New Pyrazolo[1’5’:1,6]pyrimido[4,5-d]pyridazin-4(3H)-ones Fluoroderivatives as Human A1 Adenosine Receptor Ligands

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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 A1 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 A1 in the submicromolar range (0.105–0.244 μM) and the most interesting term (compound 4c) combined an appreciable affinity for A1 (Ki = 0.132 μM) with a good selectivity toward A2A (43% inhibition at 10 μM) and A3 (46% inhibition at 10 μM).

Keywords: Adenosine receptors, A1 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, A1, A2A, A2B and A3.1 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. A1 and A3 subtypes, other that modulate calcium levels through a Gq proteins, are coupled to Gi proteins to inhibit adenyl cyclase. On the contrary, A2A and A2B 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 subtype 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 A1 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 effects8 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]pyrimidinediones11 (A), pyrimido[4,5-b]indole (B),12 and triazinobenzimidazolones (C)13 have been reported in literature as A1R antagonists (Figure 1).
In a recent paper\textsuperscript{14} we reported the synthesis and binding activities at human cloned A\textsubscript{1}, A\textsubscript{2A}, A\textsubscript{3}, and A\textsubscript{2B} 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\textsubscript{1} adenosine subtypes, with values of Ki in the submicromolar range and a good selectivity versus other adenosine receptor subtypes\textsuperscript{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\textsubscript{1} affinity and 4-F-phenyl at position 1.

![Figure 1: A\textsubscript{1} receptor antagonists](image)

### 2. Results and Discussion

#### 2.1. Chemistry

The final compounds 4-7 (4a\textsuperscript{14}) were prepared following a general synthetic procedure previously described by us\textsuperscript{15} (Scheme 1).

Isoxazolo[3,4-d]-pyridazin-7(6H)-one 1\textsuperscript{14} was condensed with the appropriate arylaldehydes (or N,N-dimethylformamide dimethyl acetal for compound 2g) to give the vinyl derivatives 2a-g (2a\textsuperscript{14}) which treated with hydrazine hydrate furnished intermediates 3a-g (3a\textsuperscript{14}) through the isoxazole opening and the following closure to pyrazole\textsuperscript{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 triethylthiophosphate 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\textsuperscript{14} for treatment with levulinic acid and in the presence of 4-(dimethylaminopyridine and of 1-[3-(dimethylaminopropyl)ethylcarbodiimide hydrochloride as coupling agent. Finally, compound 5 was obtained starting from 4c by reduction of the NO\textsubscript{2} group with SnCl\textsubscript{2}.

Scheme 2 depicts the synthesis of the final compound 10 which was obtained starting from the previously described pyrazolo[1',5':1,6]pyrimido[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\textsubscript{1}, A\textsubscript{2A} and A\textsubscript{3} receptors. The biological results are reported in Table 1 together with the values of affinity of our lead compound 4a\textsuperscript{14}. Analysis of compounds 4b and 4d in which we modified the position 9 through introduction of a Cl or an OCH\textsubscript{3} group in para of the phenyl ring led to products with A\textsubscript{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\textsubscript{2} or a NH\textsubscript{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\textsubscript{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\textsubscript{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,
confirming the results previously reported\textsuperscript{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\textsubscript{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\textsubscript{2}-Ph and a 4-NH\textsubscript{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\textsubscript{1} subtype ligands.

4. Experimental Section

All melting points were determined on a Büchi apparatus and are uncorrected. \textsuperscript{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) PhCH₂Cl, K₂CO₃, anhydrous DMF, 110 °C, 1 h.

Table 1. Binding activity at human A₁, A₂A and A₃ adenosine receptors

<table>
<thead>
<tr>
<th>Comp.</th>
<th>R</th>
<th>R₁</th>
<th>hA₁ᵃᵇ</th>
<th>hA₂Aᵃᶜ</th>
<th>hA₃ᵃᵈ</th>
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<tbody>
<tr>
<td>4a</td>
<td>Ph</td>
<td>H</td>
<td>0.252±0.060</td>
<td>45%</td>
<td>6%</td>
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<tr>
<td>4b</td>
<td>4-Cl-Ph</td>
<td>H</td>
<td>0.244±0.024</td>
<td>25%</td>
<td>30%</td>
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<tr>
<td>4c</td>
<td>4-NO₂-Ph</td>
<td>H</td>
<td>0.132±0.013</td>
<td>43%</td>
<td>46%</td>
</tr>
<tr>
<td>4d</td>
<td>4-OCH₃-Ph</td>
<td>H</td>
<td>0.233±0.007</td>
<td>50%</td>
<td>48%</td>
</tr>
<tr>
<td>4e</td>
<td>3-Furyl</td>
<td>H</td>
<td>0.886±0.085</td>
<td>56%</td>
<td>7%</td>
</tr>
<tr>
<td>4f</td>
<td>3-Pyridyl</td>
<td>H</td>
<td>0.750±0.055</td>
<td>46%</td>
<td>5%</td>
</tr>
<tr>
<td>4g</td>
<td>H</td>
<td>H</td>
<td>2.480±0.240</td>
<td>30%</td>
<td>39%</td>
</tr>
<tr>
<td>5</td>
<td>4-NH₂-Ph</td>
<td>H</td>
<td>0.105±0.010</td>
<td>0.116±0.033</td>
<td>39%</td>
</tr>
<tr>
<td>6a</td>
<td>Ph</td>
<td>i-C₃H₇</td>
<td>0.811±0.079</td>
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<td>36%</td>
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<tr>
<td>6b</td>
<td>Ph</td>
<td>n-C₄H₉</td>
<td>50%</td>
<td>25%</td>
<td>39%</td>
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<tr>
<td>6c</td>
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<td>n-C₅H₉</td>
<td>49%</td>
<td>8%</td>
<td>37%</td>
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<tr>
<td>7</td>
<td>Ph</td>
<td>-(CH₂)₂COCH₃</td>
<td>45%</td>
<td>9%</td>
<td>50%</td>
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<td>10</td>
<td></td>
<td></td>
<td>1.744±0.165</td>
<td>20%</td>
<td>30%</td>
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</tbody>
</table>

¹ 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. ² Displacement of [³H]DPCPX binding in CHO-A₁ cells membranes. ³ Displacement of [³H]NECA binding in A₂A CHO cells membranes. ⁴ Displacement of [¹²⁵I]AB-MECA binding in A₃ CHO cells membranes.

using the solvent as internal standard. Extracts were dried over Na₂SO₄, 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 (0.6 mmol), the appropriate arylaldehyde (1.5 mmol) and CH₂ONa (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)isoazolo[3,4-d]pyrazidin-7(6H)-one, 2b

Yield = 53%; mp = 217–220 °C (EtOH); ¹H-NMR (CDCl₃) δ 5.40 (s, 2H, Ph), 6.75 (d, 1H, CH=CH), 7.30–7.40 (m, 5H, Ar), 7.55 (m, 2H, Ar), 7.65 (m, 3H, 2H, Ar; H, CH=CH); MS m/z 458 [M⁺]; Anal. Calcd for C₂₆H₁₇FN₄O₄: C, 68.20; H, 3.74; N, 11.91. Found C, 68.34; H, 3.75; N, 11.92.

### 6-Benzyl-4-(4-fluorophenyl)-3-(4-nitrostyryl)isoazolo[3,4-d]pyrazidin-7(6H)-one, 2c

Yield = 73%; mp = 202–205 °C (EtOH); ¹H-NMR (CDCl₃) δ 5.40 (s, 2H, Ph), 6.90 (d, 1H, CH=CH), 7.30–7.40 (m, 5H, 4H, Ar), 7.55 (m, 2H, Ar), 7.65 (m, 3H, 2H, Ar; H, CH=CH); MS m/z 469 [M⁺]; Anal. Calcd for C₂₆H₁₇FN₄O₄: C, 66.66; H, 3.66; N, 19.96. Found C, 66.50; H, 3.67; N, 19.87.

### 6-Benzyl-4-(4-fluorophenyl)-3-(4-methoxystyryl)isoazolo[3,4-d]pyrazidin-7(6H)-one, 2d

Yield = 76%; mp = 228–230 °C (EtOH); ¹H-NMR (CDCl₃) δ 3.85 (s, 3H, OCH₃), 5.40 (s, 2H, Ph), 6.65 (d, 1H, CH=CH), 6.90 (m, 2H, Ar), 7.25–7.45 (m, 5H, Ar), 7.55 (d, 1H, CH=CH), 7.60 (m, 2H, Ar); MS m/z 454 [M⁺]; Anal. Calcd for C₂₆H₁₇FN₄O₄: C, 67.91; H, 4.89; N, 14.35. Found C, 67.91; H, 4.89; N, 14.32.

### 6-Benzyl-4-(4-fluorophenyl)-3-[2-(furan-3-yl)vinyl]isoazolo[3,4-d]pyrazidin-7(6H)-one, 2e

Yield = 77%; mp = 219–220 °C dec. (EtOH); ¹H-NMR (CDCl₃) δ 5.40 (s, 2H, CH₂Ph), 6.30 (m, 1H, Ar), 6.45 (d, 1H, CH=CH), 7.25–7.40 (m, 5H, Ar), 7.45 (m, 1H, Ar), 7.50–7.60 (m, 5H, 2H, Ar; 1H CH=CH), 7.70 (s, 1H, Ar); MS m/z 414 [M⁺]; Anal. Calcd for C₂₆H₁₉FN₄O₃: 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]isoazolo[3,4-d]pyrazidin-7(6H)-one, 2f

Yield = 16%; mp = 130–131 °C dec. (EtOH); ¹H-NMR (CDCl₃) δ 5.40 (s, 2H, CH₂Ph), 7.00 (d, 1H, CH=CH), 7.30–7.40 (m, 5H, Ar), 7.55 (m, 2H, Ar), 7.65 (m, 2H, Ar), 7.70 (d, 1H, CH=CH), 7.80 (m, 1H, Ar), 8.10 (m, 1H, Ar), 8.80 (s, 1H, Ar); MS m/z 425 [M⁺]; Anal. Calcd for C₂₆H₁₉FN₄O₃: C, 70.75; H, 4.04; N, 13.20. Found C, 70.95; H, 4.06; N, 13.16.

### 4-Amino-2-benzyl-5-[5-(4-fluorophenyl)-2H-pyrazol-3-yl]-6-(4-fluorophenyl)pyrazidin-3(2H)-one, 3b

Yield = 62%; mp = 142–143 °C (EtOH); ¹H-NMR (CDCl₃) δ 5.45 (s, 2H, CH₂Ph), 5.70 (s, 1H, Ar), 6.65 (exch br s, 2H, NH₂), 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 C₂₆H₁₇FN₄O₂: 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]pyrazidin-3(2H)-one, 3c

Yield = 72%; mp = 205–207 °C dec. (EtOH); ¹H-NMR (CDCl₃) δ 5.45 (s, 2H, CH₂Ph), 6.15 (s, 1H, Ar), 6.35 (exch br s, 2H, NH₂), 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 C₂₆H₁₉FN₄O₂: 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]pyrazidin-3(2H)-one, 3d

Yield = 30%; mp = 168–170 °C (EtOH); ¹H-NMR (CDCl₃) δ 3.85 (s, 3H, CH₃O), 5.40 (s, 2H, CH₂Ph), 6.05 (m, 1H, Ar), 6.40 (exch br s, 2H, NH₂), 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 C₂₆H₁₉FN₄O₂: 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]pyrazidin-3(2H)-one, 3e

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Yield = 38%; mp = 132–135 °C dec. (EtOH); 1H-NMR (CDCl3) δ 5.50 (s, 2H, CH2Ph), 5.90 (s, 1H Ar), 6.30 (exch br s, 2H, NH2), 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 C26H17FN6O: C, 67.48; H, 3.56; N, 14.58. Found C, 67.76; H, 3.81; N, 18.89.

Yield = 96%; mp = 165–168 °C dec (THF); 1H-NMR (CDCl3) δ 3.90 (s, 3H, OCH3), 5.55 (s, 2H, CH2Ph), 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 C26H17FN6O: C, 70.43; H, 4.24; 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]pyrazidin-4(3H)-one, 3g

Yield = 70%; mp = 190–192 °C (EtOH); 1H-NMR (CDCl3) δ 5.50 (s, 2H, CH2Ph), 5.65 (m, 1H, Ar), 6.50 (exch br s, 2H, NH2), 7.00 (m, 2H, Ar), 7.25–7.40 (m, 5H, Ar), 7.50–7.60 (m, 4H, 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 C26H17FN6O: C, 68.48; H, 4.37; N, 19.17. Found C, 68.34; H, 4.43; N, 19.13.

General Procedures for 4b-f

A mixture of compounds 3b-g (0.21 mmol), triethyl orthoformate (18 mmol) and a catalytic amount of conc. H2SO4 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]pyrazidin-4(3H)-one, 4b

Yield = 68%; mp = 246–248 °C dec (EtOH); 1H-NMR (CDCl3) δ 5.55 (s, 2H, CH2Ph), 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 C27H17ClFN6O: 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]pyrazidin-4(3H)-one, 4c

Yield = 69%; mp = 250–253 °C dec (EtOH); 1H-NMR (CDCl3) δ 5.50 (s, 2H, CH2Ph), 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 C27H17FN7O: 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]pyrazidin-4(3H)-one, 4d

To a solution of 4c (0.21 mmol) in 37% HCl (1 mL), a solution of SnCl2 (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 CH2Cl2 (3 × 15 mL) and the solvent was evaporated in vacuo affording a residue oil which was purified by column chromatography using CH2Cl2/CH3OH as eluent.

Yield = 29%; mp = >300 °C (EtOH); 1H-NMR (CDCl3) δ 5.55 (s, 2H, CH2Ph), 6.10 (s, 1H, Ar), 6.65 (exch br s, 2H, NH2), 7.20–7.40 (m, 5H, Ar), 7.30–7.40 (m, 4H, Ar), 8.05 (m, 1H, Ar), 9.45 (s, 1H, Ar); MS m/z 372 [M]+; Anal. Calcd for C20H16FN5O: 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 3a1 (0.14 mmol), the appropriate anhydride (4–9 mmol) and a catalytic amount of conc. H2SO4 was added. The mixture 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 CH2Cl2 (3 × 15 mL) and the solvent was evaporated in vacuo affording a residue oil which was purified by column chromatography using CH2Cl2/CH3OH as eluent.

Yield = 29%; mp = >300 °C (EtOH); 1H-NMR (CDCl3) δ 5.55 (s, 2H, CH2Ph), 6.10 (s, 1H, Ar), 6.65 (exch br s, 2H, NH2), 7.20–7.40 (m, 5H, Ar), 7.30–7.40 (m, 4H, Ar), 8.05 (m, 1H, Ar), 9.45 (s, 1H, Ar); MS m/z 493 [M]+; Anal. Calcd for C27H17FN7O: C, 67.29; H, 3.56; N, 14.53. Found C, 67.48; H, 3.56; N, 14.58.

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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 \text{NaHCO}_3. Compound 3a was filtered off and recrystallized from ethanol, while for compounds 6b and 6c the mixture was extracted with CH\textsubscript{2}Cl\textsubscript{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]pyrazin-4(3H)-one, 6a

Yield = 89%; mp = 217–220 °C (EtOH); \textsuperscript{1}H-NMR (CDCl\textsubscript{3}) \(\delta\) 1.60 (d, 6H, \text{CH}(\text{CH}_3)_2), 2.10 (m, 2H, CH\textsubscript{2}CH\textsubscript{2}CH\textsubscript{3}), 2.55 (m, 2H, CH\textsubscript{2}Ph), 6.05 (s, 1H, Ar), 7.30–7.50 (m, 8H, Ar), 7.60–7.80 (m, 6H, Ar), 9.80 (s, 1H, Ar); MS \(m/z\) 490 [M\textsuperscript{+}]; Anal. Calcd for C\textsubscript{31}H\textsubscript{24}FN\textsubscript{5}O: C, 71.94; H, 4.67; N, 13.53. Found C, 71.94; H, 4.67; N, 13.50.

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

Yield = 74%; mp = 188–191 °C (EtOH); \textsuperscript{1}H-NMR (CDCl\textsubscript{3}) \(\delta\) 1.15 (t, 3H, \text{CH}(\text{CH}_3)), 2.10 (m, 2H, CH\textsubscript{2}CH\textsubscript{2}CH\textsubscript{3}), 3.55 (m, 2H, CH\textsubscript{2}CH\textsubscript{2}CH\textsubscript{3}), 5.55 (s, 2H, CH\textsubscript{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\textsuperscript{+}]; Anal. Calcd for C\textsubscript{31}H\textsubscript{24}FN\textsubscript{5}O: C, 73.60; H, 4.94; N, 14.31. Found C, 73.35; H, 4.93; N, 14.35.

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

Yield = 87%; mp = 190–192 °C (EtOH); \textsuperscript{1}H-NMR (CDCl\textsubscript{3}) \(\delta\) 1.05 (t, 3H, \text{CH}(\text{CH}_3)), 1.50–1.60 (m, 2H, CH\textsubscript{2}CH\textsubscript{2}CH\textsubscript{3}), 2.00–2.10 (m, 2H, CH\textsubscript{2}CH\textsubscript{2}CH\textsubscript{3}), 3.50–3.60 (m, 2H, CH\textsubscript{2}CH\textsubscript{2}CH\textsubscript{3}), 5.55 (s, 2H, CH\textsubscript{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\textsuperscript{+}]; Anal. Calcd for C\textsubscript{31}H\textsubscript{26}FN\textsubscript{5}O: C, 73.94; H, 4.67; N, 13.91. Found C, 73.77; H, 4.51; N, 13.95.

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

A mixture of 8\textsuperscript{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); \textsuperscript{1}H-NMR (CDCl\textsubscript{3}) \(\delta\) 4.70 (s, 2H, CH\textsubscript{2}S), 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), 15.00 (exch br s, 1H, NH); MS \(m/z\) 464 [M\textsuperscript{+}]; Anal. Calcd for C\textsubscript{31}H\textsubscript{26}FN\textsubscript{5}S: C, 64.33; H, 3.24; N, 18.76. Found C, 64.13; H, 3.25; N, 18.79.

4.2. Adenosine Receptor Binding Assay\textsuperscript{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\textsubscript{1}, A\textsubscript{2\alpha}, and A\textsubscript{3} ARs we evaluated the ability of different compound concentrations to displace [\textsuperscript{125}I]8-cyclopentyl-1,3-dipropylxanthine ([\textsuperscript{125}I]DPCPX, for CHO-A\textsubscript{1}), [\textsuperscript{125}I]5’-N-ethylcarboxamidoadenosine ([\textsuperscript{125}I]NECA, for CHO A\textsubscript{2A}), or [\textsuperscript{125}I]4-amino-3-nitrobenzyl-5’-N-methylcarboxamidoadenosine ([\textsuperscript{125}I]AB-MECA, for CHO-A\textsubscript{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 $A_1$CHO 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 [3H]DPCPX (3 nM) and six different concentrations of the compounds. Non-specific binding was determined in the presence of 20 nM R-PIA. The dissociation constant ($K_d$) of [3H]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 (50 mM Tris-HCl, 10 mM MgCl$_2$, and 2 units/mL ADA, pH 7.4) in the presence of 0.14 nM NECA and six different concentrations of the synthesized compounds. Non-specific binding was determined in the presence of 100 μM DPCPX. The dissociation constant ($K_d$) of [3H]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 (50 mM Tris-HCl, 10 mM MgCl$_2$, 1 mM EDTA, and 2 units/mL ADA, pH 7.4) in the presence of 20 nM of [3H]NECA and six different concentrations of the synthesized compounds. Non-specific binding was determined in the presence of 50 μM R-PIA. The dissociation constant ($K_d$) of [3H]NECA in $A_3$ CHO cell membranes was 30 nM.

5. References


Povzetek

Članek poroča o sintezi in biološkem ovrednotenju nove serije pirazolo[1’,5’:1,6]pirimidid[4,5-d]piridazin-4(3H)-onov kot ligandov človeškega $A_1$ adenosinzkega receptorja. Triciklično ogrodje je bilo spremenjeno na položajih 6 in 9 z uvedbo majhnih alkilnih verig in substituiranih fenilov. Najbolj zanimive spojine so pokazale $K_i$ za $A_1$ v subnmimolarnem območju (0.105–0.244 μM). Najzanimivnejši del (spojina 4c) je pokazal znatno afiniteto za $A_1$ ($K_i = 0.132 μM$), skupaj z dobro selektivnostjo za $A_{2A}$ (43 % inhibicije pri 10 μM) in $A_3$ (46 % inhibicije pri 10 μM).

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