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Brief Article

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Synthesis and Biological Evaluation of 2-Alkynyl-N^6-methyl-5′-*N*-methylcarboxamidoadenosine Derivatives as Potent and Highly Selective Agonists for the Human Adenosine A_3 Receptor‡

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A new series of 2-aralkynyl-N^6-methyl-MECAs **10**−**13** were synthesized and evaluated in radioligand binding studies and in a new Eu-GTP functional assay that provides a powerful alternative to radioisotope use. The new compounds possess high affinity and selectivity for the AA_3R with N^6-methyl-2-phenylethynylMECA (**10**) showing a subnanomolar affinity and about 100000-fold selectivity vs AA_1R and $AA_{2A}R$. Furthermore, the new nucleosides showed to be full agonists, the N^6-methyl-2-(2-pyridinyl)-ethynylMECA (**13**) being the most potent in the series.

Introduction

Adenosine (Ado, Figure 1) is a naturally occurring nucleoside having a role in an ample variety of physiological and pathophysiological processes through the activation of at least four G-protein-coupled receptors (P1) cloned and classified as Ado A_1, A_{2A}, A_{2B}, and A_3 receptors (AA_1R, $AA_{2A}R$, $AA_{2B}R$, and AA_3R, respectively).[1,2] In particular, AA_3R is expressed in a broad range of tissues and couples to $G_{i/o}$ proteins leading to inhibition of adenylyl cyclase, increase of intracellular Ca^{2+} concentration, and activation of phosphoinositide 3-kinase.[3] Since this subtype is involved in a variety of key physiological processes, experimental evidence suggests that AA_3R agonists could be employed as therapeutic agents for the treatment of several pathologies.[4] Cristalli and coworkers have previously published the synthesis and biological activity of 2-aralkynyl-N^6-methoxyAdo compounds and the corresponding 5′-*N*-methyl and 5′-*N*-ethylcarboxamido derivatives (2-aralkynyl-N^6-methoxyMECAs and NECAs, respectively), which are endowed with good affinity and different degrees of selectivity for the human AA_3R.[5−7] Among them, some MECA derivatives bearing an aromatic ring directly linked to the 2-ethynyl group showed the best profile for this receptor subtype (**1**−**4**, AA_3R affinity in the low nanomolar range and selectivity ranging from 1600- to 22000-fold; for details, see Table 1).[8] Furthermore, in a previous work by the same authors it was demonstrated that the introduction of a methyl group in N^6-position of 2-phenylethynylAdo (N^6-methylPEAdo, Figure 1)[9] favored the interaction with the human AA_3R, this compound having comparable AA_3R affinity and higher selectivity than the corresponding N^6-methoxy-2-phenylethynylAdo (N^6-methoxyPEAdo, Figure 1) (N^6-methylPEAdo K_i AA_3R = 3.4 nM, selectivity AA_1R/AA_3R = 500 and $AA_{2A}R/AA_3R$ = 2500 vs N^6-methoxyPEAdo, K_i AA_3R = 3.8 nM, selectivity AA_1R/AA_3R = 300 and $AA_{2A}R/AA_3R$ = 1100).[8]

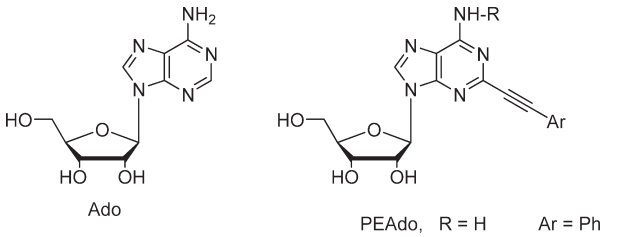

Figure 1. Structure of known potent and selective AA_3R ligands.

Starting from these observations and to find highly potent and selective AA_3R agonists, the synthesis of N^6-methyl analogues of **1**−**4** was undertaken. All of the new 2-aralkynyl-N^6-methyl-MECAs **10**−**13** were evaluated in radioligand binding studies and in functional assays. In particular, a novel time-resolved fluorometric (TRF) method, which exploits the unique fluorescence properties of lanthanide chelates and provides a powerful alternative to the assays that utilize radioisotopes, was set up to evaluate the potency of the new compounds.

Results and Discussion

Chemistry. The 2-alkynyl-N^6-methylMECA derivatives **10**−**13** were synthesized starting from 2-iodo-N^6-methylAdo (**5**), which was obtained from commercially available guanosine in four steps.[10] Protection of 2′- and 3′-hydroxyl groups of **5**, using acetone and *p*-toluenesulfonic acid at room temperature for 16 h, gave **6**, which was reacted with $KMnO_4$ in basic conditions to obtain the acid derivative **7**. Treatment of **7** with thionyl chloride in the presence of dry DMF and subsequent reaction with methylamine at 0 °C for 1 h furnished the protected 2-iodo-N^6-methylMECA **8**.

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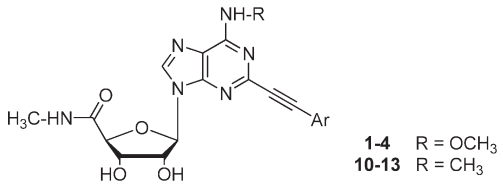
Reaction of **8** with formic acid at 50 °C for 2 h gave the deprotected 2-iodo-N^6-methylMECA (**9**) that was in turn treated with the suitable terminal alkynes, using a modification of the classical palladium-catalyzed cross-coupling reaction[10,11] to give the desired 2-alkynyl-N^6-methylMECA derivatives **10**−**13** (Scheme 1).

Biological Data. All of the new compounds were evaluated at the human recombinant adenosine receptors (ARs), stably transfected into CHO cells, utilizing radioligand binding studies (AA_1R, $AA_{2A}R$, AA_3R) or adenylyl cyclase activity assay ($AA_{2B}R$) (Table 1). Receptor binding affinity was determined using [^{3}H]CCPA (2-chloro-N^6-cyclopentyladenosine) as radioligand for AA_1R, whereas [^{3}H]NECA was used for $AA_{2A}R$ and AA_3R subtypes.[12] Most of the tested compounds were inactive at $AA_{2B}R$ (K_i > 30 μM); therefore, the $AA_{2B}R$ data are not shown in Table 1. Affinity and selectivity data of 2-aralkynyl-N^6-methoxyMECA derivatives **1**−**4** are reported in Table 1 for comparison with the corresponding new nucleosides **10**−**13**.

Table 1. Affinity Data of New Derivatives in Radioligand Binding Assays at Human AA_1R, $AA_{2A}R$, and AA_3R



1-4 R = OCH_3
10-13 R = CH_3

		K_i (nM)[a]			selectivity	
compd	Ar	AA_1R[b]	$AA_{2A}R$[c]	AA_3R[d]	AA_1R/AA_3R	$AA_{2A}R$/AA_3R
1	Ph	9140	16300	1.9	4800	8600
2	*p*-CH_3CO-Ph	53800	10400	2.5	21500	4200
3	*p*-F-Ph	3000	18700	1.9	1600	9800
4	2-Py	3990	18000	1.1	3600	16400
10	Ph	32800	41700	0.44	76500	97000
11	*p*-CH_3CO-Ph	10200	7030	0.33	31000	21500
12	*p*-F-Ph	5310	12700	0.43	12500	29500
13	2-Py	8740	24300	0.40	22000	61000

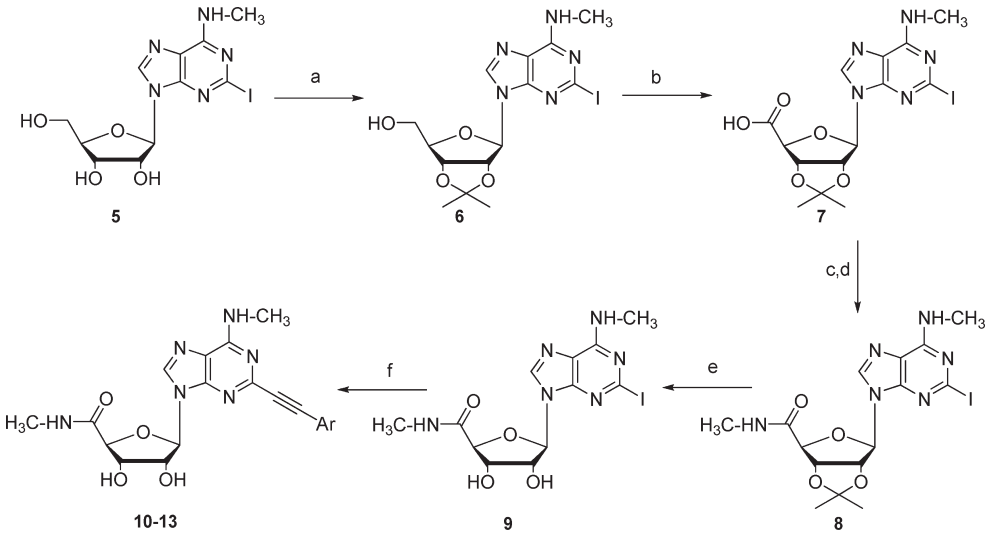
[a] 95% confidence intervals are reported in Table 2 of the Supporting Information. [b] Displacement of specific [^{3}H]CCPA binding in CHO cells, stably transfected with human recombinant AA_1R, expressed as K_i (nM). [c] Displacement of specific [^{3}H]NECA binding in CHO cells, stably transfected with human recombinant $AA_{2A}R$, expressed as K_i (nM). [d] Displacement of specific [^{3}H]NECA binding in CHO cells, stably transfected with human recombinant AA_3R, expressed as K_i (nM).

The results of binding experiments showed that replacement of the N^6-methoxy group in **1**−**4** by a methyl induced an increase of AA_3R binding affinity ranging from 3-fold (compare **13** to **4**) to 8-fold (compare **11** to **2**); in fact **10**−**13** are endowed with K_i in the subnanomolar range (0.44, 0.33, 0.43, and 0.40 nM, respectively).

Furthermore, the same substitution led to a general, although modest, decrease of affinity at the human AA_1R and $AA_{2A}R$ subtypes, resulting in a significant increase of AA_3R selectivity for the new compounds. In particular, N^6-methyl-2-phenylethynylMECA (N^6-methylPEMECA, **10**), showing K_i AA_3R = 0.44 nM and a selectivity vs AA_1R and $AA_{2A}R$ of 77 000 and 97 000, respectively, is one of the most potent and selective ligand of the human AA_3R with nucleoside structure reported so far.

Moreover, the ability of the new compounds to activate the human AA_3R, stably transfected into CHO cells, was evaluated in comparison with that elicited by the full agonist Cl-IBMECA. This measurement was carried out by utilizing the nonradioactive DELFIA Eu-GTP binding assay. The new functional method is based on fluorescence emission determination of the chelate Eu-GTP bound to AA_3R, after GDP/Eu-GTP exchange consequent to the receptor activation. Since it is the first report in which Eu-GTP functional assay is used to evaluate the AR activation, some known compounds such as the antagonist N^6-methoxy-2-(2-pyridinyl)ethynylAdo, the partial agonists N^6-methoxyPEAdo and N^6-methylPEAdo, and the full agonists **1**−**4**, whose functional activity at AA_3R has been already studied in

Scheme 1. Synthesis of Designed AA_3R Ligands[a]



10, Ar = Ph
11, Ar = *p*-CH_3CO-Ph
12, Ar = *p*-F-Ph
13, Ar = 2-Py

[a] Conditions: (a) $(CH_3)_2CO$, PTSA; (b) $KMnO_4$, KOH, H_2O; (c) $SOCl_2$, dry DMF, 50 °C; (d) CH_3NH_2, CH_2Cl_2, from −20 to 0 °C; (e) HCOOH 50%, 50 °C; (f) Ar−C≡CH, CuI, $(Ph_3P)_2PdCl_2$, Et_3N, dry DMF, room temp.

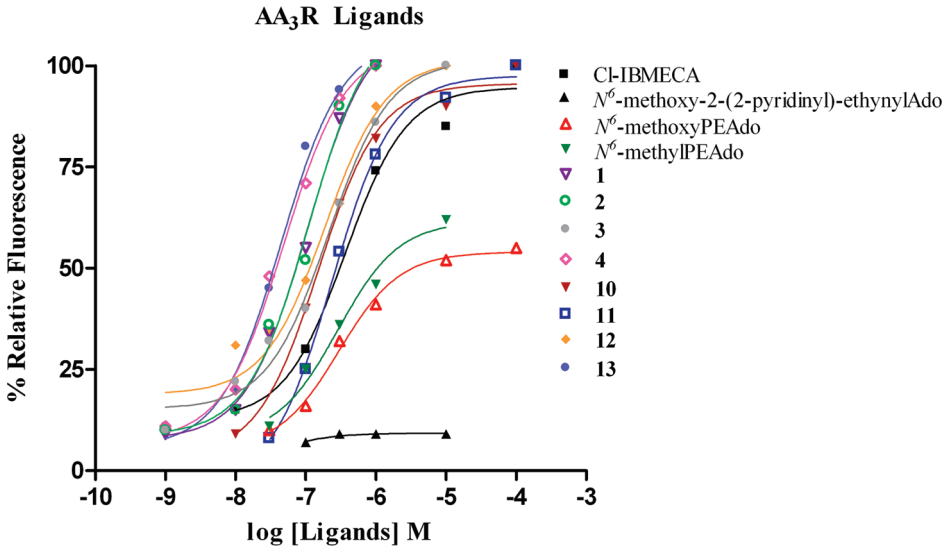


Figure 2. Concentration−response curves in Eu-GTP functional assays by using CHO cells expressing the human AA_3R. Each point represents the mean of four to five experiments performed in triplicate with a maximum SEM lower than ±10.

adenylyl cyclase experiments, were tested as reference compounds. The results obtained with the Eu-GTP assay confirmed that the three Ado derivatives are antagonists (N^6-methoxy-2-(2-pyridinyl)ethynylAdo) or partial agonists (N^6-methoxyPEAdo and N^6-methylPEAdo) showing lower ability to activate the human AA_3R than Cl-IBMECA (Figure 2). In contrast, **1**−**4** were able to activate the receptor with a relative fluorescence value comparable to that of Cl-IBMECA, taken as 100% of response, thus behaving as full agonists of the human AA_3R (Figure 2). These results are in close agreement with those obtained for the same compounds in previously reported adenylyl cyclase experiments; hence, they prove the high reliability of this nonradioactive assay. Eu-GTP methods were finally used to evaluate the new N^6-methyl derivatives **10**−**13** that proved to evoke a 100% response in Eu-GTP functional assay, confirming that 5′-*N*-alkylcarboxamidoAdo derivatives behave as full agonists of the human AA_3R.

EC_{50} values elicited by **1**−**4** and **10**−**13** were in general comparable to that of the reference compound Cl-IBMECA, with the exception of N^6-methoxy-2-(2-pyridinyl)ethynylMECA (**4**) and N^6-methyl-2-(2-pyridinyl)ethynylMECA (**13**), which are 7- to 8-fold more potent than Cl-IBMECA itself ($EC_{50} = 5.2 \times 10^{-8}$ and 4.6×10^{-8} M versus $EC_{50} = 3.6 \times 10^{-7}$ M, respectively). All data concerning percentage of response and EC_{50} of the tested ligands are reported in Table 3 of Supporting Information. These results emphasize that in the full agonist disubstituted MECA derivatives, the nature of the substituent in 2-position plays an important role in the activation of AA_3R, the presence of a 2-pyridinylethynyl group leading to an increase of potency.

Conclusion

In summary, new disubstituted MECA derivatives bearing a methyl group in the N^6-position and an aralkynyl substituent in the 2-position were designed and synthesized to find potent and selective AA_3R ligands. Binding experiments, performed in CHO cells, stable transfected with human ARs, demonstrated that the new compounds **10**−**13** possess affinity in the subnanomolar range and a very high selectivity for the target subtype, being among the most potent and selective AA_3R ligands reported so far. In particular, N^6-methyl-2-phenylethynylMECA (**10**) showed a K_i AA_3R of 0.44 nM and a remarkable selectivity vs AA_1R and $AA_{2A}R$ of 77 000 and 97 000, respectively. Furthermore, these molecules were found to behave as full agonists in a Eu-GTP functional assay, which was set up to evaluate the activation of ARs by their ligands. It is worthwhile to note that this new functional assay avoids the use of any radiolabeled compound.

Experimental Procedures

Chemistry. Melting points were determined with a Büchi apparatus and are uncorrected. ^{1}H NMR spectra were obtained with a Varian Mercury 400 MHz spectrometer; δ is in ppm and J in Hz. All exchangeable protons were confirmed by addition of D_2O. Mass spectra were recorded on an HP 1100-MSD series instrument. All measurements were performed using electrospray ionization (ESI-MS) on a single quadrupole analyzer. Thin layer chromatography (TLC) was carried out on precoated TLC plates with silica gel 60 F-254 (Fluka). For column chromatography, silica gel 60 (Merck) and silica RP-18 (Aldrich) were used. Elemental analyses were determined on Fisons Instruments model EA 1108 CHNS-O model analyzer and are within 0.4% of theoretical values. Purity of the compounds was ≥95% according to elemental analysis data.

2-Iodo-2′,3′-*O*-isopropylidene-*N*^{6}-methyladenosine (6). To **5** (5.21 g, 12.80 mmol) in dry acetone (280 mL) *p*-toluenesulfonic acid was added (24.35 g; 141.57 mmol), and the mixture was left for 16 h under stirring at room temperature. Saturated solution of $NaHCO_3$ was added to neutralize the pH, and the reaction was left under stirring 1 h. Acetone was removed under vacuum and the residue partitioned between $CHCl_3$ and H_2O. Organic extracts were dried over Na_2SO_4, filtered, and concentrated to dryness. Compound **6** was obtained, after crystallization from CH_3OH, as a white solid: 93% yield.

2-Iodo-2′,3′-*O*-isopropylidene-*N*^{6}-methyl-4′-carboxylic Acid Adenosine (7). To a suspension of **6** (4.0 g, 8.94 mmol) in 500 mL of H_2O, KOH (1.45 g) and a solution of $KMnO_4$ (4.09 g) in 120 mL of H_2O were added dropwise under stirring. The mixture was set aside in the dark at room temperature for 40 h. After cooling at 5−10 °C, the mixture was decolorized by a 35% H_2O_2 solution (10.2 mL) in 49.2 mL of H_2O, maintaining the temperature under 10 °C in an ice−salt bath. The mixture was filtered through Celite, the filtrate concentrated in vacuo to

about 50 mL and then acidified to pH 4 with 2 N HCl. The resulting precipitate was filtered off and washed with water to give **7** as a white solid: 62% yield.

2-Iodo-2′,3′-*O*-isopropylidene-N^6-methyl-5′-*N*-methylcarboxamidoadenosine (8). To **7** (0.300 g, 0.65 mmol), $SOCl_2$ (1 mL) and DMF (24 μL, 0.31 mmol) were added at 0 °C in nitrogen atmosphere. The mixture was stirred at 50 °C for 2 h. Solvent was removed under vacuum and the residue coevaporated three times with dry toluene. Then dry CH_2Cl_2 (2 mL) was added and the mixture cooled at −20 °C. CH_3NH_2 (1 mL) was added, and the mixture was left for 1 h at 0 °C. Volatiles were removed, and the residue was partitioned between H_2O and CH_2Cl_2. The organic extracts were dried over Na_2SO_4, filtered, and concentrated to dryness. The residue was purified on a flash silica gel column, eluting with *c*Hex−$CHCl_3$−CH_3OH (70:29:1 to 70:20:10). After crystallization from CH_3OH, **8** was obtained as a white solid in 44% yield.

2-Iodo-N^6-methyl-5′-*N*-methylcarboxamidoadenosine (9). To **8** (0.191 g, 0.40 mmol), a 50% solution of HCOOH (15 mL) was added, and the mixture was heated at 50 °C for 2 h. The solvent was removed under vacuum, and the residue was coevaporated three times with H_2O and then EtOH to give **9** as a white solid (crystallization from MeOH): yield 85%.

General Procedure for the Synthesis of 2-Alkynyl-N^6-methyl-5′-*N*-methylcarboxamidoadenosine Derivatives 10−13. To a solution of **9** (0.120 g, 0.28 mmol) in dry DMF (7.5 mL), triethylamine (1.12 mL), bis(triphenylphosphine)palladium dichloride (5.0 mg, 0.007 mmol), CuI (0.3 mg, 0.0015 mmol), and the suitable terminal alkyne (1.68 mmol) were added. The mixture was stirred under nitrogen atmosphere at room temperature and for the time reported in the specific description. After evaporation under vacuum and chromatography over silica gel column eluting with the suitable mixture of solvents, MECA derivatives **10−13** were obtained.

N^6-Methyl-2-phenylethynyl-5′-*N*-methylcarboxamidoadenosine (10). Reaction of **9** with phenylethyne for 40 min, followed by flash chromatography eluting with $CHCl_3$−CH_3OH (95:5) and further purification by RP-18 chromatography eluting with CH_3OH-H_2O (50:50), gave **10** as a white solid (crystallization from EtOH): 42% yield.

2-(4-Acetylphenyl)ethynyl-N^6-methyl-5′-*N*-methylcarboxamidoadenosine (11). Reaction of **9** with 4-ethynylacetophenone for 2 h followed by flash chromatography eluting with $CHCl_3$−*c*Hex−CH_3OH (80:15:5) gave **11** as a white solid (crystallization from EtOH), 93% yield.

2-(4-Fluorophenyl)ethynyl-N^6-methyl-5′-*N*-methylcarboxamidoadenosine (12). Reaction of **9** with 1-ethynyl-4-fluorobenzene for 6 h followed by flash chromatography eluting with $CHCl_3$−*c*Hex−CH_3OH (70:25:5) gave **12**, after crystallization from EtOH, as a white solid: 85% yield.

2-(2-Pyridinyl)ethynyl-N^6-methyl-5′-*N*-methylcarboxamidoadenosine (13). Reaction of **9** with 2-ethynylpyridine for 3 h, chromatography eluting with $CHCl_3$−CH_3CN−CH_3OH (80:10:10), and crystallization from EtOH gave **13** as white solid, 64% yield.

Biological Evaluation. Binding Studies. Dissociation constants of unlabeled compounds (K_i) were determined in competition experiments in 96-well microplates as described recently.[12]

Functional Assay. Compounds in Table 1 (**1−4** and **10−13**) were evaluated for receptor activity at human AA_3R, stably transfected into CHO cells, and CHO wild type utilizing DELFIA Eu-GTP (DELFIA GTP-binding kit, Perkin-Elmer Life Science) binding assay. The bound Eu-GTP was measured with time-resolved fluorometer, GENIosPro TECAN.

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Supporting Information Available: Chemical−physical data of synthesized compounds **6−13**; detailed biological protocols. This material is available free of charge via the Internet at http://pubs.acs.org.

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