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Introduction of Alkynyl Chains on C-8 of Adenosine Led to Very Selective Antagonists of the A_3 Adenosine Receptor

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Abstract—Some 8-alkynyladenosines were synthesized and evaluated for their adenosine receptor activity, utilizing radioligand binding studies (A_1, A_{2A}, A_3) or adenylyl cyclase activity assays (A_{2B}). Furthermore, the maximal induction of guanosine 5′-(γ-thio)triphosphate ([^{35}S]GTPγS) binding to G proteins and the inhibition of NECA-stimulated binding, in membranes of CHO cells which express the human A_3 receptor, were used to determine the intrinsic activity of these nucleosides at the A_3 adenosine receptor. The results showed that these new adenosine derivatives are very selective ligands for the A_3 receptor subtype and behave as adenosine antagonists, since they do not stimulate basal [^{35}S]GTPγS binding, but inhibit NECA-stimulated binding. This is the first report that adenosine derivatives, with unmodified ribose moiety, are adenosine receptor antagonists.

Introduction

Adenosine and adenosine-related molecules are involved in the regulation of many aspects of cellular metabolism. Most of adenosine actions are mediated by four extracellular receptors termed A_1, A_{2A}, A_{2B}, and A_3.[1,2] To date, only adenosine itself is approved for human use although drugs with a purinergic mechanism are an expanding therapeutic target.[3,4] However, in order to fully evaluate these prospects, selective agonists and antagonists with high affinity and potency are still required.[1]

In a search for selective adenosine receptor ligands it has been found that 2-hexynyladenosine (HEAdo) and its 5′-*N*-ethyluronamide derivative (HENECA) are endowed with high affinity for A_1 and A_{2A} adenosine receptors[5,6] (Fig. 1). Moreover, HENECA was also found to possess good affinity for A_3 receptor subtype.[7,8] More recently, we reported that 2-alkynylAdos and 2-alkynyl-N^6-alkylAdos[9] show high affinity and different degree of selectivity for human A_3 receptor.

On the other hand, the C-8 position of adenosine has been investigated in the past using few adenosine analogues with relatively short substituents and testing them only at A_1 and A_{2A} adenosine receptor subtypes.[10–14] The tested compounds resulted in generally very weak ligands and in the case of 8-alkylaminoAdos they are reported to be partial agonists at rat A_1 receptor.[12,13]

The fact that there are only few reports on the influence of a substituent in the 8-position of adenosine at A_1 and A_{2A} subtypes and no reports on such activity at A_{2B} and A_3 receptors prompted us to investigate the affinity and potency at the four adenosine receptor subtypes of adenosine analogues bearing alkynyl substituents on the C-8, also taking into account our previous experience on alkynyl chains in the 2-position of adenosine and NECA.[5,15,16]

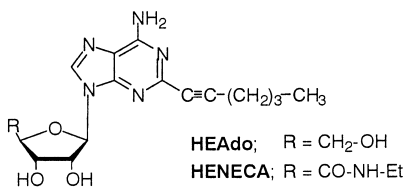

Figure 1.

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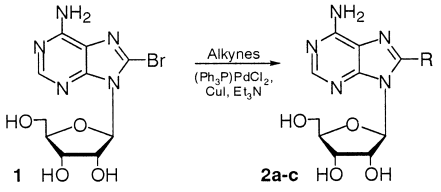
Chemistry

The synthesis of 8-substituted adenosines (**2a**–**c**) was carried out as follows starting from commercially available 8-bromoadenosine (8-BrAdo, **1**, Scheme 1). Substitution of the bromine in the 8-position of **1** with three different alkynyl chains (commercially available 1-hexyne, phenylethyne and 1-phenyl-2-proyn-1-ol) was carried out by a modification of the palladium catalyzed cross-coupling reaction. Briefly, to a solution of **1** (0.51 mmol) in dry DMF (15 mL), and Et_3N (2.3 mL) under an atmosphere of N_2 were added bis(triphenylphosphine) palladium dichloride (8.1 mg, 0.012 mmol) and CuI (0.51 mg, 0.003 mmol).

The appropriate terminal alkyne (3.1 mmol) was added and the reaction mixture was stirred under an atmosphere of N_2 at room temperature for different times depending upon the reactivity of each compound. The solvent was removed in vacuo and the residue was chromatographed on a silica gel column eluting with $CHCl_3$–MeOH (92:8) to obtain **2a** and **2b** and on a TLC plate eluting with $CHCl_3$–MeOH (85:15) to obtain **2a**, as amorphous solids.

The phenylhydroxypropyne reaction with **1** for 4 days did not give the corresponding 8-phenylhydroxypropynyl derivative but the phenylketopropenyl analogue **2c**, due to a rearrangement on the side chain. The structure of (*E*)-8-(3-keto-3-phenyl-1-propen-1-yl)-9-(β-D-ribofuranosyl)adenine (**2c**) was confirmed by ^1H NMR and ^{13}C NMR. In fact, the ^1H NMR displayed two doublets of the (*E*) double bond protons at δ 7.87 and 8.22 with a coupling constant of 15.2 Hz, while the ^{13}C NMR showed a carbonyl group signal at 188.4 ppm.



Scheme 1.

Results and Discussion

Compounds **2a**–**c** were evaluated at the human recombinant adenosine receptors, stably transfected into Chinese hamster ovary (CHO) cells, utilizing radioligand binding studies (A_1, A_{2A}, A_3) or adenylyl cyclase activity assays (A_{2B})[17] and the results are reported in Table 1.

The extent of stimulation or inhibition of [^{35}S]GTPγS binding by A_3 receptor ligands was determined as described previously[18] in membranes of CHO cells which express the human A_3 receptor. Nonspecific binding was measured in the presence of 10 μM GTPγS. NECA (1 μM) was used as a reference full agonist.

The effects of the other A_3 receptor ligands were tested at a concentration of $100 \times K_i$ (determined by inhibition of [^{3}H]NECA binding) in the absence or presence of 1 μM NECA. The results are given as percentage of the effect of 1 μM NECA after subtraction of nonstimulated binding. Basal binding was 3037 ± 121 cpm/μg protein, and binding in the presence of 1 μM NECA amounted to 4944 ± 251 cpm/μg. Data are from three independent experiments and are given as means ± SEM. The results are reported in Table 2 and Figure 2.

The results of binding and cyclase studies showed that the introduction of an alkynyl chain at the C-8 position of Ado is detrimental for the affinity and potency at A_1, A_{2A}, and A_{2B} receptors, while is more tolerated by the A_3 receptor (Table 1). In fact, compounds **2a** and **2b** exhibited A_3 affinity in the high nanomolar range

Table 2. Stimulation or inhibition of [^{35}S]GTPγS binding by NECA and **2a**–**c** in membranes of CHO cells which express the human A_3 receptor

	Concn (μM)	Agonist effect (% of NECA ± SEM)
Basal[a]		0
NECA[b]	1	100[c]
2a	65.1	0.5 ± 1.1
2a + NECA		39.0 ± 11.9
2b	78.8	0.2 ± 0.3
2b + NECA		26.8 ± 8.3
2c	983	1.9 ± 3.6
2c + NECA		43.7 ± 0.8

[a]Basal [^{35}S]GTPS binding: 3037 ± 121 cpm.
[b][^{35}S]GTPS Binding in the presence of 1 μM NECA: 4944 ± 251 cpm.
[c]100% Is the NECA-induced stimulation (4944–3037 cpm).

Table 1. Affinities of NECA and **2a**–**c** in radioligand binding assays at human A_1, A_{2A} and A_3, and in adenylyl cyclase assays at human A_{2B} adenosine receptors

	K_i or EC_{50} (μM)			
Compd	K_i (A_1)[a]	K_i (A_{2A})[b]	K_i (A_{2B})[c]	K_i (A_3)[d]
NECA	0.014 (0.006–0.028)	0.020 (0.012–0.035)	2.4 (1.83-3.41)	0.006 (0.004–0.012)
2a	> 100	> 100	> 100	0.65 (0.54–0.78)
2b	> 100	> 100	> 100	0.79 (0.42–1.48)
2c	> 100	46.6 (43.0–50.6)	> 100	9.83 (7.28–13.3)

[a]Displacement of specific [^{3}H]CCPA binding in CHO cells, stably transfected with human recombinant A_1 adenosine receptor, expressed as K_i (μM).
[b][^{3}H]NECA saturation binding in CHO cells, stably transfected with human recombinant A_{2A} adenosine receptor, is expressed as K_i (μM).[18]
[c]Measurement of receptor-stimulated adenylyl cyclase activity in CHO cells, stably transfected with human recombinant A_{2B} adenosine receptor, expressed as EC_{50} (μM).
[d][^{3}H]NECA saturation binding in CHO cells, stably transfected with human recombinant A_3 adenosine receptor, is expressed as K_i (μM).[18]

($K_i = 650$ and 790 nM, respectively), whereas they were nearly inactive at the other adenosine receptor subtypes.

On the other hand, the phenylketopropenyl analogue **2c**, although maintaining some activity at A_3 adenosine receptor, showed a reduced selectivity towards the A_{2A} subtype (K_i $A_3 = 9.83\,\mu M$ and K_i $A_{2A} = 46.6\,\mu M$).

The data obtained from the evaluation of the stimulation or inhibition of [^{35}S]GTPγS binding by compounds **2a–c** alone or in the presence of NECA, in membranes of CHO cells which express the human A_3 receptor, are even more remarkable (Table 2 and Fig. 2).

In fact, all three adenosine derivatives do not stimulate basal [^{35}S]GTPγS binding, but inhibit to various extent NECA-stimulated binding. 8-PhenylethynylAdo (8-PEAdo, **2b**) proved to be the most potent A_3 antagonist in the series with a 73% inhibition of NECA-stimulated binding and no effect on the basal [^{35}S]GTPγS binding (0.2 ± 0.3).

It is worth noting that the presence of the same alkynyl chain in the 8-position of 9-ethyladenine led to a relatively selective antagonist at adenosine A_3 subtype (9-ethyl-8-phenylethynyladenine, K_i $A_1 = 1.28\,\mu M$; K_i $A_{2A} = 0.60\,\mu M$; K_i $A_3 = 0.086\,\mu M$; IC_{50} $A_{2B} \geq 100\,\mu M$).[19]

Hence, the substitution of an alkyl chain by a ribose moiety, leaving the same base, led to a nucleoside which retains the A_3 antagonist behaviour, although with a 10-fold lower affinity [K_i $A_3 = 0.79\,\mu M$ (**2b**) vs K_i $A_3 = 0.086\,\mu M$ (9-ethyl-8-phenylethynyladenine)].

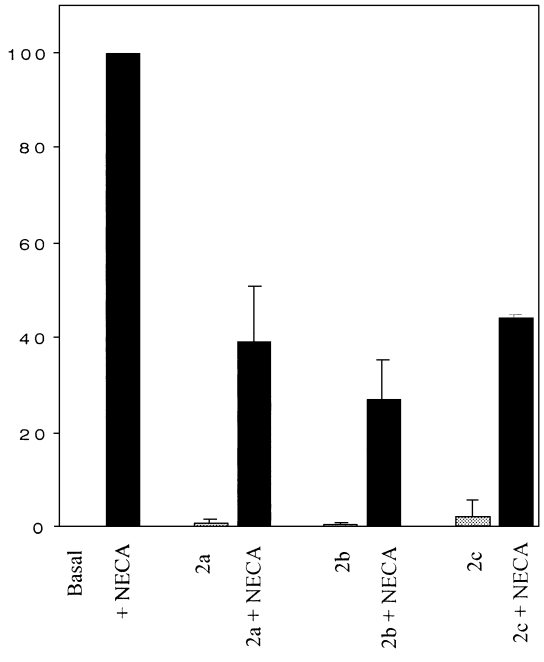


Figure 2. % Inhibition of NECA-stimulated [^{35}S]GTPγS binding by **2a–c** in membranes of CHO cells which express the human A_3 receptor.

Some preliminary studies on conformational preferences of these molecules have been performed and the main observation is that from ^{1}H NMR spectra in DMSO-d_6 the 8-alkynyladenosines prefer the *syn* conformation whereas the corresponding 2-alkynyladenosines prefer the *anti* conformation. Very similar results were obtained from the calculation of ab initio (3-21G) energies. In fact, the global minimum was found in the *syn* conformation for the 8-alkynyladenosines, and in the *anti* conformation for the 2-substituted derivatives.

Furthermore, preliminary docking experiments at the theoretical model of the human A_3 receptor[20] demonstrated that the 8-alkynyladenosines binding in the *anti* conformation, which is characteristic of adenosine receptor agonists, is not allowed. This fact is due to the steric hindrance of the alkynyl chain present at the 8-position of such molecules.

In conclusion, we described the first series of adenosine derivatives that clearly behave as adenosine antagonists, although maintaining an intact ribose in the molecular structure. Moreover, these new nucleosides are very selective ligands for the adenosine A_3 receptor.

These results are the rationale for the introduction at the 8-position of adenosine of a wider range of alkynyl chains which could improve the affinity of these molecules for the A_3 receptor to obtain a new series of potent and selective adenosine antagonists with nucleoside structure.

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