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Truncated Nucleosides as A_3 Adenosine Receptor Ligands: Combined 2-Arylethynyl and Bicyclohexane Substitutions

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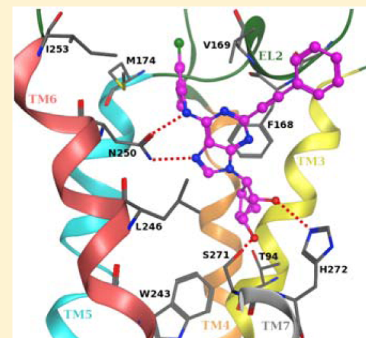
Supporting Information

ABSTRACT: C2-Arylethynyladenosine-5′-*N*-methyluronamides containing a bicyclo[3.1.0]hexane [(N)-methanocarba] ring are selective A_3 adenosine receptor (AR) agonists. Similar 4′-truncated C2-arylethynyl-(N)-methanocarba nucleosides containing alkyl or alkylaryl groups at the N^6 position were low-efficacy agonists or antagonists of the human A_3AR with high selectivity. Higher hA_3AR affinity was associated with N^6-methyl and ethyl (K_i = 3−6 nM) than with N^6-arylalkyl groups. However, combined C2-phenylethynyl and N^6-2-phenylethyl substitutions in selective antagonist **15** provided a K_i of 20 nM. Differences between 4′-truncated and nontruncated analogues of extended C2-*p*-biphenylethynyl substitution suggested a ligand reorientation in AR binding, dominated by bulky N^6 groups in analogues lacking a stabilizing 5′-uronamide moiety. Thus, 4′-truncation of C2-arylethynyl-(N)-methanocarba adenosine derivatives is compatible with general preservation of A_3AR selectivity, especially with small N^6 groups, but reduced efficacy in A_3AR-induced inhibition of adenylate cyclase.



KEYWORDS: *G protein-coupled receptor, purines, molecular modeling, structure−activity relationship, radioligand binding, adenosine receptor*

Extracellular adenosine acts in cell signaling through four subtypes of rhodopsin-like G protein-coupled receptors (GPCRs), that is, A_1 adenosine receptor (AR), A_{2A}AR, A_{2B}AR, and A_3AR.[1] The A_3AR is the least widely distributed of the subtypes in the body and has become a target in drug discovery for various disease conditions.[2−5] A_3AR-selective agonists have successfully progressed to phase 2 and 3 clinical trials for the treatment of hepatocellular carcinoma (including subsequent to hepatitis C viral infection), autoimmune inflammatory diseases, such as rheumatoid arthritis, psoriasis, and osteoarthritis, glaucoma, and dry eye disease. A_3AR agonists are also under consideration for treating uveitis, Crohn's disease, neuropathic pain, loss of skin pigmentation, lung injury, and ischemia of brain, heart, and skeletal muscle.[2,6−8] A_3AR-selective antagonists are being explored for treatment of asthma, septic shock, glaucoma, and other conditions.[9−15]

The structure−activity relationship (SAR) of nucleosides at the A_3AR has been extensively explored.[5] Selective agonists of the A_3AR are typically adenine-9-ribosides containing both 5′-*N*-methyluronamide (facilitates complete activation of A_3AR) and N^6-(3-halobenzyl) groups. A general means of enhancing A_3AR affinity and selectivity is the replacement of the flexible ribose ring with a conformationally constrained bicyclo[3.1.0]-hexane (methanocarba) ring system that enforces a receptor-preferred North (N) conformation,[16,17] as in the selective agonist **1a** (MRS3558) and its congeners (Chart 1).

The effects of truncation of adenosine derivatives at the 4′ carbon, that is, loss of the CH_2OH of adenosine, have been extensively explored in the 4′-thio, 4′-oxo, and (N)-

Chart 1. (N)-Methanocarba-adenosine Derivatives as Selective A_3AR Ligands (hAR Affinity in nM): Full Agonists in the 5′-*N*-Methyluronamide Series (1 and 3) and Truncated Nucleosides (2) That Act as A_3AR Antagonists and Partial Agonists, Including the Present Target Structures (4−21)

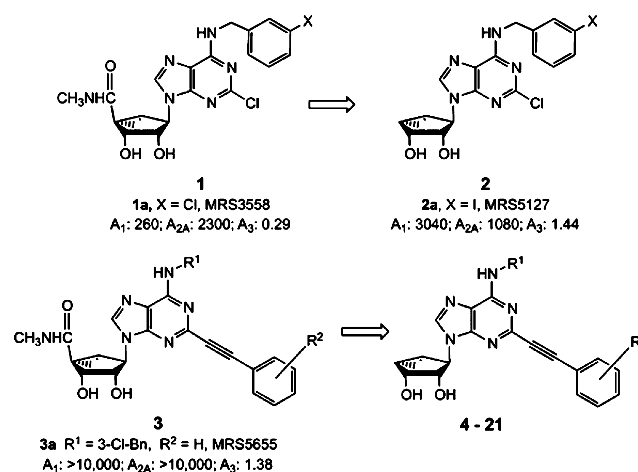


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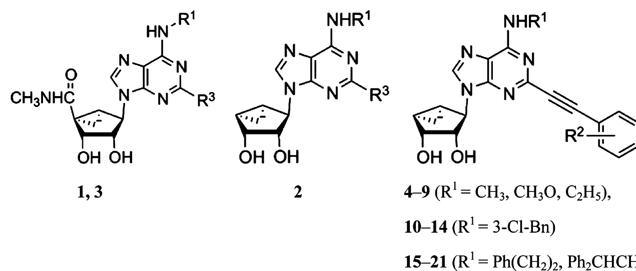
Table 1. Affinity of a Series of (N)-Methanocarba adenosine Derivatives at hARs and Functional Efficacy at the hA_3AR



Compound	R^2 or R^3	R^1	Affinity (K_i, nM) or *% inhibition*[a]			%Efficacy[b]
			hA_1	hA_{2A}	hA_3	hA_3
1[c]	Cl	3-Cl-Bn	260±60	2300±100	0.29±0.04	103±7
2a[c]	Cl	3-I-Bn	3040	1080	1.44	1.0±3.2
2b[c]	Cl	3-Cl-Bn	3070	4510	1.06	2.9±3.7
2c[d]	Cl	*trans*-2-Ph-cycloPr	1790	2010	1.30	9.7±4.1
2d[d]	C≡C-$(CH_2)_2$-CONH-$(CH_2)_2$-NH_2	3-Cl-Bn	*(15%)*	*(35%)*	404	0.9±8.5
3a[e]		3-Cl-Bn	*(20%±3%)*	*(27%±3%)*	1.35±0.30	101±5.9
3b[e]		CH_3	*(13%±6%)*	*(14%±7%)*	0.85±0.22	89.3±7.7
3c[e]	F, F	3-Cl-Bn	*(6%±4%)*	*(41%±10%)*	3.49±1.84	95.7±6.4
3d[e]	Cl	3-Cl-Bn	*(19%±2%)*	*(52%±12%)*	1.92±0.57	103±1.5
3e[e]	Cl	3-Cl-Bn	*(4%±4%)*	1790±590	4.45±1.89	91.5±11.4
3f[e]		3-Cl-Bn	*(2%±2%)*	*(0%±0%)*	3.06±1.35	89.0±4.5
4	H	Me	*(18%±1%)*	*(18%±3%)*	5.48±1.23	12.6±4.0
5	2-Cl	Me	*(35%±1%)*	*(37%±4%)*	3.20±0.91	2.9±2.1
6	H	MeO	*(17%±6%)*[f]	*(12%±7%)*[f,g]	13.0±2.0	29.8±2.8
7	2-Cl	MeO	*(30%±3%)*	*(22%±5%)*	8.52±2.86	9.0±3.7
8	H	Et	*(36%±4%)*	*(42%±4%)*	5.02±2.19	0.8±5.2
9	2-Cl	Et	*(25%±11%)*	*(17%±6%)*	5.80±2.08	-7.0±5.2
10	H	3-Cl-Bn	*(37%±4%)*[g]	680±170	39.0±20.0	13.8±5.1
11	3,4-diF	3-Cl-Bn	*(29%±1%)*	1350±190	100±30	13.6±2.3
12	2-Cl	3-Cl-Bn	2730±620	*(40%±4%)*	70.0±9.0	22.1±4.2
13	3-Cl	3-Cl-Bn	*(26%±3%)*	1800±310	210±40	4.5±4.9
14	4-Ph	3-Cl-Bn	*(48% ±4%)*	*(12%±7%)*	54.0±7.0	-3.5±3.2
15	H	$Ph(CH_2)_2$	*(30%±8%)*	*(22%±5%)*	20.0±6.0	4.1±1.2
16	2-Cl	$Ph(CH_2)_2$	*(37% ±7%)*	*(26%±10%)*	37.0±7.0	19.5±1.9
17	H	Ph_2CHCH_2	*(29%±2%)*	*(24%±4%)*	200±20	0.4±5.9
18	3,4-diF	Ph_2CHCH_2	*(14%±1%)*	*(20%±5%)*	340±20	4.0±6.4
19	2-Cl	Ph_2CHCH_2	*(26%±4%)*	*(22%±2%)*	140±30	-2.6±1.3
20	3-Cl	Ph_2CHCH_2	*(11%±3%)*	*(13%±5%)*	260±30	-7.4±3.4
21	4-Ph	Ph_2CHCH_2	*(11%±3%)*	*(13%±8%)*	1320±260	6.0±1.1

Table 1. continued

[a]Binding in membranes of CHO or HEK293 (A_{2A} only) cells stably expressing one of three hAR subtypes. The binding affinity for hA_1, A_{2A}, and A_3ARs was expressed as K_i values using agonists [^{3}H]N^6-*R*-phenylisopropyladenosine, [^{3}H]2-[*p*-(2-carboxyethyl)phenyl-ethylamino]-5′-*N*-ethylcarboxamidoadenosine, or [^{125}I]N^6-(4-amino-3-iodobenzyl)adenosine-5′-*N*-methyluronamide, respectively. A percent in parentheses refers to inhibition of binding at 10 μM. [b]Inhibition of forskolin-stimulated cyclic AMP production in hA_3AR-transfected CHO cells. At 10 μM, in comparison to the maximal effect of 10 μM 5′-*N*-ethylcarboxamidoadenosine (=100%). Selected compounds (10 μM) were evaluated for stimulation of cyclic AMP production (% of full agonist 5′-*N*-ethylcarboxamidoadenosine) in $hA_{2B}AR$-transfected CHO cells: **3c**, 28 ± 3; **3e**, 28 ± 4; **5**, 49 ± 7; **6**, 38 ± 3; **9**, 44 ± 10; **10**, 33 ± 11; **13**, 40 ± 9; and **15**, 46 ± 14. Data are expressed as means ± standard errors (*n* = 3, unless noted). [c]Data from Melman et al.[20] [d]Data from Tosh et al.[30] [e]Data from Tosh et al.[23,24] [f]Inhibition of radioligand binding at 1 μM. [g]*n* = 2.

methanocarba series of nucleosides. An effect of this truncation is typically retention of affinity at the A_3AR and reduction of affinity at other AR subtypes.[18−20] Specifically, in the (N)-methanocarba series of N^6-(3-halobenzyl) derivatives, that is, full A_3AR agonists **1**, truncation resulted in series **2** that retains hA_3AR affinity and selectivity but is typically reduced in efficacy at A_3 and A_1ARs but not $A_{2A}AR$.[21] The functional activity of **2a** (MRS5127) and its congeners range from full competitive antagonism (K_B = 8.9 nM,[20] by Schild analysis in blocking agonist-induced guanine nucleotide binding) to partial agonism of ~50% efficacy (in inhibition of adenylate cyclase). Compound **2a** was radioiodinated and studied in binding to ARs in different species,[22] and its high affinity and selectivity for the A_3AR were independent of species.

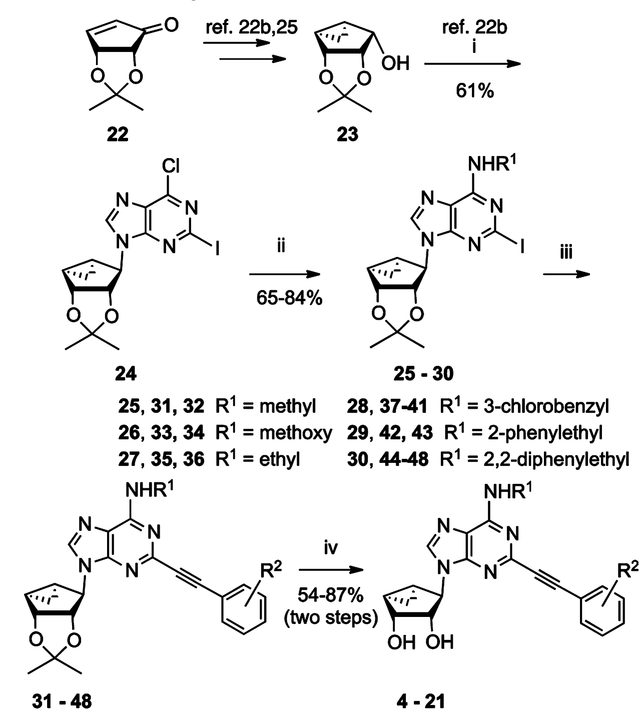
We recently demonstrated that (N)-methanocarba adenosine 5′-*N*-methyluronamide derivatives tolerate the introduction of rigid, extended C2-arylalkynyl groups.[23,24] Previously, similar groups were included in potent A_3AR agonists in the relatively flexible riboside series.[25] In the rigid (N)-methanocarba series, the much larger planar groups, such as large as 2-pyrenylethynyl, than anticipated were tolerated at the human (h) A_3AR.

The present study establishes that truncation of the 4′ group of 2-arylethynyl-(N)-methanocarba adenosine agonists in general preserves selectivity for the A_3AR, while the effects of such truncation on the relative agonist efficacy at this receptor are complex. These truncated partial agonists/antagonists of the A_3AR have favorable physicochemical properties, such as low polarity, which will likely alter pharmacokinetic behavior in vivo. The SAR in AR binding of 4′-truncated (N)-methanocarba-adenosine derivatives was characterized by Melman et al.[20] and Tosh et al.[24] The derivatives contained mainly N^6-3-halobenzyl groups and relatively flexible, straight chain alkynyl groups at the C2 position, such as **2d** (Table 1), but substitution with rigid, linear C2-arylethynyl groups was not included in the previous studies. We have explored such rigid C2 substituents in the present study and also varied the N^6 group.

The 4′-truncated analogues contain varied N^6 substitution (N^6-methyl, methoxy, or ethyl: **4−9**; N^6-3-chlorobenzyl: **10−14**; and N^6-2-phenylethyl or 2,2-diphenylethyl: **15−21**) (Table 1). With small N^6 substitution, only two phenylethynyl variations were placed at the C2 position: unsubstituted and 2-chloro, which was previously shown to promote A_3AR affinity.[23,24] However, in the N^6-3-chlorobenzyl series, several other C2-phenylethynyl substitutions were used, that is, 3,4-difluoro (**11**), 3-chloro (**13**), and 4-phenyl (**14**). N^6-Methyl and adenosines are typically more potent at the hA_3AR than rat (r) A_3AR, while N^6-(3-halobenzyl)adenosines tend to display greater species-independent selectivity.[22] Thus, it is useful to have a range of N^6 groups present.

The synthetic route used to prepare truncated (N)-methanocarba derivatives containing a C2-arylethynyl group is shown in Scheme 1. Initially, D-ribose was converted as

Scheme 1. Synthesis of Truncated (N)-Methanocarba-adenosine Analogues[a]



[a]Reagents: (i) 2-Iodo-6-chloropurine, Ph_3P, DIAD, THF, rt. (ii) R^1NH_2, Et_3N, MeOH, rt. (iii) Substituted HC≡C-Ph-R^2, Pd-$(PPh_3)_2Cl_2$, CuI, Et_3N, DMF, rt. (iv) 50% TFA (aq.), MeOH:CH_2Cl_2 (3:1), rt.

previously reported into the 2′,3′-protected intermediate cyclopentenone **22**.[27] Compound **22** was then converted stereoselectively in two steps to the alcohol **23**, which was subjected to a Mitsunobu reaction with 2-iodo-6-chloropurine to give intermediate **24**.[28,30] Nucleophilic substitution with the appropriate amine at room temperature provided the N^6-substituted intermediates **25−30**. A Sonogashira reaction[26] was then carried out with a variety of commercially available phenylalkynes, followed by acid hydrolysis of the 2′,3′-isopropylidene group to provide truncated nucleosides **4−21**.

Radioligand binding assays were performed at three hAR subtypes using standard ^{3}H- and ^{125}I-labeled nucleoside ligands of high affinity (Table 1).[20] A_3AR binding curves of the truncated nucleosides generally showed a Hill coefficient of ~1. Selected compounds in the (N)-methanocarba nucleoside series were assayed at the $hA_{2B}AR$ and found to be weakly active, as this ring system was previously found to reduce

$hA_{2B}AR$ affinity.[16] Some previously reported 2-chloro (**2a–c**) and 2-alkynyl (**2d** and **3a–f**) (N)-methanocarba-adenosine derivatives were used for comparison in the biological assays.[17,2324,29]

The N^6-methyl C2-phenylethynyl analogue **4** displayed a K_i value of 5.5 nM in binding to the hA_3AR and was nearly inactive at the hA_1AR and $hA_{2A}AR$ with only 18% inhibition of binding at 10 μM. Therefore, the degree of A_3AR selectivity of **4** was estimated to be >2000-fold. A variety of other 4′-truncated C2-arylethynyl-(N)-methanocarba nucleosides containing N^6-methoxy, N^6-ethyl, N^6-3-chlorobenzyl, N^6-2-phenylethyl, or N^6-2,2-diphenylethyl groups displayed a range of high-to-moderate affinities at the hA_3AR and generally with A_3AR selectivity. The hA_3AR affinity of these derivatives demonstrated a freedom of substitution at C2. The smaller groups at N^6 in **4–9** were associated with higher affinity at the A_3AR. Thus, N^6-methyl (**5**, K_i = 3.2 nM) and ethyl (**9**, K_i = 5.8 nM) in the set of 2-(2-chlorophenylethynyl) derivatives provided the highest hA_3AR affinity with only weak binding to A_1 and $A_{2A}ARs$, implying a selectivity of ≥1000-fold, and a N^6-methoxy derivative **7** was slightly less potent. In the N^6-3-chlorobenzyl series, the unsubstituted phenylethynyl derivative **10** was the most potent in binding at the A_3AR (K_i = 39 nM). However, unlike the 5′-*N*-methyluronamide series, halo substitution in 3,4-difluorophenylethynyl **11** and 2-chlorophenylethynyl **12** analogues significantly reduced A_3AR affinity. N^6-2-Phenylethyl substitution in C2-phenylethynyl analogue **15** provided a K_i value of 20 nM at the hA_3AR. A 2-*p*-biphenylethynyl substitution was well tolerated at the A_3AR in the N^6-3-chlorobenzyl **14** but not N^6-(2,2-diphenylethyl) **21** series.

At the hA_1AR, only 10–40% of binding inhibition was typically seen at 10 μM for a variety of substitutions, except for the 2-chlorophenylethynyl analogue **12** (K_i = 2.7 μM). However, the variability of affinity upon substitution of the arylethynyl moiety was greater at $hA_{2A}AR$ than at hA_1AR. For example, three N^6-3-chlorobenzyl compounds (**10**, **11**, and **13**) had K_i values in the range of 0.7–1.8 μM at the $hA_{2A}AR$. As in the 5′-*N*-methyluronamide series,[23,24] $hA_{2A}AR$ affinity increased substantially upon replacement with a C2-(3-chlorophenylethynyl) group in **13**, leading to only 8.6-fold selectivity in A_3AR binding.

Functional data were determined at a single, saturating concentration (10 μM) in an assay of hA_3AR-induced inhibition of the forskolin-stimulated production of adenosine 3′,5′-cyclic monophosphate (cyclic AMP) in membranes of Chinese hamster ovary (CHO) cells expressing the hA_3AR (Table 1).[28] Inhibition by 10 μM 5′-*N*-ethylcarboxamidoadenosine (NECA) was set at 100% relative efficacy. The novel truncated derivatives were generally low-efficacy partial agonists (e.g., **6**, **12**, and **16**) or antagonists of the hA_3AR. Although N^6-ethyl and 2,2-diphenylethyl derivatives tended to be antagonists of the hA_3AR, N^6-methyl derivative **4** and N^6-methoxy derivative **6** had significant residual efficacy. The efficacy of N^6-Cl-benzyl and 2-phenylethyl derivatives **10–17** varied depending on the substitution of the phenylethynyl group. The highly selective ligands **10** and **15** displayed 14% and 4% relative efficacy, respectively.

Because of the sterically bulky hydrocarbon group at the C2 position, these compounds display increased hydrophobicity in comparison to previously described truncated adenosine analogues. The large differences in steric bulk and hydrophobicity with respect to the conventional ribonucleoside series could influence the pharmacokinetics and the spectrum of biological activities at the A_3AR. For example, the cLog *P* values of potent C2-arylethynyl derivatives having N^6-methyl **5**, N^6-3-chlorobenzyl **10**, N^6-2-phenylethyl **15**, and N^6-2,2-diphenylethyl **17** substituents were 2.32, 3.76, 3.70, and 5.14, respectively. The total polar surface area of all four derivatives was 92.8 Å^2, which is within the desired range.[30] These physicochemical parameters are predicitive of bioavailability, although in vivo pharmacological and pharmacokinetic properties of these analogues have not been measured.

Flexibility of a 5′-*N*-alkyluronamido moiety of adenosine agonists, which is entirely lacking in the present analogues, is closely associated with full A_3AR activation. Consistently, these analogues have low or minimal levels of efficacy in the A_3AR-induced inhibition of adenylate cyclase, but the functional efficacy for other effector systems coupled to the same receptor has not been measured. For comparison, other truncated (N)-methanocarba-adenosine derivatives were shown to be full antagonists in a guanine nucleotide binding assay, which is relatively poorly coupled, and partial agonists in an assay of cyclic AMP.[22] The unusual shape of these nucleoside ligands might translate to ligand-dependent differences in the conformation of the nucleoside-bound A_3AR, which could have functional implications on effects induced by this receptor. There is already an indication that nucleoside ligands of the A_3AR can display a functional bias.[31]

The observation of high affinity at the A_3AR in the 5′-*N*-methyluronamido series was explained in terms of a proposed outward shift of transmembrane helix 2 (TM2) from its position in a homology model based on the agonist-bound $A_{2A}AR$ structure.[23,24] The present 4′-truncated derivatives were subjected to similar molecular modeling analysis (Supporting Information) to predict a similar 7 Å shift of TM2, similar to its position in opsin, to accommodate the bulky, extended C2 substituent. As for the 5′-*N*-methyluronamides,[23,24] the 3′- and 2′-hydroxyl groups of docked **10** formed H-bonds with Ser271 (7.42) and His272 (7.43) side chains, respectively. The side chain of Asn250 (6.55) strongly interacted with the 6-amino group and the adenine N^7 atom. Moreover, the adenine ring was anchored inside the binding site by a π–π stacking interaction with Phe168 (EL2) and strong hydrophobic contacts with Leu246 (6.51) and Ile268 (7.39). Substituents at the N^6 (TM5, TM6, and EL2) and C2 positions (TM2 and EL1) were located in hydrophobic extracellular portions of the hA_3AR binding pocket. While the 5′-*N*-methyluronamides[23,24] were able to form a H-bond with the side chain hydroxyl group of Thr94 (3.36), truncation prevented an interaction with this key residue, a putative explanation of the low efficacy profile of these 4′-truncated nucleosides.

In the 5′-*N*-methyluronamido series, an enhancement of $A_{2A}AR$ affinity was observed for 2-(3-chlorophenylethynyl) analogues, which suggested halogen–π interactions with Tyr9 (1.35) and Tyr272 (7.36) of the $A_{2A}AR$.[23,24] In the present truncated series, compound **13** contained the same C2-(3-chlorophenylethynyl) group, and the $A_{2A}AR$ affinity was enhanced, likely reflecting a common interaction with the same site on the $hA_{2A}AR$. However, 2-(3-chlorophenylethynyl) derivative **20** did not display this characteristic enhancement, suggesting a reorientation of the bound ligand, likely stemming from the bulky N^6-2,2-diphenylethyl group and the lack of stabilizing 5′-uronamido interactions.

In conclusion, we have found a new series of highly potent and selective, low-efficacy partial agonists (e.g., **6** and **16**) or antagonists (e.g., **5**, **8**, **9**, and **15**) at the A_3AR, containing

combined arylethynyl groups at the adenine C2 position and varied N^6 substitution. The binding affinities demonstrated tolerance of steric bulk on the extended C2 position substituents, allowed by a proposed plasticity of the A_3AR.[23,24] Smaller N^6 substitutuents, such as methyl and ethyl, provided higher A_3AR affinity than N^6-arylalkyl substitutuents. This difference contrasts with the analogues having an additional receptor anchor, that is, the 5′-*N*-methyluronamido group in the region of TMs 3 and 7, for which the A_3AR affinity was less dependent on the N^6 substitutuent. Overall, truncation of the 4′ group of 2-arylethynyl-(N)-methanocarba adenosine derivatives was shown to be compatible with retention of A_3AR selectivity. Substitution of the phenylethynyl group modulated the agonist efficacy (0−30% of full agonism in adenylate cyclase inhibition) at this receptor in a complex manner. These potent, truncated ligands that have improved druglike physical properties and should be useful in future pharmacological studies of the action of this receptor in disease models of the cardiovascular, inflammatory, gastrointestinal, pulmonary, and central nervous systems and in studies that relate ligand structure (and possibly receptor conformation) to functional selectivity of GPCR action.

■ ASSOCIATED CONTENT

Ⓢ Supporting Information

Procedures for chemical synthesis (with selected spectra), biological assays, and molecular modeling. This material is available free of charge via the Internet at http://pubs.acs.org.

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Author Contributions

All authors contributed to this manuscript and have given approval to its final version.

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Notes

The authors declare no competing financial interest.

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■ ABBREVIATIONS

AR, adenosine receptor; cyclic AMP, adenosine 3′,5′-cyclic monophosphate; CHO, Chinese hamster ovary; DIAD, diisopropyl azodicarboxylate; GPCR, G protein-coupled receptor; HEK, human embryonic kidney; NECA, 5′-*N*-ethylcarboxamidoadenosine; TFA, trifluoroacetic acid; TM, transmembrane helix

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