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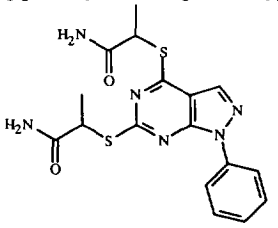
PYRAZOLO[3,4-*d*]PYRIMIDINES: C4, C6 SUBSTITUTION LEADS TO ADENOSINE A_1 RECEPTOR SELECTIVITY

Sally-Ann Poulsen and Ronald J. Quinn*

Queensland Pharmaceutical Research Institute, Griffith University, Brisbane, 4111, Australia

Abstract: Following the demonstration that substitution of 1-phenylpyrazolo[3,4-*d*]pyrimidines at C6 with thioethers containing amide moieties could effect adenosine A_1 and A_{2a} receptor selectivity, two compounds with high A_1 selectivity have been obtained by a combined C4, C6 substitution. This further demonstrates that distal moieties at C6 can effect selectivity and that C4 substitutents have an important role.

Pyrazolo[3,4-*d*]pyrimidines were identified as a general class of adenosine receptor antagonists with 4,6-bis-α-carbamoylethylthio-1-phenylpyrazolo[3,4-*d*]pyrimidine (**1**) having highest affinity at the A_1 receptor.[1,2] Since this report, our group has studied the structure-activity relationships of pyrazolo[3,4-*d*]pyrimidines in detail and have demonstrated that modifications at N1, C4, and C6 all contribute to adenosine A_1 and A_{2a} receptor binding potency and receptor subtype selectivity.[3-5]



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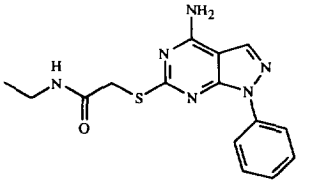

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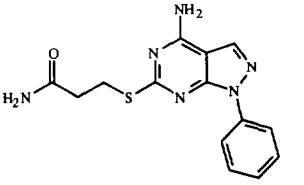

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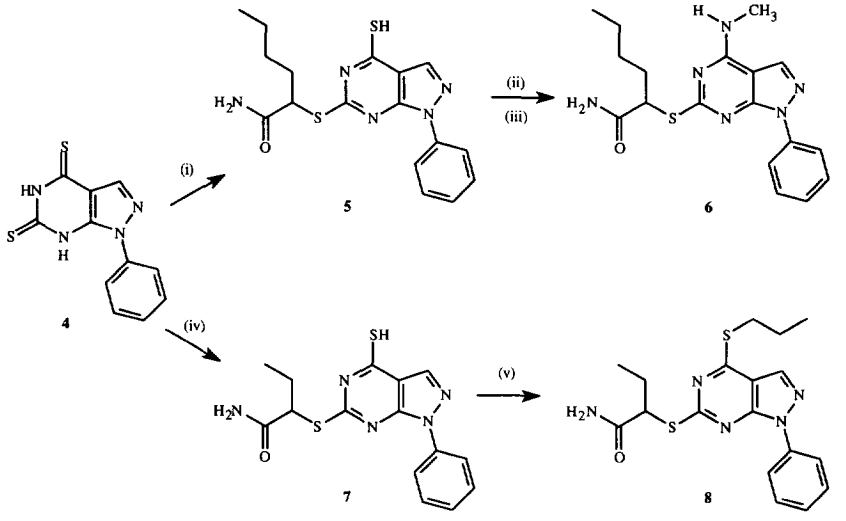
The pharmacophore model of the A_1 and A_{2a} receptor developed by ourselves[6] (a C2-N^6-C8 model[7]) has proposed that adenosine receptor ligands possess a hydrophobic binding domain which binds to a common hydrophobic binding site of the A_1 and A_{2a} receptor subtypes. The C2 substituent (of A_{2a} agonists), the N^6 substituent (of A_1 agonists) and the C8 substituent (of xanthine antagonists) are proposed to be the hydrophobic binding domains of these adenosine receptor ligands. The concept of commonality in hydrophobic residues also led to a pharmacophore model addressing only the A_1 receptor[8] (a N^6-C8 model[7]). Other models have been suggested and, more recently, receptor modelling of the A_1 receptor examined.[9,10] Enhancing receptor binding potency and selectivity via structure-activity studies of adenosine receptor ligands has concentrated on modifications of the hydrophobic substituents. We have demonstrated that there is value in modifying regions of ligands other than in their hydrophobic domain to design selective adenosine receptor ligands.[5]

2'-(4-Amino-1-phenylpyrazolo[3,4-*d*]pyrimidin-6-ylthio)-*N*-ethyl-ethanamide (**2**) had a K_i A_1 of 12.1 ± 4.5 nM and a K_i A_{2a} of 131 ± 36 nM and is 10.8 fold A_1 selective. An increase in the length of the methylene

bridge and a change to a primary amide resulted in 3'-(4-amino-1-phenylpyrazolo[3,4-*d*]pyrimidine-6-ylthio)propanamide (**3**) with a K_i A_1 of 428 ± 25 nM and a K_i A_{2a} of 101 ± 26 nM and is 4.2 fold A_{2a} selective.[5] We now report the synthesis and receptor binding at A_1 and A_{2a} receptors of 1-phenylpyrazolo[3,4-*d*]pyrimidines substituted at C6 with thioethers containing distal amide substitutents and alkyl branching and substituted at C4 with alkylamine or alkylthiol. These compounds are analogues of **1** which was shown to be an A_1 antagonist. They lack the sugar moiety which is a requisite for agonist activity.



(i) $CH_3(CH_2)_3CH(Br)CONH_2$, pyridine, rt; (ii) CH_3I, NaOH (aq), rt; (iii) CH_3NH_2 (g), EtOH, 110 °C; (iv) $CH_3CH_2CH(Br)CONH_2$, pyridine, rt; (iv) $CH_3CH_2CH_2I$, NaOH (aq), 60 °C

1-Phenyl-5H,7H-pyrazolo[3,4-*d*]pyrimidine-4,6-dithione (**4**)[3] (1.500 g, 5.76 mmol) in dry pyridine (17 mL) was treated with 2-bromohexanamide (1.12 g, 5.76 mmol, 1 equiv) in small amounts over ~20 min with continuous stirring at room temperature (rt). After 120 min the precipitate was collected and washed with ice cold water. The precipitate was refluxed in methanol and filtered while hot to remove unreacted starting material. Recrystallization of the precipitate from DMSO and water afforded pure α-(4-mercapto-1-phenylpyrazolo[3,4-*d*]pyrimidin-6-ylthio)hexanamide (**5**) in 80% yield. To **5** (0.400 g, 1.07 mmol) in NaOH (1.5 M, 10 mL, sonication) was added iodomethane (0.608 g, 4.28 mmol, 4 equiv) and the reaction mixture stirred at room temperature for 1 h. The precipitate was collected, and recrystallized from DMSO and water to give the 4-methylthio derivative in 59% yield. This methythio compound (0.190 g, 0.490 mmol) was added to ethanolic methylamine (15 mL, prepared by saturating ethanol at 0 °C with methylamine gas). The solution was placed in a bomb, sealed and heated in an oil bath at 110 °C. After 72 h the bomb was cooled to 0 °C. The product precipitated on cooling, ice cold water was added, the crude product collected and recrystallized from DMSO and water to give α-(4-methylamino-1-phenylpyrazolo[3,4-*d*]pyrimidin-6-ylthio)hexanamide (**6**) in 74% yield.[11]

To **4** (0.406 g, 1.56 mmol) in dry pyridine (10 mL) was added 2-bromobutanamide (0.259 g, 1.56 mmol, 1 equiv) in small amounts over ~15 min with continuous stirring at rt. After 120 min, work-up as above afforded α-(-4-mercapto-1-phenylpyrazolo[3,4-*d*]pyrimidin-6-ylthio)butanamide (**7**) in 73% yield. **7** (0.250 g, 0.72 mmol) was dissolved in NaOH (1.5 M, 20 mL, heated to 60 °C) and propyl iodide (1.230 g, 7.24 mmol, 10 equiv) was added with a syringe. After 1 h, the reaction mixture was cooled and the white product collected by

suction filtration. The crude product was recrystallized from chloroform to give α-(1-phenyl-4-propylthiopyrazolo[3,4-*d*]pyrimidin-6-ylthio)butanamide (**8**) in 54% yield.[12]

Table 1 Receptor binding at rat membrane adenosine A_1 and A_{2a} receptors.[13]

Compound	A_1 receptor K_i nM	A_{2a} receptor K_i nM	K_iA_{2a}/K_iA_1
6	0.74 ± 0.1	246.5 ± 41.7	331
8	29.5 ± 6.6	> 38500	> 13000

α-(4-Methylamino-1-phenylpyrazolo[3,4-*d*]pyrimidin-6-ylthio)hexanamide (**6**) is a potent ligand at the A_1 receptor. It has a K_i A_1 value of 0.74 ± 0.1 nM and is 330-fold selective for the A_1 receptor over the A_{2a} receptor. For comparison the A_1 antagonist 1,3-dipropyl-8-(3-noradamantyl)xanthine (KW-3902), which is currently undergoing clinical trials as a renal protective agent, has a K_i A_1 value of 1.3 ± 0.12 nM and is 290-fold A_1 selective.[14]

α-(1-Phenyl-4-propylthiopyrazolo[3,4-*d*]pyrimidin-6-ylthio)butanamide (**8**) is >13000-fold selective for the A_1 receptor. This selectivity has been achieved with the maintainance of most of the A_1 affinity.

The results prove the value of our approach in modifying substituents other than the hydrophobic binding domain of adenosine receptor ligands. We have generated two ligands which bind with high potency and selectivity to adenosine A_1 receptors compared to adenosine A_{2a} receptors.

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11. *Spectral data for α-(4-methylamino-1-phenylpyrazolo[3,4-d]pyrimidin-6-ylthio)hexanamide* mp 198.3-199.3 °C. ^{1}H NMR (200 MHz, DMSO-d_6): δ 0.87 (t, 3H, J = 7.2 Hz, CH_3); 1.37 (m, 4H, 2 x CH_2); 1.90 (m, 2H, βCH_2); 3.00 (d, 3H, J = 4.6 Hz, NCH_3); 4.36 (t, 1H, J = 7.2 Hz, CH); 7.16 (br s, 1H, NH); 7.35 (t, 1H, J = 7.6 Hz, H-4'); 7.54 (dd, 2H, J = 7.6, 7.6 Hz, H-3', H-5'); 7.68 (br s, 1H, NH); 8.20 (d, 2H, J = 7.6 Hz, H-2', H-6'); 8.27 (s, 1H, H-3); 8.54 (q, 1H, J = 4.6 Hz, NH_{amine}). ^{13}C NMR

(50 MHz, DMSO-d_6): δ 14.0 (CH_3); 22.1 (δCH_2); 27.0 (NCH_3); 29.5 (γCH_2); 32.6 (βCH_2); 48.5 (CH); 99.8 (C-3a); 120.4 (C-2', C-6'); 126.1 (C-4'); 129.1 (C-3', C-5'); 133.8 (C-3); 138.9 (C-1'); 153.0 (C-7a); 156.0 (C-4); 168.9 (C-6); 172.4 (C=O), IR (KBr disc) 3300, NH; 3379, NH,; 3180, NH; 1670, C=O; 1598, C=C; HRMS 370.1567. Calculated for $C_{18}H_{22}N_6OS$: 370.1576.

12. *Spectral data for α-(1-phenyl-4-propylthiopyrazolo[3,4-d]pyrimidin-6-ylthio)butanamide* mp 198.2 - 198.4 °C dec. ^1H NMR (400 MHz, DMSO-d_6): δ 1.00 (t, 3H, J = 7.2 Hz, CH_3); 1.01 (t, 3H, J = 7.2 Hz, CH_3); 1.73 (m, 2H, $SCH_2CH_2CH_3]CH_2$); 1.96 (m, 2H, CH_2); 3.36 (m, 2H, SCH_2); 4.33 (t, 1H, J = 7.2 Hz, CH); 7.24 (br s, 1H, NH); 7.38 (t, 1H, J = 7.6 Hz, H-4'); 7.56 (dd, 2H, J = 8.0/7.6 Hz, H-3', H-5'); 7.74 (br s, 1H, NH); 8.15 (d, 2H, J = 8.0 Hz, H-2', H-6'); 8.46 (s, 1H, H-3), ^{13}C NMR (50 MHz, DMSO-d_6) δ 11.9 (CH_3); 13.2 ($SCH_2CH_2CH_3$); 22.2 ($SCH_2CH_2CH_3$); 25.9 (CH_2); 30.4 ($SCH_2CH_2CH_3$); 50.6 (CH); 110.5 (C-3a); 120.9 (C-2', C 6'); 126.8 (C-4'); 129.4 (C-3', C-5'); 133.9 (C-3); 138.2 (C-1'); 151.0 (C-7a); 165.2 (C-4); 168.1 (C-6); 171.6 (C=O), IR (KBr disc) 3383, NH; 3179, NH; 1652, C=O; 1594, C=C. Anal. Calculated for $C_{18}H_{21}N_5OS_2$: C, 55.8; H, 5.5; N, 18.1; S, 16.5 Found C, 55.8; H, 5.4; N, 17.8; S, 16.6.

13. Receptor binding assays are modifications of methods already published[4,5] adapted to microtitre plates. Compounds were assessed for their ability to inhibit binding of the A_1 agonist radioligand (R)-[^3H]N^6-(phenylisopropyl)adenosine to membranes from rat whole brain at rt.[15] Receptor binding assays were carried out in 96-well microtitre plates in a final assay volume of 200 µL. Immediately prior to assay, membranes were thawed and incubated with adenosine deaminase (2I U/mL, Sigma Type VI) at 37 °C for 10 min in order to remove endogenous adenosine. Each assay contained membrane (100 µg), 1 nM (R) [^3H]N^6-PIA (Amersham, 61 Ci/mmol), incubation buffer and test compound (at least 12 concs.) in DMSO giving a final DMSO concentration of 2 % for (**7**) andr 5 % for (**9**). 2 % DMSO did not decrease control binding, while 5 % DMSO decreased control binding by 17 %. Non-specific binding was defined in the presence of 2-chloroadenosine (10 µM). The assay was incubated for 90 min at 25 °C, then filtered using a cell harvester (Tomtec Harvester 96) onto untreated Glass Fibre filtermats (Wallac Printed filtermat B, size 102 mm x 258 mm). The filtermat was dried at 60 °C for 60 min, soaked with scintillant fluid (Wallac Scintillation Products) in a bag, heat sealed and counted using a liquid scintillation counter (Wallac 1205 Betaplate liquid scintillation counter). Assays were performed three times with duplicate determinations. Results from concentration response curves were analyzed with Graphpad Inplot IV (San Diego, CA), which performs nonlinear regression on data. K_i values were calculated using the Cheng-Prussof equation,[16] using the average K_d of [^3H]PIA of 1 nM.

Compounds were assessed for their ability to inhibit the binding of the A_{2a} agonist radioligand [^3H]CGS 21680 [2-[[*p*-(2-carboxyethyl)phenethyl]amino]-5'-(ethanecarboxamido)adenosine] to rat striatal membranes.[17] Receptor binding assays were carried out in 96-well microtitre plates in a final assay volume of 250 µL. Each assay contained striatal membrane (150 µg), 5 nM [^3H]CGS 21680 (New England Nuclear 48.6 Ci/mmol), incubation buffer (50 mM Tris.HCl, 10 mM $MgCl_2$, pH 7.4) and test compound (at least 12 concs.) in DMSO, giving a final DMSO concentration of 2 % for (**7**) or 5 % for (**9**). 2 % DMSO decreased control binding by 9 %, while 5 % DMSO decreased control binding by 24 %. Non-specific binding was defined in the presence of 2-chloroadenosine (20 µM). The assay was incubated for 120 min at 25 °C. Subsequent harvesting of the assay is identical to that described for the A_1 assay. Assays were performed three times with duplicate determinations. Results from concentration response curves were analyzed with Graphpad Inplot IV (San Diego, CA). K_i values were calculated using the Cheng-Prussof equation,[16] using the average K_d of [^3H]CGS 21680 of 14.9 nM.

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