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Bioorganic & Medicinal Chemistry Letters 18 (2008) 2813–2819
Bioorganic & Medicinal Chemistry Letters

Design of (N)-methanocarba adenosine 5′-uronamides as species-independent A_3 receptor-selective agonists

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Received 19 February 2008; revised 31 March 2008; accepted 1 April 2008
Available online 4 April 2008

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 A_3 adenosine receptor (AR). However, the selectivity in mouse was greatly reduced due to an increased tolerance of this ring system at the mouse A_1AR. Therefore, we varied substituents at the N^6 and C2 positions in search of compounds that have improved A_3AR selectivity and are species independent. An N^6-methyl analogue was balanced in affinity at mouse A_1/A_3ARs, 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 A_3AR 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 A_3AR selectivity more effectively than similar chains attached at the 3-position of the N^6-benzyl group.
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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—A_1, A_{2A}, A_{2B}, and A_3—which are widely but differentially distributed throughout the body.[3] The A_3 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 A_3AR is coupled to inhibition of adenylate cyclase and also activates Akt and calcium mobilization.[8,9] Two potent and selective agonists of the A_3AR, IB-MECA **1a** and Cl-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 A_3AR 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 inflammatory bowel model.[13] Cardioprotection by selective A_3AR agonists has been extensively explored in various species (Chart 1).[14–16]

Other selective A_3AR agonists have recently been reported, based on the introduction of large substituents at the N^6 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 A_3AR agonists,[17] and 3′-amino substitution is possible.[16a] Carbocyclic nucleosides have also been developed as A_3AR 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 that is required in order to bind to and activate the receptor,

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; Functionalized congener; Carbocylic.

Keywords: Nucleoside; G protein-coupled receptor; Mouse; Adenosine receptor; Radioligand binding.

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0960-894X/$ - see front matter Published by Elsevier Ltd.
doi:10.1016/j.bmcl.2008.04.001

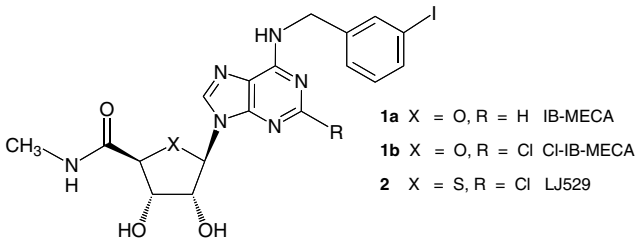


Chart 1. Prototypical selective A_3AR agonists.

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 A_3AR is maintained in combination with various structural changes at the N^6 and C2 positions, which modulate the AR selectivity profile and might otherwise reduce the A_3AR efficacy. The conformational flexibility and H-bonding in the region of the 5′-uronamide are critical factors affecting the efficacy at the A_3AR, 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 A_3AR agonists are much less selective at the A_3AR 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 A_3AR agonists displaying high affinity and selectivity that are independent of species are desired. We previously explored the SAR surrounding both N^6 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 A_3AR agonists.[22] There is a considerable evidence that appropriate modification of the N^6 and C2 positions of nucleoside agonists is tolerated at the human A_3AR, 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 A_3AR, 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 A_3AR agonists, after the substitution of the chlorine at C6 in the purine ring of **20** and **21** with a corresponding primary amine R^1NH_2, 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 coupling[26] with the corresponding alkynes followed by hydrolysis to afford agonists **15**–**17**. Agonist **14** was prepared from agonist **15** through desilylation with tetrabutylammonium fluoride. An N^6-methoxy derivative **19**, based on a recent report by Volpini et al. showing high potency at the human A_3AR of similar derivatives in the ribose series,[18] was prepared by the substitution of the 6-Cl of **20** with *O*-methylhydroxylamine followed by the 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 Sonogoshira reaction sequence using methyl hexynoate in combination with an iodo-derivatized nucleoside, either a 2-iodo (Scheme 1) or N^6-(3-iodobenzyl) (Scheme 2) derivative, provided a mixture of the product methyl ester and the corresponding carboxylic acid, which were separated by silica gel column chromatography. The primary amine congeners **18b** and **26** were prepared by the treatment of the appropriate methyl ester with excess ethylenediamine.

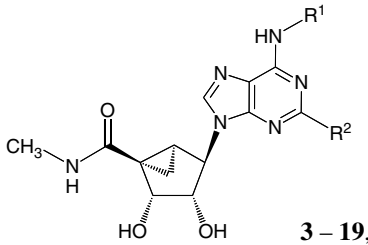
Binding assays were carried out using standard radioligands in Chinese hamster ovary (CHO) cells expressing the human A_1 or A_3ARs and in HEK293 cells expressing the human A_{2A}AR.[22] Also, the mouse ARs were expressed in HEK293 cells for binding assays.[15] A functional assay in guanine nucleotide binding ($[^{35}S]GTP\gamma S$)[20,31] in the membranes of CHO cells expressing the human A_3AR showed that **18a** is a full agonist. The pEC_{50} value of **18a** was 7.85 ± 0.15, in comparison to the pEC_{50} value of NECA of 6.46 ± 0.13.

The binding affinity at the mouse and rat A_3ARs of the N^6-methyl derivative **3** was considerably weaker than at the human A_3AR (Table 1). Thus, this compound was balanced in affinity at mouse A_1/A_3ARs, 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]

N^6-Benzyl derivatives of adenosine have previously been shown to favor selectivity at the A_3AR.[24] This observation led to the design of **1a** and **1b**. Although these two 9-riboside derivatives maintain selectivity for the mouse A_3AR, the selectivity in mouse of similar N^6-substituted benzyl (N)-methanocarba derivatives **4**–**6** was greatly reduced due to increased tolerance of this bicyclic ring system at the mouse A_1AR. Therefore, we varied substituents at the N^6 and C2 positions in an effort to reduce affinity at the mouse A_1AR. Extension of the 3-benzyl group as an alkyne in **7** reduced mouse A_1 and A_{2A}AR affinity without greatly reducing affinity at the mouse, rat, or human A_3ARs, resulting in a 57-fold selectivity for the mouse A_3AR. A 2,5-dimethoxy substitution in **8** showed only a slight enhancement of mouse A_3AR selectivity in comparison to **6**, the (N)-methanocarba equivalent of Cl-IB-MECA. Modified N^6-phenylethyl analogues **9** and **10**, although both very potent at the mouse A_3AR, were essentially nonselective in comparison to the mouse A_1AR.

Modification at the adenine C2 position was generally more beneficial than structural changes of the N^6

Table 1. Affinity of a series of (N)-methanocarba adenosine derivatives at three subtypes of ARs in various species



3 – 19, 25 – 27

Compound	R^1	R^2	Species	Affinity			Selectivity
				A_1[a] K_i (nM)	A_{2A}[a] K_i (nM) (or % inhib.)	A_3[a] K_i (nM)	A_1/A_3
1a			M[b,c]	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[b,c]	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 ± 3200	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 ± 1700	1.49 ± 0.46	10.3
			H[c,d]	260	2300	0.29	900
			R[d]	ND	ND	1.0	
5	3-Br–Bn	Cl	M	8.79 ± 0.12	6390 ± 870	0.90 ± 0.22	9.8
			H[c,d]	270	1300	0.38	710
			R[c]	ND	ND	0.76	
6[h]	3-I–Bn	Cl	M	7.32 ± 1.5	5350 ± 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_2OH)–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 ± 5700	1.72 ± 0.04	17
			H[c,d]	1600	~10,000	1.4	1100
			R[d]	ND	ND	0.87	
9	$CH_2CH(Ph)_2$	Cl	M	6.83 ± 1.5	1810 ± 581	1.67 ± 0.09	4.1
			H[c,d]	1300 ± 100	1600 ± 100	0.69 ± 0.02	1900
			R[d]	ND	ND	10 ± 4	
10	c-Pr–Ph	Cl	M	6.60 ± 1.3	38,200 ± 5300	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%)[i]	1.19 ± 0.09	83
			H[d]	610	~10,000	1.5	410
12[h]	3-Cl–Bn	I	M	210 ± 34.4	(40%)[i]	1.18 ± 0.11	178
			H[d]	2200	>10,000	3.6	610
			R[d]	ND	ND	3.9	

(*continued on next page*)

Table 1 (*continued*)

Compound	R^1	R^2	Species	Affinity			Selectivity
				A_1[a] K_i (nM)	A_{2A}[a] K_i (nM) (or % inhib.)	A_3[a] K_i (nM)	A_1/A_3
13[h]	2,5-$(OCH_3)_2$Bn	I	M	293 ± 29	(14%)[i]	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%)[i]	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%)[i]	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%)[i]	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%)[i]	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%)[i]	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–$(CH_2)_2NH_2$	M	546 ± 62	(31%)[i]	8.60 ± 1.02	64
			H	454 ± 44	(81%)[f]	2.17 ± 0.51	209
19	OCH_3	Cl	M	1160 ± 130	(2%)[i]	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%)[i]	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%)[i]	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%)[i]	4.65 ± 0.22	9.8
			H	181 ± 22	(80%)[f]	2.88 ± 0.54	63

Compounds **1a** and **1b** are 9-riboside derivatives (Chart 1).

[a] Competition radioligand binding assays using [^{125}I]N^6-(4-amino-3-iodobenzyl)adenosine-5′-*N*-methyl-uronamide (A_1 and A_3ARs) and [^{3}H]2-[p-(2-carboxyethyl)phenyl-ethylamino]-5′-*N*-ethylcarboxamidoadenosine (A_{2A}AR) were conducted with membranes prepared from HEK293 cells expressing recombinant mouse A_1, A_{2A}, or A_3ARs. At rat and human ARs, the A_1 radioligand was either [^{3}H] R-phenylisopropyladenosine or [^{3}H]2-chloro-N^6-cyclopentyladenosine. Values are expressed as means ± SEM. ND, not determined.

[b] Data from Ge et al.[15]

[c] EC_{50} 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]

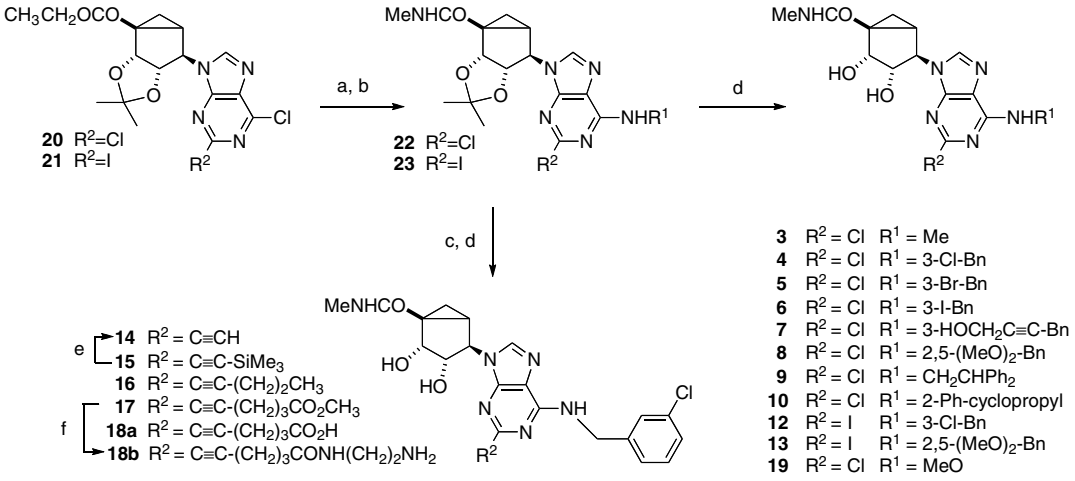
[e] Data from Kim et al.[24]

[f] Percent inhibition at 10 μM.

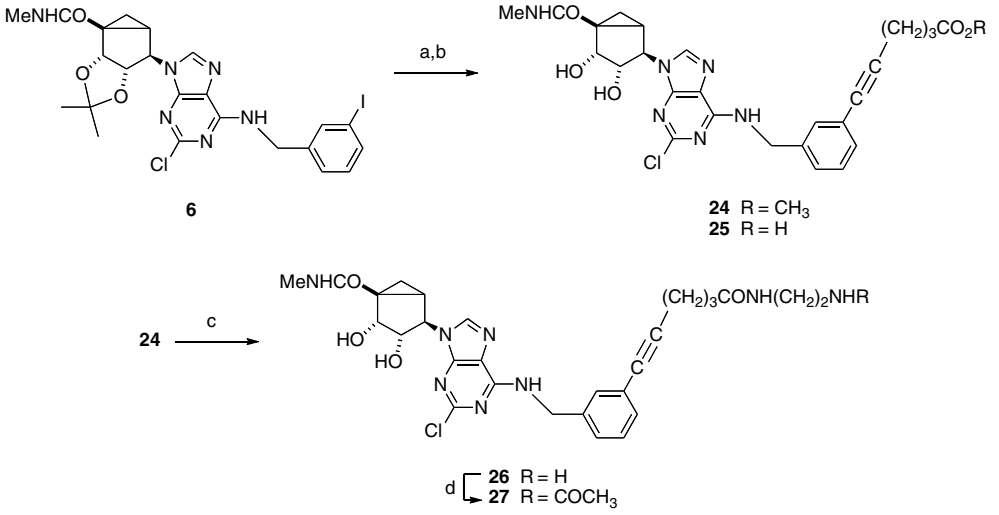
[g] Data from Lee et al.[21]

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

[i] Percent inhibition at 100 μM.



Scheme 1. Synthesis of novel (N)-methanocarba A_3AR agonists with structural variation at the 2 and N^6 positions. Note that **17** and **18a** were both isolated chromatographically from the same reaction. Reagents and condition: (a) R^1NH_2; (b) $MeNH_2$, EtOH; (c) RC≡CH, $PdCl_2(PPh_3)_2$, CuI, DMF, Et_3N; (d) TFA, MeOH, H_2O, Δ; (e) TBAF, THF; (f) ethylenediamine, MeOH.



Scheme 2. Synthesis of novel (N)-methanocarba A_3AR agonists containing functionalized alkynyl chains attached at the N^6-benzyl 3-position. Note that **24** and **25** were both isolated chromatographically from the same reaction. Reagents and condition: (a) $HC{\equiv}C(CH_2)_3COOCH_3$, $PdCl_2(PPh_3)_2$, CuI, DMF, Et_3N; (b) TFA, H_2O, MeOH, Δ; (c) ethylenediamine; (d) acetic anhydride.

substituent, with respect to mouse A_3AR selectivity. 2-Cl was replaced with small hydrophobic groups,[22] which greatly increased the selectivity for the mouse A_3AR. For example, a 2-iodo analogue **12** was 178-fold and >180,000-fold selective in binding to the mouse A_3AR in comparison to mouse A_1 and $A_{2A}AR$, respectively, with a K_i value of 1.18 nM. The corresponding 2-iodo-N^6-(2,5-dimethoxybenzyl) analogue **13** was similarly selective. Thus, the 2-iodo modification resulted in increased selectivity for the mouse A_3AR; that is, 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- to 17-fold. Other hydrophobic substituents at the C2 position, such as an ethynyl group in **14** and its trimethylsilyl adduct **15**, provided moderate mouse A_3AR selectivity. Flexible, extended ethynyl chains in **16** and **17** increased the ratio of A_3AR selectivity. 2-Alkynyl groups were previously reported to enhance the affinity of adenosine derivatives at the rat and human A_3ARs.[25,30] Compound **18a**, a long chain carboxylic acid congener, and its corresponding methyl ester **17** were highly selective for the mouse A_3AR in comparison to the A_1AR by 431- and 288-fold, respectively. Both compounds had greater A_3AR selectivity than Cl-IB-MECA **1b**, although these were less potent. A primary amino congener **18b** displayed moderate A_3AR selectivity, with a K_i value of 8.6 nM. Functionalized congeners in which a functionalized ethynyl chain was positioned on the N^6-benzyl moiety were only moderately (a carboxylic acid **25**) or weakly (a primary amine **26** and its acetyl analogue **27**) selective for the mouse A_3AR. Thus, the attachment of alkynyl chains at the 3-position of the N^6-benzyl moiety did not preserve A_3AR 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 N^6-benzyl group, to serve as a covalent linking site for conjugation, was shown previously to maintain A_3AR affinity in the series of 9-ribosides.[28,29] In the present study of the (N)-methanacarba series, a variety of adenosine derivatives bearing a 2-alkynyl group have been shown to bind potently and selectively to the A_3AR.

The (N)-methanacarba derivatives that were most potent (K_i 1–6 nM) and selective (180- to 290-fold) in binding to the mouse A_3AR 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 A_3AR; however, the affinity was intermediate, with a K_i value of 24 nM. The selectivity for the human A_3AR was >6000-fold. Thus, these C2 position-modified bicyclic nucleosides are good candidates for species-independent A_3AR agonists.

In conclusion, the selectivity (but not affinity) of (N)-methanocarba-containing nucleosides as A_3AR agonists was greatly reduced in the mouse due to increased tolerance of this ring system at the mouse A_1AR. Several analogues having varied substitution at the N^6 and C2 positions were balanced in affinity at mouse A_1/A_3ARs, 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 A_3AR 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 the retention of biological activity and receptor selectivity. Thus, we have identified novel (N)-methanocarba nucleosides that are A_3AR-selective across several species and are especially suitable for pharmacological studies in the mouse.

Acknowledgments

This research was supported in part by the Intramural Research Program of the NIH, National Institute of Diabetes and Digestive and Kidney Diseases (K.A.J.) and by NIH R01 HL077707 (J.A.A.). We thank CanFite Biopharma (Petah-Tikva, Israel) for financial support.

Supplementary data

Supplementary data (chemical synthesis, additional pharmacological procedures, and functional assay of **18a**) associated with this article can be found, in the online version, at doi:10.1016/j.bmcl.2008.04.001.

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