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4-Amino-6-alkyloxy-2-alkylthiopyrimidine Derivatives as Novel Non-nucleoside Agonists for the Adenosine A_1 Receptor

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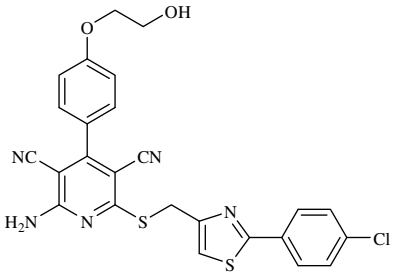
Abstract: Three 4-amino-6-alkyloxy-2-alkylthiopyrimidine derivatives (**4**-**6**) were investigated as potential non-nucleoside agonists at human adenosine receptors (ARs). When tested in competition binding experiments, these compounds exhibited low micromolar affinity (K_i values comprised between 1.2 and 1.9 μM) for the A_1 AR and no appreciable affinity for the A_{2A} and A_3 ARs. Evaluation of their efficacy profiles by measurement of intracellular cAMP levels revealed that

4 and **5** behave as non-nucleoside agonists of the A_1 AR with EC_{50} values of 0.47 μM and 0.87 μM, respectively. No clear concentration-response curves could be instead obtained for **6**, probably because this compound modulates one or more additional targets, thus masking the putative effects exerted by its activation of A_1 AR The three compounds were not able to modulate A_{2B} AR-mediated cAMP accumulation induced by the non-selective AR agonist NECA, thus demonstrating no affinity towards this receptor.

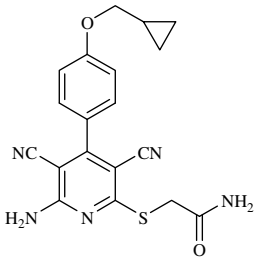
1. INTRODUCTION

The nucleoside adenosine plays a key role in a large variety of physiological and pathological processes by interacting with specific receptors. Four different subclasses of adenosine receptors (ARs) have been identified to date, A_1, A_{2A}, A_{2B}, and A_3, all belonging to the superfamily of G-protein coupled receptors (1-3). Activation of these receptors by adenosine or a synthetic agonist determines different intracellular events starting with inhibition (A_1 and A_3) or stimulation (A_{2A} and A_{2B}) of adenylate cyclase. Additional molecular mechanisms coupled to occupation of ARs by agonists are stimulation of phospholipase C (A_1, A_{2B}, and A_3), activation of potassium channels and inhibition of calcium channels (A_1) (4). Being ubiquitously distributed in tissues and organs of mammalians, ARs have been considered attractive targets for the development of agonist- and antagonist-based therapies against a wide range of pathologies, including central nervous system (CNS) disorders, cardiac arrhythmia, ischemic injuries, asthma, renal failure, and inflammatory diseases (5). Although several agonists binding selectively to each of the four ARs are available for preclinical studies, the only currently marketed AR agonists are adenosine itself, administered by intravenous infusion in the treatment of cardiovascular acute pathologies, and a synthetic derivative of adenosine, regadenoson, administered by intravenous injection as vasodilatator stress agent (6). Most of the currently known synthetic AR agonists, differently from AR antagonists, are characterized by a ribose moiety or a ribose-like moiety linked to a heterocyclic system.

The nucleoside nature of such compounds represents one of the reasons hampering their clinical use due to a low oral bioavailability and/or a short half-life. As an example, the A_1 AR agonist N^6-cyclohexyl-2'-*O*-methyladenosine (SDZ-WAG 994), administered to patients with heart failure, exhibits an oral bioavailability comprised between 30% and 50% and a terminal half-life of about 30 minutes (7). AR ligands lacking a sugar moiety have nearly always been found devoid of agonist efficacy. This has been true until the discovery of some non-nucleoside 2-amino-4-aryl-3,5-dicyanopyridines and 4-amino-6-aryl-5-cyano-2-thiopyrimidines. Representatives of such pyridine derivatives are capadenoson (**1**) and BAY 60-6583 (**2**) (Figure 1), reported as selective agonists at A_1 AR and A_{2B} AR, respectively (8). An exponent of the aforementioned pyrimidine derivatives is compound **3** (Figure 1) described as a potent selective agonist at A_{2A} AR (9,10).



1 (capadenoson)



2 (BAY 60-6583)

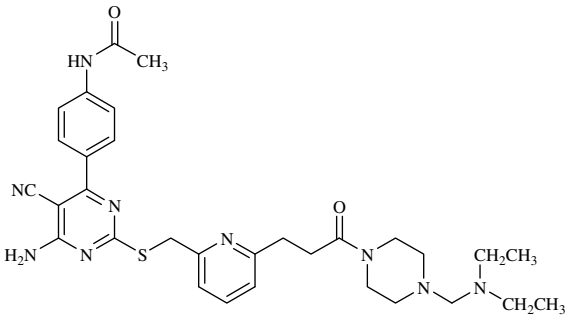


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Figure 1: Structures of compounds **1**-**3** chosen as representative of well-known non-nucleoside AR agonists.

Compound **4** (Figure 2) is a synthetic precursor of one of several 4-acylamino-6-alkyloxy-2-alkylthiopyrimidines disclosed by our research group as antagonists of the human A_3 AR (11). Compounds **5** and **6** (Figure 2) were described by some of us as potential inhibitors of Wnt signaling pathway (12). The synthesis of the above pyrimidine derivatives is summarized in Scheme 1.The resemblance of **4**-**6** with **1**-**3** led us to evaluate whether our compounds might be endowed with AR agonist efficacies, hopefully making them new reference structures in the perspective of lead-optimization studies. The present paper describes the results of such investigations.

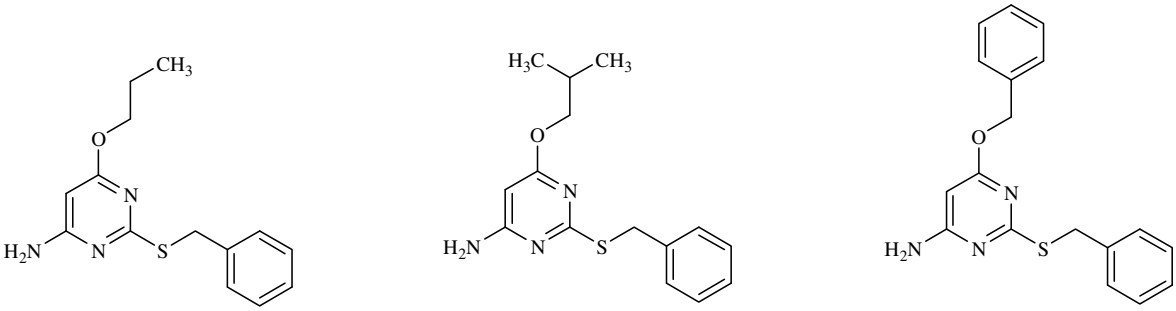
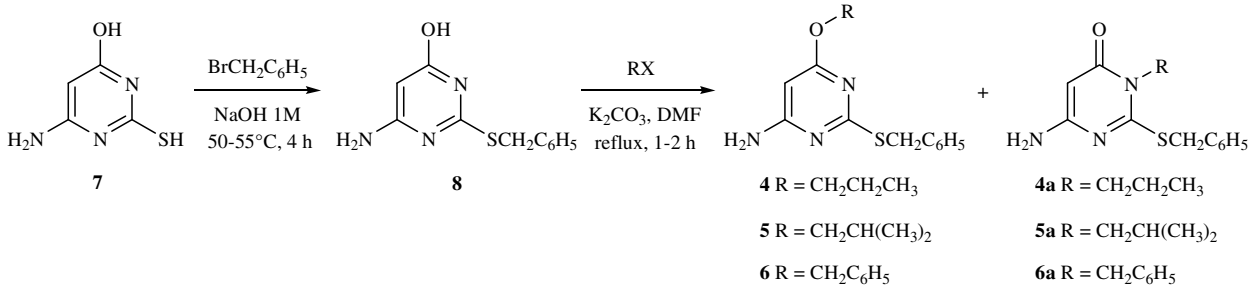


Figure 2: Structures of 4-amino-6-alkyloxy-2-benzylthiopyrimidines **4**-**6** investigated in the present work as potential non-nucleoside AR agonists.



Scheme 1: Synthesis of compounds **4**-**6**. X = Br for compounds **4** and **6**; X = I for compound **5**.

2. METHODS AND MATERIALS

The synthesis of **4** is described in ref. 11 whereas **5** and **6** are prepared as described in ref. 12 (Scheme 1). Briefly, the first step is S-alkylation of 6-hydroxy-2-mercaptopyrimidine monohydrate (**7**) by reaction with benzylbromide in NaOH 1M to give **8** (80%). This latter is collected by filtration and used, without further purification, in the next step, i.e. alkylation with an appropriate alkyl halide, in anhydrous DMF with an excess of K_2CO_3, to give **4**-**6** and the isomers **4a**-**6a**. The ratio **4**-**6**:**4a**-**6a** is about 4:1 with an overall yield comprised between 50% and 90%. Finally, **4**-**6** are isolated by flash-chromatography. The affinities of **4**-**6** towards human A_1, A_{2A} and A_3 ARs were evaluated by competition experiments, assessing the ability of the compounds to displace specific radiolabeled ligands from transfected CHO cells (13). The efficacy profiles of the compounds at human A_1 and A_{2B} ARs were evaluated by assays based on intracellular cAMP measurements in transfected CHO cells (13).

3. EXPERIMENTAL

3.1. Adenosine Receptor Binding Assays. Materials.

Each compound was routinely dissolved in DMSO and diluted with assay buffer to the final concentration, where the amount of DMSO never exceeded 2%. Percentage inhibition values of specific radiolabeled ligand binding at 1-10 μM concentration were calculated as means ± SEM of at least three determinations. [^{3}H]DPCPX and [^{3}H]NECA were obtained from DuPont-NEN (Boston, MA). ADA was from Sigma Chemical Co. (St. Louis, MO). All other reagents were from standard commercial sources and of the highest commercially available grade. CHO cells stably expressing human A_1, A_{2A}, A_{2B} and A_3 ARs were kindly supplied by Prof. K.-N. Klotz, Wurzburg University, Germany (13).

3.2. Human A_1 Adenosine Receptors.

Aliquots of cell membranes (30 μg proteins) were incubated at 25°C for 180 min in 500 μL of binding buffer (50 mM Tris-HCl, 2 mM $MgCl_2$, 2 units/mL ADA, pH 7.4) containing [^{3}H]DPCPX (3 nM) and six different concentrations of the tested compounds. Non-specific binding was determined in the presence of

50 μM R-PIA. The dissociation constant (K_d) of [^{3}H]DPCPX in human A_1 AR CHO cell membranes was 3 nM.

3.3. Human A_{2A} Adenosine Receptors.

Aliquots of cell membranes (30 μg) were incubated at 25°C for 90 min in 500 μL of binding buffer (50 mM Tris-HCl, 2 mM $MgCl_2$, 2 units/mL ADA, pH 7.4) in the presence of 30 nM of [^{3}H]NECA and six different concentrations of the tested compounds. Non-specific binding was determined in the presence of 100 μM R-PIA. The dissociation constant (K_d) of [^{3}H]NECA in human A_{2A} AR CHO cell membranes was 30 nM.

3.4. Human A_3 Adenosine Receptors.

Aliquots of cell membranes (90 μg) were incubated at 25°C for 180 min in 500 μL of binding buffer (50 mM Tris-HCl, 5 mM $MgCl_2$, 1 mM EDTA, 2 units/mL ADA, pH 7.4) in the presence of 30 nM [^{3}H]NECA and six different concentrations of the tested compounds. Non-specific binding was determined in the presence of 100 μM R-PIA (13,14).

3.5. Measurement of cAMP Levels in Human A_1 and A_{2B} AR-transfected CHO cells.

Intracellular cAMP levels were measured using a competitive protein binding method (15). CHO cells, expressing recombinant human ARs, were harvested by trypsinization. After centrifugation and resuspension in medium, cells (~ 30,000) were plated in 24-well plates in 0.5 mL of medium. After 24 h, the medium was removed, and the cells were incubated at 37°C for 15 min with 0.5 mL of DMEM in the presence of ADA (1U/mL) and the phosphodiesterase inhibitor Ro20-1724 (20 μM). The antagonist efficacy profile of the compounds at A_1 AR was evaluated by assessing their ability to counteract NECA-mediated inhibition of cAMP accumulation in the presence of 10 μM forskolin, a non-selective activator of adenylate cyclase. The compounds (10 or 50 μM) were incubated 10 min before the addiction of the agonist NECA (1 nM). The agonist efficacy profile of the compounds at A_1 AR was evaluated by assessing their ability to modulate intracellular cAMP levels in the presence of 10 μM forskolin and in the absence of standard agonists. Cells were incubated in the reaction medium (15 min at 37°C) with different concentrations of the tested compound (0.1 nM-100 μM). In the assays carried out to evaluate the antagonist efficacy profile, cells were treated with the agonist NECA (100 nM). Following incubation, the reaction was terminated by the removal of the medium and the addition of 0.4 N HCl. After 30 min, lysates were neutralized with 4 N KOH, and the suspension was centrifuged at 800 rpm for 5 min. For the determination of cAMP production, bovine adrenal cAMP binding protein was incubated with [^{3}H]cAMP (2 nM) and 50 μL of cell lysate or cAMP standard (0-48 pmol) at 0°C for 150 min in a total volume of 300 μL. Bound radioactivity was separated by rapid filtration through GF/C glass fiber filters and washed twice with 4 mL 50 mM Tris/HCl, pH 7.4. The radioactivity was measured by liquid scintillation spectrometry.

3.6. Data Analysis.

All binding and functional data were analyzed using Graph-Pad Prism 5.0 (GraphPad Software Inc., San Diego, CA). The EC_{50} values were obtained using the non-linear regression curve fitting of the concentration-response curves. The K_i values were obtained using the one-site fit curve. All values obtained are mean values of at least three different experiments each performed in duplicate.

4. RESULTS

The binding data of the 4-amino-6-alkyloxy-2-alkylthiopyrimidine derivatives **4**-**6** to human A_1, A_{2A}, and A_3 ARs, expressed as K_i values, are summarized in Table 1, along with those of DPCPX, NECA, and Cl-IBMECA, used as the reference standards. Our compounds were able to compete with the radiolabeled antagonist [^{3}H]DPCPX showing monophasic curves (Figure 3). The compounds displayed low micromolar affinity towards A_1 AR (K_i values comprised between 1.2 and 1.9 μM) and no appreciable affinity towards A_{2A} and A_3 ARs (inhibition of radioligand binding less than 20% at 10 μM). In addition, when evaluated in a functional assay, the same compounds were not able to modulate NECA-mediated cAMP accumulation in CHO A_{2B} AR transfected cells, thus demonstrating no affinity towards this receptor (percentage inhibition less than 5% at 10 μM, data not shown).

Table 1: Binding affinities of compounds **4**-**6** towards A_1, A_{2A} and A_3 ARs in CHO transfected cells.[a]

Compound	hA_1[b] K_i (nM)	hA_{2A}[c] K_i (nM)	hA_3[d] K_i (nM)
4	1,240 ± 275	> 10,000	> 10,000
5	1,945 ± 427	> 10,000	> 10,000
6	1,196 ± 280	> 10,000	> 10,000
DPCPX	0.5 ± 0.03	337 ± 28	>10,000
NECA	14 ± 4	16 ± 3	73 ± 5
Cl-IBMECA	890 ± 61	401 ± 25	0.22 ± 0.02

[a]K_i values are means ± SEM of four separate assays, each performed in triplicate. [b]Displacement of specific [^{3}H]DPCPX binding at human A_1 AR expressed in CHO cells. [c]Displacement of specific [^{3}H]NECA binding at human A_{2A} R expressed in CHO cells. [d]Displacement of specific [^{3}H]NECA binding at human A_3 AR expressed in CHO cells.

As next step, we characterized the efficacy profiles (antagonism/agonism) of **4**-**6** towards A_1 AR.

The antagonist efficacies of the compounds at this receptor were evaluated by assessing their abilities to counteract NECA-mediated inhibition of cAMP accumulation produced by forskolin, a non-selective activator of adenylate cyclase. As shown in Figure 4, none of the compounds exhibited significant effects in this assay, suggesting that they do not behave as A_1 AR antagonists.

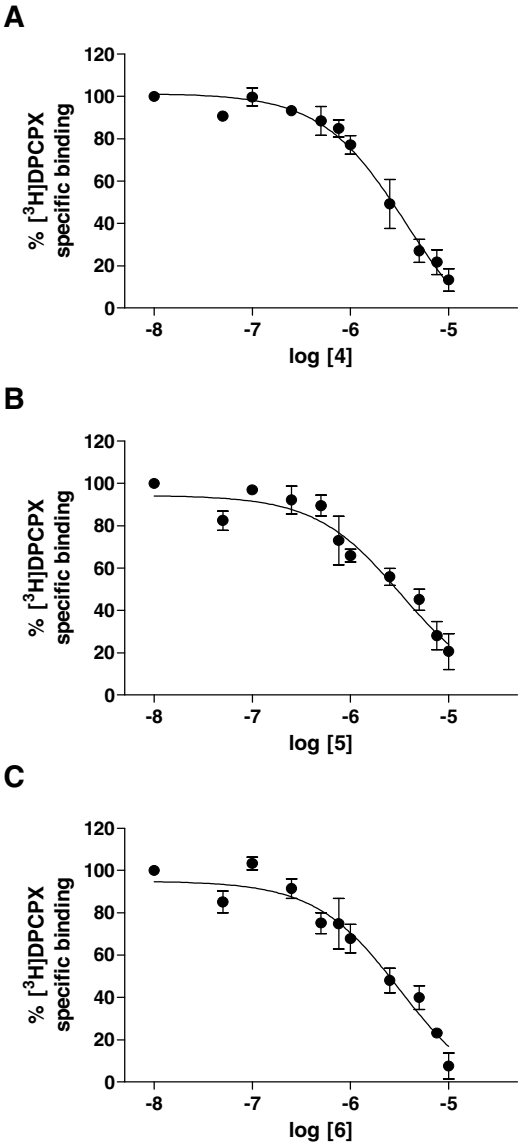


Figure 3: Displacement curves of [^{3}H]DPCPX binding by compounds **4**-**6** at human A_1 AR expressed in CHO cells. Data are expressed as percentage of [^{3}H]DPCPX specific binding and represent the mean ± SEM of three different experiments.

The agonist profiles of the compounds towards A_1 AR were evaluated by assessing their abilities to reduce intracellular cAMP levels in the presence of forskolin and in absence of any agonist. NECA was able to reduce the intracellular cAMP levels with a potency of 1.5 nM, in accordance with literature data (16). In this assay, **4** and **5** exhibited a concentration-dependent inhibition of cAMP levels (Figure 5), thus suggesting that they act as A_1 AR agonists. No clear concentration-response curves could be instead obtained for **6**, probably because this compound modulates one or more additional targets, thus masking the putative effects exerted by its activation of A_1 AR.

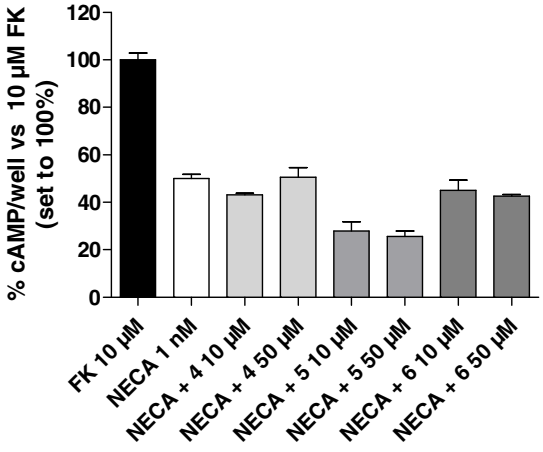


Figure 4: Effects of compounds **4**-**6** on NECA-mediated inhibition of cAMP levels in A_1 AR CHO transfected cells. Cells were treated with 1 nM NECA in the presence of 10 µM forskolin. When indicated, 10 or 50 µM of each compound was added. Intracellular cAMP levels were quantified as described in the experimental section. Data are expressed as percentage of cAMP/well versus forskolin set to 100% and represent the mean ± SEM of three different experiments.

5. DISCUSSION

The results of our studies indicate that the 4-amino-6-alkyloxy-2-benzylthiopyrimidines **4** and **5** can be regarded as the first exponents of a novel class of non-nucleoside selective A_1 AR agonists. Their EC_{50} values of 0.49 µM and 0.87 µM, respectively, are 2-2.5 fold lower than their corresponding binding affinities towards the same receptor (Table 1).

These compounds, together with **6**, were investigated by us taking into account their structural resemblance with known non-nucleoside A_1 AR agonists (Figure 2). Studies are in progress to dissect the mechanism of action of **6** underlying its unexpected biochemical behavior, quite different from those exhibited by **4** and **5**.

The therapeutic potential of the class of non-nucleoside A_1 AR agonists, now including compounds **4** and **5**, stems, as mentioned, from the key role played by A_1 AR in the organism (3,5) and the lack of pharmacokinetics-related disadvantages associated with a sugar moiety.

A comparison between the structures of **4** and **5** with those reported in literature as potent AR agonists (exemplified by **1**-**3**) suggests that requirements for a tight ligand-receptor binding might be a cyano group (at least one) and/or an aryl substituent directly attached to the pyridine/pyrimidine ring. With regard to the molecular details of such interactions, J. Robert Lane *et al.* proposed a docking model of the A_{2A} AR agonist LUF5834 (17), a pyridine analog of **1** and **2**, into the corresponding orthosteric binding site (18). According to these authors, the 2-amino and 3-cyano groups of LUF5834 are both engaged in hydrogen bonds with the Asn253 side chain of A_{2A} AR.

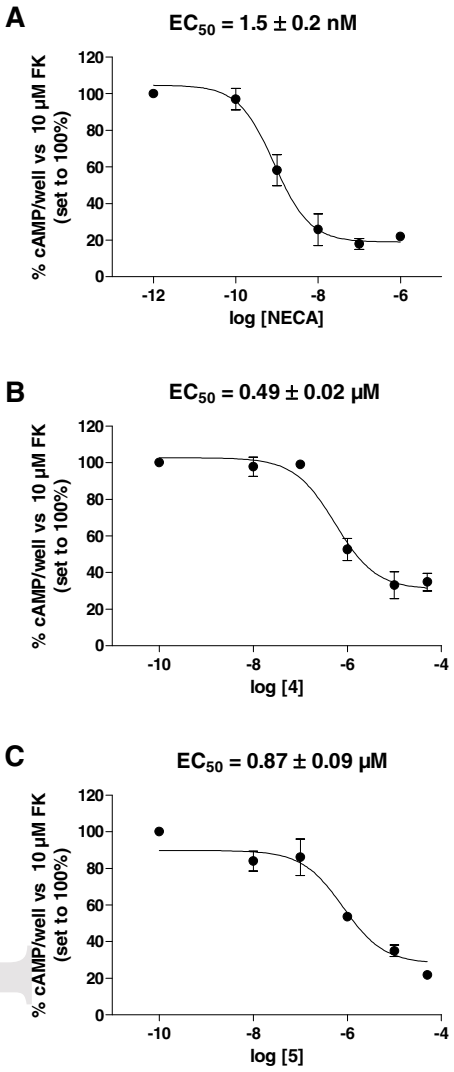


Figure 5: Effects of NECA (A), compounds **4** (B) and **5** (C) on forskolin-stimulated accumulation of cAMP in A_1 AR CHO transfected cells. Cells were treated with 10 µM forskolin in the absence or in the presence of the tested compound at different concentrations ranging from 0.01 nM to 100 µM. Intracellular cAMP levels were quantified as described in the experimental section. Data are expressed as percentage of cAMP/well versus forskolin set to 100% and represent the mean ± SEM of three different experiments.

The preliminary study described here has opened up a whole new range of investigations on the structure-activity relationships of this class of A_1 AR agonists, which are currently in progress and will be reported on in due course.

LIST OF ABBREVIATIONS

ADA, adenosine deaminase;

cAMP, 3',5'-cyclic adenosine monophosphate;

CHO, chinese hamster ovary;

Cl-IBMECA, 2-Chloro-N^6-(3-iodobenzyl)-adenosine-5'-*N*-methyluronamide;

DMEM, Dulbecco's modified Eagle's medium;

[^{3}H]DPCPX, [^{3}H]8-cyclopentyl-1,3-dipropylxanthine;

[^{3}H]NECA, [^{3}H]5'-*N*-ethylcarboxamidoadenosine;

R-PIA, *(R)*-N^6-(2-phenylisopropyl)adenosine

SEM, standard error of mean

CONFLICT OF INTEREST

The Authors confirm that this article content has no conflict of interest.

ACKNOWLEDGEMENTS

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REFERENCES

1. Fredholm, B.B., Jacobson, K.A., Klotz, K.-N., Linden, J. (2001) International Union of Pharmacology. XXV. Nomenclature and classification of adenosine receptors. Pharmacol Rev;53**:**527—532.
2. Fredholm, B.B., IJzerman, A.P., Jacobson, K.A., Linden, J., Muller, C.E. (2011) International Union of Basic and Clinical Pharmacology. LXXXI. Nomenclature and classification of adenosine receptors-an update. Pharmacol Rev;63:1—34.
3. Muller, C.E., Jacobson, K.A. (2011) Recent developments in adenosine receptor ligands and their potential as novel drugs. Biochim Biophys Acta;1808:1290—1308.
4. Schulte, G., Fredholm, B.B. (2003) Signalling from adenosine receptors to mitogen-activated protein kinases. Cell Signal;15:813—827.
5. Jacobson, K.A., Gao, Z.-G. (2006) Adenosine receptors as therapeutic targets. Nat Rev Drugs Disc;5:247—264.
6. Al Jaroudi, W., Iskandrian, A.E. (2009) Regadenoson: a new myocardial stress agent. J Am Coll Cardiol;54:1123—30.
7. Bertolet, B.D., Anand, I.S., Bryg, R.J., Mohanty, P.K., Chatterjee, K., Cohn, J.N., Khurmi, N.S., Pepine, C.J. (1996) Effects of A_1 adenosine receptor agonism using N^6-cyclohexyl-2′-*O*-methyladenosine in patients with left ventricular dysfunction. Circulation;94:1212—1215.
8. Rosentreter, U., Henning, R., Bauser, M., Kramer, T., Vaupel, A., Hubsch, W., Dembowsky, K., Salcher-Schraufstatter, O., Stasch, J.-P., Krahn, T., Perzborn, E. Substituted 2-thio-3,5-dicyano-4-aryl-6-aminopyridines and the use thereof as adenosine receptor ligands. WO2001/ 025210, April 12, 2001.
9. Kato, M., Sato, N., Okada, M., Uno, T., Ito, N., Takeji, Y., Shinohara, H., Fuwa, M. 4-Amino-5-cyanopyrimidine derivatives. WO2005/105778 A2, November 10, 2005.
10. Sato, N., Yuki, Y., Shinohara, H., Takeji, Y., Ito, K., Michikami, D., Hino, K., Yamazaki, H. Novel cyanopyrimidine derivatives. US2012/0022077 A1, January 26, 2012.
11. Cosimelli, B., Greco, G., Ehlardo, M., Novellino, E., Da Settimo, F., Taliani, S., La Motta, C., Bellandi, M., Tuccinardi, T., Martinelli, A., Ciampi, O., Trincavelli, M.L., Martini, C. (2008) Derivatives of 4-

amino-6-hydroxy-2-mercaptopyrimidine as novel, potent, and selective A_3 adenosine receptor antagonists. J Med Chem;51:1764—1770.
12. Cosimelli, B., Laneri, S., Ostacolo, C., Sacchi, A., Severi, E., Porcù, E., Rampazzo, E., Moro, E., Basso, G., Viola, G. (2014) Synthesis and biological evaluation of imidazo[1,2-*a*]pyrimidines and imidazo[1,2-*a*]pyridines as new inhibitors of the Wnt/β-catenin signaling. Eur J Med Chem;83:45—56.
13. Klotz, K.-N., Hessling, J., Hegler, J., Owman, C., Kull, B., Fredholm, B.B., Lohse, M.J. (1998) Comparative pharmacology of human adenosine receptor subtypes-characterization of stably transfected receptors in CHO cells. Naunyn-Schmiedeberg's Arch Pharmacol;357:351—359.
14. Martínez, A., Gutiérrez-de-Terán, H., Brea, J., Raviña, E., Loza, M.I., Cadavid, M.I., Sanz, F., Vidal, B., Segarra, V., Sotelo, E. (2008) Synthesis, adenosine receptor binding and 3D-QSAR of 4-substituted 2-(2'-furyl)-1,2,4-triazolo[1,5-*a*]quinoxalines. Bioorg Med Chem;16:2103—2113.
15. Nordstedt, C., Fredholm, B.B. (1990) A modification of a protein-binding method for rapid quantification of cAMP in cell-culture supernatants and body fluid. Anal Biochem;189:231—234.
16. Cordeaux, Y., IJzerman, A.P., Hill, S.J. (2004) Coupling of the human A_1 adenosine receptor to different heterotrimeric G proteins: Evidence for agonist-specific G protein activation. Br J Pharmacol;143:705—714.
17. Beukers, M.W., Chang, L.C.W., von Frijtag Drabbe Künzel, J.K., Mulder-Krieger, T., Spanjersberg, R.F., Brussee, J., IJzerman, A.P. (2004) New, non-adenosine, high-potency agonists for the human adenosine A_{2B} receptor with an improved selectivity profile compared to the reference agonist *N*-ethylcarboxamido-adenosine. J Med Chem;47:3707—3709
18. Lane, J.R., Klein Herenbrink, C., van Westen, G.J.P., Spoorendonk, J.A., Hoffmann, C., IJzerman, A.P. (2012) A Novel nonribose agonist, LUF5834, engages residues that are distinct from those of adenosine-like ligands to activate the adenosine A_{2A} receptor. Mol Pharmacol;81:475—487.