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Design, synthesis, *in silico* and *in vitro* screening of 1,2,4-thiadiazole analogues as non-peptide inhibitors of beta-secretase

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ABSTRACT

Beta-secretase is the key enzyme involved in Alzheimer's disease thus; inhibition of the enzyme can lead to a potential anti-Alzheimer drug. In the search of an effective lead candidate, we have designed non-peptide inhibitor molecules based on amino aromatic heterocyclic motifs specifically, substituted 1,2,4-thiadiazole analogues. *In silico* modelling was employed to study interaction of the designed ligands in the enzyme active site using molecular docking approach as well as for Absorption, Distribution, Metabolism and Excretion studies. The synthesized analogues were pharmacologically screened using *in vitro* FRET technique. Overall results indicate that one of the analogues, compound **8** is the most promising one against beta secretase.

1. Introduction

Beta-secretase (BACE1) has been identified as one of the main enzymes involved in the cascade of physiological events that lead to Alzheimer's disease (AD). It is a progressive, neurodegenerative disorder with acquired dementia in elderly population, resulting in severe cognitive deficits along with neuropsychiatric complications [1,2]. Currently, cholinesterase inhibitors and N-methyl-D-aspartate (NMDA) receptor modulator are the only drugs approved for treatment of AD. Although, beneficial in improving cognitive, behavioral and functional impairments, they confer modest symptomatic benefits [3]. In order to cure, it is necessary to address the molecular mechanisms that underlie the pathogenic processes, as no such existing therapy effectively stops or even cures the disease [4]. The defining characteristic hallmarks of AD are β-amyloid (Aβ) peptide deposits in senile plaques and neurofibrillary tangles (NFT), progressing to neuronal death [3]. Amyloid plaques consist mainly of fibrillary aggregates of the Aβ peptide, generated from amyloid precursor protein (APP) by the action of two aspartic proteases: β-and γ-secretases. Specifically, β-secretase (BACE1, β-site APP cleaving enzyme) 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, Aβ42 is over produced due to genetic mutations of either the APP gene or other genes and is more hydrophobic and sticky than Aβ40. Hence, fibrils of Aβ42 clump together readily to form amyloid plaques [1,3]. 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 [4–6].

BACE1 is a type I transmembrane aspartyl protease highly expressed in the brain [7]. Literature survey indicates that the active site of BACE1 has two catalytic aspartic residues (Asp32 and Asp228) [8]. The reported crystal structure of BACE1 in complex with octapeptide inhibitor OM00-3 (PDB: 1m4h); revealed eight sub sites involved in ligand-enzyme interaction [9].

Over the past decade, many BACE1 inhibitors have been reported and can be broadly categorized into two classes: peptidomimetic and non-peptide inhibitors [4,10]. Although, peptidomimetic inhibitors showed potent activity against BACE1, their relatively large molecular size, low metabolic stability and poor bioavailability render their development into therapeutic drug candidates difficult [4,11]. Hence, more efforts necessitate towards the discovery of non-peptide organic compounds with balanced ADMET properties as drug leads [12–14]. Several strategies of drug discovery have been explored e.g. substrate-based design, high-throughput screening (HTS) and fragment-based approaches [15], which lead to various scaffolds such as 2-amino-thiazole, 6-amino-imidazopyrimidine, 2-amino-quinazoline, 2-amino-pyridine, 2-amino-quinoline, 2-amino-pyrimidinone and 2-amino-isoindole [16–23]. The amino group of these compounds had key contacts with the catalytic dyad, Asp32 and Asp228.

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The reported amino aromatic heterocyclic motifs prompted us to explore amino 1,2,4-thiadiazole as pharmacophore. This nucleus is a fundamental constituent of number of synthetic products with biological activities concerning central nervous system (CNS); G-protein coupled receptors, inflammation, cardiovascular system or antibiotic activity [24]. The 3,5-diamino-1,2,4-thiadiazole and 5-amino-3-(p-toluenesulphonamido)-1,2,4-thiadiazoles have been reported to possess hypoglycemic activity and low toxicity [25]. Similarly, Huang et al. discussed cell permeable phenylurea 1,2,4-thiadiazole and diphenylurea derivatives for Alzheimer's disease [26]. Based on these guidelines; we have designed, synthesized and screened substituted 1,2,4-thiadiazoles as potential β-secretase inhibitors. All the designed series of 3-substituted-1,2,4-thiadiazol-5-amines and substituted phenyl-1,2,4-thiadiazolyl urea analogues (Fig. 1) were subjected to *in silico* ADME studies to understand their pharmacokinetic behavior.

2. Results and discussion

2.1. Molecular docking

Molecular docking studies were employed to explore the BACE1 active site (PDB: 1m4h) and to predict favourable binding pose of the designed ligands. The crystal structure of BACE1 in complex with octapeptide inhibitor OM00-3 (PDB: 1m4h, K_i: 0.3 nM) was considered for docking studies, as its potency is significantly improved compared to the inhibitor OM99-2 (K_i: 1.6 nM), hence; it provides improved versatility for inhibitor design. This inhibitor also exhibits significant binding to the subsites involved in ligand-enzyme interaction [27]. The docking protocol was validated by reproduction of binding pose of OM00-3 inhibitor in the 1m4h active site (rmsd 1.51). The octapeptide inhibitor OM00-3 of PDB 1m4h occupies S_1, S_2, S_1' and S_2' sub sites. To substantiate the protocol, some reported non-peptide BACE1 inhibitors were docked in the active site. Most of them interact with the catalytic aspartic acids, Asp32 and/or Asp228, which play a crucial role in enzymatic catalysis and inhibitor binding. All of them interact with amino acid residues occupying the S_1, S_2 and S_1' sub sites in the active site [18]. The reported crystal structure of BACE1 complexed with OM00-3 (PDB: 1m4h) has revealed amino acid residues present in the sub sites. Sub site S_1 has Leu30, Asp32, Tyr71, Gln73, Phe108, Asp228, Gly230 and S_2 consist of Tyr71, Thr72, Gln73, Gly230, Thr231, Arg235 residues [9].

Consequently, series of 3-substituted-1,2,4-thiadiazol-5-amines was docked in BACE1 active site. The analogues were oriented in the centre of BACE1 binding pocket occupying S_1 and S_2 sub sites similar to the reported docked inhibitors. The 5-amino group of the analogues interacted with Asp dyad via H-bond and electrostatic interactions, Gly230 and Thr231 residues of BACE1 whilst nitrogen's of the heterocyclic ring formed H-bonds either directly or through water molecule with Thr72 and Gln73 residues. It was observed that on changing the R_1 substituent from alkylthio with varying alkyl carbon chain upto 5 carbon atoms to methyl as well as phenyl exhibited equal number of H-bond interactions. But the alkylthio group with carbon chain length beyond 6 depicted no interaction in the BACE1 active site. The findings of amine analogues were similar to the reported amino aromatic heterocyclic motifs so it can be envisaged that these 5-amino-1,2,4-thiadiazoles successfully bind to the BACE1 catalytic site (Fig. 2) and should therefore, provide promising scaffolds for further modifications and series formation. The 3-substituted-1,2,4-thiadiazol-5-amine scaffolds were then designed as methylthio-, ethylthio-, methyl- and phenyl-1,2,4-thiadiazolyl substituted-phenyl urea analogues. It was observed that all these ligands occupy S_1, S_2 and S_3 sub sites.

Docking studies of series of phenyl-1,2,4-thiadiazolyl urea analogues indicated that substituents such as methylthio, cyano and nitro on the phenyl ring lead to similar or increase in H-bond interactions as compared to their heterocyclic scaffolds while substituents such as acetyl and chloro resulted in decreased interactions within BACE1 active site. More number of H-bonds were observed with Gln73 and Thr231 residues of BACE1 for urea analogues as compared to amine scaffolds.

2.2. Synthesis

Based on the Molecular modelling studies, two representative analogues (**6** and **7**) of the 3-alkylthio-1,2,4-thiadiazol-5-amines along with methyl and phenyl as R_1 substituent on the amine scaffold (**8** and **12**) were synthesized, characterized and further screened for activity against BACE1 (Schemes 1 and 2).

Condensation of the above synthesized 3-substituted-1,2,4-thiadiazol-5-amine scaffolds with substituted phenyl compounds resulted in four respective series of phenyl-1,2,4-thiadiazolyl urea analogues (Scheme 3). This step would further decrease basicity of the amines which would benefit them to easily cross the blood-brain barrier.

2.3. In silico ADME studies

The *in silico* ADME (Absorption, Distribution, Metabolism and Excretion) properties of designed test set analogues and the reported ones were investigated to gain an understanding of their likely behavior *in vivo*. All the four 3-substituted-1,2,4-thiadiazol-5-amines (Table 1) had low molecular weight (MW < 200 g/mol), Qplog P o/w < 2, hydrogen bond donor (HBD) < 3, hydrogen bond acceptor (HBA) < 4. These features were considered as favourable physicochemical properties of CNS drugs as highlighted in Partridge's review [28]. The Qplog P o/w parameter is an important factor to determine bioavailability of the compound and seems to be favourable. Similarly the Qplog BB values for all them were obtained in the range of −0.3 to −0.2 (Expected range: −3 to 1.2). None of them violated the Lipinski's rule of five. The QPP MDCK model is considered to be a good mimic for the blood-brain barrier (bbb) and QPP Caco model for the gut-blood barrier. The values of these analogues, were found to be great i.e. above 500 for both the models so it can be proposed that 3-substituted-1,2,

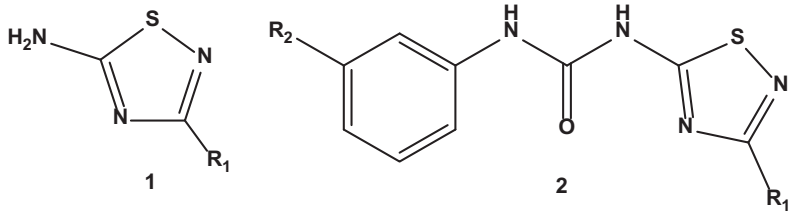


Fig. 1. 3-Substituted-1,2,4-thiadiazol-5-amine and substituted phenyl-1,2,4-thiadiazolyl urea scaffolds.

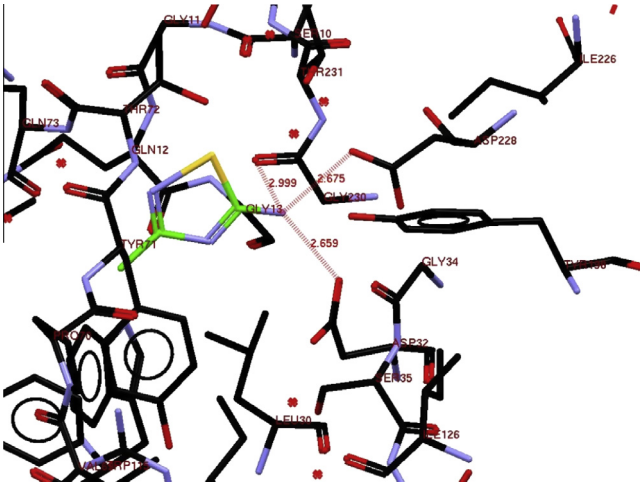


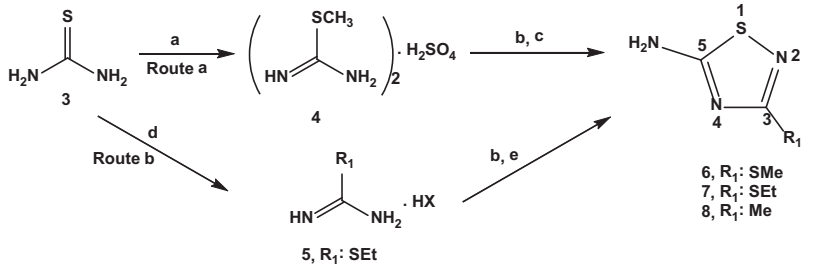
Fig. 2. Docking pose of 3-methyl-1,2,4-thiadiazol-5-amine (**8**) in BACE1 active site.

4-thiadiazol-5-amines would be absorbed to reach the target to elicit a response.

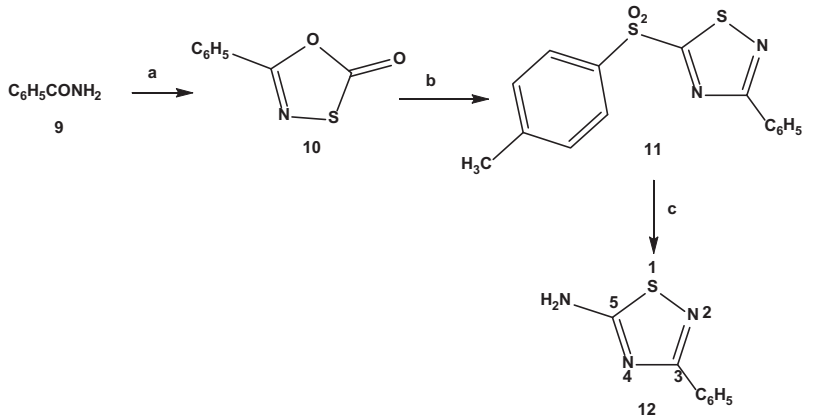
Results of *in silico* ADME studies for series of substituted phenyl-1,2,4-thiadiazolyl urea analogues were found to be in the expected range (Table 2). They possessed desirable pharmacokinetic properties such as low molecular weight (MW < 350 g/mol), hydrogen bond donor <3, hydrogen bond acceptor <7. Comparison of QPlogP o/w values for these series indicated that in **series III** lowest value of 0.6 was obtained while **series IV** listed highest value of 3.3 and the other two series exhibited moderate values in the range of 1.2–2.8. From these findings it can be proposed that **series IV** analogues were the most hydrophobic and **series III** the least. The QPlog BB values for all of them were found to be in the range of −1.5 to −0.15 (Expected range: −3 to 1.2). None of them violated the Lipinski's rule of five. The QPP MDCK and QPP Caco model values were moderate to great so it can be proposed that these analogues would be able to cross the barriers successfully.

2.4. Pharmacological screening

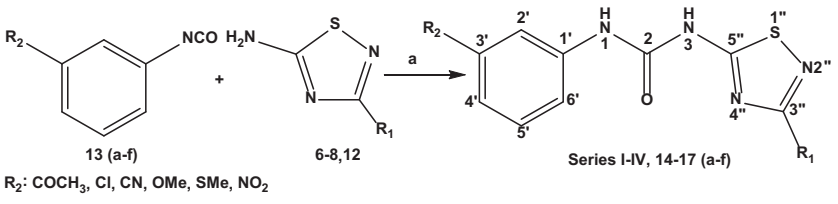
The outcome of *in vitro* BACE1 FRET (Fluorescence resonance energy transfer) inhibition study was in consensus with the molecular docking and computational results. All the 3-substituted-1,2,4-thiadiazol-5-amines, which were thought to be promising scaffolds showed moderate-to-good BACE1 inhibitor potency at



Scheme 1. Reagents: (a) $(CH_3)_2SO_4$, H_2O; (b) NaSCN, MeOH; (c) TEA, Br_2; (d) EtBr, EtOH; (e) NaOMe, Br_2.

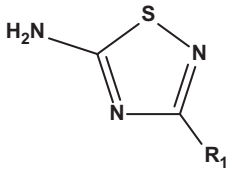


Scheme 2. Reagents: (a) ClCOSCl, toluene; (b) TsCN, decaline, 160 °C; (c) NH_3, EtOH, 60 °C.



Scheme 3. Reagents: (a) acetone, 40 °C.

Table 1
SAR of 3-substituted-1,2,4-thiadiazol-5-amines



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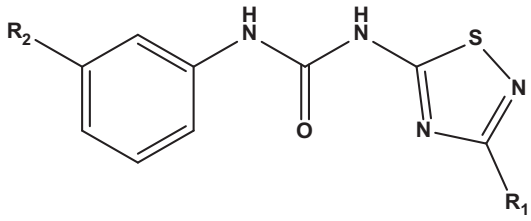
Compd	R_1	MW[a]	QPlog Po/w	HBD	HBA	QPlog BB	QPP MDCK	QPP Caco	% Inhibition ± SD at 50 μM[b]	IC_{50} (μM)[b]
6	SMe	147	0.62	2	3	−0.13	1808	989	24.35 ± 10.57	51.84
7	SEt	161	0.87	2	3	−0.25	1621	994	66.23 ± 4.29	22.21
8	Me	115	−0.13	2	3	−0.24	802	802	69.47 ± 5.29	5.96
12	Ph	177	1.82	2	3	−0.17	1158	1128	47.14 ± 8.98	58.24

[a] gm/mol.
[b] Values are the mean values of at least two experiments.

Table 2
SAR of 1-(3′-substitutedphenyl)-3-(3″-(substituted)-1″,2″,4″-thiadiazol-5″-yl)urea



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Compd	R_1	R_2	MW[a]	Qplog Po/w	HBD	HBA	QPlog BB	QPP MDCK	QPP Caco
14a	SMe	COMe	308	1.40	2	7	−0.96	635	253
14b	SMe	Cl	301	2.35	2	5	−0.15	5402	795
14c	SMe	CN	291	1.16	2	6	−1.14	400	165
14d	SMe	OMe	296	2.06	2	5	−0.39	2189	795
14e	SMe	SMe	312	2.48	2	5	−0.32	3729	795
14f	SMe	NO_2	311	1.19	2	6	−1.37	222	95
15a	SEt	COMe	322	1.74	2	7	−1.09	569	254
15b	SEt	Cl	315	2.70	2	5	−0.27	4839	798
15c	SEt	CN	305	1.50	2	6	−1.28	359	166
15d	SEt	OMe	310	2.36	2	5	−0.51	1961	798
15e	SEt	SMe	326	2.83	2	5	−0.43	3341	798
15f	SEt	NO_2	325	1.53	2	6	−1.52	198	96
16a	Me	COMe	276	0.80	2	7	−1.06	282	205
16b	Me	Cl	269	1.74	2	5	−0.27	2400	645
16c	Me	CN	259	0.58	2	6	−1.24	178	134
16d	Me	OMe	264	1.37	2	5	−0.51	973	645
16e	Me	SMe	280	1.87	2	5	−0.44	1658	645
16f	Me	NO_2	279	0.61	2	6	−1.47	98	77
17a	Ph	COMe	338	2.12	2	7	−0.99	403	307
17b	Ph	Cl	331	3.13	2	5	−0.18	3424	966
17c	Ph	CN	321	1.83	2	6	−1.18	254	201
17d	Ph	OMe	326	2.75	2	5	−0.42	1387	966
17e	Ph	SMe	342	3.26	2	5	−0.34	2364	966
17f	Ph	NO_2	341	1.96	2	6	−1.42	140	116

[a] gm/mol.

50 μM concentration. Amongst the amino thiadiazoles; analogue **8** exhibited highest IC_{50} value and values of analogue **6** and **12** were nearly equivalent (Table 1).

The *in silico* ADME observations could be correlated with their chemical structures and activity profile. It has been put forth in literature that for a hypothetical therapeutic use, compounds with QPlogP values lower than 5 should have improved drug ability properties compared to more hydrophobic analogues [29]. Thus, amongst the four series, maximum number of **series III** analogues showed BACE1 inhibition and **series IV** had least number of active analogues at 50 μM concentration.

2.5. Correlating docking and in vitro screening results of substituted phenyl-1,2,4-thiadiazolyl urea analogues

Interesting observations were seen in case of **series I** (**14a–f**), the only active analogue **14e** occupied the S_1 and S_2 sub sites while the other analogues resided in S_2 and S_3 sub sites. **14e** exhibited bidentate interactions through water molecule with Gln73 and H-bonds with other residues. The other analogues, **14c, 14d** and **14f** interacted mainly with Thr231, Thr232 and Arg235 residues of BACE1 active site. Probably interaction with basic amino acid resulted in altered activity response. Compound **16a** of **series III**

Table 3
BACE1 inhibition by analogues of series I–IV.

Compd no.	% Inhibition ± SD at 50 μM[a]
14e	41.50 ± 11.39
15c	47.00 ± 3.49
15f	29.38 ± 4.20
16c	37.49 ± 1.88
16d	19.17 ± 5.83
16e	18.16 ± 10.02
16f	17.77 ± 0.12
17e	22.09 ± 4.20

[a] Values are the mean values of at least two experiments.

also interacted with Arg235, exhibiting no inhibition of BACE1 at 50 μM concentration. **Series III** analogues, **16c** and **16f** depicted tridentate interaction with Asp32 through both nitrogen's of the urea linkage similarly; **16e** was associated in bidentate way through water molecule with Gln73. As the number of interactions had increased so their probability of BACE1 inhibition should also increase. This thought proved to be right as they all inhibited BACE1 and amongst them **16c** showed highest BACE1 inhibition.

Interpretation of docking results of **series II** was a challenging task as methylthio substituted phenyl-1,2,4-thiadiazolyl urea analogues in other series exhibited close association with the active site residues; which was not observed with compound **15e**. The cyano substituted ligand **15c** formed three H-bonds with the catalytic aspartate with good BACE1 inhibition value and **15f** exhibited tridentate interaction either direct or through water molecule with Thr231 with moderate BACE1 inhibition. In case of **series IV** analogues, only **17e** inhibited BACE1 moderately at 50 μM concentration and two nitrogen's of the urea linkage were associated with 3 H-bonds to Asp32 and one with Gly230 (Table 3).

3. Conclusion

Taken together, these studies revealed that 1,2,4-thiadiazole scaffolds appeared to be more promising than their respective urea analogues, prompting us to revisit further modifications of **7** and **8.** Analogue **8** with IC_{50} of 5.96 μM is the most potent one against BACE1. Docking studies can be employed for further designing of more powerful non-peptide BACE1 inhibitors. Encouraged by current knowledge, our effort in optimizing present hits into more potent leads will be continued based on the molecular clues from this research.

4. Experimental

4.1. Molecular docking

4.1.1. Materials

Sybyl version 8.1.1 (Tripos International, USA) running on Red Hat Enterprise Linux (RHEL) workstation was utilized for ligand preparation and its Biopolymer module for protein preparation [30]. GOLD version 4.1.1 (CCDC Ltd., UK) running on RHEL workstation was employed to carry out docking studies [31].

4.1.2. Designing of ligands

All the structures of test set ligands were sketched and subjected to energy minimization to attain gradient convergence of 0.001 kcal/mol/Å. The method employed both steepest descent and conjugate gradient minimizations with TRIPOS force field.

4.1.3. Preparation of enzyme for docking

The x-ray crystal structure of enzyme BACE1 in complex with ligand OM00-3 resolved at 2.1 Å was retrieved from protein data bank (PDB code 1m4 h). The two BACE1-inhibitor complexes in crystallographic unit were found to be essentially identical so only one monomeric unit was utilized for docking studies [32]. The inhibitor OM00-3 was extracted from the receptor along with the unwanted waters and hydrogen's to all atoms were added. Based on site points of the complexed ligand, active site of protein was defined and confirmed from the literature. The active site was defined as a sphere with a radius of 6 Å from the bound ligand. Molecular Dynamics (MD) simulation study of β-secretase complexed with the inhibitor OM99-2, suggests the protonation state of catalytic aspartate dyad in BACE1 active site to be neutral Asp32 and ionized Asp228 [33] so, net charge of −1 was set with neutral Asp32 and ionized Asp228. Only key structural water molecules were retained in the active site. The refined protein structure was subjected to minimization. The side chains were minimized using AMBER7FF99 force field to a gradient of 0.01 kcal/mol/Å.

4.1.4. Docking protocol

The default parameters in GOLD 4.1.1 were utilized for docking studies. Docking was carried out for 20 genetic algorithm (GA) runs. The docking protocol was validated by reproduction of the binding pose of OM00-3 in 1m4 h. The docked poses were ranked and scored using GoldScore. Interactions such as hydrogen bonds between ligands and the residues in the active site of BACE1 were analyzed and studied.

4.2. Synthesis

4.2.1. Materials

All chemicals and solvents used were obtained from commercial sources. The solvents were purified by established methods like distillation etc. Reactions were routinely monitored by thin layer chromatography on Merck silica gel F_{254} plates. The title compounds were purified using recrystallization and/or column chromatographic technique. Physical constants were determined in open capillary tubes in Thieles apparatus. Infrared (IR) spectra were recorded with KBr powder DRS-8000 (Diffuse Reflectance attachment) on Shimadzu IR Affinity-1 FTIR spectrophotometer. ^{1}HNMR spectra in DMSO-d6 (Dimethyl sulfoxide) solvent were recorded on Mercury Plus 300 MHz (Varian, USA) NMR spectrometer. Chemical shifts have been reported in parts per million (ppm) using tetramethylsilane (TMS) as an internal standard. Mass spectra were obtained on 410 Prostar Binary LC with 500 MS IT PDA detectors (Varian Inc., USA) mass spectrometer. Elemental analysis was performed on FLAS EA 1112 series (Thermo finnigan, Italy) CHNS (O) analyzer.

4.2.2. 3-Substituted-1,2,4-thiadiazol-5-amines (***6–8,12***)

The 3-substituted-1,2,4-thiadiazol-5-amines were synthesized as per Schemes 1 and 2.

*4.2.2.1. Synthesis of 3-(methylthio)-1,2,4-thiadiazol-5-amine (**6**).*

*4.2.2.1.1. S-methyl isothiourea sulphate (**4**).* To aqueous solution of thiourea (100 mmol), (55 mmol) of dimethyl sulphate was added and refluxed gently. On completion of the initial vigorous reaction, the mixture was refluxed for 1 h, cooled and then absolute ethanol was added and contents were filtered with suction. The residue (**4**) was washed twice with absolute ethanol and dried in air [34] (90.36%, 236 °C with decomposition).

*4.2.2.1.2. 3-(methylthio)-1,2,4-thiadiazol-5-amine (**6**).* Solution of sodium thiocyanate (65 mmol) in anhydrous methanol was cooled to 0 °C and to it s-methyl isothiourea sulphate (**4,** 25 mmol) was added with stirring. Subsequently, it was cooled to −20 °C and triethylamine (55 mmol) was added to the reaction mixture in 30 min. Further, triethylamine (50 mmol) and bromine (51 mmol)

were added simultaneously with stirring at such a rate so as to maintain the temperature below −10 °C for 1.5 h. The reaction mixture was stirred at room temperature for 2 h and poured in 500 ml of cold water and stirred for 30 min. The solid residue was filtered by using vacuum, washed with cold water and air dried. The crude product was recrystallized from water and cream coloured crystals were obtained [34,35] (62.40%, 142 °C). IR (KBr) υ: 3323, 3107 (N-H), 2204 (NCS ring), 1531 (C=N ring), 2926 (C—H) cm^{-1}. ^{1}HNMR (DMSO-d6): δ 7.99 (s, 2H), 2.48 (s, 3H). MS (acetone) [M]: 147.

*4.2.2.2. Synthesis of 3-(ethylthio)-1,2,4-thiadiazol-5-amine (**7**).*

*4.2.2.2.1. S-ethyl thiourea hydrobromide (**5**).* Mixture of thiourea (10 g, 131 mmol), ethyl bromide (16.7 g, 153 mmol) and 15 ml super dry ethanol was warmed at 55–65 °C for 3 h with occasional shaking. The reflux condenser was replaced by one set for downward distillation, and the ethanol and excess ethyl bromide were removed under vacuum. During distillation, temperature of the bath was slowly raised to the boiling point. The residual oil was poured into a 500-ml beaker and allowed to crystallize. The solid (**5**) was pulverized and dried in a desiccator [36] (92.67%).

*4.2.2.2.2. 3-(ethylthio)-1,2,4-thiadiazol-5-amine (**7**).* Solution of sodium thiocyanate (5.52 g, 68 mmol) in 45 ml of anhydrous methanol was cooled to 0 °C and then s-ethyl thiourea hydrobromide (**5**, 10 g, 54 mmol) was added with stirring. After chilling to -20 °C, cold solution of sodium in anhydrous methanol (3 g in 40 ml, 130 mmol) and cold solution of bromine in anhydrous methanol (9.03 g in 25 ml, 56.51 mmol) were added simultaneously with stirring in 2 h to the reaction mixture at such a rate so as to maintain the temperature below −10 °C. The reaction mixture was stirred at room temperature for 1 h and pH was noted, which was found to be neutral. It was further filtered by suction to remove precipitated NaBr. The methanol was removed with the use of rotary evaporator and resulting solid residue was triturated, washed twice with water and air dried. The crude product was recrystallized from water and white crystals were obtained [37] (54.65%, 96–97 °C). IR (KBr) υ: 3292, 3140 (N—H), 2183 (NCS ring), 1517 (C=N ring), 2927 (C—H) cm^{-1}. ^{1}HNMR (DMSO-d6): δ 7.99 (s, 2H), 3.05 (q, 2H, *J* = 7.3 Hz), 1.29 (t, 3H, *J* = 7.3 Hz). ^{13}CNMR (DMSO-d6): δ 182.86 (C-5), 166.19 (C-3), 24.98 (3-SCH_2—), 14.93 (3-SCH_2-CH_3). MS (acetone) [M]: 161. Anal. Calcd for $C_4H_7N_3S_2$: C, 29.81; H, 4.35; N, 26.08; S, 39.74. Found: C, 29.59; H, 4.28; N, 25.86; S, 39.17.

*4.2.2.3. Synthesis of 3-methyl-1,2,4-thiadiazol-5-amine (**8**).* Solution of sodium thiocyanate (21.06 g, 260 mmol) in 60 ml of anhydrous methanol was cooled to 0 °C. To it solution of acetamidine hydrochloride in anhydrous methanol (9.46 g in 40 ml, 100 mmol) was added with stirring. The reaction mixture was chilled to −20 °C and cold solution of sodium in anhydrous methanol (9.7 g in 140 ml, 422 mmol) and cold solution of bromine in anhydrous methanol (32 g in 60 ml, 200 mmol) were added as per the method of Section 4.2.2.2. The crude product was recrystallized using mixture of MeOH, benzene and n-hexane (0.5:1:2) and yellow crystals were obtained [37] (58.04%, 200 °C). IR (KBr) υ: 3273, 3088 (N—H), 2254 (NCS ring), 1544 (C=N ring), 2943 (C—H) cm^{-1}. ^{1}HNMR (DMSO-d6): δ 7.77 (s, 2H), 2.21 (s, 3H). MS (acetone) [M]: 115.

*4.2.2.4. Synthesis of 3-phenyl-1,2,4-thiadiazol-5-amine (**12**).*

*4.2.2.4.1. 5-phenyl-1,3,4-oxthiazol-2-one (**10**).* Mixture of benzamide (**9**, 1.6 g, 13.22 mmol), 20 ml toluene and carbonyl chlorosulphenyl chloride (1.73 g, 13.22 mmol) was carefully heated from 60 to 90 °C in round bottom flask (rbf) equipped with stirrer, reflux condenser and thermometer, till the evolution of hydrogen chloride sets in. Towards the end of reaction i.e. after about 2 h, the reaction mixture was heated to 100–110 °C and stirred for one more hour at this temperature. The reaction mixture was then concentrated under vacuum to obtain solid residue [38,39]. It was purified by column chromatography (silica, n-Hexane: Ethyl acetate, 2:1) to get pale yellow coloured compound **10** (53.16%, 68 °C). IR (KBr) υ: 1743 (C=O, γ-lactone), 1602 (C=N ring), 3064 (C—H) cm^{-1}. ^{1}HNMR (DMSO-d6): δ 7.97 (dd, 2H, *J* = 6.2, 2.5 Hz), 7.58 (td, 2H, *J* = 8.4, 3.1 Hz), 7.51 (t, 1H, *J* = 3.7 Hz).

*4.2.2.4.2. 3-phenyl-5-(1-(4-methylphenyl)sulfonyl)-1,2,4-thiadiazole (3-phenyl-5-tosyl-1,2,4-thiadiazole, **11**).* Solution of Tosyl cyanide (0.3 g, 1.68 mmol) in decaline was heated to 155–160 °C. To the resulting solution, 5-phenyl-1,3,4-oxthiazol-2-one (**10**, 0.2 g, 1.12 mmol) was added in portions over 1 h. The mixture was heated for an additional hour, then cooled to get residue [40]. It was purified through column (silica, ether: n-hexane, 1:10) (71.43%, 130 °C).

*4.2.2.4.3. 3-phenyl-1,2,4-thiadiazol-5-amine (**12**).* To the solution of 3-phenyl-5-tosyl-1,2,4-thiadiazole (**11,** 0.1 g, 0.32 mmol) in 5 ml ethanol, 50 ml of 25% ammonia solution was added and then refluxed at 60 °C. When the yellow colour of the reaction mixture dissipated then the solution was cooled and again 50 ml of 25% ammonia solution was added and refluxed at 60 °C for 16 h. The cooled reaction mixture was evaporated and the residue was purified by column chromatography (silica, n-hexane: ethyl acetate, 3:2) to get white coloured product **12** [40] (89.29%, 158–159 °C). IR (KBr) υ: 3288, 3116 (N—H), 2243 (NCS ring), 1537 (C=N ring), 3041 (C—H) cm^{-1}. ^{1}HNMR (DMSO-d6): δ 8.02 (s, 2H), 8.05 (dd, 2H, *J* = 6.6, 3.3 Hz), 7.43 (td, 2H, *J* = 8.8, 2.9 Hz), 7.46 (t, 1H, *J* = 2.9 Hz). MS (acetone) [M]: 177.

*4.2.3. Synthesis of 1-(3′-substitutedphenyl)-3-(3″-(substituted)-1″,2″,4″-thiadiazol-5″-yl)urea (**Series I–IV**) (**14–17** (**a–f**))*

Scheme 3 was followed for the synthesis of 1-(3′-substitutedphenyl)-3-(3″-(substituted)-1″,2″,4″-thiadiazol-5″-yl) urea analogues.

*4.2.3.1. General method for analogues of Series I–IV (**14–17** (**a–f**)).* To the solution of 0.2 g of respective 3-substituted-1,2,4-thiadiazol-5-amine (**6–8, 12**) in 2 ml dry acetone, equimolar solution of respective 3-substitutedphenyl isocyanate (**13** (**a–f**)) in 2 ml dry acetone was added gradually at 40 °C in 15–20 min with stirring. The reaction mixture was refluxed at 40 °C for 16 h with addition of minimum amount of dry acetone if needed. After the reflux, the reaction mixture was poured in 25–50 ml water, which resulted in formation of precipitate. The precipitate was filtered with suction, either recrystallized from ethanol or purified by column chromatography (n-hexane: ethyl acetate, benzene: acetone, chloroform: methanol) to obtain the analogues (**14–17** (**a–f**)) [25].

*4.2.3.2. 1-(3′-acetylphenyl)–3-(3″-(methylthio)-1″,2″,4″-thiadiazol-5″-yl)urea (**14a**).* Yield: 69%; 225 °C. IR (KBr) υ: 1708 (urea), 686 (C—S), 2949 (C—H) 1683 ($COCH_3$) cm^{-1}. ^{1}HNMR (DMSO-d6): δ 11.54 (s, 1H), 9.48 (s, 1H), 8.10 (t, 1H, *J* = 1.8 Hz), 7.74 (t, 1H, *J* = 1.1 Hz), 7.70 (dd, 1H, *J* = 7.7, 1.5 Hz), 7.50 (t, 1H, *J* = 7.9 Hz), 2.58 (s, 3H), 2.57 (s, 3H).

*4.2.3.3. 1-(3′-chlorophenyl)–3-(3″-(methylthio)-1″,2″,4″-thiadiazol-5″-yl)urea (**14b**).* Yield: 57%; 228 °C. IR (KBr) υ: 1714 (urea), 688 (C—S), 2926 (C—H) cm^{-1}. ^{1}HNMR (DMSO-d6): δ 11.55 (s, 1H), 9.45 (s, 1H), 7.69 (dd, 1H, *J* = 6.2, 1.5 Hz), 7.38 (d, 1H, *J* = 1.1 Hz), 7.37 (t, 1H, *J* = 7.1 Hz), 7.15 (td, 1H, *J* = 6.2, 2.1 Hz), 2.58 (s, 3H).

*4.2.3.4. 1-(3′-cyanophenyl)–3-(3″-(methylthio)-1″,2″,4″-thiadiazol-5″-yl)urea (**14c**).* Yield: 38%; 206 °C. IR (KBr) υ: 1714 (urea), 682 (C—S), 2924 (C—H), 2235 (CN) cm^{-1}. ^{1}HNMR (DMSO-d6): δ 11.65 (s, 1H), 9.19 (s, 1H), 7.97 (t, 1H, *J* = 1.6 Hz), 7.69 (td, 1H, *J* = 8.1, 1.6 Hz), 7.51 (t, 1H, *J* = 7.9 Hz), 7.45 (td, 1H, *J* = 7.7, 1.5 Hz), 2.58 (s, 3H).

*4.2.3.5. 1-(3′-methoxyphenyl)–3-(3″-(methylthio)-1″,2″,4″-thiadiazol-5″-yl)urea (**14d**).* Yield: 75%; 184 °C. IR (KBr) υ: 1703 (urea), 690 (C—S), 2926 (C—H), 2835 (OCH_3) cm^{-1}. ^{1}HNMR (DMSO-d6): δ 11.35 (s, 1H), 9.24 (s, 1H), 7.24 (t, 1H, *J* = 8.3 Hz), 7.16 (s, 1H), 7.01 (d, 1H, *J* = 7.7 Hz), 6.67 (dd, 1H, *J* = 8.2, 2.2 Hz), 3.75 (s, 3H), 2.57 (s, 3H).

*4.2.3.6. 1-(3′-methylthiophenyl)–3-(3″-(methylthio)-1″,2″,4″-thiadiazol-5″-yl)urea (**14e**).* Yield: 67%; 186 °C. IR (KBr) υ: 1705 (urea), 680 (C—S), 2924 (C—H), 688 (SCH_3) cm^{-1}. ^{1}HNMR (DMSO-d6): δ 11.44 (s, 1H), 9.28 (s, 1H), 7.45 (d, 1H, *J* = 1.5 Hz), 7.26 (t, 1H, *J* = 7.3 Hz), 7.14 (t, 1H, *J* = 7.0 Hz), 6.86 (dd, 1H, *J* = 7.5, 1.3 Hz), 2.57 (s, 3H), 2.45 (s, 3H). ^{13}CNMR (DMSO-d6): δ 176.43 (C-2), 166.14 (C-3′), 152.42 (C-1′), 140.14 (C-3″), 138.62 (C-5″), 129.29 (C-5′), 119.28 (C-6′), 115.27 (C-4′), 114.89 (C-2′), 14.63 (3″-SCH_3), 13.88 (3′-SCH_3). MS (acetone) [M + 1]: 313. Anal. Calcd for $C_{11}H_{12}N_4S_3O$: C, 42.31; H, 3.85; N, 17.95; S, 30.78. Found: C, 42.10; H, 3.45; N, 17.98; S, 31.19.

*4.2.3.7. 1-(3′-nitrophenyl)–3-(3″-(methylthio)-1″,2″,4″-thiadiazol-5″-yl)urea (**14f**).* Yield: 69%; 262 °C. IR (KBr) υ: 1714 (urea), 682 (C—S), 2937 (C—H), 1529, 1352 (NO_2) cm^{-1}. ^{1}HNMR (DMSO-d6): δ 11.76 (s, 1H), 9.81 (s, 1H), 8.54 (t, 1H, *J* = 2.2 Hz), 7.94 (dd, 1H, *J* = 8.1, 2.2 Hz), 7.85 (dd, 1H, *J* = 8.2, 2.0 Hz), 7.63 (t, 1H, *J* = 8.1 Hz), 2.58 (s, 3H).

*4.2.3.8. 1-(3′-acetylphenyl)–3-(3″-(ethylthio)-1″,2″,4″-thiadiazol-5″-yl)urea (**15a**).* Yield: 45%; 184–86 °C. IR (KBr) υ: 1710 (urea), 688 (C—S), 2927 (C—H) 1680 ($COCH_3$) cm^{-1}. ^{1}HNMR (DMSO-d6): δ 11.55 (s, 1H), 9.47 (s, 1H), 8.08 (t, 1H, *J* = 1.8 Hz), 7.68 (dd, 1H, *J* = 7.0, 2.2 Hz), 7.60 (td, 1H, *J* = 6.6, 1.1 Hz), 7.44 (t, 1H, *J* = 7.9 Hz), 3.15 (q, 2H, *J* = 7.3 Hz), 2.57 (s, 3H), 1.34 (t, 3H, *J* = 7.3 Hz).

*4.2.3.9. 1-(3′-chlorophenyl)–3-(3″-(ethylthio)-1″,2″,4″-thiadiazol-5″-yl)urea (**15b**).* Yield: 41%; 210–12 °C. IR (KBr) υ: 1701 (urea), 688 (C—S), 2924 (C—H) cm^{-1}. ^{1}HNMR (DMSO-d6): δ 11.56 (s, 1H), 9.44 (s, 1H), 7.70 (t, 1H, *J* = 1.6 Hz), 7.36 (td, 1H, *J* = 8.1, 2.1 Hz), 7.29 (t, 1H, *J* = 7.7 Hz), 7.03 (td, 1H, *J* = 7.0, 2.3 Hz), 3.159 (q, 2H, *J* = 7.1 Hz), 1.34 (t, 3H, *J* = 7.3 Hz).

*4.2.3.10. 1-(3′-cyanophenyl)–3-(3″-(ethylthio)-1″,2″,4″-thiadiazol-5″-yl)urea (**15c**).* Yield: 55%; 194 °C. IR (KBr) υ: 1697 (urea), 680 (C—S), 2924 (C—H), 2233 (CN) cm^{-1}. ^{1}HNMR (DMSO-d6): δ 11.68 (s, 1H), 9.58 (s, 1H), 7.97 (d, 1H, *J* = 1.5 Hz), 7.77 (td, 1H, *J* = 5.9, 1.7 Hz), 7.55 (t, 1H, *J* = 6.2 Hz), 7.54 (d, 1H, *J* = 6.1 Hz), 3.15 (q, 2H, *J* = 7.3 Hz), 1.34 (t, 3H, *J* = 7.3 Hz). MS (acetone) [M + 1]: 306.

*4.2.3.11. 1-(3′-methoxyphenyl)–3-(3″-(ethylthio)-1″,2″,4″-thiadiazol-5″-yl)urea (**15d**).* Yield: 85%; 158 °C. IR (KBr) υ: 1697 (urea), 692 (C—S), 2929 (C—H), 2835 (OCH_3) cm^{-1}. ^{1}HNMR (DMSO-d6): δ 11.36 (s, 1H), 9.23 (s, 1H), 7.24 (t, 1H, *J* = 8.1 Hz), 7.16 (t, 1H, *J* = 2.0 Hz), 6.92 (dd, 1H, *J* = 8.1, 1.1 Hz), 6.55 (dd, 1H, *J* = 8.1, 1.8 Hz), 3.73 (s, 3H), 3.15 (q, 2H, *J* = 7.3 Hz), 1.34 (t, 3H, *J* = 7.3 Hz).

*4.2.3.12. 1-(3′-methythiophenyl)–3-(3″-(ethylthio)-1″,2″,4″-thiadiazol-5″-yl)urea (**15e**).* Yield: 42%; 173 °C. IR (KBr) υ: 1701 (urea), 686 (C—S), 2920 (C—H), 632 (SCH_3) cm^{-1}. ^{1}HNMR (DMSO-d6): δ 11.44 (s, 1H), 9.26 (s, 1H), 7.45 (t, 1H, *J* = 1.8 Hz), 7.21 (t, 1H, *J* = 7.9 Hz), 7.14 (td, 1H, *J* = 8.1, 1.5 Hz), 6.86 (td, 1H, *J* = 8.2, 1.5 Hz), 3.15 (q, 2H, *J* = 7.3 Hz), 2.46 (s, 3H), 1.34 (t, 3H, *J* = 7.3 Hz).

*4.2.3.13. 1-(3′-nitrophenyl)–3-(3″-(ethylthio)-1″,2″,4″-thiadiazol-5″-yl)urea (**15f**).* Yield: 44%; 262 °C. IR (KBr) υ: 1687 (urea), 696 (C—S), 2926 (C—H), 1523, 1350 (NO_2) cm^{-1}. ^{1}HNMR (DMSO-d6): δ 11.77 (s, 1H), 9.79 (s, 1H), 8.56 (t, 1H, *J* = 2.2 Hz), 7.86 (dd, 1H, *J* = 6.1, 2.3 Hz), 7.77 (dd, 1H, *J* = 5.7, 1.3 Hz), 7.59 (t, 1H, *J* = 8.2 Hz), 3.16 (q, 2H, *J* = 7.3 Hz), 1.35 (t, 3H, *J* = 7.1 Hz). MS (acetone) [M + 1]: 326.

*4.2.3.14. 1-(3′-acetylphenyl)–3-(3″-(methyl)-1″,2″,4″-thiadiazol-5″-yl)urea (**16a**).* Yield: 56%; 246 °C. IR (KBr) υ: 1716 (urea), 2929 (C—H) 1660 ($COCH_3$) cm^{-1}. ^{1}HNMR (DMSO-d6): δ 11.33 (s, 1H), 9.44 (s, 1H), 8.10 (t, 1H, *J* = 1.8 Hz), 7.73 (dd, 1H, *J* = 5.9, 2.2 Hz), 7.69 (td, 1H, *J* = 7.7, 1.3 Hz), 7.49 (t, 1H, *J* = 7.9 Hz), 2.58 (s, 3H), 2.41 (s, 3H).

*4.2.3.15. 1-(3′-chlorophenyl)–3-(3″-(methyl)-1″,2″,4″-thiadiazol-5″-yl)urea (**16b**).* Yield: 62%; 236 °C. IR (KBr) υ: 1712 (urea), 2902 (C—H) cm^{-1}. ^{1}HNMR (DMSO-d6): δ 11.36 (s, 1H), 9.42 (s, 1H), 7.69 (d, 1H, *J* = 2.2 Hz), 7.39 (d, 1H, *J* = 1.8 Hz), 7.36 (t, 1H, *J* = 6.1 Hz), 7.12 (td, 1H, *J* = 6.6, 2.2 Hz), 2.40 (s, 3H).

*4.2.3.16. 1-(3′-cyanophenyl)–3-(3″-(methyl)-1″,2″,4″-thiadiazol-5″-yl)urea (**16c**).* Yield: 55%; 217–19 °C. IR (KBr) υ: 1710 (urea), 2926 (C—H), 2231 (CN) cm^{-1}. ^{1}HNMR (DMSO-d6): δ 11.49 (s, 1H), 9.57 (s, 1H), 7.97 (t, 1H, *J* = 1.6 Hz), 7.69 (td, 1H, *J* = 8.4, 2.0 Hz), 7.51 (t, 1H, *J* = 7.9 Hz), 7.45 (td, 1H, *J* = 8.2, 1.4 Hz), 2.41 (s, 3H). ^{13}CNMR (DMSO-d6): δ 176.03 (C-2), 152.33 (C-1′), 140.25 (C-5″), 139.14 (C-3″), 130.17 (C-3′), 125.67 (C-5′), 123.14 (C-4′), 121.08 (C-6′), 118.81 (C-2′), 111.64 (3′-CN), 18.54 (3″-CH_3). MS (acetone) [M + 1]: 260. Anal. Calcd for $C_{11}H_9N_5SO$: C, 50.97; H, 3.45; N, 27.03; S, 12.36. Found: C, 50.23; H, 3.44; N, 27.84; S, 12.92.

*4.2.3.17. 1-(3′-methoxyphenyl)–3-(3″-(methyl)-1″,2″,4″-thiadiazol-5″-yl)urea (**16d**).* Yield: 72%; 266 °C. IR (KBr) υ: 1708 (urea), 2927 (C—H), 2831 (OCH_3) cm^{-1}. ^{1}HNMR (DMSO-d6): δ 11.16 (s, 1H), 9.20 (s, 1H), 7.24 (t, 1H, *J* = 8.2 Hz), 7.17 (t, 1H, *J* = 2.2 Hz), 7.01 (dd, 1H, *J* = 8.1, 1.1 Hz), 6.66 (dd, 1H, *J* = 8.1, 2.2 Hz), 3.75 (s, 3H), 2.40 (s, 3H). MS (acetone) [M + 1]: 265.

*4.2.3.18. 1-(3′-methylthiophenyl)–3-(3″-(methyl)-1″,2″,4″-thiadiazol-5″-yl)urea (**16e**).* Yield: 86%; 148 °C. IR (KBr) υ: 1703 (urea), 2922 (C—H), 686 (SCH_3) cm^{-1}. ^{1}HNMR (DMSO-d6): δ 11.24 (s, 1H), 9.24 (s, 1H), 7.45 (s, 1H), 7.27 (t, 1H, *J* = 8.6 Hz), 7.23 (dd, 1H, *J* = 8.1, 1.8 Hz), 6.96 (td, 1H, *J* = 7.0, 1.6 Hz), 2.47 (s, 3H), 2.40 (s, 3H). MS (acetone) [M + 1]: 281.

*4.2.3.19. 1-(3′-nitrophenyl)–3-(3″-(methyl)-1″,2″,4″-thiadiazol-5″-yl)urea (**16f**).* Yield: 83%; 230 °C. IR (KBr) υ: 1716 (urea), 2924 (C—H), 1531, 1352 (NO_2) cm^{-1}. ^{1}HNMR (DMSO-d6): δ 11.62 (s, 1H), 9.79 (s, 1H), 8.56 (t, 1H, *J* = 2.2 Hz), 7.92 (dd, 1H, *J* = 8.2, 1.3 Hz), 7.85 (dd, 1H, *J* = 8.1, 1.1 Hz), 7.62 (t, 1H, *J* = 8.2 Hz), 2.42 (s, 3H). MS (acetone) [M + 1]: 280.

*4.2.3.20. 1-(3′-acetylphenyl)–3-(3″-(phenyl)-1″,2″,4″-thiadiazol-5″-yl)urea (**17a**).* Yield: 71%; 255 °C. IR (KBr) υ: 1712 (urea), 3020 (C—H) 1662 ($COCH_3$) cm^{-1}. ^{1}HNMR (DMSO-d6): δ 11.69 (s, 1H), 9.44 (s, 1H), 8.17 (dd, 2H, *J* = 4.6, 2.7 Hz), 8.12 (t, 1H, *J* = 1.8 Hz), 7.76 (dd, 1H, *J* = 5.9, 1.1 Hz), 7.75 (d, 1H, *J* = 7.0 Hz), 7.71 (t, 1H, *J* = 1.1 Hz), 7.53 (dd, 2H, *J* = 6.2, 1.8 Hz), 7.49 (t, 1H, *J* = 1.8 Hz), 2.59 (s, 3H).

*4.2.3.21. 1-(3′-chlorophenyl)–3-(3″-(phenyl)-1″,2″,4″-thiadiazol-5″-yl)urea (**17b**).* Yield: 54%; 216-18 °C. IR (KBr) υ: 1712 (urea), 3061 (C—H) cm^{-1}. ^{1}HNMR (DMSO-d6): δ 11.68 (s, 1H), 9.40 (s, 1H), 8.16 (dd, 2H, *J* = 6.6, 2.4 Hz), 7.51 (dd, 2H, *J* = 5.4, 2.0 Hz), 7.39 (t, 1H, *J* = 1.7 Hz), 7.34 (t, 1H, *J* = 8.0 Hz), 7.25 (t, 1H, *J* = 2.0 Hz), 7.15 (td, 1H, *J* = 6.8, 2.0 Hz), 7.03 (td, 1H, *J* = 7.1, 2.1 Hz).

*4.2.3.22. 1-(3′-cyanophenyl)–3-(3″-(phenyl)-1″,2″,4″-thiadiazol-5″-yl)urea (**17c**).* Yield: 75%; 254 °C. IR (KBr) υ: 1712 (urea), 3068 (C—H), 2231 (CN) cm^{-1}. ^{1}HNMR (DMSO-d6): δ 11.80 (s, 1H), 9.54

(s, 1H), 8.16 (dd, 2H, *J* = 4.4, 2.4 Hz), 8.00 (t, 1H, *J* = 1.2 Hz), 7.82 (t, 1H, *J* = 5.1 Hz), 7.79 (t, 1H, *J* = 2.7 Hz), 7.56 (t, 1H, *J* = 6.0 Hz), 7.54 (dd, 1H, *J* = 4.6, 1.2 Hz), 7.51 (dd, 2H, *J* = 5.4, 2.0 Hz).

*4.2.3.23. 1-(3′-methoxyphenyl)–3-(3″-(phenyl)-1″,2″,4″-thiadiazol-5″-yl)urea (**17d**).* Yield: 60%; 178–80 °C. IR (KBr) υ: 1710 (urea), 3062 (C—H), 2835 (OCH_3) cm^{-1}. ^{1}HNMR (DMSO-d6): δ 11.49 (s, 1H), 9.19 (s, 1H), 8.16 (dd, 2H, *J* = 7.7, 2.9 Hz), 7.26 (t, 1H, *J* = 8.1 Hz), 7.18 (t, 1H, *J* = 2.6 Hz), 7.15 (t, 1H, *J* = 5.1 Hz), 7.04 (d, 2H, *J* = 8.1 Hz), 6.68 (dd, 1H, *J* = 8.2, 2.4 Hz), 6.55 (dd, 1H, *J* = 8.1, 2.2 Hz), 3.73 (s, 3H).

*4.2.3.24. 1-(3′-methylthiophenyl)–3-(3″-(phenyl)-1″,2″,4″-thiadiazol-5″-yl)urea (**17e**).* Yield: 64%; 278 °C. IR (KBr) υ: 1712 (urea), 3066 (C—H), 686 (SCH_3) cm^{-1}. ^{1}HNMR (DMSO-d6): δ 11.58 (s, 1H), 9.23 (s, 1H), 8.16 (dd, 2H, *J* = 4.8, 1.7 Hz), 7.47 (td, 2H, *J* = 8.4, 1.8 Hz), 7.30 (t, 1H, *J* = 7.7 Hz), 7.26 (t, 1H, *J* = 1.6 Hz), 7.22 (t, 1H, *J* = 7.7 Hz), 6.99 (td, 1H, *J* = 7.3, 1.6 Hz), 6.86 (td, 1H, *J* = 7.7, 1.5 Hz), 2.46 (s, 3H). MS (acetone) [M + 1]: 343.

*4.2.3.25. 1-(3′-nitrophenyl)–3-(3″-(phenyl)-1″,2″,4″-thiadiazol-5″-yl)urea (**17f**).* Yield: 65%; 216 °C. IR (KBr) υ: 1707 (urea), 3043 (C—H), 1525, 1348 (NO_2) cm^{-1}. ^{1}HNMR (DMSO-d6): δ 11.90 (s, 1H), 9.75 (s, 1H), 8.56 (t, 1H, *J* = 2.0 Hz), 8.05 (td, 2H, *J* = 7.3, 2.0 Hz), 7.86 (td, 1H, *J* = 8.6, 1.4 Hz), 7.77 (td, 1H, *J* = 7.9, 1.1 Hz), 7.66 (t, 1H, *J* = 8.1 Hz), 7.59 (t, 1H, *J* = 8.1 Hz), 7.52 (dd, 2H, *J* = 5.1, 1.8 Hz).

4.3. In silico ADME studies

4.3.1. Materials

In silico ADME studies were carried out with QikProp version 3.0 (Schrodinger LLC, New York, USA) software running on Red Hat Enterprise Linux (RHEL) workstation [41].

4.3.2. Method

All the structures of test set ligands were imported from Sybyl 8.1.1 and pre-processed using Ligprep application. The processed ligands were routed to QikProp for ADME studies. The descriptors and their values for various analogues were calculated as per their default programme.

4.4. Pharmacological screening

4.4.1. Materials

BACE1 FRET (Fluorescence resonance energy transfer) inhibition study, using the M-2420 method, was performed by Department of Pharmaceutical Sciences, University of Bologna, Italy [7,42]. The BACE1 assay for some active analogues was carried out using FRET assay kit purchased from Sigma-Aldrich (Catalogue No. CS0010) [43] on Fluorolog FL3-21 (Horiba Jobinyvon) spectrofluorometer. The assay kit was validated by the manufacturer.

4.4.2. Preliminary screening

BACE1 FRET inhibition study using the M-2420 method: 5 μL of test inhibitor analogue (50 μM) or DMSO was pre-incubated with 175 μL of hrBACE1 (17.2 nM, final concentration) in 20 mM sodium acetate pH 4.5 containing 0.1% w/v CHAPS (3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate) for 1 h at room temperature. The methoxycoumarin based peptide substrate (M-2420, 3 μM, final concentration) was then added and left to react for 15 min. The fluorescence signal was measured at the excitation wavelength (λ_{ex}: 320 nm) and emission wavelength (λ_{em}: 405 nm). DMSO concentration in the final mixture was maintained below 5% v/v to guarantee no significant loss of enzyme activity. All measurements were done in duplicate and the values were calculated as the mean of $n \geqslant 2$ determinations, with standard deviation up to 10%. Fluorescence intensity with and without inhibitor was registered and compared. The background signal was measured in control well containing all the reagents, except human recombinant BACE1 (hrBACE1) and subtracted.

The % inhibition due to the presence of test analogue was calculated by the following expression: 100-(IF_i/IF_ox 100), where, IF_i and IF_o are the fluorescence intensities obtained for hrBACE1 in the presence and absence of inhibitor, respectively [7].

4.4.3. In vitro BACE1 FRET assay

4.4.3.1. Standard curve for BACE1 FRET assay. Series of concentrations of assay standard solution (100 μM) i.e. 100, 200, 300 and 500 pmol were taken along with negative (blank) and positive control. To all of them 20 μl of BACE1 substrate solution (50 μM) was added. 2 μl of BACE1 enzyme solution (0.3 unit/μl) was added to the positive control and then all the solutions were diluted with fluorescent assay buffer to final volume of 100 μl. The solutions were incubated at 37 °C for 2 h and 40 μl of stop solution was added. The fluorescence signal was measured at the excitation wavelength (λ_{ex}: 320 nm) and emission wavelength (λ_{em}: 405 nm). All the measurements were performed in duplicate. The fluorescence units (FU) of blank were subtracted from all standard signal readings. The standard curve FU was plotted against pmol present in each standard (r^2 = 0.96).

*4.4.3.2. Assay of BACE1 inhibitors (for compounds **6**–**8**, **12**).* Five different concentrations of inhibitor test analogue were considered for the assay along with negative (blank) and positive control. To all of them 20 μl of BACE1 substrate solution (50 μM) was added. 2 μl of BACE1 enzyme solution (0.3 unit/μl) was added to all except negative control and then all the solutions were diluted with fluorescent assay buffer to final volume of 100 μl. The solutions were incubated at 37 °C for 2 h and 40 μl of stop solution was added. The fluorescence signal was measured at the excitation wavelength (λ_{ex}: 320 nm) and emission wavelength (λ_{em}: 405 nm). DMSO concentration in the final solutions was maintained below 4% v/v to guarantee no significant loss of enzyme activity. All the measurements were done in duplicate.

The fluorescence intensity of blank was subtracted from all fluorescence signal readings. The% inhibition due to the presence of test analogue was calculated by the following expression: $[(IF_o–IF_i)/IF_o] \times 100$, where, IF_i and IF_o are the fluorescence intensities obtained for BACE1 in the presence and in the absence of inhibitor, respectively. The linear regression parameters were determined and the IC_{50} interpolated (GraphPad Prism 4.0, GraphPad Software Inc.) [43].

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