Abstract A series of 2-phenylethynyladenosine (PEAdo) derivatives substituted in the N6- and 4'-position was synthesised and the new derivatives were tested at the four human adenosine receptors stably transfected into Chinese hamster ovary (CHO) cells, using radioligand binding studies (A1, A2A, A3) or adenylyl cyclase activity assay (A2B). Binding studies showed that the presence of a phenyl ethynyl group in the 2 position of adenosine favoured the interaction with A3 receptors, resulting in compounds endowed with high affinity and selectivity for the A3 subtype. Additional substitution of the N6- and 4'-position increases both A3 affinity and selectivity. The results showed that the new compounds have a good affinity for the A3 receptor and in particular, the N6-methoxy-2-phenylethynyl-5'-N-methylcarboxamidoadenosine, with a Ki at A3 of 1.9 nM and a selectivity A1/A3 and A2A/A3 of 4,800- and 8,600-fold, respectively. Therefore, it is one of the most potent and selective agonists at the human A3 adenosine receptor subtype reported so far. Furthermore, functional assays of inhibition of 10 μM forskolin-stimulated cAMP production via the adenosine A3 receptor revealed that the new trisubstituted adenosine derivatives behave as full agonist of this receptor subtype. Docking analysis of these compounds was performed at a homology model of the human A3 receptor based on the bovine rhodopsin crystal structure as template, and the results are in accordance with the biological data.

Key words adenosine - adenosine agonists - adenosine receptors - agonists - G-protein-coupled receptors - homology modelling - signal transduction

Introduction

Adenosine (Ado) is an endogenous modulator of a wide range of biological functions in the nervous, cardiovascular, renal and immune systems through interaction with four receptor subtypes, termed A1, A2A, A2B and A3, which belong to the family of G-protein-coupled receptors [1]. All four Ado receptor subtypes are coupled to the enzyme adenylyl cyclase in either an inhibitory (A1 and A3 subtypes) or stimulatory manner (A2A and A2B subtypes), and their activation leads to a decrease or increase of cAMP levels, respectively. In addition, all four subtypes may positively couple to phospholipase C via different G protein subunits. In particular, the A3 Ado receptor subtype exerts its action through the stimulation of phospholipases C and D [2]. Although the physiological role of the A3 receptor subtype is not clearly clarified, due to its recent characterisation and the lack of really selective ligands for in vivo studies, it has recently attracted significant interest as novel drug target. In fact, A3 agonists may have potential as cerebroprotective and cardioprotective agents and may also be potential drugs for the treatment of asthma, as anti-inflammatory and immunosuppressive agents and in cancer therapy as cytostatics and chemoprotective compounds [3]. In many papers, it has been reported that Ado derivatives bearing in 2 position (ar)alkenyl chains showed good affinity and high degree of selectivity for the human A3 Ado receptor.
subtype [4–6]. Particularly, it has been found that the presence of a phenylethynyl group in the 2 position, combined with small substituents, such as a methyl, in the N6-position of Ado, favours interaction with A3 receptors, leading to compounds with high affinity and selectivity for the same subtype [7]. In addition, the substitution of the 4'-hydroxymethyl group of the sugar moiety with a methyl- or ethylcarboxamido substituent enhanced A3 affinity [5, 8]. Starting from these observations and with the aim of finding more potent and selective A3 Ado receptor agonists, 2-phenylethynyl Ado derivatives with a methoxy group in the N6-position were synthesised and tested at the four human Ado receptors stably transfected into Chinese hamster ovary (CHO) cells using radioligand binding studies (A1, A2A, A3) or adenylyl cyclase activity assay (A2B). The ability of the new compounds examined to inhibit 10 μM forskolin-stimulated cAMP production via the Ado A3 receptor was also studied. As genetic information and molecular modelling of this receptor subtype can facilitate the understanding and the rationalising of the drug–receptor interaction, a molecular docking analysis of these compounds was performed utilising a homology model of human A3 receptor based on the bovine rhodopsin crystal structure as template. The synthesis of these new compounds will be reported elsewhere.

Materials and methods

Biological evaluation

Binding studies

Dissociation constants of unlabelled compounds (Ki values) were determined in competition experiments in 96-well microplates, as described recently [9]. For A2A and A3 Ado receptors, the nonselective agonist [3H]NECA (30 nM and 10 nM, respectively) was utilised as radioligand. The A1-selective agonist 2-chloro-N6-[3H]cyclopentyladenosine ([3H]CCPA; 1 nM) was utilised for the characterisation of A1 receptor binding. Nonspecific binding was determined in the presence of 100 μM R-PIA and 1 mM theophylline, respectively. For details, see Klotz et al. [9]. All binding data were calculated by nonlinear curve fitting with the programme SCTFIT [10].

Adenylyl cyclase activity

Functional activity of the 2-phenylethynyladenosine (PEAdo) derivatives was determined in adenylyl cyclase experiments. Stimulation of adenylyl cyclase via A2B Ado receptors and inhibition of forskolin-stimulated adenylyl cyclase via A3 receptors were measured as described earlier [9, 11].

Molecular modelling

Computational methodologies

All molecular modelling studies were carried out on a 2 CPU (PIV 2.0–3.0 GHZ) Linux PC. Homology modelling was done using Molecular Operating Environment (MOE, version 2004.03) suite [12]. Docking studies were done with Schrodinger Macromodel (ver. 8.0) [13, 14] and Schrodinger Maestro interface.

Homology model of the human A3 receptor

A homology model of the human A3 receptor was built by using the X-ray crystal structure of the resting state bovine rhodopsin (pdb code: 1L9H [15]; available at the RCSB Protein Data Bank) with a 2.6-Å resolution as structural template. To model the human A3 receptor, the amino acid sequences of transmembrane (TM) helices of the human A3 receptor and bovine rhodopsin were aligned. In this phase, some guanine nucleotide-binding protein-coupled receptor (GPCR) highly conserved amino acid residues could work as guide, including the DRY motif (D3.49, R3.50, Y3.51, or D107, R108, Y109 respectively) and three Pro residues (P4.60, P6.50, P7.50 or P145, P189, P245, respectively). Boundaries that were identified from the X-ray crystal structure of bovine rhodopsin were applied for the corresponding sequences of the TM helices of the A3 receptor. Loop domains of the human A3 receptor were constructed by the loop search method implemented in MOE. Special care had to be given to the second extracellular (E2) loop, which folds back over TM helices. This loop limits the size of the active site, and their amino acids could be involved in direct interactions with the ligands. The presence of a conserved disulfide link between cysteines in TM domain 3 (TM3) and E2 might be the driving force to this particular fold of the E2 loop, so this domain was modelled using a rhodopsin-like constrained geometry around the E2TM3 disulfide bridge. Because of the limited sequence similarity between the human A3 receptors and the template in the C-terminal domains, only a short segment of this region was modelled. In particular, the model ends with the Ser306 residue corresponding to the Asp330 residue of the bovine rhodopsin template. Once the heavy atoms were modelled, all hydrogen atoms were added, and the protein coordinates were then minimised with MOE using the AMBER94 [16] force field. The minimisations were carried out by 1,000 steps of steepest descent followed by conjugate gradient minimisation until the RMS gradient of the potential energy was less than 0.05 kJ mol⁻¹ Å⁻¹.
Molecular docking of the human A3 receptor agonists

All agonist structures were manually docked into the hypothetical TM binding site. This receptor region has been established with the aid of published mutational data [17–20]. The searching of favourable binding conformations was conducted by a Monte Carlo Conformational Search protocol implemented in Schrodinger Macromodel. The input structure consisted of the ligand and a shell of receptor residues within the specified distance (6 Å) from the ligand. A second external shell of all the residues within a distance of 8 Å from the first shell was kept fixed. During the Monte Carlo conformational searching, the input structure was modified by random changes in user-specified torsion angles (for all input structure atoms) and molecular position (for the ligand). In this procedure, the ligand was left free to be continuously reoriented within the hypothetical binding site, and both ligand and internal shell residues conformations could be explored and reciprocally relaxed. The method consisted of 10,000 conformational search steps with MMFF94s force field [21–27]. Only unique structures within a 50 kJ mol\(^{-1}\) energy window above the found global minimum were saved. The resulting docked complexes were subjected to MMFF94s energy minimisation with Schrodinger Macromodel until the RMS of the conjugate gradient was less than 0.05 kJ mol\(^{-1}\) Å\(^{-1}\).

Results and discussion

Binding studies

The new compounds were evaluated at the human recombinant Ado receptors stably transfected into CHO cells utilising radioligand binding studies (A1, A2A, A3) or adenyl cyclase activity assay (A2B). Receptor-binding affinity was determined using \(^{[3H]}\)CCPA (2-chloro-N\(^6\)-cyclopentyladenosine) as radioligand for A1 receptors whereas \(^{[3H]}\)NECA (5’-N-ethylcarboxamidoadenosine) was used for the A2A and A3 subtypes. The relative potencies of these compounds for the A2B subtype were measured by evaluating the receptor-stimulated adenyl cyclase activity expressed as EC\(_{50}\). None of the tested compounds revealed activity at A2B receptors at concentrations up to 100 μM. The results are shown in Table 1.

PEAdo (1), 5’-N-methylcarboxamidoadenosine (PEMECA) (2), and 5’-N-ethylcarboxamidoadenosine (PENCA) (3) have been reported as reference compounds. PEAdo (1) displayed a Ki at A3 of 16 nM and a selectivity A1/A3 and A2A/A3 of 24- and 23-fold while replacement of the hydroxymethyl group in 4’-position of the sugar moiety with a methyl or ethylcarboxamido substituent increased A3 affinity. In fact, the corresponding PEMECA (2) and PENCA (3) derivatives showed increased A3 affinity and selectivity compared with PEAdo with an unmodified ribose (Table 1) [4–6, 28]. The introduction of the methoxy group in N\(^6\)-position of 1 led to an enhancement of A3 affinity of about four fold and a decrease of both A1 and A2A affinity of three and 12 fold, respectively, leading again to a considerable improvement of A3 selectivity. The N\(^6\)-methoxy-2-phenylethynylAdo (4) showed a Ki A3 = 3.8 nM and a selectivity A1/A3 and A2A/A3 of 320 and 1,100 fold, respectively [29]. Further substitution of the 4’-position of the sugar moiety of 4 enhances the affinity and/or selectivity of the corresponding compounds 5 and 6.

Thus, N\(^6\)-methoxy-2-phenylethynyl-5’-N-ethylcarboxamidoadenosine (6) showed the same affinity as the 4’-hydroxymethyl derivative 4 at A3 receptors (6; Ki A3 = 3.5 nM vs. 4; Ki A3 = 3.8 nM) combined with improved A3 selectivity (6; selectivity A1/A3 = 540 and A2A/A3 = 1,900 vs. 4; selectivity A1/A3 = 320 and A2A/A3 = 1,100) whereas an increase of both affinity and selectivity was found for

| Compound | R\(_1\) | R\(_2\) | K\(_i\)A\(_1\) | K\(_i\)A\(_2A\) | K\(_i\)A\(_3\) | A\(_1)/A\(_3\) | A\(_2A)/A\(_3\) |
|----------|--------|--------|--------------|--------------|--------------|--------------|--------------|
| 1 PEAdo [4–6] | H | CH\(_2\)OH | 391 (284–556) | 363 (285–462) | 16 (13–19) | 24 | 23 |
| 2 PEMECA | H | CONMe | 3,920 (2,390–6,430) | 1,760 (1,360–2,280) | 7.3 (4.2–13) | 540 | 240 |
| 3 PENCA | H | CONEt | 560 (480–650) | 620 (300–1,300) | 6.2 (5.1–7.5) | 90 | 100 |
| 4 | OCH\(_3\) | CH\(_2\)OH | 2,110 (893–1,630) | 4,290 (3,170–5,810) | 3.8 (2.6–5.5) | 320 | 1,100 |
| 5 | OCH\(_3\) | CONMe | 9,140 (6,680–12,500) | 16,300 (12,100–21,900) | 1.9 (1.6–2.2) | 4,800 | 8,600 |
| 6 | OCH\(_3\) | CONEt | 1,880 (1,330–2,660) | 6,660 (3,730–11,900) | 3.5 (2.9–4.2) | 540 | 1,900 |

PEAdo 2-phenylethynyladenosine, PEMECA 5’-N-methylcarboxamidoadenosine, PENCA 5’-N-ethylcarboxamidoadenosine

Table 1 Biological data of new synthesised compounds (K\(_i\), nM).

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PEAdo 2-phenylethynyladenosine, PEMECA 5’-N-methylcarboxamidoadenosine, PENCA 5’-N-ethylcarboxamidoadenosine
compound 5. In fact, the N6-methoxy-2-phenylethynyl-5'-N-methylcarboxamidoadenosine (5), showing Ki A3 = 1.9 nM and a selectivity A1/A3 and A2A/A3 of 4,800 and 8,600 fold, respectively, resulting in one of the most potent and selective agonist at the human A3 Ado receptor subtype reported so far. The ability of the new compounds examined to inhibit 10 μM forskolin-stimulated cAMP production via the Ado A3 receptor was also studied (Table 2).

In particular, the functional assay based on the evaluation of cyclic AMP levels in hA3 CHO cells shows that the N6-methoxy-PEAdo derivative 4 is a partial agonists compared with the full agonist 2-chloro-N6-(3-iodobenzyl)-5'-methylcarboxamidoadenosine (Cl-IB-MECA) as a reference [30]. On the other hand, the two N6-methoxy-PEMECA (5) and N6-methoxy-PENECA (6) derivatives show an adenylyl cyclase inhibition comparable to Cl-IB-MECA documenting that they are full A3 Ado agonists.

Molecular modelling

A molecular docking analysis of these compounds was performed at a homology model of human A3 receptor built using bovine rhodopsin crystal structure as template, with the aim of obtaining a possible rationalisation of the different binding affinities of the molecules for the human A3 receptor. The compounds have the same core structure, with different substituents in N6- and 4'-position. In particular, the different group bound to the 4' carbon of the ribose moiety seems to influence both affinity and selectivity of the compounds.

In Figure 1, the complexes of the hA3 receptor model with three agonists are shown, PEAdo (1), N6-methoxy-2-phenylethynylAdo (4) and the N6-methoxy-2-phenylethylnyl-5'-N-methylcarboxamidoAdo (5). These compounds share a common structure apart from the functionalisation of the ribose group and the nitrogen in 6-position. The docking conformations present the adenine scaffold plane almost orthogonally with respect to the receptor axis, with

| Compound       | % AC activity |
|----------------|---------------|
| 4              | 81 ± 2.3      |
| 5              | 38 ± 3.2      |
| 6              | 35 ± 4.0      |
| Cl-IB-MECA     | 40 ± 2.2      |

Shown is the percentage of activity remaining after agonist-mediated inhibition of forskolin-stimulated cyclase activity (100%) ± standard error of mean (SEM) (n = 3)

Cl-IB-MECA 2-chloro-N6-(3-iodobenzyl)-5'-methylcarboxamidoadenosine

*After 10 μM forskolin stimulation*
the 2-phenylethynyl group inserted in a space between TM3 and TM6. These conformations are in accordance with the already published docking conformation of PEAdo [31]. The presence of a methoxy group in 6-position seems to allow the ligand to interact with the receptor at two points, S247 and N250; this could explain a better affinity of N6-methoxyAdo derivatives compared with the compounds with an unmodified N-position. In addition, the 5'–N-methylcarboxamido group of the 5 derivative leads the molecule to interact with the receptor in two points, L90 and S271, compared with the corresponding hydroxy-methyl group in molecule 4, which can have only one H-bond interaction with H272.

Conclusions

A series of PEAdo derivatives substituted in the N6- and 4'-position was tested at the four human Ado receptors stably transfected into CHO cells using radioligand binding studies (A1, A2A, A3) or adenylyl cyclase activity assay (A2B). The new compounds were also examined in their ability to inhibit 10 μM forskolin-stimulated cAMP production via the Ado A3 receptor. Binding studies showed that the presence of a methoxy group in N6 of Ado derivatives favoured the interaction with A3 receptors, obtaining compounds endowed with high affinity and selectivity for the A3 subtype. Additional substitution with an alkylicarboxamido group in 4'-position increases both A3 affinity and selectivity. In particular, the N6-methoxy-2-phenylethynyl-5'-N-methylcarboxamidoadenosine (5), showed a Ki at A3 of 1.9 nM, a selectivity A1/A3 and A2A/A3 of 4,800 and 8,600, respectively, and also an ability comparable to CI-IB-MECA in inhibiting cAMP production, resulting in one of the most potent and selective agonist at the human A3 Ado receptor subtype reported so far. Docking analysis of these compounds was performed at a homology model of human A3 receptor built on the basis of the bovine rhodopsin crystal structure as template, and the results are in accordance with the biological data.

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