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Research Article

Pharmacological Evaluation and Docking Studies of 3-Thiadiazolyl- and Thioxo-1,2,4-triazolylcoumarin Derivatives as Cholinesterase Inhibitors

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Inhibition of acetylcholinesterase (AChE) and butyrylcholinesterase (BChE) is considered a promising strategy for the treatment of Alzheimer's disease (AD). This research project aims to provide a comprehensive knowledge of newly synthesized coumarin analogues with anti-AD potential. In the present work a series of 3-thiadiazolyl- and thioxo-1,2,4-triazolylcoumarins derivatives were designed, synthesized, and tested as potent inhibitors of cholinesterases. These compounds were assayed against AChE from electrophorus electricus and rabbit; and BChE from horse serum and rabbit by Ellman's method using neostigmine methylsulphate and donepezil as reference drugs. Some of the assayed compounds proved to be potent inhibitors of AChE and BChE with K_i values in the micromolar range. **4b** was found to be the most active compound with K_i value $0.028 \pm 0.002\,\mu$M and higher selectivity for AChE/BChE. The ability of **4b** to interact with AChE was further confirmed through computational studies, in which a primary binding was proved to occur at the active gorge site, and a secondary binding was revealed at the peripheral anionic site. Structure activity relationships of prepared compounds were also discussed.

1. Introduction

Alzheimer's disease (AD), Parkinson's disease, and age-related memory disorders always remain in keen interest of researchers. AD is a progressive neurodegenerative disorder that is characterized by the appearance of neurofibrillary tangles, neuritic plagues within the brain of AD patients, [1] rapid loss of synapses, and degeneration of basal cholinergic neurons [2]. Loss of cholinergic neurons causes reduction in cortical and hippocampal levels of the neurotransmitter acetylcholine (ACh) that leads to impairment in cognitive functions as well [3–5]. On the behavioral side, confusion, irritability, anger, and inability to perform body functions properly are obvious symptoms [3, 6]. As general health care system is improving globally day by day and thus proportion of older people increases, the number of AD patients is estimated to increase considerably [7]. Thus, new drugs are required for the treatment of AD. Recently it was found that AChE could accelerate the deposition phenomenon of neuritic plagues [8, 9]. Various anti-AChE agents, that is, ensaculine, donepezil, propidium, rivastigmine, and tacrine (Figure 1) have shown slight improvement in cognitive and memory disorders [10]. However, these available nitrogen containing anti-AChE drugs have certain side effects and lesser CNS permeability. So, new drugs are required for the treatment of AD with better CNS penetration and decreased toxic effects.

Natural compounds have always served as a useful source for the study of AChE inhibitory activity. A number of phytoconstituents for instance, alkaloids (indole, isoquinoline, and steroidal), pregnane glycosides (cynantrosides), stilbenes, triterpenes, [11] ursane [12], and xanthones have shown AChE inhibitory potency. These and other such examples have urged researchers to explore nature for

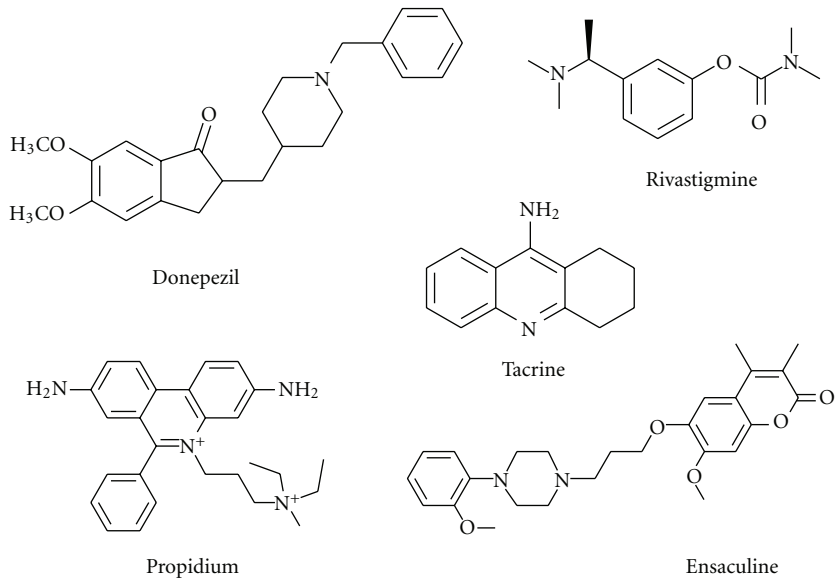
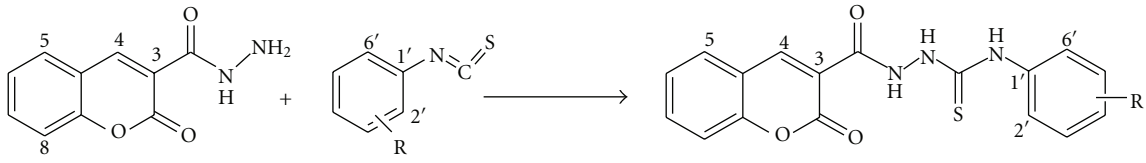


Figure 1: Chemical structures of some common FDA approved cholinesterase inhibitors.



R	2-Cl, 3-Cl, 4-Cl, 3-Me, 4-Me, 2-OMe, 3-OMe						
1	**a**	**b**	**c**	**d**	**e**	**f**	**g**

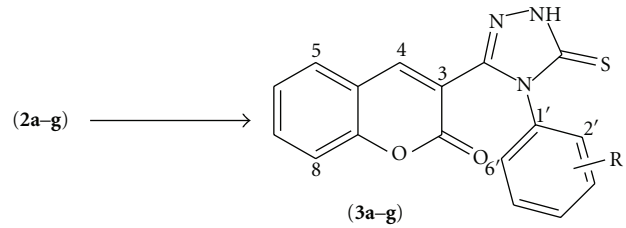
Scheme 1: Synthesis of 3-(4-Aryl-5-thioxo-4,5-dihydro-1*H*-1,2,4-triazol-3-yl)-2*H*-chromen-2-ones.

identification of active compounds against AD. Coumarins, an important class of natural compounds have been studied extensively due to their vast medicinal significance [11, 12]. They have been reported for their analgesic [13], antibacterial [14], anticancer [15], anticoagulant [16], anti-inflammatory [13], anthelmintic [17], antifungal [14], anti-hepatitis C [18], antimutagenic [19], and antituberculosis [20] activities. Coumarins have also proved as potent non-peptidic protease inhibitors [21], heat shock protein inhibitors [22], monoamine oxidase inhibitors [23], 17β-hydroxysteroid dehydrogenase (17β-HSD) type 1inhibitors [24], and TNF-α (tumor necrosis factor-alpha) inhibitors [25]. 4-methylcoumarins having different functional groups are well known for their antioxidant and radical scavenging activities [26]. Thiazole derivatives have been studied extensively due to their versatile pharmacological significance that is, they possess antitumor [27], cardiotonic [27], anti-HIV [28], analgesic and anti-inflammatory [29] activity. They are also reported as DNA-gyrase [30] and lipoxygenase enzyme inhibitors [31]. 1, 3, 4 thiadiazole nucleus is found in a number of compounds and potent due to its anti-ulcer [32], antiallergic [33] and diuretic activities [34]. The present paper focuses on synthesis of new class of coumarins which could prove as potent therapeutic moieties against progression of AD in future.

2. Results and Discussion

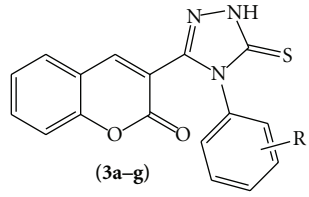
2.1. Chemistry. Coumarin-3-carbohydrazide was obtained by refluxing coumarinyl ester with hydrazine hydrate according to published procedure [32]. The thiosemicarbazides (**2a–g**) were obtained by the stirring coumarin-3-carbohydrazide and aryl isothiocyanates (**1a–g**). The IR spectra of thiosemicarbazides (**2a–g**) revealed carbonyl absorption in the range 1644–1689 cm^{-1} and that of thiocarbonyl at 1235–1268 cm^{-1}, respectively whilst the characteristic absorption bands for three secondary N-H groups appeared in the region 3143–3432 cm^{-1}. In ^{1}H NMR, the NH proton of amide type linkage appeared at δ 11.23–11.96 ppm and signal for two NH proton of thiourea type linkage at δ 9.99–11.14 ppm. The carbonyl and thiocarbonyl appeared at 164.7–167.0 and 176.2–181.7 ppm, respectively in ^{13}C NMR as well as those of the aromatic carbons (Scheme 1).

The 4,5-disubstituted-1,2,4-triazol-3-thiones (**3a–g**) were synthesized by refluxing the corresponding thiosemicarbazides (**2a–g**) in aqueous sodium hydroxide (4N)

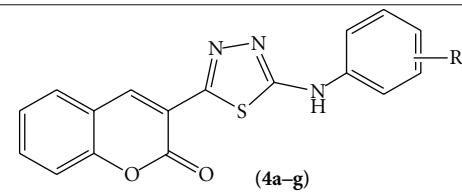


Scheme 2: Synthesis of 3-(4-Aryl-5-thioxo-4,5-dihydro-1*H*-1,2,4-triazol-3-yl)-2*H*-chromen-2-ones.

Table 1: AChE and BuChE inhibitory activities of new coumarin derivatives[a].



(3a–g)



(4a–g)

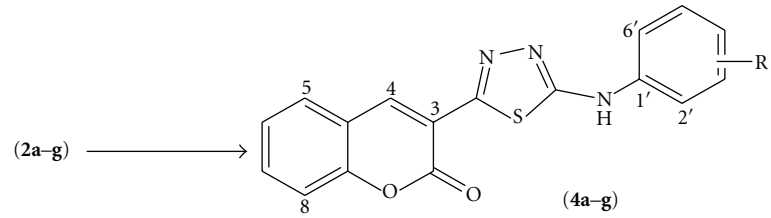
Code	Compound R	EeAChE	rAChE	hBChE	rBChE
			$K_i \pm$ SEM [μM]		
3a	2-Cl	2.42 ± 0.43	0.47 ± 0.07	0.19 ± 0.01	21.4 ± 1.6
3b	3-Cl	0.32 ± 0.07	0.60 ± 0.03	0.036 ± 0.003	65.7 ± 2.0
3c	4-Cl	0.45 ± 0.09	0.013 ± 0.002	1.34 ± 0.02	0.52 ± 0.03
3d	3-CH_3	5.77 ± 0.31	0.082 ± 0.002	0.18 ± 0.02	1.11 ± 0.05
3e	4-CH_3	18.4 ± 3.4	0.039 ± 0.002	0.13 ± 0.01	0.14 ± 0.05
3f	2-OCH_3	4.06 ± 0.34	0.061 ± 0.005	0.28 ± 0.01	0.69 ± 0.02
3g	3-OCH_3	0.98 ± 0.34	0.046 ± 0.002	7.55 ± 0.03	22.1 ± 0.2
4a	2-Cl	5.75 ± 0.78	0.031 ± 0.008	0.10 ± 0.01	1.39 ± 0.02
4b	3-Cl	0.28 ± 0.07	0.028 ± 0.002	0.54 ± 0.09	2.26 ± 0.02
4c	4-Cl	48.6 ± 4.4	0.240 ± 0.021	0.42 ± 0.01	0.35 ± 0.01
4d	3-CH_3	28.01 ± 1.63	0.017 ± 0.002	0.66 ± 0.01	0.51 ± 0.01
4e	4-CH_3	6.19 ± 0.29	0.561 ± 0.022	0.28 ± 0.02	0.46 ± 0.01
4f	2-OCH_3	7.16 ± 0.01	0.081 ± 0.001	0.23 ± 0.01	1.36 ± 0.03
4g	3-OCH_3	8.26 ± 0.01	0.021 ± 0.001	0.34 ± 0.01	1.52 ± 0.03
Neostigmine		0.056 ± 0.006	0.048 ± 0.003	0.06 ± 0.005	0.047 ± 0.006
Donepezil		0.023 ± 0.003	n.d	6.18 ± 0.34	n.d

[a] Values are expressed as the mean ± standard error of the mean of three experiments. K_i inhibitory concentration (μM) of AChE from electrophorus electricus (EeAChE) or rabbit (rAChE) and (hBChE) from horse serum or rabbit serum (rBChE).

solution (Scheme 2). The products were purified by recrystallization in aqueous ethanol. The formation of triazoles (**3a–g**) was indicated by the disappearance of broad peaks of NH and C=O groups of thiosemicarbazides and by appearance of (C=N) absorption in the range 1375–1413 cm^{-1}. In ^1H NMR, signal for N-H proton of triazoles appeared in the range of 11.13–13.94 ppm and the disappearance of signals for N-H protons confirmed the formation of the triazoles. In ^{13}C NMR the disappearance of peak due to amidic (C=O) group and the appearance of (C=N) peak in the range of 156.3–159.1 ppm was detected.

2,5-Disubstituted-1,3,4-thiadiazoles (**4a–g**) were synthesized by treating the thiosemicarbazide (**2a–g**) with concentrated polyphosphoric acid at low temperature (Scheme 3). The disappearance of broad peaks of NH groups and amidic (C=O) group of thiosemicarbazides and appearance of (C=N) absorption in the range 1415–1471 cm^{-1} was noticed in IR spectra. In ^1H NMR, signal for N-H proton of thiadiazoles appeared in the range of 11.14–11.86 ppm whilst in ^{13}C NMR the disappearance of (C=O) group peak and the appearance of (C=N) peak in the range of 157.0–159.2 ppm indicated the desired conversion.

2.2. In Vitro Inhibition Studies of AChE and BChE. The basic structure of our compound series **3a–g** is 3-(4-phenyl-5-thioxo-4,5-dihydro-1H-1,2,4-triazol-3-yl)-2H-chromen-2-one and **4a–g** series is 3-(5-(phenylamino)-1,3,4-thiadiazol-2-yl)-2H-chromen-2-one. The *in vitro* cholinesterase activity of these new molecules was determined using spectrophotometric method with neostigmine and donepezil as reference compounds (Table 1).



Scheme 3: Synthesis of 3-(5-(arylamino)-1,3,4-thiadiazol-2-yl)-2*H*-chromen-2-ones.

The K_i values suggested that most of designed compounds exhibited potent and selective inhibitory activities in nanomolar range towards cholinesterases. The most potent compounds are **3c**, **4b**, **4d**, **4c,** and **3d** on rabbit AChE than the reference compound, neostigmine. However, our investigated compounds were less active on EeAChE as compared to the reference drugs. It showed the selectivity of inhibition of the tested compounds on rAChE. Our compounds have different scaffold than neostigmine, therefore, they were more selective on both enzymes and showed more selectivity as compared to reference compound. Compound **3b** was 2-fold more active on hBChE than the neostigmine compound, but 20-fold more active than donepezil; however, other compounds were slightly less potent on the hBChE. All compounds were less potent than the reference drugs for rBChE inhibition. Varying K_i values were observed by the attachment of different substituent to the phenyl ring of **4a–g** compounds. Among the newly synthesized analogues, the most potent compound against AChE was **4b** having strong electron withdrawing (–Cl) group at metaposition. However, when −Cl is shifted to para and ortho position, activity was reduced as in the case of **4a** and **3d,** respectively. If ($-CH_3$) group is attached to para and meta positions activity was further decreased as observed in **4f** and **4c,** respectively. Reduced activity was observed when ($-OCH_3$) group is attached to phenyl group at ortho and meta position as in compounds **4g** and **4d,** respectively. Substitution of various groups to the phenyl group of **3a–g** compounds also effect inhibitory activity. **3b** showed excellent inhibitory potency when (–Cl) is attached at meta position and slightly decreased if shifted to ortho position that is, **3a**. **3g** showed good activity against AChE when ($-OCH_3$) group is attached at meta position and slightly reduced if shifted to ortho position as in case of **4e**. ($-CH_3$) group when attached at meta position showed good inhibitory activity, that is, **3f**, however remarkably reduced when shifted to para position as in case of **3e**. In general, all the tested compounds were also excellent inhibitors of butyrylcholinesterase. Among **3a–g** compounds **3b** was found to be the most potent inhibitor of BChE having (–Cl) group attached at metaposition. However, inhibitory activity was slightly reduced when –Cl is shifted to ortho and metaposition as in case of **3a** and **3c**. **4e** showed good inhibitory activity against BChE when $-OCH_3$ group is attached at ortho position and reduced if $-OCH_3$ group is shifted to meta position. ($-CH_3$) group when attached at para and metaposition showed good inhibitory activity as in compounds **3e** and **3f,** respectively. Among **4a–g** compounds, **3d** was found to be the most potent inhibitor where –Cl is attached at ortho position. However, when –Cl is shifted to meta and para position, no significant change on inhibitory potency was observed as in compounds **4b** and **4a,** respectively. In **4 g** and **4d** ($-OCH_3$) group attached at ortho and meta position, respectively, showed good inhibitory potential against both BChE enzyme isolated from horse and rabbit serum. When $-CH_3$ group was substituted at para and meta positions revealed excellent inhibitory potency as shown in **4f** and **4c** respectively.

2.3. Kinetic Characterization of AChE and BChE Inhibition. The mechanism of binding to AChE and BChE was studied by the analysis of Line-weave-Burk plots for the most potent compound **4b**. The effect of different concentrations of inhibitor (from 0–100 and 0–5 nM for acetyl, and butyrylcholinesterase, resp.) on initial velocities were investigated over a range of substrate concentrations (from 200–2500 mM). It revealed that **4b** was a competitive inhibitor of AChE and BChE (Figure 2). As K_m value was increasing and value of $V_{\max}$ remained almost same in the presence or absence of inhibitor, indication of competitive type of inhibitory mechanism against AChE and BChE.

2.4. Docking Results. In order to investigate the probable binding modes and to explain different binding affinities of the molecules, compounds were docked into binding sites of the enzymes. Newly synthesized inhibitors for AChE and BChE possessed the similar binding modes. Our docking results showed that all compounds have similar binding modes with different docking scores as shown in Table 2. Most of the compounds have been gorged into the catalytic amino acid triad [35] (Glu224, Ser225, and His 494) of AChE and (Glu225, Ser226, and His466) of BChE, respectively. The top ranked binding conformation of the most active compound of AChE and BChE is shown in Figures 3 and 4, respectively. Based on docking simulations, we can explain that strong binding affinity of **3g** with BChE is due to the hydrogen bonding of coumarin ring carbonyl moiety with N-H of His-466 residue and sulfur of triazolethoiphene with side chain of one of catalytic residue Glu225, shown in Figure 4. Similarly, the most active compound **4b** for AChE interacts with enzyme through hydrogen bonding interaction. Carbonyl moiety of coumarin ring makes a hydrogen bond with N-H of His494 residue as shown in Figure 3. It has been observed that AChE inhibitors have strong binding affinities with their enzymes over BChE inhibitors, which is in contrast to observed activities. This might be due to inabilities or poor performance of the docking scoring functions [36].

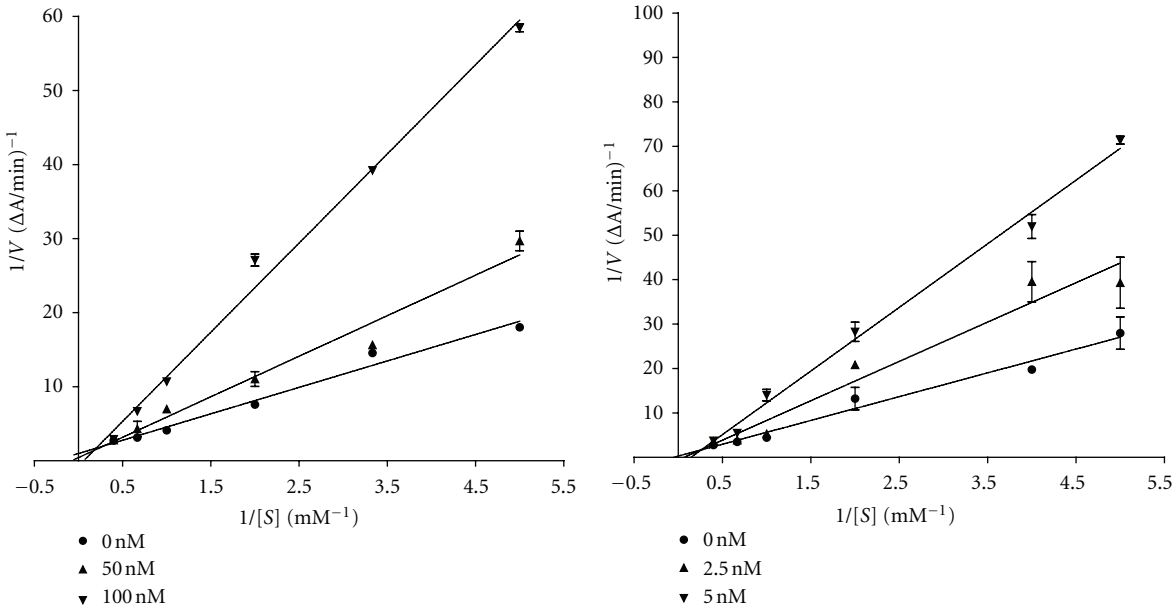


Figure 2: Lineweavere-Burk reciprocal plots of inhibition kinetics of (a) acetylcholinesterase and (b) butyrylcholinesterase by the compound **4b.** Changes in the initial velocities of the reaction were measured at different concentrations of the inhibitors (from 0–100 and 0–5 nM for acetyl and butyrylcholinesterase, resp.) by using substrates ATCI and BTCCl.

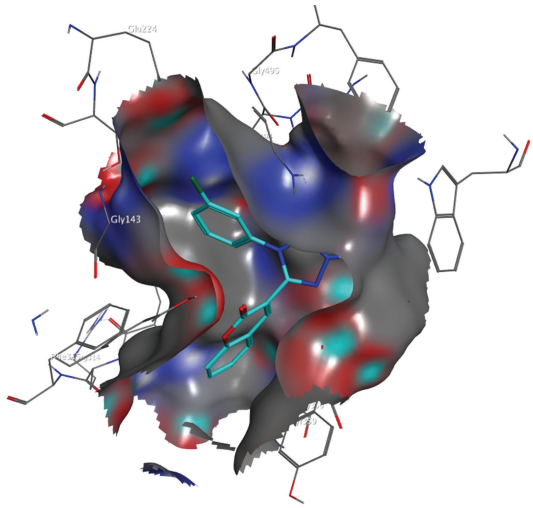


Figure 3: Binding mode of top ranked most active (**4b**) compound in the binding site of AChE enzyme.

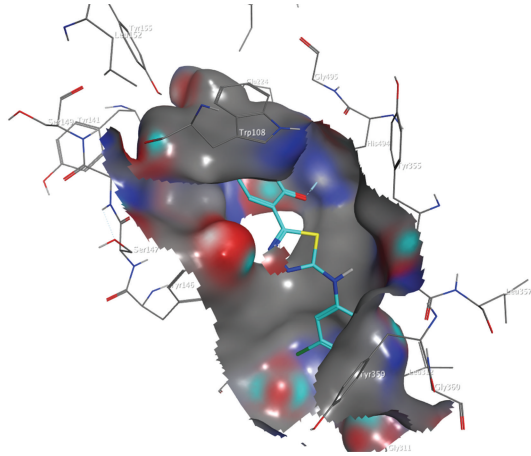


Figure 4: Binding mode of top ranked most active (**3g**) compound in the binding site of BChE enzyme.

3. Conclusion

The synthesis, biological evaluation, and molecular docking studies of fourteen novel 3-thiadiazolyl- and thioxo-1,2,4-triazolyl coumarins derivatives as potential inhibitors of cholinesterases have been reported. The cholinesterase inhibition studies revealed that all compounds were potential inhibitors of the investigated enzymes and can be used for treatment of Alzheimer's disease. Some of the investigated compounds were more potent on rabbit AChE enzyme, better than the reference drug. It was also observed that the coumarin derivatives were competitive inhibitors for the AChE and BChE enzymes. The study of their anti-cholinesterase activities might lead to a novel family of potent anti-AD compounds in near future.

4. Experimental

4.1. Chemistry

*4.1.1. Synthesis of 1,4-Disubstituted-thiosemicarbazides (**3a–g**), General Procedure.* A mixture of carbohydrazide (6.8 mmol) and substituted phenyl isothiocyanate (**1a–g**)

Table 2: Docking scores of all synthesized compounds in AChE and BChE.

Code	AChE	BChE
	Chemfitness scores	
3a	35.598	28.560
3b	34.155	29.813
3c	33.360	27.474
3d	36.365	22.734
3e	34.741	29.559
3f	37.335	29.979
3g	30.625	26.258
4a	37.172	22.609
4b	38.591	23.518
4c	38.212	26.031
4d	35.733	28.354
4e	30.520	23.943
4f	37.081	27.062
4g	31.208	23.505

(6.6 mmol) was stirred for 10–12 h at 50–60°C. After consumption of the starting materials, the mixture was cooled at room temperature. The methanol was evaporated on rotary evaporator leaving behind a crude product as oil that solidified on cooling and was recrystallized from a mixture of ethyl acetate and petroleum ether (4 : 1) to yield thiosemicarbazides(**2a–g**).

*4.1.2. 4-(2-Chlorophenyl)-1-(2-oxo-2H-chromene-3-carbonyl)thiosemicarbazide (**2a**).* Green solid (54%): m.p. 215–217°C; R_f*: 0.54; IR (KBr, cm^{-1}): 3296–3163 (NH), 1731 (C=O), 1673 (C=O), 1596, 1512 (C=C), 1268 (C=S); ^{1}H NMR (300 MHz, DMSO-d_6): δ 11.96 (s, 1H, NH-C=O), 10.05 (s, 2H, NH-C=S), 9.01 (s, 1H, C-H, H-4), 8.03 (d, 1H, J = 7.5 Hz, H-8), 7.71–7.69 (m, 1H, H-6), 7.37–7.09 (m, 3H, H-7, H-3′, H-5′), 6.99 (d, 1H, J = 8.1 Hz, H-5), 6.94–6.76 (m, 2H, H-4′, H-6′); ^{13}C NMR (75 MHz, DMSO-d_6): δ 181.2 (C=S), 166.9 (NH-C=O), 159.5 (C=O), 150.2, 135.3, 134.6, 132.1, 129.5, 126.6, 125.5, 124.9, 123.4, 122.4, 120.9, 118.6, 116.2 (Ar-Cs). Anal. Calcd. for $C_{17}H_{12}ClN_3O_3S$: C, 54.62; H, 3.24; N, 11.24, S, 8.58; Found: C, 54.54; H, 3.11; N, 11.09; S, 8.49.

*4.1.3. 4-(3-Chlorophenyl)-1-(2-oxo-2H-chromene-3-carbonyl)thiosemicarbazide (**2b**).* Yellow solid (59%): m.p. 189–191°C; R_f*: 0.54; IR (KBr, cm^{-1}): 3295–3163 (NH), 1724 (C=O), 1670 (C=O), 1590, 1508 (C=C), 1267 (C=S); ^{1}H NMR (300 MHz, DMSO-d_6): δ 11.35 (s, 1H, NH-C=O), 10.15 (s, 2H, NH-C=S), 9.00 (s, 1H, C-H, H-4), 7.68 (d, 1H, J = 7.8 Hz, H-8), 7.42–7.36 (m, 2H,H-6, H-7), 7.20 (d, J = 7.8 Hz, H-5), 6.99–6.89 (m, 2H, H-3′, H-5′), 6.84–6.79 (m, 2H, H-4′, H-6′); ^{13}C NMR (75 MHz, DMSO-d_6): δ 181.4 (C=S), 163.1 (NH-C=O), 159.3 (C=O), 156.9, 142.2, 133.6, 131.1, 129.0, 128.6, 125.4, 120.2, 119.8, 119.4, 118.7, 117.1, 116.1 (Ar-Cs). Anal. Calcd. for $C_{17}H_{12}ClN_3O_3S$: C, 54.62; H, 3.24; N, 11.24, S, 8.58; Found: C, 54.71; H, 3.17; N, 11.15; S, 8.43.

*4.1.4. 4-(4-Chlorophenyl)-1-(2-oxo-2H-chromene-3-carbonyl)thiosemicarbazide (**2c**).* Greenish solid (56%): m.p. 177–179°C; R_f*: 0.57; IR (KBr, cm^{-1}): 3341–3223 (NH), 1732 (C=O), 1670 (C=O), 1558, 1514, 1484 (C=C), 1268 (C=S); ^{1}H NMR (300 MHz, DMSO-d_6): δ 11.23 (s, 1H, NH-C=O), 11.11 (s, 2H, NH-C=S), 9.01 (s, 1H, C-H, H-4), 7.70 (d, 1H, J = 7.5 Hz, H-8), 7.50–7.22 (m, 5H, H-5, H-6, H-7, H-3′, H-5′), 7.00–6.94 (m, 2H, H-2′, H-6′); ^{13}C NMR (75 MHz, DMSO-d_6): δ 181.5 (C=S), 167.0 (NH-C=O), 163.2 (C=O), 149.3, 133.7, 131.3, 131.2, 129.1, 125.3, 124.3, 123.7, 123.4, 118.7, 117.0 (Ar-Cs). Anal. Calcd. for $C_{17}H_{12}ClN_3O_3S$: C, 54.62; H, 3.24; N, 11.24, S, 8.58; Found: C, 54.51; H, 3.32; N, 11.07; S, 8.43.

*4.1.5. 1-(2-Oxo-2H-chromene-3-carbonyl)-4-m-tolylthiosemicarbazide (**2d**).* Yellow solid (61%): m.p. 150–152°C; R_f*: 0.50; IR (KBr, cm^{-1}): 3300–3143 (NH), 1732 (C=O), 1674 (C=O), 1522, 1497 (C=C), 1236 (C=S); ^{1}H NMR (300 MHz, DMSO-d_6): δ 11.76 (s, 1H, NH-C=O), 9.99 (s, 2H, NH-C=S), 8.48 (s, 1H, C-H, H-4), 8.10 (d, 1H, J = 7.5 Hz, H-8), 7.41–7.36 (m, 1H, H-6), 7.25–7.20 (m, 2H, H-7, H-5′), 7.21–7.14 (m, 1H, H-2′), 7.01(d, 1H, J = 7.5 Hz, H-5), 6.89–6.81 (m, 2H, H-4′, H-6′), 2.31(s, 3H, CH_3); ^{13}C NMR (75 MHz, DMSO-d_6): δ 179.2 (C=S), 164.7 (NH-C=O), 157.0 (C=O), 149.4, 138.7, 137.7, 131.7, 128.3, 126.6, 126.2, 124.5, 123.3, 122.4, 120.7, 119.7, 116.4 (Ar-Cs), 21.4 (CH_3). Anal. Calcd. for $C_{18}H_{15}N_3O_3S$: C, 61.18; H, 4.28; N, 11.89, S, 9.07; Found: C, 61.11; H, 4.33; N, 11.75; S, 8.97.

*4.1.6. 1-(2-Oxo-2H-chromene-3-carbonyl)-4-p-tolylthiosemicarbazide (**2e**).* White solid (59%): m.p. 179–181°C; R_f*: 0.51; IR (KBr, cm^{-1}): 3432–3179 (NH), 1718 (C=O), 1689 (C=O), 1573, 1538, 1513 (C=C), 1259 (C=S); ^{1}H NMR (300 MHz, DMSO-d_6): δ 11.73(s, 1H, NH-C=O), 11.14 (s, 2H, NH-C=S), 9.98 (s, 1H, C-H, H-4), 7.42 (d, 1H, J = 7.5 Hz, H-8), 7.26–7.23 (m, 2H, H-7, H-6), 7.21–7.14 (m, 2H, H-2′, H-6′), 6.99 (d, 1H, J = 7.5 Hz, H-5), 6.89–6.81 (m, 2H, H-3′, H-5′), 2.30 (s, 3H, CH_3); ^{13}C NMR (75 MHz, DMSO-d_6): δ 176.2 (C=S), 165.4 (NH-C=S), 157.0 (C=O), 148.2, 137.0, 134.8, 131.8, 128.9, 126.2, 123.4, 122.7, 120.7, 119.7, 116.5 (Ar-Cs), 21.1 (CH_3). Anal. Calcd. for $C_{18}H_{15}N_3O_3S$: C, 61.18; H, 4.28; N, 11.89, S, 9.07; Found: C, 61.02; H, 4.20; N, 11.95; S, 8.92.

*4.1.7. 4-(2-Methoxyphenyl)-1-(2-oxo-2H-chromene-3-carbonyl)thiosemicarbazide (**2f**).* White solid (57%): m.p. 198–200°C; R_f*: 0.56; IR (KBr, cm^{-1}): 3326–3281 (NH), 1726 (C=O), 1644 (C=O), 1593, 1520 (C=C), 1235 (C=S); ^{1}H NMR (300 MHz, DMSO-d_6): δ 11.90(s, 1H, NH-C=O), 11.14 (s, 2H, NH-C=S), 9.97 (s, 1H, C-H, H-4), 7.88 (d, 1H, J = 7.8 Hz, H-8), 7.28–7.25 (m, 1H,H-6), 7.23–7.14 (m, 1H, H-7), 7.09 (d, 1H, J = 7.2 Hz, H-5), 7.00–6.96 (m, 2H, H-3′, H-6′), 6.93-6.86 (m, 2H, H-4′, H-5′), 3.35 (s, 3H, OCH_3); ^{13}C NMR (75 MHz, DMSO-d_6): δ 181.7 (C=S), 166.2 (NH-C=O), 161.2 (C=O), 151.6, 139.1, 136.2, 130.4, 128.2, 126.0, 125.9, 125.7, 124.5, 120.6, 120.3, 119.9, 116.6, 111.6 (Ar-Cs), 55.7 (OCH_3). Anal. Calcd. for $C_{18}H_{15}N_3O_4S$: C, 58.53; H, 4.09; N, 11.38, S, 8.68; Found: C, 58.67; H, 4.21; N, 11.24; S, 8.53.

*4.1.8. 4-(3-Methoxyphenyl)-1-(2-oxo-2H-chromene-3-carbonyl)thiosemicarbazide (**2g**).* Green solid (57%): m.p. 169–172°C; R_f*: 0.56; IR (KBr, cm^{-1}): 3324–3283 (NH), 1721 (C=O), 1652 (C=O), 1589, 1518 (C=C), 1237 (C=S); ^{1}H NMR (300 MHz, DMSO-d_6): δ 11.70 (s, 1H, NH-C=O), 10.00 (s, 2H, NH-C=S), 9.01 (s, 1H, C-H, H-4), 7.69 (d, 1H, J = 7.8 Hz, H-8), 7.40–7.37 (m, 1H, H-6), 7.30–7.17 (m, 2H, H-7, H-5), 7.00–6.91 (m, 2H, H-4′, H-5′), 6.90–6.75 (m, 2H, H-2′, H-6′), 3.76 (s, 3H, OCH_3); ^{13}C NMR (75 MHz, DMSO-d_6): δ 180.7 (C=S), 163.2 (NH-C=O), 159.4 (C=O), 150.4, 140.7, 133.7, 131.3, 129.9, 129.1, 127.5, 125.7, 124.5, 120.7, 120.0, 119.6, 117.0, 111.5 (Ar-Cs), 55.5 (OCH_3). Anal. Calcd. for $C_{18}H_{15}N_3O_4S$: C, 58.53; H, 4.09; N, 11.38, S, 8.68; Found: C, 58.45; H, 4.11; N, 11.29; S, 8.51.

*4.1.9. Synthesis of 4,5-Disubstituted-1,2,4-triazol-3(4H) thiones (**4a–g**), General Procedure.* The thiosemicarbazides (**2a–g**) (1.4 mmol) were refluxed (4-5 h) in aqueous sodium hydroxide solution (4N, 25 mL). The progress of reaction was monitored by TLC. After completion of the reaction, the reaction mixture was cooled to room temperature and filtered. The filtrate was neutralized with hydrochloric acid (4N) to precipitate the triazoles, which were filtered and recrystallized from aqueous ethanol.

4.1.10. 3-(4-(2-Chlorophenyl)-5-thioxo-4,5-dihydro-1H-1,2,4-triazol-3-yl)-2H-chromen-2-one (**3a**). Yellow solid (63%): m.p 205–207°C; R_f*: 0.61; IR (KBr, cm^{-1}): 3292 (NH), 1730 (C=O), 1555, 1477 (C=C), 1409 (C=N), 1253 (C=S); ^{1}H NMR (300 MHz, DMSO-d_6): δ 11.13 (s, 1H, NH), 9.01 (s, 1H, C-H, H-4), 7.63 (d, 1H, J = 7.8 Hz, H-8), 7.42–7.38 (m, 2H, H-6, H-7), 6.93 (d, 1H, J = 7.5 Hz, H-5), 7.11–7.08 (m, 2H, H-3′, H-5′), 7.03–6.99 (m, 1H, H-6′), 6.88–6.81 (m, 1H, H-4′); ^{13}C NMR (75 MHz, DMSO-d_6): δ 165.2 (C=S), 159.5 (C=O), 158.5 (C=N), 148.7, 133.6, 131.8, 131.1, 128.9, 128.6, 127.6, 126.4, 122.2, 121.4, 119.4, 119.1, 117.3, 116.9 (Ar-Cs). Anal. Calcd. for $C_{17}H_{10}ClN_3O_2S$: C, 57.39; H, 2.83; N, 11.81, S, 9.01; Found: C, 57.23; H, 2.71; N, 11.70; S, 8.87.

*4.1.11. 3-(4-(3-Chlorophenyl)-5-thioxo-4,5-dihydro-1H-1,2,4-triazol-3-yl)-2H-chromen-2-one (**3b**).* Yellow solid (61%): m.p. 207–209°C; R_f*: 0.61; IR (KBr, cm^{-1}): 3282 (NH), 1728 (C=O), 1553, 1482 (C=C), 1383 (C=N), 1267 (C=S); ^{1}H NMR (300 MHz, DMSO-d_6): δ 11.13 (s, 1H, NH), 9.00 (s, 1H, C-H, H-4), 7.68 (d, 1H, J = 7.8 Hz, H-8), 7.43–7.41 (m, 2H, H-6, H-7), 7.38–7.37 (m, 2H, H-4′, H-5′), 7.00 (d, 1H, J = 7.8 Hz,H-5), 6.97–6.95 (m, 2H,¯H-2′, H-6′); ^{13}C NMR (75 MHz, DMSO-d_6): δ 163.2 (C=S), 159.3 (C=O), 157.8 (C=N), 148.4, 133.7, 131.2, 130.5, 129.1, 128.6, 122.2, 120.1, 119.2, 118.6, 117.3, 117.0 (Ar-Cs). Anal. Calcd. for $C_{17}H_{10}ClN_3O_2S$: C, 57.39; H, 2.83; N, 11.81, S, 9.01; Found: C, 57.27; H, 2.69; N, 11.88; S, 8.92.

*4.1.12. 3-(4-(4-Chlorophenyl)-5-thioxo-4,5-dihydro-1H-1,2,4-triazol-3-yl)-2H-chromen-2-one (**3c**).* Green solid (66%): m.p. 204–206°C; R_f*: 0.62; IR (KBr, cm^{-1}): 3287 (NH), 1728 (C=O), 1571, 1486 (C=C), 1381 (C=N), 1269 (C=S); ^{1}H NMR (300 MHz, DMSO-d_6): δ 11.14 (s, 1H, N*H*), 9.01 (s, 1H, C-H, H-4), 7.69 (d, 1H, J = 7.8 Hz, H-8), 7.43–7.40 (m, 2H, H-6, H-7), 7.39–7.37 (m, 2H, H-3′, H-5′), 6.98 (d, 1H, J = 7.8 Hz, H-5), 6.96–6.94 (m, 2H, H-2′, H-6′); ^{13}C NMR (75 MHz, DMSO-d_6): δ 163.3 (C=S), 159.1 (C=O), 157.9 (C=N), 148.6, 133.7, 131.3, 130.4, 129.2, 128.7, 122.6, 120.0, 119.3, 118.7, 117.2, 117.0 (Ar-Cs). Anal. Calcd. for $C_{17}H_{10}ClN_3O_2S$: C, 57.39; H, 2.83; N, 11.81, S, 9.01; Found: C, 57.26; H, 2.69; N, 11.67; S, 8.92.

*4.1.13. 3-(5-Thioxo-4-m-tolyl-4,5-dihydro-1H-1,2,4-triazol-3-yl)-2H-chromen-2-one (**3d**).* Yellow solid (59%): m.p. 217-219°C; R_f*: 0.59; IR (KBr, cm^{-1}): 3281 (NH), 1725 (C=O), 1576, 1541 (C=C), 1413 (C=N), 1235 (C=S); ^{1}H NMR (300 MHz, DMSO-d_6): δ 11.15 (s, 1H, NH), 9.00 (s, 1H, C-H, H-4), 7.71–7.68 (m, 2H, H-8, H-6), 7.43–7.37 (m, 2H, H-7, H-5), 6.99–6.95 (m, 4H, H-2′, H-4′, H-5′, H-6′), 2.21 (s, 3H, CH_3); ^{13}C NMR (75 MHz, DMSO-d_6): δ 163.3 (C=S), 160.1 (C=O), 159.1 (C=N), 149.3, 133.7, 131.3, 131.1, 129.2, 128.2, 126.1, 124.6, 122.1, 120.1, 119.3, 118.6, 117.4, 117.0 (Ar-Cs), 24.3 (CH_3). Anal. Calcd. for $C_{18}H_{13}N_3O_2S$: C, 64.46; H, 3.91; N, 12.53, S, 9.56; Found: C, 64.51; H, 3.79; N, 12.42; S, 9.40.

*4.1.14. 3-(5-Thioxo-4-p-tolyl-4,5-dihydro-1H-1,2,4-triazol-3-yl)-2H-chromen-2-one (**3e**).* Yellow solid (61%): m.p. 211–213°C; R_f*: 0.58; IR (KBr, cm^{-1}): 3297 (NH), 1732 (C=O), 1557, 1513 (C=C), 1375 (C=N), 1288 (C=S); ^{1}H NMR (300 MHz, DMSO-d_6): δ 11.14 (s, 1H, NH), 9.01 (s, 1H, C-H, H-4), 7.43–7.25 (m, 4H, H-8, H-7, H-6, H-5), 7.07 (d, 2H, J = 8.1 Hz, H-2′, H-6′), 6.99 (d, 2H, J = 7.8 Hz, H-3′, H-5′), 2.23 (s, 3H, CH_3); ^{13}C NMR (75 MHz, DMSO-d_6): δ 163.5 (C=S), 159.4 (C=O), 158.6 (C=N), 149.1, 137.7, 133.6, 130.9, 129.6, 128.7, 125.5, 121.0, 119.3, 118.6, 117.0, 116.0, 112.7, 116.8 (Ar-Cs), 20.8 (CH_3). Anal. Calcd. for $C_{18}H_{13}N_3O_2S$: C, 64.46; H, 3.91; N, 12.53, S, 9.56; Found: C, 64.61; H, 3.82; N, 12.39; S, 9.41.

*4.1.15. 3-(4-(2-Methoxyphenyl)-5-thioxo-4,5-dihydro-1H-1,2,4-triazol-3-yl)-2H-chromen-2-one (**3f**).* Yellow solid (64%): m.p. 214–216°C; R_f*: 0.51; IR (KBr, cm^{-1}): 3275 (NH), 1730 (C=O), 1552, 1475 (C=C), 1410 (C=N), 1233 (C=S); ^{1}H NMR (300 MHz, DMSO-d_6): δ 13.94 (s, 1H, NH), 9.88 (s, 1H, C-H, H-4), 7.36–7.28 (m, 2H, H-8, H-6), 7.22–7.14 (m, 2H, H-5, H-7), 7.00–6.95 (m, 2H, H-3′, H-5′), 6.77–6.72 (m, 2H, H-4′, H-6′), 3.56 (s, 3H, OCH_3); ^{13}C NMR (75 MHz, DMSO-d_6): δ 168.5 (C=S), 159.1 (C=O), 156.3 (C=N), 150.9, 139.7, 132.3, 131.4, 130.7, 126.2, 123.1, 120.5, 119.8, 118.8, 117.7, 116.0, 112.7, 113.7 (Ar-Cs), 56.0 (OCH_3). Anal. Calcd. for $C_{18}H_{13}N_3O_3S$: C, 61.53; H, 3.73; N, 11.96, S, 9.13; Found: C, 61.43; H, 3.56; N, 11.88; S, 9.01.

*4.1.16. 3-(4-(3-Methoxyphenyl)-5-thioxo-4,5-dihydro-1H-1,2,4-triazol-3-yl)-2H-chromen-2-one (**3g**).* Green solid (60%): m.p. 218–220°C; R_f*: 0.51; IR (KBr, cm^{-1}): 3273 (NH), 1727 (C=O), 1551, 1477 (C=C), 1412 (C=N), 1237 (C=S); ^{1}H NMR (300 MHz, DMSO-d_6): δ 11.13 (s, 1H, NH), 9.01 (s, 1H, C-H, H-4), 7.71–7.68 (m, 2H, H-8, H-6),

7.43–7.37 (m, 2H, H-5, H-7), 7.01–6.95 (m, 2H, H-4′, H-5′), 6.70–6.36 (m, 2H, H-2′, H-6′), 3.53 (s, 3H, OCH_3); ^{13}C NMR (75 MHz, DMSO-d_6): δ 163.5 (C=S), 159.1 (C=O), 156.4 (C=N), 150.8, 139.9, 133.7, 131.3, 130.4, 126.6, 123.2, 120.7, 119.7, 118.6, 117.0, 116.1, 113.5, 112.4 (Ar-Cs), 56.7 (OCH_3). Anal. Calcd. for $C_{18}H_{13}N_3O_3S$: C, 61.53; H, 3.73; N, 11.96, S, 9.13; Found: C, 61.48; H, 3.61; N, 11.81; S, 9.16.

*4.1.17. Synthesis of 2,5-disubstituted-1,3,4-thiadiazoles (**4a–g**), General Procedure.* 1,4-Disubstituted thiosemicarbazides (**2a–g**) (1.4 mmol), in polyphosphoric acid (0.5 mL, 2.8 mmol) were stirred overnight at 70°C. After completion of reaction, the cooled solution was poured on the crushed ice. The reaction mixture was extracted with ethyl acetate (3 × 20 mL) and combined extracts were washed with sodium bicarbonate (5%) and water until the washings were neutral. The organic layer was dried with anhydrous sodium sulphate and concentrated under reduced pressure to yield the 2,5-disubstituted-1,3,4-thiadiazoles, purified by recrystallization in ethanol.

*4.1.18. 3-(5-(2-Chlorophenylamino)-1,3,4-thiadiazol-2-yl)-2H-chromen-2-one (**4a**).* Yellow solid (45%): m.p. 212–214°C; R_f*: 0.40; IR (KBr, cm^{-1}): 3278 (NH), 1725 (C=O), 1563, 1529 (C=C), 1415 (C=N); ^{1}H NMR (300 MHz, DMSO-d_6): δ 11.42 (s, 1H, NH), 8.73 (s, 1H, C-H, H-4), 7.44–7.36 (m, 4H, H-6, H-8, H-3′, H-5′), 7.04–6.96 (m, 4H, H-5, H-7, H-4′, H-6′); ^{13}C NMR (75 MHz, DMSO-d_6): δ 164.7 (C=O), 159.8 (C=N-NH), 159.2 (C=N), 149.2, 133.5, 132.6, 131.9, 130.7, 129.1, 128.5, 127.8, 127.6, 120.0, 119.8, 117.8, 117.3, 116.8 (Ar-Cs). Anal. Calcd. for $C_{17}H_{10}ClN_3O_2S$: C, 57.39; H, 2.83; N, 11.81, S, 9.01; Found: C, 57.27; H, 2.76; N, 11.72; S, 8.87.

*4.1.19. 3-(5-(3-Chlorophenylamino)-1,3,4-thiadiazol-2-yl)-2H-chromen-2-one (**4b**).* Yellow solid (44%): m.p. 210–212°C; R_f*: 0.40; IR (KBr, cm^{-1}): 3271 (NH), 1728 (C=O), 1565, 1527 (C=C), 1419 (C=N); ^{1}H NMR (300 MHz, DMSO-d_6): δ 11.15 (s, 1H, NH), 9.01 (s, 1H, C-H, H-4), 7.70–7.61 (m, 4H, H-6, H-8, H-3′, H-5′), 7.50–7.36 (m, 4H, H-5, H-7, H-2′, H-6′); ^{13}C NMR (75 MHz, DMSO-d_6): δ 163.7 (C=O), 159.9 (C=N-NH), 159.1 (C=N), 148.2, 135.5, 133.6, 132.9, 130.5, 129.2, 128.6, 127.7, 126.6, 120.2, 119.1, 117.9, 117.1, 116.3 (Ar-Cs). Anal. Calcd. for $C_{17}H_{10}ClN_3O_2S$: C, 57.39; H, 2.83; N, 11.81, S, 9.01; Found: C, 57.23; H, 2.71; N, 11.67; S, 9.09.

*4.1.20. 3-(5-(4-Chlorophenylamino)-1,3,4-thiadiazol-2-yl)-2H-chromen-2-one (**4c**).* Yellow solid (59%): m.p. 216–218°C; R_f*: 0.45; IR (KBr, cm^{-1}): 3256 (NH), 1728 (C=O), 1543, 1521 (C=C), 1432 (C=N); ^{1}H NMR (300 MHz, DMSO-d_6): δ 11.15 (s, 1H, NH), 9.01 (s, 1H, C-H, H-4), 7.69 (d, 1H, J = 7.5 Hz, H-8), 7.65–7.61 (m, 1H, H-6), 7.48 (d, 2H, J = 8.4 Hz, H-3′, H-5′), 7.42–7.36 (m, 1H,H-7), 6.98 (d, 1H, J = 8.4 Hz, H-5), 6.95(d, 2H, J = 8.1 Hz, H-2′, H-6′); ^{13}C NMR (75 MHz, DMSO-d_6): δ 163.3 (C=O), 159.1 (C=N-NH), 157.3 (C=N), 148.6, 133.7, 131.3, 130.7, 129.3, 129.1, 128.3, 127.9, 120.1, 119.3, 118.6, 116.9 (Ar-Cs). Anal. Calcd. for $C_{17}H_{10}ClN_3O_2S$: C, 57.39; H, 2.83; N, 11.81, S, 9.01; Found: C, 57.26; H, 2.96; N, 11.74; S, 8.89.

*4.1.21. 3-(5-(m-Toluidino)-1,3,4-thiadiazol-2-yl)-2H-chromen-2-one (**4d**).* Yellow solid (39%): m.p. 219–221°C; R_f*: 0.47; IR (KBr, cm^{-1}): 3198 (NH), 1730 (C=O), 1600, 1561, 1501 (C=C), 1463 (C=N); ^{1}H NMR (300 MHz, DMSO-d_6): δ 11.14 (s, 1H, NH), 9.01 (s, 1H, C-H, H-4), 7.71–7.68 (m, 2H, H-8, H-6), 7.43–7.37 (m, 2H, H-7, H-5′), 6.99–6.94 (m, 4H, H-5, H-2′, H-4′, H-6′), 2.08 (s, 3H, CH_3); ^{13}C NMR (75 MHz, DMSO-d_6): δ 163.2 (C=O), 159.1 (C=N-NH), 157.4 (C=N), 148.8, 133.7, 130.9, 130.2, 128.3, 127.1, 124.3, 121.9, 121.8, 120.1, 119.7, 119.5, 119.1, 118.6, 117.9, 117.3, 117.0, 116.7, 116.3, 116.0 (Ar-Cs), 31.1 (CH_3). Anal. Calcd. for $C_{18}H_{13}N_3O_2S$: C, 64.46; H, 3.91; N, 12.53, S, 9.56; Found: C, 64.34; H, 3.98; N, 12.38; S, 9.41.

*4.1.22. 3-(5-(p-Toluidino)-1,3,4-thiadiazol-2-yl)-2H-chromen-2-one (**4e**).* Yellow solid (56%): m.p. 191–193°C; R_f*: 0.44; IR (KBr, cm^{-1}): 3267 (NH), 1725 (C=O), 1592, 1554 (C=C), 1471 (C=N); ^{1}H NMR (300 MHz, DMSO-d_6): δ 11.14 (s, 1H, NH), 9.70 (s, 1H, C-H, H-4), 7.72–7.64 (m, 2H, H-8, H-6), 7.45–7.24 (m, 2H, H-7, H-5), 7.17–7.13 (m, 2H, H-2′, H-6′), 7.12–7.08 (m, 2H, H-3′, H-5′), 2.27 (s, 3H, CH_3); ^{13}C NMR (75 MHz, DMSO-d_6): δ 167.4 (C=O), 163.0 (C=N-NH), 157.0 (C=N), 145.8, 134.0, 131.6, 130.0, 129.0, 124.3, 121.9, 119.7, 119.5, 117.9, 117.0, 116.3 (Ar-Cs), 20.8 (CH_3). Anal. Calcd. for $C_{18}H_{13}N_3O_2S$: C, 64.46; H, 3.91; N, 12.53, S, 9.56; Found: C, 64.32; H, 3.78; N, 12.40; S, 9.45.

*4.1.23. 3-(5-(2-Methoxyphenylamino)-1,3,4-thiadiazol-2-yl)-2H-chromen-2-one (**4f**).* Yellow solid (49%): m.p. 206–208°C; R_f*: 0.51; IR (KBr, cm^{-1}): 3287 (NH), 1726 (C=O), 1594, 1544 (C=C), 1466 (C=N); ^{1}H NMR (300 MHz, DMSO-d_6): δ 11.86 (s, 1H, NH), 9.96 (s, 1H, C-H, H-4), 7.69 (d, 1H, J = 7.2 Hz, H-8), 7.43–7.38 (m, 2H, H-6, H-7), 7.04–6.85 (m, 5H, H-5, H-3′, H-4′, H-5′, H-6′), 3.87 (s, 3H, OCH_3); ^{13}C NMR (75 MHz, DMSO-d_6): δ 163.2 (C=O), 159.1 (C=N-NH), 157.3 (C=N), 148.6, 147.0, 133.7, 132.0, 129.5, 128.3, 128.0, 121.7, 121.0, 120.1, 118.6, 116.8, 116.4 (Ar-Cs), 56.5 (OCH_3). Anal. Calcd. for $C_{18}H_{13}N_3O_3S$: C, 61.53; H, 3.73; N, 11.96, S, 9.13; Found: C, 61.41; H, 3.60; N, 11.83; S, 9.04.

*4.1.24. 3-(5-(3-Methoxyphenylamino)-1,3,4-thiadiazol-2-yl)-2H-chromen-2-one (**4g**).* Yellow solid (46%): m.p. 202–204°C; R_f*: 0.51; IR (KBr, cm^{-1}): 3284 (NH), 1729 (C=O), 1584, 1545 (C=C), 1468 (C=N); ^{1}H NMR (300 MHz, DMSO-d_6): δ 11.15 (s, 1H, NH), 9.79 (s, 1H, C-H, H-4), 7.70 (d, 1H, J = 7.2 Hz, H-8), 7.43–7.37 (m, 2H, H-6, H-7), 7.99–6.85 (m, 5H, H-5, H-2′, H-4′, H-5′, H-6′), 3.56 (s, 3H, OCH_3); ^{13}C NMR (75 MHz, DMSO-d_6): δ 165.2 (C=O), 159.9 (C=N-NH), 157.7 (C=N), 147.8, 146.3, 133.6, 131.0, 129.2, 128.4, 127.7, 123.7, 121.9, 120.5, 119.6, 118.8, 116.4 (Ar-Cs), 58.6 (OCH_3). Anal. Calcd. for $C_{18}H_{13}N_3O_3S$: C, 61.53; H, 3.73; N, 11.96, S, 9.13; Found: C, 61.46; H, 3.81; N, 11.85; S, 9.02.

*R_f solvent system (petroleum ether: ethyl acetate, 4 : 1).

4.2. Materials and Methods

4.2.1. Chemicals and Materials. Acetylcholinesterase (AChE) (EC 3.1.1.7, type VI-S from Electric Eel), butyrylcholinesterase (BChE) (EC 3.1.1.8, from horse serum). AChE and BChE were also isolated from rabbit brain and serum, respectively. Acetylthiocholine iodide (ATCI), *S*-butyrylthiocholine chloride (BTCCl), 5,5′-dithio-bis(2-nitrobenzoic acid) (DTNB), neostigmine methylsulfate, donepezil, dimethylsulfoxide (DMSO), and bovine serum albumin were purchased from Sigma-Aldrich (Steinheim, Germany).

4.2.2. Isolation of AChE from Rabbit Brain and BChE from Blood Serum. AChE was isolated from rabbit brain. Heads were decapitated and brains were thawed and homogenized for 30 sec in 2.5 volumes of 1 mM EDTA, 0.32 M ice cold sucrose, 10 μM tetracain (pH7.0). The homogenates were centrifuged at 105,000 ×g for 60 min (4°C). The supernatants were removed and pellets were again homogenized in same volume of 0.2% triton phosphate buffer. After centrifugation, the supernatants were again removed and pellets were rehomogenized for 10 sec in 2.5 volumes of 1% triton phosphate buffer. The final supernatants obtained after centrifugation for 60 min at 105,000 ×g were applied to an immunosorbent affinity column at a flow rate of 6.5 mL/hr approximately [37]. BChE was obtained from rabbit blood serum by applying the techniques of centrifugation. The purified enzymes were stored at –80°C.

4.2.3. Determination of AChE and BChE Inhibitory Activities in 96-Microliter Well Plates. The inhibitory activities of coumarin derivatives were measured quantitatively by Ellman's method [38] and performed with some modifications as in Ingkaninan et al. method [39] by using 96-microliter well plate. Series of newly synthesized coumarin derivatives were tested as AChE and BChE inhibitors. Initially, each compound was dissolved in DMSO (end concentration of DMSO was less than 1% in assay) and tested at a final concentration of 1 mM or 10 μL of DMSO (as negative control) in wells for initial screening. Compounds with considerable inhibition (more than 50%) were subjected to further analysis by making their six to seven serial dilutions in an assay buffer (50 mM Tris-HCl, 0.02 M $MgCl_2 \cdot 6H_2O$, and 0.1 M NaCl at pH 8.0). Reaction mixture comprised of 50 μL of 3 mM DTNB (5,5′-dithiobis(2-nitrobenzoic acid)) in assay buffer, 10 μL of test compound, and 10 μL of AChE (0.031 IU/mL) or BChE (0.5 IU/mL). This mixture was preincubated at 25°C for 10 min. After preincubation, enzymatic reaction was started by addition of 10 μL of 10 mM MBTCCl (butyrylthiocholine chloride) or ATCI (acetylthiocholine iodide) according to the respective enzyme and mixture was incubated again for 15 min. The amount of enzymatic product was measured by the change in absorbance at 405 nm by using a microplate reader (Bio-TekELx 800, Instruments, Inc. USA). Neostigmine methylsulphate and donepezil were used as standard inhibitors. Enzyme dilution buffer consisted of 50 mM Tris-HCl containing 0.1% (w/v) BSA (pH 8). The effect of DMSO on activity of enzyme was subtracted by a negative control containing DMSO, instead of inhibitor. Each concentration was analyzed in triplicate and K_i values were calculated from IC50 values by using a nonlinear curve fitting program PRISM 5.0 (GraphPad, San Diego, California, USA).

4.2.4. Kinetic Characterization of AChE and BChE Inhibition. Kinetic characterization of AChE and BChE was performed by using Ellman's method [38]. The effect of inhibitor **4b** on varying concentration of substrate from 0.2 to 2.5 mM was investigated. Enzyme kinetic characterization studies were performed under same incubation conditions as described above by using ATCI and BTCCl as substrates and DTNB was used as chromophoric reagent. A parallel control with no inhibitor in the mixture was added for comparison. Each concentration was analyzed in triplicate; and Lineweaver-Burk ($1/V$ versus $1/[S]$) plot was constructed using Prism software.

4.2.5. Docking Studies

(1) Compounds Structures Preparation. Before performing the molecular docking simulations of small molecules inside the homology models of both protein structures, all molecules were sketched and protonated by using MOE molecules sketcher tool. The three dimensional conformations of these molecules were generated using the protonate 3D tool implemented in MOE. Finally the molecules were minimized and their charges were optimized by using the MMFF94 modified force-field.

(2) Docking Protocol. The molecular docking of compounds in generated homology models were performed by using GOLD (Genetic Optimization for ligand docking) program. GOLD uses the genetic algorithm (GA) to search full length of conformational flexibility of ligands inside the protein binding site [40]. All compounds were sketched and their ionization states were fixed using MOE. In these docking simulations, 10 Å spherical binding site was used across the His-494 and His-466 for AChE and BChE enzymes, respectively. Hydrogen atoms were also added to both model structures. During docking simulations, the protein residues remained rigid except Ser, Thr, and Tyr hydroxyl groups, in order to optimize hydrogen bonding interactions with the docked compounds. For each solution 10 GA operations were run, and best ranked solution based on the chemscore was selected for each molecule. All other parameters default values were used.

(3) Homology Models Generation. The Butyrylcholinesterase (BChE) (EC 3.1.1.8, from horse serum) and acetylcholinesterase (AChE) (EC 3.1.1.7, type VI-S from electric eel) sequences were threaded using LOMETS [41] threading programs. These programs threaded the PDB IDs (3i6 m, 1q83, 2pm8, 1qo9, 2wqz) as templates for AChE and (3i6 m, 1ea5, 2xb6, 1ea) as possible templates for BChE from PDB (protein data bank) database. In second step, continuous fragments were generated from these templates and finally used to assemble full length atomic models using a modified replica-exchange Monte Carlo simulations [42]. The loop

regions were constructed by *ab initio* modeling implemented in I-TASSER. The simulation decoys were clustered using SPICKER [43] and the cluster centroid was used as the next round of I-TASSER reassembly. The structures with the lowest energy were selected and full-atomic models were refined using fragment guided molecular dynamics. The best model based on C-score was selected and subjected for the molecular docking studies of the synthesized compounds. Before docking simulations, hydrogen atoms were added in each structure and Gasteiger charges were assigned using the UCSF chimera modeling tool.

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