

**Pharmacological and structural
investigations of the
ATP-gated ion channel receptor P2X4**

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Abstract

P2X receptors are trimeric ligand-gated ion channels comprising seven subtypes, P2X1-P2X7, which are all activated by adenosine triphosphate (ATP). These receptors are widely expressed in neuronal and non-neuronal cells. Their roles in pathophysiological processes, particularly in inflammatory diseases, make them attractive drug targets, as evidenced by the recent approval of the P2X3 receptor antagonist gefapixant for the treatment of chronic cough.

The P2X4 receptor subtype is expressed in the central nervous system, particularly on neurons and microglia, as well as on peripheral immune cells, and was shown to play a role in chronic pain, neuroinflammation and associated diseases, and in various types of cancer. The inhibition of the P2X4 receptor showed promising effects in preclinical studies of neuropathic and inflammatory pain. Since the polar nature of the P2X4 receptor ATP-binding site and its similarity to that of other P2X receptor subtypes has impeded the discovery of selective orthosteric drug-like P2X4 receptor antagonists, drug research has focused on the development of allosteric antagonists. To date, only a few potent and selective allosteric P2X4 receptor antagonists have been described, and information on their binding site(s) is sparse.

The objective of the present thesis was to advance the pharmacological and structural characterization of the P2X4 receptor, with a particular focus on identifying allosteric binding sites for P2X4 receptor antagonists. Expression and pharmacological characterization of P2X4 receptors from a variety of different species were performed, shedding light on species differences between the human P2X4 receptor and its orthologs. This study successfully established a radioligand binding assay for an allosteric P2X4 receptor antagonist, which indicated the presence of different allosteric binding sites on the P2X4 receptor. Biological investigation of nucleoside thiophosphates at P2X receptor subtypes led to the identification of novel P2X receptor agonists. These compounds may serve as pharmacological tools and provide a basis for the future development of subtype-selective agonists. The allosteric binding site of the P2X4 receptor antagonist BX430 was investigated by mutagenesis studies, indicating that BX430 may bind to the dorsal fin region of the human P2X4 receptor. A high-resolution structure of the human P2X4 receptor in complex with an allosteric antagonist was resolved by cryo-electron microscopy, which revealed an unprecedented binding site for anthraquinone derivatives.

The findings of this thesis expand the understanding of the pharmacological and structural features of the P2X4 receptor, thereby facilitating future drug development and investigations of the receptor's role in health and disease.

1. Introduction

1.1 Purinergic signaling

The concept of “purinergic signaling” was initially proposed by Geoffrey Burnstock and colleagues after identifying adenosine triphosphate (ATP) as the transmitter in non-cholinergic and non-adrenergic inhibitory nerves in guinea pig taenia coli (colon muscle).¹

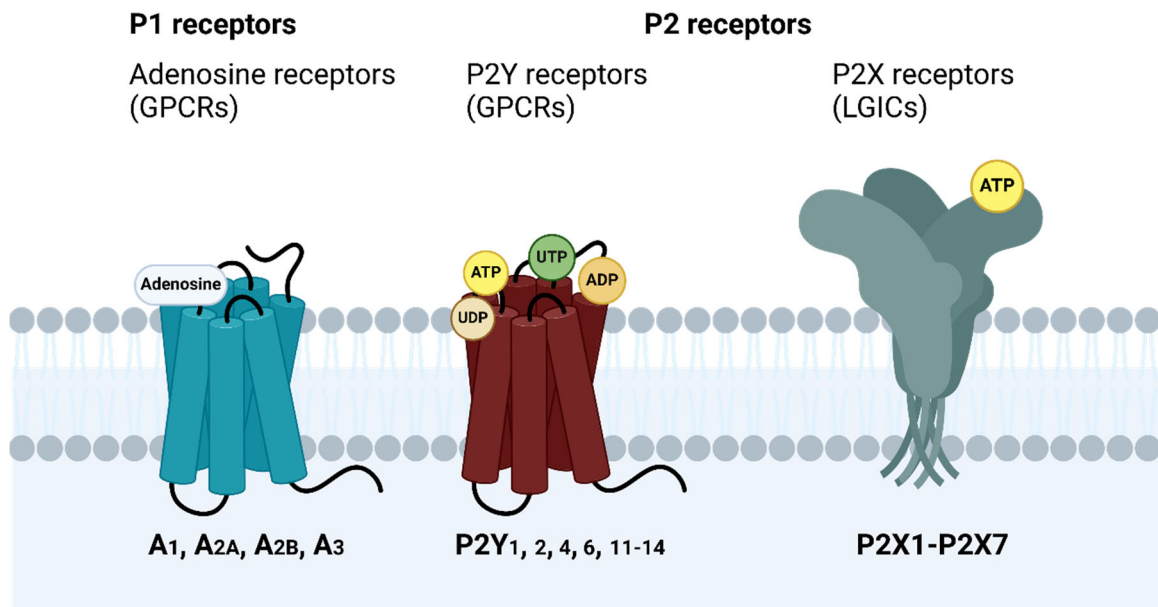


Figure 1. Schematic representation of the purinergic receptors and their endogenous agonists. Abbreviations: GPCRs, G protein-coupled receptors; LGICs, ligand-gated ion channels; ATP, adenosine triphosphate; ADP, adenosine diphosphate; UTP, uridine triphosphate; UDP, uridine diphosphate. The figure was created with BioRender.com.

A definition of purinergic receptors was published in 1976, marking a pivotal milestone in understanding purinergic neuronal transmission with ATP as an extracellular messenger.² In subsequent years, the classification of purinergic receptors was refined, leading to the pharmacological basis for distinguishing two distinct purinergic receptor families: P1 and P2 receptors (Figure 1).³ P1 receptors, which are activated by adenosine, are G protein-coupled receptors (GPCRs) that transduce signals and activate intracellular second messenger systems.⁴ The P1 receptor family comprises four different subtypes of adenosine receptors: A1, A2A, A2B, and A3.⁴ In 1985, the P2 receptor family was classified into P2X

(ligand-gated ion channels, LGICs) and P2Y receptors (GPCRs) due to differences in structural and pharmacological properties among P2 receptor subtypes.⁴ The P2 receptor family comprises seven P2X (P2X1-P2X7) and eight P2Y receptor subtypes (P2Y₁, P2Y₂, P2Y₄, P2Y₆, P2Y₁₁, P2Y₁₂, P2Y₁₃ and P2Y₁₄).⁴

1.2 P2X receptors

P2X receptors are ligand-gated ion channels that open in response to extracellular ATP, allowing the flow of cations across the cell membrane, thereby mediating various physiological and pathophysiological processes.⁵ In 1994, the first complementary DNA encoding a P2X receptor was cloned from rat vas deferens and heterologously expressed in *Xenopus* oocytes and mammalian cells, confirming that P2X receptors function as ATP-gated ion channels.⁶ All P2X receptor subunits share ATP as their endogenous agonist and show selectivity for monovalent and divalent cations (Na⁺, K⁺, and Ca²⁺).⁷ In vertebrates, seven genes encode P2X receptor subunits (P2X1-P2X7) consisting of 379–595 amino acids and sharing 40–50% identity in amino acid sequence.^{5,8}

P2X receptors assemble as functional homotrimeric complexes.^{9,10} They can also form functional and pharmacologically relevant heterotrimeric receptors.¹⁰ The P2X2/3 heterotrimer has been the focus of several studies, with evidence indicating its expression in sensory neurons and its functional role in sensory neurotransmission.¹¹ Furthermore, it was reported that P2X4 and P2X7 receptors can form heterotrimeric receptor complexes.^{12,13} However, the coexpression of P2X4 and P2X7 receptor subunits did not cause a novel electrophysiologically distinguishable P2X receptor phenotype.¹³ Other postulated P2X heterotrimers are less well investigated, and evidence for their native formation and significance is limited.¹¹ P2X6 receptors, unlike other P2X receptor subtypes, do not form functional homotrimeric complexes and require heterotrimerization, e.g., with P2X2 receptors, for proper protein folding.^{14,15}

The potency of ATP varies among P2X receptor subtypes, ranging from nanomolar concentrations for P2X1, P2X3, and P2X5 receptors to low micromolar concentrations for P2X2 and P2X4 receptors.¹⁰ Notably, the P2X7 receptor is the least sensitive subtype, requiring high micromolar to millimolar ATP concentrations for receptor activation.^{16,17} Despite structural differences between the receptor subtypes, the effect of divalent cations has been suggested to contribute to the variation in ATP sensitivity.¹⁸ Specifically, MgATP²⁻ shows lower affinity and efficacy for the P2X2 receptor than free ATP,

suggesting an inhibitory role of Mg^{2+} ions.¹⁸ Conversely, the human P2X1 and P2X3 receptors are activated by MgATP^{2-} in a way similar to free ATP.¹⁸ Nevertheless, the full extent of the effect of divalent cations on subtype-specific behavior needs further investigation.¹⁸

Also, desensitization kinetics differ between P2X receptor subtypes. P2X1 and P2X3 receptors undergo rapid desensitization within milliseconds, whereas P2X2 and P2X4 receptors exhibit desensitization rates that are 100–1,000 times slower.⁵

1.2.1 Structural investigations of P2X receptors

The elucidation of P2X receptor structures has deepened our understanding of channel gating mechanisms, desensitization, and ligand interactions. This knowledge lays the foundation for future drug design targeting P2X receptors and facilitates the identification of novel lead compounds. In addition to several structures of vertebrate P2X receptors, which will be discussed in the following chapter, a crystal structure of the invertebrate P2X receptor from the Gulf Coast tick *Amblyomma maculatum* was determined.¹⁹

1.2.1.1. P2X4 receptor

The earliest evidence for the trimeric architecture of P2X receptors was provided by cross-linking studies and blue-native polyacrylamide gel electrophoresis of P2X1 and P2X3 receptors.²⁰ Subsequently, techniques such as atomic force microscopy²¹ and electron microscopy confirmed the trimeric architecture of P2X receptors.^{22,23}

The crystallization of the truncated zebrafish P2X4 receptor in the apo form,²⁴ followed by the ATP-bound form,²⁵ marked a pivotal milestone. These structures offered the first structural insights into the ATP-induced activation mechanisms of P2X receptors and verified the previously proposed trimeric receptor structure with three ATP-binding sites (Figure 2).^{24,25} The receptor's shape resembles a chalice, with a large extracellular domain and a smaller transmembrane (TM) domain that is akin to an hourglass formed by six TM helices, two from each of the three subunits (Figure 2).²⁴ The helices are arranged antiparallel to each other, and the extracellular domain forms an equilateral triangle with protruding N-linked glycosylation moieties. The P2X4 receptor subunit displays a structure resembling that of a dolphin, with the TM helices (TM1 and TM2) forming the flukes, and the extracellular region representing the dolphin's head and body (Figure 2). The C- and N-termini are located intracellularly.²⁴ The trimeric P2X4 receptor features three non-

canonical and intersubunit ATP-binding sites located at the interface formed by the head, left flipper, and upper body of one subunit and the lower body of its neighboring subunit (Figure 2).²⁵ It was observed that ATP binding leads to a flexing of the lower body domain. This flexing directly expands the receptor's region proximal to the ion channel pore, followed by an iris-like movement of the transmembrane regions, which expands the ion-conductive pathway.²⁵

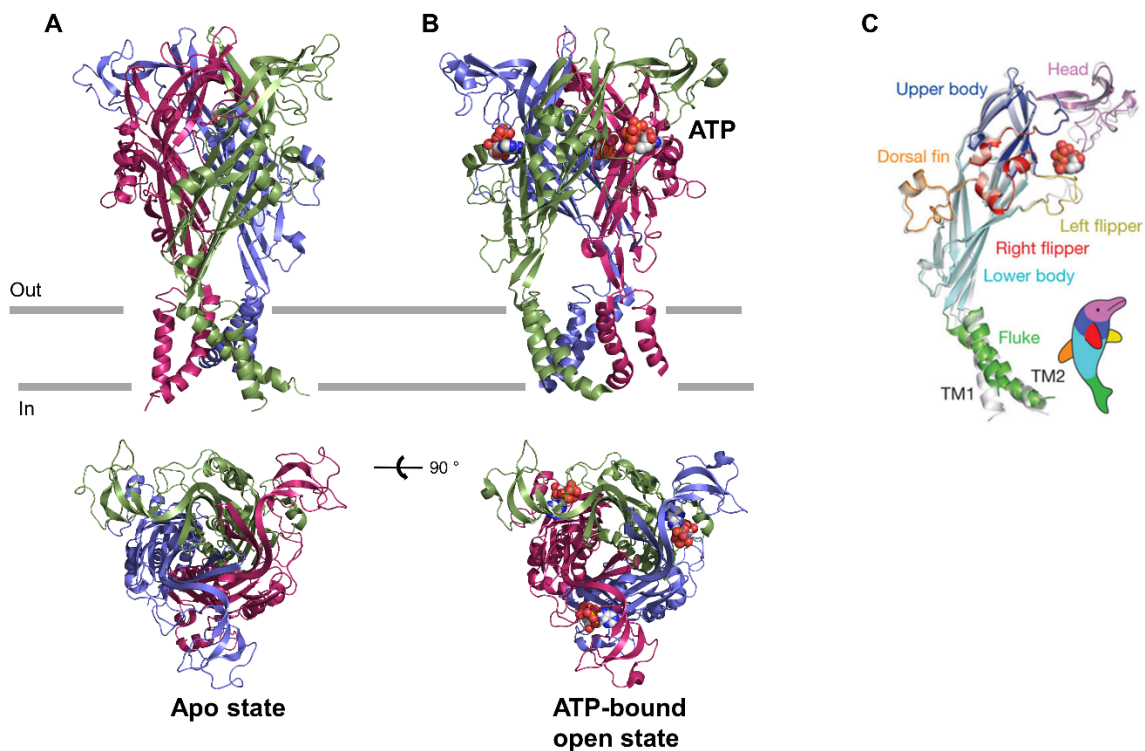


Figure 2. X-ray crystal structures of the zebrafish P2X4 receptor.²⁵ The figure displays the approximate location of the membrane bilayer with horizontal gray bars, representing the extracellular (out) and intracellular (in) environment. Both structures have truncated C- and N-termini. A. Cartoon representation of the trimeric zebrafish P2X4 receptor structure in the apo, closed state parallel to the membrane (top); view from the extracellular side (bottom). Each subunit is colored differently. Resolution: 2.9 Å. PDB ID: 4dw0. B. Cartoon representation of the trimeric zebrafish P2X4 receptor structure in the ATP-bound, open state parallel to the membrane (top); view from the extracellular side (bottom). Each subunit is colored differently. Three extracellular ATP-binding sites are visible. ATP is shown as spheres. Resolution: 2.8 Å. PDB ID: 4dw1. Protein figures in (A) and (B) were created with PyMOL 3.1.0. C. P2X4 receptor subunit with differently colored structural features resembling the body of a dolphin. Superpositions between the apo state (gray color) and the ATP-bound state using Ca positions of the protomers. ATP is shown as spheres. TM, transmembrane. Figure was taken from Hattori et al.²⁵ and reproduced with the permission of Springer Nature.

The cytidine triphosphate (CTP)-bound structure of the zebrafish P2X4 receptor unveiled further structural insights into the nucleotide specificity of P2X receptors.²⁶ CTP is a pyrimidine nucleotide that acts as a low-affinity P2X receptor agonist. Initially, it was assumed that the difference in the number of hydrogen bonds formed between nucleobases and the P2X receptors accounts for the lower affinity of CTP compared to that of ATP.²⁵ However, the structural analysis revealed that the lower affinity of CTP is more likely due to the formation of two longer hydrogen bonds between the cytosine base of CTP and the P2X4 receptor.²⁶

The cryogenic electron microscopy (cryo-EM) structures of the zebrafish P2X4 receptor in complex with two different P2X4 receptor antagonists, BX430 and BAY-1797, recently provided the first structural insights into the allosteric inhibition mechanism of the P2X4 receptor.²⁷ The binding site for both compounds was identified in the upper body domain of the receptor at the subunit interface at the top of the extracellular domain.²⁷

1.2.1.2. P2X3 receptor

X-ray crystal structures of the human P2X3 receptor were revealed for the following states: apo/resting, agonist-bound/open-pore, agonist-bound/closed-pore/desensitized, and antagonist bound/closed (Figure 3, Figure 4).²⁸ The human P2X3 receptor construct used for structural studies was truncated at the N- and C-terminus and contained three point mutations (T13P, S15V, V16I) in the N-terminus to generate a slow desensitizing receptor construct.²⁸ These mutations were introduced based on prior evidence indicating that these amino acids are critical for P2X3 receptor desensitization.^{28,29} The structure of the human P2X3 receptor exhibits the typical trimeric P2X receptor architecture, with each subunit resembling the shape of a dolphin and the characteristic ATP-binding site of P2X receptors.²⁸ The open state structure of the P2X3 receptor shows an intracellular motif, termed the “cytoplasmic cap”, that stabilizes the open state of the ion channel pore (Figure 3).²⁸ Notably, differences in the transmembrane domains and the pore architecture between the desensitized and open states are observed at the cytoplasmic surface. This led to the hypothesis that the cytoplasmic cap engages in a folding/unfolding transition during channel gating, and its stability determines the rate of receptor desensitization.²⁸ From these findings, it was further assumed that slow-desensitizing P2X receptors possess a more stable cap domain than fast-desensitizing receptor subtypes. Compared to the previously published structures of the zebrafish P2X4 receptor, the human P2X3 receptor displays

unique features, including longer transmembrane domains, different residues defining the pore constriction, and a Mg^{2+} ion bound in the head domain.²⁸

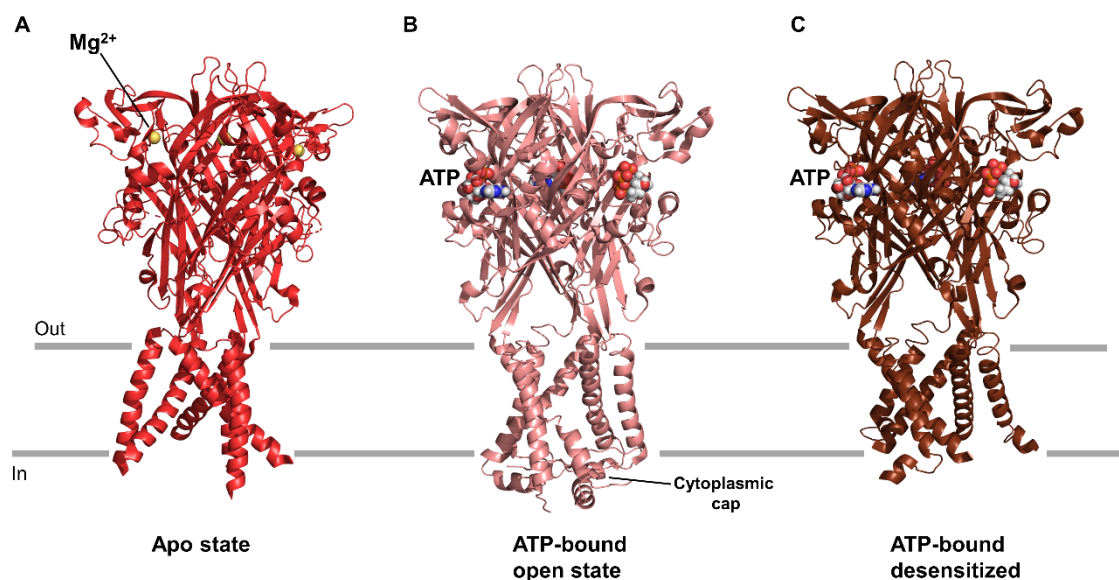


Figure 3. Cartoon representation of X-ray crystal structures of the trimeric human P2X3 receptor in different states: A. Apo state (PDB ID: 5svj),²⁸ B. ATP-bound open state (PDB ID: 5svk),²⁸ C. ATP-bound desensitized state (PDB ID: 5svl).²⁸ The figure shows the approximate location of the membrane bilayer with horizontal gray bars, representing the extracellular (out) and intracellular (in) environment. All structures are presented parallel to the membrane, and the molecules in the trimeric structures are shown as spheres. All structures have truncated C- and N-termini. Protein figures were created with PyMOL 3.1.0.

The antagonist-bound structures of the human P2X3 receptor identified an orthosteric ligand-binding pocket for two P2X receptor antagonists, trinitrophenyl-ATP (TNP-ATP) and A-317491 (Figure 4).²⁸ In contrast to ATP, the antagonists adopt a different orientation within the binding pocket, allowing deeper penetration into the binding cleft between the subunits.²⁸ Upon antagonist binding, the dorsal fin domain is prevented from moving upwards, which typically closes the binding cleft. This precludes the conformational changes that are essential for channel opening.²⁸

A druggable allosteric binding site of P2X3 receptors was elucidated in a crystal structure in complex with gefapixant (AF-219), being distinct from the ATP-binding site (Figure 4).³⁰ Gefapixant binds to a site formed by the lower body and dorsal fin domains in one

subunit, and the left flipper and lower body domains from an adjacent subunit (Figure 4).³⁰ Gefapixant's binding prevents the relative motion between the left flipper and the lower body-dorsal fin, resulting in inhibition of P2X3 receptor function.³⁰

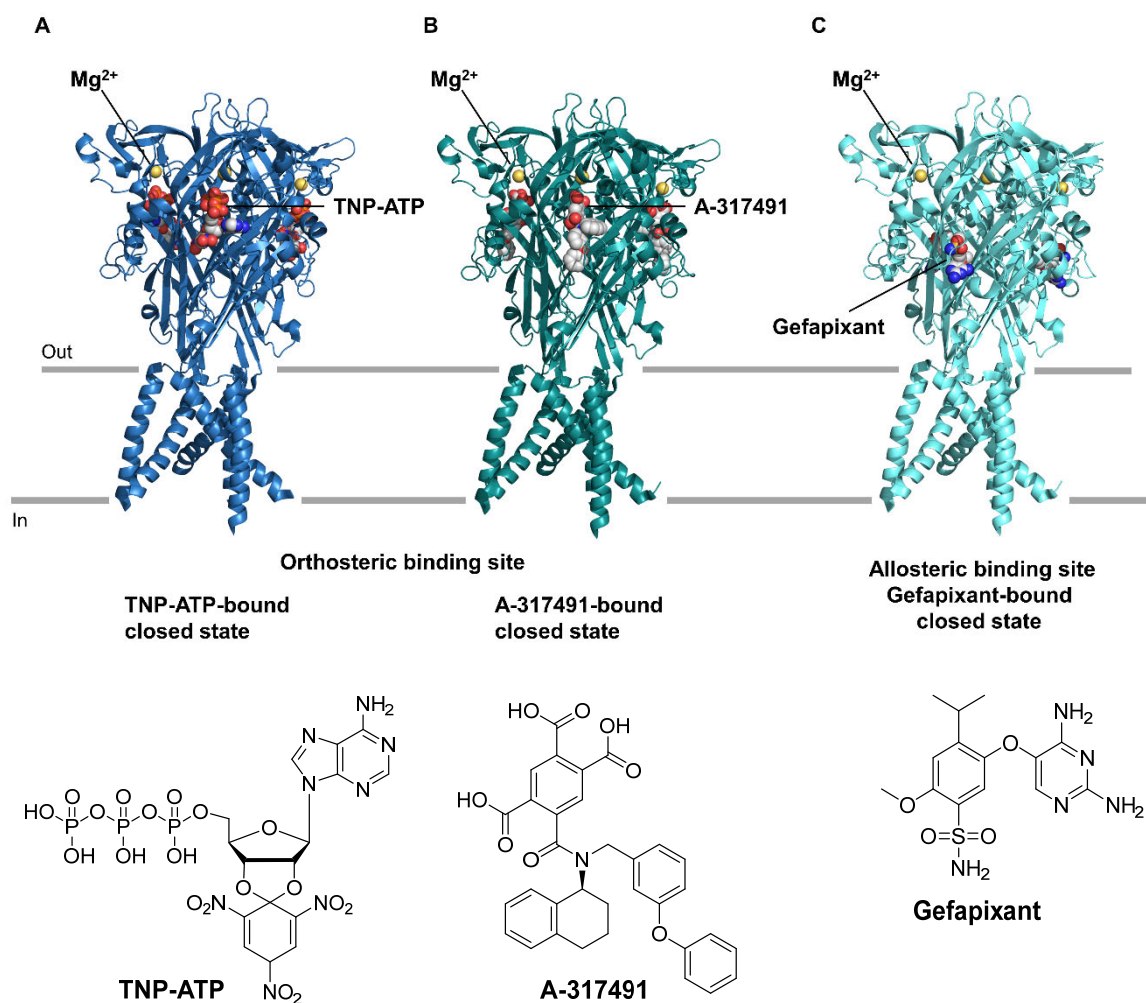


Figure 4. Cartoon representation of X-ray crystal structures of the trimeric human P2X3 receptor bound to orthosteric and allosteric antagonists. A. Top: TNP-ATP-bound closed state (PDB ID: 5svq).²⁸ Bottom: chemical structure of TNP-ATP. B. Top: A-317491-bound closed state (PDB ID: 5svr).²⁸ Bottom: chemical structure of A-317491. C. Gefapixant (AF-219)-bound closed state (PDB ID: 5yve).³⁰ The figure shows the approximate location of the membrane bilayer with horizontal gray bars, representing the extracellular (out) and intracellular (in) environment. All structures are presented parallel to the membrane, and the molecules in the structures are shown as spheres. All structures have truncated C- and N-termini. Protein figures were created with PyMOL 3.1.0.

1.2.1.3. P2X7 receptor

Structural studies of the panda P2X7 receptor confirmed its ‘dolphin-like’ shape as it was described for the zebrafish P2X4 and the human P2X3 receptor.³¹ The activation mechanism observed in the antagonist/ATP-bound state of the P2X7 receptor aligned with the proposed mechanism for P2X4 and P2X3 receptors.³¹ In the presence of five structurally distinct P2X7 receptor antagonists, the structure of the panda P2X7 receptor unveiled a novel allosteric antagonist-binding pocket (Figure 5).³¹ All P2X7 receptor antagonists share the same binding site formed between neighboring subunits, which is adjacent to the ATP-binding pocket (Figure 5).³¹ Remarkably, the equivalent pocket in the P2X4 receptor is too narrow to accommodate the smallest P2X7 receptor antagonist, A804598 (Figure 5).³¹ This indicates that distinct sizes of the allosteric pockets are responsible for the selective binding of P2X7 receptor antagonists.³¹

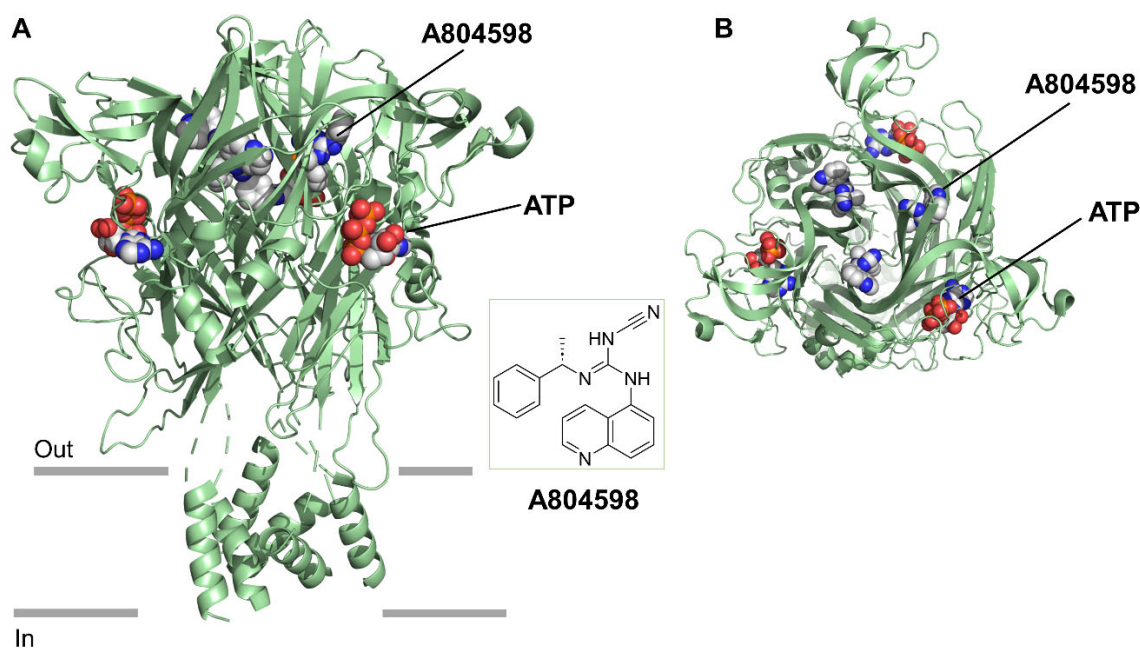


Figure 5. X-ray crystal structure of the trimeric panda P2X7 receptor in complex with the allosteric antagonist A804598.³¹ A. Cartoon representation of the overall trimeric panda P2X7 receptor structure bound to ATP/A804598 (PDB ID: 5u2h). C- and N-termini are truncated in the structure. ATP and A804598 are shown as spheres. The figure shows the approximate location of the membrane bilayer with horizontal gray bars, representing the extracellular (out) and intracellular (in) environment. The structure is presented parallel to the membrane. Chemical structure of A804598 is shown. B. Top view of the panda P2X7 receptor bound to ATP/A804598 (PDB ID: 5u2h). ATP and A804598 are shown as spheres. Protein figures were created with PyMOL 3.1.0.

The X-ray crystal structure of the chicken P2X7 receptor in the presence of the orthosteric P2X receptor antagonist TNP-ATP revealed a unique recognition pattern distinct from that observed in the structure of the human P2X3 receptor.³² The architecture of the extracellular domain of the chicken P2X7 receptor is similar to that of the ATP-bound, open state structures of P2X receptors, and the trinitrophenyl group of TNP-ATP faces towards the receptor's head and dorsal fin domains.³² In contrast, the structure of the human P2X3 receptor in complex with TNP-ATP is identical to the apo, closed state of the human P2X3 receptor.²⁸ Additionally, the trinitrophenyl group of TNP-ATP is rotated by $\sim 180^\circ$ and faces towards the lower body and left flipper domains in the human P2X3 receptor structure.²⁸ These structural differences may be due to differences in the receptor subtypes or because of different experimental conditions.³²

Cryo-EM structures of the panda P2X7 receptor in complex with two non-nucleotidic antagonists, PPADS and PPNDS (Figure 6), showed that both antagonists bind to the orthosteric binding site.³³ Structural analysis indicates that both antagonists inhibit the ATP-dependent activation of P2X7 receptors by preventing the downward movement in the left flipper domain, which is required for channel opening.

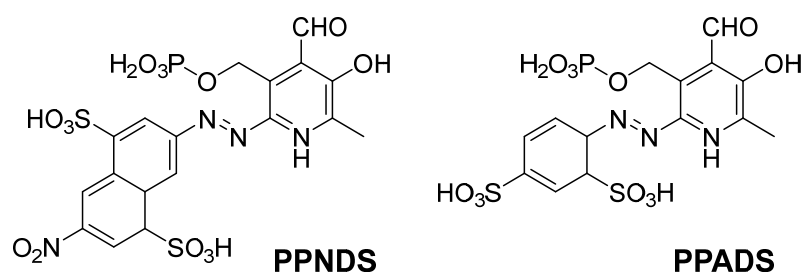


Figure 6. Chemical structures of PPNDS and PPADS.

Single particle cryo-EM was applied to investigate the structure of the full-length rat P2X7 receptor (Figure 7).³⁴ Notably, this study was the first to use a full-length P2X receptor construct for structural investigations.³⁴ Unlike other P2X receptor structures, the P2X7 receptor contains two unique cytoplasmic elements: an intracellular carboxyl-terminal cysteine-rich (C-cys) region at the end of the second transmembrane domain (TM2), named the C-cys anchor, and 120 additional residues in the C-terminus, called cytoplasmic ballast domain.^{34,35} The term “C-cys anchor” was chosen because the palmitoyl groups link this

region and the cytoplasmic end of TM2 to the membrane.³⁴ The cryo-EM structure of the rat P2X7 receptor visualizes these unique structural elements for the first time (Figure 7) and provides structural evidence that palmitoylation of the C-cys anchor modulates P2X7 receptor function, preventing it from undergoing desensitization, a common trait among the P2X receptor family.³⁴ More specifically, structural analysis allows to assume that the spatial arrangement of palmitoyl groups on four cysteine residues within the C-cys anchor, as well as one cysteine residue in the N-terminus, secures the C-cys anchor (and the N-terminus) to the membrane.³⁴ This, in turn, may restrict structural rearrangements of TM2, which are required for receptor desensitization.³⁴

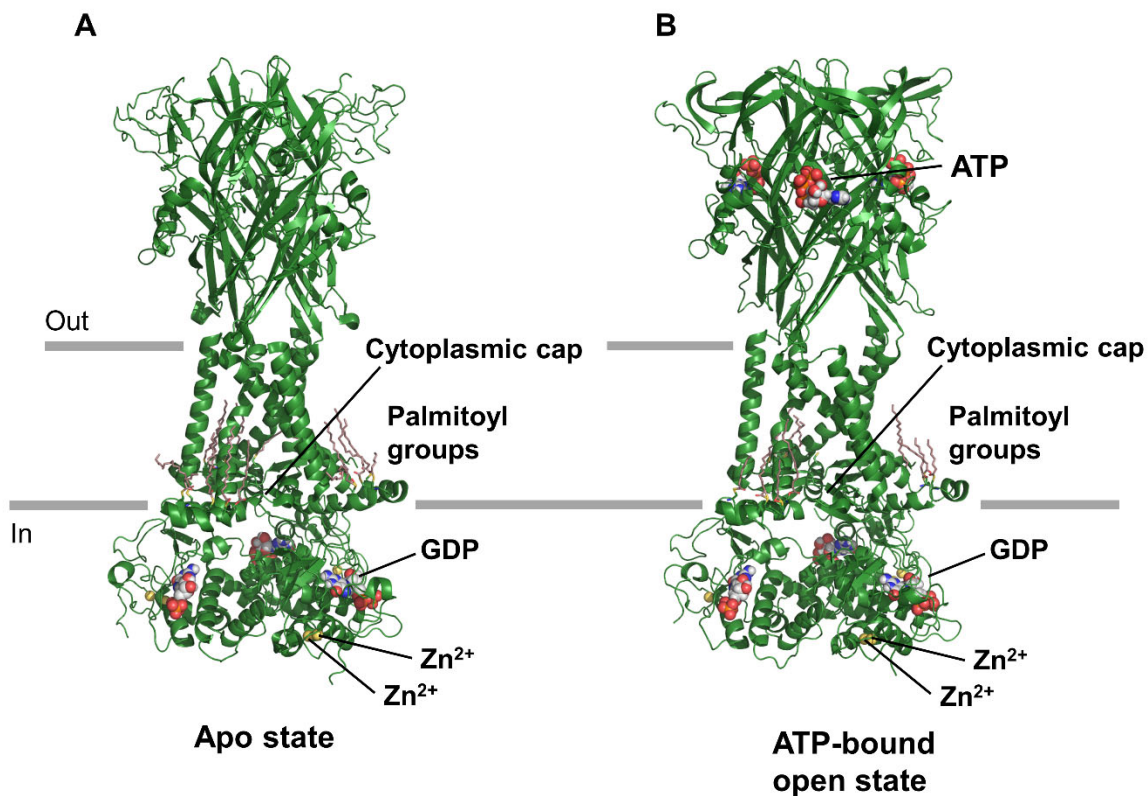


Figure 7. Cryo-EM structures of the full-length trimeric rat P2X7 receptor in cartoon representation, viewed parallel to the membrane.³⁴ The figure shows the approximate location of the membrane bilayer with horizontal gray bars, representing the extracellular (out) and intracellular (in) environment. A. Rat P2X7 receptor structure in the apo, closed state (PDB ID: 6u9v). B. Rat P2X7 receptor structure in the ATP-bound, open state (PDB ID: 6u9w). ATP and GDP (guanosine diphosphate) are shown as spheres. Palmitoyl groups are shown as sticks. Protein figures were created with PyMOL 3.1.0.

To understand the low affinity of ATP for the P2X7 receptor, the structure was compared with the ATP-bound, open structure of the human P2X3 receptor.^{28,34} Despite the virtually identical pose of ATP and the identical shape of the occupied orthosteric binding pocket in both receptors, differences in the amino acid residues interacting with ATP may account for the reduced ATP affinity of the P2X7 receptor.^{28,34} In addition, the P2X7 receptor has a narrower channel through which ATP must access the orthosteric binding pocket, rendering the receptor more shielded from its ligand.³⁴

The cryo-EM structure of the rat P2X7 receptor in complex with the agonist benzoylbenzoyl-ATP (BzATP) was recently reported, providing insights into specific ligand-protein interactions that account for the higher affinity of BzATP for the P2X7 receptor as compared to ATP.³⁶ A comparison between the ATP-bound and the BzATP-bound P2X7 receptor structures shows an open state conformation for both, with a quite similar open pore architecture.³⁶ The positions and rotamers of each amino acid directly interacting with ATP remain conserved within the orthosteric ligand-binding site, and the same direct interactions with the adenosine triphosphate core of BzATP are observed.³⁶ However, the BzATP-bound P2X7 receptor structure revealed key structural differences.³⁶ Upon binding of BzATP, three amino acid residues, Arg125, Gln143, and Ile214, which are located outside of the canonical orthosteric ligand-binding site, undergo a rearrangement to coordinate with the carboxybenzophenone moiety of BzATP.³⁶ These newly introduced ligand-receptor interactions facilitate the entry of BzATP into its binding pocket and promote the formation of a more compact pocket, which results in a slower release of BzATP.³⁶ According to structural and functional data, the high-affinity agonism of BzATP for the P2X7 receptor is driven by both an enhanced association and a reduced dissociation of BzATP with the receptor, resulting from the described ligand-receptor interactions.³⁶ The recently determined cryo-EM structures of the full-length rat P2X7 receptor in complex with six chemically diverse noncompetitive antagonists have extended the understanding of allosteric modulation of the P2X7 receptor, and identified a previously uncharacterized allosteric binding site.³⁷ The full-length rat P2X7 receptor construct provides a comprehensive view of the transmembrane and cytoplasmic domains, demonstrating that these regions remain unaffected by allosteric antagonist binding.³⁷

1.2.1.4. P2X1 receptor

Recently, high-resolution cryo-EM structures of the human P2X1 receptor in both a desensitized ATP-bound state and a closed antagonist (NF449)-bound state were determined, exhibiting the typical chalice-like shape of P2X receptors (Figure 8).³⁸

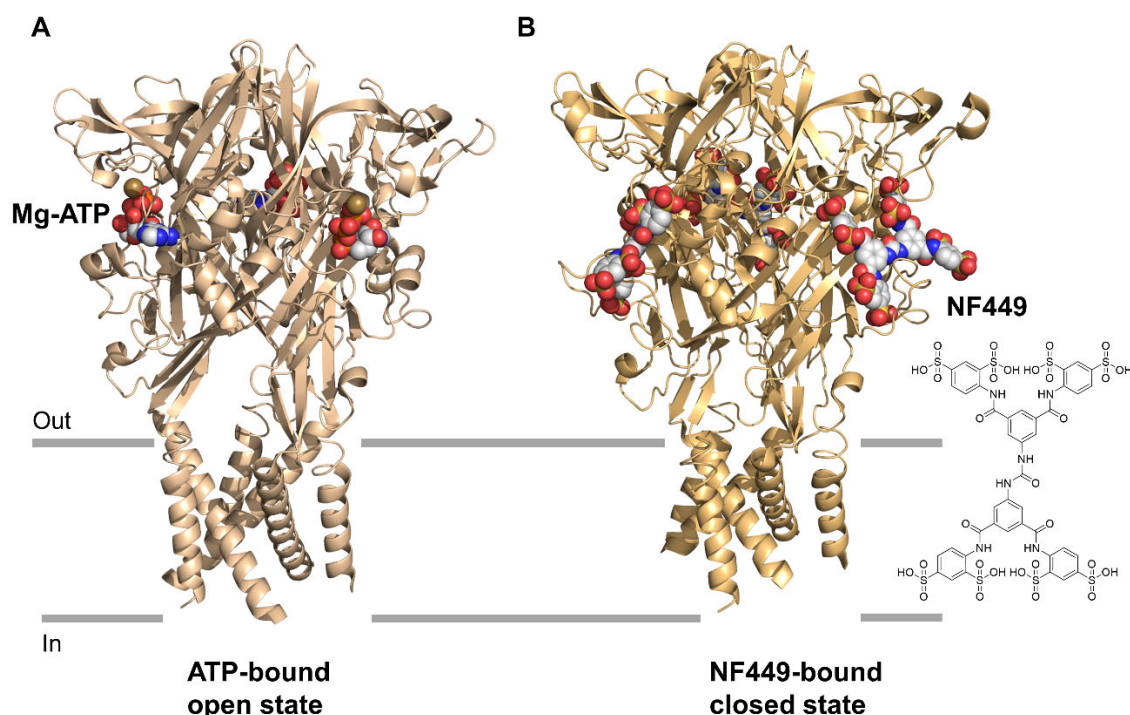


Figure 8. Cryo-EM structures of the full-length human P2X1 receptor in the ATP-bound open and the NF449-bound closed state.³⁸ A. Cartoon representation of the ATP-bound open P2X1 receptor structure (PDB ID: 9b73). ATP is shown as spheres. B. Cartoon representation of the NF449-bound closed P2X1 receptor (PDB ID: 9b95) structure. NF449 is shown as spheres. Chemical structure of NF449 is shown. The figure shows the approximate location of the membrane bilayer with horizontal gray bars, representing the extracellular (out) and intracellular (in) environment. The structures are presented parallel to the membrane. Protein figures were created with PyMOL 3.1.0.

The ATP-bound P2X1 receptor structure revealed an ATP-binding pose similar to those previously observed for P2X3, P2X4, and P2X7 receptors.^{25,28,34,38} However, unique additional interactions were identified between ATP and the P2X1 receptor, including a hydrogen bond between the amino acid residue Lys140 and the ribose sugar of ATP, which was not observed in other P2X receptor structures.³⁸ This combination of additional interactions may account for the higher affinity of ATP for the P2X1 receptor compared to

other P2X receptor subtypes.³⁸ Furthermore, the alignment of the ATP- and NF449-bound P2X1 receptor structures showed that one benzene disulfonate group of NF449 overlaps with the bound ATP molecule, thereby illustrating the mechanism by which NF449 inhibits ATP binding in a competitive manner.³⁸ The authors concluded that the high affinity and potency of NF449 for the P2X1 receptor are likely due to multiple highly polar interactions of the antagonist with its binding site.³⁸

Concurrently, another research group published cryo-EM structures of the human P2X1 receptor in the apo closed, ATP-bound desensitized, and the NF449-bound inhibited states.³⁹ To obtain the apo closed structure of the human P2X1 receptor, saturating concentrations of the antagonist NF449 were added to the cells prior to lysis to prevent binding of endogenous ATP.³⁹ Subsequently, extensive dialysis of cellular membranes was performed to remove the antagonist before detergent solubilization and reconstitution for purification.³⁹ Structural analysis of the apo state P2X1 receptor structure showed an empty ligand-binding pocket and a closed pore, which is consistent with the findings observed in other apo state P2X receptor structures.^{24,28,34,39} A comparison with the apo closed state structure of the human P2X3 receptor revealed architectural similarities between the two receptor subtypes, however notable differences in the orientations of the TM helices were observed.^{28,39} In contrast to the published structure of Bennetts et al., the NF449-bound P2X1 receptor structure revealed two NF449 molecules bound within a single binding pocket, indicating a dual-ligand supramolecular binding mode (Figure 9).^{38,39} This represents a novel feature of P2X receptor antagonists, as all other published antagonist-bound P2X receptor structures feature only a single ligand per binding pocket within each monomer.^{27,28,30,33,37,38} The total of six NF449 molecules form extensive ligand-receptor and ligand-ligand interactions, which likely explains the high potency of NF449 for the P2X1 receptor (Figure 9).³⁹ The inner NF449 molecule was described as the primary competitive inhibitor as its binding site overlaps more with the orthosteric site, thereby preserving more direct contacts with the receptor.³⁹ The second NF449 molecule was termed the “auxiliary” ligand, thought to stabilize the binding of the primary ligand and enhance its inhibitory potency.³⁹

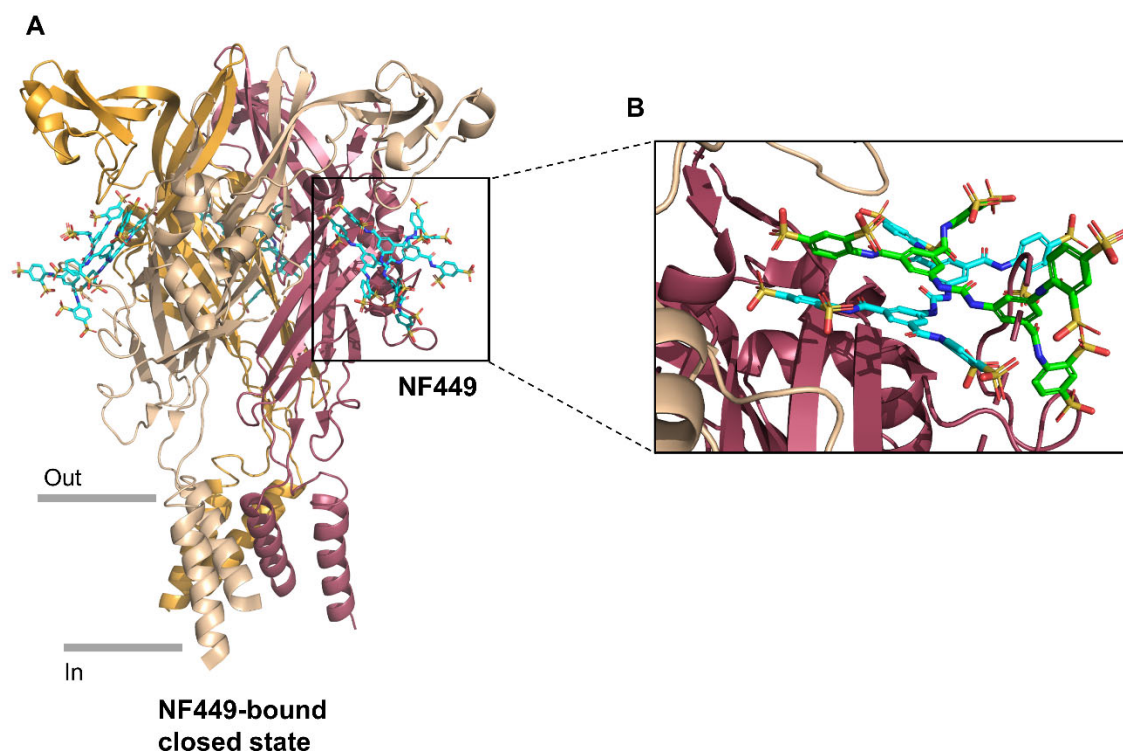


Figure 9. Cryo-EM structure of the NF449-bound closed state of the full-length human P2X1 receptor (PDB ID: 9c2c).³⁹ A. Structure of the trimeric human P2X1 receptor bound to NF449 in cartoon representation, with two NF449 molecules occupying one of three competitive ligand-binding sites. The individual protomers are colored differently. NF449 molecules are shown as sticks. The figure shows the approximate location of the membrane bilayer with horizontal gray bars, representing the extracellular (out) and intracellular (in) environment. The structure is presented parallel to the membrane. B. Magnified view from panel A, demonstrating the location of both NF449 molecules at one competitive ligand-binding site at the interface of two protomer. Protein figures were created with PyMOL 3.1.0.

1.3 P2X receptors as drug targets

The purinergic system, regulated by purines and pyrimidines, is involved in numerous physiological and pathophysiological processes across invertebrates and vertebrates.⁴⁰ In addition to its role as an intracellular energy source, ATP acts as a critical extracellular signaling mediator released from non-cholinergic and non-adrenergic inhibitory nerves.¹ In inflammatory conditions, ATP is released from damaged cells, acting as a danger signal and activating P2X receptors.⁴¹ P2X receptors are the focus of interest as novel drug targets, with each receptor subtype possessing unique functions and contributing to several pathologies.

The P2X4 receptor is widely expressed in the central and peripheral nervous systems (CNS and PNS, respectively).⁴² Following nerve injury or during neuroinflammation, P2X4 receptor expression is highly upregulated, underlining its potential as a drug target for the treatment of neuropathic pain and neurodegenerative diseases.^{42,43} Apart from that, multiple studies have suggested a contribution of the P2X4 receptor to various types of cancer.^{44–47}

The P2X7 receptor is one of the most extensively studied P2X receptor subtypes. It is predominantly expressed on immune cells and involved in inflammation and immunomodulation.^{48,49} P2X7 receptor antagonists are promising targets among others for anti-inflammatory therapy, including CNS inflammation, and for neuropsychiatric disorders, such as major depression and bipolar affective disorder.^{50,51}

P2X3 and P2X2/3 receptors are highly expressed in the nociceptive system. Multiple lines of evidence have indicated that the P2X3 receptor represents an attractive target for the treatment of visceral pain, neuropathic pain, migraine-induced pain, and cancer pain.⁵² Moreover, P2X3 and P2X2/3 receptors have been shown to be promising drug targets for the treatment of urological disorders, such as overactive bladder.⁵³ Additionally, extracellular ATP in afferents was shown to act via P2X3 and P2X2/3 receptors, leading to central processes involved in cough initiation and sensitization.⁵⁴ Gefapixant (AF-219, MK-7264) was recently approved as the first P2X3 receptor antagonist for the treatment of chronic cough.⁵⁵

The P2X2 receptor is widely expressed in CNS and PNS, and on many non-neuronal cell types, playing a central role in sensory transmission, modulation of synaptic function, and adaptation to elevated noises in the cochlea.^{56–58} In vivo studies using P2X2 knockout mice supported a role of the P2X2 receptor in mediating pain-related behavior and mechanosensory transduction within the urinary bladder.⁵⁹ These findings suggest that P2X2 receptor antagonists might be beneficial in treating pain and urinary incontinence.⁵⁹ Previous studies demonstrated that the P2X2 receptor plays an important role in the protection of hearing, as its loss of function or specific alterations in its functional properties lead to hearing loss.^{58,60} Due to this role of the P2X2 receptor in the preservation of hearing, the P2X2 receptor might be a promising therapeutic target for hearing loss.

The P2X1 receptor is predominantly expressed in smooth muscle, including the urinary bladder, vas deferens, and arteries.⁶¹ P2X1 receptor antagonists have been proposed as potential treatments for overactive bladder.⁶² In vivo studies indicated a role for the P2X1

receptor in thrombosis since it is expressed on thrombocytes, suggesting P2X1 receptor antagonists might be promising drugs for thrombosis and stroke prevention.^{63,64} The P2X1 receptor and the α_{1A} -adrenergic GPCR (adrenoceptor) are co-localized on the smooth muscle of the vas deferens, and both are involved in smooth muscle contraction, which is crucial for sperm transport during ejaculation.^{65,66} The pharmacological inhibition of the P2X1 receptor and the α_{1A} -adrenoceptor has been proposed as a promising strategy for the development of non-hormonal male contraceptives, as dual genetic deletion of both receptors in mice resulted in complete male infertility without affecting sexual behavior or function.⁶⁶

1.3.1 The concept of allosteric modulation in drug development

The orthosteric binding site of P2X receptors is mainly composed of positively charged amino acid residues (lysine and arginine) that interact with the endogenous agonist ATP.²⁵ This highly polar binding site poses challenges in developing orthosteric ligands with drug-like properties.⁶⁷ Most orthosteric ligands are polar molecules, and consequently not perorally available and unable to penetrate the blood-brain barrier.⁶⁷

In drug development, the concept of allosteric modulators has emerged as a promising approach to develop drugs overcoming the challenges associated with orthosteric ligands. A prominent example of allosteric modulation at ligand-gated ion channel receptors are benzodiazepines that positively modulate GABA_A receptors.⁶⁸

As the term suggests, allosteric modulators interact with allosteric binding sites that are distinct from the orthosteric site, recognizing the receptor's endogenous agonist.⁶⁹ Due to the binding of an allosteric modulator, the conformation of the orthosteric binding site may be altered. Depending on the effects, there are two kinds of allosteric modulators: positive allosteric modulators (PAMs) and negative allosteric modulators (NAMs). The binding of PAMs may enhance the binding affinity of the endogenous ligand to its orthosteric site followed by an increased signaling or activity, whereas the effects of NAMs may reduce the binding of the endogenous ligand to its orthosteric site. As a consequence, the signal may be reduced or the activity may be blocked.⁷⁰

Allosteric binding sites as potential drug targets offer several advantages over drugs acting via orthosteric binding sites.⁶⁷ The first advantage is the greater selectivity achievable by allosteric modulators because allosteric binding sites are less conserved than orthosteric

ones.⁶⁷ Therefore, allosteric binding sites show potential in the development of receptor subtype-selective ligands.⁷¹ The effects of PAMs are dependent on the presence of the physiological agonist. PAMs are therefore expected to have fewer side effects because they target only endogenously activated receptors. This can be described as a fine-tuning process of physiological responses.^{67,72}

1.4 P2X receptor agonists

So far, no subtype-selective agonists for P2X receptors have been described. However, several ATP derivatives and analogs were identified as P2X receptor agonists, displaying different potencies across the various receptor subtypes (Table 1, Figure 10).^{17,73,74} The respective EC₅₀ (half maximal effective concentration) values of the ATP derivatives and analogs were assessed in patch clamp or calcium influx experiments at recombinantly expressed P2X receptor subtypes (Table 1).

Despite the high conservation of the ATP-binding site in each P2X receptor subtype, the activation profile of ATP varies among P2X receptor subtypes.⁷³ ATP shows the highest potency at the human P2X1 (EC₅₀: 0.04–0.7 μ M) and P2X3 (EC₅₀: 0.04–1 μ M) receptor, followed by the human P2X2 (EC₅₀: 0.3–8 μ M) and P2X4 receptor (EC₅₀: 0.4–10 μ M).^{73–75} The human P2X7 receptor is only weakly sensitive towards ATP (EC₅₀: 541 μ M).¹⁷

α,β -Methylene-ATP (α,β -Me-ATP) is a phosphonic acid analog of ATP displaying a metabolically more stable agonist than ATP also showing highest potency at the human P2X1 (EC₅₀: 0.1–1 μ M) and P2X3 receptor (EC₅₀: 0.7 μ M).^{73,74}

ATP γ S features a thio substitution at the terminal phosphate, resulting in a metabolically more stable ATP derivative. ATP γ S shows agonistic activity at all P2X receptor subtypes, again with lowest potency at the human P2X7 receptor (EC₅₀: 138 μ M).^{73,76}

The attachment of a methylthio group to the adenine ring of ATP results in the formation of 2-methylthio-ATP (2-MeS-ATP), a compound that activates all P2X receptor subtypes, however the EC₅₀ value at the human P2X7 receptor is again very high (>100 μ M).^{73,76}

Benzoylbenzoyl-ATP (BzATP) is a commonly used P2X receptor agonist, often employed for investigations of the P2X7 receptor due to its higher potency at the human P2X7 receptor as compared to ATP.^{16,17,76} However, BzATP displays the highest potency at the human P2X1 receptor (EC₅₀: 0.02 μ M).⁷⁴

Diadenosine tetraphosphate (Ap₄A), a diadenosine polyphosphate, shows activation of the human P2X₁, P2X₂, P2X₃, and P2X₄ receptor.^{73,74} The human P2X₇ receptor is insensitive to Ap₄A activation.⁷⁴

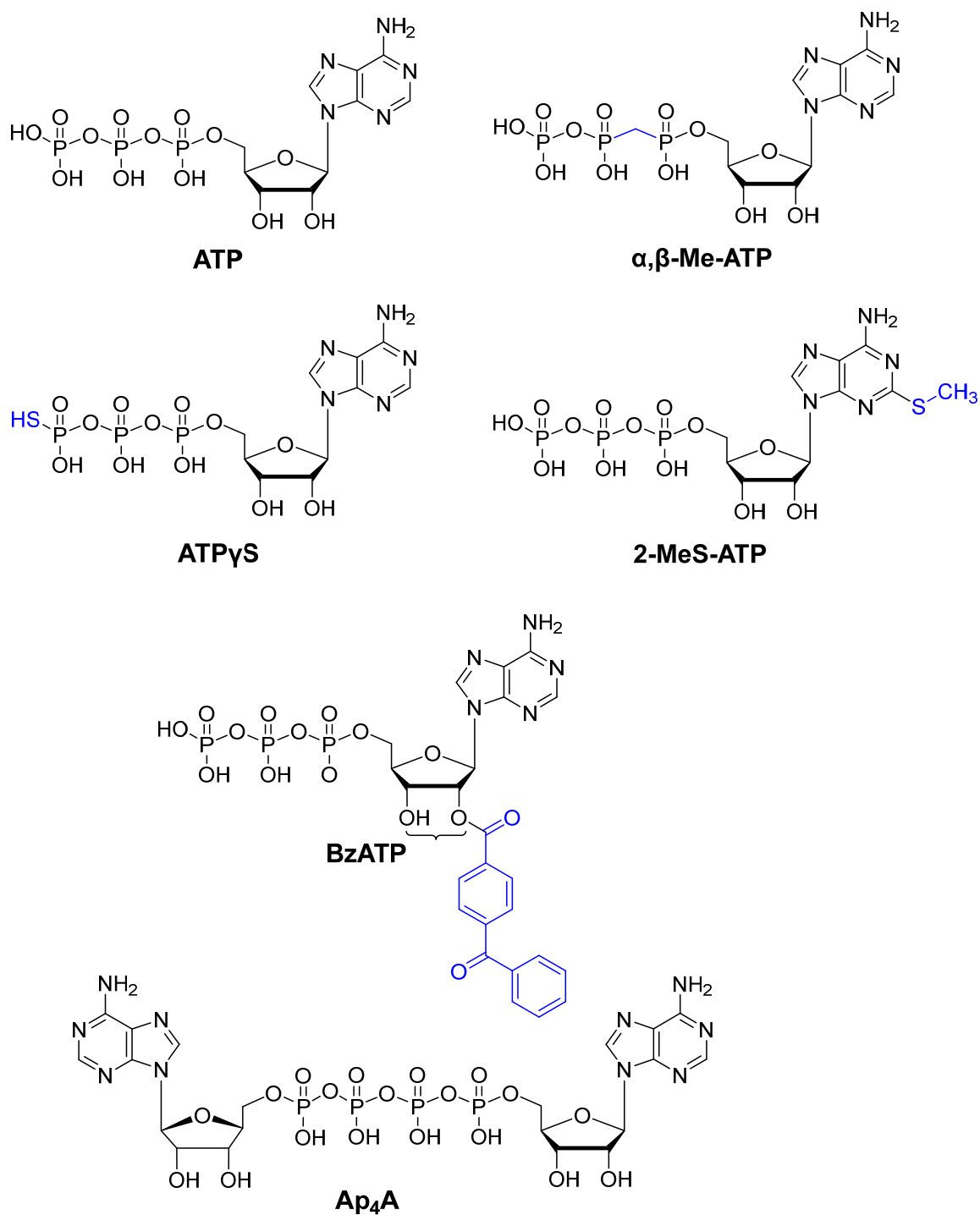


Figure 10. Structures of selected P2X receptor agonists.

Table 1. EC₅₀ values (μM) of selected P2X receptor agonists at human P2X receptor subtypes, unless otherwise noted (r, rat).^{16,17,73–76}

	P2X1	P2X2	P2X3	P2X4	P2X5	P2X7
ATP	0.04–0.7	0.3–8	0.04–1	0.4–10	0.4–10	541
α,β-Me-ATP	0.1–1	>300	0.7	0.8–8	160– >300	>300
ATPγS	2.3	1.5 (r)	0.7	11	0.5	138
2-MeS-ATP	0.05–1	1	0.4	0.3–10	10	178
BzATP	0.02	0.8	0.08	0.5–7	6–40; >500	0.4–4.7
Ap4A	0.2	15	0.5	0.1–1	0.3 (r)	>300

1.5 P2X receptor antagonists

After the identification of P2X receptors, attempts were made to develop P2X receptor antagonists to understand the receptors' biological functions. These efforts initially led to the discovery of non-selective and moderately potent P2X receptor antagonists, which initiated further efforts to develop P2X receptor antagonists with improved properties (Figure 11).

Suramin, a polysulfonated molecule, was identified as a P2X receptor antagonist with low potency and lacking subtype-selectivity.⁷⁴ Suramin acts as an antagonist at P2X1, P2X2, P2X3, and P2X5 receptors, while it is inactive at P2X4 and P2X7 receptors.^{74,77}

TNP-ATP, an ATP derivative with bulky residues, was characterized as a P2X receptor antagonist.⁷⁸ Further investigations uncovered that it is non-selective, as patch clamp recordings demonstrated its high inhibitory potency in cells expressing P2X1, P2X3, or P2X2/3 receptors (low nanomolar half-maximal inhibitory concentration (IC₅₀)) and a lower potency in cells expressing P2X2 or P2X4 receptors (micromolar IC₅₀).⁷⁹ The crystal structure of the human P2X3 receptor confirmed binding to the orthosteric site for TNP-ATP, and radioligand binding studies established a competitive binding mechanism of TNP-ATP.^{28,75}

PPADS was initially identified as a specific P2 receptor antagonist in the rabbit-isolated vas deferens.⁸⁰ Further investigations into its P2-receptor antagonism characterized it as a

non-selective P2 receptor antagonist.⁴ PPADS inhibited recombinantly expressed P2X1, P2X2, P2X3, and P2X7 receptors, but was ineffective as an antagonist at the P2X4 receptor.⁷⁴ Recent cryo-EM structures of the panda P2X7 receptor revealed the orthosteric binding site for PPADS.³³

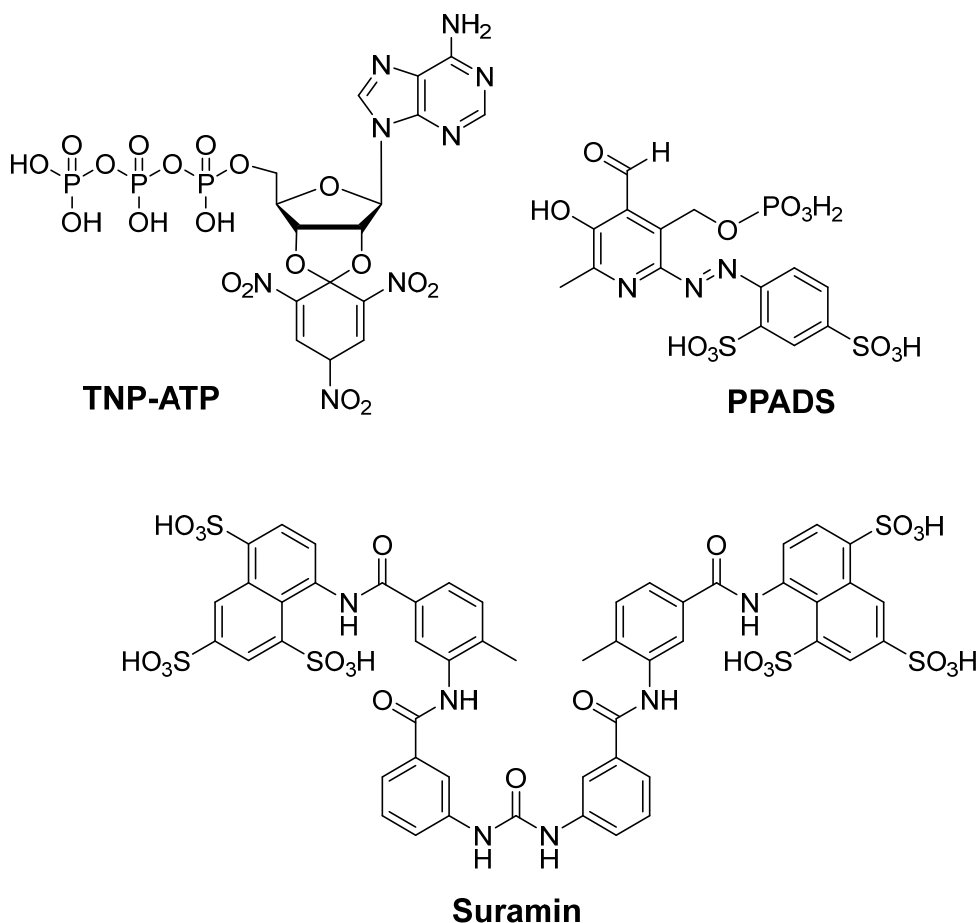


Figure 11. Structures of non-selective, competitive P2X receptor antagonists.

1.6 Allosteric modulators of P2X receptors

Allosteric modulators have been valuable in understanding the unique characteristics of each P2X receptor subtype and its functional mechanisms.

One of the most remarkable characteristics of P2X receptors is their differential allosteric modulation by metals, which can potentiate or inhibit ATP-evoked effects.^{10,81} Many attempts have been made to identify the specific amino acid residues involved in receptor modulation. Site-directed mutagenesis identified critical residues required for zinc and

copper ion effects on P2X receptors, including histidine, cysteine, or aspartate located in the extracellular domains of the receptors.⁸²

In the body, trace metals such as zinc, copper, and iron coordinate with specific amino acid residues, contribute to the catalytic activity of several CNS enzymes, and modulate the activity of several membrane channels and receptors.^{83,84} For example, iron ions are necessary elements of heme proteins, while zinc and copper ions are required for the function of various enzymes.⁸³ In terms of ion channels, zinc and copper ions modulate the activity of most ligand-gated ion channels, e.g., *N*-methyl-D-aspartate (NMDA) and α -amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA)/kainate glutamate receptors, γ -aminobutyric acid type A (GABA_A), glycine, and nicotinic receptors.⁸²

For each P2X receptor subtype, a distinct profile of modulation by metals was observed. Zinc ions inhibit the P2X₁ receptor function in a concentration-dependent manner, whereas they weakly potentiate the function of the P2X₃ receptor, with a maximal effect observed at a concentration of 10 μ M.⁸⁵ At higher concentrations, zinc ions reduce the P2X₃ receptor current, indicating the presence of two distinct allosteric sites: a high-affinity binding site exerting a positive modulatory effect, and a low-affinity site mediating negative modulation.⁸⁵ This biphasic modulation by zinc ions was also observed for the P2X₅ receptor function, suggesting again the presence of two distinct allosteric sites for zinc ions.⁸⁶ ATP-induced responses of the P2X₂ receptor are modulated in a biphasic manner by zinc and copper ions, resulting in receptor potentiation at 10–100 μ M, and in receptor inhibition at millimolar concentrations.⁸⁷ An exceptional characteristic of the P2X₄ receptor is its differential modulation by zinc and copper ions, as its function is potentiated by zinc ions but inhibited by copper ions.⁸⁸ In addition to zinc and copper ions, the P2X₄ receptor function is also modulated by cadmium, mercury, and cobalt ions. Cadmium and cobalt ions show potentiation of the P2X₄ receptor function and mercury ions exert receptor inhibition.⁸⁹ At the P2X₇ receptor subtype, the divalent cations calcium, magnesium, copper, and zinc function as negative modulators.^{90,91}

Neurosteroids are steroidal compounds synthesized *de novo* in the nervous system from cholesterol that modulate neurophysiological processes and are therefore considered as potential treatments for neurological disorders.⁹² These compounds were found to allosterically modulate various ligand-gated ion channels, such as GABA_A and glycine receptors.^{93,94} Additionally, research on the effects of neurosteroids on P2X receptor

function showed that these compounds mainly modulate P2X2 and P2X4 receptor function.^{83,95} For instance, neurosteroids such as alfaxolone, allopregnanolone, or 3 α ,21-dihydroxy-5 α -pregnan-20-one exhibit positive allosteric modulation of P2X4 receptor function, whereas pregnanolone acts as a negative allosteric modulator of the P2X4 receptor.⁹⁵ Dehydroepiandrosterone, the most abundant neurosteroid in the nervous system, was identified as a positive modulator of P2X2 receptors, with selective potentiation observed at P2X receptors containing P2X2 receptor subunits in neonatal rat dorsal root ganglion neurons.⁹⁶ Progesterone was also identified as a positive P2X2 receptor modulator, without exerting modulatory effects on P2X3 and P2X4 receptors.⁹² For the P2X7 receptor, no neurosteroid has been identified as a specific and effective modulator.⁹²

Other P2X receptor modulators include phosphoinositides (PIPs), which are key components of biological membranes.⁸³ The lipid and protein composition of the plasma membrane was shown to have a marked influence on P2X receptor activity.⁸³ For example, desensitization of the P2X2 receptor was shown to be modulated by membrane-bound PIPs, with accelerated channel desensitization in the presence of reduced membrane-bound PIPs levels.⁹⁷ It was specifically demonstrated that membrane-bound PIPs play a central role in maintaining channel activity and regulating pore dilation by interacting electrostatically with the proximal region of the P2X2 receptor's cytoplasmic C-terminal domain.⁹⁷ Specific lysine residues in the C-terminal domain of the P2X2 receptor were suggested to be critical for this observed modulation.⁹⁷ Other critical regulatory functions of membrane PIPs were described for P2X4 and P2X7 receptors.^{98,99}

Many neuronal voltage-gated and ligand-gated ion channels have been proposed as targets for ethanol action.¹⁰⁰ P2X receptors are also modulated by ethanol, but ethanol sensitivity is receptor-dependent. P2X2 and P2X4 receptors are inhibited by high concentrations of ethanol, while P2X3 receptor activity is potentiated by ethanol in a concentration-dependent manner.^{101,102} Studies demonstrated that ethanol induces P2X7 receptor expression in monocyte-derived macrophages, assuming that the P2X7 receptor may also be involved in the pathogenesis of ethanol-related diseases.¹⁰³

1.7 P2X4 receptor

This thesis focuses primarily on the P2X4 receptor subtype. In recent years, the P2X4 has become a major target of interest due to its great potential for the development of new therapies.

1.7.1 P2X4 receptor expression and function

The human P2X4 receptor gene (ENSG00000135124) is located on chromosome 12q24.32, and the protein comprises 388 amino acids.¹⁰ Within the P2X receptor family, the P2X4 receptor is a slowly desensitizing receptor subtype, exhibits high sensitivity for ATP, and it shows high Ca^{2+} permeability.¹⁰⁴ It may heteromerically assemble with P2X1, P2X2, P2X6, and P2X7 receptors, but current evidence is weak.^{11,12}

Initially discovered in rat and human brain tissue, the P2X4 receptor is widely expressed throughout the body, including CNS and PNS.^{42,105–107} It is found on neurons and microglia in the CNS, where its functions are associated with neurotransmission, neuroinflammatory signaling pathways, and the modulation of chronic pain.⁴² Additionally, the P2X4 receptor is expressed on various other immune cells, including monocytes, dendritic cells, neutrophils, eosinophils, mast cells, and T- and B-lymphocytes.¹⁰⁸ Given its wide expression in the immune system, the P2X4 receptor plays a critical role in regulating immune responses.¹⁰⁸ Beyond its functions in the CNS and the immune system, P2X4 receptors expressed by endothelial cells contribute to the regulation of vascular tone.¹⁰⁹

P2X4 receptors in human and rodent macrophages, microglia, and endothelial cells are located both on the cell surface and intracellularly.^{110,111} In contrast to other P2X receptor subtypes, P2X4 receptors on the plasma membrane are rapidly and constitutively internalized and targeted to endosomal, lysosomal, and/or lysosome-related organelles.¹¹² Two specific motifs were identified as critical for targeting P2X4 receptors to lysosomal compartments: the N-terminal di-leucine motif (L₂₂I₂₃) and the C-terminal tyrosine-based motifs (Y₃₇₂XXV and Y₃₇₈XXGL).^{111,113,114} The functions of P2X4 receptors in lysosomes have been investigated, revealing their involvement not only in membrane trafficking but also in the regulation of lysosomal functions.¹¹² Patch clamp studies with enlarged lysosomal vacuoles demonstrated that lysosomal P2X4 receptors on lysosomal membranes form functional cation channels that are activated by ATP from the luminal side.¹¹⁵ Activation of lysosomal P2X4 receptors was shown to promote P2X4 receptor-induced

endolysosomal Ca^{2+} release and endolysosomal membrane fusion, a process known to be critical for the proper functioning of lysosomes.^{112,116} Furthermore, the significant role of lysosomal P2X4 receptors was highlighted in murine models of two different pathologies. An experimental model of multiple sclerosis showed that potentiation of the P2X4 receptor function induces endo-lysosomal fusion in microglia, thereby enhancing their phagocytic capacity and facilitating efficient remyelination.¹¹⁷ In a murine model of hepatectomy, it was suggested that P2X4 receptors contribute to the protection of the liver from bile-induced damage by regulating lysosomal distribution and exocytosis at the canalicular domain.¹¹⁸

1.7.2 Structural insights into the P2X4 receptor

To obtain high-resolution structures of the P2X4 receptor, researchers investigated 35 P2X4 receptor orthologs transiently expressed in human embryonic kidney (HEK293) cells to assess stability and monodispersity.²⁴ Fluorescence detection size exclusion chromatography showed a sharp and symmetrical elution profile for the zebrafish P2X4 receptor, providing a promising candidate for resolving a crystal structure.²⁴ To improve crystallization behavior while maintaining receptor functionality, different deletions of the amino and carboxyl termini were investigated.²⁴ Additionally, three point mutations were introduced (C51F/N78K/N187R) to avoid non-native disulfide bond formation and to reduce heterogeneity due to glycosylation, respectively.²⁴ Electrophysiological experiments proved that all truncated and mutated receptor constructs are still activated by ATP, albeit with smaller current amplitudes compared to the full-length receptor, probably due to a reduced expression of the mutants.²⁴ These investigations allowed to resolve the first crystal structure of the zebrafish P2X4 receptor at 3.1 Å resolution in the apo, closed state.²⁴ A few years later, the crystal structure of the zebrafish P2X4 receptor in the ATP-bound, open state at 2.8 Å resolution was published.²⁵

As previously described, the overall architecture of the zebrafish P2X4 receptor adopts a homotrimeric and chalice-like structure with a large hydrophilic and glycosylated extracellular domain, a transmembrane domain composed of six α -helices, and short intracellular amino and carboxy termini.²⁴ Each subunit of the zebrafish P2X4 receptor resembles the shape of a dolphin, with the transmembrane helices representing the flukes and the extracellular region representing the body.²⁴

ATP-binding site

According to the ATP-bound structure of the zebrafish P2X4 receptor, three equivalent ATP-binding sites are visible, each located between the upper domain of one subunit and the lower domain of the adjacent subunit.²⁵ These intersubunit ATP-binding pockets are lined with several positively charged residues (Figure 12).

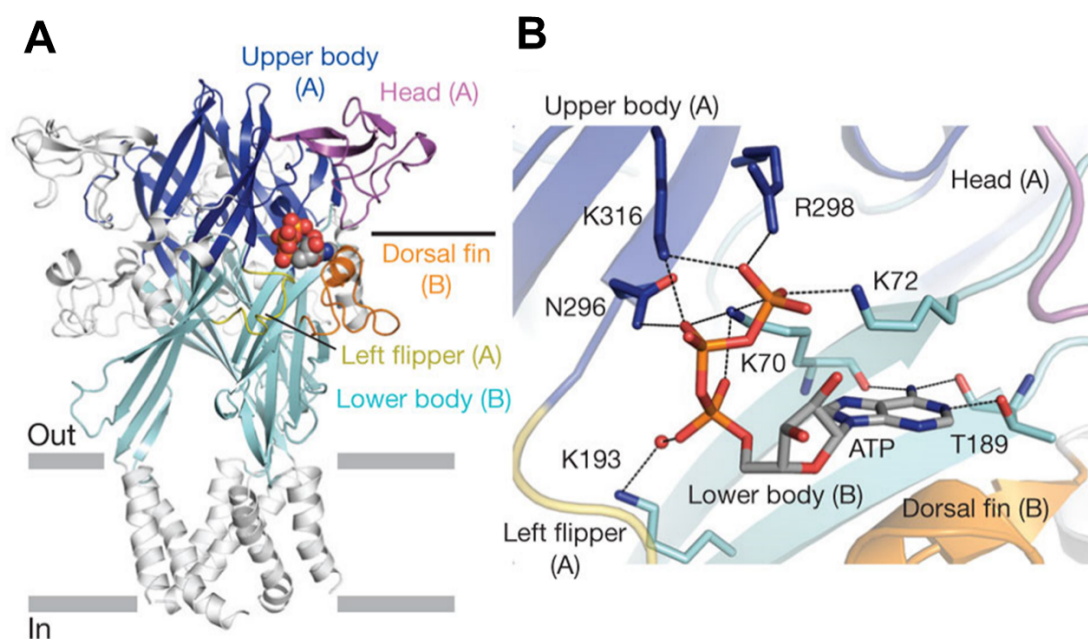


Figure 12. ATP-binding pocket.²⁵ A. X-ray crystal structure of the trimeric zebrafish P2X4 receptor with different regions forming the ATP-binding pocket, colored differently, viewed in parallel to the membrane. The figure shows the approximate location of the membrane bilayer with horizontal gray bars, representing the extracellular (out) and intracellular (in) environments. The ATP molecule is shown as spheres. (PDB ID: 4dw1). B. Close-up view of the ATP-binding pocket. Interactions between ATP and interacting amino acid residues are shown. Hydrogen bonds are shown as black dashed lines (PDB ID: 4dw1). Figures were taken from Hattori et al.²⁵ and reproduced with the permission of Springer Nature.

ATP is recognized by the upper and lower body domains through extensive hydrophilic interactions. The bound ATP molecule adopts a U-shaped structure. Key interactions include polar interactions between the negatively charged phosphates of ATP with basic amino acid residues (e.g., Lys70, Lys72, Arg298, Lys316) and polar residues (Asn296) from the upper and lower domains of the subunits (Figure 12). The adenine base of ATP is deeply embedded in the binding pocket, interacting via hydrogen bonds with Lys70 and

Thr189, and forms hydrophobic interactions with Leu191 and Ile232 (Figure 12). Lys70 plays a pivotal role as an amino acid residue in ATP-binding due to its positioning at the center of the triphosphate 'U', where its ammonium group interacts with oxygen atoms of the α -, β -, and γ -phosphate groups. Leu217 in the dorsal fin is solely responsible for recognizing the ribose ring of ATP through hydrophobic interactions, while the O2 and O3 atoms of the ribose ring remain exposed to the solvent (Figure 12).²⁵

The transmembrane pore

In the ATP-bound state of the zebrafish P2X4 receptor, an uninterrupted, continuous transmembrane pore is evident, lined mostly by TM2 amino acid residues (Leu340, Ala344, Ala347, Leu351, and Ile355) that are exposed to the putative ion permeation pathway (Figure 13). The presence of wide open lateral fenestrations within the extracellular vestibule of the receptor's open state suggests that cations could potentially access the ion channel by passing through these fenestrations (Figure 13).²⁵

The transmembrane domains

Comparing the transmembrane regions of the closed and open states, an iris-like movement of the transmembrane helices transitioning from the closed to the open state becomes apparent.^{24,25} TM1 and TM2 helices undergo rotations by $\sim 10^\circ$ and $\sim 55^\circ$ anticlockwise about a pore center axis perpendicular to the membrane plane (Figure 13).²⁵ These structural rearrangements cause the helices to move ~ 3 Å away from the central axis, thereby leading to the expansion of the ion conductive pore. In the apo state of the zebrafish P2X4 receptor, several interactions between TM2 helices, including contacts between Leu340, Leu346, and Ala347, stabilize the closed conformation of the pore. However, these stabilizing interactions are disrupted in the ATP-bound form as the transmembrane helices move away from the three-fold axis.²⁵

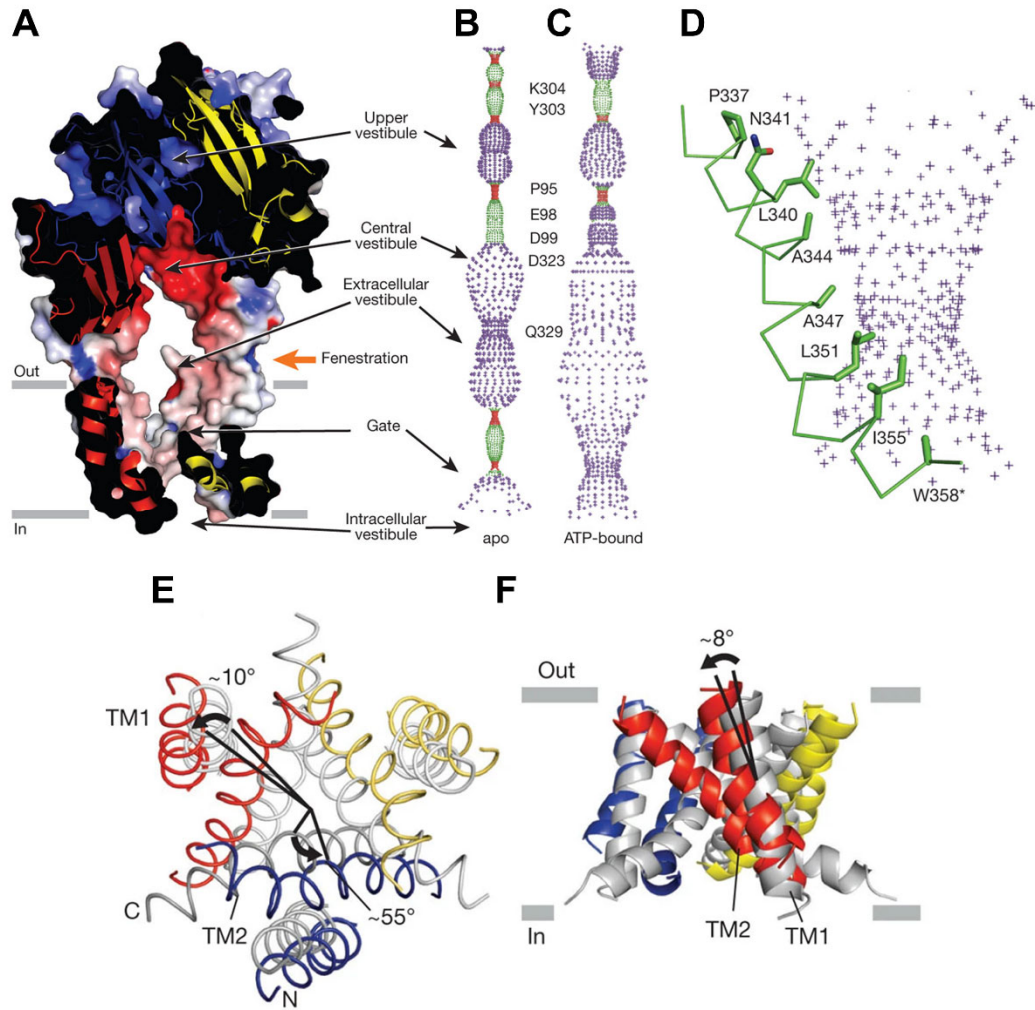


Figure 13. The transmembrane pore and structural transitions in the transmembrane domains of the zebrafish P2X4 receptor upon ATP binding.²⁵ A. A section of an electrostatic potential surface of the ATP-bound zebrafish P2X4 receptor, viewed in parallel to the membrane (PDB ID: 4dw1). Pore-lining surfaces of the apo (B) (PDB ID: 4dw0) and the ATP-bound (C) (PDB ID: 4dw1) zebrafish P2X4 receptor structures. D. Pore-lining amino acid residues of the ATP-bound zebrafish P2X4 receptor with the pore-lining surface. Residues are shown in stick representation. The transmembrane region of the apo (gray) and ATP-bound states of the zebrafish P2X4 receptor, viewed from the intracellular side (E) and parallel to the membrane (F). The figure shows the approximate location of the membrane bilayer with horizontal gray bars, representing the extracellular (out) and intracellular (in) environments. The apo state of the receptor (gray) is superimposed on the ATP-bound state using the C α positions of the trimer. The rotation of the transmembrane helices is indicated by black arrows, and black bars visualize the orientation of the helices. Figures were taken from Hattori et al.²⁵ and reproduced with the permission of Springer Nature.

Mechanism of action

The comparison between the closed and open structures of the zebrafish P2X4 receptor demonstrates ATP-induced conformational changes that finally lead to ion channel opening.²⁵ First, ATP binding promotes cleft closure between the head and dorsal fin domains of the P2X4 receptor (Figure 14). Consequently, the dorsal fin domain moves upward towards the head domain to accommodate ATP via hydrophobic interactions, while ATP displaces the left flipper away from the ATP-binding site (Figure 14). These movements of the dorsal fin and left flipper induce a simultaneous outward flexing of the lower body domain of the receptor, which expands the extracellular vestibule and thus increases the separation (Figure 14). This outward flexing of the lower body domains, in turn, promotes the opening of the ion channel pore by causing the transmembrane helices to expand in an iris-like motion (Figure 13). The upper body domains of each receptor subunit do not undergo conformational changes upon ATP binding.²⁵

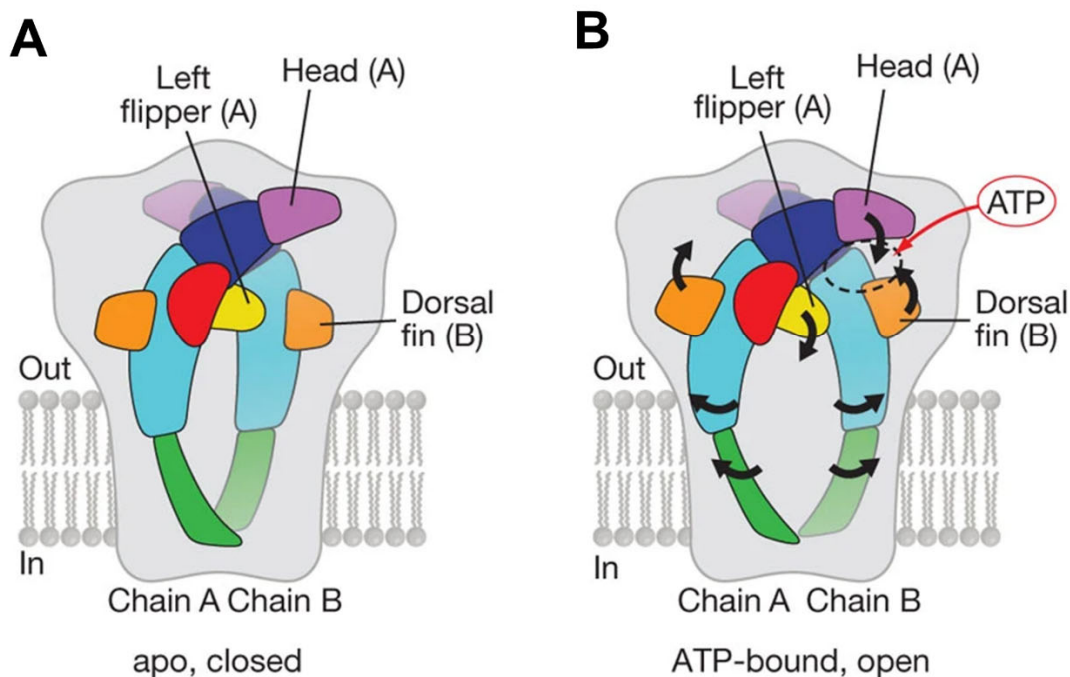


Figure 14. ATP-induced conformational changes based on the zebrafish P2X4 receptor structures in the apo and ATP-bound state.²⁵ A. B. Cartoon models of the ATP-dependent activation mechanism. The black arrows indicate the movements of the receptor domains upon ATP binding. Figures were taken from Hattori et al.²⁵ and reproduced with the permission of Springer Nature.

1.7.3 P2X4 receptor antagonists

The discovery of P2X4 receptor antagonists has faced many challenges, including sufficient potency, water solubility, and subtype-specificity. In recent years, many different studies have been undertaken to identify P2X4 receptor antagonists that are urgently needed as tools and drugs (Figure 15).

The P2X4 receptor acts as a key mediator in neuropathic pain, which is typically treated with antidepressant and antiepileptic drugs.¹¹⁹ Based on this knowledge, several studies investigated the effects of these drugs on P2X4 receptor function. Nagata et al. identified paroxetine, a monoamine uptake blocker used as antidepressant, as a P2X4 receptor antagonist.¹²⁰ Another report showed that amitriptyline, a tricyclic antidepressant, displayed no effect on the human P2X4 receptor. However, a weak effect of amitriptyline on rat and mouse P2X4 receptors was observed.¹²¹

Other compounds with reported antagonistic effects at the human P2X4 receptor include the benzodiazepine derivative 5-BDBD¹²² and its derived compounds, e.g., NP-1815-PX¹²³ and MRS4719¹²⁴, exhibiting similar inhibitory potencies at the human P2X4 receptor. NP-1815-PX was highly potent with enhanced water solubility and selectivity towards the human P2X4 receptor over other P2X receptor subtypes. The effects of NP-1815-PX were evaluated in a mouse model of colitis, and it was found to improve several inflammatory parameters.¹²⁵ MRS4719 was used as a tool compound in a mouse model of ischemic stroke, showing neuroprotective and neuro-rehabilitative effects.¹²⁴

During the investigation of *N*-substituted phenoxazine and related acridone derivatives, PSB-12054 was identified as a P2X4 receptor antagonist.¹²⁶ However, a limitation was its high lipophilicity and moderate water solubility. Subsequent refinements resulted in a more water-soluble P2X4 receptor antagonist, PSB-12062, showing selectivity for the P2X4 receptor over P2X1, P2X3 and P2X7 receptors.¹²⁶

PSB-15417¹²⁷ emerged as a highly potent, selective, and brain-permeable allosteric P2X4 receptor antagonist from a collaboration project of academia and industry. This promising tool compound was useful in pharmacological studies on P2X4 receptors in different animal models. Teixeira et al. used PSB-15417 to investigate the role of P2X4 receptors in peripheral diabetic neuropathy.¹²⁸ The results strongly suggested that the activation of P2X4 receptors in dorsal root ganglia by endogenous ATP plays a crucial part in the

maintenance of pain associated with diabetic neuropathy.¹²⁸ A structurally related allosteric P2X4 receptor antagonist, PSB-OR-2020, was recently published, also displaying inhibitory potency for the P2X4 receptor in the low nanomolar range.¹²⁹

NC-2600 (structure undisclosed) was the first human P2X4 receptor antagonist to enter clinical trials (phase I), but no further development has been reported.^{127,130}

High-throughput screening of the Bayer compound library and subsequent optimization led to a new tool compound, denoted as BAY-1797, characterized as a potent and selective small-molecule inhibitor of the P2X4 receptor.¹³¹ This molecule holds promise for exploring the role of the P2X4 receptor in inflammation, proliferation, and sensory nerve desensitization, showing anti-inflammatory and anti-nociceptive effects in mice.¹³¹

In a screening of various carbamazepine derivatives, another P2X4 receptor antagonist, *N,N*-diisopropylcarbamazepine, was identified. It was found that the carbamazepine derivative is not selective versus human P2X1 and P2X3 receptors but selective versus human P2X2 and P2X7 receptors.¹³²

The phenylurea derivative BX430 showed antagonistic properties at the human P2X4 receptor with potency in the submicromolar range.¹³³ Further experiments confirmed its selectivity for the P2X4 receptor within the P2X receptor family.¹³³ BX430 appeared to act via a noncompetitive mechanism on the P2X4 receptor by binding to an allosteric site, leading to a change in subunit conformation.¹³³ Unexpected species differences were observed, as electrophysiological experiments demonstrated an antagonistic function of BX430 at the zebrafish P2X4 receptor, but no effect was noted for the rat and mouse P2X4 receptor orthologs.¹³³ In contrast, BX430 was observed to act as an antagonist at the rat and mouse P2X4 receptors in calcium influx assays, however with lower potency than at the human receptor.¹³⁴ These discrepancies may have resulted from using much higher ATP concentrations in electrophysiological experiments than those employed in calcium influx assays, and consequently, the inhibitory effect of BX430 could not be detected in electrophysiological experiments.^{133,134}

Based on the chemical structure of BX430, compound “9o” was developed and described as a selective P2X4 receptor antagonist with an improved inhibitory potency in the submicromolar concentration range.¹³⁵

The indoline derivative indophagolin was identified as an inhibitor of autophagy by a label-free target identification strategy, namely a target-agnostic phenotypic screen using a human breast cancer cell line.¹³⁶ Subsequent thermal proteome profiling using quantitative mass spectrometry identified the P2X4 receptor as a potential target of indophagolin.¹³⁶ In calcium influx assays, indophagolin was shown to inhibit P2X4 receptor function with an IC_{50} of 2.71 μ M. Additional inhibition was observed at P2X1 and P2X3 receptors.¹³⁶

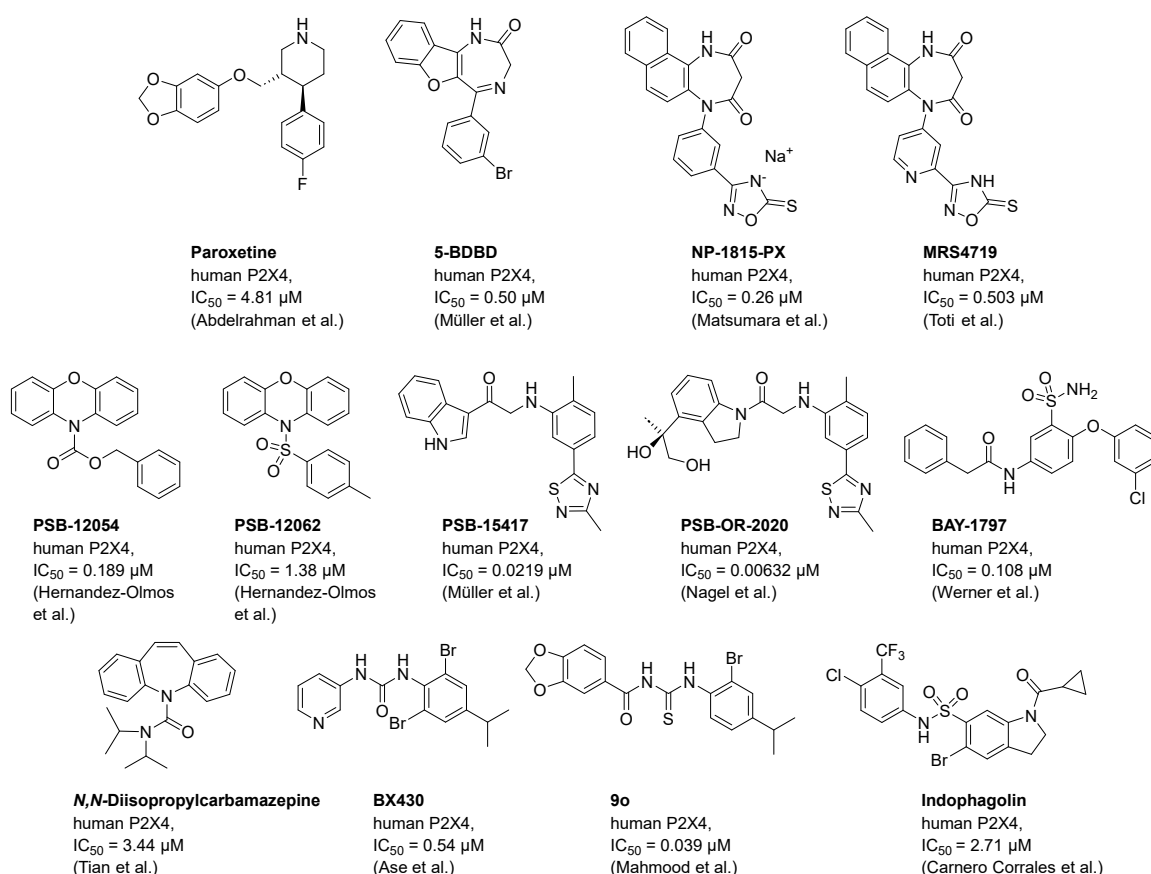


Figure 15. Structures of selected P2X4 receptor antagonists with inhibitory potencies (IC_{50} values, half-maximal inhibitory concentrations). References: Abdelrahman et al.,⁷⁵ Müller et al.,¹²⁷ Matsumara et al.,¹²³ Toti et al.,¹²⁴ Hernandez-Olmos et al.,¹²⁶ Nagel et al.,¹²⁹ Werner et al.,¹³¹ Tian et al.,¹³² Ase et al.,¹³³ Mahmood et al.,¹³⁵; Carnero Corrales et al.¹³⁶

1.7.4 Positive allosteric modulators of the P2X4 receptor

Several studies characterized ivermectin (Figure 16), a macrocyclic natural product, as a PAM of ligand-gated ion channels in vertebrates.^{137–139} Ivermectin has been used in veterinary medicine since 1981, and its antiparasitic properties have also shown promise in human medicine.¹⁴⁰ Of particular note is its efficacy in treating and preventing human onchocerciasis, commonly known as “River Blindness”.¹⁴¹ Khakh et al. investigated the effect of ivermectin on P2X4 receptors, revealing that ivermectin enhances ATP-evoked currents at homomeric P2X4 receptor channels by an allosteric mechanism.¹⁴²

Ivermectin was shown to antagonize the ethanol-mediated inhibition of the P2X4 receptor and has thus been suggested as a drug for the treatment of alcohol abuse disorders.¹⁴³ Additionally, ivermectin was employed as a tool compound in animal studies investigating the role of the P2X4 receptor in multiple sclerosis and Parkinson’s disease.⁴²

P2X4 receptor function can also be modulated by the anthraquinone derivative Cibacron Blue (Figure 16), which is the *ortho*-isomer of Reactive Blue 2.¹⁴⁴ However, this modulation is not specific for the P2X4 receptor subtype.^{145,146} Electrophysiological studies and radioligand binding experiments revealed a biphasic allosteric modulation of the rat P2X4 receptor by Cibacron Blue.^{144,145} At low concentrations (ranging from 3 μ M to 30 μ M), it activates the P2X4 receptor, whereas at high concentrations, it inhibits the receptor ($IC_{50} > 300 \mu$ M).^{144,145}

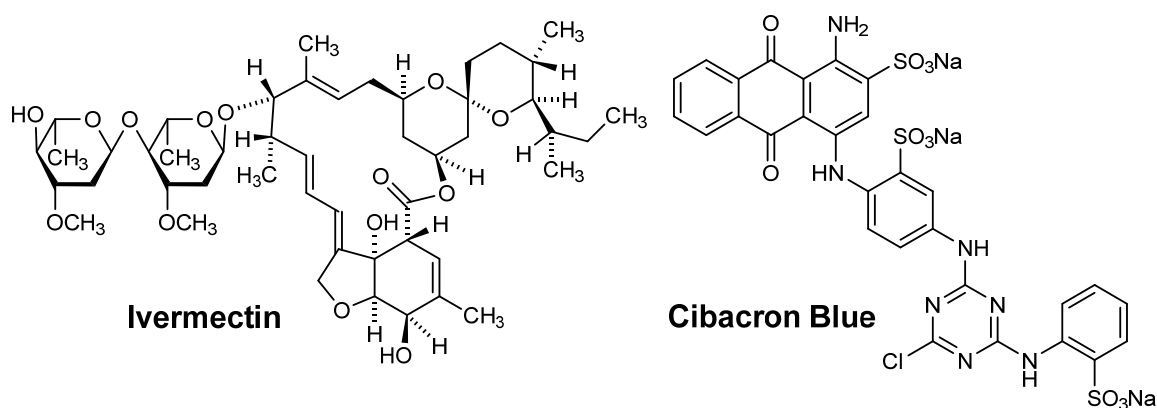


Figure 16. Chemical structure of ivermectin and Cibacron Blue.

1.7.5 Allosteric binding sites of the P2X4 receptor

Various approaches have been employed to identify allosteric binding sites of the P2X4 receptor, mainly with the aim to facilitate the development of P2X4 receptor antagonists. These approaches include mutagenesis studies, docking studies, electrophysiological experiments, calcium influx assays, and structural investigations.

As previously described, the urea derivative BX430 is a potent P2X4 receptor antagonist.¹³³ Despite efforts by several groups to identify the binding pocket of BX430, inconsistent results have been described, possibly due to variations in experimental setups or due to the existence of multiple binding sites for BX430.^{27,134,147}

Ase et al. conducted mutagenesis studies and electrophysiological analysis to investigate the putative participation of amino acids Tyr300, Asp88, and Ile312 in BX430 binding, with the phenolic group of Tyr300 proposed to forming a hydrogen bond with the keto group of BX430.¹⁴⁷ Our group generated chimeric receptors of the human P2X4 and P2X2 receptor and single P2X4 receptor mutants, and assessed BX430 inhibition using calcium influx assays.¹³⁴ The results postulated a different binding site for BX430, located at the dorsal fin of the receptor, with Ile209 as an important key residue.¹³⁴

Recently, two cryo-EM structures of the zebrafish P2X4 receptor in complex with BX430 and BAY-1797 were resolved at resolutions of 3.2 Å and 3.4 Å, respectively, identifying a common allosteric binding site for both antagonists at the subunit interface in the extracellular receptor domain (Figure 17).²⁷ Amino acid Ile312 in human P2X4 receptor (corresponding to Ile315 in zebrafish P2X4 receptor) was assumed to participate in BX430 binding, as observed in the cryo-EM structure where BX430 interacts via hydrophobic interactions with Ile315 (Figure 17).^{27,147} Thus, the previously proposed binding site of BX430 at the dorsal fin, with Ile209 as an important key residue, could not be confirmed by the cryo-EM structure.^{27,134} The cryo-EM structure revealed extensive hydrophobic interactions involving Ile212 in the zebrafish P2X4 receptor (corresponding to Ile209 in the human P2X4 receptor) and neighboring residues.²⁷ The reduced BX430 sensitivity at the P2X4-I209A receptor mutant might result from altered protein folding that indirectly affects BX430 potency.¹³⁴

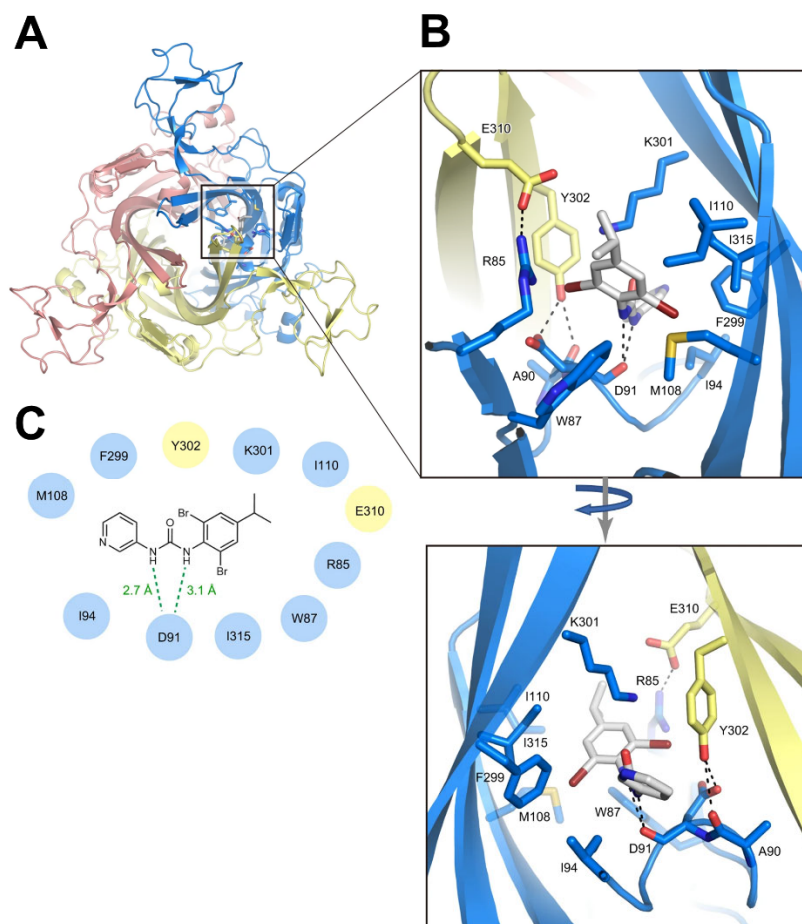


Figure 17. BX430 binding pocket.²⁷ A. Cryo-EM structure of the zebrafish P2X4 receptor in complex with BX430, viewed from the extracellular side. The stick representation in the structure shows the BX430 molecule and the amino acid residues involved in its binding from one of the three equivalent binding pockets (PDB ID: 8jv5). B. Close-up views of the BX430 binding pocket and interactions with amino acid residues. Dotted lines indicate hydrogen bonds. C. Schematic representation of the interactions between the zebrafish P2X4 receptor and BX430. Hydrogen bonds between the amino groups of BX430 and the main-chain carbonyl group (D91) of the zebrafish P2X4 receptor are shown as green dotted lines. Figures were taken from Shen et al.²⁷ The work is licensed under the [Creative Commons Attribution 4.0 International License](#).

Amino acid Ile312 in the human P2X4 receptor (corresponding to Ile315 in the zebrafish P2X4 receptor) was previously proposed to be responsible for the unexpected species differences that were observed for BX430, being a potent antagonist at the zebrafish P2X4, but not at the rat and mouse P2X4 receptors.¹⁴⁷ In the mouse and rat P2X4 receptors, Ile312 is replaced by threonine. Indeed, these results were confirmed by the cryo-EM structure of the zebrafish P2X4 receptor, which showed hydrophobic interactions between BX430 and Ile312.²⁷

An earlier study found that the dibromo-isopropylphenyl group of BX430 is oriented towards the inner side of the receptor, which contrasts with the cryo-EM structure, where it is oriented towards the outer side of the receptor.¹⁴⁷ Induced-fit docking studies of BX430 also did not align with the cryo-EM structure, suggesting that amino acids Lys298 and Tyr299 in the human P2X4 receptor do not interact with BX430.¹⁴⁸ The cryo-EM structure showed that the corresponding amino acids in the zebrafish P2X4 receptor, Lys301 and Tyr302, indeed form interactions with BX430.²⁷ Comparison of the structure of the zebrafish P2X4 receptor in its apo and BX430-bound states revealed that in the apo state, the side chain of Lys301 is oriented towards the inside of the receptor, causing steric hindrance with the pyridine group of BX430.^{24,27} Additionally, the intersubunit binding pocket in the apo state is too narrow to accommodate allosteric modulators.^{24,27} To investigate the mechanism further, molecular dynamic simulations were performed, showing that the binding pocket of the P2X4 receptor expands upon the accommodation of modulators, leading to structural changes in the head domain and a conformational stabilization of the upper body domain.²⁷ As a result, the conformational changes required for channel activation are prevented.²⁷

Although there have been discrepancies in reported findings regarding the binding site of BX430, all studies have contributed to a deeper understanding of the negative allosteric modulation of P2X4 receptors.

1.7.6 P2X4 receptor as a drug target

1.7.6.1. Neuropathic pain

Neuropathic pain is a debilitating condition resulting from lesions or diseases affecting the somatosensory nervous system. This causes widespread changes in the nervous system, including alterations in nociceptive signaling, and is characterized by spontaneous pain and an increased pain sensitivity, known as allodynia.^{119,149} Currently available analgesics are largely ineffective in treating neuropathic pain.¹⁴⁹

Accumulating evidence has indicated the pivotal role of the P2X4 receptor in the pathogenesis of neuropathic pain, thus representing a promising drug target.¹⁵⁰ Tsuda et al. demonstrated that microglia are activated in the dorsal horn of the spinal cord subsequent to peripheral nerve injury (PNI), leading to an elevated expression of the P2X4 receptor (Figure 18).⁴³ Notably, they showed that pharmacological blockade of spinal P2X4 receptors reverses tactile allodynia, a type of pain hypersensitivity caused by innocuous stimuli, which is a common symptom of nerve damage-induced pain.⁴³

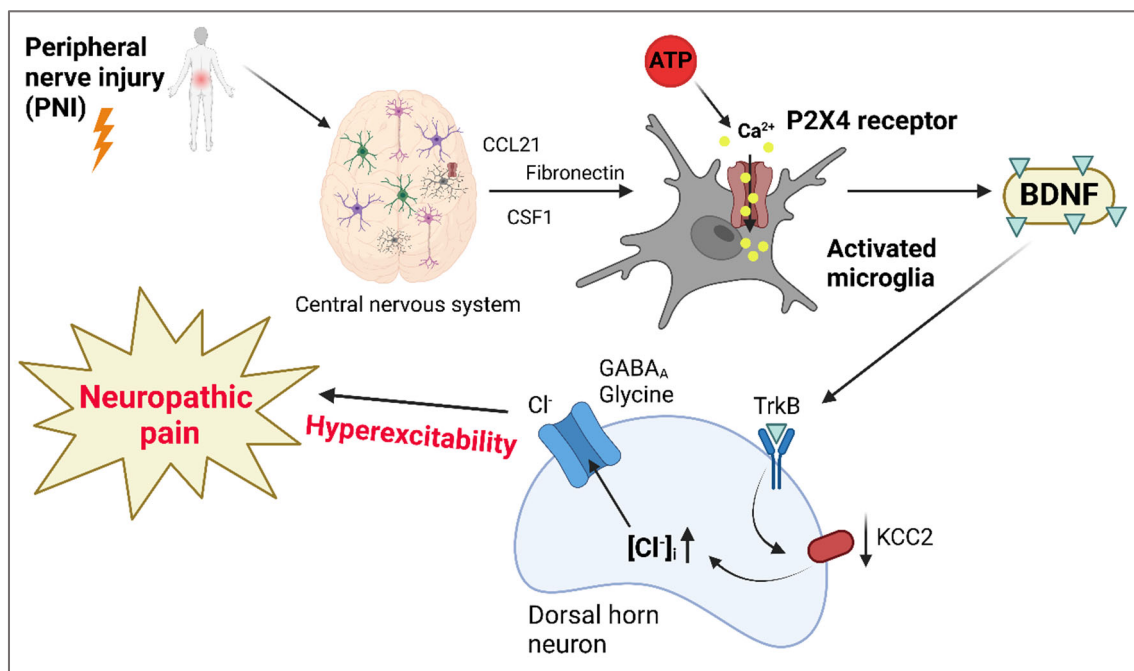


Figure 18. A schematic representation of the proposed signaling pathways underlying the pathogenesis of neuropathic pain starting from peripheral nerve injury (PNI), with microglial P2X4 receptors as key drivers. Abbreviations: CCL21, chemokine ligand 21; CSF1, colony-stimulating factor 1; ATP, adenosine triphosphate; BDNF, brain-derived neurotrophic factor; TrkB, tropomyosin-related kinase B; KCC2, K⁺/Cl⁻ cotransporter 2;

[Cl⁻]_i, intracellular chloride concentration; GABA_A, γ -aminobutyric acid type A. The references are described in the text. The figure was created with BioRender.com.

Efforts have been made to identify signaling pathways in neuropathic pain (Figure 18). The upregulation of microglial P2X4 receptor expression following PNI involves extracellular factors, including chemokine ligand 21 (CCL21),¹⁵¹ colony-stimulating factor 1 (CSF1),¹⁵² and fibronectin.¹⁵³ Stimulation of microglial P2X4 receptors was reported to promote the synthesis and release of brain-derived neurotrophic factor (BDNF).^{154,155} BDNF acts on the tropomyosin-related kinase B (TrkB) receptor located on nociceptive projection neurons, downregulating the expression of K⁺/Cl⁻ cotransporter 2 (KCC2), which maintains the transmembrane chloride gradient required for GABA_A or glycine-induced hyperpolarization.¹⁵⁶ This downregulation leads to increased intracellular chloride (Cl⁻) concentrations, causing a depolarizing shift in the anionic gradient.¹⁵⁴ Subsequently, the opening of Cl⁻ channels is triggered by the release of GABA from inhibitory interneurons after injury, leading to abnormal neuronal excitation and pain hypersensitivity.¹⁵⁴ These signaling pathways were described as being associated with neuropathic pain.¹⁵⁴ Teixeira et al. showed that the activation of P2X4 receptors is also essential in the PNS for the development of neuropathic pain, in addition to their role in the CNS.¹²⁸ In particular, they demonstrated that activation of P2X4 receptors in the dorsal root ganglia is essential for diabetes-induced neuropathic mechanical hyperalgesia, a common complication of diabetes mellitus.¹²⁸

1.7.6.2. CNS disorders

Increasing evidence indicates the involvement of neuronal and microglial P2X4 receptors, respectively, in the pathogenesis of CNS disorders. Table 2 summarizes a range of CNS disorders in which a participation of P2X4 receptors has been reported.

Table 2. P2X4 receptor function in selected CNS disorders. Abbreviations: CNS, central nervous system; A β , beta-amyloid fragment; BDNF, brain-derived neurotrophic factor; TrkB, tropomyosin-related kinase B; ALS, Amyotrophic lateral sclerosis.

CNS disorder	Features	P2X4 receptor function
Alzheimer's disease	Synaptic dysfunction and neuronal death by an overproduction of the beta-amyloid fragment (Aβ)¹⁵⁷ Progressive and irreversible neurodegeneration¹⁵⁷ Symptoms: progressive loss of memory and function impairments¹⁵⁸	Overexpression of neuronal P2X4 receptors enhances the toxic effect of Aβ and contributes to neuronal cell death¹⁵⁷ Therapeutic potential: P2X4 receptor antagonists¹⁵⁷
Multiple sclerosis	Chronic inflammatory disease of the central nervous system¹¹⁷ Demyelination, axonal damage, neurodegeneration¹¹⁷ Symptoms (wide-ranging): vision problems, muscle spasms and stiffness, mobility problems	Release of BDNF by microglial P2X4 receptors promotes remyelination¹¹⁷ Potential of P2X4 receptor signaling favors a switch of microglia/macrophage to an anti-inflammatory phenotype that leads to higher remyelination¹¹⁷ Therapeutic potential: P2X4 receptor potentiation¹¹⁷
Parkinson's disease	Degeneration of dopaminergic neurons¹⁵⁹ Symptoms: tremor, muscle stiffness, balance and coordination difficulties	P2X4 receptor activation is associated with degeneration of dopaminergic neurons¹⁵⁹ Contribution of the P2X4 receptor to neuronal autophagy through the BDNF/TrkB signaling pathway¹⁵⁹ Therapeutic potential: P2X4 receptor antagonists¹⁵⁹

CNS disorder	Features	P2X4 receptor function
Amyotrophic lateral sclerosis	Progressive motor neuron disease, degeneration of motor neurons¹⁶⁰ Neuroinflammation, cellular degeneration, accumulation of intraneuronal protein aggregates Symptoms: muscle weakness, paralysis	P2X4 receptor upregulation in degenerating motor neurons in ALS mouse and rat spinal cord, and in ALS microglia mice^{160–162} Motor neurons are reportedly protected by P2X4 receptor activation¹⁶³ Therapeutic potential: P2X4 receptor activation¹⁶³
Ischemia	Reduction in cerebral blood flow Acute release of excessive ATP Secondary brain injuries: brain edema, blood-brain-barrier leakage	Upregulation of microglial P2X4 receptors^{164,165} Modulation of inflammatory responses after stroke¹⁶⁴ Therapeutic potential: P2X4 receptor antagonists¹⁶⁶ (in the acute phase of stroke)^{164,167}
Epilepsy	Chronic neurological disorder characterized by spontaneous recurrent seizures¹⁶⁸ Dysregulation of neurotransmitter levels, abnormal neuronal activity in the brain, imbalance of excitatory and inhibitory neurotransmitters¹⁶⁸ Main symptom: life-threatening status epilepticus with continuous seizures	Upregulation of the P2X4 receptor on hippocampal microglia after status epilepticus¹⁶⁹ P2X4 receptor knock-out mice: reduction in neuronal death and microglial activation after status epilepticus, no effect on seizures¹⁶⁹ Therapeutic potential: P2X4 receptor antagonists¹⁶⁹

1.7.6.3. Cancer

P2X4 receptor function has been associated with tumorigenesis in certain cancer types, presenting a pharmaceutically significant drug target.^{44-47,170,171}

According to previous studies indicating the involvement of the P2X4 receptor in prostate cancer, He et al. investigated its distinct role in more detail.^{45,172} Quantitative reverse transcription polymerase chain reaction and calcium influx assays confirmed that the P2X4 receptor is the most highly expressed P2 receptor in prostate cancer cells.⁴⁵ Two P2X4 receptor inhibitors, 5-BDBD and PSB-12062, were tested in vivo and in vitro to evaluate the function of the P2X4 receptor in prostate cancer cell proliferation, viability, and apoptosis.⁴⁵ Indeed, the results demonstrated that P2X4 receptor inhibition significantly decreased the proliferation and viability of prostate cancer cells, although it did not affect their apoptosis.⁴⁵ Other studies corroborated the specific role of the P2X4 receptor in prostate cancer and additionally showed that direct activation of the receptor alone is sufficient to promote prostate cancer migration and invasion.¹⁷¹ Further investigation is needed to determine the specific P2X4 receptor signaling pathway involved in prostate cancer.

Upregulation of the P2X4 receptor was observed in breast cancer biopsies, primarily localized in endolysosomes.⁴⁴ Studies associated the P2X4 receptor with tumor invasion in vitro and mammary tumor growth in vivo.⁴⁴ It was also found to play a distinct role in autophagy, which is a cellular process that helps cancer cells to survive metabolic stress during the metastatic progression of breast cancer.⁴⁴ Lysosomes play a critical role in degrading intracellular components released by autophagy.^{44,173} Studies showed that the P2X4 receptor regulates autophagy and promotes mammary cancer cell survival under metabolic challenges.⁴⁴ These findings implicated that endolysosomal P2X4 receptors are critical drivers of mammary cancer progression.⁴⁴

The first observations of purinergic signaling involvement in gastric cancer pathogenesis were made by noticing changes in the expression of purinergic P2Y₂ and P2X4 receptors in tumors from gastric cancer patients.¹⁷⁰ However, a high degree of variation was observed between the analyzed tumors.¹⁷⁰ Further studies examined the gene expression of purinergic receptors on different gastric cancer cell lines, confirming a higher expression level of P2Y₂ receptors compared to P2X4 receptors.¹⁷⁴ Molecular interventions that modify P2Y₂ and P2X4 gene expression in gastric cancer-derived cell lines showed that

P2Y₂ receptor overexpression induced cell proliferation, while P2X₄ receptor overexpression had an antiproliferative effect.¹⁷⁴ These results described a potential relationship between the expression of P2Y₂ and P2X₄ receptors in gastric cancer, suggesting that the P2Y₂/P2X₄ receptor ratio may be used as a biomarker for gastric cancer.¹⁷⁴

Extracellular vesicles (EVs) play a crucial role in intercellular communication, particularly in cancer cells, which release more EVs than normal cells.¹⁷⁵ Tumor-derived EVs contain proangiogenic factors that are prominent targets in cancer therapy.¹⁷⁵ A proteomic analysis of cells stimulated with tumor-derived EVs showed an increase in P2X₄ receptor expression.¹⁷⁵ According to the study, inhibition of the P2X₄ receptor prevented the transport of lysosomal P2X₄ receptors to the cell membrane, leading to reduced tumor angiogenesis.¹⁷⁵ Based on these findings, the role of the P2X₄ receptor in the metabolism of clear cell renal cell carcinoma (ccRCC) cell lines was investigated.⁴⁶ The results showed that the P2X₄ receptor regulated the mitochondrial activity in ccRCC cells in vitro, in a patient-derived organoid model, and in vivo.⁴⁶ The P2X₄ receptor was considered a key mediator of the interaction between lysosomes and mitochondria in tumor metabolism through Ca²⁺ homeostasis, pointing to a putative therapeutic target against ccRCC.⁴⁶

The increased apoptosis of cancer cells is associated with the paracrine activation of cells within the tumor microenvironment, initiating tissue repair programs that are conducive to tumor growth.¹⁷⁶ During an investigation of possible signaling pathways in patient-derived colorectal tumor organoids, it was discovered that the mTOR (the mammalian or mechanistic target of rapamycin) pathway, a prominent pathway in cancer, is mainly involved.^{47,177} mTOR inhibitors have shown great promise in cancer therapy, but resistance mechanisms can limit their effectiveness as a cancer monotherapy.¹⁷⁰ Additional research on colorectal tumors uncovered that chemotherapy-induced colon cancer cell death led to the release of ATP, which triggered the P2X₄ receptor-mediated mTOR-dependent survival process in surrounding cancer cells.⁴⁷ Given the limitations of mTOR inhibitors in cancer therapy, the P2X₄ receptor may offer a more tolerable alternative strategy, at least as part of a combinatorial anti-cancer therapy approach.⁴⁷

Waldmann's research group aimed to discover autophagy inhibitors due to the key role autophagy plays in the pathogenesis of various diseases.^{136,178} Using a phenotypic assay on the human breast cancer cell line MCF7, they identified the indoline derivative

indophagolin as a potent inhibitor of autophagy.¹³⁶ To uncover potential targets and mechanisms of indophagolin, a technique known as thermal proteome profiling was applied.^{136,179} This technique enables the monitoring of thermal stability across the whole proteome using quantitative mass spectrometry. The P2X4 receptor was identified as a potential target of indophagolin, thereby presenting a direct link between the P2X4 receptor and autophagy that had not previously been demonstrated.¹³⁶ Subsequent calcium influx assays confirmed indophagolin's inhibitory effects on P2X4 receptor function.¹³⁶ Moreover, indophagolin was found to antagonize the function of other purinergic receptors, suggesting that its inhibition of autophagy may involve a polypharmacological mechanism.¹³⁶

1.8 Methods

1.8.1 Ion channel function and ligand interactions

Different assay systems are used to characterize P2X receptor ligands, evaluate their potency, and identify their binding sites. These assays also contribute to the understanding of P2X receptor function.

The calcium influx assay is one of the most widely applied assays to study P2X receptor function. Cell-permeable fluorescent dyes are used to measure Ca^{2+} signals induced by P2X receptors. The development of fluorescent indicators goes back to 1989.¹⁸⁰ Nowadays, the market provides various fluorescent indicators with improved properties, e.g., Fluo-4 acetoxymethyl ester (Fluo-4 AM), exhibiting high fluorescence emission and a high rate of cell permeation in vitro.¹⁸¹ These indicators consist of a Ca^{2+} -chelating moiety conjugated to a fluorogenic, fluorescein-like component. In the absence of Ca^{2+} , the fluorescence of the conjugated fluorophore is quenched by photo-induced electron transfer from the Ca^{2+} chelator.¹⁸¹ As Ca^{2+} levels rise, this phenomenon is inhibited, resulting in an increase in fluorescence intensity.¹⁸¹ In brief, the respective cells (e.g., P2X-receptor expressing cells) are incubated with the fluorescent dye solution. The acetoxymethyl ester group enables the fluorophore to enter the cells where it is hydrolyzed by esterases and, therefore, trapped inside the cell (Figure 19). Then, stimulation with an agonist leads to an influx of Ca^{2+} ions through the ion channel, and a Fluo-4- Ca^{2+} chelate complex is formed, which increases fluorescence (Figure 19). This resulting fluorescence is recorded in real-time using a fluorescence imaging plate reader. Fluorescent dyes are also suitable for photometric and

imaging applications, including flow cytometry, spectrofluorometry, or confocal laser scanning microscopy.¹⁸¹

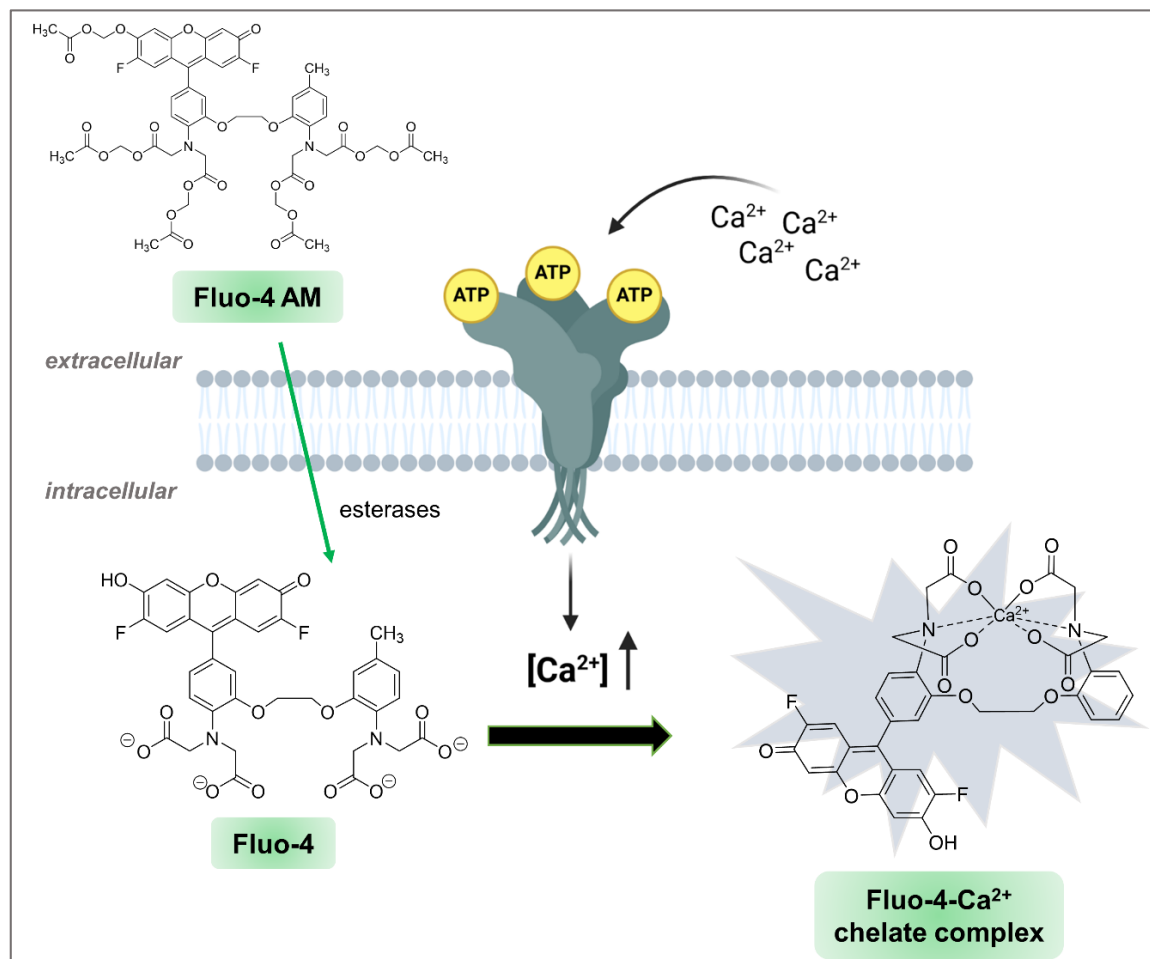


Figure 19. Schematic representation of the mechanisms in ATP-induced calcium (Ca²⁺) influx assays using the fluorescent dye Fluo-4 acetoxymethyl ester (AM). References are described in the text. Parts of the figure were created with BioRender.com.

Calcium influx assays allow the determination of functional parameters such as EC₅₀ and IC₅₀ values, for agonists and antagonists, respectively. This is achieved by measuring the calcium signal at varying concentrations of the agonist or antagonist and plotting the results to obtain a concentration-response curve. In the case of antagonists, the receptor inhibition is measured in the presence of the agonist. For agonists, it is also possible to determine the efficacy, referred to as E_{max}. E_{max} represents the maximal effect that an agonist produces in relation to a control, thus enabling the discrimination between partial and full agonists.

Calcium influx assays have proven to be extremely useful in high-throughput screening to identify P2X receptor ligands.^{75,182}

Electrophysiological measurements using patch-clamp techniques are the gold standard for examining ion channels, directly recording ion channel activity.¹⁸³ They are applied for the pharmacological testing of compounds and for determining functional parameters.¹⁸³ By forming a tight seal between a glass micropipette and the cell membrane, patch clamp recordings enable precise control and measurement of ion currents. This technique allows the recording of whole-cell or single-channel currents flowing across biological membranes through ion channels in real-time and is applicable for native and recombinantly expressed ion channels.¹⁸³ Compared to calcium influx assays, electrophysiological measurements have a much lower throughput and require complex and expensive experimental setups. Additionally, this technique is time-consuming and requires skilled operators.¹⁸³ Considering the low throughput, automated patch-clamp techniques were developed to enable the study of ion channels with much greater throughput capacities.¹⁸⁴

In drug development and in vitro pharmacology, a combination of calcium influx assays and electrophysiological experiments is commonly applied to ensure reliable and robust results.¹⁸³

Radioligand binding assays are also used to explore the pharmacological properties of P2X receptors and to identify novel modulators. However, they have not been typically used for high-throughput screening to identify P2X receptor ligands.¹⁸³ These assays focus on binding parameters and are also employed to identify binding sites and binding kinetics.

The analysis of radioligand binding assays is based on the law of mass action, which describes the interaction between one ligand molecule and one receptor molecule.¹⁸⁵ To perform radioligand binding assays, a high-affinity radioligand and a high receptor expression are required.¹⁸⁵ In the case of a filtration assay, the radioligand is combined with an unlabeled compound and a protein source (e.g., membrane preparation) and incubated until the binding equilibrium is achieved. The reaction is terminated by a filtration process that removes unbound radioligand. The remaining radioactivity resulting from the bound radioligand is measured using a liquid scintillation counter.

Different assay setups are performed to obtain parameters, including the binding affinity (K_D) and an estimate of the number of binding sites in a given tissue, the B_{max} value.¹⁸⁵ The

K_D is the equilibrium dissociation constant, which is the concentration of the radioligand that occupies 50% of the receptors.¹⁸⁵ Apart from that, important kinetic parameters can be obtained by association and dissociation assays to monitor the binding kinetics of the ligand. Competition assays are performed with a constant radioligand concentration and various concentrations of an unlabeled ligand.¹⁸⁵ The activity of the test compound is indicated by the displacement of the radioligand. These assays are applied to identify the binding site of a ligand or to determine affinities, represented as K_i values, which are equilibrium dissociation constants for the binding of the unlabeled drug. In other words, the K_i value defines the concentration of the unlabeled ligand in a competition assay that binds to half of the binding sites at equilibrium in the absence of the radioligand or other competitors.¹⁸⁵ Furthermore, radioligands are valuable tools for receptor imaging in vitro and in vivo, enabling techniques such as autoradiography, scintigraphy (SPECT, single photon emission computer tomography), and positron emission tomography (PET) studies.¹⁸⁶

1.8.2 Structural analysis of P2X receptors

Deciphering the three-dimensional structure of membrane proteins holds immense importance, facilitating structure-based drug design and enhancing our understanding of cellular processes. Over the last decades, two main techniques have emerged in the field of structural biology for membrane proteins: X-ray crystallography and cryo-EM.¹⁸⁷

X-ray crystallography has been widely considered as the primary method for determining the three-dimensional structure of proteins. The fundamental principle involves the interaction of crystalline atoms with a stream of X-rays, causing diffraction in numerous specific directions.¹⁸⁸ The resulting X-rays that are scattered by the molecule are then analyzed in terms of angles and intensities to construct a three-dimensional representation of the electron density within the crystal.¹⁸⁸ The electron density image enables the determination of the average position of the atoms in the crystal, as well as the characterization of chemical bonds, structural disorders, and various other relevant information.¹⁸⁸ X-ray crystallography begins with the challenge of coaxing the protein of interest to form crystals. The formation of well-diffracting crystals by macromolecules remains a significant obstacle in X-ray crystallography.¹⁸⁸

Cryo-EM is a newer concept in structural biology and has emerged as a revolutionary technique for resolving structures of biomolecules. In 2017, Jacques Dubochet, Joachim

Frank, and Richard Henderson were awarded the Nobel Prize in Chemistry for their work on developing cryo-electron microscopy for high-resolution structure determination of biomolecules.¹⁸⁹ Since then, significant advancements have been made in sample preparation, electron microscopes, hardware, software, and data processing algorithms to improve the resolution and efficiency of cryo-EM.¹⁸⁹ One of the main approaches in cryo-EM is the single particle analysis (SPA).¹⁸⁹ In brief, the underlying principle involves the transmission of high-energy electrons through frozen protein samples, which generates a projection image of the sample.¹⁸⁹

The process begins with the membrane protein of interest, typically overexpressed in cells, followed by solubilization and extraction from the membrane, purification, and assessment for purity and monodispersity. The standard sample preparation method in cryo-EM is plunge freezing or vitrification. This involves blotting of the protein solution onto a small carbon grid and immediately freezing it in liquid nitrogen and ethane (Figure 20).¹⁸⁹ The market offers a wide range of different grids in varying materials and sizes. Nowadays, laboratories are equipped with commercial devices to perform sample vitrification.

The sample preparation is the main bottleneck in cryo-EM and is associated with several problems.¹⁹⁰ The most common issues during sample preparation include insufficient particle density or distribution in thin ice, protein aggregation, denaturation, or complex disassembly.¹⁹⁰ Additionally, preferential particle orientation on the grid surface due to interactions with the air-water or water-support film interfaces can also pose obstacles.¹⁹⁰

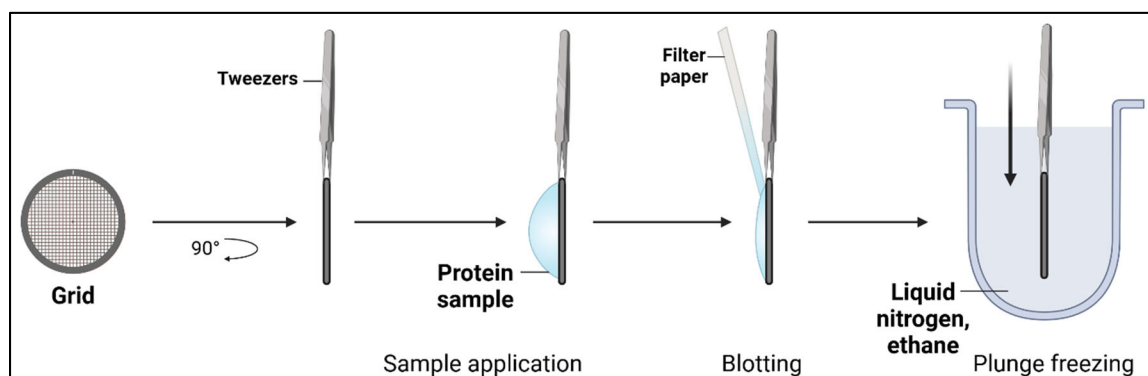


Figure 20. Simplified illustration of the plunge freezing or vitrification process in cryo-EM sample preparation. The figure was created with BioRender.com.

Prior to data acquisition, the sample undergoes an initial screening using an electron microscope to assess sample quality, including protein concentration, particle distribution, and ice thickness across the specimen grid.¹⁹⁰ Areas with an appropriate ice thickness and particle distribution are then selected for data acquisition. The sample is exposed to an electron beam, resulting in a collection of thousands of images.¹⁹⁰

These images are computationally aligned and averaged to improve the signal-to-noise ratio and to reveal higher resolution features of the particles.¹⁸⁹ Complex algorithms allow the sorting of particles into multiple classes based on their different structural appearances.¹⁸⁹ Subsequently, the spatial orientations of all two-dimensional projections are determined and the information is used to form a three-dimensional reconstruction of the protein structure.¹⁸⁹

Both X-ray crystallography and cryo-EM have their strengths and limitations. The advantages of X-ray crystallography include rapid data acquisition, high throughput, and rapid analysis of high-resolution data.¹⁸⁷ In contrast, the data collection and subsequent computational analysis in cryo-EM are more time-consuming.¹⁸⁷ The two methods rely on different physical and mathematical principles. In X-ray crystallography, crystals are exposed to a beam of X-rays, resulting in a diffraction pattern, while cryo-EM employs high-energy electrons as a source to illuminate thin specimens in a transmission electron microscope.¹⁹¹ X-ray crystallography requires large amounts of highly purified protein samples, and protein engineering may be necessary to produce highly ordered three-dimensional crystals and to enhance crystal quality.¹⁹¹ Additionally, high protein stability is essential, as crystal formation can take days or even weeks.¹⁹² In cryo-EM, lower protein amounts are sufficient, and the method does not require well-diffracting crystals, which is a significant advantage.¹⁹² Therefore, protein stability is a less significant concern in cryo-EM as compared to X-ray crystallography. Moreover, cryo-EM is more tolerant of sample quality in terms of some degree of receptor heterogeneity and the presence of contaminants, as the processing of cryo-EM images can separate molecules of receptors in the same conformation.¹⁹² However, receptor heterogeneity and contaminants would impede the formation of well-diffracting crystals in X-ray crystallography.¹⁹² Although cryo-EM sample preparation is faster and less demanding as compared to sample preparation in X-ray crystallography, it poses other challenges, as previously described.¹⁹⁰ Another difference between the methods is the lower size limit for protein samples that can be used for structure determination. The study of small membrane proteins (less than 150 kDa) by

cryo-EM is challenging due to the additional complication of the low signal-to-noise ratio of the resulting particles, while in X-ray crystallography, studying smaller membrane proteins is less problematic.¹⁹³

It is important to note that the resolution achieved in X-ray crystallography and cryo-EM is determined by different methods, which makes direct comparisons of results obtained with the two techniques challenging.¹⁸⁷ Nevertheless, both methods have had a profound impact on the study of biological macromolecules.

2. Objective of the thesis

The main objective of this thesis was to conduct a pharmacological and structural characterization of the P2X₄ receptor, with a particular emphasis on identifying allosteric binding sites for P2X₄ receptor antagonists. To accomplish this, a variety of methodologies were employed, including the development of pharmacological assays, calcium influx assays, molecular and cellular biology techniques, and structural biology methods.

Specifically, the main objectives of this thesis were:

- (i) to generate stable 1321N1 astrocytoma cell lines expressing P2X₄ receptor orthologs from a variety of pharmacologically relevant species and to analyze the pharmacology of P2X₄ receptor agonists and antagonists at the various P2X₄ receptor orthologs,
- (ii) to develop a radioligand binding assay for the human P2X₄ receptor based on a novel allosteric P2X₄ receptor antagonist,
- (iii) to identify and characterize new P2X receptor ligands as potential pharmacological tool compounds and future drug candidates,
- (iv) to identify the allosteric binding site of the negative allosteric P2X₄ receptor modulator BX430 by generating and evaluating specific human P2X₄ receptor mutants,
- (v) and finally, to resolve the structure of the human P2X₄ receptor in complex with an anthraquinone-derived P2X₄ receptor antagonist by cryo-electron microscopy.

3. Results and discussion

3.1 Manuscript I: Species differences of P2X4 receptor modulators

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3.1.1 Author contributions

The following results, presented in the form of a manuscript, were produced as a part of this thesis. The author of this thesis performed molecular biology experiments and generated stable cell lines for the human, monkey, guinea pig, pig, rabbit, and zebrafish P2X4 receptors. Most of the calcium influx assays were performed by the author, with support from Christiane Bous. In addition, the author analyzed and interpreted the data, and created all figures and tables in the manuscript, as well as the Supporting Information. The author prepared this manuscript in close collaboration with the corresponding author, Prof. Dr. Christa E. Müller.

3.1.2 Abstract

The P2X₄ receptor is an ATP-gated ion channel receptor that was proposed as a novel drug target for neuropathic pain and cancer. Animal models are indispensable to elucidate the role of receptors in health and disease, and for drug development. Here, we present a systematic analysis and comparison of P2X₄ receptors of nine different species that are relevant for basic and applied research: human, monkey, dog, guinea pig, pig, rabbit, rat, mouse, and zebrafish. 1321N1 Astrocytoma cell lines stably expressing the respective receptor were generated by retroviral transfection. Calcium influx assays were performed to assess the effects of structurally diverse P2X₄ receptor agonists and antagonists with the aim to explore species differences. Most agonists showed significantly reduced potency at mouse, rat, and zebrafish in comparison to the human P2X₄ receptor. The physiological agonist ATP was more potent at monkey, rabbit, guinea pig, and pig P2X₄ receptors (0.0285–0.0594 μ M) than at the human ortholog (0.269 μ M) and least potent at the zebrafish receptor (3,270 μ M). 2-MeS-ATP exhibited similar potency (0.290–4.50 μ M) at all investigated P2X₄ receptor species except zebrafish, and is therefore a suitable tool compound for studying P2X₄ receptors across species. The allosteric P2X₄ receptor antagonists BX430 and 5-BDBD displayed notable species differences, while the indole derivative PSB-OR-2020 blocked all investigated mammalian species with high potency (0.696–6.32 nM), exhibiting only minor species differences. These data will be valuable to support future in vitro and, in particular, in vivo studies.

3.1.3 Introduction

P2X receptors, which belong to the purinergic receptor family, are cation channels activated by the endogenous agonist ATP.¹⁰ The seven P2X receptor subtypes (P2X₁–P2X₇) are known to form functional homotrimeric or heterotrimeric receptor complexes.^{10,11}

Extracellular ATP, acting as a signaling molecule, is released by transporter-mediated mechanisms or from damaged cells and exerts proinflammatory and immunostimulatory effects.⁴¹ P2X receptor expression is often upregulated under inflammatory conditions.⁴¹ The P2X₃ receptor antagonist gefapixant has been the first P2X receptor antagonist to be approved as a therapeutic drug, namely for the treatment of chronic cough.^{55,194} Further P2X₃ receptor antagonists are currently in clinical development.^{195,196} Moreover, a P2X₇ receptor antagonist is clinically evaluated for the treatment of major depression.¹⁹⁷

The P2X4 receptor has gained attention due to its role in neuroinflammation.⁴² It is highly expressed in various cells of the central nervous system, including astrocytes, neurons, and microglia, and was first isolated from rat brain.^{42,107} Advances in its structural elucidation, including X-ray crystallography^{24,25} and cryo-EM studies²⁷ of the zebrafish P2X4 receptor confirmed its previously proposed homotrimeric structure containing three extracellular ATP-binding sites, one per receptor monomer.¹¹ These findings provided the first structural insights into the ATP-induced activation of P2X4 receptors, uncovering a receptor subunit rearrangement upon ATP binding leading to the opening of the ion channel for Na⁺, Ca²⁺, and K⁺ ions.²⁵

Several studies on P2X4 receptors in the context of neuroinflammation have identified it as a potential drug target for the treatment of various neuropathologies.⁴² Researchers have documented that the upregulation of P2X4 receptor expression on microglia is a key process in neuropathic pain, which follows nerve injury and microglial cell activation.^{155,198} A potential contribution of increased levels of P2X4 receptors in reactive microglia and neurons, respectively, has also been observed in the pathogenesis of neurodegenerative diseases such as Alzheimer's¹⁵⁷ and Parkinson's disease,¹⁵⁹ epilepsy,¹⁶⁹ multiple sclerosis,¹¹⁷ stroke,¹⁶⁷ and amyotrophic lateral sclerosis.¹⁶⁰ Moreover, P2X4 receptors appear to contribute to the pathogenesis of various types of cancer.^{44,45,174} This range of complex diseases emphasizes the potential of P2X4 receptors as drug targets.

In addition to the endogenous agonist ATP, further nucleotides have been characterized as P2X4 receptor agonists, including α,β -methylene-ATP,¹⁰⁶ 2-MeS-ATP,¹⁰⁶ BzATP,¹⁹⁹ and Ap4A (Figure 21).²⁰⁰ For some of them, significant differences in rodents and dogs as compared to human P2X4 receptors have been noted.^{42,75}

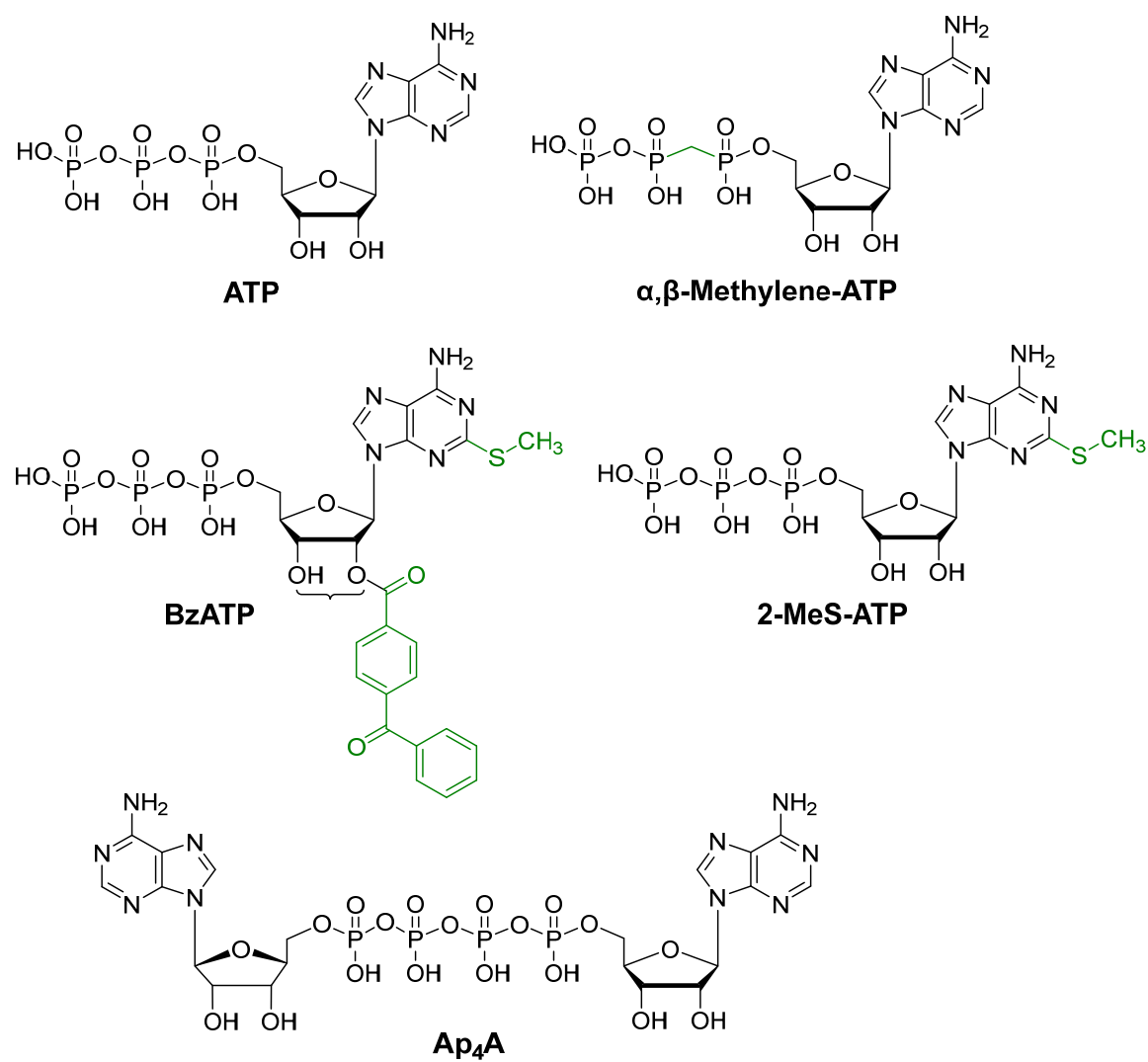


Figure 21. Structures of ATP, its derivatives, and analogs investigated in the present study.

The identification and development of P2X4 receptor antagonists has been challenging, and potent and selective P2X4 receptor antagonists have only recently been reported. Allosteric antagonists include 5-BDBD,²⁰¹ NP-1815-PX,¹²³ BAY-1797,¹³¹ BX430,¹³³ and its analogs¹³⁵ (Figure 22). Recently, indole derivatives with very high potency and selectivity for the P2X4 receptor have been developed, e.g., PSB-15417 and PSB-OR-2020 (Figure 22).^{127,129} P2X4 receptor antagonists have been essential tools for in vitro research and subsequent in vivo studies. Species differences between human and rodent P2X4 receptors have been described.^{75,133,134} For example, the benzodiazepine derivative 5-BDBD was observed to display much lower potency at mouse and rat P2X4 receptors as compared to the human ortholog.⁷⁵

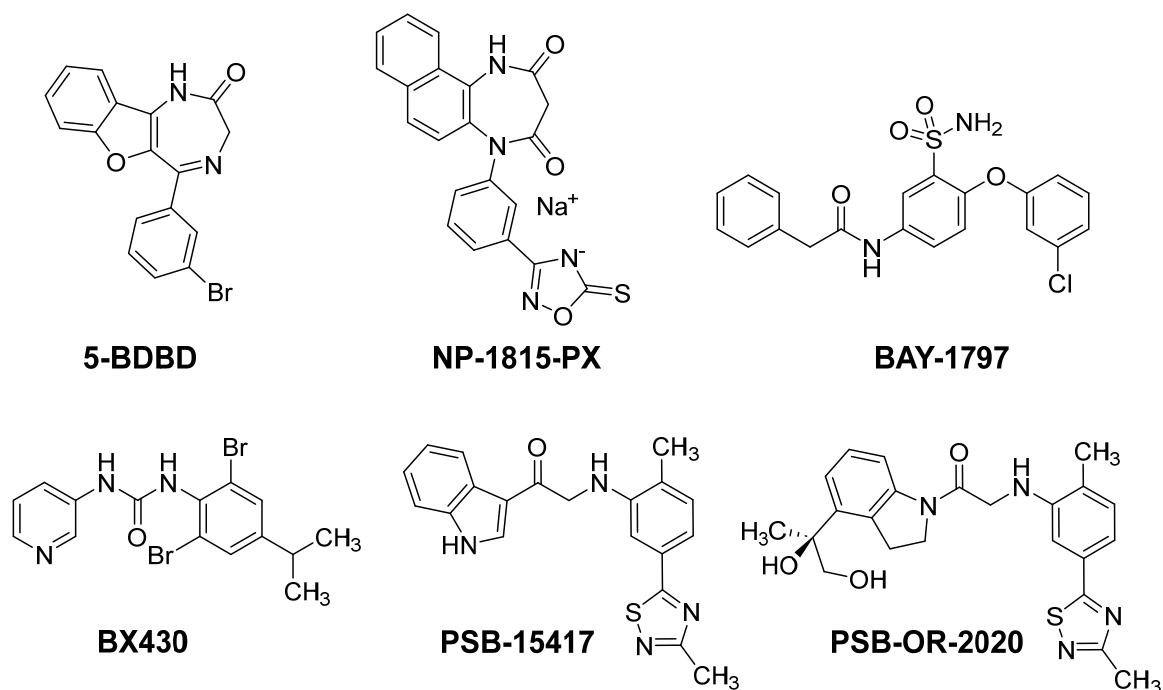


Figure 22. Structures of selected P2X4 receptor antagonists.

Animal models are indispensable in drug research and development, and continue to be the gold standard for investigating drugs before entering clinical trials.²⁰² They provide essential basic pharmacokinetic parameters and information on safety and efficacy.²⁰² Rats and mice are the most frequently used animal models due to their small size, ease of maintenance, and ability to reproduce rapidly and to mature quickly.²⁰³ Other animals used for in vivo studies include dogs, cats, rabbits, pigs, ferrets, and monkeys, as well as guinea

pigs and hamsters.²⁰⁴ Even non-mammalian animals like birds, fish, flies, and worms are regularly employed for early in vivo research.²⁰⁴ Since receptors of lower species can show enhanced stability, they are used for structural studies, as exemplified by the X-ray crystal and the recent cryo-EM structures of the zebrafish P2X4 receptor.^{24,25,27} Dogs are frequently used for investigating cardiovascular diseases because their hearts share many characteristics with those of humans at both the organ and the cellular level.²⁰⁵ The choice of the appropriate animal species typically depends on the specific research question and the targeted indication.²⁰²

While occasional data have been reported on recombinant mouse, rat, dog, bovine, and zebrafish P2X4 receptors expressed in mammalian cell lines,^{75,147,206,207} in addition to data on the human receptor, a broad and systematic study on species differences is lacking. Here, we describe such a study, including the characterization of recombinant human, monkey, dog, guinea pig, pig, rabbit, rat, mouse, and zebrafish P2X4 receptor orthologs. We generated stable recombinant 1321N1 astrocytoma cell lines for each of the P2X4 receptor orthologs and assessed the potency of a range of structurally diverse agonists and antagonists in calcium influx assays. The results revealed significant differences in the effects of P2X4 receptor agonists and antagonists at P2X4 receptor orthologs when compared to the human receptor. These data will be important for the planning and interpretation of in vivo studies on P2X4 receptors.

3.1.4 Results and discussion

Sequence homology of P2X4 receptor orthologs

A wide range of P2X4 receptor species was selected, focusing on P2X4 receptor orthologs that are of particular interest for in vivo experiments. These included primates (monkey, *Rhesus macaque (Macaca mulatta)*), mammals (dog, *Canis familiaris*; pig, *Sus scrofa*; rabbit, *Oryctolagus cuniculus*), rodents (guinea pig, *Cavia porcellus*; mouse, *Mus musculus*; rat, *Rattus norvegicus*), and zebrafish (*Danio rerio*). Investigations on mouse and rat P2X4 receptors were previously reported by our group using essentially the same expression and assay system as presented in this study.⁷⁵ Those data are included for comparison to obtain the whole picture. All of these species were selected to cover a broad range of suitable animals for early and advanced in vivo research.

Table 3. Comparison of protein sequence identity of the human P2X4 receptor with P2X4 receptors of different species.

Protein sequence identity (%) ^a									
	human ^b	mon-key ^c	dog ^d	guinea pig ^e	pig ^f	rabbit ^g	rat ^h	mouse ⁱ	zebra-fish ^j
human	100	97.4	90.0	88.9	88.9	87.6	87.4	86.9	58.3
monkey	97.4	100	89.2	88.4	88.7	87.6	87.4	86.3	58.3
dog	90.0	89.2	100	86.9	88.9	87.8	85.6	86.1	56.5
guinea pig	88.9	88.4	86.9	100	87.1	88.6	87.4	87.4	57.8
pig	88.9	88.7	88.9	87.1	100	87.1	86.9	87.4	58.0
rabbit	87.6	87.6	87.8	88.6	87.1	100	86.5	86.5	58.4
rat	87.4	87.4	85.6	87.4	86.9	86.5	100	95.6	59.1
mouse	86.9	86.3	86.1	87.4	87.4	86.5	95.6	100	57.8
zebrafish	58.3	58.3	56.5	57.8	58.0	58.4	59.1	57.8	100

^a Calculated based on the alignments of the protein sequences using multiple sequence alignment by Clustal Omega (1.2.4).²⁰⁸ UniProt entry numbers or GenBank accession number of protein sequences: ^b human (Q99571), ^c monkey (F7DXP3), ^d dog (A0A8I3Q0A9), ^e guinea pig (AFQ41091.1), ^f pig (A0A286ZYA9), ^g rabbit (G1SH28), ^h rat (P51577), ⁱ mouse (Q9JJX6), ^j zebrafish (Q98TZ0).

Comparison of the protein sequences of the P2X4 receptor orthologs with that of the human P2X4 receptor shows that the monkey receptor exhibits the highest sequence identity (97.4%), as expected, followed by the dog receptor (90.0%) (Table 3). Guinea pig and pig receptor orthologs display a sequence identity of 88.9% with the human receptor, while rabbit, rat, and mouse receptor orthologs exhibit a slightly lower degree of identity (87.6–86.9%) (Table 3). The zebrafish receptor shows the lowest sequence identity in comparison with the human receptor (58.3%) (Table 3).

The protein sequence alignments of the P2X4 receptor orthologs show that the amino acid residues that are directly involved in ATP binding are conserved between all species (Figure 23, yellow-colored amino acid residues), except for the zebrafish P2X4 receptor, which harbors an alanine residue (Ala292) instead of a serine residue (Ser189) as all the other species do. Amino acid residues within 6 Å of the ATP-binding site show variations between P2X4 receptor species (Figure 23; green-colored amino acid residues); the respective amino acid residues have been selected based on previous studies.⁷⁵ Considering these amino acid residues, all investigated P2X4 receptor orthologs harbor Ala68, except for human (Val68), monkey (Val68), and zebrafish (Val71) P2X4 receptors. Further differences are recognized for Ile218, which is replaced by valine in guinea pig and zebrafish P2X4 receptors (Val221) and by threonine in pig and rabbit P2X4 receptors (Thr221). All P2X4 receptor orthologs contain a serine residue (Ser216), except for the pig (Thr216) and zebrafish (His219) P2X4 receptor (Figure 23). The zebrafish P2X4 receptor displays even more variations in amino acid residues within 6 Å of the ATP-binding site in comparison to the human P2X4 receptor and the other species (Figure 23; green-colored amino acid residues).

Figure 23. Protein sequence alignments of human P2X4 receptor and P2X4 receptor orthologs created with Clustal Omega (1.2.4).²⁰⁸ UniProt entry number or GenBank accession number: human (Q99571), monkey (F7DXP3), dog (A0A8I3Q0A9), guinea pig

(AFQ41091.1), pig (A0A286ZYA9), rabbit (G1SH28), rat (P51577), mouse (Q9JJX6), zebrafish (Q98TZ0). Color scheme: yellow, amino acid residues directly interacting with ATP; green, amino acid residues within 6 Å distance of the ATP-binding site. The amino acid residues of the receptors' head domains are underlined in red. Dashed lines above the alignment indicate transmembrane (TM) regions (light blue: TM1; dark blue: TM2). Symbols: (*) indicates conserved residues, (:) and (.) indicate conservative and semi-conservative exchanges, and blank spaces point out non-conservative exchanges of residues between aligned ortholog sequences.

Creating P2X4 receptor-expressing cell lines

A retroviral expression system was used to generate 1321N1 astrocytoma cell lines stably expressing the human, monkey, dog, guinea pig, pig, rabbit, and zebrafish P2X4 receptor, respectively. Rat and mouse P2X4 receptor-expressing cell lines had previously been obtained.⁷⁵ 1321N1 Astrocytoma cells were selected as a host due to the absence of native expression of any functional nucleotide receptors.⁷⁴

The fluorescence signal measured in calcium influx assays applying maximal concentrations of the agonist ATP and using a Ca^{2+} -sensitive dye provided a rough estimation of the receptor expression level in the respective cell line, since it correlates with the number of functional P2X4 receptor ion channels in the cell membrane. The highest fluorescence intensity at an ATP concentration of 100 μM was measured for the dog P2X4 receptor-expressing cell line (see Supporting information, Figure 28). The lowest fluorescence intensity was observed for the rabbit P2X4 receptor-expressing cell line. Upon activation of the zebrafish P2X4 receptor, an ATP concentration of 100 μM yielded no signal, and only at 10 mM, a fluorescence signal could be observed. This is due to the very low potency of ATP at the zebrafish P2X4 receptor. Our results indicated that all P2X4 receptor species were successfully expressed in 1321N1 astrocytoma cells, with similar or only slightly varying expression levels.

Effects of agonists at P2X4 receptor orthologs

Concentration-dependent activation by ATP was subsequently determined using calcium influx assays. ATP activated each newly generated recombinant P2X4 receptor ortholog, and respective EC_{50} and EC_{80} values for ATP were calculated from concentration-response curves (Figure 24, Table 4; Supporting information, Table 7). In the comparative analysis, previously reported data on the recombinant mouse and rat P2X4 receptors determined in our group were included.⁷⁵

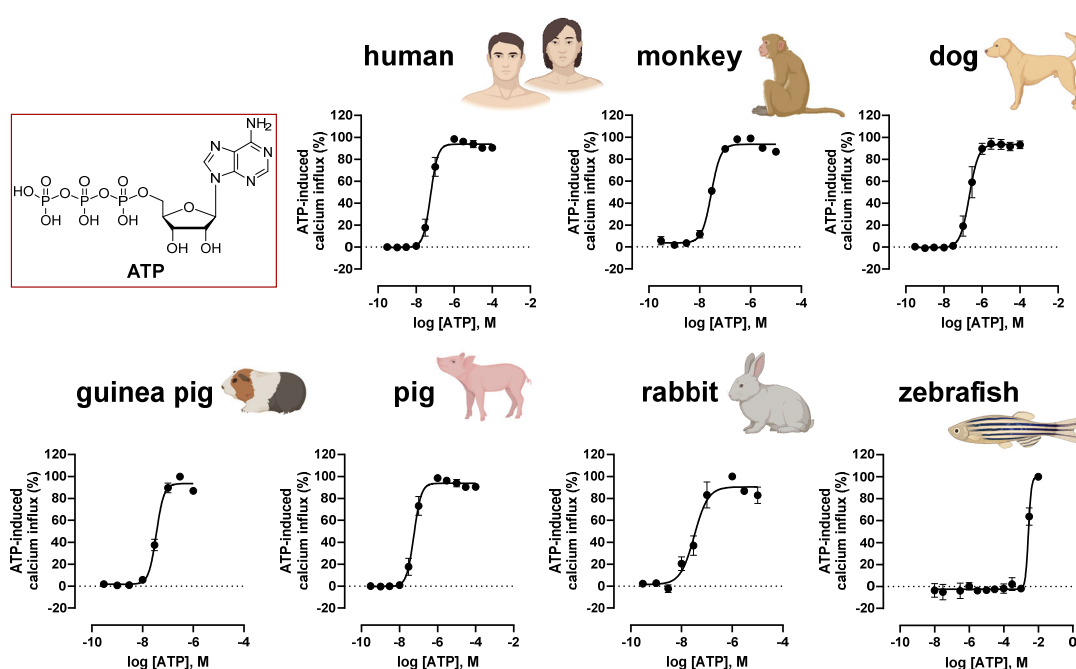


Figure 24. Concentration-response curves of ATP-induced calcium influx assays in recombinant 1321N1 astrocytoma cells stably expressing the human, monkey, dog, guinea pig, pig, rabbit, or zebrafish P2X4 receptor. Data is normalized to the highest ATP-induced effect (100%). Data represent mean $\pm$ SEM from at least three independent experiments performed in duplicates. EC_{50} values are collected in Table 4. Species images were taken from BioRender.com.

3. Results and discussion

Table 4. Potency of P2X4 receptor agonists at the human P2X4 receptor and P2X4 receptor orthologs stably expressed in 1321N1 astrocytoma cells.

	EC ₅₀ ± SEM ^a (μM)				
species	ATP	α,β-Methylene-ATP	BzATP	2-MeS-ATP	Ap4A
human	0.269 ± 0.035	1.24 ± 0.16	0.166 ± 0.012	0.478 ± 0.090	0.223 ± 0.028
monkey	0.0285 ± 0.0002****	0.651 ± 0.030 ^{ns}	0.347 ± 0.069 ^{ns}	0.390 ± 0.080 ^{ns}	0.114 ± 0.010**
dog	0.233 ± 0.069 ^{ns}	3.59 ± 1.47*	1.44 ± 0.40****	0.574 ± 0.192 ^{ns}	0.454 ± 0.034**
guinea pig	0.0371 ± 0.0043****	0.495 ± 0.164*	0.536 ± 0.074**	0.261 ± 0.072 ^{ns}	0.452 ± 0.084**
pig	0.0594 ± 0.0119***	1.83 ± 0.10 ^{ns}	0.715 ± 0.040***	0.290 ± 0.050 ^{ns}	0.224 ± 0.014 ^{ns}
rabbit	0.0296 ± 0.0096****	0.326 ± 0.023**	0.153 ± 0.050 ^{ns}	0.363 ± 0.145 ^{ns}	0.112 ± 0.014**
rat	1.72 ± 0.93 ^b	>100 ^b	>100 ^b	4.50 ± 2.03 ^b	>100 ^b
mouse	0.346 ± 0.029 ^b	>100 ^b	2.92 ± 0.21 ^b	1.35 ± 0.78 ^b	>100 ^b
zebrafish	3,270 ± 730****	>>1000 ^c	>>1000 ^c	>>1000 ^c	>>100 ^c

^a Calculated from concentration-response curves in fluorimetric calcium influx assays. All data are means ± SEM of 2–4 independent experiments performed in duplicates. Results obtained at P2X4 receptor orthologs were compared to results obtained at the human P2X4 receptor. The level of significance was calculated by an ordinary one-way ANOVA with Dunnett's post-hoc test (multiple comparisons test). Significance was expected if P value was ≤ 0.05. The significance levels were defined as follows: * P < 0.05, ** P < 0.01, *** P < 0.001, **** P < 0.0001; ns, not significant. ^b Published EC₅₀ ± SD (μM) for mouse and rat P2X4 receptors determined in fluorimetric calcium influx assays.⁷⁵ ^c no receptor activation at high concentrations (100 μM and 1000 μM).

ATP displayed a significantly higher potency at the monkey P2X4 receptor (10-fold; EC₅₀: 0.0285 μ M) as compared to the human ortholog (EC₅₀: 0.269 μ M) (Figure 24, Figure 25, Table 4). At the dog P2X4 receptor, the potency of ATP was similar to that at the human ortholog, which is consistent with previous studies.²⁰⁶ For the guinea pig, pig, and rabbit P2X4 receptors, the ATP potency was significantly higher (5- to 7-fold) than at the human receptor (Figure 24, Figure 25, Table 4). Among the investigated mammalian P2X4 receptor orthologs, ATP showed the lowest potency at the rat P2X4 receptor (EC₅₀: 1.72 μ M).⁷⁵ The mouse P2X4 receptor was activated by ATP at concentrations similar to those of the human receptor.⁷⁵ The potency of ATP at the zebrafish P2X4 receptor (EC₅₀: 3,270 μ M) was about 4 orders of magnitude lower than at the human receptor and all other investigated mammalian receptors (Figure 24, Figure 25, Table 4). Earlier electrophysiology studies had reported significantly lower ATP potency at the zebrafish receptor, with EC₅₀ values of $\sim$ 800 μ M and 274 μ M, respectively.^{24,209} Our calcium influx assays revealed an even lower ATP potency, in the millimolar range. These discrepancies might be attributed to different experimental setups or varying expression levels of the zebrafish P2X4 receptor in the employed cell lines.

As a next step, concentration-dependent calcium responses of various other P2X4 receptor agonists were determined and respective EC₅₀ values were calculated (Supporting information, Figure 29; Figure 25, Table 4). It was observed that all investigated agonists acted as full agonists with efficacies in the same range as that of ATP.

α,β -Methylene-ATP displayed an EC₅₀ value of 1.24 μ M at the human P2X4 receptor, which is comparable to results from earlier studies, determined in the same assay (EC₅₀: 0.809 μ M).⁷⁵ The EC₅₀ value of α,β -Methylene-ATP at the monkey P2X4 receptor (0.651 μ M) was similar to the value obtained at the human receptor (1.24 μ M). At the dog (EC₅₀: 3.59 μ M) and guinea pig P2X4 receptor (EC₅₀: 0.495 μ M), only minor differences in potency for α,β -methylene-ATP were observed (Table 4). In contrast, for the pig P2X4 receptor, the agonist exhibited a similar EC₅₀ value (1.83 μ M) as compared to the value at the human receptor (1.24 μ M). The potency of α,β -methylene-ATP was significantly higher (4-fold) at the rabbit P2X4 receptor than at the human ortholog. Rat and mouse as well as zebrafish P2X4 receptors were insensitive even to high concentrations of α,β -methylene-ATP (100 μ M and 1000 μ M).⁷⁵

In a previous study, the potency of BzATP at the human P2X4 receptor was reported to be $0.515\text{ }\mu\text{M}$ ⁷⁵, which is in agreement with the present results (EC_{50} : $0.166\text{ }\mu\text{M}$). At the monkey P2X4 receptor, BzATP displayed the same potency (EC_{50} : $0.347\text{ }\mu\text{M}$). At the dog P2X4 receptor, BzATP displayed somewhat lower potency (EC_{50} : $1.44\text{ }\mu\text{M}$), which is in agreement with previous results.²⁰⁶ Similarly, the potency of BzATP is somewhat lower at the guinea pig (EC_{50} : $0.536\text{ }\mu\text{M}$) and pig P2X4 receptors (EC_{50} : $0.715\text{ }\mu\text{M}$). Prior calcium influx assays had shown that the rat P2X4 receptor is insensitive towards BzATP concentrations of up to $100\text{ }\mu\text{M}$, while BzATP does activate the mouse receptor (EC_{50} : $2.92\text{ }\mu\text{M}$), however with lower potency than the human receptor.⁷⁵ The zebrafish P2X4 receptor was not activated at a BzATP concentration of $1000\text{ }\mu\text{M}$.

An EC_{50} value of $0.478\text{ }\mu\text{M}$ was determined for 2-MeS-ATP at the human P2X4 receptor, consistent with earlier results (EC_{50} : $0.286\text{ }\mu\text{M}$).⁷⁵ 2-MeS-ATP showed similar potency across the investigated P2X4 receptor species, except for the mouse and rat receptors, where its potency was somewhat lower compared to the human receptor (Figure 25, Table 4).⁷⁵ The zebrafish P2X4 receptor was not activated at a 2-MeS-ATP concentration of $1000\text{ }\mu\text{M}$.

The EC_{50} value of Ap₄A at the human P2X4 receptor was $0.223\text{ }\mu\text{M}$, which is again comparable to previous findings (EC_{50} : $0.140\text{ }\mu\text{M}$).⁷⁵ The potency of Ap₄A at the other investigated P2X4 receptors was comparable to that at the human receptor, with only minor differences (Figure 25, Table 4). The largest differences had been described for the rat and mouse receptors, both being insensitive to a high concentration of Ap₄A ($100\text{ }\mu\text{M}$).⁷⁵ Similarly, no receptor activation of the zebrafish P2X4 receptor was observed at high concentrations of Ap₄A ($100\text{ }\mu\text{M}$).

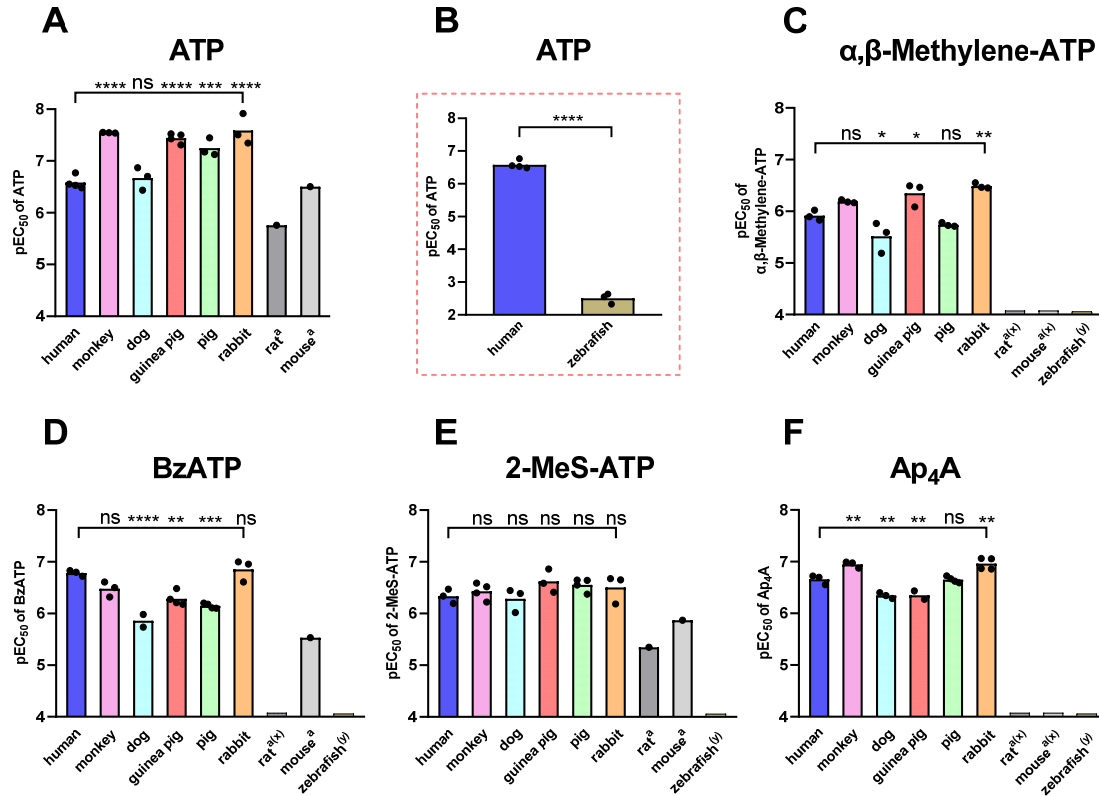


Figure 25. pEC₅₀ values of P2X₄ receptor agonists at human P2X₄ receptor and P2X₄ receptor orthologs (monkey, dog, guinea pig, pig, rabbit, rat, mouse, and zebrafish) recombinantly expressed in 1321N1 astrocytoma cells, determined by fluorimetric calcium influx assays. Data represent means from duplicates performed in 2–4 independent experiments. The values of P2X₄ receptor orthologs were compared to those for the human P2X₄ receptor. The level of significance was calculated by an ordinary one-way ANOVA with Dunnett's post-hoc test (multiple comparisons test). Significance was expected if P value was ≤ 0.05. The significance levels were defined as follows: * P < 0.05, ** P < 0.01, *** P < 0.001, **** P < 0.0001; ns, not significant. ^a Published mean values for rat and mouse P2X₄ receptors.⁷⁵ (x) no receptor activation at 100 μM; pEC₅₀ < 4. (y) no receptor activation at 100 μM/1000 μM. pEC₅₀ < 4/< 3. A. pEC₅₀ values of ATP at all investigated mammalian P2X₄ receptor orthologs. B. pEC₅₀ values of ATP at the human and zebrafish P2X₄ receptor. C. pEC₅₀ values of α,β-methylene-ATP. D. pEC₅₀ values of BzATP. E. pEC₅₀ values of 2-MeS-ATP. F. pEC₅₀ values of Ap₄A.

The systematic analysis and comparison of various P2X4 receptor species revealed largest differences in agonist potencies for mouse, rat, and zebrafish P2X4 receptors in comparison to the human P2X4 receptor. This observation aligns with the lower degree of protein sequence identity between the human receptor and those of rat (87.4%), mouse (86.9%), and zebrafish (58.3%) receptors. Remarkably, the physiological agonist ATP exhibited higher potency at the monkey, guinea pig, pig, and rabbit receptors as compared to the human P2X4 receptor, even though they share the same orthosteric binding site. Only at the dog and mouse receptors, ATP potency was similar to that at the human ortholog. In Table 5 the rank order of agonist potency at P2X4 receptor species is summarized, indicating that ATP is the most potent agonist at all mammalian orthologs, except for the human receptor. BzATP is the most potent agonist at the human receptor, however its potency is still in the same range as that of ATP and Ap₄A. α,β -Methylene-ATP exerts the lowest agonistic potency at the human receptor, as well as at monkey, dog, and pig P2X4 receptors.

Table 5. Rank order of agonist potency at P2X4 receptor species.

	Rank order of agonist potency*
human	BzATP $\cong$ Ap ₄ A $\cong$ ATP $\geq$ 2-MeS-ATP $\geq$ α,β -Methylene-ATP
monkey	ATP $>$ Ap ₄ A $\geq$ BzATP $\cong$ 2-MeS-ATP $\geq$ α,β -Methylene-ATP
dog	ATP $\geq$ Ap ₄ A $\cong$ 2-MeS-ATP $\geq$ BzATP $\geq$ α,β -Methylene-ATP
guinea pig	ATP $>$ 2-MeS-ATP $\geq$ Ap ₄ A $\cong$ α,β -Methylene-ATP $\cong$ BzATP
pig	ATP $>$ Ap ₄ A $\cong$ 2-MeS-ATP $\geq$ BzATP $\geq$ α,β -Methylene-ATP
rabbit	ATP $>$ Ap ₄ A $\cong$ BzATP $\geq$ α,β -Methylene-ATP $\cong$ 2-MeS-ATP
rat ^a	ATP $\geq$ 2-MeS-ATP $\gg$ α,β -Methylene-ATP, BzATP, Ap ₄ A
mouse ^a	ATP $>$ 2-MeS-ATP $\geq$ BzATP $\gg$ α,β -Methylene-ATP, Ap ₄ A

*Rank order was set as follows: $\cong$, up to 1.5-fold potency difference; $\geq$, 1.5–3.0-fold potency difference; $>$, from 3-fold potency difference; $\gg$ no receptor activation at high concentrations; ^a Published values.⁷⁵

All amino acid residues directly involved in ATP binding are conserved in all P2X4 receptor orthologs investigated here, except for the zebrafish P2X4 receptor (Figure 23). Therefore, another explanation has to be considered for the observed species differences. Thus, the amino acid residues within 6 Å distance of the ATP-binding site as well as those in the head domain of the P2X4 receptors were examined. In a previous study, amino acid residues within 6 Å distance of the ATP-binding site were considered to explain species differences of the rat and mouse versus the human P2X4 receptor.⁷⁵ Within this region, only a single amino acid residue is different (Ala68 in rat and mouse, Val68 in human) (Figure 23).⁷⁵ Amino acid residue Ala68 is also present in rabbit, guinea pig, pig, and dog P2X4 receptors (Figure 23), but their agonist profiles were more similar to that of the human receptor. Additional differences in amino acid residues within 6 Å distance of the ATP-binding site were noted in the guinea pig (at position 218), pig (at position 216 and 218), and rabbit (at position 218) P2X4 receptors when compared to the human receptor (Figure 23).

Since different amino acid residues near the ATP-binding site could not fully explain the observed species differences, the head domain of the receptors was additionally examined, which is known to play a critical role in agonist recognition and channel activation.^{75,210,211} The elucidation of the zebrafish P2X4 receptor structure disclosed that ATP promotes cleft closure between the head and dorsal fin domains, causing an outward flexing of the lower part of the protein, thereby allowing the ion channel pore to open.^{24,25} Molecular dynamics simulation studies suggested that the inherent dynamics of the receptor's head domain are crucial for determining ATP recognition by the orthosteric binding site.²¹² By comparing the amino acid sequences of all investigated P2X4 receptors, the amino acid residues in the head domains were found to be highly variable for all species (Figure 23, amino acid residues underlined in red).⁷⁵ Thus, variations in amino acid residues near the ATP-binding site and within the head domain of the P2X4 receptor may affect the potency of P2X4 receptor agonists or alter the conformation of the agonist binding site, explaining the observed species differences.⁷⁵

In conclusion, ATP and its derivatives and analogs show moderate to large differences depending on the investigated species compared to the human P2X4 receptor. The zebrafish P2X4 receptor requires very high, millimolar concentrations of ATP to be activated. Also, at the rat P2X4 receptor, the agonists display remarkably weak potency. All other investigated species show similar or even higher sensitivity towards ATP, and in most cases

also for ATP derivatives and analogs. 2-MeS-ATP displays the lowest degree of species differences.

Effects of P2X4 receptor antagonists at P2X4 receptor orthologs

The pharmacology of P2X4 receptor antagonists on recombinant monkey, guinea pig, pig, and rabbit P2X4 receptors has so far not been reported. In this study, the inhibition profiles of three structurally diverse P2X4 receptor antagonists, BX430 (urea derivative), 5-BDBD (benzodiazepine derivative), and PSB-OR-2020 (indole derivative) were investigated at different P2X4 receptor orthologs (Figure 26, Figure 27, Table 6). The receptors were activated by ATP at its respective EC₈₀ (for EC₈₀ values see Supporting information, Table 7). The results obtained at P2X4 receptors of different species were compared to those for the human receptor, and the data was statistically analyzed.

BX430 had been identified as a P2X4 receptor antagonist showing sub-micromolar potency at the human P2X4 receptor (IC₅₀: 0.54 μ M) determined in patch clamp studies,¹³³ which is consistent with the IC₅₀ value determined in calcium influx assays (IC₅₀: 0.127 μ M).¹³⁴ All mammalian P2X4 receptor orthologs investigated in the present study were inhibited by 10 μ M of BX430 by more than 50% (Figure 26; Supporting Information, Figure 30). Concentration-dependent inhibition revealed a 5-fold lower potency of BX430 at the monkey (IC₅₀: 0.655 μ M) as compared to the human P2X4 receptor (Figure 26, Figure 27, Table 6). BX430 also showed significantly reduced potency at the dog P2X4 receptor (14-fold; IC₅₀: 1.72 μ M), which is consistent with previous studies.²⁰⁶ The compound also exhibited somewhat reduced potency at the guinea pig P2X4 receptor (4-fold; IC₅₀: 0.447 μ M), but was similarly potent at the pig and rabbit receptors (IC₅₀ values of 0.164 μ M and 0.0977 μ M, respectively) as at the human ortholog. The same assay system was previously used to determine the potency of BX430 at the rat and mouse P2X4 receptor, displaying a 20-fold decreased potency at the rat (IC₅₀: 2.74), but a similar potency at the mouse receptor (IC₅₀: 0.286 μ M) as compared to the human receptor.¹³⁴ In previously reported electrophysiological experiments at mouse and rat receptors, the effects of BX430 could not be measured.¹³³ These differences are likely attributed to different ATP concentrations used for receptor activation (1 μ M of ATP in calcium influx assays vs. 50 μ M in electrophysiological experiments).^{133,134} At the zebrafish P2X4 receptor, the potency of BX430 was found to be 180-fold lower (IC₅₀: 23.6 μ M) compared to its potency at the human receptor (Figure 27, Table 6; Supporting information, Figure 30). Previous

studies reported that BX430 inhibited ATP-induced currents of the zebrafish P2X4 receptor with an IC_{50} value comparable to that of the human receptor.^{27,133} However, we observed a 180-fold lower potency of BX430 at the zebrafish receptor in calcium influx studies. Several studies aimed at identifying the binding site of BX430 at the human P2X4 receptor.^{134,147} The recently published cryo-EM structure of the zebrafish P2X4 receptor in complex with BX430 provides new information about the binding pocket, being located at the subunit interface in the extracellular receptor domain.²⁷ Amino acid residue Ile312 in the human P2X4 receptor (corresponding to Ile315 in the zebrafish P2X4 receptor) was previously proposed to be responsible for species differences of BX430 since the rat and mouse P2X4 receptor harbor a threonine residue at this position instead of an isoleucine residue (Figure 23).¹⁴⁷ Indeed, these results are supported by the cryo-EM structure of the zebrafish P2X4 receptor, which showed hydrophobic interactions between BX430 and amino acid residue Ile315.²⁷ Amino acid residue Ile91 was also identified to be involved in BX430 binding to the human P2X4 receptor.²⁷ In the dog P2X4 receptor, Ile91 is replaced by valine (Figure 23). Although this amino acid exchange within the assumed binding pocket of BX430 appears to be minor, it may explain the lower potency of BX430 to the dog P2X4 receptor.

5-BDBD is a P2X4 receptor antagonist of moderate potency showing an IC_{50} value of 0.990 μ M at the human P2X4 receptor determined in the present study, which is in the same range as previously reported (IC_{50} : 0.348 μ M).⁷⁵ At the monkey and dog P2X4 receptor, 5-BDBD displayed slightly higher potency (IC_{50} values of 0.322 μ M and 0.424 μ M, respectively) (Figure 26, Figure 27, Table 6). Previously published data from the dog receptor exhibited similar inhibition profile of 5-BDBD as that obtained at the human receptor.²⁰⁶ The guinea pig P2X4 receptor was not inhibited by 5-BDBD at a high concentration of 10 μ M. At the pig and rabbit P2X4 receptors, 5-BDBD exhibited similar inhibitory potency as at the human receptor. Species differences had been observed in previous studies at rodent receptors, with reduced potency of 5-BDBD at rat and mouse P2X4 receptors (IC_{50} values of 3.47 μ M and 2.04 μ M, respectively).⁷⁵ High concentrations (100 μ M) of 5-BDBD did not inhibit the zebrafish P2X4 receptor. Species differences of 5-BDBD were less pronounced than those observed for BX430. However, 5-BDBD is less potent than BX430 and poorly water-soluble, thus it is not the optimal candidate for pharmacological studies.

The indole derivative PSB-OR-2020 was recently described as a highly potent and selective P2X4 receptor antagonist with an IC_{50} value of 0.00632 μ M at the human receptor.¹²⁹ At the monkey, dog, guinea pig, pig, rabbit, rat, and mouse P2X4 receptors, PSB-OR-2020 also acted as an antagonist with low-nanomolar potencies (Figure 26, Figure 27, Table 6; Supporting information, Figure 31). It blocked the monkey receptor with 4-fold higher potency (IC_{50} : 0.00170 μ M) than the human receptor, the dog receptor with 9-fold higher potency (IC_{50} : 0.000696 μ M), and the pig and mouse receptor with 3-fold higher potency (IC_{50} : 0.00245 μ M (pig); IC_{50} : 0.00242 μ M (mouse)). The inhibitory potency of PSB-OR-2020 at the rabbit (IC_{50} : 0.00520 μ M) and rat receptor (IC_{50} : 0.00318 μ M) was found to be similar to that observed at the human receptor. At the guinea pig receptor, PSB-OR-2020 displayed the lowest potency (IC_{50} : 0.0170 μ M) compared to all other investigated mammalian species. At the zebrafish P2X4 receptor, the IC_{50} of PSB-OR-2020 was found to be in the micromolar range (IC_{50} : 23.9 μ M), 3,700-fold lower than at the human receptor (Figure 27, Table 6; Supporting information, Figure 31). The binding site of the allosteric antagonist PSB-OR-2020 is still unknown.

The importance of P2X4 receptor antagonists as pharmacological tool compounds has been demonstrated in several animal models of diseases.^{127,128,167} Our data highlight the consistently high antagonistic potency of the new indoline-derived P2X4 receptor antagonist PSB-OR-2020 across P2X4 receptors from different species with IC_{50} values between 0.7–17 nM (except for the zebrafish P2X4 receptor; IC_{50} : 23.9 μ M). Its at least 1.5–2 orders of magnitude higher potency and its moderate species differences make it the antagonist of choice for P2X4 receptor studies.

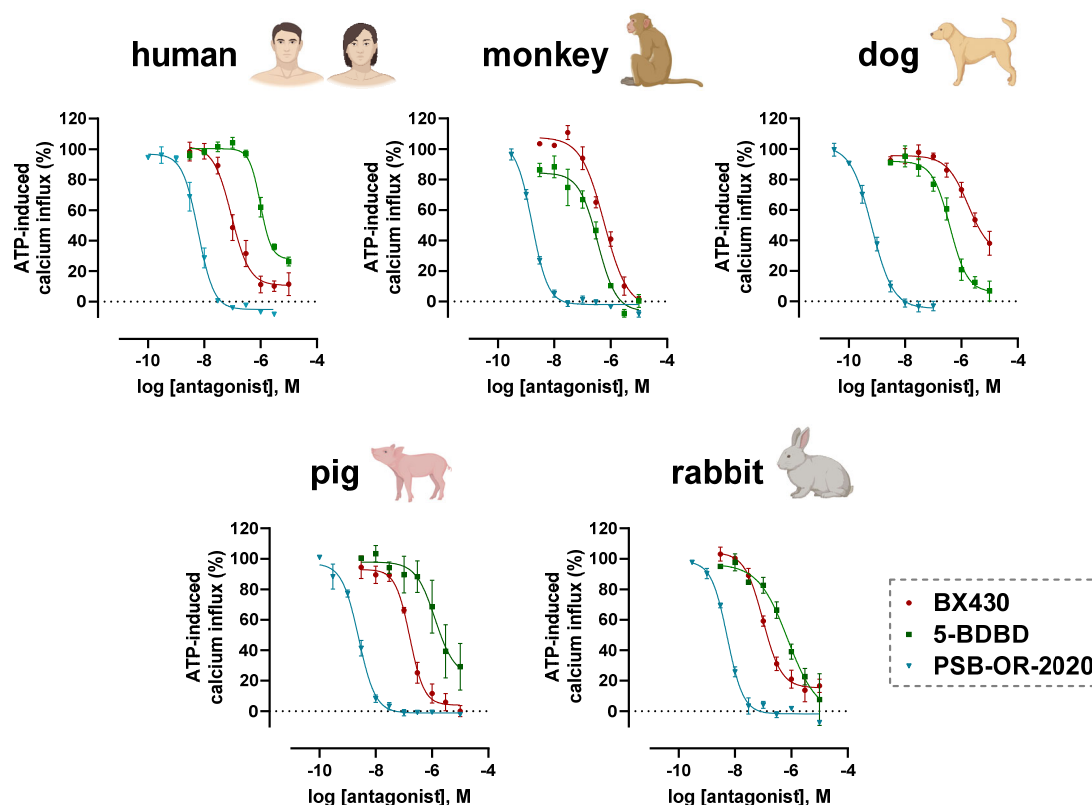


Figure 26. Inhibition curves for P2X4 receptor antagonists BX430, 5-BDBD, and PSB-OR-2020 at P2X4 receptors of selected species. Concentration-dependent inhibition of ATP-induced calcium influx (ATP at its respective EC_{80} , set at 100% in the absence of inhibitor) was determined in 1321N1 astrocytoma cells expressing the human, monkey, dog, pig, or rabbit P2X4 receptor, respectively. Data represents mean $\pm$ SEM of 2–8 independent experiments, performed in duplicates. Species images were taken from BioRender.com.

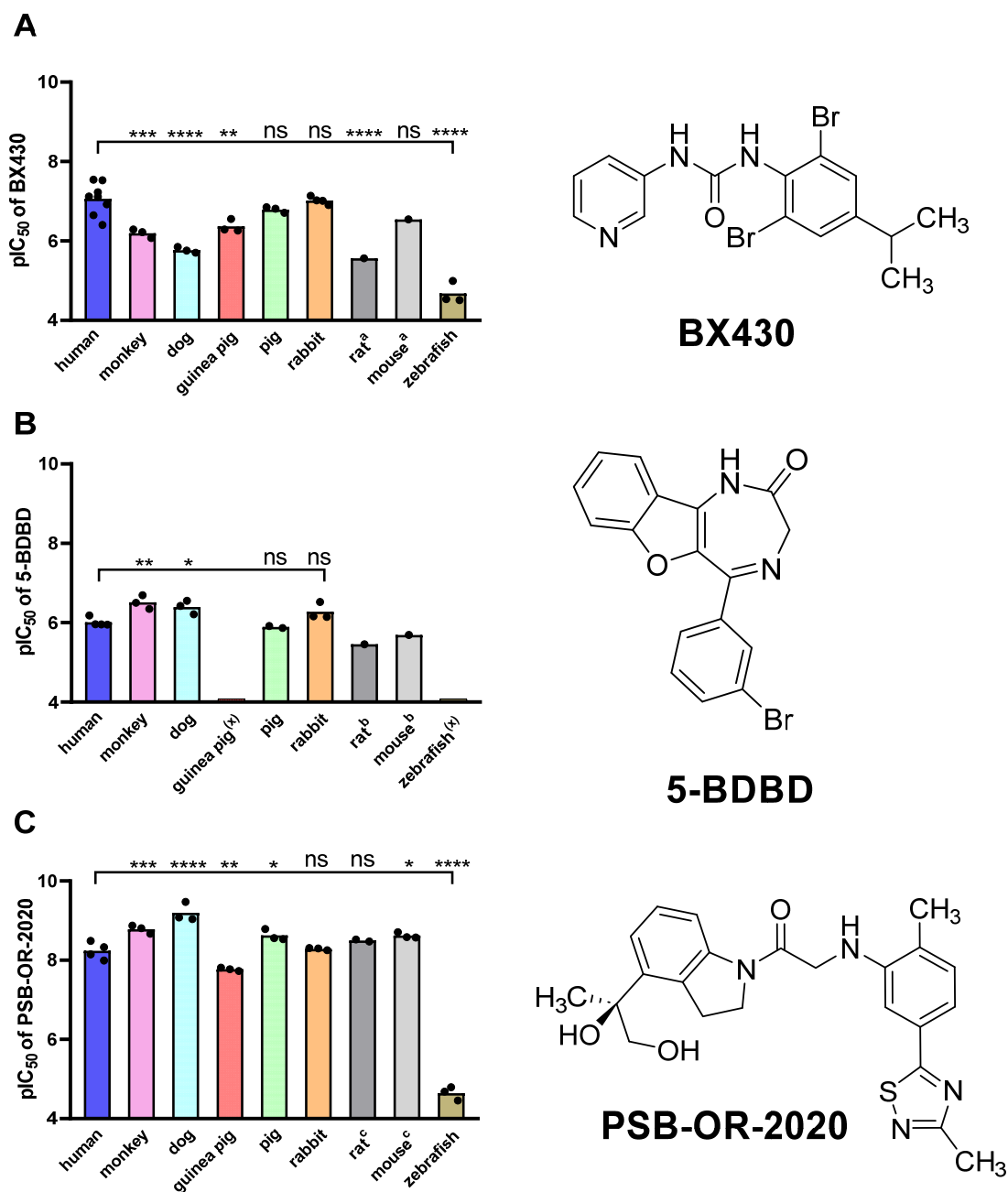


Figure 27. pIC₅₀ values of BX430, 5-BDBD, and PSB-OR-2020 at the human P2X4 receptor and at P2X4 receptor orthologs (monkey, dog, guinea pig, pig, rabbit, rat, mouse, and zebrafish) stably expressed in 1321N1 astrocytoma cells, determined by fluorimetric calcium assays. Results obtained for monkey, dog, guinea pig, pig, rabbit, (rat), and zebrafish P2X4 receptors were compared to those obtained for the human P2X4 receptor. Level of significance was calculated by an ordinary one-way ANOVA with Dunnett's post-hoc test (multiple comparisons test). Significance was expected if P value was ≤ 0.05 . The significance levels were defined as follows: * P < 0.05, ** P < 0.01, *** P < 0.001, **** P < 0.0001; ns, not significant. A. pIC₅₀ values of BX430. Data represent means from duplicates performed in 2–8 independent experiments. ^aPublished mean values.¹³⁴ B. pIC₅₀ values of 5-BDBD. Data represent means from duplicates performed in n=2–3 independent

experiments. ^(x) no receptor inhibition at high concentrations of 5-BDBD. ^b Published mean values.⁷⁵ C. pIC₅₀ values of PSB-OR-2020. Data represent means from duplicates performed in n=2–3 independent experiments. ^c These values were determined within this study.

3. Results and discussion

Table 6. Potency of selected antagonists at the human P2X4 receptor and P2X4 receptor orthologs stably expressed in 1321N1 astrocytoma cells, determined in fluorimetric calcium influx assays.

	IC ₅₀ ± SEM ^a (μM)		
species	BX430	5-BDBD	PSB-OR-2020
human	0.127 ± 0.045 ^b	0.990 ± 0.112	0.00632 ± 0.00152
monkey	0.655 ± 0.099 ^{***}	0.322 ± 0.07 ^{**}	0.00170 ± 0.00020 ^{***}
dog	1.72 ± 0.160 ^{****}	0.424 ± 0.100 [*]	0.000696 ± 0.000181 ^{****}
guinea pig	0.447 ± 0.083 ^{**}	>10	0.0170 ± 0.0010 ^{**}
pig	0.164 ± 0.016 ^{ns}	1.29 ± 0.07 ^{ns}	0.00245 ± 0.00041 [*]
rabbit	0.0977 ± 0.0107 ^{ns}	0.570 ± 0.134 ^{ns}	0.00520 ± 0.00020 ^{ns}
rat	2.74 ± 0.39 ^{**** b}	3.47 ± 2.66 ^c	0.00318 ± 0.00018 ^{ns}
mouse	0.286 ± 0.030 ^{ns b}	2.04 ± 1.56 ^c	0.00242 ± 0.00023 [*]
zebrafish	23.6 ± 6.7 ^{****}	>>100	23.9 ± 5.6 ^{****}

^a Calculated IC₅₀ values of BX430, 5-BDBD and PSB-OR-2020 ± SEM (μM) from concentration-response curves versus respective EC₈₀ of ATP; inhibition of calcium influx at 10 μM of BX430, 5-BDBD and PSB-OR-2020 ± SEM (%) (normalized to the maximum ATP-induced effect at EC₈₀). Data represent mean values ± SEM from 2–8 independent experiments performed in duplicates. ^b Published IC₅₀ value ± SEM (μM).¹³⁴ ^c Published IC₅₀ values ± SD (μM) determined in calcium influx assays.⁷⁵ The results obtained for monkey, dog, guinea pig, pig, rabbit, rat, mouse, and zebrafish P2X4 receptors were compared to those obtained for the human P2X4 receptor. Level of significance was calculated by an ordinary one-way ANOVA with Dunnett's post-hoc test (multiple comparisons test). Significance was expected if P value was ≤ 0.05. The significance levels were defined as follows: * P < 0.05, ** P < 0.01, *** P < 0.001, **** P < 0.0001; ns, not significant.

3.1.5 Conclusions

There is growing interest in the identification, characterization, and development of P2X4 receptor antagonists as tool compounds and drugs. The comprehensive analysis of P2X4 receptor orthologs, performed under identical experimental conditions, has provided insights into species-specific variations. We present the first direct comparison of recombinant monkey, dog, pig, guinea pig, rabbit, rat, mouse, and zebrafish P2X4 receptors, shedding light on species differences of P2X4 receptor agonists and antagonists. For agonists, the most remarkable potency differences were observed for the endogenous agonist ATP, possibly due to variations in amino acid residues of the receptors' head domains. Regarding P2X4 receptor antagonists, we studied structurally diverse allosteric antagonists (BX430, 5-BDBD, and PSB-OR-2020) that likely interact with different binding sites. PSB-OR-2020 is the most potent antagonist at the human as well as at all other investigated mammalian P2X4 receptor orthologs. In comparison to BX430 and 5-BDBD, PSB-OR-2020 may be the most suitable candidate for pharmacological studies due to its high potency and water solubility, moderate species differences, and high selectivity for the P2X4 receptor.

This study highlights the importance of considering species selectivity when evaluating the P2X4 receptor as a therapeutic target in animal models, given the observed variation among P2X4 receptor orthologs. Our findings offer valuable insights for further research on the P2X4 receptor as an important drug target, the development of potential drugs, and the understanding of their role in various diseases.

3.1.6 Materials and methods

P2X4 receptor agonists and antagonists

ATP was purchased from Carl Roth (Karlsruhe, Germany), α,β -methylene-ATP and Ap₄A from Sigma-Aldrich (Taufkirchen, Germany), BzATP and 2-MeS-ATP from Jena Bioscience (Jena, Germany). BX430 was purchased from Axon Medchem (Groningen, Netherlands), and 5-BDBD from Tocris (Wiesbaden-Nordenstadt, Germany). PSB-OR-2020 was synthesized by Orion Corporation (Turku, Finland).

P2X4 receptor orthologs

cDNAs of monkey, pig, rabbit, guinea pig, and dog P2X4 receptors were codon-optimized for human expression and subcloned into the retroviral pQCXIN vector at NotI and BamHI

restriction sites by BioCat GmbH (Heidelberg, Germany). Delivered plasmid DNAs were amplified at a large scale and purified using the PureLink™ HiPure Plasmid Filter Midiprep Kit (Thermo Fisher Scientific, Dreieich, Germany). Before transfection, the respective sequences were confirmed by sequencing (Eurofins Genomics, Ebersberg, Germany).

Cultivation of cells

Non-transfected 1321N1 astrocytoma cells (Sigma-Aldrich, Taufkirchen, Germany) were cultivated in Dulbecco's Modified Eagle's Medium (DMEM; Thermo Fisher Scientific, Dreieich, Germany) containing 10% fetal calf serum (FCS), penicillin (100 U/mL), and streptomycin (100 µg/mL). Stably transfected 1321N1 astrocytoma cells were grown in DMEM, 10% FCS, penicillin (100 U/mL), streptomycin (100 µg/mL), and 800 µg/mL of geneticin (G418). All cells were grown at 37 °C and 10% CO₂. FCS and antibiotics were purchased from PAN Biotech (Aidenbach, Germany).

Retroviral transfection of 1321N1 astrocytoma cells

For generating 1321N1 astrocytoma cell lines stably expressing the human P2X4 receptor or one of the P2X4 receptor orthologs (monkey, dog, guinea pig, pig, rabbit, zebrafish), a retroviral expression system was used, as described before.¹³⁴ In brief, GP+envAM-12 fibroblast-type mouse packaging cells (American Type Culture Collection (ATCC), Manassas, VA, USA) were used to produce a recombinant helper virus²¹³ by being co-transfected with 6.25 µg of the purified plasmid DNA (retroviral vector pQCXIN), 3.75 µg of a vesicular stomatitis virus G protein (VSV-G) protein DNA (in pcDNA3.1), and lipofectamine 2000 (Thermo Fisher Scientific, Dreieich, Germany) as a transfection reagent. VSV-G was used to increase infection efficiency.²¹⁴ Subsequently, non-transfected 1321N1 astrocytoma cells were infected with the virus particles containing the respective P2X4 receptor sequence. The antibiotic selection was started 48–72 h after infection using 800 µg/mL of G418.

Calcium influx assays

Transfected 1321N1 astrocytoma cells were evaluated in fluorimetric calcium influx assays. Cells were grown to 80–90% confluency, washed with phosphate-buffered saline, and detached with trypsin/ethylenediaminetetraacetic acid. Subsequently, 45,000 cells per well were seeded into a 96-well polystyrene microplate with a black and flat bottom (No. 3340, Corning, NY, USA), and incubated overnight at 37°C and 10% CO₂.

Fluo-4 acetoxymethyl ester (AM) (Thermo Fisher Scientific, Dreieich, Germany) was used as a cell-permeable Ca^{2+} -chelating fluorescent dye. At first, the medium was carefully removed. Then, the cells were loaded with dye solution containing Fluo-4 AM (3 μM) in Hanks' balanced salt solution (HBSS, Thermo Fisher Scientific, Dreieich, Germany), and 1% of the non-ionic detergent Pluronic[®] F127 (Sigma-Aldrich, Taufkirchen, Germany), and incubated for 1 h with gentle shaking. Then, the dye solution was removed carefully, and the respective antagonist (dissolved in dimethyl sulfoxide (DMSO), max. 1%), and HBSS buffer was added to the plate. For investigating agonists, only HBSS buffer was added. Transparent 96-well plates (Boettger, Bodenmais, Germany) were used for preparing agonist dilutions to induce calcium influx. For determining the inhibitory potency of antagonists, the reagent plate contained ATP at the respective EC_{80} , prepared in HBSS buffer. For assessing the potency of agonists, the reagent plate contained the respective agonist dilutions, prepared in HBSS buffer. HBSS buffer served as a negative control. After 30 min of incubation with antagonist or buffer, ATP was added, and the ATP-induced calcium influx was measured using an imaging plate reader (NOVOstar, BMG LABTECH, Ortenberg, Germany) at an excitation wavelength of 485 nm and an emission wavelength of 520 nm for 30 s at 0.4 s intervals.

Statistical analysis

Microsoft Excel and GraphPad Prism version 8.0 (GraphPad Software, San Diego, CA, USA) were used to analyze data. Nonlinear regression (sigmoidal dose-response, variable slope) was used to generate concentration-dependent curves for agonists and antagonists, and to calculate EC_{50} , EC_{80} , and IC_{50} values. The results for P2X4 receptor orthologs were compared with those for the human P2X4 receptor (fixed control group) to identify any significant differences. The level of significance was calculated by a one-way ANOVA with Dunnett's multiple comparisons test. Significance was expected if P value was ≤ 0.05 . The significance levels were defined as follows: * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, **** $P < 0.0001$.

3.1.7 Supporting information

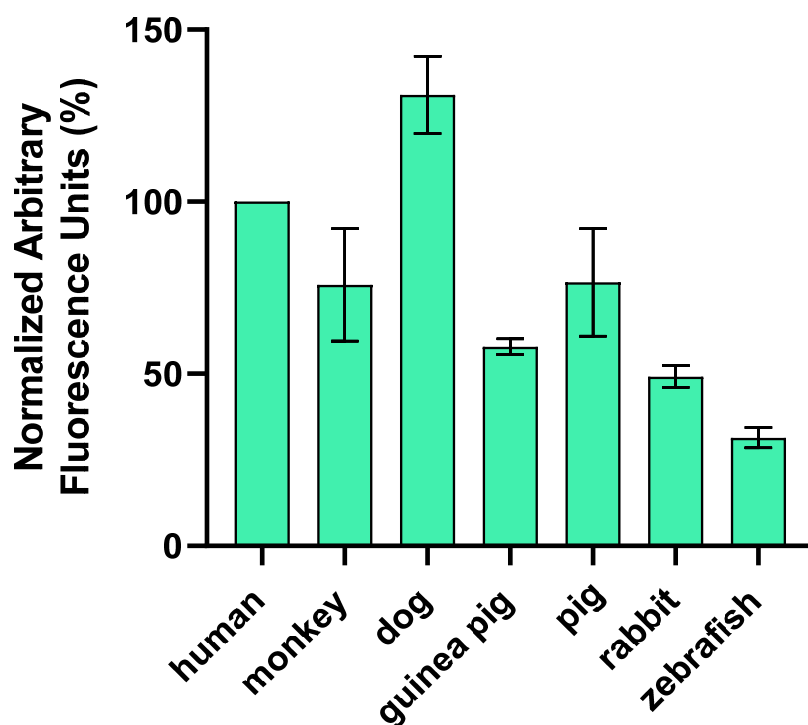


Figure 28. Representative results from fluorescence intensities measured in calcium influx assays in 1321N1 astrocytoma cells recombiantly expressing the human, monkey, dog, guinea pig, pig, rabbit, or zebrafish P2X4 receptor (45,000 cells/well). Fluoresence intensities were normalized (human: 100%). The following ATP concentrations were employed to induce calcium influx: 100 μ M of ATP for human, monkey, dog, guinea pig, pig, and rabbit receptors and 10 mM for the zebrafish receptor.

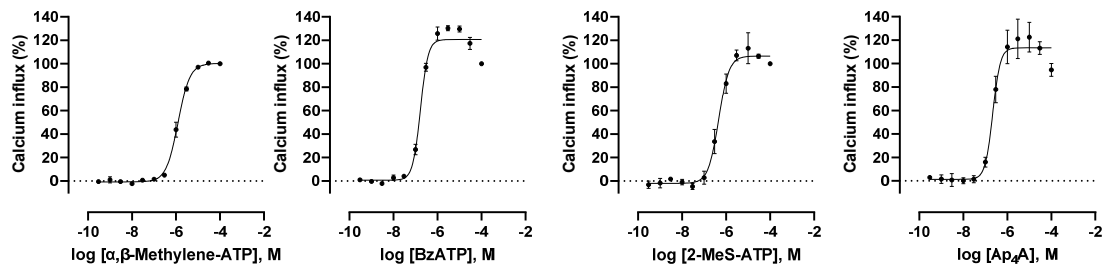
Table 7. EC₈₀ of ATP determined in calcium influx assays at P2X₄ receptor orthologs stably expressed in 1321N1 astrocytoma cells.

species	EC₈₀ ± SEM^a (μM)
human	0.558 ± 0.0574
monkey	0.0515 ± 0.0021
dog	0.443 ± 0.113
guinea pig	0.0603 ± 0.0150
pig	0.102 ± 0.025
rabbit	0.0584 ± 0.0245
zebrafish	> 1 mM

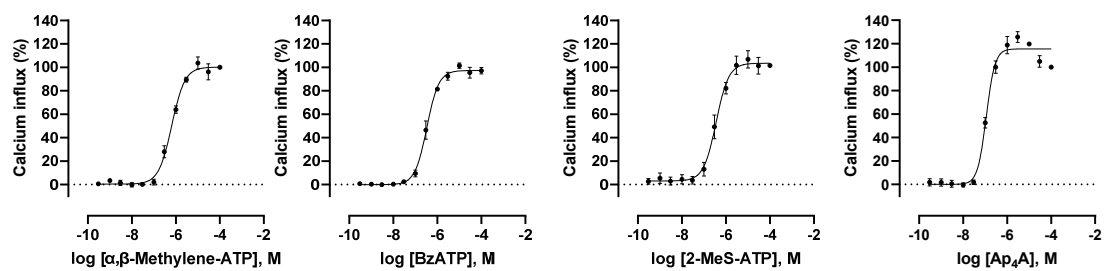
^a Calculated from concentration-response curves in fluorimetric calcium influx assays. Data represent mean values ± SEM from at least three independent experiments, performed in duplicates.

3. Results and discussion

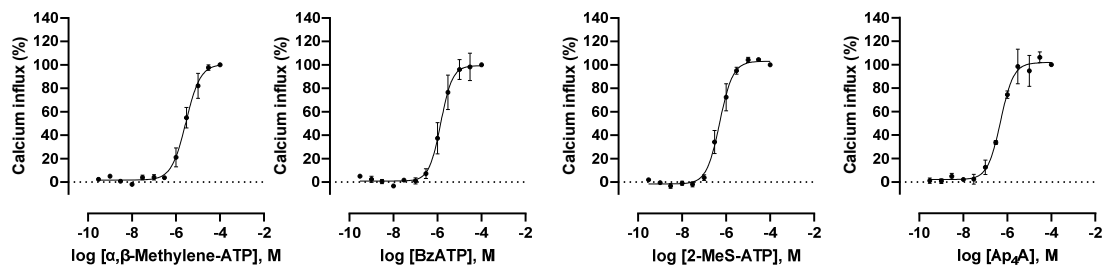
human



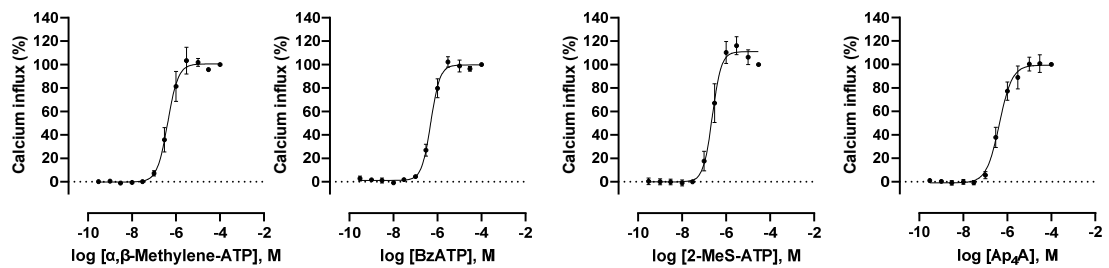
monkey



dog



guinea pig



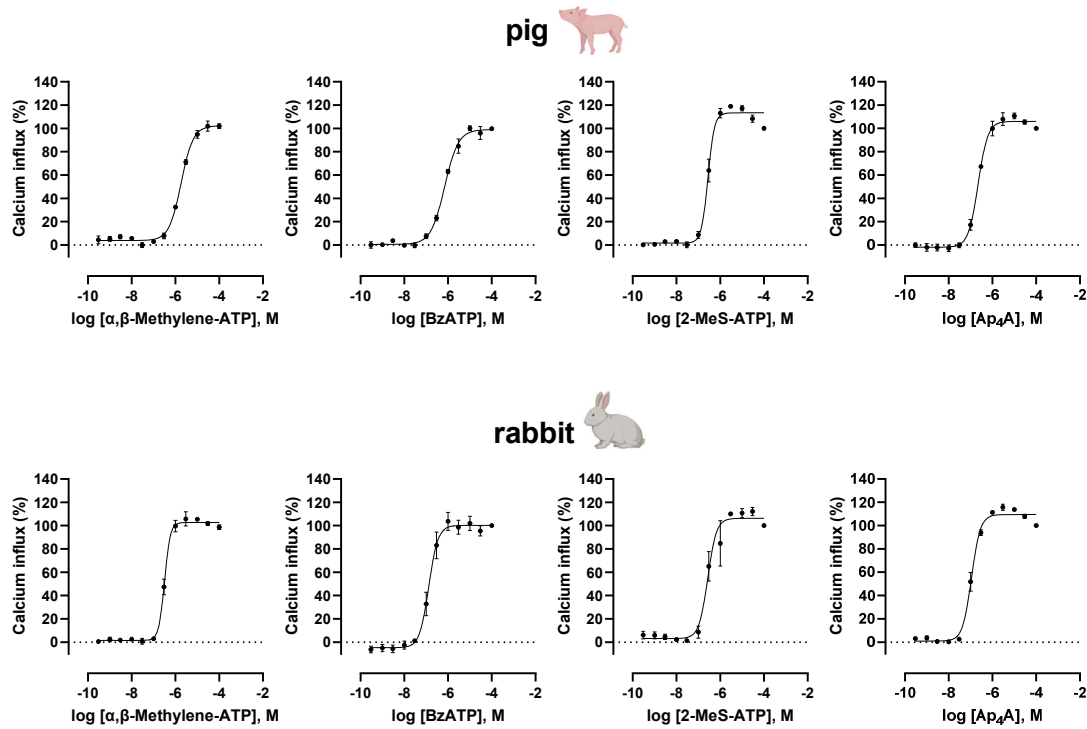


Figure 29. Concentration-response curves for selected ATP derivatives and analogs in 1321N1 astrocytoma cells stably expressing the human, monkey, dog, guinea pig, pig, and rabbit P2X4 receptor, respectively. Data represent mean $\pm$ SEM from 2–4 independent experiments, performed in duplicates. The maximal effect (calcium influx) of the respective agonist was set at 100%. Species images were taken from BioRender.com.

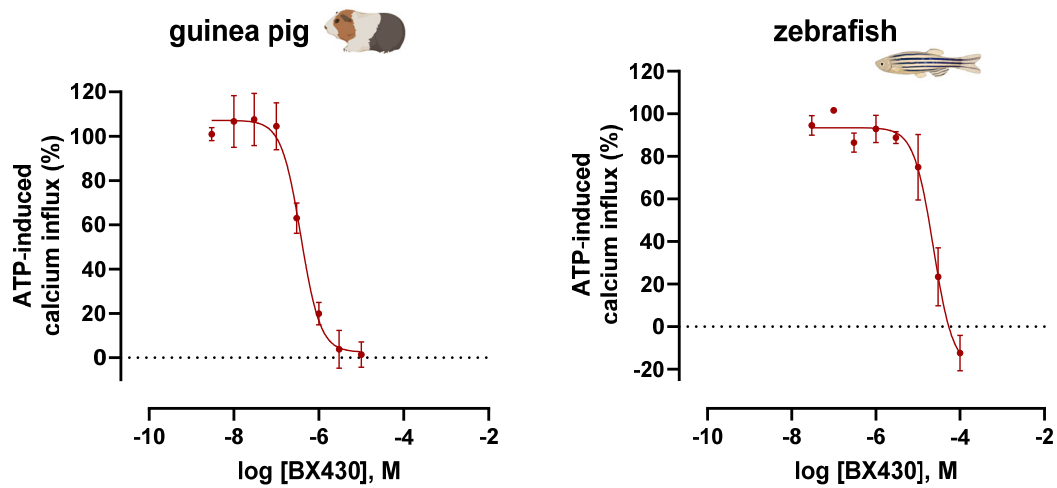


Figure 30. Inhibition curves for BX430 at guinea pig and zebrafish P2X4 receptor stably expressed in 1321N1 astrocytoma cells, respectively. Calcium influx was induced by ATP at its respective EC_{80} (set to 100% in the absence of inhibitor). Data represents mean $\pm$ SEM of three independent experiments, performed in duplicates. Species images were taken from BioRender.com.

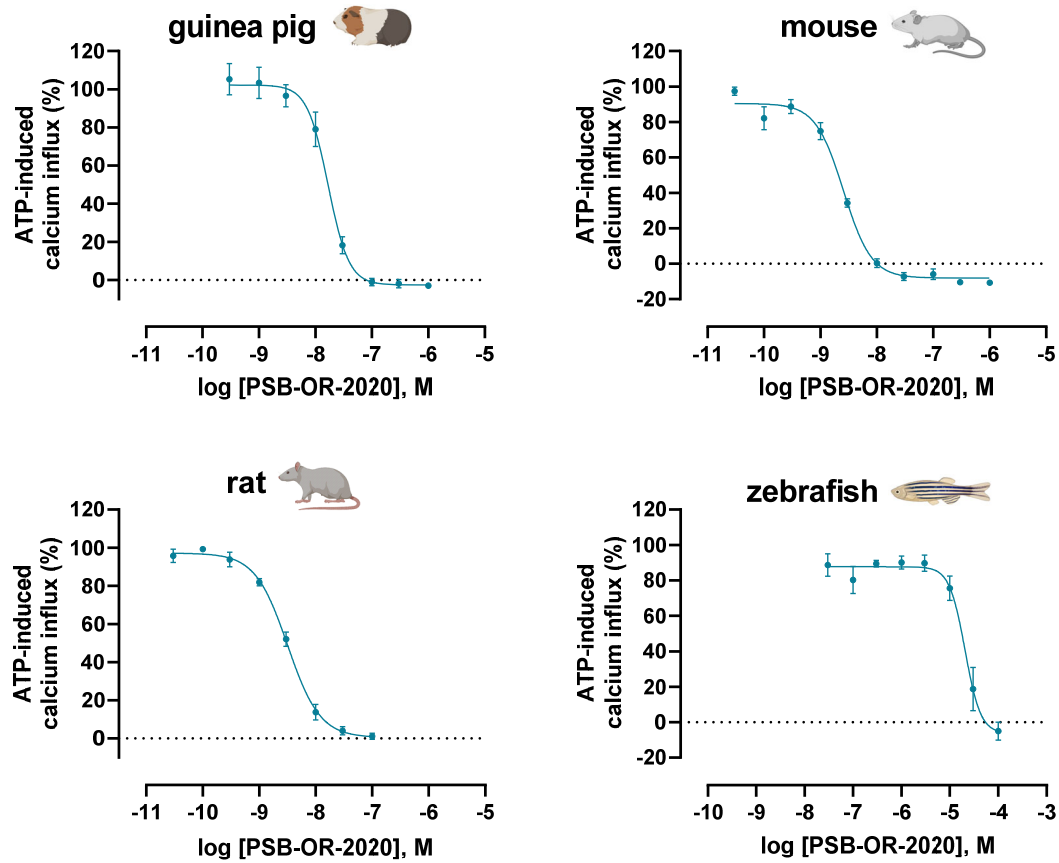


Figure 31. Inhibition curves for PSB-OR-2020 at guinea pig, mouse, rat, and zebrafish P2X4 receptors stably expressed in 1321N1 astrocytoma cells. Calcium influx was induced by ATP at its respective EC_{80} (set to 100% in the absence of inhibitor). Data represents mean $\pm$ SEM of 2–3 independent experiments, performed in duplicates or triplicates. Species images were taken from BioRender.com.

3.2 Publication I: Preparation and preliminary evaluation of a tritium-labeled allosteric P2X4 receptor antagonist

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Appendix A: Original publication and supporting information

Introduction

P2X receptors are trimeric ligand-gated ion channels with ATP as their endogenous ligand.¹⁰ Seven receptor subtypes have been identified so far, designated P2X1-P2X7, which form homotrimeric and heterotrimeric receptor complexes.^{10,11}

P2X4 receptors are expressed in the central and peripheral nervous system, on immune cells, and on endothelial cells.^{10,42,108} Researchers have provided evidence for the contribution of P2X4 receptors in the pathogenesis of various pain conditions, including inflammatory, cancer, and neuropathic pain.²¹⁵ Neuronal as well as microglial P2X4 receptors can contribute to the pathology of various diseases, including neuropathic pain, stroke, epilepsy, and Alzheimer's disease.^{119,157,164,169}

P2X4 receptor antagonists showed therapeutic efficacy in various animal models, including the alleviation of symptoms associated with neuropathic pain, inflammation, and nociception, as well as neuroprotective effects in models of ischemic injury.^{43,128,131,167} Moreover, several studies indicated the participation of P2X4 receptors in different types of cancer.^{44,171,174} These pathophysiological processes emphasize the potential of P2X4 receptors as drug targets and, in particular, the potential of P2X4 receptor antagonists as promising drug candidates.

The polar ATP-binding site of P2X4 receptors lined with basic amino acids has impeded the identification of drug-like orthosteric P2X4 receptor antagonists.^{25,67} Therefore, researchers have focused on allosteric P2X4 receptor modulators, however their development has faced other obstacles, especially in terms of high potency, water solubility, and selectivity. Despite these challenges, several allosteric P2X4 receptor antagonists have been described to date, including the urea derivative BX430, the

sulfonamide BAY-1797, the benzodiazepine derivative 5-BDBD, and the indole derivative PSB-15417.^{10,131,133}

Radioligands are powerful tools in pharmacology and drug development for studying protein-ligand interactions, identifying potential drug candidates, determining binding affinities and kinetic parameters, and assessing binding sites.^{75,144} To label the orthosteric binding sites of P2X4 receptors, the radioligand [³⁵S]ATP γ S, an ATP derivative with higher metabolic stability than ATP, was described as a suitable option.^{75,144} For the labeling of allosteric binding sites of P2X4 receptors no potent and selective antagonist radioligands have been reported so far, although attempts have been made by synthesizing radiolabeled 5-BDBD analogs for diagnostic purposes, but without success.²¹⁶

3.2.1 Publication summary

In our ongoing efforts to identify allosteric P2X4 receptor antagonists, we synthesized a series of indole derivatives exhibiting high potency, water solubility, and selectivity for the P2X4 receptor. A precursor molecule was designed based on one of the most potent antagonists of this series, which was suitable for preparing an allosteric P2X4 receptor antagonist radioligand by tritium-labeling. The resulting radioligand, designated [³H]PSB-OR-2020, was obtained in high purity (97.7%) with a specific activity of 45 Ci/mmol (1.67 TBq/mmol).

The new radioligand was employed to establish a P2X4 receptor radioligand binding assay. During the development of the radioligand binding assay, many obstacles were encountered, beginning with the initially high non-specific binding of the radioligand to glass fiber filters. By adjusting various experimental parameters, the high radioligand filter binding was successfully reduced. Cell membrane preparations from 1321N1 astrocytoma cells recombinantly expressing the human P2X4 receptor were used to determine the specific binding of [³H]PSB-OR-2020. The results indicated specific binding of the radioligand, however high non-specific binding of the radioligand to cell membranes was also observed. Despite attempts to optimize the assay conditions and to thereby increase the percentage of specific binding of the radioligand, these remained initially unsuccessful. Consequently, we generated new cell lines by transiently transfecting the human P2X4 receptor in HEK293 cells, which resulted in sufficiently high receptor expression to observe high specific binding of the radioligand, combined with low non-specific binding. The optimal conditions for the binding assay were as follows: Tris-HCl buffer (50 mM, pH 7.4)

containing 0.1% bovine serum albumin (BSA) as the assay buffer, a radioligand concentration of 10 nM (prepared in assay buffer), and a protein amount of 50–150 μ g (membrane preparations of P2X4 receptor-expressing HEK293 cells). The samples were incubated for 60 min at 4 °C with gentle shaking, filtered through glass fiber filters (type C), and washed with ice-cold Tris-HCl buffer (50 mM, pH 7.4) supplemented with 5% ethanol. Non-specific binding of the radioligand was determined in the presence of the unlabeled ligand PSB-OR-2020 (10 μ M).

With these optimized assay conditions, we performed association and dissociation experiments, revealing a kinetic K_D value of 7.06 ± 3.23 nM. The association half-life of the radioligand was 8.44 ± 2.59 min, while the dissociation half-life was 20.4 ± 2.9 min, determined at 4 °C. Subsequently, we performed homologous competition binding assays with the unlabeled ligand PSB-OR-2020, resulting in a K_D value of 20.4 ± 7.5 nM and a $B_{\max}$ value of 71.7 fmol/mg protein. The determined binding affinity of the radioligand was consistent with the IC_{50} value determined in calcium influx assays (6.32 ± 1.52 nM), demonstrating high affinity of the radioligand for the P2X4 receptor. As a subsequent step, competition assays were conducted with the endogenous agonist ATP and with structurally related (PSB-14517), and structurally diverse (5-BDBD, BX430, and BAY-1797) P2X4 receptor antagonists. High concentrations of ATP could only slightly displace the radioligand from its binding site, thereby proving an allosteric binding mode of [3 H]PSB-OR-2020. Only a partial displacement of the radioligand was caused by BX430, 5-BDBD, and BAY-1797, which may indicate the existence of different allosteric binding sites. In contrast, PSB-14517 completely displaced the radioligand, indicating that the radioligand and PSB-14517 bind to the same allosteric site of the human P2X4 receptor, both ligands being structurally related compounds.

In developing the first radioligand binding assay for the P2X4 receptor, we experienced various limitations, particularly the high non-specific binding of the radioligand and the low expression of the P2X4 receptor in the employed cell lines. To address this in future studies, it may be beneficial to employ cell lines with higher expression levels or isolated P2X4 receptor protein reconstituted into nanodiscs or amphipols. Nevertheless, we succeeded in a preliminary evaluation of the first allosteric antagonist radioligand for the P2X4 receptor, which revealed an unprecedented allosteric binding site for P2X4 receptor antagonists. [3 H]PSB-OR-2020 may become a valuable tool in P2X4 receptor pharmacology and drug development.

3.2.2 Author contributions

The author of this thesis conducted molecular biology experiments, generated P2X₄ receptor-expressing cell lines for radioligand binding assays, made cell membrane preparations, and planned and performed all radioligand binding experiments (e.g., reduction of filter binding, kinetic experiments, competition binding assays). In addition, the author analyzed and interpreted the data, and created all figures and tables in the manuscript. The author was involved in all aspects of the development of the radioligand binding assay and wrote the manuscript in close collaboration with the corresponding author, Prof. Dr. Christa E. Müller.

3.3.1 Results and discussion

The full-length human P2X4 receptor gene contained a polyhistidine tag (10x-His tag) at the C-terminus with a protein cleavage site (HRV 3C protease) and two mutated glycosylation sites (R75N, R184N). The modified human P2X4 receptor gene was expressed in *Sf9* insect cells using the Bac-to-Bac Baculovirus Expression System (Thermo Fisher Scientific, Dreieich, Germany).

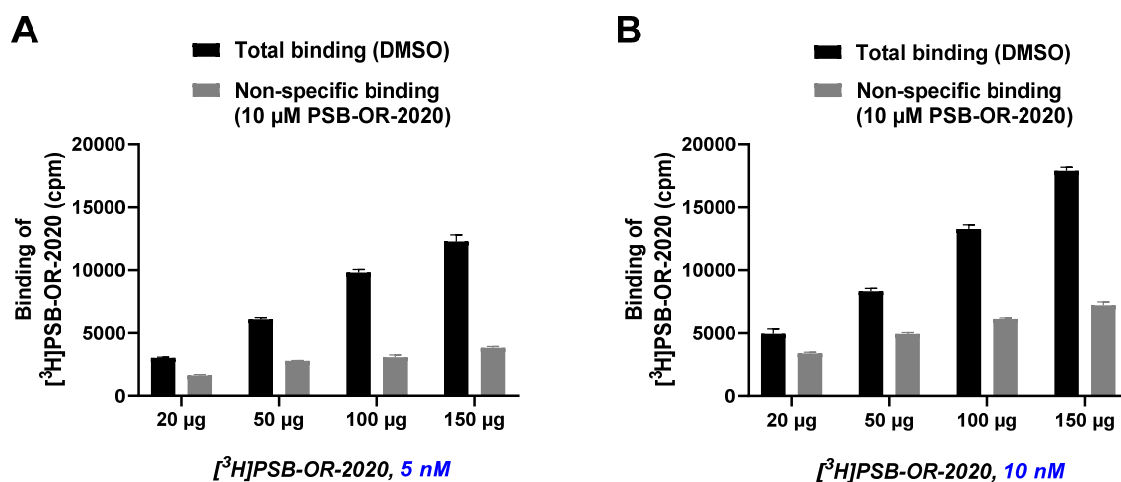


Figure 33. Total and non-specific binding of [³H]PSB-OR-2020 to cell membranes of *Sf9* insect cells recombinantly expressing the human P2X4 receptor (20–150 µg of protein/vial). Different concentrations of [³H]PSB-OR-2020 were employed, (A) 5 nM and (B) 10 nM. Total and non-specific binding were determined in the presence of DMSO, and 10 µM of unlabeled PSB-OR-2020 dissolved in DMSO, respectively.

First experiments with membrane preparations derived from *Sf9* insect cells recombinantly expressing the human P2X4 receptor were performed to investigate specific binding of [³H]PSB-OR-2020 using previously optimized assay conditions.¹²⁹ Total binding of the radioligand was determined in the presence of DMSO, while non-specific binding was determined in the presence of 10 µM of the unlabeled antagonist PSB-OR-2020 dissolved in DMSO. The first experiments indicated a concentration-dependent specific binding of [³H]PSB-OR-2020, which increased with higher protein amounts and radioligand concentrations (Figure 33). Non-specific binding of the radioligand was below 30% of total binding using 100 µg or 150 µg of protein and 5 nM of radioligand (Figure 33). These

observations suggested that the expression of the human P2X4 receptor was successful in *Sf9* insect cells, resulting in high specific binding of the radioligand.

Different cell lines and expression systems had previously been investigated, showing an increased human P2X4 receptor expression in HEK293 cells as compared to 1321N1 astrocytoma cells.¹²⁹ Cell membranes of recombinant *Sf9* insect cells indicated even higher specific binding of the radioligand, suggesting very high P2X4 receptor expression. Figure 34 shows a direct comparison of specific and non-specific radioligand binding using 10 nM of radioligand and 100 µg of protein.

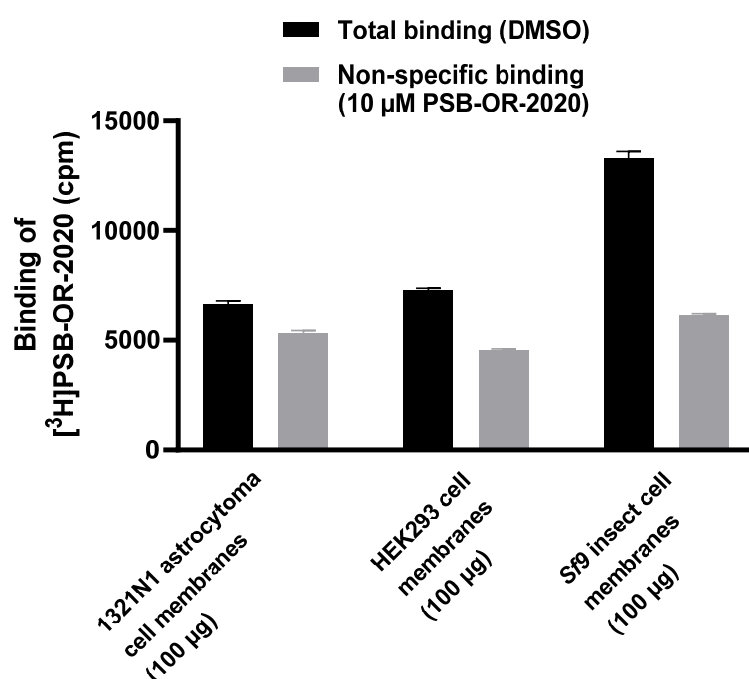


Figure 34. Comparison of total and non-specific binding of [³H]PSB-OR-2020 (10 nM) to cell membrane preparations of 1321N1 astrocytoma cells, HEK293 cells, and *Sf9* insect cells (100 µg of protein/vial, respectively) recombinantly expressing the human P2X4 receptor. Total and non-specific binding were determined in the presence of DMSO and 10 µM of unlabeled PSB-OR-2020 dissolved in DMSO, respectively.

To validate these results and to compare them with previous findings, homologous competition experiments were performed using a range of concentrations of the unlabeled P2X4 receptor antagonist PSB-OR-2020 versus constant concentration of the radioligand (5 nM) (Figure 35A). A K_D value of 3.37 ± 0.75 nM was calculated (Figure 35A). The K_D value appeared to be consistent with the IC_{50} value of the unlabeled compound PSB-OR-2020 determined in calcium influx assays at the human P2X4 receptor expressed in 1321N1 astrocytoma cells (6.32 ± 1.52 nM) and the K_D value determined in HEK293-human P2X4 receptor cell membrane preparations (20.4 ± 7.5 nM).¹²⁹

Subsequently, saturation experiments were performed with various radioligand concentrations (0.5–30 nM) to estimate K_D and B_{max} values. A K_D value of 3.28 ± 1.62 nM was calculated, which was again in agreement with previous data for the human P2X4 receptor.¹²⁹ A representative saturation curve of [³H]PSB-OR-2020 is shown in Figure 35B, indicating that binding of the radioligand to *Sy9*-P2X4 cell membrane preparations was saturable. To prevent high non-specific binding of the radioligand in subsequent experiments, a radioligand concentration of up to 10 nM would be appropriate. The estimated B_{max} value was 1660 ± 90 fmol/mg of protein.

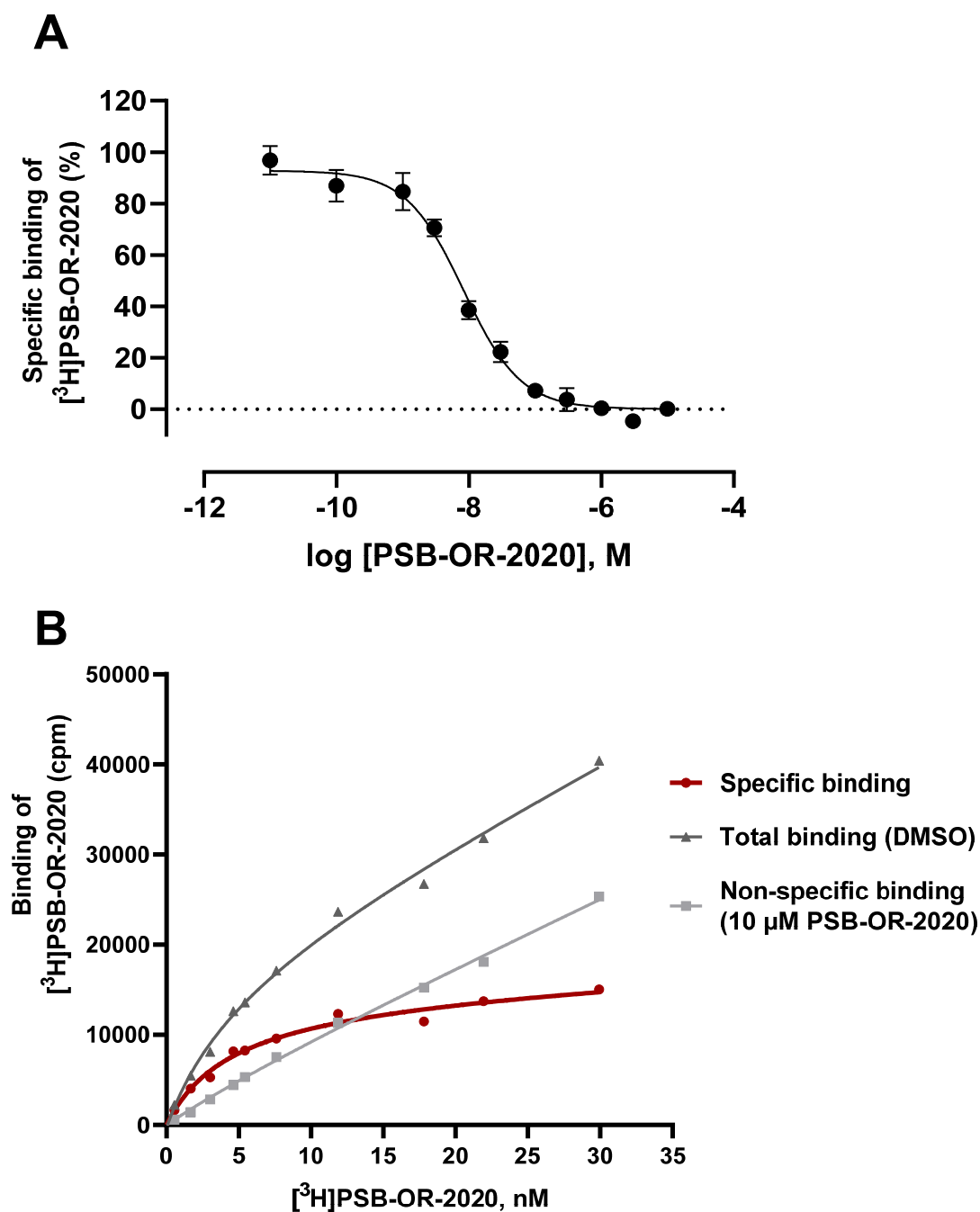


Figure 35. A. Homologous competition binding of various concentrations of PSB-OR-2020 vs. $[^3\text{H}]\text{PSB-OR-2020}$ (5 nM) using membrane preparations of *Sf9* insect cells recombinantly expressing the human P2X4 receptor (150 μg of protein/vial). Data points represent means $\pm$ SEM of four independent experiments performed in duplicates. A K_D value of 3.37 ± 0.75 nM was determined. B. Representative saturation experiment of $[^3\text{H}]\text{PSB-OR-2020}$ on *Sf9*-human P2X4 receptor cell membrane preparations (150 μg of protein/vial). Specific binding of the radioligand was determined at different concentrations (0.5–30 nM). Total and non-specific binding were determined in the presence of DMSO and 10 μM of unlabeled PSB-OR-2020 dissolved in DMSO, respectively.

Next, control experiments using membrane preparations of non-transfected *Sf9* insect cells were performed. Surprisingly, [3 H]PSB-OR-2020 showed high specific binding to native, non-transfected cell membranes (Figure 36), which was comparable to that obtained with cell membranes derived from human P2X4 receptor-expressing *Sf9* insect cells.

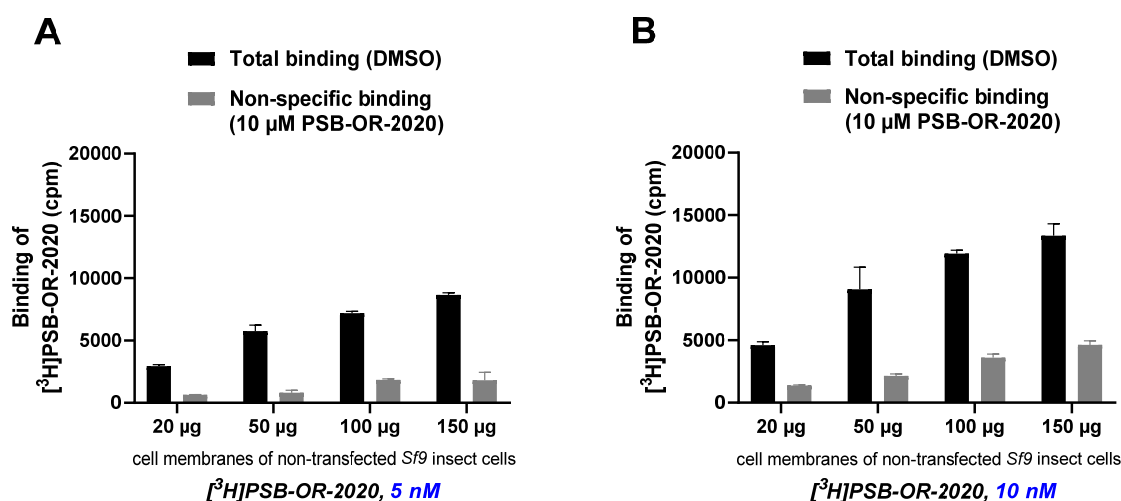


Figure 36. Total and non-specific binding of [3 H]PSB-OR-2020 to cell membranes of non-transfected *Sf9* insect cells (20–150 μ g of protein/vial). Different concentrations of [3 H]PSB-OR-2020 were employed, (A) 5 nM and (B) 10 nM. Total and non-specific binding were determined in the presence of DMSO and 10 μ M of unlabeled PSB-OR-2020 dissolved in DMSO, respectively. Membrane preparations of non-transfected *Sf9* insect cells were prepared in a buffer containing 10 mM HEPES (pH 7.5), 10 mM MgCl_2 , 20 mM KCl, and 30% (v/v) glycerol.

To exclude the possibility that buffer components might affect the specific binding of the radioligand, a new membrane preparation of non-transfected *Sf9* insect cells was prepared in 50 mM Tris-HCl (pH 7.4) buffer, and the specific binding of the radioligand was determined. However, the specific binding of [3 H]PSB-OR-2020 was identical (data not shown).

To evaluate these results further, homologous competition experiments were performed, leading to a concentration-dependent displacement of the radioligand by the unlabeled antagonist PSB-OR-2020 in cell membranes of non-transfected *Sf9* insect cells (Figure 37). A K_i value of 2.61 ± 0.60 nM was calculated.

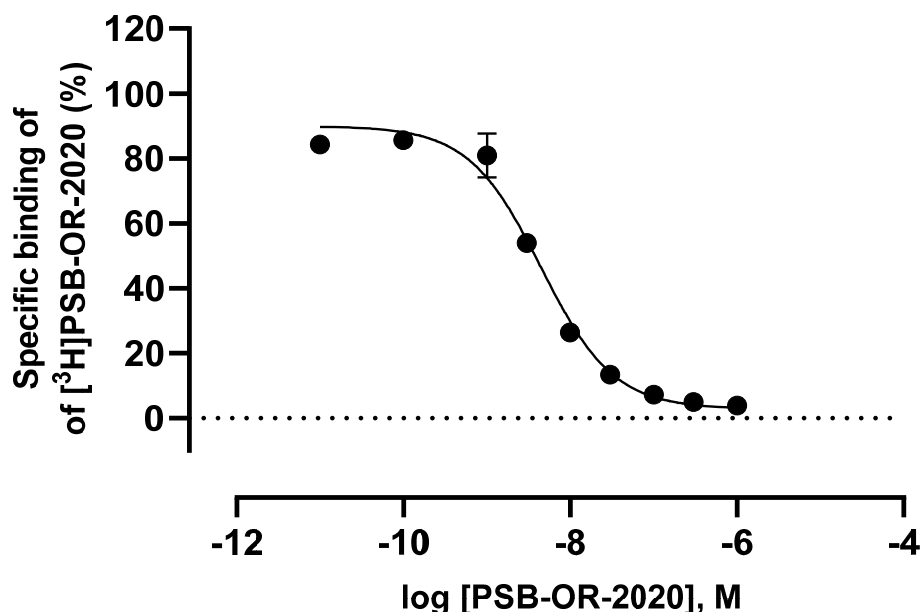


Figure 37. Homologous competition binding of different concentrations of PSB-OR-2020 vs. [^3H]PSB-OR-2020 (5 nM) using membrane preparations of non-transfected *Sf9* insect cells (150 μg of protein/vial). Data points represent means $\pm$ SEM of two independent experiments performed in duplicates. A K_i value of 2.61 ± 0.60 nM was calculated.

The results indicated that the P2X4 receptor radioligand [^3H]PSB-OR-2020 has a very high affinity for an unknown target present in *Sf9* insect cell membranes. So far, there is no evidence for native expression of any P2X receptor subtype in *Sf9* insect cells. However, it is well possible that the radioligand labels an endogenous P2X receptor in the insect cell line.

Several reports have described the use of *Sf9* insect cells to investigate the toxicity of insecticides.²²¹ It is known that voltage-gated and ligand-gated ion channels in the insect nervous system represent efficient targets of neuroactive insecticides.²²² However, it is unclear whether these receptors are expressed in *Sf9* insect cells, as these cells are not derived from central nervous system tissues. In this study, we exposed *Sf9* insect cells to a high concentration of PSB-OR-2020 (~ 10 μM) to assess potential alterations in cell growth, viability, and morphology. Untreated *Sf9* insect cells served as a control, and trypan blue staining was used to differentiate living cells from dead cells. Our preliminary experiments did not show any alterations in cell growth, viability, or morphology of *Sf9* insect cells treated with PSB-OR-2020 over a 96-hour incubation period. To characterize the effects of

PSB-OR-2020 on *Sf9* insect cells, further investigations have to be conducted, including the testing of different compound concentrations, incubation times, and toxicity.

Competition binding experiments with nicotine, imidacloprid, and ivermectin were performed to investigate whether the radioligand binds to similar targets in *Sf9* insect cells as common insecticides. Nicotine was first described in 1690 as a plant-derived insecticide, which was used in the form of tobacco extracts.²²³ It modulates nicotinic acetylcholine receptors, which are important targets for insecticides.^{223,224} These receptors are mainly expressed in the insects' central nervous system.²²⁵ Based on nicotine, several classes of insecticides have been developed, including imidacloprid, which belongs to the class of neonicotinoid insecticides.²²⁶ Imidacloprid exerts its effects through activation of postsynaptic nicotinic acetylcholine receptors.²²⁶ Additionally, ivermectin has been described as an insecticide due to its positive modulation of glutamate-gated chloride channels, which are found in insects.²²⁷ Moreover, it is a positive allosteric modulator of P2X4 receptors.²²⁸

Radioligand competition assays were performed using high concentrations of (-)-nicotine, imidacloprid, and ivermectin at cell membranes of non-transfected *Sf9* insect cells. As a control, specific binding of the radioligand was evaluated within the same experiment using 10 μ M of the unlabeled ligand PSB-OR-2020. Notably, no displacement of the radioligand by high concentrations of (-)-nicotine, imidacloprid, and ivermectin was observed (Figure 38). This shows that these compounds do not bind to the same binding site as the employed radioligand, and that they also do not modulate its binding.

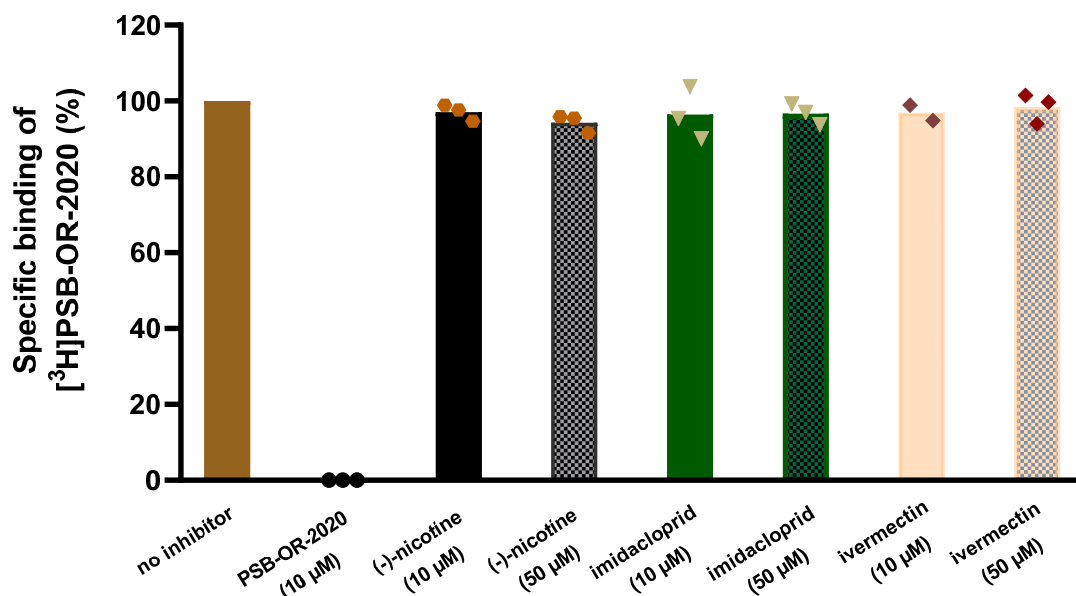


Figure 38. Specific [³H]PSB-OR-2020 binding to cell membranes derived from non-transfected *Sf9* insect cells in the absence and presence of the unlabeled ligand PSB-OR-2020, and various insecticides. Data represent the results from one experiment performed in triplicates. The final concentrations of the compounds are shown in brackets.

As *Sf9* insect cells have been mostly used as host cells for recombinant protein expression, little is known about their endogenously expressed proteins, and in particular about natively expressed ion channels. The first evidence for ion channel activity and endogenous anion channels in *Sf9* insect cells was described in 1991.²²⁹ Further studies provided evidence for endogenously expressed chloride channels, purine- and pyrimidine-selective nucleoside transporters, and proton pumps in *Sf9* insect cells.^{230–233}

Identifying the target in *Sf9* insect cells to which the P2X4 receptor radioligand binds with high affinity will require further studies. These studies should include the analysis of natively expressed proteins in *Sf9* insect cells, as well as functional and binding studies. The results of calcium influx assays could provide information regarding the presence of the P2X4 receptor in *Sf9* insect cells. Reverse transcription-quantitative polymerase chain reaction with specific primers under non-stringent conditions could be applied to amplify mRNA or RNA levels of specific genes in *Sf9* insect cells.

3.3.2 Summary and outlook

In the process of further characterizing the P2X4 receptor radioligand [^3H]PSB-OR-2020, the human P2X4 receptor was expressed in *Sf9* insect cells. In this context an interesting observation was made. First of all, the radioligand exhibited high specific binding to membranes of *Sf9* insect cells recombinantly expressing the human P2X4 receptor, with a nanomolar affinity consistent with previous studies. Surprisingly, however, we observed comparable specific binding of the radioligand as well as concentration-dependent radioligand displacement by the unlabeled ligand PSB-OR-2020 in membranes of non-transfected *Sf9* insect cells. These findings may suggest that *Sf9* insect cells express an endogenous P2X4 receptor that shows similar affinity for the radioligand as the human P2X4 receptor.

The primary objective of this study was to increase human P2X4 receptor expression in *Sf9* insect cells to enable further characterization of the radioligand. However, our results demonstrated that *Sf9* insect cell membranes natively express a high-affinity binding site for the P2X4 receptor radioligand, which will have to be further characterized. To overcome the problem of low receptor expression and high non-specific binding of the radioligand, alternative methodologies could be explored. A possible option would be to preincubate the purified His-tagged P2X4 receptor protein with metal affinity chromatography beads, which would allow to perform filtration assays.

3.3.3 Materials and methods

Materials

Chemicals were obtained from Carl Roth (Karlsruhe, Germany) or Thermo Fisher Scientific (Dreieich, Germany). The radioligand [^3H]PSB-OR-2020 (45 Ci/mmol; 1.67 TBq/mmol) was produced by custom-labeling (Pharmaron, Cardiff, UK).¹²⁹

P2X4 receptor expression in Sf9 insect cells

Design of the receptor construct

The full-length human P2X4 receptor gene contained a polyhistidine tag (10x-His tag) at the C-terminus containing a preceding human rhinovirus (HRV) 3C protease cleavage site. Two of the receptors' glycosylation sites were mutated (R75N, R184N). A baculoviral expression system (Bac-to-Bac, Thermo Fisher Scientific, Dreieich, Germany) was used to

express the P2X4 receptor in *Sf9* insect cells (Expression Systems, Davis, CA, USA). At first, the modified receptor gene was subcloned into the pFastBac1 vector (Thermo Fisher Scientific, Dreieich, Germany). All subsequent steps are outlined in detail as follows:

DNA amplification in DH5α E. coli and DNA purification

The DNA of the human P2X4 receptor construct was transformed into *Escherichia coli* (*E. coli*) strain DH5α by the heat shock method. At first, 50 µL of competent DH5α *E. coli* bacteria were thawed on ice for 10–20 min, followed by adding 5 µL of the DNA, mixed, and incubated for 30 min on ice. The heat shock was performed at 42 °C for 45 s in a water bath. After incubating the mixture on ice for a further 2 min, 100 µL of room-temperature Lysogeny broth (LB) medium were added. The DNA-bacteria mixture was then incubated at 37 °C and 350 rpm for 1 h, spread on an agar plate containing ampicillin (100 µg/mL), and incubated overnight for up to 18 h.

On the next day, single colonies were picked into fresh LB medium (5 mL) containing 100 µg/mL ampicillin and were incubated again overnight at 27 °C and 220 rpm. The DNA purification and elution was performed with the Zymo Research Plasmid Miniprep™ Kit (Zymo Research Europe, Freiburg im Breisgau, Germany) with some deviations. The final elution of the DNA was performed with 30 µL of diethyl pyrocarbonate (DEPC)-treated water.

Transformation into DH10Bac E. coli

Prior to the infection of the *Sf9* insect cell line, the human P2X4 receptor gene was transposed into the baculoviral genome using *E. coli* strain DH10Bac by the heat shock method. 50 µL of competent DH10Bac *E. coli* bacteria were thawed on ice for 10–20 min. Then, 500 ng of the purified plasmid were added and incubated on ice for 30 min. The heat shock was performed for 45 s at 42 °C in a water bath. Following a further 2 min incubation on ice, 800 µL of LB medium were added. The mixture was incubated for 4 h at 37 °C and 350 rpm. The DNA-bacteria mixture was then diluted 1:10 with LB medium, and 75 µL were spread on an agar plate containing 10 µg/mL tetracycline, 7 µg/mL gentamycin, 50 µg/mL of kanamycin, 40 µg/mL isopropyl β-D-1-thiogalactopyranoside (IPTG) and 100 µg/mL 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-Gal; VWR International, Darmstadt, Germany). The plate was wrapped in aluminum foil and incubated at 37 °C for at least 48 h.

White colonies were picked in 4 mL of fresh LB medium containing tetracycline (10 µg/mL), gentamycin (10 µg/mL), and kanamycin (50 µg/mL) and incubated overnight at 37 °C and 220 rpm. The sample was centrifuged for 10 min at 2,500 g. After removing the supernatant, 400 µL of P1 buffer (resuspension buffer containing RNase; Qiagen, Hilden, Germany) were added and the pellet was gently resuspended. Then, 400 µL of P2 buffer (lysis buffer; Qiagen, Hilden, Germany) were added and the mixture was gently inverted. After adding 400 µL of P3 buffer (neutralization buffer; Qiagen, Hilden, Germany) and gently mixing, the sample was centrifuged at 14,000 g for 15 min at 4 °C. After that, 800 µL of isopropanol were added into an empty 2 mL tube. The supernatants of the samples were carefully added to the isopropanol and inverted. After 30 min of incubation at -20 °C, the samples were again centrifuged at 14,000 g for 15 min at 4 °C to precipitate the bacmid. The supernatant was removed using a vacuum pump. Then, 500 µL of pre-chilled 70% ethanol were added, and the samples were inverted gently and again centrifuged at 14,000 g for 15 min at 4 °C. The vacuum pump was used to drain the supernatant. The last three steps were repeated once.

The resulting pellets were dried under the sterile hood for 15–20 min and 30 µL of DEPC-treated water were added. The bacmids were centrifuged at 4 °C for 10 min at 14,000 g and aliquoted under the sterile hood. Subsequently, a bacmid verification test was performed by polymerase chain reaction (PCR) and agarose gel.

PCR and agarose gel

To verify the presence of the correct insert within the bacmid, a PCR analysis of the inserted gene was performed as outlined in Table 8.

3. Results and discussion

Table 8. PCR components for bacmid verification.

Component	Volume
10x Standard Taq buffer (New England Biolabs, Frankfurt, Germany)	2.5 μ L
10 mM dNTPs (New England Biolabs, Frankfurt, Germany)	0.5 μ L
M13 forward primer (5 μ M) (5'-CCCAGTCACGACGTTGTAAAACG-3')	0.5 μ L
M13 reverse primer (5 μ M) (5'-AGCGGATAACAATTTCACACAGG-3')	0.5 μ L
Template (bacmid)	1 μ L
Taq polymerase (New England Biolabs, Frankfurt, Germany)	0.15 μ L
DEPC-treated water	19.85 μ L

Table 9. PCR program with temperature and duration.

Temperature $^{\circ}$ C	Duration
95 $^{\circ}$ C	30 s
[95 $^{\circ}$ C	30 s
58 $^{\circ}$ C	60 s
68 $^{\circ}$ C	3 min] x 25 cycles
72 $^{\circ}$ C	5 min

The PCR product was mixed with loading dye and analyzed on a 1% agarose gel (30 min at 130 V).

Sf9 insect cell cultivation

Sf9 insect cells (Expression Systems, Davis, CA, USA) were cultured in ESF921 cell culture medium (Expression Systems, Davis, CA, USA) at 27 $^{\circ}$ C and 150 rpm.

Bacmid transfection into Sf9 cells

The bacmid transfection was performed in a 24-deep well culture block under sterile conditions. The transfection sample contained 5 μ L of the bacmid sample, 100 μ L of transfection medium (Expression Systems, Davis, CA, USA), and 3 μ L of X-

tremeGENE™ HP (Sigma-Aldrich, Taufkirchen, Germany). The mixture was incubated for 15 min at room temperature and added to 2.5 mL of *Sf9* insect cells at a density of 1×10^6 cells/mL. The sample was then incubated in a thermoshaker for 96 h, at 27 °C and 400 rpm. After 96 h, the sample was centrifuged at 2,000 rpm for 15 min to harvest the virus (P0). The virus was stored at 4 °C.

Protein expression

Small-scale protein expression

Virus P0 (400 µL) was used to infect 40 mL of freshly-grown *Sf9* insect cells at $2\text{--}3 \times 10^6$ cells/mL. The cells were incubated for 48 h at 27 °C and 150 rpm. After that, the cells were precipitated by centrifugation at 4,000 g for 15 min in 50 mL falcon tubes. Virus P1 (supernatant) was stored at 4 °C. The biomass (cell pellet) was stored at -80 °C for later protein analysis.

Large-scale protein expression

Large-scale P2X4 receptor expression was performed using 1,000 mL of freshly-grown *Sf9* insect cells at $2\text{--}3 \times 10^6$ cells/mL that were infected with virus P1 (~6 mL) and incubated for 48 h at 27 °C and 150 rpm. After then, the cells were precipitated by centrifugation at 4,000 g for 30 min in 50 mL falcon tubes. The supernatant was discarded and the pellet was resuspended in 30 mL of sterile phosphate buffer (phosphate-buffered saline (PBS)) and centrifuged at 4,000 g for 20 min. The supernatant was removed and the biomass was stored at -80 °C for later protein analysis.

Sf9 insect cell membrane preparation

To prepare *Sf9* insect cell membranes, the biomass from large-scale protein expression was thawed at room temperature. All subsequent steps were carried out on ice or at 4 °C. Then, cell membrane preparations were obtained by repeated homogenization using a Dounce tissue homogenizer (VWR International, Darmstadt, Germany) and different buffers. At first, low osmotic buffer (10 mM HEPES (pH 7.5), 10 mM MgCl₂, 20 mM KCl) supplemented with ethylenediamine tetraacetic acid (EDTA)-free complete protease inhibitor cocktail tablets (Roche, Mannheim, Germany) was used, followed by centrifugation for 30–60 min at 57,000 g. The resulting pellet was again homogenized in low osmotic buffer. Then, membranes were washed twice in the same way using high osmotic buffer (10 mM HEPES (pH 7.5), 10 mM MgCl₂, 20 mM KCl, 1 M NaCl). Purified

membranes were resuspended either in resuspension buffer (10 mM HEPES (pH 7.5), 10 mM MgCl₂, 20 mM KCl, 30% (v/v) glycerol) or in 50 mM Tris-HCl buffer (pH 7.4), flash frozen with liquid nitrogen, and stored at -80 °C.

Cell membrane preparations of non-transfected cells were produced in the same way.

Protein concentration determination

Two assays, Bradford and Lowry assays, were employed to determine the protein concentration of the *Sf9* insect cell membrane preparation, two assays were employed.^{234,235} BSA was used for the calibration curve of at least five dilutions between 500 and 100 µg/mL for both assays.

The Bradford stock solution was prepared by dissolving 0.1 g of Coomassie-Brilliant Blue G in 50 mL of ethanol with 100 mL of 85% phosphoric acid, diluted to 1,000 mL with distilled water. The membrane preparations were diluted 1:10 and 1:20 in the respective buffer, and then 50 µL of every dilution was mixed with 1 mL of the Bradford stock solution. Then, the absorbance was measured at $\lambda=595$ nm using a UV/Vis spectrophotometer (DU[®]530 Life Science UV/Vis Spectrophotometer, Beckman Coulter, Krefeld, Germany).

The Lowry stock solution was prepared by mixing solution A (2% (w/v) NaHCO₃ in NaOH) and solution B (aqueous 0.5% (w/v) Cu₂SO₄ + 1% (w/v) sodium tartrate) in a 50:1 ratio. At first, 200 µL of the membrane preparations (diluted 1:10 and 1:20 in the respective buffer) were incubated with 1,000 µL of the Lowry stock solution for 20 min at room temperature. Then, 100 µL Folin & Ciocalteu's phenol reagent (18 mL Folin reagent + 90 mL distilled water) (Merck, Darmstadt, Germany) were added to the mixture, following an incubation of 30 min at room temperature. The absorbance was measured at $\lambda=500$ nm using a UV/Vis spectrophotometer (DU[®]530 Life Science UV/Vis Spectrophotometer, Beckman Coulter, Krefeld, Germany).

Radioligand binding studies

Previous optimized assay conditions were used to determine the specific binding of [³H]PSB-OR-2020 on *Sf9* insect cell membranes either being transfected with the human P2X₄ receptor or derived from non-transfected cells.¹²⁹ The detailed experimental procedures for determining specific binding of the radioligand and for homologous competition assays were described before.¹²⁹ K_D and B_{max} values were determined by using

the “competitive binding: one site – homologous” equation implemented in GraphPad Prism version 8.0 (GraphPad Software, San Diego, CA, USA).

For saturation experiments, different concentrations of [³H]PSB-OR-2020 (0.5–30 nM) were incubated with 150 µg of *Sf9*-P2X4 cell membranes and either with 5 µL of DMSO or 10 µM of unlabeled PSB-OR-2020 for determining total binding and non-specific binding, respectively. The samples were incubated for 1 h at 4 °C while gently shaking. K_D and B_{max} values were determined by using the “saturation binding: one site – specific binding” equation implemented in GraphPad Prism version 8.0 (GraphPad Software, San Diego, CA, USA).

3.4 Biological evaluation of thioisosteres of nucleoside di- and triphosphates at P2X receptor subtypes

The results of this chapter were published in:

Zhang, H.-J.; Ociepa, M.; Nassir, M.; Zheng, B.; Lewicki, S. A.; Salmaso, V.; Baburi, H.; Nagel, J.; Mirza, S.; Bueschbell, B.; Al-Hroub, H.; Perzanowska, O.; Lin, Z.; Schmidt, M. A.; Eastgate, M. D.; Jacobson, K. A.; Müller, C. E.; Kowalska, J.; Jemielity, J.; Baran, P. S. *Stereocontrolled access to thioisosteres of nucleoside di- and triphosphates*. *Nature Chemistry*, 2024, 16, 249–258.

Nucleoside diphosphates (NDPs), nucleoside triphosphates (NTPs), and their derivatives are essential biomolecules that play pivotal roles in various physiological processes, including nucleic acid synthesis, primary metabolism, energy supply, signaling, and neurotransmission.²³⁶ These biomolecules share a common structure consisting of one or two nucleosides that are attached to one or more phosphate groups, with phosphorus in the oxidation state of +5.²³⁶ In biochemistry and molecular biology, they act as enzyme substrates and receptor ligands, and contribute to the synthesis of oligonucleotides.^{236–238} Moreover, since many decades, nucleotide analogs have shown therapeutic potential as antiviral agents.^{239–241} A prominent example is the phosphoramidate drug Remdesivir (GS-5734), which exhibits a broad antiviral activity against a wide range of RNA viruses, including the ebolavirus, and, most recently, the severe acute respiratory syndrome coronavirus 2.²⁴² Two further important examples include Sofosbuvir for the treatment of chronic hepatitis C virus infections and Tenofovir to treat hepatitis B and human immunodeficiency virus infections.^{243,244} These examples demonstrate that many successful efforts have been made to develop diverse nucleotide analogs with multiple mechanisms of action to treat life-threatening viral infections. Nevertheless, using nucleotides as pharmaceutical drugs comes along with some obstacles, including resistance mechanisms, the challenge of achieving an optimal balance between efficacy and toxicity, and their rapid in vivo metabolism.²³⁹ Therefore, there is a continuing need for the development of novel nucleotide analogs with enhanced properties.

Successful strategies have been developed to address the instability of these biomolecules, including the isosteric replacement of the monophosphate moiety with phosphorothioates.

Eckstein and colleagues first described the preparation of α -thio-modified NDPs and NTPs in which a non-bridging oxygen on the α -phosphorus atom was replaced by a sulfur.^{245,246} Further studies could show that phosphorothioates are clinically validated isosteres for monophosphates with increased metabolic stability.^{247–249} They are also useful for synthesizing promising oligonucleotides and small-molecule drug candidates.^{249–251} The synthesis of phosphorothioates is particularly challenging due to low chemoselectivity, multistep reaction sequences, and problems with scalability.^{252–254} In addition, the introduction of thio groups leads to an additional chiral center at the phosphorus.^{245,255}

Recently, a reagent-controlled synthetic approach was described that allows for the stereo-controlled and scalable synthesis of thio-modified nucleotides.²⁵⁶ The invention of chiral P(V)- α -thio-di- and triphosphate transfer reagents, which can be prepared in situ and easily appended to a library of nucleoside donors, enables complete stereospecificity in the synthetic process.²⁵⁶ This results in pure R_P or S_P isomers without any P -epimerization.²⁵⁶

Using these novel transfer reagents, several stereopure thioisosteres of nucleoside di- and triphosphates were synthesized and characterized (Figure 39).²⁵⁶

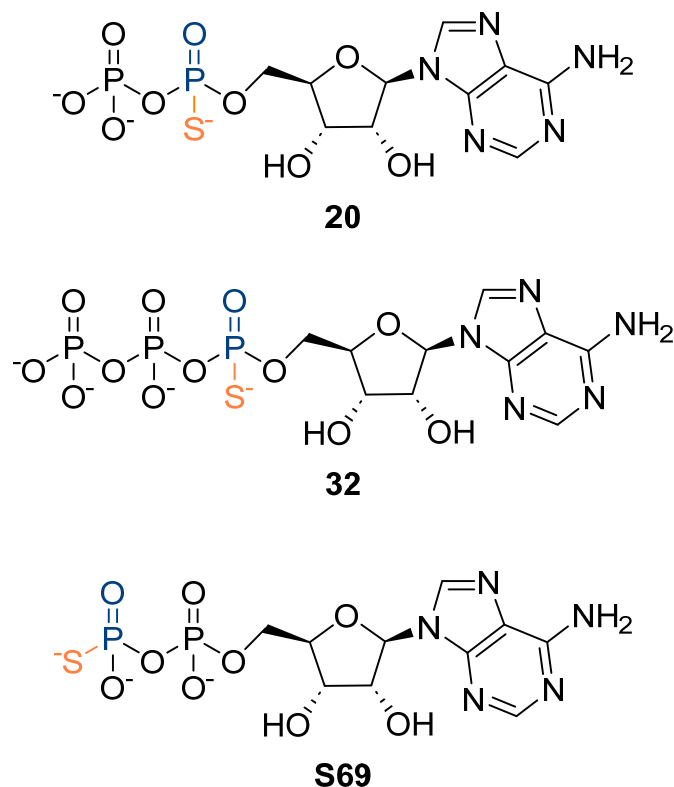


Figure 39. Chemical structures of nucleoside di- and triphosphate thioisosteres.²⁵⁶

Although the previous absence of robust synthetic routes has limited the accessibility of nucleotide thio-analogs, several biological studies have demonstrated their utility. For instance, adenosine diphosphate (ADP) thioisosteres, such as compound **20** (Figure 39), have been shown to induce human platelet aggregation, with the *S_P* isomer being five times more potent than the *R_P* isomer.²⁵⁷ Further studies described NDP thio-analogs as potent inhibitors of the pyrophosphatase/phosphodiesterase-1 enzyme with potential application for the treatment of osteoarthritis (compounds **20** and **32**, Figure 39).²⁵⁸ Additionally, several NDPs and NTPs, such as compound **20** and **32** (Figure 39), were characterized as potential P2 nucleotide receptor agonists.^{259,260} P2X receptors, which belong to the P2 receptor family, have emerged as important targets for novel drug interventions due to their involvement in many physiological and, in particular, pathophysiological processes.^{10,57}

Although several ATP derivatives and analogs were identified as P2X receptor agonists, no subtype-selective agonists have been reported to date.¹⁰ In this study, the effects of compounds (*R_P*)-**32**, (*S_P*)-**32**, (*R_P*)-**20**, (*S_P*)-**20**, and **S69** (Figure 39) were tested at human P2X1, P2X2, P2X3, and P2X4 receptors in calcium influx assays.

3.4.1 Results

Human P2X1 receptor

In order to prevent fast desensitization and to enable receptor characterization, a chimeric human P2X2/P2X1 receptor had been designed.²⁶¹ This receptor contains the entire P2X1 ectodomain, giving it the same ligand specificity as the P2X1 wildtype receptor, but with a different desensitization behavior due to the non-desensitizing portion of the P2X2 receptor.^{261,262}

All investigated compounds activated the P2X1 receptor, with the α -thio-ATP analogs (*R_P*)-**32** and (*S_P*)-**32** being the most potent ones (EC_{50} values of 79.6 ± 2.8 nM and 83.7 ± 7.8 nM, respectively) (Figure 40). The EC_{50} values were in a comparable range as the EC_{50} value of ATP (EC_{50} : 16.7 ± 2.6 nM). This indicated that an α -thiophosphate motif is well tolerated by the P2X1 receptor. In comparison, the α -thio-ADP analogs (*R_P*)-**20** and (*S_P*)-**20** were about 6-fold less potent (EC_{50} values of 459 ± 114 nM and 605 ± 147 nM, respectively). The efficacy of α -thio-ATP and α -thio-ADP analogs was between 80% and 88% at the P2X1 receptor as compared to ATP.

The β -thio-ADP analog **S69** showed the lowest potency (EC_{50} : $3,980 \pm 540$ nM) and the lowest efficacy (62%). No significant stereoselectivity was observed for the diastereomers (P value > 0.05).

Human P2X2 receptor

All investigated compounds behaved as P2X2 receptor agonists in calcium influx assays, however with different potencies and efficacies (Figure 41). Regarding potency, the α -thio-ATP analogs (R_P)-**32** and (S_P)-**32** were the most potent compounds at the P2X2 receptor (EC_{50} values of 368 ± 103 nM and 620 ± 156 nM, respectively), showing EC_{50} values comparable to that of ATP (EC_{50} : 847 ± 125 nM). These results suggested that an α -thiophosphate modification is well tolerated by the P2X2 receptor.

In comparison, the ADP analogs (R_P)-**20**, (S_P)-**20**, and **S69** were about 60-fold less potent (EC_{50} values of $21,300 \pm 4,200$ nM, $37,000 \pm 8,170$ nM, and $39,100 \pm 1,200$ nM, respectively). The efficacy was around 100% for all investigated compounds at the P2X2 receptor, except for compounds (S_P)-**20** and **S69**, displaying efficacies of less than 50%. No significant stereoselectivity was observed (P value > 0.05).

Human P2X3 receptor

To characterize the human P2X3 receptor, the non-desensitizing mutation S15V was inserted, as earlier described.²⁹ All investigated compounds showed agonistic effects at the P2X3 receptor in calcium influx assays (Figure 42). The α -thio-ATP analogs were more potent (EC_{50} of (R_P)-**32**: 22.7 ± 7.6 nM; EC_{50} of (S_P)-**32**: 112 ± 2 nM) than the α -thio-ADP analogs (EC_{50} of (R_P)-**20**: 793 ± 17 nM; EC_{50} of (S_P)-**20**: $6,240 \pm 116$ nM). The β -thio-ADP analog **S69** was the least potent (EC_{50} : $15,200 \pm 4,000$ nM) with the lowest efficacy of 18% (Figure 42). The most potent compound at the P2X3 receptor was (R_P)-**32** with a similar potency as ATP (EC_{50} of ATP: 21.6 ± 5.8 nM) and an efficacy of 92%.

Noteworthy, stereoselectivity was observed for both α -thio-ATP and α -thio-ADP analogs at the P2X3 receptor. (S_P)-**20** and (S_P)-**32** showed significantly lower potencies than their R_P isomers (P values=0.000046 and 0.001579, respectively). In the case of α -thio-ADP analogs, stereoselectivity ($R_P > S_P$) was also observed regarding efficacy (efficacy of (R_P)-**20**, 87%, and of (S_P)-**20**, 33%). These results suggested that the P2X3 receptor has a preference for the α -thio- R_P isomers.

Human P2X4 receptor

The most potent compounds at the P2X4 receptor were the α -thio-ATP analogs, with (*R*_P)-**32** exhibiting similar potency as ATP (EC₅₀ of (*R*_P)-**32**: 149 ± 38 nM; EC₅₀ of ATP: 207 ± 43 nM) (Figure 43). The potency of (*S*_P)-**32** was slightly decreased (EC₅₀: 472 ± 60 nM). Statistical analysis of EC₅₀ values indicated stereoselectivity for α -thio-ATP analogs, favoring the α -thio-*R*_P isomer (P value=0.0108).

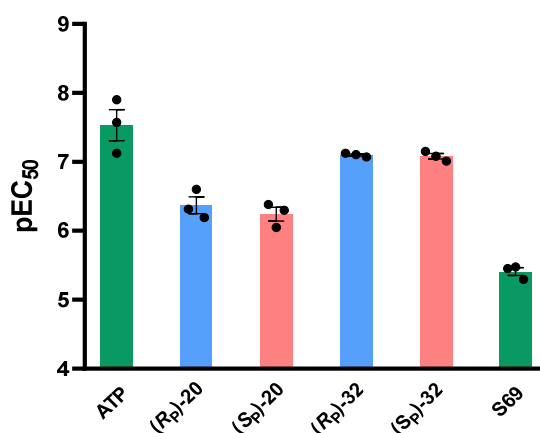
The potencies of the α -thio-ADP analogs (*R*_P)-**20** and (*S*_P)-**20** (EC₅₀ values of $4,520 \pm 510$ nM and $5,510 \pm 860$ nM, respectively) were significantly lower at the P2X4 receptor as compared to the potencies of the ATP analogs. The β -thio-ADP analog **S69** displayed the lowest potency (EC₅₀: $10,200 \pm 1,810$ nM) of all investigated compounds. Regarding efficacies, (*S*_P)-**20** and **S69** showed the lowest efficacies of 21% and 23% at the P2X4 receptor, respectively.

Human P2X1 receptor

A

Compound	EC ₅₀ ± SEM (nM)	Efficacy ± SEM (%)
ATP	16.7 ± 2.6	
(R _P)-20	459 ± 114	86 ± 9
(S _P)-20	605 ± 147	80 ± 8
(R _P)-32	79.6 ± 2.8	88
(S _P)-32	83.7 ± 7.8	86
S69	3,980 ± 540	62 ± 4

B



C

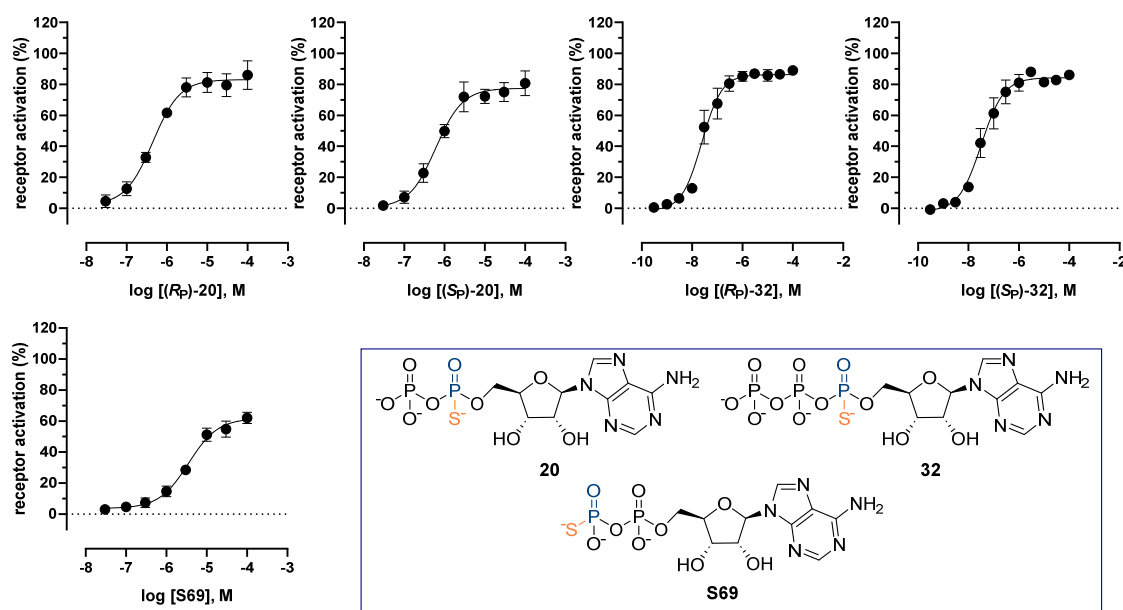


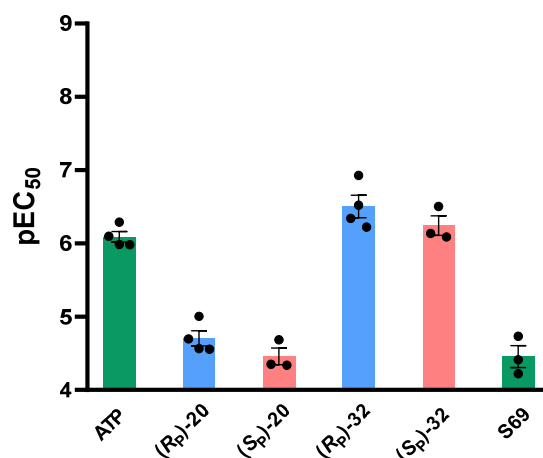
Figure 40. Biological evaluation of compounds (R_P)-20, (S_P)-20, (R_P)-32, (S_P)-32, and S69 at the human P2X1 receptor stably expressed in 1321N1 astrocytoma cells. All results were determined in fluorimetric calcium influx assays. A. Determined EC₅₀ values ± SEM (nM) and efficacies ± SEM (%), relative to the maximal ATP-induced effect (100%). B. Relative potency at the human P2X1 receptor expressed as pEC₅₀ values. The results are presented as mean values ± SEM from three independent experiments (n=3). Black dots represent results of each independent experiment, performed in duplicates. C. Concentration-dependent activation of the P2X1 receptor by compounds (R_P)-20, (S_P)-20, (R_P)-32, (S_P)-32, and S69, respectively. Receptor activation was normalized to the maximal ATP-induced effect. The results are presented as mean values ± SEM from three independent experiments (n=3), performed in duplicates.

Human P2X2 receptor

A

Compound	EC ₅₀ ± SEM (nM)	Efficacy ± SEM (%)
ATP	847 ± 125	
(R _P)-20	21,300 ± 4,200	98 ± 10
(S _P)-20	37,000 ± 8,170	48 ± 11
(R _P)-32	368 ± 103	106 ± 4
(S _P)-32	620 ± 156	101 ± 19
S69	39,100 ± 1,200	27 ± 6

B



C

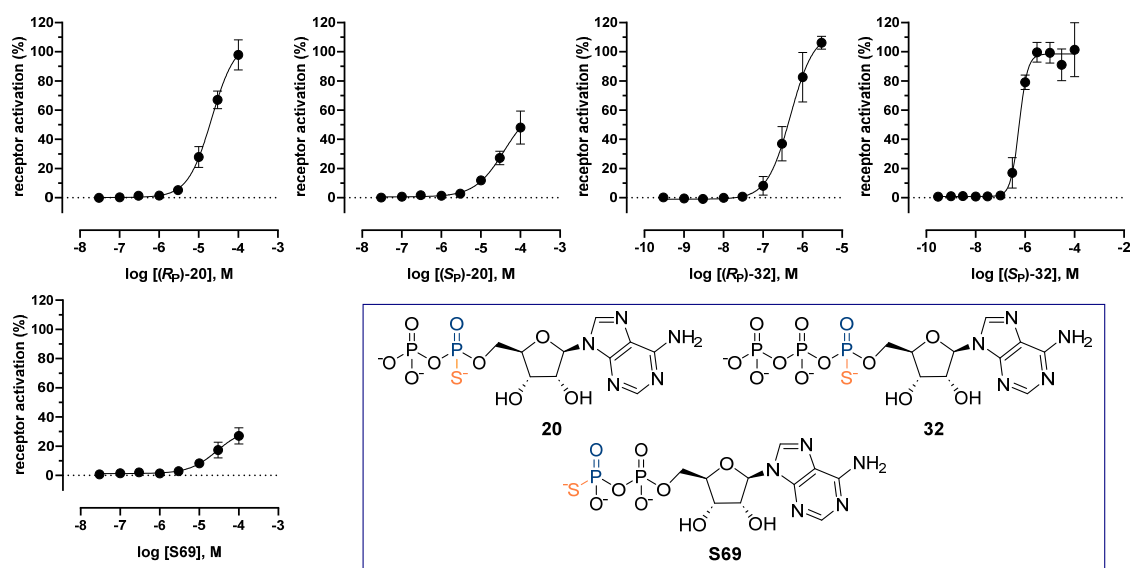


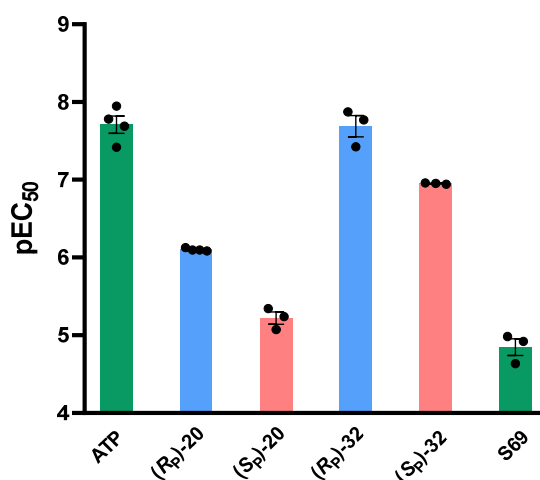
Figure 41. Biological evaluation of compounds (R_P)-20, (S_P)-20, (R_P)-32, (S_P)-32, and S69 at the human P2X2 receptor stably expressed in 1321N1 astrocytoma cells. All results were determined in fluorimetric calcium influx assays. A. Determined EC₅₀ values ± SEM (nM) and efficacies ± SEM (%), relative to the maximal ATP-induced effect (100%). B. Relative potency at the human P2X2 receptor expressed as pEC₅₀ values. The results are presented as mean values ± SEM from three independent experiments (n=3). Black dots represent results of each independent experiment, performed in duplicates. C. Concentration-dependent activation of the P2X2 receptor by compounds (R_P)-20, (S_P)-20, (R_P)-32, (S_P)-32, and S69, respectively. Receptor activation was normalized to the maximal ATP-induced effect. The results are presented as mean values ± SEM from three independent experiments (n=3), performed in duplicates.

Human P2X3 receptor

A

Compound	EC ₅₀ ± SEM (nM)	Efficacy ± SEM (%)
ATP	21.6 ± 5.8	
(R _P)-20	793 ± 17	87 ± 4
(S _P)-20	6,240 ± 116	33 ± 3
(R _P)-32	22.7 ± 7.6	92
(S _P)-32	112 ± 2	80 ± 8
S69	15,200 ± 4,000	18 ± 4

B



C

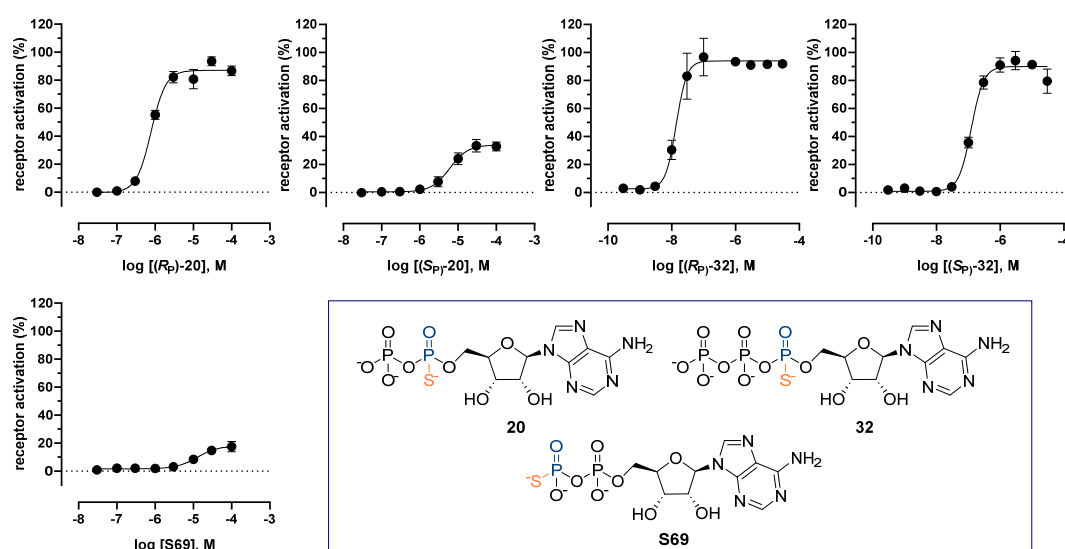


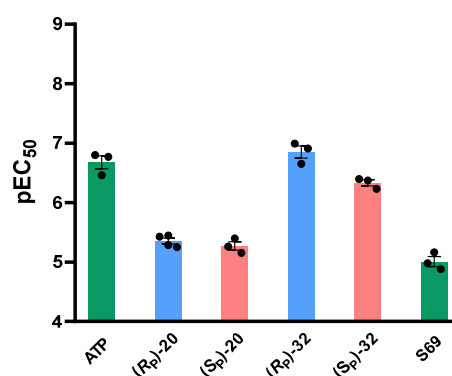
Figure 42. Biological evaluation of compounds (R_P)-20, (S_P)-20, (R_P)-32, (S_P)-32, and S69 at the human P2X3 receptor stably expressed in 1321N1 astrocytoma cells. All results were determined in fluorimetric calcium influx assays. A. Determined EC₅₀ values ± SEM (nM) and efficacies ± SEM (%), relative to the maximal ATP-induced effect (100%). B. Relative potency at the human P2X3 receptor expressed as pEC₅₀ values. The results are presented as mean values ± SEM from three independent experiments (n=3). Black dots represent results of each independent experiment, performed in duplicates. C. Concentration-dependent activation of the P2X3 receptor by compounds (R_P)-20, (S_P)-20, (R_P)-32, (S_P)-32, and S69, respectively. Receptor activation was normalized to the maximal ATP-induced effect. The results are presented as mean values ± SEM from three independent experiments (n=3), performed in duplicates.

Human P2X4 receptor

A

Compound	EC ₅₀ ± SEM (nM)	Efficacy ± SEM (%)
ATP	207 ± 43	
(R _P)-20	4,520 ± 510	93 ± 12
(S _P)-20	5,510 ± 860	21
(R _P)-32	149 ± 38	91 ± 3
(S _P)-32	472 ± 60	106
S69	10,200 ± 1,810	23 ± 2

B



C

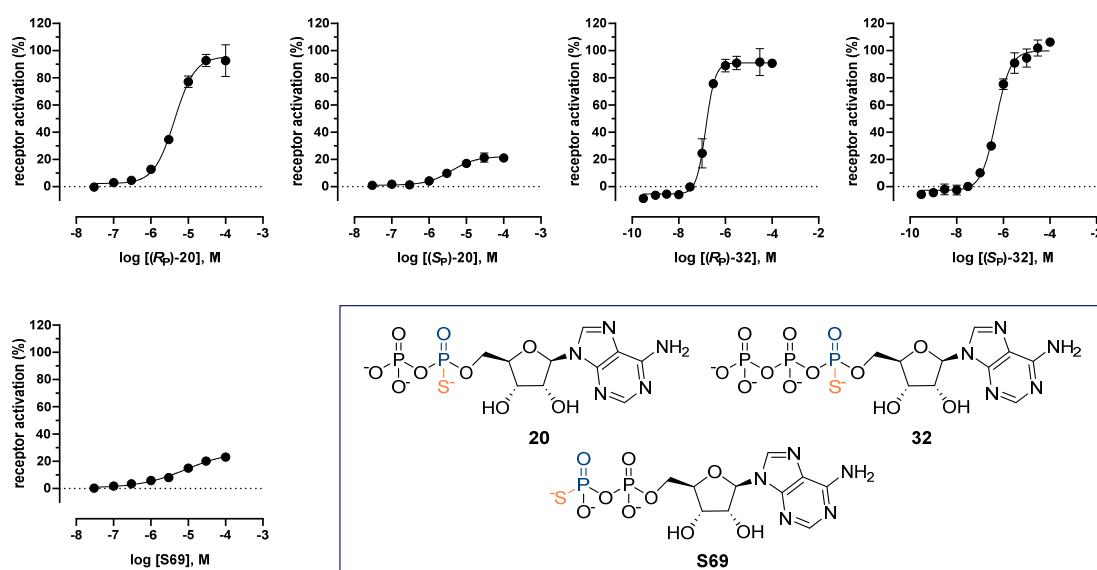


Figure 43. Biological evaluation of compounds (R_P)-20, (S_P)-20, (R_P)-32, (S_P)-32, and S69 at the human P2X4 receptor stably expressed in 1321N1 astrocytoma cells. All results were determined in fluorimetric calcium influx assays. A. Determined EC₅₀ values ± SEM (nM) and efficacies ± SEM (%), relative to the maximal ATP-induced effect (100%). B. Relative potency at the human P2X4 receptor expressed as pEC₅₀ values. The results are presented as mean values ± SEM from three independent experiments (n=3). Black dots represent results of each independent experiment, performed in duplicates. C. Concentration-dependent activation of the P2X4 receptor by compounds (R_P)-20, (S_P)-20, (R_P)-32, (S_P)-32, and S69, respectively. Receptor activation was normalized to the maximal ATP-induced effect. The results are presented as mean values ± SEM from three independent experiments (n=3), performed in duplicates.

3.4.2 Discussion

The use of nucleoside di- and triphosphates as tool compounds for studying nucleotide-dependent enzymes and receptors is limited due to their rapid *in vivo* metabolism. A novel approach enabling the stereocontrolled synthesis of nucleotide thioisosteres has offered new opportunities to obtain nucleotide analogs bearing thiophosphate groups at large scale, which are metabolically more stable.²⁵⁶

P2X receptors are important drug targets involved in a wide range of physiological and pathophysiological processes.⁵⁷ In the past decades, the focus has been on discovering positive and negative allosteric P2X receptor modulators due to challenges associated with the high polarity of the orthosteric binding site.⁶⁷ While several ATP derivatives were characterized as P2X receptor agonists, no subtype-selective agonists are currently available.⁶⁷

In this study using calcium influx assays, ATP and ADP thio-analogs (compounds **20**, **32**, and **S69**) were biologically evaluated at human P2X receptor subtypes, revealing varying potencies and efficacies. α -thio-ATP analogs (*R_P*)-**32** and (*S_P*)-**32** displayed higher potency at P2X1, P2X2, P2X3 and P2X4 receptors as compared to the potency of α -thio-ADP analogs. The highest potency of (*R_P*)-**32** and (*S_P*)-**32** was observed at P2X1 and P2X3 receptors, and the lowest potency at P2X2 and P2X4 receptors.

Overall, these results demonstrated that the α -thio-modification in ATP analogs is well tolerated by all studied P2X receptors. The β -thio-ADP analog **S69** displayed the lowest potency at all P2X receptors, indicating a limited tolerance for the β -thio-ADP analog.

Stereoselectivity was observed only at P2X3 and P2X4 receptors, both favoring the α -thio-*R_P* isomer. The potency of the α -thio-ATP analog (*R_P*)-**32** was three times higher at the P2X4 receptor and five times higher at the P2X3 receptor as compared to its (*S_P*)-stereoisomer. When compared to ATP, (*R_P*)-**32** exhibited similar potency at P2X4 and P2X3 receptors and full efficacy. Due to its similar effects to ATP, compound (*R_P*)-**32** could be considered as an alternative tool compound with increased metabolic stability to explore the roles of P2X3 and P2X4 receptors and to assess the effects of antagonists. Additionally, this compound could be a potential candidate for isotope labeling to be used in radioligand binding studies.

3.4.3 Summary and outlook

In this study, we evaluated several ATP and ADP thioisosteres, synthesized through a novel synthetic route developed by Baran and coworkers,²⁵⁶ for their effects on P2X receptors in calcium influx assays. The results indicated that an α -thio-modification in ATP analogs is well tolerated by all analyzed P2X receptors. P2X3 and P2X4 receptors displayed a preference for the α -thio- R_P isomers, demonstrating how P -stereochemistry can influence ligand-receptor interactions.

These nucleotide thioisosteres have potential as tool compounds for investigating the role of P2X receptors in vitro and in vivo. One of their major advantages is their increased metabolic stability, which greatly improves the feasibility of biological experiments. The α -thio modification of ATP analogs may be further utilized to obtain improved P2X receptor ligands.

3.4.4 Materials and methods

Chemicals

Compounds (R_P)-**32**, (S_P)-**32**, (R_P)-**20** and (S_P)-**20**, and **S69** were synthesized in the laboratory of Prof. Phil S. Baran (La Jolla, CA, USA) according to published protocols.²⁵⁶ ATP was purchased from Carl Roth (Karlsruhe, Germany).

Cell culture

Non-transfected 1321N1 astrocytoma cells (Sigma-Aldrich, Taufkirchen, Germany) were cultivated in DMEM (Thermo Fisher Scientific, Dreieich, Germany) containing 10% FCS, 100 U/mL penicillin, and 100 μ g/mL streptomycin. FCS and antibiotics were purchased from PAN Biotech (Aidenbach, Germany). Stably transfected 1321N1 astrocytoma cells were grown in DMEM, 10% FCS, penicillin (100 U/mL), streptomycin (100 μ g/mL), and 800 μ g/mL of geneticin (G418; PAN Biotech, Aidenbach, Germany). All cells were cultivated at 37 °C and 10% CO₂, except for the human P2X3-expressing cell line that was cultivated at 37 °C and 5% of CO₂. In general, the cells were cultivated in cell culture flasks until 80–90% confluency, washed with PBS, and detached with trypsin/EDTA. The trypsin reaction was terminated by adding medium, and the respective amount of cell suspension was transferred to a new cell culture flask with fresh medium.

Retroviral transfection

1321N1 astrocytoma cells were stably transfected with human P2X1, P2X2, P2X3 and P2X4 receptor, respectively, using a retroviral expression system.¹³⁴ For the expression of the P2X1 receptor, a non-desensitizing chimeric receptor construct was used, and for the P2X3 receptor, the non-desensitizing S15V mutant was inserted.^{29,261,262} All receptor DNAs were subcloned into the retroviral vector pQCXIN. At first, recombinant helper viruses were produced by using GP+envAM-12 fibroblast-type mouse packaging cells (American Type Culture Collection (ATCC), Manassas, VA, USA).²¹³ Then, 1.5×10^6 packaging cells were plated in 25 cm² cell culture flasks using DMEM with 10% FCS, penicillin (100 U/mL), and streptomycin (100 µg/mL), and incubated overnight at 37 °C and 5% CO₂. Four hours prior to transfection, the medium was exchanged for DMEM with 10% FCS without antibiotics. Then, the cells were co-transfected with 6.25 µg of the purified plasmid DNA (P2X receptor DNA in pQCXIN), 3.75 µg of a VSV-G protein DNA (in pcDNA3.1), and lipofectamine 2000 (Thermo Fisher Scientific, Dreieich, Germany) as a transfection reagent. VSV-G was used to increase infection efficiency.²¹⁴ After 12–15 h, the medium was replaced by DMEM (10% FCS, penicillin (100 U/mL), and streptomycin (100 µg/mL)) containing 30 µL of sodium butyrate (500 mM in H₂O). The cells were cultivated at 32 °C for 48 h. In the meanwhile, 5×10^5 non-transfected 1321N1 astrocytoma cells were seeded in 25 cm² culture flasks using DMEM (10% FCS, penicillin (100 U/mL), and streptomycin (100 µg/mL)). Subsequently, the virus particles containing the respective P2X receptor sequence were harvested and used to infect the non-transfected 1321N1 astrocytoma cells. Polybrene solution (4 mg/mL in H₂O) was added and the cells were incubated at 37 °C and 10% CO₂. After 2.5 h the medium was replaced for DMEM containing 10% FCS, penicillin (100 U/mL), and streptomycin (100 µg/mL). The antibiotic-based selection with G418 (800 µg/mL) was initiated after 48–72 h. The functionality of each cell line was analyzed in calcium influx assays with ATP as the agonist.

Calcium influx assays

Transfected 1321N1 astrocytoma cells were evaluated by fluorimetric calcium influx assays, as previously described.^{126,134} Fluorescent Ca²⁺-chelating dyes were employed as indicators to measure intracellular Ca²⁺ levels. For human P2X2- and human P2X4-expressing cells, Fluo-4 AM (Thermo Fisher Scientific, Dreieich, Germany) was used,

while for human P2X1- and human P2X3-expressing cells, Calcium 5 (Molecular Devices, Munich, Germany) was employed.

At first, 45,000 cells per well were seeded into a 96-well polystyrene microplate with a black and flat bottom, except for P2X3-expressing cells, for which 30,000 cells per well were seeded. The cells were incubated overnight at 37 °C and 10% CO₂ (5% CO₂ for the P2X3-expressing cell line). On the day of the assay, the medium was carefully removed, and the cells were loaded with dye solution containing Fluo-4 AM in HBSS (Thermo Fisher Scientific, Dreieich, Germany), and 1% of the non-ionic detergent Pluronic® F127 (Sigma-Aldrich, Taufkirchen, Germany), and incubated for 1 h at room temperature with gentle shaking. Calcium 5 dye was prepared in HBSS buffer, added to the P2X1- and P2X3-expressing cell lines, and then the cells were incubated for 1 h at 37 °C. For determining the EC₅₀ values of the compounds, dilution series of the respective agonists (in HBSS buffer) were prepared in transparent 96-well plates (Boettger, Bodenmais). HBSS buffer and ATP (at 100 µM and 10 µM) served as controls. The agonist-induced calcium influx was initiated by an automatic transfer of the respective agonist solution to the P2X-expressing cell line, and measured using an imaging plate reader (NOVOstar, BMG LABTECH, Ortenberg, Germany) at an excitation wavelength of 485 nm and an emission wavelength of 520 nm.

Data analysis

Microsoft Excel and GraphPad Prism version 8.0 (GraphPad Software, San Diego, CA, USA) were used to analyze data. Nonlinear regression (sigmoidal dose-response, variable slope) was used to generate concentration-dependent curves for agonists, and to calculate EC₅₀ values. Results were expressed as means ± SEM, obtained in at least three independent experiments, performed in duplicates. The results were normalized to the maximal ATP-induced effect (100% at 100/10 µM). Stereoselectivity between ATP and ADP analogs, respectively, was analyzed by using a multiple t-test. Significance was expected if P value was ≤ 0.05. The significance levels were defined as follows: * P < 0.05, ** P < 0.01, *** P < 0.001, **** P < 0.0001.

3.5 Publication II: Extracellular binding sites of positive and negative allosteric P2X4 receptor modulators

Stephanie Weinhausen, Jessica Nagel*, Vigneshwaran Namasivayam*, Claudia Spanier, Aliaa Abdelrahman, Theodor Hanck, Ralf Hausmann, and Christa E. Müller*

** These authors contributed equally to this work.*

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Appendix B: Original publication and supporting information

Introduction

Allosteric modulators are gaining increasing attention as potential therapeutic drugs.⁷⁰ Depending on the modulatory effect, receptor potentiation or inhibition, there are two types of allosteric modulators: positive allosteric modulators (PAMs) and negative allosteric modulators (NAMs).⁷⁰ They offer several advantages over orthosteric ligands. For instance, they often exhibit higher receptor subtype selectivity because their binding sites are less conserved than those of orthosteric ligands.⁶⁷ Moreover, the potential existence of several structurally distinct allosteric binding sites provides more opportunities for ligand development.⁶⁷

Therefore, efforts have been made to identify allosteric modulators for the P2X4 receptor, a trimeric ATP-gated ion channel, that is being considered as a novel drug target.¹⁰ X-ray crystal structures of the zebrafish P2X4 receptor revealed its trimeric composition, each monomer having a dolphin-like structure, with the flukes representing the transmembrane domains and the extracellular domain forming its upper body.²⁴ ATP was shown to interact with all the three binding sites of the trimeric receptor mainly via ionic interactions with several positively charged amino acid residues.^{24,25} The high polarity of the ATP-binding sites hampers the development of orthosteric ligands, especially those with oral and brain bioavailability. Furthermore, the ATP-binding site is conserved throughout the P2X receptor family, which impedes the development of receptor-selective ligands.^{83,263} Consequently, drug development has mainly focused on developing allosteric P2X4 receptor modulators.

P2X4 receptors are highly expressed in the central as well as the peripheral nervous system, on immune cells, and on endothelial cells.^{10,57,108} Several animal models demonstrated that the pharmacological blockade of P2X4 receptors is promising for the treatment of neuropathic pain, a debilitating pain condition.^{119,128} Further studies proposed a contribution of P2X4 receptors to neuropathologies and neurodegenerative diseases, different types of cancer, and peripheral immune and inflammatory processes.^{42,44,171,264,265}

Ethanol was identified as a NAM of P2X4 receptors.²⁶⁶ A number of animal studies indicated that P2X4 receptors regulate alcohol intake and preference.²⁶⁷ Positive allosteric modulation of P2X4 receptors has been proposed as a strategy for the treatment of alcohol abuse disorders, as it can reverse certain ethanol-induced behavioral effects.²⁶⁷

These wide-ranging pathophysiological processes in which the involvement of P2X4 receptors has been described emphasize the necessity for developing P2X4 receptor ligands.

In recent years, several PAMs and NAMs were described for the P2X4 receptor, including the macrocyclic natural product ivermectin (PAM), the phenylurea derivative BX430 (NAM), and the antidepressant drug paroxetine (NAM).^{120,133,142} Ivermectin is generally used as a pharmacological tool in P2X4 receptor research and has been postulated as a drug for the treatment of alcohol abuse disorders due to its P2X4 receptor-potentiating effects.^{267,268} The phenylurea derivative BX430 was identified as a potent and selective NAM of P2X4 receptors in patch clamp studies, showing submicromolar potency.¹³³ Paroxetine was developed as an antidepressant drug and belongs to the class of selective serotonin reuptake inhibitors with nanomolar potency.²⁶⁹ Animal studies investigating the pathogenesis of neuropathic pain showed that paroxetine acts as a non-competitive antagonist of P2X4 receptors at micromolar concentrations, displaying beneficial analgesic effects in neuropathic pain.^{120,269}

The elucidation of the binding sites of PAMs and NAMs would provide valuable information on protein-ligand interactions and may facilitate further drug development. So far, only limited information is available regarding the interactions of PAMs and NAMs with the P2X4 receptor.^{147,270–272}

3.5.1 Publication summary

The objective of this study was to identify and characterize potential allosteric binding site(s) of ivermectin, BX430, and paroxetine in the extracellular domain of the human P2X4 receptor. To this end, we designed a series of chimeric human P2X4(P2X2) receptors, in which selected sequences of the human P2X4 receptor were replaced by corresponding sequences of the human P2X2 receptor. The regions for the amino acid exchanges were selected based on a sequence alignment of the P2X2 and P2X4 receptors. The rationale behind generating these chimeric receptors was that ivermectin and BX430 modulate the P2X4 receptor but not the P2X2 receptor, while paroxetine was 8-fold less potent at the P2X2 receptor as compared to the P2X4 receptor. Subsequently, the chimeric receptors were stably expressed in 1321N1 astrocytoma cells and characterized in fluorimetric calcium influx assays. The effects of ivermectin, BX430, and paroxetine on ATP-induced calcium influx in the chimeric receptors were evaluated and compared to their effects at the human P2X4 wildtype receptor. The chimeric receptor approach was further supported by polymerase chain reaction-based site-directed mutagenesis, homology modeling, and docking studies.

Depending on the exchanged sequence variation in the chimeric P2X4(P2X2) receptors, ivermectin displayed a range of allosteric modulatory effects – positive, negative, or biphasic. A thorough analysis of the experimental outcomes led to the assumption that ivermectin modulates the P2X4 receptor by interacting with two distinct allosteric binding sites. This assumption was previously made during patch clamp experiments, in which ivermectin exhibited a biphasic effect at the P2X4 receptor.^{140,142,273} We identified specific regions in the dorsal fin region of the P2X4 receptor as the main, high-affinity binding site of ivermectin, with high relevance for its positive modulatory effects. Docking studies indicated that the binding of ivermectin within the cavity in the dorsal fin of the P2X4 receptor favors an upward movement of the dorsal fin region towards the ATP-binding site, leading to a potentiation of P2X4 receptor activation by ATP. The second, low-affinity binding site of ivermectin was proposed to be located within the transmembrane domains, resulting in receptor inhibition.

The effects of BX430 on the functional chimeric P2X4(P2X2) receptors led to the assumption that its binding site is also located in the receptor's dorsal fin region. Subsequent docking studies of BX430 into the human P2X4 receptor homology model

confirmed that the cavity in the dorsal fin area easily accommodated BX430. Based on these results, polymerase chain reaction-based site-directed mutagenesis was performed to generate specific P2X4 receptor mutants. The antagonistic effect of BX430 on ATP-induced calcium influx at the receptor mutants was investigated and compared to that observed at the human P2X4 wildtype receptor. The amino acid residue Ile209 was identified to likely play a role in BX430 binding by hydrophobic interactions with the phenyl ring.

In contrast, no significant effects at the chimeric P2X4(P2X2) receptors were observed for paroxetine as compared to those determined at the human P2X4 wildtype receptor, suggesting that its binding site is not located in the receptor's dorsal fin region. Since paroxetine is a basic small molecule that is positively charged at a physiologic pH value of 7.4, it might bind to the center of the cation channel, leading to receptor inhibition.

In conclusion, our comprehensive studies identified potential extracellular binding site(s) for ivermectin and BX430, indicating a common or overlapping extracellular binding site in the dorsal fin region of the P2X4 receptor. Interaction with this allosteric binding site may result in positive or negative receptor modulation. These results contribute to a more profound understanding of allosteric P2X4 receptor modulation and may be beneficial for the development of potent and selective PAMs and NAMs as potential future drugs.

3.5.2 Current research findings

Recently, the cryo-EM structure of the zebrafish P2X4 receptor in complex with BX430 was published, which identified the allosteric binding site at the intersubunit interface in the extracellular receptor domain.²⁷ Our studies had identified Ile209 of the human P2X4 receptor as an important amino acid residue for binding of BX430, however, this observation could not be confirmed by the cryo-EM structure of the zebrafish P2X4 receptor.²⁷ The authors proposed that extensive hydrophobic interactions between Ile212 in the zebrafish P2X4 receptor (corresponding to Ile209 in the human P2X4 receptor) and neighboring residues might have altered protein folding, which in turn affected BX430 potency in our mutagenesis studies.²⁷

Overall, despite the efforts of several research groups to identify the binding site of BX430, inconsistent results have been observed, possibly due to variations in experimental setups, species differences, or due to the existence of multiple binding sites for BX430 at the P2X4

receptor. Therefore, further investigations are required to elucidate the binding site of BX430.

3.5.3 Author contributions

The author of this thesis performed mutagenesis studies of the human P2X4 receptor to evaluate the negative allosteric modulation by BX430. In detail, the author designed mutagenesis primers to generate the following P2X4 receptor mutants: Y300F, I209A, T212S, D224A, and D224A_N208A. Furthermore, the author performed molecular biology experiments, generated cell lines stably expressing the receptor mutants, performed calcium influx assays at the P2X4 receptor and P2X4 receptor mutants, analyzed the data, and prepared the respective figures. The receptor mutants were selected based on previous studies performed by Dr. Stephanie Weinhausen and Dr. Vigneshwaran Namasivayam. In addition, the author evaluated the effects of paroxetine at the human P2X3 receptor in calcium influx assays.

The first preliminary draft of the manuscript, including the figures and the supporting information, was prepared by Dr. Stephanie Weinhausen, Dr. Vigneshwaran Namasivayam, and Prof. Dr. Christa E. Müller. The author of this thesis integrated the new results on BX430 (calcium influx assays at the human P2X4 receptor and corresponding receptor mutants) and paroxetine (calcium influx assays at the human P2X3 receptor) into the manuscript. Additionally, the author completely restructured, revised, and rewrote the initial manuscript, including the figures and supporting information, and finalized it for publication. All of these contributions were made in close collaboration with the corresponding author, Prof. Dr. Christa E. Müller.

3.6 Structural studies of the human P2X4 receptor

The elucidation of the three-dimensional structure of a membrane protein at near- and atomic resolutions is of great significance, as it reveals the molecular architecture, provides insights into activation and inhibition processes, and identifies the binding site of agonists and antagonists. This detailed understanding of protein-ligand interactions can facilitate the design and synthesis of novel ligands with improved physicochemical properties and potential therapeutic efficacy.

In structural biology, various techniques are employed for the solubilization and purification of recombinant proteins and subsequent analysis.

Immobilized metal ion affinity chromatography (IMAC)

Efficient protein purification is a crucial and fundamental step to enable successful structural studies. For this purpose, polyhistidine affinity tags, known as histidine (His) tags, are widely employed.²⁷⁴ His tags are amino acid motifs in proteins that can be attached to the N- or C-terminus of the desired protein and typically consist of at least six histidine residues.²⁷⁴ These residues allow for purifying recombinant proteins by immobilized metal ion affinity chromatography (IMAC).²⁷⁴ IMAC was first introduced in 1975 by Porath et al. and is based on the affinity of transition metal ions like Co^{2+} , Ni^{2+} , Cu^{2+} , and Zn^{2+} towards cysteine, histidine, and tryptophan in aqueous solution.²⁷⁵ This idea was extended to the use of metal ions immobilized on a matrix support, thereby allowing to fractionate and purify proteins in solutions.²⁷⁵ Proteins are separated based on their affinity for divalent metal ions immobilized on a support matrix. The metal ions employed in this technique are immobilized through coordinative bonds to a chelating agent, which is covalently attached to the matrix support. Common chelating agents include iminodiacetic acid and nitrilotriacetic acid.²⁷⁶ Several matrix supports with different advantages and limitations have been developed, including agarose, cellulose, and silica.²⁷⁶ Histidine exhibits the strongest interaction with immobilized metal ion matrices, as the electron donor groups on its imidazole ring readily form coordinative bonds with the immobilized transition metal.²⁷⁴

After solubilization, the protein is incubated with the affinity matrix resin to enable the binding of the His-tagged protein. Low concentrations of imidazole can be added to reduce the number of proteins that bind nonspecifically to the resin.²⁷⁴ Extensive washing steps follow to remove nonspecifically binding proteins that bind weakly to the resin. High

concentrations of imidazole can be utilized for the final elution of the His-tagged protein as it competes with the His-tag for binding to the metal cation-loaded resin.²⁷⁴

Solubilization and stabilization of membrane proteins

One of the major hurdles in studying membrane proteins is to preserve their stability and functionality in aqueous solution. Many efforts have been made to develop various methodologies to overcome these hurdles.

Detergents are most commonly used for membrane protein extraction possessing a dual hydrophobic-hydrophilic structure that resembles that of the cell membrane.²⁷⁷ The critical micelle concentration describes a detergent's specific parameter at which detergent molecules self-assemble into a micelle structure with the hydrophobic tails buried in the center, and the hydrophilic heads facing outwards.²⁷⁷ At this micellar stage, detergents can integrate into the lipid bilayer, thereby destabilizing the interactions between lipid molecules.²⁷⁷ This results in a breakdown of the membrane structure, which allows the extraction of the membrane proteins from the lipid bilayer.²⁷⁷ The detergent molecules adsorb to the hydrophobic surface of the proteins' transmembrane regions, thereby maintaining their water solubility.²⁷⁷

Following solubilization, membrane proteins are typically less stable than in native membranes, as detergents compete with specific molecular interactions between proteins and lipids, which typically determine protein stability.²⁷⁸ The use of detergents to solubilize membrane proteins has been proven to be an effective approach, however, it comes with potential obstacles and limitations, mainly due to their limited ability to maintain protein stability and function.²⁷⁷ Additionally, the presence of protein-free micelles could interfere with downstream applications.²⁷⁷

In order to provide a more stable and native-like environment for membrane proteins in aqueous solutions, short and flexible amphipathic polymers, known as amphipols, were developed.^{279,280} These polymers bind to the hydrophobic surface of membrane proteins in a noncovalent way by multiple hydrophobic contact points, resulting in an extremely slow dissociation rate of the polymer, even at extreme dilution.²⁷⁸ This is a significant advantage over detergent-protein complexes in which the protein-bound detergent molecules are in rapid equilibrium with free monomers and micelles, and dissociate upon dilution below the critical micelle concentration.²⁷⁸ In many cases, the amphipol-trapped membrane proteins

appear to be more stable than their detergent-solubilized counterparts.²⁸¹ However, the effects of amphipols on membrane protein stability and function differ from protein to protein and need to be investigated individually.²⁸¹

In cryo-EM studies, a major challenge is the preparation of cryo-EM grids with a controlled ice layer thickness, without denaturing the protein sample, and without favoring a specific orientation.²⁸² The effects of different surfactants on the preparation of cryo-EM sample grids have been investigated, demonstrating the significant advantages of amphipols over detergents.²⁸² In this particular study, amphipols were found to promote a more isotropic distribution of protein orientations, enhancing imaging quality and 3D reconstruction.²⁸²

Amphipol A8-35 (Figure 44) is one of the most widely used and most thoroughly studied amphipols.²⁷⁸ Its chemical structure features a polyacrylate chain, wherein some carboxylates remain unmodified, while others are derivatized with octylamine or isopropylamine moieties (Figure 44).^{278,279} Based on this prototype, plenty of modifications have been made to improve the functionality of the polymers.^{278,280} As A8-35 represents an extremely weak detergent, initial solubilization of membrane proteins is achieved by using conventional detergents, such as dodecyl maltoside (DDM).²⁸⁰ In addition to DDM, cholesteryl hemisuccinate (CHS) is commonly used as a membrane stabilizer for eukaryotic membrane purification.^{283,284} Subsequently, the solubilized proteins can be reconstituted into A8-35.²⁸⁰

Several cryo-EM structures of ion channels have validated the benefits of A8-35-trapped membrane proteins, including structural studies of transient receptor potential ion channels.²⁸⁵

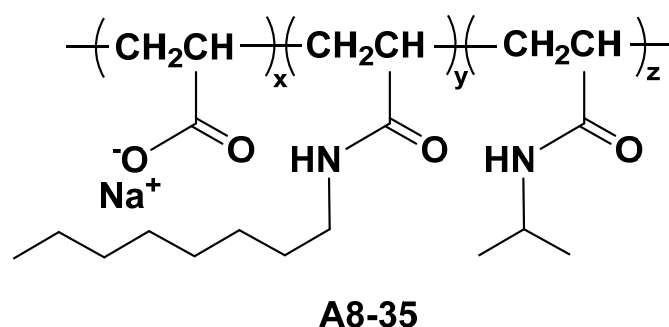


Figure 44. Chemical structure of amphipol A8-35. x, y, and z: molar percentages of each type of unit, randomly distributed along the chain. Molar ratio of monomers in mol % (mean number of monomers per molecule): ungrafted ~35%, octyl ~25%, and isopropyl ~40%. Structure and molar ratios of monomers were taken from Tribet et al.²⁷⁹

Size-exclusion chromatography (SEC)

Size-exclusion chromatography (SEC), also known as gel filtration chromatography, is a liquid chromatography technique that allows to separate molecules based on their molecular size in solution.²⁸⁶ In the field of protein analysis, SEC represents the gold standard for the qualitative and quantitative evaluation of proteins, and in particular for the evaluation of protein aggregates, dimers, trimers, and oligomers.²⁸⁶ Depending on the respective system, SEC can be used for analytical purposes, but also for protein purification. An aqueous buffer is used as the mobile phase, diffusing through the stationary phase, which consists of spherical porous particles with a carefully controlled pore size. Different SEC packaging materials are used for the stationary phase, including silica and cross-linked polymeric packaging with hydrophobic, hydrophilic, or ionic properties.²⁸⁶ The molecules are separated based on molecular size differences as they pass through the column and are eluted in the order of decreasing molecular weight. Ultraviolet (UV) detection is the predominant detection mode in SEC of proteins, as UV wavelengths of 270 nm, 275 nm, and 280 nm elicit a specific response for aromatic amino acid residues (tryptophan, tyrosine, phenylalanine).²⁸⁶

Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE)

The molecular weight of proteins can be determined by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE), in which proteins are separated according to their electrophoretic mobility.²⁸⁷ SDS is generally used as an anionic detergent to denature the proteins and to impart a negative charge that covers their intrinsic charge.²⁸⁷ This negative charge is proportional to the length of the polypeptide chain.²⁸⁷ After loaded onto a gel matrix and placed in an electric field, negatively charged protein molecules migrate towards the positively charged electrode, undergoing separation due to the molecular sieving effect, with smaller molecules moving faster.²⁸⁷ By comparing the migration distances of the proteins with those of standard proteins of known molecular weight, the molecular weight of the proteins can be estimated.²⁸⁷ Coomassie brilliant blue dye is commonly used to visualize the separated proteins, whereby the protein bands on the gel are stained in a dark blue color.²⁸⁸

Western blot

The Western blot technique, also known as immunoblotting, is an analytical method used to detect and semi-quantify proteins.²⁸⁹ After separating the protein mixture by SDS-PAGE based on their molecular weight, the protein mixture is transferred to a protein-binding membrane by using an electric field oriented perpendicular to the surface of the gel, followed by blocking non-specific proteins on the membrane with a blocking reagent (nonfat milk or BSA).²⁸⁹ Typically, the transferred protein sample is then probed with a combination of antibodies. The primary antibody binds specifically to the protein of interest, while the secondary antibody targets the host species of the primary antibody.²⁸⁹ Horseradish peroxidase (HRP)-conjugated secondary antibodies are commonly used for this purpose, generating a detectable chemiluminescent signal upon interaction with a substrate.²⁸⁹

The P2X4 receptor has emerged as an important drug target due to its contribution to various pathophysiological processes, including neuroinflammation and associated diseases, various types of pain of chronic pain, particularly neuropathic pain, and different types of cancer.^{10,42,44,47,150}

The structure of the trimeric human P2X4 receptor has not yet been elucidated. Remarkably, several researchers have succeeded in determining the structure of the zebrafish P2X4 receptor by X-ray crystallography and cryo-EM.^{24,25,27} The zebrafish P2X4 receptor shares a sequence identity of 58% with the human P2X4 receptor and exhibits a markedly different pharmacology. For instance, the potency of ATP at the zebrafish P2X4 receptor is about 200-fold lower as compared to its potency at the human P2X4 receptor.²⁴ Nonetheless, these structures have yielded valuable insights into the trimeric P2X4 receptor structure, ATP binding, and antagonist binding. All structures were obtained with truncated receptor constructs at the N- and C-termini, and with mutated glycosylation sites.^{24,25,27}

This project aimed to resolve the three-dimensional structure of the full-length human P2X4 receptor in complex with a P2X4 receptor antagonist. Initially, we sought to develop and optimize methods for protein expression on a large scale, followed by solubilization, purification, and finally, structural studies employing cryo-EM.

3.6.1 Results and discussion

Design of P2X4 receptor constructs

The initial step involved designing and cloning of various human P2X4 receptor constructs into the pFastBac1 vector (Thermo Fisher Scientific, Dreieich Germany) (Table 10) to utilize the Bac-to-Bac Baculovirus Expression System (Thermo Fisher Scientific, Dreieich, Germany). All constructs contained two mutated glycosylation sites (N75R and N184R), which had been mutated based on the receptor constructs used for the X-ray crystal structures of the zebrafish P2X4 receptor.^{24,25} An additional mutation was inserted, E307T, which was found to increase the antagonistic potency of anthraquinone derivatives.²⁹⁰ For purification, a polyhistidine tag (10x-His tag) was attached to the C-terminus, which included a preceding human rhinovirus (HRV) 3C protease cleavage site (please refer to Appendix C for protein sequences). It is known that tags can introduce steric hindrance, which can result in a less efficient enzymatic cleavage.²⁹¹ Insertion of flexible linkers before

the cleavage site has been shown to facilitate enzymatic cleavage.²⁹¹ Therefore, we incorporated a flexible Gly-Ser-Ser-Gly (GSSG) linker prior to the protease cleavage site.

The P2X4 receptor construct JN5 (see Table 10) was additionally modified at the N-terminus with an HA tag (hemagglutinin; YPYDVPDYA) and a FLAG[®] tag (DYKDDDDK) to assess the effect of these tags on protein expression. All P2X4 receptor constructs were successfully designed and confirmed by DNA sequencing.

Table 10. Human P2X4 receptor constructs for structural investigations.

P2X4 receptor construct	N-terminus	C-terminus	Additional modifications
JN3	BamHI	EcoRI HRV 3C protease cleavage site 10x-His tag	N75R, N184R, E307T GSSG linker promoter: polyhedrin
JN5	BamHI HA tag FLAG [®] tag AscI	EcoRI HRV 3C protease cleavage site 10x-His tag	N75R, N184R, E307T GSSG linker promoter: polyhedrin

Small-scale protein expression in *Spodoptera frugiperda* (*Sf9*) insect cells and purification of P2X4 receptor constructs JN3 and JN5

In order to examine the suitability of *Sf9* insect cells as an expression system for the human P2X4 receptor and to assess the level of receptor expression, two P2X4 receptor constructs with distinct modifications, JN3 and JN5, were transfected into *Sf9* insect cells and expressed in a small-scale approach (80–120 mio. *Sf9* insect cells) as described in the method section. Subsequently, the proteins were solubilized and purified in the presence of the endogenous agonist ATP at a final concentration of 5 mM. SEC and SDS-PAGE analysis were conducted to determine the protein's expression rate and verify the homogeneity of the purified protein sample.

For SEC analysis, a Sepax Nanofilm SEC-250 column (Sepax Technologies, Newark, DE, USA) with a separation range of 1.5×10^3 to 7.0×10^5 Da was used. The manufacturer's specifications and our in-house investigations indicated that large protein complexes elute between 3 and 5 min (void volume between 3.1 and 3.5 min). The analysis of the elution buffer at an absorbance of 280 nm showed no significant peaks between 3 and 5 min (Figure 45A). Therefore, subsequent SEC chromatograms are analyzed and presented exclusively within this range.

The SEC analysis of both P2X4 receptor constructs revealed two peaks at an absorbance of 280 nm (Figure 45A). For both purified proteins, the first peak eluted after ca. 3.5 min, while the second, broader peak eluted after ca. 3.9 min. Both peaks were not baseline-separated. The highest signal intensity was observed for the second peak of the protein sample JN3, with an intensity of ca. 4 milli-absorbance units (mAu). According to a BSA calibration performed with this SEC system, it was estimated that the amount of purified proteins JN3 and JN5 was less than 1 μ g.

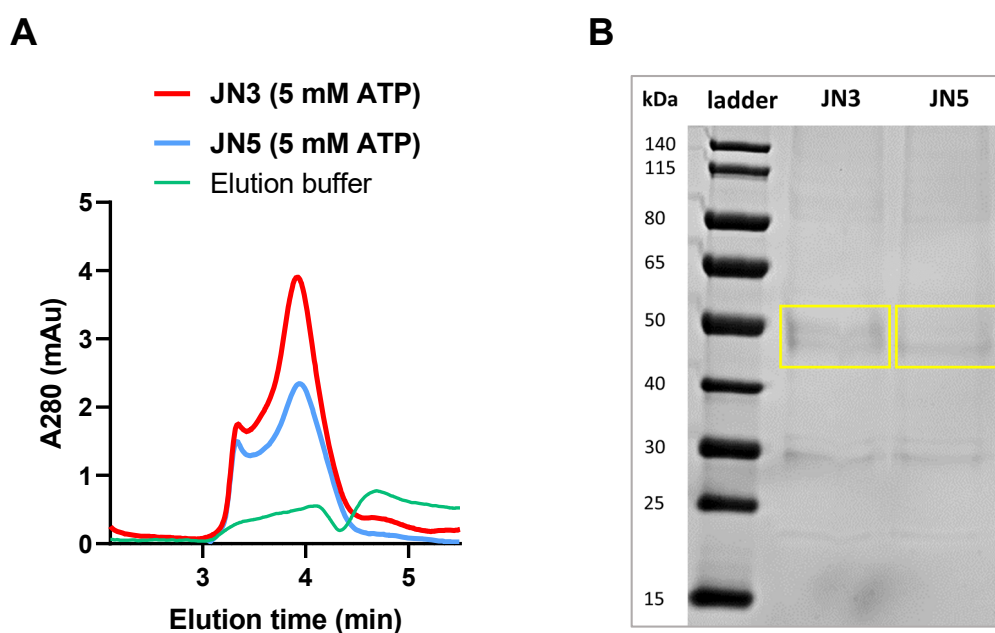


Figure 45. Analysis of purified P2X4 receptor constructs JN3 and JN5 in the presence of 5 mM ATP. Both receptor constructs were expressed in 80–120 mio. *Sf9* insect cells. A. SEC analysis of JN3 (red), JN5 (blue), and elution buffer (green) at 280 nm (mAu, milli-absorbance units). Column: Nanofilm SEC-250 (Sepax Technologies, Newark, DE, USA). B. SDS-PAGE analysis using a 10% bis-Tris gel and subsequent Coomassie blue staining of purified proteins JN3 and JN5. Ladder: PageRuler™ Prestained Protein Ladder (Thermo Fisher Scientific, Dreieich, Germany).

The first peak likely represented protein aggregates eluting at the column's void volume. The second peak may correspond to the functional trimeric P2X4 receptor structure. It is also possible that the trimeric protein eluted with the void peak, while the second peak represented dimeric or monomeric protein structures. Moreover, it is conceivable that the employed SEC column and separation system were not optimal for these protein samples. Overall, further investigations were required to evaluate the protein's stability and homogeneity.

The SDS-PAGE analysis of both purified proteins, JN3 and JN5, showed a faint protein band migrating at ~50 kDa (Figure 45B). These protein bands might correspond to the monomeric receptor structures, considering their molecular weights are 46 kDa (JN3) and 49 kDa (JN5). Additionally, faint protein bands were observed at a molecular size of ~30 kDa and ~80 kDa, respectively, which may indicate impurities in the protein sample. The protein band at ~80 kDa might also indicate the presence of larger protein complexes in the sample.

The initial results indicated the presence of protein in the samples, albeit at moderate protein concentrations.

Medium-scale expression and purification of the P2X4 receptor construct JN3

As a subsequent step, the P2X4 receptor construct JN3 was expressed in a medium-scale approach (1,000–1,500 mio. *Sf9* insect cells) to ascertain whether protein expression could be scaled up and whether these methods could be applied to larger approaches. During protein purification, ATP was added at a final concentration of 8 mM.

The SEC chromatogram (Figure 46A, black curve) shows the elution profile of the unconcentrated protein solution, which was measured directly after purification in a final sample volume of 750 μ L. The first peak eluted as a small shoulder after ca. 3.3 min, and the second peak after ca. 3.9 min, appearing as a broader peak. After freezing, thawing, and concentrating the protein sample, another SEC analysis was conducted. The elution profile of the concentrated protein sample (Figure 46A, red curve) was comparable to that of the unconcentrated sample but with an increase in signal intensity for both peaks. These results suggested a successful medium-scale expression of the P2X4 receptor construct JN3, resulting in a higher protein amount.

The SDS-PAGE analysis revealed the presence of a protein with an estimated molecular size of ~50 kDa, which is consistent with the size of the receptor's monomeric structure (46 kDa) (Figure 46B).

Overall, the medium-scale protein expression and purification of the P2X4 receptor appeared to be successful, as confirmed by SEC and SDS-PAGE analysis.

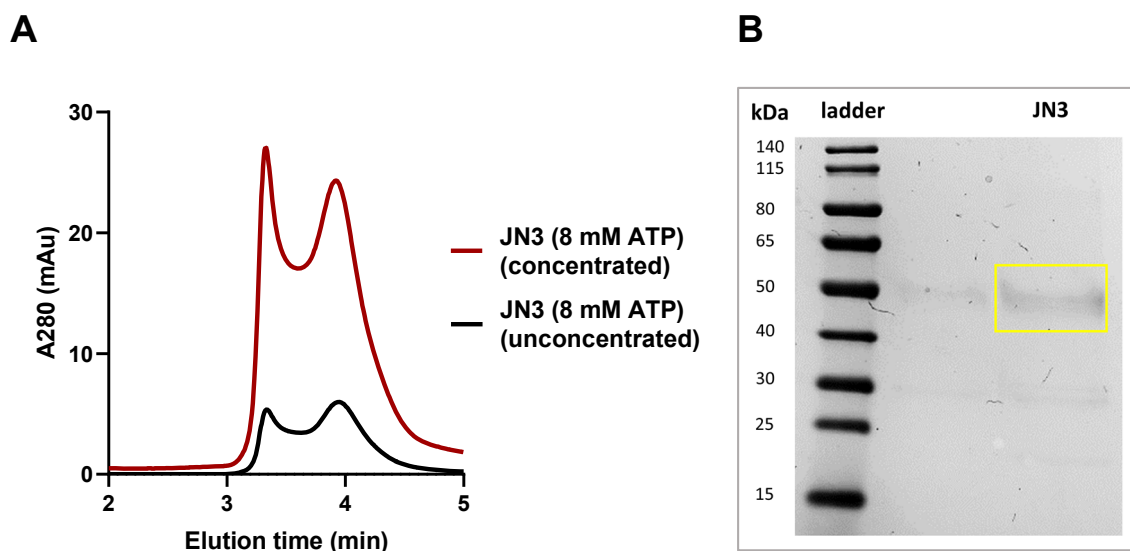


Figure 46. Analysis of the purified P2X4 receptor construct JN3, expressed in 1,000–1,500 mio. *Sf9* insect cells. Protein was purified in the presence of ATP (8 mM). A. SEC analysis of unconcentrated (black curve) and concentrated protein solution (red) at 280 nm (mAu, milli-absorbance units). Column: Nanofilm SEC-250 (Sepax Technologies, Newark, DE, USA). B. SDS-PAGE analysis using a 10% bis-Tris gel and subsequent Coomassie blue staining of unconcentrated protein solution (JN3). Ladder: PageRuler™ Prestained Protein Ladder (Thermo Fisher Scientific, Dreieich, Germany).

Western blot analysis of the P2X4 receptor construct JN3

The first results indicated a successful expression of the P2X4 receptor construct JN3 in *Sf9* insect cells. As a next step, the Western blot technique was employed to precisely analyze the protein and to confirm its identity. The protein sample was first separated by SDS-PAGE and then electrophoretically transferred to a nitrocellulose membrane. As the P2X4 receptor construct JN3 is His-tagged, an anti-His mouse antibody was used as the primary antibody. An HRP-conjugated secondary antibody was employed for chemiluminescence detection.

Chemiluminescence detection of the Western blot visualized an intensively black-colored protein band with a molecular size between 40 and 50 kDa for the P2X4 receptor construct JN3 in a small- and medium-scale expression (Figure 47). As the size of the protein's monomeric structure is 46 kDa, this band was presumed to represent the successful binding of the anti-His antibody. Less visible protein bands above 50 kDa may be due to non-specific binding of the antibody despite extensive washing steps, or could indicate the presence of larger protein complexes. In conclusion, the Western blot analysis provided further evidence for the successful expression of the P2X4 receptor construct JN3 in *Sf9* insect cells.

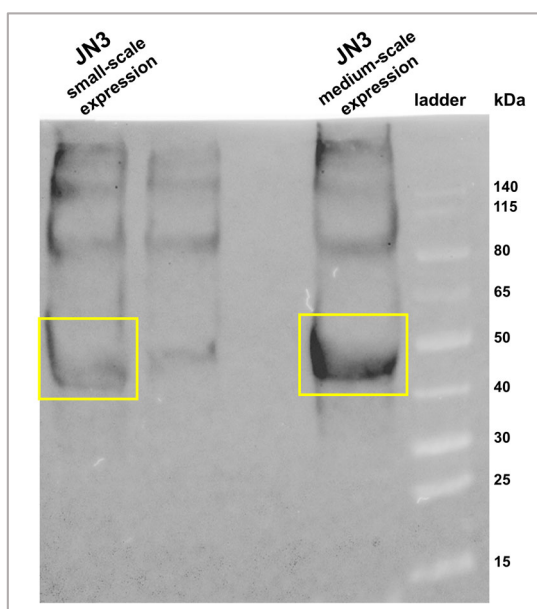


Figure 47. Western blot of purified P2X4 receptor construct JN3 after small- and medium-scale expression following an SDS-PAGE analysis using a 10% bis-Tris gel. Primary antibody: anti-His mouse antibody; secondary antibody: horseradish peroxidase (HRP) enzyme-conjugated anti-mouse antibody. Ladder: PageRuler™ Prestained Protein Ladder (Thermo Fisher Scientific, Dreieich, Germany).

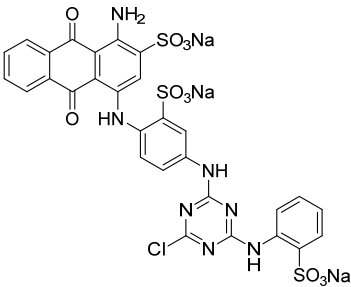
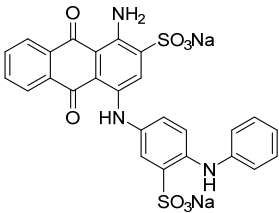
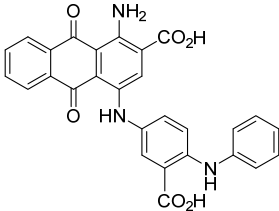
The impact of P2X4 receptor antagonists on the purification of the P2X4 receptor construct JN3

The main objective was to determine the structure of the human P2X4 receptor in complex with an allosteric P2X4 receptor antagonist to identify its binding site. To this end, the effects of two P2X4 receptor antagonists on protein stability and homogeneity were investigated.

Previous studies identified Cibacron Blue and related anthraquinone derivatives as potent P2X4 receptor antagonists.²⁹⁰ Mutagenesis studies revealed that the P2X4 receptor mutant E307T had a significant effect on the antagonists' potency (Table 11).²⁹⁰ For protein purification, two anthraquinone derivatives, PSB-0739 and PSB-0704, were selected due to their high potency at the human P2X4-E307T receptor mutant and their high water solubility, which is advantageous for structural studies of proteins. The antagonistic potency of both compounds was assessed in calcium influx assays, indicating a high potency for the human P2X4-E307T receptor mutant, which was significantly higher than for the wildtype receptor (Table 11).²⁹⁰

3. Results and discussion

Table 11. Potency of Cibacron Blue, PSB-0739, and PSB-0704 at human P2X4 wildtype receptor and P2X4-E307T receptor mutant, determined in calcium influx assays. Data represent mean values $\pm$ SEM from at least three independent experiments, performed in duplicates.

	$IC_{50} \pm SEM (\mu M)$	
	wildtype P2X4	P2X4-E307T
<i>Anthraquinone derivatives</i>		
Cibacron Blue	12.1 ± 4.2^a	0.0178 ± 0.0047^a
		
PSB-0739	8.91 ± 0.67	0.0353 ± 0.0047^a
		
PSB-0704	12.5 ± 1.9	0.0724 ± 0.0102^a
		

^a These values were determined by Dr. Stephanie Weinhausen.²⁹⁰

The P2X4 receptor construct JN3 was expressed at medium-scale, followed by protein solubilization and purification in the presence of PSB-0739 or PSB-0704 at a final concentration of 25 μM . The purified protein solutions were concentrated and subsequently analyzed by SEC.

Both purified proteins exhibited a similar elution profile, monitored at 280 nm, resulting in two peaks (Figure 48). The first peak eluted after ca. 3.3 min and appeared sharper, while

the second peak was broader and eluted after ca. 3.9 min. Both peaks were not baseline-separated. Notably, for the protein purified in the presence of PSB-0739, the first peak displayed a slightly higher signal intensity than the second peak. In contrast, for the protein sample in complex with PSB-0704, the second peak exhibited a higher signal intensity, while the first peak appeared as a smaller shoulder. Further investigations were required to determine whether the first peak observed in the chromatogram represented the trimeric P2X4 structure or protein aggregates.

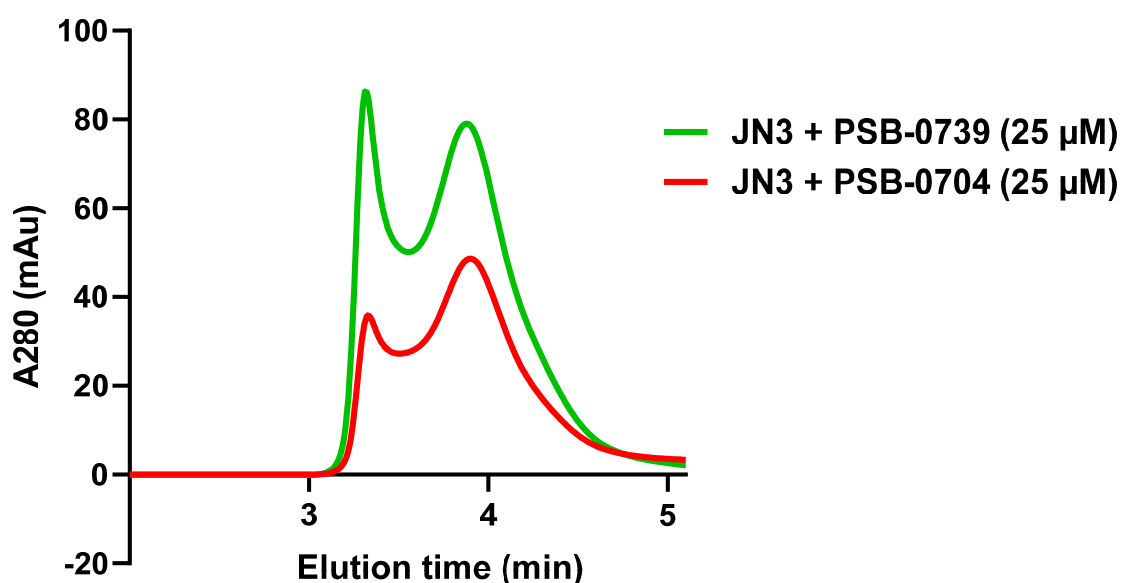


Figure 48. SEC analysis of P2X4 receptor construct JN3 in complex with PSB-0739 (green) or PSB-0704 (red) at 280 nm (mAu, milli-absorbance units). Column: Nanofilm SEC-250 (Sepax Technologies, Newark, DE, USA).

Reconstitution of the human P2X4 receptor protein into amphipol A8-35

In order to investigate the effect of amphipol (APol) A8-35 on the P2X4 receptor protein and to potentially enhance the feasibility for cryo-EM studies, the P2X4 receptor construct JN3 was reconstituted into APol A8-35. The P2X4 receptor construct JN3 was expressed at a medium-scale, solubilized with DDM/CHS, and purified in the presence of PSB-0704. Then, we sought to establish a method for the reconstitution of the receptor into A8-35, based on published protocols.²⁹² A detailed description of the method is provided in the material and method section.

As a first step, protein samples with varying P2X4 receptor protein (P2X4)/APol ratios were prepared to identify an appropriate mass ratio of the APol (Table 12). The concentration of the purified membrane protein remained constant, while the APol concentration was progressively increased.²⁹²

Table 12. Exemplary representation of the sample preparations to determine an optimal P2X4/APol mass ratio.

Mass of P2X4 (μg)	P2X4/APol mass ratio	Mass of APol (μg)
6	1:0	0
6	1:2	12
6	1:3	18
6	1:5	30

The subsequent incubation period allowed the formation of P2X4/APol/detergent complexes. In the literature, different incubation times were described, ranging from 15 min to 4 h.²⁹² In this study, the protein samples were incubated at 4 °C for 3 h upon gentle rotation.

Following incubation, an appropriate amount of Bio Beads SM2 was added to each sample, followed by an overnight incubation at 4 °C while gently rotating. Bio-Beads SM-2 are known for their effectiveness in adsorbing detergents.²⁹² Several studies have shown that APols do not adsorb onto Bio-Beads and that the optimal amount of beads should be 20 times the mass of the detergent present in the sample.²⁹²

On the next day, all samples were analyzed by SEC. All samples with different P2X4/APol ratios eluted between 3 and 5 min, monitored at an absorbance of 280 nm, displaying overlapping signal profiles, however with distinct signal intensities (Figure 49). The sample containing empty amphipols (grey curve) eluted after ca. 6 min. The black curve represents the elution profile of the protein sample in which the detergent was removed without replacement by APols, exhibiting a markedly reduced intensity of the second peak in comparison to the other samples (Figure 49).

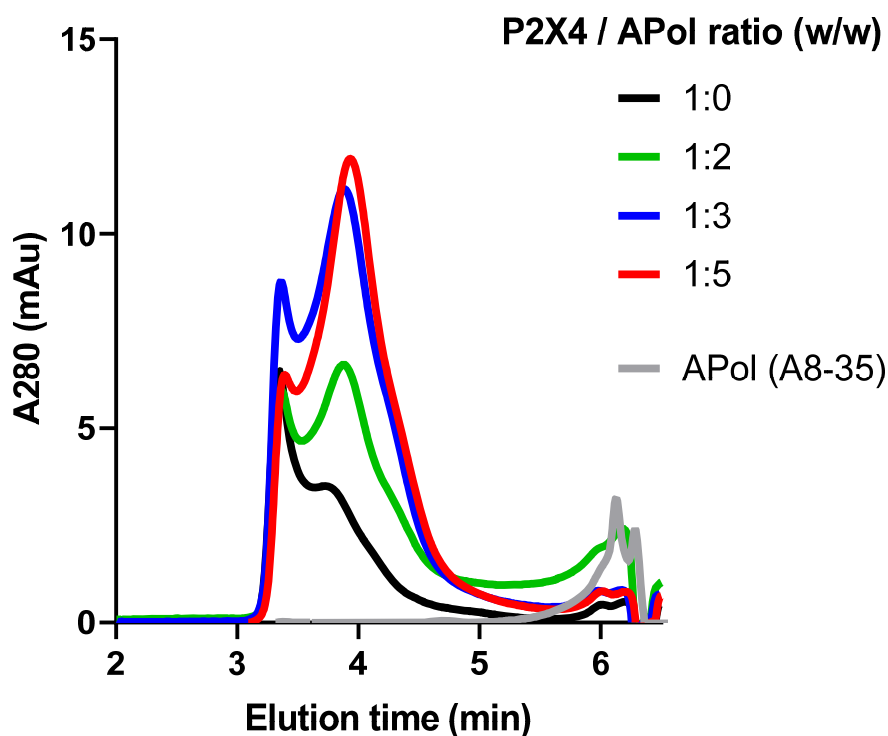


Figure 49. SEC analysis of P2X4 receptor construct JN3 in complex with PSB-0704 (25 μ M) reconstituted in different amounts of APol (A8-35) at 280 nm (mAu, milli-absorbance units). Column: Nanofilm SEC-250 (Sepax Technologies, Newark, DE, USA).

A notable observation was that a higher amount of APol resulted in a significant signal increase of the second peak, as compared to the signal intensity of the first peak. Specifically, the P2X4/APol ratios of 1:3 and 1:5 (Figure 49; blue and red curves) displayed a significantly increased intensity of the second peak compared to the sample with a P2X4/APol ratio of 1:2 (Figure 49; green curve).

These results led to the assumption that the reconstitution of the purified protein JN3 into APols was successful, and it appeared that using a larger quantity of APols is beneficial.

Cryo-EM studies

Our preliminary investigations indicated a successful expression of the P2X4 receptor in *Sf9* insect cells. However, the employed methods did not reveal whether the expression had resulted in the functional trimeric P2X4 receptor complex. To address this, we prepared a large-scale expression of the P2X4 receptor construct JN3 in 6,000–9,000 mio. *Sf9* insect cells. The protein was then solubilized in DDM/CHS, purified, and reconstituted into APol

A8-35. Solubilization and purification were performed in the presence of PSB-0704 (25 μ M). For protein analysis and the final purification step, we employed a different SEC system that enabled the separation of different fractions of the protein sample. Our previous findings indicated that P2X4 receptor expression had been relatively moderate. To prevent a significant dilution of the final protein sample, we selected the Superose 6 Increase 3.2/300 column (Cytiva, Freiburg im Breisgau, Germany), which is suitable for small sample volumes.

According to the manufacturer's instructions and previous experiments, the range between 20 and 40 min is relevant for the elution of large protein complexes (void volume at 18–19 min). Therefore, the following analysis of the SEC chromatogram was focused on this specific range. The SEC analysis of the purified and concentrated P2X4 receptor protein (JN3) revealed two peaks at an absorbance of 280 nm (Figure 50A). The first, broader peak eluted after ca. 31.2 min and exhibited a higher signal intensity than the second peak (Figure 50A).

Different peak fractions were collected, pooled, and evaluated by SDS-PAGE analysis. The pooled fractions corresponding to the first peak in the SEC chromatogram (sample 1 and sample 3) showed a distinct band on the SDS-PAGE gel, with a molecular size between 40 and 50 kDa (Figure 50B). In contrast, for the pooled fractions of the second peak (samples 2 and 4), no significant protein bands were visible on the SDS-PAGE gel (Figure 50B). These observations led us to conclude that the first peak at ca. 31.2 min corresponded to the purified P2X4 receptor protein.

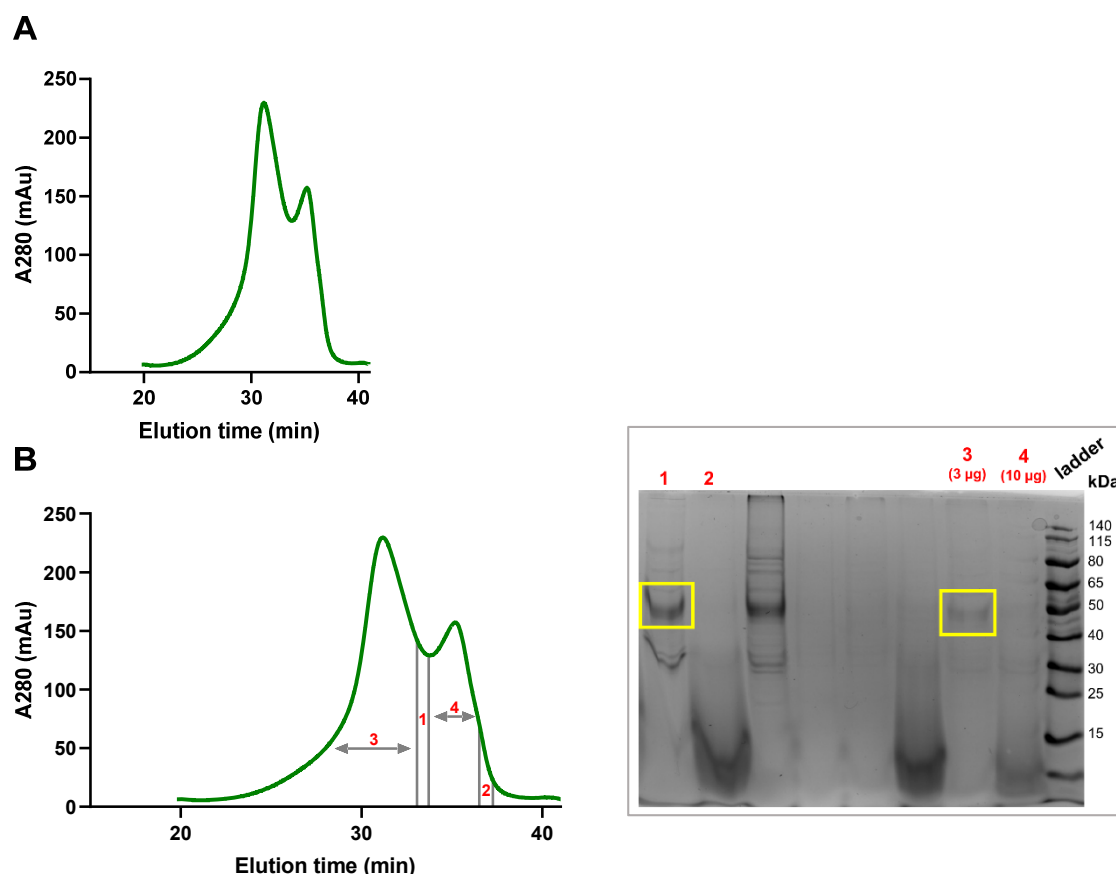


Figure 50. A. SEC analysis of purified A8-35-trapped P2X4 receptor JN3 in complex with PSB-0704 at 280 nm (mAu, milli-absorbance units). Column: Superose 6 Increase 3.2/300 (Cytiva, Freiburg im Breisgau, Germany). B. SDS-PAGE analysis using a 10% bis-Tris gel and subsequent Coomassie blue staining of different SEC peak fractions. Ladder: PageRuler™ Prestained Protein Ladder (Thermo Fisher Scientific, Dreieich, Germany).

The next step involved the preparation of cryo-EM grids to identify optimal conditions for cryo-EM sample preparation and to assess the quality of the sample for cryo-EM data acquisition. Sample preparation and data collection were carried out at StruBiTEM (Strukturbiologische Kryo-Transmissions-Elektronenmikroskopie, Cologne).

The SEC peak fraction containing the purified P2X4 receptor protein (JN3) in complex with the antagonist PSB-0704 (Figure 50B, sample 3) was concentrated to 1.4 mg/mL and applied to various grids (copper and gold), respectively. The grids were then evaluated using a 300 kV-Krios G3i Cryo-Transmission Electron Microscope equipped with a Falcon III electron detector (Thermo Fisher Scientific, Dreieich, Germany). Initial grid screening

provides information about protein particle distribution across different ice thicknesses, protein concentration, and potential ice contamination on the grid.

For the purified P2X4 receptor protein (JN3), the Quantifoil R1.2/1.3 Cu300 holey carbon grid was optimal, offering sufficient protein concentration, minimal ice contamination, and consistent particle distribution (Figure 51A). Consequently, a large dataset comprising 1,556 movies was collected. The dataset was analyzed and processed using CryoSPARC (Structura Biotechnology, Toronto, Canada) by PD Dr. Gregor Hagelüken (Institute of Structural Biology, University of Bonn). For data analysis, single-particle analysis was employed. In essence, this analysis involves the acquisition of raw cryo-EM images (micrographs) and the selection of particles, which are then subjected to 2D alignment procedures.²⁹³ Subsequently, the 3D structure of a molecule is reconstructed from a large number of its 2D projection images.²⁹³

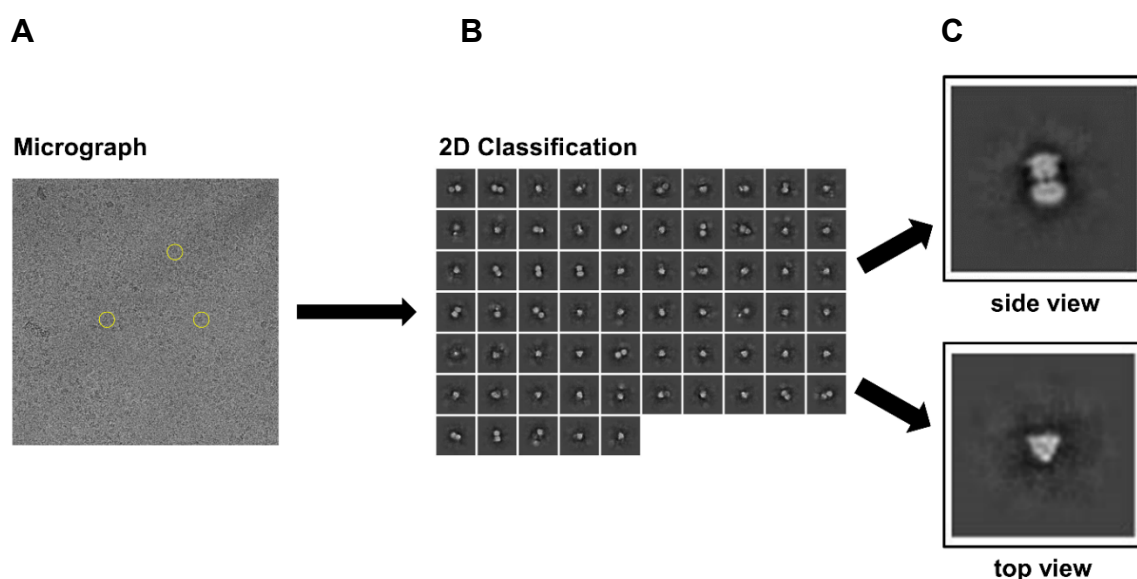


Figure 51. The cryo-EM data processing workflow using the dataset of the purified P2X4 receptor construct JN3 in complex with the antagonist PSB-0704. A. An example of a micrograph obtained from a Quantifoil R1.2/1.3 Cu300 holey carbon grid. Yellow circles illustrate exemplary protein particles. B. 2D classification was performed by PD Dr. Gregor Hagelüken using CryoSPARC (Structura Biotechnology, Toronto, Canada). C. Side and top view of the 2D classification revealing a trimeric assembly of the P2X4 receptor with a large extracellular and a smaller transmembrane domain.

In the beginning, 455,299 particles were picked and analyzed by several rounds of 2D classification to remove low-quality particles. The resulting 2D classifications revealed the trimeric assembly of the P2X4 receptor from both side and top views (Figure 50B and 50C). The large extracellular domain and the smaller transmembrane domain of the P2X4 receptor were identifiable in the 2D classifications.

Based on the remaining stack of 51,000 particles, a 3D reconstruction was performed, resulting in a volume that clearly resembled an AlphaFold 2 model of the human P2X4 receptor (Figure 52). The refinement with CryoSPARC resulted in maps with an overall resolution of ~ 8 Å (Figure 52).

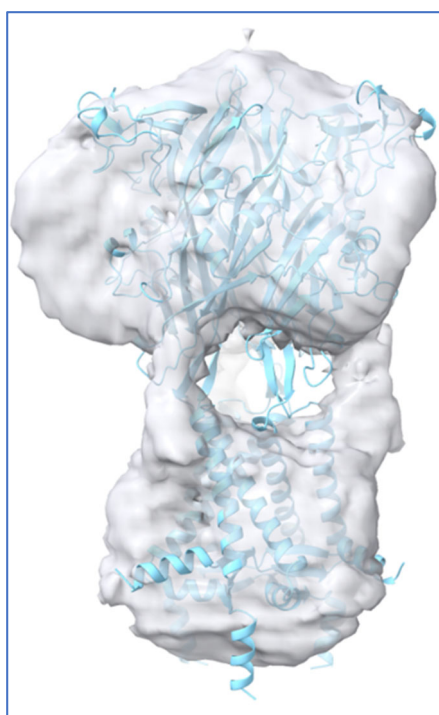


Figure 52. Superposition of the volume resulting from the 3D reconstruction of the P2X4 receptor construct JN3 in complex with PSB-0704 (overall resolution of ~ 8 Å) with an AlphaFold 2 model of the human P2X4 receptor (blue).

3.6.2 Summary and outlook

In summary, we successfully developed and optimized methods for the large-scale expression of the human P2X4 receptor in *Sf9* insect cells, followed by solubilization, purification, and reconstitution into amphipols. Initial cryo-EM studies validated the high quality of the purified P2X4 receptor protein, with 2D classifications revealing its trimeric assembly. Subsequent 3D reconstruction achieved an overall resolution of ~ 8 Å.

The first dataset was insufficient to achieve the required high resolution for the identification of the antagonist binding site. To obtain higher resolution, a larger dataset with numerous high-quality 2D images was required.

Using the optimized and validated methods, a larger cryo-EM dataset was acquired, which enabled the identification of the binding site of PSB-0704 at the human P2X4-E307T receptor. The results are described and presented in detail in Chapter 3.7 “Discovery of an allosteric binding site for anthraquinones at the human P2X4 receptor”.

3.6.3 Materials and methods

The Bac-to-Bac Baculovirus Expression System by Thermo Fisher Scientific (Dreieich, Germany) was employed to achieve high-level expression of recombinant proteins in *Sf9* insect cells. This system facilitates the generation of recombinant baculoviruses through the site-specific transposition of an expression cassette into a baculovirus shuttle vector (bacmid) propagated in *Escherichia coli* (*E. coli*). The following protocols were optimized according to the manufacturer’s manual.

Design of P2X4 receptor constructs

The full-length human P2X4 receptor gene, comprising three mutations (N75R, N184R, and E307T) and a C-terminal Rho1D4 tag in the pOET2 vector, was provided by Cube Biotech (Cologne, Germany).

To apply the Bac-to-Bac Baculovirus Expression System by Thermo Fisher Scientific, the P2X4 receptor constructs were cloned into the pFastBac1 vector (Thermo Fisher Scientific, Dreieich, Germany). The expression of the gene of interest is controlled by a polyhedrin promoter. Different P2X4 receptor constructs were prepared by applying the following methods. Primers were synthesized by Invitrogen.

Insert preparation polymerase chain reaction (PCR)

Table 13. Exemplary pipetting scheme of insert preparation PCR.

Component	Volume [μL]
5x Q5 Reaction Buffer (New England Biolabs, Frankfurt, Germany)	10
dNTPs (10 mM) (New England Biolabs, Frankfurt, Germany)	1
Forward primer (5 μ M)	2
Reverse primer (5 μ M)	2
Template DNA	0.5
Q5 [®] High-Fidelity DNA polymerase (New England Biolabs, Frankfurt, Germany)	0.5
DEPC-treated water	34

Table 14. PCR thermocycler program for insert preparation.

	Temperature [$^{\circ}$C]	Duration [s]
Initial denaturation	95	180
Denaturation	[95	30
Annealing	55	60
Elongation	68	90] x 30 cycles
Final elongation	68	600

Storage at 12 $^{\circ}$ C; Lid temperature: 105 $^{\circ}$ C***Agarose gel electrophoresis***

The PCR products were analyzed by agarose gel electrophoresis. For a 1% agarose gel, 0.5 g agarose powder were dissolved in 50 mL 1 x TAE (tris base, acetic acid, and EDTA) buffer by heating in a microwave. Then, 1 μ L of a nucleic acid dye (GelRed; Biotium, Fremont, CA, USA) was added for visualization. The entire agarose solution was poured into a gel casting tray fitted with a comb to create wells. The PCR products were mixed with loading dye (Gel Loading Dye; New England Biolabs, Frankfurt, Germany). Once the gel had solidified, the samples were pipetted into the wells. To estimate the size of the resulting DNA bands, the lambda DNA/HindIII Marker (Thermo Fisher Scientific,

Dreieich, Germany) was used. Electrophoresis was conducted at 130 V for 30–45 min in 1 x TAE running buffer. The samples were visualized in a ChemiDoc MP imaging system (Bio-Rad Laboratories, Munich, Germany).

The respective DNA band was extracted from agarose gel using the Zymoclean Gel DNA Recovery Kit (Zymo Research Europe, Freiburg im Breisgau, Germany).

Ligation

For the ligation reaction, the following reaction was set up in a microcentrifuge tube (Table 15) on ice and incubated at either room temperature for 2 h or at 16 °C overnight.

Table 15. Exemplary pipetting scheme for the ligation reaction.

Component	20 µL reaction
10x T4 DNA Ligase Buffer (New England Biolabs, Frankfurt, Germany)	10 µL
Vector DNA and insert DNA	molar ratio of 3:1
T4 DNA Ligase (New England Biolabs, Frankfurt, Germany)	1 µL
DEPC-treated water	to 20 µL

DNA amplification and purification

For DNA amplification, PCR products or purified plasmids were transformed into *E. coli* strain DH5α by the heat shock method. An aliquot of competent DH5α *E. coli* bacteria (50 µL) was thawed on ice for 10–20 min. Thereafter, 5 µL of the respective PCR product or 1 µL of purified plasmid were added, mixed, and incubated on ice for 30 min. Subsequently, a heat shock was performed at 42 °C for 45 s in a water bath or a heat block. The sample was then incubated on ice for a further 2 min, and 100 µL of room temperature LB medium were added. The DNA-bacteria mixture was then incubated for 1 h at 37 °C and 350 rpm in a heat block. The entire volume was then distributed on a LB agar plate containing ampicillin (100 µg/mL), and incubated overnight for up to 18 h at 37 °C.

On the next day, single colonies were transferred into freshly prepared LB medium (5 mL) containing 100 µg/mL ampicillin and were incubated overnight at 37 °C and 220 rpm. The

DNA purification and elution were performed with the Zymo Research Plasmid Miniprep Kit (Zymo Research Europe, Freiburg im Breisgau, Germany) with some deviations. The final elution of the DNA was performed with 30 µL of DEPC-treated water. Afterward, the concentration and purity of the DNA were determined by using a Colibri Microvolume Spectrophotometer (Berthold Technologies, Bad Wildbad, Germany).

To confirm the sequence, the samples were submitted for DNA sequencing (Eurofins Genomics, Ebersberg, Germany). Specific sequencing primers for the pFastBac1 vector were used (Table 16). Purified plasmids were stored at -20 °C.

Sequencing primers

Table 16. Sequencing primer for P2X4 receptor constructs in pFastBac1 vector.

Primer	Sequence
pBakPAC-FP forward (polyhedrin promoter)	5'-TAAAATGATAACCATCTCGC-3'
pFastBacR reverse	5'-CCTCTACAAATGTGGTATGGCTG-3'

All centrifugation steps were performed with the high-performance centrifuge Avanti JXN-26 or the centrifuge Allegra X-30 from Beckman Coulter (Krefeld, Germany).

Transformation into DH10Bac E. coli

The DH10Bac *E. coli* strain was used as the host for the pFastBac construct containing the respective P2X4 receptor gene. Following the verification of the respective DNA construct, the purified plasmids were introduced into the baculoviral genome via the heat shock method using the *E. coli* strain DH10Bac. An aliquot of competent DH10Bac *E. coli* bacteria (50 µL) was thawed on ice for 10–20 min. Then, 500 ng of the purified plasmid were added to the bacteria and incubated on ice for 30 min. The cells were heat-shocked for 45 s at 42 °C in a water bath or a heat block, followed by a two-minute incubation on ice. Subsequently, room temperature LB medium (800 µL) was added to the sample, and an incubation for 4 h at 37 °C and 350 rpm was initiated. The DNA-bacteria mixture was then diluted 1:10 with LB medium and 75 µL were spread on an agar plate containing 10 µg/mL tetracycline, 7 µg/mL gentamicin sulfate, 50 µg/mL kanamycin, 40 µg/mL IPTG (Carl Roth, Karlsruhe, Germany) and 100 µg/mL X-Gal (VWR International, Darmstadt,

Germany). The plate was covered with aluminum foil and incubated at 37 °C for at least 48 h.

Blue-white screening was applied to identify bacteria containing recombinant bacmid DNA. X-gal was used as a chromogenic substrate, and IPTG was used as a non-metabolizable analog of galactose that induces the expression of the *lacZ* gene.²⁹⁴ The baculovirus shuttle vector (bacmid) present in DH10Bac *E. coli* contains a kanamycin resistance marker, and a segment of DNA encoding the *lacZ* alpha peptide gene from a pUC-based cloning vector. The colonies formed by non-recombinant bacmids appear in a blue color due to the formation of β -galactosidase, which hydrolyzes the chromogenic substrate X-gal into a blue pigment.²⁹⁴ In contrast, colonies formed by recombinant bacmids appear in white as the expression of the *lacZ* peptide is disrupted, preventing β -galactosidase production and thus the hydrolysis of X-gal.²⁹⁴

On the following day, a single white bacterial colony was inoculated into 4 mL of freshly prepared LB medium containing tetracycline (10 μ g/mL), gentamicin sulfate (10 μ g/mL), and kanamycin (50 μ g/mL) and incubated overnight at 37 °C and 220 rpm. The following bacmid isolation and purification was performed with plasmid preparation buffers purchased from Qiagen (Hilden, Germany). The bacterial cells were centrifuged for 10 min at 2,500 g, and the supernatant was removed. The remaining pellet was then resuspended in 400 μ L of P1 buffer (resuspension buffer containing RNase). Subsequently, 400 μ L of P2 buffer (lysis buffer) were added, and the mixture was incubated for 2 min and gently inverted. After adding 400 μ L of P3 buffer (neutralization buffer) and gently mixing, the sample was centrifuged at 14,000 g for 15 min at 4 °C. The supernatants were carefully added to 800 μ L of isopropanol (room temperature) in a 2 mL Eppendorf tube and inverted. Following a 30-minute incubation at 20 °C, the samples were again centrifuged at 14,000 g for 15 min at 4 °C to precipitate the bacmid DNA. The bacmid DNA pellet was washed twice with 500 μ L of chilled ethanol (70%), dried under a laminar flow cabinet for 20 min, and finally dissolved in 30 μ L of DEPC-treated water. Subsequently, the concentration and purity of the bacmid DNA were determined using a Colibri Microvolume Spectrophotometer (Berthold Technologies, Bad Wildbad, Germany).

Bacmid verification test

To verify the presence of the gene of interest in the recombinant bacmid, a bacmid verification PCR was performed as shown below.

Table 17. PCR components for bacmid verification test.

Component	Volume
10x Standard Taq buffer (New England Biolabs, Frankfurt, Germany)	2.5 µL
dNTPs (10 mM) (New England Biolabs, Frankfurt, Germany)	0.5 µL
M13 forward primer (5 µM) (5'-CCCAGTCACGACGTTGTAAAACG-3')	0.5 µL
M13 reverse primer (5 µM) (5'-AGCGGATAACAATTCACACAGG-3')	0.5 µL
Template (bacmid)	1 µL
Taq polymerase (New England Biolabs, Frankfurt, Germany)	0.15 µL
DEPC-treated water	19.85 µL

Table 18. PCR thermocycler program for bacmid verification test.

	Temperature [°C]	Duration [s]
Initial denaturation	95	30
Denaturation	[95	30
Annealing	58	60
Elongation	68	180] x 25 cycles
Final elongation	72	300

Storage at 12 °C; Lid temperature: 105 °C

Agarose gel electrophoresis of bacmids

The resulting PCR products were further analyzed by agarose gel electrophoresis. The bacmid without a transposition has a size of ~300 bp. If the transposition was successful, a PCR product with a size of ~2,300 bp plus the size of the insert was visible.

Sf9 insect cell cultivation

Cell culture

Sf9 insect cells (Expression Systems, Davis, CA, USA) were cultured in ESF921 cell culture medium (Expression Systems, Davis, CA, USA) at 27 °C and 150 rpm. The cell culture medium was stored at 4 °C and prewarmed at room temperature before use. All works were performed under a laminar flow cabinet. Prior to use under sterile conditions, all necessary materials were wiped with 70% isopropanol to avoid contamination.

Thawing Sf9 insect cells

A frozen aliquot of *Sf9* insect cells was incubated in a clean water bath at 37 °C. If the cells were just thawed, the complete volume was transferred into a 125 cm² flask containing 20 mL cell culture medium, and incubated at 27 °C. After the cells had attached to the bottom of the flask, the medium was changed. Then, the medium was changed every three days and the cell growth was observed. After observing several cell monolayers on the surface, the cells were detached and transferred into a shaker flask containing 20–40 mL of fresh medium. The cells were incubated at 27 °C and 150 rpm, and grown up to 2–3 mio. cells/mL, after which routine cell splitting was performed.

Cultivation of Sf9 insect cells

Sf9 insect cells were split two times per week at 0.8 mio. cells/mL in 40–50 mL of fresh medium. A 1:10 dilution of the cell suspension was mixed with 0.4% trypan blue and counted using a hemocytometer. Blue-colored cells indicated dead cells, while unstained cells indicated cells that were alive. In the case of mid- and large-scale protein expression, the cells were initially grown in 200 mL of medium and then scaled up to 500 mL or more of medium.

Bacmid transfection into Sf9 insect cells

The bacmid transfection was performed in a 24-deep well culture block under sterile conditions. The transfection sample contained 5 μL of the bacmid sample, 100 μL of transfection medium (Expression Systems, Davis, CA, USA), and 3 μL of XtremeGENE™ HP (Sigma-Aldrich, Taufkirchen, Germany). The mixture was incubated for 15 min at room temperature and then added to 2.5 mL of *Sf9* insect cells at a density of 1×10^6 cells/mL. The sample was then incubated in a thermoshaker for 96 h, at 27 °C and 400 rpm. After 96 h, the sample was centrifuged at 2,000 rpm for 15 min to harvest the virus (P0). The virus was stored at 4 °C.

Protein expression***Small-scale protein expression***

For a small-scale protein expression, 400 μL of virus P0 were used to infect 40 mL of freshly-grown *Sf9* insect cells at a density of $2\text{--}3 \times 10^6$ cells/mL. The cells were incubated for 48 h at 27 °C and 150 rpm. Subsequently, the cells were precipitated by centrifugation at 4,000 g for 15 min in 50 mL tubes. The virus P1 (supernatant) was stored at 4 °C. The biomass (cell pellet) was stored at -80 °C for later protein analysis.

Medium-scale protein expression

For a medium-scale protein expression, ~6 mL of virus P1 were used to infect 1,000 mL of freshly-grown *Sf9* insect cells at a density of $2\text{--}3 \times 10^6$ cells/mL. To infect 500 mL of freshly-grown *Sf9* insect cells, ~3–4 mL of virus P1 were used. The cells were incubated for 48 h at 27 °C and 150 rpm. After that, the cells were precipitated by centrifugation at 4,000 g for 30 min. The supernatant was discarded and the pellet was resuspended in 30 mL of sterile phosphate buffer and centrifuged at 4,000 g for 20 min. The supernatant was removed and the biomass was stored at -80 °C for later protein analysis.

Membrane preparation and protein purification

All buffer compositions for membrane preparation and protein purification are shown in Table 19, Table 20, and Table 21.

Small-scale biomass

Prior to the preparation of the cell membrane, the biomass was thawed at room temperature. The following steps were carried out on ice or at 4 °C. Then, the cell membranes were disrupted by osmotic shock in 25 mL of low osmotic buffer, which was supplemented with EDTA-free complete protease inhibitor cocktail tablets (Roche, Mannheim, Germany). To homogenize the cell pellet, a Dounce tissue homogenizer (VWR International, Darmstadt, Germany) was used and ~40 strokes were performed. Afterward, the membranes were centrifuged for 30 min at 48,000 g (or 57,000 g). The supernatant was removed, and the pellet was resuspended and washed with 25 mL of high osmotic buffer using a Dounce tissue homogenizer. After subsequent centrifugation for 30 min at 48,000 g (or 57,000 g), the pellets were resuspended in 3 mL of resuspension buffer, homogenized once more, flash-frozen in liquid nitrogen, and stored at -80 °C.

Protein solubilization

All steps were carried out on ice or at 4 °C. The gravity columns and caps were washed with Millipore water in advance. After thawing the membrane resuspension on ice, it was incubated with a respective ligand for at least 30 min at 4 °C while slowly rocking. Then, 3 mL of 2x solubilization buffer were added to the membrane. The sample was incubated for 3 h at 4 °C while rocking. After the incubation, the sample was centrifuged for 30 min at 10,000 g, and the supernatants (solubilized fraction) were collected.

For His tag purification, 25 µL of washed IMAC cobalt resin beads (Takara Bio, Saint-Germain-en-Laye, France) and imidazole at a final concentration of 20 mM were added to the solubilized fraction. The sample was incubated overnight at 4 °C while slowly rocking.

On the next day, the sample was centrifuged at 100 g for 5 min to pellet the resin. The majority of the supernatant was removed, the resin was resuspended in the remaining buffer, and it was transferred into a small empty gravity flow column (Bio-Rad Laboratories, Munich, Germany). The resin was washed with 750 µL (~15 column volumes (CV)) of wash buffer I and 500 µL (~10 CV) of wash buffer II. The purified protein was eluted in three steps by adding elution buffer (1 x 25 µL, 2 x 50 µL), followed by incubation for 10 min before flow-through.

Medium- and large-scale biomass

The membrane preparation of a mid- and large-scale biomass was performed almost in the same way as described for a small-scale membrane preparation. The cell membranes were disrupted twice in 80–90 mL of low osmotic buffer (plus protease inhibitors) using a Dounce tissue homogenizer and subsequent centrifugation at 57,000 g for 30–45 min. Then, cell membranes were washed two to three times with 80–90 mL of high osmotic buffer. After the final centrifugation, the cell pellets were resuspended in 50 mL of resuspension buffer, homogenized, flash-frozen in liquid nitrogen, and stored at -80 °C until further use.

Protein solubilization

The membrane resuspension was thawed on ice. Then, it was incubated with a respective ligand for at least 30 min at 4 °C while rocking. Typically, a ligand concentration of 50 µM was used. Then, 25 mL of 2x solubilization buffer were added to the membrane (25 mL). The sample was incubated for 3 h at 4 °C while rocking. After the incubation, the samples were centrifuged for 30 min at 48,000 g, and the supernatant, containing the solubilized fraction, was collected.

For His tag purification, 750–1000 µL of washed IMAC cobalt resin beads and imidazole at a final concentration of 20 mM were added to the solubilized fraction. The samples were then rocked overnight at 4 °C.

Protein purification

All steps of the protein purification were performed on ice or at 4 °C. The gravity columns and the caps were pre-washed with Millipore water.

At first, the resin was pelleted by low-speed centrifugation at 100 g for 5 min. The majority of the supernatant was removed, and the remaining resin was resuspended and transferred into the empty gravity column (Bio-Rad Laboratories, Munich, Germany).

The resin was first washed by adding 10–15 CV of wash buffer I in different steps (e.g., 3 x 2 mL; 1 x 750 µL). The same procedure was done with wash buffer II (~10 CV; e.g., 3 x 1.5 mL, 1 x 500 µL). The amount of elution buffer for the final protein elution depends on the individual approach and the expected protein amount. In general, the purified protein is eluted by adding three to four times elution buffer (~250 µL) to the resin and incubating each step for 10 min prior to flow-through.

Buffers for membrane preparation, protein solubilization, and purification

All stock solutions and buffers were sterile-filtered before use. The buffers were stored at 4 °C.

Table 19. Buffer compositions for membrane preparation.

Membrane preparation	
Buffer	Components
Low osmotic buffer	10 mM HEPES (pH 7.5), 10 mM MgCl ₂ , 20 mM KCl
High osmotic buffer	10 mM HEPES (pH 7.5), 10 mM MgCl ₂ , 20 mM KCl, 1 M NaCl
Resuspension buffer	10 mM HEPES (pH 7.5,) 10 mM MgCl ₂ , 20 mM KCl, 30% (v/v) glycerol

Table 20. Buffer composition for the purification of small- and medium-scale biomass. DDM was purchased from Anatrache Products (OH, USA) and CHS from Sigma-Aldrich (Taufkirchen, Germany).

Small- and medium-scale protein purification	
Buffer	Components
Solubilization buffer	50 mM HEPES (pH 7.5), 800 mM NaCl, 1% (w/v) DDM, 0.2% (w/v) CHS
Wash buffer I	50 mM HEPES (pH 7.5), 800 mM NaCl, 0.1% (w/v) DDM, 0.02% (w/v) CHS, 10% (v/v) glycerol, 25 mM imidazole
Wash buffer II	50 mM HEPES (pH 7.5), 800 mM NaCl, 0.05% (w/v) DDM, 0.01% (w/v) CHS, 10% (v/v) glycerol, 50 mM imidazole
Elution buffer	25 mM HEPES (pH 7.5), 800 mM NaCl, 0.025% (w/v) DDM, 0.005% (w/v) CHS, 10% (v/v) glycerol, 220 mM imidazole

For the cryo-EM analysis, the compositions of the buffers utilized for protein solubilization and purification were slightly modified.

Table 21. Optimized buffer compositions for the purification of large-scale biomass.

Large-scale protein purification	
Buffer	Components
Solubilization buffer	50 mM HEPES (pH 7.5), 100 mM NaCl, 1.5% (w/v) DDM, 0.3% (w/v) CHS
Wash buffer I	50 mM HEPES (pH 7.5), 100 mM NaCl, 0.1% (w/v) DDM, 0.02% (w/v) CHS, 10% (v/v) glycerol, 25 mM imidazole, 20 mM MgCl ₂
Wash buffer II	50 mM HEPES (pH 7.5), 100 mM NaCl, 0.05% (w/v) DDM, 0.01% (w/v) CHS, 10% (v/v) glycerol, 40 mM imidazole
Elution buffer	25 mM HEPES (pH 7.5), 100 mM NaCl, 0.025% (w/v) DDM, 0.005% (w/v) CHS, 10% (v/v) glycerol, 220 mM imidazole

Amphipol reconstitution

The amphipol stock solution (10% w/w; 100 g/L) (A8-35; Anatrache Products, OH, USA) was prepared in Millipore water in a small glass vial, homogenized by vortexing, and stored at 4 °C. Before use, Bio-Beads SM-2 (Bio-Rad Laboratories, Munich, Germany) were washed twice with 20% (v/v) ethanol and Millipore water (5–6 times). Washed beads were stored in Millipore water at 4 °C.

After solubilization with DDM/CHS and protein purification, the protein solution was concentrated to a desired volume (e.g., 500–1000 µL) using a 100-kDa molecular weight cut-off Amicon® concentrator (Merck, Darmstadt, Germany). To ensure thorough mixing, the volume of the sample volume should be between 500 and 1000 µL. The amount of A8-35 was calculated based on the mass of protein in the sample. The respective amount of protein solution and A8-35 were added to a 1.5 mL or 2 mL Eppendorf tube and incubated for at least 4 h at 4 °C with gentle stirring. For stirring, a small magnetic stir bar was used.

After the incubation, the Bio-Beads SM-2 were dried on tissue paper to remove any excess water. Thereafter, an appropriate amount (20 x mass of detergent present in the sample) was added to the protein sample. The sample was incubated overnight at 4 °C with gentle stirring. On the next day, small gravity flow columns were pre-washed with Millipore water or SEC buffer (300–500 µL). The protein solution was then pipetted onto the column and

spun briefly in a microfuge to remove the Bio-Beads. The final protein solution was then concentrated to a desired volume or concentration using a 100-kDa molecular weight cut-off Vivaspin[®] concentrator (Sartorius, Göttingen, Germany) or an Amicon[®] concentrator (Merck, Darmstadt, Germany).

Size-exclusion chromatography (SEC)

SEC was performed with different high-performance liquid chromatography (HPLC) systems. For analytical purposes, to evaluate the protein's stability and purity, an Agilent Technologies (Waldbronn, Germany) 1260 Infinity HPLC system coupled to a UV-VIS detector was employed. The employed column was a 4.6 x 250 mm Sepax Nanofilm SEC-250 column (Sepax Technologies, Newark, DE, USA) with a pore size of 250 Å and a particle size of 5 µm. The Nanofilm SEC phase consists of a few nanometer-thick hydrophilic thin films covalently bonded to the silica. The separation range of this column is 1,500–7.0 x 10⁵ Da.

Prior to the measurement, the HPLC system was purged with Millipore water and equilibrated with the respective SEC buffer, which was sterile-filtered and stored at 4 °C. For protein samples solubilized in DDM/CHS, the buffer consisted of 25 mM HEPES (pH 7.5), 500 mM NaCl, 2% glycerol, 0.05% DDM, and 0.01% CHS. For protein samples reconstituted in A8-35, the same buffer system was used without the addition of DDM and CHS. Before the measurement, the samples were centrifuged for 10 min at 14,000 g and 4 °C. For sample injection, an autosampler compartment was used at 4 °C. The column was attached to the column oven, which was cooled down to 14 °C. Typically, 30 µL of protein solution were injected into the system. The flow rate was 0.5 mL/min. After the measurement, the column was disassembled and the HPLC system was washed with Millipore water and 70% isopropanol.

For additional purifying purposes, SEC was performed on an Agilent Technologies 1260 Infinity II (Waldbronn, Germany) HPLC system using a Superose 6 Increase 3.2/300 column (Cytiva, Freiburg im Breisgau, Germany), with an agarose-based resin and a fractionation range of 5,000–5 x 10⁶ Da. For amphipol-trapped purified proteins, the SEC buffer consisted of 20 mM HEPES (pH 7.5) and 150 mM NaCl. A maximum of 50 µL of the protein sample, which had been centrifuged beforehand, was injected into the HPLC system with a flow rate of 0.05 mL/min.

GraphPad Prism version 8.0 (GraphPad Software, San Diego, CA, USA) was used to visualize the SEC chromatograms.

SDS-PAGE

To analyze the purified protein, an SDS-PAGE was prepared with a 10% bis-Tris gel. At first, the resolving gel solution (Table 22) was prepared and poured into the gel casting (4.5 mL per gel). The gel was immediately covered with ~500 μ L of 70% isopropanol. After the resolving gel had polymerized, the isopropanol was removed with filter paper, the stacking gel solution (Table 23) was added (1.5 mL per gel), and a 10-well comb was inserted. After the stacking gel had polymerized, the gels were stored at 4 °C in tissues soaked in either water or SDS running buffer.

Table 22. Buffer compositions for SDS-PAGE resolving gel.

Resolving gel (for 4 gels)	
Component	Volume
3x gel buffer (1 M bis-Tris, pH 6.5–6.7)	6.5 mL
Acrylamide	6.5 mL
Water	6.5 mL
Ammonium persulfate (10%; freshly prepared in water)	120 μ L
Tetramethylethylenediamine	48 μ L

Table 23. Buffer compositions for SDS-PAGE stacking gel.

Stacking gel (for 4 gels)	
Component	Volume
3x gel buffer (1 M bis-Tris, pH 6.5–6.7)	2.4 mL
Acrylamide	1.6 mL
Water	5.6 mL
Ammonium persulfate (10%; freshly prepared in water)	48 μ L
Tetramethylethylenediamine	32 μ L

The purified protein sample was mixed with NuPAGE™ LDS (lithium dodecyl sulfate) (Thermo Fisher Scientific, Dreieich, Germany) sample buffer at a ratio of 3:1. The SDS running buffer contained 1 M MOPS (3-morpholinopropane-1-sulfonic acid), 20 mM EDTA, and 2% SDS. The electrophoresis chamber was assembled, filled with running buffer, and max. 30 µL of each protein sample were loaded per well. Additionally, 4 µL of prestained PageRuler™ Prestained Protein Ladder (Thermo Fisher Scientific, Dreieich, Germany) were loaded into a separate well. The electrophoresis was started at 50 V and later increased to 150 V.

Coomassie protein staining

After electrophoresis, the separated protein bands are visualized using specific dyes, such as Coomassie Brilliant Blue. The coomassie protein staining solution contained 1.25 g Coomassie Brilliant Blue R-250 (AppliChem, Darmstadt, Germany) dissolved in 670 mL Millipore water, 250 mL acetic acid, and 80 mL ethanol. After transferring the SDS-gel into a tray, coomassie protein solution was added and the staining was performed by microwave heating. To destain the gel, the gel was covered with water and heated again in the microwave. This step was repeated until the gel was almost completely destained. The separated protein bands were visualized using a ChemiDoc MP imaging system (Bio-Rad Laboratories, Munich, Germany).

Western blotting

Blotting

For the Western blot analysis, the semi-dry Trans-Blot® Turbo™ Transfer System from Bio-Rad Laboratories (Munich, Germany) was used. At first, the protein sample was separated by SDS-PAGE as described before. For the blotting, two thick whatman papers and a nitrocellulose membrane were cut to a reasonable size. The whatman papers, nitrocellulose membrane, and the SDS-gel were equilibrated in transfer buffer (48 mM Tris, 39 mM glycine, 10% ethanol, 0.1% SDS) for a few minutes. Then, the Trans-Blot® Turbo™ blotting chamber was assembled as follows:

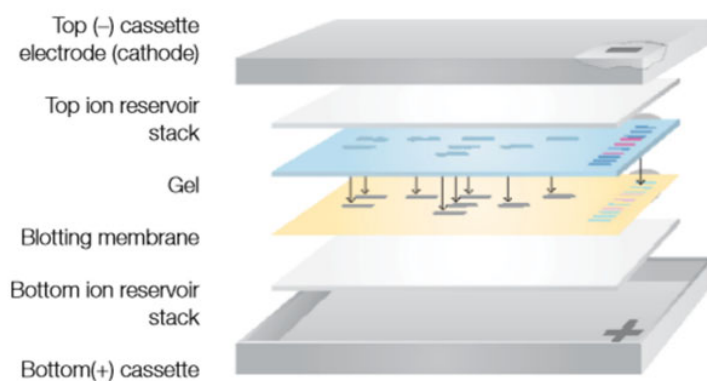


Figure 53. Assembly of the Trans-Blot[®] Turbo[™] blotting chamber. Figure taken from the Instruction Manual (Bio-Rad Laboratories, Munich, Germany).

The blotting was typically performed at 1.3 A (constant) and 20 V for ~15–20 min.

Immunodetection

At first, the membrane was incubated in a blocking buffer (5% w/v milk powder dissolved in PBS-T (1 x PBS with 0.1% Tween 20) for 45–60 min at room temperature under mild shaking conditions. After discarding the blocking buffer and rinsing the membrane with demineralized water, 8 mL of the primary antibody (1:1000 dilution of mouse 6x-His Tag Monoclonal Antibody prepared in blocking buffer) (Thermo Fisher Scientific, Dreieich, Germany) was added to the membrane. The membrane was incubated overnight at 4 °C under mild shaking conditions. On the next day, the membrane was washed with demineralized water and blocking buffer, and 10 mL of the secondary antibody (1:4000 dilution of Peroxidase-conjugated AffiniPure Goat Anti-Mouse IgG prepared in blocking buffer) (Jackson ImmunoResearch Europe, Cambridgeshire, UK) were added. After an incubation of 90 min at room temperature, the antibody was removed and the membrane was rinsed with demineralized water. Further washing steps with PBS-T were performed (4 x 5 min and 1 x 30 min). The immunodetection was performed by adding ~800 µL of the chemiluminescence substrate solution (Thermo Fisher Scientific, Dreieich, Germany) to the membrane. The picture was acquired using the multi-channel chemiluminescence mode of the ChemiDoc MP imaging system (Bio-Rad Laboratories, Munich, Germany).

TALON IMAC resin preparation

The resin is stored by the manufacturer (Takara Bio, Saint-Germain-en-Laye, France) 1:1 (v/v) in 20% ethanol. At first, a requested amount was transferred into a 15 mL tube and centrifuged at 100 g and 4 °C for 5 min. The ethanol was discarded and the beads were resuspended in an equal volume of Millipore water and centrifuged again at 100 g and 4 °C for 5 min. This procedure was repeated up to 8 times. After the last washing step, the beads were resuspended in a buffer containing 50 mM HEPES (pH 7.5), 0.5 M NaCl, 10% (v/v) glycerol, and stored at 4 °C.

Cryo-EM grid preparation

The purified P2X4-E307T protein sample (3 µL) was loaded onto various grids (Quantifoil R1.2/1.3 Cu300 holey carbon, Quantifoil R2/1+2nm C Cu200 holey carbon, and UltrAuFoil R1.2/.13 Au300), respectively, that had been glow discharged for 40 s (Diener electronic, Ebhausen, Germany). The samples were blotted for 3 s before plunge freezing into liquid ethane using a VitroBot MarkIV (Thermo Fisher Scientific, Dreieich, Germany) at 21 °C and 100% humidity.

Grid screening and data acquisition were performed using a 300 kV-Krios G3i Cryo-Transmission Electron Microscope equipped with a Falcon III electron detector (Thermo Fisher Scientific, Dreieich, Germany).

3.7 Manuscript II: Discovery of an allosteric binding site for anthraquinones at the human P2X4 receptor

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3.7.1 Author contributions

The following results, presented in the form of a manuscript, were obtained as part of this thesis. The author of this thesis developed and optimized methods for the large-scale expression of the human P2X4 receptor in *Sf9* insect cells, starting with receptor construct design using molecular biology techniques. This work was followed by protein solubilization, purification, and reconstitution into amphipols. The author prepared the final protein sample used for cryo-EM investigations, which allowed determination of the human P2X4 receptor structure in complex with an anthraquinone-based P2X4 receptor antagonist at high resolution. Cryo-EM data were analyzed by PD Dr. Gregor Hagelüken.

The author collaborated with the Institute of Structural Biology (Bonn) and the cryo-EM facilities in Cologne and Strasbourg for structure determination. The manuscript, including Supporting Information, was written and prepared by the author in collaboration with the coauthors. Preliminary pharmacological results included in the manuscript were analyzed and contextualized appropriately. The author created all figures and tables, except for those depicting structural data.

These contributions were performed in close collaboration with the corresponding authors, PD Dr. Gregor Hagelüken and Prof. Dr. Christa E. Müller, and under supervision of Prof. Dr. Christa E. Müller.

3.7.2 Abstract

P2X receptors are trimeric ATP-gated ion channels. The P2X4 receptor subtype is a promising drug target for the treatment of inflammatory diseases, neuropathic pain, and cancer. Here, the water-soluble anthraquinone derivative Cibacron Blue, previously described as a P2X4 receptor modulator, was selected as a lead structure, and structure-activity relationships were investigated. A chimeric receptor approach, combined with mutagenesis and docking studies, was applied to identify the allosteric binding site and the interacting amino acid residues. We discovered that Glu307, located in the upper body of the receptor, is prone to form an intermolecular “ionic lock” with basic amino acid residues, thereby preventing high-affinity binding of anthraquinone derivatives. Exchange of Glu307 for threonine led to a dramatic potency increase for anthraquinones in blocking P2X4 receptor function. The structure of the full-length human P2X4 receptor in complex with the anthraquinone derivative PSB-0704 was determined by cryo-electron microscopy at a resolution of 3.35 Å. This revealed an allosteric binding site in the upper body at the interface of the receptor trimer subunits, which differs from previously described allosteric sites on P2X receptors. Our results provide a rational basis for structure-based drug design towards potent and selective P2X4 receptor antagonists.

3.7.3 Introduction

ATP, the primary intracellular energy source, has emerged as a key extracellular signaling molecule, in particular in inflammatory conditions.⁴¹ ATP-induced signaling cascades are mediated by ionotropic P2X and metabotropic P2Y receptors.⁴ P2X receptors comprise seven subtypes, P2X1-P2X7, which are activated by ATP.⁴ They form functional homotrimeric receptors, but heterotrimeric receptor complexes, e.g., P2X2/P2X3, have also been described.^{10,11} Each protein subunit consists of a large extracellular domain harboring the agonist binding site and two transmembrane domains forming a cation pore that is permeable for Na⁺, K⁺, and Ca²⁺ ions.^{5,295}

P2X receptors are widely expressed in neuronal and non-neuronal cells.⁵⁷ They contribute to a number of physiological and pathological processes, including synaptic transmission, smooth muscle contraction, pain sensation, and acute and chronic inflammation.^{57,296,297} Thus, P2X receptors have emerged as attractive drug targets, whose therapeutic significance is demonstrated by the recent approval of the P2X3 receptor antagonist Gefapixant for the treatment of chronic cough.^{55,194}

The P2X4 receptor is mainly expressed in the central nervous system, particularly on neurons and microglia, and on peripheral immune cells, namely B- and T-lymphocytes, monocytes, neutrophils, and mast cells.^{42,108} The inhibition of P2X4 receptors has shown promise in preclinical studies for various pathological conditions, including neuropathic pain, epilepsy, and further peripheral and central nervous system diseases associated with inflammation.^{128,150,169,198,298} Moreover, several studies have demonstrated a role for P2X4 receptors in different types of cancer, e.g., in breast, prostate, and colon cancer proliferation and –aggressiveness.^{44,47,171}

Thus, considerable efforts have been made to develop potent and selective P2X4 receptor antagonists, however, only few compounds have been described so far.¹⁰ These include the benzodiazepine derivatives 5-BDBD,¹⁰ NP-1815-PX,¹²³ and MRS4719,¹²⁴ the urea derivative BX430,¹³³ its analog “9o”,¹³⁵ and the sulfonamide derivative BAY-1797¹³¹ (for structures, see Supporting information, Figure 64). NC-2600 (structure undisclosed) was the first P2X4 receptor antagonist to enter phase I clinical trials for neuropathic pain, but no further development has been reported.¹²⁵ In the past years, our group has developed several series of allosteric P2X4 receptor antagonists, e.g., the indole derivatives PSB-15417 and PSB-OR-2020^{126,127,129} (for structures, see Supporting information, Figure 64). The anthraquinone derivative Cibacron Blue (Figure 54a) was described as a non-selective allosteric modulator of P2X2, P2X3, and P2X4 receptors.^{144–146}

The elucidation of high-resolution structures of different P2X receptor subtypes has significantly advanced our understanding of channel architecture and opening mechanisms, processes involved in desensitization, and recognition of agonists and antagonists.^{19,24–28,30–34,36–39,299} The first high-resolution P2X receptor structures were determined by X-ray crystallography of the zebrafish P2X4 receptor in its apo state,²⁴ and its ATP-bound state.²⁵ The results confirmed the previously proposed homotrimeric structure of P2X receptors,^{9,20} with three extracellular ATP-binding sites, one on each receptor monomer. The receptor subunits resemble a leaping dolphin, where the transmembrane helices correspond to the flukes and the extracellular region to the upper body.^{24,25} Recently published cryo-EM structures of the zebrafish P2X4 receptor in complex with the negative allosteric modulators BX430 and BAY-1797 identified a common binding site for both antagonists at the top of the extracellular domain near the subunit interfaces.²⁷ Further high-resolution structures of P2X receptors, either determined by cryo-EM or X-ray crystallography, include those of the human P2X3 receptor, the panda, chicken, and rat P2X7 receptor, and

the human P2X1 receptor (see Supporting information, Table 25).^{28,30–34,36–39,299,300} Each of the determined structures has provided important and often unpredictable new insights. However, it is surprising that most P2X receptor structures have been obtained from non-primate species, and in the case of the P2X4 receptor, so far, only from zebrafish.

Anthraquinones are a major class of natural products and synthetic compounds used as therapeutic drugs, e.g., as laxatives, anti-inflammatory and antiarthritic drugs, and as anti-cancer agents.³⁰¹ The anthraquinone derivative Cibacron Blue (Figure 54a), initially used as a blue dye, was later discovered to act as an allosteric modulator of P2X receptors.^{144–146,302}

The present study reports the structure of the extracellular domain of the full-length human P2X4 receptor in complex with an allosteric anthraquinone-derived P2X4 receptor antagonist, elucidated by cryo-EM. We identified an allosteric binding site for anthraquinone derivatives, which differs from previously described allosteric sites. Our findings are based on initial computational and mutagenesis studies, supported by calcium influx and radioligand binding assays. These insights advance our molecular understanding of P2X receptors in general and of the human P2X4 receptor in particular. They may provide a basis for the development of future drugs.

3.7.4 Results

Receptor chimeras and mutagenesis

In our efforts aimed at the development and characterization of P2X4 receptor antagonists, we determined concentration-dependent effects of the anthraquinone derivative Cibacron Blue on ATP-induced calcium influx in 1321N1 astrocytoma cells stably transfected with the human P2X4 wildtype (wt) receptor. Cibacron Blue displayed a biphasic modulation of the P2X4 receptor, enhancing the ATP effect at low concentrations (up to 141%; EC₅₀: 0.243 μ M) while inhibiting it completely at higher concentrations (IC₅₀: 12.1 μ M) (Figure 54b). At the human P2X2 receptor, only an inhibitory effect was observed (IC₅₀: 13.1 μ M) (Figure 54c). To further study the different behavior of the allosteric modulator Cibacron Blue at P2X4 as compared to P2X2 receptors, and directed at the identification of the allosteric binding site for the anthraquinone derivative, we investigated the compound at a set of previously developed P2X4/P2X2 receptor chimeras.¹³⁴ Ten variable regions (v1–v10) of the human P2X4 receptor, showing large differences to those of the P2X2 receptor,

were selected based on a sequence alignment of both receptors (Figure 54d, Supporting information, Figure 65).¹³⁴ This led to the design of five chimeric (c) human P2X4 receptors in which specific variable sequences were exchanged for the corresponding sequences of the human P2X2 receptor¹³⁴, designated c1^{V71-I83}, c2-c5^{C116-T186}, c6^{N208-S216}, c7^{I218-D224}, c8^{R265-L269}, c9^{T281-P290}, and c10^{R301-Q308}.

Pharmacological characterization of the chimeras led to an unexpected discovery. The inhibitory potency of Cibacron Blue at the chimeric receptor P2X4(P2X2) c10^{R301-Q308} was dramatically increased as compared to the wt P2X4 receptor, from an IC₅₀ value of 12.1 μ M to an IC₅₀ value of 4.76 nM (2,500-fold potency increase; Figure 54e, 54f). In this chimeric receptor, the amino acids ranging from Arg301 to Gln308 of the human P2X4 receptor had been exchanged for the corresponding amino acids present in the human P2X2 receptor (Supporting information, Figure 65). The EC₅₀ value of the endogenous agonist ATP remained unaltered at the chimeric receptor P2X4(P2X2) c10^{R301-Q308} (0.862 μ M, Supporting information, Figure 66, Table 26).

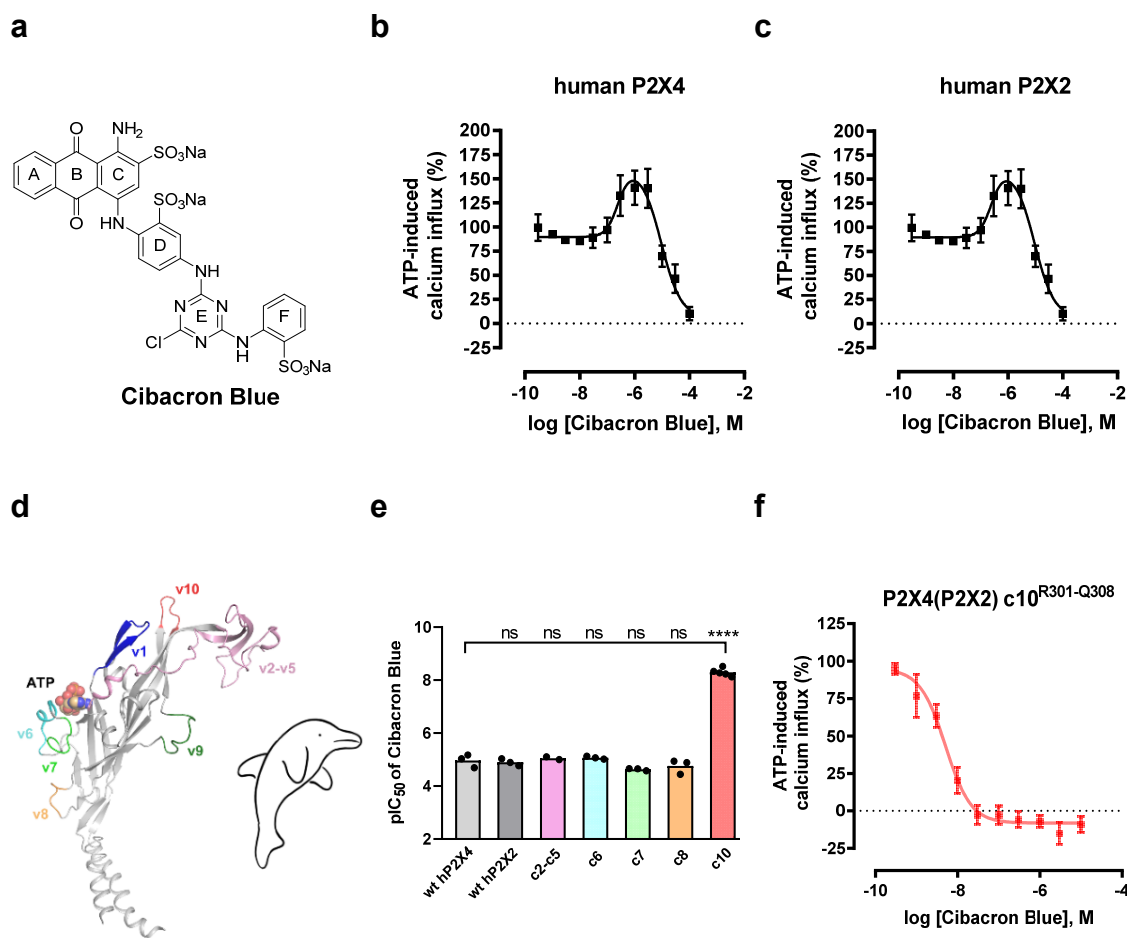


Figure 54. Pharmacological characterization of Cibacron Blue at the human wt P2X4, the human wt P2X2, and chimeric P2X4(P2X2) receptors. All receptors were stably expressed in human 1321N1 astrocytoma cells. Concentration-dependent inhibition was determined in the presence of the EC₈₀ of ATP. Data represent means $\pm$ SEM of at least three biological replicates, performed in technical duplicates. a) Chemical structure of Cibacron Blue. b) Biphasic modulation of the human wt P2X4 receptor by Cibacron Blue, determined by ATP-induced calcium influx assays. Low concentrations of Cibacron Blue increased the ATP-induced activation of the human wt P2X4 receptor, while higher concentrations of Cibacron Blue inhibited it. c) Concentration-dependent inhibition of the human wt P2X2 receptor by Cibacron Blue determined in ATP-induced calcium influx assays. d) Human P2X4 receptor subunit. Sequences that differ in the human P2X2 receptor (termed variable regions v1–v10) and the orthosteric ATP-binding site (shown as spheres) are indicated. Figure was reproduced from Weinhausen et al.¹³⁴ based on PDB ID 4dw1.²⁵ e) pIC₅₀ values of Cibacron Blue at the human wt P2X4, the human wt P2X2, and the chimeric P2X4(P2X2) receptors c2-c5^{C116-T186}, c6^{N208-S216}, c7^{I218-D224}, c8^{R265-L269}, and c10^{R301-Q308}. The chimeric receptor c1^{V71-I83} showed only minor activation by ATP (maximal effect of 4% compared to that of the human wt P2X4 receptor)¹³⁴, and the effect of Cibacron Blue could therefore not be evaluated at c1. The level of significance was calculated by a one-way ANOVA with Dunnett's multiple comparisons test. Significance was expected if P value was ≤ 0.05 . The significance levels were defined as follows: * P < 0.05, ** P < 0.01, *** P < 0.001, **** P < 0.0001, ns, not significant. f) Concentration-dependent inhibition of the chimeric P2X4(P2X2) receptor c10^{R301-Q308} mutant by Cibacron Blue.

An AlphaFold 3 (AF3)³⁰³ model of the apo human P2X4 receptor predicted that Glu307 of one receptor subunit forms ionic interactions with both Arg82 and Lys289 of the adjacent subunit (Figure 55). Arg301 may interact with the glycosylation site Asn110, an interaction that is also feasible in the N-glycosylated receptor protein. Arg82 is located in variable region v1 of the P2X4 receptor (Figure 54d), while Arg301 and Glu307 are located in variable region v10 (Figure 54d; Figure 56). Based on the AF3 model, electrostatic interactions between Glu307 and basic amino acid residues Arg82 and Lys298 result in the formation of an intermolecular “ionic lock”, which is likely responsible for the moderate potency of Cibacron Blue and related anthraquinone derivatives at the wt P2X4 receptor.

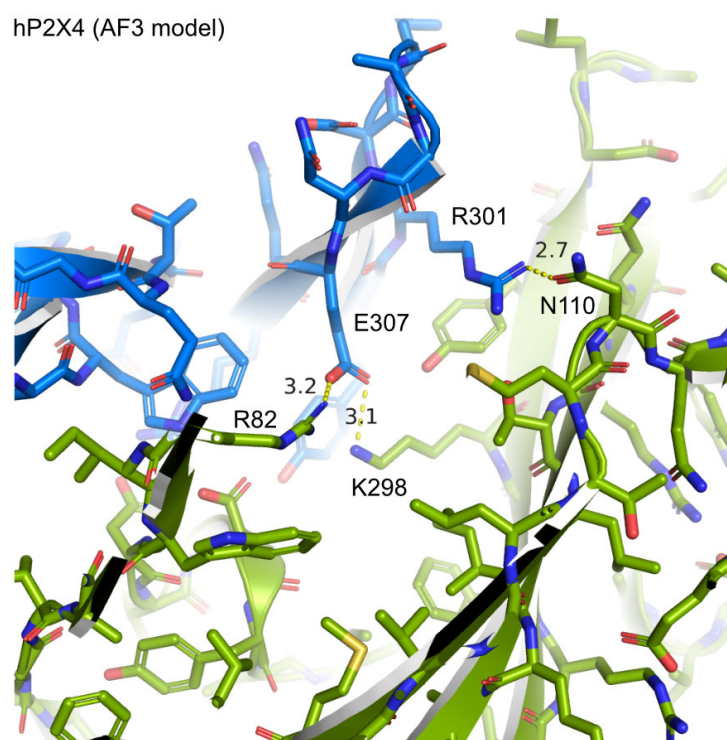


Figure 55. AlphaFold 3 (AF3) model of the apo human (h) P2X4 receptor. Glu307 is predicted to form ionic interactions with Arg82 and Lys298. Arg301 may interact with Asn110. The numbers are distances in Å.

The high potency of Cibracon Blue at the chimeric P2X4(P2X2) receptor c10^{R301-Q308} may result from the replacement of the acidic residue Glu307 in variable region v10 of the P2X4 receptor (Figure 56). Exchange of glutamate for threonine in position 307 prevents the putative ionic interaction and would make Arg301 available for direct ligand interaction, which is predicted to be the reason for the 2,500-fold increased potency of Cibacron Blue observed at the chimeric receptor P2X4(P2X2) c10^{R301-Q308}.

hP2X4_gi-116242696	IGWVFVWEKGYQETDSVV-SSVTTKVKGVAVTNTSKLGFRIWDVADYVIPAQEENSLFVM	102
hP2X2_gi-25092719	VWYVFIVQKSYQESETGPESIIITKVKGITTT----SEHKVVDVEEYVKPPEGGSVFSII	111
	: **: :*.***::: **: *****:.. :.*** :** * : . : : :	
hP2X4_gi-116242696	TNVILTMNQTLGLCPEIPD-ATTVCKSDASCTAGSAGTHSNGVSTGRVAF-NGSVKTCE	160
hP2X2_gi-25092719	TRVEATHSQTQGTCPESIRVHNATCLSDADCVAGELDMLGNGLRGTGRVFPYYQGPKTCE	171
	*. * * .*** ** * . :. * ***.**.*. . .** : ***** : : * ****	
hP2X4_gi-116242696	VAAWCPVEDDTHVPQPAFLKAAENFTLLVKNNIWYPKFNFSKRNLNITTTTLKSCIYD	220
hP2X2_gi-25092719	VFGWCPVEDGASVSQF-LGTMAPNFTILIKNSIHYPKFHFSGKNIA-DRTDGYLKRCTFH	229
	* .*****.: * * : . * ***.**.*. ******* * : * * * * :.	
hP2X4_gi-116242696	AKTDPFCPIFRLGKIVENAGHSFQDMAVEGGIMGIQVNWDCNLDRAASLCLPRYSFRRLD	280
hP2X2_gi-25092719	EASDLYCPIFKLGFIVEKAGESFTELAHKGGVIGVIINWDCDLDPASECNPKYSFRRLD	289
	: * :****:* ***.***.*** : * :****:* :*****: * * * *.*****	
	--v10--	
hP2X4_gi-116242696	TRDVEHNVSPGYNFRFAKY <u>RDLAGNEQ</u> RTLIKAYGIRFDIIVFGKAGKFDIIPMTINIG	340
hP2X2_gi-25092719	PKHVP--ASSGYNFRFAKY <u>K-INGTTT</u> RTLIKAYGIRIDVIVHGQAGKFSLIPTIINLA	346
	:. * . * *****: : * . *****:*.***.***.***.***.***.***.***.	
	variable region v10	Cibacron Blue IC₅₀ (μM)
human wt P2X4	<u>RDLAGNEQ</u> 301 307	12.1
chimeric P2X4(P2X2) c10 ^{R301-Q308}	<u>K-INGTTT</u>	0.00476

Figure 56. Sequence alignment of the P2X4 and P2X2 receptor. Top: sequence alignment of human wt P2X4 (gi-116242696) and the wt P2X2 receptor (gi-25092719) using Clustal Omega (1.2.4).²⁰⁸ Variable region v10 is colored in red. Amino acid residues predicted to be important for Cibacron Blue binding are underlined. Symbols: (*) indicates conserved residues, (:) and (.) indicate conservative and semi-conservative exchanges, and blank spaces point out non-conservative exchanges in residues between aligned sequences. Bottom: comparison of amino acid residues in variable region v10 (red) in the human wt P2X4 and the chimeric P2X4(P2X2) receptor c10^{R301-Q308}. Amino acid residues predicted to be important for Cibacron Blue binding are underlined. Inhibitory potency of Cibacron Blue (IC₅₀ (μM)) at the human wt P2X4 receptor and the chimeric P2X4(P2X2) receptor c10^{R301-Q308}, determined in calcium influx assays.

To provide further evidence for the proposed binding interactions, P2X4 receptor mutants were generated and characterized, harboring mutations in v10 (E307T, D302I, and Q308T). All mutants could be activated by ATP, and EC₅₀ values were similar to those determined at the human wt P2X4 receptor (Supporting information, Table 66). For the P2X4-E307T receptor mutant, Cibacron Blue showed a significantly higher inhibitory potency than for the wt P2X4 receptor (680-fold; IC₅₀: 0.0178 μ M (P2X4-E307T); IC₅₀: 12.1 μ M (wt P2X4)) (Figure 57a and 57b; Supporting information, Table 27), supporting our hypothesis that “ionic lock” formation, i.e. the formation of a salt bridge involving Glu307 and basic amino acid residues, prevents high-affinity binding to the wt P2X4 receptor. The inhibitory potency of Cibacron Blue at the P2X4-D302I receptor was moderately increased by about 9-fold compared to the wt P2X4 receptor (Figure 57a; Supporting information, Table 27). At the P2X4-Q308T mutant, the potency of Cibacron Blue was similar to that determined at the wt P2X4 receptor (Figure 57a; Supporting information, Table 27).

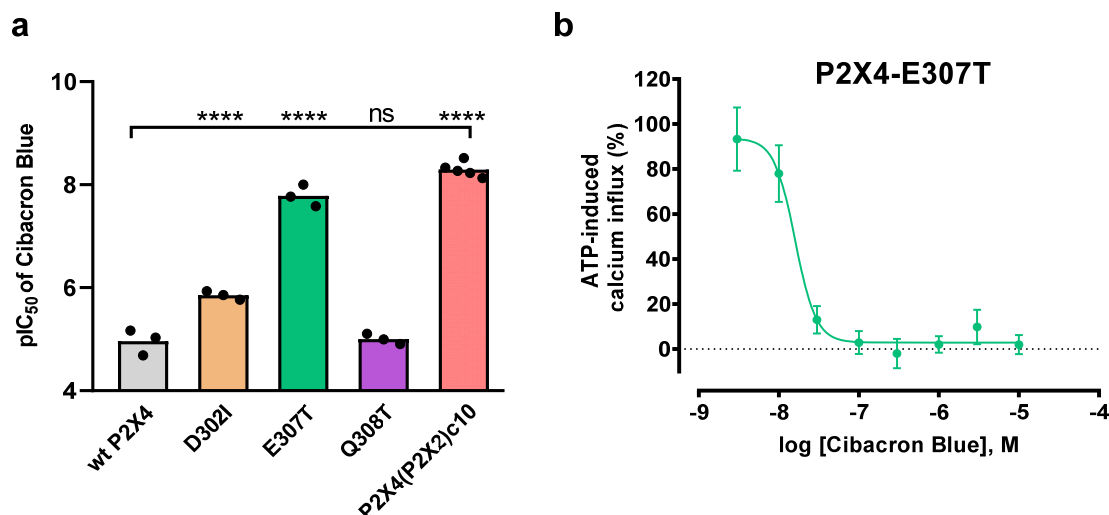
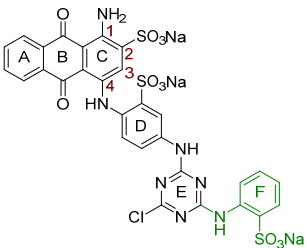
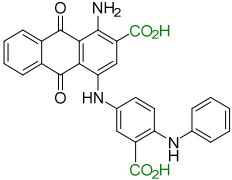
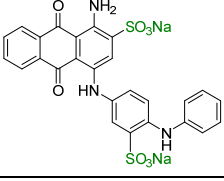
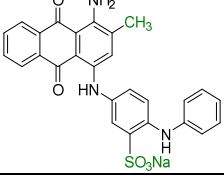
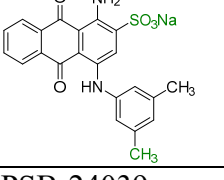
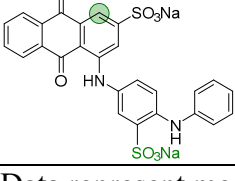


Figure 57. Potency of Cibacron Blue at P2X4 receptor mutants and chimeric P2X4(P2X2) receptor c10^{R301-Q308}. a) pIC₅₀ values of Cibacron Blue in blocking the human wt P2X4 receptor, the chimeric P2X4(P2X2) receptor c10^{R301-Q308}, and several P2X4 receptor mutants in the presence of an EC₈₀ of ATP (1–3 μ M). pIC₅₀ values were determined in fluorimetric calcium influx assays induced by ATP (at its EC₈₀). Single data points from 3–5 biological replicates, performed in technical duplicates, are shown. The level of significance was calculated by a one-way ANOVA with Dunnett’s multiple comparisons test. Significance was expected if P value was ≤ 0.05 . The significance levels were defined as follows: * P < 0.05, ** P < 0.01, *** P < 0.001, **** P < 0.0001; ns, not significant. b) Concentration-dependent inhibition by Cibacron Blue of ATP-induced activation of the human P2X4-E307T receptor mutant determined in the presence of an EC₈₀ of ATP.

These results support our hypothesis that the amino acid residue Thr307 instead of glutamate at that position is particularly important for high-affinity binding of Cibacron Blue. To validate these results and to identify a potential binding mode, further anthraquinone derivatives were tested at the P2X4-E307T receptor mutant and the chimeric P2X4(P2X2) c10^{R301-Q308} receptor (Table 24). Structure-activity relationship (SAR) studies showed that two acidic groups in the compounds, one in position 2 (ring C) and one on ring D, are beneficial for interaction with the P2X4 receptor, while ring F of Cibacron Blue (Figure 54a) was found to be dispensable (Table 24). Sulfonate or carboxylate groups at ring C and D are equally well tolerated by the P2X4 receptor, while the amino group at ring C is not required (Table 24).

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Table 24. Potency of selected anthraquinone derivatives determined by measurement of calcium influx at the human wt P2X4 receptor, the P2X4-E307T receptor mutant, and the chimeric receptor P2X4(P2X2) c10^{R301-Q308}, stably expressed in 1321N1 astrocytoma cells.

	IC ₅₀ ± SEM (μM) ^a		
	wt P2X4	P2X4-E307T	P2X4(P2X2) c10 ^{R301-Q308}
Cibacron Blue 	12.1 ± 4.2	0.0178 ± 0.0047	0.00476 ± 0.00062
PSB-0704 	12.5 ± 1.9	0.0724 ± 0.0102	n.d. ^b
PSB-0739 	8.91 ± 0.67	0.0353 ± 0.0047	0.00341 ± 0.00049
PSB-0801 	25.3 ± 0.8	0.180 ± 0.007	0.0429 ± 0.0033
PSB-09059 	20.0 ± 8.45	0.195 ± 0.014	0.266 ± 0.018
PSB-24039 	11.4 ± 0.3	0.0404 ± 0.0090	n.d. ^b

^a Data represent means ± SEM of at least three biological replicates, performed in technical duplicates. The inhibition of anthraquinone derivatives was determined in the presence of ATP at its respective EC₈₀. ^b n.d., not determined.

Thus, we had strong indications for an allosteric binding site of the P2X4 receptor that had not been previously described. To obtain unambiguous evidence, we set out to determine a cryo-EM structure.

P2X4 receptor protein expression and purification

A construct for P2X4 receptor expression was designed that would enable cryo-EM studies. We chose the full-length human P2X4 receptor sequence containing a C-terminal polyhistidine tag (10x-His tag) and three point mutations to remove potential glycosylation sites (N75R and N184R)^{24,25} and to increase the receptor's potency for anthraquinone derivatives (E307T). The receptor was overexpressed in *Sf9* insect cells, solubilized with DDM and CHS, and purified by immobilized metal affinity chromatography (IMAC). The purified protein was reconstituted into short and flexible amphipathic polymers that stabilize membrane proteins (A8-35 amphipol),^{304,278} and subsequently analyzed through single-particle cryo-EM.

To characterize the human P2X4 receptor construct used for cryo-EM studies, the solubilized fraction of the same receptor construct, but with an additional N-terminal green fluorescent protein (GFP) fusion attached for visualization, and a C-terminal Rho1D4 tag, was reconstituted into nanodiscs and evaluated by radioligand binding studies using the agonist radioligand [³⁵S]ATPγS (Figure 58a), an ATP analog with increased stability.^{75,144} A concentration-dependent inhibition of [³⁵S]ATPγS binding by the unlabeled agonist ATPγS was observed, indicating high affinity of the ligand to the P2X4 receptor reconstituted into nanodiscs, with a K_D value of 6.01 ± 0.81 nM (Figure 58a). A maximum binding capacity (B_{max} value) of 1056 ± 110 pmol/mg of protein was calculated, indicating a very high level of conformationally intact P2X4 receptors. For comparison, we performed homologous competition binding experiments using 1321N1 astrocytoma cell membranes stably expressing the human wt P2X4 receptor (Figure 58b). A K_D value of 14.1 ± 2.7 nM was determined, which is in the same range as the value determined for the purified, reconstituted P2X4-E307T receptor construct. Our results show that a high receptor density can be achieved by P2X4 receptor reconstitution into nanodiscs while retaining its bioactive conformation.

For the determination of a cryo-EM structure, we utilized amphipol A8-35 instead of nanodiscs. Both approaches can be expected to provide a similar environment, and A8-35 had been successfully employed for structure determination of ion channels.^{278,285} In

3. Results and discussion

contrast to nanodiscs, reconstitution using A8-35 does not require membrane scaffold proteins, which simplifies the procedure.^{278,305}

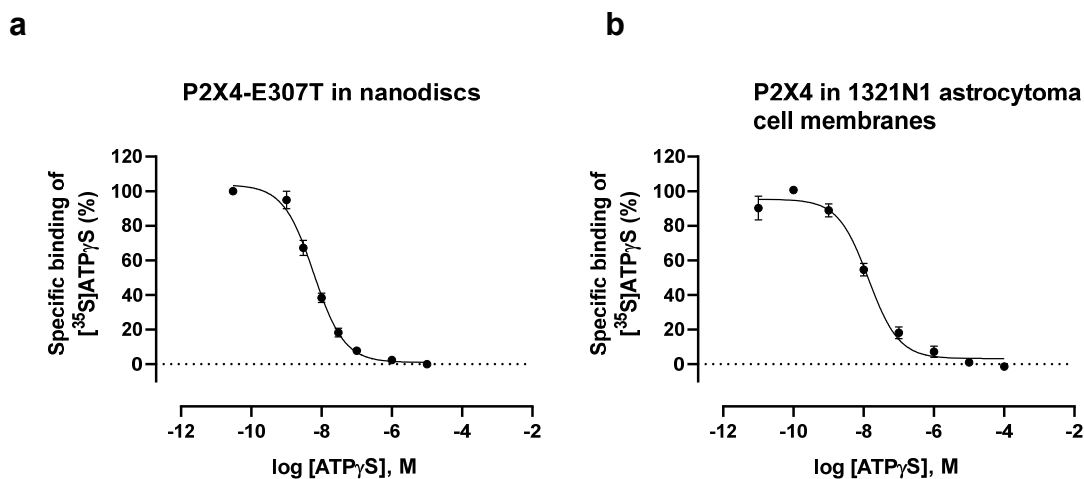


Figure 58. Radioligand binding studies using the agonist radioligand $[^{35}\text{S}]\text{ATP}\gamma\text{S}$ (1,250 Ci/mmol; 4.63×10^{13} Bq/mmol). a) Homologous competition binding experiments of a range of $\text{ATP}\gamma\text{S}$ concentrations versus 0.2 nM of $[^{35}\text{S}]\text{ATP}\gamma\text{S}$ using the human solubilized and purified P2X4-E307T receptor mutant reconstituted into nanodiscs. Radioligand binding was determined in the presence of various concentrations of unlabeled $\text{ATP}\gamma\text{S}$ in different fractions collected during gel filtration (1:25 dilutions) of the P2X4 receptor reconstituted into nanodiscs. b) Homologous competition of $\text{ATP}\gamma\text{S}$ vs. $[^{35}\text{S}]\text{ATP}\gamma\text{S}$ (0.2 nM) at the human wt P2X4 receptor recombinantly expressed in 1321N1 astrocytoma cells (15 μg of protein). Data represent means $\pm$ SEM of at least three biological replicates.

Anthraquinone derivative PSB-0704 selected for structure determination

For structural studies, the potent and water-soluble anthraquinone derivative PSB-0704 was selected (Table 24, Figure 59a). This compound is more drug-like than Cibacron Blue due to its smaller molecular weight and the exchange of sulfonate groups for carboxylate functions. PSB-0704 (Figure 59a) showed a concentration-dependent inhibition of the human wt P2X4 receptor stably expressed in 1321N1 astrocytoma cells, with an IC_{50} value of 12.5 μ M, similar to that of Cibacron Blue (Figure 54b, Figure 59b). At the P2X4-E307T receptor mutant, the potency of PSB-0704 was also strongly increased (>170 -fold, IC_{50} : 0.0724 μ M, Figure 59b).

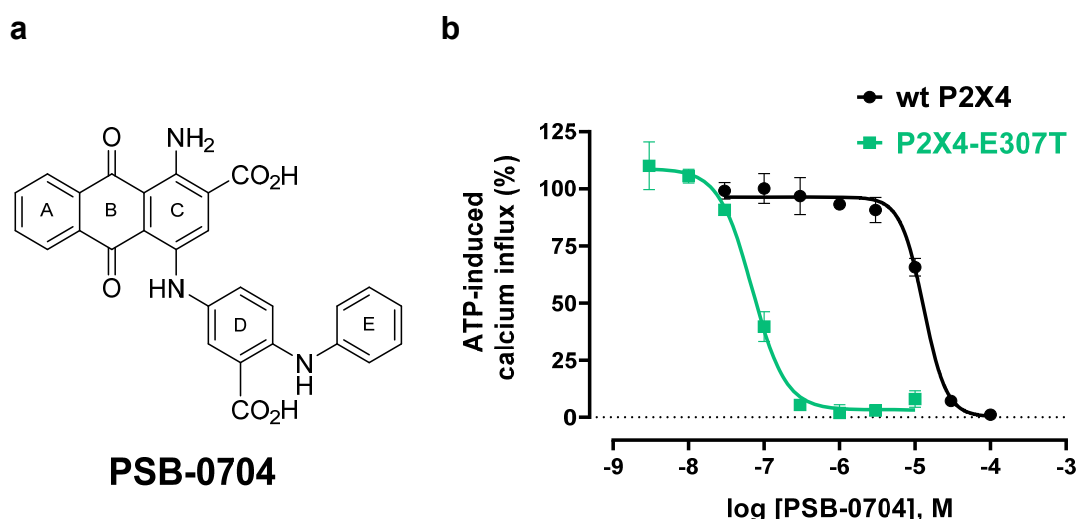


Figure 59. Characterization of the anthraquinone derivative PSB-0704 used for cryo-EM. a) Chemical structure of PSB-0704 with designation of aromatic rings (A–E). b) Concentration-dependent inhibition of ATP-induced calcium influx by PSB-0704 at the human wt P2X4 receptor (black) and the human P2X4-E307T receptor mutant (green), recombinantly expressed in 1321N1 astrocytoma cells, determined versus the EC_{80} of ATP. Data represent means $\pm$ SEM of at least three biological replicates, performed in technical duplicates.

Cryo-EM reconstruction of the human P2X4 receptor

A cryo-EM dataset (Supporting information, Table 28) of the P2X4(E307T)-PSB-0704-complex reconstituted into amphipols, was collected and processed as shown in Supporting Figure 67. Initial processing clearly revealed the characteristic C3 symmetric shape of a P2X receptor. However, the reconstructed map was difficult to interpret due to preferred orientations of the particles with a strong overrepresentation of views along the threefold symmetry axis (Supporting information, Figure 67).

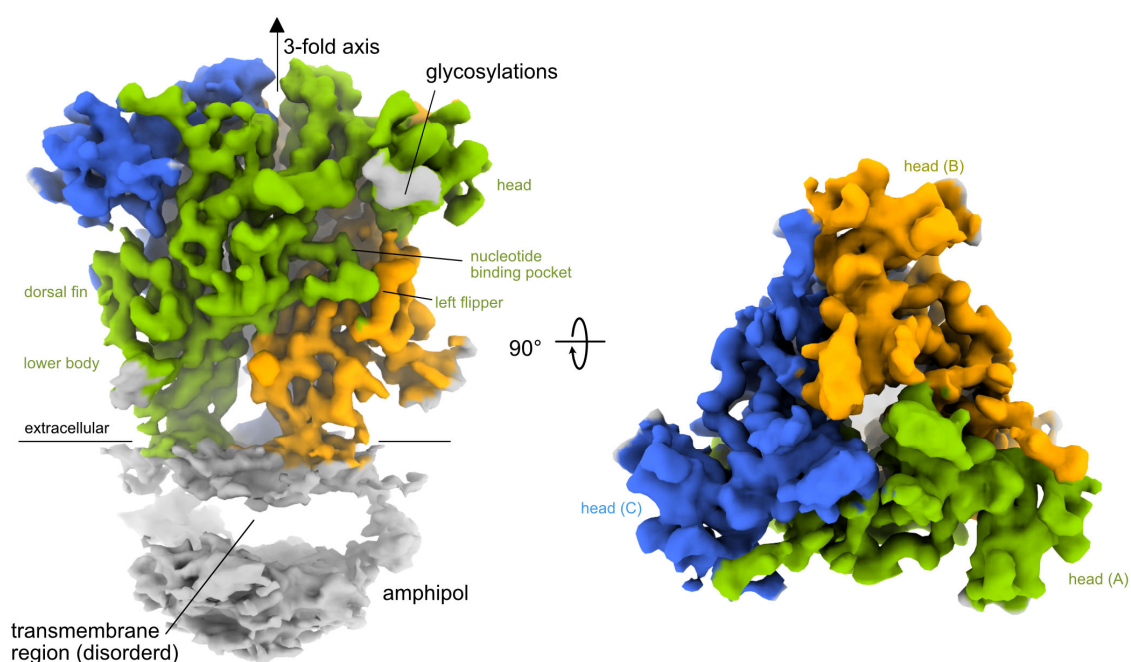


Figure 60. Structure of the human P2X4 receptor. Cryo-EM reconstruction of the human P2X4 receptor. The three protein chains of the receptor are colored in green, yellow, and blue. The C3 symmetry axis is indicated. The transmembrane region is colored gray. Side view, perpendicular to the cell membrane (left); top view from the extracellular side (right).

We reasoned that rare side views might have been sorted out during the 2D classification procedure, and therefore decided to skip the 2D classification step. Instead, all 3.6 million extracted particles were fed into three iterative heterogeneous refinement steps using the 3D reconstruction of the P2X4 receptor and five pseudo-random reconstructions. In each step, the particles that were assorted to any of the pseudo-random reconstructions were discarded (Supporting information, Figure 67b). This procedure resulted in 183,000 particles that were subjected to further 3D classifications without particle alignment and

local refinements. Finally, we succeeded in reconstructing the extracellular domain of the receptor at an overall resolution of 3.35 Å and with a more balanced distribution of viewing directions than obtained initially (Figure 60; Supporting information, Figure 67 and Figure 68).

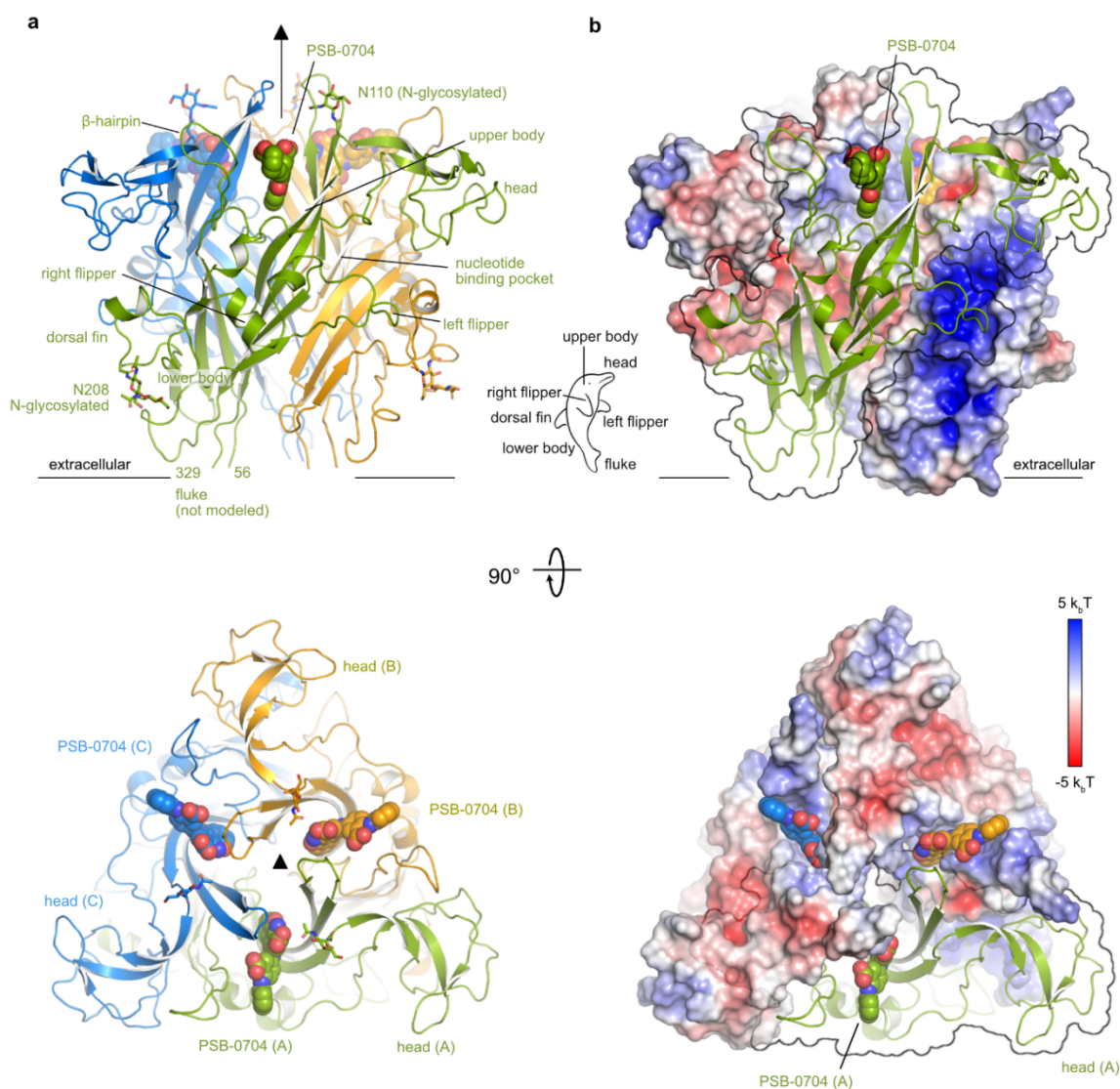


Figure 61. Cryo-EM structure of the extracellular P2X4 receptor domain showing the allosteric binding sites. a) Cartoon model of the extracellular domain of the human P2X4 receptor. The antagonist PSB-0704 is shown as spheres. The C3 symmetry axis is indicated. N-glycosylations are shown as sticks. b) Charge complementarity (calculated with Adaptive Poisson-Boltzmann Solver (APBS³⁰⁶)) of monomer interfaces in the extracellular domain stabilize the trimeric receptor. The surface of one monomer is shown as a transparent outline to visualize the surface potential in the interface areas.

An AF3³⁰³ model of the human P2X4 receptor was placed into the 3D volume, and ISOLDE³⁰⁷ was used to fit the AF3 model to the experimental map. The transmembrane helices were disordered and could not be modeled. The final structure comprises residues 56–329 of the human P2X4 receptor (Figure 61a). The overall architecture of our model reflects the typical trimeric arrangement of P2X receptors with a large extracellular domain and two (in our case disordered) transmembrane helices in each subunit. The interaction between the monomers is characterized by very large interfaces with a combined buried surface area of $\sim 3,500 \text{ \AA}^2$ between each pair of monomers and distinct charge complementarity (Figure 61b). Each monomer resembles the shape of a dolphin, consistent with previously reported P2X receptor structures²⁵ (Figure 61).

Our reconstruction revealed the known N-glycosylation sites at positions Asn110 (upper body) and Asn208 (dorsal fin). The reconstruction was of sufficient quality to add one, respectively two N-acetylglucosamine units at these positions (Figure 61). Additional density was also present at Asn153 in the head domain but it was not well enough defined to build a model of the glycosylated asparagine side chain. Further N-glycosylations are known to occur at residues Asn75 and Asn184 but, as mentioned above, these residues were mutated to arginine in our expression construct.²⁴

We used the Foldseek Search Server³⁰⁸ to compare our structural model to known structures of P2X receptors. Figures 69a and 69b (Supporting information) depict a superposition of our model with the zebrafish P2X4 receptor ortholog in both its closed and open states (PDB ID: 4dw0, 4dw1). Our model most closely resembles the closed-state apo form of the receptor, especially the central parts of the two structures at the transition between the extracellular and the membrane domain (i.e. the lower body of the dolphin) align well with a root mean square deviation (r.m.s.d.) of 1.7 Å between 447 atoms of the lower body domains. The same region is less similar to the ligand-bound open-state of the receptor (r.m.s.d. of 3.1 Å between 447 atoms of the lower body domains). We therefore conclude that our structure represents the closed state of the receptor without ATP bound (Supporting information, Figure 69c), which also had not been added during cryo-EM sample preparation. Furthermore, we noticed a concerted downward movement (i.e. towards the membrane plane) of the head domain in our structure, compared to the zebrafish structures (Supporting information, Figures 69a, 69b).

While building the structural model, we identified a large blob of extra density at the trimer subunit interface, at the back of the head domain. The antagonist PSB-0704, which had been added during protein purification and again immediately before the cryo-EM grid preparation, could be placed into this map feature with confidence (Figure 62). Presumably due to the presence of the ligand, the β -hairpin loop containing R82 is partly disordered and bent backwards compared to the AF3 model of the human P2X4 receptor without a bound ligand (Figure 61a; Supporting information, Figure 69f).

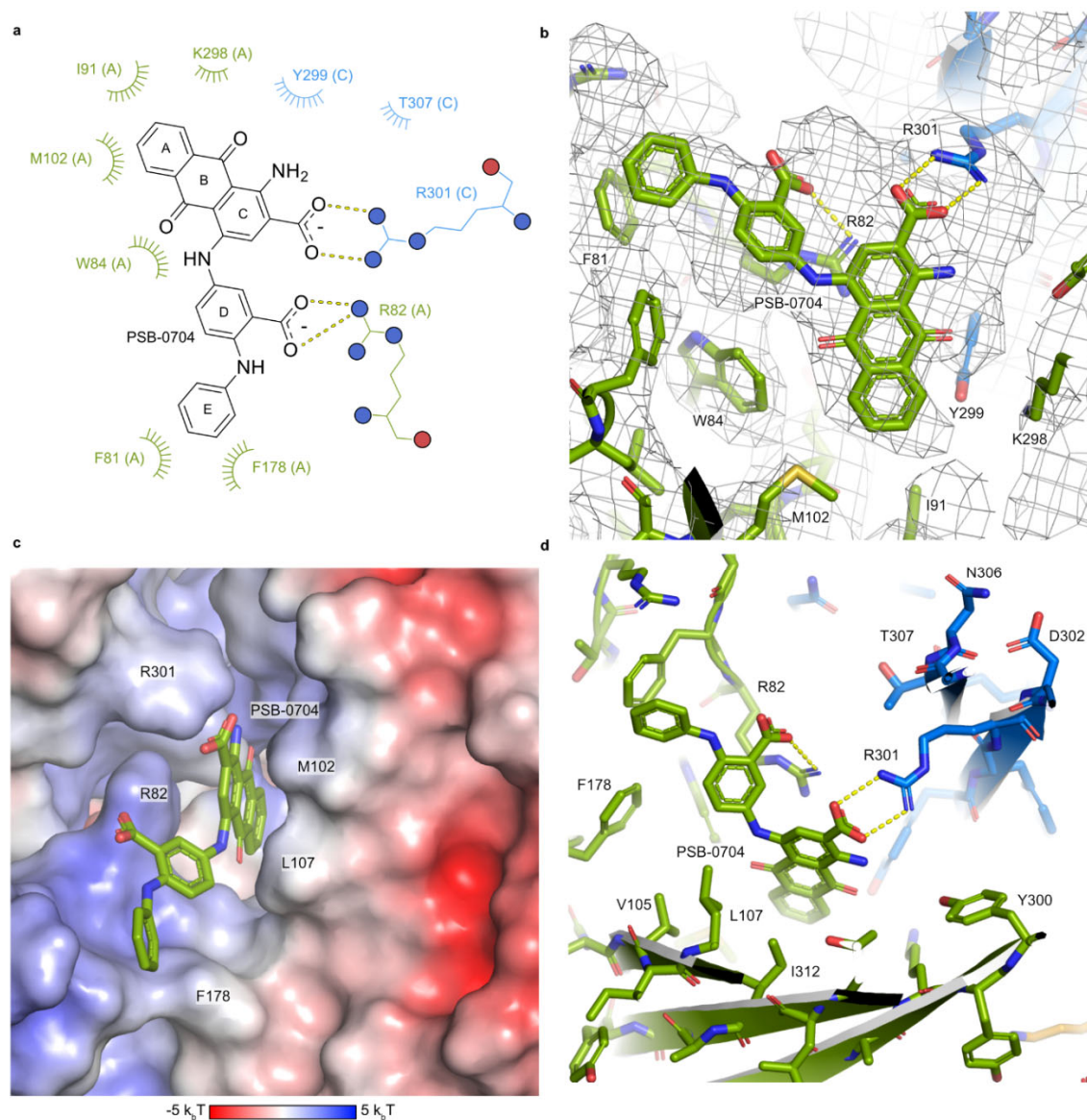


Figure 62. Molecular interactions of the antagonist PSB-0704 binding to its allosteric site at the human P2X4 receptor. a) Schematic representation illustrating the interaction between PSB-0704 and the human P2X4 receptor. Polar interactions are shown as dashed lines and hydrophobic and van-der-Waals interactions as arcs. The letters in parenthesis indicate the polypeptide chain of amino acid residues. b) Close-up of the PSB-0704 binding site with the reconstructed volume shown as a black mesh. The color scheme is the same as in Figure 61. c) Surface electrostatic potential (calculated with APBS³⁰⁶) of the ligand binding site. d) Close-up of the binding site.

The antagonist PSB-0704 engages in many hydrophobic interactions of the aromatic rings A-E with the receptor and by ionic interactions between the two carboxylate groups in rings C and D with side chains Arg301 and Arg82, respectively (Figure 62). Arg301 forms a bidentate salt bridge with the carboxylate of ring C, whereas Arg82 interacts with the carboxylate of ring D and additionally forms a cation- π stacking interaction with rings B and C (Figure 62). The amino and carbonyl oxygen substituents of ring B and C are not in close contact to any polar groups of the receptor. Note that due to the location of the binding site at the interface between two monomers, the two arginine side chains come from two adjacent chains of the trimeric receptor (Figure 62).

Mutagenesis studies had shown a significantly increased potency of Cibacron Blue and related anthraquinone derivatives, including PSB-0704, at the P2X4-E307T receptor mutant (Figures 57a, 57b, Table 24, Figure 59b). An AF3 model of the receptor together with our structure can rationalize this observation. Figure 69e (Supporting information) shows a model PSB-0704 binding to the human wt P2X4 receptor, based on the human P2X4-E307T receptor structure from this work, in which the threonine at position 307 was replaced by a glutamate residue using PyMOL, and Figure 69f (Supporting information) shows a superposition of the AF3 model of the human wt P2X4 receptor with our cryo-EM structure. In the model of the wt P2X4 receptor, Glu307 of one subunit forms salt bridge interactions with both Arg82 and Lys298 of the adjacent subunit, flanked by a hydrogen bond between Arg301 of the first subunit with Asn110 of the adjacent subunit (Figure 55). The ionic intermolecular interactions mediated by Glu307 block a significant part of the compound binding site.

3.7.5 Discussion

The P2X₄ receptor has emerged as a potential drug target, and its blockade was proposed to be beneficial for treating chronic pain, inflammatory diseases, and cancer.^{42,44,47,150,171} Since the polar nature of the ATP-binding site and its similarity to other P2X receptor subtypes has impeded the discovery of orthosteric P2X₄ receptor antagonists, drug research has been focusing on the development of negative allosteric modulators. However, up to now, only very few P2X₄ receptor antagonists have been described, most of which are only moderately potent¹⁰. Structural biology of membrane proteins has recently made huge progress; in particular, cryo-EM has become the method of choice to determine structures of P2X receptors due to the large size (>100 kDa) of the trimeric ATP-gated cation channels. To date, 47 high-resolution structures of five P2X receptor subtypes in complex with 24 different ligands have been published (see Supporting information, Table 25 for a complete list). Besides the characterization of the orthosteric ATP-binding site, this has led to the discovery of several allosteric sites for antagonists at the various P2X receptor subtypes. In Figure 63, the locations of the discovered binding sites are depicted, including that of the anthraquinone derivative PSB-0704 determined in the present study.

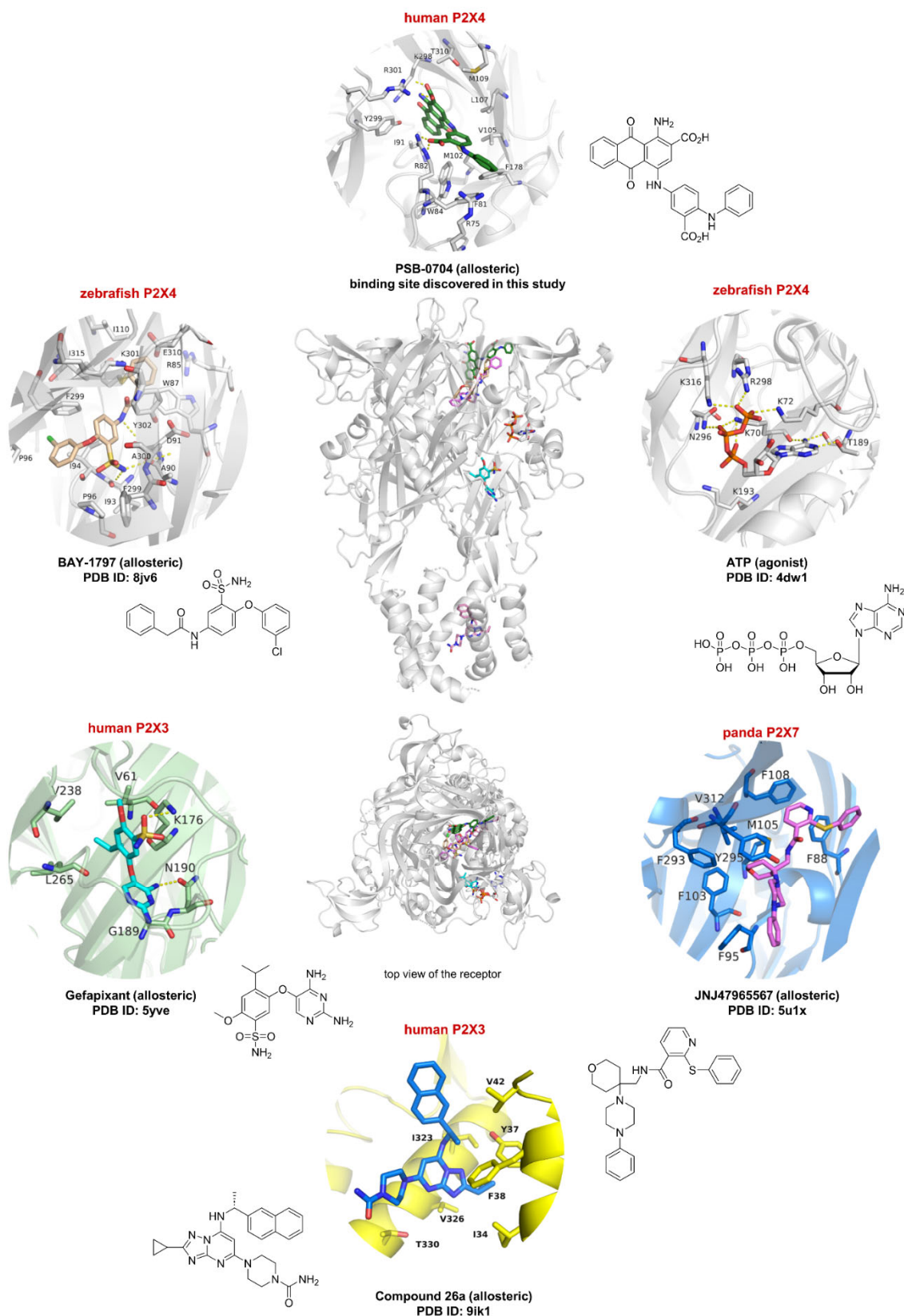


Figure 63. Allosteric binding sites of P2X receptors. Allosteric binding site of the anthraquinone derivative PSB-0704 at the human P2X4 receptor discovered in this study, compared to the orthosteric ATP-binding site and to the allosteric sites previously determined in various P2X receptor subtypes. P2X receptor side and top view showing the

allosteric binding sites. In addition, close-up views of each binding site with chemical structure of the allosteric antagonist (or ATP) and the PDB ID are depicted.

The anthraquinone derivative Cibacron Blue was first described as a moderately potent antagonist of human and rat P2X₄ receptors in patch clamp studies on receptors expressed in *Xenopus laevis* oocytes.¹⁰⁵ Later on, positive allosteric modulation was observed at lower concentrations while inhibition was found to require higher Cibacron Blue concentrations, determined at the rat P2X₄ receptor.¹⁴⁵ At rat P2X₂ receptors, the compound acted as an antagonist, while at human P2X₃ receptors it behaved as a positive allosteric modulator.^{145,146} No direct effects were observed on P2X₁ and P2X₇ receptors.¹⁴⁶ Initially, we further characterized Cibacron Blue and performed SAR studies (see Table 24). Our interest in identifying the allosteric binding site of anthraquinone derivatives was also motivated by their physicochemical properties: in contrast to other P2X₄ receptor antagonists they are well water-soluble. Investigation of a series of truncated anthraquinone derivatives related to Cibacron Blue, bearing modifications in various positions, led to the discovery that ring F was dispensable for P2X₄ receptor blockade, as was the amino group in the 1-position (see Table 24). Compounds with only one sulfonate group were less potent than those with two sulfonates (Table 24). However, the sulfonate functions could be exchanged for carboxylate (in PSB-0704), which makes the molecules more drug-like. In fact, the determined cryo-EM structure rationalizes the SAR data showing, for example, that the amino group at ring C is not important for the interaction with the P2X₄ receptor (Table 24). In our structure, the amino group is ~4 Å away from the hydroxyl group of Tyr300, which is too far to form a direct hydrogen bond (Figure 62). Aromatic amines (anilines) are regarded as potential toxicophores,³⁰⁹ and therefore these insights can be used to improve the compounds not only with regard to potency, but potentially also with regard to toxicity. The oxygen atoms of the quinone ring B have not shown any polar interactions with the receptor and thus are likely also not required. This opens up the possibility to design antagonists without the redox-reactive quinone structure by replacing it with a more stable moiety.

A combination of different, complementary approaches was applied to identify the P2X₄ receptor's binding site for anthraquinones. Chimeric receptors were studied, initially aimed at investigating and understanding the different behavior of Cibacron Blue at the P2X₄ versus the P2X₂ receptor. To this end, variable regions in both human receptor subtypes,

identified by sequence comparison, were exchanged in the human P2X4 for the corresponding amino acid residues of the human P2X2 receptor.¹³⁴ One of the chimeric P2X4(P2X2) receptor chimeras showed a strikingly different behavior in comparison to the wt receptors. Chimera c10, in which variable region v10 consisting of amino acid residues 301–308 (RDLAGNEQ) of the P2X4 receptor was exchanged by the corresponding sequence of the P2X2 receptor (K-INGTTT), exhibited a markedly increased inhibitory potency of Cibacron Blue with an IC₅₀ value of 4.76 nM determined in calcium influx assays, a 2,500-fold increase as compared to the wt P2X4 receptor (see Figures 54e, 54f). Computational studies led to the hypothesis that the glutamate Glu307 forms an ionic lock with a nearby basic amino acid residue (likely Arg82 and Lys298, but Arg301 can also not be completely excluded) thereby preventing high-affinity binding of the anthraquinone derivatives by occluding part of its binding site (Table 24). A point mutation in the P2X4 receptor, in which Glu307 was exchanged for threonine (present in P2X2) confirmed the hypothesis, Cibacron Blue still being 680-fold more potent at the P2X4(E307T) receptor mutant as compared to the wt receptor. To gain conclusive insights into the binding pocket, determination of a high-resolution structure is indispensable.

Several high-resolution X-ray and cryo-EM structures of the zebrafish P2X4 receptor have been determined.^{24,25,27} However, the zebrafish P2X4 receptor only shares 58% sequence identity with the human P2X4 receptor and exhibits a markedly different pharmacology. For instance, the potency of ATP at the zebrafish P2X4 receptor is about 700-fold lower than at the human P2X4 receptor.^{24,209} Modifications were made to improve the crystallization behavior of the zebrafish P2X4 receptor, including truncation of its amino and carboxyl termini and insertion of two single point mutations at the glycosylation sites (N78K/N187R).²⁴ The receptor construct was shown to retain functionality in electrophysiological experiments, indicating that ATP is still able to activate the receptor (ATP concentration of 1 mM), although the peak current amplitudes were observed to be smaller than those recorded from the full-length receptor.²⁴

In contrast to these drastic modifications, our structure was obtained of the full-length human P2X4 receptor construct, harboring only three single point mutations, E307T, N75R, and N184R. The latter two are mutated glycosylation sites, the same as those described for the zebrafish P2X4 receptor structure.²⁴

The obtained structure exhibited a sufficiently high overall resolution of 3.35 Å to identify the anthraquinone's binding site and interactions. The receptor showed a trimeric structure, as expected, with three well-resolved allosteric binding sites for PSB-0704, located at the trimer subunit interface, at the back of the head domain. Even the glycosylation sites are well visible, at Asn110 in the upper body, Asn208 in the dorsal fin, and Asn153 in the head domain. There are two more predicted glycosylation sites, Asn75 and Asn184 which had, however, been mutated. The reason for this was the fact that we (unsuccessfully) tried to obtain an X-ray structure as well, where heterogeneity due to inhomogeneous glycosylation patterns is interfering. The PSB-0704-bound receptor was in an inactive conformation as clearly seen by comparison with active and inactive zebrafish X-ray structures (see Supporting information, Figure 69). The antagonist induces a downward movement of the head domain in comparison to the zebrafish P2X4 structure. The β -hairpin loop containing Arg82 is bent backwards compared to the AF3 model of the human P2X4 receptor without ligand. The anthraquinone ring system of PSB-0704 forms hydrophobic and cation- π stacking interactions with the receptor. The studied mutations support the determined structure: (1) Thr307 is located near two basic amino acids, Arg82 and Arg301, which form ionic interactions with the two carboxylate groups of the antagonist. In the wt receptor, there is a glutamate (Glu307) instead of threonine, which likely forms an ionic bridge with Arg82 and additionally with Lys298 according to the computational model (see Figure 55). (2) The Q308T mutation had no effect on the potency of the anthraquinones, and indeed, the structure showed no interaction. (3) The moderate effect of the D302I mutation (9-fold higher potency of Cibacron Blue) can be explained by an interaction of Asp302 with Asn306, thereby potentially exerting conformational effects on the loop containing Arg301.

The moderately potent compounds BX430 and BAY-1797 are among the best investigated P2X₄ receptor antagonists. Both have recently been shown to share the same allosteric binding site as determined by cryo-EM in complex with the zebrafish P2X₄ receptor (see Supporting information, Figure 69d).²⁷ The allosteric site discovered for the anthraquinone PSB-0704 in the present study is in the same region, but distinct from the BX430 and BAY-1797 binding site, with which it only slightly overlaps. It is also distinct from any other allosteric binding site identified in P2X receptor subtypes so far, although the P2X₇ receptor also harbors an allosteric binding site nearby (see Figure 63 for an overview of orthosteric and allosteric P2X receptor binding sites). Compared to these two P2X₄ receptor antagonists BX430 and BAY-1797, the antagonist PSB-0704 is located further away from the membrane surface (Supporting information, Figure 69d). The fact that these three compounds have adjacent binding sites might open interesting opportunities to combine features of the different compound classes to design more potent antagonists.

Our results do not explain the biphasic behavior of Cibacron Blue at the human P2X₄ receptor. It has been observed previously that some allosteric modulators can show receptor activation or inhibition depending on concentration.¹³⁴ One explanation could be that the binding of only one (or two) molecules of Cibacron Blue in the receptor trimer leads to a conformation that enhances ATP binding and channel opening, while binding of three molecules of the modulator will induce a more dramatic conformational change drawing the subunits closer together, thereby inducing an inactive, closed receptor conformation. Another explanation could be the presence of different binding sites to which the modulator has different affinity, as postulated for some pentameric ligand-gated ion channels and their allosteric ligands.³¹⁰ These hypotheses are supported by the results obtained in radioligand binding studies using the stable ATP analog [³⁵S]ATP γ S, in which Cibacron Blue showed a biphasic competition curve.¹⁴⁴ Interestingly, this behavior is only observed for the large molecule Cibacron Blue, but not for the truncated analogs, including PSB-0704, all of which only exhibit antagonistic activity.

Cibacron Blue and closely related analogs are often promiscuous compounds interacting with a large number of proteins with moderate affinity.³⁰¹ The high-affinity interaction of the mutated P2X₄-E307T receptor with Cibacron Blue (IC₅₀: 17.8 nM) offers unique opportunities: this protein-ligand pair could be used for specifically elucidating P2X₄ receptor functions. Such an approach of chemical hijacking using an engineered protein-

ligand pair has been termed “bump-and-hole” tactic and has found broad application in basic research, in particular in chemical biology.^{311,312}

3.7.6 Conclusions

The determined structure, although not obtained for the wt P2X4 receptor but for the E307T mutant, is useful for drug design. The hypothesis of ionic lock formation has been confirmed. This offers the possibility to design compounds that disrupt the intermolecular ionic lock by interacting with Glu307, to open up a high-affinity binding site for antagonists. Introduction of a basic moiety into the structure of PSB-0704 could be a plausible strategy.

In conclusion, we discovered an allosteric binding site for anthraquinone derivatives in the full-length human P2X4 receptor, along with important interactions. The binding site is adjacent to, but not identical with a recently described allosteric P2X4 receptor binding site.²⁷ This could have relevance beyond the P2X4 receptor subtype since some anthraquinones, e.g., Cibacron Blue, have been found to also interact with several other P2X receptor subtypes.^{145,146} The presented data, along with recent literature data, allowed us to obtain and validate a model of the wt P2X4 receptor using AF3, and model the anthraquinone derivative PSB-0704 into the allosteric pocket (Supporting information, Figure 69e). Our results provide a basis that will enable and promote future drug development.

3.7.7 Materials and methods

Chimeric P2X4(P2X2) receptors

Chimeric P2X4(P2X2) receptor constructs were obtained through gene synthesis (Geneart, Life Technologies). All cDNAs were defined with attached EcoRI and BamHI restriction sites, and subcloned into the bicistronic lentiviral pLVX-IRES-mCherry plasmid (Clontech, Heidelberg, Germany), as previously described.¹³⁴ Verification of the constructed chimeric receptors was carried out by sequencing (GATC-Biotech, Ebersberg, Germany).

Receptor mutants

Receptor mutants were generated by site-directed mutagenesis with mutagenesis primers, synthesized by Invitrogen.

Transfection of 1321N1 astrocytoma cells

Two different expression systems were used for generating stable 1321N1 astrocytoma cell lines: lentiviral and retroviral expression.¹³⁴

For lentiviral expression, the genes of interest were subcloned into the pLVX-IRES-mCherry vector to coexpress the gene of interest and the mCherry gene. In brief, HEK293 T packaging cells were transfected using Xfect Reaction buffer, Lenti-X HTX Packaging-Mix, and Xfect-Polymer for virus production. Additives for lentiviral transfection were purchased from Clontech (Heidelberg, Germany). Afterwards, non-transfected 1321N1 astrocytoma cells (Sigma-Aldrich, Taufkirchen, Germany) were infected with the produced viruses. After 48–72 h, the cells were selected for fluorescence emission at 610 nm with an excitation of 561 nm. As a negative control, non-transfected 1321N1 astrocytoma cells were measured. Cells with high expression of mCherry protein were single-sorted to 96-well plates using a BD FACS Aria III.

The second method used was retroviral transfection. In this case, GP+envAM-12 fibroblast-type mouse packaging cells (American Type Culture Collection (ATCC), Manassas, VA, USA) were used to produce a recombinant helper virus. The gene of interest was subcloned into the pQCXIN vector. Briefly, GP+envAM-12 packaging cells were transfected with the plasmid containing the DNA of interest and the VSV-G protein DNA (in pcDNA3.1) to produce recombinant helper viruses. The non-transfected 1321N1 astrocytoma cells were then infected with the produced virus particles. The selection was initiated using geneticin G418 (800 µg/mL) 48–72 h after infection.

Cultivation of cells

1321N1 Astrocytoma cells stably transfected with the human wt P2X4 receptor, the human wt P2X2 receptor, the chimeric P2X4(P2X2) receptors, and the receptor mutants as well as HEK293 T and GP+envAM-12 packaging cells were cultured in DMEM (Thermo Fisher Scientific, Dreieich, Germany) supplemented with 10% FCS, penicillin (100 U/mL), and streptomycin (100 µg/mL). The medium for retrovirally-transfected cell lines contained G418 (800 µg/mL). FCS and antibiotics were purchased from PAN Biotech (Aidenbach, Germany). Stably transfected 1321N1 astrocytoma cells were incubated at 37 °C with 10% CO₂. HEK293 T and GP+envAM-12 packaging cells at 37 °C with 5% CO₂.

Chemicals

Cibacron Blue 3GA was purchased from Sigma-Aldrich (Taufkirchen, Germany) and used without further purification. The other anthraquinone derivatives, PSB-0704, PSB-0739, PSB-0801, PSB-09059, and PSB-24039 were synthesized as previously described.^{313–316} In brief, compounds PSB-0704, PSB-0739, PSB-0801, and PSB-09059 were prepared via modified Ullmann coupling reaction under microwave irradiation starting from bromaminic acid and the respective aniline derivatives.^{313–315} Compound PSB-24039 was obtained by de-amination reaction³¹⁶ of compound PSB-0739.

Calcium influx assays

To quantify intracellular Ca^{2+} levels in transfected 1321N1 astrocytoma cells, fluorimetric calcium influx assays were performed according to previously established protocols.^{75,134}

Briefly, transfected cells were plated into 96-well plates and preincubated with the fluorescent Ca^{2+} -chelating dye Fluo-4 AM (Thermo Fisher Scientific, Dreieich, Germany), dissolved in HBSS (Thermo Fisher Scientific, Dreieich, Germany) supplemented with 1% Pluronic® F127 (Sigma-Aldrich, Taufkirchen, Germany), for 1 h at room temperature. For experiments involving antagonist testing, series dilutions of antagonists (prepared either in DMSO or in HBSS buffer) were added to the cells, and incubation for 30 min was performed before the agonist ATP was added at its EC_{80} . Fluorescence imaging plate readers, Flex station (Molecular Devices, Munich, Germany), or NOVOstar (BMG LABTECH, Ortenberg, Germany), were used to measure fluorescence at an excitation wavelength of 485 nm and an emission wavelength of 520 nm.

Design of the receptor construct

The full-length human P2X4 receptor gene was subcloned into the pFastBac1 vector (Thermo Fisher Scientific, Dreieich, Germany) with a polyhedrin promoter. To the C-terminus, a polyhistidine tag (10x-His tag) was attached, which included a preceding human rhinovirus (HRV) 3C protease cleavage site. A flexible Gly-Ser-Ser-Gly (GSSG) linker was incorporated prior to the protease cleavage site. Two of the receptors' glycosylation sites were mutated (N75R and N184R) and at position 307 glutamate was mutated to threonine (E307T). No truncations were made for structure determination.

Receptor expression and purification

The modified human P2X4 receptor protein was expressed in *Sf9* insect cells (Expression Systems, Davis, CA, USA) using the Bac-to-Bac Baculovirus Expression System (Thermo Fisher Scientific, Dreieich, Germany). Freshly grown *Sf9* insect cells were infected with recombinant baculoviruses (P1) at a density of $2\text{--}3 \times 10^6$ cells ml^{-1} for 48 h at 27 °C and 150 rpm. Cell pellets were harvested by centrifugation (4,000 g) and stored at -80 °C.

All subsequent steps were carried out on ice or at 4 °C. Cell membrane preparations were obtained by repeated homogenization using a Dounce tissue homogenizer (VWR International, Darmstadt, Germany) in a hypotonic buffer (10 mM HEPES (pH 7.5), 10 mM MgCl_2 , 20 mM KCl) supplemented with EDTA-free complete protease inhibitor cocktail tablets (Roche, Mannheim, Germany), followed by centrifugation for 30–60 min at 57,000 g (twice). Then, membranes were washed in the same way using high osmotic buffer (10 mM HEPES (pH 7.5), 10 mM MgCl_2 , 20 mM KCl, 1 M NaCl) (twice). Purified membranes were resuspended in 10 mM HEPES (pH 7.5), 10 mM MgCl_2 , 20 mM KCl, 30% (v/v) glycerol, flash-frozen in liquid nitrogen, and stored at -80 °C.

Prior to receptor solubilization, cell membranes were thawed on ice and incubated in the presence of 37 μM of PSB-0704 dissolved in DMSO at 4 °C for 30 min. P2X4 receptors were then solubilized with 50 mM HEPES (pH 7.5), 100 mM NaCl, 1.5% (w/v) DDM (Anatrace Products, OH, USA), 0.3% (w/v) CHS (Sigma-Aldrich, Taufkirchen, Germany) for 3 h at 4 °C.

The solubilized fraction (supernatant) was collected after centrifugation for 30–60 min at 48,000 g. Then, 20 mM of imidazole (pH 7.5) and 0.01 mL of TALON IMAC resin beads (Takara Bio, Saint-Germain-en-Laye, France) per mL of supernatant were added and incubated for at least 3 h at 4 °C. Prior to use, TALON beads were washed with Millipore water (8 times) and stored in buffer (50 mM HEPES (pH 7.5), 0.5 M NaCl, 10% (v/v) glycerol) at 4 °C.

The resin was washed with 15–20 CV of wash buffer I containing 50 mM HEPES (pH 7.5), 100 mM NaCl, 0.1% (w/v) DDM, 0.02% (w/v) CHS, 10% (v/v) glycerol, 25 mM imidazole, 20 mM MgCl_2 , and 13 μM PSB-0704, followed by washing with wash buffer II (10–15 CV) containing 50 mM HEPES (pH 7.5), 100 mM NaCl, 0.05% (w/v) DDM, 0.01% (w/v) CHS, 10% (v/v) glycerol, 40 mM imidazole, and 15 μM PSB-0704. Finally, the protein

was eluted from the column with five CV of elution buffer (25 mM HEPES (pH 7.5), 100 mM NaCl, 0.025% (w/v) DDM, 0.005% (w/v) CHS, 10% (v/v) glycerol, 220 mM imidazole, and 15 μ M PSB-0704) and concentrated using a 100-kDa molecular weight cut-off concentrator (Amicon™; Merck, Darmstadt, Germany).

Reconstitution into amphipol

Relevant information on method development and optimization was taken from previously published protocols.²⁹² For reconstitution into amphipol A8-35, the solubilized P2X4 receptor protein was mixed with A8-35 (Anatrace Products, OH, USA) dissolved in Millipore water at a ratio of 1:10 (w:w), and the mixture was incubated for at least 4 h at 4 °C while gently stirring. The appropriate amount of A8-35 was calculated based on the mass of the protein present in the sample. For detergent removal, Bio-Beads SM-2 (Bio-Rad Laboratories, Munich, Germany) were added to the sample (20 x mass of detergent present in the sample). Prior to use, Bio-Beads were washed with 20% (v/v) ethanol and Millipore water. Detergent removal was performed overnight at 4 °C while gently stirring. After removing Bio-Beads using small chromatography columns (Bio-Rad Laboratories, Munich, Germany), the reconstituted protein was concentrated using a 100-kDa molecular weight cut-off concentrator (Amicon™; Merck, Darmstadt, Germany). For final protein purification, SEC was performed on a Superose 6 Increase 3.2/300 column (Cytiva, Freiburg im Breisgau, Germany) pre-equilibrated with SEC buffer (20 mM HEPES (pH 7.5) and 100 mM NaCl). The respective peak fractions of the trimeric human P2X4 protein reconstituted in A8-35 were collected, and concentrated using a 100-kDa molecular weight cut-off concentrator (Vivaspin; GE HealthCare, Düsseldorf, Germany). Finally, PSB-0704 dissolved in DMSO was added at a concentration of 50 μ M before grid preparation for cryo-EM studies.

Protein expression, purification, and reconstitution into nanodiscs for radioligand binding studies

The human P2X4 receptor construct used for radioligand binding studies was the same as the one used for cryo-EM studies, except for an additional N-terminal GFP tag and a C-terminal Rho1D4 tag fused by a GSSG linker, respectively.

The trimeric P2X4 receptor protein, purified in 0.05% DDM and 0.005% CHS, was incubated with membrane scaffold protein 1E3D1 (MSP1E3D1) nanodiscs (Cube Biotech,

Cologne, Germany), Bio-Beads (Bio-Rad Laboratories, Munich, Germany), cholate, and 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine (POPC) lipids (Avanti Polar Lipids, Alabaster, AL, USA) in a 1:50 (P2X4:nanodisc) ratio. After the assembly reaction, SEC was conducted on a Superose 6 Increase column (Cytiva, Freiburg im Breisgau, Germany) in 100 mM NaCl and 20 mM Tris-HCl buffer (pH 7.4) to remove empty assembled nanodiscs. Gel filtration of the solubilized GFP-tagged P2X4 receptor was performed on a Superdex 200 10/300 GL column (Cytiva, Freiburg im Breisgau, Germany). The buffer contained 10% glycerol, 150 mM NaCl, and 20 mM Na₂PO₄ (pH 7.5).

Radioligand binding studies

Radioligand binding studies were performed as previously described.⁷⁵ The radioligand [³⁵S]ATP γ S (1,250 Ci/mmol; 4.63*10¹³ Bq/mmol) was obtained from PerkinElmer (Rodgau, Germany). Homologous competition binding assays were performed with 0.2 nM [³⁵S]ATP γ S in 50 mM Tris-HCl buffer containing 1 mM EDTA (pH 7.4). Non-specific binding of [³⁵S]ATP γ S was determined in the presence of ATP (100 μ M; AppliChem, Darmstadt, Germany). Purified human P2X4-E307T-GFP receptor reconstituted in nanodiscs (various dilutions), or membrane preparations of 1321N1 astrocytoma cells expressing human P2X4 wildtype receptor were added to the samples. After an incubation of 1 h at room temperature, the incubation was terminated by rapid vacuum filtration through GF/B glass fiber filters using a Brandel 48-well harvester. The filters were rinsed three times with ice-cold Tris-HCl buffer (50 mM, pH 7.4, 3–4 mL each) to separate unbound from bound radioligand. After punching out the filters and transferring them into scintillation vials, scintillation cocktail (2.5 mL, Ultima Gold XR; PerkinElmer, Rodgau, Germany) was added. The samples were measured after 6 h of preincubation using a liquid scintillation counter (Tri-Carb 2900TR; PerkinElmer, Rodgau, Germany) for 1 min each.

Data analysis

All data from calcium influx assays and radioligand binding studies were analyzed using Microsoft Excel. The calculation of EC₅₀, EC₈₀, and IC₅₀ values from calcium influx assays was performed with GraphPad Prism (version 4.0 or 8.0, GraphPad Software, San Diego, CA, USA) by nonlinear regression with a sigmoidal dose-response equation. K_D- and B_{max}-values from radioligand binding assays were calculated by using the “one site-homologous”-equation, implemented in GraphPad Prism version 8.0. The level of significance was calculated by a one-way ANOVA with Dunnett’s post-hoc test, in case

there was a control group, and multiple comparison was required. Significance was expected if P value was ≤ 0.05 . The significance levels were defined as follows: * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, **** $P < 0.0001$.

Electron microscopy

The purified A8-35-reconstituted P2X4 receptor protein was applied onto glow-discharged EM grids (Quantifoil, Au R1.2/1.3) and plunge-frozen in liquid ethane cooled by liquid nitrogen using a Vitrobot Mark IV plunge freezer (Thermo Fisher Scientific, Dreieich, Germany). Grids were transferred to a Glacios electron microscope (Thermo Fisher Scientific, Dreieich, Germany) equipped with a Gatan K2 direct electron detector (Gatan, Pleasanton, CA, USA). For data acquisition of A8-35-reconstituted P2X4 receptor protein in complex with PSB-0704, two datasets were acquired, one with 2,994 images, and another one with 5,710 images, collected with an acceleration voltage of 200 kV, a magnification of 45,000x, a pixel size of 0.901 Å/pixel, and a defocus varied from -1.0 to -2.5 μm . The average dose rate was 46.7 e/Å². Cryo-EM data were acquired at the Institut de génétique, biologie moléculaire et cellulaire in Illkirch-Graffenstaden, France, and was supported by an iNEXT-Discovery grant.

The dataset was processed with CRYOSPARC 4.x³¹⁷ as detailed in Figure 67. Briefly, particles were picked with a blob picker job, followed by 2D classification to create templates for a template picker job, resulting in 3.6 million particles. An initial reconstruction clearly showed the P2X4 receptor but was plagued by severe resolution anisotropy. Junk particles were sorted out by heterogenous refinement jobs offering the initial reconstruction and 5 junk classes. All particles assorted to the junk classes were discarded and the procedure was repeated 2 times. This procedure was followed by 3D classification without alignment, non-uniform refinement³¹⁸ and local refinement jobs leading to a final reconstruction at 3.35 Å resolution with local resolutions in the range 2.5 Å to 12.2 Å.

The map was sharpened using DeepEMhancer.³¹⁹ An AlphaFold 3 model of the human P2X4 receptor including the ligand was placed into the 3D reconstruction and refined with ISOLDE³⁰⁷ and ChimeraX.³²⁰ B-factors were refined with PHENIX³²¹ and the model was validated with PHENIX and MOLPROBITY.³²²

3.7.8 Supporting information

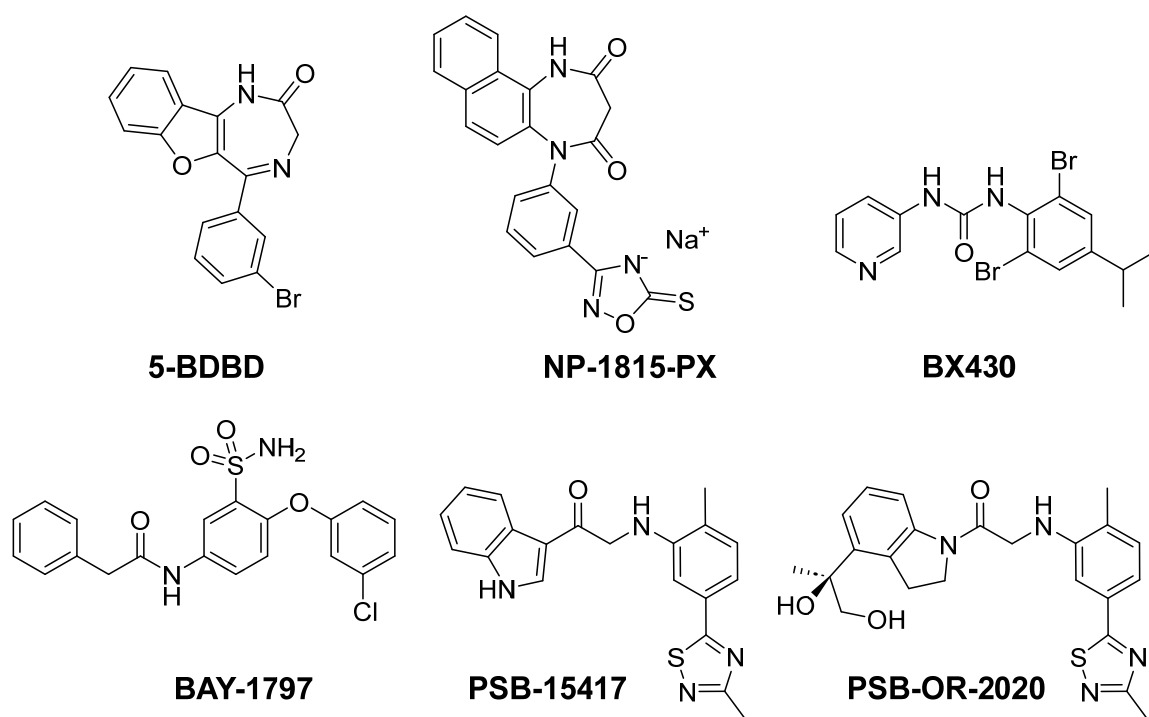


Figure 64. Structures of selected P2X4 receptor antagonists.

Table 25. Published high-resolution structures of P2X receptors, determined by cryo-EM or X-ray crystallography.

re- ceptor	species	PDB ID	state	ligand	receptor construct	method	reference
P2X4	zebrafish	3i5d 3h9v	closed, apo	/	truncated	X-ray	Kawate et al. ²⁴
	zebrafish	4dw0	closed, apo	/	truncated	X-ray	Hattori et al. ²⁵
	zebrafish	4dw1	open	ATP	truncated	X-ray	Hattori et al. ²⁵
	zebrafish	8jv5	closed	BX430	truncated	cryo- EM	Shen et al. ²⁷
	zebrafish	8jv6	closed	BAY-1797	truncated	cryo- EM	Shen et al. ²⁷
	zebrafish	5wzy	open	CTP	truncated	X-ray	Kasuya et al. ²⁶
P2X3	human	5svj 5svs 5svt	closed, apo	/	truncated	X-ray	Mansoor et al. ²⁸
	human	5svk	open	ATP	truncated	X-ray	Mansoor et al. ²⁸
	human	5svl 5svp	desensitized	ATP	truncated	X-ray	Mansoor et al. ²⁸
	human	5svm	desensitized	2-methylthio- ATP	truncated	X-ray	Mansoor et al. ²⁸
	human	5svq	closed	TNP-ATP	truncated	X-ray	Mansoor et al. ²⁸
	human	5svr	closed	A-317491	truncated	X-ray	Mansoor et al. ²⁸
	human	5yve	closed	AF-219 (gefapixant)	truncated	X-ray	Wang et al. ³⁰
	human	9ik1	closed	compound 26a	truncated	cryo- EM	Kim et al. ³⁰⁰
	human	6ah4	open	ATP, Ca ²⁺ ion	truncated	X-ray	Li et al. ²⁹⁹
	human	6ah5	open	ATP, Mg ²⁺ ion	truncated	X-ray	Li et al. ²⁹⁹
P2X7	panda	5ul1	closed, apo	/	truncated	X-ray	Karasawa et al. ³¹
	panda	5ulu	closed	A740003	truncated	X-ray	Karasawa et al. ³¹
	panda	5ulv	closed	A804598	truncated	X-ray	Karasawa et al. ³¹
	panda	5ulw	closed	AZ10606120	truncated	X-ray	Karasawa et al. ³¹
	panda	5ulx	closed	JNJ47965567	truncated	X-ray	Karasawa et al. ³¹
	panda	5uly	closed	GW791343	truncated	X-ray	Karasawa et al. ³¹
	panda	5u2h	closed	ATP/ A804598	truncated	X-ray	Karasawa et al. ³¹
	panda	8jv8	closed	PPNS	truncated	cryo- EM	Sheng et al. ³³
	panda	8jv7	closed	PPADS	truncated	cryo- EM	Sheng et al. ³³
	chicken	5xw6	closed	TNP-ATP	truncated	X-ray	Kasuya et al. ³²
	rat	6u9v	closed, apo	/	full-length	cryo- EM	McCarthy et al. ³⁴

re- ceptor	species	PDB ID	state	ligand	receptor construct	method	reference
	rat	6u9w	open	ATP	full-length	cryo- EM	McCarthy et al. ³⁴
	rat	8tr5 8v4s	closed, apo	/	full-length	cryo- EM	Oken et al. ³⁶
P2X7	rat	8trj	open	BzATP	full-length	cryo- EM	Oken et al. ³⁶
	rat	8tr6	closed	A438079	full-length	cryo- EM	Oken et al. ³⁷
	rat	8tr7	closed	A839977	full-length	cryo- EM	Oken et al. ³⁷
	rat	8tr8	closed	AZD9056	full-length	cryo- EM	Oken et al. ³⁷
	rat	8tra	closed	GSK1482160	full-length	cryo- EM	Oken et al. ³⁷
	rat	8trb	closed	JNJ47965567	full-length	cryo- EM	Oken et al. ³⁷
	rat	8trk	closed	methyl blue	full-length	cryo- EM	Oken et al. ³⁷
P2X1	human	9b73	desensitized	ATP	full-length	cryo- EM	Bennetts et al. ³⁸
	human	9b95	closed	NF449	full-length	cryo- EM	Bennetts et al. ³⁸
	human	9c2a	closed, apo	/	full-length	cryo- EM	Oken et al. ³⁹
	human	9c2b	desensitized	ATP	full-length	cryo- EM	Oken et al. ³⁹
	human	9c2c	closed	NF449	full-length	cryo- EM	Oken et al. ³⁹
P2X	gulf coast tick	5flc	open	ATP, Zn ²⁺ ion	truncated	X-ray	Kasuya et al. ¹⁹

3. Results and discussion

hP2X2_gi-25092719	MAAAQPKYPAGATARRLARGCW----	SALWDYETPKVIVVRNRLGLVYRAVQLLILLYF	-----TM1----	56
hP2X4_gi-116242696	-----MAGCCAALAAFLFEYDTPRIVLIRSRKVGLMNRVQLLILAYV	*****		43
	-----	-----v1-----		
hP2X2_gi-25092719	VWYVFIVQKSYQESSETGPSSIITKVGGIT----	SEHKVWDVEEYVKPPEGGSVFSII		111
hP2X4_gi-116242696	IGWVFWWEKGYQETDSVV-SSVTTKVGK	VAVTNTSKLGFRIWDVADYVIPAQEENSLFVM		102
	: **: *: *.***::	**: *****:..	..***: ** * : . : :	
	-----v2-----	-----v3-----	-----v4-----	
hP2X2_gi-25092719	TRVEATHSQTQGTCPESIRVHNATCLSDADCVAGELDMLGNGLRTRGRCVPYYQGPKTCE			171
hP2X4_gi-116242696	TNVILTMNQGTGL	CPEIPD-ATTVCKSDASCTAGSAGTHSNGVSTGRCVAF-NGSVKTCE		160
	*. * * .*** **	:. * ***. * . * .	..*: ***** : * *	
	-----v5-----	-----v6-----	-----v7-----	
hP2X2_gi-25092719	VFGWCPVEDGASVSQF-LGTMAPNFTILIKNSIHYPKFHFSKGNIA-DRTDGYLKRCTFH			229
hP2X4_gi-116242696	VAACWPVEDDTHVPQPAFLKAAENFT	LLVKNNIWYPKFNFSKRNLIP	NITTTYLKSC	220
	* .*****.: * * : . * *****:***. * *****:*** * : * * * * : .			
	-v7	-----v8-----		
hP2X2_gi-25092719	EASDLYCPIFKLGFIVEKAGESFTELAHKGGVIGVIINWDCDLDLPASECNPKYSFRRLD			289
hP2X4_gi-116242696	AKTDPFPCIFIRLKGIVENAGHSFQDMAVEGGIMGIQVNWDCNLD	RAASLCLPRYSFRRLD		280
	: * : **: ** * * * * * * : * : **: **: * * * * * * * * * * * * * * * * * *			
	-----v9-----	-----v10-----	-----v11-----	
hP2X2_gi-25092719	PKHVP--ASSGYNFRFAKYK-INGTTTRTLKAYGIRIDVIVHGQAGKFSLIPTIINLA			346
hP2X4_gi-116242696	TRDVEHNVS	PGYNFRFAKYRDLAGNEQ	RTLKAYGIRFDIIVFGKAGKGFDIPTMINIG	340
	:. * . * *****: : * . *****:*. * . * . * . * . * . * . * . * . * .			
	-----TM2-----			
hP2X2_gi-25092719	TALTSVGVGSFLCDWILLTFMKNKNVYSHKKFKDVKCTPSHPSGSWPVTLARVLGQAPPEP			406
hP2X4_gi-116242696	SGLALLGMATVLCDIIVLYCMKKRLYYREKKYKVEDYEQ-----	GLASELDQ-----		388
	:. * : *: .*** * * * * . * . * . * . * . * . * . * . * . * . * .			
hP2X2_gi-25092719	GHRSEDQHSPSPSGQEGQQAECGPAFPPLRPCPISAPSEQMVDTPASEPAQASTPTDPK			466
hP2X4_gi-116242696	-----			388
hP2X2_gi-25092719	GLAQL	471		
hP2X4_gi-116242696	-----	388		

Figure 65. Protein sequence alignment of the human wt P2X2 with the human wt P2X4 receptor created with Clustal Omega (1.2.4.).²⁰⁸ Stretches of non-conserved amino acid sequences were identified and labeled as variable region v1 (blue), v2–v5 (pink), v6 (cyan), v7 (green), v8 (orange), v9 (dark green), v10 (red).¹³⁴ The following chimeric (c) receptors were obtained, in which the respective colored sequence of v1–v10 was exchanged for the corresponding P2X2 receptor sequence: c1^{V71-I83}, c2–c5^{C116-T186}, c6^{N208-S216}, c7^{I218-D224}, c8^{R265-L269}, c9^{T281-P290}, and c10^{R301-Q308}, as previously described¹³⁴. Symbols: (*) indicates conserved residues, (:) and (.) indicate conservative and semi-conservative exchanges, and blank spaces point out non-conservative exchanges of residues between aligned protein sequences.

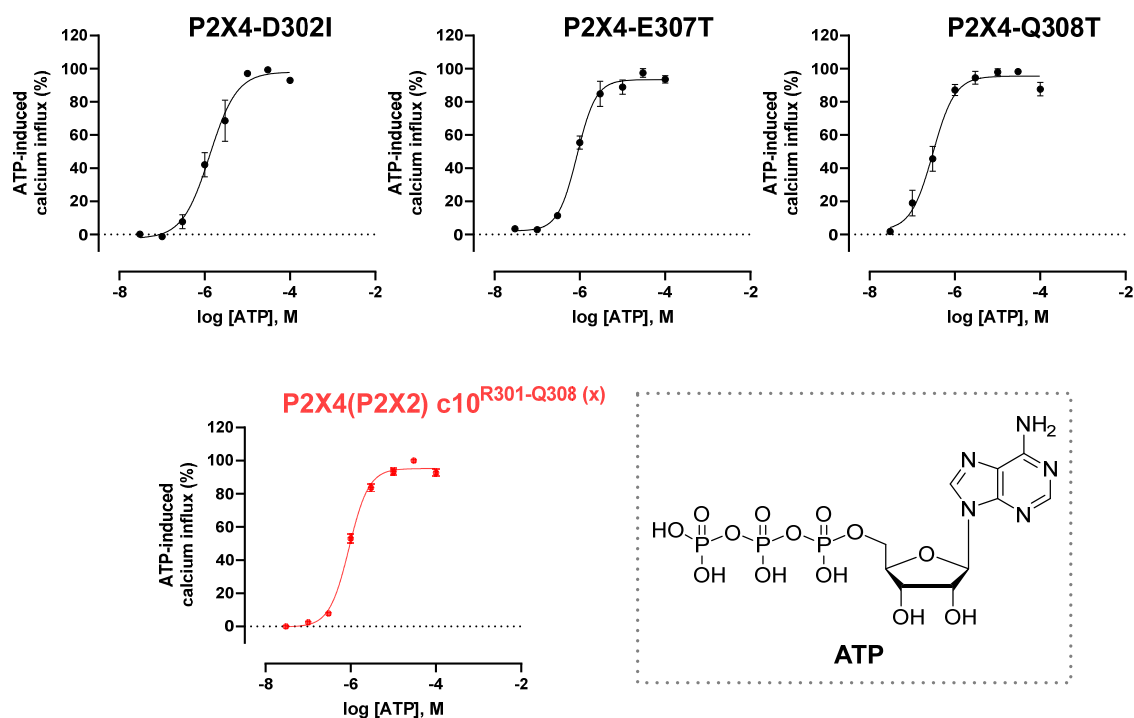


Figure 66. Concentration-dependent activation of P2X4 receptor mutants (D302I, E307T, and Q308T) and the chimeric receptor P2X4(P2X2) c10^{R301-Q308} by ATP. Receptors were stably expressed in 1321N1 astrocytoma cells. ATP-dependent receptor activation was measured in calcium influx assays. Data was normalized to the maximal ATP-induced effect (100%). Data represent means ± SEM of at least three independent biological replicates performed in technical duplicates. (x) Published results.¹³⁴

3. Results and discussion

Table 26. Potency of ATP at the human wt P2X4 receptor, the human P2X4 receptor mutants, and the chimeric receptor P2X4(P2X2) c10^{R301-Q308}, stably expressed in 1321N1 astrocytoma cells, determined by measurement of calcium influx.

	ATP EC ₅₀ (μM) ^a	ATP pEC ₅₀ ± SEM ^c	Adjusted P values ^d
wt P2X4	0.357	6.45 ± 0.04	
P2X4-D302I	1.60	5.85 ± 0.15 ^{***}	0.0007
P2X4-E307T	0.921	6.04 ± 0.03 [*]	0.0118
P2X4-Q308T	0.295	6.56 ± 0.10 ^{ns}	0.8100
P2X4(P2X2) c10 ^{R301-Q308}	0.862 ^b	6.04 ± 0.03 ^{*b}	0.0117

^a Data represent mean EC₅₀ values of ATP obtained in three biological replicates performed in technical duplicates. ^b Published value.¹³⁴ ^c Data represent pEC₅₀ values ± SEM. The level of significance was calculated by a one-way ANOVA with Dunnett's multiple comparisons test. Significance was expected if P value was ≤ 0.05. The significance levels were defined as follows: * P < 0.05, ** P < 0.01, *** P < 0.001, **** P < 0.0001; ns, not significant. ^d Adjusted P values are presented. The pEC₅₀ values of ATP at the receptor mutants were compared to those at the wt P2X4 receptor.

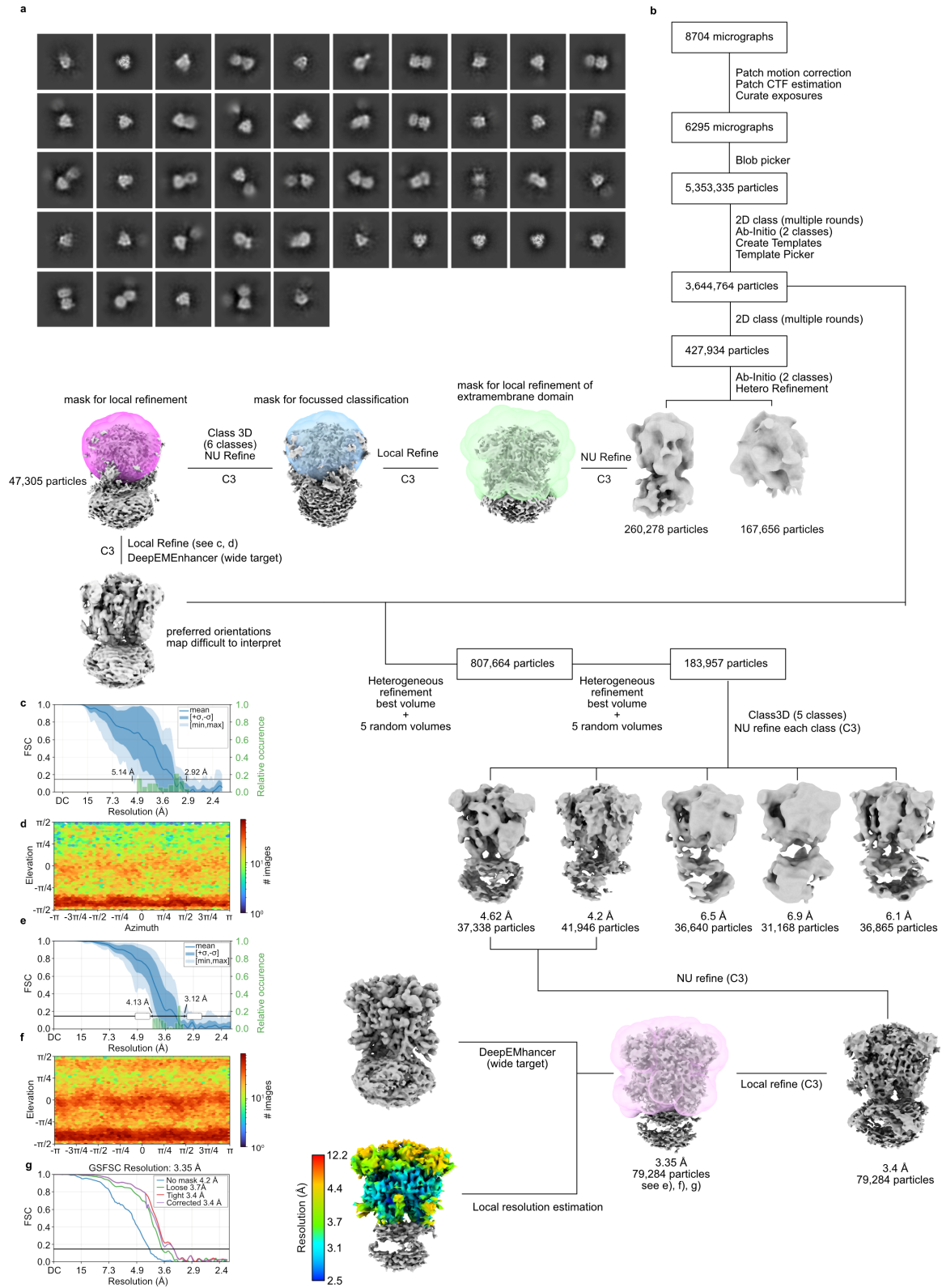
Table 27. Potency of Cibacron Blue determined in calcium influx assays at the human wt P2X4 receptor, the human wt P2X2 receptor, P2X4 receptor mutants, and the chimeric receptor P2X4(P2X2) c10^{R301-Q308}, stably expressed in 1321N1 astrocytoma cells.

	Cibacron Blue IC ₅₀ (μM) ^a	Cibacron Blue pIC ₅₀ ± SEM (n value) ^b	Adjusted P values ^c
wt P2X4	12.1	4.96 ± 0.14 (3)	
wt P2X2	13.1	4.90 ± 0.07 ^{ns} (3)	0.9971
P2X4(P2X2) c2-c5 ^{C116-T186}	8.99	5.05 ± 0.06 ^{ns} (2)	0.9933
P2X4(P2X2) c6 ^{N208-S216}	8.60	5.07 ± 0.03 ^{ns} (3)	0.9657
P2X4(P2X2) c7 ^{I218-D224}	23.6	4.63 ± 0.03 ^{ns} (3)	0.0835
P2X4(P2X2) c8 ^{R265-L269}	20.3	4.75 ± 0.16 ^{ns} (3)	0.4827
P2X4-D302I	1.42	5.85 ± 0.05 ^{****} (3)	<0.0001
P2X4-E307T	0.0178	7.78 ± 0.12 ^{****} (3)	<0.0001
P2X4-Q308T	10.4	5.00 ± 0.06 ^{ns} (3)	0.9996
P2X4(P2X2) c10 ^{R301-Q308}	0.00476	8.29 ± 0.07 ^{****} (5)	<0.0001

^a Data represent mean IC₅₀ values in μM. The inhibition of Cibacron Blue was determined in the presence of ATP at its respective EC₈₀. ^b Data represent pIC₅₀ values ± SEM with the number of experiments indicated in parentheses. The level of significance was calculated by a one-way ANOVA with Dunnett's multiple comparisons test. Significance was expected if P value was ≤ 0.05. The significance levels were defined as follows: * P < 0.05, ** P < 0.01, *** P < 0.001, **** P < 0.0001; ns, not significant. ^c Adjusted P values are presented. The pIC₅₀ values of Cibacron Blue at the receptor mutants were compared to those at the wt P2X4 receptor.

Table 28. Cryo-EM data collection, refinement and validation statistics

	EMDB-51502 (PDB 9gp7)
Data collection and processing	
Magnification	45,000x
Voltage (kV)	200
Electron exposure (e-/Å ²)	46.7
Defocus range (µm)	1.0-2.5
Pixel size (Å)	0.901
Symmetry imposed	C3
Initial particle images (no.)	3,644,764
Final particle images (no.)	79,284
Map resolution (Å)	3.35
FSC threshold	0.143
Map resolution range (Å)	12.2-2.5
Refinement	
Initial model used (PDB code)	AF-Q99571-F1
Map sharpening <i>B</i> factor (Å ²)	151
Model composition	
Non-hydrogen atoms	6636 (3x2212)
Protein residues	819 (3x273)
Ligands	3 (3x1)
<i>B</i> factors (Å ²)	
Protein (min/max/avg)	84/219/127.4
Ligand (min/max/avg)	89/164/126.9
R.m.s. deviations	
Bond lengths (Å)	0.013
Bond angles (°)	1.889
Validation	
MolProbity score	1.10
Clashscore	0.08
Poor rotamers (%)	0.7
Ramachandran plot	
Favored (%)	89.5
Allowed (%)	9.4
Disallowed (%)	1.1



tree. f) Viewing angle distribution after skipping 2D classifications. g) FSC plot of final reconstruction.

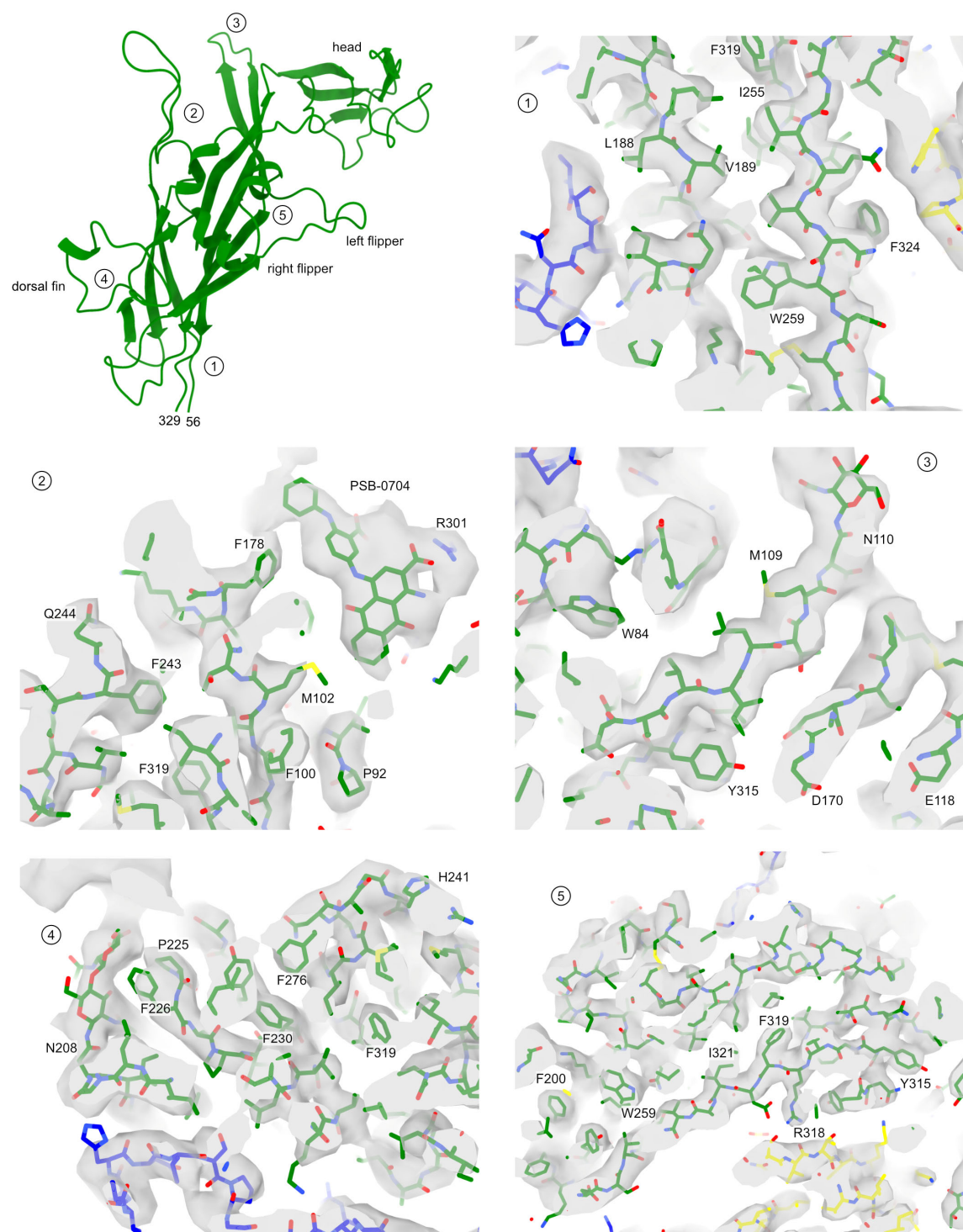


Figure 68. 3D reconstruction of the human P2X4 receptor. The top left panel shows one chain of the receptor and selected regions are indicated by numbers in circles. The remaining panels show snapshots of the 3D reconstruction of these regions (gray meshes). Selected residue numbers are indicated.

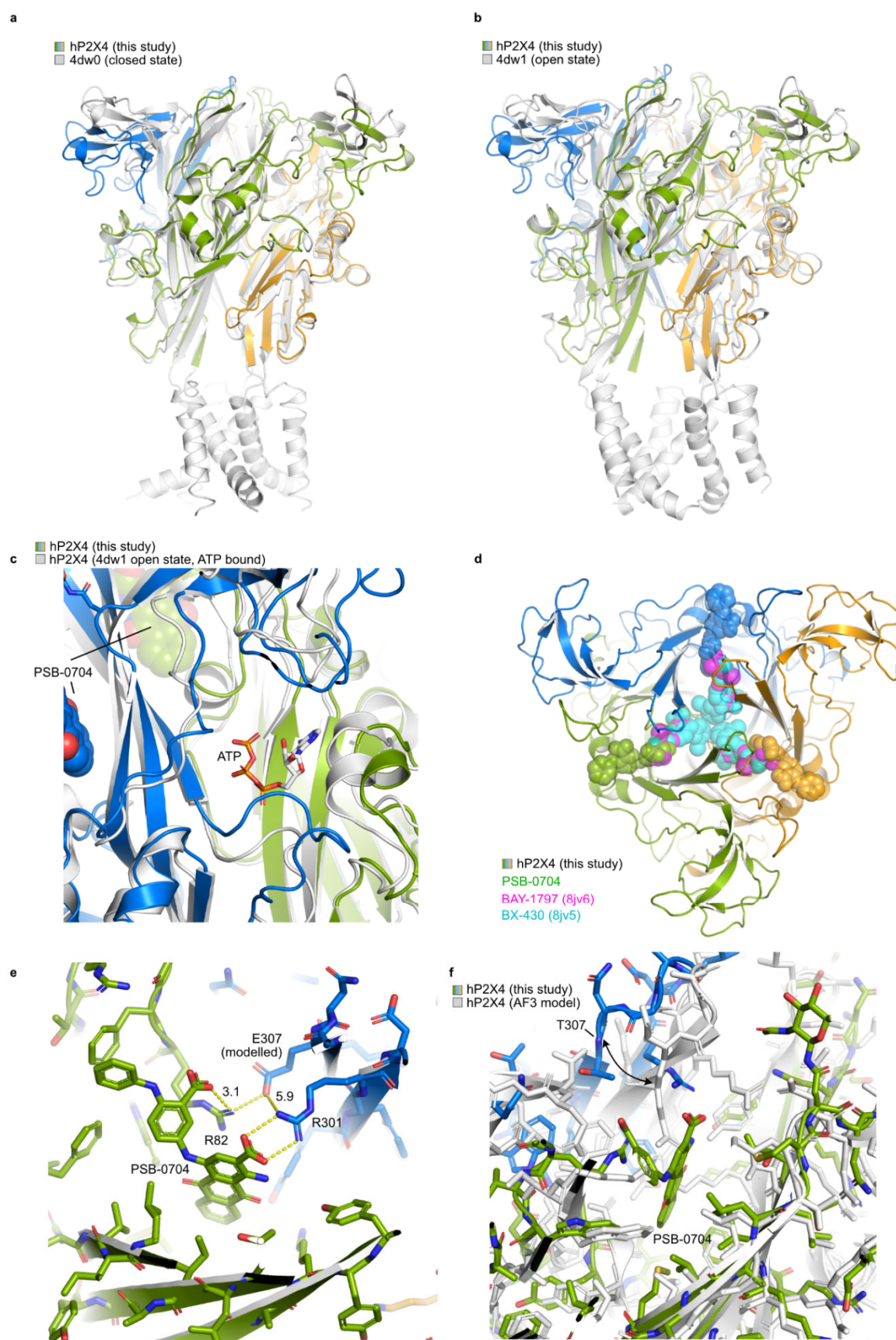


Figure 69. Comparison of the human P2X4 receptor structure with similar structures. a) Superposition of the human (h) P2X4 receptor structure (cartoon, color-coded as in the apo

closed-state structure (white cartoon) of the zebrafish homolog; b) as in a) but with the open-state structure of the zebrafish homolog. c) Detail of b) focussed on the ATP-binding site. The ATP of 4dw1 (white) is shown as sticks and the ligand PSB-0704 as spheres. d) Cartoon model of the human P2X4 receptor. The antagonist PSB-0704 is shown as green ball-and-stick model. The relative positions of the antagonists BAY-1797 and BX-430 are indicated. e) Model of PSB-0704 binding to the human wt P2X4 receptor, based on the human P2X4-E307T receptor structure from this work. The threonine at position 307 was replaced by a glutamate residue in PyMOL. The rotamer of the side chain was chosen in such a way that no severe clashes occurred. f) The cryo-EM structure of the human P2X4-E307T receptor (this work) superimposed with the AF3 model shown in Figure 55 (white).

4. Summary

The P2X4 receptor is a promising target in drug development for the treatment of neuroinflammatory and neurodegenerative diseases, chronic pain, and various types of cancer.^{10,42,44,47,150,171} The development of P2X4 receptor ligands, in particular antagonists, is of great importance to further explore the receptor's role in (patho)physiological processes with the aim to facilitate drug development. However, only few P2X4 receptor antagonists are currently available, and information on their binding sites and interactions with the receptor has been limited. This study provides valuable insights into the pharmacology and structural biology of the human P2X4 receptor.

A comprehensive analysis and comparison of various pharmacologically relevant P2X4 receptor species was conducted, involving the preparation of stable cell lines expressing the monkey, guinea pig, pig, rabbit, and zebrafish P2X4 receptor (Chapter 3.1) (Figure 70). A broad and systematic study on the differences between P2X4 receptor species had been lacking, and no prior pharmacological characterization of monkey, guinea pig, pig, and rabbit P2X4 receptors had been reported. To evaluate the effects of P2X4 receptor agonists and antagonists at the P2X4 receptor orthologs (monkey, dog, guinea pig, pig, rabbit, rat, mouse, and zebrafish), calcium influx assays were performed and compared with results obtained for the human P2X4 receptor (Figure 70). This systematic analysis showed significant species differences between the human P2X4 receptor and its orthologs for both agonists and antagonists. Most agonists were less potent at mouse, rat, and zebrafish receptors in comparison to the human P2X4 receptor. The physiological agonist ATP was more potent at monkey, rabbit, guinea pig, and pig P2X4 receptors (0.0285–0.0594 μM) than at the human ortholog (0.269 μM), and least potent at the zebrafish receptor (3,270 μM). The ATP analog 2-MeS-ATP displayed similar potency (0.290–4.50 μM) at all investigated P2X4 receptor species, except for zebrafish. Thus, 2-MeS-ATP can be considered a suitable tool compound for studying P2X4 receptors across species. Significant species differences were observed for the allosteric P2X4 receptor antagonists BX430 and 5-BDBD, while the indole derivative PSB-OR-2020 inhibited all investigated mammalian species with high potency (0.696–6.32 nM), showing only minor species differences. This information will be valuable for future preclinical in vitro and in vivo studies. The results are described and presented in a manuscript (Chapter 3.1).

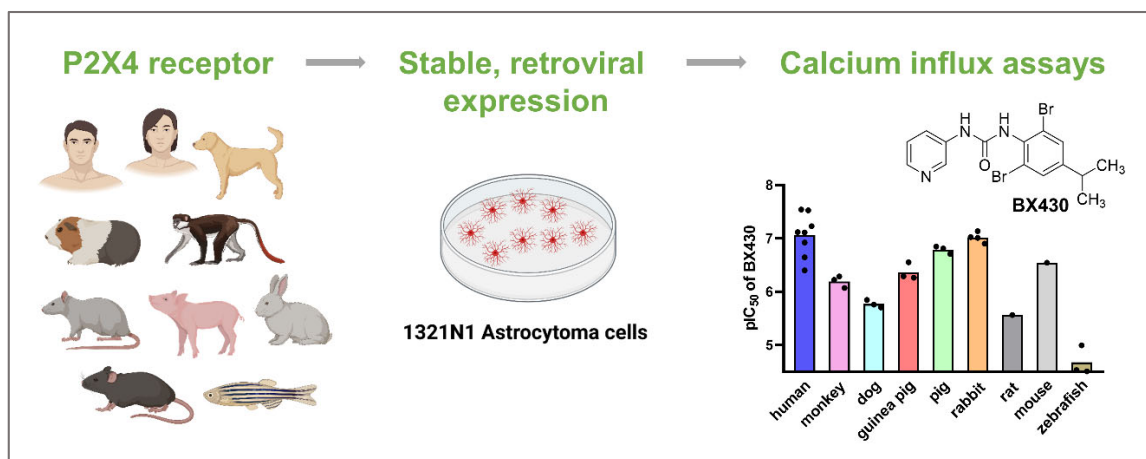


Figure 70. Study workflow to analyze and compare different P2X4 receptor species, including the generation of stable cell lines by retroviral transfection and the evaluation of P2X4 receptor agonists and antagonists using calcium influx assays. Parts of the figure were created with BioRender.com.

A radioligand binding assay was established for an allosteric P2X4 receptor radioligand derived from a potent and selective dihydroindole derivative, designated [3H]PSB-OR-2020 (Chapter 3.2) (Figure 71).¹²⁹ Various steps were conducted to increase the expression of the human P2X4 receptor using different cell lines and expression systems and to optimize assay conditions, leading to the characterization of the tritium-labeled P2X4 receptor antagonist. The highest expression of the human P2X4 receptor and a sufficiently low percentage of non-specific radioligand binding were obtained in cell membranes from HEK293 cells transiently transfected with the human P2X4 receptor. Kinetic experiments performed at 4 °C indicated an association half-life of 8.44 min and a dissociation half-life of 20.4 min, with a calculated kinetic dissociation constant (K_D value) of 7.06 nM. Homologous competition assays with the unlabeled compound PSB-OR-2020 confirmed the high affinity of the P2X4 receptor antagonist, showing a K_D value of 20.4 nM. Competition binding assays with structurally diverse P2X4 receptor antagonists, including a benzodiazepine, urea, and sulfonamide derivative, indicated the existence of different allosteric binding sites on the P2X4 receptor. This study resulted in the first successful radioligand binding assay for an allosteric P2X4 receptor antagonist, offering a promising pharmacological tool for future studies.¹²⁹ Further evaluation of the radioligand in cell membranes derived from *Sf9* insect cells suggested the possible existence of a P2X receptor in *Sf9* insect cells, which is still awaiting further characterization and cloning (Chapter 3.3).

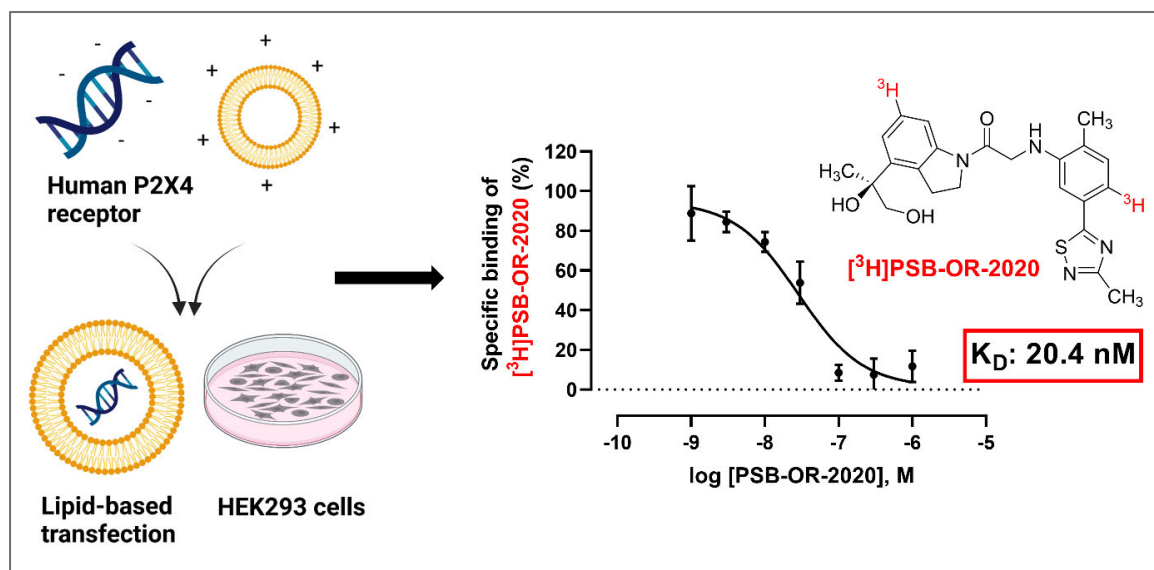


Figure 71. Development of a radioligand binding assay for the tritium-labeled allosteric P2X4 receptor antagonist $[^3\text{H}]$ PSB-OR-2020. Parts of the figure were created with BioRender.com.

The next chapter of this study describes the biological evaluation of new thioisosteres of nucleoside di- and triphosphates at human P2X1, P2X2, P2X3, and P2X4 receptors. The compounds were synthesized by the group of Phil S. Baran who developed a novel high-yielding stereoselective synthetic approach for these nucleotide analogs (Chapter 3.4).²⁵⁶ Calcium influx assays demonstrated that the thio-analogs of ATP act as potent agonists at all investigated P2X receptor subtypes, albeit with varying potencies and efficacies.²⁵⁶ These ATP thio-analogs may be considered as useful tool compounds, exhibiting higher metabolic stability than ATP, for investigating the role of P2X receptors in health and disease, and may provide a starting point for the future development of subtype-selective P2X1, P2X2, P2X3, and P2X4 receptor agonists.

The extracellular binding sites of positive and negative allosteric P2X4 receptor modulators, namely ivermectin, BX430, and paroxetine, were investigated by mutagenesis and docking studies.¹³⁴ In this study, effects of site-directed mutagenesis on the negative allosteric effects of the P2X4 receptor antagonist BX430 were evaluated (Chapter 3.5) (Figure 72). Based on previous research results, specific human P2X4 receptor mutants were generated by polymerase chain reaction-based site-directed mutagenesis and retrovirally transfected into 1321N1 astrocytoma cells. Subsequently, calcium influx assays were employed to assess the effects of BX430 on the P2X4 receptor mutants. Our results

indicated that the allosteric binding site of BX430 may be located in the dorsal fin region of the human P2X4 receptor. Key amino acid residues that are involved in the blockade of the human P2X4 receptor by BX430 were identified, including amino acid residue Ile209, which was suggested to interact with BX430 through hydrophobic interactions (Figure 72).¹³⁴

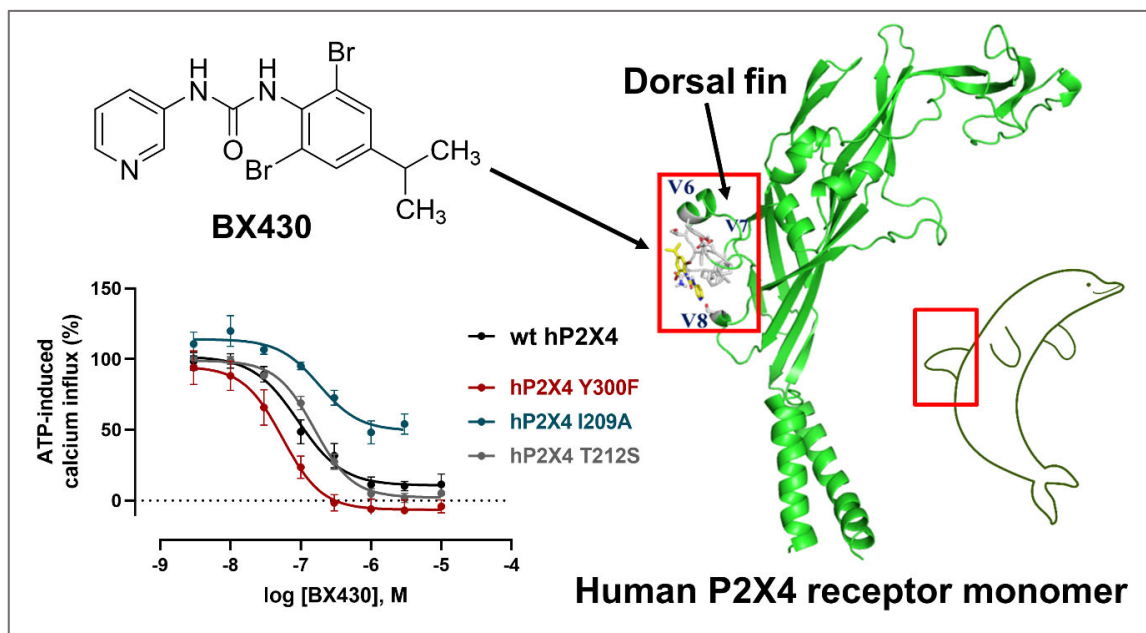


Figure 72. Summary of mutagenesis studies conducted to identify the allosteric binding site of the P2X4 receptor antagonist BX430. The illustration highlights key findings from these studies. Some elements of the figure were created with BioRender.com. The docking of BX430 into the human P2X4 receptor was performed by Dr. Vignesh Namasivayam.

A further, most significant part of the study was the determination of a cryo-EM structure of the human P2X4 receptor in complex with an anthraquinone-based antagonist. To obtain a homogeneous, highly pure, and stable P2X4 receptor protein sample, different receptor constructs were designed, and methods were developed and optimized for P2X4 receptor protein expression on a large scale in insect cells, followed by subsequent purification and reconstitution into amphipols (Chapter 3.6) (Figure 73). Following a series of optimization attempts and preliminary tests, a cryo-EM structure of the full-length human P2X4-E307T receptor in complex with an allosteric anthraquinone-based antagonist was determined at an overall resolution of 3.35 Å (Figure 73). This high-resolution structure identified an unprecedented allosteric binding site in the upper body at the P2X4 receptor trimer subunit

interface. Specific protein-ligand interactions were visualized at the atomic level, including amino acid residues Arg301 and Arg82, which are crucial for anthraquinone binding to the P2X4 receptor. These structural insights thus provide a new avenue for structure-based drug design. The results were detailed in a manuscript, complemented by pharmacological experiments (Chapter 3.7).

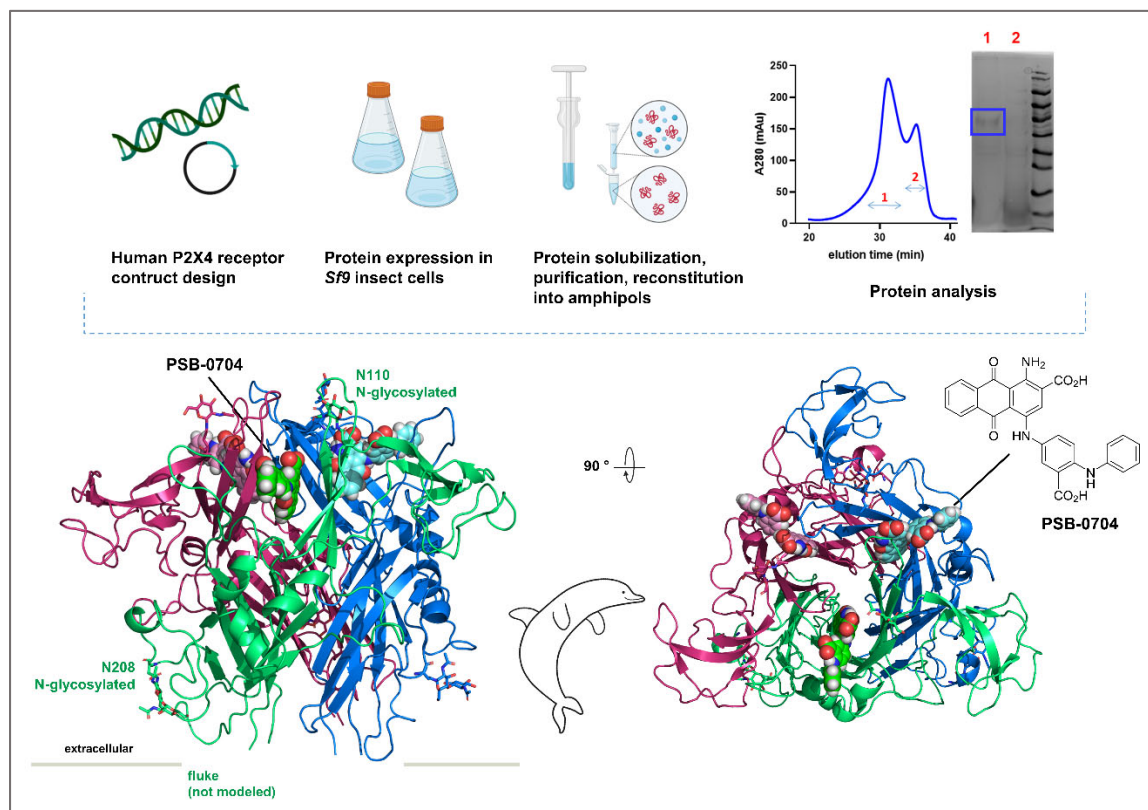


Figure 73. Method development for producing a stable, homogeneous, and pure P2X4 receptor protein for cryo-EM studies. The cartoon model depicts the extracellular domain of the human P2X4-E307T receptor in complex with the allosteric antagonist PSB-0704 (protein structure is displayed perpendicular to the membrane bilayer and from the top). PSB-0704 is shown as spheres. Glycosylation sites are shown as sticks. Protein figures were created with PyMOL 3.1.0. Some elements of the figure were created with BioRender.com.

In conclusion, this thesis provides important novel insights into the pharmacology and structural biology of the therapeutically important P2X4 receptor, which are anticipated to support future drug development.

5. List of abbreviations

2-MeS-ATP	2-Methylthio-ATP
ADP	Adenosine diphosphate
AF3	AlphaFold 3
ALS	Amyotrophic lateral sclerosis
AM	Acetoxymethyl ester
AMPA	α -Amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid
Ap₄A	Diadenosine tetraphosphate
APBS	Adaptive Poisson-Boltzmann Solver
APol	Amphipol
ATP	Adenosine triphosphate
Aβ	Beta-amyloid fragment
BDNF	Brain-derived neurotrophic factor
B_{max}	Total number of binding sites
bp	Base pairs
BSA	Bovine serum albumin
BzATP	Benzoylbenzoyl-ATP
CCL21	Chemokine ligand 21
ccRCC	Clear cell renal cell carcinoma
CHS	Cholesteryl hemisuccinate
CMC	Critical micelle concentration
CNS	Central nervous system
cpm	Counts per minute
cryo-EM	Cryogenic electron microscopy
CSF1	Colony-stimulating factor 1
CTP	Cytidine triphosphate
CV	Column volumes
DDM	n-Dodecyl- β -D-maltopyranoside
DEPC	Diethyl pyrocarbonate
DMEM	Dulbecco's Modified Eagle's Medium
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid

dNTP	Deoxynucleoside triphosphate
<i>E. coli</i>	<i>Escherichia coli</i>
EC₅₀	Half maximal effective concentration
EC₈₀	Effective concentration at 80% of the maximum possible response
EDTA	Ethylenediaminetetraacetic acid
E_{max}	Maximal efficacy
EVs	Extracellular vesicles
FCS	Fetal calf serum
Fluo-4 AM	Fluo-4 acetoxymethyl ester
FSC	Fourier shell correlation
G418	Geneticin 418
GABA_A	γ -Aminobutyric acid type A
GDP	Guanosine diphosphate
GFP	Green fluorescent protein
GPCRs	G protein-coupled receptors
HA	Hemagglutinin
HBSS	Hanks' balanced salt solution
HEK293	Human embryonic kidney 293
HEPES	4-(2-Hydroxyethyl)-1-piperazineethanesulfonic acid
His	Histidine
HPLC	High-performance liquid chromatography
HRP	Horseradish peroxidase
HRV 3C	Human rhinovirus 3C
IC₅₀	Half-maximal inhibitory concentration
IMAC	Immobilized metal ion affinity chromatography
IPTG	Isopropyl β -D-1-thiogalactopyranoside
KCC2	K ⁺ /Cl ⁻ cotransporter 2
K_D	Dissociation constant
kDa	Kilodalton
K_i	Inhibitory constant
LB	Lysogeny broth
LGICs	Ligand-gated ion channels

mAU	Milli-absorbance units
MSP1E3D1	Membrane scaffold protein 1E3D1
mTOR	Mammalian target of rapamycin
n.d.	Not determined
NAMs	Negative allosteric modulator
NDPs	Nucleoside diphosphates
NMDA	<i>N</i> -Methyl-D-aspartate
NTPs	Nucleoside triphosphates
OR	Orion Pharma
PAMs	Positive allosteric modulator
PBS	Phosphate-buffered saline
PCR	Polymerase chain reaction
PDB	Protein Data Bank
pEC₅₀	Negative logarithm of the EC ₅₀ value
PET	Positron emission tomography
pIC₅₀	Negative logarithm of the IC ₅₀ value
PIPs	Phosphoinositides
PNI	Peripheral nerve injury
PNS	Peripheral nervous system
POPC	1-Palmitoyl-2-oleoyl- <i>sn</i> -glycero-3-phosphocholine
PSB	Pharmaceutical Sciences Bonn
r.m.s.d	Root mean square deviation
RNA	Ribonucleic acid
rpm	Revolutions per minute
SAR	Structure activity relationships
SD	Standard deviation
SDS-PAGE	Sodium dodecyl sulfate polyacrylamide gel electrophoresis
SEC	Size-exclusion chromatography
SEM	Standard error of the mean
<i>Sf9</i>	<i>Sf9</i> cell line from <i>Spodoptera frugiperda</i>
SPA	Single particle analysis
SPECT	Single photon emission computer tomography
TAE	Tris base, acetic acid, and EDTA

TM	Transmembrane
TNP-ATP	Trinitrophenyl-ATP
Tris-HCl	Tris(hydroxymethyl)aminomethane hydrochloride
TrkB	Tropomyosin-related kinase B
UDP	Uridine diphosphate
UTP	Uridine triphosphate
UV	Ultraviolet
VIS	Visible
VSV-G	Vesicular stomatitis virus G protein
wt	Wildtype
X-Gal	5-Bromo-4-chloro-3-indolyl- β -D-galactopyranoside
α,β-Me-ATP	α,β -Methylene-ATP

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7. Appendices

7.1 Appendix A: Publication I - Preparation and preliminary evaluation of a tritium-labeled allosteric P2X4 receptor antagonist

This section contains the original article “Preparation and preliminary evaluation of a tritium-labeled allosteric P2X4 receptor antagonist” as it was published in *Purinergic Signalling* by Springer.¹²⁹ The publication is made available under the Creative Commons Attribution 4.0 International License. The author rights policy of the Springer publishing group allows the reuse and republish of the original article without permission or payment, as long as an appropriate acknowledgement to the original author(s) including the publication source is provided. The main article and the supporting information are included in the next pages.

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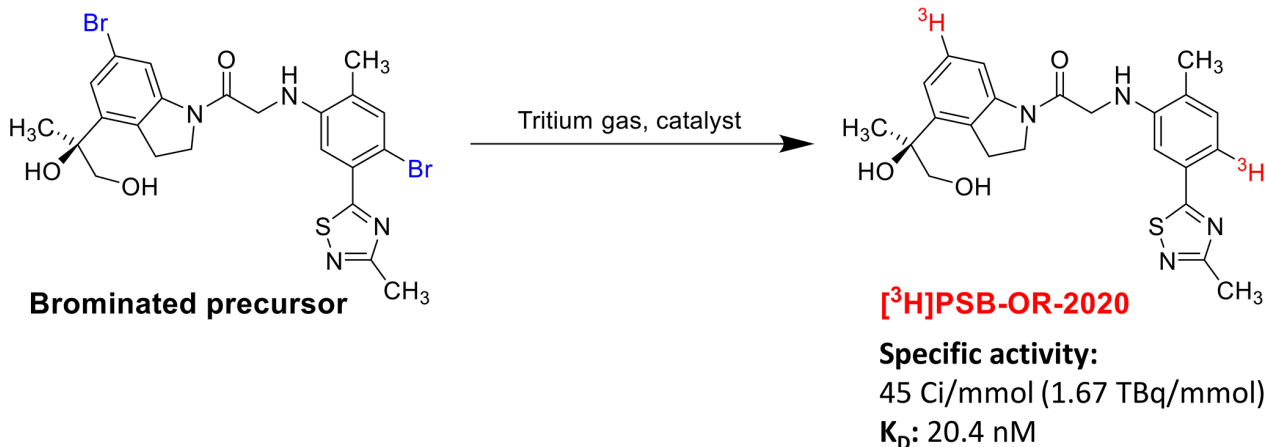
Preparation and preliminary evaluation of a tritium-labeled allosteric P2X4 receptor antagonist

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Abstract

P2X4 receptors are ATP-gated cation channels that were proposed as novel drug targets due to their role in inflammation and neuropathic pain. Only few potent and selective P2X4 receptor antagonists have been described to date. Labeled tool compounds suitable for P2X4 receptor binding studies are lacking. Here, we present a novel allosteric P2X4 receptor antagonist possessing high potency in the low nanomolar range. We describe its tritium-labeling resulting in the P2X4-selective radiotracer [³H]PSB-OR-2020 with high specific activity (45 Ci/mmol; 1.67 TBq/mmol). A radioligand binding assay was developed using human embryonic kidney (HEK293) cell membranes recombinantly expressing the human P2X4 receptor. Competition binding studies with structurally diverse P2X4 receptor antagonists revealed different allosteric binding sites indicating that the new class of P2X4 receptor antagonists, to which PSB-OR-2020 belongs, interacts with an unprecedented allosteric site. [³H]PSB-OR-2020 may become a useful tool for research on P2X4 receptors and for promoting drug development.

Allosteric P2X4 receptor radioligand



Keywords Antagonist · Ligand-gated ion channel · Negative allosteric modulator · P2X4 receptor · Radioligand

Abbreviations

ALS	Amyotrophic lateral sclerosis
AM	Acetoxymethyl ester
ATP	Adenosine 5'-triphosphate
BDNF	Brain-derived neurotrophic factor
BSA	Bovine serum albumin
Bz-ATP	2'(3')-O-(4-benzoylbenzoyl) adenosine-5'-triphosphate
CNS	Central nervous system
cpm	Counts per minute
Cryo-EM	Cryogenic electron microscopy
DMEM	Dulbecco's Modified Eagle Medium
DMSO	Dimethyl sulfoxide
EDTA	Ethylenediamine tetraacetic acid
EGTA	Ethylene glycol tetraacetic acid
FCS	Fetal calf serum
G418	Geneticin
GF/C	Glass fiber filters
HBSS	Hanks' balanced salt solution
HEK293	Human embryonic kidney type 293
HEPES	4-(2-Hydroxyethyl)-1-piperazineethanesulfonic acid
IC ₅₀	Half-maximal inhibitory concentration
K _D	Dissociation constant
PET	Positron emission tomography
PBS	Phosphate-buffered saline
SEM	Standard error of the mean
SPECT	Single photon emission computer tomography
TM	Transmembrane domain
Tris-HCl	Tris(hydroxymethyl)aminomethane hydrochloride
VSV-G	Vesicular stomatitis virus G

Introduction

P2X4 receptors belong to the P2X receptor family of ATP-activated ionotropic membrane receptors comprising seven receptor subunits, P2X1-P2X7 [1]. Crystallization and cryogenic electron microscopy (cryo-EM) structures of the zebrafish P2X4 receptor confirmed its previously proposed homotrimeric structure [2–5]. Each protein subunit is composed of two transmembrane domains (TM1 and TM2) and a large extracellular loop [2, 3]. The crystal structure of the zebrafish P2X4 receptor revealed the location of three ATP-binding sites in the extracellular domains, one in each subunit [2, 3]. Binding of the physiological agonist ATP induces receptor subunit rearrangement and ion channel opening for Na⁺, Ca²⁺ and K⁺ ions [3, 6]. In addition to homotrimeric receptors, heterotrimeric receptors exist, e.g. P2X2/3 receptors [1, 4, 7].

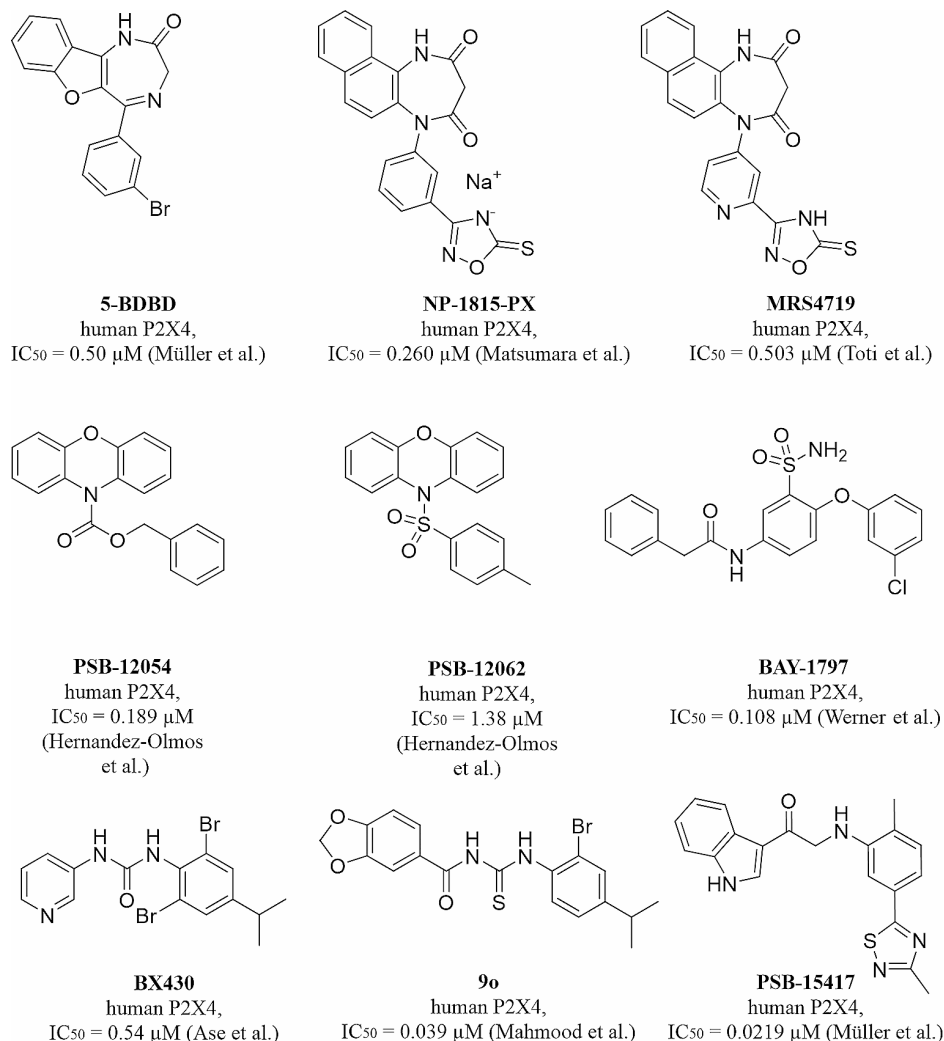
P2X4 receptors are expressed in the central and peripheral nervous system, especially in astrocytes, neurons, microglia and endothelial cells [1, 8]. A number of studies have shown that P2X4 receptors are involved in neuropathologies [9]; they appear to play a central role in neuropathic pain [10]. After peripheral nerve injury, microglial cells are activated, and P2X4 receptor expression is strongly upregulated [11–15]. This results in the release of brain-derived neurotrophic factor (BDNF) which initiates a cascade of neuroinflammatory signals resulting in pain and hypersensitivity. The pharmacological blockade of P2X4 receptors was reported to reverse tactile allodynia, a prominent symptom of neuropathic pain [11–14, 16]. P2X4 receptor blockade might also be beneficial for the treatment of other types of chronic pain, e.g. cancer pain [17], inflammatory pain [18, 19], and visceral pain [20]. An upregulation of P2X4 receptors in reactive microglia or in neurons, respectively, was also observed in Alzheimer's [21] and Parkinson's disease [22], amyotrophic lateral sclerosis (ALS) [23], epilepsy [24], multiple sclerosis [25] and stroke [26, 27]. The role of P2X4 receptors in all of these neuroinflammatory processes suggests that P2X4 receptor antagonists might be effective drugs for the treatment of associated diseases.

Besides their crucial role in neuroinflammatory diseases, P2X4 receptors have emerged as potential targets for the treatment of cancer, including breast [28], prostate [29], gastric [30], colon [31] and renal cancer [32]. Recently, P2X4 receptors have been reported to play an important role in the fine-tuning of the basal activity and the initial activation of CD4⁺ T cells [33], indicating that the receptors are involved in immune responses to pathogens and cancer cells.

The discovery of drug-like competitive P2X4 receptor antagonists is hampered by the polar ATP binding site lined with basic amino acid residues [3, 4]. However, several allosteric modulators have been identified and in some cases optimized [34–36]. P2X4 receptor antagonists include benzodiazepine derivatives, e.g. 5-BDBD and derived compounds, e.g. NP-1815-PX [37], and its analog MRS4719 [38], phenoxazine derivatives, e.g. PSB-12054 and PSB-12062 [39], sulfonamide derivatives, e.g. BAY-1797 [40], and the urea derivative BX430 and its analogs, e.g. compound 9o (Fig. 1) [41, 42].

PSB-15417 was developed as a potent, highly selective and brain-permeable allosteric P2X4 antagonist by our group showing efficacy in an animal model of neuropathic pain [14, 36]. NC-2600 (structure undisclosed) was the first P2X4 receptor antagonist to enter clinical trials (phase I) [43]. The effects of NP-1815-PX and NC-2600 were evaluated in a murine model of colitis and found to improve several inflammatory parameters [44]. MRS4719 showed neuroprotective and neuro-rehabilitative effects in a murine ischemic stroke model [38].

Fig. 1 P2X4 receptor antagonists with inhibitory potencies (IC_{50}) at the human P2X4 receptor. Müller et al. [36]; Matsumara et al. [37]; Toti et al. [38]; Hernandez-Olmos et al. [39]; Werner et al. [40]; Ase et al. [41]; Mahmood et al. [42]



Various assays have been developed for studying the potency of P2X receptor ligands. These include calcium influx assays [45], patch-clamp measurements [46], and radioligand binding assays [47, 48]. Radioligands are powerful tools in pharmacology and drug discovery for measuring direct ligand-protein interactions, determining affinities and binding kinetics, including those of unlabeled compounds by performing competition assays. Moreover, radioligands are used for receptor imaging in vitro and in vivo, for autoradiography, scintigraphy (SPECT, single photon emission computer tomography) and positron emission tomography (PET) studies. The orthosteric binding site of the P2X4 receptor can be labeled by the agonist radioligand [35 S]ATP γ S, an ATP analog with higher metabolic stability than ATP [48, 49].

Potent and selective antagonist radioligands for the labeling of allosteric P2X receptor binding sites have so far only been described for P2X3 and P2X7 receptors [47, 50].

Attempts to develop PET radioligands for the P2X4 receptor were described, based on 5-BDBD, which was radiolabeled with 11 C, 18 F, or 76 Br. However, in vitro binding studies with these radiolabeled 5-BDBD analogs have not been successful [51]. This is not surprising given the moderate potency and low water-solubility of 5-BDBD (Fig. 1).

Here, we present the development of a potent, selective allosteric antagonist radioligand for the P2X4 receptor designated [3 H]PSB-OR-2020. This radiotracer has allowed the development of the first antagonist radioligand binding assay for the human P2X4 receptor.

Results

In the search for novel scaffolds with P2X4 receptor-antagonistic activity, we had previously established assays allowing high throughput screening [49]. This led to the discovery of

Fig. 2 Concentration-dependent inhibition of ATP-induced Ca^{2+} influx by PSB-OR-2020 determined in 1321N1 astrocytoma cells stably transfected with the human P2X4 receptor. An EC_{80} of ATP was applied (300 nM) after preincubation of the cells with various concentrations of PSB-OR-2020. Data points represent means $\pm$ SEM of four independent experiments performed in duplicates. IC_{50} value: 6.32 ± 1.52 nM

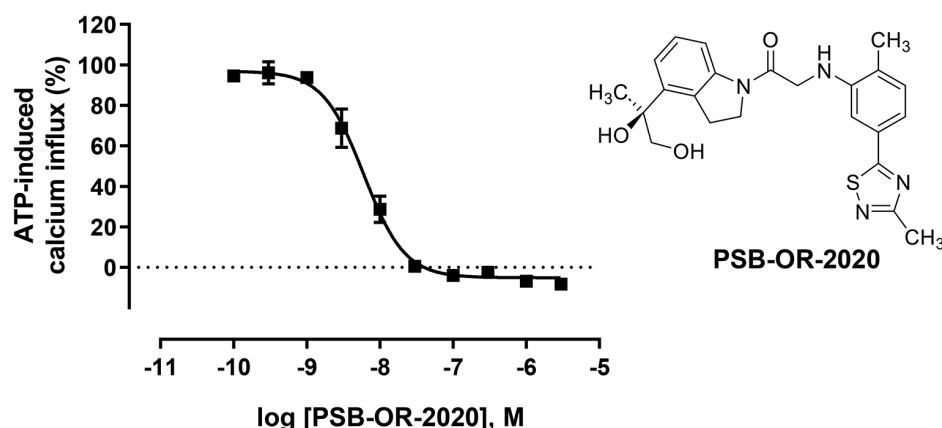


Table 1 Potency of PSB-OR-2020 as antagonist of human P2X receptor subtypes

P2X receptor subtype	$\text{IC}_{50} \pm \text{SEM}$ (or percent inhibition at indicated concentration) ^a
Human P2X4	6.32 ± 1.52 nM
Human P2X1	> 10,000 nM (9%)
Human P2X2	> 10,000 nM (6%)
Human P2X3	$2,030 \pm 480$ nM
Human P2X7	> 10,000 nM (0%) ^b

^aInhibition of ATP-induced signal (EC_{80} concentration: P2X4, 300 nM; P2X1, 100 nM; P2X2, 1,000 nM; P2X3, 100 nM) in the presence of PSB-OR-2020 (10 μM) was determined in 2–3 independent experiments performed in duplicates. Data points represent means $\pm$ SEM

^bFor activation of the human P2X7 receptor, the agonist 2'(3')-O-(4-benzoylbenzoyl)adenosine-5'-triphosphate (Bz-ATP) was used (EC_{80} concentration, 6,000 nM)

indolylcarboxymethylaniline derivatives which were subsequently optimized resulting in highly potent P2X4 receptor antagonists, e.g., PSB-15417, with IC_{50} values in the low nanomolar range determined in calcium influx assays (patent application filed in December 2022). Based on the discovery of this new scaffold, we developed a series of related dihydroindole analogs and selected one of the most potent antagonists of this series, (S)-1-(4-(1,2-dihydroxypropane-2-yl)indolin-1-yl)-2-((2-methyl-5-(3-methyl-1,2,4-thiadiazol-5-yl)phenyl)amino)ethane-1-one, designated PSB-OR-2020, for the preparation of a radioligand (see Fig. 2). The selection was also based on sufficient polarity of the compound to avoid high non-specific binding.

Characterization of the unlabeled P2X4 receptor antagonist PSB-OR-2020 in calcium influx assays

The unlabeled PSB-OR-2020 was characterized as a highly potent and selective P2X4 receptor antagonist. Calcium influx assays using 1321N1 astrocytoma cells that stably expressed the human P2X4 receptor were performed.

Concentration-dependent inhibition of ATP-induced calcium influx was observed. The employed ATP concentration corresponded to its EC_{80} value (300 nM), and an IC_{50} value of 6.32 ± 1.52 nM was determined for the antagonist PSB-OR-2020 (see Fig. 2).

The selectivity of PSB-OR-2020 versus other P2X receptor subtypes was determined in the same type of assay using 1321N1 astrocytoma cells stably expressing the respective human P2X receptor subtype (see Table 1).

At the other human P2X receptor subtypes, P2X1, P2X2, P2X3, and P2X7, inhibition of the agonist-induced signal by a high concentration of PSB-OR-2020 (10 μM) was well below 50%, except for the P2X3 receptor. Thus, a concentration-inhibition curve was determined, resulting in an IC_{50} value of $2,030 \pm 480$ nM at the P2X3 receptor. This value is about 300-fold higher than the compound's IC_{50} value at the P2X4 receptor. Thus, PSB-OR-2020 is not only a potent, but also a highly subtype-selective P2X4 receptor antagonist.

Radiolabeling

Having a P2X4 receptor antagonist with low nanomolar potency and high selectivity in hand, we designed a precursor molecule that would be suitable for catalytic hydrogenation with tritium gas ($^3\text{H}_2$) to obtain PSB-OR-2020 in ^3H -labeled form. Thus, we introduced two Br atoms, one on each aromatic ring of the scaffold, which was expected to result in sufficiently high specific radioactivity after tritiation due to the possible introduction of two tritium atoms. The final tritiation of the brominated precursor was performed by catalytic hydrogenation (custom-labeling), which led to the displacement of the bromine by tritium atoms (see Fig. 3).

^3H PSB-OR-2020 was obtained in high purity (97.7%). It showed a specific activity of 45 Ci/mmol (1.67 TBq/mmol) indicating a good tritiation efficiency (introduction of ca. 1.5 tritium atoms per molecule, for analytical details see Figs. S1 and S2 of Supporting Information).

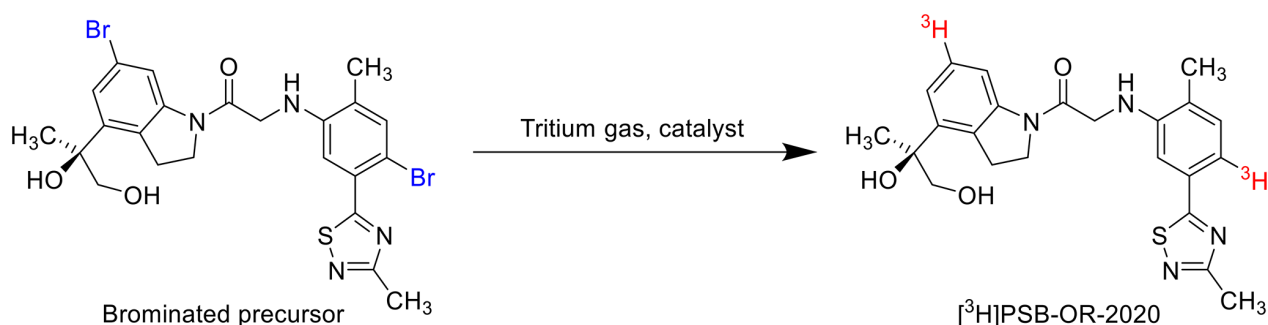


Fig. 3 Synthesis of [³H]PSB-OR-2020 by catalytic hydrogenation with tritium gas

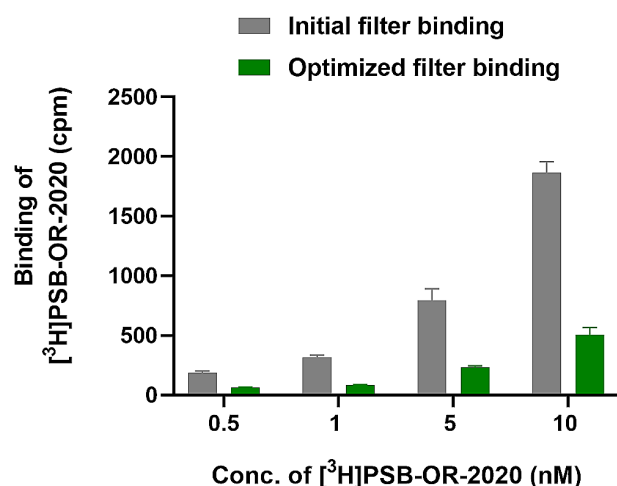


Fig. 4 Optimization of filter binding. Gray bars: initial GF/B filter binding of [³H]PSB-OR-2020 (assay buffer: ice-cold 50 mM Tris-HCl, pH 7.4; assay volume: 1 mL; washing buffer: ice-cold 50 mM Tris-HCl, pH 7.4 containing 0.1% BSA; incubation on ice for 30 min; filter was dried at room temperature for 30 min). Green bars: optimized assay conditions showing decreased non-specific radioligand binding to GF/C filters (assay buffer: ice-cold 50 mM Tris-HCl, pH 7.4 containing 0.1% BSA; assay volume: 500 μ L; washing buffer: ice-cold 50 mM Tris-HCl, pH 7.4; incubation on ice for 30 min; filter was dried at 70 $^{\circ}$ C for 60–90 min)

Development of a radioligand binding assay

As a next step, the new radioligand was utilized to establish a P2X₄ receptor radioligand binding assay. The initially observed binding of [³H]PSB-OR-2020 to GF/B glass fiber filters was relatively high (up to 2000 cpm at a radioligand concentration of 10 nM) using ice-cold Tris-HCl buffer (50 mM, pH 7.4) in a final volume of 1 mL at 4 $^{\circ}$ C, and ice-cold Tris-HCl buffer (50 mM, pH 7.4) containing 0.1% bovine serum albumin (BSA) as washing buffer. The GF/B filters were dried for 30 min at room temperature before transferring them into scintillation vials. To reduce filter binding, several experimental parameters were varied, including buffer, additives, filter types, and filter pretreatment. Reduced filter binding (to ca. 500 cpm at a radioligand concentration

of 10 nM) was achieved by using an assay buffer composed of 50 mM Tris-HCl, pH 7.4, containing 0.1% BSA, incubation at 4 $^{\circ}$ C, a washing buffer consisting of ice-cold 50 mM Tris-HCl, pH 7.4, and filtration through GF/C filters, which were dried at 70 $^{\circ}$ C for 60–90 min after harvesting (see Fig. 4). GF/C glass fiber filters are thinner and have a larger pore size of 1.2 μ m, in comparison to GF/B filters (1.0 μ m).

As a next step, experiments with membrane preparations derived from recombinant, P2X₄ receptor-expressing 1321N1 astrocytoma cells were performed. In these cells, the human P2X₄ receptor was stably expressed using a retroviral expression system [49]. Non-specific binding was determined in the presence of unlabeled PSB-OR-2020 (either 500 nM or 10 μ M). Preliminary experiments using 3 nM or 7 nM of [³H]PSB-OR-2020, respectively, indicated specific binding of [³H]PSB-OR-2020 to the P2X₄ receptor-expressing cell membrane preparations, but non-specific binding was still high (> 50% of total binding, data not shown).

To decrease non-specific radioligand binding to the cell membrane preparations, various compositions of assay and washing buffer were tested. The washing buffer was supplemented with magnesium chloride, copper chloride, ethylenediamine tetraacetic acid (EDTA), ethylene glycol tetraacetic acid (EGTA), acetic acid or sodium phosphate. Since the radioligand is well soluble in ethanol, 5% of ethanol were added to the washing buffer (ice-cold 50 mM Tris-HCl, pH 7.4) to remove unbound radioligand. Preincubation of GF/C filters in polyethylenimine solutions of different concentrations, in trimethylchlorosilane solution, or in washing buffer containing 5% of ethanol, was investigated. However, none of these modifications led to a sufficient reduction in non-specific radioligand binding, and the percentage of specific binding of [³H]PSB-OR-2020 was still too low. The radioligand's non-specific binding appeared to be mainly attributed to binding non-specifically to the membrane preparation, while filter binding was moderate after it had been optimized. The low percentage of specific radioligand binding may additionally be explained by a rather low expression level of the P2X₄ receptor.

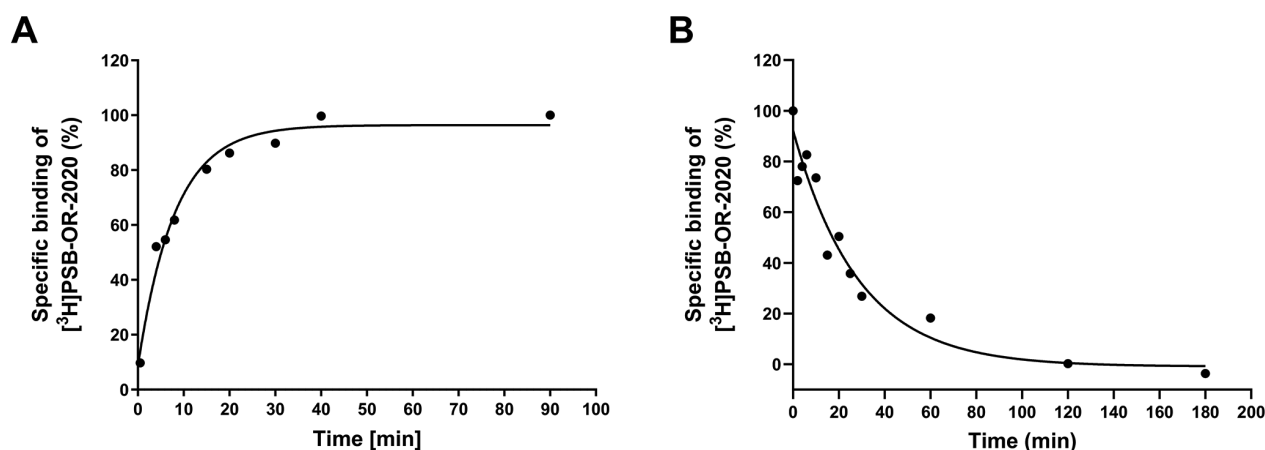


Fig. 5 Representative curves for association (**A**) and dissociation (**B**) of $[^3\text{H}]\text{PSB-OR-2020}$ (10 nM) to HEK293 cell membrane preparations recombinantly expressing the human P2X4 receptor, performed at 4 °C

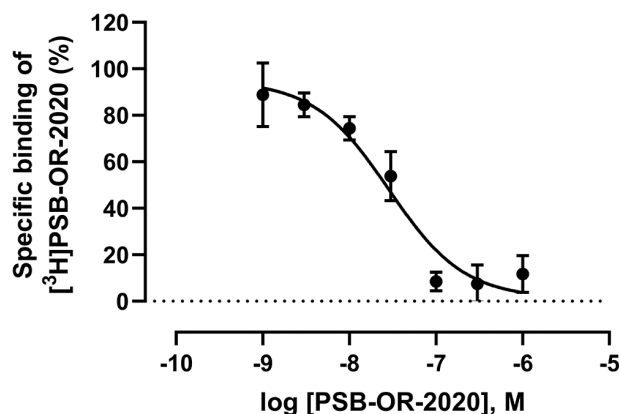


Fig. 6 Competition binding of different concentrations of PSB-OR-2020 vs. $[^3\text{H}]\text{PSB-OR-2020}$ (10 nM) using membrane preparations of HEK293 cells recombinantly expressing the human P2X4 receptor. Data points represent means $\pm$ SEM of three independent experiments performed in duplicates. K_D value of PSB-OR-2020: 20.4 ± 7.5 nM; B_{max} value: 71.7 fmol/mg of protein

Thus, new cell lines were created with the aim to obtain higher receptor expression. Membrane preparations of the resulting cell lines were used for radioligand binding studies to select the most highly expressing, and thus best suitable one. In addition to the previously described 1321N1 astrocytoma cell line, two further P2X4 receptor-expressing cell lines were studied: (i) HEK293 cells transiently expressing the human P2X4 receptor (plasmid: pQCXIN), (ii) HEK293 cells transiently expressing the human P2X4 receptor (plasmid: pcDNA3.1(-)). HEK293 cells transiently expressing the human P2X4 receptor (plasmid: pQCXIN) showed a higher expression level and resulted in a consistent, reproducible, and sufficiently low percentage of non-specific binding.

The optimal conditions for the binding assays were as follows: a final volume of 500 μL of Tris-HCl buffer (50

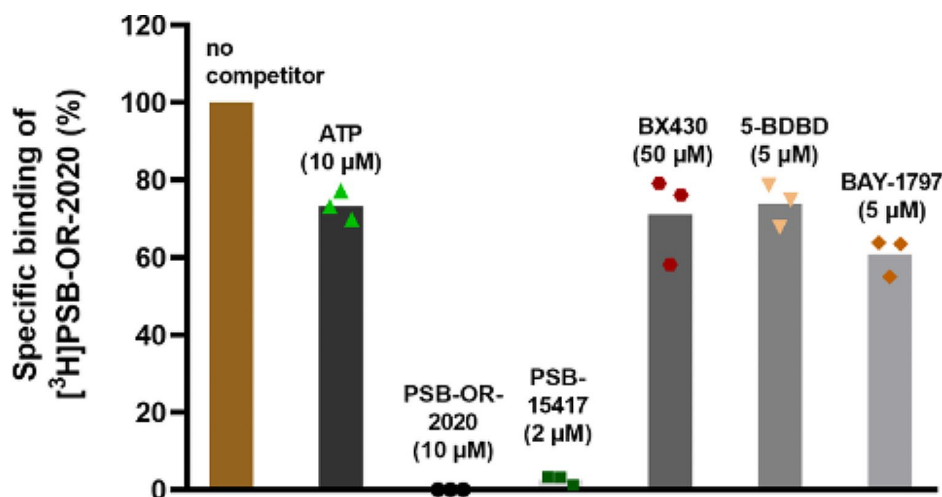
mM), pH 7.4, containing 0.1% BSA, 10 nM radioligand, 50–150 μg of protein (membrane preparation of HEK293 cells recombinantly expressing P2X4), incubation for 60 min at 4 °C, filtration through GF/C filters, and washing with ice-cold Tris-HCl buffer (50 mM), pH 7.4, containing 5% ethanol. Non-specific binding was determined in the presence of unlabeled PSB-OR-2020 (10 μM).

To characterize the binding kinetics of the radioligand, we performed association and dissociation experiments at 4 °C (see Fig. 5A and B). $[^3\text{H}]\text{PSB-OR-2020}$ associated with a half-life of 8.44 ± 2.59 min, while the dissociation half-life was 20.4 ± 2.9 min. Three independent association and dissociation experiments were performed to determine the kinetic K_D value ($k_{\text{off}}/k_{\text{on}}$) for $[^3\text{H}]\text{PSB-OR-2020}$, which was calculated to be 7.06 ± 3.23 nM.

We subsequently performed homologous competition binding studies, and determined a concentration-inhibition curve for the unlabeled ligand PSB-OR-2020 (see Fig. 6). A K_D value of 20.4 ± 7.5 nM was calculated from three independent experiments performed in duplicates. The B_{max} value was 71.7 fmol/mg of protein, and thus still not very high. The K_D value correlated well with the kinetic K_D value (7.06 ± 3.23 nM) and also with the IC_{50} value of the unlabeled PSB-OR-2020 determined in calcium influx assays (6.32 ± 1.52 nM) versus the EC_{80} concentration of the agonist ATP (300 nM).

As a next step, the effects of structurally diverse modulators of the human P2X4 receptor were studied (Fig. 7). Based on the IC_{50} values determined in calcium influx assays, and considering the compounds' solubilities, high concentrations were employed that were expected to result in a complete blockade of the receptor. PSB-15417 completely displaced the radioligand from its binding site, suggesting that the radioligand and PSB-15417 (both being structurally related compounds) bind to the same allosteric site of the

Fig. 7 Specific [^3H]PSB-OR-2020 binding to the human P2X4 receptor in the absence and presence of ATP and various P2X4 receptor antagonists utilizing membrane preparations of HEK293 cells transiently transfected with the human P2X4 receptor. Data represent means of three independent experiments performed in duplicates and triplicates. The final concentrations of the compounds are shown in brackets



human P2X4 receptor. In contrast, a high concentration of ATP only slightly displaced the radioligand from its binding site, providing evidence for an allosteric binding mode of [^3H]PSB-OR-2020 (Fig. 7). The structurally diverse P2X4 receptor antagonists BX430, 5-BDBD, and BAY-1797 (for structures see Fig. 1) also only partially displaced the radioligand from its binding site by less than 40%, even though high concentrations were employed. Thus, we assume that 5-BDBD, BX430 and BAY-1797 bind to a site or sites different from that of [^3H]PSB-OR-2020.

Discussion

Purinergic signaling is involved in many physiological processes [52]. The balance between extracellular concentrations of pro-inflammatory ATP and anti-inflammatory adenosine is crucial for homeostasis [53, 54]. Under inflammatory conditions, extracellular ATP concentrations may rise leading to the activation of P2X receptors [55]. These receptors are currently in the focus of interest as novel drug targets. The first P2X3 receptor antagonist, gefapixant, has recently been approved for the treatment of chronic cough, characterized by lung inflammation [56, 57]. P2X4 receptor antagonists are potential drug targets for inflammatory and neurodegenerative diseases including neuropathic pain [58], inflammatory pain [58], Parkinson's disease [22], Alzheimer's disease [21], multiple sclerosis [59], epilepsy [24], and cancer [28–30]. To study P2X4 receptor pharmacology, suitable tool compounds are required. Up to now, no radio- or fluorescence-labeled antagonist suitable for P2X4 receptor binding studies has been developed. This might be attributed to the fact that most of the P2X4 receptor antagonists described so far are only moderately potent and/or display low water-solubility resulting in high non-specific binding to cell membranes. In the present study,

we describe the development of the tritium-labeled P2X4 receptor antagonist [^3H]PSB-OR-2020 belonging to a novel chemical class of allosteric P2X4 receptor antagonists, and present its preliminary characterization. Major hurdles on the way to develop a binding assay were the compound's relatively high glass fiber filter binding, and its non-specific binding to cell membranes. Moreover, it was hampered by the low expression of the P2X4 receptor in the available cell lines. Nevertheless, we managed to perform kinetic studies to determine the radioligand's kinetic K_D value of 7.06 nM, and competition binding assays resulting in a K_D value of 20.4 nM. These data confirmed the high affinity of [^3H]PSB-OR-2020, which is well in agreement with its IC_{50} value obtained in calcium influx assays.

In future studies, more highly expressing cell lines, or isolated P2X4 receptor protein reconstituted into nanodiscs or amphipols, could be advantageous to further increase specific binding and to reduce non-specific binding.

Our radioligand represents an allosteric P2X4 receptor antagonist with a novel chemical structure. ATP does not compete with its binding to the receptor. Since the radioligand could also not or only partially be displaced by some previously published P2X4 receptor antagonists, including BX430, 5-BDBD, and BAY-1797, we assume that those antagonists bind to different allosteric binding sites.

Different allosteric binding sites, besides the orthosteric site, have been described for P2X receptor antagonists, as revealed by X-ray crystallography, cryo-EM, and mutagenesis studies [5, 36, 60, 61, 62]. Recently, the allosteric binding site of BX430 and BAY-1797 on the zebrafish P2X4 receptor was determined by cryo-EM to be located in the extracellular domain of the receptor, at the inter-subunit interface of the receptor trimer [5]. Our competition binding assays showed that the binding site of the new radioligand [^3H]PSB-OR-2020 at the human P2X4 receptor, to which its structurally related antagonist PSB-15417 binds, likely

differs from the allosteric binding site of the structurally different P2X₄ receptor antagonists BX430 and BAY-1797, and thus appears to occupy a novel allosteric site.

Conclusion

In conclusion, we have successfully designed and synthesized a P2X₄ receptor radioligand which allowed for the first time to establish a binding assay for this therapeutically relevant receptor. [³H]PSB-OR-2020 may become a useful tool in P2X₄ receptor pharmacology and drug development.

Methods

Chemicals

Chemicals were obtained from Roth (Karlsruhe, Germany), AppliChem (Darmstadt, Germany) or Thermo Fisher Scientific (Darmstadt, Germany). The radioligand [³H]PSB-OR-2020 (45 Ci/mmol; 1.67 TBq/mmol) was produced by custom-labeling (Pharmaron UK Ltd., Cardiff, UK), see Supporting Information.

Retroviral transfection

The 1321N1 astrocytoma cell line stably expressing the human P2X₄ receptor was generated using GP+envAM-12 fibroblast-type mouse packaging cells to produce a recombinant helper virus as described before [63]. To produce recombinant helper viruses, 6.25 µg of the plasmid DNA (human P2X₄ receptor DNA in pQCXIN) and 3.75 µg of vesicular stomatitis virus G (VSV-G) protein DNA (in pcDNA3.1) were combined. Subsequently, packaging cells were transfected using lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA) as a transfection reagent. After harvesting the virus particles, the target cells (non-transfected 1321N1 astrocytoma cells) were infected. 48–72 h after infection, the selection was started using the selection marker G418 (800 µg/mL). Cells were cultured in Dulbecco's Modified Eagle medium (DMEM) (Thermo Fisher Scientific, Darmstadt, Germany), 10% fetal calf serum (FCS) (PAN Biotech, Aidenbach, Germany), a mixture of penicillin/streptomycin (100 U/mL penicillin, 100 µg/mL streptomycin) (PAN Biotech, Aidenbach, Germany), and the selection antibiotic geneticin G418 (800 µg/mL). Cells were grown at 37 °C and 10% CO₂.

Transient transfection

HEK293 cells were transiently transfected with the human P2X₄ DNA in pQCXIN or pcDNA3.1(-). Transfection was

conducted with 10 µg of the plasmid DNA and a mixture of Opti-MEM™ medium (Thermo Fisher Scientific, Darmstadt, Germany) and lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA) that served as the transfection reagent. The selection was started after 24–72 h using DMEM, 10% FCS, a mixture of penicillin/streptomycin (100 U/mL penicillin, 100 µg/mL streptomycin) and G418 (800 µg/mL). Cells were grown at 37 °C and 5% CO₂.

Membrane preparation

Cells expressing the human P2X₄ receptor were grown to 70–90% confluence in cell culture dishes at 37 °C and 10% CO₂. After removing the medium, the cells were washed with phosphate buffer (phosphate-buffered saline (PBS)), and frozen at -20 °C. The next day, the cells were scraped off the dishes after adding 1 mL of cold hypotonic buffer (50 mM Tris-HCl, 1 mM EDTA, pH 7.4) per dish. All steps were carried out on ice. After homogenizing the cell suspension using an UltraTurrax® (IKA Labortechnik, Staufen, Germany), the homogenate was centrifuged for 10 min at 1,000 *g* and 4 °C. The supernatant was centrifuged again for 60 min at 48,000 *g* and 4 °C. The remaining pellet was resuspended in 50 mM Tris-HCl buffer, pH 7.4, and stored in aliquots at -80 °C.

Protein concentration determination with the Bradford assay [64]

To estimate the protein concentration of the membrane preparations, a Bradford assay was performed. The Bradford stock solution contained 0.1 g of Coomassie-Brilliant Blue G dissolved in 50 mL ethanol (v/v) with 100 mL of 85% phosphoric acid, diluted to 1000 mL with water. A BSA calibration curve (100 µg/mL – 500 µg/mL) was prepared in 50 mM Tris-HCl buffer, pH 7.4. The membrane preparations were diluted 1:10 and 1:20 with 50 mM Tris-HCl buffer, pH 7.4. Then, 1000 µL of the Bradford stock solution and 50 µL of the sample were mixed in a cuvette. The samples were measured at 595 nm and the final protein concentration was determined based on a BSA calibration curve.

Radioligand binding studies

Kinetic and competition binding studies using membrane preparations from HEK293 cells recombinantly expressing the human P2X₄ receptor were performed in 50 mM Tris-HCl, pH 7.4, and 0.1% BSA at 4 °C, in a final assay volume of 500 µL. The assay vials contained either 5 µL of the test compound (dissolved in dimethyl sulfoxide (DMSO)) or 5 µL of DMSO, 50 µL of [³H]PSB-OR-2020 in assay buffer (10 nM), and 50 µL of protein in 50 mM Tris-HCl buffer, pH

7.4. Non-specific and total binding of [³H]PSB-OR-2020 were determined using 10 μ M of unlabeled PSB-OR-2020 dissolved in DMSO, and 5 μ L of DMSO, respectively. To adjust a suitable amount of protein, the specific binding of the radioligand was measured for each newly generated membrane preparation. After adding the cell membrane suspension (50–150 μ g of protein), the incubation was started and lasted for 60 min at 4 °C with gentle shaking. For association experiments, 50 μ L of the cell membrane suspension were added at specific time points over a 90 min period. For dissociation experiments, the radioligand was first incubated with the cell membrane suspension for 1 h. Then, 5 μ L of unlabeled PSB-OR-2020 (10 μ M) were added at specific time points (ranging from 180 min to 0 min) to initiate dissociation. The incubation was terminated by rapid vacuum filtration using a Brandel 24-well harvester (Brandel, Gaithersburg, MD, USA), and washing with 3 $\times$ 3 mL of ice-cold washing buffer (50 mM Tris-HCl buffer, pH 7.4, containing 5% ethanol) through Whatman GF/C glass fiber filters (GE Healthcare Life Sciences Whatman™, USA). The filters were preincubated in ice-cold washing buffer for 30 min. After filtration, the filters were punched out and soaked in 2.5 mL of scintillation cocktail (ProSafe FC+, Meridian Bio-technologies Ltd., Surrey, UK) for at least 6 h before being counted using a liquid scintillation counter (Tri-Carb® 2900 TR, Perkin-Elmer, Rodgau, Germany, 53% efficiency).

Calcium influx assay

To determine the effects of the compound on P2X₄, P2X₂, and P2X₇ receptors, the agonist-mediated increases in cytosolic Ca²⁺ concentrations were measured using the fluorescent Ca²⁺ chelating dye Fluo-4 acetoxymethyl ester (AM) (Thermo Fisher Scientific). On the first day, 1321N1 astrocytoma cells (45,000 cells/well) stably expressing the respective P2X receptor were seeded into a 96-well plate (No. 3340, Corning, Kennebunk, Maine, USA), and incubated at 37 °C and 10% of CO₂. The next day, the cells were incubated with Fluo-4 AM (3 μ M) in Hanks' balanced salt solution and 1% of Pluronic® F127 (Sigma Aldrich, St. Louis, MO, USA) for 1 h at room temperature with slight shaking. For human P2X₇ receptors, a different assay buffer was used (150 mM Na-glutamate, 5 mM KCl, 0.5 mM CaCl₂, 0.1 mM MgCl₂, 10 mM D-glucose, 25 mM HEPES, pH 7.4). Calcium 5 (Molecular Devices, San Jose, CA, USA) was used as a fluorescent dye to investigate recombinantly expressed human P2X₁ and P2X₃ receptors. The cells were loaded with Calcium 5 dye in HBSS buffer, and incubated for 1 h at 37 °C and 10% CO₂ for human P2X₁, and 5% CO₂ for human P2X₃ receptors.

Following the incubation, the dye solution was carefully removed, and the antagonist (dissolved in DMSO) and HBSS buffer were added to the cells. The final DMSO concentration was 1% for human P2X₄, P2X₂, and P2X₇ receptors, and 0.5% for human P2X₁ and P2X₃ receptors. To activate the receptors, transparent 96-well reagent plates (Boettger, Bodenmais, Germany) containing the agonists dissolved in HBSS buffer (10-fold concentration of previously determined EC₈₀) were prepared. The plates were measured after 30 min of preincubation with antagonist with the imaging plate reader NOVOstar (BMG Labtech GmbH, Offenburg, Germany) at 520 nm for 30 s at 0.4 s intervals.

Data analysis

Data were analyzed using Microsoft Excel and PRISM Version 8.0 (GraphPad Prism 8, San Diego, CA).

Supplementary Information The online version contains supplementary material available at <https://doi.org/10.1007/s11302-024-10005-2>.

Author contributions J.N. and C.E.M. wrote the manuscript with contributions by all coauthors. All authors contributed to the study and reviewed the final manuscript.

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Data availability Data is provided within the manuscript or supplementary information files. The authors filed a patent on the new class of P2X₄ receptor antagonists including the radioligand described in this study.

Declarations

Ethical approval Not applicable.

Competing interests The authors filed a patent on the new class of P2X₄ receptor antagonists including the radioligand described in this study.

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Supporting Information

Preparation and preliminary evaluation of a tritium-labeled allosteric P2X4 receptor antagonist

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CAUTION-RADIOACTIVE MATERIAL

Product Specification

Pharmaron UK Ltd
The Old Glassworks
Nettlefold Road, Cardiff, CF24 5JQ
Telephone: +44(0)2920 474900

**[³H]Orion Compound****TRQ42131****1 mCi, 37 MBq**

Before using this product, please read the instructions on the use, safe handling, storage and disposal of the material.

Technical Data

Specific Activity

Determined by:

Mass Spectrometry : 45 Ci/mmol 1.67 TBq/mmol

Molecular weight (at this specific activity) : 441.7

Date of analysis : 02 April 2020

Radiochemical concentration : 1 mCi/mL 37 MBq/mL

Radiochemical purity by ultra high performance liquid chromatography : 97.7%

Column: Acquity UPLC BEH C18 1.7 µm 50 x 2.1 mm

Solvent A: 15 mM Potassium dihydrogen phosphate in water pH

Solvent B: 2.2 Acetonitrile

Gradient T(min): 0 0.5 10 12 12.5 15
%B: 5 5 85 85 5 5

Temperature: 40°C

Flow rate: 0.9mL/min

Detection: Homogeneous radiochemical detection, UV at 210nm

Chemical Identity

The material co-chromatographs with client supplied material in the above chromatographic system. The mass spectrum is consistent with the proposed structure and a non labelled reference.

Packaging and storage

The material is supplied as a solution in ethanol in a borosilicate multidose vial. Storage at -20°C is recommended.

Preparation

Manufactured to Pharmaron UK Ltd procedures.

[³H]Orion Compound was made from tritium gas by a method developed by Pharmaron UK Ltd. The material was purified by HPLC.

Figure S1. Product specification sheet of [³H]PSB-OR-2020.

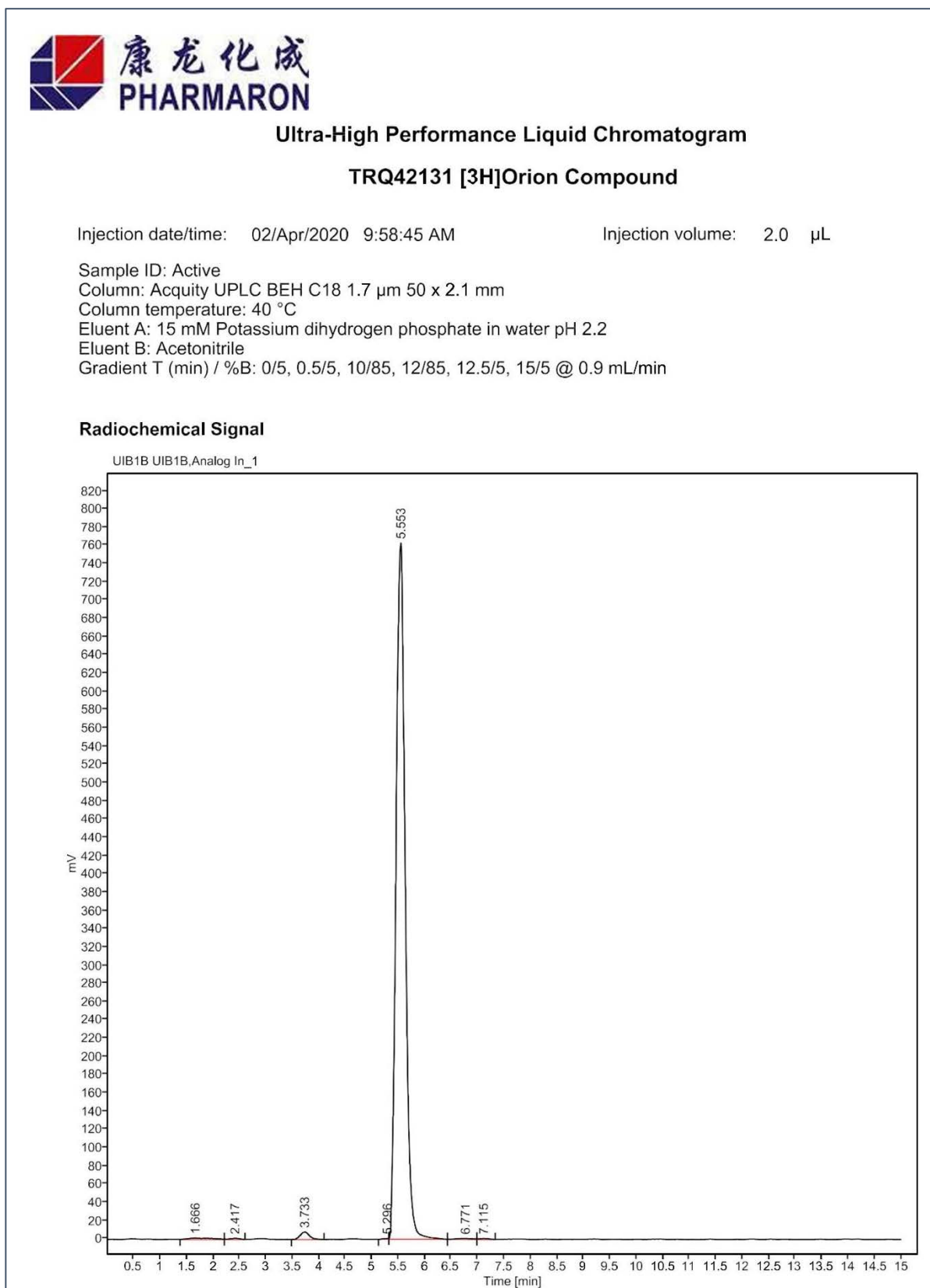


Figure S2. Ultra-high performance liquid chromatogram of [3 H]PSB-OR-2020) (page 1).



Ultra-High Performance Liquid Chromatogram

Signal: UIB1B UIB1B, Analog In_1

Name	Retention Time	Area	Area%
	1.666	49.5444	0.5
	2.417	18.0053	0.2
	3.733	100.3828	1.1
	5.296	7.5553	0.1
	5.553	9118.9207	97.7
	6.771	22.9159	0.2
	7.115	13.2217	0.1
	Sum	9330.5461	

Data file: /QC/Tritium_QC/TRQ42100 to TRQ42199/TRQ42131/Results/H3/TRQ42131_H3.rsl\TRQ42131-H3-1A_200402.dx
 Acq. method: TRQ42131_RCP.amx
 Result set: TRQ42131_H3
 Acq. operator: Lisa Edwards
 Report printed by Lisa Edwards

Figure S2. Ultra-high performance liquid chromatogram of [³H]PSB-OR-2020) (page 2).

7.2 Appendix B: Publication II - Extracellular binding sites of positive and negative allosteric P2X4 receptor modulators

The next pages contain the original article “Extracellular binding sites of positive and negative allosteric P2X4 receptor modulators” as it was published in *Life Sciences* by Elsevier.¹³⁴ According to the authors’ rights policy of the Elsevier publishing group, the authors of this Elsevier article retain the right to include it in a thesis or dissertation, as long as it is not published commercially. The main article and the supporting information are included in the next pages.



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Extracellular binding sites of positive and negative allosteric P2X4 receptor modulators

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ARTICLE INFO

Chemical compounds studied in this article:

Ivermectin (PubChem CID: 6321424)

BX430 (PubChem CID: 2810999)

Paroxetine (PubChem CID: 43815)

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Ivermectin

P2X4 receptor

BX430

Paroxetine

ABSTRACT

Aims: P2X receptors are ATP-gated ion channels which play a role in many pathophysiological conditions. They are considered as novel drug targets, particularly in the fields of pain, (neuro) inflammation, and cancer. Due to difficulties in developing drug-like orthosteric ligands that bind to the highly polar ATP binding site, the design of positive and negative allosteric modulators (PAMs and NAMs) is a promising strategy. The P2X4 receptor was proposed as a novel target for neuropathic and inflammatory pain (antagonists), and for the treatment of alcoholism (PAMs). So far, little is known about the allosteric binding site(s) of P2X4 receptors. The aim of this study was to identify the binding site(s) of the macrocyclic natural product ivermectin, the urea derivative BX430, and the antidepressant drug paroxetine that act as allosteric modulators of P2X4 receptors.

Material and methods: We generated chimeric receptors in which extracellular sequences of the human P2X4 receptor were exchanged for corresponding residues of the human P2X2 receptor, complemented by specific single amino acid residue mutants. Chimeric and mutated receptors were stably expressed in 1321N1 astrocytoma cells, and characterized by fluorimetric measurement of ATP-induced Ca^{2+} -influx. In addition, docking studies utilizing a homology model of the human P2X4 receptor were performed.

Key findings: Our results suggest a common binding site for ivermectin and BX430 in an extracellular receptor domain, while paroxetine might bind to the cation pore.

Significance: The obtained results provide a basis for the development of positive and negative allosteric P2X4 modulators with improved properties and will support future drug development efforts.

1. Introduction

P2X receptors are ligand-gated cation channels of the purine P2 receptor family [1,2]. They are activated by extracellular ATP (Fig. 1A) leading to an opening of the cation channel which is permeable for Ca^{2+} , K^{+} , and Na^{+} ions [3]. Seven P2X receptor subunits (P2X1-P2X7) exist,

which may form homo- or heteromeric assemblies of three subunits [4]. The pharmacological profiles of heterotrimers can differ from those of homotrimers, but knowledge about their relevance is restricted [5]. Five of the seven human (h) P2X subunits (P2X1-P2X4 and P2X7) can form functional homotrimeric ion channels [6]. In contrast, the human homotrimeric P2X5 receptor is nonfunctional in the majority of people

Abbreviations: AM, allosteric modulator; ATP, adenosine 5'-triphosphate; BX430, 1-(2,6-dibromo-4-isopropyl-phenyl)-3-(3-pyridyl)urea; CD39, ecto-nucleoside triphosphate diphosphohydrolase 1; DMEM, Dulbecco's Modified Eagle's Medium; DOPE, Discrete Optimized Protein Energy; EC_{50} -value, half maximal effective concentration; EC_{80} -value, effective concentration at 80 % of the maximum possible response; EDTA, ethylenediaminetetraacetic acid; FCS, fetal calf serum; G418, Geneticin 418; h, human; HCl, hydrochloric acid; HEK 293, human embryonic kidney type 293; HIV-1, human immunodeficiency virus type 1; IC_{50} -value, half-maximal inhibitory concentration; m, mouse; IVM, Ivermectin; MOE, Molecular Operating Environment; NAM, negative allosteric modulator; NCBI, National Center for Biotechnology Information; PAM, positive allosteric modulator; PCR, polymerase chain reaction; PDB, Protein Data Bank; PenStrep, penicillin, streptomycin; pEC_{50} -value, negative logarithm of the EC_{50} -value; pIC_{50} -value, negative logarithm of the IC_{50} -value; r, rat; SARS-CoV-2, severe acute respiratory syndrome coronavirus 2; SEM, standard error of the mean; T cells, thymus cells; TM, transmembrane region/domain; Tris, tris(hydroxymethyl)aminomethane; VTfM, Variable Target Function Method; wt, wild type; zf, zebrafish.

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due to the lacking of exon 10 [7]. The P2X6 receptor subunit shows a response to ATP only if it is coexpressed with P2X2 and/or P2X4 subunits [4].

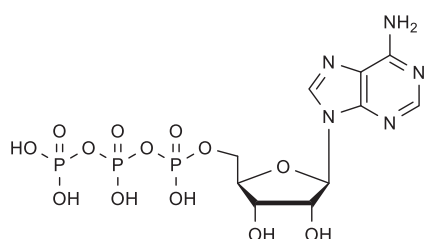
The first P2X receptor that had been crystallized was the zebrafish (zf) P2X4 receptor. X-ray crystal structures of apo (without ligand) and ATP-bound state were obtained [8,9]. The shape of each receptor subunit is reminiscent of a leaping dolphin with the transmembrane helices representing its flukes, and the extracellular domain forming its upper body [8]. The crystal structures have provided an improved understanding of the events related to ATP binding and receptor activation [10]. The negatively charged agonist ATP interacts with the three orthosteric binding sites of the receptor forming ionic interactions with several positively charged amino acid residues [8,9]. Thus, the development of orthosteric ligands, especially those with oral and brain bioavailability, might be difficult.

Several years later, X-ray crystal structures of the human P2X3 receptor in complex with the orthosteric, negatively charged antagonists TNP-ATP and A-317491 were reported, both antagonists preventing the receptor rearrangement that is required for channel opening [11].

The crystal structure of the human P2X3 receptor in complex with the negative allosteric modulator (NAM) gefapixant [12], a drug candidate for the treatment of chronic cough [13], provided insights into its allosteric binding site not far from the orthosteric site [12]. The crystal structure of the panda P2X7 receptor complexed with five different (structurally similar) antagonists provided further insights revealing a novel allosteric binding site located in a groove formed between two neighboring subunits in the upper body domain of the receptor [14,15].

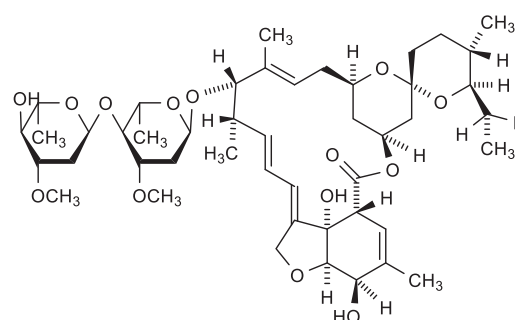
The P2X4 receptor subtype is mainly expressed in the central nervous system, especially in microglia [16] and also in glandular tissues and

A Physiological agonist



ATP (Adenosine 5'-triphosphate)

B Positive allosteric modulator



R = ethyl: Ivermectin H₂B_{1a} (main component, >90%)
R = methyl: Ivermectin H₂B_{1b} (minor component, <10%)

Ivermectin

C Negative allosteric modulators

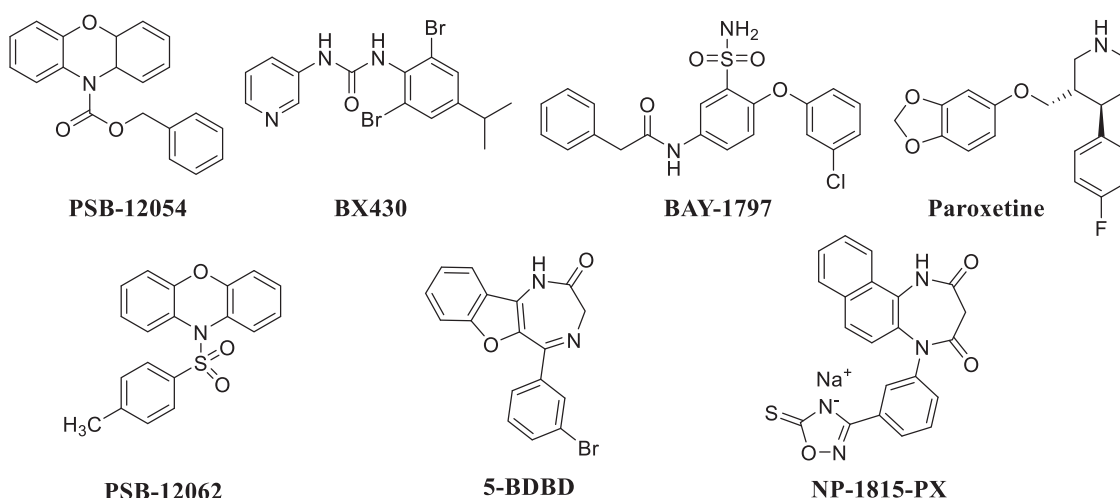


Fig. 1. Structures of P2X4 receptor ligands. A. ATP (adenosine 5'-triphosphate), the physiological agonist of the P2X4 receptor and all other P2X receptor subtypes. B. Ivermectin, a PAM of the P2X4 receptor. C. Negative allosteric modulators of the P2X4 receptor.

endothelial cells [17]. Microglial P2X4 receptors were found to be involved in the development of tactile allodynia [18] and neuropathic pain [19–21]. In case of nerve injury, spinal microglia is activated, and microglial P2X4 receptor expression is strongly upregulated [22] resulting in the release of brain-derived neurotrophic factor (BDNF) [19,22,23]. The blockade of P2X4 receptors has therefore been proposed as a promising strategy for the treatment of neuropathic pain [22,24], which has been proven in several animal models [25–28]. Further studies have proposed the contribution of P2X4 receptors to further neuroinflammatory pathologies including Alzheimer's disease [29,30], multiple sclerosis [31,32], epilepsy [33] and Parkinson's disease [34,35]. Recently, the P2X4 receptor has been described as a potential prognostic factor in prostate cancer [36]. Additionally, the P2X4 receptor appears to contribute to peripheral immune/inflammatory processes, including an increase in mast cell degranulation [37], and activation of T cells [38].

Otherwise, the activation of P2X4 receptors may be useful for the therapy of alcohol abuse disorders [39]. Ethanol induces the expression of P2X4 receptors in microglial cells [40] and acts as a NAM of P2X4 receptors [41]. Recently, CD39 (ecto-nucleoside triphosphate diphosphohydrolase 1) and the ATP-P2X4 signaling pathway were proposed as potential therapeutic targets for alcoholic steatohepatitis, an alcoholic liver disease [42].

The development of potent and selective orthosteric agonists and antagonists for the P2X4 receptor is particularly challenging because of the conserved ATP binding site throughout the P2X receptor family [5,43]. Therefore, research has focused on the identification of negative and positive allosteric P2X4 receptor modulators (NAMs or PAMs) [5,44]. In the past years, several PAMs (e.g. ivermectin, ginsenosides) and NAMs (e.g. 5-BDBD, BX430, paroxetine, NP-1815-PX, BAY-1797) with various potencies and physicochemical properties have been described for the P2X4 receptor (Fig. 1B, C) [4,44]. NC-2600 (structure undisclosed) was the first human P2X4 receptor antagonist to enter clinical trials (phase I) for the treatment of neuropathic pain, but no further development has been reported [45]. However, a recent study described anti-inflammatory effects of NC-2600 and NP-1815-PX in a murine model of colitis [46]. Additionally, PSB-12054 and PSB-12062 were discovered, displaying antagonistic effects at human P2X4 receptors, however with moderate selectivity [47]. We identified an allosteric P2X4 receptor antagonist, named PSB-15417, with nanomolar potency, brain-permeability and high selectivity versus the other P2X receptor subtypes showing efficacy in an animal model of neuropathic pain [28].

The binding sites of allosteric P2X4 receptor modulators are of great interest, but so far, only limited information is available regarding the interactions of PAMs (ivermectin) [48–50] and NAMs with the receptor [51,52]. A better knowledge of allosteric binding sites and interactions between allosteric modulators and the receptor could facilitate and advance drug development. In the present study, we focused on three allosteric modulators, the PAM ivermectin, and the NAMs BX430 [53] and paroxetine [54], which were reported to display high selectivity for P2X4 receptors versus other P2X receptor subtypes.

The macrocyclic natural product ivermectin (Fig. 1B) activates glutamate-gated chloride channels exclusively found in invertebrates, and is therefore used for the treatment of infections with ecto-parasites in human and veterinary medicine [55,56], e.g. for killing lice and ticks, mites and nematodes [57]. Besides, it is clinically used for the treatment of onchocerciasis (river blindness) [58] and lymphatic filariasis [59]. At higher concentrations, ivermectin also modulates mammalian ligand-gated ion channels including γ -aminobutyric acid type A (GABA_A) receptors, α -nicotinic receptors and glycine receptors [60].

Besides its potent PAM effects at P2X4 receptors, ivermectin was reported to additionally act as a PAM of human, but not mouse (m) or rat (r) P2X7 receptors [61]. Thus, ivermectin is generally used as an experimental tool in P2X4 receptor research [62,63], and has even been suggested as a drug for the treatment of alcohol abuse disorders due to

its P2X4 receptor-enhancing effects [39]. Recently, ivermectin was proposed for the treatment of SARS-CoV-2 (severe acute respiratory syndrome coronavirus 2) infections [64–68].

The urea derivative BX430 (Fig. 1C) was described as a potent and selective NAM of the human P2X4 receptor as determined in patch-clamp studies ($IC_{50} = 0.54 \mu M$), suggesting an interaction with the receptor from the extracellular domain [53]. At P2X4 receptors of rat and mouse, and at other P2X receptor subtypes, no significant inhibition was measured [53]. BX430 was able to inhibit the positive allosteric modulatory effect of ivermectin at the human P2X4 receptor [53].

Paroxetine (Fig. 1C) was developed as an antidepressant drug which potentially inhibits serotonin reuptake. At higher concentrations it acts as a non-competitive antagonist of P2X4 receptors ($IC_{50} = 1.87 \mu M$) showing beneficial effects in animal models of neuropathic pain [54,69].

In the present study, we designed a set of chimeric P2X4(P2X2) receptors and P2X4 receptor mutants with the aim to identify allosteric binding site(s) in the extracellular domain of the P2X4 receptor. We provide evidence for the interaction of ivermectin and BX430 with an extracellular P2X4 receptor binding site. Our results contribute to a deeper understanding of the allosteric modulation of ATP-activated P2X4 receptors and will support the development of potent and selective allosteric modulators, PAMs and NAMs, as novel drugs.

2. Materials and methods

2.1. Materials and reagents

Adenosine 5'-triphosphate disodium salt (ATP) was purchased from Roth (Karlsruhe, Germany). Ivermectin ($H_{2B_{1a}}$) was purchased from Sigma Aldrich (Darmstadt, Germany). BX430 was purchased from ThermoFisher Scientific (Waltham, MA, USA). The stock solutions of ivermectin, BX430 and paroxetine (10 mM) were prepared in dimethyl sulfoxide (Roth, Karlsruhe, Germany) and stored at $-20^{\circ}C$. Dulbecco's Modified Eagle's Medium (DMEM) for cell cultivation was purchased from ThermoFisher Scientific (Waltham, MA, USA). Fetal calf serum (FCS), Trypsin, penicillin-streptomycin (PenStrep) and Geneticin G418 were from PAN Biotech (Aidenbach, Germany). Lipofectamine 2000 was ordered at Invitrogen (Carlsbad, CA, USA). All restriction enzymes were purchased from New England Biolabs (Frankfurt, Germany).

2.2. Cultivation of cells

1321N1 astrocytoma cells stably transfected with human P2X4 wild type, chimeric P2X4(P2X2) receptors, receptor mutants as well as HEK 293 T packaging cells were grown in Dulbecco's Modified Eagle's Medium, supplemented with 10 % FCS, 1 % PenStrep (10,000 U/ml penicillin, 10 mg/ml streptomycin). 1321N1 astrocytoma cells were incubated at $37^{\circ}C$ with 10 % CO_2 , HEK 293 T cells with 5 % CO_2 .

2.3. Chimeric receptors

Chimeric P2X4(P2X2) receptors were constructed through gene synthesis (Geneart, Life technologies). All cDNAs were defined with attached EcoRI and BamHI restrictions sites. Delivered cDNAs were subcloned into the bicistronic lentiviral pLVX-IRES-mCherry plasmid (Clontech). Sequencing was performed by GATC (Konstanz, Germany).

2.4. Receptor mutants

Human P2X4 receptor mutants were generated by site-directed mutagenesis. The respective mutagenesis primers were synthesized by Invitrogen (Carlsbad, CA, USA). As a DNA template, the wt hP2X4 receptor in vector pUC19 was used. After confirming the correct mutation by sequencing, the hP2X4 receptor mutants were subcloned into the retroviral plasmid pQCXIN. Sequencing was performed by GATC (Konstanz, Germany).

2.5. Lentiviral transfection of 1321N1 astrocytoma cells

1321N1 astrocytoma cells were stably transfected with human P2X4 or chimeric P2X4(P2X2) receptors using a lentiviral transfection system. 1.5×10^6 HEK 293 T cells were plated in 25 cm² cell culture flasks using DMEM (10 % FCS, 1 % PenStrep). Four hours before transfection the medium was exchanged for DMEM (FCS, without antibiotics). The cells were infected using Xfect Reaction buffer, Lenti-X HTX Packaging-Mix and Xfect-Polymer (Clontech). After 5 h the medium was replaced by 3 ml DMEM (10 % tet-approved FCS (Clontech), 1 % PenStrep) containing 5 mM sodium butyrate. The transfected HEK 293 T cells were cultured at 32 °C and 5 % CO₂ for 48 h. The supernatant of the cell was collected and filtered. 5×10^5 1321N1 astrocytoma cells per 25 cm² flask were incubated for 2.5 h at 37 °C with 3 ml of supernatant containing 6 µl (4 mg/ml) of polybrene. After 48–72 h cells were selected for fluorescence emission at 610 nm with excitation of 561 nm. As a negative control untransfected 1321N1 astrocytoma cells were measured. Cells with high expression of mCherry protein were single sorted in 96-well plates using a BD FACS Aria III.

2.6. Retroviral transfection of 1321N1 astrocytoma cells

1321N1 astrocytoma cells were stably transfected with human P2X4, P2X1, P2X2, P2X3, P2X7, rP2X4, mP2X4 receptors and hP2X4 receptor mutants. For the expression of the hP2X3 receptor, the non-desensitizing S15V mutant was used. 1321N astrocytoma cells were stably transfected using GP+envAM-12 fibroblast-type mouse packaging cells to produce a recombinant helper virus as described before [70]. 1.5×10^6 GP+envAM-12 fibroblast-type mouse packaging cells were plated in 25 cm² cell culture flasks using DMEM (10 % FCS, 1 % PenStrep). Four hours before transfection, the medium was exchanged for DMEM (10 % FCS, without antibiotics). The transfection approach was conducted using 6.25 µg of the plasmid DNA (receptor DNA in pQCXIN) and 3.75 µg of VSV-G (vesicular stomatitis virus G protein) protein DNA. Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA) was used as a transfection reagent. After 12–15 h, the medium was replaced by DMEM (10 % FCS, 1 % PenStrep) containing 30 µl of sodium butyrate (500 mM in H₂O). The cells were cultivated at 32 °C for 48 h. 5×10^6 non-transfected 1321N1 astrocytoma cells were cultured in 25 cm² cell culture flasks using DMEM (10 % FCS, 1 % PenStrep) and then infected with the harvested virus particles. After adding polybrene solution (4 mg/ml in H₂O), the cells were incubated at 37 °C and 10 % CO₂. After 2.5 h the medium is replaced for DMEM with 10 % FCS and 1 % PenStrep. The antibiotic-based selection with G418 (800 µg/ml) started after 48–72 h.

2.7. Measurement of intracellular Ca²⁺ in transfected 1321N1 astrocytoma cells

For the measurement of intracellular Ca²⁺, the cells were prepared and the experiments were performed as described previously [47]. For all stably transfected 1321N1 astrocytoma cell lines, except for the human P2X1 and P2X3 receptor-expressing cells, the fluorescent Ca²⁺-chelating dye Fluo-4 AM (Thermo Fisher Scientific, Waltham, MA, USA) was used. For the human P2X1 and P2X3 receptor, the FLIPR® Calcium 4 or Calcium 5 Assay Kits were used (Molecular Devices, San José, CA, USA). Ivermectin, BX430 or paroxetine were added 30 min before the addition of the agonist ATP, applied at a concentration that corresponded to its maximal effect respectively at the EC₈₀. EC₈₀ concentrations of ATP at the wt P2X receptor subtypes were: hP2X4, 1 µM; hP2X1, 0.1 µM; hP2X2, 1 µM; hP2X3, 0.1 µM; hP2X7, 2000 µM. The measurement of fluorescence started directly after the addition of ATP using a fluorescence imaging plate reader, either Flex station (Molecular Devices, San José, CA, USA) or NOVOstar (BMG Labtech GmbH, Offenburg, Germany), in 96-well plates at an excitation wavelength of 485 nm and an emission wavelength of 520 nm. All calculated EC₅₀ and IC₅₀ values represent the means from at least three independent experiments.

2.8. Membrane preparations for radioligand binding assays

Membranes of 1321N1 astrocytoma cells expressing the hP2X4 receptor were prepared by scratching the cells off previously frozen cell culture dishes in ice-cold hypotonic buffer (50 mM Tris-HCl, 1 mM EDTA and 100 µM phenylmethylsulfonyl fluoride, pH 7.4 at 4 °C). The cell suspension was homogenized on ice for 20 s with an Ultra-Turrax. The homogenate was centrifuged for 20 min at 48,000g, the supernatant was discarded, and the pellet was resuspended in homogenizing buffer using the Polytron tissue disrupter (setting 5 for 5 s) and centrifuged as before. The pellet obtained was washed again by resuspension and centrifugation, then resuspended in 5 ml of homogenization buffer, and frozen at –80 °C. The frozen pellet was thawed, resuspended in 30 ml of homogenizing buffer using the Polytron tissue disrupter (setting 5 for 5 s), and centrifuged for 20 min at 48,000 g. This freeze/thaw cycle was repeated once, and the final pellet that was obtained was resuspended in assay buffer consisting of 50 mM Tris-HCl and 1 mM EDTA, pH 7.4 at 4 °C, and stored in aliquots at –80 °C.

2.9. Radioligand binding studies

All experiments were performed at room temperature in 50 mM Tris-HCl assay buffer containing 1 mM EDTA to complex divalent cations to avoid depletion of ATP through ATPase activity [71]. Assay vials containing 2.5 µl of test compound in DMSO, 100 µl of [³⁵S]ATPγS in a final concentration of 0.2 nM and 100 µl of cell membrane suspension (in a concentration of 10 µg of protein) were filled up with assay buffer to a final volume of 250 µl. Incubations of 1 h were started by the addition of receptor protein. Reactions were terminated by vacuum filtration over GF/B glass-fiber filters (GE Healthcare Life Sciences Whatman™, USA) using a Brandel 48-well harvester (Brandel, Gaithersburg, MD, USA). The filters were rinsed three times with ice-cold Tris-HCl buffer (50 mM, pH 7.4) to separate bound from free radioligand. The filters were punched out and transferred to 4 ml scintillation vials. Ultima Gold® scintillation cocktail (2.5 ml) was added and samples were counted after 6 h for 1 min each, using a liquid scintillation counter. Non-specific binding of [³⁵S]ATPγS was determined using 100 µM ATP. The experiments were performed in duplicates.

2.10. Statistical analysis

Graphpad Prism (4.0 or 8.0, Graph Pad Software, Inc., San Diego, CA, USA) was used for the calculation of EC₅₀ and IC₅₀ values by nonlinear regression with a sigmoidal dose-response equation. Results were expressed as means ± SEM, obtained in at least three independent experiments. Variations in means were tested for significance using an unpaired student's *t*-test in case results from just two groups were compared. If there was a control group and multiple comparison was required, the level of significance was calculated by an ordinary one-way ANOVA with Dunnett's post-hoc test. Significance was expected if *P* was <0.05. The significance levels were defined as follows: * *P* < 0.05, ** *P* < 0.01, *** *P* < 0.001, **** *P* < 0.0001.

2.11. Homology modeling

Homology models of human P2X4 receptors closed and open state were built based on the crystal structure of zebrafish P2X4 (PDB ID: 4DW0, 4DW1; sequence identity of 58.3 %). The sequence of the target protein, accession number GI:116242696 (UniProt ID: Q99571.2) was downloaded from NCBI server and aligned with the target sequence using the ClustalW. The resulting alignment was used as input to the MODELLER program [72] and optimized using the variable target function method (VTM). The highest rank model on the basis of Discrete Optimized Protein Energy (DOPE) score was selected for subsequent analysis.

2.12. Molecular docking

Docking simulations were performed using AutoDock 4.2 [73]. Prior to docking, protein structures were prepared using Molecular Operating Environment (MOE) [74] and input files for docking were generated using AutoDock Tools. A grid box for the docking studies was centered by including the amino acids from the regions of v6, v7 and v8, respectively. The size of the grid box was $80 \times 80 \times 110$ and the grid was centered on the amino acid residues from variable regions v6, v7 and v8. The three dimensional energy scoring grids were computed and ivermectin was docked into the binding site using the Lamarckian Genetic Algorithm in AutoDock4.2. Putative binding mode of ivermectin with the highest scoring (lowest energy) was selected by visual inspection.

3. Results and discussion

3.1. Chimeric P2X4(P2X2) receptors

We designed a series of chimeric P2X receptors, in which selected sequences of the hP2X4 receptor were replaced by corresponding sequences of the hP2X2 receptor (Fig. 2A) in order to identify and characterize potential extracellular allosteric binding sites of ivermectin, BX430, and paroxetine at the hP2X4 receptor. The rationale behind constructing these chimeric receptors was that ivermectin and BX430 act as modulators of hP2X4 receptors but do not show any significant effects on hP2X2 receptors, neither potentiation nor inhibition (Supporting Information Table 3), while paroxetine is 8-fold less potent at the hP2X2 receptor as compared to the hP2X4 receptor. The regions for the amino acid exchanges were selected on the basis of a sequence alignment of both P2X receptor subtypes.

The sequence of the hP2X7 receptor was additionally included for comparison because this was the only other P2X receptor subtype at which ivermectin had not shown any effects in our test systems (see below). Based on the alignment (Fig. 2A), we selected ten variable sequences, v1 to v10. The regions v1, and v6-v10 of the hP2X4 receptor were individually exchanged for the corresponding sequences of the hP2X2 receptor. The regions v2-v5 were combined and completely exchanged for the corresponding sequence of the hP2X2 receptor. According to the exchanged variable regions v1-v10, the resulting chimeric (c) P2X4(P2X2) receptors were designated c1, c2-c5, c6, c7, c8, c9 and c10. The chimeric receptor cDNAs were created through gene synthesis and subcloned into the pLVX-IRES mcherry vector, which is a human immunodeficiency virus type 1 (HIV-1)-based lentiviral expression vector. The seven chimeric receptors were stably expressed in 1321N1 astrocytoma cells. Single cells with high expression of red fluorescent mcherry protein were selected, isolated by fluorescence-activated cell sorting, and subsequently grown to confluence.

To characterize the various chimeric P2X4(P2X2) receptors, fluorimetric measurements of ATP-induced calcium influx were performed and compared with the results at both wildtype (wt) receptor subtypes. The wt hP2X4 and the wt hP2X2 receptors displayed EC_{50} values for ATP of $0.378 \mu\text{M}$ (P2X4) and $0.637 \mu\text{M}$ (P2X2) (Fig. 2D and Supporting Information Fig. S2). All chimeric receptors, except for c1 and c9, were significantly activated by ATP (Fig. 2E).

The exchange of the large region C116-T186 of the hP2X4 for the corresponding amino acid residues of the hP2X2 receptor (c2-c5^{C116-T186}) caused an about 7-fold reduction in the potency of ATP ($EC_{50} = 2.57 \mu\text{M}$), and the ATP effect was reduced by 50 % in comparison to the wt hP2X4 receptor. For the chimeric receptors c7^{I218-D224} and c10^{R301-Q308} a similar effect was seen with EC_{50} values of $1.14 \mu\text{M}$ (c7^{I218-D224}) and $0.862 \mu\text{M}$ (c10^{R301-Q308}), and ATP effects of 54 % (c7^{I218-D224}), and 40 % (c10^{R301-Q308}), respectively. For chimeric receptors c6^{N208-S216} and c8^{R265-L269} no significant differences in EC_{50} values were observed ($0.572 \mu\text{M}$ and $0.298 \mu\text{M}$), and ATP effects remained above 60 % (87 % and 61 %, respectively).

Chimeric receptors c1^{V71-I83} and c9^{T281-P290} showed almost no

activation by ATP with maximal effects of only 4–5 % compared to that of the wt hP2X4 receptor. Nevertheless, EC_{50} values, $4.83 \mu\text{M}$ for c1^{V71-I83} and $1.24 \mu\text{M}$ for c9^{T281-P290} (Fig. 2F), could still be estimated. They appeared to be only moderately reduced as compared to the wt hP2X4 receptor.

3.2. The positive allosteric P2X4 receptor modulator ivermectin

To investigate the effects of the allosteric modulator ivermectin at the hP2X4 receptor, concentration-response curves of the compound in the presence of $100 \mu\text{M}$ ATP were determined as ivermectin was previously described to increase the maximal response of saturating ATP concentrations [75]. This high concentration of agonist corresponds to the maximal effect of ATP at the hP2X4 receptor and at all created chimeric hP2X4(P2X2) receptors. Thus, wildtype and chimeric receptors were activated with ATP, and the effects induced by ivermectin were compared. The cells were pre-treated with ivermectin, the receptors were subsequently activated by addition of the respective ATP concentration, and the Ca^{2+} influx was fluorimetrically measured.

At the wt hP2X4 receptor, ivermectin potentiated the effect of ATP with an EC_{50} value of $0.310 \mu\text{M}$. At a high concentration of $10 \mu\text{M}$ of ivermectin, the maximal response for ATP was increased from 100 % to 167 %. In contrast, at the wt hP2X2 receptor, $10 \mu\text{M}$ ivermectin caused no potentiation of the ATP response. At the hP2X1 (74 % reduction of ATP response) and the hP2X3 receptor (49 %), high concentrations of ivermectin ($10 \mu\text{M}$) led to significant receptor inhibition (Fig. 3C, Supporting Information Table 1), while at the hP2X7 receptor neither activation nor inhibitory effects were observed in the calcium assays. This is in contrast to a published study which found positive allosteric modulation at the hP2X7 receptor [61]. Different ATP concentrations were used (1 mM versus 2 mM in our study). At these concentrations, cytotoxic effects may already be observed which could affect the observed results.

We subsequently determined IC_{50} values for ivermectin at hP2X1 and hP2X3 receptors in the presence of ATP at its EC_{80} concentration ($0.1 \mu\text{M}$). Ivermectin displayed a similar inhibitory potency at the hP2X1 and hP2X3 receptor (IC_{50} values: hP2X1 $2.27 \mu\text{M}$, hP2X3 $5.71 \mu\text{M}$).

This is the first demonstration that the PAM ivermectin can inhibit other P2X receptor subtypes. It had previously been observed that ivermectin did not affect the fractional calcium current through hP2X1 receptors which might have been expected for an inhibitor [76]. On the other hand, it was shown to negatively affect the fractional calcium current of hP2X4 receptors while potentiating ionic currents [76].

As a next step, the effects of ivermectin on the created chimeric P2X4 (P2X2) receptors were examined. To this end, concentration-response curves of ivermectin in the presence of a high concentration of ATP were determined, at which ATP displays a maximal effect. EC_{50} values were determined and compared to those obtained for the wt hP2X4 and hP2X2 receptors. A range of allosteric modulatory effects - positive, negative or biphasic - was observed depending on the exchanged sequence variation (Figs. 4 and 5).

3.2.1. Positive allosteric modulation of c2-c5^{C116-T186} and c10^{R301-Q308} chimeric P2X4(P2X2) receptors by ivermectin

At the chimeric receptors c2-c5^{C116-T186} and c10^{R301-Q308} ivermectin showed positive allosteric modulation of the ATP response with EC_{50} values of $0.119 \mu\text{M}$, and $1.15 \mu\text{M}$, respectively (Supporting Information Table 3). Ivermectin increased the maximal effect of ATP at the chimeric receptor c2-c5^{C116-T186} 2.74-fold and at the chimeric receptor c10^{R301-Q308} 1.75-fold (Fig. 4A). At both chimeric receptors ivermectin acted as an allosteric potentiator of the ATP-response, comparable with its effect at the wt hP2X4 receptor. This indicates that ivermectin is not interacting with amino acids within the regions v2-v5 and v10 of the P2X4 receptor.

A

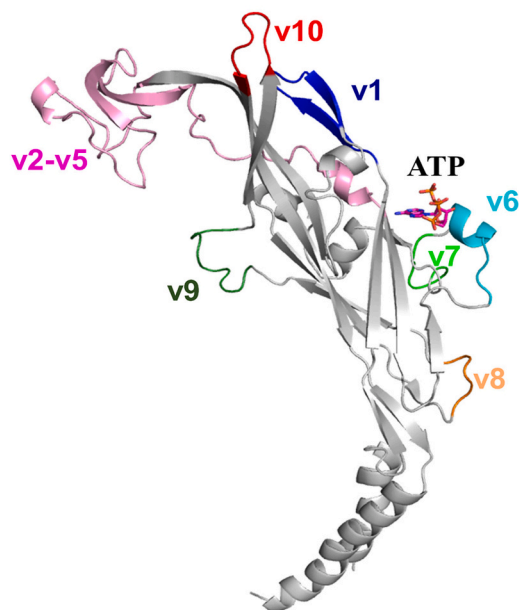
[illegible]**B**

Fig. 2. A. Sequence alignment of the human P2X4, P2X2 and P2X7 receptors. Stretches of non-conserved amino acid sequences were identified and labeled as variable regions v1 to v10. These variable regions are colored as follows: v1, blue; v2-v5, pink; v6, cyan; v7, green; v8, orange; v9, dark green; v10, red. B. The human P2X4 subunit with the variations v1-v10. C. Snake-like plot of the hP2X4 receptor. Colored residues were exchanged. The following chimeric receptors were obtained, in which the respective colored sequence was exchanged for the corresponding P2X2 receptor sequence: blue: v1, P2X4 (V71-I83 P2X2); pink: v2-v5, P2X4 (C116-T186 P2X2); cyan: v6, P2X4 (N208-S216 P2X2); green: v7, P2X4 (I218-D224 P2X2); orange: v8, P2X4 (R265-L269 P2X2); dark green: v9, P2X4 (T281-P290 hP2X2); red: v10, P2X4 (R301-Q308 P2X2). The ATP-binding sites are labeled in yellow. D. Potency of ATP determined by measurement of calcium influx at the chimeric receptor cell lines c1-c10 in comparison to the wt hP2X4 and hP2X2 receptors. E. Maximal ATP effects determined by measurement of calcium influx at the chimeric receptor cell lines c1-c10 in comparison to the wt hP2X4 and hP2X2 receptors. Carbachol (agonist of the muscarinic M3 receptor natively expressed in the cells) was used as internal reference for determining the maximal ATP-induced activation of P2X2, P2X4 and chimeric P2X4(P2X2) receptors. The maximal responses to 100 μ M of ATP were set in relation to the effect induced by 100 μ M carbachol (M3 receptor agonist) and normalized accordingly. F. Concentration-response curves of ATP at the chimeric receptors c1 and c9. Data represent means $\pm$ SEM of at least three independent experiments. The level of significance was calculated by an ordinary one-way ANOVA with Dunnett's post-hoc test: * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, **** $P < 0.0001$. Significance levels in black (*) represent the comparison between the chimeric receptors and the wt P2X4 receptor, significance levels in blue (*) represent the comparison between chimeric receptors and the wt hP2X2 receptor. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

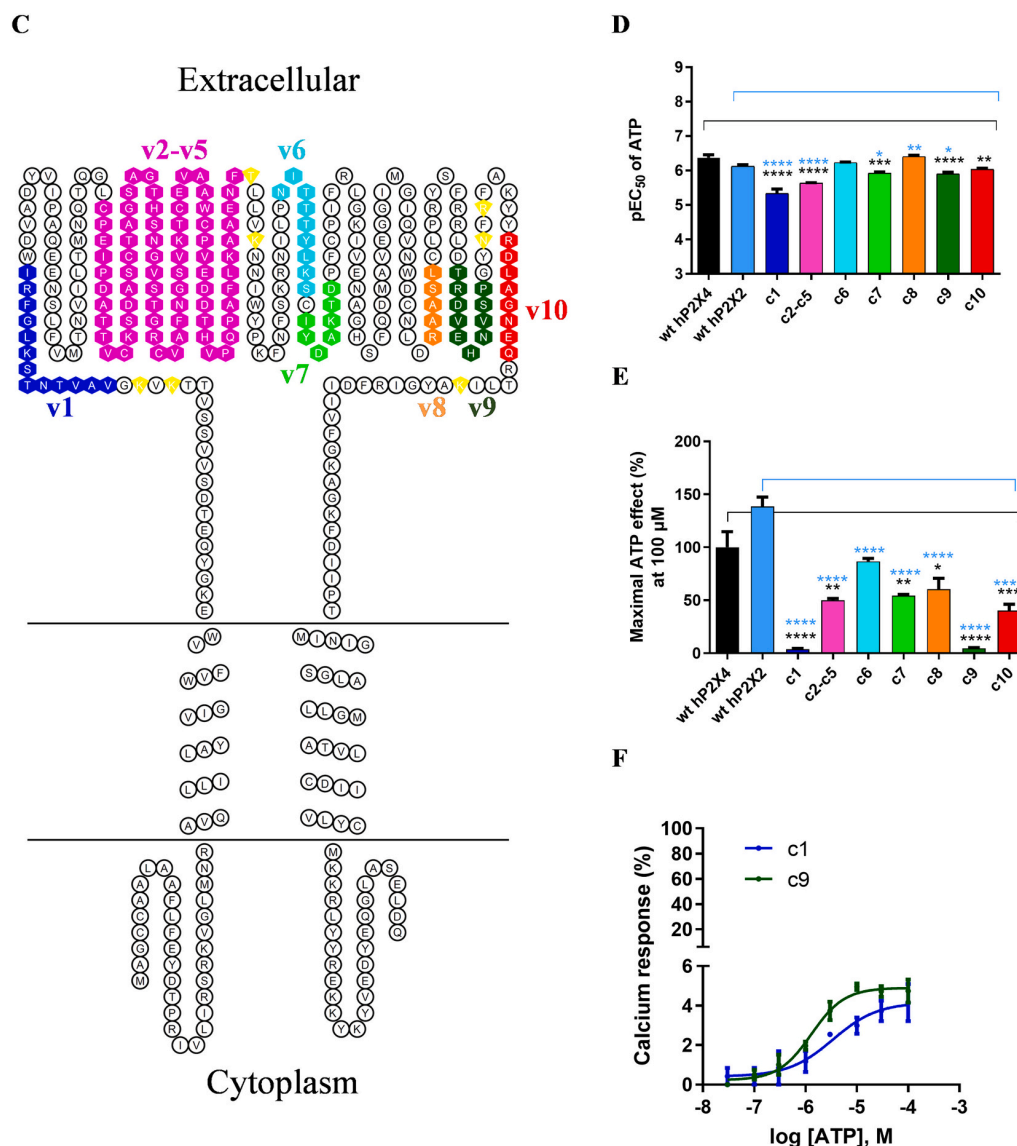


Fig. 2. (continued).

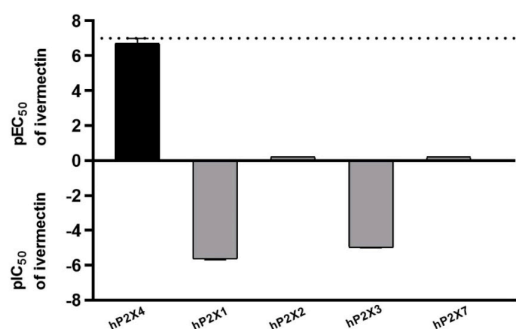
3.2.2. Positive allosteric modulation by ivermectin at c1^{V71-I83} and c9^{T281-P290} chimeric P2X4(P2X2) receptors

At chimeric receptors c1^{V71-I83} and c9^{T281-P290}, which had only shown a small degree of receptor activation (4–5 %) by the agonist ATP alone, ivermectin led to a significant gain of function (Fig. 4B). An ivermectin concentration of 10 μ M induced a 7-fold increase for the chimeric receptor c1^{V71-I83}, and a 9-fold increase for the chimeric

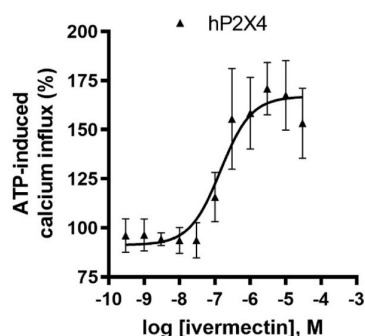
receptor c9^{T281-P290}. Both chimeras did not show significant differences in the EC₅₀ value for ivermectin compared to the wt hP2X4 receptor (Supporting Information Table 3).

In a previous study, it had been reported that a mutation of amino acid residue V291 in the zfpP2X4 receptor caused a significant increase in the EC₅₀ value for ATP while at the same time the maximal currents of ATP were strongly reduced [77]. Additionally, the authors reported that

A



B



C

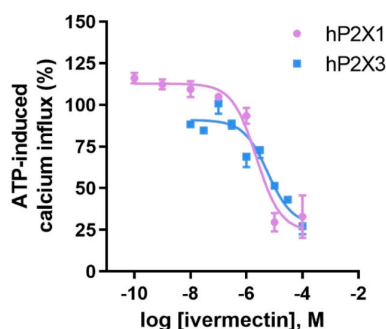


Fig. 3. A. Positive and negative allosteric modulation of P2X receptors, stably expressed in 1321N1 astrocytoma cells, by ivermectin. Potency of ivermectin determined by the measurement of ATP-induced calcium influx is presented as pEC_{50} and pIC_{50} , respectively. (EC_{80} concentrations of ATP were employed for determining the inhibitory potency of ivermectin at the P2X receptor subtypes: hP2X4, 1 μ M; hP2X1, 0.1 μ M; hP2X2, 1 μ M; hP2X3, 0.1 μ M; hP2X7, 2000 μ M). B. Positive allosteric modulation of the ATP response by ivermectin at the hP2X4 receptor. C. Concentration-dependent inhibition of ATP-induced calcium influx by ivermectin at hP2X1 and hP2X3 receptors (0.1 μ M ATP). All data are means $\pm$ SEM of at least three independent experiments.

amino acids close to the orthosteric binding site are involved in essential allosteric changes of the channel gating mechanism which are specific for the P2X4 receptor subunit. Modification of these amino acids had a large influence on the maximal response by ATP [77]. In our experiments variable region v9 which includes the amino acid residue V291 (numbering of zP2X4) was exchanged for the corresponding sequence of the wt hP2X2 receptor which led to a significant decrease in the maximal response of ATP. The variable regions v1 and v9 are both located in proximity to the orthosteric binding site. Our data obtained with the chimeric receptors c9^{T281-P290} and c1^{V71-183} support the hypothesis of essential allosteric changes near the orthosteric binding site of the hP2X4 receptor. Furthermore, our results support the importance of the variable region v9, which includes the amino acid residue V291, for receptor functionality not only of the zP2X4 receptor but also of the hP2X4 receptor. Interestingly, the almost complete loss of receptor activation due to the exchange of amino acids could be partially rescued by ivermectin.

These results show that variable regions v1 and v9 are important for P2X4 receptor functionality, but are not directly involved in PAM action since ivermectin can potentiate the activity of ATP at the corresponding chimeric receptors. ATP is still able to bind to these receptors, but the required opening movements of the channel are impeded. Our results confirm that ivermectin facilitates channel opening allowing influx in the presence of ATP in accordance with previous data [75].

3.2.3. Negative allosteric modulation at c7^{I218-D224} and c8^{R265-L269} chimeric P2X4(P2X2) receptors by ivermectin

In contrast, at the chimeric receptors c7^{I218-D224} and c8^{R265-L269}, ivermectin acted as a NAM and caused a significant inhibition of the ATP response by 44 % at c7^{I218-D224}, and by 52 % at c8^{R265-L269} (Fig. 5A, B, C, Supporting Information Table 3). The calculated IC_{50} values were 25.1 μ M and 12.1 μ M and thus several-fold higher than the EC_{50} values displayed by ivermectin at the wt hP2X4 receptor. These experimental

results raised the question why the exchange of amino acids in the wt hP2X4 receptor by corresponding sequences of the wt hP2X2 receptor in the chimeric receptors c7^{I218-D224} and c8^{R265-L269} switched the allosteric effect of ivermectin from potentiation to inhibition. Published literature had indicated that transmembrane domains as well as parts of the extracellular domain may play an important role in the positive allosteric modulation of the wt hP2X4 receptor by ivermectin [56]. Several experiments identified various amino acid residues (Trp46, Trp50, Asp331, Met336 and others) in the transmembrane domains TM1 and TM2 that are involved in channel gating [78] and interfere with the effects induced by ivermectin [39,48–50]. However, no single mutation in this region could completely abolish the effects observed upon application of ivermectin [79]. Mutations in the transmembrane domains were shown to overturn the ivermectin-induced prolonged channel deactivation and pore dilation, but not the potentiation of the maximal ATP effect [79,80]. Data by two independent research groups obtained after mutagenesis of the amino acid residue Trp46, were inconsistent [50,79]. For the single mutant Trp46A of the rP2X4 receptors, Silberberg et al. found that in the presence of ivermectin the maximal effect of ATP was increased [50]. In contrast, Zemkova et al. reported inhibition in the presence of ivermectin for the same mutant [79]. The amino acid residue Tyr42 was also postulated to be part of the ivermectin binding pocket [48], but the experimental results were difficult to interpret, because Tyr42 is a conserved residue and found to be critically involved in channel activation of the entire P2X receptor family [81]. Silberberg et al. had shown that the transfer of the entire extracellular domain of the rat P2X4 receptor to the rat P2X2 receptor did not result in a potentiation by ivermectin, while the transmembrane domains appeared to be involved in the ivermectin effects [50]. These results are not in agreement with the current results obtained at the human P2X4 receptor and might be explained by species differences between human and rat P2X4 receptors or by different experimental setups.

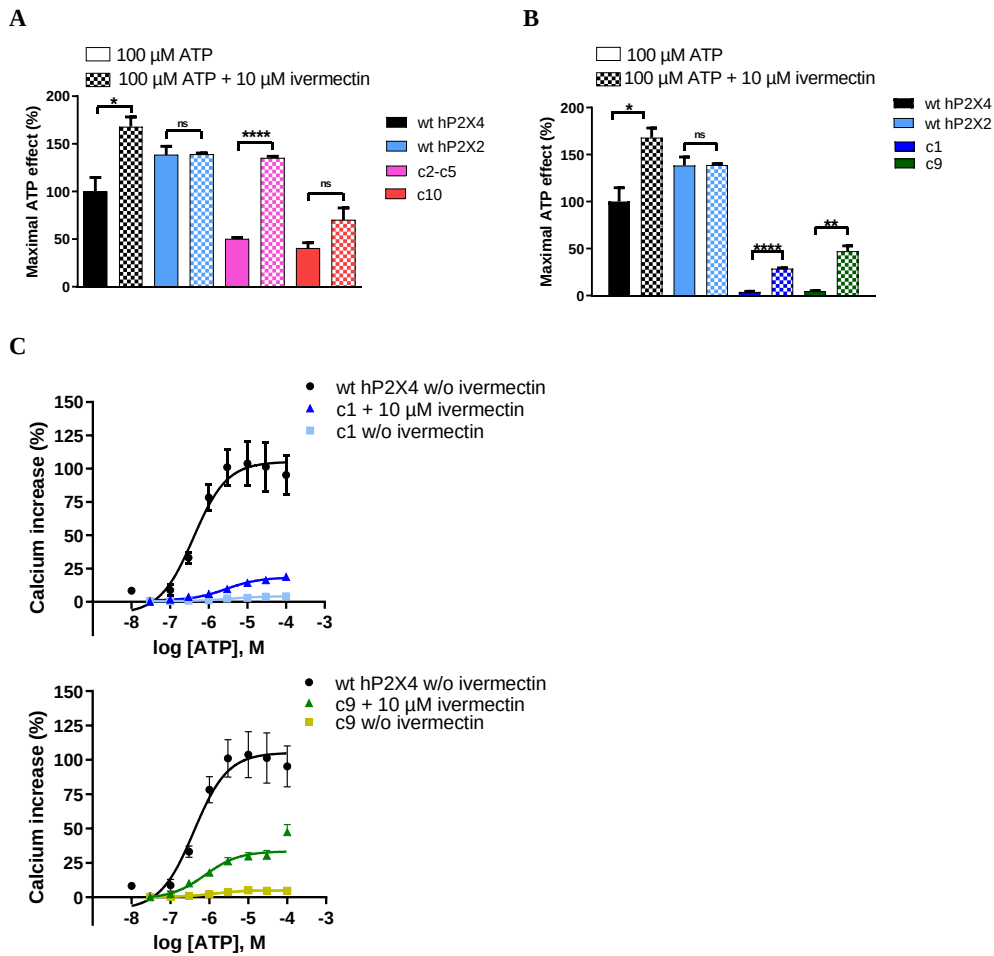


Fig. 4. Modulation of ATP response by ivermectin at chimeric P2X4(P2X2) receptors (c2-c5^{C116-T186}, c10^{R301-Q308}, c1^{V71-I83}, c9^{T281-P290}) and at wt hP2X4 and wt hP2X2 receptor stably expressed in 1321N1 astrocytoma cells. The maximal effect of ATP in the presence and the absence of 10 μM ivermectin was determined. Significance was calculated by an unpaired two tailed *t*-test: * *P* < 0.05, ** *P* < 0.01, *** *P* < 0.001, **** *P* < 0.0001. A. Positive allosteric modulation of wt hP2X4, c2-c5^{C116-T186} and c10^{R301-Q308} by ivermectin. B. Chimeric receptors c1^{V71-I83} and c9^{T281-P290} showing gain of function by ivermectin. C. Concentration-response curves of ATP at wt hP2X4 receptor without (w/o) ivermectin, at chimeric receptors c1^{V71-I83} and c9^{T281-P290} with 10 μM ivermectin and w/o ivermectin. Data represent means ± SEM of at least three independent experiments.

3.2.4. Two binding sites for ivermectin?

Our experimental results based on chimeric receptors indicate a binding site for ivermectin in the hP2X4 receptor that is formed by variable regions v6, v7 and v8 leading to positive allosteric modulation.

It might be possible that two binding sites for ivermectin are present in the P2X4 receptor, the first one within the transmembrane domains (low affinity, IC₅₀ values in the micromolar range) and another one formed by variable regions v6, v7 and v8 (EC₅₀ values in the nanomolar range). Mutations within the ivermectin binding site located in the extracellular domain (v6, v7 and v8) resulting in c6^{N208-S216}, c7^{I218-D224} and c8^{R265-L269} chimeric receptors abolish the ATP-potentiating effect of ivermectin, likely because ivermectin can then no longer bind to this site. The observed result is receptor inhibition acting by ivermectin as a NAM, rather than a PAM. In this situation, in which the amino acid residues within the extracellular high-affinity binding site are mutated through chimeric exchange, ivermectin can perhaps only bind to the low-affinity binding site within the transmembrane domains and thereby blocks opening of the receptor channel preventing calcium influx. Thus, we could demonstrate that variable extracellular regions of the P2X4 receptor participate in the mechanism of allosteric modulation by the PAM ivermectin.

Results obtained at hP2X1 and hP2X3 receptors (Fig. 3C) demonstrate negative allosteric modulation of these receptors by ivermectin. High (micromolar) concentrations of ivermectin were required for negative allosteric modulation of the hP2X1 and hP2X3 receptors. The amino acids in variable regions v6, v7 and v8 differ in the three hP2X receptor subtypes, P2X1, P2X3 and P2X4 (for sequence alignment see Supporting Information Fig. S4). Ivermectin might still be able to bind to the same region, acting as a NAM at these subtypes. Perhaps the alterations in the proposed binding region contribute to a binding site that can restrict the conformational change induced by ATP similar perhaps to the NAM gefapixant at the hP2X3 [12].

Another explanation, however, would be that it binds to the second postulated binding site within the transmembrane domains.

The amino acid residues Trp46, Asp331 and Met336, located in the transmembrane domain [39], which were previously claimed to interact with ivermectin are not P2X4 receptor-specific. Trp46 is present in hP2X1 and hP2X3 receptors, Asp331 and Met336 are present in the hP2X1 receptor, which was significantly inhibited by ivermectin (10 μM, up to 70 % inhibition). In the hP2X2 receptor, the above-mentioned amino acid residues are absent, where ivermectin showed no effect. In the hP2X7 receptor, only Asp331 is present, and the receptor was not

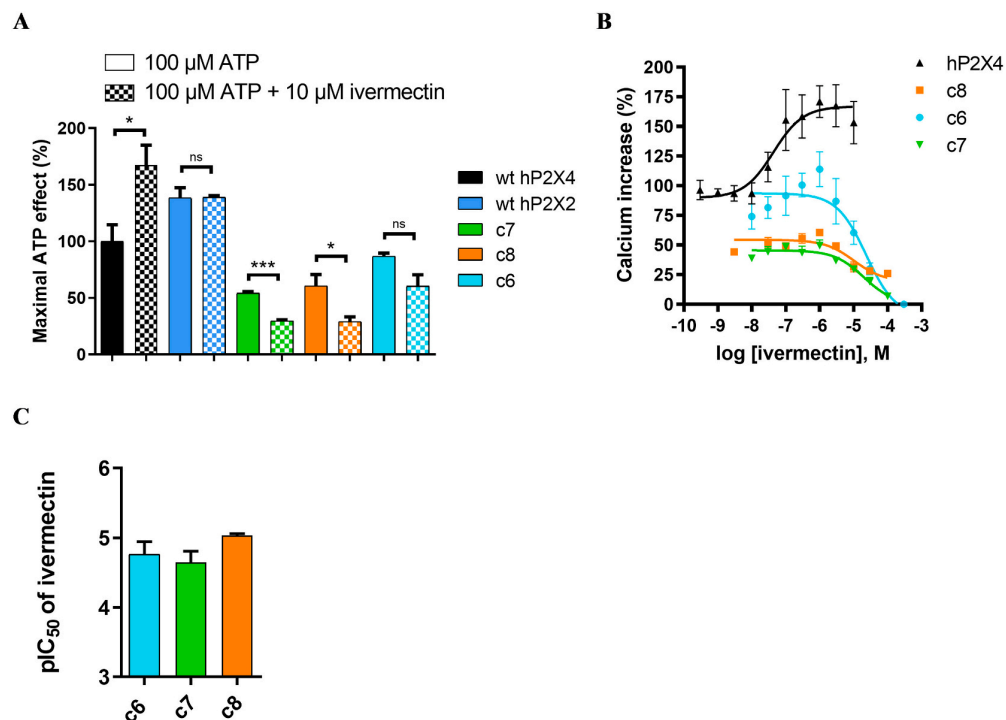


Fig. 5. Modulation of ATP response by ivermectin at chimeric P2X4(P2X2) receptors (c6^{N208-S216}, c7^{I218-D224} and c8^{R365-L269}) and at wt hP2X4 and wt hP2X2 receptor stably expressed in 1321N1 astrocytoma cells. A. Negative allosteric modulation of c6^{N208-S216}, c7^{I218-D224} and c8^{R365-L269} by ivermectin. The maximal effect of ATP in the presence and the absence of 10 μ M ivermectin was determined at the respective receptors. Significance was calculated by an unpaired two tailed *t*-test: * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$. B. Negative allosteric modulation of ivermectin at chimeric receptors c7^{I218-D224} and c8^{R365-L269} and biphasic modulation of c6^{N208-S216}. Increases of intracellular calcium (%) were set in relation to the effect obtained by 100 μ M ATP (corresponds to the maximal effect of ATP) in the absence of ivermectin at the wt hP2X4 receptor. C. pIC₅₀ values $\pm$ SEM of ivermectin in inhibiting chimeric receptors c6, c7, c8 in the presence of the respective EC₈₀ concentration of ATP. Data represent means $\pm$ SEM of at least three independent experiments.

inhibited by ivermectin in our experiments.

Our hypothesis of two ivermectin binding sites is further supported by the concentration-dependent biphasic modulation of the chimeric receptor c6 (Fig. 5B). At low concentrations ranging from 100 nM to 1 μ M of ivermectin, an enhancement of the ATP response was observed, whereas at concentrations ranging from 3 μ M to 100 μ M, the ATP effect was inhibited. At 10 μ M of ivermectin, the ATP-response was significantly decreased by 31 % (Supporting Information Table 3).

In patch-clamp experiments ivermectin had been shown to increase the channel amplitude of the hP2X4 receptor at low concentrations (EC₅₀ = 0.25 μ M), while at high concentrations (EC₅₀ = 2 μ M) it facilitated channel opening and prolonged channel deactivation [75]. Due to this biphasic effect, the existence of more than one allosteric binding site in P2X4 receptors has previously already been assumed by some authors [60,62,75]. Ivermectin had shown P2X4 receptor potentiation when it was applied extracellularly, but not intracellularly, indicating that the positive modulatory allosteric binding site has to be located in the extracellular domains of the receptor [75]. In contrast, several publications postulated the involvement of the transmembrane receptor domains in the binding of ivermectin to the P2X4 receptor [48–50], and in one study, the lateral fenestration linking the extracellular domain and the pore-forming transmembrane domains, was postulated to contribute to ivermectin binding [82]. We now provide evidence for the potential presence of two separate binding sites for ivermectin on the hP2X4 receptor, an extracellular site leading to positive allosteric modulation, and a transmembrane site of lower affinity resulting in receptor inhibition.

3.2.5. Homology modeling of the human P2X4 receptor and molecular docking of ivermectin

In order to rationalize the experimental outcome, a homology model of the P2X4 receptor was created with subsequent docking of ivermectin. Several previous studies characterizing the ATP-binding site and trying to understand the mechanism of ATP-induced channel opening led to the conclusion that a downward movement of the head domain and an upward movement of the dorsal fin and the left flipper take place [9,83]. We created a homology model of the closed-state P2X4 receptor based on the X-ray crystal structure of the zfpP2X4 apo structure, and a model of the open-state wt hP2X4 receptor based on the zfpP2X4 structure in the ATP-bound state. When ATP binds to the orthosteric site of the P2X4 receptor, a cavity is formed below the dorsal fin region. Amino acids from the three variable regions designated v6, v7 and v8, as specified in the sequence alignment, constitute the dorsal fin and are highly variable between P2X receptors.

Docking poses of ivermectin and its interaction with amino acid residues located in the region v6, v7 and v8 from the dorsal fin of the hP2X4 receptor in the open state model were observed (Fig. 6). Specifically, the binding pose with the lowest binding free energy shows an interaction of ivermectin with the amino acid residues from all three regions of the dorsal fin (v6, v7 and v8). The amino acid residues Asn208 and Thr223 are predicted to contribute to the formation of hydrogen bond interactions, and Tyr213 may form a weak aromatic interaction with ivermectin. Additionally, Ile209, Phe226, Leu269, Leu271 may contribute to hydrophobic interactions. The side chains of the amino acid residues Asp224, Ser268, and the backbone of Pro272 possibly form hydrogen bond interactions, and Phe226 is predicted to display

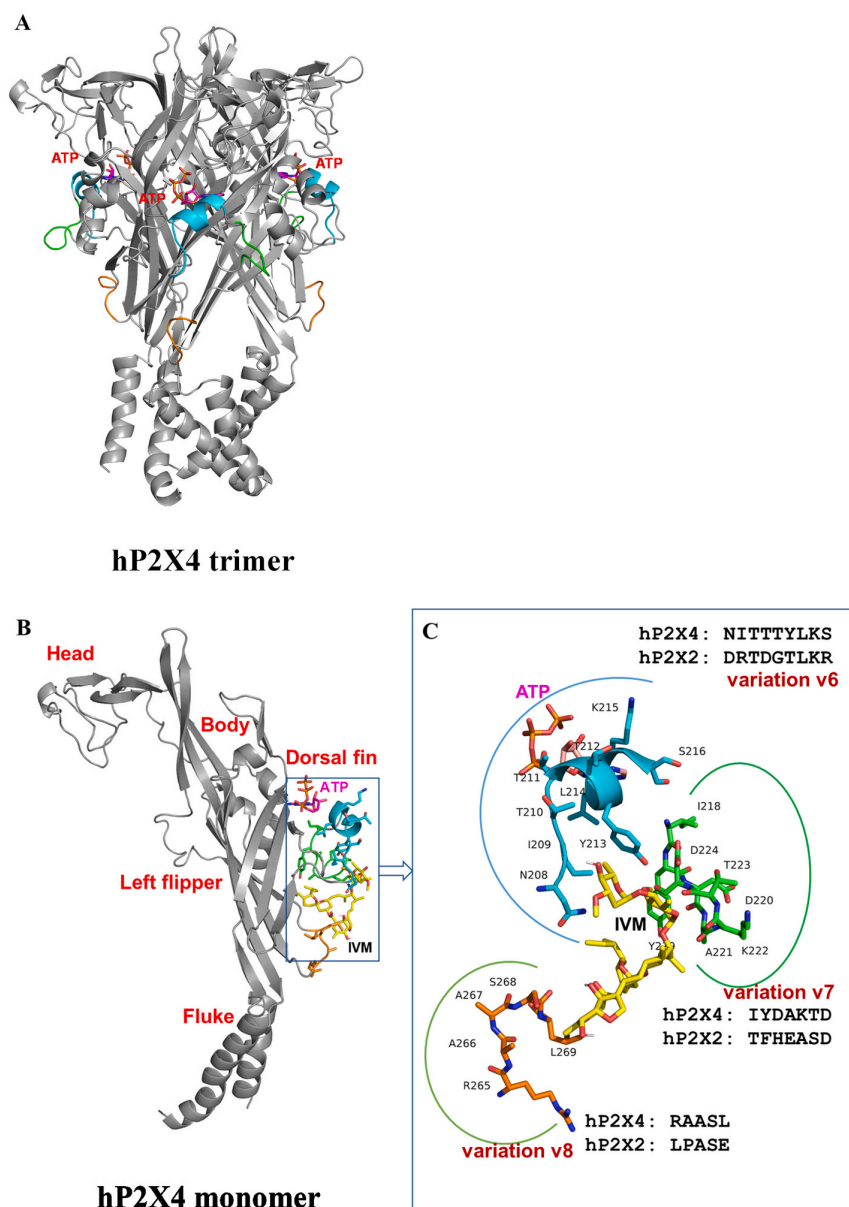


Fig. 6. Computational docking simulations on the hP2X4 receptor homology model in the open state for ivermectin (IVM). A. The hP2X4 receptor trimer (presented in ribbon model and colored in gray) with ATP (carbon atoms colored in magenta) and with variable regions of v6, v7 and v8 (highlighted in cyan, green and orange, respectively). B. The monomer of hP2X4 with ATP and the variable regions v6, v7 and v8. The lowest energy docked position of ivermectin within the homology model of the human P2X4 receptor is illustrated with carbon atoms colored in yellow and oxygen atoms colored in red. C. Ivermectin (IVM), ATP and amino acids in the regions of v6, v7 and v8 are represented as stick models. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

aromatic interactions with ivermectin. These interactions with Asp224, Phe226, Ser268 and Pro272 were considered weaker due to their greater distance ($\sim 3.5\text{--}4.0$ Å). Regarding flexible movements of these regions, we have to keep in mind that the proposed interactions are predictive. Because of conformational flexibility, for example upon receptor activation, there would be some tolerance for several interactions between amino acid residues of v6, v7 and v8 regions and the allosteric modulator ivermectin.

The predicted binding pose explains on the one hand the interactions of amino acids from the three regions, v6, v7 and v8, with ivermectin, and on the other hand, it helps to understand the mechanism of positive allosteric modulation of the hP2X4 receptor. During the process of ATP binding, the left flipper and the dorsal fin regions move upwards while the head region moves downwards. The lowest energy binding pose of ivermectin was well accommodated within the cavity, and the allosteric modulator was observed to interact with amino acids from each of the three variable regions, v6, v7 and v8. From these interactions it can be

postulated that the binding of ivermectin within the cavity in the dorsal fin favors an upward movement or extends the upward movement of the dorsal fin region towards the binding site of ATP leading to a potentiation of receptor activation by ATP (see Fig. 6).

On the basis of experimental results and docking studies, we postulate that the main binding site of ivermectin in the hP2X4 receptor is within the variable regions v6, v7 and v8 favoring an upward movement of the dorsal fin, thereby leading a potentiation of receptor activation. Our results indicate that the movement of all the three variable regions (v6, v7 and v8) is required to achieve potentiation of the hP2X4 receptor by ivermectin. The biphasic ivermectin curve obtained for the chimeric receptor c6 shows that the variable region v6 of the hP2X2 receptor combined with the variable regions v7 and v8 of the wt hP2X4 receptor displays partial ivermectin-induced receptor activation. This can be explained by the fact that four out of nine amino acid residues in the variable region v6 are common between the hP2X2 and the hP2X4 receptor and thereby possibly preserve the secondary receptor structure as

well as some interactions crucial for positive allosteric modulation.

The variable regions v7 and v8 were identified as loop regions possessing large flexibility. Thus, in the chimeric receptors c7 and c8, the exchange of amino acid residues for those of the hP2X2 receptor might alter the orientation of the amino acid residues that interact with ivermectin in the wt hP2X4 receptor. Due to the flexibility of the binding site, ivermectin might have lost the specific interaction needed for high-affinity binding in the two chimeric receptors c7 and c8. Instead, it binds to the low-affinity site within the transmembrane domain resulting in receptor inhibition.

3.3. The negative allosteric P2X4 receptor modulator BX430

To confirm the negative allosteric modulation of the P2X4 receptor by BX430, previously demonstrated in patch-clamp experiments [53], ATP-induced calcium influx assays were performed and concentration-inhibition curves of BX430 in the presence of ATP at its EC_{80} -concentration were determined at different P2X receptor subtypes. The percentage of inhibition at 10 μ M of BX430 and - where possible - IC_{50} values for BX430 were determined (see Supporting Information Table 1). At the hP2X4 receptor BX430 inhibited ATP-induced calcium influx with an IC_{50} value of 0.127 μ M (Fig. 7A, and Supporting Information Fig. S3). At the other P2X receptor subtypes no significant inhibition was observed by a high concentration of BX430 (10 μ M).

To identify the allosteric binding site of BX430, concentration-response curves were determined at all of the functional chimeric P2X4(P2X2) receptors (Fig. 7A, B). The inhibitory potency of BX430 and the percent inhibition at a high concentration of 10 μ M was calculated (see Supporting Information Table 2) and compared to the values obtained for the wt hP2X4 receptor. The antagonist led to a complete inhibition of chimeric receptors c7^{I218-D224} and c10^{R301-Q308}, and no significant difference in BX430 potency was observed at those chimeras. The chimeric receptor c2-c5^{C116-T186} was completely inhibited by the antagonist, but the IC_{50} value for BX430 was 5-fold higher in comparison to the wt hP2X4 receptor. The IC_{50} values of the antagonist were estimated to be 28.8 μ M for the c6^{N208-S216} mutant (curve was extrapolated) (200-fold higher compared to the wt hP2X4 receptor) and 0.323 μ M for the c8^{R265-L269} chimera (3-fold higher compared to the wt hP2X4 receptor). An interaction of BX430 with the regions v2-v5 is not plausible based on the homology model. The large sequence exchanges in the chimeric receptor c2-c5^{C116-T186} may have led to a conformational change of the receptor protein thereby indirectly leading to a moderate change of the interaction between the receptor and the small molecule.

3.3.1. Molecular docking of BX430

Computational docking studies of the antagonist BX430 into the closed state of the hP2X4 receptor homology model were performed to examine whether the observed cavity formed by the regions v6 to v8

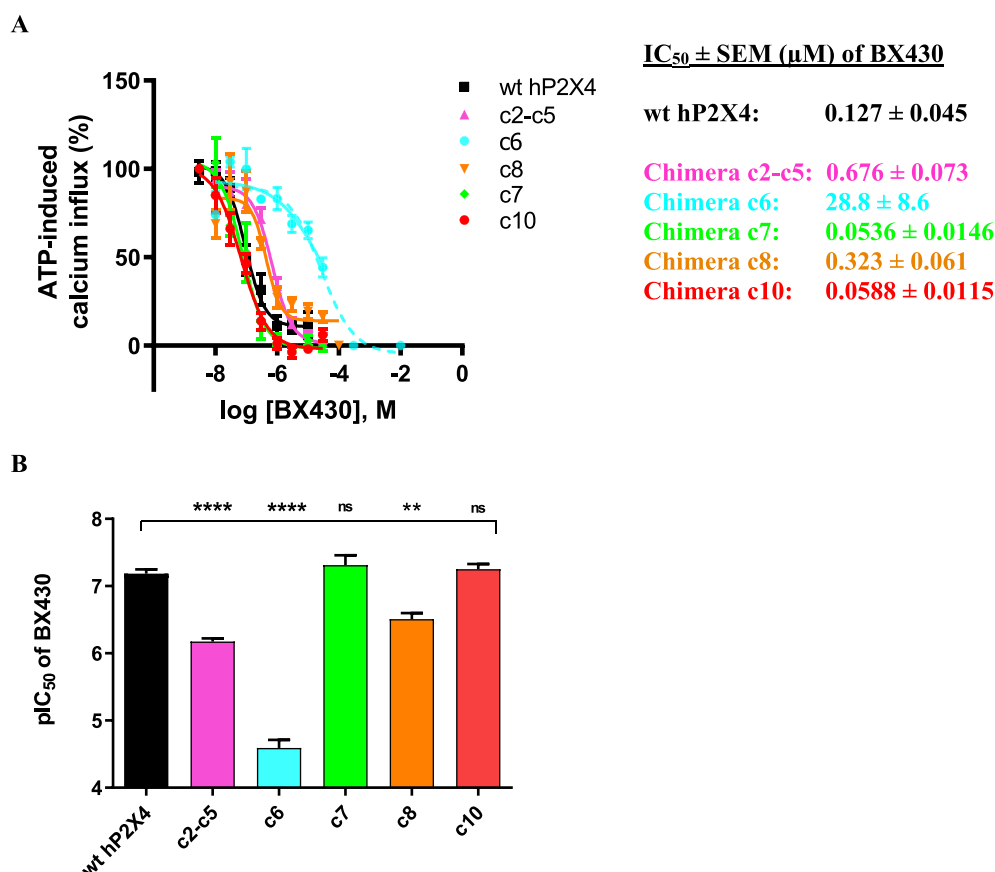


Fig. 7. Negative allosteric modulation of chimeric P2X4(P2X2) receptors and wt hP2X4 receptor mutants stably expressed in 1321N1 astrocytoma cells by BX430. **A.** Concentration-inhibition curves of BX430 at wt hP2X4 and chimeric P2X4(P2X2) receptors in the presence of the respective EC_{80} concentration of ATP. **B.** BX430 potency (pIC_{50} values $\pm$ SEM) at wt hP2X4 receptor and chimeric P2X4(P2X2) receptors determined by the measurement of ATP-induced calcium influx assay. Results were compared to the effects at wt hP2X4 receptor. The level of significance was calculated by an ordinary one-way ANOVA with Dunnett's post-hoc test: * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, **** $P < 0.0001$. Data represent the mean percentage of the ATP-induced signal $\pm$ SEM of at least three independent experiments. Curve at c6 was extrapolated.

would be a plausible binding site for BX430. BX430 was docked into the binding pocket prepared for ivermectin within the P2X4 receptor trimer. The obtained results showed that the cavity in the dorsal fin area easily accommodated BX430. It appeared that BX430 binds in proximity to the amino acid residues from variable region v6 (Fig. 8). One of the two bromine atoms on the phenyl ring may form an electrostatic interaction with Asp224. Considering the flexible nature of the region v6, the second bromine atom could also contribute to the compound's interaction with the receptor which is located at a distance ~ 2.5 Å and an angle of

$\sim 85^\circ$ from the carbonyl atom of Asn208 in the present model. The phenyl and the pyridine rings may be able to form weak aromatic-hydrogen interactions with Ile209, and Asn208, respectively (Fig. 8). The isopropyl group likely binds within the cleft formed by the amino acids Thr210 to Tyr213 present in the variable region v6. With the effective placement of the isopropyl group, the pyridine ring possibly forms a π - π interaction with Phe226. Furthermore, Ser268 from the variable region v8 is present in proximity to the pyridine ring of BX430.

Based on our docking studies, four single mutants and one double

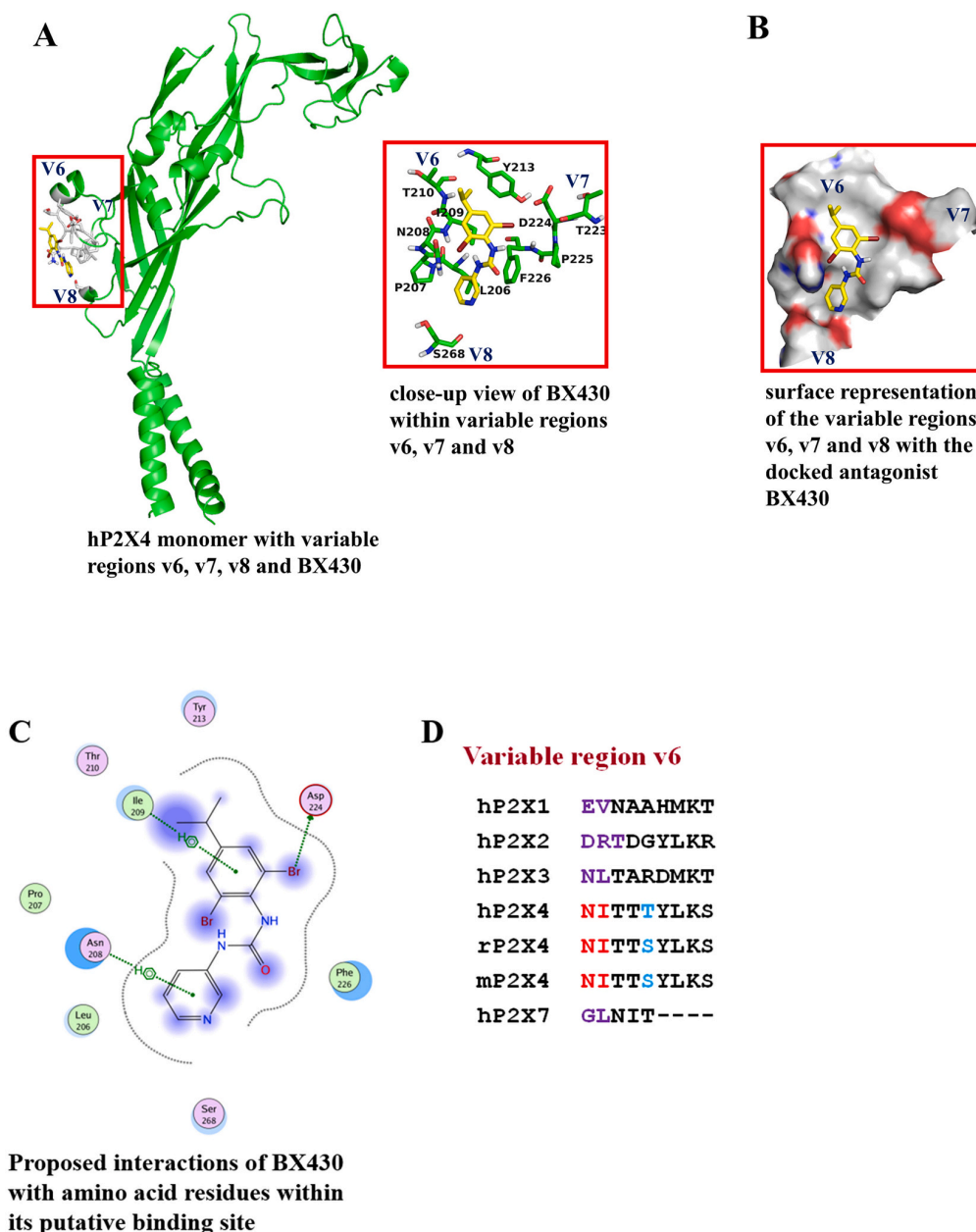


Fig. 8. Docking of the antagonist BX430 into the closed-state hP2X4 receptor homology model (docking into the binding pocket prepared for ivermectin). A. The hP2X4 receptor monomer with BX430 and interacting with variable regions v6 and v8, and close-up view of the interaction site of BX430 (represented as stick model) are shown. B. Surface representation of the hP2X4 receptor with BX430 bound within regions v6 and v8. C. 2D interaction diagram of BX430 bound to the hP2X4 receptor. Proposed interactions of BX430 with amino acids within its putative binding site. D. Alignment of variable region v6 of the human P2X receptor subtypes and of the rat and mouse P2X4 receptor.

amino acid exchange mutant (Y300F, I209A, T212S, D224A, D224A_N208A) were generated by PCR-based site-directed mutagenesis. Asn208, Ile209 and Thr212 are located in variable region v6, Asp224 in variable region v7, while Tyr300 is located next to variable region v10. Subsequently, the functionality of each cell line was investigated by ATP-induced calcium influx assays (see Supporting Information Fig. 1). The potency of ATP was determined to calculate the appropriate EC_{80} value for further experiments. The agonistic potency of ATP was similar at most of the P2X4 receptor mutants, comparable to the wt hP2X4 receptor. Exceptions were the D224A and the D224A_N208A receptor mutants, which could not be activated by ATP. These results might indicate that Asp224 in variable region v7 is important for signal transduction and thus could be interesting for

targeting by antagonists.

Next, the antagonistic effect of BX430 on ATP-induced calcium influx at the hP2X4 receptor mutants was investigated (Fig. 9A, B, Supporting Information Table 4). Compared to the wt hP2X4 receptor, the experiments showed a 10-fold higher IC_{50} value of BX430 at the I209A receptor mutant (1.21 μ M) and only partial receptor inhibition. These results fit to the previously obtained results showing a 200-fold reduced potency of BX430 at the chimeric receptor $c6^{N208-S216}$. Docking studies had predicted that Ile209 in variable region v6 is involved in BX430 binding. Our results support the assumption that strong hydrophobic interactions with Ile209 are crucial for BX430 binding.

BX430 was identified as a potent and highly selective allosteric modulator of hP2X4 receptors acting at an extracellular site [53].

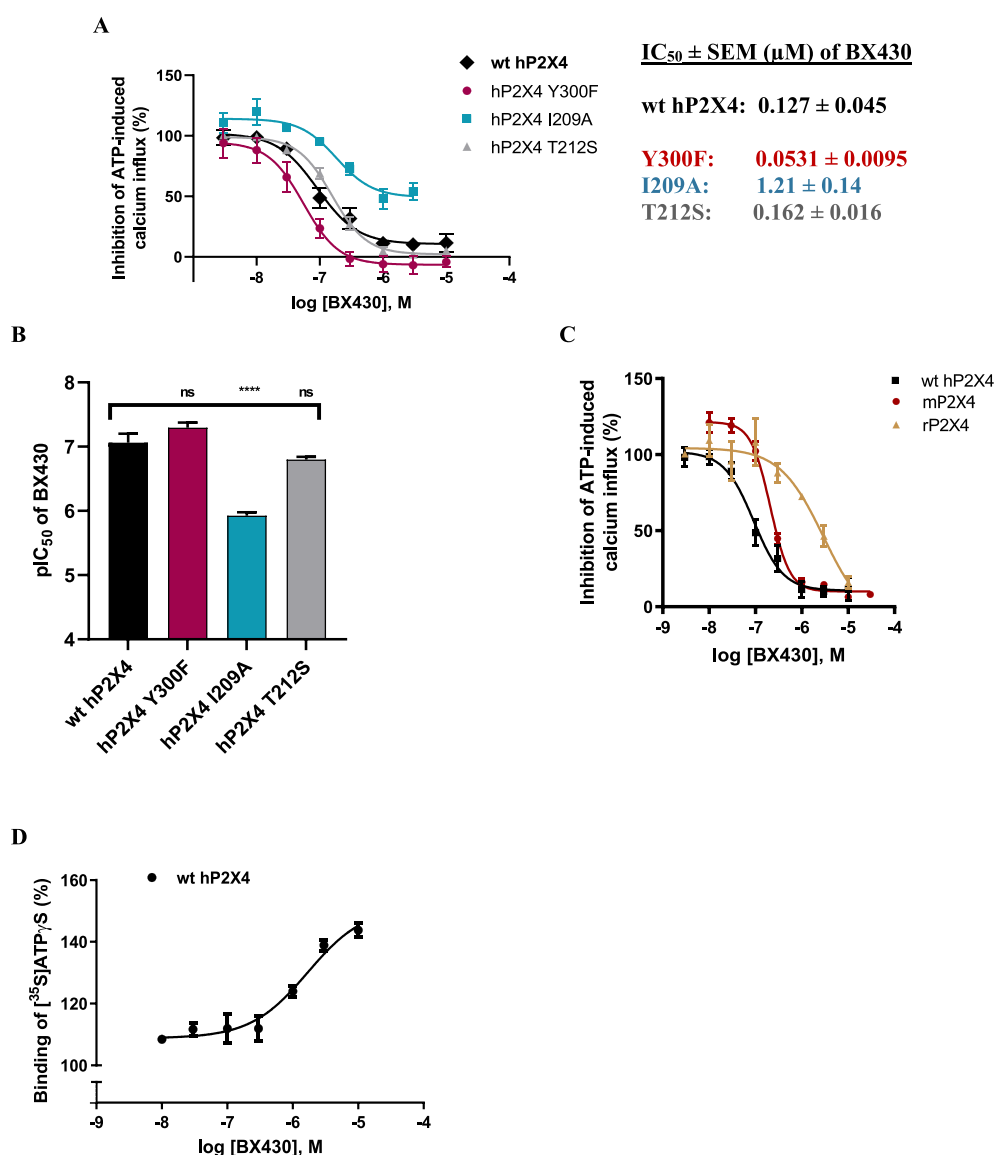


Fig. 9. Negative allosteric modulation of hP2X4 receptor mutants stably expressed in 1321N1 astrocytoma cells by BX430, species differences of BX430 and radioligand binding studies. A. Concentration-inhibition curves of BX430 at wt hP2X4 receptor and at hP2X4 receptor mutants (Y300F, I209A, T212S) in the presence of the respective EC_{80} concentration of ATP. B. BX430 potency (pIC_{50} values ± SEM) at wt hP2X4 receptor and hP2X4 receptor mutants determined by the measurement of ATP-induced calcium influx assay. Results were compared to the pIC_{50} value at wt hP2X4 receptor. The level of significance was calculated by an ordinary one-way ANOVA with Dunnett's post-hoc test: * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, $P < 0.0001$. C. Concentration-inhibition curves of BX430 at wt hP2X4, rP2X4 and mP2X4 receptors in the presence of the respective EC_{80} concentration of ATP. D. Competition experiments with BX430 versus [³⁵S]ATPγS (0.2 nM) at membrane preparations of 1321N1 cells expressing hP2X4 receptor. Data represent means ± SEM of at least three independent experiments.

Further experiments to identify the binding site of BX430 had previously led to the hypothesis that amino acids Tyr300, Asp88 and Ile312 might participate in BX430 binding; the phenolic group of Tyr300 was in fact proposed to form a hydrogen bond with the keto group of BX430 [52]. We have now created the hP2X4 Y300F receptor mutant and observed no significant difference in BX430 potency at the receptor mutant compared to the wt hP2X4 receptor (Fig. 9A, B). Thus, we could not confirm the predicted binding site and the postulated interaction [52].

BX430 showed species-selectivity for the hP2X4 receptor, its potency

being much weaker at the rat ($IC_{50} = 2.74 \mu M$, 20-fold) and slightly weaker at the mouse P2X4 receptor orthologs ($IC_{50} = 0.286 \mu M$, 2-fold) (Fig. 9C, Supporting Information Table 1). In electrophysiological experiments, in which much higher ATP concentrations had been employed (50 μM as compared to 1 μM ATP in our calcium influx experiments at the wt hP2X4 receptor corresponding to its EC_{80} value), the weak inhibitory effects of BX430 could not even be measured [53].

The alignment of the variable region v6 of the P2X4 receptor of mouse, rat and human shows a difference in one amino acid residue.

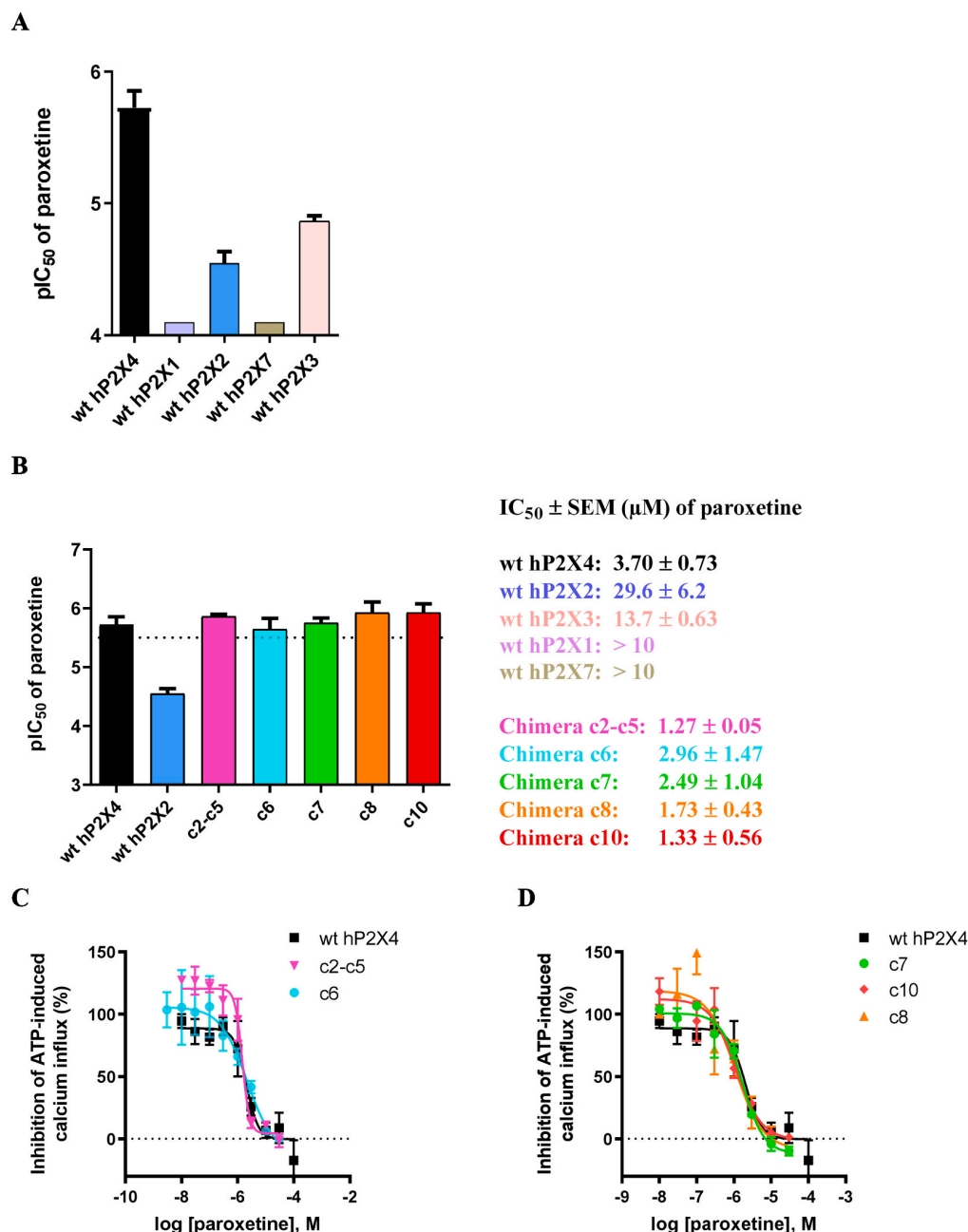


Fig. 10. Investigations of the hP2X4 receptor antagonist paroxetine. All experiments were performed with stably expressed receptors in 1321N1 astrocytoma cells. The receptors were activated with the respective EC_{80} concentration of ATP. A. $pIC_{50} \pm SEM$ values of paroxetine at wt P2X receptors determined by the measurement of ATP-induced calcium influx. B. pIC_{50} values of paroxetine at chimeric P2X4(P2X2) receptors. C. D. Concentration-inhibition curves of paroxetine at wt hP2X4 and chimeric P2X4(P2X2) receptors. Data represent means $\pm$ SEM of at least three independent experiments.

Threonine in the hP2X4 receptor is exchanged for serine in the mouse and rat receptor. This might be an explanation why BX430 affinity is reduced at mouse and rat P2X4 receptors in comparison to the human receptor. In all of the other P2X receptor subtypes the region v6 is variable. However, our mutagenesis study did not show a significant difference in potency of BX430 at the hP2X4 T212S receptor mutant compared to the wt hP2X4 receptor (Fig. 9A, B). Based on these results, there has to be another explanation for the observed species differences.

In radioligand binding studies using the orthosteric radioligand [³⁵S] ATP_γS (0.2 nM), BX430 increased the binding of the radioligand to the hP2X4 receptor by 44 % with an EC₅₀ value of 1.68 μM (Fig. 9D). Thus, BX430 led to a concentration-dependent increase of [³⁵S]ATP_γS binding to the hP2X4 receptor with an EC₅₀ value somewhat higher, but still in the same range as its IC₅₀ in functional assays. It is known that [³⁵S]ATP_γS labels an inactive desensitized state of the P2X4 channel that displays very high affinity for ATP [84]. Our results clearly prove that BX430 acts as an allosteric modulator. Interestingly, it increases binding of the agonist to the desensitized receptor, which may explain its functional inhibitory activity, stabilizing an inactive, desensitized receptor conformation.

The NAM BX430 was previously reported to inhibit the positive allosteric modulatory effect of ivermectin at the hP2X4 receptor [53]. According to our results, both compounds interact with variable region v6, indicating a common or overlapping extracellular binding site at the wt hP2X4 receptor in the dorsal fin region. Thus, interaction with this allosteric region can result in positive or negative modulation. BX430 is much smaller than ivermectin and therefore appears to interact mainly with amino acid residues in the v6 region, while for the larger ivermectin, interactions with the variable regions v6, v7, v8 have been found to contribute to its affinity.

All in all, our results provide new insights into the binding mechanism of the NAM BX430. The exchange of the variable region v6 in the hP2X4 receptor, and the specific exchange of the aliphatic Ile209 to alanine, led to a significantly reduced potency of BX430. These results support our hypothesis that variable region v6 of the hP2X4 receptor constitutes part of the binding site for BX430.

3.4. The human P2X4 receptor antagonist paroxetine

As a second, structurally different P2X4 receptor antagonist, paroxetine was investigated. The compound inhibited the wt hP2X4 receptor with an IC₅₀ value of 3.70 μM (Fig. 10), consistent with literature data [54,55]. Paroxetine was much less potent at all other investigated P2X receptor subtypes (Fig. 10A). For example, at the hP2X2 receptor, its IC₅₀ value was about 10-fold higher (29.6 μM) than at the hP2X4 receptor. At the P2X4(P2X2) chimeric receptors, paroxetine did not show any significantly altered effects compared to the wt hP2X4 receptor. Based on these results, we conclude that paroxetine does not interact with the variable regions v2-v5, v6, v7, v8 and v10.

Since paroxetine is a basic small molecule which is positively charged at a physiologic pH value of 7.4, it might bind directly to the center of the cation channel thereby plugging the ion pore and preventing the influx of calcium and sodium ions. This hypothesis still awaits its confirmation.

4. Conclusions

Our chimeric receptor approach, supported by site-directed mutagenesis, homology modeling and docking simulations, identified three variable extracellular regions in the wt hP2X4 receptor, designated v6, v7 and v8, whose exchange for those of the hP2X2 receptor exerts a dramatic effect on the ability of ivermectin to potentiate the response of ATP. We consider it reasonable to conclude that these variable regions, v6, v7 and v8, located in the dorsal fin of the receptor, have a high relevance for the mechanism of positive allosteric modulation of the P2X4 receptor by ivermectin, representing the main, high-affinity

ivermectin binding pocket. The variable region v6 likely also contributes to the binding of the NAM BX430, since a significant decrease in its potency at the chimeric receptor c6 and at c6-receptor mutants is observed. In particular, Ile209 appears to be an important amino acid residue involved in BX430 binding. In contrast, the investigated variable regions v6, v7 and v8 are likely not involved in paroxetine binding. Paroxetine, which is positively charged at a physiologic pH value, might bind inside the cation channel.

The present identification of an important allosteric site for PAMs and NAMs will be helpful for the future development of potent and selective allosteric P2X4 modulators as novel drugs, and may have implications on the development of allosteric modulators for other P2X receptor subtypes as well.

CRedit authorship contribution statement

C.E.M. conceived and designed the study. V.N. planned and performed all computational studies. S.W. and J.N. performed the majority of the experiments with contributions from A.A., C. S., T.H. and R.H. C.E.M., S.W., J.N., and V.N. wrote the manuscript with contribution from all coauthors.

Declaration of competing interest

The authors declare no conflict of interest.

Data availability

Data will be made available on request.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.lfs.2022.121143>.

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Supporting Information

Extracellular binding sites of positive and negative allosteric P2X4 receptor modulators

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Figure S4. Sequence alignment of human P2X1, P2X2, P2X3, P2X4, P2X6 and P2X7 receptors regarding variable regions v6, v7, v8 (p.13)

Table S1. Effects of the allosteric P2X4 modulators ivermectin and BX430 on different P2X receptor subtypes

Receptor h = human m = mouse r = rat	Ivermectin				BX430	
	ATP-modulation by 10 μM ivermectin (% $\pm$ SEM)	EC₅₀ $\pm$ SEM (μM)	Inhibition at 10 μM ivermectin (% $\pm$ SEM)	IC₅₀ $\pm$ SEM (μM)	Inhibition at 10 μM (% $\pm$ SEM)	IC₅₀ $\pm$ SEM (μM)
hP2X4	167 $\pm$ 17	0.310 $\pm$ 0.206	no inhibition	n.d	98 $\pm$ 6	0.127 $\pm$ 0.045
mP2X4	150 $\pm$ 6	1.66 $\pm$ 0.47	no inhibition	n.d	94 $\pm$ 2	0.286 $\pm$ 0.030
rP2X4	235 $\pm$ 7	0.107 $\pm$ 0.042	no inhibition	n.d	84 $\pm$ 4	2.74 $\pm$ 0.39
hP2X2	no potentiation	n.d	no inhibition	n.d	-1 $\pm$ 3	> 10
hP2X1	no potentiation	n.d	70 $\pm$ 5	1.72 $\pm$ 0.23	-14 $\pm$ 24	> 10
hP2X3	no potentiation	n.d	49 $\pm$ 1	17.1 $\pm$ 4.5	31 $\pm$ 8	> 10
hP2X7	no potentiation	n.d	8 $\pm$ 29 (at 20 μM)	> 10	17 $\pm$ 8	> 10

Table S2. Activation of the human P2X4 receptor, the human P2X2 receptor and chimeric P2X4(P2X2) receptors by ATP^a

Receptor		ATP	
Name	Sequence variation	EC ₅₀ ± SEM (μM)	Efficacy (% ± SEM) at 100 μM ATP
wt hP2X4	-	0.378 ± 0.087	100 ± 15
wt hP2X2	-	0.637 ± 0.047	139 ± 9
Chimera c1	P2X4(P2X2V71-I83)	(4.83 ± 1.38**** ^(*))	4 ± 1**** ^(*)
Chimera c2-c5	P2X4(P2X2C116-T186)	2.57 ± 0.03**** ^(*)	50 ± 2** ^(*)
Chimera c6	P2X4(P2X2N208-S216)	0.572 ± 0.089	87 ± 3 ^(*)
Chimera c7	P2X4(P2X2I218-D224)	1.14 ± 0.09*** ^(*)	54 ± 1** ^(*)
Chimera c8	P2X4(P2X2R265-L269)	0.298 ± 0.037	61 ± 10* ^(*)
Chimera c9	P2X4(P2X2T281-P290)	(1.24 ± 0.20**** ^(*))	5 ± 1**** ^(*)
Chimera c10	P2X4(P2X2R301-Q308)	0.862 ± 0.068**	40 ± 6**** ^(*)

^aATP-induced increases in intracellular calcium concentrations were measured in $n \geq 3$ experiments. The ATP potency and the efficacy of 100 μM ATP were determined. Carbachol (muscarinic M3 receptor agonist natively expressed in the cells) was used as internal reference for determining the maximal ATP-induced stimulation of P2X4 or chimeric P2X4(P2X2) receptors. The maximal responses to 100 μM of ATP were set in relation to the effect induced by 100 μM carbachol (M3 receptor agonist) and normalized accordingly. Results were compared to results obtained at the wt P2X4 receptor and at the wt P2X2. The level of significance was calculated by an ordinary one-way ANOVA with Dunnett's post-hoc test: * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, **** $P < 0.0001$. Significance levels colored in black (*) represent the comparison between the chimeric receptors and the wt P2X4 receptor, while significance levels in blue (*) represent the comparison between the chimeric receptors and the wt hP2X2 receptor.

Table S3. Ivermectin effect on ATP response by P2X4, P2X2 and chimeric P2X4(P2X2) receptors, recombinantly expressed in 1321N1 astrocytoma cells ^a

Receptor		Ivermectin			
Name	Sequence variation	EC ₅₀ ± SEM of ivermectin (μM)	ATP response at 100 μM (%) without ivermectin (± SEM)	ATP response at 100 μM (%) in presence of 10 μM ivermectin (± SEM)	IC ₅₀ ± SEM of ivermectin (μM)
wt hP2X4	-	0.310 ± 0.206	100 ± 15	168 ± 10*	n.d.
wt hP2X2	-	n.d.	139 ± 9	139 ± 2	n.d.
Potentiation of ATP response					
Name	Sequence variation	EC ₅₀ ± SEM of ivermectin (μM)	ATP response at 100 μM (%) without ivermectin (± SEM)	ATP response at 100 μM (%) in presence of 10 μM ivermectin (± SEM)	IC ₅₀ ± SEM of ivermectin (μM)
Chimera c2-c5	P2X4 (P2X2C116-T186)	0.199 ± 0.005	50 ± 2	135 ± 12****	n.d.
Chimera c10	P2X4 (P2X2R301Q308)	1.15 ± 0.66	40 ± 6	70 ± 12	n.d.

Gain of function for ATP					
Name	Sequence variation	EC ₅₀ ± SEM of ivermectin (μM)	ATP response at 100 μM (%) without ivermectin (± SEM)	ATP response at 100 μM (%) in presence of 10 μM ivermectin (± SEM)	IC ₅₀ ± SEM of ivermectin (μM)
Chimera c1	P2X4 (P2X2V71-I83)	1.82 ± 0.76	4 ± 1	29 ± 1****	n.d. ^b
Chimera c9	P2X4 (P2X2T281-P290)	0.356 ± 0.046	5 ± 1	47 ± 6 **	n.d.
Inhibition of ATP response					
Name	Sequence variation	EC ₅₀ ± SEM of ivermectin (μM)	ATP response (%) at 100 μM without ivermectin (± SEM)	ATP response (%) at 100 μM in presence of 10 μM ivermectin (± SEM)	IC ₅₀ ± SEM of ivermectin (μM)
Chimera c7	P2X4 (P2X2I218-D224)	n.d.	54 ± 1	30 ± 1***	25.8 ± 8.6
Chimera c8	P2X4 (P2X2R265-L269)	n.d.	61 ± 10	29 ± 5*	12.1 ± 2.9
Biphasic modulation of ATP response					
Name	Sequence variation	EC ₅₀ ± SEM of ivermectin (μM)	ATP response (%) at 100 μM without ivermectin (± SEM)	ATP response (%) at 100 μM in presence of 10 μM ivermectin (± SEM)	IC ₅₀ ± SEM of ivermectin (μM)
Chimera c6	P2X4 (P2X2N208-S216)	0.174 ± 0.112	87 ± 3	60 ± 10	20.1 ± 6.9

^a Concentration-response curves of ivermectin in the presence of 100 μ M ATP were determined at wt P2X₄, P2X₂ and chimeric P2X₄(P2X₂) receptors. The increases in intracellular calcium concentrations were measured in $n \geq 3$ experiments, and the potency of ivermectin was calculated. The ATP response at 100 μ M in the presence of 10 μ M ivermectin was compared to the ATP response without ivermectin based on the effect observed at the wt hP2X₄ receptor. The level of significance was determined by an unpaired t-test (* $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$).

^bn.d, could not be determined.

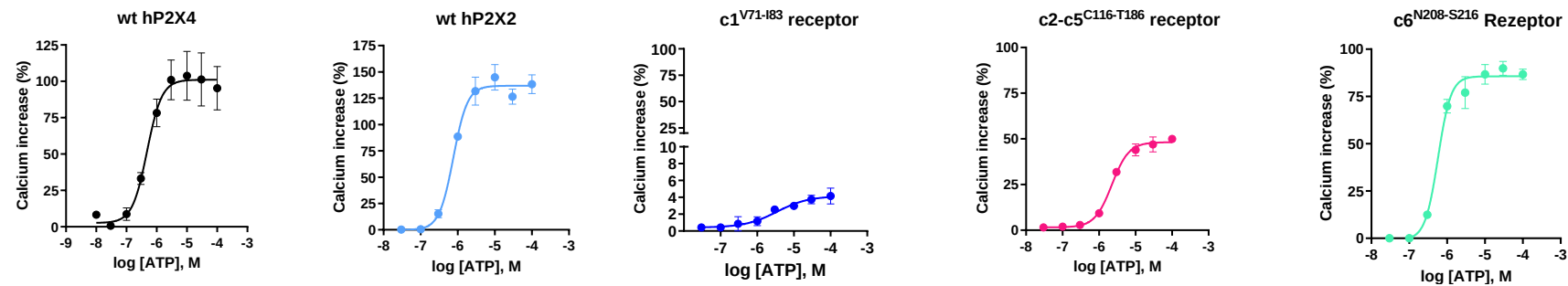
Table S4. Modulation of ATP response by the negative allosteric P2X₄ receptor modulator BX430 of the P2X₂ and chimeric P2X₄(P2X₂) receptors, and receptor mutants, recombinantly expressed in 1321N1 astrocytoma cells^a

Receptor		BX430	
Name/mutant	Sequence variation	IC ₅₀ ± SEM (μM)	Inhibition at 10 μM (% ± SEM)
wt hP2X ₄	-	0.127 ± 0.045	98 ± 6
wt hP2X ₂	-	>> 10	-1 ± 3
Chimera c2-c5	P2X ₄ (P2X ₂ C116-T186)	0.676 ± 0.073****	98 ± 6
Chimera c6	P2X ₄ (P2X ₂ N208-S216)	28.8 ± 8.6****	35 ± 5
Chimera c7	P2X ₄ (P2X ₂ I218-D224)	0.0536 ± 0.0146	97 ± 3
Chimera c8	P2X ₄ (P2X ₂ R265-L269)	0.323 ± 0.061**	77 ± 6
Chimera c10	P2X ₄ (P2X ₂ R301-Q308)	0.0588 ± 0.0115	102 ± 2
Y300F	-	0.0531 ± 0.0095	104 ± 5
I209A	-	1.21 ± 0.14**** ^b	38 ± 9
T212S	-	0.162 ± 0.016	95 ± 3

^aConcentration-response curves of BX430 in the presence of ATP at its EC₈₀ value were determined at wt hP2X₄, hP2X₂, and chimeric P2X₄(P2X₂) receptors. Increases of intracellular calcium concentrations were measured in n ≥ 3 experiments. The potency of BX430 and its percent inhibition at 10 μM were calculated and compared to the effects at the wt hP2X₄ receptor. The level of significance was calculated by an ordinary one-way ANOVA with Dunnett's post-hoc test: * < 0.05, ** P < 0.01, *** P < 0.001, **** P < 0.0001.

^bEstimated IC₅₀ value based on extrapolation of the curve.

A



B)

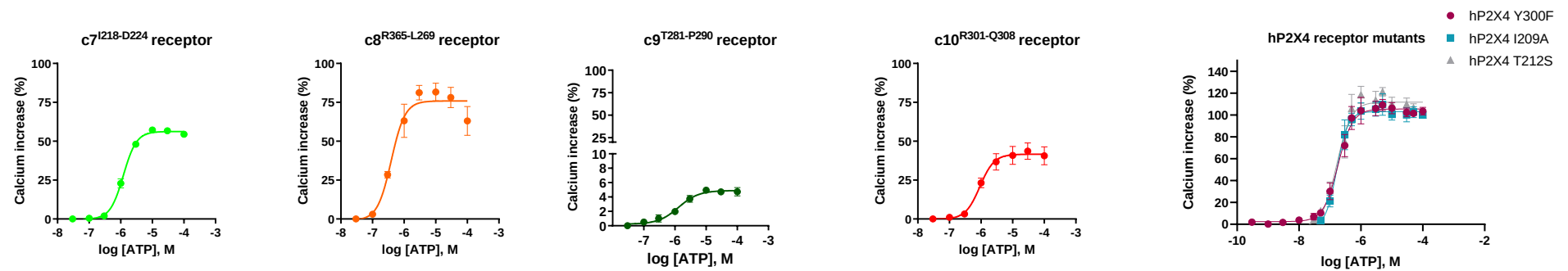


Figure S1. Concentration-response curves of ATP at human P2X4, P2X2, and chimeric P2X4(hP2X2) receptors and P2X4 receptor mutants.

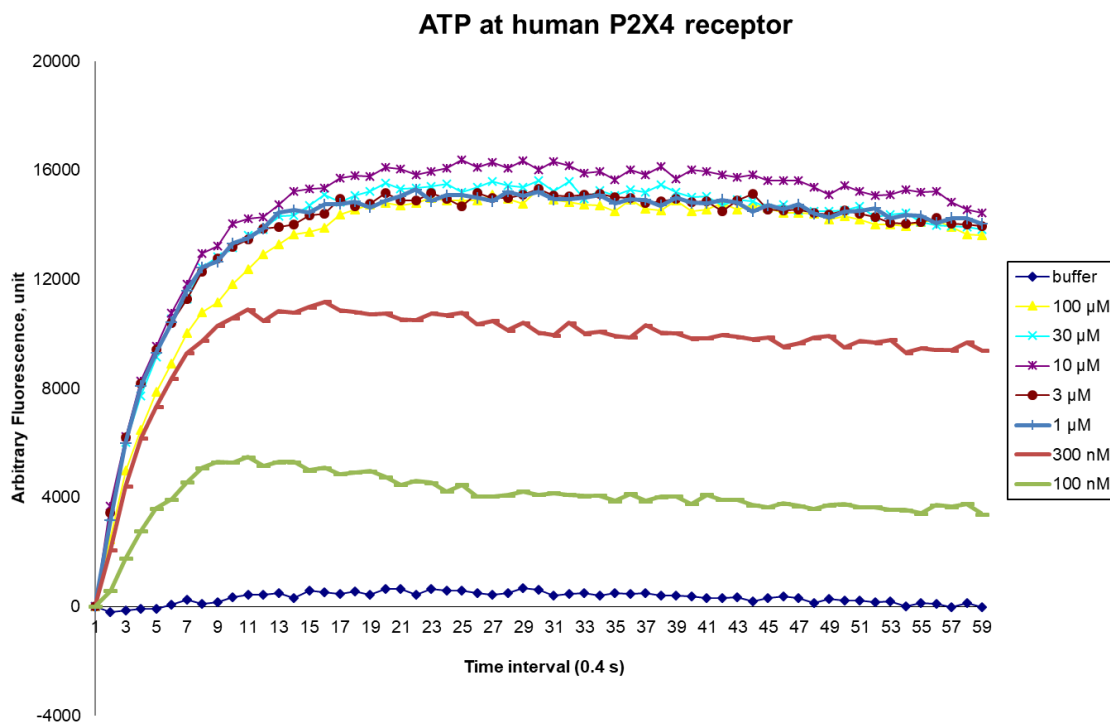


Figure S2. Typical calcium influx experiments showing the concentration-dependent increase in fluorescence by ATP at 1321N1 astrocytoma cells recombinantly expressing the human P2X4 receptor.

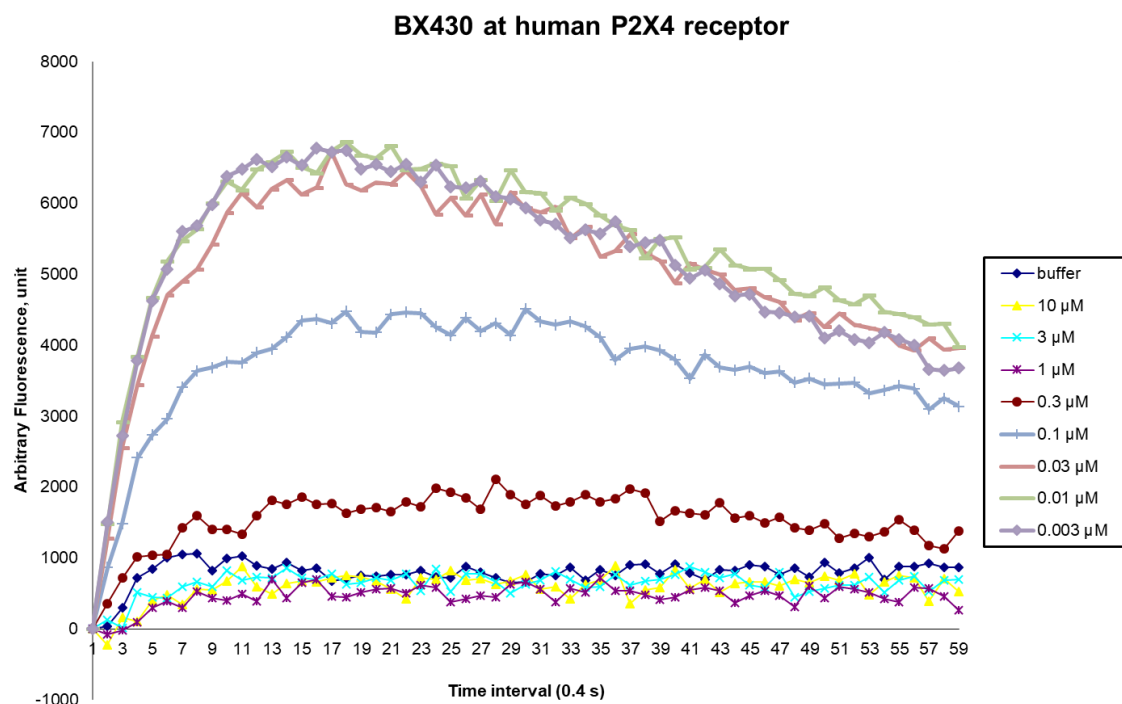


Figure S3. Typical calcium influx experiments showing the concentration-dependent fluorescence induced by ATP (EC_{80} , 1 μM) in the absence (buffer) and in the presence of various concentrations of the P2X4 receptor antagonist BX430, determined in 1321N1 astrocytoma cells recombinantly expressing the human P2X4 receptor.

Variable region v6

SP P51575 P2RX1_HUMAN	EVNAAHMKT	216
SP Q9UBL9 P2RX2_HUMAN	DRTDGYLKR	225
SP P56373 P2RX3_HUMAN	NLTARDMKT	202
SP Q99571 P2RX4_HUMAN	NITTTYLKS	216
SP 015547 P2RX6_HUMAN	TWDPTYFKH	227
SP Q99572 P2RX7_HUMAN	GLNIT----	215

Variable region v7

SP P51575 P2RX1_HUMAN	LFHKTLLH	224
SP Q9UBL9 P2RX2_HUMAN	TFHEASD	233
SP P56373 P2RX3_HUMAN	RFHPDKD	210
SP Q99571 P2RX4_HUMAN	IYDAKTD	224
SP 015547 P2RX6_HUMAN	RYEPQFS	235
SP Q99572 P2RX7_HUMAN	TFHKTQN	223

Variable region v8

SP P51575 P2RX1_HUMAN	WHVRH	269
SP Q9UBL9 P2RX2_HUMAN	LPASE	278
SP P56373 P2RX3_HUMAN	KAWDQ	255
SP Q99571 P2RX4_HUMAN	RAASL	269
SP 015547 P2RX6_HUMAN	TGDSG	280
SP Q99572 P2RX7_HUMAN	RWFHH	268

Figure S2. Sequence alignment of human P2X1, P2X2, P2X3, P2X4, P2X6 and P2X7 receptors regarding variable regions v6, v7, v8.

7.3 Appendix C: Protein sequences

Primary sequence of the human P2X4 wildtype receptor

MAGCCAALAAFLFEYDTPRIVLIRSRKVGLMNRAVQLLILAYVIGWVFVWEKGYQETDSV
VSSVTTKVKGVAVTNTSKLGFRIWDVADYVIPAQEENSLFVMTNVILTMNQTQGLCPEIP
DATTVCKSDASCTAGSAGTHSNGVSTGRCVAFNGSVKTCEVAAWCPVEDDTHVPQPAFLK
AAENFTLLVKNNIWYPKFNFSKRNILPNITTTYLKSCIYDAKTDPFCPIFRLGKIVENAG
HSFQDMAVEGGIMGIQVNWDCNLDRAASLCLPRYSFRRLDTRDVEHNVSPGYNFRFAKYY
RDLAGNEQRTLKAYGIRFDIIVFGKAGKFDIIPMTMINIGSGLALLGMATVLCDIIVLYC
MKKRLYYREKKYKYVEDYEQGLASELDQ

Primary sequence of receptor construct JN3

The following parts are colored as follows:

Point mutations

GSSG linker

HRV 3C protease cleavage site

10x-His tag

MAGCCAALAAFLFEYDTPRIVLIRSRKVGLMNRAVQLLILAYVIGWVFVWEKGYQETDSV
VSSVTTKVKGVAVT**RT**SKLGFRIWDVADYVIPAQEENSLFVMTNVILTMNQTQGLCPEIP
DATTVCKSDASCTAGSAGTHSNGVSTGRCVAFNGSVKTCEVAAWCPVEDDTHVPQPAFLK
AAE**RT**FTLLVKNNIWYPKFNFSKRNILPNITTTYLKSCIYDAKTDPFCPIFRLGKIVENAG
HSFQDMAVEGGIMGIQVNWDCNLDRAASLCLPRYSFRRLDTRDVEHNVSPGYNFRFAKYY
RDLAGN**T**QRTLKAYGIRFDIIVFGKAGKFDIIPMTMINIGSGLALLGMATVLCDIIVLYC
MKKRLYYREKKYKYVEDYEQGLASELDQ**GSSGEF****LEVLFQGP****HHHHHHHHHH**-

Protein sequence alignment of the human P2X4 wildtype receptor (1) and receptor construct JN3 (2). Sequences were aligned with Clustal Omega (1.2.4).²⁰⁸

The following parts are colored as follows:

Point mutations

GSSG linker

HRV 3C protease cleavage site

10x-His tag

(1)	MAGCCAALAAFLFEYDTPRIVLIRSRKVGLMNAVQLLLILAYVIGWVFVWEKGYQETDSV	60
(2)	MAGCCAALAAFLFEYDTPRIVLIRSRKVGLMNAVQLLLILAYVIGWVFVWEKGYQETDSV	60

(1)	VSSVTTKVKGVAVTNTSKLGFRIWDVADYVIPAQEENSLFVMTNVILTMNQTQGLCPEIP	120
(2)	VSSVTTKVKGVAVTGTSKLGFRWDVADYVIPAQEENSLFVMTNVILTMNQTQGLCPEIP	120

(1)	DATTVCKSDASCTAGSAGTHSNGVSTGRCVAFNGSVKTCCEVAACPVEDDTHVPQPAFLK	180
(2)	DATTVCKSDASCTAGSAGTHSNGVSTGRCVAFNGSVKTCCEVAACPVEDDTHVPQPAFLK	180

(1)	AAENFTLLVKNNIWYPKFNFSCRNIIPNITTTTLKSCIYDAKTDPCPIFRLGKIVENAG	240
(2)	AAENFTLLVKNNIWYPKFNFSCRNIIPNITTTTLKSCIYDAKTDPCPIFRLGKIVENAG	240

(1)	HSFQDMAVEGGIMGIQVNWDCNLDRAASLCLPRYSFRRLDTRDVEHNVSPGYNFRFAKYY	300
(2)	HSFQDMAVEGGIMGIQVNWDCNLDRAASLCLPRYSFRRLDTRDVEHNVSPGYNFRFAKYY	300

(1)	RDLAGNEQRTLIKAYGIRFDIIVFGKAGKFDIPTMINIGSGLALLGMATVLCDIIVLYC	360
(2)	RDLAGNTQRTLIKAYGIRFDIIVFGKAGKFDIPTMINIGSGLALLGMATVLCDIIVLYC	360

(1)	MKKRLYYREKKYKYVEDYEQGLASELDQ-----	388
(2)	MKKRLYYREKKYKYVEDYEQGLASELDQGSSGEFLEVLFGPHHHHHHHHHH	412

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