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Pyrazolo[1′,5′:1,6]pyrimido[4,5-*d*]pyridazin-4(3*H*)-ones as selective human A_1 adenosine receptor ligands

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ABSTRACT

A series of pyrazolo[1′,5′:1,6]pyrimido[4,5-*d*]pyridazin-4(3*H*)-ones was synthesized and tested in radioligand binding assays to determine their affinities for the human adenosine A_1, A_{2A}, A_{2B} and A_3 receptors. Results indicated that this scaffold is appropriate for adenosine receptor subtype A_1 ligands and that the best arranged groups around this scaffold are 3- and 4-pyridinyl at position 1, benzyl at position 3, hydrogen at position 6 and 3-thienyl or phenyl at position 9. The most interesting compounds showed K_i for A1 in the nanomolar range and an appreciable selectivity for other receptor subtypes.

1. Introduction

Adenosine is an endogenous and ubiquitous neuromodulator that elicits its biological functions by interacting with at least four receptor subtypes, classified as A_1, A_{2A}, A_{2B} and A_3.[1,2] All four adenosine receptors belong to the superfamily of seven transmembrane G-protein-coupled receptors and have been cloned from different species.[3]

A_1 and A_3 receptors are coupled with G_i protein, with a consequent decrease in cellular cAMP level; on the contrary, A_{2A} and A_{2B} are coupled to G_S protein and stimulate adenylate cyclase, leading to an increase in cellular cAMP levels.[4] The A_1 receptor is able to activate different messenger systems such as phospholipase C, potassium channels and inhibits calcium channels,[2] while subtype A_3 is positively coupled to phospholipase C and D.[5,6]

The potential therapeutic applications of agonists and antagonists of the various receptor subtypes have been widely investigated in these last years.[7–12] A_1 antagonists have been proposed for the treatment of central nervous system pathologies, such as Alzheimer's disease, as diuretics and as antihypertensives.[10,13–15] Selective antagonists of subtype A_{2A}, which is present in high density in the striatum,[13] but also in numerous peripheral tissue such as platelets, lymphocytes and neutrophils,[16–18] are promising drugs for the treatment of Parkinson's disease.[19] A_{2B} receptors are implicated in vascular tone, gene expression, cell growth, mast cell degranulation and hepatic glucose balance,[20–22] and are proposed as antiasthmatic and antidiabetic drugs.[23–25] Finally, antagonists of A_3 receptors, which are particularly abundant in human lung and liver[26] and seem also to be involved in cell survival regulation,[27] could be useful in the treatment of inflammatory states,[28] asthma,[29] chronic obstructive pulmonary disease,[30] cancer[31] and glaucoma.[32]

Looking at the more recent literature about polyheterocyclic derivatives, numerous series have been developed as potent and subtype selective adenosine antagonists (Fig. 1). The 3-aryl-[1,2,4]triazino[4,3-*a*]-benzimidazol(10*H*)-4-one derivative **A**[33] is a selective A_1 antagonist, displaying high affinity for the bovine A_1 subtype (K_i = 18 nM) and significant selectivity for A_{2A} (K_i >10,000 nM) and A_3 (K_i >1000 nM). The pyrazolo[4,3-*e*]-1,2,4-triazolo[1,5-*c*]pyrimidine **B** is a very potent and selective A_{2A} antagonist (hK_i = 0.048 nM) and its high affinity for the adenosine A_{2A} receptor makes it an attractive candidate for PET studies.[34] Among the A_{2B} selective antagonists, a significant example is compound **C** which has a K_i = 20 nM for the human A_{2B} subtype.[35] Finally, one of the most potent and selective A_3 antagonists

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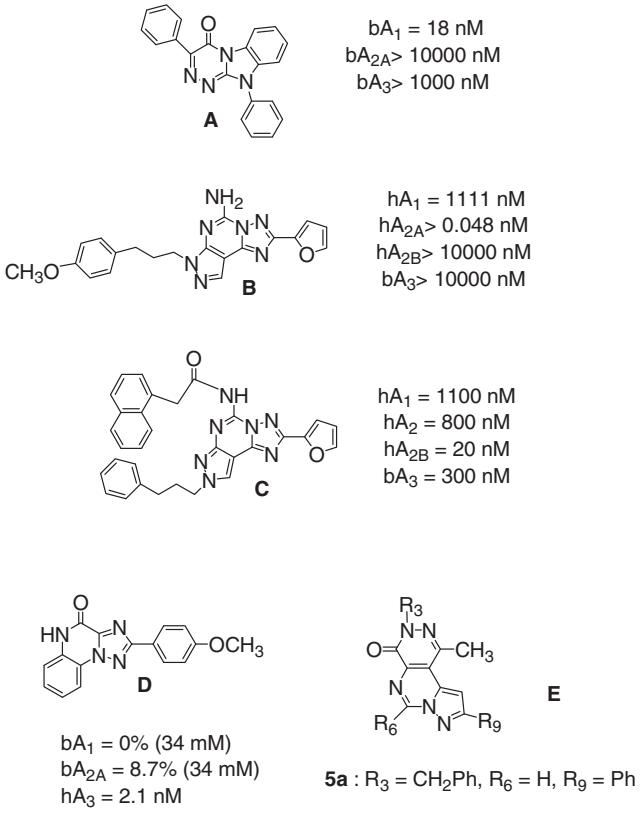


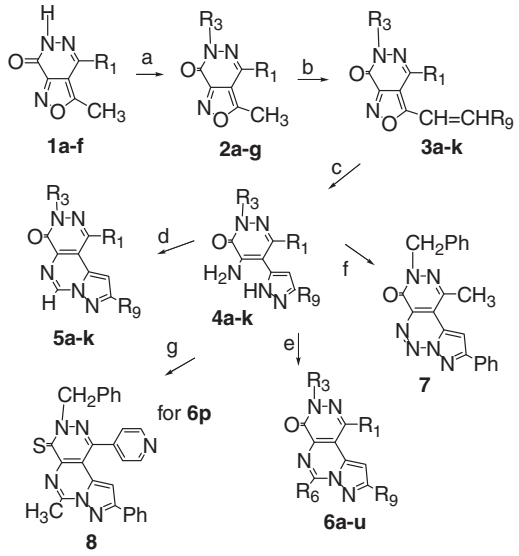
Figure 1. Adenosine receptors antagonists.

reported up till now is represented by the 1,2,4-triazolo[1,5-*a*]quinoxalin-4-one (compound **D**), synthesized by Catarzi and coworkers[36] which exhibits a $K_i = 2.1$ nM for human subtype A_3, and a complete selectivity for hA_3 versus bA_1 selectivity showing 0% inhibition of binding at 34 μM concentration at the letter subtype.

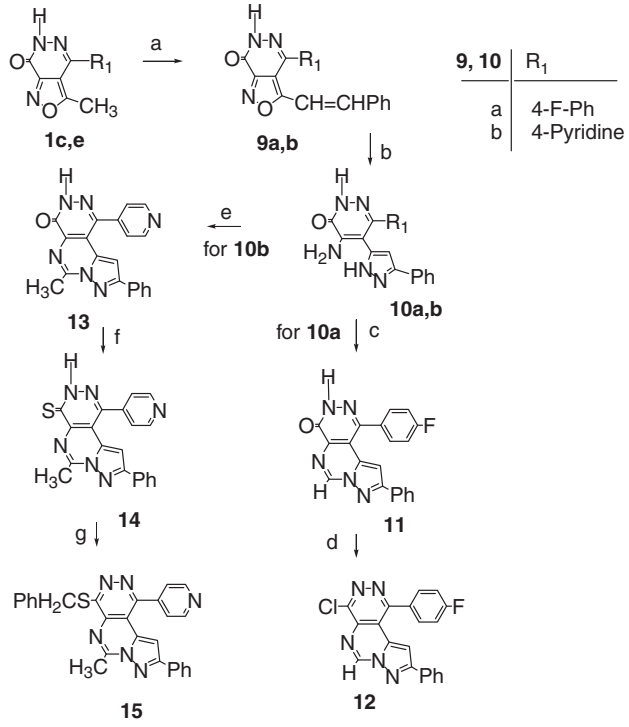
Taking into account the above reported examples and by considering the classic structural requirements proposed by Jacobson et al.[13] for adenosine receptor antagonists (planar, aromatic or π electron rich and nitrogen-containing heterocycles) we planned to test some of the pyrazolo[1′,5′:1,6]pyrimido[4,5-*d*]pyridazin-4(3*H*)-ones derivatives of **E** (Fig. 1) as adenosine receptors ligands. These compounds, synthesized in recent years in our laboratory, are potent and selective phosphodiesterase 5 (PDE5) inhibitors,[37,38] but formally also comply with the structural requirements reported for the binding to the adenosine receptors.[13] Our results have been encouraging, with some substituents showing submicromolar levels of affinity for A_1 subtype. In particular 3-benzyl-1-methyl-9-phenyl-pyrazolo[1′,5′:1,6]pyrimido[4,5-*d*]-pyridazin-4(3*H*)-one **5a**[38] was selected as the lead compound in the design synthesis of adenosine receptor ligands. Preliminarly we modified position 1, 6 and 9 of the scaffold **E** maintaining unchanged the benzyl group at position 3.

2. Chemistry

All the final compounds were synthesized as reported in Schemes 1–3, following a common three-step synthetic route previously described.[37,38] Thus isoxazolo[3,4-*d*]pyridazinones were condensed with the available arylaldehydes to give the vinyl derivatives which, in turn, were treated with hydrazine hydrate to furnish 5-pyrazolylpyridazinones intermediates. Ring closure to pyrazolo[1′,5′:1,6]pyrimido[4,5-*d*]pyridazin-4(3*H*)-ones was carried out in different conditions depending on the desired substituent at position 6.

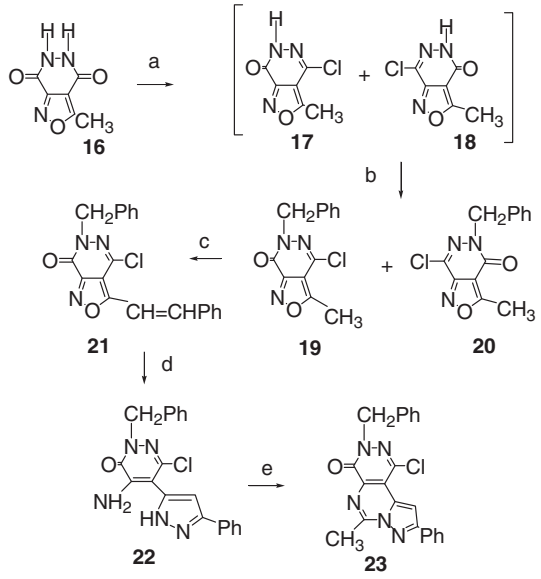


Scheme 1. Synthesis of pyrazolo[1′,5′:1,6]pyrimido[4,5-*d*]pyridazin-4(3*H*)-ones **5a–k**, **6a–u**, **7** and **8**. Reagents: (a) R_3X, DMF, K_2CO_3; (b) R_9CHO, CH_3ONa, CH_3OH; (c) NH_2NH_2, C_2H_5OH; (d) $CH(OC_2H_5)_3$, H_2SO_4, DMF; (e) $(R_6CO)_2O$; (f) CH_3COOH, $NaNO_2$; (g) Lawesson's reagent, toluene.



Scheme 2. Synthesis of pyrazolo[1′,5′:1,6]pyrimido[4,5-*d*]pyridazin-4(3*H*)-ones **12** and **15**. Reagents: (a) PhCHO, CH_3ONa, CH_3OH; (b) NH_2NH_2, C_2H_5OH; (c) $CH(OC_2H_5)_3$, H_2SO_4, DMF; (d) $POCl_3$; (e) $(CH_3CO)_2O$; (f) Lawesson's reagent, toluene; (g) benzylchloride, DMF, K_2CO_3.

In Scheme 1 the key intermediates **2a–g** (**2a**,[38] **2c**,[39] **2f**[40]) were obtained by alkylation of the 2-unsubstituted isoxazolo[3,4-*d*]pyridazinones **1a–f** with the appropriate halides, in standard conditions, as reported in literature (**1a**,[42] **1b**,[41] **1c**,**d**,[44] **1e**,[39] **1f**[43]). The 4-amino-5-pyrazolylderivatives **4a–k** (compounds **4a**,**b** and their precursors **3a**,**b** are reported in Ref. 38) were treated with triethylorthoformate, catalytic amount of concentrated sulfuric



Scheme 3. Synthesis of pyrazolo[1′,5′:1,6]pyrimido[4,5-*d*]pyridazin-4(3*H*)-ones **23**. Reagents: (a) $POCl_3$; (b) benzylchloride, DMF, K_2CO_3; (c) PhCHO, CH_3ONa, CH_3OH; (d) NH_2NH_2, C_2H_5OH; (e) $(CH_3CO)_2O$.

acid in anhydrous DMF, affording the 6-unsubstituted compounds **5a–k** (**5a**[38]). Definition of compounds **1–5** is reported in Tables 1–3.

When the ring closure of the intermediates of type **4** was performed with the appropriate anhydride under refluxing conditions, the corresponding 6-substituted tricyclic compounds **6a–u** were recovered (**6a**[37]). For compound **6p** a further transformation was performed using Lawesson's reagent in toluene to afford the final thioderivative **8**. Compound **6** is defined in Table 4.

Finally, the hexaaza-cyclopenta[*a*]naphthalen-6-one **7** was synthesized by using a saturated solution of $NaNO_2$ in glacial acetic acid.

Scheme 2 reports the synthesis of the final compounds **12** and **15** which were obtained following a procedure similar to that described in Scheme 1. After building the tricyclic system (compounds **11** and **13**), the final compounds **12** and **15** were obtained respectively by treatment with $POCl_3$ at reflux (compound **12**) and by transformation in the corresponding 4-thione derivatives with Lawesson's reagent (**14**) followed by selective alkylation at position 4 (compound **15**).

In order to insert a chlorine at position 1 (Scheme 3), we repeated the same three-step synthesis starting from the isoxazolo[3,4-*d*]pyridazin-4,7-dione **16**[45] which was treated with $POCl_3$. As expected, a mixture of the two isomers **17** and **18** was obtained; the unresolved mixture was reacted with benzyl chloride in anhydrous DMF to give the corresponding isomers **19** and **20**. After separation by column chromatography, compound **19** was submitted to the above described synthetic route.

Elemental analyses of all new compounds are reported as Supplementary data.

Table 1
Key to substituents for compounds **1a–f**

Compd **1**	R_1
a	CH_3
b	Ph
c	4-F-Ph
d	3-F-Ph
e	4-Pyridyl
f	3-Pyridyl

Table 2
Key to substituents for compounds **2a–g**

Compd **2**	R_1	R_3
a	CH_3	CH_2Ph
b	CH_3	$(CH)CH_3Ph$
c	Ph	CH_2Ph
d	4-F-Ph	CH_2Ph
e	3-F-Ph	CH_2Ph
f	4-Pyridyl	CH_2Ph
g	3-Pyridyl	CH_2Ph

Table 3
Key to substituents for compounds **3–5a–k**

Compd **3–5**	R_1	R_3	R_9
a	CH_3	CH_2Ph	Ph
b	CH_3	CH_2Ph	3-Thienyl
c	CH_3	CH_2Ph	$N(CH_3)_2$ (for **3**) and H (for **4** and **5**)
d	CH_3	$(CH)CH_3Ph$	Ph
e	Ph	CH_2Ph	Ph
f	4-F-Ph	CH_2Ph	Ph
g	4-F-Ph	CH_2Ph	3-Thienyl
h	3-F-Ph	CH_2Ph	Ph
i	3-F-Ph	CH_2Ph	3-Thienyl
j	4-Pyridyl	CH_2Ph	Ph
k	3-Pyridyl	CH_2Ph	Ph
h	3-F-Ph	CH_2Ph	Ph

Table 4
Key to substituents for compounds **6a–u**

Compd **6**	R_1	R_3	R_6	R_9
a[37]	CH_3	CH_2Ph	CH_3	Ph
b	CH_3	CH_2Ph	CH_3	H
c	CH_3	$(CH)CH_3Ph$	CH_3	Ph
d	CH_3	$(CH)CH_3Ph$	C_2H_5	Ph
e	Ph	CH_2Ph	CH_3	Ph
f	Ph	CH_2Ph	C_2H_5	Ph
g	Ph	CH_2Ph	iC_3H_7	Ph
h	Ph	CH_2Ph	nC_3H_7	Ph
i	4-F-Ph	CH_2Ph	CH_3	Ph
j	4-F-Ph	CH_2Ph	C_2H_5	Ph
k	4-F-Ph	CH_2Ph	CF_3	Ph
l	3-F-Ph	CH_2Ph	CH_3	Ph
m	3-F-Ph	CH_2Ph	C_2H_5	Ph
n	3-F-Ph	CH_2Ph	CH_3	3-Thienyl
o	3-F-Ph	CH_2Ph	C_2H_5	3-Thienyl
p	4-Pyridyl	CH_2Ph	CH_3	Ph
q	4-Pyridyl	CH_2Ph	C_2H_5	Ph
r	4-Pyridyl	CH_2Ph	nC_3H_7	Ph
s	4-Pyridyl	CH_2Ph	iC_3H_7	Ph
t	3-Pyridyl	CH_2Ph	CH_3	Ph
u	3-Pyridyl	CH_2Ph	C_2H_5	Ph

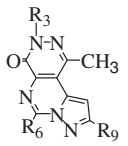
3. Biological results and discussion

All final compounds were tested in radioligand binding assays to determine their affinities for the human adenosine A_1, A_{2A}, A_{2B} and A_3 receptors and all compounds which do not inhibit the binding by 50% or more at 10 μM were considered inactive.

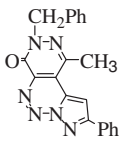
SAR analysis was performed by considering 3 series of compounds which differ in the substituent at position 1 (methyl, (fluoro)phenyl and pyridine) and the biological results are reported in Tables 5–7.

Starting the analyses from the sub-class of 1-methyl derivatives (compounds **5a–d**, **6a–d**, **7** and **23** Table 5) we can observe that the lead compound **5a**[38] showed K_i = 104.3 nM for A_1, 73-fold selectivity versus A_{2B} and no affinity for A_{2A} and A_3 (binding <50% at 10 μM); the replacement of the phenyl at R9 of **5a** with a 3-thienyl

Table 5
Binding activity at human A_1, A_{2A}, A_{2B} and A_3 adenosine receptors



5a-d, 6a-d



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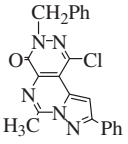
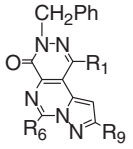


23

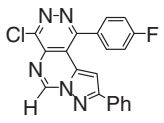
Compd	R_3	R_6	R_9	hA_1[a,b]	hA_{2A}[a,c]	hA_{2B}[a,d]	hA_3[a,e]
5a[38]	CH_2Ph	H	Ph	104.3 ± 4.7	< 50%	7560 ± 1640	<50%
5b	CH_2Ph	H	3-Thienyl	85.3 ± 2.5	7365 ± 805	4650 ± 790	<50%
5c	CH_2Ph	H	H	<50%	<50%	<50%	<50%
5d	$(CH)CH_3Ph$	H	Ph	475.2 ± 127.1	<50%	<50%	<50%
6a[37]	CH_2Ph	CH_3	Ph	3250 ± 114	<50%	<50%	<50%
6b	CH_2Ph	CH_3	H	<50%	<50%	<50%	<50%
6c	$(CH)CH_3Ph$	CH_3	Ph	3080 ± 1210	<50%	<50%	4730 ± 130
6d	$(CH)CH_3Ph$	C_2H_5	Ph	2385 ± 225	<50%	<50%	<50%
7				<50%	<50%	<50%	<50%
23				1616 ± 723	3150 ± 660	<50%	<50%
DPCPX				3.2 ± 0.2[f]	260 ± 18[f]	51 ± 1.5[f]	1300 ± 125[f]

[a] The binding activity is reported as K_i (nM) or percentage of inhibition at 10 μM; values are means ± DS of four separate assays, each performed in triplicate.
[b] Displacement of [^{3}H]DPCPX binding in CHO-A1 cells membranes.
[c] Displacement of specific [^{3}H]ZM241385 in HeLa-A_{2A} cells.
[d] Displacement of [^{3}H]DPCPX from HEK-293-A_{2B} cells.
[e] Displacement of [^{3}H]NECA in HeLa-A_3 cells.
[f] Values are reported as K_i (nM) ± SEM.

Table 6
Binding activity at human A_1 adenosine receptor



5e-i, 6e-o

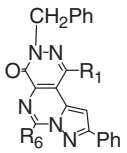


12

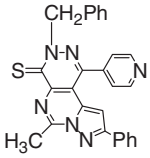
Compd[a]	R_1	R_6	R_9	hA_1[b,c]
5e	Ph	H	Ph	1432 ± 947
5f	4-F-Ph	H	Ph	252.3 ± 60.1
5g	4-F-Ph	H	3-Thienyl	122.9 ± 52.2
5h	3-F-Ph	H	Ph	92.7 ± 3.5
5i	3-F-Ph	H	3-Thienyl	188.5 ± 20.5
6e	Ph	CH_3	Ph	1595 ± 115
6f	Ph	C_2H_5	Ph	2445 ± 95
6g	Ph	iC_3H_7	Ph	<50%
6h	Ph	nC_3H_7	Ph	563.5 ± 99.5
6i	4-F-Ph	CH_3	Ph	<50%
6j	4-F-Ph	C_2H_5	Ph	<50%
6k	4-F-Ph	CF_3	Ph	<50%
6l	3-F-Ph	CH_3	Ph	3245 ± 115
6m	3-F-Ph	C_2H_5	Ph	<50%
6n	3-F-Ph	CH_3	3-Thienyl	<50%
6o	3-F-Ph	C_2H_5	3-Thienyl	<50%
12				<50%
DPCPX				3.2 ± 0.2[d]

[a] None of the compounds is able to inhibit the binding by 50% or more at 10 μM for A_{2A}, A_{2B} and A_3.
[b] The binding activity is reported as K_i (nM) or percentage of inhibition at 10 μM; values are means ± DS of four separate assays, each performed in triplicate.
[c] Displacement of [^{3}H]DPCPX binding in CHO-A1 cells membranes.
[d] Values are reported as K_i (nM) ± SEM.

Table 7
Binding activity at human A_1 and A_{2A} adenosine receptors



5j,k, 6p-u



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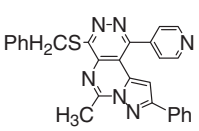

15

Compd[a]	R_1	R_6	hA_1[b,c]	hA_{2A}[b,d]
5j	4-Py	H	10.2 ± 2.3	67.9 ± 1.9
5k	3-Py	H	11.4 ± 2.2	805.1 ± 374.5
6p	4-Py	CH_3	43.6 ± 16.8	501.2 ± 238.3
6q	4-Py	C_2H_5	71.8 ± 8.9	2490 ± 290
6r	4-Py	nC_3H_7	28.3 ± 5.1	772.5 ± 216.5
6s	4-Py	iC_3H_7	192.3 ± 37.4	5190 ± 2060
6t	3-Py	CH_3	47.4 ± 5.9	2115 ± 1055
6u	3-Py	C_2H_5	180.2 ± 70.1	<50%
8			267.4 ± 14.3	572.1 ± 20.4
15			<50%	<50%
DPCPX			3.2 ± 0.2[d]	260 ± 18[d]

[a] None of the compounds is able to inhibit the binding by 50% or more at 10 μM for A_{2B} and A_3.
[b] The binding activity is reported as K_i (nM) or percentage of inhibition at 10 μM; values are means ± DS of four separate assays, each performed in triplicate.
[c] Displacement of [^{3}H]DPCPX binding in CHO-A1 cells membranes.
[d] Displacement of specific [^{3}H]ZM241385 in HeLa-A_{2A} cells.
[e] Values are reported as K_i (nM) ± SEM.

nucleus (**5b**) was associated with a small improvement in A_1 affinity (K_i = 85.3 nM) but also with a decrease of selectivity for A_{2A} and A_{2B} subtype receptors (90- and 56-fold versus A_{2A} and A_{2B}, respectively). Also for **5b** no affinity for A_3 was observed. Elimination of the phenyl group at R9 (compound **5c**) led to a dramatic reduction in affinity for all adenosine subtype receptors, this finding indicating that at position 9 the phenyl group is the optimal substituent with respect to both potency and selectivity. The introduction of a methyl at position 6 (**6a**[37]) in the lead compound **5a** led decreased affinity for the A_1 subtype, resulting in a loss of

potency of one order of magnitude (K_i = 3250 nM), while in compound **6a** elimination of the phenyl at position 9 led to an inactive compound (**6b**), according to the results above reported. The introduction of a methyl group on the benzyl at position 3 (compound **5d**, K_i = 475.2 nM) reduced the A_1 affinity by about fourfold with respect to the benzyl analogue **5a**, but increased the selectivity toward all other receptor subtypes. The introduction at position 6 of **5d** of a methyl or an ethyl group (compounds **6c** and **6d**) resulted in a loss of affinity of one order of magnitude for A_1 and, surprisingly, the appearance of affinity for the A_3 subtype in compound **6c** (K_i = 3080 nM). Finally the isosteric replacement of CH with N (compound **7**) in the pyrimidine nucleus resulted in a complete loss of A_1 affinity with respect to the deaza analogue **5a**. The same strategy applied to CH_3/Cl replacement which led to negative effects such as A_1 affinity and A_{2A} selectivity (compound **23**, A_1 K_i = 1616 nM).

The biological results of the 1-phenyl and 1-(fluoro)phenyl series (compounds **5e**–**i**, **6e**–**o** and **12**) are presented in Table 6. Only data relating to the A_1 subtype receptor are reported since none of the compounds were able to inhibit the binding by 50% or more at 10 μM for A_{2A}, A_{2B} and A_3, thus showing a very good selectivity. In the phenyl series the A_1 affinity was evaluated as a function of the nature of the alkyl group at position 6. The trend observed (*n*-Pr > H and Me > Et > *i*-Pr) suggested that a linear carbon chain is a strict requirement in this part of the molecule, **6h** (R6 = *n*-Pr, K_i = 563.5 nM) being the most active compound and **6g** (R6 = *i*-Pr) completely devoid of affinity.

In the 1-(4-fluorophenyl) subseries it emerged that keeping benzyl at position 3 and phenyl at position 9, all 6-alkyl substituted compounds (**6i**–**k**) are inactive (inhibition <50% at 10 μM). Replacement of the alkyl chain at position 6 with a hydrogen created compound **5f**, whose K_i was 252.3 nM. The substitution of the phenyl at position 9 of compound **5f** with a 3-thienyl afforded compound **5g** which showed an improved affinity for A_1 subtype receptor (K_i = 122.9 nM), in agreement with the results obtained for the 1-methyl subseries. Elimination of the benzyl chain and aromatization of the pyridazine ring led to an inactive compound (**12**). An analogue trend was observed for the series of 1-(3-fluorophenyl) derivatives **6l**–**o** and **5h**,**i**, where only the 9-phenyl derivative **5h** and the corresponding 9-(3-thienyl) derivative **5i** showed an appreciable affinity for A_1 (K_i = 92.7 and 188.5 nM respectively). All the other substitutions, with the exception of the 6-methyl derivative **6l** (K_i = 3245 nM) are not able to inhibit the binding by 50% at 10 μM.

Biological data related to 1-pyridinyl derivatives **5j**,**k**, **6p**–**u**, **8** and **15** are shown in Table 7, where also the column of A_{2A} affinity values is also reported showing that this series has appreciable binding for this adenosine subtype receptor. None of the compounds is able to inhibit the binding by 50% or more at 10 μM for A_{2B} and A_3. The analysis of 1-(4-pyridinyl) derivatives demonstrates that for this series the difference in affinity among the various 6-alkyl derivatives was significantly reduced, with the Me, Et and *n*-Pr derivatives **6p**–**r** showing good values for A_1 in the same nanomolar range (K_i = 28.3–71.8 nM). Analogously to the 1-phenyl series, the 6-unsubstituted term **5j** is the most potent compound (K_i = 10.2 nM) and the 6-branched alkyl derivative **6s** is the less active (K_i = 192.3 nM). All these compounds displayed low A1 selectivity, in particular **5j** (A_{2A}/A_1 = 7). Moreover, the essential role played by the carbonyl function at position 4 was evidenced by observing the sixfold reduced affinity for A_1 of the sulfur analogue **8**. Further evidence of the key role played by the CO was suggested by the behaviour of compound **15** in which the absence of CO was associated with the shift of the benzyl group in a neighbouring position. Also 1-(3-pyridyl) derivatives (Table 7) were active in the nanomolar range. In particular the 6-unsubstituted **5k** displayed a K_i = 11.4 nM for A_1 and good A_{2A}/A_1 selectivity (71).

Since the pyrazolo[1′,5′:1,6]pyrimido[4,5-*d*]pyridazin-4(3*H*)-ones scaffold is an appropriate system also for PDE5 inhibitors, as above mentioned,[37,38] we selected and tested on this enzyme some representative compounds. Compounds **6p**, **6t** and **6u** belonging to the 1-pyridyl series and displaying an affinity for A_1 subtype in the nanomolar range (K_i = 43.6–180.2 nM), showed a low or a very low PDE5 inhibitory activity tested at 2 μM (**6p**: 5.7%; **6t**: 29.7%; **6u**: 32.8%). On the contrary, 1-methyl derivatives **5a** and **6a**, previously published as PDE5 inhibitors[37,38] are active, with different potency, as A_1 ligands and PDE5 inhibitors (**5a**: $K_i(A_1)$ = 104.3 nM and IC_{50}(PDE5) = 180 nM; **6a**: $K_i(A_1)$ = 3250 nM and IC_{50}(PDE5) = 160 nM). This fact suggests that the nature of the substituent at position 1 of the tricyclic system is important to address the activity to PDE5 or A_1 subtype receptor (data not shown).

4. Conclusions

In conclusion, by taking all these data together, it is possible to delineate the first SAR for pyrazolopyrimidopyridazinone. It emerged that it is an appropriate system for adenosine receptor subtype A_1 ligands and that the optimal groups around this scaffold are 3- and 4-pyridinyl at position 1, benzyl at position 3, hydrogen at position 6 and 3-thienyl or phenyl at position 9. In this preliminary study we identified ligands for hA_1 adenosine subtype receptor endowed with good selectivity versus other adenosine subtypes. In the series of 1-pyridyl derivatives, compounds **5k** and **6t** displayed the best balance between potency and selectivity as well as compounds **5b** and **5h** in the series of 1-methyl and 1-fluorophenyl derivatives, respectively. Further studies are in progress to improve our knowledge of the SAR.

5. Experimental section

5.1. Chemistry

All melting points were determined on a Büchi apparatus and are uncorrected. ^{1}H NMR spectra were recorded with Avance 400 instruments (Bruker Biospin Version 002 with SGU). Chemical shifts are reported in ppm, 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 Perkin-Elmer 260 elemental analyzer for C, H, N and the results were within ±0.4% of the theoretical values, unless otherwise stated. Reagents and starting materials were commercially available.

5.1.1. General procedure for 2b, 2d–e and 2g

A mixture of isoxazolopyridazinone **1a**, **1c**–**d** and **1f** (**1a**,[42] **1c**,[44] **1d**,[44] **1f**[43]) (1.1 mmol), K_2CO_3 (2.2 mmol) and the appropriate alkylhalide (1.7–4.3 mmol) in anhydrous DMF (3 mL) was stirred at 70–90 °C for 0.5–3 h. After cooling, the mixture was diluted with cold water and the precipitate recovered by suction. For compound **2b** the reaction was carried out at 90 °C for 10 h: after dilution with cold water 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 cyclohexane/ethyl acetate 1:2 as eluent.

5.1.1.1. 3,4-dimethyl-6-(1-phenylethyl)isoxazolo[3,4-*d*]pyridazin-7(6*H*)-one, 2b. Yield = 72%; oil (purified by column chromatography using cyclohexane/ethyl acetate 1:2 as eluent); ^{1}H NMR ($CDCl_3$) δ 1.80 (d, 3H, $CHCH_3$), 2.45 (s, 3H, 4-CCH_3), 2.80 (s, 3H, 3-CCH_3), 6.40 (q, 1H, $CHCH_3$), 7.10–7.60 (m, 5H, Ar).

5.1.1.2. 6-Benzyl-4-(4-fluorophenyl)-3-methylisoxazolo[3,4-*d*]pyridazin-7(6*H*)-one, 2d. Yield = 85%; mp = 145–147 °C (EtOH); ^{1}H NMR ($CDCl_3$) δ 2.45 (s, 3H, CH_3), 5.30 (s, 2H, CH_2), 7.15–7.60 (m, 9H, Ar).

5.1.1.3. 6-Benzyl-4-(3-fluorophenyl)-3-methylisoxazolo[3,4-*d*]pyridazin-7(6*H*)-one, 2e. Yield = 85%; mp = 138–140 °C (EtOH); ^{1}H NMR ($CDCl_3$) δ 2.60 (s, 3H, CH_3), 5.35 (s, 2H, CH_2), 7.20–7.50 (m, 9H, Ar).

5.1.1.4. 6-Benzyl-3-methyl-4-pyridin-3-yl-isoxazolo[3,4-*d*]pyridazin-7(6*H*)-one, 2g. Yield = 84%; mp = 142–145 °C (EtOH); ^{1}H NMR ($CDCl_3$) δ 2.60 (s, 3H, CH_3), 5.40 (s, 2H, CH_2), 7.20–7.40 (m, 6H, Ar), 7.95 (m, 1H, Ar), 8.80 (m, 2H, Ar).

5.1.1.5. 6-Benzyl-3-(2-dimethylaminovinyl)-4-methylisoxazolo[3,4-*d*]pyridazin-7(6*H*)-one, 3c. A suspension of **2a**[38] (0.4 mmol) in *N,N*-dimethylformammide dimethyl acetal (15 mmol) was stirred at 90 °C for 15 min. After cooling the precipitate was recovered by suction.

Yield = 82%; mp = 203–206 °C (MeOH); ^{1}H NMR ($CDCl_3$) δ 1.90 (s, 3H, 4-CCH_3), 3.00 (s, 6H, $N(CH_3)_2$), 5.15 (d, 1H, CH =), 5.25 (s, 2H, CH_2), 7.20–7.35 (m, 5H, Ar), 7.55 (d, 1H, =CH).

5.1.2. General procedure for 3d–k

To a suspension of compounds **2b–g** (**2c**,[39] **2f**[40]) (0.6 mmol) and the appropriate arylaldehyde (1.2–3.6 mmol) in anhydrous methanol (2 mL), CH_3ONa (1.0–3.0 mmol) was added. The mixture was refluxed under stirring for 1–40 min. After cooling, the precipitate was recovered by suction.

5.1.2.1. 4-Methyl-6-(1-phenylethyl)-3-styrylisoxazolo[3,4-*d*]pyridazin-7(6*H*)-one, 3d. Yield = 49%; mp = 189–191 °C (EtOH); ^{1}H NMR ($CDCl_3$) δ 1.80 (d, 3H, *CH*$_3$CH), 2.60 (s, 3H, CH_3), 6.40 (q, 1H, CH_3*CH*), 7.20 (d, 1H, CH=), 7.30–7.65 (m, 10H, Ar), 7.80 (d, 1H, =CH).

5.1.2.2. 6-Benzyl-4-phenyl-3-styrylisoxazolo[3,4-*d*]pyridazin-7(6*H*)-one, 3e. Yield = 78%; mp = 241–242 °C (EtOH); ^{1}H NMR ($CDCl_3$) δ 5.40 (s, 2H, CH_2), 6.80 (d, 1H, CH=), 7.20–7.70 (m, 16H: 10H, Ar; 1H, =CH).

5.1.2.3. 6-Benzyl-4-(4-fluorophenyl)-3-styrylisoxazolo[3,4-*d*]pyridazin-7(6*H*)-one, 3f. Yield = 91%; mp = 197–199 °C (EtOH); ^{1}H NMR ($CDCl_3$) δ 5.40 (s, 2H, CH_2), 6.80 (d, 1H, CH=), 7.20–7.60 (m, 14H, Ar), 7.70 (d, 1H, =CH).

5.1.2.4. 6-Benzyl-4-(4-fluorophenyl)-3-(2-thiophen-3-yl-vinyl) isoxazolo[3,4-*d*]pyridazin-7(6*H*)-one, 3g. Yield = 85%; mp = 203–204 °C (EtOH); ^{1}H NMR ($CDCl_3$) δ 5.40 (s, 2H, CH_2), 6.60 (d, 1H, CH=), 7.00 (d, 1H, Ar), 7.15–7.60 (m, 11H, Ar), 7.65 (d, 1H, =CH).

5.1.2.5. 6-Benzyl-4-(3-fluorophenyl)-3-styrylisoxazolo[3,4-*d*]pyridazin-7(6*H*)-one, 3h. Yield = 70%; mp = 142–145 °C (EtOH); ^{1}H NMR ($CDCl_3$) δ 5.40 (s, 2H, CH_2), 6.80 (d, 1H, CH=), 7.20–7.60 (m, 14H, Ar), 7.80 (d, 1H, =CH).

5.1.2.6. 6-Benzyl-4-(3-fluorophenyl)-3-(2-thiophen-3-yl-vinyl) isoxazolo[3,4-*d*]pyridazin-7(6*H*)-one, 3i. Yield = 83%; mp = 205–207 °C (EtOH); ^{1}H NMR ($CDCl_3$) δ 5.40 (s, 2H, CH_2), 6.60 (d, 1H, CH=), 7.00 (d, 1H, Ar), 7.25–7.60 (m, 11H, Ar), 7.65 (d, 1H, =CH).

5.1.2.7. 6-Benzyl-4-pyridin-4-yl-3-styrylisoxazolo[3,4-*d*]pyridazin-7(6*H*)-one, 3j. Yield = 65%; mp = 187–189 °C (EtOH); ^{1}H NMR ($CDCl_3$) δ 5.40 (s, 2H, CH_2), 6.80 (d, 1H, CH=), 7.30–7.60 (m, 10H, Ar), 7.80 (d, 1H, =CH), 8.20 (d, 2H, Ar), 9.00 (d, 2H, Ar).

5.1.2.8. 6-Benzyl-4-pyridin-3-yl-3-styrylisoxazolo[3,4-*d*]pyridazin-7(6*H*)-one, 3k. Yield = 53%; mp = 166–169 °C (EtOH); ^{1}H NMR ($CDCl_3$) δ 5.40 (s, 2H, CH_2), 6.80 (d, 1H, CH=), 7.30–7.60 (m, 11H, Ar), 7.65 (d, 1H, =CH), 8.00 (m, 1H, Ar), 8.80–8.95 (m, 2H, Ar).

5.1.3. General procedure for 4c–k

A suspension of compounds **3c–k** (0.3 mmol) in ethanol (2–3 mL) and hydrazine hydrate (4–9 mmol) was stirred at room temperature for 0.5–7 h (compounds **4c**, **4g** and **4i** for 2–5 h at 50–90 °C). Then the mixture was concentrated in vacuo and cooled for 6–12 h: the precipitate was recovered by suction. For compound **4e**, after concentration, the mixture was diluted with water (5 mL) and, after cooling, the solid was isolated by filtration.

5.1.3.1. 4-Amino-2-benzyl-6-methyl-5-(2*H*-pyrazol-3-yl)pyridazin-3(2*H*)-one, 4c. Yield = 43%; mp = 130–132 °C (EtOH); ^{1}H NMR ($CDCl_3$) δ 2.30 (s, 3H, CH_3), 5.40 (s, 2H, CH_2), 6.30 (exch br s, 2H, NH_2), 6.50 (d, 1H, Ar), 7.25–7.45 (m, 5H, Ar), 7.60 (d, 1H, Ar), 8.70 (exch br s, 1H, NH).

5.1.3.2. 4-Amino-6-methyl-2-(1-phenylethyl)-5-(5-phenyl-2*H*-pyrazol-3-yl)pyridazin-3(2*H*)-one, 4d. Yield = 96%; mp = 197–198 °C (EtOH); ^{1}H NMR ($CDCl_3$) δ 1.80 (d, 3H, *CH*$_3$CH), 2.40 (s, 3H, 6-CCH_3), 6.20 (exch br s, 2H, NH_2), 6.40 (q, 1H, CH_3*CH*), 6.70 (s, 1H, Ar), 7.20–7.65 (m, 10H, Ar), 7.90 (exch br s, 1H, NH).

5.1.3.3. 4-Amino-2-benzyl-6-phenyl-5-(5-phenyl-2*H*-pyrazol-3-yl)pyridazin-3(2*H*)-one, 4e. Yield = 97%; mp = 118–119 °C (EtOH); ^{1}H NMR ($CDCl_3$) δ 5.40 (s, 2H, CH_2), 6.15 (exch br s, 2H, NH_2), 7.25–7.65 (m, 16H, Ar), 8.85 (exch br s, 1H, NH).

5.1.3.4. 4-Amino-2-benzyl-6-(4-fluorophenyl)-5-(5-phenyl-2*H*-pyrazol-3-yl)-pyridazin-3(2*H*)-one, 4f. Yield = 53%; mp = 137–143 °C (EtOH); ^{1}H NMR ($CDCl_3$) δ 5.40 (s, 2H, CH_2), 5.75 (s, 1H, Ar), 6.80 (exch br s, 2H, NH_2), 7.00–7.20 (m, 2H, Ar), 7.25–7.60 (m, 12H, Ar), 8.25 (exch br s, 1H, NH).

5.1.3.5. 4-Amino-2-benzyl-6-(4-fluorophenyl)-5-(5-thiophen-3-yl-2*H*-pyrazol-3-yl)-pyridazin-3(2*H*)-one, 4g. Yield = 58%; mp = 225–227 °C (EtOH); ^{1}H NMR ($CDCl_3$) δ 5.40 (s, 2H, CH_2), 5.60 (s, 1H, Ar), 6.70 (exch br s, 2H, NH_2), 7.00–7.15 (m, 3H, Ar), 7.20–7.50 (m, 9H, Ar), 8.80 (exch br s, 1H, NH).

5.1.3.6. 4-Amino-2-benzyl-6-(3-fluorophenyl)-5-(5-phenyl-2*H*-pyrazol-3-yl)pyridazin-3(2*H*)-one, 4h. Yield = 30%; mp = 167–169 °C dec. (EtOH); ^{1}H NMR ($CDCl_3$) δ 5.35 (s, 2H, CH_2), 5.95 (s, 1H, Ar), 6.90 (exch br s, 2H, NH_2), 7.00–7.65 (m, 14H, Ar), 9.20 (exch br s, 1H, NH).

5.1.3.7. 4-Amino-2-benzyl-6-(3-fluorophenyl)-5-(5-thiophen-3-yl-2*H*-pyrazol-3-yl)pyridazin-3(2*H*)-one, 4i. Yield = 39%; mp = 219–222 °C (EtOH); ^{1}H NMR ($CDCl_3$) δ 5.35 (s, 2H, CH_2), 5.95 (s, 1H, Ar), 6.80–7.50 (m, 12H, Ar), 7.60 (exch br s, 2H, NH_2), 9.10 (exch br s, 1H, NH).

5.1.3.8. 4-Amino-2-benzyl-5-(5-phenyl-2*H*-pyrazol-3-yl)-6-pyridin-4-yl-pyridazin-3(2*H*)-one, 4j. Yield = 67%; mp = 226–229 °C (EtOH/H_20 1/1); ^{1}H NMR ($CDCl_3$) δ 5.45 (s, 2H, CH_2), 5.95 (s, 1H, Ar), 6.40 (exch br s, 2H, NH_2), 7.20–7.50 (m, 10H, Ar), 7.70 (m, 2H, Ar), 8.60 (m, 2H, Ar), 9.25 (exch br s, 1H, NH).

5.1.3.9. 4-Amino-2-benzyl-5-(5-phenyl-2*H*-pyrazol-3-yl)-6-pyridin-3-yl-pyridazin-3(2*H*)-one, 4k. Yield = 45%; mp = 200–203 °C (EtOH); ^{1}H NMR ($CDCl_3$) δ 5.40 (s, 2H, CH_2), 5.70 (s, 1H, Ar), 6.60 (exch br s, 2H, NH_2), 7.20–7.50 (m, 11H, Ar), 7.80 (d, 1H, Ar), 7.95 (exch br s, 1H, NH), 8.60 (d, 1H, Ar), 8.70 (s, 1H, Ar).

5.1.4. General procedure for 5b–k

A mixture of compounds **4b–k** (**4b**[38]) (0.16 mmol), triethylorthoformate (12 mmol), anhydrous DMF (1–3 mL) and a catalytic amount of concentrated sulfuric acid was stirred at room temperature for 5–30 min. After cooling the precipitate was recovered by suction. For compounds **5h** and **5i**, the mixture was diluted with cold water (10 mL) and the solid was filtered off.

5.1.4.1. 3-Benzyl-1-methyl-9-thiophen-3-yl-pyrazolo[1′,5′:1,6]pyrimido[4,5-*d*]pyridazin-4(3*H*)-one, 5b. Yield = 81%; mp = 288–290 °C (EtOH); ^{1}H NMR ($CDCl_3$) δ 2.80 (s, 3H, CH_3), 5.40 (s, 2H, CH_2), 7.20–7.60 (m, 7H, Ar), 7.70 (m, 1H, Ar), 7.95 (d, 1H, Ar), 9.40 (s, 1H, Ar); MS (ESI) *m*/*z* 373.43 ([M+H]$^+$). Anal. ($C_{20}H_{15}N_5OS$) C, H, N.

5.1.4.2. 3-Benzyl-1-methylpyrazolo[1′,5′:1,6]pyrimido[4,5-*d*]pyridazin-4(3*H*)-one, 5c. Yield = 68%; mp = 160–163 °C (EtOH); ^{1}H NMR ($CDCl_3$) δ 2.80 (s, 3H, CH_3), 5.50 (s, 2H, CH_2), 7.10 (s, 1H, Ar), 7.20–7.40 (m, 4H, Ar), 8.20 (m, 2H, Ar), 9.40 (s, 1H, Ar). Anal. ($C_{16}H_{13}N_5O$) C, H, N.

5.1.4.3. 1-Methyl-9-phenyl-3-(1-phenylethyl)pyrazolo[1′,5′:1,6]pyrimido-[4,5-*d*]pyridazin-4(3*H*)-one, 5d. Yield = 97%; mp = 247–250 °C dec. (EtOH); ^{1}H NMR ($CDCl_3$) δ 1.90 (d, 3H, CH_3CH), 2.80 (s, 3H, CH_3), 6.60 (q, 1H, CH_3CH), 7.20–7.60 (m, 9H, Ar), 8.00 (m, 2H, Ar), 9.40 (s, 1H, Ar); MS (ESI) *m*/*z* 381.43 ([M+H]$^+$). Anal. ($C_{23}H_{19}N_5O$) C, H, N.

5.1.4.4. 3-Benzyl-1,9-diphenylpyrazolo[1′,5′:1,6]pyrimido[4,5-*d*]pyridazin-4(3*H*)-one, 5e. Yield = 72%; mp = 230–232 °C (EtOH); ^{1}H NMR ($CDCl_3$) δ 5.50 (s, 2H, CH_2), 6.20 (s, 1H, Ar), 7.25–7.80 (m, 15H, Ar), 9.45 (s, 1H, Ar); MS (ESI) *m*/*z* 429.47 ([M+H]$^+$). Anal. ($C_{27}H_{19}N_5O$) C, H, N.

5.1.4.5. 3-Benzyl-1-(4-fluorophenyl)-9-phenylpyrazolo[1′,5′:1,6]pyrimido[4,5-*d*]pyridazin-4(3*H*)-one, 5f. Yield = 82%; mp = 227–229 °C (EtOH); ^{1}H NMR ($CDCl_3$) δ 5.50 (s, 2H, CH_2), 6.20 (s, 1H, Ar), 7.25–7.60 (m, 12H, Ar), 7.80 (m, 2H, Ar), 9.40 (s, 1H, Ar); MS (ESI) *m*/*z* 447.46 ([M+H]$^+$). Anal. ($C_{27}H_{18}FN_5O$) C, H, N.

5.1.4.6. 3-Benzyl-1-(4-fluorophenyl)-9-thiophen-3-yl-pyrazolo[1′,5′:1,6]pyrimido[4,5-*d*]pyridazin-4(3*H*)-one, 5g. Yield = 78%; mp = 218–222 °C dec. (EtOH); ^{1}H NMR ($CDCl_3$) δ 5.50 (s, 2H, CH_2), 6.00 (s, 1H, Ar), 7.20–7.70 (m, 11H, Ar), 8.20 (s, 1H, Ar), 9.40 (s, 1H, Ar); MS (ESI) *m*/*z* 453.49 ([M+H]$^+$). Anal. ($C_{25}H_{16}FN_5OS$) C, H, N.

5.1.4.7. 3-Benzyl-1-(3-fluorophenyl)-9-phenylpyrazolo[1′,5′:1,6]pyrimido[4,5-*d*]pyridazin-4(3*H*)-one, 5h. Yield = 49%; mp = 202–204 °C dec. (Ether); ^{1}H NMR ($CDCl_3$) δ 5.50 (s, 2H, CH_2), 6.10 (s, 1H, Ar), 7.20–7.60 (m, 12H, Ar), 7.80 (m, 2H, Ar), 9.40 (s, 1H, Ar); MS (ESI) *m*/*z* 447.46 ([M+H]$^+$). Anal. ($C_{27}H_{18}FN_5O$) C, H, N.

5.1.4.8. 3-Benzyl-1-(3-fluorophenyl)-9-thiophen-3-yl-pyrazolo[1′,5′:1,6]pyrimido[4,5-*d*]pyridazin-4(3*H*)-one, 5i. Yield = 78%; mp = 206–208 °C dec. (EtOH); ^{1}H NMR ($CDCl_3$) δ 5.50 (s, 2H, CH_2), 6.00 (s, 1H, Ar), 7.20–7.75 (m, 12H, Ar), 9.40 (s, 1H, Ar); MS (ESI) *m*/*z* 453.49 ([M+H]$^+$). Anal. ($C_{25}H_{16}FN_5OS$) C, H, N.

5.1.4.9. 3-Benzyl-9-phenyl-1-pyridin-4-yl-pyrazolo[1′,5′:1,6]pyrimido[4,5-*d*]pyridazin-4(3*H*)-one, 5j. Yield = 82%; mp = >300 °C (EtOH); ^{1}H NMR ($CDCl_3$) δ 5.50 (s, 2H, CH_2), 6.20 (s, 1H, Ar), 7.40–7.60 (m, 8H, Ar), 7.70 (m, 2H, Ar), 7.80 (m, 2H, Ar), 8.80 (d, 2H, Ar), 9.50 (s, 1H, Ar); MS (ESI) *m*/*z* 430.46 ([M+H]$^+$). Anal. ($C_{26}H_{18}N_6O$) C, H, N.

5.1.4.10. 3-Benzyl-9-phenyl-1-pyridin-3-yl-pyrazolo[1′,5′:1,6]pyrimido[4,5-*d*]pyridazin-4(3*H*)-one, 5k. Yield = 81%; mp >300 °C dec. (Ether); ^{1}H NMR ($CDCl_3$) δ 5.40 (s, 2H, CH_2), 6.30 (s, 1H, Ar), 7.40–8.20 (m, 11H, Ar), 8.50 (m, 1H, Ar), 9.10 (m, 2H, Ar), 9.85 (s, 1H, Ar); MS (ESI) *m*/*z* 430.46 ([M+H]$^+$). Anal. ($C_{26}H_{18}N_6O$) C, H, N.

5.1.5. General procedure for 6b–d, i, j, l, m, t and u

A mixture of 4-amino-5-pyrazolyl derivatives **4c–d**, **4f**, **4h and 4k** (0.13 mmol), the appropriate anhydride (7–15 mmol) and a catalytic amount of concentrated sulfuric acid was stirred at room temperature for 15–30 min. The mixture was diluted with cold water (10 mL) and the precipitate was recovered by suction.

5.1.5.1. 3-Benzyl-1,6-dimethylpyrazolo[1′,5′:1,6]pyrimido[4,5-*d*]pyridazin-4(3*H*)-one, 6b. Yield = 47%; mp = 220–222 °C (EtOH); ^{1}H NMR ($CDCl_3$) δ 2.70 (s, 3H, 1-CCH_3), 3.00 (s, 3H, 6-CCH_3), 5.35 (s, 2H, CH_2), 7.30 (m, 1H, Ar), 7.35 (m, 4H, Ar), 8.45 (d, 1H, Ar), 8.50 (d, 1H, Ar). Anal. ($C_{17}H_{15}N_5O$) C, H, N.

5.1.5.2. 1,6-Dimethyl-9-phenyl-3-(1-phenylethyl)pyrazolo[1′,5′:1,6]pyrimido-[4,5-*d*]pyridazin-4(3*H*)-one, 6c. Yield = 75%; mp = 230–232 °C (EtOH); ^{1}H NMR ($CDCl_3$) δ 1.80 (d, 3H, CH_3CH), 2.80 (s, 3H, 1-CCH_3), 3.20 (s, 3H, 6-CCH_3), 6.60 (q, 1H, CH_3CH), 7.20–7.60 (m, 9H, Ar), 8.10 (m, 2H, Ar). Anal. ($C_{24}H_{21}N_5O$) C, H, N.

5.1.5.3. 6-Ethyl-9-phenyl-3-(1-phenylethyl)-1-methylpyrazolo[1′,5′:1,6]pyrimido-[4,5-*d*]pyridazin-4(3*H*)-one, 6d. Yield = 73%; mp = 149–152 °C (EtOH); ^{1}H NMR ($CDCl_3$) δ 1.60 (t, 3H, CH_3CH_2), 1.80 (d, 3H, CH_3CH), 2.80 (s, 3H, 1-CCH_3), 3.60 (q, 2H, CH_2CH_3), 6.60 (q, 1H, CH_3CH), 7.20–7.60 (m, 9H, Ar), 8.10 (m, 2H, Ar). Anal. ($C_{25}H_{23}N_5O$) C, H, N.

5.1.5.4. 3-Benzyl-1-(4-fluorophenyl)-6-methyl-9-phenylpyrazolo[1′,5′:1,6]pyrimido[4,5-*d*]pyridazin-4(3*H*)-one, 6i. Yield = 71%; mp = 263–264 °C (Ether); ^{1}H NMR ($CDCl_3$) δ 3.20 (s, 3H, CH_3), 5.50 (s, 2H, CH_2), 6.10 (s, 1H, Ar), 7.25–7.60 (m, 12H, Ar), 7.85 (m, 2H, Ar). Anal. ($C_{28}H_{20}FN_5O$) C, H, N.

5.1.5.5. 3-Benzyl-6-ethyl-1-(4-fluorophenyl)-9-phenylpyrazolo[1′,5′:1,6]pyrimido[4,5-*d*]pyridazin-4(3*H*)-one, 6j. Yield = 80%; mp = 267–268 °C (Ether); ^{1}H NMR ($CDCl_3$) δ 1.60 (t, 3H, CH_2CH_3), 3.55 (q, 2H, CH_2CH_3), 5.50 (s, 2H, CH_2), 6.20 (s, 1H, Ar), 7.20–7.60 (m, 12H, Ar), 7.80 (m, 2H, Ar). Anal. ($C_{29}H_{22}FN_5O$) C, H, N.

5.1.5.6. 3-Benzyl-1-(3-fluorophenyl)-6-methyl-9-phenylpyrazolo[1′,5′:1,6]pyrimido[4,5d]pyridazin-4(3*H*)-one, 6l. Yield = 68%; mp = 245–247 °C (Ether); ^{1}H NMR ($CDCl_3$) δ 3.20 (s, 3H, CH_3), 5.50 (s, 2H, CH_2), 6.15 (s, 1H, Ar), 7.25–7.60 (m, 12H, Ar), 7.70 (m, 2H, Ar); MS (ESI) *m*/*z* 461.49 ([M+H]$^+$). Anal. ($C_{28}H_{20}FN_5O$) C, H, N.

5.1.5.7. 3-Benzyl-6-ethyl-1-(3-fluorophenyl)-9-phenylpyrazolo[1′,5′:1,6]pyrimido[4,5-*d*]pyridazin-4(3*H*)-one, 6m. Yield = 77%; mp = 254–256 °C (Ether); ^{1}H NMR ($CDCl_3$) δ 1.60 (t, 3H, CH_2CH_3), 3.60 (q, 2H, CH_2CH_3), 5.40 (s, 2H, CH_2), 6.10 (s, 1H, Ar), 7.20–7.40 (m, 10H, Ar), 7.60 (m, 2H, Ar), 7.80 (m, 2H, Ar). Anal. ($C_{29}H_{22}FN_5O$) C, H, N.

5.1.5.8. 3-Benzyl-6-methyl-9-phenyl-1-pyridin-3-yl-pyrazolo[1′,5′:1,6]pyrimido-[4,5-*d*]pyridazin-4(3*H*)-one, 6t. Yield = 81%; mp = 284–286 °C (EtOH); ^{1}H NMR ($CDCl_3$) δ 3.20 (s, 3H, CH_3), 5.50 (s, 2H, CH_2), 6.20 (s, 1H, Ar), 7.30–7.80 (m, 11H, Ar), 8.00 (d,

1H, Ar), 8.90 (m, 2H, Ar); MS (ESI) *m*/*z* 444.49 ([M+H]$^+$). Anal. ($C_{27}H_{20}N_6O$) C, H, N.

5.1.5.9. 3-Benzyl-6-ethyl-9-phenyl-1-pyridin-3-yl-pyrazolo[1′,5′:1,6]pyrimido-[4,5-*d*]pyridazin-4(3*H*)-one, 6u. Yield = 91%; mp = 288–289 °C (EtOH); ^{1}H NMR ($CDCl_3$) δ 1.60 (t, 3H, CH_3CH_2), 3.60 (q, 2H, CH_3CH_2), 5.55 (s, 2H, CH_2), 6.10 (s, 1H, Ar), 7.20–7.80 (m, 11H, Ar), 8.05 (d, 1H, Ar), 9.00 (m, 2H, Ar); MS (ESI) *m*/*z* 458.51 ([M+H]$^+$). Anal. ($C_{28}H_{22}N_6O$) C, H, N.

5.1.5.10. 3-Benzyl-1-(4-fluorophenyl)-9-phenyl-6-trifluoro methylpyrazolo[1′,5′:1,6]pyrimido[4,5-*d*]pyridazin-4(3*H*)-one, 6k. A solution of **4j** (0.12 mmol) in toluene (2.5 mL) and trifluoroacetic anhydride (1 mmol) was stirred at 60 °C for 16 h. After cooling the precipitate was recovered by suction.

Yield = 68%; mp = >300 °C (EtOH); ^{1}H NMR ($CDCl_3$) δ 5.50 (s, 2H, CH_2), 6.25 (s, 1H, Ar), 7.20–7.60 (m, 12H, Ar), 7.80 (m, 2H, Ar). Anal. ($C_{28}H_{17}F_4N_5O$) C, H, N.

5.1.6. General procedure for 6e–h, 6n–s

A suspension of compounds **4e**, **4i** and **4j** (0.2–0.3 mmol) and the suitable anhydride (9–15 mmol) was refluxed under stirring for 5–60 min. Then the mixture was cooled and the solid was recovered by suction.

5.1.6.1. 3-Benzyl-1,9-diphenyl-6-methylpyrazolo[1′,5′:1,6]pyrimido[4,5-*d*]pyridazin-4(3*H*)-one, 6e. Yield = 42%; mp = 265–266 °C (EtOH); ^{1}H NMR ($CDCl_3$) δ 3.20 (s, 3H, CH_3), 5.55 (s, 2H, CH_2), 6.00 (s, 1H, Ar), 7.30–7.80 (m, 15H, Ar); MS (ESI) *m*/*z* 443.50 ([M+H]$^+$). Anal. ($C_{28}H_{21}N_5O$) C, H, N.

5.1.6.2. 3-Benzyl-1,9-diphenyl-6-ethylpyrazolo[1′,5′:1,6]pyrimido[4,5-*d*]pyridazin-4(3*H*)-one, 6f. Yield = 52%; mp = 256–257 °C (EtOH); ^{1}H NMR ($CDCl_3$) δ 1.60 (t, 3H, CH_2CH_3), 3.60 (q, 2H, CH_2CH_3), 5.50 (s, 2H, CH_2), 6.00 (s, 1H, Ar), 7.25–7.80 (m, 15H, Ar); MS (ESI) *m*/*z* 457.53 ([M+H]$^+$). Anal. ($C_{29}H_{23}N_5O$) C, H, N.

5.1.6.3. 3-Benzyl-1,9-diphenyl-6-isopropylpyrazolo[1′,5′:1,6]pyrimido[4,5-*d*]pyridazin-4(3*H*)-one, 6g. Yield = 59%; mp = 233–235 °C (EtOH); ^{1}H NMR ($CDCl_3$) δ 1.60 (d, 6H, $(CH_3)_2CH$), 4.25 (m, 1H, $(CH_3)_2CH$), 5.50 (s, 2H, CH_2), 6.05 (s, 1H, Ar), 7.20–7.80 (m,15H, Ar). Anal. ($C_{30}H_{25}N_5O$) C, H, N.

5.1.6.4. 3-Benzyl-1,9-diphenyl-6-*n*-propylpyrazolo[1′,5′:1,6]pyrimido[4,5-*d*]pyridazin-4(3*H*)-one, 6h. Yield = 45%; mp = 181–183 °C (EtOH); ^{1}H NMR ($CDCl_3$) δ 1.15 (t, 3H, $CH_2CH_2CH_3$), 2.20 (m, 2H, $CH_2CH_2CH_3$), 3.50 (t, 2H, $CH_2CH_2CH_3$), 5.50 (s, 2H, CH_2), 6.00 (s, 1H, Ar), 7.20–7.80 (m,15H, Ar); MS (ESI) *m*/*z* 471.55 ([M+H]$^+$). Anal. ($C_{30}H_{25}N_5O$) C, H, N.

5.1.6.5. 3-Benzyl-1-(3-fluorophenyl)-6-methyl-9-thiophen-3-yl-pyrazolo[1′,5′:1,6]pyrimido-[4,5-*d*]pyridazin-4(3*H*)-one, 6n. Yield = 63%; mp = 253–255 °C (EtOH); ^{1}H NMR ($CDCl_3$) δ 3.00 (s, 3H, CH_3), 5.40 (s, 2H, CH_2), 5.95 (s, 1H, Ar), 7.20–7.75 (m, 11H, Ar), 8.00 (s, 1H, Ar). Anal. ($C_{26}H_{18}FN_5OS$) C, H, N.

5.1.6.6. 3-Benzyl-6-ethyl-1-(3-fluorophenyl)-9-thiophen-3-yl-pyrazolo[1′,5′:1,6]pyrimido-[4,5-*d*]pyridazin-4(3*H*)-one, 6o. Yield = 48%; mp = 246–247 °C (EtOH); ^{1}H NMR ($CDCl_3$) δ 1.50 (t, 3H, CH_2CH_3), 3.55 (q, 2H, CH_2CH_3), 5.45 (s, 2H, CH_2), 6.00 (s, 1H, Ar), 7.20–7.80 (m, 12H, Ar). Anal. ($C_{27}H_{20}FN_5OS$) C, H, N.

5.1.6.7. 3-Benzyl-6-methyl-9-phenyl-1-pyridin-4-yl-pyrazolo[1′,5′:1,6]pyrimido-[4,5-*d*]pyridazin-4(3*H*)-one, 6p. Yield = 40%; mp = >300 °C (MeOH); ^{1}H NMR ($CDCl_3$) δ 3.40 (s, 3H, CH_3), 5.50 (s, 2H, CH_2), 6.40 (s, 1H, Ar), 7.40–7.60 (m, 8H, Ar), 7.85 (m, 2H, Ar), 8.40 (d, 2H, Ar), 9.05 (d, 2H, Ar); MS (ESI) *m*/*z* 444.49 ([M+H]$^+$). Anal. ($C_{27}H_{20}N_6O$) C, H, N.

5.1.6.8. 3-Benzyl-6-ethyl-9-phenyl-1-pyridin-4-yl-pyrazolo[1′,5′:1,6]pyrimido-[4,5-*d*]pyridazin-4(3*H*)-one, 6q. Yield = 38%; mp = 269–271 °C (EtOH); ^{1}H NMR ($CDCl_3$) δ 1.60 (t, 3H, CH_2CH_3), 3.60 (q, 2H, CH_2CH_3), 5.50 (s, 2H, CH_2), 6.20 (s, 1H, Ar), 7.30–7.80 (m,12H, Ar), 8.90 (d, 2H, Ar); MS (ESI) *m*/*z* 458.51 ([M+H]$^+$). Anal. ($C_{28}H_{22}N_6O$) C, H, N.

5.1.6.9. 3-Benzyl-9-phenyl-6-*n*-propyl-1-pyridin-4-yl-pyrazolo[1′,5′:1,6]pyrimido-[4,5-*d*]pyridazin-4(3*H*)-one, 6r. Yield = 30%; mp = 194–197 °C dec. (EtOH); ^{1}H NMR ($CDCl_3$) δ 1.10 (t, 3H, $CH_2CH_2CH_3$), 2.10 (m, 2H, $CH_2CH_2CH_3$), 3.50 (t, 2H, $CH_2CH_2CH_3$), 5.50 (s, 2H, CH_2), 6.20 (s, 1H, Ar), 7.30–7.50 (m,10H, Ar), 7.80 (d, 2H, Ar), 8.95 (d, 2H, Ar); MS (ESI) *m*/*z* 472.54 ([M+H]$^+$). Anal. ($C_{29}H_{24}N_6O$) C, H, N.

5.1.6.10. 3-Benzyl-6-isopropyl-9-phenyl-1-pyridin-4-yl-pyrazolo[1′,5′:1,6]pyrimido-[4,5-*d*]pyridazin-4(3*H*)-one, 6s. Yield = 36%; mp = 263–265 °C dec. (EtOH); ^{1}H NMR ($CDCl_3$) δ 1.60 (d, 6H, $(CH_3)_2CH$), 4.20 (m, 1H, $(CH_3)_2CH$), 5.55 (s, 2H, CH_2), 6.20 (s, 1H, Ar), 7.20–7.60 (m,10H, Ar), 7.80 (d, 2H, Ar), 8.90 (d, 2H, Ar); MS (ESI) *m*/*z* 472.54 ([M+H]$^+$). Anal. ($C_{29}H_{24}N_6O$) C, H, N.

5.1.7. 3-Benzyl-1-methyl-9-phenylpyrazolo[1,5-*c*]pyridazino[4,5-*e*][1,2,3]triazin-4(3*H*)-one, 7

To a solution of **4a**[38] in glacial acetic acid (4 mL) a saturated aqueous solution of $NaNO_2$ (1.5 mL) was added dropwise under stirring. After the mixture was cooled and the solid was recovered by suction.

Yield = 97%; mp = 253–255 °C dec. (EtOH); ^{1}H NMR ($CDCl_3$) δ 2.80 (s, 3H, CH_3), 5.40 (s, 2H, CH_2), 6.10 (s, 1H, Ar), 7.20–7.60 (m, 7H, Ar), 8.10 (s, 1H, Ar), 8.30 (m, 2H, Ar). Anal. ($C_{21}H_{16}N_6O$) C, H, N.

5.1.8. 3-Benzyl-6-methyl-9-phenyl-1-pyridin-4-yl-pyrazolo[1′,5′:1,6]pyrimido[4,5-*d*]pyridazin-4(3*H*)-thione, 8

A mixture of **6p** (0.32 mmol) and Lawesson's reagent (0.74 mmol) in toluene (10 mmol) was heated at 140 °C for 4 h. After cooling, toluene was evaporated in vacuo affording compound **8** which was purified by column chromatography using cyclohexane/ethyl acetate 1:2 as eluent.

Yield = 95%; mp = 254–258 °C dec.(EtOH); ^{1}H NMR ($CDCl_3$) δ 3.20 (s, 3H, CH_3), 6.15 (s, 2H, CH_2), 6.40 (s, 1H, Ar), 7.30–7.60 (m, 9H, Ar), 7.80 (m, 2H, Ar), 8.10 (m, 1H, Ar), 9.00 (d, 2H, Ar); MS (ESI) *m*/*z* 460.55 ([M+H]$^+$). Anal. ($C_{27}H_{20}N_6S$) C, H, N.

5.1.9. General procedure for 9a,b

Compounds **9a**,**b** were obtained from **1c**,**e** (**1c**,[44] **1e**[39]) following the general procedure described for compounds **3d**–**k**.

5.1.9.1. 4-(4-Fluorophenyl)-3-styrylisoxazolo[3,4-*d*]pyridazin-7(6*H*)-one, 9a. Yield = 92%; mp = 240–243 °C dec. (EtOH); ^{1}H NMR (DMSO) δ 6.80 (d, 1H, CH=), 7.25–7.80 (m, 10H: 9H, Ar; 1H, =CH), 12.15 (exch br s, 1H, NH).

5.1.9.2. 4-Pyridin-4-yl-3-styrylisoxazolo[3,4-*d*]pyridazin-7(6*H*)-one, 9b. Yield = 93%; mp = 268–271 °C (EtOH); ^{1}H NMR (DMSO) δ 6.90 (d, 1H, CH=), 7.35–7.80 (m, 8H: 7H, Ar; 1H, =CH), 8.85 (s, 2H, Ar), 12.95 (exch br s, 1H, NH).

5.1.10. General procedure for 10a,b

Compounds **10a**,**b** were obtained from **9a**,**b** following the general procedure described for compounds **4c**–**k**. For compound **10b** the mixture was stirred at 80 °C for 30 min.

5.1.10.1. 4-Amino-6-(4-fluorophenyl)-5-(5-phenyl-2*H*-pyrazol-3-yl)-pyridazin-3(2*H*)-one, 10a. Yield = 60%; mp = 154–157 °C dec. (EtOH); ^{1}H NMR ($CDCl_3$) δ 5.80 (exch br s, 1H, NH), 5.95 (s, 1H, Ar), 6.80 (exch br s, 2H, NH_2), 7.05–7.60 (m, 9H, Ar), 13.00 (exch br s, 1H, NH).

5.1.10.2. 4-Amino-5-(5-phenyl-2*H*-pyrazol-3-yl)-6-pyridin-4-yl-pyridazin-3(2*H*)-one, 10b. Yield = 37%; mp = >300 °C (EtOH); ^{1}H NMR ($CDCl_3$) δ 5.95 (s, 1H, Ar), 6.50 (exch br s, 2H, NH_2), 7.00 (m, 2H, Ar), 7.10–7.65 (m, 5H, Ar), 8.50 (m, 2H, Ar), 13.00 (exch br s, 1H, NH), 13.50 (exch br s, 1H, NH).

5.1.11. 1-(4-Fluorophenyl)-9-phenylpyrazolo[1′,5′:1,6]pyrimido[4,5-*d*]pyridazin-4(3*H*)-one, 11

Compound **11** was obtained from **10a** following the general procedure described for compounds **5b–k.** Yield = 78%; mp = >300 °C (EtOH); ^{1}H NMR (DMSO) δ 5.90 (s, 1H, Ar), 5.95 (s, 1H, Ar), 7.40–7.60 (m, 9H, Ar), 9.80 (s, 1H, Ar), 13.00 (exch br s, 1H, NH).

5.1.12. 4-Chloro-1-(4-fluorophenyl)-9-phenylpyrazolo[1′,5′:1,6]pyrimido[4,5-*d*]pyridazine, 12

A suspension of **11** (0.28 mmol) in $POCl_3$ (1 mL, 6.5 mmol) was stirred at 90 °C for 3 h. After cooling, the mixture was diluted with cold water and neutralized with 6 N NaOH: the final compound **12** was recovered by suction. Yield = 40%; mp = >300 °C (EtOH); ^{1}H NMR ($CDCl_3$) δ 6.40 (s, 1H, Ar), 7.20–7.50 (m, 5H, Ar), 7.70–7.90 (m, 4H, Ar), 9.50 (s, 1H, Ar). Anal. ($C_{20}H_{11}ClFN_5$) C, H, N.

5.1.13. 6-Methyl-9-phenyl-1-pyridin-4-yl-pyrazolo[1′,5′:1,6]pyrimido[4,5-*d*]pyridazin-4(3*H*)-one, 13

Compound **13** was obtained from **10b** following the general procedure described for compounds **6b–d**, **6i–j**, **6l–m**, **6t–u**. Yield = 65%; mp >300 °C (EtOH); ^{1}H NMR (DMSO) δ 3.05 (s, 3H, 6-CCH_3), 6.35 (s, 1H, Ar), 7.40 (s, 3H, Ar), 7.80–8.00 (m, 4H, Ar), 9.00 (d, 2H, Ar), 13.50 (exch br s, 1H, NH).

5.1.14. 6-Methyl-9-phenyl-1-pyridin-4-yl-pyrazolo[1′,5′:1,6]pyrimido[4,5-*d*]pyridazin-4(3*H*)-thione, 14

Compound **14** was obtained from **13** following the procedure described for compound **8:** in this case the mixture was heated at 140 °C for 24 h: after cooling the precipitate was recovered by suction.

Yield = 89%; mp = 238–242 °C dec.(DMSO); ^{1}H NMR ($CDCl_3$) δ 3.00 (s, 3H, CH_3), 5.20 (exch br s, 1H, NH), 6.30 (s, 1H, Ar), 7.00 (m, 2H, Ar), 7.40–7.90 (m, 5H, Ar), 9.00 (m, 2H, Ar).

5.1.15. 4-(Benzylthio)-6-methyl-9-phenyl-1-pyridin-4-ylpyrazolo[1′,5′:1,6]pyrimido-[4,5-*d*]pyridazine, 15

Compound **15** was obtained following the procedure described for compounds **2b**, **2d–e** and **2g**, starting from compound **14**.

Yield = 63%; mp = 260–263 °C dec. (EtOH); ^{1}H NMR ($CDCl_3$) δ 3.20 (s, 3H, CH_3), 4.75 (s, 2H, CH_2), 6.40 (s, 1H, Ar), 7.20–7.80 (m, 12H, Ar), 8.95 (d, 2H, Ar). Anal. ($C_{27}H_{20}N_6S$) C, H, N.

5.1.16. 6-Benzyl-4-chloro-3-methylisoxazolo[3,4-*d*]pyridazin-7(6*H*)-one, 19

Compound **19** was obtained following the procedure described for compounds **2b**, **2d–e** and **2g** starting from isoxazolopyridazinones **17** and **18** (the mixture of **17** and **18** intermediates was not separated): after dilution with water the suspension was extracted with ethyl acetate (3 × 15 mL) and the solvent was evaporated in vacuo to afford a crude precipitate which was purified by column chromatography using cyclohexane/ethyl acetate 2:1.

Yield = 83%; mp = 158–160 °C (EtOH); ^{1}H NMR ($CDCl_3$) δ 2.95 (s, 3H, CH_3), 5.25 (s, 2H, CH_2), 7.30–7.50 (m, 5H, Ar).

5.1.17. 6-Benzyl-4-chloro-3-styrylisoxazolo[3,4-*d*]pyridazin-7(6*H*)-one, 21

Compound **21** was obtained from **19** following the procedure described for compounds **3d–k.** Yield = 56%; mp = 224–227 °C (EtOH); ^{1}H NMR ($CDCl_3$) δ 5.30 (s, 2H, CH_2), 7.25–7.65 (m, 11H: 10H, Ar; 1H, CH=), 7.85 (d, 1H, =CH).

5.1.18. 4-Amino-2-benzyl-6-chloro-5-(5-phenyl-2*H*-pyrazol-3-yl)pyridazin-3(2*H*)-one, 22

Compound **22** was obtained from **21** following the general procedure described for compounds **4c–k**. For compound **22**, the reaction was carried out at 60 °C for 5 h and, after cooling, the crude precipitate was recovered by suction.

Yield = 38%; mp = 200–202 °C (EtOH); ^{1}H NMR ($CDCl_3$) δ 5.30 (s, 2H, CH_2), 6.95 (s, 1H, Ar), 6.80 (exch br s, 2H, NH_2), 7.20–7.70 (m, 10H, Ar), 13.00 (exch br s, 1H, NH).

5.1.19. 3-Benzyl-1-chloro-6-methyl-9-phenylpyrazolo[1′,5′:1,6]pyrimido[4,5-*d*]pyridazin-4(3*H*)-one, 23

Compound **23** was obtained from **22** following the reported general procedure of **6e–h** and **6n–s**. In this case the mixture was stirred at 100 °C for 5 h. Yield = 62%; mp = 221–223 °C (EtOH); ^{1}H NMR ($CDCl_3$) δ 3.15 (s, 3H, CH_3), 5.40 (s, 2H, CH_2), 7.20–7.60 (m, 8H, Ar), 7.85 (s, 1H, Ar), 8.10 (m, 2H, Ar). Anal. ($C_{22}H_{16}ClN_5O$) C, H, N.

5.2. Biological assays

5.2.1. Radioligand binding assays

Radioligand binding competition assays were performed in vitro using A_1, A_{2A}, A_{2B} and A_3 human receptors expressed in transfected CHO (hA_1), HeLa (hA_{2A} and hA_3) and HEK-293 (hA_{2B}) cells as previously described.[46] Briefly:

5.2.2. Human A_1 receptors

Adenosine A_1 receptor competition binding experiments were carried out in membranes from CHO-A1 cells (Euroscreen, Gosselies, Belgium) labelled with 2 nM [^{3}H]DPCPX. Non-specific binding was determined in the presence of 10 μM (R)-PIA. The reaction mixture was incubated at 25 °C for 60 min.

5.2.3. Human A_{2A} receptors

Adenosine A_{2A} receptor competition binding experiments were carried out in membranes from HeLa-A_{2A} cells labelled with 3 nM [^{3}H]ZM241385. Non-specific binding was determined in the presence of 50 μM NECA. The reaction mixture was incubated at 25 °C for 30 min.

5.2.4. Human A_{2B} receptors

Adenosine A_{2B} receptor competition binding experiments were carried out in membranes from HEK-293-A_{2B} cells (Euroscreen, Gosselies, Belgium) labelled with 35 nM [^{3}H]DPCPX. Non-specific binding was determined in the presence of 400 μM NECA. The reaction mixture was incubated at 25 °C for 30 min.

5.2.5. Human A_3 receptors

Adenosine A_3 receptor competition binding experiments were carried out in membranes from HeLa-A_3 cells labelled with 30 nM [^{3}H]NECA. Non-specific binding was determined in the presence of 100 μM (R)-PIA. The reaction mixture was incubated at 25 °C for 180 min.

After each incubation time, samples were filtered and measured in a microplate beta scintillation counter (Microbeta Trilux, Perkin-Elmer, Madrid, Spain).

5.2.6. Purification of phosphodiesterase isoenzymes

PDE5 was purified from human platelets as described by Gristwood et al.[47] Briefly, the supernatant of the cell lysate from 10^9 platelets was chromatographed by a Mono-Q ion exchange column attached to a Pharmacia FPLC system.

PDE6 was purified from bovine retinas as described by Gillespie and Beavo.[48]

The isoenzymes were characterised according to Beavo et al.[49] by selectivity and affinity, and by effect of calcium ions (10 μM) plus calmodulin (1.2 μM), cyclic GMP (5 μM) and the selective inhibitor Sildenafil. PDE5 was kept frozen at −80 °C in presence of 1 g/l bovine serum albumin until used.

5.2.7. Phosphodiesterase assay

Cyclic nucleotide phosphodiesterase activities were measured using a two step procedure according to Thompson and Strada.[50] PDE5 and PDE6 (activated by 250 μg/ml trypsin) were assayed using 0.25 μM ^{3}H-cGMP as substrate. IC_{50} values were obtained by nonlinear regression using the Prism programme by GraphPad Software. The reported values are the average of at least three independent assays. Sildenafil was used as reference substance. The drugs were dissolved in DMSO at 10^{-3} M. The effect of the solvent was taken into consideration in the calculations.

Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bmc.2010.09.043.

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