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Molecular Recognition at Purine and Pyrimidine Nucleotide (P2) Receptors

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Abstract

In comparison to other classes of cell surface receptors, the medicinal chemistry at P2X (ligand-gated ion channels) and P2Y (G protein-coupled) nucleotide receptors has been relatively slow to develop. Recent effort to design selective agonists and antagonists based on a combination of library screening, empirical modification of known ligands, and rational design have led to the introduction of potent antagonists of the P2X₁ (derivatives of pyridoxal phosphates and suramin), P2X₃ (A-317491), P2X₇ (derivatives of the isoquinoline KN-62), P2Y₁ (nucleotide analogues MRS 2179 and MRS 2279), P2Y₂ (thiouracil derivatives such as AR-C126313), and P2Y₁₂ (nucleotide/nucleoside analogues AR-C69931X and AZD6140) receptors. A variety of native agonist ligands (ATP, ADP, UTP, UDP, and UDP-glucose) are currently the subject of structural modification efforts to improve selectivity. MRS2365 is a selective agonist for P2Y₁ receptors. The dinucleotide INS 37217 potently activates the P2Y₂ receptor. UTP- γ -S and UDP- β -S are selective agonists for P2Y₂/P2Y₄ and P2Y₆ receptors, respectively. The current knowledge of the structures of P2X and P2Y receptors, is derived mainly from mutagenesis studies. Site-directed mutagenesis has shown that ligand recognition in the human P2Y₁ receptor involves individual residues of both the TMs (3, 5, 6, and 7), as well as EL 2 and 3. The binding of the negatively-charged phosphate moiety is dependent on positively charged lysine and arginine residues near the exofacial side of TMs 3 and 7.

Introduction

P2 nucleotide receptors include two families of receptors: Ligand-gated ion channels, designated P2X, and metabotropic “7TM” receptors, having seven transmembrane helical domains, designated P2Y [1]. Just as there are both ionotropic and metabotropic receptors that respond to acetylcholine, there are at least fifteen P2 receptors spanning both categories, that respond to extracellular nucleotides [2-4]. The process of cloning and identifying P2 receptors has been taking place over the last decade, and there are still a number of related orphan receptor sequences that may yet prove to be P2 receptors. The occurrence and physiological importance of these receptor systems are vast; nearly every cell type in the body expresses one or more P2 receptors, and they are involved in numerous physiological processes.

The medicinal chemistry at P2 nucleotide receptors has been relatively slow to develop due to a variety of limitations in the study of nucleotide pharmacology. Nucleotides are subject to metabolic processes, leading to both their degradation and production. This results in endogenous levels of receptor-active agents that are subject to wide fluctuations. A large family of ectonucleotidases [5] is associated with the production and degradation of P2 receptor agonists *in vivo* and consequently with the generation of the biologically-active metabolite adenosine, which can further confound interpretation of pharmacological data. In addition, the synthesis and purification of nucleotide analogues by classical chemical means can often be quite tedious, making extensive studies of structure activity relationships (SAR) difficult [6,7]. The pharmacological evaluation of newly synthesized nucleotide analogues is

also complex [8], due to the lack of high affinity radioligands for nearly all of the P2 receptor subtypes, as well as uncertainty in the correspondence between pharmacological effects and genetically-defined sequences.

The putative endogenous ligands [1,3] thought to act under physiological conditions at P2Y receptors include not only the “classical” P2 agonists ATP **1** and ADP **2**, but also the uracil nucleotides UTP **3**, and UDP **4**, dinucleotides (e.g. Ap4A **5**), and sugar nucleotides (e.g. UDP-glucose **6** and UDP-galactose **7**). Currently, the challenge is to design and synthesize not only selective, stable and bioavailable agonists, but also selective antagonists. The only known agonists are nucleotides, as no nonnucleotide, small molecule mimicks have yet been reported. The existing P2 antagonists also tend to be large and/or polyanionic molecules that display low affinity and selectivity, and are thus unsuitable as pharmaceutical lead molecules. Thus, defining a given receptor subtype pharmacologically using the existing ligand tools is usually a complex challenge. The aim of this review is to summarize major developments over recent years in the medicinal chemistry of P2 receptors, both in the identification of P2X and P2Y receptor agonists and antagonists and in understanding P2Y receptor structure.

P2X Receptor Ligands

There are seven subtypes of subunits of P2X ligand-gated ion channels [9], denoted P2X₁ - P2X₇, and each functional ion channel consists of oligomers, probably trimers. The current knowledge of the structure of P2X receptors, which is derived mainly from mutagenesis studies, is described in another chapter in this volume [10]. Both homo- and heterooligomerization seem to occur commonly, although the P2X₇ subtype exists exclusively as a homomer and the P2X₆ subtype seems never to occur as a homomer. The presence of heteromers complicates the interpretation of structure activity relationships, since each heteromeric complex would have its own pharmacological properties, some of which may be shared with either of the component subunits in homomeric form. The cationic selectivity of the P2X receptors is Na⁺, K⁺ > Ca²⁺ [11]. The distribution of the receptors is broad for many of the subtypes, and many cell types express multiple P2X subunits. The P2X₃ subunit and its heteromers with the P2X₂ subunit are of special interest in pain control, since they occur mainly on the pain pathways, thus selective antagonists may be useful for this envisioned application [12]. Other therapeutic interests related to P2X receptors are: inflammation (P2X₇), neuroprotection (P2X₇), bladder control (P2X₁), cardiac function (P2X₄), cancer (P2X₄ and P2X₇), and the central nervous system (P2X_{2,4,6}) [6].

Agonists

The compound α,β -meATP **8** distinguishes Group I receptors, i.e. P2X₁ and P2X₃ receptors, which are activated by **8** (EC₅₀ value 1 – 3 μ M), from all other P2X subtypes, which are insensitive to this agonist [11]. Another distinguishing feature is the ability of some P2X receptor subtypes (mainly Group I) to desensitize rapidly. Agonist pre-activation of P2X₁ receptors leading to desensitization, for example by **8**, has been used pharmacologically in the absence of antagonists.

The nucleoside 5'-di- and 5'-triphosphate derivatives generally diverge in activity at P2X subtypes [11]. ADP **2** elicits agonist action at the P2X₁ receptor but not at other P2X subtypes. None of the P2X receptors are activated by either AMP or adenosine, although 2-thioether substitution of the 5'-monophosphate may result in considerable activity at some P2X subtypes [13]. Both α,β -methylene, and β,γ -methylene modifications in **8** and **9a**, respectively, as well as the ω -thiophosphate group present in ATP- γ -S **10a** generally increase stability of the triphosphate group towards enzymatic hydrolysis while maintaining potency at P2X₁ receptors [11]. The L-enantiomer of β,γ -methylene-ATP **9b** activates rat P2X₁ receptors with an EC₅₀ value of 2 μ M [11]. Compound **10a** activates all P2X subtypes, except P2X₇ receptors, in the concentration range of 3 – 16 μ M. 2-Thioether substitution of the adenine moiety of ATP is known to enhance potency at various P2 receptor subtypes [13]. Thus, the *p*-aminophenylethylthio analogue (PAPET-ATP) **11** is the most potent agonist yet reported [6] of the rat P2X₃ receptor (EC₅₀ 17 nM). The effects of ribose substitution in ATP analogues on potency at P2X receptors has not been fully explored. The derivative 2'-&3'-*O*-(4-benzoyl-benzoyl)-ATP (BzATP) **12**, which is a mixture of ester species, is of nanomolar potency in activating the P2X₁ receptor [14] and, although less potent at the P2X₇ receptor, it is nevertheless the most potent reported activator of that subtype. Dinucleotides **13** of varying numbers of linked phosphate groups have been studied as agonists and partial agonists of P2X receptors [15]. Other nucleobases have been tested for activation of P2X receptors. Among the few non-adenine derivatives found to activate, 5'-CTP is an agonist of the rat P2X₃ receptor with an EC₅₀ value of 18 μ M [11].

Antagonists

Many of the “classical” antagonists of P2X receptors are highly charged polycyclic compounds. The trypanocidal drug suramin **14** and its derivatives were found to be P2X antagonists, and suramin antagonism of P2 responses is readily reversible upon washout [11]. This antagonist action was unrelated to the clinical use of suramin. The potency order of 14 at P2X receptors is (IC₅₀ in μ M): P2X₁, P2X₅ (1-4) > P2X₂, P2X₃ (10-15) > P2X₇ (78) > P2X₄, P2X₆ (>500). Lambrecht and coworkers have studied related, highly potent antagonists, selective for the P2X₁ receptor, such as NF279 **15** and NF449 **16**, which have roughly nanomolar IC₅₀ values at the rat P2X₁ receptor [16]. Newer and more potent receptor P2X receptor antagonists in this series include NF110 **17**, which shows some selectivity for the P2X₃ receptor in the guinea pig ileum (IC₅₀ = 1.7 μ M) [17].

Some antagonists derived from dyes, such as Reactive-blue 2 **18**, antagonize both P2X and P2Y receptors [18]. Reactive-blue 2 potently inhibits P2X₂ responses with an IC₅₀ value of 0.36 μ M [11]. Frahm and coworkers have introduced novel analogues of Reactive blue 2, including compounds such as **19** [18].

The SAR of antagonists derived from pyridoxal phosphate, which were also introduced by Lambrecht and coworkers, have been explored at P2X receptors [17]. The 2',4'-disulfonate derivative PPADS **20** and its 2',5'-disulfonate isomer “isoPPADS” **21** are somewhat more potent at P2X than at P2Y receptors. The pyridoxal phosphate derivative PPNDS **23** is a highly potent antagonist at the P2X₁ receptor [17]. The phosphate linkage of PPADS analogues may be replaced with more stable phosphonates [19]. For example, the

phosphonate analogue MRS 2257 **22** is a highly potent antagonist at both rat P2X₁ (IC₅₀ 5.1 nM) and P2X₃ (IC₅₀ 21.8 nM) receptors (with the receptors expressed in the *Xenopus* oocyte). Analogues in the PPADS series in which the diazo linkage has been replaced with carbon bridges have been synthesized [20]. One such analogue, MRS 2335 **24**, was roughly equipotent to the corresponding diazo derivative at the P2X₁ receptor. While high potency at P2X receptors has been achieved for PPADS derivatives, a disadvantage is the noncompetitive binding they display, accompanied by slow on- and off-rates [20].

Various nucleotide derivatives have also been found to antagonize P2X receptors. Such nucleotide antagonists are advantageous as they display more favorable binding kinetic properties than the above antagonists discussed previously. Faster on- and off-rates of binding faster than those of PPADS have been demonstrated for the antagonist TNP-ATP **25**, which binds potently to P2X₁, P2X₃, and P2X_{2/3} (heteromeric) receptors [21]. A dinucleotide derivative, Ip₅I **26**, potently antagonizes the P2X₁ receptor [22].

A novel antagonist A-317491 **27**, selective for P2X_{2/3} and P2X₃ receptors, was reported by Jarvis and colleagues [23]. The compound **27**, the *S*-enantiomer, contains three carboxylic acid groups, and is consequently of limited bioavailability. This compound reduces chronic inflammatory and neuropathic pain in the rat.

At the P2X₄ receptor, none of the commonly used antagonists bind appreciably [11]. At P2X₅ and P2X₆ receptors, antagonists have not yet been identified; this due in part to the difficulty of expressing these receptors in *Xenopus* oocytes [11]. The P2X₇ receptor occurs naturally in many mast cell cultures, and its prolonged activation induces the formation of a large pore which passes solutes up to MW ~900. Calmidazolium **28**, a calmodulin inhibitor, blocks some P2X₇ receptor responses with an IC₅₀ value of ~10 nM [11]. P2X₇ receptor antagonists have been identified among a variety of tyrosine isoquinoline derivatives [24], e.g. KN-62 **29a**, which was originally introduced as an inhibitor of CaM kinase II and thus is not receptor-selective. A closely related derivative, KN-04 **29b**, inhibits the human P2X₇ receptor but is inactive at CaM kinase II. KN-62 antagonizes the human P2X₇ receptor more potently than mouse or rat homologues. The tyrosine derivative MRS 2409 **30a** (IC₅₀ of 200 nM at hP2X₇) and its derivatives [25] act as potent antagonists of both mouse and human P2X₇ receptors. A functionalized congener approach to the design of P2X₇ receptor antagonists [25] has resulted in the amine-functionalized antagonist MRS 2483 **30b**. Baraldi and coworkers have explored the SAR in this series of P2X₇ receptor antagonists, identifying **31** as a particularly potent congener with an IC₅₀ value of 1.3 nM at hP2X₇ receptors [26].

A family of adamantane derivatives including **32** have been shown to be antagonists of the P2X₇ receptor [27,28]. Piperidine and piperazine derivatives such as **33** and **34** have been proposed as non-nucleotide P2X₇ receptor antagonists [29,30].

P2Y Receptor Ligands

P2Y receptors are 7TM receptors, which typically couple preferentially, via G_q, to phospholipase C, and lead to a rise in intracellular calcium [31]. Subclasses of P2Y

receptors have been defined based on: clustering of sequence (although, in general there exists a low homology among subtypes), ligand preference, secondary messengers, and receptor sequence analysis. The numbering system for P2Y subtypes is currently discontinuous; P2Y₁ - P2Y₁₄ receptors have been defined [3], however six of the intermediate numbered cloned receptor sequences (P2Y_{3,5,7-10}) do not correspond to distinct, mammalian subtypes of functional nucleotide receptors. A putative dinucleotide receptor [32] has not yet been cloned and in fact may not represent a molecularly distinct species. The distribution of P2Y receptors is broad, and the therapeutic interests include antithrombotic therapy, modulation of the immune system, diabetes, and treatment of cystic fibrosis and other pulmonary diseases.

P2Y₁₂, P2Y₁₃, and P2Y₁₄ receptors couple via G_i proteins and belong to a structurally distinct cluster of P2Y receptor sequences [3,31]. Stimulation of P2Y₁₂ receptors, cloned in 2002 [33], is an important pro-aggregatory signal in platelets. The most recently cloned receptor, P2Y₁₄, responds to UDP-glucose **6** and has a sequence more similar to the P2Y₁₂ and P2Y₁₃ receptors [34] than to the other P2Y subtypes [3]. The P2Y₁₄ receptor is also activated by UDP-galactose **7**. Some recently reported orphan receptors, such as GPR87 and GPR99 [35], have been noted to contain ligand recognition elements previously identified [36] for P2Y₁ receptors.

Adenine Nucleotide-Preferring P2Y Receptors: P2Y₁, P2Y₁₁, P2Y₁₂, and P2Y₁₃

Agonists

P2Y₁ and P2Y₁₃ receptors are more potently activated by ADP **2** than by ATP **1**, while at P2Y₁₁ receptors, ATP is preferred [31,34,37]. At P2Y₁₂ receptors, adenosine 5'-diphosphate derivatives activate and 5'-triphosphate derivatives antagonize. Terminal thiophosphate groups appear in many useful P2Y nucleotide agonists. For example, at P2Y₁₁ receptors, which occur primarily on hematopoietic cells, ATP- γ -S **10a** is a more potent agonist than ATP [31]. The corresponding diphosphate, ADP- β -S **10b** is a potent agonist of the P2Y₁ receptor (EC₅₀ 96 nM), although it also activates the P2Y₁₂ subtype (EC₅₀ 82 nM) [6]. The 2-position of the adenine ring has also proven to be amenable to derivatization for P2Y agonists. Most notably, the 2-alkylthio ethers [38] appear to provide high potency at the P2Y₁ receptor, which are present on platelets and on vascular endothelial cells. The 2-methylthio derivative of ADP **35** is a potent agonist (EC₅₀ in nM) at P2Y₁ (**6**), P2Y₁₂ (**1**), and P2Y₁₃ (**1**) receptors [6,34]. It is weak or inactive at P2Y_{2,4,6,11} receptors. The corresponding triphosphate, 2-MeS-ATP **36**, is a less potent and selective P2Y receptor agonist, since it also activates P2X receptors [11]. At P2Y₁₃ receptors, under optimal experimental conditions, ATP **1** and 2-MeSATP **36** are equipotent. The potency order of **36** at rat P2X receptors expressed in *Xenopus* oocytes is (EC₅₀ in μ M): P2X₃, P2X₁ (0.2-0.4) > P2X₂, P2X₆ (7-9) > P2X₅ (20) > P2X₄ (74) > P2X₇ (300).

A number of adenosine 5'-monophosphate analogues, such as HT-AMP **37** (EC₅₀ 59 nM at the turkey P2Y₁ receptor) have been found to potently activate P2Y₁ receptors [39]. The α -thio modification of AMP analogues (e.g. **38**), increases potency at the P2Y₁ receptor [40].

Such monophosphate derivatives have also been reported to inhibit ectonucleotidases, which complicates their use as P2Y receptor agonists. 5'-(1-Boranotriphosphate) derivatives such as the 2-methylthio derivative **39** have been found to potently activate the P2Y₁ receptor [41]. Two isomers of the boranophosphates were isolated. The more potent isomer of **39** displayed an EC₅₀ value of 2.6 nM at the rat P2Y₁ receptor.

Non-glycosidic substitution of the ribose moiety has also met with success in the design of ligands for P2Y₁ and other P2Y receptors. For example, a dehydroanhydrohexitol (six-membered ring) analogue **40** activates P2Y₁ receptors with an EC₅₀ value of 3.0 μM [42]. Carbocyclic and constrained carbocyclic rings have also been substituted for the ribose moiety. Some of the more useful examples of this approach are methanocarba analogues [42,43], which have conformationally constrained bicyclic carbon rings in place of the ribose moiety that may adopt one of two conformations ((N) =Northern, or (S) =Southern) depending on position of fusion of the two rings. Correlation of ring geometry with the biological activities has helped define the conformational requirements of the ribose moiety in receptor binding. The rigid methanocarba ring systems are present in the P2Y₁ receptor agonists the (N)-isomer MRS 2340 **41** and its less potent (S)-isomer MRS 2312 **42**, which have EC₅₀ values at the human P2Y₁ receptor of 52 nM and 7.2 μM, respectively [43]. The binding site of the P2Y₁ receptor displays a preference for the (N) conformation of the ribose-like ring over the corresponding Southern conformation as represented in an isomeric (S)-methanocarba series of ATP derivatives. The enhancement of P2Y₁ agonist potency upon freezing the preferred conformation in a pseudoribose ring may approach 300-fold. An example of such an enhancement is (N)-methanocarba-β,γ-methylene-ATP **43**, which is a full agonist with an EC₅₀ value at the human P2Y₁ receptor of 158 nM, while the corresponding riboside **9a** is a weak partial agonist [44]. MRS 2365 **44** is the most potent known agonist of the P2Y₁ receptor, with EC₅₀ values at the human P2Y₁ and P2Y₂ receptors of 0.4 nM and ~10 μM, respectively.

Antagonists

The introduction of potent nucleotide-derived antagonists of the P2Y₁ receptor was made possible by the observation that naturally occurring adenosine bisphosphate derivatives such as A3P5P **45** are either partial agonists or antagonists of the receptor [45]. A later generation derivative, MRS 2179 **46**, the corresponding 2-chloro analogue MRS 2216 **47**, and its (N)-methanocarba equivalent MRS 2279 **48a** were demonstrated to be high affinity competitive and selective antagonists at this subtype [42,46,47]. [³³P]MRS 2179 and [³H]MRS 2279 have been introduced as radioligands for the P2Y₁ receptor in platelets and in other tissue [47,48]. Weak antagonism by **46** of the rat P2X₁ receptor expressed in *Xenopus* oocytes was observed, however many of the known P2 receptor antagonists is magnified the potency of in this assay. Thus, for most applications, **46** and its congeners are highly selective for the P2Y₁ receptor, with inactivity demonstrated at P2Y_{2,4,6,11,12,13} and P2X_{2,3,4,7} receptors [34,46]. Unlike **46**, MRS 2216 **47** was inactive at Group 1 P2X receptors [13]. Recently, a highly potent P2Y₁ receptor antagonist in the (N)-methanocarba series, MRS 2500 **48b**, was reported (K_i = 0.79 nM at hP2Y₁). A dipeptide conjugate of adenosine **49a** was found to antagonize hP2Y₁ receptor responses with a K_B value of 4.0 μM [49]. Metabolites of hypolipidemic drugs, such as the coenzyme A conjugate of nafenopin **49b**, were found to act

as P2Y₁ receptor antagonists [55]. **49b** displayed a K_B value of 58 nM at the human P2Y₁ receptor. Raboission *et al.* have synthesized a C-nucleotide **50** that antagonized P2Y₁ receptors [50].

In addition to the approach of rigidifying the ribose moiety in a conformation that approximates the conformation preferred in receptor binding, the opposite approach – using a flexible ribose equivalent – has met with some success. Acyclic nucleotide analogues of bisphosphate antagonists, such as MRS 2298 **51**, were found to be moderately potent P2Y₁ receptor antagonists without residual agonism [51]. A related analogue containing the metabolically-stable phosphonate linkage, MRS 2496 **52**, displayed an IC₅₀ at the rat platelet P2Y₁ receptor of 0.68 μM.

A hybrid molecule SB9 **53**, consisting of PPADS-like and suramin-like components was found to potently antagonize P2Y₁ receptors [53]. Certain PPADS analogues, such as MRS 2210 **54**, display selectivity for P2Y₁ vs. P2X subtypes [54]. Recently, a cyclic depsipeptide YM-254890 **55**, a fermentation product of a *Chromobacterium* isolated from soil, was found to potently antagonize P2Y₁ receptors [64]. **55** inhibited the aggregation of platelets with an IC₅₀ of 31 nM and was inactive at P2Y₁₂ receptors. Derivatives of Acid blue 129, such as **56**, were also found to act as P2Y₁ receptor antagonists [56].

Nucleotide antagonists of high affinity for the platelet P2Y₁₂ receptor were reported, including AR-C69931MX **57** [57], which was in clinical trials as an antithrombotic agent. A nucleotide in this series (AR-C67085MX **58**) has been shown to potently activate the P2Y₁₂ receptor [58]. In this series it has been possible to substitute the unwieldy triphosphate group in this series with uncharged moieties such as short alcohols, esters, etc., thus proving that a highly anionic moiety is not needed for recognition by the P2Y₁₂ receptor. This discovery led to compounds such as AZD6140 **59**, which is an orally active P2Y₁₂ receptor antagonist of nM affinity that inhibits platelet aggregation up to 8 h after administration [59]. The presence of the 3,4-difluorophenyl group limits the metabolism of **59**.

The action of the successful antithrombotic drug clopidogrel **60** is dependent on the P2Y₁₂ receptor present on platelets. **60** produces a metabolite **61**, which acts as an irreversible P2Y₁₂ receptor antagonist [60]. Other nonnucleotide, nonhighly charged P2Y₁₂ antagonists, such as CS-747 **62**, which also acts through a metabolite, and the sulfonamide analog CT50547 **63** have been reported [61,62].

The acyclic template used in MRS 2298 **51** has been adapted to antagonists of the P2Y₁₂ receptor [52]. Upon replacement of the two phosphate groups with hydrophobic esters, such as in the dipivaloate MRS 2395 **64**, the selectivity shifted entirely from the P2Y₁ receptor to the P2Y₁₂ receptor. MRS 2395 displayed an IC₅₀ of 3.6 μM in the inhibition of ADP-induced aggregation of rat platelets. Since both receptor subtypes are integral to ADP-induced platelet aggregation, the inhibition of either P2Y₁ or P2Y₁₂ receptors impedes platelet aggregation. Analogues of ADP having neutral, hydrophobic substitutions at the ribose 2'-and 3'-hydroxyl groups and the adenine NH₂ position were found to antagonize the P2Y₂ receptor. One such analogue is INS 49266 **65**, which displayed a K_B of 361 nM in the

inhibition of platelet aggregation [63]. The agonist potencies of **65** at P2Y₁ and P2Y₂ receptors were >10 and 14 μ M, respectively.

Selective antagonists of the P2Y₁₁ or P2Y₁₃ receptors are still unknown. The P2Y₁₂ antagonist AR-C67085MX **58** acts as a potent agonist at the P2Y₁₁ receptor. The following compounds were found to antagonize action at the P2Y₁₃ receptor (IC₅₀ in μ M): suramin **14** (2.3), PPADS **20** (11.7), Ap4A **5** (0.216), and AR-C69931MX **57** (0.004) [34].

Uracil Nucleotide-Recognizing P2Y Receptors: P2Y₂, P2Y₄, P2Y₆, and P2Y₁₄

Agonists

The P2Y₂ receptor is activated nearly equipotently by UTP and ATP, but is not activated by the corresponding 5'-diphosphates, i.e. UDP and ADP. The P2Y₄, P2Y₆, and P2Y₁₄ receptor are primarily activated by uracil nucleotides. The P2Y₆ receptor is activated by uridine 5'-di- but not 5'-triphosphates, while the human P2Y₄ receptor is activated by uridine 5'-triphosphates and inhibited by adenosine 5'-triphosphates [4,65]. Uridine β -thiodiphosphate (UDP- β -S) **66** and the γ -thiophosphate (UTP- γ -S) **67** are selective agonists for P2Y₆ and P2Y₂/P2Y₄ receptors, respectively [66]. Numerous substitutions of the uracil ring of UTP have been reported to reduce potency at the P2Y₂ receptor [67]. The 5-bromo modification of UDP preserves potency at the P2Y₆ receptor [8]. The adenine dinucleotide Ap4A **5** is a potent agonist at the rat P2Y₄ receptor and is less potent than ATP at P2Y₂ receptors [8]. Some uracil dinucleotides, such as INS 365 **68**, potently activate the P2Y₂ receptor. Newer generation P2Y₂ receptor agonists such as INS 37217 **69** have been reported [68,69]. P2Y₂ receptor agonists are of clinical interest for the treatment of pulmonary and ophthalmic diseases, and possibly cancer. Ribose substitution with the (N)-methanocarba ring system has been shown to preserve the potency of both adenine and uracil nucleotides at the P2Y₂ receptor, and UTP (e.g. MRS2341 **70**) at the P2Y₄ receptor [43]. However, inclusion of the same (N)-methanocarba ring system in the corresponding 5'-diphosphate prevented activation of the P2Y₆ receptor.

Antagonists

Suramin **14** is a weak antagonist at the P2Y₂ receptor with an IC₅₀ value of 48 μ M [4]. A family of selective, heterocyclic antagonists of the P2Y₂ receptor based on thiouracil, including AR-C126313 **71a** and AR-C118925 **71b**, has been reported [70,71]. Reactive blue-2 **18** at a concentration of 100 μ M effectively blocks rat P2Y₄ receptors, but only partially blocks human P2Y₄ receptors. ATP antagonizes the human but not rat P2Y₄ receptor [69]. The isothiocyanate DIDS **72** has been shown to weakly antagonize the P2Y₆ receptor, although it is not selective since it also acts at P2X receptors [72].

Flavonoids have been identified as a new lead for the design of P2Y₂ receptor antagonists [73]. Tangeretin **73**, is a potent, noncompetitive, antagonist with an IC₅₀ value of 12 μ M. Antagonists of the P2Y₁₄ receptor are still unknown.

P2Y Receptor Structure

The structures of P2X receptors [10] are not yet amenable to molecular modeling due to the lack of a protein template. However, P2Y receptors have been successfully modeled using the high resolution structure of bovine rhodopsin as a template [36,51,74-77] by means of the homology modeling technique. Our models descend from a multiple sequence alignment based on a combined manual and automatic approach which take into account not only the primary structure of the proteins but also the 3D information deducible from the secondary and tertiary structure of the template.

As all other GPCRs, P2Y receptors are constituted by a single polypeptide chain, which crosses the cell membrane seven times, forming seven TMs connected by three extracellular (ELs) and three intracellular loops (ILs). The amino terminal region (NT) is located outside the cell, while the carboxyl terminal region (CT) is located in the cytoplasm (Figure 1). The great majority of the TM residues are arranged in α -helical structures, while in EL2 an antiparallel β -sheet is present. Especially in the regions with defined secondary structure there is a great overall similarity between all P2Y models and the template, the only significant deviation being present in very flexible NT, EL1, EL3, IL1, IL2 and IL3. As in the crystal structure of rhodopsin the alpha-helical structure of TM3, TM4 and TM6 extends into the cytoplasm for all the P2Y subtypes. At the cytoplasmic end of TM7 in all subtypes, with the exception of P2Y₂ and P2Y₁₁, the protein folds at an angle of $\sim 90^\circ$ to form a helical segment (H8) that runs parallel to the plane of the cell membrane.

Ligand affinity SAR, sequence analysis of cloned P2Y receptors and site-directed mutagenesis studies [54,74,77,78] have led to the refinement of computer-based models for ligand binding to P2Y receptors. Structural insights have been gained using molecular modeling based on a rhodopsin template in conjunction with mutagenesis to suggest recognition elements important for nucleotide binding in TMs 3, 5, 6, and 7 and in EL2 [75].

Most of the mutagenesis work in the P2Y family has been carried out on the human P2Y₁ receptor. In order to ascertain which residues of this receptor were involved in ligand recognition, individual residues of both the TMs (3, 5, 6, and 7), as well as EL 2 and 3 were mutated to alanine and various charged residues [36,77], a cluster of positively charged lysine and arginine residues near the exofacial side of TMs 3 and 7 and, to a lesser extent TM6, putatively coordinated the phosphate moieties of nucleotide agonists and antagonists. Agonists were inactive at R128A (TM3) and R310A and S314A (TM7) mutant receptors and had a markedly reduced potency at K280A (TM6) and Q307A (TM7) mutant receptors. Positively charged residues of the human P2Y₂ receptor (H262, R265, and R292 in TM6 and TM7) were similarly found to be critical for activation [76] suggesting that residues on the exofacial side of TM3 and TM7 were critical determinants of the ATP binding pocket. In contrast, there was no change in the potency or efficacy of agonists in the S317A mutant receptor, and alanine replacement of F131, H132, Y136, F226, or H277 resulted in mutant receptors that exhibited a 7- to 18-fold reduction in potency compared to that observed with the wild type receptor. These residues thus appear to serve a less important modulatory role in ligand binding to the P2Y₁ receptor.

Several charged residues in EL2 (E209) and EL3 (R287) were critical for receptor activation, suggesting that the role of the ELs in ligand recognition was as important as that of the TMs [36,77]. Moreover, energetically favorable “meta-binding sites” in the P2Y₁ receptor have been defined, involving the critical residues of the ELs. At these docking sites for nucleotides that are postulated to lie distal to the principal TM site, a ligand may bind en route to the principal TM binding site. Such secondary binding sites may then serve to guide the ligand in its approach to the TM binding site and reduce the energy barrier to ligand/receptor complex formation.

Two essential disulfide bridges in the extracellular domains of the human P2Y₁ receptor were also identified: one conserved among GPCRs and another, between the N-terminal domain and EL3, characteristic of P2Y receptors.

Since changes in the potency of 2MeSADP **35** and HT-AMP **37** paralleled the changes in potency of 2MeSATP **36** at the various mutant receptors, it appeared that the β - and γ -phosphates of the adenine nucleotides were less important than the α -phosphate in ligand/P2Y₁ receptor interactions [75]. However, T221A and T222A mutant receptors exhibited much larger reductions in triphosphate (89- and 33-fold versus wild type receptors, respectively) versus dior mono-phosphate potency, a result indicating a greater role of these TM5 residues in γ -phosphate recognition.

Taken together, the results suggest that the adenosine and α -phosphate moieties of ATP bind to the P2Y₁ receptor in the exofacial side of the cavity delimited by TM3, TM6 and TM7 and capped with EL2.

Recently, a detailed model of antagonist binding to the P2Y₁ receptor binding was presented [75]. Ligand docking in the P2Y₁ receptor model provided a hypothesis for the coordination of nucleotide antagonists in the TM regions, consistent with site-directed mutagenesis results, with a binding mode very similar to the one of the agonist. Molecular recognition in the P2Y₁ receptor of nonnucleotide antagonists such as derivatives of PPADS **20**, has also been studied [54]. The structural similarity between the potent nucleotide antagonist, MRS 2179 **46**, and nucleotide agonists suggests that receptor activation resulting in a specific conformational change depends on subtle differences between ligands [78].

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Fig. (1).

Schematic structure of showing general features of P2Y receptors, including seven TMs (indicated with Arabic numerals) and a proposed internal helix 8. The putative nucleotide binding site, based on mutagenesis and molecular modeling, experiments is indicated. Key residues found to interact with the ligand in the human P2Y₁ and P2Y₂ receptors are indicated on the helix by identifiers of the form X.YZ, where X is the helix number and YZ is the amino acid residue numbered in relation to the residue of each helix, which is most highly conserved among Group 1 GPCRs [78]. A table at the bottom gives the identity of these key residues within the family of P2Y receptors.

Chart 1.

Chart 2.

Chart 3.

Chart 4.

Chart 5.

Chart 6.

Chart 7.

Chart 8.

Chart 9.

Chart 10.

Chart 11.