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Update of P2Y Receptor Pharmacology: IUPHAR Review 27

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Abbreviations: ApoE, apolipoprotein E; BMC, bone mineral content; BMD, bone mineral density; CFTR, cystic fibrosis transmembrane conductance regulator; DUSP, dual specificity protein phosphatase; ECL, extracellular loop; EPAC, exchange protein activated by cAMP; ERKs, extracellular signal-regulated kinases; GFAP, glial fibrillary acidic protein; GSK, glycogen synthase kinase; ICL, intracellular loop; KO, knockout; MSD, musculoskeletal disorder; Mya, million years ago; PKD, protein kinase D; PLC, phospholipase C; PET, positron emission tomography; nPKC, calcium-independent protein kinase C; SNP, single nucleotide polymorphism; SS, Sjögren's syndrome; TM, transmembrane helix.

Abstract
Eight G protein-coupled P2Y receptor (P2YR) subtypes respond to extracellular adenine and uracil mono- and dinucleotides. P2YRs belong to the δ group of rhodopsin-like GPCRs and contain two structurally distinct subfamilies: $P2Y_{1, 2, 4, 6,}$ and $_{11}$ (principally G_q protein-coupled $P2Y_1$-like) and $P2Y_{12–14}$ (principally G_i protein-coupled $P2Y_{12}$-like) receptors. Brain P2YRs occur in neurons, glial cells and vasculature. Endothelial $P2Y_1$, $P2Y_2$, $P2Y_4$ and $P2Y_6$Rs induce vasodilation, while smooth muscle $P2Y_2$, $P2Y_4$ and $P2Y_6$R activation leads to vasoconstriction. Pancreatic $P2Y_1$ and $P2Y_6$Rs stimulate while $P2Y_{13}$R inhibits insulin secretion. Antagonists of $P2Y_{12}$R, and potentially $P2Y_1$R, are antithrombotic agents, and a $P2Y_2$/$P2Y_4$R agonist treats dry eye syndrome in Asia. P2YR agonists are generally pro-inflammatory, and antagonists may eventually treat inflammatory conditions. This article reviews recent developments in P2YR pharmacology (using synthetic agonists and antagonists), structure and biophysical properties (using X-ray crystallography, mutagenesis and modeling), physiological and pathophysiological roles, and present and potentially future therapeutic targeting.

Introduction and Historical Overview

Initial characterization of P2 receptor subtypes. It is now >90 years since ATP and related adenine nucleotides and nucleosides were first isolated and shown to be pharmacologically active (Drury and Szent-Györgyi, 1929). It took, however, nearly another fifty years before the receptors through which they act were defined on the basis of pharmacological criteria. Burnstock, (1978) proposed that P1 purinoceptors, i.e. adenosine receptors, had an agonist potency order of adenosine > AMP > ADP > ATP, and methylxanthines, such as theophylline and caffeine, were selective antagonists. In contrast, at P2 purinoceptors the agonist potency order was ATP > ADP > AMP > adenosine, and methylxanthines were inactive. This division received much support and was widely accepted.

Progress, thereafter, was much faster. First, Burnstock and Kennedy (1985) suggested the subdividing of P2 purinoceptors into P2X and P2Y subtypes, based on the relative agonist potency of ATP and several structural analogues in smooth muscle tissues. Shortly afterwards, Gordon (1986) identified P_{2T} purinoceptors on platelets that mediate aggregation in response to ADP, and P_{2Z} purinoceptors, which mediate ATP-induced degranulation of mast cells. Uracil nucleotides were also known to regulate cellular function and the P_{2U} R, activated by UTP and/or UDP, and in some cases, by ATP, was proposed (see Abbracchio *et al.,* 2006). P_{2D} purinoceptors sensitive to adenine dinucleotides were also identified (see Abbracchio *et al.,* 2006).

Cloning of P2Rs. Thus, numerous P2 purinoceptor subtypes had been named by the early 1990s, but their properties, distribution and physiological roles were largely unclear, as there were no selective antagonists and the endogenous agonists had complex pharmacological profiles. These uncertainties were clarified in the next few years by the cloning of multiple adenine and/or uracil nucleotide-sensitive receptors. Seven ligand-gated cation channels, including the P_{2Z} purinoceptor, were all named P2XRs, and eight GPCRs, including P_{2U} and P_{2T} purinoceptors, were called P2YRs (see Abbracchio *et al.,* 2006, 2019; Jacobson and Müller, 2016). The aim of this article is to review recent developments in our understanding of the pharmacological, biochemical and biophysical properties of P2YRs, their physiological and pathophysiological roles and how they are targeted clinically, both at present and potentially in the future. Key advances in this field since the last IUPHAR review (Abbracchio *et al.,* 2006) include X-ray crystallographic structures of two representative subtypes and many new ligand tools.

The gaps in the numbering of P2YRs reflects the historical process in which newly cloned receptors were misassigned as purinergic receptors (e.g. P2Y10). Furthermore, several related orphan receptors, GPR171 and GPR17, appear in the literature as "P2Y-like" receptors. Several but not all reports support GPR17 activation by purinergic agonists and inhibition by antagonists (Burnstock, 2017; Fumagalli *et al.*, 2016).

Selective ligand tools to study P2YRs

P2YRs are activated by mononucleotides such as ADP (**1**), ATP (**2**), UDP (**3**), or UTP (**4**), by dinucleotides like diadenosine tetraphosphate (Ap_4A, **5**) or diuridine tetraphosphate (Up_4U, **6**), and/or by nucleotide sugars such as uridine 5'-diphosphoglucose (UDP-glucose, **7**) (see Figure 1). Selective agonists and antagonists are important for studying the roles of P2YR subtypes in physiology and pathophysiology (Figures 1 and 2, Table 1, Jacobson and Müller, 2016; Conroy *et al.*, 2016; Rafehi and Müller, 2018; Abbracchio *et al.,* 2019). Their structure-activity relationship (SAR) at each P2YR subtype has been developed in detail through chemical efforts. In most studies recombinant receptor proteins have been used for the analysis. Differences in native tissues may be due to a mixture of subtypes on cells, receptor oligomerization, differences in receptor density and the metabolism of ligands by ecto-nucleotidases, such as CD39 (NTPDase-1), CD39L1 (NTPDase-2) and CD73 (NT5E).

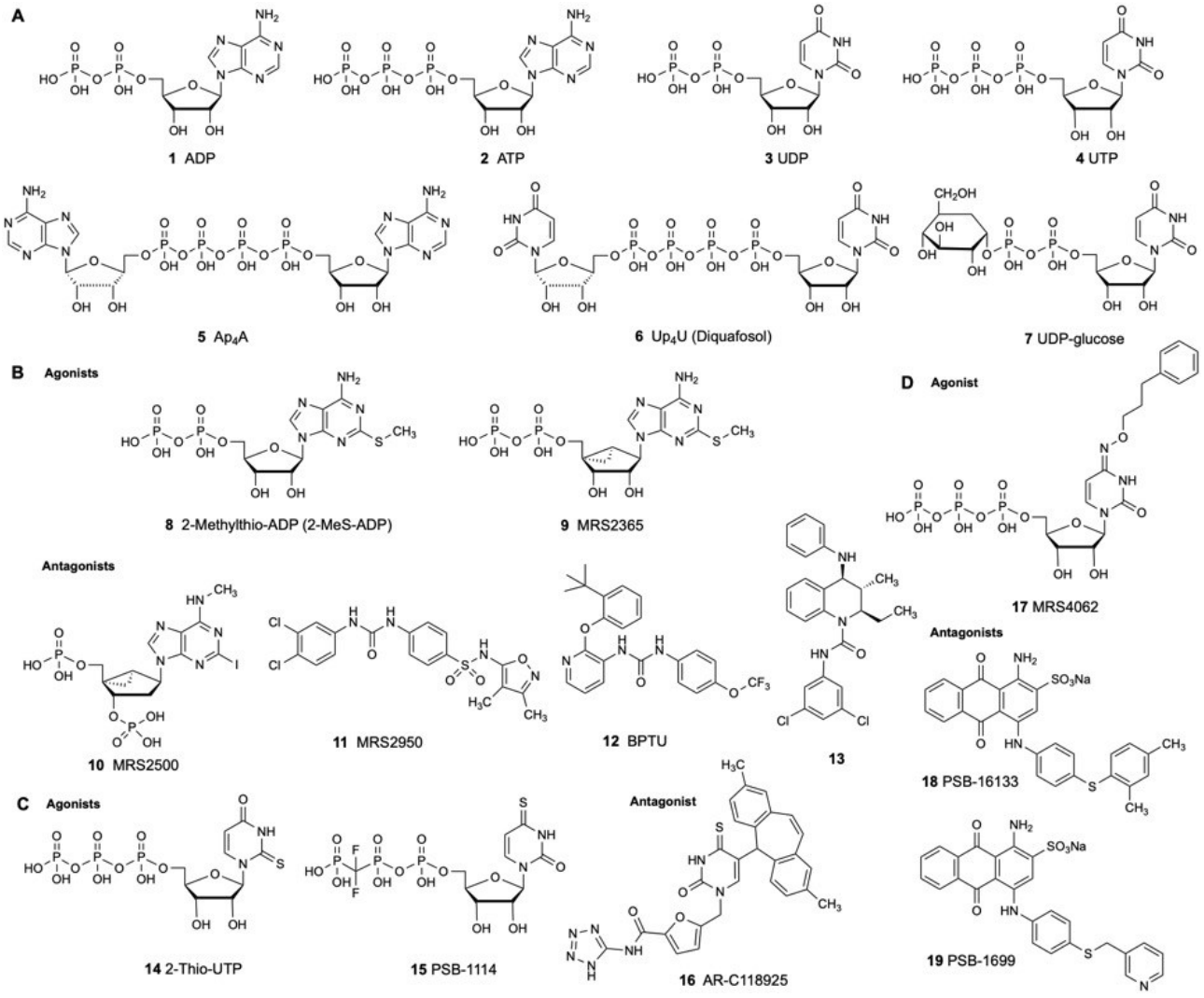

Fig. 1. P2YR ligands. **A.** Natural agonists of P2YRs. **B.** $P2Y_1R$ agonists and antagonists. **C.** $P2Y_2R$ agonists and antagonist. **D.** $P2Y_4R$ agonists and antagonists.

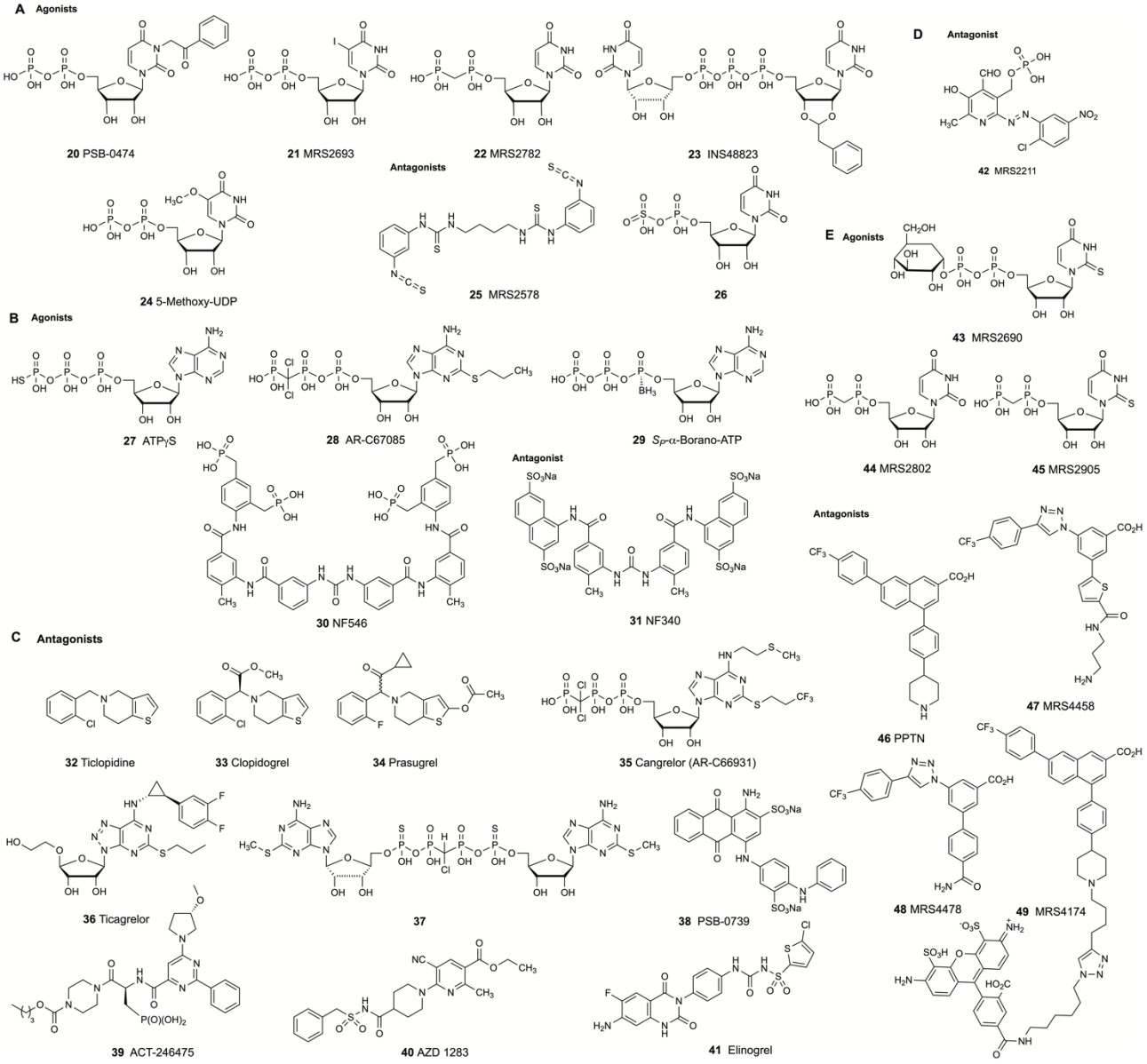

Fig. 2. P2YR ligands. **A.** $P2Y_6R$ agonists and antagonists. **B.** $P2Y_{11}R$ agonists and antagonists. **C.** $P2Y_{12}R$ agonists and antagonists. **D.** $P2Y_{13}R$ antagonist. **E.** $P2Y_{14}R$ agonists and antagonists.

$P2Y_1R$ ligands. The G_q-coupled $P2Y_1R$ is activated by ADP (**1**) and analogues including the potent agonist 2-methylthio-ADP (**8**), which is also available as a radioligand [^{3}H]2MeSADP (see Abbracchio et al., 2006, 2019). ATP is an antagonist or partial agonist at the $P2Y_1R$ (Waldo and Harden, 2004). The Northern (N)-methanocarba analogue of 2-methylthio-ADP (MRS2365, **9**) is more potent and, in addition, selective for the $P2Y_1R$ (Chhatriwala *et al.*, 2004). Selective $P2Y_1R$ antagonists have been developed. The bisphosphonate derivative MRS2500 (2-iodo-N^6-methyl-(N)-methanocarba-2′-deoxyadenosine 3′,5′-bisphosphate, **10**) is a potent and selective competitive antagonist (Hechler *et al.*, 2006). The non-nucleotide compound MRS2950 (**11**) was indentified by virtual screening the modeled receptor orthosteric site (Costanzi *et al.*, 2012). Another non-nucleotide compound, BPTU (*N*-[2-[2-(1,1-dimethylethyl)phenoxy]-3-pyridinyl]-*N*'-[4-(trifluoromethoxy)phenyl]urea, **12**) and its analogues act as an allosteric inhibitors (Zhang *et al.*, 2015a; Yuan *et al.*, 2016; Gao and Jacobson, 2017; Wang *et al.*, 2013a; Peng *et al.*, 2018).

The [^{32}P]-labeled and [^{125}I]-labeled analogues of MRS2500 are radioligands binding to P2Y$_1$Rs (Ohlmann *et al.*, 2010). An analogue of BPTU has been reported as a novel [^{18}F]-tracer for imaging of tissue P2Y$_1$R expression using positron emission tomography (PET) (Moldovan *et al.*, 2019). Tetrahydroquinolineamine derivative (**13**) was reported as a structurally novel P2Y$_1$R antagonist with binding K_i 0.5 µM (Morales-Ramos et al., 2008).

P2Y$_2$R ligands. The G_q-coupled P2Y$_2$R is activated by both ATP and UTP (see Abbracchio et al., 2006, 2019) as well as by dinucleoside polyphosphates including Ap$_4$A and Up$_4$U (diquafosol, INS365) (Xu et al., 2018). Diquafosol is approved in Japan and South Korea for the treatment of dry eye syndrome (Keating, 2015; Yamane *et al.*, 2015). 2-Thio-UTP (**14**) and related analogues are selective P2Y$_2$R agonists (El-Tayeb *et al.*, 2011; Xu *et al.*, 2018). 4-Thiouridine-5'-O-(β,γ-difluoromethylene)triphosphate (PSB-1114, **15**) is more than 60-fold selective for the P2Y$_2$R over P2Y$_4$ and P2Y$_6$Rs (El-Tayeb *et al.*, 2011).

AR-C118925 (5-[[5-(2,8-dimethyl-5*H*-dibenzo[a,d]cyclohepten-5-yl)-3,4-dihydro-2-oxo-4-thioxo-1(2*H*)-pyrimidinyl]methyl]-N-2H-tetrazol-5-yl-2-furancarboxamide, **16**) is a potent and selective antagonist for P2Y$_2$R (Kindon *et al.*, 2017; Rafehi *et al.*, 2017a, 2017c).

P2Y$_4$R ligands. The human G_q-coupled P2Y$_4$R is activated by UTP, but not by ATP or nucleoside diphosphates (see Abbracchio et al., 2006, 2019). MRS4062 (N^4-(phenylpropoxy)-CTP, **17**), MRS2927 (a 3'-deoxy-3'-fluoroglucose analogue), and N^4-(phenylethoxy)-CTP are potent P2Y$_4$R agonists (Maruoka *et al.*, 2011). PSB-16133 (**18**) and PSB-1699 (**19**) are novel P2Y$_4$R-antagonists acting at submicromolar concentrations (Rafehi *et al.*, 2017b). PSB-16133 is somewhat more potent, but PSB-1699 is more selective for the P2Y$_4$R.

P2Y$_6$R ligands. The G_q-coupled P2Y$_6$R prefers UDP as an agonist (see Abbracchio et al., 2006, 2019). Analogues including 3-phenacyl-UDP (PSB-0474, **20**; El-Tayeb *et al.*, 2011), 5-iodo-UDP (MRS2693, **21**; Ko *et al.*, 2008), α,β-methylene-UDP (MRS2782, **22**; Ko *et al.*, 2008), INS48823 (**23**, Korcok *et al.*, 2005; Rafehi and Müller, 2018), and 5-methoxy-UDP (**24**, Ginsburg-Shmuel *et al.*, 2012) are much more potent agonists.

The P2Y$_6$R is blocked by MRS2578 (N,N''-1,4-butanediylbis[N'-(3-isothiocyanato-phenyl)thiourea, **25**) (Mamedova *et al.*, 2004) and uridylyl phosphosulfate (**26**, Meltzer *et al.*, 2015).

P2Y$_{11}$R ligands. P2Y$_{11}$R couples to both G_q and G_s proteins. ATP activates human P2Y$_{11}$R, whereas ADP is a canine P2Y$_{11}$R agonist (see Abbracchio et al., 2006, 2019). Human P2Y$_{11}$R is also activated by ATPγS (**27**), the P2Y$_{12}$R antagonist 2-propylthio-β,γ-dichloromethylene-ATP (AR-C67085, **28**) and adenosine-5'-O-(α-boranotriphosphate) with a preference for the diastereomer that has an *S*-configuration at the α-phosphorus atom (compound **29**) (Ecke *et al.*, 2006; Meis *et al.*, 2010). Moreover, the suramin analogue NF546 (4,4'-(carbonylbis(imino-3,1-phenylene-carbonylimino-3,1-(4-methyl-phenylene)carbonylimino))-bis(1,3-xylene-α,α'-diphosphonic acid, **30**) has been reported to act as an agonist (Meis *et al.*, 2010). Another suramin analogue, NF340 (4,4'-(carbonylbis(imino-3,1-(4-methyl-phenylene)carbonylimino))bis(naphthalene-2,6-disulfonic acid, **31**), is a potent antagonist with a pA_2-value of 8.0 (Meis *et al.*, 2010).

P2Y$_{12}$R ligands. The G_i-coupled P2Y$_{12}$R is activated by ADP and its potent analogue 2-methylthio-ADP (**8**, Hollopeter *et al.*, 2001; see Abbracchio et al., 2006, 2019). Many P2Y$_{12}$R antagonists have been developed (Baqi & Müller, 2019). Some of them are used to reduce platelet aggregation for the prevention or treatment of cardiovascular events such as myocardial infarction or stroke. The thienopyridine compounds ticlopidine (**32**), clopidogrel (**33**, Savi *et al.*, 2006), and prasugrel (**34**, Sugidachi et al., 2000, 2016) are liver-activated prodrugs. Their active metabolites, such as R-138727 (structure not shown, Dansette *et al.*, 2015), have been shown to interact in an irreversible manner with the human P2Y$_{12}$R protein (Savi *et al.*, 2006; Algaier *et al.*, 2008; Ding *et*

al., 2009; see also Zhang *et al.*, 2014b). Competitive $P2Y_{12}R$ antagonists include the nucleotide derivatives cangrelor (**35**, AR-C69931MX, N^6-(2-methylthioethyl)-2-(3,3,3-trifluoropropylthio)-β,γ-dichloromethylene-ATP) and AR-C67085 (2-propylthio-β,γ-dichloromethylene-D-ATP, **28**) (Ingall *et al.*, 1999) as well as nucleoside derivatives such as the orally active ticagrelor (**36**, AZD6140, Springthorpe *et al.*, 2007; Hoffmann *et al.*, 2014). Ticagrelor has been reported to act as an inverse agonist under some experimental conditions (Aungraheeta *et al.*, 2016; Garcia *et al.*, 2019). A series of novel analogues of Ap_4A blocks $P2Y_{12}R$ at nanomolar concentrations; one of the most potent derivatives was compound **37** (Yanachkov *et al.*, 2016).

PSB-0739 (1-amino-9,10-dihydro-9,10-dioxo-4-[[4-(phenylamino)-3-sulfophenyl]amino]-2-anthracenesulfonic acid, **38**) is a potent and competitive non-nucleotide $P2Y_{12}R$ antagonist with a pA_2-value of 9.8 (Baqi *et al.*, 2019; Hoffmann *et al.*, 2014). Further novel $P2Y_{12}R$ antagonists include piperazinyl glutamates and derived phosphonates such as ACT-246475 (**39**, Caroff *et al.*, 2015), ethyl 6-aminonicotinate acyl sulfonamides, e.g. AZD1283 (**40**, Bach *et al.*, 2013; Zhang *et al.*, 2014b) and related sulfonamide derivatives such as elinogrel (PRT060128, **41**).The reversible and very potent antagonist ACT-246475 (Caroff *et al.*, 2014) is being tested in clinical trials for use in the prevention or treatment of cardiovascular events (Baldoni *et al.*, 2014). In addition, a variety of moderately potent $P2Y_{12}$ antagonists have been described, among them 4-benzhydrylmorpholines (Ahn *et al.*, 2016), flavonolignans (Bijak *et al.*, 2018), and salvianolic acids (Liu *et al.*, 2018). [^{3}H]PSB-0413 is an antagonist radioligand, the tritiated form of AR-C67085 (**28**), which binds to $P2Y_{12}R$ with a K_D value of 4.6 nM (Ohlmann *et al.*, 2013).

$P2Y_{13}R$ ligands. ADP and 2-methylthio-ADP (**8**) activate the G_i-coupled $P2Y_{13}R$ (Communi *et al.*, 2001; see Abbracchio et al., 2006, 2019). MRS2211 (2-[(2-chloro-5-nitrophenyl)azo]-5-hydroxy-6-methyl-3-[(phosphonooxy)methyl]-4-pyridinecarboxaldehyde, **42**) is a competitive antagonist with a pA_2-value of 6.3 (Kim *et al.*, 2005). Cangrelor, in contrast, blocks the human and the rat $P2Y_{13}R$ in a non-competitive mode of action (Fumagalli *et al.*, 2004).

$P2Y_{14}R$ ligands. UDP and sugar derivatives such as UDP-glucose and UDP-galactose are G_i-coupled $P2Y_{14}R$ agonists (see Abbracchio et al., 2006, 2019; Carter *et al.*, 2009). The analogues MRS2690 (diphosphoric acid 1-α-D-glucopyranosyl ester 2-[(4'-methylthio)uridin-5"-yl] ester, **43**), MRS2802 (α,β-difluoromethylene-UDP, **44**), and MRS2905 (α,β-methylene-2-thio-UDP, **45**) are much more potent agonists (Carter *et al.*, 2009; Das *et al.*, 2010; Abbas *et al.*, 2018).

PPTN (4-[4-(4-piperidinyl)phenyl]-7-[4-(trifluoromethyl)phenyl]-2-naphthalenecarboxylic acid, **46**) is a potent antagonist acting at the $P2Y_{14}R$ (Barrett *et al.*, 2013). Novel antagonists include MRS4458 (3-{5-[(3-aminopropyl)carbamoyl]thiophen-2-yl}-5-{4-[4-(trifluoromethyl)phenyl]-1*H*-1,2,3-triazol-1-yl}benzoic acid, **47**) and MRS4478 (4'-carbamoyl-5-{4-[4-(trifluoromethyl)phenyl]-1*H*-1,2,3-triazol-1-yl}-[1,1'-biphenyl]-3-carboxylic acid, **48**) (Yu *et al.*, 2018). MRS4174 (**49**) is a high-affinity fluorescent ligand with antagonistic properties.

The evolutionary history of P2YRs and related GPCRs

The availability of genomic sequences of human and other animal species allowed for comprehensive phylogenetic studies and meaningful analysis of the evolutionary history of the GPCR superfamily revealing several major classes of GPCRs – the rhodopsin-, adhesion/secretin-, frizzled- and glutamate-like metabotropic receptor classes (Fredriksson & Schiöth, 2005). Based on amino acid sequence similarities, P2YRs belong to the rhodopsin-like receptor class, specifically to the δ group within this class. The δ group does not only include P2YRs but also receptors for lipids, hydroxycarboxylic acids and peptides (Figure 3A).

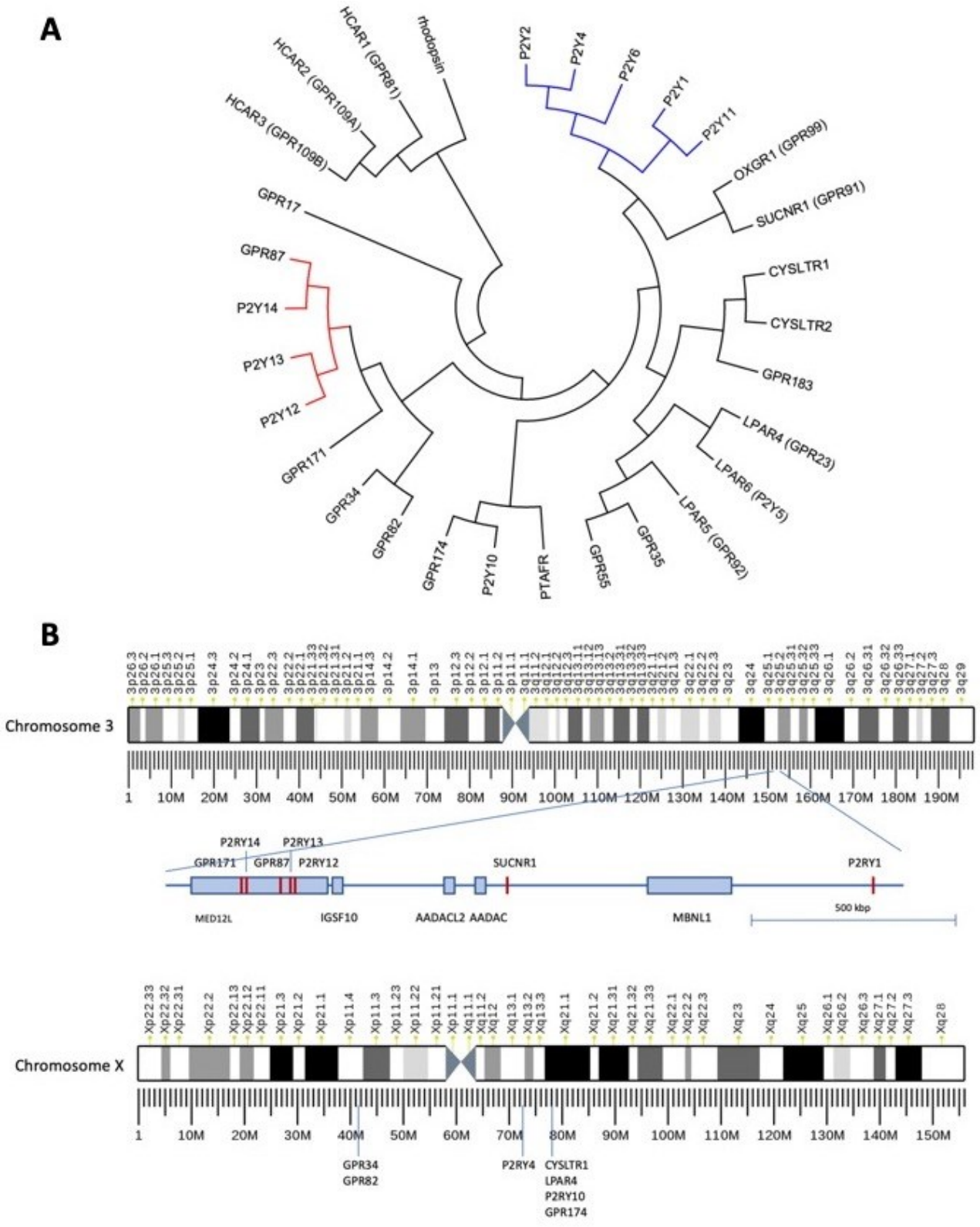

Fig. 3. **A.** Evolutionary relationships of human $P2Y_1$- and $P2Y_{12}$-like receptors of the δ group of rhodopsin-like GPCRs (Fredriksson & Schiöth, 2005). $P2Y_1$- and $P2Y_{12}$-like receptors form separated clusters of receptors indicating that they evolved independently. The evolutionary history was inferred using the Neighbor-Joining method by extending a previous analyses (Le Duc *et al.*, 2017). The optimal tree with the sum of branch length = 14.64350117 is shown. The tree is drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. The evolutionary distances were computed using the Poisson correction method and are in the units of the number of amino acid substitutions per site. The analysis involved

the amino acid sequences of 30 human receptors which were aligned by using the PAM matrix and default parameters. All positions with less than 95% site coverage were eliminated. That is, fewer than 5% alignment gaps, missing data, and ambiguous bases were allowed at any position. There were a total of 239 positions in the final dataset. Evolutionary analyses were conducted in MEGA7 (Kumar *et al.*, 2016). HCARx: hydroxycarboxylic acid receptors, LPARx: lysophosphatidic acid receptors, OXGR1: 2-oxoglutarate receptor, SUCNR1: succinate receptor, PTAFR: PAF receptor, P2Yx nucleotide receptors. **B.** Genomic clustering of P2Y-like receptors on chromosome 3 and chromosome X. Several P2YRs and other members of the δ group cluster at genomic loci, most prominent clusters at chromosome 3 and chromosome X. The $P2Y_{12}$R-like receptors are found in a MED12L gene intron at chromosome 3. Similarly, GPR34 and GPR82 are found in a reverse oriented CASK gene intron at Xp11.4. Phylogenetic analysis showed relation of cysteinyl-leukotriene receptor and lysophosphatidic acid receptors (see Figure 3A). Interestingly, CYSLTR2, LPAR6, and GPR183 are closely located at chromosome 13 (13q14.2, not shown) whereas CYSLTR1 genomically clusters with LPAR4, P2Y10, and GPR174 at the X chromosome.

The phylogenetic relation of P2YRs subdivides them into at least two groups (Figure 3A) that also correspond to their preferential G protein coupling. One group comprises $P2Y_{1, 2, 4, 6,}$ and $_{11}$ Rs (G_q-coupled $P2Y_1$R-like) while the second group contains $P2Y_{12–14}$ Rs (G_i-coupled $P2Y_{12}$R-like). The diversity of P2YR signaling pathways is discussed below in the context of neural cells. Interestingly, several other structurally related δ group GPCRs, such as the 2-oxoglutarate receptor (OXGR1, GPR99) and the succinate receptor (SUCNR1, GPR91), as well as the orphan receptors GPR34, GPR82, GPR171, and GPR87 cluster into one of both groups (Figure 3A).

The phylogenetic clustering of P2YRs correlates, at least in part, with the genomic localization of P2YRs. Chromosomal clustering in the human genome is found for $P2Y_{12-14}$R genes at chromosome 3 (Figure 3B). Here, all genes show the same orientation in a tandem-like fashion within an intron of the reverse-oriented Mediator complex subunit 12 like (Med12L) gene. Such condensed genomic localization of related genes most probably arose from multiple rounds of local gene duplications. Interestingly, SUCNR1 and $P2Y_1$R are in close genomic proximity to this $P2Y_{12}$R-like receptor gene cluster. However, both genes are in reverse orientation to the $P2Y_{12}$R cluster (Figure 3B) indicating an independent genomic history. Chromosomal clustering is also found for $P2Y_2$ and $P2Y_6$Rs at chromosome 11, the hydroxycarboxylic acid receptors HCAR1-3 at chromosome 12 and seven δ group members at chromosome X (Figure 3B).

Several P2YRs and other members of the δ group cluster have prominent genomic clusters at chromosome 3 and chromosome X. The $P2Y_{12}$-like receptors are found in an intron of the MED12L gene at chromosome 3. Similarly, GPR34 and GPR82 are found in an intron of the reverse oriented CASK gene at Xp11.4. Phylogenetic analysis showed relation of cysteinyl-leukotriene receptor and lysophosphatidic acid receptors (see Figure 3A). Interestingly, CYSLTR2, LPAR6, and GPR183 are closely located at chromosome 13 (13q14.2, not shown) whereas CYSLTR1 genomically clusters with LPAR4, P2Y10, and GPR174 at the X chromosome.

In contrast to metabotropic P2YRs, P2XRs seem to appear in evolution already in invertebrates and plants (Hou & Cao, 2016). Prototypical P2YRs, as we know them from vertebrates, seem to be an innovation that occurred first in the Cambium, because δ group GPCRs do not have orthologous sequences in invertebrates, hemichordates, and cephalochordates (Krishnan, *et al.*, 2013). Unfortunately, the "birth" of P2YRs can only be assumed at some time between the cyclostome-gnathostome split because of the lack of other recent genomes covering this evolutionary period. Because almost all P2Y- and other δ group-related sequences are present in cartilaginous and bony fish genomes these genes must have been present in the common ancestor of both fish lineages.

Most coding regions of P2YRs do not contain introns, and splice variants are rare. The genomic structure of P2YRs is highly conserved during evolution. However, introns in the coding regions of several P2YR genes newly appear in some fish species (Schöneberg *et al.*, 2007). In contrast,

introns in the 5'UTR seem to be common e.g. in $P2Y_{12}R$-like receptors, $P2Y_2$, and $P2Y_6$Rs. Obviously, human $P2Y_1R$ and $P2Y_4R$ do not contain introns in their genes. Some P2YRs contain cryptic introns in their N terminus-coding regions ($P2Y_6$, GPR87, GPR34).

The human $P2Y_{11}R$, located at chromosome 19, is an exception in many aspects. $P2Y_{11}R$ orthologs are missing in murine and chicken genomes but are present in most other birds, reptiles, and amphibians sequenced to date. In humans, $P2Y_{11}R$ transcripts exist in several variants modifying the N terminus coding region and even producing a fusion protein with an adjacent gene (PPAN) (Dreisig & Kornum, 2016). Here a chimeric transcript, characterized by the first third of PPAN exon 12 joined to $P2Y_{11}R$ exon 2, has been detected. The chromosomal synteny between $P2Y_{11}R$ and PPAN is already established in zebrafish, and fusions at the mRNA level can be found in many birds and mammals.

Structural characterization of P2YRs

The availability of numerous orthologues also allows for comparing the conservation of individual amino acid positions in P2YR proteins. Although P2YRs belong to two distinct structural and evolutionary groups (Figure 3A), as confirmed by X-ray structures (Zhang *et al.*, 2014a, 2015a), several members share the same agonist (e.g. ADP). Support for different agonist binding modes between $P2Y_1$ and $P2Y_{12}$Rs was observed in mutational analyses. While for $P2Y_1$ positions $K^{6.55}$, $Q^{7.36}$, $R^{7.39}$ (using standard residue numbering) were shown to be critical for receptor activation following agonist binding (Jiang *et al.*, 1997), positions $R^{6.55}$, $Y^{6.58}$, and $K^{7.35}$ are involved in $P2Y_{12}R$ ligand recognition (Hoffmann, *et al.*, 2014; Schmidt *et al.*, 2013). All these residues are conserved within vertebrate $P2Y_1R$ and $P2Y_{12}R$ orthologs, but are not shared between the both ADP receptors.

Phylogenetic and mutagenesis studies addressing the positions $R^{6.55}$, $Y^{6.58}$, and $K^{7.35}$ of $P2Y_{12}R$ showed that all these residues are 100% conserved among species and that most mutations of these positions interfered with receptor function (Schmidt *et al.*, 2013). However, such residue combination is also present in some GPR87, GPR171, and GPR34 orthologs (all $P2Y_{12}R$-like receptors), which are not activated by ADP or ATP. This does not rule out that these residues are involved in nucleotide binding of e.g. $P2Y_{12}R$ but it implicates additional positions which determine ligand specificity.

Structural studies of P2YRs have experienced tremendous progress in recent years. Five structures of two representative receptors $P2Y_1R$ and $P2Y_{12}R$ from two subfamilies were determined in 2014 and 2015, respectively (Zhang *et al.*, 2014a, 2014b; Zhang *et al.*, 2015a). These structures, in which the receptors are captured in complex with different ligands that vary in chemical structure and binding site, provide essential insights into ligand recognition and activation regulation of P2YRs.

The $P2Y_1R$ and $P2Y_{12}R$ structures share a canonical seven transmembrane (7TM) helical architecture of GPCRs. Like most other solved class A GPCR structures, the structures of $P2Y_{12}R$ in complex with agonists 2MeSADP and 2MeSATP and $P2Y_1R$ bound to inhibitors MRS2500 and BPTU exhibit a conserved disulfide bond connecting TM3 to the second extracellular loop (ECL2). However, this disulfide bond is not observed in the antagonist AZD1283-bound $P2Y_{12}R$ structure (Zhang *et al.*, 2014a), consistent with previous data suggesting that the ECL2 residue $Cys97^{3.25}$ may act as the covalent binding site for the active metabolites of $P2Y_{12}R$ drugs (Algaier *et al.*, 2008; Ding *et al.*, 2009). In addition to the dynamic disulfide bond, TM5 of $P2Y_{12}R$ adopts a straight and elongated conformation rather than a bent helix observed in most other GPCR structures, as the highly conserved class A GPCR residue $P^{5.50}$ that leads to a helical bend in other GPCRs (Zhang *et al.*, 2014a) is substituted by an asparagine ($N201^{5.50}$) in $P2Y_{12}R$.

Comparison of agonist- and antagonist-bound $P2Y_{12}R$ structures reveals remarkable differences in the extracellular region (Zhang *et al.*, 2014b). Compared to the $P2Y_{12}R$-AZD1283 structure, the extracellular tips of TM6 and TM7 shift towards the central axis of the TM bundle by over 10 Å and 5 Å respectively in the $P2Y_{12}R$-2MeSADP structure. The inward movement of TM6 and TM7

allows extensive interactions between the agonist and the extracellular region of the receptor. As a result, the helical bundle, the $P2Y_{12}R$ extracellular loops and N terminus undergo conformational changes, and the ligand-binding pocket shrinks to preclude antagonist binding. In contrast, the antagonist AZD1283's phenyl moiety forms a steric hindrance to impede the inward movement of TM6 in the $P2Y_{12}R$-AZD1283 structure. These structural differences suggest that the receptor extracellular region may play a role in modulating receptor activity by cooperating with the bound ligand.

The $P2Y_{12}R$ structures reveal different binding modes for the non-nucleotide antagonist AZD1283 and the nucleotide agonist 2MeSADP. The binding pockets of these two ligands vary in size and shape, with only partial overlap. AZD1283 binds to $P2Y_{12}R$ in a large open pocket formed by residues from TM3-TM7. In contrast, the nucleotide agonist is completely enclosed within the receptor ligand binding pocket. The ligand binding cavity of $P2Y_{12}R$ is separated into two sub-pockets by a barrier formed by $Y105^{3.33}$ and $K280^{7.35}$. Pocket 1 is shaped by TM3-TM7, while pocket 2 is composed of TM1-TM3 and TM7. Although pocket 2 is not occupied in the solved $P2Y_{12}R$ structures, molecular docking studies suggested that the active metabolites of $P2Y_{12}R$ antagonists such as clopidogrel many bind to pocket 2 (Zhang *et al.*, 2014a), providing new clues for the development of allosteric modulators of $P2Y_{12}R$ as anti-cardiovascular drugs with reduced side effects.

Although $P2Y_1R$ and $P2Y_{12}R$ share the same endogenous ligand ADP, the structures of these two receptors reveal (Figure 4) completely different recognition patterns for their nucleotide-like ligands (Zhang *et al.*, 2015a). In the $P2Y_1R$ structure, the nucleotide-like antagonist MRS2500 binds to the receptor in a binding pocket bordered by N terminus, ECL2 and TM6-TM7 within the TM bundle, close to the extracellular surface. The adenine ring of MRS2500 inserts into a sub-pocket formed by the N terminus, TM6 and TM7, while its two phosphate groups make extensive polar interactions with residues from N terminus, ECL2, TM2 and TM7. In the $P2Y_{12}R$-2MeSADP structure, the adenine ring of 2MeSADP reaches deep into the ligand binding pocket, making contacts with TM3 and TM4. The negatively charged diphosphate group of 2MeSADP attracts positively charged residues and hydrogen-bonding groups from N terminus, ECL2, TM3, TM6 and TM7, forming electrostatic force to stabilize the "closed" extracellular region (Jacobson *et al.*, 2015). These structural differences highlight the diversity of recognition by GPCRs.

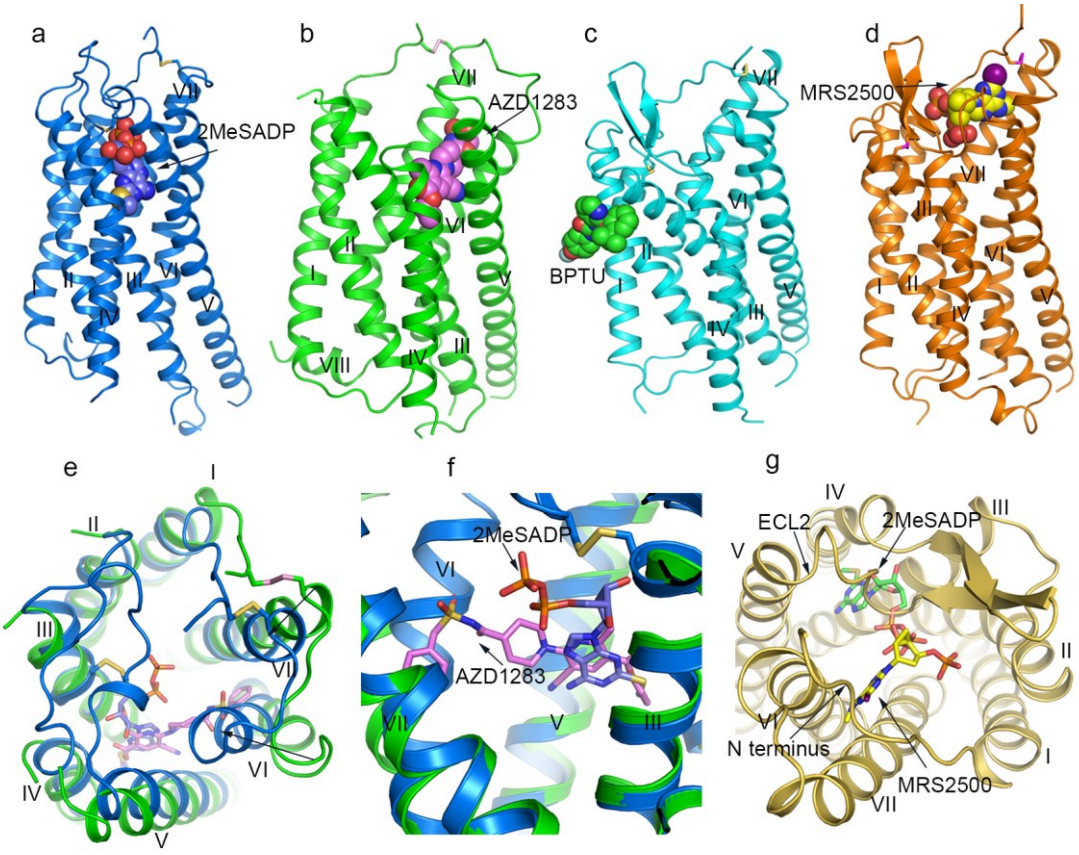

Fig. 4. Structures of $P2Y_{12}R$ and $P2Y_1R$. (a) Structure of the $P2Y_{12}R$-2MeSADP **8** complex. The $P2Y_{12}R$ is shown in blue cartoon representation. 2MeSADP is shown as blue spheres. The disulphide bonds are shown as yellow sticks. (b) Structure of the $P2Y_{12}R$-AZD1283 **40** complex. The receptor is colored green and shown in cartoon representation. AZD1283 is shown as spheres with pink carbons. The disulphide bridge is shown as pink sticks. (c) Structure of the $P2Y_1R$-BPTU complex. The $P2Y_1R$ is colored cyan and shown as cartoon. The ligand BPTU is shown as spheres with green carbons. The disulphide bonds are shown as yellow sticks. (d) Structure of the $P2Y_1R$-MRS2500 **10** complex. The $P2Y_1R$ is shown in orange cartoon representation. MRS2500 is shown as spheres with yellow carbons. The disulphide bonds are show as magenta sticks. (e) Structural comparison of the extracellular region in the $P2Y_{12}R$-2MeSADP and $P2Y_{12}R$-AZD1283 complexes. In the $P2Y_{12}R$-AZD1283 structure, the receptor is shown in green cartoon representation, the ligand AZD1283 is shown as pink sticks. In the $P2Y_{12}R$-2MeSADP structure, the receptor is shown in blue cartoon representation and 2MeSADP is shown as blue sticks. The black arrows indicate the movements of helices VI and VII in the 2MeSADP-bound structure relative to the AZD1283-bound structure. (f) Comparison of the binding pose of AZD1283 and 2MeSADP in $P2Y_{12}R$. The color scheme is the same as that in panel e. (g) Comparison of the nucleotide binding modes in $P2Y_1R$ and $P2Y_{12}R$. $P2Y_1R$ is shown in yellow cartoon representation. MRS2500 and 2MeSADP are shown as yellow sticks and green carbon, respectively.

The most striking finding in the $P2Y_1R$ structures is that the two $P2Y_1R$ inhibitors MRS2500 and BPTU occupy two disparate ligand binding pockets (Figure 4c; Zhang *et al.*, 2015a). Unlike the nucleotide-like ligand MRS2500 whose binding site is within the receptor helical bundle, the non-nucleotide antagonist BPTU binds to the receptor in a shallow binding pocket on the external interface of TM1-TM3 with the lipid bilayer, which accommodates the ligand mainly through hydrophobic interactions. This hydrophobic binding environment of BPTU is consistent with the high hydrophobicity of this ligand, and previous efforts showing that addition of any polar groups to reduce its lipophilicity decreased binding affinity to $P2Y_1R$ (Wang *et al.*, 2013a). The unique binding site of BPTU indicates that this ligand acts as an allosteric modulator of $P2Y_1R$. It most

likely inhibits receptor function by blocking the conformational change of TM1-TM3 to lock the receptor at inactive state, which is supported by ligand binding assays showing that BPTU can accelerate agonist [^{35}S]2MeSADP dissociation (Zhang *et al.*, 2015a). BPTU was the first structurally characterized selective GPCR ligand located entirely outside of the canonical ligand binding pocket of GPCRs. The finding offers new opportunities to GPCR drug discovery to target novel sites.

Currently, there is no crystal structure for pyrimidine nucleotide receptors. However, recent mutational and docking studies suggest that the conserved $R^{7.39}$ in $P2Y_1$-like receptors is also involved in UDP binding in $P2Y_6R$ (Bruser *et al.*, 2017) and agonist binding of $P2Y_2R$ (Rafehi *et al.*, 2017) indicating that the binding pocked is very similar at least within the two P2YR groups.

Signalling pathways for P2YRs

The cellular sources of nucleotide agonists of P2YRs can be cotransmission, endocytosis, release through pannexin1 (Panx1) hemichannels, lysis in cell damage, etc. (Abbracchio *et al.*, 2006; Nishimura *et al.*, 2017). The principal G-protein coupling of each of the P2YR subtypes are indicated in Table 1. However, each of the P2YRs can be associated with multiple G proteins and other signaling pathways, as was shown for $P2Y_1R$, including both G protein-dependent and independent, e.g. β-arrestin, pathways (Gao and Jacobson, 2017). Some of the P2YRs additionally couple to $G_{12/13}$ ($P2Y_2R$, $P2Y_6R$) or G_s ($P2Y_{11}R$). P2YRs regulate mitogen-activated protein kinase (MAPK) pathways and consequently affect cell survival and proliferation (Miras Portugal et al., 2019). This review emphasizes the novel contribution of nucleotide receptors to maintain cell homeostasis through the regulation of MAP kinases and phosphatases. P2YRs undergo agonist-induced desensitization and can heterodimerize with other GPCRs, such as the vascular angiotensin (Ang) II type1 receptor and the A_1 adenosine receptor (Nishimura *et al.*, 2017).

P2YRs in the nervous system

The cloning of the different P2YRs showed their presence in brain tissue. P2YRs are especially abundant in glial cells, astrocytes and microglia, but they are also present in central and peripheral neurons, oligodendrocytes and cerebral microvasculature. P2YRs together with ionotropic P2XRs mediate neurotransmission, neuron-glia interaction, regulation of cerebral blood flow, neuroprotection, or even contribute to neurodegeneration and pain transmission (Burnstock, 2017; Toth et al., 2015; Weisman, 2012). P2YRs are also present in adult stem cells, which suggests potential actions in neuroregeneration (Gómez-Villafuertes *et al.*, 2015; Stefani et al., 2018).

Astroglia from different areas, cortex, *hippocampus*, *striatum*, *cerebellum* and spinal cord, express a great variety of P2YRs: ADP selective receptors, $P2Y_1R$ and $P2Y_{13}R$, the receptor equally activated by UTP and ATP, $P2Y_2R$, and pyrimidine selective receptors $P2Y_4R$ and $P2Y_6R$ (Franke et al., 2012). Astroglial $P2Y_1R$ mediates neuron-glia interaction and calcium waves contributing to neuronal activity synchronization (Shigetomi et al., 2018). $P2Y_1R$, $P2Y_2R$ and $P2Y_{13}R$ are also present in neurons. $P2Y_1R$ and $P2Y_{13}R$ together with the ionotrotropic P2X7R regulate neuronal differentiation and are also involved in neuroprotection and neurodegeneration (Miras Portugal et al., 2019). Some P2YRs, $P2Y_1R$, $P2Y_2R$ and $P2Y_4Rs$, appear to be upregulated in pathological conditions such as brain injury, Alzheimer disease and epilepsy (Franke et al., 2012). $P2Y_1R$ antagonism is associated with cerebroprotection and might improve cognition in Alzheimer's disease (Reichenbach *et al.*, 2018 and glutamate mediated hippocampal neurodegeneration (Simões et al., 2018). $P2Y_1R$ could be also a novel candidate for the treatment of epilepsy (Alves et al., 2019). However, $P2Y_1R$ and $P2Y_{13}R$ agonists could also display neuroprotective actions against oxidative stress and glutamate excitotoxicity as reported in other experimental models (Miras Portugal et al., 2019).

$P2Y_{12}R$ exhibits a more restricted distribution, mainly located in microglia, where it plays relevant role in inflammation and neuropathic pain (Tozaki-Saitoh et al., 2008). Microglial $P2Y_{12}R$ and $P2Y_{13}R$ and astroglial $P2Y_1R$ are new players of the complex microglia-astrocyte interaction

after brain damage. After brain injury, ATP and ADP increase at extracellular space, and ADP act as a chemotactic signal recruiting microglia to damaged area via $P2Y_{12}R$ activation. Activation of microglial $P2Y_{12}R$ and $P2Y_{13}R$ releases cytokines and TNFα, which down-regulate $P2Y_1R$ in astrocytes, avoiding astroglial proliferation. Down-regulation of astroglial $P2Y_1R$ by microglia changes astrocyte phenotype from pro-inflammatory to neuroprotective phenotype, increasing reactive astrogliosis (enhances GFAP expression), glial scar formation, restoring brain blood barrier functions and suppressing leukocyte infiltration (Shinozaki et al., 2017). $P2Y_{12}R$ is also present in oligodendrocytes. Loss of $P2Y_{12}R$ could contribute to demyelination process in multiple sclerosis (Amadio et al., 2010).

As previously mentioned, P2YRs and P2XRs modulate pain transmission (Burnstock, 2017). $P2Y_1R$, $P2Y_2R$, $P2Y_{12}R$ and $P2Y_{13}R$ are expressed in nociceptive neurons and surrounding glial cells, and both pro-nociceptive and pro-analgesic effects have been proposed (Malin and Molliver, 2010). A complex regulatory mechanism for $P2Y_1R$ and $P2Y_{13}R$ of glycine transporter function in glutamatergic and GABAergic synapsis confirms the important role of these receptors as targets to modulate different pain states at the spinal cord (Jiménez et al., 2011).

Concerning the intracellular mechanisms mediating P2YR effects in neurons and glial cells, a great variety of intracellular routes have been unraveled that include, PI3K/Akt axis, glycogen synthase kinase 3 (GSK3), NFκB, JAK-STAT, MAPK, transactivation of growth factor tyrosine-kinase receptors, and cross-talk with prostanoid receptors. Among them, MAP kinase activation deserves special attention. ERKs are responsible for astrocyte proliferation and migration (Franke et al., 2012; Paniagua-Herranz et al., 2017). ERK activation is also required for neuroprotective actions displayed by $P2Y_{13}R$ in cerebellar granule neurons and astrocytes (Miras Portugal et al., 2019). Novel players in P2Y-activated signaling network encompass dual specificity protein phosphatases, DUSPs, which connect P2YRs to the inactivation mechanisms and fine-tune regulation of MAPK signaling. P2YR-mediated DUSP regulation ensures proper intensity and duration of MAPK signaling required to preserve cell survival, and to avoid the harmful impact of MAPK over-activation that can compromise cell viability and promote aberrant proliferation and differentiation rates (Miras Portugal et al., 2019). $P2Y_{13}R$ counteracts genotoxic stress by inducing the nuclear dual specificity protein phosphatase 2, DUSP2, which is responsible for the dephosphorylation of p38 in the nuclear compartment (Miras Portugal et al., 2019).

P2YRs in endocrine and exocrine function

Endocrine and exocrine tissues/organs secrete hormones, cytokines, enzymes and electrolytes and fluid, though in different direction - towards blood (endo-system) or through ducts to epithelial surfaces (exo-system). These two systems influence a whole spectrum of functions ranging from metabolism, digestion, to electrolyte/fluid homeostasis, development, growth and reproduction. The role of purinergic signaling in major endocrine glands has been recently reviewed (Bjelobaba *et al.*, 2015). Here, we focus on tissue/cells regulating metabolism: pancreatic β-islet cells, adipocytes, and the liver.

Extracellular ATP affects insulin secretion from pancreatic β-cells and the positive or negative outcome depends on glucose levels and a particular P2R expressed in a given species (Burnstock & Novak, 2013). ATP originates from nerves, insulin granules and by transport through Panx1 (Tozzi *et al.*, 2018). In rodent β-cells and pancreas, $P2Y_1$ and $P2Y_6$Rs stimulate insulin secretion (Balasubramanian *et al.*, 2013), while in mouse this effect is overshadowed by $P2Y_{13}R$ that inhibits secretion (Amisten *et al.*, 2010). In human β-cells, P2XRs were thought to be more important, but recently interest in the $P2Y_1R$ has been renewed (Khan *et al.*, 2014), not the least because one single nucleotide polymorphism (SNP) in the 3'UTR of *P2YR1* is associated with disturbance in glucose homeostasis (Todd *et al.*, 2015). In addition to classical PLC/Ca^{2+} signaling stimulating insulin release, one study revealed alternative signaling. $P2Y_1R$ agonist ADPβS stimulated cAMP/EPAC, which via PI3K inhibited Kv channels, but it is not clear whether this was mediated through the $P2Y_1$ and/or $P2Y_{11}$Rs (Zhang *et al.*, 2015b). Another study showed that the $P2Y_6R$ activates

calmodulin-AMPK to increase insulin secretion in murine MIN6 cells (Balasubramanian *et al.*, 2013). Regarding regulation of β-cells mass, $P2Y_6R$ is cytoprotective, while $P2Y_{13}R$ is pro-apototic (Burnstock & Novak, 2013). Taken together, targeting P2YRs to increase insulin secretion and β-cell mass would be a good strategy to ameliorate the course of type 1 and type 2 diabetes.

Adipose tissue releases a number of hormones and cytokines, which contribute to the regulation of appetite and satiety, fat distribution, insulin secretion and sensitivity, energy expenditure and inflammation. White adipocytes express P2YR subtypes that often have opposite effects on adipogenesis, lipogenesis, lipolysis, glucose transport, as well as on leptin and adiponectin production, reflecting adipocyte origin; therefore, the organ or body impact may differ (Tozzi & Novak, 2017). Most illustrative are studies on mice. $P2Y_1R$ deletion or its inhibition by MRS2500 reduced leptin production and secretion in lean mice, but this effect disappeared in mice on high fat diet (Laplante *et al.*, 2010). Inhibition or deletion of $P2Y_4R$ increased adiponectin secretion in cardiac adipocytes and was cardioprotective (Lemaire *et al.*, 2017). In $P2Y_2R$ knockout (KO) mice on high fat diet, there was decreased immune cell infiltration and inflammation of adipose tissue, as well as a lower fat and body weight (Merz *et al.*, 2018). In addition, hepatic steatosis was improved, and animals were protected against insulin resistance and hypercholestoremia, all indicating that the $P2Y_2R$ is promising target to combat initial phases of metabolic syndrome. Brown adipocytes are specialized in energy expenditure, they express $P2Y_{2,\ 6,\ 12}$ Rs and P2XRs, but it is not clear yet how they regulate adipocyte function (Tozzi & Novak, 2017).

Recent work shows that plasma uridine levels are influenced by adipocyte-biliary clearance (Deng *et al.*, 2017). Potentially, uridine levels could affect UDP/UTP levels, which is the case in the hypothalamus, where UDP acts on $P2Y_6R$ of AgRP neurons and promotes feeding behavior (Steculorum *et al.*, 2015). This offers a challenging opportunity to target this receptor for the treatment of diseases associated with energy balance.

The major exocrine glands include pancreas, salivary glands, liver, sweat, sebaceous and tear glands and associated epithelia. They contribute to digestion and fluid secretion, contain various ions, enzymes and mucus and can be affected in a number of diseases, such as cystic fibrosis, Sjögren's syndrome (SS) and cancer. Here, we focus on the first three glands.

Liver, the metabolic hub, also performs endocrine function (secreting e.g. IGF-1) and exocrine function (secreting bile). Not so much is known about the physiological role of purinergic signaling in endocrine function, but the role in liver diseases is well-reviewed, including the role of P2YRs on stellate cells and their relation to liver fibrosis (Lu & Insel, 2014). One key receptor is $P2Y_2R$, as KO mice are protected from liver damage and necrosis in experimental liver injury models (Ayata *et al.*, 2012). Cholangiocytes, epithelial cells lining intrahepatic ducts, produce bile containing bile acids/salts and bicarbonate. The bile secretion requires a number of Ca^{2+}-activated ion channels that are stimulated via P2YRs, and one of these, $P2Y_{12}R$, resides on the primary cilia (Masyuk *et al.*, 2008).

Pancreatic acini secrete ATP as well as digestive enzymes, but do not express active P2Rs (Haanes *et al.*, 2014). In contrast, pancreatic ducts express various P2Rs (Novak, 2011), where in particular the luminal $P2Y_2$ and $P2Y_4$Rs activate Ca^{2+}-activated Cl^- and K^+ channels (TMEM16A, cystic fibrosis transmembrane conductance regulator (CFTR), $K_{Ca}3.1$) and thereby potentiate bicarbonate and fluid secretion (Wang *et al.*, 2013c), thus fine-tune pancreatic function. In contrast, distension of ducts via ATP release and $P2Y_2R$ inhibition of $K_{Ca}1.1$ on the basolateral membrane can inhibit secretion (Wang *et al.*, 2013c). Potentially, it would be important to increase pancreatic secretion, in for example cystic fibrosis or in pancreatitis, but access of drugs to the pancreatic lumen is not feasible.

Salivary gland acini secrete amylase and fluid and here P2XRs are important. Ducts modify saliva to become hypotonic, and $P2Y_2R$ increases ductal Cl^- reabsorption via CFTR (Novak, 2011). In addition to ion channel regulation via Ca^{2+} signaling, $P2Y_2R$ trans-stimulates EGFR that promotes gland repair and regeneration (Ratchford *et al.*, 2010). However, $P2Y_2R$ is upregulated in salivary gland inflammation, as in Sjögren's syndrome (SS), and genetic ablation of $P2Y_2R$ in SS

mouse models reduced gland inflammation (Woods *et al.*, 2018). Other exocrine glands and epithelia of relevance are the tear glands and corneal epithelium. Secretion of tear glands is mainly regulated via P2XRs, but minor glands and corneal epithelium express the $P2Y_2R$ that stimulates fluid and mucin secretion. This receptor has been a successful target of the drug diquafosol in treating dry eye diseases. A similar approach to target airway epithelia hydration in cystic fibrosis patients with the related drug denufosol (INS37217) did, however, not continue past the second phase III clinical trial (Kellerman *et al*., 2008).

P2YRs in the cardiovascular system

P2YRs regulate or participate in all the physiological functions of the cardiovascular system, including heart contractility and cardiac frequency, vascular tone, release of endothelial factors, angiogenesis, smooth muscle cell proliferation, haemostasis and immunity. As such they are involved in pathological processes such as acute vascular inflammation, atherosclerosis, arterial thrombosis including myocardial infarction and cerebrovascular stroke. All the eight P2YR subtypes have been found to be expressed in the cardiovascular system, i.e. in the heart, the vasculature, including smooth muscle cells and endothelial cells or in blood cells (Nishimura *et al.*, 2017). Studies using combined techniques, such as selective antagonists, or tissue selective or global P2YR subtype deficient mice, helped to elucidate the role of the P2YRs in most aspects of the cardiovascular system.

The earliest papers on the role of adenine compounds by Drury & Szent-Gyorgy in 1929 reported on their effect on the mammalian heart. P2YRs, namely $P2Y_1$ and $P2Y_{11}$Rs, among other P2R subtypes, including P2XRs, are involved in cardiac function both indirectly via blood vessels and nerves and directly, via the cardiomyocytes (Nishimura *et al.*, 2017). These receptors have been reported to exert positive and negative inotropic effects as well as positive and negative chronotropic effects. The nucleotides also play a role in ischemic preconditioning. All known receptor subtypes have been found, by RT-PCR, in cardiomyocytes. However, the cardiac function and vascular tone were evaluated in $P2Y_1$R-deficient mice without detectable differences in wild-type mice (Gachet, unpublished work). These data do not mean that these receptors are not involved in these functions, but clearly they either only participate in the regulation of these functions or the deficient mice compensated for receptor deficiency. Studies on isolated tissues from these mice indicate a role for $P2Y_1$ and $P2Y_{11}$Rs in calcium signalling in the cardiomyocytes (Nishimura *et al.*, 2017).

More is known concerning the vascular function, and one has to subdivide it in endothelial function and smooth muscle cell function. In the endothelium, $P2Y_1$, $P2Y_2$, $P2Y_4$ and $P2Y_6$Rs are responsible for nucleotide-induced vasodilation through release of nitric oxide and prostacyclin (Nishimura *et al.*, 2017). In smooth muscle cells, $P2Y_2$, $P2Y_4$ and $P2Y_6$Rs are responsible for vasoconstriction. $P2Y_{12}$R has been found to be expressed in rat capillary endothelial cells (Simon *et al.*, 2002), while other researchers found it on smooth muscle cells where it may trigger vasoconstriction (Wihlborg *et al.*, 2004) and smooth muscle cell proliferation during atherosclerosis (Rauch *et al.*, 2010).

Indeed, in addition to their short-term effects on vascular tone, nucleotides and P2Rs are also involved in long term trophic effects on cell growth, proliferation and death which has great implications for diseases such as atherosclerosis and restenosis (Nishimura *et al.*, 2017). The $P2Y_1$R especially has been shown to play a key role during acute vascular inflammation (Zerr *et al.*, 2011) and in atherosclerosis in apolipoprotein E (ApoE)-deficient mice (Hechler *et al.*, 2008). Similar results have been reported concerning the $P2Y_2$ (Stachon et al., 2014) and $P2Y_6$Rs (Garcia et al., 2014) in vascular inflammation.

P2YRs are expressed in all blood cells including monocytes, granulocytes, dendritic cells, red blood cells and platelets. ATP and ADP are major agonists for platelets where they act on P2X1R, $P2Y_1$ and $P2Y_{12}$Rs in a coordinated fashion (Gachet, 2006). The main role of blood platelets is to ensure primary haemostasis, which is the maintenance of vessel integrity and cessation of bleeding

upon injury. While playing a major part in acute arterial thrombosis, platelets are also involved in inflammation, atherosclerosis, and angiogenesis. ADP and ATP play a crucial role in platelet activation, and their receptors are potential targets for antithrombotic drugs. $P2Y_1$ and $P2Y_{12}$Rs selectively contribute to platelet aggregation and formation of a thrombus. Owing to its central role in the growth and stabilization of a thrombus, the $P2Y_{12}$R is an established target of antithrombotic drugs such as clopidogrel, prasugrel and ticagrelor (Nishimura *et al.*, 2017).

P2YRs in immune and pulmonary function

As with P2XRs, nucleotides acting at P2YRs are generally pro-inflammatory, and their antagonists may eventually be used to treat chronic inflammatory and painful conditions. However, a duality has been noted in which the same P2YR subtype is associated with both damaging (or proinflammatory) and beneficial effects.

Immune cells all express various subtypes of P2YRs, which have been shown to be involved in the modulation of inflammation and in immune responses (Idzko *et al.*, 2014). $P2Y_2$R antagonists might be useful in treating pulmonary diseases, other chronic inflammations or cancer. The $P2Y_2$R contributes to inflammatory responses and fibrotic remodeling in allergic and inflammatory diseases, and thus $P2Y_2$R antagonists might prove useful in pulmonary and other chronic inflammatoroy diseases. $P2Y_2$R activation on neutrophils and eosinophils induces the release of IL8 and other inflammatory cytokines. $P2Y_2$R on marcophages and neutrophils, activated by damaged cells, induces phagocytosis of bacteria and apoptotic cells to promote wound healing. Migrating neutrophils release ATP from the leading edge, which can induce chemotaxis of neutrophils, dendritic cells and other immune cells. Platelets that are activated by cancerous tumors release ATP which can open the endothelial barrier to tumor cell extravasation and thus enabling metastasis, by activating an endothelial $P2Y_2$R (Schumacher *et al.*, 2013).

However, there is also justification for examining $P2Y_2$R agonists for therapeutic application. $P2Y_2$Rs on the lung epithelial surface induce Cl^- secretion and improve mucociliary clearance, raising the possibility of using an inhaled $P2Y_2$R agonist for treating cystic fibrosis. Unfortunately, efforts to gain approval of denufosol failed due to the lack of demonstrating long-term efficacy in patients, possibly due to the simultaneous pro-inflammatory effects of $P2Y_2$R activation (Kellerman *et al*., 2008).

On one hand, $P2Y_6$R activation causes release of inflammatory chemokines from immune cells and from epithelial and endothelial cells. On the other hand, $P2Y_6$R induces phagocytosis by microglial cells in the brain, which is beneficial in the removal of dying cell debris. Thus, like $P2Y_2$R, $P2Y_6$R is associated with both beneficial and detrimental effects in disease conditions.

Microglial $P2Y_{12}$R responds to ADP as a “find me” signal and induces activation and chemotaxis, suggesting a role of this receptor in neurodegeneration and pain signaling (Förster & Reiser, 2015; Tozaki-Saitoh *et al.*, 2008). Platelets, which express $P2Y_1$R and $P2Y_{12}$R, themselves play an important role not only in thrombosis but also in modulating inflammatory responses through release of inflammatory mediators or compounds with trophic activity and exposure of P-selectin, CD40, and CD40 ligand. These molecules allow interaction of platelets with immune cells and their subsequent activation with release of a range of inflammatory cytokines and exposure of platelet tissue factor (Morrell *et al.*, 2014). Therefore, in addition to acting as antithrombotics, antagonists and inhibitors of platelet P2YRs could have anti-inflammatory effects.

$P2Y_{14}$R activation promotes chemotaxis in human neutrophils, as studied in cystic fibrosis (Sesma *et al.*, 2016), the release of proinflammatory cytokines from renal intercalated cells in sterile inflammation (Azroyan, *et al.*, 2015), and the degranulation of mast cells (Gao *et al.*, 2013). Thus, $P2Y_{14}$R antagonists are being developed for anti-inflammatory applications (Junker *et al.*, 2016).

P2YRs in the musculoskeletal system

P2YRs are expressed throughout the musculoskeletal system and mediate a variety of pharmacological responses (Burnstock *et al*., 2013). Their physiological and pathophysiological

functions in skeletal muscle cells and cartilage chondrocytes, and how they might be targeted therapeutically, are still unclear, so the focus here is on bone, which not only provides support and protection for the body, but also plays a central role in Ca^{2+} homeostasis. It is a dynamic tissue that turns over continually throughout life, and so requires a balance, termed bone remodelling, between formation, controlled by osteoblasts, and resorption, controlled by osteoclasts, and coordinated by osteocytes and stem cells (Burnstock *et al*., 2013; Wang & Gartland, 2014).

Musculoskeletal disorders (MSDs) encompass numerous conditions that are characterised by pain and reduced mobility (Burnstock *et al.*, 2013). Furthermore, their incidence is increasing worldwide. MSDs are also a huge financial burden on health care systems and many treatments are only moderately efficacious and/or expensive, so MSDs are an unmet health need. P2YRs represent potentially novel therapeutic targets for treating MSDs.

P2YRs in osteoporosis. ATP, the main P2YR agonist released in bone, has been implicated in osteoarthritis, rheumatoid arthritis and cancer-induced bone diseases, but the P2X7R is the main site of action studied (Wang & Gartland, 2014). In contrast, there is evidence for the involvement of multiple P2YR subtypes in osteoporosis. This is the most common MSD, particularly in post-menopausal women. Oestrogen normally inhibits bone resorption by osteoclasts and when this action is lost post-menopause, bone remodelling becomes unbalanced. Resorption exceeds formation, making bones fragile and more likely to break. As well as stimulating P2YRs *per se*, ATP is dephosphorylated to ADP in the extracellular space by ectonucleotidases, and together, the two agonists stimulate most of the eight P2YR subtypes (see Abbracchio et al., 2006, 2019). The limited availability of potent, subtype-selective antagonists has hindered progress in determining which P2YR subtype(s) are stimulated in bone. Consequently, most advances have arisen from mouse mouse P2YR gene KO studies.

$P2Y_2R$. The P2YR2 genotype might be a useful prognostic marker for osteoporosis. Analysis of SNPs in Danish post-menopausal women found an association between the Arg312Ser SNP, which causes a $P2Y_2R$ gain-of-function, and higher bone mineral density (BMD) and lower rates of bone loss (Wesselius *et al*., 2011). A subsequent study in Dutch female fracture patients identified a higher incidence of another SNP, Leu46Pro (Wesselius *et al*., 2013). It is not known yet, however, how this SNP affects $P2Y_2R$ function.

Deleting the mouse $P2Y_2R$ gene has produced conflicting results. Higher bone mineral content (BMC) and BMD compared to wild-type animals was seen by Orriss *et al*., (2017), but Xing *et al*. (2014) reported lower bone volume and strength. Consistent with the latter, overexpression of $P2Y_2R$ in female rats increased femoral length and strength of the femoral neck, but had no effect on BMD (Ellegaard *et al*., 2017). That these disparities are due to differences in the mouse genetic background or in methodology used cannot, at present, be discounted. The recent commercial availability of AR-C118925XX, a selective, potent and competitive $P2Y_2R$ antagonist (Rafehi *et al*., 2017) may help clarify these issues.

$P2Y_{12}R$. ADP, but not ATP, stimulates $P2Y_{12}$ and $P2Y_{13}$Rs and both subtypes appear to modulate bone remodelling. $P2Y_{12}$R-KO mice showed lower pathological and age-related bone loss (Su *et al*., 2012). Consistent with this, treating wild-type mice with the selective $P2Y_{12}$R antagonist, clopidogrel, which is widely prescribed as an antithrombotic agent, inhibited osteoclast formation and increased bone mass. However, a contemporaneous cohort study of osteoporotic fracture in Danish patients prescribed clopidogrel, found a dual effect. Those receiving the clinically-recommended high dose had an increased risk of fracture, whereas a low dose was associated with a lower risk than people who had not been exposed to clopidogrel (Jørgensen *et al*., 2017). Thus clopidogrel may potentially increase the risk of osteoporosis, though it should be noted that, whilst patients with stroke have an increased risk of osteoporotic fractures, clopidogrel does not appear to increase the fracture risk (Jørgensen *et al*., 2017).

$P2Y_{13}R$. Several studies using $P2Y_{13}$R-KO mice indicate that this receptor may be protective towards bone growth. First, the mice showed reduced bone trabecular volume and rate of formation, reduced number and activity of trabecular osteoblasts and less bone loss due to lack of oestrogen

after ovariectomy (Wang *et al*., 2013b). In addition, there was greater bone formation in response to mechanical loading, possibly because the receptor mediates negative feedback of the osteogenic agonist, ATP, release from osteoblasts (Wang *et al*., 2013b). Finally, mesenchymal stem cells tended to differentiate towards adipocytes rather than osteoblasts (Biver *et al*., 2013). Thus. exercise combined with a $P2Y_{13}$ antagonist is potentially a novel treatment for osteoporosis.

$P2Y_6R$. $P2Y_6R$ is activated by UDP, which appears to be released endogenously, as $P2Y_6R$-KO mice had increased BMC, cortical bone volume, and cortical thickness in the long bones and spine, but trabecular bone was unaffected (Orriss *et al*., 2011). Thus $P2Y_6$ antagonists have potential for treating osteoporosis.

Conclusions

Discovering biomedical applications of agonists or antagonists of P2YRs has proven challenging, due to difficulties in using inherently unstable mono- and dinucleotides as pharmacological probes and the complex biological nature of ubiquitous P2YRs. Purinergic signalling pioneered by Burnstock and colleagues includes the involvement of P2YRs in all physiological systems, including (but not limited to) the following systems: CNS and peripheral nervous, endocrine and exocrine, cardiovascular, immune and musculoskeletal. Selective agonists and antagonists are important for studying the roles of P2YR subtypes in physiology and pathophysiology, and their SARs has been developed in detail through extensive chemical efforts. Nevertheless, there are not yet selective and versatile agonists and antagonists for all of the P2YR subtypes.

Phylogenetic studies have analyzed the evolutionary history of P2YRs. In sum, P2YRs evolved in early vertebrate evolution, and an expansion of P2YRs and P2YR-related sequences is found in vertebrates. X-ray crystallographic structures have been determined for members of the two P2YR subfamilies: the G_q-coupled $P2Y_1R$ (with orthosteric and allosteric antagonists) and the G_i-coupled $P2Y_{12}R$ (with orthosteric agonists and antagonists), and these structures are surprisingly structurally distinct. Phylogenetic, mutational and crystallographic studies suggest that $P2Y_1R$ and $P2Y_{12}R$ evolved independently by convergent evolution presenting the same agonist specificity but structurally different binding sites.

Nucleotides acting at P2YRs are generally pro-inflammatory, and their antagonists may eventually be used to treat chronic inflammatory conditions. However, a duality has been noted in which the same P2YR subtype is associated with both damaging (or proinflammatory) and beneficial effects. Targeting of specific P2YR subtypes has already been shown to be therapeutically useful in case of $P2Y_{12}$ antagonists as antithrombotics, and $P2Y_2$ agonists for dry eye disease. Current research data indicate that both the clinically validated P2YR subtypes and the other six subtypes have great potential for the development of new pharmacotherapeutic strategies and novel future drugs.

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Table 1. Properties of human P2YRs and key agonist/antagonist ligands (Jacobson *et al.*, 2015; Rafehi and Müller, 2018; Abbracchio *et al.*, 2019). If no compound number is given, the structure is not shown. The affinity and selectivity in other species is not shown.

Group	Subtype, gene symbol, chromo-some	Principal G protein coupling; distribution	Ligand agonist/ antagonist	pEC_{50}, pIC_{50} or pK_i	Other interactions, Subtype
$P2Y_1$-like	$P2Y_1$, P2RY1, 3q25.2	G_q; platelets, heart, skeletal muscle, brain, intestine	ADP **1**	5.09	$P2Y_{12,13}$
			2-MeSADP **8**	5.60	$P2Y_{12,13}$
			MRS2365 **9**	9.40	selective
			ADPβS	5.62	$P2Y_{12}$
			MRS2500 **10**[d]	9.02	selective
			MRS2279[d]	8.10	selective
			MRS2179[d]	6.48	selective
			MRS2298	7.20	selective
			MRS2496	5.82	selective
	$P2Y_2$, P2RY2, 11q13.4	G_q, G_i; endocrine, reproductive & immune systems, skeletal and cardiac muscle, lung, intestine	UTP **4**	7.22	$P2Y_4$
			MRS2698	8.10	selective
			ATP **2**	7.07	$P2Y_{4,11}$ + others
			Ap_4A **5**	6.1	$P2Y_{12}$ and others
			Up_4U **6**	6.68	$P2Y_4$ (6.89), $P2Y_6$ (5.94)
			PSB-1114 **15**	6.87	selective
			MRS2768	5.72	selective
			denufosol	6.66	$P2Y_4$ (6.10)
			AR-C118925 **16**	7.24	selective
	$P2Y_4$, P2RY4,	G_q, G_i;	UTP **4**	6.26	$P2Y_2$
			GTP	5.18	nonselective

	Xq13.1	placenta, brain, intestine, lung, heart, prostate, fat	MRS4062 **17**	7.64	selective
			ATP **2**[a]	6.15	$P2Y_{2,11}$ + others
			PSB-16133 **18**	6.63	selective
			PSB-1699 **19**	6.39	selective
	$P2Y_6$, P2RY6, 11q13.4	G_q; lung, heart, spleen, placenta, kidney	UDP **3**	6.28	$P2Y_{14}$ (6.80)
			Up_3U	6.57	$P2Y_2$ (5.88), $P2Y_4$ (6.06)
			MRS2957	7.92	$P2Y_2$ (6.77), $P2Y_4$ (6.10)
			PSB-0474 **20**	7.15	selective
			MRS2693 **21**	7.83	selective
			MRS2782 **22**	6.47	$P2Y_{14}$ (7.94)
			INS48823 **23**	6.90	selective
			5-MeO-UDP **24**	7.10	selective
			MRS2795	7.38	selective
			MRS2578 **25**[f]	7.43	selective
			U-phosphosulfate **26**	3.95	selective
	$P2Y_{11}$, P2RY11, 19p13.2	G_q, G_s; spleen, intestine, immunocytes	ATP **2**	4.77	$P2Y_{2,4}$ + others
			ATPγS **27**	4.62	$P2Y_{2,4}$ + others
			AR-C67085 **28**	8.5	$P2Y_{12}$
			S*p*-α-borano-ATP **29**	6.47	$P2Y_1$ (5.92)
			NF546 **30**[c]	6.27	selective
			NF157	7.35	also blocks P2X1,2,3Rs
			NF340 **31**	7.77	selective
$P2Y_{12}$-like	$P2Y_{12}$,[b] P2RY12, 3q25.1	G_i; platelets, brain, immunocytes	ADP **1**[d]	7.22	$P2Y_{1,13}$
			2-MeSADP **8**	8.3	$P2Y_{1,13}$
			Ap_4A **5**	6.0	$P2Y_4$ (5.9), $P2Y_{13}$ (6.7)
			PSB-0739 **38**	9.8	selective

			AZ11931285[d]	~9	selective
			AR-C67085 **28**[d]	8.2	$P2Y_{11}$
			AR-C69931MX **35**	9.40	$P2Y_{13}$ (8.3)
			ticagelor **36**	7.90	selective
			Ap_4A analogue **37**	6.66	$P2Y_1$ (5.67)
			ACT246475 **39**	9.00	selective
			AZD1283 **40**	7.50	selective
			elinogrel **41**	7.64	selective
	$P2Y_{13}$,[b] P2RY13, 3q25.1	G_i; spleen, brain, myeloid cells,	ADP **1**	7.94	$P2Y_{1,12}$
			2-MeSADP **8**	7.72	$P2Y_{1,12}$
			MRS2211 **42**	5.97	selective
	$P2Y_{14}$, P2RY14, 3q25.1	G_i; brain, endocrine & immune systems, muscle, lung, pancreas, intestine, kidney	UDP **3**[d]	6.80	$P2Y_6$
			UDP-glucose **7**[d]	6.45	$P2Y_2$
			MRS2690 **43**	7.31	selective
			MRS2802 **44**	7.20	selective
			MRS2905 **45**	8.70	selective
			PPTN **46**	9.36, 8.22[e]	selective
			MRS4458 **47**	6.77[e]	selective
			MRS4478 **48**	6.57[e]	selective
			MRS4147 **49**	10.10	selective

[a] ATP acts as antagonist at the human $P2Y_4R$, but an agonist at the rat or mouse $P2Y_4R$.

[b] Selective agonists not yet available.

[c] NF546 activates the $P2Y_{11}R$, although it belongs to a structural class of antagonists.

[d] Used as a radioligand, when labelled with [^{3}H], [^{32}P], [^{33}P] or [^{125}I], as appropriate.

[e] Fluorescent antagonist **49** binding assay, underestimates affinity in functional assays.

[f] Noncompetitive antagonist.