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Review

# Screening Techniques for Drug Discovery in Alzheimer's Disease

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**ABSTRACT:** Alzheimer's disease (AD) is a neurodegenerative disorder characterized by progressive and irreversible impairment of memory and other cognitive functions of the aging brain. Pathways such as amyloid beta neurotoxicity, tau pathogenesis and neuroinflammatory have been used to understand AD, despite not knowing the definite molecular mechanism which causes this progressive disease. This review attempts to summarize the small molecules that target these pathways using various techniques involving high-throughput screening, molecular modeling, custom bioassays, and spectroscopic detection tools. Novel and evolving screening methods developed to advance drug discovery initiatives in AD research are also highlighted.



# ■ INTRODUCTION

Alzheimer's disease (AD) is the main type of dementia which causes degeneration of the cells in the brain and leads to cognitive impairment and decline in independence in personal everyday activities. Currently, there are more than 55 million people suffering from dementia with 10 million new cases every year and AD contributes 60-70% of those cases. AD is characterized by neuritic plague and neurofibrillary tangles as a result of accumulation of amyloid beta (A $\beta$ ) in the brain.

 $A\beta$  protein is a central factor in AD pathology, forming extracellular deposits known as cerebral senile plaques and cerebral amyloid angiopathy (CAA).  $A\beta$  is produced through two pathways: the nonamyloidogenic pathway involves cleavage of  $A\beta$  protein precursor (APP) by  $\alpha$ - and  $\gamma$ -secretase, while the amyloidogenic pathway involves cleavage by  $\beta$ -secretase instead of  $\alpha$ -secretase.  $A\beta$  exists in various isoforms in the brain, with  $A\beta40$  and  $A\beta42$  being the predominant isoforms.  $A\beta40$  is more prevalent, but  $A\beta42$  has a higher tendency to aggregate. Detecting  $A\beta$  aggregates has been a focus of research, leading to the introduction of several improved methods in recent years.

Apart from  $A\beta$  formation as a causative agent for AD, tau pathogenesis as a neurofibrillary pathology has a better correlation with this disease. The largest neurofibrillary tangles (NFTs) were identified in the 1980s as the tau protein, coining the term "tauopathy" and establishing the hypothesis that alterations in tau might result in neurodegeneration in AD.<sup>1,2</sup> Tau not only plays a role in AD but in many other neurodegeneration diseases.<sup>3,4</sup> Under pathological conditions, tau can misfold, become hyperphosphorylated, aggregate, and disassociate from the microtubules.<sup>5</sup> Tau hyperphosphorylation was the first pathological post-translational modification of tau protein described in AD. This is a result of concerted activity by several protein kinases which is known to phosphorylate tau at nearly 40 AD related epitopes.<sup>6</sup> Thus, protein kinases have gained strong attention as druggable targets in recent AD pharmacological studies. Additionally, tyrosine phosphatases and cysteine proteases have also been targets as they display excessive neuronal activity. The tau oligomers have also been the target of many drug discoveries through the utilization of tau overexpression assays or self-association of tau monomer assays.

Along with the studies of tau pathogenesis and  $A\beta$  aggregations, the presence of other mechanisms in AD have been reported. Theories of targeting calcium homeostasis and the endogenous neurotransmitter serotonin have been suggested, in addition to neuroinflammatory response in AD. There are several reports supporting that neuroinflammation as the main neuropathological event causing neurodegeneration in AD.<sup>7–9</sup> Glial cell, including astrocytes and microglia activation contributes to inducing neurodegeneration-related inflammatory signaling pathway. However, neuroinflammatory mechanism is still debated as a consequence or a cause of neurodegeneration in AD.<sup>10</sup>

Though these are the widely accepted causes of AD, there are various hypotheses in understanding AD pathology which targets several mechanisms mainly A $\beta$ -induced neurotoxicity, tau pathogenesis, and neuroinflammatory pathways.<sup>11–14</sup> The

Received:September 14, 2023Revised:December 22, 2023Accepted:December 25, 2023Published:February 5, 2024





# Table 1. Summary of Methodology for Identifying Small Molecule Inhibitors for AD

pathway	target	main methodology	small molecule inhibitor	refs
Aβ	A $eta$ protein	OBOC γ-AApeptides	KLVFF	24
		ThT	HW-155-1	
		TEM		
		ThT assay	YIAD-0121	26
		SDS-PAGE PICUPs		
		BBB-PAMPA		
		in vivo		
		screening	MK14	27
		ThT assay		
		FP	RU-505	41
		alpha-LISA assay		
		in vivo	1 DN 01280(4  (some see  1  V))	12
		HIS FLUC EFC	LDN 0004724 (compound X)	42
		SDEED	CNE 5837	44
		STEED	obatoclax	
		HCS	various small molecules	45
		neuriteIO		10
		phenotypic screening	CO	51
		Erk1/2 inhibition screening	compounds 9, 10, and 17	59
	beta-site amyloid precursor protein [APP] cleaving enzyme 1	IMER screening assay	compound 4	34
	(BACE1)	8 7	1	
		virtual screening		
		HTS AlphaScreen	various small molecules	37
		luciferase-based screening	WAY170523	43
			CL82198	
		virtual HTS	rutin	60
		docking studies	3,4-dicaffeoylquinic acid	
		molecular dynamics simulation	nemorosone	
		MM-PBSA		65
		docking studies	derivatives of PDB	65
		FRE1 assay	inhibitor 1	66
		Срнмі	inhibitor 2	00
	huturulchalinesterase (BChF)	virtual screening (ROCS)	8012 9656	68
	butyrytenomiesterase (Denil)	CDOCKER	0012-7030	00
	apolipoprotein E4 (ApoE4)	ThT assay	EGCG	28
			idarubicin	
			PD 81723	
			epirubicin	
			indirubin	
			sulfacetamide	
			imipramine	
			olanzapine	
		FRET system	CB9032258	29
		HTS alpha-LISA assay	A03	40
	striatal-enriched tyrosine phosphatase (STEP)	HTS	various small molecules: 12	35
		STEP DIFMUP assau	compounds	
		PTS		
	nonreceptor tyrosine kinase Fyn	AlphaScreen assav for HTS	classes 1, 2, and 3	38
		BRET assav		
tau	protein kinase C (PKC)	screening custom library	Ro 31-8220	52
pathogenesis	-	· /		
	cyclin-dependent kinase 5 (CDK5)	stem cell-based phenotypic screening	LDC8	53
		HTS	ZINC6261568	61
		cell-based assay	ZINC14168360	
		docking studies		
		molecular dynamics simulations		/-
	giycogen synthase kinase-3 $\beta$ (GSK-3 $\beta$ )	virtual screening binding affinity	quinoline-based compound	67

#### Table 1. continued

pathway	target	main methodology	small molecule inhibitor	refs
	tau oligomers	HTS platform for FRET	MK-886	30
		FP-HTS	various small molecules	31
		PABPNI counter screen		
		AlphaScreen assay		
		HTS	14 small molecules	46
		affinity imaging mass spectrometry (AIMS)		
		cell-based assay screening	various small molecules	48 and 49
	caspase-6	virtual screening	various small molecules	36
		in vitro testing		
		fluorescence-based assay		
other pathways	calcium homeostasis: FAD-PS1	HTS	bepridil	47
		FRET single-cell imaging		
		kinetic assay		
	TREM2	screening	various small molecules	50
	CD33/Siglec 3	HTS phenomimetic screening	compound 1	54

heterogeneous nature of AD complicates its diagnosis, prognosis, treatment, and drug design.

In the past decade, various drug screening techniques such as high throughput screening (HTS) and the affordability of omics data sources<sup>15–18</sup> have driven much research in this area. Drug screening technology allows the use of automation to rapidly test thousands of drug-like molecules from existing biomedical platforms against many putative targets. The "hits" allow further modification and development of drugs. Recently, techniques such as artificial intelligent (AI), deep learning, and CRISPR have also been used to identify potential drugs for AD.<sup>19–22</sup> This review aims to provide a brief description and highlight the recent development of drug screening methodology used for the three main mechanisms of AD in the past decade (Table 1).

# BIOASSAY SCREENING

UV-vis Assay. To improve the detection method for measuring small molecule bindings to aggregated A $\beta$  peptides, a simple and accessible ELISA method was developed.<sup>23</sup> This ELISA method allows estimation of binding constants for small molecules from the low nanomolar to the low micromolar range, regardless of their inherent physical properties. Other techniques like surface plasmon resonance (SPR) and quartz crystal microbalance (QCM) have been previously used but require the immobilization of  $A\beta$  to a surface, potentially altering its desired conformation. The key step in the ELISA-based method involves air plasma treatment of the polystyrene surface of the plates, which helps maintain the native  $\beta$ -sheet content of the amyloid peptides during analysis (Figure 1). The relatively large size of immunoglobulin G (IgG) allows it to compete with small molecules for binding sites on the amyloid surface, making it a general competitor for various classes of molecules.

**Fluorescence Assay.** Effective inhibitors of  $A\beta$  aggregation were identified using a one-bead-one-compound (OBOC) screening process library of  $\gamma$ -AApeptides (Figure 2).<sup>24</sup> TentaGel beads were incubated with  $A\beta$ 40 peptide and then treated them with anti- $A\beta$  antibody 6E10. This was followed by the introduction of the antimouse IgG-DyLight 549 conjugate which produced strong orange fluorescence. Among over 192,000 beads, two potential hits were identified: KLVFF and HW-155-1. While KLVFF is recognized for its inhibitory effects on  $A\beta$  aggregation, HW-155-1 demonstrated superior potency in disrupting  $A\beta$  aggregation, as validated through Thioflavin T



**Figure 1.** Schematic representation of the ELISA method to determine competitive inhibition constants of small molecules with  $A\beta$  aggregates. ELISA method adapted for this study: (a) treatment of polystyrene 96 well plate with air plasma, (b) deposition of preaggregated  $A\beta$  into the wells, (c) incubated the wells with protein-free blocking buffer, (d) coincubated with fixed concentration of anti- $A\beta$  Immunoglobulin G (IgG) and increasing concentration of small molecules, (e) removed excess primary antibody, incubated secondary antibody with paranitrophenyl phosphate (p-NPP), and quantified the remaining secondary antibody using UV–vis plate reader. Adapted from ref 23. Copyright 2012 American Chemical Society.

(ThT) assay and TEM analysis, when compared to KLVF. ThT is a frequently used probe for tracking in vitro amyloid fibril formation. When bound to amyloid fibrils, ThT exhibits a strong fluorescence signal at around 482 nm under excitation at 450 nm. The fluorescence enhancement mechanism upon binding to amyloid is linked to the rotational immobilization of the central C-C bond that connects the benzothiazole and aniline ring.<sup>25</sup> Additionally, HW-155-1 demonstrated the ability to mitigate the toxicity of A $\beta$ 42 and prevent the death of N2a neuroblastoma cells. This study introduced a novel class of peptidomimetics called  $\gamma$ -AApeptides, which consist of N-acylated-N-aminoethyl



**Figure 2.** Schematic representation of OBOC screening using  $\gamma$ -AApeptides library with A $\beta$ 40 peptide. The final picture is taken under a fluorescent microscope with excitation at 550 nm and emission measured at 605 nm. Adapted with permission from ref 24. Copyright 2014 Royal Chemical Society.

amino acid units derived from  $\gamma$ -PNAs. These  $\gamma$ -AApeptides demonstrated an exceptional resistance to proteolytic degradation.

Using the ThT assay, Kim et al. initially screened various derivatives of a small molecule that they have synthesized.<sup>26</sup> Sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) analysis coupled with photoinduced cross-linking of unmodified proteins (PICUPs) was used to assess the distribution of  $A\beta$  species in the ThT assay. Then, blood brain barrier (BBB)-specific parallel artificial membrane permeability assay (PAMPA) was used to screen brain penetration in vitro. These screening methods allowed them to identify YIAD-0121 as an  $A\beta$ -dissociating small molecule, and they conducted in vivo analysis using SXFAD mice which indicated clearance of  $A\beta$  in AD brains. This study which involves relatively simple synthesis with bioassay screening method has led to the discovery of potential  $A\beta$ -dissociating molecule.

ThT assay was also used to identify small molecules that inhibit protein fibril formation using insulin as a model protein. This is based on the principle that fibril formation is observed in both Parkinson's disease and Type 2 diabetes mellitus. Screening of a small in-house library of 320 molecules from the MK library and molecular docking were utilized to identify 16 potential compounds. Through ThT assay analysis, MK14 was identified to result in fibril reduction by 80%.<sup>27</sup> However, the absorbance of MK14 in the ultraviolet region will limit its applicability in living cells. Further optimization of this molecule through molecular design and modification will see its full potential in fibril dissociating application.

Carrying one copy of apolipoprotein 4 (apoE4) increases the risk of AD by more than three times, while having two copies (homozygous) increases the risk by over 12 times. AD symptoms manifest an early onset in apoE4 carriers, and these carriers experience extensive plaque deposition, accumulation of intraneuronal  $A\beta$ , cerebral amyloid angiopathy, and poor BBB function. ApoE4 exhibits a strong binding affinity to  $A\beta$  and functions as a catalyst, hastening the formation of  $A\beta$  oligomers and fibrils, enhancing their stability, and promoting their neurotoxic effects. Moreover, in the AD brain, apoE is codeposited alongside amyloid plaques, indicating a direct interaction with  $A\beta$ .

In a study to identify small molecules that prevent the interaction between apoE4 and  $A\beta$ , ThT assay was used.<sup>28</sup> The assay was able to monitor  $A\beta$  fibrillization and study the catalytic effects of apoE4, then was optimized to be used in HTS and identify inhibitors of apoE4- $A\beta$  interaction. A drug screen was conducted using 595 compounds from the NIH Clinical

Collection (NCC) library, resulting in the identification of 134 hits. Among these hits, 87 compounds showed good BBB permeability. The dose–response effects of these 87 compounds were analyzed on the kinetics of apoE4-catalyzed  $A\beta$  fibrillization in the HTS assay. Out of the 134 hits, eight compounds (sulfacetamide, imipramine, epigallocatechin gallate (EGCG), idarubicin, PD 81723, epirubicin, olanzapine, and indirubin) were found to reduce apoE4-catalyzed  $A\beta$  fibrillization with a dose-dependent effect. Notably, EGCG, idarubicin, PD 81723, epirubicin, and indirubin inhibited the fibrillization of  $A\beta42$  alone, indicating that these compounds acted directly on  $A\beta$ . This study identified eight potential compounds that could interfere with the interaction between apoE4 and  $A\beta$ , suggesting a promising avenue for further research and drug development in the context of AD.

Fluorescence resonance energy transfer (FRET) has been devised to enhance the comprehension of the spatiotemporal regulation of diverse cellular processes. FRET entails the nonradiative transfer of energy from an initially excited donor to an acceptor. This technique is applicable for monitoring protein-protein interactions and protein conformational changes within live biological samples. The GFP-apoEeDHFR FRET was used to detect conformational changes between the two intramolecular domains of apoE.<sup>29</sup> In neurons, apoE4 reduces expression of the protein subunits of mitochondrial respiratory complexes, such as subunit 1 of complex IV (mtCOX1) and subunit of complex V, resulting in a decrease in mitochondrial respiratory function. Moreover, apoE4 has been demonstrated to disrupt neuronal function by decreasing mitochondrial motility, limiting neurite outgrowth, and inhibiting the formation of synapses. CB9032258, a derivative of phthalazinone, was able to minimize the FRET signal in GFP-apoE4-eDHFR in dose-dependent manner. Treatment of neuro-2a cells expressing apoE4 with CB9032258 resulted in the restoration of mtCOX1. Furthermore, CB9032258 treatment effectively counteracted the detrimental effects of the apoE4 domain interaction on mitochondrial motility and neurite outgrowth. CB9032258 is validated to function as an apoE4 structure corrector that can reverse the apoE4 domain interaction-dependent negative effects in neuron cell culture. The study successfully identified potent structure correctors that govern the structure of apoE4 by preventing or altering the intramolecular domain interactions.

FRET is also used as a biosensor in-cell HTS platform to detect small molecule modulation of tau oligomerization.<sup>30</sup> Small molecules (727 compounds, previously tested in clinical trials) from the NCC library were screened in combination with

the authors' in-house fluorescence lifetime plate reader which has 30 times more sensitivity than conventional fluorescence detectors. The biosensors express full-length 2N4R wild-type tau fused with green or red fluorescent proteins in HEK293 cells and allow monitoring of inter- and intramolecular tau interactions. They have identified MK-886 as a small molecule that directly binds to tau and prevents tau-elicited cell cytotoxicity at IC<sub>50</sub> of 0.523  $\mu$ M.

In order to screen potential inhibitors of mammalian suppressor of tauopathy 2 (MSUT2) RBP and RNA by fluorescence polarization (FP), fluorescent FAM (fluorescein amidite)-labeled poly(A)15 RNA (FAM-RNA) and recombinant MSUT2 ZF (CCCH zinc finger domain only) protein expressing constructs were generated. The complex exhibits a lower tumbling rate compared to free FAM-RNA, leading to a heightened emission of polarized light. Disruption of the interaction causes unbound FAM-RNA to tumble at a relatively faster rate, resulting in the emission of nonpolarized light. MSUT2 regulates the accumulation of pathological tau by binding to polyadenosine [poly(A)] tails of mRNA via its Cterminal CCCH type zinc finger domains, and loss of CCCH domain function reduces tauopathy. By inhibiting this interaction, the pathological tau accumulation can be reduced. However, poly[A] also interacts with Poly(A) Binding Protein Nuclear 1 (PABPNI), which upon inhibition can increase pathological tau accumulation. Identification of specific small molecules of polyaromatic and heterocyclic structures from the NIH Clinical Collection (700 compounds) was conducted via, first, a primary FP HTS, followed by PABPNI counter-screen, an orthogonal amplified luminescent proximity homogeneous (AlphaScreen) assay, and finally cell toxicity studies.<sup>31</sup> They have developed a drug repurposing pipeline through the identification of these small molecule inhibitors of MSUT2.

BACE1 (beta-site amyloid precursor protein [APP] cleaving enzyme (1) belongs to the aspartyl protease of the pepsin family and was first discovered in 1999. BACE1 is found both on the plasma membrane and in endosomal compartments, and it can be detected in healthy synaptic terminals as well as dystrophic neurites surrounding A $\beta$  plaques.<sup>32</sup> Functionally, BACE1 serves as the  $\beta$ -secretase enzyme responsible for cleaving the transmembrane APP. Together with secretase, it generates various A $\beta$  species. This cleavage event represents the critical step limiting A $\beta$  production. In AD brain aggregates, BACE1 contributes to the formation of an increasingly large and conformationally complex soluble region. The inhibition of BACE1 offers numerous advantages, one of which is the prevention of A $\beta$  formation at the early stage of APP processing. Various studies have provided compelling evidence, demonstrating that BACE1 inhibition leads to improved neurological function and surpasses neurological deficits observed in AD mouse models.<sup>33</sup>

Liquid chromatography method based on fluorescence detection was developed to screen for human recombinant BACE1 (hrBACE1) inhibitors. hrBACE1-immobilized enzyme reactor (hrBACE1-IMER) chromatographic method was utilized to perform a sensitive and rapid screening of 38 compounds.<sup>34</sup> These compounds were selected using a virtual screening approach. The employed substrate, M-2420, consists of a peptide chain containing the amino acid sequence (Leu-Asp) of the Swedish-mutated APP, with methoxycoumaryl and dinitrophenyl moieties serving as the fluorescent and quencher groups, respectively. Among the compounds screened using IMER, compound 4, a derivative of bis-indanone, exhibited the most noteworthy inhibition of hrBACE1-IMER activity. The IMER technique offers several advantages, including HTS of various chemical entities using a fast and practical chromatographic system, while reducing the time and cost associated with the analysis process.

Protein thermal shift assay (PTS) is a biophysical assay that detects the binding of small molecules to a recombinant protein by monitoring its melting temperature  $(T_m)$ . ThermoFluor technology, which uses a fluorescent dye, binds to the exposed hydrophobic protein core after melting, resulting in increased fluorescence. A real-time PCR system is used to incrementally heat samples over a temperature gradient and simultaneously measure fluorescence intensity. Lambert et al. used this biophysical binding assay based on the PTS technology to identify striatal-enriched tyrosine phosphatase (STEP) inhibitors.<sup>35</sup> A neuron-specific protein tyrosine phosphatases, STEP, is usually overactive in AD and other neurodegenerative diseases. High levels of STEP contribute to the cognitive deficits in AD and other neurodegenerative and neuropsychiatric disorders. More than 50,000 in-house small molecules were screened using PTS, and eventually 72 confirmed hits were identified. The STEP enzymatic activity of these hits was then tested using STEP<sub>46</sub> DiFMUP assay in 10point dose-response biochemical inhibition experiments. Finally, 12 compounds were identified as biochemically active with sub-50  $\mu$ M activity against STEP<sub>46</sub>. Further structureactivity relationship and optimization studies are required; however, these scaffolds serve as good starting points.

Caspases come from a family of cysteine proteases that play a pivotal role in programmed cell death, neuronal development, inflammation, axon pruning, and differentiation. Excess neuronal activity of caspase-6 is related to age-dependent cognitive impairment including AD. Targeting caspase-6 activity is not trivial as there are structural and functional similarities between the members of the caspase family. The LeBlanc group focused on four rare nonsynonymous missense single-nucleotide polymorphisms (SNPs) which led to substitution outside the human caspase-6 active site and affected enzyme structure and efficiency.<sup>36</sup> Three SNPs were placed at the allosteric pocket of caspase-6, and these were targeted using virtual screening and in vitro testing. They screened 57,700 small molecules from curated Chembridge and Sigma commercial libraries and narrowed the search to 40 compounds of interest based on favorable interactions at the allosteric site with the lowest energy of ligand conformation. Then, a fluorescence-based assay was used to measure recombinant active caspase-6 reaction rate in the absence and presence of the inhibitor compound. They managed to identify several inhibitors with  $IC_{50}$  and  $K_i$  values ranging from  $\sim 2$  to 13  $\mu$ M.

**Chemiluminescence Assay.** The AlphaScreen technology is designed to measure the proximity between donor and acceptor beads that are conjugated to the biomolecules of interest (Figure 3).<sup>37</sup> The binding of BACE1 to screen potential inhibitors was explored using AlphaScreen, which was employed to discover novel low-affinity inhibitors of BACE1. The authors investigated the binding of BACE1 protein to a set of smallmolecule probes derived from known BACE1 inhibitors. These probes were synthesized by incorporating a biotin PEG linker of different lengths into the hydroxyethylamine (HEA), hydantoin, and sulfamide classes of small-molecule BACE1 inhibitors. Interestingly, it was observed that a longer linker size consistently led to a higher signal in the assay. To identify BACE1 inhibitors, over 525,000 compounds were screened



**Figure 3.** Principle of AlphaScreen binding assay. The close proximity of the donor and acceptor beads generates single oxygen molecules that interact with acceptor beads to give emissions. The BACE inhibitor will reduce this signal as it competes with the biotin-labeled probe. Reproduced with permission from ref 37. Copyright 2013 Sage Journals.

using a validated AlphaScreen assay, which was miniaturized to a 1536-well format.

AlphaScreen assay suitable for HTS was also used to identify small-molecule inhibitors of the tau-Fyn SH3 interaction. Several studies have generated interest in the nonreceptor tyrosine kinase Fyn, which interacts with polyproline helices in tau through its SH3 domain.<sup>38</sup> This study proposes that targeting the tau-Fyn interaction may be sufficient to achieve the beneficial effects of tau reduction. The assay utilized purified GST-Fyn SH3 and His-Tau. For HTS, a D-tau construct was used as it represents the putative form of tau found in dendrites, where the pathogenic tau-Fyn interaction is believed to occur. Screening of 108,138 compounds identified 1852 compounds that exhibited greater than  $\sim 70\%$  inhibition. These 1852 compounds were further evaluated through dose-response assays, including the primary tau-Fyn SH3 interaction AlphaScreen assay, an AlphaScreen counter-screen to eliminate compounds that nonspecifically disrupt the system, and cellbased toxicity assays using two cell lines (LL47 and THP-1). Thirty-nine hits identified through HTS underwent assessment using a cell-based bioluminescence resonance energy transfer (BRET) assay. Out of the 39 compounds, seven showed significant activity in the BRET assay. The top hits revealed three chemical classes, with classes 1 and 2 exhibiting desirable properties and BRET activity, especially due to their low polar surface area, making them favorable for medicinal chemistry follow-up. Class 3 also demonstrated potential for medicinal chemistry efforts, and several singletons without obvious chemical similarity may serve as starting points for further modification.

The Alpha-LISA bead-based technology is based on the exclusive AlphaScreen and employs luminescent oxygenchanneling chemistry. To evaluate SirT1 protein levels, a customized Alpha-LISA assay was used in HTS, which involved screening approximately 720 compounds from the NIH Clinical Collection module. SIRT1 has been demonstrated to deacetylate neurons affected by AD in the nucleus, thereby preventing apoptotic neuron death, suggesting the protective function of SIRT1 against neurodegeneration. Overexpression of SIRT1 in the brain mitigates central nervous system AD pathologies by activating  $\alpha$ -secretase-mediated cleavage of APP. The activation of SIRT1 was also shown to inhibit NF- $\kappa$ B signaling and diminish microglia-dependent A $\beta$  toxicity.<sup>39</sup>

This collection included well-known selective serotonin reuptake inhibitor (SSRI) fluoxetine (marketed as Prozac) and N-methyl-D-aspartate (NMDA) receptor antagonist memantine (marketed as Namenda).<sup>40</sup> Among the compounds tested, A03 was found to increase SirT1 levels, whereas the well-known drugs such as fluoxetine and memantine, which act as a SSRI and NMDA receptor antagonist, respectively, did not enhance SirT1 in the presence of apoE4. Moreover, oral administration of A03 successfully reversed SirT1 deficits in the hippocampus, one of the brain regions most affected in AD. This increase in SirT1 levels in the hippocampi of apoE4-expressing AD model mice after 56 days of oral A03 treatment was associated with memory improvement. In light of the compelling evidence presented in this study, A03 emerges as a promising preclinical candidate for the development of the first apoE4-targeted brain SirT1 enhancer. The potential therapeutic impact of A03 in mitigating apoE4-related neurodegenerative processes offers hope for advancing the field of AD treatment.

Ahn et al. used FP and Alpha-LISA assays to screen a library of low molecular weight compounds that specifically block the interaction between A $\beta$  and fibrinogen, without affecting general clotting processes.<sup>41</sup> The presence of  $A\beta$  can lead to the formation of more persistent fibrin clots, which can worsen neurovascular damage and cognitive impairment in individuals with AD. Molecules that could potentially restore abnormal thrombosis and fibrinolysis and protect against vascular damage, may serve as therapeutic agents for AD patients. Over 93,000 compounds were screened using FP, which measured changes in anisotropy induced by the binding of a 5-carboxy-tetramethylrhodamine (TAMRA)-labeled A $\beta$  peptide to fibrinogen (Figure 4a). The activity of the inhibitors identified in the FP assay was independently confirmed using Alpha-LISA (Figure 4b). Among the tested compounds, five demonstrated inhibitory effects, with RU-505 exhibiting significant efficacy as an inhibitor. RU-505 was found to inhibit the interaction of both



**Figure 4.** (a) Anisotropy induced by the binding of a TAMRA-labeled  $A\beta$  peptide to fibrinogen. (b) Alpha-LISA method using donor and acceptor beads. Reproduced with permission from ref 41. Copyright 2014 The Rockefeller University Press.



**Figure 5.** SPEED screening. (a) Dot blot screening of 1047 compounds with obatoclax circled in blue which showed increased signal for  $A\beta$  with 4G8 antibody. (b) Quantitative analysis of the dot blot assay of  $A\beta$  with varying concentration of obatoclax using 4G8 antibody, confirming the alteration of the epitope. Reproduced with permission from ref 44. Copyright 2022 RSC Publishing.

 $A\beta$  monomers and oligomers with fibrinogen. RU-505 effectively restored the  $A\beta$ -induced decrease in turbidity during fibrin clot formation and significantly reduced the delay in fibrin degradation in the presence of  $A\beta$ . Importantly, it had no impact on the regular process of clot formation and fibrinolysis, while demonstrating an improvement in memory function. Long-term administration of RU-505 also reduced vascular amyloid deposits, fibrinogen infiltration, and microgliosis in the cortex of a transgenic mouse model of AD. The authors concluded that RU-505 can restore  $A\beta$ -induced altered thrombosis and delayed fibrinolysis in vitro and in vivo by inhibiting the  $A\beta$ -fibrinogen interaction.

Firefly luciferase enzyme fragment complementation (FLuc EFC) has been utilized in HTS.<sup>42</sup> This study aims to identify inhibitors of small molecule amyloid APP dimerization and study their impact on A $\beta$  generation. In this assay, inactive deletion mutants of FLuc are fused to separate APP molecules. When APP forms a dimer, the previously inactive FLuc fragments come together and form a fully functional enzyme. Chemiluminescence measurement after adding luciferin allows quantification of APP-APP interactions. This FLuc EFC assay effectively monitors APP processing and A $\beta$  production. A total of 77,440 compounds underwent screening, resulting in a final hit rate of 0.15%, with 113 compounds reducing the luciferase signal by 50% or more. Further testing revealed that out of these initial candidates, only 14 showed dose-response effects and were nontoxic. Two potent inhibitors, LDN-0128964 (Compound X) and LDN-0004724 (Compound Y), were discovered through the HTS. Compound X did not significantly affect  $A\beta$ production or APP processing but showed a slight trend toward decreasing A $\beta$ 40 and A $\beta$ 42. Compound Y significantly reduced A $\beta$ 40, A $\beta$ 42, and soluble APP $\beta$  (sAPP $\beta$ ) levels.

Luciferase-based drug screening for BACE1 identified two MMP13 inhibitors, WAY170523 and CL82198, which significantly decreased BACE1 apoEaDHpromoter-driven luciferase activity.<sup>43</sup> Interestingly, CL82198 specifically affected MMP13, leading to the control of BACE1 protein levels in various cell lines and primary cultured neurons. The study also reported elevated MMP13 levels in AD and demonstrated that altering MMP13 expression affected eukaryotic translation initiation factor 4B (eIF4B) phosphorylation at S422. This finding suggests that eIF4B activity might serve as a link between PI3K signaling and MMP13-mediated BACE1 regulation. Consequently, this supports the crucial role of eIF4B in MMP13's regulation of BACE1. Furthermore, the study demonstrated that reduced MMP13 level either via knockdown or CL82198 treatment improved spatial and associative learning memory. The activation of PI3K signaling promoted eIF4B phosphorylation at S422, facilitating the 5' UTR-dependent translation of BACE1. As a result, CL82198 reduced BACE1 protein levels and  $A\beta$  accumulation, leading to improved learning and memory in APP/PS1 mice. On the basis of these findings, the authors proposed that CL82198, as an MMP13 inhibitor closely associated with BACE1 regulation, holds therapeutic potential for AD.

Screening platform based on epitope alteration (SPEED) is a platform that offers label-free and site-specific screening for diverse functional purposes. Zhu et al. have reported the use of SPEED to screen small molecules that binds to  $A\beta$  aggregates (Figure 5).<sup>44</sup> As a proof-of-concept study,  $A\beta 1-40$  monomers were used as the target. The structure of  $A\beta$  can be classified into a hydrophilic N-terminal region (10-16 amino acid), central hydrophobic  $\beta$ 1 region (17–21 amino acid), hydrophilic turn region (22–28 amino acid), and hydrophobic  $\beta$ 2 region (29– 40/42 amino acid). A $\beta$ 17-24 was selected as the targeted epitope. This epitope targets the main core of the A $\beta$  containing KLVFF segment which has been utilized in the development of antiaggregation inhibitors and fluorescence probes based on the restriction of the rotation effect. SPEED screening was performed with a library of 1047 compounds, and antibody 4G8 was used for targeting A $\beta$ 17–24. Hits were identified based on the average chemiluminescence intensity exceeding three times the standard deviation. The initial screening yielded 7 positive results, and subsequent screening confirmed two strong hits: GNF-5837, an inhibitor of tropomyosin receptor kinase A (TrkA) which inhibits  $A\beta$  aggregation, and obatoclax, an inhibitor of the B-cell lymphoma-2 (Bcl-2) protein. This study reported several small molecules that could alter the surface properties of  $A\beta$  epitopes. Consequently, the alteration could lead to inhibition or enhancement of the antibody-epitope recognition. This work summarized that SPEED uses three main strategies which involve target epitope selection, the selection of antibody that binds to the epitope and serves as the fluorescent

marker, and finally targeted epitope screening of various small molecules.

# IMAGING TECHNIQUES

In addition to spectroscopic methods for identifying  $A\beta$  aggregates, a high content screening assay (HCS) combined with Neurite Image Quantitator (NeuriteIQ) software was used to identify small molecules that can suppress the degeneration of neuronal processes induced by  $A\beta 1-40$  (Figure 6).<sup>45</sup> The



**Figure 6.** Schematic representation of the image processing that NeuriteIQ performs using nuclei detection and segmentation method. The total average length and intensity of the neurites per neuronal cell was identified using these images. Reproduced with permission from ref 45. Copyright 2012 Elsevier.

primary screen involved testing 1040 compounds from the NINDS custom collection library II, known bioactives, in primary mouse cortical neurons exposed to oligomeric  $A\beta 1$ -40. The lengths of neurites were quantified using the NeuriteIQ software, and 42 compounds (0.4%) showed significant protection against  $A\beta 1$ -40-induced neurite loss. A secondary screen confirmed the specificity of the identified compounds and estimated their half maximal effective concentrations (EC<sub>50</sub>). It revealed that 36 (85%) of the compounds significantly protected

primary cortical neurons from  $A\beta 1$ -40-induced neurite degeneration at micromolar or lower concentrations. Notably, the identified compounds were enriched with nonsteroidal antiinflammatory drugs (NSAIDs), antihistamines, and alkylating/ DNA-damaging agents. The study demonstrated that  $A\beta$ induced neurite loss could be fully reversed in the presence of the selective peroxisome proliferator-activated receptor (PPAR) agonist 5-deoxy- $\Delta 12$ ,14-prostaglandin J2, indicating that PPAR activity alone can rescue neurite loss.

In another study, a HTS approach was employed to identify molecules that exhibit an affinity for tau deposits in the human brain.<sup>46</sup> From a small molecule library with thousands of compounds (<500 molecular weight), 14 compounds with high affinity to the AD brain were successfully identified. The study also introduced a novel screening system, utilizing affinity imaging mass spectrometry (AIMS), which enables the visualization of protein aggregation in the brain. Stainless steel microchips were utilized to hold milliliter-scale frozen brain sections, which were then exposed to solutions containing library compounds. A robot hand system was implemented to manipulate the microchips, allowing for the creation of 16,000 sections from a 1 cm<sup>3</sup> organ sample. Subsequently, the compounds demonstrating a high affinity for the brain structure were visualized using AIMS. The relative affinity for the AD brain region was assessed through a tau positive score, calculated as the ratio of the mean intensity of the AD area to that of the control area. Screening was conducted on 3200 chemical compounds from the library against brain tissue containing tau deposits, resulting in the selection of 25 compounds exhibiting affinity for AD brain sections. The 25 compounds that were identified underwent MS/MS analysis, revealing that 14 of them displayed fragment ion relevant to its precursor.

The majority of early onset familial AD (FAD) cases are linked to mutations in Presenilin genes (PS1 and PS2). These mutations result in enhancement of inositol 1,4,5-trisphosphate  $(IP_3)$  receptors sensitivity and causes calcium influx, thereby altering the intracellular calcium homeostasis. The focus on stabilizing intracellular calcium homeostasis, particularly in the endoplasmic reticulum (ER), represents an innovative target for drug discovery in AD.<sup>47</sup> Screening of compounds was conducted using the FRET single-cell-based calcium imaging technique in a fully automated high-throughput kinetic assay on the "Opera" system (PerkinElmer). To monitor both the basal calcium concentrations and the released calcium from the ER in realtime through confocal imaging, a genetically encoded FRETbased calcium probe with expanded dynamic range and fast kinetics (Yellow Cameleon 3.6 (YC3.6)) was introduced to HEK293 cells. Following the primary screen, 53 active small molecule hits were identified, and cytotoxicity assessments were conducted on these hits. Through preliminary structureactivity relationship (SAR) evaluations using the "Benchware DataMiner" software by Tripos, four lead structures were identified, which belonged to the compound classes of thiazolidine, phenothiazine, imidazole, and benzhydrol piperidinamine. The HTS led to the discovery of bepridil, a calcium antagonist drug previously known for its beneficial effects against AD by targeting both  $\beta$ - and  $\gamma$ -secretases simultaneously. In an effort to improve efficacy, 15 derivative structures similar to bepridil were synthesized. Bepridil specifically attenuated the FAD-PS1-mediated exaggerated ER calcium release. This reduction in  $A\beta$  levels was accompanied by an increase in sAPP $\alpha$  and a decrease in sAPP $\beta$  secreted fragments in a dosedependent manner.

Figure 7. Phenotypic CD33 splicing assay as suggested by Chappie et al. Adapted from ref 54. Copyright 2022 American Chemical Society.

Numerous cell-based assays have been created to explore the tau oligomerization process and assess its dissociation behavior or the prevention of its formation in the presence of smallmolecule inhibitors. Although these studies are conducted in vitro, they offer compelling evidence supporting the use of these tools for screening extensive libraries of potential small molecules. Crowe et al. utilized two cell-based assays of tau inclusion formation to screen small molecules that inhibit tau pathology.<sup>48</sup> First, a HEK293 cell-based tau overexpression assay was used to screen ~3500 pharmaceutical compounds from the National Centre for Advancing Translational Sciences (NCATS) Pharmaceutical Collection library, which gave a low hit rate. Additionally, these compounds failed in the second cellbased assay: a primary rat cortical neuron assay with physiological tau expression. They then screened the Prestwick library which consisted of 1280 mainly approved drugs, leading to a greater number of hits (32 nontoxic compounds) as tau inclusion inhibitors. The subset of these compounds also showed concentration-dependent inhibition of neuronal tau inclusion.<sup>49</sup> Using this assay, recently they have screened a library (MedChem Express Bioactive) of 8700 biologically active small molecules to reduce immunostained neuronal tau inclusions and identified 173 inhibitors of tau aggregates through activity analysis using orthogonal ELISA. Out of that, 55 compounds were confirmed inhibitors with 46 showing concentration-dependent lowering of tau inclusions. The identified small molecules were BACE1 and  $\gamma$ -secretase inhibitors.

Microglial TREM2, which is a surface receptor with rare sequence variants is identified to show increased risk for AD. Targets of small molecules from various compound libraries were screened on human myeloid cells (THP1-1) using Cellomics ArrayScan VTI HCS Reader. This device provides quantitative, kinetic, live-cell imaging and analysis which showed an increase in the expression of cell surface TREM2 protein.<sup>50</sup> Many hits showed an increase in both TREM2 and CD33 and are not included in the research since they are likely to be involved in mechanisms that stimulate cell surface protein in general. They have identified that inhibitors of kinases MEK1/2 display the strongest and most consistent increase in TREM2 protein. This finding could possibly have significant benefits if it can be achieved therapeutically.

# PHENOTYPIC SCREENING

A phenotypic small-molecule screen encompassing over 140,000 compounds were analyzed in duplicates using increased growth (optical density, OD) as the end point.<sup>51</sup> Only 30 compounds passed the scoring criteria of *Z* score >3, with half of the hits encompassing the 8-hydroxyquinoline core structure. The *Z* score was calculated using  $[OD_{600} \text{ well} - OD_{600} \text{ plate average}]/OD_{600}$  plate average. Finally, clioquinol (CQ) was identified as a clinically relevant class of compounds that can

prevent the toxic effects induced by  $A\beta$  when expressed within the secretory pathway. CQ treatment completely restored the transport of methionine uptake 1-green fluorescent protein (Mup1-GFP) from endosomes to the vacuole in strains expressing  $A\beta$ . This suggests that  $A\beta$  disrupts endosomal transport, leading to defects downstream of initial transporter internalization, which can be rescued by CQ treatment. Furthermore, CQ facilitated the degradation of metal-dependent  $A\beta$  oligomers within both the secretory and endosomal compartments. The use of a yeast model for  $A\beta$  allowed the authors to focus on the early stages of  $A\beta$  misfolding.

Protein kinase C (PKC) which is associated with memory loss, tau phosphorylation, and progression of AD can also be a target. Shim et al. screened a custom library of 120 active small molecules from the pharmaceutical inventory list at the Department of Brain Science, Asan Medical Center, to improve tau-related rough-eye phenotype in the Drosophila melanogaster model of frontotemporal dementia.<sup>52</sup> Among the hits, the previously identified potent PKC $\alpha$  inhibitor, Ro 31-8220 was investigated to reveal improvement of reduced tau phosphorylation in both in vitro and in vivo experiments. This resulted in the reversal of tau-induced memory impairment and improved the fly motor functions. Similarly, in human neuroblastoma cell lines, this small molecule reduced PKC activity and tau phosphorylation. Although Ro 31-8220 does target other cellular functions such as autophagy and apoptosis, it is still a potent inhibitor for early therapy in the disease course.

Reinhardt et al. utilized stem cell-based phenotypic screening of 44,000 compounds to identify small molecules that rescue motor neurons from neuroinflammation-induced degeneration.<sup>53</sup> They have used a previously developed phenotypic screening assay and modeled it by incorporating microglial BV2 cells. A hit series was identified consisting of 7 pyrazolotriazines that protected at least 30% of the motor neurons. They have shown that inhibition of both CDK5 and glycogen synthase kinase-3 $\beta$  (GSK-3 $\beta$ ) from the cytidine/uridine monophosphate kinase (CMPK) family is required to elicit neuroprotection. This is demonstrated in vivo using zebrafish models of motor neuron degeneration and AD. One target compound, LDC8 with an IC<sub>50</sub> of 1.4  $\mu$ M, was presumably capable of simultaneous inhibition of CDK5 and GSK3 $\beta$  and, thus, is a promising lead for development as a neuroprotective drug.

The study by Chappie et al.<sup>54</sup> focuses on CD33/Siglec 3, a cell surface receptor in the myeloid lineage, which provides protection against late-onset AD. The disease protection is credited to the alternative splicing of exon 2 of the CD33 pre-mRNA (Figure 7). A phenomimetic screening was conducted to identify a series of compounds that enhance the exclusion of CD33 exon 2. First, a HTS of the full Pfizer screening library of 3.1 million small molecules suitable for oral drugs was conducted. Further retesting and counter-screening were conducted to confirm 78 active compound hits. Then, additional



Figure 8. Molecular structure-based virtual screening to design DHP library for BACE-1 inhibitor. Adapted with permission from ref 63. Copyright 2013 Elsevier.

chemoinformatic analysis was performed to establish SAR in the assay and reduce false positives which eventually led the focus to a single compound. Thus, they have successfully shown pharmacological interventions as a tool to manipulate diseaserelated pre-mRNA splicing.

# COMPUTER-AIDED DRUG DESIGN (CADD) APPROACHES

In silico drug design, which consists of theoretical and computational approaches, can be utilized to discover novel hits or leads against selected biologically active macromolecules. Currently, computer-aided drug design (CADD) approaches, such as pharmacophore modeling, virtual screening, molecular docking, and dynamic simulation, are extensively employed in the discovery, development, and analysis of drugs and similar biologically active compounds. In the CADD approach, both structure and ligand-based pharmacophore models can identify molecules with similar activity against a specific target protein. The binding affinity of a large number of compounds with the target macromolecule can be easily evaluated through in silico molecular docking processes.<sup>55</sup>

Advancements in protein structural science technologies, such as cryo-electron microscopy and X-ray crystallography, have led to an increasing number of proteins being elucidated in three dimensional. Additionally, the prediction of protein three-dimensional structures is achievable through homology modeling. In addition to protein–ligand docking, molecular docking also covers protein–protein docking, protein–peptide docking, and other large-molecule-related docking problems using biomolecules.<sup>56</sup>

Numerous small molecule screening assays have revealed potential compounds with BACE1 inhibitory properties. CADD of natural products were utilized to design potential lead drugs as BACE1 inhibitors.<sup>57</sup> The binding region of BACE1 was analyzed using PONDR-FIT to understand the relevant amino acids involved (GLN60, GLY61, ASP80, ILE158, ILE166, ASP276, GLY278, and THR279). A screening of 61,000 Traditional Chinese Medicine (TCM) compounds from the TCM Database@Taiwan was conducted through docking studies to identify potent ligands as BACE1 inhibitors. The TCM candidates were then ranked based on various scoring functions, and ADMET (absorption, distribution, metabolism, excretion, and toxicity) prediction was employed to assess BBB penetration, CYP2D6 inhibition, and hepatotoxicity activity for all docked ligands. The results indicate that Triptofordin B1 can bind to BACE1 and may serve as a promising lead drug for the design of novel BACE1 inhibitors in the treatment of AD.

In silico chemical screening using a similarity-based approach that relied on memantine and MK-801 as reference structures was used to identify commercially available molecules that share similar feature-pair distributions as defined by Shannon entropy descriptors (SHED). A total of 1758 compounds were retrieved based on a SHED Euclidean distance cutoff of 1.5.58 Among them, 1453 and 305 compounds exhibited structural similarity to MK-801 and memantine, respectively, based on topological pharmacophoric features. Scaffold analysis was conducted by extracting the atomic frameworks of all molecules, leading to the identification of 446 scaffolds. Following visual inspection, 17 cost-effective molecules with scaffold topologies distinct from MK-801 and memantine were prioritized. These 17 selected molecules were experimentally evaluated for their ability to inhibit Erk1/2 activation induced by tissue-type plasminogen activator (tPA) treatment in hippocampal neurons. This study successfully identified novel small molecules that effectively inhibit tPA-induced Erk1/2 activation in neurons and provide high levels of neuroprotection against amyloid toxicity, a significant contributor to AD. These findings support the potential of Erk1/2 inhibitors as novel therapeutic targets for the treatment of AD.

Applying virtual HTS, 44 compounds from 500 bioactive molecules from honey, royal jelly, propolis and bee bread, wax, and venom were identified as potential inhibitors of BACE1 with favorable pharmacokinetics and pharmacodynamics properties, intestinal and oral absorption, bioavailability, BBB permeability, less skin penetration, and no inhibition of cytochrome P450 (Cyp450) inhibitors.<sup>59</sup> Docking studies using AutoDock Vina coupled with molecular dynamics simulation (GROMACS) and molecular mechanics-Poisson–Boltzmann surface area (MM-PBSA) calculations were conducted, and rutin, 3,4-dicaffeoyl-quinic acid, nemorosone, and luteolin were identified as novel inhibitors of BACE1.

The potential of 1,4-dihydropyridine (1,4-DHP) compounds as BACE1 inhibitors was previously highlighted by the Miri research lab.<sup>60–62</sup> Building on this, they conducted further investigations involving molecular modeling, synthesis, and in vitro BACE1 inhibitory activities of novel derivatives of 2,6dimethyl-3,5-bis-*N*-(aryl) carbamoyl-4-aryl-(aryl/heteroaryl)-



**Figure 9.** (a) Workflow of the virtual screening method. (b) Binding of 8012-9656 using molecular dynamic simulation. 8012-9656 is shown in cyan; important amino acid residues are presented in blue, and hydrophobic interactions are shown in pink (dotted line). Adapted with permission from ref 65. Copyright 2020 American Chemical Society.

1,4-dihydropyridine, referred to as PDB (Figure 8).63 To validate the docking procedure, ten PDB-derived BACE1 structures were utilized. These structures were selected based on their crystallographic resolutions and the similarity of cocrystallized ligands to the DHP structures. In order to understand the structural features associated with BACE-1 inhibitory activity, a series of DHP derivatives (6a-p) were synthesized. Molecular modeling studies revealed that properly positioned hydrogen bond donors/acceptors may play a crucial role in the BACE1 inhibitory activity of 3,5-bis-N-(aryl) carbamoylated DHPs. The exploration of SAR for the developed BACE1 inhibitors demonstrated the significance of the two carbamoyl NH groups in the 3,5-disubstituted aryl/heteroarylcarbamoyl-1,4-DHPs, as they form key hydrogen bonds with Asp228 and Gly230. The authors strongly suggest the potential use of DHP structures as BACE1 inhibitors without possible side effects.

Structure-based virtual screening was used to find new chemical scaffolds with better pharmacokinetic parameters and CNS permeation that target GSK-3 $\beta$  as a disease-modifying approach toward AD treatment.<sup>64</sup> The docking protocol was validated by redocking the cocrystallized ligand 2-[(cyclopropylcarbonyl)amino]-N-(4-methoxypyridin-3-yl)-pyridine-4-carboxamide into the GSK-3 $\beta$ . The top 100 ligands after the docking analysis were assessed for binding affinity using Prime/MM-GBSA critical energy calculations. The structure-based virtual HTS using a combination of the Chembridge CNS-Set library and GSK-3 $\beta$  cocrystallized structures discovered 10 small molecules through interactions, binding energies, docking score, and potential ADME profile. One quinoline-based compound was finally identified to show maximum inhibition of GSK-3 $\beta$  with an IC<sub>50</sub> of 5.10  $\mu$ M.

Rapid overlay of chemical structures (ROCS) is another 3D approach that has the potential to identify molecules that share

similarity with a target protein while possessing dissimilar underlying chemical structures, a concept known as "scaffold hopping." In ROCS, atoms are represented as three-dimensional Gaussian functions, and similarity is calculated based on volume overlaps between alignments of pregenerated molecular conformers. Virtual screening through ROCS was used to identify inhibitors of BChE (Figure 9a).<sup>65</sup> Biochemical evaluations were conducted on Chemdiv database which contains over a million compounds. Initially, 1294 compounds with scores within the top 1% were retained after the primary screening step. Subsequently, the top 186 compounds were obtained after refinement based on their interaction energy using the CDOCKER docking module in Discovery Studio 2019 (DS 2019, BIOVIA, USA), with a cutoff of -CDOCKER interaction energy >40 kcal/mol. From this process, 13 hit molecules were manually chosen based on their binding modes and structural diversity. Among these, a novel BChE inhibitor named 8012-9656 was identified (Figure 9b), which exhibited high BChE inhibition in a noncompetitive manner and displayed selectivity over AChE. The in vitro and in vivo results demonstrated that compound 8012-9656 showed promising restoration of cognitive function in the AD model.

Pharmacophore modeling is most often applied to virtual screening to identify molecules triggering the desired biological effect. A pharmacophore model can be developed through either a ligand-based approach, where a set of active molecules is overlaid to identify shared chemical features crucial for their bioactivity, or a structure-based approach, which explores potential interaction points between the macromolecular target and ligands. Molecules with distinct structures but similar pharmacophoric patterns may be inferred to interact with the same binding site on a biological target, leading to comparable biological profiles.<sup>66</sup>

Both ligand- and structure-based pharmacophore mapping were used to discover  $\gamma$ -secretase inhibitors. A total of 111 compounds known as  $\gamma$ -secretase inhibitors were screened through docking using Molegro Virtual Docker and virtual screening to find a compound having better affinity with the target protein than the established inhibitor. Compound AKOS001083915 (Pubchem CID: 24462213) demonstrated the highest affinity toward the target protein.<sup>67</sup> AKOS001083915 was found to interact with three specific amino acid residues, namely Phe218, Asp655, and Lys654, forming hydrogen bonds. Additionally, AKOS001083915 is surrounded by highly electropositive residues. To summarize, the compound AKOS001083915, identified through virtual screening, exhibits a binding affinity and relevant properties similar to that of the best-established  $\gamma$ -secretase inhibitors.

Johari et al. have identified triazine derivatives bearing a novel scaffold by first conducting 3D ligand-based pharmacophore model generation utilizing HypoGen module of the BIOVIA Discovery Studio v4.5 and subsequently structure based pharmacophore mapping to explore the binding sites of apoE4 which was the targeted pathway of the study.<sup>68</sup> This was followed by virtual screening, ADMET and density functional theory (DFT), which was used to analyze the stability of the inhibitor within the active sites of receptor, narrowed down the proposed hits as potential druglike candidates, advancing them to the subsequent stage. The binding patterns of the bestproposed hits were investigated and were subjected to molecular dynamic simulations. As a result of this study, 2 potential compounds for further experimental and clinical AD validation were identified, C13 and C15 which selectively interact with Arg112 of apoE4.

#### OVERVIEW OF EMERGING TECHNIQUES

The current techniques to identify small molecules have played a crucial role in better understanding of this disease. Several new and emerging techniques are gaining momentum in helping researchers to develop tools and knowledge about AD.

Machine learning using omics data to allow early diagnosis of AD has been a key area of investigation.<sup>21,69-74</sup> AD risk genes can be predicted using this method. For example, Binder et al. have developed a MPxgb(AD) model where they have incorporated the XGBoost algorithm that is able to generate AD-associated gene predictions.<sup>75</sup> Through this exercise they have identified FRRS1, CTRAM, SCGB3A1, FAM92B/ CIBAR2, and TMEFF21 as AD-risk genes. In a different study, millions of images of druglike molecules were used to predict their potential target.<sup>76</sup> This deep learning framework is called ImageMol which is capable of evaluating molecular properties and molecular target profiles across several data sets. This investigation was predominately conducted for identifying anti-SARS-CoV-2 molecules; however, one of the training protein target was BACE, which is a key target in AD. Deep learning using fluorescent markers have shown to improve the screening of small molecules in HCS.<sup>77</sup> A three-channel data set, 4',6-diamidino-2-phenylindole (DAPI) nuclear marker, YFPtau, and AT8-pTau, was used to train the model and an archival HCS data set was applied. The machine leaning method was able to identify compounds that can inhibit tau aggregation which are generally overlooked in traditional screening.

CRISPR technology has generated a broad precision mammalian genome regulatory toolbox, enabling gene knockout, insertion, activation, and suppression.<sup>78</sup> Several interesting reviews have been published on this topic related to AD recently.<sup>19,20,22,79</sup> One to highlight is the work by Inoue et al. where they have increased the expression of APP and/or BACE1 using CRISPR gene activation to reveal the hidden defect in  $\gamma$ -secretase in fibroblasts of FAD which results in the increased level of A $\beta$ 42.<sup>80</sup>

# CHALLENGES AND OUTLOOK OF AD

There is a high drug development failure rate in AD which prompts an urgent need to develop disease-modifying therapies that can prolong and improve the overall quality of life for patients diagnosed with early stages of AD. The complexity of AD with respect to the genetic risk factors, the pathological underpinnings, and the progressive nature requires a multitude of drug development strategies. The commencement of pathological processes long before the manifestation of initial symptoms adds an additional layer of complexity to intervention strategies.<sup>81</sup> Addressing these challenges mandates early detection and therapeutic intervention.

Due to the intricate biological mechanisms involving complex genes and proteins, AD poses a formidable challenge for researchers. While existing treatments can alleviate and temporarily impede AD symptoms, they fall short of halting the advancement of brain damage. The current approved prescription drugs do not show a good efficacy and tolerability in a wide range of patients, especially for the severe and advanced cases of AD. It is imperative to innovate and create a novel treatment strategy aimed at not only arresting the progression of AD but also effectively curing the condition.

Numerous strategies have been proposed to enhance the efficacy of AD therapy. Combining various small-molecule drugs that target distinct critical points or integrating a small-molecule drug with antibodies could potentially yield additive effects in treating AD. Another strategy involves the development of multitarget drugs (MTDs) approach which is based on combination therapy and improved pharmacokinetics, safety, and patient compliance.<sup>82</sup> Nonetheless, significant efforts are required to enhance the pharmacodynamics and effectiveness of MTDs. Moreover, a thorough understanding of AD pathology is crucial for identifying novel targets in the treatment of AD.

The progress in modeling approaches, particularly in modelinformed drug discovery and development, signifies a technological leap. This approach provides a quantitative framework for prediction and extrapolation, relying on knowledge and inference derived from integrated models encompassing compound, mechanism, and disease level data.<sup>83</sup> The primary goal is to enhance the quality, efficiency, and costeffectiveness of decision-making processes. Given the substantial failure rate in drug development, model-informed drug discovery has the potential to transform data from unsuccessful trials into valuable knowledge, thereby refining strategies in ongoing or future drug development programs.

#### CONCLUSION

Since the early 1990s when small molecule screening was introduced in the pharmaceutical industry, it has proven to be a pivotal tool which allowed not only the discovery but the development of many drugs, including those for dementia, in specific AD. Standard ELISA protocols are commonly used to quantify the binding of small molecules to  $A\beta$  aggregates. To enhance this technique, an ELISA protocol has been designed to replicate conditions found in an aqueous solution, ensuring the preservation of the desired  $A\beta$  conformation. Additionally, as

the Alpha-LISA assay operates based on intermolecular interactions, it relies on the principle that specific binding between antigens and antibodies are necessary and offers several advantages, including reduced interference, high throughput capability, heightened sensitivity, and quicker detection times. Also, a screening platform, SPEED was developed based on epitope alteration which was utilized to investigate compound epitope interaction and for small library screening.

The CADD approaches are important in assessing proteinligand interactions, allowing comprehensive analysis of scoring function, ligand flexibility, binding site characteristics, binding site radius (largest distance between a ligand and the binding site centers), presence/absence of water molecules within the site, and the number of genetic algorithms (algorithm inspired by biological evolution process). Luciferase, an enzyme utilized by various organisms for bioluminescence, notably the Firefly, serves as a vital component in multiple assays. One such assay is the luciferase fragment complementation assay and another where luciferase enzyme is utilized to evaluate the effects on protein translation. Drug discovery via HTS coupled with technological advancement in virtual screening and improvement in biological assays with the development of custom assays, FRET and BRET assays have influenced the assembly and composition of small molecule targets for AD.

However, the utilization of these technologies depends critically on the continuous effort in understanding the fundamental complicated molecular interactions that govern AD. Though  $A\beta$  neurotoxicity, tau pathogenesis, and neuroinflammatory pathways are popular routes to describe AD, many other interactions can catalyze this disease and new targets are constantly being explored. There is no doubt that screening of small molecules will continue to drive development in AD research, especially using noninvasive and affordable biomarkers with multimodal data and machine learning. Future studies should combine several preclinical screening data and available omics to further improve predictive performance of small molecules in leveraging drug development in AD.

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#### Notes

The authors declare no competing financial interest.

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