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Design and Synthesis of Novel Thiazolo[5,4-d]pyrimidine Derivatives with High Affinity for Both the Adenosine A1 and A2A Receptors, and Efficacy in Animal Models of Depression

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Abstract: New compounds with a 7-amino-2-arylmethyl-thiazolo[5,4-d]pyrimidine structure were synthesized and evaluated in vitro for their affinity and/or potency at the human (h) A1, hA2A, hA2B, and hA3 adenosine receptors (ARs). Several compounds (5, 8–10, 13, 18, 19) were characterized by nanomolar and subnanomolar binding affinities for the hA1 and the hA2A AR, respectively. Results of molecular docking studies supported the in vitro results. The 2-(2-fluorobenzyl)-5-(furan-2-yl)-thiazolo[5,4-d]pyrimidin-7-amine derivative 18 (hA1 K_i = 1.9 nM; hA2A K_i = 0.06 nM) was evaluated for its antidepressant-like activity in in vivo studies, the forced swimming test (FST), the tail suspension test (TST), and the sucrose preference test (SPT) in mice, showing an effect comparable to that of the reference anxiolytic.

Keywords: G protein-coupled receptors; adenosine A1 receptor ligands; adenosine A2A receptor ligands; thiazolo[5,4-d]pyrimidines; ligand-adenosine receptor modeling studies; depression

1. Introduction

Depression is a common debilitating illness ranked in many countries as the most prevalent of all psychiatric diseases [1]. According to the Diagnostic and Statistical Manual of Mental Disorders, depression is characterized by depressed mood and anhedonia (decreased interest in, and ability to experience, pleasure) associated with symptoms that may include significant weight gain or loss, insomnia, psychomotor agitation or retardation, fatigue or loss of energy, diminished ability to think or concentrate, and recurrent thoughts of death or suicide [2]. Moreover, depression may represent a risk factor for cancer [3], diabetes [4], and cardiovascular disorders [5,6], and shows comorbidity with several medical illnesses including serious CNS disorders such as Alzheimer’s (AD) and Parkinson’s disease (PD) [6,7], stroke [8], and chronic pain [9]. Over the years, different classes of antidepressants have been developed. However, the current treatments of depressed patients are still unsatisfactory. Particularly worrying is the fact that many patients only partially respond and some remain refractory. Moreover, commonly used medications are associated with adverse reactions difficult to tolerate including cardiovascular, anticholinergic, neurologic, gastrointestinal, and other side effects. Another big drawback of antidepressant therapy currently in use is that its full efficacy begins to appear only after 4–6 weeks of treatment or even more [10,11]. Thus, the search for novel therapeutic strategies for the treatment of depression disorders represents an important research priority.
Particular interest has been devoted to the adenosine signaling system as a new target for the treatment of depression [12,13]. Adenosine is an endogenous nucleoside present in all mammal tissues where it regulates several important functions. Adenosine exerts its actions by binding to different receptor subtypes, namely A$_1$, A$_{2A}$, A$_{2B}$, and A$_3$, all belonging to the G-protein coupled receptor family (GPCR). Upon stimulation, A$_1$ and A$_3$ adenosine receptors (ARs) inhibit adenylate cyclase activity, thus decreasing cAMP levels. Instead, A$_{2A}$ and A$_{2B}$ ARs stimulation increases adenylate cyclase activity and cAMP production [14]. In the central nervous system (CNS), adenosine plays an important role in the regulation of synaptic transmission and neuronal excitability. In the brain, several AR subtypes are present, with A$_1$ and A$_{2A}$ ARs being the most abundant. In the CNS, A$_{2A}$ ARs are localized on neurons and glial cells and are expressed in almost all districts with the highest level found in the striatum. Adenosine A$_1$ receptors are widely distributed in the brain with a high concentration, especially in the hippocampus. All these regions regulate processes such as cognition, motivation, and emotion, which appear to be altered in depressed patients [12,13].

Caffeine, which is a naturally occurring methylxanthine, is one of the most frequently used psychoactive substances. Caffeine is a weak non-selective A$_1$ and A$_{2A}$ AR antagonist, and has been demonstrated to influence behavior in classical animal models of depression [15]. In fact, caffeine after acute or repeated administration of a broad range of doses, increased mobility in the forced swim test (FST) and tail suspension test (TST). These screening tests are usually used to evaluate antidepressant drugs where the duration of immobilization is considered a measure of the depressive state. In accordance with the effects of caffeine, A$_1$ and A$_{2A}$ AR selective antagonists also provide antidepressant-like behavior. Thus, the single administration in mice of DPCPX, a selective A$_1$ AR antagonist as well as of the well-known A$_{2A}$ AR selective antagonists SCH 58261, ZM 241385, or istradefylline, exhibited antidepressant activity in the FST and TST [16–18]. Finally, it has been demonstrated that both caffeine and selective A$_1$ or A$_{2A}$ AR antagonists can enhance the antidepressant like activity of common antidepressant [16,19,20].

To conclude, it is important to mention the role of ARs in PD. In fact, much experimental data have been produced that demonstrate the efficacy of A$_{2A}$ AR antagonists in different animal models of PD [21]. Moreover, the interest in the use of A$_{2A}$ AR antagonists in PD has increased also due to their effectiveness in the treatment of non-motor symptoms of PD such as depression [22,23]. Preclinical animal models as well as clinical studies also demonstrated that non selective A$_1$ and A$_{2A}$ AR antagonists improved motor dysfunctions of PD, were neuroprotective through A$_{2A}$ AR antagonism, and may also have enhanced cognitive functions via A$_1$ AR antagonism [21,24,25].

Our efforts in the pursuit of novel AR antagonists have centered on mono- or bicyclic heteroaromatic systems. [26–30]. Within this research, an interesting class of antagonists/inverse agonists (i.e., the thiazolo[5,4-d]pyrimidine series) was identified [31–35]. Recently, we investigated the 7-amino-thiazolo[5,4-d]pyrimidine series A (Figure 1) featured by a phenyl or a furan-2yl ring at position 2 (R$^2$) combined with an aryl or heteroaryl group at position 5 (R$^5$) [34].

The results showed that compounds of series A are in general A$_1$/A$_{2A}$ AR dual antagonists and that their affinity can be modulated first by the nature of the substituent attached at position-2, and second, by that of substituents at position-5. Based on these findings, we herein report on the design and synthesis of new 7-aminothiazolopyrimidines 1–19 to obtain more potent A$_1$ and/or A$_{2A}$ AR antagonists. To this goal, compounds 1–19 were decorated at position-5 with selected substituents that in previously reported compounds of series A enhanced affinity toward A$_1$ and/or A$_{2A}$ AR (i.e., an unsubstituted or 3-CN or 3-OH substituted phenyl ring, and a heteroaryl moiety (furan-2yl or 5-methyl-furan-2yl) [34]), while at position-2, a benzyl or an ortho-substituted benzyl moiety was introduced since it improved A$_{2A}$ AR affinity in other classes of our bicyclic dual A$_1$/A$_{2A}$ antagonists [26].
2. Results and Discussion

2.1. Chemistry

The synthesis of the reported molecules 1–19 was achieved as depicted in Schemes 1 and 2.

`Scheme 1. Reagents and conditions. (a) NMP, 150 °C, 10–15 h; (b) POCl₃, 160 °C MW, 30 min; (c) NH₄OH/EtOH, reflux, 6 h.`

`Scheme 2. Reagents and conditions. (a) R⁵B(OH)₂, tetrakis, Na₂CO₃, DME/H₂O, reflux 4 h (1–5) or microwave irradiation, 160 °C, 30 min (6–19).`
The bicyclic thiazolopyrimidine core suitably decorated at position-2 was first synthesized, then introduction of the diverse substituents at position-5 was performed. Briefly, by reacting 2-aminothiole \(20\) [36] with the suitable arylacetylchloride \(21–24\) [37–39] at high temperature, the corresponding thiazolo[5,4-d]pyrimidine-5,7-dihydroxy derivatives \(25–28\) were obtained (Scheme 1). The latter were chlorinated by reaction with \(\text{POCl}_3\) under microwave irradiation to the corresponding 5,7-dichloro bicyclic derivatives \(29–32\), which in the presence of aqueous ammonia solution (33%) furnished the 7-amino-5-chloro substituted intermediates \(33–36\). Finally, replacement of the 5-chloro group of \(33–36\) with the opportune \(R^5\) substituents was achieved by reacting compounds \(33–36\) and the suitable boronic acids under Suzuki conditions (Scheme 2).

### 2.2. Binding and cAMP Assays

Compounds \(1–19\) were evaluated for their binding affinity at \(hA_1\), \(hA_{2A}\), and \(hA_3\) ARs, stably transfected in Chinese hamster ovary (CHO) cells. Moreover, they were also tested at the \(hA_{2B}\) AR subtype by determining their inhibitory effects on 5′-(N-ethylcarboxamido) adenosine (NECA)-stimulated cAMP levels in \(hA_{2B}\) CHO cells (Table 1).

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

| X | \(R_3\) | \(hA_1 K_i\) (nM) | \(hA_{2A} K_i\) (nM) | \(hA_3 K_i\) (nM) | \(hA_{2B} IC_{50}\) (nM) |
|---|---|---|---|---|---|
| 1 | H | \(C_6 H_5\) | 20 ± 4.3 | 2.9 ± 0.04 | 26.6 ± 6.0 | >30,000 |
| 2 | H | \(C_6 H_5\)-3-CN | 51.7 ± 11.7 | 4.6 ± 0.9 | 76 ± 0.9 | >30,000 |
| 3 | H | \(C_6 H_5\)-3-OH | 15.7 ± 3.4 | 7.3 ± 1.0 | 103.6 ± 21.1 | >30,000 |
| 4 | H | furan-2yl | 15.5 ± 3.6 | 1.1 ± 0.2 | 65.4 ± 12 | 794 ± 196 |
| 5 | H | furan-2yl-5-CH\(_3\) | 6.8 ± 1.2 | 0.8 ± 0.04 | 18.8 ± 1.9 | 1096 ± 228 |
| 6 | Cl | \(C_6 H_5\) | 2.8 ± 0.05 | 0.4 ± 0.04 | 143 ± 0.2 | >30,000 |
| 7 | Cl | \(C_6 H_5\)-3-CN | 72.8 ± 15 | 6.3 ± 0.18 | 295.1 ± 0.6 | 6785 ± 62 |
| 8 | Cl | furan-2yl | 3.8 ± 0.6 | 0.2 ± 0.03 | 4786 ± 5.1 | 1887 ± 175 |
| 9 | Cl | furan-2yl-5-CH\(_3\) | 0.5 ± 0.1 | 0.07 ± 0.003 | 8.5 ± 1.6 | 8847 ± 1445 |
| 10 | OCH\(_3\) | \(C_6 H_5\) | 4.5 ± 0.8 | 0.7 ± 0.1 | 37.4 ± 1 | >30,000 |
| 11 | OCH\(_3\) | \(C_6 H_5\)-3-CN | 28.4 ± 4 | 2.4 ± 0.09 | 54.2 ± 1.2 | 1536 ± 35 |
| 12 | OCH\(_3\) | \(C_6 H_5\)-3-OH | 10.2 ± 0.3 | 1.9 ± 0.4 | 79.6 ± 2.3 | 7424 ± 876 |
| 13 | OCH\(_3\) | furan-2yl | 2.4 ± 0.1 | 0.2 ± 0.03 | 48.6 ± 2.4 | 1536 ± 35 |
| 14 | OCH\(_3\) | furan-2yl-5-CH\(_3\) | 11.5 ± 2.8 | 2.0 ± 0.6 | 18.2 ± 5 | 2416 ± 545 |
| 15 | F | \(C_6 H_5\) | 1.9 ± 0.05 | 1.8 ± 0.6 | 72.1 ± 5.3 | >30,000 |
| 16 | F | \(C_6 H_5\)-3-CN | 21.5 ± 5.1 | 1.4 ± 0.3 | 95.5 ± 1.5 | 1514 ± 121 |
| 17 | F | \(C_6 H_5\)-3-OH | 0.5 ± 0.1 | 13.8 ± 0.06 | 455.9 ± 15 | 3237 ± 136 |
| 18 | F | furan-2yl | 1.9 ± 0.08 | 0.06 ± 0.02 | 93.1 ± 2.8 | 384 ± 55 |
| 19 | F | furan-2yl-5-CH\(_3\) | 5.7 ± 0.1 | 0.26 ± 0.01 | 50.2 ± 9.2 | 1695 ± 228 |
| 20 | | | 148 ± 16 | 19 ± 6.2 | 84 ± 13 | >30,000 |

\(^a\) Displacement of specific [\(^3\)H]-CCPA binding at human \(A_1\) AR expressed in CHO cells, \((\mu = 3–6)\). \(^b\) Displacement of specific [\(^3\)H]-NECA binding at human \(A_{2A}\) AR expressed in CHO cells. \(^c\) Displacement of specific [\(^3\)H]-HEMA binding at human \(A_3\) AR expressed in CHO cells. \(^d\) \(IC_{50}\) values of the inhibition of NECA-stimulated adenylyl cyclase activity in CHO cells expressing \(hA_{2B}\) AR. Data are expressed as means ± SEM. \(^e\) Reference [34].

The results showed that we reached our goal, since all final compounds \(1–19\) featured by the presence of an unsubstituted or ortho-substituted benzyl ring at position 2 of the bicyclic core, exhibited good to high binding affinities at both the \(A_1\) and \(A_{2A}\) ARs higher than those of the previously reported compound \(20\) bearing a phenyl ring at the same position. Furthermore, compounds \(1–19\) were more selective toward these two receptor...
subtypes. In fact, affinity values at the A3 AR were comparable to or higher than that of reference 20. Only three compounds (i.e., 5, 9, 18) possessed a higher affinity and among them, compound 9 showed a K_i value in the nanomolar range (K_i = 8.5 nM). Finally, all the examined compounds blocked the A2B AR with very low potencies spanning from IC_{50} = 384 nM to IC_{50} > 30,000 nM.

Analyzing the results in detail, eight compounds (5, 6, 8, 10, 13, 15, 18, 19) showed single-digit nanomolar hA1 AR K_i values (1.9 nM < K_i < 6.8 nM), while two (compound 9 and 17) fell in the subnanomolar range (K_i = 0.5 nM for both). All the other derivatives (1–4, 7, 11, 12, 14, 16) possessed good to high hA1 AR binding affinity spanning from a K_i value of 10.2 nM to one of 72.8 nM.

Regarding the affinity at the A2A AR, all the examined compounds were more active at this subtype than at the A1 AR. Moreover, compounds 1–19 exhibited hA2A AR K_i values below 13.8 nM, and for eight of them (5, 6, 8, 9, 10, 13, 18, 19), the K_i values fell in the subnanomolar range (0.06 nM < K_i < 0.8 nM). In conclusion, we identified several compounds (5, 6, 8, 9, 10, 13, 18, 19) characterized simultaneously by nanomolar and subnanomolar binding affinities for the A1 and A2A ARs, respectively. Among them, compound 18 emerged, exhibiting the highest hA1 and A2A AR affinities (A1 K_i = 1.9 nM; A2A K_i = 0.06 nM) combined with the highest selectivity toward these two receptor subtypes.

In general, better results in terms of binding affinities at both the A1 and A2A ARs were found in the ortho-substituted 2-benzyl derivative groups (6–9, 10–14, 15–19) if compared to the corresponding unsubstituted 2-benzyl derivative group (1–5). However, it is difficult to establish which substituent (Cl, OCH_3, F) on the 2-benzyl ring leads to more advantageous effects. Moreover, it seems that the presence of an unsubstituted phenyl as well as a furan-2yl or a 5-methyl-furan-2yl ring at position 5 of the bicyclic core, leads to compounds (1, 4, 5, 6, 8, 9, 10, 13, 14, 15, 18, 19), showing high affinity for both the A1 and A2A ARs regardless of the substituent at position 2.

Derivatives 5, 8–10, 13, 18 and 19, due to their high hA2A AR affinity, were also tested to assess their ability to block the hA2A AR by evaluating their effect on NECA-induced increase of cAMP accumulation in CHO cells, stably expressing hA2A ARs (Table 2). Compound 18 was also tested to evaluate its antagonist behavior at the hA1 AR by assessing its ability to counteract NECA-induced decrease of cAMP accumulation.

Table 2. Potencies of selected thiazolopyrimidines at hA2A AR.

|                | hA2A IC_{50} (nM) a | hA2A IC_{50} (nM) a |
|----------------|---------------------|---------------------|
| 5              | 88 ± 21             | 13                  |
| 8              | 39±9                | 19                  |
| 9              | 7.7 ± 1.3           | 14 ± 3.4            |
| 10             | 125 ± 33            | 18                  |
|                | (360 ± 115) b       |                     |

a IC_{50} values of the inhibition of NECA-stimulated adenyl cyclase activity in CHO cells expressing hA2A AR.
b IC_{50} values of NECA-stimulated activity in CHO cells expressing hA1 AR. Data are expressed as means ± standard errors.

The data indicated that the tested compounds were potent A2A AR antagonists showing IC_{50} values below 100 nM. In particular, 9 and 18, in accordance with their binding data, resulted in the most potent compounds possessing a potency of the low nanomolar order (IC_{50} = 7.7 and IC_{50} = 14, respectively). IC_{50} value at hA1 AR of compound 18 indicated that it behaves as a quite potent hA1 AR antagonist (IC_{50} = 360 ± 115 nM).

2.3. Molecular Docking Studies

The binding mode at the hA2A AR cavity of the newly synthesized compounds was simulated with the aid of computational tools. As a biomolecular target of docking analyses, the crystal structure of the hA2A AR in complex with the antagonist/inverse agonist ZM241385 (pdb code: 5NM4; 1.7-Å resolution [40]) was chosen. MOE (Molecular Operating Environment August 2016 [41]) software and the CCDC Gold [42] and Cresset Flare [43] docking tools were used for docking analyses. Analogue protocol was also
employed to carry out docking studies at the hA1 AR. For this task, the crystal structure of the receptor in complex with the antagonist PSB36 (pdb code: 5N2S; 3.3-Å resolution) was selected [44].

As previously made for analogues of these compounds, docking analyses of the newly synthesized molecules were performed with various docking tools to obtain a sort of average binding mode prediction at the receptor binding site. The three docking tools provided analogous results. Docking conformation of compound 18 at the hA2A AR cavity is reported in Figure 2 (see text for details).

**Figure 2.** Docking-based hypothetical binding mode of the synthesized compounds at the hA2A AR cavity; compound 18 is represented. (A) global view of the compound orientation within the receptor cavity; (B, C) detailed view of the 5- and 2-substituents, respectively, and the receptor residues located in their proximity.

Docking conformations make the new compounds being inserted in the binding cavity with the thiazolo[5,4-d]pyrimidine scaffold located between the side chains of Phe168 (EL2) and Leu249 (6.51), providing π–π interaction with these two amino acids (Figure 2). The exocyclic amine group makes polar interaction with Asn253 (6.55) and Glu169 (EL2) side chains, while the N6 atom makes an additional polar interaction again with Asn253 (6.55). The 2-substituent (an unsubstituted or substituted benzyl group) points toward the extracellular space and is located in proximity to Ile66 (2.64), Ser67 (2.65), Thr68 (2.66), Leu167 (EL2), Phe168 (EL2), Glu169 (EL2), Asp170 (EL2), His264 (EL3), Leu267 (EL3), Met270 (7.35), and Tyr271 (7.36), while the 5-substituent is located in the depth of the binding cavity in proximity to residues belonging to the TM3, TM5, and TM6 segments (Val84 (3.32), Leu85 (3.33), Thr88 (3.36), Phe168 (EL2), Met177 (5.38), Trp246 (6.48), Leu249 (6.51), His250 (6.52), Asn253 (6.55), and Ile274 (7.39)).

Considering the 2-substituent, docking results suggest that this group makes mainly non-polar interactions with residues at the entrance of the binding cavity (Figure 2C). Analogous to previously reported pyrazolopyrimidine derivatives, the insertion of substituents at the 2-position of the 2-benzyl group leads to a modulation of the hA2A AR affinity. The insertion of a fluorine or a chlorine atom in this position leads to the most potent compounds of the series. These groups have interaction with residues in their proximity such as Leu267, Met270 (7.35), and Tyr271 (7.36) (see Figure 2C). The effect of the various modifications at the 2-substituent appears fairly interpreted by the docking scoring function. Figure 3 shows the pK_i hA2A AR vs. docking score (MOE GBVI/WSA dG score) plots for groups of compounds presenting the same 5-subsituent and various 2-groups.
Figure 3. Representation of pKₐ hA₂AR vs. docking score (MOE GBVI/WSA dG score) plots for groups of compounds presenting the same 5-substituent and various 2-groups. The pKₐ data are indicated as “pA²”, while the docking scores are indicated as “S”. Panels (A–D) represent the plots for compounds presenting in the 5-position a furan-2yl ring, a 5-methyl-furan-2yl ring, a phenyl ring, and a 3-cyanophenyl ring, respectively. The docking tool appears quite efficient in assigning better docking scores to compounds endowed with higher hA₂AR affinity.

The 5-substituent may be an unsubstituted or a 5-methyl substituted furyl ring, or an unsubstituted or a meta-substituted phenyl ring. Compounds bearing an unsubstituted or a 5-methyl substituted furyl ring are generally endowed with the highest hA₂AR affinity. This may be interpreted considering that the oxygen atom of the furyl ring gives a polar interaction with the amine function of Asn253⁶.⁵⁵, even in the presence of a further methyl group at the 5-position of this substituent. In the case of the compounds bearing a 5-phenyl ring, this polar interaction is not possible and is replaced by a slightly repulsive effect between the same substituent and the above cited Asn253⁶.⁵⁵. Considering the effect of the various 5-substituents on compound arrangement within the binding site, docking results show that the conformations of compounds bearing a 5-methylfurly or a phenyl ring at the 5-position almost matched each other, even if slightly displaced compared to the conformations of the analogues bearing a 5-furyl ring. Introduction of further substituents on the 5-phenyl ring leads to a more marked rearrangement of the compounds due to steric clashes with the receptor residues.

We performed a post-docking analysis by using the IF-E 6.0 [45] SVL script tool, which calculates the per-residue interaction energies (values in kcal/mol), where negative and positive energy values are associated with favorable and unfavorable interactions with the ligand, respectively. This tool is helpful in interpreting the binding affinities at ARs in previously reported studies [26,46,47]. To analyze the effect of the presence of a furyl ring, a 5-methylfuryl ring, or a phenyl group at the 5-position, we compared nine compounds
Results show that compounds bearing a 5-methylfuryl group at the 5-position generally provide a better interaction with the binding site residues in its proximity with respect to the corresponding 5-furyl analogues, in agreement with previously observed results for pyrazolopyrimidine at the hA2A AR [26]. Furthermore, compounds bearing a 5-furyl substituent afford a better interaction with the binding site residues with respect to the corresponding 5-phenyl derivatives, analogously to what was previously observed for thiazolopyrimidines at the same receptor [47]. These data are quite in agreement with the hA2A AR affinity values. The significantly different interaction with Asn253.55 between compounds bearing an unsubstituted or substituted furyl ring and compounds bearing a 5-phenyl group can be noticed, as above described. The only discrepancy between the interaction energy data and the affinity values was observed for compounds 18 and 19, bearing at the 5-position a furyl or a 5-methylfuryl substituent. For these two compounds, hA2A AR affinity data showed the first one as the most active, while the calculated interaction energies suggest a better interaction for the latter compound.

Docking studies performed at the hA1 AR (Figure 4) show that the herein reported compounds can adopt a binding conformation highly similar to the one at the hA2A AR, making analogue interactions at the two proteins (particularly in the depth of the cavity, in proximity to the 5-substituent). Slightly different environments can be observed at the entrance of the two cavities, defined by the receptor residues in the EL2 and EL3 segments. This cavity appears slightly larger at the hA1 AR compared with the corresponding region at the hA2A AR. As a consequence, the exocyclic amine function makes a H-bond interaction with the EL2 glutamate residue at the hA2A AR (Glu169), while the corresponding residue at the hA1 AR (Glu172) appears more distant from the same amine group. Analogously, the set of EL3 residues (Ser267, Thr270.35 and Tyr271.36) of hA1 AR in proximity to the 5-substituent makes the binding site entrance a slightly larger cavity with respect to the bulkier corresponding residues of hA2A AR EL3 (Leu267, Met270.35, and Tyr271.36). These factors could be at the basis of the observation that the affinity data at the hA1 AR subtype (generally in the low nanomolar order, as indicated above) are, on average, 10-fold lower than the corresponding data at the hA2A AR, even if the comparison of the data at the two receptors shows that there is a common trend.

Table 3. Interaction energies (values in kcal/mol) between compounds bearing the same 2-substituent and a furyl (8, 18, 4), a 5-methylfuryl (9, 19, 5), or a phenyl (6, 15, 1) substitutent at the 5-position, and the binding site residues located in the proximity of the 5-position. See text for details.

| R²    | 2-furyl | 2-furyl-5-CH₃ | C₆H₅ | 2-furyl | 2-furyl-5-CH₃ | C₆H₅ | 2-furyl | 2-furyl-5-CH₃ | C₆H₅ |
|-------|---------|--------------|------|---------|--------------|------|---------|--------------|------|
| Val84 | -0.0    | -0.2         | -0.1 | -0.1    | -0.3         | -0.1 | -0.0    | -0.4         | -0.2 |
| Leu85 | -0.8    | -1.1         | -0.5 | -0.8    | -1.1         | -0.5 | -0.8    | -1.1         | -0.6 |
| Thr88 | -0.0    | -0.2         | -0.2 | -0.0    | -0.2         | -0.2 | -0.0    | -0.3         | -0.3 |
| Phe168| -5.0    | -4.9         | -5.3 | -5.7    | -5.4         | -5.8 | -3.9    | -4.0         | -2.9 |
| Met177| -2.0    | -2.3         | -0.1 | -2.0    | -2.3         | -0.1 | -2.1    | -2.3         | -0.1 |
| Trp246| -0.6    | -1.3         | -0.5 | -0.6    | -1.3         | -0.5 | -0.6    | -1.3         | -0.6 |
| Leu249| -2.5    | -2.7         | -2.1 | -2.4    | -2.7         | -2.0 | -2.5    | -2.8         | -2.0 |
| His250| 0.1     | -1.07        | -0.6 | 0.1     | -0.3         | -0.7 | 0.1     | -0.3         | -0.7 |
| Asn253| -7.0    | -6.9         | -4.9 | -6.9    | -6.9         | -4.9 | -7.3    | -7.1         | -4.4 |
| Ile274| 0.6     | 0.3          | -0.2 | 0.5     | 0.3          | -0.2 | 0.6     | 0.3          | 0.1  |
| tot   | -17.2   | -21.0        | -14.5| -17.9   | -20.2        | -15.0| -16.5   | -19.3        | -11.7|

Table 3. Interaction energies (values in kcal/mol) between compounds bearing the same 2-substituent and a furyl (8, 18, 4), a 5-methylfuryl (9, 19, 5), or a phenyl (6, 15, 1) substituent at the 5-position, and the binding site residues located in the proximity of the 5-position. See text for details.

The table shows the interaction energies (values in kcal/mol) between compounds bearing the same 2-substituent and a furyl (8, 18, 4), a 5-methylfuryl (9, 19, 5), or a phenyl (6, 15, 1) substituent at the 5-position, and the binding site residues located in the proximity of the 5-position. The table is not directly related to the text, but it provides a quantitative analysis of the interaction energies.
2.4. Behavioral In Vivo Tests

On the basis of the results obtained in the binding and cAMP assays, we selected compound 18 for evaluation in the forced swimming test (FST) and the tail suspension test (TST) in mice, which are a widely used behavioral paradigm for the evaluation of antidepressant-like activity. Moreover, compound 18 was also tested in the sucrose preference test (SPT), which is a reward-based test highly predictive for anti-anhedonia-like activity [48, 49].

2.4.1. Forced Swimming Test and Tail Suspension Test

To execute the tests, mice were divided into groups, each of which consisted of 10 animals. The tests were performed after a single p.o. administration of compound 18 at different dosages (10 mg Kg$^{-1}$ and 30 mg Kg$^{-1}$). Amitriptyline (15 mg Kg$^{-1}$) was used as the reference drug. Compounds were acutely administrated 26 min before the beginning of the experiment. In the FST (Table 4), the duration of immobility was recorded during the last 4 min of the 6 min test.

Table 4. Antidepressant-like effect of 18 in the mouse forced swimming test.

| Treatment               | Immobility Time (s) $^a$ |
|-------------------------|--------------------------|
| vehicle                 | 193.7 ± 8.9              |
| 18 10 mg kg$^{-1}$ p.o. | 184.7 ± 5.6              |
| 18 30 mg kg$^{-1}$ p.o. | 143.5 ± 9.1 **           |
| amitriptyline 15 mg kg$^{-1}$ s.c. | 149.4 ± 4.6 ** |

18 was administered per os (p.o.), amitriptyline was administered subcutaneously (s.c.). $^a$ Each value is the mean ± SEM of 10 mice per group, performed in two different experimental sets. ** $p < 0.01$ vs. vehicle treated animals.

A decrease in the duration of immobility is indicative of an antidepressant-like effect. As shown in Table 4, 18 dose-dependently induced an antidepressant-like effect comparable to that induced by the clinically used drug amitriptyline. Accordingly, a similar activity was shown in the tail suspension test (Table 5) where the immobility time was measured in
the first 2 min, when animals react to the inescapable stress, and in the last 4 min of the test, when the behavioral despair is established. Immobility was defined as the absence of any limb or body movements, except those caused by respiration. A reduction in the duration of the immobility time is indicative of an antidepressant-like effect.

Table 5. Antidepressant-like effect of 18 in the mouse tail suspension test.

| Treatment                  | Immobility Time (s) a |
|----------------------------|-----------------------|
| vehicle                    | 99.0 ± 6.8            |
| 18 10 mg kg⁻¹ p.o.         | 88.9 ± 10.16          |
| 18 30 mg kg⁻¹ p.o.         | 56.5 ± 6.1 **         |
| amitriptyline 15 mg kg⁻¹ s.c. | 61.6 ± 8.4 **       |

18 was administered per os (p.o.), amitriptyline was administered subcutaneously (s.c.). a Each value is the mean ± SEM of 10 mice per group, performed in two different experimental sets. ** p < 0.01 vs. vehicle treated animals.

2.4.2. Sucrose Preference Test

Anhedonia, or the inability to experience pleasure, is a common symptom of depression. The mood regulatory role of 18 was studied against anhedonia evaluated in the sucrose preference test in animals treated with lipopolysaccharide (LPS), a model of neuroinflammation-induced depressive-like syndrome [49]. Compound 18 (10 mg kg⁻¹ and 30 mg kg⁻¹) was daily p.o administered for five consecutive days. Twenty-four hours after the last 18 treatment, LPS 1.25 mg kg⁻¹ was intraperitoneally injected. Immediately after LPS injection, mice were placed in cages equipped with two bottles: a bottle of 2% sucrose solution and a bottle of water (unsweetened tap water). The consumption of the 2% sucrose solution was evaluated 6 h and 24 h after LPS injection. Amitriptyline (15 mg kg⁻¹) was used as the reference drug administered following the same protocol. The preference index revealed that at 30 mg kg⁻¹, 18 was able to revert the anhedonia-like behavior induced by LPS comparably to the effect of amitriptyline (Table 6).

Table 6. Antianhedonic like effect of 18 in the mouse sucrose preference test.

| Treatment                          | Sucrose Preference (%), 6 h a | Sucrose Preference (%), 24 h a |
|------------------------------------|------------------------------|-------------------------------|
| vehicle                            | 78.1 ± 2.0                   | 75.3 ± 1.8                    |
| Vehicale + LPS                     | 42.3 ± 3.1 **                | 38.0 ± 5.6 **                 |
| LPS + 18 10 mg kg⁻¹ p.o.           | 48.9 ± 6.7                   | 39.7 ± 3.7                    |
| LPS + 18 30 mg kg⁻¹ p.o.           | 67.3 ± 1.6 **                | 64.9 ± 2.6 **                 |
| LPS + amitriptyline 15 mg kg⁻¹ s.c.| 69.1 ± 4.8 **                | 70.3 ± 6.4 **                 |

18 was administered per os (p.o.), amitriptyline was administered subcutaneously (s.c.). a Each value is the mean ± SEM of 10 mice per group, performed in two different experimental sets. ** p < 0.01 vs. vehicle + LPS; ** p < 0.01 vs. vehicle treated animals.

3. Materials and Methods

3.1. Chemistry

3.1.1. General Methods

Microwave-assisted syntheses were accomplished 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 employed for analytical and preparative TLC, and for column chromatography, respectively. All melting points were registered on a Gallenkamp melting point apparatus and resulted in being uncorrected. Elemental analyses were done with a FlashE1112 Thermofinnigan elemental analyzer for C, H, N, and the results were within ± 0.4% of the theoretical values. All final compounds showed a purity not less than 95%. Compounds were named following IUPAC rules as applied by ChemDrawUltra 9.0. The IR spectra were obtained in Nujol mulls using a Perkin-Elmer Spectrum RXI spectrometer and are expressed in cm⁻¹. NMR spectra were recorded on a Bruker Avance 400 spectrometer.
(400 MHz for $^1$H NMR and 100 MHz for $^{13}$C NMR). The chemical shifts are reported in δ (ppm) and are relative to the central peak of the solvent, which was CDCl$_3$ or DMSO-$d_6$. The following abbreviations were used: s = singlet, d = doublet, t = triplet, m = multiplet, br = broad, and ar = aromatic protons. $^1$H NMR and $^{13}$C APT NMR spectra of some selected derivatives (8, 10, 11, 17, 18) are reported in the Supporting material.

3.1.2. General Procedure for the Synthesis of 25–28

To a suspension of the 5-amino-6-sulfanylpyrimidine-2,4-diol 20 [36] (8.7 mmol) in dry NMP, the suitable arylacetyl chloride 21, 22 [37], 23 [38], and 24 [39] (10.5 mmol) was added. The resulting mixture was heated at 150 °C for 10–15 h, then was cooled to rt, and diluted with cold water (100 mL), affording a precipitate, which was collected by filtration and purified by crystallization. 2-Benzylthiazolo[5,4-d]pyrimidine-5,7-diol (25).

Yield: 75%. Mp: 286–289 °C (Acetic acid). $^1$H NMR (DMSO-$d_6$): 4.29 (s, 2H, CH$_2$), 7.29–7.35 (m, 5H, ar), 11.24 (s, 1H, OH), 11.88 (br s, 1H, OH). Anal. calcd. for (C$_{12}$H$_8$N$_3$O$_5$S): C, 55.59%; H, 3.50%; N, 16.21%. Anal. found: C, 55.73%; H, 3.59%; N, 16.48%. 2-(2-Chlorobenzyl)thiazolo[5,4-d]pyrimidine (26).

Yield: 65%. Mp: >300 °C (Acetic acid/DMF). $^1$H NMR (DMSO-$d_6$): 4.41 (s, 2H, CH$_2$), 7.34–7.39 (m, 2H, ar), 7.49–7.52 (m, 2H, ar), 11.26 (s, 1H, OH), 11.89 (s, 1H, OH). Anal. calcd. for (C$_{12}$H$_8$ClN$_3$O$_5$S): C, 54.07%; H, 2.75%; N, 14.31%. Anal. found: C, 54.28%; H, 2.97%; N, 14.66%. 2-(2-Methoxybenzyl)thiazolo[5,4-d]pyrimidine-5,7-diol (27).

Yield: 50%. Mp: >300 °C (ethanol/Acetic acid). $^1$H NMR (DMSO-$d_6$): 3.81 (s, 3H, CH$_3$), 4.20 (s, 2H, CH$_2$), 6.95 (t, 1H, ar, J = 7 Hz), 7.05 (d, 1H, ar, J = 8.04 Hz), 7.29–7.34 (m, 2H, ar), 11.24 (s, 1H, OH), 11.85 (s, 1H, OH). Anal. calcd. for (C$_{13}$H$_{11}$N$_3$O$_5$S): C, 53.97%; H, 3.83%; N, 14.52%. Anal. found: C, 54.28%; H, 3.07%; N, 14.66%. 2-(2-Fluorobenzyl)thiazolo[5,4-d]pyrimidine-5,7-diol (28).

Yield: 60%. Mp: >300 °C (Acetic acid). $^1$H NMR (DMSO-$d_6$): 4.33 (s, 1H, CH$_2$), 7.20–7.26 (m, 2H, ar), 7.35–7.47 (m, 2H, ar), 11.26 (s, 1H, OH), 11.90 (s, 1H, OH). Anal. calcd. for (C$_{13}$H$_8$FN$_3$O$_5$S): C, 51.98%; H, 2.91%; N, 15.16%. Anal. found: C, 52.12%; H, 3.15%; N, 15.33%.

3.1.3. General Procedure for the Synthesis of 29–32

A suspension of the 5,7-dihydroxy derivatives 25–28 (2.5 mmol) in POCl$_3$ (10 mL) was heated at 160 °C under microwave irradiation for 30 min. The organic phase was concentrated under vacuum, then the residue was added with a mixture of ice-water (100 g) affording a precipitate that was collected by filtration and used in the next step without further purification. 2-Benzyl-5,7-dichlorothiazolo[5,4-d]pyrimidine (29).

Yield: 90%. $^1$H NMR (DMSO-$d_6$): 4.62 (s, 2H, CH$_2$), 7.34–7.43 (m, 5H, ar), 5,7-Dichloro-2-(2-chlorobenzyl)thiazolo[5,4-d]pyrimidine (30).

Yield: 65%. $^1$H NMR (DMSO-$d_6$): 4.73 (s, 2H, CH$_2$), 7.41–7.43 (m, 2H, ar), 7.54–7.56 (m, 1H, ar), 7.60–7.62 (m, 1H, ar), 6.95 (d, 1H, ar, J = 8.04 Hz), 7.34–7.41 (m, 2H, ar). 5,7-Dichloro-2-(2-methoxybenzyl)thiazolo[5,4-d]pyrimidine (31).

Yield: 90%. $^1$H NMR (DMSO-$d_6$): 3.73 (s, 3H, OCH$_3$), 4.49 (s, 2H, CH$_2$), 6.98 (t, 1H, ar, J = 7.36 Hz), 7.08 (d, 1H, ar, J = 8.16 Hz), 7.34–7.41 (m, 2H, ar). 5,7-Dichloro-2-(2-fluorobenzyl)thiazolo[5,4-d]pyrimidine (32).

Yield: 85%. $^1$H NMR (DMSO-$d_6$): 4.66 (s, 2H, CH$_2$), 7.26–7.27 (m, 2H, ar), 7.42–7.55 (m, 2H, ar).

3.1.4. General Procedure for the Synthesis of 33–36

A suspension of the 5,7-dichloro derivatives 29–32 (5 mmol) in a mixture of 33% aqueous ammonia solution (20 mL) and ethanol (15 mL) was heated at reflux for 6 h. The reaction mixture was then cooled to rt, affording a solid, which was collected by filtration. 2-Benzyl-5-chlorothiazolo[5,4-d]pyrimidin-7-amine (33).

Yield: 65%. Mp: 194–197 °C (ethanol). $^1$H NMR (DMSO-$d_6$): 4.45 (s, 2H, CH$_2$), 7.31–7.39 (m, 5H, ar), 8.16 (br s, 2H, NH$_2$). IR: 3453, 3262. Anal. calcd. for (C$_{12}$H$_8$ClN$_4$S): C,
52.08%; H, 3.28%; N, 20.24%. Anal. found: C, 52.33%; H, 3.51%; N, 20.44%. 5-Chloro-2-(2-chlorobenzyl)thiazolo[5,4-d]pyrimidin-7-amine (34).

Yield: 77%. Mp: 186–189 °C (column chromatography, eluting system: ethyl acetate/cyclohexane 5/5). 1H NMR (DMSO-d6): 4.56 (s, 1H, CH2), 7.39–7.41 (m, 2H, ar), 7.53 (d, 2H, ar, J = 7.8 Hz), 8.14 (br s, 2H, NH2). IR: 3445, 3273. Anal. calcd. for (C12H8Cl2N4S): C, 46.32%; H, 2.59%; N, 18.00%. Anal. found: C, 46.70%; H, 2.88%; N, 17.93%. 5-Chloro-2-(2-methoxybenzyl)thiazolo[5,4-d]pyrimidin-7-amine (35).

Yield: 55%. Mp: 201–203 °C (acetonic acid). 1H NMR (DMSO-d6): 3.73 (s, 3H, CH3), 6.97 (t, 1H, ar, J = 7.6 Hz), 7.07 (d, 1H, ar, J = 8.48 Hz), 7.32–7.36 (m, 2H, ar), 8.14 (br s, 2H, NH2). IR: 3437, 3277. Anal. calcd. for (C12H11ClN4OS): C, 50.90%; H, 3.61%; N, 18.26%. Anal. found: C, 51.21%; H, 3.86%; N, 18.39%. 5-Chloro-2-(2-fluorobenzyl)thiazolo[5,4-d]pyrimidin-7-amine (36).

Yield: 85%. Mp: 193–195 °C (ethyl acetate). 1H NMR (DMSO-d6): 7.21–7.28 (m, 2H, ar), 7.37–7.42 (m, 1H, ar), 7.47–7.50 (m, 1H, ar), 8.15 (s, 2H, NH2). IR: 3306, 3159. Anal. calcd. for (C12H9ClN4S): C, 48.90%; H, 2.74%; N, 19.01%. Anal. found: C, 48.66%; H, 2.99%; N, 18.75%.

3.1.5. General Procedure for the Synthesis of 1–19

To a suspension of the 5-chloro-derivatives 33–36 (1 mmol) in dimethoxethane (8.5 mL) and water (2.0 mL), the suitable boronic acids (3 mmol), tetrakis (0.1 mmol), and Na2CO3 (10 mmol) were added. The mixture was refluxed for 4 h under a N2 atmosphere (compounds 1–5, 9), or microwave irradiated at 160 °C for 30 min (compounds 6–8, 10–19). The suspension was treated with water (150 mL) and if a solid was formed (compounds 5–6, 8–9, 11, 13–15, 18–19), it was collected by filtration and dried with Na2SO4. Then, the solvent was evaporated to yield a solid which, after treatment with diethyl ether, was collected by filtration (compounds 1–4, 7, 10, 12, 16–17). The crude products were purified by chromatography and/or crystallization as specified below. 2-Benzyl-5-phenylthiazolo[5,4-d]pyrimidin-7-amine (1).

Yield: 20%. Mp: 171–173 °C (column chromatography, eluting system: ethyl acetate 3/cyclohexane 7). 1H NMR (DMSO-d6): 4.46 (s, 2H, CH2), 7.30–7.33 (m, 1H, ar), 7.37–7.42 (m, 4H, ar), 7.46–7.48 (m, 3H, ar), 7.64 (br s, 2H, NH2), 8.31–8.34 (m, 2H, ar). Anal. calcd. for (C12H12N4S): C, 67.90%; H, 4.43%; N, 17.60%. Anal. found: C, 67.63%; H, 4.72%; N, 17.95%. 3-(7-Amino-2-benzylthiazolo[5,4-d]pyrimidin-5-yl)benzonitrile (2).

Yield: 74%. Mp: 175–178 °C (column chromatography, eluting system: ethyl acetate 4/cyclohexane 6). 1H NMR (DMSO-d6): 4.48 (s, 2H, CH2), 7.29–7.33 (m, 1H, ar), 7.37–7.43 (m, 4H, ar), 7.69–7.73 (m, 1H, ar), 7.82 (br s, 2H, NH2), 7.94–7.96 (m, 1H, ar), 8.59–8.60 (m, 2H, ar). Anal. calcd. for (C19H13N2S): C, 66.45%; H, 3.82%; N, 20.39%. Anal. found: C, 66.58%; H, 4.03%; N, 20.61%. 3-(7-Amino-2-benzylthiazolo[5,4-d]pyrimidin-5-yl)phenol (3).

Yield: 55%. Mp: 179–181 °C (column chromatography, eluting system: ethyl acetate 4/cyclohexane 6). 1H NMR (DMSO-d6): 4.46 (s, 2H, CH2), 6.84–6.86 (m, 1H, ar), 7.24 (t, 1H, ar, J = 7.6 Hz), 7.27–7.31 (m, 1H, ar), 7.37–7.40 (m, 4H, ar), 7.62 (br s, 2H, NH2), 7.77–7.79 (m, 2H, ar), 9.49 (s, 1H, OH). Anal. calcd. for (C18H14N4OS): C, 64.65%; H, 4.22%; N, 16.75%. Anal. found: C, 64.87%; H, 4.45%; N, 17.07%. 2-Benzyl-5-(furan-2-yl)thiazolo[5,4-d]pyrimidin-7-amine (4).

Yield: 60%. Mp: 194–197 °C (column chromatography, eluting system: ethyl acetate 5/cyclohexane 5). 1H NMR (DMSO-d6): 4.44 (s, 2H, CH2), 6.63–6.64 (m, 1H, ar), 7.13–7.14 (m, 1H, ar), 7.31–7.41 (m, 5H, ar), 7.70 (br s, 2H, NH2), 7.82–7.83 (m, 1H, ar). Anal. calcd. for (C16H13N2OS): C, 62.32%; H, 3.92%; N, 18.17%. Anal. found: C, 62.65%; H, 4.15%; N, 18.23%. 2-Benzyl-5-(5-methylfuran-2-yl)thiazolo[5,4-d]pyrimidin-7-amine (5).

Yield: 75%. Mp: 205–208 °C (column chromatography, eluting system: ethyl acetate 5/cyclohexane 5). 1H NMR (DMSO-d6): 2.36 (s, 3H, CH3), 4.43 (s, 2H, CH2), 6.25–6.26 (m, 1H, ar), 7.03–7.04 (m, 1H, ar), 7.29–7.39 (m, 5H, ar), 7.66 (br s, 2H, NH2). Anal. calcd.
for (C_{13}H_{14}N_{4}OS): C, 63.33%; H, 4.38%; N, 17.38%. Anal. found: C, 62.99%; H, 4.42%; N, 17.42%. 2-(2-Chlorobenzyl)-5-phenylthiazolo[5,4-d]pyrimidin-7-amine (6).

Yield: 25%. Mp: 155–158 °C (column chromatography, eluting system: ethyl acetate / cyclohexane) 7). 1H NMR (DMSO-d$_6$): 4.58 (s, 2H, CH$_2$), 7.39–7.42 (m, 2H, ar), 7.46–7.48 (m, 3H, ar), 7.53–7.58 (m, 2H, ar), 7.66 (br s, 2H, NH$_2$), 8.31–8.33 (m, 2H, ar). 13C-NMR (DMSO-d$_6$): 38.23, 128.25, 128.28, 128.78, 129.03, 130.06, 130.16, 130.63, 132.35, 133.95, 135.31, 137.94, 157.08, 159.87, 164.19, 165.32. Anal. calcd. for (C$_{19}$H$_{13}$N$_3$S): C, 61.27%; H, 3.71%; N, 15.88%. Anal. found: C, 61.44%; H, 3.97%; N, 16.03%. 3-(7-Amino-2-(2-chlorobenzyl)thiazolo[5,4-d]pyrimidin-5-yl)benzonitrile (7).

Yield: 35%. Mp: 171–174 °C (column chromatography, eluting system: ethyl acetate / cyclohexane). 1H NMR (DMSO-d$_6$): 4.60 (s, 2H, CH$_2$), 7.40–7.42 (m, 2H, ar), 7.54–7.59 (m, 2H, ar), 7.71 (t, 1H, ar, J = 7.8 Hz), 7.82 (br s, 2H, NH$_2$), 7.95 (d, 1H, ar, J = 7.6 Hz), 8.59–8.63 (m, 2H, ar). 13C-NMR (DMSO-d$_6$): 38.26, 112.08, 114.62, 119.09, 128.31, 129.45, 130.10, 130.17, 130.35, 131.61, 132.38, 132.63, 133.97, 135.22, 139.06, 145.71, 157.15, 157.78, 163.98, 166.20. IR: 3483, 3375, 2228. Anal. calcd. for (C$_{14}$H$_{11}$N$_4$S): C, 60.40%; H, 3.20%; N, 18.53%. Anal. found: C, 60.56%; H, 3.33%; N, 18.71%. 2-(2-Chlorobenzyl)-5-(furan-2-yl)thiazolo[5,4-d]pyrimidin-7-amine (8).

Yield: 27%. Mp: 187–189 °C (column chromatography, eluting system: ethyl acetate / cyclohexane). 1H NMR (DMSO-d$_6$): 4.60 (s, 2H, CH$_2$), 6.63–6.64 (m, 1H, ar), 7.13–7.14 (m, 1H, ar), 7.39–7.41 (m, 2H, ar), 7.52–7.57 (m, 2H, ar), 7.69 (s, 2H, NH$_2$), 7.83 (s, 1H, ar). 13C-NMR (DMSO-d$_6$): 38.19, 112.60, 112.90, 128.28, 128.78, 130.05, 130.15, 132.38, 133.94, 135.26, 145.45, 152.51, 153.40, 157.06, 163.70, 165.10. IR: 3314, 3175. Anal. calcd. for (C$_{15}$H$_{11}$N$_4$S): C, 56.06%; H, 3.23%; N, 16.34%. Anal. found: C, 56.15%; H, 3.13%; N, 16.41%. 2-(2-Chlorobenzyl)-5-(5-methylfuran-2-yl)thiazolo[5,4-d]pyrimidin-7-amine (9).

Yield: 20%. Mp: 201–203 °C (column chromatography, eluting system: ethyl acetate / cyclohexane). 1H NMR (DMSO-d$_6$): 2.35 (s, 3H, CH$_3$), 4.55 (s, 2H, CH$_2$), 6.25–6.26 (m, 1H, ar), 7.02–7.03 (m, 1H, ar), 7.39–7.41 (m, 2H, ar), 7.52–7.57 (m, 2H, ar), 7.66 (br s, 2H, NH$_2$). IR: 3350, 3302. Anal. calcd. for (C$_{15}$H$_{14}$N$_4$OS): C, 57.22%; H, 3.67%; N, 15.70%. Anal. found: C, 57.50%; H, 3.73%; N, 15.59%. 2-(2-Methoxybenzyl)-5-phenylthiazolo[5,4-d]pyrimidin-7-amine (10).

Yield: 20%. Mp: 158–160 °C (column chromatography, eluting system: ethyl acetate / cyclohexane). 1H NMR (DMSO-d$_6$): 3.82 (s, 3H, CH$_3$), 4.37 (s, 2H, CH$_2$), 6.98 (t, 1H, ar, J = 7.4 Hz), 7.08 (d, 1H, ar, J = 8.4 Hz), 7.32–7.36 (m, 2H, ar), 7.45–7.47 (m, 3H, ar), 7.64 (br s, 2H, NH$_2$), 8.30–8.33 (m, 2H, ar). 13C-NMR (DMSO-d$_6$): 35.40, 55.91, 111.73, 121.16, 125.54, 128.24, 128.76, 128.86, 129.65, 130.54, 131.15, 138.01, 156.97, 157.50, 159.67, 164.14, 167.01. IR: 3441, 3292. Anal. calcd. for (C$_{15}$H$_{13}$N$_4$OS): C, 65.50%; H, 4.63%; N, 16.08%. Anal. found: C, 65.59%; H, 4.44%; N, 16.26%. 3-(7-Amino-2-(2-methoxybenzyl)thiazolo[5,4-d]pyrimidin-5-yl)benzonitrile (11).

Yield: 25%. Mp: 205–207 °C (column chromatography, eluting system: ethyl acetate / cyclohexane). 1H NMR (DMSO-d$_6$): 3.82 (s, 3H, CH$_3$), 4.38 (s, 2H, CH$_2$), 6.98 (t, 1H, ar, J = 7.4 Hz), 7.08 (d, 1H, ar, J = 8.4 Hz), 7.33–7.36 (m, 2H, ar), 7.70 (t, 1H, ar, J = 7.8 Hz), 7.78 (br s, 2H, NH$_2$), 7.94 (d, 1H, ar, J = 7.7 Hz), 8.59–8.63 (m, 2H, ar). 13C-NMR (DMSO-d$_6$): 35.45, 55.88, 111.73, 112.06, 119.10, 121.15, 125.45, 129.28, 129.70, 130.31, 131.16, 131.57, 132.57, 133.87, 139.13, 157.04, 157.51, 157.83, 159.76, 164.09, 166.92. IR: 3327, 3165. Anal. calcd. for (C$_{19}$H$_{14}$N$_4$O$_2$S): C, 62.62%; H, 4.43%; N, 15.37%. Anal. found: C, 62.48%; H, 4.58%; N, 15.49%. 5-(Furan-2-yl)-2-(2-methoxybenzyl)thiazolo[5,4-d]pyrimidin-7-amine (13).
Yield: 20%. Mp: 189–192 °C (column chromatography, eluting system: ethyl acetate 4/cyclohexane 6). 1H NMR (DMSO-d6): 3.81 (s, 3H, CH3), 4.35 (s, 2H, CH2), 6.63–6.64 (m, 1H, ar), 6.97 (t, 1H, ar, J = 7.4 Hz), 7.06–7.13 (m, 2H, ar), 7.32–7.36 (m, 2H, ar), 7.67 (br s, 2H, NH2), 7.82 (s, 1H, ar). IR: 3283, 3123. Anal. calcd. for (C17H14N2O2S): C, 60.34%; H, 4.17%; N, 16.56%. Anal. found: C, 60.63%; H, 4.29%; N, 16.71%. 2-(2-Methoxybenzyl)-5-(5-methylfuran-2-yl)thiazolo[5,4-d]pyrimidin-7-amine (14).

Yield: 24%. Mp: 197–199 °C (column chromatography, eluting system: ethyl acetate 4/cyclohexane 6). 1H NMR (DMSO-d6): 3.80 (s, 3H, CH3), 4.34 (s, 2H, CH2), 6.25–6.26 (m, 1H, ar), 6.95–7.08 (m, 3H, ar), 7.32–7.35 (m, 2H, ar), 7.62 (br s, 2H, NH2). 13C-NMR (DMSO-d6): 35.33, 55.87, 109.00, 111.72, 113.91, 121.11, 125.53, 128.35, 129.63, 131.12, 150.06, 153.26, 154.49, 156.92, 157.49, 163.71, 166.35. IR: 3315, 3172. Anal. calcd. for (C18H16N4O2S): C, 61.35%; H, 4.58%; N, 15.90%. Anal. found: C, 61.47%; H, 4.44%; N, 16.11%. 2-(2-Fluorobenzyl)-5-phenylthiazolo[5,4-d]pyrimidin-7-amine (15).

Yield: 45%. Mp: 192–195 °C (column chromatography, eluting system: ethyl acetate 5/cyclohexane 6/methanol 1). 1H NMR (DMSO-d6): 4.50 (s, 2H, CH2), 7.23–7.29 (m, 2H, ar), 7.38–7.53 (m, 5H, ar), 7.68 (br s, 2H, NH2), 8.31–8.33 (m, 2H, ar). IR: 3456, 3273, 3140. Anal. calcd. for (C18H13FN4S): C, 64.27%; H, 3.90%; N, 16.66%. Anal. found: C, 64.33%; H, 4.07%; N, 16.74%. 3-(7-Amino-2-(2-fluorobenzyl)thiazolo[5,4-d]pyrimidin-5-yl)benzonitrile (16).

Yield: 20%. Mp: 152–154 °C (column chromatography, eluting system: ethyl acetate 4/cyclohexane 6). 1H NMR (DMSO-d6): 4.52 (s, 2H, CH2), 7.23–7.29 (m, 2H, ar), 7.38–7.44 (m, 1H, ar), 7.49–7.53 (m, 1H, ar) 7.71 (t, 1H, ar, J = 7.8 Hz), 7.81 (br s, 2H, NH2), 7.95 (d, 1H, ar, J = 7.6 Hz). 13C-NMR (DMSO-d6): 35.04, 3331, 2233. Anal. calcd. for (C16H12F3N3S): C, 63.15%; H, 3.35%; N, 19.38%. Anal. found: C, 63.31%; H, 3.49%; N, 19.47%. 3-(7-Amino-2-(2-fluorobenzyl)thiazolo[5,4-d]pyrimidin-5-yl)phenol (17).

Yield: 35%. Mp: 172–175 °C (column chromatography, eluting system: ethyl acetate 5/cyclohexane 6). 1H NMR (DMSO-d6): 4.49 (s, 2H, CH2), 6.84–6.86 (m, 1H, ar), 7.23–7.28 (m, 3H, ar), 7.38–7.43 (m, 1H, ar), 7.50 (t, 1H, ar, J = 7.6Hz), 7.59 (br s, 2H, NH2), 7.75–7.77 (m, 2H, ar), 9.48 (s, 1H, OH). 13C-NMR (DMSO-d6): 35.36, 55.88, 111.71, 112.62, 112.70, 121.13, 125.48, 128.58, 129.67, 131.15, 152.55, 153.22, 156.94, 157.49, 163.62, 166.83. Anal. calcd. for (C16H13F3N4OS): C, 61.35%; H, 3.72%; N, 15.90%. Anal. found: C, 61.42%; H, 3.59%; N, 16.09%. 2-(2-Fluorobenzyl)-5-(furan-2-yl)thiazolo[5,4-d]pyrimidin-7-amine (18).

Yield: 35%. Mp: 173–175 °C (column chromatography, eluting system: ethyl acetate 4/cyclohexane 6/methanol 1). 1H NMR (DMSO-d6): 4.48 (s, 2H, CH2), 6.63–6.64 (m, 1H, ar), 7.14 (d, 1H, ar, J = 3.2 Hz), 7.22–7.28 (m, 2H, ar), 7.38–7.43 (m, 1H, ar), 7.50 (t, 1H, ar, J = 7.6 Hz), 7.67 (br s, 2H, NH2), 7.83 (s, 1H, ar). IR: 3310, 3134. Anal. calcd. for (C16H11F3N3OS): C, 58.89%; H, 3.40%; N, 17.17%. Anal. found: C, 59.05%; H, 3.47%; N, 17.36%. 2-(2-Fluorobenzyl)-5-(5-methylfuran-2-yl)thiazolo[5,4-d]pyrimidin-7-amine (19).

Yield: 24%. Mp: 186–188 °C (column chromatography, eluting system: ethyl acetate 4/cyclohexane 6). 1H NMR (DMSO-d6): 2.35 (s, 3H, CH3), 4.47 (s, 2H, CH2), 6.26 (s, 1H, ar), 7.03 (s, 1H, ar), 7.22–7.28 (m, 2H, ar), 7.37–7.42 (m, 1H, ar), 7.49 (t, 1H, ar, J = 7.6 Hz), 7.65 (br s, 2H, NH2). 13C-NMR (DMSO-d6): 14.06, 33.73, 109.05, 114.10, 115.96, 116.17, 124.50, 124.65, 125.33, 125.36, 128.45, 130.20, 130.28, 132.06, 132.09, 150.97, 153.42, 154.61, 157.04, 159.65, 162.09, 163.84, 164.63. IR: 3491, 3286. Anal. calcd. for (C17H13F4N4OS): C, 59.99%; H, 3.85%; N, 16.46%. Anal. found: C, 56.15%; H, 3.97%; N, 16.55%.

3.2. Pharmacological Assays

3.2.1. Membrane Preparation

CHO cells, stably expressing hARs, were grown adherently and maintained in Dulbecco’s modified Eagle’s medium (DMEM) with nutrient mixture F12 supplemented with 10% fetal bovine serum (FBS), penicillin (100 μg/mL), streptomycin (100 μg/mL), sodium pyruvate (1 mM), and geneticin (0.1 mg/mL). Cells were maintained in a humidified incubator containing 5% CO2 and 95% air.

Membranes were prepared as previously reported [34]. Briefly, cells were homogenized in cold lysis buffer (5 mM Tris HCl, 2 mM EDTA, pH 7.4), centrifuged at low speed
spin for 10 min, and subsequently, the supernatant was spun at 37,000 rpm. Membrane pellet was resuspended in the specific buffer and stored at −80 °C.

3.2.2. Radioligand Binding

The binding affinity of the novel compounds was evaluated using radioligand competition experiments in CHO cells stably transfected with hA1 AR, hA2A AR, and hA3 AR subtypes. The radioligands used were 1 nM [3H] CCPA for hA1 AR (K_D = 1.1 nM), 10 nM [3H] NECA for hA2A AR (K_D = 20 nM); 1 nM [3H] HEMADO for A3 AR (K_D = 1.5 nM). The potency at hA2B AR, expressed on CHO cells, was determined by inhibition of NECA-stimulated adenylyl cyclase activity [34].

3.2.3. GloSensor cAMP Assay

The intrinsic activity of understudy compounds was evaluated through the GloSensor cAMP assay, as described previously [50]. Briefly, cells stably expressing the hA1, hA2A, and hA2B ARs and the biosensor were incubated for 2 h at rt in equilibration medium containing 3% v/v GloSensor cAMP reagent stock solution, 10% FBS, and 87% CO2 independent medium. Afterward, cells were dispensed in the wells of a 384-well plate and the reference agonist NECA or the compounds, at different concentrations, were tested. The antagonist profile of compounds was evaluated by assessing their ability to counteract NECA-induced increase (A2A and A2B ARs) or decrease (A1 AR) of cAMP accumulation [51]. Responses were expressed as percentage of the maximal relative luminescence units (RLU).

3.2.4. Statistical Analysis

Binding data and concentration–response curves were fitted by a nonlinear regression with the Prism program (GraphPAD Prism 7 Software, San Diego, CA, USA). Each concentration was tested 3–5 times in duplicate and the K_i or IC50 (the concentration of antagonists that produces 50% inhibition of the agonist effect) values are given as the mean ± standard error (S.E.).

3.3. In Vivo Assays

3.3.1. Animals

Male CD-1 albino mice (22–25 g; Envigo, Varese, Italy) were housed in CeSAL (Centro Stabulazione Animali da Laboratorio, University of Florence, Florence, Italy) and used at least one week after their arrival. Animals were housed in 26 cm × 41 cm cages (12 mice each) and fed with a standard laboratory diet and tap water ad libitum. They were kept at 23 ± 1 °C with a 12 h light/dark cycle, light at 7 a.m. Compound 18 was administered per o.s. whereas amitriptyline was by a s.c. route following the most widely used approach reported in the literature.

3.3.2. Forced Swimming Test

Mice were individually placed into glass cylinders (height: 25 cm, diameter: 10 cm) containing 12 cm of water maintained at 22–23 °C for 6 min. Immobility was considered the animal floated in the water, in an upright position, and made only small movements to keep its head above water. The immobility time was recorded during the last 4-min of the 6-min test. A decrease in the duration of immobility is indicative of an antidepressant-like effect [52].

3.3.3. Tail Suspension Test

A piece of tape was adhered to the upper middle of the tail of each animal, creating a flap with the overlap of tape. Mice were suspended from a plastic rod mounted 50 cm above the surface by fastening the tail to the rod with adhesive tape. The duration of the test was 6 min and the immobility time was measured in the first 2 min, when animals react to the inescapable stress, and in the last 4 min of the test, when the behavioral despair
is established. Immobility was defined as the absence of any limb or body movements, except those caused by respiration.

3.3.4. LPS-Induced Anhedonia

Lipopolysaccharide (LPS) from Escherichia coli was purchased from Sigma-Aldrich, (Milan, Italy) freshly dissolved in sterile saline, and injected intraperitoneally (i.p.) at the dose of $1.25 \text{mg kg}^{-1}$ after five consecutive days of treatment with compound 18, 1 h after the last administration of the compound. Behavioral tests were performed before 6 h and 24 h after LPS administration.

3.3.5. Sucrose Preference Test

Mice were placed in cages equipped with a couple of bottles, one containing 2% sucrose solution, the second with water (unsweetened tap water). The consumption of the 2% sucrose solution was evaluated 6 h and 24 h after the beginning of the experiment. The preference index was calculated according to the following formula: preference index = volume consumed sucrose solution/(volume consumed sucrose solution + volume consumed water).

3.3.6. Statistical Analysis

Behavioral tests were performed by visual observation by researchers blinded to the treatments. Results were expressed as means ± SEM, variance was analyzed by ANOVA. A Bonferroni’s significant difference procedure was used as post-hoc comparison. $p$ values of less than 0.05 and 0.01 were considered significant. Data were analyzed using the “Origin 8.1” software.

3.4. Molecular Modeling Studies

3.4.1. Refinement of the Human A$_{2A}$AR and A$_{1}$AR Structures

The crystal structure of the hA$_{2A}$AR and hA$_{1}$AR in complex with ZM241385 (pdb code: 5NM4; 1.7-Å resolution [40]) and PSB36 (pdb code: 5N2S; 3.3-Å resolution [44]), respectively, were selected as molecular targets for molecular docking experiments. The receptor structures were checked with the Homology Modeling tool of MOE [41], by correcting the amino acidic sequence (due to the presence of some mutations and external segments within the crystallized thermostabilized receptor) to restore the wild type primary structure and by adding and energetically minimizing the hydrogen atoms.

3.4.2. Molecular Docking Analysis

All compound structures were docked into the hA$_{2A}$AR and hA$_{1}$AR binding site using three docking tools: the Induced Fit docking protocol of MOE [41], the genetic algorithm docking tool of CCDC Gold [42], and the docking tool of Cresset Flare [43]. The Induced Fit docking protocol of MOE is divided into the following stages: conformational analysis of ligands; placement; scoring; induced fit; and rescoring. Alpha HB scoring function was employed in this task. Gold tool was used with default efficiency settings through MOE interface by selecting Chemscore as scoring function. Flare docking tool was used with “accurate but slow” settings and “extra precision” quality.

3.4.3. Post Docking Analysis. Residue Interaction Analysis

The ligand–target interactions were analyzed by using the IF-E 6.0 SVL script tool [45], which calculates and displays the residue interaction forces as 3D vectors and calculates the per-residue interaction energies (negative and positive energy values being associated to favorable and unfavorable interactions, respectively). A shell of A$_{2A}$AR residues within a 10 Å distance from the ligand were considered for this analysis.
4. Conclusions

This work has led to the identification of a new set of extremely potent hA\textsubscript{1}/hA\textsubscript{2A} AR dual antagonists belonging to the 7-aminothiazolo[5,4-d]pyrimidine series. Most of the new derivatives were endowed with nanomolar and subnanomolar affinity values for the hA\textsubscript{1} and hA\textsubscript{2A}, respectively, and high selectivity versus the other ARs. The best combined activity and selectivity at the A\textsubscript{1}/A\textsubscript{2A} ARs was shown by derivative 18, which was chosen to evaluate its antidepressant-like activity. Thus, 18 was tested in in vivo models of depression (i.e., the FST and the TST), showing an efficacy comparable to that of the clinically used drug amitriptyline. Compound 18 was also tested in the sucrose preference test to evaluate its anti-anhedonia-like effect. Interestingly, 18 showed a good anti-anhedonia-like activity comparable to that of amitriptyline.

To conclude, we identified new potent hA\textsubscript{1}/hA\textsubscript{2A} AR dual antagonists belonging to the 7-amino-thiazolo[5,4-d]pyrimidine series, which can be considered as promising candidates for further pharmacological evaluation as antidepressant agents also able to contrast anhedonia.

Supplementary Materials: The following are available online at https://www.mdpi.com/article/10.3390/ph14070657/s1, \textsuperscript{1}H NMR and \textsuperscript{13}C APT NMR spectra of some selected derivatives (8, 10, 11, 17, 18).

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Institutional Review Board Statement: All animal manipulations were carried out according to the Directive 2010/63/EU of the European parliament and of the European Union council (22 September 2010) on the protection of animals used for scientific purposes. The ethical policy of the University of Florence complies with the Guide for the Care and Use of Laboratory Animals of the U.S. National Institutes of Health (NIH Publication No. 85–23, revised 1996; University of Florence assurance number: A5278-01). Formal approval to conduct the experiments described was obtained from the Italian Ministry of Health (No. 54/2014-B) and from the Animal Subjects Review Board of the University of Florence. Experiments involving animals have been reported according to ARRIVE guidelines. All efforts were made to minimize animal suffering and to reduce the number of animals used.

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