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LETTER

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Truncated (N)-Methanocarba Nucleosides as A_1 Adenosine Receptor Agonists and Partial Agonists: Overcoming Lack of a Recognition Element

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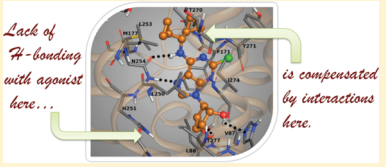
Supporting Information

ABSTRACT: A_1 adenosine receptor (AR) agonists are neuroprotective, cardioprotective, and anxiolytic. (N)-Methanocarba adenine nucleosides designed to bind to human A_1AR were truncated to eliminate 5′-CH_2OH. This modification previously converted A_3AR agonists into antagonists, but the comparable effect at A_1AR is unknown. In comparison to ribosides, affinity at the A_1AR was less well preserved than that at the A_3AR, although a few derivatives were moderately A_1AR selective, notably full agonist **21** (N^6-dicyclopropylmethyl, K_i 47.9 nM). Thus, at the A_1AR, recognition elements for nucleoside binding depend more on 5′ region interactions, and in their absence, A_3AR selectivity predominates. Based on the recently reported agonist-bound AR structure, this difference between subtypes likely correlates with an essential His residue in transmembrane domain 6 of A_1 but not A_3AR. The derivatives ranged from partial to full agonists in A_1AR-mediated adenylate cyclase inhibition. Truncated derivatives have more druglike physical properties than other A_1AR agonists; this approach is appealing for preclinical development.



KEYWORDS: G protein-coupled receptor, purines, molecular modeling, radioligand binding, adenylate cyclase

Adenosine modulates many physiological processes by activating one or more of four subtypes of G protein-coupled receptors (GPCRs).[1] The medicinal chemistry of adenosine receptors (ARs) is now well advanced in comparison to the cases of many other GPCRs, with the existence of numerous selective agonists and antagonists, allosteric modulators, prodrugs, radioligands for imaging, fluorescent probes, and macromolecular ligand conjugates.[2] A_1AR ligands have been considered clinically for a variety of conditions: agonists (diabetes, pain), partial agonists (arrhythmias), and antagonists (heart failure, renal protection).[3]

The adenosine structure has been extensively modified, on both the nucleobase and ribose, for pharmacological optimization to selectively activate ARs. One means of achieving AR subtype selectivity has been the replacement of ribose with a sterically constrained methanocarba ([3.1.0]-bicyclohexane) ring system. This bicyclic system adopts a North (N)-envelope conformation in MRS3558 (**1**) (Chart 1) to maintain a receptor-preferred conformation and enhanced affinity at the A_3AR.[6] The (N)-methanocarba modification is also tolerated at A_1AR, but without affinity enhancement. Consequently, the cardioprotective MRS3630 (**2**) containing the A_1AR-favoring N^6-cyclopentyl substituent is a mixed A_1/A_3AR agonist.[7]

Another useful modification is truncation of the ribose at the 4′ position, that is, removing the 5′-CH_2OH moiety, while retaining all other features of the ribose-like moiety and its stereochemistry. Thus, 4′-thionucleoside antagonist LJ-1251 (**3**) and 4′-oxo antagonist **4** preserve affinity and selectivity at the A_3AR, while removing the ability to induce the required conformational change for receptor activation.[8,9] Truncation of (N)-methanocarba nucleosides originally was reported to convert A_3AR agonists into selective antagonists in a guanine nucleotide binding assay.[10] Subsequently, partial agonism in a functional assay of adenosine 3′,5′-cyclic phosphate (cyclic AMP) at the G_i-coupled A_3AR was shown for MRS5127 (**5**) and congeners.[11]

In contrast to the reduced A_3AR efficacy of 5′-truncated nucleosides, at the G_s-coupled A_{2A}AR, full agonism is retained, as shown recently for the 4′-thio series.[12] The effects of truncation on A_1AR efficacy are unknown. Our major objective was to probe the effects of truncated (N)-methanocarba nucleosides at the human (h) A_1AR, both pharmacologically and with insight into the structural basis for receptor recognition. Therefore, we have incorporated N^6 substituents that are expected to promote A_1AR affinity. For example, nucleoside **6**, previously characterized at the A_3AR,[12] contains N^6-cyclopentyl, which generally produces A_1AR selectivity in the riboside series. The G_i-coupled A_1AR is more homologous in primary sequence and effector coupling to the G_i-coupled A_3AR than to the G_s-coupled A_{2A}AR. If it more closely resembles A_3AR in ligand binding and activation mechanism, the truncated analogues such as **6** will be A_1AR antagonists or partial agonists. However, if its activation more closely resembles the A_{2A}AR, then these truncated derivatives will be full A_1AR agonists. With the recent structural elucidation of an A_{2A}AR active state,[15] it is feasible to relate these findings to specific binding site interactions.

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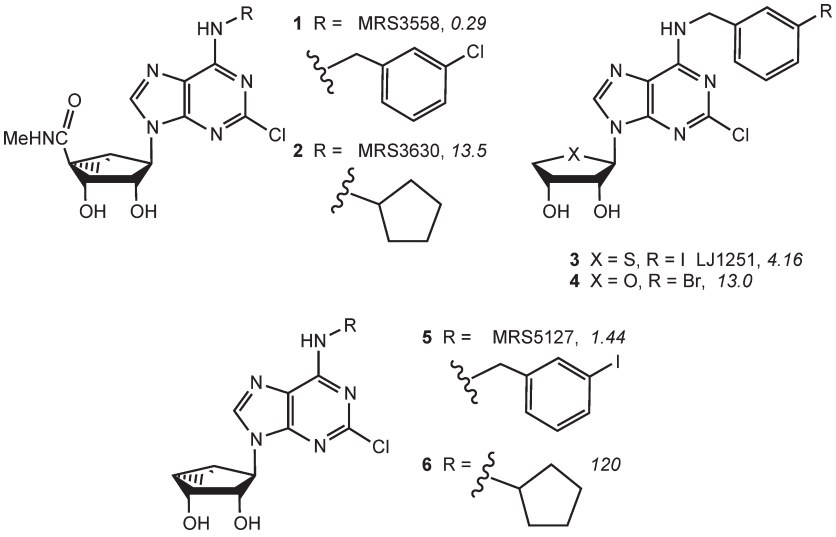
Chart 1. Structures of Representative Ribosides and Ring-Constrained Methanocarba Nucleoside Derivatives That Have Been Characterized as Agonist, Partial Agonists, and Antagonists at the A_3AR (K_i, nM, in Binding to the hA_3AR in Italics)[6−10]



RESULTS

With the objective of increasing A_1AR affinity, we explored N^6 substitution of 4′-truncated (N)-methanocarba nucleoside derivatives, which were shown previously to be selective A_3AR antagonists/partial agonists.[12] Therefore, the series included N^6-cycloalkyl (**8**−**12**) and N^6-bicycloalkyl (**13**,**14**), and N^6-acyclic alkyl and N^6-cyclopropylalkyl (**15**−**21**) substitutions associated previously with A_1AR selectivity of ribosides (Table 1).[13] Finally, certain substituted N^6-benzyladenosine derivatives, such as 2-fluorobenzyl, were reported to have enhanced affinity at the A_1AR.[23] Therefore, a variety of fluorinated and nonfluorinated N^6-benzyl derivatives (**22**−**28**) were prepared.

The synthetic route to the truncated derivatives involves nucleophilic displacement by the appropriate amine of a 6-chloroadenine group in a 2′,3′-isopropylidene-protected precursor **29** (Scheme S1 of the Supporting Information). All of the analogues contain a 2-chloro substitution of the adenine ring, which has been shown to increase affinity at either or both A_1AR and A_3AR. 2-Chloro substitution of the A_1AR agonist N^6-cyclopentyladenosine (CPA, **30**) also was shown to reduce agonist efficacy at the A_3 but not A_1AR.[14]

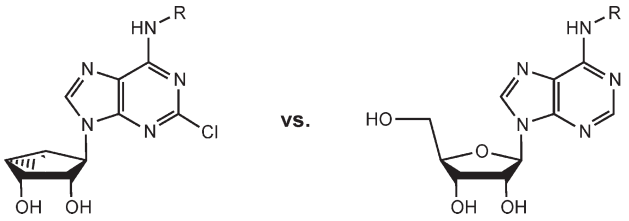
Several previously reported 2-chloro (N)-methanocarba derivatives (**1**, **2**, **5**, and **6**) were used for comparison in the biological assays (Table 1). Binding assays at three hAR subtypes were carried out using standard radioligands and membrane preparations from Chinese hamster ovary (CHO) cells (A_1 and A_3) or human embryonic kidney (HEK) 293 cells (A_{2A}) stably expressing a hAR subtype (Table 1).[12] Since activity within the class of (N)-methanocarba nucleosides was previously noted to be very weak or absent at the hA_{2B}AR,[16] we did not include this receptor in the screening protocol.

Generally, the 5′-truncated (N)-methanocarba-adenosine derivatives, in comparison to the corresponding 9-ribosides, maintained affinity at the A_3AR more effectively than at the A_1AR. Nevertheless, affinities of <100 nM at the A_1AR were achieved for certain N^6-alkyl and cycloalkyl members of this series. Among the most potently binding 2-chloro (N)-methanocarba analogues (K_i, in nM) at the hA_1AR (with K_i of the corresponding N^6 derivatized 9-ribosides at rat A_1AR in parentheses[13]) are the following: cyclobutyl **9**, 51.6 (0.7); isopropyl **17**, 72.2 (1.9); cyclopropylmethyl **19**, 68.4 (0.8); and di(cyclopropyl)methyl **21**, 47.9 (0.8). The A_1AR affinity of the N^6-ethyl analogue **15** was more substantially reduced, by 141-fold, with K_i values of 930 and 6.6 nM for the truncated and 9-riboside analogues, respectively. Other N^6 analogues that were more significantly reduced in their hA_1AR affinity in comparison to the corresponding riboside at rat A_1AR (K_i, nM) are as follows: cyclohexyl **10**, 131-fold (0.9); *endo*-norbornyl **13**, 113-fold (0.34); *exo*-norbornyl **14**, 231-fold (0.7); and 2-fluorobenzyl **22**, 800-fold (6). Because of this reduced affinity, the selectivity for the A_1AR was diminished overall. Only a few derivatives tended toward A_1AR selectivity in comparison to the A_3AR: di(cyclopropyl)-methyl **21**, 10-fold; *endo*-norbornyl **13**, 4-fold; and large cycloalkyl derivatives **10**−**12**, 2−3-fold. The degree of selectivity vs A_{2A}AR was higher: **21**, 74-fold; **13**, 30-fold. Many of the other derivatives were equipotent in binding to A_1 and A_3ARs, and the substituted N^6-benzyladenosine derivatives (**22**−**28**) were generally selective for the A_3AR, similar to previous observations with truncated N^6-benzyl derivatives.[10,12]

Functional data determined at a single concentration (10 μM) in an assay of adenylate cyclase (A_1AR-induced inhibition of cyclic AMP) are reported in Table 1. The potent and selective agonist CPA was used as the standard full agonist, and the nonselective AR agonist 5′-*N*-ethylcarboxamidoadenosine (NECA) was also a full agonist in this assay. Most of the analogues were partial agonists of the A_1AR. However, concentration−response curve for dicyclopropylmethyl analogue **21** in A_1AR-mediated inhibition of cyclic AMP indicated full agonism compared to NECA (Figure S1 of the Supporting Information). The EC_{50} values for **21** and NECA were 40.7 ± 19.7 and 10.2 ± 3.3 nM, respectively, in close agreement with the A_1AR binding affinities.

A correlation plot of the hA_3AR affinity of truncated 2-chloro-(N)-methanocarba analogues in the present study

(x-axis) vs the hA$_3$AR affinity of 2-unsubstituted adenine-7-riboside analogues (y-axis)[13]



demonstrated a parallel in these parameters (Figure 1B). The affinity at the A$_3$AR subtype was relatively well maintained (correlation coefficient of 0.55). The enhanced A$_3$AR affinity of a 5′-truncated ribonucleoside derivative was first noted for the 2-chloro-N^6-(3-iodobenzyl) derivative[16] and has been validated consistently in SAR studies that also showed lowered efficacy in this series.[8−10] However, a similar plot of A$_1$AR affinity comparing ribonucleosides and truncated ring-constrained nucleosides illustrated the trend of consistently lower affinity with truncation (Figure 1A), but without correlation of K_i values (correlation coefficient of 0.33). Therefore, in comparison to the case of ribosides, the affinity of the truncated ring-constrained analogues at the A$_1$AR was less well preserved than that at the A$_3$AR.

One derivative that tended toward A$_1$AR-selectivity, the full agonist **21**, contained a N^6-dicyclopropylmethyl group. Curiously, truncated analogues of N^6-cyclopentyl and N^6-benzyl derivatives were notably reduced in A$_1$AR affinity, even in the case of a 2-fluoro analogue **22**, a modification previously found to enhance A$_1$AR selectivity in the riboside series.[13]

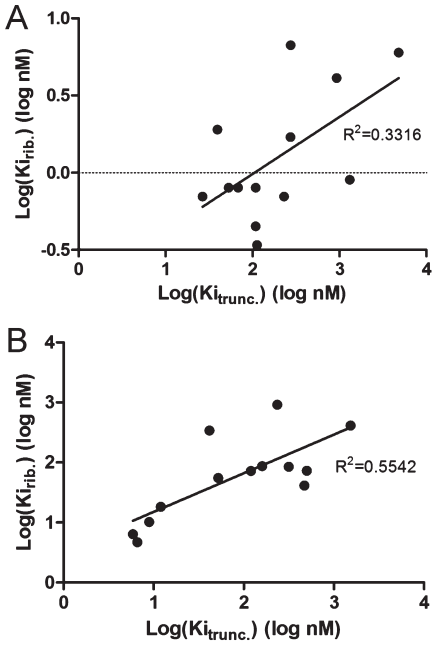


Figure 1. (A) Correlation of the affinity of truncated 2-chloro-(N)-methanocarba analogues (x-axis, at the hA$_1$AR) and 2-unsubstituted adenine-7-riboside analogues (y-axis, at the rat A$_1$AR).[13] (B) Correlation of the hA$_3$AR affinity of truncated 2-chloro-(N)-methanocarba analogues (x-axis) and 2-unsubstituted adenine-7-riboside analogues (y-axis).[13]

Compound **22** was 115-fold selective for the A$_3$AR in comparison to the A$_1$AR. A 2-fluoroethyl ether **23** was a potent and selective ligand at the A$_3$AR (K_i 10.3 nM), which might be sufficient affinity for use in radiofluorination for positron emission tomography.

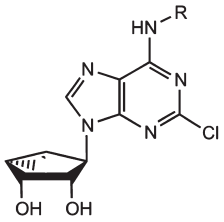
There is a general phenomenon of reduced AR efficacy by removing H-bonding groups from the ribose moiety, especially at the A$_3$AR but also at the A$_1$AR.[5,16] A precedent for reduced A$_1$AR efficacy in nucleoside derivatives is the series of xanthine 7-ribosides. The 5′-uronamide analogue 5′-N-methyl 1,3-dibutylxanthine 7-β-D-ribofuronamide (DBXRM) was a full agonist at both A$_1$ and A$_3$ARs. However, removal of the 3′-hydroxyl group produced antagonism at the rat adipocytes A$_1$AR and partial agonism at the rat A$_3$AR. In examining the question of whether 5′-truncation in the present series of rigid (N)-methanocarba analogues provides agonists or antagonists at the A$_1$AR, we found that the answer lies in the middle; that is, the agonist efficacy is partially maintained for many of the derivatives, as shown in Table 1, but the pattern is not uniform.

To examine docking of truncated (N)-methanocarba nucleosides, homology models of the hA$_1$AR and hA$_3$AR were produced from the agonist-bound A$_{2A}$AR X-ray structure (3QAK).[15] It was noted that key interactions of the nucleoside ligand with His251 in transmembrane domain (TM) 6, conserved between A$_1$AR and A$_{2A}$AR, and with Thr91 in TM3 are necessarily missing in the truncated derivatives. In the A$_{2A}$AR structure, this essential His residue, which is absent in the A$_3$AR, and Thr91 both H-bonded to a 5′-CO-NH-alkyl moiety of a cocrystallized NECA-like agonist.[15] We predict that the difference in this key His residue is related to the dramatically altered binding of the truncated ring-constrained nucleosides at A$_1$ and A$_3$ARs. Thus, in general, A$_3$AR recognition would depend more on interactions of groups other than the nucleoside 5′ substituent; A$_1$AR recognition depends more on 5′ region interactions, and in their absence, A$_3$AR selectivity predominates. This is consistent with the truncated derivatives maintaining A$_3$AR, but not A$_1$AR, affinity.

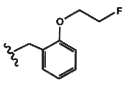
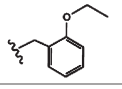
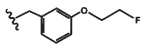
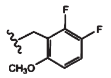
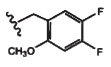
Furthermore, the hydrophilic region of the receptor associated with ribose binding is critical for activation. This step likely involves essential residues of TM3, TM6, and TM7 throughout the AR family, as in the A$_{2A}$AR.[15] Multiple H-bonding groups in this region promote agonism at the A$_3$AR,[14] such as the interactions of the 5′-CH$_2$OH of the ribose moiety or the corresponding 5′-CO-NH-alkyl in NECA-like analogues. Thus, loss of the 5′ substituent is expected to reduce AR efficacy, which it clearly does at the A$_3$AR. However, the effect at the A$_1$AR is highly variable, with relative maximal efficacies of the truncated (N)-methanocarba analogues ranging from low (20−30% in compounds **14**, **16**, and **18**) to high (80−100% in compounds **8**, **19**, and **21**). The structural basis for the full agonism of **21**, in contrast to closely related compounds such as the N^6-(3-pentyl) derivative **18**, which has greatly reduced efficacy at this subtype, must arise from conformational effects of the N^6 group.

The docking poses of nonselective agonist NECA, full agonist **21**, and low efficacy partial agonist **16** in the putative binding site of the A$_1$AR were compared (Figure 2 and Figure S2 of the Supporting Information). The H-bond interactions between the 5′-CO-NH-ethyl group of NECA and the two residues, His251(6.52) and Thr91(3.36), could lock the ribose

Table 1. Potency of a Series of Truncated (N)-Methanocarba Adenosine Derivatives at Three Subtypes of hARs and Relative Efficacy at hA_1AR



Compd	R =	Affinity K_i, nM or *(% inhibition)*[a]			% Inhibition, cyclic AMP[d]
		A_1	A_{2A}	A_3	A_1
1[b]		260±60	2300±100	0.29±0.04	
2[b]		18.3±6.3	3250±300	13.1±5.1	
5[b]		3040±610	1080±310	1.44±0.60	
7	H	350±90	3140±450	160±42	68.1±4.4
8		210±30	3700±340	12.1±3.4	79.0±18.8
9		51.6±12.6	3020±90	5.9±0.5	46.7±2.1
6[b]		109±16	1640±360	120±31	
10		140±10	2720±450	500±120	38.0±17.6
11		230±30	3930±520	560±90	48.1±10.2
12		760±110	*(43%)*	1530±60	40.1±9.0
13		82.6±15.8	2450±90	315±48	62.1±18.5
14		200±30	4080±170	236±43	27.8±6.2
15	CH_2CH_3	930±110	*(11%)*	6.6±1.6	
16	$(CH_2)_3F$	72.7±28.0	*(29%)*	32.4±6.7	23.2±2.6
17		72.2±16.4	*(39%)*	12±1	50.5±6.4

Compd	R =	Affinity K_i, nM or *(% inhibition)*[a]			% Inhibition, cyclic AMP[d]
		A_1	A_{2A}	A_3	A_1
18		78.8±15.6	3700±300	52±14	28.6±3.8
19		68.4±8.9	4410±1090	8.9±1.9	81.0±21.1
20[c]		86.8±23.7	*(41%)*	110±17	45.5±4.8
21		47.9±10.5	3950±410	470±15	94.3±5.3
22		4790±670	*(31%)*	41.5±1.0	
23		*(46%)*	*(32%)*	10.3±1.5	
24		*(34%)*	*(32%)*	15.2±3.0	
25		3580±220	*(46%)*	114±45	
26		1260±240	*(38%)*	16.5±2.8	
27		*(41%)*	*(30%)*	83.2±35.7	
28		1910±310	7510±690	40.4±13.1	

[a] Using CHO or HEK293 (A_{2A} only) cells stably expressing a hAR (Supporting Information); affinity was expressed as K_i value ($n = 3-5$) or percent inhibition of radioligand binding at 10 μM. [b] Values from refs 6, 7, and 12. **5** and **6** were prepared previously.[12] [c] **20** is a diastereomeric mixture. [d] Maximal efficacy (at 10 μM) in an A_1AR functional assay, determined by inhibition of forskolin-stimulated cyclic AMP production in AR-transfected CHO cells, expressed as percent inhibition (mean ± standard error, $n = 3-5$) in comparison to effect (100%) of full agonist CPA **30** at 10 μM. The value for NECA was 100 ± 15.

moiety in an active conformation, with the 2′,3′-hydroxyl groups of the ribose ring correctly directed toward Thr277-(7.42) and His278(7.43) in order to pull TM7 toward TM3 to efficiently activate the receptor.[15] In the absence of an interaction with His251(6.52) and/or Thr91(3.36) due to the lack of the 5′ substituent, the orientation of the rigid methanocarba moiety could be less effective in forming the H-bond interactions with Thr277(7.42) and His278(7.43) needed to attract TM7. In the case of A_3AR, the activation process could be slightly different from that for the A_1AR, due to some differences in the key residues of the binding pocket. Position 3.32 in A_3AR consists of a nonconserved bulky and hydrophobic leucine residue, while a smaller valine is present in the other AR subtypes. Residue 3.32 was close to the ribose ring of the docked NECA in both A_1AR and A_3AR binding sites. The longer side chain of Leu90(3.32) in A_3AR, in comparison to Val87(3.32) of A_1AR, could contribute to a stronger hydrophobic interaction with the methanocarba moiety of the

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5′-truncated agonists. At the same time, the different nature of the substituents at the N^6 position of the adenine ring could influence the selectivity and the efficacy of agonists at both the A_1AR and the A_3AR. The N^6 groups in the docking poses of the studied agonists lay in a region of the pocket formed by residues in the upper part of TM6 and TM7. The A_1AR and A_3AR differ in the nature of the residues lining this pocket. The variation of the affinity and potency of this series of 5′ truncated methanocarba analogues at the A_1AR indicates that the N^6 substituent can greatly affect these parameters, in some cases compensating for the lack of H-bonding interactions in the ribose 4′ region. From the docking pose of full agonist **21** in the A_1AR model (Figure 2B), the favorable interactions of the N^6-dicyclopropylmethyl substituent and the residues in the upper part of TM6 and TM7 (such as Thr257, Leu253, and Thr270) could maintain the adenine and methanocarba moieties in an efficacious active conformation with strong H-bond and hydrophobic interactions with residues in TM6, TM3, TM7, and EL2. A smaller and more flexible N^6 group, such as the 3-fluoropropyl substituent of **16**, in addition to the lack of interactions with His251(6.52) and Thr91(3.36), could negatively affect the ability to fully activate the receptor through conformational affects originating at the upper pocket of TM6 and TM7 (Figure S3 of the Supporting Information).

The physicochemical properties of nucleosides that act as AR agonists often lead to limited in vivo bioavailability. The C-log p of compound **21** is 1.41, with the optimal for small molecular pharmaceutical substances being typically 2−3.[17] The comparable parameter for the related A_1AR-selective riboside and prototypical agonist CPA is 0.14, which is less desirable. Also, the polar surface area (PSA) values for **21** and CPA are calculated to be 92.8 and 122 Å^2, respectively. Most druglike small molecules have a PSA smaller than 120 Å^2. Compound **21** has fewer hydroxyl groups than CPA, which would favor bioavailability. The molecular weight of **21** of 376 is comfortably within the preferred range. Therefore, by several criteria, the full agonist of the A_1AR **21** is more druglike than CPA.

The bioavailability of peripherally administered **21** in the brain, i.e. whether its altered physicochemical properties may facilitate its passage across the blood brain barrier, is undetermined. Many of the efforts to develop A_1 agonists have attempted to limit central nervous system (CNS) penetration to avoid central mediated side effects, but other envisioned applications of A_1 agonists depend on brain entry. In previous studies of the activity of A_1AR agonists in the CNS, only a small fraction of a peripherally administered agent crossed the blood brain barrier.[4] However, a similar attempt to alter the biodistribution by removing the 2′-hydroxyl group of CPA did not enhance brain uptake.[4]

In conclusion, truncated (N)-methanocarba adenine nucleosides display highly variable degrees of binding affinity and activation at the hA_1AR. Based on the recently reported agonist-bound AR X-ray structure, this difference between subtypes likely correlates with an essential His residue in TM6 of A_1 but not A_3AR. By overcoming the lack of an important recognition element for receptor binding, i.e., the 5′ substituent, a N^6-dicyclopropylmethyl derivative **21** was empirically identified as a moderately A_1AR selective, full agonist. This is counterintuitive given that many 5′-modified analogues are partial agonists at the A_1AR.[18] A_1AR agonists hold interest therapeutically for their cardio- and neuroprotective, antiarrhythmic, antiseizure, antilipolytic, antiglaucoma, and anxiolytic actions. It is conceivable that the expanded range of physical properties in the present series of truncated derivatives would offer pharmacokinetic advantages. Therefore, this approach is appealing for preclinical development. This hypothesis will have to be evaluated in the future in vivo studies.

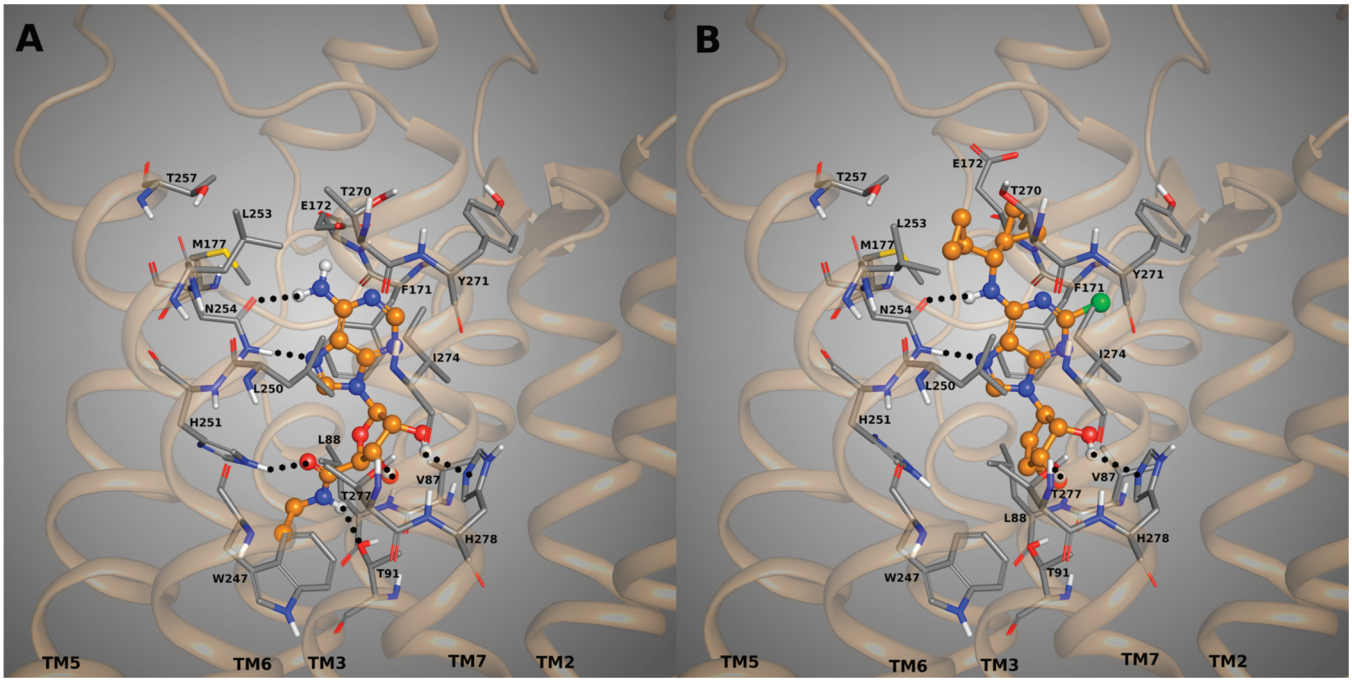

Figure 2. Docking poses of NECA (A) and truncated nucleoside **21** (B) in the binding site of the hA_1AR homology model based on the X-ray structure of an agonist-bound hA_{2A}AR, indicating the main H-bond interactions.

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■ ASSOCIATED CONTENT

Supporting Information. Synthetic procedures for compounds 7−**28**, their characterization and bioassays, and modeling procedures and results. This material is available free of charge via the Internet at http://pubs.acs.org.

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■ ABBREVIATIONS

AR, adenosine receptor; cyclic AMP, adenosine 3′,5′-cyclic phosphate; CPA, N^6-cyclopentyladenosine; CHO, Chinese hamster ovary; GPCR, G protein-coupled receptor; HEK, human embryonic kidney; MRS3558, (1′*S*,2′*R*,3′*S*,4′*R*,5′*S*)-4′-{2-chloro-6-[(3-chlorophenylmethyl)amino]purin-9-yl}-1-(methylaminocarbonyl)-bicyclo[3.1.0]hexane-2,3-diol; MRS3630, (1′*S*,2′*R*,3′*S*,4′*R*,5′*S*)-4-(2-chloro-6-(cyclopentylamino)-9*H*-purin-9-yl)-2,3-dihydroxy-*N*-methylbicyclo[3.1.0]hexane-1-carboxamide; MRS5127, (1′*S*,2′*R*,3′*S*,4′*R*,5′*S*)-4′-[2-chloro-6-(3-iodobenzylamino)-purine]-2′,3′-*O*-dihydroxybicyclo[3.1.0]hexane; NECA, 5′-*N*-ethylcarboxamidoadenosine; TM, transmembrane domain

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■ NOTE ADDED IN PROOF

The X-ray structure of NECA bound to a thermostabilized A_{2A}AR was recently solved (RCSB ID: 2YDV; Lebon et al. **2011**, *Nature*, doi:10.1038/nature10136) and is very similar to our docked pose of NECA.