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Structurally related nucleotides as selective agonists and antagonists at $P2Y_1$ receptors

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Abstract

The $P2Y_1$ receptor responds to adenine nucleotides and is present in platelets, heart, smooth muscles prostate, ovary, and brain. A selective antagonist may be useful as an antithrombotic agent. We have analyzed the binding site of this G protein-coupled receptor using ligand design, site-directed mutagenesis, and homology modeling based on rhodopsin. We have designed and synthesized a series of deoxyadenosine 3′,5′-bisphosphate derivatives that act as antagonists, or, in some cases with small structural changes, as agonists or partial agonists. The 2-position accommodates Cl or thioethers, whereas the N^6-position is limited to Me or Et. 2′-Substitution with OH or OMe increases agonist efficacy over 2′-H. Using molecular modeling of the binding site, the oxygen atoms of the ribose moiety were predicted to be non-essential, i.e. no specific H-bonds with the receptor protein appear in the model. We have, therefore, substituted this moiety with carbocylics, smaller and larger rings, conformationally constrained rings, and acyclics, with retention of affinity for the receptor. With simplified pharmacophores we are exploring the steric and electronic requirements of the receptor binding site, and the structural basis of receptor activation.

Keywords: G protein-coupled receptors; Nucleotides; $P2Y_1$; Molecular modeling

1. Introduction

Modulation of adenosine receptors (P1) and nucleotide (P2) receptors by selective agonists and antagonists [1,2] has the potential for the treatment of a wide range of diseases, including those of the cardiovascular, inflammatory, and central nervous systems. Extracellular nucleotides, principally ATP, ADP, UTP, and UDP, act through two families of membrane-bound P2 receptors: P2Y subtypes, all of which are G protein-coupled receptors (GPCRs), generally coupled to phospholipase C (PLC); and P2X subtypes, ligand-gated ion channels. Though the endogenous ligand for P2X receptors is generally ATP, P2Y receptors are activated by adenine and/or uracil nucleotides [2]. Agonists of P2 receptors are almost exclusively nucleosides and nucleotides, respectively, whereas antagonists of these receptors are structurally more diverse [1]. In comparison with the adenosine receptors, much less is known about the specific effects of P2 receptors, largely due to the lack of selective ligands. Within the family of P2Y receptors, we have focused on the $P2Y_1$ receptor, which was the first member to be cloned. The $P2Y_1$ receptor is one of three P2 receptors that occurs on the surface of platelets, and its activation has been proposed to play a role in platelet aggregation leading to thrombus formation. Thus, a selective $P2Y_1$ antagonist may prove useful as an antithrombotic agent.

We are currently designing and synthesizing novel ligands for both adenosine and P2 receptors. Methods utilized in these recent investigations include: conformationally constraining the ribose, or ribose-like, moiety of nucleosides and nucleotides to freeze a conformation that may provide favorable affinity and/or selectivity at P1 and P2 receptors [3,4]; modifying known receptor antagonists [5–7]; use of a template approach based on the pyridine family for the design of

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novel adenosine antagonists [8]; and the screening of chemical libraries in conjunction with molecular modeling [9].

2. Results and discussion

2.1. Receptor modeling as a tool in ligand development

GPCRs represent a large family of many hundreds of gene products that share a common structural motif, i.e. they contain seven membrane-spanning helical domains (TMs) [10,11]. A large fraction of currently used pharmaceutical agents consist of synthetic agents that modulate the action of GPCRs, either as agonists or antagonists. Thus, it is of great interest to explore computationally the aspects of ligand binding common to these protein targets as an aid in the design of ligands. We have used mutagenesis and molecular modeling in conjunction with chemical modification of ligands to define putative binding sites. For small molecules in general, and for the P1 and P2 receptors specifically, these sites occur within a central cavity surrounded by the TM domains. Conformational considerations of both receptors and their ligands are important in structure-based drug design.

We have used alanine scanning mutagenesis of the human $P2Y_1$ receptor to show the importance of specific residues in molecular recognition. Molecular modeling of both P1 and P2Y receptors (using a rhodopsin template) and their docked ligands, based on mutagenesis results, have been carried out to interpret the findings and to suggest new ligands [12,13]. With simplified pharmacophores we are currently exploring the steric and electronic constraints of the receptor binding site, and the structural basis of receptor activation.

Molecular modeling of cloned $P2Y_1$ receptor sequences has focused on both the seven TMs and the three extracellular loops (ELs). The features of the putative binding site identified within the TM region were consistent with both mutagenesis results and known ligand specificities. To obtain an energetically refined 3D structure of the complex, we introduced a new approach, a '*cross docking*' procedure [13], which simulated the reorganization of the native receptor induced by the ligand. In order to ascertain which residues of the human $P2Y_1$ receptor are involved in ligand recognition, we mutated individual residues of both the TMs (3, 5, 6, and 7) and ELs 2 and 3. A cluster of positively charged amino acids, Lys and Arg residues near the exofacial side of TMs 3 and 7 and to a lesser extent TM6, putatively coordinates the phosphate moieties of nucleotide agonists and antagonists [14]. Two essential disulfide bridges in the extracellular domains were identified, and several charged residues in ELs 2 (E209) and 3 (R287) were shown to be critical for receptor activation. This suggested that the role of the ELs in ligand recognition was as important as that of the TMs [15]. Moreover, using both mutagenesis and molecular modeling [15], we have defined energetically favorable 'meta-binding sites' in the $P2Y_1$ receptor, involving the critical residues of the ELs. At these nucleotide docking sites located distal to the principal TM site, a ligand may bind on its way to the principal TM binding site. These secondary binding sites may serve to guide the ligand in its approach to the TM binding site and reduce the energy barrier to complex formation. Further mutagenesis studies will be required to confirm this proposal. This phenomenon may prove to be general with the GPCR family, and may serve as the basis for modulation of the activity of endogenous ligands, perhaps using synthetic ligands that compete for binding preferentially at the 'meta-binding sites'.

2.2. Ribose-modified nucleotide analogues

We have carried out extensive chemical probing of structure–activity relationships (SARs) for nucleotides acting at the $P2Y_1$ receptor. Initially, we modified the native $P2Y_1$ receptor ligands, and tested the analogues in a functional assay (activation of PLC) at the turkey erythrocyte $P2Y_1$ receptor, which is very similar in structure to the human homologue. Using molecular modeling of the receptor binding site we predicted that there are no essential H-bonds predicted to form directly between the receptor protein and oxygen atoms of the ribose moiety of nucleotide ligands, and we have therefore removed both ether and 2′, and 3′ oxygen atoms of the ribose moiety. Based on the report by Boyer et al. [16], indicating that adenosine bisphosphates (at either 3′,5′ or 2′,5′ positions) were antagonists or partial agonists at the $P2Y_1$ receptor, a series of deoxyadenosine 3′,5′-bisphosphate derivatives acting as selective $P2Y_1$ antagonists was prepared (Fig. 1). Antagonism was defined as the ability to reverse completely the stimulation of PLC elicted by 30 nM 2-methylthioadenosine-5′-diphosphate, an agonist of nanomolar potency. In some cases the related nucleotides could be interconverted between agonists (or partial agonists) and antagonists with small structural changes [17–19]. SAR analysis indicated that the 2-position accommodated Cl or thioethers, while the N^6-position was limited to Me or Et. A 2′-substitution (OH or ether) increased agonist efficacy over 2′-H. The analogue MRS 2179 (2′-deoxy-N^6-methyladenosine-3′,5′-bisphosphate), a more potent and competitive antagonist, contained an intact 2′-deoxyribose ring with adenine modifications. The N^6-methyl and 2-chloro modifications lead to lower agonist efficacy and higher affinity, respectively. MRS 2179 also antagonized rat $P2X_1$ receptors in an electrophysiological model with an IC_{50} of 1.1 μM [20].

Other modifications included substitution of the ribose moiety with carbocylics, smaller and larger rings, conformationally constrained rings, and acyclics, with retention of affinity for the receptor. Fig. 2 summarizes various types of ribose modification of nucleotide-based $P2Y_1$ receptor antagonists. Ring expansion of the ribose in the 2-Cl-N^6-Me series led to an anhydrohexitol derivative, MRS 2283, which displayed an IC_{50} of 0.566 μM, at the turkey erythrocyte $P2Y_1$ receptor [19]. Ring contraction further emphasized the flexibility of substitution of the ribose moiety in these antagonists; the cyclobutyl derivative, MRS 2264, displayed an IC_{50} of 0.805 μM at the turkey $P2Y_1$ receptor [19].

An acyclic modification of the ribose ring also preserved affinity at the $P2Y_1$ receptor. By analogy, acyclic modification of nucleosides has proven useful for antiviral agents. The antiviral compound pencyclovir corresponds formalistically to the structural ring opening of a carbocyclic guanosine derivative. Using a similar approach, we designed the acyclic adenosine bisphosphate, MRS 2286 (2-[2-(2-chloro-6-methylamino-purin-9-yl)-ethyl]-propane-1,3-bisoxy(diammonium-phosphate)). This analogue corresponds to the carbocyclic antagonist analogue MRS 2267 (Fig. 2), in which the 2′-methylene has been omitted and a 2-Cl has been added (to increase affinity). MRS 2286 was an antagonist at the turkey $P2Y_1$ receptor with an IC_{50} value of 0.84 μM, and no agonist activity was observed [21]. Furthermore, the compound was inactive at $P2X_1$ receptors [20].

We recently discovered that one of the most generally useful modifications applicable to both P1 and P2 receptors is to lock the ribose sterically into a preferred conformation. Generally, the ribose rings of nucleosides and nucleotides may adopt a range of conformations as described by a 'pseudorotational cycle'. The Northern ((N), 2′-*exo*, 3′-*endo*) and Southern ((S), 2′-*endo*, 3′-*exo*) conformations are the most relevant to the biological activities observed for nucleosides and nucleotides in association with DNA, RNA, and various enzymes. Do the adenosine and P2 receptors prefer either one of these specific conformations of the ring?

In order to approach this question experimentally, we designed a series of P2 receptor ligands containing conformationally rigid ribose-like rings, based on carbocyclic rings. Conformational constraints were built into nucleoside and nucleotide ligands using the methanocarba approach, i.e. fused cyclopropyl and cyclopentyl rings in place of the ribose moiety. Rigid rings in the methanocarba series have defined a preference for the Northern (N) conformation of ribose at the $P2Y_1$ receptor. MRS 2268, the (N)-methanocarba analogue of 2′-deoxyadenosine-3′,5′-bisphosphate, was a potent $P2Y_1$ agonist ($EC_{50} = 155$ nM), 86-fold more potent than the corresponding Southern (S) isomer, MRS 2266. However, the corresponding 2-Cl-N^6-methyl-(N)-methanocarba analogue, MRS 2279 ((1*R*,2*S*,4*S*,5*S*)-1-[(phosphato)methyl]-4-(2-chloro-6-methylaminopurin-9-yl) bicyclo[3.1.0]-hexane-2-phosphate, Fig. 2), was a potent antagonist ($IC_{50} = 52$ nM). As with other ribose modifications, the presence of an N^6-Me group in (N)-methanocarba bisphosphate analogues transformed either a partial or full agonist into a pure antagonist, and the 2-chloro modification enhanced affinity.

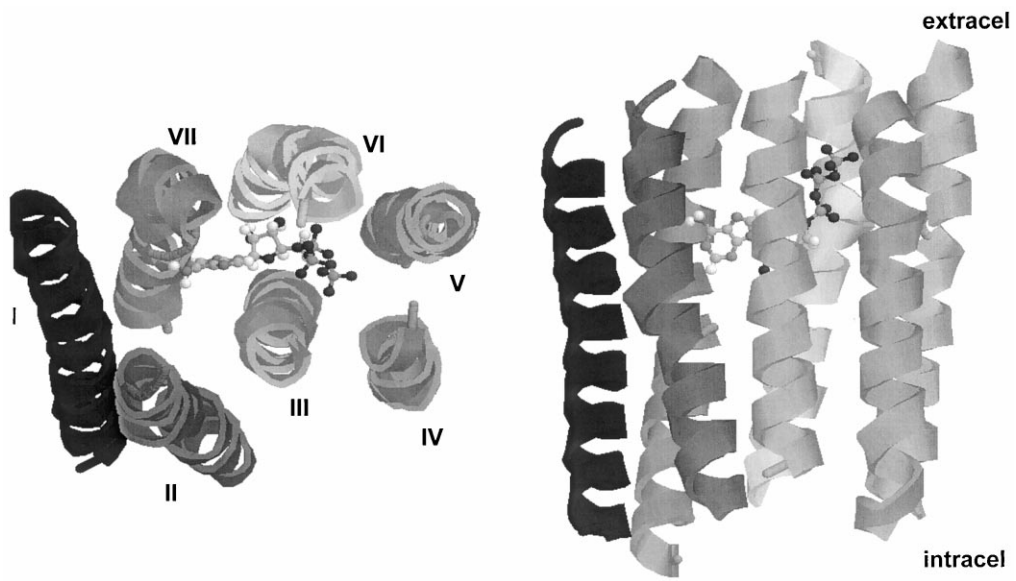

Fig. 1. Stereoview of $hP2Y_1$ transmembrane helical bundle model viewed along the helical axes from the extracellular end (left) and perpendicular to the helical axes (right) after the '*cross docking*' procedure for the $P2Y_1$–ATP complex. The adenine moiety is the portion of the ligand situated closest to TM7.

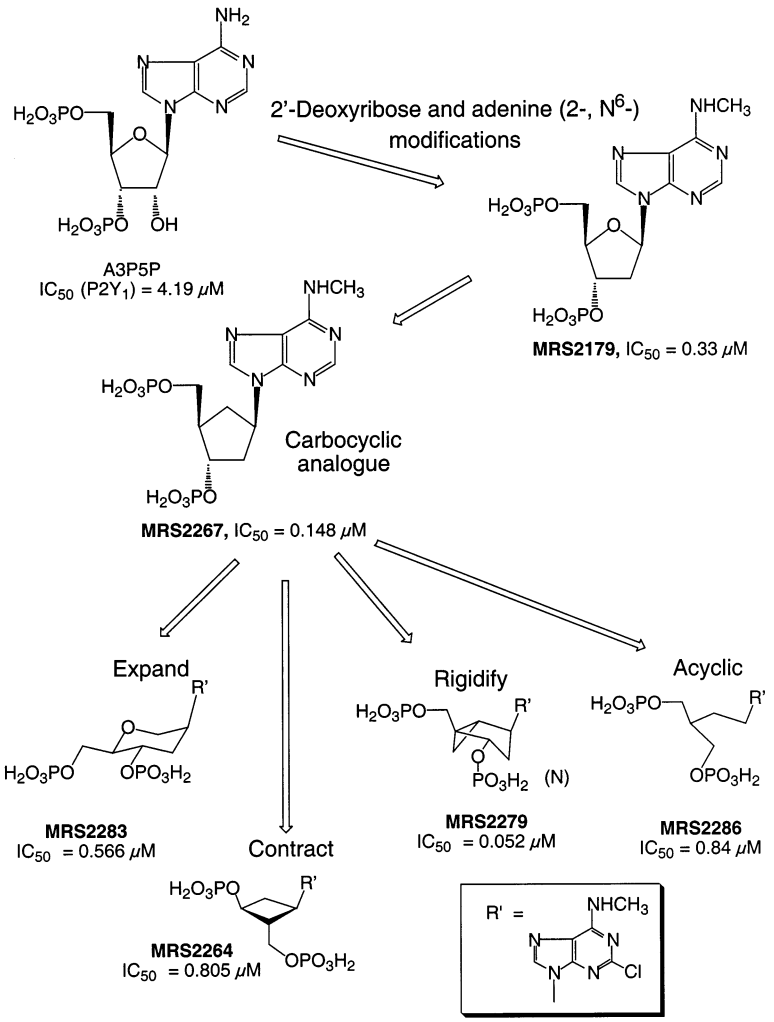

Fig. 2. Modifications of nucleotide-based ligands in the development of $P2Y_1$ receptor antagonists. IC_{50} values are for the inhibition of $P2Y_1$-receptor stimulated PLC-activity in turkey erythrocyte membranes. PLC was stimulated by 30 nM 2-methylthioadenosine-5′-diphosphate, a potent $P2Y_1$ receptor agonist.

In conclusion, we have demonstrated that the ribose moiety of $P2Y_1$ agonists and antagonists is amenable to extensive modification. We have designed and tested both conformationally rigid and structurally simplified antagonists, as well as agonist/antagonist pairs of compounds that differ in only subtle structural features. We are exploring the steric and electronic requirements of the receptor binding site, and the structural basis of receptor activation.

References

[1] B.B. Fredholm, M.P. Abbracchio, G. Burnstock, G.R. Dubyak, T.K. Harden, K.A. Jacobson, U. Schwabe, M. Williams, Towards a revised nomenclature for P1 and P2 receptors, Trends Pharmacol. Sci. 18 (1997) 79–82.

[2] K.A. Jacobson, L.J.S. Knutsen, P1 and P2 purine and pyrimidine receptors, in: M.P. Abbracchio, M. Williams (Eds.), Handbook of Experimental Pharmacology, Purinergic and Pyrimidinergic Signalling I, vol. 151/I, Springer, Berlin, 2001, pp. 129–175.

[3] E. Nandanan, S.Y. Jang, S. Moro, H. Kim, M.A. Siddiqi, P. Russ, V.E. Marquez, R. Busson, P. Herdewijn, T.K. Harden, J.L. Boyer, K.A. Jacobson, Synthesis, biological activity, and molecular modeling of ribose-modified deoxyadenosine bisphosphate analogues as P2Y1 receptor ligands, J. Med. Chem. 43 (2000) 829–842.

[4] K.A. Jacobson, X.-d. Ji, A.H. Li, N. Melman, M.A. Siddiqi, K.J. Shin, V.E. Marquez, R.G. Ravi, Methanocarba analogues of purine nucleosides as potent and selective adenosine receptor agonists, J. Med. Chem. 43 (2000) 2196–2203.

[5] Y.-C. Kim, X.-d. Ji, N. Melman, J. Linden, K.A. Jacobson, Anilide derivatives of an 8-phenylxanthine carboxylic congener are highly potent and selective antagonists at human A_{2B} adenosine receptors, J. Med. Chem. 43 (2000) 1165–1172.

[6] E. Camaioni, J.L. Boyer, A. Mohanram, T.K. Harden, K.A. Jacobson, Deoxyadenosine bisphosphate derivatives as potent antagonists at $P2Y_1$ receptors, J. Med. Chem. 41 (1998) 183–190.

[7] E. Nandanan, E. Camaioni, S.Y. Jang, Y.-C. Kim, G. Cristalli, P. Herdewijn, J.A. Secrist, K.N. Tiwari, A. Mohanram, T.K. Harden, J.L. Boyer, K.A Jacobson, Structure activity relation-

ships of bisphosphate nucleotide derivatives as $P2Y_1$ receptor antagonists and partial agonists, J. Med. Chem. 42 (1999) 1625–1638.

[8] A.-H. Li, S. Moro, N. Melman, X.-d. Ji, K.A. Jacobson, Structure–activity relationships and molecular modeling of 3,5-diacyl-2,4-dialkylpyridine derivatives as selective A3 adenosine receptor antagonists, J. Med. Chem. 41 (1998) 3186–3201.

[9] T.R. Webb, N. Melman, D. Lvovskiy, X.-d. Ji, K.A. Jacobson, The utilization of a unified pharmacophore query in the discovery of new antagonists of the adenosine receptor family, Bioorg. Med. Chem. Lett. 10 (2000) 31–34.

[10] A.M. van Rhee, K.A. Jacobson, Modelling the P2Y purinoceptor using rhodopsin as template, Drug Dev. Res. 37 (1996) 1–38.

[11] R. Osman, A. Colson, J.H. Perlman, L.J. Laakonen, M.C. Gershengorn, in: J. Wess (Ed.), Structure–Function Analysis of G Protein-Coupled Receptors, Wiley, New York, 1999, pp. 59–84.

[12] S. Moro, A.-H. Li, K.A. Jacobson, Molecular modeling studies of human A_3 adenosine receptor antagonists: structural homology and receptor docking, J. Chem. Inf. Comput. Sci. 38 (1998) 1239–1248.

[13] S. Moro, D. Guo, E. Camaioni, J.L. Boyer, T.K. Harden, K.A. Jacobson, Human P2Y1 receptor: molecular modeling and site-directed mutagenesis as tools to identify agonist and antagonist recognition sites, J. Med. Chem. 41 (1998) 1456–1466.

[14] C. Hoffmann, S. Moro, R.A. Nicholas, T.K. Harden, K.A. Jacobson, The role of amino acids in extracellular loops of the human P2Y1 receptor in surface expression and activation processes, J. Biol. Chem. 274 (1999) 14 639–14 647.

[15] S. Moro, C. Hoffmann, K.A. Jacobson, Role of the extracellular loops of G protein-coupled receptors in ligand recognition: a molecular modeling study of the human $P2Y_1$ receptor, Biochemistry 38 (1999) 3498–3507.

[16] J.L. Boyer, T. Romero-Avila, J.B. Schachter, T.K. Harden, Identification of competitive antagonists of the $P2Y_1$ receptor, Mol. Pharmacol. 50 (1996) 1323–1329.

[17] E. Camaioni, J.L. Boyer, A. Mohanram, T.K. Harden, K.A. Jacobson, Deoxyadenosine bisphosphate derivatives as potent antagonists at $P2Y_1$ receptors, J. Med. Chem. 41 (1998) 183–190.

[18] E. Nandanan, E. Camaioni, S.Y. Jang, Y.-C. Kim, G. Cristalli, P. Herdewijn, J.A. Secrist, K.N. Tiwari, A. Mohanram, T.K. Harden, J.L. Boyer, K.A. Jacobson, Structure activity relationships of bisphosphate nucleotide derivatives as $P2Y_1$ receptor antagonists and partial agonists, J. Med. Chem. 42 (1999) 1625–1638.

[19] E. Nandanan, S.Y. Jang, S. Moro, H. Kim, M.A. Siddiqi, P. Russ, V. Marquez, R. Busson, P. Herdewijn, T.K. Harden, J.L. Boyer, K.A. Jacobson, Synthesis, biological activity, and molecular modeling of ribose-modified adenosine bisphosphate analogues as $P2Y_1$ receptor ligands, J. Med. Chem. 43 (2000) 829–842.

[20] S.G. Brown, B.F. King, Y.-C. Kim, G. Burnstock, K.A. Jacobson, Activity of novel adenine nucleotide derivatives as agonists and antagonists at recombinant rat P2X receptors, Drug Dev. Res. 49 (2000) 253–259.

[21] Y.-C. Kim, C. Gallo-Rodriguez, S.Y. Jang, E. Nandanan, M. Adams, T.K. Harden, J.L. Boyer, K.A. Jacobson, Acyclic analogues of deoxyadenosine bisphosphates as $P2Y_1$ receptor antagonists, J. Med. Chem. 43 (2000) 746–755.