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Exploring the 2- and 5-positions of the pyrazolo[4,3-*d*]pyrimidin-7-amino scaffold to target human A_1 and A_{2A} adenosine receptors

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Key words: G protein-coupled receptors, A_1 and A_{2A} adenosine receptor antagonists, pyrazolo[4,3-*d*]pyrimidines, ligand-adenosine receptor modeling studies.

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[1]**Abbreviations:** AR, adenosine receptor; PD, Parkinson's disease; CCPA, 2-chloro-N(6)-cyclopentyladenosine; NECA, 5'-(N-ethyl-carboxamido)adenosine; cAMP, cyclic adenosine monophosphate; Cl-IB-MECA, 2-chloro-N6-(3-iodobenzyl)5'-(N-methylcarbamoyl)adenosine DPCPX, 8-cyclopentyl-1,3-dipropyl-xanthine; ZM241385, 4-(2-[7-amino-2-(2-furyl)[1,2,4]-triazolo[2,3-*a*][1,3,5]triazin-5-ylamino]ethyl)phenol; I-AB-MECA, N^6-(4-amino-3-iodobenzyl)-5'-(N-methylcarbamoyl)adenosine; EL2, second extracellular loop; MOE, molecular operating environment

Abstract

A new series of 7-aminopyrazolo[4,3-*d*]pyrimidine derivatives (**1-31**) were synthesized to evaluate some structural modifications at the 2- and 5- positions aimed at shifting affinity towards the human (h) A_{2A} adenosine receptor (AR) or both hA_{2A} and hA_1 ARs. The most active compounds were those featured by a 2-furyl or 5-methylfuran-2-yl moiety at position 5, combined with a benzyl or a substituted-benzyl group at position 2. Several of these derivatives (**22-31**) displayed nanomolar affinity for the hA_{2A} AR (K_i= 3.62-57 nM) and slightly lower for the hA_1 ARs, thus showing different degrees (3-22 fold) of hA_{2A} versus hA_1 selectivity. In particular, the 2-(2-methoxybenzyl)-5-(5-methylfuran-2-yl) derivative **25** possessed the highest hA_{2A} and hA_1 AR affinities (K_i= 3.62 nM and 18 nM, respectively) and behaved as potent antagonist at both these receptors (cAMP assays). Its 2-(2-hydroxybenzyl) analog **26** also showed a high affinity for the hA_{2A} AR (K_i= 5.26 nM) and was 22-fold selective versus the hA_1 subtype. Molecular docking investigations performed at the hA_{2A} AR crystal structure and at a homology model of the hA_1 AR allowed us to represent the hypothetical binding mode of our derivatives and to rationalize the observed SARs.

1. Introduction

The neuromodulator adenosine elicits its biological effects through the activation of G-protein-coupled receptors, classified as A_1, A_{2A}, A_{2B} and A_3 subtypes.[1,2] Adenosine receptors (ARs) are typically coupled to adenylyl cyclase which can be inhibited (A_1 and A_3) or activated (A_{2A} and A_{2B}), but other intracellular pathways can be modulated, depending on the cell type and on the contingent situation.

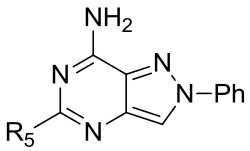
A_1 and A_{2A} ARs are coupled to mitogen-activated protein kinases (MAPK), K-ATP channel and phospholipase C. A_1 receptor is the most widely distributed subtype with the highest level in the brain, in particular in the hippocampus and prefrontal cortex, which are areas implicated in the control of emotions and cognition functions.[3-4] A_1 AR is expressed with intermediate density in peripheral organs such as heart and kidney.[1,2] Coherently, A_1 AR antagonists are sought as therapeutic agents for mental dysfunction, such as dementia and anxiety[3-5] and they have also been shown to be protectant in models of renal ischemia-reperfusion injury and vasoconstriction[5-6] although cardiac disorders have been reported as their common adverse events. A_{2A} AR subtype is extensively distributed in different organs, including heart, liver and lung, as well as in the brain where the higher density is in the striatum, nucleus acumens, cortex and hippocampus. A_{2A} AR blockade in the brain elicits protective effect in both chronic and acute neurodegenerative diseases, such as cerebral ischemia[7,8] and Parkinson's disease (PD)[9-11] respectively. Related to PD, several clinical trials have been conducted with A_{2A} AR antagonists which proved to be effective in counteracting extrapyramidal symptoms, and, recently, istradefylline has been approved for marketing in Japan.[12] Preclinical studies have also shown that PD can benefit from the use of dual A_1/A_{2A} antagonists because they reduce both motor (A_{2A}) and cognitive (A_1) deficits associated to the disease.[5,13-15]

Very recent research has demonstrated the effect of the A_{2A} AR blockade on enhancing immunologic response, highlighting that A_{2A} receptor antagonists have the potential to markedly improve anti-tumor immunity in mouse models and to promote tumor regression. Accordingly, A_{2A}

AR antagonists have been shown to enhance the effect of tumor vaccines during T cell activation, hence they might work in concert with other immune checkpoint inhibitors in cancer immunotherapy.[16-17]

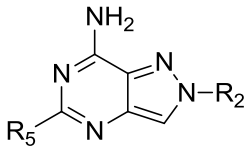
In our laboratory, much research has been addressed to the study of AR antagonists belonging to different classes [18-27] including the 2-arylpyrazolo[4,3-*d*]pyrimidine-7-amino series [25-27] (Figure 1).

Figure 1. Previously reported pyrazolo[4,3-*d*]pyrimidine derivatives **A**-**D**.



R_5= Me (**A**), Ph (**B**), CH_2Ph (**C**), $(CH_2)_3$Ph (**D**)

A recent structure-activity relationship (SAR) study on this series has highlighted that the presence of a methyl and a phenyl group at the 5-position (R_5) of the pyrazolo[4,3-*d*]pyrimidine (PP) scaffold (Figure 1, compounds **A** and **B** respectively) affords good affinity for hA_1, hA_{2A} and hA_{2B} ARs,[25] whereas a benzyl and, even better, a 3-phenylpropyl chain (Figure 1, derivatives **C** and **D**, respectively) elicit an enhancement of the A_{2A} AR affinity and selectivity.[26] Moreover, introduction of a methoxy/hydroxy group at the ortho, meta or para positions of the appended 3-phenylpropyl moiety of **D** completely reversed selectivity, affording potent and selective A_1 AR antagonists.[26] Hence, we decided to further investigate this series with the aim of obtaining more potent antagonists for the hA_{2A} AR or balanced antagonists for hA_1 and hA_{2A} ARs. Thus, we performed some modifications on the previously reported compounds **A**-**D** (compounds **1**-**31**, Figure 2) by replacing the 2-phenyl residue with aryl groups or other substituents with different lipophilicity and steric hindrance (methyl, benzyl, arylmethyl, phenethyl). Moreover, the effect of some heteroaryl moieties at the 5-position of the bicyclic scaffold was evaluated.



1-31

R_2= Me, CH_2Ph, $(CH_2)_2$-Ph

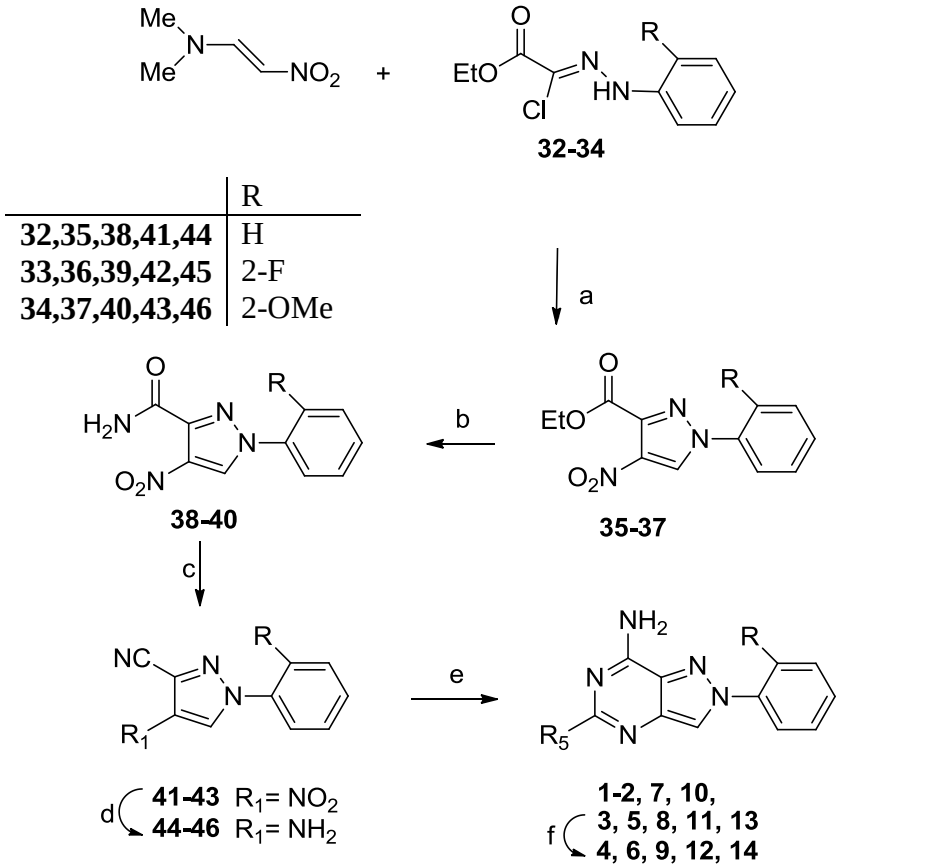
R_5= Me, Ph, $(CH_2)_{1,3}$-Ph
2-furyl, 5-methylfuran-2-yl

Figure 2. Herein reported pyrazolo[4,3-*d*]pyrimidine derivatives **1-31**.

2 Chemistry

The herein described pyrazolo[4,3-*d*]pyrimidin-7-amines **1-31** were prepared as depicted in Schemes 1-3. The pyrazolopyrimidines **1-14**, bearing an aryl moiety at the 2-position were obtained as shown in Scheme 1.

Allowing N,N-dimethyl-2-nitroetheneamine[28] to react with the suitable N_1-arylhydrazono-N_2-chloroacetates **32-33** [29-30] and **34** in chloroform, the ethyl 4-nitropyrazole-3-carboxylates **35,**[27] **36** and **37** were obtained. These compounds were transformed into the corresponding amides **38,**[27] **39** and **40** by reaction with 33% aqueous ammonia solution. Treatment of the 3-carboxamides **38-40** with phosphorous oxychloride under microwave irradiation gave the 4-nitro derivatives **41**,[27] **42** and **43** which were reduced with cyclohexene or hydrogen, in the presence of Pd/C, to provide the corresponding 4-amino derivatives **44**,[27] **45** and **46.** These compounds were cyclized by treatment with ammonium acetate and triethyl orthoacetate or the suitably synthetized ethyl iminoesters hydrochlorides [31-34] to yield the pyrazolo[4,3-*d*]pyrimidin-7-amine derivatives **1-3, 5, 7, 8, 10, 11** and **13**. The 2-(2-methoxyphenyl)-derivatives **3, 5, 8, 11** and **13** were demethylated with boron tribromide to obtain the corresponding 2-(2-hydroxyphenyl)-derivatives **4, 6, 9, 12, 14.**



	R
32,35,38,41,44	H
33,36,39,42,45	2-F
34,37,40,43,46	2-OMe

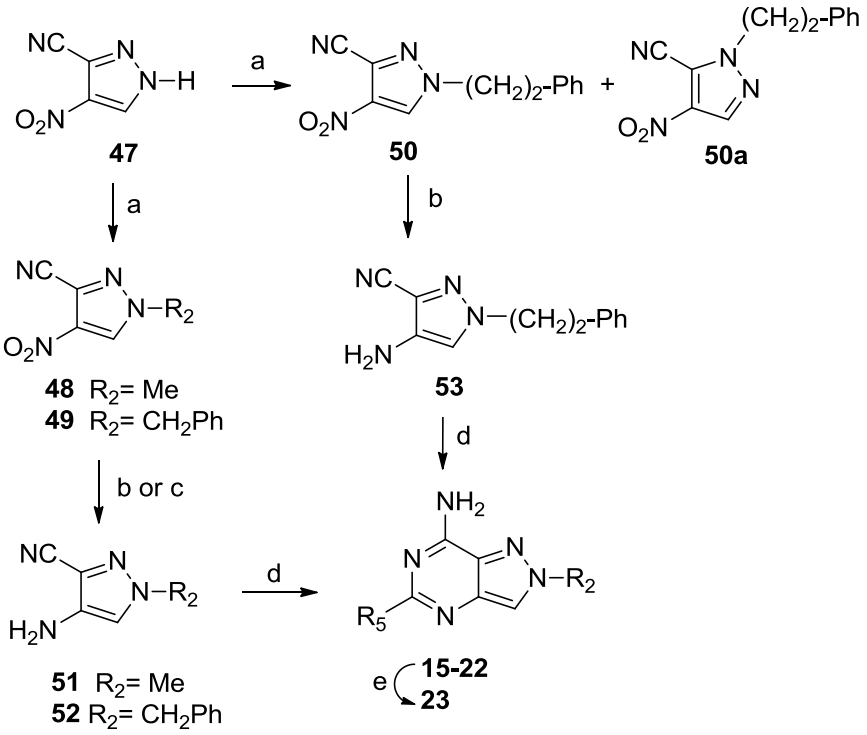
	R_5	R		R_5	R
1	Me	F	**8**	2-furyl	OMe
2	$(CH_2)_3Ph$	F	**9**	2-furyl	OH
3	$(CH_2)_3Ph$	OMe	**10**	2-(5-methylfuryl)	H
4	$(CH_2)_3Ph$	OH	**11**	2-(5-methylfuryl)	OMe
5	CH_2Ph	OMe	**12**	2-(5-methylfuryl)	OH
6	CH_2Ph	OH	**13**	2-thienyl	OMe
7	2-furyl	H	**14**	2-thienyl	OH

Scheme 1. (a) NEt_3, $CHCl_3$, mw, 140 °C; (b) 33% aqueous NH_3, r.t.; (c) $POCl_3$, mw, 150 °C; (d) cyclohexene, Pd/C, mw, 110-130 °C; (e) Me-$C(OEt)_3$ or R_5-C(OEt)NH hydrochloride, NH_4OAc, sealed tube or mw, 90-150 °C; (f) R= OMe, BBr_3, CH_2Cl_2, reflux.

The pyrazolopyrimidines **15-23**, bearing a methyl or an arylalkyl moiety at the 2-position, were obtained as depicted in Scheme 2.

The synthetic pathway started from the 4-nitropyrazole-3-carbonitrile **47** which was regioselectively alkylated with methyl iodide and benzylbromide in the presence of sodium hydride, in anhydrous tetrahydrofuran, to give the previously reported 4-nitropyrazole-3-carbonitrile derivatives **48** and **49.**[27] Alkylation of **47** with phenethylbromide, in the same conditions, afforded the 1-phenethyl-4-

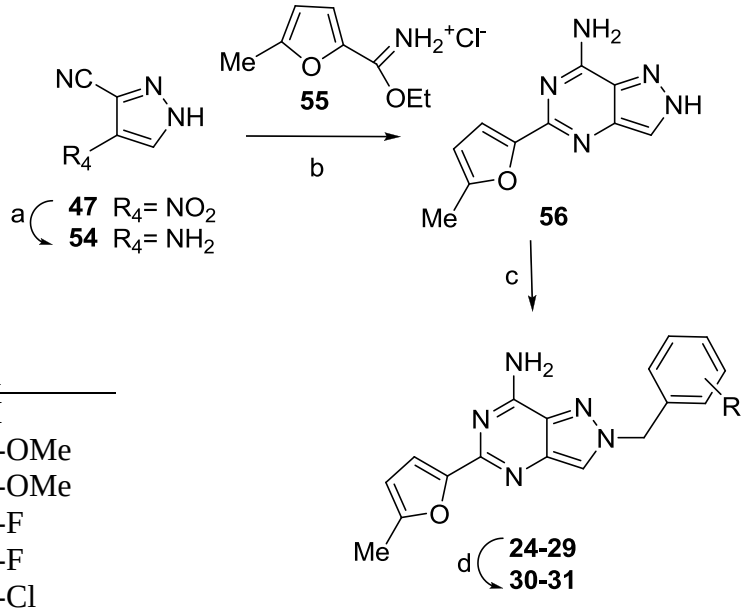
nitropyrazole-3-carbonitrile **50** as the major isomer, along with the 1-phenethyl-3-nitropyrazole-5-carbonitrile **50a** (the molar ratio between the two isomers **50** and **50a** was about 6:1 from the ^{1}H-NMR spectrum of the crude material). The structure of compound **50** was determined by means of NOESY experiments, which showed a spatial closeness of the pyrazole hydrogen atom to methylene protons.



	R_5	R_2
15	CH_2Ph	Me
16	$(CH_2)_3Ph$	Me
17	Me	CH_2Ph
18	Me	$(CH_2)_2Ph$
19	CH_2Ph	CH_2Ph
20	2-furyl	CH_2Ph
21	2-furyl	$(CH_2)_2Ph$
22	2-furyl	$CH_2C_6H_4$-2-OMe
23	2-furyl	$CH_2C_6H_4$-2-OH

Scheme 2. (a) CH_3I or Ph-$(CH_2)_n$Br, NaH, THF, room temperature (**48, 49**) or reflux (**50**); (b) H_2, Pd/C, Parr apparatus, 30 psi; (c) cyclohexene, Pd/C, mw, 150 °C; (d) Me-$C(OEt)_3$ or R_5-C(OEt)NH hydrochloride, NH_4OAc, mw at 130 °C or, for **22**, sealed tube at 120°C; (e) **22**, BBr_3, CH_2Cl_2, reflux.

The 4-nitropyrazole derivatives **48-50** were transformed into the corresponding 4-aminopyrazoles by reduction with hydrogen (**51**)[27] or cyclohexene (**52**[27] and **53**), in the presence of Pd/C. Treatment of **51-53** with triethyl orthoacetate or the suitable imminoester hydrochlorides[31-33] and ammonium acetate yielded the pyrazolopyrimidines **15-22**. Demethylation of the 2-(2-methoxybenzyl)-derivative **22** with boron tribromide afforded the respective 2-(2-hydroxybenzyl) derivative **23**. To prepare the set of pyrazolopyrimidine **24-31**, bearing the (5-methyl-furan-2-yl)- moiety at the 5-position, a new synthetic pathway was developed (Scheme 3).



	R
24	H
25	2-OMe
26	3-OMe
27	2-F
28	3-F
29	2-Cl
30	2-OH
31	3-OH

Scheme 3. (a) H_2, Pd/C, Parr apparatus, 35 psi; (b) NH_4OAc, sealed tube, 120 °C; (c) Ar-CH_2-Cl, K_2CO_3, CH_3CN/DMF, r.t. or 60 °C; (d) **25** or **26,** BBr_3, CH_2Cl_2, r.t. or reflux.

The 4-aminopyrazole-3-carbonitrile **54**,[35] obtained from the corresponding 4-nitro derivative **47**, was reacted with ammonium acetate and ethyl 5-methylfuran-2-carboximidate hydrochloride **55** to provide the 7-amino-5-(5-methyl-furan-2-yl)-2H-pyrazolo[4,3-*d*]pyrimidine **56**. This compound was transformed into the pyrazolopyrimidines **24-29** by regioselective alkylation with suitable benzyl chlorides, all commercially available, except the 2-methoxybenzyl chloride which was prepared following a reported procedure.[36] Alkylation was carried out in a mixture of

dimethylformamide/acetonitrile, in the presence of potassium carbonate. The 2-substituted structure of **24-29** was determined by means of NOESY experiments showing a spatial proximity between the pyrazole hydrogen atom and the methylene protons.

Finally, the methoxy-derivatives **25** and **26** were demethylated with boron tribromide to give the respective hydroxy-substituted compounds **30** and **31**.

3. Results and Discussion

3.1. Structure-affinity relationship studies

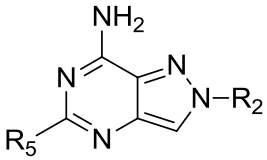
The synthesized compounds **1-31** were tested in binding assays to evaluate their affinity at cloned hA_1, hA_{2A} and hA_3 ARs, stably expressed in CHO cells. The new compounds were also tested at the hA_{2B} receptor by measuring their inhibitory effects on NECA-stimulated cAMP levels in CHO cells. The results of binding experiments and cAMP assays are reported in Table 1, where the data of compounds **A-D** are also included as references.

The reported results indicate that our aim of obtaining pyrazolopyrimidine derivatives endowed with high affinity for the hA_{2A} receptor or both hA_1 and hA_{2A} receptors has been achieved. The most interesting findings were found within the set of compounds **20-31** in which a benzyl or a substituted benzyl group at the 2-position was combined with a 2-furyl or a 2-(5-methylfuryl) moiety at position 5. Indeed, most of these derivatives (**22-31**) bind to the hA_{2A} AR with a nanomolar affinity and different degrees of selectivity (3-22 fold) versus the hA_1 ARs. Moreover, they showed good to moderate affinities for A_3 and hA_{2B} subtypes.

The first set of synthesized derivatives (**1-6**) were designed as analogues of compounds **A**, **C-D** which were modified by introduction of hydrogen bond acceptor groups (F, OMe, OH) on the ortho position of the 2-phenyl ring. This choice was based on the results of docking studies at the hA_{2A} crystal structure previously performed on the pyrazolopyrimidine series, including derivatives **A-D** (Figure 1).[25,26] In the best docking poses, these derivatives were anchored inside the binding cleft by a tight hydrogen bond network with the highly conserved residues Asn253 (TM6) and E172/169

(EL2). In particular, a hydrogen bond between the NH_2 amide moiety of Asn253 (TM6) and the N1-pyrazole atom has been evidenced. Hence, the hypothesis that a hydrogen bond acceptor group at the ortho position of the 2-phenyl ring might reinforce the anchoring of the molecule at the level of Asn253 (TM6) residue prompted us to synthesize compounds **1**-**6**. Actually, these modifications caused, on the whole, a reduction of the affinity for the hA_{2A} receptor (compare **1** to **A**; **2** and **3** to **C**; **5** and **6** to **D**) and for the other hAR subtypes.

Table 1. Binding Affinity (K_i) at hA_1, hA_{2A} and hA_3 ARs and potencies (IC_{50}) at hA_{2B} ARs.



A-D, 1-31

			Binding experiments[a] K_i (nM) or I%			**cAMP assays IC_{50}(nM) or I%**
	R_5	R_2	hA_1[b]	hA_{2A}[c]	hA_3[d]	hA_{2B}[e]
A[f]	Me	Ph	70 ±6	246 ± 23	40%	320±35
B[f]	Ph	Ph	75 ± 7	325 ± 34	48%	440 ±43
C[f]	$PhCH_2$	Ph	150 ±12	110 ±10	39%	420 ±38
D[g]	$Ph(CH_2)_3$	Ph	5.3±04	55±5	12%	42%
1	Me	C_6H_4-2-F	410 ±42	23%	8%	8%
2	$PhCH_2$	C_6H_4-2-OMe	25%	43%	163±15	1%
3	$PhCH_2$	C_6H_4-2-OH	49±4	647 ±62	6%	21%
4	Ph-$(CH_2)_3$	C_6H_4-2-F	17 ± 2	240±22	5%	5%
5	Ph-$(CH_2)_3$	C_6H_4-2-OMe	103 ± 9	34%	405 ±38	1%
6	Ph-$(CH_2)_3$	C_6H_4-2-OH	52 ± 4	21%	19%	1%
7	2-furyl	Ph	206 ±17	195±14	39%	1%
8	2-furyl	C_6H_4-2-OMe	31%	362 ± 34	37%	12%
9	2-furyl	C_6H_4-2-OH	372 ± 33	89 ± 8	36%	42%
10	2-(5-methylfuryl)	Ph	42 ± 3	99 ± 8	147±12	32%
11	2-(5-methylfuryl)	C_6H_4-2-OMe	1%	518 ± 47	297 ± 26	1%
12	2-(5-methylfuryl)	C_6H_4-2-OH	1%	147 ± 13	375 ± 32	1%
13	2-thienyl	C_6H_4-2-OMe	24%	27%	150±13	1%
14	2-thienyl	C_6H_4-2-OH	159 ± 16	412±39	209±18	28%
15	$PhCH_2$	Me	9%	1%	1%	20%
16	$Ph(CH_2)_3$	Me	36%	1%	30%	2%
17	Me	CH_2Ph	1%	1%	1%	1%

18	Me	$(CH_2)_2Ph$	10%	12%	3%	3%
19	CH_2Ph	CH_2Ph	1%	1%	15%	1%
20	2-furyl	CH_2Ph	32%	320 ± 28	2%	37%
21	2-furyl	$(CH_2)_2Ph$	12%	33%	4%	3%
22	2-furyl	CH_2-C_6H_4-2-OMe	98±8	5.37±0.39	196±17	512±49
23	2-furyl	CH_2-C_6H_4-2-OH	242±21	17±2	905±88	112±11
24	2-(5-methylfuryl)	CH_2Ph	136±12	9.23 ±0.85	269 ± 25	20%
25	2-(5-methylfuryl)	CH_2-C_6H_4-2-OMe	18±2	3.62±0.34	82 ± 7	30%
26	2-(5-methylfuryl)	CH_2-C_6H_4-2-OH	120±11	5.26±0.47	88±6	293±26
27	2-(5-methylfuryl)	CH_2-C_6H_4-3-OMe	309±8	57 ± 6	498 ± 47	354 ± 32
28	2-(5-methylfuryl)	CH_2-C_6H_4-3-OH	252±21	51 ± 5	427 ± 41	421 ± 39
29	2-(5-methylfuryl)	CH_2-C_6H_4-2-F	60±5	6.21±0.58	71±6	152±14
30	2-(5-methylfuryl)	CH_2-C_6H_4-3-F	111±10	38 ±4	122 ± 11	395 ± 38
31	2-(5-methylfuryl)	CH_2-C_6H_4-2-Cl	65±5	14±2	225±21	633±52

[a]K_i values are means ± SEM of four separate assays each performed in duplicate. Percentage of inhibition (I%) are determined at 1μM concentration of the tested compounds. [b]Displacement of specific [^{3}H]DPCPX competition binding assays to hA_1CHO cells. [c]Displacement of specific [^{3}H]ZM241385 competition binding to hA_{2A}CHO cells. [d]Displacement of specific [^{125}I]AB-MECA competition binding to hA_3CHO cells. [e]cAMP experiments in hA_{2B}CHO cells, stimulated by 200 nM NECA. Percentage of inhibition (I%) are determined at 1μM concentration of the tested compounds. [f]Ref. 25. [g] Ref. 26.

Replacement of the 5-phenyl ring of **B** with a 2-furyl (compound **7**) was more profitable because it achieved a shift of affinity, albeit small, towards the A_{2A} subtype. In fact, with respect to **B**, compound **7** bind slightly better the hA_{2A} receptor (K_i= 195 nM) and worse the hA_1 one. Insertion of the 2-(5-methyl)furyl group at the 5-position further enhanced the hA_{2A} affinity (K_i= 99 nM) but not the selectivity, compound **10** showing high and good affinity, respectively, for the hA_1 (K_i= 42 nM) and hA_3 (Ki= 147 nM) subtypes. In light of these findings, compounds **7** and **10** were modified by introduction of a 2-methoxy or 2-hydroxy group on the 2-phenyl ring. All the resulting compounds (**8, 9** and **11, 12**) showed decreased affinity for the hA_{2A} AR and even more for the hA_1 subtype. There was only one exception: the 2-(2-hydroxyphenyl) derivative **9** (R_5= 2-furyl) which was two-fold more active than **7** at the hA_{2A} AR subtype (K_i= 89 nM). A selective hA_3 AR ligand, instead, ensued from the combination of the 2-methoxyphenyl group at position 5 with a 2-thienyl

residue at the 2-position (derivative **13**), thus confirming that the 2-thienyl group is very profitable for the anchoring to the hA_3 AR,[27] unlike the 2-furyl that shifts affinity towards the hA_{2A} AR.

A dramatic reduction of affinity was obtained when the 2-phenyl ring of compounds **C** and **D** was replaced with the smaller and less lipophilic methyl group (derivatives **15** and **16**) or when the 2-phenyl ring of compound **A** was replaced with a benzyl (**17**) or phenethyl moiety (**18**). In fact, compounds **15-18** are completely inactive at both the target hA_{2A} and hA_1 receptors and also at the other hAR subtypes. The same applies to derivative **19**, characterized by the presence of a benzyl chain at both the 2- and 5-positions.

An interesting finding was achieved when the benzyl group at the 2-position was combined with the 2-furyl ring at the 5-position of the pyrazolopyrimidine scaffold to provide derivative **20**. With respect to the corresponding 2-phenyl derivative **7,** compound **20** showed higher hA_{2A} AR selectivity, even if its hA_{2A} AR affinity is slightly lower (K_i= 320 nM). Elongation of the 2-benzyl chain of **20** to 2-phenethyl dropped hA_{2A} AR affinity (compound **21**), while replacement of the 5-(2-furyl) ring of **20** with the 5-(5-methylfuran-2-yl)- substituent (compound **24**) significantly improved it. Derivative **24**, in fact, binds the hA_{2A} receptor with high affinity (K_i= 9.23 nM) and some selectivity versus both hA_1 (15-fold) and hA_3 (29-fold) subtypes, thus confirming the favorable role of the 5-(5-methylfuran-2-yl)- substituent for the hA_{2A} AR-ligand interaction (see above, **10** versus **7**).

Based on these results, both compounds **20** and **24** were modified by introduction of a methoxy or hydroxy residue at the ortho position on the benzyl group. All the resulting pyrazolopyrimidines **22, 23** (R_5= 2-furyl) and **25, 26** (R_5= 5-methylfuran-2-yl) possessed very high hA_{2A} AR affinities (3.6 nM < K_i < 17 nM), being improved with respect to those of the corresponding 2-benzyl derivatives. Moreover, the 5-(methylfuran-2-yl)- derivative **26** exhibited the highest hA_{2A} versus hA_1 AR selectivity. Hence, we sought to synthesize new compounds featured by the 5-(5-methylfuryl) residue combined with other substituted benzyl groups at position 2 (**27-31**). Insertion of a methoxy and hydroxy substituent at the meta position of the benzyl moiety afforded derivatives **27** and **28**,

whose hA_{2A} affinities were still in the low nanomolar range (K_i= 57 and 51 nM, respectively), although decreased with respect to those of the ortho substituted derivatives **25** and **26**. A similar trend of hA_{2A} affinity was found for compounds **29** and **30**, bearing the fluorine atom as hydrogen bond acceptor, respectively, at the ortho and meta position of the benzyl group. In fact, the ortho-fluorobenzyl derivative **29** displayed a higher hA_{2A} affinity (K_i= 6.21 nM) than the meta-isomer **30** (K_i= 38 nM). Finally, insertion of a chlorine atom at the ortho position on the benzyl moiety also afforded high hA_{2A} affinity (K_i= 14 nM).

In summary, the structural investigation carried out at the 5- and 2- position of pyrazolopyrimidine scaffold highlighted that high affinity for the hA_{2A} AR or for both hA_{2A} and hA_1 ARs can be achieved through the 5-(5-methylfuran-2-yl)- and 5-(2-furyl) substitution in combination with a 2-benzyl or 2-benzyl-substituted moiety.

Some selected derivatives (**22, 24-26**) were tested to assess their antagonistic potencies at hA_1 and hA_{2A} ARs. Thus, their capability to reduce the inhibitory effect of 2-chloro-N^6-cyclopentyladenosine (CCPA) in cAMP production on hA_1 CHO cells and to inhibit NECA-stimulated cAMP production on hA_{2A} CHO cells, were evaluated (Table 2).

Table 2. Antagonistic potencies of selected compounds at hA_1 and hA_{2A} ARs.

	cAMP assay IC_{50} (nM)[a]	
	hA_1	hA_{2A}
22	106 ± 10	6.21 ± 0.57
24	152 ± 14	10.91 ± 1.07
25	21 ± 2	4.35 ± 0.42
26	134 ± 12	6.42 ± 0.61

[a] cAMP experiments: the compounds were tested in hA_1CHO or hA_{2A}CHO cells in the presence of CCPA (1 nM) or NECA (10 nM), respectively. IC_{50} values are expressed as means ± SEM of four separate experiments.

The tested compounds behaved as antagonists at both hA_1 and hA_{2A} ARs with potencies well-correlated to their affinities. Among them, compounds **25** proved to be the most potent dual A_{2A} and A_1 AR antagonist, although it was able to bind also to the hA_3 AR.

3.2 Molecular modeling studies

A molecular modeling study was performed to simulate and analyse the interaction of the synthesised compounds with the binding site of the human A_{2A} AR. For this analysis, the crystal structure of the same protein solved in complex with the well-known antagonist ZM241385 (Protein Data Bank -pdb- code: 3EML; 2.6-Å resolution[37,38]) was employed. The crystal structure was re-modelled by firstly removing the external T4L segment and secondly by performing a building of missing receptor regions (the missing section of the extracellular 2 -EL2- or intracellular 3 -IL3- domains). The obtained A_{2A} AR model was checked by inspection of backbone bond lengths, angles and dihedrals, Ramachandran φ-ψ dihedral plots, and sidechain rotamer and nonbonded contact quality. These steps were performed within Molecular Operating Environment (MOE, version 2010.10) suite.[39] The A_{2A} AR structure was then used as target for the docking analysis of the synthesised derivatives by using Autodock 4.2.6 software and PyRx interface.[40-42] Docking results were then imported into MOE for post-docking energy minimization and analysis. A set of derivatives was also subjected to molecular docking analysis at a homology model of the human A_1 AR built by using the above cited A_{2A} AR crystal structure as a template.

The pyrazolopyrimidine scaffold shows structural similarity to the triazolotriazine one of the co-crystallized reference compound ZM241385. The docking results for the synthesized molecules showed two possible binding modes, one of which (binding mode 1, Figure 3A) being comparable to the one observed for ZM241385 at the A_{2A} AR crystal structure (pdb code: 3EML) and similar also to the binding mode of other pyrazolopyrimidine derivatives previously reported.[26] In details, the bicyclic scaffold is vertically oriented and positioned between residues of the EL2 and transmembrane (TM) 6 domains (Phe168 and Leu249$^{6.51}$, respectively[43]), with the 7-amino group

and the N1 nitrogen atom providing a double H-bond interaction with the Asn253$^{6.55}$ residue. The 2-substituent is oriented towards the receptor core and is located between residues of TM3, TM5, and TM6 segments (Leu85$^{3.33}$, Thr88$^{3.36}$, Met177$^{5.38}$, Asn181$^{5.42}$, Trp246$^{6.48}$, Leu249$^{6.51}$, and His250$^{6.52}$), while the 5-substituent points toward the extracellular space and is located between TM1, TM2, EL2, EL3, and TM7 residues (Tyr9$^{1.35}$, Ala63$^{2.61}$, Ile66$^{2.64}$, Leu167, Leu267, Met270$^{7.35}$, Tyr271$^{7.36}$, and Ile274$^{7.39}$).

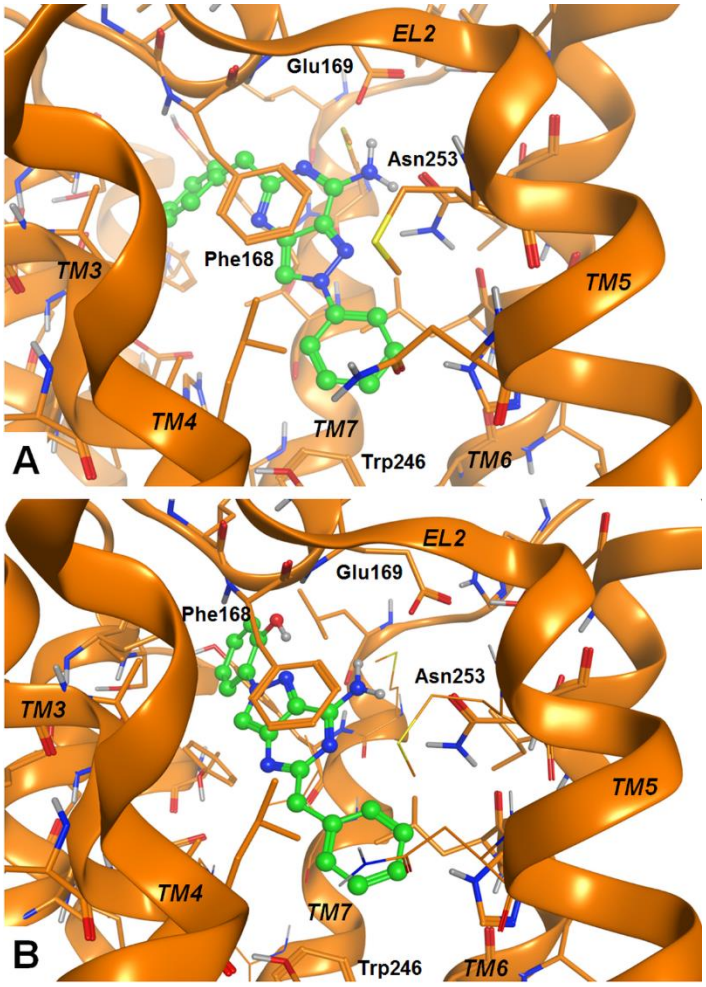


Figure 3. Docking conformations at the A_{2A} AR. Panel A. Binding mode 1 conformation (see text for details) of compound **C**. Key receptor residues and domains are indicated. Panel B. Binding mode 2 conformation of compound **3**.

In the second binding mode (binding mode 2, Figure 3B) the derivatives were located in an analogue position but were oppositely oriented considering the 2- and 5- substituents. The double

H-bond interaction with the Asn253$^{6.55}$ residue is given by the 7-amino group and the N6 nitrogen atom of the pyrazolopyrimidine moiety. In general, the derivatives bearing an arylalkyl substituent at the 2-position and an aryl group at the 5-position, or vice versa, are positioned with the more flexible arylalkyl substituent oriented towards the extracellular space. In this way, the docking poses allow the above cited interaction with the Asn253$^{6.55}$ residue, while those with the arylalkyl substituent in the depth of the binding pocket partially lose such interaction with the receptor and are associated to a significantly lower docking score. Exceptions are compounds **2**, **3**, **5**, **6**, bearing a hydroxy- or methoxy-substituted phenyl ring as 2-substituent. For these compounds, docking results showed the 2-substituent as externally oriented and the phenylalkyl group at the 5-position as located in the depth of the cavity. This binding mode still allows the 5-benzyl substituted derivatives **2** and **3** to partially interact with Asn253$^{6.55}$ and Glu169 (Figure 3B), providing a fair affinity at least for compound **3**. In the case of compounds **5** and **6** (inactive at the A_{2A} AR), the longer 5-substituent prevents the scaffold from properly interacting with the two above cited receptor residues.

The conformations predicted for compounds **20**-**31**, bearing a 2-furyl or a 2-(5-methyl)furyl group at the 5-position and an arylalkyl substituent at the 2-position, are generally belonging to the "binding mode 2" arrangement (see details of docking results for compound **25**, Figure 4).

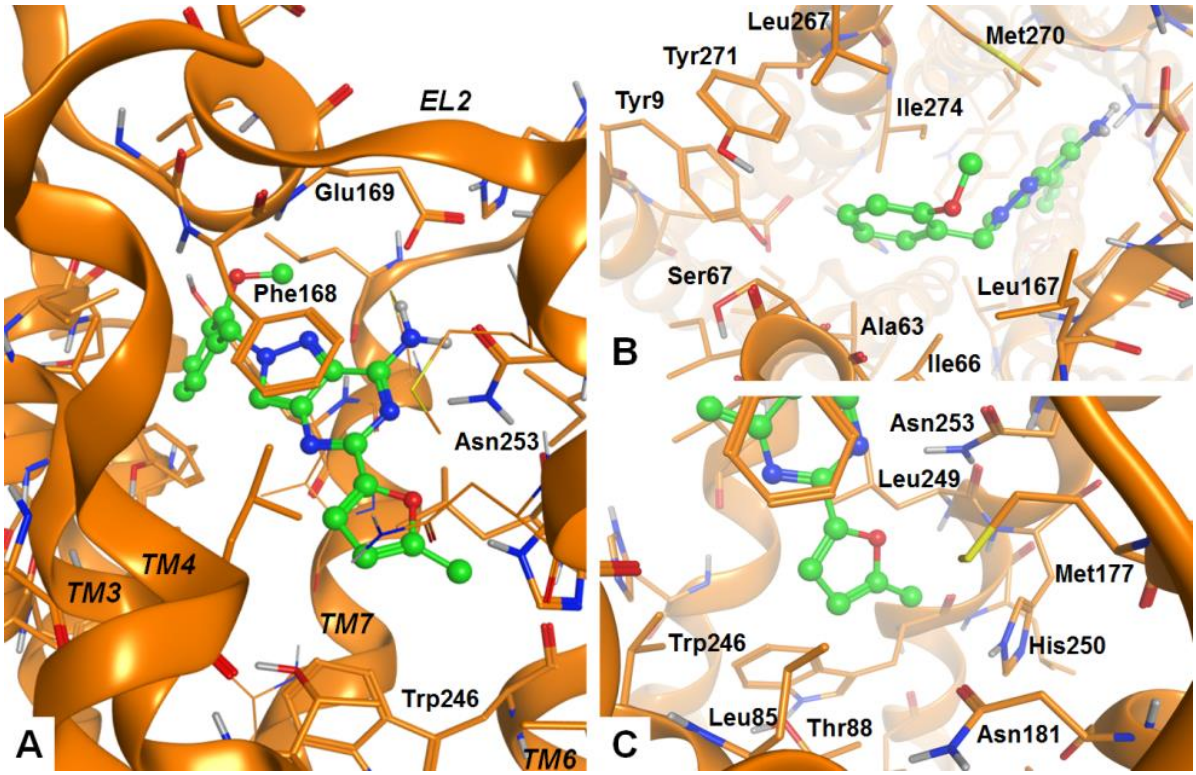


Figure 4. Docking conformation of compound **25** at the A_{2A} AR binding site. Panel A. Global view of the ligand-receptor interaction, with indication of key receptor residues. Panels B-C. Detail of the interaction between the receptor and the 2- and 5-substituents of **25**, respectively.

Interestingly, the superimposition of the top-score docking conformations of this set of molecules presents the bicyclic scaffold as occupying an almost identical position, hence not being influenced by the replacement of the 2-furyl ring with a 2-(5-methyl)furyl group. We performed a post-docking analysis of the interactions between the compounds and the receptor binding site by using the *IF-E 6.0*[44] tool that is retrievable at the SVL exchange service (Chemical Computing Group, Inc. SVL exchange: http://svl.chemcomp.com). This tool was previously employed for other analyses at ARs.[24,45,46] The script calculates and displays atomic and residue interaction forces as 3D vectors and calculates the per-residue interaction energies (values in kcal/mol), where negative and positive energy values are associated to favorable and unfavorable interactions, respectively. In this study, the analysis was focused on the interaction between the 5-substituent (2-furyl or 2-(5-methyl)furyl group) and the residues located in its proximity. For this task we employed four derivatives bearing

the same substituted benzyl group at the 2-position and a 2-furyl (**22**, **23**) or 2-(5-methyl)furyl group (**25**, **26**) at the 5-position. The results of this analysis are reported in Table 3.

Table 3. Interaction energies (values in kcal/mol) between compounds **22**, **23, 25, 26** and **8**, **9**, **11**, **12** and the binding site residues located in proximity of the 5-position of the analyzed compounds. See text for details.

res	*cpd*							
	22	**25**	**23**	**26**	**8**	**11**	**9**	**12**
Leu85	-0.51	0.07	-0.46	0.16	-0,36	-0,71	-0,44	-0,78
Thr88	-0.35	-1.02	-0.51	-1.10	-0,71	-1,03	-0,50	-0,71
Met177	-1.91	-2.13	-2.28	-2.54	-2,27	-1,19	-2,45	-1,97
Asn181	-1.29	-1.09	-1.05	-0.97	-0,89	-0,97	-0,64	-0,90
Trp246	-1.73	-1.44	-1.59	-2.04	-1,73	-0,01	-1,85	0,31
Leu249	-1.19	-1.63	-2.37	-2.15	-2,01	-1,05	-2,72	-3,30
His250	-1.52	-1.79	-1.47	-1.64	-0,80	-0,81	-1,48	-0,73
Asn253	-6.39	-6.49	-5.88	-6.24	-5,21	-5,17	-5,42	-6,04
tot	-14.88	-15.51	-15.61	-16.52	-13,98	-10,92	-15,49	-14,12

The two derivatives bearing at the 5-position a 2-(5-methyl)furyl group present a slightly better interaction with the binding site residues (located in proximity of the 5-position) respect to the corresponding 2-furyl substituted compounds. This is probably due to the mainly hydrophobic properties of the receptor residues involved in the interaction with this part of the molecules. In this sense, the presence of an additional methyl group could lead to a more favorable interaction with respect to the unsubstituted furan ring. It should be taken into account also that the presence of the additional methyl group within the 5-substituent may lead to a higher occupancy of the TM3-TM5-TM6 sub pocket and hence to a higher topological complementarity between the compounds and binding site (Figure 5A). These factors could help to depict the slightly higher affinity of the analyzed 2-benzyl-5-(5-methyl-2-furyl) substituted compounds with respect to the corresponding 5-(2-furyl) substituted analogues.

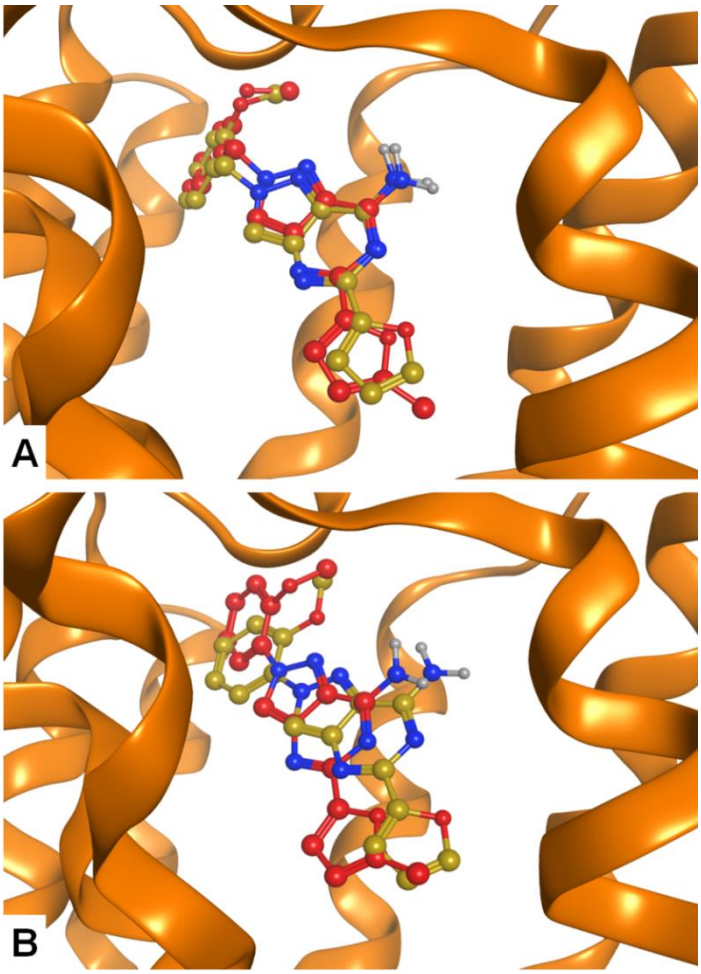


Figure 5. Comparison of docking conformations. Panel A. Superimposition of docking conformations of compounds **22** (light) and **25** (dark). Panel B. Superimposition of docking conformations of compounds **8** (light) and **11** (dark).

Derivatives bearing aryl substituents at both the 2- and 5- positions (**7**-**14**) show both the binding modes, as similarly scored by the docking tool. For all these compounds the top score conformation always presents the 2-furyl or the 2-(5-methyl)furyl oriented toward the receptor core and the 2-phenyl ring as externally located. Moreover, comparison of the binding modes of derivatives bearing the same 2-substituent and a 2-furyl or a 2-(5-methyl)furyl group at the 5-position suggests that the presence of a methyl group on the furan ring causes a slight rearrangement of the compound conformation. This can be observed in Figure 5B where superimposition of the docking conformations of compounds **8** and **11** is reported.

Differently from compounds **20**-**31,** in which the flexible 2-substituent allows the compounds to adapt their conformation to the binding pocket, in compounds **7**-**14** the 2-aryl substituents, being directly bound to the scaffold, does not permit this fitting. Hence, the presence of a methyl group on the 5-(2-furyl) residue can be a further hinder to the receptor-ligand accommodation, thus resulting generally detrimental for the compound affinity. Also in this case we performed the analysis of ligand-receptor interaction by using the above cited *IF-E 6.0* tool and by considering for comparison the compounds **8**, **9**, **11**, and **12**. The results are reported in Table 3. Both derivatives **11** and **12**, substituted at the 5-position with a 2-(5-methyl)furyl group, give weaker interactions with the binding site residues, located in proximity of the 5-position, with respect to the corresponding 2-furyl substituted compounds **8** and **9**. In addition, rearrangement of the conformation (Figure 5B) could lead to an analogue occupancy of the TM3-TM5-TM6 binding sub-pocket but also to a partial loss of the interaction with Asn253$^{6.55}$. These results seem partially in accordance with the binding data of this set of derivatives, with the exception of compound **10** where the effect on the 5-substituent appears to be different when compared with **7**. Considering the 2-substituent, binding data highlighted the higher affinity of the benzyl-substituted compounds (**20**, **22**-**31**) with respect to the phenyl-substituted analogues (**1**-**14**). As previously discussed, the more flexible benzyl substituents allow the molecules to get more deeply inserted inside the binding pocket. Regarding the substituent on the 2-benzyl or 2-phenyl moieties, we observed that the hydroxyl group at the 2-position of the aromatic ring generally engages an internal H-bond interaction with the N1 atom, while the methoxy group in the same position probably gives hydrophobic interaction with residues located at the entrance of the binding cavity (i.e. Tyr9$^{1.35}$, Ala63$^{2.61}$, Ile66$^{2.64}$, Leu167, Leu267, Met270$^{7.35}$, Tyr271$^{7.36}$, and Ile274$^{7.39}$, see Figure 4B). Docking results do not provide a clear explanation about the ten-fold decreased affinity of compounds bearing hydroxyl or methoxy group at the meta-position of the benzyl moiety with respect to the corresponding ortho-substituted derivatives (compare **27** and **28** with **25** and **26**, see Table 1). At the basis of the diverse biological behavior there could be a possible different polar interaction between the ortho- and the meta-

substituted compounds with the backbone atoms of Leu167-Phe169 residues and also a different solvation/desolvation effect of these groups that are exposed toward the external environment.

A set of derivatives (**20**-**31**) was also subjected to molecular docking analysis at a homology model of the hA_1 AR built by using the above cited 3EML hA_{2A} AR crystal structure as a template. The homology modeling and docking protocols were the same as the ones used for the refinement of the 3EML hA_{2A} AR crystal structure and the docking at the same receptor (see above). Docking results at the hA_1 AR present some similarities to those obtained at the hA_{2A} AR. The arrangement of the derivatives bearing a 2-furyl or a 2-(5-methyl)furyl group at the 5-position and a benzyl or substituted-benzyl group at position 2 is very similar at the two receptors (Figure 4 and 6) and also the role of the methyl group on the furyl moiety appears analogue. Hence, it is no particularly surprising that these compounds show affinity also for the hA_1 AR as the depth of the two binding cavities presents a high degree of conservation. Though, a series of differences among the two receptors may be observed at the entrance of the binding cavities where the position of some hydrophobic residues of the hA_{2A} AR (Leu167 in EL2, Leu267 in EL3, and Met270$^{7.35}$) is occupied by polar amino acids in the hA_1 AR (Glu170 in EL2, Ser267 in EL3, and Thr270$^{7.35}$, respectively).[47] As this sub-region is occupied by the 2-arylmethyl moiety of the docked compounds, the different chemical-physical profile of the residues in proximity (between hA_{2A} AR and hA_1 AR) may help to explain the generally higher affinity of this set of ligands for the hA_{2A} AR with respect to the hA_1 AR. Furthermore, as observed at the hA_{2A} AR, the different affinity of compounds bearing hydroxyl or methoxyl group at the ortho- or meta-position of the 2-benzyl moiety could be related to the diverse interaction with receptor residues. Docking results, in fact, suggest an interaction between the ortho-substituted derivatives and the charged amino group of Lys265 (EL3) (Figure 6). Affinity data show that the ortho-methoxy substituted derivatives (such as **25**) are more active at the hA_1 AR than their corresponding hydroxy-substituted analogues (such as **26**). This could be due to some factors. Firstly, the presence of an H-bond acceptor feature at the ortho-position, such as the methoxy (derivative **25**) appears more suitable for the interaction with Lys265 than an hydroxy H-

bond donor group (derivative **26**). Moreover, this latter substituent appears involved in an internal H-bond with the N1 atom of the scaffold, hence not presenting an optimal orientation for a strong interaction with Lys265. Instead, the methoxy group of **25**, not establishing this intramolecular bond, may assume the correct position to engage the H-bond with Lys265. Finally, the easier desolvation of the methoxy-substituted derivatives with respect to the hydroxy-substituted ones could be taken into account to explain the different receptor affinities.

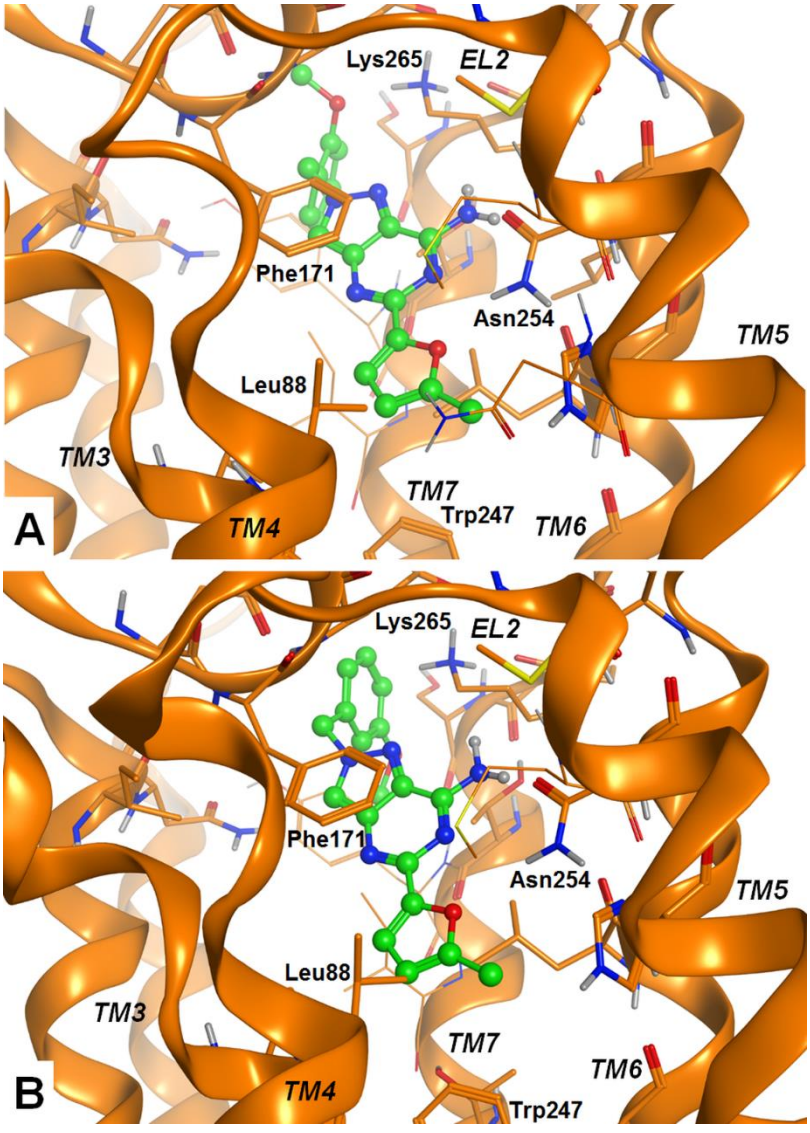


Figure 6. Docking conformation of compounds **25** (A) and **27** (B) at the hA_1 AR binding site. Key receptor residues and domains are indicated.

4. Conclusion

In the study reported here we evaluated different substituents at the 5- and 2- position of the pyrazolo[4,3-*d*]pyrimidine scaffold to shift affinity towards the hA_{2A} AR or both hA_{2A} and hA_1 ARs. Among the synthesized compounds, those featured by the 2-furyl or 5-methylfuran-2-yl moiety at position 5, combined with a benzyl or benzyl-substituted group at position 2, resulted in the most active. Several of these derivatives (**22-31**) displayed hA_{2A} AR affinities in the low nanomolar range (K_i= 3.62-57 nM) and a little lower for the hA_1 ARs (K_i = 18-234 nM), thus showing different degrees of A_{2A} versus A_1 selectivity (3-22 fold). In particular, the 2-(2-methoxybenzyl)-5-(5-methylfuran-2-yl) derivative **22** resulted in the most potent A_{2A} and A_1 AR antagonist herein reported, both in binding and in functional assays. Its 2-(2-hydroxybenzyl) analog **26** also showed a high affinity for the A_{2A} AR (K_i= 5.26 nM) and was 22-fold selective versus the A_1 subtype. Molecular docking investigations performed at the hA_{2A} AR crystal structure and at a homology model of the A_1 AR permitted us to represent the hypothetical binding mode of our derivatives as well as to rationalize the observed SARs.

5. Experimental section

5.1. Chemistry

The microwave-assisted syntheses were performed using an Initiator EXP Microwave Biotage instrument (frequency of irradiation: 2.45 GHz). Analytical silica gel plates (Merck F254), preparative silica gel plates (Merck F254, 2 mm) and silica gel 60 (Merck, 70-230 mesh) were used for analytical and preparative TLC, and for column chromatography, respectively. All melting points were determined on a Gallenkamp melting point apparatus and are uncorrected. Elemental analyses were performed with a Flash E1112 Thermofinnigan elemental analyzer for C, H, N and the results were within ± 0.4% of the theoretical values. All final compounds revealed purity not less than 95%. The IR spectra were recorded with a Perkin-Elmer Spectrum RX I spectrometer in

Nujol mulls and are expressed in cm^{-1}. NMR spectra were recorded on a Bruker Avance 400 spectrometer (400 MHz for ^{1}H NMR, 100 MHz for ^{13}C NMR). The chemical shifts are reported in δ (ppm) and are relative to the central peak of the solvent which was $CDCl_3$ or $DMSOd_6$. The following abbreviations are used: s= singlet, d= doublet, t= triplet, q= quartet, m= multiplet, br= broad and ar= aromatic protons.

5.1.1. Synthesis of Ethyl N^1-2-methoxyphenylhydrazono-N^2-chloroacetate 34.

The title compound was synthesized following the procedure previously reported to prepare compounds **32** and **33**[28,29] from the corresponding arylamines. Briefly, a mixture of 2-methoxyaniline (53 mmol) in 6 N HCl (32 mL) was cooled at -5 – 0 °C and 1 M $NaNO_2$ solution (53 mL) was added dropwise, keeping the temperature below 0 °C. The solution of the diazonium salt was poured into a cold (0 °C) solution of ethyl-2-chloroacetoacetate (53 mmol) and sodium acetate (54.2 mmol) in MeOH (200 mL), while stirring and keeping the temperature below 0 °C. The mixture was then left in a refrigerator for 16 h. The solid was collected by filtration, washed with water (200 mL) and dried.

Yield 62%; m.p.73-74 °C (cyclohexane); ^{1}H NMR ($CDCl_3$) 1.42 (t, 3H, CH_3, J = 7.1 Hz), 3.96 (s, 3H, OCH_3), 4.41 (q, 2H, CH_2, J = 7.1 Hz), 6.90-6.92 (m, 1H, ar), 6.99-7.01 (m, 2H, ar), 7.55-7.58 (m, 1H, ar), 8.83 (s, 1H, NH). Anal. Calcd. for $C_{11}H_{13}ClN_2O_3$: C, 51,47; H 5.10; N, 10.91. Found: C, 51,28; H 5.36; N, 11.22.

5.1.2. General procedure for the synthesis of ethyl 1-aryl-4-nitro-1H-pyrazole-3-carboxylates 36 and 37.

Derivative **36** and **37** were synthesized following the procedure previously described for **35**.[21] Briefly, a mixture of N,N-dimethyl-2-nitroethenamine (11 mmol), triethylamine (22 mmol) and the suitable idrazone **33** or **34** in $CHCl_3$ (5 mL) was reacted under microwave irradiation for 30 min at 140 °C. The solvent was eliminated at reduced pressure to give a black slurry. Derivative **36**

solidified upon treatment with isopropyl alcohol (2 mL). The solid obtained was collected by filtration, washed with water and recrystallized. To isolate compound **37**, the black residue was first purified by chromatography (eluent: cyclohexane/EtOAc, 3:1) and then the compound was recrystallized.

Ethyl 1-(2-fluorophenyl)-4-nitro-1H-pyrazole-3-carboxylate 36. Yield 23%; m.p. 112-114 °C (EtOH); ^{1}H NMR ($CDCl_3$) 1.45 (t, 3H, CH_3, J = 7.1 Hz), 4.52 (q, 2H, CH_2 J = 7.1 Hz), 7.31-7.37 (m, 2H, ar), 7.44-7.50 (m, 1H, ar), 7.95 (t, 1H, ar, J = 8.4 Hz), 8.72 (s, 1H, H-5). Anal. Calcd. for $C_{12}H_{10}FN_3O_4$: C, 51.62; H, 3.61; N, 15.05. Found: C, 51.44; H, 3.85; N, 15.27.

Ethyl 1-(2-methoxyphenyl)-4-nitro-1H-pyrazole-3-carboxylate 37. Yield 36%; m.p. 68-69°C (cyclohexane); ^{1}H NMR ($CDCl_3$) 1.45 (t, 3H, CH_3, J = 7.1 Hz), 3.97 (s, 3H, OCH_3), 4.52 (q, 2H, CH_2 J = 7.1 Hz), 7.10-7.14 (m, 2H, ar), 7.44 (t, 1H, ar, J = 7.9 Hz), 7.82 (d, 1H, ar, J = 7.9), 8.79 (s, 1H, H-3). Anal. Calcd. for $C_{13}H_{13}N_3O_5$: C, 53.61; H, 4.50; N, 14.43. Found: C, 53.46; H, 4.36; N, 14.60.

5.1.3. General procedure for the synthesis of 1-aryl-4-nitro-1H-pyrazole-3-carboxamides 39 and 40

Derivatives **39** and **40** were prepared following the same procedure employed to prepare **38**.[25] Briefly, a suspension of ethyl pyrazole-3-carboxylates **36** or **37** (3.0 mmol) in 33% aqueous ammonia solution (40 mL) was stirred at room temperature for about 48 h. The solid was collected by filtration, washed with water and recrystallized.

5.1.3.1. 1-(2-Fluorophenyl)-4-nitro-1H-pyrazole-3-carboxamide 39. Yield 72%; m.p. 212-213 °C (EtOH); ^{1}H NMR (DMSO-d_6) 7.41-7.48 (m, 1H, ar), 7.54-7.64 (m, 2H, ar), 7.79-7.84 (m, 1H, ar), 7.94 (br s, 1H, NH), 8.21 (br s, 1H, NH), 9.34 (s, 1H, H-5). Anal. Calcd. for $C_{10}H_7FN_4O_4$: C, 48.01; H, 2.82; N, 22.39. Found: C, 48.18; H, 2.64; N, 22.45.

5.1.3.2. 1-(2-Methoxyphenyl)-4-nitro-1H-pyrazole-3-carboxamide 40. Yield 88%; m .p. 200-202 °C (EtOAc/MeOH); ^{1}H NMR (DMSO-d_6) 3.90 (s, 3H, OCH_3), 7.15 (t, 1H, ar J = 7.8 Hz), 7.33 (d,

1H, ar, J = 8.4 Hz), 7.51 (t, 1H, ar J = 7.5 Hz), 7.64 (d, 1H, ar, J = 8.0 Hz), 7.85 (br s, 1H, NH), 8.14 (br s, 1H, NH), 9.16 (s, 1H, H-5). Anal. Calcd. for $C_{11}H_{10}N_4O_4$: C, 50.38; H, 3.84; N, 21.37. Found: C, 50.64; H, 3.75; N, 21.25.

5.1.4. General procedure for the synthesis of 1-aryl-4-nitro-pyrazole-3-carbonitrile derivatives 42-43

Compounds **42** and **43** were synthesized as previously described to prepare **41** from **38.**[25] Briefly, a suspension of the suitable amide **39** or **40** (2 mmol) in phosphorus oxychloride (5 mL) was microwave irradiated at 150 °C for 10-20 min. The excess of phosphorus oxychloride was distilled off and the residue was treated with water (about 5−10 mL). The obtained solid was collected by filtration and recrystallized.

5.1.4.1. 1-(2-Fluorophenyl)-4-nitro-1H-pyrazole-3-carbonitrile 42. Yield 96%; mp 108−110 °C (H_2O/EtOH); ^{1}H NMR ($CDCl_3$) 7.36-7.43 (m, 2H, ar), 7.49-7.58 (m, 1H, ar), 7.93 (t, 1H, ar, J = 7.9 Hz), 8.81 (s, 1H, H-5). Anal. Calcd. for $C_{10}H_5FN_4O_2$: C, 51.73; H, 2.17; N, 24.13. Found C, 51.58; H, 2.09; N, 24.32.

5.1.4.2. 1-(2-Methoxyphenyl)-4-nitro-1H-pyrazole-3-carbonitrile 43. Yield 89%; mp 134−136 °C (cyclohexane/EtOAc); ^{1}H NMR ($CDCl_3$) 4.00 (s, 3H, OMe), 7.17 (t, 2H, ar, J = 8.4 Hz), 7.51 (t, 1H, ar, J = 7.8 Hz), 7.79 (d, 1H, ar, J = 7.9 Hz), 8.89 (s, 1H, H-5). IR 2251, 1538, 1342. Anal. Calcd. for $C_{11}H_8N_4O_3$: C, 54.10; H, 3.30; N, 22.94. Found C, 54.21; H, 3.18; N, 22.78.

5.1.5. General procedure for the synthesis of 4-amino-1-aryl-1H-pyrazole-3-carbonitriles 45 and 46.

Compounds **45** and **46** were prepared as previously described for derivative **44**.[25] Briefly, a mixture of 4-nitropyrazoles **42** or **43** (2 mmol), cyclohexene (8 mmol) and 10% Pd/C (15% w/w with respect to the nitropyrazole) in EtOH (10 mL) was microwave irradiated, respectively, at 110 °C for 10 min and 130°C for 30 min. After cooling at room temperature, the catalyst was filtered off and

the solvent was evaporated at reduced pressure to give a solid which was recrystallized from suitable solvent.

5.1.5.1. 4-Amino-1-(2-fluorophenyl)-1H-pyrazole-3-carbonitrile 45. Yield 55%; m.p. 106−107 °C (H_2O/EtOH). ^{1}H NMR (DMSO-d_6) 5.05 (br s, 2H, NH_2), 7.35-7.39 (m, 1H, ar), 7.48-7.51 (m, 2H, ar), 7.67 (s, 1H, H-5), 7.77 (t, 1H, ar, J = 8.3 Hz). Anal. Calcd. for $C_{10}H_7FN_4$: C, 59.40; H, 3.49; N, 27.71. Found C, 59.58; H, 3.63; N, 27.61.

5.1.5.2. 4-Amino-1-(2-methoxyphenyl)pyrazole-3-carbonitrile 46. Yield 80%; m.p. 108−109 °C (Light petroleum ether/Et_2O). ^{1}H NMR ($CDCl_3$) 3.49 (br s, 2H, NH_2), 3.91 (s, 3H, OMe), 7.06-7.10 (m, 2H, ar), 7.38 (t, 1H, ar, J = 7.5 Hz), 7.66-7.68 (m, 2H, 1 ar + H-5). Anal. Calcd. for $C_{11}H_{10}N_4O$: C, 61.67; H, 4.71; N, 26.15. Found C, 61.48; H, 4.55; N, 26.30.

5.1.6. General procedure for the synthesis of 2-arylpyrazolo[4,3-d]pyrimidin-7-amine derivatives 1-3, 5, 7, 8, 10, 11, 13.

A mixture of the 4-aminopyrazole-3-carbonitrile derivatives **44-46** (0.84 mmol), anhydrous ammonium acetate (1.64 mmol), and triethyl orthoacetate (1.5 mmol) or ethyl imidates hydrochlorides (1.64 mmol) (R_5= CH_2Ph,[31] $(CH_2)_3Ph$,[32] 2-furyl,[33] 2-thienyl,[34] 5-methyl-furan-2-yl **55** (see **5.1.15**) was heated in a sealed tube or under microwave irradiation in the conditions described below for each compound. The obtained mixture was cooled at room temperature and treated with EtOH (0.5 mL) and diethyl ether (1-3 mL). The resulting solid was collected by filtration and washed, in sequence, with water (10-15 mL), $NaHCO_3$ saturated solution (1-2 mL) and water (5-10 mL), then dried and recrystallized. The crude compounds **5** and **7** were purified by chromatography (see below for details), then the latter was also recrystallized.

5.1.6.1. 7-amino-2-(2-fluorophenyl)-5-methyl-2H-pyrazolo[4,3-d]pyrimidine 1. The title compound was obtained by heating the reaction mixture under microwave irradiation at 130 °C for 15 min. Yield 85%; m.p. 212-214 °C (cyclohexane/EtOAc); ^{1}H NMR (DMSO-d_6) 2.37 (s, 3H, Me), 7.45 (t, 1H ar, J = 6.8 Hz), 7.54-7.62 (m, 2H, ar), 7.68 (br s, 2H, NH_2), 7.89 (t, 1H ar, J = 7.8 Hz),

8.61 (s, 1H, H-3). Anal. Calcd. for $C_{12}H_{10}FN_5$: C, 59.25; H, 4.14; N, 28.79. Found C, 59.09; H, 4.27; N, 28.58.

5.1.6.2. 7-Amino-2-(2-fluorophenyl)-5-(3-phenylpropyl)-2H-pyrazolo[4,3-d]pyrimidine 2. The title compound was obtained by heating the reaction mixture under microwave irradiation at 150 °C for 10-15 min. Yield 63%; m.p. 188-189 °C (cyclohexane/EtOAc); ^{1}H NMR (DMSO-d_6) 2.01-2.06 (m, 2H, CH_2), 2.63-2.66 (m, 4H, CH_2), 7.16-7.31 (m, 5H, ar), 7.45 (t, 1H, ar, J = 6.9 Hz), 7.54-7.59 (m, 2H, ar), 7.67 (br s, 2H, NH_2), 7.89 (t, 1H, ar, J = 7.7 Hz), 8.65 (s, 1H, H-3). Anal. Calcd. for $C_{20}H_{18}FN_5$: C, 69.15; H, 5.22; N, 20.16. Found C, 69.01; H, 5.08; N, 20.30.

5.1.6.3. 7-Amino-2-(2-methoxyphenyl)-5-(3-phenylpropyl)-2H-pyrazolo[4,3-d]pyrimidine 3. The title compound was obtained by heating the reaction mixture in sealed tube at 120 °C for 2 h. Yield 40%; m.p. 199-200 °C (cyclohexane/EtOAc); ^{1}H NMR (DMSO-d_6) 2.01-2.05 (m, 2H, CH_2), 2.63-2.65 (m, 4H, $2CH_2$), 3.87 (s, 3H, OMe), 7.20-7.33 (m, 7H, ar), 7.50-7.54 (m, 3H, 1 ar + NH_2), 7.68 (d, 1H, ar, J= 7.8 Hz), 8.50 (s, 1H, H-3). ^{13}C NMR (DMSO-d_6) 30.36, 35.38, 38.87, 56.53, 113.44, 121.16, 125.38, 126.16, 126.93, 128.69, 128.86, 129.71, 130.71, 131.33, 139.81, 142.70, 152.65, 156.26, 164.60. Anal. Calcd. for $C_{21}H_{21}N_5O$: C, 70.17; H, 5.89; N, 19.48. Found C, 70.32; H, 5.65; N, 19.35.

5.1.6.4. 7-Amino-2-(2-methoxyphenyl)-5-benzyl-2H-pyrazolo[4,3-d]pyrimidine 5. The title compound was obtained by heating the reaction mixture in sealed tube at 120 °C for 5h. The crude compound was purified by preparative TLC (eluent cyclohexane/EtOAc/MeOH, 6:4:1). Yield 65%; m.p. 141-142 °C; ^{1}H NMR (DMSO-d_6) 3.86 (s, 3H, OMe), 3.96 (s, 2H, CH_2), 7.13-7.18 (m, 2H, ar), 7.20-7.30 (m, 5H, ar), 7.52 (t, 1H, J= 7.4 Hz), 7.66-7.68 (m, 3H, 1ar + NH_2), 8.54 (s, 1H, H-3). Anal. Calcd. for $C_{19}H_{17}N_5O$: C, 68.87; H, 5.17; N, 21.13. Found C, 68.59; H, 4.95; N, 21.02.

5.1.6.5. 7-Amino-5-(2-furyl)-2-phenyl-2H-pyrazolo[4,3-d]pyrimidine 7. The title compound was obtained by heating the reaction mixture under microwave irradiation at 130 °C for 10 min. The crude derivative was purified by column chromatography (eluent cyclohexane/EtOAc 6:4). Yield 48%; m.p. 208-210 °C (EtOAc); ^{1}H NMR (DMSO-d_6) 6.62-6.63 (m, 1H, furan proton), 7.07-7.08

(m, 1H, furan proton), 7.50 (t, 1H, ar, J = 9.0 Hz), 7.63 (t, 2H, ar, J= 9.0 Hz), 7.80 (s, 1H, furan proton), 7.84 (br s, 2H, NH_2), 8.08 (d, 2H, ar, J = 9.0 Hz), 9.06 (s, 1H, H-3). IR 3335, 3150, 1591. Anal. Calcd. for $C_{15}H_{11}N_5O$: C, 64.97; H, 4.00; N, 25.26. Found C, 64.75; H, 3.88; N, 25.01.

5.1.6.6. 7-Amino-5-(2-furyl)-2-(2-methoxyphenyl)-2H-pyrazolo[4,3-d]pyrimidine 8. The title compound was obtained by heating the reaction mixture at 130 °C for 15 min in a sealed tube. Yield 38%; m.p. 295-296 °C ($MeNO_2$). ^{1}H NMR (DMSO-d_6) 3.89 (s, 3H, OMe), 6.61-6.62 (m, 1H, furan proton), 7.06-7.07 (m, 1H, H furan proton), 7.17 (t, 1H, ar, J = 7.7 Hz), 7.33 (d, 1H, ar, J = 7.5 Hz), 7.53 (t, 1H, ar, J = 8.3 Hz), 7.72 (d, 1H, ar, J = 7.8 Hz), 7.77-7.79 (m, 3H, NH_2 + 1 H furan proton), 8.64 (s, 1H, H-3). Anal. Calcd. for $C_{16}H_{13}N_5O_2$: C, 62.53; H, 4.26; N, 22.79. Found C, 62.42; H, 4.45; N, 22.65.

5.1.6.7. 7-Amino-5-(5-methylfuran-2-yl)-2-phenyl-2H-pyrazolo[4,3-d]pyrimidine 10. The title compound was obtained by heating the reaction mixture at 110 °C for 1 h in a sealed tube. Yield 38%; m.p. 257-258 °C (H_2O/EtOH); ^{1}H NMR (DMSO-d_6) 2.37 (s, 3H, Me), 6.23-6.24 (m, 1H, furan proton), 6.97-6.98 (m, 1H, furan proton), 7.49 (t, 1H, ar, J = 7.4 Hz), 7.62 (t, 1H, ar, J = 7.7 Hz), 7.79 (br s, 2H, NH_2), 8.06 (d, 2H, ar, J = 7.8 Hz), 9.00 (s, 1H, H-3). Anal. Calcd. for $C_{16}H_{13}N_5O$; C, 65.97; H, 4.50; N, 24.04. Found C, 65.75; H, 4.72; N, 24.21.

5.1.6.8. 7-Amino-5-(5-methylfuran-2-yl)-2-(2-methoxyphenyl)-2H-pyrazolo[4,3-d]pyrimidine 11. The title compound was obtained by heating the reaction mixture at 120 °C for 2 h in a sealed tube. Yield 58%; m.p. 216-217 °C (cyclohexane/EtOAc); ^{1}H NMR (DMSO-d_6) 2.36 (s, 3H, Me), 3.88 (s, 3H, OMe), 6.22-6.23 (m, 1H, furan proton), 6.95-6.96 (m, 1H, furan proton), 7.16 (t, 1H, ar, J= 7.7 Hz), 7.33 (d, 1H, ar, J= 8.4 Hz), 7.53 (t, 1H, ar, J= 7.6 Hz), 7.71-7.73 (m, 3H, 1 ar + NH_2), 8.61 (s, 1H, H-3). Anal. Calcd. for $C_{17}H_{15}N_{15}O_2$: C, 63.54; H, 4.71; N, 21.79. Found C, 63.70; H, 4.65; N, 21.68.

5.1.6.9. 7-Amino-2-(2-methoxyphenyl)-5-(2-thienyl)-2H-pyrazolo[4,3-d]pyrimidine 13. The title compound was obtained by heating the reaction mixture at 110 °C for 2 h in a sealed tube. Yield 70%; m.p. 244-245 °C ($MeNO_2$); ^{1}H NMR (DMSO-d_6) 3.89 (s, 3H, OMe), 7.13-7.19 (m, 2H, 1ar +

1 thiophene proton), 7.34 (d, 1H, ar, J= 8.4 Hz), 7.53 (t, 1H, ar, J= 8.4 Hz), 7.59-7.60 (m, 1H, thiophene proton), 7.73 (d, 1H, ar, J= 7.9 Hz), 7.79-7.80 (m, 3H, 1 thiophene proton + NH_2), 8.65 (s, 1H, H-3). Anal. Calcd. for $C_{16}H_{13}N_5OS$.

5.1.7. General procedure for the synthesis of 7-amino-2-(2-hydroxyphenyl)-pyrazolo[4,3-d]pyrimidine derivatives 4, 6, 9, 12, 14

1 M solution of BBr_3 in dichloromethane (2.60 mL) was slowly added at 0 °C, under nitrogen atmosphere, to a suspension of compounds **3, 5, 8, 11** or **13** (1.02 mmol) in anhydrous dichloromethane (20 mL). The resulting mixture was refluxed until the disappearance of the starting material (5-20 h, TLC monitoring). The organic solvent was removed under reduced pressure and the residue was diluted with water (10 mL) and neutralized with $NaHCO_3$ saturated solution. The obtained precipitate was collected by filtration, dried and purified by recrystallization, with the only exception of derivative **6** which was purified by preparative TLC (eluent cyclohexane/EtOAc/MeOH, 6:4:1).

5.1.7.1. 7-Amino-2-(2-hydroxyphenyl)-5-(3-phenylpropyl)-2H-pyrazolo[4,3-d]pyrimidine 4. Yield 73%; m.p. 243-244 °C (cyclohexane/EtOAc). ^{1}H NMR (DMSO-d_6) 2.03 (quintet, 2H, CH_2, J= 7.5 Hz), 2.63-2.67 (m, 4H, $2CH_2$), 7.01 (t, 1H, ar, J= 7.8 Hz), 7.12 (d, 1H, ar, J= 8.1 Hz), 7.16-7.34 (m, 6H, ar), 7.62 (br s, 2H, NH_2), 7.75 (d, 1H, ar, J= 8.1 Hz), 8.69 (s, 1H, H-3), 10.69 (br s, 1H, OH). Anal. Calcd. for $C_{20}H_{19}N_5O$: C, 69.55; H, 5.54; N, 20.28. Found C, 69.37; H, 5.40; N, 20.15.

5.1.7.2. 7-Amino-5-benzyl-2-(2-hydroxyphenyl)-2H-pyrazolo[4,3-d]pyrimidine 6. Yield 66%; m.p. 248-249 °C; ^{1}H NMR (DMSO-d_6) 3.95 (s, 2H, CH_2), 7.01 (t, 1H, ar, J= 7.5 Hz), 7.11 (d, 1H, ar, J= 8.1 Hz), 7.18 (t, 1H, ar, J= 7.2 Hz), 7.25-7.34 (m, 5H, ar), 7.67 (br s, 2H, NH_2), 7.73 (d, 2H, ar, J= 8.0 Hz), 8.70 (s, 1H, H-3), 10.67 (br s, 1H, OH). Anal. Calcd. for $C_{18}H_{15}N_5O$: C, 68.13; H, 4.76; N, 22.07. Found C, 68.02; H, 4.95; N, 22.25.

5.1.7.3. 7-Amino-5-(2-furyl)-2-(2-hydroxyphenyl)-2H-pyrazolo[4,3-d]pyrimidine 9. Yield 95%; m.p. 290-292 °C (2-Methoxyethanol). ^{1}H NMR (DMSO-d_6) 6.61-6.63 (m, 1H, furan proton), 7.02 (t, 1H, ar, J = 7.6 Hz), 7.07-7.08 (m, 1H, furan proton), 7.13 (d, 1H, ar, J = 7.2 Hz), 7.34 (t, 1H, ar, J = 7.4 Hz), 7.77-7.84 (m, 4H, 2 ar + NH_2), 8.80 (s, 1H, H-3), 10.71 (s, 1H, OH). ^{13}C NMR (DMSO-d_6) 111.47, 112.32, 118.03, 119.96, 125.00, 125.22, 127.53, 130.16, 130.95, 139.47, 144.49, 150.46, 152.34, 153.90, 156.17. Anal. Calcd. for $C_{15}H_{11}N_5O_2$: C, 61.43; H, 3.78; N, 23.88. Found C, 61.25; H, 3.92; N, 23.99.

5.1.7.4. 7-Amino-2-(2-hydroxyphenyl)-5-(5-methyl-furan-2-yl)-2H-pyrazolo[4,3-d]pyrimidine 12. Yield 63%; m.p. > 300 °C (isopropanol). ^{1}H NMR (DMSO-d_6) 2.36 (s, 3H, Me), 6.23-6.24 (m, 1H, furan proton), 6.95-6.96 (m., 1H, furan proton), 7.01 (t, 1H, ar, J= 7.8 Hz), 7.12 (d, 1H, ar, J= 8.1 Hz), 7.33 (t, 1H, ar, J= 7.8 Hz), 7.71-7.80 (m, 3H, 1ar + NH_2), 8.76 (s, 1H, H-3), 10.69 (s, 1H, OH). Anal. Calcd. for $C_{16}H_{13}N_5O_2$: C, 62.53; H, 4.26; N, 22.79. Found C, 62.76; H, 4.07; N, 22.98.

5.1.7.5. 7-Amino-2-(2-hydroxyphenyl)-5-(2-thienyl)-2H-pyrazolo[4,3-d]pyrimidine 14. Yield 95%; m.p. 178-179 °C ($MeNO_2$). ^{1}H NMR (DMSO-d_6) 7.05 (t, 1H, ar, J= 7.4 Hz), 7.16-7.18 (m, 2H, 1 thiophene proton + 1 ar), 7.35-7.39 (t, 1H, ar, 7.4 Hz), 7.60-7.61 (m, 1H, thiophene proton), 7.75 (br s, 2H, NH_2), 7.81-7.84 (m, 2H, 1 thiophene proton + 1 ar), 8.82 (s, 1H, H-3), 10.63 (br s, 1H, OH). Anal. Calcd. for $C_{15}H_{11}N_5OS$: C, 58.24; H, 3.58; N, 22.64. Found C, 58.03; H, 3.74; N, 22.40.

5.1.8. Synthesis of 4-Nitro-1H-pyrazole-3-carbonitrile 47.

The title compound was already known[48] but we prepared it by a different procedure, i.e. by treatment of the corresponding 3-carboxyamide (2 mmol)[48] with $POCl_3$ (5 ml) under microwave irradiation at 150 °C for 40 min. The excess of phosphorus oxychloride was distilled off and the residue was treated with water (about 5-10 mL) to give a solid which was collected and recrystallized. Yield 98%; m.p. 160−162 °C (EtOAc) %; (lit[48] 162-163°C). ^{1}H NMR (DMSO-d_6) 9.16 (s, 1H, H-5); ^{1}H NMR ($CDCl_3$) 8.39 (s, 1H, H-5). ^{13}C NMR (DMSO-d_6) 112.26, 120.88,

131.87, 137.21. Anal. Calcd. for $C_4H_2N_4O_2$: C, 34.79; H, 1.46; N, 40.57. Found C, 34.92; H, 1.32; N, 40.36.

5.1.9. Synthesis of 4-Nitro-1-phenethyl-1H-pyrazole-3-carbonitrile 50

Derivative **50** was prepared in the same conditions used to prepare compounds **48** and **49**.[27] Briefly, a solution of 4-nitro-1H-pyrazole-3-carbonitrile **47** (1.45 mmol) in anhydrous tetrahydrofuran (2 mL) was dropwise added to a suspension of sodium hydride (60% in paraffin oil, 1.74 mmol) in anhydrous tetrahydrofuran (6 mL) at 0 °C. The suspension was stirred at 0 °C for 1 h, and then a solution of phenethylbromide (2.90 mmol) in anhydrous tetrahydrofuran (1 mL) was added. The reaction was heated at reflux for about 30 h. Then the suspension was diluted with EtOAc (20 mL) and 1 M NH_4Cl (20 mL). The two phases were separated and the aqueous solution was extracted with EtOAc (30 mL for three times). The combined organic phases were anhydrified (Na_2SO_4) and the solvent evaporated at reduced pressure to give an oily residue which was chromatographed on silica gel column (eluent cyclohexane/EtOAc/MeOH, 6:4:1) to separate compound **50** from its regioisomer **50a** (molar ratio 6:1, from ^{1}H NMR spectrum of the crude oil).

5.1.9.1. 4-Nitro-1-phenylethyl-1H-pyrazole-3-carbonitrile 50. Yield 72%; m.p. 142-144 °C (EtOH). ^{1}H NMR ($CDCl_3$) 3.24 (t, 2H, CH_2, J= 6.9 Hz), 4.46 (t, 2H, CH_2 J= 6.9 Hz), 7.07 (d, 2H, ar, J= 7.7 Hz), 7.28-7.33 (m, 3H, ar), 7.85 (s, 1H, H-5). NOESY: interaction between H-5 and CH_2 protons. ^{13}C NMR ($CDCl_3$) 35.15, 57.50, 110.11, 121.21, 127.69, 128.48, 129.18, 129.76, 134.29, 135.77. Anal. Calcd. for $C_{12}H_{10}N_6O_4$: C, 59.50; H, 4.16; N, 23.13. Found C, 59.35; H, 4.02; N, 23.38.

5.1.9.2. 4-Nitro-1-phenylethyl-1H-pyrazole-5-carbonitrile *50a*. Yield 12%; m.p. 117-119 °C (cyclohexane/EtOAc)**.** ^{1}H NMR ($CDCl_3$) 3.28 (t, 2H, CH_2, J= 7.0 Hz), 4.61 (t, 2H, CH_2 J= 7.0 Hz), 7.09 (d, 2H, ar, J=7.2 Hz), 7.30-7.36 (m, 3H, ar), 8.19 (s, 1H, H-3). Anal. Calcd. for $C_{12}H_{10}N_6O_4$: C, 59.50; H, 4.16; N, 23.13. Found C, 59.37; H, 4.26; N, 23.23.

5.1.10. Synthesis of 4-Amino-1-methyl-1H-pyrazole-3-carbonitrile 51.

A mixture of the nitro derivative **48** (6.6 mmol) and 10% Pd/C (10% p/p) in EtOH (20-30 mL) was hydrogenated in a Parr apparatus at 30 psi for 18 h, then the catalyst was filtered off and the solvent was evaporated to dryness, under reduced pressure. The residue was treated with a mixture of cyclohexane (5-8 mL) and diethyl ether (1-2 mL) to give a solid which was collected by filtration and recrystallized. Yield 65%; m.p. 87-88 °C (cyclohexane/EtOAc). ^{1}H NMR (DMSO-d_6) 3.76 (s, 3H, CH_3), 4.75 (br s, 2H, NH_2), 7.20 (s, 1H, H-5). IR 3381, 3301, 2224. Anal. Calcd. for $C_5H_6N_4$: C, 49.17; H, 4.95; N, 45.88. Found C, 49.36; H, 5.10; N, 45.68.

5.1.11. Synthesis of 4-Amino-1-phenethyl-1H-pyrazole-3-carbonitriles 53

Compound **53** was prepared as previously described for **52**.[27] The nitro derivative **50** (2 mmol) was reacted with cyclohexene (8 mmol) and 10% Pd/C (15% w/w with respect to the nitropyrazoles) in the same experimental conditions described above for compounds **44-46**. Microwave irradiation was carried out at 150 °C for 10 min. After cooling at room temperature, the catalyst was filtered off and the solution was evaporated at reduced pressure to give an oily residue which did not crystallized but, being pure enough (^{1}H NMR spectrum), it was used without further purification for the next step. Yield 68%; ^{1}H NMR (DMSO-d_6) 3.06 (t, 2H, CH_2, J= Hz), 4.28 (t, 2H, CH_2, J= Hz), 4.70 (br s, 2H, NH_2), 7.16-7.21 (m, 6H, ar + H-5).

5.1.12. General procedure for the synthesis of the 7-amino-pyrazolo[4,3-d]pyrimidine derivatives 15-22

A mixture of the 4-aminopyrazole-3-carbonitrile derivatives **51-53** (0.84 mmol), anhydrous ammonium acetate (1.51 mmol), triethyl orthoacetate (1.26 mmol) or the suitable ethyl iminoesters hydrochlorides (1.26 mmol) (R_5= CH_2Ph,[31] $(CH_2)_3Ph$,[32] 2-furyl[33]) was heated under microwave irradiation at 130°C for 15 min. Compound **22** was obtained by heating the reaction mixture in a sealed tube at 120 °C for 2 h and 30 min. The obtained mixture was cooled at room temperature and

treated with diethyl ether (3-4 mL) and a few EtOH (0.5-1 mL). The resulting solid was collected by filtration, washed with water (20 mL) and $NaHCO_3$ saturated solution (1-2 mL), then dried and recrystallized. The crude compound **17** was first purified by column chromatography (eluent $CHCl_3$/MeOH/cyclohexane 8.5:1.5:0.5) and then recrystallized.

5.1.12.1. 7-Amino-5-benzyl-2-methyl-2H-pyrazolo[4,3-d]pyrimidine 15. Yield 80%; m.p. 128-129 °C (EtOAc/MeOH); ^{1}H NMR (DMSO-d_6) 4.02 (s, 2H, CH_2), 4.16 (s, 3H, CH_3), 7.24-7.31 (m, 5H, ar), 7.40 (br s, 2H, NH_2), 8.36 (s, 1H, H-3). ^{13}C NMR (DMSO-d_6) 40.87, 45.41, 123.75, 126.43, 128.60, 129.34, 130.04, 138.86, 139.76, 156.18, 162.65. IR 3350, 3151. Anal. Calcd. for $C_{13}H_{13}N_5$: C, 65.25; H, 5.48; N, 29.27. Found C, 65.02; H, 5.62; N, 29.48.

5.1.12.2. 7-Amino-2-methyl-5-(3-phenylpropyl)-2H-pyrazolo[4,3-d]pyrimidine 16. Yield 53 %; m. p. 181-182 °C (EtOAc/MeOH); ^{1}H NMR (DMSO-d_6) 1.96-2.02 (m, 2H, CH_2), 2.60 (t, 4H, CH_2 + CH_2), 4.10 (s, 3H, CH_3), 7.15-7.30 (m, 5H, ar), 7.34 (broad s, 2H, NH_2), 8.15 (s, 1H, H-3). Anal. Calcd. for $C_{15}H_{17}N_5$: C, 67.39; H, 6.41; N, 26.60. Found C 67.50; H, 6.63; N, 26.46.

5.1.12.3. 7-Amino-2-benzyl-5-methyl-2H-pyrazolo[4,3-d]pyrimidine 17. Yield 64%; m.p. 234-235 °C (EtOAc); ^{1}H NMR (DMSO-d_6) 2.32 (s, 3H, CH_3), 5.59 (s, 2H, CH_2), 7.26-7.38 (m, 7H, ar + NH_2), 8.30 (s, 1H, H-3). IR 3455, 3301, 1650. ^{13}C NMR (DMSOd_6) 26.21, 57.05, 123.42, 128.01, 128.36, 129.08, 130.71, 140.25, 155.91, 161.19. Anal. Calcd. for $C_{13}H_{13}N_5$: C, 65.25; H, 5.48; N, 29.27. Found C, 65.03; H, 5.62; N, 29.02.

5.1.12.4. 7-Amino-5-methyl-2-phenethyl-2H-pyrazolo[4,3-d]pyrimidine 18. Yield 45%; m.p. 231-232 °C (cyclohexane/EtOAc); ^{1}H NMR (DMSO-d_6) 2.31 (s, 3H, CH_3), 3.23 (t, 2H, CH_2, J= 7.1 Hz), 4.61 (t, 2H, CH_2, J= 7.1 Hz), 7.17-7.33 (m, 7H, 5ar + NH_2), 8.05 (s, 1H, H-3). ^{13}C NMR ($CDCl_3$) 25.36, 36.83, 55.91, 123.08, 127.19, 128.55, 128.77, 128.88, 136.90, 138.44, 154.69, 159.00. Anal. Calcd. for $C_{14}H_{15}N_5$: C, 66.38; H, 5.97; N, 27.65. Found C, 66.21; H, 6.16; N, 27.47.

5.1.12.5. 7-Amino-2,5-dibenzyl-2H-pyrazolo[4,3-d]pyrimidine 19. Yield 38%; m.p. 183-184 °C (cyclohexane/EtOAc); ^{1}H NMR (DMSO-d_6) 3.91 (s, 2H, CH_2), 5.60 (s, 2H, CH_2), 7.16-7.36 (m, 10H, ar), 7.42 (s, 2H, NH_2), 8.38 (s, 1H, H-3). ^{13}C NMR (DMSO-d_6) 45.80, 57.10, 123.78, 126.35,

128.04, 128.39, 128.55, 129.10, 129.34, 130.68, 137.14, 139.73, 140.03, 156.26, 163.14. IR 3435, 3314, 1659. Anal. Calcd. for $C_{19}H_{17}N_5$: C, 72.36; H, 5.43; N, 22.21. Found C 72.52; H, 5.64; N, 22.08.

5.1.12.6. 7-Amino-2-benzyl-5-(2-furyl)-2H-pyrazolo[4,3-d]pyrimidine 20. Yield 55%; m.p. 219-220 °C (EtOH); ^{1}H NMR (DMSO-d_6) 5.63 (s, 2H, CH_2), 6.58-6.60 (m, 1H, furan proton), 7.02 (d, 1H, furan proton, J= 3.4 Hz), 7.28-7.30 (m, 5H, ar), 7.58 (s, 2H, NH_2), 7.75-7.76 (m, 1H, furan proton), 8.47 (s, 1H, H). ^{13}C NMR (DMSO-d_6) 36.39, 54.83, 111.03, 112.21, 124.10, 127.04, 128.92, 129.11, 130.48, 138.29, 139.47, 144.24, 151.83, 154.04, 156.03. IR 3332, 3150, 1591. Anal. Calcd. for $C_{16}H_{13}N_5O$: C, 65.97; H, 4.50; N, 24.04. Found C, 65.84; H, 4.72; N, 23.95.

5.1.12.7. 7-Amino-5-(2-furyl)-2-(2-phenethyl)-2H-pyrazolo[4,3-d]pyrimidine 21. Yield 40%; m.p. 237-238 °C (EtOH); ^{1}H NMR (DMSO-d_6) 3.25 (t, 2H, CH_2, J= 7.2 Hz), 4.65 (t, 2H, CH_2, J=7.2 Hz), 6.58-6.60 (m, 1H, furan proton), 7.01-7.02 (m, 1H, furan proton), 7.19-7.30 (m, 5H, ar), 7.56 (s, 2H, NH_2), 7.74-7.75 (m, 1H, furan proton), 8.21 (s, 1H, H-3). IR 3242, 3163, 1650. Anal. Calcd. for $C_{17}H_{15}N_5O$: C, 66.87; H, 4.95; N, 22.94. Found C, 66.65; H, 4.73; N, 22.75.

5.1.12.8. 7-Amino-5-(2-furyl)-2-(2-methoxybenzyl)-2H-pyrazolo[4,3-d]pyrimidine 22. Yield 46%; m.p. 210-212 °C (cyclohexane/EtOAc); ^{1}H NMR (DMSO-d_6) 3.85 (s, 3H, OMe), 5.58 (s, 2H, CH_2), 6.60-6.58 (m, 1H, furan proton), 6.93 (t, 1H, ar, J=7.4 Hz), 6.98-7.02 (m, 2H, 1 ar + furan proton), 7.07 (d, 1H, ar, J= 8.2 Hz), 7.33 (t, 1H, ar, J= 8.6 Hz), 7.58 (br s, 2H, NH_2), 7.76 (d, 1H, furan proton, J = 0.8 Hz), 8.41 (s, 1H, H-3); ^{13}C NMR (DMSO-d_6) 52.65, 56.00, 111.39, 111.57, 112.32, 120.98, 124.38, 124.57, 129.82, 130.35, 130.58, 139.36, 144.40, 151.86, 153.62, 156.02, 157.23, Anal. Calcd. for $C_{17}H_{15}N_5O_2$: C, 63.54; H, 4.71; N, 21.79. Found C, 63.38; H, 4.98; N, 21.93.

5.1.13. Synthesis of 7-amino-5-(2-furyl)-2-(2-hydroxybenzyl)-2H-pyrazolo[4,3-d]pyrimidine 23

The title compound was prepared from the corresponding methoxy derivative **23** (1.02 mmol) following the procedure reported above to obtain compounds **4, 6, 9, 12, 14** but carrying out the

reaction at room temperature for 16 h. The crude derivative was purified by column chromatography (eluent cyclohexane/EtOAc/MeOH, 2:7:1). Yield 84%; ^{1}H NMR (DMSO-d_6) 5.54 (s, 2H, CH_2), 6.59-6.58 (m, 1H, furan proton), 6.78 (t, 1H, ar, J=7.2 Hz), 6.88 (d, 1H, ar, J = 7.9 Hz), 6.96 (d, 1H, ar, J = 6.7 Hz), 7.02 (d, 1H, furan proton, J = 3.2 Hz), 7.15 (t, 1H, ar, J= 7.2 Hz), 7.58 (br s, 2H, NH_2), 7.75 (s, 1H, furan proton) 8.30 (s, 1H, H), 9.88 (br s, 1H, OH); ^{13}C NMR (DMSO-d_6) 52.76, 111.06, 112.20, 115.72, 119.57, 122.99, 124.44, 129.83, 130.64, 139.63, 151.91, 154.05, 155.47, 156.04. Anal. Calcd. for $C_{16}H_{13}N_5O_2$: C, 62.53; H, 4.26; N, 22.79. Found C, 62.32; H, 4.09; N, 22.90.

5.1.14. Synthesis of 4-amino-1H-pyrazole-3-carbonitrile 54[35]

A solution of the 4-nitropyrazole derivative **47** (7.24 mmol) in methanol (200 mL) was hydrogenated in a Parr apparatus, at 30 psi for 24 h, in the presence of 10% Pd/C (10% w/w with respect to the nitro derivative). The catalyst was filtered off and the solvent was evaporated at reduced pressure to give a dark brown solid which was pure enough to be used as such for the next step. Yield 90%; ^{1}H NMR (DMSO-d_6) 4.6 (br s, 2H, NH_2), 7.22 (s 1H, H-5), 13.1 (br s, 1H, NH).

5.1.15. Synthesis of ethyl 5-methylfuran-2-carboximidate hydrochloride 55

The title compound was obtained by stirring a solution of hydrogen chloride (15.6 mmol) and the suitable nitrile (12 mmol) in absolute EtOH (15.6 mmol) at 0 °C for 1 h. The mixture was allowed to stand overnight in the refrigerator (4° C) then was treated with anhydrous diethyl ether (about 25 mL). The resulting solid was filtered off, washed with anhydrous diethyl ether (5-6 mL) and dried in vacuo over P_2O_5 in a desiccator. The imidate hydrochloride was pure enough to be characterized (^{1}H-NMR, m.p.) and used without further purification. Yield 98%; m.p. 141-143 °C. ^{1}H NMR (DMSO-d_6) 1.42 (t, 3H, CH_3, J = 7.0 Hz), 2.44 (s, 3H, CH_3), 4.59 (q, 2H, CH_2, J = 7.0 Hz), 6.56-6.57 (m, 1H, furan proton), 7.91-7.92 (m, 1H, furan proton), 11.56 (br s, 2H, NH_2^+).

5.1.16. Synthesis of 7-Amino-5-(5-methylfuran-2-yl)-2H-pyrazolo[4,3-d]pyrimidine 56. A mixture of the 4-aminopyrazole-3-carbonitrile **54** (0.92 mmol), anhydrous ammonium acetate (1.51 mmol) and ethyl 5-methylfuran-2-carboximidate hydrochloride **55** (1.38 mmol) was heated in a sealed tube at 120 °C for 2 h. The mixture was cooled at room temperature and treated with diethyl ether (3-4 mL). The resulting solid was collected by filtration, washed with water (20 mL) and $NaHCO_3$ saturated solution (1-2 mL) and then dried. The crude derivative was used for the next step without further purification. Yield 70%; ^{1}H NMR (DMSO-d_6) 2.35 (s, 3H, CH_3), 6.21-6.22 (m, 1H, furan proton), 6.93-6.94 (m, 1 H, furan proton), 7.51 (br s, NH_2), 8.07 (br s, 1H, pyrazole proton) , 12.93 (br s, 1 H, NH).

5.1.17. General procedure for the synthesis of 7-amino-2-arylmethyl-5-(5-methylfuran-2-yl)-2H-pyrazolo[4,3-d]pyrimidine 24-29

The suitable benzyl chlorides (1.95 mmol), all commercially available except the 2-methoxybenzyl chloride,[36] were added to a mixture of compound **56** (1.3 mmol) and K_2CO_3 (2.6 mmol) in acetonitrile (9 mL) and DMF (1 mL). The suspension was stirred in the condition described below for each compound and then most of the solvent was evaporated under reduced pressure. The residue was taken up with water (about 30-40 mL) and the obtained solid was collected, dried and recrystallized or purified as described below for each compound. NOESY experiments performed on **24-29** showed the spatial proximity between H-3 and CH_2 protons.

5.1.17.1. 7-Amino-5-(5-methylfuran-2-yl)-2-benzyl-2H-pyrazolo[4,3-d]pyrimidine 24. The reaction was carried out at room temperature for about 24 h. The crude solid was purified by preparative TLC (eluent $CHCl_3$/ MeOH, 9:1). Yield 49 %; m.p. 257-259 °C; ^{1}H NMR (DMSO-d_6) 2.34 (s, 3H, Me), 5.62 (s, 2H, CH_2), 6.20-6.21 (m, 1H, furan proton), 6.90-6.91 (m, 1H, furan proton), 7.29-7.37 (m, 5H, ar), 7.59 (br s, 2H, NH_2), 8.43 (s, 1H, H-3). Anal. Calcd. for $C_{17}H_{15}N_5O$: C, 63.54; H, 4.71; N, 21.79. Found C, 63.35; H, 4.93; N, 21.98.

5.1.17.2. 7-Amino-5-(5-methylfuran-2-yl)-2-(2-methoxybenzyl)-2H-pyrazolo[4,3-d]pyrimidine 25. The reaction was carried out at room temperature for about 60 h. The crude solid was purified by preparative TLC (eluent cyclohexane/ EtOAc, 6:4), then recrystallized. Yield 87%; m.p. 280-281 °C (EtOAc); ^{1}H NMR (DMSO-d_6) 2.34 (s, 3H, Me), 3.85 (s, 3H, OMe), 5.57 (s, 2H, CH_2), 6.20-6.21 (m, 1H, furan proton), 6.90-6.99 (m, 3H, 2 ar + furan proton), 7.07 (d, 1H, ar, J= 8.3 Hz), 7.33 (t, 1H, ar, J= 6.9 Hz), 7.56 (br s, 2H, NH_2), 8.29 (s, 1H, H-3). Anal. Calcd. for $C_{18}H_{17}N_5O_2$: C, 64.47; H, 5.11; N, 20.88. Found C, 64.28; H, 5.02; N, 20.71.

5.1.17.3. 7-Amino-5-(5-methylfuran-2-yl)-2-(3-methoxybenzyl)-2H-pyrazolo[4,3-d]pyrimidine 26. The reaction was carried out at room temperature for about 55 h. The crude solid was purified by preparative TLC (eluent cyclohexane/ EtOAc/MeOH, 6:1:1). Yield 85%; m.p. 257-258 °C; ^{1}H NMR (DMSO-d_6) 2.34 (s, 3H, Me), 3.74 (s, 3H, OMe), 5.58 (s, 2H, CH_2), 6.20-6.21 (m, 1H, furan proton), 6.85 (d, 1H, ar, J= 7.8 Hz), 6.89-6.91 (m, 3H, 2 ar + furan proton), 7.28 (t, 1H, ar, J= 7.8 Hz), 7.57 (br s, 2H, NH_2), 8.43 (s, 1H, H-3). Anal. Calcd. for $C_{18}H_{17}N_5O_2$: C, 64.47; H, 5.11; N, 20.88. Found C, 64.65; H, 5.36; N, 21.04.

5.1.17.4. 7-Amino-2-(2-fluorobenzyl)-5-(5-methylfuran-2-yl)-2H-pyrazolo[4,3-d]pyrimidine 27. The reaction was carried out by heating at 60 °C for about 30 h. The crude solid was purified by preparative TLC (eluent CH_2Cl_2/EtOAc/MeOH, 8:1.5:0.5). Yield 68%; m.p. 252-254 °C; ^{1}H NMR (DMSO-d_6) 2.35 (s, 3H, CH_3), 5.69 (s, 2H, CH_2), 6,20 (s, 1H, furan proton), 6.92 (d, 1H, furan proton, J= 2.9 Hz), 7.29-7.19 (m, 3H, ar), 7.44-7.40 (m, 1H, ar), 7.57 (br s, 2H, NH_2), 8.41 (s, 1H, H-3); ^{13}C NMR (DMSO-d_6) 14.05, 51.07, 108.53, 111.89, 116.15, 123.92, 124.67, 125.27, 130.95, 139.72, 152.10, 152.41, 153.33, 156.02, 159.15, 161.60. Anal. Calcd. for $C_{17}H_{14}FN_5O$: C, 63.15; H, 4.36; N, 21.66. Found C, 63.29; H, 4.50; N, 21.48.

5.1.17.5. 7-Amino-2-(3-fluorobenzyl)-5-(5-methylfuran-2-yl)-2H-pyrazolo[4,3-d]pyrimidine 28. The reaction was carried out at room temperature for about 40 h. Yield 45 %; m.p. 267-268 °C ($MeNO_2$); ^{1}H NMR (DMSO-d_6) 2.35 (s, 3H, CH_3), 5.65 (s, 2H, CH_2), 6.20-6.21 (m, 1H, furan proton), 6.92 (d, 1H, furan proton, J = 3.2 Hz), 7.11-7.19 (m, 3H, ar), 7.39-7.45 (m, 1H, ar), 7.58 (br

s, 2H, ar), 8.46 (s, 1H, H-3). Anal. Calcdd. for $C_{17}H_{14}FN_5O$: C, 63.15; H, 4.36; N, 21.66. Found C, 63.03; H, 4.61; N, 21.48.

5.1.17.6. 7-Amino-2-(2-chlorobenzyl)-5-(5-methylfuran-2-yl)-2H-pyrazolo[4,3-d]pyrimidine 29. The reaction was carried out 60 °C for about 7 h. Yield 29%; m.p. 281-283 °C (2-Methoxyethanol/EtOAc); ^{1}H NMR (DMSO-d_6) 2.35 (s, 3H, CH_3), 5.74 (s, 2H, CH_2), 6.22-6.21 (m, 1H, furan proton), 6.92 (d, 1H, furan proton, J = 3.2 Hz), 6.99 (d, 1H, ar, J= 5.8 Hz), 7.32-7.41 (m, 2H, ar), 7.53 (d, 1H ,ar, J = 6.5 Hz), 7.59 (br s, 2H, NH_2), 8.41 (s, 1H, H-3). Anal. Calcd. for $C_{17}H_{14}ClN_5O$: C, 60.09; H, 4.15; N, 20.61. Found C, 60.25; H, 3.98; N, 20.56.

5.1.18. General procedure for the synthesis of hydroxybenzyl-substituted-7-aminopyrazolo[4,3-d]pyrimidine derivatives 30 and **31**

The title compounds were prepared from the corresponding methoxy derivatives **25** and **26** (1.41 mmol) following the procedure reported above to obtain compounds **4, 6, 9, 12, 14** and carrying out the reaction for 5 h at room temperature (compound **30**) or at reflux (compound **31**). The crude derivatives were purified by preparative TLC (eluent $CHCl_3$/MeOH, 9.3:0.7 for **30,** and $CHCl_3$/MeOH, 9:1 for **31**).

5.1.18.1. 7-Amino-5-(5-methylfuran-2-yl)-2-(2-hydroxybenzyl)-2H-pyrazolo[4,3-d]pyrimidine 30. Yield 35%; m.p. > 300 °C; ^{1}H NMR (DMSO-d_6) 2.34 (s, 3H, CH_3), 5.53 (s, 2H, CH_2), 6.20-6-19 (m, 1H, furan proton), 6.77 (t, 1H, ar, J = 7. 5 Hz), 6.91-6.87 (m, 2H, 1 ar + furan proton), 6.95 (d, 1H, ar, J = 6.3 Hz), 7.18-7.14 (m, 1H, ar), 7.54 (br s, 2H, NH_2), 8.26 (s, 1H, H-3), 9.89 (br s, 1H, OH). ^{13}C NMR (DMSO-d_6) 14.04, 52.72, 108.50, 112.16, 115.71, 119.56, 123.02, 124.21, 129.83, 130.59, 139.66, 151.94, 152.48, 153.23, 155.48, 155.99. Anal. Calcd. for $C_{17}H_{15}N_5O_2$: C, 63.54; H, 4.71; N, 21.79. Found C, 63.70; H, 4.53; N, 21.90.

5.1.18.2. 7-Amino-5-(5-methylfuran-2-yl)-2-(3-hydroxybenzyl)-2H-pyrazolo[4,3-d]pyrimidine 31 Yield 25 %; m. p. 254-255 °C; ^{1}H NMR (DMSO-d_6) 2.38 (s, 3H, Me), 5.57 (s, 2H, CH_2), 6.24-6.25 (m, 1H, furan proton), 6.64 (d, 1H, ar, J= 1.8 Hz), 6.71-6.78 (m, 2H, ar), 6.95-6.96 (m, 1H,

furan proton), 7.19 (t, 1H, ar, J= 7.8 Hz), 7.64 (br s, 2H, NH_2), 8.44 (s, 1H, H-3), 9.49 (s, 1H, OH). Anal. Calcd. for $C_{17}H_{15}N_5O_2$: C, 63.54; H, 4.71; N, 21.79. Found C, 63.33; H, 4.87; N, 21.92.

5.2. Molecular Modeling Studies

Homology modeling, energy minimization, and post-docking analyses were carried out using MOE (version 2010.10) suite.[39] All ligand structures were optimized using RHF/AM1 semiempirical Calculations and the software package MOPAC implemented in MOE was utilized for these Calculations.[49] Docking experiments were performed by using Autodock 4.2.6 software and PyRx interface.[40-42]

5.2.1. Refinement of the hA_{2A} AR structure and homology modeling of the hA_1 AR

The crystal structure of the hA_{2A} AR in complex with ZM241385 were retrieved from the Protein Data Bank (http://www.rcsb.org; pdb code: 3EML; 2.6-Å resolution[37]). The structure was re-modelled by firstly removing the T4L external segment and secondly by performing a building of missing receptor regions (i.e. missing sections of EL2 or IL3 domains). The Homology Modeling tool of MOE was employed. In detail, the boundaries identified from the used hA_{2A} AR X-ray crystal structure were applied and the missing loop domains were built by the loop search method implemented in MOE. Once the heavy atoms were modelled, all hydrogen atoms were added, and the protein coordinates were then minimized with MOE using the AMBER99 force field.[50] The minimizations were performed by 1000 steps of steepest descent followed by conjugate gradient minimization until the RMS gradient of the potential energy was less than 0.05 kJ mol^{-1} $Å^{-1}$. Reliability and quality of the model were checked using the Protein Geometry Monitor application within MOE, which provides a variety of stereochemical measurements for inspection of the structural quality in a given protein, like backbone bond lengths, angles and dihedrals, Ramachandran φ-ψ dihedral plots, and sidechain-rotamer and non-bonded contact quality.

The hA_{2A} AR crystal structure was used as a template for the development of a hA_1 AR homology model. A multiple alignment of the AR primary sequences was built within MOE as preliminary

step. The boundaries identified from the used X-ray crystal structure of hA_{2A} AR were then applied for the corresponding sequences of the TM helices of the hA_1 AR. The missing loop domains were built by the loop search method implemented in MOE. Hydrogen atom addition and energy minimization steps were made as described above for the 3EML hA_{2A} AR re-modeling stage.

5.2.2. Molecular docking analysis

The compound structures were docked into the binding site of the hA_{2A} AR and the hA_1 AR using Autodock 4.2.6 software and PyRx interface.[40-42] Lamarckian genetic algorithm was employed for this analysis with the following settings: 50 runs for each ligand; 2,500,000 as maximum number of energy evaluations; 27,000 as maximum number of generations; 0.02 as rate of gene mutation and 0.8 as rate of crossover. The grid box was set with 50, 50, and 50 points in the *x*, *y*, and *z* directions, respectively, with the default grid spacing of 0.375 Å.

5.2.3. Post Docking analysis. Residue interaction analysis

The interactions between the ligands and the receptors binding site were analysed by using the *IF-E 6.0* tool[44] retrievable at the SVL exchange service (Chemical Computing Group, Inc. SVL exchange: http://svl.chemcomp.com). The program calculates and displays the atomic and residue interaction forces as 3D vectors. It also calculates the per-residue interaction energies, where negative and positive energy values are associated to favourable and unfavourable interactions, respectively. For each AR subtype, a shell of residues contained within a 10 Å distance from ligand were considered for this analysis.

5.3. Pharmacology

5.3.1. Human cloned A_1, A_{2A} and A_3 AR Binding Assay

All synthesized compounds were tested to evaluate their affinity at human A_1, A_{2A} and A_3 ARs. Displacement experiments of [^{3}H]DPCPX (1 nM) to hA_1 CHO membranes (50 μg of protein/assay) and at least 6-8 different concentrations of antagonists for 120 min at 25 °C in 50 mM Tris HCl buffer pH 7.4 were performed.[51] Non-specific binding was determined in the presence 1 μM of

DPCPX (≤ 10% of the total binding). Binding of [^{3}H]ZM-241385 (1 nM) to hA_{2A}CHO membranes (50 μg of protein/assay) was performed by using 50 mM Tris HCl buffer, 10 mM $MgCl_2$ pH 7.4 and at least 6-8 different concentrations of antagonists studied for an incubation time of 60 min at 4 °C.[52] Non-specific binding was determined in the presence of 1 μM ZM-241385 and was about 20% of total binding. Competition binding experiments to hA_3 CHO membranes (50 μg of protein/assay) were performed incubating 0.5 nM [^{125}I]AB-MECA, 50 mM Tris HCl buffer, 10 mM $MgCl_2$, 1 mM EDTA, pH 7.4 and at least 6-8 different concentrations of examined ligands for 60 min at 37 °C.[53] Non-specific binding was defined as binding in the presence of 1 μM AB-MECA and was about 20% of total binding. Bound and free radioactivity were separated by filtering the assay mixture through Whatman GF/B glass fiber filters by using a Brandel cell harvester. The filter bound radioactivity was counted by Scintillation Counter Packard Tri Carb 2810 TR with an efficiency of 58%.

5.3.2. Measurement of cyclic AMP levels in CHO cells transfected with hA_1, hA_{2A} and hA_{2B} ARs

CHO cells transfected with hAR subtypes were washed with phosphate-buffered saline, detached with tripsine and centrifuged for 10 min at 200 g. The cells ($1x10^6$ cells /assay) were suspended in 0.5 ml of incubation mixture (mM): NaCl 15, KCl 0.27, NaH_2PO_4 0.037, $MgSO_4$ 0.1, $CaCl_2$ 0.1, Hepes 0.01, $MgCl_2$ 1, glucose 0.5, pH 7.4 at 37 °C, 2 IU/ml adenosine deaminase and 4-(3-butoxy-4-methoxybenzyl)-2-imidazolidinone (Ro 20-1724) as phosphodiesterase inhibitor and preincubated for 10 min in a shaking bath at 37 °C. The potency of antagonists was determined by the inhibition of the effect of CCPA (1 nM), NECA (10 nM) or NECA (200 nM) for hA_1, hA_{2A} and hA_{2B} ARs, respectively.[54]

The reaction was terminated by the addition of cold 6% trichloroacetic acid (TCA). The TCA suspension was centrifuged at 2000 g for 10 min at 4 °C and the supernatant was extracted four

times with water saturated diethyl ether. The final aqueous solution was tested for cyclic AMP levels by a competition protein binding assay. Samples of cyclic AMP standard (0-10 pmoles) were added to each test tube containing [^{3}H] cyclic AMP and incubation buffer (trizma base 0.1 M, aminophylline 8.0 mM, 2-mercaptoethanol 6.0 mM, pH 7.4). The binding protein prepared from beef adrenals was added to the samples previously incubated at 4 °C for 150 min, and, after the addition of charcoal, was centrifuged at 2000 g for 10 min. The clear supernatant was counted in a Scintillation Counter Packard Tri Carb 2810 TR with an efficiency of 58%.

5.3.3 Data Analysis.

The protein concentration was determined according to a Bio-Rad method[55] with bovine albumin as a standard reference. Inhibitory binding constant (K_i) values were calculated from those of IC_{50} according to Cheng & Prusoff equation $K_i = IC_{50}/(1+[C^*]/K_D^*)$, where [C*] is the concentration of the radioligand and K_D^* its dissociation constant.[56] A weighted non-linear least-squares curve fitting program LIGAND[57] was used for computer analysis of inhibition experiments. IC_{50} values obtained in cyclic AMP assay were calculated by non-linear regression analysis using the equation for a sigmoid concentration-response curve (Graph-PAD Prism, San Diego, CA, U.S.A).

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Figure Captions

Figure 1. Previously reported pyrazolo[4,3-*d*]pyrimidine derivatives **A**-**D**.

Figure 2. Herein reported pyrazolo[4,3-*d*]pyrimidine derivatives **1**-**31**.

Figure 3. Docking conformations at the A_{2A} AR. A. Binding mode 1 conformation (see text for details) of compound **C**. Key receptor residues and domains are indicated. B. Binding mode 2 conformation of compound **3**.

Figure 4. Docking conformation of compound **25** at the A_{2A} AR binding site. A. global view of the ligand-receptor interaction, with indication of key receptor residues. B-C. Detail of the interaction between the receptor and the 2- and 5-substituents of **25**, respectively.

Figure 5. Comparison of docking conformations. A. superimposition of docking conformations of compounds **22** (light) and **25** (dark). B. superimposition of docking conformations of compounds **8** (light) and **11** (dark).

Figure 6. Docking conformation of compounds **25** (A) and **27** (B) at the hA_1 AR binding site. Key receptor residues and domains are indicated.