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Pharmacological characterisation and inhibitory effects of (2*R*,3*R*,4*S*,5*R*)-2-(6-amino-2-{[(1*S*)-2-hydroxy-1-(phenylmethyl)ethyl]amino}-9*H*-purin-9-yl)-5-(2-ethyl-2*H*-tetrazol-5-yl)tetrahydro-3,4-furandiol, a novel ligand that demonstrates both adenosine A_{2A} receptor agonist and adenosine A_3 receptor antagonist activity

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Abstract

The pharmacological properties of the novel ligand, (2*R*,3*R*,4*S*,5*R*)-2-(6-amino-2-{[(1*S*)-2-hydroxy-1-(phenylmethyl)ethyl]amino}-9*H*-purin-9-yl)-5-(2-ethyl-2*H*-tetrazol-5-yl)tetrahydro-3,4-furandiol (**I**), at the human adenosine receptors were investigated using Chinese hamster ovary cell lines recombinantly expressing these receptors. Functional studies were performed using a cyclic AMP-coupled reporter gene system. Binding studies were performed using membranes from these cells. The effects of ligand (**I**) were also determined on functional responses of human neutrophils and eosinophils. Ligand (**I**) had a high affinity for the adenosine A_{2A} receptor (pKi 7.8±0.2) and was a potent agonist at this receptor (pEC_{50} 9.0±0.2). Ligand (**I**) had a similar affinity for the adenosine A_3 receptor (pKi 7.8±0.1) but displayed no agonist activity, acting instead as a competitive antagonist (pA_2 8.3±0.04). Ligand (**I**) had lower affinity for adenosine A_1 and A_{2B} receptors (pKi ≤ 6) and showed relatively weak agonist activity at these receptors (pEC_{50} 7.1 at both receptors). Ligand (**I**) was a potent inhibitor of the generation of reactive oxygen species from human neutrophils and eosinophils (pEC_{50} 9.7±0.1 and 9.4±0.2 respectively). The inhibitory effect of ligand (**I**) on the release of reactive oxygen species from neutrophils was antagonised competitively by the adenosine A_{2A} receptor antagonist 9-chloro-2-(2-furanyl)-[1,2,4]triazolo[1,5-c]quinazolin-5-amine (CGS15943) with a pA_2 value (10.03±0.44) consistent with an effect on adenosine A_{2A} receptors. Ligand (**I**) also inhibited the release of granule proteins from neutrophils and eosinophils (pEC_{50} 8.7 and 8.9 respectively), albeit less potently than as an inhibitor of reactive oxygen species generation. In summary, ligand (**I**) is a potent and selective agonist for the adenosine A_{2A} receptor and a competitive antagonist at the adenosine A_3 receptor. Ligand (**I**) has potent anti-inflammatory effects on human granulocytes *in vitro*.

Keywords: (2*R*3*R*,4*S*,5*R*)-2-(6-amino-2-{[(1*S*)-2-hydroxy-1-(phenylmethyl)ethyl]amino}-9*H*-purin-9-yl)-5-(2-ethyl-2*H*-tetrazol-5-yl)tetrahydro-3 4-furandiol; Adenosine A_{2A} receptor; Adenosine A_3 receptor; Neutrophil; Eosinophil

1. Introduction

The actions of endogenous purine nucleoside adenosine are diverse and believed to be of particular importance in environments in which hypoxia, ischaemia or inflammation occurs.

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Adenosine exerts its array of pharmacological effects via at least four types of G-protein-coupled receptor, the A_1, A_{2A}, A_{2B} and A_3 receptors (Olah and Stiles, 2000). The particular role of the adenosine A_{2A} receptor in the modulation of various facets of the inflammatory response has been the subject of several recent reviews (Fredholm et al., 2002; Sitkovsky et al., 2004; Lappas et al., 2005). A range of studies in animals have implicated activation of the adenosine A_{2A} receptor by adenosine or synthetic analogues as an important mechanism of anti-inflammatory activity (Ohta and Sitkovsky, 2001; Okusa et al., 1999; Day et al., 2004; Fozard et al., 2002). In man, the adenosine A_{2A} receptor has been localised to areas of the central nervous system, vasculature and cells of the immune system (Daval et al., 1996; Svenningsson et al., 1997; Fredholm et al., 2002). It is possible that adenosine may act through A_{2A} receptors as an endogenous modulator of immune responses. Indeed, adenosine A_{2A} receptor agonists have inhibitory effects on a range of human cell types including granulocytes and mononuclear cells (reviewed by Sullivan, 2003).

The role of the human adenosine A_3 receptor is less well understood. It is difficult to predict the effects of adenosine A_3 receptor activation in man from studies in laboratory animals due to the variation between species in A_3 receptor protein sequence, pharmacology and distribution (Linden, 1994; Baraldi et al., 2000). In man, mRNA for the adenosine A_3 receptor is highest in CNS, liver and lung (Ralevic and Burnstock, 1998; Fredholm et al., 2000). The receptor is also expressed on human eosinophils, although the consequence of activation is unclear (Reeves et al., 2000). In rodents, activation of the adenosine A_3 receptor appears to give rise to both pro-inflammatory and anti-inflammatory effects (Fishman and Bar-Yehuda, 2003).

The novel ligand (2*R*,3*R*,4*S*,5*R*)-2-(6-amino-2-{[(1*S*)-2-hydroxy-1-(phenylmethyl)ethyl]amino}-9*H*-purin-9-yl)-5-(2-ethyl-2*H*-tetrazol-5-yl)tetrahydro-3,4-furandiol ((**I**); Fig. 1) was synthesised as part of a programme to create agonists that were highly selective for the adenosine A_{2A} receptor, to allow exploration of the anti-inflammatory effects mediated by this receptor (Keeling et al., 2000). In the present report we demonstrate that not only is ligand (**I**) a potent adenosine A_{2A} receptor agonist, it is also a competitive antagonist at adenosine A_3 receptors. Ligand (**I**) has potent inhibitory effects on the generation of reactive oxygen species and the degranulation of human granulocytes.

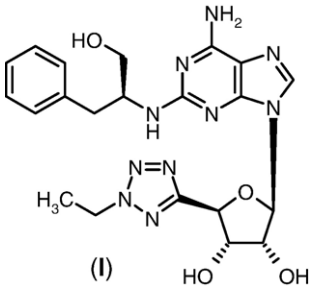

Fig. 1. Chemical structure of (2*R*,3*R*,4*S*,5*R*)-2-(6-amino-2-{[(1*S*)-2-hydroxy-1-(phenylmethyl)ethyl]amino}-9*H*-purin-9-yl)-5-(2-ethyl-2*H*-tetrazol-5-yl)tetrahydro-3,4-furandiol (**I**).

2. Methods

2.1. Preparation of recombinant cell lines

Binding and functional studies were performed using Chinese hamster ovary cells expressing human recombinant adenosine receptors (A_1, A_{2A}, A_{2B} or A_3). Chinese hamster ovary cells were transfected using a calcium phosphate transfection kit with constructs containing a gene for the human adenosine receptor (A_1, A_{2A}, A_{2B} or A_3), antibiotic resistance genes (for geneticin and hygromycin) and cAMP response elements (CRE; 6 in series) promoting the transcription of the gene for secreted placental alkaline phosphatase. Post-transfection, cells were grown in the presence of geneticin (1 g/l) and hygromycin (500 mg/l) to select for positive clones. Presence of the appropriate gene was confirmed using a reporter assay. Cells expressing adenosine receptors were then cultured in DMEM/F12 HAM (1:1) medium containing L-glutamine (2 mM), geneticin (0.5 g/l for A_1; 1 g/l for A_{2A}, A_{2B} and A_3), hygromycin (500 mg/l), foetal bovine serum (10%) and adenosine deaminase (1 unit/ml) at 37 °C in 5% CO_2.

For binding studies, cells were harvested using phosphate-buffered saline containing 5 mM EDTA and centrifuged at 500 *g* for 10 min at 4 °C. They were then resuspended in 10 × pellet volume HEPES buffer (50 mM) at pH 7.4, homogenised using an Ultra-Turrax homogeniser, centrifuged at 500 *g* for 20 min at 4 °C to remove unbroken cells and nuclei, and then the supernatant was centrifuged at 48,000 *g* for 30 min. The final pellet of cell membrane was resuspended in HEPES buffer and stored in aliquots at −80 °C.

Prior to initiation of functional (reporter gene) studies, the foetal bovine serum within the culture medium was replaced with bovine serum albumin (1 g/l) 24 h before the studies were performed (because growth factors in the foetal serum activate the cAMP response elements causing high background secreted placental alkaline phosphatase release).

2.2. Radioligand binding studies

Membranes (approximately 10 μg/tube) were incubated for 1 h at room temperature with radioligand and either ligand (**I**) or vehicle in a total volume of 200 μl. For studies using agonist radioligands, the medium included 10 mM $MgCl_2$. Radioligands used were: adenosine A_1 receptor agonist, [^{3}H] 2-chloro-N^6-cyclopentyladenosine ([^{3}H]CCPA); adenosine A_1 receptor antagonist, [^{3}H] 1,3-dipropyl-8-cyclopentylxanthine ([^{3}H] DPCPX); adenosine A_{2A} receptor agonist, [^{3}H] 2-*p*-(2-carboxyethyl) phenylethylamino-5′-*N*-ethylcarboxamidoadenosine ([^{3}H]CGS21680); adenosine A_{2B} receptor antagonist, [^{3}H] DPCPX; adenosine A_3 receptor agonist, [^{125}I] N^6-(3-iodobenzyl) adenosine-5′-*N*-methyluronamide ([^{125}I]AB-MECA). Samples were filtered using a Tomtek harvester and counted on an appropriate scintillation or gamma counter.

2.3. Functional studies using recombinant cell lines

Adenosine receptor agonist activity was determined by measuring the ability of the agonist to stimulate (A_{2A}, A_{2B}) or inhibit