2. Literature Review

2.1. Secretases

2.1.1 γ- secretase inhibitors

γ-secretase, an aspartyl protease having multi-subunits is involved in the cleavage of APP along with other type 1 transmembrane proteins (**Figure 2.1A**)(57). The ligands binding to the receptors of LIN-12/Notch family cause minimum proteolytic cleavages at two sites: the first one is in between the extracellular and transmembrane domain followed by the second one in the transmembrane domain. Presenilin (PS) is the protein required for transmembrane cleavage of β-APP(58). The catalytic core of γ-secretase is formed by PS1 and PS2 collectively (Red circle in **Figure 2.1B**). In addition to this, the three accessory proteins *i.e.,* anterior pharynx-defective 1 (APH1), nicastrin, and PS enhancer protein 2 (Pen-2) make up γ-secretase complex (**Figure 2.1B**)(59). These three essential proteins appear to be involved in maturation as well as in stability of the complex. The γ-secretase complex appears to exhibit great amount of heterogeneity, as two PS genes and two APH1 isoforms are widely found in humans. The APH1A could also be spliced alternatively into long and short forms as in APH1B and six other functional γ-secretase complexes(60). γ- secretase is a druggable target for developing orally bioavailable brain penetrant γ- secretase inhibitors (GSIs). However, the clinical development of these compounds has suffered mainly due to target-based toxicity. Further, γ-secretase acts on APP and cleaves it in its transmembrane domain resulting in production of Aβ(57).

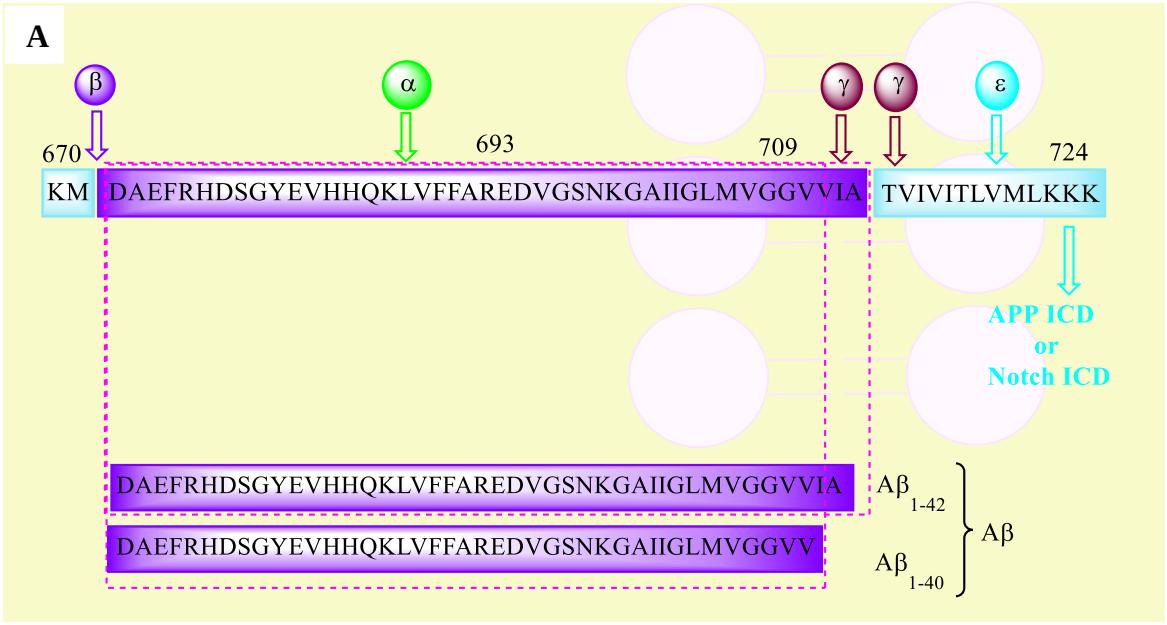
Mice with disruption of PS1 alleles were generated ($PS1^{-1}$mice) to study its effect on AD. The $PS1^{-1}$embryo exhibited an abnormal pattern of axial skeleton, spinal ganglia and these phenotypes were traced to create defects in somite segmentation and differentiation. Spatiotemporal Notch1 and *Dll1* (delta-like gene 1) gene expression are governed by PS1, which are essential for somite segmentation and maintenance of somite borders(61). Notch is a transmembrane receptor which regulates cell's fate during cell contact. Mammals have four Notch receptors and five endogenous ligands: Notch 1-4 and Delta-like 1-3 (*Dll 1-3*), Jagged 1, 2 (JAG 1, 2)(62),(63). Notch signaling plays crucial part in cell fate throughout development, proto-oncogene or tumor suppression, structure, synaptic plasticity, neuronal stem cells maintenance, as well as in neuron survival and γ-secretase is capable for cleavage of APP, Notch and other type I membrane proteins(64, 65).

The nonselective GSIs show remarkable Aβ reduction and Notch-related toxicity in therapeutic window. The 'Notch-sparing' GSIs are capable of overcoming these toxicity issues(66, 67). Recently, few novel Notch-sparing inhibitors were developed. Bms-708,163 showed lowering of plasma and CSF Aβ levels during phase I clinical trials. Begacestat, the earlier version of the Notch-sparing GSIs, binds with the adenosine triphosphate terminal of γ-secretase complex; moreover , the mode of action of second-generation Notch-sparing GSIs is unknown(68). According to the new hypothesis, these GSIs show pharmacological effects by occupying the substrate binding sites on γ-secretase, which are dissimilar among Notch and APP(68).

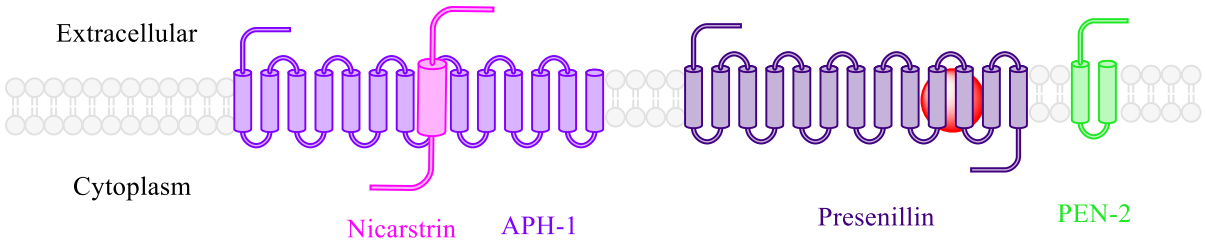
BMS-299897 was the first γ-secretase inhibitor entering into clinical trial. The compound was synthesized at Bristol-Myers Squibb but clinical data was not disclosed. It may have been abandoned in clinical trial. Secretase inhibitors like LY-450139, GSI-

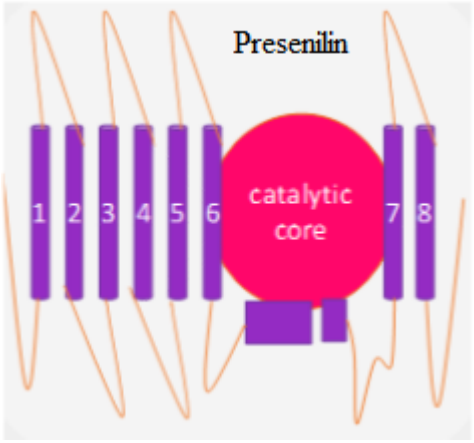
953, BMS-708163, PF-3084014, MK-0752 and E2012 are in clinical trials, but the clinical data has been published for only LY-450139. The main obstacle in design and development of more robust GSIs, is their inability to discriminate between APP and Notch thereby leading to critical issues of Notch related toxicity.

Figure 2.1 γ-secretase cleavage site and catalytic core. (A) γ-secretase, a multi-subunit aspartyl protease involve in cleaves APP intracellular domain (APP ICD) and many type 1 transmembrane proteins. (B) γ-secretase complex. It is composed of four different integral proteins: PS, Presenilin enhancer-2 (PEN-2), APH1 and Nicastrin.



B





2.1.1.1. Potent GSIs

Semagacestat (LY450139)

The action of semagacestat (LY450139) was found to be dose-dependent in decreasing $A\beta_{40}$ in brain, plasma and CSF and also in the production of $A\beta_{42}$ (**Table 2.1**). It was well tolerated and adverse actions were associated with the interference of peripheral Notch cleavage(69). LY450139 treatment showed that $A\beta_{1\text{-}40}$ in plasma decreased by 38.2%, and in CSF by 4.42 ± 9.55% (p = not significant). Higher doses of the drug may result in further decline of plasma Aβ concentrations and a measurable decrease in CSF Aβ(70). The plasma $A\beta_{40}$ accumulation was decreased by 58.2% and 64.6% in 100-mg and140-mg treated groups respectively. Although, significant decrease in CSF Aβ level was not observed, the decrease in plasma Aβ concentration was dependent on inhibition of γ-secretase(71).

The safety, tolerance and Aβ response was evaluated in a group of fifty one AD patients treated with LY-450139 for 98 days(71). The patients were randomized for treatment with placebo (n = 15) or LY-450139 (n= 36). Out of forty-three patients who accomplished the trail, seven developed skin rashes and three reported hair related abnormalities. There were no differences in cognitive or functional measures between

placebo and LY-450139-treated patients but the study was discontinued due to transient bowel obstruction in subjects.

The phase III clinical trial of LY-450139 was conducted in mild-to-moderate AD to evaluate its effect on slowing down of the disease progression. The trial was terminated before completion by recommendation from data and safety monitoring authority. It did not show any improvement in cognitive status in comparison to placebo, and patients on elevated dose had substantial worsening of practical ability. The other adverse events associated with semagacestat were skin cancers and infections(72). The wide range inhibition of all γ-secretases (more than 40 substrates), mainly Notch and also production of β-CTF, an intermediate produced during APP processing may be the possible reason for failure of semagacestat(73).

MK-0752

The GSI MK-0752 (phase II) does not differentiate among APP and Notch (**Table 2.1**). In Phase I trial, the safety, tolerability, pharmacokinetics and pharmacodynamics of single oral dose of MK-0752 between 110-1000 mg was evaluated in twenty seven healthy volunteers(74). MK-0752 was found to be safe and reached the maximum plasma levels in 3-4 hours with a $t_{1/2}$ of 20 hours. It considerably inhibited $A\beta_{1-40}$ concentration in CSF for 12 hours with peak effect of 35% in a dose of 500 mg.

MK-0752 was also being tested for the treatment of cancer. It was found to inhibit γ-secretase facilitated cleavage of Notch with IC_{50}=55 nM. The continued triggering of the Notch signal transduction was revealed in >50% of patients with T-cell acute lymphoblastic leukemia underlining its significance in pathogenesis. The preclinical trail showed that inhibition of γ-secretase quashed the T-cell acute lymphoblastic leukemia cell differentiation and prompted apoptosis by avoiding Notch cleavage, thus precluding extended stimulation of downstream pathways. The compound showed

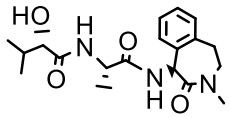
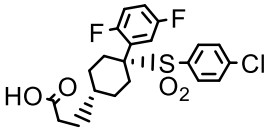
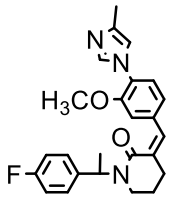
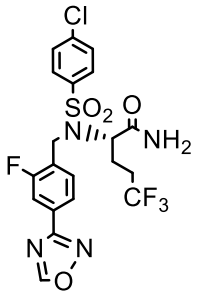
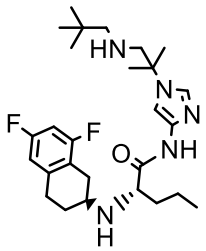
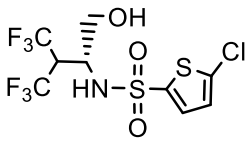
gastrointestinal toxicity and fatigue without substantial clinical activity(75). Further, the toxicity of MK-0752 was schedule dependent and dosing at an interval of one week was well tolerated, resulting in strong modulation of a Notch gene signature(76).

In pediatric brain tumors, higher levels of Notch ligand expression have been observed. Phase I trial of MK-0752 was conducted in children with persistent CNS malignancies to evaluate the maximum tolerance dose, dose-limiting toxicities, pharmacokinetics, and pharmacodynamics. It was found to be well tolerated and inhibited target at 1000 and 1400 mg/m^2/week doses in children with recurrent CNS malignancies(77). A once weekly schedule for MK-0752 is currently being explored in adults with recurrent CNS malignancies.

GSI-953 (Begacestat)

GSI-953 is a potent, selective γ-secretase inhibitor having thiophene containing sulfonamide moiety which inhibits Aβ production with low nM potency in both cellular ($A\beta_{1-42}$ IC_{50}= 15 nM) and cell-free (IC_{50}= 8 nM) assays (**Table 2.1**). The compound was found to be highly selective for the inhibition of APP cleavage in Notch cleavage assays at cellular level. In Tg2576 mice, high doses of GSI-953 significantly decreased the $A\beta_{1-40}$ levels within the brain, CSF, as well as in plasma(78). At lower doses, it caused significant reduction of $A\beta_{1-40}$ exclusively in brain and plasma but not in CSF. Essentially, the compound under discussion has been described to effect a reversal of the contextual memory deficits in Tg2576 transgenic mice. In an enhanced cell-based technique for understanding the γ-secretase enzyme activity against Notch and APP substrates, the compound begacestat was found to be equally capable of inhibiting both Notch intracellular domain (NICD) and $A\beta_{42}$ production(79).

Table 2.1 **γ-Secretase inhibitors for AD.**

Compound	Structure	Specialty	Demerits	Status
Semagacestat (LY450139)		Decreases Aβ in AD, alters the CSF peptidome in humans	In transgenic mice it showed no significant result in decreasing the brain plaque burden. Adverse events include skin cancers and infections.	Terminated in Phase III.
MK-0752		Decreases $A\beta_{1-40}$ level in healthy volunteers, now being tested for cancer.	The drug was associated with gastrointestinal toxicity and fatigue.	Terminated for AD.
E 2012		Notch sparing, gamma secretase inhibitor/ modulator without affecting Notch processing.	Lenticular opacity in rats.	
BMS-708163 (Avagacestat)		Notch sparing decreases Aβ levels in CSF of healthy volunteers.	Lack of data on brain plaque deposition in transgenic mice. and behavioral effects in animal models of AD.	Phase II
PF-3084014		Notch sparing, good brain penetration, long-lasting effects on Aβ levels in animals, no rebound effect on plasma Aβ in animals.	Lack of data on brain plaque deposition in transgenic mice, lack of data on behavioral effects in AD animal models.	Phase I
GSI-953 (Begacestat)		Improves memory in a transgenic mouse model of AD.	$A\beta_{1-40}$ level does not decrease in CSF of AD patients.	Phase II

GSI-953 is shown to have an additional 15-fold selectivity towards inhibition of APP cleavage v/s Notch cleavage and also inhibits Aβ production in both cellular ($Aβ_{42}$ IC_{50} = 15 nM) and cell-free (IC_{50} = 8 nM) assays. In Tg2576 mice, reduction of $Aβ_{41}$ levels were seen in brain, CSF, and plasma at high doses of GSI-953. Also, GSI-953 was revealed to be capable of reversing the contextual memory deficits(80). Begacestat showed promising results in phase I clinical trials and is presently undergoing clinical trials for AD treatment(81). The status of different GSIs is shown in **Table 2.1**.

BMS-708163 (Avagacestat)

BMS-708163, being developed by Bristol-Myers Squibb, is a promising aryl sulfonamide and has exhibited γ-secretase inhibitory and Notch-sparing activity (**Table 2.1**). Avagacestat displayed up to 193-fold higher selectivity for Aβ processing compared to Notch processing during preclinical studies and therefore, may possibly produce fewer adverse effects than lesser selective compounds(82). In multicenter, randomized, double-blind, placebo-controlled, single ascending dose studies, the concentrations of avagacestat elevated rapidly. Its oral administration showed a biphasic decrease in concentration with an extended terminal phase and exposures remained proportional to doses up to 200 mg. It was finely tolerable at a single dose of 800 mg, showing a biphasic effect in plasma $Aβ_{1-40}$ levels and significant adverse events were predominately absent up to 200 mg dose(83).

BMS-708163 is considered an encouraging drug candidate due to its Notch-sparing activity for the inhibition of Aβ production over Notch cleavage. It is suggested in reports that BMS-708163 undergoes direct binding with the PS-1 *N*-terminal fragment and the binding could be confronted by other pan-GSIs, although it remains unchallenged by the γ-secretase modulators (GSMs). Four different active site-directed GSI photoaffinity probes are blocked by the compound from binding to the active site.

The compound is reported to act as a nonselective γ-secretase inhibitor(84). Prodromal AD, defined by mild cognitive impairment symptoms and CSF biomarker criteria, was studied in the randomized clinical trial. Prodromal AD population receiving avagacestat or placebo had higher rates of clinical progression to dementia as compared to CSF biomarker negative participants(85). Phase II study carried out for the determination of safety and efficacy of avagacestst in prodromal AD was completely devastating. It was found that the population treated with avagacestat or placebo showed enhanced progression of dementia and brain atrophy when compared with negative CSF biomarker population(85).

PF-3084014

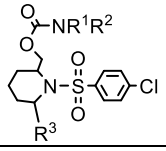
PF-3084014, a novel Notch-sparing γ-secretase inhibitor, is in due course of development (**Table 2.1**). It emerged as a viable, non-competitive and reversible human γ-secretase inhibitor showing an IC_{50} of 6.2 nM in cell free assay(82). However, in a whole-cell assay, the drug displayed an IC_{50} of 1.3 nM. It was shown to inhibit Notch signaling (rather weakly) with an IC_{50} of 19.15 nM in fetal thymus organ culture assay. The APP to Notch selectivity ratio was 1473. BBB permeability of PF-3084014 was analyzed for trans-endothelial electrical resistance (TEER) and was evaluated in both dynamic (microfluidic) and static (transwell) BBB models, either with brain endothelial cell line bEnd.3 in monoculture or in co-culture with glial cell line C6. The PF-3084014 dynamic and co-culture models resulted in lesser permeability, and appreciably higher TEER, than static and mono-culture models(86).

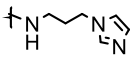
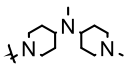
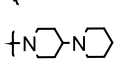
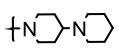
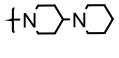
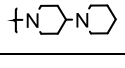
Piperidine derivatives

The piperidine and tetrahydroisoquinoline containing compounds were active as γ-secretase inhibitors. Among the piperidine derivatives, *cis*-disubstituted compounds (**1**, **2**) were approximately 5 to 10 folds more active than their *trans*-congeners (**Table 2.2**).

The cyclic amines bearing a basic nitrogen center at the right-hand along with ethyl as the R^3 substituent improved the potency of the compounds (**3**, **4**) (**Table 2.2**). Aromatic group at R^3 having mono or di-substituted fluorine decreased the IC_{50} up to double digit of decimal (**Table 2.2**).

Table 2.2 γ-Secretase inhibitors for AD.



Compound	**NR^1R^2**	**R^3**	**$A\beta_{1\text{-}40}$ IC_{50} (μM)**
1 *cis*		Me	1.97
2 *cis*		Me	0.57
3		Et	0.159
4		Et	0.034
5		2-F-Ph	0.008
6		3-F-Ph	0.003
7		2,5Di-F-Ph	0.004

Molecules were further modified in the form of conformationally restricted piperidine *N*-arylsulfonamides and orally active γ-secretase inhibitors. The conformational restriction as well as decrease in molecular weight resulted in compounds with less CYP 3A4 liability compared to early leads. CYP3A4 interaction was found in compounds containing 2,4,6-trisubstituted *N*-arylsulfonyl piperidines and the inhibition had been linked with lipophilicity and presence of basic amines(87). It was proposed that decreasing cLogP by introducing substitution such as $-OCH_3$ or -OH, on piperidine ring (R^4) could decrease the CYP3A4 interaction in the piperidine sulfonamide series. The

substitution on piperidine ring increased the IC_{50} value for the membrane $A\beta_{42}$ (310.1nm to >1000) (**Figure 2.2**). The unsubstituted triazole, imidazole or pyridine functions were mostly responsible for CYP3A4 interaction(87). GSIs lacking such groups and electronic pattern were deprived of CYP3A4 interaction. The CYP3A4 inhibition was reduced up to 0.8 µM on changing the side chain of the Piperidine *N*-arylsulfonamides(88) (**Table 2.3**).

Figure 2.2 Modification of 2,4,6-trisubstituted N-arylsulfonyl piperidines to find the agreement between CYP3A4 interaction and γ-secretase inhibition.

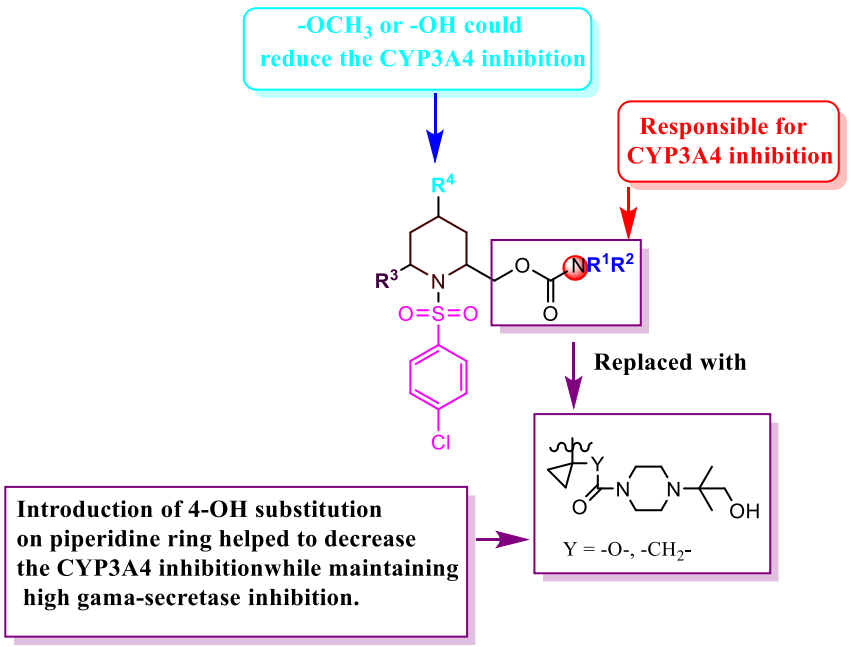
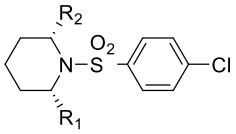
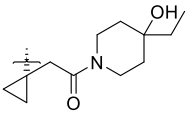
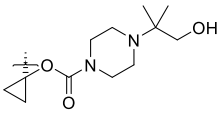
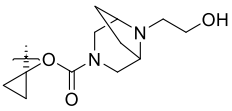
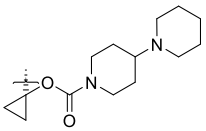
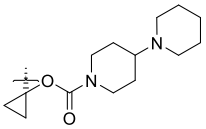
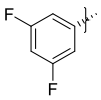
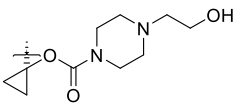
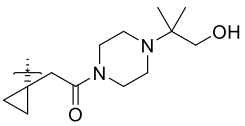
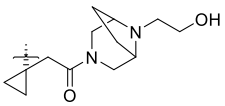
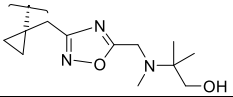


Table 2.3 Piperidine N-arylsulfonamide derivatives with IC_{50} and CYP 3A4 inhibition activity.



S.No	R_1	R_2	IC_{50}, CYP3A4 inhibition
8	$-C_2H_5$		Memb $A\beta_{40}$ IC_{50} (nM) = 3.2 Cell $A\beta_{40}$ IC_{50} (nM) = 4.3 CYP 3A4 (µM) = 1.1
9	$-C_2H_5$		Memb $A\beta_{40}$ IC_{50} (nM) = 6.9 Cell $A\beta_{40}$ IC_{50} (nM) = 8.3 CYP 3A4 (µM) = 2.2
10	$-C_2H_5$		Memb $A\beta_{40}$ IC_{50} (nM) = 3.5 Cell $A\beta_{40}$ IC_{50} (nM) = 7.7 CYP 3A4 (µM) = 2.5
11	$-C_2H_5$		Memb $A\beta_{40}$ IC_{50} (nM) = 3.5 Cell $A\beta_{40}$ IC_{50} (nM) = 9.1 CYP 3A4 (µM) = 0.8
12			Memb $A\beta_{40}$ IC_{50} (nM) = 0.3 CYP 3A4 (µM) = <0.8
13			Memb $A\beta_{40}$ IC_{50} (nM) = 2.4 CYP 3A4 (µM) = 0.06
14	$-C_2H_5$		Memb $A\beta_{40}$ IC_{50} (nM) =2.2±0.01 Cell $A\beta_{40}$ IC_{50} (nM) = 2.4±0.1 CYP 3A4 (µM) = 4.7±0.6
15	$-C_2H_5$		Memb $A\beta_{40}$ IC_{50} (nM) = 3.1±0.1 Cell $A\beta_{40}$ IC_{50} (nM) = 7.4±1.0 CYP 3A4 (µM) = 2.2
16			Memb $A\beta_{40}$ IC_{50} (nM) =9.2±1.7 CYP 3A4 (µM) = 3.1

2.1.1.2 γ- secretase modulators (GSMs)

GSMs evolved as better and safer approach for drug development against AD after the failure of GSIs in clinical trials. They interact with γ secretase through the allosteric binding site, and thus do not interfere in normal processivity of enzyme, leading to

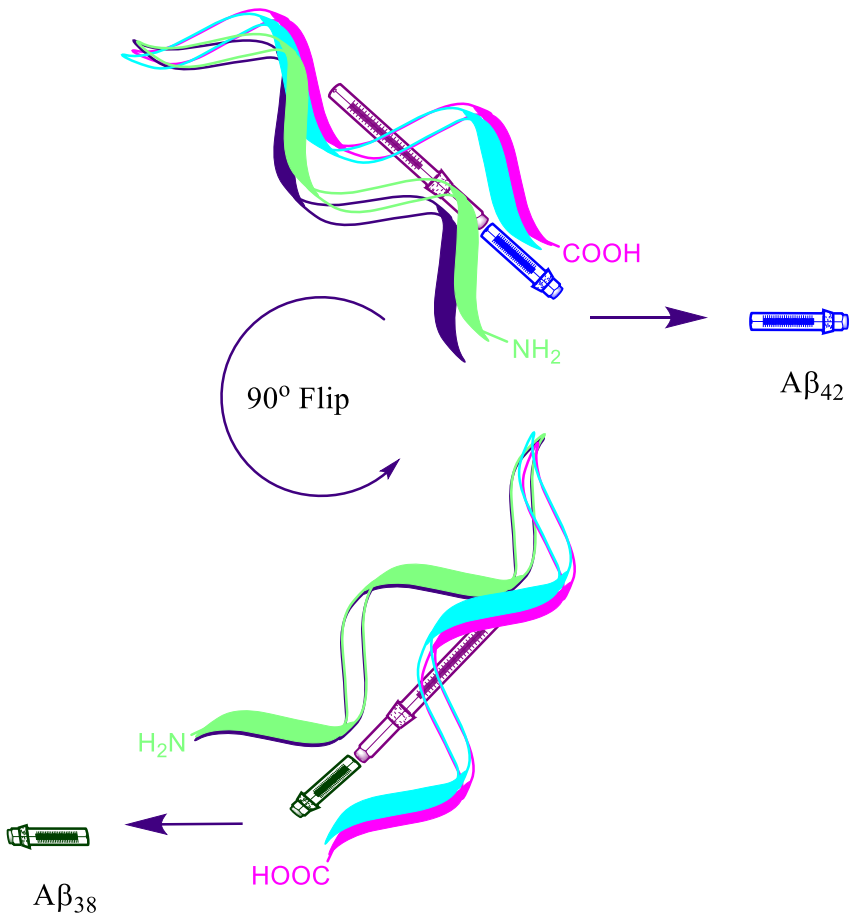
decline in Notch related toxicity. They reduce deposition of Aβ and shift the $A\beta_{42}$: $A\beta_{40}$ ratio towards the lower side to increase the smaller non-amyloidogenic Aβ species(89). Profile of Aβ peptides were found to be changed in *in-vitro* and *in-vivo* assays performed for GSMs (90, 91). GSMs increase the level of shorter Aβ peptides along with reduction in $A\beta_{42}$ production(92). The Notch inhibition by GSIs leads to many Notch signaling related abnormalities. GSMs are deprived of such Notch related side effects, thus they seem to escape the target-based toxicity. AD-associated mutations in APP and PS genes modify γ-secretase activity to favor $A\beta_{42}$ by 30%. Thus, selectively reducing the level of $A\beta_{42}$ with GSMs is an interesting alternative approach to GSI therapy. In a short clinical trial with AD patients the first GSM, Tarenflurbil, did not show efficacy. The poor blood brain barrier crossing ability of the drug was one of the major reasons behind its failure in the trial(93). There are literature evidences indicating the direct interaction of GSMs with APP rather than modulating γ-secretase(94). The new generation GSMs are preferred over the old GSMs for the treatment of AD, although, their mechanism of actions are yet to be determined.

2.1.1.2.1 First-generation GSMs

Fluorescence resonance energy transfer (FRET) based techniques provide some data towards nonsteroidal anti-inflammatory drugs (NSAIDs) based γ-secretase modulation. It was found that NSAIDs like flurbiprofen, indomethacin and *s*-ibuprofen had an allosteric effect on γ-secretase. These NSAIDs changed $A\beta_{42}$, altered PS1-APP interactions and PS1 conformation *in-vitro* and *in-vivo* studies. The PS1-APP interaction was modified allosterically by NSAIDs to direct the cleavage sites toward $A\beta_{38}$ instead of $A\beta_{42}$. The distance between the *N* and *C* termini of PS1 increased due to NSAIDs (**Figure 2.3**) and the conformational changes in PS1 shifted cleavage of APP C99 toward shorter Aβ species such as Aβ38(95, 96).

The biotinylated photoactivatable GSMs were evolved to pinpoint the target of NSAIDs. In H4 cell line, GSM photoprobes selectively interacted with APP carboxy terminus and to aggregation prone region, amino acid residues 28-36, of Aβ peptide without labeling the γ-secretase complex. Site directed mutagenesis of APP altered GSM binding affinity. Therapeutically, GSMs can alter not only production of $A\beta_{42}$ but also decrease its aggregation propensity.

Figure 2.3. Binding of GSMs at luminal side of PS induces a conformational change in the catalytic center of γ-secretase to modulate Aβ production.



2.1.1.2.2 Second-generation GSMs

The second-generation GSMs exhibit a great structural diversity. It is well established that in the pull-down assay, imidazole derived GSMs could potentially make a complex with the components of γ-secretase complex without affecting APP. The photoaffinity labeling investigation combined with limited digestion demonstrated that phenylpiperidine derived GSM-1 directly acted on transmembrane domain (TM) 1 of PS1. The binding of GSM-1 was reported to alter the substrate binding and catalytic site of γ-secretase, thereby, enhancing the production of nontoxic Aβ38. This was an indication of allosteric modulation of PS1, establishing a novel mode of action of phenylpiperidine-type GSMs (**Figure 2.4A**).

The GSM-2 exhibited reduction in $A\beta_{42}$ production in *in-vivo* mice animal model having age-dependent growth in amyloid plaques. Radio labeling studies indicated crucial role played by nascent $A\beta_{42}$ in cognitive disorders compared to plaque-associated soluble $A\beta_{42}$ (97-99). The binding of piperidine acetic acid derived GSM-1 in the region of PS1 leading to conformational change within the active site of γ-secretase was studied using fluorescence lifetime imaging microscopic technique (**Figure 2.4A, B**) (99-101). Interestingly, GSM-1 probes specifically labelled signal peptide peptidase (SPP)(84, 100, 102).

Piperidine acetic acid and E2012 GSMs specifically target PS1 but appear to take control of various sites of the γ-secretase complex. The acid GSMs mediate their action via increase in the level of $A\beta_{38}$ and decreasing the level of $A\beta_{42}$, whereas imidazole GSMs differentially decrease the levels of $A\beta_{42}$ with increase in levels of $A\beta_{38}$ and $A\beta_{37}$. It is noteworthy that imidazole GSMs exhibit multiple modes of action as depicted by NGP-555, E2012, and RO-57(98, 103). Therefore, the co-operation between GSMs and its other subtypes should be monitored carefully.

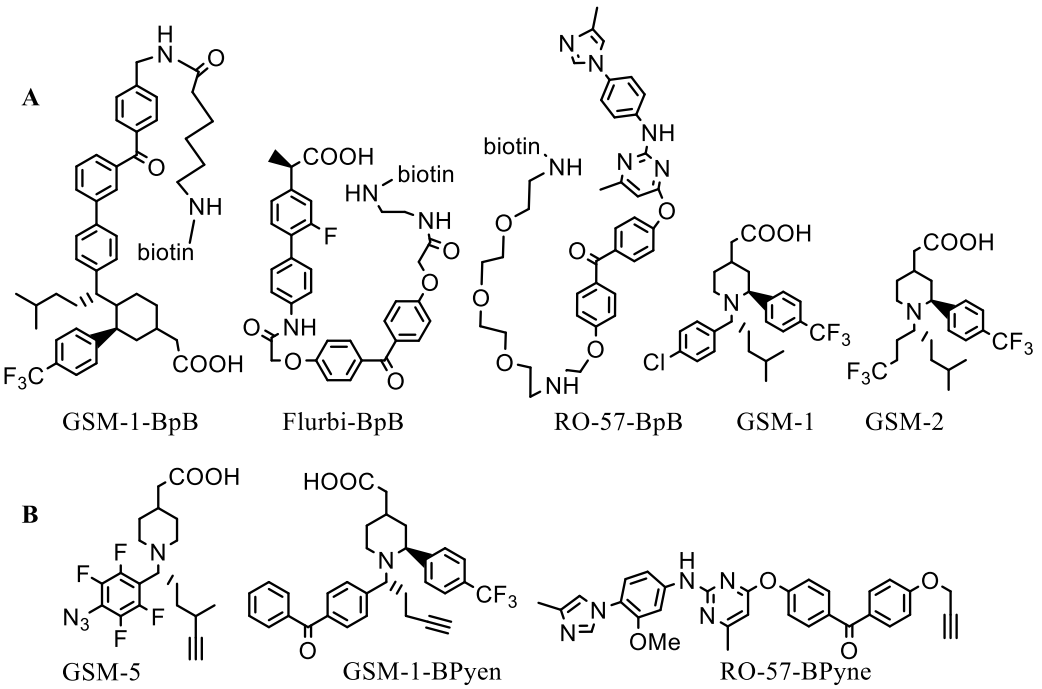
E2012, presently in phase I trial, does not interfere with Notch processing and has been claimed to decrease Aβ levels by inhibition of γ-secretase. It caused cataract upon repetitive dosing in rats which attained a peak during 70-77 days of treatment in posterior subcapsular region showing granular/punctate opaque or shiny dots lengthwise on the suture line. It was identified histologically by lenticular fiber degradation, which finally coalesced to generate a triangular or circular opacity in rat(104). Recently, several shorter Aβ isoforms *viz*. $A\beta_{1-16}$ were identified in CSF using targeted proteomics techniques. GSI treatment in AD patients produced many fold increase in $A\beta_{1-14}$, $A\beta_{1-15}$ and $A\beta_{1-16}$ in Phase II clinical trial. CSF Aβ isoform array was examined by immunoprecipitation in combination with MALDI-TOF mass spectrometry. The rise in $A\beta_{1-15}$ and $A\beta_{1-16}$ was observed while $A\beta_{1-34}$ was decreased due to treatment with GSI, LY450139. The $A\beta_{1-37}$ levels were considerably elevated in dose-dependent pattern upon treating with E2012, whereas $A\beta_{1-39}$, $A\beta_{1-40}$ and $A\beta_{1-42}$ showed downregulation. The results suggested that γ-secretase modulator E-2012 altered the cleavage site preference of γ-secretase. The growth in $A\beta_{1-37}$ may prevent $A\beta_{1-42}$ oligomerization and consequently its adverse effects.

The first-generation GSMs lack in their potential due to unaccepted neuro pharmacokinetic properties. The potency and bioavailability of second-generation GSMs have been improved by inclusion of NSAID-derived carboxylic acid and non NSAID-derived heterocyclic chemotypes(90). Carboxylic acid and heterocyclic groups derived from NSAID target PS, the catalytic subunit of γ-secretase. Natural products have also been used to improve the pharmacokinetic parameters of second-generation GSMs.

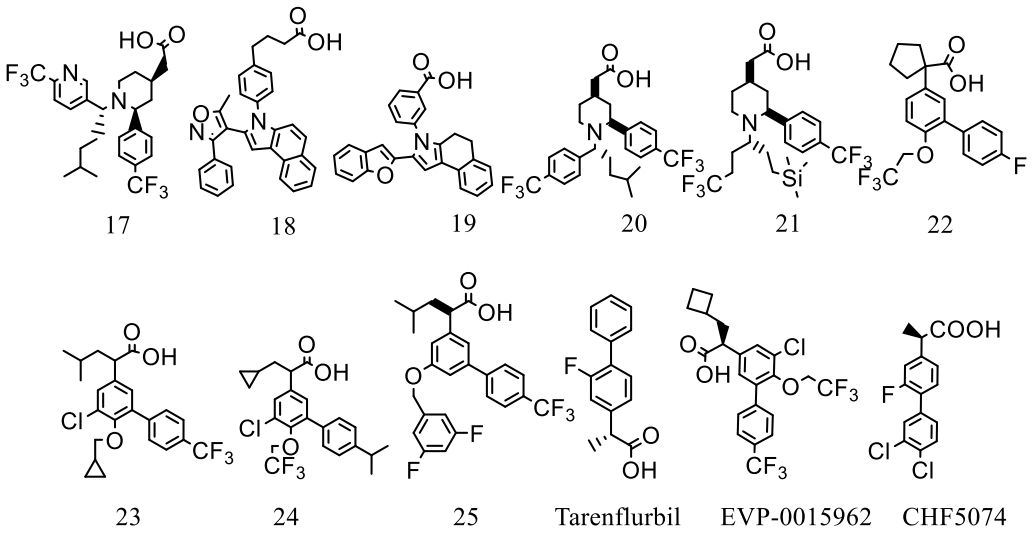
NSAID derived carboxylic acid analogs

The *in-vitro* potencies of first generation NSAIDs were very feeble and it was diffucult to get sufficient drug concentrations in brain to engage target. The main goal behind development of GSMs is to improve their potency and bioavailability.

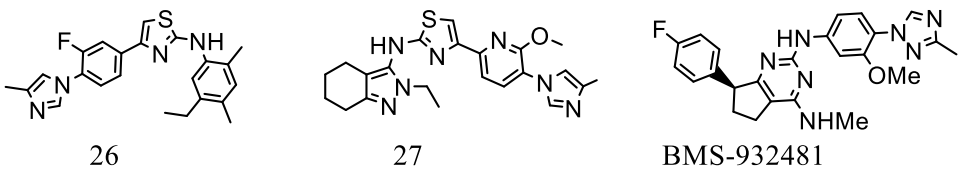
Figure 2.4. γ- secretase inhibitors and modulators. (A). NSAID derived carboxylic acid analogs (B) Non-NSAID GSMs. (C) Natural products as potent GSMs. Structures of GSM-derived photoaffinity probes containing (D) biotin (E) alkyne.



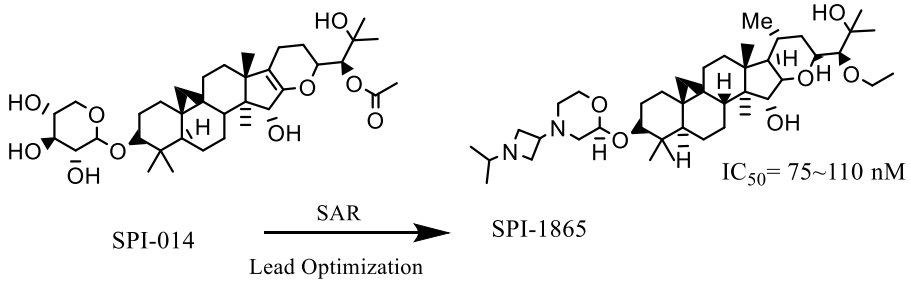
C

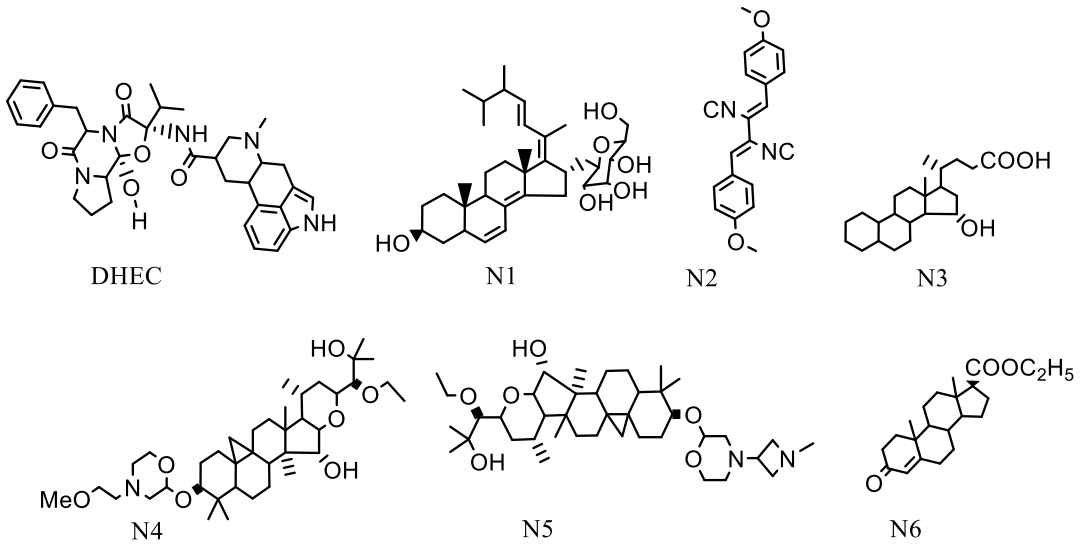

17
18
19
20
21
22
23
24
25
Tarenflurbil
EVP-0015962
CHF5074

D


26
27
BMS-932481

E


SPI-014
SAR
Lead Optimization
SPI-1865
IC50= 75~110 nM


DHEC
N1
N2
N3
N4
N5
N6

Several second generation NSAID-like GSMs with increased *in-vitro* potency and brain penetration have been reported. Piperidine-derived compound **17** was found to decrease $A\beta_{42}$, increase $A\beta_{38}$ and had no effect on the $A\beta_{40}$ level. The compound also showed better pharmacokinetic parameters in mouse, rat, and dog along with good CNS penetration in mouse (**Figure 2.4C**)(105). Few patent applications were filed including *N*-aryl indole and tetrahydroindole compounds (Myriad Genetics, Inc. US080249135. Merck & Co., Inc. WO08085301, Janssen Pharmaceutica, N.V. WO09051948; 2009). The compounds mentioned above were reported to have $A\beta_{42}$ IC_{50} values between 5-100 μM in H4 cells expressing wild-type APP695 (17-25). The novel carboxylic acid analogs *i.e.,* CHF507480 and EVP-001596281 have been probed in clinical trials. CHF5074 was dropped due to limited potency (IC_{50} value 40μM), poor drug likeness, and indigent CNS penetration (brain to plasma ratio (B:P 0.03-0.05). The details of EVP-0015962 trial are yet to be reported. It has shown improvement in *in-vitro* potency, but also suffers poor drug-like properties as other NSAID-like GSMs. Tarenflurbil is a selective $A\beta_{42}$-lowering agent which regulates γ-secretase activity and decreases $A\beta_{42}$ production without any effect on the production of $A\beta_{40}$ (**Figure 2.4C**)(90, 106, 107). Initially, it looked promising but did not show efficacy in the phase III trial(93, 108). Avagacestat (BMS708163), a selective and potent γ secretase inhibitor of $A\beta_{42}$, has an IC_{50} value of 0.27 nM and is 193-fold more selective against Notch(109).

Non NSAID imidazole GSMs.

A potential reason for the development of aminothiazole bridged aromatic (26) is its inherent lipophilicity, as highly lipophilic compounds frequently cause off-target toxicities and are difficult to develop due to their poor aqueous solubility(110). The diarylaminothiazole moiety of compound **26** was substituted with specific heterocycles which led to compound **27**. Replacement of aryl groups with certain heterocycles

improved physicochemical properties as well as kinetic and thermodynamic solubility in aqueous buffers. BMS-932481 was orally absorbed and was found to accumulate approximately three fold on multiple dosing, but further studies were discontinued due to an insufficient safety margin to test the hypothesis for the efficacy of Aβ lowering in AD (**Figure 2.4D**).

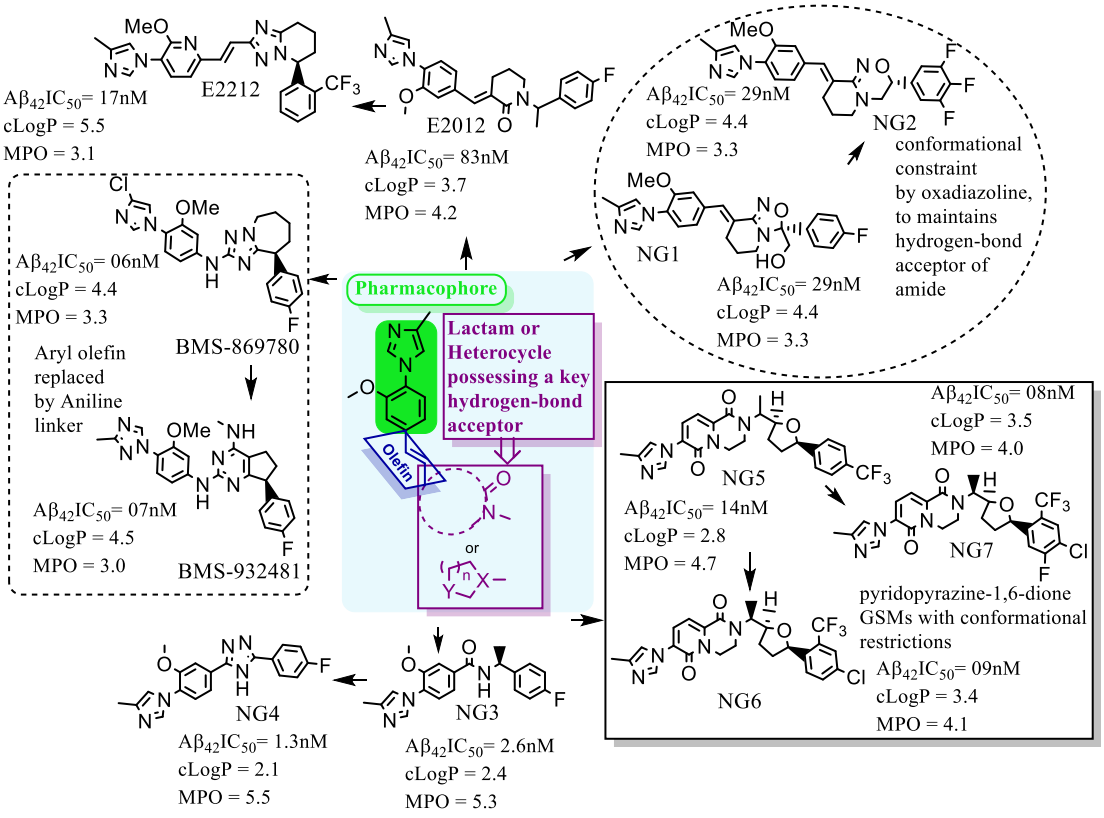
There has been a keen interest on non-carboxylic acid GSMs from research of Eisai group(111). Nearly all non-carboxylic acid GSMs possess key arylimidazole group attached via an olefin to a lactam (E2012) or a heterocycle (E2212; structure kept secret) having a major hydrogen-bond acceptor. E2012 had been a powerful early GSM (IC_{50} 83 nM) and was the first non-carboxylic acid GSM to enter clinical trials. It showed lenticular opacity in 13-weeks of rat safety study. The compound was further modified as E2212 to improve the drug-likeness. The pharmacodynamic response of E2212, measured in plasma, increased with dose and showed 54% $A\beta_{42}$ reduction (area under the curve for 0-24 h ($AUC_{0\text{-}24}$ h) of 44%) at 250 mg dose(112). Although, the structure of E2212 has never been revealed, but it is predicted by several Eisai process chemistry patents. This compound is very potent (IC_{50} 17 nM) with high MW (480), high cLogP (5.5), and a low CNS multiparameter optimization (MPO) score (3.1)(113). The safety, tolerability, pharmacokinetic, and pharmacodynamic evolution of E2212 during phase I trial established its single dose use(114). The further development of this compound has not been reported till date.

The potential nature of amide (NG1) as hydrogen-bond acceptor was achieved by conformational change between amide carbonyl and the α-methyl carbon via a cyclized hydroxyamidine (oxadiazoline). The addition of a hydroxyl group improved potency (IC_{50} 29 nM) with a decrease in cLogP (4.4 vs. 5.2). It suffered with chaotic CNS drug-like properties, as calculated by cLogP of 4 and MPO score of 3.3(115). The ring

expansion of oxadiazoline to oxadiazine afforded potent compounds with high cLogP (4.6) and low MPO scores (3.4). Further, substitution at C3 resulted in compound NG2 with good *in-vitro* potency (IC_{50} 48 nM) (**Figure 2.5**)(115, 116).

Compound BMS-869780 showed *in-vivo* efficacy in both mice and rat brains. In mice, 30 and 100 mg/kg oral doses produced $A\beta_{42}$ $AUC_{0\text{-}24h}$ reductions of 31% and 55%, respectively. Further, 10 mg/kg oral dose of the drug produced an $A\beta_{42}$ $AUC_{0\text{-}24h}$ reduction of 47% in rats(117). BMS-869780 lowered brain $A\beta_{42}$ without any evidence of Notch related side effects in rat but further studies were discontinued due to potential safety concerns. The projected daily human dose required to provide a desired $A\beta_{42}$ $AUC_{0\text{-}24h}$ reduction of 25% was very high (700 mg). The compound was further modified to typical methylimidazole with inclusion of methyltriazole (BMS-932481; IC_{50} =7 nM). Phase 1 clinical trial of BMS-932481 showed decrease in CSF $A\beta_{42}$ with a single dose of 900 mg. Lipophilic compound NG6 possessed better *in-vitro* potency (IC_{50} 9 nM), at the cost of higher MW (521), higher cLogP (3.4), and a little lower calculated MPO score (4.1). The addition of a single fluorine atom in NG6 provided compound NG7, which also demonstrated admirable potency (IC_{50} 8 nM) with MW of 538, cLogP of 3.5, and a calculated MPO score of 4.0. Although, these compounds have higher molecular weights but are predicted to possess excellent drug-like properties as calculated by their cLogP, MPO scores, and high sp^3 character (Fsp^3 = 0.4)(118) (**Figure 2.5**). However, further studies have been discontinued due to insufficient safety margins. The non NSAID GSMs should be modified further for their physiochemical properties, off target interaction and vigorously monitored for further drug design and development.

Figure 2.5. Development of Non-NSAID GSMs.



Natural products as gamma secretase modulators

GSMs found in extract of *Actaea racemose*, (black cohosh) resulted in isolation of compound SPI-014. It required nuances of medicinal chemistry to conclude SAR of SPI-014 and related semisynthetic compounds. These modifications *viz.* substitutions of both sugar and acetate moieties with more stable substitutes, enhanced drug-like properties and resulted in designing of novel drug molecule SPI-1865. However, unanticipated off-target adrenal toxicity precluded advancement of this series of compounds into clinical development(119) (**Figure 2.4E**). The atypical sterol derivative (**N1**) and the aromatic isonitriles comprising metabolite xanthocillin X dimethyl ether (**N2**) from the marine sponge-derived fungus *Dichotomomyces cejpii* were extracted. Compounds **N1** and **N2** were assessed in an AD cellular assay and were able to prevent

increased production of $A\beta_{42}$ in (2R)-2-[[9-Isopropyl-6-(N-methylanilino)purin-2-yl]amino]butan-1-ol (Aftin-5) treated cells(120). Dihydroergocristine (DHEC), a constituent of FDA approved drug, showed some promising activity. It is comprised of Leu-Val-Phe motif, which was important to bind with an allosteric site made by both Nicastrin and PS1. It competed with APP-C99 substrate for binding to the allosteric site upon binding to γ-secretase and thus affecting the processing of APP-C99(121). DHEC showed efficacy on cell line derived from AD patient by reducing Aβ level in micromolar concentration range. Further, SAR studies showed that the cyclized tripeptide moiety of DHEC was crucial for inhibiting γ-secretase (121).

While optimizing the triterpene-based GSMs, the acetate group at C_{24} position of the natural product was substituted with carbamates or ethers to obtain compounds with enhanced metabolic stability. Further, morpholines or carbamates were introduced at C_3 position to obtain desired physicochemical properties of the derivatives. The compounds thus obtained had low clearance and proper distribution into the CNS of CD-1 mice. Two of these compounds, **N4** and **N5**, were tested for pharmacodynamic effect in CD-1 mice and showed lower brain $A\beta_{42}$ level(122).

Curcumin, extracted from turmeric, is well known for its anticarcinogenic, antioxidant and anti-inflammatory properties. Recently, it has also been reported for its neuroprotective role, which might be helpful in delaying or preventing neurodegenerative diseases including AD(123). Curcumin inhibited Aβ aggregation, Aβ-induced inflammation as well as the activities of BAC and acetylcholinesterase (AChE) in *in-vitro* studies. Pyrazole analogs of curcumin were evaluated and showed promising activity against both tau and Aβ aggregation(124). Quercetin has been found to be an encouraging lead compound displaying neuroprotective activity against neurodegenerative diseases such as AD. However, the clinical application of quercetin

was impeded due to its low oral bioavailability. It reduced extracellular β-amyloidosis, tauopathy, astrogliosis and microgliosis in hippocampus and amygdala. These findings were buoyed by substantial reduction in paired helical filament (PHF), $A\beta_{1-40}$ and $A\beta_{1-42}$ levels and a decline in BACE1-mediated APP cleavage. Quercetin had been instrumental in activating sirtuins 1(SIRT1) to induce autophagy and to act as a phytoestrogen(125). Berberine, the principal therapeutic component of a venerable Chinese herb *Coptis chinensis Franch,* is traditionally used to treat diabetes. Many scientific studies have established that berberine plays a pivotal role in numerous neurological disorders including cerebral ischemic, multiple sclerosis, various neurodegenerative and neuropsychiatric disorders. It also limits the pathogenesis of extracellular amyloid plaques and intracellular NFTs and thus, has a potential for AD treatment(126, 127).

Luteolin, a flavonoid compound, was found to be useful in prevention and reduction of *cornu Ammon-1* (CA1) pyramidal cell layer in streptozotocin (STZ) induced Alzheimer's rat model. Its neuroprotective effect might be attributed due to antioxidant effect. It acts by blocking free radical products and dispersing Aβ plaques. Luteolin is a powerful and appropriate therapeutic agent for neuronal disorders, although, additional assessments including clinical trials are required to confirm the hypothesis(128). Luteolin is reported to obliterate AD-like features after traumatic brain injury (TBI) in an Aβ depositing mouse model. This study could be a benchmark for the development of natural compounds which may perhaps prevent or treat TBI with little toxic side effects(129). Rosmarinic acid (RosA), an ester of caffeic acid and 3,4-dihydroxyphenyllactic acid, is frequently seen in species of Boraginaceae family as well as in the Nepetoideae subfamily of Lamiaceae It is also reported in some species of other higher plant families, besides few fern and hornwort species. RosA has several

remarkable biological activities, viz. antiviral, antibacterial, antiinflammatory, antioxidant *etc.* Its existence in medicinal plants, herbs and spices has useful and health improving effects(130). RosA has been revealed to be effective in preventing amyloid peptide aggregation *in-vitro* in addition to delaying the disease progression in animal models. However, only paltry information is available with respect to its molecular mechanism of action(131). It was found to inhibit the formation of Aβ as well as destabilized preformed Aβ in a dose-dependent manner. The plausible action for this may be due to the presence of two 3,4 dihydroxyphenyl rings of RosA. The compact and symmetric structure might be quite suitable for specifically binding with free Aβ and subsequently hindering the polymerization of Aβ(132). Physostigmine, a pyrroloindole skeleton alkaloid, is obtained from the Calabar bean, the seeds of *Physostigma venensosum* Balf. (Leguminosae). It is a powerful, short acting and reversible inhibitor of AChE which improved cognitive functions in *in-vivo* both in normal and AD patients. But the short half-life of physostigmine limits further research although many formulations have been attempted. Lately, a controlled-release (CR) oral formulation and a skin patch were sought to overcome this problem but it showed increased incidence of adverse effects like nausea, vomiting and diarrhea. It appears that Physostigmine has little advantage over newer anticholinesterase drugs. The short half-life remains a major drawback and needs improved form of administration. Huperzine A(Hupa A), obtained from *Huperzia serrata*, acts as a potent, reversible, selective inhibitor of AChE(133). HupA is having mechanism of action similar to donepezil, rivastigmine and galantamine. When it was hybridized with tacrine and donepezil, the donepezil hybrid was less efficacious but the HupA-tacrine hybrids referred to as huprines X and Y were proficient in boosting AChE than tacrine in *in-vitro*(134). Potent, selective and reversible AChE inhibitors have been widely used

in China for the treatment of AD. Accordingly, new mechanism of action for Hupa A have been proposed during the past decade. In addition to its AChE inhibitory effect, strong, multifaceted neuroprotective effect through activating the cholinergic system and directly acting on mitochondria, have also been explored. Significant efforts have been made to optimize drug delivery system for better efficacy and safety of Hupa A therapy. Nevertheless, additional studies are still needed to corroborate its effectiveness, especially evidence from the bedside(135) (**Table 2.4**).

It was found that several acidic steroids classified as bonafide GSMs lowered $A\beta_{42}$, whereas many nonacidic steroids termed as inverse GSMs elevated $A\beta_{42}$. 5β-cholanic acid (**N3**) (IC_{50} 5.68 M) was most potent steroid GSM, and *4*-androsten-*3*-one- *17*-carboxylic acid ethyl ester (**N6**) (IC_{50} 6.25 M) was potent inverse GSM(136). It was also reported that both estrogen and progesterone were weak inverse GSMs showing added complex effects on APP processing(137). Some endogenous steroids *viz*. cholesterol, cholesterol metabolites, *etc.* might be capable of acting as GSMs and can modulate $A\beta_{42}$. Acidic steroids may function as possible therapeutic leads for drug optimization/development to treat AD(138). The carboxylic acid GSMs are found to interact with PS1-NTF, while nonacidic GSMs interact with γ-secretase components(98). Further, carboxylic acid, in acidic GSMs, forms an ionic bond with lysine amino acid residue, whereas non-acidic GSMs lack such interaction(139).

2.1.2 β-secretase inhibitor

β secretase, commonly known as β-site APP cleaving enzyme 1 (BACE1), produces toxic Aβ, which plays major role in the pathogenesis of AD. Although, BACE1 inhibitor drug development has been challenging, but several inhibitors have lately entered in clinical trials and efficacy of these inhibitors have been reported for prevention/treatment of AD.

Table 2.4. Natural products effective in AD therapy along with their mode of action.

S. No	Natural products	Source	Active constituent	Mode of action
1	Berberine	*Berberis vulgaris, Berberis aquifolium, Berberis aristata, Phellodendron amurense, Hydrastis canadensis, Tinospora cordifolia, Coptis chinensis*	Isoquinoline alkaloid	β-secretase inhibitor
2	Bilobalide	*Ginkgo biloba*	Sesquiterpenic trilactone	β-secretase inhibitor
3	Curcumin	*Curcuma longa*	Curcuminoid	Inhibit Aβ production
4	Quercetin	Apples, capers, berries, onions, red wine, black and green tea	Polyphenolic flavonol	Inhibit Aβ fibril formation and aggregation
5	Huperzine A	*Huperzia serrata*	Sesquiterpene alkaloid	α-secretase activator
6	Chitosan	Exoskeleton of crabs and shrimp (crustaceans) and cell walls of fungi	Polysaccharide	β-secretase inhibitor
7	Resveratrol	*Vitis vinifera*	Flavonoid (stilbenes)	Induces the non-amyloidogenic cleavage of APP
8	(−)Epigallocatechn-3-gallate	*Ceratonia silique, Camellia sinensis*	Flavonoid	β-secretase inhibitor
9	Luteolin	Olive oil, carrots, celery, thyme, peppers, peppermint, oregano, rosemary	Flavonoid	Decreases the expression of APP and inhibited the Aβ secretion
10	Rosmarinic acid	*Rosmarinus officinalis, Melissa officinalis, Origanum vulgare, Salvia officinalis,* peppermint, thyme	Polyphenolic carboxylic acid	Act on tumor necrosis factor-α, nuclear factor κB and Inhibit Aβ-induced cognitive impairments
11	Apigenin	Leafy vegetables and plants like artichoke, parsley, celery, basil	Bioflavonoid	Decreases copper-induced Aβ neurotoxicity

There is persistent concern about the potential mechanism-based abnormalities of these drugs. A mutation in APP [alanine-673 to valine-673 (A673V)] leads to homozygous and heterozygous carriers. The former carriers are more prone to disease while the latter remain unaffected. A673V mutation affects APP processing, leading to improved Aβ production as well as formation of amyloid fibrils in *in-vitro*. Co-incubation of wild-type and mutated peptides caused instability to Aβ-aggregates and inhibition of

amyloidogenesis and neurotoxicity. The high amyloidogenic effect due to A673V mutation in homozygous state along with its anti-amyloidogenic effect in heterozygous state leads to an autosomal recessive pattern of inheritance causing implications for genetic selection and prospective treatment of AD(140) (**Figure 2.6A**). APP gene sequence coding variation studied in 1,795 Icelanders revealed that APP gene coding mutation (A673T) protected cognitive decline in elderly people without AD. It reduced approximately 40% of Aβ generation *in-vitro.* The vigilant response of A673T substitution in AD proved the theory that inhibiting the β cleavage may shield from the disease(141).

LY2811376

LY2811376 [(*S*)-*4*-(*2,4*-difluoro-*5*-pyrimidin-*5-yl*-phenyl)-*4*-methyl-*5,6*-dihydro-*4H*-[*1,3*]thiazin-*2-yl*-amine] was generated by using fragment-based drug design. It is first non-peptidic BACE1 inhibitor with oral bioavailability which causes an Aβ-lowering effect in animals. The changes observed in biomarker levels in preclinical animal models and transformed into human, were nontoxic and tolerable in healthy volunteers. The noticeable and long-term Aβ diminutions in lumbar CSF were measured with subsequent oral dosing of 30 or 90 mg of LY2811376. A dose-dependent increase in $A\beta_{5\text{-}40}$ and decrease in $A\beta_{1\text{-}34}$ were observed on treatment with LY2811376(142). The toxicological findings observed in long-term preclinical studies prevented further progress in clinical development of this drug and thus it was discontinued in phase II(143).

MK-8931 (verubecestat)

MK-8931 is a strong, selective and structurally distinctive, novel BACE inhibitor. This drug inhibits the BACE enzyme crucial for the production of Aβ peptide. It was found to prevent the formation of amyloid plaque deposits. It is deprived of adverse effects

i.e., neurodegeneration, nerve myelination, glucose homeostasis alteration or hepatotoxicity as of other BACE inhibitors in animal models(144). In phase I clinical trial, it reduced CSF Aβ by more than 90 percent with no sign of dose limiting side effects. Further, 92% decrease in Aβ peptide concentration was observed by 100 or 500 mg single dose. In multiple dose study, 10 and 40 mg of verubecestat caused decrease in CSF Aβ peptide levels 50 and 80 percent respectively. The drug was well tolerated and ideal for once-a-day dosing due to 20 h half-life.

MK-8931underwent randomized, placebo-controlled, parallel group, double-blind Phase III clinical trials for the assessment of safety and efficacy in patients suffering from prodromal AD. The study registered 1,500 participants. The patients were randomized to receive placebo, or 12 mg or 40 mg MK-8931, once a day. The main efficacy result of study changed from baseline in CDR-SB scores following 104 weeks of treatment. The completion of the primary trial was expected by July 2017 but the results were yet not available.

LY2886721

LY2886721 showed significantly low potency against cathepsin D (off-target protease) and ten fold high potency as compared to LY2811376. The molecule was less selective against cathespine D, pepsin and renin, resulting in significantly improved protease selectivity compared to LY2811376(145). The levels of hippocampal and cortical Aβ, C99, and soluble APPβ (sAPPβ) decreased significantly after oral dosing in PDAPP transgenic mouse model. In a cannulated beagle dog model, the oral administration (1.5 mg/kg) showed major and persistent Aβ lowering in CSF. High CSF concentrations of LY2886721 suggested that BACE1 potency may be increased without conceding CNS penetration(145). The drug, LY2886721, has advanced to clinical trials due to its sturdy pharmacology and non-clinical toxicological profile. The absence of retinal epithelium

pathology, at the selected dose, has also been advancement over the previous drug LY2811376. Abnormal liver enzyme elevation was observed during the phase II study and therefore, the study was terminated (www.ClinicalTrials.govidentifier NCT01561430).

E-2609

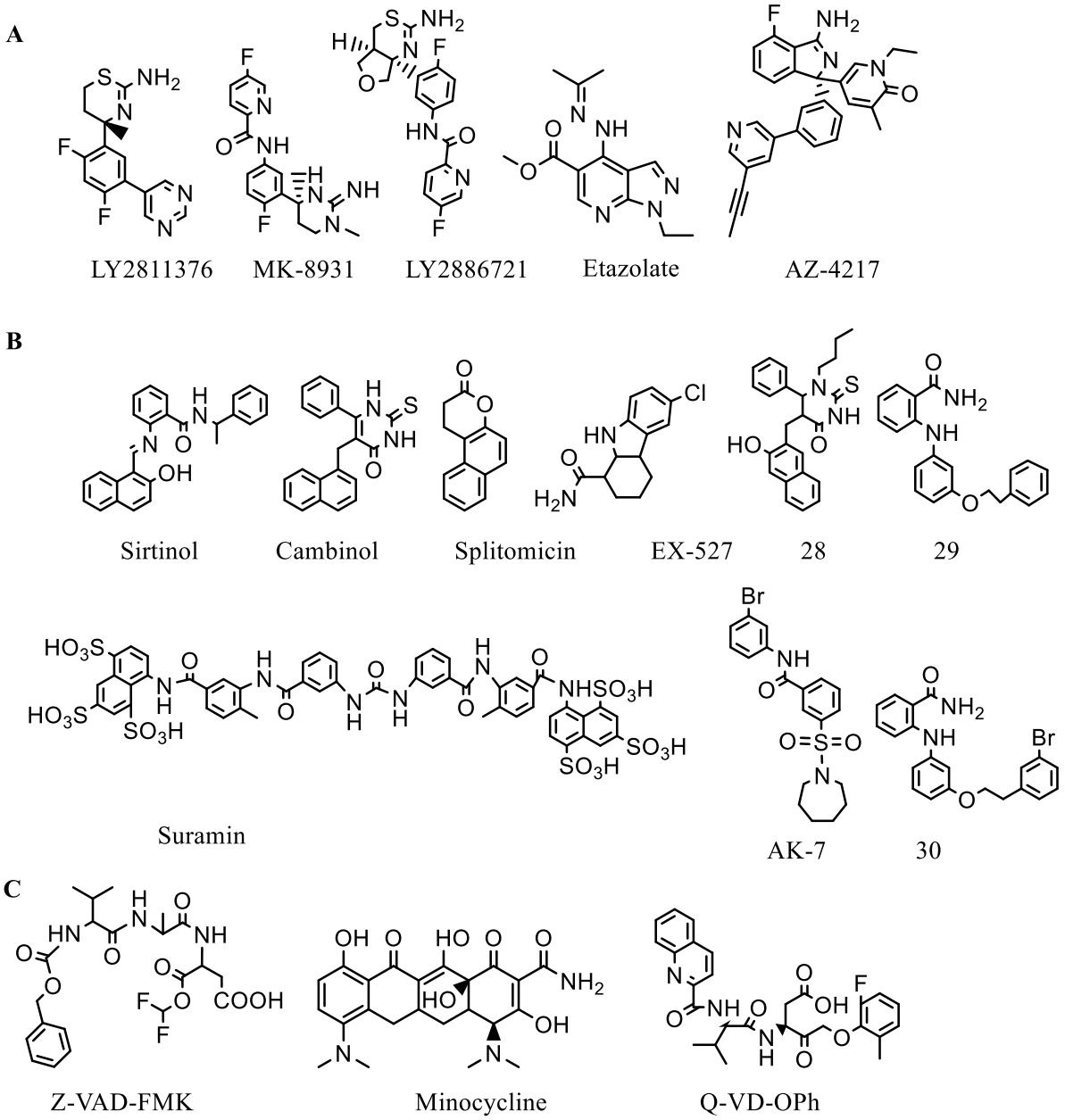
The interference of E-2609 with amyloid cascade upstream of Aβ peptide generation was the main driving factor for its development. The orally active drug has shown reduction of cerebral Aβ levels in preclinical studies. The impressive biological activity promoted to randomized, double-blind and phase I placebo-controlled clinical trials in healthy volunteers. Single oral ascending dose study amongst 73 participants and 14-days multiple oral ascending dose studies amongst 50 participants were conducted(146, 147). The single oral ascending dose study measured plasma Aβ concentrations in response to E-2609 doses ranging from 5 mg to 800 mg (nine cohorts). The multiple oral ascending dose study assessed Aβ concentrations both in plasma and CSF with E2609 doses ranging from 25 mg to 400 mg (five cohorts)(148). The plasma half-life of E2609 was found to be 12-16 h, after once-daily dosing. Together, these studies showed a robust dose-dependent reduction of Aβ concentration in CSF and plasma. The maximum dose of 400 mg in multiple oral ascending dose study led up to 85% decrease in CSF Aβ level. Further, the concentration of CSF sAPPβ was reduced, while sAPPα concentration was increased. E2609 appeared to be well tolerated and no serious adverse events were reported in either of the studies. Additionally, a single oral dose phase I trial of E2609 in patients with mild cognitive impairment or mild AD has also recently been completed (NCT01600859)(149). The other BACE1 inhibitors being investigated include AZD3293 (Roche)(150, 151), HPP854 (TransTech Pharma) and RG7129 (Roche).

The inhibitors also reported to reduce cerebral Aβ level in animal models include AZ-4217, AZD-3293, AZD3839, JNJ-54861911 and LY2811376. In cerebral amyloidosis (Tg2576), mouse model AZ-4217, showed excellent efficacy(152). AZD-3293 is in phase II/III clinical trial for prodromal-to-mild AD(153). AZD3839 (LY3314814) reduced Aβ CSF level in a phase I study(154). It showed over 1000 folds selectivity to cathepsin D than 14 folds selectivity of BACE2. It was terminated during phase I trial probably due to the human *Ether-a-go-go*-related gene (hERG) ion channel interaction(146). JNJ-54861911 was developed from hits with amino-dihydrothiazine or improved version of cyclic isothiourea. The compound crossed BBB and reduced all four forms of Aβ peptides ($A\beta_{42}$, $A\beta_{40}$, $A\beta_{38}$ and $A\beta_{37}$)(146). LY2811376 was also discontinued in the late Phase I trials due to its eye related adverse effect in which it damaged pigment epithelium.

2.1.3 *α*-Secretase

α-secretase activation emerges as distinct and potential therapeutic approach to decrease Aβ generation(155). It facilitated proteolysis of APP in non-amyloidogenic pathway and prevented Aβ formation(156). A series of membrane-bound proteases (a disintegrin and metalloprotease family) regulate the α-secretase(157). ADAM-10, ADAM-17, and ADAM-9 have been suggested as α-secretases(158). The proteases are type I integral membrane proteins and contain multi-domain in their structure. It was found that RNAi-mediated knockdown of ADAM10 suppressed entirely the APP cleavage by α-secretase in different cell lines and primary murine neurons(159). Synthetic retinoids were found to be involved in improvement of nonamyloidogenic pathway of APP.

Figure 2.6 Inhibitors and enhancers (A) Secretase inhibitors and enhancers (B) SIRT2 inhibitors (C) Caspases inhibitors.



Acitretin, a vitamin A analog, in phase II clinical trial, raises expression of ADAM-10 and it is reported to reduce Aβ levels in APP/PS-1 transgenic mice(160). It increases the mature ADAM10 stimulation process resulting in enhanced activity of α-secretase in neuroblastoma cells(160). Acitretin successfully crosses BBB and its level is not decreased by permeability glycoprotein (P-gp)(161). It has been found to be associated with severe toxicity like cheilitis, alopecia, peeling and hepatotoxicity. In absence of selective α-secretase inhibitors, coupled with regularly witnessed lethality subsequent to ablation of α-secretase encoding genes, progress in this field has been limited. The stimulation of one or more of signaling pathways concerned with α-secretase regulation may be an alternate and indirect method. Further, upregulation of α-secretase action may reduce formation of Aβ and increase assembly of sAPPα, a potential neuroprotective(162). On the other hand, the outcome of chronic upregulation observed in cleavage of other substrates mediated by α-secretase is still undefined(157). As a result, developing α-secretase enhancers continue to be an innovative and yet unexposed alternative.

2.1.3.1 α-secretase enhancers

Etazolate (EHT-0202)

Etazolate, a pyrazolopyridine analog, is involved in allosteric modulation of *gamma*-aminobutyric acid-A ($GABA_A$) receptor. It stimulates neuronal α-secretase and increases sAPPα production(163). EHT-0202 has recently been tested in subjects with mild-to-moderate AD in phase II clinical trial and displayed precognitive activity (**Figure 2.6A**)(163). The common adverse reaction of Etazolate is depression like abnormality in behavioral paradigms.

Bryostatin-1

It is a macrocyclic lactone which stimulates α-secretase activity through activation of protein kinase C and subsequent promotion of sAPPα secretion(164). Bryostatin-1 is undergoing phase II clinical trial in patients suffering from mild-to-moderate AD.

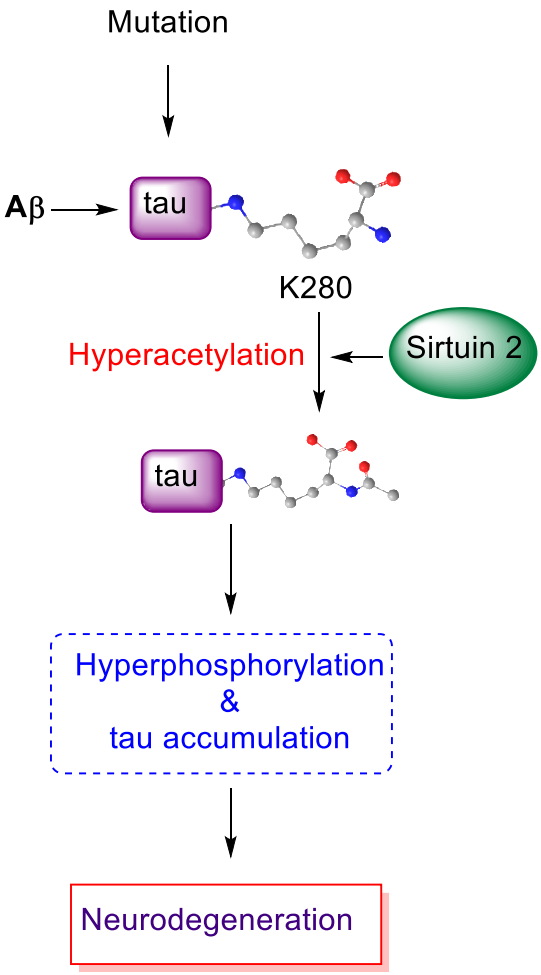
Exebryl-1

It causes modulation of α- and β-secretase activity leading to significant decrease in production of Aβ peptide. Further, its accumulation in mouse brain results in subsequent memory enhancements(165).

2.1.4 Sirtuins

Seven members of sirtuin (SIRT1-7) family are found in mammals. SIRT 1-3, 5 and 7 exhibit NAD^+-dependent deacetylase activity whereas, SIRT 4, and 6 are ADP- ribosyl-transferases(166, 167). SIRTs have been concomitant with providing protection against age-related diseases by multiple mechanisms *i.e.,* regulation of stress response, apoptosis and DNA repair(168-170). It is remarkable to note that all sirtuin substrates are not histones, in addition to that, many members of this protein family also lack deacetylase activity (171, 172). It is the phylogenetic analysis subunits from which the histone deacetylases evolved prior to histones and main purpose of these enzymes is to effect deacetylation of non-histone substrates(171, 172). Recently, SIRTs have been explored in AD, Parkinson's disease, Huntington's disease, amyotrophic spinal, bulbar muscular atrophy and lateral sclerosis through diverse techniques, which include *in-vitro* assays, cell culture, and animal models of neurodegenerative disease besides human tissue studies (**Figure 2-7**).

Figure 2.7. Roles of SIRT in AD-related neurodegeneration.



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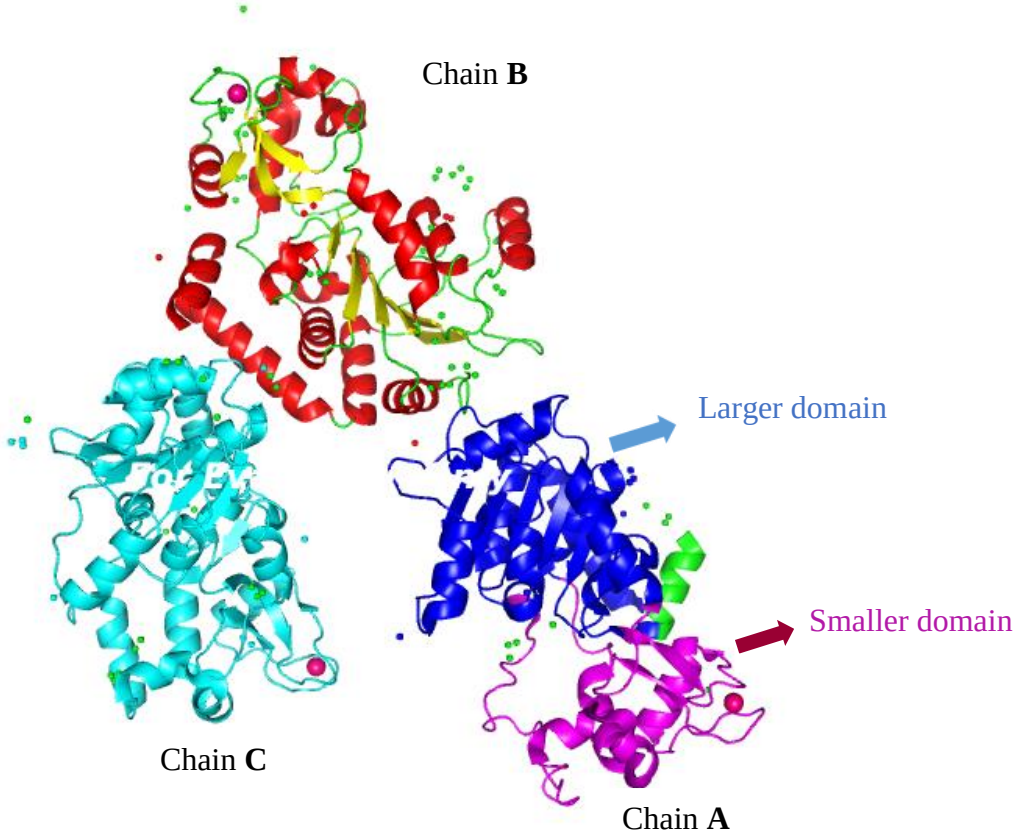
The catalytic core of SIRT2 consists of 304-amino acids and a *N*-terminal helical extension of 19-residues. The core has an elongated shape and contains two domains. The larger domain (residues 53 to 89, 146 to 186 and 241 to 356) is a modification of the Rossmann fold(173), which is present in different NAD(H)/NADP(H) binding enzymes, while its smaller domain (90 to 145 and 187 to 240) contains a zinc atom. Four crossovers of polypeptide chain serves to connect both the domains. The four crossovers together form a large groove with three loops of the large sphere as seen at the interface of two domains (**Figure 2.8**).

Figure 2.8 Structure of SIRT2; pink colored spheres show the atom (pdb id- 1j8f).



2.1.4.1 SIRT2 inhibitors

There are many SIRT2 inhibitors reported including the potent ones *i.e.*, sirtinol, combinol(174-176) and splitomicin(177-179). Most of these compounds have β-naphthol moiety and have been subjected to structural optimization. EX-527, which is among the most potent of the selective SIRT1 inhibitors, occupies nicotinamide site as well as neighboring pocket and interacts with ribose of NAD^+ or *2'-O*-acetyl-ADP ribose co-product. Crystal structure studies and biochemical assay analysis revealed that only the co-product complex, which stabilizes the closed enzyme conformation, is required for inhibition by Ex-527, as it prevents the product release(180, 181). Suramin

is slightly selective against SIRT1 over SIRT2 and SIRT3, but this compound suffers from the poor pharmacokinetic property (**Figure 2.6B**)(181, 182).

Cambinol derivatives

These SIRT inhibitors also exhibit antitumor activity in preclinical models. New *N*1-alkyl substituted derivative **28** showed increased potency and selectivity(175). The *N*-butyl moiety of the compound formed hydrophobic interactions with a narrow lipophilic channel in SIRT2 active site, a clarification that could validate its selectivity (SIRT2 *vs*. SIRT1). The cell-based assays were consistent with *in-vitro* data and showed that compound increased level of acetylated α-tubulin (**Figure 2.6B**).

2-Anilinobenzamide analogs

2-Anilinobenzamide analogs are potent SIRT2 inhibitors displaying IC_{50} values in the sub-micromolar range (compound **29**). SAR studies together with molecular modeling analyses reveal that phenethyl group of compound **29** and **30** is crucial for SIRT2 selectivity; likewise ethylene ether is essential for potency (**Figure 2.6B**).

AK-7

AK-7 is a deacetylase which targets α tubulin, histone four forkhead transcription factor. It is a benzamide derivative and a potent brain permeable SIRT2 inhibitor. The halogenated benzamide derivative exhibits *in-vitro* SIRT2 selective inhibitory activity and decreases total cholesterol levels in primary striatal neurons(183). AK-7 presents numerous problems associated with metabolism and stability. It is being considered as a lead compound for drug development, targeting cholesterol homeostasis deregulation, as is the case with AD (**Figure 2.6B**). Splitomicin, a SIRT2 lactone inhibitor active against yeast SIRT2, is reportedly inactive against hSIRT2 which is a human sirtuin isoform (178, 184). AC-93253 is another selective and potent SIRT2 inhibitor

displaying inhibitory activity at low micromolar ranges.The drug is over seven and four folds more potent than SIRT1 and SIRT3, respectively(185, 186).

2.1.4.2 SIRT1 enhancer

SIRT1 is neuroprotective and SIRT2 is involved in neurodegenerative cascade(187). SIRT1 differs from other mammalian SIRT by the extended *N*-terminal domain (NTD), and allosteric mode of activation participates in the SIRT1 activity(188). NTD contains core of three helices and is positioned differently with the catalytic domain in ligand-bound structures. Mutation studies and crystal structure of SIRT1 with resveratrol suggest that Glu-230, located in NTD, is critical for its activation. Most of the SIRT activating compounds show interaction with Glu 230(188).

Cell wall remodeling (CWR) tripeptide activates SIRT1 by an allosteric mechanism. It also activates serum SIRT1 and reduces the acetylation of p53 in neuroblastoma IMR32 cell line, resulting in prevention of cell death induced by Aβ(189). Resveratrol, cilostazol and taxifolin upregulate SIRT1 protein expression. Resveratrol, a stilbenoid present in red wine, skin of red grapes and several other plants, was recently found to decrease the risk of dementia (clinicaltrials.gov NCT01504854). It activates SIRT1, boosts adaptive immunity, controls neuroinflammation by MMP9 inhibition and prevents neurodegenerative disorders(190). Cilostazol reduces p62/SQSTM1 and increases cathepsin B action. The subsequently reduced accumulation of $A\beta_{1-42}$ in human APP Swedish mutation cells (N2aSwe) indicate the enhanced autophagosome formation. Liver kinase B1 (LKB1) dependent SIRT1 or P-AMPKα stimulation was found to be elevated by cilostazol and resveratrol. These studies suggest that cilostazol increases autophagy by stimulating SIRT1 coupled LKB1/P-AMPKα and inhibits mammalian target of rapamycin (mTOR) activation and thus resulting in decreased Aβ burden(191).

2.1.5 Caspases

Other than the neurodegenerative pathways involving Aβ toxicity and P-tau, caspases too are implicated in AD and their apoptotic cascades are the chief accelerators of cell death(192). Apoptosis can be defined as programmed cell death indicated by the slow accumulation of Aβ peptide within plaques of the brain. The disproportionate apoptosis might lead to neurodegenerative diseases, ischemic damage, autoimmune disorders and several types of cancers(193). Caspases belong to a mammalian cysteine-containing protease family of 14 members which are further classified into initiator as well as executioner caspases(192). The apoptotic cascade is involved in initiator caspase (caspase-8, 9, 10) activation through autocatalytic cleavage, consecutively, these caspases cleave and activate the executioner caspases (caspase-3, 6, 7) leading to appropriate cellular changes associated with apoptosis(192). Recent studies indicate that caspases may not be completely related with end-stage events in AD(194, 195). Besides, it has been revealed that caspase activation and cleavage of tau is concomitant with the formation of NFTs. Additionally, animal models and postmortem studies of AD patients support the hypothesis which connects executioner caspases to AD drug development.

Existing studies have suggested that a key role of caspases and apoptotic regulator Bcl-2 in AD etiology. The development of caspase inhibitors or compounds that increase expression of Bcl-2 in brain are expected to improve the disease condition. Although, development of Bcl-2 agonist is an innovative and antiapoptotic approach, no selective agonist has yet been reported(196).

2.1.5.1 Caspases inhibitors

Inhibition of apoptosis may be achieved by using caspase inhibitors *viz.* carbobenzoxy-valyl-alanyl-aspartyl-[Omethyl]fluoromethylketone (Z-VAD-FMK) (**Figure 2.6C**).

Z-VAD-FMK treatment in transgenic mice reportedly delayed both the disease onset as well as mortality. However, the drawback was its bioavailability and selectivity towards specific caspase receptors. Later, it was discontinued due to its toxic metabolite fluoroacetate which causes liver damage.

Another drug, quinolyl-valyl-*O*-methylaspartyl-[-*2,6*-difluorophenoxy]-methyl ketone (Q-VD-OPh) causes inhibition of recombinant caspases through an IC_{50} within the nanomolar range. It exhibited caspase-7 inhibition and reduced the pathological changes of tau protein in TgCRND8 mice(194). However, there was no significant difference in extracellular Aβ accumulation. Q-VD-OPh showed better progress when compared with Z-VAD-FMK in displaying greater stability, potency, cell permeability and lesser toxicity(196).

Minocycline is a semi-synthetic tetracycline analog with high lipophilicity and can easily cross the BBB(197). It showed neuroprotective activity by inhibiting cytochrome-C release and preventing activation of caspase-3. It was also revealed that apoptosis in kidney epithelial cells was prevented by minocycline through selective enhancement of Bcl-2 mRNA and protein expression (198). Additionally, treatment with minocycline had slowed down the neuronal cell death in Tg2576 mice and $A\beta_{42}$-infused rats(199). The oral bioavailability and safety have been established in AD animal models as well as in human subjects. The anti-apoptotic and anti-inflammatory functions of the drug might prove to be beneficial in AD therapy(196) (**Figure 2.6C**).

2.1.6 Glycogen synthase kinase-3 (GSK3)

GSK3, a Serine protein kinase, occurs as three isoforms, *i.e.,* GSK3α, GSK3β and GSK3β2. The α and β isoforms with 98% similarity differ in Gly rich *N*-terminal of GSK3α. GSK3β2 is a substitute linking modification of GSK3β, containing 13 amino acids inserted into the catalytic site(200). GSK3 is essentially active in cell, and a

variety of extracellular stimuli like insulin, epidermal growth factor, fibroblast growth factor and *Wnt* ligands inhibit its action(201, 202). Phosphorylation on a serine residue (Ser9 in GSK3β and Ser21 in GSK3α) located in the NTD also inhibit GSK3. It is located in NFTs and interacts with many proteins implicated in the pathogenesis and progression of AD. However, the GSK3β level is eminent in AD brain(203, 204). NFTs containing tau protein is believed to be generated by hyperphosphorylated serine and threonine residues. GSK3 is found to phosphorylate tau on multiple sites and some of which are deviated to abnormally hyperphosphorylated tau(205).

GSK3β is associated with the most common abnormalities of AD like plaques and tangles formation, decreased ACh secretion and cell degeneration. GSK3β is reported to participate in phosphorylation of microtubule binding tau protein and could also participate in the development of NFTs. GSK3β phosphorylates APP(206, 207) and get activated by neurotoxin Aβ(208). The Aβ mediated activation of GSK3β phosphorylates tau and perhaps contributes to the accumulation of hyperphosphorylated tau in AD brain(201). Additionally, the Aβ activated GSK3β, inactivates and phosphorylates pyruvate dehydrogenase thus leading to exhaustion of the ACh precursor, *i.e.*, acetyl coenzyme A. The apoptotic cascade induced by Aβ is promoted due to GSK3 signaling(209).

GSK3-mediated tau phosphorylation contributes to cognitive impairment and it is successfully explored by deletion of tau expression in GSK3βs over expressing mice(210). GSK3α or GSK3β knockdown approaches, in AD mouse models showed considerable improvement in cognitive impairment. The inactivated GSK3β human APP transgenic AD mice, when crossed with dominant-negative GSK3β transgenic mice showed improvement in learning by Morris water maze test. It also caused a reduction in Aβ load and effected a decline in tau phosphorylation in double transgenic

mice over human APP mice(211). The decreased expression of GSK3α in PDAPP (+/−) transgenic mice reduced the deficits, otherwise observed in Barnes maze test. Thus, selective inhibition of GSK3 seems to salvage cognitive impairment and attenuate neuropathology in AD rodent models.

2.4.1 GSK-3 inhibitors

Lithium, valproate and lamotrigine are enhancers of neurogenesis(212, 213) and are also investigated in various models of neurodegenerative disease for their neuroprotective and neurotrophic actions(214, 215). GSK-3 is phosphorylated by PKB in response to insulin and growth factors, thus inhibiting GSK-3 activity will modulate various cellular processes regulated by GSK-3. GSK-3 inhibitors, SB-415286 and SB-216763, are involved in protecting the neurons of central and peripheral nervous systems from cell death due to reduction in PI 3-kinase pathway activity. The neuronal cell death inhibition by these compounds has been linked to the inhibition of GSK-3 activity, coupled with GSK-3 substrates tau and β-catenin modulation(216). In the pilot study, tideglusib was investigated for safety and efficacy and was found to be well tolerated for the treatment of AD(217). The hyperactivation of GSK3 has been considered a leading cause of AD pathogenesis. In the development of GSK3 inhibitors, we should assert the requirement of the inhibitors that do not abolish the constitutive activity (**Table 2.6**)(218).

2.2 Tau based therapy: A paradigm shift

In the past two decades, AD drug discovery and development efforts had primarily been focused on amyloid cascade hypothesis. The drugs, in clinical trials, targeting Aβ are not able to improve the cognition. The enigma is slowly starting to untangle. It has long been known that twisted fibers of a protein called tau collect in brain cells of people suffering from AD, but their exact role in the disease is unclear.

On the other hand, little attention was paid to tau-based approaches until now, despite the fact that the tau pathology is a prime aspect to the disease. The other strategies targeting various features of tau pathogenesis are also being pursued so as to broaden the range of possible beneficial therapeutic tools for the treatment of AD and other tauopathies. As a matter of fact, several compounds are undergoing phase II clinical trials as we speak and numerous proof-of-concept studies are presently being planned or carried out. LMTX, a second-generation tau aggregation inhibitor, offered foremost advance over the available treatment for AD. It acted by reducing the levels of aggregated or misfolded tau proteins, which were associated with progressive neurodegeneration, the hallmark of AD. It failed in Phase III trials on fertile grounds. As it was readily absorbed and tolerated at 10-fold higher dose than Rember monotherapy.

There is evidence that both soluble (hyperphosphorylated, misfolded, and mislocalized) and fibrillar tau contribute to neuronal and synaptic dysfunction and loss. Only little evidence exists that could directly implicate tau fibrils as toxic by themselves. It is postulated that soluble forms of tau may perhaps be more toxic to neuronal and synaptic functions and ultimately leads to its degeneration. Therapeutics aimed at preventing or reversing tau aggregation may, in fact, prove deleterious by increasing the concentration of toxic soluble tau species. Tau reduction mechanisms may prove more promising avenue to pursue. In AD, discharge of some intracellular proteins like tau into extracellular space is recognized as toxic as added to neuronal cells and the toxicity depends on the extent of tau aggregation. In cultured neuroblastoma cells, tau stimulates release of intracellular calcium ions, which leads to cell death. The cascade may be mediated by interaction of muscarinic receptors with tau and release of intracellular calcium ion(219, 220).

Although, considerable limitations exist in current understanding of the physiological role of tau in neuronal function and exact steps in the pathogenesis of AD. It might be appropriate to overpower the mutant forms of tau in hereditary tauopathies through an abnormal ratio of 3R v/s. 4R tau isoforms thereby, reducing the levels of tau in AD. Many kinase inhibitors have been reported to reduce tau hyperphosphorylation, of these, two kinases GSK3β and cyclin-dependent kinase 5 (cdk5) have received particular attention, and are the primary targets for drug discovery pursuits(221).

Many of the tau-based impending avenues are worth investigating for their therapeutic potential in the treatment of AD. Tau-based therapies also play a pivotal role in the treatment of other diseases *i.e.,* Pick's disease, cortico-basal degeneration, and progressive supranuclear palsy *etc*.(222).

The tau-based therapeutics (alone or in combination) are still not productive in treating AD and other neurodegenerative disorders, but surely, the ensuing few years might bring new treatment paradigms to AD (**Table 2.5**)(223).

2.3. Calpains

Calpains proteases are a family of calcium-dependent cysteine proteases which influence and regulate cytoskeleton organization and intracellular signaling pathways. The two major isoenzymes of this class include, Calpain I and calpain II. These are present in tissues and require micromolar and millimolar levels of calcium respectively for full *in-vitro* activity, but have very similar substrate specificities(224). The vast stores of inactive calpains present in neurons while undergoing abnormal activation, cause cell injury(225, 226). It has been suggested that neurodegeneration by excitotoxicity along with other neuropathologic states involving abnormal calcium influx, is due to Calpain I activation(227-230).

Table 2.5 GSK-3 and tau-based investigational drugs in clinical development.

S.No	Drug	Clinical trial status	Mechanism of Action	Clinical Trials gov Identifier
1	Lithium Valproate	I	GSK-3 Inhibition	NCT00088387
2	Lithium carbonate	II	GSK-3 Inhibition	NCT01055392
3	Tideglusib (NP-12)	II	GSK-3 Inhibition	NCT00948259
4	Tideglusib (NP-12)	II	GSK-3 inhibitor	NCT01350362
5	Epothilone D (BMS-241027)		MT stabilizer	NCT01492374
6	Methylene blue (Rember)	II	Anti-aggregation	Na[a]
7	Leucomethylene blue (LCMX)	II	Anti-aggregation	TRx-237-005[a]
8	AADvac1	II	Active immunization	NCT01850238
9	ACI35	Ib	Active immunization	
10	C2N 8E12	I	Passive immunization	NCT02880956
11	BMS986168	I	Passive immunization	NCT02658916
12	RO 7105705	I	Passive immunization	NCT02820896

Calpain II accumulation, observed in an immunohistochemical study of AD brains, suggested a strong association of Calpain II with neuritic plaques and NFTs. Further, cdk5 responsible for neurite outgrowth and cortical lamination is activated by p35(231-233). Calpain based cleavage of p35 forms p25 and its accumulation in the brain of AD patients has been reported(234). The accumulation of p25 leads to overactivation of cdk5 and tau hyperphosphorylation which has been established in transgenic mice over-expressing p25(235, 236). According to calpain cathepsin hypothesis, lysosomal membrane disturbance mediated by calpain leads to the release of cytoplasmic cathepsins. The cathepsins release cascade represents degenerative and ischemic neuronal death(237-239).

The mitogen-activated protein kinases (MAPK) along with the extracellular signal regulated kinase 1 (ERK 1) and 2, are activated in AD and both may contribute to the hyperphosphorylation of tau(240, 241). Calpain mediates the activation of ERK 1, 2 and

ERK 1, 2-based phosphorylation of neurofilament protein sites, well-known to be changed in AD. Calpain cleavage of tau causes generation of a toxic tau fragment ~17 kD, positioned in the *N*-terminal half of tau.

Calpain activation leads to an upsurge in the level of BACE1 in AD transgenic mouse model. The transgenic mice over-expressing APP showed calpastatin deficiency which leads to heightened calpain activation, Aβ production, and simultaneous rise in mortality. Further, the increase in Aβ levels causes enhancement of Ca^{2+} influx and improved calpain activation.

Calpain activation can also be attributed to synaptic dysfunction. It involves calpain-mediated cleavage of protein kinase A (PKA) leading to decrease in its activity. The decrease in PKA activity reduces cyclic adenosine monophosphate (cAMP) regulatory element-binding protein (CREB) activation thus, impairing memory(242).

A-705253

A-705253 is benzoylalanine-derived ketoamide with improved water solubility, oral bioavailability and metabolic stability. Its administration in 3xTgAD mice diminished the cognitive impairment as well as synaptic dysfunction in dose-dependent manner. The inhibition of calpain by A-705253 lowered $A\beta_{40}$ and $A\beta_{42}$ levels and also reduced the thioflavin S-positive fibrillar Aβ deposit. These effects might be due to the down-regulation of BACE1 and up-regulation of ATP-binding cassette transporter A1 (ABCA1) expression, which contributed to reduced production of Aβ and its increased clearance. A-705253 also decreased the activation of cdk5 by inhibition of calpain resulting in diminished hyperphosphorylation of tau. The blockade of calpain also reduced activation of astrocytic and microglial responses which in turn produced AD-like pathological characteristics in aged 3xTgAD mice(243).

Pretreatment of A-705253 further decreased tau phosphorylation in hippocampal CA3 mossy fibers in both pentobarbital-induced hypothermia and acute systemic lipopolysaccharide treatment. These treatments prompted stress-induced tau hyperphosphorylation in mice. A-705253 administration in prepathogenic 3xTg-AD mice decreased the expression of calpain proteolytic p25 fragment. This resulted a decrease in activation of cdk5, a key enzyme responsible for tau hyperphosphorylation in AD brain(244, 245)

BDA-410

Administration of BDA-410, a specific calpain inhibitor, to APP/PS1 mouse, caused restoration of normal synaptic function in hippocampal cultures and hippocampal slices. Improvement in spatial working memory in addition to associative fear memory in APP/PS1 mice had been observed after BDA-410 administration. The reestablishment of normal levels of phosphorylation of transcription factor CREB by calpain inhibitors through a mechanistic redistribution of synaptic protein synapsin I is the main cause of improvement of memory by BDA-410(246, 247).

2.4. Acetylcholinesterase

AChE has been extensively explored in AD tissues since the discovery of cholinergic deficit in the disease. The alterations in AChE activity coupled with variations in its polymorphism in brain, CSF and blood were reported in patients. Enzyme co-localization in senile plaques supports its unusual features. It has also been revealed that AChE forms a stable complex together with senile plaque components by means of its peripheral anionic site (PAS). The presence of AChE amplifies neurotoxicity of amyloid components. Incidence of altered glycosylation in some forms of AChE is closely related to the occurrence of amyloid formations in AD (248).

2.4.1. Acetylcholinesterase inhibitors

Acetylcholine, a key neurotransmitter in the cortex is hydrolyzed by the enzyme AChE, so the cholinesterase inhibitors directly enhance cholinergic transmission. In the initial stages of senile plaque formation, AChE and butyrylcholinesterase (BuChE) play a pivotal role. Consequently, AChE and BuChE have been acknowledged as viable targets for effective AD management through increased acetylcholine availability in the brain regions coupled with a decline in Aβ deposition. There are several classes of cholinesterase inhibitors *i.e.* tacrine, donepezil, rivastigmine, galantamine, xanthostigmine, para-aminobenzoic acid, coumarin, flavonoid, and pyrrolo-isoxazole analogs which are being developed for the treatment of AD.

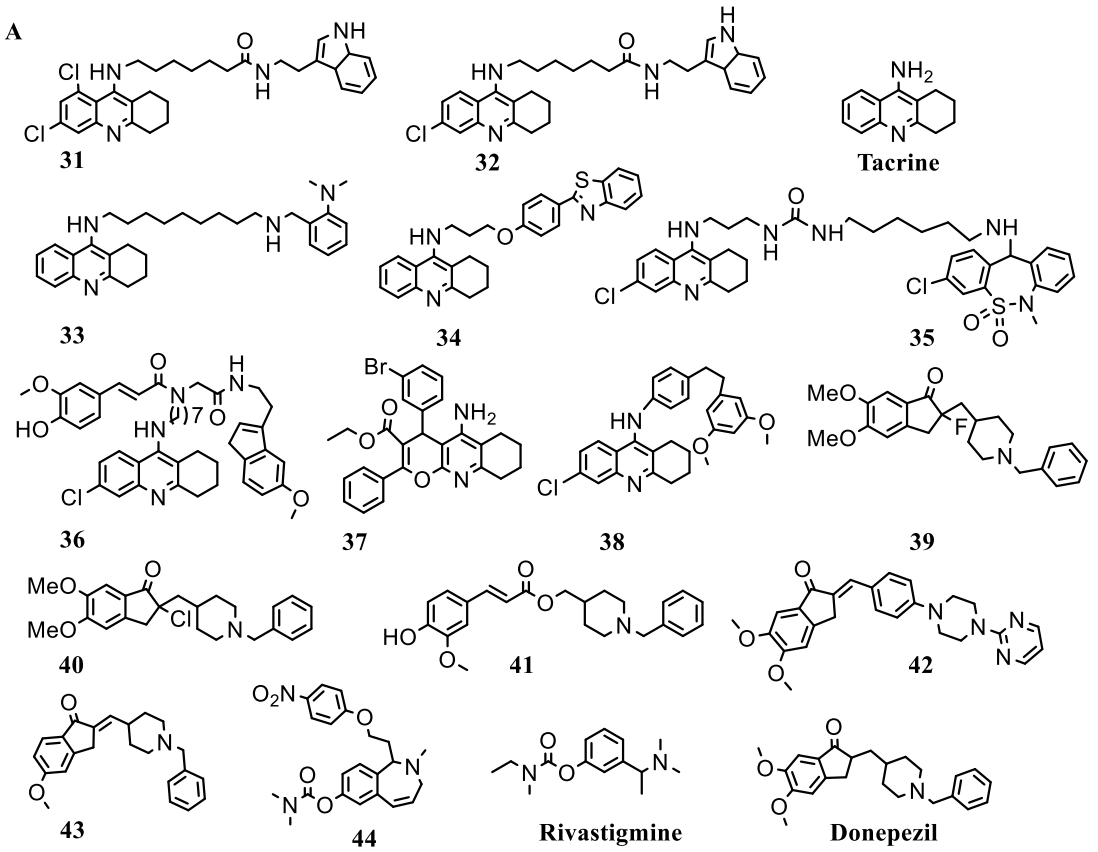
Tacrine derivatives

Tacrine (9-amino-1,2,3,4-tetrahydroacridine) (IC_{50} 167 nM) is a well-known reversible inhibitor of AChE and BuChE. Different groups were introduced to modify the structure of tacrine for the improvement of activity and elimination of hepatotoxicity(249-251). The indole ring of Trp84 present in active site of enzyme goes into binding with the acridine moiety. The changes introduced in tacrine facilitate its binding to the supplementary binding sites in AChE including the PAS. Tacrine-melatonin hybrids act as multifunctional agents in the treatment of AD wherein, 6,8-dichloro tacrine and 6-chloro tacrine stay attached to melatonin by means of a polymethylene linker(252). Compounds **31**, **32** are reported to possess IC_{50} values 0.725 and 0.008 nM respectively for AChE inhibition. Tacrine hybrids containing tacrine linked to *o*-hydroxyl and *o*-aminobenzylamine groups through polymethylene linkers were designed and synthesized by Mao *et al*. Length of methylene linker, as well as nature of the substituted amino groups attached to tacrine moiety are believed to influence the inhibitory potency of AChE. Superior AChE inhibitory effect was shown by Compound

33 (IC_{50} 0.55 nM) containing nine methylene linkers between tacrine and *O*-(*N*,*N*-dimethylamino)benzylamine(253). A series of berberinephenyl-benzoheterocyclic and tacrine-phenyl benzoheterocyclic hybrids were synthesized. The compounds containing tacrine-phenyl-benzoheterocyclic moiety displayed better AChE inhibitory activity compared to the analogs bearing berberine-phenyl benzoheterocyclic moiety. This establishes the fundamental role of the functional groups stereospecificity in AChE inhibition. Compound **34** containing tacrine and phenyl-benzofuran connected through a three methylene linker, was found to be a potent AChE inhibitor (IC_{50} 0.017 µM) (**Figure 2.9A**)(254).

The pharmacological action of tacrine is not only restricted to AChE activity, monoamine levels, ion channels but it also involves many more factors(255). Tacrine is metabolized by cytochrome P450 1A2 to various mono and dihydroxy derivatives. Some of the derivatives are less toxic and pharmacologically active, while others are further metabolized to highly reactive quinone methide form(256). Further, 7-methoxytacrine (7-MEOTA) preserves most of the pharmacological profile of tacrine with improved toxicological profile. However, AChE, BuChE inhibitory potency and some of the pharmacokinetic parameters exhibit marginal superiority to tacrine over 7-MEOTA. The muscarinic, nicotinic receptor interaction, lower toxicity, antioxidant properties and safer metabolization indicate the superiority of 7-MEOTA over tacrine(257). Tacrine changes the cellular energetics by inhibiting complex I of electron transport but 7-MEOTA devoid of such effects. The possibility of tacrine-induced adverse actions associated with the disturbance in electron transport is also proposed(258).

Figure 2.9 (A) Tacrine derivatives (31-34); Donepezil, Rivastigmine derivatives (35-38), (B) Metal chelators used in AD.



Tianeptine linked tacrine molecule showed nanomolar range IC_{50} with tacrine moiety interacting the catalytic active site (CAS) (TRP86 and HIS447) and tianeptine with PAS (TYR 72 and TYR 341) of AChE. Compound **35** with IC_{50} of 6.79 nM, underscored the importance of length of the linker between tacrine and dioxodibenzothiazepine moieties for an optimal interaction between the aromatic groups of such an inhibitor and AChE's CAS and PAS(259). Further, ferulic acid tacrine-melatonin hybrids and lipoic-tacrine-

melatonin hybrids were developed, as multi-targeted approach, for AChE inhibition along with antioxidant and neuroprotective actions. Compound **36**, a potent cholinesterase inhibitor containing feruloyl substituent with IC_{50} of 1290 nM(AChE) and 234 nM (BuChE) showed a favorable non-hepatotoxic profile compared to tacrine (260). 5-amino-2-phenyl-4H-pyrano [2, 3-b] quinoline-3-carboxylates, a polyfunctionalized tacrine-derived compound showed *in-vitro* AChE and BuChE inhibition. Compound **37**, bearing a 4-(3-bromophenyl) moiety, showed potent activity with selectivity towards AChE. The chloro/bromo substituent at *ortho* or *meta* position of 4-phenyl ring improved anticholinesterase activity (261). The anti-neuroinflammatory and antioxidant property of Resveratrol, when in combination with tacrine, resulted in the development of multi-targeted ligand approach. Compound **38** showed IC_{50} 0.8μM for AChE with lower inhibitory activity for Aβ aggregation indicating the importance of catechol moiety for this activity (262).

Donepezil, Rivastigmine

Donepezil (IC_{50} 5.7 nM) is the second drug approved by FDA for treatment of AD and is also considered to be safe and well-tolerated. It undergoes interaction with active site of AChE, along with its PAS by means of aromatic interactions(263). The indole ring of tryptophan 84 (Trp 84) present at the anionic subsite by the bottom of gorge and the indole moiety of Trp 279 next to peripheral site of enzyme, undergoes interactions with benzyl piperidine and indanone groups of donepezil respectively. *N*-benzylpiperidine derivatives with donepezil moiety, bearing a fluoro or chloro-substituents at position 2 of the indanone system (**39**, **40**) had IC_{50} 1.3 and 2 nM respectively(264, 265) (**Figure 2.9A**). Compound **41**, a feruloyl-donepezil hybrid showed IC_{50} of 0.46 μM for equine AChE along with neuron antioxidant effect (IC_{50} 16.87 μM). The hybrid also possessed *in-vitro* anti-inflammatory and metal chelating activities (266). Compound **42**,

donepezil based piperazine derivative, exhibited IC_{50} of 25 nM against AChE along with better Aβ aggregation inhibitory activity than curcumin and prevented the oxidative stress induced by H_2O_2 in SH-SY5Y cells (267). Compound **43**, a donepezil like compound showed remarkable selectivity towards human erythrocyte AChE than horse BuChE with IC_{50} of 0.043µM and 5.734 µM respectively. The compound also affected BACE1 activity without affecting cell viability (268).

Rivastigmine (IC_{50} 4,150 nM) is a widely used drug for the treatment of AD. It belongs to a new generation of cholinesterase inhibitors containing a carbamate moiety capable of forming covalent interactions with active site of enzyme. This drug shows ten folds higher affinity for brain AChE over peripheral AChE. Its preference for brain AChE besides BuChE establishes rivastigmine as a drug with a decent pharmacological profile(269). Compounds containing saturated six-membered and seven-membered rings were shown to be active against AChE inactive against SERT. Compound **44**, (IC_{50} 66 nM) the unsaturated seven-membered ring derivative containing a methyl group attached to the amine nitrogen atom was found to be most potent against AChE and SERT (**Figure 2.9A**).

Different *in-silico* approaches like docking, molecular dynamic simulation (MDS), 3D-QSAR based approaches were also used for the design and development of drugs against AD. Several plant derived secondary metabolites were screened by *in-silico* approach to target AChE enzyme. Rivastigmine, tacrine, resveratrol derivatives, terpenoids, pleiocarpine and its amino analogs were further modified using different drug design tools to improve the pharmacokinetic and pharmacodynamic properties of these molecules(270).

2.5 Noncompetitive N-methyl-D-aspartate antagonists

N-methyl-D-aspartate (NMDA), a glutamate receptor, is over activated in a tonic manner instead of phasic manner in AD. This condition causes neuronal damage and impaired synaptic plasticity. In normal resting conditions, Mg^{2+} blocks NMDA receptor and restores the normal function. In AD, Mg^{2+} is not effective enough to revoke overexcited glutamatergic system, which leads to reduced detection of significant signal involved in learning. Further, memantine takes over the physiological function of Mg^{2+} and slows down the progression of disease(35).

Memantine

Memantine has been approved for the treatment of AD in most of the countries. It is voltage dependent, uncompetitive, moderate-affinity and fast kinetics NMDA receptor antagonist(271). Memantine has a unique feature to screen the anomalous synaptic noise of AD patients without disturbing the functional stimuli of NMDA receptor. Kinetics, IC_{50}, and voltage dependence of memantine and ketamine do not differ strongly(272-274). Memantine provides protection from inhibition of mitochondrial function, decrease in blood flow to CNS and inflammation at a significant dose (1µM plasma level) (35, 275).

2.6 New mechanistic rationales for drug discovery

2.6.1 Autophagy enhancer

Small molecule enhancers of rapamycin *i.e.* (SMER)28 is an enhancer of autophagy and significantly decreases Aβ peptide levels (approx. IC_{50} of ~10 µM) and APP-CTF (approx. IC_{50} of~20 µM) in γ-secretase independent manner. The autophagic pathway involves three essential components, autophagy-related protein (Atg) 5, Beclin1, and serine/threonine-protein kinase Ulk1. These were found to participate in Aβ as well as in APP-CTF degradation. SMER28 affects Atg 5 which is a key component of the

autophagic pathway. It enhances autophagy and also takes part in the clearance of Aβ and APP-CTF. It is reported that curcumin down-regulates the expression of phosphatidylinositol 3-kinase (PI3K) and mTOR, resulting in inhibition of Aβ generation and induction of autophagy(276).

Rapamycin

Rapamycin protects neurodegeneration and is believed to enhance autophagy which facilitates clearance of Aβ aggregates(277, 278). Further, rapamycin was reported to stimulate the autophagy pathway, upregulate $A\beta_{42}$-induced Beclin-1 expression and promote PC12 cell survival(279). Activation of Beclin-1-dependent autophagy can prevent neuronal cell death, while its inhibition can promote cell death(280). As rapamycin enhances autophagy and hence can modulate APP metabolism(281). It also restrains tau hyperphosphorylation by activation of mTOR signaling, but further clinical studies need to be conducted to establish Rapamycin as a successful candidate for AD.

2.7 Miscellaneous

2.7.1 Metal chelators

Metal chelating scaffolds containing pharmacophore(s) of other AD targets enhance the pharmacological action of conjugates or hybrids. However, maintenance of metal homeostasis in AD brain is the major challenge in using metal chelating scaffolds. Copper is the main target for the development of chelators in AD due to its greatest dyshomeostasis in amyloid plaques, Aβ deposition and associated neuroinflammation(282). The chelating moieties having donor atoms and denticities like β-aminopyridines, 8-hydroxyquinolines (8HQs), diamine-based chelators, phenol amino analogs, hydroxyanthraquinones, flavonoids, amino/hydroxyl chalcones, and 3-hydroxy-4 pyridinones in various scaffolds showed promising Aβ chelation. The

important metal chelators have been nicely described in the review by Santos MA *et.al.*,(283) (**Figure 2.9B**).

8-Hydroxyquinoline

8-Hydroxyquinoline derivatives and its 5-chloro-7-iodo (CQ) analogs are frequently used in design and development of multi-targeted AD drugs. CQ showed some promising properties like BBB penetration, moderate affinity to chelates Cu (II), Zn (II) and the ability to dissolve Aβ as was evident in post-mortem of AD brains. Second generation CQ analog, PBT2 (5,7-Dichloro-2-[(dimethylamino)methyl]quinolin-8-ol), acts as copper and zinc ionophore and reduces its extracellular level resulting in decrease in metal-mediated Aβ aggregation(284-286). The Phase II clinical trials for prodromal/mild AD patients by Prana Biotechnology didn't meet the primary endpoint of statistically significant reduction in Aβ plaques in the brain. Benzo[*d*][*1,2*]selenazol-3(*2H*)-one derivatives substituted with 8-hydroxyquinolin showed significant inhibition of copper induced $A\beta_{1-42}$ aggregation and glutathione peroxidase like action. The most potent compound scavenged H_2O_2 within 200-220 min and exhibited glutathione peroxidase like activity (v_0 = 106.0 μM min^{-1})(287). The modifications at fifth (R_2) and seventh (R_3) positions of 8HQ resulted in the pharmacophore having free radical scavenging activity, monoamine oxidase B (MAO-B) and AChE inhibitory properties along with Aβ chelation. Prochelator approach masks the R_4 group with carbamate leading to site-activated compounds. *N*-propargylamine derivative (HQ1) showed higher selectivity towards AChE but dissimilar to rivastigmine and had minor BuChE inhibitory potency(288). Trehalose glycoconjugates of 8HQ showed improved water solubility in physiological condition. The conjugates also exhibited much better stability constants with metals like Cu^{2+} and Zn^{2+} when compared to 8HQ. They also reduced the toxicity of hydroxyquinoline as shown in the *in-vitro* study. Interestingly, these

molecules modulate the unusual metal-protein interactions leading to several neurodegenerative syndromes (**Figure 2.9B**)(289).

The substituted indole nucleus containing molecules like benzylpiperidine-indolylproparglamine (ASS234) showed significant metal chelating property. It considerably reduced the gliosis, Aβ burden and showed anti-apoptotic, neuroprotective as well as antioxidant abilities(290). This multipotent lead compound is ready to enter in the pre-clinical trial for AD. The major safety concern with the ASS234 is its interaction with MAO-A, that may lead to well known 'cheese effect'(291).

β-aminopyridines

N, N donor atoms in β-aminopyridines (PYN) impart selectivity towards copper. The selectivity for Cu/Zn is not obscure in hydroxyquinolines. The pharmacophoric features of selegiline and clioquinol were combined to get specific MAO-B inhibitors having antioxidant, metal chelating and anti Aβ aggregation properties. The most potent compound, PYN1, showed IC_{50} of 0.21μM (MAO-B) and antioxidant property (ORAC = 4.20)(292). Many *N*, *N*, *N*, *N* tetra donors of pyridine amines were also developed to chelate the metals efficiently(293).

2.7.2 Neurotrophic agents and their mimetics

The neurotrophin family of proteins produce series of structurally and functionally allied proteins regulating the survival and differentiation of peripheral and central neurons. It comprises of brain-derived neurotrophic factor (BDNF), nerve growth factor (NGF), neurotrophin-3 (NT-3) besides neurotrophin 4/5 (NT-4/5)(294). BDNF is found to be involved in various neuroprotective cascades like neuronal atrophy reversal, facilitation of neuron regeneration and synapse formation. It is also expressed in olfactory bulb and hippocampus, the region most impaired in AD(295). BDNF level gradually declines in AD brain(296). Infusion of glial cell line-derived neurotrophic

factor (GDNF) for nine months, during phase I trial, showed persistent benefits in Parkinson disease (297). The biggest problem with BDNF molecules is their large size, which enables them to cross the BBB. Apigenin, the non-mutagenic flavone, was found to strengthen the BDNF pathway(298). Insulin and insulin growth factor 1 (IGF1) were found to function as neurotropic agent and neuromodulator(299). Estrogen acts as a neurotropic agent and protects neurons from oxidative stress, ischemic injuries, $A\beta_{42}$ mediated impairment and thus decreases the possibility of AD. Estrogen commonly increases the risk of endometrial, ovarian and breast cancer(300).

The delivery of adeno-associated virus serotype 2 carrying NGF (AAV2-NGF) by stereotactic approach to nucleus basalis, was capable of yielding sustainable biologically significant NGF expression(301). Phase I clinical trial of AAV2-NGF carried on ten subjects, showed encouraging outcome and was extended to phase II trial(302). The degenerating neurons respond to NGF with sprouting in the axonal neuron, functional markers activation and cell hypertrophy. The NGF induced sprouting persist for ten years(303).

Idebenone, an NGF agonist, crossed the BBB and enhanced neuronal growth in cortex. Although, it improved the memory in rat model but failed in AD patients(304, 305). Tyrosine kinases A (TrkA) agonist D3 interacted at IgC2 site to cause phosphorylation and dimerization of receptor. D3, also improved short term and long term memory in mouse model(306, 307). Unfortunately, it impaired long term memory in wild, healthy mice possibly due to reduced neuronal differentiation in hippocampus(294). Derivatives of gambogic acid *i.e.* gambogic amide, dihydrogambogic acid and dimethyl gambogic acid act as TrkA agonist and prevent kainic acid mediated hippocampal neuronal injury(308, 309).

The cortical and hippocampal area of AD brain showed low levels of TrkB and BDNF(294, 310). 2-Oxa-spiro[*5,5*]-undecane molecule crafted from natural product Paecilomycine A, showed promising TrkB agonist activity in mouse and zebrafish models. The molecule acts through TrkB-MAPK-ERK-CREB-BDNF pathway(311). Deoxygedunin, a derivative of gedunin obtained from Indian neem tree, stimulated the neuronal survival and improved memory(312). TrkB receptor agonist flavonoid, 7,8-dihydroxyflavone, enhanced TrkB phosphorylation and inhibited vascular endothelial growth factor receptor (VEGFR)(313). However, the flavonoid produced neurotoxicity at concentrations 100-200 μM and was futile to keep cortico-striatal neuronal co-culture from cell death(314, 315). LM11A-31 bound competitively at p75 and led to neuroprotection. It showed impressive bioavailability, crossed BBB, possessed satisfactory half-life and reduced Aβ induced neuronal injury in hippocampal slice(316, 317),(317).

2.7.3 Receptor for advanced glycation end-products (RAGE) inhibitors

RAGE was found to be involved in the integrity maintenance of BBB(318). However, it is also participating in the transportation of Aβ in the brain. Azeliragon (TTP488), a small orally active molecule, showed inhibition of RAGE and is currently in phase III clinical trial for mild to moderate AD (NCT02080364)(147).

2.7.4 Phosphodiesterase (PDE) inhibitors

PDEs are the main target for non-amyloid based drug design and discovery. The secondary messengers, cAMP and cyclic guanosine monophosphate (cGMP) are involved actively in the maintenance of neuronal plasticity and cognition(319). PDEs hydrolyze cAMP, cGMP and are categorized as cGMP specific (PDE5, PDE6, PDE9), cAMP specific (PDE4, PDE7, PDE8) and dual acting (PDE1, PDE2, PDE3, PDE10, PDE11)(320). PDE9 isoform PDE9A is found in highest concentration in the regions of

brain involved in cognition. PDE9A hydrolyzes cGMP with highest affinity and restrict the cGMP facilitated signal transduction(319). cGMP specific PDE inhibitors are postulated to enhance cognition in AD and schizophrenia. The recently developed oral PDE inhibitor BI 409306 is in phase II trial(321).

Several PDEs inhibitors viz. rolipram (PDE4), cilostazol (PDE3) and sildenafil or tadalafil (PDE5) showed promising effect in mouse model(319). PDE4 inhibitors were effective in neuroinflammation and neurodegenerative diseases. Recently, rolipram was investigated for aging and streptozotocin induced cognitive deficit. It was found to improve the memory by anti-inflammatory, anti-cholinesterase, and anti-Aβ aggregation methods(322). Unfortunately, the serious side effect associated with PDE4 inhibitor hampered the drug development(319). Helicon Therapeutics developed PDE4 inhibitor HT-0712, which has completed the phase II trial. PDE5 inhibitor, tadalafil, is more suited for AD drug development than vardenafil or sildenafil. Tadalafil was safer and had excellent pharmacokinetic property, however, its logBB ratio was modest, -0.89 (lowest ratio accepted for CNS drug is -1)(323). PDE5 inhibitor Yonkenafil improved pathological conditions as well as cognition in transgenic AD mice. It diminished the Aβ plaque area, controlled APP processing, restored neurogenesis and prevented the abnormal triggering of microglia and astrocytes(324). PF-4447943, developed by Pfizer, was 78 times more selective for PDE9A and had logBB = -0.4, IC_{50} = 12 nM(325).

2.7.5 Apolipoprotein

Apolipoprotein E type 4 allele (*APOE ε4*) is genetically involved in the onset of AD. Approximately 25% of the population is carrier of one or more copies of *APOE ε4* allele *i.e.,* utmost significant genetic threat factor. 50% to 75% of *APOE ε4* carriers are AD patients. *APOE ε4* allele carrier faced BBB breakdown in more usual manner. Further, *APOE ε4* expression increases the Aβ plaques at earlier ages and decreases Aβ

clearance. Genetically Passive and ostensibly active immunotherapies were performed well in patients with and without *APOE* ε4 carriers(326).

2.7.6 Biomarkers

The measurement of Aβ plaques by PET, PHF tau burden estimation, resting functional connectivity and brain atrophy measurements by MRI and CSF $A\beta_{40}$, $A\beta_{42}$ phospho and total tau levels are best measured by AD biomarkers. 18F-AV1451 PET characterized the episodic memory impaired by PHF tau deposition(327). Elecsys assay has been used to measure the $A\beta_{42}$ with high precession. Neurological injuries in CNS disorders are successfully monitored by the CSF neurofilament light chain (NfL) levels. The NfL level in blood is used to monitor the neurodegenerative disorders related with tau or Aβ proteins (**Table 2.6**)(328).

Table 2.6. Biomarkers in the treatment of AD.

S.No	Target	Biomarkers
1	Tau pathology	1.1 Phospho and total tau
		1.2 Tau PET
2	Aβ plaques	2.1 $A\beta_{40}$ and $A\beta_{42}$
		2.2 Aβ PET
3	Neurodegeneration	3.1 PET ^{18}FDG
		3.2 SNAP25 and Neurogranin
		3.3 NfL
		3.4 Structural MRI
4	Neuroinflammation	4.1 CSF TREM2
		4.2 TSPO PET

^{18}FDG; fludeoxyglucose F 18, SNAP25; synaptosomal-associated protein 25, TREM2; triggering receptor expressed on myeloid cells 2, TSPO; translocator protein.

2.7.7 Non peptidic anti aggregate compounds

Homotaurine (alzhemed^{TM}) is low molecular weight, 3-aminopropane-1-sulfonic acid which mimicked the glycosaminoglycans and inhibited Aβ aggregation. It is taurine amino acid analog reported to inhibit β-sheet formation by preventing the interaction of glycosaminoglycans with Aβ. Glycosaminoglycans are involved in amyloid fibril formation, deposition and stability of plaques(329). Alzhemed inhibited the Aβ neurotoxicity, fibrillogenesis and Aβ aggregation. In the TgCRND8 transgenic mice it reduced approximately 30% of brain Aβ load(330, 331). It failed to pass phase III clinical trial for the management of AD in 2007, undertaken by Canadian company Neurochem, Inc. In quantitative determination of interference with Aβ aggregate size distribution (QIAD) assay negative result was found at 2 mM concentration of homotaurine(332, 333)

2.7.8 Hormones

α-Melanocyte stimulating hormone(α-MSH), a neuropeptide, was found to be downregulated in the CSF of AD patients. The hormone executes many functions in the normal brain *viz*. anti-inflammatory, neuroprotective effects. The treatment with α-MSH showed improvement in spatial memory, anxiety alteration and GABAergic interneurons survival in mouse model for AD. It prevented the degeneration of somatostatin subpopulation and conserved GABAergic interneurons leading to enhanced cognitive function. The α-MSH hormone has evolved as a promising therapeutic agent for AD, but its potential must be investigated in the AD patients(334). Estrogen is considered to be neuroprotective and women having early menopause may be prone for dementia(335). It was calculated that each additional month of estrogen exposure could decrease the risk of AD by 0.5%(336) but the clinical trial was inconclusive to validate this hypothesis. However, the requirement of estrogen exposure

in the years promptly after menopause, or the role of genotypes of *APOE* may reverse the results and need to be assessed (337).

Insulin and somatostatin are involved in AD and the agents that increase the effect of these hormones have therapeutic potential in AD(338). Somatostatin contributes to cognition by affecting long term memory and synaptic plasticity(339). Interestingly, the effects of octreotide, a somatostatin analog, and insulin on memory are centrally mediated(340). Octreotide acts on somatostatin type 2 receptor in brain. It has low BBB penetrating ability, but microvascular damage in the AD might increase its central effect(341, 342). The $A\beta_{1-40}$ extracellular concentration was affected in BV-2 cells on treatment with somatostatin and a negative correlation between somatostatin and Aβ was observed(343).

2.7.9 Repurposing drugs

The recent failure of drugs in clinical trials promoted a complementary approach. Therapeutic potency shown by some of anticancer, antihypertensive and insulin resistance drugs could be alluring for further drug design and discovery. Angiotensin receptor blocker (ARB) valsartan was reported to inhibit mitochondrial dysfunction, inflammation, vasoconstriction and stimulate the AChE release. Angiotensin receptor subtype 1 (AT_1) blockade by some of ARBs showed promising therapeutic effect in CNS related disorders(344). Valsartan penetrates BBB, decreases Aβ burden and in randomized controlled trials showed therapeutic benefits in comparison to placebo(364;366;367). Calcium channel blockers like nimodipine, nitrendipine, and nilvadipine might work by a novel mechanism which is independent of antihypertensive effect in AD. In *Drosophila melanogaster* model, these drugs decrease Aβ pathology(345). In randomized controlled trials, these drugs showed cognitive enhancement in AD(346, 347). Glucagon-like peptide-1 (GLP1) analog, liraglutide,

showed tau and GSK3β phosphorylation along with neuroprotective and antioxidant effects(348). Minocycline and acitretin were also tested for their anti-AD effect and it was found that both drugs were involved in up regulation of Aβ clearance (349, 350). The list of repurposing drugs in clinical trial are included in **Table 2.7**.

Table 2.7 Repurposing drugs in clinical trail.

S.No	Drug	Clinical trial status	Clinical trial gov identifier
1	Nilvadipin	III	Not confirmed
2	Liraglutide	II	NCT01469351
3	Acitretin	II	NCT01078168
4	Exenatide	II	NCT01255163