Scientific paper

New Pyrazolo[1',5':1,6]pyrimido[4,5-d]pyridazin-4 (3H)-ones Fluoroderivatives as Human A_1 Adenosine Receptor Ligands

Alessia Graziano,[1] Maria Paola Giovannoni,[1,*] Agostino Cilibrizzi,[1] Letizia Crocetti,[1] Vittorio Dal Piaz,[1] Claudia Vergelli,[1] Maria Letizia Trincavelli,[2] Claudia Martini[2] and Chiara Giacomelli[2]

[1] *Dipartimento di Scienze Farmaceutiche, via Ugo Schiff 6, 50019 Sesto Fiorentino Firenze, Italy*

[2] *Dipartimento di Psichiatria, Neurobiologia, Farmacologia e Biotecnologie, Via Bonanno 6, 56126 Pisa, Università di Pisa*

** Corresponding author: E-mail: mariapaola.giovannoni@unifi.it*
Tel +39-055-4573682; Fax +39-055-4573780

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Abstract

In this paper we report the synthesis and biological evaluation of a new series of pyrazolo[1',5':1,6]pyrimido[4,5-d]pyridazin-4(3H)-ones as human A_1 adenosine receptor ligands. The tricyclic scaffold was modified at position 6 and 9 by introducing small alkyl chains and substituted phenyls. The most interesting compounds showed Ki for A_1 in the submicromolar range (0.105–0.244 μM) and the most interesting term (compound **4c**) combined an appreciable affinity for A_1 (Ki = 0.132 μM) with a good selectivity toward A_{2A} (43% inhibition at 10 μM) and A_3 (46% inhibition at 10 μM).

Keywords: Adenosine receptors, A_1 subtype, Ligands, Pyrazolo[1',5':1,6]pyrimido[4,5-d]pyridazin-4(3H)-ones.

1. Introduction

For several years, adenosine receptors have been classified in four different subtypes, A_1, A_{2A}, A_{2B} and A_3[1]. All these receptors have been cloned from several species and have been demonstrated to differ in their primary sequence, pharmacological effects, tissue distribution and coupling to different G proteins. A_1 and A_3 subtypes, other that modulate calcium levels through a Gq proteins, are coupled to Gi proteins to inhibit adenyl cyclase. On the contrary, A_{2A} and A_{2B} receptors are primary coupled to Gs proteins and activate adenylyl cyclase causing, in turn, an increase in intracellular cAMP production.[2,3] The endogenous ligand, adenosine, interacts with all the receptor subtypes with different affinity and may elicit different effects at level of second messengers. The final effect induced by adenosine may differ in physiological and pathological conditions during which the expression levels of each subtypes are regulated and obviously these effects depend on the relative abundance of each receptor subtypes in specific tissues.

Ligand-binding properties of each adenosine receptor are primarily dictated by amino acids in the transmembrane domains of the receptors. Studies have identified certain amino acids conserved amongst adenosine receptor subtypes that are critical for ligand recognition, as well as additional residues that may differentiate between agonist and antagonist ligands and between the different receptor subtypes.[4]

The potential therapeutic applications of compounds able to bind these receptors have been investigated in recent years.[5,6] In particular antagonists for the A_1 receptor subtype may be useful for the treatment of central nervous system pathologies such as Alzheimer's and Parkinson's diseases,[7] for the treatment of congestive heart failure due to their diuretic and positive inotropic effects[2,8] and for the treatment of asthma since adenosine mediates bronchoconstriction and inflammation in the lung.[9,10]

A large number of nitrogen-containing polyheterocycles as pyrido[2,3-d]pyrimidinediones[11] (**A**), pyrimido[4,5-b]indole (**B**),[12] and triazinobenzimidazolones (**C**)[13] have been reported in literature as A_1R antagonists (Figure 1).

In a recent paper[14] we reported the synthesis and binding activities at human cloned A_1, A_{2A}, A_{2B} and A_3 adenosine receptors of a new series of compounds with pyrazolo[1',5':1,6]pyrimido[4,5-d]pyridazin-4(3H)-ones scaffold (compound **D**, Figure 1). Some of these compounds showed a good activity for hA_1 adenosine subtypes, with values of Ki in the submicromolar range and a good selectivity versus other adenosine receptor subtypes[14].

Selecting as lead the fluoroderivative **4a** (Ki = 0.252 μM) from the previous series, we report here the results of further modifications on the above scaffold at the level of positions 6 and 9 by maintaining the benzyl group unchanged at 3 necessary for A_1 affinity and 4-F-phenyl at position 1.

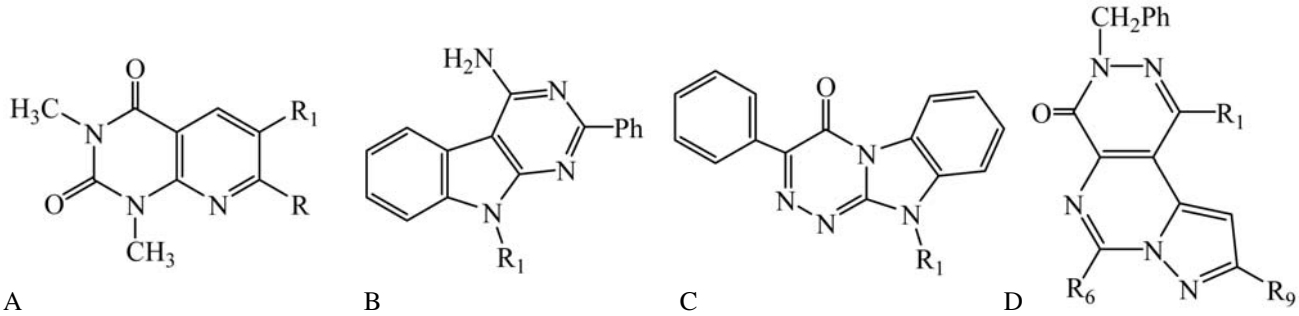


Figure 1: A_1 receptor antagonists

2. Results and Discussion

2. 1. Chemistry

The final compounds **4-7 (4a**[14]**)** were prepared following a general synthetic procedure previously described by us[15] (Scheme 1).

Isoxazolo[3,4-d]-pyridazin-7(6H)-one **1**[14] was condensed with the appropriate arylaldehydes (or N,N-dimethylformammide dimethyl acetal for compound **2g**) to give the vinyl derivatives **2a-g** (**2a**[14]) which treated with hydrazine hydrate furnished intermediates **3a-g** (**3a**[14]) through the isoxazole opening and the following closure to pyrazole[16]. Finally the cyclization to pyrazolo[1',5':1,6]pyrimido[4,5-d]pyridazin-4(3H)-one was carried out in different conditions depending on the substituent at position 6.

For the 6-unsubstituted **4a-f,** the ring closure was performed with triethylorthoformate in anhydrous DMF in the presence of catalytic amount of concentrated sulfuric acid at room temperature, whereas compounds **6a-c** were obtained with the opportune anhydride under refluxing conditions. Compound **7** was synthesized starting from 4-amino-5-pyrazolyl derivative **3a**[14] for treatment with levulinic acid and in the presence of 4-(dimethylamino)pyridine and of 1-[3-(dimethylamino)propyl]-ethylcarbodiimide hydrochloride as coupling agent. Finally, compound **5** was obtained starting from **4c** by reduction of the NO_2 group with $SnCl_2$.

Scheme 2 depicts the synthesis of the final compound **10** which was obtained starting from the previously described[14] pyrazolopyrimido[4,5-d]pyridazinone **8**, through transformation into the corresponding 4-thione derivative **9** with Lawesson's reagent followed by alkylation at position 4 with benzyl chloride in standard conditions.

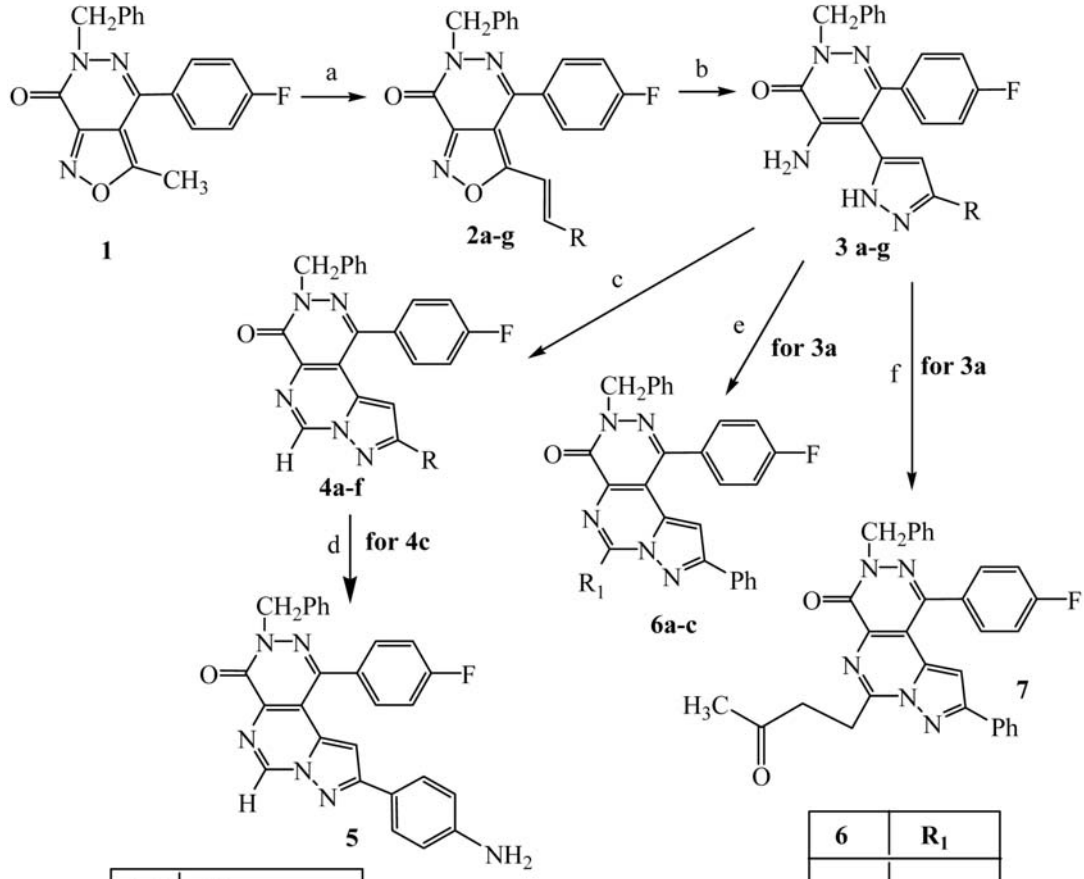
2. 2. Pharmacology

All the final compounds were investigated in radioligand binding studies to determine their affinities for human A_1, A_{2A} and A_3 receptors. The biological results are reported in Table 1 together with the values of affinity of our lead compound **4a**[14]. Analysis of compounds **4b** and **4d** in which we modified the position 9 through introduction of a Cl or an OCH_3 group in *para* of the phenyl ring led to products with A_1 affinity and selectivity comparable to that of lead **4a** (Ki = 0.244 μM and 0.233 μM for **4b** and **4d** respectively). Introduction of a NO_2 or a NH_2 group in the same position afforded compounds **4c** and **5** which had higher affinity (Ki = 0.132 μM for compound **4c** and Ki = 0.105 for **5**), but there was loss of selectivity (Ki = 0.116 μM for A_{2A}) for compound **5**.

The replacement of the phenyl at C-9 of **4a** with a 3-furyl (**4e**) or with a 3-pyridyl (**4f**) nucleus was associated with maintenance of selectivity but with of decrease in affinity for the A_1 subtype (Ki = 0.886 and 0.750 μM respectively), while elimination of the phenyl group (compound **4g**) resulted in a loss of potency of one order of magnitude (Ki = 2.48 μM) compared to **4a**.

The introduction at position 6 of short alkyl chains (compound **6a-c**) or of a functionalized alkyl chain (compound **7**) led to inactive (**6b,c** and **7**) or poorly active (**6a**, Ki = 0.811) compounds, suggesting that position 6 of system has to remain unsubstituted.

Finally compound **10**, in which the benzyl group was shifted from N-3 of pyridazinone to the neighboring 4-position, showed less activity compared to the lead **4a**,



2-4	R
a[14]	Ph
b	4-Cl-C_6H_4
c	4-NO_2-C_6H_4
d	4-OCH_3-C_6H_4
e	3-Furyl
f	3-Pyridyl
g	A[a]

[a] R = $N(CH_3)_2$ for **2a**, R = H for **3a** and **4a**

6	R_1
a	*i*-C_3H_7
b	*n*-C_3H_7
c	*n*-C_4H_9

Regent and conditions: (a) R_1CHO, CH_3ONa, CH_3OH reflux, 1–10 min.; for compound **2g:** DMDA, 110 °C, 90 min.; (b) $NH_2NH_2 \cdot H_2O$, EtOH, r.t. –50 °C, 1–3 h; (c) $CH(OC_2H_5)_3$, H_2SO_4 conc., DMF, r.t., 30 min.; (d) $SnCl_2$, 37% HCl, r.t., 20 h; (e) $(R_1CO)_2O$, H_2SO_4 conc., r.t., 10 min.; (f) levulinic acid, DMF, CH_2Cl_2, DMAP, DECDI, r.t., 6h and then reflux, 20 h.

Scheme 1. Synthesis of pyrazolo[1',5':1,6]pyrimido[4,5-d]pyridazin-4(3H)-one **4a-f, 5, 6a-c** and **7**

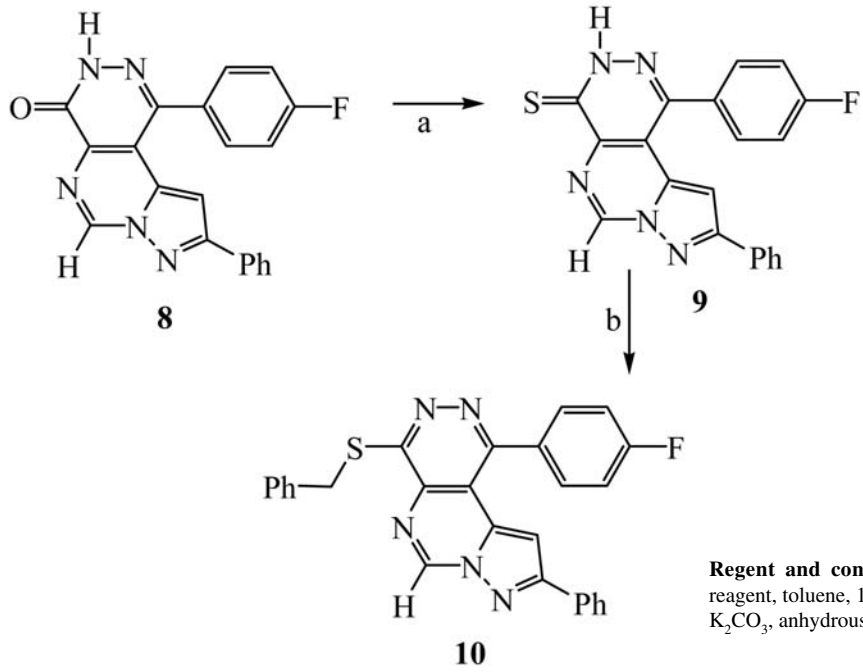
confirming the results previously reported[14] about the essential role played by carbonyl function at position 4.

3. Conclusions

In conclusion, the majority of the new tricyclic derivatives shows affinities for A_1 subtypes in submicromolar concentration range (0.105–0.886) and good selectivity versus other adenosine subtypes. As regards the modifications at position 9, the most active compounds, **4c** and **5**, bearing a 4-NO_2-Ph and a 4-NH_2-Ph group respectively, are about one-fold more potent than the lead **4a**, while the worst is the 9-unsubstituted **4g**. On the other hand the introduction of substituents at C-6 of the tricyclic scaffold led from low active to inactive compounds. Further studies are in progress in order to improve the potency and selectivity of these A_1 subtype ligands.

4. Experimental Section

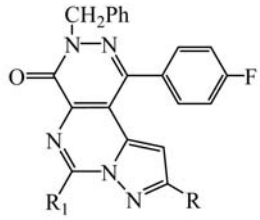
All melting points were determined on a Büchi apparatus and are uncorrected. ^{1}H-NMR spectra were recorded with an Avance 400 instruments (Bruker Biospin Version 002 with SGU). Chemical shifts are reported in ppm,



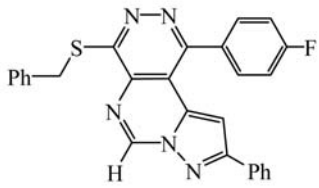
Regent and conditions: (a) Lawesson's reagent, toluene, 110 °C, 10 h (b) $PhCH_2Cl$, K_2CO_3, anhydrous DMF, 110 °C, 1 h.

Scheme 2. Synthesis of 4-(benzylthio)pyrazolo[1',5':1,6]pyrimido[4,5-d]pyridazine **10**

Table 1. Binding activity at human A_1, A_{2A} and A_3 adenosine receptors



4a-f, 5, 6a-c, 7



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Comp.	R	R_1	hA_1 [a,b]	hA_{2A} [a,c]	hA_3 [a,d]
4a[14]	Ph	H	0.252±0.060	45%	6%
4b	4-Cl-Ph	H	0.244±0.024	25%	30%
4c	4-NO_2-Ph	H	0.132±0.013	43%	46%
4d	4-OCH_3-Ph	H	0.233±0.007	50%	48%
4e	3-Furyl	H	0.886±0.085	56%	7%
4f	3-Pyridyl	H	0.750±0.055	46%	5%
4g	H	H	2.480±0.240	30%	39%
5	4-NH_2-Ph	H	0.105±0.010	0.116±0.033	39%
6a	Ph	*i*-C_3H_7	0.811±0.079	7%	36%
6b	Ph	*n*-C_3H_7	50%	25%	39%
6c	Ph	*n*-C_4H_9	49%	8%	37%
7	Ph	-$(CH_2)_2COCH_3$	45%	9%	50%
10			1.744±0.165	20%	30%

[a] The binding activity is reported as Ki (μM) or percentage of inhibition at 10 μM; values are means ± DS of four separate assay, each performed in triplicate. [b] Displacement of [^{3}H]DPCPX binding in CHO-A_1 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.

using the solvent as internal standard. Extracts were dried over Na_2SO_4, and the solvents were removed under reduced pressure. Merck F-254 commercial plates were used for analytical TLC to follow the course of reaction. Silica gel 60 (Merck 70-230 mesh) was used for column chromatography. Microanalyses were performed with a Per-

kin-Elmer 260 elemental analyzer for C, H, and N, and the results were within ±0.4% of the theoretical values, unless otherwise stated. Reagents and starting materials were commercially available.

4. 1. Preparation of Compounds

General Procedures for 2b-f

A mixture of compound **1**[14] (0.6 mmol), the appropriate arylaldehyde (1.5 mmol) and CH_3ONa (1–2 mmol) in anhydrous methanol (1–2 mL) was refluxed under stirring for 1–5 min. After cooling, the crude solid was isolated by filtration and recrystallized by ethanol.

6-Benzyl-3-(4-chlorostyryl)-4-(4-fluorophenyl)isoxazolo[3,4-*d*]pyridazin-7(6*H*)-one, 2b

Yield = 53%; mp = 217--220 °C (EtOH); ^{1}H-NMR ($CDCl_3$) δ 5.40 (s, 2H, $\underline{CH_2}$Ph), 6.75 (d, 1H, $\underline{CH}$=CH), 7.30–7.40 (m, 9H, Ar), 7.55 (m, 2H, Ar), 7.65 (m, 3H: 2H, Ar; H, CH=$\underline{CH}$); MS *m/z* 458 [M^+]; Anal. Calcd for $C_{26}H_{17}ClFN_3O_2$: C, 68.20; H, 3.74; N, 9.18. Found C, 68.34; H, 3.75; N, 9.21.

6-Benzyl-4-(4-fluorophenyl)-3-(4-nitrostyryl)isoxazolo[3,4-*d*]pyridazin-7(6*H*)-one, 2c

Yield = 73%; mp = 202--205 °C (EtOH); ^{1}H-NMR ($CDCl_3$) δ 5.40 (s, 2H, $\underline{CH_2}$Ph), 6.90 (d, 1H, $\underline{CH}$=CH), 7.30–7.40 (m, 5H, Ar), 7.45–7.55 (m, 4H, Ar), 7.65 (m, 2H, Ar), 7.70 (d, 1H, CH=$\underline{CH}$), 8.25 (d, 2H, Ar); MS *m/z* 469 [M^+]; Anal. Calcd for $C_{26}H_{17}FN_4O_4$: C, 66.66; H, 3.66; N, 11.96 Found C, 66.50; H, 3.67; N, 11.92.

6-Benzyl-4-(4-fluorophenyl)-3-(4-methoxystyryl)isoxazolo[3,4-*d*]pyridazin-7(6*H*)-one, 2d

Yield = 76%; mp = 228--230 °C (EtOH); ^{1}H-NMR ($CDCl_3$) δ 3.85 (s, 3H, OCH_3), 5.40 (s, 2H, $\underline{CH_2}$Ph), 6.65 (d, 1H, $\underline{CH}$=CH), 6.90 (m, 2H, Ar), 7.25–7.45 (m, 9H, Ar), 7.55 (d, 1H, CH=$\underline{CH}$), 7.60 (m, 2H, Ar); MS *m/z* 454 [M^+]; Anal. Calcd for $C_{27}H_{20}FN_3O_3$: C, 71.51; H, 4.45; N, 9.27. Found C, 71.34; H, 4.47; N, 9.30.

6-Benzyl-4-(4-fluorophenyl)-3-[2-(furan-3-yl)vinyl]isoxazolo[3,4-*d*]pyridazin-7(6*H*)-one, 2e

Yield = 77%; mp = 219–220 °C dec. (EtOH); ^{1}H-NMR ($CDCl_3$) δ 5.40 (s, 2H, $\underline{CH_2}$Ph), 6.30 (m, 1H, Ar), 6.45 (d, 1H, $\underline{CH}$=CH), 7.25–7.40 (m, 5H, Ar), 7.45 (m, 1H, Ar), 7.50–7.60 (m, 5H: 4H, Ar; 1H CH=$\underline{CH}$), 7.70 (s, 1H, Ar); MS *m/z* 414 [M^+]; Anal. Calcd for $C_{24}H_{16}FN_3O_3$: C, 69.73; H, 3.90; N, 10.16. Found C, 69.53; H, 3.91; N, 10.20.

6-Benzyl-4-(4-fluorophenyl)-3-[2-(pyridin-3-yl)vinyl]isoxazolo[3,4-*d*]pyridazin-7(6*H*)-one, 2f

Yield = 16%; mp = 130–131 °C dec. (EtOH); ^{1}H-NMR ($CDCl_3$) δ 5.40 (s, 2H, $\underline{CH_2}$Ph), 7.00 (d, 1H, $\underline{CH}$=CH), 7.30-7.40 (m, 5H, Ar), 7.55 (m, 2H, Ar), 7.65 (m, 2H, Ar), 7.70 (d, 1H, CH=$\underline{CH}$), 7.80 (m, 1H, Ar), 8.10 (m. 1H, Ar), 8.75 (m, 1H, Ar), 8.80 (s, 1H, Ar); MS *m/z* 425 [M^+]; Anal. Calcd for $C_{25}H_{17}FN_4O_2$: C, 70.75; H, 4.04; N, 13.20. Found C, 70.95; H, 4.06; N, 13.16.

6-Benzyl-3-(2-dimethylaminovinyl)-4-(4-fluorophenyl)isoxazolo[3,4-*d*]pyridazin-7(6*H*)-one, 2g

A suspension of **1**[14] (0.6 mmol) in N,N-dimethylformammide dimethyl acetal (22.5 mmol) was refluxed under stirring 90 min. After cooling the precipitate was recovered by suction.

Yield = 73%; mp = 162–163 °C (EtOH); ^{1}H-NMR ($CDCl_3$) δ 2.90 (s, 6H, $N(CH_3)_2$), 4.75 (d, 1H, $\underline{CH}$=CH), 5.35 (s, 2H, $\underline{CH_2}$Ph), 7.15–7.25 (m, 2H, Ar), 7.30–7.40 (m, 5H, Ar), 7.55 (m, 3H: 1H, Ar; 1H, CH=$\underline{CH}$); MS *m/z* 391 [M^+]; Anal. Calcd for $C_{22}H_{19}FN_4O_2$: C, 67.68; H, 4.91; N, 14.35. Found C, 67.91; H, 4.89; N, 14.32.

General Procedures for 3b-f

To a suspension of compounds **2b-g** (0.45 mmol) in ethanol (3–3.5 mL), 10–15 mmol of hydrazine hydrate was added and the mixture was heated at 50–70 °C for 3–4 h. After cooling the suspension was concentrated under vacuum and the solid was recovered by suction and recrystallizzed by ethanol.

4-Amino-2-benzyl-5-[5-(4-chlorophenyl)-2H-pyrazol-3-yl]-6-(4-fluorophenyl)pyridazin-3(2*H*)-one, 3b

Yield = 62%; mp = 142–143 °C (EtOH); ^{1}H-NMR ($CDCl_3$) δ 5.45 (s, 2H, $\underline{CH_2}$Ph), 5.70 (s, 1H Ar), 6.65 (exch br s, 2H, NH_2), 7.05 (m, 3H, Ar), 7.30–7.40 (m, 8H, Ar), 7.50 (m, 2H, Ar), 8.20 (exch br s, 1H, NH); MS *m/z* 472 [M^+]; Anal. Calcd for $C_{26}H_{19}ClFN_5O$: C, 66.17; H, 4.06; N, 14.84. Found C, 66.32; H, 4.07; N, 14.81.

4-Amino-2-benzyl-6-(4-fluorophenyl)-5-[5-(4-nitrophenyl)-2H-pyrazol-3-yl]pyridazin-3(2*H*)-one, 3c

Yield = 72%; mp = 205–207 °C dec. (EtOH); ^{1}H-NMR ($CDCl_3$) δ 5.45 (s, 2H, $\underline{CH_2}$Ph), 6.15 (s, 1H, Ar), 6.35 (exch br s, 2H, NH_2), 7.00–7.10 (m, 2H, Ar), 7.30–7.40 (m, 5H, Ar), 7.55 (d, 2H, Ar), 7.70 (d, 2H, Ar), 8.20 (exch br s, 1H, NH), 8.30 (d, 2H, Ar); MS *m/z* 483 [M^+]; Anal. Calcd for $C_{26}H_{19}FN_6O_3$: C, 64.73; H, 3.97; N, 17.42. Found C, 64.87; H, 3.97; N, 17.47.

4-Amino-2-benzyl-6-(4-fluorophenyl)-5-[5-(4-methoxyphenyl)-2H-pyrazol-3-yl]pyridazin-3(2*H*)-one, 3d

Yield = 30%; mp = 168–170 °C (EtOH); ^{1}H-NMR ($CDCl_3$) δ 3.85 (s, 3H, CH_3O), 5.40 (s, 2H, $\underline{CH_2}$Ph), 6.05 (s, 1H Ar), 6.40 (exch br s, 2H, NH_2), 6.95–7.05 (m, 4H, Ar), 7.30–7.40 (m, 5H, Ar), 7.55 (m, 2H, Ar), 7.65 (m, 2H, Ar), 8.20 (exch br s, 1H, NH); MS *m/z* 468 [M^+]; Anal. Calcd for $C_{27}H_{22}FN_5O_2$: C, 69.37; H, 4.74; N, 14.98. Found C, 69.56; H, 4.75; N, 14.94.

4-Amino-2-benzyl-6-(4-fluorophenyl)-5-[(5-furan-3-yl)-2H-pyrazol-3-yl]pyridazin-3(2*H*)-one, 3e

Yield = 38%; mp = 132–135 °C dec. (EtOH); ^{1}H-NMR ($CDCl_3$) δ 5.40 (s, 2H, $\underline{CH_2}$Ph), 5.90 (s, 1H Ar), 6.30 (exch br s, 2H, NH_2), 6.75 (m, 1H, Ar), 6.95–7.05 (m, 2H, Ar), 7.30–7.40 (m, 4H, Ar), 7.45 (m, 1H, Ar), 7.50 (m, 3H, Ar), 7.90 (s. 1H, Ar), 8.10 (exch br s, 1H, NH); MS *m/z* 428 [M$^+$]; Anal. Calcd for $C_{24}H_{18}FN_5O_2$: C, 67.44; H, 4.24; N, 16.38. Found C, 67.55; H, 4.24; N, 14.30.

4-Amino-2-benzyl-6-(4-fluorophenyl)-5-[(5-pyridin-3-yl-2H-pyrazol-3-yl)]pyridazin-3(2*H*)-one, 3f

Yield = 42%; mp = 238–240 °C (EtOH); ^{1}H-NMR ($CDCl_3$) δ 5.40 (s, 2H, $\underline{CH_2}$Ph), 6.20 (s, 1H, Ar), 6.40 (exch br s, 2H, NH_2), 7.30–7.40 (m, 6H, Ar), 7.50–7.60 (m, 3H, Ar), 7.90 (m, 1H, Ar), 8.10 (exch br s, 1H, NH), 8.40 (m, 1H, Ar), 8.60 (m. 1H, Ar), 9.75 (s, 1H, Ar); MS *m/z* 439 [M$^+$]; Anal. Calcd for $C_{25}H_{19}FN_6O$: C, 68.48; H, 4.37; N, 19.17. Found C, 68.34; H, 4.36; N, 19.13.

4-Amino-2-benzyl-6-(4-fluorophenyl)-5-(2H-pyrazol-3-yl)pyridazin-3(2*H*)-one, 3g

Yield = 70%; mp = 190–192 °C (EtOH); ^{1}H-NMR ($CDCl_3$) δ 5.40 (s, 2H, $\underline{CH_2}$Ph), 5.65 (m, 1H, Ar), 6.50 (exch br s, 2H, NH_2), 7.00 (m, 2H, Ar), 7.25-7.40 (m, 5H, Ar), 7.45-7.55 (m, 3H, Ar), 8.10 (exch br s, 1H, NH); MS *m/z* 362 [M$^+$]; Anal. Calcd for $C_{20}H_{16}FN_5O$: C, 66.47; H, 4.46; N, 19.38. Found C, 66.62; H, 4.45; N, 19.33.

General Procedures for 4b-f

A mixture of compounds **3b-g** (0.21 mmol), triethylorthoformate (18 mmol) and a catalytic amount of concentrated sulfuric acid in anhydrous DMF (0.5–1 mL) was stirred at room temperature for 20–30 min. The suspension was cooled and the precipitate was recovered by suction and purified by crystallization from ethanol.

3-Benzyl-9-(4-chlorophenyl)-1-(4-fluorophenyl)pyrazolo[1',5':1,6]pyrimido[4,5-*d*]pyridazin-4(3*H*)-one, 4b

Yield = 68%; mp = 246–248 °C dec (EtOH); ^{1}H-NMR ($CDCl_3$) δ 5.55 (s, 2H, $\underline{CH_2}$Ph), 6.10 (s, 1H, Ar), 7.30–7.50 (m, 7H, Ar), 7.50–7.60 (m, 4H, Ar), 7.70 (m, 2H, Ar), 9.45 (s, 1H, Ar); MS *m/z* 482 [M$^+$]; Anal. Calcd for $C_{27}H_{17}ClFN_5O$: C, 67.29; H, 3.56; N, 14.53. Found C, 67.48; H, 3.56; N, 14.58.

3-Benzyl-1-(4-fluorophenyl)-9-(4-nitrophenyl)pyrazolo[1',5':1,6]pyrimido[4,5-*d*]pyridazin-4(3*H*)-one, 4c

Yield = 69%; mp = 250–253 °C dec (EtOH); ^{1}H-NMR ($CDCl_3$) δ 5.50 (s, 2H, $\underline{CH_2}$Ph), 6.20 (s, 1H, Ar), 7.30–7.40 (m, 5H, Ar), 7.50–7.60 (m, 4H, Ar), 7.95 (d, 2H, Ar), 8.35 (d, 2H, Ar), 9.45 (s, 1H, Ar); MS *m/z* 493 [M$^+$]; Anal. Calcd for $C_{27}H_{17}FN_6O_3$: C, 65.85; H, 3.48; N, 17.07. Found C, 65.66; H, 3.48; N, 17.01.

3-Benzyl-1-(4-fluorophenyl)-9-(4-methoxyphenyl) pyrazolo[1',5':1,6]pyrimido[4,5-*d*]pyridazin-4(3*H*)-one, 4d

Yield = 96%; mp = 165–168 °C dec (THF); ^{1}H-NMR ($CDCl_3$) δ 3.90 (s, 3H, OCH_3), 5.55 (s, 2H, $\underline{CH_2}$Ph), 6.05 (s, 1H, Ar), 6.95 (d, 2H, Ar), 7.30–7.40 (m, 5H, Ar), 7.50–7.60 (m, 4H, Ar), 7.70 (d, 2H, Ar), 9.40 (s, 1H, Ar); MS *m/z* 478 [M$^+$]; Anal. Calcd for $C_{28}H_{20}FN_5O_2$: C, 70.43; H, 4.22; N, 14.67. Found C, 70.19; H, 4.23; N, 14.70.

3-Benzyl-1-(4-fluorophenyl)-9-(furan-3-yl)pyrazolo[1',5':1,6]pyrimido[4,5-*d*]pyridazin-4(3*H*)-one, 4e

Yield = 55%; mp = 219–220 °C (EtOH); ^{1}H-NMR ($CDCl_3$) δ 5.55 (s, 2H, $\underline{CH_2}$Ph), 5.90 (s, 1H, Ar), 6.75 (m, 1H, Ar), 7.30–7.40 (m, 5H, Ar), 7.50–7.60 (m, 4H, Ar), 7.70 (m, 1H, Ar), 7.85 (s, 1H, Ar), 9.40 (s, 1H, Ar); MS *m/z* 438 [M$^+$]; Anal. Calcd for $C_{25}H_{16}FN_5O_2$: C, 68.64; H, 3.69; N, 16.01. Found C, 68.81; H, 3.70; N, 16.05.

3-Benzyl-1-(4-fluorophenyl)-9-(pyridin-3-yl)pyrazolo[1',5':1,6]pyrimido[4,5-*d*]pyridazin-4(3*H*)-one, 4f

Yield = 61%; mp = 200–203 °C dec. (EtOH); ^{1}H-NMR ($CDCl_3$) δ 5.55 (s, 2H, $\underline{CH_2}$Ph), 6.35 (s, 1H, Ar), 7.30–7.40 (m, 5H, Ar), 7.50–7.60 (m, 4H, Ar), 8.10 (m, 1H, Ar), 8.80 (m, 1H, Ar), 9.00 (m, 1H, Ar), 9.30 (s, 1H, Ar), 9.50 (s, 1H, Ar); MS *m/z* 449 [M$^+$]; Anal. Calcd for $C_{26}H_{17}FN_6O$: C, 69.63; H, 3.82; N, 18.74. Found C, 69.80; H, 3.81; N, 18.79.

3-Benzyl-1-(4-fluorophenyl)pyrazolo[1',5':1,6]pyrimido[4,5-*d*]pyridazin-4(3*H*)-one, 4g

Yield = 81%; mp = 250–252 °C (EtOH); ^{1}H-NMR ($CDCl_3$) δ 5.55 (s, 2H, $\underline{CH_2}$Ph), 5.85 (m, 1H, Ar), 7.20–7.40 (m, 5H, Ar), 7.50–7.60 (m, 4H, Ar), 8.05 (m, 1H, Ar), 9.45 (s, 1H, Ar); MS *m/z* 372 [M$^+$]; Anal. Calcd for $C_{21}H_{14}FN_5O$: C, 67.92; H, 3.80; N, 18.86. Found C, 67.76; H, 3.81; N, 18.89.

9-(4-Aminophenyl)-3-benzyl-1-(4-fluorophenyl)pyrazolo[1',5':1,6]pyrimido[4,5-*d*]pyridazin-4(3*H*)-one, 5

To a solution of **4c** (0.21 mmol) in 37% HCl (1 mL), a solution of $SnCl_2$ (1.03 mmol) in 37% HCl (0.5–1 mL) was slowly added. The mixture was stirred at room temperature for 20 h. Water was then added and the mixture was neutralized with 6N NaOH. The suspension was extracted with CH_2Cl_2 (3 × 15 mL) and the solvent was evaporated *in vacuo* affording a residue oil which was purified by column chromatography using CH_2Cl_2/CH_3OH as eluent.

Yield = 29%; mp = >300 °C (EtOH); ^{1}H-NMR ($CDCl_3$) δ 5.55 (s, 2H, $\underline{CH_2}$Ph), 6.10 (s, 1H, Ar), 6.65 (exch br s, 2H, NH_2), 7.20 (d, 2H, Ar), 7.30–7.40 (m, 4H, Ar), 7.50–7.60 (m, 5H, Ar), 7.75 (d, 2H, Ar), 9.40 (s, 1H, Ar); MS *m/z* 463 [M$^+$]; Anal. Calcd for $C_{27}H_{19}FN_6O$: C, 70.12; H, 4.14; N, 18.17. Found C, 70.31; H, 4.14; N, 18.12.

General Procedures for 6a-c

A mixture of **3a**[14](0.14 mmol), the appropriate anhydride (4–9 mmol) and a catalytic amount of concen-

trated sulfuric acid was stirred at room temperature for 10 min. After cooling, the mixture was diluted with cold water (10 mL) and neutralized with $NaHCO_3$. Compound **6a** was filtered off and recrystallyzed from ethanol, while for compounds **6b** and **6c** the mixture was extracted with CH_2Cl_2, the solvent was evaporated under vacuum and the crude final compounds were purified by crystallization from ethanol.

3-Benzyl-1-(4-fluorophenyl)-6-isopropyl-9-phenylpyrazolo[1',5':1,6]pyrimido[4,5-*d*]pyridazin-4(3*H*)-one, 6a

Yield = 89%; mp = 217–220 °C (EtOH); ^{1}H-NMR ($CDCl_3$) δ 1.60 (d, 6H, CH($\underline{CH_3}$)$_2$), 4.25 (m, 1H, $\underline{CH}$(CH$_3$)$_2$), 5.50 (s, 2H, $\underline{CH_2}$Ph), 6.10 (s, 1H, Ar), 7.30–7.55 (m, 8H, Ar), 7.60–7.70 (m, 4H, Ar), 7.80 (m, 2H, Ar); MS *m/z* 490 [M$^+$]; Anal. Calcd for $C_{30}H_{24}FN_5O$: C, 73.60; H, 4.94; N, 14.31. Found C, 73.35; H, 4.93; N, 14.35.

3-Benzyl-1-(4-fluorophenyl)-9-phenyl-6-*n*-propylpyrazolo[1',5':1,6]pyrimido[4,5-*d*]pyridazin-4(3*H*)-one, 6b

Yield = 74%; mp = 188–191 °C (EtOH); ^{1}H-NMR ($CDCl_3$) δ 1.15 (t, 3H, $\underline{CH_3}$(CH$_2$)$_2$), 2.10 (m, 2H, CH$_3$$\underline{CH_2}CH_2$), 3.55 (t, 2H, CH$_3CH_2$$\underline{CH_2}$), 5.55 (s, 2H, $\underline{CH_2}$Ph), 6.10 (s, 1H, Ar), 7.30–7.50 (m, 8H, Ar), 7.60 (m, 4H, Ar), 7.80 (m, 2H, Ar); MS *m/z* 490 [M$^+$]; Anal. Calcd for $C_{30}H_{24}FN_5O$: C, 73.60; H, 4.94; N, 14.31. Found C, 73.38; H, 4.94; N, 14.28.

3-Benzyl-6-butyl-1-(4-fluorophenyl)-9-phenylpyrazolo[1',5':1,6]pyrimido[4,5-*d*]pyridazin-4(3*H*)-one, 6c

Yield = 87%; mp = 190–192 °C (EtOH); ^{1}H-NMR ($CDCl_3$) δ 1.05 (t, 3H, $\underline{CH_3}$(CH$_2$)$_3$), 1.50–1.60 (m, 2H, CH$_3$$\underline{CH_2}$(CH$_2$)$_2$), 2.00–2.10 (m, 2H, CH$_3CH_2$$\underline{CH_2}CH_2$), 3.50–3.60 (t, 2H, CH$_3CH_2CH_2$$\underline{CH_2}$), 5.55 (s, 2H, $\underline{CH_2}$Ph), 6.10 (s, 1H, Ar), 7.30–7.50 (m, 8H, Ar), 7.60–7.80 (m, 6H, Ar); MS *m/z* 504 [M$^+$]; Anal. Calcd for $C_{31}H_{26}FN_5O$: C, 73.94; H, 5.20; N, 13.91. Found C, 73.77; H, 5.21; N, 13.95.

3-Benzyl-1-(4-fluorophenyl)-6-(3-oxobutyl)-9-phenylpyrazolo[1',5':1,6]pyrimido[4,5-*d*]pyridazin-4(3*H*)-one, 7

A mixture of **3a**[14](0.27 mmol), 0.58 mmol of levulinic acid, 0.41 mmol of 4-(dimethylamino)pyridine and 0.48 mmol of 1-[3-(dimethylamino)propyl]-ethylcarbodiimide hydocloride in anhydrous CH_2Cl_2 (10.4 mL) and anhydrous DMF (1 mL), was refluxed for 20 h. After cooling CH_2Cl_2 was added (15 mL) and the organic layer was washed with 2N HCl and with 2N NaOH in turn. Evaporation in vacuum afforded the final compound **7** which was purified by column chromatography using CH_2Cl_2/CH_3OH 9.5:0.5 as eluent.

Yield = 15%; mp = 220–222 °C dec. (EtOH); ^{1}H-NMR ($CDCl_3$) δ 2.40 (s, 3H, CH_3CO), 3.35 (t, 2H, COCH$_2$$\underline{CH_2}$), 3.80 (t, 2H, CO$\underline{CH_2}CH_2$), 5.50 (s, 2H, $\underline{CH_2}$Ph), 6.10 (s, 1H, Ar), 7.30–7.40 (m, 5H, Ar), 7.40–7.50 (m, 3H, Ar), 7.55–7.65 (m, 4H, Ar), 7.80 (m, 2H, Ar); MS *m/z* 518 [M$^+$]; Anal. Calcd for $C_{31}H_{24}FN_5O_2$: C, 71.94; H, 4.67; N, 13.53. Found C, 72.18; H, 4.67; N, 13.50.

1-(4-fluorophenyl)-9-phenylpyrazolo[1',5':1,6]pyrimido[4,5-*d*]pyridazin-4(3*H*)-thione, 9

A mixture of **8**[14] (0.25 mmol) and Lawesson's reagent (1.19 mmol) in toluene (4.8 mL) was heated at 110 °C for 10 h. After cooling, the precipitate was recovered by suction.

Yield = 54%; mp = 209–210 °C dec. (EtOH); ^{1}H-NMR ($CDCl_3$) δ 6.05 (s, 1H, Ar), 7.00 (m, 3H, Ar), 7.40–7.80 (m, 6H, Ar), 9.80 (s, 1H, Ar), 15.00 (exch br s, 1H, NH); MS *m/z* 374 [M$^+$]; Anal. Calcd for $C_{20}H_{12}FN_5S$: C, 64.33; H, 3.24; N, 18.76. Found C, 64.13; H, 3.25; N, 18.79.

4-Benzylthio-1-(4-fluorophenyl)-9-phenylpyrazolo[1',5':1,6]pyrimido[4,5-*d*]pyridazina, 10

A mixture of compound **9** (0.21 mmol), K_2CO_3 (0.66 mmol) and 0.61 mmol of benzyl chloride in anhydrous DMF (1.6 mL) was heated under stirring at 110 °C for 1 h. After cooling, cold water was added and the precipitate was isolated by filtration.

Yield = 30%; mp = 281–282 °C (Ethyl acetate); ^{1}H-NMR ($CDCl_3$) δ 4.70 (s, 2H, CH_2S), 6.30 (s, 1H, Ar), 7.25–7.40 (m, 3H, Ar), 7.40–7.60 (m, 7H, Ar), 7.70–7.90 (m, 4H, Ar), 9.90 (s, 1H, Ar); MS *m/z* 464 [M$^+$]; Anal. Calcd for $C_{27}H_{18}FN_5S$: C, 69.96; H, 3.91; N, 15.11. Found C, 69.75; H, 3.90; N, 15.15 .

4. 2. Adenosine Receptor Binding Assay[17–19]

The binding activity of each compound towards adenosine receptor subtypes was calculated by competition binding experiments. To determine the affinities of the new compounds toward human A_1, A_{2A}, and A_3 ARs we evaluated the ability of different compound concentrations to displace [3H]8-cyclopentyl-1,3-dipropylxanthine ([3H]DPCPX, for CHO-A_1), [3H]5 -N-ethylcarboxamideadenosine ([3H]NECA, for CHO A_{2A}), or [125I]4-aminobenzyl-5 -N-methylcarboxamidoadenosine ([125I]AB-MECA, for CHO-A_3) binding from transfected CHO cells. Data analysis and graphic presentation were conducted using the non-linear multipurpose curve-fitting computer program Graph-Pad Prism (GraphPad, San Diego, CA). Data analysis allowed to obtain the competition curve of each compound and to calculate its affinity towards a single population of receptors expressed as Ki value. For the compounds that at 10 μM concentration showed an inhibitory effect on radioligand binding lower that 60%, the competition curve was not performed and the results were expressed as % inhibition at 10 μM.

Human A_1 Adenosine Receptors. Aliquots of cell membranes (30 μg proteins) obtained from A_1CHO cells were incubated at 25 °C for 180 min in 500 μL of buffer (50 mM Tris-HCl, 2 mM $MgCl_2$, and 2 units/mL ADA, pH 7.4) containing [^{3}H]DPCPX (3 nM) and six different concentrations of the compounds. Non-specific binding was determined in the presence of 50 μM R-PIA[20] The dissociation constant (K_d) of [^{3}H]DPCPX in A_1 CHO cell membranes was 3 nM.

Human A_{2A} Adenosine Receptors. Aliquots of cell membranes (30 μg proteins) were incubated at 25 °C for 180 min in 500 μL of buffer (50mM Tris-HCl, 2 mM $MgCl_2$, and 2 units/mL ADA, pH 7.4) in the presence of 20 nM of [^{3}H]NECA and six different concentrations of the synthesized compounds. Non-specific binding was determined in the presence of 100 μM R-PIA[20]. The dissociation constant (K_d) of [^{3}H]NECA in A_{2A} CHO cell membranes was 30 nM.

Human A_3 Adenosine Receptors. Aliquots of cell membranes (20 μg proteins) were incubated at 25 °C for 90 min in 100 μL of buffer (50mM Tris-HCl, 10mM $MgCl_2$, 1mM EDTA, and 2 units/mL ADA, pH 7.4) in the presence of 0.14 nM [^{125}I]AB-MECA and six different concentrations of the synthesized compounds. Non-specific binding was determined in the presence of 50 μM R-PIA[20]. The dissociation constant (K_d) of [^{125}I] AB-MECA in A_3 CHO cell membranes was 1.4 nM.

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Povzetek

Članek poroča o sintezi in biološkem ovrednotenju nove serije pirazolo[1',5':1,6]pirimido[4,5-d]piridazin-4(3H)-onov kot ligandov človeškega A_1 adenozinskega receptorja. Triciklično ogrodje je bilo spremenjeno na položajih 6 in 9 z uvedbo majhnih alkilnih verig in substituiranih fenilov.

Najbolj zanimive spojine so pokazale Ki za A_1 v submikromolarnem območju (0.105–0.244 μM). Najzanimivejši del (spojina **4c**) je pokazal znatno afiniteto za A_1 (Ki = 0.132 μM), skupaj z dobro selektivnostjo za A_{2A} (43 % inhibicije pri 10 μM) in A_3 (46 % inhibicije pri 10 μM).