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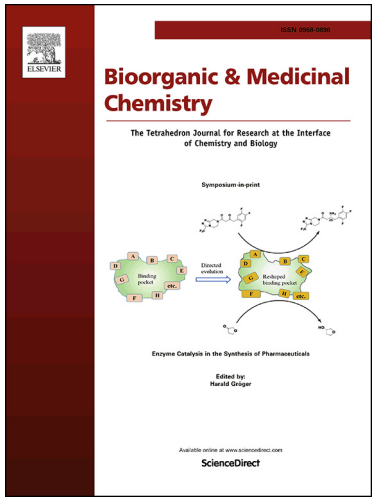

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Identification of Novel Thiazolo[5,4-d]pyrimidine Derivatives as human A_1 and A_{2A} Adenosine Receptor Antagonists/Inverse Agonists.

Flavia Varano,[a]* *Daniela Catarzi,*[a] *Matteo Falsini,*[a] *Fabrizio Vincenzi,*[b] *Silvia Pasquini,*[b] *Katia Varani,*[b] *Vittoria Colotta*[a]

[a] Dipartimento di Neuroscienze, Psicologia, Area del Farmaco e Salute del Bambino, Sezione di Farmaceutica e Nutraceutica, Università degli Studi di Firenze, via Ugo Schiff, 6, 50019, Sesto Fiorentino, Italy. [b] Dipartimento di Scienze Mediche, Sezione di Farmacologia, Università degli Studi di Ferrara, via Fossato di Mortara 17-19, 44121, Ferrara, Italy.

KEYWORDS. G protein-coupled receptors; A_{2A} adenosine receptors; inverse agonists; thiazolopyrimidine derivatives, bicyclic heteroaromatic system.

Corresponding Author

*Tel: +39 055 4573732. Fax: +39 055 4573780. E-mail: flavia.varano@unifi.it.

ABBREVIATIONS

AR, adenosine receptor; cAMP, 3',5'-cyclic adenosine monophosphate; CHO, chinese hamster ovary; DPCPX, 8-cyclopentyl-1,3-dipropyl-xanthine; I-AB-MECA, 4-[((4-amino-3-iodophenyl)methyl)amino]-5'-N-methylcarboxamidoadenosine; ZM 241385, (4-(2-[7-amino-2-(2-furil)[1,2.4] triazolo[2,3-a][1,3,5]triazin-5-ylamino] ethyl) phenol); NECA, 5'-N-ethyl-carboxamidoadenosine; AB-MECA, 4-[((4-aminophenyl)methyl)amino]-5'-N-methylcarboxamidoadenosine; TCA, trichloroacetic acid; MW, microwave.

Abstract.

In this study a new set of thiazolo[5,4-d]pyrimidine derivatives was synthesized. These derivatives bear different substituents at positions 2 and 5 of the thiazolopyrimidine core while maintaining a free amino group at position-7. The new compounds were tested for their affinity and potency at human (h) A_1, A_{2A}, A_{2B} and A_3 adenosine receptors expressed in CHO cells. The results reveal that the higher affinity of these new set of thiazolopyrimidines is toward the hA_1 and hA_{2A} adenosine receptors subtypes and is tuned by the substitution pattern at both the 2 and 5 positions of the thiazolopyrimidine nucleus. Functional studies evidenced that the compounds behaved as dual A_1/A_{2A} antagonists/inverse agonists. Compound **3**, bearing a 5-((2-methoxyphenyl) methylamino) group and a phenyl moiety at position 2, displayed the highest affinity (hA_1 K_i = 10.2 nM; hA_{2A} K_i = 4.72 nM) and behaved as a potent A_1/A_{2A} antagonist/inverse agonist (hA_1 IC_{50} = 13.4 nM; hA_{2A} IC_{50} = 5.34 nM).

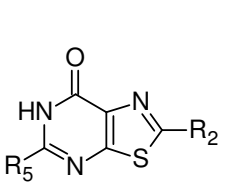
1. Introduction

The nucleoside adenosine plays important roles in many pathophysiological conditions through the modulation of cell-membrane G protein-coupled receptors known as adenosine receptors (ARs). These receptors have been extensively characterized and subdivided in four subtypes, namely A_1, A_{2A}, A_{2B} and A_3. The A_1 and A_3 ARs mediate inhibition of adenylyl cyclase, whereas adenosine A_{2A} and A_{2B} ARs induce stimulation.[1] ARs are considered very promising drug targets in many pathological conditions.[2-3] In particular, specific AR antagonists have gained considerable interest for clinical development. For example, selective A_1 AR antagonists have been of interest for renal and cardiovascular disorders,[4-5] while antagonism at the A_{2A} subtype is therapeutically effective in neurodegenerative disorders,[6-7] dermal fibrosis and scarring,[8-10] cancer[11-12] and retinal diseases.[13] The A_{2B} antagonists are promising drug candidates for the treatment of diabetes as well as asthma and chronic obstructive pulmonary disease (COPD),[14-15] while preclinical data indicate that selective A_3 AR antagonists might be useful for glaucoma, stroke, asthma, inflammation and cancer.[16] In the last decade has emerged the idea that more effective drugs for the treatment of neurodegenerative diseases such as Parkinson disease (PD), must influences multiples targets in a parallel fashion. In the field of the AR ligands, dual A_1 and A_{2A} antagonists were found to be highly active in different animal models of PD.[17] Indeed, dual A_1/A_{2A} AR antagonists would not only treat the motor symptoms of PD and potentially be neuroprotective via A_{2A} blockade, but may also show positive effects on the cognitive impairment associated with the disease by blocking the A_1 subtypes.[17]

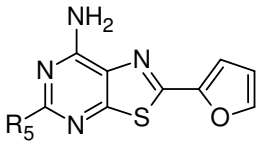
As a part of our research interest in identifying new AR ligands,[18-24] we recently investigate the thiazolo[5,4-d]pyrimidine (TP) nucleus as basis for the design of new AR antagonists (**TP A**, **TP B**, Chart 1).[25-26] Many reasons prompted us to develop this nucleus: i) the structural similarity of TP nucleus to many bicyclic cores of known AR antagonists;[6] ii) the synthetic feasibility of the TP scaffold which has made possible to synthesize a number of products that differ in the nature of the substituents at the C2, C5 and C7 positions of the TP core;[25-26] and iii) thiazolopyrimidines have been found to exhibit a range of biological activities particularly in the antimicrobial,[27]

immunosuppressive[28] and pain[29] areas. In addition, in a recent study, TP derivatives have been identified as partial agonists at the A_{2A} AR (**TP C**, Chart 1).[30] On this basis, we explored the structure-activity relationship (SAR) of the **TP B** series by introducing diverse (aryl, alkyl, alkylaryl) amino chains at the C5 position of the TP scaffold while maintaining a 2-furanyl

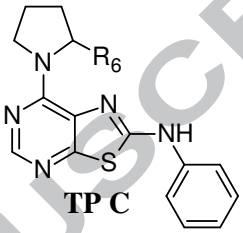
Chart 1. Previously reported thiazolo[5,4-d]pyrimidine derivatives.



TP A



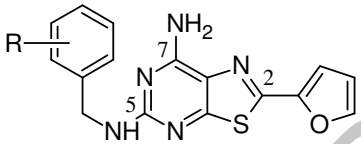
TP B



TP C

R_2 = Aryl, Het, NHaryl R_5 = CH_3, NHaryl, NHalkylaryl, NHalkyl

R_6 = H, CH_2OH



TP455 R = 2-OCH_3
KH = 3.55 fM; KL = 6.45 nM

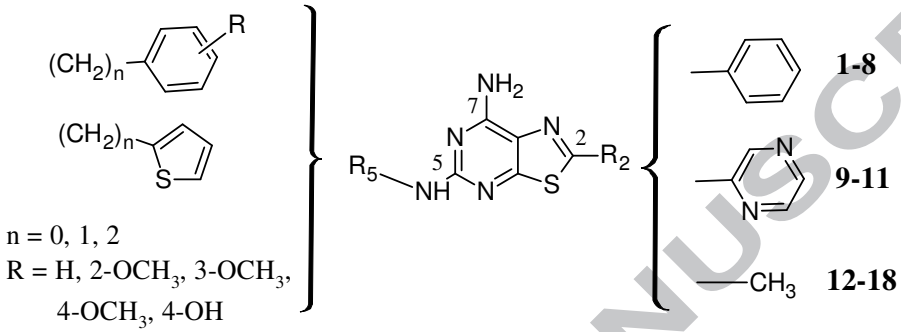
TP400 R = 3-OCH_3
KH = 5.31 fM; KL = 26 nM

and a free amino group at C2 and C7 positions, respectively. This study afforded two compounds (**TP455**, **TP450,** Chart 1) which resulted the most potent and selective A_{2A} AR inverse agonists yet reported.[26] These compounds showed an exceptional binding behavior with two K_i affinity values (KH and KL) the highest (KH) falling in the femtomolar range. In addition, **TP455** and **TP450** were also tested in murine models of acute pain exhibiting antinociceptive activity greater than that of morphine.[26] In another study, **TP455** reveals its capability to counteract the A_{2A} AR stimulated proliferation of different cancer cell lines.[31]

Due to the above encouraging results, in the present study we further explore the SARs of TP-based AR antagonists by investigating the effect of substitutions at both the C5 and C2 positions while maintaining a free amino group at C7 (compounds **1-18**, Chart 2).

The newly synthesized derivatives can be divided into three sets according to the substituent attached at the C2 position, as compounds bearing a phenyl (**1-8**), a 2-pyrazinyl (**9-11**) or a methyl (**12-18**) group.

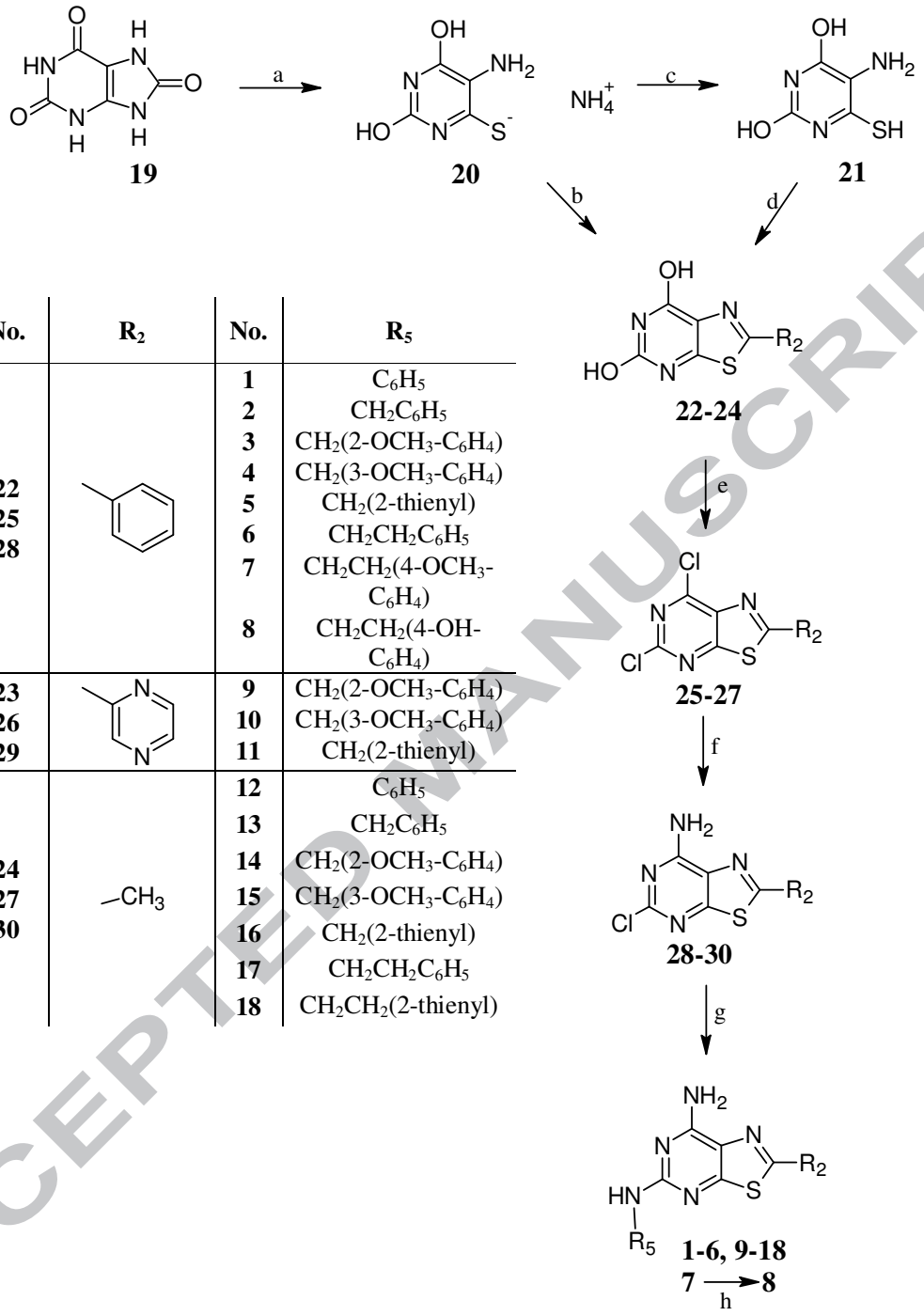
Chart 2. Newly synthesized thiazolo[5,4-d]pyrimidine derivatives.



2. Chemistry

Compounds **1-18** were prepared starting from the commercial uric acid **19**, following the synthetic procedure depicted in Scheme 1.

Thus, by reaction of the salt **20**[32] with benzoic or acetic anhydride the 2-phenyl or 2-methyl-thiazolo[5,4-d]pyrimidine-5,7-diolo derivatives **22**[33] or **24**[32] were obtained, respectively. The 5,7-diolo derivative **23**, bearing at the 2-position a 2-pyrazinyl moiety, was prepared by heating in NMP at 150 °C compound **21**[32] and 2-pyrazine carbonyl chloride prepared starting from the corresponding acid.[34] Treatment of the 5,7 diol derivatives **22-24** with $POCl_3$ gave the corresponding 5,7-dichloro compounds **25,**[35] **26, 27**[33] which were reacted with 33% aqueous solution of ammonia to afford the 7-amino-5-chloro derivatives **28-30**. Reaction of these latter with the (hetero)arylalkylamines or the arylamines of interest, under microwave irradiation at about 200 °C, provides the desired compounds **1-7**, **9-18.** Finally, the 5-(4-methoxyphenyl)ethylamino **7** was hydrolyzed to its corresponding phenol **8** by treatment with BBr_3 in CH_2Cl_2.



No.	R_2	No.	R_5
		1	C_6H_5
		2	$CH_2C_6H_5$
		3	$CH_2(2\text{-}OCH_3\text{-}C_6H_4)$
		4	$CH_2(3\text{-}OCH_3\text{-}C_6H_4)$
22		**5**	CH_2(2-thienyl)
25		**6**	$CH_2CH_2C_6H_5$
28		**7**	$CH_2CH_2(4\text{-}OCH_3\text{-}C_6H_4)$
		8	$CH_2CH_2(4\text{-}OH\text{-}C_6H_4)$
23		**9**	$CH_2(2\text{-}OCH_3\text{-}C_6H_4)$
26		**10**	$CH_2(3\text{-}OCH_3\text{-}C_6H_4)$
29		**11**	CH_2(2-thienyl)
		12	C_6H_5
		13	$CH_2C_6H_5$
24		**14**	$CH_2(2\text{-}OCH_3\text{-}C_6H_4)$
27	$-CH_3$	**15**	$CH_2(3\text{-}OCH_3\text{-}C_6H_4)$
30		**16**	CH_2(2-thienyl)
		17	$CH_2CH_2C_6H_5$
		18	CH_2CH_2(2-thienyl)

Scheme 1. Reagents and conditions: (a) $[NH_4]_2S$, KOH, H_2O, 160 °C, 16h, 60% ; (b) for **22** $(PhCO)_2O$, 170 °C, 4h, 85%, for **24** i) $(CH_3CO)_2O$, 140 °C, 1h ii) NaOH, H_2O, reflux, 1h, 80%; (c) CH_3COOH, H_2O, 80%; (d) 2-pyrazine carbonyl chloride, NMP, 150 °C, 18h, 75%; (e) for **25** and **27**: $POCl_3$, N, N dimethylaniline, 100 °C, 5h, 60-70%, for **26** $POCl_3$, 170 °C MW, 40min, 70%; (f) NH_3, H_2O, EtOH, reflux, 6-8h, 60-70%; (g) R_5NH_2, BuOH, 200 °C MW, 10-20min, 50-70%; (h) BBr_3, CH_2Cl_2, 40 °C, 24h, 60%.

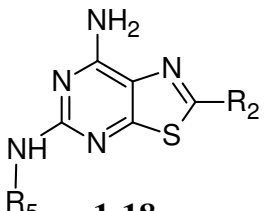
3. Results and Discussion

All final compounds (**1-18**) were tested at hA_1, hA_{2A}, hA_{2B} and hA_3 ARs expressed in CHO cells. [^{3}H]DPCPX (A_1), [^{3}H]ZM 241385 (A_{2A}) and [^{125}I]AB-MECA (A_3) were used in radioligand binding assays. Because of the lack of a suitable radioligand for the A_{2B} ARs the activity at this receptor subtype was determined through the measurement of the inhibition of NECA-stimulated adenylyl cyclase activity. Binding and potency data of the newly synthesized compounds (**1-18**) are reported in Table 1.

Concerning the hA_3 and the hA_{2B} ARs, the greater part of the newly synthesized derivatives display poor affinity with K_i and IC_{50} values above 100 nM. Compound **5**, which belong to the C2 phenyl set and bear a 5-((2-thienyl)methylamino) substituent, was the most active at both subtypes (hA_3 K_i = 112 nM, hA_{2B} IC_{50} = 72 nM).

As regards the binding results at the hA_1 AR, the most potent compounds (**2**, **3**, **4**, **5**, **18**) possess a K_i in the range 10.2-92 nM and belong to the C2 phenyl (**2**, **3**, **4**, **5**) or C2 methyl (**18**) set. Analyzing the binding data at the hA_{2A} AR it emerges that compounds **3**, **4**, **5** and **18** (hA_{2A} K_i = 4.72-61 nM) are some of the most promising also at this subtype. In addition, compounds **8**, **9** and **17** displayed good hA_{2A} binding affinities with K_i values in the range 78-96 nM.

Table 1. Affinity (K_i) and potency (IC_{50}) of the novel thiazolo[5,4-d]pyrimidines on ARs.



1-18

Affinity values obtained from displacement of specific [^{3}H]DPCPX [a], [^{3}H]ZM241383 [b] or [^{125}I]AB- MECA [c] binding to hA_1ARs, hA_{2A}ARs or A_3ARs, respectively (n = 3-6). [d] Potency (IC_{50}) in cAMP assays to hA_{2B}ARs. Percentage of inhibition (I%) is determined at 10 μM concentration of the tested compounds. Data are expressed as means ± SEM.

	R_2	R_5	hA_1AR[a] K_i (nM) (I%)	$hA_{2A}AR$[b] K_i (nM) (I%)	hA_3AR[c] K_i (nM) (I%)	$hA_{2B}AR$[d] IC_{50} (nM) (I%)
1	C_6H_5	C_6H_5	>10000 (8%)	>10000 (1%)	>10000 (1%)	>10000 (1%)
2	C_6H_5	$CH_2C_6H_5$	37 ± 4	116 ± 10	275 ± 24	358 ± 31
3	C_6H_5	CH_2(2-OCH_3-C_6H_4)	10.2 ± 1.1	4.72 ± 0.46	692 ± 67	2622 ± 224
4	C_6H_5	CH_2(3-OCH_3-C_6H_4)	32 ± 3	42 ± 4	>10000 (25%)	2387 ± 243
5	C_6H_5	CH_2(2-thienyl)	43 ± 4	28 ± 3	112 ± 10	72 ± 6
6	C_6H_5	$CH_2CH_2C_6H_5$	121 ± 13	406 ± 38	2274 ± 217	4380 ± 429
7	C_6H_5	CH_2CH_2(4-OCH_3-C_6H_4)	485 ± 37	403 ± 37	3529 ± 315	>10000 (33%)
8	C_6H_5	CH_2CH_2(4-OH-C_6H_4)	242 ± 19	96 ± 8	1612 ± 153	7524 ± 537
9	Pyrazin-2-yl	CH_2(2-OCH_3-C_6H_4)	112 ± 10	79 ± 7	725 ± 67	4017 ± 385
10	Pyrazin-2-yl	CH_2(3-OCH_3-C_6H_4)	253 ± 23	973 ± 82	749 ± 58	5013 ± 423
11	Pyrazin-2-yl	CH_2(2-thienyl))	338 ± 31	219 ± 18	165 ± 13	784 ± 62
12	CH_3	C_6H_5	547 ± 42	608 ± 51	446 ± 39	3725 ± 347
13	CH_3	$CH_2C_6H_5$	333 ± 28	532 ± 47	1912 ±176	1810 ± 168
14	CH_3	CH_2(2-OCH_3-C_6H_4)	281 ± 26	135 ± 11	782 ± 68	5538 ± 412
15	CH_3	CH_2(3-OCH_3-C_6H_4)	195 ± 18	212 ± 17	667 ± 56	3516 ± 287
16	CH_3	CH_2(2-thienyl))	531 ± 44	313 ± 28	780 ± 72	2072 ± 176
17	CH_3	$CH_2CH_2C_6H_5$	140 ± 12	78 ± 7	548 ± 44	1105 ± 97
18	CH_3	CH_2CH_2(2-thienyl)	92 ± 9	61 ± 6	1519 ± 146	134 ± 11

In the C2 phenyl set (**1-8**) binding affinity at hA_1 and hA_{2A} ARs decreases in the following order 5-phenyl-methylamino **2** > 5-phenyl-ethylamino **6** > 5-phenyl-amino **1**. These results indicated that the 5-methylamino group is the optimal linker between the TP core and the appended (hetero)aryl ring. Moreover, the presence of the 5-((2-methoxyphenyl)methylamino) group, the same present in **TP455**, afforded the most active compound (e.g compound **3**: hA_1 K_i = 10.2 nM, hA_{2A} K_i = 4.72 nM) among the herein reported TP derivatives. To note that the hA_{2A} affinity value of compound **3** (hA_{2A} K_i = 4.72 nM) is comparable to the KL affinity value of **TP455** (hA_{2A} KL = 6.45 nM). Furthermore, among the 5-phenyl-ethylamino derivatives **6-8,** compound **8** bearing an hydroxyl function in para position, showed the highest affinity at the A_{2A} AR (K_i = 96 nM). In fact, the para hydroxyl derivative **8** is about 4-fold more active than its precursor para-methoxy derivative **7** which in turn has the same affinity as the 5-phenyl-ethylamino unsubstituted **6.**

The 2-(2-pyrazinyl) derivatives **9-11** were synthesized as isosters of the corresponding C2 phenyl derivatives **3-5**. Comparison of the binding data of the two subseries clearly indicated that the substitution of the phenyl ring with a 2-pyrazinyl one is deleterious for the anchoring at the A_1 ad A_{2A} AR subtypes. However, it is interesting to remark that compound **9,** bearing a 5-((2-methoxyphenyl)methylamino) substituent, resulted the most active also among this subseries, thus confirming the beneficial effect of this 5-substituent for good hA_1 and hA_{2A} binding affinity.

In contrast, in the C2 methyl set the highest affinity at the hA_1 and hA_{2A} subtypes was observed in the 5-ethylamino substituted derivatives **17** and **18** which were, respectively, about 7 and 5 fold more active than their corresponding 5-methylamino derivatives **13** and **16**. This behavior can be explained assuming that the decreased steric hindrance at the C2 position due to the presence of a methyl group is compensated by the increased bulk of the C5 substituent. However, also in the C2 methyl set the 5-((2-methoxyphenyl)methylamino) substituted derivative **14** exhibits the higher

affinity with respect to the other 5-methylamino substituted of the same set (compare **14** vs **13**, **15**, **16**).

Compounds **3**, **4**, **5** and **18,** the most interesting in terms of affinity at the hA_1 and hA_{2A} ARs, were also evaluated for their antagonist/inverse agonist behavior toward these receptor subtypes (Table 2 and 3).

Table 2. Modulation of Forskolin-stimulated cAMP, potency (IC_{50}) and efficacy (E_{max}) of selected compounds on cyclic AMP assays in hA_1 CHO cells.[a]

	% of Forskolin-stimulated cAMP levels[b]	IC_{50} (nM)[c]	E_{max} (%)[d]
3	102±5	13.4±11.1	100±3
4	98±3	37±3	99±4
5	101±4	51±5	97±4
18	100±5	107±9	98±5

[a]Data are expressed as means ± SEM. [b]Capability of selected compounds to modulate Forskolin-stimulated cAMP levels (100%). Potency[c] and efficacy[d] of the novel compounds to antagonize CCPA (1 nM) inhibition of Forskolin-stimulated cAMP levels.

Table 3. Potency (IC_{50}) and efficacy (E_{max}) of selected compounds on cyclic AMP assays in hA_{2A} CHO cells.[a]

	IC_{50} (nM)[b]	E_{max} (%)[c]	IC_{50} (nM)[d]	E_{max} (%)[e]
3	5.34±0.47	55±4	7.63±0.61	134±13
4	48±4	52±4	66±6	128±11
5	36±4	64±5	43±4	131±11
18	83±7	58±5	102±9	124±12

[a]Data are expressed as means ± SEM. Potency ([b, d]) and efficacy ([c, e]) of selected compounds in the absence ([b, c]) or in the presence ([d, e]) of CGS 21680 (10 nM), respectively.

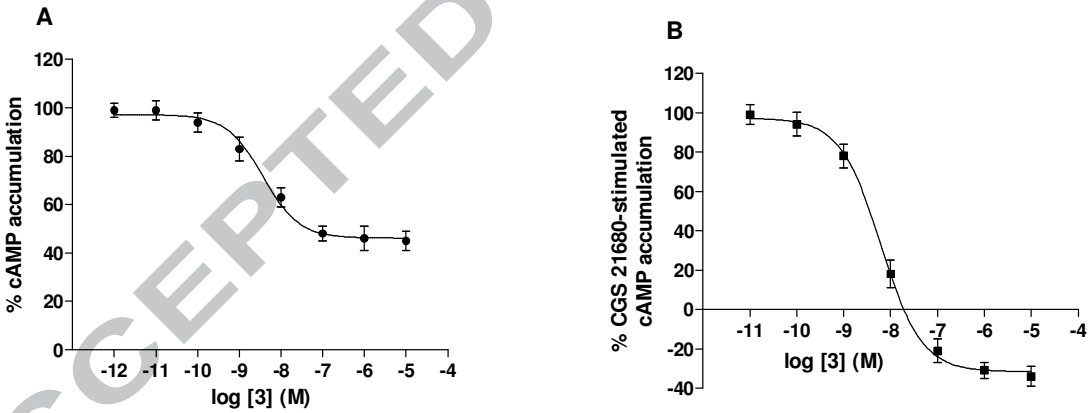
In A_1 AR CHO cells, compounds **3**, **4**, **5** and **18** were not able to modulate Forskolin-stimulated cAMP levels suggesting that they did not behave as agonists or inverse agonists. However, they

completely counteracted hA_1 AR agonist CCPA inhibition of Forskolin-stimulated cAMP levels behaving as antagonists (Table 2).

In A_{2A} AR CHO cells, the selected compounds behaved as inverse agonists being able to inhibit basal cAMP accumulation at nanomolar concentration (Table 3). Furthermore, they were able to inhibit cAMP accumulation stimulated by the selective hA_{2A} AR agonist CGS 21680 reaching values lower than those of basal production as indicated by efficacy data (Table 3).

Figure 1 reports the effect of compound **3**, the most interesting one, on cAMP assays in hA_{2A} CHO cells in the absence or in the presence of CGA 21680.

Figure 1. Inhibition of cAMP levels in hA_{2A} CHO cells by compound **3** in the absence (A) or in the presence (B) of CGS 21680 (10 nM). Data represent means ± SEM of four experiments each performed in triplicate.



In conclusion, this study allowed to deepen the SARs of the thiazolo[5,4-d]pyrimidine series as AR antagonists/inverse agonists. In particular, the herein reported results reveal that the higher affinity is toward the hA_1 and hA_{2A} AR subtypes and is tuned by the substitution pattern at both the C2 and C5 positions. In fact, it emerges that in all subseries which differ for the substituent at C2 position, the presence of a 5-((2-methoxyphenyl) methylamino) group affords good affinity for hA_1 and A_{2A}

ARs. Compound **3,** bearing a 5-((2-methoxyphenyl) methylamino) group and phenyl moiety at position 2, has shown the best affinity values for both the A_1 and A_{2A} ARs. Functional studies evidenced that the herein reported compounds are dual A_1/A_{2A} AR antagonists/inverse agonists. and thus could be useful for the therapeutic treatment of neurological disorders.

4. Experimental section

4.1.Chemistry

All the commercial available reagents and solvents were used as purchased from Sigma Aldrich (Italy), without further purification. The microwave-assisted synthesis were performed using an Initiator EXP Microwave Biotage instrument (frequency of irradiation: 2.45 GHz). Analytical silica gel plates (0.20 mm, F254, Merck, Germany), preparative silica gel plates (2 mm, F254, Merck, Germany) and silica gel 60 (70-230 mesh, Merck, Germany) were used for analytical and preparative TLC, and for column chromatography, respectively. Melting points were determined in glass capillary tubes on a Gallenkamp melting point apparatus and are uncorrected. Compounds were named following IUPAC rules as applied by ACD/ChemSketch. Elemental analyses were performed with a Flash E1112 Thermofinnigan elemental analyzer for C, H, N and the results are within ±0.4% of the theoretical values. The IR spectra were recorded with a Perkin-Elmer Spectrum RX I spectrometer in Nujol mulls and data are expressed in cm^{-1}. Proton nuclear magnetic resonance (^{1}H NMR) experiments were run on Bruker Avance 400 instrument at 400 MHz.Spectra were recorded at 300 K, using DMSO-d_6 as solvent. Chemical shifts were recorded in parts per million using the residual non-deuterated solvent as the internal standard. Data are reported as follows: chemical shift δ (ppm), multiplicity (indicated as: s, singlet; br s, broad singlet; d, doublet; t, triplet; q, quartet; m, multiplet and combination thereof), integrated intensity, coupling costants (J) in Hertz (Hz). Compounds **20**,[32] **21**,[32] **22**,[33] **24**,[32] **25**,[35] and **27**[33] were synthesized following the procedures reported in the literature.

*4.1.1. 2-(Pyrazin-2-yl)-thiazolo[5,4-d]pyrimidine-5,7-diol (**23**).* To a suspension of compound **21**[32] (3 mmol) in anhydrous NMP (6 mL), 2-pyrazine carbonyl chloride[34] (3.5 mmol) was added. The mixture was refluxed under N_2 atmosphere for 18h. After cooling the reaction mixture was diluted with water (about 100 mL) affording a solid residue which was collected by filtration and purified by crystallization. Yield: 70 %. Mp > 300 °C (ethylene glycol monomethyl ether). ^{1}H NMR: δ 8.70-8.71 (m, 1H, ar), 8.75-8.76 (m, 1H, ar), 9.27 (s, 1H, ar), 11.47 (s, 1H, OH), 12.21 (br s, 1H, OH). Anal calcd. for ($C_9H_5N_5O_2S$): C, 43.72%; H, 2.04%; N, 28.33%. Anal. found: C, 44.05%; H, 2.25%; N, 28.51%.

*4.1.2. 5,7-Dichloro-2-(pyrazin-2-yl)-thiazolo[5,4-d]pyrimidine (**26**).* A suspension of the 5,7-diolo derivative **23** (4.2 mmol) in $POCl_3$ (10 mL) was microwave irradiated at 170°C for 40min. The organics were evaporated under reduced pressure, then the residue was treated with ice-water (about 30g) affording a precipitate which was collected by filtration and used in the next step without further purication. Yield: 70 %. ^{1}H NMR: δ 8.89-8.90 (m, 1H, ar), 8.97-8.98 (m, 1H, ar), 9.54 (s, 1H, ar).

4.1.3. General procedure for the preparation of the 5-chloro-thiazolo[5,4-d]pyrimidine-7-amine derivatives **28-30**. To a suspension of the 5,7-dichloro derivatives **25-27** (4 mmol) in ethanol (10 mL), aqueous ammonia solution (33%, 15 mL) was added, and the resulting mixture was refluxed for 6-8h. The precipitate was collected by filtration and purified by crystallization.

*4.1.3.1. 5-Chloro-2-phenyl-thiazolo[5,4-d]pyrimidin-7-amine (**28**).* Yield: 70 %. Mp 288-290 °C (nitromethane). ^{1}H NMR: δ 7.60 (m, 3H, ar), 8.06-8.07 (m, 2H, ar), 8.20-8.30 (br s, 2H, NH_2). IR: 3124, 3278, 3467. Anal calcd. for ($C_{11}H_7ClN_4S$): C, 50.29%; H, 2.69%; N, 21.33%. Anal. found: C, 50.44%; H, 2.72%; N, 21.47%.

*4.1.3.2. 5-Chloro-2-(pyrazine-2yl)-thiazolo[5,4-d]pyrimidin-7-amine (**29**).* Yield: 65 %. Mp > 300 °C (acetic acid). ^{1}H NMR: δ 8.41 (br s, 2H, NH_2), 8.77-8.78 (m, 1H, ar), 8.81-8.82 (m, 1H, ar), 9.43

(s, 1H, ar). Anal calcd. for ($C_9H_5ClN_6S$): C, 40.84%; H, 1.90%; N, 31.75%. Anal. found: C, 41.11%; H, 2.22%; N, 31.88%.

*4.1.3.3. 5-Chloro-2-methyl-thiazolo[5,4-d]pyrimidin-7-amine (***30***).* Yield: 75 %. Mp 235-236 °C dec. (ethanol). ^{1}H NMR: δ 2.77 (s, 3H, CH_3), 8.10 (br s, 2H, NH_2). IR: 3136, 3296, 3359. Anal calcd. for ($C_6H_5ClN_4S$): C, 35.92%; H, 2.51%; N, 27.92%. Anal. found: C, 36.07%; H, 2.63%; N, 28.12%.

4.1.4. General procedure for the preparation of the thiazolo[5,4-d]pyrimidine-5,7-diamine derivatives **1-18**

The title compounds were prepared by reacting the 5-chloro-7-amino derivatives **28-30** (1 mmol) and the suitable amines (R_5NH_2, 3 mmol) in n-BuOH (2 mL). The reaction mixture was microwave irradiated at 200 °C for 10-20 min. The suspension was basified with aqueous KOH solution (50%), and then diluted with H_2O (about 100 mL) to afford a solid which was filtered and washed with Et_2O. The crude products were purified by chromatography and/or crystallization as specified below.

*4.1.4.1. N^5, 2-diphenyl-thiazolo[5,4-d]pyrimidine-5,7-diamine (***1***).* Yield: 80 %. Mp 245-248 °C (ethanol). ^{1}H NMR: δ 6.92 (t, 1H, ar, J = 7.2), 7.26 (t, 2H, ar, J = 7.8), 7.47-7.56 (m, 5H, 3ar + NH_2), 7.81 (d, 2H, ar, J = 7.8), 7.99 (d, 2H, ar, J = 6.6), 9.34 (s, 1H, NH). Anal calcd. for ($C_{17}H_{13}N_5S$): C, 63.93%; H, 4.10%; N, 21.93%. Anal. found: C, 64.71%; H, 4.27%; N, 22.10%.

*4.1.4.2. N^5-Benzyl-2-phenyl-thiazolo[5,4-d]pyrimidine-5,7-diamine (***2***).* Yield: 85%. Mp 207-210 °C (ethanol). ^{1}H NMR: δ 4.51 (d, 2H, J = 6.2), 7.19-7.21 (m, 2H, ar), 7.28-7.32 (m, 5H, 3 ar + NH_2), 7.50-7.52 (m, 4H, 3ar + NH), 7.92-7.94 (m, 2H, ar). IR: 3173, 3252, 3441. Anal calcd. for ($C_{18}H_{15}N_5S$): C, 64.84%; H, 4.53%; N, 21.01%. Anal. found: C, 64.63%; H, 4.39%; N, 21.15%.

*4.1.4.3. N^5-(2-Methoxybenzyl)-2-phenyl-thiazolo[5,4-d]pyrimidine-5,7-diamine (***3***).* Yield: 70%. Mp 201-204 °C (column chromatography, eluting system ethyl acetate/cyclohexane 4/6). ^{1}H NMR:

δ 3.83 (s, 3H, OCH_3), 4.49 (d, 2H, CH_2, J = 6.0), 6.88(t, 1H, ar, J = 7.3), 6.97 (d, 1H, ar, J = 8.0), 7.18-7.22 (m, 4H, 2 ar + NH_2), 7.47-7.53 (m, 4H, 3ar + NH), 7.93 (d, 2H, ar, J = 6.8). IR: 3068, 3321, 3373. Anal calcd. for ($C_{19}H_{17}N_5OS$): C, 62.79%; H, 4.71%; N, 19.27%. Anal. found: C, 62.56%; H, 4.94%; N, 19.03%.

*4.1.4.4. N^5-(3-Methoxybenzyl)-2-phenyl-thiazolo[5,4-d]pyrimidine-5,7-diamine (***4***).* Yield: 60%. Mp 169-171 °C (column chromatography, eluting system ethyl acetate/cyclohexane 3/7). ^{1}H NMR: δ 3.72 (s, 3H, OCH_3), 4.48 (d, 2H, CH_2, J = 6.1), 6.77 (dd, 1H, ar, J = 7.4, 2.0), 6.88-6.90 (m, 2H, ar), 7.19-7.23 (m, 3H, 1ar + NH_2), 7.38 (br s, 1H, NH), 7.47-7.53 (m, 3H, ar), 7.93 (d, 2H, ar, J = 7.6). Anal calcd. for ($C_{19}H_{17}N_5OS$): C, 62.79%; H, 4.71%; N, 19.27%. Anal. found: C, 62.90%; H, 4.85%; N, 19.41%.

*4.1.4.5. 2-Phenyl-N^5-(thiophen-2-ylmethyl)-thiazolo[5,4-d]pyrimidine-5,7-diamine (***5***).* Yield: 72%. Mp 186-188 °C (column chromatography, eluting system ethyl acetate/cyclohexane 3/7). ^{1}H NMR: δ 4.64 (d, 2H, CH_2, J = 6.2), 6.94-6.95 (m, 1H, ar), 7.00-7.01 (m, 1H, ar), 7.25 (br s, 2H, NH_2), 7.33 (d, 1H, ar, J = 4.9), 7.43 (br s, 1H, NH), 7.48-7.54 (m, 3H, ar), 7.94-7.95 (m, 2H, ar). IR: 3186. 3255. 3400. Anal calcd. for ($C_{16}H_{13}N_5S_2$): C, 56.61%; H, 3.86%; N, 20.63%. Anal. found: C, 56.36%; H, 4.10%; N, 20.52%.

*4.1.4.6. 2-Phenyl-N^5-(2-phenylethyl)-thiazolo[5,4-d]pyrimidine-5,7-diamine (***6***).* Yield: 75%. Mp 180-182 °C (n-butanol). ^{1}H NMR: δ 2.85 (t, 2H, CH_2, J = 7.2), 3.46-3.51 (m, 2H, CH_2), 6.87 (br s, 1H, NH), 7.18-7.32 (m, 7H, 5 ar + NH_2), 7.46-7.54 (m, 3H, ar), 7.93-7.95 (m, 2H, ar). IR: 3147, 3266. Anal calcd. for ($C_{19}H_{17}N_5S$): C, 65.68%; H, 4.93%; N, 20.16%. Anal. found: C, 65.87%; H, 5.09%; N, 20.18%.

*4.1.4.7. N^5-[2-(4-Methoxyphenyl)ethyl]2-phenyl-thiazolo[5,4-d]pyrimidine-5,7-diamine (***7***).* Yield: 67%. Mp 186-187 °C (ethanol). ^{1}H NMR: δ 2.78 (t, 2H, CH_2, J = 7.1), 3.42-3.47 (m, 2H, CH_2), 3.72 (s, 3H, CH_3), 6.81-6.87 (m, 3H, 2ar + NH), 7.16-7.19 (m, 4H, 2 ar + NH_2), 7.46-7.54 (m, 3H, ar),

7.94 (d, 2H, ar, J = 7.2). IR: 3198, 3268, 3335, 3471. Anal calcd. for ($C_{20}H_{19}N_5OS$): C, 63.64%; H, 5.07%; N, 18.55%. Anal. found: C, 63.48%; H, 4.97%; N, 18.44%.

*4.1.4.8. N^5-(2-Methoxybenzyl)-2-(pyrazin-2-yl)-thiazolo[5,4-d]pyrimidine-5,7-diamine (***9***).* Yield: 30%. Mp 276-278 °C (acetic acid). ^{1}H NMR: δ 3.83 (s, 3H, CH_3), 4.50 (d, 2H, CH_2, J = 5.6), 6.88 (t, 1H, ar, J = 7.5), 6.97 (d, 1H, ar, J = 8.0), 7.19-7.23 (m, 2H, ar), 7.31 (br s, 1H, NH), 7.39 (br s, 2H, NH_2), 8.68-8.69 (m, 2H, ar), 9.33 (s, 1H, ar). Anal calcd. for ($C_{17}H_{15}N_7OS$): C, 55.88%; H, 4.14%; N, 26.83%. Anal. found: C, 56.01%; H, 4.32%; N, 26.71%.

*4.1.4.9. N^5-(3-Methoxybenzyl)-2-(pyrazin-2-yl)-thiazolo[5,4-d]pyrimidine-5,7-diamine (***10***).* Yield: 40%. Mp 207-209 °C (acetic acid). ^{1}H NMR: δ 3.73 (s, 3H, CH_3), 4.50 (d, 2H, CH_2, J = 6.1), 6.78 (d, 1H, ar, J = 7.4), 6.89-6.91 (m, 2H, ar), 7.22 (t, 1H, ar, J = 8.0), 7.41 (br s, 2H, NH_2), 7.55 (br s, 1H, NH), 8.69-8.70 (m, 2H, ar), 9.34 (s, 1H, ar). Anal calcd. for ($C_{17}H_{15}N_7OS$): C, 55.88%; H, 4.14%; N, 26.83%. Anal. found: C, 55.94%; H, 4.07%; N, 27.00%.

*4.1.4.10. 2-(Pyrazin-2-yl)-N^5-(thiophen-2-ylmethyl)-thiazolo[5,4-d]pyrimidine-5,7-diamine (***11***).* Yield: 45 %. Mp 250-252 °C (acetic acid). ^{1}H NMR: δ 4.67 (d, 2H, CH_2, J = 6.2), 6.95 (t, 1H, ar, J = 4.1), 7.00-7.01 (m, 1H, ar), 7.33 (d, 1H, ar, J = 4.9), 7.42 (br s, 2H, NH_2), 7.57 (br s, 1H, NH), 8.70-8.71 (m, 2H, ar), 9.35 (s, 1H, ar). Anal calcd. for ($C_{14}H_{11}N_7S_2$): C, 49.25%; H, 3.25%; N, 28.72%. Anal. found: C, 48.93%; H, 3.37%; N, 29.07%.

*4.1.4.11. 2-Methyl-N^5-phenyl-thiazolo[5,4-d]pyrimidine-5,7-diamine (***12***).* Yield: 70%. Mp 214-215 °C (ethanol). ^{1}H NMR: δ 2.68 (s, 3H, CH_3), 6.89 (t 1H, ar, J = 7.3), 7.22-7.27 (m, 4H, 2 ar + NH_2), 7.78 (d, 2H, ar, J = 7.7), 9.14 (s, 1H, NH). IR: 3176, 3247, 3425. Anal calcd. for ($C_{12}H_{11}N_5S$): C, 56.01%; H, 4.31%; N, 27.22%. Anal. found: C, 56.35%; H, 4.50%; N, 27.31%.

*4.1.4.12. N^5-Benzyl-2-methyl-thiazolo[5,4-d]pyrimidine-5,7-diamine (***13***).* Yield: 80%. Mp 170-172 °C (isopropanol). ^{1}H NMR: δ 2.68 (s, 3H, CH_3) 4.47 (d, 2H, CH_2, J = 6.0), 7.00 (s, 2H, NH_2), 7.13-

7.21 (m, 2H, 1 ar + NH), 7.27-7.30 (m, 4H, ar). IR: 3186, 3326, 3425. Anal calcd. for ($C_{13}H_{13}N_5S$): C, 57.54%; H, 4.83%; N, 25.81%. Anal. found: C, 57.62%; H, 4.89%; N, 25.98%.

*4.1.4.13. N^5-(2-Methoxybenzyl)-2-methyl-thiazolo[5,4-d]pyrimidine-5,7-diamine (***14***).* Yield: 60%. Mp 215-217 °C (preparative TLC eluting system ethyl acetate/cyclohexane 6/4) ^{1}H NMR: δ 2.61 (s, 3H, CH_3), 3.82 (s, 3H, OCH_3), 4.44 (d, 2H, CH_2, J = 6.2), 6.84-6.88 (m, 2H, ar), 6.95-7.00 (m, 3H, 1 ar + NH_2), 7.16-7.22 (m, 2H, 1 ar + NH). Anal calcd. for ($C_{14}H_{15}N_5OS$): C, 55.80%; H, 5.02%; N, 23.24%. Anal. found: C, 55.61%; H, 4.96%; N, 23.15%.

*4.1.4.14. N^5-(3-Methoxybenzyl)-2-methyl-thiazolo[5,4-d]pyrimidine-5,7-diamine (***15***).* Yield: 65%. Mp 168-170 °C (preparative TLC eluting system ethyl acetate/cyclohexane 6/4) ^{1}H NMR: δ 2.61 (s, 3H, CH_3), 3.71 (s, 3H, OCH_3), 4.44 (d, 2H, CH_2, J = 6.4), 6.75-6.78 (m, 1H, ar), 6.86-6.88 (m, 2H, ar), 7.01 (s, 2H, NH_2), 7.10 (t, 1H, ar, J = 7.0), 7.12 (t, 1H, NH, J = 6.4). Anal calcd. for ($C_{14}H_{15}N_5OS$): C, 55.80%; H, 5.02%; N, 23.24%. Anal. found: C, 55.86%; H, 5.13%; N, 23.19%.

*4.1.4.15. 2-Methyl-N^5-(thiophen-2ylmethyl)-thiazolo[5,4-d]pyrimidine-5,7-diamine (***16***).* Yield: 50%. Mp 168-170 °C (preparative TLC eluting system ethyl acetate/cyclohexane 1/1) ^{1}H NMR: δ 2.63 (s, 3H, CH_3), 4.60 (d, 2H, CH_2, J = 6.4), 6.91-6.93 (m, 2H, ar), 7.04 (br s, 2H, NH_2), 7.18 (t, 1H, NH, J = 6.4), 7.30-7.31 (m, 1H, ar). IR: 3188, 3312. Anal calcd. for ($C_{11}H_{11}N_5S_2$): C, 47.63%; H, 4.00%; N, 25.25%. Anal. found: C, 47.94%; H, 4.21%; N, 25.37%.

*4.1.4.16. 2-Methyl-N^5-(2-phenylethyl)-thiazolo[5,4-d]pyrimidine-5,7-diamine (***17***).* Yield: 76%. Mp 130-133 °C (n-butanol). ^{1}H NMR: δ 2.62 (s, 3H, CH_3), 2.83 (t, 2H, CH_2, J = 7.0), 3.42-3.47 (m, 2H, CH_2), 6.62 (br s, 1H, NH), 6.97 (br s, 2H, NH_2), 7.19-7.21 (m, 1H, ar), 7.24-7.31 (m, 4H, ar). IR: 3187, 3251, 3315, 3360. Anal calcd. for ($C_{14}H_{15}N_5S$): C, 58.92%; H, 5.30%; N, 24.54%. Anal. found: C, 59.05%; H, 5.46%; N, 24.71%.

*4.1.4.17. 2-Methyl-N^5-[2-(thiophen-2-yl)ethyl]-thiazolo[5,4-d]pyrimidine-5,7-diamine (***18***).* Yield: 30 %. Mp 143-145 °C (ethyl acetate/cyclohexane). ^{1}H NMR: δ 2.62 (s, 3H, CH_3), 3.04 (t, 2H, CH_2,

J= 7.2), 3.48 (dd, 2H, CH_2, J= 13.4, 7.2), 6.69 (br s, 1H, NH), 6.90-6.99 (m, 4H, 2 ar + NH_2), 7.31-7.32 (m, 1H, ar). Anal calcd. for ($C_{12}H_{13}N_5S_2$): C, 49.46%; H, 4.50%; N, 24.03%. Anal. found: C, 49.29%; H, 4.61%; N, 23.88%.

*4.1.5. 4-[2-(7-Amino-2-phenyl-thiazolo[5,4-d]pyrimidin-5-yl)amino]ethyl]phenol (**8**).* A solution of BBr_3 in CH_2Cl_2 (1 M, 6 mL) was portion-wise added to a suspension of compound **7** (2 mmol) in anhydrous CH_2Cl_2 (40 mL). The suspension was stirred at 50 °C for one day and then was diluted with ice-water (about 90 g) and vigorously stirred for 4h. After addition of a saturated solution of $NaHCO_3$ (10 mL), the organic phase was collected, washed with water (20 mL x 2) and dried (Na_2SO_4). Evaporation at reduced pressure of the solvent afforded a solid which was purified by crystallization. Yield: 60%. Mp 205-207 °C (methanol). ^{1}H NMR: δ 2.74 (t, 2H, CH_2, J = 7.0), 3.40-3.45 (m, 2H, CH_2), 6.69 (d, 2H, ar, J = 8.0), 6.79 (br s, 1H, NH), 7.05 (d, 2H, ar, J = 8.0), 7.15 (br s, 2H, NH_2), 7.46-7.54 (m, 3H, ar), 7.94 (d, 2H, ar, J = 8.2), 9.15 (s, 1H, OH). IR: 3268, 3335, 3471. Anal calcd. for ($C_{19}H_{17}N_5OS$): C, 62.79%; H, 4.71%; N, 19.27%. Anal. found: C, 62.85%; H, 4.77%; N, 19.51%.

4.2. In vitro pharmacology.

4.2.1. Materials. [^{3}H]-DPCPX ([^{3}H]1,3-dipropyl-8-cyclopentyl-xanthine; specific activity, 120 Ci/mmol) and [^{125}I]-ABMECA ([^{125}I]4-aminobenzyl-5'-N-methyl-carboxamidoadenosine; specific activity, 2200 Ci/mmol) were obtained from Perkin Elmer Research Products (Boston, MA); [^{3}H]-ZM 241385 ([^{3}H](4-(2-[7-amino-2-(2-furil)[1,2.4] triazolo[2,3-a][1,3,5]triazin-5-ylamino] ethyl) phenol); specific activity, 17 Ci/mmol) was obtained from Biotrend (Cologne, Germany). DPCPX, NECA (N-ethylcarboxamido adenosine), AB-MECA, CGS 21680 (2-p(2-carboxyethyl) phenethylamino-5'-N-ethylcarboxamido adenosine), Forskolin, CCPA (2-Chloro-N^6-cyclopentyladenosine) and ZM 241385 were obtained from Sigma Aldrich (St. Louis, MO).

4.2.2. Cell culture and membrane preparation. Chinese Hamster Ovary (CHO) cells transfected with hA_1, hA_{2A}, hA_{2B} and hA_3 ARs were grown adherently and maintained in Dulbecco's modified Eagle's medium with nutrient mixture F12, containing 10% fetal calf serum, penicillin (100 U/ml), streptomycin (100 μg/ml), l-glutamine (2 mM), geneticine (G418; 0.2 mg/ml) at 37°C in 5% CO_2/95% air until the use in cAMP assays.[26] For membrane preparation the culture medium was removed, and the cells were washed with phosphate-buffered saline and scraped off T75 flasks in ice-cold hypotonic buffer (5 mM TrisHCl,1 mM EDTA, pH 7.4). The cell suspension was homogenized with a Polytron, centrifuged for 30min at 40000 *g* at 4°C and the resulting membrane pellet was used for competition binding experiments.[26]

4.2.3. Competition binding experiments. All synthesized compounds were tested for their affinity to hA_1, hA_{2A} and hA_3 ARs. Competition experiments to A_1 ARs were carried out incubating 1 nM [^{3}H]-DPCPX with membrane suspension (50 μg of protein/100 μl) and different concentrations of the examined compounds at 25°C for 90 min in 50 mMTrisHCl, pH 7.4. Non-specific binding was defined as binding in the presence of 1 μM DPCPX and was always < 10% of the total binding.[36] Inhibition experiments to A_{2A} ARs were performed incubating the radioligand [^{3}H]-ZM 241385 (1 nM) with the membrane suspension (50 μg of protein/100 μl) and different concentrations of the examined compounds for 60 min at 4°C in 50mMTrisHCl (pH 7.4), 10 mM $MgCl_2$. Non-specific binding was determined in the presence of ZM 241385 (1 μM) and was about 20% of the total binding.[26] Competition binding experiments to A_3ARs were carried out incubating the membrane suspension (50 μg of protein/100 μl) with 0.5 nM [^{125}I]-ABMECA in the presence of different concentration of the examined compounds for an incubation time of 120 min at 4 °C in 50 mM TrisHCl (pH 7.4), 10 mM $MgCl_2$, 1 mM EDTA. Non-specific binding was defined as binding in the presence of 1 μM ABMECA and was always < 10% of the total binding.[37] Bound and free radioactivity were separated by filtering the assay mixture through Whatman GF/B glass fiber filters using a Brandel cell harvester (Brandel Instruments, Unterföhring, Germany). The filter bound radioactivity was counted by Packard Tri Carb 2810 TR scintillation counter (Perkin Elmer).

4.2.4. Cyclic AMP assays. The potency of the examined compounds to hA_{2B} ARs were determined evaluating their capability to inhibit NECA (100 nM)-stimulated cAMP levels. In hA_1 ARs CHO cells, different concentrations of the selected compounds were incubated with Forskolin (5 μM) in the presence or in the absence of A_1 agonist CCPA (1 nM) in order to evaluate their functional behavior. Moreover, the potency of the compounds versus hA_{2A}ARs was determined studying their capability to inhibit basal levels of cAMP. The same experiments were also performed in the presence of CGS 21680 (10 nM). cAMP levels were then quantified by using the AlphaScreen cAMP Detection Kit (Perkin Elmer, Boston, USA) following the manufacturer's instructions.[38] Briefly, compounds were added to cell suspension in a half-area 96 well plate in the presence of anti-cAMP acceptor beads and incubated for 30 min. Then, streptadvidin coated donor beads and biotinylated cAMP were added and incubated for 1 hour. At the end of the experiments, plates were read with the Perkin Elmer EnSight Multimode Plate Reader.

4.2.5. Statistical analysis. The protein concentration was determined according to a Bio-Rad method with bovine albumin as a standard reference.[26] The data are expressed as the mean ± SEM of n = 4 independent experiments. Statistical analysis of the data was performed using one way ANOVA followed by Dunnett's post hoc test. Inhibitory binding constants, K_i, will be calculated from the IC_{50} values according to the Cheng and Prusoff equation: $K_i = IC_{50}/(1 + [C^*]/K_D^*)$, where [C*] is the concentration of the radioligand and K_D^* its dissociation constant.[26] IC_{50} values obtained in cAMP assays were calculated by non-linear regression analysis using the equation for a sigmoid concentration-response curve.

Author Contributions

All authors have given approval to the final version of the manuscript.

Notes

The authors declare no competing financial interest.

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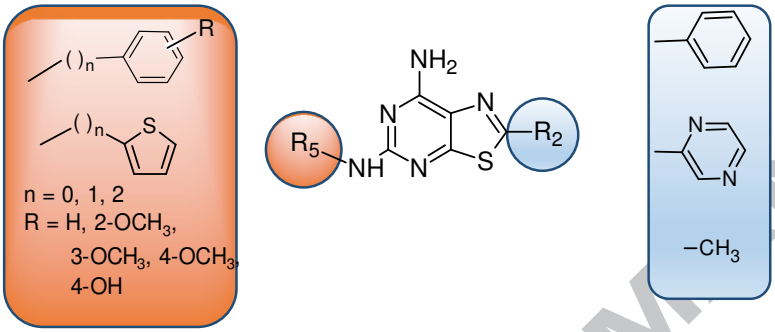
Graphical abstract



Highlights

- Synthesis of novel thiazolo[5,4-d]pyrimidine derivatives is reported
- The compounds were tested for their affinity and potency at human adenosine receptors
- The new thiazolo[5,4-d]pyrimidines are hA_1 and A_{2A} antagonists/inverse agonists