

Pharmacology of the platelet purinergic receptors

Kenneth A. Jacobson · Francesca Deflorian ·
Shilpi Mishra · Stefano Costanzi

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Abstract Platelets contain at least five purinergic G protein-coupled receptors, e.g., the pro-aggregatory P2Y₁ and P2Y₁₂ receptors, a P2Y₁₄ receptor (GPR105) of unknown function, and anti-aggregatory A_{2A} and A_{2B} adenosine receptor (ARs), in addition to the ligand-gated P2X₁ ion channel. Probing the structure–activity relationships (SARs) of the P2X and P2Y receptors for extracellular nucleotides has resulted in numerous new agonist and antagonist ligands. Selective agents derived from known ligands and novel chemotypes can be used to help define the subtypes pharmacologically. Some of these agents have entered into clinical trials in spite of the challenges of drug development for these classes of receptors. The functional architecture of P2 receptors was extensively explored using mutagenesis and molecular modeling, which are useful tools in drug discovery. In general, novel drug delivery methods, prodrug approaches, allosteric modulation, and biased agonism would be desirable to overcome side effects that tend to occur even with receptor subtype-selective ligands. Detailed SAR analyses have been constructed for nucleotide and non-nucleotide ligands at the P2Y₁, P2Y₁₂, and P2Y₁₄ receptors. The thienopyridine antithrombotic drugs Clopidogrel and Prasugrel require enzymatic pre-activation *in vivo* and react irreversibly with the P2Y₁₂ receptor. There is much

pharmaceutical development activity aimed at identifying reversible P2Y₁₂ receptor antagonists. The screening of chemically diverse compound libraries has identified novel chemotypes that act as competitive, non-nucleotide antagonists of the P2Y₁ receptor or the P2Y₁₂ receptor, and antithrombotic properties of the structurally optimized analogues were demonstrated. *In silico* screening at the A_{2A} AR has identified antagonist molecules having novel chemotypes. Fluorescent and other reporter groups incorporated into ligands can enable new technology for receptor assays and imaging. The A_{2A} agonist CGS21680 and the P2Y₁ receptor antagonist MRS2500 were derivatized for covalent attachment to polyamidoamine dendrimeric carriers of MW 20,000, and the resulting multivalent conjugates inhibited ADP-promoted platelet aggregation. In conclusion, a wide range of new pharmacological tools is available to control platelet function by interacting with cell surface purine receptors.

Keywords Purines · GPCR · Ion channel · Structure–activity relationship · Molecular modeling · Mutagenesis

Introduction: subtypes of P2X and P2Y receptors

Extracellular nucleotides activate cell surface P2 receptors which are widely distributed and participate in the regulation of nearly every physiological process, including in the immune, inflammatory, cardiovascular, muscular, and central and peripheral nervous systems [1, 2]. Closely related to these processes are the adenosine receptors (ARs), all four subtypes of which are G protein-coupled receptors (GPCRs). The P2 receptors are divided into two distinct families: fast P2X receptors (ligand-gated ion channels) and P2Y receptors (GPCRs). The P2Y receptors

K. A. Jacobson (✉) · F. Deflorian · S. Mishra
Molecular Recognition Section, Laboratory of Bioorganic
Chemistry, National Institute of Diabetes and Digestive and
Kidney Diseases, National Institutes of Health,
Bldg. 8A, Rm. B1A-19, NIH, NIDDK, LBC,
Bethesda, MD 20892-0810, USA
e-mail: kajacobs@helix.nih.gov

S. Costanzi
Laboratory of Biological Modeling, National Institute of Diabetes
and Digestive and Kidney Diseases, National Institutes of Health,
Bethesda, MD 20892, USA

respond to various naturally occurring adenine and uracil mono- and dinucleotides. The P2X receptors are more structurally restrictive in native ligand selectivity than P2Y receptors and are activated principally by ATP. These extracellular nucleotides are produced in response to tissue stress and cell damage during neurotransmitter release and as a result of hemichannel formation. The concentration of extracellular nucleotides that act as P2 receptor agonists can vary dramatically depending also on the aforementioned circumstances. Thus, the state of activation of these receptors can be highly dependent on the stress conditions or disease states affecting a given organ or tissue.

The P2X receptors consist of seven subtypes that are numbered P2X1 through P2X7. Activation of P2X receptors leads to an influx of cations such as sodium and calcium, which depolarize excitable cells and activate cytosolic enzymes, respectively. The P2X7 receptor, in addition to forming the usual cation channel, and upon prolonged agonist exposure, also opens a large pore which can pass organic cations and dye molecules. The active ligand-gated ion channels of the P2X receptors are composed of trimeric aggregates of subunits. Both heterotrimers and homotrimers have been characterized [3], and the homotrimers and heterotrimers of a given subtype may have entirely different structural requirements for agonists or antagonists. For example, the P2X1 receptor can form heteromers with the P2X2, P2X4, and P2X5 receptors [4–7].

The eight human P2Y receptor subtypes are numbered P2Y₁, P2Y₂, P2Y₄, P2Y₆, P2Y₁₁, P2Y₁₂, P2Y₁₃, and P2Y₁₄, which leaves some gaps due to premature assignment of numbers to certain putative P2Y receptors that were later shown to be either species homologs or entirely different types of GPCRs. The structures of representative native adenine and uracil (1–4 and 23–25 in Fig. 1) nucleotides that activate P2Y receptors are shown, and each of the native nucleotides may activate one or more P2Y receptor subtypes. In some cases, the same nucleotide might activate one subtype and antagonize another. The adenine nucleotide ATP 2 is a full agonist at two human P2Y subtypes (P2Y₂ and P2Y₁₁ receptors), and the corresponding diphosphate ADP 1 activates three different subtypes (P2Y₁, P2Y₁₂, and P2Y₁₃ receptors). The uracil nucleotide UTP 24 activates two subtypes (P2Y₂ and P2Y₄ receptors), while UDP 23, previously thought to activate only a single subtype (P2Y₆ receptor), is also now known to activate P2Y₁₄ receptors along with the originally designated native agonist UDP-glucose 25 [8]. The naturally occurring dinucleotide Ap₃A 3 and its homologues, including uracil derivatives, also activate various P2 receptors [9, 10]. Efforts have been made to identify other naturally occurring nucleotides that interact with known P2Y receptors and to deorphanize related GPCR sequences that have not yet been assigned a native agonist [2, 11].

Platelets contain at least five purinergic GPCRs, e.g., the ADP-activated P2Y₁ and P2Y₁₂ receptors, the P2Y₁₄ receptor (pyrimidine-selective), and A_{2A} and A_{2B} ARs, in addition to the ligand-gated P2X1 ion channel (Table 1). Co-activation of P2Y₁ and P2Y₁₂ receptors is required for the aggregatory effect of ADP [12]. The P2Y₁₂ receptor is the site of action of the important thienopyridine antiplatelet drugs Clopidogrel 29 and Prasugrel 32 (Fig. 2). Activation of the P2X1 receptor by ATP is also pro-aggregatory, but only transiently and under high shear stress conditions [13]. Antagonists of the P2X1 receptor inhibit the platelet shape change induced by α,β -meATP 5 [80]. A P2Y₁₄ receptor was also detected in platelets, but its role is undetermined [14].

The first purine receptor detected in platelets was the G_s-coupled A_{2A} AR, at which adenosine has an anti-aggregatory function [15]. The SAR of AR ligands will be covered only briefly in this review. There are a number of excellent reviews of A_{2A} AR ligands [16, 17]. Recently, a novel role in platelet aggregation was proposed for the G_s-coupled A_{2B} AR [18], which is expressed at low levels in mouse platelets [19]. This subtype is upregulated in platelets under injury or stress conditions *in vivo*, and it downregulates the expression and function of the P2Y₁ receptor by raising cyclic AMP.

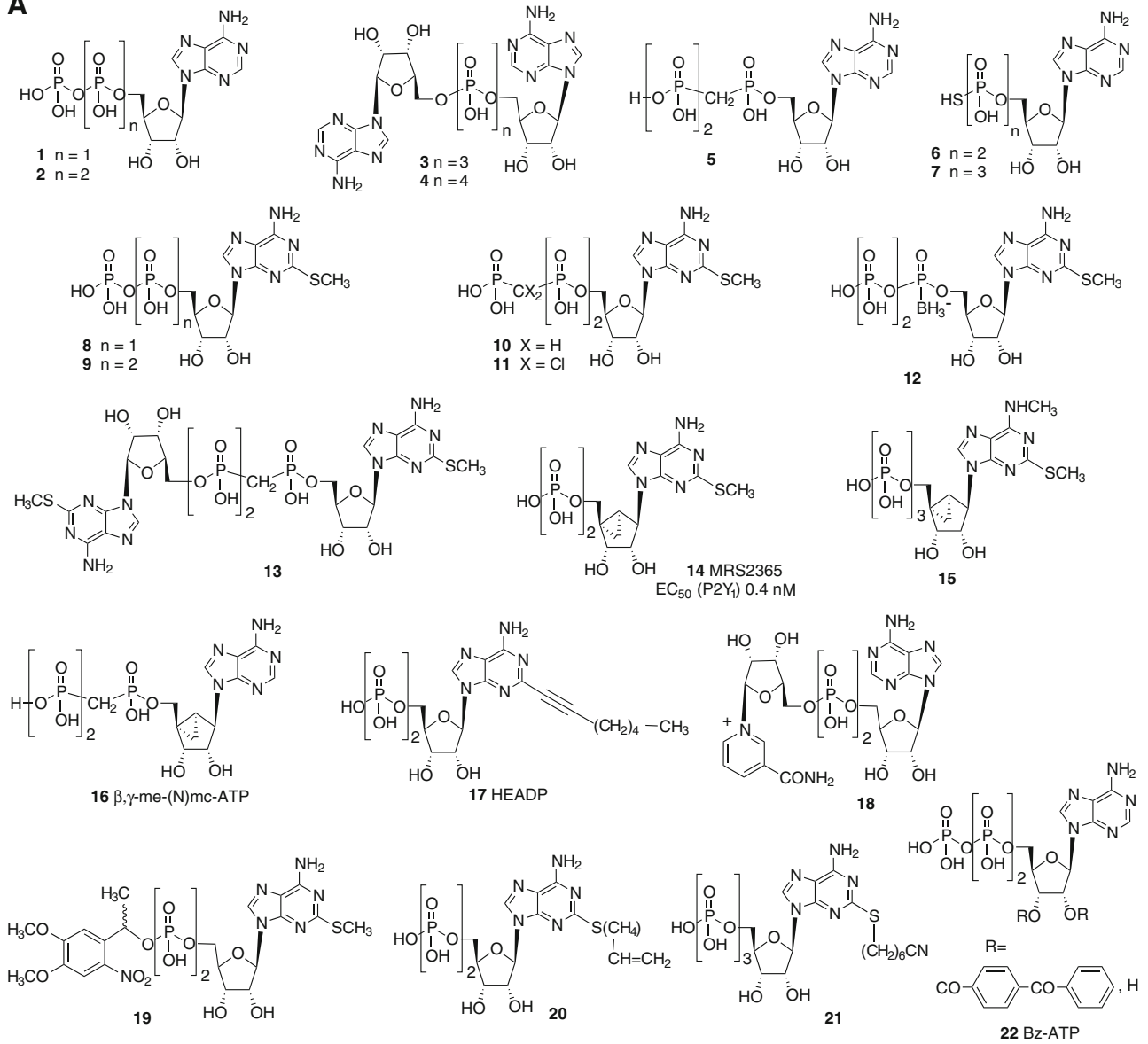
Structure and regulation of purine receptors that are expressed in platelets

The knowledge of P2X receptor structures was recently advanced with the X-ray crystallographic determination of the P2X4 subunit [20]. However, the crystal form did not contain a bound ligand, so predicting the orientation of ligands in the extracellular loop (EL) region of the P2X receptors is still subject to modeling. The X-ray structure of the P2X1 receptor in platelets has not yet been determined, but the structure–function relationships of various P2X subtypes have been probed using site-directed mutagenesis [21].

The structure, signaling, and regulation of P2Y receptors have been explored pharmacologically. Two subfamilies of P2Y₁-like (five members: P2Y₁, P2Y₂, P2Y₄, P2Y₆, and P2Y₁₁ receptors) and P2Y₁₂-like receptors (three members: P2Y₁₂, P2Y₁₃, and P2Y₁₄ receptors) have been defined. The P2Y₁ receptor was first cloned from chick brain in 1994 [22], and the platelet P2Y₁₂ receptor was first cloned in 2001 [23]. These subfamilies constitute two distinct groups based on signaling pathways and similarities in the function of key amino acid residues, but not on ligand structure [24, 25].

Fig. 1 Nucleotide derivatives that activate P2X and P2Y receptors, with emphasis on agonist ligands for studying these receptors in platelets. **a** Adenine nucleotides. **b** Uracil nucleotides. Phosphate derivatives would exist predominantly in an ionized form under physiological conditions

A



B

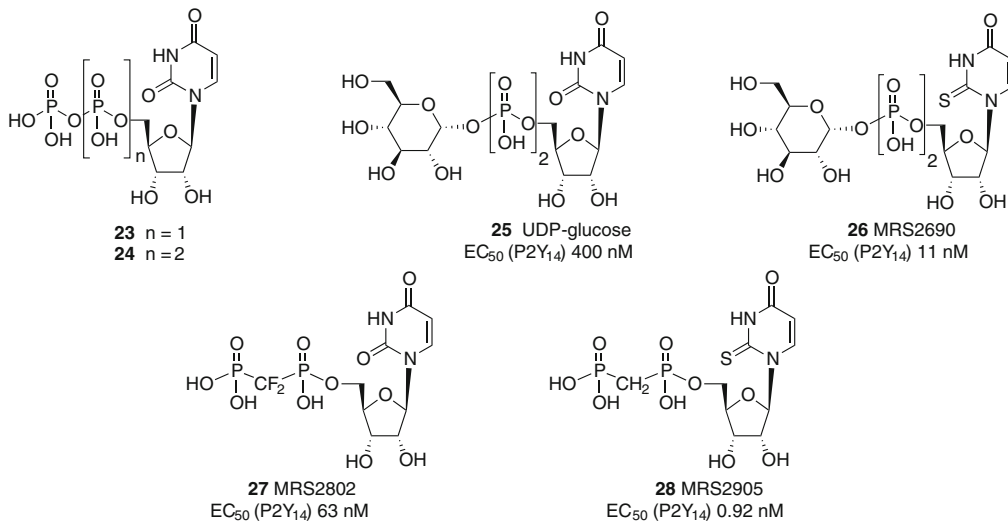
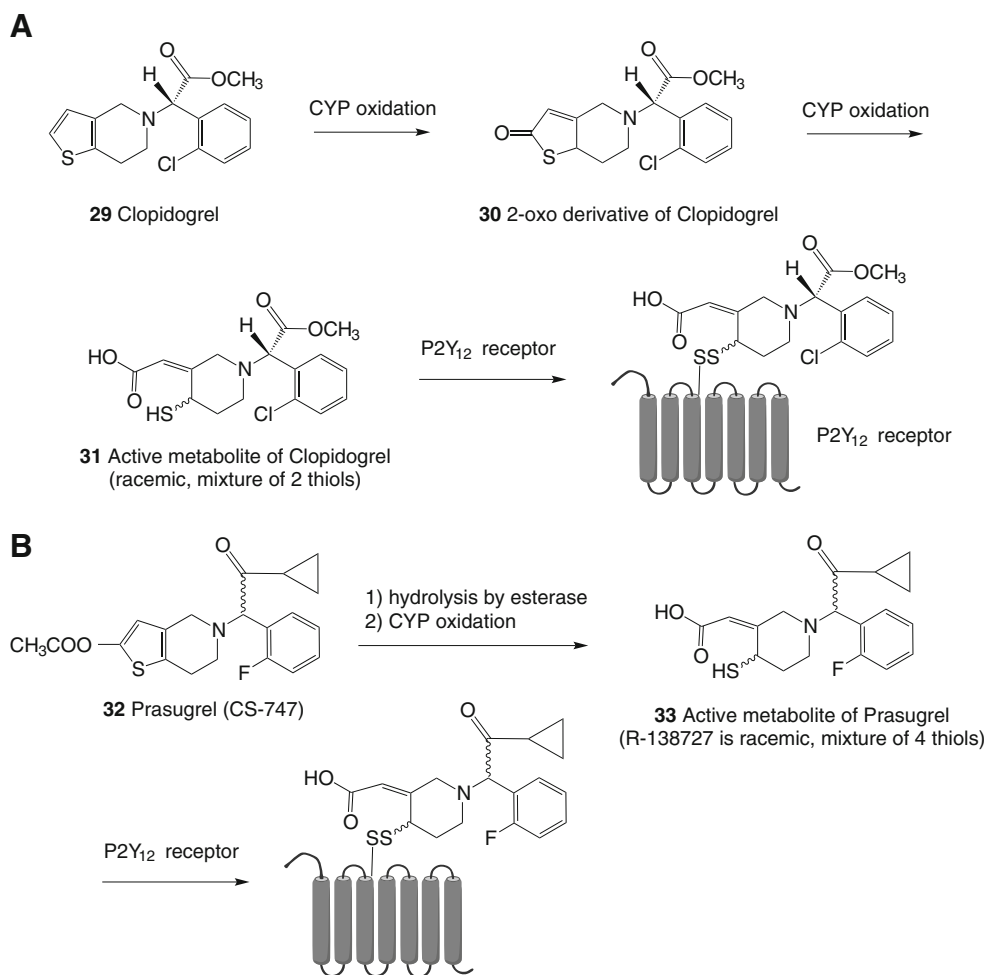


Table 1 Subtypes of platelet purine receptors and their representative ligands (potency at the human homologs, unless noted *r*=rat)

Receptor	Main distribution	Agonists (native in bold, pEC ₅₀)	Antagonists (pIC ₅₀)
P2Y₁	Brain, epithelial and endothelial cells, platelets, immune cells, osteoclasts	MRS2365 9.4 > 2-MeSADP 8.2 >>> ADPβS 7.0 > ADP 5.1 > ATP	MRS2500 9.0 > MRS2279 7.3 > MRS2179 6.5
P2Y₁₂	Platelets, brain (glial cells), microglial cells	2-MeSADP 7.9 ≥ ADP 7.2	AR-C69931MX 9.4 > AZD 6140 7.9, INS 50589 7.8 > RB2 7.6 (r) > 2-MeSAMP 4.0
P2Y₁₄	Placenta, mast cells, adipose tissue, stomach, intestine, discrete brain regions	MRS2905 8.7 > MRS2690 7.3 > UDP 6.8, UDP-glucose 6.5 > UDP-galactose 6.2	Compound 80 8.7
P2X₁	Smooth muscle, platelets, cerebellum, dorsal horn spinal neurons	BzATP 8.7 > ATP 7.3, 2-MeSATP 7.3, α,β-MeATP 6.7 (rapid desensitization) >> CTP 4.4	NF 449 9.5 > Ip ₅ I 8.8 > TNP-ATP 8.2 > Ro 0437626 > NF 279 7.7
A_{2A}	Striatum, platelets, lymphocytes	CGS21680 7.6 > adenosine 6.5	ZM241385 8.8 > SCH442416 8.4 > CSC 7.3 > theophylline 5.6 > caffeine 4.6
A_{2B}	Colon, fibroblasts, endothelial cells, astrocytes, bronchial smooth muscle, intestinal epithelial cells, mast cells, platelets	BAY 60-6583 8.0 > adenosine 4.8	PSB603 9.3 > MRS1754 8.7 > MRE2029-F20 8.3 > theophylline 5.0 > caffeine 4.5

**Fig. 2** Thienopyridines as non-nucleotide antagonists of the P2Y₁₂ receptor that require activation in vivo. Enzymatic formation of the active metabolites precedes the formation of the disulfide bond with the target P2Y₁₂ receptor on platelets. *CYP* cytochrome P450

Thus, both adenine and uracil nucleotides are found as agonists in each P2Y subfamily.

The signaling pathways of P2Y receptors have been extensively characterized. The preferential coupling of the first subfamily of P2Y₁, P2Y₂, P2Y₄, P2Y₆, and P2Y₁₁ receptors is to G_q, leading to the activation of phospholipase C β and to a rise in intracellular calcium. The P2Y₁₁ receptor alternately couples to G_s. The second subfamily of P2Y₁₂, P2Y₁₃, and P2Y₁₄ receptors couples to G_i, resulting in the inhibition of adenylyl cyclase to decrease production of cyclic AMP. Therefore, activation of the P2Y₁-like receptors leads to a rise in intracellular calcium. The amino acid residues R333 and R334 in the carboxy terminal region of the human P2Y₁ receptor were found using site-directed mutagenesis to be crucial for G_q coupling [26]. Activation of the P2Y₁ receptor can inhibit ion channels, such as the M-type K⁺ current in hippocampal neurons [27].

Desensitization of P2Y receptors in response to prolonged agonist exposure has been studied pharmacologically [28, 29]. In platelets, which express two ADP-responsive P2Y subtypes, the P2Y₁ receptor desensitizes more rapidly than the P2Y₁₂ receptor. The P2Y₁ receptor is desensitized mainly through protein kinase C-dependent processes, and the P2Y₁₂ receptor is a good substrate for the GPCR kinases leading to arrestin binding.

Factors that affect the localization of P2Y receptors within the cell have been studied. In kidney cells, the role of residues on the cytosolic C-terminal domain of the P2Y₁ receptor in basolateral sorting has been probed [30]. Deletion of this sorting signal that contains nine charged residues or alteration of the total number of charges caused a redirection of the receptor to the apical membrane. A P2Y₁-like receptor response in calcium transport has been detected on mitochondrial membranes [31].

Dimerization of GPCRs is a general phenomenon that can lead to major effects on the pharmacology of a given receptor subtype. Homodimerization of the P2Y₁ receptor expressed in human embryonic kidney (HEK)-293 cells was detected using fluorescence resonance energy transfer donor photobleaching analysis [32]. In membranes prior to agonist exposure, 44% of the P2Y₁ receptors existed in the dimeric state; upon exposure to agonist, a reversible rise to 85–100% dimerization was observed. Both monomeric P2Y₁ receptors and constitutive dimers were fully active. The terminal 19 amino acids of the cytosolic terminal region were essential for both dimerization and internalization, but activation by agonists was not reduced until >39 amino acids were removed from this region. Various heterodimers of P2Y receptors with other P2Y and non-P2Y GPCRs have been proposed. For example, a dimer of the A₁ AR and the P2Y₁ receptor was characterized [33]. The internalization of the P2Y₁₁ receptor is dependent on the co-expression of the rapidly desensitizing P2Y₁ recep-

tor, suggesting the occurrence of P2Y₁/P2Y₁₁ receptor heterodimers [34].

Molecular recognition in the P2Y₁ and P2Y₁₂ receptors, i.e., ligand binding and activation functions, has been extensively explored using mutagenesis [35–37]. Homology modeling of the P2Y receptors based on bovine rhodopsin or one of the more recent template structures followed by small molecule docking has provided insight into the possible ligand-binding modes. Comparisons of the structural characteristics and functionally important amino acid residues within the family have been described. Molecular models of these two subtypes are compared in Fig. 3. In each of the subfamilies, specific cationic, anionic, aromatic, and other residues in the helical transmembrane (TM) region and on the ELs have conserved functions in coordinating the bound nucleotide [25].

The homology model of the P2Y₁ receptor has been constructed using a two-template strategy, as described in de Castro et al. [38]. In particular, our experimentally supported rhodopsin-based P2Y₁ homology model [24] was used for the construction of almost the entire receptor, while the newly reported A_{2A} adenosine receptor structure (PDB ID: 3EML) [39] was used as the template for the conformation of the portion of second extracellular loop (EL2) downstream of C202, the conserved Cys that links EL2 to the third TM domain (TM3). The remaining portion of the loop, for which the published crystal structures of GPCRs suggest a greater variability among the receptors, was instead modeled without the use of a template. By using this approach, we obtained a model that closely resembles our previous one [24], but with a more solvent-exposed EL2. Receptor–ligand interactions were refined through Monte Carlo conformational searches, starting from our published experimentally supported binding modes [24].

Mutagenesis of the P2Y₁ receptor has concentrated on the identification of residues involved in reversible binding of agonist and antagonist ligands and its regulation. Figure 3a, b shows the human P2Y₁ receptor in complex with a selective antagonist (MRS2500) and a selective agonist ((*N*)-methanocarpa-2-MeSADP, MRS2365), respectively. The residues shown are those that, when mutated, lead to a decrease of potency of 20 times or higher of nucleotide agonists. The main differences between models of the antagonist-bound and agonist-bound complexes are shown. When the agonist is bound, K280 rotates counter-clockwise and the salt bridge between R128 and D204 breaks. The P2Y₁ homology model indicated that the ribose moiety of nucleotide ligands was situated in a hydrophilic pocket between TM3 and TM6. The studies also indicated that the adenine ring of the ligands interacts with residues from TM7 and points in the direction of TM1. Q307(7.36) (using Ballesteros numbering for each TM) [40] was found

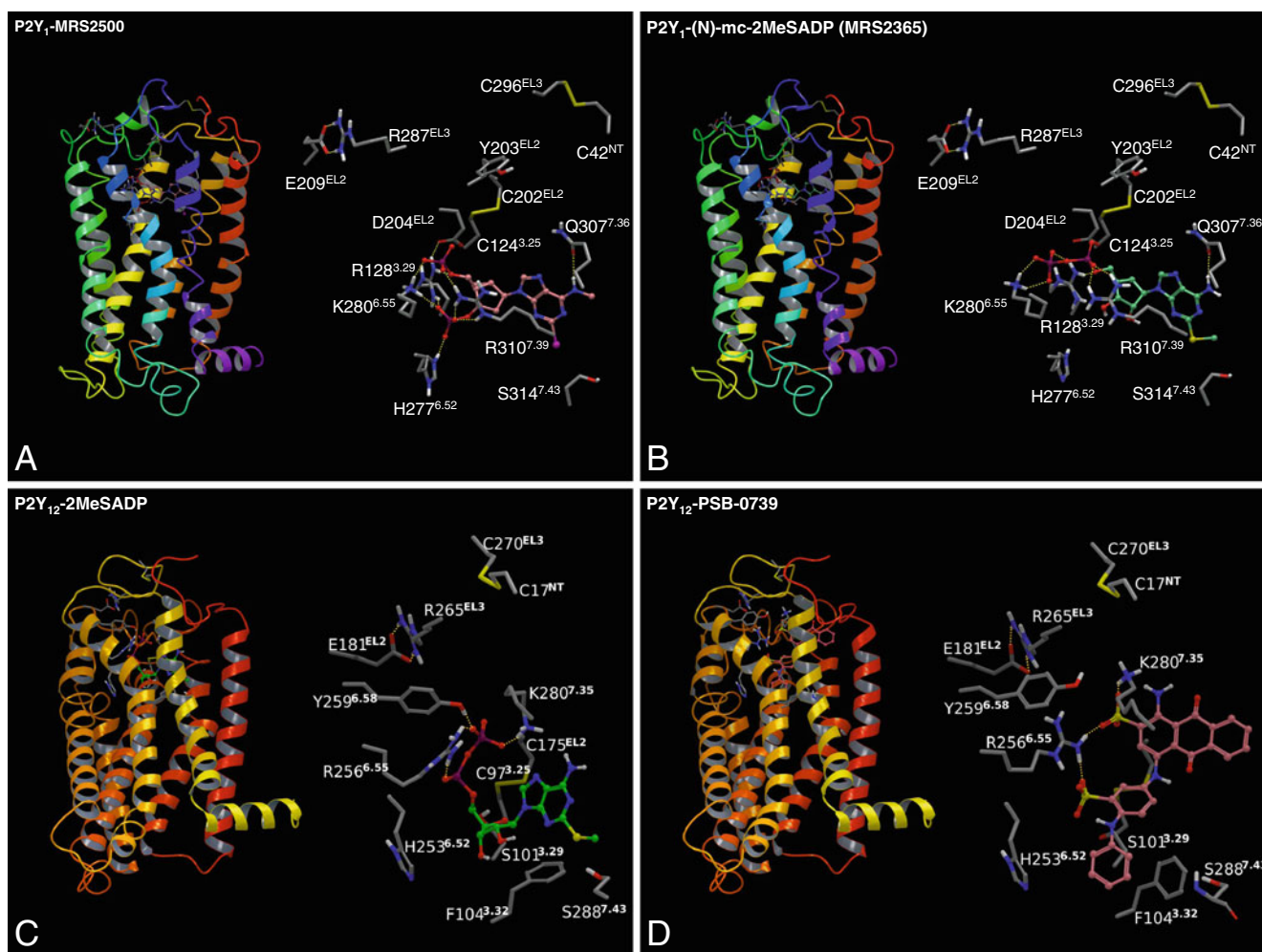


Fig. 3 **a, b** Molecular model of the human P2Y₁ receptor based on the structure of the β_2 -adrenergic receptor. The P2Y₁ receptor is shown in complex with a selective antagonist (MRS2500) (**b**) and a selective agonist (MRS2365) (**a**). The residues shown are those that, when mutated, lead to a decrease of potency of 20 times or higher. The helices are color coded as TM1 (red), TM2 (orange), TM3 (yellow), TM4 (light green), TM5 (dark green), TM6 (cyan), and TM7 (purple). **c, d** Molecular model of the human P2Y₁₂ receptor based on the structure of the A_{2A} adenosine receptor. The P2Y₁₂ receptor is shown in complex with a nonselective agonist (2-MeSADP) (**c**) and a

selective non-nucleotide antagonist (PSB-0739) (**d**). The helices are color-coded in a progression from TM1 (red) to TM7 (yellow). Two disulfide bridges and a salt bridge give rigidity to the extracellular domains of the P2Y₁ and P2Y₁₂ receptors. In the antagonist-bound state of the P2Y₁ receptor, a salt-bridge between R128 and D204 provides an additional link between TM3 and EL2. Our models suggest that agonist binding causes a disruption of this additional bridge as well as a counter-clockwise rotation (when observed from the extracellular side) of Lys280(6.55). Molecular modeling of these receptors was reported previously [24, 25, 35, 36, 38, 40, 41, 67, 89, 117]

to form a critical H-bond with the N⁶-amino group for both agonists and antagonists. The three critical cationic residues, i.e., R128(3.29), K280(6.55), and R310(7.39), appeared to form ionic interactions with both the bisphosphate chains of the antagonist and the diphosphate chain of the agonists. In particular, in the antagonist-bound complex, R128 interacted with the 5'-phosphate, K280 with both the 3' and the 5'-phosphates, and R310 with the 3'-phosphate. Two of these interactions underwent significant rearrangement upon agonist docking. In particular, R128 interacted with the α - and β -phosphates, causing the disruption of the salt bridge with D204 (in EL2) that was found in the antagonist-bound complex,

while K280 interacted with the β -phosphate, undergoing a significant rotation in the counterclockwise direction, when observed from the extracellular side. Instead, R310 underwent only a minor movement to interact with the α -phosphate group.

Mutagenesis of the P2Y₁₂ receptor has concentrated on the identification of residues involved in binding of agonist and antagonist ligands, covalent binding of thiol-reactive ligands, and desensitization. The residues R256(6.55), Y259(6.58), and, possibly, H253(6.52), as well as K280(7.35), are required for the activation of the human P2Y₁₂ receptor [37]. R256(6.55) is involved in the recognition of nucleotide

agonists and the non-nucleotide antagonist Reactive Blue-2, but not the nucleotide antagonist AR-C69931MX (Cangrelor). Hoffmann et al. [41] studied the recognition of the competitive non-nucleotide antagonist PSB-0739 at the human P2Y₁₂ receptor. The residue R256 is involved in the interaction of this and other antagonists derived from Reactive Blue-2 with the human P2Y₁₂ receptor.

The mutations F104S and S288P significantly increased agonist-induced receptor function without affecting the inhibition by AR-C69931MX [42]. R256 in TM6 and R265 in EL3 are more important for antagonist recognition than for agonist-induced activation. Compared to the wild-type P2Y₁₂ receptor, R256T/Q and/or R265W mutations, which have been detected in a patient with congenital bleeding, significantly increased the sensitivity to AR-C69931MX. Both the cytosolic side of TM3 and the exofacial side of TM5 are critical for P2Y₁₂ receptor function, which differs from the P2Y₁ receptor. R256 in TM6 and R265 in EL3 appear to play a role in antagonist recognition rather than receptor activation.

Figure 3c, d shows the human P2Y₁₂ receptor complexes with the agonist 2-MeSADP and the selective and competitive antagonist PSB-0739. Amino acid residues that were mutated corresponding to those in Fig. 3a, b are shown [43]. The three-dimensional structure of P2Y₁₂ was built by means of comparative modeling using the crystal structure of the A_{2A} adenosine receptor [39] as the template. The disulfide bridge between the N terminal and EL3, which is conserved in the P2Y family but is absent in the A_{2A} receptor structure, was created on the P2Y₁₂ model between C17 and C270. The extracellular loops were further refined. Molecular docking and Monte Carlo conformational studies were conducted with agonists and antagonists of the P2Y₁₂ receptor to understand and possibly to explain the involvement of the residues in the binding site of P2Y₁₂ receptor in the ligand recognition process.

The binding pocket for agonists and antagonists within the P2Y₁₂ receptor model is formed by residues located in TM1, TM2, TM3, TM6, and TM7. The negatively charged groups of 2-MeSADP and PSB-0739 are located in the extracellular part of the binding cavity of the P2Y₁₂ receptor, and they are surrounded by positively charged residues, including R256 in TM6 and K280 in TM7, and polar residues, including Y259 in TM6. In the complex with 2-MeSADP, the guanidinium group of R256 interacts with both phosphate groups of the agonist. The hydroxyl group of Y259 contributes to coordinate the α -phosphate group, while K280 binds to the β -phosphate group of 2-MeSADP. The residue S288(7.43) in TM7 engages in a favorable interaction with the thiomethyl group of 2-MeSADP at the bottom of the binding pocket. In the complex with the selective antagonist PSB-0739, R256 interacts directly with both the sulfonic acid groups. The

sulfonic group attached to the anthraquinone moiety interacts also with the side chain of K280. The side chain of Y259 is in proximity to the ligand, but does not interact directly with the negatively charged groups. The bottom of the binding pocket of the P2Y₁₂ receptor is characterized by a network of aromatic residues, among which are the residues H253(6.52) and F104(3.32), that embed the agonist and the antagonist molecules with favorable interactions. The S101(3.29) side chain is located in the binding pocket close to the ligands, but it is not engaged in direct interactions with either 2-MeSADP or PSB-0739.

In a direct analogy to an ionic bridge between EL2 and EL3 detected in the P2Y₁ receptor [35, 36], the P2Y₁₂ modeling suggested an ionic interaction between E181 in EL2 and R265 in EL3 that is present in both the agonist-bound and the antagonist-bound complexes. However, the question of a bridge between EL2 and TM3 is inconclusive because position 3.29 in the P2Y₁₂ receptor is S101, not an Arg as in the P2Y₁ receptor, and in EL3, the residue F177 occupies the position of D204 of the P2Y₁ receptor.

Chimeric constructs of P2Y receptors have helped establish the basis for ligand recognition, activation, and regulation. For example, a chimeric hemagglutinin-tagged human P2Y₁₂ receptor in which the cytosolic carboxy terminal region was replaced by the corresponding sequence of the P2Y₁ receptor exhibited a high level of constitutive activity [44].

Ding et al. [45] compared the differential reactivity of P2Y₁ and P2Y₁₂ receptors toward thiol reagents. Although both subtypes are encoded on the same chromosome and have similar agonist activation profiles, the thiol-reactive *p*-chloromercuribenzene sulfonic acid and the irreversibly binding metabolites (e.g., **31** and **33**) of the antiplatelet drugs Clopidogrel (Plavix[®]) **29** and Prasugrel (Effient[®]) **32** (Fig. 2) inactivated the P2Y₁₂, but not the P2Y₁ receptor. The two enantiomers of Prasugrel are similar in activity and also rapidly racemize; therefore, it is used in its racemic form. The interaction of these thiol-reactive metabolites with specific Cys residues on the human P2Y₁₂ receptor was probed by site-directed mutagenesis. There are four Cys residues in the extracellular region of the P2Y₁₂ receptor—at positions 17, 97, 175, and 270—which presumably form two disulfide pairs. It was speculated that the active metabolites of the thienopyridines **31** and **33**, which themselves are reactive thiols, formed disulfide bridges in this extracellular region, thus inactivating the receptor. The sites of covalent modification of the P2Y₁₂ receptor were suggested to be at C17 and C270 in the N-terminal domain and in EL3, respectively [45]. Algaier et al. [46] reached different conclusions about which Cys residues of the ELs of the P2Y₁₂ receptor are involved in the thiol reactivity of the active metabolite of Prasugrel, R-

138727 (2-[1-[2-cyclopropyl-1-(2-fluorophenyl)-2-oxoethyl]-4-mercapto-3-piperidinylidene]acetic acid, mixture of 4 stereoisomers) **33** [47, 48]. The mixture of (R,S) and (S,R) isomers, which is more potent than R-138727, is designated R-99224. Irreversible antagonism by R-138727 of the inhibition of forskolin-stimulated cyclic AMP production mediated by 2-MeSADP **8** was observed for both the wild-type P2Y₁₂ receptor and C17A/C270A mutant receptors. This eliminated C17 and C270 as sites for covalent modification. However, C97A and C175A constructs lost the ability to be inhibited by R-138727. These proposed sites of action of R-138727 at C97 (top of TM3) and C175 (EL2) would be closer to the ligand-binding site in the TM region of the P2Y₁₂ receptor than those thiol sites proposed by Ding et al. [45].

Mutagenesis and molecular modeling of A_{2A} AR has concentrated on the identification of residues involved in the binding of agonist and antagonist ligands [49, 50]. The X-ray structure of A_{2A} AR was reported in 2008 [39]. This advance has enabled the structure-based discovery of novel ligands of this GPCR independently by Abagyan and coworkers and by Shoichet and coworkers [51, 52]. In silico screening based on virtual docking at the A_{2A} AR of molecules that distantly resemble other AR ligands has identified antagonist molecules having novel chemotypes. Fluorescent and other reporter groups incorporated into high-affinity ligands can enable new technology for receptor assay and imaging. A fluorescence polarization assay of binding at the A_{2A} AR in HEK-293 cell membranes was reported [53]. This could serve as a model for the development of fluorescence polarization assays for other purine receptors that avoid the use of radioisotopes in ligand screening. The assay can be performed in real time in a well plate format.

Macromolecular conjugates of ligands for platelet purine receptors were recently introduced. The A_{2A} agonist CGS21680 (2-[p-(2-carboxyethyl)phenyl-ethylamino]-5'-N-ethylcarboxamidoadenosine) and the P2Y₁ receptor antagonist 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) **38** were derivatized for covalent attachment to (polyamidoamine) dendrimeric carriers of MW 20,000, and the resulting multivalent conjugates inhibited ADP-promoted human platelet aggregation [38, 54]. High affinity at the receptor was maintained in the A_{2A} AR agonist conjugates, but the multivalent conjugate of MRS2500 **38** was less potent than the parent antagonist monomer. Structural modification of these dendrimer conjugates is in progress to improve their pharmacological properties.

The mouse P2X₁, human P2Y₁, and human P2Y₁₂ receptors have all been purified to homogeneity using combinations of affinity chromatography and other column purification [55–57].

Selective ligands for the P2Y and P2X receptors in platelets

New selective agonists and antagonists have recently been identified for the P2 receptors that occur in platelets. The structures of representative nucleotide (**34–54** in Fig. 4) and non-nucleotide (**55–81** in Figs. 5 and 6) antagonists of the platelet P2 receptors are shown. Selective antagonist ligands for these receptors have been reported as a result of the systematic conversion of agonists into antagonists, the careful structural modification of known non-selective ligands, and, more recently, the screening of structurally diverse chemical libraries.

A recurrent issue in the use of typical P2 receptor ligands is the lack of specificity for a single subtype among multiple P2 receptors. Often, the complete P2 receptor selectivity profile of a given ligand is unknown. Also, many of the ligands display low bioavailability due to high molecular weight or multiple charged groups, such as phosphates and sulfonates, present in the molecules. Another drawback of many of the currently used ligands is their lability in biological systems. The use of P2 receptor ligands is also complicated by the presence of ectonucleotidases that degrade both native agonists and, often, the synthetic nucleotides that are used as agonists or antagonists [58]. Adenine nucleotides are progressively converted enzymatically to AMP and finally to adenosine, which activates its own family of four ARs. Selective inhibitors of ectonucleotidases that can serve as modulators of receptor function are being explored [59, 60]. Moreover, many P2 receptor agonists and antagonists are known to inhibit ectonucleotidases at comparable concentrations. The transformation of nucleotides can also proceed in the other direction, for example in the conversion of 5'-diphosphates to 5'-triphosphates by extracellular nucleoside diphosphokinase [61]. The purity of commercial preparations of nucleotides isolated from natural sources is variable. Thus, ATP might contain ADP and other nucleotides as contaminants. The use of synthetic and rigorously purified nucleotides minimizes this ambiguity. Finally, many of the early P2 antagonists have been found to interact intracellularly with other signaling mediators, such as G proteins at concentrations similar to those needed at P2 receptors. For example, suramin and its analogues inhibit G proteins [62]. Recently, a known P2X₁-selective antagonist (NF449, **64**) was found to inhibit signaling from the fibroblast growth factor receptor 3 [63]. In general, novel drug delivery methods, prodrug approaches, allosteric modulation, and biased agonism would be desirable to overcome side effects that tend to occur even with receptor subtype-selective ligands.

Generally, radioligand binding serves as a primary research tool for the screening of new ligands at a given

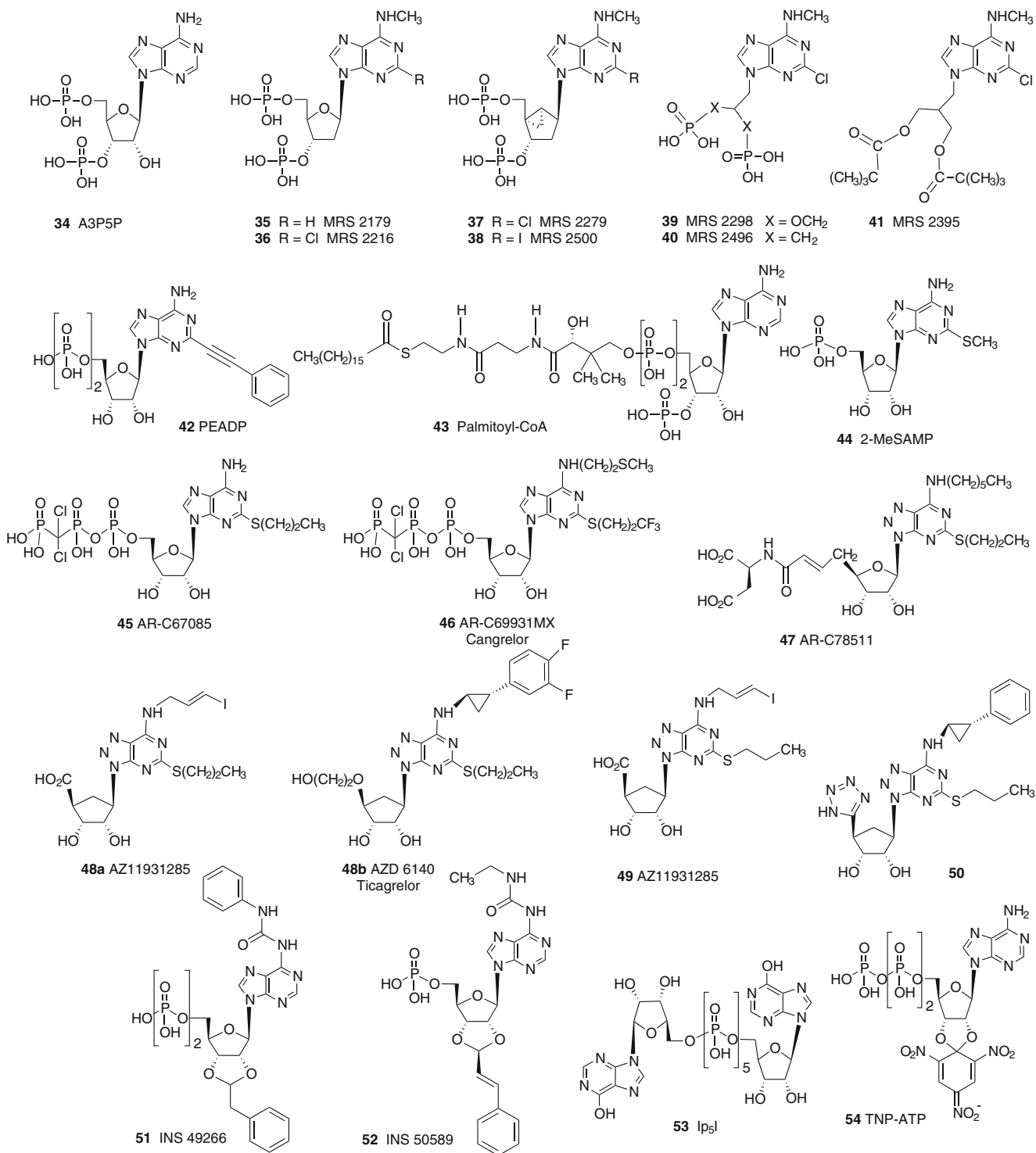


Fig. 4 Nucleotide derivatives that have been useful as antagonists in the study of the P2X and P2Y receptors in platelets. Phosphate derivatives would exist predominantly in an ionized form under physiological conditions

GPCR, but most of the P2 receptors do not have viable radioligands yet. Fortunately, the platelet purine receptors, i.e., P2Y₁, P2Y₁₂, P2X₁, A_{2A}, and A_{2B} receptors, have such high-affinity radioligand tools [17, 64–67]. Nevertheless,

much of the drug discovery at these subtypes has relied on functional assays, for example, the activation of phospholipase C by nucleotides acting at the P2Y₁ receptor expressed in 1321N1 astrocytoma cells [68].

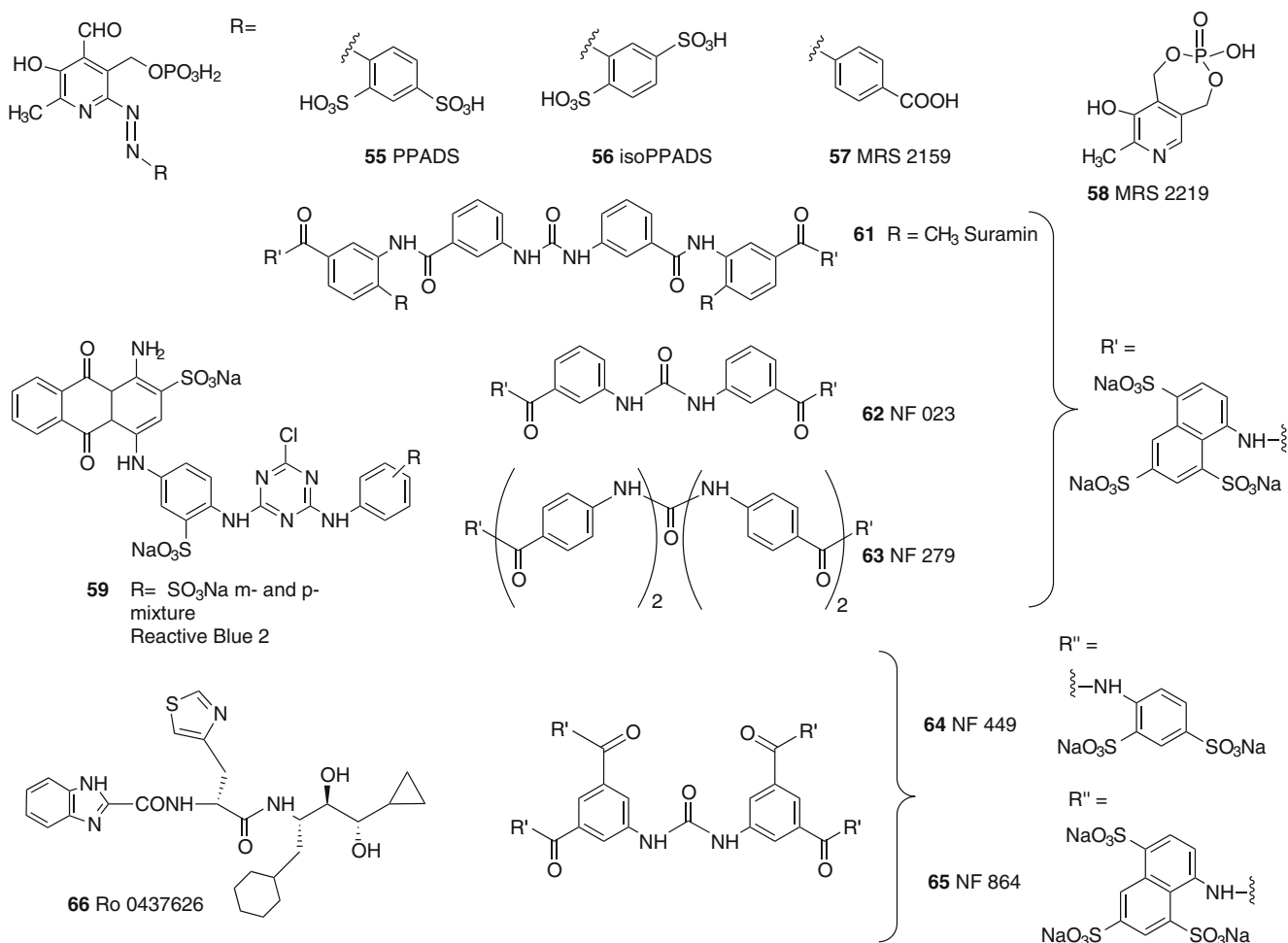


Fig. 5 Non-nucleotides antagonists: Nonselective antagonists and their derivatives and P2X₁ selective antagonists. Phosphate, sulfonate, and carboxylate derivatives would exist predominantly in ionized forms under physiological conditions

P2X₁ receptors

Novel P2X receptor ligands have been introduced for use as pharmacological probes and as potential therapeutic agents. Selective P2X receptor antagonists are of interest in pain control, depression, urinary incontinence, rheumatoid arthritis, chronic inflammation, and other conditions [69].

Non-selective P2X ligands

ATP **2**, but not UTP **24**, activates P2X receptors, and its EC₅₀ at the various subtypes varies from the low nanomolar to the high micromolar [70]. Purified ADP **1**, AMP, and adenosine are inactive at P2X receptors. 2-Methylthioadenosine 5'-triphosphate (2-MeSATP, **9**) is a potent agonist at multiple P2X receptors, for example, P2X₁ (EC₅₀=54 nM) and P2X₃ (EC₅₀=350 nM) receptors. α,β -Methyleneadenosine 5'-triphosphate (α,β -meATP, **5**) activates the P2X₁ receptor, but not the P2X₂ receptor, and its ability to rapidly desensitize the P2X₁ receptor allows it to

be used in functional pharmacological experiments in place of an antagonist. A wide variety of ATP derivatives were compared in the inhibition of specific binding of [³H] α,β -meATP at the rat urinary bladder P2X₁ receptor [66]. Among analogues with a substituted adenine ring, 2-(6-cyanoethylthio)-ATP **21** displayed a potent pIC₅₀ value of 7.24. Various nucleotide derivatives were assayed functionally at P2X receptors expressed in astrocytoma cells and oocytes [71, 72]. For example, 2', 3'-O-(4-benzoylbenzoyl) adenosine 5'-triphosphate (BzATP, **22**), which is the most potent known agonist of the P2X₇ receptor, was also found to potently activate the P2X₁ receptor. The same nucleotide also acts as an antagonist of the P2Y₁₂ receptor with an IC₅₀ of 116 μ M [45].

Until recently, the only antagonists of the P2X₁ receptor were highly charged compounds. Various negatively charged organic dyes, such as Reactive Blue-2 **59** (Fig. 5), act as non-selective and weak P2X antagonists. Reactive Blue-2 was also shown to be a potent antagonist of the P2Y₁₂ receptor with a pK_B of 7.6 [73]. The

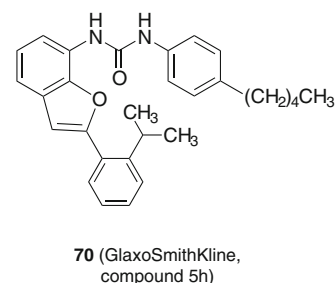
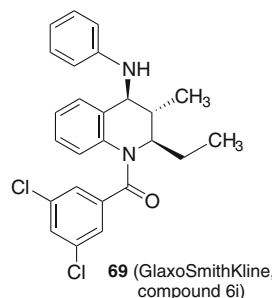
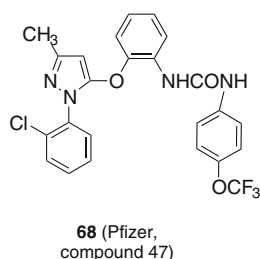
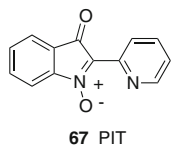
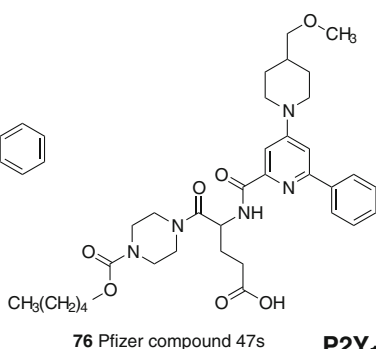
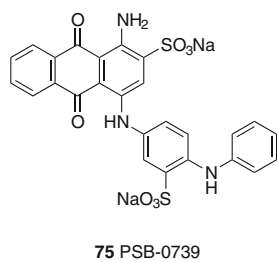
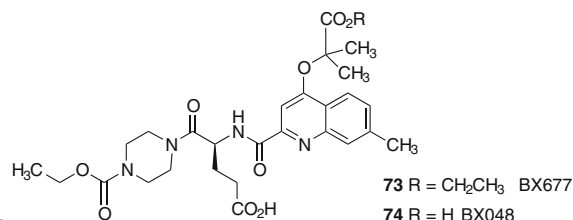
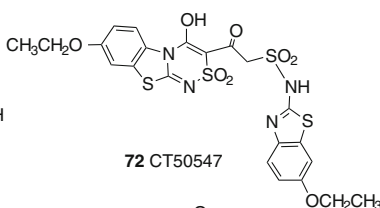
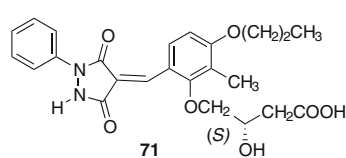
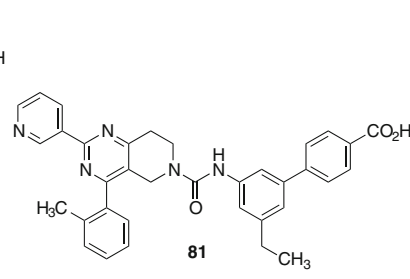
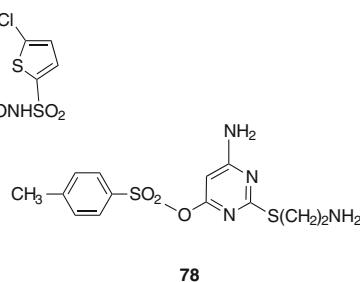
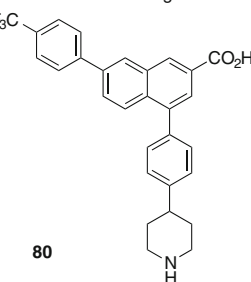
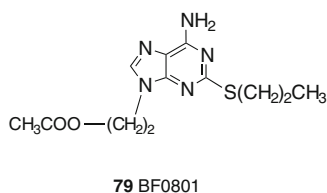
P2Y₁P2Y₁₂P2Y₁₄

Fig. 6 Selective non-nucleotides that have been useful antagonists in the study of P2Y receptors in platelets. See Fig. 2 for thienopyridine antagonists of the P2Y₁₂ receptor. Sulfonate and carboxylate derivatives would exist predominantly in ionized forms under physiological conditions

polysulfonated biphenyl derivative Evans Blue (structure not shown) acts as a P2X receptor antagonist, but it also affects other channels and amino acid binding sites [74]. Another polysulfonated derivative that has been widely used as a nonselective P2 receptor antagonist is the antiparasitic drug suramin **61**. The aryldiazo-bridged pyridoxal phosphate derivative PPADS **55** was found to inhibit P2 receptors, and its potency and selectivity have been increased by chemical modification [75]. Typically, such compounds are relatively non-subtype-selective P2X antagonists that also block some P2Y subtypes [76]. Later, a positional isomer, iso-PPADS **56**, was introduced and found to be more potent than PPADS at P2X receptors. The *p*-carboxylate analogue MRS2159 **57** is somewhat selective for the P2X₁ receptor, but also antagonizes the P2X₃

receptor. The pyridoxal phosphate derivative lacking an aryldiazo moiety MRS2219 **58** is a weak potentiator of P2X₁-mediated responses [77].

A few nucleotide derivatives have been found to block P2X receptors. For example, the dinucleotide Ip₅I **53** potently antagonizes the P2X₁ receptor [78]. TNP-ATP **54** is a potent P2X antagonist that is selective for several subtypes [79]. It antagonizes P2X₁, P2X₃, and heteromeric P2X_{2/3} receptors with IC₅₀ values of 6, 0.9, and 7 nM, respectively, and displays 1,000-fold selectivity for homomeric P2X₃ over P2X₂, P2X₄, and P2X₇ receptors.

P2X₁ antagonists of greater selectivity have been reported in compound classes both related to the known, nonselective antagonists, and to novel chemotypes. For example, the suramin analogue NF 023 **62** is a moderately

selective, competitive P2X antagonist with IC_{50} values of 0.21 and 28.9 μ M at human P2X1 and P2X3 receptors, respectively, and is inactive at the P2X2 and P2X4 receptors [80]. Another analogue NF 157 (not shown) was found to be a P2X1 antagonist, but it is not absolutely specific because it also blocks the P2Y₁₁ receptor [81]. The P2X1 potency selectivity in the suramin series of antagonists improved in later structural iterations. Other suramin derivatives that act as selective P2X1 antagonists include PPNDs, NF 279 **63**, and the more highly selective P2X1 antagonist NF 449 **64** [82, 83]. An additional high-affinity antagonist in this series is NF864 **65** (8,8',8'',8'''-(carbonylbis(imino-5,1,3-benzenetriyl-bis(carbonylimino)))tetrakis-naphthalene-1,3,5-trisulfonic acid-dodecasodium salt). The ability to inhibit the platelet P2X1 receptor displayed the following order (pA₂ in shape change): NF864 (8.49) > NF449 (7.61) > or = NF110 (7.22) > NF023 (6.11) = MK-HU1 (5.98, structure not shown) = suramin (5.76) [84]. In the series of benzimidazole derivatives introduced by Roche, a 2-carboxamide derivative Ro 0437626 **66** was found to be a selective P2X1 antagonist (IC_{50} =3 μ M) that displays >30-fold selectivity over P2X2, P2X3, and P2X2/3 receptors (IC_{50} >100 μ M) [85].

P2Y₁, P2Y₁₂, and P2Y₁₄ receptors

Selective agonist and antagonist ligands for P2Y receptors are in preclinical development for pulmonary diseases, thrombosis, and other conditions [86]. The rapidly accelerating progress in this field has already resulted in new drug candidates for pulmonary diseases, dry eye disease, and thrombosis. There is much activity in the pharmaceutical industry to identify novel antagonists of the P2Y₁₂ receptor, with the success of the antithrombotic Clopidogrel **29**, which acts as a prodrug of an irreversibly binding P2Y₁₂ receptor antagonist [87]. The P2Y₁ receptor might also prove to be a useful target for antithrombotic drugs [88]. Detailed analyses of SAR have been performed on the pro-aggregatory P2Y₁ and P2Y₁₂ receptors, for which selective nucleotide ligands have been reported. Because nucleotide analogues generally have limited bioavailability and stability in vivo, selective non-nucleotide antagonists of the P2Y₁ and P2Y₁₂ receptors are also being developed as potential antithrombotic agents. Structurally diverse chemical libraries have been screened to identify novel chemotypes to act as competitive, non-nucleotide antagonists of these subtypes, which may be optimized for selectivity and potency.

P2Y₁ receptor

ADP activates the P2Y₁, P2Y₁₂, and P2Y₁₃ receptors and therefore is of limited use in characterizing subtype-specific effects. 2-MeSADP **8** (EC_{50} =6 nM) is a more potent agonist at the P2Y₁ receptor than ADP (EC_{50} =8 μ M). 2-

MeSATP **9** and its 2-thioether congeners have also been shown to activate the P2Y₁ receptor, although depending on conditions where 5'-triphosphates might appear to be less than full agonists. ATP, itself, has been characterized as an agonist, a partial agonist, or an antagonist at the P2Y₁ receptor, depending on the model used and the level of spare receptors expressed. *N*⁶-methyl adenine nucleotides also potently activate the P2Y₁ receptor, but larger substituents on the exocyclic amine reduce potency, consistent with a small hydrophobic pocket surrounding the *N*⁶-position within the binding site of the P2Y₁ receptor. For example, *N*⁶-(2-phenylethyl)-ATP is inactive at the P2Y₁ receptor [89]. *N*⁶-Disubstituted ATP derivatives are inactive at the P2Y₁ receptor and have found application as inhibitors of ectonucleotidases, such as ARL 67156 [59].

The conformation of the ribose moiety has been the focus of recent studies of P2 receptor agonists. By comparing two isomeric conformationally restricted (i.e., rigid) equivalents of the ribose moiety in nucleotide derivatives (i.e., methanocarba ring system containing fused cyclopropane and cyclopentane rings), the favored ribose ring conformation at the P2Y₁ receptor was established [90, 91]. The North (*N*)-methanocarba analog of 2-MeSADP (MRS2365, **14**) displayed an EC_{50} of 0.4 nM as an agonist of the P2Y₁ receptor [92] and did not activate appreciably the P2Y₁₂ and P2Y₁₃ receptors. Addition of the (*N*)-methanocarba ring system to *N*⁶-methyl-ATP in **15** enhanced its potency at the turkey P2Y₁ receptor by >200-fold [91]. The preference for the (*N*)-methanocarba over the isomeric (*S*)-methanocarba nucleotide analogues was also demonstrated for the activation of the P2X1 receptor [93]. However, this conformational preference does not apply to all of the P2Y receptors; the P2Y₁₂ receptor is not activated by the (*N*)-methanocarba analogue of 2-MeSADP **14** [92].

Extension of the 2-methylthio ether on adenine nucleotides to longer alkyl and arylalkyl chains was one of the first classes of favorable modifications identified to preserve or enhance P2Y₁ receptor potency. Extended groups such as the *p*-aminophenylethylthio ether were tolerated at platelet P2Y receptors [94, 95]. Thio ethers (RS-) were found to be superior P2Y₁ receptor agonists in comparison to the corresponding oxygen ethers (RO-) or amines (RNH-) at the 2 position [96].

The presence of a (*N*)-methanocarba ring system greatly improved the stability of the phosphate ester of the AMP derivative toward hydrolysis by an ectonucleotidase [91], and the phenomenon occurred to a lesser degree for ADP and ATP derivatives. Another means of improving hydrolytic stability is the introduction of a borano group within the phosphate moiety of P2Y receptor agonists [97]. Phosphonate groups have been included in the phosphate chain to increase the stability of the nucleotide analogues. β,γ -methylene-ATP **5** itself is inactive at the human P2Y₁ receptor, but when

combined with the conformationally constrained (*N*)-methanocarba modification, the resulting analogue **16** is a potent agonist at this subtype [91]. Analogues of 2-MeSATP **9** that contain either a β,γ -methylene modification **10** or an α -borano group **12** are more stable toward hydrolysis than **9** [98]. They activate the human P2Y₁ receptor with EC₅₀ values of 80 nM and 17.2 μ M, respectively, and are inactive or only weakly active at the P2Y₂, P2Y₄, and P2Y₆ receptors. 2-MeS- β,γ -CCl₂-ATP **11** activates the P2Y₁ receptor with an EC₅₀ value of 80 nM and is resistant to a 30-min treatment with alkaline phosphatase [99].

Adenine dinucleotides, specifically diadenosine polyphosphates, are naturally occurring in secretory granules of nerve terminals. In a series of dinucleotides of varying lengths (two to six phosphates), Ap₃A **3** was found to have the highest potency at the human P2Y₁ receptor expressed in 1321N1 astrocytoma cells (pEC₅₀=7.5), and the potency was similar to ADP [100]. The compound also displayed >1,000-fold selectivity over the human P2Y₂ receptor. However, Ap₃A was also reported to activate rat P2X₁ and P2X₃ receptors [9]. The homologue Ap₄A **4**, which is a constituent of platelet dense granules, was the most potent agonist in the series at the P2Y₂ receptor, but was inactive as an agonist at the P2Y₁ receptor. Chemically synthesized Ap₄A of high purity was recently found to inhibit human platelet aggregation by antagonizing the P2Y₁ receptor (fully) and the P2Y₁₂ receptor (partially) [101]. Ap₄A was also found to be a full agonist of the P2X₁ receptor and a partial agonist of the P2Y₁₂ receptor. However, a modified analogue, di-(2-MeS)-adenosine-5',5''-P¹,P⁴, α,β -methylene-tetraphosphate **13** was found to activate the human P2Y₁ receptor with an EC₅₀ value of 0.42 μ M and was 2.5-fold more stable in human blood serum than ATP, with a *t*_{1/2} of 12.1 h [102].

Other naturally occurring nucleotides have been found to interact with the P2Y₁ receptor. For example, ADP-ribose at micromolar concentrations can act as an endogenous agonist the P2Y₁ receptor [103]. Also, extracellular nicotinamide adenine dinucleotide (NAD⁺) **18** was shown to activate the P2Y₁ receptor to induce a rise in intracellular calcium ions in transfected astrocytoma cells (EC₅₀=743 nM, efficacy of 77%, compared to ADP) [104]. NAD⁺ contains a β -blocked diphosphate group. Beta-NAD has been shown to be an inhibitory neurotransmitter that activates P2Y₁ receptors in gastrointestinal smooth muscle [105]. Curiously, another blocked diphosphate derivative **19**, in which the β -phosphate of 2-MeSADP was masked as photoreversible 2-nitroveratryl ester, did not activate the P2Y₁ receptor until irradiated to free the 5'-diphosphate. This compound acted as a caged agonist of the P2Y₁ and P2Y₁₂ receptors for use as a tool for the light-directed facilitation of platelet aggregation [106].

Many nucleotide antagonists of the P2Y₁ receptor have been introduced (Fig. 4). The initial observation was that

adenosine 3',5'-bisphosphate (A3P5P, **34**) and its naturally occurring congeners acted as partial agonists or antagonists, respectively, at the turkey and human P2Y₁ receptors [107]. Various chemical modifications of adenine nucleotides containing bisphosphate groups, for example *N*⁶-methyl 2'-deoxyadenosine 3', 5'-bisphosphate MRS2179 **35** (p*K*_B=6.99), and its 2-chloro analog MRS2216 **36** (p*K*_i=6.69), provided potent and selective P2Y₁ antagonists [108]. [³³P]MRS2179 was studied as a radioligand of the P2Y₁ receptor [109]. C-nucleoside pyrazolo[1,5-*a*]-1,3,5-triazines were prepared, and their 3',5'-bisphosphate C-nucleotide analogues are stable in vivo as P2Y₁ receptor antagonists [110].

The same conformationally constrained (*N*)-methanocarba modification of the ribose moiety that enhanced agonist action in MRS2365 **14** also favored antagonist action in nucleotide bisphosphate derivatives. For example, the 2-chloro analogue MRS2279 **37** (p*K*_B=8.10) and the 2-iodo analogue MRS2500 **38** (p*K*_B=9) were selective, high-affinity antagonists of the P2Y₁ receptor [111]. MRS2500 effectively inhibited platelet aggregation in vivo in the mouse and other species [112, 113]. The (*N*)-methanocarba antagonist [³H]MRS2279 **37** was introduced as a radioligand for the P2Y₁ receptor [114]. The higher affinity antagonist MRS2500 **38** has been prepared as a radioligand for the P2Y₁ receptor both as a ³²P form and as a ¹²⁵I form [64, 115].

Although the steric constraint of the (*N*)-methanocarba ring greatly enhanced affinity at the P2Y₁ receptor, a cyclic form was not essential for P2Y₁ receptor antagonism [116]. The acyclic bisphosphate derivative MRS2298 **39** (IC₅₀=62.8 nM) and bisphosphonate derivative MRS2496 **40** (IC₅₀=1.5 μ M) were effective inhibitors of ADP-promoted platelet aggregation with intermediate potency [112].

Costanzi et al. [117] studied QSAR of antagonists of the P2Y₁ receptor based on ligand docking models and focusing on halo and alkynyl groups at the 2 position. Other alkynyl nucleotides were evaluated at the platelet P2Y receptors. The 5'-diphosphate derivative of 2-phenylethynyladenosine (PEADP, **42**) was found to interact mainly with the platelet P2Y₁ receptor as an antagonist, while the corresponding 2-hexynyladenosine derivative (HEADP, **17**) activated the platelet P2Y₁₂ receptor, but not the P2Y₁ receptor [118].

Thiol Coenzyme A (CoA-SH) and various drug-derived CoA derivatives antagonized the human P2Y₁, but not the P2Y₂, receptor expressed in *Xenopus laevis* oocytes [119]. Palmitoyl-CoA (16:0) **43** and CoA thioester derivatives of nafenopin and ciprofibrate, two clinically relevant hypolipidemic drugs, were more potent than CoA-SH as antagonists. This phenomenon was further studied using CoA derivatives with saturated acyl groups containing 16–18 carbons to influence the platelet aggregation and Ca²⁺ mobilization induced by various P2Y agonists [120]. Palmitoyl-CoA **43** was shown to act mainly as an antagonist of the P2Y₁

receptor but also as a partial antagonist at the P2Y₁₂ receptor. Not all inhibitors of the P2Y₁ receptor are competitive with the binding of nucleotides at the receptor. For example, pyridyl isatogen (PIT) **67** was found to be an allosteric modulator of the P2Y₁ receptor [121].

The screening of structurally diverse chemical libraries has helped identify lead compounds for the development of non-nucleotide antagonists of the P2Y₁ receptor (Fig. 6). For example, the urea derivative **68** is a selective and orally bioavailable antagonist of the human P2Y₁ receptor of novel chemotype with a K_i value of 90 nM [122]. Aminobenzazole derivatives from Bristol–Myers Squibb were reported as P2Y₁ receptor antagonists [123]. Other structurally diverse antagonists of the P2Y₁ receptor have been reported. Tetrahydro-4-quinolinamines such as **69** ($K_i=70$ nM) were found to be novel P2Y₁ receptor antagonists [124]. Recently, benzofuran-substituted urea derivatives such as **70** ($K_i=140$ nM) were reported as novel P2Y₁ receptor antagonists [125].

P2Y₁₂ receptors

ADP ($EC_{50}=69$ nM) and 2-MeSADP ($EC_{50}=0.3$ nM) are potent non-selective agonists at the platelet P2Y₁₂ receptor. [³³P]2-MeSADP was utilized as a radioligand of the P2Y₁ receptor [126]. Adenine nucleotides, including 5'-monophosphates, with extended 2-alkylthio groups were found to preserve or enhance the potency as agonists at the rat C6 glioma cell P2Y₁₂ receptor [43, 127]. For example, 2-(hexenylthio)-ADP **20** displayed a pEC_{50} value of 83 nM and selectivity over the P2Y₁ receptor of 80-fold.

The SAR of antagonists of the P2Y₁₂ receptor has been extensively explored, resulting in clinical agents. Thienopyridines, notably the blockbuster antiplatelet drug Clopidogrel **29** (Fig. 2), act as liver-activated prodrugs that are irreversible inhibitors of the P2Y₁₂ receptor [128]. In order to form the P2Y₁₂ receptor antagonist species, a two-step pre-activation in vivo is required, which delays onset of action of the drug and the time required for reversal of the platelet effect after drug administration is ceased. This pre-activation process also is subject to pharmacogenomic factors, which explain the variability of the population to be effectively treated by Clopidogrel. A patient's poor clinical response to Clopidogrel can be predicted using an in vitro platelet function assay [129]. A patient's response to Clopidogrel depends partly on factors that affect its metabolism through cytochrome P450 in the liver. The presence of a reduced-function CYP2C19*2 allele or the co-administration of the proton pump inhibitor omeprazole decreases the effectiveness of Clopidogrel, and cigarette smoking increases its pre-activation. Another thienopyridine antagonist that has been in clinical trials, Prasugrel **32** (CS-747, LY640315), is a more potent P2Y₁₂ antagonist than Clopidogrel and leads to a more complete

inhibition of platelet function, but it also displays a longer bleeding time. Prasugrel, with less genetic variability than Clopidogrel, requires only one step of pre-activation in vivo [130] to the active metabolite R-138727 **33**.

As discussed above, the action of the thienopyridines depends on the covalent reaction of an active metabolite with a thiol on the P2Y₁₂ receptor. However, directly acting and reversible P2Y₁₂ receptor antagonists, both nucleotides and non-nucleotides, have also been reported. ATP, itself, has been characterized as an antagonist at the P2Y₁₂ receptor, which has enabled the introduction of various 5'-triphosphate analogs as selective receptor probes and clinical candidates. Thus, the antithrombotic nucleotide derivatives from AstraZeneca, AR-C67085 **45** and 5'-adenylic acid, *N*-[2-(methylthio)ethyl]-2-[(3,3,3-trifluoropropyl)thio]-, monoanhydride with (dichloromethylene)bis [phosphonic acid] (AR-C69931MX, Cangrelor, **46**), have been tested clinically as antithrombotic agents [131, 132]. The EC_{50} values of these P2Y₁₂ receptor antagonists are 30 μ M and 0.4 nM, respectively. Note that these two P2Y₁₂ receptor antagonists, AR-C67085MX **45** and AR-C69931MX **46**, also activate the P2Y₁₁ receptor. Ding et al. [44] reported that (*E*)-*N*-[1-[7-(hexylamino)-5-(propylthio)-3H-1,2,3-triazolo-[4,5-d]-pyrimidin-3-yl]-1,5,6-trideoxy-beta-D-ribo-hept-5-enofuranonyl]-L-aspartic acid (AR-C78511, **47**) is an inverse agonist of the P2Y₁₂ receptor, but AR-C69931MX is a neutral antagonist. [³H]2-Propylthioadenosine-5'-adenylic acid (1,1-dichloro-1-phosphonomethyl-1-phosphonyl) anhydride ([³H]PSB-0413, which is the tritiated equivalent of AR-C67085MX), is a high-affinity antagonist radioligand of the P2Y₁₂ receptor [65].

A 5'-triphosphate group in adenine nucleotides is not strictly required for P2Y₁₂ receptor antagonists. The P2Y₁₂ receptor antagonists from Inspire Pharmaceuticals, INS 49266 **51** (an ADP derivative with EC_{50} of 52 nM) and INS 50589 **52** (an AMP derivative with EC_{50} of 11 nM) [133, 134], could be considered truncated derivatives of the proven 5'-triphosphate antagonists. The potent P2Y₁₂ receptor antagonist and clinical candidate AZD 6140 **48b** (Brilinta, Ticagrelor, $pIC_{50}=7.9$) is an uncharged cyclopentyltriazolopyrimidine analogue that was developed in an extensive SAR exploration by AstraZeneca [132, 135]. Like **47**, this nucleoside derivative contains a modified base, i.e., 8-azaadenine. The antithrombotic effects of **48b** compare favorably with the thienopyridines in rat and dog models and with less risk of bleeding, possibly due to its reversible receptor binding [136]. The receptor-binding properties and functional antagonism of **48b** are complex (competitive toward 2-MeSADP, but not ADP) [67]. A related carbocyclic nucleoside derivative [¹²⁵I](1*S*,2*R*,3*S*,4*R*)-2,3-dihydroxy-4-[7-[(2*E*)-3-iodoprop-2-en-1-yl]amino]-5-(propylthio)3H-[1-3]triazolo[4,5-*d*]pyrimidin-3-yl]cyclopentane-carboxylic acid ([¹²⁵I]AZ11931285) **48a** has been proven

useful as a high-affinity antagonist radioligand of the P2Y₁₂ receptor [67]. Other uncharged analogues of nucleotides that act as potent antagonists of the P2Y₁₂ receptor are carbocyclic nucleoside tetrazole derivatives, such as **50** [137]. An uncharged acyclic adenine diester derivative MRS2395 **41** acted as a weak but selective antagonist of the P2Y₁₂ receptor (IC₅₀=3.6 μM, rat) [116]. This is in contrast to **39** and **40** which contain the same acyclic 9-alkyladenine scaffold and interact only with the P2Y₁ receptor as antagonists.

Library screening has identified novel chemotypes as antagonists of the P2Y₁₂ receptor (Fig. 6). For example, Elinogrel (PRT-128, **77**) [138], a competitive and reversible antagonist with an IC₅₀ value of 20 nM at the P2Y₁₂ receptor, is being developed as an antithrombotic agent by Portola Pharmaceuticals. Earlier, P2Y₁₂ receptor antagonists consisting of pyrazolidine-3,5-dione derivatives including **71** were reported in an abstract [139]. Tricyclic benzothiazolo[2,3-*c*]thiadiazine antagonists of the P2Y₁₂ such as CT50547 **72** (pEC₅₀=6.74, also called C1330-7) were reported [140]. An ester derivative BX 667 **73** and the corresponding free carboxylic acid BX 048 **74** (binding IC₅₀ values of 29 and 5.3 nM, respectively), which are derivatives of L-glutamic acid, reversibly inhibit the binding and functional effects of 2-MeSADP in platelets of several species [141, 142]. The functional selectivity of BX 667 and BX 048 for the P2Y₁₂ receptor in comparison to P2Y₁ and P2Y₆ receptors was demonstrated. Parlow et al. [143–145] reported piperazinyl–glutamate–pyridine derivatives such as **76** (pEC₅₀=7.82) as potent orally bioavailable P2Y₁₂ antagonists. 6-Amino-2-mercapto-3H-pyrimidin-4-one derivatives such as **78** (IC₅₀=8.1 μM) appear to antagonize the P2Y₁₂ receptor [146]. One very potent and selective competitive antagonist of the P2Y₁₂ receptor, the disulfonate derivative PSB 0739 **75**, which was derived from RB2, was recently introduced as a research tool [41, 147]. PSB-0739 was reported as the most potent competitive non-nucleotide antagonist at the human P2Y₁₂ receptor described so far (K_i=24.9 nM). BF0801 **79** is an uncharged adenine derivative that antagonizes the P2Y₁₂ receptor in platelets to inhibit aggregation with an IC₅₀ of 63.3 μM and also inhibits a phosphodiesterase [148].

P2Y₁₄ receptors

The P2Y₁₄ receptor is structurally restrictive with respect to the modification of the nucleobase, ribose, and phosphate moieties of agonist ligands. UDP-glucose **25** (EC₅₀=0.35 μM) and UDP **23** are nearly equipotent as agonists of the human P2Y₁₄ receptor. Other naturally occurring UDP-sugars activate this receptor less potently. The SAR of analogues of both endogenous agonists was recently explored in a systematic fashion [149]. When the glucose moiety is present, there is a requirement for specific

hydroxyl groups in order to potentially activate the P2Y₁₄ receptor. When the distal hexose moiety is entirely absent, very high potencies can be obtained, suggesting partly different modes of binding of the two ligand series. The 2-thiouracil modification enhances potency in both series. Thus, the 2-thio analog of UDP-glucose MRS2690 **26** is a sixfold more potent agonist for the P2Y₁₄ receptor and, unlike UDP-glucose, is inactive at the P2Y₂ receptor. Because UDP activates both the P2Y₆ and P2Y₁₄ receptors, there is a need for agonist ligands that can distinguish between these two subtypes. Stabilizing phosphonate groups have facilitated this selectivity. For example, α,β-difluoromethylene-UDP, MRS2802 **27**, is inactive at the P2Y₆ receptor and fully activates the human P2Y₁₄ receptor with an EC₅₀ of 63 nM. MRS2905 **28** displays an EC₅₀ of 2 nM at the human P2Y₁₄ receptor with a selectivity of >2,000 in comparison to the P2Y₆ receptor.

The heterocyclic antagonists of the P2Y₁₄ receptor **80** (K_i=2.2 nM) and **81** (K_i=4.0 nM) were disclosed in patents from Merck, but the full pharmacological characterization has not yet appeared in the literature [150, 151]. A prodrug approach in the structural series of compound **80** was recently reported [152].

Conclusions

Novel ligands for the purine receptor families in platelets, including both selective agents derived from known ligands and new chemotypes, are now available for use as tools in pharmacological studies. Some of these agents have entered into clinical trials. Functional properties of the P2Y₁ and P2Y₁₂ receptors were extensively explored using mutagenesis and molecular modeling, which are useful tools in drug discovery. Detailed SAR analyses have been constructed for nucleotide and non-nucleotide ligands at the P2X₁, P2Y₁, P2Y₁₂, and P2Y₁₄ receptors. There is much pharmaceutical development activity aimed at identifying newer agents to act at the P2Y₁₂ receptor that do not require enzymatic pre-activation *in vivo*. The screening of chemically diverse compound libraries has identified novel chemotypes that act as competitive non-nucleotide antagonists of the P2Y₁ receptor or the P2Y₁₂ receptor, and antithrombotic properties of the structurally optimized analogues were demonstrated. New tools have been developed for the discovery of ligands at platelet purine receptors, including *in silico* screening to identify novel antagonist chemotypes, fluorescent probes, and covalent attachment to dendrimeric carriers to produce multivalent conjugates that inhibit platelet aggregation. In conclusion, a wide range of new pharmacological tools is available to control platelet function by interacting with cell surface purine receptors.

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