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Feature

Dual-target inhibitors of cholinesterase and GSK-3β to modulate Alzheimer's disease

Junqiu He and Kin Yip Tam *

Faculty of Health Sciences, University of Macau SAR, Avenida de Universidade, Taipa, Macau SAR, China

Alzheimer's disease (AD) is a neurodegenerative disease that affects over 55 million patients worldwide. Most of the approved small-molecule drugs for AD have been designed to tackle a single pathological hallmark, such as cholinergic dysfunction or amyloid toxicity, and thus may not fully address the multifactorial nature of the disease. Inhibition of both cholinesterase and glycogen synthase kinase-3β (GSK-3β) has emerged as a promising strategy to modulate AD. However, the dual inhibition of these two targets posts challenges in molecular design: issues related to target engagements and biopharmaceutical properties in particular must be overcome. In this review, we discuss the physiopathological roles and structures of cholinesterase and GSK-3β as well as recently reported dual-target inhibitors. We critically evaluate the current status of the discovery of dual-target inhibitors of cholinesterase and GSK-3β, and highlight further perspectives.

Keywords: GSK-3b; cholinesterase; dual-target inhibitors; Alzheimer's disease; drug design strategy

Introduction

Alzheimer's disease (AD) is one of the most common neurodegenerative diseases causing dementia, with estimated 150 million cases worldwide in 2050.[p1] Four classic agents, including three cholinesterase inhibitors (donepezil, rivastigmine, galantamine) and one N-methyl-D-aspartate (NMDA) receptor antagonist (memantine), are approved for AD treatment, mainly for enhancing cognitive function and relieving symptomatic burden.[p2] In addition, there are two monoclonal antibodies, aducanumab and donanemab, recently approved for the elimination of amyloid plaques in USA,[p3],[p4] and sodium oligomannate, which was approved in China as a drug to modulate neuroinflammation by regulating the gut microbiome.[p5] Unfortunately, none of these drugs can cure AD because of its complex pathophysiology and multifactorial characteristics.

Evidence accumulated from studies of the pathophysiology of AD carried out in recent decades shows that abnormally folded amyloid β (Aβ) and hyperphosphorylated tau protein are the two hallmarks of AD. These abnormalities lead to the formation of extracellular senile plaques and intracellular neurofibrillary tangles (NFTs), followed by cholinergic neurodegeneration and neuronal loss in the brain.[p1] With the advancement of research methods and analysis, the pathological characteristics of AD, such as neuroinflammation and metabolic dysfunction, have been further elucidated.[p6] Sequencing studies have identified dozens of risk-associated genes and loci in AD patients over the years.[p7]

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Researchers working on the basis of what is known about AD pathologies have put considerable effort into drug development, with only limited success. In recent years, many attempts have been made to explore multi-target directed ligands (MTDLs), which enable the simultaneous modulation of multiple targets to tackle this complicated disorder,[p8] simply because monotherapies have failed to combat AD effectively.[p9]

Although complex pathologies such as AD post challenges, they also offer multiple therapeutic targets that can be exploited in the design of MTDLs for AD treatment. First, the classic cholinesterase seemed to be the most promising target for cognitive improvement, leading to the approval of some ChE inhibitors that provide modest benefits.[p10] Other targets based on different pathological characteristics, such as amyloid peptide or tau protein, may be exploited to achieve disease-modifying benefits.[p11] A target that regulates more than one molecular pathway and plays a cross-talk role in progressing the pathophysiology is expected to be very useful in developing a disease-modifying strategy. Glycogen synthase kinase 3β (GSK-3β) is such a target. It is ubiquitously expressed in humans, especially in the central nervous system (CNS), and plays pivotal roles in the pathogenesis of AD including Aβ production, tau phosphorylation and neuroinflammation.[p12] Dual inhibition of cholinesterase and GSK-3β could achieve symptom relief and disease modification simultaneously, and has therefore attracted considerable attention as a strategy for modulating in recent years. In particular, there are active attempts to design and evaluate such dual-target inhibitors in preclinical settings. In this review, we introduce the pathophysiological functions of these two targets and specifically highlight their interplays in the context of AD. Then, we discuss the structures of cholinesterase, GSK-3β and the recently reported dual-target inhibitors. Finally, we assess the current status of this strategy and highlight further perspectives for optimizing dual-target inhibitors to enhance the chance of successful clinical translation.

Physiology and function of cholinesterase and GSK-3β in AD

The cholinergic hypothesis put forward in 1976 is one of the earliest hypotheses for the physiopathology of AD.[p13] It suggests that acetylcholine is dramatically decreased in AD patients and that selective loss of the cholinergic system is an important feature of this disease.[p13] Acetylcholine is a major neurotransmitter in synaptic transduction and its level is regulated by enzymes that include choline acetyltransferase (ChAT) for its generation and cholinesterase (ChE) for its degradation.[p14] There are two ChEs, namely acetylcholinesterase (AChE) and butyrylcholinesterase (BuChE). AChE is mainly expressed in the CNS where it is responsible for acetylcholine hydrolysis, whereas butyrylcholinesterase predominately exists in the peripheral circulation system where it has ambiguous biological purposes besides the hydrolysis function.[p15] Moreover, ChEs can directly interact with pathophysiological hallmarks such as Aβ and can indirectly influence other risk factors such as neuroinflammation and oxidative stress in AD pathology.[p16] It has been reported that acetylcholinesterase can bind directly with presenilin-1 (PS-1), a component of γ-secretase that is involved in Aβ production by cleaving the amyloid precursor protein (APP), and thus can inhibit secretase activity.[p17] Such interactions could modulate the trafficking and maturation of AChE in Golgi regions and could favor its active form.[p18] A fragment peptide cleaved from the C-terminus of AChE was shown to be elevated in AD brain.[p19],[p20] This peptide had neurotoxic effects by mediating calcium influx and triggering a series of downstream effects, such as changes in the levels of APP, Aβ and phosphorylated tau proteins (p-Tau) through the modulation of alpha-7 nicotinic acetylcholinesterase receptor (α7nAChR).[p19],[p20] Moreover, Aβ could interact with BuChE and apolipoprotein-E (APOE), leading to the formation of soluble ultrareactive acetylcholine-hydrolyzing complexes in which the intrinsic catalytic efficiency of the ChE is allosterically modulated, thereby regulating synaptic and extracellular acetylcholine signaling.[p21]

The other target, GSK-3β, also participates in Aβ regulation, although the key role of GSK-3β in AD progression is to phosphorylate tau protein at dozens of sites (such as T181, T231 and S396), thereby accelerating the disassembly of microtubules and the formation of NFTs.[p22] Notably, the level of activated GSK-3β has been shown to be increased in AD patients.[p23] GSK-3β can modulate the production of Aβ by regulating different components of the APP processing system; for example, it can induce both γ-secretase activity through PS-1 interaction and β-secretase (BACE1) expression by activating NF-κB.[p24] It has been reported that a feedback loop between Aβ and GSK-3β activation leads to tau hyper-phosphorylation, as Aβ can disrupt the normal activity of the Wnt pathway which is supposed to exert inhibitory effects on GSK-3β.[p25],[p26] In addition, GSK-3β, a pivotal kinase in AD, has links to other abnormalities, such as the promotion of microglial response to inflammation,[p27] and participates in the regulation of autophagy and apoptosis processes leading to neurone death.[p28],[p29] Interestingly, it appears that the fate of cholinesterase could be regulated by GSK-3β. It has been reported that the hyperphosphorylation of tau that results from the overexpression of GSK-3β increased the activity and expression of AChE and decreased the level of acetylcholine in the SH-SY5Y cell line.[p30] In patients, the increased AChE activity seen in the placebo group was not observed in the group treated with a GSK-3β inhibitor.[p30] Moreover, GSK-3β has been reported to induce synaptic AChE expression and to stabilize it by co-localization in the cytosol of PC12 cells, whereas the inhibition of GSK-3β by lithium could induce rapid proteasomal degradation of the enzyme.[p31],[p32] Conversely, the activity of GSK-3β could be regulated by ChE inhibitors via α7nAChR simulation and activation of the PI3K-Akt axis.[p33] These lines of evidence highlight the complex interplay between GSK-3β and ChEs in AD. The main roles of ChEs and GSK-3β, as well as their links with AD, are summarized in Figure 1. Given the pivotal roles of ChEs and GSK-3β in AD pathophysiology, dual-target inhibition could be a promising direction in the development of treatments for this disease.[p10],[p34]

Structural characteristics of the binding sites of GSK-3β and ChE

Over the years, considerable efforts have been made to obtain structural information from complexes of small-molecule inhibitors and GSK-3β or ChE proteins (of human origin), which are well documented in the protein data bank (PDB).[p35] The structural characteristics of the binding sites have undoubtedly

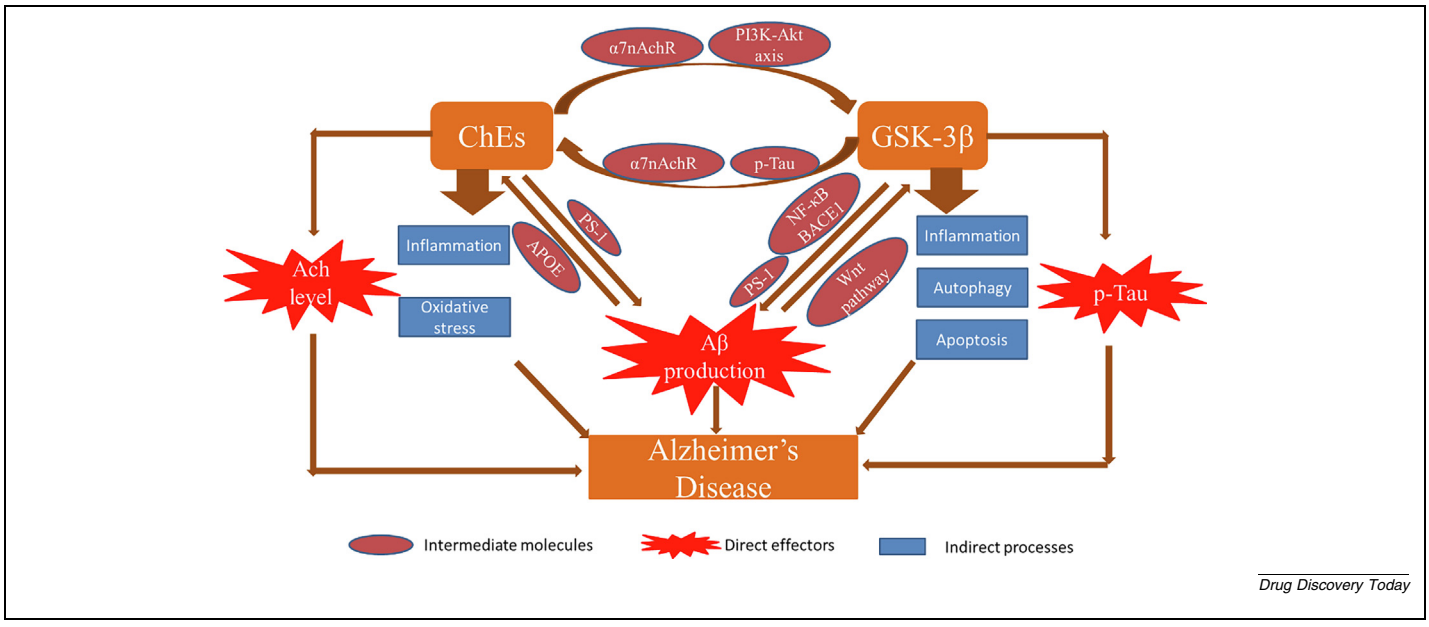


FIGURE 1

The main roles of cholinesterases (ChEs) and glycogen synthase kinase-3β (GSK-3β) in Alzheimer's disease pathology. In addition to regulating the level of acetylcholine and the phosphorylation of tau, ChEs and GSK-3β can modulate each other through multiple pathways and play important roles in amyloid β (Aβ) production. Aβ level, in turn, can modulate ChEs and GSK-3β activity. Ach, Acetylcholine; APOE, Apolipoprotein-E; BACE1, Beta-site amyloid precursor protein (APP) cleaving enzyme 1; NF-κB, Nuclear factor-κB; PI3K/AKT, Phosphoinositide 3-kinase/protein kinase B; PS-1, Presenilin-1.

informed the design of novel small molecule inhibitors (Figure 2).

As highlighted above, GSK-3β is a serine/threonine kinase, which phosphorylates a number of substrates that play important roles in neurodegenerative diseases. GSK-3β is highly expressed in the brain, particularly in neurons.[p36] The phosphorylation of substrates can occur in the Primed site or Unprimed (Axin) site of GSK-3β.[p37] It has been suggested that three binding sites are available in GSK-3β, namely, ATP, Primed and Axin sites.[p38] However, most of the reported small-molecule inhibitors in the PDB that complexed with GSK-3β were ATP competing. Only one structure reported substrate binding at the Primed site,[p39] although we are not aware of any example of small-molecule inhibitor binding to the Axin site in the PDB. Figure 2 shows the GSK-3β structure, with dotted color spheres highlighting the amino acid residues of the binding sites that interacted with known ligands (as reported in PDB). A molecular modelling approach has been used to identify additional GSK-3β binding sites that might enable allosteric modulation of the kinase,[p40] but these allosteric binding sites have not yet been confirmed by structural evidence.

With regard to ChE, AChE has a catalytic gorge that is responsible for the degradation of acetylcholine. Deep down in this gorge there is an acylation (active) site that contains the catalytic triad (Ser203/Glu334/His447) for hydrolyzing acetylcholine.[p41] This is often referred to as the catalytic active site (CAS). At the entrance of the active site, there is a peripheral active site (PAS).[p42] Trp86 is the key interacting amino acid residue of the CAS, whereas the key interacting residue of PAS is Trp286.[p42],[p43]

Although BuChE largely exists in serum, it has been reported that brain-targeted BuChE inhibitors can lead to improved cognitive performance in an animal model and can reduce Aβ level in the brain.[p44] Thus, both inhibition of AChE and modulation of BuChE are expected to be helpful in maintaining the cerebral acetylcholine level.[p45] Given the 65 % similarity in amino acid sequence, the structures of BuChE and AChE are comparable.[p46] Nevertheless, the two molecules do have subtle differences in the amino acid residues of the catalytic triads,[p46] as well as in the key interacting amino acid residues of the active site and the peripheral site. Figure 3 shows the structures of AChE and BuChE, with dotted colored spheres highlighting the amino acid residues of the binding sites that interacted with known ligands (as reported in PDB).

Dual-target inhibitors

Over the past five years, active attempts have been made to design, synthesize and identify dual-target inhibitors of cholinesterase and GSK-3β in order to modulate AD in preclinical settings (Table 1). The first reported family of dual-target inhibitors were developed by hybridizing a thiazolyl pyridine moiety with tacrine, the first approved AChE inhibitor. One inhibitor from this series **(1)** exhibited good AChE and GSK-3β inhibitory effects (IC_{50} of 6.4 ± 0.3 nM for AChE and 66.0 ± 6.2 nM for GSK-3β) and was less hepatotoxic than tacrine.[p47] Interestingly, **(1)** unexpectedly showed self-aggregation inhibition of Aβ. This inhibitor was then further optimized by simply altering the orientation of the thiazolyl pyridine moiety. This modification improved both the inhibitory effects and physicochemical properties of **(1)**, resulting in **(2)** with IC_{50} of 1.2 ± 0.1 nM for AChE and 22.0 ± 1.4 nM for GSK-3β.[p48] Molecular dynamics simulation studies revealed that the thiazolopyridine fragment bound with the ATP-binding site of GSK-3β, forming hydrogen bonds with the Val135 and Lys85 residues. Moreover, the thiazolopyridine fragment did not affect the binding of the tacrine moiety to the active site of AChE but bound to its peripheral site, so giving enhanced AChE inhibition.

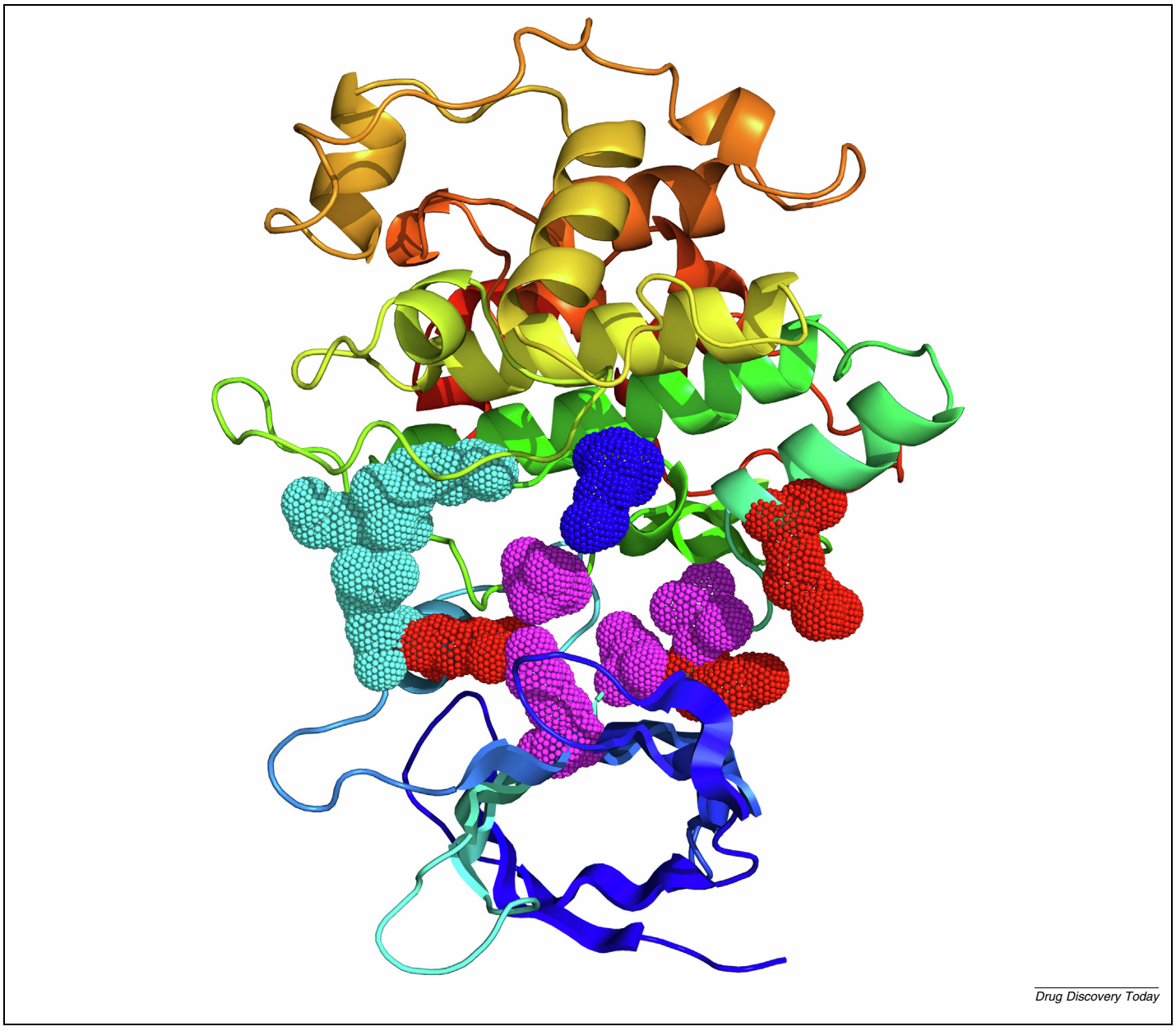


FIGURE 2

Structure of glycogen synthase kinase-3β (GSK-3β) showing the amino acid residues of the binding sites for known ligands. Cyan dotted spheres represent the known interacting residues of the Primed site (Arg96, Arg180 and Lys205; from PDB ID: 1I09[39]). Red dotted spheres represent the known interacting residues of the ATP site (Lys85, Glu97, Asp133, Try134, Val135, Arg141, Asp200; from PDB IDs: 1Q5K, 3 GB2, 3L1S, 6TCU and 7OY5). Blue dotted spheres represent the known interacting residues of the Axin site (Lys85, Asp133, Val135, Lys183 and Asp200; from [38]). The ATP and Axin binding sites share four interacting residues in the hinge region, which are represented by magenta dotted spheres (Asp133 and Val135, Lys85 and Asp200). The graphics were drawn by PyMOL using a reported GSK-3β structure (PDB ID: 4ACG).

Meanwhile, another research group synthesized a new series of tacrine–valmerin hybrids **(3)** and evaluated them *in vitro* and in cell cultures.[p49] The structure–activity relationship (SAR) for these hybrids is similar to that for **(2)**, with tacrine inhibiting AChE by binding to the CAS while the tetrahydropyridoisoindole fragment interacted with the ATP site of GSK-3β and the PAS of AChE. The representative hybrid **(3)** exhibited an improved binding affinity against GSK-3β (IC_{50} = 7.0 nM) but showed a slight drop in potency against AChE (IC_{50} = 9.5 ± 0.4 nM), suggesting that the valmerin moiety might offer a better engagement with GSK-3β when compared with the thiazolopyridine moiety. Moreover, a series of novel tacrine–pyrimidone hybrids were synthesized and identified as a potential treatment for AD. The representative compound **(4)** exhibited only moderate efficacy in target inhibition as compared with **(3)**, suggesting room for improvement in target engagement. Yet, **(4)** demonstrated neuroprotective effects in glyceraldehyde (GA)-stimulated SH-SY5Y cells and cognitive improvement in mice that had scopolamine-induced impairment.[p50]

Jiang *et al.*[p51] developed two series of dual-target inhibitors based on donepezil. One of the series was linked with a

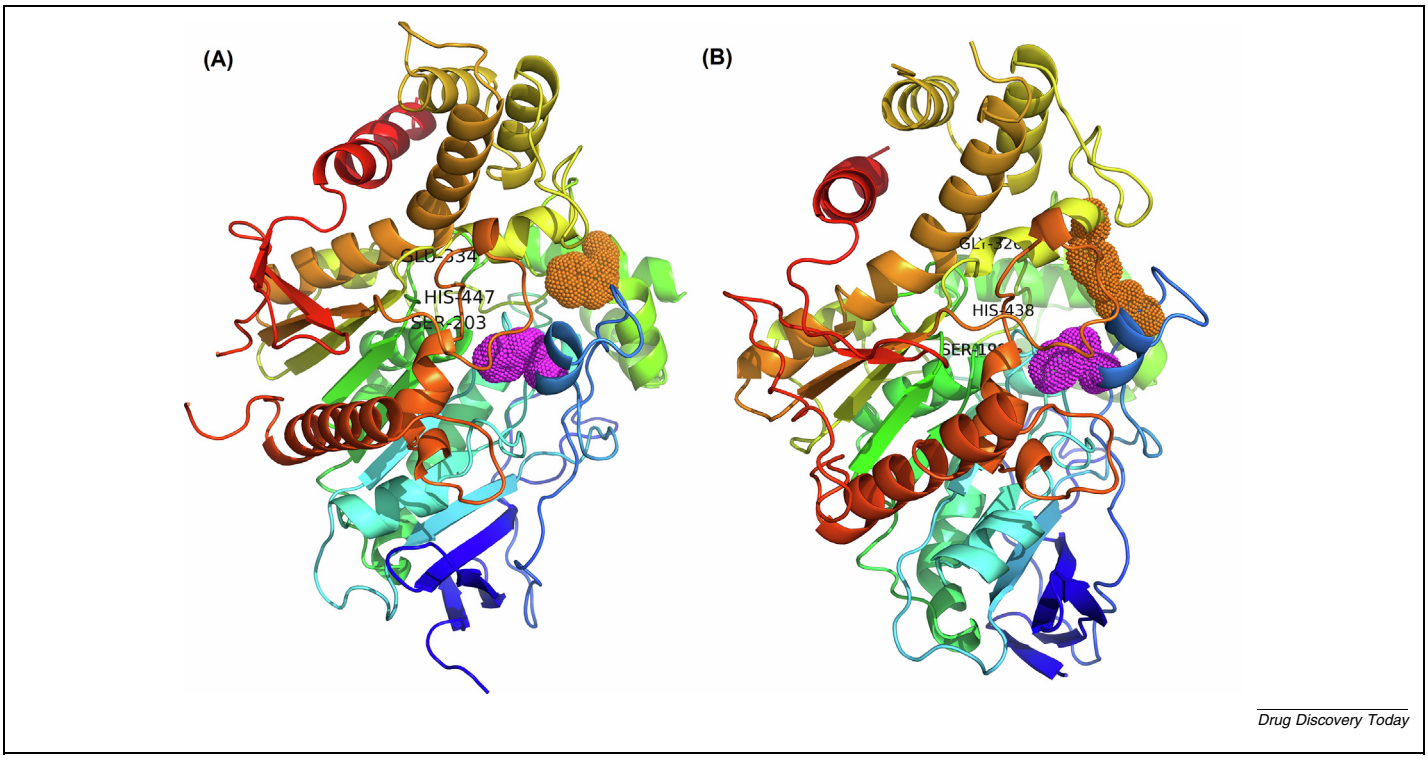


FIGURE 3

Structures of acetylcholinesterase (AChE) and butyrylcholinesterase (BuChE) showing the amino acid residues of the binding sites for known ligands. A. AChE structure (PDB ID: 4EY7) with magenta dotted and orange dotted spheres representing, respectively, the interacting residue of the active site (Trp86) and the peripheral site (Trp286) (from PDB IDs: 4E7F and 7D9P). The amino acid residues of the catalytic triad (Ser203/Glu334/His447) are labelled. **B.** BuChE structure (PDB ID: 4BDS) with magenta dotted and orange dotted spheres representing, respectively, the interacting residue of the active site (Trp82) and the peripheral site (Asp70, Tyr332) (from PDB IDs: 4BDS and 7D9P). The amino acid residues of the catalytic triad (Ser198/Glu325/His438) are labelled. The 3D view graphics were drawn by PyMOL.

thiazolopyridine moiety to produce **(5)**, which had some good drug-like properties, such as permeability across the blood–brain-barrier (BBB), but relatively poor AChE inhibitory efficacy (IC_{50} = 310.0 ± 70.0 nM).[p51] It is noted that **(5)** and **(1)** shared the same thiazolopyridine moiety as the pharmacophore of GSK-3β, but **(5)** exhibited a much lower IC_{50} of 3.0 ± 1.0 nM. This impressive IC_{50} was probably due, at least in part, to the way in which the thiazolopyridine moiety was conjugated to the linker (in **(5)** via methoxy, in **(1)** via formamide). To overcome the weak potency against AChE, which may have been due to steric hindrance from the formamide of the thiazolopyridine moiety in the binding to AChE, the same research group optimized **(5)** by replacing the thiazolopyridine moiety with an indazolyl-carboxamide or pyridyl-isonicotinamide moiety as the pharmacophore for targeting GSK-3β. This resulted in a series of novel N-(5-(piperidin-4-ylmethoxy) pyridin-3-yl) isonicotinamide analogs **(6)** that offered about 300-fold improvement in AChE inhibitory activity but about 10-fold drop-off in GSK-3β inhibition when compared with **(5)**.[p52]

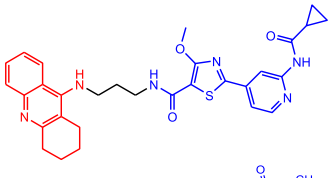
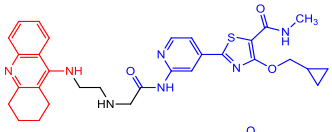
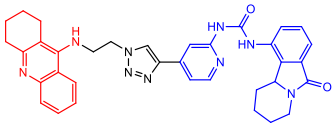
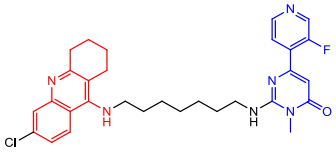
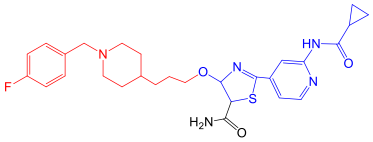
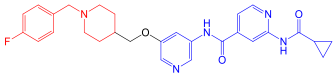
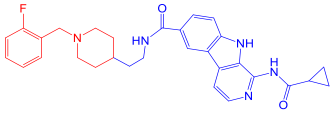
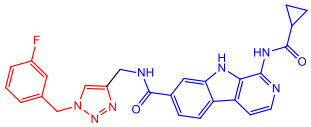
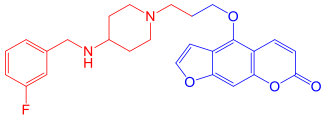
Other than synthetic small-molecule inhibitors, some chemically modified natural products or their derivatives have shown dual-inhibitory effects on AChE and GSK-3β. For example, a benzyl piperidine fragment of donepezil was conjugated with harmine,[p53] a natural alkaloid separated from the seeds of *Peganum harmala* L.. This conjugation generated a series of novel β-carboline derivatives **(7)** that had moderate dual-inhibitory potency against the two intended targets.[p54] The cyclopropylcarboxamide moiety and the pyridine ring of harmine formed the classic donor–acceptor motif, with Val135 and Lys85 in the hinge region of GSK-3β, and also bound to the PAS of AChE. Simultaneously, the benzyl piperidine fragment interacted with the CAS of AChE. Replacing the donepezil fragment with 1,2,3-triazole for CAS binding yielded another series of hybrids **(8)**, which had only moderate inhibitory potency but lower cytotoxicity in SH-SY5Y and HepG2 cells.[p55] Moreover, donepezil was hybridized with notopterol, a natural product from *Notopterygium*, resulting in moderate dual-inhibitory effects against AChE and GSK-3β **(9)**.[p56] Although the use of fragments derived from natural products might offer some improvements in cytotoxicity (such as those seen for **(8)**), the dual inhibitory effects against the intended targets are generally much weaker than those seen when both pharmacophores are derived from synthetic small-molecule fragments (as in **(3)** or **(6)**).

Current status and further perspectives

MTDLs and multi-target inhibitors for AD therapy have received considerable attention in recent years,[p57] prompted by the many failures of monotherapies in clinical evaluations.[p58] There have been some positive attempts to explore cholinergic enhancement and GSK-3β inhibition in AD trials, though none of the dual-target inhibitors that have been investigated have received regulatory approval yet. Tideglusib, a GSK-3β inhibitor, underwent

TABLE 1

Dual-target inhibitors of cholinesterases (ChEs) and glycogen synthase kinase-3β (GSK-3β) reported in the past five years. Red color highlights the pharmacophore for ChEs, whereas blue color highlights the pharmacophore for GSK3β. AChE, acetylcholinesterase; BuChE, butyrylcholinesterase.

No.	Structure	ChEs IC_{50} (nM) or % inhibition[a]	GSK-3β IC_{50} (nM) or % inhibition[b]	Ref
1		AChE: 6.4 ± 0.3 BuChE: 260 ± 32	66.0 ± 6.2	(p47)
2		AChE: 1.2 ± 0.1 BuChE: 149.8 ± 12.6	22.0 ± 1.4	(p48)
3		AChE: 9.5 ± 0.4 BuChE: 395.0 ± 27.0	7.0	(p49)
4		AChE: 51.1 ± 4.6	89.3 ± 0.1	(p50)
5		AChE: 310.0 ± 70.0 BuChE: 3410 ± 700	3.0 ± 1.0	(p51)
6		AChE: 1.0	31.0	(p52)
7		AChE: 270.0 ± 30.0 BuChE: 20,820 ± 1210	60.8 %	(p54)
8		AChE: 340.0 ± 10.0 BuChE: >10,000	1140.0 ± 50.0	(p55)
9		AChE: 58.7 %	40.3 %	(p56)

[a] % inhibition of AChE with the inhibitor at 1 μM.
[b] % inhibition of GSK-3β with the inhibitor at 10 μM.

a phase 2 clinical trial (ClinicalTrials.gov ID: NCT00948259) in which it was evaluated in AD patients with stable anticholinesterasic outcomes.(p59) Another compound, blarcamesine, a dual agonist for sigma-1 and muscarinic receptor and an inhibitor of GSK-3β, which is expected to modulate synaptic dysfunction, cholinergic neurotransmission and tauopathy, is currently in a phase 3 clinical trial for AD treatment (ANAVEX2-73, ClinicalTrials.gov ID: NCT04314934).(p8)

As summarized in Table 1, these recently reported dual-target inhibitors were originated from two pharmacophores of ChE and GSK-3β, which were conjugated by flexible linkers or direct fusion/merging. However, the pharmacophore options were limited to the known small molecules that specifically bind to the intended targets. For ChE inhibition, tacrine appears to be the preferred option because of its high anticholinesterase activity, simple structure and readiness to couple with the second pharmacophore via the primary amine group.(p60) Tacrine itself, however, showed hepatotoxicity resulting from the generation of reactive oxygen species (ROS) and glutathione depletion in the liver.(p60) It has been reported that these redox liabilities could be attenuated by introducing a second pharmacophore or other substituents with anti-oxidizing properties.(p60),(p61),(p62) This may mitigate the hepatotoxicity risk of the tacrine moiety, which should be taken into consideration when designing tacrine-based inhibitors. The alternative option was to utilize the benzyl piperidine fragment of donepezil, which exhibited a binding mode and an inhibitory effect similar to those of tacrine (Table 1). Owing

to the characteristics of the long and narrow catalytic gorge of ChE, tacrine or donepezil appear to be the best choices at present for designing dual-target inhibitors. More options, such as thiazolopyridine, valmerin or pyrimidone, are available for pharmacophore of GSK-3β, although the structures of these molecules are similar and they share a very similar mode of binding to the ATP-binding site of the kinase. In addition, a whole raft of chemotypes is presented in other known GSK-3β inhibitor molecules,[(p34),(p38),(p63)] which could provide more options to be adopted as the second pharmacophore.

The current strategy for developing dual-target inhibitors may be limited in its ability to generate promising lead-like molecules.[(p64)] In other words, simply hybridizing two pharmacophores may lead to the production of complexes that have poor physiochemical properties, suboptimal pharmacokinetics properties and/or potential toxicity liabilities, whereas chemically modified natural compounds do not necessarily exhibit good inhibitory effects against the intended targets. These issues might hinder the subsequent lead optimization and clinical translation. Although molecular hybridization could be a quick way to demonstrate the viability of dual-target inhibition, rational molecular design that is consistently supported by structural data obtained from complexes comprising inhibitors and target proteins appears to be a sensible way forward. This would help the engineering (i.e. linking, merging or fusing) of two pharmacophores into one lead-like molecule that has balanced inhibitory effects against the intended targets, which could facilitate the progression of the chemical series along the discovery pipeline.

Conclusions

Recently, dual-targeting of ChEs and GSK-3β has clearly shown some promise in preclinical AD models. Most of studies have involved the hybridization of two known pharmacophores of ChE and GSK-3β to develop inhibitors, which enabled the proof of concept. Nevertheless, it is difficult to guarantee the production of inhibitors that have good physicochemical and/or pharmacokinetics properties, which may hamper subsequent clinical translation. Although the modes through which small-molecule ligands bind to ChEs and GSK-3β are known, it would be helpful to design rationally a single lead-like molecule that contains two pharmacophores. Going forward, it is envisaged that more efforts are required in designing and discovering new dual-target inhibitors of ChE and GSK-3β that have optimal property profiles and strong target engagement.

Declarations of interest

The authors declare no conflicts of interest and have nothing to disclose.

CRediT authorship contribution statement

Junqiu He: Formal analysis, Investigation, Writing – original draft. **Kin Yip Tam:** Conceptualization, Investigation, Project administration, Writing – review & editing.

Data availability

No data was used for the research described in the article.

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Junqiu He and Kin Yip Tam *

Faculty of Health Sciences, University of Macau SAR, Avenida de Universidade, Taipa, Macau SAR, China

* *Corresponding author.*