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Further Studies on Pyrazolo[1',5':1,6]pyrimido[4,5-d]pyridazin-4(3H)-ones as Potent and Selective Human A1 Adenosine Receptor Antagonists

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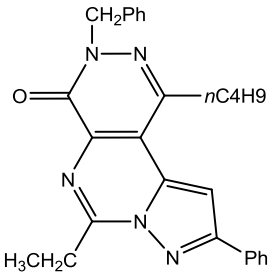
The present paper reports a new series of pyrazolo[1',5':1,6]pyrimido[4,5-d]pyridazin-4(3H)-ones which were tested in radioligand binding assays on human adenosine A_1, A_{2A} and A_3 receptors. The most interesting compound was also evaluated in cAMP functional assay and in the mouse passive avoidance test.

Graphical Abstract



16i

hA_1AR binding affinity	K_i = 1.39 nM
cAMP functional assay	IC_{50} = 2.92
Precognitive effect	10-30 mg/kg

Highlights

- Pyrazolo[1',5':1,6]pyrimido[4,5-d]pyridazin-4(3H)-ones nucleous
- Human $_{A1}$, $_{A2A}$ and $_{A3}$ adenosine receptors binding assays.
- Functional cAMP assay.
- Antiamnesic activity.

Further Studies on Pyrazolo[1',5':1,6]pyrimido[4,5-d]pyridazin-4(3H)-ones as Potent and Selective Human A_1 Adenosine Receptor Antagonists

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Abstract

A new series of pyrazolo[1',5':1,6]pyrimido[4,5-d]pyridazin-4(3H)-ones was synthesized and tested in radioligand binding assays on human A_1, A_{2A} and A_3 adenosine receptors. Most of the compounds showed high selectivity of action towards A_1 receptor and high affinity with Ki values in the low nanomolar range. The pharmacological profile of the most active molecules towards A_1 adenosine receptors was evaluated in cAMP functional assay. Compounds demonstrated their ability to completely counteract the effect of the agonist NECA, thus demonstrating their antagonist profile. Moreover, the most interesting compound, tested in the mouse passive avoidance, exhibited an antiamnesic effect at the doses of 10 and 30 mg/kg.

Keywords:

Pyrazolo[1',5':1,6]pyrimido[4,5-d]pyridazin-4(3*H*)-ones; human adenosine receptor; A_1 subtype; selectivity.

1. Introduction

Adenosine is an endogenous modulator that operates a wide variety of physiological functions in the nervous, cardiovascular, renal and immune systems, through the interaction with four different adenosine receptors (ARs) classified as A_1, A_{2A}, A_{2B} and A_3 [1-3]. All four ARs have been cloned from different species (including humans) and pharmacologically characterized[2,4].

ARs belong to G protein-coupled receptor (GPCRs) family and consist of a single polypeptide chain which passes through the membrane to form seven transmembrane helices. A_1 and A_3 receptors inhibit adenylate cyclase *via* Gi protein thus reducing the production of cyclic AMP (cAMP).Conversely the A_{2A} and A_{2B} subtypes stimulate the production of cAMP by coupling to Gs or Golf[5] protein. Nevertheless, ARs interact with other second messengers systems, such as phospholipase C (PLC) [6] (A_1, A_{2B} and A_3 subtype), phospholipase D (PLD) [7] (A_3 subtypes) and potassium and calcium channels [2,8] (A_1 subtype).

The potential therapeutic applications of selective ligands of ARs have been investigated in recent years for the treatment of several pathologies such as cardiovascular and renal diseases, CNS disorders, inflammatory and allergic disorders and cancer [9-11]. The A_1ARs are expressed at high levels in brain, cortex, cerebellum and ippocampus. Thus, the use of selective A_1 antagonists has emerged as potential target for the treatment of cognitive deficit, and for CNS disorders as Alzheimer's and Parkinson's diseases [12,13] as well as CNS-stimulatory drugs. In fact it is known that antiamnesic activities is exhibited by adenosine A_1 antagonists [14] such as DPCPX which is effective in altering memory, promoting a facilitation [15,16] and in preventing methylphenidate-triggered recognition memory impairment in adult mice [17] and 8-cyclopentyl-1,3-dimethylxanthine which exerts a positive impact on hippocampus-dependent spatial object recognition memory [18].

By contrast, the selective activation of A_1 ARs significantly impaired retention performance as demonstrated by the treatment with N6-cyclopentyladenosine (CPA) that is also able to impair memory retrieval for inhibitory avoidance task into posterior cingulate cortex [19]. Furthermore, the CPA derivative 2-Chloro-N6-cyclopentyladenosine disrupted the juvenile recognition ability of adult rats [20].

With regards to possible application of A_1AR antagonists in cardiovascular system, kidney and respiratory pathologies, different drugs are under development for the treatment of asthma [21], cardiac arrhythmia, heart failure, renal failure and hypertension [22].

Currently, the potent and selective A_1R antagonists **BG9928** (Tonapofylline) [23], **SLV320** [24] and **KW-3902** (Rolofylline) [25,26] are in clinical trials (phase II/phase III) for the treatment of congestive heart failure and renal insufficiency (Figure 1).

Our research in this field was first performed by synthesizing compounds which respected the classical structural requirements proposed by Jacobson et al. [27] for adenosine receptor antagonists. The pyrazolo[1',5':1,6]pyrimido[4,5-d]pyridazin-4(3H)-ones nucleous of our compounds proved to be an appropriate scaffold for adenosine receptor subtype hA_1 as demonstrated by the high activity and selectivity of the most interesting terms [28,29]. The various positions of the tricyclic scaffold have been investigated and results available so far showed that the optimal substituents are a pyridyl ring at position 1, benzyl at N-3, hydrogen or methyl at position 6 and a phenyl at position 9 (compounds **A** [28] is reported as example in Figure 1).

In the present work we further investigated position 1 (in which we previously inserted phenyl, 3- and 4-F-phenyl, 3- and 4-pyridyl, chlorine and methyl) [28,29] including (hetero)aryl and (cyclo)alkyl groups and maintaining at the other positions the substituents which award the

maximum activity. For some active compounds the corresponding 4-thione derivatives were synthesized in order to better understand the importance of the amide carbonyl group.

2. Chemistry

The synthetic procedure previously described[30-32] affording the final pyrazolo[1',5':1,6]pyrimido[4,5-d]pyridazin-4(3H)-ones **5a-i**, **16a-i** (**16e**[32]) and **17a-i** is depicted in Scheme 1 and Scheme 2.

In Scheme 1 isoxazolo[3,4-d]pyridazinones **1a** [33], **1b** and **1c** [34] were treated with benzyl chloride in anhydrous DMF and K_2CO_3 to give the corresponding N-benzyl derivatives **2a**, **2b** [35] and **2c**. On the bicyclic system a condensation with benzaldehyde in MeONa was performed to give rise the styril derivatives **3a-c** which, in turn, were treated with hydrazine hydrate in EtOH to afford the pyrazolo intermediates **4a-c** through reductive isoxazole ring opening and closure to pyrazole at position 5 of the pyridazinone system. To obtain the final tricyclic compounds **5a-i**, the intermediated **4a-c** were then treated with the appropriate anhydride (for 6-methyl and 6-ethyl derivatives **5b,c**, **5e,f**, **5h,i)** or with triethylortoformate, catalytic amount of concentrated sulphuric acid in anhydrous DMF (for 6-unsubstituted **5a**, **5d**, **5g**).

Scheme 2 depicts the synthetic route to introduce different substituents at position 1 of the tricyclic system such as cC_6H_{11}, cC_5H_9 and nC_4H_9. The synthesis of isoxazoles **8a-c** (**8a** [36] and **8b** [33]) and **9a-c** starting from appropriate diketones **6a-c** [37-39] and commercially available chloro(hydroximino)acetate **7** doesn't occur through a regioselective 1,3-dipolar cycloaddition, but led to a mixture of the two isomers **8** and **9**, 4-$COCH_3$ and 4-COR derivatives respectively, which were separated by column chromatography. Each isomer was transformed into the corresponding isoxazolepyridazinone of type **10** or **11** with hydrazine in EtOH and then in the N-

benzylderivative **12** or **13**. All compounds of type **8-13** were purified and completely characterized. However the separation of the two isomers **8** and **9** is very difficult and the yields are so low that limit all the synthetic route. Rather than on the pure isomer, we therefore considered it more convenient to work on the mixture of isomers **8** and **9**, to perform the closure to isoxazolepyridazinones (mixture of **10** and **11**) and to alkylate with benzyl chloride (mixture of **12** and **13**). Between these last isomers, only compounds of type **12** react with benzaldehyde because of the activated methyl group at position 3 [40], affording the stiryl derivatives **14** which are insoluble in the reaction medium and are recovered by suction. Compounds of type **14** then undergo the same sequence of reaction previously described in Scheme 1 to furnish the tricyclic compounds **16 a-i** (**16e** [32]) which were further treated with Lawesson's reagent in toluene to afford the final thioderivatives **17a-i**.

3. Pharmacology

The biological activity of compounds **5**, **16** and **17** towards adenosine receptors was evaluated by radioligand binding assays and functional experiments. Compound affinity towards human A_1, A_{2A} and A_3 adenosine receptors were reported in Tables 1 and 2. The results were obtained by competition binding experiments and expressed as Ki (nM) or inhibition percentage at 10 μM.

In addition, the pharmacological profile of two selected compounds, showing high affinity and selectivity for A_1 adenosine receptors (**16e and 16i**), together with two inactive compounds (**5c and 5f**), was evaluated by functional cAMP assay, as reported in Figure 2.

Finally, the most potent and selective compounds (**16i**) was tested to investigate its potential antiamnesic profile in the presence of amnesia induced by A_1 selective agonist (Table 3).

4. Results and discussion

SAR analyses were performed by considering two groups of compounds which at position 1 present an aromatic substituent (**5a-i**) or a (cyclo)alky group (**16a-i** and **17a-i**).

Starting from the 1-(hetero)aryl derivatives **5a-i** (Table 1) we can observe that the substitution in the lead compound **A** of the pyridine with 4-chlorophenyl or thiophene (2 or 3-thienyl) led to inactive products with a very low inhibition (I% = 8-65 at 10 μM) on all three ARs. The only exception is represented by three compounds belonging to the 1-thienyl series (**5d**, **5g** and **5e**) for which an unexpected and not yet explained activity and selectivity appears toward hA_{2A} subtype. In fact compounds **5d** and **5g** showed a Ki values of 51.0 ± 3.1 and 69.2 ± 4.4 nM respectively for hA_{2A} adenosine receptor and low affinity for hA_1 and hA_3; while compound **5e** showed an affinity towards the hA2A subtype only in the micromolar range.

On the other hand, the replacement of the aromatic at position 1 with a (cyclo)alkyl group (compounds **16a-i,** Table 2) was associated with a remarkable improvement of activity and/or selectivity. The introduction of a cyclohexyl (**16a-c**) furnished hA_1 selective ligands depending on the substituent at position 6. In fact compounds bearing a methyl or an ethyl group (**16b** and **16c**) showed a very good affinity for hA_1 subtype (Ki = 9.5±0.7 and 15.2 ±1.1 nM respectively) together with an excellent selectivity toward hA_{2A} and hA_3. Elimination of the substituent (**16a**) let to a hA_1/hA_3 mixed ligand with micromolar affinity. 1-Cyclopentyl derivatives **16d-f** showed a similar trend, being the unsubstitued term **16d** the less active and no selective (it binds all three subtype receptors with the same affinity), whereas **16e** and **16f** are more potent and selective (Ki= 6.2 ± 0.2 and 3.5 ± 0.4 nM for hA_1 respectively). The introduction at R1 of the linear chain n-butyl afforded the best results since compounds **16g-i** displayed high potency on hA_1 with Ki in the low nanomolar range (Ki=1.9 ± 0.2, 3.4 ± 0.3 and1.4 ± 0.1 nM, respectively) and a good

selectivity versus the other subtypes. The ethyl derivative **16i** is endowed with the best affinity (Ki=1.4 ± 0.1 nM) and selectivity (7% and 20% of inhibition at 10 μM for hA_{2A} and hA_3 respectively).

These data suggest that lipophilicity and a suitable steric hindrance of the groups linked at position 1 are very important requirements for the binding at hA_1 subtype receptor and the n-butyl seems the best among the substituents till now inserted. The appearance of a mild affinity in the 6-unsubstitued derivatives **16a**, **16d** and **16g** toward other subtypes receptor, in particular hA_3, could be the consequence of the lack of interaction of the alkyl group at position 6 at the binding site and a subsequent rearrangement of the molecule at the binding site (data not shown).

With the aim to better define the importance of the carbonyl at position 4, whose role has been previously investigated only in part [28], and taking into account the favorable substitutions of C=O with C=S reported in the literature [41] we synthesized the corresponding 4-thio derivatives of the active **16a-i**. The biological results of the new compounds **17a-i** have provided information not easy to interpret, as reported in Table 2. In fact, in most cases, the transformation in 4-C=S derivatives let to the loss of affinity for hA_1 subtype and only the 6-unsubstituted **17a**, **17d** and **17g**, regardless of the nature of the 1-substituent, retain the activity. The exception to this trend is represented by the 6-ethyl derivative **17f** which maintained affinity for hA_1 (Ki=4.7 ± 0.5 nM). All the active molecules of type **17** showed very good hA_1 selectivity and similarly to the series of 4-C=O, the most potent compound belongs to the 1-butyl derivatives (compound **17g**, Ki = 2.4 ± 0.2 nM). To explain the different trend of activity of the 4-C=O and 4-S=O series, it could be invoked the different steric hindrance of the sulfur-containing molecular part which could adopt different conformations with respect to the oxygen-containing derivatives, such that only the 6-unsubstituted pyrazolopyrimidopyridazin-4(3H)-ones can keep hA_1 affinity (data not shown).

The pharmacological profile of compounds **16e** and **16i**, that showed high A_1 AR affinity and selectivity, in parallel with the inactive compounds **5c** and **5f**, was further investigated by cAMP functional assay (Figure 2). The assay was performed evaluating the effect of the above products on NECA-mediated cAMP inhibition in CHO cells expressing the hA_1 receptor. The results showed that **16e** and **16i** were able to counteract the inhibitory effect of the A_1 adenosine receptor agonist NECA on cAMP production with an IC_{50} value of 5.1 ± 0.2 and 2.9 ± 0.1 nM, respectively, thus demonstrating their antagonist profile. These values are completely in accordance with hA_1 binding affinity values (Ki= 6.2 ± 0.2 and 1.4 ± 0.1 nM respectively) obtained in radioligand binding studies. Viceversa, compounds **5c** and **5f,** were completely inactive in the functional, as well as in the binding, assay confirming they were not able to bind human A_1 AR subtypes.

Finally, according with the antiamnesic activity showed by adenosine A_1 antagonist [14], compound **16i** was investigated in the mouse passive avoidance test to evaluate its antiamnesic profile and the results are showed in Table 3.

The amnesia induced by CPA (1,6 mg/kg i.p.) was prevented, by pretreatment with the A_1 selective antagonist DPCPX (3 mg kg i.p.) and with **16i** at the dose of 10 and 30 mg/kg p.o. whereas the dose of **16i** of 3 mg/kg p.o. was devoid of antiamnesic effect.

The efficacy showed by **16i** is comparable with that exhibited by DPCPX but its potency is about a third lower. Finally the examined compounds reach it maximal antiamnesic effect at the dose of 10 mg/kg p.o. since the dose three times higher is equally active.

It should be noted that all drugs under investigation elicited their modulatory effect on cognitive processes without changing either gross behavior or motor coordination, as revealed by the rota-rod test in which the number of falls in the rota-rod test progressively decreased, since mice learned how to balance on the rotating rod (data not shown). None of the drugs at the employed

doses increased the number of falls from the rotating rod in comparison with saline and CMC-treated mice (data not shown).

5. Conclusions

In conclusion, the present study provided new potent and selective hA_1 adenosine receptor antagonists belonging to the class of pyrazolo[1',5':1,6]pyrimido[4,5-d]pyridazin-4(3H)-ones. The presence of a cyclo/alkyl group at position 1 led to a series of compounds with a remarkable improvement of hA_1 AR affinity and selectivity. Compound **16i** reveals the most interesting of the series for its affinity (hA_1 Ki = 1.4±0.1 nM) and selectivity (7% and 20% of inhibition at 10 μM for hA_{2A} and hA_3 respectively). Thus, the pharmacological profile of this compound as A_1 antagonist was demonstrated by functional assay. Furthermore, the antiamnesic effect of **16i** was evaluated in the mouse passive avoidance test. The administration of acute doses of **16i** exert a pro-cognitive effect by reverting the amnesia induced by the stimulation of A_1 adenosine receptor, in accordance with its A_1 antagonism.

6. Experimental section

6.1. Chemistry

Reagents and starting materials were obtained from commercial sources. Extracts were dried over Na_2SO_4, and the solvents were removed under reduced pressure. All reactions were monitored by thin layer chromatography (TLC) using commercial plates precoated with Merck silica gel 60 F-254. Visualization was performed by UV fluorescence (λ_{max} = 254 nm) or by staining with iodine or potassium permanganate. Chromatographic separations were performed on a silica gel column by gravity chromatography (Kieselgel 40, 0.063-0.200 mm; Merck), flash chromatography (Kieselgel 40, 0.040-0.063 mm; Merck), silica gel preparative TLC (Kieselgel

60 F_{254}, 20 x 20 cm, 2 mm), or a CombiFlash® Rf System (using RediSep® Rf Silica Columns, Teledyne Isco, Lincoln, Nebraska, USA). Yields refer to chromatographically and spectroscopically pure compounds, unless otherwise stated. Compounds were named following IUPAC rules, as applied by Beilstein-Institut AutoNom 2000 (4.01.305) or CA Index Name. All melting points were determined on a microscope hot stage Büchi apparatus and are uncorrected. The identity and purity of intermediates and final compounds was ascertained through ^{1}H NMR and TLC chromatography. ^{1}H NMR and ^{13}C NMR spectra were recorded with Avance 400 instruments (Bruker Biospin Version 002 with SGU). Chemical shifts (δ) are reported in ppm to the nearest 0.01 ppm using solvent as the internal standard. Coupling constants (*J* values) are given in Hz and were calculated using 'TopSpin 1.3' software rounded to the nearest 0.1 Hz. Mass spectra (*m/z*) were recorded on an ESI-TOF mass spectrometer (Bruker Micro TOF), and reported mass values are within the error limits of ± 5 ppm mass units. Microanalyses indicated by the symbols of the elements or functions were performed with a Perkin-Elmer 260 elemental analyzer for C, H, and N, and they were within ± 0.4 % of the theoretical values.

6.1.1. General procedure for 2a and 2c. A suspension of isoxazolopyridazinone **1a**[26] or **1c**[27](1.3 mmol), K_2CO_3 (2.6 mmol) and 1.3-1.7 mmol of benzyl chloride in anhydrous DMF (2.5 mL) was stirred at 80 °C for 2-3 h. After cooling, cold water was added and the precipitate was recovered by suction.

6.1.1.1. 6-Benzyl-4-(4-chlorophenyl)-3-methyl-6H-isoxazolo[3,4-d]pyridazin-7-one [2a]. Yield = 83%; mp = 143-145 °C (EtOH); ^{1}H-NMR ($CDCl_3$) δ 2.55 (s, 3H, CH_3), 5.40 (s, 2H, CH_2Ph), 7.30-7.55 (m, 9H, Ar). ESI-MS calcd. for $C_{19}H_{14}ClN_3O_2$, 351.79; found: *m/z* 352.49 $[M+H]^+$. Anal. $C_{19}H_{14}ClN_3O_2$ (C, H, N).

6.1.2. General procedure for 3a-c. To a suspension of 1 mmol of **2a-c** (**2b**[28]) and 2.3-2.5 mmol of benzaldehyde in anhydrous MeOH (2-3 mL), MeONa (1-1.5 mmol) was added. The mixture was stirred at 60 °C for 1-30 min. After cooling, the precipitate was recovered by suction.

6.1.2.1. 6-Benzyl-4-(4-chlorophenyl)-3-styryl-6H-isoxazolo[3,4-d]pyridazin-7-one [3a]. Yield = 72%; mp = 177-179 °C (EtOH); ^{1}H-NMR ($CDCl_3$) δ 5.40 (s, 2H, *CH*$_2$Ph), 6.80 (d, 1H, *CH*=CH-Ph, J = 16.4 Hz), 7.35-7.60 (m, 14H, Ar), 7.65 (d, 1H, CH=*CH*-Ph, J = 16.2 Hz). ESI-MS calcd. for $C_{26}H_{18}ClN_3O_2$, 439.89; found: *m/z* 440.67 $[M+H]^+$. Anal. $C_{26}H_{18}ClN_3O_2$ (C, H, N).

6.1.3. General procedure for 4a-c. To a suspension of compounds **3a-c** (0.45 mmol) in EtOH (3-4 mL), 5-10 mmol of hydrazine hydrate were added and the mixture was stirred at room temperature for 2-4 h. After concentration under vacuum and addition of cold water (10-15 mL), the final products precipitate and were filtered off by suction.

6.1.3.1. 4-Amino-2-benzyl-6-(4-chlorophenyl)-5-(5-phenyl-2H-pyrazol-3-yl)-2H-pyridazin-3-one [4a]. Yield = 68%; mp = 234-235 °C (EtOH); ^{1}H-NMR ($CDCl_3$) δ 5.40 (s, 2H, *CH*$_2$Ph), 5.70 (s, 1H, Ar), 6.30 (exch br s, 2H, NH_2), 7.35-7.55 (m, 14H, Ar), 8.60 (exch br s, 1H, NH). ESI-MS calcd. for $C_{26}H_{20}ClN_5O$, 453.92; found: *m/z* 454.68 $[M+H]^+$. Anal. $C_{26}H_{20}ClN_5O$ (C, H, N).

6.1.4. General procedure for 5a, 5d and 5g. A mixture of **4a-c** (0.22 mmol), triethylorthoformate (15 mmol), anhydrous DMF (0.5-1 mL) and a catalytic amount of

concentrated sulfuric acid was stirred at room temperature for 1-2 h. After cooling the precipitate was recovered by suction and purified by crystallization with ethanol.

6.1.4.1. 3-Benzyl-1-(4-chlorophenyl)-9-phenylpyrazolo[1',5':1,6]pyrimido[4,5-d]pyridazin-4(3H)-one [5a]. Yield = 78%; mp = 219-220 °C (EtOH); ^{1}H-NMR ($CDCl_3$) δ 5.60 (s, 2H, *CH_2*Ph), 6.20 (s, 1H, Ar), 7.30-7.80 (m, 14H, Ar), 9.45 (s, 1H, Ar). ^{13}C-NMR ($CDCl_3$) δ 158.81, 156.84, 147.81, 145.21, 143.12 , 137.58, 136.01, 135.80, 131.87, 130.85, 129.18, 129.01, 128.74, 128.31, 128.01, 127.83, 125.62, 114.81, 103.81, 56.42. ESI-MS calcd. for $C_{27}H_{18}ClN_5O$, 463.92; found: *m/z* 464.73 $[M+H]^+$. Anal. $C_{27}H_{18}ClN_5O$ (C, H, N).

6.1.5. General procedure for 5b,c, 5e,f and 5h,i. A mixture of 4-amino-pyrazolyl derivatives **4a-c** (0.23 mmol) and the appropriate anhydride (11-15 mmol) was refluxed under stirring for 30 min-2 h. After cooling, the precipitate was recovered by suction. For compound **5b**, cold water was added and the mixture extracted with CH_2Cl_2 (3 x 15 mL). Removal of the solvent afforded compound **5b** which was crystallized from ethanol.

6.1.5.1. 3-Benzyl-1-(4-chlorophenyl)-6-methyl-9-phenylpyrazolo[1',5':1,6]pyrimido[4,5-d]pyridazin-4(3H)-one [5b]. Yield = 82%; mp = 235-236 °C (EtOH); ^{1}H-NMR ($CDCl_3$) δ 3.20 (s, 3H, CH_3), 5.55 (s, 2H, *CH_2*Ph), 6.20 (s, 1H, Ar), 7.30-7.85 (m, 14H, Ar). ^{13}C-NMR ($CDCl_3$) δ 157.21, 154.83, 150.18, 148.21, 144.78, 142.01, 137.33, 136.21, 135.98, 132.01, 131.01, 130.81, 129.10, 128.81, 128.10, 127.81, 127.10, 126.02, 113.98, 102.11, 57.21, 20.98. ESI-MS calcd. for $C_{28}H_{20}ClN_5O$, 477.94; found: *m/z* 478.73 $[M+H]^+$. Anal. $C_{28}H_{20}ClN_5O$ (C, H, N).

6.1.6. General procedure for 8c and 9a-c. To a cooled (0 °C) and stirred solution of EtONa, obtained from Na° (27 mmol) and anhydrous EtOH (80 mL), a solution of appropriate diketone **6a-c**[30-32] (27 mmol) in the same solvent (10-15 mL) was slowly added. Then the suspension was further cooled to -5 °C and a solution of ethyl chloro(hydroximino)acetate **7** (18 mmol) in anhydrous EtOH (20-25 mL) was added dropwise. Then the mixture was neutralized with 6N HCl, and the solvent was evaporated *in vacuo*. The residue oil was washed first with cold 0.5 N NaOH and then with cold water, and finally it was extracted with CH_2Cl_2 (3 x 20 mL). Evaporation of the solvent afforded a mixture of isomers 8 and 9 which were purified by column chromatography, using cyclohexane/ethyl acetate 4:1 as eluent, for compound **8c** and **9c** and cyclohexane/ethyl acetate 9:1 for compound **9a** and **9b**.

6.1.6.1. 5-Methyl-4-pentanoylisoxazole-3-carboxylic acid ethyl ester [8c]. Yield = 30%; oil; ^{1}H-NMR ($CDCl_3$) δ 0.90 (t, 3H, $(CH_2)_3CH_3$, J = 7.2 Hz), 1.35 (m, 2H, $(CH_2)_2CH_2CH_3$), 1,40 (t, 3H, $COOCH_2CH_3$, J = 7.2 Hz), 1.65 (m, 2H, $CH_2CH_2CH_2CH_3$), 2.65 (s, 3H, CH_3), 2.80 (t, 2H, $CH_2CH_2CH_2CH_3$, J = 7.2 Hz), 4.45 (q, 2H, $COOCH_2CH_3$, J = 7.2 Hz). ESI-MS calcd. for $C_{12}H_{17}NO_4$, 239.27; found: *m/z* 240.08 $[M+H]^+$. Anal. $C_{12}H_{17}NO_4$ (C, H, N).

6.1.7. General procedure for 10a,c and 11a-c. To a solution of the pure isoxazole **8** or **9** (**8a,b**[29,25] si lascia o si toglie avendo levato 8a-c) (2 mmol) in EtOH (2-4 mL), hydrazine hydrate (3-4 mmol) was added. The mixture was stirred at room temperature for 1-2 h, concentrated under vacuum and added of 10-15 mL of cold water to afford the solid final compounds **10a,c** and **11a-c** which were filtered off by suction.

6.1.7.1. 4-Cyclohexyl-3-methyl-6H-isoxazolo[3,4-d]pyridazin-7-one [10a]. Yield = 73%; mp = 168-171 °C (EtOH); ^{1}H-NMR ($CDCl_3$) δ 1.35-1.50 (m, 3H, cC_6H_{11}), 1.60 (m, 3H, cC_6H_{11}), 1.80 (m, 1H, cC_6H_{11}), 1.90-2.00 (m, 3H, cC_6H_{11}), 2.80 (m, 1H, cC_6H_{11}), 2.90 (s, 3H, 3-CH_3), 9.40 (exch br s, 1H, NH). ESI-MS calcd. for $C_{12}H_{15}N_3O_2$, 233.27; found: *m/z* 234.12 $[M+H]^+$. Anal. $C_{12}H_{15}N_3O_2$ (C, H, N).

6.1.8. General procedure for 12a-c and 13a-c. Compounds **12a-c** and **13a-c** were obtained following the procedure reported for compounds **2a** and **2c** starting from isoxazolopyridazinones **10a-c** (**10b**[28]) and **11a-c**. After cooling, cold water was added and the suspension was extracted with CH_2Cl_2 (3 x 15 mL). Removal of the solvent *in vacuo*, gave rise the final solid compounds.

6.1.8.1. 6-Benzyl-4-cyclohexyl-3-methyl-6H-isoxazolo[3,4-d]pyridazin-7-one [12a]. Yield = 83%; mp = 140-143 °C (EtOH); ^{1}H-NMR ($CDCl_3$) δ 1.25-1.45 (m, 4H, cC_6H_{11}), 1.55-1.65 (m, 1H, cC_6H_{11}), 1.75-1.85 (m, 1H, cC_6H_{11}), 1.90-2.00 (m, 4H, cC_6H_{11}), 2.70-2.80 (m, 1H, cC_6H_{11}), 2.85 (s, 3H, 3-CH_3), 5.30 (s, 2H, *CH_2*Ph), 7.25-7.35 (m, 3H, Ar), 7.50 (d, 2H, Ar). ESI-MS calcd. for $C_{19}H_{21}N_3O_2$, 323.39; found: *m/z* 324.18 $[M+H]^+$. Anal. $C_{19}H_{21}N_3O_2$ (C, H, N).

6.1.9. General procedure for 14a-c. Compounds **14a-c** were obtained following the same general procedure described for **3a-c**, starting from isoxazolopyridazinones **12a-c**.

6.1.9.1. 6-Benzyl-4-cyclohexyl-3-styryl-6H-isoxazolo[3,4-d]pyridazin-7-one [14a]. Yield = 72%; mp = 156-157 °C (MeOH); ^{1}H-NMR ($CDCl_3$) δ 1.30-1.70 (m, 5H, cC_6H_{11}), 1.80-1.90 (m, 1H, cC_6H_{11}), 1.95-2.10 (m, 4H, cC_6H_{11}), 2.80-2.90 (m, 1H, cC_6H_{11}), 5.30 (s, 2H, *CH_2*Ph), 7.15 (d, 1H, *CH*=CH-Ph, J = 16.2 Hz), 7.25-7.60 (m, 10H, Ar), 7.80 (d, 1H, CH=*CH*-Ph, J = 16.4

Hz). ESI-MS calcd. for $C_{26}H_{25}N_3O_2$, 411.50; found: *m/z* 412.28 $[M+H]^+$. Anal. $C_{26}H_{25}N_3O_2$ (C, H, N).

6.1.10. Alternative and more convenient synthesis of 14a-c. To a cooled (0 °C) and stirred solution of sodium ethoxide, obtained from sodium (54 mmol) and anhydrous EtOH (150 mL), a solution of the appropriate diketone **6a-c**[30-32] (54 mmol) in the same solvent (25 mL) was slowly added. Then the suspension was further cooled to -5 °C and a solution of ethyl chloro(hydroximino)acetate **7** (36 mmol) in anhydrous EtOH (40 mL) was added dropwise. Then the mixture was neutralized with 6N HCl, and the solvent was evaporated *in vacuo*. The residue oil was washed with cold 0.5 N NaOH and with cold water, and finally extracted with CH_2Cl_2 (3 x 40 mL). The solvent was evaporated, the crude oil (mixture of the isomers **8** and **9**) dissolved in 40 mL of EtOH, hydrazine hydrate (100 mmol) was added and the mixture stirred at room temperature for 1-2 h. EtOH was eliminated in vacuo and the addition of 180 mL of cold water afforded a suspension (a mixture of the isomers **10** and **11**) which was extracted with CH_2Cl_2 (3 x 30 mL). The organic layer was then evaporated and to the residue oil dissolved in anhydrous DMF (50 mL), was added with K_2CO_3 (100 mmol) and benzyl chloride (60 mmol) and the mixture was reacted for 2-3 h at 80°C. After cooling, cold water was added, the suspension was extracted with CH_2Cl_2 (3 x 30 mL) and after removal of the solvent a crude solid was recovered. In the same flask containing the solid, which is the mixture of the isomers **12** and **13**, benzaldehyde (100 mmol), MeONa (60 mmol) and anhydrous MeOH (30 mL) were added and the mixture was heated at 60°C under stirring for 1-30 min. After cooling, the precipitate (compounds of type **14**) was recovered by suction.

6.1.11. General procedure for 15a-c. Compounds **15a-c** were obtained, starting from **14a-c**, following the general procedure described for compounds **4a-c**. For compound **15b**, after addition of cold water the mixture was extracted with ethyl acetate (3 x 15 mL) and the solvent was evaporated under vacuum to afford the final compound.

6.1.11.1. 4-Amino-2-benzyl-6-cyclohexyl-5-(5-phenyl-2H-pyrazol-3-yl)-2H-pyridazin-3-one [15a]. Yield = 96%; mp = 235-238 °C dec. (Cyclohexane); ^{1}H-NMR (DMSO-d_6) δ 0.90-1.90 (m, 10H, cC_6H_{11}), 2.90-3.00 (m, 1H, cC_6H_{11}), 5.25 (s, 2H, *CH*$_2$Ph), 6.15 (s, 1H, Ar), 6.80 (m, 2H, Ar), 7.20-7.55 (m, 6H, Ar), 7.80-7.90 (m, 2H, Ar), 12.90 (exch br s, 1H, NH), 13.60 (exch br s, 2H, NH_2). ESI-MS calcd. for $C_{26}H_{27}N_5O$, 425.53; found: *m/z* 426.29 $[M+H]^+$. Anal. $C_{26}H_{27}N_5O$ (C, H, N).

6.1.12. General procedure for 16a, 16d and 16g. Compounds **16a**, **16d** and **16g** were obtained starting from **15a-c** following the same procedure described for **5a**, **5d** and **5g.**

6.1.12.1. 3-Benzyl-1-cyclohexyl-9-phenylpyrazolo[1',5':1,6]pyrimido[4,5-d]pyridazin-4(3H)-one [16a]. Yield = 98%; mp = 209-213 °C (EtOH); ^{1}H-NMR ($CDCl_3$) δ 1.30-1.75 (m, 5H, cC_6H_{11}), 1.85-1.95 (m, 1H, cC_6H_{11}), 2.00-2.15 (m, 4H, cC_6H_{11}), 3.15-3.25 (m, 1H, cC_6H_{11}), 5.50 (s, 2H, *CH*$_2$Ph), 7.25-7.40 (m, 4H, Ar), 7.50-7.60 (m, 5H, Ar), 8.05 (d, 2H, Ar), 9.45 (s, 1H, Ar). ^{13}C-NMR ($CDCl_3$) δ 157.63, 157.22, 146.74, 142.88, 136.34, 136.04, 131.00, 130.10. 129.22, 129.13, 128.49, 127.90, 126.85, 118.53, 99.86, 55.42, 42.12, 31.26, 26.63, 25.96. ESI-MS calcd. for $C_{27}H_{25}N_5O$, 435.52; found: *m/z* 436.20 $[M+H]^+$. Anal. $C_{27}H_{25}N_5O$ (C, H, N).

6.1.13. General procedure for 16b,c 16f and 16h,i. Compounds **16b,c, 16f** and **16h,i** were obtained starting from **15a-c** following the general procedure described for **5b,c**, **5e,f** and **5h,i.** For compounds **16h** and **16i** a catalytic amount of concentrated sulfuric acid was added to the reaction mixture and the reaction was carried out at room temperature for 2h. After cooling the crude precipitate was recovered by suction. Compound **16c** was extracted with CH_2Cl_2 (3 x 15 mL) from reaction mixture.

6.1.14. General procedure for 17a-i. A mixture of appropriate pyrazolopyrimidopyridazinone **16a-i** (0.3 mmol) and Lawesson's reagent (0.6 mmol) in toluene (6.5 mmol) was heated at 140 °C for 1-5 h. After cooling the solid was recovered by suction and recrystallized from EtOH.

6.1.14.1. 3-Benzyl-1-cyclohexyl-9-phenylpyrazolo[1',5':1,6]pyrimido[4,5-d]pyridazin-4(3H) -thione [17a]. Yield = 23%; mp = 258-262 °C dec. (EtOH); ^{1}H-NMR ($CDCl_3$) δ 1.40-1.80 (m, 4H, cC_6H_{11}), 1.90-2.20 (m, 6H, cC_6H_{11}), 3.20-3.40 (m, 1H, cC_6H_{11}), 6.10 (s, 2H, *CH_2*Ph), 7.25-7.40 (m, 3H, Ar), 7.50-7.65 (m, 6H, Ar), 8.05 (d, 2H, Ar), 9.50 (s, 1H, Ar). ^{13}C-NMR ($CDCl_3$) δ 173.54, 157.19, 145.94, 142.41, 136.95, 135.84, 130.80, 130.7. 129.02, 128.98, 128.17, 127.57, 127.02, 119.02, 100.15, 55.33, 41.72, 31.24, 26.58, 25.92. ESI-MS calcd. for $C_{27}H_{25}N_5S$, 451.59; found: *m/z* 4452.38 $[M+H]^+$. Anal. $C_{27}H_{25}N_5S$ (C, H, N).

6.2. Biological Assays

6.2.1 Adenosine Receptor Binding Assay.

Radioligand binding competition assays were performed using A_1, A_{2A}, and A_3 human receptors expressed in transfected CHO cells, kindly supplied by Prof. K.N. Klotz, Wurzburg University,

Germany [42,43]. [^{3}H]DPCPX, [^{3}H]NECA, and [^{125}I]AB-MECA 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.

6.2.1.1 Human A_1 Adenosine Receptors.

Aliquots of cell membranes (30 µg proteins) were incubated at 25 °C for 180 min in 500 µL of T1 buffer (50 mM Tris-HCl, 2 mM MgCl2, 2 units/mL ADA, pH 7.4) containing [^{3}H]DPCPX (3 nM, DuPont-NEN, Boston, MA) and six different concentrations of the newly synthesized compounds. Non-specific binding was determined in the presence of 50 µM R-PIA [44]. The dissociation constant (Kd) of [^{3}H]DPCPX in hA_1 CHO cell membranes was 3 nM.

6.2.1.2. Human A_{2A} Adenosine Receptors.

Aliquots of cell membranes (30 µg) were incubated at 25 °C for 90 min in 500 µL of T2 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 newly synthesized compounds. Non-specific binding was determined in the presence of 100 µM R-PIA [44]. The dissociation constant (Kd) of [^{3}H]NECA in hA_{2A} CHO cell membranes was 30 nM.

6.2.1.3. Human A_3 Adenosine Receptors.

Aliquots of cell membranes (30 µg) were incubated at 25 °C for 90 min in 100 µL of T3 buffer (50 mM Tris-HCl, 10 mM $MgCl_2$, 1 mM EDTA, 2 units/mL ADA, pH 7.4) in the presence of 1.4 nM [^{125}I]AB-MECA and six different concentrations of the newly synthesized compounds. Non-specific binding was determined in the presence of 50 µM R-PIA [44]. The dissociation constant (Kd) of [^{125}I]AB-MECA in hA_3 CHO cell membranes was 1.4 nM.

All compounds were 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 radiolabelled ligand binding at 1-10 μM concentration are means ± SEM of at least three determinations.

6.2.2. A_1 adenosine receptor functional assay.

Intracellular cyclic AMP (cAMP) levels were measured using a competitive protein binding method [44]. CHO cells, expressing recombinant human ARs, were harvested by trypsinization. After centrifugation and re-suspension in medium, cells (~ 30000) 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 Dulbecco's Modified Eagle Medium (DMEM) in the presence of adenosine deaminase (ADA) (1U/mL) and the phosphodiesterase inhibitor Ro20-1724 (20 μM). The antagonist profile of the compounds towards A_1 ARs was evaluated by assessing their ability to counteract NECA-mediated inhibition of cAMP accumulation in the presence of 1 μM forskolin, as non-selective adenylate cyclase (AC) activator. Cells were incubated in the reaction medium (15 min at 37 °C) with different concentrations of target compounds (0.1 nM- 1μM) and then were treated with the agonist [43].

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 (0-160 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.

6.2.3. In vivo studies

Animal handling was carried out according to the European Community guidelines for animal care (DL 116/92, application of the European Communities Council Directive 86/609/EEC). The ethical policy of the University of Florence conforms with the Guide for the Care and Use of Laboratory Animals of the U.S. National Institutes of Health (NIH Publication No. 85-23, revised 1996; University of Florence assurance number: A5278-01). Formal approval to conduct the experiments described herein was obtained from the animal subjects review board of the University of Florence. For the experiment described male Swiss albino mice (23-25 g) were used. The animals were fed with a standard laboratory diet and tap water ad libitum, and kept at 23 ± 1 °C with a 12 h light/dark cycle, light on at 7 a.m.

6.2.3.1. Drugs

CPA and DCPCX were dissolved in isotonic (NaCl 0.9%) saline solution while AT-11 in carboxymethylcellulose (CMC) and administered by gavage.

6.2.3.2. Passive-avoidance test

The test was performed according to the step-through method described by Jarvik and Kopp [46] with modifications. The apparatus consisted of a two-compartment acrylic box with a lighted compartment connected to a darkened one by a guillotine door. In the original method mice received a punishing electrical shock as soon as they entered the dark compartment, while in our

modified method, mice, after their entry into the dark compartment, received a punishment consisting of a fall into a cold water bath (10°C). For this purpose the dark chamber was constructed with a pitfall door. The latency times for entering the dark compartment were measured in the training test and after 24 h in the retention test. The maximum entry latency allowed in the retention session was 120 s.

6.2.43.3. Rota-rod test

The apparatus consisted of a base platform and a rotating rod of 3 cm diameter with a non-slippery surface. The rod was placed at a height of 15 cm from the base. The rod, 30 cm in length, was divided into 5 equal sections by 6 disks. Thus, up to 5 mice were tested simultaneously on the apparatus, with a rod-rotating speed of 16 r.p.m. The integrity of motor coordination was assessed on the basis of the number of falls from the rod in 30 s according to Vaught et al. [47]. Those mice scoring less than 3 and more than 6 falls in the pretest were rejected (20%).

6.2.4. Data analysis

All binding and functional data were analyzed using the non-linear regression curve fitting program GraphPad, version 5.0. IC_{50} and Ki values were directly obtained from the dose response curves. All values obtained by in vitro assays are the mean ± S.E.M of at least three different experiments, each performed in duplicate.

Results obtained by in vivo studies were expressed as the means ± S.E.M. and an analysis of variance was performed by ANOVA. A Fisher's protected least significant difference procedure was used as post-hoc comparison. Each value represents the mean of 12 mice. P values of less

than 0.05 or 0.01 were considered significant. Data were analyzed using the "Origin 7.5" software.

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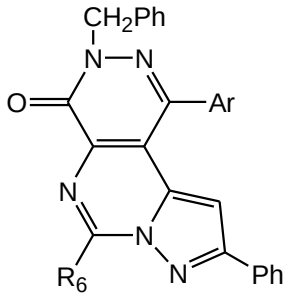
Table 1. Binding activity at human A_1, A_{2A} and A_3 ARs of compounds **5a-i**

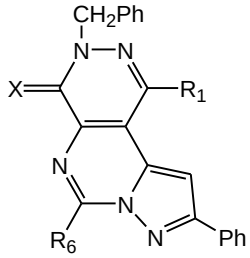


5a-i

Comp.	Ar	R_6	**hA_1** [a,b] Ki (nM) or % inhib at 10 μM	**hA_{2A}** [a,c] Ki (nM) or % inhib at 10 μM	**hA_3** [a,d] Ki (nM) or % inhib at 10 μM
5a	4-Cl-Ph	H	26%	56%	31%
5b	4-Cl-Ph	CH_3	24%	28%	25%
5c	4-Cl-Ph	CH_2CH_3	35%	10%	51%
5d	2-Thienyl	H	45%	51.0±3.1	16%
5e	2-Thienyl	CH_3	21%	2595.4±160.2	15%
5f	2-Thienyl	CH_2CH_3	16%	65%	30%
5g	3-Thienyl	H	26%	69.2±4.4	24%
5h	3-Thienyl	CH_3	8%	57%	37%
5i	3-Thienyl	CH_2CH_3	5%	41%	36%
A	3-Pyridyl	H	11.4±2.2	805.1±374.5	21%
DPCPX			3.2±0.2 [b]	260.3±18.2 [b]	-

[a] Values are means ± S.E.M of three separate experiments, each performed in duplicate.
[b] Displacement of [^{3}H]DPCPX binding in A_1 CHO cells membranes.
[c] Displacement of [^{3}H]NECA binding in A_{2A} CHO cells membranes.
[d] Displacement of [^{125}I]AB-MECA binding in A_3 CHO cells membranes.

Table 2. Binding activity at human A_1, A_{2A} and A_3 adenosine receptors of compounds **16a-i** and **17a-i**



16a-i, 17a-i

Comp.	R_1	R_6	X	**hA_1** [a,b] Ki (nM) or % inhib at 10 μM	**hA_{2A}** [a,c] Ki (nM) or % inhib at 10 μM	**hA_3** [a,d] Ki (nM) or % inhib at 10 μM
16a	cC_6H_{11}	H	O	172.5±15.2	56%	308.4±30.2
16b	cC_6H_{11}	CH_3	O	9.5±0.7	7%	35%
16c	cC_6H_{11}	CH_2CH_3	O	15.2±1.1	0%	40%
16d	cC_5H_9	H	O	32.3±3.1	75.5±7.9	64.0±2.7
16e[25]	cC_5H_9	CH_3	O	6.2±0.2	53%	41%
16f	cC_5H_9	CH_2CH_3	O	3.5±0.4	662.4±60.8	43%
16g	nC_4H_9	H	O	1.9±0.2	44%	193.2±1.4
16h	nC_4H_9	CH_3	O	3.4±0.3	46%	5%
16i	nC_4H_9	CH_2CH_3	O	1.4±0.1	7%	20%
17a	cC_6H_{11}	H	S	9.5±0.9	8%	31%
17b	cC_6H_{11}	CH_3	S	33%	0%	30%
17c	cC_6H_{11}	CH_2CH_3	S	31%	0%	42%
17d	cC_5H_9	H	S	12.9±1.2	37%	54%
17e	cC_5H_9	CH_3	S	51%	18%	13%
17f	cC_5H_9	CH_2CH_3	S	4.7±0.5	35%	24%
17g	nC_4H_9	H	S	2.4±0.2	34%	43%
17h	nC_4H_9	CH_3	S	50%	0%	16%
17i	nC_4H_9	CH_2CH_3	S	61%	3%	37%
A	3-Pyridyl	H	O	11.4±2.2	805.1±374.5	21%
DPCPX				3.2±0.2[b]	260.3±18.2[b]	-

[a] Values are means ± S.E.M. of three separate experiments, each performed in duplicate.
[b] Displacement of [^{3}H]DPCPX binding in A_1 CHO cells membranes.
[c] Displacement of [^{3}H]NECA binding in A_{2A} CHO cells membranes.
[d] Displacement of [^{125}I]AB-MECA binding in A_3 CHO cells membranes.

Table 3. Effect of **16i** in comparison with DPCPX on amnesia induced by N6-cyclopentyladenosine (CPA) in the mouse passive avoidance test

Treatment[a]	**Training session**[b]	**Retention session**[b]
CMC+SALINE	15.9 ± 3.5	97.2 ± 9.5
CMC + CPA	16.6 ± 4.1	61.2 ± 9.0*
16i 3 mg/kg + CPA	16.8 ± 4.3	56.7 ± 9.5*
16i 10 mg/kg + CPA	13.7 ± 3.9	96.1 ± 8.2§
16i 30 mg/kg + CPA	19.4 ± 3.8	88.2 ± 9.4§
DPCPX mg/kg + CPA	16.5 ± 4.2	91.9 ± 11.3§

[a] **16i** (3-30 mg/kg i.p.) and DPCPX (3 mg/kg i.p.) were administered 30 min before training session while CPA (1.6 mg/kg i.p.) was given immediately after punishment.
[b] Each value represents the mean of 10 - 12 mice. * $P < 0.01$ versus CMC-Saline treated group; §$P < 0.01$ versus CMC+CPA treated group.

Captions

Scheme 1. Synthesis of pyrazolo[1',5':1:6]pyrimido[4,5-d]pyridazin4(3H)-ones **5a-i**

Reagents and conditions: (a) $PhCH_2Cl$, anhydrous DMF, anhydrous K_2CO_3, 80 °C, 2-3 h; (b) PhCHO, MeONa, anhydrous MeOH, reflux, 1-30 min; (c) NH_2NH_2, EtOH, rt, 2-4h; (d) for R = H: $CH(OC_2H_5)_3$, H_2SO_4 conc., anhydrous DMF, reflux, 30 min-2 h; for R = CH_3 or C_2H_5 appropriate anhydride, reflux, 30 min-2 h.

Scheme 2. Synthesis of pyrazolo[1',5':1:6]pyrimido[4,5-d]pyridazin-4(3H)-ones **16a-i** and pyrazolo[1',5':1:6]pyrimido[4,5-d]pyridazin-4(3H)-thiones **17a-i**

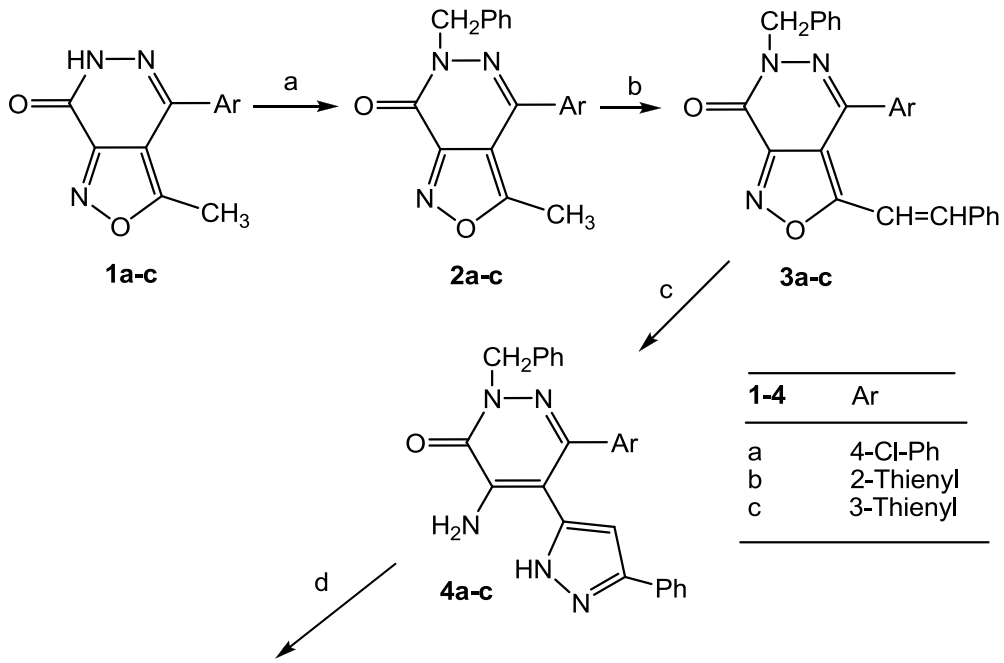
Reagents and conditions: (a) EtONa, anhydrous EtOH, 0 °C; (b) NH_2NH_2, EtOH, rt, 1-2 h; (c) $PhCH_2Br$, anhydrous DMF, anhydrous K_2CO_3, 80 °C, 2-3 h; (d) PhCHO, MeONa, anhydrous MeOH, reflux, 1-30 min; (e) NH_2NH_2, MeOH, rt, 2-4h; (f) for R = H: $CH(OC_2H_5)_3$, H_2SO_4 conc., anhydrous DMF, reflux, 30min-2h; for R = CH_3 or C_2H_5 appropriate anhydride, reflux, 30 min-2h; (g) Lawesson's reagent, toluene, 140 °C, 1-5h.

Figure Legend

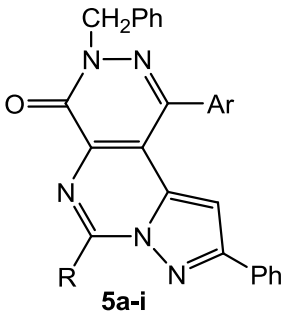
Figure 1. Selective A_1R antagonists and reference compound A

Figure 2. Effects of **16e, 16i, 5c and 5f** on NECA-mediated inhibition of cAMP accumulation in human A_1 AR-transfected CHO cells. CHO cells were treated with 1 μM forskolin and 100 nM NECA in the absence or presence of different concentrations of the compounds (1 nM-10 μM). After 15 min incubation, the reaction was stopped and the intracellular cAMP levels were quantified. The data are expressed as the percentage of the cAMP intracellular levels with respect to forskolin, which was set to 100%, and represent the mean ± SEM of at least three different experiments. Each experiments was performed in duplicate.

Scheme 1

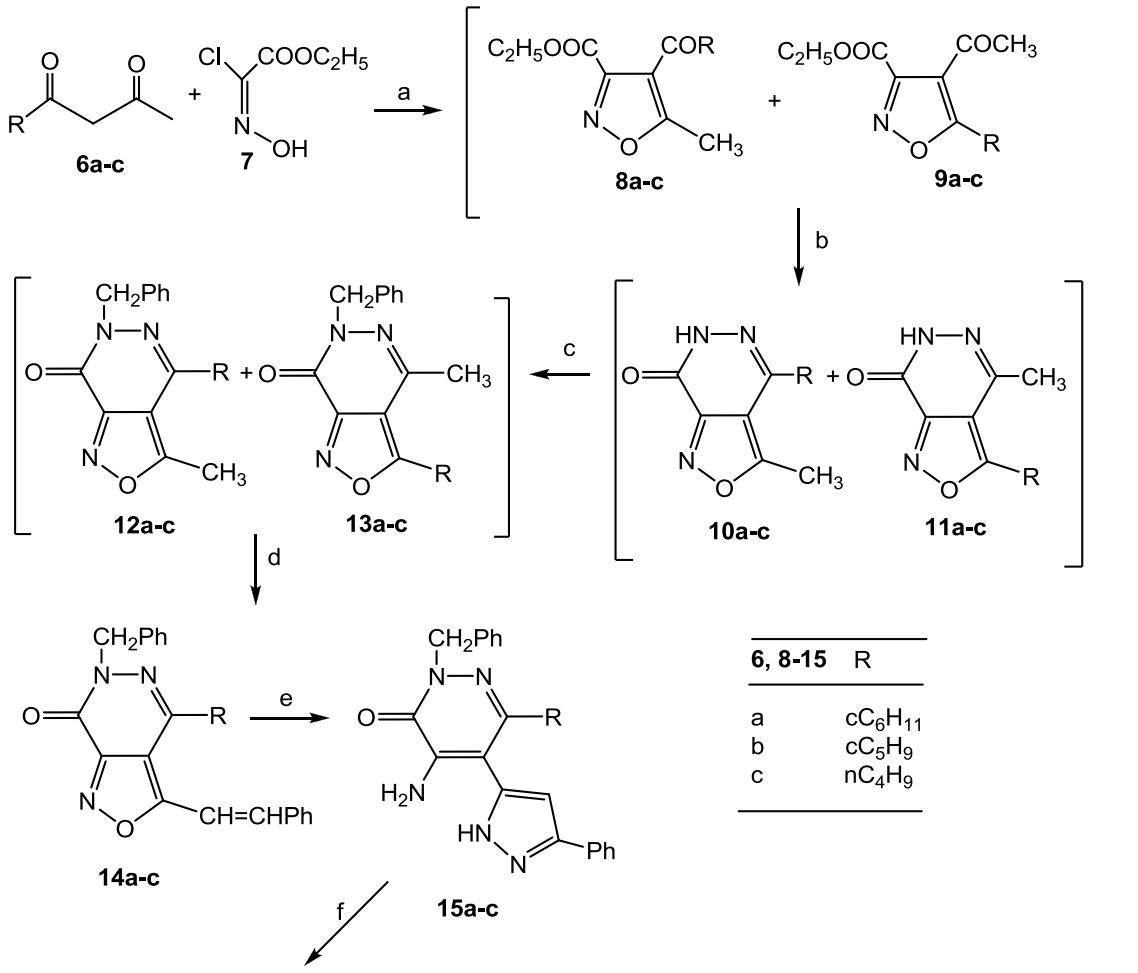


1-4	Ar
a	4-Cl-Ph
b	2-Thienyl
c	3-Thienyl



5	Ar	R
a	4-Cl-Ph	H
b	4-Cl-Ph	CH_3
c	4-Cl-Ph	CH_2CH_3
d	2-Thienyl	H
e	2-Thienyl	CH_3
f	2-Thienyl	CH_2CH_3
g	3-Thienyl	H
h	3-Thienyl	CH_3
i	3-Thienyl	CH_2CH_3

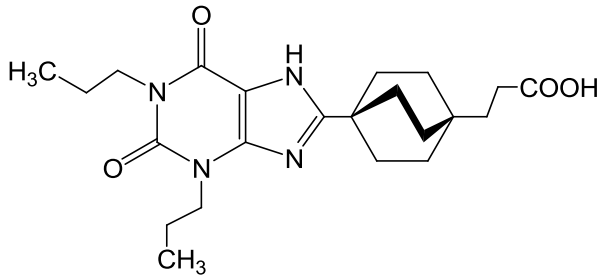
Scheme 2



6, 8-15	R
a	cC_6H_{11}
b	cC_5H_9
c	nC_4H_9

16-17	R_1	R_6
a	cC_6H_{11}	H
b	cC_6H_{11}	CH_3
c	cC_6H_{11}	CH_2CH_3
d	cC_5H_9	H
e	cC_5H_9	CH_3
f	cC_5H_9	CH_2CH_3
g	nC_4H_9	H
h	nC_4H_9	CH_3
i	nC_4H_9	CH_2CH_3

Figure 1



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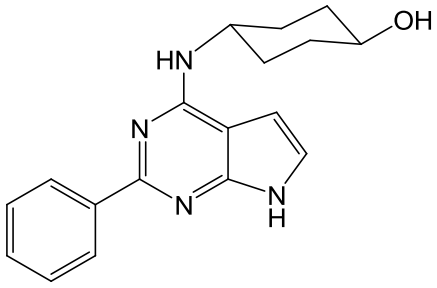

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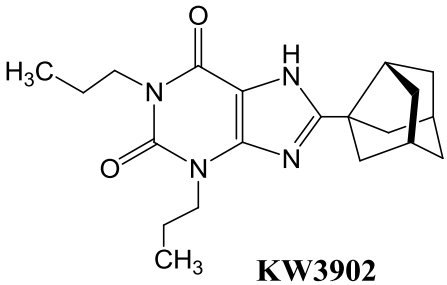

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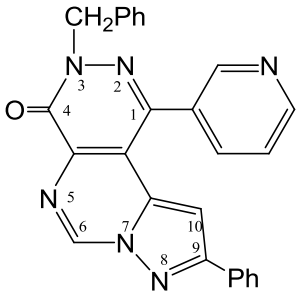
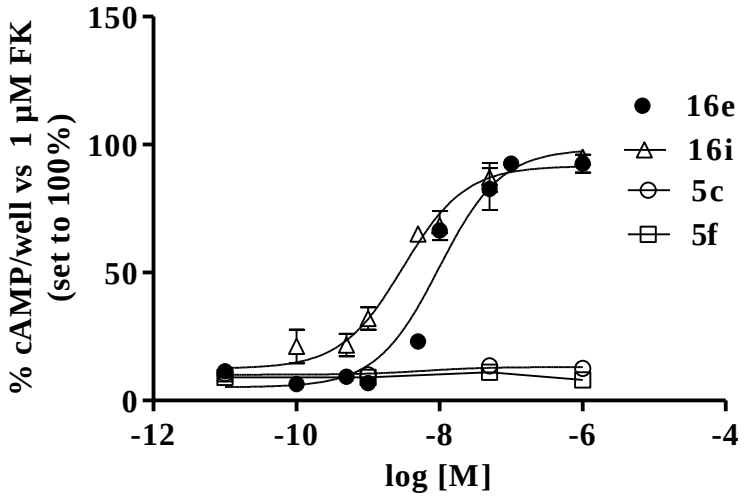


A

hA_1 = 11.4 nM
hA_{2A} = 805.1 nM
hA_3 = 21% (10 mM)

Figure 2


% cAMP/well vs 1 µM FK
(set to 100%)
150
100
50
0
-12
-10
-8
-6
-4
log [M]
16e
16i
5c
5f

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