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ORIGINAL ARTICLE

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2-Substituted *N*-ethylcarboxamidoadenosine derivatives as high-affinity agonists at human A_3 adenosine receptors

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Abstract A number of 2-substituted 5'-*N*-ethylcarboxamidoadenosine (NECA) derivatives was investigated for their affinity and selectivity at human A_3 adenosine receptors. The compounds were tested in radioligand competition studies and modulation of adenylyl cyclase activity on membranes from CHO cell lines stably transfected with the four human adenosine receptor subtypes. In binding studies the most potent compound, 2-(3-hydroxy-3-phenyl)propyn-1-yl-NECA (PHPNECA), exhibited a subnanomolar affinity for A_3 adenosine receptors with a K_i value of 0.4 nM. As opposed to the limited A_3 selectivity of PHPNECA, a 100-fold selectivity compared to both A_1 and A_{2A} receptors was found for 2-(2-phenyl)ethynyl-NECA (PENECA; K_i 6 nM). The EC_{50} values for activation of adenylyl cyclase via A_{2A} adenosine receptors were in good agreement with the respective K_i values from binding experiments. In contrast, IC_{50} values for A_1 and A_3 receptor-mediated inhibition of adenylyl cyclase were shifted to higher values compared to the respective affinities determined in radioligand competition studies. Similar discrepancies between binding and functional data have been observed for the inhibitory A_1 adenosine receptor in previous studies. Therefore, the same A_3 selectivity of PENECA compared to A_1 receptors was found in binding and adenylyl cyclase inhibition whereas the selectivity compared to A_{2A} receptors that was detected in ligand binding was obscured in the functional assay. The series of compounds presented in this study identifies 2-substitution of the purine system as a promising target for the development of A_3-selective high-affinity ligands.

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Introduction

Many important endogenous signaling molecules regulate the function of a cell via G-protein-coupled receptors. For most ligands, including adenosine, several subtypes of such receptors have been identified. Originally, A_1 and A_2 adenosine receptors were the only subtypes that were distinguished on the basis of their effector coupling and pharmacological profiles. Both subtypes modulate cAMP formation such that the A_1 subtype mediates an inhibition of adenylyl cyclase whereas A_2 receptors stimulate cyclase activity (van Calker et al. 1979; Londos et al. 1980). However, many lines of evidence pointed to the existence of additional subtypes and recent cloning of A_{2B} and A_3 receptors (Pierce et al. 1992; Salvatore et al. 1993) confirmed the existence of a total of at least four adenosine receptor subtypes (Fredholm et al. 1994). The A_{2A} and A_{2B} subtypes are both positively coupled to adenylyl cyclase (Pierce et al. 1992), whereas A_3 receptors inhibit cAMP formation (Salvatore et al. 1993) and also stimulate phospholipase C (Abbracchio et al. 1995). This further subdivision renewed the interest in the respective signaling mechanisms and the therapeutic potential of adenosine receptors (Jacobson et al. 1995; Müller and Stein 1996; Poulsen and Quinn 1998).

Although a number of high-affinity ligands for A_3 adenosine receptors has been introduced, no highly selective A_3 agonist is available yet. Typical A_3 agonists that are currently available are adenosine derivatives with a 5'-*N*-methyluronamide modification and an additional N^6-benzyl substituent (Gallo-Rodriguez et al. 1994). These and other N^6-substituted compounds suffer from the disadvantage of typically considerable A_1 affinity (Baraldi et al. 1996). Therefore, we attempted to identify selective agonists in a series of 2-substituted 5'-*N*-ethylcarboxamidoadenosine (NECA) derivatives previously shown to

Table 1 Affinity of 2-substituted NECA derivatives at A_{2A} and A_3 adenosine receptors (K_i values in nM; means of 3–4 independent experiments)

Ligand	A_{2A}		A_3 Human
	Rat	Human	
NECA	22[a]	20[d]	6.2[d]
HENECA	2.2[b]	6.4	2.4
PHPNECA	0.9[b]	7.9	0.42
PENECA	120[c]	618	6.2

[a] Data from Cristalli et al. 1992
[b] Data from Camaioni et al. 1997
[c] Data from Cristalli et al. 1995
[d] K_d value for [^{3}H]NECA from Klotz et al. 1998

have different A_{2A} potencies (Cristalli et al. 1992, 1994, 1995).

The initial observation that led us to study 2-substituted NECA derivatives was the higher affinity of NECA for A_3 adenosine receptors compared to the A_{2A} subtype (Klotz et al. 1998). In addition, the parent compound NECA binds with similar affinity to both rat and human A_{2A} receptors whereas additional 2-substitution resulted in a lower affinity for the human A_{2A} receptor (Table 1). This apparent species difference between human and rat receptors suggested that 2-substitution may provide a lead to more selective high-affinity A_3 agonists than other compounds currently available.

Materials and methods

Cell culture and membrane preparation. CHO cells stably transfected with human A_1, A_{2A}, A_{2B} or A_3 receptors, respectively, were grown and maintained in Dulbecco's Modified Eagles Medium with nutrient mixture F12 (DMEM/F12) without nucleosides, containing 10% fetal calf serum, penicillin (100 U/ml), streptomycin (100 µg/ml), L-glutamine (2 mM) and Geneticin (G-418; 0.2 mg/ml; A_{2B}, 0.5 mg/ml) at 37 °C in 5% CO_2/95% air. Cells were split two or three times weekly at a ratio between 1 : 5 and 1 : 20.

Membranes for radioligand binding experiments were prepared from fresh or frozen cells as described recently (Klotz et al. 1998). The cell suspension was homogenized in ice-cold hypotonic buffer (5 mM Tris/HCl, 2 mM EDTA, pH 7.4) and the homogenate was spun for 10 min (4 °C) at 1,000 *g*. The membranes were sedimented from the supernatant for 30 min at 100,000 *g* and resuspended in 50 mM Tris/HCl buffer, pH 7.4 (for A_3 adenosine receptors: 50 mM Tris/HCl, 10 mM $MgCl_2$, 1 mM EDTA, pH 8.25), frozen in liquid nitrogen at a protein concentration of 1–3 mg/ml and stored at –80 °C.

For the measurement of adenylyl cyclase activity a protocol with only one centrifugation step was used. The homogenate from fresh cells was sedimented for 30 min at 54,000 *g*, the resulting pellet was resuspended in 50 mM Tris/HCl, pH 7.4, and used for the adenylyl cyclase assay immediately (Klotz et al. 1998).

Binding studies. Dissociation constants of unlabeled compounds (K_i values) were determined in competition experiments in 96-well microplates as described recently (Klotz et al. 1998). For A_{2A} and A_3 adenosine receptors the nonselective agonist [^{3}H]NECA (30 nM and 10 nM, respectively) was utilized as radioligand. The A_1-selective agonist 2-chloro-*N*^{6}-[^{3}H]cyclopentyladenosine ([^{3}H]CCPA; 1 nM) was utilized for the characterization of A_1 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. (1998). All binding data were calculated by non-linear curve fitting with the program SCTFIT (De Lean et al. 1982).

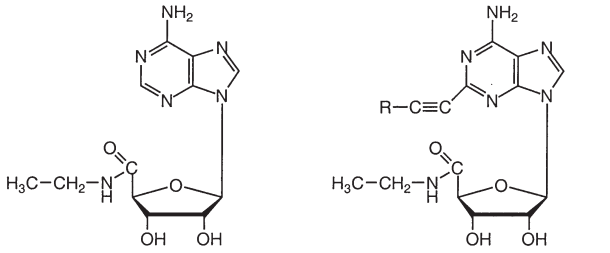
Adenylyl cyclase activity. The functional activity of the 2-substituted adenosine derivatives was determined in adenylyl cyclase experiments. The stimulation of adenylyl cyclase via A_{2A} and A_{2B} adenosine receptors was measured as described recently (Klotz et al. 1985, 1998). The inhibition of forskolin-stimulated adenylyl cyclase via A_1 and A_3 receptors followed the same procedure with minor modifications. The basal cyclase activity of CHO cells is very low, thus, inhibition was detectable only after stimulation with forskolin. A_1 and A_3 receptor-mediated inhibition was therefore measured in the presence of 30 µM forskolin. Membranes were incubated for 20 min at 37 °C in an incubation mixture containing 150,000–200,000 cpm of [α-^{32}P]ATP. All other ingredients were the same as described (Klotz et al. 1985) except EGTA and NaCl which were omitted. The EC_{50} and IC_{50} values for the stimulation and inhibition of adenylyl cyclase, respectively, were calculated with the Hill equation. Hill coefficients in all experiments were near unity.

Material. [^{3}H]NECA and [α-^{32}P]ATP were from NEN Life Science Products, Köln, Germany. All other unlabeled adenosine receptor agonists and antagonists were from RBI, Natick, Mass., USA. 2-Chloro-*N*^{6}-(3-iodobenzyl)-5'-*N*-methylcarboxamidoadenosine (Cl-IB-MECA) was provided by RBI as part of the NIMH Chemical Synthesis Program. The 2-substituted adenosine derivatives 1–14 (see Fig. 1) were synthesized as described earlier (Cristalli et al. 1992, 1994, 1995; Camaioni et al. 1997). The 96-well microplate filtration system (MultiScreen MAFC) was obtained from Millipore, Eschborn, Germany. Cell culture media and fetal calf serum were purchased from PanSystems, Aidenbach, Germany. Penicillin (100 U/ml), streptomycin (100 µg/ml), L-glutamine and G-418 were from Gibco-Life Technologies, Eggenstein, Germany. All other materials were from sources as described earlier (Klotz et al. 1985, 1998; Lohse et al. 1987).

Results

The structures of the compounds investigated in this study are shown in Fig. 1. All 2-substituted NECA derivatives tested exhibit nanomolar affinity at A_3 receptors ranging from 0.4 nM for the most potent compound PHPNECA (6) to 42 nM for compound 14 with the lowest potency (Table 2). In general, all the compounds are most potent on A_3 receptors, followed by A_1 or A_{2A} receptors. The only exceptions are the *R*-diastereomer of PHPNECA (6*R*) and compound 8 which are about three- and twofold, respectively, more potent on A_1 than on A_3 receptors. The ligand with the highest A_3 affinity identified in this series, PHPNECA, exhibits modest A_3 selectivity compared to A_1 and A_{2A} receptors (six- and sevenfold, respectively). Due to the lack of a suitable radioligand for A_{2B} receptors only functional data are available for this subtype (Table 3).

PHPNECA possesses an asymmetrical carbon in the side chain in 2-position. We investigated therefore the stereoselectivity of the respective diastereomers. Table 2 shows that the *S*-diastereomer (6*S*) is about sevenfold more potent at A_3-receptor than the *R*-form. The *S*-form is also 20-fold more potent on A_{2A} receptors whereas no difference for the diastereomers was found on A_1 receptors (Table 2) according to the data reported for rat membranes (Camaioni et al. 1997).



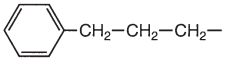
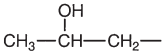
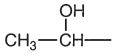
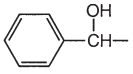
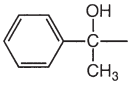
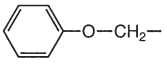
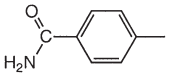
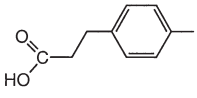
compound	R
1, HENECA	$CH_3-CH_2-CH_2-CH_2-$
2	$N\equiv C-CH_2-CH_2-CH_2-$
3	
4	
5	
6, PHPNECA	
7	
8	
9, PENECA	
10	
11	
12	
13	
14	

Fig. 1 Structures of 2-substituted NECA derivatives (*1, HENECA*, 2-hexyn-1-yl-NECA; *2*, 2-(5-cyano)pentyn-1-yl-NECA; *3*, 2-(5-phenyl)pentyn-1-yl-NECA; *4*, 2-(4-hydroxy)pentyn-1-yl-NECA; *5*, 2-(3-hydroxy)butyn-1-yl-NECA; *6*, *PHPNECA*, 2-(3-hydroxy-3-phenyl)propyn-1-yl-NECA; *7*, 2-(3-hydroxy-3-methyl-3-phenyl)propyn-1-yl-NECA; *8*, 2-(3-phenoxy)propyn-1-yl-NECA; *9*, *PENECA*, 2-(2-phenyl)ethynyl-NECA; *10*, 2-(2-furyl)ethynyl-NECA; *11*, 2-(2-thiazoyl)ethynyl-NECA; *12*, 2-(2-cyclohexen-1-yl)ethynyl-NECA; *13*, 2-(2-(4-carboxamido)phenyl)ethynyl-NECA; *14*, 2-(2-(4-propionyl)phenyl)ethynyl-NECA)

2-Substitution with alkyl chains adjacent to the triple bond (Fig. 1) results in ligands with only limited A_3 selectivity. HENECA is 25-fold more potent on A_3 receptors compared to the A_1, but only threefold more potent compared to the A_{2A} subtype (Tables 2, 3). Elongation of the alkyl chain decreases A_3 affinity (data not shown). On the other hand, 2-(3-hydroxy)butyn-1-yl-NECA (compound 5) is one of the most potent compounds at the A_3 adenosine receptor identified in this study (K_d 1.5 nM). Additional substitution with a phenyl ring increases the A_3 affinity about fourfold and yields PHPNECA, the most potent A_3 ligand identified in this series (see below). However, a phenyl substituent at the end of a longer alkyl chain as in compound 3 deteriorates the A_3 affinity about tenfold compared to the compound 2-pentynyl-NECA which lacks a phenyl (data not shown).

An aromatic substituent directly conjugated to the triple bond in the 2-substituent appears to be unfavorable for the A_{2A} receptor and, thus, results in 20- to 50-fold A_3/A_{2A} selectivity for compounds like PENECA (9), compounds 10, 11, 13 and 14 (Fig. 2). All other compounds are significantly less selective for A_3 vs. A_{2A} receptors. Figure 2 shows that hetero atoms in an aromatic ring (furyl or thiazolyl) increase the A_1 and and to a lesser degree A_{2A} affinity with only minor effects on A_3 binding, thereby reducing A_3 selectivity compared to PENECA. Para substitution of the phenyl ring in PENECA results in a small increase in A_1 affinity (Fig. 2). Compound 12, with a cyclohexenyl adjacent to the triple bond, is about fivefold less active than PENECA on all receptor subtypes (Table 2), indicating that an aromatic substituent is equally favorable for all receptors.

Compared to the most potent ligand, PHPNECA, a slightly lower affinity at A_3 adenosine receptors (K_d 6 nM) was found for PENECA. However, this compound is much less active on A_1 and A_{2A} receptors (Table 2), resulting in a ligand with the highest binding selectivity for A_3 adenosine receptors so far known. For PENECA an about 100-fold selectivity was found towards both A_1 and A_{2A} receptors (Table 2).

The agonistic activity of selected compounds was tested by stimulation of adenylyl cyclase via A_2 receptors and inhibition of adenylyl cyclase via A_1 and A_3 receptors (Table 3). All compounds tested exhibited agonistic activity at least at two of the adenosine receptor subtypes. Compounds 9 and 10 did not show agonistic activity at A_{2B} adenosine receptors in concentrations up to 100 µM (Table 3). It was excluded that these compounds acted as

Table 2 Binding affinity of 2-substituted NECA derivatives (K_i values in nM; means of 3–4 independent experiments with 95% confidence intervals *in parentheses*)

Compound	A_1	A_{2A}	A_3
NECA[a]	14 (6.4–28)	20 (12–35)[b]	6.2 (3.5–11)[b]
1, HENECA	60 (50–72)	6.4 (3.8–11)	2.4 (2.0–2.9)
2	38 (33–45)	25 (18–37)	4.7 (3.1–7.1)
3	180 (81–390)	110 (50–240)	27 (20–35)
4	40 (26–62)	14 (10–20)	4.1 (3.4–4.9)
5	3.3 (2.9–3.8)	14 (7.3–26)	1.5 (0.8–2.7)
6, PHPNECA	2.7 (1.7–4.1)	3.1 (2.4–3.9)	0.42 (0.17–1.0)
6*R, (R)*-PHPNECA	1.9 (1.8–2.1)	39 (25–59)	5.5 (3.6–8.5)
6*S, (S)*-PHPNECA	2.1 (1.2–3.7)	2.0 (1.2–3.5)	0.75 (0.52–1.1)
7	9.4 (8.5–11)	56 (28–110)	2.4 (1.9–3.1)
8	8.0 (5.9–11)	180 (110–290)	14 (10–19)
9, PENECA	560 (480–650)	620 (300–1,300)	6.2 (5.1–7.5)
10	140 (88–240)	510 (320–830)	12 (9.2–14)
11	12 (10–15)	84 (34–210)	3.7 (2.4–5.6)
12	87 (66–114)	100 (59–170)	28 (20–39)
13	200 (140–280)	470 (310–710)	9.3 (6.7–13)
14	1,000 (580–1,800)	2,200 (1,100–4,500)	42 (33–55)

[a] Data from Klotz et al. 1998
[b] K_d value for [^{3}H]NECA

Table 3 Adenylyl cyclase inhibition (A_1 and A_3 receptors) and stimulation (A_2 receptors) by 2-substituted NECA derivatives (IC_{50} or EC_{50} values, respectively, in nM; means of 3–4 independent experiments with 95% confidence intervals *in parentheses*)

Compound	A_1	A_{2A}	A_{2B}	A_3
3	10,700 (8,900–12,900)	78 (55–109)	65,000 (38,000–110,000)	3,200 (1,400–7,200)
6, PHPNECA	2,000 (640–6,200)	4.6 (3.7–5.8)	1,110 (470–2,600)	160 (69–350)
7	2,500 (1,200–5,300)	36 (28–48)	2,300 (1,700–3,100)	600 (440–810)
9, PENECA	–[a]	820 (710–950)	–	1,200 (920–1,400)
10	33,200 (18,600–59,500)	800 (770–840)	–	2,200 (1,300–3,600)
11	5,000 (2,200–11,100)	130 (120–140)	4,600 (2,200–9,500)	230 (160–350)

[a] Inactive (EC_{50} > 100,000 nM)

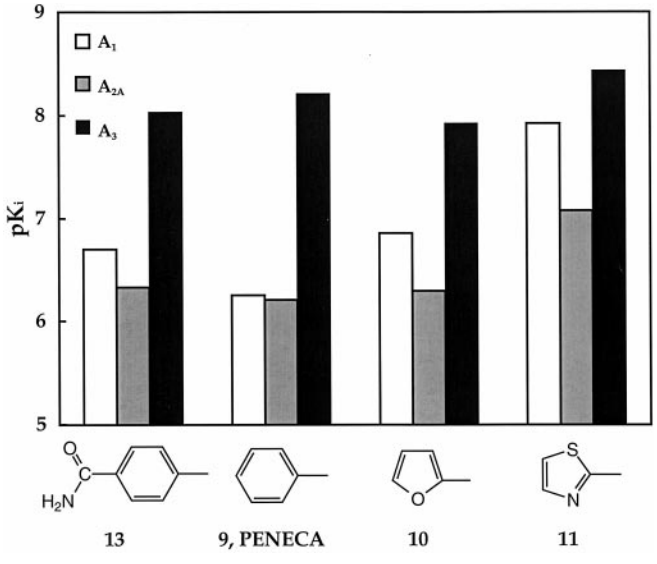


Fig. 2 Compounds with aromatic substituents at the triple bond. Comparison of the affinities at A_1, A_{2A} and A_3 receptors reveals that A_3 receptors do not have a pronounced preference for one of the substituents. However, the plain phenyl ring in PENECA results in the lowest A_1 and A_{2A} affinities and, therefore, is the ligand with the best A_3 selectivity

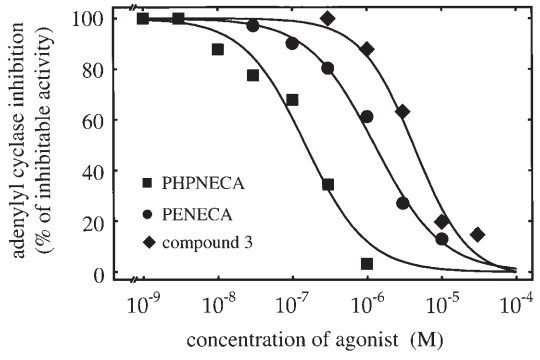


Fig. 3 A_3-receptor-mediated inhibition of forskolin-stimulated adenylyl cyclase. The curves show adenylyl cyclase inhibition for PHPNECA (■), PENECA (●) and compound 3 (◆). The respective IC_{50} values are 151 nM, 1,260 nM and 3,800 nM. Forskolin (30 µM) stimulated adenylyl cyclase 20- to 30-fold over basal activity and amounted to 68, 53 and 50 pmol/mg protein min, respectively, in the curves shown here. In all experiments for this study forskolin-stimulated cyclase was inhibited by approximately 30%–50%. The data shown here are from single experiments. For data in detail see Table 3

antagonists as they did not antagonize NECA-stimulated adenylyl cyclase activation (data not shown).

Figure 3 shows that the compounds that exhibited the highest selectivity (9), and the highest (6) and lowest potency (3) in binding experiments all inhibited adenylyl cyclase via A_3 adenosine receptors with similar efficacy. However, IC_{50} values are approximately 100-fold higher than the respective K_i values from binding experiments. A similar discrepancy was observed for A_1 receptors whereas EC_{50} values for stimulation of adenylyl cyclase via A_{2A} receptors nicely corresponded to the respective K_i values (Tables 2, 3).

The NECA derivatives with measurable activity (EC_{50} <100 μM) caused the same maximal stimulation of adenylyl cyclase via A_{2A} and A_{2B} receptors as NECA, suggesting that they are full agonists at these subtypes. The maximal inhibition achieved via A_1 receptors was similar to the inhibition observed with the prototypical A_1 agonist CCPA (data not shown).

Discussion

Most agonists with high affinity for A_3 adenosine receptors are adenosine-5'-*N*-methyluronamides with a substituted N^6-benzyl modification (Jacobson et al. 1995; Müller and Stein 1996). These compounds exhibit generally significant affinity to A_1 receptors as well, resulting in low A_3 selectivity (Table 4). The only compound with higher A_3 selectivity compared to all adenosine receptor subtypes, AB-MECA, is less potent than the "universal" ligand NECA (Table 4). In this study we present a series of NECA derivatives substituted in the 2-position with alkynyl or aralkynyl chains as a novel group of compounds as a basis for improved A_3 selectivity. Previously, such NECA derivatives have been shown to bind with high affinity to rat A_{2A} adenosine receptors (Cristalli et al. 1992, 1994, 1995; Camaioni et al. 1997). Testing some of these compounds on human A_{2A} receptors stably transfected into CHO cells (Klotz et al. 1998) revealed that their affinity was typically three- to tenfold lower than at the rat receptor, whereas the parent compound NECA was equipotent in both species (Table 1). On the other hand, NECA is more potent on human A_3 than A_{2A} receptors (Klotz et al. 1998). Interestingly, some of the 2-substitutions of NECA improved A_3 affinity, resulting in a series of potent ligands for this subtype.

The most interesting compounds identified in this study are PHPNECA and PENECA. PHPNECA binds to A_3 adenosine receptors with subnanomolar affinity (K_i 0.4 nM). It shows nanomolar affinity to A_1 and A_{2A} receptors and, therefore, limited A_3 selectivity towards these receptors. Inspection of the structure of different 2-substituents suggests that the 3-hydroxy function in PHPNECA might be important for the high affinity to the A_3 receptor of this type of ligands, since compound 5 as the ligand with the second highest A_3 affinity also bears this hydroxyl group.

For PENECA a K_i value of 6 nM was determined at A_3 receptors. However, A_1 and A_{2A} affinities are about 600 nM, resulting in a 100-fold selectivity for A_3 receptors compared to both A_1 and A_{2A} receptors. Although all aromatic systems in conjugation with the triple bond resulted in high affinity for the A_3 receptor, only PENECA with the phenyl substituent showed marked A_3 selectivity as well (Fig. 2). This distinguishes this compound from most high-affinity A_3 ligands which typically show more or less pronounced A_3/A_{2A} selectivity but very limited selectivity compared to A_1 receptors (Table 4). Interestingly, very similar affinities have been reported for IB-MECA and IAB-MECA at rat and human A_3 receptors whereas Cl-IB-MECA, which has been reported as an A_3-selective compound (Kim et al. 1994), is 30-fold less potent at the human receptor (Table 4). Consequently, it exhibits only minor A_3 selectivity in the human system.

Measurement of adenylyl cyclase activation and inhibition, respectively, with selected NECA derivatives revealed that all compounds were indeed agonists on at least two receptor subtypes. The compounds inactive at A_{2B} receptors in concentrations up to 100 μM (PENECA and compound 10) did not antagonize the NECA-stimulated adenylyl cyclase activity excluding antagonistic quality of these NECA derivatives. All compounds stimulated adenylyl cyclase via both A_2 receptor subtypes with similar efficacy as the parent compound NECA. PENECA was the only compound selected in Table 3 which showed no agonistic activity at the A_1 receptor. However, 50 μM PENECA did not diminish CCPA-induced inhibition of adenylyl cyclase, suggesting that this compound is not an antagonist at this receptor subtype. In addition, no differences in the extent of adenylyl cyclase inhibition mediated by A_1 and A_3 receptors were observed for the selected compounds with measurable activity, indicating that they are full agonists on all receptor subtypes.

A striking difference was observed between the IC_{50} values of adenylyl cyclase inhibition and the respective K_i values for both A_1 and A_3 receptors. In contrast, EC_{50} values for A_{2A}-receptor-mediated stimulation of adenylyl cyclase and K_i values from binding experiments were in good agreement. Similar differences between binding and

Table 4 A_3 binding affinity and selectivity of various adenosine receptor agonists. Selectivities are based on binding data from Klotz et al. 1998 except for Cl-IB-MECA which gave K_i values of 115 (114–116) nM, 2,100 (1,700–2,500) nM and 11.1 (9.43–13.0) nM at A_1, A_{2A} and A_3 receptors, respectively (means of 3–4 independent experiments). The EC_{50} at the A_{2B} receptor was >100 μM

	K_i A_3 (nM)	A_1/A_3	A_{2A}/A_3
CCPA	42	0.02	54
CPA	43	0.05	18
R-PIA	16	0.1	53
S-PIA	45	2	174
NECA	6	2	3
CGS 21680	67	4	0.4
AB-MECA	22	70	170
IB-MECA	1.2	3	2,100
IAB-MECA	0.6	13	740
Cl-IB-MECA	11	10	187

functional data have been noted for the inhibitory A_1 receptor in previous studies (Klotz et al. 1985; Tawfik-Schlieper et al. 1989). In these studies the IC_{50} values correspond to the respective low-affinity K_i values, suggesting that receptors in the low-affinity state caused by the presence of GTP are relevant for signal transduction. The differences in A_1 binding affinity and IC_{50} values for A_1-receptor-mediated cyclase inhibition are very similar to the differences observed for high- and low-affinity binding for prototypical adenosine receptor agonists at the human A_1 receptor in stably transfected CHO cells (Klotz et al. 1998). Given the fact that the activity of adenylyl cyclase is determined in the presence of GTP, it is clear that A_1 receptors assume the low-affinity state under the experimental conditions. The same may hold true for other inhibitory receptors like the A_3 adenosine receptor as well. Due to the lack of a radiolabeled antagonist it cannot be tested whether a respective low-affinity state for A_3 receptors corresponds to the IC_{50} values determined for cyclase inhibition mediated by this receptor subtype.

Comparison of functional and binding data for different receptor subtypes in this study shows that selective binding is not necessarily reflected by functional selectivity. The preference for a receptor subtype may even be reversed in function compared to binding as is shown at A_{2A} and A_3 receptors, e.g. for compounds 6 and 7. Although the new NECA derivatives presented in this study do not show functional selectivity for A_3 adenosine receptors, most compounds in this series exhibit binding affinities in the low nanomolar range. Thus, we discovered new lead structures for the development of selective A_3 adenosine receptor agonists with high affinity at the human receptor. We identified PHPNECA as a ligand with subnanomolar affinity yet limited selectivity. PENECA with a K_i value of 6 nM turned out to be about tenfold less potent; however, with a 100-fold selectivity compared to both A_1 and A_{2A} receptors, it is currently the most selective ligand at human A_3 adenosine receptors.

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