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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 $\mathbf{A}_{2 \mathrm{~A}}$ adenosine receptors 

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Key words: $G$ protein-coupled receptors, $\mathrm{A}_{1}$ and $\mathrm{A}_{2 \mathrm{~A}}$ adenosine receptor antagonists, pyrazolo[4,3$d$ ]pyrimidines, ligand-adenosine receptor modeling studies.

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[^1]
#### 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) $\mathrm{A}_{2 \mathrm{~A}}$ adenosine receptor ( AR ) or both $\mathrm{hA}_{2 \mathrm{~A}}$ and $\mathrm{hA}_{1}$ 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 $\mathrm{hA}_{2 \mathrm{~A}} \mathrm{AR}\left(\mathrm{K}_{\mathrm{i}}=3.62-57 \mathrm{nM}\right)$ and slightly lower for the $\mathrm{hA}_{1}$ ARs, thus showing different degrees (3-22 fold) of $\mathrm{hA}_{2 \mathrm{~A}}$ versus $\mathrm{hA}_{1}$ selectivity. In particular, the 2-(2-methoxybenzyl)-5-(5-methylfuran-2-yl) derivative $\mathbf{2 5}$ possessed the highest $\mathrm{hA}_{2 \mathrm{~A}}$ and $\mathrm{hA}_{1} \mathrm{AR}$ affinities $\left(\mathrm{K}_{\mathrm{i}}=3.62\right.$ 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 $\mathrm{hA}_{2 \mathrm{~A}} \mathrm{AR}\left(\mathrm{K}_{\mathrm{i}}=5.26\right.$ nM ) and was 22 -fold selective versus the $\mathrm{hA}_{1}$ subtype. Molecular docking investigations performed at the $\mathrm{hA}_{2 \mathrm{~A}}$ AR crystal structure and at a homology model of the $\mathrm{hA}_{1} \mathrm{AR}$ 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-proteincoupled receptors, classified as $\mathrm{A}_{1}, \mathrm{~A}_{2 \mathrm{~A}}, \mathrm{~A}_{2 \mathrm{~B}}$ and $\mathrm{A}_{3}$ subtypes. ${ }^{1,2}$ Adenosine receptors (ARs) are typically coupled to adenylyl cyclase which can be inhibited $\left(\mathrm{A}_{1}\right.$ and $\left.\mathrm{A}_{3}\right)$ or activated $\left(\mathrm{A}_{2 \mathrm{~A}}\right.$ and $\mathrm{A}_{2 \mathrm{~B}}$ ), but other intracellular pathways can be modulated, depending on the cell type and on the contingent situation.
$\mathrm{A}_{1}$ and $\mathrm{A}_{2 \mathrm{~A}}$ ARs are coupled to mitogen-activated protein kinases (MAPK), K-ATP channel and phospholipase C. $\mathrm{A}_{1}$ 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. ${ }^{3-4} \mathrm{~A}_{1} \mathrm{AR}$ is expressed with intermediate density in peripheral organs such as heart and kidney. ${ }^{1,2}$ Coherently, $\mathrm{A}_{1}$ 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. $\mathrm{A}_{2 \mathrm{~A}}$ 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. $\mathrm{A}_{2 \mathrm{~A}} \mathrm{AR}$ 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 $\mathrm{A}_{2 \mathrm{~A}} \mathrm{AR}$ 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 $\mathrm{A}_{1} / \mathrm{A}_{2 \mathrm{~A}}$ antagonists because they reduce both motor $\left(\mathrm{A}_{2 \mathrm{~A}}\right)$ and cognitive $\left(\mathrm{A}_{1}\right)$ deficits associated to the disease. ${ }^{5,13-15}$

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

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.


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 $\left(\mathrm{R}_{5}\right)$ of the pyrazolo[4,3- $d$ ]pyrimidine ( PP ) scaffold (Figure 1, compounds $\mathbf{A}$ and $\mathbf{B}$ respectively) affords good affinity for $\mathrm{hA}_{1}, \mathrm{hA}_{2 \mathrm{~A}}$ and $\mathrm{hA}_{2 \mathrm{~B}}$ ARs, ${ }^{25}$ whereas a benzyl and, even better, a 3-phenylpropyl chain (Figure 1, derivatives $\mathbf{C}$ and $\mathbf{D}$, respectively) elicit an enhancement of the $\mathrm{A}_{2 \mathrm{~A}}$ 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 $\mathrm{A}_{1} \mathrm{AR}$ antagonists. ${ }^{26}$ Hence, we decided to further investigate this series with the aim of obtaining more potent antagonists for the $\mathrm{hA}_{2 \mathrm{~A}} \mathrm{AR}$ or balanced antagonists for $\mathrm{hA}_{1}$ and $\mathrm{hA}_{2 \mathrm{~A}}$ 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.


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

## 2 Chemistry

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

Allowing $\mathrm{N}, \mathrm{N}$-dimethyl-2-nitroetheneamine ${ }^{28}$ to react with the suitable $\mathrm{N}_{1}$-arylhydrazono- $\mathrm{N}_{2}$ chloroacetates 32-33 ${ }^{29-30}$ and $\mathbf{3 4}$ in chloroform, the ethyl 4-nitropyrazole-3-carboxylates $\mathbf{3 5},{ }^{27} \mathbf{3 6}$ and 37 were obtained. These compounds were transformed into the corresponding amides $\mathbf{3 8},{ }^{27} 39$ and $\mathbf{4 0}$ 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 $\mathbf{4 1},{ }^{27} \mathbf{4 2}$ and 43 which were reduced with cyclohexene or hydrogen, in the presence of $\mathrm{Pd} / \mathrm{C}$, to provide the corresponding 4 -amino derivatives $\mathbf{4 4},{ }^{27} \mathbf{4 5}$ 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 $\mathbf{1 - 3}, \mathbf{5 , 7 , 8}, \mathbf{1 0}$, 11 and 13. The 2-(2-methoxyphenyl)-derivatives $\mathbf{3}, 5,8,11$ and 13 were demethylated with boron tribromide to obtain the corresponding 2-(2-hydroxyphenyl)-derivatives $\mathbf{4 , 6 , 9 , 1 2 , 1 4}$.




|  | R5 | R |  | R5 | R |
| :---: | :---: | :---: | :---: | :---: | :---: |
| 1 | Me | F | 8 | 2-furyl | OMe |
| 2 | $\left(\mathrm{CH}_{2}\right)_{3} \mathrm{Ph}$ | F | 9 | 2-furyl | OH |
| 3 | $\left(\mathrm{CH}_{2}\right)_{3} \mathrm{Ph}$ | OMe | 10 | 2-(5-methylfuryl) | H |
| 4 | $\left(\mathrm{CH}_{2}\right)_{3} \mathrm{Ph}$ | OH | 11 | 2-(5-methylfuryl) | OMe |
| 5 | $\mathrm{CH}_{2} \mathrm{Ph}$ | OMe | 12 | 2-(5-methylfuryl) | OH |
| 6 | $\mathrm{CH}_{2} \mathrm{Ph}$ | OH | 13 | 2-thienyl | OMe |
| 7 | 2-furyl | H | 14 | 2-thienyl | OH |

Scheme 1. (a) $\mathrm{NEt}_{3}, \mathrm{CHCl}_{3,} \mathrm{mw}, 140^{\circ} \mathrm{C}$; (b) $33 \%$ aqueous $\mathrm{NH}_{3}$, r.t.; (c) $\mathrm{POCl}_{3}, \mathrm{mw}, 150{ }^{\circ} \mathrm{C}$; (d) cyclohexene, $\mathrm{Pd} / \mathrm{C}, \mathrm{mw}, 110-130{ }^{\circ} \mathrm{C}$; (e) $\mathrm{Me}-\mathrm{C}(\mathrm{OEt})_{3}$ or $\mathrm{R}_{5}-\mathrm{C}(\mathrm{OEt}) \mathrm{NH}$ hydrochloride, $\mathrm{NH}_{4} \mathrm{OAc}$, sealed tube or mw, $90-150^{\circ} \mathrm{C}$; (f) $\mathrm{R}=\mathrm{OMe}, \mathrm{BBr}_{3}, \mathrm{CH}_{2} \mathrm{Cl}_{2}$, reflux.

The pyrazolopyrimidines $\mathbf{1 5} \mathbf{- 2 3}$, 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 $\mathbf{5 0}$ as the major isomer, along with the 1-phenethyl-3-nitropyrazole-5carbonitrile 50a (the molar ratio between the two isomers $\mathbf{5 0}$ and $\mathbf{5 0 a}$ was about $6: 1$ from the ${ }^{1} \mathrm{H}$ NMR spectrum of the crude material). The structure of compound $\mathbf{5 0}$ was determined by means of NOESY experiments, which showed a spatial closeness of the pyrazole hydrogen atom to methylene protons.


$48 \mathrm{R}_{2}=\mathrm{Me}$
$49 \mathrm{R}_{2}=\mathrm{CH}_{2} \mathrm{Ph}$

$51 \mathrm{R}_{2}=\mathrm{Me}$
$52 \mathrm{R}_{2}=\mathrm{CH}_{2} \mathrm{Ph}$

53
$\downarrow d$

$e\left\{\begin{array}{l}15-22 \\ 23\end{array}\right.$

|  | $\mathrm{R}_{5}$ | $\mathrm{R}_{2}$ |
| :--- | :--- | :--- |
| $\mathbf{1 5}$ | $\mathrm{CH}_{2} \mathrm{Ph}$ | Me |
| $\mathbf{1 6}$ | $\left(\mathrm{CH}_{2}\right)_{3} \mathrm{Ph}$ | Me |
| $\mathbf{1 7}$ | Me | $\mathrm{CH}_{2} \mathrm{Ph}$ |
| $\mathbf{1 8}$ | Me | $\left(\mathrm{CH}_{2}\right)_{2} \mathrm{Ph}$ |
| $\mathbf{1 9}$ | $\mathrm{CH}_{2} \mathrm{Ph}$ | $\mathrm{CH}_{2} \mathrm{Ph}$ |
| $\mathbf{2 0}$ | 2-furyl | $\mathrm{CH}_{2} \mathrm{Ph}$ |
| $\mathbf{2 1}$ | 2-furyl | $\left(\mathrm{CH}_{2}\right)_{2} \mathrm{Ph}$ |
| $\mathbf{2 2}$ | 2-furyl | $\mathrm{CH}_{2} \mathrm{C}_{6} \mathrm{H}_{4}-2$-OMe |
| $\mathbf{2 3}$ | 2-furyl | $\mathrm{CH}_{2} \mathrm{C}_{6} \mathrm{H}_{4}-2$-OH |

Scheme 2. (a) $\mathrm{CH}_{3} \mathrm{I}$ or $\mathrm{Ph}-\left(\mathrm{CH}_{2}\right)_{\mathrm{n}} \mathrm{Br}, \mathrm{NaH}$, THF, room temperature $\left(\mathbf{4 8}, 49\right.$ ) or reflux (50); (b) $\mathrm{H}_{2}$, $\mathrm{Pd} / \mathrm{C}$, Parr apparatus, 30 psi ; (c) cyclohexene, $\mathrm{Pd} / \mathrm{C}$, mw, $150{ }^{\circ} \mathrm{C}$; (d) $\mathrm{Me}-\mathrm{C}(\mathrm{OEt})_{3}$ or $\mathrm{R}_{5}-\mathrm{C}(\mathrm{OEt}) \mathrm{NH}$ hydrochloride, $\mathrm{NH}_{4} \mathrm{OAc}, \mathrm{mw}$ at $130^{\circ} \mathrm{C}$ or, for 22, sealed tube at $120^{\circ} \mathrm{C}$; (e) 22, $\mathrm{BBr}_{3}, \mathrm{CH}_{2} \mathrm{Cl}_{2}$, reflux.

The 4-nitropyrazole derivatives $\mathbf{4 8 - 5 0}$ were transformed into the corresponding 4-aminopyrazoles by reduction with hydrogen $(\mathbf{5 1})^{27}$ or cyclohexene ( $\mathbf{5 2}^{27}$ and $\mathbf{5 3}$ ), in the presence of $\mathrm{Pd} / \mathrm{C}$. 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 5position, a new synthetic pathway was developed (Scheme 3).


|  | R |
| :--- | :--- |
| $\mathbf{2 4}$ | H |
| $\mathbf{2 5}$ | $2-\mathrm{OMe}$ |
| $\mathbf{2 6}$ | $3-\mathrm{OMe}$ |
| $\mathbf{2 7}$ | $2-\mathrm{F}$ |
| $\mathbf{2 8}$ | $3-\mathrm{F}$ |
| $\mathbf{2 9}$ | $2-\mathrm{Cl}$ |
| $\mathbf{3 0}$ | $2-\mathrm{OH}$ |
| $\mathbf{3 1}$ | $3-\mathrm{OH}$ |



Scheme 3. (a) $\mathrm{H}_{2}, \mathrm{Pd} / \mathrm{C}$, Parr apparatus, 35 psi ; (b) $\mathrm{NH}_{4} \mathrm{OAc}$, sealed tube, $120^{\circ} \mathrm{C}$; (c) $\mathrm{Ar}-\mathrm{CH}_{2}-\mathrm{Cl}$, $\mathrm{K}_{2} \mathrm{CO}_{3}, \mathrm{CH}_{3} \mathrm{CN} / \mathrm{DMF}$, r.t. or $60^{\circ} \mathrm{C}$; (d) $\mathbf{2 5}$ or $\mathbf{2 6}, \mathrm{BBr}_{3}, \mathrm{CH}_{2} \mathrm{Cl}_{2}$, r.t. or reflux.

The 4-aminopyrazole-3-carbonitrile 54, ${ }^{35}$ obtained from the corresponding 4-nitro derivative $\mathbf{4 7}$, 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 $\mathbf{2 5}$ and 26 were demethylated with boron tribromide to give the respective hydroxy-substituted compounds $\mathbf{3 0}$ and $\mathbf{3 1}$.

## 3. Results and Discussion

### 3.1. Structure-affinity relationship studies

The synthesized compounds $\mathbf{1 - 3 1}$ were tested in binding assays to evaluate their affinity at cloned $\mathrm{hA}_{1}, \mathrm{hA}_{2 \mathrm{~A}}$ and $\mathrm{hA}_{3} \mathrm{ARs}$, stably expressed in CHO cells. The new compounds were also tested at the $\mathrm{hA}_{2 \mathrm{~B}}$ 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 $\mathrm{hA}_{2 \mathrm{~A}}$ receptor or both $\mathrm{hA}_{1}$ and $\mathrm{hA}_{2 \mathrm{~A}}$ receptors has been achieved. The most interesting findings were found within the set of compounds $\mathbf{2 0 - 3 1}$ 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 $\mathrm{hA}_{2 \mathrm{~A}} \mathrm{AR}$ with a nanomolar affinity and different degrees of selectivity (3-22 fold) versus the $\mathrm{hA}_{1}$ ARs. Moreover, they showed good to moderate affinities for $\mathrm{A}_{3}$ and $\mathrm{hA}_{2 \mathrm{~B}}$ 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 ( $\mathrm{F}, \mathrm{OMe}, \mathrm{OH}$ ) on the ortho position of the 2-phenyl ring. This choice was based on the results of docking studies at the $\mathrm{hA}_{2 \mathrm{~A}}$ 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 $\mathrm{NH}_{2}$ amide moiety of Asn253 (TM6) and the N1pyrazole 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 $\mathrm{hA}_{2 \mathrm{~A}}$ receptor (compare $\mathbf{1}$ to $\mathbf{A ; 2}$ and $\mathbf{3}$ to C; $\mathbf{5}$ and $\mathbf{6}$ to $\mathbf{D})$ and for the other hAR subtypes.

Table 1. Binding Affinity $\left(\mathrm{K}_{\mathrm{i}}\right)$ at $\mathrm{hA}_{1}, \mathrm{hA}_{2 \mathrm{~A}}$ and $\mathrm{hA}_{3}$ ARs and potencies $\left(\mathrm{IC}_{50}\right)$ at $\mathrm{hA}_{2 \mathrm{~B}}$ ARs.


A-D, 1-31

|  | $\mathrm{R}_{5}$ | $\mathrm{R}_{2}$ | $\begin{gathered} \text { Binding experiments }^{\mathrm{a}} \\ K_{i}(\mathbf{n M}) \text { or } I \% \end{gathered}$ |  |  | $\begin{aligned} & \text { cAMP assays } \\ & \mathrm{IC}_{50}(\mathrm{nM}) \text { or } \\ & \mathrm{I} \% \end{aligned}$ |
| :---: | :---: | :---: | :---: | :---: | :---: | :---: |
|  |  |  | $\mathrm{hA}_{1}{ }^{\text {b }}$ | $\mathrm{hA}_{2 \mathrm{~A}}{ }^{\text {c }}$ | $\mathrm{hA}_{3}{ }^{\text {d }}$ | $\mathrm{hA}_{2 \mathrm{~B}}{ }^{\text {e }}$ |
| $\boldsymbol{A}^{f}$ | Me | Ph | $70 \pm 6$ | $246 \pm 23$ | 40\% | $320 \pm 35$ |
| $\boldsymbol{B}^{f}$ | Ph | Ph | $75 \pm 7$ | $325 \pm 34$ | 48\% | $440 \pm 43$ |
| $C^{f}$ | $\mathrm{PhCH}_{2}$ | Ph | $150 \pm 12$ | $110 \pm 10$ | 39\% | $420 \pm 38$ |
| $D^{g}$ | $\mathrm{Ph}\left(\mathrm{CH}_{2}\right)_{3}$ | Ph | $5.3 \pm 04$ | $55 \pm 5$ | 12\% | 42\% |
| 1 | Me | $\mathrm{C}_{6} \mathrm{H}_{4}-2-\mathrm{F}$ | $410 \pm 42$ | 23\% | 8\% | 8\% |
| 2 | $\mathrm{PhCH}_{2}$ | $\mathrm{C}_{6} \mathrm{H}_{4}-2-\mathrm{OMe}$ | 25\% | 43\% | $163 \pm 15$ | 1\% |
| 3 | $\mathrm{PhCH}_{2}$ | $\mathrm{C}_{6} \mathrm{H}_{4}-2-\mathrm{OH}$ | $49 \pm 4$ | $647 \pm 62$ | 6\% | 21\% |
| 4 | $\mathrm{Ph}-\left(\mathrm{CH}_{2}\right)_{3}$ | $\mathrm{C}_{6} \mathrm{H}_{4}-2-\mathrm{F}$ | $17 \pm 2$ | $240 \pm 22$ | 5\% | 5\% |
| 5 | $\mathrm{Ph}-\left(\mathrm{CH}_{2}\right)_{3}$ | $\mathrm{C}_{6} \mathrm{H}_{4}-2-\mathrm{OMe}$ | $103 \pm 9$ | 34\% | $405 \pm 38$ | 1\% |
| 6 | $\mathrm{Ph}-\left(\mathrm{CH}_{2}\right)_{3}$ | $\mathrm{C}_{6} \mathrm{H}_{4}-2-\mathrm{OH}$ | $52 \pm 4$ | 21\% | 19\% | 1\% |
| 7 | 2-furyl | Ph | $206 \pm 17$ | $195 \pm 14$ | 39\% | 1\% |
| 8 | 2-furyl | $\mathrm{C}_{6} \mathrm{H}_{4}-2-\mathrm{OMe}$ | $31 \%$ | $362 \pm 34$ | 37\% | 12\% |
| 9 | 2-furyl | $\mathrm{C}_{6} \mathrm{H}_{4}-2-\mathrm{OH}$ | $372 \pm 33$ | $89 \pm 8$ | 36\% | 42\% |
| 10 | 2-(5-methylfuryl) | Ph | $42 \pm 3$ | $99 \pm 8$ | $147 \pm 12$ | 32\% |
| 11 | 2-(5-methylfuryl) | $\mathrm{C}_{6} \mathrm{H}_{4}-2-\mathrm{OMe}$ | 1\% | $518 \pm 47$ | $297 \pm 26$ | 1\% |
| 12 | 2-(5-methylfuryl) | $\mathrm{C}_{6} \mathrm{H}_{4}-2-\mathrm{OH}$ | 1\% | $147 \pm 13$ | $375 \pm 32$ | 1\% |
| 13 | 2-thienyl | $\mathrm{C}_{6} \mathrm{H}_{4}-2-\mathrm{OMe}$ | 24\% | 27\% | $150 \pm 13$ | 1\% |
| 14 | 2-thienyl | $\mathrm{C}_{6} \mathrm{H}_{4}-2-\mathrm{OH}$ | $159 \pm 16$ | $412 \pm 39$ | $209 \pm 18$ | 28\% |
| 15 | $\mathrm{PhCH}_{2}$ | Me | 9\% | 1\% | 1\% | 20\% |
| 16 | $\mathrm{Ph}\left(\mathrm{CH}_{2}\right)_{3}$ | Me | 36\% | 1\% | 30\% | $2 \%$ |
| 17 | Me | $\mathrm{CH}_{2} \mathrm{Ph}$ | 1\% | 1\% | 1\% | 1\% |


| $\mathbf{1 8}$ | Me | $\left(\mathrm{CH}_{2}\right)_{2} \mathrm{Ph}$ | $10 \%$ | $12 \%$ | $3 \%$ | $3 \%$ |
| :--- | :---: | :---: | :---: | :---: | :---: | :---: |
| $\mathbf{1 9}$ | $\mathrm{CH}_{2} \mathrm{Ph}$ | $\mathrm{CH}_{2} \mathrm{Ph}$ | $1 \%$ | $1 \%$ | $15 \%$ | $1 \%$ |
| $\mathbf{2 0}$ | 2-furyl | $\mathrm{CH}_{2} \mathrm{Ph}$ | $32 \%$ | $320 \pm 28$ | $2 \%$ | $37 \%$ |
| $\mathbf{2 1}$ | 2-furyl | $\left(\mathrm{CH}_{2}\right)_{2} \mathrm{Ph}$ | $12 \%$ | $33 \%$ | $4 \%$ | $3 \%$ |
| $\mathbf{2 2}$ | 2-furyl | $\mathrm{CH}_{2}-\mathrm{C}_{6} \mathrm{H}_{4}-2$-OMe | $98 \pm 8$ | $5.37 \pm 0.39$ | $196 \pm 17$ | $512 \pm 49$ |
| $\mathbf{2 3}$ | 2-furyl | $\mathrm{CH}_{2}-\mathrm{C}_{6} \mathrm{H}_{4}-2-\mathrm{OH}$ | $242 \pm 21$ | $17 \pm 2$ | $905 \pm 88$ | $112 \pm 11$ |
| $\mathbf{2 4}$ | 2-(5-methylfuryl) | $\mathrm{CH}_{2} \mathrm{Ph}$ | $136 \pm 12$ | $9.23 \pm 0.85$ | $269 \pm 25$ | $20 \%$ |
| $\mathbf{2 5}$ | 2-(5-methylfuryl) | $\mathrm{CH}_{2}-\mathrm{C}_{6} \mathrm{H}_{4}-2$-OMe | $18 \pm 2$ | $3.62 \pm 0.34$ | $82 \pm 7$ | $30 \%$ |
| $\mathbf{2 6}$ | 2-(5-methylfuryl) | $\mathrm{CH}_{2}-\mathrm{C}_{6} \mathrm{H}_{4}-2-\mathrm{OH}$ | $120 \pm 11$ | $5.26 \pm 0.47$ | $88 \pm 6$ | $293 \pm 26$ |
| $\mathbf{2 7}$ | 2-(5-methylfuryl) | $\mathrm{CH}_{2}-\mathrm{C}_{6} \mathrm{H}_{4}-3-\mathrm{OMe}$ | $309 \pm 8$ | $57 \pm 6$ | $498 \pm 47$ | $354 \pm 32$ |
| $\mathbf{2 8}$ | 2-(5-methylfuryl) | $\mathrm{CH}_{2}-\mathrm{C}_{6} \mathrm{H}_{4}-3-\mathrm{OH}$ | $252 \pm 21$ | $51 \pm 5$ | $427 \pm 41$ | $421 \pm 39$ |
| $\mathbf{2 9}$ | 2-(5-methylfuryl) | $\mathrm{CH}_{2}-\mathrm{C}_{6} \mathrm{H}_{4}-2-\mathrm{F}$ | $60 \pm 5$ | $6.21 \pm 0.58$ | $71 \pm 6$ | $152 \pm 14$ |
| $\mathbf{3 0}$ | 2-(5-methylfuryl) | $\mathrm{CH}_{2}-\mathrm{C}_{6} \mathrm{H}_{4}-3-\mathrm{F}$ | $111 \pm 10$ | $38 \pm 4$ | $122 \pm 11$ | $395 \pm 38$ |
| $\mathbf{3 1}$ | 2-(5-methylfuryl) | $\mathrm{CH}_{2}-\mathrm{C}_{6} \mathrm{H}_{4}-2-\mathrm{Cl}$ | $65 \pm 5$ | $14 \pm 2$ | $225 \pm 21$ | $633 \pm 52$ |

${ }^{a} \mathrm{~K}_{\mathrm{i}}$ values are means $\pm$ SEM of four separate assays each performed in duplicate. Percentage of inhibition (I\%) are determined at $1 \mu \mathrm{M}$ concentration of the tested compounds. ${ }^{\text {b }}$ Displacement of specific $\left[{ }^{3} \mathrm{H}\right]$ DPCPX competition binding assays to $\mathrm{hA}_{1} \mathrm{CHO}$ cells. ${ }^{c}$ Displacement of specific $\left[{ }^{3} \mathrm{H}\right]$ ZM241385 competition binding to $\mathrm{hA}_{2 \mathrm{~A}} \mathrm{CHO}$ cells. ${ }^{\mathrm{d}}$ Displacement of specific $\left[{ }^{125} \mathrm{I}\right] \mathrm{AB}-\mathrm{MECA}$ competition binding to $\mathrm{hA}_{3} \mathrm{CHO}$ cells. ${ }^{\mathrm{e}}$ cAMP experiments in $\mathrm{hA}_{2 \mathrm{~B}} \mathrm{CHO}$ cells, stimulated by 200 nM NECA. Percentage of inhibition ( $\mathrm{I} \%$ ) are determined at $1 \mu \mathrm{M}$ concentration of the tested compounds. ${ }^{\mathrm{f}}$ Ref. 25. ${ }^{\text {g }}$ Ref. 26.

Replacement of the 5-phenyl ring of $\mathbf{B}$ with a 2-furyl (compound 7) was more profitable because it achieved a shift of affinity, albeit small, towards the $\mathrm{A}_{2 \mathrm{~A}}$ subtype. In fact, with respect to $\mathbf{B}$, compound 7 bind slightly better the $\mathrm{hA}_{2 \mathrm{~A}}$ receptor $\left(\mathrm{K}_{\mathrm{i}}=195 \mathrm{nM}\right)$ and worse the $\mathrm{hA}_{1}$ one. Insertion of the 2-(5-methyl)furyl group at the 5-position further enhanced the $\mathrm{hA}_{2 \mathrm{~A}}$ affinity $\left(\mathrm{K}_{\mathrm{i}}=99 \mathrm{nM}\right)$ but not the selectivity, compound $\mathbf{1 0}$ showing high and good affinity, respectively, for the $\mathrm{hA}_{1}\left(\mathrm{~K}_{\mathrm{i}}=42\right.$ $\mathrm{nM})$ and $\mathrm{hA}_{3}(\mathrm{Ki}=147 \mathrm{nM})$ subtypes. In light of these findings, compounds $\mathbf{7}$ and $\mathbf{1 0}$ 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 $\mathrm{hA}_{2 \mathrm{~A}}$ AR and even more for the $\mathrm{hA}_{1}$ subtype. There was only one exception: the 2-(2-hydroxyphenyl) derivative $9\left(\mathrm{R}_{5}=2\right.$-furyl) which was two-fold more active than 7 at the $\mathrm{hA}_{2 \mathrm{~A}}$ AR subtype $\left(\mathrm{K}_{\mathrm{i}}=89 \mathrm{nM}\right)$. A selective $\mathrm{hA}_{3}$ 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 $\mathrm{hA}_{3} \mathrm{AR},{ }^{27}$ unlike the 2-furyl that shifts affinity towards the $\mathrm{hA}_{2 \mathrm{~A}} \mathrm{AR}$.

A dramatic reduction of affinity was obtained when the 2-phenyl ring of compounds $\mathbf{C}$ and $\mathbf{D}$ was replaced with the smaller and less lipophilic methyl group (derivatives $\mathbf{1 5}$ and 16) or when the 2phenyl 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 $\mathrm{hA}_{2 \mathrm{~A}}$ and $\mathrm{hA}_{1}$ 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 $\mathrm{hA}_{2 \mathrm{~A}} \mathrm{AR}$ selectivity, even if its $\mathrm{hA}_{2 \mathrm{~A}}$ AR affinity is slightly lower $\left(\mathrm{K}_{\mathrm{i}}=320 \mathrm{nM}\right)$. Elongation of the 2-benzyl chain of $\mathbf{2 0}$ to 2-phenethyl dropped $\mathrm{hA}_{2 \mathrm{~A}} \mathrm{AR}$ affinity (compound $\mathbf{2 1}$ ), 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 $\mathrm{hA}_{2 \mathrm{~A}}$ receptor with high affinity $\left(\mathrm{K}_{\mathrm{i}}=9.23 \mathrm{nM}\right)$ and some selectivity versus both $\mathrm{hA}_{1}$ (15-fold) and $\mathrm{hA}_{3}$ (29-fold) subtypes, thus confirming the favorable role of the 5-(5-methylfuran-2-yl)- substituent for the $\mathrm{hA}_{2 \mathrm{~A}}$ AR-ligand interaction (see above, $\mathbf{1 0}$ versus 7).

Based on these results, both compounds $\mathbf{2 0}$ and $\mathbf{2 4}$ were modified by introduction of a methoxy or hydroxy residue at the ortho position on the benzyl group. All the resulting pyrazolopyrimidines $\mathbf{2 2}$, $23\left(\mathrm{R}_{5}=2\right.$-furyl) and 25, $26\left(\mathrm{R}_{5}=5\right.$-methylfuran-2-yl) possessed very high $\mathrm{hA}_{2 \mathrm{~A}}$ AR affinities (3.6 $\mathrm{nM}<\mathrm{K}_{\mathrm{i}}<17 \mathrm{nM}$ ), being improved with respect to those of the corresponding 2-benzyl derivatives. Moreover, the 5-(methylfuran-2-yl)- derivative 26 exhibited the highest $\mathrm{hA}_{2 \mathrm{~A}}$ versus $\mathrm{hA}_{1} A R$ 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 $\mathbf{2 7}$ and $\mathbf{2 8}$,
whose $\mathrm{hA}_{2 \mathrm{~A}}$ affinities were still in the low nanomolar range ( $\mathrm{K}_{\mathrm{i}}=57$ and 51 nM , respectively), although decreased with respect to those of the ortho substituted derivatives $\mathbf{2 5}$ and $\mathbf{2 6}$. A similar trend of $\mathrm{hA}_{2 \mathrm{~A}}$ affinity was found for compounds $\mathbf{2 9}$ and $\mathbf{3 0}$, bearing the fluorine atom as hydrogen bond acceptor, respectively, at the ortho and meta position of the benzyl group. In fact, the orthofluorobenzyl derivative 29 displayed a higher $\mathrm{hA}_{2 \mathrm{~A}}$ affinity $\left(\mathrm{K}_{\mathrm{i}}=6.21 \mathrm{nM}\right)$ than the meta-isomer $\mathbf{3 0}$ $\left(\mathrm{K}_{\mathrm{i}}=38 \mathrm{nM}\right)$. Finally, insertion of a chlorine atom at the ortho position on the benzyl moiety also afforded high $\mathrm{hA}_{2 \mathrm{~A}}$ affinity $\left(\mathrm{K}_{\mathrm{i}}=14 \mathrm{nM}\right)$.

In summary, the structural investigation carried out at the 5- and 2- position of pyrazolopyrimidine scaffold highlighted that high affinity for the $\mathrm{hA}_{2 \mathrm{~A}} \mathrm{AR}$ or for both $\mathrm{hA}_{2 \mathrm{~A}}$ and $\mathrm{hA}_{1}$ 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 $\mathrm{hA}_{1}$ and $\mathrm{hA}_{2 \mathrm{~A}}$ ARs. Thus, their capability to reduce the inhibitory effect of 2 -chloro- $\mathrm{N}^{6}$ cyclopentyladenosine (CCPA) in cAMP production on $\mathrm{hA}_{1} \mathrm{CHO}$ cells and to inhibit NECAstimulated cAMP production on $\mathrm{hA}_{2 \mathrm{~A}} \mathrm{CHO}$ cells, were evaluated (Table 2).

Table 2. Antagonistic potencies of selected compounds at $\mathrm{hA}_{1}$ and $\mathrm{hA}_{2 \mathrm{~A}}$ ARs.

|  | cAMP assay $\mathrm{IC}_{50}(\mathrm{nM})^{\mathrm{a}}$ |  |
| :---: | :---: | :---: |
|  | $\mathrm{hA}_{1}$ | $\mathrm{hA}_{2 \mathrm{~A}}$ |
| $\mathbf{2 2}$ | $106 \pm 10$ | $6.21 \pm 0.57$ |
| $\mathbf{2 4}$ | $152 \pm 14$ | $10.91 \pm 1.07$ |
| $\mathbf{2 5}$ | $21 \pm 2$ | $4.35 \pm 0.42$ |
| $\mathbf{2 6}$ | $134 \pm 12$ | $6.42 \pm 0.61$ |

${ }^{\mathrm{a}}$ cAMP experiments: the compounds were tested in $\mathrm{hA}_{1} \mathrm{CHO}$ or $\mathrm{hA}_{2 \mathrm{~A}} \mathrm{CHO}$ cells in the presence of CCPA ( 1 nM ) or NECA $(10 \mathrm{nM})$, respectively. $\mathrm{IC}_{50}$ values are expressed as means $\pm$ SEM of four separate experiments.

The tested compounds behaved as antagonists at both $\mathrm{hA}_{1}$ and $\mathrm{hA}_{2 \mathrm{~A}}$ ARs with potencies wellcorrelated to their affinities. Among them, compounds $\mathbf{2 5}$ proved to be the most potent dual $\mathrm{A}_{2 \mathrm{~A}}$ and $\mathrm{A}_{1} \mathrm{AR}$ antagonist, although it was able to bind also to the $\mathrm{hA}_{3} \mathrm{AR}$.

### 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 $\mathrm{A}_{2 \mathrm{~A}} \mathrm{AR}$. 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 remodelled 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 -IL3domains). The obtained $\mathrm{A}_{2 \mathrm{~A}}$ AR model was checked by inspection of backbone bond lengths, angles and dihedrals, Ramachandran $\varphi-\psi$ dihedral plots, and sidechain rotamer and nonbonded contact quality. These steps were performed within Molecular Operating Environment (MOE, version 2010.10) suite. ${ }^{39}$ The $\mathrm{A}_{2 \mathrm{~A}}$ 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 $\mathrm{A}_{1}$ AR built by using the above cited $\mathrm{A}_{2 \mathrm{~A}}$ AR crystal structure as a template.

The pyrazolopyrimidine scaffold shows structural similarity to the triazolotriazine one of the cocrystallized 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 ZM 241385 at the $\mathrm{A}_{2 \mathrm{~A}}$ 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 Leu $249^{6.51}$, respectively ${ }^{43}$ ), with the 7 -amino group
and the N 1 nitrogen atom providing a double H -bond interaction with the Asn $253^{6.55}$ residue. The 2substituent is oriented towards the receptor core and is located between residues of TM3, TM5, and TM6 segments (Leu85 $5^{3.33}, \operatorname{Thr} 88^{3.36}$, Met177 $^{5.38}$, Asn181 $1^{5.42}, \operatorname{Trp} 246^{6.48}$, Leu249 $9^{6.51}$, and His250 ${ }^{6.52}$ ), while the 5 -substituent points toward the extracellular space and is located between TM1, TM2, EL2, EL3, and TM7 residues (Tyr ${ }^{1.35}$, Ala63 $3^{2.61}$, Ile66 ${ }^{2.64}$, Leu167, Leu267, Met2707.35, Tyr271 $1^{7.36}$, and Ile274 ${ }^{7.39}$ ).


Figure 3. Docking conformations at the $\mathrm{A}_{2 \mathrm{~A}} \mathrm{AR}$. 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 $\operatorname{Asn} 253^{6.55}$ 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 Asn $253^{6.55}$ 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 Asn $253^{6.55}$ and Glu169 (Figure 3B), providing a fair affinity at least for compound 3. In the case of compounds 5 and $\mathbf{6}$ (inactive at the $\mathrm{A}_{2 \mathrm{~A}} \mathrm{AR}$ ), 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 $\mathrm{A}_{2 \mathrm{~A}}$ 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 $\mathbf{2 5}$, 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 $I F-E$ $6.0^{44}$ 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 $\mathrm{kcal} / \mathrm{mol}$ ), 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 $(\mathbf{2 2}, \mathbf{2 3})$ or 2-(5-methyl)furyl group $(\mathbf{2 5}, \mathbf{2 6})$ at the 5-position. The results of this analysis are reported in Table 3.

Table 3. Interaction energies (values in $\mathrm{kcal} / \mathrm{mol}$ ) between compounds 22, 23, 25, 26 and $\mathbf{8}, \mathbf{9}, \mathbf{1 1}$, 12 and the binding site residues located in proximity of the 5-position of the analyzed compounds. See text for details.

| res |  | $\mathbf{2 3}$ | cpd |  |  |  |  |  |
| :---: | :---: | :---: | :---: | :---: | :---: | :---: | :---: | :---: |
|  | $\mathbf{2 2}$ | $\mathbf{2 5}$ | $\mathbf{2 3}$ | $\mathbf{2 6}$ | $\mathbf{8}$ | $\mathbf{1 1}$ | $\mathbf{9}$ | $\mathbf{1 2}$ |
| 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-TM5TM6 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 $\mathbf{8}$ (light) and $\mathbf{1 1}$ (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 2phenyl 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 $\mathbf{8}$ and $\mathbf{1 1}$ 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 $\mathbf{7 - 1 4}$ 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 $\mathbf{8}, \mathbf{9}, \mathbf{1 1}$, and $\mathbf{1 2}$. The results are reported in Table 3. Both derivatives $\mathbf{1 1}$ 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 2furyl substituted compounds $\mathbf{8}$ 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 Asn $253^{6.55}$. These results seem partially in accordance with the binding data of this set of derivatives, with the exception of compound $\mathbf{1 0}$ where the effect on the 5substituent 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 2position of the aromatic ring generally engages an internal H -bond interaction with the N 1 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 $0^{7.35}$, Tyr271 $1^{7.36}$, 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 $\mathbf{2 8}$ with $\mathbf{2 5}$ 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 $\mathrm{hA}_{1} \mathrm{AR}$ built by using the above cited 3EML $\mathrm{hA}_{2 \mathrm{~A}} \mathrm{AR}$ crystal structure as a template. The homology modeling and docking protocols were the same as the ones used for the refinement of the 3EML $\mathrm{hA}_{2 \mathrm{~A}}$ AR crystal structure and the docking at the same receptor (see above). Docking results at the $\mathrm{hA}_{1} \mathrm{AR}$ present some similarities to those obtained at the $\mathrm{hA}_{2 \mathrm{~A}} \mathrm{AR}$. 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 $\mathrm{hA}_{1} \mathrm{AR}$ 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 $\mathrm{hA}_{2 \mathrm{~A}}$ AR (Leu167 in EL2, Leu267 in EL3, and Met270 ${ }^{7.35}$ ) is occupied by polar amino acids in the $\mathrm{hA}_{1}$ 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 $\mathrm{hA}_{2 \mathrm{~A}} \mathrm{AR}$ and $\mathrm{hA}_{1} \mathrm{AR}$ ) may help to explain the generally higher affinity of this set of ligands for the $\mathrm{hA}_{2 \mathrm{~A}} \mathrm{AR}$ with respect to the $\mathrm{hA}_{1}$ AR. Furthermore, as observed at the $\mathrm{hA}_{2 \mathrm{~A}} \mathrm{AR}$, 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 $\mathbf{2 5}$ ) are more active at the $\mathrm{hA}_{1} \mathrm{AR}$ 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 N 1 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 $\mathrm{hA}_{1}$ 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 $\mathrm{hA}_{2 \mathrm{~A}} \mathrm{AR}$ or both $\mathrm{hA}_{2 \mathrm{~A}}$ and $\mathrm{hA}_{1}$ 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 $\mathrm{hA}_{2 \mathrm{~A}}$ AR affinities in the low nanomolar range ( $\mathrm{K}_{\mathrm{i}}=3.62-57 \mathrm{nM}$ ) and a little lower for the $\mathrm{hA}_{1}$ ARs $\left(\mathrm{K}_{\mathrm{i}}=18-234 \mathrm{nM}\right)$, thus showing different degrees of $A_{2 A}$ 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 $\mathrm{A}_{2 \mathrm{~A}}$ and $\mathrm{A}_{1} \mathrm{AR}$ antagonist herein reported, both in binding and in functional assays. Its 2-(2-hydroxybenzyl) analog 26 also showed a high affinity for the $\mathrm{A}_{2 \mathrm{~A}} \mathrm{AR}\left(\mathrm{K}_{\mathrm{i}}=5.26 \mathrm{nM}\right)$ and was 22 -fold selective versus the $\mathrm{A}_{1}$ subtype. Molecular docking investigations performed at the $\mathrm{hA}_{2 \mathrm{~A}} \mathrm{AR}$ crystal structure and at a homology model of the $\mathrm{A}_{1}$ 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 $\mathrm{C}, \mathrm{H}, \mathrm{N}$ and the results were within $\pm 0.4 \%$ 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 $\mathrm{cm}^{-1}$. NMR spectra were recorded on a Bruker Avance 400 spectrometer ( 400 MHz for ${ }^{1} \mathrm{H}$ NMR, 100 MHz for ${ }^{13} \mathrm{C}$ NMR). The chemical shifts are reported in $\delta$ ( ppm ) and are relative to the central peak of the solvent which was $\mathrm{CDCl}_{3}$ or $\mathrm{DMSOd}_{6}$. The following abbreviations are used: $\mathrm{s}=$ singlet, $\mathrm{d}=$ doublet, $\mathrm{t}=$ triplet, $\mathrm{q}=$ quartet, $\mathrm{m}=$ multiplet, $\mathrm{br}=$ broad and ar= aromatic protons.

### 5.1.1. Synthesis of Ethyl $\mathbf{N}^{1}$-2-methoxyphenylhydrazono- $\mathbf{N}^{2}$-chloroacetate 34 .

The title compound was synthesized following the procedure previously reported to prepare compounds $\mathbf{3 2}$ and $\mathbf{3 3}^{28,29}$ from the corresponding arylamines. Briefly, a mixture of 2methoxyaniline ( 53 mmol ) in $6 \mathrm{~N} \mathrm{HCl}(32 \mathrm{~mL})$ was cooled at $-5-0{ }^{\circ} \mathrm{C}$ and $1 \mathrm{M} \mathrm{NaNO}_{2}$ solution $(53 \mathrm{~mL})$ was added dropwise, keeping the temperature below $0^{\circ} \mathrm{C}$. The solution of the diazonium salt was poured into a cold $\left(0^{\circ} \mathrm{C}\right)$ solution of ethyl-2-chloroacetoacetate ( 53 mmol ) and sodium acetate ( 54.2 mmol ) in $\mathrm{MeOH}\left(200 \mathrm{~mL}\right.$ ), while stirring and keeping the temperature below $0{ }^{\circ} \mathrm{C}$. The mixture was then left in a refrigerator for 16 h . The solid was collected by filtration, washed with water $(200 \mathrm{~mL})$ and dried.

Yield $62 \%$; m.p. $73-74{ }^{\circ} \mathrm{C}$ (cyclohexane); ${ }^{1} \mathrm{H}$ NMR $\left(\mathrm{CDCl}_{3}\right) 1.42\left(\mathrm{t}, 3 \mathrm{H}, \mathrm{CH}_{3}, \mathrm{~J}=7.1 \mathrm{~Hz}\right), 3.96(\mathrm{~s}$, $\left.3 \mathrm{H}, \mathrm{OCH}_{3}\right), 4.41\left(\mathrm{q}, 2 \mathrm{H}, \mathrm{CH}_{2}, \mathrm{~J}=7.1 \mathrm{~Hz}\right), 6.90-6.92(\mathrm{~m}, 1 \mathrm{H}$, ar), 6.99-7.01 (m, 2H, ar), 7.55-7.58 (m, 1H, ar), $8.83(\mathrm{~s}, 1 \mathrm{H}, \mathrm{NH})$. Anal. Calcd. for $\mathrm{C}_{11} \mathrm{H}_{13} \mathrm{ClN}_{2} \mathrm{O}_{3}$ : 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 $\mathbf{3 6}$ and $\mathbf{3 7}$ were synthesized following the procedure previously described for $\mathbf{3 5}{ }^{21}$ Briefly, a mixture of $\mathrm{N}, \mathrm{N}$-dimethyl-2-nitroethenamine ( 11 mmol ), triethylamine ( 22 mmol ) and the suitable idrazone $\mathbf{3 3}$ or $\mathbf{3 4}$ in $\mathrm{CHCl}_{3}(5 \mathrm{~mL})$ was reacted under microwave irradiation for 30 min at $140{ }^{\circ} \mathrm{C}$. 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 ${ }^{\circ} \mathrm{C}$ (EtOH); ${ }^{1} \mathrm{H}$ NMR $\left(\mathrm{CDCl}_{3}\right) 1.45\left(\mathrm{t}, 3 \mathrm{H}, \mathrm{CH}_{3}, \mathrm{~J}=7.1 \mathrm{~Hz}\right), 4.52\left(\mathrm{q}, 2 \mathrm{H}, \mathrm{CH}_{2} \mathrm{~J}=7.1 \mathrm{~Hz}\right), 7.31-7.37$ $(\mathrm{m}, 2 \mathrm{H}, \mathrm{ar}), 7.44-7.50(\mathrm{~m}, 1 \mathrm{H}, \mathrm{ar}), 7.95(\mathrm{t}, 1 \mathrm{H}, \mathrm{ar}, \mathrm{J}=8.4 \mathrm{~Hz}), 8.72(\mathrm{~s}, 1 \mathrm{H}, \mathrm{H}-5)$. Anal. Calcd. for $\mathrm{C}_{12} \mathrm{H}_{10} \mathrm{FN}_{3} \mathrm{O}_{4}$ : C, $51.62 ; \mathrm{H}, 3.61 ; \mathrm{N}, 15.05$. Found: C, $51.44 ; \mathrm{H}, 3.85 ; \mathrm{N}, 15.27$.

Ethyl 1-(2-methoxyphenyl)-4-nitro-1H-pyrazole-3-carboxylate 37. Yield 36\%; m.p. 68-69 ${ }^{\circ} \mathrm{C}$ (cyclohexane); ${ }^{1} \mathrm{H}$ NMR $\left(\mathrm{CDCl}_{3}\right) 1.45\left(\mathrm{t}, 3 \mathrm{H}, \mathrm{CH}_{3}, \mathrm{~J}=7.1 \mathrm{~Hz}\right), 3.97\left(\mathrm{~s}, 3 \mathrm{H}, \mathrm{OCH}_{3}\right), 4.52(\mathrm{q}, 2 \mathrm{H}$, $\left.\mathrm{CH}_{2} \mathrm{~J}=7.1 \mathrm{~Hz}\right), 7.10-7.14(\mathrm{~m}, 2 \mathrm{H}, \mathrm{ar}), 7.44(\mathrm{t}, 1 \mathrm{H}, \mathrm{ar}, \mathrm{J}=7.9 \mathrm{~Hz}), 7.82(\mathrm{~d}, 1 \mathrm{H}, \mathrm{ar}, \mathrm{J}=7.9), 8.79(\mathrm{~s}$, 1H, H-3). Anal. Calcd. for $\mathrm{C}_{13} \mathrm{H}_{13} \mathrm{~N}_{3} \mathrm{O}_{5}$ : 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 $\mathbf{1 - a r y}$-4-nitro-1H-pyrazole-3-carboxamides $\mathbf{3 9}$ 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 $\mathbf{3 6}$ or $\mathbf{3 7}$ ( 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 ${ }^{\circ} \mathrm{C}(\mathrm{EtOH}) ;{ }^{1} \mathrm{H}$ NMR (DMSO- $\mathrm{d}_{6}$ ) 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, $1 \mathrm{H}, \mathrm{NH}$ ), 8.21 (br s, $1 \mathrm{H}, \mathrm{NH}$ ), 9.34 ( $\mathrm{s}, 1 \mathrm{H}, \mathrm{H}-5$ ). Anal. Calcd. for $\mathrm{C}_{10} \mathrm{H}_{7} \mathrm{FN}_{4} \mathrm{O}_{4}$ : 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 ${ }^{\circ} \mathrm{C}(\mathrm{EtOAc} / \mathrm{MeOH}) ;{ }^{1} \mathrm{H}$ NMR (DMSO-d $\mathrm{d}_{6}$ ) $3.90\left(\mathrm{~s}, 3 \mathrm{H}, \mathrm{OCH}_{3}\right), 7.15(\mathrm{t}, 1 \mathrm{H}$, ar J = 7.8 Hz$), 7.33(\mathrm{~d}$,
$1 \mathrm{H}, \mathrm{ar}, \mathrm{J}=8.4 \mathrm{~Hz}), 7.51(\mathrm{t}, 1 \mathrm{H}$, ar $\mathrm{J}=7.5 \mathrm{~Hz}), 7.64(\mathrm{~d}, 1 \mathrm{H}, \mathrm{ar}, \mathrm{J}=8.0 \mathrm{~Hz}), 7.85(\mathrm{br} \mathrm{s}, 1 \mathrm{H}, \mathrm{NH}), 8.14$ (br s, 1H, NH), 9.16 (s, 1H, H-5). Anal. Calcd. for $\mathrm{C}_{11} \mathrm{H}_{10} \mathrm{~N}_{4} \mathrm{O}_{4}: \mathrm{C}, 50.38 ; \mathrm{H}, 3.84 ; \mathrm{N}, 21.37$. 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 $\mathbf{4 2}$ and $\mathbf{4 3}$ were synthesized as previously described to prepare $\mathbf{4 1}$ from $\mathbf{3 8} .{ }^{25}$ Briefly, a suspension of the suitable amide 39 or 40 ( 2 mmol ) in phosphorus oxychloride ( 5 mL ) was microwave irradiated at $150{ }^{\circ} \mathrm{C}$ for $10-20 \mathrm{~min}$. The excess of phosphorus oxychloride was distilled off and the residue was treated with water (about $5-10 \mathrm{~mL}$ ). 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 $108-110{ }^{\circ} \mathrm{C}$ $\left(\mathrm{H}_{2} \mathrm{O} / \mathrm{EtOH}\right) ;{ }^{1} \mathrm{H}$ NMR $\left(\mathrm{CDCl}_{3}\right)$ 7.36-7.43 (m, 2H, ar), 7.49-7.58 (m, 1H, ar), $7.93(\mathrm{t}, 1 \mathrm{H}, \mathrm{ar}, \mathrm{J}=7.9$ $\mathrm{Hz}), 8.81$ (s, 1H, H-5). Anal. Calcd. for $\mathrm{C}_{10} \mathrm{H}_{5} \mathrm{FN}_{4} \mathrm{O}_{2}$ : 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 ${ }^{\circ} \mathrm{C}$ (cyclohexane/EtOAc); ${ }^{1} \mathrm{H}$ NMR $\left(\mathrm{CDCl}_{3}\right) 4.00(\mathrm{~s}, 3 \mathrm{H}, \mathrm{OMe}), 7.17(\mathrm{t}, 2 \mathrm{H}, \mathrm{ar}, \mathrm{J}=8.4 \mathrm{~Hz}), 7.51(\mathrm{t}$, 1 H , ar, $\mathrm{J}=7.8 \mathrm{~Hz}$ ), $7.79(\mathrm{~d}, 1 \mathrm{H}$, ar, $\mathrm{J}=7.9 \mathrm{~Hz}), 8.89(\mathrm{~s}, 1 \mathrm{H}, \mathrm{H}-5)$. IR 2251, 1538, 1342. Anal. Calcd. for $\mathrm{C}_{11} \mathrm{H}_{8} \mathrm{~N}_{4} \mathrm{O}_{3}$ : C, 54.10; H, 3.30; N, 22.94. Found C, $54.21 ; \mathrm{H}, 3.18 ; \mathrm{N}, 22.78$.

### 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 $\mathbf{4 2}$ or $\mathbf{4 3}(2 \mathrm{mmol})$, cyclohexene ( 8 mmol ) and $10 \% \mathrm{Pd} / \mathrm{C}(15 \% \mathrm{w} / \mathrm{w}$ with respect to the nitropyrazole) in $\mathrm{EtOH}(10 \mathrm{~mL})$ was microwave irradiated, respectively, at $110^{\circ} \mathrm{C}$ for 10 min and $130^{\circ} \mathrm{C}$ 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 ${ }^{\circ} \mathrm{C}\left(\mathrm{H}_{2} \mathrm{O} / \mathrm{EtOH}\right) .{ }^{1} \mathrm{H}$ NMR $\left(\mathrm{DMSO}_{6}\right) 5.05\left(\mathrm{br} \mathrm{s}, 2 \mathrm{H}, \mathrm{NH}_{2}\right), 7.35-7.39(\mathrm{~m}, 1 \mathrm{H}$, ar), 7.48-7.51 (m, 2 H , ar), 7.67 ( $\mathrm{s}, 1 \mathrm{H}, \mathrm{H}-5$ ), 7.77 (t, $1 \mathrm{H}, \mathrm{ar}, \mathrm{J}=8.3 \mathrm{~Hz}$ ). Anal. Calcd. for $\mathrm{C}_{10} \mathrm{H}_{7} \mathrm{FN}_{4}: \mathrm{C}, 59.40 ; \mathrm{H}, 3.49$; 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. $108-109{ }^{\circ} \mathrm{C}$ (Light petroleum ether/Et $\mathrm{E}_{2} \mathrm{O}$ ). ${ }^{1} \mathrm{H}$ NMR $\left(\mathrm{CDCl}_{3}\right) 3.49$ (br s, $2 \mathrm{H}, \mathrm{NH}_{2}$ ), 3.91 (s, $3 \mathrm{H}, \mathrm{OMe}$ ), 7.06-7.10 $(\mathrm{m}, 2 \mathrm{H}, \mathrm{ar}), 7.38(\mathrm{t}, 1 \mathrm{H}, \mathrm{ar}, \mathrm{J}=7.5 \mathrm{~Hz}), 7.66-7.68(\mathrm{~m}, 2 \mathrm{H}, 1 \mathrm{ar}+\mathrm{H}-5)$. Anal. Calcd. for $\mathrm{C}_{11} \mathrm{H}_{10} \mathrm{~N}_{4} \mathrm{O}$ : 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 $\mathbf{1 - 3 , 5 , 7 , 8 , 1 0 , 1 1 , 1 3 .}$

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 $(1.64 \mathrm{mmol})\left(\mathrm{R}_{5}=\mathrm{CH}_{2} \mathrm{Ph},{ }^{31}\left(\mathrm{CH}_{2}\right)_{3} \mathrm{Ph},{ }^{32}\right.$ 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 ( $1-3 \mathrm{~mL}$ ). The resulting solid was collected by filtration and washed, in sequence, with water (10-15 mL), $\mathrm{NaHCO}_{3}$ saturated solution (1-2 mL ) and water ( $5-10 \mathrm{~mL}$ ), then dried and recrystallized. The crude compounds 5 and $\mathbf{7}$ 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 $130{ }^{\circ} \mathrm{C}$ for 15 min . Yield $85 \%$; m.p. $212-214{ }^{\circ} \mathrm{C}$ (cyclohexane/EtOAc); ${ }^{1} \mathrm{H}$ NMR ( $\mathrm{DMSO}-\mathrm{d}_{6}$ ) 2.37 (s, 3H, Me), $7.45(\mathrm{t}, 1 \mathrm{H}$ ar, $\mathrm{J}=6.8 \mathrm{~Hz}), 7.54-7.62(\mathrm{~m}, 2 \mathrm{H}, \mathrm{ar}), 7.68\left(\mathrm{br} \mathrm{s}, 2 \mathrm{H}, \mathrm{NH}_{2}\right), 7.89(\mathrm{t}, 1 \mathrm{H} \mathrm{ar}, \mathrm{J}=7.8 \mathrm{~Hz})$,
8.61 (s, 1H, H-3). Anal. Calcd. for $\mathrm{C}_{12} \mathrm{H}_{10} \mathrm{FN}_{5}: \mathrm{C}, 59.25$; 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 $150{ }^{\circ} \mathrm{C}$ for $10-15 \mathrm{~min}$. Yield $63 \%$; m.p. $188-189{ }^{\circ} \mathrm{C}$ (cyclohexane/EtOAc); ${ }^{1} \mathrm{H}$ NMR (DMSO- $\mathrm{d}_{6}$ ) 2.01-2.06 $\left(\mathrm{m}, 2 \mathrm{H}, \mathrm{CH}_{2}\right), 2.63-2.66\left(\mathrm{~m}, 4 \mathrm{H}, \mathrm{CH}_{2}\right), 7.16-7.31(\mathrm{~m}, 5 \mathrm{H}, \mathrm{ar}), 7.45(\mathrm{t}, 1 \mathrm{H}, \mathrm{ar}, \mathrm{J}=6.9 \mathrm{~Hz}), 7.54-7.59$ (m, 2H, ar), 7.67 (br s, 2H, NH2), $7.89(\mathrm{t}, 1 \mathrm{H}$, ar, $\mathrm{J}=7.7 \mathrm{~Hz}$ ), $8.65(\mathrm{~s}, 1 \mathrm{H}, \mathrm{H}-3)$. Anal. Calcd. for $\mathrm{C}_{20} \mathrm{H}_{18} \mathrm{FN}_{5}$ : C, 69.15; H, 5.22; N, 20.16. Found C, $69.01 ; \mathrm{H}, 5.08 ; \mathrm{N}, 20.30$.

### 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 $120^{\circ} \mathrm{C}$ for 2 h . Yield $40 \%$; m.p. 199-200 ${ }^{\circ} \mathrm{C}$ (cyclohexane/EtOAc); ${ }^{1} \mathrm{H}$ NMR (DMSO- $\mathrm{d}_{6}$ ) 2.01-2.05 (m, 2H, CH ${ }_{2}$ ), 2.63-2.65 (m, 4H, 2CH $)$, $3.87(\mathrm{~s}, 3 \mathrm{H}, \mathrm{OMe}), 7.20-7.33(\mathrm{~m}, 7 \mathrm{H}, \mathrm{ar}), 7.50-7.54\left(\mathrm{~m}, 3 \mathrm{H}, 1 \mathrm{ar}+\mathrm{NH}_{2}\right)$, 7.68 (d, 1H, ar, J=7.8 Hz), 8.50 ( $\mathrm{s}, 1 \mathrm{H}, \mathrm{H}-3$ ). ${ }^{13} \mathrm{C}$ NMR (DMSO-d $\mathrm{d}_{6}$ ) 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 $\mathrm{C}_{21} \mathrm{H}_{21} \mathrm{~N}_{5} \mathrm{O}: \mathrm{C}, 70.17 ; \mathrm{H}, 5.89 ; \mathrm{N}, 19.48$. 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 $120^{\circ} \mathrm{C}$ for 5 h . The crude compound was purified by preparative TLC (eluent cyclohexane/EtOAc/MeOH, 6:4:1). Yield 65\%; m.p. 141-142 ${ }^{\circ} \mathrm{C}$; ${ }^{1} \mathrm{H}$ NMR ( $\mathrm{DMSO}_{\mathrm{d}}^{6}$ ) 3.86 ( $\mathrm{s}, 3 \mathrm{H}, \mathrm{OMe}$ ), $3.96\left(\mathrm{~s}, 2 \mathrm{H}, \mathrm{CH}_{2}\right), 7.13-7.18(\mathrm{~m}, 2 \mathrm{H}, \mathrm{ar})$, 7.20-7.30 (m, 5H, ar), $7.52(\mathrm{t}, 1 \mathrm{H}, \mathrm{J}=7.4 \mathrm{~Hz}), 7.66-7.68\left(\mathrm{~m}, 3 \mathrm{H}, 1 \mathrm{ar}+\mathrm{NH}_{2}\right), 8.54(\mathrm{~s}, 1 \mathrm{H}, \mathrm{H}-3)$.

Anal. Calcd. for $\mathrm{C}_{19} \mathrm{H}_{17} \mathrm{~N}_{5} \mathrm{O}: \mathrm{C}, 68.87$; 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 $130{ }^{\circ} \mathrm{C}$ for 10 min . The crude derivative was purified by column chromatography (eluent cyclohexane/EtOAc 6:4). Yield $48 \%$; m.p. 208-210 ${ }^{\circ} \mathrm{C}(\mathrm{EtOAc}) ;{ }^{1} \mathrm{H}$ NMR (DMSO- $\mathrm{d}_{6}$ ) 6.62-6.63 (m, 1H, furan proton), 7.07-7.08
$(\mathrm{m}, 1 \mathrm{H}$, furan proton), $7.50(\mathrm{t}, 1 \mathrm{H}, \operatorname{ar}, \mathrm{J}=9.0 \mathrm{~Hz}), 7.63(\mathrm{t}, 2 \mathrm{H}, \mathrm{ar}, \mathrm{J}=9.0 \mathrm{~Hz}), 7.80(\mathrm{~s}, 1 \mathrm{H}$, furan proton), $7.84\left(\mathrm{br} \mathrm{s}, 2 \mathrm{H}, \mathrm{NH}_{2}\right), 8.08(\mathrm{~d}, 2 \mathrm{H}, \mathrm{ar}, \mathrm{J}=9.0 \mathrm{~Hz}), 9.06(\mathrm{~s}, 1 \mathrm{H}, \mathrm{H}-3)$. IR 3335, 3150, 1591. Anal. Calcd. for $\mathrm{C}_{15} \mathrm{H}_{11} \mathrm{~N}_{5} \mathrm{O}: \mathrm{C}, 64.97 ; \mathrm{H}, 4.00 ; \mathrm{N}, 25.26$. 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 $130^{\circ} \mathrm{C}$ for 15 min in a sealed tube. Yield $38 \%$; m.p. 295-296 ${ }^{\circ} \mathrm{C}\left(\mathrm{MeNO}_{2}\right) .{ }^{1} \mathrm{H}$ NMR (DMSO-d ${ }_{6}$ ) $3.89(\mathrm{~s}, 3 \mathrm{H}, \mathrm{OMe})$, 6.61-6.62 (m, 1 H , furan proton), 7.06-7.07 (m, 1H, H furan proton), $7.17(\mathrm{t}, 1 \mathrm{H}, \mathrm{ar}, \mathrm{J}=7.7 \mathrm{~Hz}), 7.33(\mathrm{~d}, 1 \mathrm{H}$, ar, $\mathrm{J}=7.5 \mathrm{~Hz})$, $7.53(\mathrm{t}, 1 \mathrm{H}, \mathrm{ar}, \mathrm{J}=8.3 \mathrm{~Hz}), 7.72(\mathrm{~d}, 1 \mathrm{H}, \mathrm{ar}, \mathrm{J}=7.8 \mathrm{~Hz}), 7.77-7.79\left(\mathrm{~m}, 3 \mathrm{H}, \mathrm{NH}_{2}+1 \mathrm{H}\right.$ furan proton $)$, 8.64 (s, 1H, H-3). Anal. Calcd. for $\mathrm{C}_{16} \mathrm{H}_{13} \mathrm{~N}_{5} \mathrm{O}_{2}$ : 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 $110^{\circ} \mathrm{C}$ for 1 h in a sealed tube. Yield $38 \%$; m.p. $257-258{ }^{\circ} \mathrm{C}\left(\mathrm{H}_{2} \mathrm{O} / \mathrm{EtOH}\right) ;{ }^{1} \mathrm{H}$ NMR (DMSO- $\mathrm{d}_{6}$ ) 2.37 (s, 3H, Me), 6.23-6.24 (m, 1 H , furan proton), 6.97-6.98 (m, 1H, furan proton), $7.49(\mathrm{t}, 1 \mathrm{H}, \mathrm{ar}, \mathrm{J}=7.4 \mathrm{~Hz}), 7.62(\mathrm{t}, 1 \mathrm{H}, \mathrm{ar}, \mathrm{J}=7.7$ $\mathrm{Hz}), 7.79\left(\mathrm{br} \mathrm{s}, 2 \mathrm{H}, \mathrm{NH}_{2}\right), 8.06(\mathrm{~d}, 2 \mathrm{H}, \mathrm{ar}, \mathrm{J}=7.8 \mathrm{~Hz}), 9.00(\mathrm{~s}, 1 \mathrm{H}, \mathrm{H}-3)$. Anal. Calcd. for $\mathrm{C}_{16} \mathrm{H}_{13} \mathrm{~N}_{5} \mathrm{O} ; \mathrm{C}, 65.97 ; \mathrm{H}, 4.50 ; \mathrm{N}, 24.04$. Found C, $65.75 ; \mathrm{H}, 4.72 ; \mathrm{N}, 24.21$.
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 $120^{\circ} \mathrm{C}$ for 2 h in a sealed tube. Yield $58 \%$; m.p. $216-217{ }^{\circ} \mathrm{C}$ (cyclohexane/EtOAc); ${ }^{1} \mathrm{H}$ NMR (DMSO-d ${ }_{6}$ ) $2.36(\mathrm{~s}, 3 \mathrm{H}, \mathrm{Me})$, $3.88(\mathrm{~s}, 3 \mathrm{H}, \mathrm{OMe}), 6.22-6.23(\mathrm{~m}, 1 \mathrm{H}$, furan proton), 6.95-6.96 (m, 1H, furan proton), $7.16(\mathrm{t}, 1 \mathrm{H}, \mathrm{ar}$, $\mathrm{J}=7.7 \mathrm{~Hz}), 7.33(\mathrm{~d}, 1 \mathrm{H}, \mathrm{ar}, \mathrm{J}=8.4 \mathrm{~Hz}), 7.53(\mathrm{t}, 1 \mathrm{H}, \mathrm{ar}, \mathrm{J}=7.6 \mathrm{~Hz}), 7.71-7.73\left(\mathrm{~m}, 3 \mathrm{H}, 1 \mathrm{ar}+\mathrm{NH}_{2}\right)$, 8.61 (s, 1H, H-3). Anal. Calcd. for $\mathrm{C}_{17} \mathrm{H}_{15} \mathrm{~N}_{15} \mathrm{O}_{2}$ : 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 $110{ }^{\circ} \mathrm{C}$ for 2 h in a sealed tube. Yield $70 \%$; m.p. 244-245 ${ }^{\circ} \mathrm{C}\left(\mathrm{MeNO}_{2}\right) ;{ }^{1} \mathrm{H}$ NMR (DMSO-d ${ }_{6}$ ) $3.89(\mathrm{~s}, 3 \mathrm{H}, \mathrm{OMe}), 7.13-7.19(\mathrm{~m}, 2 \mathrm{H}, 1 \mathrm{ar}+$

1 thiophene proton), $7.34(\mathrm{~d}, 1 \mathrm{H}, \mathrm{ar}, \mathrm{J}=8.4 \mathrm{~Hz}), 7.53(\mathrm{t}, 1 \mathrm{H}, \mathrm{ar}, \mathrm{J}=8.4 \mathrm{~Hz}), 7.59-7.60(\mathrm{~m}, 1 \mathrm{H}$, thiophene proton), $7.73(\mathrm{~d}, 1 \mathrm{H}, \mathrm{ar}, \mathrm{J}=7.9 \mathrm{~Hz}), 7.79-7.80\left(\mathrm{~m}, 3 \mathrm{H}, 1\right.$ thiophene proton $\left.+\mathrm{NH}_{2}\right), 8.65$ (s, 1H, H-3). Anal. Calcd. for $\mathrm{C}_{16} \mathrm{H}_{13} \mathrm{~N}_{5} \mathrm{OS}$.
5.1.7. General procedure for the synthesis of 7-amino-2-(2-hydroxyphenyl)-pyrazolo[4,3d]pyrimidine derivatives $4,6,9,12,14$

1 M solution of $\mathrm{BBr}_{3}$ in dichloromethane ( 2.60 mL ) was slowly added at $0{ }^{\circ} \mathrm{C}$, under nitrogen atmosphere, to a suspension of compounds $\mathbf{3}, \mathbf{5}, \mathbf{8}, \mathbf{1 1}$ or $\mathbf{1 3}(1.02 \mathrm{mmol})$ 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 $(10 \mathrm{~mL})$ and neutralized with $\mathrm{NaHCO}_{3}$ saturated solution. The obtained precipitate was collected by filtration, dried and purified by recrystallization, with the only exception of derivative $\mathbf{6}$ 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 ${ }^{\circ} \mathrm{C}$ (cyclohexane/EtOAc). ${ }^{1} \mathrm{H}$ NMR (DMSO- $\mathrm{d}_{6}$ ) 2.03 (quintet, 2H, $\mathrm{CH}_{2}$, $\mathrm{J}=7.5 \mathrm{~Hz}), 2.63-2.67\left(\mathrm{~m}, 4 \mathrm{H}, 2 \mathrm{CH}_{2}\right), 7.01(\mathrm{t}, 1 \mathrm{H}, \mathrm{ar}, \mathrm{J}=7.8 \mathrm{~Hz}), 7.12(\mathrm{~d}, 1 \mathrm{H}, \mathrm{ar}, \mathrm{J}=8.1 \mathrm{~Hz}), 7.16-$ $7.34(\mathrm{~m}, 6 \mathrm{H}, \mathrm{ar}), 7.62\left(\mathrm{br} \mathrm{s}, 2 \mathrm{H}, \mathrm{NH}_{2}\right), 7.75(\mathrm{~d}, 1 \mathrm{H}$, ar, J=8.1 Hz), $8.69(\mathrm{~s}, 1 \mathrm{H}, \mathrm{H}-3), 10.69$ (br s, $1 \mathrm{H}, \mathrm{OH}$ ). Anal. Calcd. for $\mathrm{C}_{20} \mathrm{H}_{19} \mathrm{~N}_{5} \mathrm{O}: \mathrm{C}, 69.55 ; \mathrm{H}, 5.54 ; \mathrm{N}, 20.28$. 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 ${ }^{\circ} \mathrm{C} ;{ }^{1} \mathrm{H}$ NMR (DMSO-d $\mathrm{d}_{6}$ ) $3.95\left(\mathrm{~s}, 2 \mathrm{H}, \mathrm{CH}_{2}\right), 7.01(\mathrm{t}, 1 \mathrm{H}, \mathrm{ar}, \mathrm{J}=7.5 \mathrm{~Hz}), 7.11(\mathrm{~d}, 1 \mathrm{H}$, ar, $\mathrm{J}=8.1 \mathrm{~Hz}$ ), $7.18(\mathrm{t}, 1 \mathrm{H}$, ar, $\mathrm{J}=7.2 \mathrm{~Hz}), 7.25-7.34\left(\mathrm{~m}, 5 \mathrm{H}\right.$, ar), $7.67\left(\mathrm{br} \mathrm{s}, 2 \mathrm{H}, \mathrm{NH}_{2}\right), 7.73(\mathrm{~d}, 2 \mathrm{H}$, ar, $\mathrm{J}=8.0 \mathrm{~Hz}), 8.70(\mathrm{~s}, 1 \mathrm{H}, \mathrm{H}-3), 10.67(\mathrm{br} \mathrm{s}, 1 \mathrm{H}, \mathrm{OH})$. Anal. Calcd. for $\mathrm{C}_{18} \mathrm{H}_{15} \mathrm{~N}_{5} \mathrm{O}: \mathrm{C}, 68.13 ; \mathrm{H}$, 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 ${ }^{\circ} \mathrm{C}$ (2-Methoxyethanol). ${ }^{1} \mathrm{H}$ NMR (DMSO-d ${ }_{6}$ ) 6.61-6.63 (m, 1H, furan proton), 7.02 $(\mathrm{t}, 1 \mathrm{H}, \operatorname{ar}, \mathrm{J}=7.6 \mathrm{~Hz}), 7.07-7.08(\mathrm{~m}, 1 \mathrm{H}$, furan proton), $7.13(\mathrm{~d}, 1 \mathrm{H}, \mathrm{ar}, \mathrm{J}=7.2 \mathrm{~Hz}), 7.34(\mathrm{t}, 1 \mathrm{H}, \mathrm{ar}, \mathrm{J}$ $=7.4 \mathrm{~Hz}), 7.77-7.84\left(\mathrm{~m}, 4 \mathrm{H}, 2 \mathrm{ar}+\mathrm{NH}_{2}\right), 8.80(\mathrm{~s}, 1 \mathrm{H}, \mathrm{H}-3), 10.71(\mathrm{~s}, 1 \mathrm{H}, \mathrm{OH}) .{ }^{13} \mathrm{C}$ NMR (DMSO$\left.\mathrm{d}_{6}\right) 111.47,112.32,118.03,119.96,125.00,125.22,127.53,130.16,130.95,139.47,144.49$, 150.46, 152.34, 153.90, 156.17. Anal. Calcd. for $\mathrm{C}_{15} \mathrm{H}_{11} \mathrm{~N}_{5} \mathrm{O}_{2}$ : 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. > $300^{\circ} \mathrm{C}$ (isopropanol). ${ }^{1} \mathrm{H}$ NMR (DMSO-d $\mathrm{d}_{6}$ ) 2.36 (s, 3H, Me), 6.23-6.24 (m, 1 H , furan proton), 6.95-6.96 (m., 1 H , furan proton), $7.01(\mathrm{t}, 1 \mathrm{H}, \mathrm{ar}, \mathrm{J}=7.8 \mathrm{~Hz}), 7.12(\mathrm{~d}, 1 \mathrm{H}, \mathrm{ar}, \mathrm{J}=$ $8.1 \mathrm{~Hz}), 7.33(\mathrm{t}, 1 \mathrm{H}, \mathrm{ar}, \mathrm{J}=7.8 \mathrm{~Hz}), 7.71-7.80\left(\mathrm{~m}, 3 \mathrm{H}, 1 \mathrm{ar}+\mathrm{NH}_{2}\right), 8.76(\mathrm{~s}, 1 \mathrm{H}, \mathrm{H}-3), 10.69(\mathrm{~s}, 1 \mathrm{H}$, OH ). Anal. Calcd. for $\mathrm{C}_{16} \mathrm{H}_{13} \mathrm{~N}_{5} \mathrm{O}_{2}$ : 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 ${ }^{\circ} \mathrm{C}\left(\mathrm{MeNO}_{2}\right) .{ }^{1} \mathrm{H}$ NMR (DMSO-d $\left.\mathrm{d}_{6}\right) 7.05(\mathrm{t}, 1 \mathrm{H}$, ar, $\mathrm{J}=7.4 \mathrm{~Hz})$, 7.16-7.18 (m, $2 \mathrm{H}, 1$ thiophene proton $+1 \mathrm{ar}), 7.35-7.39(\mathrm{t}, 1 \mathrm{H}, \mathrm{ar}, 7.4 \mathrm{~Hz}), 7.60-7.61(\mathrm{~m}, 1 \mathrm{H}$, thiophene proton), 7.75 (br s, 2H, NH2 ), 7.81-7.84 (m, 2H, 1 thiophene proton +1 ar ), $8.82(\mathrm{~s}, 1 \mathrm{H}, \mathrm{H}-3), 10.63$ (br s, $1 \mathrm{H}, \mathrm{OH})$. Anal. Calcd. for $\mathrm{C}_{15} \mathrm{H}_{11} \mathrm{~N}_{5} \mathrm{OS}: \mathrm{C}, 58.24 ; \mathrm{H}, 3.58$; 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 $(2 \mathrm{mmol})^{48}$ with $\mathrm{POCl}_{3}(5 \mathrm{ml})$ under microwave irradiation at $150{ }^{\circ} \mathrm{C}$ for 40 min . The excess of phosphorus oxychloride was distilled off and the residue was treated with water (about $5-10 \mathrm{~mL}$ ) to give a solid which was collected and recrystallized. Yield $98 \%$; m.p. $160-162{ }^{\circ} \mathrm{C}$ (EtOAc) \%; (lit ${ }^{48} 162-163^{\circ} \mathrm{C}$ ). ${ }^{1} \mathrm{H}$ NMR (DMSO-d ${ }_{6}$ ) 9.16 (s, 1H, H-5); ${ }^{1} \mathrm{H}$ NMR $\left(\mathrm{CDCl}_{3}\right) 8.39$ (s, 1H, H-5). ${ }^{13} \mathrm{C}$ NMR (DMSO-d $\mathrm{d}_{6}$ 112.26, 120.88, 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 $47(1.45 \mathrm{mmol})$ 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 $0^{\circ} \mathrm{C}$. The suspension was stirred at $0^{\circ} \mathrm{C}$ 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 $1 \mathrm{M} \mathrm{NH}_{4} \mathrm{Cl}(20 \mathrm{~mL})$. The two phases were separated and the aqueous solution was extracted with EtOAc ( 30 mL for three times). The combined organic phases were anhydrified $\left(\mathrm{Na}_{2} \mathrm{SO}_{4}\right)$ 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 $\mathbf{5 0}$ from its regioisomer 50a (molar ratio 6:1, from ${ }^{1} \mathrm{H}$ NMR spectrum of the crude oil).
5.1.9.1. 4-Nitro-1-phenylethyl-1H-pyrazole-3-carbonitrile 50. Yield $72 \%$; m.p. $142-144{ }^{\circ} \mathrm{C}$ (EtOH). ${ }^{1} \mathrm{H}$ NMR $\left(\mathrm{CDCl}_{3}\right) 3.24\left(\mathrm{t}, 2 \mathrm{H}, \mathrm{CH}_{2}, \mathrm{~J}=6.9 \mathrm{~Hz}\right), 4.46\left(\mathrm{t}, 2 \mathrm{H}, \mathrm{CH}_{2} \mathrm{~J}=6.9 \mathrm{~Hz}\right), 7.07(\mathrm{~d}, 2 \mathrm{H}$, ar, $\mathrm{J}=7.7 \mathrm{~Hz}$ ), 7.28-7.33 (m, 3H, ar), 7.85 ( $\mathrm{s}, 1 \mathrm{H}, \mathrm{H}-5$ ). NOESY: interaction between $\mathrm{H}-5$ and $\mathrm{CH}_{2}$ protons. ${ }^{13} \mathrm{C}$ NMR $\left(\mathrm{CDCl}_{3}\right) 35.15,57.50,110.11,121.21,127.69,128.48,129.18,129.76,134.29$, 135.77. Anal. Calcd. for $\mathrm{C}_{12} \mathrm{H}_{10} \mathrm{~N}_{6} \mathrm{O}_{4}$ : 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. $117-119{ }^{\circ} \mathrm{C}$ (cyclohexane/EtOAc). ${ }^{1} \mathrm{H}$ NMR $\left(\mathrm{CDCl}_{3}\right) 3.28\left(\mathrm{t}, 2 \mathrm{H}, \mathrm{CH}_{2}, \mathrm{~J}=7.0 \mathrm{~Hz}\right), 4.61\left(\mathrm{t}, 2 \mathrm{H}, \mathrm{CH}_{2} \mathrm{~J}=7.0 \mathrm{~Hz}\right)$, $7.09(\mathrm{~d}, 2 \mathrm{H}, \mathrm{ar}, \mathrm{J}=7.2 \mathrm{~Hz}), 7.30-7.36\left(\mathrm{~m}, 3 \mathrm{H}\right.$, ar), $8.19(\mathrm{~s}, 1 \mathrm{H}, \mathrm{H}-3)$. Anal. Calcd. for $\mathrm{C}_{12} \mathrm{H}_{10} \mathrm{~N}_{6} \mathrm{O}_{4}: \mathrm{C}$, 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 $48(6.6 \mathrm{mmol})$ and $10 \% \mathrm{Pd} / \mathrm{C}(10 \% \mathrm{p} / \mathrm{p})$ in $\mathrm{EtOH}(20-30 \mathrm{~mL})$ 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 ( $5-8 \mathrm{~mL}$ ) and diethyl ether ( $1-2 \mathrm{~mL}$ ) to give a solid which was collected by filtration and recrystallized. Yield $65 \%$; m.p. $87-88{ }^{\circ} \mathrm{C}$ (cyclohexane/EtOAc). ${ }^{1} \mathrm{H}$ NMR (DMSO-d ${ }_{6}$ ) 3.76 (s, $3 \mathrm{H}, \mathrm{CH}_{3}$ ), 4.75 (br s, 2H, $\mathrm{NH}_{2}$ ), 7.20 (s, 1H, H-5). IR 3381, 3301, 2224. Anal. Calcd. for $\mathrm{C}_{5} \mathrm{H}_{6} \mathrm{~N}_{4}$ : 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 $\mathbf{5 3}$ was prepared as previously described for $\mathbf{5 2}$. ${ }^{27}$ The nitro derivative $\mathbf{5 0}(2 \mathrm{mmol})$ was reacted with cyclohexene ( 8 mmol ) and $10 \% \mathrm{Pd} / \mathrm{C}(15 \% \mathrm{w} / \mathrm{w}$ with respect to the nitropyrazoles) in the same experimental conditions described above for compounds 44-46. Microwave irradiation was carried out at $150{ }^{\circ} \mathrm{C}$ 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 ( ${ }^{1} \mathrm{H}$ NMR spectrum), it was used without further purification for the next step. Yield $68 \%$; ${ }^{1} \mathrm{H}$ NMR ( $\mathrm{DMSO}_{6}$ ) $3.06\left(\mathrm{t}, 2 \mathrm{H}, \mathrm{CH}_{2}, \mathrm{~J}=\mathrm{Hz}\right), 4.28\left(\mathrm{t}, 2 \mathrm{H}, \mathrm{CH}_{2}, \mathrm{~J}=\mathrm{Hz}\right)$, 4.70 (br s, 2H, NH2 ), 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 $\mathbf{5 1 - 5 3}$ ( 0.84 mmol ), anhydrous ammonium acetate $(1.51 \mathrm{mmol})$, triethyl orthoacetate $(1.26 \mathrm{mmol})$ or the suitable ethyl iminoesters hydrochlorides ( 1.26 mmol ) $\left(\mathrm{R}_{5}=\mathrm{CH}_{2} \mathrm{Ph}^{31}\left(\mathrm{CH}_{2}\right)_{3} \mathrm{Ph}^{32}{ }^{32}\right.$ 2-furyl $\left.{ }^{33}\right)$ was heated under microwave irradiation at $130^{\circ} \mathrm{C}$ for 15 min . Compound $\mathbf{2 2}$ was obtained by heating the reaction mixture in a sealed tube at $120^{\circ} \mathrm{C}$ for 2 h and 30 min . The obtained mixture was cooled at room temperature and
treated with diethyl ether ( $3-4 \mathrm{~mL}$ ) and a few EtOH ( $0.5-1 \mathrm{~mL}$ ). The resulting solid was collected by filtration, washed with water $(20 \mathrm{~mL})$ and $\mathrm{NaHCO}_{3}$ saturated solution $(1-2 \mathrm{~mL})$, then dried and recrystallized. The crude compound $\mathbf{1 7}$ was first purified by column chromatography (eluent $\mathrm{CHCl}_{3} / \mathrm{MeOH} /$ 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$129{ }^{\circ} \mathrm{C}(\mathrm{EtOAc} / \mathrm{MeOH}) ;{ }^{1} \mathrm{H}$ NMR (DMSO-d $\left.{ }_{6}\right) 4.02\left(\mathrm{~s}, 2 \mathrm{H}, \mathrm{CH}_{2}\right), 4.16\left(\mathrm{~s}, 3 \mathrm{H}, \mathrm{CH}_{3}\right), 7.24-7.31(\mathrm{~m}$, 5 H , ar), 7.40 (br s, 2H, NH $\mathrm{N}_{2}$ ), 8.36 ( $\mathrm{s}, 1 \mathrm{H}, \mathrm{H}-3$ ) ${ }^{13}{ }^{13} \mathrm{C}$ NMR (DMSO-d $\mathrm{d}_{6}$ ) 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 $\mathrm{C}_{13} \mathrm{H}_{13} \mathrm{~N}_{5}$ : C, $65.25 ;$ H, $5.48 ; \mathrm{N}, 29.27$. Found C, $65.02 ; \mathrm{H}, 5.62$ 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. $181-182{ }^{\circ} \mathrm{C}(\mathrm{EtOAc} / \mathrm{MeOH}) ;{ }^{1} \mathrm{H}$ NMR $\left(\mathrm{DMSO}_{-} \mathrm{d}_{6}\right) 1.96-2.02\left(\mathrm{~m}, 2 \mathrm{H}, \mathrm{CH}_{2}\right), 2.60\left(\mathrm{t}, 4 \mathrm{H}, \mathrm{CH}_{2}\right.$ $\left.+\mathrm{CH}_{2}\right), 4.10\left(\mathrm{~s}, 3 \mathrm{H}, \mathrm{CH}_{3}\right)$, 7.15-7.30 (m, 5 H , ar), 7.34 (broad s, $2 \mathrm{H}, \mathrm{NH}_{2}$ ), $8.15(\mathrm{~s}, 1 \mathrm{H}, \mathrm{H}-3)$. Anal. Calcd. for $\mathrm{C}_{15} \mathrm{H}_{17} \mathrm{~N}_{5}$ : 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$235{ }^{\circ} \mathrm{C}(\mathrm{EtOAc}) ;{ }^{1} \mathrm{H}$ NMR (DMSO-d ${ }_{6}$ ) $2.32\left(\mathrm{~s}, 3 \mathrm{H}, \mathrm{CH}_{3}\right), 5.59\left(\mathrm{~s}, 2 \mathrm{H}, \mathrm{CH}_{2}\right), 7.26-7.38(\mathrm{~m}, 7 \mathrm{H}, \mathrm{ar}+$ $\mathrm{NH}_{2}$ ), 8.30 ( $\mathrm{s}, 1 \mathrm{H}, \mathrm{H}-3$ ). IR 3455, 3301, 1650. ${ }^{13} \mathrm{C}$ NMR $\left(\mathrm{DMSOd}_{6}\right)$ 26.21, 57.05, 123.42, 128.01, 128.36, 129.08, 130.71, 140.25, 155.91, 161.19. Anal. Calcd. for $\mathrm{C}_{13} \mathrm{H}_{13} \mathrm{~N}_{5}$ : 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 ${ }^{\circ} \mathrm{C}$ (cyclohexane/EtOAc); ${ }^{1} \mathrm{H}$ NMR (DMSO- $\mathrm{d}_{6}$ ) $2.31\left(\mathrm{~s}, 3 \mathrm{H}, \mathrm{CH}_{3}\right), 3.23\left(\mathrm{t}, 2 \mathrm{H}, \mathrm{CH}_{2}, \mathrm{~J}=7.1\right.$ $\mathrm{Hz}), 4.61\left(\mathrm{t}, 2 \mathrm{H}, \mathrm{CH}_{2}, \mathrm{~J}=7.1 \mathrm{~Hz}\right), 7.17-7.33\left(\mathrm{~m}, 7 \mathrm{H}, 5 \mathrm{ar}+\mathrm{NH}_{2}\right), 8.05(\mathrm{~s}, 1 \mathrm{H}, \mathrm{H}-3) .{ }^{13} \mathrm{C}$ NMR $\left(\mathrm{CDCl}_{3}\right) 25.36,36.83,55.91,123.08,127.19,128.55,128.77,128.88,136.90,138.44,154.69$, 159.00. Anal. Calcd. for $\mathrm{C}_{14} \mathrm{H}_{15} \mathrm{~N}_{5}$ : 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. $183-184{ }^{\circ} \mathrm{C}$ (cyclohexane/EtOAc); ${ }^{1} \mathrm{H}$ NMR (DMSO-d $\mathrm{d}_{6}$ ) 3.91 ( $\mathrm{s}, 2 \mathrm{H}, \mathrm{CH}_{2}$ ), $5.60\left(\mathrm{~s}, 2 \mathrm{H}, \mathrm{CH}_{2}\right)$, 7.16-7.36 (m, 10 H , ar), 7.42 (s, 2H, NH2 $), 8.38$ (s, 1H, H-3). ${ }^{13} \mathrm{C}$ NMR (DMSO-d $\mathrm{d}_{6}$ ) 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 $\mathrm{C}_{19} \mathrm{H}_{17} \mathrm{~N}_{5}$ : 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$220{ }^{\circ} \mathrm{C}(\mathrm{EtOH}) ;{ }^{1} \mathrm{H}$ NMR (DMSO-d $\left.{ }_{6}\right) 5.63\left(\mathrm{~s}, 2 \mathrm{H}, \mathrm{CH}_{2}\right), 6.58-6.60(\mathrm{~m}, 1 \mathrm{H}$, furan proton), $7.02(\mathrm{~d}$, 1 H , furan proton, $\mathrm{J}=3.4 \mathrm{~Hz}), 7.28-7.30(\mathrm{~m}, 5 \mathrm{H}$, ar $), 7.58\left(\mathrm{~s}, 2 \mathrm{H}, \mathrm{NH}_{2}\right), 7.75-7.76(\mathrm{~m}, 1 \mathrm{H}$, furan proton), $8.47(\mathrm{~s}, 1 \mathrm{H}, \mathrm{H}) .{ }^{13} \mathrm{C}$ NMR (DMSO-d $\mathrm{d}_{6}$ ) 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 $\mathrm{C}_{16} \mathrm{H}_{13} \mathrm{~N}_{5} \mathrm{O}: \mathrm{C}, 65.97 ; \mathrm{H}, 4.50 ; \mathrm{N}, 24.04$. 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 ${ }^{\circ} \mathrm{C}(\mathrm{EtOH}) ;{ }^{1} \mathrm{H}$ NMR (DMSO-d $\left.\mathrm{d}_{6}\right) 3.25\left(\mathrm{t}, 2 \mathrm{H}, \mathrm{CH}_{2}, \mathrm{~J}=7.2 \mathrm{~Hz}\right), 4.65\left(\mathrm{t}, 2 \mathrm{H}, \mathrm{CH}_{2}\right.$, $\mathrm{J}=7.2 \mathrm{~Hz})$, 6.58-6.60 $(\mathrm{m}, 1 \mathrm{H}$, furan proton), 7.01-7.02 $(\mathrm{m}, 1 \mathrm{H}$, furan proton), 7.19-7.30 $(\mathrm{m}, 5 \mathrm{H}, \mathrm{ar})$, $7.56\left(\mathrm{~s}, 2 \mathrm{H}, \mathrm{NH}_{2}\right), 7.74-7.75(\mathrm{~m}, 1 \mathrm{H}$, furan proton), 8.21 ( $\mathrm{s}, 1 \mathrm{H}, \mathrm{H}-3$ ). IR 3242, 3163, 1650. Anal. Calcd. for $\mathrm{C}_{17} \mathrm{H}_{15} \mathrm{~N}_{5} \mathrm{O}: \mathrm{C}, 66.87 ; \mathrm{H}, 4.95 ; \mathrm{N}, 22.94$. 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 ${ }^{\circ} \mathrm{C}$ (cyclohexane/EtOAc); ${ }^{1} \mathrm{H}$ NMR (DMSO-d ${ }_{6}$ ) 3.85 (s, 3H, OMe), 5.58 (s, 2H, $\left.\mathrm{CH}_{2}\right), 6.60-6.58(\mathrm{~m}, 1 \mathrm{H}$, furan proton), $6.93(\mathrm{t}, 1 \mathrm{H}, \mathrm{ar}, \mathrm{J}=7.4 \mathrm{~Hz}), 6.98-7.02(\mathrm{~m}, 2 \mathrm{H}, 1 \mathrm{ar}+$ furan proton), $7.07(\mathrm{~d}, 1 \mathrm{H}, \mathrm{ar}, \mathrm{J}=8.2 \mathrm{~Hz}), 7.33(\mathrm{t}, 1 \mathrm{H}$, ar, $\mathrm{J}=8.6 \mathrm{~Hz}), 7.58\left(\mathrm{br} \mathrm{s}, 2 \mathrm{H}, \mathrm{NH}_{2}\right), 7.76(\mathrm{~d}, 1 \mathrm{H}$, furan proton, $\mathrm{J}=0.8 \mathrm{~Hz}$ ), $8.41(\mathrm{~s}, 1 \mathrm{H}, \mathrm{H}-3) ;{ }^{13} \mathrm{C}$ NMR $\left(\mathrm{DMSO}_{6}\right) 52.65,56.00,111.39,111.57$, $112.32,120.98,124.38,124.57,129.82,130.35,130.58,139.36,144.40,151.86,153.62,156.02$, 157.23, Anal. Calcd. for $\mathrm{C}_{17} \mathrm{H}_{15} \mathrm{~N}_{5} \mathrm{O}_{2}$ : 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 $\mathbf{2 3}$ ( 1.02 mmol ) following the procedure reported above to obtain compounds $\mathbf{4 , 6 , 9 , 1 2 , 1 4}$ but carrying out thereaction at room temperature for 16 h . The crude derivative was purified by column chromatography (eluent cyclohexane/EtOAc/MeOH, 2:7:1). Yield 84\%; ${ }^{1} \mathrm{H}$ NMR (DMSO-d ${ }_{6}$ ) 5.54 $\left(\mathrm{s}, 2 \mathrm{H}, \mathrm{CH}_{2}\right), 6.59-6.58(\mathrm{~m}, 1 \mathrm{H}$, furan proton), $6.78(\mathrm{t}, 1 \mathrm{H}, \operatorname{ar}, \mathrm{J}=7.2 \mathrm{~Hz}), 6.88(\mathrm{~d}, 1 \mathrm{H}$, ar, $\mathrm{J}=7.9$ $\mathrm{Hz}), 6.96(\mathrm{~d}, 1 \mathrm{H}$, ar, $\mathrm{J}=6.7 \mathrm{~Hz}), 7.02(\mathrm{~d}, 1 \mathrm{H}$, furan proton, $\mathrm{J}=3.2 \mathrm{~Hz}), 7.15(\mathrm{t}, 1 \mathrm{H}$, ar, $\mathrm{J}=7.2 \mathrm{~Hz})$, $7.58\left(\mathrm{br} \mathrm{s}, 2 \mathrm{H}, \mathrm{NH}_{2}\right), 7.75\left(\mathrm{~s}, 1 \mathrm{H}\right.$, furan proton) $8.30(\mathrm{~s}, 1 \mathrm{H}, \mathrm{H}), 9.88(\mathrm{br} \mathrm{s}, 1 \mathrm{H}, \mathrm{OH}) ;{ }^{13} \mathrm{C}$ 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 $\mathrm{C}_{16} \mathrm{H}_{13} \mathrm{~N}_{5} \mathrm{O}_{2}$ : 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 $10 \% \mathrm{Pd} / \mathrm{C}(10 \% \mathrm{w} / \mathrm{w}$ 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 $90 \% ;{ }^{1} \mathrm{H}$ NMR ( $\mathrm{DMSO}_{6}$ ) 4.6 (br s, 2H, $\mathrm{NH}_{2}$ ), 7.22 (s $1 \mathrm{H}, \mathrm{H}-5$ ), 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 $\mathrm{EtOH}(15.6 \mathrm{mmol})$ at $0^{\circ} \mathrm{C}$ for 1 h . The mixture was allowed to stand overnight in the refrigerator $\left(4^{\circ} \mathrm{C}\right)$ then was treated with anhydrous diethyl ether (about 25 $\mathrm{mL})$. The resulting solid was filtered off, washed with anhydrous diethyl ether ( $5-6 \mathrm{~mL}$ ) and dried in vacuo over $\mathrm{P}_{2} \mathrm{O}_{5}$ in a desiccator. The imidate hydrochloride was pure enough to be characterized ( ${ }^{1} \mathrm{H}-\mathrm{NMR}$, m.p.) and used without further purification. Yield $98 \%$; m.p. $141-143{ }^{\circ} \mathrm{C} .{ }^{1} \mathrm{H}$ NMR (DMSO-d $\mathrm{C}_{6}$ ) $1.42\left(\mathrm{t}, 3 \mathrm{H}, \mathrm{CH}_{3}, \mathrm{~J}=7.0 \mathrm{~Hz}\right), 2.44\left(\mathrm{~s}, 3 \mathrm{H}, \mathrm{CH}_{3}\right), 4.59\left(\mathrm{q}, 2 \mathrm{H}, \mathrm{CH}_{2}, \mathrm{~J}=7.0 \mathrm{~Hz}\right), 6.56-$ $6.57\left(\mathrm{~m}, 1 \mathrm{H}\right.$, furan proton), $7.91-7.92\left(\mathrm{~m}, 1 \mathrm{H}\right.$, furan proton), $11.56\left(\mathrm{br} \mathrm{s}, 2 \mathrm{H}, \mathrm{NH}_{2}{ }^{+}\right)$.
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 $\mathbf{5 4}$ ( 0.92 mmol ), anhydrous ammonium acetate ( 1.51 $\mathrm{mmol})$ and ethyl 5-methylfuran-2-carboximidate hydrochloride 55 ( 1.38 mmol ) was heated in a sealed tube at $120^{\circ} \mathrm{C}$ 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 $\mathrm{NaHCO}_{3}$ saturated solution (1-2 mL) and then dried. The crude derivative was used for the next step without further purification. Yield $70 \% ;{ }^{1} \mathrm{H}$ NMR $\left(\mathrm{DMSO}_{-} \mathrm{d}_{6}\right) 2.35\left(\mathrm{~s}, 3 \mathrm{H}, \mathrm{CH}_{3}\right), 6.21-6.22(\mathrm{~m}$, 1 H , furan proton), 6.93-6.94 (m, 1 H , furan proton), 7.51 (br s, $\mathrm{NH}_{2}$ ), 8.07 (br s, 1 H , pyrazole proton), 12.93 (br s, $1 \mathrm{H}, \mathrm{NH}$ ).

### 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 $\mathbf{5 6}(1.3 \mathrm{mmol})$ and $\mathrm{K}_{2} \mathrm{CO}_{3}(2.6 \mathrm{mmol})$ 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 $30-40 \mathrm{~mL}$ ) 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 $\mathrm{H}-3$ and $\mathrm{CH}_{2}$ 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 $\mathrm{CHCl}_{3} / \mathrm{MeOH}, 9: 1$ ). Yield $49 \%$; m.p. $257-259{ }^{\circ} \mathrm{C}$; ${ }^{1} \mathrm{H}$ NMR $\left(\mathrm{DMSO}-\mathrm{d}_{6}\right)$ $2.34(\mathrm{~s}, 3 \mathrm{H}, \mathrm{Me}), 5.62\left(\mathrm{~s}, 2 \mathrm{H}, \mathrm{CH}_{2}\right), 6.20-6.21(\mathrm{~m}, 1 \mathrm{H}$, furan proton), 6.90-6.91(m, 1 H , furan proton), 7.29-7.37 (m, 5H, ar), $7.59\left(\mathrm{br} \mathrm{s}, 2 \mathrm{H}, \mathrm{NH}_{2}\right), 8.43(\mathrm{~s}, 1 \mathrm{H}, \mathrm{H}-3)$. Anal. Calcd. for $\mathrm{C}_{17} \mathrm{H}_{15} \mathrm{~N}_{5} \mathrm{O}$ : 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 ${ }^{\circ} \mathrm{C}(\mathrm{EtOAc}) ;{ }^{1} \mathrm{H}$ NMR ( $\mathrm{DMSO}_{-} \mathrm{d}_{6}$ ) 2.34 ( $\mathrm{s}, 3 \mathrm{H}, \mathrm{Me}$ ), 3.85 ( $\mathrm{s}, 3 \mathrm{H}, \mathrm{OMe}$ ), $5.57\left(\mathrm{~s}, 2 \mathrm{H}, \mathrm{CH}_{2}\right), 6.20-$ $6.21(\mathrm{~m}, 1 \mathrm{H}$, furan proton $), 6.90-6.99(\mathrm{~m}, 3 \mathrm{H}, 2 \mathrm{ar}+$ furan proton $), 7.07(\mathrm{~d}, 1 \mathrm{H}, \mathrm{ar}, \mathrm{J}=8.3 \mathrm{~Hz}), 7.33$ (t, 1 H , ar, $\mathrm{J}=6.9 \mathrm{~Hz}$ ), 7.56 (br s, $2 \mathrm{H}, \mathrm{NH}_{2}$ ), $8.29(\mathrm{~s}, 1 \mathrm{H}, \mathrm{H}-3)$. Anal. Calcd. for $\mathrm{C}_{18} \mathrm{H}_{17} \mathrm{~N}_{5} \mathrm{O}_{2}: \mathrm{C}$, 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 ${ }^{\circ} \mathrm{C} ;{ }^{1} \mathrm{H}$ NMR (DMSO-d $\mathrm{d}_{6}$ ) 2.34 ( $\mathrm{s}, 3 \mathrm{H}, \mathrm{Me}$ ), $3.74(\mathrm{~s}, 3 \mathrm{H}, \mathrm{OMe}), 5.58\left(\mathrm{~s}, 2 \mathrm{H}, \mathrm{CH}_{2}\right), 6.20-6.21(\mathrm{~m}, 1 \mathrm{H}$, furan proton), $6.85(\mathrm{~d}, 1 \mathrm{H}$, ar, $\mathrm{J}=7.8 \mathrm{~Hz}), 6.89-6.91(\mathrm{~m}, 3 \mathrm{H}, 2 \mathrm{ar}+$ furan proton $), 7.28(\mathrm{t}, 1 \mathrm{H}, \mathrm{ar}, \mathrm{J}=7.8$ Hz ), 7.57 (br s, $2 \mathrm{H}, \mathrm{NH}_{2}$ ), 8.43 (s, 1H, H-3). Anal. Calcd. for $\mathrm{C}_{18} \mathrm{H}_{17} \mathrm{~N}_{5} \mathrm{O}_{2}$ : 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 $60^{\circ} \mathrm{C}$ for about 30 h . The crude solid was purified by preparative TLC (eluent $\mathrm{CH}_{2} \mathrm{Cl}_{2} / \mathrm{EtOAc} / \mathrm{MeOH}, 8: 1.5: 0.5$ ). Yield $68 \%$; m.p. $252-254{ }^{\circ} \mathrm{C} ;{ }^{1} \mathrm{H}$ NMR (DMSO-d ${ }_{6}$ ) $2.35\left(\mathrm{~s}, 3 \mathrm{H}, \mathrm{CH}_{3}\right), 5.69\left(\mathrm{~s}, 2 \mathrm{H}, \mathrm{CH}_{2}\right), 6,20(\mathrm{~s}, 1 \mathrm{H}$, furan proton), $6.92(\mathrm{~d}, 1 \mathrm{H}$, furan proton, $\mathrm{J}=2.9 \mathrm{~Hz}), 7.29-7.19(\mathrm{~m}, 3 \mathrm{H}, \mathrm{ar}), 7.44-7.40(\mathrm{~m}, 1 \mathrm{H}, \mathrm{ar}), 7.57\left(\mathrm{br} \mathrm{s}, 2 \mathrm{H}, \mathrm{NH}_{2}\right), 8.41(\mathrm{~s}, 1 \mathrm{H}$, 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 $\mathrm{C}_{17} \mathrm{H}_{14} \mathrm{FN}_{5} \mathrm{O}: \mathrm{C}, 63.15 ; \mathrm{H}$, 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 ${ }^{\circ} \mathrm{C}$ $\left(\mathrm{MeNO}_{2}\right) ;{ }^{1} \mathrm{H}$ NMR ( $\mathrm{DMSO}_{6}$ ) $2.35\left(\mathrm{~s}, 3 \mathrm{H}, \mathrm{CH}_{3}\right), 5.65\left(\mathrm{~s}, 2 \mathrm{H}, \mathrm{CH}_{2}\right), 6.20-6.21(\mathrm{~m}, 1 \mathrm{H}$, furan proton), $6.92(\mathrm{~d}, 1 \mathrm{H}$, furan proton, $\mathrm{J}=3.2 \mathrm{~Hz}), 7.11-7.19(\mathrm{~m}, 3 \mathrm{H}, \mathrm{ar}), 7.39-7.45(\mathrm{~m}, 1 \mathrm{H}$, ar), $7.58(\mathrm{br}$
$\mathrm{s}, 2 \mathrm{H}$, ar), 8.46 (s, 1H, H-3). Anal. Calcdd. for $\mathrm{C}_{17} \mathrm{H}_{14} \mathrm{FN}_{5} \mathrm{O}: \mathrm{C}, 63.15$; 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 $60{ }^{\circ} \mathrm{C}$ for about 7 h . Yield $29 \%$; m.p. 281-283 ${ }^{\circ} \mathrm{C}$ (2Methoxyethanol/EtOAc); ${ }^{1} \mathrm{H}$ NMR ( $\mathrm{DMSO}_{6}$ ) $2.35\left(\mathrm{~s}, 3 \mathrm{H}, \mathrm{CH}_{3}\right), 5.74\left(\mathrm{~s}, 2 \mathrm{H}, \mathrm{CH}_{2}\right), 6.22-6.21(\mathrm{~m}$, 1 H , furan proton), $6.92(\mathrm{~d}, 1 \mathrm{H}$, furan proton, $\mathrm{J}=3.2 \mathrm{~Hz}), 6.99(\mathrm{~d}, 1 \mathrm{H}, \mathrm{ar}, \mathrm{J}=5.8 \mathrm{~Hz}), 7.32-7.41(\mathrm{~m}$, $2 \mathrm{H}, \mathrm{ar}), 7.53\left(\mathrm{~d}, 1 \mathrm{H}, \mathrm{ar}, \mathrm{J}=6.5 \mathrm{~Hz}\right.$ ), 7.59 (br s, $2 \mathrm{H}, \mathrm{NH}_{2}$ ), 8.41 (s, 1H, H-3). Anal. Calcd. for $\mathrm{C}_{17} \mathrm{H}_{14} \mathrm{ClN}_{5} \mathrm{O}: \mathrm{C}, 60.09 ; \mathrm{H}, 4.15 ; \mathrm{N}, 20.61$. Found C, $60.25 ; \mathrm{H}, 3.98 ; \mathrm{N}, 20.56$.

### 5.1.18. General procedure for the synthesis of hydroxybenzyl-substituted-7-

 aminopyrazolo[4,3-d]pyrimidine derivatives $\mathbf{3 0}$ and $\mathbf{3 1}$The title compounds were prepared from the corresponding methoxy derivatives 25 and 26 (1.41 mmol ) following the procedure reported above to obtain compounds $\mathbf{4 , 6 , 9 , 1 2 , 1 4}$ 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 $\mathrm{CHCl}_{3} / \mathrm{MeOH}, ~ 9.3: 0.7$ for 30, and $\mathrm{CHCl}_{3} / \mathrm{MeOH}, 9: 1$ 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. $>300{ }^{\circ} \mathrm{C} ;{ }^{1} \mathrm{H}$ NMR ( $\mathrm{DMSO}_{-} \mathrm{d}_{6}$ ) $2.34\left(\mathrm{~s}, 3 \mathrm{H}, \mathrm{CH}_{3}\right.$ ), $5.53\left(\mathrm{~s}, 2 \mathrm{H}, \mathrm{CH}_{2}\right), 6.20-6-$ $19(\mathrm{~m}, 1 \mathrm{H}$, furan proton $), 6.77(\mathrm{t}, 1 \mathrm{H}, \mathrm{ar}, \mathrm{J}=7.5 \mathrm{~Hz}), 6.91-6.87(\mathrm{~m}, 2 \mathrm{H}, 1 \mathrm{ar}+$ furan proton $), 6.95$ $(\mathrm{d}, 1 \mathrm{H}, \mathrm{ar}, \mathrm{J}=6.3 \mathrm{~Hz}), 7.18-7.14(\mathrm{~m}, 1 \mathrm{H}, \mathrm{ar}), 7.54\left(\mathrm{br} \mathrm{s}, 2 \mathrm{H}, \mathrm{NH}_{2}\right), 8.26(\mathrm{~s}, 1 \mathrm{H}, \mathrm{H}-3), 9.89(\mathrm{br} \mathrm{s}, 1 \mathrm{H}$, $\mathrm{OH}) .{ }^{13} \mathrm{C}$ 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 $\mathrm{C}_{17} \mathrm{H}_{15} \mathrm{~N}_{5} \mathrm{O}_{2}$ : 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 ${ }^{\circ} \mathrm{C}$; ${ }^{1} \mathrm{H}$ NMR ( $\mathrm{DMSO}_{6}$ ) $2.38(\mathrm{~s}, 3 \mathrm{H}, \mathrm{Me}), 5.57\left(\mathrm{~s}, 2 \mathrm{H}, \mathrm{CH}_{2}\right), 6.24-$ $6.25(\mathrm{~m}, 1 \mathrm{H}$, furan proton), $6.64(\mathrm{~d}, 1 \mathrm{H}$, ar, $\mathrm{J}=1.8 \mathrm{~Hz}), 6.71-6.78(\mathrm{~m}, 2 \mathrm{H}, \operatorname{ar}), 6.95-6.96(\mathrm{~m}, 1 \mathrm{H}$,
furan proton), $7.19(\mathrm{t}, 1 \mathrm{H}, \mathrm{ar}, \mathrm{J}=7.8 \mathrm{~Hz}), 7.64\left(\mathrm{br} \mathrm{s}, 2 \mathrm{H}, \mathrm{NH}_{2}\right), 8.44(\mathrm{~s}, 1 \mathrm{H}, \mathrm{H}-3), 9.49(\mathrm{~s}, 1 \mathrm{H}, \mathrm{OH})$.
Anal. Calcd. for $\mathrm{C}_{17} \mathrm{H}_{15} \mathrm{~N}_{5} \mathrm{O}_{2}$ : 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 $h A_{2 A} A R$ structure and homology modeling of the $h A_{1} A R$

The crystal structure of the $\mathrm{hA}_{2 \mathrm{~A}} \mathrm{AR}$ in complex with ZM241385 were retrieved from the Protein Data Bank (http://www.rcsb.org; pdb code: 3EML; 2.6-A resolution ${ }^{37}$ ). The structure was remodelled 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 $\mathrm{hA}_{2 \mathrm{~A}}$ 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 $0.05 \mathrm{~kJ} \mathrm{~mol}^{-1} \AA^{-1}$. 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 $\varphi-\psi$ dihedral plots, and sidechain-rotamer and non-bonded contact quality. The $\mathrm{hA}_{2 \mathrm{~A}} \mathrm{AR}$ crystal structure was used as a template for the development of a $\mathrm{hA}_{1} \mathrm{AR}$ 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 $\mathrm{hA}_{2 \mathrm{~A}}$ AR were then applied for the corresponding sequences of the TM helices of the $\mathrm{hA}_{1} \mathrm{AR}$. 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 $3 \mathrm{EML} \mathrm{hA}_{2 \mathrm{~A}}$ AR re-modeling stage.

### 5.2.2. Molecular docking analysis

The compound structures were docked into the binding site of the $\mathrm{hA}_{2 \mathrm{~A}} \mathrm{AR}$ and the $\mathrm{hA}_{1} \mathrm{AR}$ 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 $0.375 \AA$.

### 5.2.3. Post Docking analysis. Residue interaction analysis

The interactions between the ligands and the receptors binding site were analysed by using the $I F-E$ 6.0 tool $^{44}$ retrievable at the SVL exchange service (Chemical Computing Group, Inc. SVL exchange: $\underline{\text { http://svl.chemcomp.com }) . ~ T h e ~ p r o g r a m ~ c a l c u l a t e s ~ a n d ~ d i s p l a y s ~ t h e ~ a t o m i c ~ a n d ~ r e s i d u e ~}$ 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 Á distance from ligand were considered for this analysis.

### 5.3. Pharmacology

### 5.3.1. Human cloned $A_{1}, A_{2 A}$ and $A_{3}$ AR Binding Assay

All synthesized compounds were tested to evaluate their affinity at human $A_{1}, A_{2 A}$ and $A_{3}$ ARs. Displacement experiments of $\left[{ }^{3} \mathrm{H}\right]$ DPCPX $(1 \mathrm{nM})$ to $\mathrm{hA}_{1} \mathrm{CHO}$ membranes ( $50 \mu \mathrm{~g}$ of protein/assay) and at least 6-8 different concentrations of antagonists for 120 min at $25^{\circ} \mathrm{C}$ in 50 mM Tris HCl buffer pH 7.4 were performed. ${ }^{51}$ Non-specific binding was determined in the presence $1 \mu \mathrm{M}$ of

DPCPX ( $\leq 10 \%$ of the total binding). Binding of $\left[{ }^{3} \mathrm{H}\right] \mathrm{ZM}-241385(1 \mathrm{nM})$ to $\mathrm{hA}_{2 \mathrm{~A}} \mathrm{CHO}$ membranes ( $50 \mu \mathrm{~g}$ of protein/assay) was performed by using 50 mM Tris HCl buffer, $10 \mathrm{mM} \mathrm{MgCl} \mathrm{mH}_{2} 7.4$ and at least 6-8 different concentrations of antagonists studied for an incubation time of 60 min at 4 ${ }^{\circ} \mathrm{C} .{ }^{52}$ Non-specific binding was determined in the presence of $1 \mu \mathrm{M} \mathrm{ZM}-241385$ and was about $20 \%$ of total binding. Competition binding experiments to $\mathrm{hA}_{3} \mathrm{CHO}$ membranes ( $50 \mu \mathrm{~g}$ of protein/assay) were performed incubating $0.5 \mathrm{nM}\left[{ }^{125} \mathrm{I}\right] \mathrm{AB}-\mathrm{MECA}, 50 \mathrm{mM}$ Tris HCl buffer, 10 mM $\mathrm{MgCl}_{2}, 1 \mathrm{mM}$ EDTA, pH 7.4 and at least 6-8 different concentrations of examined ligands for 60 $\min$ at $37{ }^{\circ} \mathrm{C} .{ }^{53}$ Non-specific binding was defined as binding in the presence of $1 \mu \mathrm{M} \mathrm{AB}$-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 $\mathbf{C H O}$ cells transfected with $h A_{1}, h A_{2 A}$ and $h A_{2 B}$

## 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 ( $1 \times 10^{6}$ cells /assay) were suspended in 0.5 ml of incubation mixture $(\mathrm{mM}): \mathrm{NaCl} 15, \mathrm{KCl} 0.27, \mathrm{NaH}_{2} \mathrm{PO}_{4} 0.037, \mathrm{MgSO}_{4} 0.1, \mathrm{CaCl}_{2} 0.1$, Hepes $0.01, \mathrm{MgCl}_{2} 1$, glucose $0.5, \mathrm{pH} 7.4$ at $37^{\circ} \mathrm{C}, 2 \mathrm{IU} / \mathrm{ml}$ 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 $37^{\circ} \mathrm{C}$. The potency of antagonists was determined by the inhibition of the effect of CCPA $(1 \mathrm{nM})$, NECA $(10 \mathrm{nM})$ or NECA $(200 \mathrm{nM})$ for $\mathrm{hA}_{1}, \mathrm{hA}_{2 \mathrm{~A}}$ and $\mathrm{hA}_{2 \mathrm{~B}}$ 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 $4^{\circ} \mathrm{C}$ 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 $\left[{ }^{3} \mathrm{H}\right]$ cyclic AMP and incubation buffer (trizma base 0.1 M , aminophylline 8.0 mM , 2-mercaptoethanol $6.0 \mathrm{mM}, \mathrm{pH} 7.4$ ). The binding protein prepared from beef adrenals was added to the samples previously incubated at $4{ }^{\circ} \mathrm{C}$ 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 $\left(\mathrm{K}_{\mathrm{i}}\right)$ values were calculated from those of $\mathrm{IC}_{50}$ according to Cheng \& Prusoff equation $\mathrm{K}_{\mathrm{i}}=\mathrm{IC}_{50} /\left(1+\left[\mathrm{C}^{*}\right] / \mathrm{K}_{\mathrm{D}} *\right)$, where $\left[\mathrm{C}^{*}\right]$ is the concentration of the radioligand and $\mathrm{K}_{\mathrm{D}} *$ its dissociation constant. ${ }^{56} \mathrm{~A}$ weighted non-linear least-squares curve fitting program LIGAND ${ }^{57}$ was used for computer analysis of inhibition experiments. $\mathrm{IC}_{50}$ 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 $A_{2 A}$ AR. A. Binding mode 1 conformation (see text for details) of compound $\mathbf{C}$. Key receptor residues and domains are indicated. B. Binding mode 2 conformation of compound 3 .

Figure 4. Docking conformation of compound $\mathbf{2 5}$ at the $\mathrm{A}_{2 \mathrm{~A}}$ 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 $\mathbf{2 5}$, 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 $\mathbf{8}$ (light) and 11 (dark).

Figure 6. Docking conformation of compounds 25 (A) and 27 (B) at the $\mathrm{hA}_{1}$ AR binding site. Key receptor residues and domains are indicated.


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[^1]:    ${ }^{1}$ 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, $\quad 8$-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, ${ }^{6}$-(4-amino-3-iodobenzyl)-5'-(N-methylcarbamoyl)adenosine; EL2, second extracellular loop; MOE, molecular operating environment

