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DIIMIDAZO[1,2-*c*:4',5'-*e*]PYRIMIDINES: ADENOSINE AGONIST ACTIVITY DEMONSTRATED BY MICROPHYSIOMETRY

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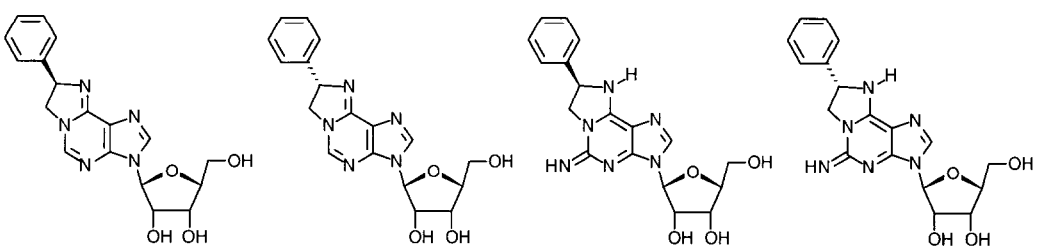
Abstract: Silicon-based microphysiometry, measuring extracellular acidification rate of cells in culture, demonstrated that a series of diimidazo[1,2-*c*:4',5'-*e*]pyrimidines were agonists at the human adenosine A_1 receptor. 5-amino-7,8-dihydro-3-ribofuranose-8-(*R*)-(phenyl)-3H-diimidazo[1,2-*c*:4',5'-*e*]pyrimidine (**2a**) had an EC_{50} of 100 μM and reached 90% of the E_{max} produced by R-PIA.

In order to retain agonist activity at adenosine receptors, only minor modification of the adenosine structure is permitted.[1] While broader modifications are tolerated at N^6 and C2, the ribose moiety cannot be extensively varied. N^6-(*R*)-(Phenylisopropyl) adenosine [R-PIA] is a potent and selective adenosine A_1 receptor agonist.[1] In a series of six diimidazo[1,2-*c*:4',5'-*e*]pyrimidines conformationally restricted N^6-N1 cyclic adenosine derivatives, we recently reported that **1a** and **1b** had high affinity for the rat A_1 receptor.[2]



1a **1b** **2a** **2b**

Compounds **1a** and **1b** lack an N^9-hydrogen atom, which may be important for receptor binding.[1,3] The corresponding 5-amino compounds **2a** and **2b** were synthesised, as tautomerism may allow availability of an N^6-hydrogen. We now report the functional responses as indicated by microphysiometry for the four compounds **1a**, **1b**, **2a** and **2b** and compare with their corresponding binding affinites.

Compounds **2a** and **2b** were synthesised starting from guanosine triacetate following the same strategy as described for **1**.[2] Guanosine triacetate was treated with phosphorous oxychloride, tetraethylammonium chloride and N,N-dimethylanilineacetonitrile at 80 °C for 15 minutes to yield the 6-chloro derivative in 70%. All subsequent steps were as previously described.[2] Amino alcohols (*R*)-phenylglycinol and (*S*)-phenylglycinol were employed for preparation of **2a** and **2b**, respectively.[4,5]

Efficacy of compounds was investigated with biosensor technology using a four channel Cytosensor® Microphysiometer (Molecular Devices, Sunnyvale, CA). In cultured cells, agonist binding to cognate receptors

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produces changes in extracellular acidification rate (ECAR), which can be monitored in real time.[6,7] The application of this method for detecting functional ligand-receptor interactions was validated using human adenosine A_1 receptors stably expressed in CHO.K1 (Chinese Hamster Ovary) cells.[8] Concentration response curves for a series of adenosine agonists were investigated[9] and agonist potency rank order was determined to be R-PIA > NECA (N-ethylcarboxyamidoadenosine) > S-PIA > CGS21680. This rank order correlates with the distinct agonist affinity order found in rat brain as shown previously in adenosine A_1 binding.[10] This rank order also correlates with agonist potency as found from experiments with [^{35}S]GTPγS in rat brains, which demonstrated cell signalling mediated through the modulation of G proteins by agonist activation of adenosine A_1 receptors[11] (Table 1). The adenosine A_1 selective antagonist DPCPX (1,3-dipropyl-8-cyclopentylxanthine) was also investigated.[9] DPCPX concentrations of 10 nM, 100 nM and 1 μM produced concentration dependent parallel rightward shifts of the R-PIA concentration response curve. Antagonism mediated by all concentrations of DPCPX was surmountable by R-PIA and Schild analysis of these results gave an r^2 value of 0.99, thus defining DPCPX as a competitive antagonist. Receptor affinity for DPCPX from microphysiometry was comparable to that found by measurements of isometric tension in organ bath experiments using rat heart muscle[12] (Table 1). These results have indicated that real time measurements of extracellular acidification rates is a fast and appropriate method for detecting functional responses from activated cells.

Table 1. Comparison of functional data for standard adenosine compounds obtained from G-protein activation and microphysiometry.

Standard Compound	[^{35}S]GTPγS binding[11,a] EC_{50} nM	Microphysiometry[b] EC_{50} nM
R-PIA	16.5 (12.7–21.4)	28 (24.6–33)
NECA	87.2 (77.8–97.8)	59 (33.9–103.3)
S-PIA	1,231 (900–1,684)	869 (498.5–1,516)
CGS21680	>100,000	6,437 (5,309–7,804)
	atria isometric tension[12,a] pA_2	Microphysiometry[b] pA_2
DPCPX	8.24 (7.33–9.42)	8.43

[a]Rat brain adenosine A_1 receptors. [b]Human adenosine A_1 receptors expressed in CHO.K1.

All four compounds showed an increase from baseline ECAR levels at 100 μM (Table 2). Peak responses recorded were reached at 6 to 7 minutes exposure time. Desensitization of cells was observed with exposures greater than 7.5 minutes with peak responses declining by approximately 50% at 20 minute exposure time. These responses are consistent with recorded time-to-peak response and observed desensitisation of the potent A_1 agonist R-PIA.

Using adenosine A_1 receptors from rat brains,[13] binding of the four compounds to adenosine A_1 receptors was observed with the following rank order : **2a** = **2b** > **1a** = **1b** (Table 2). Compounds **2a** and **2b**, containing the tautomerizable 5-amino moiety, showed higher affinity for the adenosine A_1 receptor than those analogues that lack the 5-amino group (**1a** and **1b**).

Table 2. Effects of diimidazo[1,2-*c*:4',5'-*e*]pyrimidines on [^{3}H]R-PIA binding and Extracellular Acidification Rate

Compound	Receptor Binding[a] K_i μM	Microphysiometry[b] 100 μM % of basal ECAR
1a	3.82 ± 0.41	113
1b	4.54 ± 0.47	106
2a	1.02 ± 0.12	122
2b	0.61 ± 0.19	117

[a]Rat brain adenosine A_1 receptors. [b]Human adenosine A_1 receptors expressed in CHO.K1 cells.

Compound **2a** was shown to be an agonist with an EC_{50} of 100 μM, reaching a plateau at 90% of the E_{max} produced by R-PIA. R-PIA had an EC_{50} of 33 nM (Figure 1).

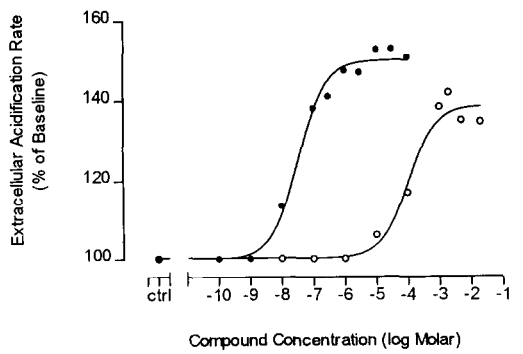

Figure 1. Stimulation of extracellular acidification rate (ECAR) mediated by R-PIA (closed circles) and **2a** (open circles) on recombinant human adenosine A_1 receptors.

The 5-amino compounds **2a** and **2b** had higher affinity at the rat A_1 receptor than the unsubstituted analogues **1a** and **1b**. Microphysiometry established that the diimidazo[1,2-*c*:4',5'-*e*]pyrimidine **2a** bearing a 3-ribose moiety mediated cellular responses through activation of the A_1 receptor, retaining the agonist properties of N^6-substituted adenosine analogues.

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4. Spectral data for **2a**: 5-amino-7,8-dihydro-3-ribofuranose-8-(*R*)-(phenyl)-3H-diimidazo[1,2-*c*:4',5'-*e*] pyrimidine.

^{1}H NMR (600 MHz, DMSO-d_6) δ 3.56–3.64 (m, 3H, 2*C$H_{5'}$ and NCHH), 3.86 (m, 1H, C$H_{4'}$), 4.10 (br. t, 1H, J = 4.0 Hz, C$H_{3'}$), 4.27 (t, 10.5 Hz, NCHH), 4.41 (br. t, 1H, J = 5.4 Hz, C$H_{2'}$), 5.11-5.20 (br. hump, 3H, OH), 5.31 (dd, 1H, J = 7.4 Hz, 10.4 Hz, CH_2CH), 5.44 (br. hump, 1H, OH), 5.68 (d, 1H, J = 5.9 Hz, C$H_{1'}$), 6.79 (br. s, 2H, NH_2), 7.26-7.36 (m, 5H, CH_{arom}), 7.86 (s, 1H, H_2). ^{13}C NMR (150 MHz, DMSO-d_6) δ 52.6 (C_7), 61.4 ($C_{5'}$), 67.0 (C_8), 70.3 ($C_{3'}$), 73.5 ($C_{2'}$), 85.0 ($C_{4'}$), 86.4 ($C_{1'}$), 112.1 (C_{1a}), 126.4 (C_{ortho}), 126.9 (C_{para}), 128.3 (C_{meta}), 134.6 (C_2), 144.6 (C_{ipso}), 148.5 (C_{10}), 150.2 (C_{3a}), 151.2 (C_5). MS (electrospray) m/z 385.5 (M^+ + 1).

5. Spectral data for **2b**: 5-amino-7,8-dihydro-3-ribofuranose-8-(*S*)-(phenyl)-3H-diimidazo[1,2-*c*:4',5'-*e*] pyrimidine.
^{1}H NMR (200 MHz, DMSO-d_6) δ 3.56–3.64 (m, 3H, 2*C$H_{5'}$ and NCHH), 3.86 (dd, 1H, J = 3.6, 7.1 Hz, C$H_{4'}$), 4.08 (br. t, 1H, J = 4.0 Hz, C$H_{3'}$), 4.26 (t, 11.0 Hz, NCHH), 4.38 (br. t, 1H, J = 5.2 Hz, C$H_{2'}$), 5.11 (br. hump, 1H, OH), 5.14 (br. hump, 1H, OH), 5.29 (dd, 1H, J = 7.5 Hz, 10.5 Hz, CH_2CH), 5.44 (br. hump, 1H, OH), 5.93 (d, 1H, J = 5.9 Hz, C$H_{1'}$), 6.77 (br. s, 2H, NH_2), 7.27–7.37 (m, 5H, CH_{arom}), 7.85 (s, 1H, H_2); ^{13}C NMR (150 MHz, DMSO-d_6) δ 52.5 (C_7), 61.7 ($C_{5'}$), 67.2 (C8), 70.2 ($C_{3'}$), 73.9 ($C_{2'}$), 85.0 ($C_{4'}$), 86.7 ($C_{1'}$), 112.1 (C_{1a}), 126.1 (C_{ortho}), 126.5 (C_{para}), 128.5 (C_{meta}), 134.3 (C_2), 144.5 (C_{ipso}), 148.1 (C_{10}), 150.1 (C_{3a}), 150.7 (C_5). MS (electrospray) m/z 385.5 (M^+ + 1).

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9. CHO.K1 cells stably expressing human adenosine A_1 receptors were cultured as a monolayer in 1:1 DMEM/F-12 (Ham's) medium supplemented with 10% fetal bovine serum and 50 IU per mL penicillin/50 μg per mL streptomycin, and maintained at 37 °C in a 5% CO_2, humidified environment. Immediately prior to experiments, near confluent cultures were detached with PBS containing EDTA.Na_2 (0.2 mg/mL) and cell viability determined by exclusion of 0.4% Trypan Blue. Cells were centrifuged at 560 g for 10 minutes at 23 °C and the pellet resuspended in running medium to 2 x 10^{-6} cells/150 μL. Running medium was prepared in advance as low buffered DMEM 5X concentrate (with L-glutamine, with 22.5 g/L D-glucose, with 550 mg/L sodium pyruvate, without sodium bicarbonate), diluted 1:4 with dH_2O, and 11.1 mL/L NaCl (4M) solution. pH was adjusted with NaOH to 7.4 at 37°C prior to filter sterilisation. DMSO to a final of 1% v/v was added on the day of experiment. Under sterile conditions, an aliquot of cell suspension was mixed with 0.8% low melting pointing agarose (agarose was maintained at 37 °C prior to use), and loaded into 3μm porous transwell capsule cups (Costar). Assembled capsule cups were mounted into Microphysiometer sensor chambers and cells allowed to establish a baseline for approximately 90 min. Running medium was maintained at 37 °C and cells were perfused at a flow rate of 100 μL/min. A 30 second interruption in flow every 2 min, allowed the determination of the ECAR from 8-28 seconds of this interval. Cells were sequentially exposed to increasing concentrations of agonist in 1% DMSO for 6-7.5 min and the peak ECAR response used to plot concentration response curves. Cells were allowed to recover for approximately 45 min between doses. Adenosine receptor agonists (R-PIA, NECA, S-PIA, CGS21680) were evaluated from three experiments, each with 14 concentration points in single determinations. DPCPX antagonist potency was determined by exposing cells to each dose level of agonist in the presence of a constant concentration of antagonist. This procedure was performed once only for three antagonist concentrations (0.01 μM, 0.1 μM, and 1 μM). Derivatives **1a**, **1b**, **2a**, and **2b** were tested at 100 μM in a single experiment. A concentration response curve was completed once with derivative **2a**. For all experiments, the ECAR was normalised to a 100% baseline value immediately prior to challenging cells with each concentration of compound and the peak responses were manually recorded. Agonist potency (EC_{50}) was determined by least squares, nonlinear regression analysis using GraphPad Prism version 2.0 (San Diego, CA) and antagonist potency (pA_2) by Schild analysis. Results for adenosine receptor ligands are expressed as the mean with 95% confidence intervals.
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