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Design, Synthesis and Pharmacological Characterization of 2-(2-Furanyl)thiazolo[5,4-*d*]pyrimidine-5,7-Diamine Derivatives: New Highly Potent A_{2A} Adenosine Receptor Inverse Agonists with Antinociceptive Activity

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

Abstract

In this study, we describe the design and synthesis of new N^5-substituted-2-(2-furanyl) thiazolo[5,4-*d*]pyrimidine-5,7-diamines (**2-18**) and their pharmacological characterization as A_{2A} adenosine receptor (AR) antagonists by using in vitro and in vivo assays. In competition binding experiments two derivatives (**13** and **14**) emerged as outstanding ligands showing two different affinity values (KH and KL) for the hA_{2A} receptor with the high affinity KH value in the femtomolar range. The in vitro functional activity assays, performed by using cyclic AMP experiments, assessed that they behave as potent inverse agonists at the hA_{2A} AR. Compounds **13** and **14** were evaluated for their antinociceptive activity in acute experimental models of pain showing an effect equal to or greater than that of morphine. Overall, these novel inverse agonists might represent potential drug candidates for an alternative approach to the management of pain.

Introduction

Pain is a prevalent problem in society and in clinical practice that is not always adequately addressed using current analgesics.[1] The unpleasant perception of pain results from a complex mechanism that involves different levels of the neuroaxis, from the periphery, via the spinal cord to higher cerebral areas.[2] For several years the neuroanatomical circuits and cellular signaling mechanisms involved in the induction of pain have been elucidated. Different mediators have been identified such as prostaglandins, bradykinin, protons, ATP, cytokines, growth factors and nitric oxide.[3] In addition, an important pathway for pain transmission is represented by N-methyl-D-aspartate receptors that are activated by glutamate released from afferent fibers.[4] The current therapeutic approaches for management of pain include but are not limited to the use of opioids even if their employment is often associated with various side effects, tolerance and potential addiction.[5] Recently, novel therapeutic strategies and pharmacological targets have been proposed with the aim of identifying alternative non-opioid analgesics for the treatment of pain. Among others, adenosine has been suggested as modulator of pain.[6]

Adenosine mediates its effects through the activation of a family of four G-protein coupled receptors (ARs) named A_1, A_{2A}, A_{2B} and A_3 ARs. ARs are widely distributed throughout the body and in the central nervous system where they modulate inflammation, locomotion, sleep, behavior and nociception.[7,8] AR signal transduction pathways are primarily linked to cyclic adenosine monophosphate (cAMP) modulation. The A_1 and A_3 ARs are negatively coupled with adenylyl cyclase and exert an inhibitory effect on cAMP production while A_{2A} and A_{2B} ARs stimulate the activity of adenylyl cyclase, inducing an increase in cAMP levels.[9,10] There is evidence that ARs are involved in several physiological processes and different pathologies, and thus their pharmacological modulation is a growing area of research in the drug discovery

process.[11, 12] In particular, the search for novel A_{2A} AR ligands has been greatly expanded and a large number of compounds have been synthesized considering their potential therapeutic and pharmacological effects in several disease states.[11-14]

The findings regarding the A_{2A} receptor signaling in pain have been controversial, with studies supporting both pro- and antinociceptive roles. Peripheral administration of A_{2A} AR agonists have been associated with nociceptive behaviours.[15] However, very low doses of A_{2A} AR agonists administered spinally have been shown to reverse neuropathic pain for weeks.[16] On the other hand, knockout mice lacking A_{2A} ARs were less sensitive to nociceptive stimuli, probably due to the A_{2A} pronociceptive receptors on sensory nerves.[17] Moreover, a well-known A_{2A} AR inverse agonist, **1** (ZM 241385)[18] (Figure 1), injected into the hind paw reduced the mechanical hyperalgesia following carrageenan in mice.[19] Double-blind studies in humans compared a single dose of analgesic plus caffeine with the same dose of the analgesic alone in the treatment of acute pain. The addition of caffeine, a non-selective AR antagonist, to a standard dose of commonly used analgesics provided a small but important increase in the proportion of participants who experienced a good level of pain relief.[20] The analgesic effects of A_{2A} AR antagonists could be due to the inhibition of A_{2A} signaling pathway involved in the modulation of glutamate release.[21] To support this hypothesis it has been demonstrated that A_{2A} knockout mice have a large reduction in the density of NMDA glutamate receptors in the spinal cord.[22] In light of these considerations, it would be valuable studying novel and more efficacious compounds acting as A_{2A} AR antagonists or inverse agonists that could represent promising candidates for the treatment of acute and chronic pain.

Over the past years, our group has been actively involved in the search for new AR antagonists belonging to different heterocyclic chemical classes.[23-31] Recently, we disclosed the

thiazolo[5,4-*d*]pyrimidine-7-one bicyclic system as a new scaffold for potent and selective hA_3 AR antagonists.[32] This encouraging result prompted us to further investigate the thiazolo[5,4-*d*]pyrimidine nucleus with the aim of obtaining potent and selective hA_{2A} AR antagonists and/or inverse agonists. Indeed, in other classes of AR ligands it is well-known that decorating a scaffold with suitable substituents in opportune positions can tune the selectivity towards a specific AR subtype. Thus, considering **1** as lead compound, we designed its thiazolo[5,4-*d*]pyrimidine analog **2** (Figure 1). Compounds **1** and **2** show similar size and shape and the same substitution pattern on the bicyclic core, i.e. the 7-amino moiety, the 2-(2-furanyl) group and the N^5-4-ethylphenol chain. Moreover, the 1,3-thiazole nucleus can be considered as a bioisostere of the 1,2,4-triazole ring of the reference **1**. Bioisosteric replacements often provide the foundation for the development of new structure-activity relationships, and ring equivalent bioisosteres have therefore been used frequently in drug discovery programs.

Thus, since the thiazolo[5,4-*d*]pyrimidine nucleus can be easily obtained following straightforward synthetic procedures, we decided to explore the structure affinity relationships by synthesizing a set of 7-amino-2-(2-furanyl)thiazolo[5,4-*d*]pyrimidine derivatives (**3-18**, Figure 1) which differ from **2** only for the chain on the 5-amino group. Derivatives **2-18** were pharmacologically evaluated at human (h) A_1, A_{2A}, A_{2B} and A_3 ARs stably expressed in CHO cells. Anticipating our results, it emerges that the thiazolo[5,4-*d*]pyrimidine nucleus is an extremely promising new scaffold for obtaining highly potent and selective hA_{2A} AR inverse agonists.

Inverse agonists stabilize the receptor in its inactive conformation and inhibit both the action of the agonists, like classical antagonists, and the constitutive activity of the receptor.[33] So, inverse agonism could represent an important aspect of drug-receptor interaction and could reach

a great therapeutic interest especially if a pathological state is worsened by constitutive activity of a receptor. It is of interest to highlight that in literature are present several papers regarding the relevant therapeutic actions of G-protein coupled receptor antagonists which successively proved to be inverse agonists.[34, 35] About this, recently it has been observed that also caffeine, in different experimental assays, behave as an inverse agonist.[36] Thus, caffeine A_{2A} AR inverse agonism may be behind some of the well-known physiological effects of this substance both in health and disease.

Hence, to help clarify the effects of A_{2A} AR inverse agonists in the modulation of pain, two thiazolo[5,4-*d*]pyrimidine derivatives (compounds **13** and **14**), showing the highest hA_{2A} AR affinity and selectivity, were evaluated in acute experimental models of pain.

Results and Discussion

Chemistry. The synthetic pathway yielding the novel compounds **2-18** is outlined in Scheme 1 and it involves the synthesis of the 7-amino-5-chlorothiazolo[5,4-*d*]pyrimidine derivative **22** starting from the readily accessible compound **19**.[37] Heating **19** with 2-furoyl chloride at 150 °C in NMP yielded the 2-(2-furanyl)thiazolo[5,4-*d*]pyrimidine-5,7-diolo **20** that was allowed to react with $POCl_3$ at reflux temperature to give the corresponding 5,7-dichloro substituted compound **21**. The latter was treated at reflux with 33% aqueous ammonia solution affording the desired 5-chloro-7-amino **22** as the only regioisomer as expected on the basis of the different reactivity of the two chlorine atoms in the thiazolopyrimidine ring system.[38]

Subsequently, reaction of the key intermediate **22** with the commercial aryl- and alkyl-amines of interest, under microwave irradiation, gave the target compounds **3-5, 7, 9-14, 16-18**. Finally, the methoxyphenyl substituted derivatives **3, 5, 7** and **14** were hydrolyzed to their corresponding phenols **2, 6, 8** and **15**, by treatment with BBr_3 in CH_2Cl_2.

Discussion. The affinity for hA_1, hA_{2A} and hA_3 AR (expressed as K_i values) and the potency for the hA_{2B} AR (expressed as IC_{50} values) of compounds **2-18** and of the reference **1** are listed in Table 1.

Compound **2** is recognized by all the hAR subtypes as similar to **1** but its hA_{2A} AR binding affinity is about 20-fold lower than that of **1**. Moreover, **2** did not show hA_{2A} versus hA_1 selectivity since it binds the two receptors with comparable affinities. The 4-methoxyphenyl substituted derivative **3**, precursor of **2**, and the corresponding unsubstituted compound **4** bind all the ARs similarly to **2**, thus showing nanomolar affinities for the hA_1 and hA_{2A} ARs. Moving the 4-hydroxy group of **2** to the 3-position gives the 3-hydroxyphenyl derivative **6** which together with its 3-methoxyphenyl precursor **5**, shows hA_{2A} AR binding affinity equal to **2**. However, differently from **2**, the 3-hydroxy- **6** and 3-methoxyphenyl substituted **5** are more active at the hA_1 AR showing K_i values in the subnanomolar range. Moreover, they also display increased hA_3 binding affinity and hA_{2B} potency with respect to the 4-hydroxyphenyl substituted **2**. The double substitution at positions 3 and 4 of the appended phenyl ring with methoxy or hydroxy groups (i.e compounds **7** and **8**, respectively) substantially do not alter the AR binding affinities which are only slightly decreased with respect to those of **2**, the only exception being the nanomolar activity of **8** at the A_{2B} subtype.

To further investigate the SARs of these new AR ligands, we decided to bring the phenyl ring of the 5-moiety far away or closer to the bicyclic core by synthesizing the N^5-phenylpropyl derivative **9** and the N^5-phenyl **10** and N^5-benzyl derivatives **11**, respectively. The N^5-phenyl derivative **10** shows a decreased binding affinity and potency at hA_1 and hA_{2A} ARs with respect to the N^5-phenylethyl **4**, thus indicating that removing the alkyl spacer is deleterious for the anchoring at these subtypes. On the contrary, the hA_1 and hA_{2A} AR affinities of the N^5-phenylpropyl **9** as well as of the N^5-benzyl derivative **11** closely resemble those of the N^5-phenylethyl **4**. It has to be noted that the N^5-benzyl derivative **11** can be considered a balanced pan-AR ligand since it binds all the ARs with nanomolar affinities. The same structural modifications performed on the triazolotriazine nucleus of the reference compound **1** lead to similar effects in terms of ARs affinity.[39] Thus, considering that the reduction of the distance by one carbon atom was well accepted by all the AR subtypes, we decided to synthesize a number of N^5-arylmethyl derivatives (**12-16)** bearing different substituents (OCH_3, OH, F) on the appended phenyl ring.

The N^5-(4-methoxyphenyl)methyl derivative **12** showed hA_1, hA_{2A} and hA_3 binding affinities and hA_{2B} potency decreased compared to those of the unsubstituted **11**, whilst the N^5-(3-hydroxyphenyl)methyl derivative **15** was a balanced pan-AR ligand as the unsubstituted **11**. On the contrary, introduction of a methoxy substituent at the 2- or 3-position of the appended phenyl ring of **11** led to derivatives **13** and **14**, respectively, that emerged as outstanding ligands. Notably, both **13** and **14** showed two different affinity values for the hA_{2A} AR with the high affinity K_i value (KH) in the femtomolar range (3.55 and 5.31 fM, respectively) and the low affinity K_i value (KL) of the nanomolar order (6.45 and 26 nM, respectively). It is worth noting that the KH values of compounds **13** and **14** are about one million times lower than their KL.

The rationale for the synthesis of N^5-(3-fluorophenyl)methyl derivative **16** was to verify how the electron-withdrawing fluorine atom in the same position of the electron-donating methoxy group of compound **14** influenced the hA_{2A} AR binding affinity. As reported in Table 1, compound **16** shows only one affinity value for the hA_{2A} AR in the subnanomolar range (0.82 nM) and is also about 10- and 40-fold A_{2A} selective over the hA_1 and hA_3 ARs respectively.

Finally, the last modification performed on the amino chain at position 5 of the thiazolo[5,4-*d*]pyrimidine core was the elimination of the aryl moiety by synthesizing the N^5-alkyl derivatives **17** and **18**. Surprisingly, the N^5-propyl **17** and the N^5-butyl derivative **18** bind all the AR subtypes similarly to the N^5-benzylsubstituted **11** despite they present lower lipophilic characteristics.

In order to demonstrate that the two affinity values of **13** and **14** were not dependent on the radioligand used, competition binding experiments by using the agonist [^{3}H]-**23** (^{3}[H]-CGS 21680, 2-[p-(2-carboxyethyl)phenethylamino]-5′-N-ethylcarboxamido adenosine;) [40] as radioligand were performed (Table 1). Notably, also in these experiments compounds **13** and **14** showed two different affinity values for the hA_{2A} AR with the KH in the femtomolar range (5.23 and 6.71 fM, respectively) and the KL of the nanomolar order (7.16 and 23 nM, respectively). In accordance, the competition curves of **13** and **14** show, in both experiments, a biphasic shape that fits better to a two-site model and can be interpreted as the interaction with two apparent binding sites (Figures 2 and 3). On the contrary, the competition binding curves of the reference compound **1** and of other selected thiazolo[5,4-*d*]pyrimidine derivatives (**2-4, 11-12, 15**) indicate the presence of only one recognition binding site as they show only one affinity value in the nanomolar range (Figure 2). In particular none of these compounds, with the exception of **13** and **14** was able to displace the radioligand at concentrations lower than 0.1 nM.

It has to be highlighted that in our experimental conditions A_{2A} ARs are present in various conformations with which **13** and **14** probably interact in a different manner. Moreover, it has been suggested that many G-protein coupled receptors are expressed on the plasmatic membranes as dimers.[41] As a consequence another hypothesis is that the A_{2A} ARs could be present as homodimers in the CHO cells affecting the affinity of the compounds. Recently, some studies on the crystallization of A_{2A} ARs coupled to Gs protein have highlighted that agonists, antagonists or inverse agonists are able to differently modify the receptor conformation.[42]

To the best of our knowledge, compounds **13** and **14**, although showing nanomolar affinities for the hA_1 and hA_3 and good potency at the hA_{2B} receptor, due to their hA_{2A} femtomolar affinity, can be considered the most potent and also selective hA_{2A} AR ligands reported so far.

To evaluate the antagonist/inverse agonist potencies of the novel compounds, we also studied their in vitro activity. In particular, the ability of compounds **2-4, 11-15** and the reference **1** to modulate cAMP production in the absence or presence of the agonist **23** was tested (Table 2). According to their extremely high hA_{2A} AR affinity, compounds **13** and **14** were very potent inverse agonists, being able to inhibit basal cAMP accumulation at picomolar concentrations (IC_{50}=1.9 and 8.3 pM, respectively,), and showing efficacy values of 63 and 41 %, respectively (Figure 4A, 4B). It is important to note that the reference compound **1** also behaved as an inverse agonist but with lower potency (IC_{50}=1.45 nM, Table 2) compared to compounds **13** and **14** (Figure 4C). The other examined compounds **2-4**, **11-12, 15** significantly reduced cAMP production in basal conditions with IC_{50} values from 25 to 187 nM (Figure 4D; Table 2). Moreover, compounds **13** and **14** were also able to inhibit cAMP production stimulated by the agonist **23** (10 nM) with high potency (IC_{50}=51 and 95 pM, respectively) (Figure 5A, 5B; Table

2). The reference compound **1** blocked the effect of the agonist with a lower potency (IC_{50}= 678 pM, Figure 5C) than those of compounds **13** and **14**.

In addition, Figure 5D reports the dose response curves of compounds **2-4, 11-12, 15** showing IC_{50} values from 16 to 123 nM (Table 2). As expected, all the compounds tested in the presence of an agonist showed an antagonist/inverse agonist profile. In particular, they reduced the cAMP accumulation, reaching values lower than those of basal production (from 116 to 145%) as indicated by Emax data (Figure 5;Table 2). Interestingly, these compounds were able to reduce not only the agonist effect but also to inhibit the constitutive activity of the receptor as indicated by the negative levels of cAMP showed in Figure 5 where the basal production is set to zero. None of the tested compounds behaved as inverse agonists for hA_1, hA_{2B} and hA_3 receptors (Table 3).

Various literature evidence suggests that A_{2A} antagonists/inverse agonists have a potential therapeutic role in the modulation of pain.[17, 19] To explore the antinociceptive activity of hA_{2A} AR inverse agonists, compounds **13** and **14** were tested in writhing and tail immersion tests in mice, in comparison with the reference compounds **1** and morphine. In the writhing test, the i.p. administration of acetic acid induced 75±12 abdominal constrictions in vehicle-treated mice. The dose-response curve of compounds **13** and **14**, **1** and morphine revealed a dose-dependent effect ($P<0.001$, one-way ANOVA). Notably, derivative **13** proved to be more potent than the reference compounds. In fact, it shows an ED_{50} of 0.0328±0.0021 mg/kg, a value 3.75-fold lower than that obtained with morphine (0.123±0.010 mg/kg) and about 42 times lower than that of **1** (1.373±0.108 mg/kg) (Figure 6 A-D). Compound **14** had antinociceptive activity with a potency similar to morphine. In the hot water tail immersion test, derivative **13** exhibited the greatest analgesic activity with an ED_{50} of 0.134±0.011 mg/kg and minimum effective dose of 0.01

mg/kg. Compound **14** and morphine showed a similar antinociceptive response while **1** had no effect until 10 mg/kg (Figure 7 A-D).

Conclusion

On the basis of previously published research and using the concept of isosterism, new 2-(2-furanyl)thiazolo[5,4-*d*]pyrimidine-5,7-diamine derivatives that behave as A_{2A} AR inverse agonists were designed and synthesized. Two compounds (**13** and **14**) have shown two different affinity values for the hA_{2A} AR with a KH value in the femtomolar range and a KL value of the nanomolar order. In accordance with their very high A_{2A} AR affinity, **13** and **14** proved to be highly potent inverse agonists, 10^3-fold more potent than the reference **1**. As a consequence, they are also the most selective A_{2A} AR inverse agonists reported so far. Moreover, **13** and **14** have shown a promising profile in terms of acute pain management being more active than morphine in two animal models of acute pain. From these results it emerges that the thiazolo[5,4-*d*]pyrimidine nucleus is a new and versatile scaffold for obtaining highly potent and selective hA_{2A} AR inverse agonists potentially useful in the treatment of pain. Of interest, the availability of selective A_{2A} AR inverse agonists could delineate future prospect for these drugs in different therapeutic areas, beside pain, such as in the treatment of neurological disorders,[13, 43] dermal fibrosis and scarring,[44-46] cancer,[47, 48] and retinal diseases.[49]

Experimental Procedures

Chemistry.

Materials and methods. 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). Silica gel plates (0.20 mm, F254, Merck, Germany), and silica gel 60 (70-230 mesh, Merck, Germany) were used for analytical 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}. Nuclear magnetic resonance (NMR) experiments were run on Bruker Avance 400 instrument (400 MHz for ^{1}H and 100 MHz for ^{13}C NMR). Spectra were recorded at 300 K, using DMSO-d_6 as solvent. Chemical shifts for ^{1}H and ^{13}C spectra were recorded in parts per million using the residual non-deuterated solvent as the internal standard. Data are reported as follows: chemical shift (ppm), multiplicity (indicated as: s, singlet; br s, broad singlet; exch, exchangeable proton with D_2O; d, doublet; t, triplet; q, quartet; m, multiplet and combination thereof), coupling costants (J) in Hertz (Hz) and integrated intensity.

4-(2-((7-Amino-2-(2-furanyl)[1,3]thiazolo[5,4-d]pyrimidin-5-yl)amino)ethyl)phenol (2). A solution of BBr_3 in CH_2Cl_2 (1 M, 1.5 ml) was, drop-by-drop, added to a suspension of

compounds **3** (0.5 mmol) in anhydrous dichloromethane (40 ml). At the end of addition, the suspension was allowed to stirr at 50 °C for 1 day. The solution was diluted with ice-water (50 g) under vigourous stirring and, after 4 hours, a saturated aqueous solution of $NaHCO_3$ (6 ml) was added. The resulting precipitate was collected by filtration, washed with water, and purified by column chromatography (eluting system ethyl acetate/cyclohexane 1:1). Yield 70%. Mp: 195-199 °C. ^{1}H NMR: δ 2.69-2.73 (m, 2H), 3.38-3.43 (m, 2H), 6.67-6.72 (m, 3H), 6.81 (br s, exch, 1H), 7.04-7.05 (m, 3H), 7.15 (br s, exch, 2H), 7.89 (s, 1H), 9.15 (s, exch, 1H). ^{13}C NMR: δ 34.88, 43.49, 109.98, 113.16, 115.52, 130.00, 130.34, 148.65, 156.00, 157.47, 160.31. Anal. calcd. for ($C_{17}H_{15}N_5O_2S$): C, 57.78%; H, 4.28%; N, 19.82%. Anal. found: C, 57.42%; H, 4.33%; N, 19.53%.

2-(2-Furanyl)-N^5-[2-(4-methoxyphenyl)ethyl][1,3]thiazolo[5,4-d]pyrimidine-5,7-diamine (3). 4-Methoxyphenetylamine (3 mmol) was added to a solution of the 5-chloro-7-amine derivative **22** (1 mmol) in n-BuOH (2 ml). The reaction mixture was microwave irradiated at 200 °C for 20 minutes, then cooled to rt and basified with aqueous KOH solution (50%, 2 ml). Addition of water (about 100 ml) afforded a solid which was collected by filtration and washed with Et_2O. The crude material was purified by column chromatography (eluting system: ethyl acetate/cyclohexane 1:1). Yield 53%. Mp: 178-180 °C. ^{1}H NMR: δ 2.75-2-78 (m, 2H), 3.41-3.46 (m, 2H), 3.72 (s, 3H), 6.72 (br s, 1H), 6.85-6.87 (m, exch 1H + 2H), 7.05 (br s, 1H), 7.16-7.18 (m, exch 2H + 2H), 7.90 (s, 1H). ^{13}C NMR: δ 34.79, 43.41, 55.43, 109.99, 113.16, 114.17, 130.10, 132.20, 148.64, 157.47, 158.06, 160.30. IR: 3208, 3267, 3382. Anal. calcd. for ($C_{18}H_{17}N_5O_2S$): C, 58.84%; H, 4.66%; N, 19.06%. Anal. found: C, 59.13%; H, 4.82%; N, 19.47%.

2-(2-Furanyl)-N^5-(2-phenylethyl)[1,3]thiazolo[5,4-d]pyrimidine-5,7-diamine (4). The title compound was obtained by reacting the 5-chloro-7-amine derivative **22** (1 mmol) with 2-phenethylamine (3 mmol) in the same experimental conditions described above to prepare derivative **3**. The crude product was purified by crystallization. Yield 80%. Mp: 196-197 °C (isopropanol). ^{1}H NMR: δ 2.82-2.86 (m, 2H), 3.47-3.50 (m, 2H), 6.72 (br s, 1H), 6.87 (br s, exch, 1H), 7.05-7.06 (m, 1H), 7.18-7.32 (m, exch 2H + 5H), 7.90 (s, 1H). ^{13}C NMR: δ 35.70, 43.21, 110.00, 113.16, 126.42, 128.73, 129.18, 140.36, 148.64, 157.48, 160.30. IR: 3201, 3263, 3461. Anal. calcd. for ($C_{17}H_{15}N_5OS$): C, 60.52%; H, 4.48%; N, 20.76%. Anal. found: C, 60.88%; H, 4.35%; N, 21.18%.

2-(2-Furanyl)-N^5-(2-(3-methoxyphenyl)ethyl)[1,3]thiazolo[5,4-d]pyrimidine-5,7-diamine (5). The title compound was obtained by reacting the 5-chloro-7-amine derivative **22** (1 mmol) with 3-methoxyphenethylamine (3 mmol) in the same experimental conditions described above to prepare derivative **3**. The crude product was purified by column chromatography (eluting system ethyl acetate/cyclohexane 1:1). Yield 57 %. Mp: 166-169 °C. ^{1}H NMR: δ 2.80-2.83 (m, 2H), 3.46-3.51 (m, 2H), 3.74 (s, 3H), 6.72-6.84 (m, 5H, exch 1H + 4H), 7.04-7.05 (m, 1H), 7.19-7.22 (m, exch 2H + 1H), 7.90 (s, 1H). Anal. calcd. for ($C_{18}H_{17}N_5O_2S$): C, 58.84%; H, 4.66%; N, 19.06%. Anal. found: C, 58.66%; H, 4.81%; N, 19.24%.

3-(2-((7-Amino-2-(2-furanyl)[1,3]thiazolo[5,4-d]pyrimidin-5-yl)amino)ethyl)phenol (6). The title compound was obtained by reacting **5** (0.5 mmol) with BBr_3 in the same experimental conditions described above to prepare derivative **2**. The crude product was purified by column chromatography (eluting system ethyl acetate/cyclohexane 1:1). Yield 47%. Mp: 206-209 °C ^{1}H NMR: δ 2.72-2.76 (m, 2H), 3.42-3.47 (m, 2H), 6.58-6.60 (m, 1H), 6.66-6.72 (m, 3H), 6.82 (br s, exch, 1H), 7.05-7.10 (m, 2H), 7.16 (br s, exch, 2H), 7.90 (s, 1H), 9.25 (s, exch 1H). Anal. calcd.

for ($C_{17}H_{15}N_5O_2S$): C, 57.78%; H, 4.28%; N, 19.82%. Anal. found: C, 57.85%; H, 4.50%; N, 20.09%.

N^5-(2-(3,4-Dimethoxyphenyl)ethyl)-2-(2-furanyl)[1,3]thiazolo[5,4-d]pyrimidine-5,7-diamine (7). The title compound was obtained by reacting the 5-chloro-7-amine derivative **22** (1 mmol) with 3,4-dimethoxyphenethylamine (3 mmol) in the same experimental conditions described above to prepare derivative **3**. The crude product was purified by crystallization. Yield 74%. Mp: 146-150 °C (nitromethane). ^{1}H NMR: δ 2.75-2.79 (m, 2H), 3.44-3.49 (m, 2H), 3.71 (s, 3H), 3.74 (s, 3H), 6.72-6.87 (m, exch 1H + 4H), 7.04-7.05 (m, 1H) 7.16 (br s, exch 2H), 7.90 (s, 1H). Anal calcd. for ($C_{19}H_{19}N_5O_3S$): C, 57.42%; H, 4.82%; N, 17.62%. Anal. found: C, 57.86%; H, 4.40%; N, 17.93%.

4-(2-((7-Amino-2-(2-furanyl)[1,3]thiazolo[5,4-d]pyrimidin-5-yl)amino)ethyl)benzene-1,2-diol (8). The title compound was obtained by reacting **7** (0.5 mmol) with BBr_3 in the same experimental conditions described above to prepare derivative **2**. The crude product was purified by crystallization .Yield 72 %. Mp: 217-220°C (acetonitrile). ^{1}H NMR: δ 2.62-2.66 (m, 2H), 6.47-6.49 (m, 1H), 6.63-6.65 (m, 2H), 6.72-6.77 (m, exch 1H + 1H), 7.04-7.05 (m, 1H), 7.15 (br s, exch 2H), 7.90 (s, 1H), 8.63 (s, exch, 1H), 8.74 (s, exch, 1H). ^{1}H NMR (+D_2O): δ 2.62-2.65 (m, 2H), 3.36-3.40 (m, 2H), 6.49-6.51 (m, 1H), 6.63-6.65 (m, 2H), 6.71-6.72 (m, 1H), 7.06-7.07 (m, 1H), 7.86 (s, 1H). Anal. calcd. for ($C_{17}H_{15}N_5O_3S$): C, 55.27%; H, 4.09%; N, 18.96%. Anal. found: C, 54.91%; H, 3.83%; N, 18.68%.

2-(2-Furanyl)-N^5-(3-phenylpropyl)[1,3]thiazolo[5,4-d]pyrimidine-5,7-diamine (9). The title compound was obtained by reacting the 5-chloro-7-amine derivative **22** (1 mmol) with 3-

phenylpropylamine (3 mmol) in the same experimental conditions described above to prepare derivative **3**. The crude product was purified by crystallization. Yield 80%. Mp: 184-187 °C (isopropanol). ^{1}H NMR: δ 1.79-1.86 (m, 2H), 2.62-2.65 (m, 2H), 3.25-3.30 (m, 2H), 6.71-6.72 (m, 1H), 6.91 (br s, exch 1H), 7.04-7.05 (m, 1H), 7.12-7.30 (m, exch 2H + 5H), 7.89 (m, 1H). ^{13}C NMR: δ 31.17, 33.16, 41.12, 109.94, 113.14, 126.11, 128.71, 128.78, 142.44, 148.67, 157.46, 160.48, 165.06. IR: 3315, 3253, 3197, 1646, 1604, 1546, 1460. Anal calcd. for ($C_{18}H_{17}N_5OS$): C, 61.52%; H, 4.88%; N, 19.93%. Anal. found: C, 61.18%; H, 4.51%; N, 20.20%.

2-(2-Furanyl)-N^5-phenyl[1,3]thiazolo[5,4-d]pyrimidine-5,7-diamine (10). The title compound was obtained by reacting the 5-chloro-7-amine derivative **22** (1 mmol) with aniline (3 mmol) in the same experimental conditions described above to prepare derivative **3**. The crude product was purified by crystallization. Yield 71%. Mp: 254-255 °C (toluene). ^{1}H NMR: δ 6.75-6.76 (m, 1H), 6.92 (t, 1H, J = 7.3), 7.12-7.13 (m, 1H), 7.26 (t, 2H, J = 7.7), 7.42 (s, exch 2H), 7.80 (d, 2H, J = 7.7), 7.93 (s, 1H). 9.29 (s, exch 1H). Anal. calcd. for ($C_{15}H_{11}N_5OS$): C, 58.24%; H, 3.58%; N, 22.64%. Anal. found: C, 58.55%; H, 3.27%; N, 22.87%.

N^5-Benzyl-2-(2-furanyl) [1,3]thiazolo[5,4-d]pyridine-5,7-diamine (11). The title compound was obtained by reacting the 5-chloro-7-amine derivative **22** (1 mmol) with benzylamine (3 mmol) in the same experimental conditions described above to prepare derivative **3**. The crude product was purified by crystallization. Yield 78%. Mp: 215-218 °C (ethyl acetate). ^{1}H NMR: δ 4.51 (d, 2H, J = 6.2), 6.72-6.73 (m, 1H), 7.04-7.05 (m, 1H), 7.18-7.22 (m, exch 2H+1H), 7.28-7.31 (m, 4H), 7.40 (br s, exch 1H), 7.90 (s, 1H). ^{13}C NMR: δ 44.58, 110. 05, 113.18, 126.89, 127.48, 128.60, 141.17, 148.59, 157.51, 160.44. Anal. calcd. for ($C_{16}H_{13}N_5OS$): C, 59.43%; H, 4.05%; N, 21.66%. Anal. found: C, 59.13%; H, 3.74%; N, 21.35%.

2-(2-Furanyl)-N^5-(4-methoxybenzyl)[1,3]thiazolo[5,4-d]pyrimidine-5,7-diamine (12). The title compound was obtained by reacting the 5-chloro-7-amine derivative **22** (1 mmol) with 4-methoxybenzylamine (3 mmol) in the same experimental conditions described above to prepare derivative **3**. The crude product was purified by crystallization. Yield 68%. Mp: 189-192 °C (ethyl acetate). ^{1}H NMR: δ 3.72 (s, 3H), 4.43 (d, 2H, J = 5.8), 6.72 (br s, 1H), 6.86 (d, 2H, J = 7.3), 7.05 (br s, 1H), 7.17 (br s, exch, 2H), 7.24-7.30 (m, exch 1H + 2H), 7.89 (s, 1H). ^{13}C NMR: δ 44.08, 55.47, 110.04, 113.16, 114.01, 128.83, 133.03, 148.61, 157.49, 158.46, 160.39. Anal. calcd. for ($C_{17}H_{15}N_5O_2S$): C, 57.78%; H, 4.28%; N, 19.82%. Anal. found: C, 58.07%; H, 4.37%; N, 20.09%.

2-(2-Furanyl)-N^5-(2-methoxybenzyl)[1,3]thiazolo[5,4-d]pyrimidine-5,7-diamine (13). The title compound was obtained by reacting the 5-chloro-7-amine derivative **22** (1 mmol) with 2-methoxybenzylamine (3 mmol) in the same experimental conditions described above to prepare derivative **3**. The crude product was purified by crystallization. Yield 81%. Mp: 254-256 °C (acetic acid). ^{1}H NMR: δ 3.83 (s, 3H), 4.47 (d, 2H, J = 6.2), 6.71-6.73 (m, 1H), 6.88 (t, 1H, J = 7.3), 6.97 (d, 1H, J = 7.8), 7.04-7.05 (m, 1H), 7.14-7.22 (m, exch 3H + 2H), 7.89-7.90 (m, 1H). ^{13}C NMR: δ 55.69, 110.04, 110.67, 113.14, 120.51, 127.46, 128.00, 128.43, 148.62, 157.05, 157.56, 160.61. Anal. calcd. for ($C_{17}H_{15}N_5O_2S$): C, 57.78%; H, 4.28%; N, 19.82%. Anal. found: C, 57.41%; H, 3.92%; N, 19.50%.

2-(2-Furanyl)-N^5-(3-methoxybenzyl)[1,3]thiazolo[5,4-d]pyrimidine-5,7-diamine (14). The title compound was obtained by reacting the 5-chloro-7-amine derivative **22** (1 mmol) with 3-methoxybenzylamine (3 mmol) in the same experimental conditions described above to prepare derivative **3**. The crude product was purified by crystallization. Yield 70%. Mp: 199-201 °C (ethyl acetate). ^{1}H NMR: δ 3.72 (s, 3H), 4.48 (d, 2H, J = 6.2), 6.71-6.73 (m, 1H), 6.77-6.78 (m,

1H), 6.88-6.90 (m, 2H), 7.04-7.05 (m, 1H), 7.19-7.23 (m, exch 2H + 1H), 7.37-7.40 (m, exch 1H), 7.90 (s, 1H). ^{13}C NMR: δ 44.59, 55.39, 110.06, 112.24, 113.17, 119.70, 129.64, 142.83, 148.60, 157.51, 159.69, 160.42. Anal. calcd. for ($C_{17}H_{15}N_5O_2S$): C, 57.78%; H, 4.28%; N, 19.82%. Anal. found: C, 58.05%; H, 4.44%; N, 19.57%.

3-(((7-Amino-2-(2-furanyl)[1,3]thiazolo[5,4-d]pyrimidin-5-yl)amino)methyl)phenol (15). The title compound was obtained by reacting **14** (0.5 mmol) with BBr_3 in the same experimental conditions described above to prepare derivative **2**. The crude product was purified by crystallization. Yield 80%. Mp: 207-209 °C (ethyl acetate). ^{1}H NMR: δ 4.43-4.45 (m, 2H), 6.58-6.60 (m, 1H), 6.71-6.73 (m, 3H), 7.05-7.10 (m, 2H), 7.23 (br s, exch, 2H), 7.36 (br s, exch, 1H), 7.89-7.90 (m, 1H), 9.25 (br s, exch, 1H). ^{13}C NMR: δ 44.45, 110.06, 113.17, 113.81, 114.13, 117.97, 129.54, 142.65, 145.52, 148.59, 157.50, 157.76, 160.35, 164.86. Anal. calcd. for ($C_{16}H_{13}N_5O_2S$): C, 56.63%; H, 3.86%; N, 20.64%. Anal. found: C, 56.97%; H, 4.01%; N, 20.97%.

N^5-(3-Fluorobenzyl)-2-(2-furanyl)[1,3]thiazolo[5,4-d]pyrimidine-5,7-diamine (16). The title compound was obtained by reacting the 5-chloro-7-amine derivative **22** (1 mmol) with 3-fluorobenzylamine (3 mmol) in the same experimental conditions described above to prepare derivative **3**. The crude product was purified by crystallization. Yield 64 %. Mp: 217-220 °C (nitromethane). ^{1}H NMR: δ 4.52 (d, 2H, J = 5.9), 6.71-6.72 (m, 1H), 7.01-7.06 (m, 2H), 7.11-7.18 (m, 2H), 7.22 (br s, exch 2H), 7.32-7.37 (m, 1H), 7.41-7.44 (m, exch 1H), 7.89-7.90 (m, 1H). Anal. calcd. for ($C_{16}H_{12}FN_5OS$): C, 56.30%; H, 3.54%; N, 20.52%. Anal. found: C, 56.59%; H, 3.81%; N, 20.89%.

2-(2-Furanyl)-N^5-propyl[1,3]thiazolo[5,4-d]pyrimidine-5,7-diamine (17). The title compound was obtained by reacting the 5-chloro-7-amine derivative **22** (1 mmol) with propylamine (3 mmol) in the same experimental conditions described above to prepare derivative **3**. The crude product was purified by crystallization. Yield 65 %. Mp: 204-207°C (ethyl acetate). ^{1}H NMR: δ 0.87-0.90 (m, 3H), 1.50-1.53 (m, 2H), 3.20-3.22 (m, 2H), 6.72 (br s, 1H), 6.83 (br s, exch 1H),7.03-7.04 (m, 1H), 7.10 (m, exch 2H), 7.89 (s ,1H). ^{13}C NMR: δ 11.95, 22.88, 43.24, 109.90, 113.13, 148.66, 157.44, 160.47, 165.06. Anal. calcd. for ($C_{12}H_{13}N_5OS$): C, 52.35%; H, 4.76%; N, 25.44%. Anal. found: C, 52.47%; H, 5.10%; N, 25.71%.

N^5-Butyl -2-(2-furanyl)[1,3]thiazolo[5,4-d]pyrimidine-5,7-diamine (18). The title compound was obtained by reacting the 5-chloro-7-amine derivative **22** (1 mmol) with butylamine (3 mmol) in the same experimental conditions described above to prepare derivative **3**. The crude product was purified by crystallization. Yield 70 %. Mp: 201-204 °C (ethyl acetate). ^{1}H NMR: δ 0.89-0.91 (m, 3H), 1.32-1.35 (m, 2H) 1.48-1.52 (m, 2H), 3.24-3.26 (m, 2H), 6.71-6.72 (m, 1H), 6.81 (br s, exch 1H),7.03-7.04 (m, 1H), 7.10 (br s, exch 2H), 7.89 (s, 1H). ^{13}C NMR: δ 14.29, 20.15, 31.83, 41.11, 109.90, 113.13, 148.67, 157.44, 160.47, 165.07. Anal. calcd. for ($C_{13}H_{15}N_5OS$): C, 53.96%; H, 5.23%; N, 24.20%. Anal. found: C, 54.28%; H, 5.40%; N, 24.58%.

2-(2-Furanyl)[1,3]thiazolo[5,4-d]pyrimidine-5,7-diol (20). To a suspension of the 5-amino-6-sulfanylpyrimidine-2,4-diol **19**[37] (10 mmol) in NMP dry, furan-2-carbonyl chloride (10 mmol) was slowly added. The resulting mixture was heated at 150 °C under N_2 atmosphere for 14 hours. The reaction mixture was then cooled to rt, diluted with cold water (100 ml) affording a precipitate which was collected by filtration and purified by crystallization. Yield 82%. Mp: >300 °C (DMSO). ^{1}H NMR: δ 6.73-6.74 (m, 1H), 7.18-7.19 (m, 1H), 7.92-7.93 (m, 1H), 11.40 (s, exch, 1H), 12.07 (s, exch, 1H). ^{13}C NMR: δ 110.76, 113.38, 130.72, 147.38, 147.95, 149.49,

150.47, 157.85. IR: 1673, 1703. Anal. calcd. for ($C_9H_5N_3O_3S$): C, 45.96% ; H, 2.14%; N, 17.86%. Anal. found: C, 46.29%; H, 2.33%; N, 18.12%.

5,7-Dichloro-2-(2-furanyl)[1,3]thiazolo[5,4-d]pyrimidine (21). To a suspension of the 5,7-diol **20** (5 mmol) in $POCl_3$ (20 ml), N,N-dimethylaniline (1.15 mL, 10 mmol) was added at rt. The resulting mixture was heated at 100 °C for 6 hours. The organic phase was concentrated under vacuum, then the crude material was taken up twice with cyclohexane (20 ml) and the organics evaporated in vacuo. The residue was added with a mixture of ice-water (100 g), affording a precipitate which was collected by filtration and used in the next step without further purification. Yield 73%. ^{1}H NMR: δ 6.89-6.91 (m, 1H), 7.66-7.67 (m, 1H), 8.17-8.18 (m, 1H). ^{13}C NMR: δ 114.38, 116.23, 142.58, 147.02, 152.80, 152.84, 158.78, 167.66.

5-Chloro-2-(2-furanyl)[1,3]thiazolo[5,4-d]pyrimidin-7-amine (22). A suspension of the 5,7-dichloro **21** (4 mmol) in a mixture of 33% aqueous ammonia solution (15 ml) and ethanol (10 ml) was heated at 85 °C for 6 hours. The reaction mixture was then cooled to rt, affording a solid, which was collected by filtration. Yield 75%. Mp: 296-300 °C dec. (2-methoxyethanol /H_2O). ^{1}H NMR: δ 6.80-6.81 (m, 1H), 7.29-7.30 (m, 1H), 8.03 (s, 1H), 8.27 (br s, exch, 2H). ^{13}C NMR: δ 112.73, 113.62, 130.33, 147.70, 152.92, 155.35, 158.12, 163.05. IR: 3136, 3298. Anal. calcd. for ($C_9H_5ClN_4OS$): C, 42.78%; H, 1.99%; N, 22.17%. Anal. found: C, 42.95%; H, 2.07%; N, 22.36%.

In vitro pharmacology.

Materials. [^{3}H]-DPCPX ([^{3}H]1,3-dipropyl-8-cyclopentyl-xanthine; specific activity, 120 Ci/mmol), [^{125}I]-ABMECA ([^{125}I]4-aminobenzyl-5'-N-methyl-carboxamidoadenosine; specific activity, 2200 Ci/mmol), [^{3}H]-**23** ([^{3}H]-2-p-(2-Carboxyethyl)phenethylamino-5′-N-ethylcarboxamido adenosine, specific activity, 40 Ci/mmol) and [^{3}H]cyclic AMP ([^{3}H]cyclic adenosine monophosphate; specific activity, 22 Ci/mmol) were obtained from Perkin Elmer Research Products (Boston, MA); [^{3}H]-**1** ([^{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, **23** and **1** were obtained from Sigma Aldrich (St. Louis, MO). Morphine was obtained from Salars (Como, Italy).

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.[50] 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 Tris HCl, 1 mM EDTA, pH 7.4). The cell suspension was homogenized with a Polytron, centrifuged for 30 min at 40000 *g* at 4°C and the resulting membrane pellet was used for competition binding experiments.[50]

Competition binding experiments. All synthesized compounds have been 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 mM Tris HCl, 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.[50] Inhibition experiments to A_{2A} ARs were performed incubating the radioligand [^{3}H]-**1** (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 50 mM Tris HCl (pH 7.4), 10 mM $MgCl_2$. Non-specific binding was determined in the presence of **1** (1 μM) and was about 20% of the total binding.[51] In addition, competition binding experiments to A_{2A} ARs were also carried out incubating the radioligand [^{3}H]-**23** (15 nM) with the membrane suspension (50 μg of protein/100 μl) and at least 12 different concentrations of the tested compounds for 90 min at 25°C in 50 mM Tris HCl (pH 7.4), 10 mM $MgCl_2$. Non-specific binding was determined in the presence of CGS 21680 (1 μM) and was about 25% of the total binding.[52] Competition binding experiments to A_3 ARs 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 Tris HCl (pH 7.4), 10 mM $MgCl_2$, 1 mM EDTA. Non-specific binding was defined as binding in the presence of 1μM ABMECA and was always < 10% of the total binding.[53] 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).

Cyclic AMP assays. CHO cells transfected with ARs were washed with phosphate-buffered saline, detached with trypsine and centrifuged for 10 min at 200 g. The pellet containing CHO cells (1x10^6 cells /sample) was suspended in 0.5 ml of incubation mixture (mM): NaCl 15, KCl 0.27, NaH_2PO_4 0.037, $MgSO_4$ 0.1, $CaCl_2$ 0.1, Hepes 0.01, $MgCl_2$ 1, glucose 0.5, pH 7.4 at 37°C, 2 IU/ml adenosine deaminase and 4-(3-butoxy-4-methoxybenzyl)-2-imidazolidinone (Ro 20-

1724) as phosphodiesterase inhibitor and preincubated for 10 min in a shaking bath at 37 °C. The potency of the examined compounds to hA_{2B} ARs were determined evaluating their capability to inhibit NECA (100 nM)-stimulated cAMP levels.[54] Moreover, the potency of the compounds versus hA_{2A} ARs was determined studying their capability to inhibit basal levels of cAMP.[55] The same experiments were also performed in the presence of agonist **23** (10 nM). Additional experiments were carried evaluating the tested compounds at the 10 μM concentration in hA_1, hA_{2B} or hA_3CHO cells to verify their effect on cAMP production in basal condition.[54] The reaction was terminated by the addition of cold 6% trichloroacetic acid (TCA). The TCA suspension was centrifuged at 2000 g for 10 min at 4°C and the supernatant was extracted four times with water saturated diethyl ether. The final aqueous solution was tested for cyclic AMP levels by a competition protein binding assay. Samples of cyclic AMP standard (0-10 pmoles) were added to each test tube containing the incubation buffer (trizma base 0.1 M, aminophylline 8.0 mM, 2 mercaptoethanol 6.0 mM, pH 7.4) and [^{3}H]-cAMP.[56] The binding protein previously prepared from beef adrenals, was added to the samples previously incubated at 4 °C for 150 min, and after the addition of charcoal were centrifuged at 2000 g for 10 min. The clear supernatant was counted in a 2810-TR Packard scintillation counter.

In vivo pharmacology.

Animals. Female CD1 mice (22-24 g) were obtained from Charles River (Milan, Italy). The animals were kept under standard environmental temperature and humidity-controlled conditions (22±2°C) with 12 h light/dark cycle with food and water *ad libitum*. The animals were acclimated to the laboratory settings for at least 1 h before testing and were used only once

throughout the experiments. All the procedures used in the present study were carried out in accordance with European Communities Council directives (86/609/EEC) and National Laws and Policies (D.L.116/92) with the authorization from the Italian Ministry for Health (4/2014-B). The experimental procedures were in agreement with the current guidelines for the care of laboratory animals and the ethical guidelines for investigations of experimental pain in conscious animals.[57]

Writhing test. The acetic acid-induced writhing response was performed after i.p. injection of 10 ml/kg of 0.6% acetic acid solution. The drugs investigated were dissolved in DMSO and further diluted in saline. The vehicle is composed by saline and 5% DMSO. A writhe is indicated by stretching of the abdomen followed by the extension of the hind limbs. The animals (8 mice per group) were placed singly in a glass cylinder and the number of writhing episode in a 30 min period was counted. Compounds were administered 15 min before injection of acetic acid solution. As expected, abdominal constrictions were not observed in saline-treated mice instead of acid acetic solution.[58] ED_{50} values were calculated by a linear regression analysis converting the data to percentage of maximum possible effect (MPE) using the following equation: 100 X (post drug response − vehicle response)/(vehicle response).

Tail immersion test. The warm-water tail immersion assay was performed using a water bath with the temperature maintained at 52°C. The drugs investigated were dissolved in DMSO and further diluted in saline. The vehicle is composed by saline and 5% DMSO. Before injecting, the baseline latency of the mice was determined. The mouse was maintained in a mouse holder, and the distal tail was then immersed in the water bath. The latency to respond to the heat stimulus with vigorous flexion of the tail was measured by means of a manual stopwatch. A 20 sec maximum cutoff time was imposed to prevent tissue damage.[59] The latency of the tail

withdrawal was then tested 15 min after compounds injection. ED_{50} values were calculated by a linear regression analysis converting the data to %MPE using the following equation: 100 X (post-drug latency – basal latency) / (cut-off latency – basal latency).

Statistical analysis. The protein concentration was determined according to a Bio-Rad method with bovine albumin as a standard reference. [53] The data are expressed as the mean ± SEM of n = 4 independent experiments for in vitro assays and n = 8-10 mice/group for in vivo assays. 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. [53] KH and KL were obtained by fitting binding data to a two sites binding model by using Graph PAD Prism (San Diego, CA, USA). IC_{50} values obtained in cyclic AMP assays were calculated by non-linear regression analysis using the equation for a sigmoid concentration-response curve. [54]

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Notes The authors declare no competing financial interest.

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Abbreviations Used

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; NECA, 5'-N-ethyl-carboxamidoadenosine; AB-MECA, 4-[((4-aminophenyl)methyl)amino]-5'-N-methylcarboxamidoadenosine; TCA, trichloroacetic acid; MPE, maximum possible effect. NNDMA, N,N-dimethylaniline; MW, microwave.

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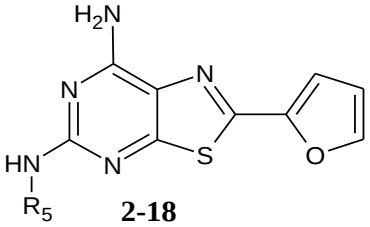
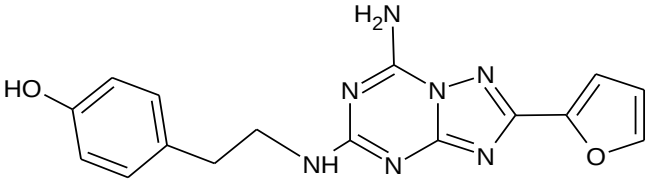
Table 1. Affinity (K_i or KH, KL) and potency (IC_{50}) of the novel compounds in comparison with reference compound **1** on ARs.



2-18



1

	R_5	hA_1AR[a] K_i (nM)	$hA_{2A}AR$[b]	$hA_{2A}AR$[c]	hA_3AR[d] K_i (nM)	$hA_{2B}AR$[e] IC_{50} (nM)
			K_i (nM) or KH* (fM) and KL** (nM)			
2	$CH_2CH_2(4\text{-}OH\text{-}C_6H_4)$	37 ± 4	18 ± 2		1884 ± 167	482 ± 41
3	$CH_2CH_2(4\text{-}OCH_3\text{-}C_6H_4)$	61 ± 5	32 ± 3		2652 ± 224	553 ± 48
4	$CH_2CH_2C_6H_5$	24 ± 3	30 ± 2		217 ± 19	389 ± 35
5	$CH_2CH_2(3\text{-}OCH_3\text{-}C_6H_4)$	0.92 ± 0.08	17 ± 2		287 ± 21	47 ± 5
6	$CH_2CH_2(3\text{-}OH\text{-}C_6H_4)$	0.23 ± 0.04	16 ± 2		76 ± 5	25 ± 3
7	$CH_2CH_2(3,4\text{-}(OCH_3)_2\text{-}C_6H_3)$	221 ± 21	91 ± 8		824 ± 76	95 ± 9
8	$CH_2CH_2(3,4\text{-}(OH)_2\text{-}C_6H_3)$	39 ± 3	40 ± 3		546 ± 48	18 ± 2
9	$CH_2CH_2CH_2C_6H_5$	49 ± 4	58± 5		1135 ± 107	1768 ± 152
10	C_6H_5	645 ± 62	782 ± 68		321 ± 28	632 58
11	$CH_2C_6H_5$	45 ± 6	22 ± 3		37 ± 3	32 ± 2
12	$CH_2(4\text{-}OCH_3\text{-}C_6H_4)$	163 ± 12	171 ± 16		381± 37	283 ± 27
13	$CH_2(2\text{-}OCH_3\text{-}C_6H_4)$	3.54 ± 0.32	3.55 ± 0.42* 6.45 ± 0.57**	5.23 ± 0.41* 7.16 ± 0.63**	36 ± 3	313 ± 29
14	$CH_2(3\text{-}OCH_3\text{-}C_6H_4)$	8.16 ± 0.72	5.31 ± 0.52* 26 ± 2**	6.71 ± 0.58* 23 ± 2**	92 ± 8	452 ± 42
15	$CH_2(3\text{-}OH\text{-}C_6H_4)$	27 ± 3	20 ± 2		55 ± 4	24 ± 3
16	$CH_2(3\text{-}F\text{-}C_6H_4)$	8.51 ± 0.76	0.82 ± 0.07		35 ± 4	103 ± 10

17	$CH_2CH_2CH_3$	64 ± 10	17 ± 2		35 ± 4	323 ± 28
18	$CH_2CH_2CH_2CH_3$	41 ± 5	8.21 ± 0.78		23 ± 3	185 ± 17
1		185 ± 14	0.91 ± 0.08	0.93 ± 0.08	683 ± 64	48 ± 5

Affinity values obtained from displacement of specific [^{3}H]DPCPX [a], [^{3}H]-**1** [b], [^{3}H]-**23** [c], or [^{125}I]AB-MECA [d] binding to hA_1ARs, hA_{2A}ARs or A_3ARs, respectively. [d] Potency (IC_{50}) in cAMP assays to hA_{2B}ARs. Data (n = 3-6) are expressed as means ± SEM.

Table 2. Potency (IC_{50}) and Efficacy (Emax) of the tested compounds in comparison with reference compound **1** on cyclic AMP assays in $hA_{2A}CHO$ cells.

compound	IC_{50} (nM) [a]	Emax (%)[b]	IC_{50} (nM)[c]	Emax (%)[d]
2	25±2	67±6	21±3	135±12
3	42±4	64±5	31±3	120±11
4	35±3	70±6	16±2	145±13
11	29±3	65±6	18±2	125±12
12	187±16	38±4	123±11	116±11
13	0.0019±0.0002	63±5	0.051±0.004	138±12
14	0.0083±0.0007	41±3	0.095±0.008	136±11
15	27±2	68±7	22±2	139±13
1	1.45±0.42	46±2	0.678±0.061	123±10

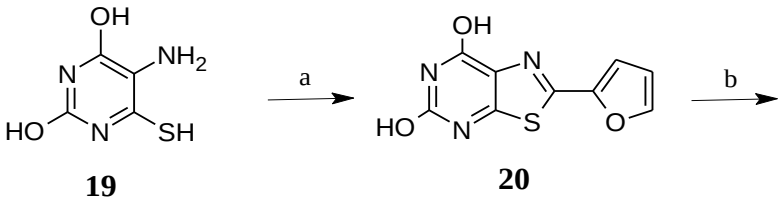
Potency (IC_{50}) [a,c] and Efficacy (Emax) [b,d] of the tested compounds in the absence [a,b] or in the presence [c,d] of agonist compound **23** (10 nM), respectively. Data are expressed as mean ± SEM.

Table 3. Capability of the tested compounds in comparison with reference compound **1** to modulate cyclic AMP production in hA_1CHO cells, hA_{2B}CHO cells and hA_3CHO cells.

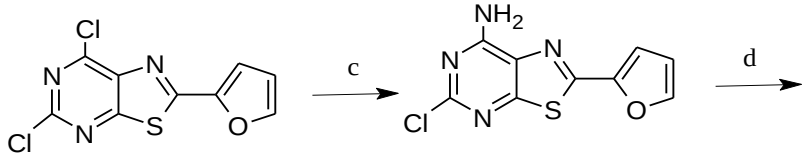
compound	% cAMP		
	hA_1CHO cells	hA_{2B}CHO cells	hA_3CHO cells
2	102±8	101±7	92±8
3	93±7	105±9	103±9
4	105±9	94±7	102±8
11	101±8	102±8	95±8
12	100±9	104±9	97±9
13	106±8	91±8	94±8
14	104±8	106±8	99±8
15	96±7	98±8	95±7
1	103±8	101±9	109±8

The data, expressed as mean ± SEM, indicate the percentage of cAMP modulation over basal level (100%) by the tested compounds at the 10 μM concentration.

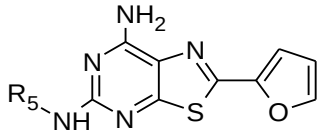
Scheme 1.[a]



19 **20**



21 **22**

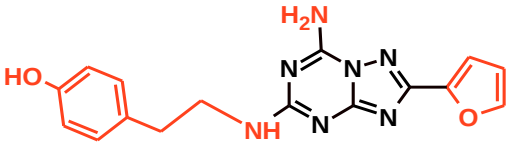


3-5, 7, 9-14, 16-18

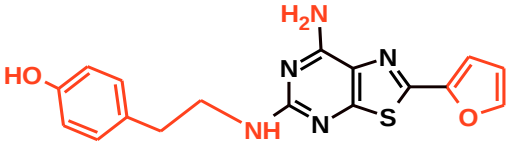
		R_5
e	**2**	$CH_2CH_2(4\text{-}OH\text{-}C_6H_4)$
	3	$CH_2CH_2(4\text{-}OCH_3\text{-}C_6H_4)$
	4	$CH_2CH_2C_6H_5$
e	**5**	$CH_2CH_2(3\text{-}OCH_3\text{-}C_6H_4)$
	6	$CH_2CH_2(3\text{-}OH\text{-}C_6H_4)$
	7	$CH_2CH_2(3,4\text{-}(OCH_3)_2\text{-}C_6H_3)$
e	**8**	$CH_2CH_2(3,4\text{-}(OH)_2\text{-}C_6H_3)$
	9	$CH_2CH_2CH_2C_6H_5$
	10	C_6H_5
	11	$CH_2C_6H_5$
	12	$CH_2(4\text{-}OCH_3\text{-}C_6H_4)$
	13	$CH_2(2\text{-}OCH_3\text{-}C_6H_4)$
e	**14**	$CH_2(3\text{-}OCH_3\text{-}C_6H_4)$
	15	$CH_2(3\text{-}OH\text{-}C_6H_4)$
	16	$CH_2(3\text{-}F\text{-}C_6H_4)$
	17	$CH_2CH_2CH_3$
	18	$CH_2CH_2CH_2CH_3$

[a] Reagents: a) 2-furoyl chloride, NMP, 150 °C. b) $POCl_3$, NNDMA, 100 °C. c) NH_3/H_2O, 85 °C. d) R_5NH_2, nBuOH, MW, 200 °C, 15 min. e) BBr_3, CH_2Cl_2, 50 °C.

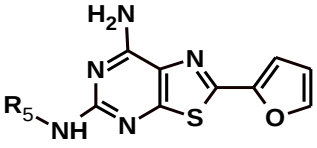
Figure 1. Reference compound **1**, its thiazolo[5,4-d]pyrimidine analog **2**, and the other newly synthesized thiazolo[5,4-d]pyrimidine derivatives **3-18**.



1 (ZM 241385)



2



3-18

$R_5 = (CH_2)_n$-aryl
n = 0, 1, 2, 3
$R_5 = (CH_2)_n$-CH_3
n = 2, 3

Figure 2. Competition curves of specific [^{3}H]-**1** binding to hA_{2A} ARs of compounds **13** (A) and **14** (B) showing the presence of two binding sites, and of reference compound **1** (C) and of other tested compounds (D) characterized by monophasic shape.

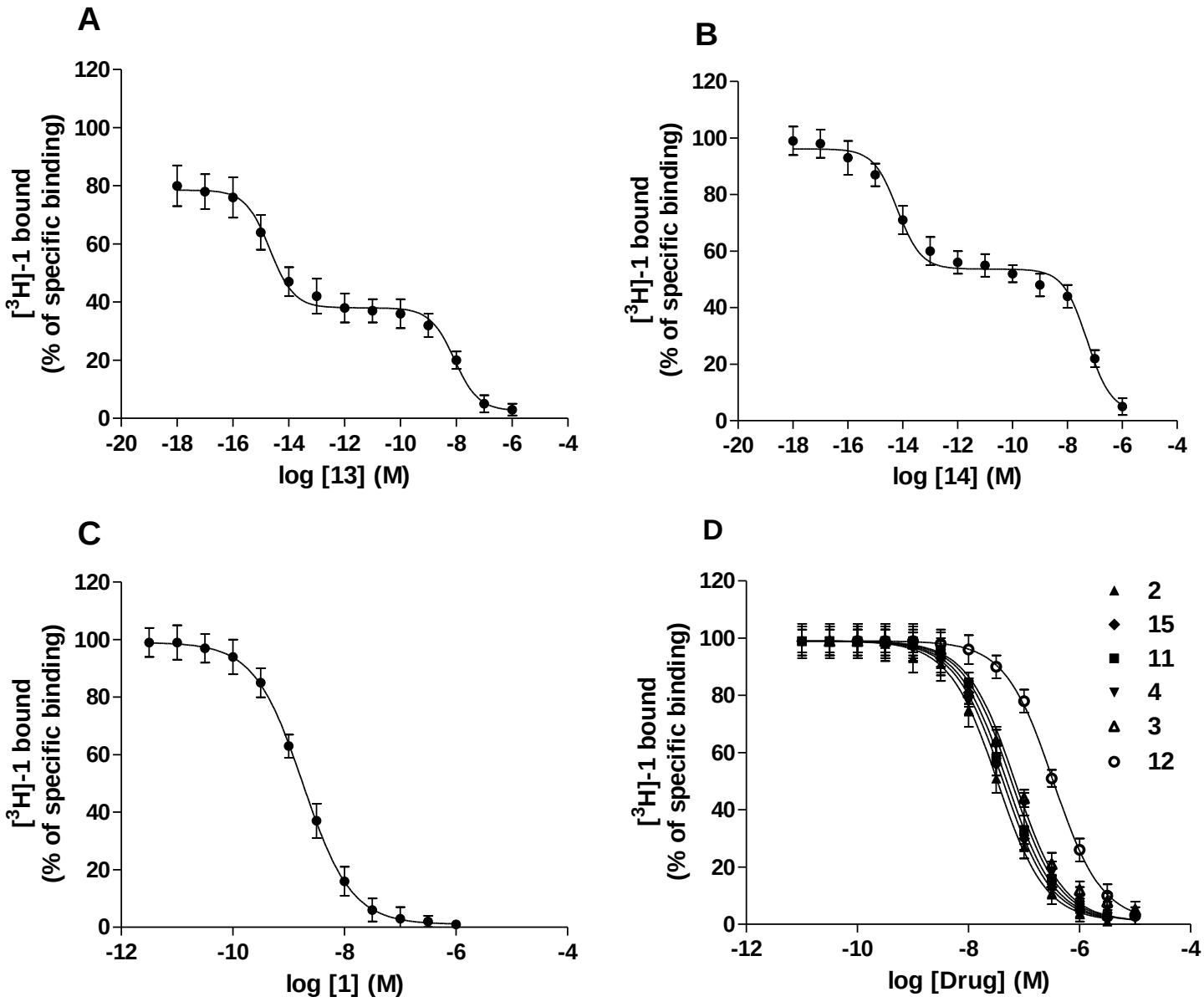


Figure 3. Competition curves of specific [^{3}H]-**23** binding to hA_{2A}ARs of compounds **13** (A) and **14** (B) characterized by biphasic shape and of reference compound **1** (C), characterized by a monophasic shape.

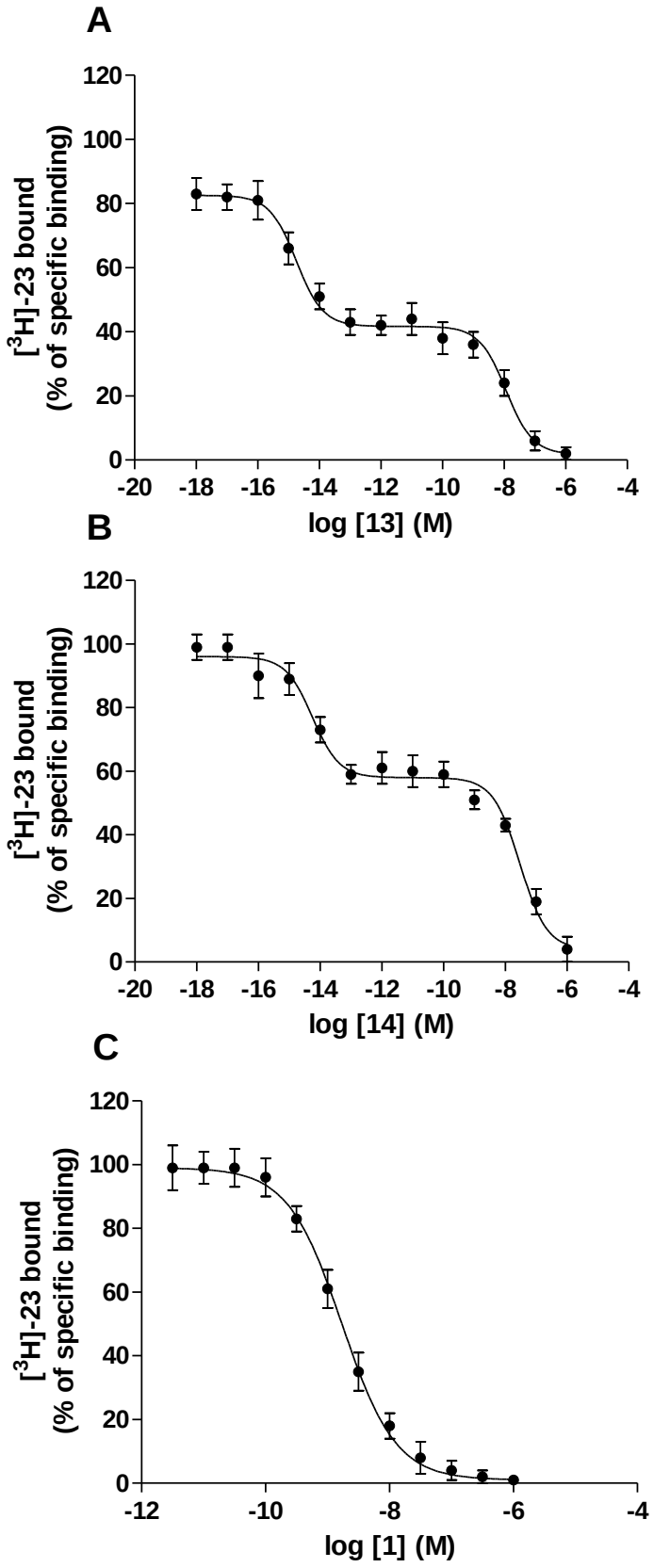


Figure 4. Inhibition of cAMP levels in hA_{2A} CHO cells by compound **13** (A), compound **14** (B), reference compound **1** (C) and by other tested compounds (D). Drug effects are expressed as a percentage of cAMP production in basal conditions.

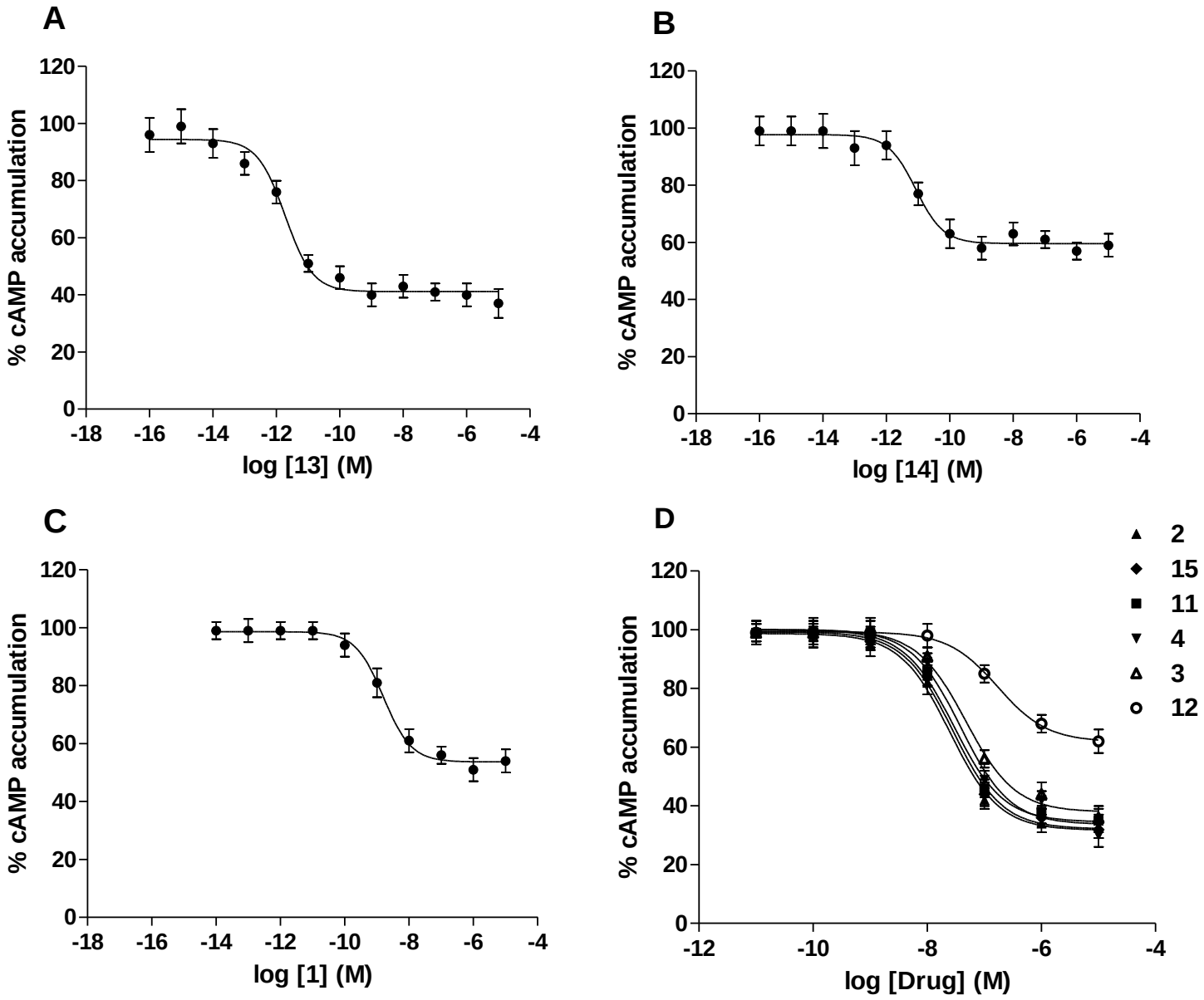


Figure 5. Inhibition of cAMP levels in hA_{2A} CHO cells by compound **13** (A), compound **14** (B), reference compound **1** (C) and by other tested compounds (D). Drug effects are expressed as a percentage of cAMP production in the presence agonist **23** (10 nM). Data represent means ± SEM of four experiments each performed in triplicate

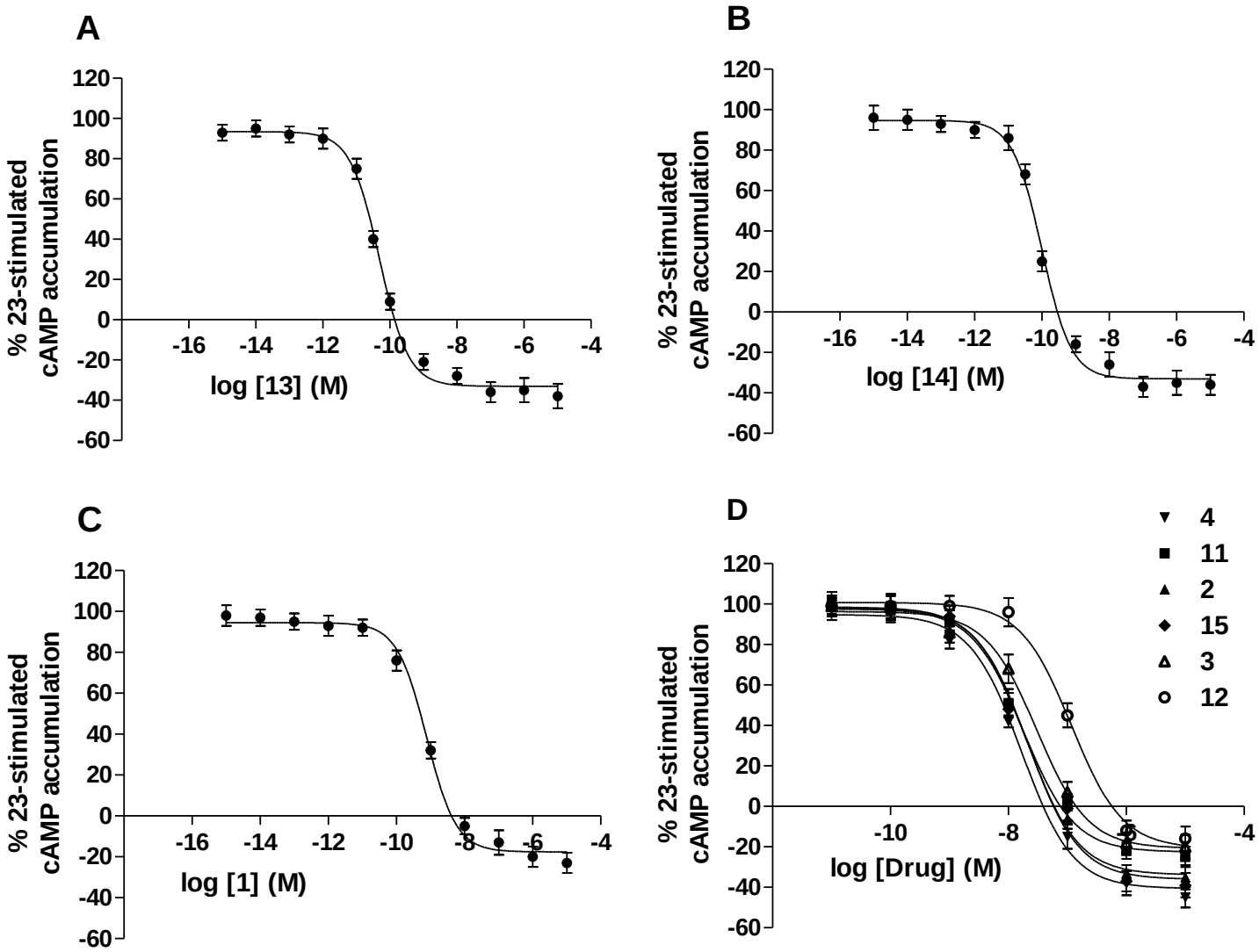


Figure 6. Analgesic effect of compound **13** (A), compound **14** (B), reference compound **1** (C) and morphine (D) in writhing test showing the effect of increasing doses in the reduction of writhing number induced by acetic acid administration. ED_{50} values are presented as means ± SEM (n=8 mice/group).

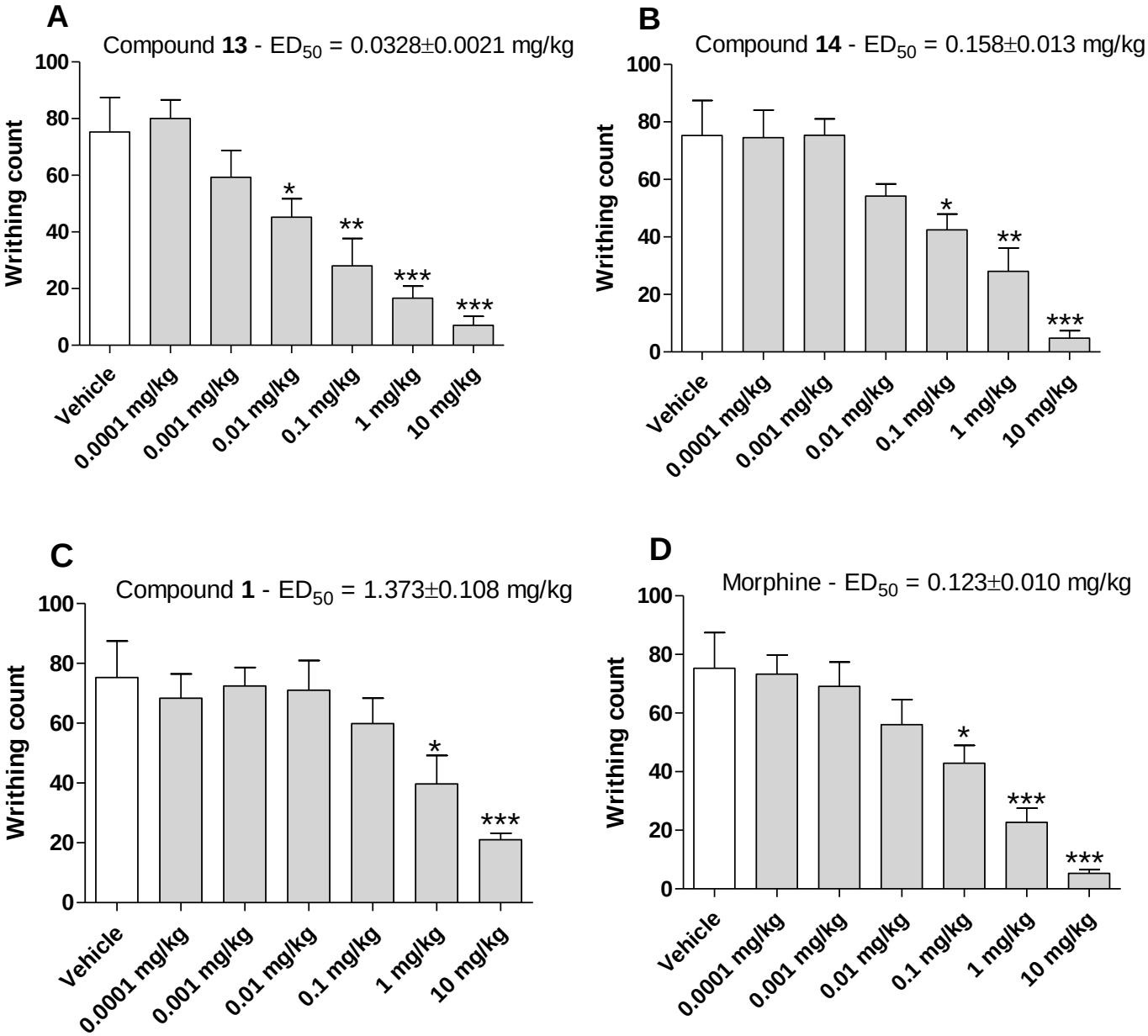
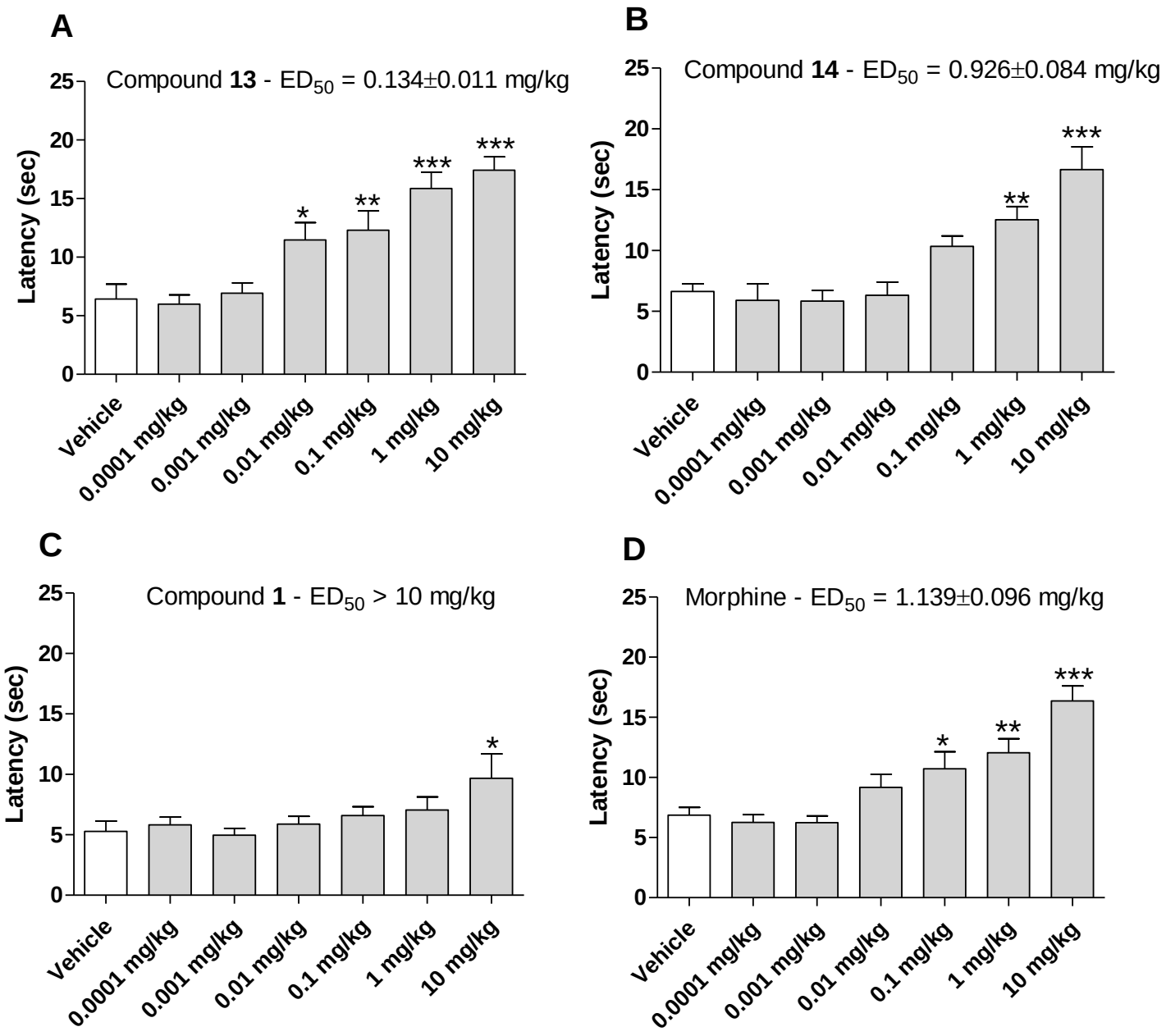


Figure 7. Anti-nociceptive effect of compound **13** (A), compound **14** (B), reference compound **1** (C) and morphine (D) in hot water tail immersion test. ED_{50} values are presented as means ± SEM (n=8 mice/group).



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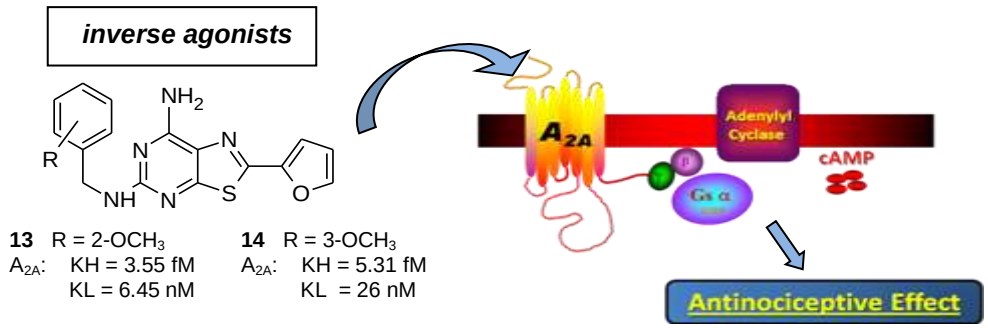

13 R = 2-OCH_3
A_{2A}: KH = 3.55 fM
KL = 6.45 nM

14 R = 3-OCH_3
A_{2A}: KH = 5.31 fM
KL = 26 nM