P2X Receptors as Drug Targets

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ABBREVIATIONS: AACBA: N-(adamantan-1-ylmethyl)-5-([(3R-amino-pyrrolidin-1-yl)methyl]-2-chloro-benzamide; A–317491: 5-[[[(3-Phenoxyphenyl)methyl][][](1S)-1,2,3,4-tetrahydro-1-naphthalenyl]amino]carbonyl]-1,2,4-benzenetricarboxylic acid; A-438079: 3-[[5-(2,3-Dichlorophenyl)-1H-tetra zol-1-yl]methyl]pyridine; A-740003: N-[1-[[Cyanooligo][5-quinolinylamino]methylene]amino]-2,2-dimethylpropyl]-3,4-dimethoxybenzeneacetamide; αβmeATP: αβmethylene-adenosine 5′-triphosphate; ADPβS Adenosine 5′-(β-thiodiphosphate); BBG: Brilliant Blue G; βγmeATP: βγmethylene-adenosine 5′-triphosphate; GSK314181A: (adamantan-1-ylmethyl)-5-[(3R-amino-pyrrolidin-1-yl)methyl]-2-chloro-benzamide; IL-1β: interleukin 1β; Iso-PPADS: pyridoxal phosphosphate-6-azophenyl-2′,5′-disulfonic acid; KN62: 4-[(2S)-2-[(5-isoquinolynlsulfonyl)methylamino]-3-oxo-(4-phenyl-1-piperazinyl)propyl] phenylosoquinoline sulfonic acid; MK-3901: N-[1(R)-(5-Fluoropyridin-2-yl)ethyl]-3-(5-methylpyridin-2-yl)-5-[5(S)-(2-pyridyl)-4,5-dihydroisoxazol-3-yl]benzamide; MRS2220: Cyclic pyridoxine-R45-monophosphate-6-azophenyl-2′,5′-disulfonic acid; NF023: 8,8′-[carbonylbis(imino-3,1-phenylen ecarboxylimino)]bis-1,3,5-naphthalene-trisulphonic acid; NF023: 8,8′-[carbonylbis(imino-3,1-phenylen ecarboxylimino)]bis-1,3,5-naphthalene-trisulphonic acid; NF449: 4,4′,4″-[carbonylbis(imino-5,1,3-benzenetriyl-bis(carboxylimino))]tetakis-1,3-benzene disulfonic acid; NF770: 7,7′-
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(carbonylbis(imino-3,1-phenylenecarbonylimino-3,1-(4-methylphenylene)carbonylimino))bis(1-methoxy-naphthalene-3,6-disulfonic acid);

NF778: 6,6′-(carbonylbis(imino-3,1-phenylenecarbonylimino-3,1-(4-methylphenylene)carbonylimino))bis(1-methoxy-naphthalene-3,5-disulfonic acid);

PPADS: pyridoxal phosphate-6-azophenyl-2′,4′-disulfonic acid; PSB-1011: disodium 1-amino-4-[3-(4,6-dichloro[1,3,5]triazine-2-ylamino)-4-sulfophenylamino]-9,10-dioxo-9,10-dihydroanthracene-2-sulfonate; PSB-12062: N-(p-methylphenylsulfonyl)phenoxazine; RO3: 5-(2-isopropyl-4,5-dimethoxy-benzyl)pyrimidine-2,4-diamine; RO4: 5-(5-iodo-2-isopropyl-4-methoxy-phenoxy)pyrimidine-2,4-diamine; TNP-ATP: 2′,3′-O-(2,4,6-trinitrophenyl)adenosine 5′-triphosphate; YO-PRO-1: 4-((3-methyl-2(3H)-benzoazolylidene)methyl)-1-(3-(trimethylammonio)propyl)quinolinium
ABSTRACT

The study of P2X receptors has long been handicapped by a poverty of small molecule tools that serve as selective agonists and antagonists. There has been progress, particularly in the past 10 years, as cell-based high throughput screening methods were applied, together with large chemical libraries. This has delivered some drug-like molecules in several chemical classes that target selectively P2X1, P2X3 or P2X7 receptors. Some of these are, or have been, in clinical trials for rheumatoid arthritis, pain and cough. Current preclinical research programs are studying P2X receptor involvement in pain, inflammation, osteoporosis, multiple sclerosis, spinal cord injury and bladder dysfunction. The determination of the atomic structure of P2X receptors in closed and open (ATP-bound) states by X-ray crystallography is now allowing new approaches by molecular modelling. This is supported by a large body of previous work using mutagenesis and functional expression, and is now being supplemented by molecular dynamic simulations and in silico ligand docking. These approaches should lead to P2X receptors soon taking their place alongside other ion channel proteins as a therapeutically important drug targets.
Introduction

Drugs acting on ion channels are among the oldest therapeutics, with quinine, procaine and curare among the best known. They also include some of the most widely prescribed, such as benzodiazepines, calcium channel blockers and sulfonlurea anti-diabetic agents. As ion channel families have been identified over the past thirty years and their genes cloned (Alexander et al., 2011), there has been a major effort to develop small molecule agonists and antagonists that are specific, selective and safe (Wickenden et al., 2012).

Three main classes of ligand-gated channels are now recognized (Fig. 1). The first is the pentameric nicotinic superfamily: these channels may show selectivity for cations or anions, and includes members gated by acetylcholine, 5-hydroxytryptamine, γ-aminobutyric acid, glycine and (in invertebrates) glutamate. The second family are tetramers, and include cation-selective channels gated by extracellular glutamate as well as others gated by intracellular cyclic nucleotides. This family also has potassium-selective members, such as the inward rectifiers which are gated by intracellular ATP by virtue of an associated sulfonylurea binding protein. The development of these receptors as drug targets has been reviewed (Bowie, 2008; Hurst et al., 2012). The third family includes two small groups, which are similar in overall membrane topology, but unrelated in primary amino acid sequence. One group includes the four subunits that form acid-sensing ion channels and as well as the four subunits that form epithelial sodium channels. The other group includes the seven P2X receptor subunits. In either group of this third family, channels are formed by assembly of three subunits, either as homotrimers or heterotrimers.

P1 and P2 receptors were first distinguished by Burnstock (1978), on the basis of whether adenosine or adenosine 5′-triphophosphate (ATP) was the more effective ligand. The P1 classification was later replaced by A, as it was realised that the preferred agonist is adenosine rather than ATP (A₁, A₂A, A₂B and A₃: Alexander et al., 2011). Burnstock and Kennedy (1985) introduced the designators P2X and P2Y, on the basis of agonist and antagonist selectivity in a range of tissues. Adenosine 5′-triphosphate (ATP) analogs in which methylene groups replaced oxygen atoms in the phosphate chain (αβmeATP and βγmeATP) tended to activate P2X receptors, whereas an adenosine 5′-diphosphate analog with a β-sulfur atom (ADPβS) was more selective for P2Y receptors. Also at this time, Gordon (1986) named the purine
receptors on platelets and mast cells P2T and P2Z, respectively. At the time of the first receptor cloning it became unambiguously clear that P2X receptors were ligand-gated ion channels (Valera et al., 1994; Brake et al., 1994), and P2Y receptors were G protein-coupled receptors (Webb et al., 1993). Receptor classification based on agonist rank orders of agonist potency (Ahlquist, 1948) and on receptor dissociation equilibrium constants (Arunlakshana and Schild, 1959) was at this time rapidly giving way to one based on protein primary structure. It was soon realised that P2Z receptors corresponded to P2X7 (Surprenant et al., 1996) and that P2T receptors were the same as P2Y12 (Hollopeter et al., 2001).

As receptors, P2X receptors are selectively activated by ATP, much less well by ADP, and not at all by AMP or adenosine or other purines (e.g. GTP) or pyrimidines (e.g. UTP and CTP). As channels, the conductive pathway is selective for cations over anions: the channel opens within a few milliseconds of ATP application, and closes within tens of milliseconds when the application is discontinued (deactivation). Ionic currents through homomeric P2X1 and P2X3 receptors decline during the application of ATP (desensitization) during tens or hundreds of milliseconds; for P2X4 and P2X2 receptors this decline occurs in seconds or tens of seconds; and for P2X7 receptors there is little decrease in the current even over several minutes. P2X receptors, most markedly P2X7 receptors, show complex gating behavior in which the permeation pathway dilates during several seconds of ATP application from a pore that allows only the passage of a small cations (Na⁺, K⁺, Ca²⁺) to one that allows permeation of larger cations (N-methyl-D-glucamine) and dyes such as ethidium and YO-PRO-1.

**P2X Antagonists**

One of the first reported antagonists of ATP receptor-mediated effects on smooth muscle contractility was the alkaloid adrenergic receptor antagonist quinidine (Burnstock, 1972). Many early nonselective ATP antagonists were high molecular weight organic molecules that included derivatives of the antiparasitic agent suramin, and various histochemical dyes (Jacobson et al., 2002). Suramin is a large polysulfonated molecule (Fig. 2) that is active at multiple targets including HIV-reverse transcriptase, vasoactive intestinal peptide receptors, G protein-coupled receptors and multiple P2 receptor subtypes (Jacobson et al., 2002). A number of truncated forms of suramin have been identified that have increased potency and
selectivity for P2X receptors (Fig. 2). For example, NF023 is approximately 10 to 20-fold more selective for P2X versus the P2Y receptors. Other suramin derivatives, including NF279 and NF449 are potent P2X<sub>1</sub> receptor antagonists (Ziyal et al., 1996; Damer et al., 1998) (Fig. 2). However, NF279 also effectively blocks slowly desensitizing P2X2 and P2X7 receptors (Donnelly-Roberts et al., 2009). Brilliant blue G (BBG; Fig. 3) is a potent antagonist at P2X<sub>7</sub> receptors with an IC<sub>50</sub> value of approximately 400 nM (Bultmann et al., 1994; Jiang et al., 2000). However, BBG was recently reported to block voltage-gated sodium channels at low micromolar concentrations (Jo and Bean, 2011).

Another structural class of P2X receptor antagonist includes derivatives of the coenzyme pyridoxal-5'-phosphate such as PPADS (Jacobson et al., 2002). PPADS is most potent at human P2X1, P2X7 and P2Y1 receptors and is approximately 10-200 fold less potent at other P2 receptor subtypes (Donnelly-Roberts et al., 2009). Numerous P2X receptor antagonists based on the PPADS template (Fig. 2) containing pyridoxal and phenyl moieties have been described that show improved potency at some P2X (e.g. P2X1 and P2X3) subtypes (Jacobson et al., 2002).

Nucleotide derivatives have also served as useful ligands to negatively modulate the effects of ATP. The agonist αβ<sub>me</sub>ATP (Fig. 2) can functionally block P2X receptor responses via rapid desensitization of the receptor (Bland-Ward and Humphrey, 1997; Burnstock, 2007). Trinitrophenyl-ATP (TNP-ATP; Fig. 2) and the corresponding di- and mono-phosphate derivatives represent the first nanomolar antagonists at P2X1, P2X3, and P2X2/3 subtypes (Virginio et al., 1998). TNP-ATP is several orders of magnitude less potent at P2X4, P2X5 and P2X7 (Virginio et al., 1998; Jacobson et al., 2006; Jarvis, 2010). TNP-ATP is rapidly metabolized, which limits its utility as a functional P2X3 receptor antagonist (Lewis et al., 1998; Donnelly-Roberts et al., 2008). Oxidized ATP is an irreversible antagonist (Jacobson et al., 2002) for the P2X<sub>7</sub> receptor at high concentrations (> 100 µM) (Donnelly-Roberts et al., 2009). Diinosine polyphosphates (e.g. P<sup>1</sup>P<sup>5</sup>-di-(inosine-5'-pentaphosphate) are highly selective antagonists for fast desensitizing P2X receptors (i.e. P2X1 and P2X3) compared to their activity at more slowly desensitizing P2X receptors (e.g. P2X2) (Jacobson et al., 2002).

The isoquinoline derivative KN-62 (Fig. 3), is an antagonist of Ca<sup>2+</sup>/calmodulin-dependent protein kinase II and a noncompetitive antagonist at P2X<sub>7</sub>
receptors at low µM concentrations (Gargett and Wiley, 1997; Donnelly-Roberts et al., 2009). KN-62 was originally reported to be a selective antagonist of human versus rodent P2X7 receptors (Humphreys et al., 1998): subsequent studies have shown that it is active at mouse P2X7 receptors, though with slower on-rates compared to human P2X7 receptors (Donnelly-Roberts et al., 2009).

Potentiators of the action of ATP at P2X receptors have also been identified. Coomassie blue, Reactive Blue 2 and the pyridoxal phosphate derivative MRS 2220 (Fig. 2) selectively enhance the effects of ATP at fast desensitizing P2X (P2X1 and P2X3) receptors (Soltoff et al., 1993; Jarvis et al., 2001; Jacobson et al., 2002). Trace metals (e.g. zinc and copper) also modulate the activity of multiple P2X receptors via allosteric mechanisms (Coddou et al., 2011).

The generation of receptor selective antagonists for individual P2X receptors has been challenging given the wide diversity of recognition sites and actions for which ATP is a crucial ligand (North, 2002; Burnstock, 2007). The molecular cloning and characterization of the two super families of P2 receptors has enabled discrete interrogations of ligand receptor interactions at individual receptor subtypes (Jacobson et al., 2002; Müller, 2010; Gum et al., 2012). Outlined below is an overview of the currently available antagonists that have potent and selective actions at P2X receptors. Their structures are shown in Figures 2 and 3.

**P2X1.** The aromatic sulfonates, NF449 and NF 279, are potent antagonists of P2X1 receptors (Rettinger et al., 2005). However, these suramin analogs likely have differences in equilibrium kinetics that can modulate their apparent pharmacology (Donnelly-Roberts et al., 2009). While NF449 selectively blocks only P2X1 receptors, NF 279 blocks P2X1, P2X2 and P2X7 receptors with similar affinities under conditions of extended (30 - 60 min) incubations (Donnelly-Roberts et al., 2009).

**P2X2.** Antagonists that have potent and selective activity for P2X2 receptors have been lacking. PPADS, iso-PPADS and TNP-ATP are active in the low micromolar range (Jacobson et al., 2006); however, TNP-ATP has nanomolar affinity for fast-desensitizing P2X1 and P2X3 receptors (Virginio et al., 1998; Jacobson et al., 2002; Jarvis, 2010). Suramin and the dihydropyridine, nicardipine, are also weak antagonists of the P2X2 receptor (Jacobson et al., 2002). Using the anthraquinone scaffold exemplified by Reactive Blue 2, Müller and colleagues generated PSB-1011, a P2X2 antagonist with high selectivity versus P2X4 and P2X7 receptors and
somewhat lower (~5-fold) selectivity versus P2X1 and P2X3 receptors (Baqi et al., 2011). Several suramin derivatives (e.g. NF770 and NF778) that are potent P2X2 antagonists have been generated based, in part, on a structural analysis of the P2X4 receptor (Wolf et al., 2011).

**P2X3.** A-317491 was the first competitive selective small antagonist that was both potent and selective for P2X3 receptors (Jarvis et al., 2002). Subsequently, other potent P2X3 antagonists have been reported, including RO-3 and RO-4, that are at least 100-fold less active across a wide range of kinases, receptors, and ion channels (Gever et al., 2006; Carter et al., 2009). The diaminopyrimidine class of P2X3 antagonists has improved physicochemical properties relative to earlier P2X3 antagonists and is likely to bind to an allosteric site on the channel (Gever et al., 2010; Ballini et al., 2011). Other recently described P2X3 antagonists include MK-3901 and AF-219 (structure undisclosed). The latter has advanced into clinical studies (Gum et al., 2012).

**P2X4.** Despite the successful crystallization of the P2X4 receptor (Kawate et al., 2009), the discovery of potent antagonists for P2X4 receptors is at an early stage. Non-selective antagonists include TNP-ATP, BBG and close analogs of the monoamine uptake blocker paroxetine (Gum et al., 2012). All of these antagonists have low affinity for the P2X4 receptor (IC$_{50}$ ~2-15 µM) and are significantly more potent at other targets (Gum et al., 2012). Recently, a phenoxazine derivative, PSB-12062, was identified as a potent (IC$_{50}$ ~ 1-2 µM), selective (~35 fold versus P2X1, P2X2, P2X3 and P2X7) and non-competitive blocker of P2X4 receptors (Hernandez-Olmos et al., 2012).

**P2X7.** There is now a large diversity of chemotypes that are potent and selective antagonists of P2X7 receptor antagonists (Guile et al., 2009; Gum et al., 2012). Many of the new antagonist pharmacophores exemplified by A-438079, A-740003, and AACBA provide tool compounds with suitable drug-like properties to enable in vivo studies (Gum et al., 2012; Keystone et al., 2012). Early P2X7 antagonists, like KN-62 and BBG show preferential activity at human or rat P2X7 receptors, respectively (Donnelly-Roberts et al., 2007; 2009). However, the apparent species differences for P2X7 antagonists diminish as a function of increased potency for the receptor (Donnelly-Roberts et al., 2009). BBG has recently been shown to potently block sodium channels and P2X4 receptors (Jo and Bean, 2011).
**Therapeutic Indications**

**Pain.** Chronic pain results from a neurochemical and phenotypic sensitization of peripheral and central sensory nerves that is characterized by increased sensitivity to painful stimuli (hyperalgesia) and the perception of pain in response to normally innocuous stimuli (allodynia) (Perl, 2007; Woolf, 2011). This sensitization process can result from acute or persistent tissue trauma and is mediated by many pronociceptive neurotransmitters and neurtrophic factors including ATP, glutamate, substance P, CGRP and proinflammatory cytokines (Basbaum et al., 2009). Exogenous ATP elicits pain in both humans and laboratory animals and enhances pain sensations associated with inflammation (Bleehen and Keele, 1977; Hamilton et al., 2000). The ability of ATP to modulate pain-associated neuronal excitability associated with nociception is mediated directly by activation of homomeric and heteromeric P2X3 receptors on central and distal processes of peripheral nerves, and indirectly by activation P2X and P2Y receptors on glial cells (astrocytes and microglia) in the periphery and spinal cord (Jarvis, 2010).

PPADS and suramin have been used to attenuate nociceptive responses in a variety of experimental pain models (Inoue et al., 2007; Jarvis, 2010). However, attribution to specific interactions with individual P2X receptors is necessarily limited by the non-selective pharmacology of these relatively weak antagonists (Jacobson et al., 2002; Donnelly-Roberts et al., 2007; 2008).

Local administration of the potent but non-selective P2X3 antagonist TNP-ATP into relevant sites has been shown to block the pro-nociceptive effects of P2 receptor agonists such as αβmeATP (Jarvis et al., 2001). Similarly, systemic administration of the selective P2X3 antagonist A-317491 produces dose-dependent effects across multiple pain modalities including neuropathic, inflammatory and nociceptive pain states (Jarvis et al., 2002). The role of homomeric and heteromeric P2X3 receptors in mediating chronic pain states is further corroborated by the ability other structurally different and potent P2X2/3 and P2X3 antagonists (e.g. RO-4 and MK-3901) to reduce nocifensive behavior in chronic pain models (Gum et al., 2012). Interestingly, AZ-2, an antagonist that is selective for only the homomeric form the P2X3 receptor, effectively reversed CFA-induced mechanical allodynia following systemic and intraplantar dosing but was ineffective when dosed intrathecally (Cantin et al., 2010; 2012).
In addition to a direct role of P2X3 receptors to modulate nociceptive sensory processing, P2X4 and P2X7 receptors on activated microglia also influence noxious sensation. P2X7(-/-) and P2X4(-/-) mice show reduced nociceptive sensitivity in neuropathic pain states compared to wild type controls and this phenotype is consistent with the anti-nociceptive phenotype of mice lacking both isoforms of interleukin-1 (α and β)(Chessell et al., 2005; Honore et al., 2009; Jarvis, 2010). Selective P2X7 receptor antagonists, A-740003 and A-438079, reduce inflammatory hyperalgesia in rodents that are not secondary to P2X7-mediated anti-inflammatory effects (see below)(Honore et al., 2006; Donnelly-Roberts et al., 2008). P2X7 receptor-selective antagonists also reduce nociception in some experimental models of neuropathic pain, an effect that is mediated, at least in part, by spinal and/or supraspinal sites of action (Inoue, 2006; McGaraughty et al., 2007).

**Arthritis.** Agonist stimulation of P2X7 receptors that are localized on macrophages and microglia opens a non-selective cation channel, and also leads to the rapid maturation and release of interleukin-1β (IL-1β)(Perregaux and Gabel, 1994; MacKenzie et al., 2001; Ferrari et al., 2006; Di Virgilio, 2006). These actions serve as a mechanistic basis for the role of P2X7 receptor in inflammation. P2X7(-/-) mice show attenuated cytokine signalling, and ATP-evoked processing of pro-IL-1β, as well as decreased signs and symptoms of arthritis in experimental models (Labasi et al., 2002). Pharmacological studies using selective antagonists also support a role for P2X7 receptors in inflammatory conditions. Pre-emptive dosing of AACBA has been shown to attenuate collagen-induced arthritis in rats (Broom et al., 2008). Further, an adamantane-based P2X7 receptor antagonist, AZD9056 (structure undisclosed) provided preliminary signals of clinical efficacy in reducing the symptoms of rheumatoid arthritis in humans; however, this effect was not maintained in a longer duration Phase II study (Keystone et al., 2012).

**Neuroprotection.** In addition to its actions as a fast neurotransmitter, ATP can also function as a danger signal in response to tissue trauma (Di Virgilio, 2006; Ferrari et al., 2006; Miller et al., 2011; Trang and Salter, 2012). Within the CNS, ATP acting at P2X receptors on both neurons and a variety of glial cells (astrocytes, oligodendrocytes and microglia) participates in the communications between neuron and glia (Tozaki-Saitoh et al., 2011). ATP can also serve as an activator of microglia cells associated with conditions of neuronal hyperexcitability and/or injury (Inoue et al., 2007; Inoue, 2006; Tozaki-Saitoh et al., 2011). Several P2 receptors, including
P2X4, P2X7, P2X12 and P2Y14, have been shown to mediate various aspects of microglial cell signalling in the context of chronic pain, spinal cord injury, multiple sclerosis and stroke (Jarvis, 2010; Tozaki-Saitoh et al., 2011). Phenotypic characterization of mice lacking some of these receptors (e.g. P2X4 and P2X7) provides evidence for a beneficial role of blocking these receptors in neuropathological conditions (Khakh and North, 2012): however, rigorous pharmacological support for these roles is not yet available (Jarvis, 2010). Activation of P2X7 receptors leads to the rapid maturation and release of the inflammatory cytokine IL-1β (MacKenzie et al., 2001) which, in turn, can exacerbate neuronal degeneration associated with infection, multiple sclerosis and stroke (Di Virgilio, 2006; Miller et al., 2011). A role for P2X7 receptors in chronic disease is further supported by the positive association of several discrete C-terminal single nucleotide polymorphisms in the P2X7 receptor with diseases such as infection, leukaemia, depression, bipolar disorder and neuropathic pain (Miller et al, 2001; Sorge et al., 2012).

**Bladder.** ATP plays a key role in mechanosensation of hollow organs (Burnstock, 2007). In this regard, homomeric and heteromeric P2X3 receptors are highly localized on lumbosacral and splanchnic nerve terminals that conduct sensations of stretch, pressure and irritation (Ford, 2012). The phenotypes of gene disrupted mice lacking P2X2, P2X3 or both receptors is characterized by reduced bladder reflexes and elevation of bladder volume thresholds (Ford, 2012). Selective P2X3 receptor antagonists including A-317491 and RO-4 reduce bladder hyperactivity in experimental models (Lu et al., 2007; Brederson and Jarvis, 2008; Ford, 2012). These data support further investigation of the roles of P2X3 receptors in overactive/unstable bladder disorders, interstitial cystitis, and benign prostatic hyperplasia (Burnstock, 2007; Ford 2012).

**Airway Hyperactivity.** The neurophysiological role of P2X3 receptors in sensory nerve function in airway passages is similar to that mediating somatic nociception (Undem and Nassenstein, 2009). This similarity has driven hypotheses concerning the involvement of P2X3 receptors in the symptoms of airway dysfunction including cough and bronchial hyperreactivity (Ford, 2012). ATP enhances citric acid-evoked and histamine-evoked cough in preclinical models, effects that can be attenuated by P2X3 selective antagonists (Kamei and Takahashi, 2006; Ford, 2012). In humans, local delivery of ATP initiates cough and bronchospasm (Basoglu et al.,...
In addition to an involvement of P2X3 receptors in neurogenic cough, activation of P2X7 receptors may contribute to inflammation associated with airway dysfunction (Ford, 2012).

**Structure-based Approaches**

P2X receptors are relatively small, trimeric membrane proteins (Figs. 1 and 4C). Each subunit (40-70 kDa) has two membrane spanning domains. The intracellular N-terminus is short (about 30 amino acids): the intracellular C-terminus is longer and quite variable among the seven different subunits. The bulk of the protein (some 270 amino acids, about 30 kDa) thus lies in the extracellular space. There are no known ancillary subunits that are required for function, although several associated proteins have been identified (Kim et al., 2001; Wilson et al., 2002; Masin et al., 2006; Chaumont et al., 2008). Our present understanding of receptor structure is based on the X-ray crystallography of closed (pdb 4DW0) and open (pdb 4DW1) zebrafish P2X4 receptors solved at better than 3Å (Kawate et al., 2009; Hattori and Gouaux, 2012) (Fig. 4A). The proteins purified for crystallization, though functional when expressed in oocytes, were truncated close to where they reach the inner membrane surface. They therefore lack the intracellular domains, and in particular the membrane proximal 15 amino acids which contain a highly conserved motif (YXXXK) in both the N- and the C-terminal regions. It seems likely that this immediately cytoplasmic region has a highly ordered structure, on account not only of its conservation but also because it has been shown to be critically involved in channel desensitization (Werner et al. 1996; Allsopp and Evans, 2011). This makes it possible that the absence of this region might alter the disposition of transmembrane domains from that adopted in the crystals studied to date.

Interpretation of the atomic detail provided by these structures is complemented by fifteen years of functional studies on receptors that have been mutated and expressed. The ATP binding pocket is formed by regions of two different subunits which provide an electropositive environment to the approaching ATP molecule (R290 and K308 on A chain; K69 and K71 on B chain; P2X2 numbering). In the open channel structure, these four positive charges interact directly with oxygen atoms on the phosphate chain (Fig. 4B). In the case of R290 an existing intra-subunit salt bridge (with E167) is replaced by a salt bridge with a γ oxygen of ATP.
(Hausmann et al., 2012). Six further conserved residues contact ATP, several through hydrophobic interactions with the ribose and adenine moiety (L186, L211, I226). Base specificity is provided by direct hydrogen bonds between -NH$_2$ and N$^1$ nitrogen atoms of adenine and the backbone and side-chain oxygens of T184 (Hattori & Gouaux, 2012)(Fig. 4B). The overall movements underlying channel opening are illustrated in Fig. 4A, in which key sections of each subunit of the P2X receptor are analogized to the body parts of a dolphin.

As the binding pocket closes around the bound ATP, the head domain descends, the left flipper retracts, and the dorsal fin rises. These movements of the left flipper and dorsal fin exert a lateral distortion on the lower body domain, flexing the walls outward through inter-subunit separation of its component $\beta$-sheets. This markedly enlarges the lateral portals between each pair of subunits (Fig. 4C), increases the diameter of the extracellular vestibule, and thus separates the outer ends of the TM domains (Fig. 4D). The pulling apart of the outer ends of the TM domains increases the diameter at the narrowest part of the permeation pathway by an iris-like movement of all six transmembrane domains. This involves significant breakage of interactions between the transmembrane domains, and must be associated with the entry of membrane lipid into the large interstices that form between their ends (Hattori and Gouaux, 2012).

The approximation of the head domain and the dorsal fin also can be observed by normal mode analysis and molecular dynamics (Jiang et al., 2012; Du et al., 2012) and can be detected by changes in the fluorescence from suitably attached tetramethylrhodamine (Lörinczi et al., 2012; Dutertre et al., 2012). In the P2X2 receptor, it allows the approach of H120 (on the underside of the head) to H123 (on the upper aspect of the dorsal fin) such that these two residues can form a zinc binding site: this was originally shown to be inter-subunit by Nagaya et al. (2005) and Tittle et al. (2007). Jiang et al. (2012) have shown recently that extracellular zinc binding at this site but in the absence of ATP, can directly promote opening of a P2X receptor which has been biased toward the open state by a minor mutation in the TM2 (P2X2[T339S]: Cao et al., 2007). We note that in the P2X7 receptor three amino acid residues of the dorsal fin are missing, corresponding perhaps to one turn of the short $\alpha$-helix. These include L211 in P2X2 (L217 in zebrafish P2X4) that contributes
directly to the ATP binding site (Fig. 4B): this might account for the very low affinity shown by P2X7 receptors for ATP.

The ATP binding pocket. Nucleotide analogues represent challenging starting points for drug development, given their likely poor stability and multiplicity of recognition sites (Jacobson et al., 2010; Gum et al., 2012). That said, two dinucleoside tetraphosphate containing molecules (diquafosol and denufosol) have sufficient drug-like properties to enable advancement into human clinical trials (Jacobson et al., 2010). The orientation of ATP within its binding site allows attachments to be made at the γ-phosphate and several such compounds retain their agonist activity (for example, P1P5-di(adenosine-5′i1pentaphosphate (Ap5A); Cinkilic et al., 2001). The 2′ and/or 3′-oxygen atoms of the ribose are also exposed to solvent, and the best studied ligands with attachments at these positions are 2′,3′-O-(benzoyl-4-benzyol)-ATP and TNP-ATP. In the former case the molecule remains an agonist activity but, except at P2X7 receptors, it is less efficacious than ATP. On the other hand, TNP-ATP is a competitive antagonist with nanomolar affinity at P2X1 and P2X3 receptors (Virginio et al., 1998). It seems likely that the protruding trinitrophenyl moiety simply prevents jaw closure (Lörinczi et al., 2012), and it will be instructive to determine by molecular docking and mutagenesis why this is more effective in the case of the P2X1 and P2X3 receptors than P2X2 and P2X4, at which it is 1000 times less effective.

The first efforts at developing novel antagonists based on atomic structure are now appearing. Wolf et al. (2011) showed that the suramin analog NF770 was a competitive antagonist at P2X2 receptors (IC50 19 nM): it was 4, 50, >10,000 and >10,000 times less potent at blocking P2X3, P2X1, P2X4 and P2X7 receptors, respectively. The high potency resulted in large part from replacing one of the three sulfonates on the naphthalene moiety with a methoxy group. Molecular docking indicated that the oxygen atom of this CH3O- group hydrogen bonded directly with R290 (chain B) while one of the other two sulfonates interacted closely with G72 (chain A). R290 is critical for ATP binding (see above) and NF770 would therefore serve as a competitive antagonist. The analog NF778 has the methoxy substitution in a different position (1-methoxy-3,5-disulfonic as distinct from 1-methoxy-3,6-disulfonic acid) and shows a much weaker interaction due to the loss of this direct hydrogen bond.
There are other inter-subunit clefts in the ectodomain that might offer attractive drug binding sites, and where conservation among P2X subtypes is less than found at the ATP binding pocket. The lateral portal itself might be considered as a potential antagonist binding, although the fact that it opens to such a large size (Fig. 3C) in the open channel might make complete blockade problematic.

**The transmembrane region.** Ivermectin is a macrocyclic lactone that finds clinical use for oncocerciasis (river blindness)(Fig. 2). It has actions on a range on ion channels (Cully et al., 1994; Krusek and Zemkova, 1994; Kause et al., 1998), but has been particularly useful in the study of P2X receptors because it potentiates currents selectively at P2X4 receptors (Khakh et al., 1999a). Given the potential role for P2X4 receptors on microglia as a therapeutic targets (Inoue et al., 2007), it is also relevant that these actions of ivermectin have been reported not only on heterologously expressed receptors but also on cultured cerebellar microglia (C8-B4 cells)(Samways et al., 2012).

Ivermectin binding involves amino acid residues on both transmembrane domains, but particularly around the outer half of TM1 (Priel and Silberberg, 2004; Silberberg et al., 2007; Jelinkova et al., 2008). Opening of the P2X receptor involves iris-like movements of the transmembrane helices, and this involves considerable rearrangements of contacts between them (Hattori and Gouaux, 2012). For example, in the closed channel a conserved aromatic side chain in the outer half of TM1 (Y42: P2X4 numbering) interacts with residues at the outer end of TM2 residues in the adjacent subunit (I332, I333, M336 in rat P2X4 receptor): this interaction breaks in the open channel as lipid enters the intervening space (Hattori and Gouaux, 2012). Somehow, bound ivermectin favours this separation of the outer ends of the TM helices, thus increasing current amplitude by retarding channel closing (Silberberg et al., 2007). Residues at the very outer end of TM1 contribute to the lower edge of the lateral "portal" through which ions enter the extracellular vestibule and thence the pore itself. These include E51 (rat P2X4: Q52 in rat P2X2, N54 in zebrafish P2X4), which is required for the relatively high fractional calcium entry shown by P2X4 receptors (Samways and Egan, 2007; Samways et al., 2011), and substitution of N54 by E in the zebra fish P2X4 receptor confers sensitivity to ivermectin (prolonged deactivation). Moreover, in addition to increasing and prolonging the current evoked by a brief application of ATP at human P2X4 receptors, ivermectin also reduces the fraction of the current carried by calcium ions (from about 14% to 8%)(Samways et
Taken together, this implies that the ivermectin binding site likely extends along the surface of TM1 from Y42 to E51: when bound it facilitates opening, impairs closing, and also screens any effect of the electronegativity at E51 on the entry of calcium ions. The distance along the surface of the helix from Y42 Cβ to E51 Cβ, taken from a homology model of the rat P2X4 receptor, is 10.4 Å which is only about half of the length of ivermectin in an extended conformation (Albers-Schönberg et al., 1981).

**The permeation pathway** Many ion channels can be directly blocked by agents that enter and bind in the permeation pathway: local anaesthetics such as lidocaine and dihydropyridine calcium channel blockers are good examples. Do such blockers exist for P2X receptors? Given that two open states are described, particularly for P2X7 receptors, it might be asked whether drugs could be developed that block one and not the other. Certainly, mutations in the permeation pathway can have this effect. In P2X4 receptor, a fast sodium-selective current (I₁) and a slower current through an NMDG-permeable pathway (I₂) can be distinguished by their time courses and reversal potentials (Khakh et al., 1999b). I₁ is abolished by the substitution G347R, whereas I₂ is abolished by the substitution G347Y)(G347 is situated one helical turn internal to the narrowest part of the channel: T339 in P2X2, A347 in zebra fish P2X4). One such candidate might be calmidazolium, which at 10 μM blocks the sodium current but not YO-PRO-1 uptake in HEK cells expressing P2X7 receptors (Virginio et al., 1998). Calmidazolium is a monovalent cation which blocks several cation permeable channels (Klockner and Isenberg, 1987; Kleene, 1994). With dimensions of 1.4 x 1.4 x 1.2 nm it may permeate P2X7 receptors in their I₂ dilated state, but not pass in the undilated state (Hattori and Gouaux, 2012). The determination of the structure of a P2X receptor in a further open state, offering a wider permeation pathway, will be important. A structural basis for a similar dynamic selectivity has recently been demonstrated for the related ASIC channel, which adopts a large (1.0 nm) non-selective, open state at pH 7.25 and smaller (0.5 x 0.7 nm) sodium-selective state at pH 5.5 (Baconguis and Gouaux, 2012).

In the case of the P2X7 receptor, it is possible that the C terminus might itself be targeted for drug discovery. In the case of the P2X2, entry into the I₂ state is associated with substantial movements of the C terminus (Eickhorst et al., 2002). There is extensive evidence that an associated intracellular signalling molecule is
engaged by the activated P2X7 receptor, and that this is important for downstream signalling such as activation of caspase and release of interleukin-1β (North, 2002). A recent approach to pain control targeted such an interaction (Sorge et al., 2012). A small peptide corresponding to residues in the cytoplasmic tail of the mouse P2X7 receptor (SLHDSPPTGQGGGYKKRRQRR: underline is 445 to 455 of the receptor, remainder is part of a cell-penetrating TAT peptide) was found to attenuate the mechanical allodynia induced by sciatic nerve injury (Sorge et al., 2012). This is an attractive avenue for drug development, but in this case presumably reflects an interaction between the peptide and the interacting protein (perhaps a Src tyrosine kinase: Iglesias et al., 2008) rather than the P2X7 receptor itself. Such approaches have the advantage of providing selectivity among P2X receptors, given that there is no relatedness among the sequence of amino acid sequences in their C termini (except for the membrane proximal region). But they offer the relative disadvantage that we currently know nothing about the structure of any P2X receptor C-terminus.

**Summary and conclusions**

It is almost twenty years since interest in these receptors accelerated sharply with the cloning of their cDNAs. There remain positives and negatives with regard to P2X receptors as drug targets. The positives include: (1) subunit diversity (human subunits are about 40% identical at the amino acid level; (2) further diversity through channel formation as heterotrimers (e.g. P2X2/3 and P2X1/5); (3) a very restricted tissue distribution for some subunits (e.g. P2X3, P2X7); and (4) a large extracellular domain that undergoes substantial conformational rearrangement on channel opening; and (5) crystal structures available for closed and open (ATP-bound) states. On the negative side, we have limited information as to the key physiological roles for P2X receptors in most physiological and pathological processes. These roles are now beginning to be worked out, with the aid of knock-out mice and small molecule tools. Although there is caution required in the use of available knock-out mice (see Kaczmarek-Hajek et al., 2012) there has been good progress in the development for small molecule tools. As this review illustrates, several such tools are now available for P2X1, P2X2/3, P2X3 and P2X7 receptors and are beginning to appear for P2X2 and P2X4 receptors. We now look forward to seeing more of these moving into animal models of disease and, of course, into trials of clinical efficacy in man.
Authorship contributions

Wrote or contributed to the writing of the manuscript: North and Jarvis
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Footnotes

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Figure legends

Fig. 1. Trimeric P2X receptor compared with tetrameric glutamate and pentameric nicotinic (Cys-loop) receptor. Left, Zebrafish P2X4 receptor (modified from pdb 4DW1). Center, Rat GluA2 receptor (modified from pdb 3KG2). Right, C. elegans glutamate-gated chloride channel (modified from pdb 3RHW). Each is shown viewed from the outside of the cell.

Fig. 2. Chemical structures of non-selective P2X receptor agonists and antagonists, modulators and antagonists at P2X1 receptors. Nucleotide-based agonists are active at multiple P2X receptor subunits. For example, BzATP is a nanomolar agonist at P2X1 and P2X3 receptors and has approximately 10,000 lower affinity for P2X7 receptors. Suramin and PPADS have been historically used as P2X receptor antagonists, but have many off-target pharmacological actions. NF449 has higher selectivity for P2X1 receptors compared to NF279 and NF023. The P2X receptor modulators (Cibacron Blue and MRS 2220) likely bind allosteric recognition sites on their respective receptors.

Fig. 3. Some receptor-selective P2X receptor antagonists. IP5I, RO-3, RO-4 likely bind allosteric recognition sites on their respective receptors. Competitive receptor antagonism has been demonstrated for TNP-ATP, A-317491, A-438079 and A-804598. Several new receptor selective antagonists for P2X2 (PSB-10211, NF770, NF778) and for P2X4 (PSB-12062) have been recently reported. AACBA is also know as GSK 314181A. See text for further details.

Fig. 4. P2X receptor structure. A, two dolphin-shaped subunits are depicted. ATP binding is associated with an upward movement of the dorsal fin (chain A, at back), a downward movement of the head region (chain B, at front) and a retraction of the left flipper (chain A, at front) in the directions shown by the arrows. The lower body (light blue) is flexed outwards, carrying with it transmembrane domains (green). Reproduced (with permission) from Hattori & Gouaux (2012). B, ATP binding pocket. Residues involved in direct interactions with ATP are shown from homology model of the rat P2X2 receptor, based on pdb 4DW1. Atoms identified are: Chain A
(blue) N288 ND2, K308 NZ, R290 NH1; chain B (pink) K71 NZ, K69 NZ, T184 OG1, I226 CG2, L186 CD2, L211 CB, K188 NZ. C, lateral portals that form the ion entry pathway into extracellular vestibule. The lateral portal between two subunits (blue and pink) is outlined by a green oval. Third subunit (yellow) is visible through the open portal. Arrowhead marks level of section shown in part D. Rat P2X2 receptor. D, dilation of outer part of transmembrane domains. Positions of TM1 (filled grey circle) and TM2 (open grey circle) are indicated. Residues shown as space-fill are I328 at outer end of TM2: dashed circle passes through Cα atoms. Rat P2X2 homology model.
Figure 3

P2X2 Antagonists

PSB-10211

NF770

NF778

P2X3 Antagonists

A-317491

MK-3901

RO-4

RO-3

P2X4 Antagonists

PSB-12062

Paroxetine

P2X7 Antagonists

Brilliant Blue G

AACBA

AstraZeneca

A-438079

A-804598

KN-62
Figure 4