Journal of Medicinal Chemistry

Article

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Benzothiazinones: A Novel Class of Adenosine Receptor Antagonists Structurally Unrelated to Xanthine and Adenine Derivatives

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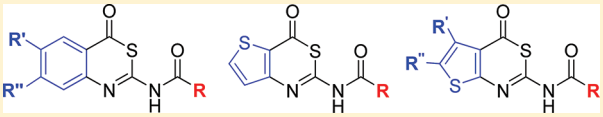
Supporting Information

ABSTRACT: 2-(Acyl)amino-4*H*-3,1-benzothiazin-4-ones and related thienothiazinones were identified as structurally novel antagonists at adenosine receptors (ARs). 6-Methyl-2-benzoylamino-4*H*-3,1-benzothiazin-4-one (**10d**) was found to be a balanced AR antagonist with affinity for all human (h) subtypes (K_i hA_1 65.6 nM; hA_{2A} 120 nM; hA_{2B} 360 nM; hA_3 30.4 nM), while in rat (r), **10d** was a highly potent A_1-selective antagonist (rA_1 7.7 nM; rA_{2A} 546 nM; rA_{2B} 679 nM, rA_3 >10000 nM). 2-(4-Methylbenzoylamino)-4*H*-3,1-benzothiazin-4-one (**10g**) was found to be a potent antagonist at human A_{2A} (68.8 nM) and A_3 ARs (23.0 nM) with high selectivity versus the other human AR subtypes. In contrast to A_1 and A_3 ARs, A_{2A} and A_{2B} ARs tolerated bulky 2-acyl substituents. *tert*-Butyl (4-oxo-4*H*-3,1-benzothiazin-2-ylcarbamoyl)benzylcarbamate (**15g**, K_i hA_{2B} 186 nM; hA_{2A} 603 nM) and 4-(4-benzylpiperazine-1-carbonyl)-*N*-(4-oxo-4*H*-3,1-benzothiazin-2-yl)benzamide (**15k**, hA_{2A} 69.5 nM; hA_{2B} 178 nM) were highly selective versus the other AR subtypes. 2-Acylamino-3,1-benzothiazin-4-ones represent novel scaffolds suitable for the development of potent and selective AR antagonists for each of the four receptor subtypes.



INTRODUCTION

Adenosine receptors (ARs) have been recognized as novel (potential) drug targets.[1,2] Four different subtypes exist, designated A_1, A_{2A}, A_{2B}, and A_3, which show a distinct expression pattern. A_1 and A_{2A} ARs are expressed in high density in certain areas of the brain, whereas A_{2B} and A_3 ARs show much lower brain expression levels.[1] While A_1 and A_3 receptors are coupled to inhibition of adenylate cyclase (AC), A_{2A} and A_{2B} receptor stimulation leads to AC activation and enhanced intracellular cAMP levels. Adenosine (**1**) itself is used for the acute treatment of supraventricular paroxysmal tachycardia, and the A_{2A}-selective agonist regadenoson (**2**) along with adenosine are applied as diagnostics for myocardial perfusion imaging (Figure 1).[2] Especially, AR antagonists are promising new drug candidates for a number of indications, including heart and renal failure (A_1), Parkinson's disease (PD), Alzheimer's disease and depression (A_{2A}), asthma and chronic obstructive pulmonary disease (A_{2B}, A_3), and glaucoma (A_3).[1,2] The xanthine derivatives caffeine (**3**) and theophylline (**4**) are the prototypic nonselective AR antagonists blocking A_1, A_{2A}, and A_{2B} receptors and thereby mediating central and cardiac stimulatory, diuretic, and antiasthmatic effects.[3] Subtype-selective ligands have been developed by modifying the xanthine substitution pattern.[3−10] Another class of AR antagonists are structurally related to adenine (**5**), the nucleobase of the physiological agonist **1**.[1,2,5,11−14] Many mono-, bi-, and tricyclic heteroaromatic structures bearing an exocyclic amino group like adenine have been found to possess AR-antagonistic activity.[1,2,11,13−15] An example is the pyrazolotriazolopyrimidine preladenant (**6**), an A_{2A} antagonist which is currently undergoing phase III clinical trials for the therapy of PD.[16,17] However, a major drawback of xanthine derivatives as well as adenine-related AR antagonists is their mostly very low water solubility.[18,19] Only recently, novel structures have been discovered by high-throughput screening of large compound libraries.[11,14] One prominent example is the benzothiazole SYN-115 (**7**), an A_{2A} AR antagonist that was developed from a screening hit.[14,20]

In the search for nonxanthine-derived, structurally non-adenine-related AR antagonists, it was the aim of this study to evaluate 2-(acyl)amino-4*H*-3,1-benzothiazin-4-ones, such as compounds **9a−b** and **10a−i** (Scheme 1), as possible candidates. Representatives of this heterocyclic class are assumed to possess biological activities because they might provide their heteroatoms as potential hydrogen bond acceptors and the fused phenyl ring for possible $\pi-\pi$ interactions. Adenosine receptor affinity of 4*H*-3,1-benzothiazin-4-ones has not been reported so far. Analogous 4*H*-3,1-benzoxazin-4-ones with a ring oxygen in place of sulfur are hydrolytically less stable.[21] 2-Amino-4*H*-3,1-benzoxazin-4-ones are inhibitors of human leukocyte elastase,[22] cathepsin G,[23] chymase,[24] C1r serine protease of the complement system,[25] and human cytomegalovirus protease.[26] 4*H*-3,1-Benzothiazin-4-ones and heterocyclic-fused analogues exhibit anticancer, antiviral, and antiproliferative activities.[27,28] It is noteworthy that, among the isomeric 4*H*-1,3-benzothiazin-4-ones, extremely effective antimycobacterial nitro derivatives have been explored and their target enzyme decaprenylphosphoryl-β-D-ribose 2′-epimerase has been identified.[29] Herein, we report on

Received: January 9, 2012
Published: March 12, 2012

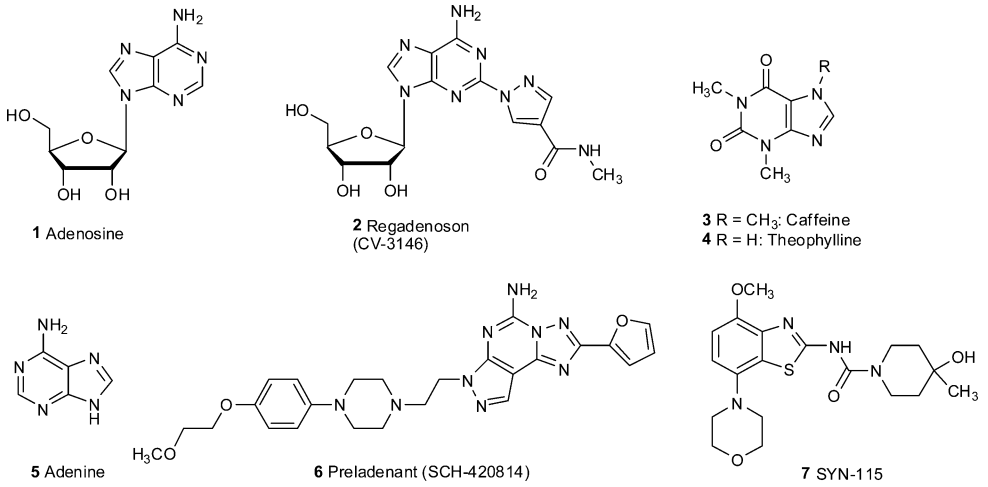
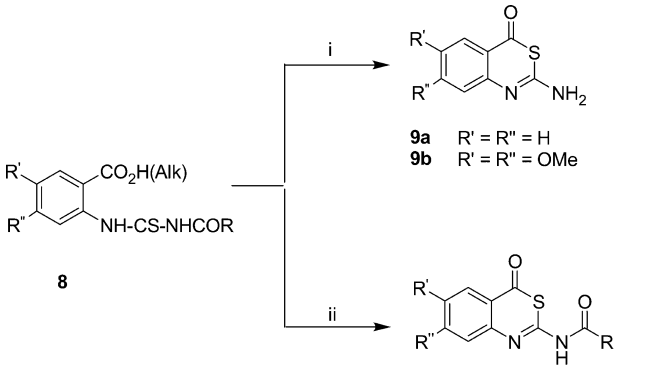


Figure 1. Structures of selected adenosine receptor ligands.

Scheme 1. Synthesis of 2-Aminobenzothiazinones 9a–b and 2-Acylaminobenzothiazinones 10a–i[a]



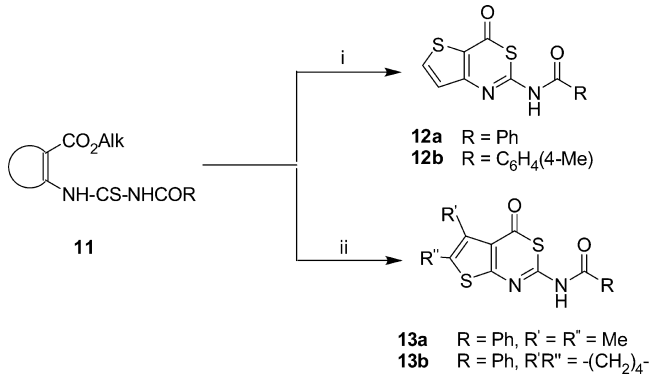
[a]Reagents and conditions: (i) (1) concd H_2SO_4, 100 °C, (2) $NaHCO_3$ (**9a** from 2-(3-benzoylthioureido)benzoic acid) or (1) concd H_2SO_4, RT, (2) $NaHCO_3$ (**9b** from methyl 2-(3-acetylthioureido)-4,5-dimethoxybenzoate; (ii) concd H_2SO_4, −8 °C–RT (**10a** and **10b** from methyl 2-(3-acetylthioureido)benzoates) or concd H_2SO_4, RT (**10c–i** from 2-(3-benzoylthioureido)benzoic acids).

the synthesis of a series of benzothiazinones and thieno analogues bearing an acylamino substitutent at position 2 as well as their thieno analogues. These heterocycles were investigated as novel ligands of the adenosine receptor subtypes A_1, A_{2A}, A_{2B}, and A_3.

RESULTS AND DISCUSSION

Chemistry. The preparation of the known 4*H*-3,1-benzothiazin-4-ones **9a–b** and **10a–g** and the products **10h–i** in the course of a cyclocondensation reaction is shown in Scheme 1.[30] The required thioureides **8** were obtained from anthranilic acids or esters and in situ generated acyl isothiocyanates. Sulfuric acid-promoted cyclization could be achieved with loss (**9a** and **9b**) or retention of the acyl residue (**10a–i**), depending on the reaction conditions. In compounds **12a–b** and **13a–b**, the fused benzene ring is exchanged for thiophene (Scheme 2). This bioisosteric replacement gave rise

Scheme 2. Synthesis of 2-Acylaminothienothiazinones 12a–b and 13a–b[a]



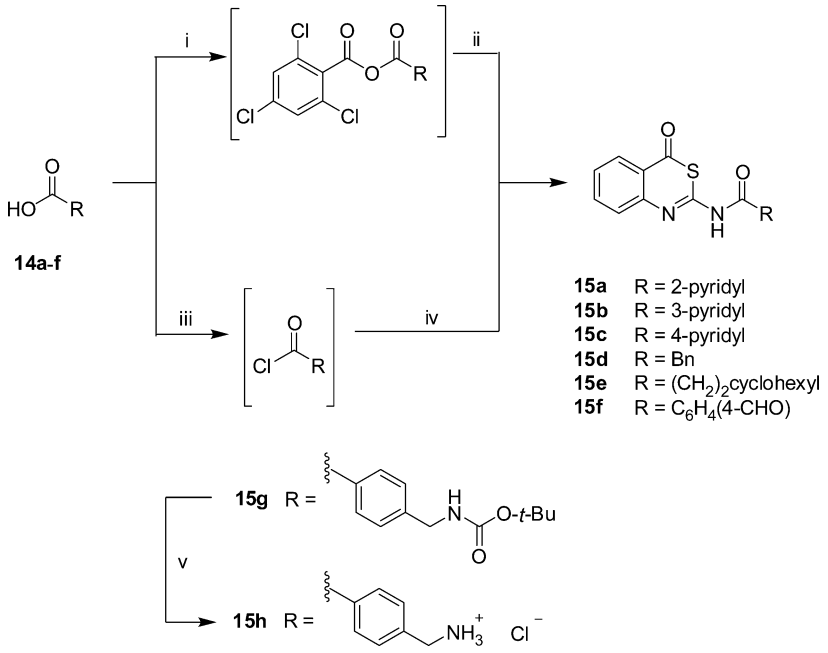
[a]Reagents and conditions: (i) methyl 2-(3-aroylthioureido)-2-thiophenecarboxylates, concd H_2SO_4, RT; (ii) from ethyl 2-(3-benzoylthioureido)-3-thiophenecarboxylates, (1) concd H_2SO_4, 90 °C, (2) benzoic anhydride, toluene, reflux.

to compounds with similar molecular geometry and, due to the electron-rich nature of thiophene, a somewhat enhanced electron density of the thiazinone fragment. Whereas the known thieno[3,2-*d*]-fused derivatives **12a** and **12b**[31] possess an unsubstituted fused thiophene ring to allow for a direct comparison with the benzothiazinones **10c** and **10g**, the [2,3-*d*]-fused compounds **13a** and **13b** bear two methyl substituents or a cycloaliphatic bridge. For the latter compounds, the formation of the NH_2-substituted thiazinones followed by rebenzoylation provided the products in better yields than the direct formation from the open-chain precursors.

The NH_2-substituted compound **9a** with preformed benzothiazinone skeleton was subjected to several acylation reactions (Scheme 3) in order to introduce further structural diversity into the acyl portion of the products. Amidations were either carried out with mixed anhydrides, obtained from the corresponding carboxylic acid and the Yamaguchi reagent (2,4,6-trichlorobenzoyl chloride),[32] or acyl chlorides, generated by the treatment of the carboxylic acid with oxalyl chloride.

dx.doi.org/10.1021/jm300029s | *J. Med. Chem.* 2012, 55, 3331–3341

Scheme 3. Synthesis of 2-Acylaminobenzothiazinones 15a–h[a]



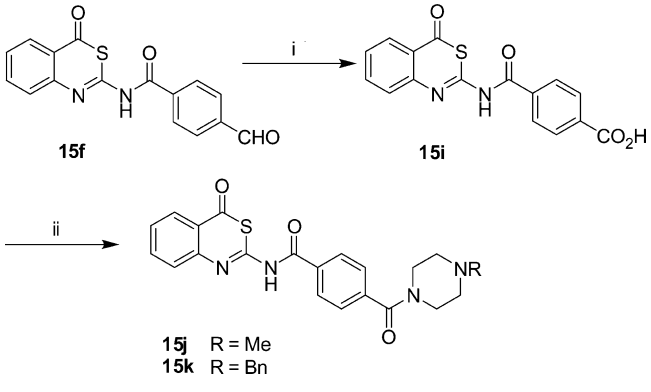
[a]Reagents and conditions: (i) (1) *N*-methylmorpholine, CH_2Cl_2, RT, (2) 2,4,6-trichlorobenzoyl chloride, RT; (ii) **9a**, pyridine, toluene, reflux (for **15a–b, 15f, 15g**); (iii) $(COCl)_2$, DMF (cat.), CH_2Cl_2, RT; (iv) **9a**, pyridine, CH_2Cl_2, reflux (for **15c–e**); (v) HCl, ethyl acetate, RT.

To synthesize **15g**, 4-(aminomethyl)benzoic acid had to be Boc-protected,[33] then coupled to the mixed anhydride and finally reacted with **9a**. Acidic carbamate deprotection of **15g** yielded the basic benzothiazinone **15h**. The following attempts have been made to attach further ionizable groups to the 2-substituent as well as to the fused benzene ring of 2-acylamino-4*H*-3,1-benzothiazin-4-ones.

Aldehyde **15f** was subjected to an oxidation reaction with oxone[34] to obtain the carboxylic acid **15i** without affecting the heterocyclic portion of the molecule (Scheme 4). Carbodiimidazole-promoted coupling of **15i** with *N*-methyl- or *N*-benzylpiperazine afforded the basic carboxamides **15j** and **15k**, respectively. These reactions were performed in DMF to overcome limitations due to the poor solubility of the starting compound. Moreover, we used an imidazole buffer system to prevent strong basic conditions and thus a possible Dimroth rearrangement of the benzothiazinone ring system.[21,30] The conditions for the preparation of **15j** and **15k** were then successfully applied to further amidation reactions in the course of this study.

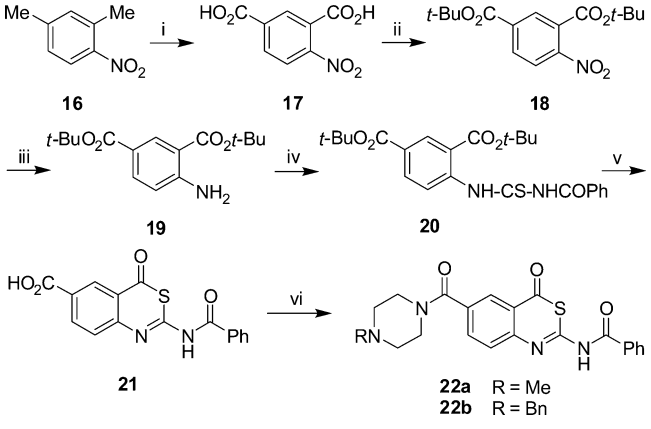
Next, we conceived a synthetic access to 2-benzoylamino-4*H*-3,1-benzothiazin-4-ones with a carboxyl group in position 6 (or 7) and, in turn, to basic amides of these carboxylic acids to increase polarity and water solubility of the compounds (Scheme 5). Oxidation of 4-nitro-*m*-xylene (**16**) with

Scheme 4. Synthesis of 2-Acylaminobenzothiazinones 15i–k[a]



[a]Reagents and conditions: (i) oxone, DMF, RT; (ii) (1) 1,1′-carbonyldiimidazole, DMF, RT, (2) *N*-methylpiperazine or *N*-benzylpiperazine (1.5 equiv), imidazole (1 equiv), HCl (2 equiv), DMF, RT.

Scheme 5. Synthesis of 2-Acylaminobenzothiazinones 21 and 22a–b[a]



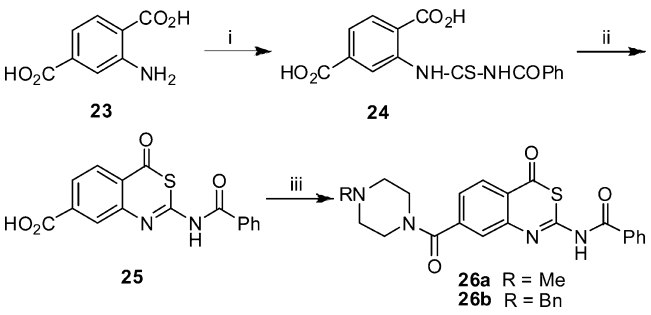
[a]Reagents and conditions: (i) $KMnO_4$, H_2O, reflux; (ii) $(COCl)_2$, DMF (cat), *tert*-BuOH, pyridine, $ZnCl_2$ (cat), RT; (iii) H_2, 10% Pd/C, EtOH, RT; (iv) benzoyl isothiocyanate, acetonitrile, RT; (v) concd H_2SO_4, RT; (vi) (1) 1,1′-carbonyldiimidazole, DMF, RT, (2) *N*-methylpiperazine or *N*-benzylpiperazine (1.5 equiv), imidazole (1 equiv), HCl (2 equiv), DMF, RT.

dx.doi.org/10.1021/jm300029s | J. Med. Chem. 2012, 55, 3331–3341

potassium permanganate was performed in boiling water whereby the oxidant was added twice to improve the yield of 4-nitroisophthalic acid (**17**).[35,36] Compound **17** was transformed via its diacyl chloride to the corresponding bis(*tert*-butyl) ester **18**, followed by catalytic hydrogenation to bis(*tert*-butyl) 4-aminoisophthalate (**19**). The subsequent reaction with in situ generated benzoyl isothiocyanate in acetonitrile afforded bis(*tert*-butyl) 4-(3-benzoylthioureido)isophthalate (**20**). The cyclization step, at which the concomitant deprotection occurred, was performed with concentrated sulfuric acid to produce the benzothiazinone-6-carboxylic acid **21**. Conversion with *N*-methyl- and *N*-benzylpiperazine gave the basic carboxamides **22a** and **22b**, respectively.

The preparation of the 7-carboxylic acid **25** and its amides **26a** and **26b** (Scheme 6) followed a similar route as for the 6-

Scheme 6. Synthesis of 2-Acylaminobenzothiazinones 25 and 26a–b[a]



[a]Reagents and conditions: (i) benzoyl isothiocyanate, acetone, RT; (ii) concd H_2SO_4, RT; (iii) (1) 1,1′-carbonyldiimidazole, DMF, RT, (2) *N*-methylpiperazine or *N*-benzylpiperazine (1.5 equiv), imidazole (1 equiv), HCl (2 equiv), DMF, RT.

functionalized derivatives. Thus, 2-aminoterephthalic acid (**23**) was suspended in acetone, and benzoyl isothiocyanate was added. Before the precipitation of the product started, some undissolved starting compound was removed by filtration to obtain 2-(3-benzoylthioureido)terephthalic acid (**24**), which could be used in the next step without further purification. Cyclocondensation afforded the benzothiazinone **25**, which was subsequently converted to the basic derivatives **26a** and **26b**.

Biological Evaluation. The new compounds were initially investigated in radioligand binding studies at rat brain adenosine A_1 and A_{2A} receptors and at human recombinant A_{2B} and A_3 receptors using cell membrane preparations.[37] Selected compounds were additionally investigated at human recombinant A_1 and A_{2A} receptors and at recombinant rat A_{2B} and A_3 receptors in order to obtain information on the affinities and selectivities of the most interesting compounds in both species, rat and human. [^{3}H]2-Chloro-N^6-cyclopentyladenosine (CCPA) was used as A_1-selective radioligand, [^{3}H]3-(3-hydroxypropyl)-7-methyl-8-(*m*-methoxystyryl)-1-propargylxanthine (MSX-2) for A_{2A} radioligand binding assays, and [^{3}H]8-(4-(4-(4-chlorophenyl)piperazine-1-sulfonyl)phenyl)-1-propylxanthine (PSB-603) as A_{2B} radioligand. For binding assays at human A_3 receptors, the A_3-selective antagonist radioligand [^{3}H]2-phenyl-8-ethyl-4-methyl-(8*R*)-4,5,7,8-tetrahydro-1*H*-imidazo[2,1-*i*]purin-5-one (PSB-11) was applied. Because [^{3}H]PSB-11 is not suitable for the labeling of rat A_3 receptors, the agonist radioligand [^{3}H]NECA was used for binding studies at rat A_3 receptors. Screening was usually performed at 10 and 1 μM, however, in A_{2B} radioligand binding studies, the highest concentration of test compounds was only 1 μM because higher concentrations often led to precipitation of the radioligand [^{3}H]PSB-603, which possesses only moderate solubility. Selected potent compounds of the present series were further investigated for their functional properties, in GTP shift assays at membrane preparations of cells recombinantly expressing human A_1 receptors, or in cAMP accumulation studies using living CHO cells recombinantly expressing human A_{2B} receptors, respectively. Results are presented in Table 1, and Figures 2 and 3. Data of standard ligands are included for comparison.

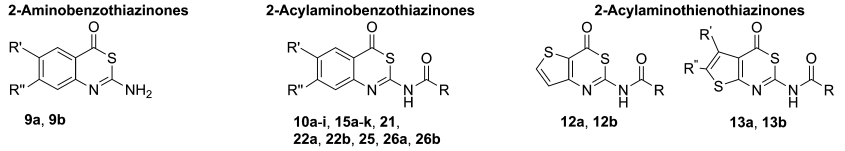
Structure–Activity Relationships. The basic structure 2-amino-4*H*-3,1-benzothiazin-4-one (**9a**) showed moderate affinity at rat A_1 and A_{2A} receptors, with K_i values in the low micromolar range and virtually no affinity for human A_{2B} and A_3 ARs. However, its A_1 and A_{2A} affinity was considerably higher than that of the nonselective standard antagonist caffeine (see Table 1). Therefore, we decided to have a closer look at the structure–activity relationships (SARs) of this new scaffold in the field of AR ligands. 6,7-Disubstitution of the benzene ring with methoxy groups (**9b**) improved affinity for A_1 ARs by 4-fold, resulting in a submicromolar K_i of 590 nM, and it also improved affinity for A_3 ARs, while no change was observed at both A_2 AR subtypes.

Next, we investigated the acylation of the exocyclic 2-amino group. Acetylation reduced affinity for the A_1 AR without much change at A_{2A} and A_{2B} ARs (compound **10a**, see Table 1). In contrast, affinity for the human A_3 AR was dramatically increased by >50-fold, resulting in a potent and A_3-selective antagonist with a K_i value of 193 nM. At rat A_3 ARs, however, **10a** was completely inactive. The same was observed for all other thiazinone derivatives of the present series which were investigated at rat A_3 ARs. Such large species differences are well-known for the A_3 ARs, and most of the known A_3 antagonists only bind to human but not to rodent A_3 ARs.[38,39] For the other AR subtypes, species differences are typically much lower due to high sequence homologies of the orthologues. 6,7-Disubstitution with methoxy groups (**10b**) led to a 5-fold reduction in A_3 affinity, while A_1 affinity was increased. Comparison of the SAR trends of **9a**/**9b** and **10a**/**10b** shows that the substituents on the 2-position and those on the 6- and 7-position are interdependent and not parallel.

The breakthrough with regard to the A_1 AR was achieved by acylation of the 2-amino function with a benzoyl group. Compound **10c** showed a K_i value of 25 nM at the rat A_1 AR and was 24-fold less potent at rat A_{2A}, 62-fold less potent at rat A_{2B}, and inactive at rat A_3 receptors. Thus, the compound showed high A_1 selectivity in rat. However, **10c** showed considerable species differences, especially at A_1 (affinity: rat > human) and A_3 ARs (human > rat), and therefore the situation at the human receptors was quite different (K_i values: 309 nM (hA_1), 25.0 nM (rA_1); 91.7 nM (hA_{2A}), 609 nM (rA_{2A}); 950 nM (hA_{2B}), 1560 nM (rA_{2B}), 86 nM (hA_3), >10000 nM (rA_3)). Thus, **10c** is nonselective in humans with highest affinities at A_{2A} and A_3 ARs. Bioisosteric replacement of the benzoyl group by a 2-, 3-, or 4-pyridinoyl residue (**15a**, **15b**, **15c**) led to much weaker or inactive compounds. The 3-pyridinoyl group (**15b**) was best tolerated, resulting in a moderately potent A_1/A_3-antagonist while the isomeric compounds **15a** and **15c** were inactive. To probe the size of the binding pocket for the N^2-acyl group, phenyl (in **10c**) was replaced by a benzyl (**15d**) or a 2-cyclohexylethyl residue (**15e**). However, both modifications led to a reduction in affinity compared to **10c**.

dx.doi.org/10.1021/jm300029s | *J. Med. Chem.* 2012, 55, 3331–3341

Table 1. Adenosine Receptor Affinities of Benzothiazinone and Thienothiazinone Derivatives and Standard Antagonists



9a, 10a,c,g,i, 15a-k R' = R'' = H
9b, 10b,f R' = R'' = OMe
10d,h R' = Me, R'' = H
10e R' = Cl, R'' = H

21 R' = CO_2H, R'' = H
22a R' = 4-methylpiperazine-1-carbonyl, R'' = H
22b R' = 4-benzylpiperazine-1-carbonyl, R'' = H
25 R' = H, R'' = CO_2H

26a R' = H, R'' = 4-methylpiperazine-1-carbonyl
26b R' = H, R'' = 4-benzylpiperazine-1-carbonyl
13a R' = R'' = Me
13b R'R'' = -$(CH_2)_4$-

		$K_i \pm$ SEM (nM)[a] human (h); rat (r)			
compd	R	A_1 vs [^{3}H]CCPA	A_{2A} vs [^{3}H]MSX-2	A_{2B} vs [^{3}H]PSB-603	A_3 vs [^{3}H]PSB-11 (h) or vs [^{3}H]NECA (r)
caffeine (**3**)		44900 (h) 41000 (r)[2,b]	23400 (h) 32500 (r)	33800 (h) 30000 (r)[2,b]	13300 (h) >100000 (r)
theophylline (**4**)		8570 (h) 14000 (r)	3680 (h) 22000 (r)	74000 (h)[2,b] 15100 (r)[2,b]	22300 (h)[2,b] 85000 (r)[2,b]
preladenant (**6**)		>1000 (h)[2,b]	0.9 (h)[2,b]	>1000 (h)[2,b]	>1000 (h)[2,b]
2-Aminobenzothiazinones					
9a		2391 ± 308 (r)	1580 ± 409 (r)	>1000 (h) (9%)[c]	>10000 (h) (33%)[c]
9b		590 ± 40 (r)	2800 ± 670 (r)	>1000 (h) (7%)[c]	2190 ± 590 (h)
2-Acylaminobenzothiazinones					
10a	Me	>10000 (h) (26%)[c] >10000 (r) (26%)[c]	>10000 (h) (0%)[c] 2390 ± 350 (r)	>1000 (h) (11%)[c]	193 ± 30 (h) >10000 (r) (18%)[c]
10b	Me	2660 ± 1160 (r)	≥10000 (r) (42%)[c]	>1000 (h) (9%)[c]	964 ± 356 (h)
10c	Ph	309 ± 17 (h) 25.0 ± 5.0 (r)	91.7 ± 16.9 (h) 609 ± 61 (r)	950 ± 150 (h) 1560 ± 260 (r)	86.0 ± 17.0 (h) >10000 (r) (3%)[c]
10d	Ph	65.6 ± 14.0 (h) 7.70 ± 3.72 (r)	120 ± 51 (h) 546 ± 119 (r)	360 ± 100 (h) 679 ± 36 (r)	30.4 ± 6.8 (h) >10000 (r) (11%)[c]
10e	Ph	>10000 (h) (17%)[c] 19.1 ± 5.3 (r)	>10000 (h) (9%)[c] ≥10000 (r) (42%)[c]	>1000 (h) (0%)[c]	≥10000 (h) (46%)[c]
10f	Ph	233 ± 54 (r)	>10000 (r) (40%)[c]	>1000 (h) (0%)[c]	>10000 (r) (0%)[c] 128 ± 23 (h)
10g	C_6H_4(4-Me)	≥10000 (h) (43%)[c] 68.7 ± 13.2 (r)	68.8 ± 6.8 (h) 656 ± 114 (r)	>1000 (h) (35%)[c]	29.0 ± 1.2 (h)
10h	C_6H_4(4-Me)	17.0 ± 0.5 (r)	1297 ± 405 (r)	≥1000 (h) (48%)[c]	69.1 ± 6.1 (h)
10i	C_6H_4(4-CO_2Me)	>10000 (r) (33%)[c]	>10000 (r) (7%)[c]	>1000 (h) (17%)[c]	503 ± 218 (h)
15a	2-pyridyl	>10000 (r) (12%)[c]	>10000 (r) (0%)[c]	>1000 (h) (7%)[c]	≥10000 (h) (38%)[c]
15b	3-pyridyl	622 ± 7 (r)	>10000 (r) (30%)[c]	>1000 (h) (8%)[c]	268 ± 55 (h)
15c	4-pyridyl	>10000 (r) (33%)[c]	>10000 (r) (0%)[c]	>1000 (h) (15%)[c]	≥10000 (h) (45%)[c]
15d	Bn	>10000 (r) (23%)[c]	1260 ± 170 (r)	>1000 (h) (40%)[c]	558 ± 157 (h)
15e	$(CH_2)_2$cyclohexyl	>10000 (r) (10%)[c]	>10000 (r) (20%)[c]	>1000 (h) (0%)[c]	>10000 (h) (30%)[c]
15f	C_6H_4(4-CHO)	415 ± 86 (r)	>10000 (r) (27%)[c]	>1000 (h) (25%)[c]	422 ± 103 (h)
15g	C_6H_4-(4-CH_2NHCO_2t-Bu)	>10000 (h) (16%)[c] >10000 (r) (32%)[c]	603 ± 190 (h) >10000 (r) (30%)	186 ± 9 (h)	>10000 (h) (38%)[c]
15h	C_6H_4(4-CH_2NH_2) × HCl	563 ± 25 (r)	2550 ± 680 (r)	>1000 (h) (0%)[c]	8480 ± 1240 (h)
15i	C_6H_4(4-CO_2H)	555 ± 52 (r)	≥10000 (r) (48%)[c]	>1000 (h) (5%)[c]	2620 ± 141 (h)
15j	4-(4-methyl-piperazine-1-carbonyl)phenyl	892 ± 149 (r)	2750 ± 280 (r)	>1000 (h) (24%)[c]	4560 ± 1620 (h)
15k	4-(4-benzyl-piperazine-1-carbonyl)phenyl	>10000 (h) (27%)[c] >1000 (r) (13%)[c]	69.5 ± 14.1 (h) 285 ± 38 (r)	178 ± 41 (h)	>1000 (h) (21%)[c]
21	Ph	>10000 (r) (5%)[c]	>10000 (r) (7%)[c]	>1000 (h) (5%)[c]	>10000 (h) (33%)[c]
22a	Ph	>10000 (r) (18%)[c]	>10000 (r) (20%)[c]	>1000 (h) (2%)[c]	6160 ± 970 (h)
22b	Ph	>10000 (r) (16%)[c]	>10000 (r) (23%)[c]	>1000 (h) (8%)[c]	≥10000 (h) (42%)[c]
25	Ph	>10000 (r) (19%)[c]	>10000 (r) (8%)[c]	>1000 (h) (1%)[c]	>10000 (h) (13%)[c]
26a	Ph	>10000 (r) (8%)[c]	>10000 (r) (12%)[c]	>1000 (h) (0%)[c]	>10000 (h) (8%)[c]
26b	Ph	>10000 (r) (13%)[c]	>10000 (r) (23%)[c]	>1000 (h) (11%)[c]	3210 ± 770 (h)
2-Acylaminothienothiazinones					
12a	Ph	42.7 ± 9.9 (r)	396 ± 60 (r)	≥1000 (h) (45%)[c]	200 ± 9 (h)
12b	C_6H_4(4-Me)	176 ± 17 (h) 50.0 ± 17.0 (r)	53.7 ± 10.4 (h) 299 ± 31 (r)	400 ± 44 (h)	56.2 ± 18.3 (h)
13a	Ph	>10000 (h) (18%)[c] 107 ± 35 (r)	286 ± 125 (h) 234 ± 65 (r)	>1000 (h) (9%)[c]	55.7 ± 15.2 (h) >10000 (r) (0%)[c]
13b	Ph	>10000 (r) (16%)[c]	>10000 (r) (10%)[c]	>1000 (h) (0%)[c]	406 ± 170 (h)

[a] $n \geq 3$ unless otherwise noted. [b] Literature data (obtained with different radioligand). [c] Percent inhibition of radioligand binding at indicated concentration.

dx.doi.org/10.1021/jm300029s | J. Med. Chem. 2012, 55, 3331−3341

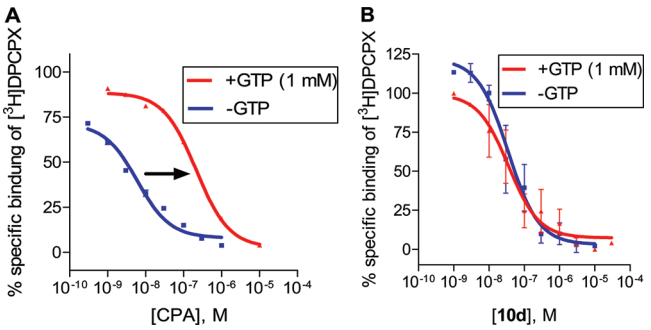


Figure 2. GTP shift assay at A_1 receptors determined in radioligand binding assays at rat brain cortical membrane preparations using the antagonist radioligand [^{3}H]DPCPX. (A) Binding curve for the full A_1 agonist N^6-cyclopentyladenosine (CPA); IC_{50} in the absence of GTP, 6.31 nM; in the presence of 1 mM GTP, 200 nM (32-fold shift). (B) Binding curve of the potent A_1 antagonist **10d** in the absence (IC_{50}, 35.1 nM) and in the presence of 1 mM GTP (IC_{50}, 35.7 nM); no significant GTP shift was observed.

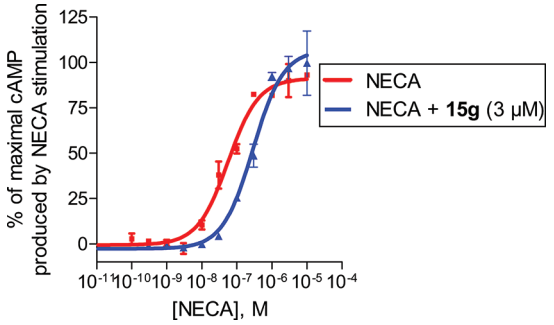


Figure 3. Agonist- (NECA-) dependent increase in cAMP accumulation in the absence and in the presence of **15g** (3 μM) in CHO cells recombinantly expressing human A_{2B} receptors. The agonist curve was shifted to right in a parallel fashion by the antagonist. A K_b value of 1238 ± 291 nM (n = 3) was calculated.

Because a 2-benzoylamino substitution (**10c**) had provided by far the best results at all AR subtypes so far, we decided to keep **10c** as the lead structure and to further study the SARs of the 2-benzoylaminothiazinone derivatives. First, we focused on effects of substituents in the 6- and/or 7-position of the benzothiazinone core structure of **10c**. The introduction of a methyl group in the 6-position yielded the most potent A_1 and A_3 antagonist of the present series (**10d**). Affinity for the human A_{2A} and A_{2B} ARs was also improved. 2-Benzoylamino-6-methyl-4*H*-3,1-benzothiazin-4-one (**10d**) is a very potent (K_i = 7.70 nM) and highly selective antagonist for rat A_1 ARs. However, its affinity is lower at human A_1 ARs (K_i = 65.6 nM). In contrast, it is relatively potent at human A_{2A} (K_i = 120 nM) and human A_3 receptors (K_i = 30.4 nM), indicating species differences not only at A_3 but also at A_1 ARs and, to a lower extent, at A_{2A} ARs for this class of compounds. In contrast to rat, compound **10d** is therefore not selective in humans. The corresponding 6-chloro-substituted derivative **10e** was slightly less potent at rat A_1 ARs than the 6-methyl analogue **10d** with a K_i value of 19.1 nM at rat A_1, and it was highly A_1 selective. Surprisingly, **10e** did not bind to human A_1 ARs. Such extreme species differences at the A_1 ARs are unprecedented. To exclude potential degradation of the compound, we prepared a fresh stock solution, confirmed the purity by LCMS (see Supporting Information) and repeated the experiments, which could be confirmed. A carboxylate function in the 6-position (compound **21**) or in the 7-position (compound **25**), respectively, was introduced to increase polarity but was not tolerated by the ARs. As an alternative strategy to improve water solubility, *N*-methyl- and *N*-benzyl- piperazine-1-carbonyl residues were introduced in the 6-position (**22a**, **22b**) or in the 7-position (**26a**, **26b**). But these relatively large substituents were not well tolerated in both positions by the ARs. 6,7-Disubstitution with the smaller methoxy residues (compound **10f**) was better tolerated by the rat A_1 and the human A_3 AR subtypes but was less potent than the parent compound **10c**.

As a next step, we investigated substitution in the *para*-position of the 2-benzoylamino residue in lead compound **10c**. A methyl residue (**10g**) had only a moderate effect on the AR affinities, slightly increasing affinity for the human A_3 AR (K_i = 29.0 nM). As a matter of fact, **10g** was the most potent A_3 antagonist of the present series. The compound showed high selectivity for the human A_3 versus the other human AR subtypes. Introducing into **10g** an additional methyl group at position 6 of the benzothiazinone ring system as in the very potent A_1 and A_3 antagonist **10d** led to compound **10h**. This dimethyl substitution was not superior to the corresponding monomethyl derivatives **10d** and **10g**. An aldehyde function in the *para*-position of the benzoylamino residue (**15f**) reduced affinity in comparison with a methyl substituent, and further oxidation to a carboxylate function (compound **15i**) led to a further reduction in affinity especially at the human A_3 AR. The corresponding methyl ester **10i** was again somewhat more potent at the human A_3AR, indicating that the A_3AR did not tolerate a negatively charged residue in that position, but **10i** was inactive at the other AR subtypes.

In a further series of compounds, larger substituents, including basic moieties, were introduced in the *para*-position of the benzoylamino residue of lead compound **10c**. The large *tert*-butyloxycarbonyl-aminomethyl substitution (compound **15g**) was only tolerated by human A_{2B} (K_i 186 nM) and to a lesser extent by human A_{2A} receptors (K_i 603 nM). Thus **15g** showed high selectivity versus the other AR subtypes. It is interesting to note that the A_{2A} and perhaps even more so the A_{2B} AR appear to have place to accommodate large substituents in the binding pocket for the 2-acylamino substituent. The aminomethyl- (**15h**) and the 1-(4-methylpiperazinyl)- (**15j**) substituted derivatives, containing basic structures with improved water solubility at physiologic pH value of 7.4, were much less potent at A_1, A_{2A}, and A_{2B} ARs in comparison with the lead structure **10c** and only weakly potent at A_3 ARs. The larger 1-(4-benzylpiperazinyl) derivative **15k**, which was inactive at A_1 and A_3 ARs, however, showed clearly higher affinity for A_{2A} and A_{2B} ARs than the lead structure **10c**. The affinity for both A_2 AR subtypes was similar (K_i = 69.5 nM at human A_{2A}, K_i = 285 nM at rat A_{2A}, K_i = 178 nM at human A_{2B}). This result confirms the finding that both A_{2A} and A_{2B} ARs appear to accommodate large substituents in the region where the acylamino residue binds.

Finally, we modified the benzothiazinone ring structure of lead compound **10c** by bioisosterically replacing the benzene by a thiophene ring (compounds **12a–b** and **13a–b**). The thieno[3,2-*d*][1,3]thiazin-4-one derivative **12a** was not superior to the corresponding benzothiazinone derivative **10c**. The 2-(*p*-methylbenzoylamino) derivative **12b** showed quite similar affinities as its benzothiazinone analogue **10g** at all AR subtypes, but **12b** tended to be somewhat more potent at the

dx.doi.org/10.1021/jm300029s | J. Med. Chem. 2012, 55, 3331–3341

human receptors than the corresponding benzothiazinone **10g**. The isomeric thieno[2,3-*d*][1,3]thiazin-4-one derivatives **13a** and **13b** bear additional substituents on the thiophene ring. While the dimethyl-substituted thienothiazinone **13a** was comparable in AR affinities to the thieno[3,2-*d*][1,3]thiazin-4-ones **12a** and **12b**, the tricyclic compound **13b** was inactive at A_1, A_{2A}, and A_3 ARs and showed only low affinity for the A_3AR.

Most AR ligands published in the literature have only been investigated in one species, that is nowadays, due to the availability of recombinant receptors, in most cases the human species. However, initial in vivo studies are typically performed in rodents, either rats or mice. It has been known for a long time that large species differences exist for A_3 antagonists between human and rodent receptors, most antagonists that are potent at human A_3AR being inactive or only weakly active at rat A_3 ARs.[1] In the present study we observed the same species differences for the new benzothiazinone scaffold. All investigated compounds showed a large preference for the human over the rat A_3 AR. Surprisingly large species differences were also observed for the A_1 AR but with the opposite preference: all benzo- and thienothiazinones investigated in both species showed a preference for the rat receptor, and in some cases the difference in affinity between the two species was >100-fold (**10g**) or even >500-fold (**10e**). In contrast, species differences at A_{2A} and A_{2B} receptors were generally smaller, and the compounds appeared to show a (mostly moderate) preference for the human receptors. Our results clearly show that AR antagonists should not only be tested at human but also at rodent receptors before they may be used as pharmacological tools in animal models.

Functional Characterization of Selected Ligands. The most potent A_1 AR antagonist identified in the series of 2-acylaminobenzothiazinones was 6-methyl-2-benzoylamino-4*H*-3,1-benzothiazin-4-one (**10d**). Therefore, we selected **10d** for investigating whether it functions as an antagonist (as expected) or as an agonist. Most AR agonists identified so far bear a ribose residue like the physiological agonist adenosine (**1**), which is lacking in the benzothiazinones. Binding of agonists is affected by the addition of GTP, which shifts the curve to the right.[40] For these experiments, the antagonist radioligand [^{3}H]DPCPX was employed. The magnitude of the shift correlates with the efficacy, full agonists showing larger shifts than partial agonists, while the binding curves of antagonists are not affected by GTP. Figure 2 shows that the full agonist N^6-cyclopentyladenosine (CPA) exhibits a large (32-fold) GTP shift, while the binding curve of **10d** remains the same in the presence as in the absence of GTP, clearly indicating that the benzothiazinone derivative is an antagonist at A_1 AR receptors.

In addition, we selected one of the most potent and selective A_{2B} antagonists identified in the present study, benzothiazinone **15g**, for functional investigations in cAMP accumulation studies at human A_{2B} ARs stably expressed in CHO cells. **15g** behaved like a competitive antagonist shifting the concentration–response curve of the agonist NECA in a parallel manner to the right. A K_b value of 1238 nM was determined (see Figure 3). Thus, the K_b value was 6.6-fold higher than the K_i value determined in radioligand binding studies at membranes preparations of the same cells. The reason for this discrepancy is unknown because normally a high correlation between K_i and K_b values at human A_{2B} receptors has been observed.[41]

CONCLUSIONS

In conclusion, we discovered a structurally novel class of AR antagonists, the 2-(acyl)amino-3,1-benzothiazin-4-ones and related thienothiazinones. A series of 33 derivatives was synthesized, and SARs were investigated in radioligand binding studies at all four AR subtypes. In addition, species differences were studied for the most potent compounds at human as compared to rat ARs. Potent antagonists were obtained for each of the four AR subtypes. Large species differences between human and rat receptors were observed, not only for the A_3 but also for the A_1 receptor subtype, indicating that one should be extremely cautious in extrapolating from one species to the other. Benzothiazinones generally showed a large preference for human over rat A_3, while the opposite preference (rat > human) was observed at the A_1 AR. One of the most potent compounds was **10d**, a balanced AR antagonist with good affinity for all human AR subtypes; in rat, however, **10d** proved to be a highly potent A_1-selective antagonist. Compound **10g** was found to be a potent antagonist at human A_{2A} and A_3 ARs with high selectivity versus the other human AR subtypes. In contrast to the A_1 and the A_3 receptor subtypes, the A_{2A} and even more so the A_{2B} receptor tolerated large, bulky 2-acyl substituents: **15g** showed a K_i value of 186 nM at hA_{2B}, 603 nM at hA_{2A} AR, and was highly selective versus the other AR subtyes. Similarly, **15k** was a potent and highly selective A_{2A}/A_{2B} antagonist (hA_{2A} 69.5 nM; hA_{2B} 178 nM). The chemical stability of some of the compounds was investigated by LCMS, and they were found to be stable at room temperature. Thus, 2-acylamino-3,1-benzothiazin-4-ones represent novel scaffolds suitable for the development of potent and selective AR antagonists for each of the four receptor subtypes.

EXPERIMENTAL SECTION

Chemistry. Melting points were determined on a Büchi 510 melting point apparatus and are uncorrected. Thin layer chromatography was performed on Merck aluminum sheets. Preparative column chromatography was performed on silica gel 60 (Acros Organics) 0.060–0.200 mm. ^{1}H and ^{13}C NMR spectra were acquired on a Bruker Avance DRX 500 spectrometer operating at 500 MHz for ^{1}H and 125 MHz for ^{13}C or on a Varian Gemini 2 instrument operating at 300 MHz for ^{1}H and 75 MHz for ^{13}C. IR spectra were recorded on a Bruker Tensor 27 FT-IR spectrometer. HRMS (ESI) spectra were recorded on a micrOTOF-Q (Bruker Daltonics) spectrometer. Elemental analyses were carried out with a Vario EL apparatus. Compounds **9a**, **10c**, **10d**, **10e**,[30] **9b**, **10b**,[42] **10a**, **10f**,[21] **10g**, **12a**, and **12b**[31] were prepared as described. Synthetic procedures and analytical data for compounds **10i**, **13b**, **15a–c**, **15e**, **15f**, **15i**, **15k**, **21**, **22b**, **25**, and **26b** are listed in the Supporting Information. Purity of the products was confirmed by TLC, elemental analysis or HRMS, and NMR spectroscopy. All tested compounds possessed a purity of not less than 95%.

*6-Methyl-2-[(4-methylbenzoyl)amino]-4H-3,1-benzothiazin-4-one (**10h**).* 2-[3-(4-Methylbenzoyl)thioureido]-5-methylbenzoic acid (984 mg, 3 mmol), prepared from 5-methylanthranilic acid and 4-methylbenzoyl isothiocyanate,[43] was dissolved in concd H_2SO_4 (3 mL) and kept at RT for 2 days. The mixture was poured into ice–water (200 mL). The precipitate was collected by filtration, extensively washed with H_2O, and dried to afford **10h** as a colorless powder (410 mg, 44%), mp 209–211 °C (EtOH). ^{1}H NMR (500 MHz, [D_6]DMSO): δ = 2.38 (s, 3H), 2.43 (s, 3H), 7.33 (d, J = 8.2 Hz, 2H), 7.57 (d, J = 8.3 Hz, 1H), 7.70 (dd, J = 8.3, 2.0 Hz, 1H), 7.85 (br s, 1H), 7.96 (d, J = 8.2 Hz, 2H), 12.12 (br s1H). ^{13}C NMR (125 MHz, [D_6]DMSO): 20.7, 21.2, 119.3, 123.9, 128.7, 129.2, 130.0, 130.5, 137.1, 137.5, 143.4, 146.0, 153.1, 167.2, 184.8. IR (KBr): ν = 1680, 1630 cm^{-1} (C=O). Anal. Calcd for $C_{17}H_{14}N_2O_2S$: C 65.79, H, 4.55, N 9.03. Found: C 65.75, H 4.62, N 9.30.

dx.doi.org/10.1021/jm300029s | J. Med. Chem. 2012, 55, 3331–3341

*N-(5,6-Dimethyl-4-oxo-4H-thieno[2,3-d][1,3]thiazin-2-yl)-benzamide (**13a**).* Ethyl 4,5-dimethyl-2-(3-benzoylthioureido)-3-thiophenecarboxylate (3.26 g, 10 mmol) was reacted with concd H_2SO_4 to obtain 2-amino-5,6-dimethyl-4*H*-thieno[2,3-*d*[1,3]thiazin-4-one (1.40 g, 66%),[44] mp 263−264 °C. A mixture of 2-amino-5,6-dimethyl-4*H*-thieno[2,3-*d*[1,3]thiazin-4-one (637 mg, 3 mmol), benzoic anhydride (1.36 g, 6 mmol), and toluene (25 mL) was refluxed for 2 h and kept at RT overnight. The precipitate was collected by suction filtration and washed with ethyl acetate to obtain **13a** as a brownish solid (460 mg, 49%), mp 248−250 °C (ethyl acetate). ^{1}H NMR (500 MHz, [D_6]DMSO): δ = 2.36 (s, 6H), 7.51−7.54 (m, 2H), 7.62−7.66 (m, 1H), 8.02−8.04 (m, 2H), 12. 40 (s, 1H). ^{13}C NMR (125 MHz, [D_6]DMSO) δ = 12.4, 13.7, 119.4, 128.6, 128.7, 128.7, 128.8, 132.0, 133.2, 157.7, 163.1, 166.9, 177.6. IR (KBr): ν = 1670, 1625 cm^{-1} (C═O). Anal. Calcd for $C_{15}H_{12}N_2O_2S_2$: C 56.94, H 3.82, N 8.85. Found: C, 57.04, H, 3.90, N 8.83.

*N-(4-Oxo-4H-3,1-benzothiazin-2-yl)-2-phenylacetamide (**15d**).* Oxalyl chloride (1.27 g, 10 mmol) and two drops of DMF were added to a solution of phenylacetic acid (1.36 g, 10 mmol) in CH_2Cl_2 (20 mL). After 2 h, the solvent was removed under reduced pressure, and the residue was dissolved in DMF (12 mL). Pyridine (1.58 g, 20 mmol) and compound **9a** (1.39 g, 7.8 mmol) were added and stirred at RT for 3 h. The mixture was poured into ice-cold 0.5 N HCl (200 mL), and the precipitate was collected by filtration. The crude product was recrystallized from EtOH to afford **15d** as a white solid (840 mg, 36%), mp 189−192 °C. ^{1}H NMR (500 MHz, [D_6]DMSO): δ = 3.77 (s, 2H), 7.24−7.28 (m, 1H), 7.31−7.34 (m, 4H), 7.51 (ddd, *J* = 8.0, 7.1, 1.3 Hz, 1H), 7.60 (dd, *J* = 8.2, 1.3 Hz, 1H), 7.87 (ddd, *J* = 8.4, 6.9, 1.6 Hz, 1H), 8.03 (dd, *J* = 7.9 1.6 Hz, 1H), 12.10 (s, 1H). ^{13}C NMR (125 MHz, [D_6]DMSO) δ = 42.3, 119.6, 124.5, 127.0, 127.3, 128.5, 129.0, 129.5, 134.6, 136.5, 147.7, 153.1, 171.7, 184.5. IR (KBr): ν = 1701, 1653 cm^{-1} (C═O). Anal. Calcd for $C_{16}H_{12}N_2O_2S$: C 64.85, H 4.08, N 9.45. Found: C, 65.00, H, 4.07, N 9.08.

*tert-Butyl (4-Oxo-4H-3,1-benzothiazin-2-ylcarbamoyl)-benzylcarbamate (**15g**).* To a solution of 4-(aminomethyl)benzoic acid (3.02 g, 20 mmol) in THF (60 mL), 1 M NaOH (22 mL) and di-*tert*-butyldicarbonate (4.80 g, 22 mmol) were added. The mixture was stirred at RT for 12 h, the organic solvent was removed under reduced pressure, the residue was diluted with water (50 mL), and 0.1 M $NaHSO_4$ was added to obtain pH 2−3. It was extracted with ethyl acetate (3 × 50 mL), dried, and evaporated to obtain 4-[(*tert*-butoxycarbonyl)aminomethyl]benzoic acid (4.52 g, 90%),[33] mp 167−168 °C. *N*-Methylmorpholine (469 mg, 4 mmol) was added to a mixture of 4-[(*tert*-butoxycarbonylamino)methyl]benzoic acid (1.0 g, 4 mmol) and CH_2Cl_2 (20 mL). After stirring for 10 min, 2,4,6-trichlorobenzoyl chloride (976 mg, 4 mmol) was added and the mixture was stirred for additional 2 h. The solvent was removed under reduced pressure, and the residue was taken up in toluene (40 mL). Compound **9a** (267 mg, 1.5 mmol) were added, the mixture was refluxed for 4 h, and the hot mixture was filtered. The filtrate was kept overnight. The precipitate was collected by suction filtration, washed with diethyl ether, and recrystallized from EtOH to afford **15g** as a colorless solid (340 g, 55%), mp 162−164 °C. ^{1}H NMR (500 MHz, [D_6]DMSO): δ = 1.40 (s, 9H), 4.20 (d, *J* = 6.0 Hz, 2H), 7.37 (d, *J* = 8.2 Hz, 2H), 7.46 (t, *J* = 5.7 Hz, 1H), 7.50−7.54 (m, 1H), 7.66 (d, *J* = 8.2 Hz, 1H), 7.86−7.90 (m, 1H), 8.00 (d, *J* = 8.2 Hz, 2H), 8.05 (d, *J* = 7.6 Hz, 1H), 12.23 (s, 1H). ^{13}C NMR (125 MHz, [D_6]DMSO) δ = 28.4, 43.4, 78.1, 119.6, 124.6, 126.9, 127.3, 128.8, 129.1, 130.5, 136.5, 145.8, 147.7, 153,9, 160.0, 176.1, 184.8. IR (KBr): ν = 1684, 1635 cm^{-1} (C═O). Anal. Calcd for $C_{21}H_{21}N_3O_4S$: C 61.30, H 5.14, N 10.21. Found: C, 61.40, H 5.32, N 9.83.

*4-Aminomethyl-N-(4-oxo-4H-3,1-benzothiazin-2-yl)benzamide hydrochloride (**15h**).* Compound **15g** (200 mg, 0.49 mmol) was suspended in ethyl acetate (10 mL), and a freshly prepared 4 M solution of HCl in ethyl acetate (15 mL) was added. The mixture was stirred for 45 min. The product was collected by suction filtration and washed with small amounts of diethyl ether and ethanol to afford **15h** as a colorless solid (160 mg, 94%), mp 238−240 °C. ^{1}H NMR (500 MHz, [D_6]DMSO): δ = 4.10 (q, *J* = 5.7 Hz, 2H), 7.53 (ddd, *J* = 7.6, 7.6, 1.3 Hz, 1H), 7.65−7.69 (m, 3H), 7.89 (ddd, *J* = 8.4, 7.1, 1.6 Hz, 1H), 8.06 (m, 3H), 8.69 (s, 3H). ^{13}C NMR (125 MHz, [D_6]DMSO) δ = 41.9, 119.6, 124.6, 127.3, 128.5, 128.9, 129.0, 132.2, 136.6, 139.3, 147.1, 154.1, 167.5, 184.8. IR (KBr): ν = 1696, 1675 cm^{-1} (C═O). HRMS-ESI *m*/*z* [*M* + H]$^+$ calcd for $C_{16}H_{13}N_3O_2S$, 312.0801; found, 312.0795.

*4-(4-Methylpiperazine-1-carbonyl)-N-(4-oxo-4H-3,1-benzothiazin-2-yl)benzamide (**15j**).* 1,1′-Carbonyldiimidazole (357 mg, 2.2 mmol) was added to a mixture of compound **15i** (653 mg, 2 mmol) and DMF (11 mL) and stirred at RT 1 h. To a solution of *N*-methylpiperazine (301 mg, 3 mmol), imidazole (136 mg, 2 mmol), DMF (2 mL), and 1,4-dioxane (2 mL), 1 mL of a 4 N solution of HCl in 1,4-dioxane was added dropwise. The two mixtures were combined, kept at RT for 2 h, and poured into satd aqueous $NaHCO_3$ solution (180 mL). The precipitate was collected by suction filtration, dried, and recrystallized from EtOH to afford **15j** as a yellow powder (110 mg, 13%), mp 245−246 °C. ^{1}H NMR (500 MHz, [D_6]DMSO): δ = 2.21 (s, 3H), 2.30 (br s, 2H), 2.38 (br s, 2H), 3.29 (br s, 2H), 3.63 (br s, 2H), 7.51 (d, *J* = 8.5 Hz, 2H), 7.53 (ddd, *J* = 7.9, 7.4, 1.0 Hz, 1H), 7.67 (dd, *J* = 8.2, 1.0 Hz, 1H), 7.89 (ddd, *J* = 8.3, 6.6, 1.6 Hz, 1H), 8.06 (dd, *J* = 8.0, 1.3 Hz, 1H), 8.10 (d, *J* = 8.5 Hz, 2H), 12.35 (s, 1H). ^{13}C NMR (125 MHz, [D_6]DMSO) δ = 41.5, 45.6, 47.0, 54.3, 54.7, 119.6, 124.6, 127.0, 127.2, 128.3, 128.9, 133.4, 136.5, 140.2, 147.1, 154.7, 167.6, 168.1, 184.8. IR (KBr): ν = 1684, 1653, 1635 cm^{-1} (C═O). HRMS-ESI *m*/*z* [*M* + H]$^+$ calcd for $C_{21}H_{20}N_4O_3S$, 409.1329; found, 409.1303.

*N-(6-(4-Methylpiperazine-1-carbonyl)-4-oxo-4H-3,1-benzothiazin-2-yl)benzamide (**22a**).* 1,1′-Carbonyldiimidazole (4 mg, 1.6 mmol) was added to a mixture of compound **21** (489 mg, 1.5 mmol), DMF (4 mL), and 1,4-dioxane (2 mL) and stirred at RT 1 h. To a solution of *N*-methylpiperazine (301 mg, 3 mmol), imidazole (136 mg, 2 mmol), DMF (2 mL), and 1,4-dioxane (2 mL), 1 mL of a 4 N solution of HCl in 1,4-dioxane was added dropwise. The two mixtures were combined, kept at RT for 2 h, and poured into satd aqueous $NaHCO_3$ solution (150 mL). The precipitate was collected by suction filtration, dried, and recrystallized from EtOH to afford **22a** as a colorless powder (240 mg, 39%), mp 264−265 °C. ^{1}H NMR (500 MHz, [D_6]DMSO): δ = 2.22 (s, 3H), 2.35 (br s, 4H), 3.58 (br s, 4H), 7.52−7.56 (m, 2H), 7.65 (t, *J* = 7.3 Hz, 1H), 7.70 (d, *J* = 8.2 Hz, 1H), 7.87 (dd, *J* = 8.2, 1.9 Hz, 1H), 8.00 (d, *J* = 1.9 Hz, 1H), 8.06 (dd, *J* = 7.6, 1.3 Hz, 2H), 12.33 (br s, 1H). ^{13}C NMR (125 MHz, [D_6]DMSO) δ = 45.6, 54.4, 66.5, 119.2, 123.2, 127.8, 128.6, 128.8, 132.4, 133.2, 134.0, 134.8, 148.0, 155.7, 167.5, 168.1, 184.7. IR (KBr): ν = 1681, 1651, 1623 cm^{-1} (C═O). HRMS-ESI *m*/*z* [*M* + H]$^+$ calcd for $C_{21}H_{20}N_4O_3S$, 409.1329; found, 409.1327.

*N-(7-(4-Methylpiperazine-1-carbonyl)-4-oxo-4H-3,1-benzothiazin-2-yl)benzamide (**26a**).* 1,1′-Carbonyldiimidazole (195 mg, 1.2 mmol) was added to a mixture of compound **25** (326 mg, 1 mmol), DMF (4 mL), and 1,4-dioxane (2 mL) and stirred at RT 1 h. To a solution of *N*-methylpiperazine (200 mg, 2 mmol), imidazole (136 mg, 2 mmol), DMF (2 mL), and 1,4-dioxane (2 mL), 1 mL of a 4 N solution of HCl in 1,4-dioxane was added dropwise. The two mixtures were combined, kept at RT for 2 h, and poured into satd aqueous $NaHCO_3$ solution (150 mL). The precipitate was collected by suction filtration, dried, and recrystallized from EtOH to afford **26a** as a colorless powder (200 mg, 49%), mp 212−214 °C. ^{1}H NMR (500 MHz, [D_6]DMSO): δ = 2.21 (s, 3H), 2.29 (br s, 2H), 2.41 (br s, 2H), 3.32 (br s, 2H), 3.65 (br s, 2H), 7.47 (dd, *J* = 8.2, 1.6 Hz, 1H), 7.51−7.54 (m, 2H), 7.55 (t, *J* = 1.6 Hz, 1H), 7.62−7.66 (m, 1H), 8.05 (dd, *J* = 8.4, 1.3 Hz, 2H), 8.09 (d, *J* = 8.2 Hz, 1H), 12.33 (br s, 1H). ^{13}C NMR (125 MHz, [D_6]DMSO) δ = 41.5, 45.6, 47.0, 54.2, 54.7, 119.7, 125.2, 125.2, 126.3, 128.6, 128.7, 132.4, 133.1, 143.4, 147.4, 155.4, 167.3, 168.0, 184.6. IR (KBr): ν = 1689, 1653 (br) cm^{-1} (C═O). Anal. Calcd for $C_{21}H_{20}N_4O_3S$: C 61.75, H 4.94, N 13.72. Found: C 61.24, H, 4.83, 13.40.

Biological Assays. *Radioligands.* Radioligands were obtained from the following sources: [^{3}H]CCPA from Amersham (58 Ci/mmol), [^{3}H]MSX-2 from Amersham (84 Ci/mmol), [^{3}H]PSB-603 from Amersham (73 Ci/mmol), [^{3}H]PSB-11 (53 Ci/mmol) from Quotient Biosearch, and [^{3}H]NECA (15.5 Ci/mmol) from Perkin-Elmer. The nonradioactive precursors

of [^{3}H]MSX-2,[45] [^{3}H]PSB-603,[4] and [3H]PSB-11[46] were synthesized in our laboratory.

Membrane Preparations. Membranes from Chinese hamster ovary (CHO) cells stably transfected with the human A_1, human A_{2A}, human A_{2B}, rat A_{2B}, and human A_3 AR were prepared as described.[4,46,47] For assays at rat A_3 ARs, commercially available membrane preparations containing the rat A_3 AR expressed in human embryonic kidney (HEK) cells were obtained from Biotrend (Cologne, Germany). Frozen rat brains obtained from Pel Freez, Rogers, Arkansas, USA, were dissected to obtain cortical membrane preparations for A_1 assays, and striatal membrane preparations for A_{2A} assays as described.[45,48,49,50]

Radioligand Binding Assays. Stock solutions of the compounds were prepared in dimethyl sulfoxide (DMSO); the final concentration of DMSO was 2.5%. The radioligand concentrations were: [^{3}H]-CCPA,[48] 0.5 nM (rat and human A_1); [^{3}H]MSX-2,[45] 1.0 nM (rat and human A_{2A}); [3H]PSB-603,[4] 0.3 nM (rat and human A_{2B}); [3H]PSB-11,[46] 0.5 nM (human A_3), [^{3}H]NECA, 10 nM (rat A_3). Binding assays were performed as described.[9,40,45,46,48] About 30−70 μg/mL of protein were used in the assays. At least three separate experiments were performed, each in duplicate or triplicate.

GTP Shift Assays. Inhibition of binding of [^{3}H]DPCPX to rat brain cortical membranes was measured in the presence and absence of 1 mM GTP according to a previously described method.[40] Unlabeled DPCPX (10 μM) was used to determine nonspecific binding. The assays were carried out under the same conditions as described for the A_1 AR competition assay.

cAMP Accumulation Assays. Assays were performed using CHO cells permanently transfected with the human A_{2B} AR and a radioactive filtration assay as described.[41]

Data Analysis. Data were analyzed using GRAPH PAD PRISM Version 4 (San Diego, CA, USA). For the calculation of K_i values by nonlinear regression analysis, the Cheng−Prusoff equation and K_D values of 0.5 nM (rat A_1), 0.61 nM (human A_1) for [^{3}H]CCPA, 8 nM for [^{3}H]MSX-2 (human and rat), 0.41 nM for [^{3}H]PSB-603 (human A_{2B}), 0.2 nM (rat A_{2B}), and 4.9 nM for [^{3}H]PSB-11 (human A_3) were used.

■ ASSOCIATED CONTENT

Supporting Information

Synthesis of additional products, analytical data, selected concentration−response curves, and results from stability studies (LCMS measurements). This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

The authors declare no competing financial interest.

■ ACKNOWLEDGMENTS

We thank Nicole Florin, Angelika Fischer, and Dieter Baumert for skilfull technical assistance. C.E.M. was funded by the German Federal Ministery for Education and Research (BMBF 01EW0911) in the frame of ERA-NET NEURON.

■ ABBREVIATIONS USED

AR, adenosine receptor; CCPA, [^{3}H]2-chloro-N^6-cyclopentyladenosine; CHO, Chinese hamster ovary; CPA, N^6-cyclopentyladenosine; DPCPX, 8-cyclopentyl-1,3-dipropylxanthine; h, human; MSX-2, [^{3}H]3-(3-hydroxypropyl)-7-methyl-8-(*m*-methoxystyryl)-1-propargylxanthine; NECA, 5′-*N*-ethylcarboxamidoadenosine; r, rat

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