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The role of 5-aryalkylamino- and 5-piperazino-moieties on the 7-aminopyrazolo[4,3-d]pyrimidine core in affecting adenosine A1 and A2A receptor affinity and selectivity profiles

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ABSTRACT

New 7-amino-2-phenylpyrazolo[4,3-d]pyrimidine derivatives, substituted at the 5-position with ary(alkyl)amino- and 4-substituted-piperazin-1-yl-moieties, were synthesized with the aim of targeting human (h) adenosine A1 and/or A2A receptor subtypes. On the whole, the novel derivatives 1–24 shared scarce or no affinities for the off-target hA3b and hA1 ARs. The 5-(4-hydroxyphenethylamino)- derivative 12 showed both good affinity ($K_i = 150$ nM) and the best selectivity for the hA2A AR while the 5-benzylamino-substituted 5 displayed the best combined hA2A ($K_i = 123$ nM) and hA1 AR affinity ($K_i = 25$ nM). The 5-phenethylamino moiety (compound 6) achieved nanomolar affinity ($K_i = 11$ nM) and good selectivity for the hA2A AR. The 5-(N4-substituted-piperazin-1-yl)-derivatives 15–24 bind the hA2A AR subtype with affinities falling in the high nanomolar range. A structure-based molecular modeling study was conducted to rationalize the experimental binding data from a molecular point of view using both molecular docking studies and Interaction Energy Fingerprints (IEFs) analysis.

Introduction

Adenosine receptors (ARs) are classified as A1, A2A, A2B and A3 subtypes and typically inhibit (A1 and A2) or activate (A2A and A2B) adenylyl cyclase. A1 receptor is highly expressed in brain areas, such as the hippocampus and prefrontal cortex, implicated in the control of emotions and cognition functions. Therefore, A1 AR antagonists are investigated as therapeutic agents for mental dysfunctions, such as dementia and anxiety. The A2A AR subtype is present in the brain with the highest concentration in the striatum, nucleus accumbens, hippocampus and cortex, and its blockade has been proven to be effective in neurodegenerative pathologies such as Parkinson’s disease (PD). The A2A AR antagonist istradefylline has been recently approved for marketing in Japan for the treatment of PD patients. In preclinical studies, dual A1/A2A antagonists have also turned out to be useful for PD therapy because they reduce both motor (A2A) and cognitive (A1) impairment associated with this pathology.

Recent studies have highlighted new therapeutic applications of A2A AR antagonists. If topically administered, they diminish scar size and promote restoration of skin integrity. A2A AR antagonists have also demonstrated efficacy in enhancing immunologic response, especially by markedly improving anti-tumor immunity in mouse models, thus promoting tumor regression. A2A AR antagonists have been shown to improve the effect of tumor vaccines during T-cell activation, and may work in concert with other immune checkpoint inhibitors in cancer immunotherapy.

In our laboratory, much research has been addressed to the study of AR antagonists belonging to different classes, including the 2-arylpurazolo[4,3-d]pyrimidine derivatives which display a broad range of affinity for the various AR subtypes, depending on the nature of the substituents at the 5- and 7-positions.
positions of the bicyclic scaffold. One recent study aimed at targeting the A₁ and A₂A ARs highlighted that the presence of a free 7-amino group, combined with a benzyl or, even better, a 3-phenylpropyl chain at the 5-position (Figure 1, compounds A and C) shifted affinity toward these two AR subtypes.

Hence, to further explore the structural requirements for addressing affinity toward the A₁ and/or A₂A ARs, various aryl(alkyl)amino- and 4-substituted-piperazin-1-yl moieties were appended at the 5-position of the scaffold (compounds 1–24, Figure 2). These substituents were selected since they are a common feature of potent A₁ and/or A₂A AR antagonists structurally correlated to our pyrazolopyrimidine derivatives (such as the triazolotriazines ZM-241385 and D, Figure 1). The pyrazolopyrimidines 1–24 were tested in binding assays to evaluate their affinity at cloned hA₁, hA₂A and hA₃ ARs, stably expressed in CHO cells. Compounds were tested in binding assays to evaluate their affinity at cloned the readily available 4-nitro-1H-pyrazole-3-carbonitrile played in Scheme 1. The latter was achieved by a cross-coupling reaction with phenylboronic acid in the presence of cupric acetate and arylation. The methoxy-substituted derivatives were a good substrate for both regioselective N-alkylation and N-amination. These substituents were selected since they are a common feature at the 5-position of the scaffold (compounds 1–24, Figure 2).

A structure-based molecular modeling study was performed on the new derivatives to rationalize the experimental binding data from a molecular point of view, using molecular docking studies in tandem with Interaction Energy Fingerprints (IEFs) analysis.

### Chemistry

The 7-amino-pyrazolo[4,3-d]pyrimidine derivatives 1–14, bearing an arylalkylamino moiety at the 5-position, were obtained as displayed in Scheme 1. The 1-phenyl (26) and 1-alkyl substituted pyrazoles (27, 28) were synthesized from a common starting compound: the readily available 4-nitro-1H-pyrazole-3-carbonitrile 25 which was a good substrate for both regioselective N-alkylation and N-arylation. The latter was achieved by a cross-coupling reaction with phenylboronic acid in the presence of cupric acetate and activated molecular sieves. The 1-phenyl-pyrazole derivative 26 was thus prepared with higher yield than those previously obtained in our laboratory through another synthetic pathway. The 1-methyl- and 1-benzyl-pyrazoles 27 and 28 were prepared from compound 25 as already described. The 4-nitropyrazolo-3-carbonitriles 26–28 were transformed into the corresponding 4-amino derivatives 29–31 by reduction with cyclohexene and Pd/C, under microwave-assisted conditions. Reaction of compounds 29–31 with isothiocyanates in anhydrous DMF yielded the corresponding S-1-substituted-3-cyano-pyrazol-4-ylthioureia derivatives 32–42. Phenyl-, 4-methoxyphenyl-, 2,4-dichlorophenyl- and benzyl-isothiocyanates were commercially available, the others were synthesized as previously reported, i.e. allowing the corresponding arylalkylamines to react with CS₂, in 30% hydrochloric acid.

Carbonitriles 26–28 were transformed into the corresponding hydroxy derivatives 4, 13 and 14 by treatment with BBr₃ in anhydrous CH₂Cl₂.
The 7-amino-pyrazolo[4,3-d]pyrimidine derivatives 15–22, bearing N-substituted-piperazine moieties at the 5-position, were obtained utilizing the synthetic route as described in Scheme 2.

Allowing the 1-phenylpyrazolo[4,3-d]pyrimidine-5,7-dione 54 to react with phosphorus oxychloride and N,N-dimethylaniline under microwave irradiation, the 5,7-dichloro-derivative 55 was prepared, which was reacted with 33% aqueous ammonia solution under microwave irradiation at 100 °C to give the 7-amino-5-chloro-pyrazolopyrimidine 56 as the only regioisomer. The 7-amino structure of 56 was expected on the basis of the well-known different mobility of the two chlorine atoms in the pyrimidine ring, also condensed with diverse heterocyclic systems32–34.

To confirm the structure, derivative 56 was treated with benzylamine in tert-butanol, in the presence of diisopropylethylamine, and the 7-amino-5-benzylaminopyrazole derivative 5, already synthesized through the unambiguous synthesis as depicted in Scheme 1, was obtained. This reaction was carried out under prolonged microwave irradiation (about 1 h at 200 °C) but conversion of derivative 56 into 5 occurred with unsatisfactory yields. The 1H NMR spectrum of the crude reaction (data not shown) displayed the presence of both the 5-benzylamino derivative 5 and the starting material 56 (ratio about 3.5:1), besides degradation compounds, thus indicating the poor reactivity of the C5 atom toward the primary benzyl ammine group. Instead, microwave-assisted reaction of the 5-chloro derivative 56 with the N-substituted piperazines 57–63, in N-methylpyrrolidone and in the presence of diisopropylethylamine, proceeded to completion, thus giving the desired pyrazolopyrimidine derivatives 15–20 with good yields (48–85%). The piperazine derivatives 57, 58, 62 and 63 were commercially available, while derivatives 59 and 61 were prepared as previously described35,36. The piperazine derivative 60 was synthesized starting from the reductive alkylation of N-Boc-piperazine 63 with 2,4,6-trifluorobenzaldehyde and triacetoxy sodium borohydride. The obtained tert-butyl 4-(2,4,6-trifluorobenzyl)piperazine-1-carboxylate was hydrolyzed with trifluoroacetic acid to give the 1-(2,4,6-trifluorobenzyl)piperazine 60, isolated as trifluoroacetate salt.

Reduction of the 2-furoyl carbonyl group of compound 20 with LiAlH4 in anhydrous THF provided derivative 22. Finally, the

\begin{table}
\centering
\begin{tabular}{|c|c|c|c|c|c|}
\hline
\textbf{R} & \textbf{R′} & \textbf{hA1} & \textbf{hA2A} & \textbf{hA3} & \textbf{hA2B} \\
\hline
1 & Ph & 67 ± 5 & 412 ± 37 & 13 ± 2 & 2% \\
2 & 4-OMe-C6H4 & Ph & 33% & 8% & 27 ± 3 & 1% \\
3 & 2,4-diClC6H3 & Ph & 1% & 1% & 61 ± 8 & 2% \\
4 & 4-OH-C6H4 & Ph & 481 ± 42 & 40% & 8% & 10% \\
5 & Ph-CH2 & Ph & 25 ± 3 & 123 ± 11 & 28 ± 3 & 1% \\
6 & Ph-CH2CH2 & Ph & 11.5 ± 1.2 & 40% & 38% & 1% \\
7 & Ph-CH2CH2CH2 & Ph & 785 ± 72 & 29% & 24% & 1% \\
8 & Ph & Me & 1% & 4% & 1% & 1% \\
9 & Ph-CH2 & Me & 9% & 1% & 20% & 1% \\
10 & Ph-CH2 & CH3Ph & 19% & 1% & 1% & 1% \\
11 & 4-OMe-C6H4-C6H4 & CH3Ph & 153 ± 11 & 26% & 19% & 1% \\
12 & 4-OH-C6H4-C6H4 & CH3Ph & 27% & 150 ± 14 & 1% & 1% \\
13 & 3,4-diOMe-C6H4-C6H4 & CH3Ph & 415 ± 39 & 189 ± 18 & 22% & 17% \\
14 & 3,4-diOH-C6H4-C6H4 & CH3Ph & 71 ± 6 & 238 ± 24 & 3% & 420 ± 38 \\
15 & Ph & – & 150 ± 12 & 110 ± 10 & 30% & 1% \\
16 & Ph-CH2 & – & 15% & 35% & 17% & 2% \\
17 & Ph & – & 5.31 ± 0.42 & 55 ± 5 & 12% & 42% \\
18 & Ph-CH2 & – & 714 & 1.6 & 743 & 75h \\
\hline
\end{tabular}
\caption{Binding affinity at hA1, hA2A and hA3 ARs and potencies at hA2B ARs.}
\end{table}

\textsuperscript{a}Ki values are means ± SEM of four separate assays each performed in duplicate. Percentage of inhibition (%) are determined at 1 μM concentration of the tested compounds.

\textsuperscript{b}Displacement of specific [1H]DPCPX competition binding assays to hA1CHO cells.

\textsuperscript{c}Displacement of specific [1H]ZM241385 competition binding to hA2ACHO cells.

\textsuperscript{d}Displacement of specific [125I]AB-MECA competition binding to hA3CHO cells.

\textsuperscript{e}CAMP experiments in hA2BCHO cells, stimulated by 200 nM NECA. Percentage of inhibition (%) are determined at 1 μM concentration of the tested compounds.

\textsuperscript{f}Ref. 24.

\textsuperscript{g}Ref. 5.

\textsuperscript{h}Ki value obtained from binding experiments at recombinant hA2B.
Table 2. Binding affinity at hA1, hA2A and hA3 ARs and potencies at hA2B ARs.

| R         | hA1 | hA2A | hA3 | hA2B |
|-----------|-----|------|-----|------|
| Ph        | 647 ± 53 | 20% | 20% | 1%   |
| CH2Ph     | 162 ± 14  | 1%   | 1%  | 1%   |
| (CH2)2Ph  | 518 ± 42  | 4%   | 40% | 1%   |
| CH2C6H2-2,4,6-F | 204 ± 18 | 24%  | 39% | 1%   |
| CH2C6H2-2,4,6-F | 193 ± 17 | 29%  | 1%  | 2%   |
| CO-2-furyl | 580 ± 47  | 16%  | 13% | 2%   |
| COOtBu    | 615 ± 49  | 33%  | 21% | 1%   |
| CH2-2-furyl| 92 ± 8    | 38%  | 5%  | 16%  |
| COCH2Bu   | 29%     | 2%   | 1%  | 2%   |
| COCH2Ph   | 429 ± 36  | 3%   | 1%  | 3%   |

\[ \text{Ki (nM) or } \% \]

\[ \text{cAMP assays } \% \]

\[^{a}K_{i}\text{ values are means ± SEM of four separate assays each performed in duplicate.}

\[^{b}\text{Percentage of inhibition (}%)\text{ are determined at 1 } \mu\text{M concentration of the tested compounds.}

\[^{c}\text{Displacement of specific [}^{[3]H}]\text{DPCPX competition binding assays to hA}_{1}\text{CHO cells.}

\[^{d}\text{Displacement of specific [}^{[3]H}]\text{ZM241385 competition binding to hA}_{2A}\text{CHO cells.}

\[^{e}\text{Displacement of specific [}^{[125]I}]\text{AB-MECA competition binding to hA}_{3}\text{CHO cells.}

\[^{f}\text{Displacement of specific [}^{[3]H}]\text{ZM241385 competition binding to hA}_{2B}\text{CHO cells.}

The results of binding experiments and cAMP assays carried out on the new 5-substituted-pyrazolopyrimidines 23–24 are displayed, respectively, in Tables 1 and 2. Table 1 also includes the affinity data of the pyrazolopyrimidines A–C and of ZM-241385 reported as references.

As expected, the new derivatives 1–24 shared scarce or no affinities for the off-target hA2B and hA3 ARs, except the 5-anilino- and 5-benzylamino derivatives 1–3 and 5, respectively which displayed nanomolar affinity for the hA2A subtype (Ki = 13–61 nM). In particular, compounds 2 and 3 are worth noting, being also highly hA2A selective.

Since the purpose of the work was to target hA1 and hA2A ARs, SAR discussion was focused on hA1 and hA2A binding data. In this respect, results of some interest have been obtained from the 5-arylalkylamino-pyrazolopyrimidines 1–14. In fact, compound 12 showed both good affinity and the best selectivity for the hA2A AR, while compounds 1, 5, 13 and 14 were able to bind both the hA1 and hA2A ARs. Moreover, a derivative having nanomolar affinity and high selectivity for the hA1 AR subtype was identified (compound 6).

The new 5-phen(alkyl)amino derivatives 1, 5 and 6 were designed as analogs of our previously reported antagonists 5-phenyl(aryl) derivatives A, B and C whose methylene linker at the 5-position of the bicyclic core was replaced with an NH. This modification, suggested by the structure of potent A2A antagonists bearing arylalkylamino moieties as key substituents11,12, was thought to change the flexibility of the 5-lateral chain and, hopefully, to increase the affinity for the targeted ARs. Actually, the NH linker enhanced the hA1 AR affinity (compare 1 and 5 to A and B, respectively) or maintained it in the nanomolar range (compare 6 to C). Instead, the hA2A AR binding was ameliorated in one case, i.e. the 5-benzylamino derivative 5 which was more active than the corresponding phenylalkyl-derivative B.

Analyzing the hA1 and hA2A AR binding data of 1–6 in detail, it can be observed that 5-phenylamino derivative 1 binds to the hA2A and hA1 AR subtypes with scarce (Ki = 412 nM) and good affinity (Ki = 67 nM), respectively. Introduction of either a 4-methoxy group or 2,4-dichloro substituents on the 5-aniline moiety of 1 (compounds 2 and 3) dropped affinity for hA1 and hA2A ARs. Instead, the presence of a 4-hydroxy residue (compound 4) reduced the hA2A affinity while conserving some ability to bind the hA1 receptor (Ki = 481 nM). Homologation of the 5-phenylamino moiety (derivative 1) to the 5-benzylamino group (derivative 5) produced some improvement in the binding activity at both hA1 (Ki = 25 nM) and hA2A ARs (Ki = 123 nM). Quite unexpectedly, homologation of the alkyl chain of compound 5, to obtain the 5-phenethylnamino- and the 5-phenylpropionamino derivatives 6 and 7, caused a drastic reduction of the hA2A AR affinity and, in the former, it increased the hA1 one, thus affording a selective hA1 receptor ligand (Ki = 11.5 nM).

Replacement of the 2-phenyl group of derivatives 1 and 5 with a methyl residue, to give compounds 8 and 9, was performed to verify whether a reduction in the volume of the molecule might permit a better accommodation inside the recognition site of the targeted hARs. This modification, instead, annulled the capability to bind the target hARs. The same detrimental effect was obtained when the 2-phenyl ring of 5 was replaced with the more flexible benzyl moiety (derivative 10).

Insertion of the para hydroxy substituent on the 5-phenethylamino moiety of derivative 6, to give compound 12, was based on the structure of the well-known potent and selective hA2A AR antagonist ZM-2413855,12 (Figure 1). Accordingly, we also thought it would be interesting to evaluate the 3,4-dihydroxy substitution (compound 14), as well as the 4-methoxy- and the 3,4-dimethoxy-substituents (derivatives 11 and 13). As expected, the presence of the 4-hydroxy group was able to shift the affinity toward the hA2A AR. In fact, the 4-hydroxy-substituted derivative 12 showed good hA2A affinity (Ki = 150 nM) and the best selectivity among all the ligands reported here. In contrast, reversed selectivity was demonstrated by the 4-methoxy derivative 11, which displayed good affinity for the hA1 AR but not for the hA2A subtype. Instead, the 3,4-dimethoxy substituted derivative 13 bound both hA1 and hA2A receptors and also the 3,4-dihydroxy derivative 14 showed quite good affinity for both the receptors, but especially for the hA1 one.

Finally, to further explore the SARs in this class of AR ligands, various N-substituted piperezine moieties were appended at the 5-position (derivatives 15–24, Table 2), in accordance with the structure of known potent and selective hA2A AR antagonists27,28.

In contrast to our expectations, none of the 5(N1-R-piperezin-1-yl) derivatives 15–24 were able to bind effectively the A2A AR while they possessed affinity for the hA1 AR subtype, falling in the high nanomolar range. The most active compounds proved to be 22 (Ki = 92 nM) and 16 (Ki = 162 nM) which bear, respectively, the (2-furyl)-methyl and 2-benzyl pendant on the N4-piperezine moiety. Introduction of halogen atoms on the benzyl moiety of 16 left almost unchanged the hA1 AR affinity (compounds 18 and 19).
while elongation of the benzyl chain decreased it (compound 17). Also the other substituents evaluated on the piperazine ring, i.e. acyl moieties (derivatives 20, 23, 24) and the tert-butoxycarbonyl group (derivative 21) did not ameliorate the hA3 AR affinities.

**Molecular modeling studies**

A structure-based molecular modeling study was conducted to rationalize the experimental binding data from a molecular point of view. Minor attention was devoted to the hA2B AR subtype, since no significant binding affinity has been estimated for any of the compounds under investigation. Docking was performed on hA1, hA2A and hA3 AR subtypes, and the resulting poses were evaluated according to the van der Waals and electrostatic interactions, as previously reported and described in detail in the "Experimental" section. Positive electrostatic and van der Waals values were used as filters to reject unfavorable docking poses. One pose for each ligand was selected on the basis of the Interaction Energy Fingerprints (IEFs) and by visual inspection.

An overview of the most favorable poses of all compounds on hA1, hA2A and hA3 ARs is reported in video SM1-SM2-SM3, included in Supplementary Material. The heat map depicted in the background reports the electrostatic and hydrophobic contributions of the residues mainly involved in binding ("ele" and "hyd" labels identify the major contribution type of the residue) by a colorimetric scale going from blue to green for negative to positive values. These crucial residues are mainly positioned on the superior half of TM6 and TM7 and EL2, and the overall binding modes of the compounds under examination are very consistent among them. Here, we describe in detail the poses of compound 1 as an example, because of its high binding affinity for all three AR subtypes taken into consideration (K<sub>i</sub> = 67 nM for hA1, K<sub>i</sub> = 412 nM for hA2A and K<sub>i</sub> = 13 nM for hA3).

![Image](image_url)
Figure 4. Comparison of the proposed binding mode of compound 1 (sky blue), compound A (pink), and reference pose of ZM-241385 (green) on hA1, hA2A and hA3 subtype receptors (panels A, B and C, respectively). Protein residues mainly involved in binding are shown as sticks (tan). The zoom makes TM1 not visible, while TM6 and TM7 are rendered in a transparent manner to give a more clear visualization of the binding site.

With regard to the hA1 AR, Glu172 (EL2) and Asn254 (6.55), represented by blue bars on electrostatic IEFs (Figure 3, panel A on the left), emerge as important residues for electrostatic contribution, together with a slight contribution of Trp247 (6.48) and His251 (6.52). Asn254 (6.55) and Glu172 (EL2) are engaged in a three hydrogen bond pattern with N1 of pyrazole and with the exocyclic amine group at position 5 of compound 1, as shown in Figure 4, panel A. The aromatic pyrazolopyrimidine scaffold is involved in a π-π stacking interaction with Phe171 (EL2), which is the only one of the residues appearing to have the strongest hydrophobic interaction on the hydrophobic IEFs (green bars in Figure 3, panel A on the right). Val87 (3.32), Leu88 (3.33), Trp247 (6.48), Leu250 (6.51), Tyr271 (7.36) and Ile274 (7.39) are also involved in significant hydrophobic contacts, with Val87 (3.32), Leu88 (3.33), Trp247 (6.48) defining the bottom of the binding pocket.

The residues involved in binding at hA2A AR are positioned equivalently to those just described for the hA1 subtype. Glu169 (EL2) and Asn253 (6.55) are involved in hydrogen bonds and Phe168 (EL2) makes a π-π stacking interaction, as can be seen in Figure 4, panel B. Trp246 (6.48) and His250 (6.52), together with Glu169 and Asn253, give stabilizing electrostatic contributions to the binding of 1, while Leu85 (3.33), Leu167 (EL2), Phe168 (EL2), Trp246 (6.48), Leu249 (6.51), Tyr271 (7.36) are interested by hydrophobic contacts (Figure 3, panel B).

The binding of compound 1 to the hA3 subtype mainly engages Trp243 (6.48), Ser247 (6.52) and Asn250 (6.55) for electrostatic interactions, and Leu91 (3.33), Phe168 (EL2), Val169 (EL2), Trp243 (6.48), Leu246 (6.51), Leu264 (7.35), Tyr265 (7.36), Ile268 (7.39) for hydrophobic interactions, as can be seen in Figure 3, panel C. In this case only Asn250 can be involved in the hydrogen bond network (Figure 4, panel C), since in the A3 AR the position equivalent to Glu172 of the hA1, and Glu169 of the A2A AR is occupied by Val169, which cannot establish a hydrogen bond with the amino group at position 5 of compound 1.

Most of the poses resemble the conformation that ZM-241385 assumes in the binding site of the hA2A AR crystal structure and of hA1 and hA3 AR models. The benzene ring at position 2 occupies the position of the furan ring of ZM-241385, the 7-amino-pyrazolopyrimidine scaffold is well superimposed on the reference 7-amino-triazolotriazine and the aroylalkylamino group at position 5 points in the same direction as the para-hydroxyphenyl-ethylamino fragment. The similarity of the binding modes confirms the expectation provided by the IEFs comparison between 1 and ZM-241385 (Figure 3, panels A, B and C). To quantitatively compare the calculated IEFs profiles, two novel analyses have been proposed called RMSD and RMSD_trend analysis (see the Experimental Section for more details). In the case of derivative 1 both RMSD and RMSD_trend between electrostatic and hydrophobic IEFs on each hAR subtype are quite low. However, the electrostatic RMSD (2.18 kcal/mol) and the electrostatic RMSD_trend (0.47 kcal/mol) for the hA3 subtype are higher than the values observed for hA2A and hA1 ARs. This does not seem to fit with the low Kᵢ (67 nM) for the hA1 receptor; however, it appears that the major unfavorable contribution is provided by Glu170, which may probably be corrected by a slight rotation of the phenyl group of the compound.

Subsequently, we compared the binding behavior of compound 1 to that of its analog derivative A (Kᵢ = 150 nM for hA1, Kᵢ = 110 nM for hA2A and I% = 39 at 1 μM for hA3), having a methylene instead of the NH linker at the 5-position. The IEFs comparison did not allow a complete rationalization of the different selectivity profiles of compounds 1 and A (Figure SM1). Electrostatic RMSD and RMSD_trend Values on the hA3 receptor (1.10 and 0.18 kcal/mol, respectively) are higher than those of compound 1 (0.60 and 0.06 kcal/mol, respectively), in accordance with the lower potency of derivative A (I% = 39% at 1 μM) compared with 1 (Kᵢ = 13 nM). On the other hand, we have to honestly observe that also compound A presents higher RMSD and RMSD_trend values (1.49 and 0.18 kcal/mol, respectively) on the hA2A receptor as compared with compound 1 (0.96 and 0.08 kcal/mol, respectively), but in this case the affinity of the former (110 nM) is higher than that of the latter (412 nM). The result of the IEFs comparison is confirmed by the similarity of the binding modes of derivatives 1 and A at all receptor binding sites, as reported in Figure 4 (panels A, B and C). In this case docking is not sufficient to rationalize the difference in binding affinities. In fact, the mere examination of the final state of the binding process may not be sufficient to explain differences in the activity or selectivity profiles. The presence of water molecules and the entropic effect are only two among the pool of binding contributions that we are not taking into consideration during our docking simulations.

Similar considerations can be made observing the results of IEFs comparison for all the dataset compounds on the different AR subtypes (Figure 5). We would have expected to find blue and red rectangles associated with good and bad binders, respectively, but
this prevision was not satisfied: a major similarity of the IEFs between the target and the reference compounds are not always related to good binding affinity of the ligand. However, an interesting example is provided by compounds 8, 9, and 10, which have no affinity for any of the receptors. Red rectangles cross horizontally almost the whole hydrophobic RMSD and RMSD\textsubscript{trend} table, meaning that there is a considerable loss in the binding hydrophobic contribution in comparison with the reference. As a control experiment, ZM-241385 has been docked into the three AR subtypes, the IEFs have been computed for the selected poses and compared with that of the reference pose of ZM241385: as expected, the electrostatic and hydrophobic RMSD and RMSD\textsubscript{trend} values are close to zero (Figure 5).

The 5-(N\textsubscript{4}-R-piperazin-1-yl) compounds 15–24 are hA\textsubscript{1} AR selective. These derivatives find a steric hindrance in the hA\textsubscript{3} binding site and the van der Waals values of the selected poses are positive (as indicated by exclamation points in Figure 5). However, from the IEFs comparison analysis (Figure 5), we would have predicted a hA\textsubscript{2A} versus hA\textsubscript{1} selectivity (blue versus red rectangles). In fact, while at the hA\textsubscript{2A} binding site the predicted poses of these compounds behave like ZM-241385, at the hA\textsubscript{1} binding site they deviate a little from the reference position, losing some of the canonical interactions (Video SM1-SM2). Interestingly, this diversification in a gain for compounds 16, 17, 18, 19, and 22: the protonated amine at position 4 of the piperazine moiety is involved in an ionic interaction with Glu170 (EL2), which is confirmed by a highly negative electrostatic contribution reported on the heat map in the background of Video SM1. The absence of a negatively charged residue at a position equivalent to Glu170 on hA\textsubscript{2A} (Leu167) and hA\textsubscript{3} (Gln167) receptors may be associated with the hA\textsubscript{1} selectivity of these compounds.

**Conclusion**

The herein reported structural investigation was carried out to identify new antagonists targeting the hA\textsubscript{2A} AR or both the hA\textsubscript{1}/hA\textsubscript{2A} ARs. Hence, various arylalkylamino- and 4-substituted-piperazin-1-yl moieties were appended at the 5-position of the pyrazolo[4,3-d]pyrimidine scaffold. The 4-hydroxyphenylethylamino group was the most profitable, since the ZM-241385-based compound 12 showed both good hA\textsubscript{2A} affinity (K\textsubscript{i} = 150 nM) and the highest selectivity among all the ligands reported here. The 5-benzylamino moiety (compound 5) achieved the best combined hA\textsubscript{2A} (K\textsubscript{i} = 123 nM) and hA\textsubscript{1} affinity (K\textsubscript{i} = 25 nM) while the 5-phenethylamino pendant (compound 6) afforded nanomolar affinity (K\textsubscript{i} = 11 nM) and good selectivity for the hA\textsubscript{1} AR. The 5-(N\textsuperscript{4}-substituted-piperazin-1-yl) derivatives 15–24 were inactive at the hA\textsubscript{2A} AR while the hA\textsubscript{1} affinities spanned the high nanomolar range. These outcomes provide new insights about the structural requirements of our pyrazolopyrimidine series for hA\textsubscript{2A}- and hA\textsubscript{1}-receptor ligand interaction. Nevertheless, the obtained results do not prompt us to synthesize further derivatives of this series featured by 5-arylalkyamino- and 5-piperazino- moieties.

A structure-based molecular modeling study was conducted to rationalize the experimental binding data from a molecular point of view using molecular docking studies in tandem with Interaction Energy Fingerprints (IEFs) analysis. Moreover, to quantitatively compare IEFs profiles and, consequently, to address the similarity of the binding modes of different compounds in different receptor subtypes, two novel analyses have been proposed, called RMSD and RMSD\textsubscript{trend} analyses. Even if we are conscious that the simple inspection of the final state of the binding process may not be sufficient to explain differences in the activity or selectivity profiles, these novel tools can facilitate the mode of representation and interpretation of the docking data obtained by analyzing simultaneously several compounds against different receptor subtypes.

**Experimental section**

**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 a purity not less than 95%. The IR spectra were recorded with a Perkin-Elmer Spectrum RXI spectrometer in Nujol mulls and are expressed in cm⁻¹. The ¹H NMR spectra were obtained with a Bruker Avance 400 MHz instrument. The chemical shifts are reported in δ (ppm) and are relative to the central peak of the solvent which was CDCl₃ or DMSO-d₆. The assignment of exchangeable protons (OH, and NH) was confirmed by addition of D₂O. The following abbreviations are used: s = singlet, d = doublet, t = triplet, m = multiplet, br = broad and ar = aromatic protons.

4-Nitro-1-phenyl-1H-pyrazole-3-carbonitrile 26, 26

The title compound was prepared with a different procedure from that previously described by us.²⁶ Briefly, phenylboronic acid (2.4 mmol), cupric acetate (1.8 mmol) and activated 4 Å molecular sieves (750 mg) were added to a solution of 4-nitro-1H-pyrazole-3-carbonitrile (1.2 mmol) in anhydrous dichloromethane (8 mL) and pyridine (2.4 mmol). The mixture was stirred at room temperature, under air, in a loosely capped flask for two days, then it was diluted with chloroform (20–30 mL) and filtered through celite. The solution was evaporated at reduced pressure to give a solid which was collected by suction, washed with water and then cyclohexane and recrystallized. Yield 75%; m.p. 143–145 °C (cyclohexane/EtOH); ¹H NMR (DMSO-d₆) 7.54–7.63 (m, 3H, ar), 7.74–7.76 (m, 2H, ar), 8.71 (s, 1H, H-5).

General procedure for the synthesis of 3-substituted-1-(3-cyano-1-phenyl-1H-pyrazol-4-yl)thioureas 32–42

The commercially available phenyl-, 4-methoxyphenyl- and benzyl-isothiocyanates or the suitably synthesized phenylenethynyl, 4-methylenethynyl, 3,4-dimethylenethynyl, phenylypropyl-isothiocyanates were added to a solution of the 1-substituted-4-amino-pyrazole-3-carbonitriles 29–31, 26 (1.64 mmol) in anhydrous DMF (1.5 mL). The mixture was stirred at room temperature for 3–4 h (compounds 32–34, 38, 39), for 16 h (compounds 35, 40, 42) and for 24 h (compounds 36, 37, 41).

The obtained dark slurry was treated with water (20 mL) and, in the case of compounds 33–35 and 39, a solid precipitated which was collected by filtration. For derivatives 32, 36–38, 40–42, the aqueous mixture was extracted with EtOAc (30 mL ×3). The combined organic extracts were anhydried (Na₂SO₄) and the solvent evaporated at reduced pressure. The obtained solid was treated with Et₂O (5–10 mL) and isolated by filtration. Crude compound 42 was purified by column chromatography (elucent cyclohexane/EtOAc/MeOH 6:4:1). Derivatives 32, 38, 40, as well as 42, were unstable upon recrystallization, hence they were used as such for the next step.

1-(3-Cyano-1-phenyl-1H-pyrazol-4-yl)-3-phenylthiourea 32

Yield 89%; ¹H NMR (DMSO-d₆) 7.19 (t, 1H, ar, J = 7.4 Hz), 7.36 (t, 2H, ar, J = 7.6 Hz), 7.46 (t, 1H, ar, J = 7.4 Hz), 7.51 (d, 2H, ar, J = 7.6 Hz), 7.58 (t, 2H, ar, J = 7.5 Hz), 7.88 (d, 2H, ar, J = 7.7 Hz), 8.97 (s, 1H, pyrazole proton), 9.68 (br s, 1H, NH), 10.04 (br s, 1H, NH).

1-(3-Cyano-1-phenyl-1H-pyrazol-4-yl)-3-(4-methoxyphenyl)thiourea 33

Yield 95%; m.p. 165–167 °C (cyclohexane/EtOAc); ¹H NMR (DMSO-d₆) 3.75 (s, 3H, OCH₃), 6.95 (d, 2H, ar, J = 8.9 Hz), 7.33 (d, 2H, ar, J = 8.9 Hz), 7.46 (t, 1H, ar, J = 7.6 Hz), 7.58 (t, 2H, ar, J = 7.3 Hz), 7.89 (d, 2H, ar, J = 7.6 Hz), 8.95 (s, 1H, pyrazole proton), 9.56 (br s, 1H, NH), 9.89 (br s, 1H, NH). Anal. Calc. for C₁₈H₁₅N₅S.

1-(3-Cyano-1-phenyl-1H-pyrazol-4-yl)-3-(2,4-dichlorophenyl)thiourea 34

Yield 98%; m.p. 166–169 °C (cyclohexane/EtOAc); ¹H NMR (DMSO-d₆) 7.44–7.60 (m, 5H, ar), 7.80 (s, 1H, ar), 7.96 (d, 2H, ar, J = 7.9 Hz), 9.02 (s, 1H, pyrazole proton), 9.82 (br s, 1H, NH), 9.98 (br s, 1H, NH). Anal. Calc. for C₁₉H₁₃Cl₂N₅S.
Evaporation of the anhydrified (Na₂SO₄) organic phase gave a
1H NMR (DMSO-d₆) 2.81 (t, 2H, CH₂), 7.24 (t, 2H, CH₂, J = 7.5 Hz), 2.39–2.72 (m, 5H, OCH₃ + CH₂), 3.75 (s, 3H, OCH₃), 6.76 (d, 1H, ar, J = 8.1 Hz), 6.84 (s, 1H, ar), 6.89 (d, 1H, ar, J = 8.2 Hz),
7.46 (t, 1H, ar, J = 7.2 Hz), 7.58 (t, 2H, ar, J = 7.1 Hz), 7.86 (d, 2H, ar, J = 8.0 Hz), 8.04 (br s, 1H, NH), 8.91 (s, 1H, pyrazole proton), 9.48 (br s, 1H, NH). Anal. Calc. for C₁₂H₁₄N₂O₂S.

3-[(Cyano-1-phenylpyrazolo-4-yl)-4] -N'-phenyl-S-methylisothiourea 43 Yield 86%; 1H NMR (DMSO-d₆) 2.56 (s, 3H, CH₃), 2.97 (t, 2H, CH₂, J = 7.3 Hz), 3.75 (s, 3H, OCH₃), 4.29 (s, 2H, CH₂, J = 7.3 Hz), 6.91 (d, 2H, ar, J = 9.5 Hz), 7.24 (d, 2H, ar, J = 9.5 Hz), 7.43 (t, 1H, ar, J = 9.5 Hz), 7.57 (t, 2H, ar, J = 9.3 Hz), 7.98 (d, 2H, ar, J = 9.3 Hz), 8.11 (s, 1H, NH), 8.85 (s, 1H, pyrazole proton).

General procedure for the synthesis of 5-aryl(alkyl)amino-7-amino-2H-
pyrazolo[4,3-d]pyrimidine derivatives 1–3, 5–12
A mixture of the suitable S-methylisothioureas 43–53 (1 mmol) and NH₄Cl (20 mmol) in formamide (2 mL) was microwave irradiated at 110 °C for 20 min, then treated with NaHCO₃ until pH 7 and the obtained solid was collected by filtration. The crude derivatives were purified by recrystallization, except compounds 1, 6, 7, 10, 11 which were first purified by column chromatography or preparative TLC (see below for details).

7-Amino-5-phenylamino-2-phenyl-2H-pyrazolo[4,3-d]pyrimidine 1
Purified by column chromatography (EtO₂/ cyclohexane/ EtOAc 3:1:1). Yield 66%; m.p. 252–254 °C (EtOH). 1H NMR (DMSO-d₆) 6.86
7-Amino-5-(4-methoxyphenyl)amino-2-phenyl-2H-pyrazolo[4,3-d]pyrimidine 11 Purified by column chromatography (cyclohexane/EtOAc/MeOH 6:4:1). Yield 58%; m.p. 142–145°C (cyclohexane/EtOAc). ¹H NMR (DMSO-d₆) 2.73 (t, 2H, CH₂, J = 7.3 Hz), 3.38–3.41 (m, 2H, CH₂), 3.73 (s, 3H, OCH₃), 6.03 (br s, 1H, NH), 6.69 (2d, 2H, ar, J = 8.9 Hz), 7.04 (d, 2H, ar, J = 8.9 Hz), 7.22 (br s, 2H, NH₂), 7.41 (t, 1H, ar, J = 7.3 Hz), 7.57 (t, 2H, ar, J = 7.6 Hz), 7.98 (d, 2H, ar, J = 7.9 Hz), 8.51 (s, 1H, H-3). Anal. Calc. for C₂₀H₂₀N₂O₂.

**General procedure for the synthesis of the pyrazolo[4,3-dipyr imidine-7-amine derivatives 4, 13 and 14**

To a suspension of the methoxy-substituted pyrazolopyrimidine derivatives 2, 11 and 12 (1.02 mmol) in anhydrous CH₂Cl₂ (20 mL), a 1 M BBr₃ solution (2.60 mL for 2, 11 and 5.2 mL for 12) in CH₂Cl₂ was added at 0°C, under nitrogen atmosphere. The mixture was stirred at room temperature for 20–24 h (compounds 4, 13 or 16h (compound 14)), then was diluted with water (10 mL) and neutralized with NaHCO₃ saturated solution. The organic solvent was removed under reduced pressure and the obtained precipitate was collected by filtration and recrystallized. The crude derivative 4 was first purified by column chromatography (eluent CHCl₃/MeOH 9:1) and then recrystallized.

7-Amino-5-(4-hydroxyphenyl)amino-2-phenyl-2H-pyrazolo[4,3-dipyr imidine 12 Purified by preparative TLC (cyclohexane/EtOAc/MeOH 6:4:1). Yield 45%; m.p. 100–102°C (H₂O/MeOH). ¹H NMR (DMSO-d₆) 2.79 (t, 2H, CH₂, J = 7.2 Hz), 3.43–3.48 (m, 2H, CH₂), 3.72 (s, 3H, OCH₃), 3.74 (s, 3H, OCH₃), 6.10 (br s, 1H, NH), 6.75 (d, 1H, ar, J = 6.5 Hz), 6.84–6.87 (m, 2H, ar), 7.27 (br s, 2H, NH₂), 7.30 (t, 1H, ar, J = 7.2 Hz), 7.56 (2d, 2H, ar, J = 7.8 Hz), 7.97 (d, 2H, ar, J = 7.8 Hz), 8.51 (s, 1H, H-3). Anal. Calc. for C₁₉H₁₈N₂O₂.

7-Amino-5-(3,4-dihydroxyphenyl)amino-2-phenyl-2H-pyrazolo[4,3-dipyr imidine 13 Yield 89%; m.p. 241–244°C (EtOH/EtOAc). ¹H NMR (DMSO-d₆) 2.73 (t, 2H, CH₂, J = 7.3 Hz), 3.38–3.40 (m, 2H, CH₂), 6.03 (br s, 1H, NH), 6.68 (2d, 2H, ar, J = 8.3 Hz), 7.04 (d, 2H, ar, J = 8.3 Hz), 7.22 (br s, 2H, NH₂), 7.41 (t, 1H, ar, J = 7.4 Hz), 7.56 (t, 2H, ar, J = 7.7 Hz), 7.97 (d, 2H, ar, J = 7.8 Hz), 8.51 (s, 1H, H-3), 9.14 (s, 1H, OH). Anal. Calc. for C₁₉H₁₈N₂O₂.

7-Amino-5-(3,5-dihydroxyphenyl)amino-2-phenyl-2H-pyrazolo[4,3-dipyr imidine 14 Yield 50%; m.p. 242–243°C (EtOH). ¹H NMR (DMSO-d₆) 2.65 (t, 2H, CH₂, J = 7.1 Hz), 3.37–3.41 (m, 2H, CH₂), 6.03 (t, 1H, NH, J = 5.8 Hz), 6.59–6.47 (d, 1H, ar, J = 8.0 Hz), 6.63–6.66 (m, 2H, ar), 7.23 (br s, 2H, NH₂), 7.40 (t, 1H, ar, J = 7.4 Hz), 7.56 (t, 2H, ar, J = 7.6 Hz), 7.97 (d, 2H, ar, J = 7.7 Hz), 8.51 (s, 1H, H-3), 8.62 (br s, 1H, OH), 8.74 (br s, 1H, OH). Anal. Calc. for C₁₉H₁₈N₂O₂.

**Synthesis of 5,7-dichloro-2-phenyl-2H-pyrazolo[4,3-dipyr imidine 55**

A suspension of the pyrazolopyrimidine-5,7-dione derivative 54 (2 mmol) and N,N-dimethylaniline (3.95 mmol) in phosphorus...
The excess of phosphorus oxychloride was distilled off under reduced pressure and the residue was treated with water (about 5 – 10 mL). The crude product was collected by filtration and recrystallized. Yield 96%; m.p. 252–254 °C (EtOH). 1H NMR (DMSO-d6) 7.62 (t, 1H, ar, J = 9.1 Hz), 7.69 (t, 2H, ar, J = 9.3 Hz), 8.17 (d, 2H, ar, J = 9.1 Hz), 9.69 (s, 1H, H-3). Anal. Calc. for C11H6N4Cl2.

Synthesis of 7-amino-5-chloro-2-phenyl-2H-pyrazolo[4,3-d]pyrimidine 16
A mixture of the 5-chloro-pyrazolopyrimidine derivative 15 (0.41 mmol), the suitable N-substituted piperazine (0.82 mmol) and ethyldiisopropylamine (0.49 mmol) in N-methylpyrrolidone (2 mL) was heated by microwave irradiation in the presence of lithium aluminium hydride (1 mmol) in anhydrous THF (5 mL) at 0 °C. The suspension was stirred for 16 h at room temperature, then treated with water (10 mL). The organic phase was anhydrified and the solvent was removed at reduced pressure to give a solid which was purified by chromatography (column or preparative TLC, as reported in the procedures). The solid which precipitated was collected by filtration, purified by chromatography (column chromatography: eluent cyclohexane/EtOAc 7:3). Yield 53%; m.p. 203–205 °C. 1H NMR (DMSO-d6) 2.47 (br s, 4H, piperazine protons), 3.57 (2H, CH2), 3.70 (br s, 4H, piperazine protons), 7.24 (t, 2H, ar, J = 6.2 Hz), 7.41–7.60 (m, 7H, ar, H-1, H-3, H-2), 7.87 (m, 1H, furan proton), 7.99 (d, 2H, ar, J = 7.6 Hz), 8.60 (s, 1H, H-3). Anal. Calc. for C22H19N6O2.

Tert-butyl 4-(7-amino-2-phenyl-2H-pyrazolo[4,3-d]pyrimidin-5-yl)piperazine 1-carboxylate 21
The reaction mixture was microwave irradiated at 150 °C for 1 h and 30 min. Column chromatography: eluent ethyl acetate/MeOH 6:4. Yield 76%; m.p. 169–171 °C. 1H NMR (DMSO-d6) 1.43 (s, 9H, t-But), 3.34 (m, 4H, piperazine protons), 7.24 (t, 2H, ar, J = 7.3 Hz), 7.40–7.46 (m, 3H, 1 ar, +NH2), 7.57 (t, 2H, ar, J = 7.5 Hz), 8.00 (d, 2H, ar, J = 8.4 Hz), 8.59 (s, 1H, H-3). Anal. Calc. for C21H21N5O2.

Synthesis of 2-phenyl-5-(4-(methyl-2-furyl)piperazin-1-yl)-2H-pyrazolo[4,3-d]pyrimidin-7-amine 22
A solution of the 5-(4-(methyl-2-furyl)piperazin-1-yl) derivative 20 (1 mmol) in anhydrous THF (5 mL) was added to a suspension of LiAlH4 (3 mmol) in anhydrous THF (20 mL) at 0 °C. The suspension was stirred for 16 h at room temperature, then treated with water (10 mL) and the solid which precipitated was filtered off. The clear solution was diluted with water (about 30 mL) and extracted with ethyl acetate (3 x 20 mL). The organic phase was anhydrified and the solvent was removed at reduced pressure to give a solid which was purified by preparative TLC (elucent: cyclohexane/ethyl acetate 1:1). Yield 65%; m.p. 198–200 °C. 1H NMR (DMSO-d6) 2.40 (s, 6H, piperazine protons), 3.68 (m, 4H, piperazine protons), 6.80 (t, 1H, H-7), 7.00 (d, 2H, ar, J = 7.3 Hz), 7.24 (t, 2H, ar, J = 7.3 Hz), 7.40–7.46 (m, 3H, 1 ar, +NH2), 7.57 (t, 2H, ar, J = 7.5 Hz), 8.00 (d, 2H, ar, J = 8.4 Hz), 8.59 (s, 1H, H-3). Anal. Calc. for C22H21N7O2.
Synthesis of 1-(2,4,6-trifluorobenzyl)piperazinium trifluoroacetate 60

A solution of N-(Boc)piperazine 63 (2.06 mmol) and 2,4,6-trifluorobenzaldehyd (1.87 mmol) in anhydrous CH2Cl2 (20 mL) was stirred at room temperature for 1.5 h, then triacetoxy sodium borohydride (7.47 mmol) was added portion wise. The mixture was refluxed for 48 h, then treated with iced water (10 mL) and diluted with CH2Cl2 (15 mL). The aqueous phase was extracted with CH2Cl2 (10 mL × 3) and the organic phases were collected and anhydrified (Na2SO4). Evaporation of the solvent at reduced pressure gave the crude tert-butyl-4-(2,4,6-trifluorobenzyl)piperazinium-1-carboxylate which was purified by preparative TLC (elucent: CH2Cl2/acetonitrile/cyclohexane, 9:1:1) and obtained as a yellow oil. Yield 91%; 1H NMR (CDCl3) 1.46 (s, 9H, tert-But), 2.41 (br s, 2H, NH2) ppb. This derivative was then transformed into the title compound as follows. A solution of concentrated trifluoroacetic acid (2.5 mL) was stirred at room temperature for 1 h. Then, 3,3-dimethylbutiryl chloride (1.17 mmol) or phe- nalacetyl chloride (1.17 mmol) was added and the solution was stirred at room temperature for 3 h, then the solvent and the excess of the acid were removed at reduced pressure. The residue was treated with Et2O (5 mL) to give a solid which was collected by filtration and dried. The crude compound was used for the next step without further purification. Yield 67%; 1H NMR (CDCl3) 2.29–3.31 (m, 4H, piperazine protons), 3.49–3.52 (m, 4H, piperazine protons), 4.12 (s, 2H, CH2), 6.73 (t, ar, J = 7.8 Hz).

Synthesis of 4-(7-amino-2-phenyl-2H-pyrazolo[4,3-d]pyrimidin-5-yl)piperazinium-1-ium trifluoroacetate 64

The title compound was obtained by treatment of compound 21 (1.04 mmol) with trifluoroacetic acid, in the conditions described previously to prepare compound 60 from the corresponding N-Boc-derivative. The crude compound was used directly for the next step without purification. Yield 68%; 1H NMR (DMSO-d6) 2.29–3.29 (m, 4H, piperazine protons), 3.98–4.02 (m, 4H, piperazine protons), 7.51 (t, 1H, ar, J = 7.3 Hz), 7.63 (t, 2H, ar, J = 7.8 Hz), 8.03 (d, 2H, ar, J = 8.2 Hz), 8.69 (s, 1H, H-3), 9.25 (br s, 2H, NH2)ppb.

General procedure for the synthesis of S-(4-acylpiperazin-1-yl)-substituted-2H-pyrazolo[4,3-d]pyrimidin-7-amines 23 and 24

A mixture of derivative 64 (0.98 mmol) and triethylamine (1.96 mmol) in anhydrous THF (20 mL) was stirred at room temperature for 1 h. Then, 3,3-dimethylbutiryl chloride (1.17 mmol) or phenylacetyl chloride (1.17 mmol) was added and the solution was stirred at room temperature for 5 h or 3 h, respectively. The mixture was diluted with water (15 mL) and extracted with EtOAc (20 × 3 mL). The organic phase was anhydrified (Na2SO4) and the solvent evaporated at reduced pressure to give a solid which was taken up with cyclohexane and EtOAc, collected by filtration and purified by column chromatography (elucent CHCl3/MeOH 10:0.5 for compound 23, MeOH for derivative 24).

7-Amino-2-phenyl-5-(4-(3,3-dimethylbutiryl)piperazin-1-yl)-2H-pyra- zolo[4,3-d]pyrimidine 23 Yield 32%; m.p. 178–180 °C. 1H NMR (DMSO-d6) 1.02 (s, 9H, t-But), 2.29 (s, 2H, CH2), 3.58–3.60 (m, 4H, piperazine protons), 3.69–3.72 (m, 4H, piperazine protons), 7.42–7.46 (m, 3H, 1 ar + NH2), 7.59 (t, 2H, ar, J = 7.4 Hz), 8.00 (d, 2H, ar, J = 7.9 Hz), 8.60 (s, 1H, H-3). Anal. Calc. for C21H27N7O.

7-Amino-2-phenyl-5-(4-phenylacetylpiperazin-1-yl)-2H-pyrazolo[4,3-d]pyrimidine 24 Yield 69%; m.p. 207–209 °C (CH3NO3). 1H NMR (DMSO-d6) 3.52–3.57 (m, 4H, piperazine protons), 3.60–3.66 (m, 4H, piperazine protons), 3.77 (s, 2H, CH2), 7.21–7.34 (m, 5H, ar), 7.42–7.50 (m, 3H, 1 ar + NH2), 7.57 (t, 2H, ar, J = 7.9 Hz), 7.98 (d, 2H, ar, J = 8.2 Hz), 8.59 (s, 1H, H-3). Anal. Calc. for C23H23N7O.

Molecular modeling studies

Software overview

MOE suite (Molecular Operating Environment, version 2015.1001) was used to perform most general molecular modeling operations. Docking simulations were performed using the GOLD (Genetic Optimization for Ligand Docking, version 5.2) suite45. Quantum mechanical calculation of PM3 charges was carried out with the software MOPAC41 as implemented in the MOE suite.

Analyses of docking poses in terms of energy calculation and visual inspection were executed taking advantage of the MOE suite.

Molecular modeling studies have been performed on a 8 CPU (Intel® Xeon® CPU E5-1620 3.70 GHz) linux workstation.

Three-dimensional structures of adenosine receptors

Among all the available crystallographic structures of hA2A AR co-crystallized with a ligand in the orthosteric binding site, we opted for a complex with the antagonist ZM-241385 because of the structural similarity of its (1,2,4)triazolo[1,5,a][1,3,5]triazin-5,7-ylidiamine scaffold with the (pyrazolo[4,3-d]pyrimidin-5,7-yl)idiamine scaffold of the compounds under investigation. The crystallo- graphic structure identified with 4EIY PDB code42 was selected among all the structures co-crystallized with ZM-241385, because of its highest resolution (1.80 Å).

Since to date there are no crystallographic structures available for hA2A and hA3 ARs, we retrieved from the Adenosinland web-platform43,44 previously developed by our research group, their homology models constructed using 4EIY structure as template. Those models were constructed in the presence of ZM-241385 as environment for induced fit, so the resulting structures consist in complexes between each AR subtype and the antagonist ZM-241385.

The residues are identified according to the generic Ballesteros Weinstein numbering system45.

Molecular docking

Three-dimensional structures of ligands were built taking advantage of the MOE-Builder tool and ionization states were predicted using the MOE-Protonate-3D tool46. Ligand structures were subjected to MMFF94* energy minimization until the root mean square (rms) gradient fell below 0.05 kcal mol−1 Å−1. GOLD docking tool47 was selected as conformational search program and GoldScore as scoring function, thanks to a docking benchmark study previously carried out in our laboratory38,47. For each compound, 10 docking runs were performed on each receptor subtype, searching in a sphere of 20 Å radius centered on the coordinates of the center of mass of ZM-241385 in complex with the receptor. Along with the compounds under investigation, docking simulations were conducted also for ZM-241385 as a reference example.

After computing atomic partial charges both of ligand poses, using PM3/ESP method, and receptors, using Amber10EHT force field, electrostatic and van der Waals contributions to the binding energy were calculated with MOE.
Interaction energy fingerprints (IEFs)

Individual electrostatic and hydrophobic interactions, hereinafter identified as IEele and IEhyd, respectively, were computed between ligand poses and each protein residue involved in binding. Both these contributions were computed using MOE and, in particular, IEele were calculated as non-bonded electrostatic interactions energy term of the force field, so they are expressed in kcal/mol. Instead, IEhyd were computed as contact hydrophobic surfaces and are associated to an adimensional score (the higher the better). The data obtained by this analysis were reported in a graphic, called Interaction Energy Fingerprints (IEFs), representing residues (x-axis) in the form of equally high rectangles rendered according to a colorimetric scale. As regards IEele, colors from blue to red represent energy values ranging from negative to positive values; for IEhyd, colors from white to dark green depict scores going from 0 to positive values. More precisely, we retrieved the coordinates of the center of mass of ZM-241385 in the structure of each AR subtype complex. Only residues within 10 Å from this point were retained as belonging to the binding site, and plotted in the IEFs.

Interaction Energy Fingerprints comparison (IEFs comparison)

A new method has been introduced to evaluate docking results, which rests on the observation that ligands able to bind the same site of a protein often share a similar interaction pattern, too. The new method consists in the comparison of the IEFs of the pose of a candidate ligand (herein after called “docked”) with the IEFs of a ligand whose bound conformation is considered known (herein after called “reference”).

A quantitative estimation of the similarity of IEFs is computed as root mean square deviation (RMSD) between per residue interaction energies of the docked and the reference poses, both for electrostatic and hydrophobic interactions. This would inform about the average divergence of the docked from the reference: in particular a high RMSD value corresponds to large differences.

So far, there is no information about the direction of the divergence thus, along with RMSD, another analysis, named RMSDtrend, has been proposed. This consists of the sum of differences between per residue interaction energies of the docked and the reference, weighted by the number of residues of the binding site. A more favorable interaction energy profile would correspond to a negative RMSDtrend in the case of electrostatic and hydrophobic interactions, while, for hA1 and hA3 ARs, we used the homology models, that are receptor-ZM-241385 complexes, since they were constructed considering ZM-241385 as environment for induced fit.

An additional graph was added, which allows to compare electrostatic and hydrophobic IEFs RMSDs and RMSDtrend for different ligands on the different AR subtypes. RMSD and RMSDtrend for the ligands (y-axis) on the various receptors (x-axis) were reported on a heat map, where they are represented by a colorimetric scale going from red to blue from unfavorable to favorable values. Finally, if a ligand presents blue rectangles on all receptors, it is expected to be “non-selective”, otherwise red and blue rectangles should describe lower and higher stability values, respectively, among the different receptor subtypes.

MMsDocking video maker

To facilitate the visualization and analysis of data obtained from the docking simulations, we have implemented a in-house tool, named MMsDocking video maker, for the automated production of a video that shows the most relevant docking data, such as docking poses, per residue IEhyd and IEele data, experimental binding data and scoring values. Videos were mounted using MEncoder 48 starting from images obtained with the following procedure: the heat maps in the background were drawn with GNUPLot 4.6 49 starting from per residue IEhyd and IEele data computed with MOE. 2d depictions of compounds were generated using the open-source cheminformatics toolkit RDKit 50. Representations of docking poses within the binding site were constructed using CHIMERA 51.

Pharmacological assays

Human cloned A1, A2A and A3 AR binding assay

All synthesized compounds were tested to evaluate their affinity at human A1, A2A and A3 ARs. Displacement experiments of [1H]DPCPX (1 nM) to hA1 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. Non-specific binding was determined in the presence of 1 μM of DPCPX (≤10% of the total binding). Binding of [1H]ZM-241385 (1 nM) to hA2A CHO membranes (50 μg of protein/assay) was performed by using 50 mM Tris-HCl buffer, 10 mM MgCl2 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 hA3 CHO membranes (50 μg of protein/assay) were performed incubating 0.5 nM [125I]-AB-MECA, 50 mM Tris-HCl buffer, 10 mM MgCl2, 1 mM EDTA, pH 7.4 and at least 6–8 different concentrations of examined ligands for 60 min at 37 °C 52. 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%.

Measurement of cyclic AMP levels in CHO cells transfected with hA2A AR

CHO cells transfected with hA2A AR subtypes were washed with phosphate-buffered saline, diluted trypsin and centrifuged for 10 min at 200 g. The cells (1 × 10⁶ cells/assay) were suspended in 0.5 ml of incubation mixture (mM): NaCl 15, KCl 0.27, Na₂HPO₄
Data analysis

The protein concentration was determined according to a Bio-Rad method53 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 \(K_d\) is its dissociation constant55. A weighted non-linear least-squares curve fitting program LIGAND56 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).

Disclosure statement

The authors report no declarations of interest.

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