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Discovery of benzothiazole-based adenosine A_{2B} receptor antagonists with improved A_{2A} selectivity

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ABSTRACT

The highly potent but modestly selective *N*-(2-amino-4-methoxy-benzothiazol-7-yl)-*N*-ethyl-acetamide derivative **2** was selected as the starting point for the design of novel selective A_{2B} antagonists, due to its excellent potency, and good drug-like properties. A series of compounds containing nonaromatic amides or ureas of five- or six-membered rings, and also bearing an *m*-trifluoromethyl-phenyl group (shown to impart superior potency) were prepared and evaluated for their selectivity against the A_{2A} and A_1 receptors. This work resulted in the identification of compound **30**, with excellent potency and high selectivity against both A_{2A} and A_1 receptors.

Adenosine is an autocoid that mediates protective effects under normal physiological conditions through four G-protein coupled receptors, namely the A_1 and A_3 receptors (which are coupled to G_i and G_o proteins, respectively), and the A_{2A} and A_{2B} receptors (which are coupled to G_s proteins).[1] Interestingly, adenosine's agonist potency is determined to be significantly varied at its four receptors, which were individually expressed in Chinese Hamster Ovary (CHO) cells: 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] Thus, the low affinity A_{2B} receptor is believed to remain silent at normal physiological state, and become activated when under elevated extracellular adenosine levels during chronic high oxidative stress conditions, such as hyperglycemia or mast-cell activation. Antagonists of the A_{2B} receptor have therefore been evaluated as potential thearapeutic agents in models of diabetes, asthma, COPD, and pulmonary fibrosis.[3–9]

As discussed in an earlier communication,[10] our initial SAR exploration of A_{2B} receptor antagonists, starting with 7-*N*-acetamide-4-methoxy-2-aminobenzothiazole 4-fluorobenzamide (compound **1**, Fig. 1), led to the identification of the 7-*N*-ethyl derivative **2** with good A_{2B} potency, and modest selectivity versus A_{2A} (ca. six-fold) and A_1 (ca. 12-fold) receptors (Table 1).[11,12] Furthermore, incorporation of a *m*-trifluoromethyl-benzyl substituted pyrazole regioisomer (which was previously reported to provide a potency boost towards A_{2B} in other A_{2B} antagonists)[13] resulted in the identification of compound **3**, which displayed even greater potency towards A_{2B}, albeit with a complete loss of selectivity versus the A_{2A} and A_1 receptors

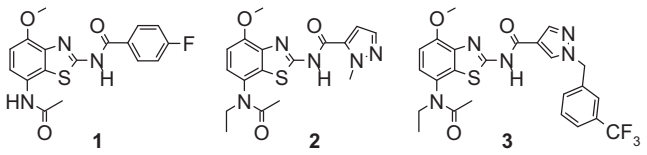

Figure 1. Structures of compounds **1**–**3**.

(Table 1), in contrast to what was observed with the xanthine series.[13]

The good in vitro potency of compound **2**, as well as its good rodent PK profile,[10] prompted us to further characterize this compound in a number of in vitro selectivity and safety assays. We were pleased to find that compound **2** did not show any significant activity (defined as >50% inhibition at 10 μM) against a panel of 63 receptors, enzymes and ion channels tested at MDS Panlabs (Bothell, WA). In addition, compound **2** exhibited virtually no inhibition (IC_{50} >50 μM) of CYP450 enzymes (2C9, 2C19, 2D6 and 3A4), as well as a low risk of QT interval prolongation (hERG, IC_{50} >100 μM). Furthermore, compound **2** did not show any genotoxicity liability in the microsuspension version of the Ames test in the absence and/or presence of an exogenous metabolic activation system (S9).

In summary, compound **2** possessed all the desired characteristics for a pre-clinical candidate (excellent A_{2B} potency, desirable PK properties, absence of in vitro toxicological liabilities), but lacked sufficient selectivity against A_1 and A_{2A} receptors. Thus, we focused our efforts on trying to improve the one remaining shortcoming of our aminobenzothiazole A_{2B} antagonists, namely sub-optimal adenosine receptor selectivity, while maintaining their excellent

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Table 1
Functional and binding activities of three 7-*N*-acetamide-4-methoxy-2-aminobenzothiazole amides (compounds **1**–**3**)

Compound	A_{2B} cAMP (IC_{50}, nM)	A_1 binding (K_i, nM)	A_{2A} binding (K_i, nM)	A_{2B} binding (K_i, nM)
1	350	1600	250	130
2	21	100	51	8
3	6	6	3	4

potency. We chose to keep the core structure of compounds **2** and **3** [i.e., the *N*-(2-amino-4-methoxy-benzothiazol-7-yl)-*N*-ethyl-acetamide moiety] intact, while further modifying the right hand (amide) portion of the molecules. As previously described,[10] amides of simple five- or six-membered aromatic and heteroaromatic rings did not produce significant breakthroughs in terms of selectivity against A_1 and A_{2A} receptors. Aiming to take advantage of the *m*-trifluorophenyl moiety in compound **3** (in order to maintain A_{2B} potency), we therefore decided to synthesize amides or ureas of non-aromatic, five- or six-membered rings (compounds **13–30**), in hopes that the added nonplanar structural elements would impart better selectivity to our A_{2B} antagonists. Thus, all new compounds were designed to contain a nonaromatic spacer (piperidine, pyrrolidine, aminocyclohexane, or aminocyclopentane), followed by a linker (carbonyl, sulfonyl, or methylene) group, connecting the core aminobenzothiazole to the *m*-triflurometh ylphenyl moiety required for potency (Fig. 2).

The amides of nonaromatic five- or six-membered rings (compounds **13**–**27**) were synthesized as shown in Scheme 1, via a three step sequence starting from intermediate **4** (which was prepared on a multi-gram scale as previously described).[10] The N-Boc-protected amino acids **5**–**8** were coupled to starting material **4** (step I. amide coupling) using either standard coupling procedures (CDI in THF), or alternatively via activation of the acids with 1-methylimidazole and *p*-toluenesulfonyl chloride, followed by treatment with amine **4**. The N-Boc protecting groups of the resulting amides were then removed upon treatment with trifluoroacetic acid or 1N HCl (step II. Boc-deprotection). Finally, the resulting unmasked amines were further derivatized (step III. N-capping) by either (i) acylation with 3-trifluoromethyl-benzoyl chloride (**9**) or (ii) sulfonylation with 3-trifluoromethyl-benzenesulfonyl chloride (**10**) or (iii) reductive amination with 3-trifluoromethyl-benzaldehyde (**11**), to produce the A_{2B} antagonists **13**–**27**.

Additionally, a few piperidine urea derivatives (compounds **28**–**30**) were also prepared, via the conversion of starting material **4** to the corresponding *p*-nitrophenyl carbamate via treatment with **12**, followed by the displacement of *p*-nitrophenol with an appropriately substituted amine, as shown in Scheme 2.

The new analogs thus prepared were then profiled in the A_{2B} cAMP assay[11] as well as A_1 and A_{2A} receptor binding assays (Tables 2–5).[12] As there were no commercially available membrane preparations for A_{2B} receptors, only a selected number of the more interesting compounds were tested in the A_{2B} binding assay[14] The data are summarized below.[15]

Initially, we chose to evaluate analogs bearing five-membered ring nonaromatic spacers (i.e., spacers derived from **5** or **6**, Table 3), as they were the 'structurally closest' analogs to compounds **2** and **3**. For synthetic ease, the carbonyl linker was used to connect the spacers to the *m*-triflurophenyl group. In the case of pyrrolidine derivatives, we noted a slight preference for the (*R*)-enantiomer (compound **13**) over the corresponding (*S*)-enantiomer (compound **14**) in the A_{2B} cAMP assay. We were especially pleased to see that the more potent enantiomer **13,** while maintaining a similar potency to compound **2**, showed much improved A_{2A} binding selectivity (K_i >2 μM), without losing its modest A1 selectivity.

Interestingly, when the spacer length was increased by one atom, the selectivity profiles of the compound were drastically altered. The chiral *cis*- and *trans*-3-*N*-Boc-amino-cyclopentane analogs **15** and **16** (which retained the same preferred stereochemistry as **13** at the carbon attached to the benzothiazole core) showed a

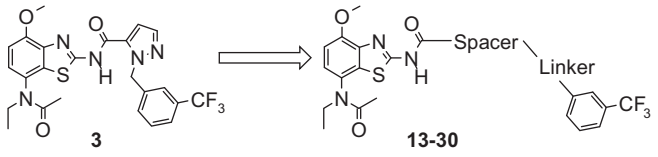
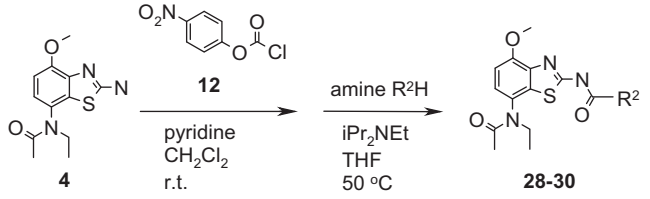
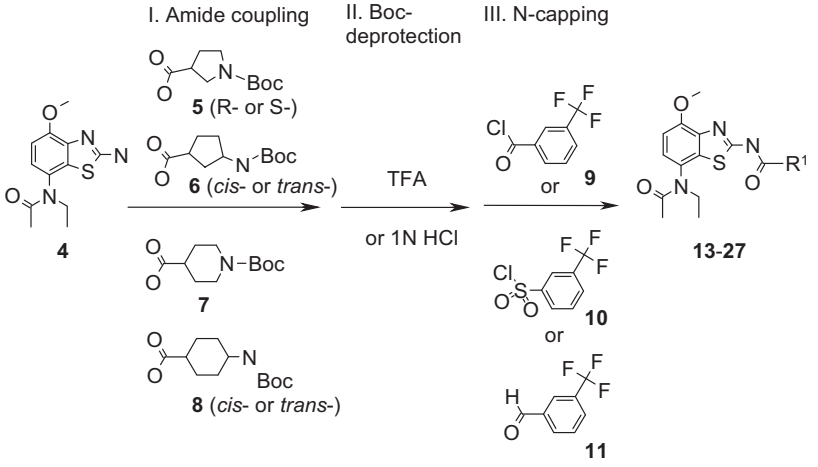


Figure 2. Design of compounds **13**–**30**.

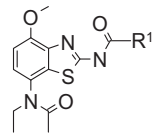


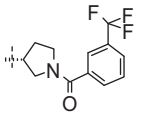
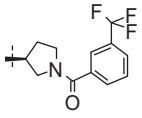
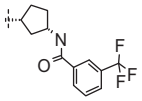
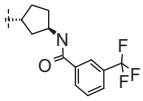
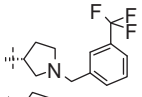
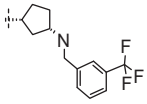
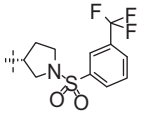
Scheme 2. Synthesis of various *N*-(2-amino-4-methoxy-benzothiazol-7-yl)-*N*-ethyl-acetamide ureas (compounds **28**–**30**).



Scheme 1. Synthesis of various *N*-(2-amino-4-methoxy-benzothiazol-7-yl)-*N*-ethyl-acetamide amides (compounds **13**–**27**).

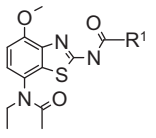
Table 2
Functional and binding activities of various 7-(acetyl-ethyl-amino)-4-methoxy-benzothiazol-2-yl amides (compounds **2**, **13**–**19**)

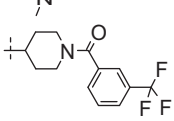
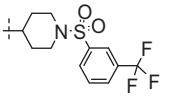
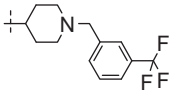
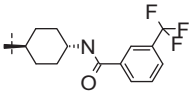
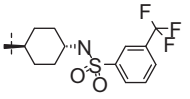
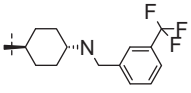
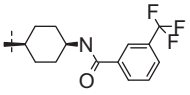
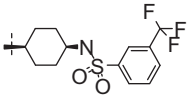


Compound	R^1	A_{2B} cAMP (IC_{50}, nM)	A_1 binding (K_i, nM)	A_{2A} binding (K_i, nM)
2		21	100	51
13		14	100	2040
14		41	120	710
15		8	61	81
16		8	31	31
17		72	NT[a]	NT[a]
18[b]		19	220	180
19		42	220	600

[a] NT: not tested.
[b] Compound was prepared as the trifluoroacetate salt.

Table 3
Functional and binding activities of various 7-(acetyl-ethyl-amino)-4-methoxy-benzothiazol-2-yl amides (compounds **2**, **18**–**25**)



Compound	R^1	A_{2B} cAMP (IC_{50}, nM)	A_1 binding (K_i, nM)	A_{2A} binding (K_i, nM)
2		21	100	51
20		17	2	230
21		49	78	1040
22		44	10	1000
23		28	7	470
24		13	6	200
25[a]		30	67	280
26		26	20	52
27		16	46	110

[a] Compound was prepared as the trifluoroacetate salt.

slight improvement (two- to three-fold) in A_{2B} cAMP potency, when compared with **2** or **13**. Unfortunately, this modest gain in potency was accompanied by significant losses in A_{2A} selectivity for both compounds.

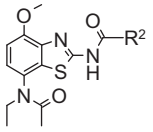
We then procceded to prepare the amine analogs **17** and **18** to investigate the effects of a methylene linker in conjunction with the five-membered ring spacers. Disappointingly, replacement of the amide in **13** with the corresponding amine in **17** resulted in a five-fold loss in A_{2B} cAMP potency. In the case of the one-carbon extended analogs **15** and **18**, going from a carbonyl linker to a methylene linker resulted in a slight benefit in terms of selectivity against the A_1 receptor, although with an accompanied loss in A_{2B} cAMP potency. Finally, the sulfonamide analog **19**, although of similar potency (within two-fold) in the A_{2B} cAMP assay, provided no additional benefits in terms of selectivity against A_1 and and A_{2A}, when compared to compound **13**.

Next, we turned our attention to A_{2B} antagonists bearing six-membered ring nonaromatic spacers (Table 4, compounds **20**–**22**), which were prepared from *N*-Boc-piperidine-4-carboxylic acid (**7**). While these three analogs maintained comparable potency in A_{2B} cAMP assay and significantly improved A_{2A} selectivity when compared with **2**, they suffered from complete loss of A1 selectivity.

Analogs **23**–**27**, which were synthesized from either *trans*- or *cis*-3-*N*-Boc-amino-cyclohexanecarboxylic acid (**8**), were next evaluated. All of these derivatives were of similar potency as compound **2** in the A_{2B} cAMP assay, and most offered improved A_{2A} binding selectivity (with the exception of **26**). Unfortunately, the poor A_1 selectivity for these compounds rendered them unsuitable for further studies.

Finally, we chose to evaluate a limited number of antagonists which contained nonaromatic, six-membered ring urea spacers in place of the amide moiety. Three of the most potent ureas are shown in Table 4. We were pleased to discover that compared to compound **2**, ureas **28**–**30** are of similar potency in the A_{2B} cAMP assay, but have significantly improved A_{2A} selectivity. In the case of sulfone-containing analogs **19**, **21**, **27**, and **30**, we have noted a trend towards a simultaneous loss of affinity to both A_1 and A_{2A} receptors, while maintaining potency at the A_{2B} receptor. Most importantly, urea **30**, which is equipotent to compound **2** in the A_{2B} cAMP assay, not only displayed significantly improved A_{2A}

Table 4
Functional and binding activities of various 7-(acetyl-ethyl-amino)-4-methoxy-benzothiazol-2-yl amide and ureas (compounds **2**, **27**–**32**)



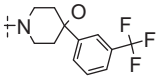
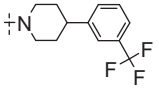
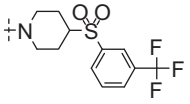
Compound	R^2	A_{2B} cAMP (IC_{50}, nM)	A_1 binding (K_i, nM)	A_{2A} binding (K_i, nM)
2		21	100	51
28		8	19	210
29		18	5	240
30		20	690	530

Table 5
Functional/binding activities of selected 7-(acetyl-ethyl-amino)-4-methoxy-benzothiazol-2-yl analogs

Compound	A_{2B} cAMP (IC_{50}, nM)	A_1 binding (K_i, nM)	A_{2A} binding (K_i, nM)	A_{2B} binding (K_i, nM)
2	21	100	51	8
13	14	100	2040	18
25	30	67	280	18
28	8	19	210	5

selectivity (10-fold improvement over compound **2**), but also exhibited a superior A_1 selectivity profile (ca. seven-fold improvement over compound **2**).

In summary, starting with compound **2**, and through investigation of nonaromatic amide derivatives of A_{2B} antagonists containing the *N*-(2-amino-4-methoxy-benzothiazol-7-yl)-*N*-ethyl-acetamide core structure, we have been able to identify several highly potent A_{2B} antagonists with very good A_{2A} selectivity Table 5. Moreover, extension of our SAR exploration into similar urea derivatives led to the identification of compound **30**, which displayed excellent A_{2B} potency, as well as good A_{2A} and A_1 selectivity. Future communications will describe further attempts in improving the A_1 and A_{2A} selectivities, as well as in vivo studies with selected A_{2B} antagonists.

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11. *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.
12. *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 a 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 a 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 a 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.
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14. Over the course of our studies on various classes of A_{2B} antagonists, 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 (data not shown). Thus, we felt comfortable to use the more readily available cAMP assay as a measure of both A_{2B} potency and for the assessment of selectivity.
15. The values reported are the average of at least two separate experiments, where typically the duplicate values were within two-fold of each other.