Pergamon

Bioorganic & Medicinal Chemistry Letters 9 (1999) 2413–2418

BIOORGANIC & MEDICINAL CHEMISTRY LETTERS

SELECTIVE A_1-ADENOSINE RECEPTOR ANTAGONISTS IDENTIFIED USING YEAST *SACCHAROMYCES CEREVISIAE* FUNCTIONAL ASSAYS

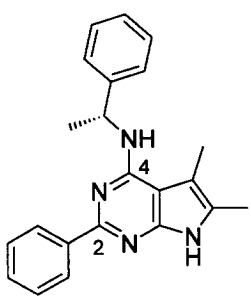
Robert M. Campbell, Craig Cartwright, Wei Chen, Yong Chen, Emir Duzic, Jian-Min Fu, Michelle Loveland, Ron Manning, Bryan McKibben, Christopher M. Pleiman, Lauren Silverman, Joshua Trueheart, David R. Webb, Vicki Wilkinson, David J. Witter, Xiaobing Xie, Arlindo L. Castelhano,*

Cadus Pharmaceutical Corporation, 777 Old Saw Mill River Road, Tarrytown, NY, 10591

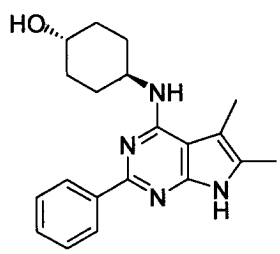
Received 25 May 1999; accepted 12 July 1999

Abstract: Evaluation of a biased "library" of pyrrolo[2,3-d]pyrimidines using yeast-based functional assays expressing human A_1- and A_{2a}-adenosine receptors, led to the A_1 selective antagonist **4b**. A direct correlation between yeast functional activity and binding data was established. Practical compounds with polar residues at C-4 of the pyrrolopyrimidine system required H-bond donor functionality for high potency.

The development of cell-based assays formatted for high-throughput screening can lead to rapid identification of potential drug molecules in chemical libraries.[1] Mammalian and yeast cell-based assays provide an *in vitro* setting that mimics the normal physiological milieu for evaluation of biochemical interactions, bioavailability, and cytotoxicity.[2] Yeast-based assays are an attractive screening tool as they are easy to manipulate, inexpensive to run, grow rapidly, and are tolerant to solvents.[2] With G-protein coupled receptor targets, yeast also offer the advantage of a null background in that only one mammalian receptor is present in the cell. Adenosine receptors provide the unique opportunity to evaluate the interface of the yeast technology with lead discovery. Indeed, we show that yeast functional activity tightly correlates with radioligand binding data and **4b** has been identified as a novel and selective human A_1-adenosine antagonist.



4a



4b

The pursuit of selective agonists and antagonists for the four known subtypes of adenosine receptors (A_1, A_{2a}, A_{2b}, A_3)[3] promises to lead to treatments for disorders of the CNS, renal, and cardiovascular systems with

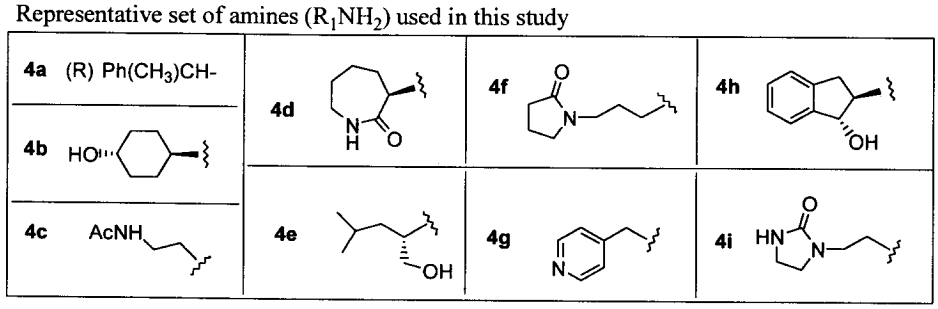
0960-894X/99/$ - see front matter
PII: S0960-894X(99)00398-4

several compounds in clinical evaluation.[3,4] Selective A_1 antagonists may be useful in blocking bronchoconstriction in asthmatics.[3c,5] The less characterized A_{2b} receptor[6] is also gaining prominence because of its putative role in mast cell activation and the potential relevance of this action on asthma.[7] Selective antagonists of this receptor have not yet been reported.

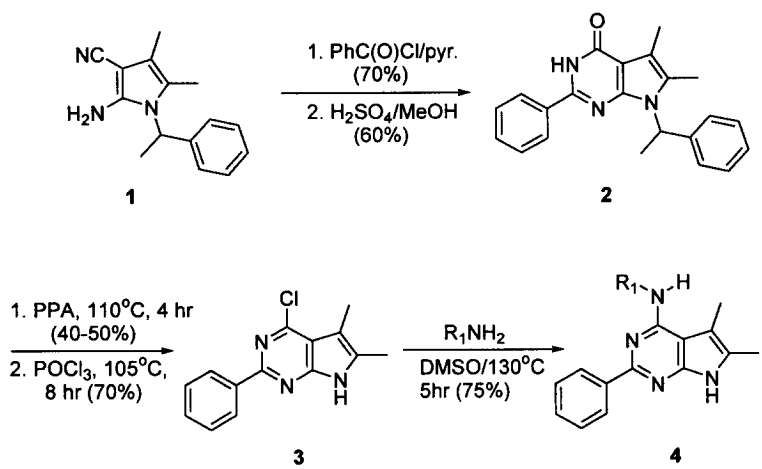
Signaling of human adenosine G-protein coupled receptors has been reconstituted in the yeast *Saccharomyces cerevisae* to yield a facile growth response or a reporter gene readout[8] and used to screen a series of pyrrolo[2,3-d]pyrimidines. Using traditional radioligand binding assays, Muller *et al.*[9] found the pyrrolo[2,3-d]pyrimidine **4a**[9b] to be a potent and selective rat A_1 receptor antagonist exhibiting a K_i of 6.7 nM and a selectivity >4,000 over the rat A_{2A} receptor. Although this level of selectivity is noteworthy, **4a** is highly insoluble. Moreover, the potency and selectivity at the cognate human receptors are modest (Table 1). Nevertheless, we felt that the pyrrolo[2,3-d]pyrimidine core unit would provide a good template for adenosine receptors[10] and assembled a "library" of forty-eight compounds with phenyl at C-2 and various polar elements at C-4 to improve water solubility.

Chemistry: The preparation of the pyrrolo[2,3-d]pyrimidine "library", outlined in Scheme 1, follows the literature method[9b] with key improvements. The one pot assembly of the pyrrole starting material **1** from malononitrile, 1-phenylethylamine, and 3-hydroxy-2-butanone proceeded uneventfully in near quantitative yield. However, cyclization of the N-benzoyl intermediate to the pyrimidinone **2** was problematic using P_2O_5.[9b] Use of 10% conc. H_2SO_4 in refluxing methanol gave better results with **2** isolated in ~60% yield as a precipitate upon cooling. Pyrimidinone **2** was then added portion-wise to polyphosphoric acid at ~110°C with *effective* stirring for 4 hr. On cooling, the resulting syrup was poured into ice water and neutralized with KOH to yield a precipitate in 50% yield. Chlorination in neat $POCl_3$ (25 equiv.) at 105°C for 8 hr followed by concentration in vacuo and water quench, gave the 4-chloropyrimidine **3**, the key intermediate used in the library synthesis. This material is highly insoluble in most organic solvents but in DMSO at 130°C it dissolved and reacted with a set of forty-eight primary amines (2-10 equiv. of amine depending on availability and cost) on a 0.1 mmole scale in >75% yield. Isolation of the desired product **4** involved partitioning between water and ethyl acetate followed by concentration and passage through a short plug of silica gel.

Representative set of amines (R_1NH_2) used in this study



Scheme 1. General Synthesis of pyrrolo[2,3-d]pyrimidine adenosine antagonists



Results and Discussion: The search for drug molecules commonly begins with radioligand binding assays followed by functional activity studies in mammalian cells. However, binding does not discriminate between compounds having mixed agonist or antagonist properties and many early leads fail in functional studies.[11] A better approach would be to identify candidate drug molecules that exhibit the desired function in a dose-dependent manner followed by binding studies to confirm a receptor-mediated event. In this study, *Saccharomyces cerevisiae* strains containing the human adenosine A_1 and A_{2a} receptors[8] were formatted for high throughput screening. Concurrently, radioligand binding assays were performed on membranes from yeast cells transformed with human A_1 receptor and membranes from HEK293 cells stably expressing the human A_{2a} receptor. The established antagonist DPCPX[12] was used as a standard.

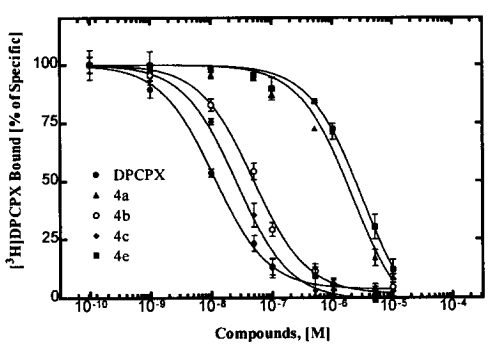


Figure 1. Competition binding profiles of selected compounds for human adenosine A_1 receptors in membrane homogenates from transformed yeast.

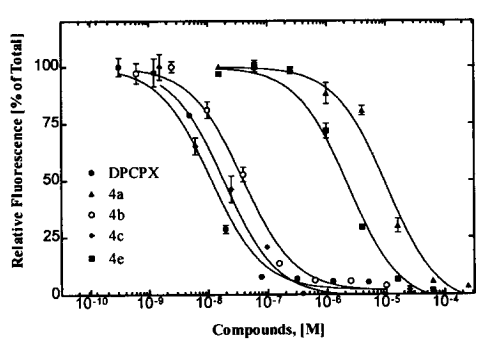


Figure 2. Competition profiles of selected compounds in a yeast functional assay (yeast transformed with the human adenosine A_1 receptor and FUS1-lacZ.

The affinity of the library components for these receptors was assessed by performing competition binding studies against the A_1 and A_{2a} selective ligands, [^{3}H]DPCPX and [^{3}H]CGS-21680,[12] respectively. Typical dose-dependent curves in binding and yeast were generated with active compounds (Figures 1 and 2). The activity observed for the DPCPX standard is comparable to literature reports.[3,12] The rank order of potency observed in the radioligand binding studies is consistent with that observed in the yeast-based functional assays (Table 1). A correlation between yeast and binding IC_{50} data (using either yeast or mammalian membranes) is apparent (Table 1).[13] Using linear regression analysis of yeast vs. binding data for the entire series, r^2 values of 0.93 and 0.88 were obtained for A_1 and A_{2a}, respectively. This indicates that these compounds not only bind adenosine receptors, but exhibit functional antagonist activity.

Table 1. Activities of selected pyrrolo[2,3-d]pyrimidines from a *biased* library in yeast functional assays and radioligand binding assays

Entry	cLog P	(M^++1)	Yeast IC_{50} (nM)		Binding IC_{50} (nM)		Binding K_i (nM)	
			hA_1	hA_{2a}	hA_1	hA_{2a}	hA_1	hA_{2a}
DPCPX	2.5	-	13 ± 1	261 ± 15	14 ± 1	217 ± 22	7 ± 0.5	70 ± 5
4a	6.1	343.2	1607 ± 373	3876 ± 399	1981 ± 22	61% ± 7 [a]	981 ± 55	61% ± 7 [a]
4b	4.4	337.2	40 ± 5	1618 ± 74	49 ± 2	1540 ± 107	25 ± 2	754 ± 59
4c	3	324.5	24 ± 3	48 ± 4	24 ± 2	48 ± 13	12 ± 1	23 ± 7
4d	3.7	350.2	1256 ± 116	2108 ± 185	1535 ± 136	2060 ± 220	771 ± 93	962 ± 48
4e	5	339.5	1754 ± 210	2684 ± 340	1910 ± 597	3405 ± 940	951 ± 303	1136 ± 57
4f	3.5	364.2	5647 ± 1181	58% ± 8 [a]	75% ± 8 [a]	54% ± 1 [a]	75% ± 8 [a]	54% ± 1 [a]
4g	4.4	330.5	222 ± 14	2010 ± 178	262 ± 75	1711 ± 270	132 ± 38	811 ± 173
4h	5.1	371.1	1696 ± 425	3218 ± 475	1810 ± 512	59% ± 4 [a]	896 ± 255	59% ± 4 [a]
4i	2.9	351.3	422 ± 47	725 ± 88	364 ± 151	604 ± 199	144 ± 67	215 ± 98

[a] % inhibition @ 10 μM; Data is represented as Mean ± SEM from at least three independent experiments

The two "hits" from the *biased* library, **4b** and **4c**, were unexpected since previous work[9a,b] with the cognate rat receptors indicated that aromatic hydrophobes at C_4 such as the phenylethyl moiety provided the best binding and selectivity for rat A_1.[14] The activity of **4b** and **4c** was dependent upon a critical hydrogen bond and the presence of an H-bond donor; hydroxyl in the cyclohexane ring or amido in the ethyl chain. Absence of this functionality abrogated activity, indicative of a strong H-bond contributing ~2-3 Kcal/mole to binding. In the cyclohexyl case, the preferred disposition of amino and alcohol groups was *trans*-1,4. The corresponding *cis*-1,4 isomer or the acetate derivative of **4b** were less active.[15] The required position and orientation of the H-bond donor was also apparent in **4d**, **4f**, and **4i** which mimic a *cis*-amide geometry and position H-bond donor/acceptor capabilities at various distances from the 4-amino pyrrolopyrimidine nucleus.

In summary, screening with yeast-based assays expressing two adenosine receptors led to the identification of **4b** and **4c** as novel antagonists from a *biased* "library" of pyrrolopyrimidines and a direct correlation between functional activity and binding.

Yeast Functional Assays: Assays were conducted with a final volume of 100 μL in 96-well microtiter plates such that a final concentration of 2% DMSO was achieved in all wells. Initial library screening was at 1 and 10 μM and subsequent compound profiling at eight concentrations. To each microtiter plate, 10 μL of 20% DMSO was added to "Control" and "Total" wells while 10 μM of test compound in 20% DMSO was added to "Unknown" wells. Subsequently, 10 μL of NECA (625 nM for A_1R, 10 nM for $A_{2a}R$) were added to "Total" and "Unknown" wells; 10 μL of dH_2O was added to the "Control" wells. In the final addition, 80 μL of yeast strain, CY12660 (A_1) or CY8362 (A_{2a}), were added to all wells. All plates were then agitated briefly and allowed to incubate for 4 h at 30°C. Fluorescein digalactopyranoside (FDG), a β-galactosidase substrate, was added to all wells at 20 μL/well (final concentration = 80 μM). Plates were shaken for approx. 2min and then incubated at 37°C for 30 min. At the end of the incubation, β-Galactosidase activity was stopped using 20 μL/well of 1M Na_2CO_3 and all plates shaken for approx. 2min. Plates were then read for relative fluorescence intensity as determined using a Wallac Victor I fluorometer.

Resynthesis of library hits:

***N*-(*trans*-4-Hydroxycyclohexyl)-5,6-dimethyl-7*H*-pyrrolo[2,3-d]pyrimidin-4-amine (4b).** Chloride **3** (0.5 g, 1.94 mmol) reacted with *trans*-4-aminocyclohexanol (2.23 g, 19.4 mmol) in DMSO (10 mL) at 130 °C for 5 hr to afford **4b** (0.45 g, 69%) after flash chromatography (SiO_2; 2:1 Hexane:EtOAc): mp 197-199.5 °C; ^1H NMR (200 MHz, $CDCl_3$) δ 10.60 (s, 1H), 8.40-8.44 (dd, 2H, J = 2.2, 8.0, Hz), 7.26-7.49 (m, 3H), 4.88 (d, 1H, J = 8.0 Hz), 4.32-4.38 (m, 1H), 3.68-3.79 (m, 1H), 2.29 (s, 3H), 2.08 (s, 3H), 1.25-1.59 (m, 8H); MS (ES): 337.2 (M^+ + 1); Anal. ($C_{20}H_{24}N_4O$) C, H, N.

***N*-(2-Acetamidoethyl)-5,6-dimethyl-7*H*-pyrrolo[2,3-d]pyrimidin-4-amine (4c).** Proceeding in the same manner as above, the displacement of chloride **3** (0.5 g, 1.94 mmol) with *N*-acetylethylenediamine (1.98 g, 19.4 mmol) in DMSO (10 mL) afforded **4c** (0.42 g, 67%) after flash chromatography (SiO_2; 2:1 Hexane:EtOAc): mp 135 °C (dec); ^1H NMR (200 MHz, $CDCl_3$) δ 10.76 (s, 1H), 8.36-8.43 (dd, 2H, J = 1.0, 7.0 Hz), 7.44-7.47 (m, 3H), 5.63 (br t, 1H), 3.96 (m, 2H), 3.59 (m, 2H), 2.31 (s, 3H), 1.97 (s, 3H), 1.72 (s, 3H); MS (ES) 324 (M^+ + 1); Anal. ($C_{18}H_{21}N_5O$) C, H, N.

References and Notes:

1. Broach, J. R.; Thorner, J. High-throughput screening for drug discovery. *Nature* **1996**, *384*, 14-16.
2. Silverman, L.; Campbell, R.; Broach, J. R. New assay technologies for high-throughput screening. *Current Opinion in Chemistry and Biology* **1998**, *2*, 397-403.
3. (a) Muller, C. E. A_1-adenosine receptor antagonists. *Exp. Opin. Ther. Patents* **1997**, *7*, 419-440. (b) Muller, C. E.; Stein, B. Adenosine receptor antagonists: structures and potential therapeutic applications. *Curr. Pharm. Des.* **1996**, *2*, 501-530. (c) DeNinno, M. P. Chapter 11. Adenosine. *Annu. Rep. Med. Chem.* **1998**, *33*, 111-120. (d) Poulsen S.-Ann; Quinn, R. J. Adenosine Receptors: New Opportunities for Future Drugs. *Bioorg. & Med. Chem.* **1998**, *6*, 619-641.

4. (a) Pfister J. R.; Belardinelli, L.; Lee, G.; Lum, R. T.; Milner, P. Stanley, W. C.; Linden, J.; Baker, S. P.; Schreiner, G. Synthesis and biological evaluation of the enantiomers of the potent and selective A_1-adenosine antagonist 1,3-dipropyl-8-[2-(5,6-epoxynorbornyl)]-xanthine. *J. Med. Chem.* **1997**, *40*, 1773-1778. (b) Kim, Y.-C.; de Zwart, M.; Chang, L.; Moro, S.; Kunzel, J. K. von F. D.; Melman, N.; Ijzerman, A. P.; Jacobson, K. A. Derivatives of the Triazoloquinazoline adenosine antagonist (CGS 15943) having high potency at the human A_{2a} and A_3 receptor subtypes. *J. Med. Chem.* **1998**, *41*, 2835-2845 and references therein. (c) Van Calenbergh, S.; Kunzel, J. K. von F. D.; Blaton, N. M.; Peeters, O. M.; Rozenski, J.; Van Aerschot, A.; De Bruyn, A.; De Keukeleire, D.; Ijzerman, A. P.; Herdewijn, P. N^6-Cyclopentyl-3'-substituted-xylofuranosyladenosines: A new class of non-xanthine adenosine A_1 receptor antagonists. *J. Med. Chem.* **1997**, *40*, 3765-3772.
5. Nyce, J. W.; Metzger, W. J. DNA antisense therapy for asthma in an animal model. *Nature* **1997**, *385*, 721-725.
6. Auchampach, J. A.; Jin, X.; Wan, T. C.; Caughey, G. H.; Linden, J. Canine mast cell adenosine receptors: Cloning and expression of the A_3 receptor and evidence that degranulation is mediated by the A_{2B} receptor. *Molecular Pharmacology* **1997**, *52*, 846-860.
7. Feoktistov, I.; Polosa, R.; Holgate, S. T.; Biaggioni, I. Adenosine A_{2B} receptors: A novel therapeutic target in asthma? *TiPS* 1998, *19*, 148-153.
8. Silverman, L. *et al.* unpublished results. A patent application describing this methodology has been filed.
9. (a) Muller, C. E.; Hide, I.; Daly, J. W.; Rothenhausler, K.; Eger, K. 7-Deaza-2-phenyladenines: Structure-Activity relationships of potent A_1 selective adenosine receptor antagonists. *J. Med. Chem.* **1990**, *33*, 2822-2828. (b) Muller, C. E.; Geis, U.; Grahner, B.; Lanzner, W.; Eger, K. Chiral pyrrolo[2,3-d]pyrimidine and pyrimido[4,5-b]indole derivatives: Structure-activity relationships of potent, highly stereoselective A_1-adenosine receptor antagonists. *J. Med. Chem.* **1996**, *39*, 2482-2491.
10. A similar stragety has been applied to kinase inhibitors, see Gray, N. S.; Wodicka, L.; Thunnissen, A.-M. W. H.; Norman, T. C.; Kwon, S.; Espinoza, F. H.; Morgan, D. O.; Barnes, G.; LeClerc, S..; Meijer L.; Kim, S.-H.; Lockhart, D. J.; Schultz, P. G. Exploiting chemical libraries: Structure, and genomics in the search for kinase inhibitors. *Science*, **1998** *281*, 533-538.
11. A particular case is the work on C5a antagonists, see for example Konteatis, Z. D.; Siciliano, S. J.; Van Riper G.; Molineaux, C. J.; Pandya, S.; Fischer, P.; Rosen, H.; Mumford, R. A.; Springer, M. S. Development of C5a receptor Antagonists – Differential loss of functional responses. *The Journal of Immunology* **1994**, *153*, 4200-4205.
12. An initial study with the following reference compounds indicated that the yeast-based assays are of sufficient sensitivity to discriminate receptor subtype specificity: 8- [4-[[[[(2-aminoethyl)-amino]carbonyl]methyl]oxy] phenyl]-1,3-dipropylxanthine (XAC), 1,3-dipropyl-8-cyclopentylxanthine (DPCPX), 9-chloro-2-(2-furanyl)-5,6-dihydro-[1,2,4]-triazolo[1,5]quinazolin-5-iminemonomethane-sulfonate (CGS-15943), 5'-N-ethyl carboxa- midoadenosine (NECA), N^6-(R-phenylisopropyl)adenosine (R-PIA), N^6-(3-iodobenzyl)-5'-(methylcarboxa- moyl)adenosine (IB-MECA), 2-[[*p*-(2-carboxyethyl)-phenethyl]amino]-5'-N-ethylcarboxamidoadenosine (CGS21680), and Alloxazine).
13. The 1,2-regioisomer of **4b** (mixture of four stereoisomers from *cis,trans*-2-aminocyclohexanol) exhibited higher activity in the A_1 yeast assay (IC_{50} = 96 nM) than in the A_1 binding assay (IC_{50} = 838 nM); a discrepancy perhaps attributed to this being a compound mixture.
14. N^6-(3-hydroxypropyl)adenosine has been reported to show A_1 selectivity, see Bruns, R. F.; Lu, G. H.; Pugsley, T. A "Characterization of the A_2 Adenosine Receptor Labeled by [^{3}H]NECA in Rat Striatal Membranes. *Mol. Pharmacol.* **1986**, *29*, 331-346. The corresponding N-(3-hydroxypropyl)-5,6-dimethyl-7*H*-pyrrolo[2,3-d]pyrimidin-4-amine exhibited a binding IC_{50} = 220 nM vs. A_1 receptor.
15. The steric demand of the cyclohexyl moiety in **4b** introduced selectivity for A_1 by ~ 400 vs. A_{2A} and ~10 fold vs. A_3. Subsequent lead optimization of **4b** led to CDS-096370, a potent and selective human A_1 antagonist (yeast EC_{50} = 9.4 nM; binding IC_{50} = 7.3 nM, K_i = 3.3 nM) with a cLog P ~3.3; a superior profile to that of **4a**. The synthesis and further biological activity of CDS-096370 will be described in a full paper.