British Journal of Pharmacology (1996) 117, 1645–1652

KW-3902, a selective high affinity antagonist for adenosine A_1 receptors

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1 We demonstrate that 8-(noradamantan-3-yl)-1,3-dipropylxanthine (KW-3902) is a very potent and selective adenosine A_1 receptor antagonist, assessed by radioligand binding and cyclic AMP response in cells.

2 In rat forebrain adenosine A_1 receptors labelled with [^{3}H]-cyclohexyladenosine (CHA), KW-3902 had a K_i value of 0.19 nM, whereas it showed a K_i value of 170 nM in rat striatal A_{2A} receptors labelled with [^{3}H]-2-[*p*-(2-carboxyethyl)-phenethylamino]-5′-N-ethylcarboxamidoadenosine (CGS21680), indicating 890 fold A_1 receptor selectivity versus the A_{2A} receptor. KW-3902 at 10 μM showed no effect on recombinant rat A_3 receptors expressed on CHO cells.

3 Saturation studies with [^{3}H]-KW-3902 revealed that it bound with high affinity (K_d = 77 pM) and limited capacity (B_{max} = 470 fmol mg^{-1} of protein) to a single class of recognition sites. A high positive correlation was observed between the pharmacological profile of adenosine ligands inhibiting the binding of [^{3}H]-KW-3902 and that of [^{3}H]-CHA.

4 KW-3902 showed potent A_1 antagonism against the inhibition of forskolin-induced cyclic AMP accumulation in DDT1 MF-2 cells by the A_1-selective agonist, cyclopentyladenosine with a dissociation constant (K_B value) of 0.34 nM. KW-3902 antagonized 5′-N-ethylcarboxamidoadenosine-elicited cyclic AMP accumulation via A_{2B} receptors with a K_B value of 52 nM.

5 KW-3902 exhibited marked species-dependent differences in the binding affinities. The highest affinity was for the rat A_1 receptor (K_i = 0.19 nM) and these values for guinea-pig and dog A_1 receptors were 1.3 and 10 nM, respectively.

Keywords: 8-(Noradamantan-3-yl)-1,3-dipropylxanthine (KW-3902); adenosine receptors; A_1 and A_2 receptors; A_1 antagonists; cyclic AMP; DPCPX

Introduction

Adenosine modulates a number of physiological effects by stimulating specific cell surface receptors. On the basis of both molecular pharmacological and biochemical studies these receptors have been divided into A_1, A_{2A}, A_{2B} and A_3 adenosine receptor subtypes. The A_1 and A_3 receptors mediate inhibition, and the A_{2A} and A_{2B} receptors stimulation of adenylate cyclase activity (Van Calker *et al.*, 1979; Linden *et al.*, 1991; Zhou *et al.*, 1992). Functional studies of each subtype both *in vitro* and *in vivo* have progressed by use of selective agonists and antagonists. Furthermore, several agents selective for adenosine A_1 or A_2 receptor have provided possible means of treating renal disease, ischaemic bradyarrhythmias, sleep apnoea and cognitive disorders, ischaemic conditions by a reversal of the vascular steal phenomenon and erythrocytosis following renal transplantation (Collis & Hourani, 1993).

KW-3902 [8-(noradamantan-3-yl)-1,3-dipropylxanthine] (Figure 1) is a newly synthesized adenosine A_1 receptor antagonist (Shimada *et al.*, 1992). Recently, Mizumoto *et al.* (1993) have reported that KW-3902 has potent diuretic and renal protective activities in rats at very low doses. These activities of KW-3902 have also been observed in dogs but at significantly higher doses than in rats (Kobayashi *et al.*, 1993).

As yet the *in vitro* pharmacological properties of KW-3902 have not been described in detail. Furthermore, the reason why such a big species-dependent difference in pharmacological activities has been observed remains to be elucidated. This paper describes the binding properties of KW-3902 in brain membrane A_1 receptors labelled with [^{3}H]-N^6-cyclohexyladenosine ([^{3}H]-CHA), compared to those of other selective A_1 receptor ligands. We then used [^{3}H]-KW-3902 to determine the binding properties of the compound in rat forebrain. Functional A_1 and A_{2B} receptor antagonism of the compound in DDT1 MF-2 and Jurkat cells was also investigated in terms of the cyclic AMP signalling system. We further describe differences in the binding properties of KW-3902 among rat, guinea-pig and dog and discuss the reason for the species-dependent difference in the *in vivo* pharmacological activities.

Methods

[^{3}H]-CHA and [^{3}H]-KW-3902 binding studies

Forebrain tissues from Wistar rats, Hartley guinea-pigs and beagle dogs were homogenized in ice-cold 50 mM Tris-HCl

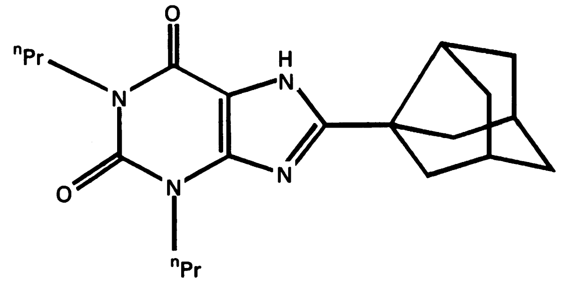

Figure 1 Structure of 8-(noradamantan-3-yl)-1,3-dipropylxanthine (KW-3902).

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buffer, pH 7.7 in a Polytron homogenizer (setting 5, 30 s). The homogenate was centrifuged at 50,000 *g* for 10 min at 4°C, and the pellet was washed twice in fresh buffer. The pellet was resuspended in the buffer supplemented with 2 units ml^{-1} adenosine deaminase. Following a 30 min incubation at 37°C, the suspension was recentrifuged at 50,000 *g* for 10 min at 4°C. The final pellet was kept frozen until the binding assay experiments. No difference in binding kinetics was found between stored and fresh tissue. To label mammalian brain A_1 receptors, we used [^{3}H]-CHA as one of the best A_1 receptor radioligands available. Binding of [^{3}H]-N^6-cyclohexyladenosine ([^{3}H]-CHA) to A_1 receptors was measured by a modification of the method described by Bruns *et al.* (1980). Incubation was for 90 min at 25°C, in the assay mixture containing 50 mM Tris-HCl buffer, pH 7.7, forebrain membranes, and 1 nM [^{3}H]-CHA in rat and guinea-pig or 6 nM [^{3}H]-CHA in dog for displacement studies. N^6-(**R**)-phenylisopropyladenosine (**R**-PIA; 10 μM) was added to determine the non-specific binding. Binding reactions were terminated by filtration through Whatman GF/C filters under reduced pressure using a MT-24 cell harvester (Brandel, MD, U.S.A.). Filters were washed three times with ice-cold buffer (5 ml) and placed in scintillation vials, and bound radioactivity was determined in a TRI-CARB 4530 Packard liquid scintillation counter (Packard Instrument Co., CT, U.S.A.). 8-(Noradamantan-3-yl)-[^{3}H]-1,3-dipropylxanthine ([^{3}H]-KW-3902) binding was measured by the same procedure as for [^{3}H]-CHA binding with the exception that incubation was for 60 min and 100 μM **R**-PIA was used to determine the non-specific binding. The concentration of 100 μM **R**-PIA was required to displace fully [^{3}H]-KW-3902 specific binding since the A_1 receptor agonist showed a shallow inhibition curve in the case of the [^{3}H]-KW-3902 binding.

In the saturation studies, mammalian forebrain membranes were incubated with 10 to 12 different concentrations of [^{3}H]-CHA ranging from 0.2 to 5 nM in rat, from 0.5 to 22 nM in guinea-pig and from 1 to 33 nM in dog; or with [^{3}H]-KW-3902, ranging from 0.01 to 0.7 nM. For the displacement studies, 7 to 15 different concentrations of inhibitor were included in the incubation buffer containing [^{3}H]-CHA as described.

[^{3}H]-CGS21680 binding study

Striatal tissues from Wistar rats were homogenized in ice-cold 50 mM Tris-HCl buffer, pH 7.7. The homogenate was centrifuged at 50,000 *g* for 10 min at 4°C, and the pellet was washed in fresh buffer. Binding of [^{3}H]-2-[*p*-(2-carboxyethyl)-phenethylamino]-5'-*N*-ethylcarboxamidoadenosine (CGS21680) to rat striatal membranes was measured by a modification of the method described by Jarvis *et al.* (1989). The assay mixture contained 50 mM Tris-HCl buffer, pH 7.7, striatal membranes, 4 nM [^{3}H]-CGS21680, 10 mM $MgCl_2$, and 0.1 unit ml^{-1} adenosine deaminase. Incubation was for 120 min at 25°C. Nonspecific binding was defined in the presence of 100 μM N^6-cyclopentyladenosine (CPA). Termination of the reaction and determination of the bound radioactivity were carried out by the same procedure as for [^{3}H]-CHA binding. The binding assays were performed in duplicate with seven concentrations of each drug. Protein concentrations were determined by the method of Lowry *et al.* (1951) with bovine serum albumin used as the reference standard.

[^{125}I]-4-aminobenzyl-5'-N-methylcarboxamidoadenosine (AB-MECA) binding study

Two primers (5'-CGGAATTCCGATGAAAGCCAACAATACC-3' and 5'-CGGAATTCCGCTACTCAGTAGTCTGTTC-3') were made, based on the published DNA sequence of the rat adenosine A_3 receptor (Zhou *et al.*, 1992). Rat kidney mRNA was subjected to a RT-PCR amplification reaction. The resulting 963-bp fragment was subcloned into the EcoRI site of pBluescriptII SK(−) (Stratagene CA, U.S.A.). The DNA sequence obtained was confirmed to be the sequence as reported. The cDNA was inserted into the expression vector pAGE110, a dhfr transcription unit containing a version of pAGE107 (Miyaji *et al.*, 1990). For stable expression, the vector was transfected into Chinese hamster ovary (CHO) cells. CHO cells were maintained in MEMα-2000 medium containing 5% dialyzed foetal bovine serum, G418 (0.3 mg ml^{-1}), methotrexate, (50 nM), penicillin (100 units ml^{-1}) and streptomycin (100 μg ml^{-1}). The binding of [^{125}I]-AB-MECA to A_3 receptor membranes from transfected CHO cells was carried out as described by Olah *et al.* (1994). The assays were conducted at 37°C for 90 min in the assay mixture containing 50 mM Tris-HCl, 10 mM $MgCl_2$, 1 mM EDTA, pH 8.26 buffer (50/10/1 buffer), 3 units ml^{-1} adenosine deaminase, cell membranes and 0.1 nM [^{125}I]-AB-MECA. Non-specific binding was determined by the presence of 100 μM **R**-PIA. Binding reactions were terminated by filtration through Whatman GF/B filters presoaked in 0.3% polyethylenimine using a MT-24 cell harvester. Filters were washed three times with 50/10/1 buffer (3 ml) and bound radioactivity was measured in a γ-counter (COBRA, Packard Instrument Co., CT, U.S.A.).

Binding of various radioligands

Binding of [^{3}H]-nitrobenzylthioinosine, [^{3}H]-2-([2',6'-dimethoxy]-phenoxyethylamino)methylbenzodioxan (WB-4101), [^{3}H]-clonidine, [^{3}H]-dihydroalprenolol, [^{3}H]-flunitrazepam, [^{3}H]-(**R**)-(+)-8-chloro-2,3,4,5-tetrahydro-3-methyl-5-phenyl-1H-3-benzazepin-7-ol hemimaleate (SCH23390), [^{3}H]-spiperone, [^{3}H]-muscimol, [^{3}H]-mepyramine, [^{3}H]-tiotidine, [^{3}H]-quinuclidinylbenzilate, [^{3}H]-(−)-nicotine, [^{3}H]-8-hydroxy-2(di-n-propylamino)tetralin, [^{3}H]-ketanserin, and [^{3}H]-quipadine to the adenosine transporters (Verma & Marangos, 1985), to α_1- and α_2-adrenoceptors (Greenberg *et al.*, 1976), β-adrenoceptors (U'Prichard *et al.*, 1978), benzodiazepine receptors (Braestrup & Squires, 1977), dopamine D_1 receptors (Billard *et al.*, 1984), dopamine D_2 receptors (Leysen & Gommeren, 1981), $GABA_A$ receptors (Williams & Risley, 1979), histamine H_1 receptors (Chang *et al.*, 1979), histamine H_2 receptors (Gajtkowski *et al.*, 1983), muscarinic M_1 acetylcholine receptors (Bloom *et al.*, 1987), nicotinic acetylcholine receptors (Lippiello & Fernandes, 1988), 5-HT_{1A} receptors (Gozlan *et al.*, 1983), 5-HT_2 receptors (Leysen *et al.*, 1981) respectively, were measured according to the methods described previously.

Cyclic nucleotide phosphodiesterase (PDE) assay

PDE isoenzymes, calmodulin-dependent PDE (PDE I), cyclic GMP-stimulated PDE (PDE II), cyclic GMP-inhibited PDE (PDE III), cyclic AMP-specific PDE (PDE IV) and cyclic GMP-specific PDE (PDE V) were prepared from dog trachea according to Torphy & Cieslinski (1990). PDE activity was measured by a two-step procedure previously described by Kincaid & Manganiello (1988).

Cell culture

DDT1 MF-2 cells, a hamster vas deferens cell line, and Jurkat cells, a human T cell line were obtained from the American Type Culture Collection. DDT1 MF-2 cells were grown in a flask containing Dulbecco's modified Eagle's medium (DMEM) with 2.5% foetal bovine serum, 2.5% horse serum, 100 units ml^{-1} penicillin, 100 μg ml^{-1} streptomycin and 2 mM glutamine. Jurkat cells were cultured in RPMI1640 medium with 10% foetal bovine serum, antibiotics and 2 mM glutamine.

Assay of cyclic AMP level in DDT1 MF-2 and Jurkat cells

DDT1 MF-2 cells were harvested by centrifugation for 5 min at 800 *g*, washed twice in assay medium (serum-free DMEM buffered with 20 mM HEPES, pH 7.4, supplemented with 0.1% BSA). The cells were suspended in the same buffer supplemented with 3 units ml^{-1} adenosine deaminase and 30 μM rolipram (phosphodiesterase inhibitor) and then preincubated for 10 min at 37°C. Assays were initiated by transferring the suspension of DDT1 MF-2 cells (1×10^5 cells) into different concentrations of drugs and/or 30 μM forskolin. Incubation was for 10 min at 37°C, and was terminated by heating at 95°C for 2 min. After centrifugation, cyclic AMP content in the supernatant was measured with a cyclic AMP radioimmunoassay kit from Yamasa (Chiba Japan). In Jurkat cells, assays were performed as described for DDT1 MF-2 cells, with the exception that RPMI1640 medium buffered 20 mM HEPES, pH 7.0 was used as the assay medium, and adenosine deaminase and forskolin were not used.

Data analysis

Computer analyses (EBDA and LIGAND (Munson & Rodbard, 1980) were used to evaluate the dissociation constant (K_d value) and receptor density (B_{max} value). A partial F test was used to determine whether the binding was better fitted by a one- or two-site model (Munson & Rodbard, 1980). IC_{50} values and Hill coefficients were determined from computerization of the logit-log curves. The Cheng-Prusoff equation (Cheng & Prusoff, 1973) was used to calculate K_i values from IC_{50} values. Statistical significance of the K_d and B_{max} changes were determined with a Scheffe type test. Data represent means ± standard errors (s.e.).

Chemicals

[^{3}H]-N^6-cyclohexyladenosine (CHA, specific activity, 1024.9 GBq $mmol^{-1}$) and [^{3}H]-2-[p-(2-carboxyethyl)-phenethylamino]-5′-N-ethylcarboxamidoadenosine (CGS21680, 1465.2 GBq $mmol^{-1}$) were purchased from New England Nuclear (Boston, MA, U.S.A.). [^{125}I]-4-aminobenzyl-5′-N-methylcarboxamidoadenosine (AB-MECA, 74 TBq $mmol^{-1}$) was purchased from Amersham International plc. (Buckinghamshire, England). 8-(Noradamantan-3-yl)-[^{3}H]-1,3-dipropylxanthine ([^{3}H]-KW-3902, 1980 GBq $mmol^{-1}$; radiochemical purity, 98%) was tritiated by Amersham International plc. (Buckinghamshire, England). CGS21680 was from Research Biochemicals, Inc. (Natick, MA, U.S.A.). N^6-cyclopentyladenosine (CPA), N^6-cyclohexyladenosine (CHA), 5′-N-ethylcarboxamidoadenosine (NECA), N^6-(**R**)-phenylisopropyladenosine (**R**-PIA), adenosine deaminase, forskolin and methotrexate (MTX) were from Sigma Chemical Co. (St. Louis, MO, U.S.A.). KW-3902, 8-cyclopentyl-1,3-dipropylxanthine (DPCPX), 9-chloro-2-(2-furyl)[1,2,4]-triazolo[1,5-c]quinazolin-5-amine (CGS15943), xanthine amine congener (XAC), 8-(3,4-dimethoxystyryl)-1,3-dipropyl-7-methylxanthine (KF17837S) and rolipram were synthesized at the Medicinal Chemistry Department of the Pharmaceutical Research Laboratories (Shimada *et al.*, 1992). All cell culture supplies and G418 (Geneticin) were from GIBCO-BRL (Gaithersburg, MD, U.S.A.). Other reagents were from standard commercial sources.

Results

Affinities of KW-3902 for rat brain adenosine A_1 and A_{2A} receptors

KW-3902 concentration-dependently displaced [^{3}H]-CHA binding to adenosine A_1 receptors on rat forebrain membranes (Figure 2a). The K_i value was 0.19 ± 0.042 nM. Reference xanthine antagonists, DPCPX and XAC, and nonxanthine antagonist, CGS15943 had K_i values of 0.49 ± 0.060 nM, 1.1 ± 0.043 nM, and 2.1 ± 0.49 nM, respectively (Figure 2a). Scatchard analysis showed that KW-3902 caused a significant change in the K_d and B_{max} values, indicating that the inhibition mode is a mixed type (Figure 2b). The apparent K_d value of [^{3}H]-CHA increased from control values of 1.7 ± 0.09 nM ($n = 4$) to 5.7 ± 0.15 nM ($n = 3$) in the presence of 1 nM KW-3902 ($n = 3$, $P < 0.01$). The maximal number of binding sites was reduced from 270 ± 15 fmol mg^{-1} of protein to 196 ± 10 fmol mg^{-1} of protein ($n = 4$, $P < 0.05$) in the presence of 0.3 nM KW-3902 and to 166 ± 12 fmol mg^{-1} of protein ($n = 3$, $P < 0.01$) in the presence of 1 nM KW-3902. KW-3902 displaced [^{3}H]-CGS21680 binding to the rat striatal A_{2A} receptor with a K_i value of 170 ± 16 nM (Table 1). KW-3902 was

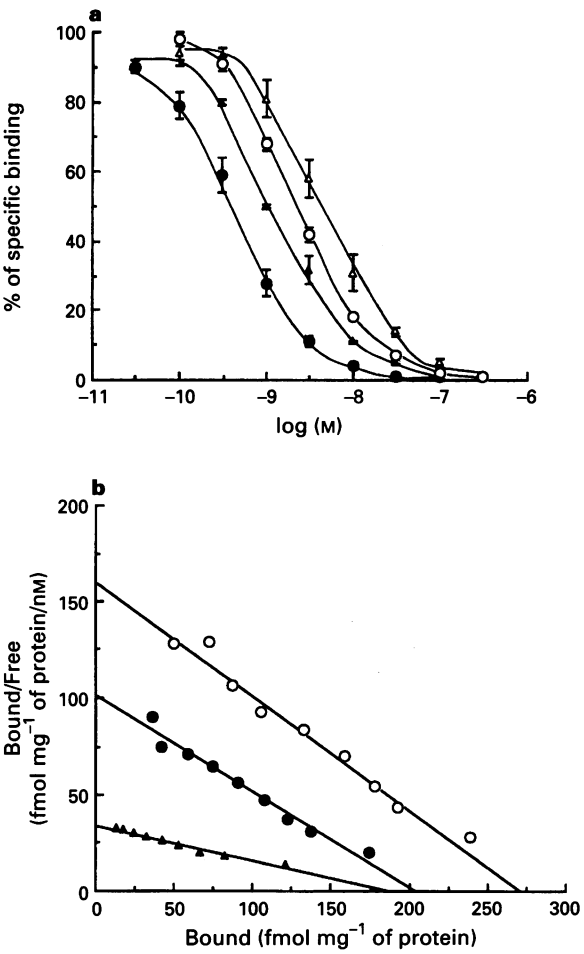

Figure 2 (a) Concentration-inhibition curves of KW-3902 and other adenosine antagonists versus 1 nM [^{3}H]-CHA in rat forebrain membranes: (●) KW-3902; (▲) DPCPX; (○) XAC; (△) CGS15943. (b) Competition of KW-3902 for [^{3}H]-CHA binding to adenosine A_1 receptors on rat forebrain membranes. Scatchard analysis of [^{3}H]-CHA binding in the presence or absence of KW-3902: (○) control; (●) in the presence of 0.3 nM KW-3902; (▲) in the presence of 1 nM KW-3902. Values are means ± s.e. of three or four separate experiments in duplicate.

Table 1 Affinities of KW-3902 and other compounds for adenosine A_1 and A_{2A} receptors in rat brain membranes

Compound	K_i (*nM*) *A_1 receptor*	*A_{2A} receptor*	K_i *ratio* *A_{2A}/A_1*
KW-3902	0.19 ± 0.042	170 ± 16	890
DPCPX	0.49 ± 0.060	120 ± 9.1	240
XAC	1.1 ± 0.043	14 ± 4.0	13
CGS15943	2.1 ± 0.49	0.39 ± 0.13	0.19

Rat forebrain (A_1) or striatal (A_{2A}) membranes were incubated with 1 nM [^{3}H]-CHA or 4 nM [^{3}H]-CGS21680, respectively, in the presence of various concentrations of compounds. Values are means ± s.e. of three separate experiments performed in duplicate.

therefore 890 fold selective for A_1 versus A_{2A} receptors in rat brain membranes. KW-3902, DPCPX, XAC and CGS15943 up to 10 μM did not inhibit [^{125}I]-AB-MECA binding in membranes from CHO cells expressing the rat adenosine A_3-receptor.

[^{3}H]-KW-3902 binding in rat forebrain membranes

After 60 min incubation at 25°C, 1 nM [^{3}H]-KW-3902 bound to rat forebrain membranes with specific binding amounting to approximately 80% of total binding (Figure 3a). Kinetic time course experiments revealed that the binding of [^{3}H]-KW-3902 reached equilibrium after approximately 10 min and was reversed by the addition of 100 μM **R**-PIA (Figure 3b). Association and dissociation kinetic rate constants determined as described by McPherson (1985), were as follows: $k_{obs}=0.41\pm0.022$ min^{-1}, $k_{-1}=0.019\pm0.00099$ min^{-1} $t_{1/2}=37\pm2.0$ min, $k_{+1}=0.68\pm0.088$ min^{-1} nM^{-1} and $K_d=0.030$ nM. In saturation studies, [^{3}H]-KW-3902 bound to a single site with a B_{max} of 470 ± 23 fmol mg^{-1} of protein and K_d of 0.077 ± 0.0055 nM (Figure 4a).

Concentration-inhibition curves for adenosine receptor antagonists versus [^{3}H]-KW-3902 were steep, with Hill coefficients near 1.0, whereas adenosine receptor agonists had shallower curves with Hill coefficients between 0.53 and 0.60 (Table 2). The K_i values of the antagonists for displacement of [^{3}H]-KW-3902 binding were in good agreement with those of [^{3}H]-CHA binding. The affinities of the agonists were one order of magnitude lower for [^{3}H]-KW-3902 binding than for [^{3}H]-CHA binding. However, the order of potencies of the agonists was the same in both radioligand assays; **R**-PIA > CHA > NECA > > CGS21680. The Hill coefficient of the agonist **R**-PIA in [^{3}H]-KW-3902 binding was 0.63 ± 0.051 (Figure 4b). The data analyzed by the LIGAND programme fit significantly a model for two classes of non-interacting sites ($P<0.001$ vs. a model for single class), giving a high affinity site with K_{iH} of 2.7 nM and a low affinity site with K_{iL} of 64 nM. As shown in Figure 4b, inclusion of 100 μM GTP shifted the inhibition curve of **R**-PIA to the right and the Hill coefficient to 0.86 ± 0.051. The K_i values of **R**-PIA altered from 17 ± 0.98 nM in the absence of GTP to 85 ± 13 nM in the presence of GTP. On the other hand, GTP did not affect the inhibition curve and the K_i value for KW-3902 (K_i; 0.10 ± 0.019 nM and n_H; 0.97 ± 0.12 in the absence of GTP, K_i; 0.10 ± 0.021 nM and n_H: 1.25 ± 0.020 in the presence of GTP, Figure 4b).

Effect of KW-3902 on cyclic AMP accumulation in DDT1 MF-2 and Jurkat cells

The adenosine A_1 receptor function has been defined by its ability to inhibit cyclic AMP production. In DDT1 MF-2 cells, the adenosine A_1 selective agonist, CPA, markedly inhibited cyclic AMP accumulation in the presence of 30 μM forskolin, consistent with the previous report by Schachter & Wolfe (1992) (Figure 5a). Although DDT1 MF-2 cells express both

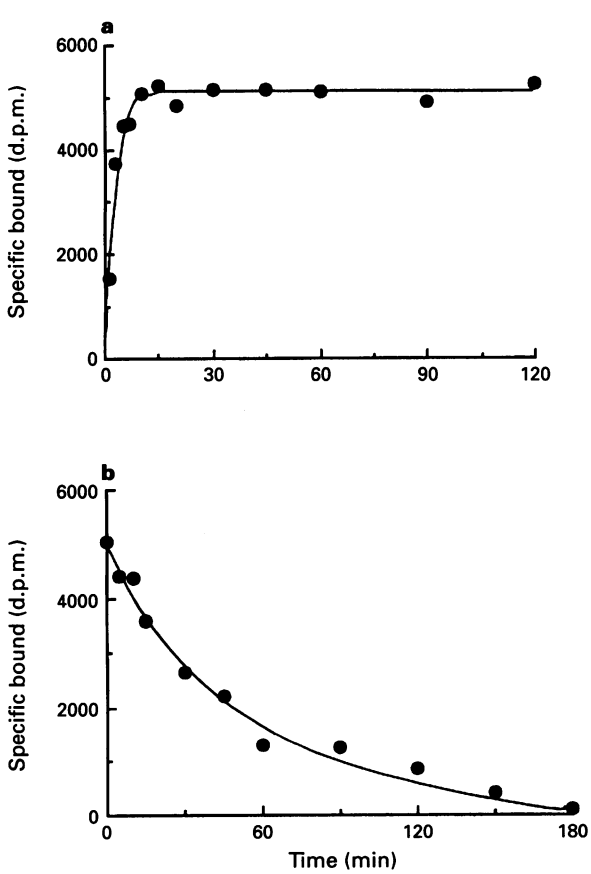

Figure 3 Kinetics of [^{3}H]-KW-3902 binding to rat forebrain membranes: representative curves of association (a) and dissociation (b). Dissociation was initiated by the addition of 100 μM unlabelled **R**-PIA after a 60 min incubation of ligand with the tissue at 25°C. Association and dissociation kinetic rate constants were; $k_{obs}=0.41\pm0.022$ min^{-1}, $k_{-1}=0.019\pm0.00099$ min^{-1} from a $t_{1/2}=37\pm2.0$ min, and $k_{+1}=0.68\pm0.088$ min^{-1} nM^{-1}. Values indicate means ± s.e. obtained from at least three separate experiments.

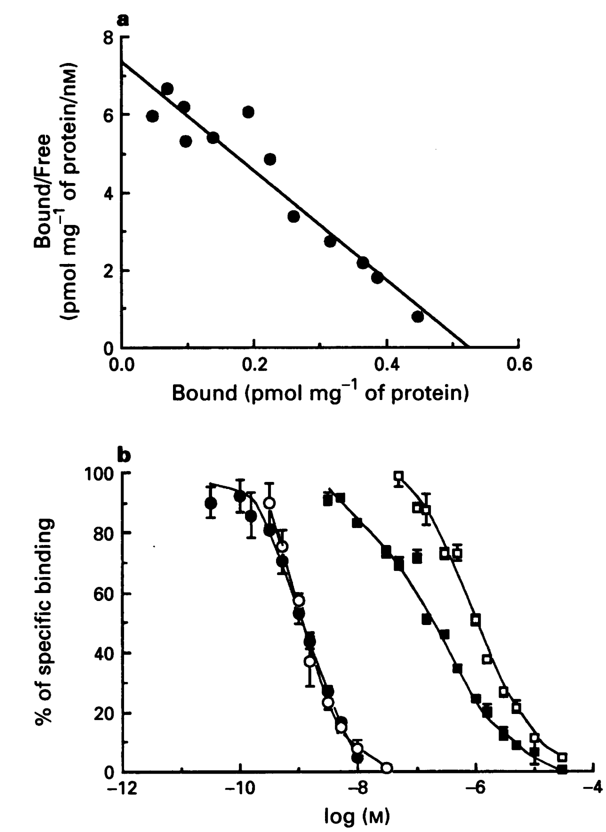

Figure 4 (a) Representative Scatchard plot for [^{3}H]-KW-3902 binding to rat forebrain membranes. A K_d value of 0.077 ± 0.0055 nM and a B_{max} value of 470 ± 23 fmol mg^{-1} protein were determined. These K_d and B_{max} values indicate means ± s.e. from four experiments. (b) Competition of [^{3}H]-KW-3902 binding to rat forebrain membranes by **R**-PIA (□, ■) and KW-3902 (○, ●). Specific binding of [^{3}H]-KW-3902 in the presence (open symbols) and in the absence (closed symbols) of 100 μM GTP was determined at various concentrations of **R**-PIA or KW-3902. Data points represent means ± s.e. from at least three separate experiments conducted in duplicate.

adenosine A_1 and A_2 receptors, A_1 receptors are much higher in density than A_2 receptors (Ramkumar *et al.*, 1990). Thus, in the presence of the A_1 agonist CPA from 10^{-10} M to 10^{-7} M, DDT1 MF-2 cells show only a pharmacological character of typical A_1 receptors. Nanomolar concentrations of KW-3902 shifted the dose-response curve for CPA to the right (Figure 5a). Schild analysis indicated an apparent K_B value of 0.34 ± 0.042 nM with a slope factor of 1.40 ± 0.058. KW-3902 alone had no effect on either the basal cyclic AMP level (3.2 ± 0.53 pmol/10^6 cells) or the forskolin-stimulated cyclic AMP levels (67 ± 7.9 pmol/10^6 cells).

To evaluate its effect on the A_{2B} receptor, the actions of KW-3902 on 5'-N-ethylcarboxamidoadenosine (NECA)-induced cyclic AMP accumulation in Jurkat cells were examined. As shown in Figure 5b, KW-3902 caused a rightward shift of the NECA dose-response curve and showed an apparent K_B value of 52 ± 5.2 nM with a slope factor of 1.47 ± 0.092. The K_B value was comparable to that of DPCPX (55 ± 7.3 nM, slope factor 1.08 ± 0.17, Figure 5c). KW-3902 had no effect on the basal cyclic AMP levels (0.90 ± 0.10 pmol/10^6 cells). These results indicated that KW-3902 showed approximately 150 times more potent antagonism for the A_1 receptor response than for the A_{2B} receptor response without any partial agonist activity.

Affinities of KW-3902 for adenosine A_1 receptors in guinea-pig and dog forebrain membranes

Using membranes prepared from forebrains of rat, guinea-pig and dog, we investigated species differences in adenosine A_1 receptors (Table 3). The data on the equilibrium binding studies in each species fit to a single-site binding model, suggesting homogeneity for [^{3}H]-CHA binding sites within each species (data not shown). Referred to the K_d value for [^{3}H]-CHA in rat forebrain membranes (1.2 ± 0.19 nM), the K_d value for the

Table 2 Comparison of the inhibition by various adenosine agonists and antagonists of the binding of [^{3}H]-KW-3902 and [^{3}H]-CHA to rat forebrain membranes

	[^{3}H]-KW-3902 K_i *(nM)*	n_H	*[^{3}H]-CHA* K_i *(nM)*
Agonists			
R-PIA	13 ± 2.9	0.57 ± 0.016	1.1 ± 0.12
CHA	21 ± 4.4	0.60 ± 0.028	1.1 ± 0.5
NECA	61 ± 11	0.53 ± 0.022	5.7 ± 1.6
CGS21680	> 10 000	ND[a]	420 ± 2.9
Antagonist			
KW-3902	0.10 ± 0.012	0.94 ± 0.024	0.19 ± 0.042
DPCPX	0.27 ± 0.013	0.97 ± 0.019	0.49 ± 0.060
XAC	0.57 ± 0.099	1.00 ± 0.020	1.1 ± 0.043
CGS15943	1.2 ± 0.21	1.04 ± 0.13	2.1 ± 0.49
KF17837S	190 ± 33	0.92 ± 0.048	390 ± 68

Values are means ± s.e. of three to five separate experiments performed in duplicate.
[a]ND: not determined

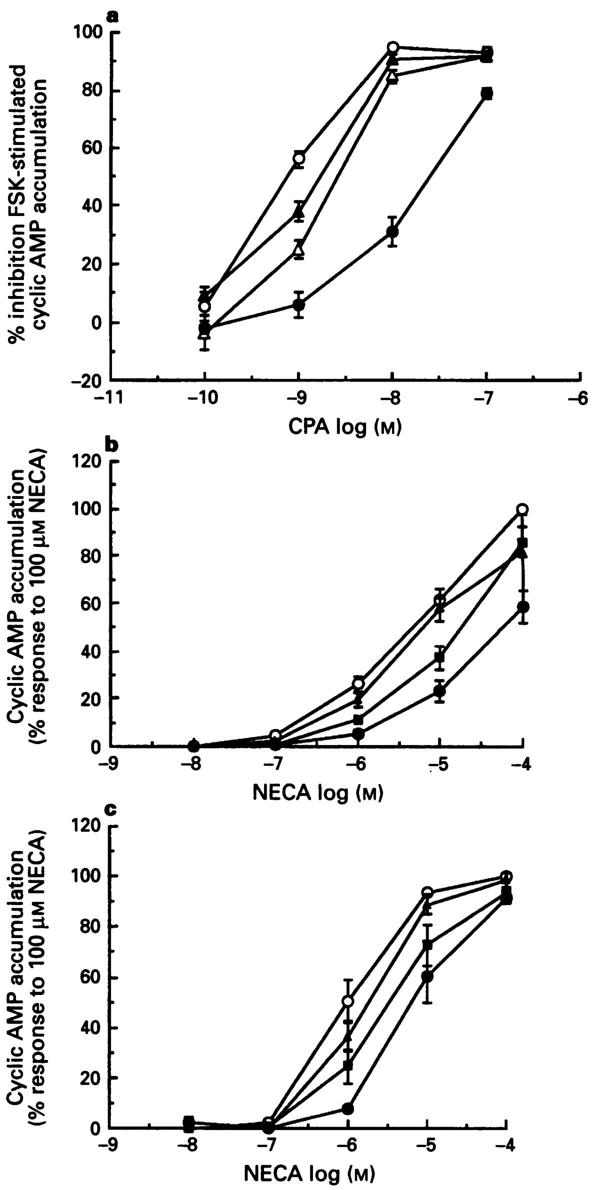

Figure 5 (a) Antagonism by KW-3902 of the ability of CPA to inhibit forskolin-stimulated cyclic AMP accumulation in DDT1 MF-2 cells: (○) control; (▲) in the presence of 0.3 nM KW-3902; (△) in the presence of 1 nM KW-3902; (●) in the presence of 3 nM KW-3902. The results are expressed as percentage of the 30 µM forskolin-stimulated cyclic AMP levels (67 ± 7.9 pmol/10^6 cells). The basal cyclic AMP levels are 3.2 ± 0.53 pmol/10^6 cells. (b and c) Antagonism by KW-3902 (b) and DPCPX (c) of NECA-elicited cyclic AMP accumulation in Jurkat cells: (○) control; in the presence of 30 (▲), 100 (■) and 300 (●) nM compounds. The results are expressed as percentage of the 100 µM NECA-elicited cyclic AMP levels (KW-3902; 70 ± 21 pmol/10^6 cells, DPCPX; 49 ± 1.4 pmol/10^6 cells). The basal cyclic AMP levels are 0.90 ± 0.10 pmol/10^6 cells in (b) and 0.81 ± 0.17 pmol/10^6 cells in (c) respectively. Values are means ± s.e. of three separate experiments performed in duplicate.

Table 3 Equilibrium binding constants of [^{3}H]-CHA binding to mammalian forebrain and the inhibition constants of KW-3902 and other adenosine antagonists

Species	K_d *(nM)* *[^{3}H]-CHA*	K_i *(nM)* *KW-3902*	*DPCPX*	*CGS15943*	*XAC*
Rat	1.2 ± 0.19	0.19 ± 0.042	0.49 ± 0.06	2.1 ± 0.49	1.1 ± 0.043
Guinea-pig	7.5 ± 0.28	1.3 ± 0.12	6.4 ± 0.35	9.6 ± 0.44	11 ± 0.28
Dog	28 ± 5.2	$10 + 2.6$	17 ± 6.5	8.2 ± 1.1	16 ± 1.8

Values are means ± s.e of three to five separate experiments performed in duplicate.

guinea-pig membranes was 7.5 ± 0.28 nM and that for the dog forebrain membranes was 28 ± 5.2 nM. The K_i values of KW-3902 for [^{3}H]-CHA binding in rat, guinea-pig, and dog were 0.19 ± 0.042, 1.3 ± 0.12, and 10 ± 2.6 nM, respectively. Accordingly, the activities of KW-3902 for guinea-pig and dog were 6.8 fold and 53 fold lower than the activity for rat, respectively (Table 3).

Affinities of KW-3902 for other receptors and phosphodiesterases

KW-3902 had no effects on the adenosine transporters, on α_1- and α_2-adrenoceptors, and β-adrenoceptors, benzodiazepine receptors, dopamine D_1 receptors and dopamine D_2 receptors, $GABA_A$ receptors, histamine H_1 receptors and histamine H_2 receptors, muscarinic M_1 acetylcholine receptors, nicotinic acetylcholine receptors, 5-HT_{1A} receptors and 5-HT_2 receptors at a concentration of 10 μM (Table 4).

KW-3902 had no effects on all 5 isoenzymes of cyclic nucleotide phosphodiesterase from dog trachea at concentrations up to 10 μM (data not shown).

Discussion

KW-3902 has very potent diuretic and renal protective effects in rats (Mizumoto *et al*., 1993). In the present studies, we have characterized KW-3902 in various *in vitro* assays. KW-3902 showed the most potent activity for A_1 receptors among the A_1 antagonists including the well known A_1-selective antagonist, DPCPX (Bruns *et al*., 1987; Lohse *et al*., 1987). We used the radioligand [^{3}H]-CHA to label mammalian brain A_1 receptors, because DPCPX has been reported to act as an antagonist at A_{2B} receptors with a K_B value of about 10^{-8} M (Brackett & Daly, 1994). In the study of [^{3}H]-CHA binding to rat brain membrane A_1 receptors, KW-3902 bound to a single class of sites at the receptors as indicated by pseudo-Hill slopes of 0.99 ± 0.013 (Figure 2a). The competition binding studies of KW-3902 showed a mixed type of inhibition (Figure 2b). The decrease in B_{max} together with the increase in K_d suggests that KW-3902 binds at least at two different sites on the A_1 receptor, one site is likely to be the site the radioligand binds. The other site remains unclear.

The sites of KW-3902 binding have been further characterized by using the newly prepared radioligand, [^{3}H]-KW-3902. [^{3}H]-KW-3902 showed a high affinity binding to rat

Table 4 Radioligand binding profile of KW-3902

Receptor	*% inhibition (10 µM)*
Adenosine transporter	−3
Adrenaline α_1	5
Adrenaline α_2	3
Adrenaline β	23
Benzodiazepine	2
Dopamine D_1	7
Dopamine D_2	4
$GABA_A$	0
Histamine H_1	4
Histamine H_2	3
Muscarinic M_1 ACh	3
Nicotinic ACh	4
5-HT_{1A}	−1
5-HT_2	4

Assays were run according to standard protocol documented in references. Rat or guinea-pig brain membranes were used as receptor sources. All assays were validated using appropriate reference standards. Values are means of three separate experiments performed in duplicate.

forebrain membrane. The K_d value was 0.077 ± 0.0055 nM and the B_{max} value 470 ± 23 fmol mg^{-1} of protein (Figure 2b). This K_d value was consistent with the affinity of KW-3902 for the [^{3}H]-CHA binding site. The B_{max} value was similar to the values of [^{3}H]-XAC and [^{3}H]-DPCPX binding to the A_1 receptor (Jacobson *et al*., 1986; Lohse *et al*., 1987). The order of potencies of adenosine agonists and antagonists in competing for [^{3}H]-KW-3902 binding agreed well with those in the adenosine A_1 receptor binding study with [^{3}H]-CHA (Table 2). The competition curves for the agonists versus the binding of [^{3}H]-KW-3902 were biphasic in the absence of GTP; e.g. K_{iH} of 2.7 nM and K_{iL} of 64 nM for **R**-PIA. The K_{iH} was similar to the K_i for [^{3}H]-CHA binding (1.1 nM) suggesting that the high affinity site was corresponding to the A_1 receptor recognized by [^{3}H]-CHA. The addition of GTP resulted in rightward shift of the inhibition curve for **R**-PIA with a Hill coefficient of 0.86 (Figure 4b). The inhibition curve of the antagonist KW-3902 had no effect either in the presence or absence of GTP. The rightward shift of the agonist competition curve, but no shift of the antagonist curve, by GTP indicated that the [^{3}H]-KW-3902 binding sites were coupled to G-proteins. The K_i value of **R**-PIA in the presence of GTP corresponded to the K_{iL}. This suggests that **R**-PIA recognizes both the GTP-associated low affinity state and the GTP-dissociated high affinity state. These results demonstrated that [^{3}H]-KW-3902 specifically labelled adenosine A_1 receptors in rat forebrain membrane.

KW-3902 had a 890 fold binding selectivity for the A_1 over the A_{2A} receptor in rat brain and showed no effect on recombinant rat adenosine A_3 receptor expressed on CHO cells at a concentration of 10 μM. Since no good binding assays for the A_{2B} receptor have been developed due to the lack of specific ligands for these receptors, we have not yet determined the binding affinity of KW-3902 for the A_{2B} receptor. We alternatively examined the functional assays for the A_1 and the A_{2B} receptors using DDT1 MF-2 cells and Jurkat cells, respectively. KW-3902 potently antagonized the CPA-induced cyclic AMP decrease in the presence of 30 μM forskolin in intact DDT1 MF-2 cells which express A_1 receptors (Figure 5a). The antagonism was not competitive with a slope factor of 1.40 ± 0.058 from the Schild plot and it showed a mixed type inhibition at the binding assay using rat brain membranes. A similar inhibition type has been noted for PACPX (Burnstock & Hoyle, 1985), although the cause remains unclear. The apparent K_B value (0.34 nM) corresponded to the binding affinity for the A_1 receptor in rat brain membrane (K_i value: 0.19 nM). KW-3902 inhibited NECA-elicited cyclic AMP accumulation in Jurkat cells (Figure 5b), a response via A_{2B} receptors (Kvanta *et al*., 1991) but again the inhibition was not competitive, with a slope factor of 1.47 ± 0.092 from the Schild plot. The apparent K_B value of KW-3902 for the A_{2B} receptor antagonism was 52 ± 5.2 nM, indicating that KW-3902 has a 150 fold selectivity for the A_1 over the A_{2B} receptor. Furthermore, the compound had substantially no affinity for the other 14 receptors and 5 phosphodiesterase isoenzymes. The apparent K_B value of KW-3902 in Jurkat cells was almost equal to that of DPCPX (55 ± 7.3 nM, Figure 5c), one of the most potent and selective A_1 antagonists reported so far. Recently, DPCPX has been reported to antagonize the cyclic AMP response in NIH 3T3 fibroblast cells via A_{2B} receptors with the K_B value of 17 ± 3 nM (Brackett & Daly, 1994), comparable to the value in Jurkat cells. The K_i values of DPCPX were 0.49 ± 0.060 nM for [^{3}H]-CHA binding and 120 ± 9.1 nM for [^{3}H]-CGS21680 binding rat, respectively (Table 1). The selectivity ratios of DPCPX for the A_1 receptor binding were 240 versus the A_{2A} receptor and 110 versus the A_{2B} receptor whereas the ratios of KW-3902 were 890 versus the A_{2A} and 270 versus the A_{2B} receptor. Altogether, KW-3902 turns out to be the most potent and selective A_1 antagonist.

A_1 receptors in DDT1 MF-2 cells have been reported to inhibit adenylate cyclase via pertussis toxin-sensitive G-proteins, G_{i2} and/or G_{i3} (Gerwins *et al*., 1990; Gerwins & Fredholm, 1991). KW-3902 therefore appears to antagonize the CPA-induced cyclic AMP decrease via a pertussis toxin-sensitive G protein in DDT1 MF-2 cells. In addition, the rightward shift of the **R**-PIA inhibition curve caused by GTP in the [^{3}H]-KW-3902 binding experiments indicated that adenosine A_1 receptors labelled with [^{3}H]-KW-3902 were coupled to G-proteins. However, it has been suggested that diuretic and renal protective effects of KW-3902 *in vivo* did not involve a pertussis toxin-sensitive mechanism (Mizumoto *et al.*, 1993). Previously van der Ploeg *et al.* (1992) reported that intraventricular injection of the toxin had no effect on A_1 receptor binding in any brain region studied, as evaluated by autoradiography. Clarifying what mechanism underlies the pertussis toxin-insensitive activities of KW-3902 *in vivo* requires further investigation.

Species-dependent differences have been found in the potencies of antagonists for the A_1 receptor binding (Table 3). Several other xanthine antagonists have already been reported to show higher binding affinity for rat A_1 receptors than for guinea-pig A_1 receptors (Ferkany *et al.*, 1986; Ukena *et al.*, 1986). We further demonstrated that the affinities of xanthine antagonists for A_1 receptors were lower in dog than in guinea-pig. Thus KW-3902 showed K_i values of 0.19 ± 0.042, 1.3 ± 0.12, and 10 ± 2.6 nM for rat, guinea-pig, and dog A_1 receptors, respectively (Table 2). KW-3902 also exhibited species difference in diuretic activity, i.e. the threshold dosages having significant diuretic activity were 0.001 mg kg^{-1} p.o. in rat and 1 mg kg^{-1} p.o. in dog (Kobayashi *et al.*, 1993). The difference in the A_1 affinity of KW-3902 apparently contributed to the difference in the diuretic activities. In addition, preliminary pharmacokinetic studies of KW-3902 showed that the maximum concentration of the compound in serum was much higher in rats than in dogs (unpublished data). The big difference in diuretic activities between rats and dogs therefore appears to be due to the binding affinities together with the pharmacokinetics of KW-3902.

Adenosine has been reported to act on the kidney to cause various functional changes. They include control of glomerular filtration rate, renin secretion from the juxtaglomerular cell, transport throughout the length of the nephron, intracellular Ca^{2+} in cortical collecting duct, and the arginine vasopressin induced cyclic AMP accumulation in medullary collecting duct (McCoy *et al.*, 1993; Spielman & Thompson, 1982; Spielman & Arend, 1991). These adenosine actions might be involved in the diuretic and renal protective effects of KW-3902 via the A_1 receptor. Further studies are necessary to clarify the mechanism of action of KW-3902.

In conclusion, KW-3902 is the most potent and specific antagonist at adenosine A_1 receptors described to date. In addition [^{3}H]-KW-3902 is a useful radioligand for adenosine A_1 receptors. KW-3902 should facilitate further studies on physiopathological role of the adenosine A_1 receptor but also have a potential clinical use for the treatment of oedema and renal failure.

We thank Miss Taeko Suzuki and Miss Mayumi Saki for the expert assistance. We thank Dr Akiko Saito for providing transfected CHO cells. We are grateful to Dr Tadashi Hirata for encouragement and support.

References

BILLARD, W., RUPERTO, V., CROSBY, G., IORIO, L.C. & BARNETT, A. (1984). Characterization of the binding of ^{3}H-SCH23390, a selective D-1 receptor antagonist ligand, in rat striatum. *Life Sci.*, **35**, 1885–1893.

BLOOM, J.W., HALONEN, M., SEAVER, N.A. & YAMAMURA, H.I. (1987). Heterogeneity of the M_1 muscarinic receptor subtype between peripheral lung and cerebral cortex demonstrated by the selective antagonist AF-DX 116. *Life Sci.*, **41**, 491–496.

BRACKETT, L.E. & DALY, J.W. (1994). Functional characterization of the A_{2b} adenosine receptor in NIH 3T3 fibroblasts. *Biochem. Pharmacol.*, **47**, 801–814.

BRAESTRUP, C. & SQUIRES, R.F. (1977). Specific benzodiazepine receptors in rat brain characterized by high-affinity [^{3}H]diazepam binding. *Proc. Natl. Acad. Sci. U.S.A.*, **74**, 3805–3809.

BRUNS, R.F., DALY, J.W. & SNYDER, S.H. (1980). Adenosine receptors in brain membranes: Binding of N^6-cyclohexyl[^{3}H]adenosine and 1,3-diethyl-8-[^{3}H]-phenylxanthine. *Proc. Natl. Acad. Sci. U.S.A.*, **77**, 5547–5551.

BRUNS, R.F., FERGUS, J.H., BADGER, E.W., BRISTOL, J.A., SANTAY, L.A., HARTMAN, J.D., HAYS, S.J. & HUANG, C.C. (1987). Binding of the A_1-selective adenosine antagonist 8-cyclopentyl-1,3-dipropylxanthine to rat brain membranes. *Naunyn-Schmied. Arch. Pharmacol.*, **335**, 59–63.

BURNSTOCK, G. & HOYLE, C.H. (1985). PACPX- a substituted xanthine-antagonizes both the A_1 and A_2 subclasses of the P_1 purinoceptor: antagonism of the A_2 subclass is competitive but antagonism of the A_1 subclass is not. *Br. J. Pharmacol.*, **85**, 291–296.

CHANG, R.S.L., TRAN, V.T. & SNYDER, S.H. (1979). Heterogeneity of histamine H_1-receptors: Species variations in [^{3}H]mepyramine binding of brain membranes. *J. Neurochem.*, **32**, 1653–1663.

CHENG, Y.-C. & PRUSOFF, W.H. (1973). Relationship between the inhibition constant (K_i) and the concentration of inhibitor which causes 50% inhibition (IC_{50}) of an enzymatic reaction. *Biochem. Pharmacol.*, **22**, 3099–3108.

COLLIS, M.G. & HOURANI, S.M.O. (1993). Adenosine receptor subtypes. *Trends Pharmacol. Sci.*, **14**, 360–366.

FERKANY, J.W., VALENTINE, H.L., STONE, G.A. & WILLIAMS, M. (1986). Adenosine A1 receptors in mammalian brain: Species differences in their interactions with agonists and antagonists. *Drug Dev. Res.*, **9**, 85–93.

GAJTKOWSKI, G.A., NORRIS, D.B., RISING, T.J. & WOOD, T.P. (1983). Specific binding of ^{3}H-tiotidine to histamine H_2 receptors in guinea pig cerebral cortex. *Nature*, **304**, 65–67.

GERWINS, P. & FREDHOLM, B.B. (1991). Glucocorticoid receptor activation leads to up-regulation of adenosine A_1 receptors and down-regulation of adenosine A_2 responses in DDT_1 MF-2 smooth muscle cells. *Mol. Pharmacol.*, **40**, 149–155.

GERWINS, P., NORDSTEDT, C. & FREDHOLM, B.B. (1990). Characterization of adenosine A_1 receptors in intact DDT_1 MF-2 smooth muscle cells. *Mol. Pharmacol.*, **38**, 660–666.

GOZLAN, H., MESTIKAWY, S.EI, PICHAT, L., GLOWINSKI, J. & HAMON, M. (1983). Identification of presynaptic serotonin autoreceptors using a new ligand: ^{3}H-PAT. *Nature*, **305**, 140–142.

GREENBERG, D.A., U'PRICHARD, D.C. & SNYDER, S.H. (1976). Alpha-noradrenergic receptor binding in mammalian brain: differential labeling of agonist and antagonist states. *Life Sci.*, **19**, 69–76.

JACOBSON, K.A., UKENA, D., KIRK, K.L. & DALY, J.W. (1986). [^{3}H]Xanthine amine congener of 1,3-dipropyl-8-phenylxanthine: an antagonist radioligand for adenosine receptors. *Proc. Natl. Acad. Sci. U.S.A.*, **83**, 4089–4093.

JARVIS, M.F., SCHULZ, R., HUTCHISON, A.J., DO, U.H., SILLS, M.A. & WILLIAMS, M. (1989). [^{3}H]CGS21680, a selective A_2 adenosine receptor agonist directly labels A_2 receptors in rat brain. *J. Pharmacol. Exp. Ther.*, **251**, 888–893.

KINCAID, R.L. & MANGANIELLO, V.C. (1988). Assay of cyclic nucleotide phosphodiesterase using radiolabeled and fluorescent substrates. In *Methods in Enzymology*. ed. Corbin J.D. & Johnson, R.A. Vol. **159**, pp. 457–462. New York: Academic Press.

KOBAYASHI, T., MIZUMOTO, H. & KARASAWA, A. (1993). Diuretic effects of KW-3902 (8-(noradamantan-3-yl)-1,3-dipropylxanthine), a novel adenosine A_1 receptor antagonist, in conscious dogs. *Biol. Pharmacol. Bull.*, **16**, 1231–1235.

KVANTA, A., JONDAL, M. & FREDHOLM, B.B. (1991). CD3-dependent increase in cyclic AMP in human T-cells following stimulation of the CD2 receptor. *Biochim. Biophys. Acta*, **1093**, 178–183.

LEYSEN, J.E. & GOMMEREN, W. (1981). Optimal conditions for [^{3}H]apomorphine binding and anomalous equilibrium binding of [^{3}H]apomorphine and [^{3}H]spiperone to rat striatal membranes: Involvement of surface phenomena versus multiple binding sites. *J. Neurochem.*, **36**, 201–219.

LEYSEN, J.E., NIEMEGEERS, C.J.E., VAN NUETEN, J.M. & LADURON, P.M. (1981). [^{3}H]ketanserin (R41468), a selective ^{3}H-ligand for serotonin$_2$ receptor binding sites. *Mol. Pharmacol.*, **21**, 301–314.

LINDEN, J., TUCKER, A.L. & LYNCH, K.R. (1991). Molecular cloning of adenosine A_1 and A_2 receptors. *Trends Pharmacol. Sci.*, **12**, 326–328.

LIPPIELLO, P.M. & FERNANDES, K.G. (1988). Identification of putative high affinity nicotinic receptors on cultured cortical neurons. *J. Pharmacol. Exp. Ther.*, **246**, 409–416.

LOHSE, M.J., KLOTZ, K.-N., LINDENBORN-FOTINOS, J., REDDINGTON, M., SCHWABE, U. & OLSSON, R.A. (1987). 8-Cyclopentyl-1,3-dipropylxanthine (DPCPX) – a selective high affinity antagonist radioligand for A_1 adenosine receptors. *Naunyn-Schmied. Arch Pharmacol.*, **336**, 204–210.

LOWRY, O.H., ROSEBROUGH, N.J., FARR, A.L. & RANDALL, R.J. (1951). Protein measurements with the Folin phenol reagent. *J. Biol. Chem.*, **193**, 265–275.

MCCOY, D.E., BHATTACHARYA, S., OLSON, B.A., LEVIER, D.G., AREND, L.J. & SPIELMAN, W.S. (1993). The renal adenosine system: structure, function and regulation. *Semin. Nephrol.*, **13**, 31–40.

MCPHERSON, G.A. (1995). Analysis of radioligand binding experiments: A collection of computer programs for the IBM PC. *J. Pharmacol. Methods*, **14**, 213–228.

MIYAJI, H., MIZUKAMI, T., HOSOI, S., SATO, S., FUJIYOSHI, N. & ITOH, S. (1990). Expression of human beta-interferon in Namalwa KJM-1 which was adapted to serum-free medium. *Cytotechnology*, **3**, 133–140.

MIZUMOTO, H., KARASAWA, A. & KUBO, K. (1993). Diuretic and renal protective effects of 8-(noradamantan-3-yl)-1,3-dipropylxanthine (KW-3902), a novel adenosine A_1-receptor antagonist, via pertussis toxin insensitive mechanism. *J. Pharmacol. Exp. Ther.*, **266**, 200–206.

MUNSON, P.J. & RODBARD, D. (1980). LIGAND, a versatile computerized approach for characterization of ligand-binding systems. *Anal. Biochem.*, **107**, 220–239.

OLAH, M.E., GALLO-RODRIGUEZ, C., JACOBSON, K.A. & STILES, G.L. (1994). ^{125}I-4-aminobenzyl-5′-N-methylcarboxamidoadenosine, a high affinity radioligand for the rat A_3 adenosine receptor. *Mol. Pharmacol.*, **45**, 978–982.

RAMKUMAR, V., BARRINGTON, W.W., JACOBSON, K.A. & STILES, G.L. (1990). Demonstration of both A_1 and A_2 adenosine receptors in DDT1 MF-2 smooth muscle cells. *Mol. Pharmacol.*, **37**, 149–156.

SCHACHTER, J.B. & WOLFE, B.B. (1992). Cyclic AMP differentiates two separate but interacting pathways of phosphoinositide hydrolysis in the DDT_1-MF_2 smooth muscle cell line. *Mol. Pharmacol.*, **41**, 587–597.

SHIMADA, J., SUZUKI, F., NONAKA, H. & ISHII, A. (1992). 8-Polycycloalkyl-1,3-dipropylxanthines as potent and selective antagonists for A_1-adenosine receptors. *J. Med. Chem.*, **35**, 924–930.

SPIELMAN, W.S. & AREND, L.J. (1991). Adenosine receptors and signaling in the kidney. *Hypertension*, **17**, 117–130.

SPIELMAN, W.S. & THOMPSON, C.I. (1982). A proposed role for adenosine in the regulation of renal hemodynamics and renin release. *Am. J. Physiol.*, **242**, F423–F435.

TORPHY, T.J. & CIESLINSKI, L.B. (1990). Characterization and selective inhibition of cyclic nucleotide phosphodiesterase isozymes in canine tracheal smooth muscle. *Mol. Pharmacol.*, **37**, 206–214.

UKENA, D., JACOBSON, K.A., PADGETT, W.L., AYALA, C., SHAMIM, M.T., KIRK, K.L., OLSSON, R.O. & DALY, J.W. (1986). Species differences in structure-activity relationships of adenosine agonists and xanthine antagonists at brain A_1 adenosine receptors. *FEBS Lett.*, **209**, 122–128.

U'PRICHARD, D.C., BYLUND, D.B. & SNYDER, S.H. (1978). (±)-[^{3}H]Epinephrine and (−)-[^{3}H]dihydroalprenolol binding to β_1- and β_2-noradrenergic receptors in brain, heart, and lung membranes. *J. Biol. Chem.*, **253**, 5090–5102.

VAN CALKER, D., MÜLLER, M. & HAMPRECHT, B. (1979). Adenosine regulates via two different types of receptors, the accumulation of cyclic AMP in cultured brain cells. *J. Neurochem.*, **33**, 999–1005.

VAN DER PLOEG, I., PARKINSON, F.E. & FREDHOLM, B.B. (1992). Effect of pertussis toxin on radioligand binding to rat brain adenosine A_1 receptors. *J. Neurochem.*, **58**, 1221–1229.

VERMA, A. & MARANGOS, P.J. (1985). Nitrobenzylthioinosine binding in brain: an interspecies study. *Life Sci.*, **36**, 283–290.

WILLIAMS M. & RISLEY, E.A. (1979). Characterization of the binding of [^{3}H]muscimol, a potent γ-aminobutyric acid agonist, to rat brain synaptosomal membranes using a filtration assay. *J. Neurochem.*, **32**, 713–718.

ZHOU, Q.-Y., LI, C., OLAH, M.E., JOHNSON, R.A., STILES, G.L. & CIVELLI, O. (1992). Molecular cloning and characterization of an adenosine receptor: The A_3 adenosine receptor. *Proc. Natl. Acad. Sci. U.S.A.*, **89**, 7432–7436.

(Received August 30, 1995
Revised December 18, 1995
Accepted December 22, 1995)