The *in vitro* pharmacology of ZM 241385, a potent, non-xanthine, A_{2a} selective adenosine receptor antagonist

¹S.M. Poucher, J.R. Keddie, P. Singh, S.M. Stoggall, P.W.R. Caulkett, G. Jones & ²M.G. Collis

Cardiovascular and Metabolism Department, ZENECA Pharmaceuticals, Mereside, Alderley Park, Macclesfield, Cheshire, SK10 4TG

1 This paper describes the *in vitro* pharmacology of ZM 241385 (4-(2-[7-amino-2-(2-furyl) [1,2,4]-triazolo[2,3-a][1,3,5]triazin-5-yl amino]ethyl) phenol), a novel non-xanthine adenosine receptor antagonist with selectivity for the A_{2a} receptor subtype.

2 ZM 241385 had high affinity for A_{2a} receptors. In rat phaeochromocytoma cell membranes, ZM 241385 displaced binding of tritiated 5'-N-ethylcarboxamidoadenosine (NECA) with a pIC₅₀ of 9.52, (95% confidence limits, c.l., 9.02–10.02). In guinea-pig isolated Langendorff hearts, ZM 241385 antagonized vasodilatation of the coronary bed produced by 2-chloroadenosine (2-CADO) and 2-[p-(2-carboxyethyl) phenethylamino]-5'-N-ethylcarboxamidoadenosine (CGS21680) with pA₂ values of 8.57 (c.l., 8.45–8.68) and 9.02 (c.l., 8.79–9.24) respectively.

3 ZM 241385 had low potency at A_{2b} receptors and antagonized the relaxant effects of adenosine in the guinea-pig aorta with a pA₂ of 7.06, (c.l., 6.92–7.19).

4 ZM 241385 had a low affinity at A₁ receptors. In rat cerebral cortex membranes it displaced tritiated R-phenylisopropyladenosine (R-PIA) with a pIC₅₀ of 5.69 (c.l., 5.57–5.81). ZM 241385 antagonized the bradycardic action of 2-CADO in guinea-pig atria with a pA₂ of 5.95 (c.l., 5.72–6.18).

5 ZM 241385 had low affinity for A₃ receptors. At cloned rat A₃ receptors expressed in chinese hamster ovary cells, it displaced iodinated aminobenzyl-5'-N-methylcarboxamido adenosine (AB-MECA) with a pIC₅₀ of 3.82 (c.l., 3.67–4.06).

6 ZM 241385 had no significant additional pharmacological effects on the isolated tissues used in these studies at concentrations three orders of magnitude greater than those which block A_{2a} receptors. At 10 µM it displayed only minor inhibition of the bradycardic effects in guinea-pig atria to some concentrations of carbachol. At 10 µM, ZM 241385 had a small inhibitory effect on relaxant effects of isoprenaline in guinea-pig aortae but no effect on sodium nitrite-induced relaxation. ZM 241385 (100 µM) was without effect on phenylephrine-induced tone in guinea-pig aortae.

7 ZM 241385 (10 µM) had no inhibitory effect on rat hepatocyte phosphodiesterase types I, II, III and IV but caused a small inhibition of the calcium calmodulin-activated type I enzyme.

8 ZM 241385 is the most selective adenosine A_{2a} receptor antagonist yet described and is therefore a useful tool for characterization of responses mediated by A₂ adenosine receptors.

Keywords: Adenosine receptors: A₁ and A₂ receptors: A₂ antagonists; ZM 241385; (4-(2-[7-amino-2-(2-furyl) [1,2,4]triazolo[2,3-a][1,3,5]triazin-5-yl amino]ethyl) phenol)

Introduction

Adenosine P₁ purinoceptors were originally subdivided into A₁, which are coupled to adenylate cyclase in an inhibitory manner, and A₂, which are coupled to adenylate cyclase in a stimulatory manner (Londos & Wolff, 1977; Van Calcar *et al.*, 1979; Bruns *et al.*, 1980). The A₂ receptor has been further subdivided into two classes (Daly, 1985) based upon the observations that some A₂ receptors have EC₅₀ values for adenosine in the high nanomolar range and some in the micromolar range (Londos *et al.*, 1982). The high affinity A₂ receptor exists in the striatum and nucleus accumbens and their activation can be measured in broken cell adenylate cyclase assays, whereas the low affinity A_{2b} receptor exists in almost all areas of the brain and their activity can be measured by adenosine 3':5'-cyclic monophosphate (cyclic AMP) accumulation in brain slices, but not in broken cell preparations (Premont *et al.*, 1979). The high affinity, A_{2a} receptor was further characterized by [³H]-5'-N-ethylcarboxamidoadenosine ([³H]-NECA) binding in rat striatal membranes (Bruns *et al.*, 1986). The agonist, 2-[p-(2-carboxyethyl)-phenethylamino]-5'-N-ethyl-carboxamidoadenosine, CGS21680, displays higher affinity for the

A_{2a} receptor than the A_{2b} (Jarvis *et al.*, 1989) and has been used widely to investigate and characterize the A_{2a} receptor subtype. More recently, an additional adenosine receptor sub-type (A₃) has been characterized (Abbracchio *et al.*, 1993; Collis & Hourani, 1994).

The search for selective adenosine receptor antagonists, which will enable a more complete understanding of the role of the various sub-types of adenosine receptor in physiological responses, has provided a significant challenge. The xanthine, PD115199 (1,3-dipropyl-8-N-[2-dimethylamino]ethyl]-N-methyl-4,2,3,6,7-tetrahydro-2,6-dioxo)-benzenesulphonamidexanthine), although having higher affinity for the A_{2a} binding site than the A_{2b} binding site, is equipotent at A_{2a} and A₁ binding sites (Bruns *et al.*, 1987b). PD116948 (8-cyclopentyl-1,3-dipropylxanthine) has higher affinity for the A₁ receptor than for A_{2a} binding sites in rat brain studies (Bruns *et al.*, 1987a,b; Jarvis *et al.*, 1989) and clearly demonstrated A₁-selective antagonism in guinea-pig isolated tissues (Collis *et al.*, 1989). To date, the most A_{2a}-selective xanthine antagonist is 8-(3-chlorostyryl)-1,3-dipropylxanthine which in binding studies demonstrates A_{2a}:A₁ selectivity of 520 fold but only 22 fold in functional tests (Jacobson *et al.*, 1993). The compound is however, of limited utility, for functional assays due to its limited solubility (Jacobson *et al.*, 1993). A number of non-xanthine adenosine receptor antagonists have been described,

¹ Author for correspondence.

² Present address: Pfizer Central Research, Sandwich, Kent.

characterized primarily by binding studies. CGS15943 (5-amino-9-chloro-2-(2-furyl)-pyrazolo-[4,3-e]1,2,4-triazolo[1,5-c]quinazoline) has high affinity for the A_{2a} receptor (K_i 3 nM) but modest A_{2a}:A₁ selectivity (7 fold) (Williams *et al.*, 1987). Dionisotti *et al.* (1994) used CGS15943 as a chemical starting point for the synthesis of 5-amino-8-(4-fluorobenzyl)-2-(2-furyl)-pyrazolo-[4,3-e]1,2,4-triazolo[1.5-c]pyrimidine (8FB-PTP), which in binding assays has a K_i at A₂ receptors of 0.074 nM and 28 fold A₂:A₁ selectivity. However, in functional assays this compound loses its selectivity and is indeed modestly A₁ receptor-selective.

Therefore, based upon functional assays there are currently no A_{2a} selective, antagonists available. The following paper describes the *in vitro* pharmacology of ZM 241385 (4-(2-[7-amino-2-(2-furyl) [1, 2, 4]triazolo[2, 3-a][1,3,5]triazin-5-yl amino]ethyl) phenol), a non-xanthine adenosine receptor antagonist. Affinity of the compound for A_{2a}, A₃ and A₁ binding sites was assessed in binding assays with membranes from a rat pheochromocytoma (PC12) cell line (Williams *et al.*, 1986), rat cloned A₃ receptors expressed in chinese hamster ovary cell line (Olah *et al.*, 1994) and in rat cortex (Bruns *et al.*, 1987a) respectively. Evaluation of antagonism of adenosine receptor-mediated responses was further investigated in guinea-pig atria (A₁ receptor, Collis, 1983), guinea-pig Langendorff hearts (A_{2a} receptor, Ueeda *et al.*, 1991) and guinea-pig isolated aortae (A_{2b} receptor, Hargreaves *et al.*, 1991; Martin, 1992).

Methods

Ligand binding at the A₁ site in rat cerebral cortex

Alderley Park rats were killed by cervical dislocation and crude membranes from cerebral cortex were prepared by a modification of the method of Lohse *et al.* (1984). Briefly, the frontal cortex was removed and immediately homogenized in 50 mM Tris-HCl, pH 7.4. Membrane pellets were produced by a series of centrifugation and resuspension steps and finally resuspended in ice-cold buffer and stored under liquid nitrogen until required. Tritiated N⁶(S-2-phenylisopropyl)adenosine (R-PIA, 35–60 Ci mmol⁻¹, final concentration 10 nM) binding studies were carried out in assay mixtures buffered with 50 mM Tris-HCl, pH 7.4 at room temperature. All studies were carried out in the presence of adenosine deaminase (2 u ml⁻¹) to remove endogenous adenosine.

For both the A₁ and A_{2a} binding assays the saturation isotherms were analysed with a binding analysis programme 'LIGAND' (Munson & Rodbard, 1980). The pIC₅₀ was determined for all compounds.

Ligand binding at the A_{2a} site in rat PC12 cell membranes

PC12 cells were originally obtained from the Beatson Institute, Glasgow and were later maintained in the Tissue Culture Unit at ZENECA Pharmaceuticals. PC12 membranes were prepared by the same method as used for the cortex membranes, frozen and stored under liquid nitrogen until required. Binding studies were carried out by use of the same method as for the rat cortex but with tritiated NECA (50 µl, 20–40 Ci mmol⁻¹, final concentration 40 nM) instead of R-PIA (Hide *et al.*, 1991).

Ligand binding at the cloned rat A₃ receptor

The rat A₃ adenosine receptor was cloned and expressed into a CHO cell line as described by Zhou *et al.* (1992). Synthesis and radioligand binding of N⁶-(3-[¹²⁵I]iodo-4-aminobenzyl-5'-N-methylcarboxamido adenosine ([¹²⁵I]-AB-MECA) assays for the rat A₃ receptor were undertaken as described previously (Olah *et al.*, 1994).

Adenosine A₁-receptor mediated chronotropic effects in guinea-pig atria

Dunkin Hartley guinea-pigs (male 250–400 g) were killed by cervical dislocation and their atria removed and immersed in Krebs solution. The atrial pairs were mounted in organ baths containing oxygenated Krebs solution (95% O₂/5% CO₂) at 37°C (Collis, 1983). The nucleoside transport inhibitor, dipyridamole (10 µM) was present in the Krebs solution since the agonist, 2-chloroadenosine (2-CADO) has been shown to be a substrate for the transporter (Collis & Brown, 1983; Jarvis *et al.*, 1985). Adenosine deaminase (2 u ml⁻¹) was added to remove endogenous tissue adenosine. The spontaneously beating atria were placed under a resting tension of 1 g and allowed to equilibrate for 50 min with continuous overflow. 2-CADO (range 0.01 µM–10 µM) was administered to produce a slowing of atrial rate before and after incubation of test compound for 30 min (ZM 241385, 3 µM–30 µM). The affinity of ZM 241385 (10 µM) for atrial muscarinic receptors was determined using carbachol (0.01 µM–3 µM) concentration-response curves.

Concentration-response curves to 2-CADO were plotted as % inhibition of rate against log concentration 2-CADO. Concentration-ratios were calculated from the EC₅₀ concentration of 2-CADO in the absence and presence of a potential antagonist. The pA₂ values of each antagonist studied were determined by Schild plot analysis (Arunkalakshana & Schild 1959). Where the Schild plot slope was not significantly different from unity the slope gradient was constrained to one for generation of pA₂ values.

Adenosine A_{2b} receptor-mediated vasorelaxation in guinea-pig aortae

Sections (3–5 mm) of guinea-pig thoracic aorta were mounted (under 1 g resting tension) in organ baths containing oxygenated Krebs solution (95% O₂/5% CO₂) at 37°C, pre-contracted with 3 µM phenylephrine for assessment of adenosine receptor-mediated vasorelaxation responses (Collis & Brown, 1983). The nucleoside transport inhibitor, dipyridamole (10 µM) and the adenosine deaminase inhibitor erythro-9-(2-hydroxy-3-nonyl) adenine hydrochloride (EHNA, 10 µM) were present in the Krebs solution. Adenosine was added cumulatively (0.1 µM–1 mM) both before and after 30 min incubation with the test compound. The antagonists used did not affect the contractile response to phenylephrine. Additional concentrations of antagonist were investigated in fresh aortae. The specificity of ZM 241385 (10 µM) at the adenosine receptor, in this preparation, was determined by use of isoprenaline (1 nM–10 µM) and sodium nitrite (0.1 µM–1 mM) concentration-response curves before and after the adenosine antagonist.

Adenosine A_{2a}-receptor mediated vasodilatation in guinea-pig Langendorff hearts

Assessment of activity at functional A_{2a} receptors was undertaken as described by Ueeda *et al.* (1992) with some modifications. Male guinea-pigs (300–550 g) were killed and the heart rapidly removed and placed in cold, millipore-filtered modified (high potassium to arrest the contracting hearts) Krebs solution (composition in mM: NaCl 108, KCl 14.8, KH₂PO₄ 1.2, MgSO₄ 1.2, NaHCO₃ 25, glucose 11, CaCl₂ 2.8, sodium pyruvate 2.2) i.e. equimolar substitution of KCl for NaCl. Contractions stopped within a few seconds and the heart was set up and perfused in the Langendorff mode with high potassium Krebs solution (at a temperature of 37°C and gassed with a mixture of 95% O₂/5% CO₂) at a constant flow rate of 3.5 ml min⁻¹ and allowed to equilibrate for at least 20–45 min before the start of the experiment.

After equilibration, the coronary vasculature was constricted with an infusion of a near maximal dose of phenylephrine (0.3 µM) producing a coronary perfusion pressure of 55–60 mmHg which remained stable for 60 min. Concentra-

tion-response curves were then constructed to the adenosine analogues: NECA, CGS21680, 2-CADO and R-PIA. Antagonist potency was determined in paired Langendorff hearts. One group served as the vehicle control for responses to 2-CADO or CGS21680 whilst separate preparations were challenged with 2-CADO or CGS21680, 30 min following administration of a single concentration of antagonist.

Phosphodiesterase studies

The activity of ZM 241385 was determined against a range of phosphodiesterase enzymes from rat hepatocytes and compared with the activity of theophylline. Isolated hepatocytes were prepared from fed male Sprague Dawley rats and incubated as described by Heyworth *et al.* (1983). Cells (3–5 mg dry weight ml⁻¹) were pre-incubated at 37°C for 20 min, with constant gassing (95% O₂/5% CO₂), before use. Cyclic AMP phosphodiesterase activity was measured by a modification of the two-step procedure of Thompson & Appleman (1971). All assays were performed at 30°C in the presence of cyclic AMP (1 µM).

Compounds used

4-(2-[7-amino-2-(2-furyl) [1,2,4]triazolo[2,3-a][1,3,5]triazin-5-yl amino]ethyl) phenol (ZM 241385), 8-cyclopentyl-1-3-dipropylxanthine (PD116948), and 2-[p-(2-carboxyethyl)-phenethylamino]-5'-N-ethyl-carboxamidoadenosine (CGS21680) were synthesized by Dr Geraint Jones, Mr Peter Caulkett and Dr Mike Block at Zeneca Pharmaceuticals. Dipyridamole (Persantin) was obtained from Boehringer Ingelheim, Bracknell, UK. R-phenylisopropyladenosine (R-PIA), 5'-N-ethylcarboxamidoadenosine (NECA) and erythro-9-(2-hydroxy-3-nonyl) adenine hydrochloride (EHNA) were obtained from Research Biochemicals Inc. St Albans, Herts. Adenosine, 2-chloroadenosine (2-CADO), theophylline, 8-phenyltheophylline (8PT), 8-phenylsulphotheophylline (8PST), adenosine deaminase (Type IV), carbachol, isoprenaline, sodium nitrite and phenylephrine hydrochloride were all obtained from Sigma Chemical Co., Poole, Dorset. Tritiated NECA was obtained from Amersham International, Little Chalfont, Bucks. Tritiated R-PIA was obtained from New England Nuclear, Dreieich, Germany. ZM 241385 was made up in a stock solution at 10⁻² M in dimethylsulphoxide (DMSO) and diluted with distilled water as required.

Results

Ligand binding at the A₁ site in rat cerebral cortex

The order of agonist affinity obtained for binding of agonist ligands in the rat cerebral cortex was R-PIA > NECA = 2-

CADO > > CGS 21680 (Table 1), which is consistent with the binding site being of the A₁ subtype. The A₁-selective antagonist, PD116948, also displayed high affinity (pIC₅₀ = 8.34) in this system, confirming the A₁ subtype binding site (Table 2). In contrast to PD116948, ZM 241385 displayed low affinity in competing with tritiated R-PIA (pIC₅₀ = 5.69).

Ligand binding at the A₂ site in rat PC12 membranes

The order of agonist affinity obtained at the A₂ binding site in the PC12 membranes was NECA > CGS21680 > 2-CADO = R-PIA. This is consistent with the adenosine receptor high affinity A_{2a} binding site (Table 1). In competition studies the non-selective antagonists, 8PST and 8PT demonstrated relatively low affinity in competing with tritiated NECA from PC12 binding sites (Table 2). The A₁-selective antagonist, PD116948 (pIC₅₀ = 6.75) was less potent than ZM 241385 (pIC₅₀ = 9.52) which was clearly the most potent antagonist studied in this assay. In binding studies, ZM 241385 demonstrated A_{2a}:A₁ selectivity of 6,761 fold (range, 28,183–1,622).

Ligand binding at the cloned rat A₃ receptor

ZM 241385 displayed a very low affinity at the A₃ adenosine receptor cloned from rat and expressed in CHO cells with a pIC₅₀ of 3.82 (c.l., 3.67–4.06, n = 3). The compound therefore demonstrated A_{2a}:A₃ selectivity of over 500,000 fold (range 2,218,196–91,200) in binding assays.

A₁ receptor antagonism in guinea-pig atria

The agonist potency order in the guinea-pig atria was R-PIA = NECA > 2-CADO > > CGS21680 (CGS21680 having a very low potency, EC₅₀, > 30,000 nM), consistent with A₁ receptor subtype (Table 1). 8PST and 8PT both demonstrated relatively low potency in the atria whereas PD116948 (pA₂ = 8.40) was the most potent of the xanthines at this receptor.

ZM 241385 demonstrated-concentration dependent, rightward parallel displacement of the 2-CADO concentration-response curve (Figure 1). The Schild plot slope was not different from unity (1.04); with the Schild slope constrained to 1 a pA₂ value of 5.95 (c.l., 5.72–6.18) was obtained.

A_{2b} receptor antagonism in guinea-pig aortae

The relative potency of the agonists i.e. NECA > 2-CADO > R-PIA confirms the presence of an A₂ receptor in the guinea-pig aorta; however, the lack of any significant activity of CGS21680 (Table 1) indicates that the A₂ receptor in this preparation is not of the A_{2a} subtype but is the A_{2b} subtype. 8PST and 8PT demonstrate low affinity in the aorta

Table 1 Comparison of the affinity and activity of selected adenosine receptor agonists in binding and functional assays

Agonists	Rat cortex binding IC ₅₀ (nM) (n = 2–9) Mean (c.l.)	Rat PC12 binding IC ₅₀ (nM) (n = 2–9) Mean (c.l.)	G-pig atria EC ₅₀ (nM) (n = 4–12) Mean (c.l.)	G-pig aorta EC ₅₀ (nM) (n = 8–17) Mean (c.l.)	G-pig Langendorff EC ₅₀ (nM) (n = 4–7) Mean (c.l.)
CGS21680	20,527 (20,520–20,534)	335 (331–339)	> 30,000	> 20,000	4.5 (3.2–7.9)
NECA	88 (55–121)	62 (61–63)	363 (190–708)	312 (255–369)	10.2 (7.9–15.8)
2-CADO	92	913	2570 (1202–5623)	3250 (2030–4470)	86.6 (65–115)
R-PIA	15.2 (12.4–17.9)	989 (983–995)	331 (95–1175)	9200 (7330–11070)	115.5 (100–158)

Mean values of potency for specific agonists are presented. Where the value has been determined three or more times the 95% confidence limit (c.l.) was calculated from the *t* value for 5% probability and the standard error of the mean value. For abbreviations, see text.

Table 2 Comparison of the affinity and activity of selected adenosine receptor agonists in binding and functional assays

Antagonists	Rat cortex binding pIC_{50} (n=4) Mean (c.l.)	Rat PC12 binding pIC_{50} (n=2-6) Mean (c.l.)	G-pig atria pA_2 (n=6-15) Mean (c.l.)	G-pig aorta pA_2 (n=6-19) Mean (c.l.)	G-pig Langendorff pA_2 or $[pK_b]$ (n=3-20) Mean (c.l.)
8PST	NT	4.5	5.35 (5.14-5.55)	5.34 (5.21-5.48)	6.20 (5.99-6.42)
8PT	NT	4.5	6.30 (6.13-6.41)	6.50 (6.3-6.71)	NT
PD116948	8.34 (8.14-8.57)	6.75	8.40 (8.3-8.5)	6.69 (6.45-6.93)	[7.10] (6.96-7.15)
ZM 241385	5.69 (5.57-5.81)	9.52 (9.02-10.02)	5.95 (5.72-6.18)	7.06 (6.92-7.19)	8.57 (8.45-8.68)

All figures quoted for functional assays are pA_2 values determined by Schild plot analysis with the slope constrained to unity when not significantly different from unity. pK_b is the 'apparent' pK_b calculated as an estimate of antagonist potency when only a single concentration of antagonist was used and assumes that the antagonist behaves as a competitive antagonist with a Schild plot slope of unity. NT=not tested. For abbreviations, see text.

(pA_2 =5.35 and 6.03 respectively) which compares with their similar low affinity in the atria and confirms their non-selectivity at A_1 and A_2 receptors in functional models.

ZM 241385 produced rightward parallel displacements of the adenosine concentration-response curve, without any effect on the maximum response therefore showing competitive antagonism, giving a pA_2 value of 7.06 (c.l., 6.92-7.19, slope of 1.07 and constrained to unity) from the Schild Plot (Figure 2). Following ZM 241385, low concentrations of adenosine (<3 μ M) resulted in a small, 20% increase in tone, likely to be due to activation of A_1 adenosine receptor subtypes (Stogdall & Shaw, 1990), before producing vasorelaxant activity.

A_{2a} receptor antagonism in guinea-pig Langendorff hearts

The potency order of agonists in lowering coronary perfusion pressure was CGS21680 > NECA > 2-CADO > R-PIA (Table 1). The adenosine receptor subtype involved in this response is therefore consistent with the A_{2a} sub-type.

The antagonist potencies in attenuating the effect of 2-CADO in this preparation (ZM 241385 > PD116948 > 8PST) parallel those seen in the PC12 binding studies (Table 2). 8PST (pA_2 =6.20) demonstrated a relatively low affinity in all preparations whilst PD116948 demonstrated approximately a ten fold greater potency than 8PST in the Langendorff prepara-

Figure 1 Effect of ZM 241385 on bradycardic effects of 2-CADO in guinea-pig isolated atria. (a) Each point represents the mean $\pm$ s.e.mean. Dose-response curve for 2-CADO in the absence ($\circ$) and presence of ZM 241385: 3×10^{-6} M ($\bullet$), 10^{-5} M ($\blacktriangle$) and 3×10^{-5} M ($\blacklozenge$). (b) Schild regression plot for antagonism of 2-CADO-induced bradycardia in guinea-pig atria by ZM 241385 ($\blacklozenge$ 95% confidence limits to the pA_2 value).

Figure 2 Effect of ZM 241385 on relaxant effects of adenosine in phenylephrine-constricted guinea-pig isolated aorta. (a) Each point represents the mean $\pm$ s.e.mean. Dose-response curve for adenosine in the absence ($\circ$) and presence of ZM 241385: 3×10^{-7} M ($\bullet$), 10^{-6} M ($\blacktriangle$) and 3×10^{-6} M ($\blacklozenge$). (b) Schild regression plot for antagonism of adenosine-induced vasorelaxation in guinea-pig aorta by ZM 241385 ($\blacklozenge$ 95% confidence limits to the pA_2 value).

tion. The most potent antagonist however, was ZM 241385 which produced concentration-dependent parallel rightward shifts in the 2-CADO-mediated responses consistent with competitive antagonism ($pA_2 = 8.57$, c.l., 8.45–8.68, slope of 1.1, not different from unity, Figure 3).

ZM 241385 also showed high antagonist potency ($pA_2 = 9.02$, c.l., 8.80–9.20, slope 0.79, not different from unity) in attenuating the effects of the A_{2a} selective agonist CGS21680 in this guinea-pig Langendorff heart preparation, further demonstrating the selectivity of ZM 241385 for the A_{2a} receptor (Figure 4).

Specificity of ZM 241385

ZM 241385 (10 μ M) produced a small (1.4 fold) rightward shift in the bradycardic response to the muscarinic agonist, carbachol, on guinea-pig aorta. ZM 241385 had no effect on re-

laxation induced by sodium nitrite, indicating lack of effect on cyclic GMP pathways at this concentration. ZM 241385 (100 μ M) did not inhibit the constrictor effect of phenylephrine on guinea-pig aorta, indicating a lack of effect at α_1 -adrenoceptors.

ZM 241385 (10 μ M) was inactive against several rat hepatocyte cyclic AMP phosphodiesterase enzymes (Table 3); however, activity at this concentration was seen against the calcium/calmodulin activated type I phosphodiesterase. At a much higher concentration (1 mM i.e. 100,000 fold more than the pA_2 at the A_{2a} receptor) ZM 241385 inhibited a wider range of hepatocyte cyclic AMP phosphodiesterase isozymes. The effects of ZM 241385 were broadly similar to those of theophylline (Table 3).

Figure 3 Effect of ZM 241385 on the vasorelaxant response of 2-CADO in phenylephrine-constricted, K⁺-arrested, guinea-pig isolated Langendorff hearts. (a) Each point represents the mean $\pm$ s.e.mean, $n = 4$. Dose-response curve for 2-CADO in the absence ($\circ$) and presence of ZM 241385: 10⁻⁸ M ($\blacksquare$), 5 $\times$ 10⁻⁸ M ($\blacktriangle$), 10⁻⁷ M ($\bullet$), 5 $\times$ 10⁻⁷ M ($\square$) and 10⁻⁶ M ($\triangle$). CPP = coronary perfusion pressure. (b) Schild regression plot for antagonism of 2-CADO-induced depressor response in guinea-pig Langendorff hearts by ZM 241385 ($\diamond$ 95% confidence limits to the pA_2 value).

Figure 4 Effect of ZM 241385 on the vasorelaxant effects of the A_{2a}-selective agonist CGS21680 in phenylephrine-constricted, arrested, guinea-pig isolated Langendorff hearts. (a) Each point represents the mean $\pm$ s.e.mean, $n = 4$. Dose-response curve for CGS21680 in the absence ($\circ$) and presence of ZM 241385: 5 $\times$ 10⁻⁹ M ($\blacktriangle$), 1.5 $\times$ 10⁻⁸ M ($\bullet$), 5 $\times$ 10⁻⁸ M ($\square$) and 10⁻⁷ M ($\triangle$). CPP = coronary perfusion pressure. (b) Schild regression plot for antagonism of CGS 21680-induced depressor response in guinea-pig Langendorff hearts by ZM 241385 ($\diamond$ 95% confidence limits to the pA_2 value).

Table 3 Inhibition of hepatocyte cyclic AMP phosphodiesterases by ZM 241385 and theophylline

Isoform	Compound			
	Theophylline (1 mM)	Theophylline (10 μ M)	ZM 241385 (1 mM)	ZM 241385 (10 μ M)
IBMX-insensitive type I	NI	NI	NI	NI
Cyclic GMP-activated type II	59 $\pm$ 2	NI	47 $\pm$ 2	NI
Cyclic GMP-inhibited type III	NI	NI	NI	NI
Ca ²⁺ /CaM-activated type I	30 $\pm$ 1	22 $\pm$ 2	73 $\pm$ 1	45 $\pm$ 3
Ro 1724-sensitive type IV	48 $\pm$ 2	NI	57 $\pm$ 3	NI

Results are the means ($\pm$ standard error) from six determinations in three separate enzyme preparations. All assays were undertaken with a concentration of 1 μ M cyclic AMP. NI, not inhibited ($< 5\%$ change in activity). For abbreviations, see text.

Discussion

The present data show that the non-xanthine, ZM 241385, is a competitive antagonist with high affinity (in the nanomolar range) at the adenosine A_{2a} receptor subtype. In addition, the compound has low affinity at the A₁ and A₃ receptor subtypes and shows 400–1000 fold selectivity for A_{2a} over A₁ receptors in isolated tissues from the guinea-pig and 6700 fold selectivity for A_{2a} over A₁ and 5000,000 fold selectivity for A_{2a} over A₃ binding sites.

In both binding and functional preparations containing A_{2a} receptors, ZM 241385 showed high affinity. Affinity of ZM 241385 in the PC12 membrane preparation was however an order of magnitude higher than when 2-CADO was used as the agonist in the guinea-pig Landendorff heart. This difference was less evident when CGS21680 was used in the guinea-pig Langendorff heart, (pA₂ = 8.57 with 2-CADO and 9.02 with CGS 21680). This implies a mixed population of A_{2a} and A_{2b} receptor subtypes in the guinea-pig heart, but this could not be detected by changes in the Schild plot slope (which was not different from unity for either agonist). ZM 241385 demonstrated low affinity in both A₁ preparations studied. In some studies, marked differences in affinity have been noted at A₁ receptors in ligand binding studies on cell membranes from different species (Ukena *et al.*, 1986). Although binding studies were performed only in rat membranes and functional studies were performed only in guinea-pig isolated preparations, no significant difference was noted between affinities calculated in rat and guinea-pig tissue. The lack of species difference observed for the affinity of ZM 241385 for A₁ and A_{2a} receptors suggests that it can be used to classify adenosine receptors in a number of species. Recently an A₃ receptor subtype, which induces hypotension, has been described based upon studies with N⁶-2-(4-aminophenyl)ethyladenosine in pithed rats; the effect is resistant to antagonism with the xanthine, 8PST (Fozard & Carruthers, 1993). The affinity of ZM 241385 at the A₃ receptor was 500,000 fold lower than at the A_{2a} receptor subtype.

The degree of A_{2a} subtype selectivity shown by ZM 241385 exceeds that reported for other xanthine and non-xanthine adenosine receptor antagonists such as 8-(3-chlorostyryl)-1,3-dipropylxanthine, CGS15943 and 8FB-PTP. 8-(3-chlorostyryl)-1,3-dipropyl-xanthine, currently the most A_{2a}:A₁ selective antagonist described, was not tested in the current series of experiments, and therefore a direct comparison with it was not possible. In binding studies however 8-(3-chlorostyryl)-1,3-dipropyl-xanthine is reported to have a pK_i of 7.3 at A_{2a} sites on striatal membranes and A_{2a}:A₁ selectivity of 520 fold but is only 22 fold selective in functional tests (Jacobson *et al.*, 1993). In comparison, ZM 241385, was 19 times more selective than 8-(3-chlorostyryl)-1,3-dipropyl-xanthine in functional assays. In addition to its limited selectivity in functional studies, the lack of information of activity of 8-(3-chlorostyryl)-1,3-dipropyl-xanthine at A_{2b} receptor subtypes (Jacobson *et al.*,

1993) may limit the use of the compound in determining the individual A₂ adenosine receptor subtype contribution to either *in vitro* or *in vivo* functional responses. CGS 15943 has affinity for the A_{2a} receptor (K_i 3 nM) but only 7 fold A_{2a}:A₁ selectivity (Williams *et al.*, 1987). Although not assayed in all functional tests, we have found CGS 15943 to be equipotent in both atria (A₁) and aorta (A_{2b}) (pA₂ 7.85, c.l., 7.59–8.10 and pA₂ 7.56, c.l., 7.38–7.74 respectively, unpublished results) compared to 13 fold A_{2b}:A₁ selectivity seen with ZM 241385. Rollins *et al.* (1994) have also recently reported CGS 15943 to be 4 fold A_{2b}:A₁ selective in binding studies with cloned human receptors. In binding assays, 8FB-PTP which has a K_i at A_{2a} receptors of 0.074 nM and 28 fold A_{2a}:A₁ selectivity, loses all A_{2a} selectivity in functional assays (Dionisotti *et al.*, 1994). ZM 241385 had 30–80 fold selectivity for A_{2a} over A_{2b} receptors. ZM 241385 is not unique with respect to this selectivity as PD115199 has been reported to have a similar degree of selectivity for A_{2a} over A_{2b} receptors (Hargreaves *et al.*, 1991). PD115199 however shows little selectivity for A_{2a} relative to A₁ receptors (2.4 fold, Bruns *et al.*, 1987b).

ZM 241385 had no additional pharmacological effects at concentrations which blocked A_{2a} adenosine receptors. The minor inhibitory effects on carbachol-induced bradycardia and isoprenaline-induced aortic relaxation occurred at concentrations 3–4 orders of magnitude greater than those required to block A_{2a} receptors. A number of adenosine receptor antagonists inhibit a range of phosphodiesterases at concentrations close to those which block adenosine receptors, e.g. theophylline (Smellie *et al.*, 1979). ZM 241385 showed similar potency to theophylline as a cyclic AMP phosphodiesterase inhibitor, but because it is four orders of magnitude more potent than theophylline as an A₂ receptor antagonist (pA₂ for theophylline = 4.6) it is unlikely that phosphodiesterase inhibitory effects will complicate interpretation of its effects.

In conclusion, ZM 241385 is a novel, non-xanthine A_{2a}-selective adenosine receptor antagonist in binding studies in rat receptors and guinea-pig isolated tissue preparations. ZM 241385 is free of non-specific activity at concentrations several orders of magnitude greater than the pA₂ value for A_{2a} receptors. It is therefore, the most selective A_{2a} adenosine receptor antagonist yet described and should prove a useful tool with which to evaluate the role of A_{2a} adenosine receptors in pharmacological preparations.

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