeting tauopathy

The development of tau pathology is a complex multifactorial process, presenting multiple points where therapeutic intervention is possible. Several reports have suggested that most of these pathological tau events in AD can be targeted with small molecules, such as alteration of tau post-translational modification (hyperphosphorylation, acetylation, truncation, glycosylation etc.), prevention of tau aggregation and improvement of tau degradation (Fig. 2) [56,57].

3.1. Agents modulating tau post-translational modifications

3.1.1. Modulation of tau phosphorylation

3.1.1.1. Phosphatase activators. In contrast with inhibiting tau kinases, activation of protein phosphatases may prove to be a more practical approach for developing a single therapeutic agent against multiple distinct protein kinases implicated in tau hyperphosphorylation [58]. Phosphoprotein phosphatase 2A (PP2A), the key tau phosphatase accounting for over 70% of tau dephosphorylation [59]. In AD brain the activities of PP2A and protein phosphatase 5 (PP5) toward tau are significantly decreased. PP2A acts as a trimer composed of a catalytic subunit (PP2A Cα or Cβ) and a scaffolding or structural subunit (PP2A A, PR65α, or PR65β) that together form the core enzyme, and one of several regulatory subunits (PP2A B, e.g., B55α) [60]. The binding of selective 'B' subunits and other regulators contributes to PP2A substrate specificity and subcellular localization, aimed at preventing unwanted its promiscuous phosphatase activity [61]. Increasing the activity of an enzyme is typically a challenging task; however, the regulatory interactions of PP2A do provide opportunities to increase its activity. Generally, three potential strategies could be highlighted: 1. Inhibition of an inhibitory interaction. 2. Modulation of post-translation modifications of PP2A. 3. Allosteric activation.

Sodium selenate (Na_2SeO_4) is negatively charged anionic compound that activates PP2A *in vitro* and *in vivo*, reverses memory deficits and reduces tau phosphorylation in animal models of AD [62]. PP2A activation by sodium selenate appears to be selective towards phosphorylated tau (p-tau) *in vivo*. In a Phase IIa randomized control trial (Australian and New Zealand Clinical Trials Registry, ID: ACTRN12611001200976), sodium selenate treatment at doses up to 30 mg per day for 24 weeks was safe and well-tolerated in patients with mild to moderate AD, and showed some benefits on diffusion magnetic resonance imaging [63]. Later, the selenium concentrations in serum and cerebrospinal fluid (CSF) taken from patients participating in this Phase IIa trial were measured and showed that sodium selenate supplementation at a high or supranutritional dose induced an increase in selenium uptake into the central nervous system (CNS) [64]. Memantine (**1**, Fig. 3), an antagonist of *N*-methyl-D-aspartate receptors in the brain, received European marketing approval in 2002 and U.S. Food and Drug Administration (FDA) approval in 2003 for AD treatment [65]. In addition, memantine reduces tau hyperphosphorylation *in vitro* as a cationic PP2A activator by blocking I2/SET inhibition of PP2A. In the absence of I2/SET, memantine does not activate PP2A, but in the presence of I2/SET, memantine restored phosphatase activity towards p-tau [66]. The apolipoprotein E mimetic, COG112 (acetyl-RQIKIWFQNRRMKWKKCLRVRLASHLRKLRKRLL-amide), also increases PP2A activity by inhibiting its interaction with SET [67]. In animal models of AD, ApoE-mimetic peptides reduce tau phosphorylation, behavioral deficits, plaques, and tangles [68]. Similar to COG112, Fingolimod (FTY720; **2**, Fig. 3), a sphingosine-1-phosphate receptor agonist, disrupts SET-PP2A interactions, resulting in increased cellular PP2A activity [69]. Fingolimod is an immunomodulatory drug, mostly used for treating multiple sclerosis. In AD animal models it exhibited anti-inflammatory and

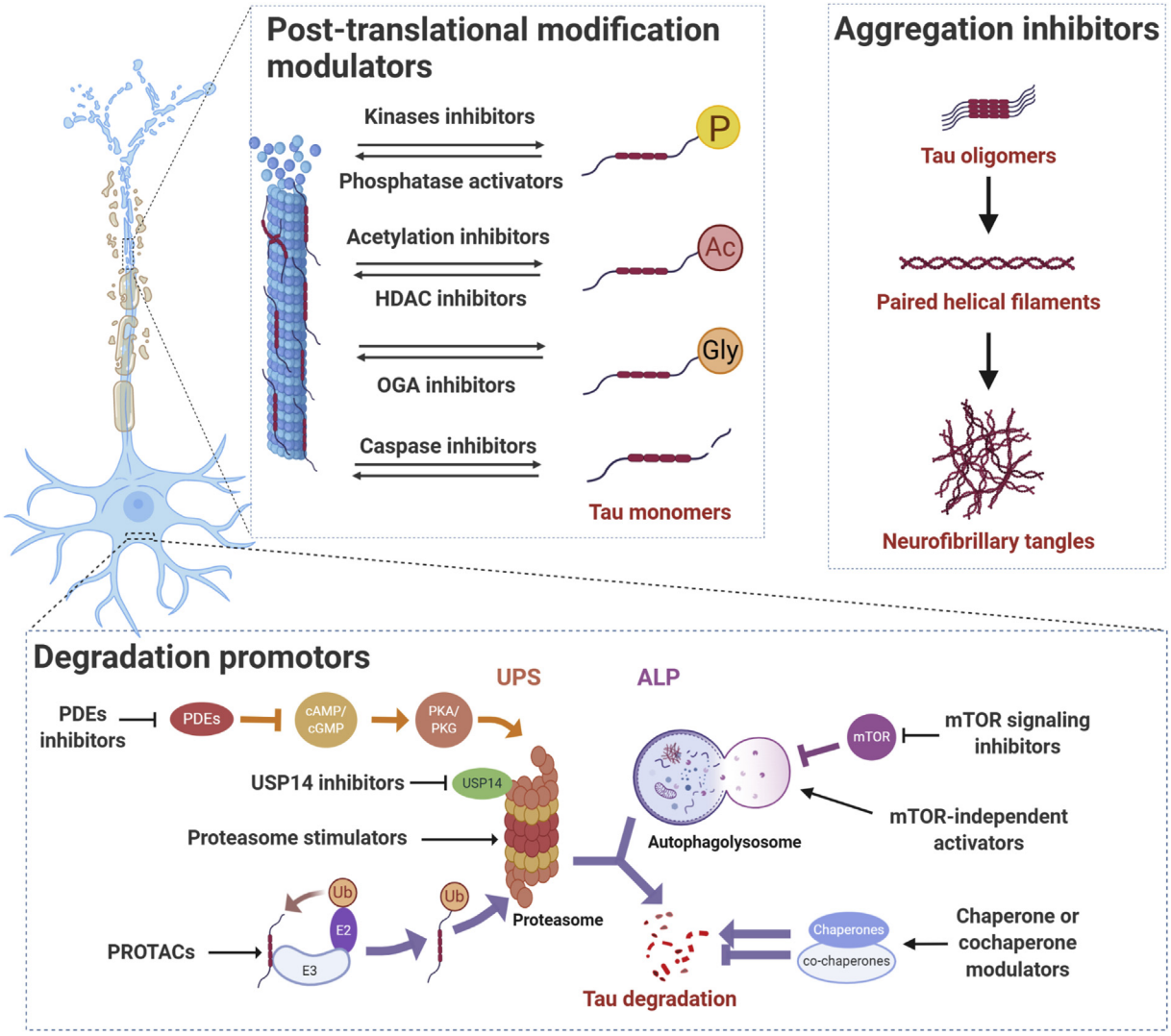

Fig. 2. Potential therapeutic targets of anti-tau molecules in Alzheimer's disease. Small molecules targeting tau in AD preclinical or clinical development include tau post-translational modification modulators (kinases inhibitors, phosphatase activators, acetylation inhibitors, HDAC inhibitors, OGA inhibitors and caspase inhibitors), aggregation inhibitors and degradation promotors (PDEs inhibitors, USP14 inhibitors, proteasome stimulators, PROTACs, mTOR signaling inhibitors, mTOR-independent ALP activators and chaperone or cochaperone modulators). AD, Alzheimer's disease; P, phosphate; Ac, acetyl group, Gly, glycosyl group; HDAC, histone deacetylase; OGA, *O*-GlcNAcase; UPS, ubiquitin-proteasome system; ALP, autophagy-lysosome pathway; PDEs, phosphodiesterases; cAMP, cyclic adenosine monophosphate; cGMP, cyclic guanosine monophosphate; PKA, cAMP-protein kinase A; PKG, cGMP-protein kinase G; USP14, ubiquitin specific peptidase 14; PROTACs, proteolysis targeting chimeric molecules; Ub, ubiquitin; E2, E2 ubiquitin-conjugating enzyme; E3, E3 ubiquitin ligase; mTOR, mammalian target of rapamycin. Created with BioRender.com.

neuroprotective effect associated with improved spatial learning and memory [70]. SEW2871 (**3**, Fig. 3), a more selective sphingosine-1-phosphate receptor agonist than FTY720, can reduce tau-Ser262 phosphorylation in rat hippocampal slices [71]. Another PP2A inhibitory protein, Cancerous Inhibitor of PP2A (CIP2A) plays an important role in disease-related phosphorylation of tau/APP and tau pathology/Aβ overproduction through inhibiting PP2A in AD brain [72]. In cell models of AD, genistein (**4**, Fig. 3) ameliorated tau hyperphosphorylation through repressing the inhibitory effect of CIP2A on PP2A [73]. Several molecules increase PP2A activity and decrease tau phosphorylation via inhibition of pathways leading to proteasomal degradation of PP2A. Metformin, a drug used to treat diabetes, can decrease tau phosphorylation in a PP2A-dependent manner, by disrupting the interaction of PP2A C subunit with specific regulatory subunits, which normally promotes PP2A degradation [74]. Resveratrol (**5**, Fig. 3) induces dephosphorylation of tau by interfering with the MID1-PP2A complex [75]. The increase in PP2A activity is caused by decreased expression of the MID1 ubiquitin ligase that mediates ubiquitin-specific modification and degradation of the catalytic subunit of PP2A [75].

The activity of PP2A is regulated by several post-translational modifications, including phosphorylation and methylation [76]. Cornel iridoid glycoside (Fig. 3) which mainly comprises of morroniside (**6**) and loganin (**7**) elevated PP2A activity via inhibiting PP2Ac demethylation resulting in inhibition of tau hyperphosphorylation [77]. Allosteric activators of PP2A include several distinct classes of molecules with ceramide analogues such as sphingosine, sphingosine phosphate, ceramide phosphate, 1-*O*-methyl-C6-ceramide, compounds being best studied [78]. Other PPA2 activators such as dithiolethione, xylulose-5-phosphate, eicosanoyl-5-hydroxytryptamide, taurolidine, 1,8-naphthyridines have been studied in cell and *in vivo* models, however their ability to reduce tau hyperphosphorylation was not documented [79–83].

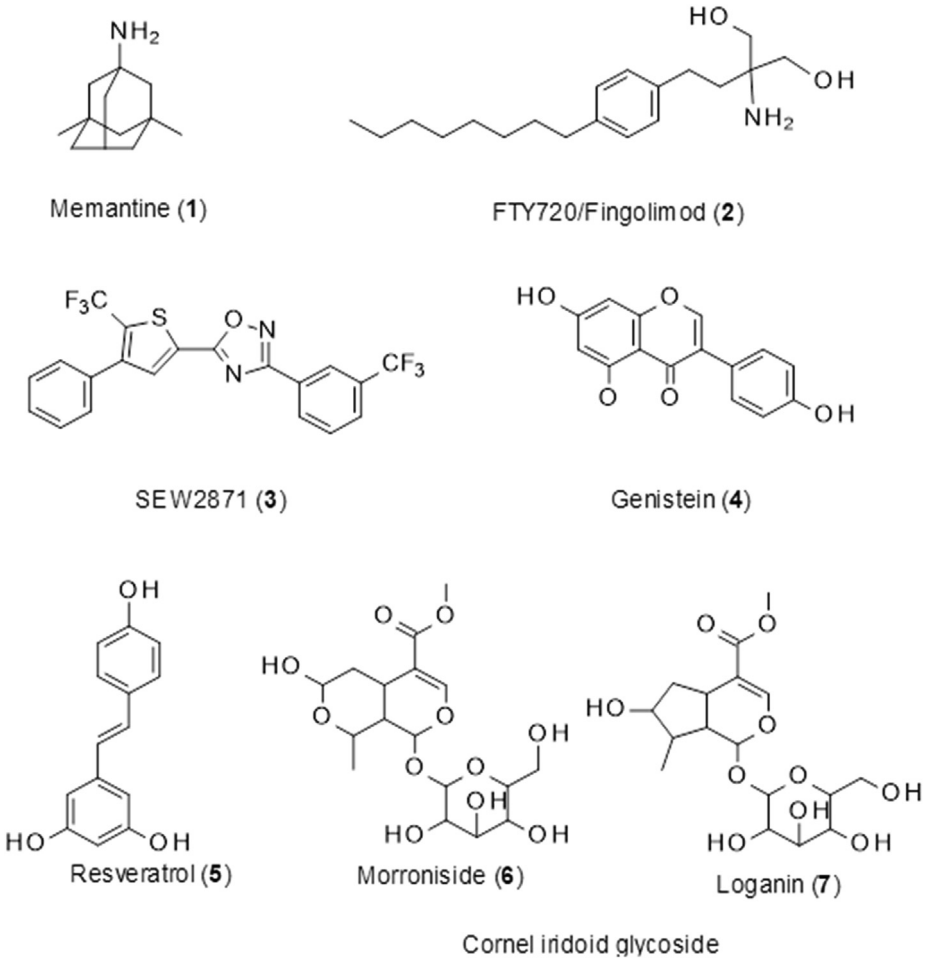

Fig. 3. Phosphatase activators.

3.1.1.2. Kinases inhibitors. Protein kinases are a ubiquitous group of enzymes catalyzing the transfer of phosphoryl group from a phosphate donor (usually ATP) to a receptor substrate, which are serine, threonine, tyrosine or histidine residues of proteins [84,85]. Several different kinases which are involved in tau phosphorylation can be largely divided into three classes: proline-directed kinase, non-proline-directed kinase and tyrosine protein kinases. These kinases including glycogen synthase kinases 3 alpha and beta (GSK3α, GSK3β), cyclin dependent kinase 5 (CDK5), mitogen-activated protein kinase family (MAPKs), leucine-rich repeat kinase 2 (LRRK2), Akt (protein kinase B), c-Abelson (c-Abl), dual-specificity tyrosine phosphorylation-regulated kinases (DYRK1A) and Fyn lead to phosphorylation of tau which loses its ability to bind and stabilize the microtubules leading to formation of dysfunctional tau [86]. There are several nicely written reviews summarizing the developments of protein kinase inhibitors as potential AD therapeutics [87–92]. Herein, we cover, in brief details, the kinases inhibitors which are currently undergoing clinical trials.

Up till now, two of the GSK3β inhibitors, tideglusib (**8**, Fig. 4) (NP031112) and lithium, and one of the Fyn inhibitor, saracatinib (**9**, Fig. 4) (AZD0530), have been evaluated in clinical studies. Tideglusib is an orally available, small-molecule drug of the thiadiazolidinone class. In a Phase IIa trial (NCT00948259) in 30 mild to moderate AD patients already on cholinesterase inhibitor treatment for several months, tideglusib (400–1000 mg/day) was generally well tolerated except for a transient increase in serum transaminase levels and produced positive trends in Mini-Mental Status Examination, Alzheimer's Disease Assessment Scale-cognitive subscale, Geriatric Depression Scale, and Global Clinical Assessment without statistical significance in this small sample [93] However, in a subsequent Phase IIb trial (NCT01350362) in 306 mild to moderate AD patients on cholinesterase inhibitor and/or memantine treatment for several months, tideglusib produced no clinical benefit [94]. Lithium is primarily used for treatment of bipolar disorder. Multiple cohort and case-control studies suggest an association between lithium treatment and dementia risk reduction or reduced dementia severity [95–103]. A meta-analysis of three randomized controlled Phase II studies indicates that lithium treatment may have beneficial effects on cognitive performance in patients with mild cognitive impairment (MCI) and AD [104–107]. Recently, a new randomized clinical trial in older adults with amnestic MCI suggested that long-term lithium treatment attenuated cognitive and functional decline in amnestic MCI, and modified AD-related CSF biomarkers [108]. Another randomized controlled trial with low-dose lithium treatment plans to assess the agitation in AD patients at the first time [109]. If lithium demonstrates efficacy in this trial, a Phase III study will be conducted to warrant the lithium treatment for AD. The Fyn inhibitor, saracatinib (AZD0530) was reported to be safe and well tolerated in a Phase Ib

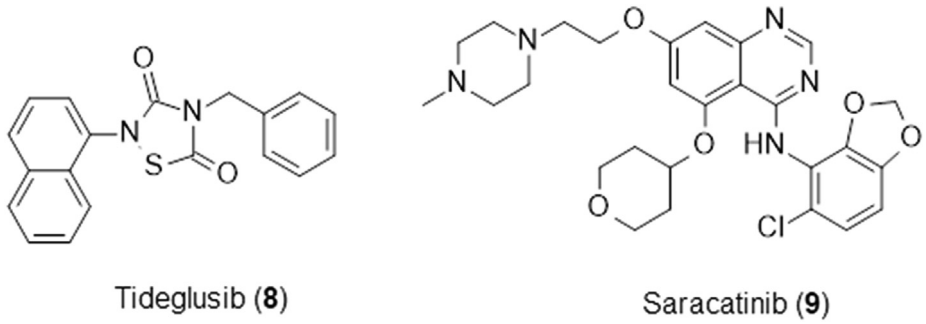

Fig. 4. Kinases inhibitors.

clinical trial (NCT01864655) in patients with mild-to-moderate AD, and could achieve substantial CNS penetration with oral dosing at 100–125 mg [110]. Later, in a multicenter Phase IIa randomized clinical trial (NCT02167256) in 159 mild AD patients, saracatinib treatment did not slow the decline in cerebral metabolic rate for glucose and had no effect on total brain or ventricular volume but did show trends for slowing the reduction in hippocampal volume and entorhinal thickness [111].

3.1.2. Modulation of tau acetylation

3.1.2.1. Acetylation inhibitors. Tau acetylation at lysine 174 (ac-K174) is an early change in AD brains and a critical determinant in tau homeostasis and toxicity in mice [112]. Salsalate (**10**, Fig. 5), a prodrug of salicylate (**11**), is one of the small-molecule non-steroid ant-inflammatory drugs and inhibits acetyltransferase p300-induced tau acetylation [112]. In HEK293 cells, salicylate reduced acetylation of H2AK5, a well-established substrate of p300, in a dose-dependent manner (1.25–20 mM). In rat primary cortical neurons infected with a lentiviral vector encoding wild-type human tau, salicylate (10 mM, 24 h) reduced levels of p300, ac-K174 and AT8-positive p-tau relative to total tau. Consistent with the notion that ac-K174 inhibits tau degradation, reduction of ac-K174 with salicylate (5 mM) enhanced tau turnover in primary neurons infected with a lentiviral vector encoding wild-type human tau. In the PS19 transgenic mouse model of frontotemporal dementia (FTD), salsalate administration (225 mg/kg, oral gavage once daily for 2–3 months) after disease onset inhibited brain p300 activity, reduced total tau levels, and induced a reduction in ac-K174 relative to total tau levels, and a trend of reduction in AT8-positive p-tau. Salsalate treatment also prevented hippocampal atrophy and rescued tau-induced spatial learning and memory deficits. Pharmacokinetic analysis showed that salsalate (600 mg/kg, gavage injection) penetrated into the brain of wild-type C57B6 mice and gave rise to relatively stable salicylate levels over 8 h. The tau-lowering and neuroprotective effects of salsalate/salicylate were diminished in neurons and mice expressing K174 mutant tau, suggesting that ac-K174 inhibition is a mediator of salicylate/salsalate's tau-lowering and protective effects [112]. For clinical trials, a Phase Ib, 12-month study (NCT03277573) of salsalate in patients with mild to moderate AD is expected to be completed by July 21, 2021 [113]. The purpose of this study is to test the safety, tolerability, pharmacokinetics, pharmacodynamics, and preliminary efficacy of salsalate in patients with mild to moderate AD. Approximately 40 subjects will be randomized 1:1 to placebo or active (two salsalate tablets twice daily, 3000 mg total daily).

C646 (**12**, Fig. 5) is a pyrazolone-containing small-molecule inhibitor of p300 with a Ki of 400 nM [114]. In primary cortical neurons, C646 (20 μM) eliminated ac-tau and AT8-positive p-tau within 2 h and decreased total tau levels within 8 h.

The glyceraldehyde-3-phosphate dehydrogenase nitrosylation inhibitor CGP3466B (omigapil) (**13**, Fig. 5) abrogated $A\beta_{1-42}$-induced tau acetylation, spatial memory impairment, and locomotor dysfunction in mice at the dose of 2.5 mg/kg through intraperitoneal (i.p.) injection [115]. Recently, omigapil has been proved to be safe and well-tolerated in children and adolescents with congenital muscular dystrophy in a Phase I clinical trial (NCT01805024) [116].

The nicotinamide adenosine dinucleotide-dependent class-III protein deacetylase SirT1 is one of the major enzymes involved in deacetylation of tau [117]. A03 (**14**), a known selective serotonin reuptake inhibitor called alaproclate (**14**, Fig. 5), induced dose-response increases of neuroprotective SirT1 in N2a cells stably expressing full-length ApoE4 with an EC_{50} of 2 μM [118]. Moreover, A03 increased SirT1 levels in ApoE4-transfected human A172 glioblastoma cells. In 5XFAD-ApoE4 AD model mice, subcutaneous administration of A03 (10 mg/kg/day) for 56 days increased SirT1 levels in the hippocampus and elicited cognitive improvement while inducing no observed toxicity. In addition, pharmacokinetic study shows that A03 is orally bioavailable and brain penetrant. The SirT1 enhancing effects of A03 may decrease acetylated tau levels and abrogate tau pathology, which needs direct evidence in the future studies.

3.1.2.2. Histone deacetylase (HDAC) inhibitors. Acetylation of tau on KXGS motifs inhibits phosphorylation on this same motif, and also prevents tau aggregation [119]. In AD patients and in a mouse model of tauopathy (rTg4510), KXGS motifs are hypoacetylated and hyperphosphorylated [119]. Histone deacetylase (HDAC) 6 is the enzyme responsible for the deacetylation of tau's KXGS motifs. ACY-738 (**15**, Fig. 6) selectively inhibits HDAC6 with an IC_{50} of 2 nM and efficiently crosses the blood-brain barrier (BBB). In FVB non-transgenic mice, subcutaneous injection of ACY-738 for 3 days (0.5 mg/kg) decreased p-tau species, as well as PHF in the brain and increased acetylation of tau on KXGS motifs [119]. In primary neurons transduced with human P301L tau-AAV, ACY-738 treatment (1 μM, 24 h) inhibited HDAC6 activity and led to a significant reduction in tau levels and a striking decrease in phosphorylation at Ser-324 [120].

Another highly BBB-penetrating HDAC6 inhibitor, CKD-504, selectively inhibits HDAC6 with an IC_{50} of 72.2 nM. In brain organoids formed from AD patient-derived induced pluripotent stem cells (iPSCs), CKD-504 (5 μM, 4 h) decreased the amounts of total and AT8-positive p-tau [121]. In AD-like pathology$^{APP\ \&\ Tau}$ mice produced by a cross between 5xFAD and JNPL3 mice, CKD-504 treatment (1 or 2.5 mg/kg, i.p. twice a day for 4 months beginning at 4.5 months of age) reduced the amount of total tau and p-tau, rescued synaptic pathologies and cognitive impairment [121].

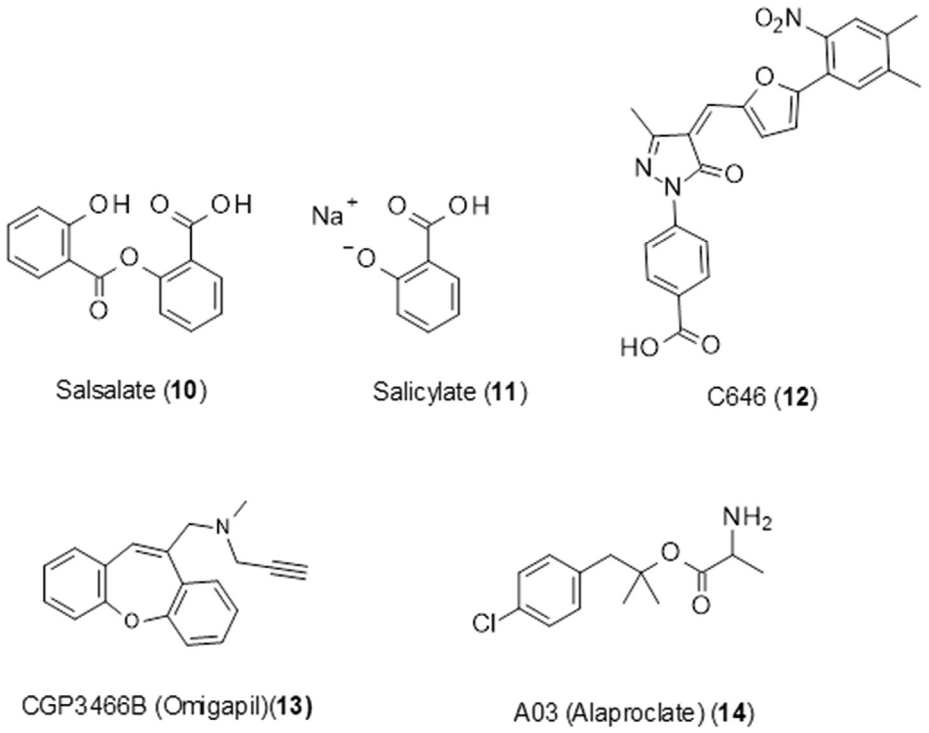

Fig. 5. Acetylation inhibitors.

Furthermore, HDAC6 inhibition by CKD-504 increased the interactions of tau with chaperones and E3 ligases in AD brain organoids and AD-like pathology$^{APP\&Tau}$ mice, resulting in accelerated proteasomal degradation of tau [121]. Now, CKD-504 is in Phase I trials for Huntington's disease (NCT03713892) with aims to assess the safety, tolerability, pharmacokinetics and pharmacodynamics of single and multiple ascending oral doses of CDK-504 compared to placebo in healthy Korean and Caucasian adult subjects [122].

In addition, Zeb et al., developed a validated pharmacophore, generated from the structure of human HDAC6 in complex with trichostatin A, and integrated virtual screening, molecular docking and molecular dynamics simulation to identify novel inhibitors of HDAC6 [123]. Finally, three novel hit inhibitors (Fig. 6) of HDAC6 were recommended, namely glycodeoxycholic acid (**16**), PubChem ID: 38028580 (**17**), and PubChem ID: 16399643 (**18**). Further studies need to explore their effects on tau acetylation and tau-pathogenesis.

RGFP-966 (**19**, Fig. 6) is a brain-penetrant and selective HDAC3 inhibitor. In HEK-293 cells overexpressing APP with Swedish mutation (HEK/APP_{SW}), RGFP-966 (10 μM, 48 h) increased histone H3 and H4 acetylation and decreased p-tau. In the 3xTg AD mouse model, repeated administration of RGFP-966 (3 and 10 mg/kg) reversed pathological tau phosphorylation in the brain and improved spatial learning and memory. Furthermore, in primary neurons derived from iPSCs obtained from AD patients, RGFP-966 decreased tau acetylation in patient 1 with no effect on tau phosphorylation, whereas it decreased tau hyperphosphorylation in patient 2 with no effect on tau acetylation [124]. Larger follow-up studies are required to elucidate this finding.

3.1.3. *Modulation of tau glycosylation (O-GlcNAcase inhibitors)*

Nucleocytoplasmic glycosylation by *O*-linked *N*-acetylglucosamine to serine and threonine residues (*O*-GlcNAc) can compete with phosphorylation at certain sites of tau, block tau pathological hyperphosphorylation and hinder its subsequent oligomerization [125]. *O*-GlcNAcylation is dynamically regulated by *O*-GlcNAc transferase, the enzyme that transfers GlcNAc to proteins, and *O*-GlcNAcase (OGA), the enzyme that removes GlcNAc from proteins. Current studies have focused on the development of OGA inhibitors to increase tau *O*-GlcNAc for halting disease progression of tauopathies including AD.

Identification of PUGNAc (**20**, Fig. 7) was the starting point for the development of OGA inhibitors [126]. In okadaic acid-treated differentiated PC12 cells that stably expressed human tau_{441}, treatment with PUGNAc at 50 μM for 3 h significantly induced tau *O*-GlcNAcylation, and reduced the phosphorylation levels of tau at Ser199, Ser202, Thr205, Thr212, Ser214, Ser262 and Ser396. In okadaic acid-treated metabolically active slices from adult rat brains, PUGNAc (0.2 mM) increased protein *O*-GlcNAcylation and decreased tau phosphorylation at Ser199, Thr212, Thr217, Ser262, Ser 396 and Ser422 [127]. However, the lack of selectivity over the functionally related human lysosomal β-hexosaminidase hindered the further development of PUGNAc [128]. To increase the selectivity for OGA, NAG-thiazoline (**21**) and NButGT (**22**) were developed (Fig. 7) [129,130]. Acute or chronic treatment of rats and mice with NButGT increased *O*-GlcNAc levels throughout all tissues without perturbing insulin sensitivity or altering glucohomeostasis [131].

Later, Thiamet-G (**23**, Fig. 7), a potent and selective inhibitor of OGA (Ki = 20 nM for human OGA), was designed and synthesized by Yuzwa et al., in 2008 [132]. In differentiated PC-12 cells, Thiamet-G inhibited *O*-GlcNAcase in both a dose and time-dependent manner and decreased tau phosphorylation at Thr231 and Ser396 at 100 μM for 4 h [132]. In 21-week-old male rats, oral administration of Thiamet-G at a dose of 200 mg/kg/d for one day

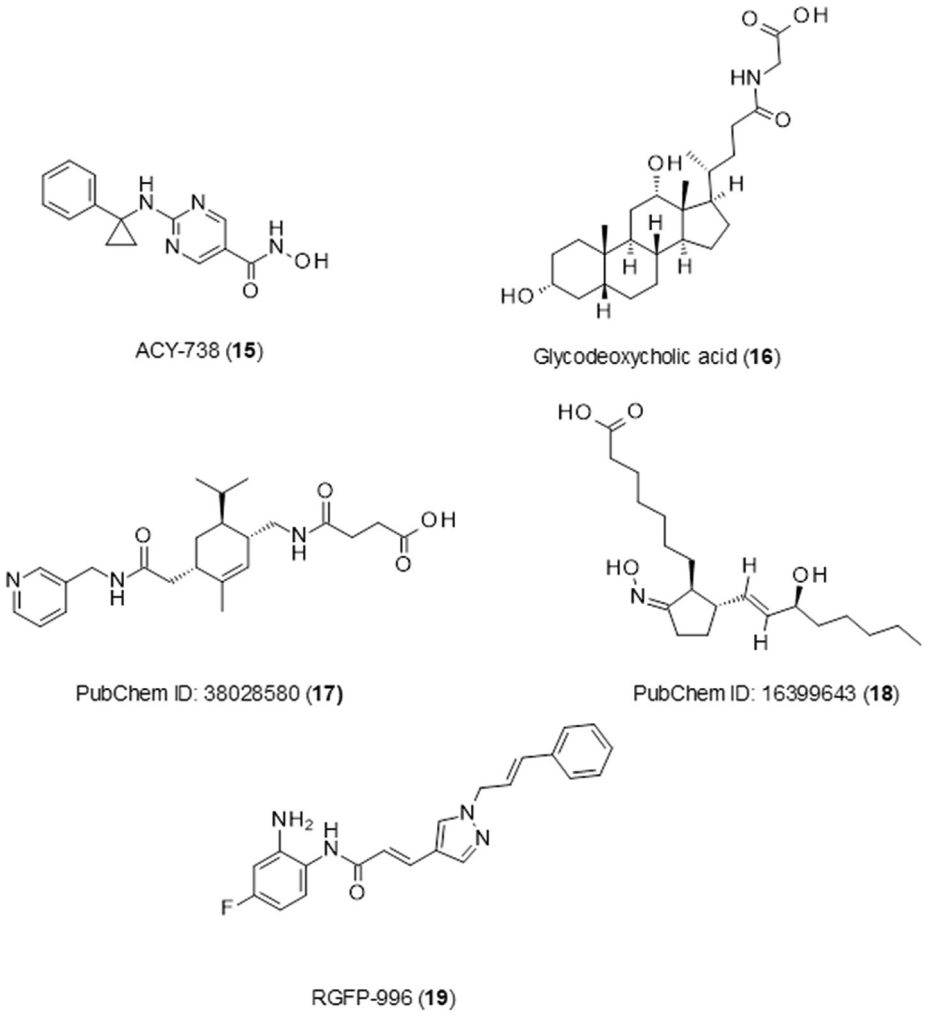

Fig. 6. Histone deacetylase inhibitors.

efficiently increased *O*-GlcNAc and reduced tau phosphorylation at Thr231, Ser396 and Ser422 in both rat cortex and hippocampus [132]. Bilateral injection of Thiamet-G into the lateral ventricle of human tau_{441} transgenic mice (175 mg/mouse) increased protein *O*-GlcNAcylation, decreased tau phosphorylation at Thr181, Thr212, Ser214, Ser262/Ser356, Ser404 and Ser409, but increased tau phosphorylation at Ser199, Ser202, Ser396 and Ser422 in the mouse brain [133]. However, chronic treatment of hemizygous JNPL3 tau transgenic mice with Thiamet-G (p.o., 500 mg/kg/d for 36 weeks) increased tau *O*-GlcNAc, hindered formation of tau aggregates, decreased neuronal cell loss without apparent adverse effects, but did not alter tau phosphorylation [125]. Consistently, Graham et al., found that acute treatment of rTg4510 mice with Thiamet-G (p.o., 500 mg/kg/d for 1 d) transiently reduced tau phosphorylation at S202/205, S262, S396 and S356, whereas long-term treatment with Thiamet-G (p.o., 500 mg/kg/d for 4 months) strongly increased tau *O*-GlcNAcylation, reduced the number of dystrophic neurons, and protected against the formation of pathological tau species without altering the phosphorylation of non-pathological tau [134]. Recently, Hastings et al., and Wang et al., also proved that chronic treatment of rTg4510 mice with Thiamet-G (p.o., 500 mg/kg/d) prevented brain atrophy and led to a significant reduction of aggregated tau and several p-tau species in the insoluble fraction of rTg4510 mouse brain and total tau in CSF [135,136]. Importantly, Thiamet-G acts within brain through a mechanism involving stimulation of autophagy through an mTOR-independent pathway without obvious toxicity, which aids the brain in combatting the accumulation of toxic protein species [137].

However, the clinical utility of Thiamet-G is limited by its poor CNS penetration profile. Based on its structure, Selnick et al., developed a highly potent and selective OGA inhibitor, MK-8719 (**24**, Fig. 7), with optimized physicochemical parameters to increase CNS exposure [138]. Preclinical data confirmed that subchronic administration of MK-8719 significantly increased protein *O*-GlcNAcylation, reduced pathological tau accumulation, decreased inflammatory marker expression and attenuated brain weight loss and forebrain volume loss in Tg4510 mouse brain [139]. The Phase I study in 16 healthy volunteers shows that MK-8719 is safe and well tolerated at single doses up to 1200 mg in subjects. Its efficacy was also assessed by measuring *O*-GlcNAcylated protein levels in peripheral blood mononuclear cells, which increased in a dose-dependent manner [140]. In 2016, FDA granted orphan drug designation to MK-8719 for the treatment of progressive supranuclear palsy (PSP), one of the neurodegenerative tauopathies related to AD [141].

Similarly, ASN120290, previously known as ASN-561, potently inhibits OGA and penetrates the BBB better than Thiamet-G. Interestingly, the chemical structure of ASN120290 is not revealed. In JNPL3 mice treated with ASN120290 for 5 d, the levels of *O*-GlcNAcylated tau rose dose-dependently up to 12-fold higher

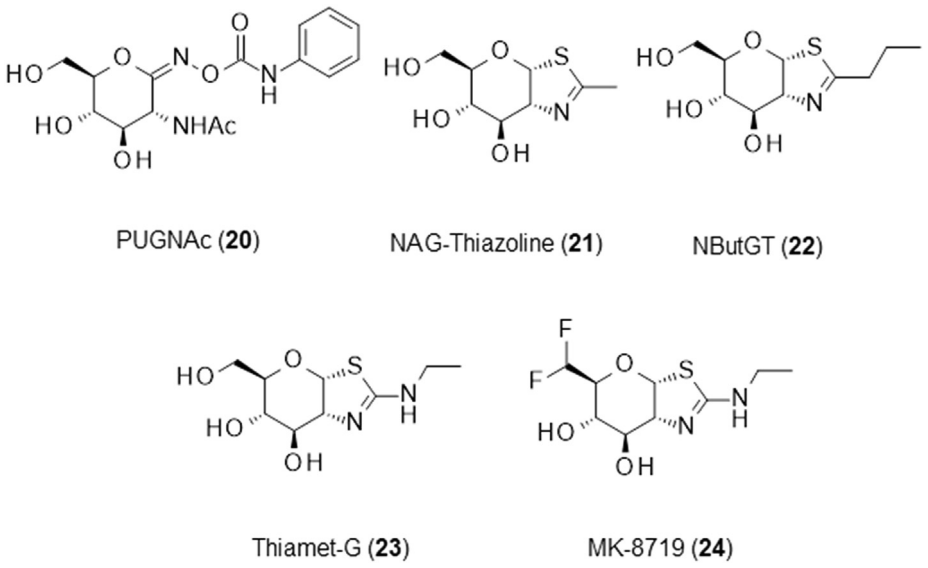

Fig. 7. O-GlcNAcase inhibitors.

than that in vehicle-treated mice [142]. In P301S mice, treatment with ASN120290 at 100 mg/kg for 3.5 months boosted tau *O*-GlcNAcylation, decreased tau phosphorylation and developed fewer PHFs in cortex [143]. A Phase I safety and tolerability study of oral ASN120290 was conducted in healthy adult and aged volunteers in 2017 and shows that this drug is well tolerated without severe adverse events. In 2018, FDA granted an orphan drug designation to ASN120290 for the treatment of the primary tauopathy, PSP [144].

3.1.4. Modulation of tau truncation (caspase inhibitors)

Numerous proteases have been shown to proteolyze tau under pathological and physiological conditions, including caspases [22], calpains [145], thrombins [146], cathepsins [147], aminopeptidases [148] and human high temperature requirement serine protease A1 [149]. The cleavage of tau by caspase-3, caspase-6, calpains, and thrombins leads to the production of toxic tau fragments, whereas the cleavage of tau by aminopeptidases, human high temperature requirement serine protease A1 and in some circumstances caspase-3 may facilitate tau degradation and protect neurons [150]. Studies have focused on the development of caspase inhibitors to reduce toxic fragments of tau.

Benzyloxycarbonyl-valine-alanine-aspartate-fluoromethylket one (Z-VAD-FMK) (**25**, Fig. 8) was developed as a caspase inhibitor for therapeutic use. Unfortunately, the metabolism of Z-VAD-FMK produces the highly toxic fluoroacetate *in vivo* [151]. However, as a tool for basic research it is a great success. In murine neuroblastoma N2a cells overexpressing human 2N4R tau, the pan-caspase inhibitors, Z-VAD (OMe)-FMK (**26**, Fig. 8) and ApoBlock, at 50 μM significantly diminished tau proteolysis triggered by staurosporine and prevented the generation of conformationally altered and aggregated tau recognized by the MC1 conformational antibody [152]. This suggests that therapeutic agents aimed at inhibiting caspase-mediated tau cleavage may prove beneficial in slowing cleavage and aggregation, thus potentially halting tau pathology and disease progression.

Quinolyl-valyl-*O*-methylaspartyl-(-2, 6-difluorophenoxy)-methyl ketone (Q-VD-OPh) (**27**, Fig. 8), a broad-spectrum caspase inhibitor, is less toxic than Z-VAD-FMK even at very high concentrations [153]. Q-VD-OPh is able to cross the BBB and has promising potential in several models of human disease [154]. In TgCRND8 mice, chronic treatment of with Q-VD-OPh (i.p., 10 mg/kg, three times a week) prevented caspase-7 activation and limited caspase-cleaved fragments of tau in the brain [155].

Another caspase inhibitor minocycline (**28**, Fig. 8) (10 μM and 20 μM) prevented Aβ-induced neuronal death and caspase-3 activation, and reduced caspase-3 cleavage of tau in primary cortical neurons [156,157]. In tangle-forming transgenic mice (htau line), minocycline treatment (i.p., 10 mg/kg/d for 14 d) reduced caspase-3 activation, lowered the generation of caspase-cleaved tau fragments, decreased p-tau levels and insoluble tau aggregates [156]. However, two years of minocycline treatment does not modify cognitive or functional decline in patients with mild AD in a randomized clinical trial and 400 mg of minocycline is poorly tolerated in this population [158].

3.2. Tau aggregation inhibitors

Several small molecules have been identified that can inhibit tau aggregation *in vitro*, including methylene blue, curcumin derivatives, N744, rhodanines, aminothienopyridazines (ATPZs), and other classes.

3.2.1. Methylene blue

Methylene blue (**29**, Fig. 9) is a phenothiazine that has been widely used to treat malaria and methemoglobinemia. Multiple studies have revealed that it prevents tau aggregation or dissolve existing aggregates to interfere with downstream pathological consequences of aberrant tau in tauopathies including AD and other neurodegenerative diseases [159–161]. Rember TM (TRx-0014) is a purified, proprietary formulation of methylene blue, the chloride salt of the oxidized form of methylthioninium. A Phase II study (NCT00515333) in mild or moderate AD indicated a minimum safe and effective dose was identified as 138 mg/day, but dose-dependent dissolution and absorption limitations restricted its use at a higher dose of 228 mg/day [162]. In the meantime, TauRx had developed a second-generation compound, LMTM (TRx0237) (**30**, Fig. 9), which is a stabilized and reduced form of methylthioninium with better absorption and tolerability. However, one Phase II (NCT01626391) and two Phase III (NCT01689233 and NCT01689246) clinical trials of LMTM failed to slow cognitive or functional decline in subjects with mild to moderate AD

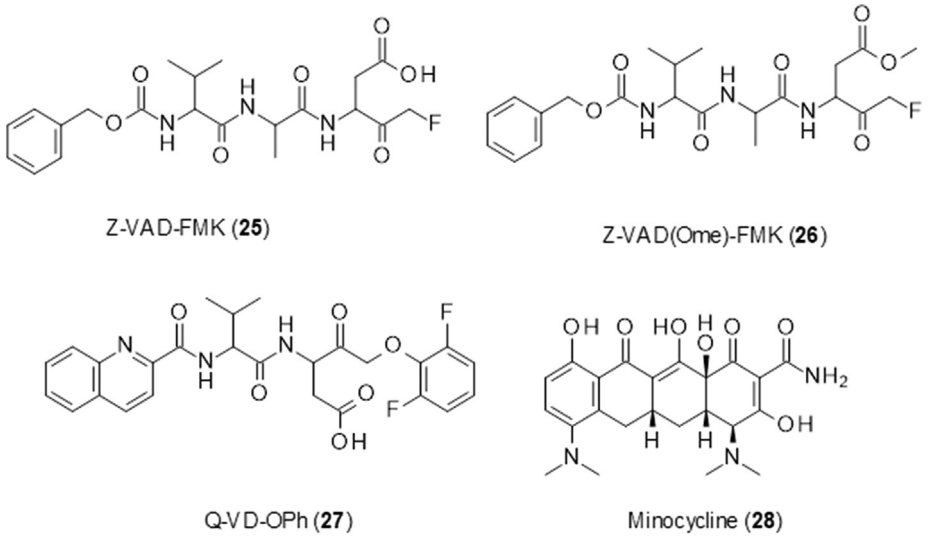

Fig. 8. Caspase inhibitors.

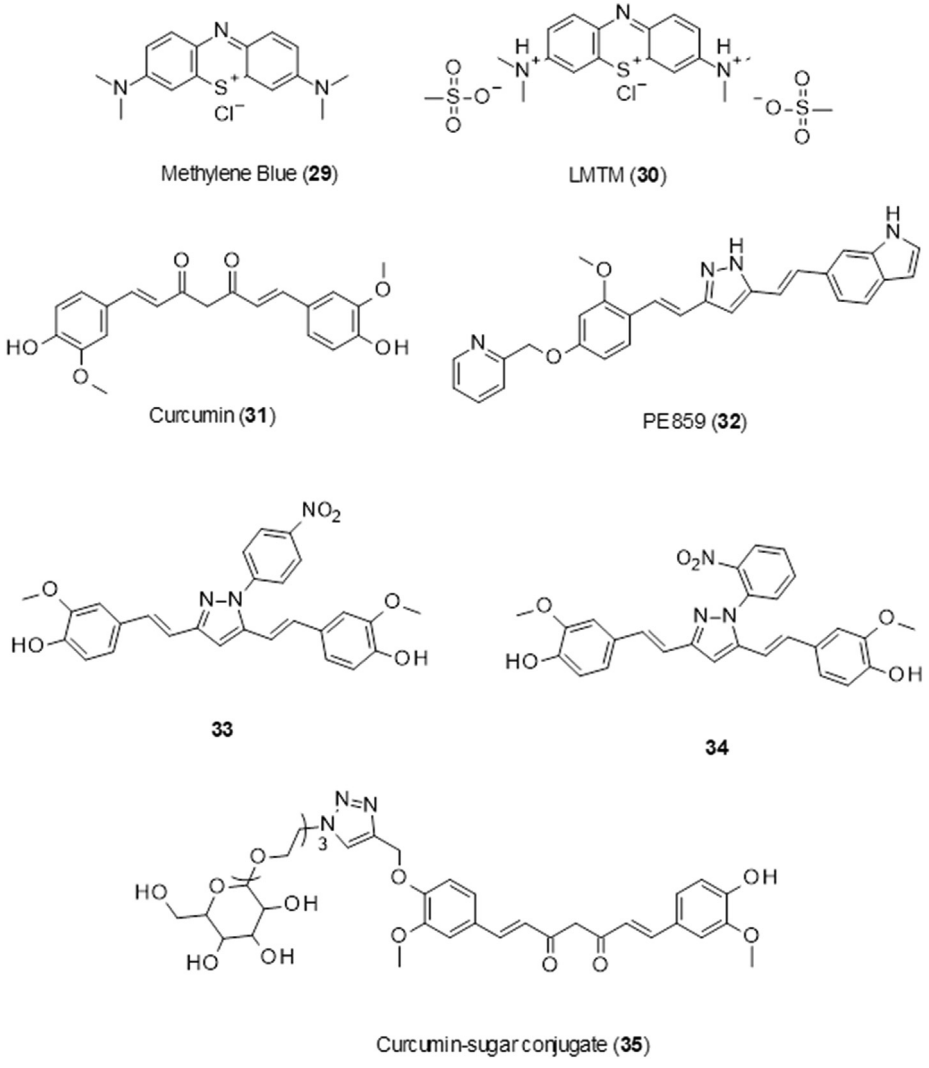

Fig. 9. Methylene blue and curcumin derivatives as tau aggregation inhibitors.

[163,164]. In 2018, TauRx started another Phase III trial (NCT03446001) aiming to determine the safety and efficacy of LMTM treatment (16 mg/day and 8 mg/day) in subjects with AD, and this study will be completed in 2022 [165].

3.2.2. Curcumin and its derivatives

Curcumin (**31**, Fig. 9) is a natural polyphenol produced by *Curcuma longa* plants and has many potentially neuroprotective properties, such as antioxidant, anti-inflammatory and anti-protein-aggregate effects [166]. Curcumin binds to the β-pleated sheet in the PHFs of tau [167] and prevent tau aggregation [168]. Several Phase I and II clinical trials (NCT01001637, NCT00164749, NCT00099710, NCT01811381, NCT00595582, NCT01383161) of curcumin were conducted in subjects with AD or MCI [169]. A recent meta-analysis suggested that curcumin appears to be more effective in improving cognitive function in the elderly than in improving symptoms of AD and schizophrenia [170]. Further high-quality trials of curcumin in AD with larger sample sizes need to be performed for the reliable assessment.

Okuda et al., designed, synthesized and evaluated curcumin derivatives as dual inhibitors of Aβ and tau aggregation. One of the novel curcumin derivatives, PE859, (3-[(1E)-2-(1H-indol-6-yl) ethenyl]-5-[(1E)-2-[2-methoxy-4-(2-pyridylmethoxy) phenyl] ethenyl]-1H-pyrazole (**32**, Fig. 9), came up as a potent inhibitor of Aβ and tau aggregation with IC_{50} value of 1.2 ± 0.2 μM and 0.66 ± 0.13 μM, respectively along with a better pharmacokinetic profile than curcumin [171]. The probable mechanism of their action is to break the β-sheet structure of Aβ or tau aggregates, which is similar to curcumin.

Curcumin has metal chelation properties and to minimize its chelation propertycurcumin derived pyrazoles and isoxazoles were synthesized by replacement of the 1,3-dicarbonyl moiety and compound **33** and **34** (Fig. 9) showed tau aggregation inhibition at low micromolar concentrations. It is also observed that the aryl ring of the *N*-aryl pyrazoles when topped up with an electron-withdrawing group as in the case of compound **33** and **34** possessed increased inhibition of tau aggregation by 2-100-fold [172]. In addition, Dolai et al., synthesized and studied "clicked" sugar-derivatives of curcumin which possess high bio-efficacy in modulating Aβ and tau aggregation. The sugar-curcumin conjugate (**35**, Fig. 9) inhibited Aβ aggregation at 8 nM and tau aggregation at 0.1 nM concentrations, which is far more effective than curcumin [173].

3.2.3. N744

Chirita et al., screened a library of small molecules for inhibition of tau fibrillization against arachidonic acid induced htau40 assembly by fluorescence-based assay. N744 (**36**, Fig. 10), a charged molecule of Congo red family, was identified as inhibitor and its ability to antagonize tau fibrillization was further determined by transmission electron microscopy against htau40 (4 μM) induced by arachidonic acid (75 μM). N744 inhibited the aggregation of tau filaments by interfering with their nucleation at stoichiometric concentrations relative to total tau with an IC_{50} value of 294 ± 23 nM. Further, N744 promoted the tau filament disaggregation with first order kinetics. N744 did not inhibit the Aβ and α-synuclein aggregation when assessed for its selectivity for tau over Aβ and α-synuclein. Thus, *in vitro* results offered N744 as pharmacological approach for inhibition of tau fibrillization in neurodegeneration [174].

Necula et al., further studied this tau fibrillization inhibitor to investigate its mechanism of action and potential for testing in biological models. They studied *in vitro* interactions between N744 and full length four repeat tau using transmission electron microscopy and fluorescence spectroscopy. Analysis of N744 effect on tau aggregation kinetics revealed that N744 decreased the total filament length without inhibiting filament nucleation [175].

3.2.4. Rhodanines

Bulic et al., investigated a series of substituted rhodanines for their ability to inhibit tau aggregation and disaggregate filaments *in vitro* and in a cell model of a neuroblastoma cell line. Initially, they performed high-throughput screen and identified rhodanine derivative as hit because of its appreciable activity in screening assay. Then to further optimize the structure, central core heterocycle rhodanine was replaced with other heterocycles and compounds were assessed for tau aggregation inhibition. The rhodanine containing derivatives appeared to be the most potent. Further, the importance of the carboxylic acid was investigated by either by esterifying or replacing it with an imidazole or a benzimidazole. All such modifications led to reduced disassembly activity. The effect of the linker length between carboxylic acid and rhodanine core was also ascertained, appreciable increase in inhibitory activity was observed with increase in distance up to two carbon bonds. Subsequently, biaryl part was studied and results revealed that the heteroaromatic side chain tolerated variations whereas modifications on furan diminished the activity. Compound RH-1 (**37**, Fig. 10) emerged as the most potent derivative of the series with nanomolar range for inhibition and disaggregation. All synthesized derivatives were also variedly active in murine neuroblastoma N2a cell assay and were not or very weakly cytotoxic [176].

3.2.5. Aminothienopyridazines

In an attempt to discover novel orally bioavailable ATPZs of tau aggregation inhibitors, Ballatore et al., synthesized and evaluated a series of new analogues that are both effective inhibitors of tau fibrillization and display significant brain-to-plasma exposure ratios after administration to mice. Compound **38** and **39** (Fig. 10) were found to cross BBB and reach to the brain at the concentrations above 800 ng/g (i.e., >2 μM) 1 h after dosing at a dose of 5 mg/kg. The results suggest that ATPZs may generate novel orally bioavailable clinical drug candidates against neurodegenerative tauopathies [177]. Further derivatives of ATPZ were synthesized and evaluated in *in-vivo* settings and compound **40** (Fig. 10), 5-amino-N cyclopropyl-3-(4-fluorophenyl)-4-oxo-3,4-dihydro thieno [3,4-*d*]pyridazine-1-carboxamide, was reported to be well-tolerated with no notable side-effects at an oral dose of 50 mg/kg/day in rodent model. Moreover, the compound **40** possessed oral bioavailability of ~60%, good water solubility and non-specific brain tissue binding which renders it as a potential clinical drug candidate from this class of tau aggregation inhibitors [178].

3.2.6. Other classes

George et al., screened an aqueous extract of cinnamon and found that it possesses moderate tau aggregation inhibitory activity *in vitro*. Further experiments led to identification of cinnamaldehyde (**41**, Fig. 11) and oxidized form of epicatechin (**42**, Fig. 11) as inhibitors of tau aggregation *in vitro* due to their interaction with the two cysteine residues in tau. Mass spectrometry of a synthetic peptide, SKCGS, representing the actual tau sequence, identified the thiol group as reacting with cinnamaldehyde and epicatechin. The interaction of cinnamaldehyde with tau cysteines was reversible and the presence of cinnamaldehyde did not impair the biological function of tau in tubulin assembly *in vitro*. Additionally, they revealed that epicatechin can sequester highly reactive and toxic byproducts of oxidation such as acrolein which together with its reversible interaction with cysteines makes it a potential anti-tauopathy compound [179].

Seventeen oligo heteroaromatic compounds were rapidly synthesized via a one-pot, 3- or 4-component coupling procedure

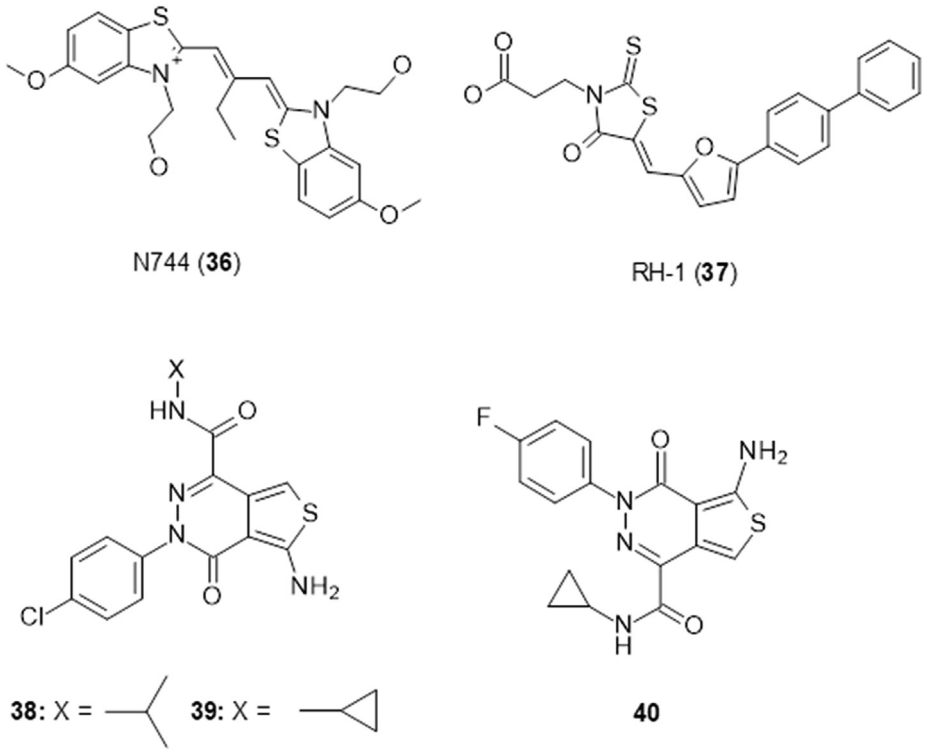

Fig. 10. Rhodanines and aminothienopyridazines as tau aggregation inhibitors.

(Knoevenagel condensation) approach. Evaluation showed that compounds **43** and **44** (Fig. 11) were the potent inhibitors for Aβ and tau aggregations (**43**: IC_{50} 0.38 mM, 0.29 mM against Aβ, tau, respectively, **44**: IC_{50} 0.55 mM, 0.30 mM against Aβ, tau, respectively) [180].

Karakani et al., reported that crocin (**45**, Fig. 11) can inhibit the aggregation of human tau. In the presence of crocin, the β-structure/random coil ratio of tau under fibril condition decreased significantly. The plausible mechanism of anti-tau-aggregation of crocin could be related to its chemical structure that consists of three parts containing a polyene hydrocarbon chain, carbonyl groups and β-D-gentiobiosyl at both ends. The partial negative charge of carbonyl groups can likely interact with positive residues such as Lysine and Arginine. On the other hand, positive residue especially lysine exists in hexapeptide aggregation cores of protein (275VQIINK280 and 306VQIVYK311) and it plays a critical role in the self-assembly of tau into abnormal fibrils [181].

Lin et al., while aiming for identification of GSK3β kinase inhibitors assayed sixteen compounds by luminescent kinase assay and a cell assay using HEK293 cells expressing DsRed-tagged ΔK280 in the repeat domain of tau (tauRD). The compounds VB-003 which is a potent GSK3β inhibitor and VB-008 (**46**, Fig. 11) (AM404, an anandamide transport inhibitor) showed reduced tau aggregation with IC_{50} values of 0.25 and 5.4 μM, respectively and showed potency by increased expression of phospho-GSK3β (Ser9) and by reduced endogenous tau phosphorylation at the sites of Ser202, Thr231, and Ser396. VB-008, but not VB-003, in the ΔK280 tauRD-DsRed SH-SY5Y cells, enhanced HSPB1 and GRP78 expression, increased ΔK280 tauRD-DsRed solubility, and increased neurite outgrowth and performed best by the end of the study [182].

Frenkel-Pinter et al., demonstrated the promising therapeutic approach of *N*-(3-chloro-1,4-dihydro-1,4-dioxo-2-naphthalenyl)- L-tryptophan (Cl-NQTrp) (**47**, Fig. 11) which is an effective inhibitor of tau aggregation *in vitro* and in *vivo*. Larvae of drosophila treated with Cl-NQTrp led to a significant 70% decrease in total tau levels and significant amelioration of tauopathy-engendered phenotypes through inhibition of the accumulation of tau in the eye tissue of treated flies. This suggests that Cl-NQTrp is also an effective aggregation inhibitor of tau *in vivo* [183].

3.3. Agents promoting tau degradation

The two main protein degradation pathways in neurons are the ubiquitin-proteasome system (UPS) and the autophagy-lysosome pathway (ALP), and their lowered efficiency has been implicated in tauopathies. Multiple studies suggest that enhancing the activities of UPS or ALS to facilitate pathological tau clearance may be effective for the prevention and treatment of AD.

3.3.1. UPS activators

In the context of the UPS, acceleration the rate of tau degradation could be achieved through stimulating proteasome-mediated proteolysis, inhibiting ubiquitin specific peptidase 14 (USP14), enhancing cAMP or cGMP signaling systems by phosphodiesterases (PDEs) inhibitors or increasing the recruitment of ubiquitin machinery to tau via proteolysis targeting chimeric molecules (PROTACs).

3.3.1.1. Proteasome stimulators. Several studies have shown that small molecules which increase the activity or levels of the 20S proteasome could enhance the clearance of tau *in vitro* and in cell culture. In 2017, Jones et al., discovered a new class of molecules, such as chlorpromazine (**48**, Fig. 12), capable of enhancing 20S proteolytic activity via a ligand-20S proteasome interaction, which induces the selective degradation of disordered proteins, α-synuclein and tau, over structured proteins *in vitro* and in glioblastoma (U87-MG) cells [184]. Later, Njomen et al., generated a small molecule TCH-165 (**49**, Fig. 12), which can regulate the dynamic

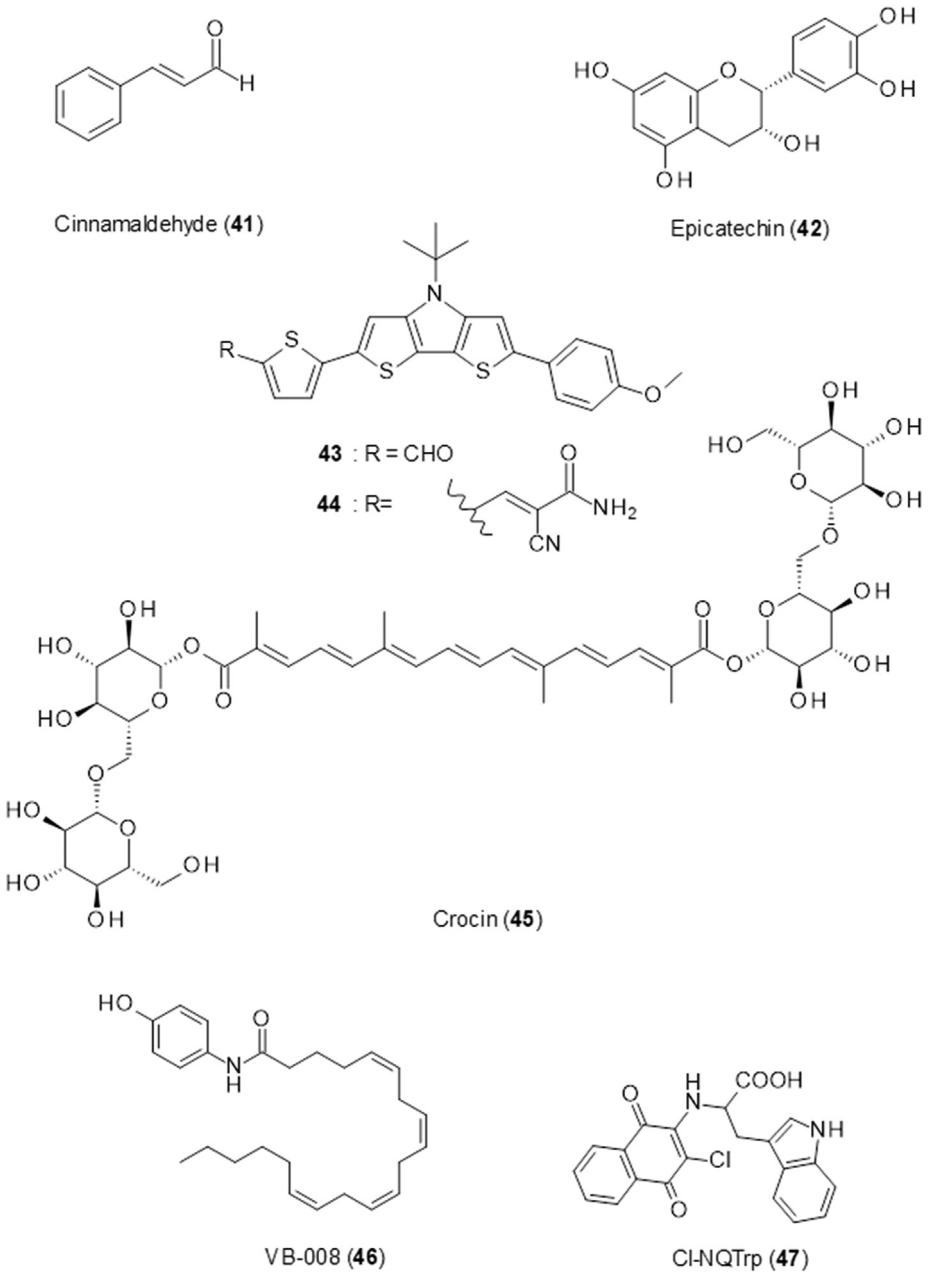

Fig. 11. Miscellaneous tau aggregation inhibitors.

equilibrium between the 20S and 26S proteasome complexes, favoring 20S-mediated tau degradation [185]. Further *in vivo* studies need to explore the therapeutic potential of 20S activation.

3.3.1.2. USP14 inhibitors. USP14 is a proteasome-associated deubiquitinating enzyme which can inhibit the degradation of ubiquitin-protein conjugates [186,187]. A small-molecule selective inhibitor of USP14, known as IU1 (**50**, Fig. 12), increased proteasome activity and reduced overexpressing tau levels dose dependently in murine embryonic fibroblasts, with a strong effect at 50 μM [186]. But in rat cerebral cortical neurons, IU1 induced calpain mediated tau cleavage which is not relevant to USP14 inhibition [188]. Recently, Boselli et al., developed a more potent inhibitor, IU1-47 (**51**, Fig. 12), with a 10-fold increase in affinity for USP14 over IU1 [189]. In primary neurons, IU1-47 enhanced the degradation of wild-type tau, pathological tau mutants P301L and P301S, and the A152T tau variant. The effect of IU1-47 on proteasome-mediated tau degradation was also proved to be dependent on lysine 174 of tau [189].

3.3.1.3. PDEs inhibitors. cAMP-protein kinase A (PKA) and cGMP-protein kinase G (PKG) signaling systems activate 26S proteasomes through phosphorylation and enhance the degradation of misfolded proteins, such as tau [190,191]. PDEs inactivate cAMP or cGMP by degrading their phosphodiester bond. Recent studies have shown that PDEs inhibition can be a strategy to promote the clearance of abnormal tau by increasing cAMP or cGMP signaling which enhance proteasome activity.

Rolipram (**52**, Fig. 13) is a selective PDE4 inhibitor developed initially as an antidepressant drug [192]. Myeku et al., showed that exposure of cortical brain slices from rTg4510 early-stage mice (3–4

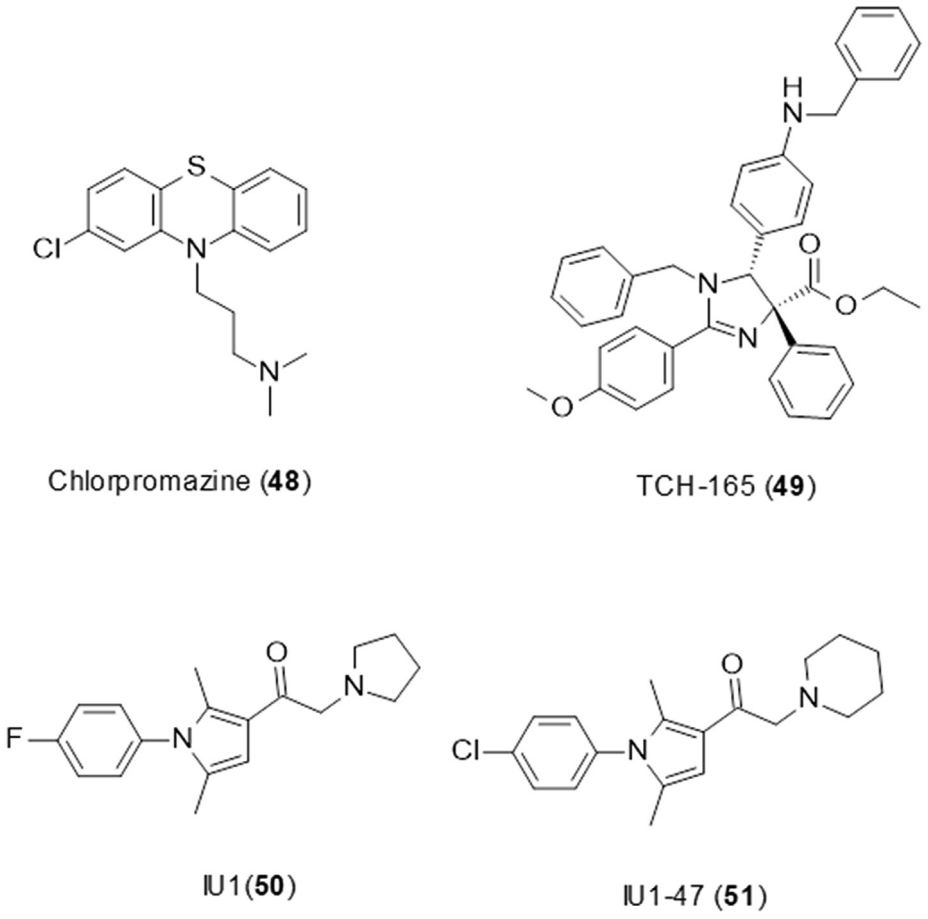

Fig. 12. Proteasome stimulators and USP14 inhibitors.

months of age) to rolipram enhanced proteasome activity via cAMP-PKA and reduced amounts of total and insoluble tau, including p-tau [193]. *In vivo*, rolipram treatment (0.03 mg/kg, i.p. twice daily for 21 days) enhanced proteasome activity, reduced accumulation of total and p-tau and p62, and improved cognition in rTg4510 mice with early-stage disease [193].

BPN14770 (**53**, Fig. 13), an allosteric inhibitor of PDE-4D, was designed by Burgin et al., with reduced potential to cause emesis which is a dose-limiting side effect of existing active site-directed PDE4 inhibitors [194]. In humanized PDE-4D mice treated with intrahippocampal microinjection of oligomeric $A\beta_{1-42}$, BPN14770 treatment (0.003 mg/kg, oral gavage for 14 days) reduced memory impairment, damage to nerve morphology, deficits in synaptic proteins, and impaired neurological signaling [195]. In 2015 and 2016, two Phase I clinical trials (NCT02648672 and NCT02840279) were conducted to evaluate single and multiple oral doses of BPN14770 in the total of 109 healthy male and female adults, including elderly subjects [196,197]. The results showed that BPN14770 is safe and orally bioavailable in single and multiple dose studies and has cognitive benefits on working memory at lower doses in healthy elderly subjects in multiple dose trial [198]. In 2019, a Phase II clinical trial (NCT03817684) of BPN14770 in 255 patients with early Alzheimer's disease was conducted [199]. Even there was no significant change in the Assessment of Neurological Status-Delayed Memory Index (RBANS-DMI) with BPN14770 treatment, a subgroup analysis of patients in the 25 mg group with above-median Clinical Dementia Rating Sum of Boxes (CDR-SB) scores at baseline indicated a signal for improvement on the CDR-SB [200]. These results warrant further clinical studies of BPN14770.

A selective PDE3 inhibitor, cilostazol (**54**, Fig. 13) is a medication used to treat intermittent claudication in peripheral vascular disease. Schaler et al., showed that i.p. administration of cilostazol at 3 mg/kg twice daily for 30 days enhanced proteasome function via the cAMP/PKA pathway, promoted the clearance of abnormal tau, and attenuated tauopathy and cognitive decline in early-stage tauopathy in rTg4510 mice [201]. Several clinical studies, including pilot studies [202,203], retrospective studies [204,205], case control studies [206] and cohort studies [207], suggested that cilostazol may reduce the risk to develop dementia in AD patients. In 2011, a Phase IV clinical trial (NCT01409564) was initiated by the Seoul National University Hospital in Korea [208]. Its results showed that cilostazol treatment added to donepezil may delay the decline in regional cerebral metabolism in AD with white matter lesions compared with donepezil monotherapy [209]. Later in 2015, a Phase II clinical trial (NCT02491268) of cilostazol in 200 participants was conducted for the prevention of conversion from MCI to dementia [210] and it will be completed by December 2020.

Sildenafil (**55**, Fig. 13) is a medication used to treat erectile dysfunction and pulmonary arterial hypertension and acts by blocking PDE5, an enzyme that promotes breakdown of cGMP. Recently, VerPlank et al., showed that sildenafil treatments (1 and 10 μM) increased proteasome activity and the rate of pathological tau clearance, and reduced morphological abnormalities in zebrafish overexpressing Dendra-tau-A152T [191]. Several studies indicate that sildenafil can improve cognition in AD animal models [211]. In AD patients, a single 50 mg dose of sildenafil improved cerebral vascular

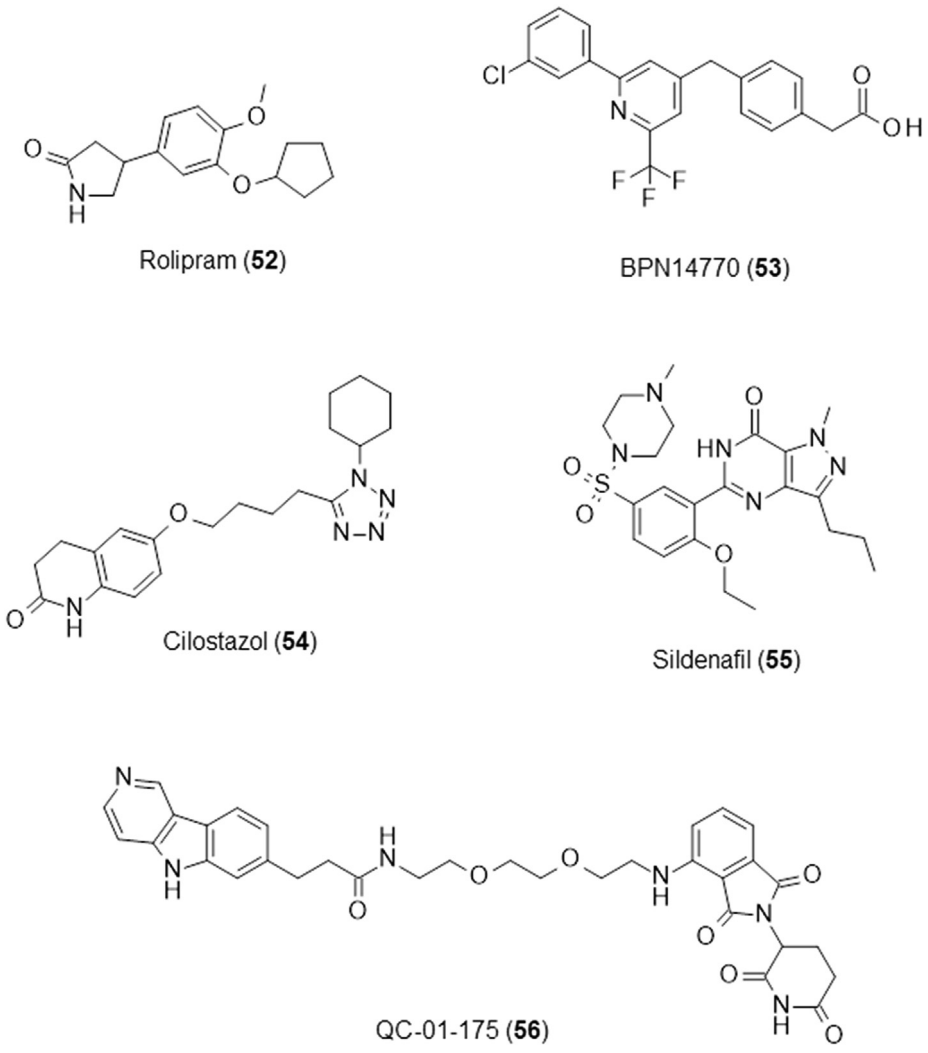

Fig. 13. Phosphodiesterase inhibitors and PROTAC (QC-01-175).

and metabolic function [212], and decreased hippocampal spontaneous neural activity [213]. To assess clinical effects of sildenafil in AD patients, a randomized control trial should be undertaken [211].

3.3.1.4. PROTACs. Another emerging approach is the use of PROTACs. PROTACs are hetero-bifunctional molecules that contain a ligand for recruiting an E3 ligase, a linker, and another ligand to bind with the target protein (such as tau), resulting in prey ubiquitination and degradation by proteasomes [214]. In 2016, Chu et al., reported that synthesized multifunctional PROTACs peptide TH006 increased tau poly-ubiquitination and effectively induced tau degradation in N2a and SH-SY5Y cells, and reduced tau levels in primary neurons and in the brain of 3xTg-AD mice [215]. In 2018, a new peptide PROTAC which recruits the Keap1-Cul3 ubiquitin E3 ligase complex to tau was developed [216]. This peptide PROTAC could downregulate the intracellular tau levels in different tau over-expressed cell lines (SH-SY5Y, N2a and PC-12 cell lines) and promote the Keap1-dependent poly-ubiquitination and proteasome-dependent degradation of tau. In 2019, positron emission tomography tracers of tau were transformed into the development of new classes of PROTACs and a lead PROTAC QC-01-175 (**56**, Fig. 13) was designed to engage both tau and Cereblon (CRBN), a substrate-receptor for the E3-ubiquitin ligase $CRL4^{CRBN}$ [217]. QC-01-175 promoted tau clearance in human neuronal cell models of tauopathy (A152T and P301L) and was more effective at degrading tau species found in FTD patient-derived neurons than that from healthy controls. QC-01-175-mediated tau degradation is proteasome-dependent and occurs via $CRL4^{CRBN}$ binding, but not autophagy. To develop new therapeutic strategies for AD, a recent patent provided several new PROTACs with a tau binding moiety and an E3 ubiquitin ligase binding moiety such as lenalidomide or thalidomide [218]. These compounds induced tau ubiquitination and degraded hyperphoshorylated tau and total tau in human tau-A152T neurons and tau-P301L neurons. Among these compounds, QC-01-175 can also be absorbed into the blood and cross the BBB. Further clinical trials of PROTACs for tau clearance is required to determine if they could be used as a therapeutic strategy for AD.

3.3.2. Autophagy activators

In addition to enhancing UPS, a number of autophagy activators have been shown to be effective to facilitate tau degradation and ameliorate AD.

3.3.2.1. Targeting the mTOR-dependent pathways. The mammalian target of rapamycin (**57**, Fig. 14) (mTOR) signaling pathway is one of the major negative regulators of autophagy. Rapamycin, an FDA approved drug for the prevention of organ transplant rejection and the treatment of lymphangioleiomyomatosis, inhibits mTOR

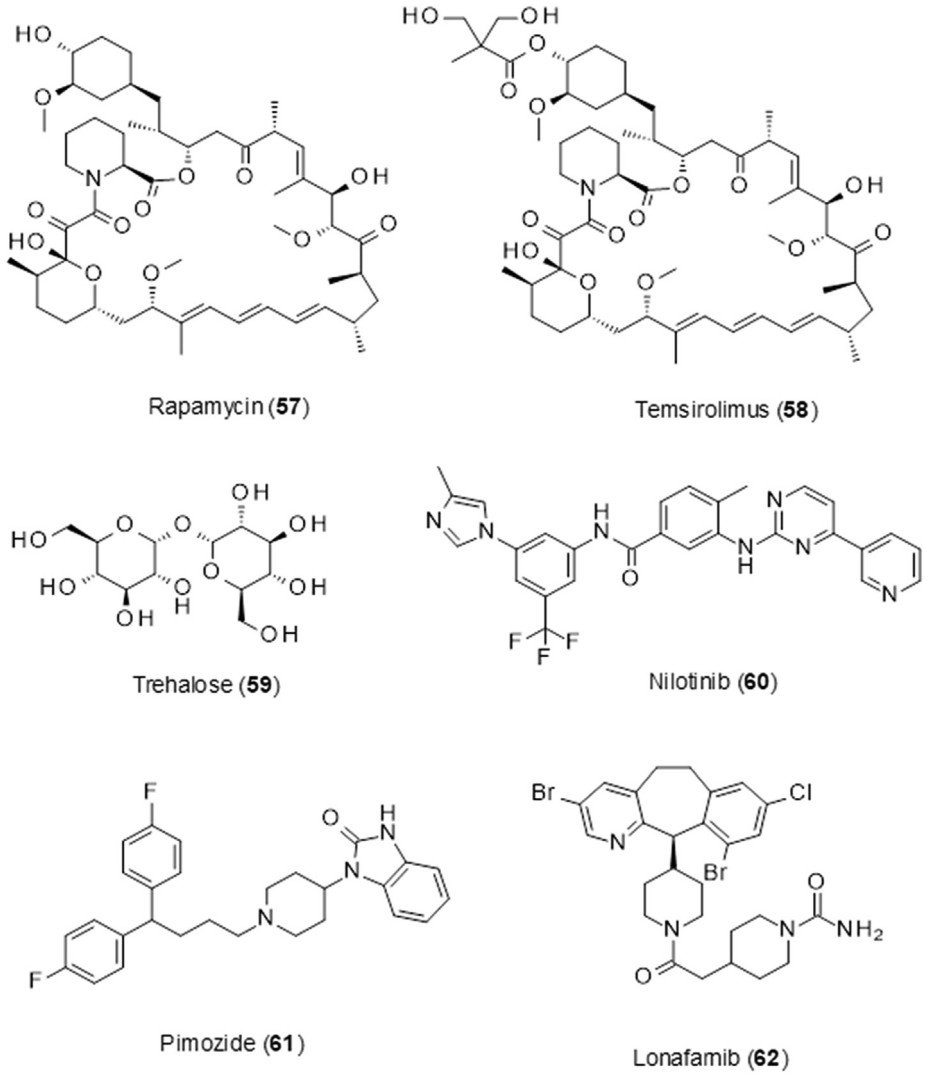

Fig. 14. Autophagy activators.

signaling pathway and clears tau through autophagy induction. In a *Drosophila* model expressing wild-type or mutant tau R406W, rapamycin facilitated the clearance of insoluble tau by induction of autophagy [219]. In 3 × Tg-AD mice, rapamycin restored mTOR signaling in the brains, reduced p-tau (Thr181) and total tau levels by increasing autophagy induction, and rescued early learning and memory deficits [220,221]. Similarly, rapamycin treatment lowered accumulation of the autophagy associated proteins, and reduced tau phosphorylation and insoluble tau in the brain of P301S mutant tau transgenic mice [222,223]. Recently, an early Phase I study (NCT04200911) of rapamycin was started to evaluate its safety and feasibility in older adults with MCI or early AD and will be completed in 2020 [224].

Rapamycin analog, temsirolimus (**58**, Fig. 14), is an FDA-approved anti-cancer drug. Like rapamycin, temsirolimus upregulates autophagy through inhibition of the mTOR pathway, and has been shown to enhance autophagic clearance of hyperphosphorylated tau in okadaic acid-incubated SH-SY5Y cells and in the brain of P301S transgenic mice, and rescue spatial learning and memory impairments [225]. Later, Frederick et al., also suggested that temsirolimus could lead to a stimulation of macroautophagy and decrease of sarkosyl-insoluble tau levels in the mutant tau Tg30 transgenic mice [226].

3.3.2.2. Targeting the mTOR-independent pathways. Autophagy activation mediated by mTOR-independent pathways is another option for autophagy-based AD therapy. Trehalose (**59**, Fig. 14), a safely consumed disaccharide sugar, enhances the removal of misfolded tau in several neurodegenerative models through autophagy activation. In N2a cells expressing the repeat domain of tau with the FTDP-17 mutation ΔK280, trehalose activated the autophagy pathway and reduced tau aggregation and cytotoxicity [227]. In primary rat cortical neurons, trehalose activated autophagy and reduced total tau and p-tau levels [227]. In parkin deleted/tau overexpressing (G272V, P301L and R406W) mice, trehalose treatment (1% in the drinking water) increased autophagy markers and decreased tau pathology in the hippocampus, striatum and cortex [228]. In the human mutant P301S tau mice, trehalose (2% in the drinking water) stimulated autophagy and decreased the amount of insoluble tau [229]. Even though there are several clinical trials of trehalose in oculopharyngeal muscular dystrophy, fatty liver disease, dry eye and vascular aging, there has not been any clinical studies of trehalose in AD yet.

Lithium not only inhibits GSK3β activity but also upregulates autophagy by inhibition of inositol monophosphatase, which reduces *myo*-inositol-1,4,5,-triphosphate signaling [230]. In transgenic mice overexpressing human mutated tau (P301L), oral

administration of lithium chloride (LiCl) for 4 months decreased phosphorylated tau, soluble tau and NFTs levels, increased the number of LC3-positive autophagosome-like puncta and reduced p62 levels [231].

Methylene blue, which has been known to directly inhibit tau aggregation, can also induce autophagy through activation of 5′ adenosine monophosphate-activated protein kinase (AMPK) signaling [232]. Congdon et al., have shown that methylene blue modulated autophagy markers (p62, cathepsin D, BECN1 and LC3-II) and reduced aggregated and p-tau in organotypic slice cultures from JNPL3 mice which express the longest human tau isoform containing the P301L mutation [233]. Further study in CHO cells expressing human tau showed that BECN1 knockdown eliminated the effects of methylene blue on total tau levels, indicating that autophagy induced by methylene blue plays a significant role in controlling tau levels. *In vivo*, treatment with methylene blue (0.02, 2 and 20 mg/kg, oral gavage) for two weeks altered autophagy markers and resulted in a significant decrease in the levels of total tau and p-tau in male JNPL3 mice [233]. In rTg4510 mice, direct hippocampal infusion of methylene blue (1 mM) for 28 days also reduced total and p-tau levels and reversed spatial learning deficits [234].

Nilotinib (**60**, Fig. 14) (AMN107) is a second-generation Abelson (Abl) tyrosine kinase inhibitor which used as a medication to treat chronic myelogenous leukemia [235]. Hebron et al., demonstrated that nilotinib treatment (i.p., 10 mg/kg daily for 3 weeks) could promote autophagic clearance of p-tau in TauP301L mice [236]. In a single-center, Phase II clinical trial (NCT02947893) in 37 participants with mild to moderate AD, nilotinib was safe and well-tolerated although more instances of agitation, aggression, and irritability were noted with the 300 mg dose [237]. Treatment with 150 mg or 300 mg nilotinib daily resulted in 3.5−4.7 nM CSF levels of nilotinib and reduced the level of CSF $A\beta_{1\text{-}42}$, $A\beta_{1\text{-}40}$, and p-tau, lowered CNS amyloid burden, and attenuated hippocampal volume loss in patients with AD compared with placebo. However, there were no differences between the placebo and nilotinib groups on clinical, cognitive, functional, or behavioral outcomes, suggesting that a larger, longer, multicenter Phase III study need to be conducted to examine its potential efficacy [237].

Pimozide (**61**, Fig. 14), an antipsychotic drug of the diphenylbutylpiperidine class, increased autophagic flux through activating AMPK-Unc-51 like autophagy activating kinase 1 (ULK1) axis, but not of mTOR in SH-SY5Y cells and mouse embryo fibroblasts [238]. Further studies showed that pimozide reduced levels of abnormally p-tau aggregates in SH-SY5Y cells transfected with GFP-tau [238]. *In vivo*, pimozide treatment (10 mg/kg/day, i.p.) for 30 days induced autophagy activation through the AMPK-ULK1 axis, reduced levels of tau oligomers and aggregates, and rescued memory deficits of TauC3 mice expressing a caspase-cleaved form of tau [238]. In addition, lonafarnib (**62**, Fig. 14), a farnesyltransferase inhibitor, induced autophagy through activation of lysosomes, prevented the formation of tau inclusions, and attenuated behavioral abnormalities in rTg4510 mice [239]. To date, there have been no clinical studies of pimozide and lonafarnib in AD.

3.3.3. Chaperone and co-chaperone modulators

Molecular chaperones, such as heat shock protein 90 (Hsp90) and Hsp70 including heat shock cognate protein 70 (Hsc70), and their co-chaperones, such as carboxyl terminus of the Hsc70-interacting protein (CHIP), play an important role in tau degradation through the UPS or chaperone mediated autophagy (CMA). In UPS, the CHIP-Hsc70 complex is the tau E3 ligase, with UbcH5B as the E2 enzyme, and can induce tau ubiquitination leading tau degradation by 26S proteasome [240,241]. But Hsp90 can interact with CHIP-Hsc70 complex and refold tau for aggregation [242]. Instead of macroautophagy, CMA is another type of autophagy that degrades substrate proteins with a lysosomal targeting motif [243]. After the recognition by Hsc70, the substrate proteins bind to the lysosome-associated membrane protein type 2A at the lysosomal membrane and then across the membrane into the lysosomal lumen for degradation [244]. Full-size tau is not normally amenable to degradation via CMA, but the truncated mutant tau can bind to the lysosomal membrane as one of the CMA substrates. However, the mutant tau could not translocate into the lysosomal lumen resulting in CMA blockage and the formation of tau oligomers directly at the lysosomal surface [245]. Therefore, molecular chaperones Hsp70 and Hsp90 and their co-chaperones could be the potent targets to treat tauopathy in AD.

3.3.3.1. Hsp70. A high-throughput screening system revealed several compounds that inhibited or activated the activity of Hsp70 [246]. Hsp70 activation was thought to increase the clearance of tau by UPS. Unexpectedly, Hsp70 inhibitors dramatically and rapidly lower tau levels, whereas Hsp70 activators stabilize tau both in cells and brain tissue [247]. The Hsp70 inhibitors reducing tau levels are mainly in three chemical classes: phenothiazines (methylene blue and azure C), flavones (myricetin) and rhodacyanines (MKT-077, YM-01, YM-08, JG-48 and JG-98). The methylene blue (**29**), its demethylated analog, azure C (**63**, Fig. 15), and the flavonol, myricetin (**64**, Fig. 15), inhibited human Hsp70 by >80% with IC_{50} values of 83, 11, and 12 μM, respectively [248]. Methylene blue, azure C and myricetin significantly reduced total tau and p-tau levels in HeLa cells overexpressing human tau. Ubiquitination of tau was evident in HEK tau transfectants following treatment with azure C, suggesting that the tau reduction was mediated by proteasomal degradation. These reductions were also observed for endogenous tau levels in both human [BE (2)-M17 and SH-SY5Y] and murine (N2a) neuroblastoma cell lines. In organotypic brain slices from mice engineered to express mutant human tau (rTg4510) and age-matched wild-type littermates, tau levels were significantly reduced following 3 h of treatment with either methylene blue or azure C (50 μM). *In vivo*, azure C (2 μL of 10 mM, hippocampal injection) significantly and uniformly reduced soluble tau and pS396/S404 tau levels in the hippocampus of 4-month-old rTg4510 mice.

MKT-077 (**65**, Fig. 15), a Hsc70 inhibitor belonging to the class of rhodacyanines, reduced tau levels in HeLaC3 cells with an EC_{50} value of ~8 μM [249]. As a derivative of MKT-077, YM-01 (**66**, Fig. 15) improves the potency to reduce tau levels. In the human neuroblastoma BE (2)M17 and HeLaC3 cells, YM-01 dramatically reduced tau levels by ~75% with EC_{50} values between 1.5 and 0.9 μM [249]. YM-01 also reduced p-tau and total tau levels in primary hippocampal neurons and brain slice cultures derived from rTg4510 tau transgenic mice [249]. However, MKT-077 and YM-01 could not penetrate the BBB, limiting their use in the CNS as clinical candidates. Another analog, YM-08 (**67**, Fig. 15), was then developed. Although YM-08 is less effective than MKT-077 and YM-01 in anti-tau assays, it is BBB permeable and can be quickly cleared from the kidney, reducing the opportunity for renal damage [250].

As another analog of MKT-077, JG-48 (**68**, Fig. 15) reduced tau levels by ~50% at 30 μM in HeLaC3 which had been stably transfected with human 4R0N tau [251]. In SH-SY5Y neuroblastoma cells, JG-48 (10 and 30 μM) decreased endogenous total and p-tau levels as well. In hippocampal brain slices from the rTg4510 transgenic mouse model, JG-48 (10, 30 and 100 μM) reduced the levels of total and p-tau. Furthermore, tau levels in tubulin-positive neurons were significantly reduced by JG-48 treatment (10 μM) [251]. Another analog JG-98 (**69**, Fig. 15) also reduced tau levels in Hela-V5-Tau cells and DIV15 cortical primary neurons at 10 μM [252]. Recently, another Hsp70 inhibitor, VER-155008 (**70**, Fig. 15) (i.p. 10 μmol/kg/day, 18 days), was shown to significantly reduce

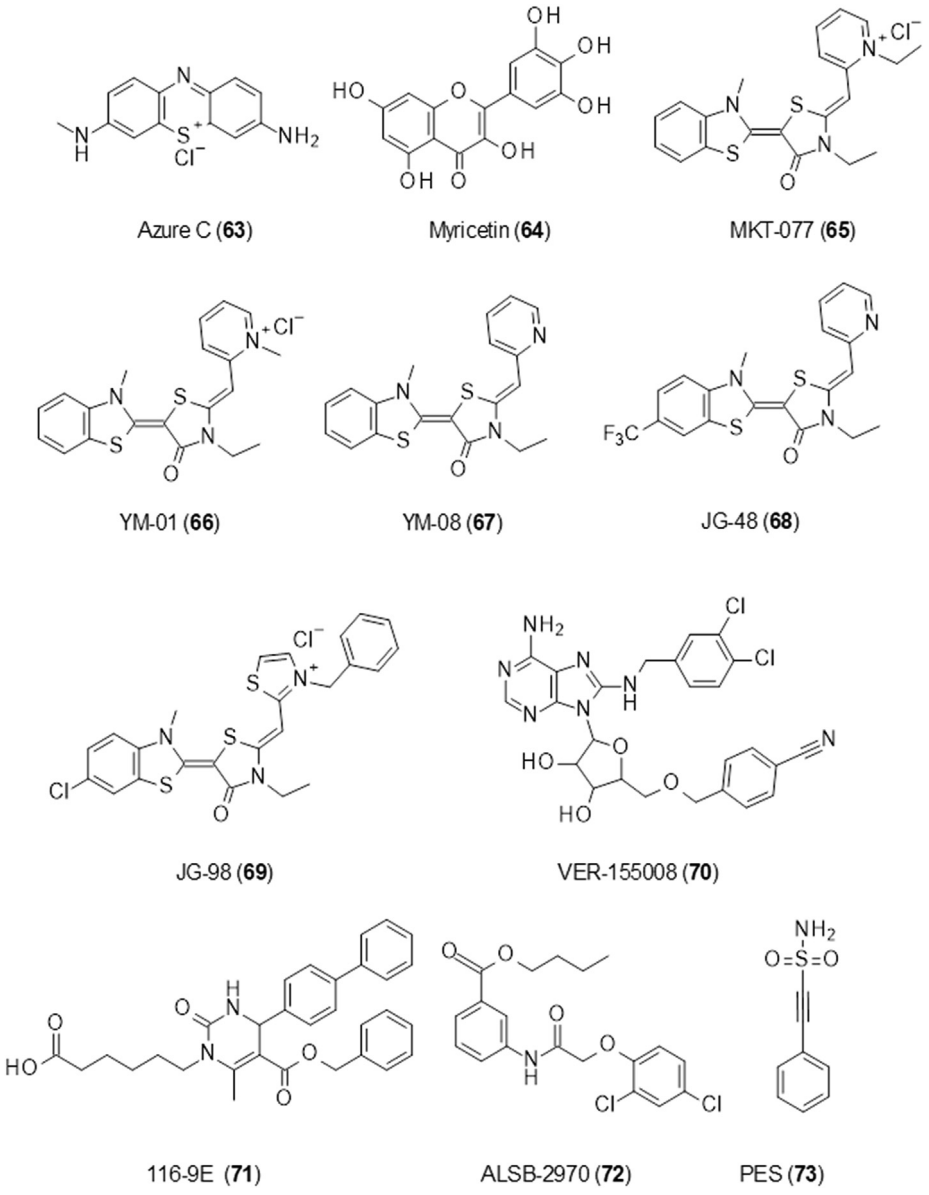

Fig. 15. Hsp70 modulators.

amyloid plaques and PHF tau accumulation in 5 × FAD mice [253]. Furthermore, VER-155008 penetrated into the brain after i.p. administration, suggesting that VER-155008 can act in the brain *in situ*.

Methylene blue which has been identified as a potent Hsp70 inhibitor and an autophagy inducer, also inhibits tau aggregation *in vitro* [159]. Based on the known pleiotropy of methylene blue, Martin et al. [254], investigated the anti-tau efficacy of additional scaffolds with Hsp70 inhibitory activity. They found compounds with activity against both Hsp70 activity and tau aggregation *in vitro* can predict tau-lowering activity at greater than 90% when both cytotoxicity and PAINS (Pan Assay Interference Compounds) classification are accounted for. In this research, Martin et al., examined the tau-lowering capability of several published Hsp70 inhibitors and found that phenothiazine and rhodacyanine compounds, with high anti-tau aggregation potency, reduced tau levels at all concentrations (3–30 μM) in HEK293T cells overexpressing tau. However, dihydropyrimidine (116-9E) (**71**), phenoxy-*N*-arylacetamide (ALSB-2970) (**72**), sulfonamide (2-phenylethynesulfonamide, PES) (**73**, Fig. 15) and flavonol (myricetin) scaffolds, with low anti-tau aggregation potency, only lowered tau levels at the highest concentration tested, 30 μM..

3.3.3.2. Hsp90. Hsp90 has been implicated in allowing for the accumulation of aberrant tau species which can result in neuronal death. There are many classes of Hsp90 inhibitors (Fig. 16) involving in tau regulation, such as geldanamycin analogues (17-AAG) (**74**), purine class i.e. EC102 (**75**), PU-DZ8 (**76**) and PU24FCl (**77**), dihydropyridine derivatives (LA1011) (**78**), novobiocin analogues (KU32) (**79**), and celastrol type (celastrol) (**80**).

17-AAG is a less toxic analog of the geldanamycin which inhibits HSP90 by interfering with its ATP-binding site. At 200 nM, it reduced p-tau levels (pT181, AT8 and AT270) in primary neurons derived from the cerebral cortices of embryonic day 17 (E17) mouse embryos [255]. In COS-7 cells overexpressing human wild-type tau or P301L mutation, 17-AAG (0.4–0.8 μM) resulted in the decreased

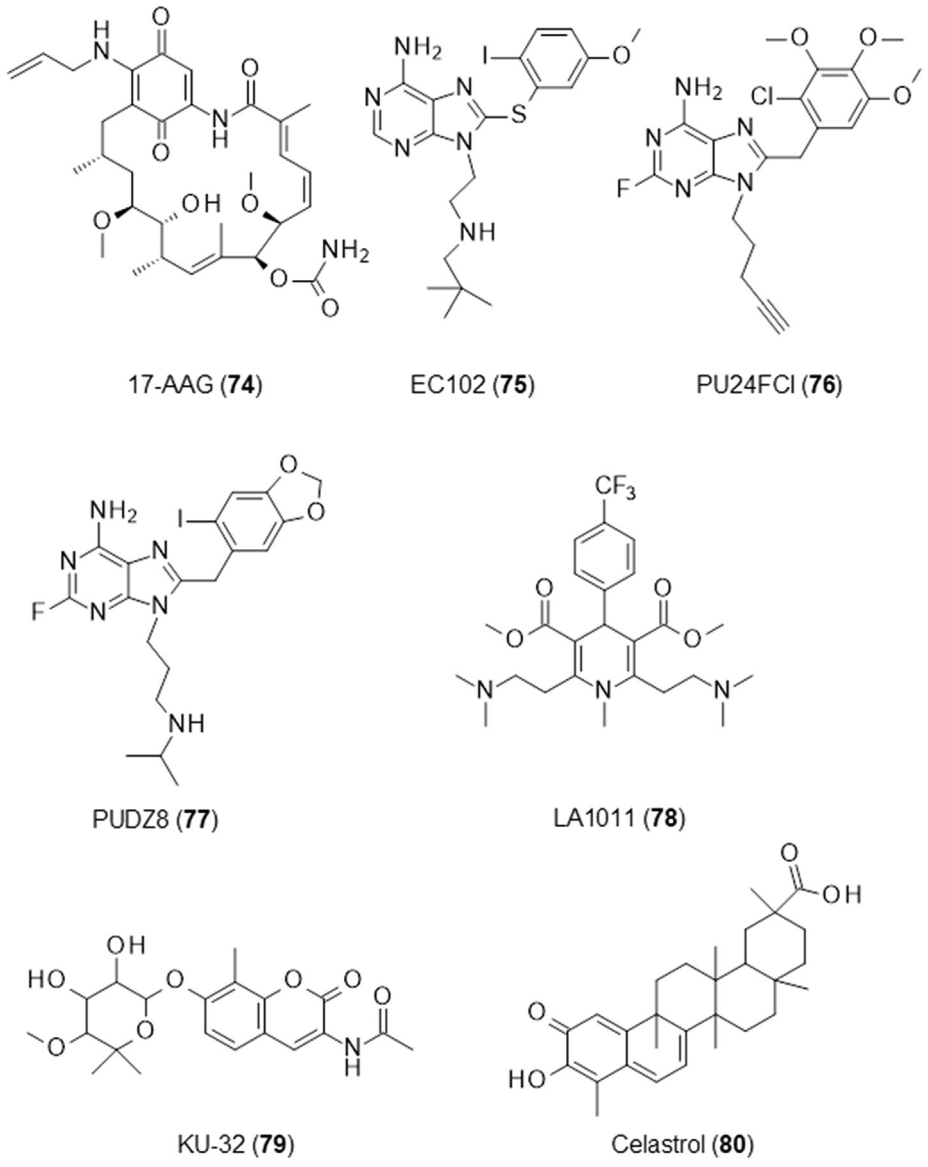

Fig. 16. Hsp90 modulators.

levels of mutant tau while leaving levels of wild-type tau unaffected [256].

As one of the purine-based Hsp90 inhibitors, EC102 was shown to enhance degradation of abnormal p-tau in P301L tau-expressing cells [257,258]. EC102 also reduced total tau and p-tau levels both in cultured human HeLa cells expressing V5-tau (1 μM EC102) and in transgenic Htau mice (200 mg/kg EC102, i.p. once daily for 7 days), and it can cross the BBB following i.p. administration [259]. PU24FCl, another purine-based Hsp90 inhibitor, was developed by the group of Chiosis G. PU24FCl (5–10 μM) decreased p-tau levels in primary embryonic cortical neurons and lowered phosphorylated and mutant tau levels in COS-7 cells [256]. PU-DZ8 is a higher potency water-soluble derivative of PU24FCl and can permeate the BBB. A single dose of PU-DZ8 (75 mg/kg, i.p.) administered to tau transgenic mice (JNPL3 line) resulted in a significant reduction of mutant tau levels [256].

KU-32 is a novel, novobiocin-based Hsp90 inhibitor and can reduce tau levels at 20 μM in HeLa V5-Tau cells [252]. Dihydropyridine derivatives bind to the C-terminal and middle-domain of Hsp90 and activate its ATPase activity, which in turn compromises the chaperone activity of Hsp90 [260]. As one of the dihydropyridine derivatives, LA1011 (i.p., 3 mg/kg, once a day for 6 months) decreased the NTF number in an APP × PS1 AD mouse model and effectively improved the spatial learning and memory functions in wild-type and AD mice [261].

Celastrol, a natural triterpene compound isolated from the plant family *Celastraceae*, interacts with Hsp90 C-terminal domain and inhibits its ATPase activity without blocking the ATP-binding pocket [262]. Cao et al., demonstrated that celastrol (600 nM) decreased p-tau (S396 and S199/202) and Hsp90 induced by $A\beta_{1\text{-}42}$ in SH-SY5Y cells [263]. However, celastrol had no effect on the ubiquitination of tau, expression of Hsf1 and Hsp70, and the interaction between Hsp70 and tau or Hsp70 and CHIP induced by $A\beta_{1\text{-}42}$. The results suggest that celastrol inhibition of tau hyperphosphorylation is not dependent on the cause of HSF-1/Hsp70/CHIP-mediated ubiquitination of tau.

3.3.3.3. Co-chaperones. CHIP plays an important role in the removal of p-tau. Deletion of CHIP in mice leads to the accumulation of non-aggregated, ubiquitin-negative, hyperphosphorylated

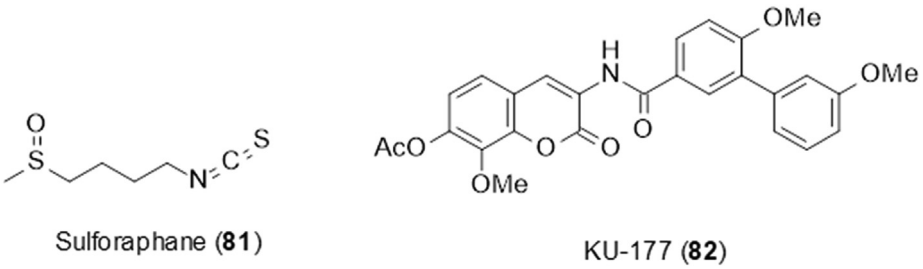

Fig. 17. Co-chaperone modulators.

tau species, which also be supported in *Caenorhabditis elegans* and cell culture systems using RNA interference for *CHIP* (*chn-1*) [264]. Oral gavage of sulforaphane (**81**, Fig. 17) (10 or 50 mg/kg/d, 6 days a week for 8 weeks) increased CHIP and Hsp70 levels, reduced total and p-tau levels, and ameliorated memory deficits in 3 × Tg-AD mice [265]. Furthermore, in CHIP-deficient primary neurons derived from 3 × Tg-AD mice, sulforaphane failed to clear tau, suggesting that CHIP might be involved in sulforaphane-mediated tau clearance [265].

The Hsp90 co-chaperone Aha1 can increase tau fibril formation resulting in insoluble tau accumulation by stimulating Hsp90 ATPase activity [266]. KU-177 (**82**, Fig. 17) (10 μM) inhibited the binding of Aha1 to Hsp90 in PC3-MM2 and HEK cells and reduced Hsp90/Aha1-mediated insoluble P301L tau accumulation in HEK (iHEK)-P301L cells [266]. This indicates that Aha1 may be a promising target for the development of therapeutics directed toward reducing tau aggregation.

The overexpression of FK506-binding protein 51 (FKBP51), a Hsp90 co-chaperone, reduces tau ubiquitination in HeLa cells stably overexpressing V5-tagged wild-type human tau [267]. Further *in vitro* studies indicated that FKBP51 and Hsp90 synergistically prevent tau degradation by the 20S proteasome and produce non-amyloid, Thioflavin T-negative tau oligomers [268]. These results suggest that strategies aimed at attenuating FKBP51 levels or the interaction between FKBP51 and Hsp90 have the potential to be therapeutically relevant for tauopathies. The group of Hausch F. has recently developed selective FKBP51 inhibitors, SAFit1 and SAFit2, and proved that these inhibitors could enhance neurite outgrowth and have antidepressant-like effects in male C57BL/6 mice [269]. A recent study showed that SAFit2 could also reduce alcohol consumption and reinstatement of conditioned alcohol effects in mice [270]. However, there has not been any studies of these FKBP51 inhibitors in tauopathy.

Co-chaperone BCL2-Associated Athanogene 2 (BAG2) plays a vital role in clearing tau tangles from neurons. The BAG2/Hsp70 complex is tethered to the microtubule and this complex can capture and deliver tau to the proteasome for ubiquitin-independent degradation [271]. Curcumin (**31**) significantly up-regulated BAG2 levels and downregulated abnormally p-tau (AT8) in primary rat cortical neurons at 12.5 μM, suggesting a possible role of curcumin-induced BAG2 expression in the reduction of p-tau levels [272]. *In vivo*, curcumin treatment (500 ppm curcumin in PMI 5015) suppressed soluble tau dimers in aged human tau expressing transgenic mice [273]. Overall, except methylene blue and curcumin, other molecules which target molecular chaperones or co-chaperones and reduce tauopathies do not have any related clinical studies in AD patients to date.

4. Tau-oriented multi-target directed ligands

It is well established that AD is a complex multifactorial disease including Aβ and tau misfolding/aggregation components, decreased cholinergic signaling, CNS inflammation, energy metabolism deficiency, oxidative stress, dysregulation of brain metal homeostasis, synaptic loss and neurodegeneration. Genetic background and environmental risk factors also contribute to the AD progression. Many small molecules targeting tau are based on the "one drug-one target" paradigm and have gone through various stages of preclinical and clinical development in the treatment of AD. However, until now the single-target drugs have not been successful in AD clinical trials. MTDLs are single molecules capable of interacting with multiple targets. Designing and evaluating MTDLs could be a promising research strategy for developing a cure for multi-factorial AD.

Some MTDLs are hybrid molecules with two or more pharmacophores modulating multiple biological targets. Shogaol-huprine hybrid (**83**, Fig. 18) and levetiracetam-huprine hybrid (**84**, Fig. 18) possess multi-target activities including the inhibition of tau aggregation. Perez-Areales et al., designed and synthesized novel class of shogaol-huprine hybrid (**83**) [274]. These hybrid compounds were evaluated in *in vitro* assays for their inhibitory activity against human acetylcholinesterase (AChE) and butyrylcholinesterase and antioxidant activity (ABTS+, DPPH and Folin-Ciocalteu assays), and in intact *E. coli* cells for their $A\beta_{1-42}$ and tau anti-aggregation activity. In another study, Sola et al., synthesized a series of heptamethylene-linked levetiracetam-huprine and levetiracetam-(6-chloro)tacrine to evaluate their activity against Aβ, tau, and cholinergic pathologies and Aβ-induced epilepsy [275]. They found the inhibition activity against human AChE and butyrylcholinesterase in an *in vitro* assay and amyloid aggregation model of *E. coli* which were incubated with moderately potent Aβ and tau aggregating agents. The putative effect of levetiracetam-huprine hybrid (**84**) on epileptiform activity is complemented with a direct effect on amyloid and tau pathologies, neuroinflammation, and cholinesterases in transgenic APP/PS1 mice. Hybrid molecules targeting GSK3β and AChE or beta-site amyloid precursor protein cleaving enzyme 1 (BACE1) or HDACs have also been developed. Oukoloff et al., synthesized and evaluated a series of tacrine (AChE) -valmerin (GSK3α/β) hybrids with a linker containing a 1,2,3-triazole moiety [276]. In these hybrid molecules, compound **85** (Fig. 18) showed the most promising potencies *in vitro*, inhibiting both human AChE and GSK3α/β in the nanomolar range (9.5 and 7 nM, respectively). Moreover, in cell lines they exhibited low cytotoxicity and a good ability to penetrate the BBB without interacting with efflux pumps such as P-gp. The first class of BACE1/GSK3β dual inhibitors were reported by Prati et al. that based on a 3,4-dihydro-1,3,5-triazin-2(1H)-one skeleton, with a guanidino

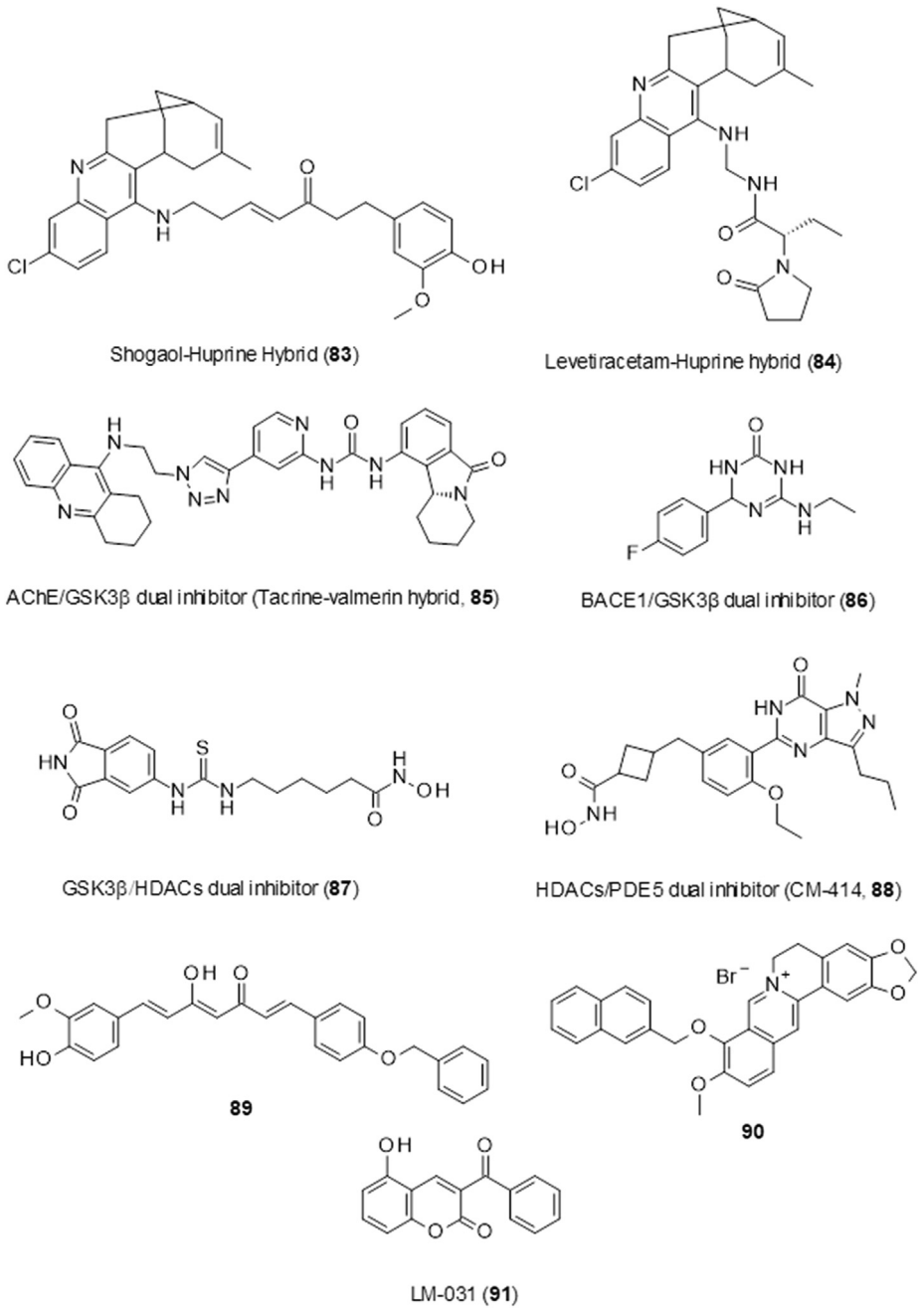

Fig. 18. Tau oriented multi-target directed ligands.

motif (BACE1) and a cyclic amide group (GSK3β) [277]. The hit compound **86** (Fig. 18) potentially inhibited BACE1 and GSK3β with IC_{50} value of 16 and 7 μM, respectively and displayed cellular neuroprotective and neurogenic effects without neurotoxicity. In mice, compound **86** reached a maximum concentration (1.50 ng/mg) 30 min after i.p. administration (10 mg/kg). De Simone et al., developed the first class of dual GSK3β/HDACs inhibitors possessing a hydroxamic acid as the zinc-binding group (HDACs) and the phthalimide moiety as cap group (GSK3β) [278]. Among them compound **87** (Fig. 18) showed the best biochemical profile with low molecular weight and high solubility. In addition, Cuadrado-Tejedor et al., discovered a new molecule, CM-414 (**88**, Fig. 18), which acts as a dual inhibitor of PDE5 and HDACs [279]. CM-414 rescued the impaired long-term potentiation evident in hippocampal slices from APP/PS1 mice. In Tg2576 mice, chronic treatment with CM-414 (40 mg/kg, i.p., 4 weeks) diminished brain Aβ and p-tau levels, increased dendritic spine density on hippocampal neurons, and ameliorated cognitive deficits.

Another group of MTDLs are natural products and their derivatives. Natural products are synthesized by living organisms in nature and have evolved for optimal interactions with biological macromolecules. As an essential source of drug discovery, natural products also have shown to affect multiple pathological pathways including tau in AD, such as resveratrol (**5**) and curcumin (**31**). But the low bioavailability and less clinical efficacy limit their therapeutic use. Therefore, efforts have been taken to design novel MTDLs based on natural templates with less drawbacks. For example, various curcumin derivatives have been synthesized and

evaluated, and they can inhibit tau/Aβ aggregation, AChE/BACE1 activities, and chelate metals and remove free radicals [280]. Moreover, Di Martino et al., discovered a new class of curcumin derivatives and the promising candidate (**89**, Fig. 18) inhibited BACE1 and GSK3β with IC_{50} value of 0.97 and 0.90 μM, respectively and endowed with neuroprotective potential and brain permeability [281]. Another example is the berberine derivatives. In the study of Sobolova et al., berberine core was substituted at position 9-*O* of its aromatic ring region, and these derivatives, especially the top-ranked compound (**90**, Fig. 18), showed multi-targeted profile inhibiting tau/Aβ aggregation, prolyl oligopeptidase, AChE and butyrylcholinesterase [282]. In addition, Lin et al., examined the ability of licochalcone A and its five derivatives in cell assays for inhibiting tau aggregation, oxidation, and providing neuroprotection [283]. Among them, LM-031 (**91**, Fig. 18) showed high potency in cell assays, reduced tau and Aβ levels in the hippocampus and cortex, and rescued cognitive deficits in streptozocin-induced hyperglycemic 3 × Tg-AD mice. In parallel artificial membrane permeability assay, LM-031 was categorized as the high BBB permeable compound [283].

Now the development of MTDLs is in preclinical stage. Even though MTDLs are able to modulate multiple targets, their clinical trials may face more challenge. Because most of MTDLs are designed by combing different pharmacophores, their molecular weight, solubility and permeability have to be optimized for absorption and BBB penetration.

5. Summary and future perspective

As summarized in this review, there are numerous therapeutic small molecules under investigation to reduce tau pathology in AD. These molecules include tau post-modification modulators, aggregation inhibitors and degradation promotors. Currently, most of them are in preclinical stage, and only fourteen molecules enter clinical phases, namely LMTM, Rember TM, curcumin, BPN14770, cilostazol, nilotinib, minocycline, lithium, tideglusib, saracatinib, sodium selenate, AZP2006 [284], salsalate and rapamycin (Fig. 19). Even though there are no therapeutic candidates obtaining the final success in AD clinical trials, the recent Phase III trial of LMTM (NCT03446001) is still ongoing.

Given the complex pathology of AD, tau-oriented MTDLs are developed based on the structures of small molecule tau therapeutics, and their emerging preclinical research provide promising future for the treatment of multi-factorial AD [285,286]. That is also one of the areas our lab is working on [287,288]. Another multi-target approach, the combination therapy, is beginning to be

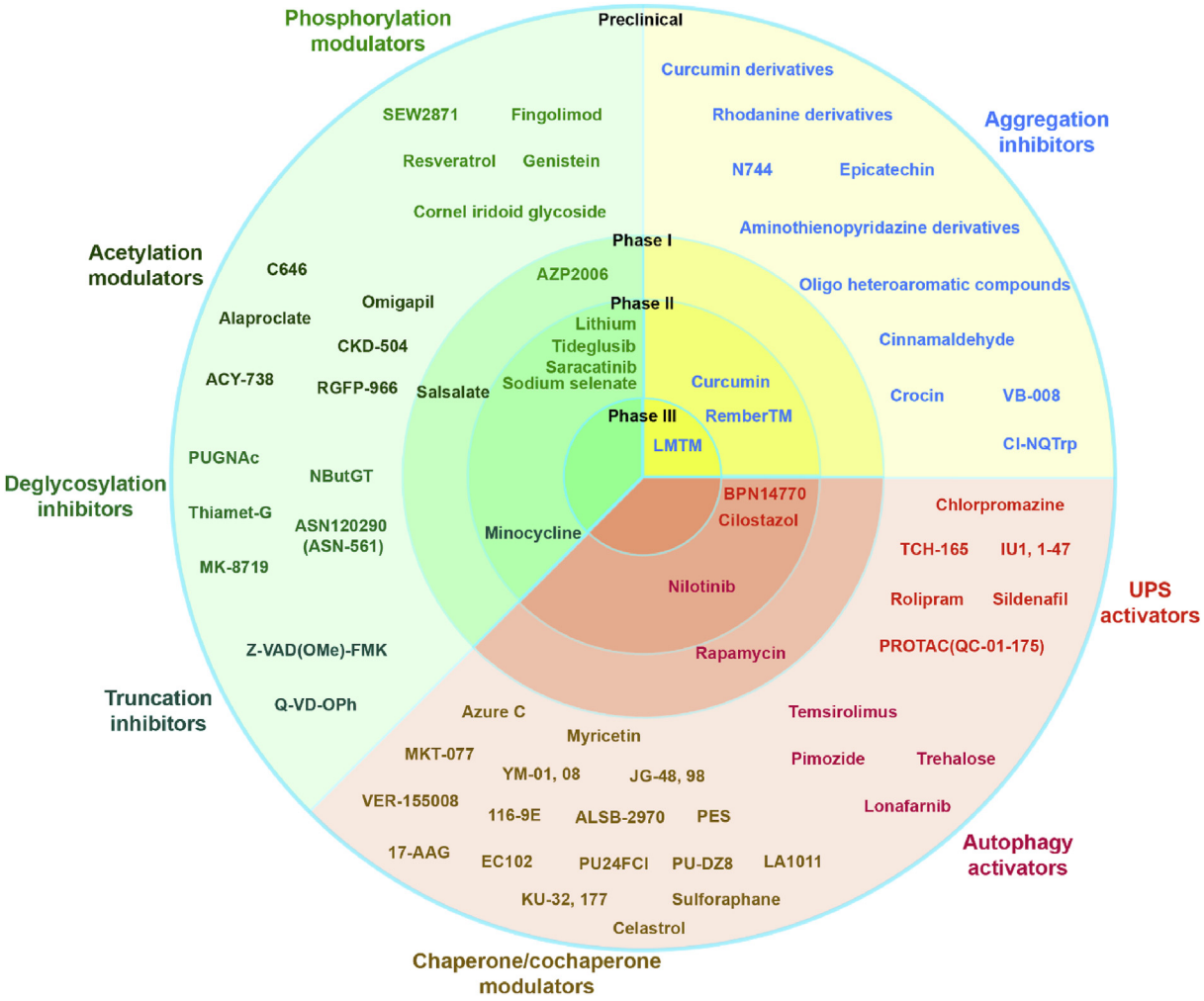

Fig. 19. Small molecules targeting tau in preclinical and clinical trials of Alzheimer's disease. Small molecules targeting tau in AD preclinical or clinical development include tau post-translational modification modulators (phosphorylation modulators, acetylation modulators, deglycosylation inhibitors and truncation inhibitors), aggregation inhibitors and degradation promotors (UPS activators, autophagy activators and chaperone/cochaperone modulators). AD, Alzheimer's disease; UPS, ubiquitin-proteasome system.

explored and can be tailored to each patient, as is the current HIV treatment. For example, by combining drugs targeting tau, Aβ, CNS inflammation and cholinergic system, one could simultaneously address these four pathologies in AD. However, it is challenging to choose the right combination for superior therapeutic effect and side-effect profile. This approach will also increase the risk of possible drug-drug interactions and reduce patient compliance and therapy adherence. As the development of computational methodologies in drug discovery, we believe that the complicated multi-target approaches can be realized for AD treatment.

Another reason for the clinical failures is that the drug cannot reaching the human target *in vivo*. Nanoparticles-mediated drug delivery systems could improve drug solubility and bioavailability, facilitate drug delivery to the brain, and favor multiple drug loading and higher loading capacity [289]. Interestingly, Sonawane et al., reported a novel role of the protein-capped metal (cadmium sulfide) nanoparticles in inhibiting tau aggregation [290]. Thus, nanoparticles render as superior alternatives for AD treatment.

Importantly, AD neurodegeneration is the result of a several-step process and involves a host of environmental and genetic factors. It is crucial to choose optimal treatments in different stages of AD progression and for each individual patient. Future clinical trials should be empowered by the robust use of biomarkers such as neuroimaging [291], blood and CSF biomarkers, proteomic and genomic AD biomarkers and innovative trial designs [292]. For the pre-symptomatic group disease preventive clinical trials are also ongoing [293]. Future AD clinical trials should also include more sophisticated approaches of "personalized medicine" accounting to patient genetic background, lifestyle risk factors, AD biomarkers profile and neuropsychological evaluation profile.

The up-to-date advances in the development of small molecules targeting tauopathy provide hope for AD treatment in combination with drugs targeting other pathologies. Meanwhile, the active investigation will also unravel the complexities of tauopathy and its interactions with other AD targets, which will give us a better understanding of AD mechanisms for future drug discovery. Moreover, development of small molecules targeting tau would also hold better chance to be efficient in primary tauopathies such as FTD-tau, corticobasal degeneration, PSP or traumatic brain injury where molecular pathology landscape is dominated by the misfolded and aggregated tau. No matter how many difficulties in the development of AD treatment, we believe that the successful therapeutic approach for AD will be found depending on the establishment of critical AD targets, development of effective agents and conduction of rigorous clinical trials.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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