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Synthesis and biological evaluation of 2-aminothiazoles and their amide derivatives on human adenosine receptors. Lack of effect of 2-aminothiazoles as allosteric enhancers

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Abstract—A number of 2-aminothiazoles (**2a–e**) and their amide derivatives (**4–10**) were prepared. The 2-aminothiazoles themselves were tested as allosteric enhancers of agonist binding to human adenosine A_1 receptors. In a variety of experimental set-ups the compounds did not show any such effect, in contrast to earlier findings by another research group. Subsequently the 2-aminothiazoles were used as intermediates in the synthesis of a number of amide derivatives of either aromatic (**4–6**) or aliphatic nature (**7–10**). Some of the compounds emerged as moderately active antagonists on human adenosine A_1 and/or A_{2A} receptors with lower or negligible potency at adenosine A_3 receptors.
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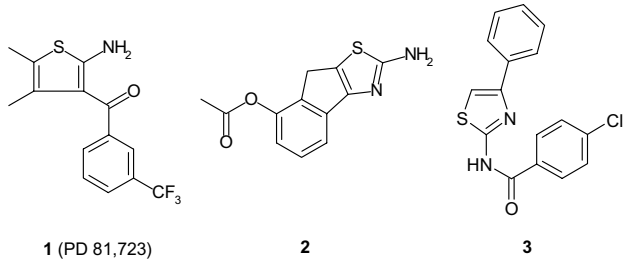
1. Introduction

Adenosine receptors are membrane-bound proteins belonging to the large family of G protein-coupled receptors. The four subtypes of adenosine receptors are the target for the endogenous ligand adenosine and as such they play an important role in intra- and intercellular communication. Their important role in physiology (e.g., in cardiac and metabolic homeostasis, in inflammation, and sleep/wake control mechanisms) has evoked the interest of medicinal chemists who, over the years, have generated a vast library of synthetic ligands interacting with these adenosine receptors.[1]

Among these ligands an interesting class of the so-called 'allosteric enhancers' of adenosine binding and function at the adenosine A_1 receptor was discovered next to 'classic' agonists and antagonists. The reference compound is PD 81,723,[2] a 2-amino-3-aroylthiophene (**1**, Fig. 1), which served as a template for a range of related structures with similar characteristics.[3–6] Although the receptor binding site for these allosteric modulators has not been elucidated yet, it is believed that such compounds indeed bind to another site on the receptor than the primary ligand binding site for adenosine and synthetic derivatives thereof.



1 (PD 81,723) **2** **3**

Figure 1. Chemical structures of putative allosteric enhancers (**1**, **2**) and antagonist (**3**) for adenosine A_1 receptors.

Interestingly, in a recent report a novel class of allosteric enhancers was identified. Certain 2-aminothiazoles were described to be potent enhancers of agonist binding surpassing the potency of PD 81,723 with **2** being the most active (Fig. 1).[7] Our attention was drawn by these findings, also because we had previously reported on a series

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of functionalized 2-aminothiazoles as adenosine receptor antagonists (e.g., **3** in Fig. 1).[8]

We therefore decided to synthesize and study a number of known as well as new 2-aminothiazoles with respect to their activity on adenosine receptors, including hybrid structures of compounds **2** and **3**. We were not able to confirm the allosteric enhancing capacity of the 2-aminothiazoles. Some of the compounds, however, proved to be antagonists for the adenosine receptors and showed affinity in the higher nanomolar range.

2. Results

2.1. Chemistry

To prepare the compounds we applied a known synthetic route with minor modifications.[7,9] 2-Aminothiazolium salts were prepared by a one-step synthesis from the appropriate ketones, thiourea, and iodine. 2-Aminothiazoles (**2a**–**e**) were obtained from the above HI salts after alkaline treatment. The syntheses of amide derivatives of 2-aminothiazoles (**4a**–**e**–**6a**–**e**; **7b**–**10b**) were achieved by condensation reactions of 2-aminothiazoles (**2a**–**e**) with the appropriate acylchlorides either under classic[8] or microwave conditions, which allowed us to prepare the compounds in approximately 20 min instead of 2–5 h. Optimized yields were not aimed for, and were in the range of 13–99% (Schemes 1 and 2).

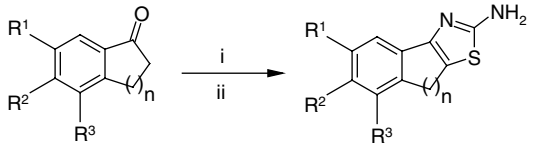
2.2. Biology

All compounds were initially characterized in equilibrium radioligand displacement studies at a final concentration of 1 μM on human adenosine A_1, A_{2A}, and A_3 receptors (Table 1). For A_1 adenosine receptors both a radiolabeled agonist ([^{3}H]CCPA) and a radiolabeled antagonist ([^{3}H]DPCPX) were used. Compounds **5c**, **4d**, **9b**, and **10b** significantly inhibited [^{3}H]DPCPX binding, that is, they displaced the radioligand for more than 50% (Table 1). We determined the apparent affinity of these compounds (Table 2). Of the four compounds **9b** proved to be the most potent with a K_i value of 144 ± 30 nM. Interestingly none of the compounds appeared to enhance [^{3}H]CCPA binding.

Subsequently, all compounds were studied on adenosine A_{2A} receptors with [^{3}H]ZM241385 as the radiolabeled antagonist. Compounds **4d**, **6d**, **8b**–**10b** significantly (i.e., >50%) inhibited [^{3}H]ZM241385 binding at the single concentration of 1 μM (Table 1). Of these eight compounds **8b** proved to be the most active with a K_i value of 262 ± 48 nM (Table 2). The compounds were also studied on adenosine A_3 receptors with [^{125}I]AB-MECA as the radiolabeled agonist. None of the compounds displaced the radioligand by more than 50% (Table 1), and hence we refrained from determining their K_i values.

Selected 2-aminothiazoles **2b** and **2c** (at concentrations of 10 μM) were used for further characterization as putative allosteric enhancers by studying their influence on association and dissociation kinetics of the adenosine A_1 receptor agonist radioligand [^{3}H]CCPA. The association kinetics of [^{3}H]CCPA were slightly affected by the two compounds with negligible effects on its dissociation characteristics. Compounds **2b** and **2c** appeared to increase the extent of association somewhat. For compound **2b** the highest value was 121%, for **2c** it was 110%, compared to the control of 93%. The rate constants for the two processes did not differ significantly between the treatment protocols (Fig. 2, Table 3).

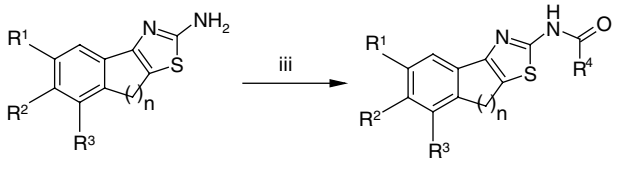
Finally the most active compounds in the adenosine A_1 receptor binding studies were also analyzed in a functional assay, that is, their influence on the cAMP production in cells expressing the human adenosine A_1 receptor. Compounds **9b** and **10b** were capable of blocking the effect induced by the reference agonist N^6-cyclopentyladenosine (CPA), demonstrating their antagonistic activity (Fig. 3).



2a-e

a: n = 1, $R^1 = R^2 = R^3$ = H; **b**: n = 2, $R^1 = R^2 = R^3$ = H;
c: n = 3, $R^1 = R^2 = R^3$ = H; **d**: n = 2, $R^1 = R^2$ = H, R^3 = OMe;
e: n = 2, $R^1 = R^2$ = OMe, R^3 = H

Scheme 1. Reagents and conditions: (i) iodine, thiourea, heating at 100 °C, (ii) NaOH.



2a-e **4a-e – 6a-e; 7b-10b**

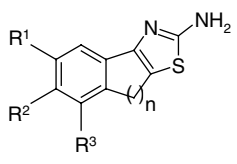
4a-e: R^4 = Ph; **5a-e**: R^4 = *p*NO_2-Ph; **6a-e**: R^4 = *p*Cl-Ph; **7b**: R^4 = Me;
8b: R^4 = Et; **9b**: R^4 = *t*Bu; **10b**: R^4 = *c*Pe

Scheme 2. Reagents and conditions: (iii) R^4–COCl, EtN_3, 1,4-dioxane.

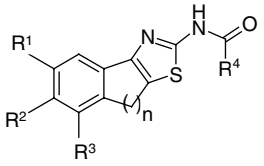
3. Discussion

Recently 2-aminothiazoles were introduced as a new class of agonist allosteric enhancers of adenosine A_1 receptors.[7] We first synthesized and analyzed such 2-aminothiazoles, both known (**2a**–**c**) and new derivatives (**2d**, **2e**). At a concentration of 1 μM none of these five compounds appeared to affect significantly either radiolabeled antagonist ([^{3}H]DPCPX) or radiolabeled agonist ([^{3}H]CCPA) binding to the adenosine A_1 receptor. Particularly the lack of effect on [^{3}H]CCPA binding surprised us, since an increase in its binding had been anticipated due to the presence of a putative allosteric enhancer. We then decided to study the influence of two compounds also reported by Chordia et al.[7] (**2a**

Table 1. Binding affinity of compounds **2a–e**, **4a-e–6a–e**, and **7b–10b** at human A_1, A_{2A}, and A_3 adenosine receptor subtypes (concentration of compounds was 10^{-6} M)



2a-e



4a-e – 6a-e; 7b-10b

Compd.	*n*	R^1	R^2	R^3	R^4	% Specific binding of radioligand remaining			
						A_1		A_{2A}	A_3
						[^{3}H]DPCPX	[^{3}H]CCPA	[^{3}H]ZM241385	[^{125}I]-AB-MECA
2a	1	H	H	H	—	101	96	100	105
2b	2	H	H	H	—	104	95	101	103
2c	3	H	H	H	—	96	103	90	97
2d	2	H	H	OMe	—	93	97	87	105
2e	2	OMe	OMe	H	—	100	98	96	105
4a	1	H	H	H	Ph	51	81	59	68
5a	1	H	H	H	*p*-NO_2–Ph	58	85	61	92
6a	1	H	H	H	*p*-Cl–Ph	88	93	82	86
4b	2	H	H	H	Ph	74	87	66	66
5b	2	H	H	H	*p*-NO_2–Ph	70	81	54	80
6b	2	H	H	H	*p*-Cl–Ph	81	86	69	71
4c	3	H	H	H	Ph	55	87	50	67
5c	3	H	H	H	*p*-NO_2–Ph	48	90	52	67
6c	3	H	H	H	*p*-Cl–Ph	60	94	51	65
4d	2	H	H	OMe	Ph	39	75	43	58
5d	2	H	H	OMe	*p*-NO_2–Ph	100	92	86	80
6d	2	H	H	OMe	*p*-Cl–Ph	65	79	44	57
4e	2	OMe	OMe	H	Ph	101	100	95	73
5e	2	OMe	OMe	H	*p*-NO_2–Ph	97	109	100	90
6e	2	OMe	OMe	H	*p*-Cl–Ph	97	109	93	102
7b	2	H	H	H	Me	84	89	54	65
8b	2	H	H	H	Et	70	82	43	54
9b	2	H	H	H	*t*-Bu	18	47	34	69
10b	2	H	H	H	*c*-Pe	21	54	40	88

Data are expressed as means from two independent experiments performed in duplicate; individual values varied less than 10% for [^{3}H]CCPA binding, less than 15% for [^{3}H]DPCPX binding, and less than 20% for [^{3}H]ZM241385 and [^{125} I]-AB-MECA binding. The results are given as percentage specific binding of radioligand remaining, where total control binding is 100% and nonspecific binding is 0%.

Table 2. Effects of various compounds as displacing agents of [^{3}H]DPCPX and [^{3}H]ZM241385 binding to human adenosine A_1 and A_{2A} receptors

Compound	A_1 K_i (nM)	A_{2A} K_i (nM)
4c	—	1040 ± 411
5c	801 ± 73	—
4d	374 ± 73	369 ± 67
6d	—	410 ± 60
8b	—	262 ± 48
9b	144 ± 30	343 ± 96
10b	171 ± 15	274 ± 17

and **2b**) on the association and dissociation kinetics of [^{3}H]CCPA. Alteration of the dissociation kinetics in particular is regarded as experimental evidence for allosteric modulation.[10] However, at a concentration of 10 μM we did not observe any effect on the dissociation kinetics with identical $t_{1/2}$ and k_{off} values under all conditions (Fig. 2B, Table 3). This finding is in contrast with data by Chordia et al.,[7] who reported a significant retardation of the dissociation kinetics with EC_{50} values of 38 and 17 μM for **2a** and **2b**, respectively. The reason for this discrepancy is unclear to us, although slight differences in experimental conditions may be a trivial explanation (e.g., the use of a different radioligand, [^{125}I]-N^6-aminobenzyladenosine rather than [^{3}H]CCPA). The association kinetics of [^{3}H]CCPA were somewhat affected by the two compounds. The extent of association was increased by approximately 20% without an effect on k_{on} values (Fig. 2A, Table 3). The association kinetics had not been studied by Chordia et al.[7]

We then focused our attention to the resemblance between these aminothiazole structures and a 2-amino-4-phenylthiazole template that we had used before to obtain adenosine receptor antagonists (e.g., compound **3** in Fig. 1). From research in our group it had been established that 4-phenyl-2-(phenylcarboxamido)-1,3-thiazole derivatives are indeed antagonists for the adenosine A_1 receptor with little effect on adenosine A_{2A} and A_3 receptors.[8] Later Jung et al. identified a series of related 4-(4-methoxyphenyl)-2-aminothiazole derivatives as potent

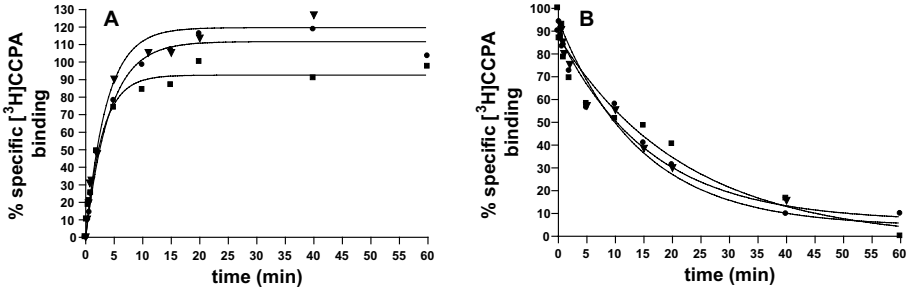


Figure 2. Association (A) and dissociation kinetics (B) of [^{3}H]CCPA binding to and from human adenosine A_1 receptors. Data are from a representative experiment performed in duplicate. Radioligand binding is expressed as percentage of specific binding. Concentration of compounds **2b** (▼) and **2c** (●) was 10 μM. Control (■) was without allosteric modulators.

Table 3. Association and dissociation kinetic parameters of [^{3}H]CCPA binding to and from human adenosine A_1 receptors in the presence or absence of **2b** and **2c** (10 μM)

	Association		Dissociation	
	$t_{1/2}$ (min)	k_{on} (nM^{-1} min^{-1})	$t_{1/2}$ (min)	k_{off} (min^{-1})
Control	1.9 ± 0.2	0.4 ± 0.03	11.8 ± 3.2	0.06 ± 0.02
2b	2.4 ± 0.3	0.3 ± 0.05	11.1 ± 1.2	0.06 ± 0.01
2c	2.7 ± 0.2	0.3 ± 0.03	10.7 ± 0.4	0.07 ± 0.01

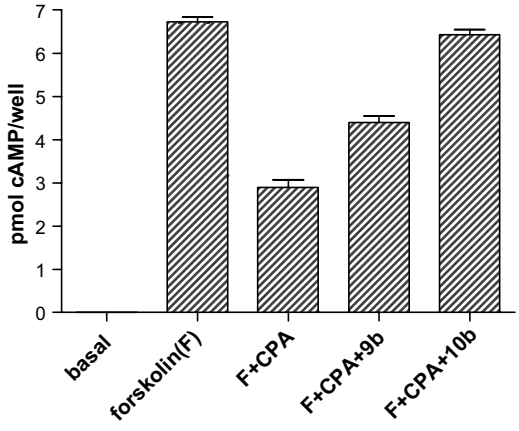


Figure 3. Modulation of forskolin-induced cAMP production in CHO cells expressing the human adenosine A_1 receptor. CPA (100 nM) reduced the forskolin-induced cAMP production by approximately 60%. This reduction was reversed by the addition of either **9b** (partially) or **10b** (fully), both at 10 μM.

and selective human adenosine A_3 receptor antagonists.[11] A further extension of the chemical structure with a 5-pyridin-4-yl substituent also provided a number of potent adenosine A_3 receptor antagonists.[12]

With this in mind we prepared a number of amides (**4**–**10**) from the above 'bridged' 2-aminothiazoles (**2a**–**e**).

The amide derivatives were also tested on all three adenosine receptor subtypes at a concentration of 1 μM. It appeared that in contrast to the aminothiazoles **2a**–**e** some of the amides showed significant affinity for adenosine A_1 and/or A_{2A} receptors (Table 1). We determined K_i values if the compounds in the initial screen displaced more than 50% of the radioligand. The affinities were all in the range from 100 nM to 1 μM (Table 2), suggesting that these compounds were modestly active on either the adenosine A_1 or A_{2A} receptor or on both. From the three compounds that were tested on both receptors (**4d**, **9b**, and **10b**) we learned that selectivity for either one of the receptors was negligible. It is interesting to compare compound **3**, synthesized and tested before with a K_i value of 18 nM on (rat) adenosine A_1 receptors,[8] with compounds **6a**–**c** that show only little affinity for the (human) adenosine A_1 receptor. Apparently, bridging the thiazole and phenyl rings with 1–3 methylene moieties and thus rigidifying the structures is not favorable for affinity toward adenosine A_1 receptors. The most potent compounds (**9b** and **10b**) contained aliphatic rather than (substituted) benzoyl amides. These two compounds were also tested in a cAMP assay demonstrating their antagonistic characteristics. In view of their structure, that is without a ribose group, this was as expected, although we have recently reported on ribose lacking compounds that behave as agonists for adenosine receptors.[13]

4. Conclusion

In our experiments 2-aminothiazoles (**2a**–**e**) did not behave as allosteric enhancers of agonist binding to adenosine A_1 receptors as suggested by Chordia et al.[7] We therefore suggest to examine and reconsider the allosteric activity of this type of compounds. Amides derived from the 2-aminothiazoles (**4**–**10**) were also tested in radioligand binding studies and some of them proved to have modest affinity for adenosine A_1 and/or adenosine A_{2A} receptors. They were classified as antagonists based on the results obtained in a functional assay of cAMP production.

5. Experimental

5.1. Materials and methods

5.1.1. Chemicals. [^{3}H]DPCPX (128 Ci/mmol) and [^{125}I]AB-MECA (approx. 2000 Ci/mmol) were purchased from Amersham Pharmacia Biosciences (The Netherlands). [^{3}H]CCPA (54.9 Ci/mmol) was obtained from NEN (The Netherlands). DPCPX and CPA were from Sigma-RBI (The Netherlands). [^{3}H]ZM 241385 was obtained from Tocris Cookson (United Kingdom),

[^{3}H]cAMP was obtained from Perkin Elmer Life Sciences (The Netherlands). HEK 293 cells stably expressing the human adenosine A_3 receptor were a gift from Dr. K.-N. Klotz (University of Würzburg, Germany).

All compounds made were tested in radioligand binding assays to determine their affinities at the human adenosine A_1, A_{2A}, and the A_3 receptors. All other chemicals, including starting materials, were from standard sources and of the highest purity commercially available.

5.1.2. Instruments and analysis. ^{1}H NMR spectra were measured at 200 MHz with a Bruker AC 200 or Bruker DMX 600 spectrometer. ^{13}C NMR spectra were measured at 50 or 150 MHz. Chemical shifts for ^{1}H and ^{13}C are given in ppm (δ) relative to tetramethylsilane (TMS) as internal standard, coupling constants are given in Hz. Melting points were determined with a Büchi capillary melting point apparatus and are uncorrected. Combustion analyses of new target compounds were performed by the analytical department of the Gorlaeus Laboratories, Leiden University (The Netherlands) and are within 0.4% of theoretical values unless otherwise specified.

5.2. General procedure for the preparation of 2-aminothiazoles[7,9]

Thiourea (3.0 equiv) and iodine (1.1 equiv) were added to a solution of the appropriate ketone in absolute ethanol (1.0 mmol/2 mL). The mixture was heated for 2–3 h in an open vessel at 100 °C, which yielded 2-aminothiazole hydroiodide salts. 2-Aminothiazoles were obtained from the above hydroiodides by alkaline treatment (5% NaOH), extraction (CH_2Cl_2), and evaporation under reduced pressure. The crude products were purified by column chromatography on silica gel and recrystallized afterwards.

5.2.1. 8*H*-Indeno(1,2-*d*)thiazol-2-ylamine (2a). Starting material was 1-indanone (4.5 mmol, 0.59 g). The eluent for column chromatography was 1% methanol in CH_2Cl_2. The product was recrystallized from CH_2Cl_2, to give off-white crystals. Yield: 0.72 g, 85%. Mp: 202–207 °C. Lit.[9] Mp: 213–214 °C. MS (ESI) *m/z*: 189.1 $[M+H]^+$. ^{1}H NMR ($CDCl_3$): δ 3.70 (s, 2H, CH_2), 5.16 (s, 2H, NH_2), 7.15–7.37 (m, 2H, Ar), 7.45 (d, 1H, J = 7.3 Hz, Ar), 7.57 (d, 1H, J = 7.3 Hz, Ar). ^{13}C NMR (MeOD): δ 32.9, 118.6, 124.6, 125.3, 127.4, 138.4, 146.6, 156.4, 175.7. Anal. Calcd for $C_{10}H_8N_2S$: C, 63.80; H, 4.28; N, 14.88. Found: C, 63.78; H, 4.10; N, 15.07.

5.2.2. 4,5-Dihydro-naphto(1,2-*d*)thiazol-2-ylamine (2b). Starting material was α-tetralone (4.5 mmol, 0.66 g). The product was recrystallized from diisopropylether, to give white crystals. Yield: 0.72 g, 79%. Mp: 129–131 °C. Lit.[9] Mp: 133–134 °C. ^{1}H NMR ($CDCl_3$): δ 2.85–2.90 (m, 2H, CH_2), 3.00–3.08 (m, 2H, CH_2), 5.04 (s, 2H, NH_2), 7.10–7.29 (m, 3H, Ar), 7.68 (d, 1H, J = 7.3 Hz, Ar). ^{13}C NMR (DMSO): 21.1, 28.5, 117.6, 122.2, 126.3, 126.7, 127.7, 131.7, 134.3, 144.4, 166.7. Anal. Calcd for $C_{11}H_{10}N_2S$: C, 65.32; H, 4.98; N, 13.85. Found: C, 65.00; H, 4.56; N, 14.00.

5.2.3. 5,6-Dihydro-4*H*-benzo(6,7)cyclohepta(1,2-*d*)thiazol-2-ylamine (2c).[7] Starting material was 1-benzosuberon (6.3 mmol, 1.01 g). The eluent for column chromatography was diisopropylether. The product was recrystallized from CH_2Cl_2 to give the product as off-white crystals. Yield: 0.72 g, 53%. Mp: 129–131 °C. ^{1}H NMR ($CDCl_3$): δ 2.07–2.20 (m, 2H, CH_2), 2.80–2.78 (m, 4H, CH_2), 4.89 (s, 2H, NH_2), 7.13–7.31 (m, 4H, Ar), 7.91 (d, 1H, J = 7.3 Hz, Ar). ^{13}C NMR (MeOD): 26.6, 30.9, 34.9, 123.0, 127.1, 129.3, 130.0, 135.5, 141.8, 145.5, 167.5. Anal. Calcd for $C_{12}H_{12}N_2S$: C, 66.63; H, 5.59; N, 12.95. Found: C, 66.46; H, 5.80; N, 13.11.

5.2.4. 6-Methoxy-4,5-dihydro-naphto(1,2-*d*)thiazol-2-ylamine (2d). Starting material was 5-methoxy-1-tetralone (11.3 mmol, 2.00 g). The eluent for column chromatography was 5% methanol in CH_2Cl_2. The product was recrystallized from chloroform–methanol = 80:20, to give the product as white crystals. Yield: 1.40 g, 53%. Mp: 196–198 °C. ^{1}H NMR ($CDCl_3$): δ 2.79–2.87 (m, 2H, CH_2), 2.99–3.08 (m, 2H, CH_2), 3.85 (s, 3H, OCH_3), 4.94 (s, 2H, NH_2), 6.79 (d, 1H, J = 8.0 Hz, Ar), 7.17–7.26 (m, 1H, Ar), 7.36 (d, 1H, J = 7.3 Hz, Ar). ^{13}C NMR ($CDCl_3$): 19.7, 20.7, 55.1, 109.2, 115.0, 119.3, 121.9, 126.9, 131.9, 156.0, 166.8. Anal. Calcd for $C_{12}H_{12}N_2OS$: C, 62.04; H, 5.21; N, 12.06. Found: C, 61.88; H, 5.00; N, 12.16.

5.2.5. 7,8-Dimethoxy-4,5-dihydro-naphto(1,2-*d*)thiazol-2-ylamine (2e). Starting material was 6,7-dimethoxy-1-tetralone (8.2 mmol, 1.69 g). The eluent for column chromatography was 5% methanol in CH_2Cl_2. The product was recrystallized from chloroform–methanol = 90:10, to give the product as white crystals. Yield: 0.69 g, 32%. Mp: 236–227 °C. Lit.[14] Mp: 225–228 °C. ^{1}H NMR ($CDCl_3$): δ 2.78–2.86 (m, 2H, CH_2), 2.97–3.01 (m, 2H, CH_2), 3.91 (d, 6H, J = 8.78 Hz, $2CH_3$), 4.89 (s, 2H, NH_2), 6.72 (s, 1H, Ar), 7.29 (s, 1H, Ar). ^{13}C NMR ($CDCl_3$): 21.5, 28.4, 55.6, 55.7, 106.3, 111.5, 116.8, 124.2, 126.8, 144.1, 147.2, 147.4, 167.0. Anal. Calcd for $C_{13}H_{14}N_2O_2S$: C, 59.52; H, 5.38; N, 10.68. Found: C, 59.25; H, 5.10; N, 10.74.

5.3. General procedure for the syntheses of the amides

Method A: Triethylamine (1.1 equiv) and the appropriate acid chloride (1.5 equiv) were added to a solution of 2-aminothiazole in 1,4-dioxane (1 mmol/3–4 mL). The mixture was refluxed for 2–5 h. After the reaction was completed the mixture was extracted with CH_2Cl_2 and washed with water. The water layer was extracted with CH_2Cl_2. The organic layers were collected and dried on $MgSO_4$. The products were purified by column chromatography and recrystallized.[8]

Method B (microwave-assisted chemistry): Triethylamine (1.1 equiv) and the appropriate acid chloride (1.5 equiv) were added to a solution of 2-aminothiazole in 1,4-dioxane (1 mmol/2.5–3 mL). Conditions: prestirring 120 s, temperature 140 °C, time 1200 s, normal sample absorption, no fixed hold time. Work up and purification of the products were the same as described in Method A.

5.3.1. *N*-(8*H*-Indeno[1,2-*d*]thiazol-2-yl)-benzamide (4a). Scale: 1.0 mmol. Eluent for column chromatography was CH_2Cl_2. The product was recrystallized from ethylacetate–ethanol = 95:5, to give white crystals. Yield: 0.22 g, 75%. Mp: 221–223 °C. ^{1}H NMR ($CDCl_3$): δ 3.86 (s, 2H, CH_2), 7.16–47.26 (m, 2H, Ar), 7.33–7.55 (m, 5H, Ar), 7.98 (d, 2H, J = 9.5 Hz, Ar), 10.86 (br s, 1H, NH). ^{13}C NMR ($CDCl_3$): δ 32.3, 118.2, 124.5, 124.8, 126.5, 128.6, 130.7, 131.8, 132.5, 136.3, 145.3, 155.2, 162.7, 165.0. Anal. Calcd for $C_{17}H_{12}N_2OS{\cdot}0.1H_2O$: C, 69.41; H, 4.18; N, 9.52. Found: C, 69.26; H, 3.91; N, 9.67.

5.3.2. *N*-(8*H*-Indeno[1,2-*d*]thiazol-2-yl)-4-nitro-benzamide (5a). Scale: 1.0 mmol. Eluent for column chromatography was CH_2Cl_2. The product was recrystallized from *N*,*N*-dimethyl-formamide and after recrystallization the crystals were washed three times with a solution of petroleum ether–methanol = 95:5, to give orange crystals. Yield: 0.29 g, 86%. Mp: 270–275 °C. ^{1}H NMR (DMSO-d_6): δ 3.95 (s, 2H, CH_2), 7.25–7.44 (m, 4H, Ar), 7.61 (d, 4H, J = 8.0 Hz, Ar), 11.89 (br s, 1H, NH). ^{13}C NMR (DMSO-d_6): δ 32.5, 123.8, 125.3, 127.0, 128.3, 128.6, 129.8, 131.2, 132.0, 135.6, 146.1, 149.7, 166.8, 167.1. Anal. Calcd for $C_{17}H_{11}N_3O_3S{\cdot}1.0H_2O$: C, 57.46; H, 3.69; N, 11.82. Found: C, 57.42; H, 4.00; N, 12.19.

5.3.3. *N*-(8*H*-Indeno[1,2-*d*]thiazol-2-yl)-4-chloro-benzamide (6a). Scale 0.50 mmol. Eluent for column chromatography was CH_2Cl_2. The product was recrystallized from chloroform–ethanol = 75:25, to give white crystals. Yield: 0.16 g, 98%. Mp: 232–235 °C. ^{1}H NMR ($CDCl_3$): δ 3.87 (s, 2H, CH_2), 7.18–7.34 (m, 3H, Ar), 7.38–7.45 (m, 3H, Ar), 7.50–7.53 (m, 2H, Ar), 10.43 (br s, 1H, NH). ^{13}C NMR (DMSO-d_6): δ 30.0 (CH_2), 118.0, 125.1, 125.2, 126.9, 128.3, 128.7, 130.1, 130.9, 137.0, 137.6, 146.0, 162.3, 164.0, 175.5. Anal. Calcd for $C_{17}H_{11}ClN_2OS{\cdot}0.5H_2O$: C, 60.80; H, 3.60; N, 8.34. Found: C, 60.74; H, 3.20; N, 8.55.

5.3.4. *N*-(4,5-Dihydro-naphto[1,2-*d*]thiazol-2-yl)-benzamide (4b). Scale 1.0 mmol. Eluent for column chromatography was CH_2Cl_2. The product was recrystallized from CH_2Cl_2, to give off-white crystals. Yield: 0.30 g, 98%. Mp: 119–123 °C. ^{1}H NMR ($CDCl_3$): δ 2.95–3.13 (m, 4H, CH_2), 7.13–7.28 (m, 3H, Ar), 7.35–7.63 (m, 4H, Ar), 7.95–8.00 (m, 2H, Ar). ^{13}C NMR ($CDCl_3$): 21.3, 28.8, 122.2, 124.4, 126.7, 127.6, 128.2, 128.4, 130.4, 131.8, 132.4, 134.5, 157.3, 165.0, 188.1. Anal. Calcd for $C_{18}H_{14}N_2OS{\cdot}1.0H_2O$: C, 66.64; H, 4.97; N, 8.64. Found: C, 66.35; H, 5.15; N, 8.84.

5.3.5. *N*-(4,5-Dihydro-naphto[1,2-*d*]thiazol-2-yl)-4-nitro-benzamide (5b). Scale: 1.0 mmol. Eluent for column chromatography was CH_2Cl_2. The product was recrystallized from ethylacetate, to give yellow crystals. Yield: 0.33 g, 94%. Mp: 246–248 °C. ^{1}H NMR ($CDCl_3$): δ 1.58 (s, 2H, CH_2), 3.04 (s, 2H, CH_2), 7.06–7.13 (m, 2H, Ar), 7.42–7.55 (m, 2H, Ar), 7.94 (d, 2H, J = 8.8 Hz, Ar), 8.14 (d, 2H, J = 8.8 Hz), 11.21 (br s, 1H, NH). ^{13}C NMR (DMSO-d_6): δ 20.8, 28.2, 122.1, 123.6, 124.6, 127.0, 127.1, 128.2, 129.8, 130.9, 134.9, 137.8, 143.8, 149.7, 156.2, 163.7. Anal. Calcd for $C_{18}H_{13}N_3O_3S$: C, 61.53; H, 3.73; N, 11.96. Found: C, 61.43; H, 3.53; N, 12.09.

5.3.6. *N*-(4,5-Dihydro-naphto[1,2-*d*]thiazol-2-yl)-4-chloro-benzamide (6b). Scale: 1.0 mmol. Eluent for column chromatography was CH_2Cl_2. The product was recrystallized from ethanol, to give off-white crystals. Yield: 0.28 g, 82%. Mp: 204–205 °C. ^{1}H NMR ($CDCl_3$): δ 2.95–3.11 (m, 4H, $2CH_2$), 7.01–7.26 (m, 5H, Ar), 7.41–7.50 (m, 1H, Ar), 7.65–7.72 (m, 2H, Ar), 11.32 (s, 1H, NH). ^{13}C NMR (DMSO-d_6): δ 20.8, 28.3, 122.1, 124.3, 127.0, 128.1, 128.7, 130.1, 130.9, 131.0, 134.8, 137.5, 143.6, 156.5, 164.0. Anal. Calcd for $C_{18}H_{13}ClN_2OS$: C, 63.43; H, 3.84; N, 8.22. Found: C, 63.53; H, 3.65; N, 8.58.

5.3.7. *N*-(4,5-Dihydro-naphto[1,2-*d*]thiazol-2-yl)acetamide (7b). Scale: 1.0 mmol. Eluent for column chromatography was CH_2Cl_2. The product was recrystallized from methanol, to give white crystals. Yield: 0.22 g, 90%. Mp: 214–216 °C. Lit.[9] Mp: 233–234 °C. ^{1}H NMR ($CDCl_3$): δ 2.12 (s, 3H, CH_3), 2.93–3.12 (m, 4H, $2CH_2$), 7.15–7.42 (m, 3H, Ar), 7.67–7.80 (m, 1H, Ar), 10.20 (br s, 1H, NH). ^{13}C NMR ($CDCl_3$): δ 21.5, 22.9, 28.8, 122.1, 124.3, 126.8, 127.1, 127.9, 130.6, 134.7, 143.2, 157.5, 168.3. Anal. Calcd for $C_{13}H_{12}N_2OS$: C, 63.91; H, 4.95; N, 11.47. Found: C, 63.62; H, 5.19; N, 11.07.

5.3.8. *N*-(4,5-Dihydro-naphto[1,2-*d*]thiazol-2-yl)-propionamide (8b). Scale: 1.0 mmol. Eluent for column chromatography was petroleum ether–ethylacetate = 80:20. The product was recrystallized from ethanol, to give off-white crystals. Yield: 0.23 g, 89%. Mp: 147–148 °C. ^{1}H NMR ($CDCl_3$): δ 2.20–2.30 (m, 3H, CH_3), 2.72–2.78 (m, 4H, $2CH_2$), 2.91 (t, 2H, J = 7.3, 6.6 Hz, CH_2), 7.16–7.29 (m, 2H, Ar), 7.35–7.63 (m, 1H, Ar), 7.93 (d, 1H, J = 7.3 Hz, Ar). ^{13}C NMR ($CDCl_3$): δ 8.8, 21.3, 28.9, 29.0, 122.1, 124.2, 126.8, 127.1, 127.8, 130.6, 134.6, 143.2, 157.5, 172.0. Anal. Calcd for $C_{14}H_{14}N_2OS$: C, 65.09; H, 5.46; N, 10.84. Found: C, 65.33; H, 5.26; N, 10.96.

5.3.9. *N*-(4,5-Dihydro-naphto[1,2-*d*]thiazol-2-yl)-2,2-dimethyl-propionamide (9b). Scale: 1.0 mmol. Eluent for column chromatography was 12.5% ethylacetate in petroleum ether. The product was recrystallized from 1% *n*-hexane in methanol, to give off-white crystals. Yield: 0.25 g, 87%. Mp: 133–135 °C. ^{1}H NMR ($CDCl_3$): δ 1.34 (s, 9H, $3CH_3$), 2.89–3.08 (m, 4H, $2CH_2$), 7.13–7.31 (m, 3H, Ar), 7.70 (d, 1H, J = 7.3 Hz, Ar), 9.91 (br s, 1H, NH). ^{13}C NMR ($CDCl_3$): δ 21.2, 26.9, 28.7, 38.9, 122.2, 124.0, 126.8, 127.0, 127.7, 130.6, 134.5, 143.0, 156.9, 176.3. Anal. Calcd for $C_{16}H_{18}N_2OS$: C, 67.10; H, 6.34; N, 9.78. Found: C, 66.79; H, 5.94; N, 9.85.

5.3.10. *N*-(4,5-Dihydro-naphto[1,2-*d*]thiazol-2-yl)-cyclopentanecarboxamide (10b). Scale: 1.0 mmol. Eluent for column chromatography was *tert*-butyl methyl ether–petroleum ether = 1:1. The product was recrystallized from methanol, to give off-white crystals. Yield: 0.21 g, 70%. Mp: 148–150 °C. ^{1}H NMR ($CDCl_3$): δ 1.25–1.42

(m, 4H, $2CH_2$), 1.68–1.97 (m, 4H, $2CH_2$), 2.54–2.69 (m, 1H, CH), 2.79–3.09 (m, 4H, $2CH_2$), 7.14–7.32 (m, 3H, Ar), 7.73 (d, 1H, $J = 7.3$ Hz, Ar), 11.37 (br s, 1H, NH). ^{13}C NMR ($CDCl_3$): δ 21.2, 25.6, 28.9, 30.1, 45.0, 122.2, 124.0, 127.1, 127.7, 130.5, 134.6, 143.0, 157.9, 174.6, 182.3. Anal. Calcd for $C_{17}H_{18}N_2OS$: C, 68.41; H, 6.08; N, 9.39. Found: C, 68.15; H, 5.85; N, 9.51.

5.3.11. *N*-(5,6-Dihydro-4*H*-benzo[6,7]cyclohepta[1,2-*d*]-thiazol-2-yl)-benzamide (4c). Scale: 1.0 mmol. Eluent for column chromatography was CH_2Cl_2. The product was recrystallized from 1,4-dioxane–*n*-hexane = 80:20, to give white crystals. Yield: 0.24 g, 74%. Mp: 60–62 °C. ^{1}H NMR ($CDCl_3$): δ 2.16–2.29 (m, 2H, CH_2), 2.71–2.77 (m, 2H, CH_2), 2.94 (t, 2H, $J = 7.3$ Hz, CH_2), 7.10–7.21 (m, 3H, Ar), 7.27–7.34 (m, 1H, Ar), 7.41–7.50 (m, 1H, Ar), 7.73–7.81 (m, 3H, Ar). ^{13}C NMR ($CDCl_3$): δ 25.1, 29.9, 33.6, 126.1, 127.2, 127.4, 128.0, 128.9, 129.9, 130.3, 131.9, 132.0, 133.0, 133.6, 140.4, 143.9, 156.8, 165.5, 171.4. Anal. Calcd for $C_{19}H_{16}N_2OS{\cdot}1.0\ C_4O_2$: C, 67.45; H, 5.98; N, 6.91. Found: C, 67.54; H, 5.94; N, 6.81.

5.3.12. *N*-(5,6-Dihydro-4*H*-benzo[6,7]cyclohepta[1,2-*d*]-thiazol-2-yl)-4-nitro-benzamide (5c). Scale: 0.90 mmol. Eluent for column chromatography was CH_2Cl_2. The product was recrystallized from ethylacetate–methanol = 1:1, to give dark yellow crystals. Yield: 0.22 g, 67%. Mp: 163–164 °C. ^{1}H NMR ($CDCl_3$): δ 2.18–2.24 (m, 2H, CH_2), 2.60–2.66 (m, 2H, CH_2), 2.93 (t, 2H, $J = 7.3$ Hz, CH_2), 7.00–7.05 (m, 4H, Ar), 7.73 (d, 2H, $J = 8.8$ Hz, Ar), 7.97 (d, 2H, $J = 8.8$ Hz, Ar). ^{13}C NMR ($CDCl_3$): δ 24.6, 30.2, 33.0, 122.8, 126.1, 127.3, 127.6, 127.9, 128.3, 128.7, 133.3, 137.7, 140.3, 143.9, 149.2, 156.9, 164.4. Anal. Calcd for $C_{19}H_{15}N_3O_3S{\cdot}0.8H_2O$: C, 60.08; H, 4.41, N, 11.06. Found: C, 60.06; H, 4.79; N, 11.09.

5.3.13. *N*-(5,6-Dihydro-4*H*-benzo[6,7]cyclohepta[1,2-*d*]-thiazol-2-yl)-4-chloro-benzamide (5d). Scale: 0.80 mmol. Eluent for column chromatography was CH_2Cl_2. The product was recrystallized from CH_2Cl_2, to give colorless crystals. Yield: 0.28 g, 99%. Mp: 180–182 °C. ^{1}H NMR ($CDCl_3$): δ 2.19–2.29 (m, 2H, CH_2), 2.71–2.77 (m, 2H, CH_2), 2.95 (t, 2H, $J = 7.8$ Hz, CH_2), 7.05–7.21 (m, 6H, Ar), 7.51–7.70 (m, 2H, Ar), 11.66 (br s, 1H, NH). ^{13}C NMR ($CDCl_3$): δ 24.9, 30.0, 33.4, 126.0, 127.4, 127.5, 128.0, 128.6, 130.6, 133.5, 138.0, 140.3, 144.0, 156.9, 165.1. Anal. Calcd for $C_{19}H_{15}ClN_2OS$: C, 64.31; H, 4.26; N, 7.89. Found: C, 64.10; H, 4.13; N, 8.04.

5.3.14. *N*-(6-Methoxy-4,5-dihydro-naphto[1,2-*d*]thiazol-2-yl)-benzamide (4d). Scale: 1.0 mmol. Eluent for column chromatography was 1% methanol in CH_2Cl_2. The product was recrystallized from diisopropylether, to give light yellow crystals. Yield: 0.07 g, 21%. Mp: 177–179 °C. ^{1}H NMR ($CDCl_3$): δ 2.93–3.13 (m, 4H, $2CH_2$), 3.84 (s, 3H, OCH_3), 6.75–6.80 (m, 1H, Ar), 7.13–7.63 (m, 5H, Ar), 7.87–9.91 (m, 2H, Ar), 10.57 (br s, 1H, NH). ^{13}C NMR ($CDCl_3$): δ 20.9, 55.4, 109.6, 115.2, 122.4, 124.8, 127.1, 128.4, 131.6, 132.0, 132.3, 143.8, 156.2, 157.1, 165.2. Anal. Calcd for $C_{19}H_{16}N_2O_2S{\cdot}0.2H_2O$: C, 67.12; H, 4.86; N, 8.24. Found: C, 67.09; H, 4.91; N, 8.51.

5.3.15. *N*-(6-Methoxy-4,5-dihydro-naphto[1,2-*d*]thiazol-2-yl)-4-nitro-benzamide (5d). Scale: 1.0 mmol. Eluent for column chromatography was petroleum ether–ethylacetate = 70:30. The product was recrystallized from CH_2Cl_2–methanol = 90:10, to give yellow crystals. Yield: 0.05 g, 13%. Mp: 267–268 °C. ^{1}H NMR ($CDCl_3$): δ 2.94–3.08 (m, 4H, $2CH_2$), 3.84 (s, 3H, OCH_3), 6.67–6.72 (m, 1H, Ar), 6.97–7.10 (m, 2H, Ar), 7.86–7.92 (m, 2H, Ar), 8.08 (d, 2H, $J = 8.8$ Hz, Ar), 11.37 (br s, 1H, NH). ^{13}C NMR (DMSO-d_6): δ 20.2, 20.6, 55.5, 110.1, 115.0, 122.1, 123.6, 124.5, 127.5, 129.8, 130.0, 131.7, 137.8, 149.6, 149.8, 155.4, 156.3, 163.5. Anal. Calcd for $C_{19}H_{15}N_3O_4S{\cdot}0.3H_2O$: C, 59.00; H, 4.06; N, 10.86. Found: C, 58.86; H, 3.81; N, 11.19.

5.3.16. *N*-(6-Methoxy-4,5-dihydro-naphto[1,2-*d*]thiazol-2-yl)-4-chloro-benzamide (6d). Scale: 1.0 mmol. Eluent for column chromatography was 1% methanol in CH_2Cl_2. The product was recrystallized from ethanol, to give light brown crystals. Yield: 0.06 g, 16%. Mp: 216–218 °C. ^{1}H NMR ($CDCl_3$): δ 2.93–3.12 (m, 4H, $2CH_2$), 3.86 (s, 3H, OCH_3), 6.71–6.75 (m, 1H, Ar), 7.02 (t, 1H, $J = 7.3$ Hz, Ar), 7.13–7.26 (m, 3H, Ar), 7.66–7.71 (m, 2H, Ar), 11.26 (br s, 1H, NH). ^{13}C NMR ($CDCl_3$): δ 20.8, 21.0, 55.4, 109.6, 114.9, 122.4, 124.8, 127.0, 128.3, 128.8, 130.7, 131.3, 138.3, 143.8, 156.3, 157.5, 164.9. Anal. Calcd for $C_{19}H_{15}ClN_2O_2S$: C, 61.54; H, 4.08; N, 7.55. Found: C, 61.46; H, 4.19; N, 7.95.

5.3.17. *N*-(7,8-Dimethoxy-4,5-dihydro-naphto[1,2-*d*]thiazol-2-yl)-benzamide (4e). Scale: 1.0 mmol. Eluent for column chromatography was 0.5% methanol in CH_2Cl_2. The product was recrystallized from ethanol, to give off-white crystals. Yield: 0.18 g, 49%. Mp: 180–182 °C. ^{1}H NMR ($CDCl_3$): δ 2.99 (s, 4H, $2CH_2$), 3.88 (d, 6H, $J = 4.38$ Hz, $2OCH_3$), 6.74 (s, 1H, Ar), 7.25 (d, 1H, $J = 4.38$ Hz, Ar), 7.40–7.89 (m, 3H, Ar), 7.91 (d, 2H, $J = 6.58$ Hz, Ar), 10.28 (br s, 1H, NH). ^{13}C NMR ($CDCl_3$): δ 21.5, 28.4, 55.6, 55.8, 105.9, 111.3, 119.3, 122.4, 123.6, 127.0, 127.2, 128.5, 131.8, 132.4, 143.9, 147.4, 147.7, 156.5, 164.6. Anal. Calcd for $C_{20}H_{18}N_2O_3S$: C, 65.56; H, 4.95; N, 7.64. Found: C, 65.38; H, 4.96; N, 7.96.

5.3.18. *N*-(7,8-Dimethoxy-4,5-dihydro-naphto[1,2-*d*]thiazol-2-yl)-4-nitro-benzamide (5e). Scale: 1.0 mmol. Eluent for column chromatography was ethylacetate–petroleum ether = 1:1. The product was recrystallized from 2% methanol in CH_2Cl_2, to give dark red crystals. Yield: 0.08 g, 20%. Mp: 269–273 °C. ^{1}H NMR ($CDCl_3$): δ 2.95 (s, 4H, $2CH_2$), 3.79 (s, 6H, $2OCH_3$), 6.94 (s, 1H, Ar), 7.32 (s, 1H, Ar), 8.28–8.39 (m, 4H, Ar), 12.11 (br s, 1H, NH). ^{13}C NMR 600 MHz (DMSO-d_6): δ 20.9, 27.7, 55.5, 55.6, 106.2, 112.4, 123.5, 126.5, 127.2, 127.3, 129.7, 129.9, 130.2, 134.3, 147.5, 147.8, 149.0, 149.5, 165.8, 175.6. Anal. Calcd for $C_{20}H_{17}N_3O_5S{\cdot}1.8H_2O$: C, 54.12; H, 4.68; N, 9.47. Found: C, 53.86; H, 4.65; N, 9.85.

5.3.19. *N*-(7,8-Dimethoxy-4,5-dihydro-naphto[1,2-*d*]thiazol-2-yl)-4-chloro-benzamide (6e). Scale: 1.0 mmol. Eluent for column chromatography was 2% methanol in CH_2Cl_2. The product was recrystallized from 2% ethanol in CH_2Cl_2, to give light brown crystals. Yield: 0.29 g, 72%. Mp: 190–192 °C. ^{1}H NMR ($CDCl_3$): δ 2.99 (s, 4H, $2CH_2$), 3.93 (d, 6H, $J = 4.38$ Hz, $2OCH_3$), 6.78 (s, 1H, Ar), 7.28 (d, 1H, $J = 8.04$ Hz, Ar), 7.44–7.50 (m, 2H, Ar), 7.98–8.08 (m, 2H, Ar). ^{13}C NMR ($CDCl_3$): δ 21.4, 28.4, 55.6, 55.7, 105.9, 111.2, 122.2, 122.7, 127.3, 128.6, 129.0, 130.1, 130.9, 138.8, 143.0, 147.8, 157.9, 164.1, 170.5. Anal. Calcd for $C_{20}H_{17}ClN_2O_3S{\cdot}1.3H_2O$: C, 56.61; H, 4.66; N, 6.60. Found: C, 56.60; H, 4.88; N, 6.67.

5.4. Radioligand binding assays

For displacement experiments, membranes of CHO cells expressing recombinant human adenosine A_1 receptors (20 μg of protein for [^{3}H]CCPA and 10 μg of protein for [^{3}H]DPCPX), were incubated at 25 °C for 60 min with ~1.0 nM of [^{3}H]CCPA, or ~1.6 nM of [^{3}H]DPCPX, and a fixed concentration or increasing concentrations of the compounds, in a final volume of 0.4 mL Tris–HCl buffer. Nonspecific binding was measured in the presence of 10 μM DPCPX ([^{3}H]CCPA) or 10 μM CPA ([^{3}H]DPCPX). Binding reactions were terminated by dilution with ice-cold 50 mM Tris–HCl buffer. Samples were then filtered through Whatman GF/B glass-fiber filters using a Brandell cell harvester or a Millipore manifold. Filters were washed three times with 2–3 mL of the same buffer. Bound radioactivity was measured in a liquid scintillation counter (LKB Wallac) after the addition of 3.5 mL of scintillation liquid (Emulsifier-Safe, Packard).

In kinetic studies, the association of the radiolabeled agonist [^{3}H]CCPA (1 nM) was started by addition of the membranes (30 μg) to the radioligand, in the presence or absence of either **2b** or **2c**. To study dissociation of [^{3}H]CCPA, membranes were preincubated with [^{3}H]CCPA (1 nM) at 25 °C for 60 min. Dissociation of [^{3}H]CCPA was then initiated by the addition of 100 μM 8-cyclopentyltheophylline (CPT) in the presence or absence of either **2b** or **2c**. Samples were handled as mentioned before.

The conditions for adenosine A_{2A} receptor binding were slightly different. In competition experiments, membranes of HEK293 cells expressing recombinant human A_{2A} receptors (22.5 μg of protein) were incubated with ~1.7 nM [^{3}H]ZM241385 for 120 min at 25 °C. Nonspecific binding was measured in the presence of 100 μM CPA.

For A_3 receptor displacement experiments, membranes of HEK293 cells expressing recombinant human A_3 receptors (20 μg of protein), were incubated at 37 °C for 60 min with ~0.1 nM of [^{125}I]AB-MECA, and a fixed concentration of the compound, in a final volume of 0.2 mL of 50 mM Tris/10 mM $MgCl_2$/1 mM EDTA/0.01% CHAPS buffer (pH 8.26 at 5 °C). Nonspecific binding was measured in the presence of 100 μM R-PIA. Binding reactions were terminated by dilution with ice-cold buffer. Samples were then filtered through Whatman GF/B glass-fiber filters using a Brandell cell harvester. Filters were washed three times with 2–3 mL of the same buffer. Bound radioactivity was measured in a Minaxi γ Auto-gamma® 5000 series gamma counter.

Compounds **9b** and **10b** were tested in functional assays for their ability to influence the levels of cAMP in the test system. CHO cells expressing the human adenosine A_1 receptor were grown overnight as a monolayer in 24-well tissue culture plates (400 μL/well; 2×10^5 cells/well). cAMP generation was performed in Dulbecco's Modified Eagles Medium (DMEM)/*N*-2-hydroxyethylpiperazin-*N*′-2-ethansulfonic acid (HEPES) buffer (0.60 g HEPES/50 mL DMEM pH 7.4). Each well was washed twice with HEPES/DMEM buffer (250 μL), and the following added, adenosine deaminase (0.8 IU/mL), rolipram (50 μM), cilostamide (50 μM). This was then incubated for 30 min at 37 °C followed by the introduction of the compound of interest. After a further 10 min of incubation, forskolin was added (10 μM). After a subsequent 15 min, incubation was stopped by aspirating the assay medium and by adding 200 μL of ice-cold 0.1 M HCl. The amount of cAMP was determined by competition with [^{3}H]cAMP for protein kinase A (PKA). Briefly, the sample, approximately 1.8 nM [^{3}H]cAMP, and 100 μL PKA solution were incubated on ice for 2.5 h. The incubations were stopped by rapid dilution with 2 mL of ice-cold Tris–HCl buffer (pH 7.4) and bound radioactive material was then recovered by filtration through Whatman GF/C filters. Filters were additionally rinsed with 2×2 mL Tris–HCl buffer and then the radioactivity counted in Packard Emulsifier Safe scintillation fluid (3.5 mL). All data reflect three independent experiments performed in duplicate.

5.5. Statistical analysis

Binding parameters were estimated using a nonlinear regression curve-fitting program (GraphPad Prism 4, GraphPAD Software Inc. San Diego, CA, USA). K_D values of the radioligands were 1.6, 1.0, 1.0, and 5.0 nM for [^{3}H]DPCPX, [^{3}H]CCPA, [^{3}H]ZM 241385, and [^{125}I]AB-MECA, respectively. Data are expressed as mean ± SEM for the number of experiments indicated.

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