

# Nucleotide and Nucleoside Receptors



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Brian King is a Senior Lecturer in the Department of Physiology, and Andrea Townsend-Nicholson a Lecturer in the Department of Biochemistry and Molecular Biology. They share a common interest in purine signalling at recombinant and native P1 and P2 receptors and have worked together over the last seven years on the isolation and characterisation of nucleotide receptors.

### Introduction

Extracellular nucleotide signalling molecules, such as ATP, ADP, UTP and UDP, work through specific ionotropic (P2X) or metabotropic (P2Y) cell-surface receptors. The use of any ligand as a signalling molecule necessitates a mechanism for its inactivation and so, purine nucleotides are hydrolysed to adenosine, a nucleoside that acts through metabotropic adenosine (P1) receptor subtypes, to exert physiological effects in almost all organ systems. Inosine, formed by the deamination of adenosine, has also been shown to have agonist activity at nucleoside receptors. Furthermore, dinucleotides are signalling molecules in their own right – not only at P2 receptors, but also at specific  $A_p_n$  receptors – and, through degradation, can generate nucleosides and nucleotides to broaden their extracellular targets.

Seven P2X, eight P2Y and four adenosine receptor proteins have been cloned and characterised. Despite the ubiquity of both purinergic ligands and purinoceptors, the differential expression of purinoceptor subtypes and ligand-metabolising enzymes allows for the generation of very specific physiological responses in particular cell types or tissues. It is for this reason that nucleotide and nucleoside receptors have now become therapeutic targets for the treatment of conditions as diverse as pain, urinary incontinence, stroke and depression, and the field of purinoceptor research has expanded significantly in the last ten years. In this brief review, we examine a number of the pharmacological tools currently available for the study of nucleotide and nucleoside receptors.

### P2X Receptors

P2X receptors (P2XRs) are ligand-gated ion-channels (LGICs) widely distributed amongst various tissue types, including autonomic, central, enteric and sensory neurons, cochlear and retinal cells, endothelium and epithelium, vascular and visceral smooth muscle, heart and developing skeletal muscle, bone, haemopoietic cells. These LGICs serve to control the excitability of their host cells through two processes: i) influx of extracellular sodium ions to elicit depolarisation, and ii) influx of extracellular calcium ions to activate internal enzymes.

### P2XR assembly

Seven building blocks, or subunit proteins, are involved in the construction of P2XRs. These seven building blocks – the P2X<sub>1</sub> to P2X<sub>7</sub> subunits – have been cloned from human and rat cDNA libraries and, in some cases, also from mouse, guinea-pig, chick and zebrafish libraries.<sup>1,2</sup> Few structural differences have been noted between species orthologues of the same P2X subunit. However, the degree of homology can be as little as 27% between different subunits of the same species.<sup>1</sup> Furthermore, the P2X genes are complex and, during transcription, can undergo alternative splicing which, subsequently, generates variant subunit proteins that are either non-functional, dominant negative or modulatory.

Each of the subunit proteins can form homomeric P2XR assemblies, although there appear to be difficulties forming P2X<sub>5</sub> receptors<sup>3</sup> and P2X<sub>6</sub> receptors.<sup>4</sup> For such difficulties, post-translational modification of subunit proteins – particularly glycosylation – has a strong influence on the functionality of membrane-trafficked P2XRs.<sup>5,6,7</sup> It is currently believed that either three or six glycosylated subunits make a functional P2XR, by forming either a stretched trimer or a hexamer of conjoined trimers.<sup>2,8</sup> In addition to homomeric assemblies of identical subunits, P2X proteins also form heteromeric assemblies by using two or three building blocks. Where two P2X subunits are involved, immunoprecipitation studies have predicted up to 11 heteromeric receptor subtypes.<sup>9</sup> Here, an interesting pattern has emerged – P2X<sub>7</sub> subunits are most discerning and only will form homomeric receptors, whereas P2X<sub>5</sub> subunits are most promiscuous and will heteropolymerize with all P2X subunits (except P2X<sub>7</sub>). Other P2X subunits can polymerise with some, but not all, P2X subunits. As yet, no one has undertaken a systematic study of heteromeric assemblies involving three different subunits, although such assemblies must surely occur in native tissues, since a number of tissues (e.g. dorsal root ganglia, autonomic ganglia and smooth muscle cells) possess in abundance transcripts and proteins for several P2X subunits.

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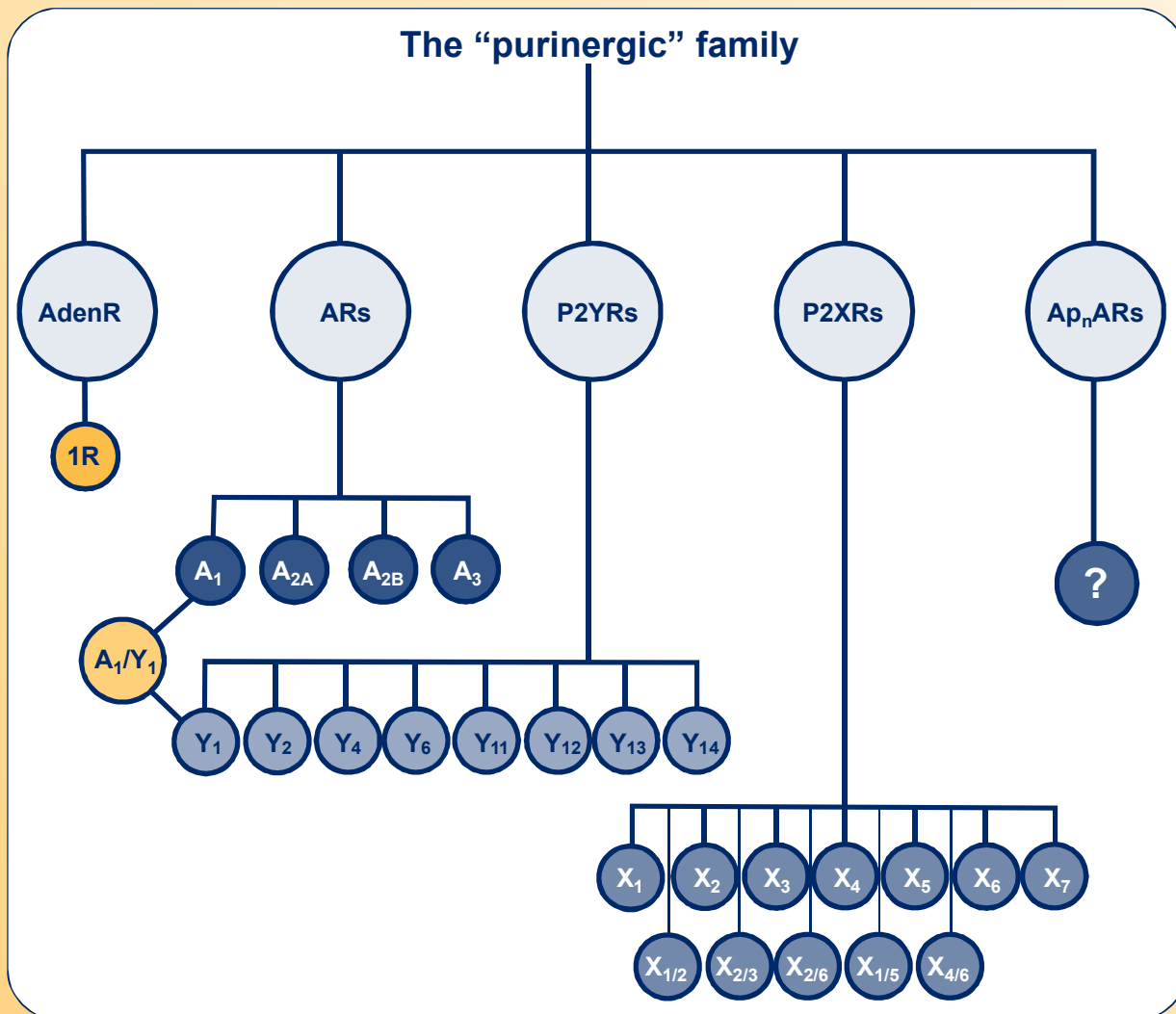
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**Figure 1. The purinoceptor family of cell-surface receptors**

The purinoceptor family, at this point in time, comprises 5 structurally and pharmacologically distinct groups – the adenine receptor group (AdenR), adenosine receptors (ARs), metabotropic nucleotide receptors (P2YRs), ionotropic nucleotide receptors (P2XRs) in their homomeric and heteromeric forms, and dinucleotide receptors ( $Ap_nAR$ ). The last group ( $Ap_nAR$ s) might eventually be subdivided into ionotropic and metabotropic subgroups, for which there is pharmacological, but not yet structural, evidence. Finally, members of the AR and P2YR groups appear to heterodimerise, although only the  $A_1/Y_1$  complex has been studied functionally.



To date, seven homomeric P2XRs (P2X<sub>1</sub> to P2X<sub>7</sub>) and five heteromeric assemblies (P2X<sub>1/2</sub>, P2X<sub>1/5</sub>, P2X<sub>2/3</sub>, P2X<sub>2/6</sub> and P2X<sub>4/6</sub>) have been characterised by their biophysical and pharmacological properties. Operational differences between many of these P2XR assemblies are surprisingly subtle. Also, most tissues express both homomeric and heteromeric P2XRs of a similar pharmacological nature. As a result, it is an inordinately difficult process to determine the identity of native P2XRs. At this point in time, it is inadvisable to categorise native P2XRs as P2X<sub>1</sub>, P2X<sub>2</sub>, P2X<sub>3</sub>, etc. and, instead, they should be associated with a particular phenotype (e.g. P2X<sub>1</sub>-like, P2X<sub>2</sub>-like, P2X<sub>3</sub>-like, etc.). The roles of several P2X receptor subunits in native channels have been examined by the generation of knockout mice. Null mutations for the P2X<sub>1</sub>,<sup>10</sup> P2X<sub>3</sub><sup>11</sup> and P2X<sub>7</sub><sup>12</sup> subunits, and overexpressing P2X<sub>1</sub> transgenic mice,<sup>13</sup> have been described, implicating P2X receptors in processes as diverse as fertility, thrombosis, pain and cytokine production *in vivo*.

#### P2XR agonism

All of the known P2X assemblies are activated by ATP, which is the most potent of the naturally-occurring nucleoside triphosphates (NTPs). Other NTPs can work at certain receptor subtypes (e.g. CTP at recombinant P2X<sub>1</sub>, P2X<sub>4</sub> and P2X<sub>5</sub>, GTP at P2X<sub>5</sub>, and UTP at P2X<sub>3</sub>), but none are as potent as ATP

itself. Neither ADP nor AMP (when purified of contaminating ATP) will activate P2X receptors, except at homomeric assemblies of P2X<sub>1</sub> splice variants (exon 6 deleted) which are activated by ADP and not ATP,<sup>14</sup> and at homomeric P2X<sub>7</sub> receptors which will respond to both ADP and AMP (within a period of time) after being activated first by ATP.<sup>15</sup> The naturally-occurring diadenosine polyphosphates ( $Ap_nA$ , n = 3-7) and closely-related dinucleotides ( $Ap_nG$ , n = 3-6) also activate P2XRs, but none with either the potency or efficacy of ATP. Members of the diadenosine polyphosphate family are so close in potency and efficacy at P2X assemblies that these substances cannot be used reliably (and alone) as discriminators of P2X subtypes. The diphosphates,  $Ap_2A$  and  $Ap_2G$ , are not agonists at P2XRs, in keeping with the relative inactivity of ADP.

Amongst recombinant P2XRs, the potency of ATP can vary enormously, with EC<sub>50</sub> values ranging from 50 nM to 300 μM, depending on subunit composition.<sup>1,2,16-20</sup> Although mutational analyses have been carried out, a structural basis has not been clearly established for this observed variability in agonist potency. A common ATP recognition site has been mapped for P2X<sub>1</sub>,<sup>21</sup> and P2X<sub>2</sub>,<sup>22</sup> subunits, and this site is shared by the remaining P2X subunits. However, other modulatory factors must clearly come into consideration. For instance, agonist potency is

**Table 1. Key ligands for homomeric P2X receptors**

Subtype	Agonists (pEC <sub>50</sub> )	Antagonists (pIC <sub>50</sub> )	Potentiators (pEC <sub>50</sub> /pK <sub>a</sub> )	Inhibitors (pIC <sub>50</sub> /pK <sub>i</sub> )	References
rP2X <sub>1</sub>	ATP (7.0) <b>2-MeSATP (7.0)</b> ATPγS (6.2) Ap <sub>6</sub> A (6.0) Ap <sub>6</sub> G (5.7) α,β-meATP (5.5) Bz-ATP (4.6) CTP (4.4)	<b>NF 449 (9.5)</b> <b>NF 279 (7.7)</b> <b>NF 023 (6.7)</b> <b>Suramin (5.7)</b> Ip <sub>5</sub> I (8.5) TNP-ATP (9.0) MRS 2257 (8.3) MRS 2159 (8.0) <b>PPNDS (7.8)</b> <b>PPADS (6.9)</b> RB-2 (5.7)  (h*) A-317491 (4.9)	–	H <sup>+</sup> (6.3) Zn <sup>2+</sup> (6.0) Gd <sup>3+</sup> (6.5)	1, 2, 3, 17-20, 25-30, 88, 89
rP2X <sub>2</sub>	ATP (5.3) <b>2-MeSATP (5.1)</b> ATPγS (5.1) Ap <sub>4</sub> A (4.8) α,β-meATP (3.0)	RB-2 (6.4) <b>NF 279 (6.4)</b> BBG (5.9) TNP-ATP (5.9) <b>PPADS (5.8)</b> <b>Suramin (5.0)</b>  (h*) A-317491 (4.33)	H <sup>+</sup> (7.1) Zn <sup>2+</sup> (5.0) Cu <sup>2+</sup> (4.8) Cd <sup>2+</sup> (3.5)	La <sup>3+</sup> (5.0) Gd <sup>3+</sup> (5.0) Ca <sup>2+</sup> (1.1)	1, 2, 3, 17-20, 25-30, 90-94
rP2X <sub>3</sub>	<b>2-MeSATP (6.7)</b> ATP (5.9) ATPγS (5.9) Ap <sub>5</sub> A (5.5) Ap <sub>5</sub> G (5.6) α,β-meATP (5.7) UTP (4.0)	A-317491 (7.6) <b>NF 279 (5.8)</b> <b>NF 449 (5.6)</b> <b>Suramin (5.4)</b> <b>NF 023 (5.0)</b> TNP-ATP (9.5) MRS 2257 (7.7) <b>MRS 2159 (6.9)</b> <b>PPADS (6.7)</b> Ip <sub>5</sub> I (5.5) RB-2 (4.3)	Zn <sup>2+</sup> (5.0)  (h*) RB-2 (5.9)	H <sup>+</sup> (6.0) Ca <sup>2+</sup> (1.8)	1, 2, 3, 17-20, 88, 89, 95, 96
rP2X <sub>4</sub>	ATP (5.4) <b>2-MeSATP (3.6)</b> CTP (3.5)  partial agonist: Ap <sub>4</sub> A (5.5) α,β-meATP (4.2)	TNP-ATP (4.8) BBG (3.9)  (h*) BBG (5.5) (h*) <b>PPADS (4.6)</b>	<b>Ivermectin (6.6)</b> Cibacron blue (5.5) <b>Propofol (4.3)</b> Zn <sup>2+</sup> (5.9) Cd <sup>2+</sup> (5.0)	H <sup>+</sup> (6.8)	1, 2, 3, 17-20, 25-30, 97-103
rP2X <sub>5</sub>	ATP (6.4) <b>2-MeSATP (6.4)</b> ATPγS (6.5) Bz-ATP (5.9) α,β-meATP (6.0) Ap <sub>4</sub> A (6.6) GTP (4.6) CTP (4.3)	<b>PPADS (6.7)</b> TNP-ATP (6.3) <b>Suramin (5.8)</b> RB-2 (4.7)	Zn <sup>2+</sup> (4.4)	H <sup>+</sup> (5.5) Ca <sup>2+</sup> (3.0)	1, 2, 3, 17-20
rP2X <sub>6</sub>	ATP (6.2)	–	–	–	7
rP2X <sub>7</sub>	BzATP (5.2) <b>2-MeSATP (5.0)</b> ATP (3.4)  (m*) ADP (2.7) (m*) AMP (2.3)	BBG (8.0) <b>PPADS (4.3)</b> TNP-ATP (~ 4.3)  (h*) <b>KN-62 (7.4)</b> (h*) KN-04 (~ 6.0)		H <sup>+</sup> (6.1) Cu <sup>2+</sup> (6.5) Zn <sup>2+</sup> (4.9) Mg <sup>2+</sup> (3.3) Ca <sup>2+</sup> (2.5) Halide ions	1, 2, 3, 15, 17-20, 31-33, 104

(Bold text denotes compounds available from Tocris)

Potency indices for agonists, etc. given as -log<sub>10</sub> EC<sub>50</sub> (pEC<sub>50</sub>) values. Data for human (h\*) and mouse (m\*) isoforms are given for some key ligands.

strongly affected in one way or another by extracellular H<sup>+</sup> ions (pH) and extracellular Zn<sup>2+</sup> ions at all the known P2XR subtypes. Other extracellular ions, including Na<sup>+</sup>, Ba<sup>2+</sup>, Ca<sup>2+</sup>, Cd<sup>2+</sup>, Cu<sup>2+</sup>, Mg<sup>2+</sup>, Mn<sup>2+</sup>, Al<sup>3+</sup>, Gd<sup>3+</sup>, La<sup>3+</sup> and halide anions, also can affect ATP potency to different degrees at recombinant P2XRs. Some modulators will work through key structural elements in P2X subunits, others through the ionization state of ATP, and some through the biophysical and biochemical properties of the cell membrane itself.

In addition to NTPs and dinucleotides, there are a number of synthetic analogues that will activate recognized P2XRs. Best known are the 2-thioalkyl derivatives (e.g. 2-MeSATP), methylene-phosphonate derivatives (e.g.  $\alpha,\beta$ -meATP) and modified ribose derivatives (e.g. 2',3'-BzATP). None of these compounds is more potent than ATP, and none clearly discriminates between P2X subtypes. However, their potencies relative to ATP and other naturally-occurring nucleotides (see above) help establish agonist profiles for each recombinant P2XR. This knowledge, in turn, can help to determine the dominant population of native P2XRs in whole tissues.

The channel properties are clearly different for P2XR subtypes.<sup>2</sup> In the presence of ATP or other agonist, some activate and inactivate within several seconds (e.g. P2X<sub>1</sub>, P2X<sub>3</sub>, P2X<sub>5</sub>, P2X<sub>1/2</sub>, P2X<sub>1/5</sub>), some over tens of seconds (P2X<sub>2</sub>, P2X<sub>4</sub>, P2X<sub>6</sub>, P2X<sub>2/3</sub>, P2X<sub>2/6</sub>, P2X<sub>4/6</sub>) and, once activated, the P2X<sub>7</sub> receptor does not inactivate at all. The reversal potential for ion currents falls in the region of -5 to 0 millivolts for all P2XRs, but some subtypes, particularly P2X<sub>2</sub>, P2X<sub>2/3</sub>,

P2X<sub>4</sub> and P2X<sub>7</sub>, show time-dependent changes in channel permeability and shifts in reversal potential.<sup>23,24</sup> This effect is most profound for P2X<sub>7</sub> receptors, which can become more permeable to larger cations (e.g. NMDG<sup>+</sup>) and, later, undergo a channel-to-pore conversion to allow the passage of large dye molecules (e.g. ethidium or YO-PRO). In contrast to permeability changes, some drugs can cause an apparent gain-of-function at P2XRs, by increasing the number of ion-channels available for activation. This is most obvious for homomeric P2X<sub>4</sub> and heteromeric P2X<sub>4/6</sub> receptors, which are modified allosterically by ivermectin. Furthermore, human P2X<sub>3</sub> and rat P2X<sub>4</sub> receptors are modified in a similar way by Cibacron blue (one of two isomers of Reactive blue 2). General anaesthetics (propofol) and ethanol can also modify some P2XR subtypes.

### P2XR antagonism

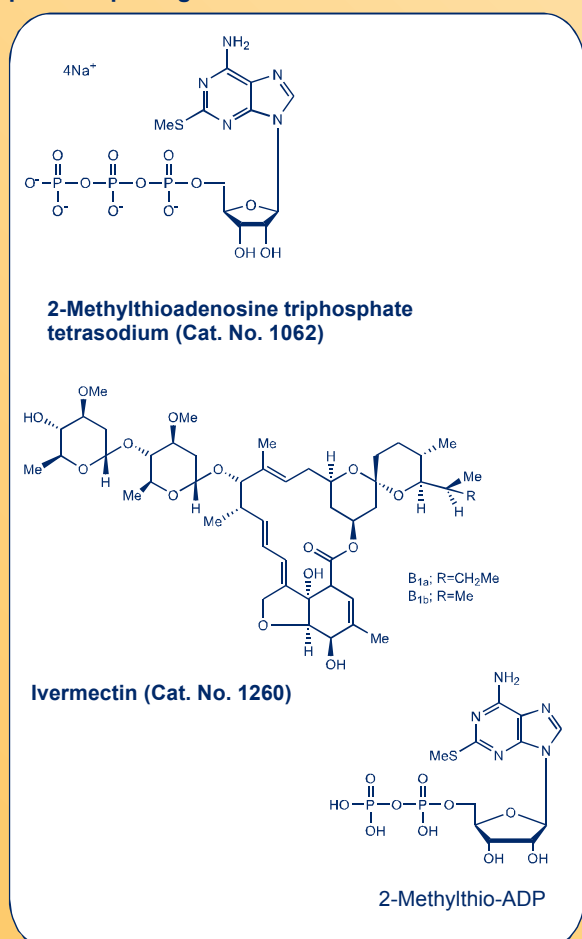
Significant advances have been made in the identification of P2X subtype-selective antagonists. Two lines of chemistry have dominated, based on either suramin or PPADS as the template for highly potent derivatives.<sup>25</sup> The major suramin analogues include NF023, NF279 and NF449, the last showing subnanomolar blocking activity at the P2X<sub>1</sub> subtype (IC<sub>50</sub>, 0.3 nM) and being 1000-fold more potent here than at P2X<sub>3</sub> receptors.<sup>25,26</sup> The major PPADS analogues include isoPPADS, PPADS, MRS2159 and MRS2257.<sup>20,25</sup> The phosphonate analogue, MRS2257, is 14-fold more potent than PPADS at P2X<sub>1</sub> (IC<sub>50</sub>, 5 nM) and 10-fold more potent than PPADS at P2X<sub>3</sub> (IC<sub>50</sub>, 22 nM).<sup>27</sup> Both key compounds – NF449 and MRS2257 – are relatively inactive at the other P2XR (and P2YR) subtypes. Furthermore, these potent derivatives no longer show the worst side-effects of suramin (e.g. G-protein inhibition) and of PPADS (e.g. ATPase inhibition). Other lines of chemistry, involving the nucleotide template, also have proven fruitful. The ribose-modified derivative, TNP-ATP, shows nanomolar blocking activity at P2X<sub>1</sub> (IC<sub>50</sub>, 1 nM) and P2X<sub>3</sub> (IC<sub>50</sub>, 0.3 nM) and almost as potent at heteromeric P2X<sub>2/3</sub> (IC<sub>50</sub>, 11 nM), yet 1000-fold less potent at other P2XR subtypes (when compared to P2X<sub>3</sub> activity).<sup>28</sup> The newer dinucleotide antagonist, Ip<sub>5</sub>l, provides greater discrimination and is a potent blocker only at P2X<sub>1</sub> (IC<sub>50</sub>, 3 nM), not as potent at P2X<sub>3</sub> (IC<sub>50</sub>, 3  $\mu$ M) and is inactive at P2X<sub>2/3</sub>.<sup>29</sup> Ip<sub>5</sub>l is not an antagonist of P2X<sub>2</sub>, P2X<sub>4</sub> and P2X<sub>5</sub> receptors.

The most recent advances in P2XR drug design include the competitive antagonist, A-317491, which blocks P2X<sub>3</sub> (IC<sub>50</sub>, 22 nM) and P2X<sub>2/3</sub> (IC<sub>50</sub>, 92 nM; pA<sub>2</sub>, 6.63).<sup>30</sup> This non-nucleotidic drug shows analgesic properties *in vivo*, presumably through the blockade of native P2X<sub>3</sub>- and P2X<sub>2/3</sub>-like receptors on primary afferent fibres. A-317491 is markedly less potent at P2X<sub>1</sub> (IC<sub>50</sub>, 10  $\mu$ M) and P2X<sub>2</sub> (IC<sub>50</sub>, 47  $\mu$ M), whilst it is inactive at P2X<sub>4</sub> receptors and P2YR subtypes.

Another notable antagonist is Coomassie Brilliant blue G (BBG), which blocks rat P2X<sub>7</sub> (IC<sub>50</sub>, 10 nM) and human P2X<sub>7</sub> (IC<sub>50</sub>, 265 nM).<sup>31</sup> This drug also blocks human P2X<sub>4</sub> (IC<sub>50</sub>, 3  $\mu$ M) and, therefore, is the most potent antagonist known for this receptor subtype. Unfortunately, BBG is also active at P2X<sub>2</sub> receptors (IC<sub>50</sub>, 1  $\mu$ M) and, so, it cannot readily discriminate between P2X<sub>2</sub> and P2X<sub>4</sub> receptors when used alone. However, a broad range of antagonists work at P2X<sub>2</sub> receptors (e.g. suramin, PPADS, Reactive blue 2, TNP-ATP) which, otherwise, are largely ineffective at P2X<sub>4</sub> receptors.

The isoquinoline derivative, KN-62, is a potent blocker of human P2X<sub>7</sub> (IC<sub>50</sub>, 34 nM) but is inactive at the rat

**Figure 2. Chemical structures of some purinoceptor agonists and modulators**



(Bold text denotes compounds available from Tocris)

P2X<sub>7</sub> receptor.<sup>32</sup> The related compound, KN-04, also blocks human P2X<sub>7</sub> and, of the two, is slightly less potent (pA<sub>2</sub>: 7.31 vs 8.10).<sup>33</sup> KN-62, but not KN-04, is an inhibitor of CaM kinase II. Since both analogues act as non-competitive antagonists at P2X<sub>7</sub>, their blocking actions here are considered to be CaMKII-independent.

The anthraquinone dye, Reactive blue 2, is a racemic mixture of Cibacron blue and Basilen blue. Each isomer has broad antagonist activity at recombinant P2XR<sub>s</sub> – also some P2YR<sub>s</sub> – and, therefore, its utility to discriminate between P2XR and P2YR subtypes is questionable. Other dyes, such as Acid blue, Trypan blue, Uniblue, Evans blue, can block P2X<sub>1</sub>-like receptors in rat vas deferens but their selectivity for P2X<sub>1</sub> (over other recombinant P2XR subtypes) has not been fully investigated.

## P2Y Receptors

P2Y receptors (P2YRs) are G protein-coupled receptors (GPCRs) broadly distributed amongst various tissue types including autonomic, central, enteric and sensory neurons, glia and astrocytes, endothelium and epithelium, vascular and visceral smooth muscle, heart and developing skeletal muscle, soft tissues (kidney, liver, lung, pancreas, prostate and thymus), bone, haemopoietic cells. The P2YRs serve multiple functions in their host cells, working through two major processes: i) activation of intracellular signalling cascades, via the catalytic G protein  $\alpha$ -subunit, and ii) modulation of membrane ion-channels, via regulatory G protein  $\beta\gamma$ -subunits. A third, less understood, process involves the physical interaction of P2YRs with membrane proteins in their close proximity.

### P2YR structure

The P2YRs are heptahelical proteins of structural similarity to the rhodopsin GPCR template. Fourteen proteins – P2Y<sub>1</sub> to P2Y<sub>14</sub> – have been linked to the P2YR family, and most have been cloned from human cDNA libraries.<sup>34,35</sup> In some cases, orthologues of P2YR subtypes have been cloned from bovine, canine, chick, mouse, rat, skate, turkey and *Xenopus* libraries.<sup>36</sup> These P2Y proteins represent some of the shortest GPCRs (328 to 532 residues in length) found in vertebrate species. P2Y proteins possess seven hydrophobic regions (TMI-VII) – believed to form  $\alpha$ -helices – that pass through the cell membrane and are connected to an extracellular N-terminus (21-51 residues in length) and intracellular C-terminus (16-217 residues in length). For the membrane-spanning regions (TMI-VII), alignment of protein sequences reveals 17 to 61% identity amongst the P2YR family.<sup>34,35</sup> The P2YR genes, unlike their P2XR counterparts, do not normally undergo alternative splicing. However, examples of P2YR polymorphisms have been reported, where a mutation has occurred in a single codon (e.g. an arginine-cysteine transition in P2Y<sub>2</sub>).<sup>37</sup> Also, fusion proteins have been identified as the result of intergenic splicing (e.g. P2Y<sub>11</sub> and SSF1 genes).<sup>38</sup>

Each P2Y protein is believed to exist in the cell membrane as a monomer coupled to a single G protein. Suggestions of homodimeric assemblies of P2Y proteins have been mooted, but there is no concrete evidence for such. However, compelling evidence has been presented for heterodimeric assemblies of recombinant P2Y<sub>1</sub> and A<sub>1</sub> proteins.<sup>39</sup> The occurrence of such heterodimers has not been confirmed in whole tissues, although their likely existence could explain the phenomenon of the native P3 receptor (where ATP and adenosine are equipotent agonists).

The accepted monomeric proteins (that function as a nucleotide receptor) include P2Y<sub>1</sub>, P2Y<sub>2</sub>, P2Y<sub>4</sub>, P2Y<sub>6</sub>, P2Y<sub>11</sub>, P2Y<sub>12</sub>, P2Y<sub>13</sub> and newly-named P2Y<sub>14</sub> (formerly called the UDP-glucose receptor).<sup>40</sup> Missing receptors in the above sequence can be accounted for in three ways: i) inclusion of non-mammalian P2YRs (e.g. chick P2Y<sub>3</sub>, and *Xenopus* P2Y<sub>8</sub>), ii) inclusion of orphan receptors (e.g. P2Y<sub>5</sub>, P2Y<sub>9</sub>, P2Y<sub>10</sub>) which are structurally similar to known P2YRs, and iii) misidentification of GPCRs (e.g. P2Y<sub>7</sub> is a leukotriene B<sub>4</sub> receptor). The eight accepted P2YRs are diverse in their operational and pharmacological profiles – and even show major differences between species orthologues – making the identification of native P2YRs a very difficult process.<sup>41</sup> However, in the last 5 years, a new generation of drugs, notably antagonists, has helped to identify some P2YR subtypes, *in vivo*. Null mutations in P2Y receptor subtypes have also helped identify roles for known P2Y subtypes *in vivo*, with knockout mice having been generated for P2Y<sub>1</sub>,<sup>42,43</sup> P2Y<sub>2</sub>,<sup>44</sup> P2Y<sub>4</sub><sup>45</sup> and P2Y<sub>12</sub>.<sup>46</sup>

### P2YR agonism

A wide range of naturally-occurring nucleoside triphosphates (NTPs) is active at P2YRs. Few receptors (only P2Y<sub>1</sub>) are activated by ATP alone and, even here, this NTP can behave as either a partial agonist or antagonist.<sup>34</sup> ATP fully activates other P2YRs including P2Y<sub>2</sub>, some isoforms of P2Y<sub>4</sub> and P2Y<sub>11</sub>, but each of these subtypes is activated additionally by other NTPs in an equipotent manner.<sup>34,35</sup> In this respect, UTP will fully activate all isoforms of P2Y<sub>2</sub>, all isoforms of P2Y<sub>4</sub> and, as shown recently, human P2Y<sub>11</sub>.<sup>47</sup> UTP can also activate P2Y<sub>6</sub>, either partially or fully, depending on the receptor reserve. CTP, GTP and ITP will fully activate mouse and rat isoforms of P2Y<sub>4</sub>. Furthermore, nucleoside diphosphates (NDPs) are important agonists at P2YRs (in sharp contrast to P2XR<sub>s</sub>). ADP is a full agonist at P2Y<sub>1</sub>, dog isoform of P2Y<sub>11</sub>, P2Y<sub>12</sub> and P2Y<sub>13</sub>, and a partial agonist at isoforms of P2Y<sub>6</sub>. UDP is an agonist at all isoforms of P2Y<sub>6</sub>, and both IDP and UDP at isoforms of P2Y<sub>13</sub>. A most unusual agonist, UDP-glucose, is the preferred and main agonist of P2Y<sub>14</sub> isoforms. The naturally-occurring diadenosine polyphosphates (Ap<sub>n</sub>A, n = 2-6) activate a number of P2YRs.<sup>48,49</sup> None is more potent than either NTPs or NDPs, and none is clearly discriminatory for P2YR subtypes.<sup>48</sup>

For recombinant and native P2YRs, the absolute potency of NTPs, NDPs and Ap<sub>n</sub>As is firmly dependent on the levels of receptor expression. Therefore, typical EC<sub>50</sub> values are not easily defined for specific agonists at particular P2YR subtypes, without prior knowledge of receptor number. Furthermore, relative agonist potencies at recombinant P2YRs cannot be easily assimilated with agonist data from native P2YRs, since, at the latter, the naturally occurring agonists are susceptible to degradation and transformation by local extracellular enzymes. Most of the naturally occurring P2YR agonists are rapidly broken down and, at this point in time, there are few inhibitors to check the actions of these extracellular enzymes, *in situ*. For recombinant P2Y receptors, little in the way of duplicatory work has been carried out on the known modulators of agonist activity at P2XR<sub>s</sub> (i.e. pH, extracellular Zn<sup>2+</sup>, other divalent and trivalent cations etc.) to help with the problem of native P2YR identification.

A number of synthetic nucleotides and dinucleotides can activate the recombinant P2YRs. The phosphorothioates (ADP $\beta$ S, UDP $\beta$ S and UTP $\gamma$ S) are notable because they are resistant to breakdown and show some selectivity for P2YR subtypes. ADP $\beta$ S is

Table 2. Key ligands for monomeric P2Y receptors

Subtype	Agonists (pEC <sub>50</sub> )	Antagonists (pIC <sub>50</sub> )	References
hP2Y <sub>1</sub>	2-MeSADP (8.7-7.9) <b>2-MeSATP (8.5-6.9)</b> 2-HT-AMP (6.4), ADP (8.0-6.6) ATP (6.5-5.0), ATPγS (6.4) ADPβS (6.1-6.0)  ( <i>r</i> *) 2-MeSADP (9.2), ADP (8.1) ( <i>t</i> *) 2-MeSADP (7.6), ADP (6.3)	MRS 2279 (7.3) <b>MRS 2179 (6.5)</b> A3P5P (6.0), <b>PPADS (5.4)</b> [BzATP (pK <sub>i</sub> , 5.4), <b>2-MeSATP (pK<sub>i</sub>, 5.2)</b> , ATP (pK <sub>i</sub> , 5.3)]  ( <i>t</i> *) <b>Suramin (pA<sub>2</sub>, 5.8)</b>	34, 35, 41, 55-57, 105-115
hP2Y <sub>2</sub>	UTP (7.7-6.2), UTPγS (6.6) 5-BrUTP (5.7), ATP (7.1-6.6) ATPγS (6.2-5.8), GTP (4.9) Up <sub>4</sub> U (7.0), dCp <sub>4</sub> U (6.7) AP <sub>4</sub> A (6.6-6.1)	<b>Suramin (pA<sub>2</sub>, 4.9-4.3)</b>	34, 35, 37, 41, 48, 52, 53, 107, 116-118
hP2Y <sub>4</sub>	UTP (7.6-5.6), Up <sub>4</sub> U (6.4) dCp <sub>4</sub> U (6.1), UTPγS (5.8) 5-BrUTP (4.8-4.3) ATP (5.4-4.4), GTP (5.2) ITP (5.1-4.0)  ( <i>r</i> *) ATP (5.9-5.7), Ap <sub>4</sub> A (5.5) ( <i>m</i> *) ATP (6.4-6.2)	<b>PPADS (4.8)</b> ATP (pK <sub>b</sub> , 6.1)  ( <i>r</i> *) RB-2 (4.7) ( <i>m</i> *) RB-2 (4.3)	34, 35, 41, 48, 52, 53, 60, 107, 119-121
hP2Y <sub>6</sub>	UDPβS (7.6), UDP (7.0-6.5) Up <sub>3</sub> U (6.7), 5-BrUDP (6.1) UTP (5.2-5.0), IDP (4.5) ADP (4.5-4.2)  ( <i>r</i> *) UDP (8.2-6.7) ( <i>m</i> *) ADP (7.4)	RB-2 (4.5)	34, 35, 41, 52, 122-127
hP2Y <sub>11</sub> (cAMP assay)	AR-C67085 (5.8), ATPγS (5.5-4.6) dATP (5.1-5.0), BzATP (5.1-4.2) ATP (4.9-4.2) <b>2-MeSATP (4.6-4.3)</b> α,β-meATP (4.1-3.9) β,γ-meATP (3.7)  ( <i>c</i> *) 2-MeSADP (6.8)	<b>Suramin (4.8)</b> AMPS (3.5-2.5)	34, 35, 50, 51, 128-134
hP2Y <sub>12</sub>	<b>2-MeSATP (10-8.5)</b> 2-MeSADP (10-7.9) ADP (7.4-6.5), ADPβS (7.0-6.4) ATP (6.2-5.9)  ( <i>r</i> *) 2-MeSADP (8.7) ADP (7.1)	AR-C69931 (7.6) C-1330-7 (7.4), RB-2 (5.9) 2-MeSAMP (5.9-5.3) <b>Suramin (5.4)</b>  ( <i>r</i> *) AR-C67085 ( <i>r</i> *) BzATP (5.3-5.0)	34, 35, 135-138
hP2Y <sub>13</sub>	ADP (8.0), 2-MeSADP (7.9) <b>2-MeSATP (7.1)</b> , ADPβS (7.4) ATP (6.6), IDP (6.3)  ( <i>m</i> *) ADP (8.4) ( <i>m</i> *) IDP (8.0)	–	139, 140
hP2Y <sub>14</sub>	UDP-glucose (7.1) UDP-galactose (6.4) UDP- <i>N</i> -acetylglucosamine (6.1)  ( <i>r</i> *) UDP-glucose (7.6) ( <i>m</i> *) UDP-glucose (7.7)	–	141, 142

(Bold text denotes compounds available from Tocris)

Potency indices for agonists, etc. given as  $-\log_{10} EC_{50}$  (pEC<sub>50</sub>) values. Data are given as a range of reported values, to make allowance for the effects of receptor reserve on drug potency. Data for canine (*c*\*), mouse (*m*\*), rat (*r*\*) and turkey (*t*\*) isoforms are given for key ligands. Some ligands (e.g. ATP) have been reported as either agonists or antagonists at some P2Y subtypes (e.g. P2Y<sub>1</sub> and P2Y<sub>4</sub>).

a potent agonist at P2Y<sub>1</sub> and, whilst also active at a few P2XR subtypes,<sup>18</sup> can still be used judiciously. UDPβS is a potent agonist at P2Y<sub>6</sub>, whereas UTPγS is useful as a stable agonist of P2Y<sub>2</sub> and P2Y<sub>4</sub> receptors. However, at this point in time, neither UDPβS nor UTPγS is commercially available. The 2-thioalkyl derivatives (2-MeSADP and 2-MeSATP) are potent agonists at the P2Y<sub>1</sub> subtype, but are not selective and also activate the known isoforms of P2Y<sub>11</sub>, P2Y<sub>12</sub> and P2Y<sub>13</sub>. The ribose-modified derivative, BzATP, is active at P2Y<sub>2</sub> and P2Y<sub>11</sub>, the latter also being potently activated by AR-C67085 (2-propylthio-β,γ-dichloromethylene-D-ATP)<sup>50</sup> and β,γ-methylene ATP.<sup>51</sup> The synthetic dinucleotides, Cp<sub>n</sub>U and Up<sub>n</sub>U, are potent agonists at most of the pyrimidine-activated P2YRs (P2Y<sub>2</sub>, P2Y<sub>4</sub> and P2Y<sub>6</sub>) and, importantly, are much more stable than UTP and UDP *in vivo*.<sup>52,53</sup> These uridine-based dinucleotides are not yet commercially available.

Regardless of how they are activated, the recombinant P2YRs show specific patterns of intracellular signalling. P2Y<sub>1,2,4,6</sub> and P2Y<sub>11</sub> receptors couple strongly to G<sub>q</sub> and activate the PLCβ/IP<sub>3</sub> pathway to release intracellular Ca<sup>2+</sup> in common heterologous expression systems.<sup>34,35</sup> However, P2Y<sub>2,4</sub> and P2Y<sub>6</sub> will couple secondarily to pertussis toxin-sensitive G<sub>i/o</sub> to liberate regulatory β,γ-subunits and inactivate cation channels in specialised expression systems (e.g. mammalian sympathetic neurons). P2Y<sub>11</sub> will also couple to G<sub>s</sub> to raise intracellular cAMP levels in various expression systems and some cell lines (e.g. HL-60). A recent report describes a P2Y<sub>6</sub> receptor-mediated elevation of cAMP.<sup>54</sup> Whether this is achieved, as for P2Y<sub>11</sub>, by direct coupling through G<sub>s</sub> is unclear, although it should be

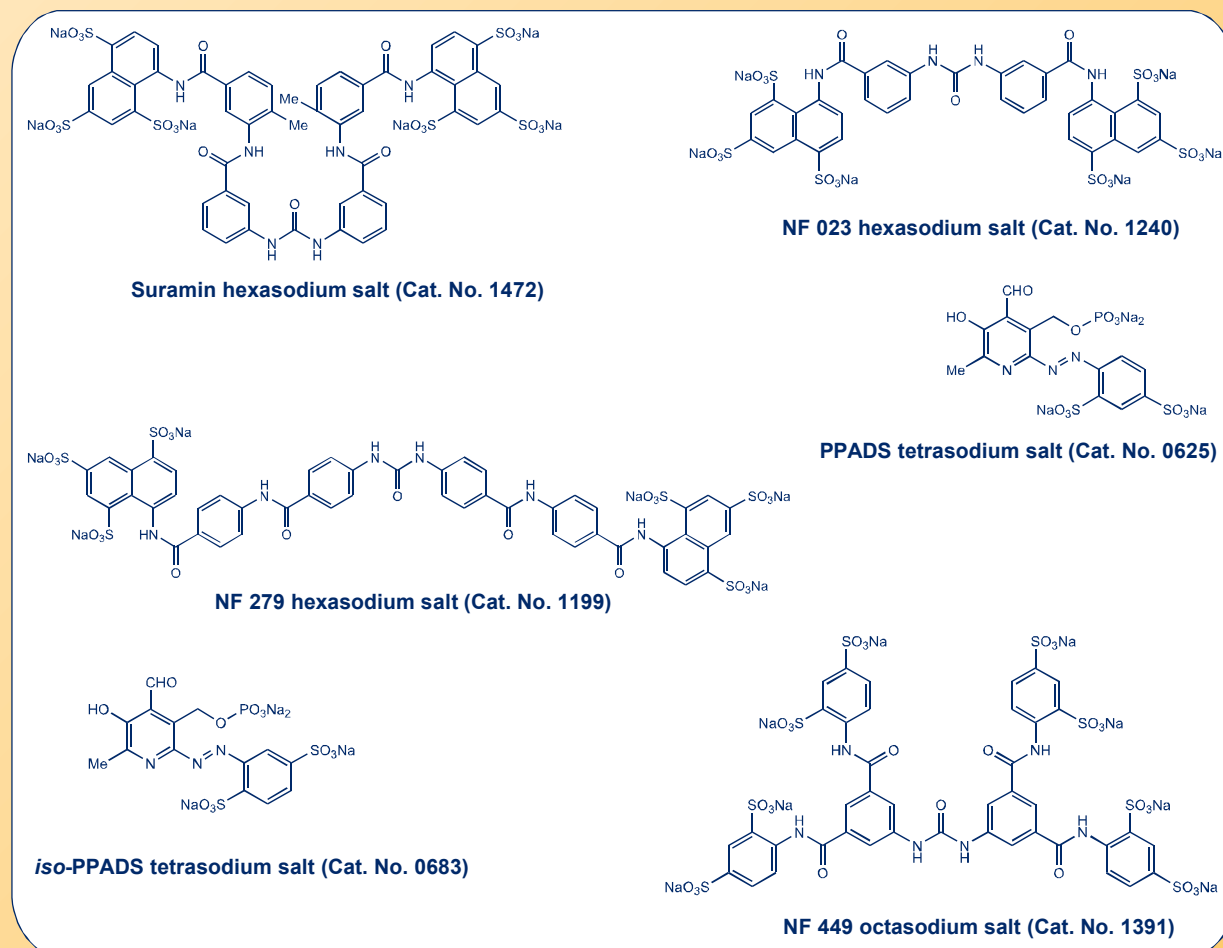
noted that the P2Y<sub>1</sub> receptor failed to elevate cAMP levels in the same expression system. The P2Y<sub>12,13</sub> and P2Y<sub>14</sub> receptors, which share common structural motifs, couple to G<sub>i</sub> to lower cAMP levels.<sup>40</sup> Thus, the pattern of GPCR signalling and range of agonists affecting a particular P2YR are seen as key elements in the identification of native P2YRs. This is particularly true for native P2Y<sub>12</sub> which, otherwise, has been called the G<sub>i</sub>-coupled P2Y<sub>ADP</sub> receptor of blood platelets.

### P2YR antagonism

For only a few P2YR subtypes, significant advances have been made in the identification of selective antagonists. The most potent P2Y<sub>1</sub> antagonists are structural analogues of adenosine bis-phosphates (e.g. MRS2179 and MRS2279)<sup>55</sup> with MRS2279 (and [<sup>3</sup>H]-MRS2279) showing nanomolar blocking activity at both native and recombinant P2Y<sub>1</sub> receptors.<sup>56,57</sup> For P2Y<sub>12</sub>, structural analogues of ATP (AR-C67085 and AR-C69931) show nanomolar blocking activity.<sup>58</sup> Clopidogrel, once rendered active by liver metabolism, is also an effective blocker of native and recombinant P2Y<sub>12</sub> receptors.<sup>59</sup> Since P2Y<sub>1</sub> and P2Y<sub>12</sub> receptors co-exist in blood platelets, the identification of P2Y subtype-selective antagonists has helped considerably in understanding the processes of blood clot formation and thrombolysis.

There are no potent and selective antagonists for the pyrimidine-activated P2YRs, although suramin (P2Y<sub>2</sub> and P2Y<sub>11</sub>), Reactive blue 2 (RB-2, P2Y<sub>4</sub> and P2Y<sub>6</sub>) and PPADS (P2Y<sub>4</sub>, P2Y<sub>6</sub> and P2Y<sub>11</sub>) show blocking activity at micromolar concentrations and higher.<sup>34</sup> At human P2Y<sub>4</sub>, ATP itself is considered to be a competitive antagonist (pA<sub>2</sub>, 6.15).<sup>60</sup> No antagonists

**Figure 3. Chemical structures of some purinoceptor antagonists**



(Bold text denotes compounds available from Tocris)

have been reported for P2Y<sub>13</sub> and P2Y<sub>14</sub> receptors. One of the biggest challenges currently facing the P2YR field is the identification of further selective blockers for P2Y<sub>2,4,6,11,13</sub> and <sub>14</sub> subtypes.

### Adenosine Receptors

The identification and characterisation of adenosine receptors (ARs) preceded that of the nucleotide receptors. The discovery that adenosine could affect numerous physiological systems was made as early as 1929,<sup>61</sup> although the suggestion that adenosine acts through specific membrane receptors came fifty years later,<sup>62</sup> with two different forms of receptor being identified five years after that.<sup>63</sup> In 1989, cDNAs encoding two different AR subtypes were isolated<sup>64</sup> and, shortly after, the A<sub>3</sub> subtype was identified.<sup>65</sup> Although the A<sub>1</sub>, A<sub>2A</sub> and A<sub>2B</sub> subtypes had been identified through pharmacological and functional studies prior to their molecular cloning, the existence of the A<sub>3</sub> receptor had not. ARs are occasionally referred to as P1 receptors, most typically when being compared directly with their nucleotide receptor cousins, the P2 receptors.

#### AR structure

Four different AR subtypes, A<sub>1</sub>, A<sub>2A</sub>, A<sub>2B</sub> and A<sub>3</sub>, have been identified. All are members of the rhodopsin-like family of GPCR, and all four AR subtypes have been cloned from a large number of different species. At 318 amino acids in length, the A<sub>3</sub> subtype is the shortest whilst A<sub>2A</sub> is the longest (412 residues). Their N-termini are relatively short (7-13 residues in length), as are their C-termini (32-120 residues). In the transmembrane domains (TMI-TMVII), human ARs share 39-61% sequence identity with each other and 11-18% identity with P2YRs. It is interesting to note that P2Y<sub>11</sub> is as closely related, in terms of sequence identity, to the A<sub>2B</sub> receptor (18%) as it is to P2Y<sub>12</sub> (17%), P2Y<sub>13</sub> (17%) or P2Y<sub>14</sub> (17%) receptors. Each of the four human AR genes contains an intron within the coding region – located immediately after the end of the third transmembrane domain – although alternative splicing within the coding region has not been observed. Polymorphisms have been observed in the A<sub>1</sub><sup>66</sup> and the A<sub>2A</sub><sup>67</sup> receptors; polymorphic A<sub>2A</sub> receptors have been implicated in panic disorders.<sup>68</sup>

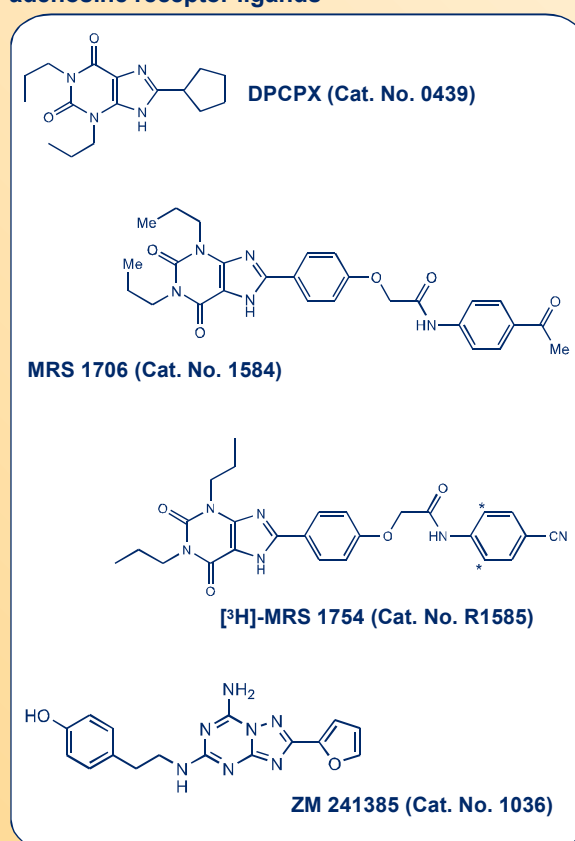
ARs couple principally to adenylate cyclase. A<sub>1</sub> and A<sub>3</sub> are negatively coupled to adenylate cyclase through the G<sub>i/o</sub> protein  $\alpha$ -subunits, whereas A<sub>2A</sub> and A<sub>2B</sub> are positively coupled to adenylate cyclase through G<sub>s</sub>. The human A<sub>2B</sub> receptor has also been observed to couple through G<sub>q/11</sub> to regulate phospholipase C activity<sup>69</sup> and the A<sub>3</sub> receptor may interact directly with G<sub>s</sub>.<sup>70</sup> For the most part, the pharmacology of AR orthologues is quite similar, except for A<sub>3</sub> receptor subtypes, where significant differences in pharmacology and distribution have been observed.<sup>71</sup>

The diverse physiological effects mediated by the different AR subtypes, particularly modulation of the cardiovascular, immune and central nervous systems, have been confirmed by transgenic knockout mice. Null mice have been generated for each of the A<sub>1</sub>, A<sub>2A</sub> and A<sub>3</sub> receptors<sup>72-75</sup> and, in all knockout animals generated, the ARs in question do not appear to play a critical role during development. Knockout mice have yet to be described for the A<sub>2B</sub> receptor subtype. In contrast to knockout studies, overexpression of either A<sub>1</sub> or A<sub>3</sub> subtypes in transgenic mice has a cardioprotective effect,<sup>76,77</sup> although it would appear that, under certain conditions, overexpression of the A<sub>3</sub> subtype can give rise to an embryonic lethal phenotype.<sup>78</sup>

#### AR Agonism

Pharmacological tools for the study of ARs are much more precise than those currently available for the study of P2 receptors. Whilst a number of subtype-selective agonists are known, care must be taken when using these at high concentrations as their selectivity is limited. The affinity of adenosine at the different human ARs has been determined in functional assays<sup>79</sup> with the following EC<sub>50</sub> values obtained: A<sub>1</sub>, 0.31  $\mu$ M; A<sub>2A</sub>, 0.7  $\mu$ M; A<sub>2B</sub>, 24  $\mu$ M; A<sub>3</sub>, 0.29  $\mu$ M. These values indicate that the A<sub>1</sub>, A<sub>2A</sub> and A<sub>3</sub> receptors can be activated by physiological concentrations of adenosine, whereas pathophysiological concentrations of adenosine are required to activate the A<sub>2B</sub> receptor. Inosine, another endogenous ligand of ARs, activates rodent receptors with K<sub>i</sub> values of 15-25  $\mu$ M, but is a weak partial agonist at the human A<sub>3</sub> receptor.<sup>80</sup>

**Figure 4. Chemical structures of some key adenosine receptor ligands**



(Bold text denotes compounds available from Tocris)

All of the known AR agonists are closely related to adenosine in structure, with few modifications permitted.<sup>81</sup> Alteration or opening of the ribose ring drastically reduces affinity. The hydroxyl group at the 2'-position is needed for both affinity and activity, whilst removal of the 3'- and 5'-hydroxyl groups leads to partial agonists with reasonably high affinity. Substitution at the 5'-position of the ribose is permitted and NECA (5'-N-ethylcarboxamido-adenosine) is a potent, non-selective AR agonist. NECA is also the most potent A<sub>2B</sub> receptor agonist with a K<sub>i</sub> of approximately 300 nM at the human A<sub>2B</sub> receptor, compared with K<sub>i</sub>'s of 14 nM at A<sub>1</sub>, 20 nM at A<sub>2A</sub> and 6.2 nM at the A<sub>3</sub> receptor. The amino group attached to the C6-position of the purine ring is essential for agonist activity, but substitution of one of the two hydrogen atoms in this group tends to give rise to A<sub>1</sub>-selective agonists of high potency. The most selective agonist for the A<sub>1</sub> subtype is CCPA (2-chloro-N<sup>6</sup>-cyclopentyladenosine), which is approximately 50-fold selective in human and over 600-fold selective in rat than at the A<sub>3</sub> receptor subtype. Modification at the C2-position of the purine

**Table 3. Key ligands for adenosine receptors**

Subtype	Agonists	Antagonists	References
<b>hA<sub>1</sub></b>	<b>CCPA (9.1, 9.4-9.6*)</b> ( <i>R</i> )-PIA (8.7, 8.9-9.3*) IAB-MECA (8.1, 7.7*) <b>NECA (7.9, 7.9-8.4*)</b> <b>CI-IB-MECA (6.9, 6.1*)</b> <b>CGS 21680 (6.5, 5.5*)</b> Adenosine (6.5 <sup>1</sup> )	<b>DPCPX (9.1, 9.5-9.7*)</b> <b>CGS 15943 (8.5, 7.7-8.2*)</b> XAC (7.5, 8.4-8.6*) <b>ZM 241385 (6.6, 5.7*)</b> SCH 58261 (6.5, 6.9*) MRS 1754 (6.4, 7.8*) MRE3008F20 (6.0, 5.0*)	79, 82, 83
<b>hA<sub>2A</sub></b>	<b>NECA (7.7, 7.7-8.0*)</b> <b>CGS 21680 (7.6, 7.7*)</b> IAB-MECA (6.3, 6.7*) Adenosine (6.2 <sup>1</sup> ) ( <i>R</i> )-PIA (6.1, 6.1-6.9*) <b>CI-IB-MECA (5.7, 6.3*)</b> <b>CCPA (5.6, 5.4*)</b>	SCH 58261 (9.2, 8.6*) <b>ZM 241385 (9.1, 9.5*)</b> XAC (9.0, 7.3-7.6*) <b>CGS 15943 (8.4, 8.5-8.9*)</b> <b>DPCPX (6.9, 6.5*)</b> MRE3008F20 (6.9, 5.7*) MRS 1754 (6.3, 6.2*)	79, 82
<b>hA<sub>2B</sub></b>	<b>NECA (6.4-6.5)</b> Adenosine (4.6 <sup>1</sup> ) ( <i>R</i> )-PIA (4.5-5.4) <b>CCPA (4.4)</b> <b>CGS 21680 (3.4)</b>	MRS 1754 (8.7) XAC (7.9) <b>CGS 15943 (7.8)</b> <b>ZM 241385 (7.5)</b> <b>DPCPX (7.3)</b> MRE3008F20 (5.7)	79, 82
<b>hA<sub>3</sub></b>	IAB-MECA (9.2, 8.9*) <b>NECA (8.2, 6.6*)</b> <b>CI-IB-MECA (8.0, 9.5*)</b> ( <i>R</i> )-PIA (7.8, 6.7-6.8*) <b>CCPA (7.4, 6.6*)</b> <b>CGS 21680 (7.2, 6.2*)</b> Adenosine (6.54 <sup>1</sup> )	MRE3008F20 (9.5, > 5.0*) <b>CGS 15943 (7.3, &gt; 4.0*)</b> XAC (7.0, > 4.0*) MRS 1754 (6.2) <b>DPCPX (5.4, &gt; 5.0*)</b> SCH 58261 (5.0) <b>ZM 241385 (5.0, 3.8*)</b>	79, 82

(Bold text denotes compounds available from Tocris)

Potency indices for agonists, etc. are given as either  $-\log_{10} K_i$  (pK<sub>i</sub>) or  $-\log_{10} EC_{50}$  (pEC<sub>50</sub>)<sup>1</sup> values. Data for rat (*r*\*) receptors are given for some key ligands.

ring can give some selectivity for A<sub>2</sub> receptors depending on the size of the substituent. CGS 21680, the most selective A<sub>2A</sub> agonist, is substituted both at the 5'-position of the ribose and the C2-position of the purine ring. Although CGS 21680 is approximately 26-fold selective for the rat A<sub>2A</sub> receptor, it is less than 3-fold selective for the human A<sub>2A</sub> receptor, compared to its actions at the A<sub>3</sub> subtype. Whilst IAB-MECA is approximately 13-fold selective for both the human and rat A<sub>3</sub> receptors, CI-IB-MECA is 11-fold selective for the human and ~ 1400-fold selective for the rat A<sub>3</sub> receptor. CI-IB-MECA is substituted at the 5', C2- and N<sup>6</sup>-positions. As mentioned previously, there is no selective A<sub>2B</sub> agonist available.

Radiolabelled agonists used for the characterisation of ARs in binding assays include: [<sup>3</sup>H]-CCPA, [<sup>3</sup>H]-NECA, [<sup>3</sup>H]-CHA, [<sup>3</sup>H]-PIA, [<sup>3</sup>H]-CPA, [<sup>3</sup>H]-CGS 21680, [<sup>125</sup>I]-I-AB-MECA and [<sup>125</sup>I]-APNEA. Affinities (K<sub>i</sub> values) for AR agonists have been determined using both whole-cell assays and membrane preparations, and in competition against either radiolabelled agonists or antagonists.<sup>82</sup> Two affinity states, one high and one low, are observed for agonists when binding assays are performed using membrane preparations, whereas a single K<sub>i</sub> value, corresponding to the low affinity site observed in membrane binding assays, is determined in whole-cell assays.<sup>83,84</sup> This difference is most likely due to high intracellular levels of GTP present in intact cells. Agonist affinities observed in functional assays, represented as the EC<sub>50</sub> values, vary widely as a function of expression levels and coupling efficiency to G proteins. The affinity of a given agonist at a particular receptor subtype can vary widely depending

on experimental conditions. As a consequence, the use of selective antagonists is a far more reliable means of characterising endogenous AR responses.

#### AR antagonists

Caffeine is the most frequently consumed psychotropic drug in the world, and the consumption of coffee and tea beverages leads to significant plasma concentrations of caffeine, theophylline, theobromine and other methylxanthine compounds.<sup>85</sup> These methylxanthines look very much like adenosine, but without the ribose moiety. In general, methylxanthines are weak AR antagonists yet also can act through other mechanisms such as phosphodiesterase inhibition. For adenylate cyclase assays, IBMX (3-isobutyl-1-methylxanthine) is frequently included as a phosphodiesterase inhibitor in non-adenosinergic receptor systems (such as the P2Y family). ARs and P2YRs are frequently expressed in the same cells; the functional characterisation of heterodimeric assemblies between these two families of GPCRs<sup>39</sup> may have been impeded by the use of AR antagonists in assays of P2YR function.

Modification of methylxanthines, at certain positions, has led to some subtype-selective AR antagonists.<sup>81</sup> DPCPX (8-cyclopentyl-1,3-dipropylxanthine; also referred to as CPX) is an A<sub>1</sub> receptor antagonist with subnanomolar affinity (K<sub>d</sub> = 0.8 nM at the human A<sub>1</sub> receptor).<sup>83</sup> Unfortunately, the K<sub>i</sub> of DPCPX at the human A<sub>2B</sub> receptor is approximately 50 nM, which makes the fold-selectivity of this compound for the A<sub>1</sub> receptor less than two orders of magnitude. The most selective A<sub>2B</sub> receptor antagonist is MRS1754 (~ 200-

fold). The most selective A<sub>2A</sub> and A<sub>3</sub> antagonists are not xanthine derivatives. SCH58261 and ZM241385 are subnanomolar A<sub>2A</sub> antagonists in human, but ZM241385 is more selective at the rat A<sub>2A</sub> receptor. MRE 3008F20 is the most selective human A<sub>3</sub> receptor antagonist, although this compound is essentially inactive at the rat receptor. MRS 1523 is reasonably selective for the A<sub>3</sub> receptor in rat<sup>60</sup> and also blocks this receptor subtype in frog.<sup>70</sup> Radiolabelled antagonists used for the characterisation of AR subtypes in binding assays include: [<sup>3</sup>H]-DPCPX, [<sup>3</sup>H]-SCH58261, [<sup>3</sup>H]-ZM241385, [<sup>125</sup>I]-ZM241385, [<sup>3</sup>H]-MRS1754, [<sup>125</sup>I]-ABO and [<sup>3</sup>H]-XAC. XAC (xanthine amine congener) is a good non-selective antagonist at human ARs (A<sub>1</sub>, 29 nM; A<sub>2A</sub>, 1 nM; A<sub>2B</sub>, 7.3 nM; A<sub>3</sub>, 92 nM), as is the non-xanthine compound CGS 15943 (A<sub>1</sub>, 3.5 nM; A<sub>2A</sub>, 4.2 nM; A<sub>2B</sub>, 16 nM; A<sub>3</sub>, 51 nM). The use of methylxanthines as AR antagonists has always been plagued by their activity at other sites. Methylxanthines are also direct inhibitors of PI3K lipid kinase (p110 delta in particular) activity, as is the non-xanthine adenosine receptor antagonist CGS 15943 (IC<sub>50</sub> < 10 μM at p110 delta).<sup>86</sup> Care should be exercised in the use of AR antagonists at high concentrations.

### Closing remarks

The identification, in 1992, of the A<sub>3</sub> receptor was the first hint that the number of molecular entities encoding nucleoside and nucleotide receptors would

greatly exceed that predicted by pharmacological and functional studies. This has been true in the case of the P2Y receptors, where eight human subtypes have already been identified at the molecular level and probably more remain to be cloned. This has also been true in the case of the P2X receptors, where the complexity of subunit assembly has revealed at least 12 subtypes of ATP-gated ion-channels and more remain to be characterised. Additionally, the molecular cloning of a receptor for adenine<sup>87</sup> adds a further dimension to the purinoceptor family (Figure 1).

The use of recombinant systems, particularly *Xenopus laevis* oocytes (the only expression system known to be devoid of functional P1 and P2 receptors), has enabled the operational profile of individual receptor subtypes to be determined. Such information is of great utility in attempting to unravel the ways nucleoside and nucleotide receptors interact with each other and with other proteins *in vivo*. There is still much to do in the search for subtype selective agonists and antagonists, but ligand selectivity is now achievable for some subtypes – with the promise of more to follow. We hope that the ligands identified in this review will help investigators to explore nucleotide and nucleoside signalling *in vivo*, with greater confidence.

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# Adenosine and Purinergic Receptor Ligands available from Tocris

## Adenosines

### Agonists

1063	CGS 21680 HCl*	A <sub>2A</sub> agonist
1705	2-Chloro-N <sup>6</sup> -cyclopentyladenosine	Potent, selective A <sub>1</sub> agonist
1104	2-CI-IB-MECA	Highly selective A <sub>3</sub> agonist
1702	N <sup>6</sup> -Cyclopentyladenosine	Potent, selective A <sub>1</sub> agonist
1579	HEMADO	High affinity selective A <sub>3</sub> agonist
1066	IB-MECA	A <sub>3</sub> selective agonist
1691	NECA	Adenosine receptor agonist

### Antagonists

1699	CGS 15943	Potent adenosine receptor antagonist
0486	1,3-Dipropyl-8-phenylxanthine	A <sub>1</sub> selective antagonist
0439	DPCPX	A <sub>1</sub> selective antagonist
R439	[ <sup>3</sup> H]-DPCPX	Radiolabelled form of (0439)
1217	MRS 1220	Highly potent, selective hA <sub>3</sub> antagonist
1584	MRS 1706	Potent and selective A <sub>2B</sub> antagonist
R1585	[ <sup>3</sup> H]-MRS 1754	Selective hA <sub>2B</sub> antagonist radioligand (analogue of 1584)
1359	VUF 5574	Potent, selective hA <sub>3</sub> antagonist
1036	ZM 241385	Potent, highly selective A <sub>2A</sub> antagonist
R1036	[ <sup>3</sup> H]-ZM 241385	Radiolabelled form of (1036)

### Other

0481	Dilazep 2HCl	Adenosine uptake inhibitor
0691	Dipyridamole	Adenosine transport inhibitor
1363	PD 81723	Allosteric potentiator of A <sub>1</sub> receptors

## Purinergics

0845	Evans Blue tetrasodium salt	Selective P2X purinergic antagonist
1260	Ivermectin	Modulates glutamate/GABA-activated Cl <sup>-</sup> channels
1277	KN-62	Non-competitive P2X <sub>7</sub> antagonist
1062	2-Methylthioadenosine triphosphate tetrasodium salt	P2 purinergic agonist
1388	MRS 2159	Potent, highly selective P2X <sub>1</sub> antagonist
0900	MRS 2179 tetraammonium salt	Selective P2Y <sub>1</sub> antagonist
1203	MRS 2219	Potentiates P2X <sub>1</sub> -mediated responses
1204	MRS 2220	P2X <sub>1</sub> antagonist
1240	NF 023 hexasodium salt	Selective, competitive P2X antagonist
1199	NF 279 hexasodium salt	Potent selective P2X <sub>1</sub> antagonist
1391	NF 449 octasodium salt	Highly selective P2X <sub>1</sub> antagonist
1682	PIT	P2Y ligand; displays mixed antagonism/potentialiation
0625	PPADS tetrasodium salt	P2 purinergic antagonist
0683	iso-PPADS tetrasodium salt	P2X purinergic antagonist
1309	PPNS	Potent, selective P2X <sub>1</sub> antagonist
1472	Suramin hexasodium salt	Non-selective P2 antagonist

\*Local regulations may restrict the sales of this product in certain territories. Please consult your local Tocris Cookson office or distributor for further details.

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