Running title: P2X7 in pathological conditions

Title: P2X7 receptors as a therapeutic target

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Abstract

P2X7 receptor is an ATP gated cation channel that upon agonist interaction leads to cellular influx of Na^+ and Ca^{2+} and efflux of K^+. P2X7 is expressed by a wide variety of cells and its activation mediates a large number of biological processes like inflammation, neuromodulation, cell death or cell proliferation and it has been associated to related pathological conditions including infectious, inflammatory, autoimmune, neurological and musculoskeletal disorders and, in the last years, to cancer. This chapter describes structural features of P2X7, chemical properties of its agonist, antagonist and allosteric modulators and summarise recent advances on P2X7 receptor as therapeutic target in the aforementioned diseases. We also give an overview on recent literature suggesting that P2X7 single nucleotide polymorphisms could be exploited as diagnostic biomarkers for the development of tailored therapies.

Introduction

The P2X7 receptor for extracellular adenosine triphosphate (ATP) is an ion channel belonging to the family of P2X receptors. As extracellular messenger, ATP engages seven P2X receptors subtypes. These proteins are cation selective channels, assembling as either homo- or hetero-trimers (North & Jarvis, 2013). Each receptor subunit consists of a short N-terminus, two transmembrane domains divided by a long extracellular loop, responsible for ligand interaction, and a C-terminal tail varying in length depending upon P2X subtype (MacKenzie, Surprenant, & North, 1999). With its 595 aminoacids, P2X7 is the largest protein of the P2X family, mainly due to its long C-terminal domain, conferring it the ability of gating a large nonselective pore (Surprenant, Rassendren, Kawashima, North, & Buell, 1996). Opening of this pore is coupled to the well-known P2X7 cytotoxic activity usually triggered by high (i.e. mM) pharmacological ATP concentrations (Falzoni et al., 1995). On the contrary, basal tonic P2X7 activation mediated by endogenous ATP release was associated to a trophic function of the receptor (Baricordi et al., 1999; Adinolfi et al., 2005). The *p2rx7* gene is located on the long arm of chromosome 12 at 12q24.31 and its transcription initiation site has been identified at adenine -91 (Zhou, Luo, Qi, Li, & Gorodeski, 2009). It has many polymorphic variants and isoforms that can modulate its function leading to an increased or a decreased activity. Over 1500 single nucleotide polymorphisms (SNPs) are reported in NCBI SNP database, the majority of which are nonsynonymous, intronic or missense SNPs. Among these, a small number has been related to receptors activity including 10 loss of function (LOF) and 3 gain of function (GOF) variants (Table 1) . Some SNPs were also located in regulatory regions including the upstream promoter of the gene (Bartlett, Stokes, & Sluyter, 2014). Alternative splice variants of P2X7 have been identified both in man

(Cheewatrakoolpong, Gilchrest, Anthes, & Greenfeder, 2005) and in rodent (Masin et al., 2012; Nicke et al., 2009). The truncated receptors, resulting from alternative splicing, in humans have been associated to cell proliferation (Adinolfi et al., 2010) and cancer development (Feng, Li, Wang, Zhou, & Gorodeski, 2006; Giuliani et al., 2014). [Insert Table 1 here]

P2X7 receptor ligands

Several P2X ligands have been developed to date (Coddou, Yan, Obsil, Huidobro-Toro, & Stojilkovic, 2011; Lambertucci et al., 2015; Muller, 2015), with the agonists consisting in derivatives obtained mainly by slight modifications of the endogenous agonist ATP (i.e. αβ-methylene-ATP or αβ-meATP, βγ-methylene-ATP or βγ-meATP, adenosine-5'-O-(3-thiotriphosphate or ATP-gamma-thio or ATPγS, and 2-methylthio-ATP or 2-meSATP). Further ATP analogues presenting agonist activity were developed by modification at the sugar moiety, like 2'(3')-O-4-benzoylbenzoyl)-ATP (BzATP) (Evans et al., 1995) or the so-called "acyclic nucleotides" (Volpini et al., 2009) (Figure 1). The inhibitors belong to different structural classes, ranging from small drug-like molecules to large polyanionic compounds. Various series of positive and negative allosteric modulators were also reported. [Insert Figure 1 here]

Agonists and ATP-competitive antagonists

The agonists and ATP-competitive antagonists of the P2X7 play their role by interacting with the receptor at the ATP binding site and by activating or inhibiting receptor's activity, respectively. The recent publication of the crystal structures of the zebrafish P2X4 (zP2X4) receptor in the presence and absence of ATP at 2.8 and 2.9 Å resolution provided structural detail of the P2X receptor structure, consisting in a chalice-shaped trimeric architecture, and on the location and topology of the ATP

binding sites (Grimes & Young, 2015; Hattori & Gouaux, 2012; Kawate, Michel, Birdsong, & Gouaux, 2009). These receptor regions are located at the interface between two P2X monomers and the interaction with the endogenous ligand ATP causes a conformation rearrangement that takes to pore opening and to ion flux. Molecular modelling studies have been made to develop structural models of the P2X receptors aimed at analysing the role of some residues found critical for receptor function by mutagenesis (Browne, Jiang, & North, 2010; Chataigneau, Lemoine, & Grutter, 2013; Evans, 2010; Hausmann, Kless, & Schmalzing, 2015). *In silico* studies were also performed to analyse the ATP binding cavity of these proteins and to simulate the interaction with agonists or antagonists (Dal Ben et al., 2015; Hattori & Gouaux, 2012; Kaczmarek-Hajek, Lorinczi, Hausmann, & Nicke, 2012; Lorinczi et al., 2012; Riedel, Wiese, Leichsenring, & Illes, 2012; Wolf et al., 2011). The P2X7 receptor shares the majority of the residues building the ATP binding cavity with all the other P2X subtypes (Figure 2) [Insert Table 2 here]. Nevertheless, some non-conserved aminoacids provide topological and chemical-physical properties to the P2X7 binding pocket that differentiate it from the corresponding region of the other P2Xs. In detail, the depth of the ATP binding pocket presents the highest degree of conservation, with a significant number of positively charged residues (arginines and lysines) that provide the main binding region for the negatively charged ligands (Figure 2A, the binding site of the human P2X1 receptor). At the entrance of the pocket there is the highest degree of variability, ranging from P2X subtypes presenting a relevant number of positively (i.e. P2X1) or positively/negatively (i.e. P2X2 or P2X3) charged aminoacids to the P2X7 whose binding site entrance contains several hydrophobic aromatic residues (Figure 2C, the binding site of the human P2X7 receptor). Furthermore, a serine residue conserved in human and rat P2X1-5

receptors is replaced by a tyrosine or a phenylalanine in P2X7 pocket (Tyr288 and Phe288 in the hP2X7 and rP2X7, respectively). The result of this aminoacid substitution and of the different profile of the binding pocket entrance is that the P2X7 cavity results smaller and less polar with respect to the corresponding region of the other P2X subtypes (Figure 2B-D, comparison of the binding sites of the human P2X1 and P2X7 represented as molecular surfaces) (Dal Ben et al., 2015).

Agonists

The above-described structural and chemical features of the ATP cavity of the P2X7 receptor make this receptor unique among the other P2Xs as per agonist interaction. In detail, the endogenous ligand ATP interacts with the P2X7 receptors at high micromolar to low millimolar level while it activates the other P2X subtypes with nanomolar-low micromolar EC50 values. BzATP, a molecule obtained through modification of the adenine base or the sugar moiety of ATP, is the most potent P2X7 agonist developed to date, with a low micromolar EC50 data at the human receptor. BzATP consists of a mixture of the 3'- and 2'-benzoyl-benzoyl esters of ATP (Figure 1). This compound activates P2X1 and P2X3 at low nanomolar level, presenting also micromolar potency at the other P2X subtypes. The higher potency at P2X7 of BzATP as compared to other ATP analogues was interpreted with the aid of molecular modelling studies, which highlighted the role of aromatic residues like Phe218 and Tyr288 (Figure2) in the ligand-receptor interaction (Dal Ben et al., 2015). General P2X agonists can be ranked according to the potency at the P2X7 as follows: BzATP >> ATP > ATPγS > 2-MeSATP >> α,β-meATP. Despite their diffused use as pharmacological tools to analyze the pathophysiological role of the receptor in cellular and animal models, none of these molecules are selective P2X7 ligands,

(Donnelly-Roberts, Namovic, Han, & Jarvis, 2009; Hibell, Kidd, Chessell, Humphrey, & Michel, 2000; Hibell et al., 2001).

Antagonists

In general, antagonists of the various P2X receptors are small or large molecules presenting several negative charges able to interact with the positively charged residues within the ATP binding cavity (Figure 3) [Insert Figure 3 here]. Classical P2X antagonists consist for example in large and polyanionic molecules like suramin or suramin-like derivatives, where the different number and localization of negative charges within the molecule influence the activity at the various P2X receptors leading to IC50 data in the sub-nanomolar level. Two P2X antagonists were developed starting from the endogenous ligand ATP and modifying its ribose moiety by insertion of a trinitrophenyl substituent in the 2'-3' position or by oxidization and breakage of the ribose ring to obtain two aldehyde functions in the 2' and 3' positions (TNP-ATP and oATP, respectively; Figure 3). While TNP-ATP is active at low nanomolar level at P2X1 and P2X3 receptors but possesses also high micromolar potency as P2X7 antagonist, oATP is an irreversible antagonist of the P2X7 receptor possibly due to the formation of Schiff bases by lysine residues of the receptor binding cavity and the aldehyde functions of the ligand. Besides the above-described molecules, the ATP-competitive P2X7 antagonists are generally small and non-charged drug-like compounds, like the disubstituted azoles and cyanoguanidines derivatives reported in literature (Honore et al., 2006; Nelson et al., 2006). The first molecules of these series were developed by Abbott Laboratories and consisted of a set of tetrazole derivatives presenting a phenyl and a benzyl substituent. Among these compounds, A-438079 shows an IC50 of 0.13 µM and 0.32 µM at the human and rat P2X7 receptors, respectively, in the calcium (Ca^{2+}) influx assays and 0.20 µM in the

YO-PRO assay (Nelson et al., 2006), presenting also high selectivity versus other P2X and P2Y receptors (Donnelly-Roberts et al., 2009). The two substituents as well as the central tetrazole ring, were further modified using various strategies (Nelson et al., 2006; Carroll et al., 2007). These modifications led to the development of A-839977 (Figure 3), with an IC50 of 0.02-0.150 μM at recombinant human, rat, and mouse P2X7 receptors (Florjancic et al., 2008; Friedle, Curet, & Watters, 2010; Honore et al., 2009). This compound is able to block agonist induced IL-1β release and to give anti-hyperalgesic effects in an inflammatory model of pain in mice. The first relevant cyanoguanidine derivative reported in literature as P2X7 antagonist was the compound A-740003 (Figure 3) (Honore et al., 2006), developed again by Abbott Laboratories and presenting IC50 values of 0.040 and 0.020 μM at both human and rat P2X7 receptor, respectively. This molecule resulted selective versus other P2X and P2Y receptors and proved to be efficacious in *in vivo* models of neuropathic pain, neuroblastoma and melanoma (Perez-Medrano et al., 2009; Amoroso et al., 2015; Adinolfi et al., 2015). Modifications of this molecule were made at the t-butyl-substituted carbon spacer that was replaced with cyclic moieties (i.e. piperazine). Few obtained derivatives showed comparable potency to A-740003 (Morytko et al., 2008) and some potential for the treatment of rheumatoid arthritis and other inflammatory conditions, even due to their metabolic stability and favorable pharmacokinetic properties (Betschmann et al., 2008). Further optimization of this series of compounds led to the development of the compound A-804598 (Figure 3), showing, with the Ca^{2+} influx assay, an IC50 of 0.0109, 0.0099, and 0.0089 μM at the human, rat, and mouse P2X7 receptors, respectively, and a good selectivity versus the other P2X and P2Y receptors (Donnelly-Roberts et al., 2009). The detail at molecular level of the interaction between these molecules and the P2X7 receptor is lacking as no crystal

structures of the P2X7 receptor have been reported to date. Molecular modelling studies tried to simulate the binding of the above described P2X7 antagonists at the ATP binding cavity of the protein (Dal Ben et al., 2015) (Figure 4). [Insert Figure 4 here]

Allosteric modulators

The activity of the P2X receptors may be regulated by a variety of allosteric modulators (AM) both endogenous (i.e. ions like Mg^{2+} or Ca^{2+} (Coddou, Stojilkovic, & Huidobro-Toro, 2011), lipid metabolites (Bernier, Ase, & Seguela, 2013), and steroids (De Roo, Rodeau, & Schlichter, 2003)) and synthetic. These chemical entities are able to bind the receptor at sites different from the ATP-binding cavity, changing the conformation or stabilizing P2X7 tridimensional arrangement and hence to increase (positive AM) or decrease (negative AM) the effect of the endogenous ligand ATP (Coddou et al., 2011; Evans, 2009; Gunosewoyo & Kassiou, 2010; Mehta et al., 2014; Muller, 2015). The advantage of using these molecules instead of the orthosteric ligands is that the allosteric modulators modify an underway physiological function (the activation by endogenous ligand when and where it occurs) and hence their activity is site- and event-specific, while orthosteric ligands lead to a general activation or block of the receptor in the whole organism. Furthermore, the allosteric ligands are generally more subtype selective as they don't bind the conserved ATP cavity but they act at different regions of the receptor trimer. On the other hand, the availability of P2X structure makes easier the *in silico* design of orthosteric ligands, while the localization and the chemical or topological features of the allosteric binding sites is obscure requiring a screening effort for their identification. In general the synthetic non-competitive ligands of the P2X7 reported to date (in patents and/or in literature) are negative AMs and belong to various structural classes (Figure 5),

some relevant examples being given by Brilliant Blue G (BBG), AZD9056, KN-62, AZ-11645373, AZ-10606120, GW791343, GSK314181A, GSK1482160, CE-224,535, AFC-5128, JNJ-479655, and EVT-401 (Alves, Bezerra, Faria, Ferreira, & da Silva Frutuoso, 2013; Friedle et al., 2010; Guile et al., 2009; Kaczmarek-Hajek et al., 2012; Mehta et al., 2014; North & Jarvis, 2013). These compounds present nanomolar/micromolar potency at the P2X7. [Insert Figure 5 here]

P2X7 in inflammation

Due to its widespread expression on immune cells and to its ability to cause release of pro-inflammatory cytokines, the best recognized function of P2X7 receptor is to participate in phlogistic reactions. Inflammation is an essential immune response to noxious conditions that aims to restore homeostasis and participates in the pathogenesis of several diseases (Medzhitov, 2010). Indispensable elements in its progression are PAMPs/ MAMPs (pathogen-associated molecular patterns/ microbe-associated molecular patterns), DAMPs (damage-associated molecular patterns), PRRs (pattern recognition receptors), cells of innate and adaptive immunity and the inflammasomes. Inflammasomes are large intracellular multiprotein complexes involved in the cleavage and maturation of phlogistic mediators (Guo, Callaway, & Ting, 2015) having a central role in the aetiology of inflammatory disorders. During inflammation, extracellular ATP is released as DAMP, and P2X7 receptor, activated by this nucleotide, switches on the inflammasome and causes the release of several proinflammatory cytokines such as IL-1β, IL-18, IL-1α, IL-36α (Di Virgilio, 2013; Bartlett et al., 2014). Of interest, a protein-protein interaction between P2X7 and NLR family, pyrin domain containing 3 (NLRP3), a component of the inflammasome platform, has been recently demonstrated (Franceschini et al., 2015) and the activity of Colchicine on inflammasome-linked pathologies has been attributed to P2X7

down-modulation (Marques-da-Silva, Chaves, Castro, Coutinho-Silva, & Guimaraes, 2011; Leung, Yao Hui, & Kraus, 2015). Inflammasome-P2X7 crosstalk and their involvement in phlogistic conditions have been extensively covered by recent reviews (Di Virgilio, 2013; Di Virgilio, 2015) and are therefore out of the scope of present overview. However, P2X7 receptor acts also through inflammasome-independent mechanisms leading to release of prostaglandins causing fever (Ivanov & Romanovsky, 2004) and inflammatory pain (Samad, Sapirstein, & Woolf, 2002). P2X7 antagonism blocks autacoids and IL-1β release, emerging as a possible substitute of molecules targeting cyclooxygenase-2 (COX-2) activity, like aspirin and other nonsteroidal anti-inflammatory drugs (Barbera-Cremades et al., 2012). Inflammation is one of the main causes of several pathologies, including everything from asthma and rheumatoid arthritis to mental deterioration and cancer. Different P2X7 antagonists or allosteric modulators were tested in pharmacological studies and clinical trials for inflammation-related diseases (Table 2). In this chapter, we summarise recent advances on P2X7 receptor as therapeutic target in infectious, inflammatory, autoimmune, neurological, musculoskeletal disorders and cancer and on the analysis of its variants as possible disease biomarkers. [Insert Table 2 here]

P2X7 receptor in infectious diseases

Professional phagocytes and antigen presenting cells are long known for expressing a functional P2X7 receptor (Falzoni et al., 1995). In particular, P2X7 is involved in host mechanisms to remove microorganisms that parasites macrophages such as *Mycobacteria*, *Leishmania* and *Chlamydia* (Miller et al., 2011). *Mycobacterium tuberculosis* is a facultative intracellular pathogen, causative agent of human tuberculosis (TB) that is able to survive and replicate in phagosomes within macrophages, by blocking the phagosome-lysosome fusion (Vergne et al., 2005).

ATP evoked P2X7 activation triggers phagosome-lysosome fusion (Fairbairn, Stober, Kumararatne, & Lammas, 2001; Coutinho-Silva et al., 2003) and it gives rise to apoptosis of mycobacterial- infected macrophages (Placido et al., 2006). Several genetic studies confirmed a role for P2X7 in inducing mycobacterial killing, showing that LOF polymorphisms in the human gene are involved in increased susceptibility to *M. tuberculosis* infection. The most common P2X7 polymorphism analyzed in correlation to TB lies within exon 13 at position 1513 and results in the expression of a non-functional P2X7 receptor in macrophages from homozygous subjects (Gu et al., 2001). Analyzing independent patients cohorts from Southeast Asia (Fernando et al., 2007), Mexico (Nino-Moreno et al., 2007), Russia (Mokrousov, Sapozhnikova, & Narvskaya, 2008) and North India Punjabi (Sharma et al., 2010) different investigators demonstrated a strong association with the 1513A>C SNP and susceptibility to extra-pulmunary and pulmonary TB. However, other groups did not confirmed this association (Li et al., 2002; Sambasivan, Murthy, Reddy, Vijayalakshimi, & Hasan, 2010; Xiao et al., 2010) and two meta-analyses published in 2010 and 2011 reported contrasting data. Xiao et al., found a contribution of the polymorphism to all forms of TB (Xiao et al., 2010), whereas Wang et al., did not find any association between 1513A>C SNP and pulmonary TB risk (Wang, Xiao, Lan, Mao, & Chen, 2011). Finally, a more recent analysis of the effect of P2X7 1513A>C on the risk of TB, indicates that this polymorphism contributes to TB susceptibility only in Asians (Wu et al., 2014), suggesting that population diversity, combined with environmental factors could be the source of the observed P2X7 heterogeneity. Similarly, additional studies analyzing the association between TB and the P2X7 promoter SNP -762C>T showed a protective (Li et al., 2002) or a predisposing (Sambasivan et al., 2010) role of this variant, depending upon the

considered population. An additional explanation for the contrasting data coming from SNPs analysis could be also attributed to the role of P2X7 receptor in the pathogenesis of TB caused by hypervirulent mycobacteria. Indeed, Amaral and colleagues recently demonstrated that P2X7 contributes to the formation of pulmonary necrotic lesions caused by highly virulent mycobacteria strains as *p2rx7* null mice were almost completely devoid of granulomatous pneumonia induced by mycobacterial infection (Amaral et al., 2014). In the case of other intracellular parasites such as *Chlamydia* and *Leishmani*a, the P2X7 receptor is clearly protective. Indeed, *p2rx7* ablation significantly increased chlamydial infection of vaginal epithelial cell (Darville et al., 2007) and rendered macrophages unable to eliminate *Leishmania* (Chaves et al., 2009; Chaves, Marques-da-Silva, Monteiro, Canetti, & Coutinho-Silva, 2014).

P2X7 Receptor in Osteoporosis

Osteoporosis is a progressive skeletal disease that leads to low bone mass and microarchitectural deterioration of bone tissue, causing an enhancement of bone fragility and an increased fracture risk (Kanis, 2000). It is a multi-factorial disease resulting from a combination of genetic and environmental factors that affect peak bone mass and rate of bone loss (Jorgensen et al., 2012). In bone, multiple P2X and P2Y receptors are functionally expressed by both osteoblasts and osteoclasts and their activation regulates cellular proliferation and apoptosis, subsequently modulating bone formation and resorption in the bone microenvironment (Bowler et al., 2001; Agrawal et al., 2010; Gartland et al., 2003; Gartland, Hipskind, Gallagher, & Bowler, 2001; Jorgensen et al., 2002). Panupinthu and colleagues demonstrated that in osteoblasts P2X7 receptor stimulates proliferation and enhances mineralization (Panupinthu et al., 2008). Following studies confirmed this hypothesis suggesting that

P2X7 will activate the ERK (Liu et al., 2008), PI3K (Grol, Zelner, & Dixon, 2012) and NFATc1 (Giuliani et al., 2014) pathways leading to osteoblast growth. The role of P2X7 in bone-resorbing osteoclasts is still controversial as it has been suggested that P2X7 participates in osteoclastogenesis by affecting cell fusion (Pellegatti, Falzoni, Donvito, Lemaire, & Di Virgilio, 2011) but osteoclasts from *p2rx7* null mice are normal in number and dimensions (Ke et al., 2003; Gartland, Buckley, Hipskind, Bowler, & Gallagher, 2003). The interest on P2X7 receptor function in bone biology was fostered by pioneering studies on *p2rx7* null mice that demonstrated a unique skeletal phenotype, revealing a major role for the P2X7 receptor in bone formation and remodeling. Ke et al. demonstrated that *p2rx7* lacking mice are endowed with significant reduction in total and cortical bone content and periosteal circumference in femurs, reduced periosteal bone formation and increased trabecular bone reabsorption in tibias (Ke et al., 2003). Moreover, Li and colleagues demonstrated a central role for P2X7 in mechanical loading induced bone formation (Li, Liu, Ke, Duncan, & Turner, 2005) and in callus remodeling (Li, Meyer, Duncan, & Turner, 2009). These data attracted increasing interest on the role of P2X7 in osteoporosis. Most performed studies tried correlating P2X7 SNPs to osteoporosis susceptibility or risk of bone fracture. In particular, Ohlendorff and colleagues found an association between P2X7 LOF SNPs (1513A>C, 1729T>A) and increased vertebral fracture risk in a post-menopausal women cohort (Ohlendorff et al., 2007). Following studies confirmed these data by correlating LOF P2X7 SNPs (946G>A, 1729T>A, 151+1:G-T, 1513A>C, 474G>A) to increased rate of bone loss and fracture risk and/or reduced bone mineral density both in men and post-menopausal women (Gartland et al., 2012; Jorgensen et al., 2012; Husted et al., 2013). Furthermore, they found associations between GOF variants (1405A>G and 1068G>A) and lower vertebral fracture

incidence 10 years after menopause (Jorgensen et al., 2012; Husted et al., 2013), allowing to consider GOF variants as protective against bone loss. However, a recent report by Wesselius and colleagues, while confirming an association of the GOF SNP 1068G>A with increased lumbar spine bone density, reported contrasting results for two other GOF variants (489C>T and 1405A>G) which correlated respectively with decreased femoral neck bone mineral density values and osteoporosis in men (Wesselius et al., 2013). These data suggest that to clarify the role of P2X7 in osteoporosis further studies should explore the effect of the presence of multiple LOF and GOF SNPs in the same patient.

P2X7 Receptor in rheumatoid arthritis

Rheumatoid arthritis (RA) is a widespread chronic systemic inflammatory disorder affecting approximately 1% of the worldwide population (Alamanos, Voulgari, & Drosos, 2006) and it is characterized by chronic inflammation and destruction of bone and cartilage in diarthrodial joints (Choy & Panayi, 2001). Yet to date, the causes of RA are not completely known, but a complex interaction among hormonal, environmental, immunological and genetic factors has been postulated (Davidson & Diamond, 2001). The activation of P2X7 receptor has been associated with downstream-signalling pathways that lead to release of a number of inflammatory mediators, such as IL-1β, IL-18 and consequently IL-6, IL-8 and TNF-α (Di Virgilio, 2013) that are, in turn key molecules involved in the pathogenesis of RA. Based on this evidence, different P2X7 blocking drugs were evaluated in clinical trials on RA and related conditions. Tested molecules includes AZD9056 (ClinicalTrials.gov code: NCT00520572), CE-224535 (ClinicalTrials.gov code: NCT00418782 and NCT00628095), GSK1482160 (ClinicalTrials.gov code: NCT00849134) and EVT-401 (Table 2). However, although all molecules were generally well tolerated, in the

first phases of the trials, they failed to provide significant therapeutic efficacy against RA. Two studies also tried associating P2X7 SNPs to RA susceptibility (Portales-Cervantes et al., 2012; Al-Shukaili et al., 2011). Among the analyzed polymorphisms (489C>T, 946G>A, 1068G>A, 1096C>G, 1513A>C) only the homozygous GOF 1068AA variant was identified as susceptibility gene locus, resulting two fold higher in the RA group compared to controls (Portales-Cervantes et al., 2012; Al-Shukaili et al., 2011). Contrasting results came from the association of P2X7 SNPs with secretion of inflammatory cytokines and RA biomarkers. Indeed, while 489C>T genotype increased IL-1β production, it caused a decrease in IL-18 secretion (Portales-Cervantes et al., 2012). Moreover, LOF polymorphism 1513A>C correlated with increased levels of negative prognostic factors in RA (Al-Shukaili et al., 2011).

P2X7 receptor in diabetes

Increasing literature attributed a role to P2X7 receptor in both type1 (T1D) and type 2 diabetes (T2D) aetiology and complications and suggested that receptors blockade could be useful for anti-diabetic therapeutic intervention (Fotino, Vergani, Fiorina, & Pileggi, 2015). Different studies have suggested that P2X7 could modulate the immune response mediating pancreatic β cell degeneration in mice models of TD1 (Coutinho-Silva, Robson, Beales, & Burnstock, 2007; Chen et al., 2011) but the results are far to be conclusive and it remains to be elucidated whether P2X7 plays a role in the pathophysiology of TD1 also in the clinical settings. Of interest, targeting P2X7 with its antagonist oxidized ATP proved a successful strategy in reducing the rejection of transplanted pancreatic β islets, which represents an emerging therapeutic option for TD1 patients. A major problem with grafted islets is the immune response causing their rejection that is successfully impaired by P2X7 blockade (Vergani et al., 2013). T2D is generally evolving from a previous condition of obesity requiring

adaptation of the β cells to compensate to the higher insulin demand. Glas et al. proposed that obese patients undergo an increase in P2X7 expression in β cells leading to increased IL-1β secretion that will be lost upon TD2 onset (Glas et al., 2009). Accordingly, recent reports correlated the expression of P2X7 on monocytes with increased inflammatory parameters in T2D patients (Wu et al., 2015) and the GOF 1068G>A P2X7 SNP with increased insulin sensitivity and secretion (Todd et al., 2015). Finally, diabetes associated symptoms such as vascular damage (Solini et al., 2004) and increased neuropathic pain (Ursu et al., 2014) have also been ascribed to increased P2X7 activity.

P2X7 receptor in neurological disorders

Several cell types of the nervous system, including astrocytes, oligodendrocytes, microglia, Schwann cells (Sperlagh, Vizi, Wirkner, & Illes, 2006), and some neuronal populations (Lenertz, Gavala, Zhu, & Bertics, 2011), express P2X7 receptor. In the central nervous system (CNS) activation of P2X7 leads to the release of the major excitatory neurotransmitters glutamate and ATP (Sperlagh et al., 2002). The receptor is involved both in the differentiation of neuronal progenitor cells toward the gliogenic phenotype, during development (Tsao, Chiu, & Sun, 2013), and in their cell death in the adult brain (Sperlagh & Illes, 2014). Moreover, spinal cord injury is associated with prolonged P2X7 receptor activation, leading to neuronal excitotoxicity (Wang et al., 2004). Several reports associate P2X7 receptors with a number of neurological disorders, such as multiple sclerosis, Alzheimer's and Huntington's disease, amyotrophic lateral sclerosis and epilepsy. However, despite P2X7 potential as druggable target, at present, there are no P2X7 antagonists or allosteric modulators in clinical trials for neurological diseases, due to the difficulty of

synthesize brain penetrant and safe molecules (Volonte, Apolloni, Skaper, & Burnstock, 2012).

Multiple Sclerosis

Multiple sclerosis (MS) is a neurological disorder of the CNS that is characterized by demyelination, oligodendroglial death, inflammation and consequent neurodegeneration (Ransohoff, Hafler, & Lucchinetti, 2015). In the CNS, oligodendrocytes and their precursors are responsible for myelin sheath formation and are among the first cells lacking their correct function in multiple sclerosis. P2X7 receptor is responsible for ATP mediated cell death in mature rat cortical oligodendrocytes and their precursors both *in vitro* and *in vivo* (Matute et al., 2007; Volonte et al., 2012). Moreover, in MS patients-derived specimens, the expression of the receptor is elevated in axons tracts preceding lesions, suggesting that P2X7-mediated cell death could be associated with newly forming lesions. Interestingly, different P2X7 blockers proved efficacious in reduce demyelination and improve axonal conduction. P2X7 pharmacological modulators BBG and oATP ameliorated the symptoms associated with chronic autoimmune encephalomyelitis, an animal model of MS (Matute et al., 2007; Volonte et al., 2012). Inflammatory reactions causing tissue damage are another important cause of MS symptoms (Steinman, 1996). P2X7 receptor, expressed by activated monocytes infiltrating axonal lesions areas, has been attributed a role in causing phlogistic reactions leading to neurodegeneration. Indeed, MS associated inflammation can be down-modulated by Glatiramer Acetate, a drug currently used to reduce the frequency of MS relapses, through reduction of P2X7 activity (Caragnano et al., 2012). Of interest, Gu and colleagues recently reported and association between P2X7 receptor variant and MS in a large cohort of patients. The 946G>A polymorphism confers a 1.8-fold protective

effect on MS risk. The authors suggested that the rare P2X7 LOF SNP would protect against MS development thanks to reduced receptor pro-inflammatory activity (Gu et al., 2015).

Alzheimer's disease

Alzheimer's disease (AD) is a chronic and irreversible neurodegenerative disease and the principal cause of senile dementia. Central events in AD aetiology are the formation and accumulation of extracellular amyloid β (Aβ)3 plaques and consequent microglia-activated inflammatory reactions (Koffie et al., 2012; Pooler, Noble, & Hanger, 2014). Activity of P2X7 receptor, expressed by microgliar cells, was shown to be upregulated by (Aβ)3 that acted as both agonist and positive allosteric modulator of the receptor. Accordingly, in a mice model of AD, P2X7 showed a pivotal role in IL-1β release as is lost almost halved the production of the cytokine (Sanz et al., 2009). In a similar experimental setting, microglial-P2X7, stimulated by (Aβ)3, increases production of reactive oxygen species leading to neuronal damage (Lee, Won, Gwag, & Lee, 2011). Moreover, prolonged activation of microglial P2X7 receptor leads to neuronal injury upon co-culture of microglia and neurons (Skaper et al., 2006). P2X7 is also involved in (Aβ)3 production as receptor activation by ATP and BzATP stimulates soluble fragment of amyloid precursor protein (sAPPα) release from several neural cells, and its modulation (by BBG, oATP and A-438079) or knockdown inhibits sAPPα shedding (Delarasse, Auger, Gonnord, Fontaine, & Kanellopoulos, 2011). An additional investigation has demonstrated that blockade of P2X7 receptor by BBG in an *in vivo* mouse model of Familial AD reduces the amyloid plaques formation in brain hippocampal structures, and this reduction is mediated by an increase in α-secretase activity, fundamental for the proteolytic processing of APP (Miras-Portugal et al., 2015). Interestingly, the P2X7 SNP

489C>T was found to be significantly less frequent in AD patients compared to control, suggesting a possible protective role of this receptor variant (Sanz et al., 2014).

Huntington's disease

A possible role of P2X7 receptor has been hypothesized also for Huntington's disease (HD), a hereditary neurodegenerative condition caused by a CAG triplet-repeat expansion coding for a polyglutamine sequence in the huntingtin protein (Myers et al., 1993). Díaz-Hernández and colleagues showed that levels of P2X7 receptor are increased in the brain of HD mouse models and mutant-huntingtin-expressing neurons are more vulnerable to P2X7-mediated apoptosis. Moreover, *in vivo* administration of the P2X7 negative allosteric modulator BBG leads to a decreased neuronal apoptosis, but this therapy is beneficial only in advanced stages of disease progression (Diaz-Hernandez et al., 2009).

Amyotrophic Lateral Sclerosis

Amyotrophic lateral sclerosis (ALS) is a progressive neurodegenerative disease that affects motor neurons in the brain and in the spinal cord that provide voluntary movements and muscle control, causing disability, muscle weakness and death. The most common form is characterized by mutations in superoxide dismutase 1 (SOD1), responsible for converting superoxide radicals to oxygen and hydrogen peroxide (Borchelt et al., 1994; Sea et al., 2015). The primary cause of motor neurons degeneration is still unknown, but inflammation and oxidative stress are known to be implicated in the pathogenesis of ALS and contribute to neurodegeneration (Philips & Robberecht, 2011). P2X7 receptor has been shown to be upregulated and able to increase pro-inflammatory response in *in vitro* ALS microglia (Yiangou et al., 2006; Apolloni et al., 2013). Accordingly, in an ALS mice model (SOD1-G93A), blockade

of P2X7 by BBG delays the pathogenesis of the disease, decreases microgliosis and reduces motor neuron loss, improving motor performance and inflammatory parameters (Apolloni et al., 2014). However, the same group had also demonstrated that in contrast to data obtained with pharmacological blockers, ablation of *p2rx7* in SOD1-G93A mice aggravates gliosis and motor neurons death (Apolloni, Amadio, Montilli, Volonte, & D'Ambrosi, 2013). These data suggests that P2X7 receptor could play multiple roles in the pathogenesis of ALS depending upon the kinetics of its activation, implying that the choice of the appropriate time window of therapeutic intervention with P2X7 blockers will be pivotal to improve motor neuron survival (Apolloni et al., 2014). Consistent with P2X7-dependent neurodegeneration and neuroinflammation, clemastine a CNS-penetrant H1 receptor antagonist and promising drug for the treatment of both MS and ALS, was shown to downregulate P2X7 expression in lumbar spinal cord of SOD1-G93A mice (Apolloni, Fabbrizio, Parisi, Amadio, & Volonte, 2014).

Epilepsy

Epilepsy is a chronic neurological disorder with a range of aetiologies and comorbidities, characterized by continuous seizures due to a change in neuronal excitation/inhibition balance, leading to alteration of motor control, sensory perception and behaviour (Kaila, Ruusuvuori, Seja, Voipio, & Puskarjov, 2014). ATP is an important neuromodulator in the CNS; it is stored by secretory and synaptic vesicles and released from damaged cells (Burnstock, 2008; Abbracchio, Burnstock, Verkhratsky, & Zimmermann, 2009). It is well known that seizures cause large decrease in cerebral ATP (Klein & Olsen, 1947), and the activation of neuronal pathways increases ATP release (Dale & Frenguelli, 2009). P2X7 receptor is activated during status epilepticus (SE), a dangerous state of continuous seizures, and

increased levels of this receptor have been found in hippocampus of epileptic animals (Vianna et al., 2002). It has been demonstrated that P2X7 is upregulated in the neurons of the neocortex after SE and its inhibition by the antagonist A-438079 decreases seizures and protects neocortex against damage. P2X7 blockade by BBG, reduces seizure-induced cell death in the same murine model (Jimenez-Pacheco et al., 2013). Further proof of P2X7 cytotoxic role in epilepsy, came from a recent paper demonstrating that receptor activation, during seizures, causes astroglial death through a PARP-1 dependent mechanism (Kim, Ko, & Kim, 2015). Interestingly, Soni and colleagues have recently reported a crosstalk between purinergic and glutamatergic pathways in epilepsy. Indeed, while P2X7 is neurotoxic, glutamate transporters activity is protective against SE (Kong et al., 2012). The authors demonstrated that, in a rat model of epilepsy, the modulation of glutamate transporter -1 and the inhibition of P2X7 alone and in combination prevent seizures and restore oxidative defence and acethylcholinesterase activity (Soni, Koushal, Reddy, Deshmukh, & Kumar, 2015).

P2X7 Receptor in Mood Disorders

Bipolar disorders, major depressive disorders and anxiety disorders are included in a large group of common depression conditions defined as affective mood disorders and characterized by alteration in mood that modifies thoughts, emotions and behaviors (Lindsay, Sykes, McDowell, Verreault, & Laurin, 2004; Somers, Goldner, Waraich, & Hsu, 2006). Considering that P2X7 is implicated in Ca^{2+} signaling and neurotransmitter release and that alterations in intracellular concentration of Ca^{2+} have been observed in patients affected by mood disorders (Wasserman et al., 2004), in the last years *p2rx7* gene has been proposed as a candidate locus of susceptibility to affective mood disorders (Shink et al., 2005). Thanks to several genetic association

studies, a possible link between P2X7 variants and the pathogenesis of mood disorders has been found. Nevertheless, the majority of results are still contradictory. Three large studies carried out in European and French Canadian populations associated P2X7 partial loss of function with different mood disorders. Indeed, in this cohorts P2X7 LOF SNP 1405A>G strongly correlated with susceptibility to bipolar I or II disorder, recurrent major depression and both bipolar- and unipolar-affective disorders (Barden et al., 2006; Lucae et al., 2006; McQuillin et al., 2009). Further studies did not confirm these results. Two different groups, analyzing large cohorts of European patients suffering from bipolar I disorders and unipolar recurrent major depression, found no association of these diseases either with P2X7 1405A>G (Green et al., 2009; Viikki et al., 2011) or with P2X7 489C>T. P2X7 variants failed to associate also with rates of remission after therapeutic intervention with either serotonin selective reuptake inhibitors or electroconvulsive therapy (Viikki et al., 2011). Accordingly, a recent meta-analysis that used a case-control design, suggests that P2X7 1405A>G SNP may not contribute to the risk of affective mood disorders overall (Feng, Zhang, Li, & Liu, 2014). Nevertheless, a significant association of these polymorphisms with mood disorders was found in family-based cohorts (Feng et al., 2014). Future studies, collections of larger cohorts and appropriate matched controls are warranted in order to clarify or validate the association of P2X7 SNPs with affective mood disorders.

P2X7 potential in oncologic treatment

In recent years P2X7 emerged as potent modulator of oncogenic responses including: promotion of cancer cell growth (Adinolfi et al., 2012; Amoroso et al., 2015) and migration (Jelassi et al., 2011; Jelassi et al., 2013), host immune reactions to tumour development (Adinolfi et al., 2015; Hofman et al., 2015; Bianchi et al., 2014) and

cancer-associated pain (Falk et al., 2015). For these reasons both P2X7 agonism and antagonism have been proposed as strategy for cancer treatment (Adinolfi, Capece, Amoroso, De Marchi, & Franceschini, 2015). The idea of administering ATP to oncologic patients as anti-tumoral came from the assumption that P2X7 receptor would mediate cancer cell death through its known pro-apoptotic/ necrotic function. Indeed, mice administration of a very high concentration of ATP (25 mM) has been proved to cause reduction of tumour dimensions in two xenogeneic models of cancer (Shabbir, Thompson, Jarmulowiczc, Mikhailidis, & Burnstock, 2008; White, Knight, Butler, & Burnstock, 2009). However, to our knowledge, these findings were never extended to immune-competent models were one should take into account the effects of the main degradation product of ATP, which is the immunosuppressive agent adenosine, well recognized for its oncogenic activity (Young, Mittal, Stagg, & Smyth, 2014). Moreover, clinical trials designed to test the effect of ATP administration to cancer patients gave contrasting results depending upon cancer tested, drug concentration and route of administration, the only positive effect being an improvement of the quality of life in terminal non-small-cell lung cancer patients (Agteresch, Burgers, van der Gaast, Wilson, & Dagnelie, 2003; Beijer et al., 2009; Beijer et al., 2010; Beijer et al., 2010). Finally, recent reports strongly support a role for ATP, through either P2X2, P2X3, P2X4 or P2X7 itself in cancer associated pain (Kaan et al., 2010; Hansen et al., 2012; Ye et al., 2014; Jin, Wang, Zuo, Yang, & Liu, 2014; Yang et al., 2015; Falk et al., 2015; Franceschini & Adinolfi, 2014) suggesting that administration of ATP at high doses could even increase cancer dependent pain sensation. On the other hand, a therapeutic approach based upon P2X7 blockers administration could prove more efficacious, especially in those numerous cancers were P2X7 over-expression has been reported (Adinolfi et al., 2015; Burnstock & Di

Virgilio, 2013; Adinolfi, Amoroso, & Giuliani, 2012). Indeed, various P2X7 inhibitors and antagonist reduce cancer cell growth or spreading when administered either intra-tumour or systemically in preclinical animal models of cancer. The antagonists and inhibitors used includes oATP (Adinolfi et al., 2012; Hattori et al., 2012), BBG (Vazquez-Cuevas et al., 2014) AZ10606120 (Adinolfi et al., 2012; Adinolfi et al., 2015; Amoroso et al., 2015), A-740003 (Adinolfi et al., 2015; Amoroso et al., 2015), A-438079 (Jelassi et al., 2011) but also P2X7 blocking antibodies (Ren et al., 2010) and traditional Chinese medicine compounds that act as partial antagonists at the receptor (Jelassi et al., 2013). Cancer models tested comprise colon (Adinolfi et al., 2012), breast (Jelassi et al., 2011) and ovarian carcinoma (Vazquez-Cuevas et al., 2014), neuroblastoma (Amoroso et al., 2015), melanoma (Adinolfi et al., 2012; Adinolfi et al., 2015) and glioma (Ryu, Jantaratnotai, Serrano-Perez, McGeer, & McLarnon, 2011). Of interest, a phase I clinical trial in basal cell carcinoma performed by Biosceptre with an anti-PX7 antibody retrieved good results causing a reduction in the tumour lesions in 65% of the tested patients (source Biosceptre 2014). Although, the company claims that their antibody (BIL -010t) will be targeting a yet to be defined non-functional P2X7, it was never specified whether this is a splice or polymorphic variant of the receptor. Therefore, it is tempting to speculate that BIL-010t will block P2X7 activity comparably to what done by the antagonists in the animal models tested (Adinolfi et al., 2012; Amoroso et al., 2015). Different biochemical pathways were shown to be affected by P2X7 in oncogenesis including those regulating energy production (Amoroso, Falzoni, Adinolfi, Ferrari, & Di Virgilio F., 2012; Amoroso et al., 2015), tumour vascularization (Adinolfi et al., 2012; Amoroso et al., 2015) and extracellular matrix degradation (Jelassi et al., 2011). We have recently demonstrated that, in neuroblastoma, P2X7 acts as positive

modulator of the PI3K/Akt/HIF1α axis favouring tumour cell growth and vascularization (Amoroso et al., 2015). Interestingly, no addictive effect was seen when PI3K/Akt blocking drugs and P2X7 antagonist were co-applied, suggesting that P2X7 is an upstream modulator of this biochemical pathway (Amoroso et al., 2015). The Akt axis is one of the best-recognized mediators of P2X7-dependent oncogenic phenotype. Indeed, Akt activation sustained by P2X7 was associated to the effect of statins in preventing pancreatic and prostate cancer spreading (Mistafa & Stenius, 2009; Ghalali, Wiklund, Zheng, Stenius, & Hogberg, 2014). These data were corroborated by two recent studies (Qiu et al., 2014; Xia, Yu, Tang, Li, & He, 2015) which proposed Akt to be central in P2X7 mediated cancer invasiveness, demonstrating that is via this kinase that P2X7 modulates the expression of invasion related proteins such as E-cadherin and matrix metalloprotease-3 and 13 (Qiu et al., 2014; Xia et al., 2015). Transforming growth factor beta (TGFβ) is a component of tumour microenvironment that affects cancer cell migration and was shown to influence vesicular release of ATP and activation of P2X7, causing cytoskeletal re-arrangements leading to cancer cell movement (Takai et al., 2012; Takai, Tsukimoto, Harada, & Kojima, 2014). Of interest, also this phenomenon is dependent upon variations in intracellular Ca^{2+} and PI3K activation (Hattori et al., 2012). P2X7 triggered Ca^{2+} rise was known for long to result in cell proliferation (Adinolfi et al., 2005) also causing activation of the Ca^{2+} sensitive nuclear factor NFATc1 (Adinolfi et al., 2009; Adinolfi et al., 2010). Overexpression of P2X7 receptor was accompanied by increased NFATc1 activity also in cancer models (Adinolfi et al., 2012; Giuliani et al., 2014). Among the paths associated to P2X7-dependent tumour growth, the HIF1-α/vascular endothelial growth factor (VEGF) axis plays a central role. HIF1-α and its target gene VEGF are central in ensuring nutrients to cancer cells

through vascularization. P2X7 enhances tumour vascularization in experimental models of colon carcinoma, melanoma and neuroblastoma (Adinolfi et al., 2012; Amoroso et al., 2015) causing an increase in both HIF1-α activity and VEGF secretion (Amoroso et al., 2012; Adinolfi et al., 2012; Amoroso et al., 2015). Moreover, administration of the VEGF antagonist Avastin in tumour bearing mice reverts P2X7-dependent growth advantage (Adinolfi et al., 2012). Hypoxia has been shown to regulate P2X7 expression though HIF1-α leading to increased cancer cell mobility (Tafani et al., 2011). Solini et al. recently suggested that hyper-activity of P2X7 and VEGF receptor 2 (VEGFR2) would worsen prostate cancer evolution and demonstrated that the association of loss of function polymorphisms in both VEGFR2 and P2X7 correlates to favourable prognosis in prostate cancer (Solini et al., 2015). P2X7 overexpression was associated with poor outcome and metastatic dissemination of different malignancies including chronic lymphocytic and acute leukaemia (Adinolfi et al., 2002; Chong et al., 2010), papillary thyroid carcinoma (Gu et al., 2010) and stage IV neuroblastoma (Amoroso et al., 2015). Several studies also tried to correlate P2X7 LOF or GOF SNPs with chronic lymphocytic leukaemia (Wiley et al., 2002; Thunberg et al., 2002; Ke et al., 2003), and multiple myeloma (Paneesha et al., 2006; Vangsted et al., 2014), but with contrasting results, probably due to the analysis of a limited number of SNPs and to lack of receptor function characterization. Divergent reports also came from laboratories working on glioma with some groups attributing a proliferative role to P2X7 (Ryu et al., 2011; Braganhol et al., 2015) and others claiming an opposite behaviour (Fang et al., 2013). Morrone and collaborators recently published intriguing results associating a high expression of P2X7 with the ability of glioblastoma patients to respond to radiotherapy. It was already known that irradiation caused ATP secretion (Ohshima et al., 2010; Gehring

et al., 2012) Gehring et al. demonstrated that lack of P2X7 receptor prevents response to radiations in an *in vivo* model of glioblastoma and associated high P2X7 expression with good prognosis for patients undergoing radiotherapy (Gehring et al., 2015). Of interest, also the administration of cytotoxic chemotherapy causes a release of ATP that activates P2X7 receptor on dendritic cells stimulating host cancer-specific immune responses. This phenomenon is known as immunogenic cell death and its currently being exploited to improve oncological treatments (Fucikova et al., 2015; Pol et al., 2015). The critical activity of P2X7 in anti-tumoral immune cell activation is further supported by two recent studies demonstrating increased growth of experimental tumours in *p2rx7* null mice versus wild type controls (Adinolfi et al., 2015; Hofman et al., 2015). Interestingly, both studies reported reduced immune infiltrating cells at the tumour bed in *p2rx7* lacking animals. Therefore, although systemic administration of P2X7 antagonist in immunocompetent mice bearing P2X7-positive tumours proved efficacious in reducing tumour growth in immune-competent mice (Adinolfi et al., 2015; Amoroso et al., 2015), one should take into account the possible action of these drugs on the immune system when treating specific malignancies. Treatment with P2X7 blocking drugs could be tailored to patients of those oncological conditions such as neuroblastoma, acute leukaemia and papillary thyroid carcinoma whereby a clear association between P2X7 expression and negative prognosis has been demonstrated. Moreover, in the case of neuroblastoma when we compared the effect of pharmacological blockade of P2X7 in xenogeneic (immune compromised) versus syngeneic (immune competent) mice we retrieved an increased efficacy of receptor's antagonist in the immune responsive model especially in regard to reduction in VEGF content and blood vessels number (Amoroso et al., 2015). These data, together with the proved function of P2X7 in immune cells favouring

tumour development (Bianchi et al., 2014; Bergamin et al., 2015), support the hypothesis that in certain oncological settings blockade of P2X7 on host cancer-infiltrating cells could prove detrimental for tumour growth.

Conclusions and future perspectives

In conclusion, the reported literature suggests that P2X7 receptor is involved in the pathogenesis of several diseases, ranging from inflammatory to autoimmune disorders and from altered neurorological conditions to cancer, suggesting a high pharmacological potential for P2X7 blocking drugs in a broad range of settings, especially in disorders where other clinical trials have failed. An increasing number of studies reported preclinical *in vivo* evidence for the efficacy of P2X7 antagonist in conditions as different as multiple and amyotrophic lateral sclerosis, Alzheimer's disease, epilepsy, pain and cancer, prompting the use of this drugs, which have already been shown to be safe and well tolerated in humans, also in the clinical settings.

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Abbreviations

(Aβ)3, extracellular amyloid β; AD, Alzheimer's disease; ALS, amyotrophic lateral sclerosis; AM, allosteric modulators; ATP, adenosine triphosphate; BD, bipolar disorder; BBG, Brilliant Blue G; BzATP, 2'(3')-O-4-benzoylbenzoyl)-ATP; Ca^{2+}, calcium; CNS, central nervous system; CLL, chronic lymphocytic leukaemia; COX-2, cyclooxygenase-2; DAMPs, damage-associated molecular patterns; GOF, gain of function; HD, Huntington's disease; HIF1-α, hypoxia-inducible factor 1-alpha; LOF, loss of function; LS BMD, lumbar spine bone mineral density; MAMPs. microbe-

associated molecular patterns; MDD, major depressive disorder; MM, multiple myeloma; MS, multiple sclerosis; NFATc1, nuclear factor of activated T cells complex 1; NLRP3, NLR family, pyrin domain containing 3; sAPPα, soluble fragment of amyloid precursor protein; SNPs, single nucleotide polymorphisms; PAMPs, pathogen-associated molecular patterns; PRRs, pattern recognition receptors; RA, rheumatoid arthritis; SE, status epilepticus; SOD 1, superoxide dismutase 1; T1D, type 1 diabetes; T2D, type 2 diabetes; TB, tuberculosis; TGFβ, transforming growth factor beta; VEGF, vascular endothelial growth factor; VEGFR2, VEGF receptor 2.

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Tables

Table 1.

Table 1. Principal non-synonymous SNPs in the *p2rx7* gene associated to disease.

dbSNP ID*	Nucleotide Base Change	Amino acid Change	Effect of Minor Allele	Associated Conditions	References
rs3751143	1513A>C	E496A	LOF	Risk of bone fracture; decreased LS BMD; familial CLL; conflicting results on susceptibility to tuberculosis	Gu et al., 2001; Ohlendorff et al., 2007; Wesselius et al., 2013; Husted et al., 2013; Dao-Ung et al., 2004; Fernando et al., 2007; Xiao et al., 2010
rs1653624	1729T>A	I568N	LOF	Risk of bone fracture	Wiley et al., 2003; Ohlendorff et al., 2007; Jorgensen et al., 2012
rs28360457	946G>A	R307Q	LOF	Increased rate of bone loss; Protection against MS.	Gu et al., 2004; Jorgensen et al., 2012; Gartland et al., 2012;
rs2230911	1096C>G	T357S	LOF	No association with RA	Shemon et al., 2006; Portales-Cervantes et al., 2012; Al-Shukaili et al., 2011
rs208294	489C>T	H155Y	GOF	No association with MDD and RA; protection against AD	Cabrini et al., 2005; Viikki et al., 2011 Portales-Cervantes et al., 2012; Al-Shukaili et al., 2011; Sanz et al., 2014
rs1718119	1068G>A	A348T	GOF	Lower vertebral fracture incidence; increased LS BMD values	Cabrini et al., 2005; Jorgensen et al., 2012; Wesselius et al., 2013
rs2230912	1405A>G	Q460R	LOF	BD, MDD, Osteoporosis	Cabrini et al., 2005; Barden et al., 2006; Lucae et al., 2006; McQuillin et al., 2009; Jorgensen et al., 2012; Wesselius et al., 2013
rs28360447	474G>A	G150R	LOF	Increased risk of osteoporosis	Denlinger et al., 2006; Wesselius et al., 2013
rs35933842	151+1G>T	-	LOF	Increased risk of MM	Skarratt et al., 2005; Vangsted et al., 2014

Table 2.

Table 2. International Clinical Trials for P2X7

Status	Main ID	Study Title	Condition
Not recruiting	NCT02293811	Decoding of the expression of tumor suppressor P2RX7 in inflammatory and malignant colonic mucosa	Crohn disease-associated colorectal adenocarcinoma
Completed, Not recruiting	NCT00628095	Study of CE-224,535 a twice daily pill to control rheumatoid arthritis in patients who have not totally improved with methotrexate	Rheumatoid arthritis
Terminated, Not recruiting	NCT00471120	Feasibility study: accuracy of biomarker in detection of endometrial cancer	Uterine cancer; Endometrial cancer
Recruiting	NCT00293189	Gene-polymorphisms in the P2X7 gene in patients with osteoporotic fractures	Hip fractures
Active, Not recruiting	NCT02082821	A P2X7R single nucleotide mutation promotes chronic allograft vasculopathy	Cardiac allograft vasculopathy
Completed	NCT00697983	Cohort study on associations between purinergic receptor SNPs and osteoporosis risk	Osteoporosis
Completed	NCT00849134	First time in human study evaluating the safety, tolerability, pharmacokinetics, pharmacodynamics and the effect of food of single assending doses of GSK1482160	Inflammatory pain
Phase II	NCT00520572	A 6-month randomised, double-blind, open arm comparator, phase IIb, with AZD9056, in patients with rheumatoid arthritis (RA)	Rheumatoid arthritis

Figure legends

Figure 1. Chemical structure of main P2X7 receptor agonists.

Figure 2. ATP binding site of the human P2X1 (A-B) and P2X7 (C-D) receptor. (A-C): Detailed view of the P2X1 and P2X7 ATP binding cavities. Ball and sticks residues represent the P2X conserved aminoacids. (B-D): Same binding cavities represented as molecular surfaces. Dark and light colours indicate polar and non-polar regions, respectively.

Figure 3. Chemical structure of main P2X7 receptor antagonists.

Figure 4. Simulated binding mode of A-839977 (A) and A-804598 (B) with the human P2X7 binding site (represented as molecular surface, with dark and light regions indicating polar and non-polar regions, respectively).

Figure 5. Chemical structure of main P2X7 receptor allosteric modulators.