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Functionalized Congeners of A_3 Adenosine Receptor-Selective Nucleosides Containing a Bicyclo[3.1.0]hexane Ring System†

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Abstract

(N)-Methanocarba nucleosides containing bicyclo[3.1.0]hexane replacement of the ribose ring previously demonstrated selectivity as A_3 adenosine receptor (AR) agonists (5′-uronamides) or antagonists (5′-truncated). Here, these two series were modified in parallel at the adenine C2 position. N^6-3-Chlorobenzyl-5′-*N*-methyluronamides derivatives with functionalized 2-alkynyl chains of varying length terminating in a reactive carboxylate, ester, or amine group were full, potent human A_3AR agonists. Flexibility of chain substitution allowed the conjugation with a fluorescent cyanine dye (Cy5) and biotin, resulting in binding K_i values of 17 and 36 nM, respectively. The distal end of the chain was predicted by homology modeling to bind at the A_3AR extracellular regions. Corresponding L-nucleosides were nearly inactive in AR binding. In the 5′-truncated nucleoside series, 2-Cl analogues were more potent at A_3AR than 2-H and 2-F, functional efficacy in adenylate cyclase inhibition varied, and introduction of a 2-alkynyl chain greatly reduced affinity. SAR parallels between the two series lost stringency at distal positions. The most potent and selective novel compounds were amine congener **15** (K_i = 2.1 nM) and truncated partial agonist **22** (K_i = 4.9 nM).

Introduction

Selective agonist and antagonist ligands of the A_3 adenosine receptor (AR[a]), a G-protein-coupled receptor (GPCR), have been reported.[1] A_3AR antagonists are proposed for the

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[a]Abbreviations: AR, adenosine receptor; cAMP, adenosine 3′,5′-cyclic phosphate; CCPA, 2-chloro-N^6-cyclopentyladenosine; CHO, Chinese hamster ovary; Cl-IB-MECA, 2-chloro-N^6-(3-iodobenzyl)-5′-*N*-methylcarboxamidoadenosine; DMEM, Dulbecco's modified Eagle's medium; EDTA, ethylenediaminetetraacetic acid; GPCR, G-protein-coupled receptor; HATU, 2-(1*H*-7-azabenzotriazol-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate methanaminium; HEK, human embryonic kidney; I-AB-MECA, 2-[*p*-(2-carboxyethyl)phenylethylamino]-5′-*N*-ethylcarboxamidoadenosine; NECA, 5′-*N*-ethylcarboxamidoadenosine; DIPEA, diisopropylethylamine; DCM, dichloromethane; DMF, *N*,*N*-dimethylformamide; HEPES,4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; HRMS, high resolution mass spectroscopy; PET, positron emission tomography; TEA, triethylamine; TLC, thin layer chromatography.

Supporting Information Available: Synthetic procedures for compounds **30, 13–16, 10, 7, 35, 37, 20, 21, 41, 42, 46, 47, 49, 22**, and **25** and their characterization; UV–visible spectra of **17a, 17b**, and **18**; docking view of a reference agonist in the hA3AR homology model. This material is available free of charge via the Internet at http://pubs.acs.org.

treatment of cancer, inflammation, glaucoma, and asthma.[2–5] A_3AR agonists are already in clinical trials for liver cancer, rheumatoid arthritis, psoriasis, and dry eye disease,[6,7] with additional envisioned applications for bowel inflammation, ischemia, and other autoimmune inflammatory disorders.[8–10]

Selective A_3AR antagonists encompass both non-purine heterocyclic antagonists[1,11] and nucleoside[12,13] antagonists. Nucleoside-derived A_3AR antagonists have the advantage of a species-independent pharmacological profile;[4] i.e., they tend to maintain A_3AR selectivity in both human and murine species, while most of the non-purine antagonists are selective for the human (h) but not rat (r) A_3AR. Modification of the ribose moiety of adenosine derivatives, and to a lesser extent N^6 and C2 substituents, has been shown to reduce agonist efficacy and thus be useful for converting A_3AR agonists into antagonists.[12,13]

Both the binding and selectivity of adenosine derivatives as prototypical A_3AR agonists can be substantially increased by replacing the flexible ribose scaffold with a rigid bicyclo[3.1.0] hexane ring system resulting in a class of carbocyclic nucleosides related to **1** (Chart 1).[14–16] These (N)-methanocarba adenosine agonists have been found to possess high potency and enhanced selectivity for the A_3AR,[11] and an agonist of this class (MRS3558, **1a**) has been shown to display antiarthritic activity and protection in a model of lung ischemia.[17,18] The N^6-3-iodobenzyl derivative **1b** (MRS1898) was recently radioiodinated to form an A_3AR agonist radioligand that can be used selectively in murine and in hA_3AR binding studies.[19]

We describe in this work two new series of selective A_3AR ligands that extend recent reports, i.e., agonists and antagonists of the (N)-methanocarba family, derived through largely parallel modifications at the adenine C2 position.[20,21] Thus, the new agonists contained a 5′-*N*-methyluronamide group, and this amide group was removed entirely in the truncated series. The principle of lowering the relative efficacy of full agonists by removing a flexible 5′-amide group to produce, in some cases, hA_3AR antagonists was shown for 4′-thionucleosides by Jeong et al. and for (N)-methanocarba analogues by Melman et al. (i.e., **2a–c**).[13,20] The stereospecificity of binding to the ARs was also probed with the preparation of corresponding L-nucleosides. In both series, the conformationally restricted (N)-methanocarba scaffold was retained, and most of the analogues contained a substituted 6-(benzylamino) moiety. The most structurally diverse modifications were made at the adenine C2 position, with the addition of extended alkynyl chains terminating in a carboxylic, ester, or amino group, following a functionalized congener approach.[22,23] This terminal functionalized group was remote from the minimal pharmacophore required for recognition at the primary binding site of the receptor. The intended application of the functional groups is for covalent coupling to various other moieties, including spectroscopic reporter groups, without losing biological activity.

Results

Chemical Synthesis

In this study, we have elaborated and extended functionalization at the C2 position of previously reported (N)-methanocarba nucleoside derivatives, which were shown to be selective A_3AR agonists[15,16,21] and antagonists.[20] The series of 5′-*N*-methyluronamides (Chart 1, **II**) are similar to the reported agonists **1**, and the series of truncated nucleosides (**I**) are similar to the reported antagonists **2**. Here, these two series were modified in parallel at the adenine C2 position.

The synthetic route to the 5′-*N*-methyluronamide (*N*)-methanocarba derivatives **5–16** is shown in Scheme 1A. The synthesis of the 2′,3′-protected intermediate **27**, containing an N^6-(3-chlorobenzyl) group, from L-ribose was previously reported.[24] This 2-iodo intermediate

was then subjected to Sonogashira coupling[25] with the corresponding alkyne esters. The products **28–30** of varying alkynyl chain length were deprotected to liberate the 2′,3′-hydroxyl groups to provide nucleosides **8–10**, followed by hydrolysis of the methyl ester to afford agonists **5–7**. Alternatively, compounds **28–30** were aminolyzed using the appropriate alkyldiamine in excess, and the product amines were deprotected to yield the amine functionalized agonists **11–16** of varying chain length.

The amino and carboxylic derivatives contained chemically functionalized, extended alkynyl chains to serve as functionalized congeners for conjugation to other biologically active moieties or to carriers. [23,26] Several representative biologically active conjugates for the detection and characterization of the A_3AR were prepared as shown in Scheme 1B. Biotin conjugates of varying length **17a** and **17b** were synthesized from the amine **11**, which was the analogue having the shortest chain in the homologous series of amine congeners. The biotin conjugate **17a** was prepared by a HATU coupling with biotin, and the extended chain analogue **17b** was prepared from the corresponding biotin-*ε*-aminocaproyl active ester. The fluorescent derivative **18** similarly was prepared by treating **11** with the active ester of a chain-derivatized Cy5 cyanine dye.[27]

Selected L-enantiomers **19–21** of the 5′-*N*-methyluronamide methanocarba agonist series were also prepared, as shown in Scheme 2. The isopropylidene intermediate **31** was prepared from D-ribose as previously reported. A Mitsunobu condensation[21] with 2-iodo-6-chloropurine provided **32**, which was first converted to the N^6-(3-chlorobenzyl) derivative **33** and then treated with excess methylamine to yield **34**. This 2-iodo intermediate was subjected to a Sonogashira coupling[25] with the corresponding alkyne esters to provide compounds **35–37** of varying alkynyl chain length. Esters **35** and **37** were aminolyzed with ethylenediamine followed by hydrolysis of the acetonide group with 10% TFA to provide compounds **20** and **21**. In the case of compound **36**, the 2′,3′-isopropylidene group was first deprotected, and hydrolysis of the ester afforded the carboxylic acid derivative **19**.

Scheme 3 shows the new synthetic route used to prepare the truncated (N)-methanocarba derivatives **22–26**. While the previous study of truncated (N)-methanocarba nucleosides as antagonists of the hA_3AR depended on a low yielding decarboxyation step, an alternative simple synthetic approach was designed. The protected cyclopentenone **38** was stereoselectively reduced to the corresponding alcohol **39**,[28] which was then subjected to cyclopropanation to yield the glycosyl donor **40** for the truncated nucleosides and therefore converged on the previously reported route.[20] Compound **40** was subjected to a Mitsunobu condensation[20] with the appropriate C2 substituted 6-chloropurine derivative to give the 2-H (**41**) and 2-halopurine (**42, 43**) derivatives. The 2-1 derivative **44** was prepared in a similar manner and was substituted with 3-chlorobenzylamine at the 6 position to give **45**, which serves as a precursor for 2-alkynyl derivatives in the truncated series. This route afforded the isopropylidene-protected truncated nucleosides **46–49**, which were subsequently deprotected to provide compounds **22–25** for biological testing.

A truncated 2-iodo intermediate **45** was subjected to Sonogashira coupling[25] with methyl pentynate to give compound **50**, which upon amination with ethylenediamine and subsequent acid hydrolysis provided the truncated nucleoside **26** containing an amine functionalized chain.

Pharmacological Activity

Several previously reported 2-chloro-(N)-methanocarba agonists (**1a, 1b, 3, 4, 6, 9**, and **12**) and antagonists (**2a–c**) were used for comparison in the biological assays (Table 1). Binding assays at three hAR subtypes were carried out using standard radioligands[29–31] and membrane preparations from Chinese hamster ovary (CHO) cells (A_1 and A_3) or human

embryonic kidney (HEK293) cells (A_{2A}) stably expressing a hAR subtype (Table 1).[32,33] 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. Functional data were determined in an assay of adenylate cyclase (A_3AR-induced inhibition).[34] Functional data for selected compounds in a functional assay consisting of A_3AR-induced stimulation of guanine nucleotide binding are reported in Table 1.[35]

Compounds **5–15** in the (N)-methanocarba 5′-*N*-methyluronamide series (series A), which had functionalized 2-alkynyl chains terminating in a carboxylic acid, ester, or amino group, were potent A_3AR ligands. These derivatives having charged, polar, and chemically reactive groups at the end of a chain were intended for structural probing of distal regions of the ligand binding site of the receptor and for the design of specialized high-affinity ligands, such as fluorescent probes.

The optimal chain length for A_3AR affinity among carboxylic acid derivatives occurred with the previously reported compound **6**,[21] which had three methylenes in the chain. The corresponding ester derivative **9**[21] was also the most potent in the series of homologous esters **8–10**. A shorter chain carboxylic acid derivative **5** was considerably less potent than other members of the series in binding to the hA_3AR.

The carboxylic acid derivatives were then extended by amide linkage to diaminoalkyl groups. In the series of resulting terminal amino derivatives **11–13**, the length of the carboxyl alkyl chain was varied, and in the series of **11** and **14** and **15** the length of the diaminoalkyl chain was varied. While **12** displayed an atypically high A $_1$AR affinity (K_i = 454 nM) and **13** an atypically high $A_{2A}AR$ affinity (K_i = 270 nM), most of the members of these series were highly selective for the A_3AR. The K_i values at the hA_3AR of the primary amino derivatives **11–15** of varying chain length were in the range of 2–3 nM. The most potent and selective novel member of this series, an amine derivative **15** (Figure 1, Table 1), was 360 and > 5000-fold selective in binding to the hA_3AR in comparison to hA_1 and $A_{2A}ARs$, respectively. Compound **16**, containing two free amino groups in the chain, one primary and another secondary, was slightly reduced in affinity at the A_3AR with a K_i value of 15.4 nM.

As functionalized congeners, these long chain derivatives were intended for conjugation to carrier moieties for receptor detection and characterization, in a manner that did not prevent receptor recognition.[22,23] A biotin conjugate **17b** derived from amine **11** in the series of 5′-*N*-methyluronamide derivatives displayed a K_i value at the hA_3AR of 58 nM (Figure 1) and selectivity of > 170-fold and 66-fold in comparison to the A_1 and $A_{2A}AR$, respectively. A shorter analogue **17a** lacking the ε-aminocaproyl spacer chain was slightly more potent in hA_3AR binding with a K_i value of 36 nM. A fluorescent conjugate **18** containing the sulfonated carbocyanine dye Cy5[27] was potent and selective in A_3AR binding. The fluorescent excitation and emission maxima of **18** in aqueous medium, 646 and 660 nm, respectively, were characteristic of Cy5 dye conjugates.

Various compounds were examined for the ability to inhibit the production of adenosine 3′, 5′-cyclic phosphate (cAMP) in membranes of CHO cells expressing the hA_3AR (Table 1).[34] Inhibition by 10 μM NECA (**51**, 5′-*N*-ethylcarboxamidoadenosine) was set at 100% relative efficacy. The (N)-methanocarba 5′-*N*-methyluronamide derivatives (series A) were consistently full agonists. Full concentration response curves for compounds **17a** and **18** provided IC_{50} values in hA_3AR-mediated inhibition of adenylate cyclase of 2.50 ± 0.42 and 1.09 ± 0.28 nM, respectively (Figure 2A). Thus, this series provided many new potent and selective A_3AR agonists, and alkynyl chain derivatization at the C2 position appears to be an

effective strategy for covalent attaching sterically bulky groups while maintaining A_3AR recognition and activation.

A series of L-nucleosides was prepared as enantiomers of members of the potent agonist functionalized congeners (series B). Compounds **19**, **20**, and **21** were the enantiomers of the potent A_3AR agonists **5**, **11**, and **13**, respectively. We confirmed that at submicromolar concentrations no substantial AR binding activity was seen with these L-enantiomers, confirming stereoselectivity of binding at the ARs. At 10 μM, **19–21** were weak or inactive in binding at the A_1 and A_{2A}ARs, and at the A_3AR they tended to be slightly more potent in binding. These L-nucleosides induced partial activation of the A_3AR at 10 μM, as confirmed in the cAMP functional assay in which compounds **20** and **21** activated to the degree of 26% and 52%, respectively. Nevertheless, these L-nucleosides can be used at nanomolar concentrations in pharmacological studies as inactive control compounds of similar physicochemical properties, by analogy to the use of inactive enantiomers of other GPCRs.[36] At these concentrations, interactions with ARs were lacking. In the truncated 4′-thionucleoside class of A_3AR antagonists, stereospecificity of binding was similarly demonstrated.[45]

The effects of modification at the C2 position were explored in the truncated series C. In the 5′-*N*-methyluronamide derivatives (series A), even the presence of a long chain at the C2 position did not interfere with the binding and activation of the A_3AR. However, in the series of nucleosides that were truncated at the 4′-carbon, variability in both binding affinity and efficacy at the hA_3AR was observed, depending on the nature of the C2 substitution. Compounds **22–24**, lacking a functionalized chain at the C2 position, were potent and selective A_3AR ligands with binding K_i values of 5–11 nM. Removal of the 2-C1 substitution in N^6-benzyl-type 4′-truncated derivatives (e.g., **22**, **23**) or its replacement with 2-F (e.g., **24**) maintained A_3AR selectivity but reduced the A_3 AR affinity by 5- to 10-fold. Replacement of 2-C1 with 2-H either had no effect (cf. **2a** and **22**) on affinity at the A_{2A}AR or moderately reduced it (cf. **2c** and **23**), and affinity at the A_1AR was moderately increased. The most A_3AR selective truncated compound was the 2-unsubstituted N^6-(3-chlorobenzyl) derivative **22** with 330-fold and 940-fold selectivity in comparison to the A_1 and A_{2A}AR, respectively.

An amine functionalized congener **26** in the truncated series, with a K_i value of 403 nM, proved to be weaker in binding to the A_3AR than those analogues with less sterically bulky substitution at the C2 position. However, it nevertheless displayed moderate selectivity of > 20-fold in comparison to both the A_1 and A_{2A}ARs. This derivative was analogous to compound **11** in the 5′-*N*-methyluronamide series, which was 160-fold more potent in binding to the A_3AR.

A 5′-truncated N^6-cyclopentyl derivative **25**, by analogy to compound **4**,[37] was intended to display enhanced affinity at both A_1 and A_3ARs but not at the A_{2A}AR. However, the loss of the 5′-*N*-methyluronamide group reduced affinity at A_1 and A_3ARS by 6- and 32-fold, respectively. Thus, N^6-benzyl substitution of the (N)-methanocarba nucleosides preserved the AR selectivity profile upon truncation better than an N^6-cyclopentyl group.

Selected compounds in the present series were tested in binding at the rA_3AR. *The* K_i values (nM) were determined to be the following: **6**, 66.5 ± 3.9; **15**, 8.68 ± 0.85; **18**, 9.62 ± 1.50; **22**, 20.3 ± 2.0. Thus, the high A_3AR affinity was maintained in a murine species.

The A_3AR efficacy in the cAMP functional assay varied greatly in this series of truncated (N)-methanocarba derivatives. Compounds **2b** and **2c**, which were reported as A_3AR antagonists based on an assay of [^{35}S]GTPγS binding,[20] were partial agonists in the assay of adenylate cyclase (Figure 2B). Both partial (e.g., **22**) and nearly full agonism (e.g., **23** and

25) were observed for the novel truncated nucleosides in the cyclase assay. Replacement of the 2-Cl of the truncated analogues **2b** with hydrogen in **22** resulted in a 58% relative efficacy, which was comparable to 46% for **2b**. The 2-H **23** and 2-F **24** N^6-(3-iodobenzyl) analogues displayed relative efficacy of 81% and 19%, respectively. Compound **25** inhibited the production of cAMP via the hA_3AR to the same extent as **51** and was therefore a full agonist as judged by adenylate cyclase inhibition.

A homology model of the hA_3AR based on the $A_{2A}AR$ X-ray structure[38,39] was used to study the putative interactions between the chain-functionalized agonist **15** and the A_3AR. The binding mode of **15** obtained after InducedFit docking revealed the following binding mode of the ligand (Figure 3). The 2′- and 3′-hydroxyl groups were located in proximity to Ser271 (7.42) and His272 (7.43) and could form H-bonds with these residues. The oxygen atom of the 5′-*N*-methylcarboxamido moiety was involved in H-bonding with the side chain hydroxyl group of Thr94 (3.36). Both N^6-amino group and the N7 atom of the adenine ring formed H-bonds with Asn250 (6.55). A $\pi-\pi$ interaction was observed between the adenine ring of **15** and Phe168 (EL2). The ligand–receptor interactions observed in the present model were in good agreement with the data of site-directed mutagenesis and with our previously published models of ARs, including the studies of AR agonists docked to the $A_{2A}AR$ crystal structure.[38,40] In the model obtained, the 3-chlorobenzyl ring was located in the hydrophobic pocket formed by Val169 (EL2). Met172(EL2), Met174(EL2), Met177(5.38), and Ile253(EL3).

The long chain substituent at the C2 position of 15 was oriented toward the extracellular part of the receptor, which is a prerequisite in functionalized congeners.[23] A similar orientation of the 2-propynyl fragment of **15** was previously suggested for C2 substituted agonists of the A_{2B} receptor.[41,42] The carbonyl oxygen of the amide group of the C2 substituent of **15** was H-bonded to the side chain NH_2-group of Gln 167 (EL2), and consequently the NH-group of the C2 substituent of **15** was oriented toward the hydroxyl group of Tyr265 (7.36) (Figure 3). The terminal amine of the ligand interacted with negatively charged Glu258 (EL3), and it was also H-bonded to the backbone oxygen atom of Val259 (EL3).

Discussion

In the present work and in a previous study,[20] parallel patterns of SAR in receptor binding were observed in the two series of 5′-$CONHCH_3$ (full agonist) and truncated (N)-methanocarba nucleosides, in cases with substituted N^6-benzyl groups and small substituents present at the adenine C2 position. This parallel behavior was not maintained in a derivative bearing a chain at the C2 position.

The replacement of the adenine 2-Cl with H in the series of substituted N^6-benzyl-(N)-methanocarba agonists containing the 5′-$CONHCH_3$ group was previously shown to have only minor effects on AR binding.[16] Specifically, it moderately reduced affinity at the A_1 and $A_{2A}ARs$ without a significant reduction at the A_3AR (cf. **1a** and **3**). In the present 5′-*N*-methyluronamide series with more elaborate modification at the C2 position, the preservation of potent and selective binding to the hA_3AR was demonstrated.

The flexibility of chain substitution at C2 allowed the conjugation with sterically bulky moieties. This distal region of the nucleosides was predicted by homology modeling to be at A_3AR extracellular regions and consequently free from the steric constraints present at the pharmacophore binding region. Thus, this series of highly potent and selective A_3AR agonist functionalized congeners is suitable for conjugation to prosthetic groups for radiolabeling, fluorescent detection, avidin complexation, and polymeric coupling with

retention of biological activity. The functionalized congener approach was already applied to 1,4-dihydropyridine derivatives as antagonists of the hA_3AR.[26]

Specifically, agonists in this series were conjugated with biotin and a fluorescent dye with retention of high A_3AR affinity and selectivity. The fluorescent conjugate **18** was a highly potent, full agonist with a K_i values at the hA_3AR and the rA_3AR of 17.2 and 9.6 nM, respectively. The fluorescent dye Cy5, which was incorporated covalently in **18**, has favorable properties for use in biological systems including near-infrared imaging, with excitation and emission maxima at 649 and 670 nm, respectively, and with a relatively high quantum yield of 0.28.[27] Another fluorescent agonist of the hA_3AR having lower affinity than **18** was used in fluorescence correlation spectroscopy to demonstrate that the receptor exists in heterogeneous microdomains of individual living cells.[43]

In our previous study of truncated (N)-methanocarba nucleosides,[20] the docking of two representative structures in a rhodopsin-based homology model of the hA_3AR indicated that both (N)-methanocarba agonists and nucleoside-derived antagonists were able to bind in a very similar mode in the receptor binding site, which is common to other A_3AR-selective nucleosides, such as the prototypical agonist 2-chloro-N^6-(3-iodobenzyl)-5′-*N*-methylcarboxamidoadenosine (Cl-IB-MECA).[20] We have shown that the SAR parallels between these two series were less stringent with respect to distal modification, e.g., when a long functionalized chain was attached at the C2 position. Thus, in the truncated series the attachment of a functionalized amino chain at the C2 position greatly reduced A_3AR affinity. Therefore, among the 2-alkynyl nucleosides in this study, only those containing a 5′-*N*-methyluronamide group, i.e., full agonists, were suitable as functionalized congeners.

Truncation of the 5′-$CONHCH_3$ moiety of A_3AR agonists in the (N)-methanocarba class was again demonstrated as a means of reducing the relative efficacy of A_3AR-selective agonists to produce partial agonism, as indicated in the cyclase assay. A previous study of 2-triazolo derivatives of adenosine demonstrated modulation of the relative efficacy at the A_3AR by structural changes at the 2 position.[12c] In the truncated series, the remaining efficacy at the hA_3AR, as was evident in a cAMP functional assay, ranged from small (e.g., **24** and **26**) to substantial (e.g., **23**).

We compared selected compounds in the ability to stimulate the A_3AR using multiple functional criteria. When stimulation of [^{35}S]GTPγS binding was used as a criterion of receptor activation, truncated (N)-methanocarba nucleosides (e.g., **2a**–**c**) reported by Melman et al.[20] did not induce a substantial activation of the A_3AR, with < 10% of the full agonism. However, in the current study using a different functional assay that measured inhibition of the production of cAMP to detect antagonism, considerable activity in the truncated series was seen. The efficacy in the cyclase assay generally exceeded the efficacy in [^{35}S]GTPγ S binding. Other truncated nucleosides in the 4′-thio series were A_3 AR antagonists in both [^{35}S]GTPγS and cAMP assays.[44] Recently, truncated nucleosides in the 4′-oxo series were demonstrated to be A_3 AR antagonists in the cAMP assay.[45]

In the truncated series, removal of the 2-Cl substitution or replacement with 2-F in **24** moderately reduced the A_3AR affinity but retained high selectivity. The introduction of fluorine in **24** was intended for possible radiofluorination as a means of preparing tracers for in vivo PET imaging of the A_3AR.[47] One member of the previously reported truncated (N)-methanocarba nucleoside series **2b**, (1*R*,2*R*,3*S*,4*R*,5*S*)-4-(6-(3-bromobenzylamino)-2-chloro-9*H*-purin-9-yl)bicyclo-[3.1.0]hexane-2,3-diol (MRS5147), was recently labeled with the short-lived positron-emitter ^{76}Br to form a selective hA_3AR radioligand of high affinity for use in positron emission tomography (PET). This ligand was intended for imaging of the A_3 AR in murine as well as primate species, because high affinity binding was maintained at

the rA_3AR. Binding of these truncated (N)-methanocarba nucleosides at the A_3AR was competitive,[20] and therefore, the related derivatives in this study should be useful as receptor probes.

In conclusion, two highly potent and selective series of nucleoside ligands containing the (N)-methanocarba ring system have been synthesized and characterized as A_3AR agonists, partial agonists, and antagonists. Furthermore, in the 5′-*N*-methyluronamide series but not in the truncated series, the functionalized congener approach was found to maintain potency and selectivity at the hA_3AR and therefore provides an entry into high affinity molecular probes. Thus, the binding and activation profiles of the truncated analogues indicated the biological interdependence of substitution at the C2, 5′, and N^6 positions. Potent agonist ligands containing biotin and fluorescent dye moieties should be useful in future pharmacological studies of the localization, association, regulation, and internalization of the A_3AR and might provide an alternative to the use of radioligands for this receptor.

Experimental Section

Chemical Synthesis. Materials and Instrumentation

D-Ri-bose, L-ribose, and other reagents and solvents were purchased from Sigma-Aldrich (St. Louis, MO). Alcohol derivative **31** was prepared as reported.[201] H NMR spectra were obtained with a Varian Gemini 300 spectrometer using $CDCl_3$ and CD_3OD as solvents. Chemical shifts are expressed in δ values (ppm) with tetramethylsilane (δ 0.00) for $CDCl_3$ and water (δ 3.30) for CD_3OD. TLC analysis was carried out on aluminum sheets precoated with silica gel F_{254} (0.2 mm) from Aldrich. All target compounds are ≥95% pure as determined by HPLC. HPLC mobile phases consisted of the following: for system A, linear gradient solvent system of CH_3CN/triethyl ammonium acetate from 5/95 to 60/40 in 20 min and flow rate of 1.0 mL/min; for ystem B, linear gradient solvent system of CH_3CN/ tetrabutyl ammonium phosphate from 20/80 to 60/40 in 20 min and flow rate of 1.0 mL/min. Low-resolution mass spectrometry was performed with a JEOL SX102 spectrometer with 6 kV Xe atoms following desorption from a glycerol matrix or on an Agilent LC/MS 1100 MSD, with a Waters (Milford, MA) Atlantis C18 column. High resolution mass spectroscopic (HRMS) measurements were performed on a proteomics optimized Q-TOF-2 (Micromass-Waters) using external calibration with polyalanine, unless otherwise noted. Observed mass accuracies are those expected on the basis of known performance of the instrument as well as trends in masses of standard compounds observed at intervals during the series of measurements. Reported masses are observed masses uncorrected for this time-dependent drift in mass accuracy.

(1′*S*,2′*R*,3′*S*,4′*S*,5′*S*)-4′-[6-(3-Chlorobenzylamino)-2-(4-methoxycarbonyl-l-butynyl)-9-yl]-2′, 3′-*0*-isopropylidenebicyclo[3.1.0]-hexane-1′-carboxylic Acid *N*′-Methylamide (28)

To a solution of compound **27** (52 mg, 0.087 mmol) in anhydrous DMF (1.5 mL), $PdCl_2(PPh_3)_2$ (12 mg, 0.017 mmol), CuI (2 mg, 0.010 mmol), methyl-ω-pentynate (39 mg, 0.347 mmol), and then triethylamine (0.12 mL, 0.86 mmol) were added. The reaction mixture was stirred at room temperature overnight. Solvent was evaporated under vacuum, and the residue was purified on flash silica gel column chromatography (CH_2Cl_2/MeOH = 30:1) to give compound **28** (40 mg, 78%) as a foamy syrup. ^1H NMR (CD_3OD, 300 MHz) δ 8.16 (s, 1H), 7.42–7.44 (m, 4H), 5.81 (d, *J* =*6.7* Hz, 1H), 5.02 (s, 1H), 4.82 (m, 2H), 3.78 (s, 3H), 2.85 (s, 3H), 2.72–281 (m, 5H), 2.09–2.14 (m, 1H), 1.55 (s, 3H), 1.41 (t, *J*=5.1 Hz, 1H), 1.29 (s, 3H), 0.82–0.96 (m, 1H). HRMS calculated for $C_{29}H_{32}ClN_6O_5$ $(M + H)^+$: 579.2105: found 579.2123.

(1′*S*,2′*R*,3′*S*,4′*S*,5′*S*)-4′-[6-(3-Chlorobenzylamino)-2-(4-(*β*-aminoethyl aminocarbonyl)-1-butynyl)-9-yl]-2′,3′-dihydroxybicyclo [3.1.0]hexane-1′-carboxylic Acid *N*-Methylamide (11)

To a solution of compound **28** (20 mg, 0.034 mmol) in methanol (0.3 mL), ethylenediamine (1.5 mL) was added. The mixture was stirred overnight at room temperature. Solvent was evaporated, and the residue was roughly purified on flash silica gel column chromatography. The aminated product was dissolved in methanol (1.5 mL) and 10% trifluoroacetic acid (1.5 mL) and heated at 70 °C for 15 h. Solvent was evaporated and the residue was purified on flash silica gel column chromatography (CH_2Cl_2/MeOH/NH_4OH = 10:1:0.1) to give compound **11** (15 mg, 79%). ^{1}H NMR (CD_3OD, 300 MHz) δ 8.09 (s, 1H), 7.39 (s, 1H), 7.27–7.32 (m, 3H), 4.99 (d, J = 6.6 Hz, 1H), 4.78– 4.91 (m, 2H), 3.97 (d, J = 6.6 Hz, 1H), 3.33–3.36 (m, 4H), 2.87 (s, 3H), 2.75–2.80 (m, 4H), 2.53 (t, J = 7.2 Hz, 2H), 2.59–3.41 (m, 1H), 1.88 (t, J = 4.5 Hz, 1H), 1.34–1.43 (m, 1H). HRMS calculated for $C_{27}H_{31}ClN_6O_2Na$ $(M + Na)^+$: 589.2068; found 589.2054.

(1′*S*,2′*R*,3′*S*,4′*S*,5′*S*)-4′-[6-(3-Chlorobenzylamino)-2-(4-methoxycarbonyl)-1 -butyn yl)-9-yl]-2′,3′ -dihydroxybicyclo [3.1.0] hexane-1′-carboxylic Acid *N*-Methylamide (8)

A solution of compound **28** (67 mg, 0.115 mmol) in methanol (3 mL) and 10% triflromethanesulfonic acid (2 mL) was heated at 70 °C overnight. Solvent was evaporated and the residue was purified on flash silica gel column chromatography (CH_2Cl_2/MeOH = 20:1) to give compound **8** (46 mg, 75%). ^{1}H NMR (CD_3OD, 300 MHz) δ 8.13 (s, 1H), 7.40 (s, 1H), 7.22–7.45 (m, 3H), 5.10 (d, J = 6.3 Hz, 1H), 4.80–4.86 (m, 2H), 4.03 (d, J = 6.6 Hz, 1H), 3.63 (s, 3H), 2.86 (s, 3H), 2.76–2.85 (m, 2H), 2.46–2.58 (m, 2H), 2.05–2.10 (m, 1H), 1.77–1.83 (m, 1H), 1.35–1.40 (m, 1H), 0.82–0.96 (m, 1H). HRMS calculated for $C_{26}H_{28}ClN_6O_5$ $(M + H)^+$: 539.1731; found 539.1743.

(1′*S*,2′*R*,3′*S*,4′*S*,5′*S*)-4′-[6-(3-Chlorobenzylamino)-2-(4-hydroxycarbonyl)-l-butynyl)-9-yl]-2′, 3′-dihydroxybicyclo[3.1.0]hexane-1′-carboxylic Acid *N*′-Methylamide (5)

To a solution of ester **8** (30 mg, 0.055 mmol) in methanol (1.5 mL), 1 M solution of potassium hydroxide (1 mL) was added. The mixture was stirred at room temperature overnight. The reaction mixture was neutralized with acetic acid, and solvent was evaporated, and the residue was purified on flash silica gel column chromatography (CH_2Cl_2/MeOH = 10:1) to give compound **5** (23 mg, 80%). ^{1}H NMR (CD_3OD, 300 MHz) δ 8.06 (s, 1H), 7.38 (s, 1H), 7.24–7.35 (m, 3H), 5.14 (d, J= 5.8 Hz, 1H), 4.79–4.86 (m, 2H), 4.03 (d, J = 6.3 Hz, 1H), 2.87 (s, 3H), 2.42–2.80 (m, 4H), 2.04– 2.32 (m, 1H), 1.82 (t, J =4.8 Hz, 1H), 1.36–1.40 (m, 1H), 0.84– 0.97 (m, 1H). HRMS calculated for $C_{25}H_{26}ClN_6O_5$ $(M + H)^+$: 525.9562; found 525.9583.

(1′*S*,2′*R*,3′*S*,4′*S*,5′*S*)-4′-[6-(3-Chiorobenzylamino)-2-(*N*-biotinyl(*β*-aminoethylaminocarbonyl)-1-butynyl)-9-yl]-2′, 3′-dihydroxybicyclo[3.1.0]hexane-1′-carboxylic Acid *N*-Methylamide (17a)

To a solution of compound **11** (4 mg, 0.007 mmol) in dry DMF (0.5 mL), biotin (1.89 mg, 0.0077 mmol), HATU (3.2 mg, 0.0084 mmol), and DIEA (1.6 μL, 0.009 mmol) were added. The mixture was stirred at room temperature overnight. Solvent was evaporated and the residue was purified on flash silica gel column chromatography (CH_2Cl_2/MeOH/NH_4OH =5:1:0.1) to give compound **17a** (3.5 mg, 62%). ^{1}H NMR (CD_3OD, 300 MHz) δ 8.14 (s, 1H), 7.43 (s, 1H), 7.29–7.39 (m, 3H), 5.07 (d, J = 6.9 Hz, 1H), 4.78–4.86 (m, 2H), 4.48–4.52 (m, 1H), 4.28–4.32 (m, 1H), 4.04 (d, J =6.3 Hz, 1H), 3.70–3.82 (m, 2H), 3.24–3.31 (m, 1H), 3.08–3.23 (m, 1H), 2.91 (s, 3H), 2.70–2.83 (m, 4H), 2.56 (t, J =7.5 Hz, 2H), 2.04–2.15 (m, 2H), 1.89 (t, J =5.1 Hz, 1H), 1.33–1.70 (m, 10H), 0.84–1.02 (m, 1H). HRMS calculated for $C_{37}H_{46}ClN_{10}O_6S$ $(M + H)^+$: 793.3011; found 793.3030.

(1′*S*,2′*R*,3′*S*,4′*S*,5′*S*)4′-[6-(3-Chlorobenzylamino)-2-(*N*-biotinyl{5-aminopentanyl}(*β*-aminoethylaminocarbonyl)-1-butynyl)-9-yl]-2′,3′-dihydroxybicyclo[3.1.0]hexane-1′-carboxylic Acid *N*-Methylamide (17b)

To a solution of compound **11** (3.4 mg, 0.0059 mmol) in DMF (0.5 mL), sulfo-NHS-LC-biotin (10 mg, 0.017 mmol, Pierce) and 10 μL of triethyl amine were added. The mixture was stirred overnight at room temperature. Solvent was evaporated and the residue was purified on flash silica gel column chromatography (CH_2Cl_2/MeOH/NH_4OH = 7:1:0.1) to give compound **17b** (2.9 mg, 55%). ^{1}H NMR (CD_3OD, 300 MHz) δ 7.99 (s, 1H), 7.29 (s, 1H), 7.19–7.32 (m, 3H), 4.93 (d, J = 5.4 Hz, 1H), 4.70–4.80 (m, 2H), 4.36–4.42 (m, 1H), 4.16–4.22 (m, lH), 3.88 (d,J = 6.9 Hz, 1H), 3.42–3.62 (m, 2H), 3.03–3.15 (m, 4H), 2.89 (s, 3H), 2.56–2.86 (m, 6H), 2.41 (t, J = 6.6 Hz, 1H), 1.96–2.13 (m, 5H), 1.15–1.16 (m, 14H), 0.74–0.88 (m, 1H). $C_{43}H_{57}C1N_{11}O_7S$ $(M+H)^+$: 906.3852; found 906.3878.

(1′*S*,2′*R*,3′*S*,4′*S*,5′*S*)-4′-[6-(3-Chlorobenzylamino)-2-(*N*-cyaiune(*β*-aminoethylaminocarbonyl)-1-butynyl)-9-yl]-2′,3′-dihydroxybicyclo[3.1.0]hexane-1′-carboxylic Acid *N*-Methylamide (18)

To a solution of compound **11** (1.69 mg, 0.0029 mmol) in DMF (0.3 mL), Cy5 fluorescent dye (2.36 mg, 0.0029 mmol) and bicarbonate buffer (60 μL) were added. The mixture was stirred at room temperature overnight. The reaction mixture was covered with aluminum foil in order to protect from light. Solvent was evaporated and the residue was purified on flash silica gel column chromatography (CH_2Cl_2/MeOH/NH_4OH = 3:1:0.1) to give compound **18** (3.2 mg, 89%) as a dark-blue syrup. ^{1}H NMR (CD_3OD, 300 MHz) δ 8.21–8.42 (m, 1H), 8.08 (s, 1H), 7.85–7.93 (m, 2H), 7.29–7.46 (m, 6H), 6.28–6.45 (m, 1H), 5.07 (d, J = 6.9 Hz, 1H), 4.84–4.86 (m, 2H), 3.97–4.20 (m, 3H), 3.53 (t,J = 6.0Hz, 2H), 3.12 (t,J = 6.0Hz, 2H), 2.89 (s, 3H), 2.49 (t,J =7.5 Hz, 2H), 2.52–2.64 (m, 2H), 2.01–2.17 (m, 2H), 1.56–1.89 (m, 9H), 1.22–1.49 (m, 10H), 0.86–0.96 (m, 1H). HRMS calculated for $C_{60}H_{68}ClIN_{10}O_{11}S_2$ (M^+): 1203.4199; found 1203.4175.

(1′*R*,2′*S*,3′*R*,4′*R*,5′*R*)-4′-(6-Chloro-2-iodopurin-9-yl)-2′,3′-isopropylidenebicyclo[3.1.0]hexane-1′-carboxylic Acid Ethyl Ester (32)

To a solution of triphenylphosphine (0.357 g, 1.361 mmol) and 6-chloro-2-iodopurine (0.286 g, 1.019 mmol) in dry THF (4 mL), DIAD (0.26 mL, 1.359 mmol) was added. The mixture was stirred at room temperature for 10 min. A solution of compound **31** (0.165 g, 0.681 mmol) in THF (2 mL) was added to the reaction mixture, and the mixture was further stirred overnight at room temperature. Solvent was evaporated and the residue was purified on flash silica gel column chromatography (hexane/ethyl acetate = 3:1) to give the product **32** (0.2 g, 58%) as a foam. ^{1}H NMR ($CDCl_3$, 300 MHz) δ 1.31–1.37 (m, 4H), 1.56 (s,6H), 1.77–1.80 (m, 1H), 2.10–2.22 (m, 1H), 4.19–4.27 (m, 2H), 4.77 (d, J = 14.2 Hz, 1H), 4.91 (s, 1H), 5.83 (d, J = 15.3 Hz, 1H),7.97(s, 1H). HRMS calculated for $C_{17}H_{19}ClIN_4O_4$ (M + H)$^+$: 505.0061; found 505.0073.

(1′*R*,2′*S*,3′*R*,4′*R*,5′*R*)-4′-[6-(3-Chlorobenzylamino)-2-iodopurin-9- yl]-2′,3′-isopropylidenebicyclo[3.1.0]he xane-1′-carboxylic Acid Ethyl Ester (33)

To a solution of compound **32** (0.273 g, 0.54 mmol) in anhydrous methanol (5 mL), triethylamine (1 mL, 7.569 mmol) and 3-chlorobenzylamine (0.33 mL, 2.697 mmol) were added. The mixture was stirred overnight at room temperature. Solvent was evaporated and the residue was purified on flash silica gel column chromatography (hexane/ethyl acetate = 1:1) to give the product **33** (0.255 g, 78%) as a foamy solid. ^{1}H NMR ($CDCl_3$, 300 MHz) δ 7.57 (s, 1H), 7.20–7.25 (m, 4H), 6.03–6.15 (m, 1H), 5.83 (d, J = 4.45 Hz, 1H), 5.28 (s, 1H), 4.65– 4.88 (m, 3H), 4.11–4.22 (m, 2H), 2.18–2.23 (m, 1H), 1.22–1.80 (m, 7H), 1.10–1.21 (m, 4H). HRMS calculated for $C_{24}H_{26}ClI$-N_5O_4 (M + H)$^+$: 610.0639; found 610.0649

(1′*R*,2′*S*,3′*R*,4′*R*,5′R)-4′-[6-(3-Chlorobenzylamino)-2-iodopurin-9-yl]-2′-,3′-isopropylidenebicyclo[3.1.0]hexane-1′-carboxylic Acid-*N*-Methylamide (34)

To a solution of compound **33** (0.258 g, 0.423 mmol) in methanol (5 mL), 40% methylamine (4 mL) was added. The mixture was stirred at room temperature for 2 days. Solvent was evaporated and the residue was purified on flash silica gel column chromatography (CH_2Cl_2/MeOH = 40:1) to give the product **34** (0.165 g, 66%) as a foam. ^{1}H NMR($CDCl_3$, 300 MHz) δ 7.58 (s, 1H), 7.25–7.37 (m, 4H), 6.52 (bs, 1H), 5.65 (d, J = 6.9 Hz, 1H), 4.73–4.83 (m, 3H), 2.97 (s, 3H), 2.04–2.07 (m,1H), 1.62–1.82 (m, 1H), 1.55 (s, 3H), 1.22–1.33 (m, 5H). HRMS calculated for $C_{23}H_{25}ClIN_6O_3$ $(M + H)^+$: 595.0643: found 595.0649.

(1′*R*,2′*S*,3′*R*,4′*R*,5′*R*)-4′-[6-(3-Chlorobenzylamino)-2-(4-hydroxycarbonyl)-1-butynyl)-9-yl]-2′,3′-dihydroxybicyclo[3.1.0]hexane-1′-carboxylic Acid *N*-Methylamide (19)

To a solution of compound **34** (32 mg, 0.053 mmol) in anhydrous DMF (1.0 mL), $PdCl_2(PPh_3)_2$ (7 mg, 0.01 mmol), CuI (2 mg, 0.01 mmol), methyl-ω-hexynate (27 mg, 0.214 mmol), and then triethylamine (0.014 mL, 0.10 mmol) were added. The reaction mixture was stirred at room temperature overnight. Solvent was evaporated under vacuum, and the residue was roughly purified on flash silica gel column chromatography. To a solution of the product in methanol (1 mL), 10% TFA (1 mL) was added, and the mixture was heated at 70 °C for 7 h. Solvent was evaporated under vacuum, the crude reaction mixture was further dissolved in methanol (1 mL), and 1 M solution of KOH (1 mL) was added, and the mixture was stirred at room temperature overnight. Reaction mixture was neutralized with acetic acid, solvent was evaporated under vacuum, and the residue was purified on flash silica gel column chromatography (CH_2Cl_2/MeOH = 20:1) to give compound **19** (18 mg, 62%) as a colorless powder. ^{1}H NMR (CD_3OD, 300 MHz) δ 7.97 (s, 1H), 7.30 (s, 1H), 7.14– 7.22 (m, 3H), 4.97 (d, J = 6.7 Hz, 1H), 4.73 (brs, 3H), 3.90 (d, J = 6.6 Hz, 1H), 2.75 (s, 3H), 2.42 (t, J = 7.2 Hz, 2H), 2.28 (t, J = 7.2 Hz, 2H), 1.96 (m, 1H), 1.83 (m, 3H), 1.75 (m, 1H), 1.25 (m, 1H). HRMS calculated for $C_{26}H_{28}ClN_6O_5$ $(M + H)^+$: 539.1731: found 539.1724.

(1*R*,2*R*,3*S*,4*S*,5*S*)-2,3-*O*-Isopropylidene-2,3,4-trihydroxybicyclo[3.1.0]hexane (40)

To a solution of compound **39** (0.773 g, 4.95 mmol) in dry CH_2Cl_2 (25 mL), Et_2Zn (19.8 mL, 1 M solution in hexane) was added at 0 °C. The mixture was stirred for 15 min. After 15min, CH_2I_2 (3.2 mL, 39.57 mmol) was added at the same conditions and then the reaction mixture was stirred at room temperature overnight. After completion of reaction, it was quenched with saturated ammonium chloride solution and the aqueous layer was extracted with CH_2Cl_2. The combined organic layer was dried (Na_2SO_4), filtered, and evaporated. The residue was purified on flash silica gel column chromatography (hexane/ethyl acetate = 3:1) to give compound **40** (0.710 g, 85 %) as a colorless syrup. ^{1}H NMR ($CDCl_3$, 300 MHz) δ 4.88 (t, J = 6.3 Hz, 1H), 4.46 –4.54 (m, 2H), 2.37 (d, J = 9.3 Hz, 1H), 1.83 – 1.86 (m, 1H), 1.61–1.66 (m, 1H), 1.55 (s,3H), 1.29 (s,3H), 0.95– 0.99 (m, 1H), 0.62–0.68 (m, 1H). HRMS calculated for $C_9H_{15}O_3$ $(M + H)^+$: 171.0943; found 171.0954.

(1′*R*,2′*R*,3′*S*,4′*S*,5′*S*)-4′-(6-Chloro-2-iodopurine)-2′,3′-*O*-isopropylidene-2′,3′-dihydroxybicyclo[3.1.0]hexane (44)

To a solution of triphenylphosphine (132 mg, 0.5 mmol) and 6-chloro-2-iodopurine (141 mg, 0.50 mmol) in dry THF (2 mL), DIAD (0.1 mL, 0.50 mmol) was added. The mixture was stirred at room temperature for 10 min. A solution of compound **31** (43 mg, 0.252 mmol) in THF (2 mL) was added to the reaction mixture, and the mixture was further stirred overnight at room temperature. Solvent was evaporated and the residue was purified on flash silica gel column chromatography (hexane/ethyl acetate = 3:1) to give the product **44** (65 mg, 61%) as a foam. ^{1}H NMR ($CDCl_3$, 300 MHz) d 8.07 (s, 1H), 5.37 (t, J = 2.1 Hz, 1H), 4.67 (d, J = 5.7Hz, 1H), 2.13–2.18 (m, 1H), 1.65–1.69 (m, 2H), 1.27 (s, 3H), 1.26 (s, 3H),

0.96–1.01 (m, 2H). HRMS calculated for $C_{14}H_{15}ClIN_4O_2$ $(M + H)^+$: 432.9927; found 432.9928.

(1′*R*,2′*R*,3′*S*,4′*S*,5′*S*)-4′-[6-(3-Chlorobenzylamino)-2-iodopurine]-2′,3′- *O*-isopropylidene-2′,3′-dihydroxybicyclo[3.1.0]hexane (45)

To a solution of compound **44** (0.105 g, 0.243 mmol) in anhydrous methanol (5 mL), triethylamine (0.4 mL, 3.38 mmol) and 3-chlorobenzylamine (0.15 mL, 1.21 mmol) were added. The mixture was stirred overnight at room temperature. Solvent was evaporated and the residue was purified on flash silica gel column chromatography (hexane/ethyl acetate = 1:1) to give the product **45** (0.096 g, 74%) as a foam. ^{1}H NMR ($CDCl_3$, 300 MHz) δ 7.63 (s, 1H), 7.37 (s, 1H), 7.27–7.26 (m, 3H), 6.25 (bs, 1H), 5.34 (t, J = 5.7Hz, 1H), 4.94 (s,2H), 4.64 (d, J =6.9Hz, 1H), 2.06–2.09 (m, 1H), 1.61–1.69 (m,2H), 1.54(s,3H), 1.26(s,3H), 0.88–0.96 (m, 2H). HRMS calculated for $C_{21}H_{22}ClIN_5O_2$ $(M + H)^+$: 538.0497; found 538.0507.

(1′*R*,2′*R*,3′*S*,4′ *S*,5′ *S*)-4′-[6-(3-Iodobenzylamino)purine]-2′,3′-dihydroxybicyclo[3.1.0]hexane (23)

A solution of compound **46** (32 mg, 0.063 mmol) in MeOH (2 mL) and 10% trifluoroacetic acid (1.5 mL) was heated at 70 °C overnight. Solvent was evaporated and the residue was purified on flash silica gel column chromatography (CH_2Cl_2/MeOH =30:1) to give compound **23** (23 mg, 79%). ^{1}H NMR (CD_3OD, 300 MHz) δ 8.34 (s, 1H), 8.32 (s, 1H), 7.8 (s, 1H), 7.64 (d,J = 8.1 Hz, 1H), 7.42 (d, J =7.5 Hz, 1H), 7.13 (t, J= 7.8 Hz, 1H), 4.90 (s, 2H), 4.72 (t, J= 6.3 Hz, 2H), 3.96 (d, J = 6.6 Hz, 1H), 1.96–2.04 (m, 1H), 1.67–1.72 (m, 1H), 1.30–1.34 (m, 1H), 0.75–0.83 (m, 1H). HRMS calculated for $C_{18}H_{19}IlN_5O_2$ $(M +H)^+$: 464.0596; found 464.0584.

(1′*R*,2′*R*,3′*S*,4′*S*,5′*S*)-4′-[6-(3-Iodobenzylamino)-2-fluoro-purine]-2′,3′-dihydroxybicyclo[3.1.0]hexane (24)

To a solution of compound **42** (10 mg, 0.03 mmol) in anhydrous methanol (0.5 mL), triethylamine (0.06 mL, 0.42 mmol) and 3-iodobenzylamine (0.02 mL, 0.15 mmol) were added. The mixture was stirred overnight at room temperature. Solvent was evaporated, and the residue was roughly purified on flash silica gel column chromatography. The product was dissolved in methanol (1 mL) and 10% TFA (0.5 mL), and the reaction mixture was heated at 70 °C for 6 h. Solvent was evaporated and the residue was purified on flash silica gel column chromatography (hexane/ ethyl acetae = 1:1) to give compound **24** (8 mg, 55%). ^{1}H NMR (CD_3OD, 300 MHz) δ 8.13 (s, 1H), 7.78 (s, 1H), 7.63 (dd, J = 7.8 Hz, 1H), 7.41 (d, J = 7.8 Hz, 1H), 7.11 (t, J = 7.8 Hz, 1H), 4.76 (s, 1H), 4.71–4.76 (m, 2H), 3.92–3.96 (m, 2H), 1.96–2.01 (m, 1H), 1.64–1.67 (m, 1H), 0.73–0.91 (m, 2H). HRMS calculated for $C_{18}H_{18}FIN_5O_2$ $(M + H)^+$: 482.0473; found 482.0489.

(1′*R*,2′*R*,3′*S*,4′*S*,5′*S*)-4′-[6-(3-Chlorobenzylamino)-2-(4-methoxycarbonyl-1-butynyl)-9-yl]-2′,3′-*O*-isopropylidenebicyclo[3.1.0]-hexane (50)

To a solution of compound **45** (43 mg, 0.08 mmol) in anhydrous DMF (1.5mL),$PdCl_2(PPh_3)_2$ (11 mg,0.015mmol), CuI (1.5 mg, 0.007 mmol), methyl-ω-pentynate (35 mg, 0.31 mmol), and then triethylamine (0.11 mL, 0.80 mmol) were added. The reaction mixture was stirred at room temperature overnight. Solvent was evaporated under vacuum and the residue was purified on flash silica gel column chromatography (hexane/ ethyl acetate = 1:1) to give compound **50** (30 mg, 74%) as a foamy syrup. ^{1}H NMR ($CDCl_3$, 300 MHz) 7.98 (s, 1H), 7.32– 7.21 (m, 4H), 6.19 (bs, 1H), 5.31 (t, J = 5.24, 1H), 5.12 (s, 2H), 4.60 (d, J= 7.2 Hz, 1H), 3.78 (s, 3H), 2.69 –2.84 (m, 4H), 2.06– 2.14 (m, 1H), 1.62–1.72 (m, 1H), 1.56 (s,3H), 1.25 (s,3H), 0.88– 1.04 (m, 2H). HRMS calculated for $C_{27}H_{29}ClN_5O_2$ $(M + H)^+$: 522.9953; found 522.9967.

(1′ *R*,2′ *R*,3′*S*,4′*S*,5′*S*)-4′ -[6-(3-Chlorobenzylamino)-2-(4-*β*-aminoethylaminocarbonyl)-1-butynyl)-9-yl]-2′,3′-dihydroxybicyclo [3.1.0]hexane (26)

To a solution of compound **50** (25 mg, 0.047 mmol) in methanol (0.4 mL), ethylenediamine (1.5 mL) was added. The mixture was stirred overnight at room temperature. Solvent was evaporated, and the residue was roughly purified on flash silica gel column chromatography. The aminated product was dissolved in methanol (1.5 mL) and 10% trifluoroacetic acid (1.0 mL), and the mixture was heated at 70 °C for 15 h. Solvent was evaporated and the residue was purified on flash silica gel column chromatography (CH_2Cl_2/MeOH = 5:1) to give compound **26** (14 mg, 58%). ^{1}H NMR (CD_3OD, 300 MHz) δ 8.12 (s, 1H), 7.25–7.39 (m, 4H), 5.97 (s, 1H), 4.70 (t, J = 6.3 Hz, 1H), 3.95 (d, J = 6.6 Hz, 1H), 3.77 (t, J = 6.3 Hz, 2H), 2.94 (m, 2H), 2.52 (t, J = 7.8 Hz, 2H), 1.97–2.01 (m, 1H), 1.68–1.72 (m, 2H), 1.30–1.35 (m, 2H), 0.89–0.91 (m, 2H), 0.78–0.82 (m, 1H). HRMS calculated for $C_{25}H_{29}ClN_7O_3$ $(M + H)^+$: 510.2000; found 510.2020.

UV–Visible Spectra of Biotinyl (17) and Fluorescent (18) Probes and Fluorescence Spectrum of 18

UV–visible spectra were measured on an HP diode array spectrophotometer, model no. 8452A. The concentration of each was 40 μM in pH 7.5 aqueous buffer (50 mM Tris-HCl containing 10 mM $MgCl_2$). The parameters for the compounds are as follows: **17a**, λ 276 nm (ε = 9890 L/(mol·cm)); **17b**, λ 276 nm (ε = 7400 L/(mol·cm)); 18, λ 274 nm (ε = 16 100 L/(mol·cm)), λ 650 nm (ε = 44900 L/(mol·cm)). Fluorescence spectra were measured on a PerkinElmer LS503: excitation λ_{max} = 646 nm, emission λ_{max} = 660 nm.

Receptor Binding and Functional Assays

[^{3}H]2-Chloro-N^6-cyclopentyladenosine (**52**, [^{3}H]CCPA, 42.6 Ci/mmol) was a custom synthesis product (Perkin-Elmer). [^{125}I]N^6-(4-Amino-3-iodobenzyl)adenosine-5′-N′-methyluronamide (**53**, [^{125}I]I-AB-MECA, 2200 Ci/mmol) and [^{3}H](2-[p-(2-carboxyethyl)-phenylethylamino]-5′-N′-ethylcarboxamidoadenosine) (**54**, [^{3}H] CGS21680, 40.5 Ci/mmol) were purchased from Perkin-Elmer Life and Analytical Sciences (Boston, MA). Test compounds were prepared as 5 mM stock solutions in DMSO and stored frozen.

Cell Culture and Membrane Preparation

CHO cells stably expressing the recombinant hA_1, hA_3, and rA_3Rs and HEK-293 cells stably expressing the hA_{2A}AR were cultured in Dulbecco's modified Eagle medium (DMEM) and F12 (1:1) supplemented with 10% fetal bovine serum, 100 units/mL penicillin, 100μg/mL streptomycin, and 2 μmol/mL glutamine. In addition, 500 μg/mL Geneticin was added to the A_{2A} media, while 800 μg/mL hygromycin was added to the A_1 and A_3 media. After being harvested, cells were homogenized and suspended in PBS. Cells were then centrifuged at 240*g* for 5 min, and the pellet was resuspended in 50 mM Tris-HCl buffer (pH 7.5) containing 10 mM $MgCl_2$. The suspension was homogenized and was then ultracentrifuged at 14,330 *g* for 30 min at 4 °C. The resultant pellets were resuspended in Tris buffer and incubated with adenosine deaminase (3 units/mL) for 30 min at 37 °C. The suspension was homogenized with an electric homogenizer for 10 s, pipetted into 1 mL vials, and then stored at −80 °C until the binding experiments. The protein concentration was measured using the BCA protein assay kit from Pierce Biotechnology, Inc. (Rockford, IL).[49]

Binding Assays

Into each tube in the binding assay was added 50 μL of increasing concentrations of the test ligand in Tris-HCl buffer (50 mM, pH 7.5) containing 10 mM $MgCl_2$, 50 μL of the appropriate agonist radioligand, and finally 100 μL of membrane suspension. For the A_1AR

(22 μg of protein/tube) the radioligand used was [^{3}H]**51** (final concentration of 3.5 nM) or [^{3}H]**52** (final concentration of 1.0 nM). For the A_{2A}AR (20 μg/tube) the radioligand used was [^{3}H]**53** (10 nM). For the A_3AR (21 μg/tube) the radioligand used was [^{125}I]**54** (0.34 nM). Nonspecific binding was determined using a final concentration of 10 μM **51** diluted with the buffer. The mixtures were incubated at 25 °C for 60 min in a shaking water bath. Binding reactions were terminated by filtration through Brandel GF/B filters under reduced pressure using a M-24 cell harvester (Brandel, Gaithersburg, MD). Filters were washed three times with 3 mL of 50 mM ice-cold Tris-HCl buffer (pH 7.5). Filters for A_1 and A_{2A}AR binding were placed in scintillation vials containing 5 mL of Hydrofluor scintillation buffer and counted using a Perkin-Elmer liquid scintillation analyzer (Tri-Carb 2810TR). Filters for A_3AR binding were counted using a Packard Cobra II γ-counter. The K_i values were determined using GraphPad Prism for all assays.

cAMP Accumulation Assay

Intracellular cAMP levels were measured with a competitive protein binding method.[34] CHO cells that expressed the recombinant hA_3AR were harvested by trypsinization. After centrifugation and resuspended in medium, cells were planted in 24-well plates in 1.0 mL medium. After 24 h, the medium was removed and cells were washed three times with 1 mL of DMEM, containing 50 mM HEPES, pH 7.4. Cells were then treated with the agonist **51** or test compound in the presence of rolipram (10 μM) and adenosine deaminase (3 units/mL). After 45 min forskolin (10 μM) was added to the medium, and incubation was continued for an additional 15 min. The reaction was terminated by removing the supernatant, and cells were lysed upon the addition of 200 μL of 0.1 M ice-cold HCl. The cell lysate was resuspended and stored at −20 °C. For determination of cAMP production, 100 μL of the HCl solution was used in the Sigma direct cAMP enzyme immunoassay following the instructions provided with the kit. The results were interpreted using a Bio-Tek Instruments ELx808 Ultra microplate reader at 405 nm.

Molecular Modeling

The Prime program of the Schrodinger package[48] was utilized to build a new molecular model of the hA_3AR. The recently reported model of the complex of the nonselective AR agonist **51** docked to the crystal structure of the A_{2A}AR was used as a template for modeling of the A_3AR.[38] The sequence alignment of the A_{2A} and A_3ARs was performed with Prime, taking into account positions of highly conserved amino acid residues. The default values of all Prime parameters were used. The position of full agonist **51** inside the A_{2A} receptor was taken into account during the modeling. Thus, the resulting model of the A_3AR contained **51** prepositioned inside the receptor. The Glide program of the Schrodinger package was used to redock **51** to the A_3AR model obtained. The receptor grid generation was performed for the box with a center in the centroid of **51** in its initial position. The size of the box was determined automatically. The extra precision mode (XP) of Glide was used for the docking. The ligand scaling factor was set to 1.0. The geometry of the ligand binding site of the complex of A_3AR with **51** docked was optimized. The binding site was defined as **51** and all amino acid residues located within 5 Å from **51**. All A_3AR residues located within 2 Å from the binding site were used as a shell. The following parameters of energy minimization were used: OPLS2005 force field; water was used as an implicit solvent; a maximum of 5000 iterations of the Polak–Ribier conjugate gradient minimization method was used with a convergence threshold of 0.01 kJ·mol^{-1} ·A^{-1}. Another reference nucleoside, 5′-*N*′-methylcarboxamidoadenosine, was also docked in this hA_3AR homology model (Supporting Information).

The A_3AR model obtained was utilized to study a binding mode of amine congener **15** using InducedFit docking implemented in the Schrödinger package. The grid generation was

performed for the box with a side of 32 Å with a center in the centroid of **51**. The default values were used for other parameters.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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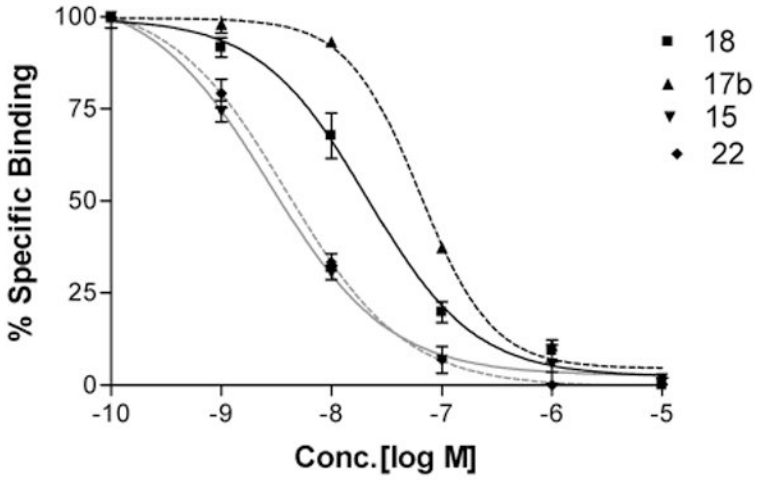


Figure 1.

Inhibition of radioligand binding by (N)-methanocarba nucleoside analogues in membranes of CHO cells expressing the human A_3AR. Inhibition curves are shown for the agonist analogues containing a 5′-*N*-methyluronamide group and compounds **15** (amine functionalized congener), **17b** (biotinylated probe), and **18** (fluoresecent probe) and for the truncated partial agonist **22**. All of the analogues shown were highly selective for the A_3AR in comparison to the A_1 and A_{2a}ARs.

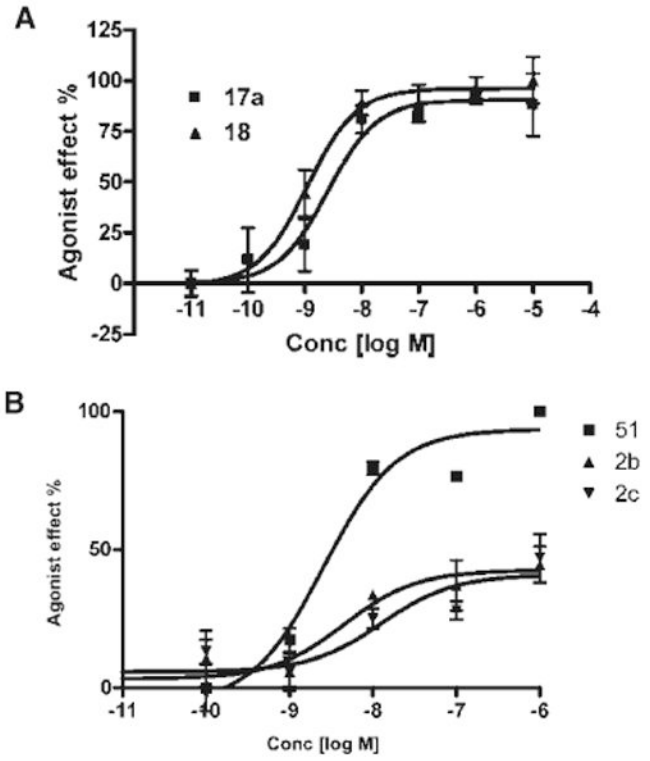


Figure 2.

Functional agonism by the tested in an assay of adenylate cyclase in membranes of CHO cells expressing the hA_3AR. Each experiment was repeated three times. (A) Activity of the *5′-N*-methyluronamide (N)-methanocarba analogues **17a** and **18**. The EC_{50} values of **17a** and **18** were 2.50 ±0.42 and 1.09 ±0.28 nM, respectively. (B) Activity of the truncated (N)-methanocarba analogues **2b** and **2c** at the hA_3AR. The full agonist **51** was included for comparison (representing 100% efficacy). The percent relative efficacy of **2b** and **2c** was 46 ± 4% and 44 ± 6%, respectively. The EC_{50} values of **2b** and **2c** were 4.2± 0.6 and 12± 1 nM, respectively.

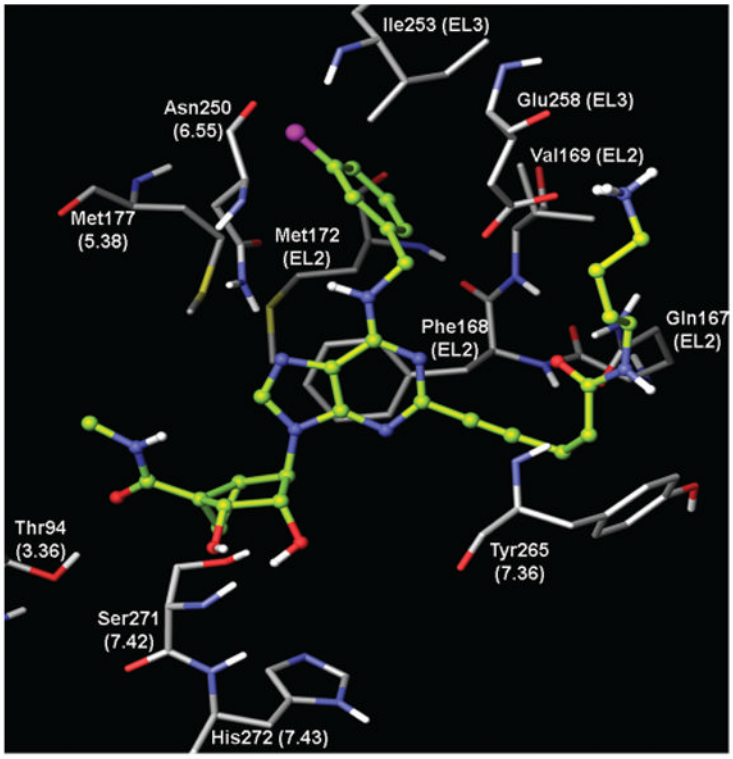
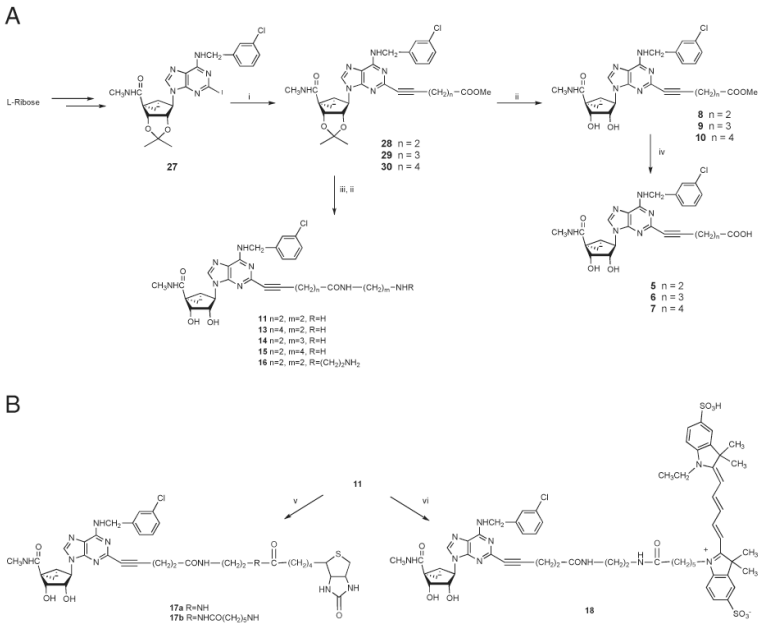
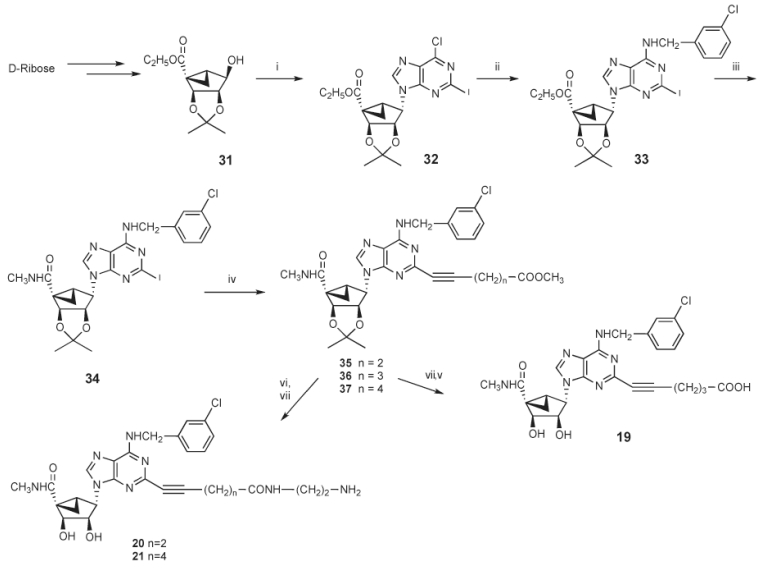


Figure 3.
Binding mode of amine congener **15** in the 5′-*N*-methyluronamide series obtained after InducedFit docking to the A_3AR homology model built based on the crystal structure of the A_{2A}AR.



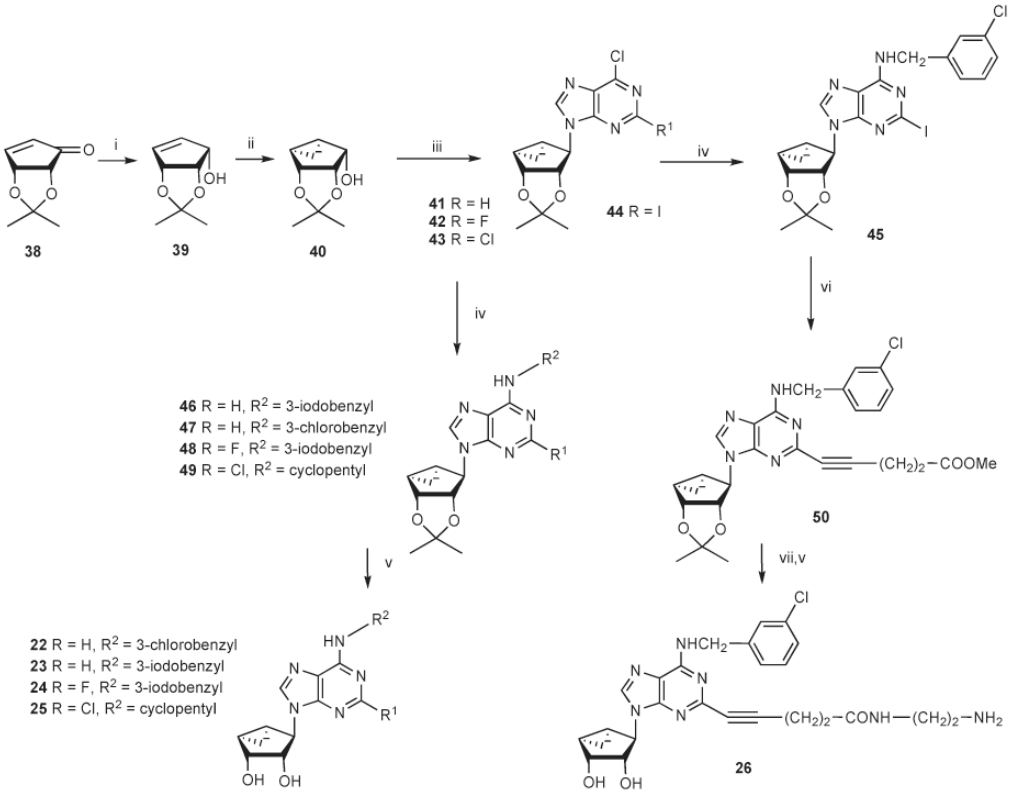
Scheme 1a.

[a] (A) (i) $HC{\equiv}C(CH_2)_nCOOMe$, $Pd(PPh_3)_2Cl_2$, CuI, Et_3N, DMF, room temp; (ii) 10% TFA, MeOH, 70 °C; (iii) appropriate alkyldiamine, MeOH, room temp; (iv) KOH, MeOH, room temp. (B) (v) For **17a**: biotin, HATU, DIEA, DMF, room temp. For **17b**: biotin-ε-aminocaproyl *N*-succinimidyl ester, Et_3N, DMF, room temp. (vi) *N*-succinimidyl ester of chain-derivatized Cy5 cyanine dye, bicarbonate buffer, DMF.



Scheme 2 a.

[a] (i) 2-Iodo-6-chloropurine, Ph_3P, DIAD, THF, room temp; (ii) 3-chlorobenzylamine, Et_3N, MeOH, room temp; (iii) 10% methylamine, MeOH, room temp; (iv) $HC{\equiv}C(CH_2)_nCOOMe$, $Pd(PPh_3)_2Cl_2$, CuI, Et_3N, DMF, room temp; (v) KOH, MeOH; (vi) ethylenediamine, MeOH, room temp; (vii) 10% TFA, MeOH, 70 °C.



Scheme 3a.

[a] (i) $NaBH_4$, $CeCl_3 \cdot 7H_2O$, MeOH, 0 °C; (ii) Et_2Zn, CH_2I_2, CH_2Cl_2, room temp; (iii) 2-substituted-6-chloropurine, Ph_3P, DIAD, THF, room temp; (iv) 3-iodo- or 3-chlorobenzylamine or cyclopentylamine, Et_3N, MeOH, room temp; (v) 10% TFA, MeOH, 70 °C; (vi) $HC{\equiv}C(CH_2)_2COOMe$, $Pd(PPh_3)_2Cl_2$, CuI, Et_3N, DMF, room temp; (vii) ethylenediamine, MeOH, room temp.

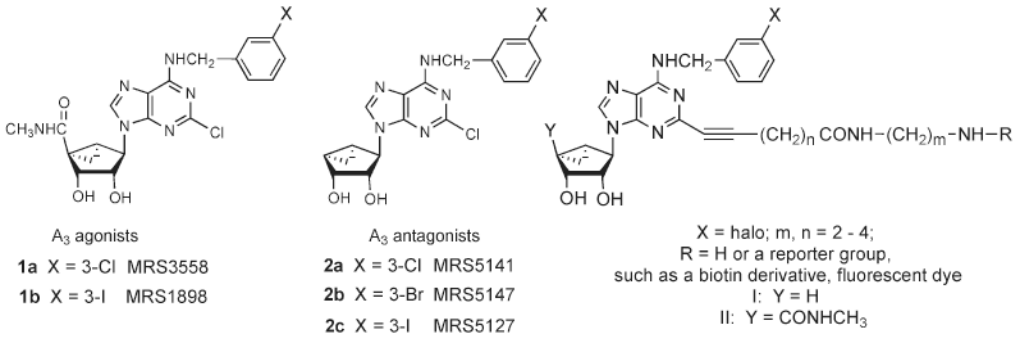


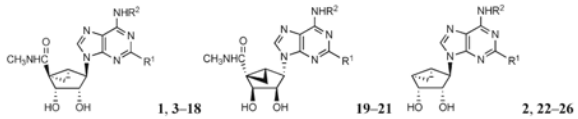
Chart 1.

(N)-Methanocarba Derivatives of Adenosine as A_3AR-Selective Agonists (**1a, 1b)** and Antagonists (**2a, 2b**)[a]

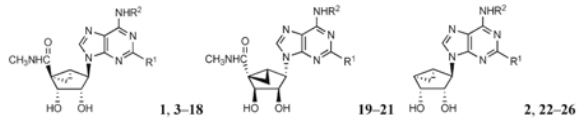
[a]General formulas **I** and **II** represent two parallel structural series included in the present study. The agonist corresponding to X = Cl, *n* = 3, *m* = 2, R = H in series **II** was already reported.[21]

Table 1

Potency of a Series of (N)-Methanocarba Adenosine Derivatives at Three Subtypes of Human ARs and the Functional Efficacy at the A_3AR



	structure		affinity (K_i, nM) or % inhibition[a]			
compd	**R^1**	**R^2**	**A_1**	**A_{2A}**	**A_3**	**% efficacy,[b] A_3**
		Series A				
1a[c]	Cl	3-Cl-Bn	260 ± 60[h]	2300 ±100	0.29 ± 0.04	103 ± 7
1b	Cl	3-I-Bn	136 ± 22[d,h]	784 ± 97[d]	1.5 ± 0.2[c]	100[c]
3[c]	H	3-I-Bn	700 ± 270[h]	6200 ± 100	2.4 ± 0.5	100
4[c]	Cl	cyclopentyl	18.3 ± 6.3[h]	3250 ± 300	3.7 ± 0.9	101
5	$C{\equiv}C(CH_2)_2COOH$	3-Cl-Bn	(17 ± 4%)	(24 ± 12%)	61.1 ± 35.8	106 ±18
6[c,e]	$C{\equiv}C(CH_2)_3COOH$	3-Cl-Bn	14,900 ± 3500[h]	(43%)	2.38 ± 0.56	ND
7	$C{\equiv}C(CH_2)_4COOH$	3-Cl-Bn	(11 ± 5%)	(38 ± 2%)	12.4 ± 1.8	74.4 ± 5.1
8	$C{\equiv}C(CH_2)_2COOCH_3$	3-Cl-Bn	(41 ± 0%)	(43 ± 5%)	5.5 ± 0.3	90.0 ± 6.2
9[c]	$C{\equiv}C(CH_2)_3COOCH_3$	3-Cl-Bn	482 ± 23[h]	(49%)	1.17 ± 0.27	ND
10	$C{\equiv}C(CH_2)_4COOCH_3$	3-Cl-Bn	(20 ± 7%)	3540 ± 1370	11.1 ± 2.3	85.0 ± 10.2
11	$C{\equiv}C(CH_2)_2CONH(CH_2)_2NH_2$	3-Cl-Bn	2320 ± 520	550 ± 83	2.5 ± 0.5	96.9 ± 6.8
12[c]	$C{\equiv}C(CH_2)_3CONH(CH_2)_2NH_2$	3-Cl-Bn	454 ± 44[h]	(81%)	2.17 ± 0.51	ND
13	$C{\equiv}C(CH_2)_4CONH(CH_2)_2NH_2$	3-Cl-Bn	(47 ± 3%)	277 ± 33	3.4 ± 0.7	100 ± 2
14	$C{\equiv}C(CH_2)_2CONH(CH_2)_3NH_2$	3-Cl-Bn	(31 ± 9%)	979 ± 181	3.1 ± 0.4	97.9 ± 18.4
15	$C{\equiv}C(CH_2)_2CONH(CH_2)_4NH_2$	3-Cl-Bn	(30 ± 2%)	766 ± 109	2.1 ± 0.4	102 ± 13
16	$C{\equiv}C(CH_2)_2CO[NH(CH_2)_2]_2NH_2$	3-Cl-Bn	(33 ± 2%)	890 ± 110	15.4 ± 4.4	87.6 ± 21.2
17a	$C{\equiv}C(CH_2)_2CONH(CH_2)_2NH$-biotin	3-Cl-Bn	(1 ± 1%)	(51 ± 2%)	36.4 ± 5.6	84.5 ± 12.0
17b	$C{\equiv}C(CH_2)_2CONH(CH_2)_2NH\text{-}CO(CH_2)_5NH$-biotin	3-Cl-Bn	(12 ± 4%)	(47 ± 11%)	57.7 ± 16.2	107 ± 18
18	$C{\equiv}C(CH_2)_2CONH(CH_2)_2NH\text{-}CO\text{-}(CH_2)_5Cy5$[g]	3-Cl-Bn	(36 ± 3%)	4730 ± 1020	17.2 ± 3.1	94.4 ± 9.6



	structure		affinity (K_i, nM) or % inhibition[a]			
compd	**R[1]**	**R[2]**	**A_1**	**A_{2A}**	**A_3**	**% efficacy,[b] A_3**
		Series B				
19	C≡C(CH$_2$)$_3$COOH	3-Cl-Bn	(0%)	(9 ± 2%)	5270 ± 1090	ND
20	C≡C(CH$_2$)$_2$CONH(CH$_2$)$_2$NH$_2$	3-Cl-Bn	(33 ± 7%)[h]	(8 ± 1%)	(30 ± 1%)	25.6 ± 10.9
21	C≡C(CH$_2$)$_4$CONH(CH$_2$)$_2$NH$_2$	3-Cl-Bn	(36 ± 5%)[h]	(5 ± 1%)	4630 ± 1060	52.0 ± 9.4
		Series C				
2a[d]	Cl	3-Cl-Bn	3070 ± 1500[h]	4510 ± 910	1.06 ± 0.36	2.9 ± 3.7[f]
2b[d,e]	Cl	3-Br-Bn	1760 ± 1010[h]	1600 ± 480	0.73 ± 0.30	46 ± 4,5.8 ± 0.8[f]
2c[d,e]	Cl	3-I-Bn	3040 ± 610[h]	1080 ± 310	1.44 ± 0.60	44 ± 6, 1.0 ± 3.2[f]
22	H	3-Cl-Bn	1600 ± 150	4520 ± 830	4.9 ± 0.7	58.3 ± 8.0
23	H	3-I-Bn	839 ± 68	2330 ± 680	11.0 ± 2.2	80.8 ± 19.0
24	F	3-I-Bn	513 ± 4	4000 ± 780	10.7 ± 0.9	19.0 ± 3.0
25	Cl	cyclopentyl	109 ± 16	1640 ± 360	120 ± 31	95.1 ± 4.6
26	C≡C(CH$_2$)$_2$CONH(CH$_2$)$_2$NH$_2$	3-Cl-Bn	(15 ± 2%)	(35 ± 6%)	404 ± 67	0.9 ± 8.5

[a] All experiments were done on CHO or HEK293 (A_{2A} only) cells stably expressing one of four subtypes of human ARs. The binding affinity for A_1, A_{2A}, and A_3 ARs was expressed as K_i values (n = 3–5) and was determined by using agonist radioligands ([^{3}H]**51**, [^{3}H]**54**, or [^{125}I]**53**, respectively), unless otherwise noted. A percent in parentheses refers to inhibition of radioligand binding at 10 μM.

[b] Unless otherwise noted, the efficacy at the human A_3AR was determined by inhibition of forskolin-stimulated cAMP production in AR-transfected CHO cells. At a concentration of 10 μM, in comparison to the maximal effect of **51** (= 100%) at 10 μM. Data are expressed as mean ± standard error (n = 3). ND, not determined.

[c] Values from Melman et al.; Lee et al.; Tchilibon et al.[15,16,21]

[d] Values from Melman et al.[20]

[e] **2b**, MRS5147; **2c**, MRS5127; **6**, MRS5151.[21,33]

[f] A_3AR functional assay consisted of stimulation of [^{35}S]GTPγS binding at 10 μM, expressed as a percentage of the full effect induced by 10 μM **51** (100 ± 5%).

[g] Structure given in Scheme 1B.

^{h}K_i at A_1AR binding determined using [^{3}H]**52**.