Supporting Information

Structure-Activity Relationships of Truncated D- and L-4'-Thioadenosine Derivatives as Species-Independent A_3 Adenosine Receptor Antagonists

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Content

Pharmacological Methods

[^{125}I]N^6-(4-amino-3-iodobenzyl)adenosine-5′-*N*-methyluronamide (I-AB-MECA; 2000 Ci/mmol), [^{3}H]CCPA (2-chloro-N^6-cyclopentyladenosine, 42.6 Ci/mmol), [^{3}H]CGS21680 (2-[*p*-(2-carboxyethyl)phenylethylamino]-5′-*N*-ethylcarboxamido-adenosine, 47 Ci/mmol) and [^{3}H]cyclic AMP (40 Ci/mmol) were from Amersham Pharmacia Biotech (Buckinghamshire, UK).

Cell culture and membrane preparation

Adherent mammalian cells stably transfected with cDNA encoding the appropriate human AR were cultured in DMEM supplemented with 10% fetal bovine serum, 100 Units/mL penicillin, 100 μg/mL streptomycin, 2 μmol/mL glutamine and 800 μg/mL geneticin. The recombinant A_1, A_{2B}, and A_3 ARs were expressed in CHO (Chinese hamster ovary) cells (Klotz *et al*., *Naunyn-Schmiedeberg's Arch. Pharmacol.* **1998**, *357*, 1-9) and the A_{2A} AR in HEK-293 (human embryonic kidney) cells (Kim *et al*., *J. Med. Chem.* **2000**, *43*, 1165-1172). Cells were harvested by trypsinization. After homogenization and suspension, cells were centrifuged at 500 *g* for 10 min, and the pellet was re-suspended in 50 mM Tris·HCl buffer (pH 7.4) containing 10 mM $MgCl_2$. The suspension was homogenized with an electric homogenizer for 10 sec, and was then re-centrifuged at 20,000 *g* for 20 min at 4 °C. The resultant pellets were resuspended in buffer in the presence of 3 units/mL adenosine deaminase, and the suspension was stored at -80 °C until the binding experiments. The protein concentration was measured using the Bradford assay (Bradford *et al*., *Anal. Biochem.* **1976**, *72*, 248-254).

Cyclic AMP accumulation assay

Intracellular cyclic AMP levels were measured with a competitive protein binding method.[18,19] CHO cell that expressed recombinant the human or rat A_3AR or the human A_1 or A_{2B}AR were harvested by trypsinization. After centrifugation and resuspended in medium, cells were planted in 24-well plates in 1.0 mL medium. After 24 h, the medium was removed and cells were washed three times with 1 mL DMEM, containing 50 mM HEPES, pH 7.4. Cells were then treated with agonists and/or test compounds in the presence of rolipram (10 μM) and adenosine deaminase (3 units/mL). After 45 min forskolin (10 μM) was added to the medium, and incubation was continued an additional 15 min. The reaction was terminated by removing the supernatant, and cells were lysed upon the addition of 200 μL of 0.1 M ice-cold HCl. The cell lysate was resuspended and stored at -20°C. For determination of cyclic AMP production, protein

kinase A (PKA) was incubated with [^{3}H]cyclic AMP (2 nM) in K_2HPO_4/EDTA buffer (K_2HPO_4, 150 mM; EDTA, 10 mM), 20 μL of the cell lysate, and 30 μL 0.1 M HCl or 50 μL of cyclic AMP solution (0-16 pmol/200 μL for standard curve). Bound radioactivity was separated by rapid filtration through Whatman GF/C filters and washed once with cold buffer. Bound radioactivity was measured by liquid scintillation spectrometry.

[^{35}S]GTPγS binding assay

[^{35}S]GTPγS binding was measured by a variation of the method described (Jacobson *et al*., *J. Med. Chem.* **2000**, *43*, 2196-2203). Each assay tube consisted of 200 μL buffer containing 50 mM Tris HCl (pH 7.4), 1 mM EDTA, 1 mM $MgCl_2$, 1 μM GDP, 1 mM dithiothreitol, 100 mM NaCl, 3 U/ml ADA, 0.2 nM [^{35}S]GTPγS, 0.004% 3-[(3-cholamidopropyl) dimethylammonio]propanesulfonate (CHAPS), and 0.5% bovine serum albumin. Incubations were started upon addition of the membrane suspension (CHO cells stably expressing the native human A_3AR, 5 μg protein/tube) to the test tubes, and they were carried out in duplicate for 30 min at 25°C. The reaction was stopped by rapid filtration through Whatman GF/B filters, pre-soaked in 50 mM Tris HCl, 5 mM $MgCl_2$ (pH 7.4) containing 0.02% CHAPS. The filters were washed twice with 3 mL of the same buffer, and retained radioactivity was measured using liquid scintillation counting. Non-specific binding of [^{35}S]GTPγS was measured in the presence of 10 μM unlabelled GTPγS.

Statistical analysis

Binding and functional parameters were calculated using Prism 4.0 software (GraphPAD, San Diego, CA, USA). IC_{50} values obtained from competition curves were converted to K_i values using the Cheng-Prusoff equation (Cheng and Prusoff, *Biochem. Pharmacol.* **1973***, 22*, 3099-3108). Data were expressed as mean ± standard error.

Elemental Analyses Data

Compound **9**: Anal. Calcd for $C_{12}H_{13}ClN_4O_2S$: C, 46.08; H, 4.19; N, 17.91; S, 10.25 Found: C, 46.44; H, 4.08; N, 17.99; S, 10.12.
Compound **10**: Anal. Calcd for $C_9H_9ClN_4O_2S$: C, 39.64; H, 3.33; N, 20.54; S, 11.76. Found: C, 39.87; H, 3.12; N, 20.14; S, 11.57.

Compound **7a**: Anal. calcd for $C_{10}H_{13}N_5O_2S$: C, 44.93; H, 4.90; N, 26.20; S, 12.00. Found: C, 44.89; H, 5.08; N, 25.85; S, 11.73.
Compound **7b**: Anal. Calcd for $C_{16}H_{16}FN_5O_2S$: C, 53.17; H, 4.46; N, 19.38; S, 8.87. Found: C, 53.42; H, 4.47; N, 19.49; S, 8.50.
Compound **7c**: Anal. Calcd for $C_{16}H_{16}ClN_5O_2S$: C, 50.86; H, 4.27; N, 18.53; S, 8.49. Found: C, 51.51; H, 4.32; N, 18.48; S, 8.35.
Compound **7d**: Anal. Calcd for $C_{16}H_{16}BrN_5O_2S$: C, 45.51; H, 3.82; N, 16.58; S, 7.59. Found: C, 45. 57; H, 3.83; N, 16.58; S, 7.57.
Compound **7e**: Anal. Calcd for $C_{16}H_{16}IN_5O_2S$: C, 40.95; H, 3.44; N, 14.92; S, 6.83. Found: C, 41.04; H, 3.43; N, 14.82; S, 6.81.
Compound **7f**: Anal. Calcd for $C_{16}H_{15}BrClN_5O_2S$: C, 42.07; H, 3.31; N, 15.33; S, 7.02. Found: C, 42.08; H, 3.30; N, 15.57; S, 7.01.

Compound **7g**: Anal. Calcd for $C_{16}H_{15}ClIN_5O_2S$: C, 38.15; H, 3.00; N, 13.90; S, 6.37. Found: C, 38.10; H, 3.40; N, 13.76; S, 6.33.