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4-Substituted-7-*N*-alkyl-*N*-acetyl 2-aminobenzothiazole amides: Drug-like and non-xanthine based A_{2B} adenosine receptor antagonists

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ABSTRACT

7-*N*-Acetamide-4-methoxy-2-aminobenzothiazole 4-fluorobenzamide (compound **1**) was chosen as a drug-like and non-xanthine based starting point for the discovery of A_{2B} receptor antagonists because of its slight selectivity against A_1 and A_{2A} receptors and modest A_{2B} potency. SAR exploration of compound **1** described herein included modifications to the 7-*N*-acetamide group, substitution of the 4-methoxy group by halogens as well as replacement of the *p*-flouro-benzamide side chain. This work culminated in the identification of compound **37** with excellent A_{2B} potency, modest selectivity versus A_{2A} and A_1 receptors, and good rodent PK properties.

Adenosine is an autocoid produced in many tissues, which mediates various functions through four G-protein coupled receptors (GPCRs), namely A_1, A_{2A}, A_{2B}, and A_3. The A_1 and A_3 receptors are coupled to G_i and G_o proteins, respectively, while the A_{2A} and A_{2B} receptors are coupled to G_s proteins.[1] Due to these differences in receptor function, adenosine signals an increase in intracellular cAMP levels via its action through the A_{2A} and A_{2B} receptors, and a decrease in cAMP levels through the A_1 and A_3 receptors. In addition, adenosine increases intracellular calcium ion levels via the A_{2B} receptor through its coupling to G_q proteins. Adenosine's agonist potency at its four receptors individually expressed in Chinese Hamster Ovary (CHO) cells was determined to be, A_3 (EC_{50} = 0.29 μM) ~ A_1 (EC_{50} = 0.31 μM) > A_{2A} (EC_{50} = 0.7 μM) ≫ A_{2B} (EC_{50} = 24 μM).[2] Based on this relative order of adenosine agonist potency, it is believed that the A_{2B} receptor remains silent under normal physiological conditions and is activated as a consequence of elevated extracellular adenosine levels during chronic, high oxidative stress conditions, such as hyperglycemia and mast-cell activation. For example, in an asthmatic lung exposed to an allergen, the increased levels of adenosine signal through the A_{2B} receptor which mediates its proinflammatory effects.[3,4] CVT-6883 (Fig. 1), a potent and selective A_{2B} receptor antagonist was found to be efficacious in various animal models of asthma, COPD and pulmonary fibrosis.[5–7] Researchers at Eisai, using specific agonists and antagonists of adenosine receptors, illustrated the key role of A_{2B} receptor antagonism in inhibiting hepatic glucose production.[8] Based on the SAR of 2-alkynyl-8-aryl-9-methyladenines, they identified a series of A_{2B} receptor antagonists that were efficacious in lowering fasting and fed glucose levels in KK-Ay mice,[9] a well recognized model of type 2 diabetes. Thus, the potential utility of adenosine A_{2B} receptor antagonists for the treatment of asthma and type 2 diabetes encouraged us to seek novel antagonists.

The majority of potent A_{2B} receptor antagonists reported to date are based on xanthine or adenine core structures.[10] In our search for drug-like, non-xanthine based A_{2B} receptor antagonists, we utilized a series of previously disclosed A_{2A} receptor antagonists, 4-methoxy-7-aryl (**I**) and 4-methoxy-7-morpholino (**II**) 2-aminobenzothiazoles as the starting point.[11] During exploratory studies with 4-methoxy-7-substituted 2-aminobenzothiazoles, we systematically modified the 7-substituent to determine whether A_{2B}-selectivity could be achieved within the series but disappointingly found that almost all the analogs bearing aromatic, heteroaromatic and heterocyclic 7-substituents were potent A_{2A}-selective antagonists (data not shown). We then narrowed our focus on 7-*N*-acetyl analog **1** which is unique in possessing slight A_{2B}-selectivity over A_1 and A_{2A} receptors (Table 1). With the goal of improving the A_{2B} potency and selectivity of **1**, we discuss in this communication the results of our SAR studies which included modifications to the 7-*N*-acetamide group, substitution of the 4-methoxy group by halogens as well as replacement of the *p*-flouro-benzamide side chain (Fig. 1). This work culminated in the identification of compound **37**, which incorporates excellent A_{2B} potency, modest selectivity versus A_{2A} and A_1 receptors, and good rodent PK properties.

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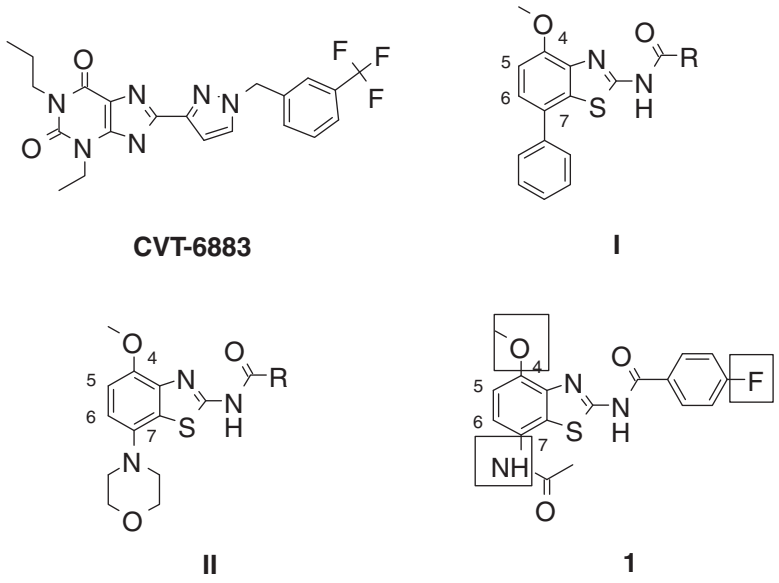
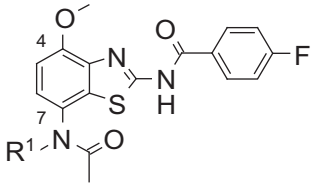


Figure 1. Structures of CVT-6883, compounds **I-II** and **1**.

Table 1
Functional and binding activities of various 7-N-alkylated 7-*N*-acetamide-4-methoxy-2-aminobenzothiazole 4-fluorobenzamides (compounds **1**, **6**–**19**)



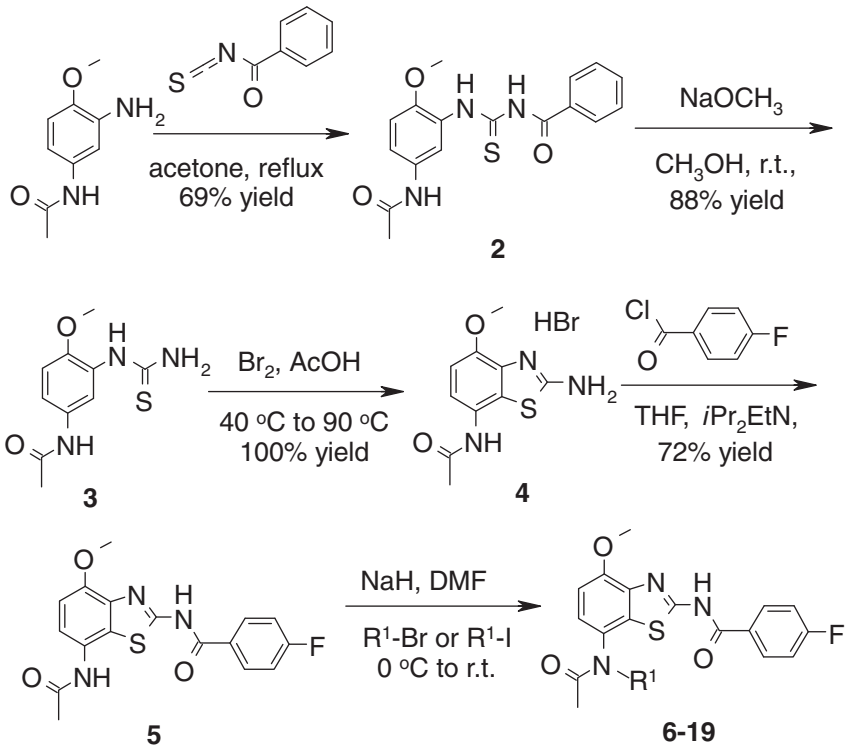
Compound	R^1 =	A_{2B} cAMP (IC_{50}, nM)	A_1 Binding[a] (K_i, nM)	A_{2A} Binding[b] (K_i, nM)	A_{2B} Binding[c] (K_i, nM)
1	H (7-*N*-acetyl)	350	1600	250	130
6	CH_3	15	66	39	22
7	CH_2CH_3	12	66	22	13
8	$CH_2CH(CH_3)_2$	23	37	3	NT[d]
9	$CH(CH_3)_2$	19	NT[d]	NT[d]	NT[d]
10	CH_2CF_3	40	42	35	12
11	CHF_2	230	30	61	NT[d]
12	CH_2CH_2OH	77	450	98	NT[d]
13	$CH_2CH_2CH_2OH$	42	100	29	NT[d]
14	$CH_2CH_2OCH_3$	33	110	38	NT[d]
15	$CH_2CO_2CH_3$	8	23	5	NT[d]
16	CH_2CO_2H	6000	2600	2200	NT[d]
17	CH_2CONH_2	60	560	160	NT[d]
18	$CH_2CONHCH_3$	350	790	330	NT[d]
19	$CH_2CON(CH_3)_2$	600	1600	480	NT[d]

[a] Binding affinity for the A_1 receptor was determined by competition for binding sites labeled by ^{3}H-DPCPX (4.8 nM) in commercial (Euroscreen) A_1 membranes.
[b] Binding affinity for the A_{2A} receptor was determined by competition for binding sites labeled by ^{3}H-ZM241385 (5 nM) in commercial (Perkin–Elmer) A_{2A} membranes.
[c] Binding affinity for the A_{2B} receptor was determined by competition for binding sites labeled by ^{3}H-ZM241385 (30 nM) in whole cells (CHO) expressing A_{2B} receptors.
[d] NT: not tested.

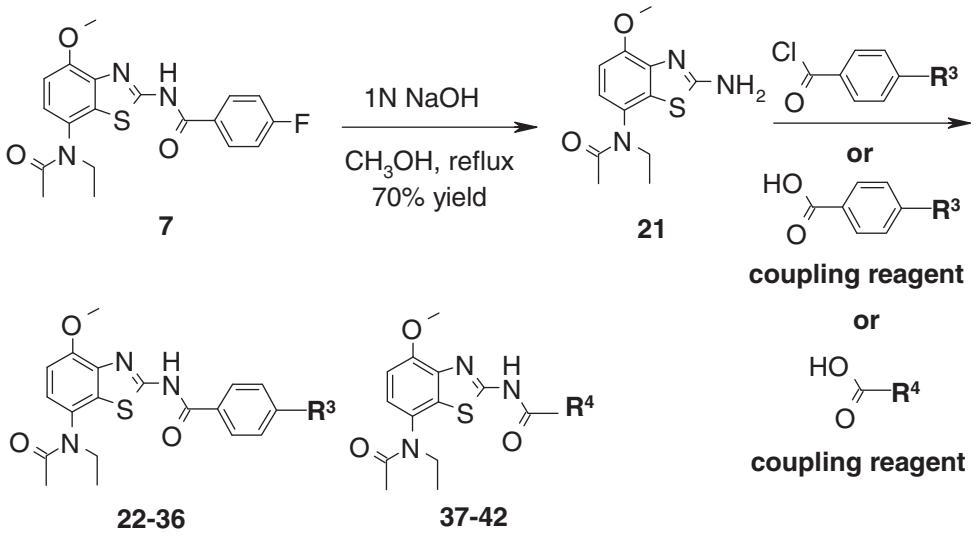
Various 7-N-alkylated analogs (compounds **6**–**19**) were prepared according to Scheme 1. Coupling of commercially available *N*-(3-amino-4-methoxy-phenyl)-acetamide with benzoyl isothiocyanate[12] gave benzoylated thiourea **2**. De-benzoylation of compound **2** was achieved using sodium methoxide in methanol[13] and the resulting thiourea **3** was cyclized with bromine in acetic acid[14] to give 4-methoxy-7-*N*-acetamide 2-aminobenzothiazole (**4**). The first three steps in the synthetic scheme could be carried out on a multi-gram scale and did not require chromatography. The *p*-fluorobenzoyl group was coupled to the 2-aminobenzothiazole core using the corresponding acid chloride to give compound **5**. Treatment of compound **5** with 2.5 equiv of sodium hydride in *N*,*N*-dimethylformamide generated the corresponding dianion and the 7-*N*-acetamide anion, being more nucleophilic than the 2-aminobenzothiazole benzamide anion, was preferentially alkylated when treated with 1 equiv of alkylating agent at 0 °C to give compounds **6**–**19**.

The corresponding 4-Cl analog (compound **20**) of compound **7** was made in an analogous manner to that shown in Scheme 1 starting with commercially available *N*-(3-amino-4-chloro-phenyl)-acetamide.

Various aryl and heteroaryl amides of 7-*N*-ethyl-acetamide-4-methoxy-2-aminobenzothiazole (compounds **22**–**42**) were prepared according to Scheme 2. Treatment of compound **7** with 1 N sodium hydroxide/methanol (1:1) under reflux for 2–3 days gave *N*-7-(acetyl-ethyl-amino)-4-methoxy-2-aminobenzothiazole (**21**).



Scheme 1. Synthesis of various 7-N-alkylated 7-*N*-acetamide-4-methoxy-2-aminobenzothiazole 4-fluorobenzamides (compounds **6**–**19**).



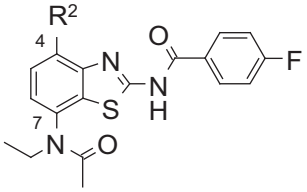
Scheme 2. Synthesis of various amides of 7-*N*-ethyl-acetamide-4-methoxy-2-aminobenzothiazole (compounds **22**–**42**).

Various amides (compounds **22**–**42**) were synthesized by reacting compound **21** with either the corresponding acid chloride or the corresponding carboxylic acid and an amide coupling reagent (selected from 1,1′-carbonyldiimidazole, benzotriazole-1-yl-oxy-tris-(dimethylamino)-phosphonium hexafluorophosphate or 1-methylimidazole/*p*-toluenesulfonyl chloride[15]).

All the new analogs prepared were tested in the A_{2B} cAMP assay[16] and the most interesting analogs were further tested in A_1, A_{2A}, and A_{2B} receptor binding assays.[17] The data from the above assays are summarized in Tables 1–4. The values reported are the average of at least two separate experiments and it is typical for duplicate values to be within twofold of each other.

As shown in Table 1, introduction of simple alkyl groups such as methyl (compound **6**), ethyl (compound **7**), *i*-butyl (compound **8**), and *i*-propyl (compound **9**) to the 7-*N*-acetamide moiety of compound **1** led to significant improvements in A_{2B} cAMP potency. The most A_{2B} potent analog, compound **7** (7-*N*-ethyl, cAMP IC_{50} = 12 nM) is about fivefold selective over A_1 (A_1, K_i = 66 nM vs A_{2B}, K_i = 13 nM) and almost equipotent between A_{2B} and A_{2A} (A_{2A}, K_i = 22 nM vs A_{2B}, K_i = 13 nM). Introduction of fluorine atoms such as trifluoromethyl (compound **10**) led to a slight drop in A_{2B} cAMP potency without any gain in A_1 and A_{2A} selectivity, compared to compound **7**. Unexpectedly, replacement of two hydrogens of the 7-*N*-methyl of compound **6** by fluorines (difluoromethyl group,

Table 2
Functional and binding activities of 4-substituted 7-*N*-ethyl-acetamide-2-aminobenzothiazole 4-fluorobenzamides (compounds **7** and **20**)



Compound	R^2 =	A_{2B} cAMP (IC_{50}, nM)	A_1 Binding[a] (K_i, nM)	A_{2A} Binding[b] (K_i, nM)	A_{2B} Binding[c] (K_i, nM)
7	OCH_3	12	66	22	13
20	Cl	23	30	15	NT[d]

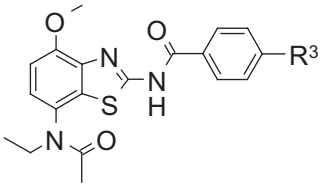
[a,b,c,d] See footnotes to Table 1.

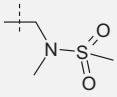
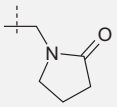
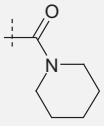
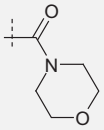
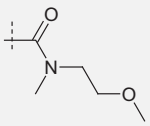
compound **11**) led to a 15-fold drop in A_{2B} cAMP potency without any significant change in A_1 and A_{2A} binding affinity, compared to compound **6**.

Introduction of polar groups such as hydroxy and methoxy to the 7-*N*-alkyl group (compounds **12**–**14**) led to slight drops (two to sixfold) in A_{2B} cAMP potency and this approach was not pursued further. Introduction of a methyl ester to the 7-*N*-alkyl group gave compound **15** which is of similar A_{2B} potency in the cAMP assay to compound **7**. In an attempt to improve selectivity against A_1 and A_{2A} receptors and increase the metabolic stability of compound **15**, its methyl ester was converted to the corresponding acid (compound **16**) and amides (compounds **17**–**19**). Unfortunately, these analogs all showed significant decreases in potency in the A_{2B} cAMP assay.

Investigation of SAR at the 4-position of the 2-aminobenzothiazole core was carried out next. Our previous explorations at the

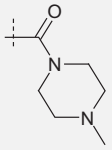
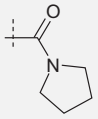
Table 3
Functional and binding activities of various *p*-substituted benzamides of 4-methoxy-7-*N*-ethyl-acetamide-2-aminobenzothiazole (compounds **7**, **22**–**36**)



Compound	R^3 =	A_{2B} cAMP (IC_{50}, nM)	A_1 Binding[a] (K_i, nM)	A_{2A} Binding[b] (K_i, nM)	A_{2B} Binding[c] (K_i, nM)
7	F	12	66	22	13
22	SO_2CH_3	36	390	92	19
23	CN	28	71	50	10
24	CO_2CH_3	35	185	170	15
25	$NHSO_2CH_3$	37	110	46	22
26		19	410	71	4
27		28	400	27	14
28		19	150	77	8
29		28	130	80	23
30		21	78	66	18
31		16	300	100	4
32		21	160	76	7
33		13	180	26	13

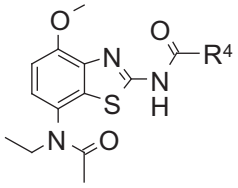
(*continued on next page*)

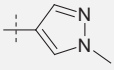
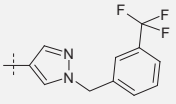
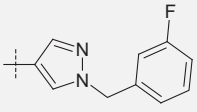
Table 3 (*continued*)

Compound	R^3 =	A_{2B} cAMP (IC_{50}, nM)	A_1 Binding[a] (K_i, nM)	A_{2A} Binding[b] (K_i, nM)	A_{2B} Binding[c] (K_i, nM)
34		38	120	89	18
35		21	185	84	NT[d]
36		16	50	68	NT[d]

[a,b,c,d] See footnotes to Table 1.

Table 4
Functional and binding activities of various amides of 7-*N*-ethyl-acetamide-2-aminobenzothiazole (compounds **37**–**42**)



Compound	R^4 =	A_{2B}, cAMP (IC_{50}, nM)	A_1 Binding[a] (K_i, nM)	A_{2A} Binding[b] (K_i, nM)	A_{2B} Binding[c] (K_i, nM)
37		21	100	51	8
38		7	35	18	NT[d]
39		21	NT[d]	NT[d]	NT[d]
40		29	NT[d]	41	19
41		6	6	3	4
42		12	9	3	6

[a,b,c,d] See footnotes to Table 1.

Table 5
PK parameters of compound **37** after iv and po administration to Han Wistar Rats

Compound	PK Profile[a] (iv, 2.5 mg/kg)			PK Profile[a] (po, 50 mg/kg)			*F* (%)
	CL (mL/min/kg)	V_d (L/kg)	AUC extrap. (ng h/mL)	C_{max} (ng/mL)	AUC extrap. (ng h/mL)	$t_{1/2}$ (h)	
37	46	3.0	929	7420	74,400	2.1	400

[a] A dose of compound **37** was either intravenously (2.5 mg/kg, DMA/PEG 400/40% HPBCD/H_2O) injected into the tail vein of male Han Wistar rat ($n = 4$) or orally (50 mg/kg, Capmul PG8) administered using an intubation tube ($n = 4$). Plasma samples were collected up to 24 h after intravenous or oral administration. The plasma concentrations of compound **37** were determined by LC–MS.

4-position of a structurally simpler 2-aminobenzothiazole template (without 7-substitution) showed that 4-substituents such as –OCH_3, –Cl, and –F gave analogs with the best A_{2B} potency (data not shown). Therefore, the 4-OCH_3 moiety of compound **7** was replaced by 4-Cl and disappointingly, the resulting compound **20** showed a slight drop in A_{2B} cAMP potency and no gain in selectiv-

ity against A_1 or A_{2A} receptors (Table 2). The 4-F analog of compound **7** was also prepared and was less potent than compound **7** in A_{2B} cAMP assay (data not shown). The 4-F series was therefore not pursued further. Based on the results of our SAR at the 4- and 7-positions of 2-aminobenzothiazole core, compound **7** with 4-OCH_3 and 7-*N*-ethyl-acetamide substitutions was selected as having the best combination of A_{2B} potency and selectivity against A_1 and A_{2A} receptors for further optimization.

Thus, the *p*-fluoro group of the benzamide side chain of compound **7** was replaced by a diverse set of substituents such as methylsulfone (**22**), cyano (**23**), *N*-methanesulfonamide (**25**), methyl ester (**24**) and its corresponding amides (**31**–**36**), five-membered heteroaromatics (**28**–**30**), together with *N*-methyl-*N*-methanesulfonamide (**26**) and pyrrolidinone (**27**) both extended by a methylene spacer. Interestingly, all the above analogs showed similar potency in the A_{2B} cAMP assay (Table 3, IC_{50} within 13–38 nM range) and similar A_{2B} receptor binding affinity (K_i within 4–23 nM range).[18] In terms of A_1 and A_{2A} selectivity, although most compounds in Table 3 have moderate A_1 selectivity (A_1, K_i >100 nM), only two of them showed A_{2A} K_i equal to or above 100 nM (compounds **24** and **31**).

Moving away from a phenyl ring in the amide region, five-membered heteroaromatic rings were explored and the data for those analogs are shown in Table 4. Compound **37**, incorporating 2-methyl-2*H*-pyrazole, exhibited good potency in the A_{2B} cAMP assay and reasonable selectivity against A_1 (about 12-fold) and A_{2A} (about sixfold). Slight variations in the pyrazole ring of compound **37** led to compounds **38**–**40** which are similar to compound **37** in A_{2B} potency, but do not offer an advantage in A_1 and A_{2A} receptor selectivity. It was reported at the time of our investigation that *m*-F and *m*-CF_3 benzyl-pyrazol-4-yl groups imparted good A_{2B} affinity and selectivity in the 1,3-diethyl and 1,3-dipropyl derivatives in the xanthine class of compounds.[19] We therefore prepared analogs **41** and **42** with similar side chains in our series. Although compounds **41** and **42** are very potent in A_{2B} assays, they did not show adenosine receptor subtype selectivity, and thus were not profiled further.

Based on A_{2B} potency and selectivity against A_1 and A_{2A}, some of the analogs described above (compounds **6**, **7**, **22**, **26**, **27**, **34**, and **37**) were tested for PK exposure via oral route of administration (formulation: 2% Klucel/0.1% Tween 80 aqueous suspension) in C57 mice at 50 mg/kg dose. Of the seven compounds tested, only compounds **6**, **7**, and **37** showed plasma drug exposures (C_{max} and AUC, data not shown) that warranted further profiling. Compound **37** was studied more thoroughly in a rat PK study and was found to have moderate volume of distribution, moderately high clearance and excellent oral bioavailability (Table 5).[20]

In summary, 7-*N*-acetamide-4-methoxy-2-aminobenzothiazole 4-fluorobenzamide (compound **1**) was chosen as a drug-like and non-xanthine based starting point for the discovery of A_{2B} receptor antagonists because of its slight selectivity against A_1 and A_{2A} receptors and modest A_{2B} potency. SAR exploration of **1** included modifications to the 7-*N*-acetamide group, substitution of the 4-methoxy group by halogens, as well as replacement of the *p*-flouro-benzamide side chain. This work culminated in the identification of compound **37** with excellent A_{2B} potency, modest selectivity versus A_{2A} and A_1 receptors, and good rodent PK properties. In vivo pharmacological evaluation of compound **37** and further attempts to improve its A_1 and A_{2A} selectivity will be described in future publications.

Acknowledgments

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References and notes

1. Fredholm, B. B.; Ijzerman, A. P.; Jacobson, K. A.; Klotz, K. N.; Linden, J. *Pharmacol. Rev.* **2001**, *53*, 527.
2. Fredholm, B. B.; Irenius, E.; Kull, B.; Schulte, G. *Biochem. Pharmacol.* **2001**, *61*, 443.
3. Wilson, C. N. *Br. J. Pharmacol.* **2008**, *155*, 475.
4. Holgate, S. T. *Br. J. Pharmacol.* **2005**, *145*, 1009.
5. Kalla, R. V.; Zablocki, J. *Purinergic Signalling* **2009**, *5*, 21.
6. Sun, C.-X.; Zhong, H.; Mohsenin, A.; Morschl, E.; Chunn, J. L.; Molina, J. G.; Belardinelli, L.; Zeng, D.; Blackburn, M. *J. Clin. Invest.* **2006**, *116*, 2173.
7. Mustafa, S. J.; Nadeem, A.; Fan, M.; Zhong, H.; Belardinelli, L.; Zeng, D. *J. Pharmacol. Exp. Ther.* **2007**, *320*, 1246.
8. Harada, H.; Asano, O.; Kawata, T.; Inoue, T.; Horizoe, T.; Yasuda, N.; Nagata, K.; Murakami, M.; Nagaoka, J.; Kobayashi, S.; Tanaka, I.; Abe, S. *Bioorg. Med. Chem.* **2001**, *9*, 2709.
9. Harada, H.; Asano, O.; Hoshino, Y.; Yoshikawa, S.; Matsukura, M.; Kabasawa, Y.; Niijima, J.; Kotake, Y.; Watanabe, N.; Kawata, T.; Inoue, T.; Horizoe, T.; Yasuda, N.; Minami, H.; Nagata, K.; Murakami, M.; Nagaoka, J.; Kobayashi, S.; Tanaka, I.; Abe, S. *J. Med. Chem.* **2001**, *44*, 170.
10. Kalla, R. V.; Zablocki, J.; Tabrizi, M. A.; Baraldi, P. G. *Handb. Exp. Pharmacol.* **2009**, *193*, 99.
11. Flohr, A.; Jakob-Roetne, R.; Norcross, R. D.; Riemer, C. WO 2003043636; CAN 139:6865 and related patents.
12. Sarkis, G. Y.; Faisal, E. D. *J. Heterocycl. Chem.* **1985**, *22*, 137.
13. Rajappa, S.; Advani, B. G.; Sreenivasan, R. *Indian J. Chem.* **1980**, *19B*, 536.
14. Alanine, A.; Flohr, A.; Miller, A. K.; Norcross, R. D.; Riemer, C. WO 2001097786; CAN 136:69803.
15. Wakasugi, K.; Iida, A.; Misaki, T.; Nishii, Y.; Tanabe, Y. *Adv. Synth. Catal.* **2003**, *345*, 1209.
16. *A_{2B} cAMP assay*: CHO cells were stably transfected with human A_{2B} receptor and cultured under 5% CO_2/95% O_2 atmosphere at 37 °C in DMEM and DMEM/F-12 (1:1 mixture) medium (Invitrogen) with 10% fetal calf serum (Invitrogen), 100 U/mL penicillin (Invitrogen), 100 U/mL streptomycin (Invitrogen), 1 mg/mL G418 (Invitrogen) and 0.2 mg/mL Hygromycin B (Invitrogen). Experimental cultures were grown overnight as a monolayer in 384-well tissue culture plates (0.06 mL/well–7500 cells/well). Each well was washed once with 0.1 mL of Krebs buffer. To each well was added 50 μL of Krebs buffer containing 100 μM of the phosphodiesterase inhibitor 4-(3-butoxy-4-methoxybenzyl)-2-imidazolidinone, 100 nM NECA (Sigma–Aldrich), 0.02% BSA Fraction V (Roche Biochemicals), the test compound (appropriate concentration). The final concentration of DMSO was 1.1%. After incubation for 20–25 min, the wells were emptied and blotted on paper towel to remove residual solution. The HitHunter cAMP Assay Kit from DiscoverX for adherent cells was used for lysing the cells and measuring cAMP concentrations.
17. *Binding assays*: (a) Human A_1 membrane receptors (Euroscreen) were diluted in assay buffer (HEPES 50 mM, NaCl 100 mM and $MgCl_2$ 1 mM) to yield final concentration of 10 μg/well. The test compounds (10 μL) and 40 μL of [^{3}H]-DPCPX ligand (4.8 nM final conc., Perkin–Elmer), were added to 96-well polypropylene plates (Becton Dickinson) followed by addition of membranes (150 μL) and incubation at room temperature for 1 h on an orbital shaker. (b) Human A_{2A} membrane receptors (Perkin–Elmer) were diluted in assay buffer (HEPES 50 mM, EDTA 1 mM) to yield final concentration of 8.5 μg/well. The test compounds (10 μL) and 40 μL of [^{3}H]-ZM241385 ligand (5 nM final) were added to 96-well polypropylene plates (Becton Dickinson) followed by addition of membranes (150 μL) and incubation at room temperature for 1 h on an orbital shaker. (c) For human A_{2B} receptor, whole cells (CHO cells) expressing the receptor were used. Confluent (80%) T75 flasks were harvested mechanically and frozen in aliquots of 1 mL. On the day of assay, a single vial was suspended in 25 mL of assay buffer. The test compounds (10 μL) and [^{3}H] ZM241385 ligand 40 μL (30 nM final) were added to 96-well polypropylene plates followed by addition of cell suspension (150 μL) and incubation at room temperature for 1 h on an orbital shaker. Reactions were harvested using 96-well MultiScreen FB plates (0.5% polyethyleneimine-treated) and MultiScreenHTS vacuum manifold (Millipore). Plates were air dried followed by addition of scintillation fluid and read on MicroBeta counter (Perkin–Elmer). (d) Human A_3 receptor binding data was not obtained routinely because its membrane preparations are not commercially available in the US due to patent restrictions. Only compound **37** from the manuscript was tested in A_3 binding assay and its K_i in A_3 was determined to be 80 nM.
18. A strong (r^2 = 0.64) and linear relationship was observed between the potency of A_{2B} antagonists for the cAMP (IC_{50}) and binding assay (K_i) across a wide range of potencies (single digit nM–μM cAMP IC_{50} values) and across multiple structural subtypes (over 300 compounds were analyzed, data not shown).

19. Elzein, E.; Li, X.; Kalla, R.; Perry, T.; Palle, V.; Varkhedkar, V; Maa, T.; Nguyen, M.; Wu, Y.; Maydanik, V.; Lustig, D.; Leung, K.; Zeng, D.; Zablocki, J. *Abstracts of Papers*, 227th National Meeting of the American Chemical Society, Anaheim, CA, Mar 28–Apr 1, 2004; American Chemical Society: Washington, DC, 2004; MEDI-251.

20. A likely explanation for the greater than 100% oral bioavailability is saturation of clearance since the oral dose is twenty times higher than the iv dose. However, we cannot rule out other possibilities such as precipitation at the injection site or insufficient sampling following iv administration and the consequent failure to fully capture the distribution phase.