1521-0111/88/2/220–230$25.00
MOLECULAR PHARMACOLOGY

http://dx.doi.org/10.1124/mol.114.095711
Mol Pharmacol 88:220–230, August 2015

MINIREVIEW

Nucleotides Acting at P2Y Receptors: Connecting Structure and Function

Kenneth A. Jacobson, Silvia Paoletta, Vsevolod Katritch, Beili Wu, Zhan-Guo Gao, Qiang Zhao, Raymond C. Stevens, and Evgeny Kiselev

Molecular Recognition Section, Laboratory of Bioorganic Chemistry, National Institutes of Diabetes and Digestive and Kidney Diseases, National Institutes of Health, Bethesda, Maryland (K.A.J., S.P., Z.-G.G., E.K.); The Bridge Institute, Dana and David Dornsife School of Letters, Arts, and Sciences, University of Southern California, Los Angeles, California (V.K., R.C.S.); and Chinese Academy of Sciences Key Laboratory of Receptor Research, Shanghai Institute of Materia Medica, Chinese Academy of Sciences, Shanghai, China (B.W., Q.Z.)

Received September 11, 2014; accepted April 2, 2015

ABSTRACT

Eight G protein–coupled P2Y receptor (P2YR) subtypes are important physiologic mediators. The human P2YRs are fully activated by ATP ($P2Y_2$ and $P2Y_{11}$), ADP ($P2Y_1$, $P2Y_{12}$, and $P2Y_{13}$), UTP ($P2Y_2$ and $P2Y_4$), UDP ($P2Y_6$ and $P2Y_{14}$), and UDP glucose ($P2Y_{14}$). Their structural elucidation is progressing rapidly. The X-ray structures of three ligand complexes of the G_i-coupled $P2Y_{12}R$ and two of the G_q-coupled $P2Y_1Rs$ 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 $P2Y_1$, $P2Y_2$, $P2Y_4$, and $P2Y_{12}Rs$. Each P2YR subtype has the ability to be activated by structurally bifunctional agonists, such as dinucleotides, typically, dinucleoside triphosphates or tetraphosphates, and nucleoside polyphosphate sugars (e.g., UDP glucose) as well as the more conventional mononucleotide agonists. A range of dinucleoside polyphosphates, 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 G_q-coupled $P2Y_1$-like ($P2Y_1$, $P2Y_2$, $P2Y_4$, $P2Y_6$, and $P2Y_{11}$) and three G_i-coupled $P2Y_{12}$-like ($P2Y_{12}$–$P2Y_{14}$) 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 ($P2Y_2$ and $P2Y_{11}$), ADP ($P2Y_1$, $P2Y_{12}$, and $P2Y_{13}$), UTP ($P2Y_2$ and $P2Y_4$), UDP ($P2Y_6$ and $P2Y_{14}$), and UDPG ($P2Y_{14}$). At increased concentrations, there are some additional crossovers in the activation patterns, such as UDPG acting as a full agonist at $P2Y_2R$ (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) $P2Y_4R$ (Kennedy et al., 2000).

P2YRs are widespread in the body and involved in the regulation of nearly all systems, notably, immune, skeletomuscular, digestive, nervous, endocrine, cardiovascular, pulmonary, gastrointestinal, and renal systems (Abbracchio et al., 2006).

This work was supported by the National Institutes of Health National Institute of Diabetes and Digestive and Kidney Diseases [Grant Z01-DK031126-08].
dx.doi.org/10.1124/mol.114.095711.

ABBREVIATIONS: 2MeSADP, 2-methylthioadenosine 5′-diphosphate; Ap_4A, P^1-(5′-adenosinyl)-P^4-(5′-adenosinyl)-tetraphosphate; AZD1283, ethyl 6-(4-[(benzylsulfonyl)carbamoyl]piperidin-1-yl)-5-cyano-2-methylnicotinate; BPTU, 1-(2-[2-(tert-butyl)phenoxy]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*,4′*S*,5′*S*)-4-(2-iodo-6-methylamino-purin-9-yl)-1-[(phosphato)-methyl]-2-(phosphato)-bicyclo[3.1.0]hexane; P2YR, P2Y receptor; SAR, structure activity relationship; TM, transmembrane helix; UDPG, UDP glucose; Up_4A, P^1-(5′-adenosinyl)-P^4-(5′-uridinyl)-tetraphosphate.

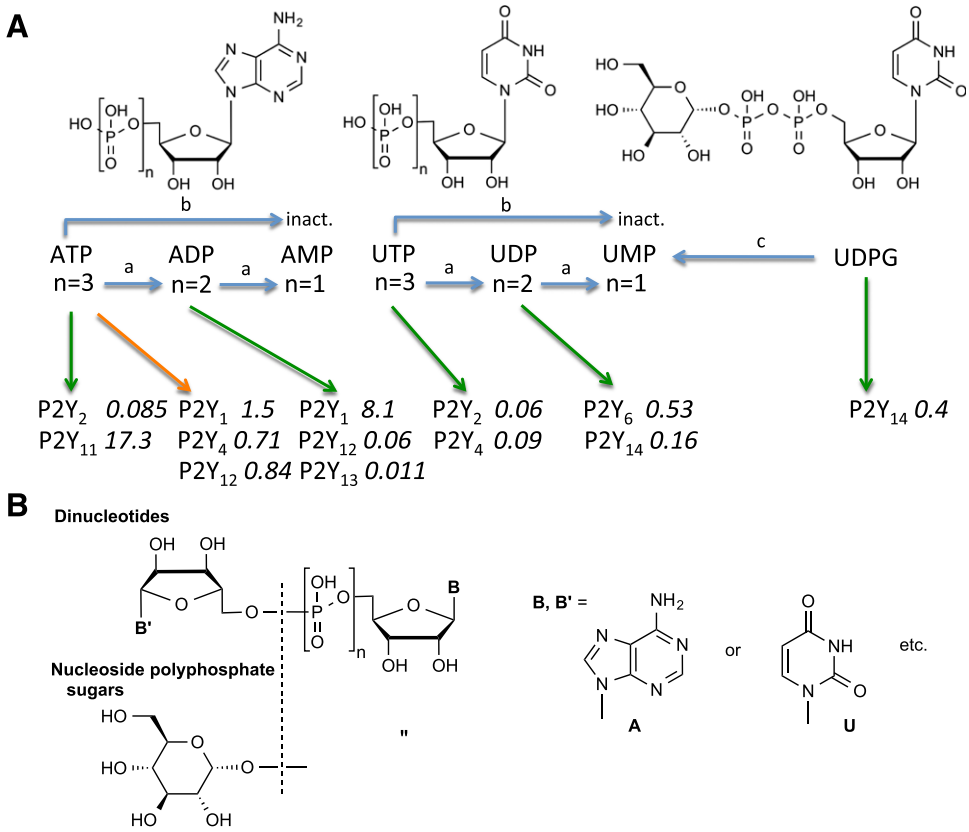


Fig. 1. (A) Action at P2YRs of nucleotides released from cells (e.g., ATP, UTP, and UDPG) and their conversion outside the cell to 5′-diphosphates, which act at different P2YRs, and/or to 5′-monophosphates, which are inactive (inact.). 5′-nucleotidase catalyzes the final conversion of AMP to adenosine, which acts at its own set of four GPCRs (adenosine A_1, A_{2A}, A_{2B}, and A_3 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). EC_{50} or IC_{50} values (μM) at human P2YRs from measurement of adenylate cyclase or phospholipase C activity are indicated in italics. Weaker interactions, such as UDP, as an agonist of $P2Y_2R$ (~10) or $P2Y_6R$ (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 Up_4A and Ap_4A, 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).

The broad distribution of P2YRs and the multiplicity of effects of each subtype 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 triphosphates or tetraphosphates and nucleoside polyphosphate sugars (e.g., UDPG). The concentrations of dinucleotides achieved in the extracellular medium are often sufficient to activate a range of P2YRs; thus, this is a physiologically relevant component of the purinergic system (Rapaport and Zamecnik, 1976). For example, the endogenous levels of P^1-(5′-adenosinyl)-P^4-(5′-adenosinyl)-tetraphosphate (Ap_4A) and related dinucleotides vary in response to stress, and they participate in 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. For example, P^1-(5′-adenosinyl)-P^4-(5′-uridinyl)-tetraphosphate (Up_4A) is released from the vascular endothelium to induce vasoconstriction and has been explored in various biologic contexts, such as $P2Y_2R$-induced migration of smooth muscle cells and activation of enteric neuronal $P2Y_1R$ (Wiedon et al., 2012; Durnin et al., 2014). Diadenosine polyphosphates, such as Ap_4A, are plentiful in platelet granules and secretory granules of nerve terminals. They contribute either directly or after cleavage to ADP/ATP to thrombus formation and participate in synaptic transmission (Zamecnik et al., 1992; Pintor et al., 2000).

Now with the availability of structural information on the P2Y family and mutagenesis data on the role of specific amino acid residues in ligand binding and/or receptor activation for a few of the P2YRs (Fig. 2), it is feasible to compare the recognition pattern for the various types of agonists more systematically within the P2YR family. Until recently, the empirically

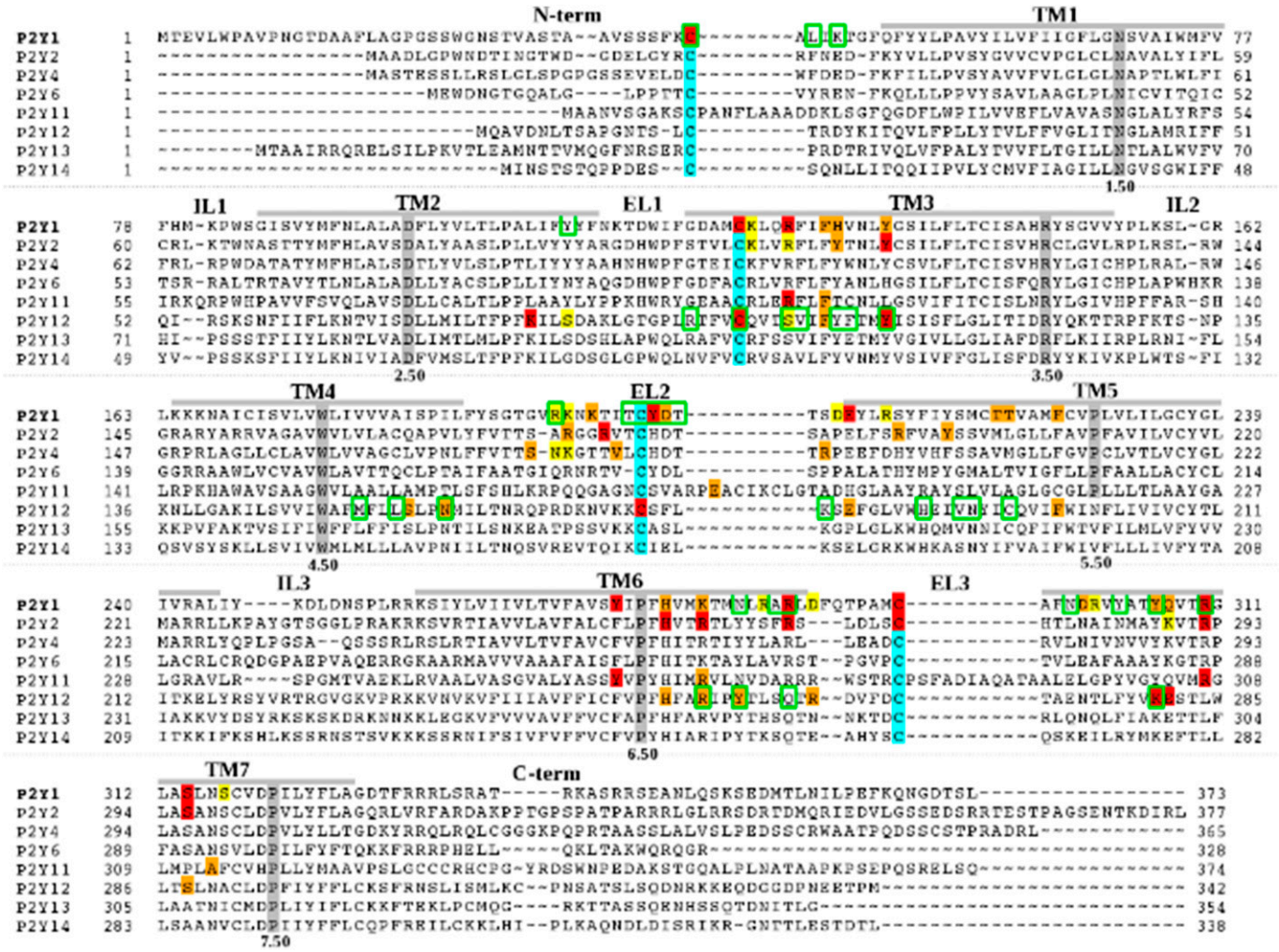


Fig. 2. Sequence alignment of the human P2YRs. Residues that have been identified using site-directed mutagenesis, as involved in ligand binding and/or receptor activation at $P2Y_1R$ (Abbracchio et al., 2006; Zhang et al., 2015), $P2Y_2R$ (Erb et al., 1995; Hillmann et al., 2009), $P2Y_4R$ (Herold et al., 2004), $P2Y_{11}R$ (Zylberg et al., 2007), and $P2Y_{12}R$ (Hoffmann et al., 2008; Mao et al., 2010; Ignatovica 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 $P2Y_{12}R$ or within 3 Å from the crystallographic pose of MRS2500 at $P2Y_1R$ 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.

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 $P2Y_1$-like receptor subfamily or other second messengers, such as cAMP, rather than binding affinity because only three of the P2YRs ($P2Y_1$, $P2Y_{12}$, and $P2Y_{14}$ receptors) have radioligands available.

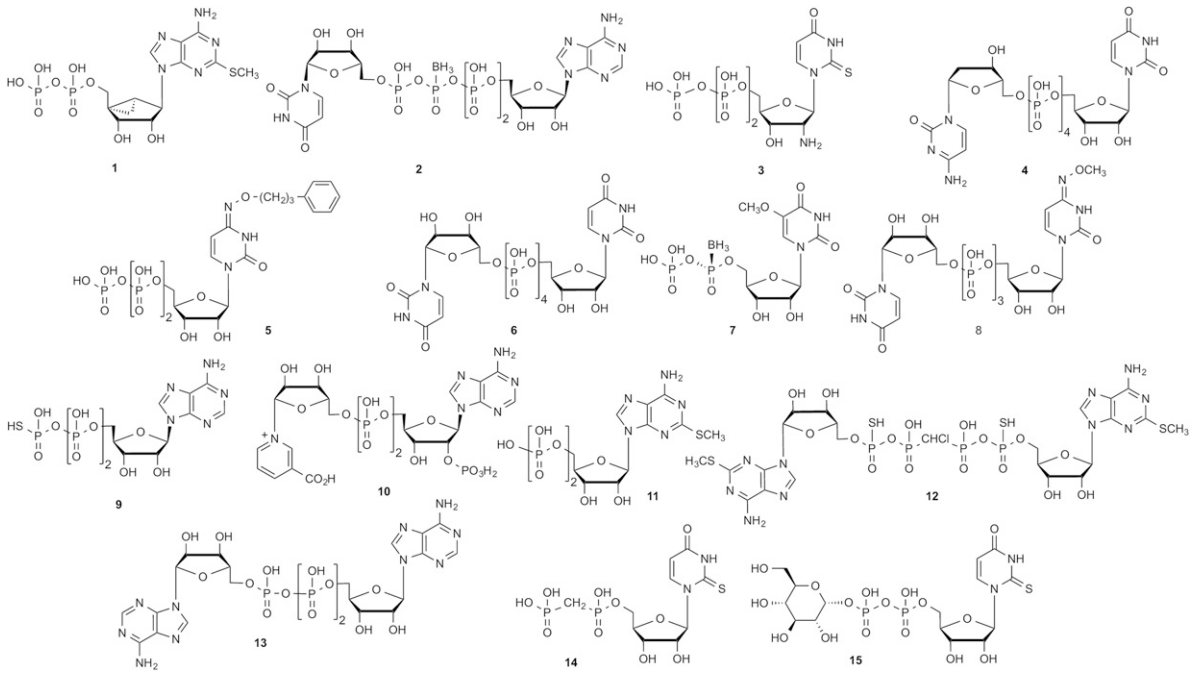
Ecto-nucleotidases and other enzymes are involved in the interconversion of nucleotides that act as P2YR ligands (Fig. 1A) and finally by 5′-nucleotidase for the conversion to adenosine, which acts at its own set of four GPCRs. Recently, the structures of 5′-nucleotidase and ecto-nucleoside triphosphate 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 $P2Y_{12}$ (antithrombotic antagonists) (Ferri et al., 2013) and $P2Y_2$ (agonist treatment of dry eye,

TABLE 1

Representative examples of different types of synthetic or natural ligands (agonists, unless noted), either mononucleotide or dinucleotide (or nucleotide sugar), for each of the P2YRs

The potency (nM) was measured in functional assays at the human P2YRs.



P2YR	Synthetic Agonist (Mononucleotide), Potency	Bifunctional Ligand, Potency	References
	nM		
$P2Y_1$	**1**, MRS2365, 0.4	**2**, $Up_4(\beta$-B)A, A isomer, 500	Houston et al., 2008; Yelovitch et al., 2012
$P2Y_2$	**3**, MRS2698, 8.0	**4**, INS37217, 220	Houston et al., 2008; Yerxa et al., 2002
$P2Y_4$	**5**, MRS4062, 26	**6**, INS365 (Up_4U)[a], 130	Ko et al., 2008; Maruoka et al., 2011
$P2Y_6$	**7**, 5-OMe-UDPαB, 8	**8**, MRS2957, 12	Maruoka et al., 2010; Haas et al., 2014
$P2Y_{11}$	**9**, ATP-γ-S, 24,000	**10**, NAADP, 64,000	Djerada and Millart, 2013
$P2Y_{12}$	**11**, 2MeSADP[b], 5	**12**, compound 17 (R/S)[c], 13	Zhang et al., 2002; Yanachkov and Wright, 2010
$P2Y_{13}$	**11**, 2MeSADP[b], 19	**13**, Ap_3A, 72	Zhang et al., 2002
$P2Y_{14}$	**14**, MRS2905, 2.0	**15**, MRS2690, 70	Das et al., 2010

NAADP, nicotinic acid adenine dinucleotide phosphate.

[a]INS365 also activates $P2Y_2R$ (EC_{50} = 210 nM).

[b]2MeSADP activates $P2Y_1R$ (EC_{50} = 6.6 nM), $P2Y_{12}R$, and $P2Y_{13}R$ (also used as a high affinity ^3H- or ^{33}P-radioligand) (Takasaki et al., 2001).

[c]Antagonist.

which is approved in Japan) (Lau et al., 2014). Two of the three $P2Y_{12}R$ antagonists in use as antithrombotics (thienopyridines) are actually prodrugs of irreversibly receptor-binding thiols and therefore have clinical limitations. The attempt to use $P2Y_2$ 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 $P2Y_1R$ has provided such agents, which are also being evaluated as potential antithrombotics (Yang et al., 2014). Also, the SAR of nucleotide antagonists is particularly advanced for the platelet ADP receptors $P2Y_1$ and $P2Y_{12}$. 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 Up_4U [P^1-(5′-uridinyl)-P^4-(5′-uridinyl)-tetraphosphate] and uridine 5′-tetraphosphate glucose are tolerated at $P2Y_2$, $P2Y_4$, and $P2Y_6$ receptors. Only $P2Y_2R$ readily accepts either A or U as the nucleobase in 5′-triphosphate agonists. ATP binds to human $P2Y_4R$ as an antagonist. Alternate nucleobases are sometimes recognized at P2YRs, but at much higher concentrations, for example, IDP as an agonist at $P2Y_{12}R$ (EC_{50} = 3.18 μM) and $P2Y_{13}R$ (EC_{50} = 0.552 μM) (Lazarowski et al., 1995). ITP and GTP act as agonists at $P2Y_4R$, with EC_{50} values of 7.38 and 6.59 μM (intracellular Ca^{+2}), respectively (Kennedy et al., 2000). Some 2-alkylthio derivatives of AMP interact with $P2Y_1$ and $P2Y_{12}$ receptors as agonists (Boyer et al., 1996b), whereas 2MeSAMP [2-methylthioadenosine 5′-monophosphate] is a $P2Y_{12}R$ antagonist (Zhang et al., 2002).

At $P2Y_2$ and $P2Y_4$ receptors, UMP and UDP are inactive, but some of their analogs activate these receptors as well as UMP analogs at $P2Y_6R$ (El-Tayeb et al., 2011). Steric constraint of the ribose ring using a bicyclo[3.1.0]hexane (methanocarba) ring system has demonstrated a strong preference for $P2Y_6R$ for

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 $P2Y_6$, but not $P2Y_2$ and $P2Y_4$ receptors (Haas et al., 2014). Thiocarbonyl substitution of the uracil 2 or 4 position is variably tolerated at the $P2Y_2$, $P2Y_4$, and $P2Y_{14}$ receptors. 2′- or 3′-deoxynucleotides are not well tolerated as P2YR agonists. Methylene 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 $P2Y_1R$; a pure stereoisomer of the 2-Cl member of that series displayed an EC_{50} of 7 nM (Azran et al., 2013). Although $P2Y_6R$ 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 C=O substitution, are tolerated at $P2Y_2$, $P2Y_4$, and $P2Y_6$ 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 $P2Y_1$, $P2Y_2$, $P2Y_4$, and $P2Y_{12}$ receptors. Some of these structures are shown in Fig. 3. A3p5p **16** was identified as a partial agonist of human $P2Y_1R$, 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 2′,5′) reduced its efficacy at $P2Y_1R$. N1 was not required for recognition (e.g., 1-deaza analog **19**), and several other modifications, N^6-methylation, and removal of the 2′-OH, further reduced the efficacy, leading to antagonists, such as MRS2179 **18** (Houston et al., 2008). N^6-dimethylation or the addition of *N*-alkyl groups larger than ethyl greatly reduced affinity at $P2Y_1R$, 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 bisthiophosphates 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 $P2Y_1R$-preferred conformation (Kim et al., 2003). Halo (by IC_{50}; I < Cl < F), methyl, methythio, and methylseleno substitution at the C2 position preserved high affinity (Costanzi et al., 2007). The presence of the N^6-methyl

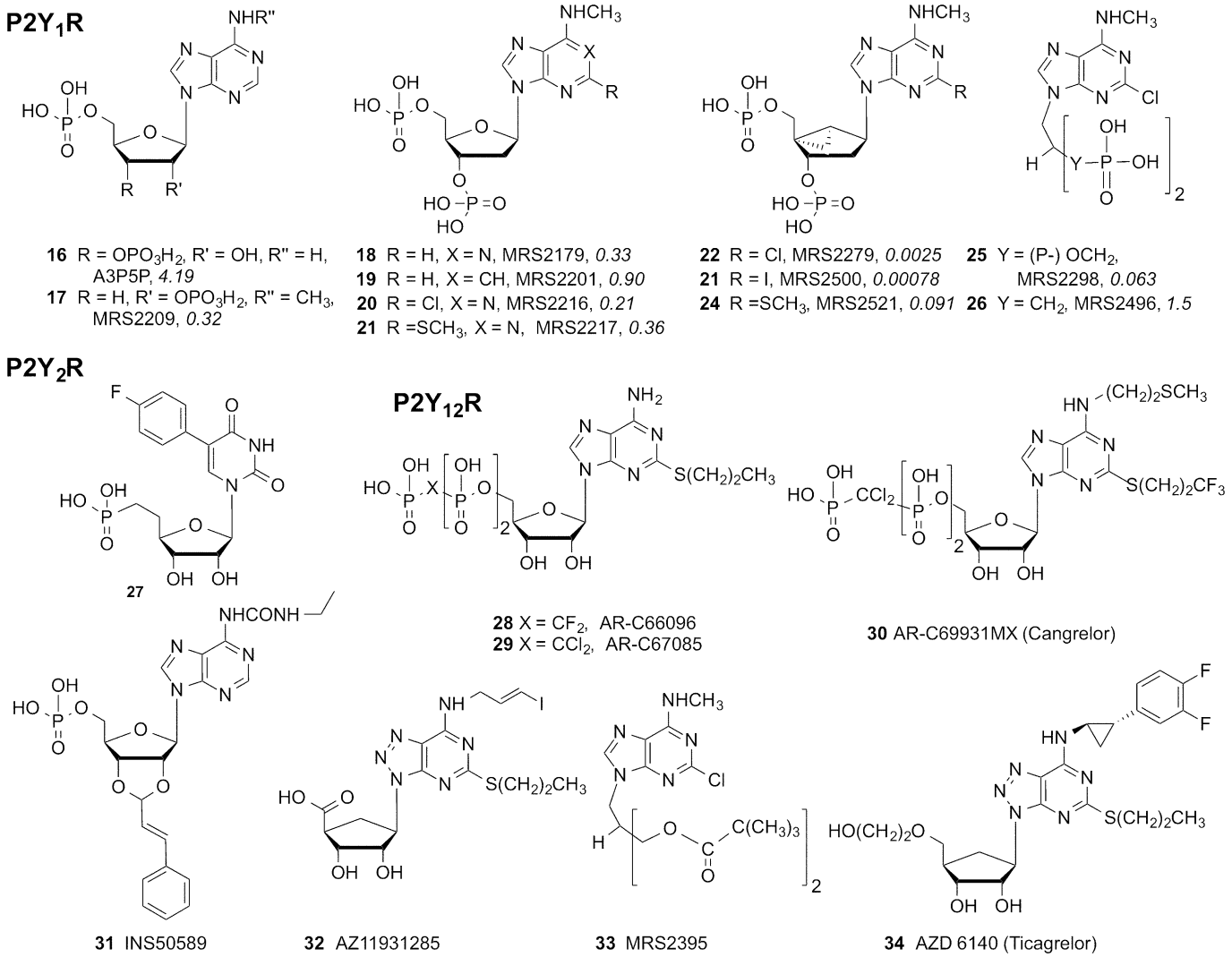


Fig. 3. Structures of nucleotide and nucleotide-like antagonists and partial agonists of P2YRs (IC_{50} values in micromolar at the human $P2Y_1R$ are shown in italics).

group in (1′*R*,2′*S*,4′*S*,5′*S*)-4-(2-iodo-6-methylamino-purin-9-yl)-1-[(phosphato)-methyl]-2-(phosphato)-bicyclo[3.1.0]hexane (MRS2500) **23** (also used as a high affinity ^{3}H- or ^{125}I-radioligand) enhanced the antagonist affinity by 16-fold. The same (*N*)-bicyclic ring in $P2Y_1R$ 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 $P2Y_1R$ must have some flexibility. The uracil phosphonate **27** appears to be an allosteric partial agonist, with selectivity for $P2Y_2R$, but additional characterization of this compound is required (Cosyn et al., 2009).

At $P2Y_{12}R$, 5′-triphosphates were found to be partial agonists or, in some cases, antagonists (Kauffenstein 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 $P2Y_{12}R$ antagonism. **29** has been used as a high affinity radioligand, [^{3}H]PSB-0413 (Ohlmann et al., 2013). Conversely, there are studies showing that ATP seems to be a full $P2Y_{12}R$ agonist (Schmidt et al., 2013). Simplifications of the unwieldy triphosphate group are possible. Monophosphate derivative **31** (Douglass et al., 2008) and carboxyl derivative **32**, which was used as a ^{125}I-radioligand (van Giezen et al., 2009), are $P2Y_{12}R$ antagonists. Even uncharged nucleotide-like derivatives, such as acyclic diester **33** and carbocyclic 8-aza derivative **34** (ticagrelor, now approved as an antithrombotic), act as reversibly binding $P2Y_{12}R$ antagonists.

Toward a Systematic Characterization of the SAR of Dinucleotides at P2YRs

Distinct biologic activities are associated with dinucleotides acting at P2YRs, and both $P2Y_1$- and $P2Y_{12}$-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, Ap_4A was found to activate the recombinant human $P2Y_4R$ (Lazarowski et al., 1995). At $P2Y_{12}R$, which is involved in ADP-induced platelet aggregation, the series of Ap_nA has been studied. In certain conditions, Ap_4A 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 (Ap_5A) 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 ^{31}P and ^{1}H 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 $P2Y_{14}R$ (originally called GPR105) was UDPG (Chambers et al., 2000). Other related UDP sugars have considerable potency at $P2Y_{14}R$, 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-phosphate of the $P2Y_1$/$P2Y_{12}$ agonist 2-methylthioadenosine 5′-diphosphate (2MeSADP) is esterified with a photocleavable *o*-nitrobenzyl alcohol, receptor activity is lost (Gao et al., 2008).

The pharmacological properties within the series of Np_nN (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 $P2Y_1$-like subfamily was studied systematically (Shaver et al., 2005). Using highly purified analogs, the rank order of agonist potencies in general was $Np_3N >> Np_4N$, Np_2N at $P2Y_1$ and $P2Y_6$ receptors, and $Np_4N >> Np_3N > Np_2N$ at $P2Y_2$ and $P2Y_4$ 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, Ap_4A was reported to be either inactive (Patel et al., 2001) or active at $P2Y_1R$. Recent studies have expanded the SAR of dinucleotide analogs, including boranophosphates, 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 disassociated 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 Ap_4A, a dimer of ADP, at $P2Y_{12}R$. Analogs of Up_4A and Ap_4A have been studied at the recombinant $P2Y_1R$ (e.g., **2**) and platelet $P2Y_{12}R$ (e.g., **12**), whereas analogs of Up_4U (e.g., **3** and **4**) have been studied at $P2Y_2R$ and $P2Y_4R$. Up_3U [P^1-(5′-uridinyl)-P^3-(5′-uridinyl)-triphosphate] and its derivatives (e.g., **8**) have demonstrated high potency at $P2Y_6R$. Ap_3A **13** clearly activates $P2Y_{13}R$, whereas higher diadenosine polyphosphate homologs are inactive (Zhang et al.,

2002). Nicotinic acid adenine dinucleotide phosphate (**10**) is an endogenous agonist of $P2Y_{11}R$. β-Nicotinamide adenine dinucleotide is released from sympathetic nerve terminals and appears to activate $P2Y_1R$ and $P2Y_{11}R$ (Moreschi et al., 2006; Mutafova-Yambolieva et al., 2007; Klein et al., 2009). The production and enzymatic stability of an endogenous $P2Y_{14}R$ agonist, UDPG **14**, was studied (Lazarowski et al., 2003). UDPG was also used as a ^{3}H-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 $P2Y_{14}R$ potency is highly sensitive to changes in sugar functional groups and stereochemistry (Ko et al., 2009). Thus, at $P2Y_{14}R$, 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′-tetraphosphate sugars and uridine triphosphates each have distinct SAR patterns at $P2Y_4R$ (Maruoka et al., 2011) and uridine 5′-diphosphate sugars, such as **15**, and uridine 5′-diphosphates, such as **14**, each have distinct patterns at $P2Y_{14}R$ (Das et al., 2010).

Dinucleotides have been the focus of pharmaceutical development. Diuridine polyphosphates have been explored as drug candidates by virtue of activating $P2Y_2R$ (tetraphosphates) or $P2Y_6R$ (triphosphates). Inspire Pharmaceuticals introduced the former as a candidate for the treatment of cystic fibrosis (**4**, INS37217) (Deterding et al., 2007), which displayed exceptional stability to nucleotidases but later lacked efficacy in clinical trials, and reported selective dinucleotide antagonists of $P2Y_{12}R$ (Douglass et al., 2008). A simple diuridine tetraphosphate (**6**, INS365) is approved for the treatment of dry eye disease in Japan but not in the United States and is roughly equipotent at $P2Y_2R$ and $P2Y_4R$ (Lau et al., 2014). A diuridine triphosphate $P2Y_6R$ agonist (**8**, MRS2957) 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 $P2Y_6R$ 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-Aranguez et al., 2012). Ip_4I [P^1-(5′-inosyl)-P^4-(5′-inosyl)-tetraphosphate] was the most efficacious in the inosine series, with a 26% reduction in intraocular pressure and an EC_{50} 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 Ip_5I [P^1-(5′-inosyl)-P^5-(5′-inosyl)-pentaphosphate] as the most potent at P2X1R (North and Jarvis, 2013).

Freilinger et al. have recently reported analogs of Ap_4A that are potent and selective antagonists of the platelet $P2Y_{12}R$ 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 Ap_4A 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 $P2Y_{12}R$ without action at $P2Y_1R$ or P2X1R.

Structural Characterization of P2YRs

Mutagenesis studies have identified residues in various P2YRs that are likely involved in ligand recognition, regulation,

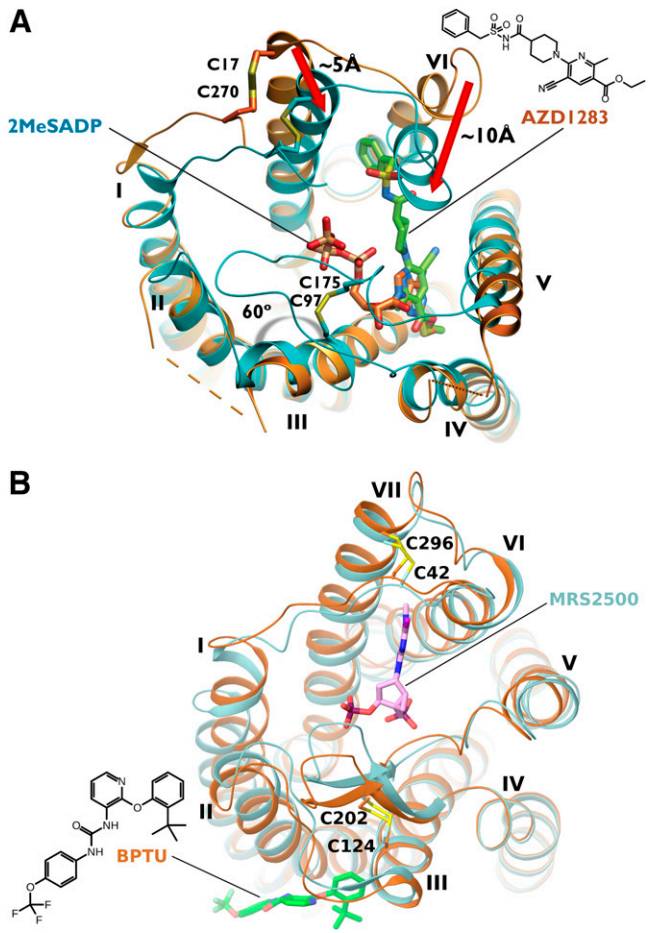


Fig. 4. (A) Human $P2Y_{12}R$ X-ray structures in complex with AZD1283 (the non-nucleotide antagonist is shown in green carbon sticks, and the receptor is shown in orange ribbons) and 2MeSADP (the nucleotide full agonist is shown in orange carbon sticks, and the receptor is shown in cyan ribbons) (Zhang et al., 2014a,b). (B) Human $P2Y_1R$ X-ray structures in complex with the antagonists MRS2500 (the nucleotide antagonist is shown in pink carbon sticks, and the receptor is shown in cyan ribbons) and BPTU (the allosteric antagonist is shown in green carbon sticks, and the receptor is shown in orange ribbons) (Zhang et al., 2015).

and/or receptor activation (Fig. 2) (Brinson and Harden, 2001; Costanzi et al., 2004; Abbracchio et al., 2006). Given the requirement for negatively charged groups in all agonists thus far identified, the presence of many positively charged amino acid residues in the outer regions of P2YRs suggests direct interaction with nucleotide ligands. The requirement for specific positively charged residues in the recognition of agonists of P2YRs has been well documented (see Fig. 2).

The role of extracellular loops (ELs) in recognition at P2YRs was indicated in several mutagenesis studies (Jacobson et al., 2012). Single amino acid replacements of $P2Y_1R$ and $P2Y_2R$ led to this conclusion. For example, a single Asp residue (D204) in $P2Y_1R$ was found by Ala mutation to be essential for activation by ADP analogs, and even Glu, Asn, and Gln replacement of this residue failed to restore recognition (Moro et al., 1999). Meta-binding sites, which refer to transient complexes of nucleotide ligands as they approach the principal binding site, have been proposed for $P2Y_1R$. Chimeric $P2Y_1/P2Y_6Rs$, 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 $P2Y_{12}R$ complexes with the non-nucleotide antagonist ethyl 6-(4-[(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 structure than in the nucleotide 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 $P2Y_{12}R$. 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 ($NH\alpha$ of C175, $Y105^{3.33}$, $Y259^{6.58}$, and $Q263^{6.62}$), 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 contrast, 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 $P2Y_{12}R$. Formation of this disulfide bond in the nucleotide complexes requires an ~60° rotation of TM3 as compared with the AZD1283 structure. Adenine of 2MeSADP and the pyridine ring of AZD1283 form a π-π interaction with $Y105^{3.33}$. The methylthio group is situated between TMs 3–5.

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 $P2Y_{12}R$. 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 $P2Y_{12}R$. Similar to the most closely related 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 $P2Y_{12}R$ 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 $P2Y_1R$ complexes with the nucleotide antagonist MRS2500 **23** and with a non-nucleotide

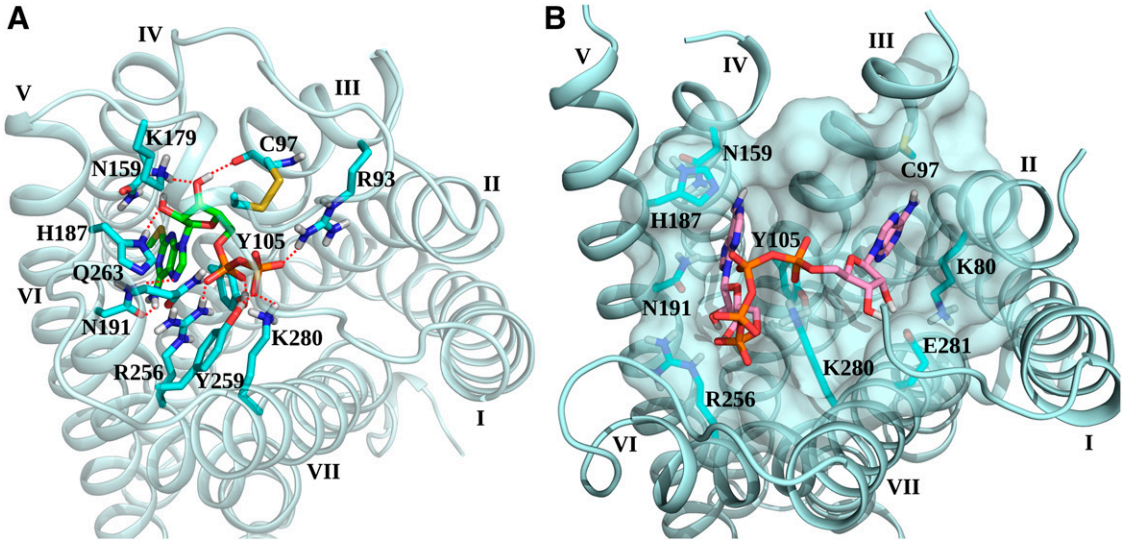


Fig. 5. $P2Y_{12}R$ structures and binding of mononucleotides and dinucleotides. (A) Top view of the crystallographic pose of 2MeSADP (green carbon sticks) at $P2Y_{12}R$ (Zhang et al., 2014b). Side chains of some residues important for ligand recognition are displayed (cyan carbon sticks). H-bonds and ionic interactions are pictured as red dotted lines. (B) Top view of the theoretical docking pose of Ap_4A (pink carbon sticks) at the antagonist-bound $P2Y_{12}R$ 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-[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 $P2Y_{12}R$ structures. Thus, the modeling of the two subfamilies of P2YRs requires distinct structural approaches and assumptions. In both $P2Y_1R$ 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 $P2Y_{12}R$-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 $R310^{7.39}$ and makes hydrogen bonds with T205 in EL2 and $Y306^{7.35}$. The 3′-phosphate is coordinated by the N-terminus and EL2 and by the phenol groups of $Y^{2.63}$ and $Y^{7.32}$. $N^{6.58}$ 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. $R287^{6.62}$ and L44 were on opposite sides of the adenine moiety, and a π-π stacking, as in $P2Y_{12}R$, was lacking. The N^6 methyl group of MRS2500 was inserted between TMs 6 and 7, forming hydrophobic interactions with $A286^{6.61}$ and $N299^{7.28}$. The (*N*)-methanocarba ring contacts the phenyl group of Y203 in ECL2, which is essential for binding of **23**. Although the two antagonist-bound $P2Y_1R$ protein structures were very similar, the non-nucleotide antagonist BPTU binds to a novel site on the exterior of the 7TM bundle, and its allosteric antagonism was shown by dissociation kinetics of [^{3}H]2MeSADP. The increase in the dissociation rate of this agonist induced by BPTU was lost when this unusual allosteric site, which is located outside of the TM region and in contact with the phospholipid bilayer, was blocked by a mutation that sterically interfered with this binding region. Mutagenesis indicated that mutually exclusive residues are essential for the two antagonists and suggested that the agonist 2MeSADP may be bound in a similar fashion as **23**. The Y306F mutant receptor lost affinity for both agonist and antagonist nucleotide ligands but not BPTU.

Although an unequivocal orientation for dinucleotides at $P2Y_{12}R$ 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 $P2Y_{12}R$ 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 Ap_4A in $P2Y_{12}R$ based on the structure of its AZD1283 complex. In particular, the docking pose of Ap_4A at the antagonist-bound $P2Y_{12}R$ 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 $P2Y_{12}R$ structure revealed that the proposed orientation of the nucleotide moiety of Ap_4A 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 $P2Y_{12}R$ 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 available crystal structures, and further molecular modeling studies can help to obtain a more realistic orientation of dinucleotides in the binding site. The 5′-phosphate of $P2Y_1R$-bound MRS2500 points downward toward a sterically limited region. Thus, the mode of dinucleotide binding to $P2Y_1R$ is not apparent from the currently available structure.

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 $P2Y_1R$ and three structures of $P2Y_{12}R$, which will be especially useful in characterizing recognition at the subfamilies of $P2Y_1R$-like and $P2Y_{12}R$-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 P2YR 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 P2YR subtypes is now partly understandable structurally with the observation that $P2Y_{12}R$, and potentially other P2YRs have more than one binding cleft in the upper part of the TM region. However, a structural explanation for the recognition of dinucleotides at $P2Y_1R$ is still lacking.

Authorship Contributions

Performed data analysis: Paoletta.

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

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Address correspondence to: Dr. Kenneth A. Jacobson, Laboratory of Bioorganic Chemistry, National Institutes of Diabetes and Digestive and Kidney Diseases, NIH, Bldg. 8A, Rm. B1A-19, Bethesda, MD 20892-0810. E-mail: kajacobs@helix.nih.gov