Cyclic AMP-dependent inhibition of human neutrophil oxidative activity by substituted 2-propynylcyclohexyl adenosine A_{2A} receptor agonists

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1 Novel 2-propynylcyclohexyl-5'-N-ethylcarboxamidoadenosines, *trans*-substituted in the 4-position of the cyclohexyl ring, were evaluated in binding assays to the four subtypes of adenosine receptors (ARs). Two esters, 4-{3-[6-amino-9-(5-ethylcarbamoyl-3,4-dihydroxy-tetrahydro-furan-2-yl)-9H-purin-2-yl]-prop-2-ynyl}-cyclohexanecarboxylic acid methyl ester (ATL146e) and acetic acid 4-{3-[6-amino-9-(5-ethylcarbamoyl-3,4-dihydroxy-tetrahydro-furan-2-yl)-9H-purin-2-yl]-prop-2-ynyl}-cyclohexylmethyl ester (ATL193) were > 50 × more potent than 2-[4-(2-carboxyethyl)phenethylamino]-5'-N-ethylcarboxamidoadenosine (CGS21680) for human A_{2A} AR binding. Human A_{2A} AR affinity for substituted cyclohexyl-propynyladenosine analogues was inversely correlated with the polarity of the cyclohexyl side chain. There was a comparable order of potency for A_{2A} AR agonist stimulation of human neutrophil [cyclic AMP]_i, and inhibition of the neutrophil oxidative burst. ATL146e and CGS21680 were ~equipotent agonists of human A₃ ARs.

2 We measured the effects of selective AR antagonists on agonist stimulated neutrophil [cyclic AMP]_i and the effect of PKA inhibition on A_{2A} AR agonist activity. ATL193-stimulated neutrophil [cyclic AMP]_i was blocked by antagonists with the potency order: ZM241385 (A_{2A}-selective) > MRS1220 (A₃-selective) > > N-(4-Cyano-phenyl)-2-[4-(2,6-dioxo-1,3-dipropyl-2,3,4,5,6,7-hexahydro-1H-purin-8-yl)-phenoxy]-acetamide (MRS1754; A_{2B}-selective) ≈ 8-(N-methylisopropyl)-amino-N⁶-(5'-endohydroxy-endonorbornyl)-9-methyladenine (WRC0571; A₁-selective). The type IV phosphodiesterase inhibitor, rolipram (100 nM) potentiated ATL193 inhibition of the oxidative burst, and inhibition by ATL193 was counteracted by the PKA inhibitor H-89.

3 The data indicate that activation of A_{2A}ARs inhibits neutrophil oxidative activity by activating [cyclic AMP]_i/PKA.

British Journal of Pharmacology (2001) **132**, 1017–1026

Keywords: A_{2A} adenosine receptors; neutrophil; oxidative burst; cyclic AMP-dependent protein kinase

Abbreviations: ATL146a, 4-{3-[6-amino-9-(5-ethylcarbamoyl-3,4-dihydroxy-tetrahydro-furan-2-yl)-9H-purin-2-yl]-prop-2-ynyl}-cyclohexanecarboxylic acid; ATL146e, 4-{3-[6-amino-9-(5-ethylcarbamoyl-3,4-dihydroxy-tetrahydro-furan-2-yl)-9H-purin-2-yl]-prop-2-ynyl}-cyclohexanecarboxylic acid methyl ester; ATL193, acetic acid 4-{3-[6-amino-9-(5-ethylcarbamoyl-3,4-dihydroxy-tetrahydro-furan-2-yl)-9H-purin-2-yl]-prop-2-ynyl}-cyclohexylmethyl ester; ATL2037, 5-{6-amino-2-[3-(4-hydroxymethyl-cyclohexyl)-prop-1-ynyl]-purin-9-yl}-3,4-dihydroxy-tetrahydro-furan-2-carboxylic acid ethylamide; BW-1433, 8-(4-carboxyethylphenyl)-1,3-dipropylxanthine; CGS21680, 2-[4-(2-carboxyethyl)phenethylamino]-5'-N-ethylcarboxamidoadenosine; HE-NECA, 5-(6-amino-2-hex-1-ynyl-purin-9-yl)-3,4-dihydroxy-tetrahydro-furan-2-carboxylic acid ethylamide; MRE0470, 2-[6-amino-2-(N'-cyclohexyl-methylene-hydrazino)-purin-9-yl]-5-hydroxymethyl-tetrahydro-furan-3,4-diol; MRS1220, N-(9-chloro-2-furan-2-yl-[1,2,4]triazolo[1,5-c]quinazolin-5-yl)-2-phenyl-acetamide; MRS1754, N-(4-cyano-phenyl)-2-[4-(2,6-dioxo-1,3-dipropyl-2,3,4,5,6,7-hexahydro-1H-purin-8-yl)-phenoxy]-acetamide; NECA, 5'-N-ethylcarboxamidoadenosine; rolipram, 4-(3'-cyclopentyl-4'-methoxyphenyl)-2-pyrrolidone; WRC0571, 8-(N-methylisopropyl)amino-N⁶-(5'-endohydroxy-endonorbornyl)-9-methyladenine; XAC, 8-(4-((2-aminoethyl)aminocarbonylmethoxy)phenyl)-1,3-dipropylxanthine; ZM241385, 4-(2-[7-amino-2-[2-furyl][1,2,4]triazolo[2,3-a][1,3,5]triazin-5-yl-amino]ethyl)-phenol

Introduction

There are four subtypes of G protein coupled adenosine receptors (ARs), A₁, A_{2A}, A_{2B} and A₃. A_{2A} ARs have been extensively characterized in striatum (Rosin *et al.*, 1998), coronary arteries (Belardinelli *et al.*, 1998) and platelets

(Dionisotti *et al.*, 1996). A_{2A}AR agonists have been reported to produce anti-inflammatory responses mediated by receptors on neutrophils (Cronstein *et al.*, 1983; Sullivan *et al.*, 1999; reviewed by Gessi *et al.*, 2000) and other types of inflammatory cells (reviewed by Sullivan & Linden, 1998). Among the most potent and selective agonists of A_{2A}ARs are the 2-substituted amine, 2-[4-(2-carboxyethyl)phenethylamino]-5'-N-ethylcarboxamidoadenosine (CGS21680) (Hutchison

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et al., 1989) the hydrazines, (Niiya *et al.*, 1992) and the 2-alkynyls, e.g. 5-(6-amino-2-hex-1-ynyl-purin-9-yl)-3,4-dihydroxy-tetrahydro-furan-2-carboxylic acid ethylamide (HE-NECA) (Cristalli *et al.*, 1992) (Figure 1). In this study we describe the properties of new compounds designed to combine the cyclohexyl element of a very active hydrazine, MRE0470, and the alkynyl element of HE-NECA to produce the first 2-(4-substituted-cyclohexyl)propynyl NECAs, 4-{3-[6-amino-9-(5-ethylcarbamoyl-3,4-dihydroxy-tetrahydro-furan-2-yl)-9H-purin-2-yl]-prop-2-ynyl}-cyclohexanecarboxylic acid methyl ester (ATL146e) and acetic acid 4-{3-[6-amino-9-(5-ethylcarbamoyl-3,4-dihydroxy-tetrahydro-furan-2-yl)-9H-purin-2-yl]-prop-2-ynyl}-cyclohexylmethyl ester (ATL193); Figure 1). We observed that the nature of the 4-substitution markedly affects the potency and selectivity of these compounds.

We and others have observed that both A_{2A} AR and A₃ AR agonists have anti-inflammatory activities *in vitro*

(Bouma *et al.*, 1997; Bowlin *et al.*, 1997; Cronstein *et al.*, 1990; Sullivan *et al.*, 1999) and *in vivo* (Bowlin *et al.*, 1997; Hasko *et al.*, 1998; Hasko *et al.*, 1996; Salvatore *et al.*, 2000; Sullivan *et al.*, 1999; Szabo *et al.*, 1998). Because there is potential cross-reactivity between binding of adenosine agonists to A_{2A} ARs and A₃ ARs, we have used the new A_{2A} AR agonists and selective receptor antagonists to confirm the neutrophil AR subtype responsible for inhibiting neutrophil oxidative activity. Since there is disagreement in the literature concerning the role of [cyclic AMP]_i and cyclic AMP-dependent protein kinase (PKA) in adenosine inhibition of the neutrophil oxidative burst (Cronstein *et al.*, 1988; Fredholm *et al.*, 1996; Sullivan *et al.*, 1995; 1999), we used the phosphodiesterase inhibitor, rolipram, and the PKA inhibitor, N-[2-(p-bromocinnamylamino)ethyl]-5-isoquinoline sulfonamide (H-89), to further investigate the role of cyclic AMP as a mediator of the A_{2A}AR response in neutrophils. The data indicate that ATL146e and ATL193 are very potent inhibitors of neutrophil oxidative activity and that they signal predominantly through A_{2A}AR_S, cyclic AMP and PKA.

Methods

Materials

4-(2-[7-amino-2-[2-furyl][1,2,4]triazolo[2,3-a][1,3,5]triazin-5-yl-amino]ethyl)-phenol (ZM241385) (Poucher *et al.*, 1995) was a gift from Simon Poucher, Astra-Zeneca Pharmaceuticals, Cheshire, U.K. Stock solutions (1 mM and 10 mM in dimethyl sulphoxide [DMSO]) were stored at -20°C. Recombinant human tumor necrosis factor- α (TNF) was a gift from Dainippon Pharmaceutical Co. Ltd Osaka, Japan (specific activity = 600 pg u⁻¹). Stock solutions were made in Hanks balanced salt solution (HBSS)-0.1% human serum albumin (HAS) at 2 $\times$ 10⁵ u ml⁻¹, aliquoted into single day samples and frozen at -70°C. Rolipram (4-[3'-cyclopentyl-4'-methoxyphenyl]-2-pyrrolidone) was a gift from Berlex Laboratories (Cedar Knolls, NJ, U.S.A.). Stock solutions (10 mM) were made in DMSO and stored at -70°C. 8-(4-carboxyethenylphenyl)-1,3-dipropylxanthine (BW1433) was a gift from Susan Daluge (Glaxo-Wellcome, Research Triangle Park, NC, U.S.A.). N-(4-cyano-phenyl)-2-[4-(2,6-dioxo-1,3-dipropyl-2,3,4,5,6,7-hexahydro-1H-purin-8-yl)-phenoxy]-acetamide (MRS1754) (Kim *et al.*, 2000) and, N-(9-chloro-2-furan-2-yl-[1,2,4]triazolo[1,5-c]quinazolin-5-yl)-2-p henyl-acetamide (MRS1220) (Jacobson, 1998) were gifts from Kenneth Jacobson (National Institutes of Health; Bethesda, MD, U.S.A.). 8-(N-methylisopropyl)amino-N⁶-(5'-endohydroxy-endonorbornyl)-9-methyladenine (WRC0571) (Martin *et al.*, 1996) and, 2-[6-amino-2-(N'-cyclohexylmethylenehydrazino)-purin-9-yl]-5-hydroxymethyl-tetrahydro-furan-3,4-dio (MRE0470) (Martin *et al.*, 1997) were gifts from Pauline Martin (Discovery Therapeutics; Richmond, VA, U.S.A.). CGS21680 was purchased from Research Biochemicals Incorporated (Natick, MA, U.S.A.). Neutrophil isolation medium (Ficoll-Hypaque) was purchased from ICN Biomedicals (Aurora, OH), Accurate Chemicals and Scientific (Westbury, NY, U.S.A.), and Cardinal Associates (Santa Fe, NM, U.S.A.). The following reagents were purchased from Sigma Chemical (St. Louis, MO, U.S.A.), luminol, 8-(4-((2-aminoethyl) aminocarbonyl methoxy)phenyl)-1,3-di-

Figure 1 Molecular structures of some A_{2A} AR agonists discussed in the study.

propylxanthine (XAC) and formyl-methionyl-leucyl-phenylalanine (fMLP). Adenosine deaminase was purchased from Boehringer Mannheim (Indianapolis, IN, U.S.A.), and Sigma Chemical. N-[2-(p-bromocinnamylamino)ethyl]-5-isoquinolinesulfonamide (H-89), was purchased from Calbiochemical (La Jolla, CA, U.S.A.).

Synthesis of novel 2-cyclohexyl-propynyl-NECAs

The synthesis of ATL146e involved the coupling of 2-iodo-5'-(N-ethylcarboxamido) adenosine with the acetylenic methyl-(cyclohexylcarboxylate). This synthetic strategy has been described previously (Cristalli *et al.*, 1992) and (Matsuda *et al.*, 1992) for the making 2-alkynyl A_{2A} AR agonists and has the value of enabling considerable synthetic manipulation of the adenosine nucleus. The principal focus of our search for novel A_{2A} agonists was modifying the adenosine C2-position to affect selectivity and potency. The conversion of the adenosine 5'-position into an ethyl carboxamide was retained because it improves A_{2A} selectivity and precludes the possible formation of phosphorylated metabolites from the action of adenosine kinase at this position. Studies of A_{2A} structure-activity relationships suggest that these sites exhibit a substantial level of independence—that is, each site may be modified independently with an additive effect on activity. Other analogues we have synthesized include the de-esterified hydrolysis product of ATL146e, 4-{3-[6-amino-9-(5-ethyl-carbamoyl-3,4-dihydroxy-tetrahydro-furan-2-yl)-9H-purin-2-yl]-prop-2-ynyl}-cyclohexanecarboxylic acid (ATL146a), and the inverse ester of ATL146e (ATL193) and its hydrolysis product, 5-{6-amino-2-[3-(4-hydroxymethyl-cyclohexyl)-prop-1-ynyl]-purin-9-yl}-3,4-dihydroxy-tetrahydro-furan-2-carboxylic acid ethylamide (ATL2037).

The novel 2-alkynyladenosines (2-AAAs) designed and evaluated in this report required the coupling of a series of terminal alkynes to 2-iodo 5'-N-ethylcarboxamidoadenosine (NECA) (Cristalli *et al.*, 1992) utilizing triethylamine (TEA) and copper (I) iodide (CuI) in dimethylformamide (DMF)/Acetonitrile with catalytic Pd(triphenylphosphine)[PPh₃]₄ in a classical Sonogashira cross-coupling fashion. These novel alkynes were realized from modification of commercially available *trans*-1,4-cyclohexanedimethanol. This diol was first mono-protected with tertbutyldimethylsilyl chloride (TBSCl) under classical conditions using imidazole and DMF as solvent. The remaining free hydroxyl group was then activated by formation of the tosylate using p-toluenesulphonyl (TosCl) and pyridine in chloroform. This tosylate was then displaced with the acetylene group through the use of lithium acetylide. After deprotection with tetrabutylammonium fluoride (TBAF), the alkyne precursor to ATL2037 was complete. This compound was then either acetalated with acetic anhydride to form the precursor to ATL193, or oxidized *via* Jones oxidation to form the acid precursor ATL146a. Finally, the ATL146e precursor was formed through esterification of ATL146a with trimethylsilyl (TMS) diazomethane.

To a solution of 2-Iodo NECA (0.025 mmol) and alkyne (0.045 mmol) in DMF/acetonitrile (0.5 ml ml⁻¹) was added TEA (0.76 mmol), Pd(PPh₃)₄ (0.0038 mmol), CuI (0.0046 mmol), and PPh₃ (0.0038 mmol). The clear yellow solution was heated to 60°C for 12 h under nitrogen to yield a brown/black solution. The solvent was then removed *en*

vacuo to yield a syrup that was purified *via* silica gel column chromatography using 6% methanol/chloroform to yield pure products. These products were characterized *via* ¹H NMR, ¹³C NMR and MS. Purity was confirmed *via* LC/MS and was greater than 95% for all compounds tested.

Human neutrophil preparation

A one-step Ficoll-Hypaque separation procedure (Ferrante & Thong, 1980) was used to purify human neutrophils from normal heparinized (10 u ml⁻¹) venous blood yielding approximately 98% neutrophils; >95% viable as determined with trypan blue containing <50 pg ml⁻¹ of endotoxin. Following separation, the neutrophils were washed with Hank's balanced salt solution three times.

Radioligand binding assays

Our binding methodology has been described previously (Sullivan *et al.*, 1999). In brief, all four subtypes of recombinant human ARs were stably expressed in HEK-293 cells (A₃, A_{2A}, and A_{2B} receptors) or CHO-K1 cells (A₁ receptors). Crude membrane preparations from these transfected cells were diluted in HE buffer (50 mM HEPES; 1 mM EDTA pH 7.4) at concentrations between 2–35 µg tube⁻¹ in 50 µl. Dilutions of agonist and antagonist compounds were prepared in HE plus ADA (2 u ml⁻¹), MgCl₂ (10 mM), and an appropriate radioligand (¹²⁵I-ABA for A₁ and A₃ receptors, ¹²⁵I-ZM241385 for A_{2A} receptors and ¹²⁵I-ABOPX for A_{2B} receptors). The final concentrations in binding assays are ADA (1 u ml⁻¹) and MgCl₂ (5 mM). Diluted agonists or antagonists (50 µl) were added to each membrane sample (50 µl), and the tubes incubated for 2 h at room temperature and then filtered through glass fibre filters and counted. Samples were assayed in triplicate. Non-specific binding was measured in the presence of XAC (50 µM) (A₁, A_{2A}, and A_{2B} receptors) or BW1433 (10 µM) (A₃ receptors).

Neutrophil intracellular cyclic AMP concentrations [*cyclic AMP*]_i

Purified human neutrophils were incubated in HBSS containing 0.1% HSA (2 × 10⁶ PMN 250 µl⁻¹) and ADA (1 u ml⁻¹), ±ATL146e, ATL146a, ATL193, or CGS21680, plus rolipram (1 µM), and ±ZM241385 (100 nM) for 15 min at 37°C in a shaking water bath. The reaction was stopped with 0.5 ml of 0.15 N HCl. The cells were pelleted by centrifugation and 0.5 ml samples were frozen for cyclic AMP analysis in the University of Virginia Radioimmunoassay Core Laboratory.

Neutrophil oxidative activity (luminol-enhanced chemiluminescence)

Activated neutrophils emit light from unstable high-energy oxygen species produced by the plasma membrane associated NADPH oxidase and metabolized by cytoplasmic and granule enzymes. The light signal from activated neutrophils can be enhanced by the addition of luminol to the samples. Luminol-enhanced emission of light is stimulated by singlet oxygen, a reactive oxygen species, dependent on both the

production of superoxide and mobilization of myeloperoxidase from primary granules (DeChatelet *et al.*, 1982).

Purified neutrophils ($1 \times 10^6 \text{ ml}^{-1}$) were incubated in HBSS containing 0.1% HSA (1 ml), plus adenosine deaminase (1 u ml^{-1}), $\pm$ ATL193, ATL2037, CGS21680, ATL146e or ATL146a, $\pm$ ZM241385, $\pm$ H-89, $\pm$ rolipram (100 nM), and with TNF α (10 u ml^{-1}) for 30 min at 37°C in a shaking water bath. Then luminol ($1 \times 10^4 \text{ M}$) enhanced f-met-leu-phe ($1 \mu\text{M}$)-stimulated chemiluminescence was read with a Chronolog Photometer (Chronolog Corp., Havertown, PA, U.S.A.) at 37°C for 8 min. Chemiluminescence is reported as relative peak light emitted (=height of the curve) compared to TNF α -primed fMLP-stimulated control samples.

A_{2A}AR agonist bioactivity in human plasma

Heparinized (10 u ml^{-1}) platelet free human plasma was prepared from normal blood by differential centrifugation and then frozen (-80°C). ATL193 and ATL146e ($15 \mu\text{l}$) were added to 1.5 ml aliquots of plasma from 1 mM stock solutions in DMSO and incubated at 37°C in a 5% CO_2 environment. Aliquots ($200 \mu\text{l}$) were sampled at 0, 15, 30, 60, 120, and 240 min and immediately added to 1.8 ml of cold acetone to arrest compound metabolism and to precipitate plasma proteins. Following centrifugation, the supernatants were stored at -20°C . The acetone was dried in a Speed Vac (Savant, Farmingdale, NY, U.S.A.) and residual compound reconstituted in PBS and assayed by competition for ^{125}I -ZM241385 binding to recombinant human A_{2A}ARs (see radioligand binding assays above). The concentrations of ATL193 and ATL146e present were calculated from an appropriate standard curve run concurrently with the samples. Control serum samples containing vehicle did not compete for ^{125}I -ZM241385 binding to A_{2A}ARs .

Statistical analysis Binding and functional data values (mean, SEM, EC_{50} and K_i) were determined using GraphPad PRISM (San Diego, CA, U.S.A.). Schild analysis (Schild, 1957) was used to calculate pA_2 values of inhibitors.

Results

Design of novel A_{2A}AR agonists

Selectivity of adenosine analogues has been conferred by modifying the 5'-hydroxyl of adenosine to make 5'-*N*-ethylcarboxamine and by adding various 2-substitutions. CGS21680 (Figure 1) is an adenosine analogue that is substituted on the 5' ribose with *N*-ethylcarboxamide and on the 2 position with amino-ethyl-phenyl-propionic acid. This compound has emerged as the most widely used A_{2A} -selective agonist (Jarvis *et al.*, 1989). Among a series of 2-hydrazino adenosine analogues, a very potent compound was a 2-cyclohexyl derivative, MRE0470 (Niiya *et al.*, 1992). Of the various 2-substituted compounds that have been synthesized, 2-propynyl compounds such as HE-NECA appear to have high potency and selectivity (Cristalli *et al.*, 1992). These considerations led us to synthesize 2-propynyl-cyclohexyl NECAs with various substitutions on the 4 position of the cyclohexyl ring.

Comparison of binding and functional activities of A_{2A} agonists

The effects of cyclohexyl substitutions had not been explored previously. We found that these compounds, particularly ATL146e and ATL193, were among the most potent and selective A_{2A} agonists yet synthesized. The polarity of the 4-cyclohexyl substituent has a large effect on affinity (see Figure 2 and Table 1). The ester, ATL146e, was >40 times more potent at human A_{2A}ARs than its de-esterified acid metabolite, ATL146a, or CGS21680. Compared to the ATL146e, the inverse ester, ATL193, had similar affinity, but the de-esterified metabolites differ in affinity; the moderately polar alcohol, ATL2037, was more potent than the more polar acid, ATL146a.

Although the novel A_{2A}AR agonist, ATL146e bound to transfected human A_{2A}ARs with 4.8, 13 and 46 fold greater affinity than NECA, MRE0470 and CGS21680, respectively (Figure 2 and Table 1), its absolute potency to uncoupled recombinant human A_{2A}ARs was moderate; the K_i was 83 nM . CGS21680 and MRE0470 are widely recognized as A_{2A}AR selective compounds based on their relative binding affinities for A_1 and A_{2A} receptors in rat brain and based on

Figure 2 Competition by A_{2A} AR agonists for binding of ^{125}I -ZM241385 to recombinant human A_{2A} ARs. (A) ATL146e, ATL146a and CGS21680. (B) ATL193 and ATL2037. Membranes ($2 \mu\text{g protein tube}^{-1}$) prepared from HEK-293 cells stably transfected with human A_{2A} ARs were incubated with ^{125}I -ZM241385 (0.5 nM) and various concentrations of competing compounds. Specific binding was $>80\%$ of total binding. Each point is the mean $\pm$ s.e.mean of triplicate determinations. Similar results were obtained in two or more additional experiments. K_i values are reported in Table 1.

Table 1 Binding to membranes derived from cells stably transfected with recombinant human ARs. Each value is the mean $\pm$ s.e.mean of 3–10 replicate experiments

Receptor	A_{2A}	A_3	A_{2B}	A_1
Radioligand (affinity)*	^{125}I -ZM241385 (low)	(^{125}I -ABA) (high)	(^{125}I -ABOPX) (low)	(^{125}I -ABA) (high)
Agonists		K_1 (nM) [fold less affinity than ATL146e]		
ATL146e	83 $\pm$ 20 [1]	45 $\pm$ 15 [1]	> 10,000	77 $\pm$ 12 [1]
ATL146a	3820 $\pm$ 1249 [46]	29 $\pm$ 10 [0.6]	> 10,000	189 $\pm$ 19 [2.4]
ATL193	187 $\pm$ 25 [2.3]	231 $\pm$ 91 [5.1]	> 10,000	71 $\pm$ 14 [0.9]
ATL2037	694 $\pm$ 69 [8.4]	76 $\pm$ 6 [1.7]	> 10,000	19 $\pm$ 1 [0.2]
NECA	401 $\pm$ 106 [4.8]	32 $\pm$ 9 [0.7]	2200 $\pm$ 532	2.0 $\pm$ 1.4 [0.02]
MRE0470	1090 $\pm$ 252 [13]	41 $\pm$ 8 [0.9]	> 10,000	113 $\pm$ 21 [1.7]
CGS21680	3820 $\pm$ 733 [46]	82 $\pm$ 18 [1.8]	> 10,000	316 $\pm$ 59 [4.1]
Antagonists		K_1 (nM)		
WRC0571	4110 $\pm$ 2530	> 10,000	> 10,000	5.6 $\pm$ 0.2
MRS1220	25 $\pm$ 2	1.3 $\pm$ 0.1	355 $\pm$ 60	231 $\pm$ 23
ZM241385	1.9 $\pm$ 0.5†	774 $\pm$ 124	33 $\pm$ 10†	250 $\pm$ 89†
MRS1754	508 $\pm$ 62	570 $\pm$ 151	2.2 $\pm$ 0.3	403 $\pm$ 16

*Agonists are known to bind to two affinity states of G protein coupled receptors. Low affinity binding reflects receptors uncoupled from G proteins. High affinity refers to receptor-G protein complexes that are selectively detected with agonist radioligands. †(Sullivan *et al.*, 1999).

functional assays, such as coronary dilator potency (Shryock *et al.*, 1998). We evaluated for the first time the affinity of various ligands to all four subtypes of recombinant human ARs in competition radioligand binding assays. None of the agonists appear to be highly selective for human A_{2A} ARs over A_1 ARs or A_3 ARs (Table 1). However, these binding parameters are not a direct measure of functional potency, since agonist affinity is influenced by G protein coupling and spare receptors in tissues. The relative potency at A_{2A} ARs is probably underestimated (see Discussion). Functionally, the potency of ATL146e to inhibit oxidative burst in human neutrophils (EC_{50} = 0.32 nM, Figure 3) was 260 fold greater than the potency for binding to human A_{2A} ARs (K_1 = 83 nM; Table 1). The potency orders in assays of binding to human A_{2A} ARs, neutrophil cyclic AMP accumulation and inhibition of oxidative burst in all cases were the same: ATL146e > ATL193 > NECA > ATL2037 > MRE0470 > CGS21680 $\approx$ ATL146a (Figures 2 and 3, Table 1). In contrast, ATL146e and ATL193 had comparable binding affinity compared to CGS21680 and ATL146a for binding to human A_3 ARs (Table 1). ATL146e and ATL193 had $\sim$ 30 fold lower affinity for human A_1 ARs compared to NECA, and all of the agonists tested had relatively low affinity for human A_{2B} ARs. These results suggest that inhibition of the oxidative burst in neutrophils by ATL146e and ATL193 are mediated by A_{2A} ARs.

The results of cyclic AMP assays with selective antagonists are consistent with the conclusion that the agonists are acting through A_{2A} ARs. The affinity order of antagonists for human A_{2A} ARs was ZM241385 > MRS1220 > MRS1754 > WRC0571 (Table 1). ZM241385 (100 nM) selectively blocks A_{2A} and not A_3 ARs; WRC0571 selectively blocks A_1 and not A_{2A} receptors; and MRS1754 is a highly selective antagonist of human A_{2B} ARs (Kim *et al.*, 2000). Agonist activity at A_{2A} ARs and A_{2B} ARs stimulate adenylyl cyclase through coupling to G_s proteins. In contrast, agonist activity at A_1 and A_3 ARs inhibit adenylyl cyclase through coupling to G_i

proteins. Hence, it would be predicted that A_{2A} and A_{2B} AR antagonists will decrease cyclic AMP accumulation stimulated by their respective receptor agonists and that A_1 and A_3 AR antagonism would result in increased [cyclic AMP]_i in response to A_1 and A_3 AR agonist stimulation, respectively. The accumulation of cyclic AMP stimulated by ATL193 was potently blocked by ZM241385 (100 nM) and not by 1 μ M WRC0571 nor by MRS1754 (Figure 4). It is significant that the A_3 AR-selective antagonist, MRS1220, has only moderate selectivity (19 fold) for A_3 over A_{2A} ARs. If used at concentrations in the range of 100 nM–1 μ M, MRS1220 will not distinguish well between responses mediated by human A_{2A} and A_3 receptors and consequently (as would be predicted of an A_{2A} AR antagonist but not an A_3 AR antagonist) decreased ATL193-stimulated cyclic AMP accumulation. In contrast, ZM241385 is 407 fold selective for human A_{2A} over A_3 ARs, and produces selective A_{2A} blockade of [cyclic AMP]_i stimulated by ATL 193 in the range of 100 nM–1 μ M. ZM241385 also antagonized ATL193 inhibition of neutrophil oxidative activity (Figure 5). Schild analysis of the data indicated a pA_2 = 8.86 (K_D = 1.4 nM; Figure 5, inset) which agrees closely with the K_1 of ZM241385 for binding to A_{2A} ARs (1.9 nM).

The role of cyclic AMP and cyclic AMP-dependent kinase (PKA) in mediating the functional effects of A_{2A} AR agonists

Phosphodiesterase (PDE) type IV is the most abundant isozyme found in inflammatory cells. Selective PDE IV inhibitors such as rolipram (Schneider *et al.*, 1986) increase cellular [cyclic AMP]_i by blocking the breakdown of cyclic AMP to 5' AMP. Although we have observed that higher concentrations of rolipram decrease neutrophil oxidative activity (Sullivan *et al.*, 1995), rolipram (100 nM) alone had little effect on TNF α -primed fMLP-stimulated human neutrophil oxidative activity, but synergistically decreased the oxidative burst of neutrophils when added with sub-

Figure 3 Inhibition of the neutrophil oxidative burst and stimulation of neutrophil [cyclic AMP]_i by agonists of A_{2A} ARs. (A) ATL146e, ATL146a, and CGS21680. (B) ATL193 and ATL2037. Luminol-enhanced chemiluminescence was assayed in human neutrophils ($1 \times 10^6 \text{ ml}^{-1}$) primed with TNF α (10 u ml^{-1} ; 30 min; 37°C) and then stimulated with fMLP ($1 \mu\text{M}$; 10 min; 37°C) $\pm$ A_{2A} AR agonists + ADA (1 u ml^{-1}). The results are reported as peak chemiluminescence as per cent of a TNF-primed control. Each point is the mean $\pm$ s.e.mean, $n=4-6$ experiments. The EC₅₀s for decreasing oxidative activity are: ATL146e = 0.3 nM; ATL193 = 0.8 nM; ATL146a = 16.7 nM; ATL2037 = 4.4 nM; and CGS21680 = 9.2 nM. Inset: Human neutrophils ($8 \times 10^6 \text{ ml}^{-1}$) were incubated (15 min; 37°C) with $1 \mu\text{M}$ rolipram and 1 u ml^{-1} ADA $\pm$ A_{2A} AR agonists. Neutrophil [cyclic AMP]_i was measured by radioimmunoassay. Each point (cyclic AMP pmol ml⁻¹) is the mean $\pm$ s.e.mean ($n=6$). The EC₅₀ values for increased neutrophil [cyclic AMP]_i are: ATL146e = 7.1 nM; ATL193 = 5.6 nM; ATL146a = 19.7 nM; ATL2037 = 8.6 nM and CGS21680 = 17.1 nM.

optimal concentrations of ATL193. The EC₅₀ was shifted 20 fold from 0.81 nM to 0.04 nM upon the addition of 100 nM rolipram (Figure 6). The PKA inhibitor H-89 (Chijiwa *et al.*, 1990) did not affect TNF α -primed fMLP-stimulated human neutrophil oxidative activity (Figure 7), did not prime neutrophils in the absence of TNF α (data not shown), did not bind to human A_{2A} receptors (data not shown), but did completely counteract the inhibitory effect of ATL193 (10 nM) on the neutrophil oxidative burst (Figure 7). The EC₅₀ of H-89 for reversing ATL193 (10 nM)-inhibited neutrophil oxidative burst was 2.2 μM.

A_{2A}AR agonist bioactivity in human plasma

We observed that both ATL146e and ATL193 lost bioactivity when incubated in human plasma with a half-life of 55 and 41 min, respectively (Figure 8). While almost all of the

Figure 4 Competitive antagonism of ATL193-stimulated neutrophil cyclic AMP accumulation. (A) ZM241385 (100 nM). (B) ATL193 ($1 \mu\text{M}$), WRC0571, MRS1754, MRS1220, and ZM241385 (10–1000 nM). Human neutrophils ($8 \times 10^6 \text{ ml}^{-1}$) were incubated (15 min; 37°C) with $1 \mu\text{M}$ rolipram and 1 u ml^{-1} ADA $\pm$ ATL193 (0.01–100 nM) and $\pm$ adenosine receptor antagonists (WRC0571, MRS1754; MRS1220 or ZM241385; 10–1000 nM). Neutrophil [cyclic AMP]_i was measured by radioimmunoassay. Each point (cyclic AMP pmol ml⁻¹) is the mean $\pm$ s.e.mean ($n=6$).

Figure 5 Effect of ZM241385 on ATL193-inhibited neutrophil oxidative activity. Luminol-enhanced chemiluminescence was assayed in neutrophils ($1 \times 10^6 \text{ ml}^{-1}$) primed with TNF α (10 u ml^{-1} ; 30 min; 37°C) and then stimulated with fMLP ($1 \mu\text{M}$; 10 min; 37°C) $\pm$ ATL193 (0.01 nM–100 nM) $\pm$ ZM241385 + ADA (1 u ml^{-1}). The results are reported as percent TNF-primed control. Each point is the mean $\pm$ s.e.mean ($n=6$). Inset: Schild analysis, $pA_2=8.86$.

ATL146e bioactivity was lost after 240 min, ATL193 activity reached a plateau at about one-third of the initial bioactivity (Figure 8). These results are consistent with the possibility that ATL146e and ATL193 are de-esterified to the acid, ATL146a and the alcohol, ATL2037, respectively.

Figure 6 Synergistic effects of rolipram and ATL193 on the oxidative burst in human neutrophils. Luminol-enhanced chemiluminescence was measured in human neutrophils ($1 \times 10^6 \text{ ml}^{-1}$) primed with TNF α (10 u ml^{-1} ; 30 min; 37°C) and then stimulated with fMLP ($1 \mu\text{M}$ 10 min; 37°C) $\pm$ ATL193 (0.01 nM – 100 nM) $\pm$ rolipram (100 nM) + ADA (1 u ml^{-1}). The results are reported as per cent of TNF-primed control. Each point is the mean $\pm$ s.e.mean ($n=4-5$).

Figure 7 H-89 counteracts ATL193-inhibited neutrophil oxidative activity. Luminol-enhanced chemiluminescence was measured in human neutrophils ($1 \times 10^6 \text{ ml}^{-1}$) primed with TNF α (10 u ml^{-1} ; 30 min; 37°C) and then stimulated with fMLP ($1 \mu\text{M}$; 10 min; 37°C) $\pm$ ATL193 (10 nM) $\pm$ H-89 (100 nM – $10 \mu\text{M}$) + ADA (1 u ml^{-1}). The results are reported as percent of a TNF-primed control. Each point is the mean $\pm$ s.e.mean ($n=6$).

Discussion

We have investigated the properties of novel 2-propynylcyclohexyl NECAs as potent and selective agonists of human A_{2A} ARs. These compounds combine the best features of a series of 2-substituted hydrazine and 2-substituted propynyl compounds to make 2-propynyl-cyclohexyl derivatives. Of interest was the new finding that the substituent on the 4-position of the cyclohexyl ring had a profound effect on binding affinity to human A_{2A} ARs. In particular, the ester, ATL146e, had >40 times higher affinity than the acids, ATL146a and CGS21680. Our hypothesis for the present studies was that the novel A_{2A} -selective agonists would inhibit pro-inflammatory neutrophil activity by binding A_{2A} receptors *via* a cyclic AMP-dependent mechanism.

Based on radioligand binding assays, none of the compounds we evaluated, including ATL146e or CGS21680, were highly selective for human A_{2A} receptors over human A_1 and A_3 receptors. This incongruity can be explained by

Figure 8 Kinetics of declines in the bioactivity of ATL146e and ATL193 in human plasma. (A) ATL146e. (B) ATL193. ATL193 and ATL146e ($15 \mu\text{l}$ of 1 mM) were added to 1.5 ml aliquots of human plasma and incubated (37°C ; $5\% \text{ CO}_2$) for 0–240 min. Aliquots ($200 \mu\text{l}$) were transferred to 1.8 ml of cold acetone at each time point. Following removal of precipitated proteins and rapid evaporation bioactivity was assayed by competitive radioligand binding to A_{2A} ARs. Each point is the mean $\pm$ s.e.mean ($n=3-4$).

the fact that agonists bind to two affinity states of G protein coupled receptors. Low affinity binding sites reflect agonist binding to receptors uncoupled from G proteins, and high affinity sites reflect binding to receptor-G protein complexes that are selectively detected with agonist radioligands. A_{2A} ARs are poorly coupled to Gs in striatum and particularly to overexpressed recombinant receptors in transfected cells (Luthin *et al.*, 1995). These uncoupled receptors are best detected with antagonist radioligands. Binding of agonists to uncoupled receptors was measured in competition with antagonist radioligands, ^{125}I -ZM241385 for A_{2A} ARs and ^{125}I -ABOPX for A_{2B} ARs. This and possible spare receptors on neutrophils may account for the large discrepancy (>250 fold) between A_{2A} agonist ligand K_i values and EC_{50} values to inhibit neutrophil oxidative activity. In contrast, agonist binding to the Gi/o coupled A_1 and A_3 ARs detects primarily GTP-sensitive high affinity binding sites of these well-coupled receptor-G protein complexes.

The potency order of both agonists (ATL146e $>$ ATL193 $>$ NECA $>$ ATL2037 $>$ MRE0470 $>$ CGS21680 $\approx$ ATL146a) and antagonists (ZM241385 $>$ MRS1220 $>$ MRS1754 $\approx$ WRC057) in neutrophil functional assays (i.e. oxidative activity and [cyclic AMP] $_i$) correlate well with the potency order of these compounds in binding assays. These data support the conclusion that the agonists act through A_{2A} ARs

to increase neutrophil [cyclic AMP]_i and decrease the oxidative burst, rather than through A₃ARs (where ATL146e $\approx$ CGS21680 in binding affinity) or A₁ARs (where NECA > ATL146e and ATL2037 > ATL193 in binding affinity). Although the A_{2A} AR agonists are potent inhibitors of the neutrophil oxidative burst, they do not completely inhibit the oxidative burst. This suggests that some of the oxidative burst is resistant to inhibition by A_{2A} AR agonist binding. This is consistent with our previous observations (Sullivan, *et al.*, 1995) that A_{2A} AR agonists do not decrease unstimulated neutrophil oxidative activity nor oxidative activity induced by some stimuli (e.g. phorbol myristate acetate). These data indicate that the neutrophil oxidase is not directly affected by A_{2A} AR binding, but rather upstream process(es) such as coupling of the f-met-leu-phe receptor to its signalling pathway (Cronstein *et al.*, 1992a) may be affected.

In addition to A_{2A}ARs, neutrophils express A₁ARs (Cronstein *et al.*, 1992b), and possibly A₃ receptors that may control human neutrophil degranulation (Bouma *et al.*, 1997). Unlike binding to A_{2A}ARs, when adenosine binds to A₁ARs the effects are pro-inflammatory, resulting in increased adhesion of neutrophils to endothelial cells (Cronstein *et al.*, 1992b), augmented phagocytosis (Salmon & Cronstein, 1990), and enhanced stimulation of oxidative burst by immune complexes (Salmon & Cronstein, 1990). In contrast, 2-propynylcyclohexyl adenosine receptor agonists displayed only anti-inflammatory activity, substantiating their selectivity for A_{2A}ARs.

Whereas agonist binding to either A_{2A} or A_{2B} ARs stimulates adenylyl cyclase *via* G_s coupling, agonist binding to A₁ and A₃ ARs signal *via* G_i coupling to decrease adenylyl cyclase activity (Ongini & Fredholm, 1996). 2-Propynylcyclohexyl adenosine receptor agonists only stimulated neutrophil [cyclic AMP]_i, and this effect was selectively blocked by the A_{2A} AR antagonist, ZM241385. Consistent with the expectation of little binding by the new compounds to human A_{2B} ARs, the selective A_{2B}AR antagonist MRS1754 had little effect on agonist-stimulated [cyclic AMP]_i.

Like A_{2A}AR agonists, other agents that stimulate neutrophil cyclic AMP accumulation also decrease neutrophil oxidative activity (Bengis-Garber & Gruener, 1996). However, the roles of cyclic AMP and cyclic AMP-dependent PKA in the A_{2A} receptor-mediated anti-inflammatory effects of adenosine on neutrophil function are controversial. There are conflicting reports regarding the correlation between adenosine-stimulated [cyclic AMP]_i and effects on neutrophil function (Cronstein *et al.*, 1988). Supporting a role for cyclic AMP is the finding of synergistic effects of A_{2A}AR agonists and type IV phosphodiesterase inhibitors (Sullivan *et al.*, 1995). However, another study reported additive activity (Cronstein *et al.*, 1988). In addition, some PKA inhibitors only partially counteract inhibition of neutrophil stimulated oxidative activity (Mueller *et al.*, 1992). Hence, the role of cyclic AMP in the A_{2A} adenosine receptor anti-inflammatory response in neutrophils is unresolved.

In the present study, we observed a correlation between stimulation of neutrophil [cyclic AMP]_i and inhibition of the oxidative burst by the substituted 2-propynylcyclohexyl adenosine agonists. Although there is a correlation between neutrophil [cyclic AMP]_i and inhibition of the oxidative burst, the EC₅₀ for decreasing the oxidative burst is

consistently lower than the EC₅₀ for increasing neutrophil [cyclic AMP]_i. This suggests the possibility that effector function stimulated by A_{2A} AR agonists may also act through cyclic AMP/PKA independent pathway(s). It has been recently reported that stimulation of β -adrenergic receptors on thymocytes in addition to activating PKA, can stimulate through G_s and the Src family tyrosine kinase Lck to induce apoptosis independent of cyclic AMP/PKA (Gu *et al.*, 2000). It is not known if neutrophils have analogous Src family tyrosine kinase dependent cyclic AMP independent pathway(s) stimulated by A_{2A} AR agonists.

The effects of ATL193 on neutrophil oxidative activity were markedly potentiated by the type IV PDE inhibitor rolipram (Figure 6), and ATL193 suppression of the oxidative burst was completely counteracted by the PKA inhibitor H-89 (Figure 7). These data taken together strongly indicate that the substituted 2-propynylcyclohexyl adenosine agonists are anti-inflammatory predominantly by increasing neutrophil [cyclic AMP]_i. Possible effects of PKA activation in neutrophils include phosphorylation of Rap1A, preventing incorporation of cytochrome b₅₅₈ into the NADPH oxidase complex (Bokoch *et al.*, 1991), and phosphorylation of RhoA (Laudanna *et al.*, 1997) resulting in diminished phospholipase D activity (Thibault *et al.*, 2000) and reduced oxidase activity (Frank *et al.*, 1994). In addition, PKA activation causes reduced fMLP-stimulated phosphorylation of the NADPH oxidase component p47 phox upstream of protein kinase C (Bengis-Garber & Gruener, 1996).

We observed that the binding affinities and functional activities of the relatively nonpolar compounds, ATL146e and ATL193, were much greater at human A_{2A} ARs than their more polar de-esterified products, ATL146a and ATL2037 or the polar agonist CGS21680 (Figure 1). The rapid decay of bioactivity of ATL146e and ATL193 in human plasma are consistent with the possibility that serum esterase(s) convert the esters to the corresponding acid, ATL146a, and alcohol, ATL2037, respectively. ATL146a had little detectable residual bioactivity in plasma since ATL146a had 46 times less binding affinity than ATL146e for A_{2A}ARs. The residual activity of ATL2037 in plasma could be because it bound to A_{2A}ARs with only 3.6 fold lower affinity than ATL193.

Several anti-inflammatory responses occur when A_{2A}ARs are activated on neutrophils. Adenosine and A_{2A}AR agonists decrease expression of adhesion molecules (Wollner *et al.*, 1993), which results in decreased neutrophil adherence to matrix proteins (Sullivan *et al.*, 1999) and to vascular endothelium (Cronstein *et al.*, 1992b). A_{2A}AR agonists reduce the oxidative activity of both suspended and adherent neutrophils (Cronstein *et al.*, 1983; De La Harpe & Nathan, 1989; Sullivan *et al.*, 1999), and inhibit neutrophil degranulation (Richter, 1992).

There is also evidence *in vivo* that adenosine is anti-inflammatory by binding to A_{2A}ARs. For example, methotrexate appears to act by increasing adenosine concentrations within inflamed tissues. This endogenous adenosine binds to neutrophil A_{2A}ARs and attenuates the inflammatory response (Cronstein *et al.*, 1993). Moreover, we have observed that the A_{2A}AR agonist, MRE0470, inhibits endotoxin-induced pleocytosis and blood brain barrier permeability in a rat model of bacterial meningitis (Sullivan *et al.*, 1999). In recent studies we have shown that ATL146e can protect rat

kidneys from ischaemia/reperfusion induced damage (Okusa *et al.*, 1999), and lung reperfusion injury in a rabbit transplantation model (Ross *et al.*, 1999).

We conclude that substituted 2-propynylcyclohexyl adenosine agonists, ATL146e and ATL193, inhibit the neutrophil oxidative burst by binding predominantly to A_{2A} ARs and act principally by a mechanism mediated by cyclic AMP-dependent PKA. Hence, compounds in this class may have therapeutic potential for reducing inflammation due to infection or ischaemia.

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(Received September 29, 2000
 Revised November 23, 2000
 Accepted December 8, 2000)