Antagonism of the ATP-gated P2X7 receptor: a potential therapeutic strategy for cancer

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Abstract

The P2X receptor 7 (P2X7R) is a plasma membrane receptor sensing extracellular ATP associated with a wide variety of cellular functions. It is most commonly expressed on immune cells and is highly upregulated in a number of human cancers where it can play a trophic role in tumorigenesis. Activation of this receptor leads to the formation of a non-selective cation channel, which has been associated with several cellular functions mediated by the PI3K/Akt pathway and protein kinases. Due to its broad range of functions, the receptor represents a potential therapeutic target for a number of cancers. This review describes the range of mechanisms associated with P2X7R activation in cancer settings and highlights the potential of targeted inhibition of P2X7R as a therapy. It also describes in detail a number of key P2X7R antagonists currently in pre-clinical and clinical development, including oxidised ATP, Brilliant Blue G (BBG), KN-62, KN-04, A740003, A438079, GSK1482160, CE-224535, JNJ-54175446, JNJ-55308942, and AZ10606120. Lastly, it summarises the *in vivo* studies and clinical trials associated with the use and development of these P2X7R antagonists in different disease contexts.

Keywords Purinergic receptors · P2X7 receptor · Antagonists · Inflammation · Cancer

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Introduction

Purinergic receptors are a family of membrane-bound proteins that act as signalling receptors [1]. Whilst only discovered in 1972 [2], and subsequently debated in the literature, they are now commonly accepted as their own class of receptors that mediate intercellular and intracellular communication via ionic transport [3]. They are activated by derivatives of purines, which are some of the most primitive molecular messengers in the animal kingdom [4]. These receptors have also been shown to be conserved in a number of different animals, including simple

unicellular organisms [5], and are therefore hypothesised to be some of the earliest evolved signalling receptors [4–6]. Purinoceptors are divided into 2 main categories: P1 receptors that are specifically activated by adenosine and P2 receptors that are activated by a range of purine and pyrimidine nucleotides [1, 7]. P2 receptors are further subdivided into P2Y and P2X receptors, with P2Y receptors being a subfamily of G protein coupled receptors and P2X receptors a subfamily of ligand-gated ion channels [8]. These different receptor subtypes are differentially expressed in specific tissues, with effectively all tissues expressing varying types and degrees of purinoceptors [1].

Whilst purinergic signalling was originally investigated for its roles in neurotransmission, neuromodulation, and chemoattraction, recent studies have shifted to investigating potential trophic roles of these receptors associated with cell proliferation and differentiation [3, 9]. One of the most widely studied purinoceptors in this context is the P2X7 receptor (P2X7R), a ligand-gated ion channel that is activated by extracellular ATP [10]. In physiological settings of controlled ion concentrations and membrane potential polarization, P2X7R allows controlled influx of Na^+ and Ca^{2+} and efflux of K^+ . It is formed by 3 homologous subunits, each containing 2 transmembrane spanning domains connected to a large extracellular domain, that assemble to form a homotrimer [10]. Unlike other types of P2X purinoceptors, P2X7R has been described to not only function as an ion channel but also form a non-selective pore that allows movement of molecules up to ~900 Da through the cell membrane (Fig. 1) [10, 11].

P2X7R is present in nearly all tissues and organs [12, 13], with high expression shown in the majority of immune cell types, including monocytes/macrophages, microglia, T-cells,

B-cells, dendritic cells, natural killer cells, and mast cells [10, 14, 15]. Increased expression is also seen in a wide range of cancer cells [12, 16], indicating that alterations in receptor expression and function may be modulated in different disease contexts compared to normal tissue [10]. Since its discovery however, the exact function of P2X7R has been controversial. There are multiple conflicting studies initially describing pro-apoptotic and cytolytic roles for the receptor, before more recent demonstrations of trophic roles [17, 18]. Prior to isolation and characterisation of P2X7R, initial studies investigating the role of extracellular ATP suggested that changes observed due to extracellular ATP were induced not by ATP itself, but by an ‘ATP receptor’ [19, 20]. This receptor, initially termed P2Z, was later renamed to P2X7R due to its homology with other P2X receptors [21]. These early studies highlighted that extracellular ATP causes the cell membrane to become permeable and induce changes within the cell that could lead to apoptosis [22, 23]. More contemporary literature has revealed both trophic and pro-apoptotic functions. It is now accepted that P2X7R stimulation can activate a variety of cellular functions, including cell division, increased energy metabolism, and functions to do with inflammation and immunity. The exact functions of the receptor are thought to be cell and context dependant, relying also on the surrounding microenvironment and level of stimulation from extracellular ATP [10, 24].

The dual conductance states of P2X7R, the ion channel and pore, possess different functions [11]. Efflux of K^+ through the P2X7R channel is associated with production of various cytokines, whilst pore conductance has been historically described as pro-apoptotic [25]. This was shown through in vitro

Fig. 1 The dual conductance states of P2X7R upon activation with extracellular ATP. P2X7R is initially inactive (left), until stimulated with extracellular ATP which binds between the extracellular regions of subunits, the ‘ATP binding pocket’. This induces a conformational

change of the complex to allow influx of Na^+ and Ca^{2+} and efflux of K^+ (middle). Upon sustained stimulation with high levels of extracellular ATP, a larger conductance pore is formed that allows movement of proteins of up to 900 Da in size (right) across the membrane

investigations in which continuous stimulation of the P2X7R resulted in continuous pore opening, loss of membrane integrity, and inevitably cell death [17, 26]. However, recent research has postulated that under physiological and ‘regulated’ conditions (when stimulated by locally released ATP), in the absence of supramaximal or sustained stimulation, the P2X7R pore can induce a trophic response without the cytotoxic effects of an indiscriminate large pore opening [26, 27]. Therefore, the true nature of P2X7R pore in various physiological and pathological states needs further characterisation and the majority of understood physiological functions are mediated by controlled ion movement [11].

P2X7R in Cancer

The expression of P2X7R has been investigated in a wide range of human cancers, including gastric, neuroendocrine, kidney, ovarian, uterine, breast, pancreatic, lung, skin, and brain cancer [18, 28–36]. Studies using cell lines and biopsies of these cancers have demonstrated differing results depending on the cancer and even the method of investigation used. Whilst some studies demonstrate decreased P2X7R expression when compared to normal tissue, the majority of studies show increases in mRNA and/or protein levels, making it difficult to characterise a consistent role of P2X7R in the cancer context [33]. Of these, multiple studies specifically demonstrate that increased P2X7R expression is associated with worsening disease prognosis, shown in clear cell renal carcinoma [37], invasive lobular carcinoma and ductal carcinoma [34], endothelial cancers [36], prostate cancer [35], colorectal cancer [38], and neuroblastoma [31], highlighting the potential of P2X7R as a biomarker of disease severity in these cancers. It has also been shown that splice variants of P2X7R have been associated with reduced leukocyte infiltration in tumours, shown in patients with lung adenocarcinoma, further highlighting the involvement of P2X7R in the cancer setting [39]. Whilst P2X7R functionality is not fully assessed in every cancer type, some cancers studies have demonstrated that the receptor retains its functionality and is able to drive processes such as increased tumour cell survival via several different mechanisms [14, 16, 18, 40]. Several other splice variants of P2X7R have also been identified in recent years, such as P2X7 isoform B and nP2X7 with studies increasingly indicating them as potential biomarkers and therapeutic targets [41].

P2X7R has been shown to play a key role in cancer cell division and tumour invasiveness. It does this via inflammatory mechanisms involving the release of various cytokines/chemokines from both the cancer cells themselves as well as nearby immune cells [14]. The receptor is thought to be stimulated by ATP secreted from nearby cells or from necrotic

regions of solid tumours [42, 43] to modify the tumour microenvironment and promote tumour growth [44]. The release of pro-inflammatory cytokines and proteins from both cancer and immune cells can increase cell proliferation, whilst also being able to induce trophism through changes in cell metabolism, angiogenesis, and extracellular matrix invasion. It has also been demonstrated that some pro-inflammatory pathway components can be upregulated in the tumour setting, such as the P2X7R itself, caspase-1, and IL-1 β , as shown in pancreatic cancer [45]. This could indicate that in conjunction with the trophic and cell survival processes that occur within tumour cells, in the tumour microenvironment there may be aberrant upregulations of the ‘inflammatory’ functions attributed to P2X7R which can further lead to increased tumour cell survival and proliferation. Whilst the involvement of P2X7R in cancer growth and metabolism is not fully understood, P2X7R has been linked with several key pathways and processes.

Signalling pathways linked to the P2X7R

As mentioned, activation of P2X7R is controlled by extracellular ATP, which can act as a potent signalling molecule through activation of purinergic signalling receptors [46]. Release of ATP from surrounding cells and necrotic tumour regions can activate P2X7R and has been shown to induce several trophic functions [42–44]. Along with this, P2X7R itself has been shown to control constitutive release of extracellular ATP through the P2X7R pore, as shown in neuroblastoma cells [47] and B16 melanoma cells [48]. This highlights a potential positive feedback mechanism by which P2X7R controls release of ATP and contributes to the overall level of extracellular ATP that can act to amplify purinergic receptor activation in surrounding cells.

Activation of P2X7R has been linked to the PI3K-Akt key signalling pathway [31]. This pathway is composed of a molecular cascade that links multiple oncogenes, receptors, and cellular functions, and is potentially the most commonly activated signalling pathway in human cancers [49]. Activation of this pathway results in multiple downstream cellular functions including protein synthesis, glycogen synthesis, cell growth, cell survival, transcription, and angiogenesis [50]. Multiple human cancers have demonstrated mutations in this pathway, with these mutations potentially representing an essential event necessary for tumorigenesis [51, 52]. P2X7R has been shown to promote activation of this pathway, though the mechanism is not fully understood [53]. One study indicated that Ca²⁺ influx is involved in activation of PI3K [54]; therefore, it is possible that P2X7R-dependent Ca²⁺ influx leads to release of growth factors via pathways such as NF- κ B induced transcription, which bind the receptor tyrosine kinase (RTK) to activate PI3K signalling [50].

Expression of the P2X7R has also been linked to increased angiogenesis, with studies showing the number of blood vessels increasing by 3–4 fold in two different animal models, both a xenograft tumour mouse model using HEK293 cells transfected with human P2X7R and a synergistic mouse model using CT26 murine colorectal carcinoma cells transfected with mouse P2X7R [18]. This was largely attributed to VEGF (vascular endothelial growth factor) secretion as both the transfected HEK293 cells and the transfected CT26 murine colorectal carcinoma cells showed diffuse VEGF staining, whilst the mock/wild-type tumours were virtually negative [18]. In a separate study, P2X7R silencing using a shRNA and antagonism with AZ10606120 and A740003 both induced an almost 50% reduction in HIF α protein levels and subsequent VEGF secretion in the CAN neuroblastoma cell line [31]. Activation of the PI3K/Akt pathway has been shown to increase VEGF secretion through both hypoxia-inducible factor 1 (HIF-1) and HIF-1 independent mechanisms, as well as modulate expression of angiogenesis factors such as nitric oxide and angiopoietins, implicating this pathway as a link between P2X7R activation and increased angiogenesis [55].

It has also been demonstrated that activation of the PI3K/Akt pathway can result in upregulation of P2X7R expression in neuroblastoma cells, with this upregulated expression increasing survival of neuroblastoma cells under growth factor restricted conditions [56]. This is due to P2X7R expression previously shown to increase proliferation and decrease apoptosis in growth limiting conditions [57]. Whilst this growth promoting activity is not fully understood, it has been associated with a number of mechanisms including increased release of VEGF, and therefore subsequent recruitment of cancer and immune cells [18, 58], facilitation of extracellular matrix invasion through release of mature proteases [59], improved energy metabolism via increasing mitochondrial potential [57], and upregulation of glycolytic enzymes and increasing intracellular glycogen stores [60].

Along with the effects of PI3K-Akt signalling, P2X7R activation has also been associated with activation of kinase proteins including several mitogen activated protein kinases (MAPK), protein kinase C (PKC), and cellular sarcoma tyrosine kinase (c-Src) [10]. Specifically, P2X7R mediated movement of Ca²⁺ has been shown to activate several MAPK proteins including ERK1/2 [61] and c-Jun-N-terminal kinases (JNK) [62], as well as PKC [63]. These play roles including regulation of RNA translocation, transcription factors, cell division, and proliferation [61, 64]. P2X7R has also been shown to potentially act as a substrate for some protein kinases, with some tyrosine residues shown to be phosphorylated by c-SRC [65], which may initiate functions such as membrane blebbing [66]. P2X7R activation has also been shown to maintain CDC42 activity, a GTPase of the Rho family that has a demonstrated role in several cancer cell functions including morphological changes that increase invasiveness [59, 67].

These interactions represent some of the signalling pathways that are associated with activation of P2X7R, and the broad range of interactions that P2X7R activation directly causes. The subsequent broad range of trophic functions that are induced, highlight the potential broad range of effects that targeting of P2X7R could modulate.

P2X7R antagonism as a potential therapeutic strategy

Considering the wide variety of functions that P2X7R activation can induce, antagonism of the receptor has been investigated in a number of disease contexts including in a number of different cancer types [42]. This is due to P2X7R's inflammatory functions as well as its role in promoting cancer cell growth, which make it an appealing therapeutic target [18, 33, 59, 68]. However, studies have also highlighted potential anti-cancer effects of P2X7R associated with extracellular ATP inducing cell death [16, 69, 70]. These conflicting results in cancer settings highlight that the role of P2X7R activation is highly variable depending on the cancer type and other varying factors [59]. Several studies have demonstrated that growth of experimental tumours can be strongly inhibited by targeting of P2X7R [14]. Therefore, it is clear that P2X7R antagonists hold promise as effective therapeutic agents, depending on the cancer type, with a number currently in pre-clinical and clinical development (Table 1) [14]. A range of P2X7R antagonists and their effects in different studies are described and discussed in detail below.

Oxidised ATP

Oxidised ATP is a potent irreversible, competitive antagonist of the P2X7R and one of the earliest and most widely used in exogenous settings [71]. It functions as a Schiff-base forming reagent, allowing for selective modification of lysine residues in the vicinity of the ATP-binding site to block receptor function [72] and inhibits both the P2X7R channel and pore conductance states [27]. The antagonistic behaviour of oxidised ATP was demonstrated through ablation of ATP induced Ca²⁺ influx in most cell types, indicative of blocking of the P2X7R transmembrane ionic conductance [73]. Most of the early studies conducted presumed oxidised ATP to have moderate specificity for P2X7R; however, it was subsequently shown that oxidised ATP also reduced intracellular signalling, independent of P2X7R. This was demonstrated in human 1321N1 astrocyte cultures, a cell line known to lack purinergic signalling receptors, where oxidised ATP treatment potently reduced NF κ B activation and IL-8 release despite the absence of P2X7R [73]. Oxidised ATP was used to target P2X7R in an in vivo mouse model of islet allograft rejection and was demonstrated to preserve islet grafts [74]. However, it was subsequently shown that similar results occurred in P2X7R^{-/-} recipients, again indicating that P2X7R was not

Table 1 Summary of the in vitro and in vivo cancer studies as well as clinical trials of P2X7R antagonists

Antagonist	Disease	Model	Main effect
Oxidised ATP	Colon carcinoma	BALB/c mice inoculated with CT26 colon carcinoma cells; both wild-type and overexpressing mouse P2X7R	Intratumour injection of oxidised ATP (600 µmol/L) resulted in reduced tumour size and growth of CT26-mP2X7R tumours, but had no effect on CT26-wt tumours [18].
	Mesothelioma	Malignant pleural mesothelioma (MPM) cell lines; SV-40Tag, MSTO-211H, IST-MES2, and MPP89	Administration of oxidised ATP resulted in a significant decrease in cell proliferation in all cell lines and a significant increase in LDH release in all cell lines except IST-MES2 cells [26].
Brilliant Blue G	Glioma	Sprague–Dawley rats implanted with C6 glioma cells into the striatum	Intraperitoneal injection of BBG (50 mg/kg) resulted in up to a 52% reduction in tumour size in all areas examined compared to control [29].
KN-62	Pancreatic cancer	BALB/c nude mice inoculated with MIA PaCa-2 pancreatic cancer cells	Intratumour injection of KN-62 (200 nM) and ATP (300 µM) abolished increased tumour proliferation seen with ATP alone, however did not reduce tumour proliferation compared to control [88].
	Mammary cancer	MDA-MB-435s breast cancer cell line	Administration of KN-62 (1 µM) inhibited P2X7R mediated ion currents, ethidium uptake, and calcium influx which are all measures of P2X7R functionality, as well as abolished ATP induced cancer cell migration and invasion [89].
A740003	Mammary cancer	MDA-MB-435s breast cancer cell line	Administration of A740003 (300 nM) inhibited P2X7R mediated ion currents and abolished ATP induced cancer cell migration and invasion [89].
	Osteosarcoma	BALB/c nude mice inoculated with HOS/MNNG osteosarcoma cancer cells	Intraperitoneal injection of A740003 (0.025 mg/kg) reduced tumour size by 50% compared to control, and also attenuated growth seen with BzATP (2.5 mg/kg) stimulation [53].
	Neuroblastoma	Nu/nu mice subcutaneously injected with human ACN neuroblastoma cells and AlbinoJ mice injected with Neuro2A mouse neuroblastoma cancer cells	Intraperitoneal injection every second day with A740003 (5 µM) caused an ~40% reduction in overall tumour size in nu/nu mice, and an ~50% reduction in overall tumour size in AlbinoJ immunocompetent mice [31].
A438079	Mammary cancer	4T1 mouse mammary cancer cell line	Administration of A438079 (10 µM) inhibited P2X7R mediated ion current, ethidium uptake, calcium influx, cancer cell migration, and matrix degradation/invasion [59].
	Mammary cancer	BALB/cj mice inoculated with 4T1 mouse mammary cancer cells genetically modified to express luciferase	Intraperitoneal injection of 230 µL of 3 mg/mL A438079 every second day was shown to reduce tumour growth by a factor of 2 and reduce metastatic progression [59].
GSK-1482160	Clinical Trial of Safety	First-in-human single blind, randomized, placebo-controlled trial	Data collected from this trial was analysed via PK/PD modelling and showed that it was not possible to achieve the > 90% inhibition of IL-1β release necessary, whilst maintaining a sufficient safety margin. The compound was therefore discontinued [100].
CE-224535	Clinical Trial for Rheumatoid Arthritis	Phase IIA, randomized, double-blind, placebo-controlled, parallel-group, multicentre study	Twice daily oral administration of CE-224,535 (500 mg) was shown to have adequate safety and tolerability, but was not efficacious when compared to placebo for treatment of rheumatoid arthritis [102].
JNJ-54175446	Clinical Trial for Major Depressive Disorder	Randomised, placebo-controlled, double-blind trial	Currently in progress, results not available [108].
JNJ-55308942	Clinical trial	Phase I double-blind, placebo-controlled, randomized single and multiple ascending dose study	Clinical trial completed, results not yet posted [110].
AZ10606120	Pancreatic cancer	Athymic nu/nu mice injected in the pancreas with PancTu-1 Luc pancreatic cancer cells	Intraperitoneal injections every second day of AZ10606120 (5 mg/kg) did not reduce overall tumour size postmortem, but did decrease overall tumour deviation and tumour growth rate [30].
	Pancreatic cancer	PancTu-1 Luc pancreatic cancer cell line	Administration of AZ10606120 (10 µM) inhibited cell proliferation, reduced cell migration, and reduced collagen production/deposition [30].
	Mesothelioma		Intratumour injections of AZ10606120 (0.7 mg/kg) resulted in up to 50% reduction in tumour size in

Table 1 (continued)

Antagonist	Disease	Model	Main effect
		Nu/nu mice subcutaneously inoculated with MSTO-211H or IST-MES2 mesothelioma cells	MSTO-211H tumours and accelerated tumour regression in IST-MES2 tumours [26].
	Mesothelioma	Malignant pleural mesothelioma (MPM) cell lines; SV-40Tag, MSTO-211H, IST-MES2, and MPP89	Administration of AZ10606120 (300 nM) resulted in a significant decrease in cell proliferation in all cell lines except MPP89 cells, though caused a significant increase in LDH release in MPP89 and SV-40Tag cells [26].
	Mammary cancer	4T1 mouse mammary cancer cell line	Administration of AZ10606120 (300 nM) resulted in inhibition of P2X7R mediated ion currents, ethidium uptake, calcium influx, cancer cell migration, and matrix degradation/invasion [59].
	Mammary cancer	BALB/cj mice inoculated with 4T1 mouse mammary cancer cells genetically modified to express luciferase	Intraperitoneal injection of 100 µL of 300 nM AZ10606120 every second day was shown to reduce tumour growth and metastatic progression [59].
	Neuroblastoma	Nu/nu mice subcutaneously injected with human ACN neuroblastoma cells and AlbinoJ mice injected with Neuro2A mouse neuroblastoma cancer cells	Intraperitoneal injection every second day of AZ10606120 (300 nM) caused an ~40% reduction in overall tumour size in nu/nu mice, and an ~50% reduction in overall tumour size in AlbinoJ immunocompetent mice [31].
	Melanoma	C57BL/6 mice inoculated with B16 melanoma cancer cells	Intratumour injections of AZ10606120 (300 nmol/L) caused strong inhibition of tumour growth and a large reduction in VEGF staining [18].

the only target of oxidised ATP action [74]. These studies demonstrate that the anti-inflammatory actions of oxidised ATP are mediated, at least in part, by interactions with cell receptors and pathways independent of P2X7R [72].

In cancer settings, oxidised ATP treatment had demonstrated a significant reduction in tumour growth and size in mouse CT26 colon cancer mouse models (where CT26 cells were transfected with P2X7R), as measured using histological techniques [18]. It was also shown to both reduce cell proliferation and increase LDH release in a human mesothelioma cell line [26]. Whilst these are promising anti-cancer results, due to the demonstrated off target effects as well as studies highlighting its potential cardiovascular toxicity [75], oxidised ATP is no longer seen as a specific P2X7R antagonist. New more selective P2X7R antagonists are now available, though due to its potency oxidised ATP can still be used in various in vitro assays where receptor functionality is being studied. However, its utility in in vivo settings and in future human clinical trials remains limited.

Brilliant Blue G (BBG)

Brilliant Blue G is a derivative of a commonly used synthetic food dye compound known as FD&C Blue 1 which was found to be a highly selective P2X7R antagonist [76]. As it was previously used as a food additive, it is considered non-toxic and approved by the FDA in other contexts [77]. BBG binds an intersubunit allosteric pocket of P2X7R in a non-competitive manner and inhibits ATP-evoked currents with an IC_{50} of ~300 nM [78]. Due to its safety and ability to penetrate the blood-

brain barrier, it was subsequently investigated as a potential P2X7R antagonist and has been investigated in a number of neurological and inflammatory diseases [75]. BBG has been shown to attenuate multiple disease characteristics present in a rat model of Parkinson's disease, including deficits in locomotion and memory [79]. In the same study it was demonstrated to reduce the degree of gliosis [79]. It has been shown to reduce serum IFN- γ and tissue inflammation in a humanised in vivo murine model of Graft versus Host Disease [80]. It has also been shown to improve motor recovery in rat models of thoracic spinal cord injury, as well as reduce local activation of astrocytes and microglia [75]. In a dose-dependent manner, BBG was also shown to produce an anti-depressive like effect in a validated chronic depression model in rats [81].

Similar to oxidised ATP, BBG was also demonstrated to be non-selective for P2X7R as it was shown to also block the human P2X4R in the micromolar concentration range [82]. In the cancer setting, BBG is less characterised, but has been shown in an in vivo C6 glioma mouse model to reduce tumour size by 52% compared to controls [29]. These studies show the potential anti-inflammatory and possible anti-cancer effects of BBG as a P2X7R antagonist in different disease contexts. However, its demonstrated effects on the human P2X4R highlight the need for further understanding of its mechanism of action before it can be applied as a potential therapy.

KN-62 and KN-04

Isoquinoline derivatives KN-62 and KN-04 are structural analogues of each other that were both found to antagonise the

P2X7R [83]. KN-62 is a potent and selective inhibitor of the Ca^{2+} /calmodulin-dependant protein kinase II (CaMKII) and was used to investigate the effects of CaMKII on extracellular ATP along with KN-04, an inactive analogue [84]. However, they were both found to completely inhibit ion channel fluxes when used at concentrations in the nanomolar range (~500 nM) and were almost equipotent. This indicated that CaMKII was not involved in the extracellular ATP associated ion channel fluxes and it was subsequently determined that they were acting by inhibiting P2X7R [84]. It was shown that efficacy was different depending on experimental conditions with KN-62 acting as a full antagonist upon ATP stimulation, but only a partial antagonist when stimulated with the agonist 2'(3')-O-(4-benzoylbenzoyl)adenosine 5'-triphosphate (BzATP) [83]. Similar to BBG, KN-62 and KN-04 both structurally inhibit the P2X7R by binding to an intersubunit allosteric binding site near the top of the extracellular region, adjacent to the ATP binding site [78]. It was also shown that both KN-62 and KN-04 only partially block non-selective cation movement, indicating that even at micromolar ranges (1 μM) these compounds only partially block P2X7R pore formation [85]. KN-62 has been used in the *Entamoeba histolytica* parasite to characterise P2X7R involvement in NLRP3 inflammasome activation and IL-1 β production [86], and used in human monocyte derived dendritic cells to show invariant natural killer T cell (iNKT) induced dendritic cell Ca^{2+} signalling [87].

Due to their use as a CaMKII inhibitor, and that they do not completely inhibit P2X7R pore formation even at micromolar concentrations, these compounds have not been extensively investigated as therapies and are instead utilised to characterise mechanistic pathways associated with P2X7R. In cancer settings, KN-62 has been shown to abolish increased tumour proliferation induced by ATP stimulation in an in vivo patient derived xenograft mouse model of pancreatic cancer, but did not reduce tumour proliferation compared to unstimulated controls [88]. KN-62 was also investigated in vitro in MDA-MB-435s cells, a highly aggressive breast cancer cell line. KN-62 treatment was shown to abolish a number of functions in these cells including P2X7R mediated ion currents, ethidium uptake, Ca^{2+} influx, and ATP induced cell migration and invasion [89]. A number of other isoquinoline derivatives have also been created and investigated as P2X7R antagonists based on the structure of KN-62, but require further characterisation to be considered as potential therapies [90].

A740003 and A438079

A740003 and A438079 were characterised around the same time and were both investigated in the context of neuropathic pain [91, 92]. A740003 is a cyanoguanidine derivative whilst A438079 is a tetrazole derivative. Both were originally thought to be competitive inhibitors of P2X7R, but have since been further

characterised as allosteric inhibitors [91–93]. Whilst they are different compounds, they act in the same way on P2X7R and have shown identical changes in efficacy under multiple mutation variations of the allosteric binding pocket, indicating that they bind in very similar configurations [94]. Cyanoguanidine and tetrazole derivatives have the highest potency and selectivity for P2X7R in comparison to other P2X and P2Y receptors, with A740003 having an IC_{50} of 40 nM and A438079 having an IC_{50} of 130 nM for the human P2X7R [91, 92, 95].

In disease contexts, A74003 has been evaluated as a potential radioligand for tracing neuroinflammation as demonstrated by synthesis and uptake in an in vivo healthy rat model [96]. A438079 has been shown to reduce inflammation in an in vivo mouse model of salivary gland exocrinopathy [97] and reduce pain signalling in both in vitro and in vivo experiments in a rat model of pathological pain via chronic constriction injury [98]. In the cancer setting, A740003 has been shown to reduce ATP induced ion currents and cancer cell invasion and migration in vitro in the MDA-MB-435s human breast cancer cell line [89]. In vivo it has been shown to reduce tumour size by ~50% in a mouse model of osteosarcoma [53] and has been used in both a xenograft mouse model of neuroblastoma using the human ACN neuroblastoma cell line, where intraperitoneal injection caused an ~40% reduction in tumour mass, as well as in an immunocompetent mouse model using the mouse Nervo2A neuroblastoma cell line, causing an even stronger reduction of ~50% in tumour mass [31]. A438079 has been demonstrated in vitro to inhibit P2X7R mediated ion current, ethidium uptake, calcium influx, cancer cell migration, and matrix degradation/invasion in 4T1 mouse mammary cancer cells [59]. It has also been shown to reduce tumour growth and metastatic progression in vivo in BALB/cj mice implanted with genetically modified 4T1 cells mammary cancer cells expressing luciferase activity [59]. These antagonists highlight how different chemical derivatives can inhibit P2X7R via a similar mechanism and have been used in a range of disease contexts in which P2X7R is involved, with potential to be applied to a range of cancers.

GSK1482160

GSK1482160 is a pyroglutamic acid amide analogue that was synthesised as a potent P2X7R antagonist [99]. It demonstrated an excellent safety profile and potency in in vivo rat models of both chronic joint pain as a model of inflammatory pain and chronic constriction injury as a model of neuropathic pain [99]. Based on these results it was subsequently investigated for safety in a human clinical trial [100]. A pharmacokinetics/pharmacodynamics characterization was conducted in this trial to determine a suitable dose that would have the desired pharmacological profile, with results showing that it was not possible to achieve > 90% inhibition of IL-1 β whilst maintaining a sufficient safety margin. Therefore, it was

discontinued for further development as a therapy for chronic inflammatory pain [100]. Interestingly, GSK1482160 also showed an ability to penetrate into the central nervous system (CNS), making it potentially useful in neurological contexts [14]. This has been utilised to become a potential biomarker of neuroinflammation through radiolabelling. GSK1482160 can be radiolabelled with ¹¹C, to create ¹¹C-GSK1482160, which can subsequently bind the P2X7R to measure expression [101]. Initial characterization of this compound has shown high affinity for the P2X7R and favourable association–disassociation kinetics, highlighting its potential use as a novel radioligand [101]. This highlights the potential uses of pharmacologically synthesised and developed P2X7R antagonists, even if found to not be efficacious in specific disease clinical trials, to still have uses in other contexts whether that be as biomarkers or as therapies in other diseases.

CE-224535

CE-224535 is another P2X7R antagonist that progressed to a Phase IIA human clinical trial to investigate safety and efficacy in patients with inadequately controlled rheumatoid arthritis [102]. It was originally discovered from high-throughput screening and was found to have moderate P2X7R antagonistic activity [103]. It was then subsequently optimized through various chemical processes until it was developed into CE-224535, which showed excellent pharmacokinetics and safety [103]. Although CE-224535 has not been thoroughly investigated in cancer settings, it has been used in other conditions. In the rheumatoid arthritis human trial, whilst it was shown to be well tolerated and have high bioavailability in patients over a 12-week period, it was determined to be no better than placebo for any efficacy outcome variable, including the primary endpoint measure, the American College of Rheumatology 20% (ACR20) response rate at 12 weeks [102]. It was therefore concluded that this agent was not an effective treatment for rheumatoid arthritis [102]. This highlights that whilst antagonists can have high efficacy in *in vitro* and *in vivo* analysis, they may still be ineffective in the human setting. Considering the proven safety and bioavailability of CE-224535, it may still hold promise for use in other contexts such as inflammatory pain or cancer; however, no recent studies have been completed.

JNJ-54175446 and JNJ-55308942

Multiple P2X7R antagonists that can penetrate the CNS have been developed by Janssen Pharmaceuticals including JNJ-47965567, JNJ-42253432, JNJ-54175446, and JNJ-55308942 [104]. These compounds were synthetically developed and optimised sequentially, to create potential clinically viable P2X7R antagonists for CNS diseases. JNJ-47965567 was developed initially and showed high selectivity and

affinity for P2X7R via the allosteric binding pocket [93], as well as moderate efficacy in attenuating inflammation in an *in vivo* rat model of neuropathic pain [105]. These characteristics allowed it to be used to help probe the central role of the P2X7R in CNS pathophysiology [105]. JNJ-42253432 was then subsequently developed and showed significant improvement over JNJ-47965567 in terms of pharmacokinetics, pharmacodynamics, oral bioavailability, and CNS penetration [106]. It was also found to be efficacious at a much lower dose at attenuating BzATP induced IL-1 β release *in vitro* from human blood and monocytes. In contrast, it showed no efficacy in an *in vivo* rat model of neuropathic pain, which when reviewed alongside the results from the discontinued trials for GSK1482160 and CE-224535, raises questions about the utility of P2X7 receptor antagonists as analgesics [100, 102, 106].

Subsequent studies then synthesised JNJ-54175446, which was optimised as a clinical candidate for the treatment of mood disorders [107]. A Phase II randomised control clinical trial using this antagonist as a therapy for major depressive disorder is currently ongoing, based on the rationale of a link between inflammation and stress-related depression [108]. Whilst JNJ-54175446 represented a clear clinical candidate, studies continued to optimise the compound to increase solubility and modulate its bioavailability properties [109]. This led to the development of a second clinical candidate, JNJ-55308942, which demonstrated high solubility, as well as good tolerability margins and *in vivo* safety [109]. A Phase I clinical trial was recently completed (results not published) investigating the safety, tolerability, and pharmacokinetics of JNJ-55308942 in human patients [110].

AZ10606120

AZ10606120 is a P2X7R antagonist that has recently been investigated in a number of different cancer settings as a potential therapy, with pre-clinical data showing no signs of toxicity *in vivo* [45]. Similar to most previously described antagonists, it binds to the P2X7R via an allosteric binding pocket, restricting the receptor to induce structural changes that occur upon ATP binding [111]. AZ10606120 has been used in a number of *in vitro* and *in vivo* cancer settings. It showed reduced BrdU incorporation, indicating inhibition of cell proliferation and drastic reductions in cell migration as measured by wound assay in pancreatic cancer PancTu-1 Luc cells *in vitro* [30]. It also reduced tumour growth rate over time in an *in vivo* mouse model of pancreatic cancer, though there was no difference in endpoint tumour volume [30]. It was also ineffective at blocking metastasis; however, it did significantly reduce tumour fibrosis by reducing production/deposition of collagen [30]. AZ10606120 has also been investigated in mesothelioma, where it inhibited cell proliferation and increased LDH release in a number

of malignant pleural mesothelioma (MPM) cell lines [26]. It also significantly inhibited tumour invasion and growth when administered either subcutaneously or intraperitoneally in an *in vivo* xenograft mouse model of mesothelioma, with an overall ~50% decrease in tumour size post-mortem [26]. In mammary cancer, AZ10606012 was shown *in vitro* to inhibit P2X7R mediated ion current, ethidium uptake, calcium influx, cancer cell migration, and matrix degradation/invasion in 4T1 mouse mammary cancer cells similar to A438079 [59]. It was also shown *in vivo* to reduce tumour growth and increase survival time in BALB/cj mice implanted with genetically modified 4T1 cells mammary cancer cells expressing luciferase activity [59]. AZ10606120 has been used in neuroblastoma to reduce tumour size in an *in vivo* xenograft mouse model of neuroblastoma using the human ACN neuroblastoma cell line, where intraperitoneal injection caused an ~40% reduction in tumour mass [31]. It was also used in an *in vivo* syngeneic mouse model that used mouse Neuro2A cells and resulted in an even greater reduction in tumour size than the xenograft model, indicating that a functioning immune system may function to increase efficacy of AZ10606120 [31]. Similarly, AZ10606120 has been used in an *in vivo* mouse model of melanoma, where it strongly inhibited tumour growth and caused large reduction in VEGF secretion and subsequently a decrease in number of blood vessels [18]. Lastly, AZ10606120 was also shown to reduce cell number *in vitro* in cultures of high-grade glioblastoma derived from patient samples [32]. These studies highlight the efficacy of AZ10606120 mediated P2X7R inhibition at reducing tumour size in multiple different cancer models and demonstrates how the anti-inflammatory and anti-cancer effects caused by P2X7R antagonism can affect completely different cancers by targeting common inflammatory processes.

Natural P2X7R antagonists

Finally, the antagonists previously described are all synthetic compounds specifically developed to target P2X7R. Several naturally occurring compounds have also been shown to antagonise P2X7R with the potential to be therapeutically valuable. This includes compounds such as Rhein, Emodin, Teniposide, and Baicalein which have all been demonstrated to inhibit P2X7R function in different contexts [112–116]. Whilst these compounds all show P2X7R antagonising properties, there has been limited studies completed in the context of cancer, with only Emodin specifically being investigated in breast cancer cells and shown to limit cancer cell invasion [117]. Therefore, whilst these naturally occurring P2X7R antagonists do have potential as cancer therapeutics, a large amount of data in cancer contexts is still required.

Conclusion

P2X7R is highly expressed in several cancers and has various functions, thought to be both important in tumour growth, invasion, and angiogenesis, as well as potentially induce cell death in different cancer contexts. It exerts its pro-tumour effects via various intracellular signalling cascades as well as intercellular changes mediated by various cytokines, chemokines, proteases, and other signalling cascades. Several P2X7R antagonists have been developed and utilised in a range of cancers with mostly anti-tumour effects. Considering the range of processes that P2X7R influences, and its high presence in multiple cancer types, P2X7R antagonism represents a key potential therapy for a variety of cancers that are commonly difficult to effectively treat. Here we summarise the most widely used and developed antagonists of the receptor reported in pre-clinical studies and clinical trials, to highlight the effects and benefits seen with P2X7R inhibition. Results of these studies have demonstrated that P2X7R antagonism is generally well tolerated; however, the major limitation has been translating the efficacy shown *in vitro* and *in vivo* animal studies to human trials. Therefore, future studies are needed to optimise properties such as bioavailability, mode of administration, dosage, and efficacy to maximise the potential of these antagonists. With continued research, optimisation, and testing, P2X7R antagonists could become key therapeutics in multiple cancer settings in need of new therapies.

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Code availability Not applicable.

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Declarations

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