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Extended N^6 substitution of rigid C2-arylethynyl nucleosides for exploring the role of extracellular loops in ligand recognition at the A_3 adenosine receptor

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ABSTRACT

2-Arylethynyl-(N)-methanocarba adenosine 5′-methyluronamides containing rigid N^6-(*trans*-2-phenylcyclopropyl) and 2-phenylethynyl groups were synthesized as agonists for probing structural features of the A_3 adenosine receptor (AR). Radioligand binding confirmed A_3AR selectivity and N^6-1*S*,2*R* stereoselectivity for one diastereomeric pair. The environment of receptor-bound, conformationally constrained N^6 groups was explored by docking to an A_3AR homology model, indicating specific hydrophobic interactions with the second extracellular loop able to modulate the affinity profile. 2-Pyridylethynyl derivative **18** was administered orally in mice to reduce chronic neuropathic pain in the chronic constriction injury model.

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X-ray crystallographic structures of the A_{2A} adenosine receptor (A_{2A}AR) in complex with both agonists and antagonists[1–3] can serve as suitable templates for accurately predicting the ligand interactions of other AR subtypes. We now focus on sterically bulky and hydrophobic N^6 substituents on adenosine derivatives that are associated with high affinity at the A_3AR. Adenosine derivatives that selectively activate the A_3AR are in clinical trials for autoimmune inflammatory diseases and liver cancer; thus, there is considerable interest in drug discovery at this receptor.[4]

Many studies have established the effects of diverse substitution of the exocyclic amine of adenosine on the AR subtype selectivity, with predominantly hydrophobic N^6 groups found to be best suited for high affinity.[5–7] For example, the structure activity relationship (SAR) of the N^6-(2-*trans*-phenylcyclopropyl) group and its substituted analogues was studied in monosubstituted adenosine derivatives (e.g., **1**, **2**, Chart 1), which tended toward selectivity at the A_3AR.[8] The steric constraint and stereochemical definition of the N^6-(2-*trans*-phenylcyclopropyl) group, especially in combination with rigid ribose substitutions, make it possible to study the structural implications of this conformationally constrained substituent in A_3AR recognition. Here we use a recent homology model[9] of the human (h) A_3AR to probe the structural basis for the effects on binding affinity in this class of agonists.

We previously explored a class of constrained (N)-methanocarba adenosine-5′-methylamide nucleosides, including 2-chloro derivatives (e.g., **3**)[10] and C2-arylethynyl analogues (e.g., **4**),[9,11] which are very potent and selective full agonists of the A_3AR. N^6-Benzyl-type substitution is often used because of its selective enhancement of A_3AR affinity. Here, we have synthesized new N^6-(2-phenylcyclopropyl)-substituted analogues in this extended methanocarba series and characterized them pharmacologically. We already reported the A_3AR selectivity of one analogue, **5** (R^1 = H) in which a rigid ribose-like (N)-methanocarba scaffold[10] was combined with a sterically restricted N^6-(2-phenylcyclopropyl) substitution, based on our earlier study of SAR in the 9-riboside series.[8] The corresponding 4′-truncated (N)-methanocarba nucleoside containing a racemic N^6-(2-phenylcyclopropyl) substitution was found to be a selective A_3AR antagonist (K_i 1.3 nM).[12] The 1*S*,2*R* diastereoisomer of **2** was ~10-fold more potent in binding to the hA_3AR (K_i 11.2 nM) than the corresponding 1*R*,2*S* isomer. This N^6 substituent was also combined with C2-cyano or carboxamide substitution in a subsequent 9-riboside series resulting in

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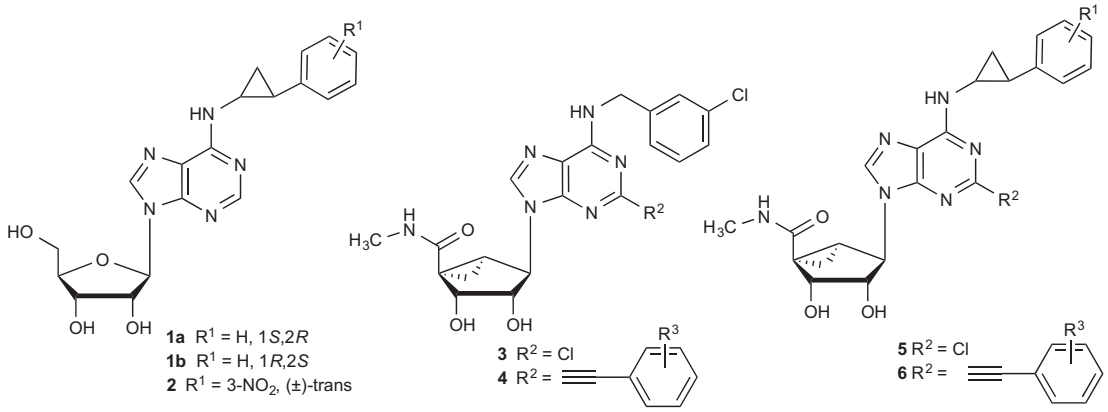


Chart 1. Progression of SAR studies of A_3AR agonists containing bulky N^6 substituents, such as *trans*-2-phenylcyclopropyl, and modification of the ribose as a ring-constrained bicyclic system.

a moderate reduction of A_3AR affinity.[13] In our earlier study of ribosides,[8] there was a 38-fold stereoselectivity of **1a** versus **1b** in A_3AR binding, and a smaller ratio was observed for stereoisomers of a 3-nitro derivative **2**. A large species difference was associated with the N^6-(2-phenylcyclopropyl) group, such that affinity was greatly enhanced in progressing from rat to human.

The current expanded series (general formula **6**) combined rigid C2-arylethynyl and N^6-(2-phenylcyclopropyl) groups. This extended, sterically defined N^6 substitution allows for the exploration of the outer regions of the A_3AR by docking of the ligands to homology models of the receptor. Thus, the effects of the interaction of different N^6 substituents with the normally flexible extracellular loops (ELs) were analyzed by detailed modeling.

Chronic neuropathic pain (NP) is an important unsolved medical need, which is associated with nerve injury and often with diseases such as cancer and diabetes.[14,15] We recently reported that A_3AR agonists have unanticipated efficacy in vivo in various models of chronic but not acute pain.[1] Although N^6-(2-phenylcyclopropyl) substitution in the riboside series greatly reduces the affinity at the murine A_3ARs,[24] we studied one of the new analogues in a mouse model of chronic pain resulting from constriction injury (CCI).[16]

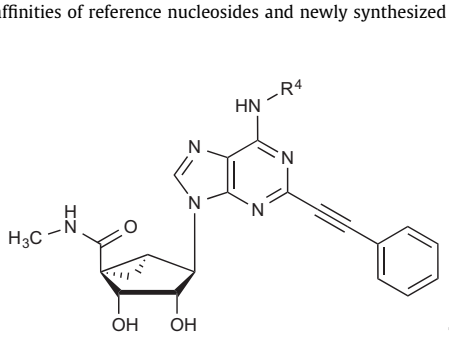
The nucleoside derivatives **9**–**19** (Table 1) were synthesized, characterized and examined in AR binding assays. Compounds **1**, **4**, **5**, **7** and **8**, prepared earlier,[8–11] were included as reference compounds. To synthesize N^6-(2-phenylcyclopropyl) derivatives, protected intermediate **20**[9] was sequentially treated with the appropriate 2-phenylcyclopropylamine to yield **21a**–**h** followed by methylamine to provide **22a**–**h** (Scheme 1). Then, intermediates **22a**–**h** were subjected to Sonogashira coupling with the appropriate arylacetylene in the presence of $PdCl_2(Ph_3P)_2$, CuI and triethylamine to give protected intermediates **23a**–**j**, which upon acid hydrolysis gave the target compounds **10**–**19**. The synthesis of 6-NH_2 derivative **9** will be reported elsewhere. Although most of the entries in Table 1 contain a mixture of stereoisomers, compounds **16**–**19** correspond to products with well-defined (1*R*,2*S* or 1*S*,2*R*) stereochemistry of the N^6-(2-phenylcyclopropyl) group. Compound **15** is the racemic form of pure diastereoisomers **16** and **17**.

The binding assays were based on widely used radioligands using membranes of CHO cells expressing the hA_1AR ([^{3}H]**24**) or hA_3AR ([^{125}I]**26**) or HEK293 cells expressing the hA_{2A}AR ([^{3}H]**25**).[17–19] Most of the nucleosides bound to the hA_3AR with low nanomolar affinity and with minimal binding to the hA_1AR and hA_{2A}AR. Although the A_3AR affinity range of the newly synthesized 2-arylethynyl compounds was somewhat less than the reference 2-chloro-(N)-methanocarba nucleoside **5**, the selectivity remained high, with the significant reduction in affinity at the A_1 and A_{2A}ARs. The unsubstituted, racemic N^6-(2-phenylcyclopropyl) derivative **10** retained a higher A_3AR affinity than the ring-substituted analogues **11**–**15**. However, the 3,4-difluorophenyl analogue **15** was very selective for the A_3AR, and the fluoro substitution was intended to diminish aromatic oxidation in vivo.[25] A comparison of the A_3AR affinity of diastereomeric pairs showed a 4-fold preference for the 1*S*,2*R* diastereoisomer in the pair of 2-phenylethynyl derivatives **16** and **17**, but there was no difference in A_3AR affinities of the 2-(2-pyridylethynyl) diastereomeric pair **18** and **19**.

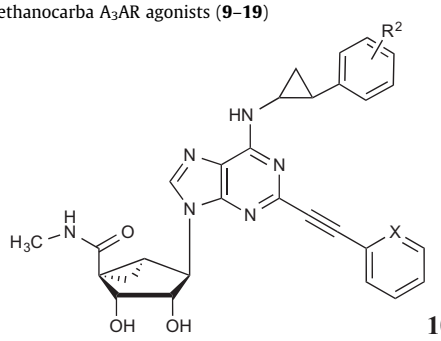
Therefore, we have used a combination of adenine substitutions and a ribose-like scaffold, all of greatly reduced conformational freedom, to help analyzing ligand recognition in the outer regions of the A_3AR. The environment of the receptor-bound N^6-(2-phenylcyclopropyl) group was explored by docking to an A_3AR homology model. We used our previously-reported homology model of the hA_3AR,[9,11] which was based on a hybrid A_{2A}AR-β_2 adrenergic receptor template. The general methodology used for homology modeling (MOE homology modeling tool)[20] and ligand docking (Glide module of the Schrödinger Suite)[17] has been described before,[9,11] and specific methodological details are reported in the Supporting Information. In the present study, after the first round of docking to the initial A_3AR homology model and selection of the best docking pose for derivative **7**, we performed refinement of the portion of the second EL in contact with the ligand (from Gln167 to Arg173), using the Prime module of the Schrödinger Suite,[21] to optimize its conformation around the N^6 substituent. This step was followed by a second round of docking of all derivatives to the optimized model, to identify the final proposed docking poses. All of the final nucleosides were also subjected to docking simulations at a hA_{2A}AR crystal structure (PDB ID: 2YDV)[3] to explore the reasons for ligand selectivity.

As expected, docking results at the hA_3AR for all the analyzed derivatives showed the pseudo-sugar moiety, adenine N7 and exocyclic NH participating in highly conserved H-bonding interactions with key residues of the binding site (Fig. 1), such as Thr94 (3.36), Asn250 (6.55), Ser271 (7.42) and His272 (7.43) (numbers in parenthesis follow the Ballesteros–Weinstein notation).[22] The same H-bonding network has been observed in the agonist-bound hA_{2A}AR crystal structures and is supposed to be important for receptor activation.[2,3] Another important binding feature for AR ligands, both agonists and antagonists, is the π–π stacking interaction with an aromatic residue in EL2 (Phe168 at the hA_3AR). Thus, considering that our quite rigid compounds showed all these critical interactions when docked to the A_3AR model, we could analyze in more detail the possible interactions with the less defined EL regions of the receptor. Docking results showed the rigid C2-arylethynyl

Table 1
Structures and binding affinities of reference nucleosides and newly synthesized (N)-methanocarba A_3AR agonists (**9**–**19**)



4,7-9



10-19

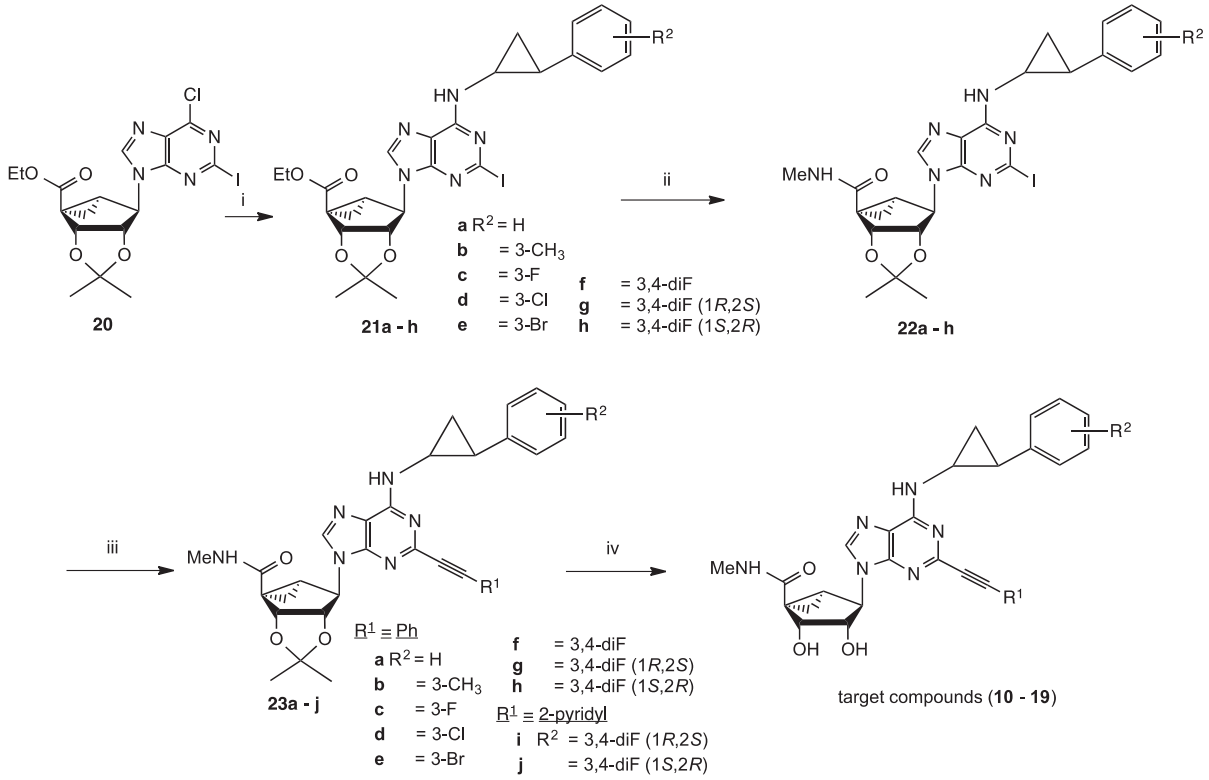
Compd	X	R^2 or R^4	Configuration of N^6 group	A_1AR[a] % *inhibition* or K_i (nM)	$A_{2A}AR$[a] % *inhibition* or K_i (nM)	A_3AR[a] K_i (nM)
1[b,c]	[d]	[d]	(±)-*trans*	124 ± 30 nM	2530 ± 720 nM	0.86 ± 0.09
4[b]	[d]	3-Cl-PhCH$_2$	[d]	*20 ± 3%*	*27 ± 3%*	1.34 ± 0.30
5[b,c] (R^1 = H)	[d]	[d]	(±)-*trans*	770 ± 50 nM	4800 ± 200 nM	0.78 ± 0.06
7[b]	[d]	Ph-(CH$_2$)$_2$	[d]	*11 ± 3%*	*32 ± 4%*	1.23 ± 0.57
8[b]	[d]	CH$_3$	[d]	*13 ± 6%*	*14 ± 7%*	0.85 ± 0.22
9	[d]	H	[d]	*28 ± 4%*	1200 nM	1.51 ± 0.41
10	CH	H	(±)-*trans*	*2 ± 1%*	*43 ± 8%*	6.16 ± 0.22
11	CH	3-CH$_3$	(±)-*trans*	*8 ± 4%*	*53 ± 6%*	17.6 ± 1.3
12	CH	3-F	(±)-*trans*	*18 ± 10%*	*0 ± 0%*	16.5 ± 1.9
13	CH	3-Cl	(±)-*trans*	*6 ± 3%*	*39 ± 5%*	11.2 ± 4.2
14	CH	3-Br	(±)-*trans*	*17 ± 4%*	*32 ± 18%*	14.5 ± 1.0
15	CH	3,4-DiF	(±)-*trans*	*11 ± 6%*	*6 ± 3%*	20.2 ± 2.1
16[e]	CH	3,4-DiF	1*R*,2*S*-*trans*	*11 ± 6%*	*0 ± 0%*	16.9 ± 3.3
17[e]	CH	3,4-DiF	1*S*,2*R*-*trans*	*16 ± 1%*	*0 ± 0%*	4.55 ± 1.63
18	N	3,4-DiF	1*R*,2*S*-*trans*	*19 ± 8%*	*25 ± 5%*	8.18 ± 2.22
19	N	3,4-DiF	1*S*,2*R*-*trans*	*26 ± 1%*	*41 ± 1%*	7.31 ± 1.74

[a] Binding was performed as described[11] using membranes prepared from HEK293 (A_{2A} only) or CHO cells stably expressing one of three hAR subtypes. The binding affinities for A_1, A_{2A} and A_3ARs were determined using agonist radioligands [^{3}H]N^6-*R*-phenylisopropyladenosine ([^{3}H]**24**), [^{3}H]2-[*p*-(2-carboxyethyl)phenyl-ethylamino]-5′-*N*-ethylcarboxamido-adenosine ([^{3}H]**25**), or [^{125}I]N^6-(4-amino-3-iodobenzyl)adenosine-5′-*N*-methyluronamide ([^{125}I]**26**), respectively. Data (n = 3–4) are expressed as K_i values. A percent in italics refers to inhibition of binding at 10 μM. Stock solutions of N^6-(2-phenylcyclopropyl) derivatives in DMSO were stored at −80 °C.
[b] Compounds **1**,[8] **4**,[9] **5**,[10] **7**[11] and **8**[9] were reported earlier.
[c] See Chart 1 for structure.
[d] Not applicable.
[e] Compound **16**, MRS7030; **17**, MRS7034.

substituent of the synthesized compounds to be directed towards the extracellular side in proximity of TM2; in fact, this group could be accommodated in the binding site only after outward movement of TM2, as previously described.[9,11] On the other hand, the different N^6 substituents of the reported derivatives are in proximity of EL2, and even though the recognition region for the N^6 group is on the outer regions, it involves mainly hydrophobic residues at the hA_3AR. In fact, at this receptor the exocyclic amino group of the ligands is in proximity of a small, secondary (side) pocket delimited by EL2 (residues Val169, Ser170, Val171, Met172, Arg173, Met174 and Met 151). This side pocket is able to well accommodate substituents such as N^6-(3-Cl-benzyl) and N^6-phenylethyl (see docking poses of corresponding compounds **4** and **7** in Fig. 1). The good fit of these substituents with this region can explain the very high affinity of such compounds at the hA_3AR. These two derivatives also showed high affinity for the mouse A_3 subtype, in agreement with the observation that the mA_3AR can accommodate these N^6 substituents in the same region, as previously reported,[11] even though the surrounding residues are different. One key difference is the presence of an Arg in the murine A_3 subtypes (both mouse and rat) in place of Val169 in the hA_3AR. This bulkier and positively charged residue can determine a more difficult fit of bulkier and/or more rigid N^6 groups. This observation can explain the substantially lower affinity of compound **1**, with the N^6-(2-phenylcyclopropyl) group, at the rA_3AR as compared to the hA_3AR.[8]

Residues in EL2 seem to be implicated also in A_{2A} versus A_3 selectivity, as suggested before by docking studies.[23] At the $hA_{2A}AR$, the glutamic acid (Glu169) present at this position interacts through H-bonds with both agonists (e.g., at unsubstituted 6-NH_2 groups) and antagonists, as highlighted by crystallographic data.[1–3] As we observed previously, the insertion of an extended C2-arylethynyl substituent considerably reduces the affinity at the A_{2A} subtype.[9] However, the only C2-arylethynyl derivative showing a good docking pose at the $hA_{2A}AR$ was compound **9** (Fig. S1, Supporting Information), in agreement with its binding affinity of ~1 μM at this subtype. This compound is the only one of the series presenting an unsubstituted exocyclic amino group, which can interact with Glu169 in EL2 through a H-bond.

At the hA_3AR, the N^6-(2-phenylcyclopropyl) group of the C2-arylethynyl compounds can still be accommodated in the region surrounded by EL2, even though there is a cost in affinity. This is probably due to the increased rigidity compared to the N^6-phenylethyl derivatives (compare compound **10**, K_i hA_3AR = 6.16 nM, and compound **7**, K_i hA_3AR = 0.85 nM). Moreover, binding data from both the ribose series and (N)-methanocarba series showed binding stereoselectivity for this substituent in several isomeric pairs with preference for the 1*S*,2*R* diastereoisomer. This can be explained considering that the 1*S*,2*R* isomers could better fit the EL2-delimited side pocket as compared to the 1*R*,2*S* isomers, as shown by the docking poses of compounds **16** and **17** in Figure 1. On the other hand, no difference in affinity was observed for the N^6-(2-phenylcyclopropyl) C2-(2-pyridylethynyl) isomers **18** and **19**. This seems to be related to a compensatory effect due to the formation of an additional H-bond between the pyridine nitrogen and the backbone amino group of Phe168 in EL2 of the hA_3AR (Fig. S2, Supporting Information).



Scheme 1. Synthesis of N^6-phenylcyclopropyl (N)-methanocarba derivatives **10**–**19**. Reagents and conditions: (i) R^2-phenylcyclopropyl-NH_2, Et_3N, MeOH, rt; (ii) 40% $MeNH_2$, MeOH, rt; (iii) $HC{\equiv}CR^1$, $Pd(PPh_3)_2Cl_2$, CuI, Et_3N, DMF, rt; (iv) 10% TFA, MeOH, 70 °C.

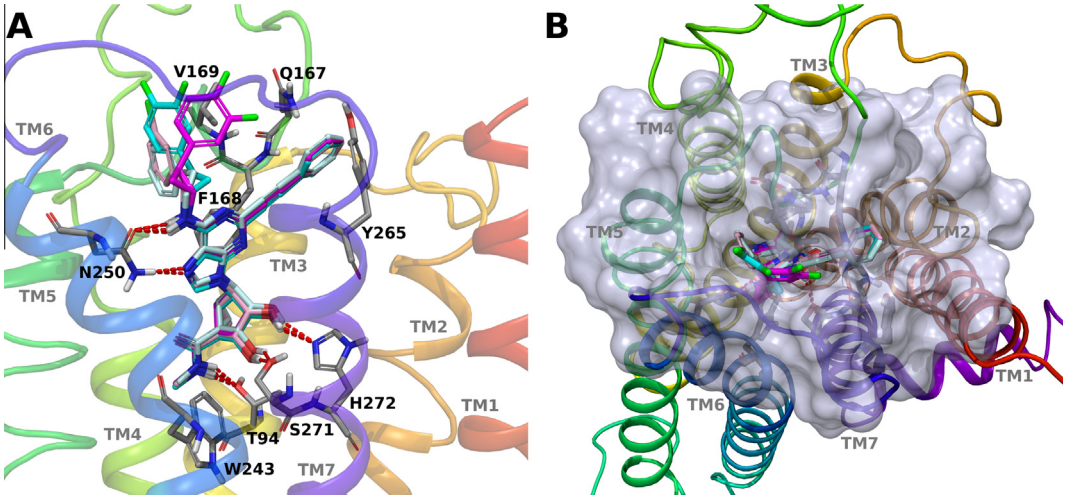


Figure 1. Putative binding modes of selected (N)-methanocarba derivatives obtained after docking simulations at the hA_3AR model. (A) Side view of the receptor binding site. Ligands are shown in sticks: compound **4** (pink carbons), compound **7** (pale cyan carbons), compound **16** (magenta carbons) and compound **17** (cyan carbons). Side chains of some amino acids important for ligand recognition are highlighted (grey carbon sticks). H-bonding interactions are pictured as dotted lines. Nonpolar hydrogen atoms are not displayed. (B) Top view of the receptor binding site. Semi-transparent surface of binding site's residues is displayed. Color scheme as in panel A.

The importance of the ELs in ligand recognition is emphasized in the modeling results. In fact, even though compounds have the ability to form all the crucial interactions with key residues in the lower part of the binding site, N^6 and C2 substituents can modulate their affinity by interacting with the aqueous-exposed outer region of the binding site. In general, the hA_3AR is able to tolerate bulkier and/or more rigid substitutions at these positions as compared to the murine A_3ARs and the $hA_{2A}AR$, because of the differences present in EL2. Therefore, the hA_3AR can accommodate several different-sized N^6 groups, i.e. methyl, 3-Cl-benzyl, phenylethyl and 2-phenylcyclopropyl, in a hydrophobic region delimited by EL2, maintaining good affinity. Moreover, different N^6- and C2-substituents seem to influence each other depending on their complementarity with the binding site. In fact, the presence of a N^6-(2-phenylcyclopropyl) group is more tolerated in absence of an extended C2-arylethynyl, while the presence of both rigid groups makes it more difficult to accommodate the compound in the cavity (compare compound **5**, K_i hA_3AR = 0.78 nM, and compound **10**, K_i hA_3AR = 6.16 nM). The C2 substituent can also have a compensatory effect due to the formation of additional

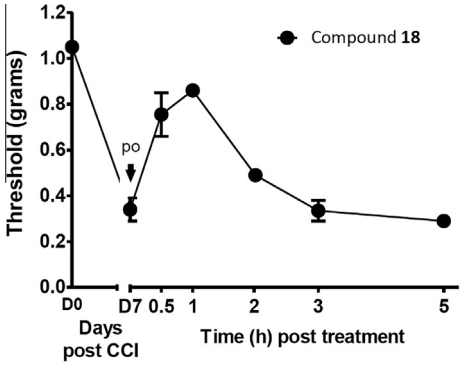


Figure 2. Activity of **18** in a CCI model of chronic neuropathic pain (mechanoallodynia), performed as described (po, oral administration).[24] Paw withdrawal threshold is shown as a function of time. The nucleoside was administered by gavage at the time of peak pain (7 days after chronic constriction injury).

interactions as observed in the C2-(2-pyridylethynyl) series. The modulatory action of these two substituents can be related both to their ability to properly orient the ligand in the binding site so that it can form optimal interactions with key residues, and to their possible role in influencing the conformation of residues in EL2 by modulating their interaction with the ligand. This is understandable considering that a highly conserved interaction in AR binding is the adenine π–π stacking interaction with the conserved Phe in EL2; therefore, possible conformational changes determined by rigidified substituents interacting with the loop could change the orientation of this Phe and its interaction with the ligand.

Although the molecular weight of this chemical series is outside the optimal range for oral bioavailability (MW 558; *c*log*P* 2.63; total polar surface area 134 Å), one derivative **18** was tested in the chronic constriction injury (CCI) pain model after oral administration, following the general method previously described.[24] It displayed considerable activity in reversing chronic neuropathic pain (Fig. 2). This likely reflects the very high A_3AR affinity and selectivity of this compound series. The peak effect occurred 1 h post-administration.

In conclusion, we have identified a group of highly rigidified and spatially extended nucleosides that were shown to be selective A_3AR agonists. Rigid substituents at N^6 and C2 positions, each of which was previously established as being conducive to A_3AR binding, were combined. The activity of a representative compound in a clinically relevant in vivo pain model was demonstrated. Given these pharmacologically important properties, the conformational analysis of receptor binding was analyzed. The steric constraints of this molecular series allow us to probe the environment and propose specific amino acid interactions when receptor-bound. The conformationally constrained N^6 group in docking to an A_3AR homology model formed clear hydrophobic interactions with the normally flexible EL2. The conformational mobility of residues in this loop in the closely related A_{2A}AR structures was noted, based on a comparison of agonist-bound and antagonist-bound structures.[2] This study introduces sterically well-defined ligands that have rigid projections that can define the conformations of the A_3AR in the TM and outer regions that are required to accommodate these ligands. This study introduces tools that will aid in the understanding of the role of receptor plasticity (in particular within the EL regions) in receptor recognition and activation.

Acknowledgments

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Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bmcl.2014.06.006.

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