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Polypharmacology of conformationally locked methanocarba nucleosides

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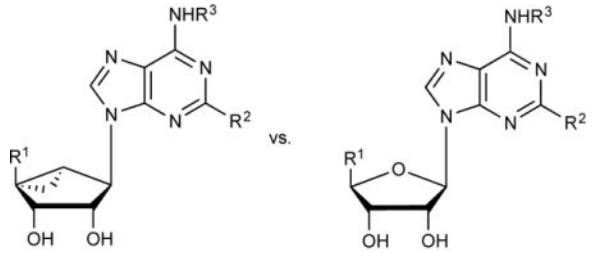
Abstract

A single molecular scaffold can be adapted to interact with diverse targets, either separately or simultaneously. Nucleosides and nucleotides in which ribose is substituted with bicyclo[3.1.0]hexane are an example of a versatile drug-like scaffold for increasing selectivity at their classical targets: kinases, polymerases, adenosine and P2 receptors. Also, by applying structure-based functional group manipulations, rigidified adenosine derivatives can be repurposed to satisfy pharmacophoric requirements of various GPCRs, ion channels, enzymes and transporters, initially detected as off-target activities. Recent examples include $5HT_{2B}$ serotonin receptor antagonists and novel dopamine transporter allosteric modulators. This directable target diversity establishes rigid nucleosides as privileged scaffolds.

Graphical abstract



Introduction

It is now recognized that many pharmaceuticals on the market for central nervous system (CNS) diseases, cancer and other conditions hit multiple targets [1–3]. The pharmacological spectrum of a given compound can contribute to its net biological benefit in a disease state or detract from it through undesired side-effects [4]. Thus, it is important to assess and direct, if possible, the multiple actions of a compound or compound class. Here, we analyze in detail the polypharmacology of a class of nucleoside derivatives that were initially

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introduced as antiviral agents [5,6] and as selective ligands of purine receptors in the cell membrane [7]. Structural modification within this class can direct a given compound toward multitarget action or a single interaction, either at the original receptor or through previously undetected mechanisms.

The term ‘privileged scaffold’ was coined by Evans *et al.* [8,9] as a core structure that can be adapted to different protein targets by functionalization, early examples of which were benzodiazepines, indoles and 1,4-dihydropyridines [10]. This concept has been explored widely for chemically diverse scaffolds, especially flat heterocyclic systems. Benzodiazepines, otherwise known as allosteric enhancers at the γ-aminobutyric acid $(GABA)_A$ ionotropic receptor, can be functionalized to achieve high-affinity binding at various G-protein-coupled receptors (GPCRs) of interest such as cholecystokinin (CCK) receptors [8].

Biologically relevant chemical space is immense, and various attempts have been made to chart and categorize it [11–13]. Within that space, privileged structures, fragments or scaffolds have been identified by diverse screening and by design, including combinatorial design of bicyclic structures [14–16]. Purine nucleobases were previously identified as privileged structures for medicinal chemistry [14,15]. We and others have modified nucleoside derivatives to expand the range of their target proteins and to shift their selectivity. It is now apparent that nucleosides can serve as privileged scaffolds to bind to diverse proteins, and the many nucleoside drugs approved for therapy testify to the pharmacological versatility of this scaffold [17].

Conventional targets of nucleosides and small nucleotides

Nucleosides and nucleotides constitute a large class of drug-like molecules. Many nucleosides have proven their favorable physicochemical properties for use in humans in anticancer and antiviral therapies [17], which account for more than 30 pharmaceuticals currently on the market, mostly in those two categories. Complex nucleoside derivatives are also useful as antibiotics [18]. Therapies based on oligonucleotides and aptamers are also under development [19,20]. Various other nucleoside drugs, such as kinase inhibitors, can mimic a substrate and compete for a common binding site on their targets.

Naturally occurring extracellular nucleosides and nucleotides bind to and activate a variety of cell surface receptors that have diverse and important signaling roles in the body [21–23]. These receptors include family A (rhodopsin-like) GPCRs [i.e., adenosine receptors (ARs) and P2Y receptors (P2YRs)] and ligand-gated ion channels [i.e., P2X receptors (P2XRs)]. There are four AR subtypes and eight subtypes of P2YRs, which can function as monomeric GPCRs, as dimeric species in some cases or as higher order aggregates. The P2YRs have two subfamilies: $P2Y_1$R-like G_q-coupled and $P2Y_{12}$R-like G_i-coupled. In addition, there are seven P2XR subunits that form obligatory trimeric channels activated by ATP. The heterotrimeric or homotrimeric composition of each determines a characteristic pharmacology. Purine and pyrimidine nucleotides are important in the activation of P2YRs, whereas ARs and P2XRs are principally activated by adenine nucleosides and nucleotides, respectively. The concentrations of extracellular nucleosides and nucleotides, and

consequently the levels of endogenous stimulation of these receptors, are controlled by enzymatic, transport and channel processes and by cell damage causing the release of these ligands from intracellular sources. The production and degradation of extracellular nucleosides and nucleotides along with their signaling functions through 19 receptors can be considered a 'purinome' [24]. Purinergic signaling through these receptors, transporters and enzymes has a role in most physiological processes and constitutes a major system for homeostatic control in the body. This has led to numerous therapeutic concepts, such as selective A_3AR activation for cancer, inflammatory disease, chronic neuropathic pain and other conditions [25,26].

Our studies of nucleoside polypharmacology have focused recently on methanocarba nucleoside and nucleotide analogs, in which the tetrahydrofuryl core of the ribose ring system is replaced with a rigid bicyclo[3.1.0]hexane. This ring system was first introduced in nucleosides by Marquez and colleagues, and applied to conformational control of substrates of kinases and for transporters, aptamers and oligonucleotides [5,6]. There are two isomeric forms in the methanocarba series, depending on the fusion site of the cyclopropane ring: North (N)-methanocarba and South (S)-methanocarba (Fig. 1). These constrained [rings act as bioisosteres of ribose to pre-establish the conformation that is preferred by the target protein(s) or nucleic acids, thus lowering energy barriers for binding. The bicyclo[3.1.0] hexane ring lacks a furanose-type oxygen, which in some cases offers stabilizing effects. However, compared with the other locked nucleosides such as locked nucleic acids (LNAs) (containing an extra methylene bridge connecting the 4′ carbon and 2′ oxygen as an ether) [27], methanocarba rings feature the following advantages in addition to conformational rigidity: (i) availability of 2′ and 3′ hydroxyl groups for interaction with biomolecules, which are essential for ribose-like behavior; (ii) less sterically crowded than LNAs around the furanose plane.

The synthesis of these conformationally locked nucleosides requires long synthetic routes, the various stages of which are described in detail elsewhere [28–32]. Optimization of the synthetic approaches has made this nucleoside class more synthetically tractable and allowed stereochemical purity to be achieved. Common intermediates allow the introduction of diverse functional groups that can direct the polypharmacology of this compound class. The (S)-methanocarba nucleosides were initially prepared and tested as a mixture of enantiomers [39], but in 2008 the synthesis of the pure enantiomer series was reported [33], which enabled conformational studies.

Receptor targets

The methanocarba ring constraint was shown to be useful for designing ligands of ARs, P2YRs and P2XRs, as well as antiviral and anticancer compounds (Fig. 2). For example, a potent agonist of the A_3AR, MRS5980 (**1**), contains a (N)-methanocarba ring [34]. MRS5980 and its congeners were demonstrated to have drug-like properties in ADMET tests, including oral bioavailability [34]. The high affinity (N)-methanocarba agonist MRS5980 (**1**) was twofold and 32-fold more-potent in binding to the human (*h*) and mouse (*m*)A_3AR, respectively, than the corresponding ribose derivative MRS7294 (**2**) [35]. Similarly, the N^6-propyl equivalents of **1** and **2** (not shown) displayed even more

pronounced difference; the binding affinity of the (N)-methanocarba analog was sixfold higher than the ribose equivalent at the *h*A_3AR and 88-fold in affinity higher at the *m*A_3AR [35]. At the rat *(r)*A_3AR, agonist affinity was better maintained with respect to *h*A_3AR in the methanocarba series compared with the ribose series [7]. Selectivity for the A_3AR over the other ARs, especially the A_{2A}AR, was also increased by this ring modification. Thus, the (N)-methanocarba nucleosides, simple and hypermodified, either preserved or enhanced the affinity at the A_3AR in multiple species.

The (N)- and (S)-methanocarba rings were systematically incorporated in most of the native nucleotide ligands of the P2YR family. At the $P2Y_1R$, the (N) conformer was highly favored, whereas at the $P2Y_6R$, which is also coupled to G_q-protein, the (S) conformer was highly favored [28]. MRS2365 (**4**) was enhanced in affinity (K_i = 0.4 nM) as well as selectivity as an agonist of the $P2Y_1R$, a platelet receptor that is important in aggregation, in comparison to its ribose analog 2-methylthio-adenosine 5′-diphosphate (2-MeSADP, K_i ~ 100 nM). Compound **4** activated only the $P2Y_1R$, whereas 2-MeSADP, like the native agonist ADP, additionally activated the G_i-coupled $P2Y_{12}R$ and $P2Y_{13}R$. In fact, neither the (N)- or (S)-methanocarba analogs of the native agonists of the G_i-coupled P2YR subfamily activated those receptors, perhaps because of steric hindrance of the cyclopropyl ring by a conserved $Val^{3.30}$ residue in TM3 [36]. However, at the G_q-coupled $P2Y_6R$, the (N) analog of native agonist UDP was inactive, whereas the (S) analog, MRS2795 (**5**, enantiomerically pure), was 7-fold more potent than UDP in a functional assay [37]. Thus, even within the same GPCR family that has a relatively high sequence identity (33% for *h*$P2Y_1R$ and *h*$P2Y_6R$ compared with 21% for *h*$P2Y_1R$ and *h*$P2Y_{12}R$), the conformational preference of the ribose was variable and dramatic, leading to increased selectivity of the methanocarba nucleotide analogs.

A 2′-deoxy-methanocarba nucleotide, (1′*R*,2′*S*,4′*S*,5′*S*)-4-(2-iodo-6-methylamino-purin-9-yl)-1-[(phosphato)-methyl]-2-(phosphato)-bicyclo[3.1.0]hexane (MRS2500, **3**) is the most potent known competitive antagonist for the ADP-activated $P2Y_1R$ and has antithrombotic activity [38]. The X-ray structure of the $P2Y_1R$ in complex with **3** confirmed the (N) conformation of this inhibitor (Fig. 3). The nucleotide binding site was located almost exclusively in the receptor's extracellular region, which is unusual for an orthosteric ligand binding region (i.e., the same site at which a native agonist binds). The hydrophobic nucleobase of **3** was inserted in a hydrophobic pocket at the top of the binding site between the extracellular tips of transmembrane helix (TM)6 and TM7 and the N-terminal domain. The hydrophilic and charged, phosphorylated pseudoribose moiety was anchored at the bottom of the binding site, coordinated by residues in the second extracellular loop (EL2). The phosphate moieties at 3′ and 5′ positions contributed to ligand recognition by associating with positively charged and H-bonding amino acids of the receptor's outer regions, which was confirmed by site-directed mutagenesis [38].

Four subtypes of P2XRs in primary rat neurons (homotrimeric P2X1, P2X2 and P2X3Rs and a heteromeric P2X2/3R) also displayed a strong preference for the (N)-methanocarba analog of ATP (**6**) compared with the (S)-methanocarba analog **7** (racemic), which was weak or inactive [39]. An (N)-methanocarba equivalent (MRS2339, **8**) of 2-chloro-AMP that

activates a P2 × 4R on cardiac myocytes is an experimental drug for treating heart failure [40].

Kinase and RNA targets

Marquez and colleagues have extensively characterized rigidified nucleosides and nucleotides at diverse kinases and polymerases [5,6]. Although in general (S)-methanocarba isomers are the preferred substrates for nucleoside kinases and (N) isomers for nucleotide kinases, in many cases the nonpreferred isomers are metabolized at biologically significant rates, depending on the substrate and enzyme variant. For instance, *h*-deoxycytidine kinase and nucleoside-diphosphate kinases (NDPKs) readily phosphorylate (S)- and (N)-methanocarba-cytidine [41], but there is no human counterpart that metabolizes (N)-methanocarbathymidine (N-MCT, **10**; Fig. 2). Nevertheless, N-MCT is a potent inhibitor of herpes simplex virus (HSV) [42] because of its species-dependent selectivity as a substrate for HSV thymidine kinases (HSV-TKs). It is also being evaluated for treatment of Kaposi's-sarcoma-associated herpes virus [43], and a clinical trial of N-MCT was initiated for shingles [44]. Hence, in a diseased state the combination of properties of nucleoside/nucleotide-metabolizing enzymes (i.e., viral and cellular kinases and polymerases) was exploited to turn N-MCT into a potent HSV inhibitor [45,46]. Similarly, most polymerases [e.g., *h*DNA polymerase and HIV reverse transcriptase (HIV-RT)] incorporated only (N)-methanocarba nucleoside triphosphates [47–49]. A qualitative study of locked nucleotides as substrates of ectonucleotidases also suggested some degree of bias. The *r*NTPDase1 hydrolyzed (N)- and (S)-isomers at about half the rate of ATP, whereas *r*NTPDase2 did not hydrolyze (N)-methanocarba-ATP (**6**) [50]. The corresponding (S)-isomer **7** was hydrolyzed three-times slower than ATP by *r*NTPDase2. Also, both the isomers of methanocarba-AMP were relatively stable to 5′-ectonucleotidase (CD73) [50].

Recently, (N)- and (S)-methanocarba nucleosides MRS4203 **11** and MRS4380 **12** were also introduced as inhibitors of adenosine kinase (ADK), another conventional purine target [51]. Inhibitors of *h*ADK have pronounced antiepileptic and antiepileptogenic effects [52]. Compounds **11** and **12** were comparable in potency to a standard ADK carbocyclic nucleoside inhibitor A-134974 [51]. A year after the first report of methanocarba nucleosides by Marquez and colleagues [53], Altmann *et al.* independently disclosed that the stability of oligodeoxynucleotide heteroduplexes involving (N)-methanocarba-T (T^N) increased, and the (S)-methanocarba-T destabilized the heteroduplex [54,55]. Later, the stability of oligonucleotides having multiple T^N was found to be additive in nature [5,56]. This led to a study on RNAi of siRNAs with T^N modifications. Compared with LNAs, in addition to the increased thermal and serum stability of siRNA-duplexes in A-form, the North locked T^N congeners were less subject to innate immunostimulation but with comparable gene-silencing activities [57].

Unconventional nucleoside targets

Recent findings suggest that the utility of nucleosides and nucleotides extends beyond the classical targets, especially among analogs that contain a sterically constrained substitution of ribose [58]. In some cases, a secondary activity, such as antioxidant, can be engineered

into the nucleoside or nucleotide [59]. In other cases, the nucleobase alone displays a spectrum of activities (e.g., adenine derivatives that hit multiple targets). Therefore, methanocarba nucleosides and nucleotides are particularly suited for the exploration of polypharmacology, which needs to be considered in the biological characterization of any nucleoside or nucleotide. Furthermore, the rigidity of this scaffold facilitates the 3D exploration of ligand–protein interactions.

The methanocarba ring system is one of many constrained small ring systems that have been applied to conformational control in drug design. This is a general modification of nucleosides and nucleotides that can enhance high-affinity interactions with a variety of protein targets. The methanocarba modification has been shown to increase the affinity or selectivity, or both parameters, compared with ribose at a target such as a GPCR by enforcing a specific conformation that approximates the target-preferred conformation, such as at a GPCR [24]. Thus, drug-like methanocarba AR ligands have been repurposed to satisfy the pharmacophoric requirements of various GPCRs and other protein targets. The ability to introduce diverse chemical functionality at multiple sites in this privileged scaffold class provides the ability to adapt to different biological targets. By systematically identifying off-target sites as minor activities of nucleosides and then enhancing those activities by stepwise structural modification, this approach can be considered as scaffold repurposing.

Representative nucleosides, designed for interaction with ARs, were screened in radioligand binding assays at 53 diverse off-target activities performed by the Psychoactive Drug Screening Program (PDSP) at the University of North Carolina. The weak offtarget hits at non-nucleoside receptors and transporters included the α- and β-adrenergic receptors, serotonin receptors, δ-opioid receptor (DOR), sigma receptors, the translocator protein (TSPO), among others. A prototypical methanocarba nucleoside training set of ten congeneric compounds was used to probe the orientation of these ligands in selected off-target proteins (e.g., GPCRs) for which structural information is available. This led to SAR studies of nucleoside derivatives at the off-target GPCRs and other protein targets. For example, the potent agonist of *h* and *m*A_3AR MRS5698 (**13**, K_i 3 nM) and its congeners additionally bound to the rat TSPO in the 200–300 nM range. Thus, this systematic effort revealed a modest number of unanticipated interactions of these rigidified and highly substituted nucleosides and their substructures with diverse off-target sites.

Subsequent studies focused on particular off-target hits to enhance the activity and/or selectivity at these sites as well as to minimize the activity at the previous on-target site (i.e., the Ars). Being conformationally constrained, (N)-methanocarba derivatives increase awareness of the spatial environment at diverse receptors of known structure. In some cases, we have arrived at self-consistent computational models for recognition of nucleosides at these unconventional targets. These nucleosides are not pan-assay interference compounds (PAINS) because each compound has no more than a few diverse interactions. However, the versatility of substitution extends the relevance of this chemical class to diverse targets. Thus, we have expanded the range of target proteins that interact with nucleoside derivatives.

Off-target activity of nucleosides at other (non-adenosine) GPCRs

A detailed examination of the polypharmacology of methanocarba nucleosides and their derivatives revealed moderate cross reactivity with other non-purine GPCRs, usually at higher concentrations. This cross reactivity could be modulated depending on the ligand functionalization. The SARs of the (N)-methanocarba nucleoside derivatives that were synthesized to achieve selective activation of a subtype of the ARs (e.g., A_3AR) were analyzed with respect to off-target GPCR activities. The contributions to the new SAR of large hydrophobic N^6 groups, adenine nitrogens, 5′-functionalization or extended C^2-alkynyl groups were explored. Thus, functional group substitution on the methanocarba adenosine derivatives was customized to favor each class of new targets.

The ARs belong to the rhodopsin-like α-branch GPCRs, and other members include the biogenic amine receptors. In an initial study of polypharmacology, various off-target activities of AR agonists were found at the biogenic amine receptors, with K_i values as low as 61 nM. To design nucleosides that are selective for the biogenic amine receptors, it was necessary to incorporate the SAR features favoring these off-target receptors, to deselect the well-defined activity at the ARs. Some nucleoside derivatives synthesized in the context of AR activity interacted weakly with $5HT_{2B}$/$5HT_{2C}$ serotonin receptors (Table 1). This simplified table indicates the typical contributions of each structural feature to the overall affinity of these nucleosides at the indicated target. The SAR of these (N)-methanocarba adenosine derivatives was then probed to design related molecules that were even more potent in binding at $5HT_{2B}$ and $5HT_{2C}$ receptors than our original fortuitous hits. Using computational docking and molecular dynamics, putative interactions of these rigid nucleosides with $5HT_{2B}$ and $5HT_{2C}$ receptors were predicted. Functional assays demonstrated that the nucleosides were antagonists at serotonin receptors. N^6-dicyclopropylmethyl 5′-methylamide (N)-methanocarba derivative MRS7185 (**15**;) was 170-fold selective in functional assays as an antagonist of the $5HT_{2B}$R compared with the $5HT_{2C}$R. The corresponding 5′-ethyl ester MRS7221 (**14**) bound with higher affinity but not selectivity at the $5HT_{2B}$R.

The pharmacokinetics of **15** and the methyl ester homolog of **14** demonstrated prolonged exposure *in vivo.* The ester derivative was shown to be eliminated slowly in the rat, and therefore not rapidly hydrolyzed by esterases. Nucleoside derivatives typically do not readily cross the blood–brain barrier, and thus many of the (N)-methanocarba nucleosides described in this work are expected to attain much higher concentrations in the periphery than in the brain. Peripherally acting $5HT_{2B}$R antagonists might be useful for protection of liver and heart tissue because activation of the $5HT_{2B}$R causes fibrosis in these tissues.

The orientation of the methanocarba nucleosides in each target protein, such as a GPCR, can be analyzed by molecular modeling or in the best case by X-ray crystallography, which aids in their subsequent derivatization. In different GPCRs, the relative orientation of the pseudoribose and the nucleobase can be similar or even reversed. Docking and molecular dynamics (MD) analyses of the rigid adenosine derivative MRS7185 (**15**) at h$5HT_{2B}$R suggested a binding mode with ligand inserted deeply in the TM bundle and lying almost parallel to the membrane plane (a). The adenine core of the ligand engaged in a **π-π**

stacking interaction with Phe340$^{6.51}$ (superscript refers to a numbering convention for GPCRs that identifies the TM and the relationship of the residue to the most conserved point in that helix), the N^6-dicyclopropylmethyl moiety pointed toward TM5 and TM6 by establishing hydrophobic contacts with, among other residues, Met218$^{5.39}$.

In modeling the interactions of nucleosides in diverse GPCRs it is important to consider the part played by water molecules. Following MD simulations of MRS7185 (**15**) in the $5HT_{2B}R$, the ribose moiety was anchored in the binding site by extended water-mediated H-bond interactions. Indeed, as depicted in b, several tightly bound water molecules connected the 2′- and 3′-OH groups to the conserved Asp135$^{3.32}$, among other residues. A water molecule anchored the 5′-carbonyl group to the side chain of Gln359$^{7.32}$ (acting as H-bond donor) and the Leu209^{EL2} backbone (acting as H-bond acceptor). This hypothetical binding mode enabled us to rationalize the greater affinity of this nucleoside series for the $h5HT_{2B}R$. Indeed, the $h5HT_{2C}R$ features a Glu$^{7.32}$ residue in place of Gln$^{7.32}$ of $5HT_{2B}R$ and a shorter EL2. The Glu$^{7.32}$ side-chain could not act as a H-bond donor and therefore would not enable a H-bond network to occur in the $h5HT_{2C}R$ as described above for the $h5HT_{2B}R$. The shorter EL2 in the $h5HT_{2C}R$ is expected to affect the 3D arrangement of the downstream region of the loop as well as of the extracellular tip of TM5, where the two key residues binding to the 5′-carbonyl group in $h5HT_{2B}R$, namely Leu209^{EL2} (conserved) and Met$^{5.39}$ (occurring as Val$^{5.39}$ in $h5HT_{2C}R$), are located. A direct comparison of $h5HT_{2B}R$ binding between (N)-methanocarba nucleosides and the corresponding ribosides indicated that the bicyclic ring system enhanced affinity at this nonpurine receptor. MRS5698 **13** also bound to the δ-opioid receptor with a K_i value of 2.44 μM. Thus, the opioid receptor system is also a potential target family for (N)-methanocarba nucleosides. Other GPCRs that recognize various (N)-methanocarba nucleosides in the μM range are α_{2A}, α_{2B}, α_{2C} and β_3 adrenergic receptors.

Nucleoside off-target activity at other (non-nucleoside) transporters

Cell surface transporters for neurotransmitters, such as biogenic amines, ions, metabolites and nucleosides, belong to the large family of solute carrier (SLC) membrane transport proteins, which consists of >300 members. Two of the 52 families of SLC proteins are transporters for adenosine and other nucleosides [i.e., the equilibrative transport (ENT, SLC29) and sodium-coupled concentrative transport (CNT, SLC28) proteins]. The SAR of adenosine and its derivatives at the ENTs and, especially, at the CNTs is more restrictive than the corresponding SAR at ARs. ENT1 is potently inhibited by the (N)-methanocarba nucleoside equivalents of known inhibitors, such as the (N)-methanocarba derivative MRS1942 (**9**). (S) conformers either inhibit or are less potent substrates at ENTs and CNTs, and the permeability in CNTs is greater for (N) conformers than for (S) conformers.

Unexpectedly, a few of the nucleoside analogs interacted with the dopamine transporter (DAT, SLC6A3), a member of the same SLC membrane transporter family as ENTs. DAT is the protein target of cocaine and its blockade -the source of cocaine's behavioral stimulant effects. However, a strange phenomenon was noted in the initial radioligand binding results at *h*DAT – (N)-methanocarba nucleosides dramatically increased radioligand binding (up to sevenfold the control value) rather than inhibiting it. In collaboration with the Janowsky

laboratory, we characterized the nucleosides as novel allosteric DAT modulators, at a previously unaccessed site on the DAT protein – not the same (orthosteric) site where cocaine-like molecules (tropanes) bind. These nucleosides () increased the affinity of radioligands at DAT and inhibited DAT-mediated dopamine uptake. Thus, they have a complex mixture of positive allostery with respect to tropanes and negative allostery with respect to functional activity of DAT in the absence of tropanes. MRS5980 (**1**), a 5′-methylamide that also potently activates the A_3AR, displayed an EC_{50} value of 35 nM in enhancing DAT binding. Some compounds were also found to interact with the norepinephrine transporter (NET, SLC6A2), which is an important target for treating pain and mood disorders. The mode of interaction at NET appears to be similar to DAT (i.e., radioligand binding enhancement at the orthosteric site and transport inhibition). The SAR of this chemical series was then explored to design related molecules that were even more potent in enhancing binding at DAT than the original hits. The corresponding 5′-methyl ester, MRS7292 (**16**), and 5′-ethyl ester, MRS7232 (**17**), were equipotent to the amide 1 at hDAT and even more potent than **1** in enhancing binding at *h*NET. The nucleosides also demonstrated probe dependence depending on which class of DAT radioligands was used – a characteristic of allosteric modulators. At *m*DAT, **16** and **17** were selective in comparison to *m*A_3AR. A feature that was particularly important for transporter interaction was an extended C^2 group terminating in a 5-bromo- or 5-chlorothienyl group; an unsubstituted alkynylthienyl group was considerably less potent. Also, an N^6-methyl group was strongly favored over larger groups. As with the *h*$5HT_{2B}$R, the (N)-methanocarba modification enhanced DAT interaction in comparison to the corresponding riboside. The ribose equivalent of **16** (not shown) was weaker in DAT interaction (EC_{50} 127 nM). Thus, new nucleoside analogs were synthesized that were especially potent and efficacious in their interaction with DAT, and they tended to be less potent than earlier compounds at the original target of ARs. Potential applications of peripherally acting allosteric inhibitors of DAT and NET might be similar to the current use of biogenic amines such as dopamine (i.e., as cardiac inotropic agents and to increase kidney/splanchnic circulation). Other non-GPCR targets to which various (N)-methanocarba nucleosides bind in the μM range are the σ_χ and σ_2 receptors and an ion channel 5_3 serotonin receptor. However, these interactions have not been optimized in this chemical series.

Does endogenous adenosine interact with the 5HT2B and $5HT_{2C}$Rs or with DAT? The affinity of adenosine has not been determined at these proteins; however, 2-chloroadenosine, often taken as a close mimic of adenosine, lacks significant binding affinity at these sites. In fact, no off-target actions of 2-chloroadenosine at 10 μM were found in the standard PDSP screen. Therefore, it is unlikely that endogenous adenosine also interacts with serotonin receptors or other off-target sites described here.

Relationship of nucleoside ligands to other privileged structures

How do nucleosides compare to other scaffolds that were deemed privileged? Most small molecules approved as pharmaceuticals as well as the contents of currently available chemical libraries contain flat heterocycles, and it is suggested that adding three-dimensionality could provide greater utility in hitting protein targets. With respect to the nucleobase, adenine adds hydrophobic character; adenine was reported to be the most

frequently appearing bicyclic structure in approved drugs. An advantage of nucleosides over the nucleobases as privileged scaffolds is that they contain separate domains of predominately either sp^2 or sp^3 atoms. Therefore, methanocarba nucleosides combine flat and 3D characteristics and are desirable as privileged structures because they contain planar (nucleobase) and 3D (ribose or ribose-like) components. The concept of combining these features has already been noted in the literature. For example, Kombarov *et al.* described an approach to drug discovery using privileged structures termed 'BioCores', which, like nucleosides, contain pairs of saturated and aromatic heterocyclic moieties.

For ARs, these two domains (i.e., the adenine and ribose) have separate functions in AR recognition. The adenine moiety with its C^2 and N^6 substituents corresponds to the address portion of the ligands, and the ribose moiety is responsible for AR activation, which could be called the message portion (a). This amphiphilic feature and the high degree of rigidity, especially of the methanocarba nucleosides, can be useful in predicting an energetically favorable binding mode in the binding site of a given protein. Moreover, the divergent physicochemical properties of these two moieties can delineate preferred binding regions in the canonical GPCR binding site in the central cavities of rhodopsin-like GPCRs, which are often amphiphilic. This hybrid feature of nucleosides could be advantageous for interaction with a wide range of proteins in addition to the purine receptors.

Concluding remarks

Nucleosides are well represented as pharmaceuticals and have proven to be a generally well-tolerated drug class. We focus on a subcategory of nucleosides (and nucleotides) that introduces a significant steric constraint on the ribose moiety, which has the effect of enhancing pharmacological properties (e.g., potency and selectivity) and directing their activity at conventional and unconventional targets. The scope of action of these conformationally locked nucleosides has now been extended through embracing their potential usefulness for diverse targets that normally do not recognize ribonucleosides. Thus, the rigid methanocarba nucleoside scaffold that has been well explored at purine receptors, enzymes and transporters has been repurposed to satisfy the pharmacophoric requirements of unrelated GPCRs and transporters, such as biogenic amine carrier proteins. For example, this effort provided novel, selective $5HT_{2B}R$ antagonists and the first allosteric modulators of the dopamine transporter, in some cases providing an array of structural information for interaction with a family of ligand substructures. These novel ligands might be useful as antifibrotic agents ($5HT_{2B}R$ antagonists) or inotropic agents (peripheral DAT modulators). The systematic correlation of protein interaction of the ligands with specific amino acid residues and regions of receptors, for example, could eventually lead to predicting multitarget interactions of new analogs within the same ligand family. Methanocarba nucleosides could be considered a privileged scaffold given their expansive applicability to many drug areas, but clearly they are not generally promiscuous compounds. The repurposing of this chemically well-explored scaffold to new and diverse biological targets could serve as an example for similar analyses for other drug and compound classes.

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Biographies

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Highlights

- Weak off-target activity of nucleosides at novel targets can be optimized.
- Conformationally constrained nucleosides are privileged scaffolds.
- The affinity and selectivity at conventional nucleoside targets can be enhanced.
- Unconventional targets are the dopamine transporter and non-purine GPCRs.

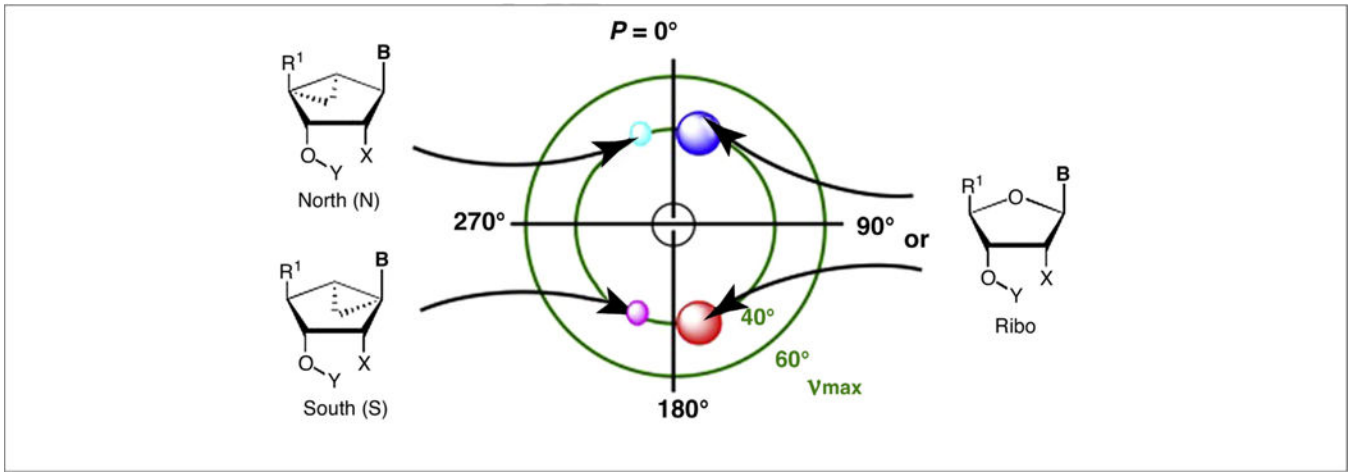


FIGURE 1.

Relationship between ribose ring structure and favored conformations as depicted on the pseudorotational cycle, made by a mathematical formula to describe all twists of the ribose ring [5]. P = pseudorotational angle; ν = out of plane angle. On right side: red circle = region of North (N) conformation in nature; with cyan and link circles representing the conformations of the methanocarba rings (left side). Typically: B = nucleobase; Y = H; X = OH. R^1 can be oxymethylene or carbonyl moieties.

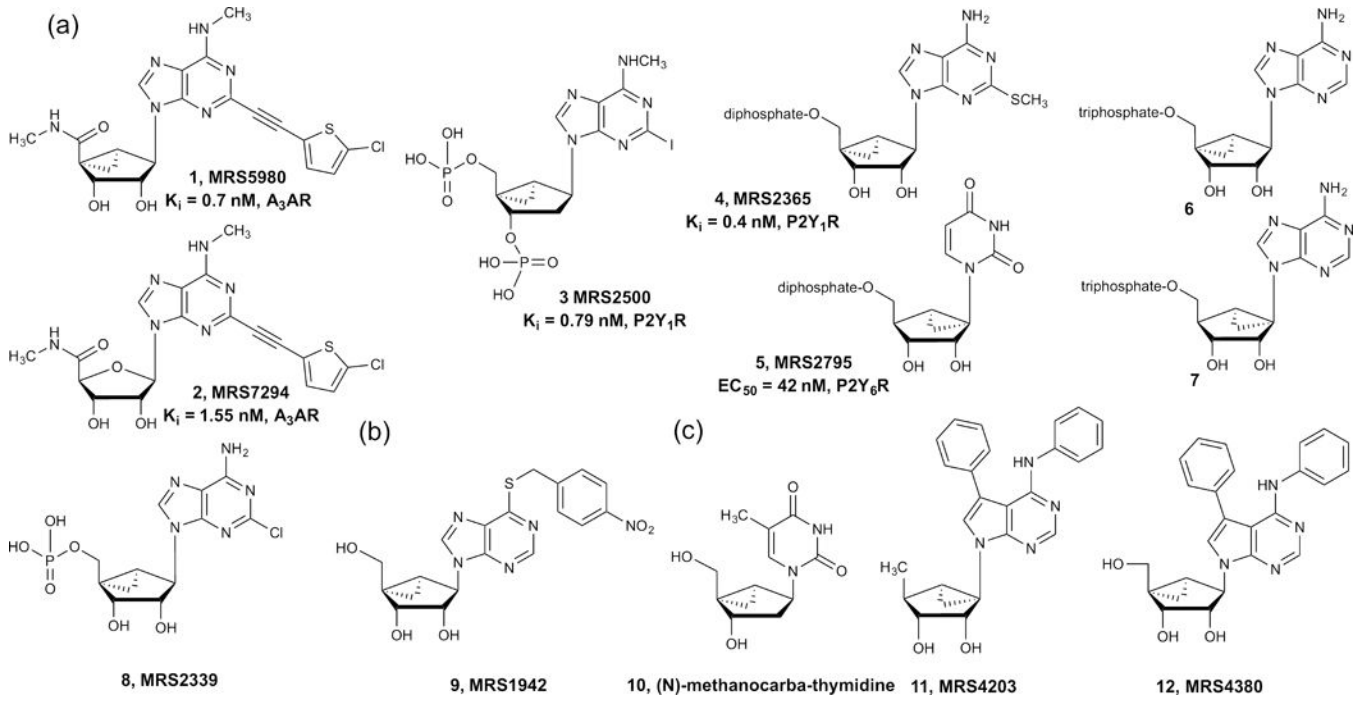


FIGURE 2.

Structures of methanocarba nucleosides that interact with: **(a)** $P2Y_1R$, **(b)** ENT1, **(c)** polymerases and kinases [35,45,62].

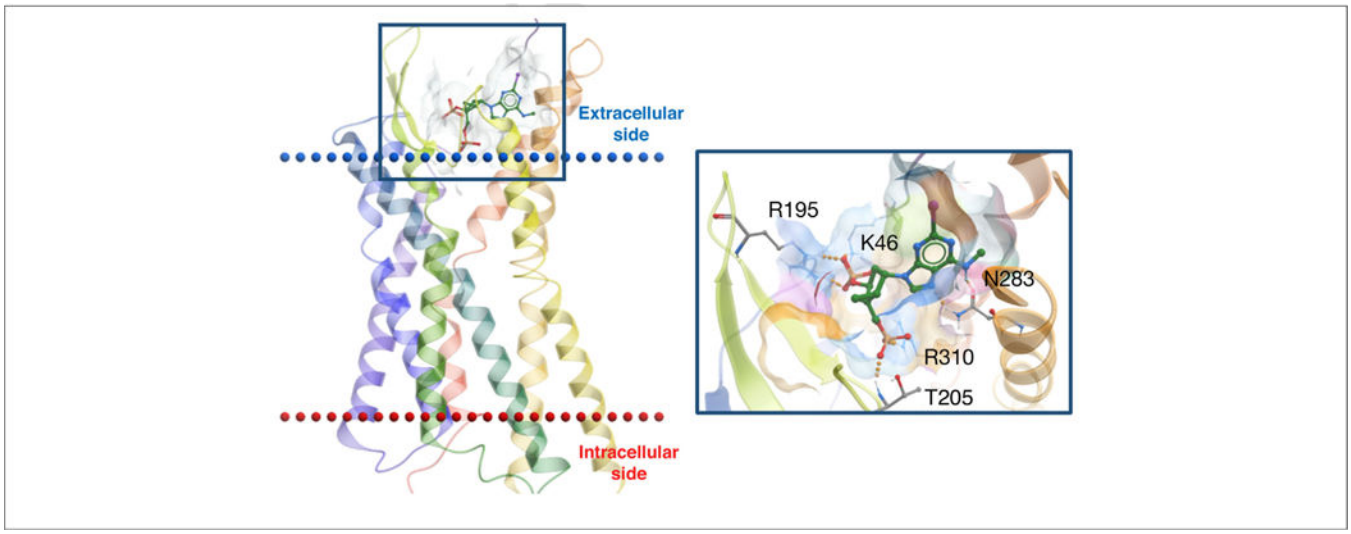


FIGURE 3.

X-ray structure of the hP2Y$_1$R showing the binding mode of orthosteric antagonist MRS2500 (**3**) [38]. In this inactive state, the 3′-phosphate is coordinated by K46 (EL1) and R195 (EL2), the 5′-phosphate deeper in the binding site by T205 (EL2) and R310 (7.39) and N^6 by N283 (6.58).

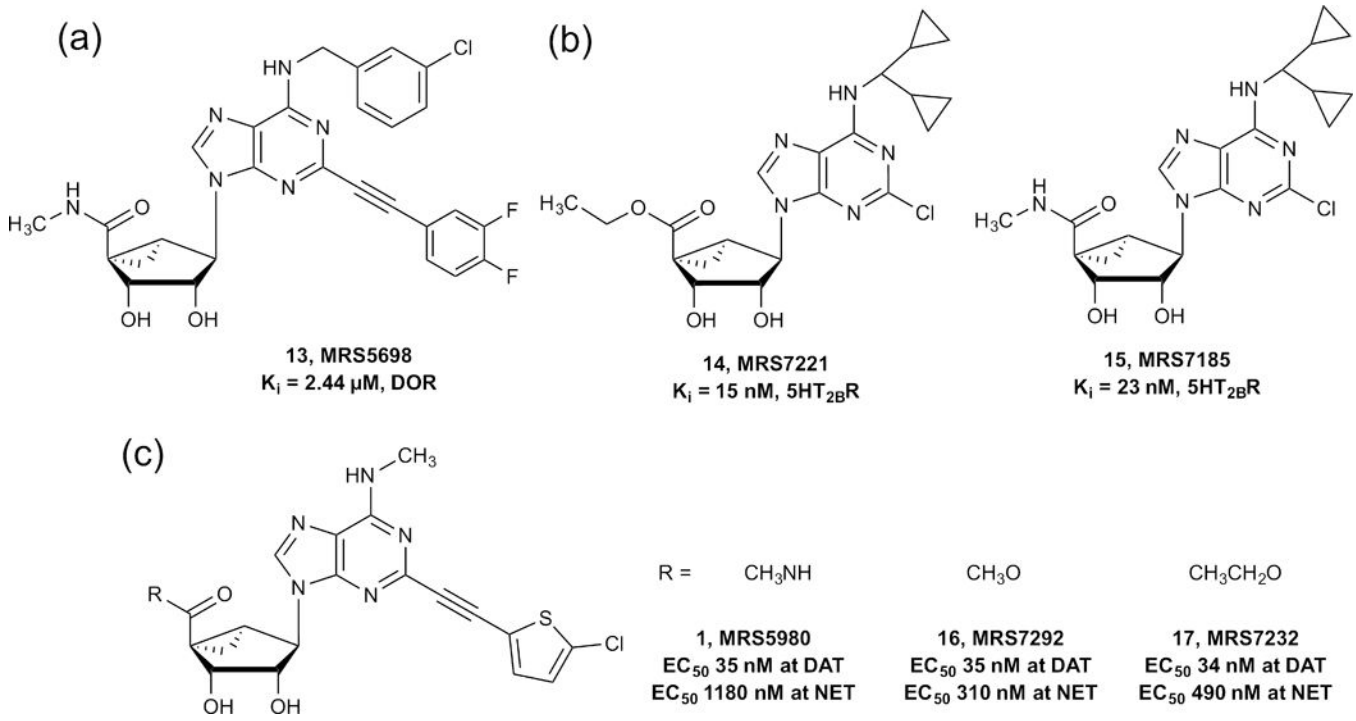


FIGURE 4.

(a) Structures of (N)-methanocarba nucleosides that interact with conventionally non-purine sites. (a) DOR, **(b)** $5HT_2Rs$, **(c)** DAT.

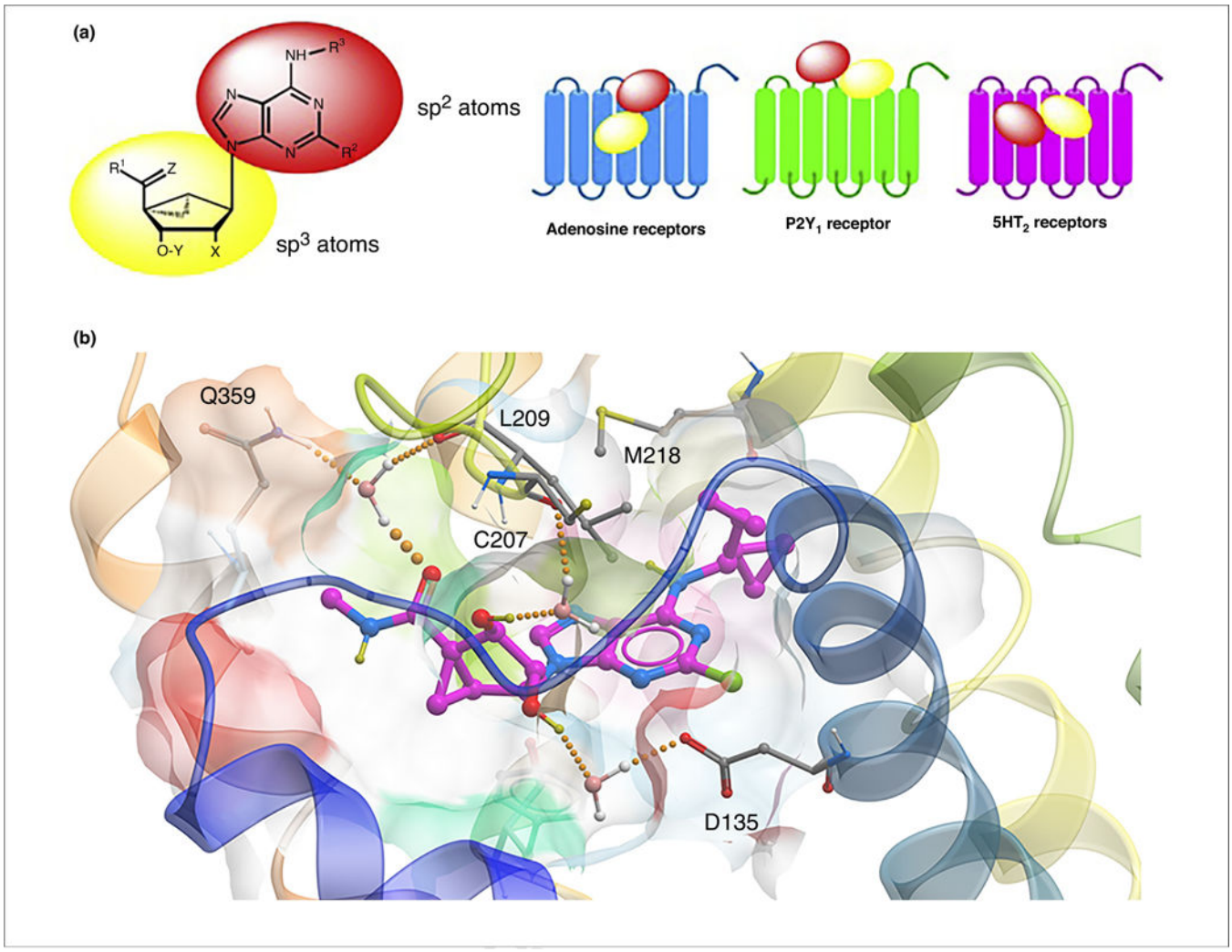
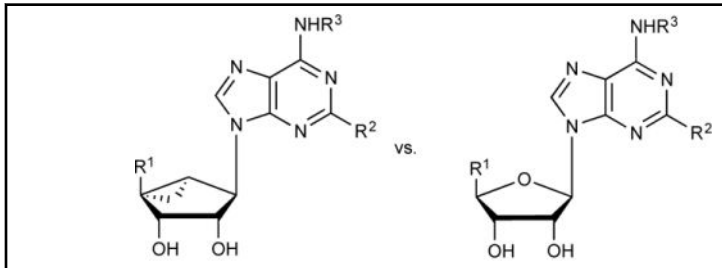


FIGURE 5.

(a) Separate roles of the two moieties in rigid nucleosides in recognition at ARs and other sites, as illustrated for the general case of (N)-methanocarba adenosine derivatives. The relative orientations in the binding sites of ARs (based on X-ray structures of other nucleosides bound in the A_{2A}AR), $P2Y_1$R (from X-ray structure of the MRS2500 (**3**) complex) and in the $5HT_{2B}$R, as predicted following induced fit docking and molecular dynamics simulations, are contrasted. Typical substituents: X = H, phosphate; Y = H, OH; Z = H_2, O; R^1, R^2, R^3 = H, alkyl, O-alkyl; ethynyl, etc. (**b**) Hypothetical binding mode of antagonist MRS7185 (**15**) at the h$5HT_{2B}$R. The H-bonding contacts (all through water bridges) are: 5′-carbonyl with L209 backbone (EL2) and Q359 (7.32), the 2′-hydroxyl to D135 (3.32) and the 3′-hydroxyl to C207 backbone (EL2). M218 (5.39) forms a hydrophobic contact with the N^6 group.

Table 1

Interactions of representative adenosine derivatives with receptors and transporters. This simplified table indicates the typical contributions of each structural feature to the overall affinity of these nucleosides at the indicated target, with the beneficial gain in binding affinity indicated as +++ > ++ > + > −. Values were determined from several examples and are not necessarily general to all examples.



A B

Structural feature	**hA$_1$AR**	**hA$_3$AR**	**mA$_3$AR**	**h5HT$_{2B/2C}$**	**hDAT**	**hNET**
References:	62, 35, 72	62, 35, 72	62	58, 62	35, 72	35, 72
Ribose modifications (A vs. B), R^1 at 5′						
A (N)-methanocarba	+++	+++	+++	+++	+++	+
5′-CONHCH$_3$	+++	+++	+++	++	+++	+
5′-COOMe or 5′-COOEt	++	++[a]	−	++	+++	+
5′-COOPr	ND	++	+	+	+++	−
5′-COO-*i*-Pr	ND	+	−	+ (2C)	−	−
5′-COOH	ND	+	−	+	+	−
5′-CONH-(CH$_2$)$_2$-NH$_2$	−	−	−	+	+	−
4′-truncated	+	++[a]	+	+	−	ND
B 9-riboside (CH$_2$OH)	+++	++	+	++	++	+
Adenine modifications: R^2 at C2; R^3 at N^6						
C2-Cl	+++	+++	+++	++	−	−
C2-C≡C-(5-Cl-thien-2-yl)	−	+++	++	+	+++	+
N^6-Me	++	+++	++	+	+++	+

Structural feature	hA_1AR	hA_3AR	mA_3AR	$h5HT_{2B/2C}$	hDAT	hNET
N^6-Pr	++	+++	++	ND	++	–
N^6-CH(cPr)$_2$	+++	++	ND	+++	–	–
N^6-CH$_2$Ph	++	+++	+++	++	–	–
N^6-(Me)$_2$	–	+	+	++	–	–
1-deaza	+++	+++	++	+	–	–

ND, not determined.

[a] reduces agonist efficacy.