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Structure–activity relationships of thiazole and thiadiazole derivatives as potent and selective human adenosine A_3 receptor antagonists

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Abstract—4-(4-Methoxyphenyl)-2-aminothiazole and 3-(4-methoxyphenyl)-5-aminothiadiazole derivatives have been synthesized and evaluated as selective antagonists for human adenosine A_3 receptors. A methoxy group in the 4-position of the phenyl ring and *N*-acetyl or propionyl substitutions of the aminothiazole and aminothiadiazole templates displayed great increases of binding affinity and selectivity for human adenosine A_3 receptors. The most potent A_3 antagonist of the present series, *N*-[3-(4-methoxy-phenyl)-[1,2,4]thiadiazol-5-yl]-acetamide (**39**) exhibiting a K_i value of 0.79 nM at human adenosine A_3 receptors, showed antagonistic property in a functional assay of cAMP biosynthesis involved in one of the signal transduction pathways of adenosine A_3 receptors. Molecular modeling study of conformation search and receptor docking experiments to investigate the dramatic differences of binding affinities between two regioisomers of thiadiazole analogues, (**39**) and (**42**), suggested possible binding mechanisms in the binding pockets of adenosine receptors.

1. Introduction

Extracellular adenosine regulates a number of physiological functions by activation of specific cell membrane receptors, classified as A_1, A_{2A}, A_{2B}, and A_3 receptors, which belong to the super family of G-protein coupled receptors.[1,2] Adenosine A_3 receptors, linked to Gi proteins decreasing cAMP production upon receptor activation, have been determined to show important roles in physiological systems, including protective actions against myocardial and brain ischemia,[3,4] antiproliferative effects through cell cycle arrest and the Wnt signaling pathway,[5–7] and airway inflammation with high receptor expression in lung mast cells in mice,[8,9] implicating a potential drug target for ischemia, cancer and asthma. Furthermore, A_3 receptor antagonists may be useful in the treatment of glaucoma.[10]

Since the cloning of adenosine A_3 receptors from several species in the early 1990s, many efforts have been devoted to develop agonists and antagonists of the receptors, resulting in the design of potent and selective antagonists. Unlike typical xanthine core antagonists of other adenosine receptor subtypes, several diverse heterocyclic compound classes of selective antagonists for A_3 receptors have been developed. Triazolonaphthyridine,[11] 1,4-dihydropyridines,[12,13] pyridines,[14,15] pyrans,[16] triazoloquinazolines,[17,18] flavonoids,[19] triazolopyrimidines,[20,21] triazolopurines,[22] isoquinolines and quiazolines,[23] imidazopurinones,[24] pyridopurinediones,[25] have been synthesized and identified as adenosine A_3 receptor antagonists. Recently, thiazole and thiadiazole analogues have been described as the possible core skeletons of A_3 receptor antagonists with moderate affinity and selectivity.[26,27] In this study, we report new findings of great improvement of the scaffold derivatives with subnanomolar affinity at human adenosine A_3 receptors and high subtype selectivity through an SAR (structure–activity relationship) study combined with molecular modeling approaches.

2. Results and discussion

2.1. Chemistry

The general syntheses of various regioisomers of 4-(4-methoxyphenyl)-2-aminothiazole and 3-(4-methoxy-

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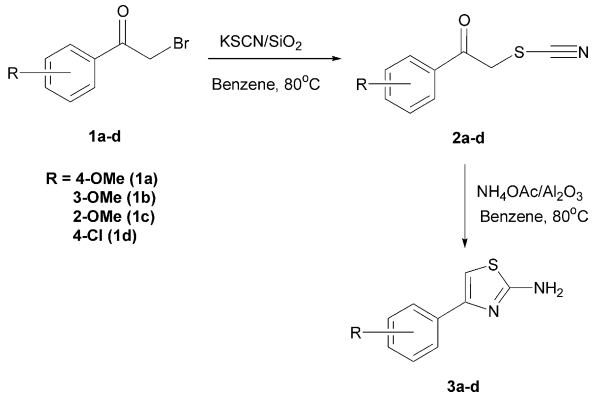
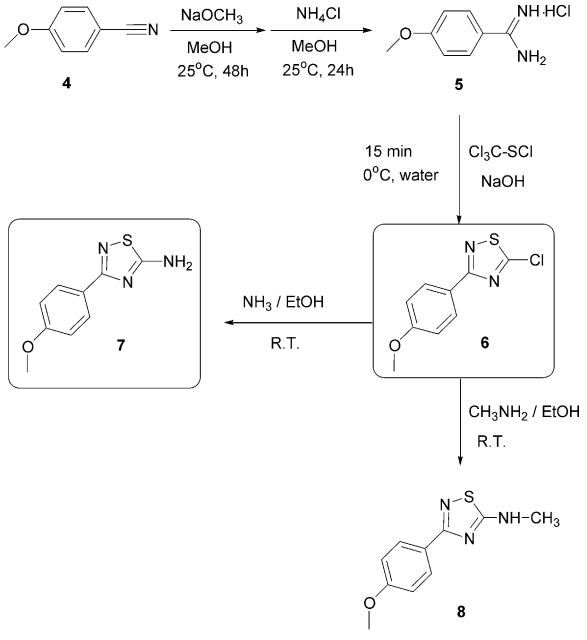
phenyl)-5-aminothiadiazole derivatives are depicted in Schemes 1–3. 2-Aminothiazole analogues (**3a–d**) were prepared from the cyclization of 2-thiocyanoacetophenones in the presence of alumina supported ammonium acetate as described by Kodomari et al. (Scheme 1).[28]

In order to synthesize the 5-amino[1,2,4]thiadiazole derivative **7**, 4-methoxybenzonitrile was converted to the 4-methoxybenzamidine hydrochloride derivaive **5**, followed by a cyclization reaction using perchloromethyl mercaptan[29] in basic aqueous solution to afford the chlorothiadiazole intermediate **6**. Substitution reaction of **6** with ammonia and methylamine gave **7** and **8**, respectively (Scheme 2).

Another regioisomer, 5-amino[1,3,4]thiadiazole derivative **10** was prepared from the reaction of *p*-anisaldehyde with thiosemicarbazide to give intermediate **9**, followed by cyclization in the presence of ferric chloride in aqueous solution (Scheme 3).[30] The synthesized and commercially available aminothiazole and aminothiadiazole compounds were subjected in the standard acylation protocols including coupling reagents with acids or anhydrides, or acid chlorides with pyridine as base to afford various derivatives **11–43** to be tested in adenosine receptor binding assays.



Scheme 1. Synthesis of 2-aminothiazole skeleton.

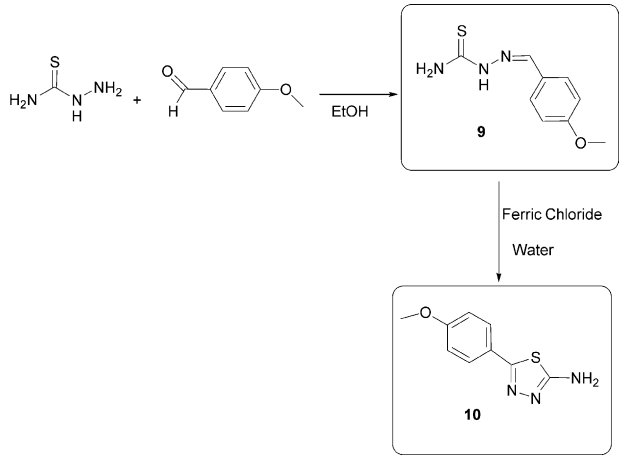


Scheme 2. Synthesis of 5-amino[1,2,4]thiadiazole skeleton.

3. Biological activity

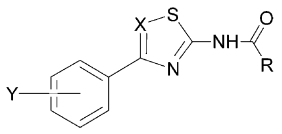
The binding affinity of each compound at four different adenosine receptor subtypes was determined as K_i values in radioligand binding assays at rat cortical or recombinant human A_1 receptors versus [^{3}H]-(*R*)-PIA ([^{3}H]-(*R*)-N^6-(phenylisopropyl)adenosine) and recombinant human A_{2A} receptors versus [^{3}H]-CGS 21680 ([^{3}H]-2-[4-(2-carboxyethyl)phenyl]ethylamino-5′-(*N*-ethylcarbamoyl)adenosine).[31,32] Affinity at recombinant human A_3 receptors expressed in CHO cells[33] was determined using [^{125}I]AB-MECA (N^6-(4-amino-3-[^{125}I]iodobenzyl)-5′-(*N*-methyl carbamoyl)-adenosine.[34]

The representative thiadiazole analogue reported earlier by the IJzerman's group[27] was *N*-(3-phenyl-1,2,4-thiadiazole-5-yl)-4-methoxybenzamide (LUF5417) with Ki values of 0.032, 2.3, and 0.082 μM at rat A_1, rat A_{2A}, and human A_3 receptors, respectively. In the course of SAR studies of the derivatives of thiazole and thiadiazole templates, we found that 4-methoxyphenyl and aliphatic acyl group substitutions displayed large enhancement of the affinity and selectivity for human adenosine A_3 receptors compare to the parent compound, LUF5417. For example, **11**, a 5-acetamido analogue of phenylthiazole showed a K_i value of 18.3 nM at human adenosine A_3 receptors with high selectivity versus human A_1 and A_{2A} receptors and **16** with 4-methoxy substitution of the phenyl group of **11**, displayed a 6-fold increase of binding affinity at A_3 receptors with a K_i value of 3 nM, while maintaining high selectivity (Table 1). Structural variations and binding assays following aliphatic and aromatic acyl substitutions at the 5-amino group identified a slightly more potent analogue, **20**, which contained one more methylene unit than **16** and displayed a K_i value of 2.4 nM. Although most of the aromatic acyl substituted derivatives **26–35**

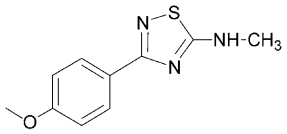


Scheme 3. Synthesis of 5-amino[1,3,4]thiadiazole skeleton.

Table 1. Binding affinities of thiazole and thiadiazole derivatives at adenosine receptors



(11-35,37-41,LUF5417)



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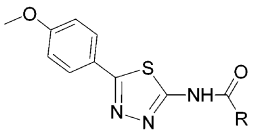

(42,43)

Compd	R	X	Y	K_i (nM) or % inhibition at 10 μM		
				Human A_1	Human A_{2A}	Human A_3
LUF5417[27]	4-$OCH_3C_6H_4$	N	H	32 (rat A_1)	2,300 (rat A_{2A})	82±4
11	CH_3	CH	H	59%	74%	18.3±4.2
12	$(CH_3)_3CO$	CH	H	11%	31%	5,100±1,130
13	$NCCH_2$	CH	H	50%	68%	204±17
14	CH_3	CH	4-Cl	0%	27%	50.9±23.5
15	$C_6H_5CH_2$	CH	4-Cl	0%	0%	100
16	CH_3	CH	4-CH_3O	35%	33%	3.0±0.8
17	CH_3	CH	3-CH_3O	34%	61%	4.1±1.8
18	CH_3	CH	2-CH_3O	18%	63%	82±21
19	CF_3	CH	4-CH_3O	0%	16%	530±91
20	CH_3CH_2	CH	4-CH_3O	22%	47%	2.4±0.6
21	$CH_3CH_2CH_2$	CH	4-CH_3O	49%	48%	7.8±1.5
22	$(CH_3)_2CH$	CH	4-CH_3O	49%	56%	16.3±4.1
23	$NCCH_2$	CH	4-CH_3O	33%	40%	24.3±3.9
24	$(CH_3)_3C$	CH	4-CH_3O	69%	20%	31.9±4.2
25	$(CH_3)_3O$	CH	4-CH_3O	0%	0%	3,260±340
26	C_6H_5	CH	4-CH_3O	0%	14%	28.7±3.3
27	$C_6H_5CH_2$	CH	4-CH_3O	17%	27%	14.2±3.0
28	$C_6H_5CH_2CH_2$	CH	4-CH_3O	0%	28%	29.1±9.3
29	*p*-$CH_3OC_6H_4CH_2$	CH	4-CH_3O	0%	0%	1,160±190
30	*p*-$CH_3OC_6H_4CH_2CH_2$	CH	4-CH_3O	0%	12%	28.6±7.3
31	$(C_6H_5)_2CH$	CH	4-CH_3O	0%	18%	526±193
32	$(C_6H_5)_2CHCH_2$	CH	4-CH_3O	0%	17%	400
33	2-Furan	CH	4-CH_3O	41%	35%	31.5±1.3
34	Thiophene-2-CH_2	CH	4-CH_3O	10%	7%	32.3±7.1
35	2-Thiophene	CH	4-CH_3O	21%	23%	69.3±10.0
36				11%	0%	373±74
37	CH_3	N	H	56%	78%	2.3±0.2
38	$C_6H_5CH_2$	N	H	19%	73%	79±26
39	CH_3	N	4-CH_3O	24%	28%	0.79±0.18
40	$C_6H_5CH_2$	N	4-CH_3O	7%	18%	23.8±0.3
41	CH_3CH_2	N	4-CH_3O	47%	3530±600	1.13±0.14
42	CH_3			0%	0%	4,690±1,380
43	$C_6H_5CH_2$			0%	0%	>100,000

showed lower binding affinities than **16** or **20**, the binding affinities were two-digit nanomolar K_i values except for **29**, **31**, and **32**, which possessed an extra phenyl group, suggesting a hydrophobic binding pocket of limited space in the receptor for the recognition of the acylamino moieties.

In an effort to further enhance the A_3 binding affinity, we applied the acetamido functionality to a thiadiazole template. *N*-(3-Phenyl-[1,2,4]thiadiazol-5-yl)-acetamide, **37**, showed an 8-fold increase of binding affinity, with a K_i value of 2.3 nM at A_3 receptors, than the corresponding thiazole derivative, **11**. Thus, we could assume that 4-methoxyphenyl substitution would afford a further increase of affinity, which encouraged us to explore synthesizing 3-(4-methoxy-phenyl)-[1,2,4]thiadiazol-5-ylamine, **7**. An acetylated derivative of **7**, that is, **39**, was characterized as a selective and potent antagonist for human A_3 receptors with subnanomolar affinity, K_i value of 0.79 nM. The corresponding propionyl analogue, **41**, which was expected to be slightly more potent than **37** based on a comparision in the thiazole series of **16** and **20**, showed a K_i value of 1.13 nM at A_3 receptors, again with high subtype selectivity. Interestingly, the affinity of another regioisomer of **39**, *N*-(2-(4-methoxyphenyl)-[1,3,4]thiadiazol-5-yl)-acetamide, **42** was dramatically decreased to a K_i value of 4.7 μM.

In terms of species differences, we attempted to determine the receptor binding affinity at rat A_1 receptors, since the parent compound LUF5417 displayed high affinity at the receptors. However, most of the derivatives displayed very weak binding affinities with K_i values >50 μM at the rat A_1 and A_{2A} receptors. Also binding to the rat adenosine A_3 receptors was weak. K_i values were 3.03 μM for compound **33** and >10 μM for compounds **17**, **39** and **40**.

4. Molecular modeling

In order to explain the different binding affinities of two regioisomers, **39** and **42**, we calculated the thermodynamically stable conformations. From the result of

the PM3 method, the lowest HF (Heat of Formation) of 1,2,4- and 1,3,4-thiadiazole compounds, **39** and **42**, were −20.09 and 12.17 kcal/mol, respectively. Thus, the most potent one, **39**, was more thermodynamically stable than the other, poor binding isomer **42** by >30 kcal. The lowest energy conformers of both derivatives were used for docking to the human adenosine A_3 receptor.[41]

We used an automated docking procedure (FlexiDock) with manual adjustment to determine the most energetically favorable binding location and orientation of the antagonist **39** (Fig. 1A). The result of FlexiDock applied to **39** displayed the possibility of a few complex models clustering within small energy differences. The complex model with the lowest energy among several docking models was well correlated with SAR and selectivity data in binding to the human A_3 receptor. The bound conformation of the amide showed *trans*-geometry with 4 kcal higher energy than the lowest one with a *cis*-amide bond obtained using the PM3 method. The high affinity antagonist **39**, a 1,2,4-thiadiazole derivative, interacted through the H-bonding of the CO group with Q167 in EL2, which was a unique residue in human A_3 receptors in relation to other subtypes of adenosine receptors; The homologous residue was E for A_1, L for A_2 adenosine receptors (Fig. 1). This hydrophilic interaction appears to explain why compound **39** is selective for the human adenosine A_3 receptors. In addition, $S181^{5.42}$ formed H-bonding with the *N* atom of the thiadiazole ring. The amino acid corresponding to $S181^{5.42}$ in other adenosine receptor subtypes was N. This additional H-bonding to the thiadiazole ring, absent with the thiazole derivatives, could contribute to the increase of human adenosine A_3 receptor affinity. The thiadiazole and phenyl rings were surrounded by many hydrophobic amino acids; $F168^{EL2}$, $F182^{5.43}$, $I186^{5.47}$, and $L246^{6.51}$. The methyl group of the 4-methoxy phenyl ring substituent showed a hydrophobic interaction with $I186^{5.47}$ and $W243^{6.48}$. $S247^{6.52}$, which corresponds to H in other adenosine receptor subtypes, and was very close to the O of the methoxy group. The distance between the oxygen atom of the methoxy group and the hydroxyl group of S was 4.2 Å. The methyl of the acetamide was oriented toward EL2, indicating a hydrophobic interaction of methyl with $I253^{6.58}$. In proximity of the terminal methyl group, there was S170 in EL2, suggesting that a hydrophilic group in preference to a hydrophobic group, aromatic ring or aliphatic chain, might increase the binding affinity to the human adenosine A_3 receptor.

In the case of the 1,3,4 analogue **42**, there was no H-bonding in its complex when docked in the binding site of its isomer **39**, correlating with its binding data; that is, about 6000-fold decrease of the binding affinity for the human adenosine A_3 receptor (Fig. 1B). The total energy of its complex was 9 kcal higher than that of the complex of the 1,2,4-analogue **39**. Thus, the molecular modeling results supported the docking of the 1,2,4-thiadiazole **39** being energetically more favorable than the 1,3,4-derivative **42**.

Thus, the result of FlexiDock was consistent with the current binding results. However, to confirm the reliability of the docked complexes, additional mutational experiments and more SAR data are needed, because theoretically there are other possible models of complexes within small energy differences.

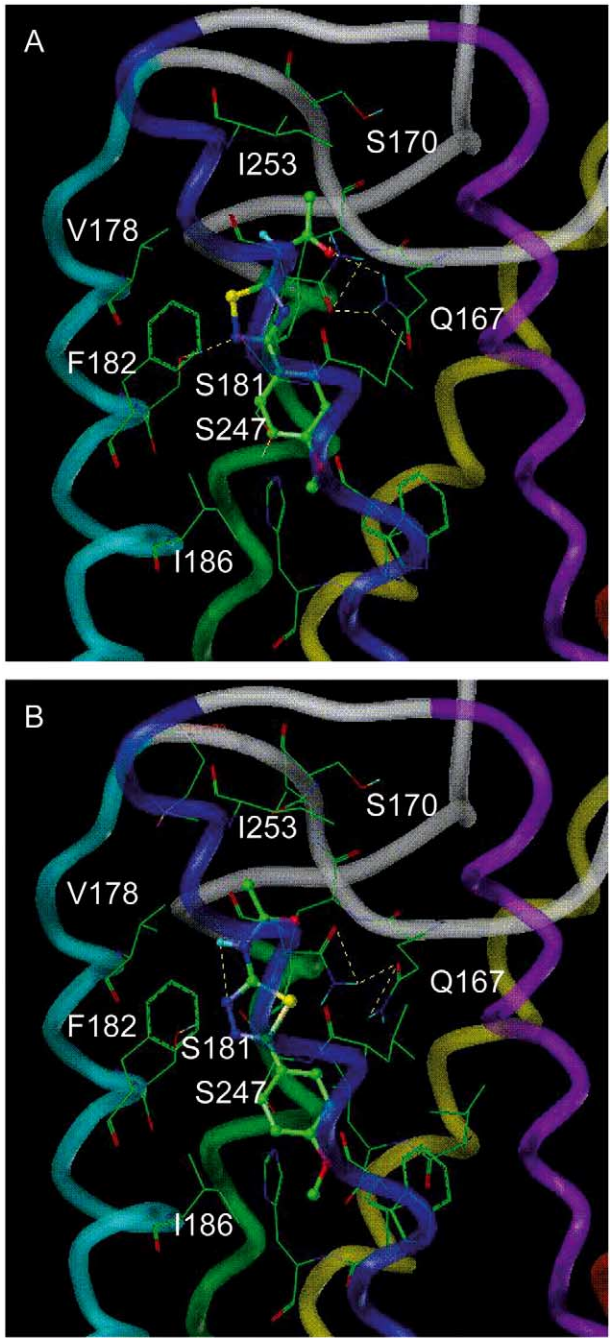

Figure 1. The complex of the human adenosine A_3 receptors with two regioisomers in the putative binding site of the receptor model. (A) a 1,2,4-thiadiazole compound **39** and (B) 1,3,4-thiadiazole analogue **42**. All ligands are displayed as ball-and-stick models, and the side chains of human A_3 receptors are shown as line models. The H-bonding between ligand and the receptor is displayed in yellow. The human A_3 receptor is represented by a tube model with a different color for each TM domain (TM3 in yellow, TM4 in green, TM5 in cyan, TM6 in blue, TM7 in purple).

The adenosine receptor sequence alignment indicated that most of the amino acids in the putative binding site within 5Å of the A_3-selective antagonist **39** were

conserved among adenosine receptors. Highly conserved amino acids were T94$^{3.36}$, V178$^{5.39}$, F168^{EL2}, F182$^{5.43}$, W243$^{6.48}$, and N250$^{6.55}$. However, some variable residues among the four subtypes of ARs, H95$^{3.37}$, Q167^{EL2}, S170^{EL2}, S247$^{6.52}$, and I253 $^{6.58}$, were located near R, X, and Y positions of the thiazole compound. Thus, variation of substituents in proximity to R, X and Y positions would be expected to increase the affinity and selectivity for the human A_3 subtype through additional H-bonding or hydrophobic interactions.

4.1. Functional assay of cAMP biosynthesis

The most potent derivative, **39**, was further evaluated in a functional assay, which measured the antagonism of the inhibitory effect on cAMP production via Gi protein mediated signal transduction system upon activation of the adenosine A_3 receptor. Compound **39** displayed functional antagonistic properties with antagonism of inhibition of cAMP production by Cl-IB-MECA, a selective A_3 receptor agonist in a concentration-dependent manner with K_B value of 1.7 nM (Fig. 2).

5. Conclusion

In summary, we have identified antagonists for the human adenosine A_3 receptor having subnanomolar affinity and high selectivity versus other subtype receptors through structure–activity relationship studies of aminothiazole and aminothiadiazole as the lead templates. An assay of cAMP production also demonstrated functional inhibition by the most potent antagonist **39**, and speculation of a binding mode of thiadiazole isomers was suggested by molecular docking experiments with a human adenosine A_3 receptor model built from homology modeling of the X-ray crystal structure of rhodopsin. The potent human adenosine A_3 receptor antagonists developed in this study will be useful to investigate biological significance of adenosine receptors and to develop therapeutics for the treatment of inflammatory disease, such as asthma, and glaucoma.

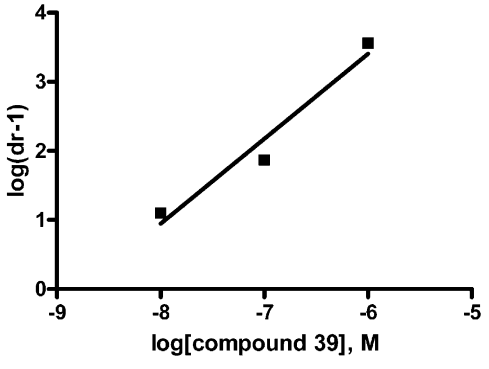

Figure 2. Antagonism by compound **39** of the inhibition of cyclic AMP production elicited by 100 nM Cl-IB-MECA in CHO cells stably transfected with the human adenosine A_3 receptors. The experiment was performed in the presence of 10 μM rolipram and 3 units/mL adenosine deaminase. Forskolin (10 μM) was used to stimulate cyclic AMP levels. The level of cAMP corresponding to 100% was 220±30 pmol mL^{-1}. The K_B value for compound **39** was calculated to be 1.7 nM.

6. Experimental

6.1. Chemistry

Proton nuclear magnetic resonance spectroscopy was performed on a Bruker AVANCE 600, JEOL JNM-LA 300WB spectrometers and spectra were taken in DMSO-d_6 or $CDCl_3$. Unless noted, chemical shifts are expressed as ppm downfield from tetramethylsilane as the internal standard, and J values are given in Hz. Mass spectroscopy was carried out on an MALDI-TOF and FAB instrument. All thiazole and thiadiazole derivatives showed one spot on thin layer chromatography (Merck silica gel 60; F_{254}, 0.25 mm). Elemental analyses determined on a Flash EA 1112series (CE Instruments) analyzer. Elemental and high-resolution mass analyses were performed in Seoul Branch Analytical Laboratory of Korea Basic Science Institute.

6.1.1. General procedure for the synthesis of 2-amino-4-[methoxy (or 4-chloro)-phenyl]-thiazole analogues (3). 1 mmol of 2-bromo-methoxy(or 4-chloro)acetophenone, 5 mmol of KSCN/SiO_2, and 18 mmol of NH_4OAc/Al_2O_3 were added in 15 mL of dry benzene. After stirring at 90 °C for 16 h, the yellow mixture was filtered and dried under reduced pressure. The residue was purified by silica gel column chromatography ($CHCl_3$/MeOH, 50:1–30:1) to afford the desired compound (**3a–d**). Yield 60%.

6.1.2. 5-Amino-3-(−4′-methoxyphenyl)-1,2,4-thiadiazole (7). Was synthesized from 4-methoxybenzonitrile **4**. A 100 mL 2-necked round-bottom flask was charged with 30 mL of anhydrous MeOH, 1.0 g (7.5 mmol) of the 4-methoxybenzonitrile **4**, and 0.04 g (0.75 mmol) of sodium methoxide. The contents of the flask were protected from moisture and stirred magnetically for 48 h. Then, 0.4 g (7.5 mmol) of NH_4Cl was added and stirring was continued for 24 h. Unreacted NH_4Cl was filtered, and methanol was stripped from the filtrate to afford the crude product, 4-methoxybenzamidine **5**, which was washed ether to remove unreacted 4-methoxybenzonitrile and used for the next step without further purification. 2.2 g (14.6 mmol) of **5** was dissolved into 50 mL water and mixed with 1 mL of sodium dodecylsulfate (as emulsion) and 3.2 mL (29.2 mmol) of perchloromethyl mercaptan. To the mixture was added a solution of 1.6 g of NaOH in 50 mL of water (0.04 M) dropwise, maintaining the temperature under 10 °C. Stirring was continued for another 15 min. The product color was changed to the orange-yellow, which was washed with cold water and dried under reduced pressure. The crude product was purified by silica gel column chromatography (hexanes–EtOAc, 30:1) to give 5-chloro-3-(4′-methoxyphenyl)-1,2,4-thiadiazole **6** (Yield 10%). ^{1}H NMR (600 MHz, DMSO-d_6) δ 3.84 (3H, s, CH_3O), 7.10 (2H, d, J = 7.2 Hz, phenyl-3H), 8.11 (2H, d, J = 7.2 Hz, phenyl-2H); MS [MALDI-TOF] m/z = 226.7 (M). Compound **6** was converted to 5-amino-3-(4′-methoxyphenyl)-1,2,4-thiadiazole **7** (Yield 34%) and 5-methylamino-3-(4′-methoxyphenyl)-1,2,4-thiadiazole **8** (Yield 74%) in ethanolic ammonia and methylamine at room temperature, respectively. **7:** ^{1}H NMR (300 MHz, DMSO-d_6) δ 3.80 (3H, s, CH_3O), 7.01 (2H, d, J = 9.0 Hz, phenyl-3H), 7.97 (2H, s, NH_2), 8.00 (2H, d, J = 9.0

Hz, phenyl-2H); MS [MALDI-TOF] $m/z = 207.2$ (M); **8**: ^{1}H NMR (300 MHz, DMSO-d_6) δ 2.97 (3H, d, $J = 4.8$, CH_3), 3.84 (3H, s, CH_3O), 7.02 (2H, d, $J = 9.0$ Hz, phenyl-3H), 8.04 (2H, d, $J = 9.0$ Hz, phenyl-2H), 8.42 (1H, s, NH); MS [MALDI-TOF] $m/z = 221.2$ (M).

6.1.3. 5-Amino-2-(4′-methoxyphenyl)-1,3,4-thiadiazole (10). 10 g (0.11 mol) of thiosemicarbazide and 13.3 g (0.11 mol) of *p*-anisaldehyde were dissolved in 30 mL ethanol. The mixture was stirred at room temperature for 7 h and filtered under reduced pressure. The residue was washed with ethanol to remove the side-products, and dried under vacuum oven to afford 19.83 g of **9** (86%). ^{1}H NMR (600 MHz, DMSO-d_6) δ 3.85 (3H, s, CH_3O), 6.92 (2H, d, $J = 8.4$ Hz, phenyl-3H), 7.02 (1H, s, NCH), 7.60 (2H, d, $J = 8.4$ Hz, phenyl-2H), 7.85 (2H, s, NH_2), 10.07 (1H, s, SCNH); MS [MALDI-TOF] $m/z = 209.3$ (M). A mixture of 1.67 g (8 mmol) of **9**, 360 mL of water and 6.0 g (22 mmol) of ferric chloride hexahydrate was stirred and heated at 80–90 °C for one-half hour, then filtered while hot. The filtrate was concentrated under reduced pressure and the residue was treated with 10% aqueous ammonia to an alkaline solution. The insoluble material was filtered, dried and purified by silica gel column chromatography ($CHCl_3$–MeOH, 20:1). Yield 5.6%. **10:** ^{1}H NMR (300 MHz, DMSO-d_6) δ 3.80 (3H, s, CH_3O), 7.00 (2H, d, $J = 9.0$ Hz, phenyl-3H), 7.29 (2H, s, NH_2), 7.66 (2H, d, $J = 9.0$ Hz, phenyl-2H); MS [MALDI-TOF] $m/z = 207.3$ (M).

6.1.4. General procedure for the preparation of *N*-acyl substituted thiazole and thiadiazole derivatives. Method A. A mixture of appropriate aminothiazoles or aminothiadiazoles (1 mmol), pyridine (2 mmol) and acyl chloride (3 mmol) in 5 mL of anhydrous dichloromethane was stirred at room temperature for 1 h. The solvent was removed under reduced pressure, and the residue was partitioned between saturated ammonium chloride solution (30 mL) and $CHCl_3$ (3×20 mL). The organic layer was dried on Na_2SO_4 and evaporated under vacuum, and the residue was purified by silica gel column chromatography (hexanes–EtOAc, 3:1) to afford the product as solid (**11**, **14**–**18**, **20**–**22**, **24**, **26**–**29**, **31**, **33**–**43**).

6.1.5. Method B. The appropriate aminothiazole (0.242 mmol) was dissolved in absolute EtOH (3 mL), and ethyl cyanoacetate (0.726 mmol) and sodium ethoxide (0.726 mmol) were added. The mixture was refluxed for 3 h, then, after cooling, the solvent was removed under reduced pressure and the crude product was purified by column chromatography on silica gel ($CHCl_3$/MeOH, 50:1) to afford the product as solid (**13** and **23**).

6.1.6. Method C. To the appropriate aminothiazole (0.2 mmol) in 3 mL of absolute dichloromethane was added dimethylaminopyridine (0.3 mmol) and corresponding anhydride (0.4 mmol). The mixture was stirred at room temperature for 3 h and the solvent was removed under reduced pressure. The residue was purified by column chromatography on silica gel (hexanes–EtOAc, 6:1) to afford the final products (**12, 19** and **25**).

6.1.7. Method D. A mixture of 40 mg of 2-amino-4-(4α-methoxyphenyl)-thiazole **3a** (0.19 mmol), 74.4 mg of EDAC (0.388 mmol), 71.1 mg of dimethylaminopyridine (0.582 mmol) and appropriate acid (0.388 mmol) in 3 mL of anhydrous dichloromethane was stirred at room temperature for 2 h. The solvent was removed under reduced pressure, and the residue was partitioned between saturated ammonium chloride (30 mL) and $CHCl_3$ (3×20 mL). The organic layer was dried on Na_2SO_4 and evaporated under vacuum. The residue was purified by column chromatography on silica gel (hexanes–EtOAc, 6:1) to afford the final products as solids (**30** and **32**).

6.1.8. *N*-(4-Phenyl-thiazol-2-yl)-acetamide (11). Yield 20%; ocher yellow powder; ^{1}H NMR (600 MHz, $CDCl_3$) δ 1.94 (3H, s, CH3), 7.14 (1H, d, $J = 7.0$ Hz, phenyl-4H), 7.34 (1H, s, thiazole-3H), 7.41 (2H, dd, $J = 6.5$, 7.0 Hz, phenyl-3H), 7.81 (2H, d, $J = 6.5$ Hz, phenyl-2H), 10.67 (1H, s, NH); HRMS calcd for $C_{11}H_{10}N_2OS$ (MH^+) 219.0514, found 219.0511.

6.1.9. (4-Phenyl-thiazol-2-yl)-carbamic acid *tert*-butyl ester (12). Yield 19.1% orange-yellow powder; ^{1}H NMR (600 MHz, $CDCl_3$) δ 1.61 (9H, s, $(CH_3)_3CO$), 7.08 (1H, d, $J = 7.0$ Hz phenyl-4H), 7.37 (1H, s, thiazole-3H), 7.39-7.41 (2H, dd, $J = 6.5$, 7.0 Hz, phenyl-3H), 7.89 (2H, d, $J = 6.5$ Hz, phenyl-2H), 11.45 (1H, s, NH). HRMS calcd for $C_{14}H_{16}N_2O_2S$ (MH^+) 277.0932, found 277.0933.

6.1.10. 2-Cyano-*N*-(4-phenyl-thiazol-2-yl)-acetamide (13). Yield 52% ochre yellow powder; ^{1}H NMR (600 MHz, $CDCl_3$) δ 3.1 (2H, s, CH_2), 7.26 (1H, s, thiazole-3H), 7.41–7.44 (1H, d, $J = 7.6$ Hz, phenyl-4H), 7.47–7.50 (2H, dd, $J = 7.1$, 7.6 Hz, phenyl-3H) 7.79 (2H, d, $J = 7.1$ Hz, phenyl-2H), 12.61 (1H, s, NH). HRMS calcd for $C_{12}H_9N_3OS$ (MH^+) 244.0466, found 244.0461.

6.1.11. *N*-[4-(4-Chloro-phenyl)-thiazol-2-yl]-acetamide (14). Yield 86.1% white powder; ^{1}H NMR (300 MHz, DMSO-d_6) δ 2.16 (3H, s, CH_3), 7.50 (2H, d, $J = 8.4$ Hz, phenyl-3H), 7.67 (1H, s, thiazole-3H), 7.90 (2H, d, $J = 8.4$ Hz, phenyl-2H), 12,31 (1H, s, NH); MS [MALDI-TOF] $m/z = 252.2$ (M). Anal. calcd for $C_{11}H_9ClN_2OS$: C, 52.28; H, 3.59; N, 11.08. Found: C, 51.96; H, 3.44; N, 11.27.

6.1.12. *N*-[4-(4-Chloro-phenyl)thiazol-2-yl]-2-phenyl-acetamide (15). Yield 98.6% light green powder; ^{1}H NMR (300 MHz, DMSO-d_6) δ 3.76 (2H, d, $J = 7.5$ Hz, $CH_2C_6H_5$), 7.14–7.48 (5H, m, phenyl-4H + phenyl-3H + phenyl 2H), 7.50 (2H, d, $J = 8.7$ Hz, phenyl-3H), 7.68 (1H, s, thiazole-3H), 7.90 (2H, d, $J = 8.7$ Hz, phenyl-2H), 12.52 (1H, s, NH). HRMS calcd for $C_{17}H_{13}ClN_2OS$ (MH^+) 329.0437, found 329.0433.

6.1.13. *N*-[4-(4-Methoxy-phenyl)-thiazol-2-yl]-acetamide (16). Yield 20% light green powder; ^{1}H NMR (300 MHz, DMSO-d_6) δ 3.77 (3H, s, CH_3), 6.97 (2H, d, $J = 8.7$ Hz, phenyl-3H), 7.41 (1H, s, thiazole-3H), 7.80 (2H, d, $J = 8.7$ Hz, phenyl-2H), 12.19 (1H, s, NH). HRMS cald for $C_{12}H_{12}N_2O_2S$ (MH^+) 249.0619, found 249.0627.

6.1.14. *N*-[4-(3-Methoxy-phenyl)-thiazol-2-yl]-acetamide (17). Yield 62.1% white powder; ^{1}H NMR (300 MHz, DMSO-d_6) δ 2.16 (3H, s, CH_3), 3.79 (3H, s, CH_3O), 6.90 (1H, d, $J=8.1$ Hz, phenyl-6′·H), 7.33 (1H, t, $J=8.1$ Hz, phenyl-5′·H), 7.43-7.48 (2H, m, phenyl-2′H + phenyl-4′H), 7.63 (1H, s, thiazole-3H), 12.26 (1H, s, NH). HRMS calcd for $C_{12}H_{12}N_2O_2S$ (MH^+) 249.0619, found 249.0618.

6.1.15. *N*-[4-(2-Methoxy-phenyl)-thiazol-2-yl]-acetamide (18). Yield 99.3% white powder; ^{1}H NMR (300 MHz, DMSO-d_6) δ 2.16 (3H, s, $COCH_3$), 3.91 (3H, s, CH_3O), 7.03 (1H, t, $J=7.5$ Hz, phenyl-5′ H), 7.13 (1H, d, $J=7.5$ Hz, phenyl-6′ H), 7.28 (1H, dd, $J=7.5$, 7.8 Hz, phenyl-4′ H), 7.62 (1H, s, thiazole-3H), 8.05 (1H, d, $J=7.8$ Hz, phenyl-3′ H), 12.18 (1H, s, NH); MS [MALDI-TOF] $m/z=248.2$ (M). Anal. calcd For $C_{12}H_{12}N_2O_2S$: C, 58.05; H, 4.87; N, 11.28. Found: C,57.93; H, 4.49; N, 11.08.

6.1.16. 2,2,2-Trifluoro-*N*-[4-(4-methoxy-phenyl)-thiazol-2-yl]-acetamide (19). Yield 20.7% white powder; ^{1}H NMR (300 MHz, DMSO-d_6) δ 3.79 (3H, s, CH_3O), 7.03 (2H, d, $J=9.0$ Hz, phenyl-3H), 7.57 (1H, s, thiazole-3H), 7.82 (2H, d, $J=9.0$ Hz, phenyl-2H), 14.12 (1H, broad s, NH); MS [MALDI-TOF] $m/z=302.1$ (M). Anal. calcd for $C_{12}H_9F_3N_2O_2S$: C, 47.68; H, 3.00; N, 9.27. Found: C, 47.38; H, 2.80; N, 9.08.

6.1.17. *N*-[4-(4-Methoxy-phenyl)-thiazol-2-yl]-propionamide (20). Yield 74.7% white powder; ^{1}H NMR (300 MHz, DMSO-d_6) δ 1.1 (3H, t, $J=7.5$ Hz, CH_3), 2.46 (2H, q, $J=7.5$ Hz, CH_2), 3.78 (3H, s, CH_3O), 6.97 (2H, d, $J=9.0$ Hz, phenyl-3H), 7.42 (1H, s, thiazole-3H), 7.83 (2H, d, $J=9.0$ Hz, phenyl-2H), 12.24 (1H, s, NH); MS [MALDI-TOF] $m/z=262.4$ (M). Anal. calcd for $C_{13}H_{14}N_2O_2S$: C, 59.52; H, 5.38; N, 10.68. Found: C, 59.43; H, 5.52; N, 10.39.

6.1.18. *N*-[4-(4-Methoxy-phenyl)-thiazol-2-yl]-butyramide (21). Yield 82.2% white powder; ^{1}H NMR (300 MHz, DMSO-d_6) δ 0.90 (3H, t. $J=7.3$ Hz, CH_3), 1.59–1.66 (2H, m, CH_2CH_3), 2.41 (2H, t, $J=7.3$ Hz, $COCH_2$), 3.78 (1H, s, CH_3O), 6.99 (2H, d, $J=4.8$ Hz, phenyl-3H), 7.43 (1H, s, thiazole-3H), 7.82 (2H, d, $J=4.8$ Hz, phenyl-2H), 12.36 (1H, s, NH); MS [MALDI-TOF] $m/z=276.1$ (M). Anal. calcd for $C_{14}H_{16}N_2O_2S$: C, 60.85; H, 5.84; N, 10.14. Found: C, 60.53; H, 5.91; N, 9.92.

6.1.19. *N*-[4-(4-Methoxy-phenyl)-thiazol-2-yl]-isobutyramide (22). Yield 71.6% white crystals; ^{1}H NMR (300 MHz, DMSO-d_6) δ 1.10 (6H, d, $J=7.2$ Hz, $CH(CH_3)_2$), 2.75 (1H, m, $CH(CH_3)_2$), 3.78 (1H, s, CH_3O), 6.99 (2H, d, $J=8.7$ Hz, phenyl-3H), 7.43 (1H, s, thiazole-3H), 7.82 (2H, d, $J=8.7$ Hz, phenyl-2H), 12.18 (1H, s, NH); MS [MALDI-TOF] $m/z=276.7$ (M). Anal. calcd for $C_{14}H_{16}N_2O_2S$: C, 60.85; H, 5.84; N, 10.14. Found: C, 60.71; H, 5.89; N, 10.01.

6.1.20. 2-Cyano-*N*-[4-(4-methoxy-phenyl)-thiazol-2-yl]-acetamide (23). Yield 91.2% ocher yellow powder; ^{1}H NMR (300 MHz, DMSO-d_6) δ 3.79 (3H, s, CH_3O), 4.05 (2H, d, $J=3.6$ Hz, CH_2), 6.98 (2H, d, $J=8.4$ Hz, phenyl-3H), 7.52 (1H, s, thiazole-3H), 7.82 (2H, d, $J=8.4$ Hz, phenyl-2H), 12.56 (1H, s, NH). HRMS calcd for $C_{13}H_{11}N_3O_2S$ (MH^+) 274.0572, found 274.0583.

6.1.21. *6.1.21.N*-[4-(4-Methoxy-phenyl)-thiazol-2-yl]-2,2-dimethyl-propionamide (24). Yield 95% white crystals; ^{1}H NMR (300 MHz, DMSO-d_6) δ 1.25 (9H, s, $C(CH_3)_3$), 3.78 (3H, s, CH_3O), 6.99 (2H, d, $J=12.0$ Hz, phenyl-3H), 7.43 (1H, s, thiazole-3H), 7.85 (2H, d, $J=12.0$ Hz, phenyl-2H), 11.79 (1H, s, NH); MS [MALDI-TOF] $m/z=289.6$ (M). Anal. calcd for $C_{15}H_{18}N_2O_2S$: C, 62.04; H, 6.25; N, 9.65. Found: C, 62.33; H, 6.34; N, 9.43.

6.1.22. [4-(4-Methoxy-phenyl)-thiazol-2-yl]-carbamic acid *tert*-butyl ester (25). Yield 12% orange-yellow crystals; ^{1}H NMR (300 MHz, $CDCl_3$) δ 1.60 (9H, s, $OC(CH_3)_3$), 3.84 (3H, s, CH_3O), 6.93 (2H, d. $J=8.4$ Hz, phenyl-3H), 7.26 (1H, s, thiazole-3H), 7.80 (2H, d, $J=8.4$ Hz, phenyl-2H), 11.44 (1H, s, NH). HRMS calcd for $C_{15}H_{18}N_2O_3S$ (MH^+) 307.1038, found 307.1032.

6.1.23. *N*-[4-(4-Methoxy-phenyl)-thiazol-2-yl]-benzamide (26). Yield 56.1% white powder; ^{1}H NMR (300 MHz, DMSO-d_6) δ 3.79 (3H, s, CH_3O), 6.99 (2H, d, $J=8.7$ Hz, phenyl-3H), 7.51–7.64 (3H, m, COC_6H_5-H3 + COC_6H_5-H4), 7.53 (1H, s, thiazole-3H), 7.87 (2H, d, $J=8.7$ Hz, phenyl-2H), 8.14 (2H, d, $J=8.1$ Hz, COC_6H_5-H2), 12.83 (1H, s, NH). HRMS calcd for $C_{17}H_{14}N_2O_2S$ (MH^+) 311.0776, found 311.0773.

6.1.24. *N*-[4-(4-Methoxy-phenyl)-thiazol-2-yl]-2-phenyl-acetamide (27). Yield 41.8% white powder; ^{1}H NMR (300 MHz, DMSO-d_6) δ 3.79 (2H, s, CH_2), 3.79 (3H, s, CH_3O), 6.97 (2H, d, $J=8.7$ Hz, phenyl-3H), 7.24-7.35 (5H, m, C_6H_5-2H + C_6H_5-3H + C_6H_5-4H), 7.44 (1H, s, thiazole-3H), 7.83 (2H, d, $J=8.7$ Hz, phenyl-2H), 12.47 (1H, s, NH). HRMS calcd for $C_{18}H_{16}N_2O_2S$ (MH^+) 325.0932, found 325.0931.

6.1.25. *N*-[4-(4-Methoxy-phenyl)-thiazol-2-yl]-3-phenyl-propionamide (28). Yield 80.6% white powder; ^{1}H NMR (300 MHz, DMSO-d_6) δ 2.76 (2H, t, $J=8.1$ Hz, $CH_2C_6H_5$), 2.90 (2H, t, $J=8.1$ Hz, $COCH_2$), 3.78 (3H, s, CH_3O), 6.99 (2H, d, $J=8.7$ Hz, phenyl-3H), 7.21-7.29 (5H, m, C_6H_5-2H + C_6H_5-3H + C_6H_5-4H), 7.43 (1H, s, thiazole-3H), 7.80 (2H, d, $J=8.7$ Hz, phenyl-2H), 12.25 (1H, s, NH). HRMS calcd for $C_{19}H_{18}N_2O_2S$ (MH^+) 339.1089, found 339.1081.

6.1.26. 2-(4-Methoxy-phenyl)-*N*-[4-(4-methoxy-phenyl)-thiazol-2-yl]-acetamide (29). Yield 61.1% lemon-yellow powder; ^{1}H NMR (300 MHz, DMSO-d_6) δ 3.69 (2H, s, $COCH_2$), 3.73 (3H, s, $CH_3OC_6H_4CH_2$), 3.78 (3H, s, CH_3O), 6.88 (2H, d, $J=8.4$ Hz, $CH_2C_6H_4OCH_3$-3H), 6.97 (2H, d, $J=8.7$ Hz, thiazole-C_6H_4-3H), 7.25 (2H, d, $J=8.4$ Hz, $CH_2C_6H_4OCH_3$-2H), 7.44 (1H, s, thiazole-3H), 7.83 (2H, d, $J=8.7$ Hz, thiazole-C_6H_4-2H), 12.32 (1H, s, NH); MS [MALDI-TOF] $m/z=354.7$ (M). Anal. calcd for $C_{19}H_{18}N_2O_3S$: C, 64.39; H, 5.12; N, 7.90. Found: C, 64.13; H, 5.01; N, 7.88.

6.1.27. 3-(4-Methoxy-phenyl)-*N*-[4-(4-methoxy-phenyl)-thiazol-2-yl]-propionamide (30). Yield 43.4% gray-brown powder; ^{1}H NMR (300 MHz, DMSO-d_6) δ 2.50 (2H, t, J = 7.5 Hz, $CH_2C_6H_4OCH_3$), 2.71 (2H, t, J = 7.5 Hz, $COCH_2$), 3.70 (3H, s, $CH_3OC_6H_4CH_2$), 3.78 (3H, s, CH_3O), 6.86 (2H, d, J = 8.4 Hz, $CH_2C_6H_4OCH_3$-3H), 6.99 (2H, d, J = 8.7 Hz, thiazole-C_6H_4-3H), 7.16 (2H, d, J = 8.7 Hz, thiazole-C_6H_4-2H), 7.43 (1H, s, thiazole-3H), 7.82 (2H, d, J = 8.4 Hz, $CH_2C_6H_4OCH_3$-2H), 12.36 (1H, s, NH); MS [MALDI-TOF] m/z = 367.8(M). Anal. calcd for $C_{20}H_{20}N_2O_3S$: C, 65.20; H, 5.47; N, 7.60. Found: C, 64.87; H, 5.40; N, 7.66.

6.1.28. *N*-[4-(4-Methoxy-phenyl)-thiazol-2-yl]-2,2-diphenylacetamide (31). Yield 39.5% white powder; ^{1}H NMR (300 MHz, DMSO-d_6) δ 3.78 (3H, s, CH_3O), 5.37 (1H, s, $CH(C_6H_5)_2$), 6.99 (2H, d, J = 8.7 Hz, $CH_3OC_6H_4$-3H), 7.22–7.40 (10H, m, $CH(C_6H_5)_2$), 7.48 (1H, s, thiazole-3H), 7.82 (2H, d, J = 8.7 Hz, $CH_3OC_6H_4$-2H), 12.67 (1H, s, NH). HRMS calcd for $C_{24}H_{20}N_2O_2S$ (MH^+) 401.1245, found 401.1243.

6.1.29. *N*-[4-(4-Methoxy-phenyl)-thiazol-2-yl]-3,3-diphenyl-propionamide (32). Yield 79.3% light-brown crystals; ^{1}H NMR (300 MHz, DMSO-d_6) δ 3.26 (2H, d, J = 8.1 Hz, CH_2), 3.78 (3H, s, CH_3O), 4.61 (1H, s, $CH(C_6H_5)_2$), 6.99 (2H, d, J = 8.7 Hz, $CH_3OC_6H_4$-3H), 7.14–7.2 (5H, m, phenyl-2H + phenyl-3H + phenyl-4H), 7.25–7.33 (5H, m, phenyl-2H + phenyl-3H + phenyl-4H), 7.39 (1H, s, thiazole-3H), 7.78 (2H, d, J = 8.7 Hz, $CH_3OC_6H_4$-2H), 12.67 (1H, s, NH); MS [MALDI-TOF] m/z = 413.8 (MS). Anal. calcd for $C_{25}H_{22}N_2O_2S$: C, 72.44; H, 5.35; N, 6.76. Found: C, 72.26; H, 5.62; N, 6.58.

6.1.30. Furan-2-carboxylic acid [4-(4-methoxy-phenyl)-thiazol-2-yl]-amide (33). Yield 97.8% light-brown powder; ^{1}H NMR (300 MHz, DMSO-d_6) δ 3.79 (3H, s, CH_3O), 6.74 (1H, dd, J = 1.5, 2.1 Hz, furan-3H), 7.0 (2H, d, J = 9.0 Hz, phenyl-3H), 7.51 (1H, s, thiazole-3H), 7.71 (1H, d, J = 1.5 Hz, furan-2H), 7.88 (2H, d, J = 9.0 Hz, phenyl-2H), 8.02 (1H, d, J = 2.1 Hz, furan-4H), 12.61 (1H, s, NH). HRMS calcd for $C_{15}H_{12}N_2O_3S$ (MH^+) 301.0569, found 301.0571.

6.1.31. *N*-[4-(4-Methoxy-phenyl)-thiazol-2-yl]-2-thiophen-2-yl-acetamide (34). Yield 11.7% light-gray powder; ^{1}H NMR (300 MHz, DMSO-d_6) δ 3.79 (3H, s, CH_3O), 4.03 (2H, s, CH_2), 6.98–7.12 (2H, m, thiophen-4H + thiophen-2H), 7.15 (2H, d, J = 8.7 Hz, phenyl-3H), 7.42 (1H, t, J = 1.5 Hz, thiophen-3H), 7.47 (1H, s, thiazole-3H), 7.98 (2H, d, J = 8.7 Hz, phenyl-2H), 12.51 (1H, s, NH). HRMS calcd for $C_{16}H_{14}N_2O_2S_2$ (MH^+) 331.0497, found 331.0492.

6.1.32. Thiophene-2-carboxylic acid [4-(4-methoxy-phenyl)-thiazol-2-yl]-amide (35). Yield 18% light-gray powder; ^{1}H NMR (300 MHz, DMSO-d_6) δ 3.79 (3H, s, CH_3O), 6.99 (2H, d. J = 8.7 Hz, phenyl-3H), 7.26 (1H, dd, J = 2.7, 3.9 Hz, thiophenyl-3H), 7.52 (1H, s, thiazole-3H), 7.87 (2H, d, J = 8.7 Hz, phenyl-2H), 7.98 (1H, d, J = 3.9 Hz, thiophenyl-2H), 8.31 (1H, d, J = 2.7 Hz, thiophenyl-4H), 12.87 (1H, s, NH). HRMS calcd for $C_{15}H_{12}N_2O_2S_2$ (MH^+) 317.0340, found 317.0347.

6.1.33. [3-(4-Methoxy-phenyl)-[1,2,4]-thiadiazol-5-yl]-methyl-amine (36). Yield 74% white powder; ^{1}H NMR (300 MHz, DMSO-d_6) δ 2.95 (3H, d, J = 4.5 Hz, CH_3), 3.80 (3H, s, CH_3O), 7.01 (2H, d, J = 9.0 Hz, phenyl-3H), 8.03 (2H, d, J = 9.0 Hz, phenyl-2H), 8.43 (1H, broad s, NH). HRMS calcd for $C_{10}H_{11}N_3OS$ (MH^+) 222.0623, found 222.0623.

6.1.34. *N*-(3-Phenyl-[1,2,4]thiadiazol-5-yl)-acetamide (37). Yield 67.8% white crystals; ^{1}H NMR (300 MHz, DMSO-d_6) δ 2.27 (3H, s, CH_3), 7.51 (1H, d, J = 5.1 Hz, phenyl-4H), 7.54 (2H, d, J = 4.5 Hz, phenyl-2H), 8.16 (2H, dd, J = 4.5, 5.1 Hz, phenyl-3H), 13.10 (1H, broad s, NH); MS [MALDI-TOF] m/z = 219.0 (M). Anal. calcd for $C_{10}H_9N_3OS$: C, 54.78; H, 4.14; N, 19.16. Found: C, 54.48; H, 4.01; N, 19.02.

6.1.35. 2-Phenyl-*N*-(3-phenyl-[1,2,4]thiadiazol-5-yl)-acetamide (38). Yield 41.2% white powder; ^{1}H NMR (300 MHz, DMSO-d_6) δ 3.91 (2H, s, CH_2), 7.21–7.36 (5H, m, phenyl-2H + phenyl-3H + phenyl-4H), 7.51 (1H, d, J = 2.1 Hz, phenyl-4H), 7.54 (2H, d, J = 4.5 Hz, phenyl-2H), 8.17 (2H, dd, J = 2.1, 4.5 Hz, phenyl-3H), 12.93 (1H, s, NH). HRMS calcd for $C_{16}H_{13}N_3OS$ (MH^+) 296.0799, found 296.0791.

6.1.36. *N*-[3-(4-Methoxy-phenyl)-[1,2,4]thiadiazol-5-yl]-acetamide (39). Yield 74.2% white powder; ^{1}H NMR (300 MHz, DMSO-d_6) δ 2.26 (3H, s, CH_3), 3.82 (3H, s, CH_3O), 7.04 (2H, d, J = 8.7 Hz, phenyl-3H), 8.07 (2H, d, J = 8,7 Hz, phenyl-2H), 13.05 (1H, s, NH). HRMS calcd for $C_{11}H_{11}N_3O_2S$ (MH^+) 250.0572, found 250.0571.

6.1.37. *N*-[3-(4-Methoxy-phenyl)-[1,2,4]thiadiazol-5-yl]-2-phenyl-acetamide (40). Yield 81.0% white powder; ^{1}H NMR (300 MHz, DMSO-d_6) δ 3.82 (3H, s, CH_3O), 3.90 (2H, s, CH_2), 7.05 (2H, d, J = 9.0 Hz, phenyl-3H), 7.25–7.36 (5H, m, C_6H_5-2H + C_6H_5-3H + C_6H_5-4H), 8.08 (2H, d, J = 9.0 Hz, phenyl-2H), 13.25 (1H, s, NH). HRMS calcd for $C_{17}H_{15}N_3O_2S$ (MH^+) 326.0885, found 326.0889.

6.1.38. *N*-[3-(4-Methoxy-phenyl)-[1,2,4]thiadiazol-5-yl]-propionamide (41). Yield 52.0% white powder; ^{1}H NMR (300 MHz, DMSO-d_6) δ 1.10 (3H, t, J = 7.5 Hz, CH_3), 2.53 (2H, q, J = 7.5 Hz, CH_2), 3.82 (3H, s, CH_3O), 7.04 (2H, d, J = 9.0 Hz, phenyl-3H), 8.08 (2H, d, J = 9.0 Hz, phenyl-2H), 12.97 (1H, s, NH). HRMS calcd for $C_{12}H_{13}N_3O_2S$ (MH^+) 264.0728, found 264.0731.

6.1.39. *N*-[5-(4-Methoxy-phenyl)-[1,3,4]thiadiazol-2-yl]-acetamide (42). Yield 79.0% white powder; ^{1}H NMR (300 MHz, DMSO-d_6) δ 2.20 (3H, s, CH_3), 3.83 (3H, s, CH_3O), 7.08 (2H, d, J = 9.0 Hz, phenyl-3H), 7.88 (2H, d, J = 9.0 Hz, phenyl-2H), 12.51 (1H, broad s, NH); MS [MALDI-TOF] m/z = 248.9 (M). Anal. calcd for $C_{11}H_{11}N_3O_2S$: C, 53.00; H, 4.45; N, 16.86. Found: C, 52.97; H, 4.33; N, 16.98.

6.1.40. *N*-[5-(4-Methoxy-phenyl)-[1,3,4]thiadiazol-2-yl]-2-phenyl-acetamide (43). Yield 62.3% white powder; ^{1}H

NMR (300 MHz, DMSO-d_6) δ 3.81 (3H, s, CH_3O), 3.90 (2H, s, CH_2), 7.05 (2H, d, $J = 9.0$ Hz, phenyl-3H), 7.26–7.35 (5H, m, C_6H_5-2H + C_6H_5-3H + C_6H_5-4H), 7.84 (2H, d, $J = 9.0$ Hz, phenyl-2H), 12.87 (1H, broad s, NH). HRMS calcd for $C_{18}H_{16}N_2O_2S$ (MH^+) 325.0932, found 325.0933.

6.2. Pharmacology

[^{125}I]N^6-(4-Amino-3-iodobenzyl)adenosine-5′-*N*-methyluronamide (I-AB-MECA; 2000 Ci/mmol), [^{3}H]-(*R*)-PIA ([^{3}H]-(*R*)-N^6-(phenylisopropyl)adenosine), [^{3}H]-CGS 21680 ([^{3}H]-2-[4-(2-carboxyethyl)phenyl]ethylamino]-5′-(*N*-ethylcarbamoyl)adenosine), and [^{3}H]cyclic AMP (40 Ci/mmol) were from Amersham Biosciences (Piscataway, NJ). All other reagents including Cl-IB-MECA (N^6-(3-iodobenzyl)-2-chloro-adenosine-5′-*N*-methyluronamide) were obtained from Sigma (St. Louis, MO, USA).

CHO (Chinese Hamster Ovary) cells expressing the recombinant human A_1, A_3 adenosine receptors and HEK293 cells expressing the recombinant human adenosine A_{2A} receptors were cultured in Dulbecco Modified Eagle's Medium (DMEM) and F12 (1:1) supplemented with 10% fetal bovine serum, 100 units/mL penicillin, 100 μg/mL streptomycin, 2 μmol/mL glutamine and 800 μg/mL geneticin. After homogenization and suspension, cells were centrifuged at 500 *g* for 10 min, and the pellet was re-suspended in 50 mM Tris–HCl buffer (pH 8.0) containing 10 mM $MgCl_2$. The suspension was homogenized with an electric homogenizer for 10 s, and was then re-centrifuged at 20,000*g* for 20 min at 4 °C. The resultant pellets were resuspended in buffer containing 3 U/mL adenosine deaminase, and the suspension was stored at −80 °C. Striatal and forebrain tissues from Wistar rats were homogenized in ice-cold 50 mM TrisHCl buffer, pH 7.4, using an electric homogenizer. The homogenate was centrifuged at 20,000*g* for 10 min at 4 °C, and the pellet was washed in fresh buffer. The final pellet was stored at −80 °C until the binding experiments. The protein concentration was measured using the Bradford assay.[35]

For binding to human A_1 receptors, [^{3}H]R-PIA (2 nM) was incubated with membranes (40 μg/tube) from CHO cells stably expressing human A_1 receptors at 25 °C for 60 min in 50 mM Tris–HCl buffer (pH 7.4; $MgCl_2$, 10 mM) in a total assay volume of 200 μL. Nonspecific binding was determined using 10 μM of N^6-cyclopentyladenosine. For human A_{2A} receptor binding, membranes (20 μg/tube) from HEK-293 cells stably expressing human A_{2A} receptors were incubated with 15 nM [^{3}H]CGS21680 at 25 °C for 60 min in 200 μL 50 mM Tris–HCl, pH 7.4, containing 10 mM $MgCl_2$. 5′-*N*-ethyluronamidoadenosine (10 μM) was used to define nonspecific binding. Reaction was terminated upon filtration over GF/B filters.

For binding to recmbinant A_3 receptors, each tube contained 50 μL membrane suspension (20 μg protein), 25 μL of [^{125}I]I-AB-MECA (0.5 nM), and 25 μL of increasing concentrations of the test ligands in Tris–HCl buffer (50 mM, pH 8.0) containing 10 mM $MgCl_2$, 1 mM EDTA. Nonspecific binding was determined using 10 μM Cl-IB-MECA in the buffer. The mixtures were incubated at 25 °C for 60 min. Binding reactions were terminated by filtration through Whatman GF/B filters under reduced pressure using a MT-24 cell harvester (Brandell, Gaithersburgh, MD, USA). Filters were washed three times with 9 mL ice-cold buffer. Radioactivity was determined in a Beckman 5500B γ-counter.

Intracellular cyclic AMP levels were measured with a competitive protein binding method.[36,37] CHO cells expressing human or rat adenosine A_3 receptors were harvested following trypsinization. After centrifugation and resuspended in medium, cells were planted in 24-well plates in 0.5 mL medium. After 24 h, the medium was removed and cells were washed three times with 1 mL DMEM, containing 50 mM HEPES, pH 7.4. Cells were then treated with agonists and/or test compounds in the presence of rolipram (10 μM) and adenosine deaminase (3 U/mL). After 45 min forskolin (10 μM) was added to the medium, and incubation was continued an additional 15 min. The reaction was terminated by removing the supernatant, and cells were lysed upon the addition of 200 μL of 0.1 M ice-cold HCl. The cell lysate was resuspended and stored at −20 °C. For determination of cyclic AMP production, protein kinase A (PKA) was incubated with [^{3}H]cyclic AMP (2 nM) in K_2HPO_4/EDTA buffer (K_2HPO_4, 150 mM; EDTA, 10 mM), 20 μL of the cell lysate, and 30 μL 0.1 M HCl or 50 μL of cyclic AMP solution (0–16 pmol/200 μL for standard curve). Bound radioactivity was separated by rapid filtration through Whatman GF/C filters and washed once with cold buffer. Bound radioactivity was measured by liquid scintillation spectrometry.

6.3. Molecular modeling

All calculations were performed on a Silicon Graphics (Mountain View, CA) Octane workstation (300 MHz MIPS R12000 (IP30) processor). All ligand structures were constructed with the use of the Sketch Molecule of SYBYL 6.9.[38] A conformational search of antagonists **39** and **42** was performed by grid search, rotating three rotatable bonds in 60° increments and the amide bond at 0° or 180°. A random search was also performed, using the following options for all rotatable bonds; 3000 iterations, 3-kcal energy cutoffs, and no chirality checking. In all cases, MMFF force field[39] and charge were applied with the use of distance-dependent dielectric constants and the conjugate gradient method until the gradient reached 0.05 kcal·mol^{-1}·Å^{-1}. Representative conformers selected from clusters of low-energy conformers obtained in the conformational search were reoptimized by semiempirical molecular orbital calculations with the PM3 method in the MOPAC 6.0 package.[40]

A human adenosine A_3 receptor model, which was previously built using homology modeling[41] from the X-ray structure of bovine rhodopsin with 2.8 Å resolution[42] was used for docking studies. For a brief explanation of this method, multiple-sequence alignment data of selected GPCRs were used for the construction of

human adenosine A_3 receptor TM domains..[43] For the model of the second extracellular loop, EL2, two beta-sheet domains in rhodopsin were first aligned, including the disulfide bond between Cys83 and Cys166, and then other components were added or deleted. To construct the N-terminal region and the intra- and extracellular loops, each alignment was manually adjusted to preserve the overall shape of the loop. *N*-acetyl and *N*-methyl amide groups blocked the N-terminal and C-terminal regions, respectively, to prevent electrostatic interactions. All helices with backbone constraints and loops were separately minimized. After combining, all structures were reminimized initially with backbone constraints in the secondary structure and then without any constraints. The Amber all-atom force field[44] with a fixed dielectric constant of 4.0 was used for all calculations, terminating when the conjugate gradient reached 0.05 kcal·mol^{-1}·Å^{-1}.

For the conformational refinement of the adenosine A_3 receptor, the optimized structures were then used as the starting point for subsequent 50-ps MD, during which the protein backbone atoms in the secondary structures were constrained as in the previous step. The options of MD at 300 K with a 0.2-ps coupling constant were a time step of 1fs and a nonbonded update every 25 fs. The lengths of bonds with hydrogen atoms were constrained according to the SHAKE algorithm.[45] The average structure from the last 10-ps trajectory of MD was reminimized with backbone constraints in the secondary structure and then without all constraints as described above.

For a starting point for FlexiDock,[46] the superimposition between **39** and MRS1220 (9-chloro-2-(2-furyl)[1,2,4]triazolo[1,5-c]quinazolin-5-phenylacetamide),[18] both A_3 selective antagonists, was performed. All heteroatoms except H and S atoms of **39** were fitted into the bound conformation of MRS1220 as a template. Flexible docking was facilitated through the FlexiDock utility in the Biopolymer module of SYBYL 6.9. During flexible docking, the ligand and the side chains of hydrophilic amino acids in the putative binding site were defined as rotatable bonds. After the hydrogen atoms were added to the receptor, atomic charges were recalculated by using Kollman All-atom for the protein and Gasteiger–Hückel for the ligand. H-bonding sites were marked for all residues in the active site and ligands with H-bond donor or acceptor. Ligands were variously pre-positioned in the putative binding cavity guided by several superimposition results. Default FlexiDock parameters were set at 3000-generations for genetic algorithms. To increase the binding interaction, the torsion angles of the side chains within 5 Å of the ligands were manually adjusted from the results of FlexiDock. Finally, the complex structure was minimized by using an Amber force field with a fixed dielectric constant (4.0), until the conjugate gradient reached 0.1 kcal·mol^{-1}·Å^{-1}.

Several possible docking modes of **39**, which were consistent with the SAR study, were selected from the energetically favorable models. The most similar conformation of antagonist **42** compared to the bound conformation of antagonist **39** was chosen and superimposed into the putative binding sites of several possible docking models. The energies of the two regioisomer complexes were compared.

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