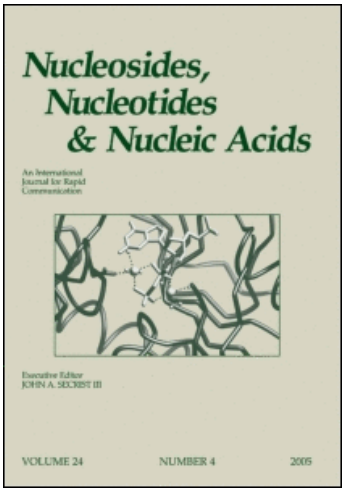

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Action of Nucleosides and Nucleotides at 7 Transmembrane-Spanning Receptors

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ACTION OF NUCLEOSIDES AND NUCLEOTIDES AT 7 TRANSMEMBRANE-SPANNING RECEPTORS

Kenneth A. Jacobson, Stefano Costanzi, Soo-Kyung Kim, Eunjoo Roh, Bhalchandra V. Joshi, Susanna Tchilibon, Heng T. Duong, and Zhan-Guo Gao □ *Molecular Recognition Section, Laboratory of Bioorganic Chemistry, National Institute of Diabetes, Digestive and Kidney Diseases, National Institutes of Health, Bethesda, Maryland, USA*

□ *Ribose ring-constrained nucleosides and nucleotides to act at cell-surface purine recesptors have been designed and synthesized. At the $P2Y_1$ nucleotide receptor and the A_3 adenosine receptor (AR) the North envelope conformation of ribose is highly preferred. We have applied mutagenesis and rhodopsin-based homology modeling to the study of purine receptors and used the structural insights gained to assist in the design of novel ligands. Two subgroups of P2Y receptors have been defined, containing different sets of cationic residues for coordinating the phosphate groups. Modeling/mutagenesis of adenosine receptors has focused on determinants of intrinsic efficacy in adenosine derivatives and on a conserved Trp residue (6.48) which is involved in the activation process. The clinical use of adenosine agonists as cytoprotective agents has been limited by the widespread occurrence of ARs, thus, leading to undesirable side effects of exogenously administered adenosine derivatives. In order to overcome the inherent nonselectivity of activating the native receptors, we have introduced the concept of neoceptors. By this strategy, intended for eventual use in gene therapy, the putative ligand binding site of a G protein-coupled receptor is reengineered for activation by synthetic agonists (neoligands) built to have a structural complementarity. Using a rational design process we have identified neoceptor-neoligand pairs which are pharmacologically orthogonal with respect to the native species.*

Keywords Purines; Neoceptor; Mutagenesis; Pyrimidines; Molecular modeling

INTRODUCTION

Extracellular adenosine, purine nucleotides (such as ATP and ADP), and pyrimidine nucleotides (such as UTP and UDP) act as neurotransmitters/modulators.[1,2] These ubiquitous signaling molecules

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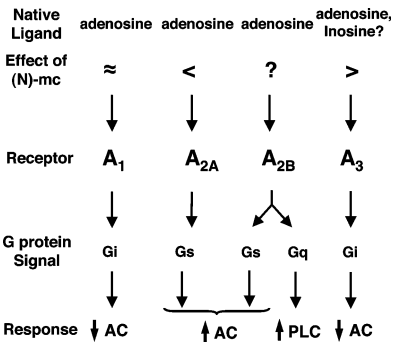


FIGURE 1 The 4 known subtypes of receptors for extracellular adenosine and their principal intracellular signaling systems. Also shown is the general effect on potency at each subtype of substitution of the ribose moiety of the native ligand with the (N)-methanocarba ring system. PLC = phospholipase C. AC = adenylate cyclase.

modulate the function of diverse mammalian cell types and tissues under both normal and pathophysiological conditions. Receptors for extracellular nucleosides and nucleotides have been characterized through medicinal chemical, molecular biological, and pharmacological approaches.

The 4 subtypes of adenosine receptors (ARs), denoted A_1, A_{2A}, A_{2B}, and A_3, are all 7 transmembrane-spanning (7TM) receptors, which couple to G proteins (Figure 1). ARs are involved in many of the body's cytoprotective functions. Activation of the A_1AR decreases the oxygen demand of a stressed organ, for example, by slowing the heart rate. Conversely, activation of the A_{2A}AR tends to increase its oxygen supply, for example, by vasodilation and through inhibition of platelet aggregation. A_{2B}AR activation has an angiogenic effect. A_{2A}AR antagonists are under development as anti-Parkinson's agents.[3] Activation of the cardiac A_3AR preconditions cardiac myocytes against ischemic damage[4] and protects against toxicity, for example, in the case of the cardiotoxic effects of the anticancer drug doxorubicin.[5] A_3AR antagonists are of interest as potential antiglaucoma agents.[6].

The nucleotide receptors, denoted P2, comprise both ligand-gated ion channels (7 subtypes of P2X receptors) and 7TM receptors (8 subtypes of P2Y receptors). The distribution of P2Y receptors is broad, and the therapeutic interests include antithrombotic therapy, modulation of the immune system and cardiovascular system, and treatment of cystic fibrosis and other pulmonary diseases.[1] Subclasses of P2Y receptors (Figure 2) have been defined based on clustering of sequences (in general, low similarity among subtypes), ligand preference, second messenger coupling, and receptor sequence analysis.[7] The $P2Y_1$, $P2Y_2$, $P2Y_4$, $P2Y_6$, and $P2Y_{11}$ receptors couple preferentially to the stimulation of phospholipase Cβ, and the $P2Y_{12}$, $P2Y_{13}$, and $P2Y_{14}$ receptors couple preferentially to the inhibition of adenylate cyclase. $P2Y_1$ and $P2Y_{12}$ receptors occur on the surface of platelets and both act in concert to promote aggregation in response to ADP, and consequently, their antagonists are of interest as antiplatelet agents.[8]

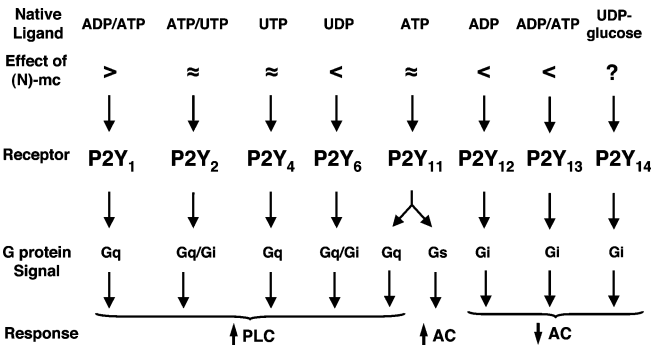


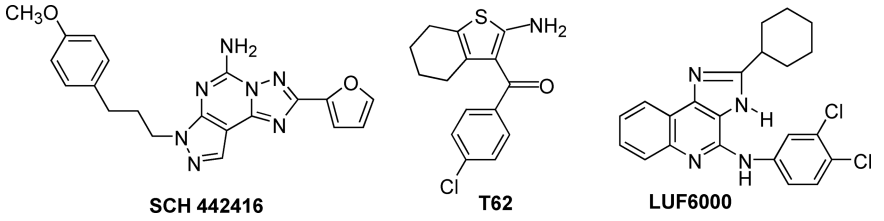
FIGURE 2 The 8 known subtypes of 7TM receptors for extracellular nucleotides and their principal intracellular signaling systems. Also shown is the general effect on potency at each subtype of substitution of the ribose moiety of the native ligand with the (N)-methanocarba ring system. PLC = phospholipase C. AC = adenylate cyclase.

USE OF HOMOLOGY MODELING TO STUDY 7TM RECEPTOR STRUCTURE

Because crystallographic structural determination of nearly all 7TM receptors is not yet feasible, we achieved the structure-function analysis of adenosine[9] and P2Y receptors[7] by indirect means, using mutagenesis and homology modeling, based on a template of the high-resolution structure of rhodopsin. Furthermore, we used the structural insights gained in this modeling to assist in the design of novel ligands. 3D homology models of the human A_{2A} (1UPE), A_3 (1OEA), $P2Y_1$ (1Y36), $P2Y_2$ (1Z8E), $P2Y_6$ (2B6R), and $P2Y_{12}$ (1Y9C) receptors are available from RCSB Protein Data Bank (Rutgers, The State University of New Jersey, USA).

We used molecular modeling based on a rhodopsin template in conjunction with mutagenesis to identify recognition elements important for nucleotide binding at the $P2Y_1$ and other P2Y receptors. In order to ascertain which residues of the $P2Y_1$ receptor were involved in ligand recognition and activation, individual residues of the TMs (3, 5, 6, and 7) and ELs 2 and 3 were mutated to Ala and various charged residues. A cluster of positively charged lysine and arginine residues near the exofacial side of TMs 3, 6 and 7 putatively coordinate the phosphate moieties of nucleotide agonists and antagonists.[7,10] The 2 subgroups of P2Y receptors (either $P2Y_1$–like or $P2Y_{12}$–like) contain different sets of cationic residues that function in the coordination of the 5′-di- or triphosphate groups. At the $P2Y_1$ receptor, it has been possible to convert nucleotide agonists (5′-diphosphate derivatives) into antagonists by separating the diphosphate moiety into bisphophates attached at the 3′ and 5′ positions, or alternately at the 2′ and 5′ positions. Based on structure activity relationship (SAR) studies, there is a small hydrophobic pocket present at the N^6 binding region of the $P2Y_1$ receptor.

Ligand development at the ARs, initially focusing on xanthine derivatives as antagonists, now has been greatly expanded to include many classes of structurally diverse heterocyclic molecules.[11] One class of such antagonists that shows selectivity for the A_{2A}AR is the triazolopyrimidines,[3] such as the highly potent SCH442416.[12] Agonist ligands for the ARs are almost exclusively nucleoside derivatives. Positive allosteric modulators of the A_1AR (such as T62) and the A_3AR (such as LUF6000) also are known.[13,14]



SCHEME 1

The ligand recognition within the putative binding site of the ARs also has been probed through extensive SAR studies, mutagenesis and molecular modeling.[2] The hydrophobic environment surrounding the purine ring of AR agonists as bound in the putative A_{2A}AR model is defined mainly by residues of TM5 and TM6.[9] This region is very similar to the putative binding region of hydrophobic triazolopyrimidine antagonists. An exocyclic amino group is common to both adenosine agonists and typical heterocyclic antagonists such as triazolopyrimidines, and in the putative binding site the amines of both types of ligands are H-bonded with a residue that is highly conserved among ARs, corresponding to $N253^{6.55}$ of the A_{2A}AR.

The subtype selectivity of adenosine derivatives as agonists has been probed extensively, principally through modification of the N^6-amine moiety (where large hydrophobic groups tend to produce A_1AR and A_3AR-selectivity) and the C2-position (where large hydrophobic groups tend to produce A_{2A}AR-selectivity). The ribose moiety is less amenable to the addition of steric bulk, although substitution of the 5′-alcohol with amides, ethers, or other hydrophilic groups has been explored. The putative binding region of the ribose moiety of agonists is lined with predominantly hydrophilic residues in TM3 and TM7. Important interactions revealed by modeling the binding of the highly potent but nonselective agonist NECA (5′-*N*-ethylcarboxamidoadenosine) to the A_{2A}AR include additional H-bonds between the 3′-OH and $H278^{7.43}$ and between the 5′-amide group and residues $T88^{3.36}$ and $S277^{7.42}$. In A_3AR binding of NECA the *anti*-conformation of the glycosidic bond was favored,[9] while the A_{2A}AR complex showed a preference for the high *anti*-conformation.

It should be noted that the only current crystallographic structures of rhodopsin to serve as a template for 7TM receptor structure are of the

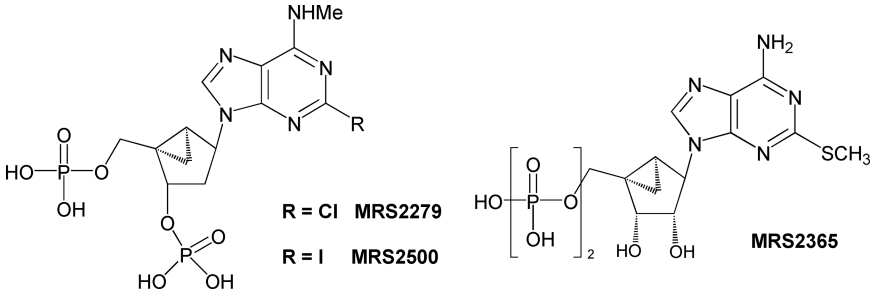
inactive (resting) state, which is more applicable to modeling antagonist docking. However, the model has proven useful for studying agonist ligands as well, and has helped to elucidate which local conformational changes may be involved in activation of the receptor.[15] For example, for docking of NECA and other agonists to the A_3AR model, binding in the region of the 5′-substituent induced a movement of the side-chain of W243$^{6.48}$. This conserved aromatic residue has been described as a "rotamer switch" for activation of a variety of 7TM receptors,[16] including the β-adrenergic receptor, A_3AR, and $P2Y_1$ receptors. Its mutation to Ala in all of the latter (purinergic) subtypes has been shown to preclude activation of the receptor by agonist but not binding of the same ligands.

USE OF RING-CONSTRAINED NUCLEOSIDES/NUCLEOTIDES TO DEFINE CONFORMATIONAL PREFERENCES AT ARs AND P2Y RECEPTORS

Medicinal chemists frequently utilize the approach of conformationally constraining otherwise flexible molecules to probe the "active" conformations and to increase ligand affinity by overcoming energy barriers needed to attain this preferred conformation. In collaboration with V. Marquez of the National Cancer Institute, we have synthesized nucleoside and nucleotide analogues containing novel rigid ring systems in place of ribose as ligands for the adenosine and P2 nucleotide receptors.[7,8,17] The focus on conformational factors of the ribose or ribose-like moiety allows introduction of general modifications that lead to enhanced potency and selectivity at certain subtypes of these receptors. One ring system selected for this purpose is the methanocarba (bicyclo[3.1.0]hexane) ring system, which has been incorporated in either of 2 isomeric forms that adopt either a North (N) or South (S) envelope conformation.[18] We combined these ribose modifications with known enhancing modifications at other positions on the molecule to explore the structure-activity relationships.

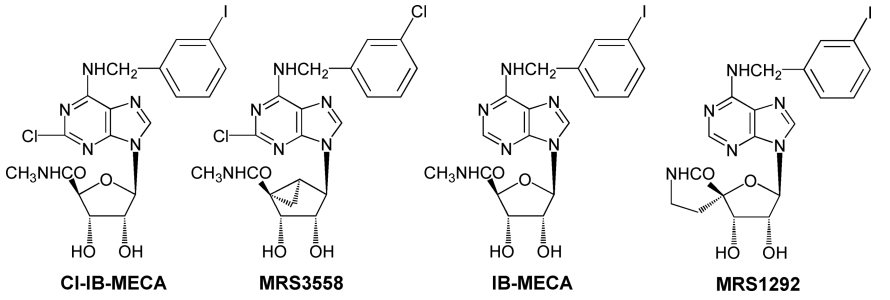
Nucleotide Receptors: Nucleotide derivatives containing the (N)-methanocarba ring system show that this pseudoribose conformation is highly preferred over the (S) conformation for both agonists and antagonists at the $P2Y_1$ receptor. For agonist derivatives, enhancement of potency by hundreds-of-fold has been reported.[19] Using this observation we have designed and synthesized selective modulators of $P2Y_1$ receptors, among which is the first general-use radioligand for $P2Y_1$ receptor, [^{3}H]MRS2279 ((1′R,2′S,4′S,5′S)-4-(2-chloro-6-methylamino-purin-9-yl)-1-[(phosphato)-methyl]-2-(phosphato)-bicyclo[3.1.0]-hexane), and its more potent (K_i = 0.78 nM) 2-iodo analogue MRS2500.[8] This antagonist is completely specific for $P2Y_1$ versus other P2Y subtypes and has been radiolabeled.[20,21] MRS2365, the (N)-methanocarba analogue of the potent, but less selective

2-MeSADP, is a highly selective for the $P2Y_1$ receptor in comparison to $P2Y_{12}$ and $P2Y_{13}$ subtypes.[22]



SCHEME 2

The respective ring-constrained nucleotide analogues were examined at other P2 receptors.[23] At $P2Y_2$ and $P2Y_{11}$ receptors, there is also a preference for the (N)-conformation of ATP over the corresponding (S), although there was no observed potency enhancement over the ribose-containing nucleotides. At $P2Y_4$ receptor the (N)-methanocarba analogue of UTP (the native agonist) was equipotent to the riboside. However, the conformational requirements of the ribose moiety in binding to the $P2Y_6$ receptor were very different from those of the abovementioned receptor subtypes; that is, the (N)-methanocarba analogue of uridine 5′-diphosphate (native agonist) was inactive.[23] Recently, the preference for a ribose South (S) conformation at the $P2Y_6$ receptor was predicted by modeling and confirmed experimentally.[24]



SCHEME 3

Nucleoside Receptors: We have systematically probed the effects of base and ribose substitution of adenosine on both the affinity and intrinsic efficacy at the A_3AR. IB-MECA and its more selective 2-chloro analogue, Cl-IB-MECA, are potent A_3AR agonists used widely as pharmacological tools. IB-MECA has entered clinical trials for the treatment of cancer and rheumatoid arthritis.[25,26]

Nucleoside binding to and activation of the A_3AR are separate processes and appear to have distinct structural requirements. There is no correlation between the affinity of a given nucleoside derivative in binding to the A_3AR with its ability to fully versus partially activate the receptor (Table 1). N^6-benzyl and 2-chloro substituents on the adenine moiety reduce the intrinsic efficacy. The intrinsic efficacy of N^6-(2-phenylethyl) derivatives is extremely sensitive to substitution of the phenyl ring and the β-methylene carbon. SAR studies also indicate that flexibility in the ribose 5′-region is a prerequisite for A_3AR activation, perhaps in concert with rotation of TM6. Thus, with proper manipulation of groups at the N^6 and/or ribose moieties, a high affinity agonist may be converted into a selective A_3AR antagonist.

Based on these observations we have designed new nucleoside antagonists of the A_3AR, such as the rigid spirolactam MRS1292.[27] MRS1292 binds potently and selectively to the rat and human A_3ARs, but does not activate the receptors. Modeling/mutagenesis of adenosine receptors has focused on distinct residues related to ligand binding and the intrinsic efficacy in adenosine derivatives and on a conserved Trp residue (6.48), which is involved in the activation process (see "rotamer switch," above). The docking of MRS1292 to the A_3AR model is not accompanied by rotation of this residue, as occurs with nucleoside agonists, consistent with its action as antagonist. Moreover, the affinity and selectivity of MRS1292 occurs across species, unlike most other heterocyclic antagonists of the A_3AR reported. This allows its use in nonprimate experimental animals used as clinical models. For example, MRS1292 applied directly to the eye in mice has been shown to be effective in reducing intraocular pressure, which is predictive of its utility as an antiglaucoma agent.[6]

We also have designed and synthesized novel, ring-constrained agonists of the A_3AR based on the (N)-methanocarba ring system. Curiously, the conformational requirements of the ribose moiety in binding to the A_3AR are very similar to those of the $P2Y_1$ receptor; that is the (N) envelope conformation is highly preferred. This led to the introduction of MRS3558 ((1′R,2′R,3′S,4′R,5′S)-4-{2-chloro-6-[(3-chloro- phenylmethyl)amino]purin-9-yl}-1-(methylaminocarbonyl)bicyclo-[3.1.0]-hexane-2,3-diol) as a full agonist with subnanomolar potency at the A_3AR and its congeners as full agonists with nanomolar potency at the A_3AR.[17] We have recently explored in detail the SAR of MRS3558 and related congeners as A_3AR agonists[17] and its utility in treating lung injury.[28] However, in order to achieve full efficacy at the A_3AR, a 5′-uronamide moiety is needed. The corresponding 5′-alcohol is an antagonist of the A_3AR. We have speculated that the freely rotating 5′-uronamide is able to make and break H-bonds, which constitutes a necessary degree of flexibility during the receptor activation step.

TABLE 1 Binding affinities of mono-substituted adenosine derivatives (either N^6-substituted, **1–9**, or 2-ether-substituted, **10–16**) at the human A_3AR expressed in CHO cells and maximal agonist effect (inhibition of forskolin-stimulated adenylate cyclase) at 10 μM using a reference value for Cl-IB-MECA of 100%).[32,33]

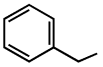
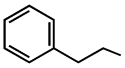
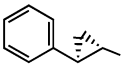
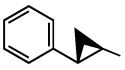
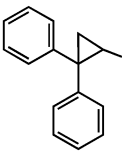
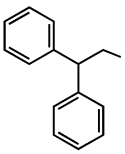
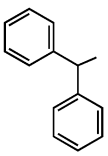
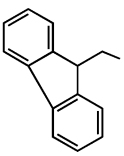
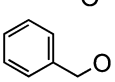
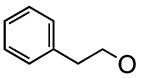
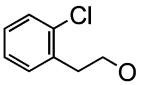
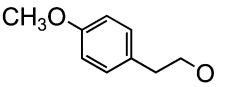
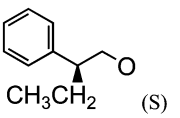
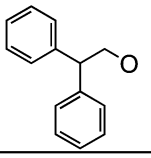
Compound	*Substitution*	*pK_i at A_3AR^a*	*% Activation*
N^6-Modified analogues			
1		7.83	102
2		7.38	55
3		8.68	84
4	 (S,R)	9.20	100
5	 (R,S)	7.62	87
6		7.04	100
7		8.41	0
8		5.48	87
9		9.04	99
2-Ether analogues			
10		6.44	32
11		6.93	17

TABLE 1 Binding affinities of mono-substituted adenosine derivatives (either N^6-substituted, **1–9**, or 2-ether-substituted, **10–16**) at the human A_3AR expressed in CHO cells and maximal agonist effect (inhibition of forskolin-stimulated adenylate cyclase) at 10 μM using a reference value for Cl-IB-MECA of 100%). [32,33] (*Continued*)

Compound	*Substitution*	*pK_i at A_3AR[a]*	*% Activation*
12		7.27	71
13		6.84	1
14		6.98	91
15		6.76	0
16		7.27	0

[a]A_3AR binding experiments were performed with membranes prepared from adherent CHO cells stably transfected with cDNA encoding the human A_3 receptor, using as radioligand [^{125}I]N^6-(4-amino-3-iodobenzyl)adenosine-5′-*N*-methyluronamide ([^{125}I]I-AB-MECA; 2000 Ci/mmol) at a final concentration 0.5 nM, in Tris·HCl buffer (50 mM, pH 8.0) containing 10 mM $MgCl_2$, 1 mM EDTA. Nonspecific binding was determined using 10 μM Cl-IB-MECA. The mixtures were incubated at 25°C for 60 minutes.

NEOCEPTORS: ENGINEERING OF RECEPTORS FOR GENE THERAPY

Although selective agonists of several of the ARs have been known for years, their use as pharmaceutical agents has been impeded by undesirable side effects of exogenously administered adenosine derivatives. In spite of clinically useful protective properties of adenosine agonists observed in experimental animals, such as protection against ischemic damage and suppression of excessive inflammation, none of the selective synthetic agonists has yet been approved for human use. The only adenosine agonist currently in clinical use is adenosine itself, for the treatment of supraventricular tachycardia and as an aid in cardiac imaging.

Since ARs are widespread in the body, in order to overcome inherent nonselectivity of activating the native receptors using synthetic agonists, we have introduced the concept of neoceptors by which the putative ligand binding site of a 7TM receptor is reengineered for activation by

synthetic agonists (neoligands) built to have a structural complementarity. This is a molecular-modeling approach to receptor engineering by which a mutant receptor (neoceptor) is designed for selective activation by a novel synthetic ligand (neoligand) at concentrations that do not activate the native receptor. An amino acid residue of the receptor and a functional group of the ligand moiety thought to be in close proximity can be modified in a complementary fashion so that the two groups exhibit a novel mode of interaction, for example reversing the polarity in a salt bridge or introducing unique hydrogen bonding sites. If a stabilizing interaction exists between these 2 groups, an increase in affinity is expected at the mutant receptor relative to the wild type. This strategy is intended for eventual use in gene therapy and also may be useful in mechanistic elucidation, using neoceptor-neoligand pairs that are pharmacologically orthogonal with respect to the native species. Neoceptors have so far been applied successfully to A_{2A} and A_3ARs.[9,29–31]

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