

# Leukocyte P2 Receptors: A Novel Target for Anti-inflammatory and Anti-tumor Therapy

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**Abstract:** P2 receptors are a class of plasma membrane receptors ligated by extracellular nucleotides and expressed ubiquitously throughout the body. Two main families are known: P2X and P2Y. P2X are ligand (ATP)-gated channels, while P2Y are G-protein-coupled seven membrane-spanning receptors. The P2X and the P2Y subfamilies comprise seven and eight members, respectively. While ATP is the only known physiological ligand of P2X receptors, P2Y receptors are known to be also activated by ADP, UTP, UDP and UDP-glucose in a subtype-specific manner. Several P2 subtypes are expressed by leukocytes where they have been implicated in a host of different responses ranging from chemotaxis to differentiation, from proliferation to cytotoxicity, from secretion of inflammatory mediators to cell fusion. However, until recently there was no *in vivo* proof of the participation of P2 receptors in inflammatory or proliferative disorders and, in addition, few pharmacological modulators of P2 function were available. During the last two years animal and human studies have produced preliminary but nevertheless compelling evidence in support of an important function of P2 receptors in inflammation and hematological tumors. Importantly, selective blockers of these receptors have been synthesized, thus paving the way to the possible development of P2-targeted anti-inflammatory and anti-tumoral therapies.

## INTRODUCTION

It is taking time, but eventually the concept that adenosine 5'-triphosphate (ATP), the universal intermediate of energy transactions, the key player in all aspects of intracellular metabolism, also plays a role as an extracellular messenger is being accepted even by most skeptic opponents. This was not an easy path, and if it had not been for Geoff Burnstock's prophetic vision and stubborn determination, we would still be ignorant of a vital and ubiquitous pathway of cell-to-cell signalling. While it has been appreciated for a few decades that adenosine is an important inhibitory mediator in the cardiovascular [1, 2] and central nervous [3] systems, and more recently also in the inflammatory and immune system [4, 5], very few were open to grant the status of extracellular messenger to the compound that, upon sequential de-phosphorylation, produces adenosine, i.e. ATP.

It is now clear that the presence of ATP in the extracellular space is rather the rule than the exception since this compound may be released not only as a consequence of cell damage or cell death (the cytoplasmic ATP concentration is in the 5-10 mM range), but also via non-lytic pathways that may involve constitutive vesicular transport [6], regulated exocytosis [7, 8], and non-vesicular secretion via membrane pathways such as connexins [9, but see 10], members of the P2X subfamily (P2X<sub>7</sub>) [11], or the

multidrug resistance protein (MDR) [12]. There is general agreement that in the pericellular space, under resting conditions, cells keep an ATP concentration in the nanomolar range (1-100 nM) [13-15]. Upon stimulation, or in the event of cell damage, the level that extracellular ATP may reach is uncertain [see 16-19], as it depends on a number of as yet poorly known factors such as (a) the intrinsic ATP-releasing ability of the given cell type, (b) the concomitant activation/inhibition of plasma membrane ecto-ATPases expressed on the ATP-releasing cell or on neighbouring, bystander, cells, (c) the presence of ATP-degrading enzymes in the extracellular fluid, and (d) the rate of equilibration with the extracellular fluid. Furthermore, it is possible that ATP release occurs into protected compartments [20], such as those that may form at sites of close cell-to-cell interaction. In this latter case, ATP may reach micromolar or even higher concentrations.

## P2 RECEPTOR SUBFAMILIES

P2 receptors (P2R) transduce signals carried by extracellular nucleotides. According to the most recent nomenclature of IUPHAR (International Union of Pharmacology), they are classified into P2Y and P2X. P2Y receptors (P2YR), also referred to as "metabotropic", are coupled via G-proteins to changes in intracellular Ca<sup>2+</sup> ([Ca<sup>2+</sup>]<sub>i</sub>) homeostasis and/or cAMP concentration [21]. P2XR, also referred to as "ionotropic", are poorly selective cation channels made by the assembly of the same (homo-oligomers) or different (hetero-oligomers) subunits. Thus far, seven P2X subunits have been cloned [22, 23]. The main signal-transducing system activated by P2XR stimulation involves changes in the intracellular cation concentration, but other pathways have also been identified recently [24, 25].

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## P2Y RECEPTORS

As of now, eight subtypes are included in the P2YR subfamily: 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 P2Y<sub>14</sub>. P2YR are included in the superfamily of rhodopsin-like, G-protein-coupled receptors with seven transmembrane domains. The size goes from 333 (P2Y<sub>13</sub>) to 379 (P2Y<sub>6</sub>) aminoacids, for a predicted molecular mass of 36 to 42 kDa [26]. Based on the degree of structural relatedness, two P2Y subgroups have been identified: the first includes P2Y<sub>1</sub>, P2Y<sub>2</sub>, P2Y<sub>4</sub>, P2Y<sub>6</sub> and P2Y<sub>11</sub>, and the second P2Y<sub>12</sub>, P2Y<sub>13</sub> and P2Y<sub>14</sub> [21]. It is likely that the final number of members of the two subgroups will exceed this list as several orphan G-protein-coupled receptors with substantial sequence identity to known P2YR have been identified. Some of these perspective new members of the P2Y family retain amino acid motifs that are thought to be necessary for ATP binding and receptor activity in their sixth (TM6) and seventh (TM7) trans membrane stretches (TM6 H-X-X-R/K motif, and TM7 Y-Q/K-X-X-R or K-E-X-X-L motif). Agonist selectivity is also a discriminant feature: P2Y<sub>1</sub>, P2Y<sub>11</sub>, P2Y<sub>12</sub> and P2Y<sub>13</sub> are mainly responsive to ATP and ADP, while P2Y<sub>2</sub>, P2Y<sub>4</sub> and P2Y<sub>6</sub> respond also (or mainly) to UTP and UDP [21, 27]. According to the latest review of the IUPHAR committee for receptor nomenclature the receptor for UDP-glucose has also been added to the list of P2YR (P2Y<sub>14</sub>) [21]. Most P2YR couple to G<sub>q</sub> and activate phospholipase C, with the generation of inositoltrisphosphate (IP<sub>3</sub>) and increase in [Ca<sup>2+</sup>]<sub>i</sub>. Exceptions are P2Y<sub>12</sub>, P2Y<sub>13</sub> and P2Y<sub>14</sub> which do not activate phospholipase C. This in turn leads to the activation of additional downhill pathways such as protein kinase C, phospholipase A2, phospholipase D, mitogen-activated protein (MAP) kinase, ion channel opening [27, 28]. Adenylate cyclase activity can be either stimulated or inhibited, depending on the P2YR subtype [29]. Preliminary data suggest that P2Y<sub>14</sub> also couples to G proteins, possibly G<sub>i/o</sub>, but the precise subtype is as yet unknown. P2YR have been thought so far to function as monomers, however recent findings suggest that this might not be the case, as it has been reported that P2Y<sub>1</sub>R and the adenosine A<sub>1</sub>R dimerize, with the resultant oligomeric receptor exhibiting a distinct pharmacology [30, 31].

### P2Y<sub>1</sub>

The P2Y<sub>1</sub>R has been cloned from several species and has a broad tissue distribution [27, 29]. Physiological agonist is ADP, while ATP has a much lower intrinsic activity that may vary from competitive antagonism to full agonism [32, 33]. The principal appeal of P2Y<sub>1</sub>R stems from the role it plays in platelet activation [34, 35]. The P2Y<sub>1</sub> knock-out mouse shows a distinct phenotype characterized by decreased platelet aggregation, increased bleeding time and resistance to thromboembolism [36, 37]. The main signal transduction pathway depends on the activation of phospholipase C and related downhill events, but there is hint to an involvement of Rho-dependent kinase (ROCK) [38, 39]. An additional intriguing second messenger-generating system is the direct interaction of the P2Y<sub>1</sub> C-terminal domain with plasma membrane ion channels [40]. This property is also shared by other P2YR such as P2Y<sub>2</sub>, P2Y<sub>6</sub>, and P2Y<sub>11</sub>, but not P2Y<sub>4</sub>. The pharmacological profile

of P2Y<sub>1</sub> activation is the following: 2-MeSADP > 2-MeSATP ≥ ADP >> ATP. , -meATP, , -meATP and UTP are inactive. A potent antagonist, MRS 2279, has been recently introduced [41].

### P2Y<sub>2</sub>

The P2Y<sub>2</sub>R was originally cloned from a mouse neuroblastoma cell line [42] and later shown to be widely expressed in rodents and human tissues [43]. Major interest for possible therapeutical applications has focused on the role of P2Y<sub>2</sub> in the control of fluid secretion in the airways and in the eye. P2Y<sub>2</sub>R is positively coupled to opening of Ca<sup>2+</sup>-sensitive Cl<sup>-</sup> channels that control fluid secretion in epithelial cells, and phospholipase C activation. Coupling to inward rectifier K<sup>+</sup> channels has also been described [44]. A *p2y2*<sup>-/-</sup> mouse has been generated and UTP-dependent Cl<sup>-</sup> measured in different tissues [45]. It appears that P2Y<sub>2</sub>R is the dominant receptor mediating nucleotide-stimulated fluid secretion from the airways (trachea), while it has a lesser role in the gallbladder and none in the jejunum. ATP and UTP are equipotent, while ADP, UDP, 2-MeSATP and , -meATP are inactive. P2Y<sub>2</sub> has become a focus of intense industrial activity that has led to the synthesis of several stable compounds with agonist activity (see below) [46].

### P2Y<sub>4</sub>

The P2Y<sub>4</sub>R is activated by UTP but not UDP. ATP is an agonist at the rat and a weak competitive antagonist at the human P2Y<sub>4</sub>R [47]. This receptor is expressed in several tissues, but main interest stems from its expression by secretory epithelial cells and skin, thus suggesting a potential role for this receptor in the control of fluid secretion and wound healing [48-50]. ADP, ATP S and , -meATP are weak agonists.

### P2Y<sub>6</sub>

The P2Y<sub>6</sub>R is a selective UDP receptor. ADP is a weak agonist while ATP is fully inactive. This receptor is widely distributed, but its main interest arises from its localization to immune cells, where it appears to be involved in several important immune-mediated responses [19, 51, 52]. Coupling to intracellular second messengers occurs *via* phospholipase C and activation of plasma membrane K<sup>+</sup> channels.

### P2Y<sub>11</sub>

This was thought to be the only strictly ATP-selective P2YR so far identified in human tissue [27, 53], but recent evidence suggests that UTP may also be an agonist [54]. With the recent demonstration that dendritic cell (DC) differentiation can be modulated by extracellular nucleotides [55], the P2Y<sub>11</sub>R has become increasingly appealing as there is strong evidence that it is the P2R involved in such a modulatory activity [56-58]. Besides immune cells, P2Y<sub>11</sub> is also present in the intestine and in the kidneys [53]. It is coupled to both the phosphoinositide pathway and adenylyl cyclase, with the unique property among P2YR to induce an increase in the cAMP concentration. The rank of potency

is ATP S = BzATP > ATP > 2 MeS-ATP >> ADP. UDP is inactive.

### P2Y<sub>12</sub>

This is the long-sought platelet ADP-sensitive receptor that has eluded identification for many years, until it was finally cloned in 2001 [59-61]. P2Y<sub>12</sub> is the target of the thienopyridines ticlopidine and clopidogrel, and of a number of new drugs developed as anti-thrombotic agents. ADP is the preferred agonist while ATP is an antagonist. A patient was identified with a bleeding disorder who carries a defect in the gene coding for P2Y<sub>12</sub> [59, 62]. Mice deleted of the *p2y12* gene have a normal phenotype but an increased bleeding time and reduced platelet aggregation in response to ADP, thrombin and collagen [63]. The P2Y<sub>12</sub>R negatively couples to adenylate cyclase and is mainly expressed on platelets and megakaryocytes, but is also present in the brain [59, 64].

### P2Y<sub>13</sub>

The P2Y<sub>13</sub>R was cloned and expressed in 2001 and shown to correspond to orphan receptor GPR86 (also known as GPR94) [65, 66]. It has a high affinity for ADP and a close homology to P2Y<sub>12</sub>. P2Y<sub>13</sub> is predominantly expressed in spleen, lymph nodes, bone marrow and brain, thus a role in immune response and hematopoiesis has been suggested [65]. 2MeS-ADP is as potent as ADP at the human P2Y<sub>13</sub>R and triphosphate nucleotides are inactive [66].

### P2Y<sub>14</sub>

In 2000 a receptor was cloned selectively activated by UDP-glucose and other UDP-sugars [67]. This receptor was later shown to have a high degree of structural relatedness with P2Y<sub>12</sub> and P2Y<sub>13</sub> and has now been included in the P2Y subfamily as P2Y<sub>14</sub> [21]. The identification of a receptor for UDP-sugars raises intriguing questions as to the possible role of these molecules in intercellular signaling [68]. It was long thought that UDP-glucose and UDP-galactose were exclusively involved in intermediary metabolism of carbohydrates, but it is clear that this view has to be reconsidered. The relevance of P2Y<sub>14</sub> is stressed by its wide distribution, especially in cells of hematopoietic origin [67, 69]. Recently, Lee *et al.* have shown that within hematopoietic cells, P2Y<sub>14</sub> identifies a subpopulation with stem-cell like multipotential long-term culture capability [70]. The following sugars had agonist activity at the heterologously expressed receptor: UDP-glucose, UDP-galactose, UDP-glucuronic acid and UDP-acetylglucosamine.

## P2X RECEPTORS

P2XR are multimeric structures made by the assembly of the same (homo-oligomers) or different (hetero-oligomers) subunits, seven of which have been cloned (P2X<sub>1-7</sub>). At variance with P2YR, for which the discovery of further members of the family is anticipated on the basis of sequence homology with orphan receptors identified in mouse and human genome, there are no reports of additional homologous P2X sequences, a finding suggesting that the

total and final number of the members of the P2X subfamily is seven. P2X subunit size ranges from 379 (rat P2X<sub>6</sub>) to 595 amino acids (human and rat P2X<sub>7</sub>). Splice variants have been reported for all P2XR but not P2X<sub>7</sub> [23]. Two stretches are present in the amino acid sequence of sufficient length to span the plasma membrane, and separated by a bulky domain that is thought to face the extracellular space. In the extracellular domain 10 cysteines and 6 glycosylation sites have been identified. A membrane topology has been proposed with a large extracellular region flanked by two transmembrane stretches and the N and C termini both located on the cytoplasmic side of the plasma membrane [71, 72]. The COOH terminal regions are the most divergent in sequence, and this might be relevant for expression, assembly and functional properties of the different P2X subunits [73-75]. Current evidence suggests that P2X subunits aggregate in trimers to form homomeric or heteromeric channels (homotrimers or heterotrimers) [76, 77]. Formation of hexamers has also been reported [77].

By immunoprecipitation studies, it has been shown that most P2X subunits can co-assemble in an heterologous expression system with the notable exception of P2X<sub>7</sub> [78]. Heteromerization confers to the receptor functional features that reflect the contribution of the individual subunits. Native heteromeric receptors have been found in certain tissues such as sensory neurons, sympathetic ganglion cells and brain neurons [79]. Some P2X subunits appear to have a preferential localization in the nervous system (i.e. P2X<sub>2</sub>, P2X<sub>3</sub> and P2X<sub>5</sub>), while others (i.e. P2X<sub>1</sub>, P2X<sub>4</sub> and P2X<sub>7</sub>) are more ubiquitously distributed. P2X<sub>6</sub> expression has so far been demonstrated in rat superior cervical ganglia, rat brain, human lymphocytes and skeletal muscle [23, 80, 81]. In contrast to P2YR, which are activated by a fairly broad range of di- or tri-phosphate uridine or adenine nucleosides, the only known physiological ligand of P2XR is ATP, different ATP analogues though have different potency at the various P2XR subtypes.

### P2X<sub>1</sub>

This is the main P2XR responsible for ATP-dependent contraction of smooth muscle cells. Thus, it appears to have a central role in the contraction of vas deferens, urinary bladder, and arteries and veins isolated from different tissues [82-84]. Mice lacking P2X<sub>1</sub> have much reduced fertility due to deficient vas deferens contraction, and thus faulty ejaculatory function [83]. Bladder contractility is also reduced. Rather interestingly, blood pressure seems not to be affected. Currents through P2X<sub>1</sub> quickly decline in the continuous presence of ATP, thus this receptor is the prototypic fast-desensitizing P2XR. The rank order of potency is BzATP > 2-Me-SATP ≥ ATP > , -meATP. This receptor is blocked fairly selectively by pyridoxal-phosphate-6-azophenyl-2',4'-disulfonic acid (PPADS) and by 2', 3'-O-(2,4,6-trinitrophenyl)-ATP (TNP-ATP) [85].

### P2X<sub>2</sub>

The P2X<sub>2</sub>R is expressed in sensory neurons and may be involved in pain sensation (co-assembled with P2X<sub>3</sub>, see below). In contrast to P2X<sub>1</sub>, P2X<sub>2</sub> is a slowly-desensitizing

receptor. In some conditions, P2X<sub>2</sub> undergoes a progressive increase in permeability during protracted exposure to ATP, a behaviour reminiscent of the progressive dilation observed in P2X<sub>7</sub>. No selective agonists or antagonists of P2X<sub>2</sub> are currently available. 2-Me-SATP and ATP S are equipotent, while , -meATP and , -meATP are inactive.

### P2X<sub>3</sub>

This seems to be the main P2X subunit involved in nociception. It is expressed in sensory neurons and in the brain, while it is absent from smooth muscle [79, 86]. Mouse deleted of the *p2x3* gene show reduced pain sensation and bladder hyporeflexia. The potency order is BzATP >> 2-Me-SATP > ATP = , -meATP. Convincing evidence suggests that sympathetic neurons and primary sensory neurons express P2X<sub>2/3</sub> heteromers, that share the pharmacological profile of homomeric P2X<sub>3</sub>, and the slow desensitizing behaviour of P2X<sub>2</sub> homomers.

### P2X<sub>4</sub>

The P2X<sub>4</sub>R has been cloned from several excitable and non-excitable tissues [87]. P2X<sub>4</sub> undergoes a progressive increase in conductance when application of the stimulus is protracted, suggesting that it might undergo a transition into a non-selective pore similar to that typically described for

P2X<sub>7</sub>, and to a lesser extent P2X<sub>2</sub>. At variance with other P2XR, P2X<sub>4</sub> is relatively insensitive to blockade by PPADS.

### P2X<sub>5</sub>

The P2X<sub>5</sub>R is mainly expressed in the nervous and immune system. A human full length cDNA has not yet been isolated [88]. The activation profile is ATP > 2-Me-SATP > ADP. P2X<sub>5</sub> is reported to form functional heteromers with P2X<sub>1</sub> [89].

### P2X<sub>6</sub>

This appears to be a silent receptor as no currents can be recorded when the P2X<sub>6</sub> cDNA is expressed in oocytes or HEK292 cells (North *et al.*, 2002). mRNA of this receptor, originally designated P2XM, was isolated from peripheral lymphocytes and skeletal muscle [81].

### P2X<sub>7</sub>

This is the P2R previously known as P2Z [90]. It has been isolated from rat, mouse and human tissues, and it is widely distributed. This is the prototypical cytotoxic P2X receptor, and the one for which the ability to form a large non-selective pore upon sustained stimulation has been more convincingly demonstrated (see also P2X<sub>2</sub> and P2X<sub>4</sub>) [91].

**Table I. Preferred Physiological Agonist, Size and Signal Transduction Mechanism of Mammalian P2 Receptors**

Subtype	Agonist	Amino acid number	Signal transduction	
P2Y <sub>1</sub>	ADP	362	IP3	cAMP
P2Y <sub>2</sub>	UTP, ATP	373	IP3	
P2Y <sub>4</sub>	UTP	352	IP3	
P2Y <sub>6</sub>	UDP	379	IP3	
P2Y <sub>11</sub>	ATP	371	IP3	cAMP
P2Y <sub>12</sub>	ADP	342		cAMP
P2Y <sub>13</sub>	ADP	334	IP3	cAMP
P2Y <sub>14</sub>	UDP-glucose	338	IP3	
P2X <sub>1</sub>	ATP	399	Ion currents	
P2X <sub>2</sub>	ATP	472	Ion currents	
P2X <sub>3</sub>	ATP	397	Ion currents	
P2X <sub>4</sub>	ATP	388	Ion currents	
P2X <sub>5</sub>	ATP	455	Ion currents	
P2X <sub>6</sub>	ATP	379	Ion currents	
P2X <sub>7</sub>	ATP	595	Ion currents Protein-protein interaction	

Cloning of an additional P2YR, P2Y<sub>15</sub>, was recently reported [204]. This finding is waiting for final approval and validation by IUPHAR. At P2Y<sub>1</sub> and P2Y<sub>12</sub> ATP may act as an antagonist. P2Y<sub>13</sub> mediates inhibition of adenylate cyclase at low levels of activation and stimulation at high levels. Splice variants of P2X<sub>2</sub> and P2X<sub>4</sub> have been identified [205-207]. P2XR may assemble as heterooligomers (P2X<sub>2</sub>/P2X<sub>3</sub>, P2X<sub>1</sub>/P2X<sub>5</sub>, P2X<sub>4</sub>/P2X<sub>6</sub>).

92]. The rank order of potency is BzATP >> ATP > ATP S >> ADP. The P2X<sub>7</sub>R is a low-affinity receptor ( $k_m > 500 \mu\text{M}$  in the presence of physiological concentrations of  $\text{Ca}^{2+}$  and  $\text{Mg}^{2+}$ ), and this has been a matter of concern ever since it was characterized, to the point that doubts were raised as to the physiological relevance of ATP-mediated activation of this receptor. Now, a study by Seman and co-workers [93] in mouse T lymphocytes suggests that in fact another endogenous ligand (nicotinamide adenine dinucleotide, NAD) besides ATP is also able to trigger P2X<sub>7</sub>R opening via ADP-ribosylation probably of the receptor itself.

The human P2X<sub>7</sub>R is reversibly blocked by nanomolar concentrations of 1-(N,O-bis(1,5-isoquinolinesulfonyl)-N-methyl-L-tyrosyl-4-phenylpiperazine (KN-62) [94], and by several of its analogues [95]. Another useful non-competitive, irreversible blocker is oxidized ATP [96], a compound that is thought to form Schiff bases with unprotonated lysins in the vicinity of the ATP binding site. During the last few years P2X<sub>7</sub> has become the focus of intense research activity due to its involvement in a host of responses spanning from cytotoxicity to stimulation of proliferation, from membrane blebbing to activation of transcription factors, from release of cytokines to stimulation of NO production [92]. Considerable effort has been put into the dissection of the signal transduction cascade and the identification of proteins that co-assemble with P2X<sub>7</sub> [24].

#### WHY ARE P2 RECEPTORS A POTENTIAL TARGET IN THE TREATMENT OF INFLAMMATORY DISEASES AND NEOPLASIA?

Although the proposal to use ATP as an anti cancer drug dates back to the beginning of 1980 [97, 98], these early studies posed several serious mechanistic problems and did not suggest a logical and rationale approach. This scenario has changed only with the molecular cloning of P2R and the discovery that all immune cells exhibit robust expression of most if not all P2YR or P2XR subtypes [92]. These findings were further strengthened by the observation that some tumours have unusually high levels of expression of some P2XR subtypes, a feature that renders these tumours eminently sensitive to ATP-mediated cytotoxicity. Very recent *in vitro* and *in vivo* data have further emphasized the potential of P2R in inflammation and cancer by showing on one hand that extracellular ATP at low concentrations may shape the differentiation of DC [99], and on the other that interference with systems involved in the degradation of extracellular ATP severely affects the development of inflammation [100]. Both modulation of DC differentiation and the more general effect on the inflammatory response depend on a complex interplay between P2YR, P2XR and ectoATPase (CD39). This is a significant shift with respect to the current view centred on P2X<sub>7</sub> as the main P2 player in inflammation and immunomodulation.

#### P2 Receptors and Inflammation

Inflammation is a complex homeostatic mechanism that allows detection of injurious agents, elimination of foreign organisms and restoration of tissue integrity [101, 102]. Traditionally, little attention has been paid to P2R by

investigators in the fields of inflammation and immunity until four different groups started an indepth investigation of P2R function in lymphocytes and myelomonocytic cells [103-106]. It soon appeared clear that P2R, besides being responsible for a striking modulation of cell morphology and viability, could also affect myeloid and lymphoid cell maturation. In turn, we now know that P2R expression is finely tuned during leukocyte differentiation [see 107 for a recent review].

Among P2R, P2Y<sub>1</sub> and P2Y<sub>2</sub> appears to be important in chemotaxis of macrophages, granulocytes and mast cells [108-111]. Besides chemotaxis, extracellular nucleotides have several additional stimulatory effects on polymorphonuclear leukocytes, such as stimulation of superoxide anion generation [112], granule exocytosis [113], increased membrane expression of CD11b/CD18 adhesion molecule [114] and IL-8 secretion [115]. However, P2YR involved in these latter responses are incompletely characterized. P2Y<sub>1</sub>R and P2Y<sub>2</sub>R have an important activating role also in macrophages, where they are probably the receptors responsible for nucleotide-dependent activation of inducible nitric oxide synthase (iNOS) [116-117] and stimulation of phospholipid metabolism [118], respectively. Recently, the P2Y<sub>6</sub>R has attracted interest as its stimulation by UDP in THP-1 cells leads to IL-8 gene expression and IL-8 secretion [19]. During the last two years immunologists have payed increasing attention to the P2Y<sub>11</sub>R. This receptor seems to be involved in granulocyte differentiation [119, 120], and more intriguingly in the maturation of DC derived from blood mononuclear phagocytes. DC are key participants in immunity as they are the most potent antigen-presenting cells, and the only cell type endowed with the ability to initiate the primary immune response [121, 122]. DC are normally quiescent (immature) until they meet an antigen (or a bacterial product). At this point, they start a complex process of maturation that allows them to interact and activate naive T helper (T<sub>H</sub>) lymphocytes, and thus initiate the immune response. DC can drive the differentiation of T<sub>H</sub> cells into either T<sub>H1</sub> or T<sub>H2</sub> subtypes, two lymphocyte populations with widely differing abilities to shape the subsequent immune response. T<sub>H1</sub> lymphocytes stimulate innate immunity, enhance phagocytic and bactericidal activity of phagocytes and favour the development of chronic inflammatory response, while T<sub>H2</sub> lymphocytes mainly stimulate adaptive immunity and drive the synthesis of the immunoglobulins IgA and IgE [123]. Several recent papers have provided clear evidence in support of an important role of extracellular nucleotides in the acquisition of a mature phenotype by DC [56, 58, 99, 124-129]. DC matured in the presence of ATP differentiate via an alternative pathway characterised by up-regulation of costimulatory molecules, but lack of production of IL-12 and of pro-inflammatory chemokines and cytokines. As a result of impaired IL-12 production, ATP-pulsed DC have a reduced ability to drive type 1 differentiation of naive T<sub>H</sub> lymphocytes, rather favouring development of T<sub>H2</sub> lymphocytes. Accordingly, ATP pulsed-DC are less efficient for recruiting T<sub>H1</sub> than T<sub>H2</sub> lymphocytes. Overall, this translates into a decreased ability of DC matured in an ATP-containing environment to initiate and sustain chronic

inflammation, a process dependent on the expansion of T<sub>H1</sub> lymphocytes [55].

The importance of the ATP content of the microenvironment in which DC mature has been underlined by a recent study by Mizumoto and co-workers [100] reporting an altered ability to produce a contact hypersensitivity response in mice deleted of the principal plasma membrane ecto-ATPase of skin Langerhans cells (these are the DC of the epidermis). In these knock-out mice lack of nucleotide-hydrolysing activity leads to a chronic accumulation of ATP in the pericellular space. Persistence of unphysiologically-elevated levels of pericellular ATP might affect Langerhans cell functions either by causing P2Y receptor desensitization or by promoting a T<sub>H2</sub>-skewing maturation (this type of maturation inhibits the development of contact hypersensitivity which is a disease typically dependent on T<sub>H1</sub> cells). Rather interestingly, but not unexpectedly, the CD39 knock-out mice develop a strong acute dermatitis if locally challenged with irritants [100]. This response appears to be due to the fast and massive release of ATP from injured keratinocytes and is likely mediated *via* P2X<sub>7</sub>R. This brings us to a discussion of the prototypic inflammatory P2R: P2X<sub>7</sub>.

The P2X<sub>7</sub>R, previously known as P2Z, is the P2R that has been more convincingly associated with stimulation of inflammation and modulation of immune response [92, 130-133]. Considerable evidence supports the view that P2X<sub>7</sub>R blockade might reduce the release of the pro-inflammatory cytokines IL-1, IL-18 and TNF [18, 131, 132, 134, 135], and thus down-regulate inflammation. This was confirmed by experiments in mice deleted of P2X<sub>7</sub>R that showed both reduced cytokine production and decreased inflammation when challenged with intraperitoneal injections of ATP or of anti-collagen antibodies [136, 137]. Interest was also raised in the possible role played by extracellular nucleotides in sustaining inflammation in the central nervous system by stimulating cytokine and chemokine release from astrocytes or microglial cells [132, 138-141], although *in vivo* experiments seem not to support a direct role of P2X<sub>7</sub> in ischemic or excitotoxic brain damage [142]. The possible application of P2X<sub>7</sub> agonists in anti-mycobacterial therapy has also stirred interest, given that macrophages stimulated *via* P2X<sub>7</sub> show an enhanced ability to kill intracellular *Mycobacterium tuberculosis* [143, 144], a process dependent on an increased rate of phagosome-lysosome fusion [145, 146]. Very recently, ATP was shown to inhibit chlamydial infection through a similar mechanism [147]. This observation further stresses the relevance of P2X<sub>7</sub>-targeted drugs in fighting infections by intracellular parasites. Extracellular ATP might have a wider role in pathogen-macrophages interactions, as suggested by a recent paper showing that factors secreted by *Vibrio cholerae* require extracellular ATP to exhibit significant toxicity towards macrophages and mast cells [148]. P2X<sub>7</sub> is implicated in this case. It has been proposed that killing of *Candida albicans* induced by histatins (antimicrobial molecules secreted in the saliva of humans and higher primates) is dependent on the release of ATP and the activation of a P2X<sub>7</sub>-like receptor [149]. The possible participation of P2X<sub>7</sub> in membrane fusion events is also highlighted by the observation that

mononuclear phagocytes that express high levels of this receptor spontaneously fuse in culture, and inhibition of P2X<sub>7</sub> blocks formation of multinucleated giant cells *in vitro* (multinucleated giant cells are frequently found in inflammatory granulomas, but their function and the mechanism of formation are obscure) [150-152]. While the *in vivo* role of the P2X<sub>7</sub>R in cell fusion and granuloma formation is yet to be investigated, we are intrigued to notice that monocytes from patients affected by granulomatous diseases show an enhanced susceptibility to P2X<sub>7</sub> agonists [153].

An unavoidable (and utterly unpleasant) accompanying symptom of inflammation is pain. P2XR are involved in pain sensation, especially with the P2X<sub>3</sub> homomer and P2X<sub>2/3</sub> heteromer [154-158]. These and other reports point to a more likely role of the P2X<sub>3</sub>R in chronic inflammatory and neuropathic rather than acute pain. According to these authors, tonic ATP release from injured tissue, or from nerve terminals, triggers a tonic activation of the P2X<sub>3</sub>R of sensory endings, which then signal pain sensation [159]. Rather unexpectedly, the P2X<sub>4</sub>R has also been recently implicated in pain sensation. Tsuda *et al.* [160] have shown that pharmacological blockade of this receptor abolished tactile allodynia after peripheral nerve injury in rats. These authors went further to show that after nerve injury P2X<sub>4</sub>R expression increased in microglia, thus converting these cells into a hyperactive phenotype. Increased release of cytokines or other pro-inflammatory factors from hyperactivated microglia might then be responsible for sensitization of sensory afferents to otherwise innocuous stimuli. Recently a role for P2X<sub>7</sub> in arthritic pain has been suggested, although exclusively on the basis of antagonist studies [162].

## P2 Receptors and Cancer

The main interest in this field stems from the perspective of using ATP (or its stable derivatives) as anti cancer agents. The rationale is a) to push the differentiation of immature, proliferating, neoplastic cells toward a terminally-differentiated form, or b) to kill cancer cells (hopefully in a selective fashion). These two effects can in principle be achieved by modulating P2YR or P2XR function, respectively. During these last few years it has been observed that the P2X<sub>7</sub>R may also function as a growth-promoting receptor. This has suggested a third perspective for P2R as anti cancer targets: the use of P2X<sub>7</sub>R inhibitors as cytostatic drugs.

There is evidence that in the presence of extracellular nucleotides the human myelogenous cell lines HL-60 and U937, or the mouse M1 myelomonocytic cell line, are stimulated to acquire phenotypic characteristics of differentiated phagocytes [163, 164]. The ability of extracellular nucleotides (in particular ATP) to drive cell differentiation has been confirmed in several different *in vitro* models, and has been ascribed to P2Y<sub>11</sub> [120]. Scattered observations also suggest that some epithelial cancers (e.g. prostate cancer, oesophageal cancer, colorectal cancer), may be sensitive to growth inhibition mediated by P2R [165-168]. A similar growth-inhibitory effect was described in human breast tumour cells [169]. Besides its

differentiating or cytostatic effect, it was known for several years that extracellular ATP can be frankly cytotoxic [91, 105, 106, 170, 171]. The mechanism of ATP-mediated cytotoxicity was conclusively established thanks to the functional characterization and finally the molecular cloning of the responsible P2 receptor [90, 130, 172]. It is now clear that susceptibility to the cytotoxic effect of ATP is conferred by expression of a fully functional P2X<sub>7</sub>R. Sustained ATP-dependent opening of the P2X<sub>7</sub>R pore is the prime cause of ATP-dependent cell death, whether in the presence or absence of extracellular Ca<sup>2+</sup>, and whether it occurs by necrosis or apoptosis [173]. The ATP effect on tumor cell growth cannot always be clearly assigned to a pure cytostatic or mixed cytostatic/cytotoxic effect, and it is sometimes complicated by a paradoxical stimulation of growth at high concentrations [174].

Despite *in vitro* sparse data and the lack of insight into the mechanism of the differentiating/cytostatic/cytotoxic action of extracellular ATP, a few *in vivo* attempts to use ATP as anti cancer drug have been carried out, starting with the early work of Rapoport [175]. More recently, beneficial effects were shown for ATP infusion in patients with advanced non-small-cell lung cancer, probably *via* a mechanism unrelated to its known activity at P2R, but rather dependent on a general, ill-defined, non-specific effect on the nutritional status of the patients [176-178].

Recent studies investigating expression and function of the P2X<sub>7</sub>R have paved the way to a more rationale strategy for the use of P2R as perspective drug targets. In 2002 two groups reported on the association between P2X<sub>7</sub>R expression and function and the clinical course of chronic lymphocytic leukemia (CLL) [179, 180]. In the first study [179], two populations of CLL patients, one with an aggressive and the other with an indolent course of the disease, were screened for P2X<sub>7</sub>R expression and function. Patients with the aggressive variant had a disease requiring therapy, with a shorter than 12-month lymphocyte doubling time, or a disease progression through the Rai staging system. Those with the indolent course never required cytotoxic therapy, had a stable blood count and no disease progression. Patients with the evolutive disease showed a higher expression level of the P2X<sub>7</sub>R that correlated with a higher basal and ATP-stimulated Ca<sup>2+</sup> influx. Furthermore, incubation in the presence of ATP decreased spontaneous proliferation of lymphocytes from patients with the evolutive but not the indolent variant. In the second study [180], the prevalence of a loss-of-function mutation (A1513C causing a Glu to Ala substitution at position 496) in the P2X<sub>7</sub>R was studied in individuals with CLL and healthy controls. It was found that the A1513C mutation was three fold more frequent in CLL patients than in the healthy controls. Furthermore, two families in which father-son and sister-sister were affected by CLL, were also investigated for the presence of this loss-of-function mutation. Both the father and son, and the two sisters who were affected by CLL turned out to carry the A1513C mutation. Thus the study by Wiley *et al.*, [180] concluded that loss of P2X<sub>7</sub> function contributes to the pathogenesis of CLL, probably by decreasing the susceptibility to apoptosis of leukemic

lymphocytes (this would favour the accumulation of neoplastic lymphocytes in the circulation). A natural mutation (P451L) that decreases P2X<sub>7</sub>R function has also been recently identified in the mouse strain C57BL/6 [181].

The work by Adinolfi *et al.* [179] and Wiley *et al.* [180] established a strong link between a very relevant human disease (CLL is the most frequent adult hematopoietic tumour in the western world) and P2X<sub>7</sub>, but suggested an opposite pathogenetic role for this receptor. The picture was clarified and the opposite views reconciled by two later contributions that helped to put the role of P2X<sub>7</sub> in leukemia in the right perspective [182, 183]. Thunberg *et al.*, [182] correlated the A1513C *p2x7* R-gene polymorphism with the mutation status of the genes encoding the variable portion of the immunoglobulin heavy chain (V<sub>H</sub>) (a prognostic index in CLL) and with the overall survival of 170 CLL patients. Their main finding was that the presence of the A1513C mutation correlated with a longer survival (better prognosis), especially in those patients that also had mutated V<sub>H</sub> genes. Di Virgilio and Wiley in a recent Commentary [183] critically scrutinized these reports, and proposed two hypothesis that try to reconcile these contrasting results. On the one hand, P2X<sub>7</sub> might behave as a growth-promoting receptor, since decreased function correlates with a longer survival. Previous *in vitro* data obtained with P2X<sub>7</sub>-transfected cells support this view [11, 184]. Odd as it may seem, a positive role for P2X<sub>7</sub> in supporting cell proliferation was also found in proliferative vitreoretinopathy, a retinal disease characterised by abnormal proliferation of Muller cells, the main macroglial cells within vertebrate retina [185]. On the other, P2X<sub>7</sub> might be required for the physiological removal of lymphocytes from circulation. Poor function of this receptor in individuals who inherit the A1513C allele might allow a faster accumulation of neoplastic lymphocytes in the blood, which on the one hand leads to an earlier onset of the disease, but on the other also allows an earlier diagnosis, thus allowing a better treatment and a longer survival. It must be stressed that, while heavy expression of the P2X<sub>7</sub>R by CLL lymphocytes is so far undisputed, the association of a given polymorphism in the *p2x7* gene with a distinct clinical course in CLL, and therefore the diagnostic and prognostic relevance, is still a matter of hot debate [186, 187]. In any case, it is clear that these studies establish for the first time a clear and undisputable link between a P2R and cancer at the molecular, biochemical and epidemiological level.

Association between P2X<sub>7</sub> and cancer is further strengthened by studies demonstrating a strong expression of this receptor in specimens from several human solid tumours. Barden and co-workers reported extensive labelling for the P2X<sub>7</sub>R in melanoma cells [188], prostate cancer [189], and breast cancer [190]. It is worth mentioning that in prostate specimens P2X<sub>7</sub> was also present in apparently normal epithelial cells, but with a distinct pattern of expression, compared to cancer cells. On the contrary, in the breast the P2X<sub>7</sub>R was exclusively present in carcinomatous cells. The P2X<sub>7</sub>R is also heavily expressed by basal and squamous cell carcinoma [191]. Very interestingly, both ATP and the strong P2X<sub>7</sub>R agonist benzoyl ATP exhibited a

strong *in vitro* cytotoxic activity on the squamous cell carcinoma line A431. This latter observation, in keeping with the demonstration by Adinolfi *et al.* [179] of the potent *in vitro* cytostatic effect of ATP on B lymphocytes from patients with the aggressive CLL variant, raise the issue of P2X<sub>7</sub>-targeted anti-cancer therapy.

Scattered literature data support the use of P2R receptor agonists to inhibit cancer cell growth [167, 168, 170, 179, 192, 193]. However, we think that the most straightforward evolution of a P2X<sub>7</sub>-based innovative therapy resides probably in the design and synthesis of potent, long-lived and selective P2X<sub>7</sub> agonists that may allow efficient killing of neoplastic cells in the blood or in the tissues. The strong cytotoxic effect of P2X<sub>7</sub> agonists can also be exploited to potentiate the effect of more conventional cytotoxic agents used for anti tumour therapy, and for the *in vitro* purging of marrow cells to be used for autologous transplantation [194]. Alternatively, P2X<sub>7</sub> blockers might turn out to be useful for slowing down proliferation of P2X<sub>7</sub>-expressing cells [11]. In addition, it is important to point out that there are very few prognostic indicators so far available in CLL, thus we anticipate that the analysis of *p2x7* R-gene polymorphisms will have growing importance in predicting the clinical outcome of these patients.

#### ANTI-INFLAMMATORY AND ANTI TUMOUR DRUGS TARGETED TO P2 RECEPTORS

This is a painful issue. Identification of novel, potent and selective pharmacophores that interact with P2R has lagged behind the biochemistry, physiology, and molecular and cell biology. There are no P2R-targeted compounds suitable for anti-inflammatory or anti-cancer therapy that have been introduced in clinical use. A large number of P2 agonists or antagonists have been synthesized and tested *in vitro* (see 26 for a recent extensive review), but we believe that it is fair to say that the medicinal chemistry of P2R-based anti-inflammatory or anti-tumour compounds is yet to be written. We will focus here only on a few that either have been taken through preliminary clinical trials or appear to be particularly interesting in inflammation and cancer.

The most systematic approach to the development of selective P2 agonist/antagonist is the one initiated several years ago by Inspire Pharmaceuticals that has lead to the introduction of several P2Y<sub>2</sub> selective agonists and antagonists based on dinucleotides and dinucleotides derivatives that are extremely resistant to activity of ectonucleotidases and other nucleotide-metabolizing enzymes. Inspire is also developing nucleotide-like substances named nucleotoids in which replacement of given functional groups improves bioavailability without impairment of receptor selectivity. Some of the compounds developed by Inspire are currently being tested to improve mucociliary clearance in cystic fibrosis, chronic obstructive pulmonary disease, or to improve collection of mucus from upper airways and thus facilitate the diagnosis of lung cancer. One of the Inspire compounds (INS37217) is also being tested for treatment of allergic rhinitis [195]. Several poorly selective P2 antagonists have been tested with some success *in vivo* as anti-nociceptive agents (e.g. Evans blue, Trypan blue, reactive red, pyridoxal-5'-phosphate-6-

azophenyl-2',4'-disulfonic acid also abbreviated (PPADS), trinitrophenyl-ATP (TNP-ATP)), and thus they might be helpful in the control of inflammatory pain [see 196 for review].

Recent interest has been raised by a class of isoquinoline derivatives epitomized by 1-(N,O-bis(1,5-isoquinoline-sulfonyl)-N-methyl-L-tyrosyl-4-phenylpiperazine (KN-62) [94]. This compound blocks ATP-induced, P2X<sub>7</sub>-dependent ethidium bromide influx into human leukemic lymphocytes with a IC<sub>50</sub> value of 13 nM [197]. Human macrophages have proven to be very useful for the evaluation of KN-62 which is, to the best of our knowledge, the most potent antagonist for the human P2X<sub>7</sub> receptor with an IC<sub>50</sub> of 51 nM and complete inhibition at 500 nM. Recently, our group has studied the effect of conformational restriction of KN-62 [198] by the synthesis of conformationally constrained KN-62 analogue, shown in Fig. 1 with formula 2, where the tyrosine backbone was replaced with the 1,2,3,4-tetrahydroisoquinoline (TIC) that can be considered as a "cyclic tyrosine". Unfortunately, this constrained form of KN-62 completely lost antagonist properties with respect to the parent compound, and this confirms that an extended rather than folded conformation of tyrosine is preferred at the P2X<sub>7</sub>R. In the structure of KN-62, the importance for binding capacity to the P2X<sub>7</sub>R of the isoquinoline-5-sulfonyl moieties linked to the nitrogen and to the hydroxyl group of the N-methyltyrosine has been studied by the synthesis of further analogues (Fig. 1, compounds 3-5) [199]. Unfortunately, none of the compounds 3-5 is able to maintain the same antagonist activity as the parent compound KN-62, and this means that the isoquinoline-5-sulfonyl moiety is essential for the activity. In addition, the presence of the methyl group on the -nitrogen is well tolerated without loss of antagonism.

We have performed a systematic structure-activity analysis on the phenylpiperazine moiety trying to select the better substitution on the phenyl ring [95]. From this study we have obtained compounds 6 and 7 which showed 30 and 10-fold higher activity, respectively, than the parent compound KN-62. IC<sub>50</sub> for inhibition of ATP-stimulated IL-1 release from human macrophages was in the 2-3 nM range [95]. Along the same line of research, in a study in which three positions of KN 62 have been systematically modified, Jacobson *et al.* have identified MRS 2306 as slightly more potent with respect the parent compound [200].

Tyrosine derivatives synthesized by Jacobson and coworkers and by our group do not represent good ideal candidates for the development of oral drugs, due to their high molecular weight (>700), high lipophilicity (clog P>6) and the presence of metabolically labile sulphonate groups. AstraZeneca has recently disclosed a series of thiazole-2,4-diones and cyclic imides, exemplified by general structure 1 and 2, derived by a hit derivative discovered through high-throughput screening (HTS) of a compound collection [201]. Using the HTS and the consecutive Hit-to-Lead (HtL) strategy, AstraZeneca has also reported a series of adamantanes with general formula 3, where 2-chloro- or 2-chloro-3/5/6 substituted benzamides had activity as P2X<sub>7</sub> antagonists [202].



## CONCLUSIONS AND FUTURE DIRECTIONS

P2R made their start as drug targets in a minor key. Few believed that receptors for extracellular nucleotides might become of importance in drug development. Contrary to this belief, increasing awareness of the participation of P2R in the modulation of leukocyte functions and cell growth suggests several innovative approaches to the therapy of inflammatory and neoplastic diseases. Now, we rely on medicinal chemistry for key advances in P2R-targeted drug discovery.

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