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Synthesis and pharmacology of pyrido[2,3-*d*]pyrimidinediones bearing polar substituents as adenosine receptor antagonists

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Abstract—Amino-substituted pyrido[2,3-*d*]pyrimidinediones have previously been found to bind to adenosine A_1 and A_{2A} receptors in micromolar concentrations. The present study was aimed at studying the structure–activity relationships of this class of compounds in more detail. Most of the investigated compounds were provided with polar substituents, such as ethoxycarbonyl groups and basic amino functions, in order to improve their water-solubility. The compounds were synthesized starting from 6-amino-1,3-dimethyluracil via different reaction sequences involving (cyano)acetylation, Vilsmeier formylation, or reaction with diethyl ethoxymethylenemalonate (EMME). The most potent and selective compound of the present series was 6-carbethoxy-1,2,3,4-tetrahydro-1,3-dimethyl-5-(2-naphthylmethyl)aminopyrido[2,3-*d*]pyrimidine-2,4-dione (**11c**) with a K_i value of 5 nM at rat and 25 nM at human A_1 receptors. The compound was more than 60-fold selective versus A_3 and more than 300-fold selective versus A_{2A} receptors. It showed an over 300-fold improvement with respect to the lead compound. In GTPγS binding studies at membranes of Chinese hamster ovary cells recombinantly expressing the human adenosine A_1 receptor, **11c** behaved as an antagonist with inverse agonistic activity. A regioisomer of **11c**, 6-carbethoxy-1,2,3,4-tetrahydro-1,3-dimethyl-7-(2- naphthylmethyl)aminopyrido[2,3-*d*]pyrimidine-2,4-dione (**7a**) in which the 2-naphthylmethylamino substituent at position 5 of **11c** was moved to the 7-position, was a relatively potent (K_i = 226 nM) and selective (>20-fold) A_3 ligand. In the series of compounds lacking an electron-withdrawing ethoxycarbonyl or cyano substituent in the 6-position, compounds with high affinity for adenosine A_{2A} receptors were identified, such as 1,2,3,4-tetrahydro-1,3-dimethyl-5-(1-naphthyl)aminopyrido[2,3-*d*]pyrimidine-2,4-dione **16b** (K_i human A_{2A} = 81.3 nM, K_i human A_1 = 153 nM, and K_i human A_3 > 10,000 nM).

1. Introduction

Adenosine is formed within cells of all mammalian organisms by hydrolysis of adenine nucleotides or from *S*-adenosylhomocysteine and can be released to act at specific receptors which belong to the family of G-protein-coupled receptors.[1] Extracellular metabolism of adenine nucleotides can also produce adenosine. Four adenosine receptor (AR) subtypes have been cloned and characterized from various species including humans and rat: A_1, A_{2A}, A_{2B}, and A_3.[2] Adenosine acting at adenosine receptors modulates a variety of important physiological processes and exhibits central nervous system (CNS) depressant, cardiodepressant, antidiuretic, and immunomodulatory effects, among others.[1,2] The AR subtypes A_1 and A_{2A} are 'high-affinity subtypes' since they are usually activated by low, nanomolar concentrations of adenosine.[2–4] In many tissues, for example, in certain brain areas, A_1 and A_{2A} receptors appear to be tonically activated. Selective antagonists for the AR subtypes are being developed as potential new drugs.[3–9] Selective A_1 antagonists may be useful as kidney-protective diuretics, for the treatment of congestive heart failure due to their diuretic and positive inotropic effects, and have potential for the treatment of brain diseases, such as depression or dementia.[5,6] A large body of evidence has been accumulated that selective A_{2A} antagonists can be very effective in the symptomatic treatment of Parkinson's disease and may

Keywords: Adenosine receptor antagonists; Pyrido[2,3-*d*]pyrimidines; Inverse agonist; A_1-selectivity; Structure–activity relationships; Gould–Jacobs reaction; Vilsmeier formylation.
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exhibit neuroprotective effects.[7,8] A_3 AR antagonists might be therapeutically useful for the acute treatment of stroke, for glaucoma, and also as antiasthmatic and antiallergic drugs.[9]

A large number of AR antagonists have been developed during the past 30 years, initially based on the xanthine derivatives theophylline and caffeine as lead structures, and later on from various families of heterocyclic compounds many of which are structurally more or less related to adenine.[5,10] Despite considerable effort by pharmaceutical companies, no selective adenosine receptor antagonist has made it to the market as yet. After initial unsuccessful clinical trials with first-generation A_1 AR antagonists, there is now a renewed high interest in this class of compounds.[11–21] Up to now, the non-selective AR antagonists theophylline and caffeine are still the only AR antagonists that are used as drugs. Reasons for the slow progress in the development of drugs that block ARs may be unfavorable pharmacokinetic properties. One major problem is the low water-solubility of many of these mostly highly lipophilic compounds featuring flat, aromatic ring systems, which limits the absorption of the compounds from the gut.[5,10,22] In addition, intermolecular clustering via hydrogen bonding and π-stacking results in water-insoluble compounds which exhibit poor brain penetration. In the present study, we combined features of the xanthine derivatives theophylline and caffeine (the dimethylpyrimidinedione ring) with an amino-substituted heterocycle typical for non-xanthine AR antagonists, in our case 2-aminopyrimidine, or 4-aminopyrimidine, respectively. The structural features of the resulting aminopyrido[2,3-*d*]pyrimidine-2,4-diones include a planar nitrogen-containing hetero-bicyclic ring system with a monosubstituted exocyclic amino function which is characteristic of several classes of potent non-xanthine adenosine receptor antagonists[1,2,5,10,22,23] (Fig. 1).

Earlier work in our laboratories involved the synthesis of 5-aminopyrido[2,3-*d*]pyrimidine-2,4-diones and their preliminary screening at ARs.[24,25] Background of those studies had been the observation that some members of this class of compounds produced positive inotropic effects as determined in guinea pig left atria.[26–30] In order to elucidate their molecular mechanism of action, we had investigated their affinity for adenosine receptors. A few 5-amino-1,3-dimethylpyrido[2,3-*d*]pyrimidine-2,4-diones were identified that showed affinity and selectivity for A_1 ARs.[24,25] Based on those preliminary findings, we have now synthesized a larger series of derivatives with variations in the substitution pattern of the pyridine ring. The new compounds were investigated in radioligand binding assays at adenosine A_1, A_{2A}, and A_3 receptors in order to get more insight into the structure–activity relationships of pyrido[2,3-*d*]pyrimidinediones, taking N^5-butyl-6-cyano-1,3-dimethyl-pyrido[2,3-*d*]pyrimidine-2,4-dione as a lead structure (Fig. 1). Particular emphasis was laid on the introduction of polar groups, such as ethoxycarbonyl and moderately basic amino functions, which would increase water-solubility but at the same time still allow brain penetration.

2. Results and discussion

2.1. Chemistry

For the preparation of our target compounds we developed different approaches commonly applied to the synthesis of pyrido[2,3-*d*]pyrimidine derivatives. Starting from 6-amino-1,3-dimethyluracil (**1**) 7-aminopyrido[2,3-*d*]pyrimidines **3** were prepared using a two-step procedure via Vilsmeier formylation, nucleophilic substitution with methylamine, and subsequent condensation of the pyrimidine **2** with acetonitrile derivatives (Fig. 2).[31]

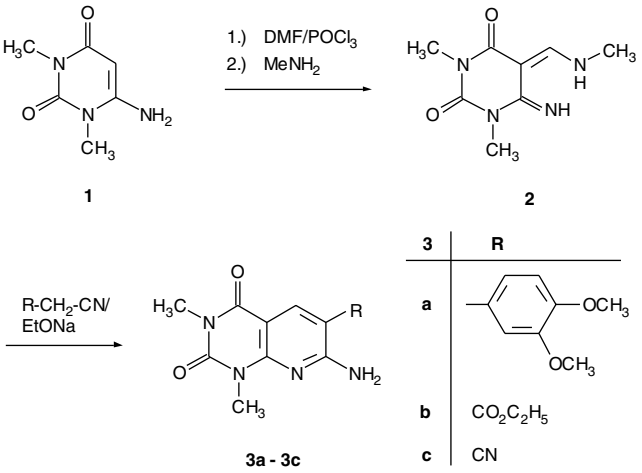


Figure 2. Synthesis of 7-amino-substituted pyrido[2,3-*d*]pyrimidine-2,4-diones.

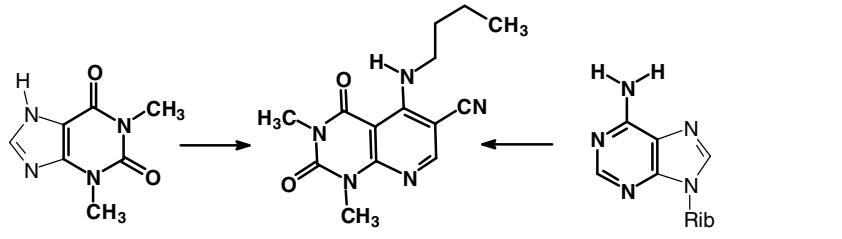


Figure 1. Lead structure and its structural relationship to xanthine and adenine derivatives.

Another synthetic route involved the condensation of **1** and diethyl ethoxymethylenemalonate (EMME) **4** to give the anellated pyridone **5** followed by Vilsmeier reaction for introduction of chlorine in position 7 and subsequent nucleophilic substitution to yield the 7-aminopyrido[2,3-*d*]pyrimidines **7** (Fig. 3).[28]

The reaction of 6-aminopyrimidine **1** with EMME has been widely used for the synthesis of 6-carbethoxy-5-oxopyrido[2,3-*d*]pyrimidines[32,33] and further quinolone antimicrobial agents (Fig. 4).[34] Thus, it has been shown that the condensation of **1** and EMME in ethanol containing one equivalent of sodium ethoxide gave in the normal Gould–Jacobs manner the desired enamine **8**, thermal cyclization of which afforded in refluxing diphenyl ether the pyrido[2,3-*d*]pyrimidine **9** in excellent yield.[28]

Quinolones can frequently be chlorinated using phosphorus oxychloride without any solvent;[30] unfortunately, this procedure failed when applied to **9**. But we found that treatment with $POCl_3$ in DMF under Vilsmeier conditions provided the desired 5-chloro derivative **10**.[28] As expected, nucleophilic displacement of the halogen atom by the appropriate amine generally occurred in high yield after 30 min of reaction in refluxing ethanol yielding the 5-aminopyrido[2,3-*d*]pyrimidines **11**. The relative ease of displacement is presumably due to the effect of the carboxylic acid ester together with the carbon amide group adjacent to the reaction center. The alkyl side chain was selected on the basis of results previously obtained in the 4-aminoquinoline series[30] and consisted of an aralkyl group with optional hydroxyl on the alkyl portion. The 5-amino substituent turned out to be of great importance for high AR affinity of the present series of compounds. Furthermore, we wanted to obtain more information about the role of the electron-withdrawing group in the 6-position and decided to employ a hydrogen atom or cyano group. Recently, we had described the synthetic pathway outlined in (Fig. 5).[28] Introduction of the keto function in position 5 (compounds **12**[35] and **13**[36]) was followed by ring closure using Vilsmeier formylation (compounds **14** and **15**[37]) and subsequent introduction of amino groups yielded the desired pyrido[2,3-*d*]pyrimidines **16**.

2.2. Biological activity

The compounds were investigated in radioligand binding studies at A_1 ARs of rat brain cortical membranes using the A_1-selective radioligand [^{3}H]2-chloro-N^6-cyclopentyladenosine ([^{3}H]CCPA)[38] and at A_{2A} ARs of rat brain striatal membranes using the A_{2A}-selective radioligand [^{3}H]3-(3-hydroxypropyl)-7-methyl-8-(*m*-methoxystyryl)-1-propargylxanthine ([^{3}H]MSX-2).[39] Additional experiments were performed at human recombinant A_1, A_{2A}, and A_3 adenosine receptors stably expressed in Chinese hamster ovary (CHO) cells. For A_3 binding studies the A_3-selective radioligand [^{3}H]8-ethyl-4-methyl-2-phenyl(8*R*)-4,5,7,8-tetrahydro-1*H*-imidazo[2,1-*i*]-purin-5-one[40] ([^{3}H]PSB-11) was applied. Functional properties of the most potent compounds were investigated in [^{35}S]GTPγS binding studies at human recombinant A_1 receptors expressed in CHO cells.

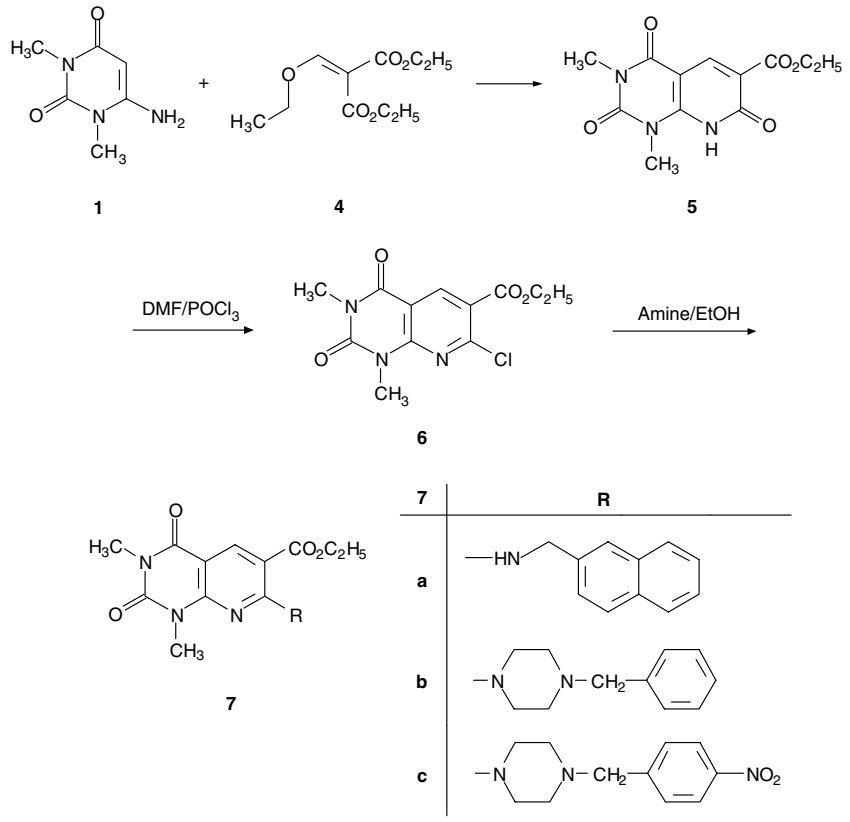


Figure 3. Synthesis of N^7-substituted 7-aminopyrido[2,3-*d*]pyrimidine-2,4-diones.

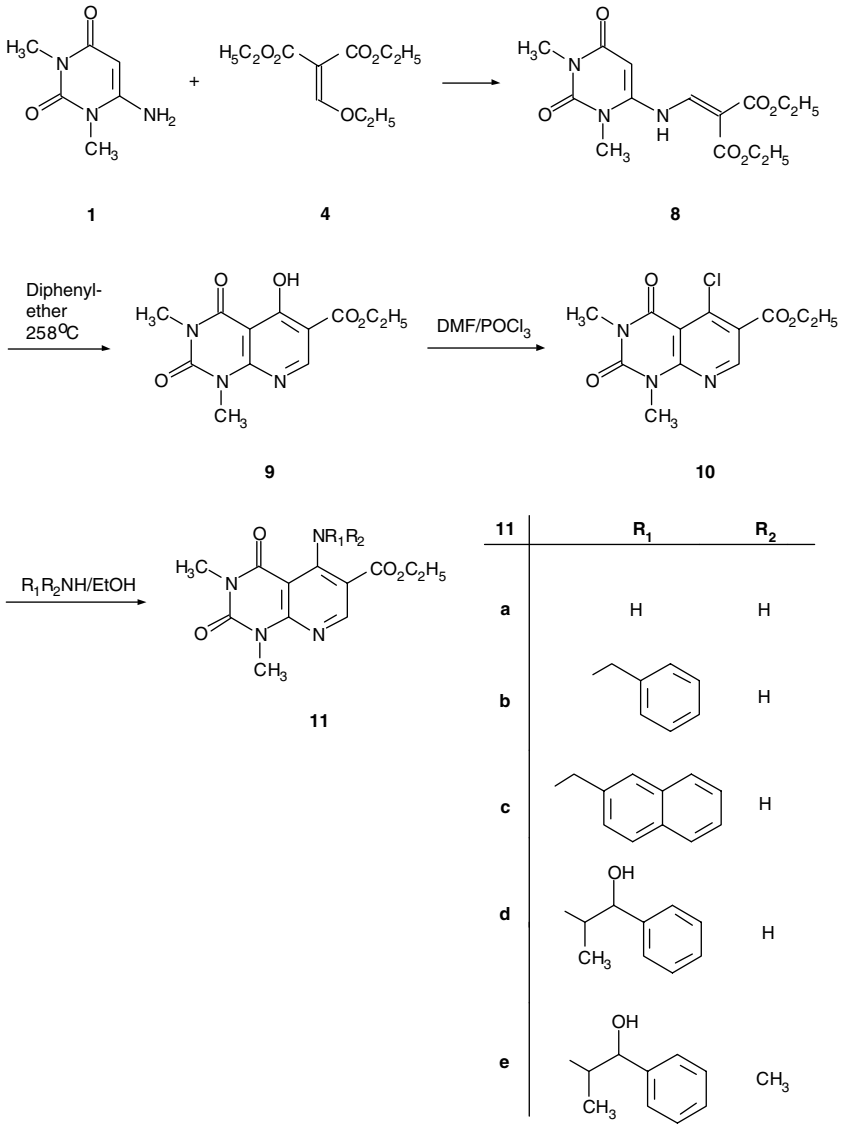


Figure 4. Synthesis of 5-amino-substituted 6-carbethoxypyrido[2,3-*d*]pyrimidine-2,4-dione.

2.2.1. Affinity in different species: rat and human adenosine receptors. For initial screening of the compounds we used native rat brain tissues expressing the A_1 or A_{2A} AR in high density. Rats and mice are frequently used animals for in vivo testing of drugs and therefore it is essential to know the affinity of new ligands for rodent receptors. Since the ultimate target of new ligands are the human receptors, either as pharmacological tools used in human tissues or cell lines, or for diagnostic or therapeutic purposes in patients, the affinity at human receptors is also important to know. Only moderate species differences have been reported for A_1 and A_{2A} ARs between rat and humans due to the high degree of sequence homology.[2,5,22] Only the A_3 AR usually shows larger species differences.[2,9] Because rat brain and most other rat tissues express only a very low density of A_3 ARs, only human recombinant A_3 ARs were employed. Depending on the affinity at rat A_1 and A_{2A} receptors, selected compounds were additionally investigated at human recombinant A_1 and A_{2A} ARs. In fact, the set of pyridopyrimidinediones assayed at rat and human ARs showed only moderate species differences. K_i values were in all cases in a similar range. The compounds appeared to be somewhat less potent at human A_1 as compared to rat A_1 ARs (ca. 3- to 5-fold) and slightly more potent at human A_{2A} as compared to rat A_{2A} ARs (e.g., no significant difference for **7b**, ca. 3-fold difference for **16b**).

2.2.2. Structure–activity relationships. Pyridopyrimidine derivatives **3a**–**3c** and **7a**–**7c** (Table 1) contain an amino group in the 7-position. Compounds **3b**–**3c** bearing an unsubstituted amino group with small, electron-withdrawing substituents in the neighboring 6-position (ethoxycarbonyl, **3b**; cyano, **3c**) exhibited affinity for A_1 and A_{2A} receptors at low micromolar concentrations, but showed only very low affinity for A_3 ARs. Both compounds were slightly more potent at A_1 than at A_{2A} receptors. The ethoxycarbonyl substituent (compound **3b**) was superior to a cyano group (compound **3c**). However, the introduction of a bulky, lipophilic 3,4-dimethoxyphenyl group in the 6-position was not well tolerated

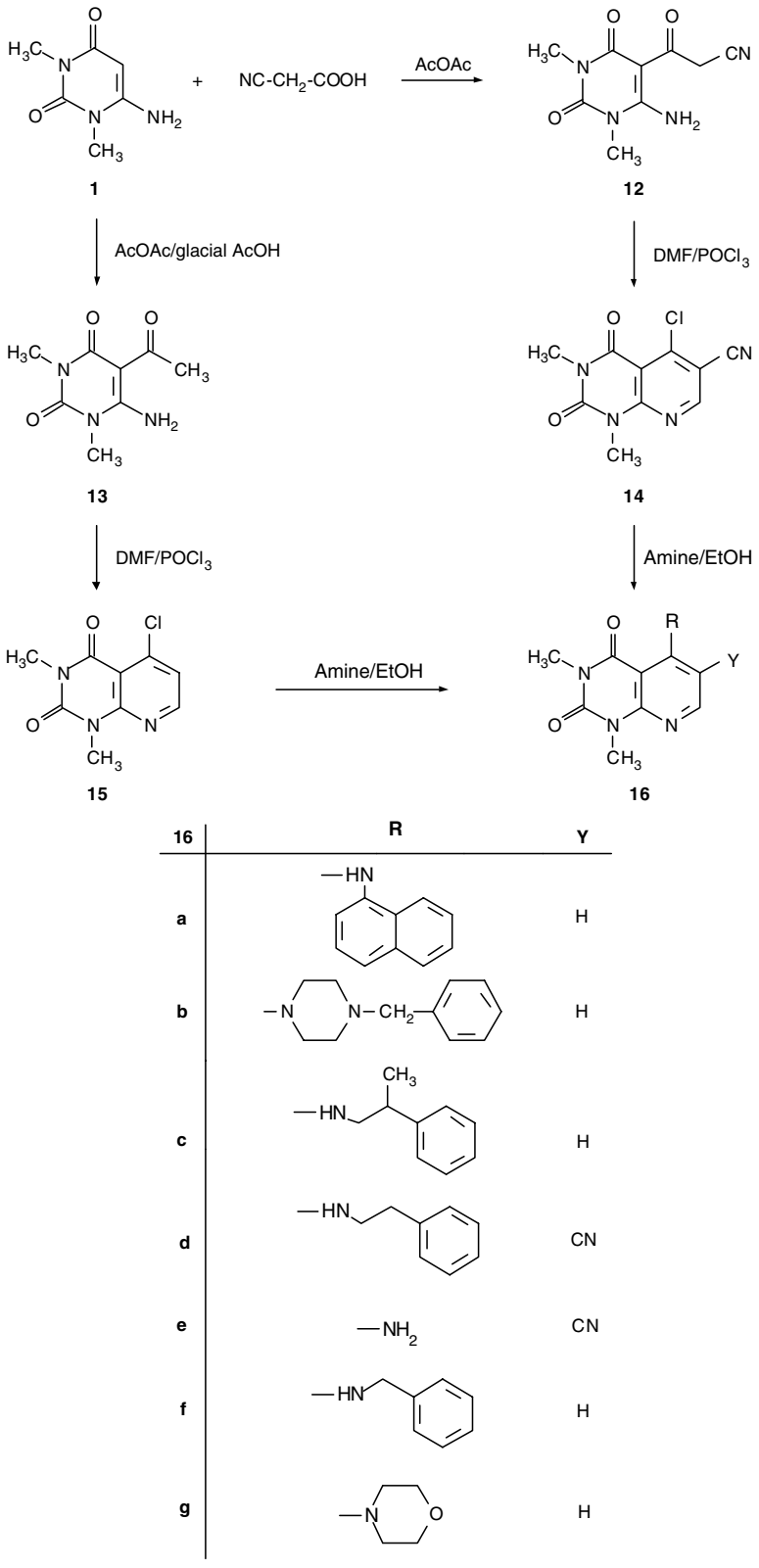
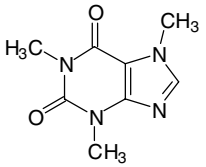
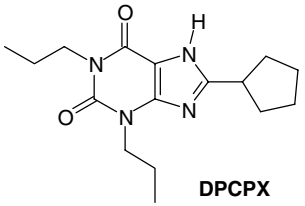
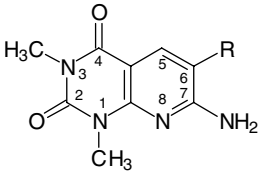
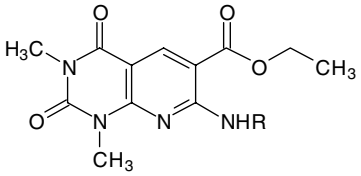
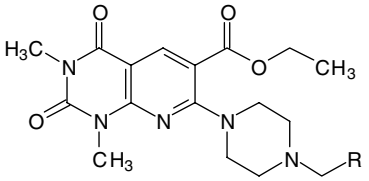
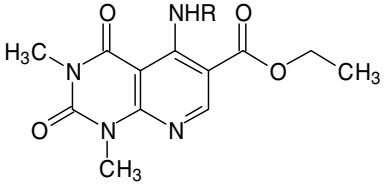
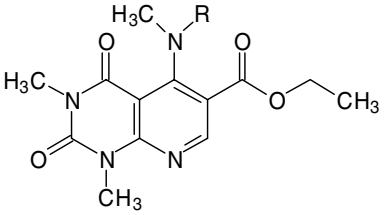


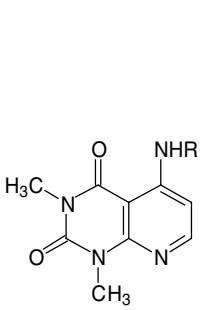
Figure 5. Synthesis of 5-amino-substituted 6-unsubstituted or 6-cyano-substituted pyrido[2,3-*d*]pyrimidine-2,4-diones.

by the receptors (compound **3a**). In the next step, the ethoxycarbonyl group in position 6 was combined with various substituents on the 7-amino group (**7a**–**7c**). A bulky 2-naphthylmethyl substituent was tolerated by the A_1 AR but resulted in a less potent compound (**7a**) compared to the corresponding unsubstituted **3b**. However, the 2-naphthylmethyl group increased A_3 affinity to a large extent. Compound **7a** exhibited a K_i value of 230 nM at human A_3 ARs and was more than 20-fold selective versus the other AR subtypes. Despite its polar

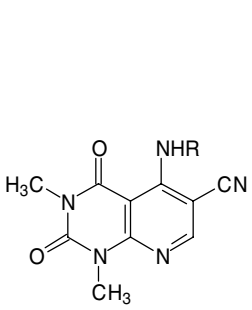
Table 1. Affinities of amino-substituted pyrido[2,3-*d*]pyrimidinediones and standard ligands for adenosine receptors

Compound	R	A_1 K_i ± SEM (μM) versus [^{3}H]CCPA rat cortex (n = 3)[a]	A_1 K_i ± SEM (μM) versus [^{3}H]CCPA hA$_1$-CHO (n = 3)[a]	A_{2A} K_i ± SEM (μM) versus [^{3}H]MSX-2 rat striatum (n = 3)[a]	A_{2A} K_i ± SEM (μM) versus [^{3}H]MSX-2 hA$_{2A}$-CHO membranes (n = 3)[a]	A_3 K_i ± SEM (μM) versus [^{3}H]PSB-11 hA$_3$-CHO membranes (n = 3)[a]
		 **Caffeine**	 **DPCPX**	 **3a-c**		
Caffeine	—	18.8 ± 5.6	44.9 ± 6.2	32.5 ± 8.0	23.4 ± 7.1[b]	n.d.
DPCPX	—	0.0005 ± 0.0002	0.0030 ± 0.0008	0.157 ± 0.006	0.060 ± 0.045[b]	0.243 ± 0.056
3a	3,4-Dimethoxyphenyl	>1.0 (19 ± 1% inhibition)[c]	n.d.	≫1.0 (1 ± 1% inhibition)[c]	n.d.	≫1.0 (0% inhibition)[c]
3b	Ethoxycarbonyl	1.23 ± 0.13	n.d.	5.50 ± 2.04	n.d.	>10 (29 ± 2% inhibition)[c]
3c	Cyano	4.07 ± 0.35	n.d.	ca. 10 (46 ± 6% inhibition)[c]	n.d.	>10 (15 ± 2% inhibition)[c]
		 **7a**	 **7b-c**			
7a	2-Naphthylmethyl	5.2 ± 2.5[d]	>10 (29% inhibition)[c]	>10 (23 ± 5% inhibition)[c]	n.d.	0.226 ± 0.110
7b	Phenyl	2.50 ± 0.17	n.d.	2.23 ± 0.22	2.00 ± 1.02	1.83 ± 0.68
7c	*p*-Nitrophenyl	1.60 ± 0.27	n.d.	6.10 ± 7.30[d]	n.d.	0.793 ± 0.029
		 **11a-d**	 **11e**			

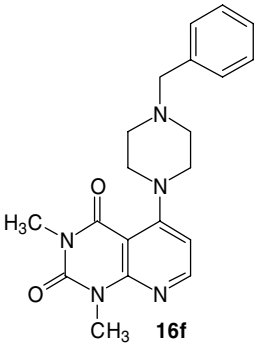
11a	H	5.25 ± 0.15	n.d.	3.98 ± 0.95	n.d.	⩾10 (37 ± 15% inhibition)[c]
11b	Benzyl	⩾1.0 (46 ± 3% inhibition)[c]	n.d.	>1.0 (23 ± 3% inhibition)[c]	n.d.	4.65 ± 0.44
11c	2-Naphthylmethyl	0.00497 ± 0.00082	0.0251 ± 0.0041	>10 (35 ± 2% inhibition)[c]	n.d.	1.54 ± 0.58
11d	(1*RS*,2*RS*)1-Methyl-2-hydroxy-2-phenylethyl	1.90 ± 0.25	n.d.	⩾10 (41 ± 5% inhibition)[c]	n.d.	⩾10 (43 ± 1% inhibition)[c]
11e	(1*RS*,2*RS*)1-Methyl-2-hydroxy-2-phenylethyl	5.83 ± 0.26	n.d.	>10 (13 ± 3% inhibition)[c]	n.d.	⩾10 (34 ± 1% inhibition)[c]



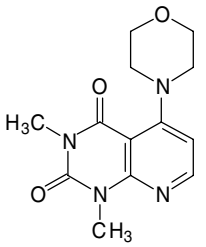
16a-c



16d,e



16f



16g

16a	Benzyl	>1.0 (27 ± 3% inhibition)[c]	n.d.	>1.0 (10 ± 2% inhibition)[c]	n.d.	9.18 ± 2.43
16b	1-Naphthyl	0.0383 ± 0.0088	0.153 ± 0.009	0.217 ± 0.037	0.0813 ± 0.0303	>10 (26 ± 6% inhibition)[c]
16c	(*RS*)-Phenylisopropyl	0.300 ± 0.025	1.063 ± 0.182	4.13 ± 2.24	n.d.	4.50 ± 0.28
16d	H	5.20 ± 0.10[d]	n.d.	>10 (23 ± 5% inhibition)[c]	n.d.	⩾10 (38 ± 5% inhibition)[c]
16e	Phenethyl	0.540 ± 0.066	1.44 ± 0.16	6.03 ± 2.08	n.d.	4.30 ± 1.16
16f	—	≫1.0 (3% inhibition)[c]	n.d.	≫1.0 (0% inhibition)[c]	n.d.	≫1.0 (8 ± 4% inhibition)[c]
16g	—	≫1.0 (0% inhibition)[c]	n.d.	≫1.0 (1% inhibition)[c]	n.d.	≫1.0 (0% inhibition)[c]

[a] Unless otherwise noted.
[b] Determined versus [^{3}H]CGS21680 as radioligand; data for antagonists determined with [^{3}H]CGS21680 have been shown to be similar to those determined with [^{3}H]MSX-2.[39]
[c] Percent inhibition of radioligand binding at the indicated concentration (1 or 10 μM) depending on the solubility of the compound.
[d] $n = 2$.

ethoxycarbonyl substituent, compound **7a** showed only moderate water-solubility. In order to increase water-solubility, piperazinyl-substituted compounds **7b** and **7c** were prepared containing a basic, protonatable nitrogen atom. Both compounds, the benzylpiperazinyl (**7b**) and the *p*-nitrobenzylpiperazinyl derivative (**7c**), showed A_1, A_{2A}, and A_3 affinity in the low micromolar concentration range; they were of similar potency to the unsubstituted parent compound **3b** at A_1 and A_{2A} ARs; however, they exhibited a higher affinity for A_3 receptors. Thus, the substitution of the 7-amino group gave no advantage with respect to A_1 or A_{2A} AR affinity, but it increased A_3 affinity and even led to A_3-selective compounds, such as **7a**.

A series of isomeric derivatives was investigated in which the amino group occupies the 5-position instead of the 7-position (compounds **11a**–**11e**). These compounds were analogs of the previously investigated derivatives[24,25] (Fig. 1). Compound **11a**, the isomer of the 7-amino derivative **3b** featuring an unsubstituted 5-amino group, was similar in potency to **3b** at all three receptor subtypes. Substitution of the amino group with a 2-naphthylmethyl residue, however, had a dramatic effect: compound **11c** was a very potent A_1 antagonist (vide infra) with a K_i value of 4.97 nM at the rat A_1 receptor and 25 nM at the human A_1 AR. The compound showed very high selectivity versus the rat A_{2A} AR (>400-fold) and the A_3 AR (>60-fold); it was more than 1000-fold more potent at the A_1 AR than its regioisomer **7a**. All analogs bearing bulky substituents at N^5 showed low affinity for the A_{2A} AR. A benzyl (**11b**) or a 1-methyl-2-hydroxy-2-phenylethyl substituent (**11d**) resulted in much weaker A_1 antagonists than the 2-naphthylmethyl substitution in **11c**. N-Methylation of **11d** yielding **11e**, which lacks the hydrogen bond donor function of the 5-amino group, further decreased AR affinity. The human A_3 AR best tolerated a 2-naphthylmethyl or a benzyl substituent at the 5-amino group, but the affinities were in the low micromolar range at best (**11c**: K_i = 1.54 μM).

Subsequently, a further series of 5-amino-substituted pyridopyrimidinediones was investigated, in which the 6-position remained unsubstituted (**16a**–**16c**, **16f**, **16g**) or was substituted by a cyano group (**16d**, **16e**; see Table 1). Replacement of the ethoxycarbonyl group by a cyano group did not alter A_1 and A_3 affinity but reduced A_{2A} affinity (compare **16d/11a**). Additional substitution on the amino group with a bulky phenethyl residue (**16e**) led to a moderately potent (K_i rat A_1 = 540 nM, human A_1 = 1.44 μM), only weakly A_1-selective AR antagonist.

In the series of 6-unsubstituted pyridopyrimidinediones, substitution of the 5-position by a benzylpiperazinyl (**16f**) or a morpholinyl residue (**16g**) resulted in totally inactive compounds. Both compounds are lacking a free NH group that may be required in this position for hydrogen bonding with the receptor. As seen with the previous series of 6-ethoxycarbonyl derivatives, a 5-benzylamino residue was not well tolerated by the receptors (**16a**, compare with **11b**), it was best tolerated by the A_3 AR (K_i = 9.18 μM). However, phenylisopropyl (2-methyl-2-phenylethyl) substitution of the 5-amino group, a substituent that had resulted in high A_1 affinity and selectivity when attached to the exocyclic amino group of adenosine—a standard A_1 agonist is (*R*)-phenylisopropyladenosine (*R*-PIA)[1–4]—was much better tolerated: compound **16c** had a K_i value of 300 nM at rat A_1, 1.06 μM at human A_1, 4.13 μM at rat A_{2A}, and 4.50 μM at human A_3 ARs (Table 1). The best compound in this series of 6-unsubstituted 5-amino-pyridopyrimidines was **16b** bearing a 1-naphthyl substituent at the 5-amino group, which was also the only arylamino-substituted compound in this series. It showed high A_1 affinity (K_i 38 nM rat, 103 nM human) and was also relatively potent at A_{2A} receptors (K_i 217 nM rat, 80 nM human). The compound was virtually inactive at human A_3 ARs. Thus, **16b** is a balanced A_1/A_{2A} AR antagonist and could serve as a lead structure for the development of A_{2A}-selective compounds, which are of high interest as novel drugs for Parkinson's disease.[7] Thus, an aryl substituent at the exocyclic amino group may be advantageous for A_1 as well as A_{2A} receptor affinity.

The structure–activity relationships are in accordance with A_1 AR pharmacophore models[5,11,23] that postulate the requirement of a hydrogen bond donor in proximity to a hydrogen bond acceptor, in addition to a hydrophobic substituent. Figure 6 shows a possible binding mode of a prototypical A_1-selective AR antagonist (1,3-dipropyl-8-cyclopentylxanthine, DPCPX) and a prototypical A_1 agonist N^6-cyclopentyladenosine (CPA) in comparison with that of compound **11c**. The lipophilic cyclopen-

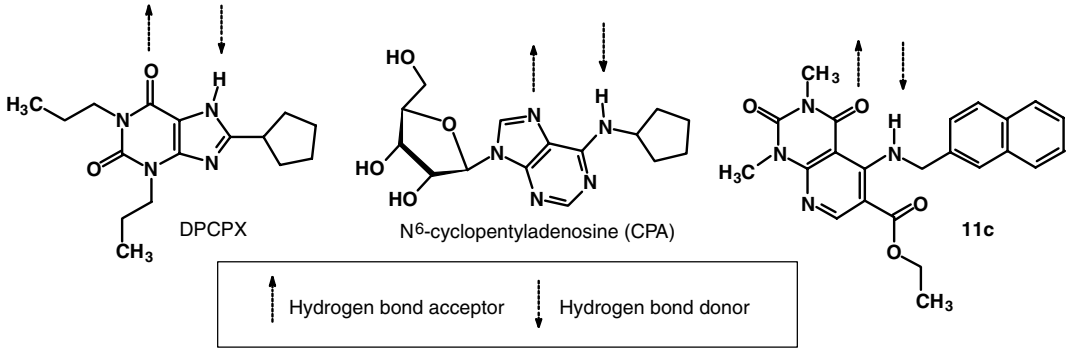


Figure 6. Putative binding mode of pyrido[2,3-*d*]pyrimidindione derivative **11c** with respect to the A_1-selective xanthine antagonist DPCPX and the A_1-selective agonist CPA.

tyl group in DPCPX and CPA may correspond to the 2-naphthylmethyl residue in **11c**. Surprisingly, replacement of the 2-naphthylmethyl by a benzyl substituent (in **11b**) abolished affinity for the A_1 receptor.

2.2.3. [^{35}S]GTPγS binding studies. The two most potent A_1 AR ligands of the present series of pyridopyrimidinediones, **11c** and **16b**, were investigated in [^{35}S]GTPγS binding studies at membrane preparations of CHO cells recombinantly expressing the human A_1 AR in order to obtain information about their functional properties. The results are shown in Figure 7. The A_1-selective AR agonist N^6-cyclopentyladenosine (CPA) and the A_1-selective AR antagonist DPCPX, that has previously been shown to act as an inverse agonist,[41] were included as standard compounds for comparison. The binding of agonists to G_i protein-coupled receptors (GPCR) can be modulated by GTP upon its binding to the G-protein. The addition of GTP, for example, has been shown to cause a rightward shift of the competition curve for agonists but not for antagonists at the G_i-coupled A_1 AR.[42] Conversely, A_1 AR agonists can also modulate GTP binding to the G-protein. This may be measured in radioligand binding assays using [^{35}S]GTPγS, a radiolabeled, stable analog of GTP.[43] The A_1 AR agonist CPA caused a concentration-dependent increase in [^{35}S]GTPγS binding with an EC_{50} value of 3.5 nM (Fig. 7), which corresponds well with the K_i value of CPA obtained in radioligand binding experiments at human A_1 ARs (K_i = 2.3 nM).[44] So-called neutral antagonists would not have any influence on [^{35}S]GTPγS binding, while inverse agonists lead to a reduction in [^{35}S]GTPγS binding.[41] While agonists are believed to bind preferentially to the active receptor conformation(s), neutral antagonists are thought to have no preference for either conformation and inverse agonists to bind preferentially to inactive conformation(s).[41] As previously described, the inverse agonist DPCPX showed a decrease in [^{35}S]GTPγS binding with an EC_{50} value of 2.4 nM consistent with its affinity for human A_1 ARs (K_i = 3.9 nM).[44] Both investigated pyridopyrimidinediones, **11c** and **16b**, did not enhance [^{35}S]GTPγS binding but led to a significant inhibition. The maximal effect was lower than that of DPCPX (ca. 70% of the DPCPX effect), which is one of the most efficacious inverse agonists of A_1 ARs described to date.[41] These experiments clearly show that the pyridopyrimidinediones are antagonists at A_1 ARs with inverse agonistic potency.

3. Conclusions

In conclusion, we have studied the structure–activity relationships for pyrido[2,3-*d*]pyrimidinediones as ligands of adenosine receptors and identified a novel highly potent and selective A_1 antagonist, 6-carbethoxy-1,2,3,4-tetrahydro-1,3-dimethyl-5-(2-naphthyl)methylaminopyrido[2,3-*d*]pyrimidine-2,4-dione (**11c**). It is 360-fold more potent than our lead compound identified in a previous study[24] (Fig. 1). Furthermore, a regioisomer of **11c**, 6-carbethoxy-1,2,3,4-tetrahydro-1,3-dimethyl-7-(2-naphthyl)methylaminopyrido[2,3-*d*]pyrimidine-2,4-dione (**7a**) in which the 2-naphthylmethylamino-substituent at position 5 of **11c** was moved to the 7-position, was a relatively potent (K_i = 226 nM) and selective (>20-fold) A_3 AR ligand. Both compounds have an ethoxycarbonyl residue in the 6-position. In the series of compounds lacking a substituent in that position, compounds with good affinity for the A_{2A} AR were identified, such as the non-selective 1,2,3,4-tetrahydro-1,3-dimethyl-5-(1-naphthyl)aminopyrido[2,3-*d*]pyrimidine-2,4-dione (**16b**, K_i human A_{2A} = 81.3 nM, K_i human A_1 = 153 nM, K_i human A_3 > 10,000 nM). Compound

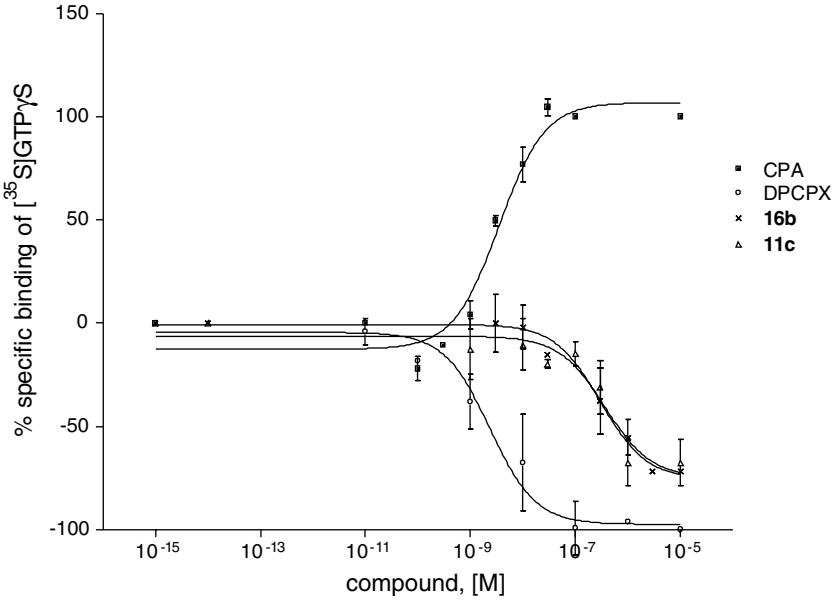


Figure 7. [^{35}S]GTPγS binding studies of selected pyridopyrimidinediones **11c** and **16b** at human recombinant adenosine A_1 receptors expressed in Chinese hamster ovary cells (normalized data with respect to DPCPX as full inverse agonist set at −100% = maximal inhibition). The maximal stimulation by the full agonist CPA was set at +100%. Data points are means of at least three independent experiments ± SEM.

16b could serve as a new lead structure for the development of A_{2A}-selective AR antagonists.

4. Experimental

4.1. Synthetic procedures

4.1.1. General procedures. Elemental analyses were performed at the Microanalytical Laboratory of the Institute of Inorganic Chemistry, University of Kiel. Melting points are uncorrected and were recorded with a Büchi 510 melting point apparatus. IR spectra (KBr pellet) were measured on a Perkin-Elmer FT-IR 16 PC spectrometer. The ^{1}H NMR spectra (internal standard: Me_4Si) were recorded in DMSO-d_6 on a Bruker ARX 300 spectrometer (δ given in ppm, J in Hz). Macherey-Nagel Polygram® SIL G/UV$_{254}$ on plastic sheets was used for TLC monitoring.

Compounds **2**, **3b**, and **3c**,[31] **5**,[33] **8**, **9**,[32] **10**, **11a**, **11b**, **14**, and **16e**,[28] **12**,[35] **13**,[36] **15**[37] are described in the literature.

4.1.2. 7-Amino-6-(3,4-dimethoxyphenyl)-1,3-dimethyl-1,2,3,4-tetrahydropyrido[2,3-*d*]pyrimidine-2,4-dione (3a). To a solution of 5,4 g of Na in 800 ml of ethanol were added 113.7 g (0.58 mol) of the pyrimidine **2** and 113.4 g (0.64 mol) of 3,4-dimethoxyphenylacetonitrile. The resulting slurry was heated at reflux for 3 h, during which time a homogeneous solution slowly was formed. Toward the end of the period a yellow precipitate began to separate. The mixture was cooled and the precipitate of crude **3a** was filtered and washed with diethyl ether. After this has been recrystallized from 95% ethanol, 109.8 g (61%) of **3a**, mp 272 °C was obtained. IR (KBr, ν cm^{-1}): 1675 (–CO–, lactam), 1730 (–CO–, lactam), 1755 (–CO–, ester). ^{1}H NMR (DMSO-d_6, δ ppm): 3.25 (s, 3H, N-CH$_3$), 3.50 (s, 3H, N-CH$_3$), 3.79 (s, 6H, 2 x OCH$_3$), 6.94 (m, 3H-arom, 2H, NH$_2$), 7.71 (s, 1H, H-5).

$C_{17}H_{18}N_4O_4$ (342.70): Calcd C, 59.70; H, 5.30; N, 16.38. Found: C, 57.92; H, 5.44; N, 15.58.

4.1.3. 6-Carbethoxy-7-chloro-1,3-dimethyl-1,2,3,4-tetrahydropyrido[2,3-*d*]pyrimidine-2,4-dione (6). To DMF (5.0 ml) at 0 °C was added phosphorus oxychloride (15.0 ml) dropwise with stirring. To this solution was added the pyrido[2,3-*d*]pyrimidine **5** (2.0 g, 7.2 mmol) and after 5 min the mixture was heated at 65 °C for 3 h. On pouring the reaction mixture into ice water, the crude product precipitated was collected by filtration and washed with petroleum ether. Recrystallization from ethanol afforded compound **6** (0.7 g, 60%). Mp: 133.8 °C (ethanol), IR (KBr, ν cm^{-1}): 1650 (–CO–, lactam), 1720 (–CO–, lactam), 1755 (–CO–, ester). ^{1}H NMR (DMSO-d_6, δ ppm): 1.37 (t, J = 6.0 Hz, 3H, CH$_3$), 3.38 (s, 3H, N-CH$_3$), 3.65 (s, 3H, N-CH$_3$), 4.39 (q, J = 7.0 Hz, 2H, CH$_2$), 9.05 (s, 1H, H-5).

$C_{12}H_{12}ClN_3O_4$ (297.70): Calcd C, 48.53; H, 4.07; N, 14.15. Found: C, 48.79; H, 4.12; N, 14.75.

4.1.4. Amino-substituted 1,3-dimethyl-1,2,3,4-tetrahydropyrido[2,3-*d*]pyrimidine-2,4-diones 7, 11, 16 (general procedure). A solution of 2 mmol of the chloro derivatives **6**, **10**, **14**, and **15**, respectively, and 20 mmol of the appropriate amine in 40 ml of ethanol was refluxed for 30 min. After cooling at 0 °C, the crude product was collected by filtration, washed with ice-cold ethanol, and recrystallized from ethanol.

4.1.5. 6-Carbethoxy-1,2,3,4-tetrahydro-1,3-dimethyl-7-(2-naphthyl)methylaminopyrido[2,3-*d*]pyrimidine-2,4-dione (7a). 1 mmol of the educt was used. Yield: 334 mg, 80%; mp: 197 °C; IR (KBr, ν cm^{-1}): 1667 (–CO–, lactam), 1688 (–CO–, lactam), 1707 (–CO–, ester), 3325 (NH). ^{1}H NMR (CDCl$_3$, δ ppm): 1.32 (t, J = 7.0 Hz, 3H, CH$_3$), 3.33 (s, 3H, N-CH$_3$), 3.60 (s, 3H, N-CH$_3$), 4.25 (q, J = 7.0 Hz, 2H, CH$_2$), 5.17 (t, J = 7.0 Hz, 2H, CH$_2$), 7.10–7.35 (m, 1H-arom), 7.37–7.50 (m, 4 H-arom), 7.74–7.85 (d, 2H-arom), 7.99 (d, J = 7.0 Hz, 1H-arom), 8.80 (s, 1H, H-5), 8.96–9.08 (br s, 1H, NH).

$C_{23}H_{22}N_4O_4$ (418.45): Calcd C, 66.08; H, 5.26; N, 13.40. Found: C, 66.96; H, 5.48; N, 12.75.

4.1.6. 6-Carbethoxy-7-benzylpiperazino-1,2,3,4-tetrahydro-1,3-dimethylpyrido[2,3-*d*]pyrimidine-2,4-dione (7b). 1 mmol of the educt was used. Yield: 174 mg, 40%; mp: 95 °C; IR (KBr, ν cm^{-1}): 1661 (–CO–, lactam), 1707 (–CO–, ester). ^{1}H NMR (CDCl$_3$, δ ppm): 1.32 (t, J = 7.0 Hz, 3H, CH$_3$), 3.23 (s, 3H, N-CH$_3$), 3.31 (s, 4H, 2× CH$_2$), 3.44 (s, 3H, N-CH$_3$), 3.55 (m, 6H, 3× CH$_2$), 4.25 (q, J = 7.0 Hz, 2H, CH$_2$), 7.32 (s, 5H-arom), 8.32 (s, 1H, H-5).

$C_{23}H_{27}N_5O_4$ (437.42): Calcd C, 63.21; H, 6.18; N, 16.02. Found: C, 62.27; H, 6.18; N, 15.88.

4.1.7. 6-Carbethoxy-1,2,3,4-tetrahydro-1,3-dimethyl-7-(*p*-nitrobenzyl)piperazinopyrido[2,3-*d*]pyrimidine-2,4-dione (7c). Yield: 501 mg, 52%; mp: 164 °C; IR (KBr, ν cm^{-1}): 1653 (–CO–, lactam), 1695 (–CO–, lactam), 1700 (–CO–, ester). ^{1}H NMR (CDCl$_3$, δ ppm): 1.32 (t, J = 7.0 Hz, 3H, CH$_3$), 3.23 (s, 3H, N-CH$_3$), 3.45 (s, 3H, N-CH$_3$), 3.58 (m, 4H, 2× CH$_2$), 3.73 (s, 2H, CH$_2$), 4.27 (q, J = 7.0 Hz, 2H, CH$_2$), 7.63 (d, 2H-arom), 8.20 (d, 2H-arom), 8.34 (s, 1H, H-5).

$C_{23}H_{26}N_6O_6$ (482.87): Calcd C, 57.31; H, 5.39; N, 17.42. Found: C, 57.09; H, 5.49; N, 17.60.

4.1.8. 6-Carbethoxy-1,2,3,4-tetrahydro-1,3-dimethyl-5-(2-naphthyl)methylaminopyrido[2,3-*d*]pyrimidine-2,4-dione (11c). Yield 454 mg, 54%; mp: 145 °C; IR (KBr, ν cm^{-1}): 1645 (–CO–, lactam), 1702 (–CO–, lactam), 1710 (–CO–, ester). ^{1}H NMR (CDCl$_3$, δ ppm): 1.21 (t, 3H, CH$_3$), 3.17 (s, 3H, N-CH$_3$), 3.46 (s, 3H, N-CH$_3$), 4.17 (q, 2H, CH$_2$), 4.79 (d, 2H, CH$_2$), 7.45–7.57 (m, 4H-arom), 7.86–7.99 (m, 3H-arom), 8,45 (s, 1H-arom), 10,20 (s, 1H, NH).

$C_{23}H_{22}N_4O_4$ (418.85): Calcd C, 66.08; H, 5.26; N, 13.14. Found: C, 65.94; H, 5.33; N, 13.45.

4.1.9. 6-Carbethoxy-1,2,3,4-tetrahydro-5-(1-hydroxy-1-phenylprop-2-yl)amino-1,3-dimethylpyrido[2,3-*d*]pyrimidine-2,4-dione (11d). 1 mmol of the educt was used. Yield: 191 mg, 45%; mp: 146 °C; IR (KBr, ν cm^{-1}): 1644 (–CO–, lactam), 1690 (–CO–, lactam), 1705 (–CO–, ester), 3440 (NH). ^1H NMR ($CDCl_3$, δ ppm): 1.12 (d, 3H, CH_3), 1.38 (t, J = 7.0 Hz, 3H, CH_3), 2,40 (s, 1H, OH), 3.43 (s, 3H, N-CH_3), 3.64 (s, 3H, N-CH_3), 3.88–4.07 (m, 1H, CH), 4.29–4.45 (m, 2H, CH_2), 4.95 (s, 1H, CH), 7.24–7.35 (m, 5H-arom), 8.50 (s, 1H, H-7), 10.30 (d, 1H, NH).

$C_{22}H_{36}N_4O_5$ (426.52): Calcd C, 62.02; H, 6.10; N, 13.14. Found: C, 60.11; H, 5.76; N, 13.43.

4.1.10. 6-Carbethoxy-1,2,3,4-tetrahydro-5-[(1-hydroxy-1-phenylprop-2-yl)methyl]amino-1,3-dimethylpyrido[2,3-*d*]pyrimidine-2,4-dione (11e). 1 mmol of the educt was used. Yield: 127 mg, 30%; mp: 122 °C; IR (KBr, ν cm^{-1}): 1651 (–CO–, lactam), 1690 (–CO–, lactam), 1708 (–CO–, ester), 3419 (NH). ^1H NMR ($CDCl_3$, δ ppm): 0.97 (d, 3H, CH_3), 1.39 (t, J = 7.0 Hz, 3H, CH_3), 3.12 (s, 3H, N-CH_3), 3.48 (s, 3H, N-CH_3), 3.70–3.76 (m, 4H, CH, N-CH_3), 4.31–4.46 (m, 2H, OCH_2), 4.86 (s, 1H, OH), 5.00 (s, 1H, OCH), 7.20–7.30 (m, 5H-arom), 8.72 (s, 1H, H-7).

$C_{21}H_{24}N_4O_5$ (426.52): Calcd C, 61.21; H, 6.82; N, 13.59. Found: C, 62.08; H, 6.25; N, 12.97.

4.1.11. 5-Benzylamino-1,2,3,4-tetrahydro-1,3-dimethylpyrido[2,3-*d*]pyrimidine-2,4-dione (16a). 1 mmol of the educt was used. Yield: 133 mg, 45%; mp: 121 °C; IR (KBr, ν cm^{-1}): 1670 (–CO–, lactam), 1690 (–CO–, lactam), 1720 (–CO–, ester), 3330 (NH). ^1H NMR ($CDCl_3$, δ ppm): 3.21 (s, 3H, N-CH_3), 3.46 (s, 3H, N-CH_3), 3.30–3.69 (m, 4H, $(CH_2)_2$), 6.52 (d, J = 6.0 Hz, 1H, H-6), 7.23–7.31 (m, 5H-arom), 8.06 (s, J = 6.0 Hz, 1H, H-7), 9.14 (br s, 1H, NH).

$C_{16}H_{16}N_4O_2$ (296.41): Calcd C, 64.92; H, 5.44; N, 18.92. Found: C, 63.35; H, 4.96; N, 19.45.

4.1.12. 1,2,3,4-Tetrahydro-1,3-dimethyl-5-(1-naphthyl)aminopyrido[2,3-*d*]pyrimidine-2,4-dione (16b). 1 mmol of the educt was used. Yield: 104 mg, 30%; mp: 232 °C; IR (KBr, ν cm^{-1}): 1670 (–CO–, lactam), 3330 (NH). ^1H NMR ($CDCl_3$, δ ppm): 3.22 (s, 3H, N-CH_3), 3.53 (s, 3H, N-CH_3), 4.96 (d, J = 7.0 Hz, 2H, CH_2), 6.60 (d, J = 6.0 Hz, 1H, H-6), 7.42–7.49 (m, 2H-arom), 7.55–7.63 (m, 2H-arom), 7.88–7.91 (m, 1H-arom), 7.97–8.00 (dd, J = 7.0 Hz, 1H-arom), 8.08–8.12 (m, 2H, H-7, 1H-arom), 9.55 (t; 1H, NH).

$C_{20}H_{18}N_4O_2$ (346.41): Calcd C, 68.67; H, 4.82; N, 16.87. Found: C, 69.66; H, 5.35; N, 15.87.

4.1.13. 1,2,3,4-Tetrahydro-1,3-dimethyl-5-(2-phenylpropyl)aminopyrido[2,3-*d*]pyrimidine-2,4-dione (16c). 1 mmol of the educt was used. Yield: 155 mg, 50%; mp: 131,7 °C; IR (KBr, ν cm^{-1}): 1640 (–CO–, lactam), 1689 (–CO–, lactam), 1720 (–CO–, ester), 3362 (NH). ^1H NMR ($CDCl_3$, δ ppm): 1.62 (d, J = 7.0 Hz, 3H, CH_3), 3.27 (s, 3H, N-CH_3), 3.45 (s, 3H, N-CH_3), 4.83 (quintet, J = 7.0 Hz, 1H, CH), 6.30 (d, J = 6.0 Hz, 1H, H-6), 7.06–7.31 (m, 5H-arom), 7.97 (d, J = 6.0 Hz, 1H, H-7), 9.58 (d, 1H, NH).

$C_{17}H_{18}N_4O_2$ (310.21): Calcd C, 65.86; H, 5.85; N, 18.07. Found: C, 65.68; H, 5.87; N, 18.26.

4.1.14. 5-Amino-6-cyano-1,2,3,4-tetrahydro-1,3-dimethylpyrido[2,3-*d*]pyrimidine-2,4-dione (16d). Yield: 189 mg, 41%; mp: 151 °C; IR (KBr, ν cm^{-1}): 1670 (–CO–, lactam), 1690 (–CO–, lactam), 1720 (–CO–, ester), 3330 (NH). ^1H NMR ($CDCl_3$, δ ppm): 3.24 (m, J = 7 Hz, 3H, N-CH_3, CH_2), 3.48 (s, 3H, N-CH_3), 7.86 (s, J = 7.0 Hz, 1H, H-7), 8,52 (br s, 1H, NH), 9.02 (s, 1H, NH).

$C_{10}H_9N_5O_2$ (231.46): Calcd C, 64.48; H, 5.07; N, 20.89. Found: C, 63.57; H, 5.18; N, 20.59.

4.1.15. 6-Cyano-1,2,3,4-tetrahydro-1,3-dimethyl-5-(2-phenylethyl)aminopyrido[2,3-*d*]pyrimidine-2,4-dione (16e). Yield: 509 mg, 76%; mp: 151 °C; IR (KBr, cm^{-1}): 2999 (w, C═C, aromatic), 2219 (s-m, CN), 1704 (–CO–, lactam), 1647 (–CO–, lactam), 1599 (–CO–, ester). ^1H NMR ($CDCl_3$, ppm): 3.25-3.29 (m, J = 7.0 Hz, 5H, N-CH_3, CH_2), 3.38 (s, 3H, N-CH_3), 4.56 (m, J = 7.0 Hz, 2H, CH_2), 6.45 (d, J = 7.0 Hz, 1H, H-6), 7.29–7.34 (m, 5H-arom), 9.54 (br s, 1H, NH).

$C_{18}H_{17}N_5O_2$ (335.41): Calcd C, 64.48; H, 5.07; N, 20.89. Found: C, 63.57; H, 5.18; N, 20.59.

4.1.16. 5-Benzylpiperazino-1,2,3,4-tetrahydro-1,3-dimethylpyrido[2,3-*d*]pyrimidine-2,4-dione (16f). Yield: 365 mg, 50%; mp: 130 °C; IR (KBr, ν cm^{-1}): 2940 (w, C═C, arom), 2813 (w, C═C, arom), 1691 (s, –CO–, lactam), 1651 (s, –CO–, lactam). ^1H NMR ($CDCl_3$, δ ppm): 3.21 (s, J = 7.0 Hz 5H, N-CH_3, N-CH_2), 3.30 (s, 5H, N-CH_3, N-CH_2), 3.49 (m, 4H, N-$(CH_2)_2$), 6.73 (d, J = 7.0 Hz, 1H, H-6), 7.24–7.30 (m, 5H-arom), 8.19 (d, J = 7.0 Hz, 1 H, H-7).

$C_{20}H_{23}N_5O_2$ (365.52): Calcd C, 65.93; H, 6.13; N, 19.51. Found: C, 68.85; H, 6.04; N, 19.23.

4.1.17. 1,2,3,4-Tetrahydro-1,3-dimethyl-5-morpholinopyrido[2,3-*d*]pyrimidine-2,4-dione (16g). Yield: 176 mg, 32%; mp: 184 °C; IR (KBr, ν cm^{-1}): 1670 (–CO–, lactam), 1690 (–CO–, lactam), 1720 (–CO–, ester), 3330 (NH). ^1H NMR ($CDCl_3$, δ ppm): 3.25 (s, 7H, N-CH_3, $N(CH_2)_2$), 3.57 (s, 3H, N-CH_3), 3.78 (t, J = 7.0 Hz, 4H, $(CH_2)_2$), 6.78 (d, J = 6.0 Hz, 1 H, H-6), 8.26 (s, J = 6.0 Hz, 1H, H-7).

$C_{13}H_{16}N_4O_3$ (276.42): Calcd C, 56.57; H, 5.84; N, 20.30. Found: C, 55.92; H, 6.08; N, 20.22.

4.2. Receptor radioligand binding studies

4.2.1. Materials. [^{3}H]CCPA (54.9 Ci/mmol) and [^{35}S]GTPγS (1250 Ci/mmol) were from NEN Life Sciences, [^{3}H]MSX-2 (85 Ci/mmol) and [^{3}H]PSB-11 (53 Ci/mmol) were obtained from Amersham. The non-radioactive precursors of [^{3}H]MSX-2 (MSX-1)[45] and [^{3}H]PSB-11 (PSB-10)[46] were synthesized in our laboratory.

4.2.2. Membrane preparations. Frozen rat brains obtained from Pel Freez®, Rogers, AR, USA, were dissected to obtain cortical membrane preparations for A_1 assays and striatal membrane preparations for A_{2A} assays as described.[46,47] CHO cells recombinantly expressing the human adenosine A_1, A_{2A}, and A_3 receptors were a gift from Dr. K.-N. Klotz and grown as described.[44] Membrane preparations were obtained as previously described.[44]

4.2.3. Adenosine receptor binding assays. Stock solutions of the compounds were prepared in dimethylsulfoxide (DMSO), the final concentration of DMSO in the assays being 2.5%. The radioligands and their concentrations were as follows: [^{3}H]CCPA, 0.5 nM (A_1), [^{3}H]MSX-2, 1 nM (A_{2A}), and [^{3}H]PSB-11, 0.5 nM (A_3). Binding assays were performed essentially as described.[39,47] Membranes (ca. 70 μg protein/mL) were preincubated for 20 min with 0.12–0.22 IU/mL of adenosine deaminase in order to remove endogenous adenosine. Curves were determined using 6–7 different concentrations of test compounds spanning 3 orders of magnitude. At least three separate experiments were performed, each in triplicate. For non-linear regression analysis, the Cheng–Prusoff equation and K_D values of 0.2 nM (rat A_1) and 0.6 nM (human A_1), respectively, for [^{3}H]CCPA,[38] 8 nM (rat A_{2A}) and 7 nM (human A_{2A}), respectively, for [^{3}H]MSX-2,[39] and 4.9 nM (human A_3) for [^{3}H]PSB-11[40] were used to calculate K_i values from IC_{50} values.

4.2.4. [^{35}S]GTPγS binding assays. [^{35}S]GTPγS binding assays were performed as previously described[43] by incubating membrane preparations of recombinant CHO cells expressing the human A_1 AR (5 μg per tube) with 0.5 nM [^{35}S]GTPγS in a total volume of 200 μl in 50 mM Tris–HCl buffer, pH 7.4, containing 1 mM EDTA, 5 mM $MgCl_2$, 1 mM dithiothreitol, 10 μM GDP, 100 mM NaCl, 2 IU/ml adenosine deaminase, 0.5% bovine serum albumin, and different concentrations of test compounds. Non-specific binding was determined in the presence of 10 μM of unlabeled GTPγS. After 1 h of incubation at room temperature, the assay was filtered through glass-fiber filters (Whatman GF/B), which had been presoaked for 30 min in ice-cold filtration buffer, on a Brandel cell harvester, followed by three washing steps with ice-cold 50 mM Tris–HCl containing 5 mM $MgCl_2$, pH 7.4. The punched-out filters were immediately transferred to mini-vials and incubated with 2.5 ml of Ultima Gold® scintillation cocktail (Canberra Packard) for 9 h before counting in a liquid scintillation counter Tricarb 2100TR (Canberra Packard). Data were analyzed using GraphPad Prism®, version 3.0 (GraphPad, San Diego, California, USA). Experiments were carried out in triplicate in at least three independent experiments.

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