Bioorganic & Medicinal Chemistry 18 (2010) 2195–2203

Contents lists available at ScienceDirect

Bioorganic & Medicinal Chemistry

journal homepage: www.elsevier.com/locate/bmc

2-Amino-5-benzoyl-4-phenylthiazoles: Development of potent and selective adenosine A_1 receptor antagonists

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ARTICLE INFO

Article history:
Received 18 November 2009
Revised 26 January 2010
Accepted 29 January 2010
Available online 4 February 2010

Keywords:
Thiazole
Adenosine receptor
Adenosine A_1 receptor antagonist
Structure–activity relationships

ABSTRACT

A series of 2-amino-5-benzoyl-4-phenylthiazole derivatives was investigated in radioligand binding studies at adenosine receptor (AdoR) subtypes with the goal to obtain potent and A_1-selective antagonists. Acylation of the 2-amino group was found to be crucial for high A_1 affinity. The best compound of the present series was 2-benzoylamino-5-*p*-methylbenzoyl-4-phenylthiazole (**16m**) showing a K_i value of 4.83 nM at rat and 57.4 nM at human A_1 receptors combined with high selectivity versus the other AdoR subtypes. The compound behaved as an antagonist in GTP shift assays at A_1 receptors. Compound **16m** may serve as a new lead structure for the development of second-generation non-xanthine-derived A_1 antagonists which have potential as novel drugs.

1. Introduction

Adenosine receptors (AdoR) are widely distributed in the human body and of considerable interest as targets for therapeutic intervention. They are subdivided into four subtypes, A_1, A_{2A}, A_{2B} and A_3, which have been cloned from various species, including humans.[1] These receptors are currently investigated as drug targets, for example, for the treatment of cardiovascular disorders, renal diseases, hypertension, Parkinson's disease, Alzheimer's disease, asthma, chronic obstructive pulmonary disease (COPD), inflammatory and allergic disorders and cancer.[2,3]

$AdoA_1R$ are expressed in the central nervous system (CNS) as well as in peripheral tissues. In the central nervous system high levels can be found in brain, cortex, cerebellum, hippocampus, and the dorsal horn of the spinal cord.[1] Activation of central $AdoA_1R$ leads to sedation, anticonvulsive and anxiolytic effects.[1] Therefore, A_1 antagonists may be useful as CNS-stimulatory drugs and have been suggested for the treatment of cognitive deficits.[4] In the lung adenosine mediates bronchoconstriction, inflammation, increased endothelial cell permeability, and mucin production.[5] $AdoA_1R$ antagonists are therefore currently in development for the treatment of asthma.[5] In the heart $AdoA_1R$ are located on sinoatrial and atrioventricular nodes and mediate negative chronotropic, dromotropic, and inotropic effects.[1] $AdoA_1R$ antagonists could be used to treat cardiac arrhythmia and congestive heart failure.[6] In the kidney activation of $AdoA_1R$ leads to vasoconstriction, reduction of glomerular filtration rate, inhibition of renin secretion, and inhibition of neurotransmitter release. Antagonizing these effects would help to treat renal failure, renal dysfunction, nephritis, hypertension and edema.[7]

Xanthine derivatives represent the first and most established class of AdoR antagonists.[1,8] Theophylline (**1**, Fig. 1) has been used in the treatment of asthma for more than 60 years although side effects are common at doses that are needed for bronchodilation. Theophylline is a weak, non-selective AdoR antagonist and, in addition, an inhibitor of different families of phosphodiesterases (PDE).[8] Therefore, **1** shows a low therapeutic index and drug levels have to be closely monitored.

Efforts have been made to develop more potent and selective xanthine derivatives as AdoR antagonists. Modification of the xanthine structure, in particular at the 8-position, led to derivatives provided with higher affinity and better subtype-selectivity than theophylline.[1,8,9] A drawback of many xanthine derivatives is their

Abbreviations: AdoR, adenosine receptor(s); CADO, 2-chloroadenosine; [^{3}H]CCPA, [^{3}H]2-chloro-N^6-cyclopentyladenosine; CHO, Chinese hamster ovary; CPA, N^6-cyclopentyladenosine; DMSO, dimethylsulfoxide; DPCPX, 8-cyclopentyl-1,3-dipropylxanthine; gp, guinea pig; h, human; m, mouse; [^{3}H]MSX-2, [^{3}H](*E*)-3-(3-hydroxypropyl)-8-(2-(3-methoxyphenyl)vinyl)-7-methyl-1-prop-2-ynyl-3,7-dihydropurine-2,6-dione; NECA, 5′-(*N*-ethylcarboxamido)adenosine; *R*-PIA, (*R*)-N^6-phenylisopropyladenosine; PSA, polar surface area; [^{3}H]PSB-11, [^{3}H]2-phenyl-8-ethyl-4-methyl-(8*R*)-4,5,7,8-tetrahydro-1*H*-imidazo[2.1-*i*]purin-5-one; [^{3}H]PSB-603, [^{3}H]8-(4-(4-(4-chlorophenyl)piperazine-1-sulfonyl)phenyl)-1-propylxanthine; r, rat; s, sheep; TRIS, tris(hydroxymethyl)aminomethane.

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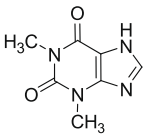
doi:10.1016/j.bmc.2010.01.072



1 Theophylline

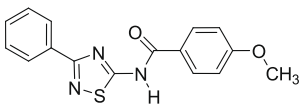
K_i values[9] (nM; h = human, r = rat):

hA_1 = 6770	rA_1 = 14000
hA_{2A} = 6700	rA_{2A} = 22000
hA_{2B} = 9070	rA_{2B} = 15100
hA_3 = 22300	rA_3 = 85000

Figure 1. Structure of theophylline—a therapeutically used non-selective adenosine receptor antagonist.

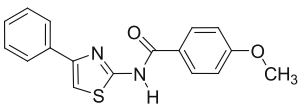
low water-solubility which may limit their bioavailability.[10] In order to overcome the low water-solubility of xanthines, prodrug approaches have been pursued.[10–13]

To provide alternatives research has focussed on the search for non-xanthine-based structures as AdoR antagonists.[1–3] Various heterocycles, including adenine, pyrrolo[2,3-*d*]pyrimidine, pyrimido[4,5-*b*]indole, pyridopyrimidinediones, imidazopurines, and naphthyridine derivatives have been characterized as $AdoA_1R$ antagonists.[14–19] All of these heterocyclic structures can be envisaged as analogs of adenine—the nucleobase partial structure of adenosine lacking the ribose moiety.[3] Molecular modeling and screening efforts have recently identified novel structures with AdoR-antagonistic activity: 4-phenyl-1,3-thiazole and 3-phenyl-1,2,4-thiadiazole derivatives.[20] *N*-(3-Phenyl-1,2,4-thiadiazol-5-yl)-4-methoxybenzamide (LUF 5417, **2**, Fig. 2) exhibited high affinity for $AdoA_1R$ and $AdoA_3R$ and much lower affinity for $AdoA_{2A}R$. The affinity of *N*-(4-phenylthiazol-2-yl)-4-methoxybenzamide (LUF 5433, **3**, Fig. 2) for the $AdoA_1R$ was lower, but it was somewhat selective versus the $AdoA_3R$.[20] Press et al. developed a series of phenyl-substituted 4-phenyl-5-pyridin-4-yl-thiazoles and investigated these compounds at all four AdoR subtypes.[21] Structure–activity relationship studies identified *N*-[5-pyridin-4-yl-4-(3,4,5-trimethoxyphenyl)thiazol-2-yl]acetamide (**4**) as the most potent compound of the series; it is an $AdoA_3R$ antagonist exhibiting subnanomolar potency with 1000-fold selectivity versus the other AdoR subtypes (Fig. 2).[21] A series of 2-amino-5-benzoyl-4-(2-furyl)thiazoles was synthesized and tested by Cole et al. leading to the identification of selective $AdoA_{2A}R$ antagonists as potential therapeutics for Parkinson's disease.[22] The best compound was a 2-amino-5-benzoyl-4-(2-furyl)thiazole derivative substituted with a 2-thienylethyl residue at the 2-amino function (**5**, Fig. 2). The K_i value at human $AdoA_{2A}R$ was 12 nM, and **5** was >500-fold selective versus A_1.



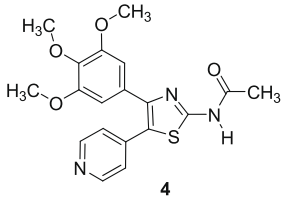
2 LUF5417

$rA_1 = 32$[20]
$rA_{2A} = 2340$[20]
$hA_3 = 82$[20]



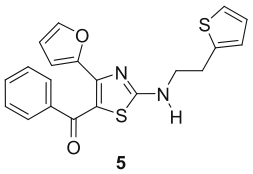
3 LUF5433

$rA_1 = 76$[20]
$rA_{2A} = 1900$[20]
$hA_3 = 670$[20]



4

$hA_1 > 10000$[21]
$hA_{2A} > 10000$[21]
$hA_{2B} = 3200$ (K_B value)[21]
$hA_3 = 0.4$[21]



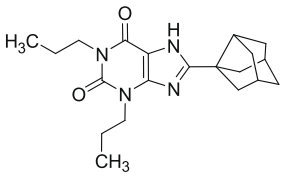
5

$hA_1 > 6700$[22]
$hA_{2A} = 12$[22]

Figure 2. Thiadiazole and thiazole derivatives with high affinity for adenosine receptors and their K_i values (nM) at AdoR subtypes (h = human, r = rat).

Our goal was to develop novel potent AdoR antagonists with selectivity for the A_1 receptor subtype, structurally unrelated to xanthines or adenines, but based on a 4-phenylthiazole scaffold.

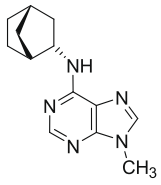
Several A_1 antagonists have been or are being evaluated in various phases of clinical trials or are in preclinical development. The xanthine derivative rolofylline (KW3902, **6**, Fig. 3), an A_1 antagonist of the first generation, had shown promising results in a pilot phase III study in patients with acute heart failure, but in a recently published larger phase III study (PROTECT) it did not show significant improvement over placebo.[6,23] The adenine derivative N-0861 (**7**, Fig. 3) has been in clinical studies for the therapy of heart failure. Two other A_1 antagonists, FK-453 (**8**) and FK-838 (**9**) have been undergoing clinical trials for acute renal failure and hypertension, respectively.[24,25] Tonapofylline (BG9928, **10**, Fig. 3), a xanthine derivative of the second generation, is a potent oral $AdoA_1R$ antagonist undergoing clinical investigation in heart failure.[26] For SLV320 (**11**), a potent and selective $AdoA_1R$ antagonist, phase II studies in the treatment of congestive heart failure have been completed.[27] The xanthine derivative L-97-1 (**12**) is in preclinical development as an oral drug for the treatment of asthma.[28]



6 KW3902

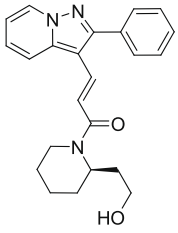
Rolofylline

$hA_1 = 0.295$[29]
$rA_1 = 0.19$[30]
$mA_1 = 0.145$[29]
$gpA_1 = 1.1$[31]
$rA_{2A} = 170$[30]
$gpA_{2A} = 230$[31]



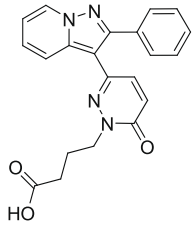
7 N-0861

$gpA_1 = 375$[31]
$gpA_{2A} = 3750$[31]
$sA_3 = 41.4$[32]



8 FK-453

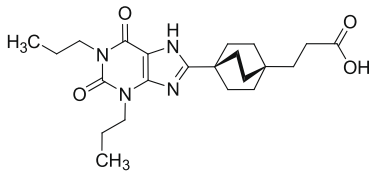
$hA_1 = 0.490$[29]
$rA_1 = 17.2$ (IC_{50} value)[24]
$mA_1 = 0.550$[29]
$gpA_1 = 0.56$ (IC_{50} value)[24]
$rA_{2A} = 11300$ (IC_{50} value)[24]
$gpA_{2A} = 1180$ (IC_{50} value)[24]



9 FK-838

$hA_1 = 12.0$[29]
$rA_1 = 6.61$[33]
$mA_1 = 10.2$[29]
$gpA_1 = 10.0$[29]
$rA_{2A} = 1202$[33]

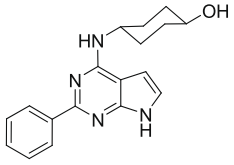
Figure 3. A_1-Selective adenosine receptor antagonists in clinical development and their K_i values (nM) at AdoR subtypes (gp = guinea pig, h = human, m = mouse, r = rat, s = sheep).[29–35]



10 BG9928

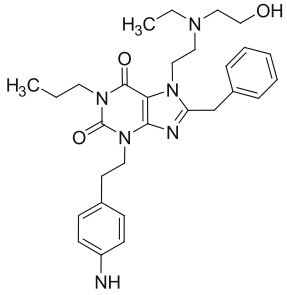
Tonapofylline

$hA_1 = 7.4$[34] $rA_1 = 1.3$[34]
$hA_{2A} = 6410$[34] $rA_{2A} = 2440$[34]
$hA_{2B} = 90$[34]
$hA_3 = 10000$[34]



11 SLV320

$hA_1 = 1.00$[27] $rA_1 = 2.51$[27]
$hA_{2A} = 398$[27] $rA_{2B} = 501$[27]
$hA_{2B} = 3981$[27]
$hA_3 = 200$[27]



12 L-97-1

$hA_1 = 0.58$[35]
$hA_{2A} > 100$ (IC_{50} value)[35]
$hA_{2B} > 100$ (IC_{50} value)[35]

Fig. 3 (*continued*)

We have now investigated the structure–activity relationships of a series of 2-amino-5-benzoyl-4-phenyl-1,3-thiazole derivatives in order to develop compounds with high affinity and subtype-selectivity for $AdoA_1R$, but with novel structural features unrelated to classical A_1 antagonists.

2. Results and discussion

2.1. Chemistry

The synthesis of **16g** (Scheme 1) had previously been described.[36] Further thiazole derivatives were prepared in analogy to the described procedure.[36] Diethylamine (**13**) was reacted with benzonitrile in the presence of aluminum chloride to form *N*,*N*-diethylbenzimidamide (**14**, Scheme 1).[37] Reaction of **14** with the appropriate isothiocyanate in hexane or tetrahydrofuran led to the formation of **15a–f**.[38]

The final products, thiazole derivatives **16a–r**, were prepared from **15a to 15f** according to a protocol described by Rajappa et al.[36] Acetophenone derivatives were treated with equimolar amounts of bromine to obtain the corresponding bromoacetophenone derivatives. These were subsequently treated with **15a–f** in acetonitrile to yield the desired 5-benzoyl-substituted thiazole derivatives **16a–r** (Scheme 1) after reaction times of a few hours in acceptable to good yields ranging from 28% to 75%. Since the reactions were performed under mild conditions and the solid thiazole derivatives **16a–r** precipitated from the reaction medium as pure products, chromatographic purification was not required. The structures of the synthesized compounds were confirmed by ^1H NMR spectroscopy, mass spectroscopy, and for most products IR spectra were also obtained. For selected compounds, ^{13}C NMR spectra were recorded and elemental analyses were determined (see Section 4). Purity was further determined by HPLC-ESI-UV/MS analysis and found to be >95% in all cases.

2.2. Biological investigations

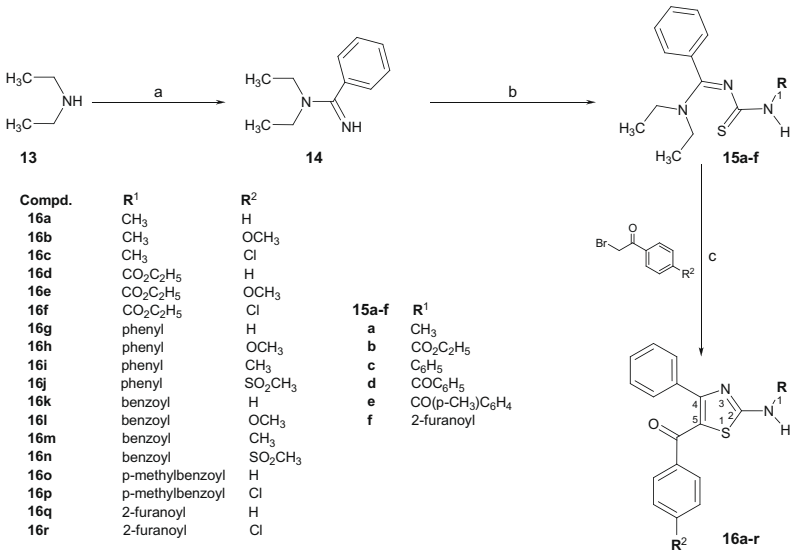
Receptor-radioligand binding studies at rat A_1 and A_{2A}, human A_1 and A_{2A} (for selected compounds) as well as human A_{2B} and A_3 receptors were performed as previously described.[39] Initially, the 2-amino-5-benzoyl-4-phenylthiazoles were screened at a single concentration of 1 μM. For compounds that inhibited radioligand binding by more than 50% full concentration–inhibition curves were recorded and K_i values were determined. All results are collected in Table 1.

2.3. Structure–activity relationships

A systematic modification of two positions in the 2-amino-5-benzoyl-4-phenylthiazole scaffold was performed with the goal to enhance $AdoA_1R$ affinity in this series. Various substituents (methyl, ethylcarboxylate, phenyl, benzoyl, *p*-methylbenzoyl, 2-furanoyl) were introduced at the 2-amino function. All 2-methylaminothiazole derivatives (**16a–c**) were inactive at the four AdoR subtypes at the test concentration of 1 μM. Likewise, a phenyl substitution (**16g–j**) yielded compounds that were mostly inactive at AdoR. Only 5-benzoyl-substituted derivative **16g** showed some affinity for the A_{2B} receptor (K_i 337 nM) and appeared to be selective for that receptor subtype. However, when the amino group was acylated rather than methylated or phenyl-substituted, higher affinity for AdoR was observed: the ethyl carbamates **16d–f** showed significant AdoR affinity, compounds **16d** (5-benzoyl derivative) and **16e** (5-*p*-methoxybenzoyl derivative) being most potent at the $AdoA_1R$ with K_i values of 72.9 nM (**16d**), and 69.9 nM (**16e**), respectively. Both compounds were considerably weaker (>20-fold) at $AdoA_{2A}R$, but only slightly weaker at $AdoA_3$ than at A_1R. The 5-benzoyl derivative 16d was also quite potent at the $AdoA_{2B}R$ (K_i 391 nM).

The best N^2-substituents in the present series with respect to high A_1 affinity were the benzoyl (e.g., **16k**) and the 2-furanoyl (see e.g., **16q**) residue. 2-Benzoylamino-5-benzoyl-4-phenylthiazole (**16k**) showed a K_i value of 9.51 nM for the rat $AdoA_1R$ and was very selective versus the other AdoR subtypes. The corresponding 2-(2-furanoyl)amino-substituted derivative was similarly potent (K_i 5.77 nM), but somewhat less selective. If the N^2-benzoyl residue was *p*-methylated (**16o**) the affinity was abolished. Thus, the SARs are steep at the *p*-position of the benzoyl residue and a methyl residue is already too large to be accommodated in the binding pocket.

In the next step we investigated substituents in the *p*-position of the benzoyl residue in order to potentially achieve an increase in A_1 affinity and selectivity of the compounds. Introduction of a *p*-methoxy residue into bis-benzoyl derivative **16k** yielding 2-benzoylamino-5-*p*-methoxybenzoyl-4-phenyl-thiazole **16l** resulted in a compound that was similarly potent as the parent compound (K_i 7.15 nM for **16l**, K_i 9.51 nM for **16k**). Compound **16l** was highly

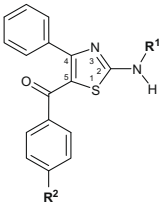


Compd.	R^1	R^2
16a	CH_3	H
16b	CH_3	OCH_3
16c	CH_3	Cl
16d	$CO_2C_2H_5$	H
16e	$CO_2C_2H_5$	OCH_3
16f	$CO_2C_2H_5$	Cl
16g	phenyl	H
16h	phenyl	OCH_3
16i	phenyl	CH_3
16j	phenyl	SO_2CH_3
16k	benzoyl	H
16l	benzoyl	OCH_3
16m	benzoyl	CH_3
16n	benzoyl	SO_2CH_3
16o	p-methylbenzoyl	H
16p	p-methylbenzoyl	Cl
16q	2-furanoyl	H
16r	2-furanoyl	Cl

15a-f	R^1
a	CH_3
b	$CO_2C_2H_5$
c	C_6H_5
d	COC_6H_5
e	$CO(p\text{-}CH_3)C_6H_4$
f	2-furanoyl

Scheme 1. Synthesis of thiazole derivatives. Reagents and conditions: (a) (1) benzonitrile, $AlCl_3$, 0–8 °C; (2) 120–150 °C; (b) R^1-NCS, 0–5 °C, 2–5 h; (c) bromoacetophenone derivatives, acetonitrile, 0 °C, 5 h.

Table 1
Affinities of thiazole derivatives and standard compounds for adenosine receptors



Compound	R^1	R^2	K_i ± SEM (nM) (or % inhibition of radioligand binding ± SEM at 1 μM) (n = 3)			
			A_1[d] versus [^{3}H]CCPA	A_{2A}[d] versus [^{3}H]MSX-2	A_{2B}[e] versus [^{3}H]PSB-603	A_3[e] versus [^{3}H]PSB-11
CPA[a]	—	—	0.8[40]	2470 ± 1310[39]	n.d.	3250 ± 950[41]
DPCPX[b]	—	—	0.90[42]	157[39]	n.d.	243 ± 56[43]
Caffeine[c]	—	—	41000 ± 6000[44]	32500[39]	33800 ± 1200[39]	>100000[45]
16a	CH_3	H	>1000[f]	>1000[f]	>1000[f]	>1000[f]
16b	CH_3	OCH_3	>1000[f]	>1000[f]	>1000[f]	>1000[f]
16c	CH_3	Cl	>1000[f]	>1000[f]	>1000[f]	>1000[f]
16d	$CO_2C_2H_5$	H	72.9 ± 13.9	1640 ± 680	391 ± 74	206 ± 167
16e	$CO_2C_2H_5$	OCH_3	69.9 ± 7.6	1840 ± 300	>1000[f]	98.8 ± 2.1
16f	$CO_2C_2H_5$	Cl	ca.1000 (41 ± 8)	>1000[f]	>1000[f]	487 ± 163
16g	Phenyl	H	>1000[f]	>1000[f]	337 ± 39	>1000[f]
16h	Phenyl	OCH_3	>1000[f]	>1000[f]	>1000 (39 ± 9)	>1000[f]
16i	Phenyl	CH_3	>1000[f]	>1000[f]	>1000[f]	>1000[f]
16j	Phenyl	SO_2CH_3	>1000[f]	>1000[f]	>1000[f]	>1000[f]
16k	Benzoyl	H	9.51 ± 2.72	3030 ± 110	>1000[f]	354 ± 107
16l	Benzoyl	OCH_3	7.15 ± 0.21	>1000[f]	>1000[f]	ca.1000 (56 ± 14)
16m	Benzoyl	CH_3	4.83 ± 1.06 57.4 ± 8.3[e]	>1000[f] 6250 ± 1970[e]	>1000[f]	2160 ± 880
16n	Benzoyl	SO_2CH_3	>1000[f]	>1000[f]	>1000[f]	>1000[f]
16o	*p*-Methylbenzoyl	H	>1000[f]	>1000[f]	>1000 (40 ± 13)	>1000[f]
16p	*p*-Methylbenzoyl	Cl	>1000[f]	>1000[f]	>1000[f]	>1000[f]
16q	2-Furanoyl	H	5.77 ± 0.93 54.3 ± 1.2[e]	662 ± 40 348 ± 70[e]	>1000[f]	208 ± 52
16r	2-Furanoyl	Cl	12.0 ± 1.5	>1000[f]	>1000[f]	619 ± 63

[a] N^6-Cyclopentyladenosine.
[b] 8-Cyclopentyl-1,3-dipropylxanthine.
[c] 1,3,7-Trimethylxanthine.
[d] Rat receptors unless otherwise indicated (A_1: rat brain cortex; A_{2A}: rat brain striatum).
[e] Human recombinant receptor expressed in CHO cells.
[f] Inhibition of radioligand binding was < 30% at a concentration of 1 μM.

selective versus the other AdoR subtypes. Introduction of a smaller methyl group (in **16m**) was advantageous: the affinity was increased by twofold and—more importantly—selectivity versus the $AdoA_3R$ was increased in comparison with **16k**. Compound **16m** was the most potent compound of the present series showing a K_i value of 4.83 nM at the rat $AdoA_1R$. An even larger methylsulfonyl substitution in the *p*-position of the 5-benzoyl residue abolished affinity. This showed that small, apolar residues were best tolerated at that position. A chlorine atom—introduced into the 2-(2-furanoyl)amino derivative (compound **16r**) reduced affinity by about twofold and thus had a similar effect as a methoxy group investigated in the 2-benzoylamino series (compound **16l**).

The most potent A_1 antagonists of the present series, **16m** and **16q**, were additionally investigated at *human* $AdoA_1$ and A_{2A} receptors in order to study potential species differences. Both compounds were about 10-fold less potent at human as compared to rat A_1 receptors. Nevertheless, **16m** and **16q** are potent $AdoA_1R$ ligands exhibiting K_i values of 57.4 nM (**16m**), and 54.3 nM (**16q**), respectively, for the human A_1 receptor. Especially **16m** is not only a very potent, but in addition a highly selective A_1 antagonist. In humans, its selectivity is >100-fold versus $AdoA_{2A}$ and $A_{2B}R$, and 38-fold versus A_3R.

2.4. GTP shift experiments

In order to investigate whether the best compounds **16m** and **16q** were antagonists or agonists at $AdoA_1R$ GTP shift experiments were performed. The so-called GTP shift is an in vitro parameter indicating intrinsic activity.[10] GTP can cause an uncoupling of the $AdoA_1R$ from the G_i protein leading to a shift of the receptor from the high- to the low-affinity state for agonists such as N^6-cyclopentyladenosine (CPA). The affinities of compounds **16m** and **16q** versus the antagonist radioligand [^{3}H]DPCPX at rat brain cortical membranes were determined in the absence and in the presence of 100 μM GTP. Results are listed in Table 2. The addition of GTP resulted in a significant rightward shift of the binding curve of the full agonist CPA (21.9-fold shift). For **16m** and **16q** no significant shifts of the binding curves in the presence of GTP could be observed. Therefore, they could be considered as antagonists at $AdoA_1R$ in our test system. Owing to the structural similarity of all compounds in this series we suppose that they are all antagonists (Fig. 4).

2.5. Structural comparison of 2-amino-5-benzoyl-4-phenylthiazole with xanthine derivatives

The described thiazole derivatives constitute a new class of A_1-selective AdoR antagonists which are unrelated to the structures of the classical A_1-selective xanthine derivatives, such as DPCPX. However, a closer look at the structural features of compound **16k** and a comparison with DPCPX showed that both classes of compounds exhibit similarities which could explain their binding to the same target structure (Fig. 5).

Table 2
Affinities (IC_{50} values at rat $AdoA_1R$ the presence and absence of GTP) and GTP shifts of compounds **16m** and **16q** in comparison with the agonist N^6-cyclopentyladenosine

Compound	IC_{50} ± SEM (nM)[a] (*n* = 3)		
	− GTP	+ 100 μM GTP	GTP shift[b]
16m	22.1 ± 3.4	22.3 ± 5.0	0.99 ± 0.07
16q	25.4 ± 6.4	28.8 ± 0.9	1.2 ± 0.3
CPA (agonist)	7.09 ± 0.31	154 ± 13	21.9 ± 2.9

[a] Displacement of [^{3}H]DPCPX from rat cortical membranes.
[b] The affinities for the $AdoA_1R$ were determined in the absence and presence of 100 μM GTP. The GTP shifts were calculated by dividing the IC_{50} values determined in the presence of GTP by those measured in the absence of GTP.

Both compounds have a similar size and shape. Furthermore, they are characterized by the same number of hydrogen bond acceptors (3) and hydrogen bond donors (1), which are found in similar, overlapping positions. Additional common features are the presence of three lipophilic domains—occupied by phenyl rings in the case of the benzoylaminothiazoles, and (cyclo)alkyl residues in the xanthine derivative. According to current models of adenosine receptors and their ligand binding site the 8-substituent of xanthines (e.g., the cyclopentyl ring in DPCPX, or the propionic acid-substituted bicyclo[2.2.2]octanyl residue in tonapofylline (**10**)) is oriented toward the cell surface.[46] The benzoylamino substituent in the 2-position of the thiazole derivatives is likely to point into the same direction. Like in xanthines, there should be room for further substitution at the benzoylamino residue. However, a methyl group in the *p*-position was detrimental (compounds **16o** and **16p**). This may be explained by the fact that only polar substituents are tolerated due to the hydrophilic nature of the extracellular cell surface. In future studies, this hypothesis should be examined by introducing polar groups into the 2-benzoylamino residue.

Thus, the well-known SARs for A_1-selective xanthine derivatives may be used for the future optimization of the new benzoylaminothiazole series. The calculated polar surface area (PSA) values are 59 Å^2 for **16m** and 72 Å^2 for **16q** indicating that the compounds have potential for oral absorption and may even be able to penetrate into the brain.[47]

3. Conclusions

A series of 18 2-amino-5-benzoyl-4-phenyl-1,3-thiazole derivatives was synthesized and pharmacologically evaluated at the AdoR subtypes A_1, A_{2A}, A_{2B} and A_3. Compounds **16d**, **16e**, **16k**–**m**, **16q** and **16r** showed affinities for $AdoA_1R$ in the nanomolar concentration range. GTP shift experiments indicated that compound **16m** and **16q** behave as antagonists at $AdoA_1R$. The most potent and selective $AdoA_1R$ antagonist identified in this study was 2-benzoylamino-5-*p*-methylbenzoyl-4-phenylthiazole (**16m**). This compound may serve as a new lead structure for the development of second-generation, non-xanthine-based $AdoA_1R$ antagonists, which are structurally unrelated to adenine.

4. Experimental section

4.1. General information

All commercially available reagents were obtained from various producers (Acros, Aldrich, Fluka, Merck, and Sigma) and used without further purification. The reactions were monitored and the purity of the compounds was checked by thin layer chromatography (TLC) using aluminum sheets with Silica Gel 60 F_{254} (Merck). Mass spectra were recorded on an API 2000 (Applied Biosystems, Darmstadt, Germany) mass spectrometer (turbo ion spray ion source) coupled with an HPLC system (Agilent 1100) using a Phenomenex Luna 3 μ C18 column. ^{1}H and ^{13}C NMR spectra were performed on a Bruker Avance 500 MHz spectrometer. DMSO-d_6 or $CDCl_3$ were used as solvents as indicated below. Shifts are given in ppm relative to the remaining protons of the deuterated solvents used as internal standard (^{1}H, ^{13}C). Infrared spectra were recorded on a Buck Scientific M-500 IR spectrophotometer. Elemental analyses were determined on a Heraeus Carlo Erba 1108 elemental analyzer. Melting points were measured on a Büchi 510 melting point apparatus and are uncorrected. The purity of the compounds was checked by dissolving 1 mg/mL in MeOH containing 2 mM ammonium acetate. A sample of 10 μL was injected into an HPLC instrument (Agilent 1100) using a Phenomenex Luna 3 μ C18

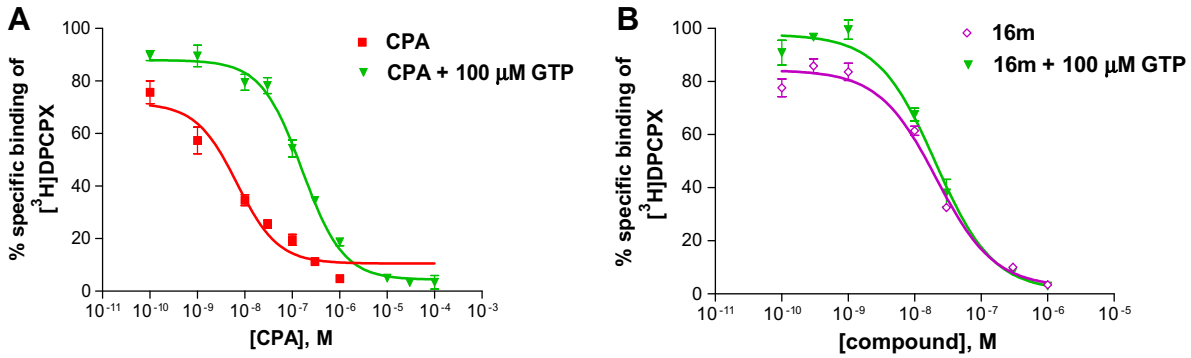


Figure 4. Concentration–inhibition curves of the agonist CPA and compound **16m** in the absence and presence of 100 µM GTP at rat AdoA$_1$R of brain cortex. [^{3}H]DPCPX (0.4 nM) was used as radioligand. Data points are means ± SEM of three separate experiments performed in triplicate.

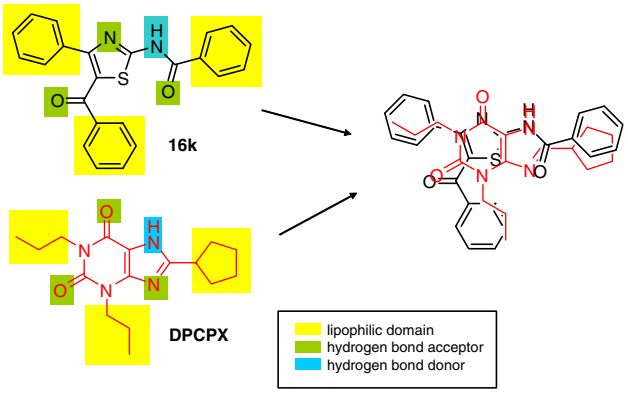


Figure 5. Structural comparison of the benzoylaminothiazole derivative **16k** and the xanthine derivative DPCPX, both of which are potent and selective AdoR antagonists.

column. Elution was performed with a gradient of water: methanol (containing 2 mM ammonium acetate) from 60:0 to 40:100 for 20 min followed by 10 min of 100% MeOH at a flow rate of 250 µL/min. UV absorption for each compound was detected at 254 nM. The purity of the products was generally ⩾95%. PSA values were calculated using molinspiration property engine v2009.01.

Benzoyl isothiocyanate and furanoyl isothiocyanate were synthesized following the procedure developed by Reeves et al.[48] The preparation of *p*-methylbenzoyl isothiocyanate and ethoxycarbonyl isothiocyanate was performed as previously described.[49,50] Methyl and phenyl isothiocyanate were obtained from commercial sources.

4.2. General procedure for the synthesis of *N*′-carbamothioyl-*N*,*N*-diethylbenzimidamide derivatives 15a–f

A solution of 0.0123 mol of isothiocyanate derivative in hexane or THF was cooled to 0 °C and to it was added 0.0123 mol of N,N-diethylacetamidine (**14**) in hexane or THF (15 mL) during 5 min. The solution was stirred at 0 °C for 2–5 h, and the separated solid was filtered off to give the products **15a–f**.

4.3. General procedure for the preparation of thiazole derivatives 16a–r

To a solution of 1 mmol of **15** in 15 mL of acetonitrile was added 1 mmol of bromoacetophenone derivative at 0 °C and the solution was stirred for 5 h. Product **16** precipitated and was filtered off and dried.

4.3.1. (2-(Methylamino)-4-phenylthiazol-5-yl)(phenyl)methanone (16a)

^{1}H NMR (500 MHz, DMSO-d_6) δ 2.91 (s, 3H), 6.70 (m, 2H), 7.10–7.30 (m, 6H), 7.40 (m, 2H), 8.5 (s, 1H). IR (KBr cm^{-1}); 3201, 2910, 1613, 1579, 1516, 1476, 1444, 1400, 1337, 1285, 1183, 1115, 1073, 1000, 932, 902, 852, 782, 637. LC/ESI-MS negative mode 293 ([M−H]$^-$), positive mode 295 ([M+H]$^+$). Mp 201 °C. Yield 32%.

4.3.2. (4-Methoxyphenyl)(2-(methylamino)-4-phenylthiazol-5-yl)methanone (16b)

^{1}H NMR (500 MHz, DMSO-d_6) δ 2.91 (s, 3H), 3.7 (s, 3H), 6.70 (m, 2H), 7.10–7.29 (m, 5H), 7.36–7.41 (m, 2H), 8.5 (s, 1H). IR (KBr cm^{-1}); 3197, 2930, 1598, 1520, 1479, 1446, 1405, 1332, 1254, 1180, 1161, 1144, 1111, 1049, 1026, 903, 863, 827, 790, 754, 712, 665. LC/ESI-MS negative mode 323 ([M−H]$^-$), positive mode 325 ([M+H]$^+$). Mp 220 °C. Yield 28%.

4.3.3. (4-Chlorophenyl)(2-(methylamino)-4-phenylthiazol-5-yl)methanone (16c)

^{1}H NMR (500 MHz, DMSO-d_6) δ 3.1 (s, 3H), 7.0–7.3 (m, 7H), 7.4 (m, 2H), 8.8 (s, 1H). IR (KBr cm^{-1}); 3197, 2900, 1609, 1519, 1466, 1398, 1188, 1143, 1108, 1045, 1014, 948, 926, 900, 855, 828, 783, 747, 679. Elemental Anal. Calcd for $C_{17}H_{13}ClN_2OS$ (328.82): C, 62.10; H, 3.99; N, 8.52. Found: C, 61.70; H, 4.37; N, 8.54. LC/ESI-MS negative mode 327, 330 ([M−H]$^-$), positive mode 329 ([M+H]$^+$). Mp 240 °C. Yield 28%.

4.3.4. Ethyl 5-benzoyl-4-phenylthiazol-2-ylcarbamate (16d)

^{1}H NMR (500 MHz, DMSO-d_6) δ 1.27 (t, J = 7.25 Hz, 3H), 4.26 (q, J = 7.25 Hz, 2H), 7.15 (m, 2H), 7.20 (m, 1H), 7.21–7.26 (m, 2H), 7.33 (m, 2H), 7.41 (m, 1H), 7.52 (m, 2H), 12.30 (s, 1H). ^{13}C NMR (125 MHz, DMSO-d_6) δ 14.4, 62.3, 124.8, 127.9, 128.2, 128.8, 129.1, 129.6, 132.4, 134.3, 137.8, 154.1, 155.3, 162.2, 189.0. IR (KBr cm^{-1}); 3163, 2910, 1731, 1622, 1589, 1554, 1524, 1482, 1444, 1335, 1306, 1232, 1160, 1065, 912, 862, 765, 724, 645. LC/ESI-MS negative mode 351 ([M−H]$^-$), positive mode 353 ([M+H]$^+$). Mp 165 °C. Yield 68%.

4.3.5. Ethyl 5-(4-methoxybenzoyl)-4-phenylthiazol-2-ylcarbamate (16e)

^{1}H NMR (500 MHz, $CDCl_3$) δ 1.13 (t, J = 7.25 Hz, 3H), 3.76 (s, 3H), 4.10 (q, J = 7.25 Hz, 2H), 6.67 (m, 2H), 7.17 (m, 3H), 7.41 (m, 2H), 7.64 (m, 2H). ^{13}C NMR (125 MHz, $CDCl_3$) δ 14.1, 55.4, 62.9, 113.3, 125.9, 128.0, 128.8, 129.5, 130.1, 132.0, 133.9, 153.0, 153.3, 161.8, 163.2, 187.9. LC/ESI-MS negative mode 381 ([M-H]$^-$), positive mode 383 ([M+H]$^+$). Mp 180 °C. Yield 71%.

4.3.6. Ethyl 5-(4-chlorobenzoyl)-4-phenylthiazol-2-ylcarbamate (16f)

^{1}H NMR (500 MHz, DMSO-d_6) δ 1.27 (t, J = 7.1 Hz, 3H), 4.26 (q, J = 7.1 Hz, 2H), 7.16 (m, 2H), 7.22–7.27 (m, 3H), 7.31 (m, 2H), 7.51 (m, 2H), 12.35 (s, 1H). ^{13}C NMR (125 MHz, DMSO-d_6) δ 14.4, 62.4, 124.8, 128.0, 128.2, 128.9, 129.8, 131.0, 134.2, 136.6, 137.1, 154.1, 155.8, 162.6, 188.0. IR (KBr cm^{-1}); 3265, 3071, 2978, 2934, 1701, 1620-, 1547, 1475, 1400, 1310, 1238, 1175, 1118, 1064, 1015, 910, 866, 830, 773, 739, 706, 669. LC/ESI-MS negative mode 385 ([M−H]$^-$), positive mode 387 ([M+H]$^+$). Mp 180 °C. Yield 54%.

4.3.7. Phenyl(4-phenyl-2-(phenylamino)thiazol-5-yl)methanone (16g)

^{1}H NMR (500 MHz, DMSO-d_6) δ 7.14–7.07 (m, 1H), 7.11–7.19 (m, 5H), 7.32–7.42 (m, 5H), 7.44–7.46 (m, 2H), 7.65–7.67 (m, 2H), 10.88 (s, 1H). Elemental Anal. Calcd for $C_{22}H_{16}N_2OS$ (356.44): C, 74.13; H, 4.52; N, 7.86. Found: C, 74.06; H, 4.98; N, 7.86. LC/ESI-MS negative mode 355 ([M−H]$^-$), positive mode 357 ([M+H]$^+$). Mp 193 °C. Yield 61%.

4.3.8. (4-Methoxyphenyl)(4-phenyl-2-(phenylamino)thiazol-5-yl)methanone (16h)

^{1}H NMR (500 MHz, DMSO-d_6) δ 3.71 (s, 3H), 6.72–6.75 (m, 2H), 7.02–7.06 (m, 1H), 7.17–7.24 (m, 3H), 7.34–7.40 (m, 4H), 7.48–7.51 (m, 2H), 7.65–7.68 (m, 2H), 10.78 (s, 1H). IR (KBr cm^{-1}); 3178, 2950, 1609, 1555, 1520, 1477, 1453, 1425, 1337, 1215, 1168, 1118, 1072, 1025, 912, 854, 760, 713, 665, 639. Elemental Anal. Calcd for $C_{23}H_{18}N_2O_2S$ (386.47): C, 71.48; H, 4.69; N, 7.25. Found: C, 71.24; H, 5.12; N, 7.19. LC/ESI-MS negative mode 385 ([M−H]$^-$), positive mode 387 ([M+H]$^+$). Mp 185 °C. Yield 39%.

4.3.9. (4-Phenyl-2-(phenylamino)thiazol-5-yl)(*p*-toluyl)methanone (16i)

^{1}H NMR (500 MHz, DMSO-d_6) δ 2.22 (s, 3H), 6.99–7.06 (m, 3H), 7.14–7.22 (m, 3H), 7.35–7.40 (m, 6H), 7.65–7.67 (m, 2H) 10.82 (s, 1H). IR (KBr cm^{-1}); 3178, 2920, 1669, 1563, 1519, 1474, 1330, 1220, 1168, 1116, 1021, 914, 861, 787, 748, 704, 668. LC/ESI-MS negative mode 369 ([M−H]$^-$), positive mode 371 ([M+H]$^+$). Mp 198–199 °C. Yield 34%.

4.3.10. (4-(Methylsulfonyl)phenyl)(4-phenyl-2-(phenylamino)-thiazol-5-yl)methanone (16j)

^{1}H NMR (500 MHz, CDCl$_3$) δ 3.1 (s, 3H), 7.0 (m, 3H), 7.2–7.3 (m, 3H), 7.4 (m, 2H), 7.55–7.60 (m, 6H), 11.04 (s, 1H). IR (KBr cm^{-1}); 3275, 2930, 1665, 1541, 1513, 1414, 1340, 1313, 1209, 1148, 1087, 956, 813, 712, 652. LC/ESI-MS negative mode 433 ([M−H]$^-$), positive mode 435 ([M+H]$^+$). Mp 215 °C. Yield 75%.

4.3.11. *N*-(5-Benzoyl-4-phenylthiazol-2-yl)benzamide (16k)

^{1}H NMR (500 MHz, DMSO-d_6) δ 7.18–7.24 (m, 3H), 7.24–7.28 (m, 2H), 7.41–7.46 (m, 3H), 7.56–7.60 (m, 4H), 7.67 (m, 1H), 8.15 (m, 2H), 13.23 (s, 1H). ^{13}C NMR (125 MHz, DMSO-d_6) δ 125.1, 128.0, 128.3, 128.6, 128.8, 128.9, 129.3, 129.7, 131.6, 132.7, 133.2, 134.4, 137.8, 154.8, 160.7, 166.1, 189.4. IR (KBr cm^{-1}); 3168, 2939, 1668, 1532, 1468, 1330, 1286, 1177, 1072, 936, 871, 799, 694. LC/ESI-MS negative mode 383 ([M−H]$^-$), positive mode 385 ([M+H]$^+$). Mp 205 °C. Yield 48%.

4.3.12. *N*-(5-(4-Methoxybenzoyl)-4-phenylthiazol-2-yl)benzamide (16l)

^{1}H NMR (500 MHz, CDCl$_3$) δ 3.77 (s, 3H), 6.71 (m, 2H), 7.18 (m, 3H), 7.44 (m, 2H), 7.52 (t, J = 7.72, 2H), 7.62 (t,1H), 7.68 (m, 2H), 7.96 (m, 2H). ^{13}C NMR (125 MHz, CDCl$_3$) δ 55.4, 113.4, 126.3, 127.5, 128.2, 128.8, 129.1, 129.3, 130.2, 131.3, 132.1, 133.3, 134.0, 153.1, 159.6, 163.4, 164.7, 188.0. IR (KBr cm^{-1}); 3165, 2944, 1668, 1596, 1473, 1338, 1249, 1177, 1093, 1027, 904, 872, 839, 788, 757, 714, 672, 608. Elemental Anal. Calcd for $C_{24}H_{18}N_2O_3S$ (414.48): C, 69.55; H, 4.38; N, 6.76. Found: C, 69.39; H, 4.59; N, 6.93. LC/ESI-MS negative mode 413 ([M−H]$^-$), positive mode 415 ([M+H]$^+$). Mp 180–182 °C. Yield 59%.

4.3.13. *N*-(5-(4-Methylbenzoyl)-4-phenylthiazol-2-yl)benzamide (16m)

^{1}H NMR (500 MHz, CDCl$_3$) δ 2.29 (s, 3H), 7.01 (m, 2H), 7.14 (m, 3H), 7.38 (m, 2H), 7.47 (t, J = 7.72 Hz, 2H), 7.57 (m, 3H), 7.88 (d, J = 7.88 Hz, 2H). ^{13}C NMR (125 MHz, CDCl$_3$) δ 21.6, 126.2, 127.6, 128.1, 128.8, 128.9, 129.1, 129.4, 129.8, 131.1, 133.4, 133.6, 134.9, 143.6, 153.3, 160.0, 164.7, 189.0. IR (KBr cm^{-1}); 3280, 2934, 1675, 1630, 1528, 1475, 1401, 1339, 1287, 1238, 1150, 1120, 1087, 1029, 952, 900, 838, 782, 698, 669. Elemental Anal. Calcd for $C_{24}H_{18}N_2O_2S$ (398.48): C, 72.34; H, 4.55; N, 7.03. Found: C, 72.08; H, 4.76; N, 6.98. LC/ESI-MS negative mode 397 ([M−H]$^-$), positive mode 399 ([M+H]$^+$). Mp 180 °C. Yield 34%.

4.3.14. *N*-(5-(4-(Methylsulfonyl)benzoyl)-4-phenylthiazol-2-yl)benzamide (16n)

^{1}H NMR (500 MHz, CDCl$_3$) δ 3.14 (s, 3H), 7.15 (m, 3H), 7.35 (m, 2H), 7.7 (m, 7H), 8.14–8.17 (m, 2H), 13,30 (s, 1H). IR (KBr cm^{-1}); 3275, 2939, 1674, 1631, 1527, 1472, 1399, 1339, 1240, 1150, 1088, 1027, 954, 900, 837, 746, 649. LC/ESI-MS negative mode 461 ([M−H]$^-$), positive mode 463 ([M+H]$^+$). Mp 270–271 °C. Yield 29%.

4.3.15. *N*-(5-Benzoyl-4-phenylthiazol-2-yl)-4-methylbenzamide (16o)

^{1}H NMR (500 MHz, DMSO-d_6) δ 2.39 (s, 3H), 7.17–7.28 (m, 5H), 7.36–7.38 (m, 2H), 7.40–7.45 (m, 3H), 7.57–7.59 (m, 2H), 8.05–8.06 (m, 2H), 13.11 (s, 1H). IR (KBr cm^{-1}); 3333, 2925, 1669, 1624, 1576, 1537, 1474, 1446, 1337, 1299, 1237, 1198, 1166, 1086, 1073, 904, 720, 641. Elemental Anal. Calcd for $C_{24}H_{18}N_2O_2S$ (398.48): C, 72.34; H, 4.55; N, 7.03. Found: C, 72.13; H, 4.97; N, 6.98. LC/ESI-MS negative mode 397 ([M−H]$^-$), positive mode 399 ([M+H]$^+$). Mp 202–209 °C. Yield 59%.

4.3.16. *N*-(5-(4-Chlorobenzoyl)-4-phenylthiazol-2-yl)-4-methylbenzamide (16p)

^{1}H NMR (500 MHz, DMSO-d_6) δ 2.39 (s, 3H), 7.19–7.22 (m, 2H), 7.24–7.30 (m, 3H), 7.36–7.40 (m, 4H), 7.54–7.56 (m, 2H), 8.05–8.06 (m, 2H), 13.14 (s, 1H). IR (KBr cm^{-1}); 3319, 2925, 1655, 1625, 1587, 1540, 1517, 1472, 1402, 1338, 1284, 1197, 1085, 1013, 902, 838, 738, 643. Elemental Anal. Calcd $C_{24}H_{17}ClN_2O_2S$ (432.92): C, 66.58; H, 3.96; N, 6.47. Found: C, 66.23; H, 4.36; N, 6.40. LC/ESI-MS negative mode 431 ([M−H]$^-$), positive mode 433 ([M+H]$^+$). Mp 265–267 °C. Yield 59%.

4.3.17. *N*-(5-Benzoyl-4-phenylthiazol-2-yl)furan-2-carboxamide (16q)

^{1}H NMR (500 MHz, DMSO-d_6) δ 6.76 (m, 1H), 7.15–7.28 (m, 5H), 7.38–7.45 (m, 3H), 7.57 (m, 2H), 7.76 (m, 1H), 8.05 (m, 1H), 13.20 (s, 1H). ^{13}C NMR (125 MHz, DMSO-d_6) δ 112.6, 117.6, 125.1, 127.9, 128.2, 128.8, 129.2, 129.6, 132.6, 134.3, 137.8, 145.2, 148.0, 154.7, 156.4, 160.1, 189.3. IR (KBr cm^{-1}); 3294, 2934, 1668, 1616, 1530, 1465, 1380, 1332, 1296, 1175, 1098, 1030, 951, 867, 782, 723, 637. LC/ESI-MS negative mode 373 ([M−H]$^-$), positive mode 375 ([M+H]$^+$). Mp 190 °C. Yield 41%.

4.3.18. *N*-(5-(4-Chlorobenzoyl)-4-phenylthiazol-2-yl)furan-2-carboxamide (16r)

^{1}H NMR (500 MHz, DMSO-d_6) δ 6.76 (m, 1H), 7.18–7.22 (m, 2H), 7.26 (m, 1H), 7.27 (m, 2H), 7.37 (m, 2H), 7.54 (m, 2H), 7.78 (m, 1H), 8.06 (m, 1H), 13.20 (s, 1H). ^{13}C NMR (125 MHz, DMSO-d_6) δ 112.6,

117.6, 125.0, 128.0, 128.2, 128.9, 129.8, 131.0, 134.2, 136.5, 137.2, 145.2, 148.1, 155.2, 156.4, 160.4, 188.2. IR (KBr cm^{-1}); 3236, 2934, 1685, 1665, 1630, 1589, 1539, 1486, 1336, 1230, 1163, 1106, 1089, 1014, 905, 875, 762, 704, 670. LC/ESI-MS negative mode 407, 410 ($[M-H]^-$), positive mode 409, 412 ($[M+H]^+$). Mp 200 °C. Yield 41%.

4.4. Radioligand binding assays

For competition experiments [^{3}H]2-chloro-N^6-cyclopentyladenosine ([^{3}H]CCPA, 48 Ci/mmol, 1 nM), [^{3}H](*E*)-3-(3-hydroxypropyl)-8-(2-(3-methoxyphenyl)vinyl)-7-methyl-1-prop-2-ynyl-3,7-dihydropurine-2,6-dione ([^{3}H]MSX-2, 84 Ci/mmol, 1 nM), [^{3}H]8-(4-(4-chlorophenyl)piperazine-1-sulfonyl)phenyl)-1-propylxanthine ([^{3}H]PSB-603, 73 Ci/mmol, 0.3 nM), and [^{3}H]2-phenyl-8-ethyl-4-methyl-(8*R*)-4,5,7,8-tetrahydro-1*H*-imidazo[2.1-*i*]purin-5-one ([^{3}H]PSB-11, 53 Ci/mmol, 1 nM) were used as A_1, A_{2A}, A_{2B} and A_3 radioligands, respectively. For GTP shift experiments at rat adenosine A_1 receptors [^{3}H]8-cyclopentyl-1,3-dipropylxanthine ([^{3}H]DPCPX, 120 Ci/mmol, 0.4 nM) was used as a radioligand. Radioligands were commercially available or custom-labeled by GE Healthcare from appropriate precursors as described.[39] 2-Chloroadenosine (CADO), 5′-(*N*-ethylcarboxamido)adenosine (NECA), (*R*)-N^6-phenylisopropyladenosine (*R*-PIA) and GTP were obtained from Sigma. Membranes of rat brain cortex ($AdoA_1R$), rat brain striatum ($AdoA_{2A}R$) and CHO cells expressing either the human adenosine A_1, A_{2A}, A_{2B} or A_3 receptor were prepared according to described methods.[18] Binding assays at A_1, A_{2A} and A_3 receptors were carried out using 96-well-plates in a total volume of 200 μL assay buffer (50 mM TRIS–HCl, pH 7.4) containing 100 μL of membrane protein suspension and 50 μL of radioligand solution in the presence of 50 μL of various concentrations of test compound. Nonspecific binding was determined in the presence of 10 μM CADO in $AdoA_1R$ assays, 50 μM NECA in $AdoA_{2A}R$ assays, or 100 μM *R*-PIA in $AdoA_3R$ assays. The membrane preparations were preincubated for 20 min with adenosine desaminase (3 μL of a solution containing 2 U/mL per mg of protein). Incubation was carried out at rt for 90 min in $AdoA_1R$ assays, for 30 min in $AdoA_{2A}R$ assays, and for 60 min in $AdoA_3R$ assays. Incubation was terminated by rapid filtration using a Brandel 96-channel cell harvester (Brandel, Gaithersburg, MD) through Whatman GF/B glass fiber filters. Filters were rinsed three times with 2 mL each of ice-cold TRIS–HCl buffer (50 mM, pH 7.4) and incubated for 10 h with 50 μL of scintillation cocktail (UltimaGold, Microscint 20™ Perkin–Elmer) per well before radioactivity was counted in a liquid scintillation counter (TopCount NXT, Packard, Perkin–Elmer). $AdoA_{2B}R$ binding assays were carried out in a total volume of 500 μL containing 25 μL of test compound dissolved in 50% DMSO/50% TRIS–HCl buffer (50 mM, pH 7.4), 275 μL TRIS–HCl buffer (50 mM, pH 7.4), 100 μL radioligand solution, and 100 μL of membrane suspension. Nonspecific binding was determined in the presence of 10 μM DPCPX. The assay was incubated for 75 min at rt. The A_{2B} assay was filtered through GF/B glass fiber filters using a 48-channel cell harvester, and filters were washed four times with ice-cold TRIS-HCl buffer (50 mM, pH 7.4) containing 0.1% bovine serum albumin (BSA). Then filters were transferred to vials and incubated for 9 h with 2.5 mL of scintillation cocktail (Beckman Coulter). Radioactivity was counted in a liquid scintillation counter (Tricarb 2700TR) with a counting efficiency of 54%. Experiments were performed three times, each in triplicate. GTP shift experiments were performed under the same conditions as described above for $AdoA_1R$ assay except for the use of the radioligand (see above), the determination of the nonspecific binding using 10 μM DPCPX.

Data were analyzed with GraphPad Prism, Version 3.0 (GraphPAD, San Diego, CA, USA). For the calculation of K_i values by nonlinear regression analysis, the Cheng–Prusoff equation and K_D values of 0.2 nM (rat $AdoA_1R$) and 0.61 nM (human $AdoA_1R$) for [^{3}H]CCPA, 0.28 nM (rat $AdoA_1R$) for [^{3}H]DPCPX, 8 nM (rat $AdoA_{2A}R$) and 7.3 nM (human $AdoA_{2A}R$) for [^{3}H]MSX-2, 0.41 nM (human $AdoA_{2B}R$) for [^{3}H]PSB-603 and 4.9 nM (human $AdoA_3R$) for [^{3}H]PSB-11 were used.[39]

Acknowledgements

Marion Schneider is gratefully acknowledged for recording LC/MS spectra and Sabine Terhart-Krabbe for recording NMR spectra. We thank Karen Schmeling for skillful technical assistance. A. E.-T. is grateful for support by the DAAD (STIBET scholarship).

Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bmc.2010.01.072.

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