MINIREVIEW

Nucleotides Acting at P2Y Receptors: Connecting Structure and Function

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ABSTRACT

Eight G protein–coupled P2Y receptor (P2YR) subtypes are important physiologic mediators. The human P2YRs are fully activated by ATP (P2Y2 and P2Y11), ADP (P2Y1, P2Y12, and P2Y13), UTP (P2Y2 and P2Y4), UDP (P2Y6 and P2Y13), and UDP glucose (P2Y13). Their structural elucidation is progressing rapidly. The X-ray structures of three ligand complexes of the Gi-coupled P2Y12R and two of the Gq-coupled P2Y1Rs were recently determined and will be especially useful in structure-based ligand design at two P2YR subfamilies. These high-resolution structures, which display unusual binding site features, complement mutagenesis studies for probing ligand recognition and activation. The structural requirements for nucleotide agonist recognition at P2YRs are relatively permissive with respect to the length of the phosphate moiety, but less so with respect to base recognition. Nucleotide-like antagonists and partial agonists are also known for P2Y1, P2Y2, P2Y4, and P2Y12Rs. Each P2YR subtype has the ability to be activated by structurally bifunctional agonists, such as dinucleotides, typically, dinucleoside triphosphates or tetraposphates, and nucleoside polynucleotide sugars (e.g., UDP glucose) as well as the more conventional mononucleotide agonists. A range of dinucleoside polynucleotides, from triphosphates to higher homologs, occurs naturally. Earlier modeling predictions of the P2YRs were not very accurate, but recent findings have provided much detailed structural insight into this receptor family to aid in the rational design of new drugs.

Introduction

The discovery and cloning of the P2Y family of G protein–coupled receptors (GPCRs), which respond to a range of extracellular nucleotides, has spawned a vast array of biologic studies (Webb et al., 1993; Abbracchio et al., 2006). These eight receptors can be divided into two subfamilies based on sequence homology and second messengers: five Gq-coupled P2Y1-like (P2Y1, P2Y2, P2Y4, P2Y6, and P2Y13) and three Gi-coupled P2Y12-like (P2Y12–P2Y14) receptors. The first native agonists of P2Y receptors (P2YRs) with recognized biologic effects were ATP and ADP, and later, UTP, UDP, and UDP-glucose (UDPG) were found to activate various P2YRs (Fig. 1A). Thus, the diversity of purine and pyrimidine nucleotide agonists of this family is broader than for most other GPCR families, which typically respond to a single molecule endogenous agonist. The correspondence of the principal native agonists to human P2YR subtypes is ATP (P2Y2 and P2Y11), ADP (P2Y1, P2Y12, and P2Y13), UTP (P2Y2 and P2Y4), UDP (P2Y6 and P2Y14), and UDPG (P2Y14). At increased concentrations, there are some additional crossovers in the activation patterns, such as UDPG acting as a full agonist at P2Y2R (Ko et al., 2009). ATP may act as an antagonist or partial agonist at several P2YR subtypes, including antagonism at the human (but not rat) P2Y4R (Kennedy et al., 2000). P2YRs are widespread in the body and involved in the regulation of nearly all systems, notably, immune, skeletal muscular, digestive, nervous, endothocrine, cardiovascular, pulmonary, gastrointestinal, and renal systems (Abbracchio et al., 2006).

ABBREVIATIONS: 2MeSADP, 2-methylthioadenosine 5′-diphosphate; Ap4A, P1′-(5′-adenosinyl)-P4′-(5′-adenosinyl)-tetraphosphate; AZD1283, ethyl 6-(4-[(benzyloxysulfonyl) carbamoy]piperidin-1-yl)-5-cyano-2-methylnicotinate; BPTU, 1-[(2-[tert-butyl]phenoxo)pyridin-3-yl]-3-[4-(trifluoromethoxy)phenyl]urea; CD39, ecto-nucleoside triphosphate diphosphohydrolase; EL, extracellular loop; GPCR, G protein–coupled receptor; MRS2500, [1′R,2′S,3′S,4,5′S]-4-(2-iodo-6-methylamino-purin-9-yl)-1-[(phospho)-methyl]-2-(phospho)-bicyclo[3.1.0]hexane; P2YR, P2Y receptor; SAR, structure activity relationship; TM, transmembrane helix; UDPG, UDP glucose; Up4A, P1′-(5′-adenosinyl)-P4′-(5′-uridinyl)-tetraphosphate.
The broad distribution of P2YRs and the multiplicity of effects throughout the body, which are often both protective and damaging, make this system both highly attractive and challenging for drug discovery and development (Jacobson and Boeynaems, 2010).

In addition to the conventional mononucleotide (i.e., nucleoside 5'-polyphosphate) agonists, each of the eight P2YR subtypes has the ability to be activated by structurally bifunctional nucleotides, principally, dinucleotides (Jankowski et al., 2009). They are bifunctional in the respect that the receptor binding site would have to accommodate two nonphosphate end groups, such as nucleoside moieties, linked through a phosphate or polyphosphate moiety (Fig. 1B). Such bifunctional nucleotides typically would include dinucleoside polyphosphates, from triphosphates to higher homologs, occurs naturally. For example, P1-(5'-adenosinyl)-P4-(5'-uridinyl)-tetraphosphate (Up4A) is released from the vascular endothelium to induce vasoconstriction and has been explored in various contexts, such as P2Y2R-induced migration of smooth muscle cells and extracellular signaling in many tissues and cells, ranging from bacterial to human (Schlüter et al., 1994; Monds et al., 2010).

This phenomenon of broader agonist recognition beyond the mononucleotide agonists was not discovered in a systematic manner for each P2YR subtype, but rather stemmed from the observation that dinucleotides are naturally occurring substances having considerable biologic activity (Miras-Portugal et al., 1999). A range of dinucleoside polyphosphates, from triphosphates to higher homologs, occurs naturally. A range of dinucleoside polyphosphates, from triphosphates to higher homologs, occurs naturally. For example, P1-(5'-adenosinyl)-P4-(5'-uridinyl)-tetraphosphate (Up4A) is released from the vascular endothelium to induce vasoconstriction and has been explored in various contexts, such as P2Y2R-induced migration of smooth muscle cells and extracellular signaling in many tissues and cells, ranging from bacterial to human (Schlüter et al., 1994; Monds et al., 2010).

Fig. 1. (A) Action at P2YRs of nucleotides released from cells (e.g., ATP, UTP, and UDPG) and their conversion outside the cell to 5'-diphosphates, which act at different P2YRs, and/or to 5'-monophosphates, which are inactive (inact.). 5'-nucleotidase catalyzes the final conversion of AMP to adenosine, which acts at its own set of four GPCRs (adenosine A1, A2A, A2B, and A3 receptors). There is a redundancy of ligands that activate various P2YR subtypes. The nucleotides may act as full agonists (green arrows) or variably partial agonists and antagonists (orange arrow). EC50 or IC50 values (nM) at human P2YRs from measurement of adenylyl cyclase or phospholipase C activity are indicated in italics. Weaker interactions, such as UDP, as an agonist of P2Y1R (∼10) or P2Y6R (16) are not shown. The enzymatic conversions are catalyzed by ecto-nucleotidases (blue arrows): (a) ecto-nucleoside triphosphate diphosphohydrolases (CD39s) act on either 5'-triphosphates or 5'-diphosphates; (b) ecto-nucleotide pyrophosphatase/phosphodiesterases convert 5'-trimonophosphates to 5'-monophosphates; (c) NPP1 and NPP3 hydrolyze UDPG to produce UMP. (B) Naturally occurring dinucleotides (n = 2–7; B is a nucleobase) are shown schematically and described in detail later in the text. The dinucleotides, such as Up4A and Ap4A, may either act directly on P2YRs, in some cases, or be converted by ecto-nucleotide pyrophosphatase/phosphodiesterases to active mononucleotides, such as ADP. P2YR potencies of simple dinucleotides are reported (Shaver et al., 2005).
detected dual recognition at the P2YRs of mononucleotides and dinucleotides has lacked a structural explanation.

Medicinal Chemistry of P2YRs: Focus on Nucleotides

This review emphasizes the action of nucleotides, most of which in this context are P2YR agonists. The characterization of nucleotides as receptor ligands is challenging due to their pharmacological lability, low bioavailability, nonselectivity in activating specific P2YRs, and difficulties in chemical synthesis. Potency values in medicinal chemical studies often reflect either activation of phospholipase C within the P2Y1-like receptor subfamily or other second messengers, such as cAMP, rather than binding affinity because only three of the P2YRs (P2Y1, P2Y12, and P2Y14 receptors) have radioligands available.

Ecto-nucleotidases and other enzymes are involved in the interconversion of nucleotides that act as P2YR ligands (Fig. 1A) and finally by 5'-nucleotidase for the conversion to adenosine, which acts at its own set of four GPCRs. Recently, the structures of 5'-nucleotidase and ecto-nucleoside triphosphate diphosphohydrolase (CD39) were determined using X-ray crystallography (Heuts et al., 2012; Zimmermann et al., 2012). The structures of some of the other enzymes involved in processing purine receptor ligands, such as ecto-nucleotide pyrophosphatase/phosphodiesterase-1, have also been determined (Jansen et al., 2012). Inhibition or activation of these enzymes is an appealing means of indirectly modulating the activation of the receptors at which the nucleotides and the nucleoside adenosine act. This is an alternative approach to the design of directly acting receptor ligands, either orthosteric or allosteric. The polyphosphate moiety of synthetic nucleotide ligands may contain substitution at limited positions: methylene or halomethylene bridges or P-thio or P-borano substitution (Table 1), all of which can reduce their enzymatic degradation.

The only P2YR subtypes that are currently targeted by pharmaceutical agents are P2Y12 (antithrombotic antagonists) (Ferri et al., 2013) and P2Y2 (agonist treatment of dry eye, Fig. 2. Sequence alignment of the human P2YRs. Residues that have been identified using site-directed mutagenesis, as involved in ligand binding and/or receptor activation at P2Y1R (Abbracchio et al., 2006; Zhang et al., 2015), P2Y2R (Erb et al., 1995; Hillmann et al., 2009), P2Y11R (Herold et al., 2004), P2Y14R (Zylberg et al., 2007), and P2Y12R (Hoffmann et al., 2008; Mao et al., 2010; Ignatovic et al., 2012; Zhang et al., 2014a,b) are highlighted with different colors: residues whose mutation can have a major effect on ligand binding and/or receptor activation (red); residues whose mutation modulates ligand binding and/or receptor activation (orange); and residues whose mutation has a minor or no effect on ligand binding and/or receptor activation (yellow). Residues within 3 Å from the crystallographic pose of 2MeSADP at P2Y12R or within 3 Å from the crystallographic pose of MRS2500 at P2Y2R are circled in green. The most highly conserved residue among GPCRs of each helix is highlighted in gray. Cysteine residues involved in disulfide bridges are highlighted in cyan.
which is approved in Japan (Lau et al., 2014). Two of the three P2Y12R antagonists in use as antithrombosis (thienopyridines) are actually prodrugs of irreversibly receptor-binding thiols and therefore have clinical limitations. The attempt to use P2Y2 agonists in the treatment of cystic fibrosis unfortunately failed in clinical trials (Deterding et al., 2007).

Most of the P2YRs still lack uncharged, drug-like antagonists. However, recent extensive exploration of the structure activity relationship (SAR) at P2Y1R has provided such agents, which are also being evaluated as potential antithrombics (Yang et al., 2014). Also, the SAR of nucleotide antagonists is particularly advanced for the platelet ADP receptors P2Y1 and P2Y12. Several reviews have cataloged the variety of nucleotide and non-nucleotide ligands of P2YRs in detail (Brunschweiger and Müller, 2006; Houston et al., 2008; Jacobson et al., 2012).

The structural requirements for P2YR nucleotide recognition are relatively permissive with respect to the length of the phosphate moiety, but less so with respect to base recognition. Nucleoside polyphosphates beyond 5'-triphosphates, e.g., uridine 5'-tetraphosphate, are also reported to activate various P2YR subtypes (Ko et al., 2008). Bifunctional agonist analogs of Up4U [P1\(^1\)-(5'-uridinyl)-P4\(^4\)-(5'-uridinyl)-tetraphosphate] and uridine 5'-tetraphosphate glucose are tolerated at P2Y4 and P2Y6 receptors. Only P2Y2 readily accepts either A or U as the nucleobase in 5'-triphosphate agonists. ATP binds to human P2Y4R as an antagonist. Alternate nucleobases are sometimes recognized at P2YRs, but at much higher concentrations, for example, IDP as an agonist at P2Y12R (EC\(_{50} = 5.31 \mu M\)) and P2Y13R (also used as a high affinity \(^3\)H- or \(^33\)P-radioligand) (Takasaki et al., 2001). A\(^\text{ antagonist.}\)

### Table 1

<table>
<thead>
<tr>
<th>P2YR</th>
<th>Synthetic Agonist (Mononucleotide), Potency</th>
<th>Bifunctional Ligand, Potency</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>P2Y1</td>
<td>1, MRS2365, 0.4</td>
<td>2, Up4(β-B)A, A isomer, 500</td>
<td>Houston et al., 2008; Yelovitch et al., 2012</td>
</tr>
<tr>
<td>P2Y2</td>
<td>3, MRS2698, 8.0</td>
<td>4, INS37217, 220</td>
<td>Houston et al., 2008; Yerxa et al., 2002</td>
</tr>
<tr>
<td>P2Y4</td>
<td>5, MRS4062, 26</td>
<td>6, INS365 (Up4U(^a)), 130</td>
<td>Ko et al., 2008; Maruoka et al., 2011</td>
</tr>
<tr>
<td>P2Y6</td>
<td>7, 5-OMe-UDP(^b)-B, 8</td>
<td>8, MRS2957, 12</td>
<td>Maruoka et al., 2010; Haas et al., 2014</td>
</tr>
<tr>
<td>P2Y11</td>
<td>9, ATP-γ-S, 24,000</td>
<td>10, NAADP, 64,000</td>
<td>Djerada and Millard, 2013</td>
</tr>
<tr>
<td>P2Y12</td>
<td>11, 2MeSADP(^b), 19</td>
<td>12, compound 17 (R/S)(^c), 13</td>
<td>Zhang et al., 2002; Yanachkov and Wright, 2010</td>
</tr>
<tr>
<td>P2Y13</td>
<td>11, 2MeSADP(^b), 19</td>
<td>13, Ap1A, 72</td>
<td>Zhang et al., 2002</td>
</tr>
<tr>
<td>P2Y14</td>
<td>14, MRS2905, 2.0</td>
<td>15, MRS2690, 70</td>
<td>Das et al., 2010</td>
</tr>
</tbody>
</table>

\(^a\)NAADP, nicotinic acid adenine dinucleotide phosphate.

\(^b\)INS365 also activates P2Y1R (EC\(_{50} = 6.6 \mu M\)).

\(^b\)2MeSADP activates P2Y1, P2Y12, and P2Y13R (also used as a high affinity \(^3\)H- or \(^33\)P-radioligand) (Takasaki et al., 2001).

\(^c\)Antagonist.
the south (S) conformation over the north (N) (Maruoka et al., 2010). Substitution of the uracil 5 position, e.g., with iodo or methoxy, is tolerated at P2Y6, but not P2Y2 and P2Y4 receptors (Haas et al., 2014). Thiocarbonyl substitution of the uracil 2 or 4 position is variably tolerated at the P2Y2, P2Y4, and P2Y14 receptors. 2’- or 3’-deoxynucleotides are not well tolerated as P2YR agonists. Methylenes or halomethylene bridges, such as in antagonist 12 or agonist 14, are tolerated at some of the P2YRs (Das et al., 2010; Yelovitch et al., 2012). Boronation of the α-phosphate of ADP derivatives is conducive to activity at the P2Y1R; a pure stereoisomer of the 2-Cl member of that series displayed an EC50 of 7 nM (Azran et al., 2013). Although P2Y6R prefers UDP over UTP, various 5’-triphosphate analogs have proven to be potent (Maruoka et al., 2010). 4-Alkoxyimino groups on the pyrimidine ring, which preserve a double bond character in a C5O substitution, are tolerated at P2Y2, P2Y4, and P2Y6 receptors, and this has allowed the attachment of long-chain fluorophores through that linkage (Jayasekara et al., 2014).

Furthermore, nucleotide-like antagonists and partial agonists are also known for P2Y1, P2Y2, P2Y4, and P2Y12 receptors. Some of these structures are shown in Fig. 3. A3p5p 16 was identified as a partial agonist of human P2Y1R, a key finding that was later optimized by extensive structural modification (Boyer et al., 1996a). Thus, the separation of the two phosphate moieties of ADP and attachment to ribose as bisphosphates (either 3’,5’ or 3’,2’) reduced its efficacy at P2Y1R. N1 was not required for recognition (e.g., 1-deaza analog 19), and several other modifications, N6-methylation, and removal of the 2’-OH, further reduced the efficacy, leading to antagonists, such as MRS2179 18 (Houston et al., 2008). N6-dimethylation or the addition of N-alkyl groups larger than ethyl greatly reduced affinity at P2Y1R, suggesting the presence of a small hydrophobic pocket in the receptor, with a requirement for NH as an H-bond donor. Replacement of the 3’,5’-bisphosphates with bistriphosphates also greatly reduced affinity. Halogen 20 or small thioethers 21 were tolerated at the C2 position. Substitution of the ribose ring with an (N)-methanocarba ring system, as in 22-24, greatly enhanced potency in the antagonist series by maintaining a P2Y1R-preferred conformation (Kim et al., 2003). Halo (by IC50: I < Cl < F), methyl, methythio, and methylseleno substitution at the C2 position preserved high affinity (Costanzi et al., 2007). The presence of the N6-methyl

Fig. 3. Structures of nucleotide and nucleotide-like antagonists and partial agonists of P2YRs (IC50 values in micromolar at the human P2Y1R are shown in italics).
group in (1'R,2'S,4'S,5'S)-4-(2-iodo-6-methylamino-purin-9-yl)-1-[(phospho)-methyl]-2-(phospho)-bicyclo[3.1.0]hexane (MRS2500) 23 (also used as a high affinity 3H or 125I-radioligand) enhanced the antagonist affinity by 16-fold. The same (N)-bicyclic ring in P2Y1R agonists, such as 1, was also greatly potency enhancing, which suggests a common binding site for nucleotide antagonists and agonists at this receptor, along with other SAR parallels. Curiously, although rigidity of the ribose enhanced pharmacological properties, acyclic ribose substitutes (25, 26) were also tolerated with micromolar affinities as long as two charged phosphate or phosphonate groups were present. Thus, the binding site for the anionic moieties in P2Y1R must have some flexibility. The uracil phosphonate 27 appears to be an allosteric partial agonist, with selectivity for P2Y12R, but additional characterization of this compound is required (Cosyn et al., 2009).

At P2Y12R, 5′-triphosphates were found to be partial agonists or, in some cases, antagonists (Kaufenstein et al., 2004; Springthorpe et al., 2007). In platelets, triphosphates and triphosphate mimics, such as 28-30, inhibit ADP-induced aggregation, which is consistent with P2Y12R antagonism. 29 has been used as a high affinity radioligand, 3H]PSB-0413 (Ohlmann et al., 2013). Conversely, there are studies showing that ATP seems to be a full P2Y12R agonist (Schmidt et al., 2013). Simplicifications of the unwieldy triphosphate group are possible. Monophosphate derivative 31 (Douglas et al., 2008) and carboxyl derivative 32, which was used as a 125I-radioligand (van Giezen et al., 2009), are P2Y12R antagonists. Even uncharged nucleotide-like derivatives, such as acyclic diester 33 and carbocyclic 8-aza derivative 34 (ticagrelor, now approved as an antiplatelet), act as reversibly binding P2Y12R antagonists.

**Toward a Systematic Characterization of the SAR of Dinucleotides at P2YRs**

Distinct biologic activities are associated with dinucleotides acting at P2YRs, and both P2Y1- and P2Y12-like subfamilies are represented. Zamecnik et al. published early reports on both the chemistry and biology of dinucleoside polyphosphates (Zamecnik et al., 1992). Using recombinant P2YRs, the actions of dinucleotides have been studied systematically at individual molecular targets. For example, Ap3A was found to activate the recombinant human P2Y1R (Lazarowski et al., 1995). At P2Y12R, which is involved in ADP-induced platelet aggregation, the series of ApnA has been studied. In certain conditions, ApnA appears to be either an agonist, antagonist, or partial agonist (Chang et al., 2010). Diadenosine polyphosphates are also known to activate P2X ion channels. For example, diadenosine pentaphosphate (Ap5A) activates P2X receptors on human cerebrocortical synaptic terminals (Delicado et al., 2006).

Dinucleoside polyphosphates tend to be more stable than mononucleotides at the cell surface because they are not substrates of the ecto-nucleotidases, such as CD39, which cleaves the terminal P-O-P bond from nucleoside 5′-polyphosphates (Kukulski et al., 2011). However, dinucleoside polyphosphates are hydrolyzed by NPP4, which is expressed on the surface of vascular endothelial cells and elsewhere (Albright et al., 2012). Fischer et al. studied diadenosine polyphosphates as inhibitors of nucleotide pyrophosphatase/phosphodiesterases and agonists of various P2YRs (Yelovitch et al., 2012). The inclusion of a borano group in place of OH at a specific location on the polyphosphate moiety was found to have a major enhancing effect on potency and enzymatic stability. The borano substitution of an asymmetric phosphate may also create a new chiral center, e.g., 7, which necessitates separation of diastereomers, and 31P and 1H NMR can be used to determine the relative configuration.

We include in the scope of this review terminal sugar derivatives, which are related structurally to dinucleotides. The first recognized native ligand of what is now designated P2Y14R (originally called GPR105) was UDPG (Chambers et al., 2000). Other related UDP sugars have considerable potency at P2Y14R, and UDP itself is now known to be one of the cognate ligands of this receptor (Carter et al., 2009). There are other reported examples of nucleoside polyphosphates as potent P2YR ligands, in which the terminal phosphate is blocked with a simple aromatic or aliphatic moiety (Das et al., 2010). However, in some cases, blocking the terminal phosphate moiety of a nucleoside 5′-diphosphate or 5′-triphosphate can lead to a great reduction in activity. For example, if the β-phosphoryl of the P2Y1/P2Y12 agonist 2-methylthioadenosine 5′-diphosphate (2MeSATP) is esterified with a photocleavable o-nitrobenzyl alcohol, receptor activity is lost (Gao et al., 2008).

The pharmacological properties within the series of NpnA (dinucleotides with base N and polyphosphate length n) also vary considerably with the value of n. The potency of various dinucleoside polyphosphates to induce a rise in intracellular calcium in 1321N1 astrocytoma cells heterologously expressing P2YRs of the P2Y1-like subfamily was studied systematically (Shaver et al., 2005). Using highly purified analogs, the rank order of agonist potencies in general was Np4N > Np3N > Np2N > Np1N at P2Y1 and P2Y4 receptors, and Np4N > Np3N > Np2N > Np1N at P2Y2 and P2Y4 receptors. However, the results are inconsistent with earlier reports that may not purely reflect their potency in activating a given P2YR, and species differences may exist. For example, ApnA was reported to be either inactive (Patel et al., 2001) or active at P2Y1R. Recent studies have expanded the SAR of dinucleotide analogs, including boronophosphates, at P2YRs (Maruoka et al., 2011; Yelovitch et al., 2012).

The length of the polyphosphate chain required for activation of each P2YR in some cases is highly limited, i.e., with narrow SAR requirements, suggesting that specific interactions with the receptor are involved. Thus, the distal terminal moiety, i.e., either a nucleoside or sugar, with respect to the primary pharmacophore, is not likely to be dissociated from the constraints of the receptor protein. Rather than have complete conformational freedom in the extracellular space, this terminal moiety appears to occupy a secondary binding region that reflects specific interactions with amino acid residues on the receptor.

Representative dinucleotides and related bifunctional compounds (i.e., blocked on both ends of the polyphosphate chain with a phosphodiester) that potently interact with each of the P2YRs are shown in Table 1. The dinucleotides found to modulate P2YRs are often symmetric tail-to-tail dimers of the principal native ligands, such as Ap3A, a dimer of ADP, at P2Y12R. Analogs of UppA and ApnA have been studied at the recombinant P2Y1R (e.g., 2) and platelet P2Y12R (e.g., 12), whereas analogs of Upp4U (e.g., 3 and 4) have been studied at P2Y1R and P2Y12R. Upp4U [P′-[(5′-uridinyl)-P′-[(5′-uridinyl)-triphosphate] and its derivatives (e.g., 8) have demonstrated high potency at P2Y1R. Ap5A 13 clearly activates P2Y1R, whereas higher diadenosine polyphosphate homologs are inactive (Zhang et al.,
Nicotinic acid adenine dinucleotide phosphate (10) is an endogenous agonist of P2Y11R. β-Nicotinamide adenine dinucleotide is released from sympathetic nerve terminals and appears to activate P2Y1R and P2Y11R (Moreschi et al., 2006; Mutafova-Yambolieva et al., 2007; Klein et al., 2009). The production and enzymatic stability of an endogenous P2Y11R agonist, UDPG, was studied (Lazarowski et al., 2003). UDPG was also used as a 3H-radioligand (Brunschweiger and Müller, 2006). It is cleaved by nucleotide pyrophosphatase/phosphodiesterases but is stable to the action of several ecto-nucleotidases, such as CD39, which hydrolyze mononucleotides. UDPG release accompanies trafficking of proteins to the cell surface.

Each P2YR subtype has a characteristic SAR for the nucleoside moiety that is not necessarily in parallel between the mononucleotide and dinucleotide series. SAR analysis of mononucleotide pharmacophores at P2YRs is better characterized than for the terminal ends of P2YR-active dinucleotides. In some cases, there is freedom of substitution, and in other cases, the activity is highly dependent on subtle structural changes. For example, if a terminal glucose or other sugar is present on the β-phosphate of UDP, the P2Y11R potency is highly sensitive to changes in sugar functional groups and stereochemistry (Ko et al., 2009). Thus, at P2Y11R, UDP sugars seem to have a different SAR from 5′-diphosphates. Many of the simple UDP analogs are equipotent or more potent than UDPG. However, when present, the terminal β-sugar has specific structural requirements that can greatly reduce potency, and when absent, there is no detrimental effect in general on potency, which suggests a defined binding site for the distal end of UDPG on the receptor. Also, uridine 5′-triphosphate sugars and uridine triphosphates each have distinct SAR patterns at P2Y11R (Maruoka et al., 2011) and uridine 5′-diphosphate sugars, such as 15, and uridine 5′-diphosphates, such as 14, each have distinct patterns at P2Y11R (Das et al., 2010).

Dinucleotides have been the focus of pharmaceutical development. Diuridine polyphosphates have been explored as drug candidates by virtue of activating P2Y2R (tetraphosphates) or P2Y12R (triphosphates). Inspire Pharmaceuticals introduced the former as a candidate for the treatment of cystic fibrosis (INS37217) (Deterding et al., 2007), which displayed exceptional stability to nucleotidases but later lacked efficacy in clinical trials, and reported selective dinucleotide antagonists of P2Y12R (Douglass et al., 2008). A simple diuridine tetraphosphate (6, INS9365) is approved for the treatment of dry eye disease in Japan but not in the United States and is roughly equipotent at P2Y12R and P2Y12R (Lau et al., 2014). A diuridine triphosphate P2Y6R agonist (8, MRS29577) has been shown to increase insulin release from mouse β-islet cell cultures in a glucose-dependent manner and protect against apoptosis induced by TNFα (Balasubramanian et al., 2013). Both actions of P2Y6R might be favorable in cases of diabetes.

Miras-Portugal, Pintor, and colleagues studied the effects of dinucleoside polyphosphates in the nervous system and eye (Castany et al., 2011). Diinosine polyphosphates have been proposed as antiglaucoma agents based on their activation of P2YRs when applied to the corneal surface (Guzman-Aranguex et al., 2012). Ip5I [P3′-(5′-inosyl)P3′-(5′-inosyl)-pentaphosphate] was the most efficacious in the isosine series, with a 26% reduction in intraocular pressure and an EC50 value of 0.63 μM, but the P2YR subtype involved was not determined. One complication in interpreting the biologic activity in this series is that diinosine polyphosphates also act as antagonists of P2X receptors, with Ip5I [P3′-(5′-inosyl)-P3′-(5′-inosyl)-pentaphosphate] as the most potent at P2X1R (North and Jarvis, 2013).

Freilinger et al. have recently reported analogs of Ap4A that are potent and selective antagonists of the platelet P2Y12R and have an antithrombotic action (Yanachkov and Wright, 2010; Chang et al., 2012). Some of the analogs have enhanced stability in biologic systems due to the inclusion of methylene or halomethylene bridges between several phosphorus atoms. Because the thiophosphate group of 12 is a stereocenter, clarification of the biologic implications of this stereochemistry was needed. Pure diastereomers of a monochloromethylene diphosphonate derivative of Ap4A were separated chromatographically and characterized biologically (Chang et al., 2014). One of the isomers was clearly the most potent in inhibiting platelet aggregation through antagonism of P2Y12R without action at P2Y1R or P2X1R.

**Structural Characterization of P2YRs**

Mutagenesis studies have identified residues in various P2YRs that are likely involved in ligand recognition, regulation,
is mainly empty. The structures revealed major conformational
pocket 1, which is delimited by transmembrane helices (TMs)
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prominent in the antagonist-bound structure than in the nu-
binding site suggests two distinct subpockets, which are more
structural insight into this receptor family. The shape of the
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agonist 2MeSATP [2-methylthioadenosine 5
11
nucleotide full agonist 2MeSADP

carbamoyl]piperidin-1-yl)-5-cyano-2-methylnicotinate (AZD1283),
with the non-nucleotide antagonist ethyl 6-(4-[(benzylsulfonyl)
dimensional structural context.

effort to understand these mutagenesis findings in a three-
GPCR structural templates, until recently, has impeded the
relatively low structural homology of P2YRs to the available
Meta-binding sites, which refer to transient complexes of
nucleotide ligands as they approach the principal binding site,
have been proposed for P2Y1R. Chimeric P2Y7/P2Y6Rs, both of
which respond to nucleoside 5'-diphosphates, indicated the role
of several ELs in ligand selectivity (Hoffmann et al., 2004). The
relatively low structural homology of P2YRs to the available
GPCR structural templates, until recently, has impeded the
effort to understand these mutagenesis findings in a three-
dimensional structural context.

The high-resolution X-ray structures of the P2Y12R complexes
with the non-nucleotide antagonist ethyl 6-[(benzylsulfonyl)
carbamoyl]piperidin-1-yl)-5-cyano-2-methylnicotinate (AZD1283),
nucleotide full agonist 2MeSADP 11, and nucleotide partial
agonist 2MeSATP [2-methylthioadenosine 5’-triphosphate] were
reported (Fig. 4A) (Zhang et al., 2014a,b), providing major
structural insight into this receptor family. The shape of the
binding site suggests two distinct subpockets, which are more
prominent in the antagonist-bound than in the nucleo-
tide complexes. Both AZD1283 and 2MeSADP bind in
pocket 1, which is delimited by transmembrane helices (TMs)
3–7, whereas pocket 2, which is delimited by TMs 1, 2, 3, and 7,
is mainly empty. The structures revealed major conformational
changes in the binding pocket between nucleotides and
AZD1283-bound complexes of P2Y12R. Thus, the negatively
charged phosphate groups of the nucleotide ligands attract
positively charged (R19, R93 3.21, R256 6.55, and K280 7.35) and
hydrogen-bonding groups (NHα of C175, Y105 3.33, Y259 5.58,
and Q263 6.63), and the extracellular regions of TMs 6 and 7 are
bent inward toward the bound ligand, further enclosing the
ligand (Fig. 5A). The entrance to this pocket is completely
blocked by the ELs, suggesting that ligand access to its binding
site requires high plasticity of the extracellular region. In con-
trast, the non-nucleotide antagonist AZD1283 stabilizes a wide-
open structure of the pocket by pushing TM6 and TM7 outward
with its phenyl group. The long EL2 appears to be flexible and
lacking a well defined three-dimensional conformation in the
AZD1283 complex. Moreover, the disulfide bond connecting TM3
with EL2, which is highly conserved among family A GPCRs, is
missing, contributing to the open conformation of the binding site
in the AZD1283-bound structure of P2Y12R. Formation of this
disulfide bond in the nucleotide complexes requires an ~60°
rotation of TM3 as compared with the AZD1283 structure.

The classic ionic lock of many GPCRs, which is a pair of
oppositely charged residues in TMs 3 and 6 and holds the
receptor in an inactive conformation, is absent in P2Y12R. The
potential sodium-binding site, which is associated with a highly
conserved Asp residue in TM2 of class A GPCRs (Katritch et al.,
2014), is conserved in P2Y12R. Similar to the most closely re-
lated PAR1 structure, which contains a sodium ion in the
crystal structure, P2YRs have a second Asp residue in TM7 that
can participate in the coordination of cations. The crystallized
P2Y12R construct has an Asp-to-Asn mutation in this TM7
position, which improved the purified yield of the protein but
apparently reduced sodium ion binding.

The crystal structures of the P2Y1R complexes with the
nucleotide antagonist MRS2500 23 and with a non-nucleotide

Fig. 5. P2Y12R structures and binding of mononucleotides and dinucleotides. (A) Top view of the crystallographic pose of 2MeSADP (green carbon
sticks) at P2Y12R (Zhang et al., 2014b). Side chains of some residues important for ligand recognition are displayed (cyan carbon sticks). H-bonds and
ionic interactions are pictured as red dotted lines. (B) Top view of the theoretical docking pose of Ap4A (pink carbon sticks) at the antagonist-bound
P2Y12R structure (Zhang et al., 2014a). Side chains of some residues in contact with the ligand are displayed (cyan carbon sticks). Semitransparent
surface of binding site’s residues is displayed in pale cyan.
allosteric antagonist 1-(2-[(tert-butyl)phenoxy]pyridin-3-yl)-3-[4-(trifluoromethoxy)phenyl]urea (BPTU) (Chao et al., 2013) were recently determined (Fig. 4B) (Zhang et al., 2015). The two binding sites were dramatically different from each other and from the P2Y12R structures. Thus, the modeling of the two subfamilies of P2YRs requires distinct structural approaches and assumptions. In both P2Y1R structures, two disulfide bonds were present, connecting the N-terminus to TM7 and TM3 to EL2. The nucleotide-binding site is situated in the EL region, above the region corresponding to P2Y12R-bound nucleotides. This position is reminiscent of an allosteric site of the muscarinic m2 receptor that is above the orthosteric site (Kruse et al., 2013). The 5'-phosphate of MRS2500 is coordinated by R310<sup>23</sup> and makes hydrogen bonds with T205 in EL2 and Y306<sup>7,35</sup>. The 3'-phosphate is coordinated by the N-terminus and EL2 and by the phenol groups of Y<sub>2.65</sub> and Y<sub>7.32</sub>. N<sub>6.58</sub> coordinates the N6 and N7 groups of the adenine moiety, and a small 2-iodo group is complementary to a small subpocket in the N-terminal segment, including the main chain carbonyl of C42. R287<sup>30</sup> and L44 were on opposite sides of the adenine moiety, and a π-π stacking, as in P2Y<sub>12</sub>R, was lacking. The N<sub>4</sub> methyl group of MRS2500 was inserted between TMs 6 and 7, forming hydrophobic interactions with A286<sup>35</sup> and N299<sup>28</sup>. The (N)-methanocarba ring contacts the phenyl group of Y203 in ECL2, which is essential for binding of 23. Although the two antagonist-bound P2Y<sub>1</sub>R protein structures were very similar, the non-nucleotide antagonist BPTU binds to a novel site on the upper part of the TM region. However, a structural explanation for the recognition of dinucleotides at P2Y<sub>1</sub>R is still lacking.

**Conclusions**

The structural understanding of the interaction of nucleotide ligands with P2YRs has been greatly advanced with the resolution of two antagonist-bound structures of P2Y<sub>1</sub>R and three structures of P2Y<sub>12</sub>R, which will be especially useful in characterizing recognition at the subfamilies of P2Y<sub>1</sub>R-like and P2Y<sub>12</sub>R-like receptors. With new structural data on the P2YR family currently available, it will be possible to use more rational design processes to explore the SAR of different classes of nucleotides at P2YRs.

At each of the P2Y<sub>2</sub> subtypes, both mononucleotides and dinucleotides can act; thus, both naturally occurring and synthetically optimized dinucleoside polyphosphates can serve as agonist or antagonist ligands at various P2YRs. There are several preclinical drug candidates based on this phenomenon. The empirical observation of very specific patterns of SAR of dinucleotides or nucleoside phosphosugars at each of the P2Y<sub>2</sub> subtypes is now partly understandable structurally with the observation that P2Y<sub>1</sub>R<sub>2</sub> and potentially other P2Y<sub>2</sub>Rs have more than one binding cleft in the upper part of the TM region. However, a structural explanation for the recognition of dinucleotides at P2Y<sub>1</sub>R<sub>1</sub> is still lacking.

**Authorship Contributions**

Performed data analysis: Paoletta.

Wrote or contributed to the writing of the manuscript: Jacobson, Kiselow, Katritch, Wu, Gao, Zhao, Stevens.

**References**


Brunsweigewer A and Müller CE (2006) P2 receptors activated by uracil nucleotides and from the P2Y<sub>12</sub>R structures. Thus, the modeling of the two subfamilies of P2YRs. With new structural data on the P2YR family currently available, it will be possible to use more rational design processes to explore the SAR of different classes of nucleotides at P2YRs.

Although an unequivocal orientation for dinucleotides at P2Y<sub>1</sub>R<sub>1</sub> and other P2YRs is still undetermined, the recently solved crystallographic structures suggest the accommodation of both nucleoside moieties of dinucleotides in the unusual bifurcated cavity at P2Y<sub>12</sub>R and possibly at other P2YRs (Trujillo et al., 2015). This is consistent with docking studies showing dinucleotide ligands reaching both subpockets in the bound state (Zhang et al., 2014a). Figure 5B shows a hypothetical docking of ApPyA in P2Y<sub>12</sub>R based on the structure of its AZD1283 complex. In particular, the docking pose of ApPyA at the antagonist-bound P2Y<sub>12</sub>R shows one nucleotide moiety accommodated in pocket 1 and the other one in pocket 2, with the phosphate groups interacting with positively charged residues on TMs 6 and 7. The subsequent resolution of the agonist-bound P2Y<sub>12</sub>R structure revealed that the proposed orientation of the nucleotide moiety of ApPyA in pocket 1 is different from the one observed for 2MeSADP in the crystal (Fig. 5A). In fact, residues stabilizing the orientation of the ribose and base have different conformations in the two P2Y<sub>12</sub>R structures: Lys179 in EL2; Cys97 in TM3 because of the missing disulfide bond; and His187 and Asn191 in TM5. This indicates that the receptor conformation that binds dinucleotides may probably combine some of the structural features observed in the two crystallographic structures, and further molecular modeling studies can help to obtain a more realistic orientation of dinucleotides in the binding site. The 5'-phosphate of P2Y<sub>1</sub>R-bound MRS2500 points downward toward a sterically limited region. Thus, the mode of dinucleotide binding to P2Y<sub>1</sub>R is not apparent from the currently available structure.


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