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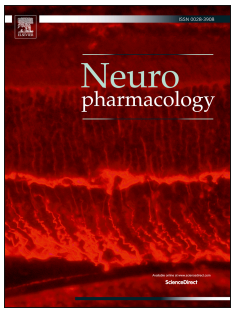

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Ronald Sluyter, Reece A. Sophocleous, Leanne Stokes

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P2X receptors: insights from the study of the domestic dog

Ronald Sluyter[a,b,*], Reece A. Sophocleous[a,b], Leanne Stokes[c]

[a] *Illawarra Health and Medical Research Institute, Wollongong, NSW, 2522, Australia*

[b] *Molecular Horizons and School of Chemistry and Molecular Bioscience, University of Wollongong, Wollongong, NSW, 2522, Australia*

[c] *School of Pharmacy, University of East Anglia, Norwich, Norfolk, NR4 7TJ, UK*

* Corresponding author. Illawarra Health and Medical Research Institute, Wollongong, NSW, 2522, Australia.

E-mail address: rsluyter@uow.edu.au (R Sluyter)

ORCID

Ronald Sluyter 0000-0003-4909-686X

Reece A. Sophocleous 0000-0002-8339-9090

Leanne Stokes 0000-0003-4013-6781

ABSTRACT

Fifty years ago, the late Geoffrey Burnstock described the concept of purinergic nerves and transmission bringing into existence the broader concepts of purinergic signaling including P2X receptors. These receptors are trimeric ligand-gated cation channels activated by extracellular adenosine 5'-triphosphate (ATP). P2X receptors have important roles in health and disease and continue to gain interest as potential therapeutic targets in inflammatory, neurological, cardiovascular and many other disorders including cancer. Current understanding of P2X receptors has largely arisen from the study of these receptors in humans and rodents, but additional insights have been obtained from the study of P2X receptors in the domestic dog, *Canis familiaris*. This review article will briefly introduce purinergic signaling and P2X receptors, before detailing the pharmacological profiles of the two recombinant canine P2X receptors studied to date, P2X7 and P2X4. The article will then describe the current state of knowledge concerning the distribution and function of the P2X receptor family in dogs. The article will also discuss the characterization of single nucleotide polymorphisms in the canine *P2RX7* gene, and contrast this variation to the canine *P2RX4* gene, which is largely conserved between dogs. Finally, this article will outline published examples of the use of dogs to study the pharmacokinetics of P2X7 and P2X3 antagonists, and how they have contributed to the preclinical testing of antagonists to human P2X7, CE-224,535, and human P2X3, Gefapixant (AF-219, MK-7264) and Eliapixant (BAY 1817080), with Gefapixant gaining recent approval for use in the treatment of refractory chronic cough in humans.

Keywords: Purinergic receptor; Purinergic signaling; Ligand-gated ion channel; Extracellular adenosine 5'-triphosphate; Canine; Companion animal

Abbreviations: ADP, adenosine 5'-diphosphate; ATP, adenosine 5'-triphosphate; ATPγS, adenosine-5′-O-(3-thio) triphosphate; BzATP, 2′(3′)-O-(4-benzoylbenzoyl) ATP; EC_{50}, half-maximal effective concentration; IC_{50}, half-maximal inhibitory concentration; LPS,

lipopolysaccharide; MDCK, Madin-Darby canine kidney; α,β-meATP, α,β-methylene ATP; 2meATP, 2-methylthio-ATP; SNP, single nucleotide polymorphism; TNP-ATP, 2,4,6-trinitrophenol-ATP

1. Introduction

In 1972 the late Geoffrey Burnstock described the concepts of purinergic nerves and transmission (Burnstock, 1972), giving rise to the now well-accepted concept of purinergic signaling evident in all mammalian cells and tissues (Burnstock, 2012). In its simplest form purinergic signaling describes the role of nucleotides as extracellular signaling molecules (Burnstock, 1972), but has come to be more fully described as a cell signaling network comprised of P1 receptors activated by extracellular adenosine, and P2 receptors typically activated by extracellular adenosine 5'-triphosphate (ATP) and other nucleotides, regulated by the extracellular metabolism, and release and uptake of nucleosides and nucleotides (Huang, et al., 2021). The cell surface receptors are divided into P1 and P2 receptors (Burnstock, 1978). P1 receptors, more commonly known as adenosine receptors, are G protein-coupled receptors activated by adenosine and comprise four subtypes (A_1, A_{2A}, A_{2B} and A_3) (IJzerman, et al., 2022). P2 receptors are further classified as P2Y and P2X receptors (Burnstock and Kennedy, 1985). P2Y receptors are G protein-coupled receptors activated by various nucleotides, comprising eight subtypes ($P2Y_1$, $P2Y_2$, $P2Y_4$, $P2Y_6$, $P2Y_{11}$, $P2Y_{12}$, $P2Y_{13}$ and $P2Y_{14}$) (Jacobson, et al., 2020). P2X receptors are trimeric ligand-gated ion channels activated by ATP formed from the homomeric or heteromeric assembly of seven P2X subunits (P2X1-P2X7), with the homomeric assembly of P2X6 rarely observed (Illes, et al., 2021).

P2X receptors are widely expressed on most cell types (Burnstock and Knight, 2004) and play important roles in physiology and pathophysiology from the hematopoietic (Sluyter, 2015) and immune (Di Virgilio, et al., 2018) systems, to the nervous, cardiovascular, gastrointestinal, musculoskeletal and renal systems (Burnstock, 2017), as well as cancer (Di Virgilio, et al., 2021). In addition to the natural ligand ATP, P2X receptors can be experimentally activated by a variety of

synthetic ATP analogs (Coddou, et al., 2011, Illes, et al., 2021). This list includes 2′(3′)-O-(4-benzoylbenzoyl) (BzATP), often misdescribed as a specific P2X7 agonist but which can also activate P2X1, P2X2, P2X3 and P2X4 at sub-micromolar concentrations, and P2X5 and P2X6 at micromolar concentrations similar to that of P2X7. While the non-hydrolyzable analog α,β-methylene ATP (α,β-meATP) can activate P2X1, P2X3, P2X4 and P2X5 at sub-micromolar concentrations, and P2X6 at micromolar concentrations, but it is considered a full agonist at P2X1 and P2X3 only. P2X5 can also be fully activated by adenosine-5′-O-(3-thio) triphosphate (ATPγS), with this compound a partial agonist of the remaining P2X receptors. 2-Methylthio-ATP (2MeATP) is a partial agonist of each P2X receptor, with P2X1-P2X3 and P2X5 activated at sub-micromolar concentrations, and P2X4 and P2X7 activated at micromolar concentrations (Coddou, et al., 2011, Illes, et al., 2021). Such pharmacological differences have been useful in identifying native P2X receptors in cells and tissues, but this approach is limited when multiple P2 receptors are present, or where the pharmacological profile of a given P2 receptor in one species differs to the receptor ortholog in another species. This can be largely addressed through use of the many specific P2X receptor antagonists now available (Müller and Namasivayam, 2021).

Most knowledge regarding P2X receptors has been through the study of those in cells and tissues from humans and rodents. However, the domestic dog, *Canis familiaris*, which represents important models of disease and preclinical drug testing (Schulte and Arlt, 2022, Ward and Osenkowski, 2022), and forms an integral part of society as companion, assistance and working animals (Cunningham-Smith and Emery, 2020, MacLean, et al., 2021) has provided novel insights and offers new opportunities to better understand P2X receptors. Notably, dogs provided early physiological insights to purinergic signaling *in vivo*, predominately in relation to its roles in the cardiovascular system (Burnstock and Kennedy, 1986, Chiba and Yang, 2003, Pelleg, et al., 1987), to which Burnstock contributed a small number of studies (De Mey, et al., 1979, Houston, et al., 1987), aligning with his long standing interest in comparative physiology (Burnstock, 1975). This article, which is part of a special issue celebrating 50 years since the publishing of the seminal review of purinergic nerves and

transmission by Burnstock (Burnstock, 1972), will review insights gained from the study of the pharmacology, expression and function of canine P2X receptors, and the use of dogs in the preclinical evaluation of P2X receptor antagonists for potential use in people. This review will largely focus on studies over the past two decades, coinciding with first reports of the sequencing of the canine genome, which included the gene sequences of the seven canine P2X subunits (Kirkness, et al., 2003, Lindblad-Toh, et al., 2005).

2. Recombinant canine P2X receptors

The heterologous expression of recombinant P2X receptors in mammalian cells has greatly advanced the field of purinergic signaling. To date, recombinant canine P2X receptors and their pharmacology have only been described for P2X7 (Table 1) and P2X4 (Table 2).

Recombinant P2X7 was originally cloned and studied from a non-disclosed dog breed (Michel, et al., 2009, Roman, et al., 2009) and then from an English Springer Spaniel (Bartlett, et al., 2017, Liang, et al., 2015, Spildrejorde, et al., 2014a). Another group has investigated a recombinant canine P2X7, but the origins of this receptor was not disclosed (Bhattacharya, et al., 2013, Chrovian, et al., 2018, Letavic, et al., 2017). These studies combined reveal that activation of canine P2X7 results in membrane currents, Ca^{2+} fluxes and organic cation uptake, including $choline^{+}$ and fluorescent dyes, similar to that of human P2X7. The first comparison of canine and human P2X7 revealed that both receptors are stimulated by ATP and BzATP, with similar half-maximal effective concentration (EC_{50}) values between species (Roman, et al., 2009). Unexpectedly, this study reported that BzATP was partial agonist of canine P2X7, opposing comparisons of endogenous human and canine (English Springer Spaniel) P2X7, where ATP was a partial agonist compared to BzATP (Stevenson, et al., 2009). Subsequently, ATP was revealed to be a partial agonist of the recombinant English Springer Spaniel P2X7 compared to BzATP (Spildrejorde, et al., 2014a). Reasons for this discrepancy between the two recombinant canine P2X7 remain unknown. One possible explanation is that the difference in the amino acid residue at position 103 alters BzATP or ATP sensitivity, with a (mutant) leucine

present in the original canine P2X7 and a (wildtype) phenylalanine present in the English Springer Spaniel P2X7 (Roman, et al., 2009, Spildrejorde, et al., 2014a). However, this residue forms part of the allosteric drug binding pocket in the extracellular loop of giant panda and human P2X7 (Allsopp, et al., 2018, Bin Dayel, et al., 2019, Karasawa, et al., 2017) reducing the likelihood that this residue alters agonist sensitivity. Recombinant canine P2X7 can also be partially activated by ATPγS but not by adenosine 5'-diphosphate (ADP), uridine 5'-triphosphate or αβ-meATP (Spildrejorde, et al., 2014a).

Numerous studies have directly compared various P2X7 antagonists, including BBG (Jiang, et al., 2000), KN-62 (Gargett and Wiley, 1997), A438079 (McGaraughty, et al., 2007) and AZ11645373 (Stokes, et al., 2006), against recombinant canine and human P2X7, which share remarkably similar antagonist profiles (Table 1). Half-maximal inhibitory concentration (IC_{50}) values are similar for a large number of selective P2X7 antagonists, as well as the less selective P2X7 antagonists BBG and KN-62 (Bhattacharya, et al., 2013, Chrovian, et al., 2018, Letavic, et al., 2017, Michel, et al., 2009, Roman, et al., 2009). Consistent with earlier studies, which reported the inhibition of human and murine P2X7 by amiloride analogs (Chessell, et al., 1998, Nuttle and Dubyak, 1994, Wiley, et al., 1990), 6-furopyridine hexamethylene amiloride was recently shown to be a nonselective P2X7 antagonist, impairing recombinant English Springer Spaniel and human P2X7 with similar IC_{50} values (Cuthbertson, et al., 2022). BBG, KN-62, AZ10606120 and AZ11645373 can also impair recombinant English Springer Spaniel P2X7 with similar IC_{50} values (Spildrejorde, et al., 2014a) to that described elsewhere (Table 1) but other P2X7 antagonists are yet to be tested against the receptor from English Springer Spaniels. Probenecid can also impair recombinant English Springer Spaniel P2X7 but at a relatively high IC_{50} value (Bartlett, et al., 2017) and similar to that of human P2X7 (Bhaskaracharya, et al., 2014). It remains unknown if this drug, used occasionally to reduce renal excretion to maintain antibiotic concentrations in dogs (Barza, et al., 1975), impairs P2X7 when used in these animals. Finally, Mg^{2+} and *N*-[4-(1-methylethyl)phenyl]-4-[2-(3-pyridinyl)ethyl]-1-phthalazinamine can near-completely impair P2X7 in erythrocytes from English Springer Spaniels at

10 mM (Stevenson, et al., 2009) and 10 μM (Shemon, et al., 2008), respectively, but these compounds are yet to be studied with recombinant canine P2X7.

The study of recombinant canine P2X4, which was generated based on the known canine *P2RX4* gene sequence, has been reported in one study (Sophocleous, et al., 2020a). Like canine P2X7, canine recombinant P2X4 displays ATP-induced membrane currents and Ca^{2+} fluxes similar to that of human P2X4 and with comparable EC_{50} values between species (Sophocleous, et al., 2020a). However, studies to date have been unable to demonstrate that this receptor can mediate ATP-induced dye uptake (Sophocleous and Sluyter, *unpublished results*) as originally demonstrated for rat P2X4 (Khakh, et al., 1999a, Virginio, et al., 1999) and more recently human P2X4 (Dhuna, et al., 2019). One possible explanation for this difference is that only a green fluorescent protein tagged canine P2X4 has been studied to date, suggesting that this fusion protein is not able to fully dilate to allow the passage of organic cations through its pore. Recombinant canine P2X4 can also be partially activated by BzATP but not ADP (Sophocleous, et al., 2020a). This study showed that commercial preparations of ADP can activate canine and human P2X4, but also that this effect was mediated by contaminating amounts of ATP, as previously observed in studies of other P2 receptors (Mahaut-Smith, et al., 2000, Micklewright, et al., 2018). It should also be noted that the EC_{50} value for BzATP at canine P2X4 is two-fold less than that observed for human P2X4 (Sophocleous, et al., 2020a), which may reflect differences in amino acid residues with sequence identity between these receptors at 90%. Consistent with earlier reports that the veterinary anti-parasitic agent ivermectin is a positive modulator of human and rodent P2X4 activation (Khakh, et al., 1999b, Priel and Silberberg, 2004), this compound can also increase canine P2X4 activation with a similar EC_{50} value to that for ivermectin against human P2X4 (Sophocleous, et al., 2020a). Whether P2X4 contributes to ivermectin-mediated neurotoxicity observed in some dog breeds remains unknown (Sophocleous, et al., 2022). Finally, it should be noted that ivermectin is a positive modulator of human P2X7 but has limited effect against murine or rat P2X7 (Nörenberg, et al., 2012), while its action against canine P2X7 has not been reported.

To date only one study has directly compared P2X4 antagonists, including 2,4,6-trinitrophenol-ATP (TNP-ATP) (Virginio, et al., 1998) and 5-BDBD (Balázs, et al., 2013), against recombinant canine and human P2X4 (Sophocleous, et al., 2020a). Recombinant canine P2X4 displays a similar antagonist profile to that of recombinant human P2X4, with similar IC_{50} values for the non-selective P2X antagonist, TNP-ATP and the selective P2X4 antagonist 5-BDBD (Sophocleous, et al., 2020a). Likewise, similar IC_{50} values between canine and human P2X4 were observed for duloxetine and paroxetine (Sophocleous, et al., 2020a), both of which are selective serotonin reuptake inhibitors used to treat various mood disorders in people (Bourin, et al., 2001, Müller, et al., 2008), but also inhibit human and rodent P2X4 (Nagata, et al., 2009, Yamashita, et al., 2016) and P2X7 (Dao-Ung, et al., 2015, Wang, et al., 2016). Reports of accidental exposure to duloxetine and paroxetine in dogs indicate that these drugs can be safely used in the companion animals (Fitzgerald and Bronstein, 2013). Thus, these drugs may have potential uses in inhibiting P2X4 (or P2X7) in canine disorders where these receptors may play a role, based on studies in humans and rodents, such as neuropathic or inflammatory pain (Inoue and Tsuda, 2021, Sophocleous and Sluyter, 2023).

Collectively, the above studies indicate that canine P2X7 and P2X4 share very similar pharmacological profiles to human P2X7 and P2X4, suggesting that dogs may serve as appropriate models to test P2X7 and P2X4 antagonists developed for use in people. Conversely, P2X7 and P2X4 antagonists that can be used safely to treat human disorders may also have therapeutic benefits against similar disorders in dogs. It remains to be determined if other canine P2X receptors share similar antagonist profiles to the corresponding human P2X receptors, but the recent development of a number of P2X antagonists for use in humans (Dane, et al., 2022) highlights the potential application of such compounds in dogs and other companion animals.

3. Endogenous canine P2X receptors

The cardiovascular effects of ATP administration into dogs have been long appreciated (Burnstock and Kennedy, 1986, Chiba and Yang, 2003, Pelleg, et al., 1987) and provided the first evidence of

P2X receptors, as well as P2Y and adenosine receptors, in dogs. Although it is beyond the scope of this article to review these seminal studies, except for two reports where the P2X receptors involved were identified (Xu, et al., 2005, Zhou, et al., 2010), the *ex vivo* study of cells and tissues has helped determine the distribution and function of P2X receptors in dogs (Tables 3 and 4). Nevertheless, the distribution and function of most P2X receptors on many different cell types (including those listed Tables 3 and 4) remain to be explored and established.

Arguably, the first direct evidence of a P2X receptor in dog cells was established by Parker and Snow (Parker and Snow, 1972, Parker, et al., 1977), and subsequently by others (Elford, 1975, Romualdez, et al., 1976), who observed that addition of extracellular ATP to canine erythrocytes could induce Na^+ and K^+ fluxes in these cells, a process that could lead to membrane hyperpolarisation (Parker, et al., 1977). Three decades later, it was shown directly that canine erythrocytes express P2X7 (Sluyter, et al., 2007a) and that activation of this receptor could lead to cation fluxes (Shemon, et al., 2008, Sluyter, et al., 2007a, Stevenson, et al., 2009), as well as to phosphatidylserine exposure and hemolysis (Faulks, et al., 2016, Sluyter, et al., 2007a). P2X7 is also present on canine T and B cells and monocytes (Jalilian, et al., 2012a, Sluyter, et al., 2007a, Spildrejorde, et al., 2014a, Stevenson, et al., 2009). Notably, the functional activity of P2X7 in canine erythrocytes is 40 times that of human erythrocyte P2X7 (Romualdez, et al., 1976, Sluyter, et al., 2007a), despite somewhat similar P2X7 activity on canine lymphocytes and monocytes (Jalilian, et al., 2012a, Sluyter, et al., 2007a, Stevenson, et al., 2009). Corresponding to the increased P2X7 channel activity on canine erythrocytes compared to human erythrocytes, ATP-induced phosphatidylserine exposure on erythrocytes is six times greater on those from dogs than humans, while 24 hour incubation with ATP does not induce hemolysis in human erythrocytes despite doing so in canine erythrocytes (Faulks, et al., 2016, Sluyter, et al., 2007a, Sluyter, et al., 2007b, Sophocleous, et al., 2015). The difference in P2X7 activity between canine and human erythrocytes is most likely due to greater P2X7 expression in the former compared to the latter cells, as indicated by semi-quantitative immunoblotting studies (Sluyter, et al.,

2007a). However, the relative amount of P2X7 expression in erythrocytes from both species is yet to be compared using more quantitative approaches.

mRNA encoding transcripts for P2X1 P2X4 and P2X7 are present in canine DH82 macrophages, with functional expression of P2X4 (and $P2Y_2$) detected in these cells (Sophocleous, et al., 2020b). P2X7 protein is present in lipopolysaccharide (LPS)-primed canine monocytes (Jalilian, et al., 2012a). Notably, P2X7 activation can induce the release of interleukin-1β from LPS-primed canine monocytes (Jalilian, et al., 2012a) and whole blood (Bartlett, et al., 2017, Roman, et al., 2009, Spildrejorde, et al., 2014b) indicating that many of the pathways downstream of P2X7 and other P2X receptors observed in humans and rodents most likely occur in dogs. Finally, more recent studies of canine erythrocytes and leukocytes have revealed that the presence of P2X7 extends beyond the initial reports of P2X7 in these cells from English Springer Spaniels (Shemon, et al., 2008, Sluyter, et al., 2007a, Stevenson, et al., 2009) to other breeds (Bartlett, et al., 2017, Faulks, et al., 2016, Jalilian, et al., 2012a, Roman, et al., 2009, Spildrejorde, et al., 2014a, Spildrejorde, et al., 2014b).

Within the nervous system, mRNA encoding transcripts for either P2X2, P2X3, P2X4, P2X5 or P2X7 are present in the brain (Lee, et al., 2005), with presence of protein only shown for P2X7 (Truvé, et al., 2016). The cellular identity of P2X receptors within the brain remains to be determined but are likely to be present on various cell types, including neurons, microglia and astrocytes, based on studies in humans and rodents (Burnstock, 2015). P2X7 protein is also present in various forms of canine brain tumours (Truvé, et al., 2016), the only study to date reporting P2X receptor expression in dog cancers. P2X7 protein has also been reported on neurons from myenteric plexus of the ileum and colon (SchÄfer, et al., 2018), but the presence of this receptor in neurons remains questionable (Illes, et al., 2017, Miras-Portugal, et al., 2017). Of note, preliminary data suggests that P2X7 is not present in the enteric nervous system but rather in macrophages and glia associated with this tissue in mice (Jooss, et al., 2022a, Jooss, et al., 2022b) suggesting that P2X7 may also be associated with these non-neuronal cells in the intestinal tract of dogs.

As stated above the effect of ATP on the cardiovascular system has long been appreciated. In particular, it is well known that administration of ATP stimulates the vagal reflex to slow the heart rate of dogs (Belhassen and Pelleg, 1984). Furthermore, another study demonstrated that the P2X receptor antagonist TNP-ATP, but not the P2X1 and P2X3 antagonist diinosine pentaphosphate, completely blocked the vagal reflex stimulated by intracranial ATP in dogs (Xu, et al., 2005). The authors attributed these effects of TNP-ATP to the inhibition of P2X2/P2X3, but this compound can impair all P2X receptors other than P2X7 (Illes, et al., 2021). Thus, analysis of which P2X receptors are present in the vagal afferent nerve terminals of dogs is required to support these findings. P2X3 mRNA and protein has been detected in carotid body of dogs, with its knockdown shown to reduce blood pressure (Xue, et al., 2021), as first shown in rats (Pijacka, et al., 2016). To the best of our knowledge, this provides the first report of the targeting a specific P2X receptor in dogs to alter physiological or pathophysiological processes and supports the therapeutic potential of targeting these receptors in veterinary medicine. Finally, P2X4 is thought play a role in heart failure, with the P2X agonist MRS2339 shown to improve heart function in dogs subject to rapid pacing-induced heart failure (Zhou, et al., 2010). These effects were thought to be mediated by P2X4 activation, as over-expression of cardiac myocyte P2X4 in a mouse model of heart failure had similar effects to MRS2339 in dogs (Zhou, et al., 2010). The P2X receptor specificity of MRS2339 remains obscure, as recognised by others (Coddou, et al., 2011) and with the developers of MRS2339 ascribing its activity towards P2X4 on the basis of P2X4 expression in cardiac myocytes but not ruling out the possibility that MRS2339 targets P2X4-containing heteromeric receptors (Kumar, et al., 2010). Nevertheless, P2X4 remains a potential drug target in cardiovascular disease in humans (Bragança and Correia-de-Sá, 2020) and by extension in dogs.

In addition to the above studies of P2X receptors in the canine myenteric plexus, which also forms part of the gastrointestinal system, mRNA encoding transcripts for P2X2, P2X3, P2X4 or P2X5 (but not P2X7) have been detected in the circular and/or longitudinal muscle layers of the colon from dogs, as well as from myocytes of these muscle layers (Lee, et al., 2005). ATP stimulation of these

tissues resulted in circular and longitudinal muscle contraction, which appeared to be mediated predominately by activation of P2X2 and P2X4, or P2X3, respectively (Lee, et al., 2005). Parallel conclusions were made for ATP-induced inward currents in myocytes from these muscle layers (Lee, et al., 2005).

The expression of P2X receptors is not limited to gastrointestinal muscles, with P2X1 and P2X4 present in gracilis (adductor) muscle (Delorey, et al., 2012). These receptors were thought to decrease vascular conductance, the ease with which blood flows through vascular bed at a given pressure difference (Joyce, et al., 2019), in response to α,β-meATP infusion (Delorey, et al., 2012). Although α,β-meATP is a known agonist of P2X1 and P2X4 (as well as P2X3), this nucleotide displays species specificity against P2X4, being a potent agonist of human but not rodent P2X4 (Illes, et al., 2021). Thus, future studies should determine which canine P2X receptors are activated by α,β-meATP.

The study of P2X receptors in the renal system of dogs, is confined to reports of P2X7 expression and activity in Madin-Darby canine kidney (MDCK) cells. mRNA encoding P2X7 transcripts and P2X7 protein has been detected in MDCK cells (Jalilian, et al., 2012b, Rodat-Despoix, et al., 2013, Turner, et al., 2007) and cysts derived from these cells (Turner, et al., 2007). P2X7 activation in MDCK cells results in inward currents and Ca^{2+} fluxes (Rodat-Despoix, et al., 2013), but relatively low amounts of dye uptake (Jalilian, et al., 2012b). Moreover, P2X7 activation promoted the epithelial to mesenchymal transition of MDCK cells to myofibroblasts in a mitogen-activated protein kinase 1-dependent fashion (Zuccarini, et al., 2017). In contrast, P2X7 activation appears to have no role in the growth of MDCK cell-derived cysts, with autocrine ATP effects largely attributed to P2Y receptors (Turner, et al., 2007).

Finally, functional studies, based on the agonist profile, indicate a role for P2X7 activation in the proliferation of canine adipose-derived stem cells (Roszek, et al., 2017). However direct evidence for P2X7 expression or the use of P2X7 antagonists in this study was lacking. In this regard, murine adipose-derived stem cells are reported to express functional P2X receptors but not P2X7 (Forostyak,

et al., 2016). These contrasting findings may reflect differences between species and/or cell culture conditions, or indicate the presence of an alternate P2X receptor in these canine cells.

4. Polymorphic variants of canine P2X receptors

The study of recombinant and native canine P2X7 and P2X4 has allowed the investigation of naturally occurring single nucleotide polymorphisms (SNPs) in dogs. Genetic sequence analyses of over 100 different dogs (and multiple breeds) have revealed that the canine *P2RX7* gene encodes at least five non-synonymous SNPs: F103L, R270C, R365Q, L440F and P452S (Sophocleous, et al., 2020c, Spildrejorde, et al., 2014a) (Fig. 1). Molecular modeling of the canine P2X7 subunit reveals the sites of these SNPs (Fig. 2), but the direct impacts of these SNPs on P2X7 structure or structurally related receptor functions are yet to be investigated experimentally.

Of the five non-synonymous, R270C in homozygous dosage results in a near-complete loss of P2X7 activity (Spildrejorde, et al., 2014a) and appears to be restricted to dogs of Cocker Spaniel pedigree (Sophocleous, et al., 2020c, Spildrejorde, et al., 2014a). Molecular modeling indicates that R270C resides in close proximity (<5 Å) to a disulfide bridge (C260-C269) in the extracellular loop (Fig 2C), potentially disrupting receptor conformation and leading to a loss of activity. Notably, a SNP encoding a change in the equivalent site in human P2X7 (R270H) is associated with reduced pain sensitivity in people (Sorge, et al., 2012), however it remains to be determined if Cocker Spaniels carrying the SNP for R270C also have reduced pain sensitivity.

F103L and R365Q remain possible partial loss-of-function mutations (Spildrejorde, et al., 2014a), but this needs verification. F103L has been associated with increased glioma susceptibility in dogs (Truvé, et al., 2016), as well as with brachycephalic dog breeds, especially those of Bulldog ancestry (Sophocleous, et al., 2020c), which are known to be more susceptible to glioma (Song, et al., 2013). Collectively, this supports a role for P2X7 in glioma development as indicated for human P2X7 (Bergamin, et al., 2019, Kan, et al., 2020, Matyśniak, et al., 2022). However, reduced P2X7 activity is typically associated with reduced glioma progression, so it remains unclear how the potential partial

loss-of-function mutation F103L promotes glioma development in dogs. Moreover, F103 (Fig. 2B), as discussed above, is a key residue within the allosteric drug binding pocket in the extracellular loop. Canine P2X7 containing this SNP is inhibited by numerous P2X7 antagonists (Bartlett, et al., 2017, Cuthbertson, et al., 2022, Spildrejorde, et al., 2014a), but whether this SNP alters antagonist sensitivity remains to be determined. R365Q appears unique to dogs of Labrador Retriever ancestry (Sophocleous, et al., 2020c) but the significance of this finding remains to be investigated. R365Q resides within the region of the C-terminus (Fig. 2D) that contains the so-called C-cys anchor, which facilitates cell surface expression and prevents desensitization of P2X7 due to palmitoylation of cysteine residues in this region (Gonnord, et al., 2009, McCarthy, et al., 2019). Whether, R365Q alters the propensity of these cysteine residues to be palmitoylated and to subsequently reduce receptor cell surface expression or promote receptor desensitization to partially reduce P2X7 activity remains to be investigated.

L440F remains a possible partial gain-of-function mutation but was only observed in the recombinant English Springer Spaniel P2X7 (Spildrejorde, et al., 2014a) and does not appear in databases (NCBI dbSNP, EMBL-EBI European Variation Archive and iDOG), so may represent a cloning artefact. P452S is over-represented in non-brachycephalic dog breeds (Sophocleous, et al., 2020c), but does not appear to impact P2X7 activity (Spildrejorde, et al., 2014a). A SNP encoding a change in the equivalent site in murine P2X7 (P451L) to P452S in dogs, results in reduced function in P2X7 (Adriouch, et al., 2002, Young, et al., 2006) but not in human P2X7 (Adamczyk, et al., 2015). Collectively, these studies support the notion that additional amino acid residues neighbouring the P451 amino acid residue in mice contribute to its overall effect on P2X7 activity (Sluyter and Stokes, 2014). Both L440F and P452S are located within the C-terminus (Fig. 2E) in the structure forming the so-called cytoplasmic ballast that contains guanosine nucleotide and Zn^{+} binding sites (McCarthy, et al., 2019), which may regulate P2X7 signaling, and P2X7 processing and trafficking, respectively (Mansoor, 2022). However, direct interaction of these molecules with the amino acid residues at the

equivalent positions to L440F and P452S has not been reported (Mansoor, 2022, McCarthy, et al., 2019).

In contrast to the canine *P2RX7* gene, no non-synonymous SNPs were observed in the canine *P2RX4* gene from genetic sequence analyses of over 100 different dogs (Sophocleous, et al., 2020a), many of which were the same as those from the study of canine *P2RX7* (Sophocleous, et al., 2020c, Spildrejorde, et al., 2014a). This finding is consistent with the human *P2RX4* gene containing less non-synonymous SNPs (Stokes, et al., 2011) than the human *P2RX7* gene (Stokes, et al., 2010). However, it should be noted that three non-synonymous SNPs in the canine *P2RX4* gene, A9D, R231C and L345V, have been reported in databases (NCBI dbSNP, EMBL-EBI European Variation Archive and iDOG), but many more non-synonymous SNPs are listed in the canine *P2RX7* gene in such databases. This high amount of sequence similarity in the *P2RX4* gene between dogs suggests the canine P2X4 remains a druggable target in most if not all breeds. Finally, in addition to the association of the canine *P2RX7* gene mutation F103L with glioma susceptibility discussed above, a widespread mutation in the *P2RY12* gene encoding the platelet $P2Y_{12}$ receptor, in Greater Swiss Mountain dogs, results in post-operative hemorrhage in this breed (Boudreaux and Martin, 2011, Flores, et al., 2017). This further highlights the potential physiological and pathophysiological importance of SNPs and mutations in P2 receptors in dogs.

5. Pharmacokinetics and safety of P2X receptor antagonists in dogs

Dogs have served as valuable animal models to test the pharmacokinetics and safety of numerous compounds (Fleischer, et al., 2008). However, published reports of such studies with P2X drugs in dogs are limited, with all but two relating to the preclinical testing of P2X7 antagonists (Table 5). Of these, the P2X7 antagonist CE-224,535 showed excellent pharmacokinetics and safety in dogs (Duplantier, et al., 2011), which led to its clinical testing in patients with rheumatoid arthritis where it demonstrated acceptable toxicity and tolerability but (unfortunately) limited efficacy (Stock, et al., 2012). Several P2X7 antagonists, developed by Janssen, have also been studied in dogs,

demonstrating acceptable pharmacokinetics and safety (Chrovian, et al., 2018, Letavic, et al., 2017, Swanson, et al., 2016, Ziff, et al., 2016). Of these compounds, JNJ-54175446, which was shown to penetrate the blood-brain barrier of dogs (Letavic, et al., 2017), has since undergone clinical testing in healthy subjects (Recourt, et al., 2020, Timmers, et al., 2018). Results from these studies revealed that JNJ-54175446 was well-tolerated and could penetrate the blood-brain barrier of people, indicating its potential use in the treatment of mood or other neurological disorders.

Dogs have also been used in the preclinical testing of P2X3 antagonists (Table 5). Gefapixant (AF-219, MK-7264), which impairs both P2X2 and P2X2/P2X3 (Richards, et al., 2019), is approved for the treatment of refractory chronic cough or unexplained chronic cough (Markham, 2022). Although details concerning the testing of Gefapixant in dogs have not been published, unpublished findings in the first clinical trial using this drug reported that Gefapixant at high doses could precipitate in the kidneys of dogs, and at higher doses could lead to nausea and reduced food consumption indicating that the use of Gefapixant in people with poor renal function may be limited (Abdulqawi, et al., 2015). The approval of Gefapixant for use in people has supported the clinical testing of another P2X3 antagonist, Eliapixant (BAY 1817080) in healthy subjects (Friedrich, et al., 2022, Klein, et al., 2022) and leading to its approval for testing in refractory chronic cough (Morice, et al., 2021). Another study has reported the testing of first- and second-generation P2X3 antagonists (MK-3901 and compound 21, respectively) in dogs (Ginnetti, et al., 2018), but these compounds do not appear to have progressed to clinical testing.

6. Conclusions

Since the description of purinergic nerves and purinergic transmission by the late Geoffrey Burnstock 50 years ago, purinergic signaling has become a widely accepted concept leading to the first approval of a P2X antagonist, Gefapixant, for use in people in refractory chronic cough. Following on from pioneering studies on the effects of ATP in the cardiovasculature of dogs, recombinant P2X4 and P2X7 have been characterized, with the functional expression of at least P2X1, P2X2, P2X3, P2X4

and P2X7 established in dogs. Preclinical testing of P2X antagonists has led to the testing of several P2X antagonists in dogs including Gefapixant. Collectively, the study of dogs has provided additional insights into the role of P2X receptors in mammalian physiology and pathophysiology. To gain further insights from dogs, future studies should characterize recombinant versions of the remaining canine P2X receptors. In addition, these receptors along with that of P2X4 and P2X7 can be used to study the structure-function relationships of these receptors and any discovered isoforms, as well as canine heteromeric P2X receptors. Moreover, as a result of breed development and the unintended consequence of disease gene selection and ensuing breed-specific disorders, many of which parallel human disorders (Parker, et al., 2010), further studies are required to identify and characterise potential SNPs or other forms of variation within the genes coding for canine P2X receptors to better understand the role of these receptors in health and disease. In addition, further studies examining in detail the distribution and function of P2X receptors in dogs are required to advance the health and well-being of both dogs and humans.

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Author contributions

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Figure legends

Fig. 1. Polymorphic variants of canine P2X7 and their association with phenotype or breed. Protein alignment of sequences from canine or human P2X7 illustrating the relative position of five non-synonymous SNPs (bold and underline) reported in canine P2X7 (canine P2X7 numbering). R270C in Cocker Spaniels is associated with either P452 or 452S, and R365Q in Labrador Retrievers is associated with either F103 or 103L and/or P452 or 452S (Sophocleous, et al., 2020c). A non-synonymous SNP in human P2X7, commonly reported as R270H (bold and shaded), corresponds to the R270C SNP in the equivalent site of canine P2X7. Sequences from: canine, National Center for Biotechnology Information (NCBI) (NM_001113456.1); canine, GlaxoSmithKline (GSK) from a non-closed breed (Roman, et al., 2009); canine, University of Wollongong (UOW) from an English Springer Spaniel (Spildrejorde, et al., 2014a); canine, brachycephalic and canine, non-brachycephalic (Sophocleous, et al., 2020c); canine, Cocker Spaniel (Sophocleous, et al., 2020c, Spildrejorde, et al., 2014a); canine, Labrador Retriever (Sophocleous, et al., 2020c, Spildrejorde, et al., 2014a); and human, NCBI (Y09561.1).

Fig. 2. Molecular model of the canine P2X7 subunit. (A) The full length canine P2X7 subunit (NCBI) was modelled with the I-TASSER protein structure prediction suite (Yang and Zhang, 2015, Zheng, et al., 2021) using the cryo-electron microscopy structure of rat P2X7, PDB ID: 6u9v (McCarthy, et al., 2019) as a template. The model returning the highest C-score (0.61) was selected, with an estimated TM-score of 0.80 ± 0.09 and root mean square deviation of 6.4 ± 3.9 Å. Sections of the canine P2X7 subunit are colored as depicted by the dolphin-like structure (inset) of the P2X subunit (Kawate, et al., 2009), including the head (blue), upper body (violet), right (magenta) and left (yellow) flippers, dorsal fin (red), lower body (grey), transmembrane domain-spanning fluke (green), and the cytosolic region (not shown on inset; white). Boxed areas indicate regions containing polymorphic amino acid residues. (B-E) Close-up images of boxed areas, with polymorphic amino acid residues (B) F103L (PHE103), (C) R270C (ARG270), (D) R365Q (ARG365), and (E) L440F (LEU440) and

P452S (PRO452) shown as green ball and stick structures. Amino acid residues within 5 Å of the residue of interest are also labeled. Images were produced using Mol* (Sehnal, et al., 2021).

Table 1
Pharmacological profile of recombinant P2X7 receptors

Compound	Canine P2X7	Human P2X7
Agonists[1]	*pEC50*	*pEC50*
ATP	2.5	2.6
BzATP[2]	3.3	3.4
ATP[3]	3.6	ND[4]
BzATP	4.9	ND
ATPγS[3]	3.4	ND
Antagonists[5]	*pIC50*	*pIC50*
A438079[6]	5.4	6.0
A804598[6]	7.5	7.7
AZ10606120	7.2	8.6
AZ11645373	7.4	7.5
BBG	7.3	6.5
6-FPHMA	5.9	5.8
GW791343	6.7	7.0
GSK1271360	7.3	8.0
GSK314181	7.3	8.4
GSK361390	7.4	8.1
JNJ-47965567[6]	8.5	8.3
JNJ-54175446[6]	7.9	8.8
JNJ-55308942[6]	7.7	8.0
KN62	8.0	7.6
Probenecid[7]	3.8	3.7

[1]Values for ATP and BzATP from (Roman, et al., 2009) and for ATP, BzATP and ATPγS from (Spildrejorde, et al., 2014a).
[2]Partial agonist compared to ATP.
[3]Partial agonist compared to BzATP.
[4]ND, not determined.
[5]Values from (Roman, et al., 2009) except A438079, A804598 and JNJ-47965567 from (Bhattacharya, et al., 2013), 6-furopyridine hexamethylene amiloride (6-FPHMA) from (Cuthbertson, et al., 2022), JNJ-54175446 from (Letavic, et al., 2017), JNJ-55308942 from (Bhattacharya, et al., 2018), AZ11645373 from (Michel, et al., 2009) and probenecid from (Bartlett, et al., 2017, Bhaskaracharya, et al., 2014).
[6]pIC_{50} determined using BzATP (not ATP) as agonist.
[7]Respective pIC_{50} for canine P2X7 compared to human P2X7 not obtained from same study.

Table 2
Pharmacological profile of a recombinant P2X4 receptor

Compound	Canine P2X4	Human P2X4
Agonists[1]	*pEC$_{50}$*	*pEC$_{50}$*
ATP[2]	6.6	6.7
BzATP	3.4	5.0
Antagonists[1]	*pIC$_{50}$*	*pIC$_{50}$*
BX 430	5.1	5.7
5-BDBD	5.2	5.3
Duloxetine	4.8	4.8
Paroxetine	4.9	4.1
TNP-ATP	5.1	5.4

[1]Values from (Sophocleous, et al., 2020a).
[2]ATP responses of canine and human P2X4 potentiated by ivermectin by 111% and 137%, respectively (Sophocleous, et al., 2020a).

Table 3
mRNA and protein expression of endogenous canine P2X receptors

Cell or tissue	P2X receptor(s)	mRNA	Protein	Reference(s)
Haematopoietic system				
Erythrocytes	P2X7		+	(Sluyter, et al., 2007a)
Monocytes (LPS-primed)	P2X7	+	+	(Jalilian, et al., 2012a)
Macrophages (DH82 cells)	P2X1, P2X4, P2X7	+	+ (P2X4)	(Sophocleous, et al., 2020b)
Nervous system				
Brain	P2X2, P2X3, P2X4, P2X5, P2X7	+		(Lee, et al., 2005)
Brain (cerebrum)	P2X7	+	+	(Truvé, et al., 2016)
Brain tumour (astrocytoma, glioblastoma, oligoastrocytoma or oligodendroglioma)	P2X7	+	+	(Truvé, et al., 2016)
Carotid body	P2X3	+	+	(Xue, et al., 2021)
Neurons (ileum myenteric plexus)	P2X7		+	(SchÄfer, et al., 2018)
Neurons (colon myenteric plexus)	P2X7		+	(SchÄfer, et al., 2018)
Cardiovascular system	P2X1, P2X4		+	(Delorey, et al., 2012)
Muscle (gracilis) arteries	P2X3	+	+	(Xue, et al., 2021)
Carotid body	P2X3	+	+	(Xue, et al., 2021)
Gastrointestinal system				
Muscle (gracilis)	P2X1, P2X4		+	(Delorey, et al., 2012)
Muscle (circular colon)	P2X2, P2X3, P2X4	+		(Lee, et al., 2005)
Muscle (longitudinal colon)	P2X2, P2X3, P2X4, P2X5	+		(Lee, et al., 2005)
Myocytes (circular colon)	P2X2, P2X3, P2X4	+		(Lee, et al., 2005)
Myocytes (longitudinal colon)	P2X3, P2X5	+		(Lee, et al., 2005)
Renal system				
Kidney cells (MDCK cells)	P2X7	+	+	(Jalilian, et al., 2012b, Rodat-Despoix, et al., 2013, Turner, et al., 2007)
Renal cysts (MDCK cells)	P2X7	+		(Turner, et al., 2007)

Table 4
Functional expression of endogenous canine P2X receptors

Cell or tissue	P2X receptor(s)	Assay	Agonist(s)	Antagonist(s)	Reference(s)
Haematopoietic system					
Erythrocytes	P2X7	$^{22}Na^{+}$, $^{24}Na^{+}$, $^{42}K^{+}$ or $^{86}Rb^{+}$ fluxes	BzATP > ATP > 2meSATP > ATPγS	Oxidized ATP, BBG, KN-62, Compound P[1], Mg^{2+}	(Elford, 1975, Parker and Snow, 1972, Parker, et al., 1977, Romualdez, et al., 1976, Shemon, et al., 2008, Sluyter, et al., 2007a, Stevenson, et al., 2009)
Erythrocytes	P2X7	[*Methyl*-^{14}C]choline^{+} uptake	ATP	KN-62, Mg^{2+}	(Sluyter, et al., 2007a, Stevenson, et al., 2009)
Erythrocytes	P2X7	Membrane hyperpolarisation	ATP		(Parker, et al., 1977)
Erythrocytes	P2X7	PS exposure and hemolysis	BzATP > ATP	AZ10606120	(Faulks, et al., 2016, Sluyter, et al., 2007a)
Lymphocytes (B and T cells)	P2X7	Ethidium^{+} or YO-PRO-1^{2+} uptake	BzATP > ATP	KN-62	(Jalilian, et al., 2012a, Stevenson, et al., 2009)
Monocytes	P2X7	Ethidium^{+} or YO-PRO-1^{2+} uptake	BzATP > ATP	KN-62, A438079	(Jalilian, et al., 2012a, Sluyter, et al., 2007a, Spildrejorde, et al., 2014a, Stevenson, et al., 2009)
Monocytes (LPS-primed)	P2X7	Interleukin-1β release	ATP	A438079	(Jalilian, et al., 2012a)
Blood (LPS-primed)	P2X7	Interleukin-1β release	ATP	A438079, AZ10606120, GSK314181, GSK361390, KN-62, probenecid	(Bartlett, et al., 2017, Roman, et al., 2009, Spildrejorde, et al., 2014b)
Macrophages (DH82 cells)	P2X4	Ca^{+} influx	ATP[2]	5-BDBD, paroxetine TNP-ATP	(Sophocleous, et al., 2020b)

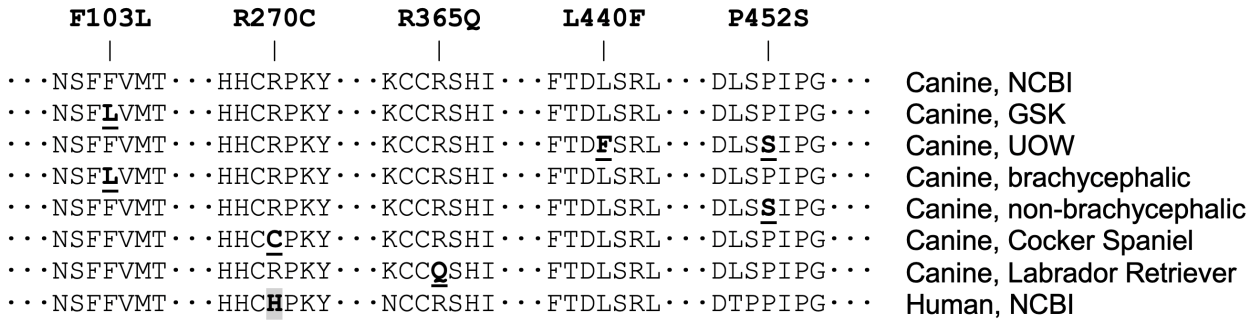
Cardiovascular system					
Muscle (gracilis)	P2X1, P2X4	Reduced vascular conductance (*in vivo*)	α,β-meATP[3]		(Delorey, et al., 2012)
Vagal sensory nerve	P2X2/P2X3	Reduced vagal reflex (*in vivo*)	ATP	TNP-ATP	(Xu, et al., 2005)
Carotid body	P2X3	Reduced blood pressure		*P2rx3* knockdown	(Xue, et al., 2021)
Muscle (cardiac)	P2X (P2X4)	Improved heart function	MRS2339[4]		(Zhou, et al., 2010)
Gastrointestinal system					
Muscle (circular colon)	P2X2, P2X4	Muscle contraction	ATP		(Lee, et al., 2005)
Myocytes (longitudinal colon)	P2X3	Muscle contraction	ATP, α,β-meATP		(Lee, et al., 2005)
Myocytes (circular colon)	P2X2, P2X4	Inward currents	ATP = 2MeSATP > α,β-meATP		(Lee, et al., 2005)
Myocytes (longitudinal colon)	P2X3	Inward currents	α,β-meATP = ATP > 2MeSATP		(Lee, et al., 2005)
Renal system					
Kidney cells (MDCK cells)	P2X7	Ethidium^{+} uptake	BzATP, ATP	A438079, KN-62	(Jalilian, et al., 2012b)
Kidney cells (MDCK cells)	P2X7	Mesenchymal transition	BzATP	Oxidized ATP	(Zuccarini, et al., 2017)
Kidney cells (ciliated MDCK cells)	P2X7	Inward currents, Ca^{2+} influx	ATP[5]	Oxidized ATP, suramin	(Rodat-Despoix, et al., 2013)
Other					
Adipose-derived stem cells	P2X7	Proliferation	BzATP > ATP > adenosine		(Roszek, et al., 2017)

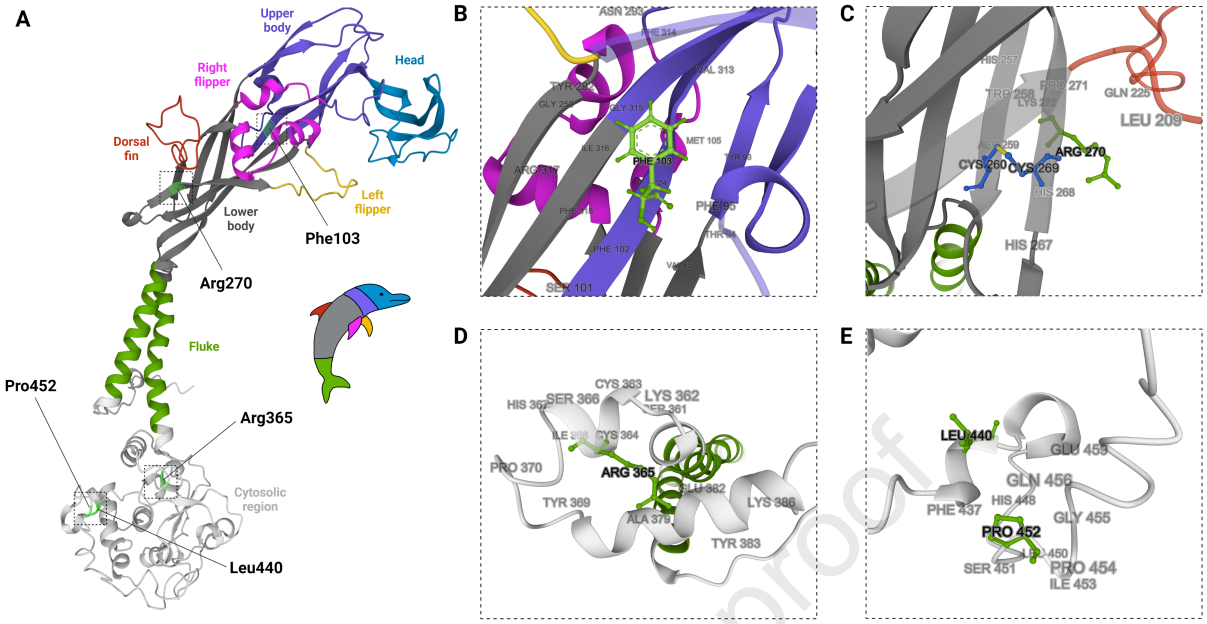
[1]*N*-[4-(1-Methylethyl)phenyl]-4-[2-(3-pyridinyl)ethyl]-1-phthalazinamine.
[2]Potentiated by ivermectin.
[3]α,β-MeATP infused at rest and during exercise.
[4]MRS2339 infused in rapid pacing-induced heart failure.
[5]Exogenous ATP in response to sheer-stress or endogenous ATP.

Table 5
Published studies reporting pharmacokinetics and safety of P2X7 and P2X3 antagonists in dogs

Compound[1]	CL (mL/min/kg)[2]	V_{ss} (L/kg)	$t_{1/2}$ (h)	*F* (%)	Safety	Reference
GlaxoSmithKline						
Compound 2	14	0.9	0.7	69	NR	(Abberley, et al., 2010)
Compound 18	2	0.8	5.7	100	NR	(Abberley, et al., 2010)
Pfizer						
Compound 28	3.8	0.6	3.8	84	NR	(Duplantier, et al., 2011)
Compound 33 (CE-224,535)	12	0.61	0.46	59	Safe	(Duplantier, et al., 2011)
Janssen						
Compound 1 (JNJ-54166060)	5.5	3.6	11.9	>100	Safe	(Swanson, et al., 2016)
Compound 20	8	2.3	NR	35	NR	(Ziff, et al., 2016)
Compound 11	33	3.3	2.4	65	NR	(Letavic, et al., 2017)
Compound 12	1.4	1.3	11.0	70	NR	(Letavic, et al., 2017)
Compound 13	21	1.8	0.7	86	NR	(Letavic, et al., 2017)
Compound 14 (JNJ-54175446)	0.9	2.5	32	164	Safe	(Letavic, et al., 2017)
Compound 29	3.8	2.6	8.8	123	Safe	(Chrovian, et al., 2018)
Compound 35 (JNJ-55308942)	0.8	1.4	21.2	98	Safe	(Chrovian, et al., 2018)
Afferent						
Gefapixant (AF-219, MK-7264)	NR	NR	NR	NR	Safe	(Abdulqawi, et al., 2015)
Merck						
MK-3901	NR	NR	NR	68	NR	(Ginnetti, et al., 2018)
Compound 21	7.7	NR	3.9	63	NR	(Ginnetti, et al., 2018)

[1]P2X7 antagonists other than Gefapixant and MK-3901 (both P2X3 antagonists).
[2]CLP, clearance; V_{ss}, Volume of distribution at steady state; $t_{1/2}$ (h), *in vivo* half-life; *F*, bioavailability; NR, not reported.





Highlights

- Studies of the domestic dog have provided new insights about P2X receptors.
- Recombinant forms of canine P2X4 and P2X7 receptors have been studied.
- Native forms of various canine P2X receptors have been studied *in vitro*.
- Genetic variants of the canine *P2RX7* gene but not *P2RX4* gene are common.
- Dogs have been used to study P2X3 and P2X7 receptor antagonists *in vivo*.