Full Paper

Synthesis of Novel Pyrido[3,2-*e*][1,2,4]triazolo[1,5-*c*]pyrimidine Derivatives: Potent and Selective Adenosine A_3 Receptor Antagonists

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A series of novel pyrido[3,2-*e*][1,2,4]triazolo[1,5-*c*]pyrimidine derivatives **5** was prepared from 2-amino-3-cyano-4-trifluoromethyl-6-phenylpyridine **1** in two steps via formation of iminoether **3** followed by reaction with different aroylhydrazides **4**. Representative products **5** were evaluated for their affinity towards all four subtypes of human adenosine receptors. Compounds 2-(3-fluorophenyl)-8-phenyl-10-(trifluoromethyl)pyrido[3,2-*e*][1,2,4]triazolo[1,5-*c*]pyrimidine (**5b**), 2-(furan-2-yl)-8-phenyl-10-(trifluoromethyl)pyrido[3,2-*e*][1,2,4]triazolo[1,5-*c*]pyrimidine (**5d**), and 2-(furan-2-yl)-5-methyl-8-phenyl-10-(trifluoromethyl)pyrido[3,2-*e*][1,2,4]triazolo[1,5-*c*]pyrimidine (**5j**) showed high affinity for the A_3 receptors, with K_i values of 8.1, 10.4, and 12.1 nM, respectively, and were >1000-fold selective versus all other adenosine receptor subtypes.

Keywords: Adenosine receptors / 2-Aminonicotinonitrile / Aroyl hydrazide / Orthoacetate / Orthoformate

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Introduction

Adenosine receptors (ARs) designated as A_1, A_{2A}, A_{2B}, and A_3, belong to the superfamily of G protein-coupled receptors (GPCRs), which modulate a wide range of biological functions including CNS, cardiac, and immune suppressive functions [1, 2]. A_{2A} ARs, for example, are present in high density in the basal ganglia of the brain where they are co-expressed with dopamine receptors forming heteromeric receptor complexes [3]. A_{2A} receptors are also present on tissues of the peripheral system, e.g., on platelets [4], lymphocytes [5], neutrophils [6, 7], monocytes, macrophages, and mast cells. A_1 and A_{2A} ARs are generally 60% identical within the transmembrane domains [8–10] and represent potential pharmacological targets for the treatment of asthma, psychosis, chronic inflammation, anxiety, and neurodegenerative disorders [11–13]. Thus, discovery and development of adenosine receptor antagonists have been an attractive field of research from the perspective of identifying new drugs for the treatment of widespread disorders such as inflammation, asthma, and Parkinson's disease. Efforts to find selective ligands for A_{2A} receptors led to the identification of pyrazolotriazolopyrimidines such as SCH 58261 (5-amino-7-(2-phenylethyl)-2-(2-furyl)pyrazolo[4,3-*e*]1,2,4-triazolo[1,5-*c*]pyrimidine) and SCH 63390 (5-amino-7-(3-phenylpropyl)-2-(2-furyl)pyrazolo[4,3-*e*]1,2,4-triazolo[1,5-*c*]pyrimidine) [14]. Compounds which possess hydrophilic groups in the *para*- and *ortho*-position of the aromatic ring have been found to be potent and selective for A_{2A} ARs [15, 16]. Like A_{2A} ARs, A_{2B} ARs

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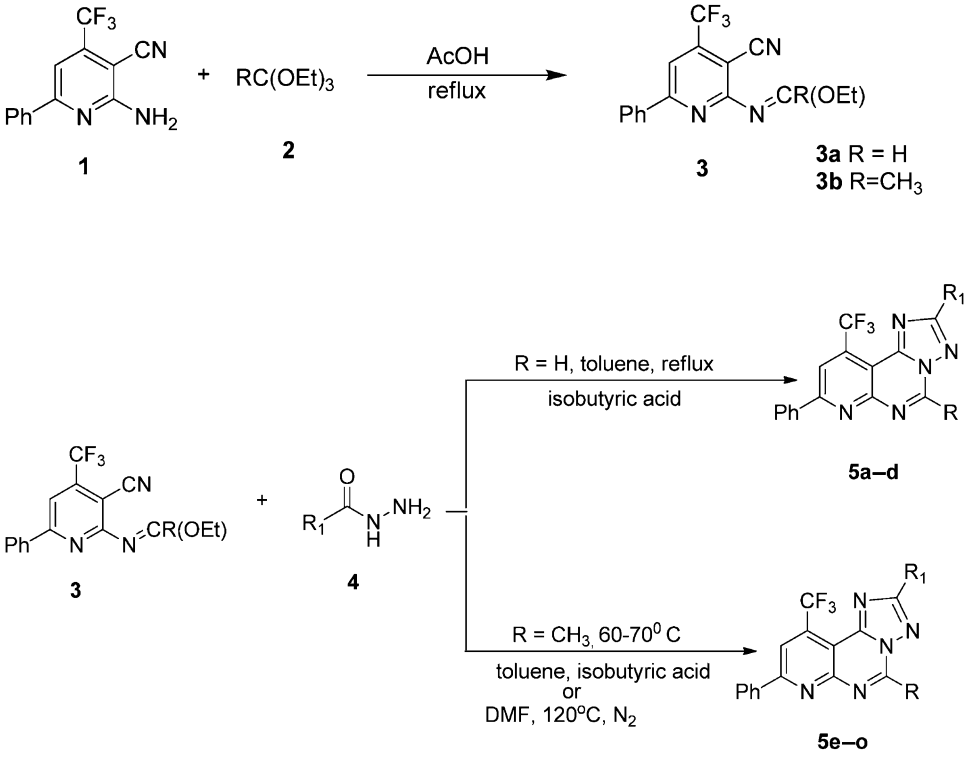
stimulate adenylate cyclase activity in the presence of adenosine. In contrast, A_1 and A_3 ARs inhibit adenylate cyclase, and A_3 antagonists have been proposed as novel anticancer drugs [17], e.g., for the treatment of human glioblastomas. Efforts to find selective ligands acting as antagonists at A_3 ARs led to the discovery of various structures, such as KF-26777, MRS-1191, MRS-1220, MRS-1334, MRS-1523, MRS-3777, MRE-3005-F20, MRE-3008-F20, PSB 10, PSB-11, OT-7999, and VUF-5574 with high affinity [20, 21]. However none have so far been successfully evaluated in clinical trials, which may partially be attributed to their poor pharmacokinetic profile. Recent studies report that the pyrrolo[3,4-*e*][1,2,4]triazolo[1,5-*c*]pyrimidine and the pyrazolo-[4,3-*e*][1,2,4]triazolo[1,5-*c*]pyrimidine structures have emerged as useful templates for targeting A_3 ARs [22–24]. Thus, based on our recent results in the synthesis of such compounds [18, 19, 25], we focussed our attention on the preparation of pyrido-[3,2-*e*][1,2,4]triazolo[1,5-*c*]pyrimidine derivatives as bioisosteric modification of the pyrrolo[3,4-*e*]/pyrazolo[4,3-*e*][1,2,4]triazolo-[1,5-*c*]pyrimidine structure, and evaluated the newly designed and synthesized compounds in radioligand binding studies for their affinity towards A_1, A_{2A}, A_{2B}, and A_3 ARs and we thereby identified promising new compounds.

Results and discussion

Chemistry

The 2-amino-3-cyano-4-trifluoromethyl-6-phenylpyridine **1** [22] was reacted with triethylorthoformate/orthoacetate **2** in the presence of catalytic amounts of acetic acid at reflux temperature for 8 h yielding the imine ether derivatives **3** [23]. The compounds **3a** and **3b** were independently reacted with different aroylhydrazides in toluene using catalytic amounts of isobutyric acid at reflux temperature forming products **5a–d** while at 60–70 °C in toluene or at 120 °C in DMF products **5e–o** were obtained. The sequence of the reaction is mainly an amination on the imine carbon followed by cyclization of the amine with the nitrile carbon to result in products **5**. The reaction of compound **3a** with various aroylhydrazides is found to be sluggish. The structures of the synthesized compounds were confirmed by ^{1}H and ^{13}C NMR spectroscopy, in addition to HPLC analysis coupled to electrospray ionization mass spectrometry (LC/ESI-MS), which was also used to determine the purity that was in all cases shown to be >95%.

The reactions outlined in Scheme 1 and products collected are presented in Table 1.



Scheme 1. Preparation of pyrido[3,2-*e*][1,2,4]triazolo[1,5-*c*]pyrimidine derivatives **5**.

Table 1. Physical properties of pyrido[3,2-*e*][1,2,4]triazolo[1,5-*c*]pyrimidine derivatives **5**.

S. no	Compd. no.	R	R_1	mp (°C)	Yield (%)
1	**5a**	H	C_6H_5	248	54
2	**5b**	H	3-FC_6H_4	273	60
3	**5c**	H	3-ClC_6H_4	256	45
4	**5d**	H	2-furyl	250	56
5	**5e**	CH_3	C_6H_5	263	70
6	**5f**	CH_3	3-FC_6H_4	290	73
7	**5g**	CH_3	3-ClC_6H_4	275	81
8	**5h**	CH_3	4-ClC_6H_4	276	73
9	**5i**	CH_3	4-FC_6H_4	264	68
10	**5j**	CH_3	2-furyl	296	65
11	**5k**	CH_3	2-ClC_6H_4	259	74
12	**5l**	CH_3	2-OHC_6H_4	274	68
13	**5m**	CH_3	4-$NO_2C_6H_4$	269	64
14	**5n**	CH_3	2-thienyl	271	69
15	**5o**	CH_3	3-pyridyl	284	74

Radioligand binding studies

The selected pyrido[3,2-*e*][1,2,4]triazolo[1,5-*c*]pyrimidine derivatives (**5b**, **5d–j**) were tested for their ability to displace [^{3}H]2-chloro-N^6-cyclopentyladenosine ([^{3}H]CCPA) from A_1 AR in rat cortical membranes and [^{3}H]MSX-2 from A_{2A} AR in rat striatal membranes, [^{3}H]PSB-603 from human recombinant A_{2B} ARs and [^{3}H]PSB-11 from human recombinant A_3 ARs. The radioligand binding affinities of the compounds (**5b**, **5d–j**) were expressed as K_i values or in percent inhibition (at 10 μM (A_1 and A_{2A}), or at 1 μM (A_{2B} and A_3) concentration, respectively). The binding affinity data of the compounds are presented in Table 2.

All the investigated compounds did not show binding affinity towards either A_1 or A_{2A} ARs at 10 μM or towards A_{2B} ARs at 1.0 μM concentration. However, compounds **5b**, **5d**, and **5j** were found to show high binding affinity towards A_3 ARs with K_i values of 8.1, 10.4, and 12.1 nM, respectively. Figure 1 shows the radioligand competition binding curves of the most potent compounds **5b**, **5d**, and **5j**. Primarily it appeared that the presence of a furyl substituent on the triazole ring of compound **5d** (10.4 nM) and **5j** (12.1 nM) promotes binding affinity towards the A_3 receptor. This was not surprising since it had been shown in recent X-ray crystallographic studies of the adenosine A_{2A} receptor in complex with the furyl-substituted triazolotriazine derivative ZM241385 (4-(2-(7-amino-2-(2-furyl)[1,2,4]triazolo[2,3-*a*][1,3,5]-triazin-5-yl)amino)ethyl)phenol) that an asparagine residue in transmembranal helix 6 (N253 corresponding to $N^{6.55}$) formed a hydrogen bond with the oxygen atom of the furanyl residue [26, 27].

This asparagine residue is conserved in all four AR subtypes. Therefore it is likely that a similar interaction can be found in A_3ARs. In order to investigate the necessity of the furanyl residue, it was replaced with fluoro/chlorophenyl substituents. The 3-fluorophenyl was found to yield very high affinity as well, with compound **5b** displaying a K_i value of 8.1 nM. Thus, the furyl residue is not an essential substituent at the triazole ring. Further optimization of the substituent is currently in progress to identify even more promising compounds.

Conclusion

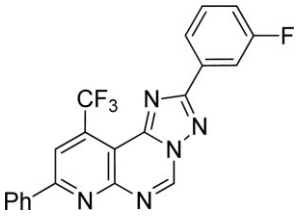
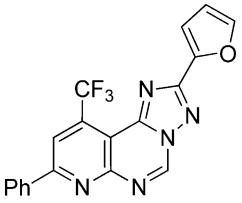
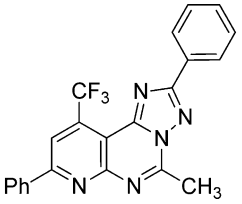
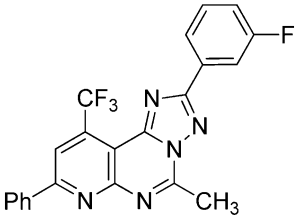
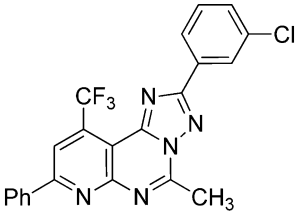
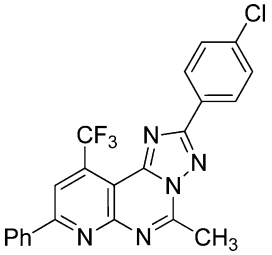
A series of novel pyrido[3,2-*e*][1,2,4]triazolo[1,5-*c*]pyrimidine derivatives **5** was prepared and evaluated for affinity in radioligand binding studies towards all adenosine receptor subtypes, A_1, A_{2A}, A_{2B}, and A_3. Compounds **5b**, **5d**, and **5j** showed high selectivity for A_3 versus all other AR subtypes with K_i values of 8.1, 10.4, and 12.1 nM, respectively. This new class of A_3 antagonists shows promising properties and further investigations appear warranted.

Experimental

General

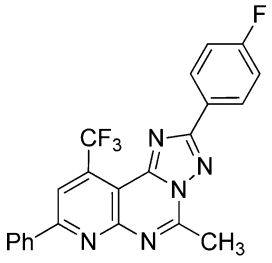
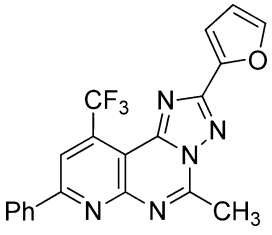
Melting points of all the compounds were recorded on a Casia-Siamia (VMP-AM) melting point apparatus and are uncorrected. IR spectra were recorded on a Perkin-Elmer FT-IR 240-C spectrophotometer using KBr discs. ^{1}H NMR and ^{13}C NMR spectra were recorded on 400, 300, and 75 MHz spectrometer, respectively in $CDCl_3$ and CF_3COOD using TMS as an internal standard. Electron impact (EI) and chemical ionization mass spectra were recorded on a VG 7070 H instrument at 70 eV. All high-resolution spectra were recorded on QSTARXL hybrid MS/MS system (Applied Biosystems, USA) under electron spray ionization. All the reactions were monitored by thin layer chromatography (TLC) on precoated silica gel 60 F_{254} (mesh) plates; spots were visualized with UV light. Merck silica gel (60–120 mesh) was used for column

Table 2. Activity data of compound **5** on radioligand binding affinity towards adenosine receptors.

Code	Compound	A_1 rat brain cortical membranes [^{3}H]CCPA $K_i \pm$ SEM (nM) ($n = 3$) (% inhibition ± SEM at 10 μM) ($n = 2$)[a)]	A_{2A} rat brain striatal membranes [^{3}H]MSX-2 $K_i \pm$ SEM (nM) ($n = 3$) (% inhibition ± SEM at 10 μM) ($n = 2$)[a)]	A_{2B} human recombinant [^{3}H]PSB-603 $K_i \pm$ SEM (nM) ($n \geq 3$) (% inhibition ± SEM at 1 μM) ($n = 2$)[a)]	A_3 human recombinant [^{3}H]PSB-11 $K_i \pm$ SEM (nM) ($n \geq 3$) (% inhibition ± SEM at 1 μM) ($n = 2$)[a)]
5b		>10000 (13 ± 10)	>10000 (−74 ± 27)	>1000 (−4 ± 17)	8.10 ± 0.96 ($n = 5$)
5d		3600 ± 2180	113 ± 50	>1000 (17 ± 13)	10.4 ± 1.60 ($n = 4$)
5e		>10000 (38 ± 1)	>10000 (−77 ± 2)	>1000 (8 ± 3)	>1000 (44 ± 3)
5f		>10000 (2 ± 11)	>10000 (−162 ± 9)	>1000 (−5 ± 1)	>1000 (30 ± 3)
5g		>10000 (6 ± 6)	>10000 (−52 ± 2)	>1000 (−26 ± 9)	>1000 (21 ± 6)
5h		>10000 (−6 ± 4)	>10000 (−51 ± 11)	>1000 (−1 ± 3)	>1000 (46 ± 6)

(*Continued*)

Table 2. (*Continued*)

Code	Compound	A_1 rat brain cortical membranes [^{3}H]CCPA $K_i \pm$ SEM (nM) ($n = 3$) (% inhibition ± SEM at 10 μM) ($n = 2$)[a)]	A_{2A} rat brain striatal membranes [^{3}H]MSX-2 $K_i \pm$ SEM (nM) ($n = 3$) (% inhibition ± SEM at 10 μM) ($n = 2$)[a)]	A_{2B} human recombinant [^{3}H]PSB-603 $K_i \pm$ SEM (nM) ($n \geq 3$) (% inhibition ± SEM at 1 μM) ($n = 2$)[a)]	A_3 human recombinant [^{3}H]PSB-11 $K_i \pm$ SEM (nM) ($n \geq 3$) (% inhibition ± SEM at 1 μM) ($n = 2$)[a)]
5i		3640 ± 2560	>10000 (−22 ± 9)	>1000 (−9 ± 2)	137 ± 7 ($n = 4$)
5j		>10000 (9 ± 5)	>10000 (−6 ± 6)	>1000 (8 ± 3)	12.1 ± 1.6 ($n = 4$)

[a)] Unless otherwise noted.

chromatography. Radioligand binding affinity of representative pyrido[3,2-*e*][1,2,4]triazolo[1,5-*c*]pyrimidine derivatives was evaluated towards A_1, A_{2A}, A_{2B}, and A_3 ARs.

Preparation of ethyl *N*-[3-cyano-6-phenyl-4-(trifluoromethyl)-2-pyridyl]imido formate (**3a**) was done as described in [23]. Preparation of ethyl *N*-[3-cyano-6-phenyl-4-(trifluoromethyl)-2-pyridyl]ethane imidate (**3b**) was performed as described in [23].

*General procedure for the preparation of 2-aryl-8-phenyl-10-(trifluoromethyl)pyrido[3,2-e][1,2,4]triazolo[1,5-c]-pyrimidine (**5a–d**)*

Compound **3a** (142.4 mg, 0.47 mmol) and aroylhydrazide (0.47 mmol) were dissolved in toluene (3 mL) along with catalytic amount of isobutyric acid (0.15 mL) and stirred at 60–70°C for 2 h; as a result, solid separated from the solution. Toluene was removed by filtration and washing was done with $CHCl_3$. The crude product was dissolved on heating in toluene (5 mL) and refluxed for 10–12 h, cooled to room temperature and toluene was removed under vacuum. The crude product residue was purified by passing through a column packed with silica gel 60–120 using 10% ethyl acetate in *n*-hexane.

*2,8-Diphenyl-10-(trifluoromethyl)pyrido[3,2-e][1,2,4]-triazolo[1,5-c]pyrimidine (**5a**)*

White solid, yield: 100 mg (54.4%), mp: 248 C, IR (KBr) cm^{-1}: 1598 (C=N), ^{1}H NMR ($CDCl_3$) δ: 7.5–7.7 (m, 6H, Ar–H). 8.2–8.4 (m, 4H, Ar–H), 8.7 (s, 1H, Ar–H), 9.6 (s, 1H, Ar–H). EI-mass: 391 (M^+), HRMS *m/z* calcd. for $C_{21}H_{13}N_5F_3$ ($[M+H]^+$): 392.1123. Found 392.1131.

*2-(3-Fluorophenyl)-8-phenyl-10-(trifluoromethyl)pyrido-[3,2-e][1,2,4]triazolo[1,5-c]pyrimidine (**5b**)*

Brown solid, yield: 116 mg (60.2%), mp: 256 C, IR (KBr) cm^{-1}: 1596 (C=N), ^{1}H NMR ($CDCl_3$) δ: 7.16–7.23 (m, 1H, Ar–H), 7.46–7.63 (m, 4H, Ar–H.), 8.03–8.10 (d, 1H, $J = 8.4$, Ar–H), 8.15–8.20 (d, 1H, $J = 7.74$, Ar–H), 8.28–8.36 (m, 2H, Ar–H), 8.46 (s, 1H, Ar–H), 9.53 (s, 1H, Ar–H). ESI-mass: 410 (M^++H), HRMS *m/z* calcd. for $C_{21}H_{12}N_5F_4$ ($[M+H]^+$): 410. 1029. Found 410.1037.

*2-(3-Chlorophenyl)-8-phenyl-10-(trifluoromethyl)pyrido-[3,2-e][1,2,4]triazolo[1,5-c]pyrimidine (**5c**)*

Yellow solid, yield: 90 mg (45%), mp: 273 C, IR (KBr) cm^{-1}: 1598 (C=N), ^{1}H NMR ($CDCl_3$) δ: 7.5 (m, 2H, Ar–H), 7.5–7.7 (m, 3H, Ar–H), 8.2–8.4 (m, 4H, Ar–H), 8.25 (s, 1H, Ar–H), 9.5 (s, 1H, Ar–H). EI-mass: 425 (M^+), HRMS *m/z* calcd. for $C_{21}H_{12}N_5ClF_3$ ($[M+H]^+$): 426.0733. Found 426.0742.

*2-(Furan-2-yl)-8-phenyl-10-(trifluoromethyl)pyrido[3,2-e]-[1,2,4]triazolo[1,5-c]pyrimidine (**5d**)*

White solid, yield: 100 mg (55.8%), mp: 250 C, IR (KBr) cm^{-1}: 1599 (C=N), ^{1}H NMR ($CDCl_3$) δ: 6.59–6.62 (dd, 1H, $J = 3.77$ and 1.51 =C–H), 7.33–7.39 (d, 1H, $J = 3.77$, =C–H), 7.56–7.62 (m, 3H,

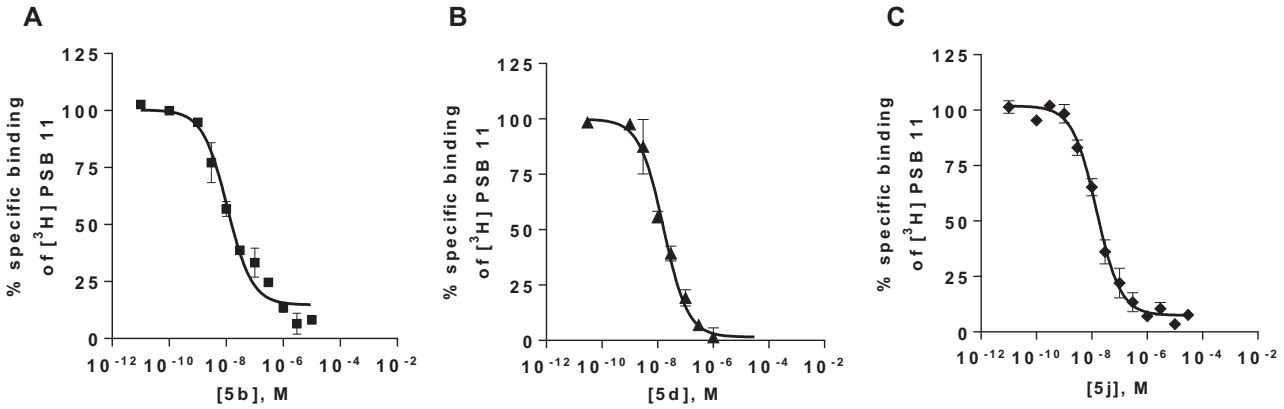


Figure 1. Competition binding curves of compounds **5b**, **5d**, and **5j** at the human A_3AR expressed in CHO cell membranes versus [^{3}H]PSB-11.

Ar–H), 7.67 (d, 1H, $J = 1.51$, =C–H), 8.29–8.34 (m, 2H, Ar–H), 8.44 (s, 1H, Ar–H), 9.51 (s, 1H, Ar–H). ESI-mass: 404 (M^++Na), HRMS *m/z* calcd. for $C_{19}H_{10}N_5OF_3$ ([M+Na]$^+$): 404.0735. Found 404.0743.

*General procedure for the preparation of 2-aryl-5-methyl-8-phenyl-10-(trifluoromethyl)pyrido[3,2-e][1,2,4]triazolo-[1,5-c]pyrimidine (**5e–j**)*

Compound **3b** (142.65 mg, 0.45 mmol) and aroylhydrazide (0.45 mmol) were dissolved in toluene (3 mL) and catalytic amount of isobutyric acid (0.15 mL) was added. After stirring at 60–70°C for 3 h, a solid separated from the solution. The solid was filtered and washed with hot ethanol. The crude product was dried and purified by passing through a column packed with silica gel 60–120 mesh using 10% ethyl acetate in *n*-hexane.

*5-Methyl-2,8-diphenyl-10-(trifluoromethyl)pyrido[3,2-e]-[1,2,4]triazolo[1,5-c]pyrimidine (**5e**)*

White solid, yield: 127 mg (69.6%), mp: 263 C, IR (KBr) cm^{-1}: 1629 (C=N), ^{1}HNMR (CD Cl_3) δ: 3.23 (s, 3H, CH_3), 7.47–7.57 (m, 6H, Ar–H), 8.28–8.32 (m, 2H, Ar–H), 8.36–8.40 (m, 3H, Ar–H). ^{13}C NMR (CF_3COOD, 75 MHz): δ 20.84, 111.54, 122.72 (q, $J = 276.74$ Hz), 122.98, 129.57, 130.19 (2-carbons), 130.96 (6-carbons), 131.06, 132.65 (2-carbons), 134.36, 137.88, 145.05 (q, $J = 37.41$ Hz), 149.38, 161.45, 170.20. ESI-mass: 406 (M^++H), HRMS *m/z* calcd. for $C_{22}H_{15}N_5F_3$ ([M+H]$^+$): 406.1279. Found 406.1271.

*2-(3-Fluorophenyl)-5-methyl-8-phenyl-10-(trifluoromethyl)-pyrido[3,2-e][1,2,4]triazolo[1,5-c]pyrimidine (**5f**)*

White solid, yield: 140 mg (73.5%), mp: 290 C, IR (KBr) cm^{-1}: 1624 (C=N), ^{1}H NMR ($CDCl_3$) δ: 3.22 (s, 3H, CH_3), 7.15–7.24 (m, 1H, Ar–H), 7.49–7.62 (m, 4H, Ar–H), 8.02–8.13 (dd, 1H, $J = 1.51$ and 8.6 Ar–H), 8.16–8.23 (d, 1H, $J = 7.74$, Ar–H), 8.27–8.36 (m, 2H, Ar–H), 8.4 (s, 1H, Ar–H). ^{13}C NMR (CF_3COOD, 75 MHz): 20.86, 111.88, 116.77, 117.08, 120.93, 121.22, 122.69 (q, $J = 278.94$ Hz), 123.05, 126.07, 130.99 (2-carbons), 132.06, 132.69 (2-carbons), 132.82, 137.95, 145.35 (q, $J = 36.86$ Hz), 149.45, 161.36, 161.70, 167.07, 169.34. ESI-mass: 424 ([M+H]$^+$), HRMS *m/z* calcd. for $C_{22}H_{14}N_5F_4$ ([M+H]$^+$): 424.1185. Found 424.1177.

*2-(3-Chlorophenyl)-5-methyl-8-phenyl-10-(trifluoromethyl)-pyrido[3,2-e][1,2,4]triazolo[1,5-c]pyrimidine (**5g**)*

White solid, yield: 160 mg (81%), mp: 275 C, IR (KBr) cm^{-1}: 1623 (C=N), ^{1}H NMR ($CDCl_3$) δ: 3.23 (s, 3H, CH_3), 7.46–7.49 (m, 2H, Ar–H), 7.54–7.59 (m, 3H, Ar–H), 8.27–8.33 (m, 3H, Ar–H), 8.36 (s, 1H, Ar–H), 8.4 (s, 1H, Ar–H). ^{13}C NMR (CF_3COOD, 75 MHz): δ 20.92, 111.84, 114.02, 122.67 (q, $J = 276.19$ Hz), 123.11, 128.27, 129.89, 130.83, 131.01 (2-carbons), 131.72, 132.26, 132.71 (2-carbons), 134.04, 137.26, 137.99, 145.35 (q, $J = 37.41$ Hz), 149.39, 161.33, 161.58, 168.81. EI-mass: 439 (M^+), HRMS *m/z* calcd. for $C_{22}H_{14}N_5F_3Cl$ ([M+H]$^+$): 440.0889. Found 440.0882.

*2-(4-Chlorophenyl)-5-methyl-8-phenyl-10-(trifluoromethyl)-pyrido[3,2-e][1,2,4]triazolo[1,5-c]pyrimidine (**5h**)*

White solid, yield: 145 mg (73.4%), mp: 276 C, IR (KBr) cm^{-1}: 1628 (C=N), ^{1}H NMR ($CDCl_3$) δ: 3.23 (s, 3H, CH_3), 7.49–7.58 (m, 5H, Ar–H), 8.35–8.40 (m, 5H, Ar–H). ^{13}C NMR (CF_3COOD, 75 MHz): δ 20.56, 122.87, 123.27 (q, $J = 277.84$ Hz), 128.29, 130.92 (3-carbons), 131.28 (3-carbons), 131.50 (3-carbons), 132.59 (3-carbons), 137.90, 141.15, 145.09 (q, $J = 37.41$ Hz), 149.33, 161.51, 169.50. ESI-mass: 440 (M^++H), HRMS *m/z* calcd. for $C_{22}H_{14}N_5F_3Cl$ ([M+H]$^+$): 440.0889. Found 440.0875.

*2-(4-Fluorophenyl)-5-methyl-8-phenyl-10-(trifluoromethyl)-pyrido[3,2-e][1,2,4]triazolo[1,5-c]pyrimidine (**5i**)*

Orange yellow, yield: 130 mg (68.28%), mp: 264 C, IR (KBr) cm^{-1}: 1596 (C=N), ^{1}H NMR ($CDCl_3$) δ: 3.21 (s, 3H, CH_3), 7.17–7.24 (t, 2H, $J = 8.4$, Ar–H), 7.52–7.61 (m, 3H, Ar–H), 8.27–8.34 (m, 2H, Ar–H), 8.35–8.44 (m, 3H, Ar–H). ^{13}C NMR (CF_3COOD, 75 MHz): δ 20.79, 111.64, 117.86, 118.13, 122.72 (q, $J = 275.64$ Hz), 122.97, 126.18, 130.95 (4-carbons), 132.54, 132.63 (4-carbons), 137.84, 145.16 (q, $J = 37.96$ Hz), 149.44, 161.46, 166.21, 169.57. ESI-mass: 424 (M^++H), HRMS *m/z* calcd. for $C_{22}H_{14}N_5F_4$ ([M+H]$^+$): 424.1185. Found 424.1267.

*2-(Furan-2-yl)-5-methyl-8-phenyl-10-(trifluoromethyl)-pyrido[3,2-e][1,2,4]triazolo[1,5-c]pyrimidine (**5j**)*

White solid, yield: 115 g (64.68%), mp: 296 C, IR (KBr) cm^{-1}: 1599 (C=N), ^{1}H NMR ($CDCl_3$) δ: 3.2 (s, 3H, CH_3), 6.58–6.62 (m, 1H, =C–H),

7.31–7.38 (d, 1H, $J = 3.2$, =C–H), 7.52–7.58 (m, 3H, Ar–H), 7.66 (m, 1H, =C–H), 8.25–8.35 (m, 2H, Ar–H), 8.38 (s, 1H, Ar–H). ^{13}C NMR (CF_3COOD, 75 MHz): δ 21.13, 111.49, 114.57, 119.15, 122.64 (q, $J = 276.19$ Hz), 123.17, 130.75, 131.08 (3-carbons), 132.68 (3-carbons), 138.10, 145.25, 145.61 (q, $J = 37.96$Hz), 148.80, 149.74, 161.08, 161.84. ESI-mass: 396 (M^++H), HRMS *m/z* calcd. for $C_{20}H_{13}N_5OF_3$ ($[M+H]^+$): 396.1072. Found 396.1082.

*General procedure for the preparation of 2-aryl-5-methyl-8-phenyl-10-(trifluoromethyl)pyrido[3,2-e][1,2,4]triazolo-[1,5-c]pyrimidine (**5k–o**)*

To a solution of ethyl-3-cyano-6-phenyl-4-(trifluoromethyl)pyridine-2-ethanamidate **3b** (2.40 mmoles) in *N*,*N*-dimethyl formamide (5 mL) was added aroylhydrazide (2.7 mmol) and the resulting mixture was heated at 120°C for 2–4 h, under a nitrogen atmosphere. After completion of reaction, it was filtered, washed with hot ethanol, and dried. The crude product was further purified by passing through a column packed with silica gel 60–120 mesh using 10% ethylacetate in *n*-hexane.

*2-(2-Chlorophenyl)-5-methyl-8-phenyl-10-(trifluoromethyl)-pyrido[3,2-e][1,2,4]triazolo[1,5-c]pyrimidine (**5k**)*

Yield: 0.780 g (74%), mp: 258–260 C, IR (KBr) cm^{-1}: 1594 (C=N), ^{1}H NMR (400 MHz, $CDCl_3$) δ: 3.16 (s, 3H, CH_3), 7.36–7.37 (m, 2H, Ar–H), 7.49–7.52 (m, 4H, Ar–H), 8.15–8.16 (m, 1H, Ar–H), 8.23–8.26 (m, 2H, Ar–H), 8.34 (s, 1H, Ar–H) ppm. ^{13}C NMR (CF_3COOD, 75 MHz): δ 20.81, 111.16, 112.26, 117.40, 122.45 (q, $J = 277.01$ Hz), 128.85 (2-carbons), 128.98, 129.83, 130.90, 132.12, 132.48 (2-carbons), 132.95, 133.08, 134.37, 135.95, 136.78, 143.68 (q, $J = 37.23$ Hz), 151.80, 156.41, 167.56. MS [ES^+ (*m/z*, % relative intensity)]: 480.1 (M^++1+K, 30), 482.2 (M^++2+K, 10), 462.1 (M^++1+Na, 100), 464.1 (M^++2+Na, 25), 440.1 (M+H^+, 30), 442.2 (M^++2, 6).

*2-[5-Methyl-8-phenyl-10-(trifluoromethyl)pyrido[3,2-e]-[1,2,4]triazolo[1,5-c]pyrimidin-2-yl]phenol (**5l**)*

Yield: 0.680 g (68%), mp: 272–275 C, IR (KBr) cm^{-1}: 3030 (–OH), 1621 (C=N), ^{1}H NMR (400 MHz, $CDCl_3$) δ: 3.18 (s, 3H, CH_3), 6.93–6.99 (m, 2H, Ar–H), 7.33–7.42 (m, 2H, Ar–H), 7.53–7.54 (m, 2H, Ar–H), 8.22 (m, 1H, Ar–H). 8.27–8.29 (m, 2H, Ar–H), 8.44 (s, 1H, Ar–H), 11.10 (s, 1H, OH). ^{13}C NMR (CF_3COOD, 75 MHz): δ 20.78, 113.38, 119.20, 122.44 (q, $J = 277.84$ Hz), 129.02 (2-carbons), 130.97 (2-carbons), 132.12 (2-carbons), 132.47 (2-carbons), 136.10,137.18, 138.77, 145.73 (q, $J = 37.41$ Hz), 152.18, 155.38, 158.12, 158.64, 160.36, 166.60. MS [ES^+ (*m/z*, % relative intensity)]: 422.1 (M^++H, 100).

*5-Methyl-2-(4-nitrophenyl)-8-phenyl-10-(trifluoromethyl)-pyrido[3,2-e][1,2,4]triazolo[1,5-c]pyrimidine (**5m**)*

Yield: 0.640 g (64%), mp: 268–270 C, IR (KBr) cm^{-1}: 1629 (C=N), ^{1}H NMR (400 MHz, $CDCl_3$): δ 3.33 (s, 3H, CH_3), 7.52–7.54 (m, 3H, Ar–H), 8.28–8.30 (m, 2H, Ar–H), 8.34–8.36 (m, 2H, Ar–H), 8.45 (s, 1H, Ar–H), 8.52–8.55 (m, 2H, Ar–H). ^{13}C NMR (CF_3COOD, 75 MHz): δ 20.81, 111.84, 122.47 (q, $J = 275.64$ Hz), 123.12, 125.95 (2-carbons), 130.72, 130.90 (2-carbons), 131.06 (2-carbons), 132.39 (2-carbons), 136.80, 137.60, 145.01 (q, $J = 37.96$ Hz), 149.43, 151.43, 151.81, 161.19, 161.54, 167.40. MS [ES^+ (*m/z*, % relative intensity)]: 451.1 (M^++H, 100).

*5-Methyl-8-phenyl-2-(thiophen-2-yl)-10-(trifluoromethyl)-pyrido[3,2-e][1,2,4]triazolo[1,5-c]pyrimidine (**5n**)*

Yield: 0.685 g (69%), mp: 270–272 C, IR (KBr) cm^{-1}: 1627 (C=N), ^{1}H NMR (400 MHz, $CDCl_3$) δ: 3.20 (s, 3H, CH_3), 7.18–7.20 (m, 1H, HC-thienyl), 7.51–7.52 (m, 1H, HC-thienyl), 7.55–7.59 (m, 3H, Ar–H), 8.02–8.04 (m, 1H, HC-thienyl), 8.30–8.32 (m, 2H, Ar–H), 8.39 (s, 1H, Ar–H). ^{13}C NMR (CF_3COOD, 75 MHz): δ 20.60, 110.75, 122.44 (q, $J = 275.09$ Hz), 122.68, 128.89, 130.24, 130.77 (2-carbons), 131.47, 132.12 (2-carbons), 132.49, 132.87, 133.11, 137.06, 143.72 (q, $J = 37.41$ Hz), 148.98, 149.06, 160.36, 165.47. MS [ES^+ (*m/z*, % relative intensity)]: 412.1 (M^++H, 100).

*5-Methyl-8-phenyl-2-(pyridin-3-yl)-10-(trifluoromethyl)-pyrido[3,2-e][1,2,4]triazolo[1,5-c]pyrimidine (**5o**)*

Yield: 0.722 g (74%), mp: 283–285 C, IR (KBr) cm^{-1}: 1625 (C=N), ^{1}H NMR (400 MHz, $CDCl_3$) δ: 3.24 (s, 3H, CH_3), 7.57–7.59 (m, 3H, Ar–H), 8.26–8.28 (m, 2H, Ar–H), 8.30–8.33 (m, 2H, Ar–H), 8.42 (s, 1H, Ar–H), 8.82–8.83 (m, 2H, Ar–H). ^{13}C NMR (CF_3COOD, 75 MHz): 21.02, 109.45, 112.06, 121.79 (q, $J = 276.19$ Hz), 123.83, 126.80, 129.72, 131.15, 131.60, 132.10, 135.56 (5-carbons), 144.03 (3-carbons), 145.05 (q, $J = 37.96$ Hz), 158.18, 158.57. MS [ES^+ (*m/z*, % relative intensity)]: 407.1 (M^++H, 100).

Radioligand binding studies

Materials

[^{3}H]CCPA ([^{3}H]2-chloro-N^6-cyclopentyladenosine) was obtained from NEN Life Sciences (58 Ci/mmol), [^{3}H]MSX-2 ([^{3}H]3-(3-hydroxypropyl)-7-methyl-8-(*m*-methoxystyryl)-1-propargylxanthine) from Amersham (84 Ci/mmol), [^{3}H]PSB-603 (8-(4-(4-(4-chlorophenyl)-piperazine-1-sulfonyl)phenyl)-1-propylxanthine) from GE Healthcare (73 Ci/mmol), and [^{3}H]PSB-11 ([^{3}H]-8-ethyl-4-methyl-2-phenyl-(8*R*)-4,5,7,8-tetrahydro-1*H*-imidazo[2,1-*i*]-purin-5-one) from Quotient Biosciences (53 Ci/mmol). The precursors of the radioligands were synthesized at the University of Bonn [24, 28, 29].

Membrane preparations for A_1, A_{2A}, A_{2B}, and A_3 receptor assays

Frozen rat brains were obtained from PelFreez (Rogers, AR, USA). Rat brains were dissected to obtain cortical membrane preparations for A_1 and striatal membrane preparations for A_{2A} assays as previously described [28, 30–34]. Membranes of CHO cells expressing the human A_{2B} receptor or the human A_3 receptor were prepared by scraping the cells off the previously frozen cell culture dishes in ice-cold hypotonic buffer (5 mM Tris–HCl, 2 mM EDTA, pH 7.4). The cell suspension was homogenized on ice for 20 s with an Ultra-Turrax and spun down for 10 min (4°C) at 1000*g*. The supernatant was subsequently centrifuged for 60 min at 48000*g*. The obtained membrane pellets were re-suspended in 10 mL of 50 mM Tris–HCl buffer, pH 7.4 and centrifuged once more under the same conditions. Then the membrane pellets were re-suspended and homogenized in the required amount of 50 mM Tris–HCl buffer, pH 7.4 to obtain a protein concentration of 1–3 mg/mL. The protein concentration was determined by the method of Lowry et al. using bovine serum albumin as a standard reference. Aliquots of the membrane preparation (1 mL each) were stored at −80°C until they were used in the binding assays.

A_1 and A_{2A} radioligand binding assays

Binding assays for A_1 and A_{2A} were performed essentially as described in the literature [29, 30]. Stock solutions of the

compounds were prepared in dimethyl sulfoxide (DMSO), the final concentration of DMSO in the assay being 2.5%. Membrane preparations (30 μg/vial) were incubated in 96-well plates with 0.5 nM [^{3}H]CCPA (rat A_1) or 1.0 nM [^{3}H]MSX-2 (rat A_{2A}) in 50 mM Tris–HCl, pH 7.4 in a total volume of 200 μL. Incubation was carried out at room temperature for 90 (rat A_1) or 30 min (rat A_{2A}), respectively. Nonspecific binding was determined in the presence of 10 μM CADO (2–chloroadenosine) for rat A_1 or 10 μM CGS15943 (9-chloro-2-(2-furanyl)-[1,2,4]triazolo[1,5-*c*]quinazolin-5-amine) for rat A_{2A}. The incubation was terminated by rapid filtration through GF/B glass filters (Whatman, Dassel, Germany) using a Brandel cell harvester (Brandel, Gaithersburg, MD). Scintillation cocktail was added to the filter plates (40 μL Microscent-20 per well) and after an incubation of 9 h filter-bound radioactivity was measured by liquid scintillation counting.

A_{2B} radioligand binding assays

Competition experiments with [^{3}H]PSB-603 were performed in a final volume of 1000 μL containing 10 μL of test compound, 790 μL of buffer (50 mM Tris–HCl, pH 7.4), 100 μL of radioligand solution (final concentration 0.3 nM), and 100 μL of membrane preparation (30 μg protein per vial, pre-incubated with 2 U ADA/mg protein). The final DMSO concentrations did not exceed 1%. Nonspecific binding was determined in the presence of 10 μM 8-cyclopentyl-1,3-dipropylxanthine (DPCPX). After an incubation time of 75 min at room temperature, the assay mixture was filtered through GF/B glass fiber filters using a Brandel harvester (Brandel). Filters were washed four times (3–4 mL each) with ice-cold 50 mM Tris–HCl buffer, pH 7.4, containing 0.1% bovine serum albumin (BSA) and transferred to mini vials. Two milliliters scintillation cocktail (Ultima Gold, Canberra Packard) was added and after an incubation of 9 h radioactivity was counted in a liquid scintillation counter at an efficiency of 54%.

A_3 radioligand binding assays

Binding studies with the A_3AR radioligand [^{3}H]phenyl-8-ethyl-4-methyl-(8*R*)-4,5,7,8-tetrahydro-1*H*-imidazo[2,1-*i*]purine-5-one ([^{3}H]-PSB-11, 1 nM) were performed in Tris–HCl buffer (50 mM Tris, pH 7.4) containing 100 μg of protein per vial (pre-incubated with 2U ADA/mg protein) in a final volume of 400 μL. Nonspecific binding was determined in the presence 100 μM *R*-PIA (N^6-[(*R*)-phenylisopropyl]adenosine). After 45 min of incubation at room temperature to allow equilibrium to be reached, bound and free radioactivity were separated by filtering the assay mixture through Whatman GF/B glass fiber filters using a Brandelcell harvester (Brandel). Filters were rinsed three times with 2 mL of ice-cold Tris–HCl buffer (50 mM, pH 7.4) each and incubated for 9 h with scintillation cocktail (Ultima Gold, Canberra Packard) before radioactivity was counted by liquid scintillation counting. The compounds that inhibited the specific [^{3}H]PSB-11 binding by >50% at a concentration of 1 μM in the initial screening were subsequently re-analyzed at a range of concentrations to determine their IC_{50} values. Curves were determined using seven to nine different concentrations of test compounds spanning 3 orders of magnitude.

Data analysis

Data were analyzed using Graph Pad PRISM version 6.0 (San Diego, CA). For nonlinear regression analysis, the Cheng–Prusoff equation and K_D values of 0.2 nM (rat A_1) for [^{3}H]CCPA and 4.9 nM (human A_3) for [^{3}H]PSB-11, respectively, were used to calculate K_i values from IC_{50} values.

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