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Rational Design of Sulfonated A₃ Adenosine Receptor-Selective Nucleosides as Pharmacological Tools to Study Chronic Neuropathic Pain

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Abstract

(N)-Methanocarba (bicyclo[3.1.0]hexane)-adenosine derivatives were probed for sites of charged sulfonate substitution, which precludes diffusion across biological membranes, e.g. blood brain barrier. Molecular modeling predicted that sulfonate groups on C2-phenylethynyl substituents would provide high affinity at both mouse (m) and human (h) A₃ adenosine receptors (ARs), while a *N*⁶-*p*-sulfo-phenylethyl substituent would determine higher hA₃AR vs. mA₃AR affinity. These modeling predictions, based on steric fitting of the binding cavity and crucial interactions with key residues, were confirmed by binding/efficacy studies of synthesized sulfonates. *N*⁶-3-Chlorobenzyl-2-(3-sulfophenylethynyl) derivative **7** (MRS5841) bound selectively to h/m A₃ARs (K_i hA₃AR 1.9 nM) as agonist, while corresponding *p*-sulfo isomer **6** (MRS5701) displayed mixed A₁/A₃AR agonism. Both nucleosides administered i.p. reduced mouse chronic neuropathic pain that was ascribed to either A₃ or A₁/A₃ARs using A₃AR genetic deletion. Thus, rational design methods based on A₃AR homology models successfully predicted sites for sulfonate incorporation, for delineating adenosine's CNS vs. peripheral actions.

Keywords

Molecular modeling; G protein-coupled receptor; neuropathic pain; purines; radioligand binding; adenosine receptor

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Supporting Information Available:

Modeling Figures S1 and S2, reaction Scheme S1, selected spectra of synthesized compounds and 3D coordinates of the modeled hA₃AR and mA₃AR complexes with **7**. This material is available free of charge via the Internet at <http://pubs.acs.org>.

Introduction

G protein-coupled receptors (GPCRs), which constitute ~4% of the human genome's protein production, remain a fruitful area of pharmaceutical research due to their implication in many pathophysiological processes. The neuromodulator adenosine acts through four closely related GPCRs: A₁, A_{2A}, A_{2B} and A₃ adenosine receptors (ARs).¹ The A₃ subtype was the last member of the AR family to be cloned and has become a target in drug discovery for various disease conditions.² Agonists of A₃AR appear to be efficacious, without serious adverse effects, in clinical trials for treatment of hepatocellular carcinoma (**1b**) and autoimmune inflammatory diseases (**1a**) such as rheumatoid arthritis, psoriasis, and dry eye disease.^{3–7} A₃AR agonists are also under consideration for treating conditions of the central nervous system (CNS) and peripheral nervous system.^{3,13} Additionally, A₃AR-selective antagonists have been explored in models of glaucoma, septic shock, asthma and other diseases.^{8–12}

Recently, the treatment of chronic neuropathic pain was proposed as a novel potential application of A₃AR agonists.¹³ In mouse models, A₃AR agonists blocked the development of mechanically- and chemotherapy-induced neuropathic pain in a dose-dependent fashion and significantly augmented the analgesic effects of currently used analgesics. The ability of adenosine to reduce pain by activating A₁ and A_{2A} ARs has been known for decades, but the clinical development of A₁AR and A_{2A}AR agonists is largely restricted to local delivery due to the risk of cardiovascular side effects.¹⁴ Conversely, the activation of A₃AR in humans by potent, selective, and orally bioavailable A₃AR agonists, is not associated with cardiac or hemodynamic side effects and could represent a viable therapeutic strategy for chronic pain of distinct etiologies.^{13,15} There is evidence for both central and peripheral sites of action of A₃AR agonists in protection against chronic neuropathic pain, and there is a need for new pharmacological tools to explore this phenomenon.

The structure activity relationship (SAR) of nucleosides at the A₃AR has been extensively explored, and modification of the adenosine structure has centered on the adenine C2 and N⁶ positions and on the ribose moiety (Chart 1).^{2,16} 2-Chloro-N⁶-(3-iodobenzyl)adenosine-5'-N-methyluronamide (CI-IB-MECA, **1b**) was the first potent and highly selective A₃AR agonist reported.¹⁷ The replacement of the flexible ribose tetrahydrofuryl ring with a conformationally constrained bicyclo[3.1.0]hexane (methanocarba) ring system, which enforces a North (N)-envelope conformation, maintains or enhances affinity at A₁ and A₃ARs with respect to the ribosides, while decreasing affinity at the A_{2A}AR (compound **2**).^{18,19}

The truncation of nucleosides at the 4'-carbon, i.e. removal of the 5'-group, is another extensively explored modification in various chemical series and generally results in retention of A₃AR affinity and selectivity with concurrent reduction in efficacy at A₃AR, generating antagonists or low efficacy agonists (compound **3**).^{20–23} In this truncated series, the extent to which the efficacy is reduced to provide either partial agonists or antagonists can depend on the species, the effector system being measured and the level of receptor expression. Such nucleoside derivatives that are classified as antagonists in one effector system (e.g. cAMP) might show greatly increased efficacy by other measures or in the presence of positive allosteric modulators.²⁴

We recently demonstrated that the introduction of rigid, extended C2-arylalkynyl groups in (N)-methanocarba adenosine derivatives, both substituted with a 5'-N-methyluronamide and 4'-truncated, is tolerated at the human (h) A₃AR (compound **4**).^{25,26} Previously, C2-phenylalkynyl groups were included in potent A₃AR agonists in the relatively flexible 9-ribose series.²⁷ Other groups that enhance affinity and selectivity in the SAR of

nucleosides at the A₃AR include hydrophobic substituents at the N⁶ position, such as N⁶-(3-halobenzyl) groups or small N⁶-alkyl groups.^{17,27} N⁶-Methyl derivatives are typically more potent at the hA₃AR than rat (r) A₃AR, while N⁶-(3-halobenzyl)adenosines tend to display greater species-independent A₃ selectivity.²⁸

Using the known SAR data for nucleosides at the A₃AR, this study probes possible sites of insertion of a sulfonate group in the (N)-methanocarba-adenosine series with the aim of developing novel A₃AR ligands to be used as pharmacological tools. A negatively charged sulfonate substitution of small molecules is one means of excluding diffusion across biological membranes such as the blood brain barrier.^{29–31} Such potent and selective A₃AR agonists or antagonists would be useful in delineating in vivo effects in the CNS from those in the periphery, depending on the route of administration.

Previously investigated sulfonated nucleosides for ARs include N⁶-(*p*-sulfophenyl)adenosine as a moderately potent and selective A₁AR agonist and recently reported C2-(*p*-sulfophenyl)ethylthio-adenosine as an A_{2A}AR agonist.^{29,30} Moreover, sulfonated derivatives of 1,3-dialkylxanthines (on a C8-phenyl ring) act as peripherally selective A₁AR antagonists because they were found not to penetrate into brain after intraperitoneal (i.p.) injection in mice.³¹ However, our previous attempt to develop sulfonated A₃AR ligands through the introduction of an aryl sulfonate group on a N⁶-benzyl substituent of adenosine-5'-*N*-methyluronamide was unsuccessful leading to compounds with very low affinity at rARs.³²

Consequently, this study uses currently available three-dimensional (3D) information about the AR family to predict sites at which sulfonate substitution is tolerated or even potency enhancing. We apply receptor-based molecular modeling approaches to test in silico sites for the introduction of permanently charged sulfonate groups in both the 5'-*N*-methyluronamide series and the 4'-truncated series of 2-phenylethynyl (N)-methanocarba adenosines for use as A₃AR ligands. We consider not only the binding site features of the hA₃AR but also of the mouse (m) A₃AR, with the intent of developing compounds with species-independent A₃ affinity so that they can be useful in in vivo studies frequently performed in mouse models.^{8,10,13}

In the present study, nucleosides with various substitutions at the C2 and N⁶ positions were synthesized, guided by insights from molecular modeling. The receptor binding properties and functional effects of these rationally designed sulfonated ligands at both h and mA₃ARs have been characterized. Even though their in vivo pharmacokinetics has not been quantified, based on physicochemical considerations, we expect that these permanently anionic compounds will not easily diffuse across biological membranes, resulting in useful probes for delineating the CNS vs. peripheral actions of adenosine. Moreover, two isomeric sulfonate derivatives with different pharmacological profiles have been tested in a well-characterized mouse model of neuropathic pain to determine their efficacy in reversing the development of mechano-allodynia.

Results

Molecular Modeling

In order to design sulfonated nucleoside ligands for the A₃AR belonging to the relatively rigid (N)-methanocarba series, we started from the SAR data already available for this class of compounds and from the available 3D information regarding the AR family. So far, the only published crystallographic data for this family of four GPCRs was obtained from the hA_{2A}AR, whose structure has been solved in complex with both antagonists and agonists.^{33,34} Therefore, to study the putative interactions of (N)-methanocarba nucleosides

with the A₃AR, we built homology models of the h and mA₃AR subtypes based on their close homology to the A_{2A}AR X-ray structures.

As previously reported, the C2-arylalkynyl (N)-methanocarba derivatives were unable to fit inside the binding pocket of the initial hA₃AR model, based on the agonist-bound hA_{2A}AR X-ray structure, due to the bulkiness of the substituents at the C2 position.^{25,26} However, a hybrid model of the A₃AR predicted that a reorganization of the helical bundle, mimicking the structures of opsin and the β_2 adrenergic receptor through a proposed outward movement of transmembrane helix (TM) 2, was needed to accommodate the inflexible and linearly extended C2-arylalkynyl group.

Therefore, we performed docking studies of several (N)-methanocarba nucleosides (compounds **5–20**) using homology models of the h and mA₃ARs based on a hybrid template where the extracellular part of TM2 was modeled following the structure of the β_2 adrenergic receptor in an active state.³⁵ In these hybrid models as compared to the A₃AR models based exclusively on the A_{2A}AR crystal structure, the extracellular terminal of TM2 was shifted outward, which created a larger pocket for the accessibility of C2 substituents of the present (N)-methanocarba nucleosides.²⁵

Alignment of the h and mA₃AR sequences (~73% identity) together with an analysis of the docking results revealed that the lower (cytosolic) part of the transmembrane binding site is highly conserved among species, while significant differences are present in the residues of the very upper part of the TMs and of the extracellular loops (ELs, Figure S1, Supporting Information). Notably, a hydrophobic valine residue in EL2 of the hA₃AR (Val169) is mutated to a bulkier and positively charged arginine in the mA₃AR (Arg170), giving a very different electrostatic and steric character to the entrance of the A₃AR binding site (Figure S2, Supporting Information). Residues at this position of the ARs were shown by mutagenesis studies and crystallographic data to be involved in the binding of ligands (e.g. Glu169 in EL2 of the hA_{2A}AR)^{33,36} and were proposed to determine selectivity among different AR subtypes.³⁷

The performed molecular docking simulations of (N)-methanocarba derivatives showed a common binding mode of the sulfonated compounds inside the h and mA₃AR binding pockets (Figures 1 and 2), at least with respect to the adenine and ribose moieties. This binding mode presented most of the key interactions previously observed in the crystal structures and docking poses of nucleosides at the ARs.^{25,34} In particular, the 3'- and 2'-hydroxyl groups formed H-bonds with the side chains of Ser 7.42 (Ser271 in hA₃AR and Ser272 in mA₃AR) and His 7.43 (His272 in hA₃AR and His273 in mA₃AR), respectively; while the 5'-N-methyluronamide chain was interacting through a H-bond with the side chain hydroxyl group of Thr 3.36 (Thr94 in hA₃AR and Thr95 in mA₃AR). The side chain of conserved Asn 6.55 (Asn250 in hA₃AR and Asn251 in mA₃AR) strongly interacted with these compounds through two H-bonds involving the 6-amino group and the N⁷ atom of the adenine ring. Moreover, the adenine core was anchored inside the binding site by a π - π stacking interaction with a phenylalanine in EL2 (Phe168 in hA₃AR and Phe169 in mA₃AR) and strong hydrophobic contacts with Leu 6.51 (Leu246 in hA₃AR and Leu247 in mA₃AR) and Ile 7.39 (Ile268 in hA₃AR and Ile269 in mA₃AR). It has to be noted that the lack of a 5'-methyluronamide chain prevented the 4'-truncated (N)-methanocarba derivatives from interacting with the threonine residue in TM3, resulting in a missing H-bonding interaction (Figure 2).

In the binding mode described above, the extended substituents at the N⁶ and C2 positions were located in the less ordered extracellular portion of the hA₃AR binding pocket and therefore were judged to be suitable sites for insertion of a sulfonate group on these (N)-

methanocarba derivatives. Thus, we designed *N*⁶-3-chlorobenzyl derivatives with a *p*- or *m*-sulfo-phenylethynyl group at the C2 position (**6** and **7**) and *N*⁶-*p*-sulfo-phenylethyl C2-phenylethynyl derivatives (**16** and **18**) and docked them at our validated h and mA₃AR models.

Figure 1 shows the hypothetical binding modes of compounds **6** and **7** inside the h and mA₃AR binding pockets. The sulfonated phenylethynyl substituent at the C2 position of these analogs was oriented toward TM2 and EL1 and was anchored by favorable hydrophobic interactions with residues of the upper part of the binding site. Moreover, either the *m*- or *p*-sulfonate group on the C2 substituent was predicted to form H-bonds with Gln167 (EL2) and Tyr265 (7.36) at the hA₃AR (Figure 1, panels A and B). Both of these residues differed in the mA₃AR sequence, and in this case the sulfonate groups of compounds **6** and **7** would interact with Ser74 (EL1) or His168 (EL2) and Arg170 (EL2) (Figure 1, panels C and D). Considering the fact that the binding modes of compounds **6** and **7** presented all the key interactions important for anchoring the (N)-methanocarba series with a good accommodation of the C2 group, both *m*- and *p*- insertion of a sulfonate group on the C2-phenylethynyl substituent should be tolerated for the binding at both h and mA₃ARs.

On the other hand, in the binding poses of compounds **16** and **18** at the h and mA₃ARs (Figure 2), the sulfonated *N*⁶-phenylethyl substituent was accommodated in a side pocket surrounded by TM5, TM6 and EL2. Residues delimiting this area diverge between h and mA₃ARs, being Val169 (EL2), Met172 (EL2), Arg173 (EL2), Met174 (5.35), Met177 (5.38) and Ile253 (6.58) at the hA₃AR, and Arg170 (EL2), Val173 (EL2), Ser174 (EL2), Leu175 (5.35), Met178 (5.38) and Ser254 (6.58) at the mA₃AR. At the hA₃ subtype, the sulfonated *N*⁶ substituent of compounds **16** and **18** fit well in this area and formed ionic interactions with Arg173 (EL2) and a H-bond with the backbone of Met174 (5.35), while the rest of the ligand structure interacted with all the crucial residues in the deeper regions of the binding site (Figure 2, panels A and B). At the mA₃AR this sulfonate group formed ionic interactions with Arg170 (EL2) and with another arginine residue located in the upper part of EL2 (Arg152). However, these *N*⁶-*p*-sulfo-phenylethyl derivatives **16** and **18** were unable to strongly interact with Asn251 (6.55) because the adenine core was shifted to accommodate the bulky *N*⁶ substituent (Figure 2, panels C and D). The difference in the behavior of these compounds between the h and mA₃AR subtypes, seemed to be mainly related to the mutation Val169/Arg170 in EL2 and to the resulting different orientations of ligands in the binding sites. Therefore, we expected the *N*⁶-*p*-sulfo-phenylethyl derivatives to be relative less potent at the mA₃AR as compared to the hA₃AR. The detrimental effect of the putative missing interaction with Asn251 (6.55) for these derivatives at the mA₃AR would be even more relevant for the affinity of the 4'-truncated compound **16**, due to the additional missing H-bond with the threonine in TM3 (Figure 2, panel D).

To analyze in a more quantitative way the observed different interactions of these derivatives at the h and mA₃ARs, we calculated per-residue interaction energies from the reported ligand-receptor complexes. Figure 2, panel E, highlights the contributions to the interaction energy of selected key residue of the binding sites. In agreement with the analysis of the docking poses, the favorable contribution to the interaction energy of Asn 6.55 was much stronger for the complexes of compounds **16** and **18** with the hA₃AR as compared to the mA₃AR. While, the contribution of Thr 3.36 was significantly weaker, at both the h and mA₃ARs, for the 4'-truncated compound **16** as compared to the 5'-methylluronamide compound **18**.

Chemical Synthesis

The synthetic routes used to prepare the new sulfonated and nonsulfonated (N)-methanocarba adenosine analogues **6**, **7**, **17**, and **18** (Scheme 1) and truncated derivative **16** (Scheme 2) are shown. The synthetic approaches followed the general schemes reported by Tosh et al.^{25,26} Intermediate **19** was subjected to sequential displacement of the 6-Cl group in the presence of appropriate amines at room temperature followed by aminolysis of the 5' ester with 40% methylamine solution provided the corresponding amide derivatives (**23–25**). Sonogashira coupling of iodo derivatives (**23–25**) with sulfonated and non-sulfonated phenylacetylenes in the presence of PdCl₂(Ph₃P)₂, CuI, triethylamine and subsequent acid hydrolysis of the isopropylidene group afforded the final nucleoside derivatives (**6**, **7**, **17** and **18**). The intermediate **31** (Scheme 2) in the truncated series, was treated with 4-(2-aminoethyl)benzenesulfonic acid in the presence of triethylamine following a similar sequence including a displacement reaction at 6-Cl as in compound **19** (Scheme 1) to provide compound **32**. Sonogashira coupling of **32** with phenylacetylene followed by acid hydrolysis with aqueous trifluoroacetic acid yielded compound **16**. The sulfonated phenylacetylenes **38** (*p*) and **39** (*m*) used in the preparation of **6** and **7**, respectively, were prepared as shown in Scheme S1 (Supporting Information).

Pharmacological Evaluation

The synthesized analogues (Table 1) were assayed using radioligand binding at three hAR subtypes and at three mA₃AR subtypes using standard ³H and ¹²⁵I-labeled nucleoside ligands of high affinity (**40 – 42**).^{25,26} The hA₁AR and hA₃AR were stably expressed in CHO cells, and the hA_{2A}AR and the mA₃ARs were stably expressed in HEK293 cells. The (N)-methanocarba ring modification was previously shown to reduce potency at the A_{2B}AR, therefore these nucleosides were not assayed at this AR subtype.³⁸ The data for hA₃AR binding for **5** and **8–15** were already published,²⁶ and the data for these compounds at the mA₃AR were determined here. Functional potency and efficacy at the A₃AR have been determined for several derivatives including the sulfonated (N)-methanocarba derivatives and for some previously reported analogs (Figure 3).

The previously reported *N*⁶-3-chlorobenzyl C2-phenylethynyl derivative **5** displayed identically high affinity and selectivity at both the h and mA₃ARs (K_i hA₃AR = 1.3 nM; K_i mA₃AR = 1.2 nM), while being nearly inactive at the A₁ and A_{2A}ARs (h and m). The C2-*p*-sulfo-phenylethynyl analogue **6** displayed a K_i value of 11.1 nM in binding to the hA₃AR and also a comparable affinity at the mA₃AR (K_i = 9.7 nM). However, *m*-sulfo-substitution on the C2-phenylethynyl group was associated with slightly higher affinity at the hA₃AR as compared to the mA₃AR (compound **7**, K_i hA₃AR = 1.9 nM; K_i mA₃AR = 11.3 nM).

At the A₁AR, only 10 to 50% of binding inhibition was seen at 10 μM for the majority of the tested (N)-methanocarba derivatives with few exceptions. In particular, the C2-*p*-sulfo-phenylethynyl derivative **6** showed enhanced affinity at the hA₁AR (K_i = 383 nM) and even higher affinity at the mA₁AR (K_i = 35.1 nM).

A *N*⁶-*p*-sulfo-phenylethyl group was tolerated at the hA₃AR in both the 5'-*N*-methyluronamide and 4'-truncated series (uronamide **18**, K_i hA₃AR = 12.1 nM; truncated compound **16**, K_i hA₃AR = 30.2 nM). However, this substitution reduced mA₃AR affinity, especially in the 4'-truncated series (**18**, K_i mA₃AR = 71.1 nM; **16**, K_i mA₃AR = 3.92 μM).

Functional data were determined in assays of A₃AR-induced inhibition of the forskolin-stimulated production of cAMP in CHO cells stably expressing the hA₃AR or in HEK293 cells stably expressing the mA₃AR (Table 1, Figure 3). As expected, 4'-truncated derivatives were generally low-efficacy partial agonists or antagonists of the hA₃AR, while 5'-*N*-

methyluronamide analogs showed high efficacy at this subtype. In the truncated series the efficacy varied depending on the substitution of the C2-phenylethynyl group and on the N^6 substituent. Full curves for functional potency at the mA₃AR (EC_{50} values in nM) were determined for C2 *p*-sulfonate **6** (13 ± 11), C2 *m*-sulfonate **7** (2.60 ± 0.89), uncharged truncated N^6 -phenylethyl derivative **14** and the corresponding 5'-*N*-methyluronamide N^6 -phenylethyl derivative **17** (3.33 ± 2.5) in comparison to standard riboside **1b** (0.33 ± 0.18). Only **14** displayed significantly less (~14% of the level of **1b**) than full efficacy.

For compounds **6** and **7** functional potency and efficacy at the mA₁AR were also determined in assays of A₁AR-induced inhibition of the production of cAMP in HEK293 cells expressing the mA₁AR (Figure 4). The *m*-sulfo analogue **7** was completely inactive up to 10 μ M, and the corresponding *p*-sulfo isomer **6**, the only derivative showing good affinity at the mA₁AR, was a moderately potent partial agonist at the mA₁AR (EC_{50} 495 ± 52 nM, with maximal efficacy 49% of full agonist **44**).

In vivo studies

The prototypical A₃AR agonists N^6 -(3-iodobenzyl)adenosine-5'-*N*-methyluronamide (IB-MECA, **1a**), **1b** and an (N)-methanocarba derivative **2** were shown to reverse the development of neuropathic pain following chronic constriction injury (CCI) in mice via an A₃AR-mediated mechanism.¹³ Using the same model, the *p*- and *m*-sulfo derivatives **6** and **7** also reversed mechano-allodynia. When peak mechano-allodynia developed on day 7 (D7) following CCI of the mouse sciatic nerve,³⁹ i.p. administration of either agonist (3 μ mol/kg, $n=6$), but not vehicle (5% DMSO in saline, $n=6$), reversed mechano-allodynia, with a maximal effect within 1 h (Figure 5A, B). As was demonstrated for the prototypical agonists **1a** and **1b**,¹³ the *p*- and *m*-sulfo derivatives **6** and **7** lacked effect on Paw Withdrawal Thresholds (PWT, g) in contralateral paws (data not shown). Intrathecal administration of the A₃AR selective antagonist 5-propyl-2-ethyl-4-propyl-3-(ethylsulfanylcabonyl)-6-phenylpyridine-5-carboxylate (**45**, MRS1523, 3 nmol, $n=5$), had no effect on the anti-allodynic actions of systemic agonist **7** (3 μ mol/kg, $n=5$, Figure 5C). The effects of A₃AR-selective agonist **7** were completely lost in the A₃AR^{-/-} mice ($n=4$) whereas those of mixed A₁AR/A₃AR agonist **6** were significantly but not completely abrogated ($n=4$, Figure 5D).

Discussion

This study is an example of applying the new structural insights into GPCRs to substitution of functional groups on existing ligand scaffolds. Previously, such efforts would be performed entirely empirically, and now it is possible to predict interactions and the effects on pharmacological potency using homology modeling and ligand docking.

The previously observed SAR of the (N)-methanocarba series at the A₃AR can be rationalized based on steric fitting of the binding cavity and crucial interactions with key binding site residues (Figure 6). The use of hybrid A₃AR models helps to explain the high A₃AR affinity and selectivity associated with the insertion of a C2-phenylethynyl group that can be accommodated after outward movement of TM2. Different hydrophobic arylalkyl substituents, including substituted phenylethyl groups or longer rigid groups, at the N^6 position can be accommodated in a side pocket delimited by EL2 and are tolerated for A₃AR affinity. Truncation of the 5'-*N*-methyluronamide moiety results in a missing H-bonding interaction with a Thr in TM3, key residue for agonist binding, and determines a slight decrease in affinity and a significant efficacy reduction at the A₃ subtype.

The present C2-phenylethynyl analogues include both full agonists containing a 5'-*N*-methyluronamide and the corresponding 4'-truncated antagonists (or low efficacy agonists).

In the truncated series the N^6 -phenylethyl group, in comparison to other N^6 substitution, tended to preserve interspecies selectivity at the A_3AR . Even the widely used N^6 -(3-halobenzyl) group in *p*-biphenylethynyl **13** did not result in high A_3AR selectivity in the mouse, as it did in human. Curiously, this compound bound to the mA_1AR with nearly as high affinity as at the mA_3AR . Similarly, a truncated N^6 -methyl analogue **8** was relatively weak and nonselective between A_1AR and A_3AR .

Sulfonated analogues of otherwise uncharged receptor ligands have been shown to restrict diffusion across biological membranes and are invariably associated with increased aqueous solubility.³¹ Sulfonation inhibits penetration into the brain, and such charged compounds will most likely also have very limited oral bioavailability. There are no previous reports of potent and selective A_3AR agonists having a sulfonate group, although such pharmacological tools are needed for the delineation of effects of this receptor in the central and peripheral nervous systems. An earlier attempt to introduce a sulfonate group, which is essentially permanently charged at physiological pH, in A_3AR agonists failed.³² Nevertheless, our molecular modeling predicted that an aryl sulfonate group placed on an extended substituent at the C2 position would be tolerated in binding to the h and mA_3AR , while a N^6 -*p*-sulfo-phenylethyl substituent would determine higher h A_3AR affinity as compared to the mA_3AR . The present set of analogues contains several aspects of conformational rigidity that increase confidence in the molecular docking: rigidity of the ribose-like moiety and a rigid rod-like aryethynyl group at the C2 position. The precision of the homology modeling, based on the X-ray structure of the closely related $A_{2A}AR$, allowed predictions to be made for comparing the two species and three different positions of sulfonate substitution, as a result of predicted interactions with amino acid residues in the extracellular regions of the A_3AR . The great degree of sequence divergence for the A_3AR subtype between rodents and other mammalian species can lead to differences in ligand binding properties. Therefore, the construction of both h and mA_3AR models proved to be useful in designing ligands with species-independent A_3AR affinity.

In particular, docking results showed how the insertion of a sulfonate group on the C2-phenylethynyl substituent was well tolerated at A_3AR s of both species. In fact, despite the presence of different residues in EL2 and EL1 between h and mA_3AR s, the region around the C2-substituent is open to the extracellular side in both h and mA_3AR models and could easily accommodate a sulfonate group at either the *p*- or *m*- position of the phenyl ring.

Much stricter are the requirements regarding the N^6 substituents to have compounds with high inter-species A_3AR s affinity. Due to the Val 169/Arg 170 substitution in EL2 between h and mA_3AR s, respectively, only the h A_3AR could tolerate several different N^6 substituents without loss in affinity. Conversely, at the mA_3AR a good steric fit between the N^6 group and the side pocket delimited by EL2 and TMs 5 and 6 seemed to be required to place the ligand in a binding conformation able to strongly interact with all the key residues of the binding site. Therefore, compound **18**, bearing a sulfonate group at the *p*- position of the N^6 -phenylethyl substituent, showed optimal interactions with the h A_3AR but weaker contacts at the mA_3AR , due to missing H-bonds of the adenine moiety with Asn251 in TM6. Insertion of a sulfonate group on a N^6 -phenylethyl substituent, was also examined in the truncated series of nucleosides, which are known to display greatly reduced efficacy at the A_3AR . In this case, the effect of the more difficult accommodation of the N^6 group and consequent weaker interaction with Asn 6.55 at the mA_3AR is even more marked due to the already missing interaction with a Thr in TM3. A truncated nucleoside **16** with a sulfonate on an N^6 -phenylethyl group was a selective, low efficacy partial agonist at the h but not m A_3AR .

Thus, it seems that the N^6 substituent can either compensate for the missing interactions of the 4'-truncated series in case of optimal fit with the extracellular region of the binding site, as previously observed for the A_1 AR,⁴⁰ or increase the detrimental effect of the truncation on the affinity in case of difficult accommodation in the extracellular region, as observed here.

We have confirmed modeling predictions by chemical synthesis of the designed molecules and binding and efficacy studies. Moreover, two of these sulfonated compounds were tested in a mouse model of neuropathic pain following our initial report of the beneficial effect of A_3 AR activation in such conditions.¹³ Compound **6** was found to activate both A_1 and A_3 ARs, while an isomeric nucleoside **7**, in which the sulfonate was moved from the *p*- to *m*-position on the C2-phenylethynyl group, activated only the A_3 AR. The A_3 AR selectivity of **7** in comparison to the A_1 AR and A_{2A} AR was >1000-fold in the mouse and >10,000-fold in the human. The structural basis for the interaction of **6** with the h or mA $_1$ AR has not been probed in this study. However, we can speculate that for some particular C2-arylethynyl derivatives a similar outward movement of TM2, as observed in the A_3 AR, can occur also in the A_1 AR. This receptor, in fact, does not present a disulfide bond network constraining the TM2-EL1-TM3 region as observed in the A_{2A} AR crystal structure. Therefore, in particular cases the A_1 AR could undergo conformational changes when binding to ligands bearing bulkier C2-groups.

The ability of agonists of the A_1 AR or the A_3 AR to reduce or prevent neuropathic pain has been studied previously.^{13,14,41,42} The sulfonated agonists are introduced here as a means of separating the peripheral and central effects of AR agonists, depending on the route of administration. Upon i.p. administration, the mixed agonist **6** elicited a more pronounced reduction in neuropathic pain than the A_3 AR-selective agonist **7** following CCI. We ascribe the protection by **6** to a combination of A_1 and A_3 AR-dependent effects. Additionally, the selective A_3 AR antagonist **45**, given centrally, was unable to block the anti-allodynic effects of agonist **7**, further supporting that the site of action of sulfonated agonist **7** as administered is restricted to the periphery. The same antagonist administered systemically was completely effective in blocking the anti-allodynic effects of A_3 AR agonist **1a** in the identical CCI model.¹³ However, antagonists of the A_1 AR and A_{2A} AR were completely ineffective in blocking the protective action of **1a**. The attenuation and loss, respectively, of the beneficial actions of agonists **6** and **7** in A_3 AR^{-/-} mice is consistent with their measured AR selectivity in both binding and functional assays.

The presence and role of the A_3 AR in the CNS is subject to contradictory findings. Attempts to label the A_3 AR binding sites autoradiographically in the brain have not succeeded using a potent nonselective labeled agonist in the presence of selective antagonists for other ARs.⁴³ Ravid and coworkers found expression of the A_3 AR in the mouse brain.⁴⁴ The A_3 AR is also known to be expressed in astrocytes and microglial cells.^{45,46} Using knockout mice, the A_3 AR was shown to influence heart rate, motor activity and body temperature, even though the level of expression in the brain is likely low.⁴⁷ It appears that protection against neuropathic pain by A_3 AR agonists has both central and peripheral components.⁴⁸ The effects of low doses of **1a** in CCI mice to reverse mechano-allodynia had both peripheral and spinal sites of action that were attenuated by intraplantar or intrathecal injection of **45**. Thus, the introduction of potent A_3 AR agonist probes designed to be precluded from crossing the blood brain barrier promises to be useful in delineating the effects of activating central vs. peripheral ARs.

The isomeric compounds **6** and **7** are very close in physicochemical properties and likely in vivo distribution. The cLog P of both is 1.15, and the total polar surface area is 176 Å², which is outside the range of molecules that readily cross the blood brain barrier. In addition

to the exclusion from readily crossing biological membranes, there is the dimension of either very high selectivity for the A₃AR in **7** or the feature of dual activation of the mA₁AR and mA₃AR in **6**. This near equivalence of high affinity to the exclusion of the A_{2A}AR is present in the mouse and to a lesser extent in the human. Therefore this pair of isomeric nucleosides can be used together as pharmacological tools.

Conclusions

Molecular modeling has provided a detailed window into the binding site interactions in the ARs. This insight has guided the process of refining the selectivity of A₃AR agonists in recent studies, and now we extend the analysis to provide useful pharmacological tools that have increased water solubility and are selective for compartments in the body because they are not expected to diffuse readily across phospholipid bilayers. The ability of molecular modeling now to predict the likely pharmacological effects of functional group substitution on a ligand scaffold is a useful and labor-saving approach in the exploration of SAR at GPCRs.

Experimental Section

Molecular Modeling

hA₃AR and mA₃AR homology models—Homology models of the hA₃ and mA₃ ARs were built based on a hybrid template structure following a strategy previously described²⁵ and using the alignment and the homology modeling tools implemented in the MOE suite.⁴⁹ To build these models, an agonist-bound hA_{2A}AR crystal structure (PDB ID: 3QAK)³⁴ was used as a template for the entire A₃AR structure except for the extracellular terminus of TM2 (residues from Val63 to Ser73 at the hA₃AR and from Val64 to Ser74 at the mA₃AR) and EL1 (residues from Leu74 to Tyr81 at the hA₃AR and from Leu75 to Tyr82 at the mA₃AR). The X-ray structure of the hβ₂ adrenergic receptor in complex with the Gs protein (PDB ID: 3SN6),³⁵ after superimposition with the hA_{2A}AR crystal structure, was used as template to build the extracellular terminus of TM2. No structural template was used for the modeling of EL1.

In particular, h and mA₃ARs sequences were retrieved from the UniProt database⁵⁰ and aligned against the sequence of the hybrid template, taking into account the highly conserved residues in each TM domain and following the numbering scheme by Ballesteros and Weinstein.⁵¹ Then, A₃AR homology models were built using the automated Homology Modeling protocol implemented in the MOE suite. The previously published docking pose of compound **11** inside the A₃AR²⁶ was taken into account during the modeling, following the so called Ligand Based Homology Modeling approach.⁵² The AMBER99 forcefield has been used for protein modeling and the Protonate 3D methodology has been used for protonation state assignment.⁵³ The final model has been refined through energy minimization until an RMS gradient of 0.1 kcal/mol Å. Model's stereochemical quality was checked using several tools (Ramachandran plot; backbone bond lengths, angles and dihedral plots; clash contacts report; rotameric strain energy report) implemented in the MOE suite.

Molecular docking of (N)-methanocarba derivatives at the A₃AR models—

Compounds structures were built using the builder tool implemented in the MOE suite⁴⁹ and subjected to energy minimization using the MMFF94x force field, until a RMS gradient of 0.05 kcal/mol Å. Molecular docking of the ligands at the A₃AR models was performed by means of the Glide⁵⁴ package part of the Schrödinger suite.⁵⁵ The docking site was defined using key residues in the binding pocket of the A₃AR models, namely Phe (EL2), Asn (6.55), Trp (6.48) and His (7.43), and a 20Å × 20Å × 20Å box was centered on these

residues. Docking of ligands was performed in the rigid binding site using the XP (extra precision) procedure. The top scoring docking conformations for each ligand were subjected to refinement using the Prime MM-GBSA module implemented in the Schrödinger suite.⁵⁶ The Prime side-chain sampling was performed on all the residues within a 6 Å of the ligand. The refined model for each ligand was chosen as final binding conformation. Finally, to calculate per-residue interaction energies, selected ligands were scored in place in their refined complexes, using the Glide module.

Chemical Synthesis

Materials and Instrumentation—All reagents and solvents were purchased from Sigma-Aldrich (St. Louis, MO). ¹H NMR spectra were obtained with a Bruker 400 spectrometer using CDCl₃ and CD₃OD as solvents. Chemical shifts are expressed in δ values (ppm) with tetramethylsilane (δ 0.00) for CDCl₃ and water (δ 3.30) for CD₃OD. TLC analysis was carried out on glass sheets precoated with silica gel F254 (0.2 mm) from Aldrich. All the final sulfonate nucleoside compounds were purified by HPLC with a Luna 5 μmRP-C18(2) semipreparative column (250 mm × 10.0 mm; Phenomenex, Torrance, CA) and using the following conditions: flow rate of 2 mL/min; 10 mM trifluoroacetic acid-water (TFA-H₂O)-CH₃CN from 80:20 to 30:70 in 20–40 min. The purity of final nucleoside derivatives was checked using a Hewlett–Packard 1100 HPLC equipped with a Zorbax SB-Aq 5 μm analytical column (50 × 4.6 mm; Agilent Technologies Inc., Palo Alto, CA). Mobile phase: linear gradient solvent system, 5 mM TBAP (tetrabutylammonium dihydrogenphosphate)–CH₃CN from 80:20 to 0:100 in 13 min; the flow rate was 0.5 mL/min. Peaks were detected by UV absorption with a diode array detector at 230, 254, and 280 nm. All derivatives tested for biological activity showed >95% purity by HPLC analysis (detection at 254 nm). 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 noted. Observed mass accuracies are those expected based on 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.

4-((6-(3-Chlorobenzylamino)-9-((1S,2R,3S,4R,5S)-3,4-dihydroxy-5-(methylcarbamoyl)bicyclo[3.1.0]hexan-2-yl)-9H-purin-2-yl)ethynyl)benzenesulfonic acid (6)—PdCl₂(PPh₃)₂ (2.5 mg, 0.003 mmol), CuI (1.0 mg, 0.004 mmol), 3-ethynyl benzenesulfonic acid (37, 20 mg, 0.11 mmol) and triethylamine (25 μL, 0.18 mmol) were added to a solution of compound **23** (10.8 mg, 0.018 mmol) in anhydrous DMF (1 mL), and stirred at room temperature for overnight. Solvent was evaporated under, vacuum and the residue was roughly purified on flash silica gel column chromatography. The resulting compound was dissolved in methanol (1 mL) and 10% trifluoromethane sulfonic acid (1 mL) and heated at 70 °C for 5 h. Solvent was evaporated under, vacuum and the residue was purified on semi preparative HPLC (retention time 30 min) to give the compound **27** (7.6 mg, 69%) as a syrup. ¹H NMR (CD₃OD, 400 MHz) δ 8.15 (s, 1H), 7.88 (d, *J* = 8.4 Hz, 2H), 7.71 (d, *J* = 8.4 Hz, 2H), 7.46 (s, 1H), 7.38–7.26 (m, 3H), 5.09 (d, *J* = 6.4 Hz, 1H), 4.91 (s, 1H), 4.83 (d, *J* = 6.4 Hz, 1H), 2.84 (s, 3H), 2.14–2.10 (m, 1H), 1.89–1.86 (m, 1H), 1.40–1.36 (m, 1H). HRMS calculated for C₂₈H₂₆N₆O₆SCl (M + H)⁺: 609.1323; found 609.1312.

3-((6-(3-Chlorobenzylamino)-9-((1S,2R,3S,4R,5S)-3,4-dihydroxy-5-(methylcarbamoyl)bicyclo[3.1.0]hexan-2-yl)-9H-purin-2-

yl)ethynyl)benzenesulfonic acid (7)—Compound **7** (65%) was prepared from compound **23** following the same method for compound **6**, except using **38**. Semipreparative HPLC retention time 28 min. ¹H NMR (CD₃OD, 400 MHz) δ 8.13 (s, 1H), 7.90 (d, *J* = 7.6 Hz, 1H), 7.72 (d, *J* = 7.6 Hz, 1H), 7.54–7.46 (m, 3H), 7.38–7.26 (m, 3), 5.10 (d, *J* = 6.0 Hz, 1H), 4.91 (s, 1H), 4.05 (d, *J* = 6.0 Hz, 1H), 2.84 (s, 3H), 2.13–2.10 (m, 1H), 1.89–1.86 (m, 1H), 1.41–1.38 (m, 1H). HRMS calculated for C₂₈H₂₄N₆O₆SCl (M – H)⁺: 607.1167; found 607.1161.

4-(2-(9-((1S,2R,3S,4R,5R)-3,4-Dihydroxybicyclo[3.1.0]hexan-2-yl)-2-(phenylethynyl)-9H-purin-6-ylamino)ethyl)benzenesulfonic acid (16)—

Compound **16** (93%) was prepared from compound **32** following the same method for compound **17**. ¹H NMR (CD₃OD, 400 MHz) δ 8.34 (s, 1H), 7.77 (d, *J* = 8.0 Hz, 2H), 7.69–7.66 (m, 2H), 7.47–7.46 (m, 3H), 7.40 (d, *J* = 8.0 Hz, 2H), 4.71 (t, *J* = 6.3 Hz, 1H), 4.01 (s, 1H), 3.93 (d, *J* = 6.4 Hz, 1H), 3.10 (t, *J* = 6.8 Hz, 2H), 2.02–2.00 (m, 1H), 1.74–1.68 (m, 1H), 1.38–1.32 (m, 1H), 0.82–0.79 (m, 1H). HRMS calculated for C₂₇H₂₄N₅O₅S (M – H)⁺: 530.1498; found 530.1495.

(1S,2R,3S,4R,5S)-2,3-Dihydroxy-N-methyl-4-(6-(phenethylamino)-2-(phenylethynyl)-9H-purin-9-yl)bicyclo[3.1.0]hexane-1-carboxamide (17)—

PdCl₂(PPh₃)₂ (5.8 mg, 0.008 mmol), CuI (1.0 mg, 0.004 mmol), phenylacetylene (27 μL, 0.25 mmol) and triethylamine (30 μL, 0.41 mmol) were added to a solution of compound **24** (24.6 mg, 0.041 mmol) in anhydrous DMF (1 mL), and stirred at room temperature for overnight. Solvent was evaporated under, vacuum and the residue was roughly purified on flash silica gel column chromatography. The resulting compound was dissolved in methanol (2 mL) and 10% trifluoromethane sulfonic acid (2 mL) and heated at 70 °C for 5 h. Solvent was evaporated under, vacuum and the residue was purified on flash silica gel column chromatography (CH₂Cl₂:MeOH = 25:1) to give the compound **7** (15 mg, 71%) as a syrup. ¹H NMR (CD₃OD, 400 MHz) δ 8.10 (s, 1H), 6.78–6.75 (m, 2H), 7.47–7.44 (m, 3H), 7.33–7.26 (m, 4H), 7.21–7.17 (m, 1H), 5.05 (d, *J* = 5.2 Hz, 1H), 4.89 (s, 1H), 4.01 (d, *J* = 5.2 Hz, 1H), 3.90 (br s, 2H), 3.02 (t, *J* = 7.2 Hz, 2H), 2.84 (s, 3H), 2.13–2.09 (m, 1H), 1.89–1.87 (m, 1H), 1.41–1.37 (m, 1H). HRMS calculated for C₂₉H₂₉N₆O₃ (M + H)⁺: 509.2301; found 509.2291.

4-(2-(9-((1S,2R,3S,4R,5S)-3,4-Dihydroxy-5-(methylcarbamoyl)bicyclo[3.1.0]hexan-2-yl)-2-(phenylethynyl)-9H-purin-6-ylamino)ethyl)benzenesulfonic acid (18)—

Compound **18** (63%) was prepared from compound **25** following the same method for compound **6**. ¹H NMR (CD₃OD, 400 MHz) δ 8.18 (s, 1H), 7.76 (d, *J* = 7.6 Hz, 2H), 7.70–7.67 (m, 2H), 7.48–7.46 (m, 3H), 7.39 (d, *J* = 8.4 Hz, 2H), 5.06 (d, *J* = 6.8 Hz, 1H), 4.90 (s, 1H), 4.03 (d, *J* = 6.8 Hz, 1H), 3.92 (br s, 2H), 3.09 (t, *J* = 6.8 Hz, 2H), 2.84 (s, 3H), 2.14–2.11 (m, 1H), 1.89–1.86 (m, 1H), 1.42–1.39 (m, 1H). HRMS calculated for C₂₉H₂₇N₆O₆S (M – H)⁺: 587.1713; found 587.1696.

(1S,2R,3S,4R,5S)-Ethyl –(2,3-O-isopropylidene)-4-(2-iodo-6-(phenylethylamino)-9H-purin-9-yl)bicyclo[3.1.0]hexane-1-carboxylate (21)—

Phenylethylamine (0.14 mL, 1.12 mmol) and triethylamine (0.4 mL, 2.2 mmol) were added to a solution of compound **19** (114 mg, 0.22 mmol) in anhydrous methanol (4 mL) and stirred at room temperature for overnight. Solvent was evaporated under, vacuum and the residue was purified on flash silica gel column chromatography (hexane:ethyl acetate=1:1) to give the compound **21** (109 mg, 82%) as foamy solid. ¹H NMR (CD₃OD, 400 MHz) δ 7.93 (s, 1H), 7.27 (d, *J* = 4.4 Hz, 4H), 7.21–7.18 (m, 1H), 5.82 (d, *J* = 7.2 Hz), 4.91 (s, 1H), 4.80 (d, *J* = 6.4 Hz), 3.79–3.76 (m, 4H), 2.96 (t, *J* = 7.2 Hz, 2H), 2.25–2.21 (m, 1H), 1.65–

1.61 (m, 1H), 1.53–1.49 (m, 4H), 1.34 (t, $J = 7.2$ Hz, 3H), 1.27 (s, 3H). HRMS calculated for $C_{25}H_{29}IN_5O_4$ ($M + H$)⁺: 590.1264; found 590.1286.

(1*S*,2*R*,3*S*,4*R*,5*S*)-Ethyl –(2,3-*O*-isopropylidene)-4-{2-iodo-6-(4-(2-aminoethyl) benzene sulfonic acid)-9*H*-purin-9-yl}bicyclo[3.1.0]hexane-1-carboxylate (22)

—Compound **22** (78%) was prepared from compound **19** following the same method for compound **21**. ¹H NMR (CD₃OD, 400 MHz) δ 7.91 (s, 1H), 7.78 (d, $J = 6.0$ Hz, 2H), 7.35 (d, $J = 6.0$ Hz, 2H), 5.82 (d, $J = 7.2$ Hz), 4.91 (s, 1H), 4.80 (d, $J = 5.6$ Hz), 3.77 (br s, 2H), 3.15 (t, $J = 6.9$ Hz, 2H), 3.05 (t, $J = 6.4$ Hz, 2H), 2.26–2.22 (m, 1H), 1.65–1.61 (m, 1H), 1.53–1.47 (m, 4H), 1.34 (t, $J = 7.2$ Hz, 3H), 1.29 (s, 3H). HRMS calculated for $C_{25}H_{29}IN_5O_7S$ ($M + H$)⁺: 670.0832; found 670.0851.

(1*S*,2*R*,3*S*,4*R*,5*S*)-(2,3-*O*-Isopropylidene)-4-(2-iodo-6-(phenylethylamino)-9*H*-purin-9-yl)-*N*-methylbicyclo[3.1.0]hexane-1-carboxamide (24)

—40% Methylamine solution (2 mL) was added to a solution of compound **21** (65 mg, 0.11 mmol) in methanol (2 mL) and stirred at room temperature for 24 h. Solvent was evaporated under vacuum and the residue was purified on flash silica gel column chromatography (CH₂Cl₂:MeOH=40:1) to give the compound **43** (41 mg, 65%) as a syrup. ¹H NMR (CD₃OD, 400 MHz) δ 7.95 (s, 1H), 7.27 (d, $J = 4.4$ Hz, 4H), 7.20–7.17 (m, 1H), 5.72 (d, $J = 6.8$ Hz, 1H), 4.92 (s, 1H), 4.83 (d, $J = 6.0$ Hz, 1H), 3.76 (br s, 2H), 2.96 (t, $J = 7.2$ Hz, 2H), 2.9 (s, 3H), 2.15–2.13 (m, 1H), 1.54–1.49 (m, 4H), 1.40 (t, $J = 5.2$ Hz, 1H), 1.30 (s, 3H). HRMS calculated for $C_{24}H_{28}IN_6O_3$ ($M + H$)⁺: 575.1268; found 575.1266.

(1*S*,2*R*,3*S*,4*R*,5*S*)-(2,3-*O*-Isopropylidene)-4-(2-iodo-6-(4-(2-aminoethyl) benzene sulfonic acid)-9*H*-purin-9-yl)-*N*-methylbicyclo[3.1.0]hexane-1-carboxamide (25)

—Compound **25** (68%) was prepared from compound **22** following the same method for compound **24**. ¹H NMR (CD₃OD, 400 MHz) δ 7.93 (s, 1H), 7.78 (d, $J = 6.0$ Hz, 2H), 7.35 (d, $J = 6.0$ Hz, 2H), 5.71 (d, $J = 7.2$ Hz, 1H), 4.92 (s, 1H), 4.83 (d, $J = 5.6$ Hz, 1H), 3.80 (br s, 2H), 3.12 (t, $J = 7.6$ Hz, 2H), 2.9 (s, 3H), 2.17–2.12 (m, 1H), 1.54–1.49 (m, 4H), 1.39 (t, $J = 5.3$ Hz, 1H), 1.30 (s, 3H). HRMS calculated for $C_{24}H_{26}IN_6O_6S$ ($M + H$)⁺: 653.0679; found 653.0677.

(1*S*,2*R*,3*S*,4*R*,5*S*)-(2,3-*O*-Isopropylidene)-4-(2-iodo-6-(4-(2-aminoethyl) benzene sulfonic acid)-9*H*-purin-9-yl)-bicyclo[3.1.0]hexane (32)

—Compound **32** (76%) was prepared from compound **31** following the same method for compound **21**. ¹H NMR (CD₃OD, 400 MHz) δ 8.02 (s, 1H), 7.78 (d, $J = 6.8$ Hz, 2H), 7.35 (d, $J = 6.8$ Hz, 2H), 5.36 (t, $J = 6.4$ Hz, 1H), 4.95 (s, 1H), 4.69 (d, $J = 7.2$ Hz, 1H), 3.80 (br s, 2H), 3.16 (t, $J = 6.4$ Hz, 2H), 2.05–2.02 (m, 1H), 1.72–1.67 (m, 1H), 1.51 (s, 3H), 1.25 (s, 3H), 0.93–0.87 (m, 2H). HRMS calculated for $C_{22}H_{23}N_5O_5SI$ ($M - H$)⁺: 596.0465; found 596.0441.

3-((Trimethylsilyl)ethynyl)benzenesulfonic acid (36)—PdCl₂(PPh₃)₂ (110 mg, 0.15 mmol), CuI (15 mg, 0.15 mmol), trimethylsilylacetylene (0.66 mL, 4.7 mmol) and triethylamine (1.0 mL, 7.8 mmol) were added to a solution of compound **34** (252 mg, 0.78 mmol) in anhydrous DMF (5 mL), and stirred at room temperature for overnight. Solvent was evaporated under vacuum and the residue was purified on flash silica gel column chromatography (CH₂Cl₂:MeOH:TFA = 10:1:0.1) to give the compound **36** (133 mg, 67%) as a syrup. ¹H NMR (CD₃OD, 400 MHz) δ 7.91 (s, 1H), 7.84–7.76 (m, 1H), 7.56–7.42 (m, 2H), 0.21 (s, 9H). HRMS calculated for $C_{11}H_{13}O_3SSi$ ($M - H$)⁺: 253.0355; found 253.0361.

4-((Trimethylsilyl)ethynyl)benzenesulfonic acid (37)—Compound **37** (69%) was prepared from compound **35** following the same method for compound **36**. ¹H NMR

(CD₃OD, 400 MHz) δ 7.80 (d, J = 7.2 Hz, 2H), 7.49 (d, J = 7.2 Hz, 2H), 0.25 (s, 9H). HRMS calculated for C₁₁H₁₃O₃SSi (M – H)⁺: 253.0355; found 253.0355.

3-Ethynylbenzenesulfonic acid (38)—Tetrabutylammonium fluoride (1.9 mL, 1M solution in THF) was added to a solution of compound **36** (406 mg, 1.59 mmol) in THF and stirred at room temperature for 2 h. Solvent was evaporated and the residue was purified on flash silica gel column chromatography (CH₂Cl₂:MeOH:TFA = 8:1:0.1) to give the compound **37** (235 mg, 81%) as a colorless powder. ¹H NMR (CD₃OD, 400 MHz) δ 7.85–7.76 (m, 2H), 7.48–7.37 (m, 2H), 3.60 (s, 1H). HRMS calculated for C₈H₅O₃S (M – H)⁺: 180.9959; found 180.9958.

4-Ethynylbenzenesulfonic acid (39)—Compound **39** (84%) was prepared from compound **37** following the same method for compound **38**. ¹H NMR (CD₃OD, 400 MHz) δ 7.73 (d, J = 8.0 Hz, 2H), 7.45 (d, J = 8.0 Hz, 2H), 3.53 (s, 1H). HRMS calculated for C₈H₅O₃S (M – H)⁺: 180.9959; found 180.9956.

Binding Studies

[³H]R-*N*⁶-Phenylisopropyladenosine (**40**, [³H]R-PIA, 63 Ci/mmol), [³H](2-[*p*-(2-carboxyethyl)phenyl-ethylamino]-5'-*N*-ethylcarboxamido-adenosine) (**41**, [³H]CGS21680, 40.5 Ci/mmol) and [¹²⁵I]*N*⁶-(4-amino-3-iodobenzyl)adenosine-5'-*N*-methyluronamide (**42**, [¹²⁵I]I-AB-MECA, 2200 Ci/mmol) were purchased from Perkin–Elmer Life and Analytical Science (Boston, MA). Test compounds were prepared as 5 mM stock solutions in DMSO and stored frozen. Pharmacological standards **1b** (A₃AR agonist), adenosine-5'-*N*-ethylcarboxamide (**43**, NECA, nonselective AR agonist) and 2-chloro-*N*⁶-cyclopentyladenosine (**44**, CCPA, A₁AR agonist) were purchased from Tocris R&D Systems (Minneapolis, MN).

Cell Culture and Membrane Preparation—CHO cells stably expressing the recombinant hA₁ and hA₃ARs and HEK293 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, 100 µg/mL streptomycin, and 2 µmol/mL glutamine. In addition, 800 µg/mL geneticin was added to the A_{2A} media, while 500 µg/mL hygromycin was added to the A₁ and A₃ media. After harvesting, 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 MgCl₂. The suspension was homogenized and was then ultra-centrifuged at 14,330 *g* for 30 min at 4 °C. The resultant pellets were resuspended in Tris buffer, incubated with adenosine deaminase (3 units/mL) for 30 min at 37 °C. The suspension was homogenized with an electric homogenizer for 10 sec, 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).⁵⁷

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 MgCl₂, 50 µL of the appropriate agonist radioligand, and finally 100 µL of membrane suspension. For the A₁AR (22 µg of protein/tube) the radioligand used was [³H]**40** (final concentration of 3.5 nM). For the A_{2A}AR (20 µg/tube) the radioligand used was [³H]**41** (10 nM). For the A₃AR (21 µg/tube) the radioligand used was [¹²⁵I]**42** (0.34 nM). Nonspecific binding was determined using a final concentration of 10 µM **43** 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 a 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 A₁ 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 A₃AR binding were counted using a Packard Cobra II γ -counter. The K_i values were determined using GraphPad Prism for all assays.

Similar competition binding assays were conducted using HEK293 cell membranes expressing mARs using [¹²⁵I]**42** to label A₁ or A₃ARs and [³H]**41** to label A_{2A}ARs. IC₅₀ values were converted to K_i values as described.⁵⁸ Nonspecific binding was determined in the presence of 100 μ M **43**. *cAMP accumulation assay*: Intracellular cAMP levels in CHO cells expressing the recombinant hA₃AR were measured using an ELISA assay.⁵⁹ Cells were first harvested by trypsinization. After centrifugation and resuspended in medium, cells were planted in 96-well plates in 0.1 mL medium. After 24 h, the medium was removed and cells were washed three times with 0.2 mL DMEM, containing 50 mM HEPES, pH 7.4. Cells were then treated with the agonist (10 μ M **43** for hA₃AR) or test compound in the presence of rolipram (10 μ M) and adenosine deaminase (3 units/mL). After 30 min forskolin (10 μ M) 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 100 μ L of 0.1 M ice-cold HCl. The cell lysate was resuspended and stored at -20°C. For determination of cAMP production, 50 μ L of the HCl solution was used in the Amersham cAMP Enzyme Immunoassay following the instructions provided with the kit. The results were interpreted using a SpectroMax M5 Microplate reader (Molecular Devices, Sunnyvale, CA) at 450 nm.

Similar cAMP assays were conducted with HEK293 cells expressing the mA₁AR or mA₃AR. HEK293 cells were detached from cell culture plates, resuspended in serum-free DMEM containing 25 mM HEPES (pH 7.4), 1 unit/ml adenosine deaminase, 4-(3-butoxy-4-methoxyphenyl)methyl-2-imidazolidone (Tocris, Ro 20,1724, 20 mM) and 300 nM 8-[4-[4-(4-chlorophenyl)piperazine-1-sulfonyl]phenyl]-1-propylxanthine (Tocris, PSB603, 300 nM) inhibit A_{2B}ARs expressed endogenously in HEK293 cells, and then transferred to polypropylene tubes (2 $\times$ 10⁵ cells/tube). The cells were co-incubated with forskolin (10 μ M) and AR ligands for 15 min at 37°C with shaking, after which the assays were terminated by adding 500 μ L 1 N HCl. The lysates were centrifuged at 4000 $\times$ g for 10 min. The cAMP concentration was determined in the supernatants using a competitive binding assay, as previously described.⁵⁹ EC₅₀ and E_{max} values were calculated by fitting the data to: $E = E_{min} + (E_{max} - E_{min}) / (1 + 10^{x - \log EC_{50}})$.

In vivo Studies

Experimental animals—Male CD-1 mice (25–30 g) from Harlan (Indianapolis, IN) were housed 4–5 (for mice) per cage in a controlled environment (12 h light/dark cycles) with food and water available *ad libitum*. Male A₃AR knockout mice (A₃AR^{-/-}) (1) were provided by Merck & Co., Inc. (Whitehouse Station, NJ) and used to support A₃AR-mediated effects. Sex, age and weight-matched C57BL/6J wild-type (WT; Harlan, Indianapolis, IN) mice were used as controls. The A₃AR^{-/-} mice are healthy, fertile and exhibit no differences in the development of any organ systems.⁶⁰ Experiments were performed in accordance with International Association for the Study of Pain, NIH guidelines on laboratory animal welfare and Saint Louis University Institutional Animal Care and Use Committee recommendations. Experimenters were blinded to treatment conditions in all experiments.

CCI model of neuropathic pain—CCI to the sciatic nerve of the left hind leg in mice was performed under general anaesthesia using the well characterized Bennett model.³⁹

Briefly, mice (weighing 25–30 g at the time of surgery) were anesthetized with 3% isoflurane/100% O₂ inhalation and maintained on 2% isoflurane/100% O₂ for the duration of surgery. The left thigh was shaved, scrubbed with Nolvasan (Zoetis, Madison, NJ), and a small incision (1–1.5 cm in length) was made in the middle of the lateral aspect of the left thigh to expose the sciatic nerve. The nerve was loosely ligated around the entire diameter of the nerve at three distinct sites (spaced 1 mm apart) using silk sutures (6.0). The surgical site was closed with a single muscle suture and a skin clip. Pilot studies established that under our experimental conditions peak mechano-allodynia develops by D5-D7 following CCI. Test substances or their vehicles were given i.p. at peak mechano-allodynia (D7).

Statistical analysis for in vivo experiments—Data are expressed as mean $\pm$ SEM for *n* animals. Behavioral data were analyzed by two-way ANOVA with Bonferroni comparisons. Significant differences were defined at a *P* < 0.05. All statistical analysis was performed using GraphPad Prism (v5.03, GraphPad Software, Inc., San Diego, CA).

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations used

AR	adenosine receptor
cAMP	adenosine 3',5'-cyclic monophosphate
CCI	chronic constriction injury
CHO	Chinese hamster ovary
CNS	central nervous system
DMEM	Dulbecco's modified Eagle medium
EL	extracellular loop
GPCR	G protein-coupled receptor
HEPES	2-[4-(2-hydroxyethyl)piperazin-1-yl]ethanesulfonic acid
HEK	human embryonic kidney
NECA	5'-N-ethylcarboxamidoadenosine
PBS	phosphate buffered saline
PWT	Paw Withdrawal Threshold
RMS	root-mean-square
SAR	structure-affinity relationship
TM	transmembrane helix

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Figure 1.

Putative binding modes of sulfonated (N)-methanocarba derivatives obtained after docking simulations: (A) compound **6** at the hA₃AR; (B) compound **7** at the hA₃AR; (C) compound **6** at the mA₃AR; (D) compound **7** at the mA₃AR. Side chains of some amino acids important for ligand recognition are highlighted. H-bonding and ionic interactions are pictured as dotted lines. Hydrogen atoms are not displayed. Part of TM7 is omitted.

Figure 2.

Putative binding modes of sulfonated (N)-methanocarba derivatives obtained after docking simulations: (A) compound **18** at the hA₃AR; (B) compound **16** at the hA₃AR; (C) compound **18** at the mA₃AR; (D) compound **16** at the mA₃AR. Side chains of some amino acids important for ligand recognition are highlighted. H-bonding and ionic interactions are pictured as dotted lines. Hydrogen atoms are not displayed. Part of TM7 is omitted. (E) Per-residue interaction energy graph. Interaction energy values (in kcal/mol) between the ligand and selected key residues of the binding site, calculated from the binding poses reported in panels A–D.

Figure 3. Functional agonism by selected nucleosides, (A) *p*-sulfonate **6**, (B) *m*-sulfonate **7**, (C) truncated *N*⁶-phenylethyl derivative **14**, (D) *N*⁶-phenylethyl 5'-*N*-methyluronamide derivative **17**, in an assay of inhibition of forskolin-stimulated cAMP accumulation with HEK293 cells expressing the mA₃AR. Concentration-effect curves with potent A₃AR agonist **1b** are included for comparison. Data are the mean $\pm$ SEM, $n = 3-5$.

Figure 4.

Functional agonism by mixed A₁/A₃AR agonist **6** and selective A₃AR agonist **7** in an assay of inhibition of forskolin-stimulated cAMP accumulation with HEK293 cells expressing the mA₁AR. Concentration-effect curve with reference full agonist **44** is included for comparison. Data are the mean $\pm$ SEM, n = 4–7.

Figure 5.

Mixed A₁/A₃AR agonist **6** (n = 6) and selective A₃AR agonist **7** (n = 5) reverse CCI-induced neuropathic pain (A, B), measured as described.¹³ Mechano-allodynia developed by D7 after CCI of the sciatic nerve, which was reversed by i.p. administration (3 μmol/kg, in 5% DMSO in saline) of the sulfonated nucleosides. (C) Intrathecal administration of the selective A₃AR antagonist **45** (3 nmol, n=5), did not block the effects of systemic **7**, excluding the spinal cord as a site of action of this sulfonate-containing agonist. In male A₃AR knock-out mice (D), only **6** (i.p., 3 μmol/kg, n = 2) retained the ability to produce a small protective effect. The protective effect of **7** by (i.p., 3 μmol/kg, n = 3) was completely eliminated. **P* < 0.001 vs. D0; †*P* < 0.01 and ††*P* < 0.001 vs. D7.

Figure 6. General requirements of high affinity binding to the A₃AR for the (N)-methanocarba series rationalized towards molecular modeling analysis, showing putative interactions of sulfonate groups.

Scheme 1.

Synthesis of new sulfonated and nonsulfonated (N)-methanocarpa-adenosine analogues containing the 5'-N-methyluronamide group. Reagents: (i) $R^1\text{NH}_2$, Et_3N , MeOH, rt; (ii) 40% MeNH₂, MeOH, rt; (iii) $\text{HC}\equiv\text{CAr}$, $\text{Pd}(\text{PPh}_3)_2\text{Cl}_2$, CuI, Et_3N , DMF, rt; (iv) 10% TFA, MeOH, 70°C.

Scheme 2.

Synthesis of new sulfonated, truncated (N)-methanocarpa-adenosine analogue **16**. Reagents:

(i) 4-SO₃H-Ph(CH₂)₂NH₂, Et₃N, MeOH, rt; (ii) HC≡C-Ph, Pd(PPh₃)₂Cl₂, CuI, Et₃N, DMF, rt; (iii) 10% TFA, MeOH, 70°C.

Chart 1.
Nucleosides as selective A₃AR ligands.

Potency of 5'-N-methyluronamido and 4'-truncated (N)-methanocarpa adenosine derivatives at hARs and mARs and functional efficacy at the A₃ARs.

Table 1

Compd	Structure		Species	Affinity (K _i , nM) or % inhibition ^{a,b}			% Efficacy ^c	
	R ¹	R ²		A ₁	A _{2A}	A ₃	A ₃	A ₃
5 ^d	3-Cl-Bn	H	<i>h</i>	(20%±3%)	(27%±3%)	1.34±0.30	101±5.9	
			<i>m</i>	(50%±5%)	(2%±1%)	1.23±0.14	ND	
6	3-Cl-Bn	4-SO ₃ H	<i>h</i>	383±75	(23%±3%)	11.1±1.6	96.8±5.7	
			<i>m</i>	35.1±5.5	(14%±4%)	9.68±0.15	95.7±19.1	
7	3-Cl-Bn	3-SO ₃ H	<i>h</i>	(16%±3%)	(7%±6%)	1.90±0.03	98.2±6.7	
			<i>m</i>	(15%±2%)	(1%±1%)	11.3±1.9	89.3±7.1	
8 ^d	Me	H	<i>h</i>	(18%±1%)	(18%±3%)	5.48±1.23	12.6±4.0	
			<i>m</i>	3800±780	(8%±3%)	1530±240	ND	
9 ^d	Et	H	<i>h</i>	(36%±4%)	(42%±4%)	5.02±2.19	0.8±5.2	
			<i>m</i>	(49%±6%)	(49%±2%)	1480±170	ND	
10 ^d	Et	2-Cl	<i>h</i>	(25%±11%)	(17%±6%)	5.80±2.08	-7.0±5.2	
			<i>m</i>	(47%±8%)	(11%±1%)	(50%±9%)	ND	
11 ^d	3-Cl-Bn	H	<i>h</i>	(37%±4%) ^s	680±170	39.0±20.0	13.8±5.1	
12 ^d	3-Cl-Bn	3-Cl	<i>h</i>	(26%±3%)	1800±310	210±40	4.5±4.9	
13 ^d	3-Cl-Bn	4-Ph	<i>h</i>	(48%±4%)	(12%±7%)	54.0±7.0	-3.5±3.2	
			<i>m</i>	1110±220	(0%)	255±77	ND	
14 ^d	Ph(CH ₂) ₂	H	<i>h</i>	(30%±8%)	(22%±5%)	20.0±6.0	4.1±1.2	
			<i>m</i>	(39%±6%)	(13%±2%)	480±90	14.3±6.1	
15 ^d	Ph ₂ CHCH ₂	2-Cl	<i>h</i>	(26%±4%)	(22%±2%)	140±30	-2.6±1.3	
			<i>m</i>	(23%±1%)	(16%±%)	(54%±3%)	ND	

Compd	Structure		Species	Affinity (K _i , nM) or % inhibition ^{a,b}			% Efficacy ^c
	R ¹	R ²		A ₁	A _{2A}	A ₃	
16	4-SO ₃ H- Ph(CH ₂) ₂	H	<i>h</i>	(10%±5%)	(15%±3%)	30.2±4.3	7.2
			<i>m</i>	(18%±2%)	(1%±2%)	3920±1190	ND
17	Ph(CH ₂) ₂	H	<i>h</i>	(11%±3%)	(32%±4%)	1.23±0.57	105.3±9.8
			<i>m</i>	(25%±5%)	(1%±1%)	8.75±2.12	114.6±14.6
18	4-SO ₃ H- Ph(CH ₂) ₂	H	<i>h</i>	(9%±5%)	(1%±1%)	12.1±1.0	93.8±7.1
			<i>m</i>	(7%±2%)	(0%)	71.1±13.0	ND

^a Binding in membranes prepared from CHO or HEK293 (A_{2A} only) cells stably expressing one of three hAR subtypes. The binding affinity for A₁, A_{2A} and A₃ARs was expressed as K_i values (n = 3–4) using agonist radioligands [³H]N⁶-R-phenylisopropyladenosine **40**, [³H]2-[p-(2-carboxyethyl)phenyl-ethylamino]-5'-N-ethylcarboxamido-adenosine **41**, or [¹²⁵I]N⁶-(4-amino-3-iodobenzyl)adenosine-5'-N-methyluronamide **42**, respectively. A percent in parentheses refers to inhibition of binding at 10 μM.

^b Binding in membranes prepared from HEK293 cells stably expressing one of three mAR subtypes. Radioligand used were [¹²⁵I]N⁶-(4-amino-3-iodobenzyl)adenosine-5'-N-methyluronamide **43** (A₁ and A₃ARs) and [³H]2-[p-(2-carboxyethyl)phenyl-ethylamino]-5'-N-ethylcarboxamido-adenosine **41** (A_{2A}AR). The data (n = 3–4) are expressed as K_i values. A percent in parentheses refers to inhibition of binding at 10 μM.

^c Efficacy, expressed as a percentage of the maximal effect of either 5'-N-ethylcarboxamido-adenosine **43** (hA₃ARs) or 2-chloro-N⁶-(3-iodobenzyl)adenosine-5'-N-methyluronamide **1b** (mA₃ARs) to inhibit forskolin-stimulated cAMP production, was determined in cAMP assays using hA₃AR-transfected CHO cells or mA₃AR-transfected HEK cells. In studies with the hA₃AR, maximal efficacies of **1b** and test compounds were estimated by measuring the extent of inhibition of forskolin-stimulated cAMP accumulation produced each at a concentration of 10 μM. In studies with the mA₃AR, maximal efficacies of **1a** and test compounds were determined from concentration-effect curves. Data are expressed as mean±SEM (n = 3–5).

^d Compounds **5** and **8–15** were prepared and tested for binding at the hARs in Ref. 26.

ND – not determined.