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Structural investigation on thiazolo[5,4-d]pyrimidines to obtain dual-acting blockers of CD73 and adenosine A_{2A} receptor as potential antitumor agents.

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Abstract.

Adenosine pathway, including its generating enzyme (CD73) and its receptors represents a key target for cancer immunotherapy. Here we aimed to search for novel compounds able to co-target the CD73 and the A_{2A} adenosine receptor (A_{2A} AR) as dual-blockers of adenosine generation and activity. The design project was to combine in the same molecule the thiazolo[5,4-d]pyrimidine core, an essential pharmacophoric feature to block the A_{2A} AR, with a benzenesulfonamide group which is a characteristic group of CD73 inhibitors. Most of the reported compounds resulted in inverse agonists of the human (h) A_{2A} AR endowed with high affinity, selectivity and potency. However they were weak inhibitors of CD73 enzyme. Nevertheless, this study can be considered as a starting point to develop more active compounds.

KEYWORDS. Thiazolo[5,4-d]pyrimidine, adenosine A_{2A} receptor inverse agonists, CD73 inhibitors, cancer immunotherapy, antitumor agents

In recent years cancer immunotherapy has represented a novel, high potential strategy for oncological treatment. In particular, researchers' attention was focused on the immune checkpoint targeting therapy, which aims to interfere with the negative feedback mechanism activated by the tumor on immune cells, resulting in suppression of the physiological anti-tumor immune response [1]. In addition to the well known anti-CTLA4 and anti-PD1/PDL1 monoclonal antibodies (mAbs) [2], the adenosine pathway has recently been proposed as an important target to restore the anti-tumor immune response [3]. In fact, as a consequence of hypoxia and extracellular stress, in solid tumor microenvironment (TEM) an accumulation of ATP occurs that is converted into adenosine by the ectonucleotidases CD39 and CD73 [4]. Adenosine interacts with G-protein-coupled adenosine receptors (ARs), classified as A_1, A_{2A}, A_{2B} and A_3 based on their affinity for adenosine and on their signal transduction pathway [5]. Indeed, physiological concentration of extracellular adenosine (0.3-1000 nM), while it is high enough to activate both A_1 and A_{2A} ARs, is not sufficient for the low affinity A_{2B} and A_3 subtypes. All ARs are primarily coupled to adenylyl cyclase, which is inhibited (A_1 and A_3) or activated (A_{2A} and A_{2B}), thus reducing or enhancing cAMP levels [6-7]. It is well documented that activation of ARs can produce a large variety of effects in different tissues and organs. In particular, one of the important activities of adenosine is inflammation suppression through a direct inhibitory effect on diverse immune cells that express the A_{2A} AR [8]. Thus, increased production of adenosine within the hypoxic microenvironment of inflamed tissue has been shown to inhibit overactive immunosuppressive cells from attacking healthy tissues. However, in the hypoxic TME, elevated concentration of adenosine activates A_{2A} AR on immune cells, thus causing suppression of the antitumor immune response [3-4]. Moreover, the A_{2A} AR is expressed also on tumor cells and its stimulation induces and increases cell proliferation, survival, chemotaxis and migration, thus favoring tumor growth [7-8].

In the TME stressed conditions increase of extracellular adenosine concentration is also able to activate the low affinity A_{2B} AR subtype. Compelling evidence suggests that A_{2B} AR plays an important role in mediating the pro-tumor effects of adenosine, since its selective blockade can inhibit

tumor growth in some tumor murine models [9-10]. For this reason compounds able to antagonize the A_{2B} ARs or both A_{2A} and A_{2B} ARs, may be considered as potential therapeutic agents in cancer treatment.

CD73 is a glycophosphatidylinositol (GPI)-anchored homodimeric protein that contains two Zn^{2+} ions as cofactors in its active site, and is classified as a metallophosphatase. The enzyme catalyzes the dephosphorylation of purine and pyrimidine ribo- and deoxyribonucleoside monophosphates to the corresponding nucleosides. CD73 has high affinity for adenosine monophosphate (AMP) and catalyzes its conversion to the bioactive adenosine, thus it is an important regulator of the adenosine signaling pathway [11-12]. The underlying regulatory role of CD73 renders this enzyme a promising therapeutic target in many pathological conditions [13]. In particular, inhibition of CD73, overexpressed in TME and mainly responsible for the high concentration of adenosine, has proved useful in counteracting adenosine-induced tumor immunosuppression through A_{2A} ARs. Indeed, inhibition of CD73 by small molecules or antibodies has been shown to reduce tumor growth and metastasis [14-16].

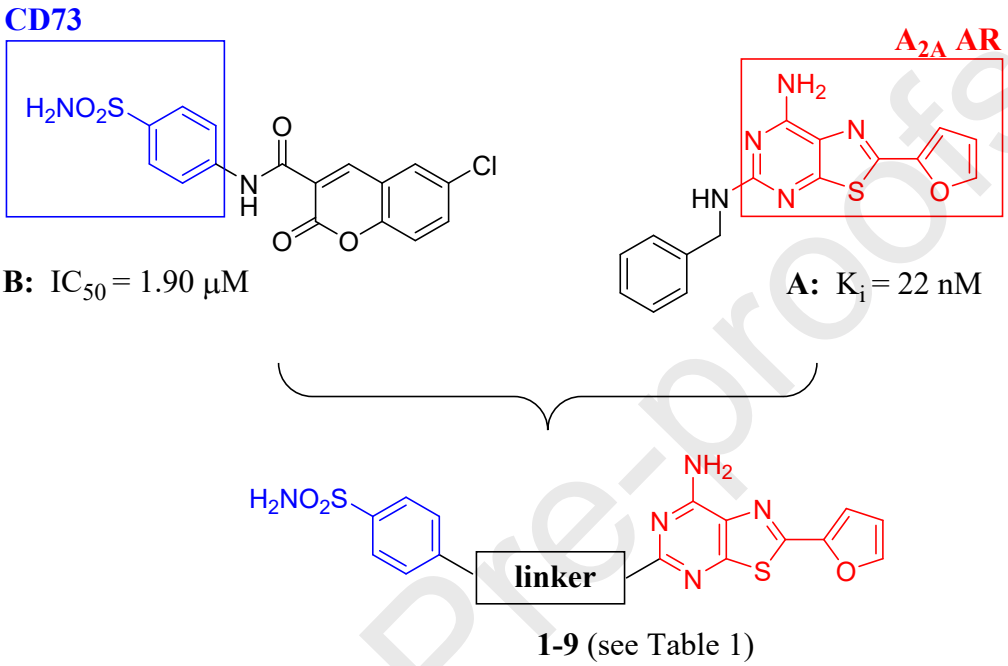
Taking into account these considerations, it is evident that the adenosine pathway, including its generating enzymes and its receptors, may represent a key target for cancer therapy, acting in TEM to restore an efficient anti-tumor immune response.

Thus, the purpose of this study was to develop novel compounds as potential anti-tumor drugs, able to co-target the CD73 and the A_{2A}AR to block at the same time adenosine generation and its activity. This working plan was also supported by the studies of Young et al. [17] which demonstrated that the anti-tumor activity, derived from combined inhibition of CD73 and A_{2A} AR signaling, is more powerful than blockade of each target alone.

Multi-target compounds can be obtained by using different strategies of rational drug design. One of these is molecular hybridization through which new chemical entities are obtained by combining into a single molecule two or more pharmacophoric units from different bioactive compounds [18]. Following this design approach, and with the aim to find A_{2A}AR/CD73 bifunctional blockers, we

carried out the synthesis of a series of compounds based on two active scaffolds, namely thiazolo[5,4-d]pyrimidines and benzenesulfonamides (Figure 1).

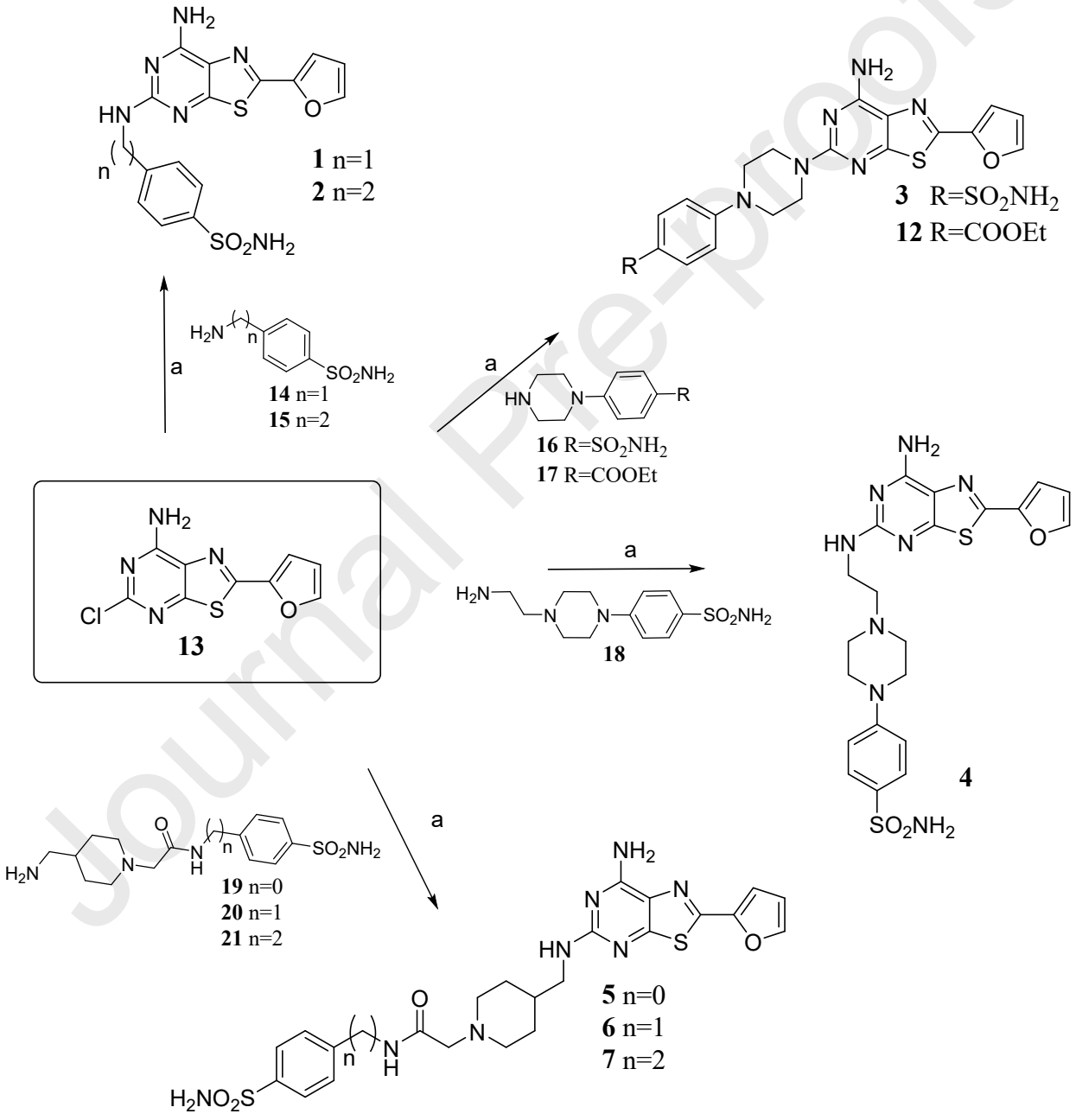
Figure 1. Design strategy to obtain dual blockers of CD73/hA_{2A} AR.



Recently, as a part of our efforts to find novel AR antagonists [19-29], we identified the thiazolo[5,4-d]pyrimidine as a privileged scaffold to obtain potent and selective hA_{2A} AR antagonists/inverse agonists (see compound **A** as an example) [21, 25-26]. On the other hand, benzenesulfonamide represents a characteristic group of some potent CD73 inhibitors reported in the literature (see compound **B** as an example) [30]. The SO_2NH_2 moiety seems to interact with a Zn^{2+} ion present into the catalytic site of the enzyme thus reinforcing the inhibitory activity of the compounds. Thus, novel 7-amino-2-(2-furanyl)thiazolo[5,4-d]pyrimidine derivatives **1-9** all bearing a terminal benzensulfonamide group, attached to the 5 position of the bicyclic core through linkers of different length and flexibility, were synthesized. Moreover, to broaden structure affinity/activity relationship studies of this new class of dual-acting agents, compounds **10-11** and **12**, bearing as terminal group of the 5-substituent a saccharinyl **10-11** or an ethyl benzoate **12** moiety, were synthesized.

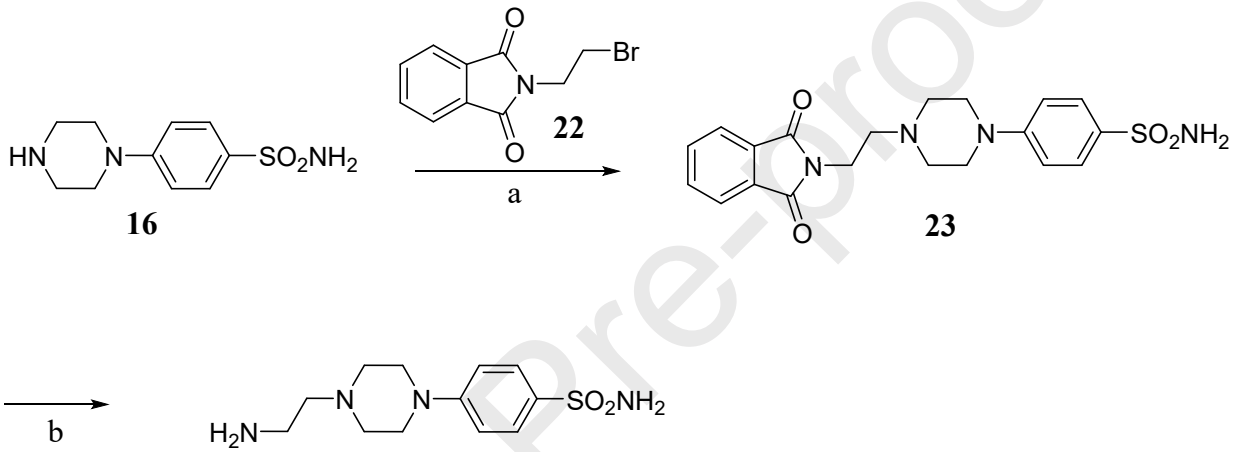
All the newly synthesized thiazolopyrimidines **1-12** were evaluated both for their affinity at hARs expressed in Chinese Hamster Ovary (CHO) cells, and for their inhibitory activity at human recombinant CD73 enzyme.

Compounds **1-7, 12** were prepared according to the procedure depicted in Scheme 1, which involves as starting material the 5-chloro-7-amino **13** previously synthesized by us [21].



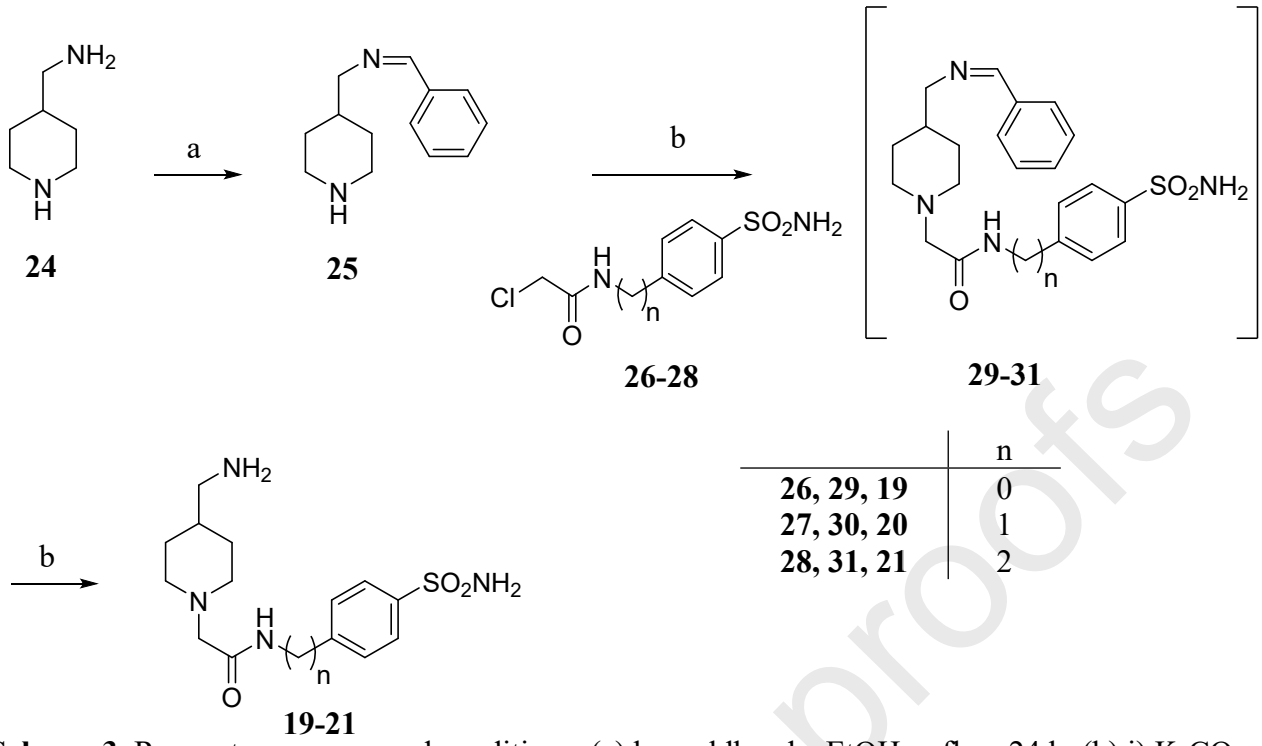
Scheme 1. Reagents and conditions: (a) n-BuOH, 200 °C MW, 20 min, 23-87% yield.

Displacement of the 5-chlorine atom of **13** by amines **14-21** was obtained by microwave irradiation of a mixture of derivative **13** and an excess of the proper amine in butanol. While the 4-aminomethyl-**14** and 4-(2-aminoethyl)-benzensulfonamide **15**, and the N^1-substituted piperazine derivatives **16-17** were commercial (**14-15**) or prepared according to literature (**16-17**) [31-32], the others amines **18-21** were synthesized as shown in Schemes 2 and 3. Briefly, the 4-(piperazin-1-yl)benzenesulfonamide **16** [31] was reacted with N-(2-bromoethyl)phtalimide **22** to yield the N-(2-ethylsubstituted)-phtalimide **23** which furnished the corresponding ethyl amine **18** by hydrazinolysis (Scheme 2).



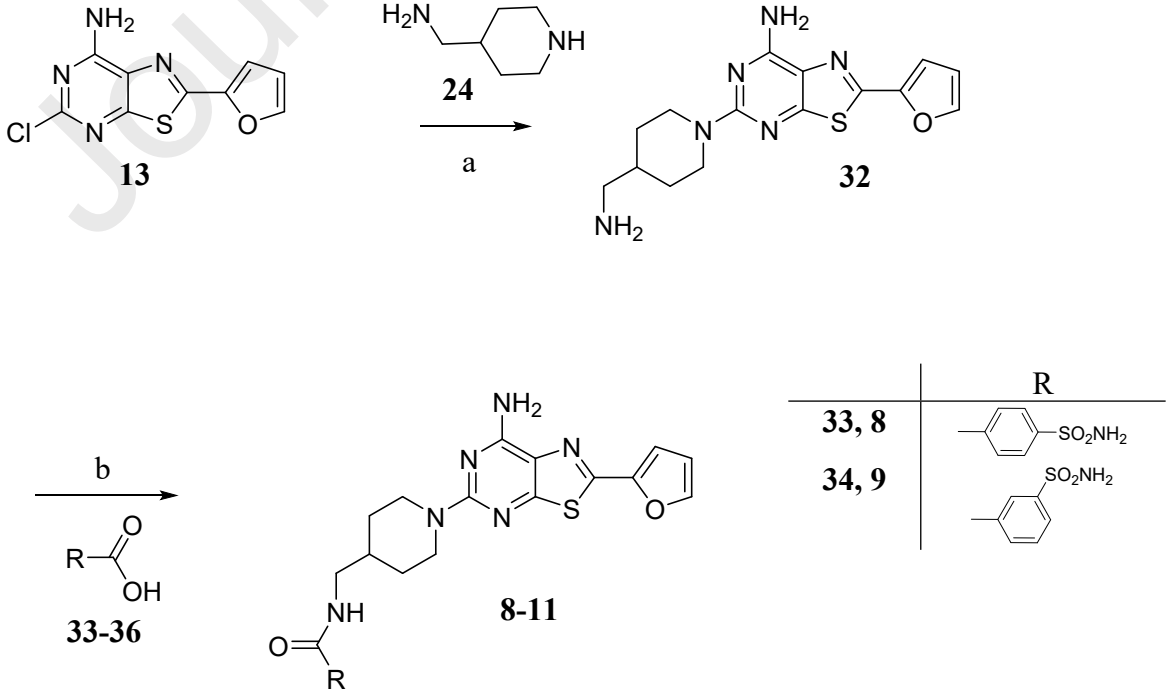
Scheme 2. Reagents and conditions: (a) Et_3N, CH_3CN, reflux, 12 h, 90% yield; (b) $NH_2NH_2 \cdot H_2O$, MeOH, reflux, 2 h, 80% yield.

Reaction of 4-aminomethylpiperidine **24** and benzaldehyde in absolute ethanol gave the imino derivative **25** [33] which was then reacted with the proper chloroacetamido derivative **26-28** [34] to yield the corresponding N-substituted piperidine derivatives **29-31** which were not isolated because they were unstable (Scheme 3).

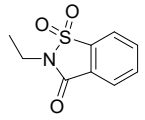


Scheme 3. Reagents and conditions: (a) benzaldheyde, EtOH, reflux, 24 h; (b) i) K_2CO_3, CH_3COCH_3, rt, 12 h; ii) oxalic acid, CH_2Cl_2/H_2O, 50 °C, 3 h, 55-75% yield.

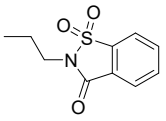
Hydrolysis of the protecting imino group of **29-31** was performed in acidic aqueous medium to furnish the desired 4-aminomethyl-piperidine derivatives **19-21**. Scheme 4 reports the synthetic procedure to prepare the thiazolo[5,4-d]pyrimidine derivatives **8-11** bearing at position 5 of the bicyclic core a 1-piperidinyl moiety.



35, 10

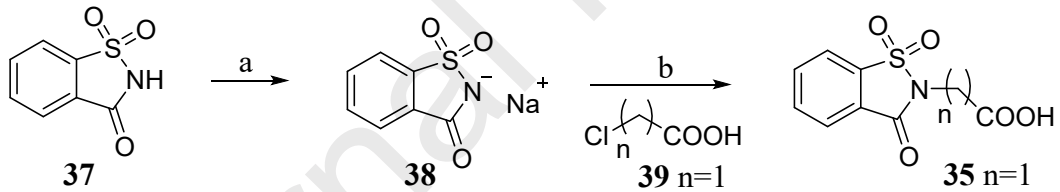


36, 11



Scheme 4. Reagents and conditions: (a) n-BuOH, 200 °C, MW, 20 min, 85% yield; (b) DMAP, EDCI, BtOH, DMF, rt, 5 h, 40-90% yield.

Thus, the 5-chloro-7-amino **13** was reacted with the commercial 4-aminomethylpiperidine **24**, under microwave irradiation at 200 °C, to furnish derivative **32**. Reaction of the latter with the proper carboxylic acids **33-36**, in the presence of EDCI, DMAP, and hydroxybenzotriazole, yielded the corresponding amide derivatives **8-11**. While the sulfamoylbenzoic acids **33** and **34** were commercial, the N-(saccharinyl) acids **35** and **36** were synthesized as reported in Scheme 5.



Scheme 5. Reagents and conditions: (a) MeONa, MeOH, reflux, 30 min; (b) sealed tube, 110 °C, 3h, 30-50% yield.

Briefly, commercial saccharin **37**, after deprotonation with sodium methoxide, was reacted with the appropriate chloroacid **39-40** to give the corresponding carboxylic acids **35-36** with high yields.

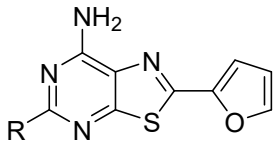
Derivatives **1-12** were evaluated for their affinity to hA_1, hA_{2A}, and hA_3 ARs stably expressed in CHO cells by using competition binding assays. Binding experiments at hA_1 and hA_3 ARs were performed to establish the A_{2A} selectivity of the tested compounds versus A_1 and A_3 AR subtypes.

Due to the lack of a suitable radioligand for the A_{2B} ARs the activity at this receptor subtype was determined by measuring the inhibition of NECA-stimulated adenylyl cyclase activity in hA_{2B} CHO cells. Binding and potency data of the newly synthesized compounds **1-12** and of the reference thiazolo[5,4-d]pyrimidine derivative **A**, are reported in Table 1.

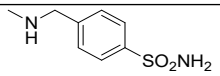
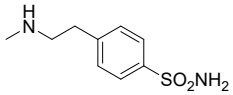
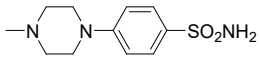
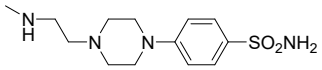
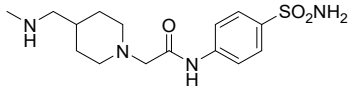
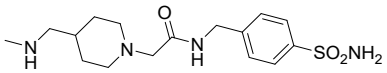
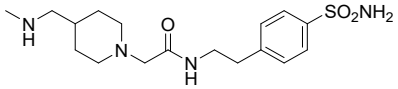
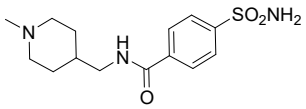
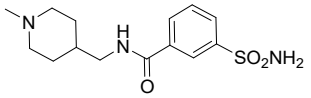
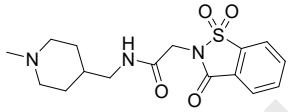
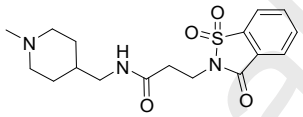
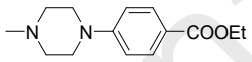
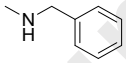
Several compounds (**2-7**, **9**) display high affinity for the hA_{2A} subtype ($2.02<K_i< 87$ nM), while the most (compounds **1-12**) show a poor affinity for the hA_1 AR (K_i values > 100 nM), except derivative **4** which was able to bind this subtype with a K_i falling in the nanomolar range (K_i = 84 nM). None of the reported derivatives binds the hA_3 AR with the exception of **3** which displayed some affinity (K_i=582 nM). Finally, the majority of compounds do not interact with the hA_{2B} AR subtype apart from four (**1-2**, **6-7**) which displayed high (**1** IC_{50}=28 nM; **2** IC_{50}=34 nM) or good potencies (**6** IC_{50}=146 nM; **7** IC_{50}=156 nM).

Detailed analysis of the binding results revealed that the aminomethyl- and the aminoethyl-benzenesulfonamide derivatives **1** and **2**, which differ only for the length of the chain between the bicyclic core and the benzenesulfonamide residue, displayed a very different binding behavior. In fact, the ethylamino derivative **2** shows a hA_{2A} AR affinity about 50 fold higher than that of its lower homologue **1**. In contrast, the length of the chain does not influence the potency at the hA_{2B} AR. Indeed, compounds **1-2** showed IC_{50} values falling in the nanomolar range. It is worth noting that the aminoethyl-benzensulfonamide derivative **2** while is equiactive to the reference compound **A** at the hA_{2B} AR, it is 5-fold more active at the A_{2A} subtype. Moreover, it shows hA_1 and hA_3 AR binding affinities falling in the micromolar range, as to be considered a very interesting dual hA_{2A}/hA_{2B} AR antagonist, highly selective versus the hA_1 and hA_3 AR subtypes (more than 200-fold).

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



1-12; A

compd	R	hA_1AR[a] K_i (nM)	$hA_{2A}AR$[b] K_i (nM)	$hA_{2B}AR$[c] IC_{50} (nM) (I%)	hA_3AR[d] K_i (nM) (I%)
1		236±18	295±23	28±3	3,458±303
2		1,326±256	5.73±0.48	34±3	1,874±158
3		387±32	86±7	>10,000 (12%)	582±49
4		89±8	2.02±0.18	>10,000 (30%)	>10,000 (22%)
5		119±9	57±5	>10,000 (28%)	>10,000 (15%)
6		687±48	87±7	146±13	>10,000 (27%)
7		603±56	60±6	156±15	>10,000 (29%)
8		5,703±487	488±42	>10,000 (9%)	>10,000 (12%)
9		568±53	41±4	>10,000 (17%)	>10,000 (31%)
10		1,671±28	488±39	>10,000 (5%)	>10,000 (6%)
11		1,068±28	639±55	>10,000 (12%)	>10,000 (22%)
12		4,536±312	279±23	>10,000 (38%)	2,679±221
A[e]		45±6	22±3	32±2	37±3

Affinity values obtained from displacement of specific [^{3}H]DPCPX [a], [^{3}H]ZM241383 [b] or [^{125}I]AB-MECA [d] binding to hA_1ARs, hA_{2A}ARs or A_3ARs, respectively (n=3–6). [c] 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. [e] Ref. 21.

In derivatives **3** and **4** the benzenesulfonamide group was attached through a piperazine moiety directly linked to the thiazolopyrimidine system (**3**) or spaced by an ethylamino chain (**4**). This structural difference makes the more flexible and longer derivative **4** about 40 fold more active than the more rigid and shorter compound **3**. Indeed, the aminoethylpiperazin substituted **4** displays the highest hA_{2A} AR affinity (K_i hA_{2A}=2.02 nM) among the herein reported compounds. Furthermore, it was more active and selective toward the hA_{2A} AR with respect to the reference **A**.

Compounds **5-7** possess the same 2-(4-(aminomethyl)piperidin-1-yl)acetamide fragment at 5-position, which is directly linked to the benzenesulfonamide moiety (derivative **5**) or through an alkyl chain of different length (derivatives **6**, **7**). These compounds show a similar good affinity for the hA_{2A} AR indicating that, in this set, moving away the benzenesulfonamide residue from the bicyclic core doesn't seem to influence the binding to this receptor. On the contrary, distancing the benzenesulfonamide group while decreases the hA_1 AR affinity, increses that for the A_{2B} subtype. Indeed, also compounds **6** and **7** can be considered dual hA_{2A}/hA_{2B} AR antagonists less potent than **2**.

Derivatives **8** and **9**, which exhibit the benzenesulfonamido moiety connected to the bicyclic core by a N-(piperidin-4ylmethyl)acetamide group, differ only for the position (para or meta) of the sulfonamide residue. While the para-sulfonamide substituted derivative **8** binds scarcely the hA_{2A} AR, the corresponding meta-substituted **9** displays a good binding affinity (K_i = 41 nM).

Compounds **10-11**, showed a low hA_{2A} AR binding affinity probably due to an excessive hindrance of the appended saccharinyl moiety.

Finally, when the sulfonamide group of derivative **3** was substituted with a carboxyethyl moiety, a drop of hA_{2A} AR affinity was obtained. In fact, the carboxyethyl derivative **12** exhibit a 3 fold lower affinity than that of the corresponding sulfonamide compound **3**.

All the reported compounds **1-12** were assayed against CD73, in order to evaluate their capability to block also adenosine generation (Table 2). In general, the tested derivatives showed weak inhibitory activity, whereas compounds **6**, **9**, and **10** were devoid of effects on CD73 activity.

In vitro activity of some selected compounds (**2-4, 12)** was also studied, in order to evaluate their antagonist/inverse agonist profile toward the hA_{2A} AR. In particular, the ability of the tested compounds to modulate cAMP production was tested. As shown in Table 3, derivatives **2-4** and **12** behaved as inverse agonists being able to inhibit basal cAMP accumulation at nanomolar concentration.

Table 2. Percent of CD73 activity in the presence of 0.1 mM of compounds **1-12**.[a]

compound	% Activity CD73	compound	% Activity CD73
1	85±5 (2)	**7**	90±20 (2)
2	84±21 (2)	**8**	97±5 (2)
3	81±8 (5)	**9**	100±6 (2)
4	89±1.6 (2)	**10**	100±15 (2)
5	90±4 (2)	**11**	80±5 (2)
6	100±13 (2)	**12**	77±4 (2)

[a] Data are expressed as means ± SEM. The number of assay are presented in the parentheses.

Table 3. Potency (IC_{50}) of selected compounds on the reduction of cyclic AMP levels in CHO cells expressing hA_{2A} AR.

Compounds	Potency (IC_{50} nM)	Pharmacological behaviour
2	5.20±0.46	Inverse agonist
3	58±5	Inverse agonist
4	0.34±0.03	Inverse agonist
12	334±28	Inverse agonist
A [a]	29±3	Inverse agonist

[a] Ref. 21. Data are expressed as means ± SEM.

In accordance with the binding results, compounds **2** and **4** displayed higher potencies than those of **3** and **12**, and also in this assay compound **4** is the most active showing a potency value in the subnanomolar range (IC_{50}=0.34 nM). Moreover, compounds **2** and **4** resulted inverse agonists more potent than the reference compound **A**.

In conclusion, the reported compounds were hA_{2A} AR inverse agonists endowed with high affinity, selectivity and potency for this receptor subtype. However, they were weak CD73 inhibitors. Furthermore, compounds with an intriguing dual hA_{2A}/hA_{2B} AR inverse agonist/antagonist profile (**2**, **6**-**7**) were identified, compound **2** being also highly selective versus hA_1 and hA_3 AR subtypes. These results allow us to use these compounds as lead structure to design more active derivatives

characterized by dual inhibition of the adenosine producing enzyme CD73, and of adenosine receptors.

Notes

The authors declare no competing financial interest. All authors have given approval to the final version of the manuscript.

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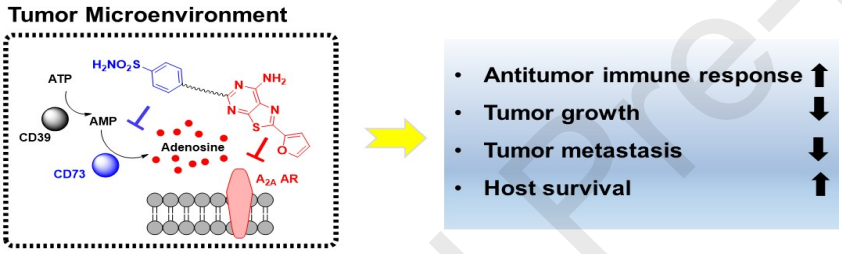
Declaration of interests

☒ The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

☐The authors declare the following financial interests/personal relationships which may be considered as potential competing interests:

Graphical abstract



Highlights

- The synthesis of novel 7-amino-thiazolo[5,4-d]pyrimidine derivatives is reported
- Compounds were tested for their affinity and potency at human adenosine receptors
- Compounds were tested for their inhibitory activity at recombinant human CD73