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A potential therapeutic role for P2X7 receptor (P2X7R) antagonists in the treatment of inflammatory diseases

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Abstract

Introduction—The P2X7 receptor (P2X7R) has an important role in inflammation and immunity, but until recently, clinical application has been limited by a lack of specific antagonists. Recent studies using P2X7R knockout (KO) mice and specific receptor antagonists have shown that the P2X7R is an important therapeutic target in inflammatory diseases.

Areas covered—We have reviewed the current literature on the role of the P2X7R in inflammatory diseases, focusing on potential therapeutic applications of selective P2X7R antagonists as an anti-inflammatory agent. Particular emphasis has been placed on the potential role of P2X7R in common inflammatory diseases. The latest developments in phase I and II clinical trials of P2X7R antagonists are covered.

Expert opinion—Recent studies using gene KO mice and selective P2X7R antagonists suggest that P2X7R is a viable therapeutic target for inflammatory diseases. However, efficacious P2X7R antagonists for use in clinical studies are still at an early stage of development. Future challenges include: identifying potential toxicity and side effects of treatment, timing of treatment initiation and its duration in chronic inflammatory conditions, optimum dosage, and development of a functional assay for P2X7R that would help to guide treatment.

1. Introduction

1.a. Purinergic receptors

An important early conceptual shift proposed by Burnstock in 1970 was that intracellular adenosine triphosphate (ATP) might have an additional role outside cells (extracellular ATP) as a signalling molecule and the neurotransmitter in so-called non-cholinergic, non-adrenergic (NANC) neurotransmission. Since then our understanding of the role of ATP as an extracellular messenger (purinergic cell signalling) that is not confined to the nervous system has increased dramatically; many roles for purinergic receptors in health and disease have now been identified.

In 1978, Burnstock sub-classified the purinergic receptors into P1 and P2 receptors: adenosine acting on P1 receptors, whereas ATP and its breakdown products, ADP and AMP, acting on P2 receptors. Burnstock and Kennedy later proposed a further sub-classification of the P2 receptors, dividing them into P2X and P2Y receptors: P2X receptors are inotropic ligand-gated non-selective cation channel receptors and P2Y receptors are G protein coupled receptors; there are currently seven P2X subtypes and eight P2Y subtypes [1].

Declaration of Interest

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The P2X7 receptor (P2X7R) is a distinct member of the P2X subclass, as its downstream signalling is coupled to pro-inflammatory cascades. It is a 595-amino acid polypeptide with two membrane-spanning domains and has a long intracellular C-terminus compared with the other P2X receptors [2]. This receptor is expressed on many types of cells, the most studied being macrophages and monocytes, and it has a key role in regulating cell survival and release of mature IL-1 β and IL-18 cytokines [3].

1.b. IL-1 β , IL-18, and caspase-1

IL-1 is a central player in the inflammatory cascade. It is produced by many types of cells, including activated monocytes and macrophages. It has a variety of effects on its target cells by activation of signal transduction pathways, such as MAPK and NF- κ B, resulting in upregulation of several gene products in the inflammatory cascade, such as COX-2, IL-6, chemokines, and cellular adhesion molecules. IL-1 has two isoforms, IL-1 α and IL-1 β , which bind to the same receptors and are biologically active [4,5].

Inflammatory stimuli, especially LPS, engage with the TLR4 receptor of T cells, which activates MAPK and/or NF κ B signalling cascades, resulting in the synthesis of pro-IL-1 β . Pro-IL-1 α is constitutively expressed and does not require TLR stimulation [6]. Both IL-1 α and IL-1 β are produced as 31 kDa precursors that are stored within the cell cytosol. The pro-IL-1 β precursor remains within the cytosol and is cleaved to its mature form through the action of caspase-1 (or interleukin 1 β Converting enzyme). Pro-IL-1 β is also cleaved into its active form by other enzymes such as serine proteases (e.g., proteinase 3) and metalloproteinases (MMP-2 and MMP-9). IL-1 α processing is via the Ca-dependent protease calpain [7]. IL-1 α has similar biological activity in its precursor and cleavage product forms; in contrast, IL-1 β is only active once it is cleaved to its 17 kDa mature form. Caspase-1 is crucial for the processing of intracellular pro-IL-1 β ; although extracellular pro-IL-1 β can be processed by a number of different proteases during inflammation [3,8].

Caspase-1 is itself produced from the constitutively expressed 45 kDa pro-enzyme, pro-caspase, which is also found in the cytoplasm; it requires post-translational processing to form 20 and 10k Da forms of active caspase-1 [8]. Activation of caspase-1 occurs following assembly of an intracellular complex known as the 'inflammasome'. The NALP3 inflammasome is a multiprotein complex containing NALP3 (cryopyrin), apoptosis-associated speck-like protein (ASC), and caspase-1, which oligomerise on cell activation [9]. Proteolytic activation of IL-1 β occurs within the inflammasome protein complex. However, the exact mechanism of inflammasome formation and activation is not fully understood, since it can be triggered by different mechanisms in different cell types. Mature IL-1 β may also be released into the extracellular space by exocytosis in small vesicles or as a result of loss of membrane integrity [10].

In addition to IL-1, IL-18 is a key mediator in the host response to infection and the inflammatory response [4,11]. IL-18 is also constitutively produced as a precursor, pro-IL-18 [11]. Pro-IL-18 is cleaved by either caspase-1 or proteinase-3 (cf. pro IL-1 β above) into its active form, which is released into the extracellular space along with mature IL-1 β . Extracellular release of active IL-1 β and IL-18 is dependent on ATP-sensitive P2X7R activation. Contact with LPS alone is insufficient for extracellular release of IL-1 β and IL-18; any one of a number of different stimuli, including extracellular ATP, nigericin, bacterial toxins, hypotonic stress, and T cells are usually required. The best-established stimulus to IL-1 β and IL-18 post-translational processing and release is ATP acting via the P2X7R [3,11].

Experimental evidence supporting an attenuated inflammatory response with the use of P2X7R antagonists, or in P2X7R KO mice, may be directly related to the inhibition of IL-1 β

release. However, the attenuated inflammatory response may also be related to the lack of induction of other inflammatory mediators. IL-1 β may induce or release nitric oxide, COX-2, superoxide products, and other pro-inflammatory mediators [6,12], which must be considered when interpreting experimental data.

1.c. P2X7R

P2X7R expression was originally defined in macrophages and monocytes [13], and has since been characterised in several different cell types. The expression and activity of P2X7R in monocytes and macrophages is significantly upregulated in response to inflammatory stimuli, including LPS. Unlike other P2X receptors, the P2X7 receptor is distinct because its downstream signalling is coupled to pro-inflammatory cascades [2]. During active inflammation, the local concentrations of extracellular ATP are raised, which is thought to be mainly a consequence of cell damage and lysis. To activate the P2X7R *in vitro*, extracellular concentrations of ATP in the range of 1mM are required, in contrast to concentrations of 100 μ M needed to activate other P2 receptors. The ATP molecule binds to and activates P2X7, resulting in pore formation. This leads to K⁺ efflux from the cell, which is a crucial step in inflammasome assembly. Macrophages treated with ATP in medium containing KCl (rather than NaCl) failed to activate and release IL-1 β , suggesting that an ATP-induced K⁺ efflux from the cell is necessary for release of mature IL-1 β , IL-1 α , and IL-18. In addition to K⁺ efflux from the cell, there is an influx of Ca²⁺, which is also required for the release of active IL-1 β (Figure 1) [3,10]. Prolonged activation of the P2X7R results in irreversible pore formation and allows the non-selective passage of ions and hydrophilic solutes of up to 900Da; this can result in colloido-osmotic lysis and cell death by apoptosis or necrosis [3]. Pore formation is also thought to allow entry of bacterial products (pathogen associated molecular proteins, PAMPs) and extracellular ATP into the cell, which drives inflammasome formation [14].

Oxidised ATP (oATP) is a potent, but unselective, P2X7R antagonist and it can attenuate inflammatory responses independent of P2 receptor blockade [15], which must be considered when interpreting much of the experimental data referred to in this review; oATP has been one of the most widely used P2X7R antagonists. More selective P2X7R antagonists exist and they have been used successfully in *in vitro* experiments (Table 1), and in Phase 1 and Phase 2 clinical trials (Table 2).

1.d. Genetics of the P2X7R in human disease

SNPs are the most common form of genetic variation in the human genome. SNPs are now widely used to identify genetic associations and thereby disease-causing genes, but may in themselves have altered gene function. The *P2X7* gene is located on chromosome 12q24.31, spanning 53kb [16]; it is highly polymorphic with 32 identified SNPs within it. There are four non-synonymous *P2X7* SNPs that form five haplotypes in 99.9% of Caucasians [17]. These SNPs may lead either to loss or gain of function mutations. For instance, the SNP rs1653624 results in the amino acid substitution of isoleucine to asparagine at residue 568. This substitution prevents trafficking and normal cell surface membrane expression of the receptor [17,18]; it is uncommon in Caucasian populations and has an estimated allelic frequency of 0.026. Population studies of mapping and linkage disequilibrium have revealed a *P2X7* SNP (rs22309012) that renders increased susceptibility to either bipolar disorder or major depressive illness. However, the functional effect of this SNP on P2X7R is yet to be determined [19,20]. Other conditions linked to P2X7R gene polymorphisms include tuberculosis (TB) susceptibility, resistance to infection with *Chlamydia trachomatis*, and increased fracture risk in post-menopausal women [17].

1.e. The P2X7R and cell survival

There is evidence to support the role of the P2X7R in cell survival and growth. The growth-promoting function of P2X7R seems at odds with its established role in apoptosis; however, this receptor is expressed at a high level in some malignancies, which may be consistent with its growth-promoting role [21]. The apparent anti-apoptotic property of P2X7R is based on studies using oATP [21,23] and KN-93 [25]. Work by Adinolfi et al has demonstrated that basal activation of P2X7R (via the autocrine/paracrine release of extracellular ATP) promotes cell growth. P2X7R-transfected HEK293 cells have greater amounts of intracellular ATP, higher mitochondrial resting potential, and increased basal mitochondrial Ca^{2+} compared with HEK293 mock transfected cells. P2X7R transfected HEK293 cells are able to grow in the absence of serum [22]. This growth-promoting function of the P2X7R is dependent on pore formation, since it does not occur in cells transfected with a truncated form of the P2X7R that cannot form a pore. The same authors showed that in P2X7R transfected HeLa cells an ATP challenge resulted in mitochondrial fragmentation and subsequent cell death [23]. HEK293 P2X7R expressing cells were also resistant to apoptosis induced by C2-ceramide, staurosporin, or intracellular Zn^{2+} chelation. This survival advantage was dependent on NFATc1 activation, the main intracellular growth-promoting pathway mediated by increases in ER Ca^{2+} concentration [23].

It is controversial whether P2X7R are expressed at the plasma membrane of neutrophils [24]. The anti-apoptotic property of the P2X7 receptor has been described in neutrophils of patients with rheumatoid arthritis (RA) [26]. Serum amyloid A (SAA) purified from the plasma of patients with RA, or recombinant SAA, suppressed both spontaneous and α -FAS (CD95) induced-neutrophil apoptosis of human neutrophils *in vitro*; oATP inhibited this SAA-mediated anti-apoptotic effect.

Nagaoka et al have also demonstrated an anti-apoptotic action of P2X7R [25]. LL-37, an antimicrobial cathelicidin peptide (see later) has been shown to suppress neutrophil apoptosis in association with reduced activity of caspase-3. This effect was blocked by oATP and KN93 (a more selective P2X7 receptor antagonist). Future work with more selective P2X7R antagonists will be needed to clarify the role of P2X7R in neutrophils.

Although these studies have increased our understanding of the potentially bi-functional (pro- and anti-apoptotic) P2X7R, it is still not clear if these effects can occur in all cell types expressing P2X7R, or what factors determine the balance between pro- and anti-apoptosis.

1.f. LL-37

Although ATP is a P2X7R agonist, causing IL-1 β processing and its release from LPS-primed macrophages and monocytes, the concentration of ATP (0.5-5mM) required to induce IL-1 β release *in vitro* is believed to be far higher than is normally present in the extracellular space of tissues, at least under physiological conditions [27]. There are several studies suggesting that human cathelicidin-derived peptide, LL-37, may be a P2X7R agonist. Cathelicidins are an integral part of the innate immune system. Human cationic antimicrobial protein-18 (hCAP18) is the only known cathelicidin in humans [28]. In neutrophils, hCAP18 is stored as a precursor molecule and cleaved by proteases in the neutrophilic granules to release the active C-terminal peptide LL-37 [29]. This is a potentially important finding, but further work is necessary to assess to what extent the effect of LL-37 is P2X7R-dependent.

In the presence of LL-37, the concentration of ATP required to activate P2X7 is approximately 4000-fold less than the usual concentration of ATP needed to release the same amount of IL-1 β [30]. LL-37 has a well-established antimicrobial function, exerting direct antimicrobial activity against both Gram-positive and Gram-negative organisms, and

neutralising LPS [31]. LL-37 treatment of LPS-primed monocytes leads to caspase-1 activation and an increase in IL-1 β release via P2X7R in a dose-dependent manner [30]. LL-37 treatment of LPS-primed monocytes also results in a transient release of ATP, peaking after 5-10 minutes at a concentration of ~150nM [30]; the mechanism of this is not clear. LL-37-mediated release of IL-1 β from monocytes may be independent of extracellular ATP. The addition of apyrase (to rapidly degrade ATP) to LL-37 primed monocytes did not significantly alter the effect on IL-1 β release, and addition of exogenous ATP did not increase further the amount of IL-1 β released.

2. P2X7R and inflammatory diseases

2.a P2X7R and renal disease

Within the rat kidney, P2X7R is expressed at very low levels under normal conditions [32]; however, several studies have reported upregulation of renal P2X7R expression under various pathological conditions *in vivo* and *in vitro*. Harada et al reported that rat P2X7R mRNA levels are very low in normal isolated glomeruli and in cultured mesangial cells [33]. P2X7R protein was detectable in unstimulated mesangial cells only by immuno-electron microscopy (EM). P2X7R mRNA was induced by TNF- α and linked to mesangial cell apoptosis.

P2X7R expression in renal diseases was first described in a rat model of hypertension and diabetes mellitus [34]. In normal rat kidneys, consistent with Harada's earlier findings, P2X7R was detected at very low levels in glomeruli [33], whereas in a rat transgenic model of renin-dependent hypertension there was an increase in P2X7R expression at 12 weeks. Similarly, rats with streptozotocin-induced diabetes had significant P2X7R detectable in their glomeruli at 6 and 9 weeks. At the mRNA level this corresponded to a ten-fold increase in expression of P2X7R. Immunohistochemistry and immuno-electron microscopy studies of diabetic rats showed that P2X7R were expressed mainly in glomerular podocytes (also with some expression in endothelial cells and mesangial cells).

The role of P2X7R and TGF- β in extracellular matrix deposition was demonstrated in a mouse model of unilateral ureteral obstruction (UUO) [35]. Immunohistochemistry demonstrated P2X7R expression in the epithelial cells of the renal cortex in KO mice on day 7 only. Gonclaves et al demonstrated that myofibroblast number and collagen deposition were significantly reduced in P2X7R KO mouse compared with wildtype mice in the UUO model. There was a similar reduction in TGF- β protein in the KO mice. Furthermore, at day 14, kidneys from the P2X7R KO mice showed reduced numbers of infiltrating macrophages and had less tubular cell apoptosis. The tubulo-interstitial damage and subsequent fibrosis associated with UUO were attenuated in P2X7R KO mice, suggesting a role for tubular P2X7R in renal inflammation and fibrosis.

Taylor et al demonstrated perhaps the most striking evidence for a pathogenic role of P2X7R in a rodent model of experimental glomerulonephritis (GN). They confirmed that P2X7R is normally expressed at very low levels in rat and mouse kidney. In their mouse model of accelerated nephrotoxic nephritis, an increased expression of this receptor was demonstrated, along with an increase in glomerular apoptotic cells. In renal biopsies of patients with lupus nephritis, there was also increased glomerular, and some tubular, expression of P2X7R. In a rat model of antibody-mediated GN, the onset of glomerular P2X7R expression coincided with the onset of proteinuria [36].

Following this finding the same group of investigators demonstrated that P2X7R KO mice were significantly protected from antibody-mediated GN [37]. P2X7R KO mice exposed to nephrotoxic serum had reduced glomerular macrophage infiltration, less fibrin deposition,

less glomerular thrombosis, a reduction in proteinuria, and protection of overall renal function (with a smaller rise in serum creatinine concentration). IL-1 β levels were too low for quantification, but CCL2 (also known as MCP-1) levels were reduced in P2X7R KO mice compared with wildtype controls.

Treatment with a selective P2X7R antagonist (A438079) also reduced the glomerular expression of CCL2, the number of glomerular macrophages, the severity of glomerular injury, and proteinuria.

These data provide evidence for a pathogenic role of P2X7R in inflammatory GN, as well as a possible therapeutic role for a selective P2X7R receptor antagonist in the treatment of renal inflammatory diseases.

2.b. P2X7R and respiratory disease

As already mentioned, the P2X7R gene is located on chromosome 12q24, which also contains multiple genes linked to respiratory function [16,38], so it is perhaps not surprising that the P2X7R has been implicated in respiratory disease. Chronic inflammatory reactions in lung tissue are characterized by the formation of multinucleated giant cells (MGC), as seen in patients with sarcoidosis and TB. The ability of monocytes from patients with sarcoidosis and TB to form MGC *in vitro* is abolished by the presence of the non-selective P2X7R antagonist α ATP, suggesting a role for P2X7R [39]. Polymixin B, which stimulates P2X7R pore formation in rat alveolar macrophages, caused a dose-dependent increase in the formation of MGC *in vitro*. The Polymixin B-stimulated alveolar cells increased in number and had a greater tendency to fuse with adjacent cells, resulting in a significant increase in MGC formation. Pre-treatment with α ATP abrogated the stimulatory effect of polymixin B [40]. These findings suggest a role for P2X7R in MGC formation during chronic inflammatory reactions. Functional P2X7R have also been demonstrated in human lung mast cells.

P2X7R is important in the defence mechanism against mycobacterium tuberculosis [41]. *In vitro* studies have shown that mycobacteria can survive following phagocytosis by macrophages, and that the crucial bactericidal step is P2X7R-mediated apoptosis of the macrophage; having a loss-of-function SNP results in a 3.5-fold increase in the risk of latent TB reactivation in a South-East Asian population in Australia [41], and in a Mexican population [42].

Prolonged exposure to high oxygen concentrations results in inflammation of lung tissue and in acute lung injury [43], which is related to an increase in IL-1 β levels. Murine alveolar macrophages exposed to hyperoxia *in vitro* and *in vivo* demonstrated activation of P2X7R, inflammasome formation, release of IL-1 β and induction of caspase-1, and IL-1 β cleavage [44]. This effect was enhanced by the addition of the P2X7R agonist ATP, and inhibited by the P2X7R antagonist α ATP. The release of IL-1 β was absent *in vitro* on silencing the short hairpin RNA of inflammasome components, demonstrating the role of the inflammasome in P2X7R-mediated inflammation in hyperoxia.

Extracellular ATP is upregulated in the airways of patients with chronic obstructive pulmonary disease (COPD), bronchial asthma, and pulmonary fibrosis, and may also contribute to the pathogenesis of these diseases [45-47]. P2X7R activity has been linked to virus-induced loss of asthma control in patients with mild- to moderate bronchial asthma and who were followed after the onset of a naturally occurring common cold through to convalescence [48]. Whole-blood P2X7R pore function inversely correlated with asthma symptoms. P2X7R pore function in whole blood was assessed by the fold change in Bz-ATP-stimulated YO-PRO-1 uptake relative to the buffer control. Furthermore, P2X7R pore

activity from whole blood samples correlated positively with the peak in nasal lavage neutrophil counts, suggesting that this whole blood P2X7R pore functional assay may identify patients with asthma-associated loss-of-function P2X7R genotypes. Consistent with these findings, Muller et al demonstrated upregulation of P2X7R in bronchoalveolar lavage (BAL) macrophages and in peripheral blood eosinophils during acute and chronic asthmatic airways inflammation in mice and humans [46]. The functional significance of P2X7R was demonstrated by the use of the specific receptor antagonist KN62, which attenuated allergic airway inflammation in rats. P2X7R-deficient dendritic cells (DC) showed a reduced capacity to induce Th2 immunity in a DC-driven murine model of allergic airway inflammation. Inhibition of this receptor on haematopoietic cells (e.g. DCs or eosinophils) may prove a novel therapeutic target in asthma treatment.

Similar findings have been described in cigarette smoke (CS)-induced lung inflammation and emphysema *in vivo*. Extracellular ATP contributes to the development of CS-induced lung inflammation and emphysema through activation of P2X7R. Upregulation of P2X7R in blood and airway neutrophils, alveolar macrophages, and in whole lung tissue of mice with CS-induced lung inflammation, has been demonstrated in an *in vivo* model [45]. Selective intrapulmonary inhibition of P2X7R reduced CS-induced lung inflammation and prevented the development of emphysema. Consistent with this finding, P2X7R KO mice showed reduced pulmonary inflammation following acute CS exposure. These results raise the possibility that targeting P2X7R might also be a therapeutic option in the management of COPD.

Patients with pulmonary fibrosis have elevated levels of ATP in BAL samples. In a bleomycin-induced murine model of pulmonary inflammation and fibrosis, Riteau et al found increased levels of extracellular ATP in BAL fluid [47]. The use of apyrase resulted in a significant reduction in bleomycin-induced inflammatory cell recruitment, and in local IL-1 β levels. Conversely, the use of the more stable analogue of ATP, ATP γ S, increased inflammation. P2X7R KO mice were protected from bleomycin-induced lung inflammation and had reduced markers of tissue fibrosis, including lung collagen deposition. *In vitro* studies also demonstrated the release of ATP from pulmonary epithelial cells exposed to bleomycin. The release of ATP was reduced by selective P2X7R antagonists (A438079 and A740003) *in vitro*.

ATP has been implicated in an animal model of ventilator-associated lung injury [49]. High-pressure mechanical ventilation was found *in vivo* to increase broncho-alveolar lavage ATP concentrations, whereas lactate dehydrogenase concentrations were unchanged, which makes ATP release from cell lysis unlikely. Furthermore, intra-tracheal ATP increased lung water content, implicating nucleotides in mechanical ventilation-associated pulmonary oedema. However, a functional role for P2X7R in this setting requires further work.

2.c. P2X7R and rheumatological disease

High levels of purines and pyrimidine nucleotides have been identified in the synovial fluid of patients with rheumatoid arthritis (RA) [50]. Activated synovial fibroblasts (type B synoviocytes) are involved in the pathogenesis of cartilage damage in RA [51]. Nucleotides have been shown to produce joint inflammation through the production of local cytokines, including IL-1 β , TNF- α , IL-2, and IL-6 [52].

Functional P2X7R on human rheumatoid synoviocytes has been demonstrated *in vivo* [53]. BzATP, a relatively selective P2X7R agonist, induced an upregulation of IL-6 mRNA and protein expression, which was diminished in the presence of oATP. The functional capacity of P2X7R to form large pores was absent in this cell type, as demonstrated by the failure of

BzATP to induce significant uptake of YO-PRO. The effect of BzATP-induced IL-1 β production by synoviocytes has not been reported.

Peripheral blood mononuclear cells isolated from patients with RA produce significantly higher amounts of IL-1 β in response to ATP in the presence of LPS [54]; although the degree of P2X7R upregulation after 24 hours of ATP incubation did not vary between mononuclear cells from normal controls and from patients with RA. However, it remains possible that mononuclear cells of patients with RA are more responsive to ATP stimulation due to a genetic polymorphism in the P2X7R gene.

Cathepsins are lysosomal proteases that play a role in inflammatory and degenerative arthropathies, leading to joint destruction [55]. Cathepsins are involved in the degradation of proteins expressed by the MHC class II processing pathway, and in the proteolytic removal of invariant chain (Ii), a critical regulator of MHC class II function. Mice lacking cathepsins show a defect in Ii degradation in antigen presenting cells, and presentation of exogenous proteins *in vivo*, conferring protection from collagen-induced arthritis [56].

P2X7R activation of mouse bone marrow-derived macrophages (BMDM) results in release of lysosomal cathepsins that degrade collagen matrix. The release of these cathepsins is inhibited by P2X7R antagonists (AZ10573295 and AZ11648720) and is absent in P2X7R KO mice, suggesting a functional role for P2X7R in their release [57]. However, the lysosomal release of cathepsins was found to be independent of release of IL-1 β .

P2X7R antagonists have been shown to limit joint destruction and pain in a rodent model of RA. Arthritis was induced by the local injection of Freund's complete adjuvant into the hind paws of rats. Administration of oATP into inflamed paws of rats with arthritis relieved oedema and inflammatory pain within 48 hours [58]. The reduction in inflammatory pain was mediated by downregulation of P2X7R in peripheral nerve endings and epithelial cells, and it was independent of immune cell recruitment.

Neutrophil apoptosis is an important part of the regulation of inflammation [59]. The role of the P2X7R is less well-established in neutrophils. P2X7R may have an additional and indirect role in the mediation of inflammatory arthritis via anti-apoptotic signalling in neutrophils. Serum amyloid A (SAA) protein is an acute phase reactant that often correlates with active joint inflammation, and it is elevated in many patients with RA [60]. SAA purified from patients with RA suppresses α -CD95 (FAS) mAb-induced human neutrophil apoptosis *in vitro* [26]; the P2X7R antagonist oATP prevented this inhibitory effect, which suggests that SAA promotes neutrophil survival via a P2X7R-dependent mechanism (see earlier - section 1.d.).

2.d. P2X7R and neurological disease

Microglial cells are the primary immune cells of the CNS. Unsurprisingly, they express a number of P2 receptors activated by nucleotides that release cytokines, including IL-1 β and IL-6 [61]. Microglia play an important part in the immune system of the CNS by acting as scavenger cells. Activation of P2X7R by ATP in microglial cells results in the release of autolysosomes into the extracellular space, providing a mechanism for the clearance of intracellular pathogens [62].

P2X7R is upregulated around beta- amyloid plaques in a mouse model of Alzheimer's disease [12]. P2X7R also plays a role in the generation of superoxide in microglia. Thus, P2X7R has been implicated in the pathophysiology of Alzheimer's disease and other neurodegenerative conditions through ATP-mediated cortical cell death and superoxide release.

Wang *et al* have demonstrated a downregulation of P2X7R in oligodendrocyte precursor cells in hypoxic brain injury [63], which may indicate a role for this receptor in hypoxic brain injury. In a recent paper by Domercq et al supporting this idea, ATP released during ischemia, and subsequent activation of P2X7R, was linked to white matter loss following ischaemic injury [64].

P2X7R pathway disruption also results in amelioration of chronic inflammatory and neuropathic pain [65,66]. Work in P2X7 knock out mice has demonstrated the lack of an exaggerated response to mechanical stimuli (allodynia and hyperalgesia) during chronic inflammation in P2X7 KO mice [65]. In addition to the expected attenuation in IL-1 β production, P2X7 KO mice had reduced IL-6, TNF α , and MCP-1 production. There has been extensive research on the anti-noiceptive properties of P2X7R antagonists, which is beyond the scope of this article.

3. Conclusion

Laboratory and clinical research to date has provided promising results for the clinical application and safety of P2X7R antagonists. Further study of SNP of P2X7R in the susceptibility and severity to diseases, and their interaction with antagonist may be the next step. The challenges ahead are to identify appropriate therapeutic applications for P2X7R antagonists, increase our understanding of the physiology and pathophysiology of the P2X7R, and to develop more selective and potent P2X7R agonists and antagonists.

4. Expert opinion

Finding therapeutic agents that are able to act rapidly to prevent any irreversible organ damage in inflammatory diseases remains a challenge, as many patients suffer from inflammatory diseases that are resistant to current therapies. The need to find an efficacious agent with limited adverse effects, either as a first-line or adjunctive therapy, is important. There is growing evidence to support the role of P2X7R in inflammatory diseases (Table 1), particularly from studies of P2X7R KO mice. A large number of antagonist studies have been carried out using non-selective or poorly selective antagonists like oATP. Therefore, the results obtained with these antagonists need to be interpreted with some caution. Recently, intervention studies with more selective P2X7R antagonists, such as A438079, have provided results supporting the importance of P2X7R as a potential therapeutic target. Thus, more selective P2X7R antagonists are likely to prove promising therapeutic tools (Table 2).

4.1. P2X7R and sepsis

Sepsis is a common and serious clinical condition, with an incidence of 700,000 cases and a mortality of 200,000 cases per year in the United States [67]. The importance of caspase-1 and secretion of mature IL-1 β in sepsis have been studied in rodent models of sepsis [68,69], and studies suggest a key role for the regulation/inhibition of IL-1 β release as a therapeutic strategy [70].

In an *in vivo* model of sepsis, LPS-induced vascular smooth muscle hyporeactivity has been shown to be mediated by P2X7R activation and subsequent IL-1 β release, followed by an increase in iNOS expression, and increased levels of NO [71]. Therefore, P2X7R antagonism might be beneficial in states of vasodilatory shock.

However, a large Phase 3 randomised clinical trial has failed to support the benefit of the recombinant human IL-1 receptor antagonist in the treatment of patients with severe sepsis [72]. Although this finding does not preclude the use of a P2X7R antagonist in sepsis, as

there are actions over and above IL-1 β release that could be beneficial in sepsis. An immunomodulatory agent that can interfere with more than one arm of the sepsis response, such as a P2X7 receptor antagonist, may have promise if given to the right patient group and at the right time. For example, apoptosis of lymphocytes is associated with a worse outcome in sepsis [73,74] and blocking apoptosis has been shown to result in improved survival in experimental models of sepsis [74,75]. T lymphocytes express functional P2X7R [76] and blocking these receptors with P2X7R antagonists to prevent apoptosis might confer a survival advantage in sepsis.

There is also a significant association between sepsis severity, mitochondrial dysfunction, and outcome [77]. BzATP stimulation of P2X7R transfected HEK293 and HeLa cells results in loss of the mitochondrial membrane potential with subsequent fragmentation of mitochondria and cell death [22]. Mitochondrial fragmentation was absent in cells lacking P2X7R. Thus, it is plausible that a P2X7R antagonist could have a role in the preservation of mitochondrial function in sepsis.

4.2 P2X7R antagonist in renal disease

Renal P2X7R upregulation has been described in both acute and chronic diseases, including glomerulonephritis (GN), ureteric obstruction, diabetes mellitus, and hypertension [34-36]. P2X7R KO mice are protected against GN, and a selective P2X7 receptor antagonist (A438079) confers protection in a rat model of GN [37]. This supports a pathologic role for P2X7R in acute kidney injury in GN. In addition, mesangial cells express P2X7R in response to TNF- α , suggesting a role for P2X7R in inflammatory renal disease [33].

Under basal conditions, renal P2X7R may have a role in maintaining normal cell turnover through the regulation of cell growth and apoptosis [33,78]. The possible functional roles of the P2X7R in inflammation include apoptosis, cytokine production, loss of functional receptors, and fibrosis. There is good evidence to support a role for apoptosis of renal tubular cells in experimental models of ischaemic and endotoxic renal injury [79,80]. In experimental GN, there is increased glomerular expression of caspase-3 and pro-apoptotic genes p53 and bax, with no change in the anti-apoptotic gene bcl-2 [36,81]. Mesangial cells can also undergo apoptosis on exposure to BzATP *in vitro* [33]. Glomerular cell apoptosis may lead to scar formation as part of healing, with subsequent glomerulosclerosis [82,83]. Thus, blockade of P2X7R to prevent apoptosis in these conditions could be protective.

Urinary IL-18 is a sensitive marker of AKI in sepsis, ischaemia, and post-operatively [84]. Caspase-1-KO mice are protected from renal ischaemic injury, and effect that was dependent on impaired IL-18 processing and an associated decrease in renal neutrophil infiltration [85]. Caspase-1 inhibition resulted in decreased IL-18 protein and IFN- γ mRNA, a reduction in histological injury, preservation of renal function, and increased survival [86].

The role of IL-1 β in renal ischaemic reperfusion injury and endotoxaemia is also well established. Furuchi et al demonstrated that IL-1 β stimulation of renal tubular cells *in vitro* results in increased MCP-1 and MIP-1 α production [87]. *In vivo* this is related to an increased inflammatory cell infiltrate in the kidney during ischaemia-reperfusion injury. In a GN model, P2X7R expression correlated with IL-1 β gene expression, with a renal protective effect of P2X7R antagonism [36]. P2X7R blockade may decrease IL-1 β and IL-18 production, leading to renal dysfunction in sepsis or ischaemia-reperfusion injury.

Caspase-1-deficient mice are protected from AKI in endotoxaemia [88]. This protective effect was independent of tubular cell apoptosis, neutrophil infiltration, caspase-3 activity, and calpain activity. This suggests that inhibition of IL-1 β and IL-18 production may have renal protective effects over and above apoptosis and inflammatory cell infiltration. Schmidt

et al have demonstrated that IL-1 β administered to mice results in decreased renal function and expression of functional renal transporters, including the epithelial sodium channel (ENaC), the renal outer medullary potassium channel (ROMK), and the Na⁺/K⁺-ATPase [89]. LPS-induced downregulation of sodium transporters was unaltered in cytokine-deficient mice, supporting a role for IL-1 β in renal dysfunction. Similar results were seen after ischaemia-reperfusion injury in the kidney.

Following an episode of inflammation, fibrosis often follows and P2X7R has been shown to promote TGF- β -induced fibrosis in a model of UUO [35]. Mesangial cell proliferation mediates glomerular scarring by secretion of extracellular matrix components and pro-inflammatory cytokines [83,90]. Prevention of scar formation and glomerulosclerosis may help to preserve renal structure and function.

Given the evidence that P2X7R plays a part in renal inflammation, it seems likely that blockade of P2X7R will help to preserve renal structure and function in renal inflammatory diseases.

4.3 P2X7R and antibody production

The role of P2X7R in IL-1 and IL-18 release and in cell death is now well established. The nephrotoxic nephritis model provides evidence that P2X7R may affect antibody production. P2X7R is primarily found on antigen presenting cells, including dendritic cells and macrophages [13]. In the accelerated nephrotoxic nephritis model of GN there was a small decrease in the (autologous) mouse anti-sheep IgG deposition in the glomeruli of P2X7R KO mice [37]. This raises the possibility that P2X7R is also involved in antibody production. This possibility requires further investigation with, for example, the use of an autoantigen-induced model of autoimmune GN. This may indicate that P2X7R is also a potential target in treating autoimmune disease.

4.4 Current use of P2X7R antagonists in clinical trials

Phase 1 and phase 2 clinical trials investigating the safety and efficacy of the P2X7R antagonists are currently underway (Table 2). There have not been any serious concerns regarding the safety profile of the existing P2X7R antagonists. Phase 2 clinical trials have investigated the use of P2X7R antagonists in inflammatory bowel disease, rheumatoid arthritis, and chronic obstructive airway disease. The main side effects have included gastrointestinal upset (diarrhoea and nausea), dizziness, and headaches. These results, though not conclusive, are encouraging. Current P2X7R antagonists have proven to be safe and well tolerated in humans. Although some of P2X7R antagonists have been withdrawn from further clinical development because of insufficient clinical efficacy, a new generation of antagonists is entering early stage clinical studies (Table 2).

Factors that may have limited efficacy up to now may be due to drug pharmacokinetics and pharmacodynamics. P2X7R polymorphisms may be another factor to consider. Until we know exactly where P2X7R sits in the inflammatory pathway, and what redundancy there might be, it is difficult to be sure in what situations antagonising this receptor will be most effective.

In future drug development, the affinity of P2X7R antagonists for different polymorphic forms of P2X7R may need investigation and correlation with efficacy. With a better understanding of the underlying disease processes and more drug development, the prospects for P2X7 targeted therapies are still encouraging.

Article Highlights

- The P2X7 receptor (P2X7R) has an important role in inflammation and immunity.
- There are several emerging functions of the P2X7R that require further understanding
- Recent studies using P2X7R knockout (KO) mice and specific receptor antagonists have shown that the P2X7R is an important therapeutic target in inflammatory diseases, including glomerulonephritis
- Clinical trials of P2X7R antagonists for inflammatory arthritis, inflammatory lung disease, and inflammatory bowel disease have progressed to Phase I and Phase II
- There are several other potential applications of the P2X7R antagonists, such as the amelioration of the pro- inflammatory phase of sepsis

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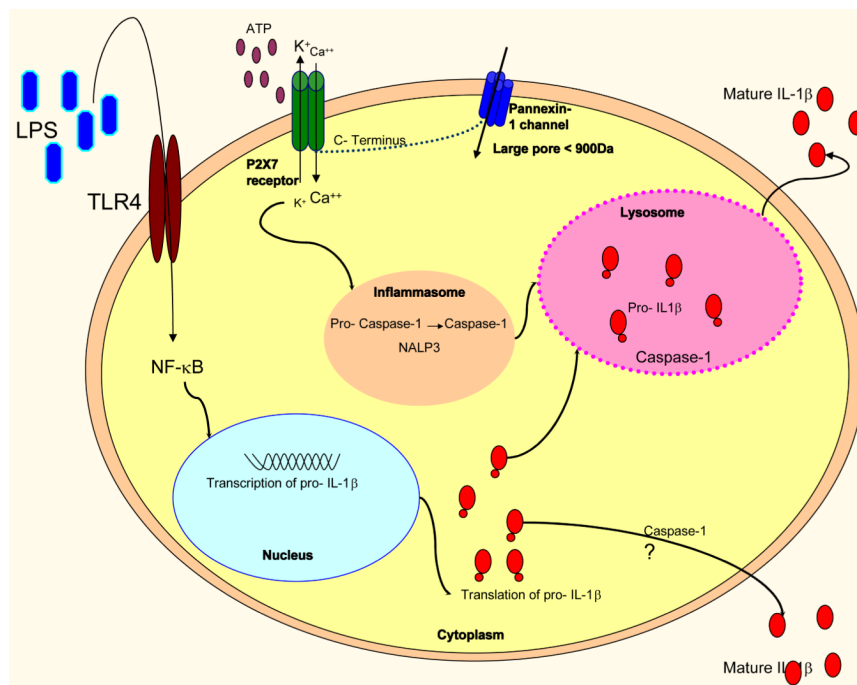


Figure 1. Mechanism of action of the P2X₇ receptor

LPS engages with the Toll receptor 4 (TLR4). This results in activation of NFκB and nuclear transcription of IL-1β. IL-1β mRNA is subsequently translated into the precursor molecule pro-IL-1β within the cytoplasm [1]. IL-18 is transcribed and translated constitutively [2].

LPS 'primes' the P2X₇R. On contact with ATP and/or LL37, the P2X₇R forms a non-selective ion pore [2]. This allows the efflux of K⁺ and the influx of Ca²⁺ [3]. The change in membrane potential and intracellular Ca²⁺ and K⁺ results in the assembly of the inflammasome and drives the conversion of pro-caspase-1 into mature caspase-1. Pro-IL-1β and caspase-1 are incorporated into the lysosome. Pro-IL-1β is converted into mature IL-1β by the action of caspase-1 within the lysosome and is released into the extracellular environment [4].

Caspase-1-dependent processing of pro-IL-1β may occur in the cytosol following activation of P2X₇-receptor and may be released via mechanisms independent of the lysosomal pathway (marked with '?') [5,6]. The P2X₇ receptor is coupled to a dye uptake pore, pannexin-1, which opens on prolonged stimulation of the P2X₇R [7]. Opening of the pannexin pore allows the passage of hydrophilic solutes into the cell. This is thought to result in cell death [1].

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Table 1

P2X₇R and inflammatory diseases

P2X ₇ R and renal disease						
Reference	Disease/ Stimulation	P2X7 expression (Cell type)	<i>in vivo</i> / <i>in vitro</i>	Species	KO/ Antagonist	
Turner [32]	None	None	<i>In vivo</i>	Rat	-	
Harada [33]	TNF- α	Mesangial cells	<i>In vitro</i>	Rat	-	
Goncalves [35]	Unilateral ureteric obstruction	Tubular epithelial cells	<i>In vivo</i>	Mouse	KO	
Vonend [34]	HTN	Glomeruli	<i>In vivo</i>	Rat	-	
	DM	Glomerular podocytes	<i>In vivo</i>	Rat	-	
Turner [36]	Experimental glomerulonephritis	Glomeruli and macrophages	<i>In vivo</i>	Mouse Rat		
Turner [36]	Lupus Nephritis	Glomerular	<i>In vivo</i>	Humans	-	
Taylor [91]	Experimental Glomerulonephritis		<i>In vivo</i>	Rat Mouse	Selective antagonist A438079 KO	
Gauer [92]	None	Collecting duct alpha- and beta-intercalated cells	<i>In vivo</i>	Humans	-	
P2X ₇ R and respiratory disease						
Reference	Disease/stimulation	P2X7 expression (Cell type)	<i>in vivo</i> / <i>in vitro</i>	Species	KO/ Antagonist	
Mizuno [39]	Con A	Monocytes	<i>In vitro</i>	Humans	oATP	
Lemaire [93]	Polymixin B	Alveolar macrophages	<i>In vitro</i>	Rat	oATP	
Wareham [40]	ATP	Lung mast cells	<i>In vitro</i>	Human	AZ11645373	
Fernando [41]	ATP-mediated mycobacterial killing	Macrophages	<i>In vitro</i>	Human	Loss of function SNP	
Kolliputi [44]	Hyperoxia	Alveolar macrophages	<i>in vitro and in vivo</i>	Mice	oATP	
Dellinger [48]	Asthma	'Whole blood'	<i>in vivo</i>	Human	-	
Muller [46]	Acute and chronic asthmatic airways	Dendritic cells Bronchial lavage macrophages and peripheral blood	<i>In vivo</i>	Mice and humans	KO oATP, KN62 (in mice)	

P2X ₇ R and renal disease						
Reference	Disease/ Stimulation	P2X ₇ expression (Cell type)	<i>in vivo</i> / <i>in vitro</i>	Species	KO/ Antagonist	
Lucatelli [45]	Cigarette smoke (CS)-induced lung inflammation and emphysema	Blood and airway neutrophils, alveolar macrophages, and in whole lung tissue	<i>In vitro</i>	Mice	KO KN62	
Riteau [47]	Bleomycin-induced lung inflammation and fibrosis	Respiratory epithelial cells	<i>In vivo</i> <i>In vitro</i>	Mice	KO In vitro A438079 and A740003	
P2X ₇ R and rheumatological disease						
Reference	Disease/stimulation	P2X ₇ expression (Cell type)	<i>in vivo</i> / <i>in vitro</i>	Species	KO/ Antagonist	
Caporali [53]	Patients with RA	Synoviocytes	<i>In vivo</i>	Human	-	
Al-Shukaiti [54]	Patients with RA	Peripheral blood mononuclear cells produced more IL-1 β	<i>In vitro</i>	Human	-	
Lopez-Castejon [57]	ATP-induced cathepsin release	Macrophages (Human) Bone marrow-derived macrophages (mice) release of lysosomal cathepsins	<i>In vitro</i>	Humans Mice	Antagonist (AZ10573295 and AZ11648720) KO	
Dell'Antonio [94]	Arthritis was induced by the local injection of Freund's complete medium	Peripheral nerve endings, and in epithelial cells	<i>In vivo</i>	Rats	oATP	
Christenson [26]	SAA purified from patients with RA suppresses a-CD95 (FAS) mAb-induced human neutrophil apoptosis	Neutrophil	<i>In vitro</i>	Human	oATP	

Table 2

Summary of Clinical studies (Phase I and Phase II) investigating P2X₇ receptor antagonists

Study design	Study aim	Patients included	Intervention	Primary Outcome Result
An Open, Randomised, 2-way Crossover Study (AZD9056; Astra Zeneca) (Study code: D1520C05285) [1] (2003)	To establish the absolute bioavailability of AZD9056 in tablet formulation (200 mg) compared with an intravenous formulation (25 mg)	12 healthy Volunteers	AZD9056 was provided as two 100 mg tablets for oral administration, and as a 100 mL intravenous (iv) infusion of 25 mg.	The mean absolute bioavailability was estimated as 62% There was evidence of secondary peak at 5 hours post-dose in the mean plasma concentration profiles for both the tablet and the iv formulations. The geometric mean t _{1/2} was approximately the same for each formulation (18 hours for iv and 16 hours for oral). The average plasma clearance was 29 L/h, and the average volume of distribution was 735 L. The median t _{max} was shorter for the iv infusion (2 hours) than for the oral tablet (3 hours).
A Phase I, Double-Blind, Placebo-Controlled, Randomised, Group Comparative Study (AZD9056; Astra Zeneca) Study code: D1520C05216 [2] (2004)	To Investigate the Safety, Tolerability, PK and PD of Multiple Ascending Doses of AZD9056 in Healthy Volunteers	48 healthy volunteers	11 patients were in the 100 mg dose group (Cohort A), and 12 each to two 400 mg dose groups (Cohorts B and C) and the 600 mg dose group (Cohort D) for 10 consecutive days.	Thirteen subjects discontinued from the study. Two discontinuations were due to AEs (one in Cohort B and one in Cohort C). The subject in Cohort B subsequently experienced a SAE, and as a result the remainder of Cohort B were also discontinued from the study
An Open, Non-Randomised, Crossover Study (AZD9056; Astra Zeneca) (Study Code D1520C00002) [3] (2005)	To Investigate the Effect of AZD9056 (50 mg Oral Dose for 5 Days) on Cytochrome P450 3A Using the Probe Drug Midazolam (7.5 mg Oral and 1 mg Intravenous Dose)	12 male and female healthy volunteers	AZD9056 administered orally as a single 50 mg tablet midazolam administered orally as a 7.5 mg tablet and as an IV bolus of 1 mg At Visit 2, subjects were administered a single 7.5 mg tablet of midazolam. Six hours later, they were administered 1 mg midazolam by iv bolus injection. At Visit 3, subjects were administered 50 mg AZD9056 for 5 days. On Day 5, they were also administered 7.5 mg oral midazolam and 1 mg iv midazolam, as for Visit 2. There was a washout period of 6 days between Visits 2 and 3.	Results suggest weak inhibition of CYP3A by ADZ9056 The estimate of t _{1/2} , (20.5 hours) suggests that steady state for AZD9056 would be achieved by Day 4 to 5
Phase II RCT-, double blind, placebo-controlled,	<u>Primary outcome:</u> Pain control <u>Secondary outcomes:</u> Safety and tolerability	Patients with OA of the knee 404 patients enrolled and 108 patients were randomised (54 in each	AZD9056 given for 28 days as a daily oral single dose (200mg for first 7	No significant difference in pain assessment. Discontinuation rate due to intolerability

Study design	Study aim	Patients included	Intervention	Primary Outcome Result
parallel group, multicentre international study (AZD9056; Astra Zeneca) (Study code: D1522C00001) [4] (2005)		arm). 87 patients completed the study	days followed by 400mg for next 21 days)	similar. 1 serious AE in ADZ group (viral illness) AEs: GI disturbance, headache
Phase II Clinical study RCT-, double blind, placebo- controlled, parallel group, multicentre international study (CREATE Study, 2007) (AZD9056; Astra Zeneca) (Study code: D1520C05287) [5,6] (2006)	Efficacy and tolerance ACR and DAS28 scores ESR Physician assessment of disease activity Patient assessment of physical function Pain, fatigue, and duration of morning stiffness	75 patients with RA receiving background treatment with methotrexate and/ or sulphasalazine	An ascending dose study to compare the efficacy and tolerance of 2 doses of orally administered ADZ9056 (100mg and 400mg) over placebo over a one month period	<i>ADZ9056 400mg was associated with significantly more patients achieving ACR 20, clinically relevant improvements in DAS28, HAQ, and reduced swollen and tender joints compared with placebo.*</i> No significant improvement in systemic surrogate markers of disease activity (ESR, CRP). ADZ-9056 was well-tolerated-main AEs were gastrointestinal (nausea, diarrhoea, and vomiting)
A Phase 1, Double Blind Study NCT00446784 [7] (2007) (CE 224, 535; Pfizer)	The Safety And PK Of Multiple Doses Of CE 224,535 PK of CE 224, 535 and methotrexate	Subjects With Rheumatoid Arthritis Receiving Methotrexate	Multiple doses of CE 224,535 administered to patients with RA who have had at least 4 weeks of methotrexate	Study completed
A 2 Week, Randomized, Double Blind, Placebo And Positive Controlled, Parallel Group, Multicenter Study Of CE-224,535 NCT00418782 [8] CE 224, 535 (2007)	To evaluate the analgesic and anti inflammatory efficacy and safety of CE 224,535 versus placebo and naproxen treatment in patients with OA knee pain. Safety, tolerability, PK and PD of oral multiple doses of CE 224,535	Subjects With Osteoarthritic Pain Of The Knee	Administration of CE 224,535 or placebo and naproxen in patients with OA knee pain for 2 weeks	Results of interim analysis indicate lack of efficacy when compared to placebo
Phase II RCT-, double blind, placebo-controlled, parallel group, multicentre international study (AZD9056; Astra Zeneca) (Study code: D1521C00002) [9] (2007)	<u>Primary outcome:</u> Lung function tests, Exercise tolerance, Symptoms, Baseline dyspnoea, Inflammatory markers <u>Secondary outcomes:</u> Safety and tolerability	Patients with moderate to severe COPD 190 patients were enrolled and 134 patients were randomised-66 to active treatment and 68 to placebo	AZD9056 given for 28 days as a daily oral single dose (400mg)	No difference in either primary or secondary outcomes
Phase IIa/ III Clinical study RCT- randomised, double blind, placebo-controlled, parallel group, multicentre international study (CE-224,535; Pfizer)	ACR 20, 50 and 70 scores DAS28 score PK Adverse events Components of ACR responses	Patients with active RA who have not completely improved with methotrexate	Twice daily oral CE-224,535 500mg over a 12- week time period	Study completed

Study design	Study aim	Patients included	Intervention	Primary Outcome Result
NCT 00628095 [10] (2008)				
A Phase 1, Randomized, 4-Period, 4-Sequence Cross-Over NCT00782600 [11] CE 224, 535 (2008)	Study Of The PK Of 3 Durations Of Release Of A Controlled Release Formulation And A Single Dose Of An Immediate Release Oral Suspension Of CE-224,535	Normal Healthy Volunteers	Administration of a controlled release formulation and a single dose of an immediate release oral suspension of CE-224,535	Study completed
Phase I Ib randomised, double blind, open arm comparator, parallel group, multicentre international study (AZD9056; Astra Zeneca) NCT00520572 [12] (2008)	ACR 50 and ACR 70 response Disease activity score (DAS 28) Health assessment questionnaire-disability index (HAQ DI)	Patients with RA with active disease who are receiving a background of methotrexate or sulphasalazine	Once daily oral ADZ9056 (4 doses- 50mg, 100mg, 200mg, and 400mg) administered for 6 months compared to patients receiving etanercept (25mg IM twice weekly) or placebo	ACR 20 response at 6 months (treatment: ACR 20 score/ serious adverse events (%))- ADZ9056 50mg: 23/ 3.17 ADZ9056 100mg: 26/ 0 ADZ9056 200mg: 23/ 1.59 ADZ9056 400mg: 21/ 1.56 Placebo: 21/ 1.54 Etanercept: 42/ 3.13
Phase I, single centre, non-randomised, open-label study (AZD9056; Astra Zeneca) NCT00736606 study [13] (2008)	To assess the PK of both AZD9056 (steady state) and simvastatin (single dose) when co-administered in healthy volunteers	12 healthy volunteers	One single dose of simvastatin 40mg 400mg once daily of oral ADZ9056 with a single dose of 40mg of simvastatin on day 7	Study completed
Phase I randomised double-blind, placebo-controlled, 2- period cross over study (AZD9056; Astra Zeneca) NCT00700986 [14] (2008)	Retinal function as measured by electroretinography Visual acuity, contrast sensitivity, and colour vision Visual evoked potential Psychomotor testing	12 healthy volunteers	Single dose of oral ADZ9056 800mg	Study completed
Phase II RCT-, double blind, placebo-controlled, parallel group, multicentre international study (AZD9056; Astra Zeneca) (Study Code D8830C00002) [15] (2008)	<u>Primary outcome:</u> Change in CDAI (Crohn's disease activity index) <u>Secondary outcomes:</u> Reduction in CDAI by at least 70 points Time to remission Time in study SF-36 and IBD questionnaire	Patients with active Crohn's disease affecting the ileum and/ or colon 40 patients were enrolled and 30 patients were randomised-20 to active treatment and 10 to placebo	AZD9056 given for 28 days as a daily oral single dose (200mg)	<i>Statistically significant decrease in CDAI in ADZ group*</i> Proportion of patients in remission and with clinical response was greater in the ADZ group Significant improvement in the IBDQ score in the ADZ group AE: Abdominal pain (no difference between groups)
Phase I double blind, placebo-controlled study of EVT-401 (Evotec; 2008) [16]	To evaluate the compound's safety, tolerability, pharmacokinetic profile, and pharmacodynamic effects	96 Healthy male volunteers	Single ascending dose study	Safe and well-tolerated
An Open-Label Two-Part Randomized, Crossover	The PK Of CE-224,535 Administered	Healthy Volunteers	Four treatment periods to take either different forms of the pill(s) or the same	Study completed

Study design	Study aim	Patients included	Intervention	Primary Outcome Result
Study NCT00838058 [17] CE 224, 535 (2009)	As Controlled And Immediate Release Formulations		form either after fasting or eating a meal	
Phase I open- label, randomised, 2 cohort, 2 period crossover study (AZD9056; Astra Zeneca) NCT00908934 [18] (2009)	To assess the relative bioavailability of a new formulation of AZD9056 using PK variables Cmax and AUC compared to the previous phase III to phase IIb formulations of AZD9056	24 healthy volunteers	AZD9056 formulation Single dose of 50mg or 400mg of both the previous and new formulations	Study completed
An Open-label, Randomized, 2-cohort, 2-period Crossover Study. (AZD9056; Astra Zeneca) (Study Code D1520C00004) [19] (2009)	To Assess the Relative Bioavailability of the Phase III to the Phase IIb Formulation of AZD9056 in Healthy Male and Female Subjects	24 Healthy subjects, aged 18 to 55 years	Subjects received either 50mg or 400mg of AZD9056	AZD9056 50 mg and 400 mg from both test and reference formulations appeared to be well tolerated
A Randomised, Double-Blind (with Open Comparator Etanercept Limb), Placebo-Controlled, Phase IIb, Multicentre Study (AZD9056; Astra Zeneca) (Study Code D1520C00001) [20] (2009)	To Evaluate the Efficacy of 4 Doses of AZD9056 Administered for 6 Months on the Signs and Symptoms of Rheumatoid Arthritis in Patients with Active Disease Receiving Background Methotrexate or Sulphasalazine	658 patients were enrolled into the study; 385 of whom were subsequently randomised to study treatment (64 patients assigned to etanercept and each of the AZD9056 dose levels and 65 patients to placebo	4 doses of AZD9056 (50, 100, 200 and 400 mg) or etanercept for 6 months	Each of the AZD9056 treatment groups was similar to the placebo group in terms of the ACR20 response rates at week 24. The observed effect in the open-label etanercept arm was clearly distinguishable from placebo and consistent with expectations. A similar pattern was seen for the ACR50 and ACR70.
Phase I randomised, single-blind, parallel assignment, safety study NCT00849134 [21] (GSK1482160; GlaxoSmithKline) (2010)	To monitor the safety parameters, adverse events, PK and PK of single ascending doses of GSK1482160 and to make a preliminary assessment of the effect of food Measuring the amount of inhibition of inflammatory chemical release from blood following dosing with GSK1482160	32 healthy volunteers	Multiple doses of the drug (or placebo) with or without a high fat meal	Study completed
Study design	Study aim	Patients included	Intervention	Primary Outcome Result

Abbreviations

PK: Pharmacokinetics

PD: Pharmacodynamics

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11. A phase 1, randomized, 4-period, 4-sequence cross-over study of the pharmacokinetics of 3 durations of release of a controlled release formulation and a single dose of an immediate release oral suspension of ce-224,535 in normal healthy volunteers. Clinicaltrials.Gov identifier: Nct00782600. 2009. Available at: <http://clinicaltrials.gov/ct2/show?term=CE-+224%2C+535&rank=4>
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