Chapter One

General Introduction
1.1 Alzheimer’s Disease

Alzheimer’s disease (AD) is a progressive, neurodegenerative disorder and the most common form of acquired dementia in elderly population. This epidemic neurodegenerative disorder is the fourth leading cause of death in developed nations. It claims millions of victims per year, not just erasing the memory of the patient, but effecting severe implications on the social environment. The symptoms of the disease include memory loss, confusion, impaired judgement, personality changes, disorientation and loss of language skills.\(^1\) AD is a common age-related dementia, distinct from vascular dementia associated with brain infarction.\(^2\) It may progress to a totally vegetative state. Atrophy of cortical and sub cortical area is associated with deposition of \(\beta\)-amyloid (A\(\beta\)) protein in the form of senile plaques and formation of neurofibrillary tangles (NFT). There is marked cholinergic deficiency in the brain, though other neurotransmitter systems are also affected.\(^3\)

Two forms of AD exist:

(i) **Familial Alzheimer’s disease (FAD):** In this multiple family members may genetically inherit this disease. In FAD mutations of the amyloid precursor protein (APP) gene or other genes that control amyloid processing have been discovered. It accounts for only a small portion (less than 10\%) of AD and has early onset, occurs between ages 30 to 60.

(ii) **Sporadic Alzheimer’s disease (SAD):** One or few members of a family develop the disease. It affects roughly 2\% of those 65 years of age, with the incidence roughly doubling every 5 years up to the age 90 at which the incidence is over 50\%.\(^2,\(^4\)

1.1.1 Molecular Causes of AD

In November 1906, pathologist Alois Alzheimer’s seminal report stated the defining characteristic hallmarks of the disease to be \(\beta\)-amyloid (A\(\beta\)) peptide deposits in senile plaques and neurofibrillary tangles (NFT), consisting mainly of paired helical filaments of abnormally phosphorylated tau (\(\tau\)) protein. As the disease progresses, neuronal death appears. In particular, cholinergic neurons and synapses of the basal forebrain are selectively lost, accounting for the development of cognitive impairments.\(^5\)
Various approaches proposed to confront AD pathogenesis were as follows:

(i) **Cholinergic hypothesis:** In 1976 loss of choline acetyltransferase (CAT) in the nucleus basalis was the first biomarker of AD. In AD, CAT activity in the cortex and hippocampus is reduced considerably (30-70%) and also there is decreased density of nicotinic acetylcholine receptors in the cerebral cortex. It was observed that muscarinic acetylcholine receptors play an important role in learning & memory, so cholinergic enhancement will improve cognitive functions in AD. With this perspective, Acetylcholinesterase (AChE) inhibitors were the first FDA-approved drugs for the treatment of AD. These drugs were presumed to prolong the lifetime of released acetylcholine. Cholinesterase inhibitors (AChEi) produce small improvements in cognitive and global assessments, but do not address the severe mortality in the final stages of AD.⁶

(ii) **N-methyl-D-aspartate (NMDA) receptor modulator:** Memantine hydrochloride, was first made in 1960 by Ely Lilly as an anti-diabetic agent, protects neurons against over activation of N-methyl-D-aspartate receptors and was approved for the treatment of moderate to severe Alzheimer’s disease in 2002 as first of its class.⁵ This NMDA receptor antagonist appears to block excitotoxicity of the transmitter glutamate in a non-competitive and use-dependent manner.³,⁶

(iii) **Amyloid cascade hypothesis:** Compelling evidence suggests that Aβ secretion is the triggering event in the pathogenesis of AD and the β aggregation may be an important secondary event linked to neurodegeneration. The amyloid beta (Aβ) peptide clusters into amyloid plaques on the outside surface of neurons of the brain, which ultimately leads to the killing of neurons and dementia. Amyloid plaques, which are found in the post-mortem brain of Alzheimer’s disease patients, consist mainly of fibrillar aggregates of the Aβ peptide. The Aβ peptides, which differ in length from 38 to 42 amino acids, are generated from the amyloid precursor protein (APP) by the action of two aspartic proteases: β- and γ-secretases. APP is normal neuron membrane protein thought to be a natural neuroprotective agent induced by neuronal stress or injury and protects neurons from glutamate.

Specifically, β-secretase (BACE1, β-site APP cleaving enzyme) in rate limiting step mediates the primary cleavage of APP from N-terminal side, generating sAPPβ and membrane bound C-terminal APP fragment (C99), which on cleavage
by γ-secretase (BACE2, γ-site APP cleaving enzyme) liberates the Aβ peptide (Aβ40 or Aβ42) and an APP intracellular domain (AICD). In AD, the 42 amino acid amyloid beta peptide (Aβ42) is overproduced due to genetic mutations of either the APP gene or other genes. Aβ42 is more hydrophobic & sticky than 40 amino acid amyloid beta peptide (Aβ40). Hence, fibrils of Aβ42 clump together readily to form amyloid plaques. The α-secretase (α-site APP cleaving enzyme) acts on APP to release the larger extracellular domain as soluble APP (sAPPα), which serves various poorly understood trophic functions. 1,5 (Figure 1.1.1)

![Diagram showing processing of APP by secretases.](image)

**Figure 1.1.1: Processing of APP by secretases.**

Following amyloid plaque formation two processes play an important role in causing the death of neurons, inflammation and neurofibrillary tangles (NFT). The two major types of brain cells: astrocytes and microglia participate in the inflammatory response. Astrocytes become more numerous in AD and these cells on activation produce prostaglandin/arachidonic acid mediated inflammation. Activated microglial cells produce damaging free radicals. The activities of astrocytes & microglia lead to the death of neurons. Τ is an important protein which maintains the structural integrity of microtubules. Substances for nutrient and cell regulation are transported along microtubules within neurons. In AD the phosphorylated tau proteins bind to each other, tying themselves in knots known as NFT. Neurons full of NFT rather than functional microtubules soon die. 2,4

Since β-secretase mediated cleavage of APP is the first and rate limiting step of the amyloidogenic processing pathway, BACE1 inhibition can be considered as a
prominent therapeutic target for treating AD by diminishing Aβ peptide formation in AD patients.7 Thus, BACE1 represents a key target protein in the development of new potential drugs for the non-symptomatic treatment of Alzheimer’s disease.8

(iv) Lipoproteins and Cholesterol: Apoproteins are the lipoproteins that transport cholesterol. Apolipoprotein E (ApoE) mobilizes cholesterol in the central nervous system (CNS) and is important for repair of damaged neurons & synapse plasticity. Mutations in gene, that code for the lipid transport protein ApoE4, predispose to AD, probably because expression of abnormal ApoE4 proteins facilitates the aggregation of Aβ peptides.2,4,5

(v) Inflammation: Neuroinflammation of CNS cells has been recognized as an invariable feature of all neurodegenerative disorders. In AD, microglia are activated by Aβ to produce inflammatory cytokines like Inter Leukin-1β (IL-1β) & Tumour Necrosis Factor-alpha (TNF-α), chemokines and neurotoxins that are potentially toxic and therefore may contribute to neuronal inflammation & degeneration.4,5

(vi) Oxidative stress: Oxidative damage is present within the brain of AD patients and is observed within every class of biological macromolecules. Oxidative injury may develop secondary to excessive oxidative stress resulting from Aβ-induced free radicals, mitochondrial abnormalities, inadequate energy supply, inflammation or altered antioxidant defences. Oxidative stress is thought to have a causative role in the pathogenesis of AD.5

(vii) Metal ion dyshomeostasis: As related to the oxidative damage hypothesis, there is general acceptance that redox active metals can contribute to excess production of damaging reactive oxygen species (ROS) through Fenton’s chemistry. Besides creating oxidative stress, copper, together with other metal ions, influences the protein aggregation processes that are critical in most neurodegenerative diseases. APP and Aβ are able to bind and reduce copper, which forms a high-affinity complex with Aβ, promoting its aggregation, and Aβ neurotoxicity depends on catalytically generated H2O2 by Aβ-copper complexes in vitro. Moreover, copper, together with zinc and iron, is accumulated in the amyloid deposits of AD brains, which are partially disassembled by metal chelators.4,5
1.2 Secretases

1.2.1 α-Secretase

α-secretase cleaves the normal neuron membrane protein (APP) to release a large fragment of soluble APP (sAPPα) which is not amyloidogenic and serves various poorly understood trophic functions. Thus, more efforts were directed to the study of β- and γ-secretases as they are involved in the amyloidogenic processing pathway.²

1.2.2 β-Secretase

The cloning and identification of BACE1 (memapsin 2) was first reported in 1999. The bilobal structure of BACE1 has the conserved general folding of the aspartic proteases super family and more precisely the pepsin subfamily. Other members of aspartyl family include HIV protease, pepsin, renin and cathepsin D. The crystal structures of BACE1 confirm that the active site is a long cleft for the substrate recognition with two catalytic aspartic residues (Asp32 and Asp228).⁹ Memapsin 2 is a type I transmembrane aspartyl protease highly expressed in the brain, produced by neurons having an optimum pH of 4.5 and is localized in acidic sub cellular compartments of the cell.¹⁰ BACE1 is anchored to the membrane via its transmembrane domain (455-480) and may occur as a dimer. BACE1 consists of an NH₂-terminal protease domain, a connecting strand, a transmembrane region, and a cytosolic domain.⁸ It has a pro-peptide domain, which is cleaved by furin like proteases to form mature enzyme. The C-terminal transmembrane domain of BACE1 is not strictly required for activity, but the localisation of both enzyme and substrate in the same membrane enhances kinetics and specificity. The C-terminal truncation seems to influence enzyme kinetics even in the absence of membranes. BACE1 maturation requires cysteine formation (Cys216/Cys420, Cys278/Cys443 and Cys330/Cys380), N-glycosylation and propeptide removal. Cysteine mutants undergo impaired maturation, but obtain catalytic activity. The Cys330/Cys380 bridge was found to be the most important. Like other aspartic proteases, it consists of two structural domains (N- and C-terminus), that define the active site along with a hairpin like structure or “flap” region, which covers the active site.⁶
Homology with pepsin-like aspartyl proteases is reflected in the similar folding pattern of BACE1 with extensive β-sheet organization and proximal location of the two catalytic aspartyl residues for peptide bond cleavage. However, the C-terminal lobe is larger than other aspartyl proteases and critical disulfide bridge links the C-terminal region to the body of the molecule. The active site of memapsin 2 is more open and less hydrophobic than that of other human aspartic proteases.

During catalysis, the flap opens to allow the substrate to enter into the active site and then closes to promote catalysis. Finally, the flap re-opens to allow diffusion of the hydrolyzed products from the active site. BACE1 catalyzes the cleavage of substrate such as peptide amide bond via general acid-base mechanism. These aspartic peptidases have Asp-Thr-Gly sequences lining the active site and contain water molecule bound between the two catalytically active aspartate residues. This substrate water molecule is activated by the aspartic acids for nucleophilic attack on the substrate amide carbonyl to generate the tetrahedral intermediate, which is observed as a low energy species in molecular dynamic ab initio calculations. Finally, the tetrahedral intermediate collapses resulting in the release of hydrolyzed products and the enzyme is restored for another cycle of catalysis. (Figure 1.2.1)

Figure 1.2.1: BACE1 catalytic mechanism.
Molecular Dynamics (MD) simulation study of β-secretase complexed with the inhibitor OM99-2, suggests the protonation state of catalytic aspartate dyad in the BACE1 active site to be neutral Asp32 and ionized Asp228. They are adequately positioned by Ser35 and Thr231 respectively, in enzymatic catalysis and inhibitor binding. Generally it is observed that in the catalytic hydrolysis of a peptide by aspartic proteases such as pepsin, penicillopepsin, renin and HIV-1 protease, the two active-site aspartyl residues should operate cooperatively with net charge of -1.\textsuperscript{13}

As far as only few mammalian aspartic proteases exist, it could be emphasized that the number of enzymes that could be cross-inhibited and lead to toxic side effects is restricted. Moreover, after analyzing the phenotype of young BACE1 knockout mice, it was found that these were healthy and fertile and display reduced Aβ levels. This was crucial for development of assay methods and animal models.\textsuperscript{6}

\subsection*{1.2.3 \textit{γ}-Secretase}

The identity of γ-secretase was for a long time a subject of debate and the detailed structure is still unknown. There is a close relation to the Notch pathway, which is important in embryonic development.\textsuperscript{6} Unfortunately, inhibition of γ-secretase is associated with toxicity issues in both animal models and in clinic, mainly because γ-secretase is an unusual transmembrane protease complex, which involves four components including presenilin. As presenilins are also involved in Notch cleavage, specific inhibitors, which do not affect Notch pathway, are required, since this latter pathway plays an important role in development stages. For this reason, β-secretase appears to be a therapeutic promise.\textsuperscript{14}

\subsection*{1.3 Current Therapies for Alzheimer’s Disease}

Recent advances in understanding the process of neurodegeneration in Alzheimer’s disease (AD) have yet to result in therapies able to retard it. Currently, cholinesterase inhibitors and NMDA receptor modulator are the only drugs approved for treatment of AD, though many drugs have been claimed to improve cognitive performance and several new approaches are being explored.\textsuperscript{2}
1.3.1 Cholinesterase inhibitors

Five drugs that are currently in the market; to be used as acetylcholinesterase inhibitors (AChEIs) are as follows:

(i) Tacrine

It is the first centrally acting AChEI drug to be introduced for treatment of AD. In clinical trials, it produced significant improvement in memory, attention, reason and language. Frequent side effects and hepatotoxicity have restricted its use.

(ii) Donepezil

This cerebroselective and reversible anti-AChE produces measurable improvements in several cognitive as well non-cognitive scores in AD. This benefit is ascribed to elevation of Acetyl Choline (ACh) level in the cortex, especially in the surviving neurons that project from basal forebrain to cerebral cortex and hippocampus. It has distinct advantage of single dose as compared to rivastigmine and galantamine which need twice daily dosing.

(iii) Rivastigmine

This carbamate derivative of physostigmine inhibits both AChE and BuChE (Butyryl choline esterase), but is more selective for the G1 isoform of AChE that predominates in certain areas of the brain. In clinical trials an average of 3.8 point improvement in Alzheimer’s disease Assessment Scale (ADAS-cog) has been obtained as compared to placebo.
(iv) **Galantamine**

It is a natural alkaloid which selectively inhibits cerebral AChE and has some direct agonistic action on nicotinic receptors as well. Effects are comparable to others.

All of them exhibit mild cholinergic side effects. They afford modest symptomatic benefit in AD.² ³

1.3.2 **NMDA receptor modulator**

**Memantine**

This new N-methyl-D-aspartate (NMDA) receptor antagonist, related to amantadine has been found to slow the functional decline in moderate to severe AD, but benefits in milder disease are unclear. It is better tolerated than ACHEIs used in AD. Side effects are constipation, tiredness, headache and drowsiness. The current strategy to use memantine hydrochloride in combination therapy with the AChEI donepezil was concluded from the outcome of a 24 week trial against placebo. The combinations showed further decline, but the magnitude of these effects was modest.³ ⁶
Although beneficial in improving cognitive, behavioural, and functional impairments, the above mentioned classes of drugs are unable to address the molecular mechanisms that underlie the pathogenic processes.\textsuperscript{5} Thus, a causal therapy is still in utter demand, as no existing therapy effectively stops or even cures the disease.\textsuperscript{6}

1.3.3 β-Secretase inhibitors

Over the past decade, many BACE1 inhibitors have been reported and these inhibitors can be broadly divided into two classes: peptidomimetic and non-peptide or pseudopeptide inhibitors. The peptidomimetic inhibitors were the analogues of the natural substrates of BACE1 and these included hydroxyethylenes, statines, norstatines and hydroxyethylamines.

Several types of BACE1 inhibitors which have been reported so far are as follows:

(i) Hydroxyethylene-based BACE1 Inhibitors: Tang and co-workers at the University of Oklahoma, reported the peptidic hydroxyethylene-based first potent BACE1 inhibitor OM99-2 (1) (Ki: 1.6 nM, IC\textsubscript{50}: 0.002 \textmu M.) and it was co-crystallized with BACE1 for resolving the three-dimensional structure of the enzyme-inhibitor complex.\textsuperscript{10, 15} From its crystal structure, the nature of the sub-pockets in the BACE1 active site was revealed and eight such pockets were known to be involved.\textsuperscript{9} Modification in OM99-2 structure resulted in OM00-3 (2) (Ki: 0.31 nM), which is still the most potent inhibitor of β-secretase.\textsuperscript{16} (PDB: 1m4h) (Figure 1.3.1) The small interaction of the C-terminal end of the octapeptides OM99-2 (1) and OM00-3 (2) with the enzyme inspired the design of shorter peptidic inhibitor by Chen et al. (3) (IC\textsubscript{50}: 35 nM).
Figure 1.3.1: PDB structure 1m4h of β-secretase complexed with inhibitor OM00-3.
(ii) Statin and Homostatine BACE1 Inhibitors: These types of inhibitors have been mostly developed by Elan and Pharmacia & Upjohn. The compounds demonstrate dose-dependent and mechanism-specific reduction of Aβ in human embryonic kidney (HEK) cells e.g. Elan compound 4 (IC50: 1 nM, HEK-293 IC50: 1 μM) and 5 (IC50: 30 nM, HEK-293 IC50: 3 μM). Kiso inhibitor 6 (KMI-370, IC50: 3.4 nM) featured a short C-terminal dicarboxylic acid and displayed high activity in vitro and in vivo (BACE1-HEK293 cells EC50: 0.20 μM).

Pr: n-propyl group.
(iii) **Hydroxyethylamine (HEA) BACE1 Inhibitors:** The HEA dipeptide isostere is a known motif of the aspartyl protease inhibitors, notably from early research on HIV protease and renin inhibitors. The pharmaceutical industries such as Elan (7), Pharmacia, Glaxo, Astex (8, IC\textsubscript{50}: 200 nM), Sunesis (9), Bristol Myers Squibb, and Takeda (10, IC\textsubscript{50}: 99 nM) have made important contributions with closely related core structures. Several patent applications have been disclosed. The Elan compounds have lost a good part of their peptidic origin, which is mandatory to obtain sufficient oral absorption and blood brain barrier penetration.

![Chemical Structures](image-url)
Pr: n-propyl group, Et: ethyl group, Me: methyl group.

To overcome the historical problems associated with peptide like structures such as low oral bioavailability, poor blood brain barrier permeability, and susceptibility to P-glycoprotein transport, focus was shifted towards the identification of non-peptide or pseudopeptide inhibitors.\textsuperscript{6,17}

(iv) **Non-peptide Inhibitors:** Different classes of non-peptidic potent BACE1 inhibitors have been reported by different pharmaceutical companies such as Vertex, Actelion Pharmaceuticals, De Novo Pharmaceuticals, Elan and Pharmacia. In 2001, Takeda disclosed the first BACE1 inhibitor to be aminooethyl-substituted tetralin. In 2002, Vertex reported the halogen-substituted biphenyl piperazines and similar biphenylated amines were revealed by Actelion. Vertex also proposed the first 3D pharmacophore map of BACE1 (Figure 1.3.2) to guide the design and optimization of inhibitors. Other reported BACE1 inhibitors include acylguanidines, isophthalic acid derivatives, amino aromatic heterocyclic motifs, and arylpiperazines. (Figure 1.3.3). The non-peptide inhibitors will be discussed in depth in the Literature survey chapter of thesis.

From this survey, it appears that the ideal properties of a BACE1 inhibitor for clinical use are: (i) ability to potently and effectively lower Aβ in transgenic mice, (ii) CNS penetration, (iii) oral bioavailability, (iv) good pharmacokinetic profile and finally (v) selectivity against other aspartyl proteases.\textsuperscript{6,18}

The thesis highlights the design and synthesis of non-peptide inhibitors of β-secretase.
Figure 1.3.2: Vertex inhibitor and 3D BACE1 pharmacophore map.

(HB: H-bonding moiety interactions with the active site residues of BACE1,
HPB: Hydrophobic moiety interactions with BACE1 sub sites.)
1.3.3 γ-secretase inhibitors

Peptidic PS1 inhibitors, like Merck’s L-685,458 (11) are still the most potent inhibitors and were patented prior to publication in scientific journals. To this date, very little structural information is available for the γ-secretase complex. Therefore, selective, non-peptidic γ-secretase inhibitors had to be provided by high throughput screening (HTS) efforts. Non-peptidic inhibitors of γ-secretase are known from the patents by Elan, Eli Lilly, Bristol Myers Squibb and DuPont. Elan’s DAPT (12), (HEK IC₅₀: 20 nM) was developed from N-dichlorophenylalanine lead, and the phenylglycine and the difluorophenylacetic acid are crucial for activity. Bristol Myers Squibb and Merck disclosed 1000 derivatives of
4-Chloro-N-(2, 5-difluorophenyl)-benzenesulfonamides (13). 500 of these were reported to be very good inhibitors of γ-secretase. The activity clustered around the core structure (13), with a wide variation of the R substituent to modulate bioavailability. The difluorophenacyl-caprolactam derivative (14), stemming from Scios/DuPont cooperation, proved to be the most potent compound (IC<sub>50</sub>: 0.3nM). Boehringer-Ingelheim disclosed diaminopyrimidines (15) as non-peptidic inhibitors of γ-secretase with IC<sub>50</sub>: 4 to 1000 nM.<sup>5</sup> (Figure 1.3.4)

![Chemical structures](image)

**Figure 1.3.4:** Some γ-Secretase inhibitors.
1.3.5 Non-steroidal anti-inflammatory drugs (NSAIDs)

Few NSAIDs may reduce Aβ42 formation by modulation of γ-secretase. Promising results were obtained with some COX1 inhibitors. Any derivatization of the NSAIDs carboxylic acid results in loss of this activity. (R)-Flurbiprofen (16), (Aβ40/42 IC50: 307 μM, 10 and 25 mg/kg/d) elicits non-selective reductions in both Aβ1-40 and Aβ1-42 plasma levels, and was found to be toxic. Sulindac sulfide (17), (Aβ40/42 IC50: 40-60 μM) and ibuprofen (18), (Aβ40/42 IC50: 200 μM) were found to be neither toxic nor efficacious at doses up to 50 mg/kg/d.²,⁶

1.3.6 Immunological approach

An ingenious new approach was taken by Schenk et al. (1999), who immunized AD transgenic mice with Aβ protein and found that this not only prevented plaque formation but actually reversed it, suggesting an immunological approach that might be used in humans. However, clinical trials with an antibody directed against Aβ showed that it tends to cause CNS inflammation, so the project was abandoned.²
1.3.7 Metal chelating agents
The amoebicidal drug Clioquinol is a metal chelating agent that causes regression of amyloid deposits in animal models of AD and is currently undergoing clinical trials.\(^2\)

![Clioquinol](image)

1.3.8 Nerve growth factor
Shortage of growth factors (particularly nerve growth factor) may contribute to the loss of forebrain cholinergic neurons in AD. Implanting cells engineered to secrete nerve growth factor are under investigation.\(^2\)

1.4 1,2,4-Thiadiazoles
During the last decade, 1,2,4-thiadiazoles have been the subject of great interest because of their biological activities. This nucleus is a fundamental constituent of a number of synthetic products with biological activities concerning central nervous system (CNS), G-protein coupled receptors, inflammation, cardiovascular system or antibiotic activity.

Thiadiazole related compounds have been successfully described as potential drugs for the treatment of Alzheimer's disease. The antioxidant and muscarinic receptor binding properties of 3-(thiadiazolyl) pyridine-1-oxide compounds (19) were reported and a family of 1,2,4-thiadiazolidinone derivatives containing the N-benzylpiperidine fragment (20) has also shown acetylcholinesterase inhibitory activity. Moreover in a series of 1,2,4-thiadiazoles bearing a mono or bicyclic amine at C5 (21), the ring represents an ester mimic in the binding of muscarinic ligands capable of displaying high receptor affinity over a wide efficacy range. Recently, the thiadiazolidin-3,5-dione (TDZD) derivatives (22) were reported as
the first non-ATP competitive inhibitors of glycogen synthase kinase 3b (GSK-3b). This enzyme has emerged as one of the most attractive therapeutic targets for the development of selective inhibitors as new promising drugs for unmet pathologies including Alzheimer’s disease, stroke, bipolar disorders, chronic inflammatory processes, cancer and diabetes type II.\(^9\) (Figure 1.4.1)

![Chemical structures](image)

**Figure 1.4.1: 1,2,4-thiadiazole derivatives with CNS activity.**

The usefulness of 1,2,4-thiadiazole as pharmacophore in medicinal chemistry has prompted us to explore this system for our research work.

### 1.4.1 Synthesis of 1,2,4-Thiadiazoles

The parent compound 1,2,4-thiadiazole was first synthesized in 1955 by sequence of reactions, but the route remains relatively inaccessible for the preparation of derivatives. In the older literature, 1,2,4-thiadiazoles have been indexed as azosulfimes or as perthiocyanates. The 1,2,4-thiadiazole ring may be built up from simple fragments mainly by use of the general approaches. They are classified according to the nature of the fragments from which the heterocyclic ring will be ultimately formed:

a) **Type A Syntheses (NCS + CN):** This synthesis involves the oxidation of compounds containing the thiocarbamoyl group such as thioamides, thioureas to form substituted 1,2,4-thiadiazoles.
b) **Type B Syntheses** (NCN + CS): It comprises of cyclization's involving a two stage condensation between a compound incorporating an amidino group (including amidoximes, amidines, iso(thio)ureas, guanidines, etc.) and one containing a thio carbonyl group (e.g. carbon disulfide, thiocyanogen, thiocyanate, isothiocyanate esters and halogenated methane sulfenyl chlorides). Recent contributions have made this a remarkably versatile approach.

c) **Type C Syntheses** (NCNCS): The oxidative cyclization of compounds incorporating the amidinothiono group [-C (=NH) NH-CS-] continues to provide the most versatile route to substituted 1,2,4-thiadiazoles.

d) **Type D Syntheses** (CNCSN): The condensation of N-chloro amidines and potassium methyl cyanoiminodithiocarbonate in chloroform below 5°C yields substituted 1,2,4-thiadiazolines in one stage.

e) **Type E Syntheses** (CNCS + N): The interaction of thiobenzoyl isocyanate and nitrosobenzene yields the 1,2,4-thiadiazolin-3-ones with elimination of oxygen.

Type B syntheses have been used to synthesize the 3-substituted-5-amino-1,2,4-thiadiazoles.

(i) **Syntheses from Amidines (Type B syntheses)**

In 1954, Goerdeler introduced a general synthesis of 1,2,4-thiadiazoles from amidines. This versatile method has since been widely extended and used for synthesis of variety of 1,2,4-thiadiazole derivatives. In this method, the amidine or N-halogenoamidine is converted to its N-thiocyanato derivative on reaction with thiocyanate. The derivative *in situ*, in the presence of bromine and sodium methoxide, cyclizes spontaneously to form the desired 3-substituted-5-amino-1,2,4-thiadiazoles in good yields. (Figure 1.4.2) The special feature of the present synthesis is the attachment of the thiocyanate group at nitrogen instead of a carbon atom, which seems to enhance its reactivity. By employing suitable analogues of amidines in this general synthesis, various 3-substituted-5-amino-1,2,4-thiadiazoles can be readily prepared, e.g. isothioureas can form the corresponding 3-alkylthio-5-amino-1,2,4-thiadiazoles in good yields.
1.4.2 Properties of 1,2,4-thiadiazole and its analogues

They possess aromatic character and are classified as $\pi$-excessive sulphur-containing heteroaromatic compounds. They are weakly basic and the basicity decreases further on introduction of substituent such as 3-alkylthio or 3-alkyl sulfonyl. Substituents in the 3- and 5-position exert a marked stabilizing effect on the heterocyclic nucleus towards acids, alkalis, oxidizing and reducing reagents. Spectroscopic evidence suggest strongly that 3-substituted-5-amino-1,2,4-thiadiazole exist in enamine (23) rather than ketimine (24) form.$^{20, 21}$ (Figure 1.4.3)
23 Enamine 24 Ketimine

Figure 1.4.3: Tautomerism in 3-Substituted-5-amino-1,2,4-thiadiazoles.

The research work encompasses the 3-substituted-5-amino-1,2,4-thiadiazoles and its ureido derivatives.

1.5 Pharmacological Evaluation
There is a great deal of interest in the discovery of selective non-peptide BACE1 inhibitors with a new chemical skeleton, suited for central nervous system penetration and endowed with more appropriate pharmacokinetic properties. Therefore, the selection of appropriate methods for screening and characterization of BACE1 inhibitors is crucial.

The most common method used to study in vitro BACE1 activity and inhibition is the fluorescence resonance energy transfer (FRET) assay. FRET methods are widely used because they offer a homogeneous and sensitive assay in multiwell format that is easily adopted also for high-throughput screening (HTS). BACE1 FRET assay can be performed with a number of peptide-based substrates, without the presence of a preferential substrate.8

1.5.1 In vitro FRET assay
BACE1 in vitro activity and inhibition are usually measured by using fluorescence resonance energy transfer (FRET) technology. Synthetic peptides having a fluorophore (donor group) & a quencher (acceptor group), and bearing the Swedish mutated sequence of APP (scissile bond -Leu ~ Asp instead of -Met ~ Asp-) are used as substrates; the Swedish mutated sequence is preferred to the wild-type form, as it is cleaved by BACE1 faster. The principle of the BACE1
FRET assay is as follows: The FRET peptide substrate consists of a fluorescence donor on one end and a quenching acceptor on the other. The intrinsic fluorescence of the intact substrate is dramatically reduced because of intramolecular resonance energy transfer to the quenching group. Upon enzymatic cleavage, the energy transfer is disrupted and the full quantum yield of the donor is restored. Enzyme activity is linearly related to the increase in fluorescence. (Figure 1.5.1) In the same way, the gain of fluorescence is lost by inhibition, due to the presence of the test compound.

![Figure 1.5.1: Principle of FRET Assay.](image)

To have high assay sensitivity, in addition to a high rate of substrate conversion, a fluorophore with a high quantum yield of fluorescence and donor-acceptor pairs with effective resonance energy transfer are required. A high emission signal is desired for a low substrate concentration in order to be able to appreciate and rank a vast range of inhibitor potencies, using low enzyme concentrations. In this regard, (7-methoxycoumarin-4-yl) acetyl and dinitrophenyl as a fluorophore-quencher pair combines high fluorescence intensity with good quenching efficiency, better than the 5-[(2-aminoethyl)amino]naphthalene-1-sulfonic acid (EDANS) and 4-(4-dimethylaminophenylazo)benzoic acid (DABCYL) pair, which was shown to provide only limited sensitivity because of the low fluorescence intensity of the dansyl group.

Fluorescence data can be easily correlated to enzyme activity and inhibition. To estimate IC$_{50}$ values, according to the Michaelis theory, fluorescence intensities with and without the inhibitor are compared and the percent inhibition due to the
presence of test compounds is calculated. The background signal is usually measured in control wells containing all the reagents, except BACE1 and is subtracted.⁸