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Molecular Recognition at Adenine Nucleotide (P2) Receptors in Platelets

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Abstract

Transmembrane signaling through P2Y receptors for extracellular nucleotides controls a diverse array of cellular processes, including thrombosis. Selective agonists and antagonists of the two P2Y receptors present on the platelet surface—the G_q-coupled P2Y₁ subtype and the G_i-coupled P2Y₁₂ subtype—are now known. High-affinity antagonists of each have been developed from nucleotide structures. The (N)-methano-carba bisphosphate derivatives MRS2279 and MRS2500 are potent and selective P2Y₁ receptor antagonists. The carbocyclic nucleoside AZD6140 is an uncharged, orally active P2Y₁₂ receptor antagonist of nM affinity. Another nucleotide receptor on the platelet surface, the P2X₁ receptor, the activation of which may also be proaggregatory, especially under conditions of high shear stress, has high-affinity ligands, although high selectivity has not yet been achieved. Although α,β -methylene-adenosine triphosphate (ATP) is the classic agonist for the P2X₁ receptor, where it causes rapid desensitization, the agonist BzATP is among the most potent in activating this subtype. The aromatic sulfonates NF279 and NF449 are potent antagonists of the P2X₁ receptor. The structures of the two platelet P2Y receptors have been modeled, based on a rhodopsin template, to explain the basis for nucleotide recognition within the putative transmembrane binding sites. The P2Y₁ receptor model, especially, has been exploited in the design and optimization of antagonists targeted to interact selectively with that subtype.

Keywords

G protein-coupled receptors; P2Y receptors; agonist; antagonist; mutant

Adenine nucleotides influence platelet aggregation by interacting with three different receptors: P2Y₁ and P2Y₁₂, two metabotropic receptors coupled to G_{aq} and G_{ai}, respectively, and P2X₁, an ionotropic receptor that mediates rapid calcium influx.^{1,2} Simultaneous stimulation of the two P2Y receptors in platelets induces aggregation; stimulation of each alone is insufficient for full aggregation.³ P2Y₁ receptor activation is particularly associated with the initial shape change of platelets in response to ADP.^{3,4} P2X₁

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receptor activation may also be proaggregatory, especially under conditions of high shear stress.⁵

Medicinal chemical studies of P2 receptors are largely concerned with the development of selective small molecules as agonists or antagonists of a given receptor. Rational approaches to the design of such ligands take into account the structure of the target biopolymers, in other words, G protein-coupled receptors (GPCRs) (P2Y receptors) or ligand-gated ion channels (P2X receptors). The structural differences among the three platelet nucleotide receptors can be exploited to design compounds targeted to interact with each single receptor regulating the distinct functions that they play in the process of aggregation. This article will summarize the structures of known selective and partially selective ligands for the three nucleotide receptors found on the surface of platelets and will provide insights into the microscopic interactions within the binding sites of these receptors. The structural aspects of the receptors are especially focused on the P2Y₁ receptors,⁶⁻⁸ which have been characterized by detailed mutagenesis⁹ and molecular modeling based on the structural template of bovine rhodopsin.

STRUCTURE OF P2Y AND P2X RECEPTORS

Human P2Y receptors are a family of nucleotide-activated GPCRs that comprise at least eight different subtypes with different selectivity, for example, for adenine versus uracil nucleotides and for 5'-diphosphates versus 5'-triphosphates. Both P2Y₁¹⁰ and P2Y₁₂^{11,12} are selectively activated by the nucleotide adenosine diphosphate (ADP, **1**).

By means of a phylogenetic analysis we delineated two clearly distinct subgroups of P2Y receptors: the G_q-coupled subtypes (i.e., P2Y₁, P2Y₂, P2Y₄, P2Y₆, and P2Y₁₁) and those coupled to G_i (i.e., P2Y₁₂, P2Y₁₃, and P2Y₁₄).⁶ Therefore, functional effects described later that are mediated by the P2Y₁ and P2Y₁₂ receptors refer to activation of phospholipase C (PLC) and inhibition of adenylate cyclase, respectively.

P2Y receptors have been successfully modeled^{6,8,13} by homology modeling based on the high resolution structure of bovine rhodopsin¹⁴ as a template. Our most recent models are derived from a multiple sequence alignment based on a combined manual and automatic approach that takes into account not only the primary structure of the proteins, but also the three-dimensional information deducible from the secondary and tertiary structure of the template.

As for all other GPCRs, P2Y₁ and P2Y₁₂ receptors consist of single polypeptide chains that cross the cell membrane seven times, forming seven helical transmembrane domains (TMs) connected by three extracellular (ELs) and three intracellular loops (ILs). The amino terminal region (NT) is located outside the cell, and the carboxyl terminal region (CT) is located in the cytoplasm. According to our models,⁶ at the cytoplasmic end of TM7 both of the receptors fold at an angle of approximately 90 degrees to form a helical segment (homologous to H8 in rhodopsin)¹⁴ that runs parallel to the plane of the cell membrane.

The role of the ELs in ligand recognition and receptor activation has been studied.⁷ Two essential disulfide bridges in the extracellular domains of the human P2Y₁ receptor were

also identified by site-directed mutagenesis⁷: one connecting EL2 with the upper part of TM3 (conserved among most GPCRs and confirmed by the crystal structure of bovine rhodopsin)¹⁴ and another between the N-terminal domain and EL3. On the basis of a bioinformatic study, we proposed the existence of the same disulfide bridges for the other subtypes of P2Y receptors, including P2Y₁₂.⁶

The putative binding site of the human P2Y₁ receptor has been extensively studied by means of site-directed mutagenesis. To ascertain which residues of this receptor were involved in ligand recognition, individual residues of the TMs and ELs were mutated to Ala.^{7,9} A cluster of positively charged amino acid side chains in TMs 3, 6, and 7 were proposed to form the counter ions to the negatively charged diphosphate or triphosphate moiety. Site-directed mutagenesis has validated this prediction and further indicated several uncharged hydrophilic residues that may coordinate the nucleobase. Thus, agonists, such as 2-methylthio-adenosine-5'-diphosphate (2-MeSADP **4**) were inactive at R128(3.29)A and R310(7.39)A and at S314(7.43)A mutant P2Y₁ receptors and had a markedly reduced potency at K280(6.55)A and Q307(7.36)A mutant P2Y₁ receptors.

After the construction of the rhodopsin-based homology models, we performed mutagenesis-driven automatic docking experiments at P2Y₁ and P2Y₁₂ receptors. The results suggested that ADP binds to the P2Y₁ and P2Y₁₂ receptors on the exofacial side of the cavity delimited by TM1, TM2, TM3, TM6, and TM7 and capped with EL2 (Fig. 1). We proposed the existence of two different sets of three basic amino acids involved in the phosphate coordination for the two subgroups. In the case of the P2Y₁ subgroup, in good agreement with the available mutagenesis data, these residues were R3.29, K/R6.55, and R7.39. Among them, only R6.55 was common to both subgroups.⁶ In the P2Y₁₂ subgroup, in fact, the role of R3.29 in TM3 seemed to be fulfilled by a Lys residue in EL2, whereas the R7.39 in TM7 seemed to be substituted by K7.35 located within the same TM but at a distance of four residues, in other words, one helical turn in the exofacial direction.

Ligand docking at P2Y₁ and P2Y₁₂ receptors provided a hypothesis for the coordination of nucleotide-like antagonists in the TM regions, consistent with site-directed mutagenesis results, with a binding mode very similar to that of the agonist.^{7,13} Molecular recognition in the P2Y₁ receptor of non-nucleotide antagonists, such as derivatives of pyridoxal-phosphate-6-azophenyl-2',4'-disulphonic acid (PPADS) **20**, has also been studied.¹⁵

P2X receptors are composed of seven subtypes of subunits (P2X₁₋₇), and each functional ion channel consists of oligomers, probably trimers.¹⁶ Both homo- and hetero-oligomerization seem to occur commonly. For example, the P2X₁ receptor is known to form heteromers with P2X₂ and P2X₅ receptors.¹⁷ Each subunit consists of a single polypeptide chain 399 amino acids in length containing two TMs connected by a large EL, which is likely endowed with five disulfide bridges and four glycosylation sites. The N-terminal and the C-terminal domains are cytoplasmic. The structures of P2X receptors are not yet amenable to homology modeling because of the lack of a protein template. Of the two TMs of the P2X₁ receptor, the second TM (proximal to the C-terminus) is predicted to be of a more regular α -helical structure.¹⁸ Extensive mutagenesis has been performed to identify residues involved in ligand recognition in P2X₁¹⁸ and other P2X receptors.^{19,20}

P2Y₁ AND P2Y₁₂ RECEPTOR LIGANDS

Agonists

The P2Y₁ and P2Y₁₂ receptors are activated by adenine nucleotides, but not by uracil nucleotides. The P2Y₁ receptor is activated more potently by ADP **1** than by ATP **2**.¹⁰ The P2Y₁ and P2Y₁₂ receptors are not activated by either adenosine monophosphate (AMP) or adenosine. ADP is a full agonist, whereas ATP appears to be a partial agonist in some models of P2Y₁ receptors. At P2Y₁₂ receptors, ADP derivatives activate and ATP derivatives antagonize. Thus, ATP inhibits ADP-induced human platelet aggregation with a K_i value of about 20 mM.²¹ Terminal thiophosphate groups appear in many useful nucleotide agonists of P2Y receptors present in platelets. For example, ADP-β-S **3** is a potent agonist of both the P2Y₁ receptor (EC₅₀ [median effective concentration] of 96 nM) and the P2Y₁₂ subtype (EC₅₀ 82 nM).² ATP-γ-S is a slightly more potent agonist of the P2Y₁ receptor than ATP.²

The 2 position of the adenine nucleobase of adenosine-5'-phosphate derivatives has also proved to be amenable to derivatization for P2Y agonists, whereas substitution at the 8 position greatly reduces potency.²¹ Most notably, the 2-alkylthio ethers in ADP and ATP series²² appear to provide high potency at the P2Y₁ receptor. The corresponding O-ethers are less potent. The 2-methylthio derivative of ADP **4** is a potent agonist (EC₅₀ in nM) at P2Y₁ (**6**), P2Y₁₂ (**1**), and P2Y₁₃ (**1**) receptors^{2,23} and an important pharmacological probe for studying these subtypes. The 2-methylthio derivative of ADP **4** was recently found to be three orders of magnitude more potent than ADP was at the purified human P2Y₁₂ receptor.²⁴ 2-Azido-ADP and 2-chloro-ADP are more potent than ADP is in inducing aggregation of human platelets. Only limited substitution is allowed at the N⁷ position of adenine nucleotides acting as inducers of platelet aggregation.

Several adenosine 5'-monophosphate analogues, such as 2-hexylthioadenosine-5'-monophosphate (HT-AMP) **5** (EC₅₀ 59 nM at the turkey P2Y₁ receptor) have been found to potently activate P2Y₁ receptors.²⁵ The α-thio modification of AMP analogues (e.g., **6**) increases potency at the P2Y₁ receptor,²⁶ and agonist activation of PLC is maintained. Such 2-modified AMP derivatives have also been reported to inhibit ectonucleotidases, which complicates their use as P2Y receptor agonists. Adenosine-5'-(1-boranotriphosphate) derivatives such as the 2-methylthio derivative **7** (EC₅₀ 2.6 nM at the rat P2Y₁ receptor) have been found to potently activate the P2Y₁ receptor,²⁷ indicating that -BH₂ may substitute for an ionic oxygen of the α-phosphate when bound to the P2Y₁ receptor binding site.

Riboside-based nucleotides are subject to deglycosylation reactions. Nonglycosidic substitution of the ribose moiety leading to greater chemical stability has recently been the focus of structure activity relationship (SAR) studies of nucleotide ligands for the P2Y₁ receptor. For example, a dehydroanhydrohexitol (six-membered ring) analogue **8** was found to activate P2Y₁ receptors with an EC₅₀ value of 3.0 μM. N⁶-methylation of this derivative leads to P2Y₁ receptor antagonism.

Carbocyclic and constrained carbocyclic rings have also been substituted in place of the ribose moiety. Some of the more useful examples of this approach are the methanocarba ring system^{28,29} used in place of the ribose moiety. This ring system features fused carbon rings (3 and 5 membered), which are constrained in either a northern (N) or southern (S) ring conformation depending on position of fusion of the two rings. Correlation of ring geometry with the biological activities has helped define that the binding site of the P2Y₁ receptor prefers the (N) conformation of the ribose-like ring over the corresponding (S) conformation. For example, the (S)-isomer of ATP, MRS 2312 **10**, with an EC₅₀ value of 7.2 μM, is pronouncedly less potent than the corresponding (N)-isomer MRS 2340 **9**, which displays an EC₅₀ value of 52 nM at the human P2Y₁ receptor.²⁹ The enhancement of P2Y₁ agonist potency on freezing the preferred conformation in a pseudoribose ring may approach 300 fold. An example of such an enhancement is (N)-methanocarba-β,γ – methylene-ATP, which is a full agonist with an EC₅₀ value at the human P2Y₁ receptor of 158 nM, whereas the corresponding riboside is a weak partial agonist. (N)-methanocarba-2-MeS-ADP, MRS 2365 **11**, with an EC₅₀ value of 0.4 nM, is the most potent known agonist of the human P2Y₁ receptor and is highly selective for that subtype versus P2Y₁₂ receptors and other subtypes.^{30,31} Thus, compound **11** may be used for selective P2Y₁ receptor activation in platelets.

The adenosine 5'-monophosphate analogue MRS2055, *N*⁶-methyl-2-[(5-hexenyl)thio]-AMP, appears to activate the C6 glioma cell P2Y₁₂ receptor considerably more potently than does the P2Y₁ receptor (EC₅₀ 3.2 μM at the rat P2Y₁₂ receptor versus > 100 μM at the turkey P2Y₁ receptor).^{22,25}

Antagonists

The introduction of potent nucleotide-derived antagonists of the P2Y₁ receptor was made possible by the observation that naturally occurring adenosine bisphosphate derivatives such

as A3P5P **12** are either partial agonists or antagonists of the receptor in various species.³² A later generation 2'-deoxy-*N*⁶-methyl derivative, MRS 2179 **13** and the corresponding 2-chloro analogue MRS 2216 **14** were found to be high-affinity antagonists of the P2Y₁ receptor.²⁸ Furthermore, MRS 2216 was shown to be inactive at the rat P2X₁ receptor.³³ In the riboside bisphosphate series of P2Y₁ receptor antagonists, the 2-methyl and 2-ethyl substituents have been found to be particularly advantageous.³⁴ At the *N*⁷ position of the riboside bisphosphates, the pattern of P2Y₁ receptor affinity as a function of substitution is methyl > ethyl ≫ propyl, and larger alkyl groups or acylation (e.g., with benzoyl) lead to inactivity. Thus, there appears to be a small hydrophobic pocket at this position of the nucleotide binding site of the receptor. The (N)-methanocarbo equivalent of MRS 2216, that is, MRS 2279 **15**, was demonstrated to be a high-affinity competitive and selective antagonist at this subtype.^{35–37} This antagonist competitively inhibited ADP-promoted aggregation of human platelets with a K_B value of 8.9 nM.³⁵ The high affinity of this analogue having a rigid (N)-methanocarba ring system, which has a 2'-endo envelope conformation, indicated the conformational preference in the antagonist binding site of the receptor. [³³P]MRS 2179 and [³H]MRS 2279 have been introduced as the first generally useful high-affinity radioligands for the P2Y₁ receptor in platelets and in other tissue.^{36,37} The selectivity of these bisphosphate derivatives for the P2Y₁ receptor makes them attractive probes for pharmacological studies, with inactivity demonstrated at P2Y_{2,4,6,11,12,13} and P2X_{2,3,4,7} receptors.^{33,35} Weak antagonism was observed by compound **13**, but not compound **14**, of ion flux at the rat P2X₁ receptor expressed in *Xenopus* oocytes.³³ However, this activity may not be relevant to other pharmacological models, because many of the known P2 receptor antagonists are unusually potent in this assay. MRS2279 in micromolar concentrations was also found to antagonize the rat P2X₁ receptor in the same electrophysiological model. Thus, for most applications, compound **13** and its congeners are essentially specific for the P2Y₁ receptor. Recently, a highly potent P2Y₁ receptor antagonist in the (N)-methanocarba series, MRS 2500 **16**, was reported (K_i = 0.78 nM at hP2Y₁).³⁸ This antagonist inhibited aggregation of human platelets with a median inhibitory concentration (IC₅₀) value of 0.95 nM.³⁹ Raboisson et al⁴⁰ have synthesized a C-nucleotide bisphosphate **17** that antagonized P2Y₁ receptors.

In addition to the approach of rigidifying the ribose moiety in a conformation that approximates the conformation preferred in receptor binding, the opposite approach—using a flexible ribose equivalent—has met with some success. Acyclic nucleotide analogues of bisphosphate antagonists, such as MRS 2298 **18** (IC₅₀ 0.48 μM at the turkey P2Y₁ receptor), were found to be moderately potent P2Y₁ receptor antagonists without residual agonism. The number of methylene groups adjacent to the adenine moiety in MRS 2298 (*i.e.*, one) has been optimized. Chains longer than two methylene units in this series greatly reduced potency at the P2Y₁ receptor. In addition, the introduction of steric constraints in the form of cyclopropyl rings and olefinic groups in the alkyl moiety failed to enhance antagonistic properties. An analogue related to MRS 2298, except that it contains the metabolically stable phosphonate groups in place of the phosphates, was MRS 2496 **19**. By antagonizing the P2Y₁ receptor, compound **19** displayed an IC₅₀ in the inhibition of aggregation of rat platelets of 0.68 μM.⁴² Curiously, nucleotides having a cyclic ribose equivalent may act as

either P2Y₁ agonist or antagonist, depending on substituent groups, whereas the acyclic structures have been found to act only as P2Y₁ antagonists.

Moderately potent, nonnucleotide antagonists of the P2Y₁ receptor are numerous although selectivity is generally less than with the nucleotide antagonists. The azo-linked pyridoxal phosphate derivative PPADS **20** is a mixed P2X/P2Y antagonist.⁴³ Another nonselective P2 antagonist of mixed selectivity is the polysulfonate suramin,² which inhibits P2X₁ (IC₅₀ 1 μM), P2X₂ (10 mM), P2X₃ (3 μM), P2X₅ (4 μM), P2Y₂ (48 μM), and P2Y₁₂ (4 μM) receptors. Certain analogues of PPADS **20**, such as the nonsulfonated derivative MRS 2210 **21**, display selectivity for P2Y₁ versus P2X sub-types.¹⁵ Compound **21** is a competitive antagonist of the human P2Y₁ receptor, with a K_B value derived by Schild analysis of 2.4 μM. A hybrid molecule SB9 **22**, consisting of PPADS- and suramin-like components, was found to potently antagonize P2Y₁ receptors.⁴⁴ Another large polysulfonated aromatic molecule, reactive blue 2, inhibited the human P2Y₁ receptor with a K_B value of 1.6 mM.¹⁵ Recently, a cyclic depsipeptide YM-254890 **23**, a fermentation product of a *Chromobacterium* isolated from soil, was found to potently antagonize P2Y₁ receptors.⁴⁵ Compound **23** inhibited the aggregation of platelets with an IC₅₀ of 31 nM, and it was inactive at P2Y₁₂ receptors. Derivatives of acid blue 129, such as compound **24**, were also found to act as P2Y₁ receptor antagonists.^{46,47}

A variety of structurally diverse, non-nucleotide antagonists of the P2Y₁ receptor have been shown to be noncompetitive inhibitors. For example, pyridyl isatogen tosylate (PIT) **46**, which has been explored as a possible allosteric modulator of the receptor,⁴⁸ was found to be a P2Y₁-selective antagonist in recombinant P2Y receptors systems.⁴⁹ It reduced the maximal effect of 2-MeSADP in stimulation of PLC with an IC₅₀ of 0.14 mM but had no effect on the binding of [³H]MRS2279. MRS 2576 **47**, a diisothiocyanate derivative, was found to be a potent insurmountable (and possibly irreversible by virtue of the reactive isothiocyanate groups) antagonist of human P2Y₁, as well as of other P2Y receptors.⁵⁰ Covalent affinity labels of any receptor P2X or P2Y that bind with high affinity have yet to be developed.

Nucleotide antagonists of very high affinity for the platelet P2Y₁₂ receptor are under development, including AR-C69931MX **25**,⁵¹ which was already evaluated in clinical trials as an antithrombotic agent. A nucleotide in this series (AR-C67085MX **26**) has been shown to potently activate the P2Y₁₁ receptor.⁵² It has been possible to substitute the unwieldy triphosphate group in this series with uncharged moieties such as short alcohols, esters, and so on, thus proving that a highly anionic moiety is not needed for recognition by the P2Y₁₂ receptor. This discovery led to compounds such as AZD6140 **27**, which is an orally active P2Y₁₂ receptor antagonist of nM affinity that inhibits platelet aggregation up to 8 hours after administration.⁵³ The presence of the 3,4-difluorophenyl group limits the metabolism of **27**. According to our modeling studies,⁶ the 3,4-difluorophenyl ring of the N⁶-substituent of **27** formed an aromatic interaction with F77(2.48). A residue notation in the form X.XX useful for drawing analogy to the same position in other receptors was used.⁶ This residue was unique to P2Y₁₂ and could be one of the reasons for the high P2Y₁₂ selectivity of this antagonist.

"P" = monophosphate

Among non-nucleotide antagonists, reactive blue 2 is highly potent at the P2Y₁₂ receptor with an IC₅₀ of 25 nM.² PPADS **20**, however, is inactive at the P2Y₁₂ receptor.

The action of the successful antithrombotic drug clopidogrel **28** (and its clinical predecessor ticlopidine, which is also a thienopyridine derivative) depends on the P2Y₁₂ receptor present on platelets.⁵⁴ During hepatic metabolism, compound **28** is transformed into a free thiol **29**, which acts as an irreversible P2Y₁₂ receptor antagonist. Thus, clopidogrel itself is not active in vitro and must be administered either intravenously or orally. The related compound,

CS-747 **30**, also acts as a P2Y₁₂ antagonist through a metabolite.⁵⁵ The sulfonamide analog CT50547 **31** has also been reported to antagonize the P2Y₁₂ receptor.⁵⁶ Other non-nucleotide antagonists of the P2Y₁₂ receptor,⁵⁷ especially those acting directly on the receptor, are currently the subject of high throughput screening efforts.

The acyclic scaffold (attached at the 9-position of adenine) used in the P2Y₁ receptor antagonist MRS 2298 **18** has been adapted to relatively weak antagonists of the P2Y₁₂ receptor.⁴² Upon replacement of the two phosphate groups with hydrophobic esters, such as in the dipivaloate MRS 2395 **32**, the selectivity shifted entirely from the P2Y₁ receptor to the P2Y₁₂ receptor. MRS 2395 displayed an IC₅₀ of 3.6 μM in the inhibition of ADP-induced aggregation of rat platelets. Because both receptor subtypes are integral to ADP-induced platelet aggregation, the inhibition of either P2Y₁ or P2Y₁₂ receptors impedes platelet aggregation. Analogues of ADP having neutral, hydrophobic substitutions at the ribose 2'- and 3'-hydroxyl groups and the adenine NH₂ position were found to antagonize the P2Y₁₂ receptor. One such analogue is INS 49266 **33**, which has the unusual feature of a urea group at the N⁶-position and an acetal group at the ribose hydroxyls; it displayed a K_B of 361 nM in the inhibition of platelet aggregation.⁵⁸ The agonist potencies of **33** at P2Y₁ and P2Y₂ receptors were > 10 and 14 μM, respectively.

P2X₁ RECEPTOR LIGANDS

Agonists

Among the seven subtypes of P2X receptors, ATP is the most potent at the P2X₁ receptor, with an EC₅₀ value of 56 nM.² One means of enhancing the stability of the triphosphate moiety of ATP and ADP derivatives is to substitute the bridge oxygen atoms with methylene and halomethylene units.^{21,59,60} In this capacity, the -CF₂ bridge is a close electronic mimic of the oxygen bridge, based on the electron withdrawing properties of fluorine, which lower the pK_a values of the resultant difluoro-methylene phosphonates.⁶⁰ The methylene-bridged nucleotide α,β-meATP **34** distinguishes group I receptors, in other words, P2X₁ and P2X₃ receptors, which are activated by **34** (EC₅₀ value 1 to 3 μM), from all other P2X subtypes, which are insensitive to this agonist.⁶¹ Another distinguishing feature is the ability of some P2X receptor subtypes (mainly group I) to desensitize rapidly in response to agonists such as **34**, which has been used in pharmacological studies of the P2X₁ receptor in place of true antagonists. At the P2Y₁ and P2Y₁₂ receptors, α,β-meATP is inactive.

The nucleoside 5'-diphosphate and 5'-triphosphate derivatives generally diverge in activity at P2X subtypes.⁶¹ Although much weaker than ATP, ADP **2** elicits agonist action at the P2X₁ receptor (EC₅₀ 10 μM) but not at other P2X subtypes.² The P2X₁ receptor is not activated by either AMP or by adenosine. Both α,β-methylene and β,γ-methylene, modifications of ATP, as well as the ω-thiophosphate group present in ATP-γ-S **35** generally increase stability of the triphosphate group toward enzymatic hydrolysis while maintaining potency at P2X₁ receptors.⁶¹ The L-enantiomer of β,γ-methylene-ATP **36** activates rat P2X₁ receptors with an EC₅₀ value of 2 μM.⁶¹ The effect of ribose substitution in ATP analogues on potency at P2X receptors has not been fully explored. The derivative 2'-&3'-O-(4-benzoyl-benzoyl)-ATP (BzATP) **37**, which is a mixture of ester species, is of nanomolar potency in activating the P2X₁ receptor.⁶² At higher concentrations compound

37 also activates the P2X₇ receptor, where it is one of the most potent agonists known. Adenine dinucleotides activate the P2X₁ receptor.⁶³ For example, the EC₅₀ of Ap₄A is 182 nM. Selective agonism by dinucleotides of group I P2X receptors (including P2X₁) depends on a single adenine moiety.⁶³ Curiously, Ap₄A and its more hydrolytically stable methylene-bridged and halomethylene-bridged analogues inhibit platelet aggregation.⁶⁰

2-Alkylthio ethers of ATP, such as 2-[2-(4-nitro-phenylethylthio)]-ATP (PAPET),²² retain agonist properties at the P2X₁ receptor.³³ HT-AMP **5** is a weak partial agonist (EC₅₀ 0.84 μM, about 50% efficacious) at the rat P2X₁ receptor.³³

Antagonists

Many of the “classic” antagonists of P2X receptors are highly charged polycyclic compounds. The trypanocidal drug suramin **37** and its derivatives were found to be P2X antagonists in the micromolar range. Suramin antagonism of P2 responses is readily reversible upon washout.⁶³ This antagonist action was unrelated to the clinical use of suramin. The potency order of compound **37** at P2X receptors is P2X₁, P2X₅ (IC₅₀ 1 to 4 μM) > P2X₂, P2X₃ (10 to 15 μM) > P2X₇ (78 μM) > P2X₄, P2X₆ (>500 μM). Lambrecht and coworkers⁶⁴ have studied related, highly potent antagonists, selective for the P2X₁ receptor, such as NF279 **38** and NF449 **39**, which have roughly nanomolar IC₅₀ values at the rat P2X₁ receptor.⁶⁵ The structure-activity relationship (SAR) in this polysulfonate series at the P2X₁ receptor has been explored.⁶⁶

The SAR of antagonists derived from pyridoxal phosphate, which were also introduced by Lambrecht and coworkers,⁶⁴ have been explored at P2X receptors. The 2',4'-disulfonate derivative PPADS **20** and its 2',5'-disulfonate isomer "isoPPADS" **40** are somewhat more potent at P2X than they are at P2Y receptors. The pyridoxal phosphate derivative PPNDS **41** is a highly potent antagonist at the P2X₁ receptor.⁶⁴ The phosphate linkage of PPADS analogues may be replaced with more stable phosphonates.⁶⁷ For example, the phosphonate analogue MRS 2257 **42** is a highly potent antagonist at rat P2X₁ (IC₅₀ 5.1 nM) receptors (with the receptors expressed in the *Xenopus* oocyte). Analogues in the PPADS series in

which the diazo linkage has been replaced with carbon bridges have been synthesized.⁶⁸ One such analogue, MRS 2335 **43**, was roughly equipotent to the corresponding diazo derivative at the P2X₁ receptor. Although high potency at P2X receptors has been achieved for PPADS derivatives, a disadvantage is the noncompetitive binding they display, accompanied by slow on and off rates.⁶⁸

Various nucleotide derivatives have also been found to antagonize the P2X₁ receptor. Such nucleotide antagonists are advantageous because they display more favorable binding kinetic properties than do the polyanionic, non-nucleotide antagonists discussed previously. Faster on and off rates of binding faster than those of PPADS **20** have been demonstrated for the antagonist TNP-ATP **44**, which binds potently to P2X₁ and P2X₃ receptors and to heteromeric P2X_{2/3} and P2X_{1/5} receptors.⁶⁹ A dinucleotide derivative, Ip5I **45**, potently antagonizes the P2X₁ receptor.⁷⁰

CONCLUSION

The three subtypes of P2 receptors present on platelets are among the most highly developed from the perspective of medicinal chemistry among the P2X and P2Y families. Each has potent and somewhat selective antagonists and a variety of potent agonists. Highly selective antagonists of P2Y₁ and P2Y₁₂ receptors of nanometer affinity have been developed, largely as derivatives of agonist structures. The P2X₁ receptor is rapidly desensitized by the action of potent nucleotide agonists, and directly acting competitive antagonists, such as polysulfonates, are also known. The development of even more potent and selective ligands for modulating platelet function is underway. Indirectly obtained structural knowledge (from sequence analysis, modeling, mutagenesis, and ligand docking) of the P2Y₁ receptor has aided in the development of such ligands. A putative ligand binding site in the TM region and its specific recognition elements, such as positively charged Lys and Arg residues that serve as counterions to the negatively-charged phosphate group, have been identified.

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Objectives: On completion of this article, the reader should be able to (1) understand the structure of the P2Y and P2X receptors on platelets, and (2) recognize the potential for the development of potent agonists and antagonists to these receptors.

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Figure 1.

Modeled structures of the putative binding sites of P2Y1 and P2Y12 receptors.⁶ The putative nucleotide binding site as viewed from the plane of the plasma membrane with a docked ADP molecule, based on mutagenesis and molecular modeling experiments is shown. Key residues found to interact with the ligand in the human P2Y1 and P2Y12 receptors are indicated. To the left of each detailed structure is a smaller three-dimensional representation of the receptor including seven TMs (color coded) and the connecting loops. Color of TMs: cyan (TM1), orange (TM2), green (TM3), magenta (TM4), blue (TM5), red (TM6), gray

(TM7). The orientation of the entire receptor relative to the membrane is the same as for each detailed binding site model.

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