







Article

# 2-Aryladenine Derivatives as a Potent Scaffold for Adenosine Receptor Antagonists: The 6-Morpholino Derivatives

Filipe Areias <sup>1,2,3,†</sup>, Carla Correia <sup>1,†</sup>, Ashly Rocha <sup>1</sup>, Sofia Teixeira <sup>1</sup>, Marián Castro <sup>2,4</sup>, Jose Brea <sup>2,4</sup>, Huabin Hu <sup>5</sup>, Jens Carlsson <sup>5</sup>, Maria I. Loza <sup>2,4</sup>, M. Fernanda Proença <sup>1</sup> and M. Alice Carvalho <sup>1,\*</sup>

- <sup>1</sup> Centre of Chemistry of University of Minho (CQUM), Campus de Gualtar, Universidade do Minho, 4710-057 Braga, Portugal; filipeareias@yahoo.com.br (F.A.); carlabcpereira@gmail.com (C.C.); ashlyrochaa@gmail.com (A.R.); id9191@alunos.uminho.pt (S.T.); fprouenca@quimica.uminho.pt (M.F.P.)
- <sup>2</sup> Center for Research in Molecular Medicine and Chronic Diseases (CiMUS), Universidade de Santiago de Compostela, Avda de Barcelona, E-15782 Santiago de Compostela, Spain; marian.castro@usc.es (M.C.); pepo.brea@usc.es (J.B.); mabel.loza@usc.es (M.I.L.)
- <sup>3</sup> School of Chemical Sciences & Engineering, Yachay Tech University, Yachay City of Knowledge, Urcuqui 100119, Ecuador
- <sup>4</sup> Instituto de Investigación Sanitaria de Santiago de Compostela (IDIS), Travesía da Choupana s/n, E-15706 Santiago de Compostela, Spain
- <sup>5</sup> Science for Life Laboratory, Department of Cell and Molecular Biology, Uppsala University, SE-75124 Uppsala, Sweden; huabin.hu@icm.uu.se (H.H.); jens.carlsson.lab@gmail.com (J.C.)
- \* Correspondence: mac@quimica.uminho.pt
- † These authors contributed equally to this work.

**Abstract:** A set of 2-aryl-9-H or methyl-6-morpholinopurine derivatives were synthesized and assayed through radioligand binding tests at human A<sub>1</sub>, A<sub>2A</sub>, A<sub>2B</sub>, and A<sub>3</sub> adenosine receptor subtypes. Eleven purines showed potent antagonism at A<sub>1</sub>, A<sub>3</sub>, dual A<sub>1</sub>/A<sub>2A</sub>, A<sub>1</sub>/A<sub>2B</sub>, or A<sub>1</sub>/A<sub>3</sub> adenosine receptors. Additionally, three compounds showed high affinity without selectivity for any specific adenosine receptor. The structure-activity relationships were made for this group of new compounds. The 9-methylpurine derivatives were generally less potent but more selective, and the 9H-purine derivatives were more potent but less selective. These compounds can be an important source of new biochemical tools and/or pharmacological drugs.

**Keywords:** G protein-coupled receptors; adenine derivatives; adenosine receptor antagonists; 2-arylpurine derivatives; structure-activity relationship



**Citation:** Areias, F.; Correia, C.; Rocha, A.; Teixeira, S.; Castro, M.; Brea, J.; Hu, H.; Carlsson, J.; Loza, M.I.; Proença, M.F.; et al. 2-Aryladenine Derivatives as a Potent Scaffold for Adenosine Receptor Antagonists: The 6-Morpholino Derivatives. *Molecules* **2024**, *29*, 2543. <https://doi.org/10.3390/molecules29112543>

Academic Editors: Igor Ivanov and Zhangguo Gao

Received: 8 February 2024

Revised: 6 May 2024

Accepted: 24 May 2024

Published: 28 May 2024



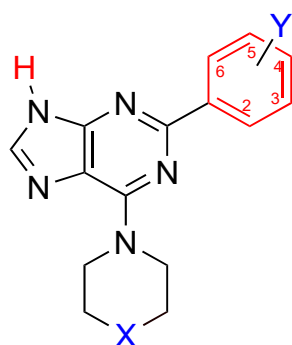
**Copyright:** © 2024 by the authors. Licensee MDPI, Basel, Switzerland. This article is an open access article distributed under the terms and conditions of the Creative Commons Attribution (CC BY) license (<https://creativecommons.org/licenses/by/4.0/>).

## 1. Introduction

Adenosine, a purine nucleoside composed of an adenine linked to a ribose via a  $\beta$ -N9-glycosidic bond, modulates many physiological conditions related to neurological, immunologic, and cardiovascular systems [1,2]. Adenosine receptors, namely A<sub>1</sub>, A<sub>2A</sub>, A<sub>2B</sub>, and A<sub>3</sub>, belong to the G protein-coupled receptor (GPCR) superfamily and are widely recognized as attractive targets for the design and development of new therapeutic agents against different clinical disorders [3–5]. The activation of the A<sub>1</sub> and A<sub>3</sub> receptors inhibits adenylyl cyclase activity through the action of G<sub>i/o</sub> proteins and, as a result, intracellular cyclic AMP (cAMP) decreases, while the A<sub>2A</sub> and A<sub>2B</sub> receptors increase cAMP production through the action of G<sub>s</sub> proteins [6]. So, it is of therapeutic importance to synthesize new drugs active on adenosine receptors A<sub>1</sub>, A<sub>3</sub>, and, additionally, dual A<sub>1</sub>/A<sub>3</sub> to study the synergistic effect between these two receptor subtypes as they preferentially couple to the same G proteins [2]. Indeed, there are many patents for adenosine receptor ligands, and some of these ligands are already in clinical trials or waiting for FDA approval [4,5,7]. For example, A<sub>1</sub> ligands are useful for glaucoma, heart failure, angina, seizures, ischemia, depression, obesity, asthma, renal protection, edema associated with congestive heart failure, atrial arrhythmias, type II diabetes, neuropathic pain, neuroprotection, atrial fibrillation,

tachycardia, cardioprotection, and sleep regulation [1,8–12]. In addition, A<sub>3</sub> ligands are useful for liver regeneration, hepatitis, psoriasis, rheumatoid arthritis, inflammation, dry eye syndrome, fibrotic diseases, neurodegeneration, ischaemia, asthma, chronic obstructive pulmonary disease, glaucoma, and cancer [13–20], with A<sub>3</sub> antagonist PBF-677 (Palobiofarma SL), that has reached clinical trials for glaucoma, ulcerative colitis, and eosinophilic esophagitis [21,22]. Finally, dual A<sub>1</sub>/A<sub>3</sub> ligands can be useful as potential therapeutics for treating glaucoma, kidney failure, pulmonary diseases, and Alzheimer's disease [23,24]. Despite the fact that several adenosine receptor ligands are already in clinical trials, achieving selectivity is still a challenge. Additionally, a number of side effects were reported for A<sub>1</sub> antagonists in the literature, such as an increase in the frequency of seizures, strokes [25,26], dizziness, nausea, transient hypertension, and transient hypotension [27] in treated patients, which justifies the search for new high-affinity and selective drugs.

In our previous work, we synthesized and identified a new scaffold for adenosine receptor antagonists based on the adenine nucleus (Figure 1) [28]. Highly potent and selective compounds were identified as having a piperidinyl group in C-6, a proton in N-9, and several selected aryl groups in C-2 of the purine nucleus. Indeed, the structure-activity relationship (SAR) study indicated that an aryl unit at C-2 is crucial for activity, and a proton at N-9 increased potency. Furthermore, a piperidinyl group in C-6 instead of a 4-*N*-methylpiperazinyl group led to purines with higher or similar affinity for the adenosine receptors A<sub>1</sub> and A<sub>3</sub> but with higher selectivity.



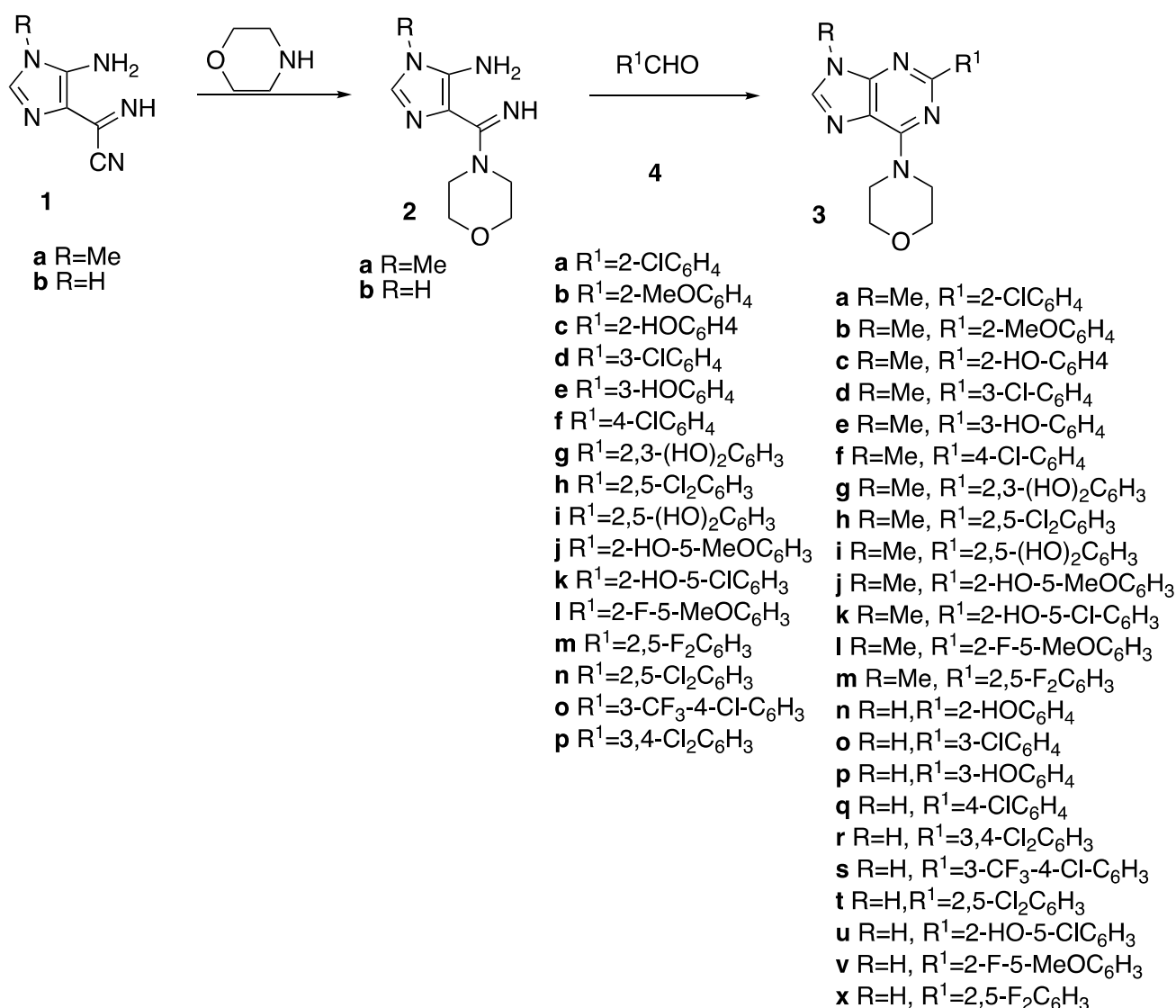
**Figure 1.** The adenosine receptor antagonists scaffold identified in our previous work.

As an attempt to increase the affinity and selectivity of the compounds obtained in our previous work and to establish the importance of group X (Figure 1), herein, we synthesized new 6-morpholino purine derivatives substituted with a proton or a methyl group in N-9 and a selection of aryl substituents in the C-2 position of the purine nucleus. The results will allow us to complete the SAR study for the adenine-based scaffold identified in our previous publication.

## 2. Results and Discussion

### 2.1. Chemistry

The target compounds **3a–x** were synthesized through the synthetic approach described in Scheme 1. The starting reagents **1** [29] and **2** [28,30] were obtained following the procedures described in previous work. Briefly, the commercially available diamino-maleonitrile was treated with triethyl orthoformate, under reflux. The solid obtained was reacted with the convenient amine followed by treatment with a base to generate imidazoles **1**. The compounds **2** were obtained in excellent yield through reaction of **1** with the excess of morpholine, in acetonitrile, at room temperature.



**Scheme 1.** Synthetic approach to 2-aryl-adenine derivatives 3a–x.

The products **3** were obtained through reaction of compounds **2** with different aldehydes **4** following a previously described methodology [28,30]. The experimental conditions used in each reaction mainly depended on the aldehyde. Generally, when imidazoles **1** were reacted with non-phenolic aldehydes **4**, the reactions were performed in basic medium. Considering that compounds **2** are temperature-sensitive, the reactions always started at low to moderate temperature. When the reactions were carried out under those conditions (Method A), they were slower, but the products **3** were precipitated pure from solution and isolated through simple filtration from the reaction medium. In order to accelerate the reactions, most of them started at moderate temperature, but, when the TLC showed the absence of imidazoles **2**, the temperature was increased and the reactions were monitored by TLC until the spots assigned to the intermediates were absent. In these cases, the reactions led to black-greyish solids that required purification. The purification was achieved through filtration of a dichloromethane solution of the compounds through a silica gel column.

To obtain the phenolic derivatives, the reactions were performed in an acidic medium until complete consumption of the reagents (evidenced by TLC) and then continued in a basic medium at a temperature between 40 and 80 °C. In these reactions, degradation of the reaction mixture also occurred when higher temperatures were used. The pure

products were obtained following the purification procedure described above for the other derivatives. The new compounds were fully characterized by usual techniques ( $^1\text{H}$  and  $^{13}\text{C}$  NMR spectra are presented in the supporting information).

## 2.2. Pharmacology

All compounds synthesized in this work have a morpholine group as a substituent at C-6, an H atom or a methyl group at N-9, and different substituted aryl groups at C-2 of the purine nucleus. The compounds (**3a–x**) were assessed through radioligand binding assays at all human adenosine subtype receptors ( $A_1$ ,  $A_{2A}$ ,  $A_{2B}$ , and  $A_3$ ) expressed in mammalian cell lines. The percentage of inhibition ( $\%_{\text{inhib}}$ ) of radioligand binding was determined for all compounds at 10  $\mu\text{M}$  concentration. Those compounds showing a  $\%_{\text{inhib}}$  higher than 80% were tested at different concentrations to determine their affinities (calculated as  $pK_i$ ) at the diverse receptors. Table 1 shows the binding affinities of all the synthesized compounds **3** at adenosine receptors  $A_1$ ,  $A_{2A}$ ,  $A_{2B}$ , and  $A_3$ .

**Table 1.** Binding affinities of compounds **3** at all human adenosine receptors expressed as  $\%_{\text{inhib}}$  at 10  $\mu\text{M}$  or  $pK_i$ . Values are reported as mean  $\pm$  SEM of three experiments with duplicate measurements.

	R	R <sup>1</sup>	A <sub>1</sub>	A <sub>2A</sub>	A <sub>2B</sub>	A <sub>3</sub>
a	Me	2-ClC <sub>6</sub> H <sub>4</sub>	36 $\pm$ 1%	11 $\pm$ 2%	22 $\pm$ 5%	14 $\pm$ 2%
b	Me	2-MeOC <sub>6</sub> H <sub>4</sub>	22 $\pm$ 1%	12 $\pm$ 5%	12 $\pm$ 2%	36 $\pm$ 5%
c	Me	2-HOC <sub>6</sub> H <sub>4</sub>	<b>6.51 <math>\pm</math> 0.11</b>	<b>6.47 <math>\pm</math> 0.05</b>	<b>5.70 <math>\pm</math> 0.07</b>	6 $\pm$ 5%
d	Me	3-ClC <sub>6</sub> H <sub>4</sub>	<b>6.80 <math>\pm</math> 0.07</b>	15 $\pm$ 4%	2 $\pm$ 2%	25 $\pm$ 4%
e	Me	3-HOC <sub>6</sub> H <sub>4</sub>	<b>6.16 <math>\pm</math> 0.21</b>	37 $\pm$ 1%	34 $\pm$ 3%	52 $\pm$ 4%
f	Me	4-ClC <sub>6</sub> H <sub>4</sub>	37 $\pm$ 4%	42 $\pm$ 5%	15 $\pm$ 4%	31 $\pm$ 5%
g	Me	2,3-(HO) <sub>2</sub> C <sub>6</sub> H <sub>3</sub>	33 $\pm$ 0.3%	25 $\pm$ 2%	22 $\pm$ 1.5%	42 $\pm$ 3%
h	Me	2,5-Cl <sub>2</sub> C <sub>6</sub> H <sub>3</sub>	41 $\pm$ 2%	2 $\pm$ 3%	22 $\pm$ 3%	8 $\pm$ 1%
i	Me	2,5-(HO) <sub>2</sub> C <sub>6</sub> H <sub>3</sub>	<b>6.36 <math>\pm</math> 0.15</b>	<b>6.25 <math>\pm</math> 0.12</b>	<b>6.32 <math>\pm</math> 0.01</b>	53 $\pm$ 5%
j	Me	2-HO-5-MeOC <sub>6</sub> H <sub>3</sub>	<b>6.28 <math>\pm</math> 0.17</b>	<b>5.91 <math>\pm</math> 0.11</b>	<b>5.78 <math>\pm</math> 0.03</b>	19 $\pm$ 11%
k	Me	2-HO-5-ClC <sub>6</sub> H <sub>3</sub>	<b>6.91 <math>\pm</math> 0.08</b>	<b>6.15 <math>\pm</math> 0.03</b>	55 $\pm$ 1%	53 $\pm$ 4%
l	Me	2-F-5-MeOC <sub>6</sub> H <sub>3</sub>	61 $\pm$ 4%	42 $\pm$ 1%	27 $\pm$ 2%	<b>5.28 <math>\pm</math> 0.11</b>
m	Me	2,5-F <sub>2</sub> C <sub>6</sub> H <sub>3</sub>	<b>6.28 <math>\pm</math> 0.13</b>	37 $\pm$ 2%	30 $\pm$ 4%	25 $\pm$ 3%
n	H	2-HOC <sub>6</sub> H <sub>4</sub>	<b>7.19 <math>\pm</math> 0.10</b>	<b>6.97 <math>\pm</math> 0.07</b>	<b>6.86 <math>\pm</math> 0.10</b>	<b>6.59 <math>\pm</math> 0.06</b>
o	H	3-ClC <sub>6</sub> H <sub>4</sub>	<b>7.75 <math>\pm</math> 0.14</b>	<b>7.33 <math>\pm</math> 0.07</b>	<b>7.12 <math>\pm</math> 0.13</b>	<b>7.40 <math>\pm</math> 0.14</b>
p	H	3-HOC <sub>6</sub> H <sub>4</sub>	<b>7.49 <math>\pm</math> 0.06</b>	<b>6.26 <math>\pm</math> 0.12</b>	60 $\pm$ 6%	<b>6.33 <math>\pm</math> 0.08</b>
q	H	4-ClC <sub>6</sub> H <sub>4</sub>	<b>7.02 <math>\pm</math> 0.07</b>	56 $\pm$ 3%	50 $\pm$ 4%	<b>7.47 <math>\pm</math> 0.05</b>
r	H	3,4-Cl <sub>2</sub> C <sub>6</sub> H <sub>3</sub>	29 $\pm$ 1%	22 $\pm$ 5%	35 $\pm$ 3%	<b>7.15 <math>\pm</math> 0.08</b>
s	H	3-CF <sub>3</sub> -4-ClC <sub>6</sub> H <sub>3</sub>	43 $\pm$ 5%	13 $\pm$ 5%	8 $\pm$ 5%	<b>6.02 <math>\pm</math> 0.04</b>
t	H	2,5-Cl <sub>2</sub> C <sub>6</sub> H <sub>3</sub>	<b>5.97 <math>\pm</math> 0.11</b>	44 $\pm$ 2%	26 $\pm$ 1%	<b>7.16 <math>\pm</math> 0.17</b>
u	H	2-HO-5-ClC <sub>6</sub> H <sub>3</sub>	<b>7.05 <math>\pm</math> 0.09</b>	35 $\pm$ 1%	<b>6.21 <math>\pm</math> 0.08</b>	37 $\pm$ 1%
v	H	2-F-5-MeOC <sub>6</sub> H <sub>3</sub>	38 $\pm$ 2%	45 $\pm$ 4%	<b>6.40 <math>\pm</math> 0.16</b>	<b>7.83 <math>\pm</math> 0.16</b>
x	H	2,5-F <sub>2</sub> C <sub>6</sub> H <sub>3</sub>	<b>8.23 <math>\pm</math> 0.06</b>	<b>7.15 <math>\pm</math> 0.05</b>	<b>7.44 <math>\pm</math> 0.11</b>	<b>7.63 <math>\pm</math> 0.12</b>

Analysis of the data presented in the Table 1 shows that at 10  $\mu\text{M}$ , several compounds presented a percentage inhibition higher than 80%. The  $pK_i$  was determined, being in the range of  $5.28 \leq pK_i \leq 8.23$  (**3l**, **3x**). Both series, R = H and R = Me, gave ligands with high affinity to the four receptors subtypes; however, higher affinities are observed for derivatives with R = H (**3d** vs. **3h**, **3c** vs. **3n**, **3e** vs. **3p**, **3k** vs. **3u**, **3l** vs. **3v**, and **3m** vs. **3x**). The higher potency of derivatives with a proton at N-9 agrees with the results published in our previous work [28], but the increase in potency of N-9-methyl derivatives having a morpholine unit at C-6 may indicate that the morpholine ring is interacting with the target through the oxygen atom. When we look for selectivity, we find selective ligands either having a methyl group at N-9 (**3d**, **3e**, **3l** and **3m**) or a proton (**3p**, **3r**, **3s**, **3t** and **3v**). Additionally, the ligands with a methyl group at N-9 show high potency mainly for the  $A_1$  receptor (**3c**, **3d**, **3e**, **3i**, **3j**, **3k**, and **3m**), and ligands with a proton at N-9 presented high potency for both the  $A_1$  and  $A_3$  receptors (**3n–3x**). To evaluate the influence of the

$R^1$  group on potency and selectivity against the four receptors, we analyzed first the  $R^1 =$  chloro-derivatives, compounds **3a**, **3d**, **3f**, **3h**, **3o**, **3q**, **3r**, **3s**, and **3t**.

In this set, the most potent and selective compound for the  $A_1$  receptor was **3d**, with  $R^1 = 3\text{-ClC}_6\text{H}_4$  [ $pK_i (A_1) = 6.80 \pm 0.07$ ]. Compounds **3r** ( $R^1 = 3,4\text{-Cl}_2\text{C}_6\text{H}_3$ ) and **3s** ( $R^1 = 3\text{-CF}_3\text{-4-ClC}_6\text{H}_3$ ) were highly potent and selective for the  $A_3$  receptor. Compound **3q** ( $R^1 = 4\text{-ClC}_6\text{H}_4$ ) showed dual selectivity for  $A_1/A_3$  receptors. These results seem to indicate that the presence of an electron-withdrawing chlorine atom at the *meta* position together with a hydrophobic methyl group at N-9 interacts efficiently with the  $A_1$  receptor (**3d**). However, when the hydrophilic hydrogen was at N-9, a promiscuous ligand resulted (**3o**). The presence of a chlorine atom at the *para* position and a proton at N-9 interacts efficiently with receptors  $A_1$  and  $A_3$  (**3q**). Compound **3q** is a potential dual ligand for  $A_1/A_3$  receptors with therapeutic interest, as  $A_1$  and  $A_3$  receptors, principally coupled to  $G_{i/o}$  proteins, can simultaneously inhibit the adenylyl cyclase activity [6,31,32].

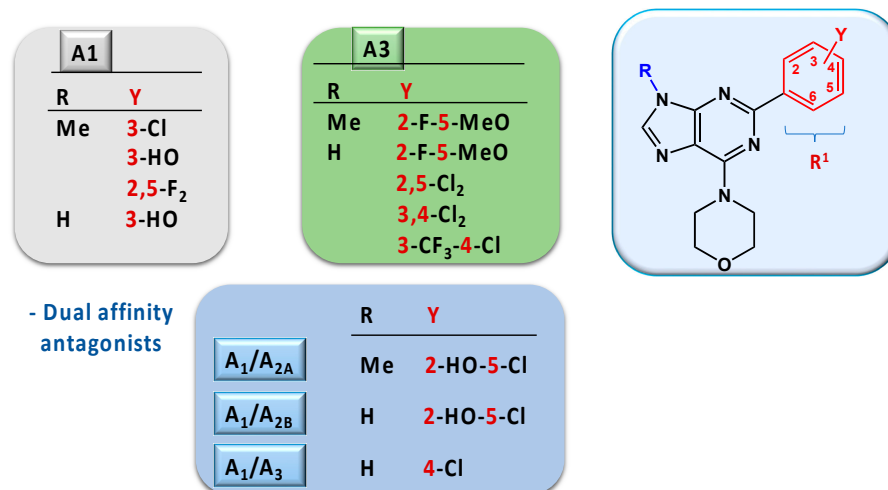
In addition, selectivity was achieved when a second chlorine or a  $\text{CF}_3$  group was simultaneously at the *meta* position (**3r**, **3s**), suggesting volume constraints at the  $A_1$ ,  $A_{2A}$ , and  $A_{2B}$  pockets interacting with the ligands' *meta* position. This hypothesis is also supported by the lower  $pK_i$  observed for compound **3s** ( $R^1 = 3\text{-CF}_3\text{-4-Cl-C}_6\text{H}_3$ ;  $pK_i = 6.02 \pm 0.04$ ) when compared to the  $pK_i$  of compound **3r** ( $R^1 = 3,4\text{-Cl}_2\text{C}_6\text{H}_3$ ;  $pK_i = 7.15 \pm 0.08$ ).

The sub-set of derivatives with hydroxyl groups at *ortho* or *meta* positions of the  $R^1$  group (**3c**, **3e**, **3g**, **3i**, **3j**, **3k**, **3n**, **3p**, and **3u**) will be analyzed. Generally, a hydroxyl group at *ortho* or *meta* positions of  $R^1$  led to very potent but not selective ligands (**3c**, **3i**, **3j**, **3k**, **3n**, and **3u**), except compounds **3e** and **3p**, which have a hydroxyl group at the *meta* position of  $R^1$  and showed selectivity for the  $A_1$  receptor. Derivative **3d** ( $R^1 = 3\text{-ClC}_6\text{H}_4$ ) also showed selectivity for the  $A_1$  receptor. These results suggest that an electron-withdrawing group produced through the inductive effect at the *meta* position of  $R^1$  interacts efficiently with a polar moiety at the  $A_1$  receptor. Comparing the affinity values between **3b** ( $R^1 = 2\text{-MeOC}_6\text{H}_4$ ) and **3c** ( $R^1 = 2\text{-HOC}_6\text{H}_4$ ), the increase of affinity of **3c** suggests that a hydrogen-bond donor (HBD) group at the *ortho* position of  $R^1$  is important for the interaction with the adenosine receptors  $A_1$ ,  $A_{2A}$ , and  $A_{2B}$ . However, the possibility of a hydrogen-bond-acceptor (HBA) interaction of the ligand with the target should also be considered. Hydroxyl and methoxyl groups may interact that way with the targets; however, if the receptor pockets have volume constraints, this will explain the lack of activity of derivative **3b** and support the hypothesis of HBD interaction. Furthermore, compound **3c** has almost the same submicromolar affinity for the  $A_1$  and  $A_{2A}$  receptors. This result indicates that **3c** is a dual ligand for  $A_1/A_{2A}$  receptors. Dual affinity for the  $A_1/A_{2A}$  receptors was also observed for ligand **2k**. These ligands could have medicinal interest, as compounds with dual affinity at  $A_1/A_{2A}$  receptors have shown therapeutic efficacy in animal models of Parkinson's disease [33,34]. On the other hand, compound **3u** showed a dual affinity for receptors  $A_1/A_{2B}$ . A comparison of affinities registered for **3c**, **3d**, and **3e** with **3n**, **3o**, and **3p** suggests more hydrophilic or small pockets at receptors to accommodate the group at N-9 of the ligands. Additionally, compound **3e** ( $R^1 = 3\text{-HOC}_6\text{H}_4$ ) is a very potent and selective ligand for the  $A_1$  receptor with  $pK_i = 6.16 \pm 0.21$ , while compound **3g** ( $R^1 = 2,3\text{-(HO)}_2\text{C}_6\text{H}_3$ ) has a low affinity for all of the receptors. This may indicate that an intramolecular hydrogen bond between the hydroxyl groups of the ligand precludes the interaction with the target, leading to low activity.

Finally, the fluoro derivatives (**3l**, **3m**, **3v**, and **3x**) presented high potency and selectivity for receptors  $A_1$  and  $A_3$ , except compound **3x**, which showed no selectivity. Compounds **3l** and **3v** with  $R^1 = 2\text{-F-5-MeOC}_6\text{H}_3$  were selective to the  $A_3$  receptor, with  $pK_i = 5.28 \pm 0.11$  and  $pK_i = 7.83 \pm 0.16$ , respectively. Compound **3v** also showed a high affinity for receptor  $A_{2B}$  ( $pK_i = 6.40 \pm 0.16$ ). These results suggest volume restrictions in the pockets of receptors  $A_{2B}$  and  $A_3$  to lodge the methyl group at N-9 of the ligand, with the available space in receptor  $A_3$  bigger than in receptor  $A_{2B}$ . Compound **3m** showed high affinity and selectivity for the  $A_1$  receptor, while compound **3x** showed higher potency

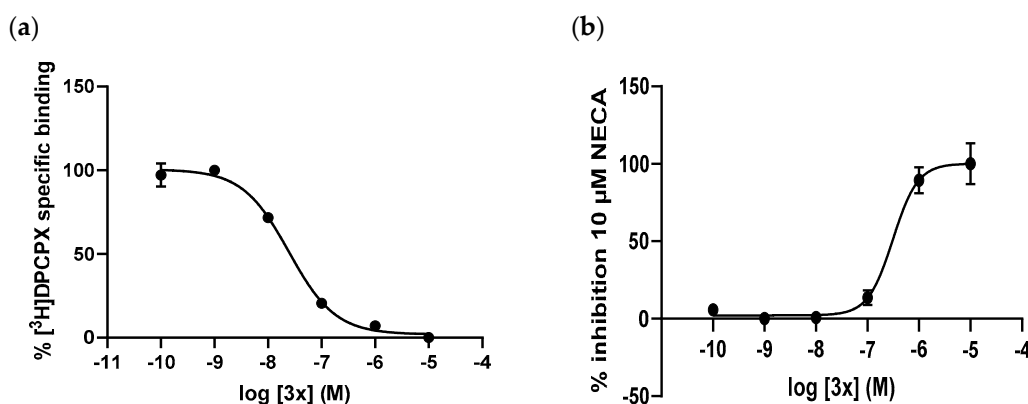
for all receptors but no selectivity. These results also support the hypothesis that there is limited space in the receptors' pockets to accommodate bulky groups at N-9. The difference in the selectivity of compounds **3l** and **3m** may indicate that receptor A<sub>3</sub> has a hydrophobic pocket to accommodate the substituent MeO present at the *meta* position of the ligand, while receptor A<sub>1</sub> does not. This hypothesis is also supported by the results obtained for ligands **3v** and **3x**. Figure 2 represents a SAR model that summarizes the results.

#### - Selective ligand antagonists for adenosine receptors



**Figure 2.** Structure-activity relationship of 2-aryl-9-substituted-6-morpholino purine derivatives for the adenosine receptor antagonists.

The most potent compounds for A<sub>1</sub> (**3x**;  $pK_i = 8.23 \pm 0.06$ ; Figure 3a) and A<sub>3</sub> (**3v**;  $pK_i = 7.83 \pm 0.16$ ) receptors were selected and tested in intracellular cAMP tests to study their functional activity. The agonist/antagonist behavior of the two compounds selected was determined by assessing their effect on the modulation of cAMP levels by the receptor agonist NECA. Figure 3b shows the result of a representative test for the antagonist potency of compound **3x** at A<sub>1</sub> receptor. The results confirmed that the compounds **3v** and **3x** are antagonists of the A<sub>3</sub> and A<sub>1</sub> receptors, and their antagonist potencies, expressed as  $pK_B$  values, were  $8.24 \pm 0.11$  and  $8.25 \pm 0.16$ , respectively (Table 2). Based on their structural similarity, we can generalize that all compounds of this work are antagonists of adenosine receptors.



**Figure 3.** (a) Competition binding curve for compound **3x** at A<sub>1</sub> receptors. (b) Concentration-response curve of compound **3x** in the presence of 10 μM of NECA at human A<sub>1</sub> receptor expressed in CHO cells. Points represent the mean  $\pm$  standard deviation (vertical bars) of duplicate measurements.

**Table 2.** Antagonist potency ( $pK_B$ ) of the most potent compounds at human  $A_1$  and  $A_3$  adenosine receptors in cAMP tests. Values are reported as mean  $\pm$  SEM of three experiments with duplicate measurements.

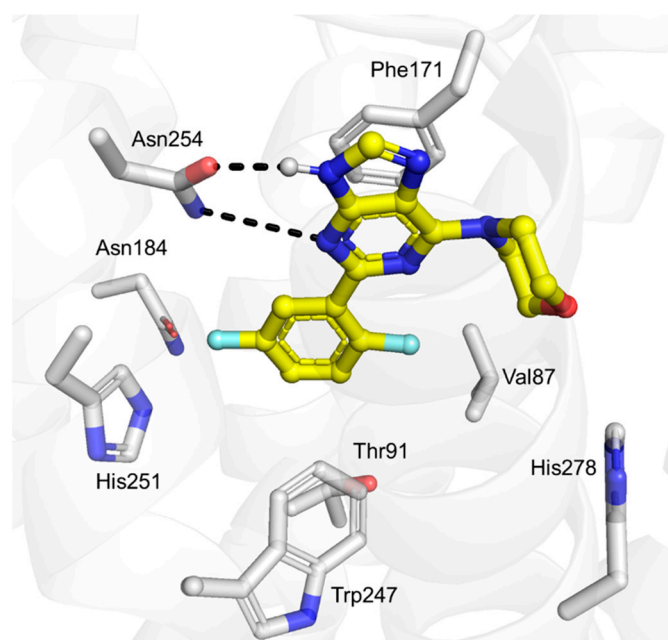
Compounds	Potency ( $pK_B$ )	
	$A_1$	$A_3$
<b>3v</b>	N.D.	$8.24 \pm 0.11$
<b>3x</b>	$8.25 \pm 0.16$	N.D.

N.D.—Not determined.

In this work, we synthesized highly potent and selective hits based on the scaffold for adenosine receptor antagonists identified in our previous publication [28]. These results indicate that the purines substituted with a morpholine group at C-6, a hydrogen atom or a methyl group at N-9, and a specific aryl group at C-2 of purine nucleus, gave high affinity and selective antagonists for adenosine  $A_1$  and  $A_3$  receptors, as well as dual antagonists for receptors  $A_1/A_{2A}$ ,  $A_1/A_{2B}$ , and  $A_1/A_3$ . In addition, four compounds (**3i**, **3j**, **3n**, and **3o**) showed high affinity, although they were not selective for any specific adenosine receptor subtype.

### 2.3. Docking Studies

Compound **3x** was computationally docked to the orthosteric site of the  $A_1$  receptor using a crystal structure representing an inactive conformation (PDB accession code: 5UEN) [35]. The ligand was predicted to form hydrogen bonds with Asn254 and  $\pi$ - $\pi$  stacking with Phe171, which are interactions characteristic of adenosine receptor antagonists (Figure 4) [36]. The predicted binding mode of compound **3x** in the  $A_1$  receptor also explained observed SARs. For example, replacing the fluorine at the 5-position of the phenyl ring with a methoxy group led to a substantial loss of affinity at the  $A_1$  receptor ( $pK_i < 5$ , compound **3v**). Consistent with this observation, increasing the size of this substituent in our model of the complex led to clashes with Asn184 and His251.



**Figure 4.** Predicted binding mode of compound **3x** in the orthosteric site of the  $A_1$  receptor. The receptor is depicted as a grey cartoon, with the ligand and key side chains represented as sticks. Hydrogen bonds are shown as black dashed lines.

### 3. Experimental Protocols

#### 3.1. Chemistry

The imidazoles **1** used in this work were synthesized according to previously described procedures [29], and compounds **2** were synthesized according to the procedure described in [28,30]. Solvents and other commercially available chemicals were used as shipped. The melting points (m.p.) were determined with a Gallenkamp melting point apparatus, and they are uncorrected. The reactions were followed by thin layer chromatography (TLC) using Silica Gel 60 F<sub>254</sub> (Merck, Darmstadt, Germany) plates with detection by UV light. The NMR spectra were obtained on a Varian Unity Plus (<sup>1</sup>H: 300 MHz, <sup>13</sup>C: 75 MHz) or on a Bruker Avance III NMR spectrometer (<sup>1</sup>H: 400 MHz, <sup>13</sup>C: 100 MHz) including the <sup>1</sup>H and <sup>13</sup>C bidimensional correlation spectra (HMBC and HMQC). For solutions, [D<sub>6</sub>]-DMSO residual [D<sub>6</sub>]-DMSO ( $\delta_{\text{H}} = 2.49$  ppm) or [D<sub>6</sub>]-DMSO ( $\delta_{\text{C}} = 39.5$  ppm) was used as the internal standard at 298 K. Chemical shifts ( $\delta$ ) were reported in parts per million (ppm) and the coupling constants, *J*, were presented in hertz (Hz). The purity of all tested compounds was higher than 95% according to elemental analysis, which was reported to be within 0.4% of the calculated values. The IR spectra were recorded with a FT-IR Bomem MB 104 using nujol mulls and NaCl cells. Elemental analyses were performed with a LECO CHNS-932 instrument.

#### 3.2. General Procedure for the Synthesis of **3a–x**

**Method A:** To a suspension of imidazole **2** in ethanol, an ethanol/acetonitrile mixture, or DMSO, the aldehyde **4** (1.1–1.5 equivalents) was added, followed by triethylamine (10 equivalents). The reaction was kept at room to moderate temperature and followed by TLC. Otherwise, when the TLC indicated the absence of the starting reagent but the presence of intermediates, the reaction continued at a higher temperature. When the TLC showed the absence of intermediate spots, the solvent was removed in the rotary evaporator, and an off-white solid was isolated after the addition of a small amount of ethanol to the residue. When the residue showed a dark color, it was dissolved in DCM. The solution was filtered through a column of silica gel (0.5 cm high), and the column was washed with an extra 30 mL of DCM. The resulting solution was concentrated until dry, diethyl ether was added to the oil, and an off-white solid was precipitated. The solid was filtered and washed with cold diethyl ether.

**Method B:** To a suspension of imidazole **2** in ethanol, the aldehyde **4** (1.1–1.5 equivalents) was added, followed by trifluoroacetic acid (1.3–2.0 equivalents). The reaction was kept at 22 °C under a magnetic stirrer until the TLC showed consumption of starting reagent **2**. The solvent was eliminated in the rotary evaporator, dimethyl sulfoxide and/or ethanol was added, followed by triethylamine (10 equivalents), and the reaction continued at 40–80 °C. When the TLC indicated the end of the reaction, the solvents were removed in the rotary evaporator, and the product was precipitated through the addition of water. The isolated solid was dissolved in a mixture of DCM/THF/EtOH and purified through dry flash chromatography using DCM as the elution solvent.

##### 3.2.1. 4-(2-(2-Chlorophenyl)-9-methyl-9H-purin-6-yl)morpholine **3a**

Method A: Compound **2a** (0.18 g, 0.89 mmol) in EtOH, aldehyde **4a** (1.1 eq.), and Et<sub>3</sub>N (10 eq.) was kept at 22 °C for 20 days. Product **3a** was isolated as an off-white solid (0.12 g, 0.28 mmol, 31%); m.p. = 99–101 °C; Found: C, 58.28; H, 4.86; N, 21.25. C<sub>16</sub>H<sub>16</sub>ClN<sub>5</sub>O requires C, 58.27; H, 4.89; N, 21.24; IR (Nujol mull)  $\nu_{\text{max}}$ : 1584 cm<sup>-1</sup>; <sup>1</sup>H (300 MHz, DMSO-d<sub>6</sub>)  $\delta$ : 8.21 (s, 1H), 7.75–7.72 (m, 1H), 7.53–7.50 (m, 1H), 7.45–7.38 (m, 2H), 4.23 (br s, 4H), 3.75 (s, 3H), 3.71 (t, *J* = 4.5 Hz, 4H); <sup>13</sup>C (75 MHz, DMSO-d<sub>6</sub>)  $\delta$ : 157.6, 152.7, 151.6, 141.4, 138.7, 131.6, 131.5, 130.1, 129.9, 126.8, 117.7, 66.2, 45.2, 29.6.

##### 3.2.2. 4-(2-(2-Methoxyphenyl)-9-methyl-9H-purin-6-yl)morpholine **3b**

Method A: Compound **2a** (0.13 g, 0.64 mmol) in DMSO, aldehyde **4b** (1.1 eq.), and Et<sub>3</sub>N (10 eq.) was kept at 60 °C for 6 days. Product **3b** was isolated as an off-white



solid (0.05 g, 0.23 mmol, 36%); m.p. = 114–116 °C; Found: C, 62.10; H, 5.92; N, 21.15.  $C_{17}H_{19}N_5O_2 \cdot 0.2H_2O$  requires C, 62.08; H, 5.90; N, 21.30; IR (Nujol mull)  $\nu_{max}$ : 1698, 1587, 1577  $cm^{-1}$ ;  $^1H$  (300 MHz, DMSO- $d_6$ )  $\delta$ : 8.15 (s, 1H), 7.50 (dd,  $J = 7.7$  Hz,  $J = 1.8$  Hz, 1H), 7.37 (td,  $J = 7.8$  Hz,  $J = 1.8$  Hz, 1H), 7.09 (d,  $J = 7.8$  Hz, 1H), 6.99 (t,  $J = 7.8$  Hz, 1H), 4.20 (br s, 4H), 3.80–3.50 (m, 10H).  $^{13}C$  (75 MHz, DMSO- $d_6$ )  $\delta$ : 158.4, 157.2, 152.8, 151.7, 149.9, 130.6, 130.1, 129.7, 120.0, 112.5, 66.2, 55.9, 45.2, 29.5.

### 3.2.3. 2-(9-Methyl-6-morpholino-9H-purin-2-yl)phenol 3c

Method A: Compound **2a** (0.08 g, 0.38 mmol) in a mixture of EtOH/CH<sub>3</sub>CN (1/1) and aldehyde **4c** (1.5 eq.) was kept at 11 °C for 17 days. Product **3c** was isolated as an off-white solid (0.08 g, 0.25 mmol, 66%); m.p. = 210–212 °C (Found: C, 61.17; H, 5.52; N, 21.00.  $C_{16}H_{17}N_5O_2 \cdot 0.1H_2O$  requires C, 61.45; H, 5.49; N, 22.40); IR (Nujol mull)  $\nu_{max}$ : 3097, 1591, 1530, 1519  $cm^{-1}$ ;  $^1H$  (300 MHz, DMSO- $d_6$ )  $\delta$ : 13.52 (s, 1H, OH), 8.38 (dd,  $J = 8.4$  Hz,  $J = 1.8$  Hz, 1H), 8.21 (s, 1H), 7.33 (td,  $J = 7.8$  Hz,  $J = 1.8$  Hz, 1H), 6.91 (t,  $J = 7.8$  Hz, 1H), 6.89 (d,  $J = 7.8$  Hz, 1H), 4.25 (br s, 4H), 3.78 (s, 4H), 3.77 (s, 3H);  $^{13}C$  (75 MHz, DMSO- $d_6$ )  $\delta$ : 159.4, 157.5, 152.4, 149.7, 141.4, 132.0, 129.0, 119.3, 118.6, 117.8, 117.2, 66.1, 45.4, 29.6.

### 3.2.4. 4-(2-(3-Chlorophenyl)-9-methyl-9H-purin-6-yl)morpholine 3d

Method A: Compound **2a** (0.31 g, 1.46 mmol) in EtOH, aldehyde **4d** (1.1 eq.), and Et<sub>3</sub>N (10 eq.) was kept at 20 °C for 12 days and then at 70 °C for 21 h. The product **3d** was isolated as an off-white solid (0.35 g, 1.06 mmol, 73%); m.p. = 160–162 °C (Found: C, 58.04; H, 4.76; N, 21.18.  $C_{16}H_{16}ClN_5O$  requires C, 58.27; H, 4.89; N, 21.24); IR (Nujol mull)  $\nu_{max}$ : 1690, 1575  $cm^{-1}$ ;  $^1H$  (300 MHz, DMSO- $d_6$ )  $\delta$ : 8.31 (m, 2H), 8.17 (s, 1H), 7.48–7.50 (m, 2H), 4.26 (br s, 4H), 3.76 (br s, 4H), 3.73 (s, 3H);  $^{13}C$  (75 MHz, DMSO- $d_6$ )  $\delta$ : 155.3, 153.0, 151.9, 141.5, 140.4, 133.2, 130.2, 129.6, 127.1, 126.3, 118.4, 66.2, 45.3, 29.5.

### 3.2.5. 3-(9-Methyl-6-morpholino-9H-purin-2-yl)phenol 3e

Method B: Compound **2b** (0.22 g, 1.10 mmol) in EtOH, aldehyde **4e** (1.1 eq.), and TFA (2 eq.) was kept at 22 °C for 3 days. Then, the reaction continued in DMSO and Et<sub>3</sub>N (10 eq.) at 60 °C for 2 days. The product **3e** was isolated as an off-white solid (0.23 g, 0.75 mmol, 67%); m.p. = 230–232 °C (Found: C, 61.67; H, 5.52; N, 22.64.  $C_{16}H_{17}N_5O_2$  requires C, 61.72; H, 5.50; N, 22.49); IR (Nujol mull)  $\nu_{max}$ : 3281, 1578, 1505  $cm^{-1}$ ;  $^1H$  (300 MHz, DMSO- $d_6$ )  $\delta$ : 9.45 (s, 1H, OH), 8.16 (s, 1H), 7.84 (br.s, 2H), 7.24 (t,  $J = 8.4$  Hz, 1H), 6.82 (d,  $J = 7.8$  Hz, 1H), 4.28 (br s, 4H), 3.77 (s, 3H), 3.75 (m, 4H);  $^{13}C$  (75 MHz, DMSO- $d_6$ )  $\delta$ : 157.3, 156.8, 152.9, 152.0, 141.1, 139.7, 129.1, 118.6, 118.1, 116.7, 114.5, 66.2, 45.1, 29.4.

### 3.2.6. 4-(2-(4-Chlorophenyl)-9-methyl-9H-purin-6-yl)morpholine 3f

Method A: Compound **2a** (0.34 g, 1.57 mmol) in EtOH, aldehyde **4f** (1.1 eq.), and Et<sub>3</sub>N (10 eq.) was kept at 21 °C for 8 days and then at 70 °C for 6 h. The product **3f** was isolated as an off-white solid (0.25 g, 0.75 mmol, 48%); m.p. = 226–228 °C (Found: C, 57.99; H, 4.86; N, 21.13.  $C_{16}H_{16}ClN_5O$  requires: C, 58.27; H, 4.89; N, 21.24); IR (Nujol mull)  $\nu_{max}$ : 1706, 1587, 1571  $cm^{-1}$ ;  $^1H$  (300 MHz, DMSO- $d_6$ )  $\delta$ : 8.40 (d,  $J = 8.4$  Hz, 2H), 8.17 (s, 1H), 7.52 (d,  $J = 8.4$  Hz, 2H), 4.28 (br s, 4H), 3.79 (s, 3H), 3.74 (m, 4H);  $^{13}C$  (75 MHz, DMSO- $d_6$ )  $\delta$ : 155.7, 152.9, 151.0, 141.4, 137.1, 134.5, 129.4, 128.3, 118.2, 66.2, 45.1, 29.5.

### 3.2.7. 3-(9-Methyl-6-morpholino-9H-purin-2-yl)benzene-1,2-diol 3g

Method B: Compound **2a** (0.15 g, 0.69 mmol) in EtOH, aldehyde **4g** (1.1 eq.), and TFA (2 eq.) was kept at 22 °C for 3 days. Then, the reaction continued in a mixture of DMSO/EtOH (0.1/1) and Et<sub>3</sub>N (10 eq.) at 80 °C for 6 h. The product **3g** was isolated as an off-white solid (0.14 g, 0.43 mmol, 62%); m.p. = 254–256 °C (Found: C, 58.87; H, 5.55; N, 21.02.  $C_{16}H_{17}N_5O_3$  requires C, 58.71; H, 5.23; N, 21.39); IR (Nujol mull)  $\nu_{max}$ : 3415, 1588, 1569  $cm^{-1}$ ;  $^1H$  (300 MHz, DMSO- $d_6$ )  $\delta$ : 13.73 (s, 1H, OH), 8.83 (br s, 1H, OH), 8.19 (s, 1H), 7.83 (dd,  $J = 8.0$  Hz,  $J = 1.5$  Hz, 1H), 6.84 (dd,  $J = 7.5$  Hz,  $J = 1.5$  Hz, 1H),

6.70 (t,  $J = 7.8$  Hz, 1H), 4.23 (br s, 4H), 3.77 t,  $J = 5.4$  Hz, 7H);  $^{13}\text{C}$  (75 MHz, DMSO- $d_6$ )  $\delta$ : 157.9, 152.3, 149.8, 148.3, 146.1, 141.3, 119.5, 119.1, 117.9, 117.6, 117.3, 66.0, 45.4.

### 3.2.8. 4-(2-(2,5-Dichlorophenyl)-9-methyl-9H-purin-6-yl)morpholine **3h**

Method A: Compound **2a** (0.25 g, 1.22 mmol) in EtOH, aldehyde **4h** (1.1 eq.), and  $\text{Et}_3\text{N}$  (10 eq.) was kept at 40 °C for 4 days and then at 50 °C for 10 days. The product **3h** was isolated as an off-white solid (0.18 g, 0.50 mmol, 41%); m.p. = 141–142 °C (Found: C, 52.64; H, 4.11; N, 19.45.  $\text{C}_{16}\text{H}_{15}\text{Cl}_2\text{N}_5\text{O}$  requires C, 52.76; H, 4.15; N, 19.23); IR (Nujol mull)  $\nu_{\text{max}}$ : 3048, 1585  $\text{cm}^{-1}$ ;  $^1\text{H}$  (300 MHz, DMSO- $d_6$ )  $\delta$ : 8.22 (s, 1H), 7.78(d,  $J = 2.7$  Hz, 1H), 7.56 (d,  $J = 8.4$  Hz, 1H), 7.50 (dd,  $J = 8.4$  Hz,  $J = 2.4$  Hz, 1H), 4.23 (br s, 4H), 3.76 (s, 3H), 3.72 (t,  $J = 4.5$  Hz, 4H);  $^{13}\text{C}$  (75 MHz, DMSO- $d_6$ )  $\delta$ : 156.3, 152.7, 151.5, 141.6, 140.0, 131.9, 131.4, 130.9, 130.5, 129.7, 117.9, 66.2, 45.2, 29.6.

### 3.2.9. 2-(9-Methyl-6-morpholino-9H-purin-2-yl)benzene-1,4-diol **3i**

Method B: Compound **2a** (0.20 g, 0.96 mmol) in EtOH, aldehyde **4i** (1.1 eq.), and TFA (2 eq.) was kept at 22 °C for 5 h. Then, the reaction continued in a mixture of DMSO/EtOH (0.1/1) and  $\text{Et}_3\text{N}$  (10 eq.) at 60 °C for 3 days. The product **3i** was isolated as an off-white solid (0.20 g, 0.61 mmol, 64%); m.p. = 288–290 °C (Found: C, 57.21; H, 5.09; N, 20.61.  $\text{C}_{16}\text{H}_{17}\text{N}_5\text{O}_3 \cdot 0.5\text{H}_2\text{O}$  requires C, 57.14; H, 5.36; N, 20.83); IR (Nujol mull)  $\nu_{\text{max}}$ : 3353, 1574, 1500  $\text{cm}^{-1}$ ;  $^1\text{H}$  (300 MHz, DMSO- $d_6$ )  $\delta$ : 12.81 (s, 1H, OH), 8.88 (s, 1H, OH), 8.20 (s, 1H), 7.80 (d,  $J = 2.7$  Hz, 1H), 6.77 (dd,  $J = 8.6$  Hz,  $J = 2.7$  Hz, 1H), 6.72 (d,  $J = 8.7$  Hz, 1H), 4.25 (br s, 4H), 3.77 (t,  $J = 3.6$  Hz, 7H);  $^{13}\text{C}$  (75 MHz, DMSO- $d_6$ )  $\delta$ : 157.6, 152.4, 152.2, 149.9, 149.2, 141.4, 119.8, 119.3, 117.7 (2C), 114.1, 66.1, 45.4, 29.7.

### 3.2.10. 4-Methoxy-2-(9-methyl-6-morpholino-9H-purin-2-yl)phenol **3j**

Method B: Compound **2a** (0.15 g, 0.70 mmol) in EtOH, aldehyde **4j** (1.2 eq.), and TFA (2 eq.) was kept at 22 °C for 2 days. Then, the reaction continued in EtOH and  $\text{Et}_3\text{N}$  (10 eq.) at 25 °C for 5 days. The product **3j** was isolated as an off-white solid (0.14 g, 0.43 mmol, 62%); m.p. = 146–148 °C (Found: C, 59.75; H, 5.67; N, 20.42.  $\text{C}_{17}\text{H}_{19}\text{N}_5\text{O}_3$  requires C, 59.81; H, 5.61; N, 20.52); IR (Nujol mull)  $\nu_{\text{max}}$ : 3011, 1594, 1578  $\text{cm}^{-1}$ ;  $^1\text{H}$  (300 MHz, DMSO- $d_6$ )  $\delta$ : 12.99 (s, 1H, OH), 8.20 (s, 1H), 7.89 (d,  $J = 3.2$  Hz, 1H), 6.97 (dd,  $J = 8.8$  Hz,  $J = 3.2$  Hz, 1H), 6.83 (d,  $J = 8.8$  Hz, 1H), 4.23 (s, 4H), 3.77 (m, 7H);  $^{13}\text{C}$  (75 MHz, DMSO- $d_6$ )  $\delta$ : 157.2, 153.4, 152.4, 151.5, 149.8, 141.4, 119.3, 118.7, 117.9, 117.8, 112.6, 66.0, 55.4, 45.4, 29.6.

### 3.2.11. 4-Chloro-2-(9-methyl-6-morpholino-9H-purin-2-yl)phenol **3k**

Method B: Compound **2a** (0.13 g, 0.64 mmol) in EtOH, aldehyde **4k** (1.1 eq.), and TFA (2 eq.) was kept at 22 °C for 2 days. Then, the reaction continued in a mixture of DMSO/EtOH (0.2/1) and  $\text{Et}_3\text{N}$  (10 eq.) at 60 °C for 6 days. The product **3k** was isolated as an off-white solid (0.104 g, 0.41 mmol, 64%); m.p. = 179–180 °C (Found: C, 55.65; H, 4.50; N, 20.00.  $\text{C}_{16}\text{H}_{16}\text{ClN}_5\text{O}_2$  requires C, 55.58; H, 4.66; N, 20.25); IR (Nujol mull)  $\nu_{\text{max}}$ : 3016, 1589, 1528  $\text{cm}^{-1}$ ;  $^1\text{H}$  (300 MHz, DMSO- $d_6$ )  $\delta$ : 13.59 (s, 1H, OH), 8.24 (d,  $J = 2.8$  Hz, 1H), 8.20 (s, 1H), 7.32 (dd,  $J = 8.8$  Hz,  $J = 2.8$  Hz, 1H), 6.90 (d,  $J = 8.8$  Hz, 1H), 4.20 (s, 4H), 3.77 (t,  $J = 4.8$  Hz, 7H);  $^{13}\text{C}$  (75 MHz, DMSO- $d_6$ )  $\delta$ : 158.0, 156.0, 152.2, 149.5, 141.5, 131.4, 127.7, 122.2, 120.5, 119.1, 118.0, 66.0, 45.3, 29.6.

### 3.2.12. 4-(2-(2-Fluoro-5-methoxyphenyl)-9-methyl-9H-purin-6-yl)morpholine **3l**

Method A: Compound **2a** (0.16 g, 0.77 mmol) in EtOH, aldehyde **4l** (1.3 eq.), and  $\text{Et}_3\text{N}$  (10 eq.) was kept at 22 °C for 1 day and then at 60 °C for 4 days. The product **3l** was isolated as an off-white solid (0.15 g, 0.44 mmol, 57%); m.p. = 102–104 °C (Found: C, 58.76; H, 4.93; N, 19.99.  $\text{C}_{17}\text{H}_{18}\text{FN}_5\text{O}_2 \cdot 0.2\text{H}_2\text{O}$  requires C, 58.86; H, 5.31; N, 20.19); IR (Nujol mull)  $\nu_{\text{max}}$ : 3122, 1598, 1579  $\text{cm}^{-1}$ ;  $^1\text{H}$  (300 MHz, DMSO- $d_6$ )  $\delta$ : 8.19 (s, 1H), 7.52 (dd,  $J = 3.2$  Hz,  $J = 6.4$  Hz, 1H), 7.18 (dd,  $J = 8.0$  Hz,  $J = 6.9$  Hz, 1H), 7.01 (dt,  $J = 6.9$  Hz,  $J = 2.4$  Hz, 1H), 4.23 (br s, 4H), 3.80–3.70 (m, 10H).  $^{13}\text{C}$  (75 MHz, DMSO- $d_6$ )  $\delta$ : 155.2 (d,  $J = 4$  Hz), 154.9

(d,  $J = 2$  Hz), 154.7 (d,  $J = 244$  Hz), 153.4, 151.7, 141.4, 127.7 (d,  $J = 11$  Hz), 117.8, 117.3 (d,  $J = 24$  Hz), 115.9 (d,  $J = 2$  Hz), 115.8 (d,  $J = 8$  Hz), 66.2, 55.6, 45.2, 29.5.

### 3.2.13. 4-(2-(2,5-Difluorophenyl)-9-methyl-9H-purin-6-yl)morpholine **3m**

Method A: Compound **2a** (0.31 g, 1.48 mmol) in EtOH, aldehyde **4m** (1.1 eq.), and Et<sub>3</sub>N (10 eq.) was kept at 40 °C for 4 days and then at 50 °C for 6 days. The product **3m** was isolated as an off-white solid (0.30 g, 0.91 mmol, 61%); m.p. = 143–145 °C (Found: C, 57.90; H, 4.77; N, 20.97. C<sub>16</sub>H<sub>15</sub>F<sub>2</sub>N<sub>5</sub>O requires C, 58.00; H, 4.56; N, 21.14); IR (Nujol mull)  $\nu_{\text{max}}$ : 3083, 1616, 1578 cm<sup>-1</sup>; <sup>1</sup>H (400 MHz, DMSO-d<sub>6</sub>)  $\delta$ : 8.20 (s, 1H), 7.80–7.79 (m, 1H), 7.34–7.30 (m, 1H), 4.24 (br s, 4H), 3.77 (s, 3H), 3.73 (t,  $J = 4.5$  Hz, 4H); <sup>13</sup>C (100 MHz, DMSO-d<sub>6</sub>)  $\delta$ : 157.7 (dd,  $J = 230$  Hz,  $J = 6$  Hz), 156.5 (dd,  $J = 246$  Hz,  $J = 4$  Hz), 154.2 (dd,  $J = 5$  Hz,  $J = 2$  Hz), 152.9, 151.6, 141.6, 128.5 (dd,  $J = 11$  Hz,  $J = 4$  Hz), 118.4 (dd,  $J = 25$  Hz,  $J = 9$  Hz), 118.0, 117.5 (dd,  $J = 24$  Hz,  $J = 9$  Hz), 128.3 (dd,  $J = 25$  Hz,  $J = 2$  Hz), 66.2, 45.2, 29.5.

### 3.2.14. 2-(6-Morpholino-9H-purin-2-yl)phenol **3n**

Method B: Compound **2b** (0.30 g, 1.55 mmol) in EtOH, aldehyde **4c** (1.1 eq.), and TFA (1.5 eq.) was kept at 22 °C for 4.5 h. Then, the reaction continued in DMSO and Et<sub>3</sub>N (10 eq.) at 40 °C for 10 days. The product **3n** was isolated as an off-white solid (0.15 g, 0.52 mmol, 34%); (Found: C, 60.43; H, 5.12; N, 23.71. C<sub>15</sub>H<sub>15</sub>N<sub>5</sub>O<sub>2</sub> requires C, 60.60; H, 5.09; N, 23.56); <sup>1</sup>H (400 MHz, DMSO-d<sub>6</sub>)  $\delta$ : 13.64 (s, 1H), 13.23 (br s, 1H), 8.35 (dd,  $J = 8.4$  Hz,  $J = 2.0$  Hz, 1H), 8.20 (s, 1H), 7.31 (dt,  $J = 8.4$  Hz,  $J = 2.0$  Hz, 1H), 6.90 (m, 2H), 4.28 (br s, 4H), 3.78 (m, 4H); <sup>13</sup>C (100 MHz, DMSO-d<sub>6</sub>)  $\delta$ : 159.4, 157.5, 152.5, 149.9, 139.0, 131.8, 128.9, 119.4, 118.6, 117.6, 117.2, 66.1, 45.5.

### 3.2.15. 4-(2-(3-Chlorophenyl)-9H-purin-6-yl)morpholine **3o**

Method A: Compound **2b** (0.14 g, 0.72 mmol) in EtOH, aldehyde **4d** (1.1 eq.), and Et<sub>3</sub>N (10 eq.) was kept at 40 °C for 10 days. The product **3o** was isolated as an off-white solid (0.10 g, 0.32 mmol, 44%); m.p. = 265–267 °C (Found: C, 56.21; H, 4.26; N, 21.66. C<sub>15</sub>H<sub>14</sub>ClN<sub>5</sub>O.0.3H<sub>2</sub>O requires C, 56.10; H, 4.55; N, 21.81); IR (Nujol mull)  $\nu_{\text{max}}$ : 3103, 1587, 1571 cm<sup>-1</sup>; <sup>1</sup>H (300 MHz, DMSO-d<sub>6</sub>)  $\delta$ : 13.16 (s, 1H, NH), 8.18 (s, 1H), 8.55–7.48 (m, 2H), 8.32–8.28 (m, 2H), 4.29 (br s, 4H), 3.76 (t,  $J = 4.8$  Hz, 4H); <sup>13</sup>C (75 MHz, DMSO-d<sub>6</sub>)  $\delta$ : 155.2, 152.9, 152.7, 140.7, 139.4, 133.1, 130.2, 129.3, 127.0, 126.1, 118.3, 66.2, 45.1.

### 3.2.16. 3-(6-Morpholino-9H-purin-2-yl)phenol **3p**

Method B: Compound **2a** (0.31 g, 1.58 mmol) in EtOH, aldehyde **4e** (1.1 eq.), and TFA (1.5 eq.) was kept at 22 °C for 7 days. Then, the reaction continued in DMSO and Et<sub>3</sub>N (10 eq.) at 50 °C for 1 month. The product **3p** was isolated as an off-white solid (0.24 g, 0.81 mmol, 51%); (Found: C, 60.53; H, 5.02; N, 23.52. C<sub>15</sub>H<sub>15</sub>N<sub>5</sub>O<sub>2</sub> requires C, 60.60; H, 5.09; N, 23.56); <sup>1</sup>H (300 MHz, DMSO-d<sub>6</sub>)  $\delta$ : 13.07 (br s, 1H), 9.45 (br s, 1H), 8.13 (s, 1H), 7.77–7.79 (m, 2H), 7.23 (t,  $J = 7.8$  Hz, 1H), 6.82 (ddd,  $J = 7.8$  Hz,  $J = 3.3$  Hz,  $J = 0.9$  Hz, 1H), 4.28 (br s, 4H), 3.76 (m, 4H); <sup>13</sup>C (75 MHz, DMSO-d<sub>6</sub>)  $\delta$ : 157.3, 156.9, 152.9, 152.6, 139.9, 138.8, 129.2, 118.5, 117.9, 116.6, 114.4, 66.3, 45.1.

### 3.2.17. 4-(2-(4-Chlorophenyl)-9H-purin-6-yl)morpholine **3q**

Method A: Compound **2b** (0.31 g, 1.58 mmol) in EtOH, aldehyde **4f** (1.1 eq.), and Et<sub>3</sub>N (10 eq.) was kept at 20 °C for 13 days and then at 50 °C for 21 h. The product **3q** was isolated as an off-white solid (0.15 g, 0.47 mmol, 30%); m.p. = 284 °C (dec.) (Found: C, 57.00; H, 4.51; N, 22.05. C<sub>15</sub>H<sub>14</sub>ClN<sub>5</sub>O requires C, 57.06; H, 4.47; N, 22.18); IR (Nujol mull)  $\nu_{\text{max}}$ : 1583 cm<sup>-1</sup>; <sup>1</sup>H (300 MHz, DMSO-d<sub>6</sub>)  $\delta$ : 13.13 (s, 1H), 8.34 (s, 1H), 8.16 (d,  $J = 9.0$  Hz, 2H), 7.50 (d,  $J = 9.0$  Hz, 2H), 4.28 (br s, 4H), 3.75 (m, 4H); <sup>13</sup>C (75 MHz, DMSO-d<sub>6</sub>)  $\delta$ : 155.8, 153.0, 152.5, 139.0, 137.3, 134.4, 129.3, 128.3, 118.1, 66.2, 45.3.

**3.2.18. 4-(2-(3,4-Dichlorophenyl)-9H-purin-6-yl)morpholine 3r**

Method A: Compound **2b** (0.31 g, 1.57 mmol) in EtOH, aldehyde **4p** (1.1 eq.), and Et<sub>3</sub>N (10 eq.) was kept at 40 °C for 2 days and then at 50 °C for 6 days. The product **3t** was isolated as an off-white solid (0.18 g, 0.51 mmol, 33%); m.p. > 300 °C (Found: C, 51.28; H, 3.91; N, 19.93. C<sub>15</sub>H<sub>13</sub>Cl<sub>2</sub>N<sub>5</sub>O requires C, 51.45; H, 3.74; N, 20.00); IR (Nujol mull)  $\nu_{\text{max}}$ : 3010, 1606, 1584, 1561 cm<sup>-1</sup>; <sup>1</sup>H (300 MHz, DMSO-d<sub>6</sub>)  $\delta$ : 13.05 (br s, 1H), 8.45 (d, *J* = 2.1 Hz, 1H), 8.29 (dd, *J* = 8.7 Hz, *J* = 2.1 Hz, 1H), 8.20 (s, 1H), 6.90 (d, *J* = 8.7 Hz, 1H), 4.29 (br s, 4H), 3.76 (t, *J* = 4.8 Hz, 4H); <sup>13</sup>C (75 MHz, DMSO-d<sub>6</sub>)  $\delta$ : 154.5, 152.9, 139.3, 139.1, 132.2, 131.2, 130.6, 128.9, 127.5, 118.2, 66.2, 45.2.

**3.2.19. 4-(2-(4-Chloro-3-(trifluoromethyl)phenyl)-9H-purin-6-yl)morpholine 3s**

Method A: Compound **2b** (0.27 g, 1.38 mmol) in EtOH, aldehyde **4o** (1.1 eq.), and Et<sub>3</sub>N (10 eq.) was kept at 22 °C for 7 days and then at 40 °C for 5 days. The product **3u** was isolated as an off-white solid (0.28 g, 0.76 mmol, 55%); m.p. = 248–250 °C (Found: C, 50.00; H, 3.53; N, 18.28. C<sub>16</sub>H<sub>13</sub>ClF<sub>3</sub>N<sub>5</sub>O requires C, 50.00; H, 3.39; N, 18.25); IR (Nujol mull)  $\nu_{\text{max}}$ : 3191, 1587, 1575 cm<sup>-1</sup>; <sup>1</sup>H (300 MHz, DMSO-d<sub>6</sub>)  $\delta$ : 13.24 (s, 1H, NH), 8.72 (d, *J* = 2.1 Hz, 1H), 8.60 (dd, *J* = 8.4 Hz, *J* = 2.1 Hz, 1H), 8.21 (s, 1H), 7.82 (d, *J* = 8.4 Hz, 1H), 4.30 (br s, 4H), 3.76 (t, *J* = 4.5 Hz, 4H); <sup>13</sup>C (75 MHz, DMSO-d<sub>6</sub>)  $\delta$ : 156.3, 152.9, 152.4, 139.3, 137.9, 132.6, 131.8, 131.6 (q, *J* = 1Hz), 126.6 (q, *J* = 1Hz), 126.6 (q, *J* = 31 Hz), 126.0 (q, *J* = 6 Hz), 122.9 (q, *J* = 271 Hz), 118.3, 66.2, 45.1.

**3.2.20. 4-(2-(2,5-Dichlorophenyl)-9H-purin-6-yl)morpholine 3t**

Method A: Compound **2b** (0.28 g, 1.45 mmol) in EtOH, aldehyde **4h** (1.1 eq.), and Et<sub>3</sub>N (10 eq.) was kept at 22 °C for 6 days and then at 40 °C for 6 days. The product **3r** was isolated as an off-white solid (0.21 g, 0.60 mmol, 41%); m.p. = 259–261 °C (Found: C, 51.26; H, 3.69; N, 19.98. C<sub>15</sub>H<sub>13</sub>Cl<sub>2</sub>N<sub>5</sub>O requires C, 51.45; H, 3.74; N, 20.00); IR (Nujol mull)  $\nu_{\text{max}}$ : 3090, 1602, 1586, 1561 cm<sup>-1</sup>; <sup>1</sup>H (300 MHz, DMSO-d<sub>6</sub>)  $\delta$ : 13.23 (br s, 1H, NH), 8.22 (s, 1H), 7.76 (d, *J* = 2.1 Hz, 1H), 7.57 (d, *J* = 8.7 Hz, 1H), 7.50 (dd, *J* = 8.7 Hz, *J* = 2.1 Hz, 1H), 4.24 (br s, 4H), 3.72 (t, *J* = 4.5 Hz, 4H); <sup>13</sup>C (75 MHz, DMSO-d<sub>6</sub>)  $\delta$ : 156.3, 152.7, 152.0, 140.2, 139.3, 131.9, 131.4, 131.0, 130.4, 129.6, 117.7, 66.2, 45.2.

**3.2.21. 5-Chloro-2-(6-morpholino-9H-purin-2-yl)phenol 3u**

Method B: Compound **2b** (0.18 g, 0.91 mmol) in EtOH, aldehyde **4k** (1.1 eq.), and TFA (1.0 eq.) was kept at 22 °C for 2 days. Then, the reaction continued in EtOH and Et<sub>3</sub>N (10 eq.) at 40 °C for 24 days. The product **3s** was isolated as an off-white solid (0.08 g, 0.24 mmol, 26%); m.p. > 300 °C. (Found: C, 54.33; H, 4.23; N, 21.31. C<sub>15</sub>H<sub>14</sub>ClN<sub>5</sub>O<sub>2</sub> requires C, 54.31; H, 4.25; N, 21.11); <sup>1</sup>H (400 MHz, DMSO-d<sub>6</sub>)  $\delta$ : 13.76 (s, 1H), 13.32 (br s, 1H), 8.27 (d, *J* = 2.8 Hz, 1H), 8.23 (s, 1H), 7.34 (dd, *J* = 8.8 Hz, *J* = 2.8 Hz, 1H), 6.94 (d, *J* = 8.8 Hz, 1H), 4.27 (br s, 4H), 3.78 (t, *J* = 4.8 Hz, 4H); <sup>13</sup>C (100 MHz, DMSO-d<sub>6</sub>)  $\delta$ : 158.2, 156.2, 152.5, 149.6, 139.3, 131.4, 127.7, 122.2, 120.7, 119.2, 117.9, 66.1, 45.4.

**3.2.22. 4-(2-(2-Fluoro-5-methoxyphenyl)-9H-purin-6-yl)morpholine 3v**

Method A: Compound **2b** (0.35 g, 1.80 mmol) in EtOH, aldehyde **4l** (1.1 eq.), and Et<sub>3</sub>N (10 eq.) was kept at 20 °C for 19 days and then at 40 °C for 7 days. The product **3v** was isolated as an off-white solid (0.17 g, 0.52 mmol, 29%); m.p. = 261–263 °C (Found: C, 57.99; H, 4.89; N, 20.98. C<sub>16</sub>H<sub>16</sub>FN<sub>5</sub>O<sub>2</sub> requires C, 58.35; H, 4.90; N, 21.27); IR (Nujol mull)  $\nu_{\text{max}}$ : 1579, 1500 cm<sup>-1</sup>; <sup>1</sup>H (300 MHz, DMSO-d<sub>6</sub>)  $\delta$ : 13.17 (s, 1H), 8.18 (s, 1H), 7.50 (dd, *J* = 6.6 Hz, *J* = 3.6 Hz, 1H), 7.17 (dd, *J* = 10.5 Hz, *J* = 9.0 Hz, 1H), 7.02 (dt, *J* = 3.6 Hz, *J* = 3.3 Hz, 1H), 4.24 (br s, 4H), 3.77 (s, 3H), 3.72 (m, 4H); <sup>13</sup>C (75 MHz, DMSO-d<sub>6</sub>)  $\delta$ : 154.7 (d, *J* = 244 Hz), 155.2 (d, *J* = 5 Hz), 155.0 (d, *J* = 2 Hz), 152.9, 152.3, 139.2, 127.8 (d, *J* = 12 Hz), 117.7, 117.4 (d, *J* = 25 Hz), 116.0 (d, *J* = 8 Hz), 115.7 (d, *J* = 2 Hz), 66.2, 55.6, 45.1.

### 3.2.23. 4-(2-(2,5-Difluorophenyl)-9H-purin-6-yl)morpholine 3x

Method A: Compound **2b** (0.31 g, 1.59 mmol) in EtOH, aldehyde **4m** (1.1 eq.), and Et<sub>3</sub>N (10 eq.) was kept at 35 °C for 1 month. The product **3x** was isolated as an off-white solid (0.28 g, 0.88 mmol, 56%); m.p. = 260–262 °C (Found: C, 56.78; H, 4.00; N, 21.89. C<sub>15</sub>H<sub>13</sub>F<sub>2</sub>N<sub>5</sub>O requires C, 56.78; H, 4.13; N, 22.07); IR (Nujol mull)  $\nu_{\text{max}}$ : 1581, 1500 cm<sup>-1</sup>; <sup>1</sup>H (300 MHz, DMSO-d<sub>6</sub>)  $\delta$ : 13.22 (s, 1H), 8.19 (s, 1H), 7.75–7.80 (m, 1H), 7.29–7.33 (m, 2H), 4.24 (br s, 4H), 3.73 (br s, 4H); <sup>13</sup>C (75 MHz, DMSO-d<sub>6</sub>)  $\delta$ : 157.8 (dd,  $J = 240$  Hz,  $J = 4$  Hz), 156.6 (dd,  $J = 250$  Hz,  $J = 4$  Hz), 154.2 (d,  $J = 3$  Hz), 152.9, 152.2, 139.3, 128.7 (dd,  $J = 11$  Hz,  $J = 8$  Hz), 118.4 (dd,  $J = 25$  Hz,  $J = 9$  Hz), 117.9, 117.4 (dd,  $J = 23$  Hz,  $J = 11$  Hz), 117.3 (dd,  $J = 23$  Hz,  $J = 5$  Hz), 66.2, 45.2.

### 3.3. Radioligand Binding Assays

The %<sub>inhib</sub> of specific radioligand binding at the receptors by the compounds was tested at the concentration of 10  $\mu$ M at all adenosine receptors following the conditions described below. Competition binding curves at several receptors were made by testing six different concentrations (ranging from 10 nM to 100  $\mu$ M) for all of the compounds showing an %<sub>inhib</sub>  $\geq$  80%.

#### 3.3.1. Human A<sub>1</sub> Receptor

Competition binding experiments of adenosine A<sub>1</sub> receptor were made with membranes from CHO-A<sub>1</sub> cells (Euroscreen, Brussels, Belgium). Membranes were defrosted and suspended in an incubation buffer with 10 mM of MgCl<sub>2</sub>, 100 mM of NaCl, 20 mM of Hepes, 2 UI/mL of adenosine deaminase, and pH = 7.4. Each well of a GF/C multiscreen plate (Millipore, Madrid, Spain), prepared in duplicate, contained 15  $\mu$ g of protein, 2 nM of [<sup>3</sup>H]DPCPX, and the tested compound. Non-specific binding was calculated with 10  $\mu$ M of (R)-PIA. The reaction mixture was incubated at room temperature (25 °C) for 60 min, after which samples were filtered and measured in a microplate beta scintillation counter (Microbeta Trilux, Perkin Elmer, Madrid, Spain).

#### 3.3.2. Human A<sub>2A</sub> Receptor

Competition binding experiments of adenosine A<sub>2A</sub> receptor were made in membranes from HeLa-A<sub>2A</sub> cells, which were provided by Dr. Mengod (Departament de Neuroquímica, Institut d'Investigacions Biomèdiques de Barcelona, CSIC-IDIBAPS, Barcelona, Spain). Membranes were defrosted and suspended in an incubation buffer with 1 mM of EDTA, 50 mM of Tris-HCl, 10 mM of MgCl<sub>2</sub>, and 2 UI/mL of adenosine deaminase, with pH = 7.4. Each well of a GF/C multiscreen plate (Millipore, Madrid, Spain), prepared in duplicate, contained 3 nM of [<sup>3</sup>H]ZM241385, 10  $\mu$ g of protein, and the tested compound. Nonspecific binding was calculated with 50  $\mu$ M of NECA. The reaction mixture was incubated at room temperature (25 °C) for 30 min, after which samples were filtered and measured in a microplate beta scintillation counter (Microbeta Trilux, Perkin Elmer, Madrid, Spain).

#### 3.3.3. Human A<sub>2B</sub> Receptor

Competition binding experiments of adenosine A<sub>2B</sub> receptor were made in membranes from HEK-293-A<sub>2B</sub> cells (Euroscreen, Brussels, Belgium). Membranes were defrosted and suspended in incubation buffer with 1 mM of EDTA, 10 mM of MgCl<sub>2</sub>, 0.1 mM of benzamide, 50 mM of Tris-HCl, 10  $\mu$ g/mL of bacitracin, and 2 UI/mL of adenosine deaminase, with pH = 6.5. Each reaction well, prepared in duplicate, contained 35 nM of [<sup>3</sup>H]DPCPX, 18  $\mu$ g of protein, and the tested compound. Non-specific binding was calculated with 400  $\mu$ M of NECA. The reaction mixture was incubated at room temperature (25 °C) for 30 min, after which samples were filtered through a multiscreen GF/C microplate and measured in a microplate beta scintillation counter (Microbeta Trilux, Perkin Elmer, Madrid, Spain).

### 3.3.4. Human A<sub>3</sub> Receptor

Competition binding experiments of adenosine A<sub>3</sub> receptor were made in membranes from HeLa-A<sub>3</sub> cells, which were provided by Dr. Mengod (Departament de Neuroquímica, Institut d'Investigacions Biomèdiques de Barcelona, CSIC-IDIBAPS, Barcelona, Spain). Membranes were defrosted and suspended in incubation buffer with 1 mM of EDTA, 5 mM of MgCl<sub>2</sub>, 50 mM of Tris-HCl, and 2 UI/mL of adenosine deaminase, with pH = 7.4. Each reaction well of a GF/B multiscreen plate (Millipore, Madrid, Spain), prepared in triplicate, contained 90 µg of protein, 30 nM of [<sup>3</sup>H]NECA, and the tested compound. Non-specific binding was determined with 100 µM of (R)-PIA. The reaction mixture was incubated at room temperature (25 °C) for 180 min, after which samples were filtered and measured in a microplate beta scintillation counter (Microbeta Trilux, Perkin Elmer, Madrid, Spain).

## 3.4. Functional Studies

### 3.4.1. Human A<sub>1</sub> Receptor

The agonist/antagonist behavior of tested compounds at A<sub>1</sub> receptor was determined in CHO-A<sub>1</sub> cells by measuring reversion induced by the tested compound of 10 µM of NECA-mediated inhibition of forskolin-stimulated cAMP production. Cells grown in 96-well plates with growth medium containing dialyzed fetal bovine serum were washed 2 times with F-12 nutrient mixture medium containing 25 mM of HEPES and 20 µM of the phosphodiesterase inhibitor rolipram, with pH = 7.4. After this time, the tested compounds were preincubated for 15 min in test medium at different concentrations (ranging from 0.1 nM to 10 µM). After this time, 10 µM of NECA and 3 µM of forskolin were added to each well. The incubation was continued for 15 min, and the reaction was stopped with a lysis buffer from the cAMP enzyme immunoassay kit (GE Healthcare, Chicago, IL, USA). Cell lysates were put in a plate with anti-IgG and anti-cAMP in the presence of cAMP-peroxidase during 60 min. Then, the wells were washed 4 times with the wash buffer from the kit, and TMB was added to each well and incubated for 60 min. The peroxidase reaction was stopped with 1 M of sulphuric acid, and the cAMP amount was determined from the optical density at 450 nm (Tecan M100 reader).

### 3.4.2. Human A<sub>3</sub> Receptor

The agonist/antagonist behavior of tested compounds at A<sub>3</sub> receptor was determined in CHO-A<sub>3</sub> cells, which were in-house generated at BioFarma Research Group (Department of Pharmacology, University of Santiago de Compostela, Spain), by measuring reversion induced by the tested compound of 10 µM of NECA inhibition of forskolin-stimulated cAMP production. Cells grown in 96-well plates with growth medium containing dialyzed fetal bovine serum were washed 2 times with DMEM F-12 nutrient medium containing 25 mM of HEPES, 30 µM of rolipram, with pH = 7.4. After this time, the tested compounds were preincubated for 15 min in test medium at different concentrations (ranging from 0.1 nM to 10 µM). Finally, 10 µM of NECA and 10 µM of forskolin were added to each well. The incubation lasted 15 min, and cAMP was determined through an enzyme immunoassay (Perkin Elmer) for Human A<sub>1</sub> receptor.

## 3.5. Data Analysis

Concentration response curves were fitted with Prism 10.1.2 (Graph Pad, San Diego, CA, USA). Radioligand binding pK<sub>i</sub> values were extrapolated from the equation

$$pK_i = -\log(IC_{50}/(1 + ([L]/K_D)))$$

where IC<sub>50</sub> is the concentration that displaced specific radioligand binding at 50%, extrapolated from non-linear fitting, [L] is the concentration of radioligand employed, and K<sub>D</sub> is the dissociation constant of the radioligand. In cAMP studies, K<sub>B</sub> values were derived by

employing the derivation of the Cheng-Prusoff equation, reported by Leffand Dougall in 1993 [37].

$$K_B = IC_{50}/(2 + (A/(EC_{50})^n)^{1/n} - 1);$$

where A is the concentration of the agonist employed to stimulate the receptor,  $EC_{50}$  is the potency of the agonist for stimulating the receptor, and n is the Hill slope of the antagonist's concentration-response curve.

### 3.6. Docking Studies

The molecular docking calculations were carried out using the Molecular Operating Environment (MOE) software (version 2022.02, <https://www.chemcomp.com>, accessed on 25 April 2024) and the crystal structure of the  $A_1$  receptor in complex with the ligand DU1 (PDB accession code: 5UEN) [35]. The receptor was prepared by removing non-protein atoms and adding missing side chains. Engineered mutations were reverted back to the wild-type sequence based on the side chain rotamers determined through MOE. The protonation states of ionizable residues were set to the most probable at physiological pH. The binding site was defined based on the co-crystallized ligand, and default parameters in MOE were used in the docking calculations.

## 4. Conclusions

In summary, several 6-morpholino-purine derivatives with different substituents in C-2 and a methyl or a hydrogen at N-9 of the purine ring were synthesized and pharmacologically tested at human  $A_1$ ,  $A_{2A}$ ,  $A_{2B}$ , and  $A_3$  adenosine receptor subtypes. Four of the twenty-three synthesized purines showed high affinity and selectivity for  $A_1$  receptors, and five showed high affinity and selectivity for  $A_3$  receptors. Three compounds (**3k**, **3q**, and **3u**) showed a dual affinity for  $A_1/A_{2A}$ ,  $A_1/A_3$ , and  $A_1/A_{2B}$  receptors, respectively. In addition, three compounds showed high affinity without selectivity for any specific adenosine receptor subtype (**3n**, **3o**, and **3x**). SAR studies showed that the substituent groups in C-2, C-6, and N-9 positions affect the potency and selectivity of the compounds for adenosine receptors. To generate highly potent antagonist ligands for adenosine receptors, a morpholine group and a hydrogen atom must be present in C-6 and N-9, respectively. Indeed, 9H-purines bind some 5–358 times more strongly than their corresponding 9- $CH_3$ -purines for the adenosine receptors. The substituted aryl group in C-2 is very important for governing the selectivity towards the adenosine receptor subtypes. Additionally, our results suggested that the  $A_3$  receptor subtype presents a larger hydrophobic pocket than that of the  $A_1$  receptor subtype, as  $A_3$  accommodates bigger substituent groups Y present at the *meta* position of substituent  $R^1$ . In conclusion, several purine-based compounds are described herein, some with high affinity and antagonist selectivity for  $A_1$ ,  $A_3$ , dual  $A_1/A_{2A}$ ,  $A_1/A_{2B}$ , and  $A_1/A_3$  receptors.

**Supplementary Materials:** The following supporting information can be downloaded at: <https://www.mdpi.com/article/10.3390/molecules29112543/s1>,  $^1H$  and  $^{13}C$  NMR spectra of representative synthesized compounds.

**Author Contributions:** Conceptualization, F.A., M.F.P. and M.A.C.; formal analysis, M.C., J.B., J.C. and M.A.C.; funding acquisition, J.C., M.I.L., M.F.P. and M.A.C.; investigation, F.A., C.C., A.R., S.T., M.C., J.B. and H.H.; methodology, M.C. and M.A.C.; project administration, M.I.L., M.F.P. and M.A.C.; supervision, J.C., M.F.P. and M.A.C.; writing—original draft, F.A.; writing—review and editing, M.A.C. All authors have read and agreed to the published version of the manuscript.

**Funding:** This research was funded by the Portuguese Fundação para a Ciência e Tecnologia/Ministério da Educação e Ciência (MEC) through funds in the framework of the Strategic Funding of CQUM (UID/QUI/00686/2020), (UID/QUI/00686/2018), FEDER funds through the Operational Programme for Competitiveness Factors (COMPETE 2020), Programa Operacional de Competitividade e Internacionalização (POCI) (POCI-01-0145-FEDER-031354) Rede Nacional de RMN (PINFRA/22161/2016), and PhD grants to Carla Correia and Ashly Rocha (SFRH/BD/22270/2005; SFRH/BD/85937/2012), Xunta de Galicia (ED431C 2022/20), and the European Regional Development Fund (ERDF). Filipe Areias

gratefully acknowledges the Post-PhD grant from the Portuguese FCT (SFRH/BPD/26106/2005). J.C. received funding from the Olle Engkvist Foundation (219-0154) and the Swedish Research Council (2021-4186). The LECO instrument for elemental analysis TruSpec Micro CHN was purchased within the PT-OPENSREEN project (NORTE-01—0145-FEDER-085468), financed by CCDR-N.

**Institutional Review Board Statement:** Not applicable.

**Informed Consent Statement:** Not applicable.

**Data Availability Statement:** Data are contained within the article and Supplementary Materials.

**Conflicts of Interest:** The authors declare no conflicts of interest.

## References

1. Batra, R.; Jain, V.; Sharma, P. Adenosine: A partially discovered medicinal agent. *Future J. Pharm. Sci.* **2021**, *7*, 214. [[CrossRef](#)] [[PubMed](#)]
2. Borea, P.A.; Gessi, S.; Merighi, S.; Vincenzi, F.; Varani, K. Pharmacology of adenosine receptors: The state of the art. *Physiol. Rev.* **2018**, *98*, 1591–1625. [[CrossRef](#)] [[PubMed](#)]
3. Jamwal, S.; Mittal, A.; Kumar, P.; Alhayani, D.M.; Al-Aboudi, A. Therapeutic potential of agonists and antagonists of A1, A2a, A2b and A3 adenosine receptors. *Curr. Pharm. Des.* **2019**, *25*, 2892–2905. [[CrossRef](#)] [[PubMed](#)]
4. Effendi, W.I.; Nagano, T.; Kobayashi, K.; Nishimura, Y. Focusing on adenosine receptors as a potential targeted therapy in human diseases. *Cells* **2020**, *9*, 785. [[CrossRef](#)] [[PubMed](#)]
5. Ijzerman, A.P.; Jacobson, K.A.; Müller, C.E.; Cronstein, B.N.; Cunha, R.A. International union of basic and clinical pharmacology. CXII: Adenosine receptors: A further updates. *Pharmacol. Rev.* **2022**, *74*, 340–372. [[CrossRef](#)] [[PubMed](#)]
6. Müller, C.E.; Jacobson, K.A. Recent developments in adenosine receptor ligands and their potential as novel drugs. *Biochim. Biophys. Acta Biomembr.* **2011**, *1808*, 1290–1308. [[CrossRef](#)] [[PubMed](#)]
7. Borah, P.; Deka, S.; Mailavaram, R.P.; Deb, P.K. P1 receptor agonists/antagonists in clinical trials-potential drug candidates of the future. *Curr. Pharm. Des.* **2019**, *25*, 2792–2807. [[CrossRef](#)] [[PubMed](#)]
8. Gao, Z.-G.; Tosh, D.K.; Jain, S.; Yu, J.; Suresh, R.R.; Jacobson, K.A. A1 adenosine receptor agonists, antagonists, and allosteric modulators. In *The Adenosine Receptors*; Borea, P., Varani, K., Gessi, S., Merighi, S., Vincenzi, F., Eds.; Springer International Publishing: Cham, Germany, 2018; pp. 59–89.
9. Stockwell, J.; Jakova, E.; Cayabyab, F. Adenosine A1 and A2A Receptors in the brain: Current research and their role in neurodegeneration. *Molecules* **2017**, *22*, 676. [[CrossRef](#)] [[PubMed](#)]
10. Pak, E.S.; Cha, J.J.; Cha, D.R.; Kanasaki, K.; Ha, H. Adenosine receptors as emerging therapeutic targets for diabetic kidney disease. *Kidney Res. Clin. Pract.* **2022**, *41*, S74–S88. [[CrossRef](#)] [[PubMed](#)]
11. Ramos-Barbon, D.; Brienza, N.S.; Bigorra Rodríguez, T.; Mateus Medina, É.F.; Gich Saladich, I.; Puntos Rodríguez, M.; Antonijoan Arbos, R.M.; Quirce Gancedo, S.; Castro Palomino Laria, N.; Castro Palomino Laria, J. PBF-680, an oral A1 adenosine receptor antagonist, inhibits the late allergic response (LAR) in mild-to-moderate atopic asthmatics: A Phase-IIa trial. *Eur. Respir. J.* **2020**, *56*, 4784. [[CrossRef](#)]
12. Nguyen, A.T.N.; Tran, Q.L.; Baltos, J.-A.; McNeill, S.M.; Nguyen, D.T.N.; May, L.T. Small molecule allosteric modulation of the adenosine A1 receptor. *Front. Endocrinol.* **2023**, *14*, 1184360. [[CrossRef](#)] [[PubMed](#)]
13. Sousa, J.B.; Fresco, P.; Diniz, C.; Goncalves, J. Adenosine receptor ligands on cancer therapy: A review of patent literature. *Recent Pat. Anticancer. Drug Discov.* **2018**, *13*, 40–69. [[CrossRef](#)] [[PubMed](#)]
14. Kim, Y.; Kim, D.M.; Son, W.C.; Moon, B.G. A selective adenosine A3 receptor antagonist, HL3501, has therapeutic potential in preclinical liver and renal fibrosis models. *Vivo* **2022**, *36*, 2186–2193. [[CrossRef](#)] [[PubMed](#)]
15. Baraldi, P.G.; Romagnoli, R.; Saponaro, G.; Baraldi, S.; Tabrizi, M.A.; Preti, D. A3 adenosine receptor antagonists: History and future perspectives. In *A3 Adenosine Receptors from Cell Biology to Pharmacology and Therapeutics*; Borea, P.A., Ed.; Springer Science+Business Media B.V.: Dordrecht, The Netherlands, 2010; pp. 121–147.
16. Spinaci, A.; Buccioni, M.; Dal Ben, D.; Maggi, F.; Marucci, G.; Francucci, B.; Santoni, G.; Lambertucci, C.; Volpini, R. A3 adenosine receptor antagonists with nucleoside structures and their anticancer activity. *Pharmaceuticals* **2022**, *15*, 164. [[CrossRef](#)] [[PubMed](#)]
17. Jacobson, K.A.; Merighi, S.; Varani, K.; Borea, P.A.; Baraldi, S.; Aghazadeh Tabrizi, M.; Romagnoli, R.; Baraldi, P.G.; Ciancetta, A.; Tosh, D.K.; et al. A3 adenosine receptors as modulators of inflammation: From medicinal chemistry to therapy. *Med. Res. Rev.* **2018**, *38*, 1031–1072. [[CrossRef](#)]
18. Spinozzi, E.; Baldassarri, C.; Acquaticci, L.; Del Bello, F.; Grifantini, M.; Cappellacci, L.; Riccardo, P. Adenosine receptors as promising targets for the management of ocular diseases. *Med. Chem. Res.* **2021**, *30*, 353–370. [[CrossRef](#)] [[PubMed](#)]
19. Kim, Y.; Yang, J.; Kim, J.Y.; Lee, J.M.; Son, W.C.; Moon, B.G. HL3501, a novel selective A3 adenosine receptor antagonist, lowers intraocular pressure (IOP) in animal glaucoma models. *Transl. Vis. Sci. Technol.* **2022**, *11*, 30. [[CrossRef](#)] [[PubMed](#)]
20. Barkan, K.; Lagarias, P.; Stampelou, M.; Stamatis, D.; Hoare, S.; Safitri, D.; Klotz, K.N.; Vrontaki, E.; Kolocouris, A.; Ladds, G. Pharmacological characterisation of novel adenosine A3 receptor antagonists. *Sci. Rep.* **2020**, *10*, 20781. [[CrossRef](#)] [[PubMed](#)]
21. Guglielmi, P.; Carradori, S.; Campestre, C.; Poce, G. Novel therapies for glaucoma: A patent review (2013–2019). *Expert Opin. Ther. Pat.* **2019**, *29*, 769–780. [[CrossRef](#)] [[PubMed](#)]



22. Lee, J.; Kim, S.; Kim, D.; Ahn, K.H.; Lee, G.B.; Kim, D.; Hwang, H.S. Compounds antagonizing A3 adenosine receptor, method for preparing them, and medical-use thereof. WO2017123058A1, 20 July 2017.
23. Petrelli, R.; Torquati, I.; Kachler, S.; Luongo, L.; Maione, S.; Franchetti, P.; Grifantini, M.; Novellino, E.; Lavecchia, A.; Klotz, K.N.; et al. 5'-C-ethyl-tetrazolyl-N 6-substituted adenosine and 2-chloro-adenosine derivatives as highly potent dual acting A1 adenosine receptor agonists and A3 adenosine receptor antagonists. *J. Med. Chem.* **2015**, *58*, 2560–2566. [[CrossRef](#)]
24. Abdelrahman, A.; Yerande, S.G.; Namasivayam, V.; Klapschinski, T.A.; Alnouri, M.W.; El-Tayeb, A.; Müller, C.E. Substituted 4-phenylthiazoles: Development of potent and selective A1, A3 and dual A1/A3 adenosine receptor antagonists. *Eur. J. Med. Chem.* **2020**, *186*, 111879. [[CrossRef](#)]
25. Massie, B.M.; O'Connor, C.M.; Metra, M.; Ponikowski, P.; Teerlink, J.R.; Cotter, G.; Weatherley, B.D.; Cleland, J.G.F.; Givertz, M.M.; Voors, A.; et al. Rolofylline, an adenosine A1-receptor antagonist, in acute heart failure. *N. Engl. J. Med.* **2010**, *363*, 1419–1428. [[CrossRef](#)]
26. Teerlink, J.R.; Iragui, V.J.; Mohr, J.P.; Carson, P.E.; Hauptman, P.J.; Lovett, D.H.; Miller, A.B.; Piña, I.L.; Thomson, S.; Varosy, P.D.; et al. The safety of an adenosine A1-receptor antagonist, rolofylline, in patients with acute heart failure and renal impairment. *Drug Saf.* **2012**, *35*, 233–244. [[CrossRef](#)]
27. Mitrovic, V.; Seferovic, P.; Dodic, S.; Krotin, M.; Neskovic, A.; Dickstein, K.; de Voogd, H.; Bocker, C.; Ziegler, D.; Godes, M.; et al. Cardio-renal effects of the A1 adenosine receptor antagonist SLV320 in patients with heart failure. *Circ. Hear. Fail.* **2009**, *2*, 523–531. [[CrossRef](#)]
28. Areias, F.; Correia, C.; Rocha, A.; Brea, J.; Castro, M.; Loza, M.I.; Proença, M.F.; Carvalho, M.A. 2-Aryladenine derivatives as a potent scaffold for A1, A3 and dual A1/A3 adenosine receptor antagonists: Synthesis and structure-activity relationships. *Bioorganic Med. Chem.* **2019**, *27*, 3551–3558. [[CrossRef](#)]
29. Alves, M.J.; Booth, B.L.; Proença, M.F.J.R.P. Synthesis of 5-amino-4-(cyanoformimidoyl)-1H-imidazole: A reactive intermediate for the synthesis of 6-carbamoyl-1,2-dihydropurines and 6-carbamoylpurines. *J. Chem. Soc. Perkin Trans. 1* **1990**, *1*, 1705–1712. [[CrossRef](#)]
30. Correia, C.; Carvalho, M.A.; Proença, M.F. Synthesis and in vitro activity of 6-amino-2,9-diarylpurines for Mycobacterium tuberculosis. *Tetrahedron* **2009**, *65*, 6903–6911. [[CrossRef](#)]
31. Jacobson, K.A. Introduction to adenosine receptors as therapeutic targets. In *Adenosine Receptors in Health and Disease*; Wilson, C.N., Mustafa, S.J., Eds.; Springer: Berlin/Heidelberg, Germany, 2009; pp. 1–24.
32. Giorgi, I.; Nieri, P. Adenosine A 1 modulators: A patent update (2008 to present). *Expert Opin. Ther. Pat.* **2013**, *23*, 1109–1121. [[CrossRef](#)]
33. Shook, B.C.; Rassnick, S.; Osborne, M.C.; Davis, S.; Westover, L.; Boulet, J.; Hall, D.; Rupert, K.C.; Heintzelman, G.R.; Hansen, K.; et al. In vivo characterization of a dual adenosine A2A/A1 receptor antagonist in animal models of Parkinson's disease. *J. Med. Chem.* **2010**, *53*, 8104–8115. [[CrossRef](#)]
34. Jung, J.; Lee, Y.; Moon, A.-N.; Ann, J.; Jeong, J.J.; Do, N.; Lee, J. Discovery of Novel Dual Adenosine A2A and A1 receptor antagonists with 1H-Pyrazolo[3,4-d]pyrimidin-6-amine core scaffold as anti-Parkinson's disease agents. *Pharmaceuticals* **2022**, *15*, 922. [[CrossRef](#)]
35. Glukhova, A.; Thal, D.M.; Nguyen, A.T.; Vecchio, E.A.; Jörg, M.; Scammells, P.J.; May, L.T.; Sexton, P.M.; Christopoulos, A. Structure of the adenosine A1 receptor reveals the basis for subtype selectivity. *Cell* **2017**, *168*, 867–877. [[CrossRef](#)]
36. Jacobson, K.A.; Gao, Z.; Matricon, P.; Eddy, M.T.; Carlsson, J. Adenosine A 2A receptor antagonists: From caffeine to selective non-xanthines. *Br. J. Pharmacol.* **2022**, *179*, 3496–3511. [[CrossRef](#)]
37. Leff, P.; Dougall, I.G. Further concerns over Cheng-Prusoff analysis. *Trends Pharmacol. Sci.* **1993**, *14*, 110–112. [[CrossRef](#)]

**Disclaimer/Publisher's Note:** The statements, opinions and data contained in all publications are solely those of the individual author(s) and contributor(s) and not of MDPI and/or the editor(s). MDPI and/or the editor(s) disclaim responsibility for any injury to people or property resulting from any ideas, methods, instructions or products referred to in the content.