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Design of (N)-Methanocarba Adenosine 5'-Uronamides as Species-Independent A₃ Receptor-Selective Agonists

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Abstract

2-Chloro-5' -N-methylcarboxamidoadenosine analogues containing the (N)-methanocarba (bicyclo [3.1.0]hexane) ring system as a ribose substitute display increased selectivity as agonists of the human A₃ adenosine receptor (AR). However, the selectivity in mouse was greatly reduced due to an increased tolerance of this ring system at the mouse A₁AR. Therefore, we varied substituents at the N⁶ and C2 positions in search of compounds that have improved A₃AR selectivity and are species independent. An N⁶-methyl analogue was balanced in affinity at mouse A₁/A₃ARs, with high selectivity in comparison to the A_{2A}AR. Substitution of the 2-chloro atom with larger and more hydrophobic substituents, such as iodo and alkynyl groups, tended to increase the A₃AR selectivity (up to 430-fold) in mouse and preserve it in human. Extended and chemically functionalized alkynyl chains attached at the C2 position of the purine moiety preserved A₃AR selectivity more effectively than similar chains attached at the 3 position of the N⁶-benzyl group.

Keywords

nucleoside; G protein-coupled receptor; mouse; adenosine receptor; radioligand binding

Adenosine is a protective mediator that has been described as a general endogenous signal for tissue protection and regeneration and even “the signal of life”.^{1,2} Adenosine activates four different receptor subtypes - A₁, A_{2A}, A_{2B}, and A₃ - which are widely but differentially distributed throughout the body.³ The A₃ adenosine receptor (AR) is located in some neurons, astrocytes, various immune cell populations (neutrophils, eosinophils, mast cells) and potentially muscle cells and endothelial cells.⁴⁻⁷ The A₃AR is coupled to inhibition of adenylate cyclase and also activates Akt and calcium mobilization.^{8,9} Two potent and selective agonists of the A₃AR, IB-MECA **1a** and CI-IB-MECA **1b**, are currently in Phase II clinical trials for the treatment of rheumatoid arthritis, several other autoimmune inflammatory diseases, and cancer.¹⁰⁻¹² Protective mechanisms in the envisioned disease applications of A₃AR agonists appear to be a downregulation of the NF-κB system, common to both arthritis and cancer treatments,¹² and a widespread correction of gene dysregulation induced in an

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inflammatory bowel model.¹³ Cardioprotection by selective A₃AR agonists has been extensively explored in various species.¹⁴⁻¹⁶

Other selective A₃AR agonists have recently been reported, based on introduction of large substituents at the N⁶ and C2 positions and modification of the ribose ring, particularly at the 4' and 5' positions.¹⁶⁻¹⁹ For example, the 4'-thio analogue **2** is among the most selective known A₃AR agonists,¹⁷ and 3'-amino substitution is possible.¹⁶ Carbocyclic nucleosides have also been developed as A₃AR agonists.²⁰⁻²² Conformationally constrained methanocarba (bicyclo[3.1.0]hexane) nucleoside analogues were used to determine that the biologically active conformation of the ribose ring, i.e. that required in order to bind to and activate the receptor, corresponds to a North (N) conformation. Furthermore, the addition of a 5'-N-methyl or ethyl uronamide group assures that the efficacy of the nucleoside to activate the A₃AR is maintained in combination with various structural changes at the N⁶ and C2 positions, which modulate the AR selectivity profile and might otherwise reduce the A₃AR efficacy. The conformational flexibility and H-bonding in the region of the 5'-uronamide are critical factors affecting the efficacy at the A₃AR, as deduced from structure activity relationship (SAR) studies and from rhodopsin-based dynamic molecular modeling and ligand docking.²³

In this study, we report that certain known A₃AR agonists are much less selective at the A₃AR in the mouse than in other species, such as human, and are therefore unsuitable for use alone as definitive pharmacological probes in mouse. Thus, additional A₃AR agonists displaying high affinity and selectivity that are independent of species are desired. We previously explored the SAR surrounding both N⁶ and C2 positions of 5'-N-methylcarboxamido adenosine analogues that contained an (N)-methanocarba ring system as a ribose substitute, with the objective of designing potent, and selective A₃AR agonists.²² There is considerable evidence that appropriate modification of the N⁶ and C2 positions of nucleoside agonists is tolerated at the human A₃AR, while SAR at the mouse ARs has not been systematically explored.^{3, 16-19, 23-25}

Table 1 lists the structures of the adenosine derivatives that were assayed for binding affinity at ARs from several species. Compounds **3** – **12**, previously synthesized and evaluated at human and rat ARs,^{21, 22} were evaluated at the mouse ARs. Based on indications that the 2-iodo derivative **12** maintained selectivity at the mouse A₃AR, compounds **13** – **19** and **25** – **27** were synthesized. Synthetic routes to the novel derivatives are shown in Schemes 1 and 2. In the synthetic route to each of the new A₃AR agonists, after substitution of the chlorine at C6 in the purine ring of **20** and **21** with a corresponding primary amine R¹NH₂, the 5'-ester group was aminolyzed with methylamine solution. Resultant compounds of type **22** or **23** were either hydrolyzed to afford agonists **3-10**, **12**, **13**, **19**, or underwent Sonogashira coupling²⁶ with corresponding alkynes followed by hydrolysis to afford agonists **15** – **17**. Agonist **14** was prepared from agonist **15** through desilylation with tetrabutylammonium fluoride. An N⁶-methoxy derivative **19**, based on a recent report by Volpini et al. showing high potency at the human A₃AR of similar derivatives in the ribose series,¹⁸ was prepared by substitution of the 6-Cl of **20** with *O*-methylhydroxylamine followed by deprotection of the 2' and 3' hydroxyl groups.

Some of the 2-alkynyl derivatives contained chemically functionalized, extended alkynyl chains to serve as functionalized congeners for conjugation to other biologically active moieties or to carriers.²⁷⁻²⁹ These included carboxylic acids **18a** and **25**, amines **18b** and **26**, and an acetylamino derivative **27**. The Sonogashira reaction sequence using methyl hexynoate in combination with an iodo-derivatized nucleoside, either a 2-iodo (Scheme 1) or N⁶-(3-iodobenzyl) (Scheme 2) derivative, provided a mixture of the product methyl ester and corresponding carboxylic acid, which were separated by silica gel column chromatography.

The primary amine congeners **18b** and **26** were prepared by treatment of the appropriate methyl ester with excess ethylenediamine.

Binding assays were carried out using standard radioligands in Chinese hamster ovary (CHO) cells expressing the human A₁ or A₃ARs and in HEK293 cells expressing the human A_{2A}AR.²² Also, the mouse ARs were expressed in HEK293 cells for binding assays.¹⁵ A functional assay in guanine nucleotide binding ([³⁵S]GTPγS)^{20,31} in membranes of CHO cells expressing the human A₃AR showed that **18a** is a full agonist. The pEC₅₀ value of **18a** was 7.85 ± 0.15, in comparison to the pEC₅₀ value of NECA of 6.46 ± 0.13.

The binding affinity at the mouse and rat A₃ARs of the N⁶-methyl derivative **3** was considerably weaker than at the human A₃AR (Table 1). Thus, this compound was balanced in affinity at mouse A₁/A₃ARs, with high selectivity in comparison to the mouse A_{2A}AR. Other agonists having this mixed AR selectivity were explored for their cardioprotective properties.²⁹

N⁶-Benzyl derivatives of adenosine have previously been shown to favor selectivity at the A₃AR.²⁴ This observation led to the design of **1a** and **1b**. Although these two 9-riboside derivatives maintain selectivity for the mouse A₃AR, the selectivity in mouse of similar N⁶-substituted benzyl (N)-methanocarba derivatives **4** – **6** was greatly reduced due to increased tolerance of this bicyclic ring system at the mouse A₁AR. Therefore, we varied substituents at the N⁶ and C2 positions in an effort to reduce affinity at the mouse A₁AR. Extension of the 3-benzyl group as an alkyne in **7** reduced mouse A₁ and A_{2A}AR affinity without greatly reducing affinity at the mouse, rat, or human A₃ARs, resulting in a 57- fold selectivity for the mouse A₃AR. A 2,5-dimethoxy substitution in **8** showed only a slight enhancement of mouse A₃AR selectivity in comparison to **6**, the (N)-methanocarba equivalent of Cl-IB-MECA. Modified N⁶-phenylethyl analogues **9** and **10**, although both very potent at the mouse A₃AR, were essentially nonselective in comparison to the mouse A₁AR.

Modification at the adenine C2 position was generally more beneficial than structural changes of the N⁶ substituent, with respect to mouse A₃AR selectivity. 2-Cl was replaced with small hydrophobic groups,²² which greatly increased the selectivity for the mouse A₃AR. For example, a 2-iodo analogue **12** was 178-fold and >180,000-fold selective in binding to the mouse A₃AR in comparison to mouse A₁ and A_{2A}AR, respectively, with a K_i value of 1.18 nM. The corresponding 2-iodo-N⁶-(2,5-dimethoxybenzyl) analogue **13** was similarly selective. Thus, the 2-iodo modification resulted in increased selectivity for the mouse A₃AR; i.e., the selectivity ratio of 2-iodo compounds (**12** and **13**) was increased over that of the corresponding 2-chloro analogues (**4** and **8**, respectively) by 11 – 17-fold. Other hydrophobic substituents at the C2 position, such as an ethynyl group in **14** and its trimethylsilyl adduct **15**, provided moderate mouse A₃AR selectivity. Flexible, extended ethynyl chains in **16** and **17** increased the ratio of A₃AR selectivity. 2-Alkynyl groups were previously reported to enhance affinity of adenosine derivatives at the rat and human A₃ARs.^{25,30} Compound **18a**, a long chain carboxylic acid congener, and its corresponding methyl ester **17** were highly selective for the mouse A₃AR in comparison to the A₁AR by 431- and 288-fold, respectively. Both compounds had greater A₃AR selectivity than Cl-IB-MECA **1b**, although they were less potent. A primary amino congener **18b** displayed moderate A₃AR selectivity, with a K_i value of 8.6 nM. Functionalized congeners in which a functionalized ethynyl chain was positioned on the N⁶-benzyl moiety were only moderately (a carboxylic acid **25**) or weakly (a primary amine **26** and its acetyl analogue **27**) selective for the mouse A₃AR. Thus, the attachment of alkynyl chains at the 3 position of the N⁶-benzyl moiety did not preserve A₃AR selectivity as well as the placement of similar chains at the adenine C2 position.

The use of an alkynyl substituent at the 3-position of an N⁶-benzyl group, to serve as a covalent linking site for conjugation, was shown previously to maintain A₃AR affinity in the series of

9-ribosides.^{28,29} In the present study of the (N)-methanocarba series, a variety of adenosine derivatives bearing a 2-alkynyl group have been shown to bind potently and selectively to the A₃AR.

The (N)-methanocarba derivatives that were most potent (K_i 1 – 6 nM) and selective (180 – 290-fold) in binding to the mouse A₃AR were, in order of decreasing affinity: **12**, **13**, **17**, and **16**. Compound **18a** was the most selective novel agonist in this study at the mouse A₃AR; however, the affinity was intermediate, with a K_i value of 24 nM. The selectivity for the human A₃AR was >6000-fold. Thus, these C2 position-modified bicyclic nucleosides are good candidates for species-independent A₃AR agonists.

In conclusion, the selectivity (but not affinity) of (N)-methanocarba-containing nucleosides as A₃AR agonists was greatly reduced in the mouse due to increased tolerance of this ring system at the mouse A₁AR. Several analogues having varied substitution at the N⁶ and C2 positions were balanced in affinity at mouse A₁/A₃ARs, with high selectivity in comparison to the A_{2A}AR. Substitution of the 2-chloro atom with larger and more hydrophobic substituents, such as iodo and alkynyl groups, tended to increase the A₃AR selectivity in mouse and preserve it in human. The carboxylic acid **18a** and primary amino **18b** derivatives are good candidates for use as functionalized congeners for covalent conjugation with retention of biological activity and receptor selectivity. Thus, we have identified novel (N)-methanocarba nucleosides that are A₃AR-selective across several species and are especially suitable for pharmacological studies in the mouse.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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ABBREVIATIONS

AR

adenosine receptor

CGS21680

2-[p-(2-carboxyethyl)phenylethylamino]-5' -N-ethylcarboxamido-adenosine

CHO

Chinese hamster ovary

Cl-IB-MECA

2-chloro-*N*⁶-(3-iodobenzyl)-5' -*N*-methylcarboxamidoadenosine

CPA

*N*⁶-cyclopentyladenosine

DMEM

Dulbecco's modified Eagle's medium

I-AB-MECA

*N*⁶-(4-amino-3-iodobenzyl)-5' -*N*-methylcarboxamidoadenosine

NECA

5' -*N*-ethylcarboxamidoadenosine

Chart 1.
Prototypical selective A₃AR agonists.

Scheme 1.

^a Synthesis of novel (N)-methanocarba A₃AR agonists with structural variation at the 2 and N⁶ positions. Note that **17** and **18a** were both isolated chromatographically from the same reaction.

^a **Reagents & conditions:** a) R¹NH₂; b) MeNH₂, EtOH; c) RC≡CH, PdCl₂(PPh₃)₂, CuI, DMF, Et₃N; d) TFA, MeOH, H₂O, Δ; e) TBAF, THF; f) ethylenediamine, MeOH.

Scheme 2.

^a Synthesis of novel (N)-methanocarba A₃AR agonists containing functionalized alkynyl chains attached at the N⁶-benzyl 3 position. Note that **24** and **25** were both isolated chromatographically from the same reaction.

^a **Reagents & conditions:** a) $\text{HC}\equiv\text{C}(\text{CH}_2)_3\text{COOCH}_3$, $\text{PdCl}_2(\text{PPh}_3)_2$, CuI , DMF , Et_3N ; b) TFA , H_2O , MeOH , Δ ; c) ethylenediamine; d) acetic anhydride.

Affinity of a series of (N)-methanocarba adenosine derivatives at three subtypes of ARs in various species. Compounds **1a** and **1b** are 9-ribose derivatives.

3 – 19, 25 – 27

Table 1

Compound	R ¹	R ²	Species	A ₁ ^a K _i , nM	Affinity A _{2A} ^a K _i , nM (or % inhib.)	A ₃ ^a K _i , nM	Selectivity A ₁ /A ₃
1a			M ^{bc}	5.9	~1000	0.087	68
			H	49.3±3.7	93.1±4.2	1.74±0.36	28 ^d
			R ^e	54	56	1.1	49
1b			M ^{bc}	35	~10,000	0.18	190
			H ^d	220	5400	1.4	160
			R ^e	820	470	0.33	2500
3	CH ₃	Cl	M	55.3±6.0	20,400±3,200	49.0±3.9	1.1
			H	2100±1700	(6%) ^{d,f}	2.2±0.6	950
			R	805 ^g	>10,000 ^g	160±30 ^d	5.0
4^h	3-Cl-Bn	Cl	M	15.3±5.8	10,400±1,700	1.49±0.46	10.3
			H ^{cd}	260	2300	0.29	900
			R ^d	ND	ND	1.0	
5	3-Br-Bn	Cl	M	8.79±0.12	6,390±870	0.90±0.22	9.8
			H ^{cd}	270	1300	0.38	710
			R ^c	ND	ND	0.76	
6^h	3-I-Bn	Cl	M	7.32±1.5	5,350±860	0.80±0.14	9.2
			H ^c	136	784	1.5	91
			R	83.9 ^g	1660 ^g	1.1	76
7	3-(C≡C-CH ₂ OH)-Bn	Cl	M	111±22	(11%) ⁱ	1.94±1.1	57.2
			H ^d	2600±300	(56%) ^f	2.9±0.7	900
			R ^d	ND	ND	1.6±0.6	
8	2,5-(OCH ₃) ₂ Bn	Cl	M	29.0±3.3	44,700±5,700	1.72±0.04	17
			H ^{cd}	1600	~10,000	1.4	1100
			R ^d	ND	ND	0.87	
9	CH ₂ CH(Ph) ₂	Cl	M	6.83±1.5	1.810±581	1.67±0.09	4.1
			H ^{cd}	1300±100	1600±100	0.69±0.02	1900

				R ^d	ND	Affinity	10 ^{±4}	Selectivity
						ND		
10	c-Pr-Ph		Cl	M	6.60±1.3	38,200±5,300	2.79±0.89	2.4
				H, ^{c,d}	770±50	4800±200	0.78±0.06	990
11	3-Cl-Bn		SCH ₃	M	98.9±18.8	(32%) ^f	1.19±0.09	83
				H ^d	610	~10,000	1.5	410
12^h	3-Cl-Bn		I	M	210±34.4	(40%) ^f	1.18±0.11	178
				H ^d	2200	>10,000	3.6	610
13^h	2,5-(OCH ₃) ₂ -Bn		I	R ^d	ND	ND	3.9	
				M	293±29	(14%) ^f	1.51±0.36	194
				H	3070±820	(35%) ^f	1.30±0.27	2360
14	3-Cl-Bn		C≡CH	M	45.6±7.9	(41%) ^f	0.85±0.08	53.6
				H	174±23	(48%) ^f	1.30±0.38	134
15	3-Cl-Bn		C≡C-Si(CH ₃) ₃	M	159±22	(20%) ^f	4.46±0.57	35.6
				H	160±40	(52%) ^f	0.98±0.14	160
16	3-Cl-Bn		C≡C(CH ₂) ₂ -CH ₃	M	1390±430	(42%) ^f	6.06±1.21	229
				H	1040±83	(80%) ^f	0.82±0.20	1300
17	3-Cl-Bn		C≡C(CH ₂) ₃ -COOCH ₃	M	1340±330	(50%) ^f	4.65±0.53	288
				H	482±23	(49%) ^f	1.17±0.27	412
18a^h	3-Cl-Bn		C≡C(CH ₂) ₃ -COOH	M	10,500±1900	(8%) ^f	24.4±3.1	431
				H	14,900±3500	(43%) ^f	2.38±0.56	6260
18b^h	3-Cl-Bn		C≡C(CH ₂) ₃ -CONH-	M	546±62	(31%) ^f	8.60±1.02	64
				H	454±44	(81%) ^f	2.17±0.51	209
			(CH ₂) ₂ NH ₂					
19	OCH ₃		Cl	M	1,160±130	(2%) ^f	877±149	1.3
				H	265±45	(2%) ^f	149±15	1.8
25	3-(C≡C(CH ₂) ₃ -COOH)-Bn		Cl	M	703±71	(5%) ^f	14.4±2.5	49
				H	320±31	(14%) ^f	17.1±1.2	19
26	3-(C≡C(CH ₂) ₃ -CON(CH ₂) ₂ -NH ₂)-Bn		Cl	M	151±18	(39%) ^f	11.9±2.4	13
				H	271±23	(58%) ^f	5.21±0.91	52
27	3-(C≡C(CH ₂) ₃ -CONH-(CH ₂) ₂ NH-COCH ₃)-Bn		Cl	M	45.4±3.4	(68%) ^f	4.65±0.22	9.8
				H	181±22	(80%) ^f	2.88±0.54	63

^a Competition radioligand binding assays using [¹²⁵I]N⁶-(4-amino-3-iodobenzyl)adenosine-5'-N-methyl-uronamide (A1 and A3ARs) and [³H]2-[p-(2-carboxyethyl)phenyl-ethylamino]-5'-N-ethylcarboxamidoadenosine (A2AAR) were conducted with membranes prepared from HEK293 cells expressing recombinant mouse A1, A2A, or A3ARs. At rat and human ARs, the A1 radioligand was either [³H]R-phenylisopropyladenosine or [³H]2-chloro-N⁶-cyclopentyladenosine. Values are expressed as the mean ± SEM. ND, not determined.

^b Data from Ge et al.¹⁵

^c EC₅₀ value in activation of the A_{2B}AR (human or mouse, as indicated) is ≥10 μM.^{15,22}

^d Data from Tchilibon et al.²²

^e Data from Kim et al.²⁴

^f Percent inhibition at 10 μM.

^g Data from Lee et al.²¹

i, Percent inhibition at 100 μ M.

h **4**, MRS3558; **6**, MRS1898; **12**, MRS3609; **13**, MRS5128; **18a**, MRS5151; **18b**, MRS5166.