MAPPING THE BINDING SITE OF P2X7 RECEPTOR ANTAGONISTS

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Abstract

Mapping the binding site of P2X7 receptor antagonists-Anfal Bin Dayel

The P2X7 receptor (P2X7R) is a ligand gated ion channel activated by high concentrations of ATP that are usually found in conditions of stress, cellular damage and disease. Selective P2X7R antagonists therefore may have therapeutic potential for treatment of several pathological conditions. Understanding how P2X7R antagonists act on the receptor is useful to facilitate drug design. In this thesis the molecular basis for several selective P2X7R antagonists has been investigated. This was done by using a range of chimeras and mutations between antagonist sensitive hP2X7R and antagonist insensitive hP2X1R. This work showed that the selective P2X7R antagonist AZ11645373 binds at the allosteric pocket between two adjacent subunits away from the orthosteric ATP binding pocket. Molecular docking of AZ11645373 is in line with effects of mutations on the sensitivity to the antagonist. The allosteric binding mode of AZ11645373 explains variation in antagonist sensitivity between human and rat P2X7Rs. Comparing the effects of allosteric point mutations of AZ11645373 with other previously characterized allosteric inhibitors demonstrated that there is a similar reduction in sensitivity with the F88A, T90V, D92A, F103A and V312A mutations. These allosteric mutations were used as a signature to identify the site of action for P2X7R antagonists ZINC58368839, KN-62, Brilliant Blue G and Calmidazolium. The combination of signature allosteric point mutations and molecular docking propose that ZINC58368839 has a mode of binding similar to AZ11645373. However, the binding site for the large molecule P2X7R antagonists KN-62, Brilliant Blue G and Calmidazolium appear to be more complex, because they not only bind at the allosteric site but also extend into the central cavity. In conclusion, this thesis has enhanced our knowledge of selective P2X7R antagonists and advanced a template to develop allosteric P2X7R inhibitors.
Publications

Papers


Abstract

Anfal F Bin Dayel, Richard J Evans. Mapping the binding site for the P2X7 receptor antagonist AZ11645373. Poster presentation at the Italian-German purine club meeting 2017, Italy, Rome.

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Abbreviations

**A-438079** 3-[[5-(2,3-dichlorophenyl)-1H-tetrazol-1-yl]methyl]pyridine

**A-70003** [N-(1-((cyanoimino)(5-quinolinylamino)methyl)amino)-2,2-dimethylpropyl]-2-(3,4-dimethoxyphenyl) acetamide

**Aβ** Amyloid β

**ADP** Adenosine diphosphate

**ATP** Adenosine 5'-triphosphate

**ATPγS** Adenosine-5'-O-(3-thio) triphosphate

**ANOVA** Analysis of variance

**AZ10606120** N-[2-(2-[2-hydroxyethyl]amino)ethyl]amino]-5-quinolinyl]-2-tricyclo[3.3.1.1^{3,7}]dec-1-ylacetamide

**AZ11645373** [1-[[3'-Nitro[1,1'-biphenyl]-4-yl]oxy] methyl]-3-(4-pyridinyl) propyl]-2,4-thiazolidinedione

**BzATP** 2'(3')-O-(4-Benzylobenzoyl) adenosine 5'-triphosphate

**CaMKII** Calcium-calmodulin dependent protein kinase II

**cAMP** Cyclic adenosine monophosphate

**cDNA** Complementary DNA

**CNS** Central nervous system

**CTP** Cytidine triphosphate

**DAG** Diacylglycerol

**DMEM** Dulbecco's Modified Eagle's medium

**EC_{50}** Half maximal effective concentration of agonist

**EC_{90}** Concentration of agonist that induced 90% of the maximal response

**E-coli** Escherichia-coli

**FSEC** Fluorescence detection size exclusion chromatography

**GFP** Green fluorescent protein
<table>
<thead>
<tr>
<th>Term</th>
<th>Definition</th>
</tr>
</thead>
<tbody>
<tr>
<td>HEK293 cell</td>
<td>Human embryonic kidney 293 cell</td>
</tr>
<tr>
<td>HIF1-α</td>
<td>Hypoxia inducible factor 1-alpha</td>
</tr>
<tr>
<td>hP2X7R</td>
<td>Human P2X receptor</td>
</tr>
<tr>
<td>IC₅₀</td>
<td>Half maximal inhibitory concentration of antagonist</td>
</tr>
<tr>
<td>IL-1β</td>
<td>Interleukin-1β</td>
</tr>
<tr>
<td>IL-6</td>
<td>Interleukin-6</td>
</tr>
<tr>
<td>IL-8</td>
<td>Interleukin-8</td>
</tr>
<tr>
<td>IP₃</td>
<td>Inositol triphosphate</td>
</tr>
<tr>
<td>JNJ-54175446</td>
<td>4-Methyl-6,7-dihydro-4H-triazolo[4,5-c]pyridine</td>
</tr>
<tr>
<td>KN-62</td>
<td>[4-[(2S)-2-[(5-isoquinolinylsulfonyl) (methyl amino)-3-oxo-3-(4-phenyl-1-piperazinyl)propyl]phenyl isoquinoline sulfonic acid ester</td>
</tr>
<tr>
<td>KO</td>
<td>Knockout</td>
</tr>
<tr>
<td>Ms</td>
<td>Millisecond</td>
</tr>
<tr>
<td>MTS</td>
<td>Methanethiosulfonate</td>
</tr>
<tr>
<td>MTS-TPAE</td>
<td>2-[(Tripentylammonium)ethyl Methanethiosulfonate Bromide</td>
</tr>
<tr>
<td>MW</td>
<td>Molecular weight</td>
</tr>
<tr>
<td>NANC</td>
<td>Non-aderenergic non-cholinergic</td>
</tr>
<tr>
<td>NEAA</td>
<td>Nonessential amino acid</td>
</tr>
<tr>
<td>NF449</td>
<td>(4,4′,4″,4‴- (carbonyl bis (imino-5,1,3 benzenetriylbis(carbonylimino)))tetrakis-benzene-1,3-disulfonic acid)</td>
</tr>
<tr>
<td>nfP2X7R</td>
<td>Non-pore functional P2X7 receptor</td>
</tr>
<tr>
<td>NLRP3</td>
<td>Nucleotide like receptor family Pyrin domain member 3 inflammasome</td>
</tr>
<tr>
<td>NMDG</td>
<td>N-methyl-D-glutamine</td>
</tr>
<tr>
<td>NO₂</td>
<td>Nitro</td>
</tr>
<tr>
<td>oATP</td>
<td>Oxidized adenosine 5’-triphosphate</td>
</tr>
<tr>
<td>PBMCs</td>
<td>Peripheral blood mononuclear cells</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Definition</td>
</tr>
<tr>
<td>--------------</td>
<td>------------</td>
</tr>
<tr>
<td>PCR</td>
<td>Polymerase chain reaction</td>
</tr>
<tr>
<td>Pd</td>
<td>Panda</td>
</tr>
<tr>
<td>PLC</td>
<td>Phospholipase C</td>
</tr>
<tr>
<td>PNS</td>
<td>Peripheral nervous system</td>
</tr>
<tr>
<td>PolyA</td>
<td>Polyadenylated</td>
</tr>
<tr>
<td>PPADS</td>
<td>Pyridoxal phosphate-6-azophenyl-2-4-disulfonic acid</td>
</tr>
<tr>
<td>P2XR</td>
<td>Purinoreceptor 2X receptor</td>
</tr>
<tr>
<td>P2YR</td>
<td>Purinoreceptor 2Y receptor</td>
</tr>
<tr>
<td>RFU</td>
<td>Relative fluorescence units</td>
</tr>
<tr>
<td>rP2X7R</td>
<td>Rat P2X receptor</td>
</tr>
<tr>
<td>Rpm</td>
<td>Rotations per minute</td>
</tr>
<tr>
<td>S</td>
<td>Second</td>
</tr>
<tr>
<td>SEM</td>
<td>Standard error of the mean</td>
</tr>
<tr>
<td>SNP</td>
<td>Single nucleotide polymorphism</td>
</tr>
<tr>
<td>SOC</td>
<td>Super optimal broth with catabolite repression</td>
</tr>
<tr>
<td>TEVC</td>
<td>Two-electrode voltage clamp</td>
</tr>
<tr>
<td>THP-1</td>
<td>Tamm-horsefall protein-1</td>
</tr>
<tr>
<td>TM1</td>
<td>The first tranmembrane</td>
</tr>
<tr>
<td>TM2</td>
<td>The second tranmembrane</td>
</tr>
<tr>
<td>TNF-α</td>
<td>Tumor necrosis factor-α</td>
</tr>
<tr>
<td>TNP-ATP</td>
<td>2’, 3’-O-(2, 4, 6-trinitrophenyl)- adenosine 5’-triphosphate</td>
</tr>
<tr>
<td>UDP</td>
<td>Uridine diphosphate</td>
</tr>
<tr>
<td>UTP</td>
<td>Uridine 5’-triphosphate</td>
</tr>
<tr>
<td>VEGF</td>
<td>Vascular endothelial growth factor</td>
</tr>
<tr>
<td>WT</td>
<td>Wild type</td>
</tr>
</tbody>
</table>
YO-PRO-1 quinolinium, 4-[(3-methyl-2(3H)-benzoxazolylidene) methyl]-1-[3-(triethylammonio) propyl] – diiodide

Zf Zebra fish

ZINC58368839 N-cycloheptyl-N-methyl-2-(5-nitro-1H-indol-1-yl) acetamide
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References
Chapter 1: Introduction

1. Introduction

The idea of purinergic transmission was proposed by Geoffrey Burnstock (Burnstock, 1972). It is now well known from a variety of studies that adenosine 5'-triphosphate (ATP) can act at ligand gated ion channel P2X receptors (P2XRs) and G-protein coupled P2Y receptors (P2YRs). To date, seven subunits of mammalian P2XRs have been identified (P2X1-P2X7), which assemble to form homomers or heteromers with a wide range of physiological and pathological roles. P2X7 receptors (P2X7Rs) have distinct properties, most notably requiring high concentrations of ATP to activate the receptor and this usually only occurs in conditions of stress and cell damage. As a result of this, P2X7Rs contribute to a range of disease processes and are novel target for a range of therapeutic strategies. The first part of this introduction is a brief overview of the history and types of purinergic receptors.

1.1. Adenosine 5'-triphosphate (ATP)

ATP was isolated from muscle extracts by Lohmann and Fiske (Lohmann, 1929, Fiske and Subbarow, 1929). The first role described for ATP was as an intracellular energy carrier. In terms of structure, ATP consists of an adenine base, a ribose sugar and a chain of three phosphate groups (figure 1.1) (Baddiley et al., 1949). Energy is generated during hydrolysis of ATP to adenosine diphosphate (ADP). It is produced by different process such as glycolysis, the citric acid cycle and oxidative phosphorylation (Lipmann, 1941). Usually high levels of ATP (mM) are found within cells (Bonora et al., 2012). It is co-stored in synaptic vesicles with other neurotransmitters such as acetylcholine and noradrenaline (Sperlagh et al., 1997, Hoyle, 1996).

![Figure 1.1. Structure of ATP. ATP consists of an adenine, a ribose and three phosphate groups.](image-url)
1.1.1. History of ATP as a transmitter

The first study suggesting that ATP has an extracellular role was by Drury and Szent–Györgyi in 1929. They observed that injection of ATP and/or adenosine into the guinea pig reduced the heart rate and produced vasodilation (Drury and Szent-Györgyi, 1929). Subsequently in 1948, it was reported that injection of ATP into the ventricle caused ataxia and muscular weakness (Emmelin and Feldberg, 1948). In the 1950s, Holton reported that following nerve stimulation ATP was released and produced vasodilation (Holton and Holton, 1954) and that ATP was released from sensory nerves (Holton, 1959).

In 1963, Burnstock and his co-workers were examining neurotransmission in the intestinal smooth muscle of guinea pig taenia coli. Following blockade of adrenergic receptors by bretylium and muscarinic receptors by atropine, they noticed a residual neurogenic component that produced hyperpolarization and relaxation. This was termed non-adrenergic non-cholinergic (NANC) neurotransmission (Burnstock et al., 1963). This residual component was blocked by tetrodotoxin that prevents nerve conduction, demonstrating that NANC mediates the neurogenic inhibitory junction potentials (Burnstock et al., 1964). The effects of NANC were mimicked by the application of ATP, leading to the proposal of ATP as a neurotransmitter.

The concept of ATP as a neurotransmitter was supported by studies showing that the ATP is co-released with other neurotransmitters. In the peripheral nervous system (PNS), it was shown that ATP was released with noradrenaline from sympathetic nerves and acetylcholine from parasympathetic nerves (Su et al., 1971, Burnstock, 1978). In the central nervous system (CNS), ATP has been shown to be co-released with all classical transmitters such as dopamine, glutamate, GABA and others (Burnstock, 2004a). ATP is not only released from neuronal cells but it can be also released from non-neuronal cells. It was demonstrated that ATP was released from non-neuronal vascular cells (e.g. platelets) and non-vascular cells (e.g. astrocytes). Furthermore, ATP can be released under mechanical stress as well as cell death and hypoxia (Bodin and Burnstock, 2001). After it is released, ATP is broken down by several ectonucleotidase enzymes to adenosine which is then taken back up in to the nerve terminal to be resynthesized and restored in vesicles (Zimmermann, 2001).
1.2. Purine Receptors

Purinergic receptors were proposed in 1978 and classified into two types, P1 receptors for adenosine and P2 receptors for ATP/ADP (Burnstock, 1978). P2 receptors were then divided into two types dependent on their pharmacological properties into P2X receptors (P2XRs) and P2Y receptors (P2YRs) (Burnstock and Kennedy, 1985). Subsequently cloning of P2 receptors classified the receptors into two types based on their structure; P2XR ligand gated ion channels and P2YR G-protein coupled receptors (Abbracchio and Burnstock, 1994).

1.2.1. P1 Receptors

P1 receptors are commonly called adenosine receptors (Fredholm et al., 2001). The cloning of P1 receptors identified 4 different subtypes (A₁, A₂A, A₂B and A₃) (Libert et al., 1989). These are G protein coupled receptors. The A₁ and A₃ receptors are coupled to Gᵢ proteins while A₂A and A₂B receptors are coupled to Gₐ proteins (Freissmuth et al., 1991, Olah, 1997, Pierce et al., 1992, Palmer et al., 1995). The activation of the adenosine receptor subtypes coupled to Gₐ leads to increased cyclic adenosine monophosphate (cAMP) production by the stimulation of adenylate cyclase, while the activation of the adenosine receptor subtypes coupled to Gᵢ leads to decreased cAMP levels by inhibition adenylate cyclase (Burnstock, 2007). Adenosine receptors show a wide distribution throughout the body and have a range of physiological and pathological roles. For example; (i) A₁ receptors activation has been shown to reduce pain sensation (Borghi et al., 2002) (ii) A₂B receptors are expressed on the lung and contribute to asthma (Feoktistov and Biaggioni, 1995) and (iii) A₂A and A₃ receptors activation reduced inflammatory responses (Salvatore et al., 2000, Jordan et al., 1997).

1.2.2. P2 Receptors

(i) P2Y Receptors

The first complementary DNAs encoding P2YRs were isolated in 1993 from embryonic chick brain and mouse neuroblastoma cells (Lustig et al., 1993, Webb et al., 1993). Eight different subtypes have been cloned (P2Y₁, P2Y₂, P2Y₄, P2Y₆, P2Y₁₁, P2Y₁₂, P2Y₁₃ and P2Y₁₄). According to the subtype, P2YRs are stimulated by different
types of nucleotides such as ADP, ATP, uridine diphosphate (UDP) or uridine 5’-triphosphate (UTP). All P2YR subtypes are G-protein coupled receptors (Abbrachio et al., 2006). The eight P2YRs are classified into two groups dependent on the signal transduction. The first group is coupled to G_q proteins (P2Y_1, P2Y_2, P2Y_4, P2Y_6 and P2Y_11). The second group is coupled to G_i proteins (P2Y_12, P2Y_13 and P2Y_14) (Abbrachio et al., 2006). The G_q protein coupled P2YR subtypes stimulate phospholipase C (PLC) and generate inositol triphosphate (IP_3) and diacylglycerol (DAG). The G_i protein coupled P2YR subtypes inhibit adenylate cyclase and reduce cAMP levels.

P2YRs are highly expressed in endothelial cells, platelets, pancreatic β-cells, immune cells, brain, kidney tubules and osteoblasts (Loubatieres-Mariani and Chapal, 1988, Eltze and Ullrich, 1996, Kunapuli and DANIEL, 1998, MacKenzie et al., 1999, Hoebertz et al., 2002). This widespread expression has a diversity of functions in the cardiovascular system, respiratory system, immune system and bone. Therefore P2YRs could be used in the treatment of different conditions including cardiovascular disease, osteoporosis, diabetes, and cystic fibrosis (Erlinge, 2011). Some P2YRs antagonists are used clinically, for instance the P2Y_12R antagonist clopidogrel has anti-coagulant effects (Savi and Herbert, 2005). For general reviews of P2YRs function see (Abbrachio et al., 2006, von Kuegelgen and Hoffmann, 2016).

(ii) P2X Receptors

Seven mammalian P2XR subunits (P2X1R to P2X7R) have been cloned. The first two subunits were isolated in 1994 and named P2X1R and P2X2R (Valera et al., 1994, Brake et al., 1994). The other subunits were isolated and named (P2X3R-P2X7R). These subunits assemble to form homotrimeric or heterotrimeric channels (North, 2002). The sequence of amino acids varies between P2XR subunits, with ~35 to 50% similarity (Kaczmarek-Hájek et al., 2012). The length is also different between P2XR subunits, the shortest is the P2X6R with 379 amino acids and the longest is the P2X7R with 595 amino acids (table 1.1) (North, 2002).
P2XRs are non-selective ion channels activated by ATP binding leading to an influx of Na\(^+\) and Ca\(^{2+}\) and efflux of K\(^+\), and membrane depolarization (Burnstock and Verkhratsky, 2012). The receptors show a wide distribution throughout the body. This indicates that P2XRs have multiple functional roles. P2XRs are structurally distinct from other ligand gated ion channels such as cysteine loop receptors and inotropic glutamate receptors. P2XRs have two transmembrane domains (TM1 and TM2), a large extracellular domain and intracellular amino (N) and carboxyl (C)-termini (Coddou et al., 2011).

The following sections will be presented (i) the general properties of homomeric and heteromeric P2XRs, with a focus on the P2X7R; (ii) the native distribution, physiological and pathological roles of P2XRs; (iii) pre-crystal and crystal structure of P2XRs; and (iv) P2XR antagonists, with a focus on P2X7R antagonists.

<table>
<thead>
<tr>
<th>Receptor</th>
<th>The first isolated cloning</th>
<th>Length of amino acid</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>P2X1R</td>
<td>Rat vas deferens</td>
<td>399</td>
<td>(Valera et al., 1994)</td>
</tr>
<tr>
<td>P2X2R</td>
<td>Rat pheochromocytoma cells</td>
<td>472</td>
<td>(Brake et al., 1994)</td>
</tr>
<tr>
<td>P2X3R</td>
<td>Rat dorsal root ganglion</td>
<td>397</td>
<td>(Chen et al., 1995, Lewis et al., 1995)</td>
</tr>
<tr>
<td>P2X4R</td>
<td>Rat superior cervical ganglion</td>
<td>388</td>
<td>(Bo et al., 1995, Buell et al., 1996)</td>
</tr>
<tr>
<td>P2X5R</td>
<td>Rat celiac ganglion</td>
<td>417</td>
<td>(Collo et al., 1996)</td>
</tr>
<tr>
<td>P2X6R</td>
<td>Rat superior cervical ganglion</td>
<td>379</td>
<td>(Collo et al., 1996)</td>
</tr>
<tr>
<td>P2X7R</td>
<td>Rat brain</td>
<td>595</td>
<td>(Surprenant et al., 1996)</td>
</tr>
</tbody>
</table>

Table 1.1. The first isolated cloning and length of amino acids of P2X receptors.
1.3. General properties of homomeric P2X receptors

The properties of P2XRs depend on the subunit composition and show variations resulting from amino acid differences. The section below summarizes the properties of recombinant homomeric P2XRs in human embryonic kidney (HEK) 293 cells and Xenopus oocytes. These properties include sensitivity to agonists, the time-course of the response to ATP and sensitivity to divalent cations.

1.3.1. Agonists

Agonist sensitivity can be measured as the concentration required to produce fifty percentage of the maximum effect (EC$_{50}$). ATP is a physiological agonist for all P2XRs. However, the sensitivity of this agonist differs between P2XR subunits. (i) P2X1 and P2X3Rs have a high ATP potency with an EC$_{50}$ concentration of < 1 µM (Chen et al., 1995, Garcia-Guzman et al., 1997b). (ii) The P2X2R has an EC$_{50}$ that ranges from 10 to 60 µM (Brake et al., 1994, Evans et al., 1995). (iii) P2X4, P2X5 and P2X6Rs have EC$_{50}$ concentrations ~ 10 to 15 µM (Buell et al., 1996, Collo et al., 1996). (iv) The P2X7R has a low ATP sensitivity with an EC$_{50}$ concentration of > 100 µM in normal physiological solution (Surprenant et al., 1996). The reason for the decreased ATP sensitivity at P2X7Rs is due in part to the inhibitory effects of divalent cations (see section 1.3.3). In contrast to ATP, breakdown products such as ADP are ineffective at P2XRs (Burnstock, 2004b). In addition, nucleoside triphosphates such as cytidine triphosphate (CTP) can act as a weak agonist at P2XRs (Burnstock, 2004b).

ATP analogues can be used to distinguish between P2XR subunits. For instance, α,β-methylene-ATP (α,β-meATP) is effective at P2X1 and P2X3Rs, with an EC$_{50}$ of ~ 1 µM (Evans et al., 1995, Chen et al., 1995). Adenosine-5‘-O-(3-thio) triphosphate (ATP$_{3S}$) is effective at all P2XRs except the P2X7R (Burnstock, 2004b). Furthermore, 2(3’)-O-(4-Benzoylbenzoyl) adenosine-5‘-triphosphate (BzATP) is the most potent agonist at the P2X7R and is ~10 fold more potent than ATP, with an EC$_{50}$ of concentration ~ 10 µM in normal physiological solution (Surprenant et al., 1996).
1.3.2. Time-course of the response to ATP evoked currents

P2XR subtypes can also be distinguished based on the time-course of the response to ATP. This can be divided into the activation and desensitization phase. The activation phase is a rapid rise of inward current evoked by the agonist application (North, 2002). Most of P2XRs have a fast activation and produce a reproducible response with a rapid rise time. However, the rise time of the P2X7R differs from other P2XRs (table1.2) (see properties of P2X7R section 1.4.1).

During sustained agonist application the inward current may decay to baseline; which is referred to as desensitization (North, 2002). The desensitization rate is different between P2XR subunits (figure1.2) (table1.2). P2X1 and P2X3Rs show fast desensitization and decay by 50% within 100-300 milliseconds (ms) during agonist application (Valera et al., 1994, Lewis et al., 1995). In contrast, the P2X2R show slow desensitization with < 15% decay during a 2-seconds (s) agonist application (Evans et al., 1996). P2X4 and P2X6Rs show intermediate desensitization, ~ 60% of the current remaining at the end of 2s agonist application (Collo et al., 1996, Garcia-Guzman et al., 1997a). At the P2X7R, the inward current evoked by ATP does not desensitize with sustained agonist application, indeed it may continue to increase (see facilitation section 1.4.1) (North, 2002).

<table>
<thead>
<tr>
<th>Receptor</th>
<th>Activation</th>
<th>10-90% rise time</th>
<th>Desensitization</th>
<th>% remaining at 2 seconds</th>
</tr>
</thead>
<tbody>
<tr>
<td>P2X1R</td>
<td>Fast</td>
<td>7 ms</td>
<td>Fast</td>
<td>0%</td>
</tr>
<tr>
<td>P2X2R</td>
<td>Fast</td>
<td>500 ms</td>
<td>Slow</td>
<td>78%</td>
</tr>
<tr>
<td>P2X3R</td>
<td>Fast</td>
<td>50 ms</td>
<td>Fast</td>
<td>90%</td>
</tr>
<tr>
<td>P2X4R</td>
<td>Biphasic</td>
<td>400 ms</td>
<td>Intermediate</td>
<td>64%</td>
</tr>
<tr>
<td>P2X5R</td>
<td>Fast</td>
<td>14 ms</td>
<td>Slow</td>
<td>87%</td>
</tr>
<tr>
<td>P2X6R</td>
<td>Fast</td>
<td>14 ms</td>
<td>Intermediate</td>
<td>60%</td>
</tr>
<tr>
<td>P2X7R</td>
<td>Biphasic</td>
<td>20 s*</td>
<td>Non</td>
<td>-</td>
</tr>
</tbody>
</table>

Table 1.2. The time-course of the response to ATP at the P2X receptors.
Figure 1.2. Time-course of the response to ATP evoked currents. (a) P2X receptor inward currents induced by sustained ATP application. P2X receptors expressed in HEK293 cells and activated by ATP (10 µM for P2X1R and P2X3R, 100 µM for P2X2R and P2X4R and 3 mM for P2X7R), $\tau_{\text{des}}$ (desensitization time-constant) (images taken from Coddou et al., 2011).
1.3.3. Effect of divalent cations and protons

The sensitivity to divalent cations varies between P2XR subunits. In normal physiological solution, divalent cations particularly magnesium (Mg$^{2+}$) can bind to ATP$^{4-}$ and form the MgATP$^{2-}$ complex. The effect of this complex is not the same between P2XRs. It was reported that the MgATP$^{2-}$ activated responses at the P2X1 and P2X3Rs. In contrast, MgATP$^{2-}$ inhibited responses at the P2X2, P2X4 and P2X7Rs (Valera et al., 1994, Li et al., 2013). For many P2XRs the removal of divalent cations from the external solution can enhance the response. For instance, at the P2X7R, the removal of Mg$^{2+}$ increased current amplitude 6 to 8 fold and increased ATP sensitivity ~ 2 fold (Surprenant et al., 1996, Rassendren et al., 1997b).

Furthermore, there is variation in pH sensitivity between P2XRs. For example, acidification decreasing extracellular pH from ~7 to 6 has been shown to decrease the peak current amplitude of P2X1, P2X3, P2X4 and P2X7Rs. Whereas, P2X2R responses were potentiated at acidic pH (table 1.3) (Stoop et al., 1997, Virginio et al., 1997).

<table>
<thead>
<tr>
<th>Receptor</th>
<th>Magnesium</th>
<th>pH ~ 8 (alkalinization)</th>
<th>pH ~ 6 (acidification)</th>
</tr>
</thead>
<tbody>
<tr>
<td>P2X1R</td>
<td>No effect</td>
<td>No effect</td>
<td>~ 40% decrease current</td>
</tr>
<tr>
<td>P2X2R</td>
<td>10 µM: ~ 95% decrease current</td>
<td>~ 90% decrease current</td>
<td>~3 fold increase current</td>
</tr>
<tr>
<td>P2X3R</td>
<td>No effect</td>
<td>No effect</td>
<td>~ 30% decrease current</td>
</tr>
<tr>
<td>P2X4R</td>
<td>10 µM: ~ 90% decrease current</td>
<td>No effect</td>
<td>~ 80% decrease current</td>
</tr>
<tr>
<td>P2X5R</td>
<td>1.8 mM: decrease current</td>
<td>No effect</td>
<td>~4 fold decrease ATP potency</td>
</tr>
<tr>
<td>P2X7R</td>
<td>1 mM: ~ 60% decrease current</td>
<td>Increase current</td>
<td>~ 50% decrease current</td>
</tr>
</tbody>
</table>

Table 1.3. The effect of magnesium and protons at the P2X receptors.
1.4. P2X7 receptors

1.4.1. Properties of P2X7 receptors

The P2X7R has unique properties that distinguish it from the other P2XRs. One of these is that P2X7Rs are activated by high concentrations of ATP with an EC\textsubscript{50} value $\sim$ 0.1-1 mM in normal physiological solution. This concentration of ATP is not present in normal cells and it is higher than other P2XR subtypes (EC\textsubscript{50} $\sim$1-10 µM) (Abbracchio et al., 2009). Therefore, it seems likely that P2X7Rs have minimal activity in resting conditions. In contrast, under pathological conditions, extracellular ATP concentrations can increase to mM levels and then activate P2X7Rs on a range of cell types (Sperlágh and Illés, 2014).

Another feature of the P2X7R that distinguishes it from other P2XRs is the time-course of the response to ATP. Unlike other P2XRs, the P2X7R response to initial agonist application is commonly slow. This slow onset gets faster by repeated agonist application; that is called facilitation of the receptor (Roger et al., 2008). The facilitation is a signature in the P2X7R as prolonged stimulation can increase the current amplitude and agonist sensitivity as well as speed up the time-course. The intracellular C-terminus domain plays an important role in the P2X7R facilitation. It was reported that removing residues from 419 to 595 in this domain increased current amplitude upon the initial agonist application (Surprenant et al., 1996). This indicates that the current facilitation of the P2X7R can be modified by truncation in the C-terminal domain. Another method to modify the P2X7R current facilitation is discussed in section 1.4.3.

1.4.2. Permeability of P2X7 receptors

Early studies showed that mM ATP can open large pores in immune cells especially mast cells and lymphocytes (Cockcroft and Gomperts, 1979). The receptor was termed the P2Z receptor which is termed now as the P2X7R (Surprenant et al., 1996). Studies also showed that activation of P2X7Rs can lead to direct permeation of large cations such as N-methyl-D-glutamine (NMDG) (MW: 195 Da) and large organic dyes such as ethidium bromide (MW: 314 Da) and (quinolinium, 4-[(3-methyl-2(3H)-benzoxazolylidene) methyl]-1-[3-(triethylammonio) propyl] - diiodide (YO-PRO-1) (MW: 375 Da). These dyes were also seen to permeate P2X2 and P2X4Rs (Virginio et al., 1999).
It was originally thought that large cations and dyes permeate by dilating the pore during prolonged or repeated agonist application. The P2X7R pore dilation was supported based on the observation that the permeability of large organic cations such as NMDG appeared to increase during prolonged agonist applications (Virginio et al., 1999, Khakh et al., 1999). However, this pore dilation model has been questioned by other studies. Li et al. (2015) showed that time dependent NMDG permeability changes in whole cell patch clamp recordings result from non-intended cations accumulation and therefore changed the intracellular ion concentrations (Li et al., 2015). Furthermore, Riedel et al. (2007) found that the single channel conductance of the hP2X7R with several ions including NMDG was unchanged during sustained stimulation, suggesting that the hP2X7R channel does not dilate during sustained stimulation (Riedel et al., 2007).

Similarly, Harkat et al. (2017) found that agonist binding opens an NMDG permeable channel in milliseconds. These studies demonstrate that P2X7Rs immediately open a pore which is a wide enough for large organic cations and dyes to permeate (Harkat et al., 2017). Interestingly, it was seen that other P2XR subtypes including P2X1, P2X2, P2X3 and P2X4Rs are also permeant to NMDG, suggesting that the open pore state may be a common feature of P2XRs (Evans et al., 1996, Harkat et al., 2017).

1.4.3. **Species differences human versus rat**

The human (h) P2X7R was cloned from monocytes (Rassendren et al., 1997b). Although the amino acid sequence of the hP2X7R is 80% identical with the rat (r) P2X7R, there are several differences in properties between these two species. Firstly, differences in agonist sensitivity, with the hP2X7R being 10 to 25 fold less sensitive to ATP and BzATP than the rP2X7R (Rassendren et al., 1997b). Secondly, differences are also apparent in the sensitivity to P2X7R antagonists, which are discussed in detail in section 1.8. Thirdly, the current facilitation differs between hP2X7 and rP2X7Rs, with 5 fold slower current facilitation observed in the hP2X7R (Roger et al., 2010). The reason for this difference is that the rP2X7R C-terminal domain contains a novel calcium/calmodulin binding motif between isoleucine residue at position 541(I541) and serine residue at position 560 (S560) (Roger et al., 2008). This binding motif is missing in the hP2X7R and resulted in current facilitation slower than the rP2X7R, suggesting that current facilitation of the hP2X7R is not dependent on calcium (Roger et al., 2010). The calcium independent facilitation was supported by Allsopp and Evans (2015) who
demonstrated current facilitation in calcium free solution (Allsopp and Evans, 2015). However, the facilitation/speeding was reversible and returned to an initial slow onset following a 10 minutes ATP free period (Allsopp and Evans, 2015).

Chimeras and mutations between the human and rat P2X7R were generated to determine if the C-terminal calmodulin binding motif was involved in facilitation (Roger et al., 2010). The human with rat C-terminal P2X7R increased current amplitude ~ 3 fold compared to the wild type (WT) hP2X7R. The rat with human C-terminal chimera produced current facilitation similar to the WT rP2X7R. Mutations within the C-terminal calmodulin binding motif were also used to identify residues that could have a role in facilitation of the P2X7R. Three residues in the C-terminal calmodulin binding motif are variant between the human and rat P2X7R at positions 541, 552 and 559. The human to rat triple mutations (T541I, C552S, G559V) produced current amplitudes ~ 3 fold higher than the first current amplitude and the current facilitation of the WT hP2X7R. This indicates that increasing current amplitude at the human with C-terminal rat P2X7R chimera was determined by the T541I, C552S, G559V triple mutation (Roger et al., 2010). The evidence presented in this section shows that the current facilitation of the hP2X7R can be modified by using the intracellular C-terminal chimera. It can also be modified by an intracellular amino (N)-terminal chimera (hP2X7-2Nβ chimera), which is discussed in more detail in (section 3.1.1) and (section 3.1.2).

1.4.4. Variants of P2X7 receptors

(i) P2X7 splice variants

The full length P2X7R (P2X7A) consists of 13 exons. Variants occur when there is truncation/alternative of one or more of the 13 P2X7A exons. In humans, there are nine splice variants from P2X7B-P2X7J (Cheewatrakoolpong et al., 2005, Feng et al., 2006, Adinolfi et al., 2010). Most of these variants are non-functional except the P2X7B variant, which has intron 10 with a stop codon leading to form a short C-terminus. P2X7B is highly expressed in the immune and nervous system. This receptor has been seen to be up regulated in inflammation and cell death (Adinolfi et al., 2010).

In rodents, there are three splice variants which are P2X7K, P2X7 13B and P2X7 13C. P2X7K is a functional variant with an alternative exon 1 produced receptor with an alternative amino terminal and first transmembrane domain. It was seen that P2X7K
increased agonist sensitivity ~ 8 fold compared to the P2X7A (Nicke et al., 2009). P2X7 13B and 13C are variants with alternative exon 13 and result in truncated or alternate C termini, respectively. Compared with P2X7A, the agonist evoked currents were smaller at the P2X7 13B and P2X7 13C receptors (Masin et al., 2012).

(ii) P2X7 single nucleotide polymorphisms

Single nucleotide polymorphisms (SNPs) can be defined as a change of a single base pair in a gene. A number of SNPs have been identified in the hP2X7R gene. Some of these result in loss of function such as SNP at position 568, which is located within a trafficking motif. It was seen that isoleucine to asparagine polymorphism on the P2X7R (I568N) prevents normal trafficking to the cell membrane (Wiley et al., 2003). Other P2X7R SNPs result in gain of function such as SNP at position 348, which is located at the end of second transmembrane. It was reported that the alanine to threonine polymorphism on P2X7R (A348T) increased P2X7R responses and increased interleukin-1β secretion ~ 3 fold, suggesting that inheritance of A348T may be associated with inflammation diseases such as rheumatoid arthritis (Stokes et al., 2010, Al-Shukaili et al., 2011).

Several lines of evidence show that SNPs have been associated with different conditions. For example, the loss of function SNP (E496A) increased susceptibility to tuberculosis infection by ~ 50%, enhanced bone density in postmenopausal women and decreased risk of heart disease (Fernando et al., 2007, Ohlendorff et al., 2007, Gidlöf et al., 2012). On the other hand, the gain of function SNP (Q406A) increased in patients with bipolar disorder and autoimmune diseases such as rheumatoid arthritis (Lucae et al., 2006, Lester et al., 2013). These studies indicate that the P2X7R is a target for prevention and/or treatment of various types of diseases.
1.5. **General properties of heteromeric P2X receptors**

Many cells in the body express more than one type of P2XR subunit. This can lead to the production of heteromeric channels with pharmacological properties distinct from homomeric channels. The first functional heteromer characterized was the P2X2/3R with a mixture of properties from both subunits. This heteromer has the time-course of the P2X2R but the agonist sensitivity of the P2X3R (Lewis et al., 1995, Spelta et al., 2002).

Recombinant studies identified potential heteromers by co-immunoprecipitation (Torres et al., 1999). However, this method does not give information about the subunit interaction. Therefore, other methods are required to prove the existence of heterotrimeric P2XRs. For example, cross-linking and atomic force microscopy have been used to demonstrate the existence of heterotrimeric P2X2/3Rs and P2X2/6Rs (Jiang et al., 2003, Barrera et al., 2007). Heteromeric P2XRs have been described between two different subunits and unclear whether three different subunits can assemble to form a functional receptor. The properties of the heteromers are discussed below.

**P2X1/2 heteromers**

Studies have supported the co-immunoprecipitation of P2X1/2Rs (Aschrafi et al., 2004). The co-expressed of P2X1/2R shows rapid desensitization similar to the P2X1R. However, this heteromer differs from P2X1 homomer in terms of sensitivity to extracellular pH. The agonist sensitivity of P2X1 homomers is decreased at low pH whereas the agonist sensitivity at the P2X1/2 heteromer is increased under acidic condition (Brown et al., 2002).

**P2X1/4 heteromers**

Co-expression of P2X1/4 leads to novel properties in *Xenopus* oocytes (Nicke et al., 2005). Like the P2X1R, the P2X1/4 heteromer has high sensitivity to α,β-meATP. However the time-course of this heteromer resembled the P2X4R, with intermediate desensitization (Saul et al., 2013).
P2X1/5 heteromers

Several studies have suggested the formation of P2X1/5 heteromers (Lê et al., 1999, Torres et al., 1998c). The P2X1/5R heteromer has the ATP sensitivity of the P2X1R but the time-course of the P2X5R (Haines et al., 1999). A native receptor corresponding to P2X1/5 was represented in submucosal arterioles and cortical astrocytes (Surprenant et al., 2000, Lalo et al., 2008).

P2X2/3 heteromers

P2X2/3 trimers are highly expressed in sensory neurons and dorsal root ganglia cells (Lewis et al., 1995). This high expression has a crucial role in pain sensation (Cook et al., 1997). The P2X2/3R shows the α,β-meATP sensitivity of the P2X3R but the slow desensitization and pH sensitivity of the P2X2R (Lewis et al., 1995, Stoop et al., 1997, Spelta et al., 2002).

P2X2/5 heteromers

P2X2/5 are found co-expressed in the brain, spinal cord and dorsal root ganglia (Compan et al., 2012). The P2X2/5R has properties resemble the homomeric P2X7R, which may cause pore dilatation and membrane blebbing (Compan et al., 2012). Unlike to other P2XRs, this heteromer is insensitive to α,β-meATP (Saul et al., 2013).

P2X2/6 heteromers

The P2X6R alone cannot form functional homomers. However studies showed that co-expression of this receptor with P2X2Rs can form functional heteromers (Saul et al., 2013). The properties of heteromeric P2X2/6Rs are different from the homomeric P2X2Rs including agonist and pH sensitivity. In contrast to P2X2 homomers, which show potentiation of current amplitude at low pH, the current amplitude at the P2X2/6 heteromer is inhibited under acidic condition (King et al., 2000, Stoop et al., 1997). Co-expression of the P2X2 and P2X6 has been showed in the thalamus, pineal gland and neuronal stem cells (Collo et al., 1996, Schwindt et al., 2011).
**P2X4/6 heteromers**

P2X4/6R subunits are widely distributed in the hippocampus and the cerebellum (Soto et al., 1996). This receptor has properties similar to the homomeric P2X4R in terms of ATP sensitivity. However, the sensitivity to α,β-meATP was increased ~3 fold compared to the P2X4R (Lê et al., 1998).

**P2X4/7 heteromers**

There is debate about whether P2X4 and P2X7 receptors can form heteromers or homomeric complexes. The existence of heterotrimeric P2X4/7Rs has been suggested by measuring the surface expression levels. It was seen that the expression of P2X4R is increased ~2 fold when it is co-expressed with P2X7R, indicating interaction between two subunits (Guo et al., 2007). On the other hand, cross-linking analysis and atomic force microscopy showed that interaction between P2X4R and P2X7R occurs through homomers rather than heteromers (Antonio et al., 2011). The co-expression of P2X4 and P2X7Rs has been found in airway ciliated cells and parotid acinar cells (Ma et al., 2006, Casas-Pruneda et al., 2009). The properties of P2X4/7R co-expressed are quite similar to the P2X7R, including low sensitivity to ATP agonist and sensitivity to Brilliant Blue G and KN-62 P2X7R antagonists (Ma et al., 2006).
1.6. Physiological and pathological roles of P2X receptors

P2XRs are widely distributed throughout the mammalian body. Many physiological and pathological roles of P2XRs have been identified by using knockout (KO) animal models. These roles have often been confirmed with potent selective antagonists. An overview P2XRs functions will be presented in the following pages, with a focus on the P2X7R.

P2X1 receptors

The P2X1R is widely expressed in smooth muscle cells such as the vas deferens, urinary bladder, arteries and blood cells particularly platelets (Valera et al., 1994, Chan et al., 1998, Mulryan et al., 2000, Vial and Evans, 2000, Vial et al., 1997). In the vas deferens, the activation of P2X1Rs is responsible for sixty percent of the contraction (Mulryan et al., 2000). A study showed that the fertility of male P2X1R knockout mice was decreased by ninety percent (Mulryan et al., 2000). This may indicate that P2X1R antagonists are potential target for the development of male contraceptive.

In renal arteries, the P2X1R has a role in the auto regulation of renal blood flow. It was demonstrated that the auto regulation process in P2X1 knockout mice is significantly reduced (Inscho et al., 2003). This is supported by studies using P2X1R antagonists. For example, PPADS inhibited kidney auto regulation of renal blood flow (Osmond and Inscho, 2010).

Furthermore, P2X1Rs were demonstrated to be involved in platelet function and P2X1R knockout mice had reduced thrombosis and platelet aggregation in animal models with normal bleeding time (Hechler et al., 2003). This was supported by using P2X1R antagonists. For example, NF449 (10 mg/kg) reduced platelet aggregation by ~ 15% in mouse models of thrombosis (Hechler et al., 2005).
**P2X2 receptors**

P2X2Rs are highly expressed in the central and peripheral nervous system (Nörenberg and Illes, 2000, Burnstock and Knight, 2004). In the CNS, P2X2Rs are found in the cerebral cortex, cerebellum and medulla oblongata (Kidd et al., 1995, Simon et al., 1997, Kanjhan et al., 1999). In the PNS, P2X2R are found in sensory and autonomic ganglion neurons (Vulchanova et al., 1997, Xiang et al., 1998). P2X2Rs are also expressed in non-neuronal cells, such as skeletal muscle, smooth muscle and cardiac muscle (Ryten et al., 2004, Jiang et al., 2005, Hansen et al., 1999).

The high expression of P2X2R in the CNS has a diversity roles in synaptic transmission including learning and motor function (Gever et al., 2006). The P2X2R has also been shown to be involved in sensory functions such as odorants and taste (Spehr et al., 2004, Finger et al., 2005). P2X2 and P2X3Rs knockout mice abolished responses to the gustatory nerves which are involved in taste (Huang et al., 2011). Furthermore, this receptor contributes to auditory transduction with a significant role in hearing. P2X2Rs are found in epithelial cells lining the cochlea that include sensory hair cells (Housley et al., 1999). Under high sound levels, P2X2R null mice with no hair cell synapse had lost hearing function but not in wild type mice (Housley et al., 2013).

**P2X3 receptors**

P2X3Rs are mainly expressed on sensory neurons (Vulchanova et al., 1997, Cheung and Burnstock, 2002). They have been shown to play a crucial role in nociceptive pathway (Cook et al., 1997, North, 2004). This role was identified by studies using P2X3R knockout mice. For example, mice lacking P2X3Rs revealed markedly reduced pain sensation after injection with formalin and ATP (Cockayne et al., 2000, Souslova et al., 2000). This was supported by using selective P2X3Rs antagonist in rat models of pain. For example, A-317491 reduced pain sensation in the rat induced thermal hyperalgesia, suggesting that selective P2X3R antagonists are potential target for the treatment of pain (Jarvis et al., 2002).
In addition to the role of P2X3R in nociception, P2X3R knockout mice also showed impaired urinary bladder function with significantly increased bladder capacity and reduced voiding frequency (Cockayne et al., 2000). These effects are due to a reduced response to pelvic afferents in the mice lacking P2X3Rs (Vlaskovska et al., 2001). Furthermore, it was also shown that the absence of the P2X3R impaired peristalsis in the intestine (Bian et al., 2003).

Recently, human studies reported that P2X3R antagonist AF-219 reduced cough by 75% in patients suffering from chronic cough. The antitussive effect of P2X3R antagonist might be due to reduced response of airway vagal afferents (Abdulqawi et al., 2015).

**P2X4 receptors**

P2X4Rs are widely distributed throughout the mammalian body including the central nervous system, peripheral nervous system, smooth muscle, skeletal muscle, reproductive organs, epithelial cells and endothelial cells (Kaczmarek-Hájek et al., 2012). This wide distribution indicates a variety of potential physiological roles.

The most commonly described role of P2X4Rs is in the pathogenesis of inflammatory and neuropathic pain. P2X4R expression is upregulated in microglia following spinal cord injury (Tsuda et al., 2003). Mice lacking the P2X4R display reduced pain sensation after injection with formalin (Guo et al., 2005). Furthermore, it was demonstrated that the P2X4-deficient mice showed a reduction in inflammatory mediators such as prostaglandin E2 (Ulmann et al., 2010).

The role of P2X4Rs in vascular endothelial cells has been investigated by using P2X4 knockout mice. P2X4R(-/-) mice revealed abnormal endothelial cell responses to flow including calcium influx (Yamamoto et al., 2006). In addition, It was demonstrated that the absence of the P2X4R increased blood pressure and reduced production of vasodilator nitric oxide (Yamamoto et al., 2006). This indicates that P2X4Rs have role in regulation of blood pressure.

P2X4Rs are also found in cardiac muscle cells and their activation increased cardiac contractility (Mei and Liang, 2001). Overexpression of P2X4Rs may be beneficial in cardiac diseases. For instance, in heart failure animal models,
overexpression of the P2X4R showed improved cardiac function and prolonged survival, suggesting that P2X4R could be target in the treatment of heart disease (Yang et al., 2004).

**P2X5 receptors**

P2X5Rs are expressed in the brain, skeletal and cardiac muscle, kidney and testis (Kaczmarek-Hájek et al., 2012). In humans this receptor is highly expressed in the immune system (Lê et al., 1997). However, most humans express a P2X5 splice variant that is non-functional due to truncation of ATP binding site and TM2, suggesting that P2X5R does not have a crucial role in humans (Kotnis et al., 2010).

In rats, P2X5Rs have been found to play a role in cell differentiation such as skeletal muscle cells. It was seen that activation of P2X5R inhibits proliferation and stimulates differentiation of rat skeletal muscle cells (Ryten et al., 2002). Further studies showed that activation of P2X5R may be involved in the proliferation and differentiation of different types of cancer cells such as prostate cancer (Calvert et al., 2004).

**P2X6 receptors**

P2X6Rs are found in the central nervous system especially cerebellum (e.g. Purkinje cells) (Collo et al., 1996). They are also found in sensory ganglia, skeletal muscle, bronchial epithelia and salivary gland epithelial cells (Xiang et al., 1998, Meyer et al., 1999, Collo et al., 1996, Worthington et al., 1999). This receptor is upregulated in patients with heart disease (Banfi et al., 2005). To date, there is limited information about the physiological roles of the P2X6R as this receptor is the only P2XR subtype that does not form homomers. It seems likely that P2X6Rs are obligated to be co-expressed with other P2XR subtypes such as P2X2 or P2X4Rs to form functional heteromers (King et al., 2000, Lê et al., 1998). A study showed that P2X6Rs are expressed in the distal convoluted tubule of the kidney (de Baaij et al., 2014). However, P2X6 knockout mice had no obvious abnormalities from wild type mice in regulation of renal electrolyte (de Baaij et al., 2016). Therefore, the physiological roles of P2X6Rs remain unclear.
P2X7 receptors

P2X7Rs are widely distributed in immune cells including mast cells, monocytes, lymphocytes, leukocytes and dendritic cells (Labasi et al., 2002, Sluyter et al., 2001, Berchtold et al., 1999). P2X7Rs are also found in the central and peripheral nervous system, including microglia, astrocytes, oligodendrocytes, schwann and satellite cells (Franke et al., 2001, Chen et al., 2008). Furthermore, these receptors are present in epithelial cells, endothelial cells, osteoblasts and osteoclasts (Garcia-Marcos et al., 2006, Ramirez and Kunze, 2002, Gartland et al., 2001, Jørgensen et al., 2002). The extensive expression of the P2X7R indicates that this receptor has a potential role in a variety of conditions, which are discussed in detail below.

(i) Inflammation

The P2X7R is highly expressed on immune cells. The activation of P2X7Rs in response to inflammation is associated with the release of pro-inflammatory cytokines such as interleukin-1β (IL-1β), interleukin-6 (IL-6) and tumor necrosis factor-α (TNF-α) (Di Virgilio, 2007). During inflammation, the P2X7R is activated by high concentrations of ATP resulting in an influx of Ca^{2+} and Na^{+} and the efflux of K^{+}. Reduced levels of intracellular K^{+} promotes the formation of the nucleotide like receptor family Pyrin domain member 3 (NLRP3) inflammasome. This stimulates caspase-1 that cleaves pro-IL-1β and pro-IL-18 to form IL-1β and IL-18. These inflammatory cytokines are released from the cells and contribute to the inflammatory response (Bartlett et al., 2014).

Several studies show that P2X7R knockout mice have attenuated agent-induced inflammation. For example, P2X7R knockout mice have attenuated IL-1β and TNF-α productions by ~ 2 fold after injection with lipopolysaccharide, suggesting that P2X7R antagonists can reduce inflammatory responses (Mingam et al., 2008). This was supported by studies using P2X7R antagonists in animal models of inflammation. For example, Brilliant Blue G (100 mg/kg) reduced IL-1β production by ~ 30% in a mouse model of inflammation (Csölle and Sperlagh, 2010). Furthermore, 1 μM of AZ11645373 inhibited release of pro-inflammatory interleukin-8 (IL-8) by ~ 60% in endothelial cells treated with oxidized phospholipid (Oskolkova et al., 2017).
P2X7Rs have also play a role in rheumatoid arthritis, which is a chronic inflammatory disorder characterized by the destruction of joint, cartilage and bone (Choy and Panayi, 2001). Studies showed collagen degradation in WT mice after injection with anti-collagen antibody induced arthritis, but not in P2X7R knockout mice (Labasi et al., 2002). The P2X7R antagonist has contributed to improved symptoms in mice models of inflammatory arthritis. For example, oxidized ATP and A-438079 significantly reduced hyperalgesia and cartilage erosion (Fulgenzi et al., 2008, Ardissone et al., 2011, Westlund et al., 2012). In view of these studies, one may summarize that a P2X7R antagonist is a possible target for treatment of inflammation.

(ii) Neuropathic pain

The P2X7R is highly expressed on microglia. This expression is upregulated in neuropathic pain that leads to release of cytokines such as IL-1β (Chu et al., 2010, Chessell et al., 2005). The role of P2X7R in neuropathic pain was identified by using P2X7R knockout mice. For example, Chessell et al. (2005) demonstrated that hypersensitivity to thermal stimuli was reduced in P2X7R knockout mice but not in wild type mice (Chessell et al., 2005). This was confirmed by using P2X7R antagonists in animal models. For example, Fulgenzi et al. (2008) demonstrated that oxidized ATP reduced hyperalgesia in a mouse model of neuropathic pain (Fulgenzi et al., 2008). Furthermore, Chu et al. (2010) showed that Brilliant Blue G inhibited the upregulation of microglia P2X7R and reduced pain sensation in a rat model of neuropathic pain (Chu et al., 2010).

Interestingly, genetic studies demonstrated an association between the P2X7R and pain. Impaired pore formation by the P451L mutation in the mouse P2X7R gene reduced pain sensation (Sorge et al., 2012). In humans, P2X7R SNPs that result in loss of function were associated with decreased pain sensation. For example, following mastectomy, women that carried a histidine at position 270 (H270) had a lower pain sensitization than women with arginine at this position (R270) (Sorge et al., 2012). In contrast, P2X7R SNPs that produce gain of P2X7R function were associated with increased pain sensation. For example, in diabetic neuropathy, patients that had a tyrosine at position 155 (Y155) had more pain than those had histidine at this position (H155).
These studies indicate that P2X7Rs have a possible role in the treatment of pain.

(iii) Neurological diseases

P2X7Rs are widely expressed on cells in the central nervous system including microglia, oligodendrocytes, schwann and satellite cells (Franke et al., 2001, Chen et al., 2008). A much-debated question is whether P2X7Rs are expressed in neurons and astrocytes under normal conditions (Illes et al., 2017). Several groups have reported neuronal P2X7Rs at presynaptic terminals by using electrophysiological methods (Deuchars et al., 2001, Miras-Portugal et al., 2003). However, this has been disputed by other studies. Sim et al. (2004) reported that a lack of neuronal P2X7R immunoreactivity in WT and KO mice (Sim et al., 2004). Recently, Kaczmarek-Hajek et al. (2018) using mouse models with P2X7R driven from its native promoter showed no signal was found on neurons (Kaczmarek-Hajek et al., 2018). A similar debate exists about expression of P2X7R in astrocytes, which have been reported in vitro with no evidence in vivo (Sperlágh and Illes, 2014).

A number of animal studies demonstrate that P2X7Rs are upregulated with neurological diseases including Alzheimer’s disease, Huntington’s disease, Parkinson’s disease, epilepsy and multiple sclerosis. Therefore, the P2X7R is a possible target for treatment of these diseases.

A. Alzheimer’s disease

Alzheimer’s disease is a chronic neurodegenerative disease resulting from accumulation of extracellular amyloid β (Aβ) (Delarasse et al., 2011). A study using a mouse model following injection of Aβ showed that the WT mice increased IL-1β cytokine production. However, this production was reduced ~ 8 fold in P2X7R knockout mice (Sanz et al., 2009). Furthermore, the P2X7R antagonist Brilliant Blue G when administered to Alzheimer’s disease model mice reduced the number and size of amyloid plaques in brain hippocampal structures, indicating that P2X7R antagonists have possible roles in the treatment of Alzheimer’s disease (Miras-Portugal et al., 2015). Interestingly, it was seen that the P2X7R SNP 489T is less frequent than 489C in Alzheimer’s disease patients. The presence of the 489T may stimulate phagocytosis activity of P2X7R and then elimination of extracellular Aβ (Sanz et al., 2014).
B. Huntington’s disease

Huntington’s disease is a hereditary neurodegenerative disorder that results from a triple repeat coding for a polyglutamine sequence in the huntingtin protein (Myers et al., 1993). It was demonstrated that P2X7Rs are upregulated ~ 2-3 fold in the brain of Huntington’s disease mice models and the P2X7R antagonist Brilliant Blue G prevented neuronal apoptosis and enhanced motor coordination (Díaz-Hernández et al., 2009).

C. Parkinson’s disease

Parkinson’s disease is a motor disorder accompanied by reduction of dopaminergic neurons in the substantia nigra (Bernheimer et al., 1973). Blockade of P2X7R by A-438079 in a rat model of Parkinson’s disease prevented depletion of dopamine stores but not reduced dopaminergic cell loss (Marcellino et al., 2010).

D. Epilepsy

Studies using epilepsy models showed that P2X7Rs are upregulated in astrocytes, neurons and microglial cells (Kim et al., 2009, Doná et al., 2009). In animal models, status epilepticus was induced in mice by injection of kainic acid. It was demonstrated that pretreatment of mice with the P2X7R antagonist A-438079 decreased the duration of seizure by 58% and decreased seizure-induced neuronal damage by 60% (Engel et al., 2012). It was also demonstrated that A-438079 reduced seizure severity by ~ 70% in a mouse epilepsy model (Jimenez-Pacheco et al., 2013).

E. Multiple sclerosis

Multiple sclerosis is a chronic neurodegenerative disorder characterized by inflammation, demyelination, oligodendrocyte damage, and neuronal death (Amadio et al., 2011). P2X7Rs are upregulated 30% in the experimental autoimmune encephalomyelitis mice model of multiple sclerosis (Grygorowicz et al., 2010). Studies showed that P2X7R deficient mice have ameliorated the signs associated with autoimmune encephalomyelitis (Matute et al., 2007, Sharp et al., 2008). Furthermore, several P2X7R antagonists such as Brilliant Blue G showed reduced demyelination, improved neuron conduction and reduced oligodendrocyte death by ~ 10 fold (Matute et al., 2007).
(iv) Osteoporosis

Osteoporosis is skeletal disease manifested by low bone mass making bone fragile and susceptible to fracture (Kanis and Glüer, 2000). P2X7Rs are expressed on osteoblasts and osteoclasts (Gartland et al., 2001, Jørgensen et al., 2002). The role of P2X7Rs in bone formation and remodeling was identified by studies using P2X7R knockout mice. For example, Ke et al. (2003) showed that P2X7R knockout mice reduced bone formation and increased bone resorption (Ke et al., 2003). Panupinuthu et al. (2008) showed that activation P2X7R enhanced mineralization and increased bone formation (Panupinuthu et al., 2008). Moreover, Li et al. (2009) demonstrated that P2X7R null mice reduced callus bone formation by 30% (Li et al., 2009). Together these studies provide insights into the role of P2X7R in bone formation and remodeling.

The role of P2X7Rs in bone was supported by studies using SNPs. In animal studies, mice that carried proline at position 451 showed more pore formation with stronger bone and higher levels of collagen than the mice that carried leucine at this position that were defective in pore formation (Syberg et al., 2012). This indicates that the P2X7R pore formation is associated with osteoporosis. In human studies, several other P2X7R SNPs have been demonstrated in osteoporosis. For example, the P2X7R SNP (E496A) that results in loss of function is associated with decreased bone density and increased fracture risk in postmenopausal women (Ohlendorff et al., 2007). However, the P2X7R SNP (A348T) that results in gain of function is associated with increased bone density and decreased fracture risk (Husted et al., 2013), suggesting that P2X7R gain of function SNPs might be used as protective against bone fracture.

(v) Cancer

There is an association between P2X7R expression and cancer, with high levels of this receptor found in various cancer types, suggesting that P2X7Rs could be used as a cancer biomarker (Adinolfi et al., 2002, Li et al., 2006, Zhang et al., 2004). Overexpression of P2X7R in animal cancer cells is usually accompanied by increased cell proliferation, production of high levels of vascular endothelial growth factor (VEGF) and increased hypoxia inducible factor 1-alpha (HIF1-α) activity (Adinolfi et al., 2012, Amoroso et al., 2015). For example, activation of P2X7Rs in breast cancer was seen to increase cell migration by 35% and cell invasion by 150% (Jelassi et al., 2011). Furthermore, P2X7Rs enhanced tumour vascularization in animal models of various
cancer types resulting in increased in both VEGF and HIF1-α activity (Adinolfi et al., 2012, Amoroso et al., 2015).

A number of animal studies show that P2X7R antagonists have a possible role in treatment of different cancer types. For example, A-740003 and KN-62 reduced cancer cell immigration by 5% (Jelassi et al., 2011). In the glioma brain tumor animal model, Brilliant Blue G reduced tumor size by ~ 50% (Ryu et al., 2011). Furthermore, in a neuroblastoma mouse model, A-740003 and AZ10606120 reduced tumor growth by ~ 40% and reduced in both VEGF secretion and HIF1-α activity (Amoroso et al., 2015).

In recent years, there is growing evidence that shows the role of P2X7R antibodies in treatment of cancer. The first P2X7R antibody for the treatment of basal cell carcinoma is BIL010t that is currently in a phase I clinical trial. It was reported that the BIL010t antibody decreased lesion size in 65% of patients (Gilbert et al., 2017). This sheep polyclonal antibody acts against the non-functional form of P2X7R, which is up regulated in response to the high levels of ATP in tumor cells (Gilbert et al., 2019).

Furthermore, there is mounting evidence showing that P2X7R SNPs play a major role in different types of cancer such as chronic lymphocytic leukemia. The P2X7R is expressed in lymphocytes and this expression is upregulated in patients with chronic lymphocytic leukemia (Adinolfi et al., 2002). It was seen that loss of function P2X7R SNP E496A reduced lymphocytes in chronic lymphocytic leukemia by ~ 50%. Furthermore, survival for the chronic lymphocytic leukemia patients carried E496 was found to be lower than those carried A496 in P2X7Rs (Wiley et al., 2002). These animal and human studies indicate that the P2X7R is a potential target for treatment of different types of cancer.

Together, these studies show that P2XR subtypes are widely distribution in the body. It seems likely that all cell types expressed at least one P2XR subtype. This wide distribution showed a variety of functional roles of the P2XR subtypes. Animals and human studies demonstrated that P2XR subtypes are therapeutic target for the treatment of different conditions. In order to know the molecular basis of the variances in properties of P2XRs, particularly P2X7Rs, it is important to understand the structure of P2XRs.
1.7. **Structure of P2X receptors**

Initial biochemical and molecular studies provided the basic information about the structure of P2XRs. Subsequently, crystallization of several P2XRs has provided high-resolution images of the channel in various states. In this section will be presented molecular information on P2XRs before and after the crystallization.

1.7.1. **Pre-crystal structure of P2X receptors**

Expression cloning of P2XRs showed that these receptors do not share amino acid sequence with other ion channels, indicating that P2XRs are novel family of ligand gated ion channels. The amino acid sequence of the first two P2XRs was analyzed by hydrophobicity plot. Sequence analysis showed two hydrophobic regions in each subunit that were long enough to cross the plasma membrane, suggesting that P2XRs have two transmembrane domains (TM1 and TM2) (Brake et al., 1994, Valera et al., 1994). The amino-terminal did not have a leader signal peptide, suggesting that this terminal was intracellular (Brake et al., 1994). This is supported by an immunofluorescence study which showed that N- and C-terminal antibodies only detected protein after permeabilization of the cell, suggesting that both termini were intracellular (Torres et al., 1998b). Valera *et al.* (1994) showed that the molecular weight of the P2X1R was higher than that predicted, suggesting post-translational modification of the receptor (glycosylation) (Valera et al., 1994). The consensus sequence for glycosylation is (asparagine (N)-any residue (X)-threonine or serine (T/S). Brake *et al.* (1994) predicted that three potential N-glycosylation sites are located in the extracellular domain (Brake et al., 1994). Subsequent work showed that N-linked glycosylation is important for the cell surface expression (Torres et al., 1998a). Removal of three potential N-glycosylation sites at positions 182, 239 and 298 reduced cell surface expression and reduced the molecular weight, supporting the topology prediction of the P2XR and that glycosylation sites are located on the extracellular domain (Torres et al., 1998b). Together, these studies predicted that P2XRs have two transmembrane domains, a large extracellular loop and intracellular N- and C- termini.

This topology of the P2XR is different from other extracellular ligand transmitter gated ion channels. For instance, cysteine loop receptors with four transmembrane domains (Barnard, 1992) and inotropic glutamate receptors with three transmembrane
domains (Rosenmund et al., 1998). On the other hand, the topology of the P2XR is similar to the epithelial sodium channel (Snyder et al., 1994) and the acid sensing ion channels (Saugstad et al., 2004). However these channels do not share amino acid sequence homology of the P2XR, indicating that P2XRs are distinct from other ligand gated ion channels.

**Extracellular domain**

Mutagenesis studies in the extracellular domain were used to extend the understanding of P2XRs. One of the interesting features of this domain is presence of ten conserved cysteine residues for all mammalian P2XRs (Clyne et al., 2002, Ennion and Evans, 2002a). Ennion *et al.* (2002) suggested that these residues form five disulfide bonds, which are contributed in receptor trafficking to the cell surface (Ennion and Evans, 2002a). Furthermore, mutations showed several conserved amino acid residues in the extracellular loop that are important for ATP sensitivity (Ennion et al., 2000). Residues involved in ATP potency and position of ATP binding site are discussed in detail in section 1.7.3.

**Transmembrane domains**

Mutagenesis studies showed that the transmembrane domains line the pore of receptor and form the channel gate. For example, Rassendren *et al.* (1997) identified residues in the TM2 region forming ionic pore of the receptor by using individual cysteine mutations. They measured any changes in channel activity after application of cysteine reagent methanethiosulfonates (MTS). The rational is if the introduced cysteine residue was accessible to MTS, covalently bound MTS would reduce/block the channel activity. When MTS was applied, three cysteine mutants (I328C, N333C and T336C) showed significant reduction in ATP evoked current, indicating that TM2 lines pore of the receptor (Rassendren et al., 1997a).
**Intracellular domains**

The intracellular N-terminus is a short part (~30 amino acids), which is approximately similar in length for all P2XRs and contains a consensus site for protein kinase C (threonine (T) - any residue (X)-lysine or arginine (K/R)) (Kaczmarek-Hájek et al., 2012). Mutations showed that the threonine residue contributed to the time-course of the P2X1R and P2X2R (Boué-Grabot et al., 2000, Ennion and Evans, 2002b). For example, threonine to alanine point mutation at position 18 (T18A) (P2X1R numbering) produced ~10 fold faster desensitization than the wild type P2X1R (Ennion and Evans, 2002b).

The intracellular C-terminus is variable in the length and ranges from ~30 residues in P2X6R to ~215 amino acids in P2X7R (Coddou et al., 2011). This terminal contains a YXXXK motif (tyrosine (Y)-any residues (XXX)-lysine (K)), which is conserved in all P2XRs. This motif is located close to TM2 in all P2XRs except the P2X7R where there are eighteen amino acids (cysteine rich region) in between TM2 and this motif. The motif is involved in regulation of surface expression of P2XRs and mutations within this motif reduced the trafficking of P2XRs (Chaumont et al., 2004).

**Trimeric assembly of P2X receptors**

Early studies suggested that three molecules of ATP are needed to open P2XR channels and predicted that the P2XR formed as a trimer (Bean, 1990). This was supported by co-expression studies of P2XRs. The trimeric architecture of P2XRs was supported by three lines of evidence (i) cross-linking and blue native polyacrylamide gel electrophoresis (Nicke et al., 1998); (ii) atomic force microscopy (Barrera et al., 2005); and (iii) electron microscopy (Mio et al., 2005, Young et al., 2008). The trimeric architecture of P2XRs was confirmed by crystallization of the receptor, which is discussed in more detail below.
1.7.2. Crystal structure of the P2X receptor

Currently, there are crystal structures from different species and different states including for P2X3R, P2X4R and P2X7Rs. The structures from crystallization were consistent with predictions made from mutagenesis and biochemical studies. The first crystal structure of a P2XR in the closed state was the zebra fish (zf) P2X4R (Kawate et al., 2009). The zfP2X4R was selected after screening a range of P2X orthologous by fluorescence detection size exclusion chromatography (FSEC).

The zfP2X4R was the best candidate for crystallization, which had a sharp and symmetrical peak profile in FSEC. In order to improve the stability of purified protein for the crystallization, this receptor was modified by truncation of several residues from the intracellular N- and C-terminal domains (27 residues from the N-terminus and 8 residues from the C-terminus (ΔzfP2X4-A)). In addition, three point mutations at positions C51F, N78K and N187R were included (ΔzfP2X4-B) (C51F mutation to avoid formation of non-native disulphide bond and N78K and N187R mutations to remove glycosylation sites) (Kawate et al., 2009). The optimization of this structure took ~ seven years.

Electrophysiological recording showed that both the ΔzfP2X4-A and ΔzfP2X4-B constructs were functional and activated by ATP. However, the ATP sensitivity was decreased compared to the wild type zfP2X4R (EC₅₀ ~ 1 mM for ΔzfP2X4-A and ΔzfP2X4-B and 800 µM for wild type zfP2X4R). In addition, the peak current amplitudes were smaller than the wild type zfP2X4R, suggesting that modification of the receptor had changed P2XR properties (Kawate et al., 2009). The ΔzfP2X4-B structure was further modified by removing 24 residues from the C-terminus (ΔzfP2X4-C) to increase the resolution from 3.1Å to 2.9Å. Subsequently, the zfP2X4R crystal structure in the open state was reported with resolution of 2.8Å (Hattori and Gouaux, 2012).

Overall structure

Crystallization of the zfP2X4R shows three subunits that wrap around each other, with a large extracellular domain, two transmembrane domains and short intracellular N- and C-termini (figure 1.3a). Each of the subunits has a dolphin like shape, with the transmembrane domain forming the fluke and the extracellular domain forming the body.
The body domain is characterized by β-stands and consists of head, dorsal fin, right and left flipper (figure 1.3b).

The truncation in the intracellular domain was a drawback of the zfP2X4R crystal structures that means the intracellular information on the zfP2X4R structure was not obtained. However, the hP2X3R crystal structure has been published recently and was less truncated and thus provides more information on the intracellular domain (figure 1.4a) (Mansoor et al., 2016). In this detailed study of intracellular domain, the first crystal structure of a P2XR in the agonist-bound desensitized state was published (Mansoor et al., 2016).

The Extracellular domain

The interfaces between subunits occur predominantly in the extracellular region. The main parts of contact are body-to-body, body to head and dorsal fin to left flipper (Kawate et al., 2009). There are highly conserved residues in the body domain, suggesting a common mode of assembly in all P2XRs. On the other hand, there are less conserved residues in the head, the left flipper and dorsal fin, suggesting that the variation in these regions may be responsible for differences in properties between P2XRs (Kawate et al., 2009). The extracellular domain is stabilized by five disulfide bonds (SS1-SS5), which are formed between ten conserved cysteine residues. This is consistent with predictions in mutagenesis studies (Clyne et al., 2002, Ennion and Evans, 2002a). The first three bonds (SS1, SS2 and SS3) are located in the head region whereas the fourth (SS4) and the fifth (SS5) bonds are located in the dorsal fin and the lower body, respectively (Kawate et al., 2009).

The transmembrane domain

The transmembrane domains consist of six α-helices; each of the three subunits has two TM α-helices. They twist to the left through the membrane in the absence of agonist with the TM2 regions crossing over each other, resulting in closing the pore of the channel (Kawate et al., 2009). The zfP2X4R crystal structure showed that TM2 lines the pore with residues L340, A344, A347, L351 and I355 (Hattori and Gouaux, 2012). These residues play a crucial role in the permeability of ions as had been predicted in
previous cysteine accessibility studies (Rassendren et al., 1997a, Egan et al., 1998). Recently, the hP2X3R crystal structure showed that TM2 lines the pore with residue I323 (L340 for zfP2X4R numbering) compressed the extracellular boundary of the gate in the closed state and residues T330 (A347 for zfP2X4R numbering) defined the intracellular boundary of the gate (Mansoor et al., 2016).

**The intracellular domain**

The intracellular domain is missing in zfP2X4R crystal structures. Therefore, the hP2X3R crystal structure is the only candidate to extend our understanding on the intracellular region. The hP2X3R crystal structure starts from aspartic acid residue at position 6 (D6) to threonine residue at position 364 (T364). Three point mutations in intracellular N-terminal at positions 13, 15 and 16 (T13P, S15V and V16I) were generated to slow desensitization of the hP2X3R (Mansoor et al., 2016). These mutations were selected based on previous mutagenesis studies showing that the intracellular N-terminal is important for the time-course (Hausmann et al., 2014).

The open state structure of the hP2X3R revealed the intracellular domains that were truncated in the zfP2X4 structure. The intracellular domain in the hP2X3R crystal structure was termed the cytoplasmic cap, which contained two β-strands from the N-terminus and one β-strand from the C-terminus. These three β-stands were located under the transmembrane domain capping the surface of the pore (figure 1.4) (Mansoor et al., 2016). The cytoplasmic cap was seen only in the agonist-bound open state structure (figure 1.8). Three point mutations in the intracellular N-terminal domain make the cap structure more stable. However, in the closed state and agonist-bound desensitized state, the residues forming the cytoplasmic cap are not seen although being present in the protein. This suggested that the cytoplasmic cap is stabilized in the open state and is most likely to be unassembled in case of closed and desensitized states (Mansoor et al., 2016). Although the hP2X3R crystal structure provides structural information about intracellular domains in the open state, the structure still remains unclear in case of closed and desensitized states.
Figure 1.3. The crystal structure of the zebrafish P2X4 receptor. (a) Trimeric structure of the P2XR, each subunit twists around each other. (b) Individual zfP2X4R subunit which has a dolphin-like shape with head, body, left and right flipper, dorsal fine and fluke (images taken from Hattori and Gouaux, 2012).
Figure 1.4. The crystal structure of the hP2X3 receptor in the ATP-bound opened state. (a) Comparison of hP2X3R structure (green) to zP2X4R structure (grey) (b) Individual hP2X3R subunit which has a dolphin-like shape with cytoplasmic cap (images taken from Mansoor et al., 2016).
1.7.3. Agonist binding sites

ATP activates all P2XR subtypes thus it was likely that the binding sites were composed of conserved residues between all P2XR subtypes. Mutagenesis studies at the conserved residues were used to investigate the location of ATP binding site at the P2XR. It was seen that positively charged residues (K69, K70, R292 and K309) (P2X1R numbering) contributed to ATP potency, indicating that these residues were involved in binding the negative charges of ATP (Jiang et al., 2000b, Ennion et al., 2000). Furthermore, aromatic residues F185 and F291 and polar amino acids T186 and N290 also contributed to agonist action (Roberts and Evans, 2004, Roberts and Evans, 2006).

Marquez-Klaka et al. (2007) showed that residues involved ATP binding site are located at the interface between two neighboring subunits (Marquez-Klaka et al., 2007).

After the crystal structure of the zfP2X4R in the absence of ATP was published, it was seen that all of residues identified by mutagenesis lined a putative pocket between two subunits. Residues K70, K72, F188 and T189 from one subunit and N296, F297, R298 and K316 from other subunit (P2X4R numbering) (Kawate et al., 2009). Some of these residues such as F188 and F297 are located away from the pocket in the absence of ATP, suggesting that ATP binding is associated with conformational changes in the P2XR and thus making the residues more close together to enable binding (Kawate et al., 2009). The crystallization therefore confirmed several mutagenesis studies, indicating that mutagenesis approach is useful to develop models of ligand binding sites.

The location of the ATP binding site was well defined after the crystal structure of the zfP2X4R in the presence of ATP was published (Hattori and Gouaux, 2012). This crystal structure confirmed the putative ATP pocket suggested in 2009. In addition, the orientation of the ATP binding site was seen in this structure, which is approximately 40Å from the extracellular boundary of the transmembrane regions. It was found that ATP has a novel U-shaped structure in the pocket. Residue K70 locates in the center of the ATP molecule, which allow for interactions with oxygen atoms of α, β and γ phosphate groups. The β phosphate formed interaction with N296 and K316 while γ phosphate interacted with K72, R298 and K316 (figure 1.5) (Hattori and Gouaux, 2012). The adenine base of ATP was buried in the ATP pocket, which interacted with the side chain of T189 and oxygen atoms of K70 and T189. In addition, it was seen that the adenine ring interacted with hydrophobic amino acid residues, which are leucine (L) 191 in the lower body and isoleucine (I) 232 in the dorsal fine. These residues are conserved.
in all P2XR subunits. The ribose sugar of ATP is interacted with L217 in the dorsal fine (Hattori and Gouaux, 2012). More recently, it was reported that the weak agonist CTP has a binding site similar to ATP (Kasuya et al., 2017).

Early studies were interested in determining the number of ATP molecules required for channel opening. For example, it was seen that the best-fit concentration response curve of ATP was with three molecules of ATP binding, suggesting that three molecules of ATP are needed to open P2XR channels (Bean, 1990). This is supported by crystal structures that show trimeric assembly with three ATP molecules bound (Hattori and Gouaux, 2012). However, Stelmashenko et al. (2012) found that ATP binding at two sites is enough to open the P2XR channel (Stelmashenko et al., 2012).

1.7.4. Ionic pathway

The structure of the zfP2X4R in the closed state suggested two possible pathways for ionic permeation. The first pathway is that cations entered through the three-fold axis of symmetry, which extends the length of the extracellular domain. The second pathway is that cations entered through fenestrations, which are located above transmembrane domains at the extracellular vestibule (Kawate et al., 2009). In the ATP-bound opened state of zfP2X4R structure, the pathway through three-fold axis of symmetry is too narrow for ions to pass while the pathway through the fenestrations are wide open for ions to pass (figure 1.6) (Hattori and Gouaux, 2012). Mutagenesis studies supported that ions entered through the fenestrations (Kawate et al., 2011). Furthermore, the hP2X3R crystal structure showed that the cap in the open state prevents the ion flow through the central axis and revealed that cytoplasmic cap and TM2 helices form a triangular shaped cytoplasmic fenestration that might from a pathway for ions to pass. This pathway was supported by molecular dynamic stimulations which showed that ions can enter through the extracellular fenestrations and exist through the cytoplasmic fenestrations (figure 1.8) (Mansoor et al., 2016). However, biochemical and functional studies are required to substantiate the model.
Figure 1.5. Location of the agonist binding site. (a) Homology model of the zfP2X4R showing the position of ATP binding sites in sphere representation. The right hand panel shows the residues interact with ATP molecule (images taken from Hattori and Gouaux, 2012).
Figure 1.6. The transmembrane pore of the zfIP2X4 receptor in the closed and open states. (a) The zfIP2X4R shows that four vestibules (upper, central, extracellular and intracellular) which are located on the molecular three fold axis. The extracellular fenestration indicated by the orange arrow. (b) Pore-lining surface in the closed state and (c) in the opened state. Each colour represents a different radius from the centre of the pore channel (red colour; < 1.15Å, green colour; 1.15-2.3Å and purple colour; > 2.3Å (images taken from Hattori and Gouaux, 2012)).
1.7.5. **Gating cycle of the P2X receptor channel**

Comparison of crystal structures in the ATP-unbound closed state, ATP-bound open state and desensitized states demonstrated conformational changes of the receptor upon ATP binding (Kawate et al., 2009, Hattori and Gouaux, 2012, Mansoor et al., 2016). ATP binding moves the left flipper outward and stimulates cleft closure between the head and dorsal fin. The narrowing of the cleft results from downward movement of the head and upward movement of the dorsal fin (figure 1.7) (Hattori and Gouaux, 2012). This leads to an outward flexing movement of the lower body domains and expands the extracellular vestibule.

Because the lower body domains directly couple to the transmembrane domains, TM1 and TM2 helices moved outward in an iris-like movement. This movement leads to opening the pore of the receptor and thus ions can flow into the cells. The movement of the transmembrane domains was demonstrated by several studies. For example, Cao *et al.* (2007) and Cao *et al.* (2009) identified several point mutations in the transmembrane domain that produced spontaneous channel activation. They found that these mutations directly opened the channel even in the absence of an agonist, suggesting there is inherent movement in the closed state (Cao *et al.*, 2007, Cao *et al.*, 2009). Interestingly, the frequency of channel opening at these mutations might be increased by using zinc binding in the orthosteric site (Jiang *et al.*, 2012). It is clear to see there is a relationship between the agonist-binding site and the pore.

The first crystal structure of a P2XR in desensitized state was the hP2X3R (Mansoor *et al.*, 2016). It shows that the extracellular architecture in the open and desensitized states are similar, whereas the transmembrane domains and pore structure are significantly different (Mansoor *et al.*, 2016). The transition from the open to desensitized state is associated with TM2 recoiling upward. This movement allowed the channel pore to close. In the desensitized state, the N-terminus is located in the opposite direction of the backbone away from the channel pore, suggested that cytoplasmic cap ruptures in this state (figure 1.8) (Mansoor *et al.*, 2016).

In summary, the crystallization of several P2XRs has been of particular interest in the understanding of P2XRs structure and function. However, these crystal structures are not full length receptors. This indicates that the gating cycle of P2XRs is not fully explained.
Figure 1.7. Gating cycle of the P2X receptor. (a) Mechanism of activation of the P2XR during ATP binding. The black arrows indicate the direction of movement (images taken from Hattori and Gouaux, 2012).
Figure 1.8. A model showing the mechanism of P2XRs activation, desensitization and ion access (Ions enter through the extracellular fenestration indicated by red star and exist through the cytoplasmic fenestration indicated by blue star) (images taken from Mansoor et al., 2016).
1.8. **P2X receptors antagonists**

As mentioned in section 1.6, the activation of P2XRs has been associated with several pathological conditions that was clearly seen for the P2X7R. It was also demonstrated that inhibition of P2X7Rs might have therapeutic potential in the treatment of a variety of conditions. Therefore, it is important to develop potent and selective P2X7R antagonists. Understanding of how P2X7R antagonists bind on the receptor is useful to facilitate drug design.

Currently, several potent and selective P2X7R antagonists have been identified and demonstrated species and subtype selectively, which is presented in the following pages. The molecular basis for some of these antagonists was identified by mutagenesis studies such as AZ10606120, A-70003 and A-438079 (Allsopp et al., 2017, Allsopp et al., 2018) (see section 4.1.1). The molecular basis of P2X7R antagonists is supported by the panda (pd) P2X7R crystal structures with five different P2X7R antagonists (Karasawa and Kawate, 2016). However, the molecular basis for other P2X7R antagonists remains unclear such as AZ11645373, Brilliant Blue G, Calmidazolium, KN-62 and ZINC58368839.

1.8.1. **AZ11645373**

AZ11645373 [1-[[3'-Nitro [1,1'-biphenyl]-4-yl]oxy] methyl]-3-(4-pyridinyl) propyl]-2,4-thiazolidinedione is one of the cyclic imides that was reported by AstraZeneca laboratories as a potent selective hP2X7R antagonist (figure 1.9). This compound inhibited BzATP induced ethidium bromide uptake in the human tamm-horsefall protein-1 (THP-1) monocyte cell line, with an IC₅₀ value ~ 20 nM (Alcaraz et al., 2003).

AZ11645373 has also been characterized in several other cellular assays including membrane currents, calcium influx and YO-PRO-1 uptake (Stokes et al., 2006). It was seen that AZ11645373 is a highly potent antagonist at the hP2X7R, with IC₅₀ values of 5 to 90 nM depending on the assays. However, its more than 500 fold less effective at the rP2X7R (less than 50% inhibition at 10µM) and greater than 1000 fold less sensitive at the hP2X1R (less than 10% inhibition occurring at 10µM) (Stokes et al., 2006). In addition, AZ11645373 is ineffective at other P2XRs at concentration up to 10µM. This demonstrates species difference (human versus rat) and subtype selectivity. It was also seen that AZ11645373 had similar levels of affinity by using either ATP or
BzATP agonists. The inhibition by AZ11645373 at the hP2X7R was slowly reversible, with only a 10% recovery of ATP evoked current after 15-30 min washout. This antagonist can inhibit the ATP-induced release of interleukin-1β with an IC\textsubscript{50} value of 90 nM AZ11645373, suggested that AZ11645373 has a therapeutic potential in inflammation (Stokes et al., 2006).

To identify the mechanism of action of AZ11645373 at the hP2X7R, Michel \textit{et al.} (2009) examined the effect of AZ11645373 after pre-treatment with the orthosteric antagonist (decavanadate agent) in an ethidium accumulation study. It was seen that AZ11645373 inhibition was not surmountable by using decavanadate, suggesting that AZ11645373 does not act at the ATP binding site (Michel et al., 2009). Furthermore, in a radio ligand-binding study, Michel \textit{et al.} (2009) examined the effect of AZ11645373 after pre-treatment with the allosteric antagonist (AZ10606120). It was seen that AZ11645373 produced an inhibitory effect at the hPX7R similar to AZ10606120. This suggested that AZ11645373 has a mode of action similar to the AZ10606120 (Michel et al., 2009). Mutagenesis studies were used to identify where AZ11645373 was binding at the hP2X7R. It was seen that mutation at position 95, which mutated phenylalanine (F) to leucine (L) in the hP2X7R reduced AZ11645373 sensitivity by ~10 fold, with an IC\textsubscript{50} ~ 100 nM, suggested that AZ11645373 had an allosteric effect (Michel et al., 2009).

![Chemical structure of AZ11645373.](image)

\textbf{Figure 1.9. Chemical structure of AZ11645373.}
1.8.2. Brilliant Blue G

Brilliant blue G was characterized as a P2X7R antagonist in 1989 (figure 1.10) (Soltoff et al., 1989). It is a potent antagonist at the rP2X7R, with an IC$_{50}$ value of 10 nM. However, it is more than 1000 fold less effective at most other P2XRs (P2X1, P2X3, P2X4, P2X1/5 and P2X2/3) and more than 150 fold less effective at the rP2X2R (Jiang et al., 2000a).

Brilliant blue G was shown to be more sensitive at the rat than human P2X7Rs which inhibited BzATP induced currents with IC$_{50}$ values of 10 and 200 nM at the rP2X7R and hP2X7R, respectively (Jiang et al., 2000a). This inhibition did not recover following 20 minutes washout. IC$_{50}$ concentrations for Brilliant blue G inhibition of rP2X7R were similar at different concentrations of BzATP (30 and 100 µM), suggested that Brilliant blue G is non-competitive antagonist acts distinct from the agonist binding site (Jiang et al., 2000a).

One of the features of Brilliant blue G is that the potency of this antagonist varies between different assays. Compared with mammalian P2X7Rs, Brilliant blue G was only seen to block calcium influx at the rP2X7R with an IC$_{50}$ concentration of ~ 8 µM. This antagonist inhibited BzATP induced YO-PRO-1 uptake at the mouse, rat and human P2X7Rs with IC$_{50}$ concentration of ~ 0.5, 0.6 and 2 µM respectively (Donnelly-Roberts et al., 2009). The discrepancy of Brilliant blue G potency at P2X7Rs may be related to differences in incubation time between YO-PRO-1 uptake and calcium influx assays. The YO-PRO-1 uptake was longer than calcium influx assay with an incubation time 60 min versus 3 min, respectively. It was found that extended incubation time from 3 to 60 min on calcium influx assay increased the potency of Brilliant blue G at the rP2X7R from ~ 8 to 0.7 µM (Donnelly-Roberts et al., 2009).

![Figure 1.10. Chemical structure of Brilliant Blue G.](image)

Figure 1.10. Chemical structure of Brilliant Blue G.
1.8.3. Calmidazolium

Calmidazolium is an isoquinoline derivative and acts as a calcium-calmodulin dependent protein kinase II (CaMKII) inhibitor (figure 1.11). It acted as a selective P2X7R antagonist (Virginio et al., 1997). It is more effective at the rat than human P2X7R with IC_{50} concentrations ~ 13 nM and 100 nM, respectively (Virginio et al., 1997, Chessell et al., 1998a).

The effect of Calmidazolium was completely different between ionic current and dye uptake. It inhibited BzATP evoked currents in HEK293 cells expressing the rP2X7R, with an IC_{50} concentration of ~ 13 nM. This inhibition was slowly reversible with 50-90% recovery of BzATP evoked currents after 10 minutes of washing. The inhibition was non-competitive which reduced maximum currents without change in agonist EC_{50} value at different concentration of antagonist, suggested that Calmidazolium is allosteric antagonist at the P2X7R. In contrast, this antagonist at concentration up to 10 µM had no effect on BzATP induced YO-PRO-1 uptake at the rP2X7R. It is clear to see that Calmidazolium only inhibits currents but not dye uptake (Virginio et al., 1997).

![Figure 1.11. Chemical structure of Calmidazolium.](image)

1.8.4. KN-62

KN-62, [4-[(2S)-2-[(5-isoquinolinylsulfonyl)(methylamino)-3-oxo-3-(4-phenyl-1-piperazinyl)propyl]phenyl isoquinoline sulfonyl acid ester, is an isoquinoline derivative and acted as a potent antagonist of CaMKII, 1 µM of KN-62 inhibited protein kinase by ~ 80% (figure 1.12) (Tokumitsu et al., 1990). KN-62 was shown to be more effective at hP2X7Rs than rP2X7Rs. At the hP2X7R, co-application of this antagonist (1-1000 nM) inhibited ATP induced ethidium bromide uptake in a concentration dependent manner, with an IC_{50} concentration of 13 nM (Gargett and Wiley, 1997).
contrast, at the rP2X7R, KN-62 up to 3 µM had no effect on ATP induced ethidium bromide uptake, showing that this antagonist was insensitive at the rP2X7R. The inhibition of KN-62 at the hP2X7R and lack effect at the rP2X7R was also seen in ionic currents. KN-62 (3-300 nM) inhibited ATP evoked currents at the hP2X7R with an IC50 concentration ~ 35 nM. This inhibition was partially reversible, with ~ 60% recovery of ATP evoked current after 10 minutes of washing (Humphreys et al., 1998).

To identify the mechanism of action of KN-62, Michel et al. (2000) examined the effect of KN-62 in combination with an orthosteric antagonist such as oxidized ATP (oATP). It was reported that KN-62 inhibition was non-surmountable effect of oATP, as the KN-62 was not attenuated the inhibition effect of oATP. This suggested that KN-62 has mode of action different from oATP and may acts as a non-competitive antagonist at the hP2X7R (Michel et al., 2000). This was supported by mutagenesis studies. It was reported that mutation of phenylalanine at position 95 to leucine (F95L) in the hP2X7R reduced KN-62 sensitivity by ~ 3 fold and mutation of the serine at position 86 to glycine (S86G) increased KN-62 sensitivity by ~ 4 fold. These residues are distinct from ATP binding site, suggested that KN-62 act as allosteric inhibitor (Michel et al., 2008).

![Chemical structure of KN-62](image)

**Figure 1.12. Chemical structure of KN-62.**
1.8.5. ZINC67825876, ZINC58368839 and ZINC09315614

A study by Caseley et al. (2016) identified three novel hP2X7R antagonists by structure-based screening. These antagonists were ZINC67825876, ZINC58368839 and ZINC09315614 (Caseley et al., 2016). All three antagonists were effective at the hP2X7R with micromolar potency but less effective at the rP2X7R (less than 20% inhibition at 10µM). In addition, ZINC58368839 and ZINC09315614 were ineffective at the hP2X4R and rP2X3R. However, ZINC67825876 was effective at these subtypes; with 10 µM ZINC67825876 producing ~ 50% and 70% inhibition at the hP2X4R and rP2X3R, respectively (Caseley et al., 2016).

The potency of these antagonists at the hP2X7R was characterized by measuring Ca\(^{2+}\) responses, currents and YO-PRO-1 uptake in HEK293 cells. All three antagonists inhibited BzATP induced Ca\(^{2+}\) responses with IC\(_{50}\) concentrations of 5.1, 4.8 and 3.2 µM for ZINC67825876, ZINC58368839 and ZINC09315614 respectively. Similarly, ZINC67825876 and ZINC58368839 inhibited BzATP induced currents, with IC\(_{50}\) values of 0.35 and 1.2 µM, respectively. The current inhibition by ZINC67825876 and ZINC58368839 was completely reversed upon washout. However, ZINC09315614 at concentration up to 10 µM had no effect on BzATP induced currents at the hP2X7R, showing this antagonist was not effect on ion channel. In addition, co-application of all these antagonists (0.3-30 µM) inhibited the BzATP induced YO-PRO-1 uptake at the hP2X7R in a concentration dependent manner, with IC\(_{50}\) concentrations of 1.8, 1 and 0.8 µM for ZINC67825876, ZINC58368839 and ZINC09315614, respectively. Therefore, the inhibition by these antagonists could have a significant effect on the large pore formation of the P2X7R. Furthermore, these antagonists strongly decreased ATP induced cell death by ~ 50% (Caseley et al., 2016).

The structures of ZINC67825876, ZINC58368839 and ZINC09315614 are shown in figure 1.13. All three antagonists contain an N-methyl amide group, with NO\(_2\) group at one side and a bulky ring at other side. It was seen that replacement of the NO\(_2\) group with CH\(_3\) reduced ZINC58368839 sensitivity. This suggested that nitrogen in this compound is required for a hydrogen bond with residue such as Q143 in the agonist binding site. A molecular docking study with these compounds predicted that the Y288 residue interacts with the bulky ring and Q143 interacts with NO\(_2\) group (Caseley et al., 2016).
1.8.6. AZ10606120

AZ10606120, N-[2-((2-[hydroxyethyl]amino)ethyl)amino]-5-quinolinyl]-2-tricyclo[3.3.1.1<sup>3,7</sup>]dec-1-ylacetamide, was reported by AstraZenca laboratories as a P2X7R antagonist (figure 1.14) (Alcaraz et al., 2003, Guile et al., 2009). AZ10606120 is highly potent antagonist at both human and rat P2X7Rs. However, it is greater than 1000 fold less effective at other P2XRs. This antagonist inhibited ATP induced ethidium bromide uptake at the human and rat P2X7Rs, with IC<sub>50</sub> concentrations of ~3 and 30 nM, respectively (Roman et al., 2009).

![Chemical structure of AZ10606120](image)

Figure 1.14. Chemical structure of AZ10606120.
1.8.7. A-740003

A-740003, \([N-(1-((cyanoimino) (5-quinolinylamino) methyl) amino)-2,2-dimethylpropyl)-2-(3,4-dimethoxyphenyl) acetamide],\) is a cyanoguanidine derivatives and a potent selective P2X7R antagonist (figure 1.15). It inhibited BzATP induced YO-PRO-1 uptake at the human and rat P2X7Rs, with an IC\(_{50}\) concentration ~ 100 nM (Honore et al., 2006). However, it is more than 1000 fold less effective at other P2XRs (Honore et al., 2006).

1.8.8. A-438079

A-438079, 3-[[5-(2,3-dichlorophenyl)-1H-tetrazol-1-1yl]methyl]pyridine is a tetrazole derivatives reported as a potent selective P2X7R antagonist (figure 1.15) (McGaraughty et al., 2007). A-438079 inhibited BzATP induced YO-PRO-1 uptake at the human and rat P2X7Rs with IC\(_{50}\) values of ~ 1000 and 100 nM, respectively (Donnelly-Roberts et al., 2009). However, it is greater than 1000 fold less effective at other P2XRs (McGaraughty et al., 2007).

![A-740003](image)

![A-438079](image)

Figure 1.15. Chemical structure of A-740003 and A-438079
1.8.9. PPADS

More than twenty years ago, pyridoxal phosphate-6-azophenyl-2-4-disulfonic acid (PPADS) was shown as an antagonist of P2XRs in the rabbit vas deferens (Lambrecht et al., 1992). PPADS is a non-selective P2XR antagonist and acts at most P2XR subtypes. It was seen to inhibit at the P2X1, P2X2, P2X3 and P2X5Rs with IC$_{50}$ concentrations range from 1 to 3 µM (Evans et al., 1995, Lewis et al., 1995, Collo et al., 1996). However, it was ineffective at the P2X4 and P2X6Rs, with an IC$_{50}$ value of more than 100 µM (Collo et al., 1996).

Early studies characterized PPADS as a weak P2X7R antagonist. It inhibited BzATP induced currents at the rat and human P2X7Rs with IC$_{50}$ values of ~ 45 and 60 µM, respectively (Surprenant et al., 1996, Rassendren et al., 1997b). However, PPADS was later characterized as a potent P2X7R antagonist. It inhibited BzATP induced YO-PRO-1 uptake at the rat and human P2X7Rs with IC$_{50}$ values of ~ 100 and 10 nM, respectively (Hibell et al., 2001). The discrepancy in PPADS potency between studies might be related to the use of different protocols.

In terms of structure, PPADS has three negative charges, one charge in the phosphate group and two charges in polystyrene sulfonate groups (figure 1.16). As this antagonist is a charged compound like ATP, it might will bind in the orthosteric binding sites. To identify the mechanism of action of PPADS, Michel et al. (2000) showed that PPADS reduced the inhibition effect of oATP (orthosteric antagonist), and this suggested that PPADS has a binding site similar to oATP (Michel et al., 2000). Michel et al. (2007) showed that the inhibitory effect of PPADS was not surmountable by using radio ligand AZ10606120 (allosteric agent), which means the PPADS inhibition was increased when the concentration was increased, suggesting that PPADS has an orthosteric action (Michel et al., 2007). This was supported by mutagenesis studies showed that mutations in allosteric sites such as F95L had no effect on PPADS sensitivity (Michel et al., 2009). Recently, a combination of the biochemical, mutagenesis and molecular docking showed that PPADS binds at the orthosteric sites (Huo et al., 2018).
1.9. Structural basis of P2X receptor antagonists

In the last two years, there have been major advances in understanding the molecular basis of antagonist action. To date, three independent binding sites for antagonists have been identified and structurally characterized (figure 1.17). Understanding the molecular basis of antagonist action/binding sites can provide a great opportunity for P2XR drug design.

1.9.1. Orthosteric antagonist binding site

In 2016, hP2X3R crystal structures with two P2X3R competitive antagonists, 2′, 3′-O-(2, 4, 6-trinitrophenyl)- adenosine 5′-triphosphate (TNP-ATP) and A-317491 have been established. TNP-ATP is potent antagonist at the P2X3R, with an IC\( _{50} \) value of ~1 nM (Virginio et al., 1998). A-317491 is a selective antagonist at the P2X3 with an IC\( _{50} \) value of ~100 nM (Jarvis et al., 2002).

It was seen that both agonist and the antagonists occupy the orthosteric binding pocket at the interface between two subunits. Compared to ATP, the antagonists bind deeper into the binding cleft in a Y-shaped structure and acting as the trunk. The trunk in both antagonists makes hydrophobic interactions with phenylalanine residue at position 174 (F174), which is located deep in the orthosteric pocket. By binding in the deep pocket, both antagonists prevent the ATP induced upward movement of the dorsal fin to close the binding cleft, hindering the conformational changes required for channel opening. It seems that the structure of hP2X3R in antagonist bound state is similar to the closed state (Mansoor et al., 2016). Kasuya et al. (2017) identified another orthosteric binding site for TNP-ATP in the chick P2X7R, which is distinct from the hP2X3R site.

![Chemical structure of PPADS](source_image_url)
It is likely that each P2XR subtype has a specific binding site even in conserved ATP binding site (Kasuya et al., 2017) (figure 1.18).

1.9.2. Allosteric antagonist binding site

The pdP2X7R crystal structures with five P2X7R selective antagonists bound, (A740003, A804598, AZ10606120, GW791343 and JNJ47965567), showed the first allosteric binding pocket in the P2X7R (Karasawa and Kawate, 2016). To improve the stability of this structure, 21 residues (Δ1-21) from the N-termini and 240 residues (Δ360-600) from the C-termini were removed and five point mutations (N241S, N284S, V35A, R125A and E174K) were introduced. Compared to hP2X7R, the pdP2X7R showed slower deactivation with no current facilitation after repeated agonist applications.

In overall structure, it was seen that the crystal structure of the pdP2X7R was similar to zfP2X4R (Kawate et al., 2009, Hattori and Gouaux, 2012). The allosteric binding pocket of the P2X7R is located behind the ATP binding pocket and surrounded by residues mainly from β-strands (β4, β13 and β14) in the upper body domains (figure 1.19). It was seen that antagonist binding makes hydrophobic interactions mainly with residues F95, F103, M105, F293 and V312, which are located deep within the allosteric pocket. To validate the antagonist receptor interactions, mutagenesis studies were used. Consistent with the crystal structure, alanine point mutations at position F95, F103, M105, F293 and V312 increased IC50 value of P2X7 antagonists, supporting that these residues contributed in antagonist binding (Karasawa and Kawate, 2016). The allosteric binding site in the pdP2X7R crystal structure was consistent with chimeras and mutagenesis studies, which are discussed in chapter 4.

To gain insight into how P2X7R antagonists act at the receptor, it was proposed that the inter-subunit cavity might have a role in functional regulation. Compared to zfP2X4R and hP2X3R, the inter-subunit cavity formed by β13 and β14 is wider in pdP2X7R. Four cysteine point mutations in the allosteric pocket were generated (F103C, K110C, T308C and I310C) to identify the contribution of the inter-subunit cavity in pdP2X7R activation. Cysteine reactive agent 2-(Tripentylammonium) ethyl Methanethiosulfonate Bromide (MTS-TPAE) was used to measure the channel activity. It was hypothesized that cysteine residues with MTS-TPAE should interfere with the conformational changes required for channel opening, therefore resulting in reduced
channel activity. When MTS-TPAE was used in the absence of agonist, cysteine mutations reduced peak current amplitude, suggesting that the covalently bound MTS-TPAE at these residues prevent the conformational changes required for channel opening. In contrast, when MTS-TPAE was used in the presence of agonist, these mutations had no significant effect on current amplitude. This indicated that four residues in the antagonist binding pocket are more accessible to cysteine reactive agent in the apo state than in the open state and suggested that the antagonist binding pocket is narrowed during agonist binding. MTS-TPAE used in the presence or absence of agonist, did not change current amplitude for cysteine mutants of the corresponding residues in the P2X4R (Karasawa and Kawate, 2016).

The pdP2X7R crystal structure suggested that the inter-subunit cavity in the upper domain shrinks during activation. This signal allows the lower body domain to expand further, which is necessary for channel opening. In antagonist-bound state, a turret-like structure was locked without shrinking. Therefore, the movement associated with pore lining transmembrane helixes was insufficient to open the channel (figure 1.20) (Karasawa and Kawate, 2016).

The second allosteric binding site was published in the hP2X3R, which is distinct from the pdP2X7R site (Wang et al., 2018). The hP2X3R allosteric binding site is located at the interface of the left flipper, lower body and dorsal fin domains. Binding of P2XR small molecule antagonists such as AF-219 to this allosteric site could affect the movement of the left flipper, lower body and dorsal fin, supporting that this movement is required for channel opening (figure 1.21) (Wang et al., 2018).

In summary, this chapter has given an overview about the properties and physiological roles of P2XRs, with focus on the P2X7R. It has also shown several crystal structures for P2XRs that form in different species and different states. Although there are a major advance in understanding the molecular basis of antagonist action, the binding site and/or mode of action for other antagonists remain unclear.
Figure 1.17. Antagonist binding sites of P2X receptors. Antagonists binding sites is shown as sphere. (a) Orthosteric antagonist binding site of hP2X3R with A-317491. (b) Allosteric binding site of pP2X7R with JNJ4785567. (c) Allosteric binding site of hP2X3R with AF219. (images taken from Schmid R, Evans RJ. 2019).
Figure 1.18. Orthosteric binding site of the hP2X3 receptor. (a) ATP binding sites. (b) TNP-ATP binding site. (c) A-317491 binding site (images taken from Mansoor et al., 2016).
Figure 1.19. Allosteric binding pocket of the P2X7 receptor. (a) Cartoon representation of the panda (pd) P2X7 receptor, the box indicates location of the upper body domains in (b) and (c). (b) The upper body domains showing A804598 binding sites (spheres) with ATP binding pocket (orange dotted lines). (c) The top of view of the pdP2X7R with A804598 binding pocket (green dotted lines) and ATP binding pocket (orange dotted line) (images taken from Karasawa and Kawate, 2016).
Figure 1.20. Activation and inhibition of P2X7 receptors. The top of view of the P2X7R. In open state-agonist bound, the antagonist binding pocket and the turret narrow through channel activation. In closed state-antagonist bound, antagonist closed conformation by hindering the movement of the inter-subunit (images taken from Karasawa and Kawate, 2016).
Figure 1.21. The structure of hP2X3 receptor with AF-219. (a) The structure of AF-219 bound on the hP2X3R. (b & c) Close up of the AF-219 binding site. AF-219 is shown as stick representation at the interface of the left flipper, lower body and dorsal fine (images taken from Wang et al., 2018).
1.10. Thesis aims

The aim of this thesis was to map the binding site of the AZ11645373 antagonist action at the P2X7R. This was done by mutagenesis between antagonist sensitive hP2X7R and antagonist non-sensitive hP2X1R. Residues determined by these methods were used to create the homology model of the AZ11645373 binding site at the hP2X7R. These residues were also used to provide insight into insensitivity of the AZ11645373 at the rP2X7R. Finally, signature mutations were used to study the site of action of other P2X7R antagonists. Understanding the molecular basis of antagonists at the P2X7R is useful to rational drug design with therapeutic potential for various diseases.
Chapter 2: Materials and Methods

2.1. Molecular Biology

2.1.1. The Wild type P2X receptors

The wild type (WT) hP2X1R, hP2X7R and rP2X7R complementary DNAs (cDNAs) were available in the lab. The hP2X1R cDNA was cloned from the human bladder (Ennion et al., 2000). The hP2X7R was a gift of Dr Lin-Hua Jiang, (University of Leeds, U.K). The rP2X7R cDNA was cloned from rat microglial cells (Chessell et al., 1998b). All P2XR cDNAs were cloned into the pcDNA3.1 vector which includes an ampicillin resistance gene, a Mlul restriction enzyme, T7 promoter site and a Polyadenylated (PolyA) tail. The ampicillin resistance gene used to select the appropriate plasmid in *Escherichia-coli* (E-coli). The plasmid has a Mlul restriction enzyme site to linearize the DNA to produce the mRNA. The vector also contains a T7 promoter site to facilitate protein expression and a PolyA tail to protect mRNA from exonucleases (Ennion and Evans, 2002a) (figure 2.1).

![Plasmid map of pcDNA3.1(+)](image)

**Figure 2.1. Plasmid map of pcDNA3.1(+)** (El-Ajouz, 2011). The human cytomegalovirus (CMV) promoter and the simian virus 40 (SV40) promoter are shown in green. The ampicillin resistance gene ((Amp (R)) and neomycin resistance gene ((Neo (R)) are shown in yellow. The T7 promoter in blue and polyadenylated (( poly (A)) tail in red. Other restriction enzymes in purple and site of replication pUC ori in black.
2.1.2. Chimeras

The main background used for most of the studies was the hP2X7-2Nβ chimera. This chimera was previously generated by Dr. Allsopp and replaced the section of the pre-TM1 amino region of the hP2X7R with the corresponding region of the hP2X2R (residues 16-26) (Allsopp and Evans, 2015).

A range of hP2X7-2Nβ/hP2X1 chimeric receptors were also available in the lab (Allsopp et al., 2017). The swapping region of chimeras were generated by two polymerase chain reactions (PCR). The first reaction generated a mega primer. This mega primer amplified the region of interest of the hP2X1R with hP2X7-2NβR overhanging ends. The second reaction inserted the mega primer into the hP2X7-2Nβ template receptor. This generated the chimera which contained a region of the hP2X1R exchanged into the hP2X7-2NβR (figure 2.2).

**Figure 2.2. Generation of hP2X7-2Nβ/hP2X1 chimeras.** To generate a mega primer, forward and reverse primers were used in the first PCR reaction which contained 25 base pairs of the hP2X7-2NβR and 25 base pairs of the hP2X1R on the hP2X1R template. To generate a chimera, the mega primer amplified the hP2X7-2NβR which contained the region of interest of the hP2X1R in the second PCR reaction.
2.1.3. Point Mutations

Point mutations were generated by PCR. Forward and reverse primers incorporating the mutation were selected using the primerX program (www.bioinformatics.org/primerx) and each of these primers contained at least 25 base pairs and 40% to 60% of GC base. For example the mutation of lysine at position 81 to proline (K81P) in the hP2X7-2Nβ.

Forward primer for K81P mutant (5’ to 3’)
GAG AAT GGA GTG CCT AAG TTG GTG CAC

Reverse primer for K81P mutant (3’ to 5’)
GTG CAC CAA CTT AGG CAC TCC ATT CTC

Primers (Sigma) were reconstituted to 1 nmol/µl and then diluted to a working concentration of 10 pmol/µl in nuclease free water. The QuickChange™ mutagenesis kits (Stratagene) was used for point mutations. Each of PCR reaction contained:

1 µl template P2X7-2Nβ receptor (50 ng)
1 µl forward primer (100 ng)
1 µl reverse primer (100 ng)
0.5 µl dNTPs (200 µM)
5 µl 10X Pfu reaction buffer
1 µl Pfu Turbo DNA polymerase (2.5 unit/µl)
40.5 µl nuclease free water

The PCR was set up in a Techne Genius thermocycler with the following programme:

<table>
<thead>
<tr>
<th>Step</th>
<th>Temperature</th>
<th>Time</th>
</tr>
</thead>
<tbody>
<tr>
<td>Initial denaturation</td>
<td>95°C</td>
<td>3 minutes</td>
</tr>
<tr>
<td>Denaturation</td>
<td>95°C</td>
<td>30 seconds</td>
</tr>
<tr>
<td>Annealing</td>
<td>55-75°C</td>
<td>60 seconds</td>
</tr>
<tr>
<td>Elongation</td>
<td>68°C</td>
<td>16 minutes</td>
</tr>
<tr>
<td>Final elongation</td>
<td>68°C</td>
<td>10 minutes</td>
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<tr>
<td></td>
<td></td>
<td>16 cycles</td>
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</tbody>
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To see if the PCR reaction was successful, 5 µl of the sample was run on a 1% agarose gel with 2 µl ethidium bromide. The positive reaction band would be detected under UV light, while the negative reaction only primer dimer would be detected. If no positive band was detected the reaction was repeated with different annealing temperatures from 55 °C to 75°C. If the positive reaction was detected the remaining of PCR sample was treated with 1 µl of DpnI restriction enzyme (Agilent Technologies) at 37°C for 1 hour. This enzyme was used to digest the template methylated DNA. After that, the enzyme was inactivated by heating at 80°C for 10 minutes and then the PCR product were transformed into *E.coli* as described below.

### 2.1.4. Transformation and DNA Extraction

XL-1 blue super competent cells (Stratagene, Agilent Technologies) were used for the DNA transformation. 25 µl of XL-1 blue super competent cells were added to a 15 ml falcon tube on ice with 1 µl of the DpnI treated PCR product. The cells were incubated for 30 minutes on ice and heat shocked in a 42°C water bath for 45 seconds followed by a further 2 minutes incubation on ice. Under sterile conditions, 250 µl of super optimal broth with catabolite repression (SOC) medium (Invitrogen) was added to the falcon tube to increase efficiency of transformation. The tube was then incubated at 37°C in a shaker at 250 rotations per minute (rpm) for 1 hour. After 1 hour incubation, 100 µl of the product was spread on the LB agar plate which contained 50 µg/ml ampicillin and incubated overnight at 37°C. If the transformation was successful colonies would be detected the next day. At least two colonies on each plate were picked and added individually to 5 ml LB Broth with 15 µl ampicillin (50 mg/ml stock). The tubes were then incubated overnight at 37°C in a shaking incubator at 250 rpm. The following day 500 µl of the LB culture was added into an Eppendorf tube and spun down for 2 minutes. The supernatant was removed and 500 µl of the LB /glycerol mixture was added. The sample was re-suspended by vortex and placed at -80°C as a glycerol stock. The remainder of the LB culture was used for DNA extraction by Wizard® Plus SV Minipreps DNA Purification System (Promega). The concentration of DNA was measured by a nanodrop spectrophotometer (50-200 ng).
2.1.5. DNA Sequencing

Mutations were verified by sending extracted DNA with four primers (two forward primers and two reverse primers) to Protein and nucleic acid chemistry (PNAACL), University of Leicester and analyzed by SeqMan II software (DANSTAR).

2.1.6. mRNA Synthesis

mRNA was generated from the plasmid DNA. On the first day, DNA was linearized in 100 µl digests that contained 86 µl DNA (50-200 ng), 10 µl buffer H and 4 µl MluI-1 enzyme (Roche) and incubated at 37°C for 4 hours. This product contained the T7 promoter site and the polyA tail. Then, 1 µl proteinase K (20 mg/ml) and 5 µl 10% SDS were added to the product and incubated at 50°C for 1 hour. This treatment of product was used to remove RNase and transcription inhibitors. 100 µl of phenol: chloroform: isoamyl alcohol (25:24:1) was added for purification of nucleic acid. After that, the product from the top layer was transferred to a new Eppendorf tube containing 200 µl of 100% of ethanol and left overnight at -20°C to precipitate. On the second day, the tubes were centrifuged at 13000 rpm at 4°C for 90 minutes. After that the ethanol was gently removed, the pellet was washed with 1 ml of 70% of ethanol and centrifuged at 13000 rpm at 4°C for 5 minutes. All ethanol was removed by drying the tube at 50°C for approximately 2-3 minutes. The pellet was re suspended in 6 µl nuclease free water.

The mMessage mMachaine T7 transcription Kit (Ambion) was used to synthesize mRNA. For that 2 µl of reaction buffer, 10 µl of NTP/CAP ribonucleic mix and 2 µl of T7 polymerase enzyme mix were added to 6 µl of pellet dissolved and incubated at 37°C for 2 hours. 1 µl of Turbo DNase was added and then incubated at 37°C for 30 minutes. To precipitate the RNA 30 µl of lithium chloride was added and the sample stored at -20°C overnight. On the third day, the tubes were centrifuged at 13000 rpm at 4°C for one hour. Then the ethanol was gently removed and the RNA pellet was washed with 1 ml of 70% of ethanol and centrifuged at 13000 rpm at 4°C for 5 minutes. All the ethanol was gently removed and the tube was dried at 50°C for approximately 2-3 minutes. The concentration of RNA was measured by a nanodrop spectrophotometer after the pellet was resuspended in 3-6 µl of nuclease free water. Finally, the RNA was diluted to a final concentration of 1µg/µl.
2.2. Expression in *Xenopus laevis* oocytes

P2XRs RNA’s were injected into *Xenopus laevis* oocytes. Manually defolliculated *Xenopus laevis* oocytes at stage V were used; at this stage, half the oocyte was dark brown and the other half was a light cream color. This characteristic was used for the determination of stage V. Oocytes were injected with 50 nl (50 ng) of cRNA using an inject + Matic microinjector (J. Alejandro. Gaby, inject Matic, Geneva, Switzerland) and kept at 16°C in Barth's solution (in mM; 88 NaCl, 1 KCl, 0.41 CaCl₂, 0.33 Ca(NO₃)₂, 1 MgSO₄, 2.4 NaHCO₃ and 10 HEPES, pH=7.4 supplemented with 50µg/ml gentamicin, 50µg/ml tetracycline and 1mM sodium pyruvate). Oocytes media was changed daily, before recording 3-7 days later.

2.3. Electrophysiological recording

Two-electrode voltage clamp (TEVC) was used to measure membrane currents from oocytes. Oocytes were bathed in a divalent free solution (in mM; 96 NaCl, 2 KCl, 5 sodium Pyruvate, 5 HEPES, 0.1 flufenamic acid, pH 7.5). The thin wall electrodes were made from capillary glass with diameter 1.5 mm (TW150F-4 World Precision Instruments). The tip of electrodes 1-2 µM diameter were prepared by using two stage puller (Narishige, Japan). Electrodes were filled with 3M KCl and had resistances between 0.1-0.2MΩ. Membrane currents were recorded from oocytes at a holding potential of -60 mV using a Gene Clamp 500B amplifier with a Digidata 1322A analog to digital converter and pClamp 8.2 acquisition software (Molecular devices, Menlo Park, CA, USA). The ATP (disodium salt, Sigma) was applied via a U-tube perfusion system for 10 seconds. This was repeated at 5 minutes for the hP2X7-2Nβ and 7 minutes for the hP2X1 receptor. After reproducible responses to a maximal concentration of ATP were recorded, lower concentrations of ATP were applied. Then, a full sigmoidal concentration response curve was generated. AZ11645373 (Tocris), Brilliant Blue G (Sigma), Calmidazolium chloride (Sigma), KN-62 (Adooq Bioscience), PPADS (Sigma) and ZINC58368839 (Alomone) antagonists were tested against an EC₉₀ concentration of ATP to standardize any shift in ATP potency at chimeras/mutants. The antagonists were perfused alone for 5 minutes before being co-applied with an EC₉₀ concentration of ATP.
for 10 seconds. Each concentration of antagonist was applied twice and inhibition had reached steady state.

2.4. Cell culture

Human embryonic kidney (HEK) 293 cells were grown in Dulbecco's Modified Eagle's medium (DMEM) with 10% fetal bovine serum and 1% nonessential amino acids (NEAAs). The cells were incubated in growth media at 37°C in a humidified atmosphere of 95% air and 5% CO₂. For transfection, the cells were split following 0.05% trypsin enzyme into 6 well plates. When they were at 80-90% confluent the cells were transfected with 3.5 µg DNA of P2X7R plus 0.5 µg DNA of green fluorescent protein (GFP) as an indication of transfection efficiency and 10 µl of lipofectamine 2000 as a transfection reagent (Invitrogen) in 500 µl of reduced serum-media Opti-MEM. After 48 hours of incubation, the cells were then plated onto 96 well clear bottom black-walled plates that had coated with ploy-D-lysine polymer (BD Biosciences) to help the cells to adhere in the plate. The plates were incubated at 37°C, 95% of air and 5% CO₂ overnight.

2.5. Ethidium Bromide Uptake

The media was removed and transfected cells were washed with sucrose buffer (in mM; 280 sucrose, 5.6 KCL, 0.5 CaCl₂, 10 HEPES, 10 glucose and 5 N-methyl-D-glucamine, pH=7.4). Cells were then incubated with or without P2X7R antagonists (1 nM- 1µM) for 40 minutes followed by ethidium bromide (20 µM). After agonist addition ATP (disodium salt, Sigma) (1mM), ethidium bromide uptake was assessed by FlexStation fluorometric plate reader with an excitation wavelength of 525 nm and an emission wavelength of 610 nm. GFP levels were used to assess the DNA transfection efficiency at excitation wave length 395 nm and emission wave length 509 nm.
2.6. Data analysis

All experiments were repeated at least 3 times. Individual normalized concentration response curves were fitted with the Hill equation (variable slope):

\[ Y = \frac{(X)^{H} \times M}{(X)^{H} + (EC_{50})^{H}} \]

where \( Y \) is response, \( X \) is the agonist concentration, \( H \) is the Hill coefficient, \( M \) is the maximum response and \( EC_{50} \) is the concentration of ATP evoking 50% of the maximum response. Some data in the rP2XRs the concentration response curve were fitted to the % of maximum mean response. The \( pEC_{50} \) was used to determine ATP potency. This value is a negative logarithm of the concentration of ATP needed to activate 50% of the maximal response. The software used was GraphPad Prism 6 (GraphPad software Inc., San Diego, USA). Data points were presented as the mean ± standard error of the mean (SEM). Any significant differences between the means were determined by one-way analysis of variance (ANOVA) test followed by Dunnett's multiple comparison test to compare the means versus the control, or unpaired student's t test to compare the two different means as appropriate. Inhibition curves were fitted with the same formula, with IC\(_{50}\) values instead with EC\(_{50}\) values, where IC\(_{50}\) is the concentration of antagonist inhibiting the EC\(_{90}\) of ATP by 50%. Inhibition curves are fitted to mean normalized data. The \( pIC_{50} \) value was used to determine antagonist potency. This value is a negative logarithm of the concentration of antagonist inhibiting the EC\(_{90}\) of ATP by 50%. Visualization of residues were plotted in a homology model of the P2X7 receptor by using PyMOL (Molecular Graphics System, Version 1.2r3, Schrodinger, LLC).

2.7. Molecular docking

The homology models of the five series hP2X7R antagonists were generated by Dr. Ralf Schmid (Department of Molecular and Cell Biology, University of Leicester) and ranked in MODELLER 9.15 software (Webb and Sali, 2017). The structures of antagonist bound pdP2X7Rs (Protein Data Bank Identifiers: 5U1U, 5U1V, 5U1W, 5U1X, 5U1Y) were used as templates (Karasawa and Kawate, 2016). The same process was used to generate rP2X7R homology models.

AZ11645373, Brilliant Blue G, KN-62, Calmidazolium, and ZINC58368839 were docked into hP2X7R homology models using Rosetta Ligand (Davis and Baker,
2009). The centre of the allosteric docking site was started from aspartic acid at position 92 (D92), whereas the orthosteric docking site was started from lysine at position 64 (K64). Ten thousand docking poses were generated for each ligand. The best 10% of the docking poses (based on the lowest Rosetta energy) were clustered by using cpptraj module (Combs et al., 2013). The clustering results were then visualised in PyMol.
Chapter 3: Using of hP2X7/1 chimeras to study the molecular basis of the antagonist AZ11645373

3.1. Introduction

P2X7R antagonists have therapeutic potential in the treatment of a variety of conditions. Properties of P2X7Rs, in particular their facilitation, complicate drug screening. This can be overcome by using the intracellular amino terminal chimera (P2X7-2Nβ). The concept of the P2X7-2Nβ chimera will be discussed in more detail below.

3.1.1. Amino terminal chimeras and time-course

The time-course of ATP evoked currents is different between P2XR subtypes. A chimeric approach showed that the intracellular amino terminus plays a crucial role in the regulation of the time-course. The WT P2X1R showed rapid desensitization (~ 5% of current remaining at the end of the 20 seconds agonist application) whereas the P2X2R showed little decline (~ 76% of current remaining at the end of the 20 seconds agonist application) (Allsopp and Evans, 2011). In order to understand the molecular basis of these differences chimeric receptors were generated. Chimeras that swapped the extracellular region or both transmembrane domains between the P2X1R and P2X2R had no effect on the rate of desensitization. This indicates that the agonist binding site and the pore region of the receptor are not responsible for the variations in time-course. In contrast, swapping the intracellular amino terminal domain of the P2X1R with the corresponding domain of the P2X2R (P2X1-2N chimera) slowed desensitization ~ 3 fold. The reciprocal of this chimera on the P2X2R (P2X2-1N) produced rapid desensitization similar to the WT P2X1R. These results show that the intracellular amino terminus makes an important contribution to regulation of the time-course of channel opening (Allsopp and Evans, 2011).

Sub chimeras that swapped smaller sections of the amino terminal were also tested to identify variations in the intracellular amino terminus domain that contribute to desensitization. Swapping the first 16 residues (1-16) in intracellular amino terminus domain (P2X1-2Nα and P2X2-1Nα chimeras) had no effect on the rate of desensitisation. However, swapping residues (16-30) just before TM1 (P2X1-2Nβ and P2X2-1Nβ chimeras) slowed the time-course for P2X1-2Nβ chimera and speeded the time course for P2X2-1Nβ chimera. Point mutations within Nβ region (16-30) were tested to identify
specific residues contributing to desensitization. It was identified that the variant residues at position 17 and 20-23 are responsible for slowing the time-course of P2X1-2Nβ and speeding the time-course of P2X2-1Nβ (Allsopp and Evans, 2011). This demonstrates that the Nβ region (16 residues) before the first transmembrane segment plays a crucial role in the regulation of the time-course.

To test whether the amino terminal was involved in regulation of the time-course with other P2XR subtypes, further chimeras between P2X7R and P2X2Rs were generated. Compared to other P2XR subtypes, the P2X7R current has two phases. The first response to agonist application (1mM of ATP) is characterized by a slow rise time ~ 20 seconds and the inward current continues to grow during 60 seconds of agonist application. The second response to agonist application is characterized by a rapid rise time ~ 5 seconds. This speeding of the time-course by repeated agonist application is called facilitation (Surprenant et al., 1996, Roger et al., 2008, Roger et al., 2010) (see properties of P2X7R in section 1.4). Allsopp et al. (2015) showed that swapping the intracellular amino terminal region of the hP2X7R with the corresponding region of hP2X2R (hP2X7-2N chimera) led to speeding in the rise time (Allsopp and Evans, 2015). Sub chimeras that divided the amino terminal domain into small sections were tested and interestingly it was identified that the 11 amino acids (residue 16-26) before the first transmembrane segment (Nβ region) had a major role in current facilitation. Swapping of the Nβ region between hP2X7R and hP2X2R (hP2X7-2Nβ chimera) produced an ~ 8 fold increase in ATP sensitivity (with an EC_{50} concentration ~ 200 µM and ~ 30 µM in divalent free solution at the WT and hP2X7-2Nβ, respectively). The first response to ATP at the hP2X7-2Nβ chimera was ~ 40 fold faster than the initial agonist application at the WT hP2X7R and ~ 12 fold faster on repeated agonist application of the WT hP2X7R. There was an ~ 75% decrease in current amplitude at the hP2X7-2Nβ chimera. Western blot analysis showed that it does not result from a channel expression, indicating that the reduction in current amplitude reflects an effect on channel gating (Allsopp and Evans, 2015). Point mutations within the Nβ region identified that residues at position 16 and 23 are responsible for changing properties in the hP2X7-2Nβ chimera (Allsopp and Evans, 2015).
3.1.2. The Advantage of using the hP2X7-2Nβ chimera as a background for the P2X7R pharmacological studies

The facilitation phenomenon in the P2X7R is a potential confounding factor for efficient pharmacological screening of P2XRs as repeated long applications of ATP are required to give reproducible responses. This can be overcome by replacing the Nβ region of the hP2X7R with the corresponding part of the hP2X2R. It was demonstrated that hP2X7-2Nβ chimera removed facilitation and gave readily reproducible responses to agonist application with a fast time-course. As this chimera has only changed the intracellular amino terminal domain, it is unlikely to have an effect on antagonist sensitivity. This is supported by previous studies that showed that the chimera hP2X7-2Nβ had no effect on the sensitivity of AZ10606120, A740003 and A438079 (Allsopp et al., 2017, Allsopp et al., 2018). This chimera had not been tested with AZ11645373, therefore this chapter characterizes the action of AZ11645373 at the hP2X7-2Nβ chimera.

3.1.3. The use of hP2X7-2Nβ/1 chimeras to investigate the molecular basis of AZ11645373 antagonist action

AZ11645373 was first described as a P2X7R antagonist in 2003 (see section 1.8.1) (Alcaraz et al., 2003). It is effective at the hP2X7R with an IC₅₀ value of 90 nM. Interestingly, it is more than 500 fold less effective at the rat P2X7R (Stokes et al., 2006). A previous study examined differences in the extracellular domain to identify residues that can contribute to the species difference in AZ11645373 action. Mutation at position 95 of phenylalanine in hP2X7R to leucine in the rP2X7Rs (F95L) reduced AZ11645373 antagonist sensitivity by 10 fold (Michel et al., 2009). However the Michel et al. (2009) study did not test all variant residues between human and rat P2X7Rs in the extracellular domain and other residues must also contribute to the remaining 50 fold difference. When I started my thesis, this was the only molecular information available about how this antagonist acted at the P2X7R. Within 6 months of starting my project, Caseley et al. (2015) used molecular docking centred on F95 to postulate AZ11645373 binding in the upper vestibule of the extracellular region with several residues including Y295, Y299, K297 and K300 (Caseley et al., 2015). This region is distinct from the inter-subunit
allosteric site identified by Allsopp et al. (2017) and (2018) for other P2X7R antagonists. The Caseley et al. (2015) study did not test predications in mutagenesis studies.

A molecular docking study with another P2X7R antagonist AZ10606120 identified potential orthosteric and allosteric antagonist binding sites at the apex of the receptor (distinct from the upper vestibule site suggested by Caseley et al. (2015)) (Allsopp et al., 2017). This P2X7 antagonist is selective for P2X7Rs over other P2XR subtypes (Michel et al., 2007, Guile et al., 2009). Due to the large differences between hP2X7R and hP2X1R, Allsopp et al. (2017) generated a range of chimeras on the hP2X7-2Nβ background which replaced regions of hP2X7R (sensitive to AZ10606120) in the extracellular domain with the corresponding region of the hP2X1R (insensitive to AZ10606120) (figure 3.7). These hP2X7-2Nβ/1 chimeras tested the contribution of variant residues lining the putative orthosteric and allosteric antagonist binding pockets. Studying hP2X7-2Nβ/1 chimeras showed that AZ10606120 acts at an inter-subunit allosteric site (Allsopp et al., 2017) that was published independently at the same time with an antagonist bound panda P2X7R crystal structure (Karasaki and Kawate, 2016). These chimeras were also used to identify variant regions contributing to antagonist action of A740003 and A438079 (Allsopp et al., 2018). Therefore hP2X7-2Nβ/1 chimeras will enable the contribution of variant parts in the extracellular region to AZ11645373 antagonist sensitivity to be determined.

3.1.4. Chapter Aims

In order to test whether the hP2X7-2Nβ chimera could be used to study the pharmacological properties of AZ11645373, it was first essential to characterize the effect of this antagonist at the hP2X7-2Nβ chimera. In addition, it was important to determine the effect of the AZ11645373 at the WT hP2X1R to validate that the chimera approach using P2X1 regions (P2X7/1) can be used. Subsequently the effects of AZ11645373 antagonist at the hP2X7-2Nβ/1 chimeras, that had previously been made by Dr. Allsopp (Allsopp and Evans, 2015), were determined to provide insight to the molecular basis of AZ11645373 action.
3.2. Results

3.2.1. Characterization of ATP currents at hP2X7-2Nβ and WT hP2X1 receptors

ATP evoked concentration dependent inward currents at the hP2X7-2Nβ and hP2X1Rs. At the hP2X7-2NβR, the peak amplitude to a maximal concentration of ATP (300 µM) was 744 ± 40.17 nA (figure 3.1a). The responses had a pEC₅₀ value of 4.63 ± 0.07 (EC₅₀ ~ 25 µM) in divalent free solution with a Hill slope 1.7 ± 0.07 (figure 3.1c). At the hP2X1R the peak amplitude of a maximal response to ATP (100 µM) was 8627 ± 472.93 nA (figure 3.1b). Compared to the hP2X7-2NβR, ATP was approximately 16 fold more potent at the hP2X1R with a pEC₅₀ value of 5.84 ± 0.14; p < 0.01 (EC₅₀ ~ 1.5 µM) in divalent free solution and a Hill slope 1.17 ± 0.3 (figure 3.1c).

There were marked differences in the time-course of response between hP2X1R and hP2X7-2NβR. The rise time (10% to 90% of the peak response) at a maximum ATP concentration of the hP2X1R (97.79 ± 3.1 ms) was approximately 14 fold faster than the hP2X7-2NβR (1374.87 ± 136.87 ms; p < 0.01) (figure 3.2a). For the hP2X1R the inward current returned back to baseline during continued agonist application (called desensitization), the peak current decayed to 50% in 0.76 ± 0.04 s and 4.22 ± 0.41% of the current remained at the end of a 10 second. In contrast at the hP2X7-2NβR 77.25 ± 2% of the peak current remained at the end of a 10 second ATP application (figure 3.2b).

3.2.2. The effect of AZ11645373 at the hP2X7-2Nβ receptor

A range of hP2X7/1 chimeras have been useful for determining the site of antagonist action (Allsopp et al., 2017). These chimeras were made on the hP2X7-2Nβ background (Allsopp and Evans, 2015). To determine the site of action of AZ11645373 by using hP2X7/1 chimeras, it was first necessary to make sure that the antagonist has a similar action at 2Nβ P2X7Rs.

Co-application of AZ11645373 (0.001-10 µM) with an EC₉₀ concentration of ATP (100 µM) inhibited the agonist evoked current in a concentration dependent manner (figure 3.3b & d). A second application of AZ11645373 produced a similar level of inhibition showing that the effect had equilibrated. AZ11645373 was effective at the hP2X7-2NβR; 1µM inhibited response by approximately 90% and had a pIC₅₀ of 7.47 ± 0.06 (IC₅₀ ~ 30 nM) in divalent free solution (Hill slope of 0.96 ± 0.14) (figure 3.3d).
AZ11645373 had an effect on the time-course to ATP responses at the hP2X7-2NβR. There was an approximately 2 fold slowing in the rise time of the hP2X7-2NβR with the maximum concentration of AZ11645373 (10 μM) (3.9 ± 1.39 s in ATP alone and 8.90 ± 0.46 s in ATP and AZ11645373, p < 0.05) (figure 3.4a). The inhibitory effect of AZ11645373 did not recover following 20 minutes washout at the hP2X7-2NβR (figure 3.5) (the % of remaining current at 20 minutes after washout of 1μM AZ11645373 was 8.69 ± 1.27%).

3.2.3. The effect of AZ11645373 at the WT hP2X1 receptor

The effect of AZ11645373 at the WT hP2X1R was tested. Co-application of AZ11645373 (10-10000 nM) with an EC90 concentration of ATP (10 μM) had no effect on ATP evoked currents, showing the hP2X1R is insensitive to this antagonist (figure 3.3c, d). The maximum concentration of AZ11645373 (10 μM) had no effect on the rise time of the hP2X1 receptor (88.5 ± 3.5 ms in ATP alone and 85.3 ± 4.6 ms in ATP and AZ11645373) (figure 3.4b). The lack effect of hP2X1R confirmed the previous study of Stokes et al. (2006). Therefore the P2X1R can be used as a donor for chimeras to study AZ11645373 action.
Figure 3.1. Concentration dependence of ATP evoked currents at the hP2X7-2Nβ and hP2X1 receptors. (a & b) Representative traces showing the effect of different ATP concentrations (µM) on hP2X7-2NβR and WT hP2X1R expressed in *Xenopus* oocytes. 5-7 minutes was given between each application. Black bars indicate 10 seconds of ATP application. (c) Concentration response curves for the hP2X7-2NβR (black) and WT hP2X1R (blue), pEC\(_{50}\) values are given (n > 3). The red dotted line corresponds to the EC\(_{90}\) concentration of ATP used for antagonist studies. Unpaired student’s t test was used. **, p < 0.01. Data are shown as mean ± S.E.M.
Figure 3.2. Time course of ATP evoked currents at the hP2X7-2NβR and hP2X1 receptors.
(a) Representative trace showing the rise time of hP2X7-2NβR. Rise time is the time to increase currents from 10% to 90%. In the right hand panel, a comparison of the rise time at hP2X7-2NβR and hP2X1R. (b) Representative hP2X1R trace current showing the peak of current of hP2X1R decayed to baseline during a 10 second agonist application. In the right hand panel, % of current remaining of hP2X1R and hP2X7-2NβR at the end of a 10 second agonist application. Unpaired student’s t test was used. **, p < 0.01; ****, p < 0.0001. Data are shown as mean ± S.E.M (n ≥ 3).
Figure 3.3. AZ11645373 inhibition at hP2X7-2Nβ and hP2X1 receptors. (a & b) Representative traces showing the effect of AZ11645373 on currents evoked by an EC₉₀ concentration of ATP in Xenopus oocytes expressing hP2X7-2NβR and WT hP2X1R. 5 minutes was given between each application. Bars indicate 10 seconds of ATP application and ATP co-application with AZ11645373 following 5 minutes pre-incubation with the indicated concentration of compound. (c) AZ11645373 inhibition curves at an EC₉₀ concentration of ATP. Black dotted line corresponds to pIC₅₀, which was used to measure AZ11645373 potency. Data are shown as mean ± S.E.M (n ≥ 3).
Figure 3.4. Rise time of ATP evoked currents at hP2X7-2Nβ and hP2X1 receptors in the presence of AZ11645373. (a) Representative trace showing the effect of ATP alone (100 µM) and with AZ11645373 (10 µM) at the hP2X7-2NβR. In the right hand panel, a comparison of the rise time of the hP2X7-2NβR to ATP alone and ATP with AZ11645373. (b) Representative trace showing the effect of ATP alone (10 µM) and with AZ11645373 (10 µM) on the rise time of hP2X1R. Black bars indicate 10 seconds of ATP application and ATP co-application with AZ11645373 following 5 minutes pre-incubation. In the right hand panel, a comparison of the rise time of the hP2X1R in to ATP alone and ATP with AZ11645373. Unpaired student's t test was used. Stars indicate a significant difference. *, p < 0.05. Data are shown as mean ± S.E.M (n ≥ 3).
Figure 3.5. The inhibitory effect of AZ11645373 on ATP evoked currents at the hP2X7-2Nβ receptor was not reversed by 20 minutes washout. (a) Representative traces showing the inhibitory effect of AZ11645373 was not reversed by 20 minutes washout. 5 minutes was given between each application. Bars indicate 10 seconds of ATP application and ATP co-application with AZ11645373 following 5 minutes per-incubation. (b) % of remaining currents up to 20 minutes after washout of 1µM AZ11645373. Data are shown as mean ± S.E.M (n ≥ 3).
3.2.4. The effect of AZ11645373 at hP2X7-2Nβ/1 receptor chimeras

AZ11645373 is a selective antagonist for hP2X7Rs. Comparing the amino acid sequence of the hP2X7R with hP2X1R, there are several variations in the extracellular region that are distributed on the surface of the receptor. In addition the P2X7R has several unique variant residues in the extracellular region. Of particular note, there are seven amino acids inserted between β stands at region 73-79, a four amino acids deletion in the dorsal fin, a three amino acids insertion in the left flipper and other individual variant residues (figure 3.6 a).

Previously hP2X7-2Nβ/1 chimeras were used to identify the allosteric binding site of AZ10606120, A740003 and A438079 (Allsopp et al., 2017, Allsopp et al., 2018). These hP2X7-2Nβ/1 chimeras focus on the orthosteric binding site and putative allosteric antagonist binding pockets (figure 3.6 c). Chimeras, which replaced sections of the extracellular domain of the hP2X7-2NβR (sensitive to AZ11645373) with the corresponding regions of the hP2X1R (insensitive to AZ11645373) were used (figure 3.7 a). Two chimeras around the orthosteric site were examined which are 210-217 in the dorsal fin and 279-285 in the left flipper. One deletion (73-79 deletion) and eight chimeras around the allosteric sites were tested (81-88, 89-94, 105-114, 112-118, 122-128, 164-168, 170-177 and 295-310) (figure 3.7 b). Chimera 295-310 was the largest (16 amino acids) while chimera 164-168 was the smallest (5 amino acids). Chimera 295-310 had eleven variant residues, while 89-94 had only two variant residues. The EC\textsubscript{90} concentration of ATP at the hP2X7-2Nβ/1 chimeras was determined previously by Allsopp and Evans in the initial characterization of chimeras for AZ10606120 study (table 3.7 c) (Allsopp et al., 2017).
Figure 3.6. Location of amino acid differences between human P2X7 receptor and human P2X1 receptor in the extracellular loop. (a) Amino acid sequence line up of the extracellular region of hP2X7R and hP2X1R. Black residues are conserved amino acids, grey residues are variant amino acids and raspberry residues are unique residues in the P2X7R. (b) Homology model of the hP2X7R with the three subunits shown in colour, subunit A (pale cyan), subunit B (wheat) and subunit C (grey). Conserved residues in the extracellular loop are shown in black, unique residues in the P2X7R are shown in raspberry (compared with all hP2XR) and variant residues are shown as the colour of the subunit. (c) Potential orthosteric and allosteric binding site(s) of the P2X7R antagonist at one subunit. ATP (black), orthosteric (green) and allosteric (red) binding pockets in the P2X7R.
Figure 3.7. Location of extracellular chimeras on the hP2X7-2Nβ receptor (a) Amino acid sequence line up of hP2X7R (top) and hP2X1R (bottom) of the extracellular region. Conserved amino acids are shown in black, variant amino acids are in grey and unique amino acids in the P2X7R are in raspberry (compared with all hP2XRs). Regions of chimeras are shown by coloured boxes. (b) Position of extracellular regions swapped to generate chimeras shown in the homology model of hP2X7R. ATP binding sites are shown in the black dotted ring. The right hand panel shows the top of view of the 73-79 deletion (firebrick), 81-88 (red), 89-94 (salmon), 105-114 (orange), 112-118 (yellow-orange), 122-128 (purple), 164-168 (green), 170-177 (marine), 210-217 (yellow), 279-285 (blue) and 295-310 (magenta) chimeras. (c) The table shows the number of conserved, variant and unique residues in the hP2X7R and also shows the ATP sensitivity at each chimeras (*pEC50 of ATP taken from Allsopp et al., 2017).
The effect of AZ11645373 on the hP2X7-2Nβ/1 receptor chimeras around the orthosteric binding sites

In order to determine whether AZ11645373 bound at the orthosteric site, two chimeras 210-217 and 279-285 were tested. Swapping the dorsal fin at the base of the receptor (210-217 chimera) had no effect on AZ11645373 sensitivity, pIC$_{50}$ = 7.43 ± 0.13 (figure 3.8 a, b, c & e). Previous studies showed that swapping the left flipper of the hP2X7-2NβR with the corresponding region of the hP2X1R (279-285 chimera) changed ATP sensitivity and modified action of known allosteric antagonists (Allsopp et al., 2017, Allsopp et al., 2018). The 279-285 chimera was approximately 27.5 fold less sensitive to AZ11645373 than the hP2X7-2NβR, 1 µM AZ11645373 inhibited currents by approximately 55%, pIC$_{50}$ = 6.03 ± 0.15, p < 0.0001 (figure 3.8 d & f). This indicates that changes in the left flipper modified both ATP and AZ11645373 action. In order to understand this complicated effect, the 279-285 region was studied in more detail.

Characterization of the hP2X7-2Nβ/1 chimera by Allsopp et al. (2017) showed that swapping the 279-285 region increased ATP sensitivity approximately 30 fold (Allsopp et al., 2017). This effect could be related to a change in the length of the left flipper and/or variant residues (figure 3.8a). In order to determine whether changing the length of the left flipper by removal of three amino acids (279-285 chimera) accounted for the increased ATP sensitivity, Allsopp et al. (2018) examined another chimera that swapped the corresponding sequence with the hP2X4R (TRDVE) (this hP2X4 chimera does not shorten the amino acid sequence) (figure 3.10a). It was reported that exchange with the region 280-284-X4 increased ATP sensitivity approximately 10 fold, indicating that the variant residues in the 280-284 region contribute to ATP sensitivity (Allsopp et al., 2018).

There are two residues in the 280-284 region of the left flipper that are unique in the P2X7R (D280 and N284) and three residues that are removed in the PX1R (K281, T282 and T283) (figure 3.10a). In order to identify the contribution of individual residues in the left flipper to ATP action, alanine point mutations within region 280-284 were made. The negative charged aspartic acid at position 280 was interesting as mutation to alanine (D280A) increased ATP sensitivity 10 fold, pEC$_{50}$ = 5.63 ± 0.07, p < 0.0001 (figure 3.9b &c). This value is equivalent to the hP2X4 chimera, indicating that a unique residue at position 280 is responsible for increasing ATP sensitivity. Removal of the positively charged lysine at position 281 (K281A) reduced ATP sensitivity 3.5 fold,
pEC$_{50}$ = 4.08 ± 0.03, p < 0.0001. The threonine to alanine mutation at position 283 (T283A) decreased ATP sensitivity approximately 6 fold, pEC$_{50}$ = 3.86 ± 0.08, p < 0.0001. The T282A and N284A mutations had no effect on ATP potency (figure 3.9). It seems that the effect on ATP sensitivity may be related to structural change on ATP binding. A homology model of the hP2X7R shows that the unique residue D280 is located near to K193 and R206 residues that may affect the channel gating (figure 3.12a). The K193 is conserved residue in all P2XRs and R206 is conserved residue between hP2X7R and hP2X1R. Neither alanine nor cysteine mutations at position 193 (K193A) and (K193C) were functional. Removal of the positively charged arginine at position 206 (R206A) had no effect on ATP sensitivity. This indicates that R206 mutation does not contribute to ATP sensitivity.

The effect of the length and individual residues were also determined for AZ11645373. The 280-284-X4 chimera had no effect on AZ11645373 sensitivity, pIC$_{50}$ = 7.29 ± 0.26, indicating that shortening of the left flipper does not contribute to AZ11645373 action (figure 3.10c & d). For completeness AZ11645373 inhibition was also tested at the point mutations of the left flipper around the orthosteric binding site to determine which residues were important for AZ11645373 sensitivity. The point mutations K281A, T282A, T283A and N284A had no effect on AZ11645373 sensitivity, demonstrating that individual residues in the left flipper do not contribute to AZ11645373 action. However the D280A reduced AZ11645373 sensitivity 8 fold, pIC$_{50}$ = 6.56 ± 0.19, p < 0.01 (figure 3.11).

To make sure if there are any other residues around the orthosteric site that may have an effect on AZ11645373 sensitivity, we examined AZ11645373 at additional residues lining the orthosteric binding site. Removal of the positively charged arginine at position 206 (R206A) had no effect on AZ11645373 sensitivity. At the top of the P2X7 left flipper there is a unique tyrosine residue at position 288. The tyrosine to serine mutation at position 288 (Y288S) had no effect on AZ11645373 action. Other residues around the orthosteric pocket are Y291 and Q143. The tyrosine to phenylalanine mutation at position 291 (Y291F) and glutamine to lysine mutation at position 143 (Q143K) had no significant effect on AZ11645373 potency (figure 3.12). These results demonstrate that eight residues around the orthosteric pocket do not contributing to the antagonist action of AZ11645373 and only one residue in the orthosteric pocket contributes to
AZ11645373 sensitivity (table 3.1). It seems that AZ11645373 does not act at the orthosteric site.

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Table 3.1. ATP and AZ11645373 sensitivity on the point mutations around the orthosteric antagonist binding site. The table shows the pEC₅₀ values of ATP, pIC₅₀ values of AZ11645373 and fold shift relative to the hP2X7-2NβR. A star indicate a significant shift in EC₅₀ and IC₅₀ from the hP2X7-2NβR. *, p < 0.05; **, p < 0.01; ****, p < 0.0001. Arrows indicate an increasing ATP/AZ11645373 sensitivity. Data are shown as mean ± S.E.M (n ≥ 3).
Figure 3.8. Effect of chimeras around the orthosteric binding site that change the length of the left flipper and dorsal fin. (a) Amino acid sequence of hP2X7R and hP2X1R in the 210-217 and 279-285 regions. Conserved amino acids are shown in black, variant amino acids are in grey and unique residues in the P2X7R are in raspberry. (b) Position of extracellular regions swapped to generate chimeras around the orthosteric pocket shown in the homology model of the hP2X7R. ATP binding sites are shown by the black dotted ring. Position of chimera in the dorsal fin (210-217 [yellow]) and the left flipper (279-285 [blue]). (c & d) Traces of current recorded from 210-217 & 279-285 at 1 µM of AZ11645373. There was a 5 minute recovery between each application. Bars indicate 10 seconds of ATP application or ATP application with AZ11645373 following 5 minutes pre-incubation with the indicated concentration of compound. (e & f) AZ11645373 inhibition curves at 210-217 & 279-285 chimeras. ****, p < 0.0001; (n=3-5).
Figure 3.9. ATP sensitivity at point mutations of the left flipper (280-284). (a) Homology model of the hP2X7R showing the position of point mutations in the left flipper (D280, K281, T282, T283 and N284 [green]). (b) ATP sensitivity curve at D280A, K281A and T283A point mutation. The hP2X7-2NβR is shown as a black dotted line. (c) The pEC50 values of ATP at the 280-284 point mutations. Any significant differences from the wild type hP2X7-2NβR were determined by one-way ANOVA test followed by Dunnett’s test. Stars indicate a significant difference from the hP2X7-2NβR. ****, p < 0.0001. Data are represented as mean ± S.E.M (n= 3-5).
Figure 3.10. AZ11645373 inhibition at the 280-284-X4 chimera in the left flipper around the orthosteric antagonist binding sites. (a) Amino acid sequence of hP2X7R and hP2X4R. Conserved amino acids are shown in black, variant amino acids are in grey and unique residues in the P2X7R are in raspberry. (b) Homology model of hP2X7R showing the position of extracellular region swapped between hP2X7R and hP2X4R in the left flipper to generate 280-284 chimera (blue). (c) Trace of current recorded from 280-284-X4 at 1 µM of AZ11645373. There was a 5 minutes recovery between each application. Bar indicates 10 seconds of ATP application or ATP application with AZ11645373 following 5 minutes pre-incubation with the indicated concentration of compound. (d) AZ11645373 inhibition curves at 280-284-X4 chimera. The hP2X7-2NβR is shown as black dotted line, (n=3-5).
Figure 3.11. AZ11645373 inhibition at point mutations of the left flipper (280-284). (a) Homology model of the hP2X7R showing the position of the mutant which has a significant effect on AZ11645373 sensitivity (D280A [green]) and mutants which have no effect on AZ11645373 sensitivity (K281A, T282A, T283A and N284A [grey]). (b) AZ11645373 inhibition curve at D280A point mutation. The hP2X7-2NβR is shown as a black dotted line. (c) Summary of pIC₅₀ values of AZ11645373 at D280A, K281A, T282A, T283A and N284A. Any significant differences from the wild type hP2X7-2NβR were determined by one-way ANOVA test followed by Dunnett’s test. A three fold change from hP2X7-2NβR is indicated by the dotted line. Stars indicate a significant difference from the hP2X7-2NβR. **, p < 0.01. Data are represented as mean ± S.E.M (n= 3-5).
Figure 3.12. AZ11645373 inhibition at additional residues lining the orthosteric binding site
(a) Position of the left flipper region (grey) and residues around the orthosteric pocket (orange) shown in the homology model of hP2X7R. ATP binding sites are shown in black dotted ring. In the right hand panel, residue where mutation decreased AZ1164537 sensitivity is shown in green (D280A) and residues where mutation had no effect on AZ11645373 sensitivity are shown in grey.
(b) Summary of pIC$_{50}$ values of AZ11645373 at Q143K, R206A, Y288S and Y291F. A three fold change from hP2X7-2NβR is indicated by dotted line. Data are represented as mean ± S.E.M (n= 3-5).
3.2.6. The effect of AZ11645373 on the hP2X7-2Nβ/1 receptor chimeras around the allosteric binding sites

To test the contribution of variation around the allosteric pocket at the apex of the receptor to antagonist action, one deletion (residues 73-79) and eight chimeras around the allosteric binding pocket (81-88, 89-94, 105-114, 112-118, 122-128, 164-168, 170-177, and 295-310) were tested (figure 3.13 a & b). Previous work showed there is no significant change in ATP potency for most of these chimeras (7/8 unchanged) (table 3.2) (Allsopp et al., 2017).

The AZ11645373 sensitivity of the 73-79 deletion and the chimeras 105-114, 122-128, 164-168 and 170-177 was the same as the hP2X7-2Nβ parent receptor, pIC$_{50}$ = 7.82 ± 0.10, 7.11 ± 0.0009, 7.47 ± 0.16, 7.09 ± 0.15 and 7.68 ± 0.17 respectively (figure 3.14). There was a modest approximately 4 fold reduction in AZ11645373 sensitivity at the 295-310 and 81-88 chimeras, pIC$_{50}$ = 6.85 ± 0.05, p < 0.05 and pIC$_{50}$ = 6.85 ± 0.14, P < 0.05 respectively. AZ11645373 sensitivity was reduced approximately 8 fold at the 112-118 chimera; 1 µM AZ11645373 inhibited currents by approximately 83%, pIC$_{50}$ = 6.59 ± 0.1, p < 0.001 (figure 3.15). The most striking effect was at the 89-94 chimera AZ11645373 (1 µM) had no effect on ATP responses indicating a more than 1000 fold reduction in sensitivity (figure 3.15). These results demonstrate variation in the allosteric pocket underline antagonist sensitivity. Overall the results of this chapter indicate that regions 81-88, 89-94, 112-118, and 295-310 play a significant role in AZ11645373 antagonist sensitivity and support an allosteric mode of action (figure 3.16).
Table 3.2. ATP and AZ11645373 sensitivity at the hP2X7-2Nβ/1 chimeras around allosteric and orthosteric binding sites.

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<td>4.5</td>
<td>6.85 ± 0.05 *</td>
<td>4.2</td>
</tr>
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</table>

Table 3.2. ATP and AZ11645373 sensitivity at the hP2X7-2Nβ/1 chimeras around allosteric and orthosteric binding sites. The table shows the pEC_{50} values of ATP (Allsopp et al., 2017), pIC_{50} values of AZ11645373 and fold shift relative to the hP2X7-2NβR. A star indicate a significant shift in EC_{50} and IC_{50} from the hP2X7-2NβR. *, p < 0.05; **, p < 0.01; ***, p < 0.001; ****, p < 0.0001. Arrows indicate an increasing ATP/AZ11645373 sensitivity. Data are shown as mean ± S.E.M (n ≥ 3).
Figure 3.13. Location of chimeras around the potential allosteric binding site. (a) Position of extracellular regions swapped to generate chimeras around the allosteric pocket are shown in the homology model of hP2X7R for one subunit. (b) The top of view of the 73-79 deletion (firebrick), 81-88 (red), 89-94 (salmon), 105-114 (orange), 112-118 (yellow), 122-128 (purple), 164-168 (green), 170-177 (marine) and 295-310 (magenta) chimeras.
Figure 3.14. AZ11645373 inhibition at the chimeras around the potential allosteric binding site. (a) The top view of the 73-79 deletion (firebrick), 105-114 (orange), 122-128 (purple), 164-168 (green) and 170-177 (marine) chimeras. The allosteric binding sites in the P2X7 receptor are shown as black cartoon representation. (b) Traces of current recorded from 73-79 deletion, 105-114, 122-128, 164-168 and 170-177 in response to EC₉₀ of ATP (100 µM) (unfilled circle) and ATP with 1 µM of AZ11645373 (filled circle). There was 5 minutes between applications. Bar indicates 10 seconds of ATP application or ATP application with AZ11645373 following 5 minutes pre-incubation. (c) AZ11645373 inhibition curves at 73-79 deletion, 105-114, 122-128, 164-168 and 170-177 chimeras. The hP2X7-2Nβ is shown as black dotted line, (n=3-5).
Figure 3.15. AZ11645373 inhibition at the 81-88, 89-94, 112-118 and 295-310 chimeras around the allosteric binding sites. (a) The top of view of the 81-88 (red), 89-94 (salmon), 112-118 (yellow-orange), 295-310 (magenta) chimeras. The allosteric binding sites in the P2X7 receptor are shown as black cartoon representation. (b) Traces of current recorded from 81-88, 89-94, 112-118 and 295-310 in response to EC₉₀ of ATP (100 µM) (unfilled circle) and ATP with 1 µM of AZ11645373 (filled circle). There was 5 minutes between each application. Bars indicate a 10 seconds of ATP application or ATP application with AZ11645373 following 5 minutes pre-incubation. (c) AZ11645373 inhibition curves at 81-88, 89-94, 112-118 and 295-310 chimeras. The hP2X7-2Nβ is shown as black dotted line. A star indicate a significant shift in IC₅₀ from the hP2X7-2Nβ. One way analysis of variance (ANOVA) test was used followed by Dunnett’s test. *, p < 0.05; ***, p < 0.001; ****, p < 0.0001; (n=3-5). Data are presented as mean ± S.E.M.
Figure 3.16. Summary of the effects of AZ11645373 at chimeras around the allosteric binding site. (a) Position of chimeras around allosteric pocket which have a significant effect on AZ11645373 sensitivity (81-88 [red]), (89-94 [salmon]), (112-118 [yellow-orange]) and (295-310 [magenta]) and no effect on AZ11645373 sensitivity (73-79 deletion, 105-114, 122-128, 164-168, 170-177 [grey]). (b) Summary of pIC_{50} values of AZ11645373 at chimeric hP2X7-2Nβ receptors. Any significant differences from the wild type hP2X7-2NβR in either direction were determined by one-way ANOVA test followed by Dunnett’s test. A three fold change from hP2X7-2Nβ is indicated by dotted lines. Stars indicate a significant difference from the hP2X7-2Nβ receptors. *, p < 0.05; ***, p < 0.001; ****, p < 0.0001. Data are presented as mean ± S.E.M (n=3-5).
3.3. Discussion

The initial experimental work of this thesis was to determine whether the modified P2X7R (hP2X7-2NβR) could be used as a template for chimeras to study the molecular basis of the AZ11645373 binding site. This was done with electrophysiological recording in the Xenopus laevis oocytes expression system. This work confirmed that ATP is effective at the hP2X7-2NβR in divalent free buffer, with an EC₅₀ concentration of approximately 25 µM, consistent with that reported by Allsopp et al. (2017). ATP was effective at the hP2X1R with an EC₅₀ concentration of approximately 1 µM in free divalent buffer. This is line with that reported by Li et al. (2013).

This study also shows that the pharmacological properties of the hP2X7-2NβR for AZ11645373 antagonist are similar to WT hP2X7R. At the hP2X7-2NβR, co application of AZ11645373 inhibited the ATP evoked current in a concentration dependent manner with an IC₅₀ value of 30 nM in free divalent buffer. The sensitivity of AZ11645373 at the hP2X7-2NβR is similar to that reported by Stokes et al. (2006) who showed that AZ11645373 inhibited the ATP evoked current at the WT hP2X7R with an IC₅₀ value 90 nM in low divalent buffer (Stokes et al., 2006). The slight difference in the value of AZ11645373 potency between hP2X7-2Nβ and hP2X7Rs may be related to using different conditions of the divalent buffer.

The sensitivity of AZ11645373 at the hP2X7-2NβR and lack of effect at the hP2X1R was consistent with a previous study (Stokes et al., 2006). The greater than 1000-fold difference in AZ11645373 inhibition between the hP2X7-2NβR (sensitive) and the hP2X1R (insensitive) was the starting point for my thesis. The variation in antagonist sensitivity between P2XR subunits is due to variations of amino acids in the extracellular region. Therefore, chimeras which replaced regions of hP2X7-2Nβ with the corresponding regions of hP2X1R were used to identify variant regions contributing to antagonist action. Molecular modelling identified potential orthosteric and allosteric binding pockets in the P2X7Rs. These hP2X7-2Nβ/1 chimeras had previously been used to identify the allosteric binding sites for AZ10606120, A740003 and A438079 (Allsopp et al., 2017, Allsopp et al., 2018).

One interesting feature of orthosteric chimeras in the left flipper was changed ATP sensitivity. It was reported that 279-285 chimera and 280-285-X4 chimera increased ATP sensitivity ~ 30 fold and 10 fold, respectively (Allsopp et al., 2017, Allsopp et al.,
In this study removing the unique aspartic acid residue at position 280 (D280A) makes the hP2X7R ~10 fold more sensitive to ATP, demonstrating an effect on ATP binding. Previous studies have shown that the movement of the left flipper is associated with the channel opening upon ATP binding (Karasawa and Kawate, 2016, Zhao et al., 2014). A homology model of the hP2X7R shows that D280 residue is located between two basic residues (K193 and R206) (figure 3.12a) and molecular dynamics suggested that D280 residue can make a salt bridge with K193 and R206. This salt bridge prevents the movement of the left flipper and thus impacts the channel gating (Allsopp et al., 2018). The relationship between the movement of the left flipper and channel gating could explain that the removing of the salt bridge by the D280A mutation is associated with increased ATP sensitivity.

Another interesting feature of the orthosteric chimeras was that changing in the left flipper by using 279-285 chimera, showed a reduction in AZ11645373 sensitivity. This finding was also reported by Allsopp et al. (2018) who found that the 279-285 chimera produced an 10 and 30 fold decrease in sensitivity for A740003 and A438079 respectively (Allsopp et al., 2018). Point mutations within the left flipper showed that D280A was responsible for reduction of AZ11645373 sensitivity. However there are several reasons to think that AZ11645373 does not act at the orthosteric site. Firstly, the orthosteric chimera (279-285) reduced AZ11645373 action and also had a significant effect for the allosteric antagonists A740003 and A438079. This suggests that changing in the left flipper by using chimera 279-285 may modify the allosteric pocket. Secondly, chimeras and most of the point mutations in the left flipper changed ATP sensitivity, indicating conformational change on channel gating and thus may modify the allosteric pocket. Thirdly, most of the point mutations around the orthosteric site (8/9 mutations) had no effect on AZ11645373 sensitivity. It seems that AZ11645373 ruled out an orthosteric binding site. Fourthly, our results showed that sensitivity of AZ11645373 was predominantly changed with allosteric chimeras (4 chimeras affected); only one orthosteric chimera affected. Therefore it can be suggested that AZ11645373 was more likely to be allosteric rather than orthosteric. This is supported by P2X7Rs protection study suggested that AZ11645373 does not act at the ATP binding site. Michel et al. (2009) examined the effect of P2X7R antagonists such as AZ11645373 and PPADS after pre-treatment with a decavanadate agent as an orthosteric antagonist. It was seen that AZ11645373 inhibition was not surmountable by using decavanadate, which means the
inhibitory effect of AZ11645373 was not changed when the concentration of a decavanadate was increased. This suggested that AZ11645373 has a mode/site of action different from decavanadate. In contrast, PPADS inhibition was surmountable by using decavanadate, the inhibitory effect of PPADS was decreased when the concentration of decavanadate antagonist was increased. This competition effect indicates that PPADS and decavanadate antagonists have a similar mode of action (Michel et al., 2009).

The most striking finding in the current study was that four chimeras around the allosteric pocket (81-88, 89-94, 112-118 and 295-310) had a significant effect on AZ11645373 sensitivity. The greatest change was seen at the 89-94 chimera which produced a more than 1000 fold decrease in AZ11645373 sensitivity. These chimeras had effects on the other P2X7R antagonists. Allsopp et al. (2017) reported that 81-88, 89-94 and 112-118 chimeras had effect on AZ10606120 action. It was also reported that 89-94, 112-118 and 295-310 chimeras had effect on the sensitivity of A740003 and A438079 (Allsopp et al., 2017, Allsopp et al., 2018). The allosteric site of AZ11645373 is also supported by a radio ligand binding study which used AZ10606120 as an allosteric antagonist. It was reported that AZ11645373 produced an inhibitory effect at the hP2X7R similar to AZ10606120, indicating that AZ11645373 may bind at the allosteric binding sites (Michel et al., 2009). It seems most likely that AZ11645373 binds to an allosteric site.

In summary, this chapter characterized the effect of ATP and AZ11645373 at the hP2X7-2Nβ and hP2X1Rs. The results were consistent with previous studies suggesting that these receptors are appropriate for the chimera study. Studying chimeras which replaced regions of the extracellular domain of hP2X7-2NβR (sensitive to AZ11645373) with the corresponding regions of the hP2X1R (insensitive to AZ1145373) was useful to identify regions contributing to antagonist action. Our results demonstrate several regions of the chimeras that may underline antagonist sensitivity and suggest most likely an allosteric binding site. However the orientation of AZ11645373 with the allosteric pocket needs to be determined. In the chapter 4 sub chimeras and point mutations within these regions of chimeras have been used to identify specific residues that could responsible for antagonist sensitivity.
Chapter 4: Using sub chimeras and point mutation to identify the binding site of AZ11645373

4.1. Introduction

The use of hP2X7-2Nβ/1 chimeras in chapter 3 demonstrated that variations in the allosteric pocket underlie antagonist sensitivity. In order to build and test a model of antagonist action, the contributions of residues lining the allosteric pocket needs to be determined. The generation of sub chimeras and point mutations in this pocket would be useful to identify residues that are involved in determining AZ11645373 sensitivity. Previous mutagenesis studies on P2X7R antagonists have been used to highlight residues that contribute to antagonist binding and are discussed in more detail below.

4.1.1. Mutagenesis studies on AZ10606120, A740003 and A438079 at the P2X7 receptor

Mutagenesis studies established the binding mode of a range of P2X7R antagonists at the allosteric pocket (Allsopp et al., 2017, Allsopp et al., 2018). For instance, the molecular basis of AZ10606120 binding was identified at the hP2X7R by a range of point mutations that swapped individual residues of hP2X7R to the corresponding residue in the hP2X1R (Allsopp et al., 2017). These point mutations tested the contribution of variant residues lining allosteric antagonist pocket. Studying hP2X7/1Rs by mutagenesis highlighted several hydrophobic residues that contributed to AZ10606120 sensitivity. These mutations were also used to identify residues contributing to antagonist action of A740003 and A438079 (Allsopp et al., 2018).

Studies on AZ10606120, A740003 and A438079 showed a common feature of the allosteric binding pocket. Most of the point mutations (17/18) located in the middle and base regions of the allosteric binding site had major effects on antagonist sensitivity. Residues that showed the greatest effects (10-1000 fold reduction in antagonist sensitivity) were located at positions F88, D92, F95, F103, M105 and F108 (figure 4.1). It was suggested that mutations at these positions served as a finger print for allosteric antagonist binding (Allsopp et al., 2018). However, there were also specific differences between the antagonists, indicating that each P2X7 antagonist has a different binding
mode. For instance, removal of the P2X7R unique insertion residues 73-79 at the entrance to the pocket reduced antagonist sensitivity of AZ10606120 ~ 40 fold (Allsopp et al., 2017) but had no significant effect on A740003 and increased antagonist sensitivity of A438079 ~ 4 fold (Allsopp et al., 2018). Molecular docking showed that AZ10606120 binds towards the entrance of the allosteric pocket but not in case of the A740003 and A438079 (Allsopp et al., 2018) and explains the difference in the effects of the chimeras.

4.1.2. Chapter Aims

The results from the initial hP2X7-2Nβ/1 chimeras in chapter 3 identified the effects of swapping regions between hP2X7R and hP2X1R around the allosteric pocket and suggested that residues within 81-88, 89-94, 112-118 and 295-310 regions of the allosteric pocket are important for the AZ11645373 action. In this chapter point mutations within these regions were used to identify the contribution of individual residues in the allosteric pocket which are responsible for AZ11645373 action. The results are then used to validate models of the AZ11645373 binding site at the hP2X7R.

4.2. Results

From the homology model of the hP2X7R, residues in the allosteric pocket can be classified into residues lining the allosteric pocket and residues facing away from the allosteric pocket (figure 4.2). To determine the role of variant residues in the allosteric pocket point mutations were tested that replaced individual residues of the hP2X7-2NβR with the corresponding residues of the hP2X1R. For conserved residues in the allosteric pocket individual alanine or cysteine point mutations were studied.
Figure 4.1. “Finger print” for allosteric inhibition of P2X7 receptors. (a) Homology model of the hP2X7R showing residues around the allosteric pocket which have significant effects on AZ10606120, A438079 and A740003 sensitivity. (b) Residues F88, F95, F103, M105 and F108 is shown in one subunit.
Figure 4.2. Location of point mutations within allosteric chimeras. (a) Homology model of the hP2X7R showing chimeras around the allosteric pocket which have a significant effect on AZ11645373 sensitivity (81-88 [red]), (89-94 [salmon]), (112-118 [yellow]) and (295-310 [magenta]) and no effect on AZ11645373 sensitivity [grey]. (b) Residues lining the allosteric pocket. (c) Residues that are not directly facing the allosteric pocket.
4.2.1. The effect of AZ11645373 on the 81-88 sub chimeras

AZ11645373 sensitivity was reduced at the 81-88 chimera, to determine which residues are important for sensitivity; two sub-chimeras 81-84 and 85-88 (figure 4.3a & b) that divided the region into smaller sections were tested. The 81-84 chimera had an approximately 20 fold increase in AZ11645373 sensitivity with a pIC$_{50}$ value of 8.77 ± 0.44; p < 0.01 (figure 4.3c & e). However the 85-88 chimera produced an approximately 7 fold decrease in AZ11645373 action with a pIC$_{50}$ value of 6.61 ± 0.10; p < 0.05 (figure 4.3d & f) (table 4.1). Due to the shift seen in the sensitivity to AZ11645373 inhibition of the 81-84 and 85-88 chimeras, individual point mutations that replaced residues of the hP2X7-2NβR with the corresponding residues of the hP2X1R were tested.

<table>
<thead>
<tr>
<th>ATP</th>
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<td>pEC$_{50}$ ± S.E.M</td>
<td>Fold Shift</td>
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<tr>
<td>hP2X7-2Nβ</td>
<td>4.63 ± 0.07</td>
</tr>
<tr>
<td>73-79 deletion</td>
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<tr>
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</tr>
<tr>
<td>81-84</td>
<td>4.88 ± 0.05</td>
</tr>
<tr>
<td>85-88</td>
<td>4.64 ± 0.07</td>
</tr>
<tr>
<td>89-94</td>
<td>4.35 ± 0.09</td>
</tr>
<tr>
<td>105-114</td>
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</tr>
<tr>
<td>112-118</td>
<td>4.59 ± 0.06</td>
</tr>
<tr>
<td>122-128</td>
<td>4.33 ± 0.06</td>
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<tr>
<td>164-168</td>
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</tr>
<tr>
<td>170-177</td>
<td>4.56 ± 0.01</td>
</tr>
<tr>
<td>210-217</td>
<td>4.53 ± 0.03</td>
</tr>
<tr>
<td>279-285</td>
<td>5.33 ± 0.19 ****</td>
</tr>
<tr>
<td>280-284-X4</td>
<td>5.64 ± 0.07 ****</td>
</tr>
<tr>
<td>295-310</td>
<td>3.98 ± 0.01 **</td>
</tr>
</tbody>
</table>

Table 4.1. ATP and AZ11645373 sensitivity at the hP2X7-2Nβ/1 chimeras around allosteric and orthosteric binding sites. The table shows the pEC$_{50}$ values of ATP (Allsopp et al., 2017), pIC$_{50}$ values of AZ11645373 and fold shift relative to the hP2X7-2NβR. Stars indicate a significant shift in EC$_{50}$ and IC$_{50}$ from the hP2X7-2NβR. *, p < 0.05; **, p < 0.01; ***, p < 0.001; ****, p < 0.0001. Arrows indicate increase ATP/AZ11645373 sensitivity. Data are shown as mean ± S.E.M (n ≥ 3).
Figure 4.3. AZ11645373 inhibition at sub chimeras of the 81-88 region. (a) Amino acid sequence line up of the hP2X7R (top) and hP2X1R (bottom) of the 81-88 region. Conserved amino acids are shown in black and variant amino acids are in grey. The 81-84 chimera is shown in a blue box and the 85-88 chimera in a purple box. (b) Top of view of the 81-84 (blue) and 85-88 (purple) chimeras in one subunit. The allosteric binding site in the hP2X7R is shown in black cartoon representation. (c & d) Traces of currents recorded from 81-84 and 85-88 at 100 µM of ATP and 1 µM of AZ11645373. There was 5 minutes between each application. Bars indicate 10 seconds of ATP application and ATP application with AZ11645373 following 5 minutes pre-incubation with the indicated concentration of compound. (e & f) AZ11645373 inhibition curves at 81-84 and 85-88 chimeras. The hP2X7-2Nβ is shown as black dotted line. Stars indicate a significant shift in IC₅₀ from hP2X7-2Nβ receptor. One way analysis of variance (ANOVA) test was used followed by Dunnett’s test. *, p < 0.05; **, p < 0.01; (n= 3-5). Data are presented as mean ± S.E.M.
4.2.2. The effect of AZ11645373 on the 81-84 point mutations

The 81-84 region is located at the entrance of the allosteric pocket. Comparison of the hP2X7R and hP2X1Rs in this region shows there are three variant amino acids (K81, K82 and V84) and one conserved amino acid (L83) (figure 4.4a & b). Residues K82 and V84 line the allosteric pocket while residues K81 and L83 face away from the allosteric pocket (figure 4.2). Point mutations at K82G and V84G had no effect on AZ11645373 sensitivity. However the K81P mutant, which mutated a positively charged lysine to a non-polar proline, reduced AZ11645373 sensitivity by approximately 4 fold, pIC$_{50}$ = 6.83 ± 0.2, p < 0.05. In addition the leucine to alanine mutation at position 83 (L83A) reduced AZ11645373 sensitivity by approximately 5 fold, pIC$_{50}$ = 6.72 ± 0.04, p < 0.01 (figure 4.4c &d). This indicates that AZ11645373 binding is reduced by a change in polarity at position 81 and a reduction the size of the residue at position 83.

4.2.3. The effect of AZ11645373 on the 85-88 point mutations

There are three amino acids in the 85-88 region that are variant between hP2X7R and hP2X1Rs (H85, S86 and F88) (figure 4.5a). This region is located at the entrance of the allosteric pocket (figure 4.5b). Residues S86 and F88 line the allosteric pocket while residues H85 and V87 face away from the allosteric pocket (figure 4.2). Removing the positively charged histidine at position 85 (H85P) reduced AZ11645373 sensitivity approximately 4 fold, pIC$_{50}$ = 6.91 ± 0.06, p < 0.05. The serine at position 86 was interesting as mutation to glutamine (S86Q) produced a modest approximately 4 fold decrease in AZ11645373 sensitivity, pIC$_{50}$ = 6.86 ± 0.06; p < 0.05, whereas mutation to alanine (S86A) had no significant effect on the potency of AZ11645373. This indicates that the size of residues at this position may be important. The F88W mutation (conservative substitution which maintains the aromatic side chain) produced an approximately 27 fold decrease in AZ11645373 sensitivity, pIC$_{50}$ = 6.04 ± 0.1, p < 0.0001. A similar reduction was seen at the F88A mutation with pIC$_{50}$ = 6.05 ± 0.1, p < 0.0001 (figure 4.5c &d). These results highlight the importance of individual residues in the 85-88 region that contribute to AZ11645373 binding.
Figure 4.4. AZ11645373 inhibition at the 81-84 point mutations. (a) Amino acid sequence line up of the hP2X7R (top) and hP2X1R (bottom) of the region 81-84. The conserved amino acid at position 83 is shown in black and variant amino acids at positions 81, 82 and 84 are shown in grey. (b) Top of view of the 81-84 (blue) chimera in one subunit. The allosteric binding site in the hP2X7R is shown in black cartoon representation. The right hand panel shows a sphere and stick representation of the 81-84 chimera. Residues where mutation decreased AZ11645373 sensitivity are shown in blue and residues where mutation had no effect on AZ11645373 sensitivity are shown in grey. (c) AZ11645373 inhibition curves at K81P, K82G, L83A and V84G. hP2X7-2Nβ is shown as a black dotted line. (d) The pIC₅₀ values of AZ11645373 at the K81P, K82G, L83A and V84G mutations. A three fold change from hP2X7-2NβR is indicated by the dotted line. Stars indicate a significant shift in IC₅₀ from hP2X7-2NβR. One way analysis of variance (ANOVA) test was used followed by Dunnett’s test. *, p < 0.05; **, p < 0.01; (n= 3-5). Data are presented as mean ± S.E.M.
Figure 4.5. AZ11645373 inhibition at the 85-88 point mutations. (a) Amino acid sequence line up of the hP2X7R (top) and hP2X1R (bottom) of the region 85-88. The conserved amino acid at position 87 is shown in black and variant amino acids at positions 85, 86 and 88 are shown in grey. (b) Top of view of the 85-88 (purple) chimera in one subunit. The allosteric binding site in the hP2X7R is shown in black cartoon representation. The right hand panel shows a sphere and stick representation of the 85-88 chimera. Residues where mutation decreased AZ11645373 sensitivity are shown in purple and the residue where mutation had no effect on AZ11645373 sensitivity is shown in grey. (c) AZ11645373 inhibition curves at H85P, S86Q, S86A, F88W. The hP2X7-2Nβ is shown as a black dotted line. (d) The pIC₅₀ values of AZ11645373 at the H85P, S86Q, S86A, F88W and F88A mutations. A three fold change from hP2X7-2NβR is indicated by the dotted line. Stars indicate a significant shift in IC₅₀ from hP2X7-2NβR. One way analysis of variance (ANOVA) test was used followed by Dunnett's test. *, p < 0.05; ****, p < 0.0001; (n= 3-5). Data are presented as mean ± S.E.M.
4.2.4. The effect of AZ11645373 on the 89-94 point mutations

The 89-94 chimera has two unique variant residues at positions 90 and 94. These are threonine in the P2X7R and valine in all other P2XR (Figure 4.6a). Both T90 and T94 are located in the α-helix at the base of the allosteric antagonist binding pocket. However these residues face away from the allosteric pocket (Figure 4.2c). The threonine to valine mutation at position 90 (T90V) produced an approximately 12 fold decrease in AZ11645373 action, pIC50 = 6.4 ± 0.08, p < 0.0001. Mutation to serine at this position (T90S) reduced AZ11645373 sensitivity by approximately 7.5 fold, pIC50 = 6.59 ± 0.1, p < 0.0001. However the threonine to alanine mutation (T90A) had no effect on AZ11645373 sensitivity (Figure 4.6c) (Figure 4.8b). The threonine at position 94 was interesting as mutation to valine (T94V) abolished the antagonist action of AZ11645373 (1 µM of AZ11645373 had no effect on ATP responses). Other substitutions were tested at T94 and gave an approximately 39 fold and 19 fold decreases in AZ11645373 potency for T94S and T94A, respectively (Figure 4.6d) (Figure 4.8b).

Within the 89-94 region there are four conserved residues between the hP2X7R and the hP2X1R (D89, A91, D92 and Y93) which are located in the α-helix at the base of the allosteric antagonist binding site (Figure 4.7a). These residues face away from the allosteric pocket (Figure 4.2c). It was reported that the D89A and D89C mutants to remove the conserved negative charge at position 89 (aspartic acid at all P2XR) were non-functional (Allsopp et al., 2017). The alanine to cysteine mutation at position 91 (A91C) reduced AZ11645373 sensitivity by approximately 17 fold, pIC50 = 6.24 ± 0.07, p < 0.0001. Mutation to remove a negative charge at position 92 (aspartic acid at all P2XR) (D92A) and (D92G) reduced AZ11645373 sensitivity by 30 and 25 fold p < 0.0001 respectively. Interestingly the D92R mutant, which mutated a negatively charged aspartic acid to a positively charged arginine, abolished the antagonist action of AZ11645373 demonstrating a more than 1000 fold reduction in AZ11645373 sensitivity. The tyrosine to alanine mutation at position 93 (Y93A) produced an approximately 5.5 fold decrease in AZ11645373 sensitivity, pIC50 = 6.73 ± 0.08, p < 0.01 (Figure 4.7c) (Figure 4.8). Therefore residues within the 89-94 region are highly important for the conformation of the allosteric antagonist binding site although these residues do not directly face the allosteric pocket, suggesting a structural role on the integrity of the pocket.
Figure 4.6. AZ11645373 inhibition at T90 and T94 point mutations. (a) Amino acid sequence line up of the hP2X7R (top) and hP2X1R (bottom) of the region 89-94. Conserved amino acids are shown in black and unique variant amino acids at position 90 and 94 are shown in raspberry. (b) Top of view of the 89-94 (salmon) chimera in one subunit. The allosteric binding site in the hP2X7R is shown in black cartoon representation. The right hand panel, mutants that decreased AZ11645373 sensitivity are shown in salmon. (c & d) AZ11645373 inhibition curves at (T90V, T90A, T90S) and (T94V, T94A, T94S). The hP2X7-2Nβ is shown as a black dotted line. Stars indicate a significant shift in IC₅₀ from hP2X7-2NβR. One way analysis of variance (ANOVA) test was used followed by Dunnett’s test. ***, p < 0.001; ****, p < 0.0001; (n= 3-5). Data are presented as mean ± S.E.M.
Figure 4.7. AZ11645373 inhibition at A91, D92 and Y93 point mutations. (a) Amino acid sequence line up of the hP2X7R (top) and hP2X1R (bottom) of the extracellular region (89-94 region). Conserved amino acids are shown in black and unique variant amino acids at position 90 and 94 are shown in raspberry. (b) Top of view of the 89-94 (salmon) chimera in one subunit. The allosteric binding site in the hP2X7R is shown in black cartoon representation. The right hand panel, mutants that decreased AZ11645373 sensitivity are shown in salmon. (c) AZ11645373 inhibition curves at the A91C, (D92A, D92G and D92R) and Y93A. The hP2X7-2Nβ is shown as a black dotted line. Stars indicate a significant shift in IC₅₀ from hP2X7-2NβR. One way analysis of variance (ANOVA) test was used followed by Dunnett’s test. **, p < 0.01; ****, p < 0.0001; (n= 3-5). Data are presented as mean ± S.E.M.
Figure 4.8. Summary of the effect of AZ11645373 on the 89-94 point mutations. (a) Top view of the 89-94 chimera (salmon) in one subunit. The allosteric binding site in the hP2X7R is shown in black cartoon representation. (b) Summary of pIC$_{50}$ values of AZ11645373 at the 89-94 point mutations. A three fold change from hP2X7-2NβR is indicated by the dotted line. Stars indicate a significant shift in IC$_{50}$ from hP2X7-2NβR. One way analysis of variance (ANOVA) test was used followed by Dunnett's test. **, p < 0.01; ***, p < 0.001; ****, p < 0.0001; (n= 3-5). Data are presented as mean ± S.E.M.
4.2.5. The effect of AZ11645373 on the other residues in the allosteric pocket

There is a conserved aromatic amino acid at position 95 (phenylalanine in hP2X7R and hP2X1R) (figure 4.9a). The F95 residue lines the allosteric pocket (figure 4.2b). A previous study showed that mutation of the phenylalanine to leucine (non-polar amino acid) in the hP2X7R, had an allosteric effect (Michel et al., 2009). Similarly, in this study the F95L mutant reduced AZ11645373 sensitivity by approximately 250 fold, pIC$_{50}$ = 5.07 ± 0.46, p < 0.0001. Other mutations including aromatic and non-aromatic amino acids substitution were generated at position 95 to determine the importance of side chain properties at this position. Removing the aromatic residue at position 95 (F95A) had a greater effect than F95L and produced a more than 250 fold decrease in AZ11645373 sensitivity. The F95Y mutant, which changed the residue from a polar to a non-polar aromatic residue, reduced AZ11645373 sensitivity by 7 fold, pIC$_{50}$ = 6.63 ± 0.01, p < 0.001. However, the more conservative mutation F95W had no effect on AZ11645373 sensitivity. Mutation of the F95H was non-functional (figure 4.9c &d).

Residues P96 and L97 face away from the allosteric pocket (figure 4.2c). Mutating the proline at position 96 (conserved at all P2X7Rs) (P96A), abolished the antagonist action of AZ11645373 (1 µM) indicating a more than 1000 fold reduction in sensitivity (figure 4.9c & d). There is an unique leucine residue at position 97 in the hP2X7R (L97). The alanine mutation at this position was non-functional (Allsopp et al., 2017). However the leucine to proline mutation (L97P) produced an approximately 32 fold decrease in AZ11645373 action, pIC$_{50}$ = 5.96 ± 0.1, p < 0.0001 (figure 4.9c & d). These results indicate that P96 and L97 residues are important for AZ11645373 antagonist binding although both of these residues do not line the allosteric pocket, suggesting that these residues are most likely to influence the structure of the allosteric pocket.

Residues F103, M105, F108, K110 and T111 line the allosteric pocket (figure 4.2b). The phenylalanine at position 103 was important as mutation to alanine (F103A) reduced AZ11645373 sensitivity by 37 fold, pIC$_{50}$ = 5.9 ± 0.01, p < 0.0001. Mutation of the conserved methionine at position 105 (M105A) had no effect on the AZ11645373 sensitivity. Mutation to remove the aromatic phenylalanine at position 108 (F108C) produced a modest 5 fold reduction in AZ11645363 action. Mutation to remove the
positive charge at position 110 (K110Y) increased sensitivity of AZ11645373 by 16 fold, pIC$_{50}$ = 8.67 ± 0.01, p < 0.0001. Residue T111 conserved in all hP2XRs had no effect on AZ11645373 sensitivity (figure 4.10).

4.2.6. The effect of AZ11645373 at the 112-118 point mutations

There are five variant amino acids between hP2X7R and hP2X1R in the 112-118 region (figure 4.11a). Residues E112, G113, E115 and R117 line the allosteric pocket whereas residue L118 face away from the allosteric pocket (figure 4.2a & b). Neutralizing the negatively charged glutamic acid at position 112 (E112C) and the positively charged arginine at position 117 (R117C) had no effect on AZ11645373 sensitivity. However glycine to cysteine mutation at position 113 (G113C) reduced AZ11645373 sensitivity by 7 fold. Neutralizing the negative charge glutamic acid at position 115 (E115C) produced an approximately 17 fold decrease in AZ11645373 sensitivity, pIC$_{50}$ = 6.24 ± 0.02, p < 0.0001 (figure 4.11c &d). Neither cysteine nor tyrosine mutations at position 118 were functional.

Within 112-118 region there are two residues that are conserved in all hP2XRs, and theses line the allosteric pocket (Q114 and Q116). Cysteine mutation at position 114 (Q114C) was non-functional. However glutamine to cysteine mutation at position 116 (Q116C) had a modest 5 fold reduction in AZ11645373 sensitivity (figure 4.11d).

4.2.7. The effect of AZ11645373 at the 295-310 point mutations

Within the 295-310 region there are several residues that are variant between the hP2X7R and hP2X1R (figure 4.12a). Residues Y295, K297, Y298, T308 and I310 line the allosteric pocket while residues Y299, V304, E305 and K306 face away from the allosteric pocket (figure 4.2). At position 295, mutation to remove the aromatic residue (Y295A) or mutation with conservative aromatic residue (Y295F) had no effect on AZ11645373 action. The arginine at position 297 is conserved in all P2XRs (K297). Neither alanine nor cysteine mutations at position 297 were functional. The arginine to glycine mutation (K297G) had no significant effect on AZ11645373 sensitivity. There was no significant effect on AZ11645373 sensitivity at Y298, Y299, V304, E305, K306 and T308. The alanine mutation at position 310 (I310A) produced a significant decrease
of approximately 22 fold in AZ11645373 action, pIC$_{50}$ = 6.12 ± 0.06, p < 0.0001. Residue V312 (conserved between hP2X7R and hP2X1R) lines the allosteric pocket. Alanine mutant at position 312 (V312A) reduced AZ11645373 sensitivity by 19 fold, pIC$_{50}$ = 6.19 ± 0.01, p < 0.0001 (figure 4.12).
Figure 4.9. AZ11645373 inhibition at F95, P96 and L97 point mutations. (a) Amino acid sequence line up of the hP2X7R (top) and hP2X1R (bottom) of the region 95-97. The conserved amino acids are shown in black and a unique variant amino acid at position 97 is shown in raspberry. (b) Top of view of the F95, P96 and L97 in one subunit. The allosteric binding site in the hP2X7R is shown in black cartoon representation. The right hand panel shows stick representation at F95, P96 and L97. Point mutants which have a significant effect are shown as pink (F95, P96 and L97). (c) AZ11645373 inhibition curves at the F95A, F95L, F95Y, F95W, P96A and L97P. The hP2X7-2Nβ is shown as a black dotted line. (d) The pIC₅₀ values of AZ11645373 at the F95L, F95W, F95Y and L97P. A three fold change from hP2X7-2NβR is indicated by the dotted line. Stars indicate a significant shift in IC₅₀ from hP2X7-2NβR. One way analysis of variance (ANOVA) test was used followed by Dunnett's test. **, p < 0.01; ****, p < 0.0001; (n= 3-5). Data are presented as mean ± S.E.M.
Figure 4.10. AZ11645373 inhibition at F103, M105, F108, K110 and T111 point mutations. (a) Amino acid sequence line up of the hP2X7R (top) and hP2X1R (bottom) of the region 103-111. The conserved amino acids are shown in black and variant amino acids are shown in grey. (b) Top of view of the F103, M105, F108, K110 and T111 in one subunit. The allosteric binding site in the hP2X7R is shown in black cartoon representation. The right hand panel shows a sphere and stick representation. Residues where mutation changed AZ11645373 sensitivity are shown in pink and residues where mutation had no effect on AZ11645373 sensitivity are shown in grey. (c) AZ11645373 inhibition curves at the F103A, M105A, F108C, F108Y, T111S and K110Y. The hP2X7-2Nβ is shown as a black dotted line. (d) The pIC₅₀ values of AZ11645373 at the F103A, M105A, F108C, F108Y, K110Y and T111S. A three fold change from hP2X7-2NβR is indicated by the dotted line. Stars indicate a significant shift in IC₅₀ from hP2X7-2NβR. One way analysis of variance (ANOVA) test was used followed by Dunnett's test. *, p < 0.05; ****, p < 0.0001; (n= 3-5). Data are presented as mean ± S.E.M.
Figure 4.11. AZ11645373 inhibition at the 112-118 point mutations. (a) Amino acid sequence line up of the hP2X7R (top) and hP2X1R (bottom) of the region 112-118. The conserved amino acids are shown in black and variant amino acids are shown in grey. (b) Top of view of the 112-118 (yellow) chimera in one subunit. The allosteric binding site in the hP2X7R is shown in black cartoon representation. The right hand panel shows a sphere and stick representation at the 112-118 chimera. Residues where mutation decreased AZ11645373 sensitivity are shown in yellow and residues where mutation had no effect on AZ11645373 sensitivity are shown in grey. (c) AZ11645373 inhibition curves at the E112C, G113C, E115C and Q116C. The hP2X7-2Nβ is shown as a black dotted line. The pIC₅₀ values of AZ11645373 at the E112C, G113C, E115C, Q116C and R117C. A three fold change from hP2X7-2NβR is indicated by the dotted line. Stars indicate a significant shift in IC₅₀ from hP2X7-2NβR. One way analysis of variance (ANOVA) test was used followed by Dunnett’s test. *, p < 0.05; **, p < 0.01; ****, p < 0.0001; (n= 3-5). Data are presented as mean ± S.E.M.
Figure 4.12. AZ11645373 inhibition at the 295-310 point mutations. (a) Amino acid sequence line up of the hP2X7R (top) and hP2X1R (bottom) of the region 295-310. The conserved amino acids are shown in black and variant amino acids are shown in grey. (b) Top of view of the 295-310 (magenta) chimera in one subunit. The allosteric binding site in the hP2X7R is shown in black cartoon representation. The right hand panel shows sphere and stick representation at the 295-310 chimera. Residues where mutation decreased AZ11645373 sensitivity are shown in magenta and residues where mutation had no effect on AZ11645373 sensitivity are shown in grey. (c) AZ11645373 inhibition curves at the Y295F, I310A, T308A and V312A. The hP2X7-2Nβ is shown as a black dotted line. (d) The pIC50 values of AZ11645373 at the 295-310 point mutations. A three fold change from hP2X7-2Nβ is indicated by the dotted line. Stars indicate a significant shift in IC50 from hP2X7-2NβR. One way analysis of variance (ANOVA) test was used followed by Dunnett's test. ****, p < 0.0001; (n= 3-5). Data are presented as mean ± S.E.M.
All point mutations that were tested on AZ11645373 sensitivity are shown in table 4.2.

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**Table 4.2. ATP and AZ11645373 sensitivity on the point mutations around the allosteric antagonist binding site.** The table shows the pEC<sub>50</sub> values of ATP (Allsopp et al., 2017), pIC<sub>50</sub> values of AZ11645373 and fold shift relative to the hP2X7-2NβR. Stars indicate a significant shift in EC<sub>50</sub> or IC<sub>50</sub> from the hP2X7-2NβR. **, p < 0.01; ***, p < 0.001; ****, p < 0.0001. Arrows indicate an increasing ATP/AZ11645373 sensitivity. Data are shown as mean ± S.E.M (n ≥ 3).
4.3. Discussion

The aim of this chapter was to map the binding site of AZ11645373. This was done by mutagenesis and complements with molecular docking undertaken by Dr. Ralf Schmid, which is discussed in more detail below.

4.3.1. Molecular docking of AZ11645373

The molecular docking of AZ11645373 into the hP2X7R was generated by Dr. Ralf Schmid. Both stereoisomers of AZ11645373 act as the hP2X7R inhibitor and because it is not clear if the R or S stereoisomer, both isomers were used in molecular docking. It was seen that AZ11645373 prefers an allosteric binding site over binding to the orthosteric site consistent with the effects of mutations on antagonist sensitivity (table 4.3).

In the representative docking pose for the major cluster of AZ11645373(S) sites the thiazolidinedione and nicotine groups of AZ11645373 deep in the allosteric pocket whereas the aromatic nitro group of AZ11645373 sites at the entrance of the pocket. This pose suggested that the side chain of lysine at position 110 (K110) contributed in hydrogen bonding to the nitro (NO$_2$) group of AZ11645373. Residues where mutation has the greatest effects on AZ11645373 affinity are consistent with proposed binding pose, demonstrating the hydrophobic environment for the AZ11645373. The docking pose also suggested aromatic interactions between the AZ11645373 nitrobenzene group and phenylalanine at position 88 (F88) and between the AZ11645373 nicotine group and phenylalanine at positions 95 (F95) and (F103) (figure 4.13). Most of effects of point mutations on AZ11645373 can be explained in the context of the proposed binding pose (except L97P mutant). This mutation may induce indirect effect on the shape of the allosteric pocket.

<table>
<thead>
<tr>
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<th>Rosetta interface scores (mean ± S.E.M)</th>
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</tr>
</thead>
<tbody>
<tr>
<td>AZ11645373(R) allosteric</td>
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</tr>
<tr>
<td>AZ11645373(R) decoy</td>
<td>-13.70 ± 1.10</td>
<td>543</td>
</tr>
<tr>
<td>AZ11645373(S) allosteric</td>
<td>-16.70 ± 0.80</td>
<td>211</td>
</tr>
<tr>
<td>AZ11645373(S) decoy</td>
<td>-13.60 ± 1.10</td>
<td>407</td>
</tr>
</tbody>
</table>

Table 4.3. Rosetta interface scores for ligand docking. (Data from Dr. Ralf Schmid).
Figure 4.13. Representative binding pose for AZ11645373 in the hP2X7R. (a) View from the top of the extracellular domains along the central axis perpendicular to the membrane. The P2X7R model is shown as cartoon with the three subunits highlighted in light blue, light pink and grey; AZ11645373 is shown as spheres. (b) As in (a), but 90 degrees rotated. (c) Zoom into the proposed AZ11645373 binding site, one subunit (light blue in a and b) is omitted for clarity. Residues K110 (blue - increase), I310, F88, V312, D92, A91, T94, F103, F95, P96, L97 (red – decrease or no inhibition) where mutations showed the most significant effects on AZ11645373 are shown as sticks.
4.3.2. Allosteric binding model for AZ11645373

The combination of mutagenesis and molecular docking provides a validated model that AZ11645373 binds at the base region of the allosteric pocket, consistent with antagonist-bound pdP2X7R crystal structures (Karasawa and Kawate, 2016). The allosteric binding mode of AZ11645373 suggests that this P2X7R antagonist acts to stop the movement or closure of the allosteric pocket which is associated with movement required for P2X7R channel opening (see section 1.9.2). This mode of action is in agreement with other allosteric inhibitors (Karasawa and Kawate, 2016, Allsopp et al., 2017, Allsopp et al., 2018).

However, this finding differs from that proposed by Caseley et al. (2015) who used molecular docking centred on F95 to predict AZ11645373 binding in the upper vestibule of the extracellular region of the receptor nearby the orthosteric site with several residues including Y295, Y299, K297 and K300 (Caseley et al., 2015). Caseley and co-workers did not test their model extensively and the results of the current study do not support it. It is notable that residue F95 is present in both allosteric pocket and upper vestibule models. However, it seems likely that AZ11645373 binds in the allosteric pocket rather than the upper vestibule model. As our results showed twenty mutants in the allosteric pocket alter AZ11645373 sensitivity (figure 4.14). The allosteric site of AZ11645373 was confirmed by molecular docking which showed ten of these mutants are directly involved in AZ11645373 binding (figure 4.13). These mutants in the allosteric pocket can be classified into residues lining the allosteric pocket that are likely to have direct effect with AZ11645373 and residues facing away from the allosteric pocket that are most probably to have an indirect effect on the shape of the allosteric pocket (figure 4.14 a & b).
Figure 4.14. Effects of point mutations in the allosteric pocket on AZ11645373 sensitivity. (a) Individual point mutation of residues lining the allosteric pocket with 3-10 fold change in AZ11645373 sensitivity are shown in orange, 10-100 fold change in green and > 100 fold change in blue (same colour coding in the panel b and c). (b) Individual point mutation of residues that are not directly facing the allosteric pocket. (c) The fold change in AZ11645373 sensitivity. A three fold change from hP2X7-2NβR is indicated by the dotted line. One way analysis of variance (ANOVA) test was used followed by Dunnett's test. Data are presented as mean ± S.E.M (n= 3-5).
(i) Residues lining the allosteric pocket

The current study found that point mutations of aromatic residue at F88, F95, F103 and F108 significantly reduced AZ11645373 sensitivity. Interestingly, molecular docking showed aromatic interactions between AZ11645373 nitrobenzene group and F88 and between AZ11645373 nicotine group and F95 and F103. These aromatic interactions cannot occur at F88A, F95A and F103A mutants that may explain decreased AZ11645373 sensitivity (figure 4.13c and 4.14a). A similar result was seen for residues at the base of the pocket where decrease in side chain size reduced AZ11645373 sensitivity (e.g. I310A and V312A), probably by reducing the pocket size. Compared with other allosteric inhibitors, three of these mutations (F88A, F103A and V312A) significantly reduced AZ10606120, A-70003 and A-438079 sensitivity (figure 4.15) (table 4.4) (Allsopp et al., 2017, Allsopp et al., 2018). It can thus be suggested that residues F88, F103 and V312 are common features of the P2X7R allosteric binding sites.

Another important finding was that K110Y mutant at the entrance of the pocket increased AZ11645373 sensitivity. Molecular docking showed that lysine residue at position 110 (K110) is contributing to hydrogen bonding to the nitro group of AZ11645373. More hydrogen bonding by tyrosine hydroxyl group in the K110Y mutant could interpret increased AZ11645373 sensitivity. Compared with other allosteric inhibitors, this mutant also produced a more than 10 fold increase in AZ10606120 sensitivity but had no effect on the sensitivity to A-70003 and A-438079 (Allsopp et al., 2017, Allsopp et al., 2018). It seems likely that binding pose of AZ11645373 similar to AZ10606120 expands the entrance of the allosteric pocket. This contrasts to that A-70003 and A-438079 which bind deeper in the allosteric pocket (Allsopp et al., 2018).

(ii) Residues facing away from the allosteric pocket

Point mutations of the unique threonine residues at position 90 (T90V) and position 94 (T94V) have significant effects on the AZ11645373 action (>10 fold). It is interesting to note that the facing of these residues are away from the allosteric pocket. This means that T90 and T94 do not have direct interactions with AZ11645373. It is possible therefore that their mutations have structural effects on the integrity of the allosteric pocket. This finding was also reported by Allsopp et al. (2017) and (2018) who found that T90V and T94V mutations produced the greatest decrease in sensitivity for
AZ10606120, A-70003 and A-438079 (table 4.4) (figure 4.15) (Allsopp et al., 2017, Allsopp et al., 2018). Molecular dynamic stimulations of WT-hP2X7R showed that T94 forms persistent hydrogen bond with the carbonyl group of T90 and T90 interacts with the hydroxyl group of Y93. These interactions cannot occur in the T90V and T94V mutations. This is due to the short α-helix being less persistent in T90V and T94V stimulations (~30%) compared to the WT-hP2X7R stimulations (~60%) (Allsopp et al., 2017). It seems likely that reducing the dynamic effect of the short α-helix is associated with a reduction of the allosteric pocket volume and that could affect sensitivity of AZ11645373.

One interesting find is that the A91C mutant decreased AZ11645373 sensitivity ~17 fold. A possible explanation for this effect might be that the cysteine side-chain has a longer volume than the alanine side-chain (small methyl group) or the cysteine side-chain is more polar than the alanine side-chain. This mutant had also a significant effect at other P2X7R antagonists. It was reported that A91C produced (5-30 fold) decrease in sensitivity for AZ10606120, A-70003 and A-438079 (figure 4.15) (Allsopp et al., 2017, Allsopp et al., 2018).

Another important finding was that D92A mutant decreased AZ11645373 sensitivity ~30 fold. This mutant had also a greatest effect on other P2XR antagonists. Allsopp et al. (2017) and (2018) reported that D92A mutant produced a more than 100 fold decrease in sensitivity for AZ10606120, A-740003 and A-438079 (figure 4.15) (Allsopp et al., 2017, Allsopp et al., 2018). Molecular dynamic stimulations of WT-hP2X7R showed that D92 interacts with Y298. This interaction stabilized the β-strand that lines the allosteric pocket and that cannot occur in D92A mutant (Allsopp et al., 2018). It seems likely that the destabilization of the allosteric pocket by D92A mutants is associated with decreased antagonist sensitivity.

This study also found that point mutation of proline residue at position 96 had a large effect (P96A) on AZ11645373 sensitivity (>1000 fold). This result may be explained by the fact that proline is rigid structure and removing this rigidity could interpret decreased AZ11645373 sensitivity. However this finding differs from that reported by Allsopp et al. (2017) who found that P96A mutant had no effect on AZ10606120 sensitivity (figure 4.15) (Allsopp et al., 2017). This indicates that each allosteric inhibitor has specific binding sites.
On the other hand, there are several point mutants that do not directly bind the AZ11645373. These mutants alter AZ11645373 sensitivity possibly by changing the structure of the allosteric pocket. For instance, K81P and H85P mutations at the entrance of the allosteric pocket reduced AZ11645373 sensitivity ~ 4 fold. Restricting pocket access by proline rigid structure may explain this effect. This finding was also reported by Allsopp et al. (2018) who found that H85P mutation produced a modest decrease in sensitivity for A-70003 ~ 5 fold (Allsopp et al., 2018). A similar result was seen for S86Q mutant at the entrance of the allosteric pocket where an increase the size of the side-chain reduced AZ11645373 sensitivity, probably by reducing pocket access. This also accords with that reported by Allsopp et al. (2017) who found that S86Q mutant reduced AZ10606120 sensitivity by ~ 9 fold (Allsopp et al., 2017). Other residues in the allosteric pocket where a decrease in side-chain size (e.g. G113C, E115C and Q116C) reduced AZ11645373 sensitivity, probably by reduce allosteric pocket size.

According to these data, we can conclude that there are common features of the P2X7R allosteric binding sites. Compared the effects of mutations of the residues around the allosteric pocket of AZ11645373 with AZ10606120, A-70003 and A-38079 shows a consistent decrease in sensitivity at mutations F88A, T90V, D92A, F103A and V312A. These mutations could be used as a “signature” to identify binding site for other P2X7R antagonists (see chapter 6). However, it is important to note that the allosteric model of AZ11645373 binding sites is not fully consistent with other allosteric inhibitors. It is observed that there are variations between the effects of point mutants (e.g. M105A, K110Y, K297G and I310A) (figure 4.15). This observation may support the idea that each allosteric inhibitor has specific binding sites.

In summary, mutagenesis studies in this chapter mapped the binding site of the P2X7R selective antagonist AZ11645373 at the base region of the allosteric pocket with several hydrophobic residues. The allosteric model of AZ11645373 binding sites in the hP2X7R was confirmed by molecular docking. One of the interesting features of AZ11645373 is that it shows species selectivity at P2X7Rs and is > 500 fold less effective at the rat isoform (Stokes et al., 2006). If the model of AZ11645373 binding is correct, the amino acid variations between the rat and human P2X7Rs must determine the difference in antagonist sensitivity. Our data showed five variants residues between the human and rat P2X7R around the allosteric pocket at positions K81, S86, F95, F108 and V312 that may underline the variation in AZ11645373 sensitivity. To validate the
The allosteric model of AZ11645373 binding and the effects of these variations were systematically determined, see chapter 5. Our results also demonstrate five point mutations (F88A, T90A, D92A, F103A and V312A) on the hP2X7R that can be used as a signature for allosteric inhibitors. In chapter 6, these five point mutations have been used to identify the binding site of KN-62, ZINC58368839, Brilliant Blue G and Calmidazolium.

<table>
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<tr>
<th></th>
<th>AZ10606120 pIC50 ± S.E.M</th>
<th>Shift</th>
<th>AZ10606120 pIC50 ± S.E.M</th>
<th>Shift</th>
<th>AZ10606120 pIC50 ± S.E.M</th>
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<td>2Nβ</td>
<td>8.12 ± 0.06</td>
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<td>7.13 ± 0.05</td>
<td>-</td>
<td>6.03 ± 0.06</td>
<td>-</td>
</tr>
<tr>
<td>K81P</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
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<td>L83A</td>
<td>9.10 ± 0.18****</td>
<td>←9.5</td>
<td>6.54 ± 0.02****</td>
<td>3.6</td>
<td>6.08 ± 0.05</td>
<td>1.0</td>
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<td>H85P</td>
<td>7.87 ± 0.01</td>
<td>1.8</td>
<td>6.43 ± 0.02****</td>
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<td>5.95 ± 0.02</td>
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<td>S86Q</td>
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<td>-</td>
<td>-</td>
<td>-</td>
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<td>1.6</td>
<td>7.10 ± 0.03</td>
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<td>6.00 ± 0.06</td>
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<td>F88A</td>
<td>6.74 ± 0.08****</td>
<td>24.0</td>
<td>5.30 ± 0.03****</td>
<td>67.6</td>
<td>Insensitive****</td>
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<td>13.2</td>
<td>6.35 ± 0.04****</td>
<td>6.0</td>
<td>5.33 ± 0.01****</td>
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<td>A91C</td>
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<td>5.58 ± 0.03****</td>
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<td>4.68 ± 0.02****</td>
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<td>D92A</td>
<td>5.27 ± 0.04****</td>
<td>708</td>
<td>5.13 ± 0.02****</td>
<td>100.4</td>
<td>Insensitive****</td>
<td>&gt;1000</td>
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<td>Y93A</td>
<td>7.53 ± 0.02****</td>
<td>3.9</td>
<td>7.05 ± 0.05</td>
<td>1.2</td>
<td>5.42 ± 0.10****</td>
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<tr>
<td>T94V</td>
<td>6.89 ± 0.08****</td>
<td>17.0</td>
<td>6.41 ± 0.03****</td>
<td>5.3</td>
<td>Insensitive****</td>
<td>&gt;1000</td>
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<tr>
<td>F95A</td>
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<td>←2.3</td>
<td>6.03 ± 0.05****</td>
<td>12.5</td>
<td>Insensitive****</td>
<td>&gt;1000</td>
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<td>P96A</td>
<td>7.86 ± 0.08</td>
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<td>6.48 ± 0.06****</td>
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<td>L97P</td>
<td>7.64 ± 0.07****</td>
<td>3.0</td>
<td>6.43 ± 0.02****</td>
<td>5.0</td>
<td>6.85 ± 0.01****</td>
<td>←6.6</td>
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<tr>
<td>F103A</td>
<td>6.51 ± 0.02****</td>
<td>40.7</td>
<td>6.19 ± 0.02****</td>
<td>8.7</td>
<td>Insensitive****</td>
<td>&gt;1000</td>
</tr>
<tr>
<td>M105A</td>
<td>6.12 ± 0.02****</td>
<td>100</td>
<td>4.87 ± 0.1****</td>
<td>180</td>
<td>5.77 ± 0.09</td>
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<td>F108C</td>
<td>7.78 ± 0.06</td>
<td>2.2</td>
<td>6.00 ± 0.20****</td>
<td>13.5</td>
<td>4.98 ± 0.06**</td>
<td>11.0</td>
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<td>K110Y</td>
<td>Insensitive****</td>
<td>&gt;10</td>
<td>7.02 ± 0.06</td>
<td>1.3</td>
<td>6.10 ± 0.04</td>
<td>←1.2</td>
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<td>Y295A</td>
<td>8.74 ± 0.08****</td>
<td>←4.2</td>
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<td>2.1</td>
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<td>2.3</td>
</tr>
<tr>
<td>K297G</td>
<td>Insensitive****</td>
<td>&gt;1000</td>
<td>7.87 ± 0.08**</td>
<td>←5.0</td>
<td>7.30 ± 0.05****</td>
<td>←18.9</td>
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<td>Y298A</td>
<td>7.88 ± 0.07</td>
<td>1.7</td>
<td>5.68 ± 0.30****</td>
<td>28.0</td>
<td>5.18 ± 0.12*</td>
<td>7.0</td>
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<tr>
<td>Y299C</td>
<td>8.10 ± 0.06</td>
<td>1.0</td>
<td>6.97 ± 0.13</td>
<td>1.4</td>
<td>6.60 ± 0.38</td>
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<td>V304C</td>
<td>8.54 ± 0.03</td>
<td>←2.6</td>
<td>6.95 ± 0.02</td>
<td>1.5</td>
<td>7.15 ± 0.16****</td>
<td>←13.4</td>
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<tr>
<td>E305A</td>
<td>8.69 ± 0.04</td>
<td>←3.7</td>
<td>7.58 ± 0.06</td>
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<td>6.67 ± 0.09</td>
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<tr>
<td>K306C</td>
<td>8.30 ± 0.07</td>
<td>←1.5</td>
<td>6.50 ± 0.03*</td>
<td>4.3</td>
<td>5.80 ± 0.08</td>
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Table 4.4. AZ10606120, A-740003 and A-38079 sensitivity on the point mutations around the allosteric antagonist binding site. The table shows the pIC\(_{50}\) values of AZ10606120, A-740003 and A-38079 and fold shift relative to the hP2X7-2N\(\beta\)R. Stars indicate a significant shift in IC\(_{50}\) from the hP2X7-2N\(\beta\)R. *, p < 0.05; **, p < 0.01; ***, p < 0.001; ****, p < 0.0001. Arrows indicate an increasing sensitivity. Data are shown as mean ± S.E.M (n ≥ 3). (These data are taken from Allsopp et al. (2017) and (2018)).

<table>
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<tr>
<th>Mutations</th>
<th>pIC(_{50}) (Mean ± S.E.M)</th>
<th>Fold Shift</th>
<th>pIC(_{50}) (Mean ± S.E.M)</th>
<th>Fold Shift</th>
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<tbody>
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<td>T308C</td>
<td>8.95 ± 0.02</td>
<td>← 6.8</td>
<td>6.95 ± 0.04</td>
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<tr>
<td>I310A</td>
<td>8.61 ± 0.04****</td>
<td>← 3.0</td>
<td>6.91 ± 0.07</td>
<td>1.7</td>
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<tr>
<td>V312A</td>
<td>7.73 ± 0.05**</td>
<td>2.5</td>
<td>6.42 ± 0.08**</td>
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Figure 4.15. Comparison of effects of point mutations in the allosteric pocket on the sensitivity to antagonist AZ11645373, AZ10606120, A-740003 and A-438079. (a) The fold change in antagonist sensitivity for four P2X7R antagonist. A three fold change from hP2X7-2NβR is indicated by the dotted line. Data for AZ10606120, A-740003 and A-438079 taken from Allsopp et al., 2017 and Allsopp et al., 2018.
Chapter 5: Using rat to human point mutations to test the model of AZ11645373 binding site

5.1. Introduction

Several P2X7R antagonists including AZ11645373 show differences in sensitivity between species (human versus rat). It was reported that AZ11645373 is a highly potent antagonist at the hP2X7R (IC$_{50}$ ~ 90 nM). However, it is more than 500 fold less effective at the rP2X7R (less than 50% inhibition at 10 µM) (Stokes et al., 2006). In order to identify amino acids responsible for species differences in antagonist effects, Michel et al. (2009) generated individual point mutants between the human and rat P2X7Rs. They identified that the human to rat point mutation at position 95, which mutated phenylalanine to leucine (F95L) in the hP2X7R, reduced AZ11645373 sensitivity by ~ 10 fold, indicating that the variation at position 95 contributed to species differences (Michel et al., 2009). However, Michel et al. (2009) did not test all variant residues between the human and rat P2X7Rs and other residues are also likely to contribute to the remaining 50 fold difference in sensitivity.

It is important to note that inducing sensitivity at a receptor (i.e. gain of function) is more difficult than reducing sensitivity of the receptor. This is clearly seen for the KN-62 antagonist, which is effective at the hP2X7R (1 µM inhibited response by more than 90%) but had no effect at the rP2X7R up to 10 µM (Michel et al., 2008). It was reported that the human to rat point mutation at position 95 (F95L) reduced KN-62 sensitivity by ~ 3 fold, but the rat to human point mutation at position 95 (L95F) did not increase KN-62 sensitivity (Michel et al., 2008).

5.1.1. Chapter Aims

The use of point mutations at the hP2X7R in chapter 4 allowed a model of the allosteric AZ11645373 binding site to be proposed. If this model is correct, the amino acid variations between the human and rat P2X7Rs around the allosteric pocket should determine the difference in AZ11645373 sensitivity. The results from point mutations at the hP2X7R in chapter 4 highlighted several residues in the allosteric pocket contributing to AZ11645373 sensitivity. Five of these residues at positions 81, 86, 95, 108 and 312 are variant between the human and rat P2X7Rs (figure 5.1). To study further their
contribution to AZ11645373 sensitivity we generated individual point mutations of these residues at the rP2X7R, replacing residues of the rP2X7R (insensitive to AZ11645373) with the corresponding residues of the hP2X7R (sensitive to AZ11645373). Replacing these residues can be used to “humanize” the rP2X7R and potentially rescue AZ11645373 sensitivity. In addition, human to rat point mutations were generated. Results from these studies will be useful to further validate the allosteric model of AZ11645373 binding site.

5.2. Results

5.2.1. Characterization of ATP currents at the rat P2X7 receptor

The ATP potency at the WT rP2X7R was determined. Responses to ATP applications at the rP2X7R were reproducible. After reproducible responses to a maximal concentration of ATP (1 mM), lower concentrations of ATP (10-300 µM) were applied to generate concentration response curve.

ATP evoked concentration dependent inward currents at the rP2X7R. The peak amplitude to a maximal concentration of ATP (1 mM) was 4294 ± 253.87 nA (figure 5.2a). The response had a pEC₅₀ value of 4.05 ± 0.01 (EC₅₀ ~ 90 µM) in divalent free solution with a Hill slope 1.23 ± 0.01 (figure 5.2b).
Figure 5.1. Location of amino acid differences between human and rat P2X7 receptors. (a) Amino acid sequence line up in the extracellular region of the hP2XR and rP2XR. Black residues are conserved amino acids, grey residues are variant amino acids and red residues are variant amino acids in the allosteric pocket (at positions 81, 86, 95, 108 and 312). (b) Homology model of the differences between hP2X7R and rP2X7R. Conserved residues are shown in black, variant residues are shown in grey and variation residues in the allosteric pocket are shown in red. The right hand panel shows the top of view.
Figure 5.2. Concentration dependence of ATP evoked currents at the wild type rat P2X7 receptor. (a) Representative traces showing the effect of different ATP concentrations (µM) on the rP2X7R expressed in Xenopus oocytes. 3-5 minutes was given between each application. Black bar indicates 10 seconds of ATP application. (b) Concentration response curves for the rP2X7R, pEC₅₀ value is given (n > 3). The red dotted line corresponds to the EC₉₀ concentration of ATP used for antagonist studies. Data are shown as mean ± S.E.M.
5.2.2. The effect of AZ11645373 at the rat P2X7 receptor

The effect of AZ11645373 at the rP2X7R was tested. Co-application of AZ11645373 (1-1000 nM) with an EC$_{90}$ concentration of ATP (300 µM) had no effect on ATP evoked currents, showing the rP2X7R is insensitive to this antagonist (figure 5.3a & b). The lack effect of rP2X7R confirmed the previous study of Stokes et al. (2006).

5.2.3. The effect of AZ11645373 at the rat/human P2X7 receptor

In contrast to the hP2X7R, AZ11645373 is ineffective at the rP2X7R. Comparing the amino acids sequence between the two species, there are five variant residues in the allosteric pocket contributed in AZ11645373 sensitivity on the hP2X7R. These residues are located at positions 81, 86, 95, 108 and 312. In order to test whether rP2X7Rs can be made more sensitive to AZ11645373, rat to human individual point mutations were generated (T81K, G86S, L95F, Y108F and A312V) (figure 5.1a).

Firstly, we characterized the effect of ATP at the rat/human P2X7R mutants. Responses to ATP applications at these mutations were not reproducible. Therefore the first response to an ATP application at different concentrations were recorded and then mean amplitudes analysed from (n > 8) oocytes to generate concentration response curves. Compared to the WT rP2X7R (pEC$_{50}$ = 4.05 ± 0.03), the T81K and G86S mutations had no effect on ATP potency, pEC$_{50}$ = 3.45 ± 0.01 and 4.01 ± 0.08, respectively. The L95F, Y108F and A312V showed an approximately 5-10 fold reduction in ATP sensitivity, with pEC$_{50}$ values 3.30 ± 0.07, p < 0.05, 3.13 ± 0.13, p < 0.01 and 3.08 ± 0.15, p < 0.01, respectively (figure 5.4).

The effect of AZ11645373 on the rat/human P2X7R point mutation was tested. To do this, the first response to co-application of EC$_{90}$ concentration of ATP (300 µM) with AZ11645373 at different concentrations (1-1000 nM) were recorded and then mean amplitudes analysed from (n > 8) oocytes to generate AZ11645373 inhibition curves. AZ11645373 was still ineffective at T81K and G86S rat P2X7R mutants. The point mutations at L95F and A312V resulted in AZ11645373 sensitivity equivalent to the hP2X7-2NßR, pIC$_{50}$ = 7.52 ± 0.02 and 7.5 ± 0.18, respectively. The Y108F mutant partially rescued AZ11645373 sensitivity, pIC$_{50}$ = 6.1 ± 0.1, p < 0.0001 (23 fold less than
hP2X7-2NβR) (figure 5.5). This indicates that AZ11645373 affinity is increased by replacing residues of the rat P2X7R at positions 95, 108 and 312.

5.2.4. The effect of AZ11645373 at the human/rat P2X7 receptor

In reciprocal studies we generated human to rat individual point mutations (K81T, S86G, F95L, F108Y and V312A) on the hP2X7-2NβR background. Any effect on ATP sensitivity at the human to rat P2X7R mutants was determined. Compared to the hP2X7-2NβR (pEC$_{50}$ = 4.63 ± 0.07), the K81T, S86G, F108Y and V312A mutants had no effect on ATP sensitivity, pEC$_{50}$ = 4.6 ± 0.01, 4.7 ± 0.1, 4.6 ± 0.2 and 4.4 ± 0.03, respectively. The phenylalanine to leucine mutation at position 95 (F95L) decreased ATP sensitivity approximately 5 fold, pEC$_{50}$ = 3.9 ± 0.03, p < 0.0001 (figure 5.6).

The effect of AZ11645373 at the human/rat P2X7R point mutations was tested. The point mutations at the K81T, S86G and F108Y had little effect on AZ11645373 sensitivity, pIC$_{50}$ = 6.8 ± 0.03, 6.9 ± 0.2 and 7.1 ± 0.1, respectively. In contrast, AZ11645373 sensitivity was decreased approximately 250 fold at the F95L mutant (pIC$_{50}$ = 5.1 ± 0.5, p < 0.0001) and at the V312A mutant there was approximately 20 fold decrease in sensitivity (pIC$_{50}$ = 6.2 ± 0.01, p < 0.001) (figure 5.7) (table 5.1). The reciprocal studies confirm that F95 and V312 are important for AZ11645373 antagonist binding.
Figure 5.3. Lack of AZ11645373 inhibition at the wild type rat P2X7 receptor. (a) Representative traces showing the effect of AZ11645373 (1 µM) on currents evoked by an EC₉₀ concentration of ATP in *Xenopus* oocytes expressing WT rP2X7R. 3 minutes was given between each application. Black bar indicates 10 seconds of ATP application and ATP co-application with AZ11645373 following 3 minutes pre-incubation with the indicated concentration of compound. (b) AZ11645373 inhibition curve at an EC₉₀ of ATP. Data are shown as mean ± S.E.M (n > 3).
Figure 5.4. ATP sensitivity at the rat/human point mutations (T81K, G86S, L95F, Y108F and A312V). (a) Histogram showing the peak amplitude (µA) of the first response to ATP application at different concentrations (µM) on L95F mutant. (b) ATP sensitivity at L95F point mutation. The rP2X7R is shown as a black dotted line. (c) The pEC$_{50}$ values of ATP at the T81K, G86S, L95F, Y108F and A312V point mutations. Any significant differences from the rP2X7R were determined by one-way ANOVA test followed by Dunnett's test. Stars indicate a significant difference from the rP2X7R. *, p < 0.05; **, p < 0.01. Data are represented as mean ± S.E.M (n > 8).
Figure 5.5. The effect of AZ11645373 at the rat/human P2X7 point mutations. (a) homology model of variant residues in allosteric pocket between rat and human P2X7 receptors. The right hand panel shows residues which have effect on AZ11645373 sensitivity (L95F, Y108F and A312V) shown as stick red representation. (b) AZ11645373 inhibition curves at rat to human point mutations. The hP2X7-2Nβ is shown as black dotted line. The rP2X7R is shown as black line. Stars indicate a significant shift in IC₅₀ from hP2X7-2Nβ receptor. One way analysis of variance (ANOVA) test was used followed by Dunnett’s test. ****, p < 0.0001; (n > 8 ). Data are presented as mean ± S.E.M.
Figure 5.6. ATP sensitivity at the human/rat point mutations (K81T, S86G, F95L, F108Y and V312A). (a) homology model of variant residues in the allosteric pocket between hP2X7 and rP2X7R (red colour). (b) ATP sensitivity at F95L point mutation. The hP2X7-2NβR is shown as a black dotted line. (c) The pEC\textsubscript{50} values of ATP at the K81T, S86G, F95L, F108Y and V312A point mutations. Any significant differences from the hP2X7-2NβR were determined by one-way ANOVA test followed by Dunnett’s test. Stars indicate a significant difference from the hP2X7-2NβR. ****, \( p < 0.0001 \). Data are represented as mean ± S.E.M (\( n \geq 3 \) ).
Figure 5.7. Variant residues in the allosteric binding pocket contribute to AZ11645373 sensitivity. (a) homology model of variant residues in the allosteric pocket between hP2X7 and rP2X7R (red colour). The right hand panel shows that variant residues in red stick representation (81, 86, 95, 108, 312). (b) AZ11645373 inhibition curves at human to rat point mutations. The hP2X7-2Nβ is shown as black dotted line. The rP2X7R is shown as black line. Stars indicate a significant shift in IC₅₀ from hP2X7-2Nβ receptor. One way analysis of variance (ANOVA) test was used followed by Dunnett's test. ***, p < 0.001; ****, p < 0.0001; (n ≥ 3). Data are presented as mean ± S.E.M.
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<td>pIC(_{50}) ± S.E.M</td>
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Rat to human mutations

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Human to rat mutations

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Table 5.1. ATP and AZ11645373 sensitivity at the human/rat and rat/human point mutations. The table shows the pEC\(_{50}\) values of ATP, pIC\(_{50}\) values of AZ11645373 and fold shift relative to the hP2X7-2N\(\beta\)R. Stars indicate a significant shift in EC\(_{50}\) and IC\(_{50}\) from the hP2X7-2N\(\beta\)R. *, p < 0.05; **, p < 0.01; ***, p < 0.001; ****, p < 0.0001. Arrows indicate an increasing ATP/AZ11645373 sensitivity. Data are shown as mean ± S.E.M.
5.3. Discussion

Species differences between human and rat P2X7Rs (Stokes et al., 2006) were confirmed in the present study for the antagonist AZ11645373. The main aim of this chapter was to identify residues in the allosteric pocket that are responsible for the species variations in AZ11645373 sensitivity and further validate the model proposed in chapter 4. This was done by (i) rat to human point mutations, (ii) human to rat point mutations and (iii) molecular docking of AZ11645363 at the rP2X7R.

In the current study, there is a difference between the WT rP2X7R and rat to human point mutations. It was found that there was a lack of reproducible responses of the rP2X7R mutants in the *Xenopus laevis* oocytes expression system. The reason for this difference is unclear but it may suggest that mutations changed the gating properties of the receptor.

The present study showed that rat to human point mutations did not make the receptor ATP sensitivity equivalent to the hP2X7-2NβR. It was found that the sensitivity of rat to human point mutations (EC\textsubscript{50} \sim 100 to 800 µM) was 4-32 times less than the hP2X7-2NβR (EC\textsubscript{50} \sim 25 µM). These findings were expected as the hP2X7-2NβR is more sensitive than the WT hP2X7R. Allsopp and Evans (2015) reported that the hP2X7-2NβR produced an \sim 8 fold increase ATP sensitivity, compared to the WT hP2X7R (Allsopp and Evans, 2015). Therefore, as in this study used hP2X7-2NβR instead to the WT hP2X7R, it may not expect that ATP sensitivity at rat P2X7R mutations increased to the human construct sensitivity.

The present study also showed that some rat to human point mutations reduced ATP sensitivity. It was found that L95F, Y108F and A312V was \sim 6-9 times less than the WT rP2X7R. Although these mutations are located at the allosteric pocket away from orthosteric ATP binding site, these mutations decreased ATP sensitivity, possibly by reducing movement of the allosteric pocket that is associated with the channel opening upon ATP binding.

It is interesting to note that only the mutations at position 95 did not have reciprocal effects on ATP sensitivity. It was seen that both L95F and F95L mutants decreased ATP sensitivity \sim 5 fold. Position 95 is present at the base region of the allosteric pocket nearby the orthosteric site; therefore mutations at this position may change the conformation of both the allosteric and orthosteric pockets or may change the ability of movement in these regions associated with ligand binding.
There are five variant residues between the human and rat P2X7Rs in the allosteric pocket at positions 81, 86, 95, 108 and 312 that may underlie species differences in AZ11645373 sensitivity. Rat to human point mutation at these positions showed that AZ11645373 sensitivity could be increased by three “humanizing” residues at positions 95 (L95F), 108 (Y108F) and 312 (A312V). These results strongly support the allosteric model of AZ11645373 binding sites (figure 5.5a). The allosteric site of AZ11645373 was also confirmed by molecular docking where the rP2X7R binding poses have higher scores compared to the hP2X7R. The docking scores support that rat has lower potency than human P2X7Rs (table 5.2) (figure 5.8).

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<td>-15.40 ± 0.70</td>
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**Table 5.2. Rosetta interface scores for ligand docking.** (Data from Dr. Ralf Schmid).
Figure 5.8. Representative binding pose for AZ11645373 in the hP2X7R. (a) View from the top of the extracellular domains along the central axis perpendicular to the membrane. The P2X7R model is shown as cartoon with the three subunits highlighted in light blue, light pink and grey; AZ11645373 is shown as spheres. (b) As in (a), but 90 degrees rotated. (c) Zoom into the proposed AZ11645373 binding site, one subunit (light blue in a and b) is omitted for clarity. Residues K110 (blue - increase), I310, F88, V312, D92, A91, T94, F103, F95, P96, L97 (red – decrease or no inhibition) where mutations showed the most significant effects on AZ11645373 are shown as sticks (d) as in (c), F95, V312 and Y108, residues where P2X7R rat to human, and human to rat mutations affected AZ11645373 sensitivity are shown as spheres.
In the rP2X7R, molecular docking predicts that AZ11645373 sits deep at the base region of the allosteric pocket (figure 5.8d). This model showed that AZ11645373 does not have any direct interactions with residues at the entrance of the allosteric pocket including T81 and G86. Mutagenesis studies showed that T81K and G86S mutants had no effect on AZ11645373 sensitivity. These findings broadly support the molecular docking model. Other support was that L95F and A312V mutants increased AZ11645373 sensitivity to that equivalent to the hP2X7-2NβR. Furthermore, the Y108F mutant partially increased AZ11645373 sensitivity. Molecular docking showed that several residues lining the allosteric pocket including L95, Y108 and A312 are directly involved in AZ11645373 binding (figure 5.8d). Residues L95 and A312 are present more deeper than Y108 that may explain that L95F and A312 mutants produced more change in AZ11645373 sensitivity than Y108F mutant. These mutants alter AZ11645373 sensitivity possibly by interaction directly with AZ11645373 or changing the structure of the allosteric pocket. For instance, L95F mutant has aromatic interaction between phenylalanine at position 95 and nicotine group of AZ11645373. The aromatic interaction may also appear between phenylalanine at position 108 and nitro group of AZ11645373. These aromatic interactions may interpret increased AZ11645373 sensitivity at L95F and Y108F mutants. A similar result was seen for A312V mutant in the allosteric pocket where an increase the size of the side-chain increased AZ11645373 sensitivity, possibly by increased pocket size. Taken together, rat to human point mutations and molecular docking at the rP2X7R are strongly support the allosteric model of AZ11645373.

The allosteric model of AZ11645373 was further supported by reciprocal studies. One might have expected that each point mutation increased AZ11645373 sensitivity at the rP2X7R; the reciprocal mutant in the hP2X7R would abolish activity. However, this expectation is not in the case as our results showed only reciprocal mutants F95L and V312A reduced AZ11645373 sensitivity by ~250 fold and 20 fold respectively. This may suggest that species variations in AZ11645373 sensitivity are associated with the combined local environment. It may be that phenylalanine at position 95 and valine at position 312 have predominant inhibitory effects on AZ11645373. This means if F95 or V312 is present in receptor displays inhibition. For F95L mutant this finding is consistent with that of Michel et al. (2009) who reported that F95L mutant reduced AZ11645363 sensitivity ~10 fold (Michel et al., 2009). However, it is notable that F95L mutant in this study reduced AZ11645373 sensitivity more than Michel et al. (2009) study. The
difference in the value of AZ11645373 reduction may be related to using different type of experiments. The present study assessed AZ11645373 sensitivity at the F95L mutant by recording ATP induced currents but Michel et al. (2009) study was used ethidium accumulation experiments. Another possible explanation for this is that F95L mutant in this study form hP2X7-2NβR background instead to the WT hP2X7R. Interestingly, F95L mutant also produced a more than 3 fold decrease in KN-62 sensitivity (Michel et al., 2008). It is possible therefore that F95L mutant may have a more generalized effect on the integrity of the allosteric pocket.

In summary, this chapter provides further support for our proposed allosteric model of AZ11645373 binding by three lines of evidence (i) rat/human point mutations (ii) human/rat point mutations and (iii) molecular docking of AZ11645373 at the rP2X7R. Our work showed that the presence of F95 and V312 are important for AZ11645373 binding. This suggests that these residues might be used to investigate antagonist that acts at the allosteric site.
Chapter 6: Use of “signature” allosteric point mutants to study the site of action of other P2X7R antagonists

6.1. Introduction

6.1.1. The concept of “signature” mutants within the allosteric pocket for several P2X7R antagonists

The use of mutagenesis in combination with molecular docking at P2X7Rs in chapters 4 and 5 demonstrated that AZ11645373 binds at the same allosteric site as AZ10606120, A-740003 and A-438079 (Allsopp et al., 2017, Allsopp et al., 2018). Comparing the effects of allosteric point mutations on AZ11645373 with AZ10606120, A-740003 and A-438079 demonstrates that there is a similar reduction in sensitivity at the F88A, T90V, D92A, F103A and V312A mutations. These mutations may therefore represent a “signature” for other P2X7R antagonists that bind allosterically (figure 6.1).

The molecular basis of binding for several selective P2X7R antagonists including KN-62, ZINC58368839, Brilliant Blue G and Calmidazolium is unclear (see section 1.8). Therefore, the signature mutations might be useful to identify the mode of binding for these antagonists. Recently, it was shown that the non-selective P2XR antagonist PPADS binds at the orthosteric site (Huo et al., 2018). Therefore, PPADS can be used as a negative control for the allosteric signature point mutations.

6.1.2. Chapter Aims

In this chapter, “signature” mutants were used to identify whether the P2X7R antagonists KN-62, ZINC58368839, Brilliant Blue G and Calmidazolium act at the allosteric site. To do this it was first essential to characterize the effect of these antagonists at the hP2X7-2NβR and rP2X7R by measuring ATP induced currents in *Xenopus* oocytes. The antagonists were further characterized by measuring ethidium bromide uptake at the hP2X7R and rP2X7R expressed in HEK293 cells. Subsequently, the effects of these antagonists at defined mutants in the allosteric pocket were determined in electrophysiological studies.
Figure 6.1. “Signature” allosteric point mutants. (a) Homology model of the hP2X7R showing residues around the allosteric pocket which have significant effects on AZ11645373, AZ10606120, A438079 and A740003 sensitivity. (b) Residues F88, T90, D92, F103 and V312 is shown in one subunit.
6.2. Results

6.2.1. Characterization of ATP dye uptake at human and rat P2X7 receptors

The characterization of ATP currents at the hP2X7-2NβR and rP2X7R was presented in chapters 3 and 5. In this chapter, the effect of ATP on ethidium bromide uptake at the hP2X7R and rP2X7R was determined as many P2X7R antagonists have been studied using this assay.

6.2.1.1. Measurements of human and rat P2X7 receptors dye uptake

Previous studies demonstrated that P2X7R mediated dye uptake was not observed in *Xenopus* oocytes (Petrou et al., 1997). Therefore, to study effects on dye uptake, P2X7Rs were expressed in HEK293 cells and ATP induced ethidium bromide uptake recorded. It was also reported that ethidium bromide uptake at the hP2X7-2NβR was reduced by ~ 5 fold compared to the hP2X7R (Allsopp and Evans, 2015). Therefore, we used the hP2X7R for the dye uptake experiments.

Initially, we optimized the buffer solution. In sucrose solution (280 mM), ethidium bromide dye uptake at 20 minutes was raised at the hP2X7R and rP2X7R by ~ 4 to 5 fold compared to either sodium chloride (140 mM) or potassium chloride buffer (140 mM) (p < 0.0001), consistent to that reported previously (Jiang et al., 2013, Allsopp and Evans, 2015) (figure 6.2). Therefore, we used the sucrose buffer to optimize the response in the assay of P2X7R mediated dye uptake.

ATP evoked concentration dependent ethidium bromide uptake at the hP2X7R and rP2X7R. At the hP2X7R, the responses had a pEC$_{50}$ value of 4.03 ± 0.08 (EC$_{50}$ ~ 90 µM) in sucrose buffer with a Hill slope 1.3 ± 0.1 (figure 6.3 a & c). At the rP2X7R, the responses had a pEC$_{50}$ value of 3.95 ± 0.14 (EC$_{50}$ ~ 100 µM) in sucrose buffer with a Hill slope 1.2 ± 0.2, similar to that reported in chapter 5 (figure 6.3 b & c).
Figure 6.2. Sucrose buffer induces ethidium bromide dye uptake at the wild type human and rat P2X7 receptors. (a & b) Representative Flexstation responses showing ATP-evoked ethidium bromide (20 µM) dye uptake at the human (a) and rat (b) P2X7Rs for 20 minutes under various buffer conditions [sucrose (280 mM), NaCl (140 mM) and KCl (140 mM) buffers]. Arrow indicate the addition of ATP (1 mM) at 120 seconds. (c & d) Histogram showing the relative fluorescence units (RFU) change under various buffer conditions. A star indicate a significant change from the sucrose buffer. Data are shown as mean ± S.E.M. ****, p < 0.0001; (n=6).
Figure 6.3. Concentration dependence of ATP induced ethidium bromide uptake at the human P2X7 and rat P2X7 receptors. (a & b) Representative Flexstation responses showing the effect of different ATP concentrations (µM) at hP2X7R and rP2X7R expressed in HEK293 cells for 20 minutes. Arrow indicates the addition of ATP at 120 second. (c) Concentration response curves for the hP2X7R (black) and rP2X7R (blue), pEC₉₀ values are given (n = 6). The red dotted line corresponds to the EC₉₀ concentration of ATP used for antagonist studies. Data are shown as mean ± S.E.M (n ≥ 6).
6.2.2. Characterization of P2X7R antagonists at human and rat P2X receptors

The sensitivity of P2X7R antagonists was characterized at the hP2X7-2NβR and rP2X7R by measuring ATP induced currents in Xenopus oocytes and for comparison by measuring ATP evoked ethidium bromide uptake in HEK293 cells.

6.2.2.1. The effect of AZ11645373 at the human and rat P2X7 receptor

In HEK293 cells expressing the hP2X7R, co-application of AZ11645373 (0.001-1 µM) with an EC90 concentration of ATP (300 µM) inhibited agonist evoked ethidium bromide uptake with a pIC50 of 7.3 ± 0.14 (IC50 ~ 50 nM) in sucrose buffer and a Hill slope 1 ± 0.2. AZ11645373 inhibited ATP evoked currents in oocytes expressing the hP2X7-2NβR with similar affinity (pIC50 value 7.47 ±0.06) (figure 6.4 a, c & e). At the rP2X7R, AZ11645373 up to 10 µM had no effect on ATP evoked current or ethidium bromide uptake further confirming the insensitivity of the rP2X7R to this antagonist (figure 6.4 b, d & f).

6.2.2.2. The effect of PPADS at the human and rat P2X7 receptor

The effect of PPADS on ATP evoked currents and ethidium bromide uptake at the hP2X7R and rP2X7R was tested (figure 6.5). At the hP2X7-2NβR, co-application of PPADS (0.001-1 µM) with an EC90 concentration of ATP (100 µM) inhibited the agonist evoked current with a pIC50 of 6.68 ± 0.03 (IC50 ~ 200 nM) in divalent free solution and a Hill slope of 1.1 ± 0.2 (figure 6.5 a & e). This inhibition was slowly reversible (the % of remaining current at 30 minutes after washout of 1 µM PPADS was 12.93 ± 0.84%) (figure 6.6). PPADS was a more potent antagonist of ATP evoked ethidium bromide uptake in HEK293 cells expressing the hP2X7R, with a pIC50 of 7.45 ± 0.08; p < 0.001 (IC50 ~ 35 nM) in sucrose buffer and a Hill slope of 1.3 ± 0.08 (figure 6.5 c & e). At the rP2X7R, co-application of PPADS (0.01-1 µM) with an EC90 concentration of ATP (300 µM) inhibited the agonist evoked currents with a pIC50 of 6.4 ± 0.3 (IC50 ~ 400 nM) and a Hill slope of 0.7 ± 0.09 (figure 6.5 b & f). In HEK293 cells expressing the rP2X7R, co-application of PPADS (0.01-100 µM) with an EC90 concentration of ATP (1 mM) inhibited the agonist evoked ethidium bromide uptake with a pIC50 of 6.25 ± 0.1 (IC50 ~ 560 nM) in sucrose buffer and a Hill slope of 1.3 ± 0.3 (figure 6.5 d & f). These results show that for the rP2X7R the IC50 concentration for inhibition of current was not significantly different from the IC50 concentration for inhibition of ethidium bromide uptake.
Figure 6.4. AZ11645373 inhibition at human and rat P2X7 receptors. (a & b) Representative traces showing the effect of AZ11645373 on currents evoked by an EC₉₀ concentration of ATP in *Xenopus* oocytes expressing hP2X7-2NβR and rP2X7R. 5 minutes was given between each application. Bars indicate 10 seconds of ATP application and ATP co-application with AZ11645373 following 5 minutes pre-incubation with the indicated concentration of compound. (c & d) Representative Flexstation responses showing the effect of AZ11645373 on dye uptake induced by an EC₉₀ concentration of ATP in HEK293 cells expressing hP2X7R and rP2X7R for 20 minutes. Arrow indicates the addition of ATP at 120 second. (e & f) AZ11645373 inhibition curves for ATP induced currents (blue) and ethidium bromide uptake (red). pIC₅₀ was used to measure AZ11645373 potency. Data for currents are shown as mean ± S.E.M (n ≥ 3). Data for ethidium bromide uptake are shown as mean ± S.E.M (n ≥ 6).
Figure 6.5. PPADS inhibition at human and rat P2X7 receptors. (a & b) Representative traces showing the effect of PPADS on currents evoked by an EC₉₀ concentration of ATP in *Xenopus* oocytes expressing hP2X7-2NβR and rP2X7R. 5 minutes was given between each application. Bars indicate 10 seconds of ATP application and ATP co-application with PPADS following 5 minutes pre-incubation with the indicated concentration of compound. (c & d) Representative Flexstation responses showing the effect of PPADS on dye uptake induced by an EC₉₀ concentration of ATP in HEK293 cells expressing hP2X7R and rP2X7R for 20 minutes. Arrow indicates the addition of ATP at 120 second. (e & f) PPADS inhibition curves for ATP induced currents (blue) and ethidium bromide uptake (red). pIC₅₀ was used to measure PPADS potency. Unpaired student's t test was used.***, p < 0.001. Data for currents are shown as mean ± S.E.M (n ≥ 3). Data for ethidium bromide uptake are shown as mean ± S.E.M (n ≥ 6).
Figure 6.6. The inhibitory effect of PPADS on ATP evoked currents at the hP2X7-2Nβ receptor was not reversed by 30 minutes washout. (a) Representative traces showing the inhibitory effect of PPADS was not reversed by 30 minutes washout. 5 minutes was given between each application. Bars indicate 10 seconds of ATP application. ATP co-application with PPADS following 5 minutes pre-incubation. (b) % of remaining currents up to 30 minutes after washout of 1µM PPADS. Data are shown as mean ± S.E.M (n ≥ 3).
6.2.2.3. The effect of KN-62 at the human and rat P2X7 receptors

KN-62 inhibited ATP evoked currents in oocytes expressing the hP2X7-2NβR with a pIC\textsubscript{50} value of 7.66 ± 0.23 (IC\textsubscript{50} ~ 20 nM) in divalent free solution and a Hill slope of 0.35 ± 0.003 (figure 6.7 a & e). The inhibitory effect of KN-62 did not recover following 20 minutes washout at the hP2X7-2NβR (figure 6.8). Ethidium bromide uptake evoked by ATP (300 µM) was inhibited with a similar IC\textsubscript{50} value and a Hill slope of 0.86 ± 0.15 (figure 6.7 c & e). At the rP2X7R, KN-62 up to 1 µM had no effect on ATP evoked current and ethidium bromide uptake, showing the rP2X7R is insensitive to this antagonist (figure 6.7 b, d & f).

6.2.2.4. The effect of ZINC58368839 at the human and rat P2X7 receptors

ZINC58368839 inhibited currents evoked by 100 µM ATP in a concentration dependent manner at the hP2X7-2NβR, with a pIC\textsubscript{50} of 6.35 ± 0.17 (IC\textsubscript{50} ~ 450 nM) in divalent free solution and a Hill slope of 0.94 ± 0.3 (figure 6.9 a & e). This inhibition was reversible within 40 minutes of washout (figure 6.10). Co-application of ZINC58368839 (0.001-10 µM) with an EC\textsubscript{90} concentration of ATP (300 µM) inhibited ethidium bromide uptake at the hP2X7R in a concentration dependent manner, with a pIC\textsubscript{50} of 6.1 ± 0.12 (IC\textsubscript{50} ~ 800 nM) in sucrose buffer and a Hill slope of 0.6 ± 0.09 (figure 6.9 c & e). At the rP2X7R, co-application of ZINC58368839 (0.01-1 µM) with an EC\textsubscript{90} concentration of ATP had no effect on ATP evoked currents and ethidium bromide uptake, showing the rP2X7R is insensitive to this antagonist (figure 6.9 b, d & f). The lack effect of rP2X7R confirmed the previous study of Caseley et al. (2016).
Figure 6.7. KN-62 inhibition at human and rat P2X7 receptors. (a & b) Representative traces showing the effect of KN-62 on currents evoked by an EC₉₀ concentration of ATP in *Xenopus* oocytes expressing hP2X7-2NβR and rP2X7R. 5 minutes was given between each application. Bars indicate 10 seconds of ATP application and ATP co-application with KN-62 following 5 minutes pre-incubation. (c & d) Representative Flexstation responses showing the effect of KN-62 on dye uptake induced by an EC₉₀ concentration of ATP in HEK293 cells expressing hP2X7R and rP2X7R for 20 minutes. Arrow indicates the addition of ATP at 120 second. (e & f) KN-62 inhibition curves for ATP induced currents (blue) and ethidium bromide uptake (red). pIC₅₀ was used to measure KN-62 potency. Data for currents are shown as mean ± S.E.M (n ≥ 3). Data for ethidium bromide uptake are shown as mean ± S.E.M (n ≥ 6).
Figure 6.8. The inhibitory effect of KN-62 on ATP evoked currents at the hP2X7-2Nβ receptor was not reversed by 20 minutes washout. (a) Representative traces showing the inhibitory effect of AZ11645373 was not reversed by 20 minutes washout. 5 minutes was given between each application. Bars indicate 10 seconds of ATP application and ATP co-application with AZ11645373 following 5 minutes pre-incubation. (b) % of remaining currents up to 20 minutes after washout of 1µM AZ11645373. Data are shown as mean ± S.E.M (n ≥ 3).
Figure 6.9. ZINC58368839 inhibition at human and rat P2X7 receptors. (a & b) Representative traces showing the effect of ZINC58368839 on currents evoked by an EC₉₀ concentration of ATP in Xenopus oocytes expressing hP2X7-2NβR and rP2X7R. 5 minutes was given between each application. Bars indicate 10 seconds of ATP application and ATP co-application with ZINC58368839 following 5 minutes pre-incubation with the indicated concentration of compound. (c & d) Representative Flexstation responses showing the effect of ZINC58368839 on dye uptake induced by an EC₉₀ concentration of ATP in HEK293 cells expressing hP2X7R and rP2X7R for 20 minutes. Arrow indicates the addition of ATP at 120 seconds. (e & f) ZINC58368839 inhibition curves for ATP induced currents (blue) and ethidium bromide uptake (red). pIC₅₀ was used to measure ZINC58368839 potency. Data for currents are shown as mean ± S.E.M (n ≥ 3). Data for ethidium bromide uptake are shown as mean ± S.E.M (n ≥ 6).
Figure 6.10. The inhibitory effect of ZINC58368839 on ATP evoked currents at the hP2X7-2Nβ receptor was reversed by 40 minutes washout. (a) Representative traces showing the inhibitory effect of ZINC58368839 was reversed by 40 minutes washout. 5 minutes was given between each application. Bars indicate 10 seconds of ATP application and ATP co-application with ZINC58368839 following 5 minutes pre-incubation. (b) % of remaining currents up to 40 minutes after washout of 1μM ZINC58368839. Data are shown as mean ± S.E.M (n ≥ 3).
6.2.2.5. The effect of Brilliant Blue G at the human and rat P2X7 receptors

At the hP2X7-2NβR, currents in response to ATP (100 µM) were inhibited by Brilliant Blue G with a pIC$_{50}$ of $6.37 \pm 0.09$ (IC$_{50}$ ~ 430 nM) in divalent free solution and a Hill slope of $0.7 \pm 0.1$ (figure 6.11 a & e). The inhibitory did not recover following 20 minutes washout at the hP2X7-2NβR (figure 6.12) (the % of remaining current at 20 minutes after washout of 1 µM Brilliant Blue G was $23.3 \pm 2.3 \%$). Co-application of Brilliant Blue G (0.01-10 µM) with an EC$_{90}$ concentration of ATP (300 µM) at the hP2X7R inhibited the agonist evoked ethidium bromide uptake with similar a pIC$_{50}$ value $6.33 \pm 0.25$ (IC$_{50}$ ~ 470 nM) in sucrose buffer and a Hill slope of $0.4 \pm 0.06$ (figure 6.11 c & e).

At the rP2X7R, co-application of Brilliant Blue G (0.01-1 µM) with an EC$_{90}$ concentration of ATP (1 mM) inhibited the agonist evoked ethidium bromide uptake with a pIC$_{50}$ of $6.4 \pm 0.1$ (IC$_{50}$ ~ 400 nM) in sucrose buffer and a Hill slope of $1.34 \pm 0.24$ (figure 6.11 d & f). Brilliant Blue G was a more potent antagonist of ATP evoked currents with a pIC$_{50}$ of $8.09 \pm 0.2$; p < 0.0001 (IC$_{50}$ ~ 8 nM) in divalent free solution and a Hill slope of $0.5 \pm 0.09$ (figure 6.11 b & f).

6.2.2.6. The effect of Calmidazolium at the human and rat P2X7 receptors

At the hP2X7-2NβR, co-application of Calmidazolium (0.01-1 µM) with an EC$_{90}$ concentration of ATP (100 µM) inhibited the agonist evoked currents in a concentration dependent manner with a pIC$_{50}$ of $6.46 \pm 0.36$ (IC$_{50}$ ~ 350 nM) in divalent free buffer and a Hill slope of $0.65 \pm 0.07$ (figure 6.13 a & e). The inhibitory effect of Calmidazolium did not recover following 20 minutes washout at the hP2X7-2NβR (figure 6.14). At the rP2X7R, Calmidazolium inhibited ATP evoked currents with a pIC$_{50}$ of $7.75 \pm 0.1$ (IC$_{50}$ ~ 16 nM) in divalent free buffer and a Hill slope of $0.35 \pm 0.03$, indicating that this antagonist was more potent at the rP2X7R (figure 6.13 b & f). In contrast, Calmidazolium (0.01-10 µM) had no effect on amplitude of ATP evoked ethidium bromide uptake in the HEK293 cells expressing the hP2X7R and rP2X7R (figure 6.13 c & d). These results indicate that Calmidazolium was ineffective at inhibiting ATP evoked dye uptake, similar to that reported previously (Virginio et al., 1997), suggesting differences in the mechanism of ATP currents and ethidium bromide influx.
Figure 6.11. Brilliant Blue G inhibition at human and rat P2X7 receptors. (a & b) Representative traces showing the effect of Brilliant Blue G on currents evoked by an EC₉₀ concentration of ATP in *Xenopus* oocytes expressing hP2X7-2NβR and rP2X7R. 5 minutes was given between each application. Bars indicate 10 seconds of ATP application and ATP co-application with Brilliant Blue G following 5 minutes pre-incubation with the indicated concentration of compound. (c & d) Representative Flexstation responses showing the effect of Brilliant Blue G on dye uptake induced by an EC₉₀ concentration of ATP in HEK293 cells expressing hP2X7R and rP2X7R for 20 minutes. Arrow indicates the addition of ATP at 120 second. (e & f) Brilliant Blue G inhibition curves for ATP induced currents (blue) and ethidium bromide uptake (red). pIC₅₀ was used to measure Brilliant Blue G potency. Unpaired student’s t test was used. ****, p < 0.0001. Data for currents are shown as mean ± S.E.M (n ≥ 3). Data for ethidium bromide uptake are shown as mean ± S.E.M (n ≥ 6).
Figure 6.12. The inhibitory effect of Brilliant Blue G on ATP evoked currents at the hP2X7-2Nβ receptor was not reversed by 20 minutes washout. (a) Representative traces showing the inhibitory effect of Brilliant Blue G was not reversed by 20 minutes washout. 5 minutes was given between each application. Bars indicate 10 seconds of ATP application and ATP co-application with Brilliant Blue G following 5 minutes pre-incubation. (b) % of remaining currents up to 20 minutes after washout of 1µM Brilliant Blue G. Data are shown as mean ± S.E.M (n ≥ 3).
Figure 6.13. Calmidazolium inhibition at human and rat P2X7 receptors. (a & b) Representative traces showing the effect of Calmidazolium on currents evoked by an EC₉₀ concentration of ATP in *Xenopus* oocytes expressing hP2X7-2NβR and rP2X7R. 5 minutes was given between each application. Bars indicate 10 seconds of ATP application and ATP co-application with Calmidazolium following 5 minutes pre-incubation with the indicated concentration of compound. (c & d) Representative Flexstation responses showing the effect of Calmidazolium on dye uptake induced by an EC₉₀ concentration of ATP in HEK293 cells expressing hP2X7R and rP2X7R for 20 minutes. Arrow indicates the addition of ATP at 120 second. (e & f) Calmidazolium inhibition curves for ATP induced currents (blue) and ethidium bromide uptake (red). pIC₅₀ was used to measure Calmidazolium potency. Data for currents are shown as mean ± S.E.M (n ≥ 3). Data for ethidium bromide uptake are shown as mean ± S.E.M (n ≥ 6).
Figure 6.14. The inhibitory effect of Calmidazolium on ATP evoked currents at the hP2X7-2Nβ receptor was not reversed by 20 minutes washout. (a) Representative traces showing the inhibitory effect of Calmidazolium was not reversed by 20 minutes washout. 5 minutes was given between each application. Bars indicate 10 seconds of ATP application and ATP co-application with Calmidazolium following 5 minutes pre-incubation. (b) % of remaining currents up to 20 minutes after washout of 1µM Calmidazolium. Data are shown as mean ± S.E.M (n ≥ 3).
Characterization of P2X7R antagonists on ionic current and dye uptake in hP2X7Rs and rP2X7Rs are shown in table 6.1.

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Table 6.1. AZ11645373, PPADS, KN-62, ZINC58368839, brilliant blue G and calmidazolium sensitivity at human and rat P2X7 receptors. The table shows the pIC₅₀ values of AZ11645373, PPADS, KN-62, ZINC58368839, brilliant blue G and calmidazolium on currents and dye uptake at hP2X7Rs and rP2X7Rs. Stars indicate a significant difference. ***, p < 0.001; ****, p < 0.0001. Data are shown as mean ± S.E.M (n ≥ 3).
6.2.3. Effects of signature allosteric point mutations on P2X7 receptor antagonists

The signature allosteric point mutations (F88A, T90V, D92A, F103A and V312A) were generated on hP2X7-2N\(\beta\)R background. Therefore, the effects of these mutations on P2X7R antagonists were tested by measuring ionic currents as this will allow direct comparisons to be made for a range of antagonists.

(i) PPADS

Compared to the hP2X7-2N\(\beta\)R (pIC\(_{50}\) = 6.70 ± 0.03), there was no significant change in PPADS sensitivity at the F88A, T90V, D92A, F103A and V312A mutations, pIC\(_{50}\) = 6.8 ± 0.03, 6.7 ± 0.03, 6.8 ± 0.1, 6.9 ± 0.03 and 6.8 ± 0.09 respectively (figure 6.15). The lack effect of these allosteric point mutations on PPADS sensitivity confirms that PPADS binds at the orthosteric site, consistent with that published recently (Huo et al., 2018).

(ii) KN-62

The phenylalanine to alanine mutation at position 88 (F88A) had no significant effect on the potency of KN-62. The threonine to valine mutation at position 90 (T90V) produced an approximately 200 fold decrease in KN-62 action, pIC\(_{50}\) = 5.4 ± 0.2, p < 0.001. A similar reduction was seen at the V312A mutation with pIC\(_{50}\) = 5.3 ± 0.2, p < 0.001. The most striking effect was at the D92A and F103A mutations that abolished the antagonist action of KN-62 (1 µM) demonstrating a more than 1000 fold reduction in sensitivity (figure 6.16).

(iii) ZINC58368839

The phenylalanine to alanine mutation at position 88 (F88A) had no significant effect on the potency of ZINC58368839. The threonine to valine mutation at position 90 (T90V) produced an approximately 6 fold decrease in ZINC58368839 action, pIC\(_{50}\) = 5.6 ± 0.04, p < 0.01. A similar reduction was seen at the V312A mutation, pIC\(_{50}\) = 5.7 ± 0.1, p < 0.05. Interestingly, D92A and F103A mutations abolished the antagonist action of ZINC58368839 (1 µM of ZINC58368839 had no effect on ATP responses), indicating a more than 1000 fold reduction in ZINC58368839 sensitivity (figure 6.17). These results demonstrate that residues T90, D92, F103 and V312 in the allosteric pocket contribute to ZINC58368839 binding.
Figure 6.15. PPADS inhibition at point mutations in the allosteric pocket. (a) Homology model of the hP2X7R showing residues in the allosteric pocket which have a significant effect on AZ11645373 sensitivity (F88A, T90V, D92A, F103A, V312A [red]). In the right hand panel, residues where mutation had no effect on PPADS sensitivity are shown in grey. (b) PPADS inhibition curves at F88A, T90V, D92A, F103A and V312A. The hP2X7-2Nβ is shown as a black dotted line. (c) The pIC₅₀ values of PPADS at the F88A, T90V, D92A, F103A and V312A mutations. A three fold change from hP2X7-2NβR is indicated by the dotted line. Data are presented as mean ± S.E.M.
Figure 6.15. PPADS inhibition at point mutations in the allosteric pocket. (a) Homology model of the hP2X7R showing residues in the allosteric pocket which have a significant effect on AZ11645373 sensitivity (F88A, T90V, D92A, F103A, V312A [red]). In the right hand panel, residues where mutation had no effect on PPADS sensitivity are shown in grey. (b) PPADS inhibition curves at F88A, T90V, D92A, F103A and V312A. The hP2X7-2Nβ is shown as a black dotted line. (c) The pIC₅₀ values of PPADS at the F88A, T90V, D92A, F103A and V312A mutations. A three fold change from hP2X7-2Nβ is indicated by the dotted line. Data are presented as mean ± S.E.M.
Figure 6.17. ZINC58368839 inhibition at point mutations in the allosteric pocket. (a) Homology model of the hP2X7R showing residues in the allosteric pocket which have a significant effect on AZ11645373 sensitivity (F88A, T90V, D92A, F103A, V312A [red]). In the right hand panel, residues where mutation decreased ZINC58368839 sensitivity are shown in red and residues where mutation had no effect on ZINC58368839 sensitivity are shown in grey. (b) ZINC58368839 inhibition curves at F88A, T90V, D92A, F103A and V312A mutations. The hP2X7-2Nβ is shown as a black dotted line. (c) The pIC₅₀ values of ZINC58368839 at F88A, T90V, D92A, F103A and V312A mutations. A three fold change from hP2X7-2NβR is indicated by the dotted line. Stars indicate a significant shift in IC₅₀ from hP2X7-2NβR. One way analysis of variance (ANOVA) test was used followed by Dunnett's test. *, p < 0.05; **, p < 0.01; ****, p < 0.0001; (n= 3-5). Data are presented as mean ± S.E.M.
(iv) **Brilliant Blue G**

The Brilliant Blue G sensitivity at the F88A and T90V mutations was the same as the hP2X7-2NβR, pIC$_{50}$ = 6.1 ± 0.2 and 6.4 ± 0.1 respectively. The most obvious effect was at the D92A, F103A and V312A mutations where Brilliant Blue G (1 µM) had no effect on ATP responses indicating a more than 1000 fold reduction in sensitivity (figure 6.18). These results demonstrate that residues D92, F103 and V312 in the allosteric pocket contribute to Brilliant Blue G binding.

(v) **Calmidazolium**

Interestingly, F88A, T90V, D92A, F103A and V312A mutations abolished the antagonist action of Calmidazolium (1 µM of Calmidazolium had no effect on ATP responses), indicating that these residues contribute to Calmidazolium binding (figure 6.19).
Figure 6.18. Brilliant Blue G inhibition at point mutations in the allosteric pocket (a) Homology model of the hP2X7R showing residues in the allosteric pocket which have a significant effect on AZ11645373 sensitivity (F88A, T90V, D92A, F103A, V312A [red]). In the right hand panel, residues where mutation decreased Brilliant Blue G sensitivity are shown in red and residues where mutation had no effect on Brilliant Blue G sensitivity are shown in grey. (b) Brilliant Blue G inhibition curves at F88A, T90V, D92A, F103A and V312A. The hP2X7-2Nβ is shown as a black dotted line. (c) The pIC₅₀ values of Brilliant Blue G at F88A, T90V, D92A, F103A and V312A mutations. A three fold change from hP2X7-2NβR is indicated by the dotted line. Stars indicate a significant shift in IC₅₀ from hP2X7-2NβR. One way analysis of variance (ANOVA) test was used followed by Dunnett's test. ****, p < 0.0001; (n ≥ 3). Data are presented as mean ± S.E.M.
Figure 6.19. Calmidazolium inhibition at point mutations in the allosteric pocket. (a) Homology model of the hP2X7R showing residues in the allosteric pocket which have a significant effect on AZ11645373 sensitivity (F88A, T90V, D92A, F103A, V312A [red]). In the right hand panel, residues where mutations decreased Calmidazolium sensitivity are shown in red. (b) Calmidazolium inhibition curves at F88A, T90V, D92A, F103A and V312A. The hP2X7-2Nβ is shown as a black dotted line. (c) The pIC₅₀ values of Calmidazolium at F88A, T90V, D92A, F103A and V312A mutations. A three fold change from hP2X7-2NβR is indicated by the dotted line. Stars indicate a significant shift in IC₅₀ from hP2X7-2NβR. One way analysis of variance (ANOVA) test was used followed by Dunnett's test. ****, p < 0.0001; (n ≥ 3). Data are presented as mean ± S.E.M.
All signature allosteric point mutations that were tested on sensitivity of P2X7R antagonists are shown in (table 6.2) and (figure 6.20).

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<td>5.40 ± 0.20 *** 199.5</td>
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<tr>
<td>D92A</td>
<td>Insensitive **** 1000</td>
<td>3</td>
<td></td>
</tr>
<tr>
<td>F103A</td>
<td>Insensitive **** 1000</td>
<td>3</td>
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<tr>
<td>V312A</td>
<td>5.30 ± 0.20 *** 251.1</td>
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<td>T90V</td>
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<tr>
<td>D92A</td>
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<td>3</td>
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<td>Insensitive**** 1000</td>
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<td>V312A</td>
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<tr>
<td>F88A</td>
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<tr>
<td></td>
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<td>Fold shift</td>
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<td>3</td>
</tr>
<tr>
<td>T90V</td>
<td>Insensitive****</td>
<td>1000</td>
<td>3</td>
</tr>
<tr>
<td>D92A</td>
<td>Insensitive****</td>
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<tr>
<td>V312A</td>
<td>Insensitive****</td>
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**Calmidazolium**

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<tr>
<td>T90V</td>
<td>6.40 ± 0.10</td>
<td>1</td>
<td>3</td>
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Table 6.2. AZ11645373, PPADS, KN-62, ZINC58368839, brilliant blue G and clamidazolium sensitivity on the signature allosteric point mutations. The table shows the pIC₅₀ values of AZ11645373, PPADS, KN-62, ZINC58368839, brilliant blue G and clamidazolium and fold shift relative to the hP2X7-2NβR. Stars indicate a significant shift in IC₅₀ from the hP2X7-2NβR. *, p < 0.05 **, p < 0.01; ***, p < 0.001; ****, p < 0.0001. Arrows indicate an increasing sensitivity. Data are shown as mean ± S.E.M (n ≥ 3).
Figure 6.20. Comparison of effects of residues in the allosteric pocket on the sensitivity to the P2X7 antagonists AZ11645373, KN-62, ZINC58368839, Brilliant Blue G and Calmidazolium. (a) homology model of allosteric binding pocket with AZ11645373 antagonist shown in red stick representation. (b) Graph showing the fold shift in P2X7 antagonist sensitivity for five antagonists. A three fold change in antagonist sensitivity is indicated by dotted line.
6.2.4. Molecular docking provides models of KN-62, ZINC58368839, Brilliant Blue G and Calmidazolium in accord with mutagenesis studies

The molecular docking of P2X7R antagonists was generated by Dr. Ralf Schmid. To see how the molecular docking on its own could give evidence for allosteric binding site and to find reasonable binding poses, P2X7R antagonists (PPADS, KN-62, ZINC58368839, Brilliant Blue G and Calmidazolium) were docked at the allosteric site and orthosteric site (as decoy) (table 6.3). Interestingly, docking scores of KN-62, ZINC58368839, Brilliant Blue G and Calmidazolium prefer an allosteric binding site in accord with mutagenesis studies (figure 6.21). In full accord with no effect of allosteric point mutations on PPADS sensitivity, docking poses of PPADS in both allosteric and orthosteric sites revealed weak scores. This indicates that PPADS is unlikely to bind at the allosteric site.

ZINC58368839 (MW ~ 329.4) has approximately the same molecular weight as AZ11645373 (MW ~ 463.5) and the docking pose showed that ZINC58368839 occupies the allosteric site similar to AZ11645373 at the apex of the receptor. The cycloheptane group of ZINC58368839 binds deeply in the allosteric pocket at residues F103 and V312, whereas the indole group of ZINC58368839 sits at the entrance of the allosteric pocket at residue F88. This pose also suggested that the side chain of lysine at position 110 (K110) contributed in hydrogen bonding of the NO2 group on the indole group of ZINC58368839, similar to that reported on AZ11645373. As AZ11645373 binding mode, three molecules of ZINC58368839 bind in structurally similar to the trimeric receptor.

KN-62, Brilliant Blue G and Calmidazolium have molecular weights higher than AZ11645373 and ZINC58368839. Therefore, it is difficult to imagine how these larger compounds bind in the allosteric pocket in the same way. Because of the bigger size molecular docking for these antagonists is hindered. However, docking scores of KN-62 prefer an allosteric binding site in accord with the effect of T90V, D92A, F103A and V312A mutations on the sensitivity of KN-62. The phenyl group of KN-62 binds deeply in the pocket at residues F103 and V312. One of the quinolone group binding at the top of the allosteric at residue F88 and other quinolone group is facing toward the central cavity.
Both stereoisomers of Calmidazolium were used in docking. Docking scores of Calmidazolium favour an allosteric binding site but there were no difference between the R and S stereoisomers. The binding poses for both stereoisomers are similar and bind at the same place with the dichlorophenyl groups binding the allosteric pocket whereas the imidazole and chlorophenyl groups bind in the central cavity.

The binding pose of Brilliant Blue G is similar to that of KN-62 and Calmidazolium, it occupies the allosteric pocket and the central cavity. The binding pose shows no direct interact with F88 but F103 is near to ethoxy phenyl group. This could explain why the F103A mutant, but not F88A mutant, had effect on the Brilliant Blue G sensitivity.

To conclude, binding poses for KN-62, Brilliant Blue G and Calmidazolium bind in the allosteric pocket in accord with mutagenesis studies. Furthermore, these three P2X7R antagonists are also occupying the central cavity. In terms of structure, it seems unlikely for KN-62, Brilliant Blue G and Calmidazolium that three molecules bind similar modes to the trimeric P2X7R. This indicates that these antagonists have bind mode differ from the AZ11645373.

<table>
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<th>Rosetta interface scores (mean ± S.E.M)</th>
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<td>PPADS allost.</td>
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<td>128</td>
</tr>
<tr>
<td>PPADS decoy</td>
<td>-10.10 ± 1.60</td>
<td>157</td>
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<tr>
<td>KN-62 allost.</td>
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<tr>
<td>KN-62 decoy</td>
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<tr>
<td>Calmidazolium(R) allost.</td>
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<tr>
<td>ZINC58368839 decoy</td>
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<td>144</td>
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Table 6.3. Rosetta interface scores for ligand docking. (Data from Dr. Ralf Schmid).
Figure 6.21. Use of allosteric pocket “signature mutants” to investigate the site of action for Brilliant Blue G, KN-62, Calmidazolium, ZINC58368839 and PPADS. (a) Concentration dependent inhibition by P2X7 antagonists of response to an EC<sub>90</sub> concentration of ATP for P2X7-2Nβ (black), F88A (firebrick), T90V (orange), D92A (dark yellow), F103A (green) and V312A (purple). (b) Top panel: P2X7R overview with “signature mutants” residues shown as spheres, colours as in (a). Other panels: view from the top of the extracellular domains along the central axis perpendicular to the membrane. The P2X7R model is shown as cartoon with the three subunits highlighted in light blue, light pink and grey with the docked pose of the antagonist shown as spheres. (c) Top panel: surface representation of entrance to allosteric pocket. Other panels: zoom into the proposed binding site, one subunit (light blue in (b)) is omitted for clarity. Residues F88, T90, D92, F103 and V312 and the respective antagonist are shown as sticks (colours as in (a) and (b)).
6.3. Discussion

The activity of P2X7Rs can be evaluated by measuring ion influx and dye uptake. In chapters 3 and 5, the sensitivity of ATP at the hP2X7-2NβR and rP2X7R was characterized by measuring ATP induced currents in *Xenopus laevis* oocytes. For further characterization of P2X7Rs, the sensitivity of ATP at the hP2X7R and rP2X7R was assessed by measuring ATP evoked ethidium bromide uptake in HEK293 cells. Firstly, the ethidium bromide uptake analysis was optimized by selecting a suitable buffer solution. This study found that ethidium bromide uptake was smaller by 4-5 fold in an extracellular sodium solution compared to sucrose buffer, consistent to that reported previously (Allsopp and Evans, 2015). A similar result was seen for the effect of extracellular potassium on P2X7R mediated dye uptake in HEK293 cells. However, this is in contrast to that reported by Ou et al. (2018) who found that ethidium bromide uptake was higher in an extracellular potassium compared to sodium buffer in human peripheral blood mononuclear cells (PBMCs) (Ou et al., 2018). This discrepancy may be related to using different expression systems. The current results suggest that ethidium bromide competes with sodium and potassium to flow through the P2X7R and in their absence (sucrose buffer) can flow more easily into the cell. These findings suggest that sucrose buffer is the best buffer condition for assay of P2X7R dye uptake in HEK293 cells.

The present study showed that ATP potency at P2X7Rs was similar when measured by ionic currents or dye uptake. It was found that ATP induced dye uptake at the hP2X7R in sucrose buffer with an EC$_{50}$ ~ 90 µM, similar to that reported by Allsopp et al. (2015) who found that ATP induced current at the hP2X7R in free divalent buffer with an EC$_{50}$ ~ 200 µM (Allsopp and Evans, 2015). It was also found that ATP induced dye uptake at the rP2X7R in sucrose buffer (EC$_{50}$ ~ 100 µM), similar to that effect on current in free divalent buffer (EC$_{50}$ ~ 90 µM). It is notable that ATP sensitivity was not changed by using these different buffers. According to these results, we can infer that ATP sensitivity at P2X7Rs on ionic currents were similar to the dye uptake suggesting a common mechanism of action and supporting the idea that ethidium flows directly through the P2X7R pore (Browne et al., 2013, Harkat et al., 2017). However, it should be pointed out that in physiological solutions containing divalent cations that ATP sensitivity is ~ 2.5 fold lower (EC$_{50}$ concentrations for ATP at the hP2X7R in normal divalent solution was ~ 1.8 mM and 720 µM in low divalent solution) (Stokes et al.,
2006) giving the signature low agonist sensitivity and likelihood that it is inactive under normal condition.

The current study found that the effects of the P2X7R antagonists (AZ11645373, PPADS, KN-62, ZINC58368839 and Brilliant Blue G) on ionic currents were similar to those on the dye uptake with the exception of Calmidazolium. Calmidazolium inhibited ATP induced currents at the hP2X7-2NβR and rP2X7R in a concentration dependent manner with IC\textsubscript{50} values of 350 nM and 16 nM respectively, but had no effect on ethidium bromide uptake. This finding is consistent to that reported previously (Virginio et al., 1997). Previous studies showed that large organic dyes can pass through P2X7R channel (Browne et al., 2013, Harkat et al., 2017). However, this may not be the main route of dye influx as Calmidazolium reduced ionic currents by 60-80% but had no effect on dye uptake. It can thus be suggested that there is an additional route for dye influx that is independent of P2X7R channel opening. Previously it had been suggested that pannexin hemi-channels were involved in dye uptake (Pelegrin and Surprenant, 2006). However, there are several cases where P2X7R mediated dye uptake is seen in the absence of pannexin channels (Pelegrin and Surprenant, 2009) thus the mechanism of Calmidazolium resistant dye uptake still remains unanswered.

The aim of this chapter was to identify P2X7R antagonists that bind at the allosteric site. This was done by using signature allosteric point mutants (figure 6.20) and complements the molecular docking (figure 6.21) undertaken by Dr. Ralf Schmid. The current study used PPADS as a negative control for the signature allosteric point mutations. It was found that F88A, T90V, D92A, F103A and V312A mutants had no effect on PPADS sensitivity. These results strongly support the orthosteric model of PPADS binding site. The orthosteric site of PPADS was also confirmed by molecular docking where the docking pose of PPADS in the allosteric site had a weaker score compared to the other P2X7R antagonists (table 6.3). This finding was also reported recently by Huo et al. (2018) who found that in P2X1R the PPADS binds in proximity of the ATP binding site (Huo et al., 2018).
6.3.1. Allosteric binding model for ZINC58368839

The combination of signature allosteric point mutants and molecular docking provides a model showing ZINC58368839 binds at the base of the allosteric pocket, similar to AZ11645373 (this study). The allosteric site of ZINC58368839 is consistent with antagonist-bound pdP2X7R crystal structures (Karasawa and Kawate, 2016). However, this finding differs from that proposed by Caseley et al. (2016) who used molecular docking to predict ZINC58368839 binding in the orthosteric ATP binding pocket surrounded by glutamine residue at position 143 (Q143) and tyrosine residue at position 288 (Y288) (Caseley et al., 2016). Caseley et al. (2016) study used the zfP2X4R based model. It is important to note that the zfP2X4R allosteric pocket is considerably smaller than the pdP2X7R (Karasawa and Kawate, 2016). Therefore, it is perhaps not surprising that they did not find the allosteric site in their docking studies.

In the current study, several lines of evidence suggest that ZINC58368839 binds at the allosteric site rather than the orthosteric site. Firstly, most of signature allosteric point mutants (4/5) diminished ZINC58368839 sensitivity. Secondly, the molecular docking binding pose of ZINC58368839 in the allosteric site had a higher score than found for docking into the orthosteric site rendering the orthosteric docking poses unlikely. Thirdly, the allosteric site of ZINC58368839 was also confirmed by molecular docking which showed that residues where mutation has the significant effects on ZINC58368839 sensitivity are involved in ZINC58368839 binding. A good illustration of this is that F103A and V312A mutants at the base region of the allosteric pocket significantly reduced ZINC58368839 sensitivity and molecular docking showed that ZINC58368839 cycloheptane group binds deeply in the allosteric pocket at residues F103 and V312. This may explain that mutations at these residues reduced ZINC58368839 sensitivity. It was also found that although T90V and D92A mutants are not directly binding the ZINC58368839, these mutations significantly reduced ZINC58368839 sensitivity, possibly by changing the structure of the allosteric pocket.

6.3.2. Binding models for Brilliant Blue G, KN-62 and Calmidazolium

The molecular weight of KN-62, Brilliant Blue G and Calmidazolium are higher than the other allosteric P2X7R antagonists that reported in this study (AZ11645373 and ZINC58368839) or reported previously (Karasawa and Kawate, 2016, Allsopp et al.,
This raises question of whether allosteric modes might be possible for Brilliant Blue G, KN-62 and Calmidazolium.

(i) Brilliant Blue G

There are similarities between Brilliant Blue G and PPADS. Brilliant Blue G has two negative charges in sulphate groups; this is similar to PPADS which has three negative charges, one charge in the phosphate group and two charges in sulphate groups. Furthermore, both Brilliant Blue G and PPADS show a non-competitive mode of action which occurred without change in agonist $EC_{50}$ value (Jiang et al., 2000a, Hibell et al., 2001). These similarities may suggest that Brilliant Blue G has an orthosteric mode of action similar to the PPADS. However, there are reasons to think that Brilliant Blue G does not act at the orthosteric site. The current study found that the signature allosteric point mutations have a significant effect on the Brilliant Blue G sensitivity. The greatest change was seen at the D92A, F103A and V312A mutations which produced more than 1000 fold decrease in Brilliant Blue G sensitivity. These results support that Brilliant Blue G binds at the allosteric site and are consistent with molecular docking showed that Brilliant Blue G binds at the allosteric site expanding into the central cavity.

(ii) KN-62

One interesting finding was that the most signature allosteric point mutants had large effects on KN-62 sensitivity. The greatest change was seen at T90V, F103A, D92A and V312A mutations that produced a more than 100 fold decrease in KN-62 sensitivity. These results are strongly support that KN-62 binds allosterically. The allosteric site of KN-62 is also supported by combination study that used oATP as an orthosteric antagonist. It was reported that KN-62 inhibition was non-surmountable on the effect of oATP, as the KN-62 did not attenuate the inhibitory effect of oATP. This suggested that KN-62 has a mode of action different from oATP that binds at the orthosteric site (Michel et al., 2000). It is interesting to note that KN-62 inhibited ATP evoked current and dye uptake with a Hill slope less than 1, suggesting that KN-62 may have more than one mode of binding. This is supported by molecular docking showing that KN-62 not only bind at the allosteric site but can also occupy the central cavity.
(iii) Calmidazolium

Another important finding was that all signature allosteric point mutants abolished Calmidazolium action. It was found that F88A, T90V, D92A, F103A and V312A produced more than 1000 fold decrease in Calmidazolium sensitivity. These results strongly support that Calmidazolium binds at the allosteric site. As KN-62, Calmidazolium inhibited ATP evoked current with a Hill slope less than 1. It is possible therefore that Calmidazolium has more than one mode of binding. This also accords with molecular docking which showed that Calmidazolium can not only bind at the allosteric site but also extends into the central cavity.

According to these data, we can conclude that P2X7R antagonists including Brilliant Blue G, KN-62 and Calmidazolium have binding modes that differ from AZ11645373 and ZINC58368839. This difference may explained by the fact of these three P2X7R antagonists are big molecules. It is possible therefore that Brilliant Blue G, KN-62 and Calmidazolium bind at more than one site in the receptor.

6.3.3. Diagnostic features of action at the allosteric site

It is notable that signature allosteric point mutations show different effects dependent on the P2X7R antagonist. For instance, F88A mutant decreased sensitivity of AZ11645373 and Calmidazolium but had no effect on sensitivity of KN-62, ZINC58368839 and Brilliant Blue G. Furthermore, T90V mutant decreased sensitivity of AZ11645373, KN-62, ZINC58368839 and Calmidazolium but had no effect on sensitivity for Brilliant Blue G. These results support the idea that each allosteric inhibitor has a specific interactions important for high affinity binding. However, comparing the effects of allosteric point mutations for eight allosteric inhibitors (five in this study: AZ11645373, KN-62, ZINC58368839 Brilliant Blue G and Calmidazolium) and (three from previous studies: AZ10606120, A-740003 and A-438079) (Allsopp et al., 2017, Allsopp et al., 2018) shows a consistent decrease in sensitivity at mutations D92A, F103A and V312A. It can thus be suggested that these three mutations can be used as a diagnostic for antagonists that bind at the allosteric pocket.
In summary, signature allosteric point mutations and molecular docking in this chapter identified that ZINC58368839 binds at the base region of the allosteric pocket, similar to allosteric site of AZ11645373. However, the binding site of KN-62, Brilliant Blue G and Calmidazolium appears to be more complex, which not only bind allosteric site but also extend into the central cavity. Interestingly, this study has identified D92A, F103A and V312A mutations as diagnostic of an allosteric mode of action.
Chapter 7: General discussion

The activation of P2X7Rs has been associated with several pathological conditions including inflammation, neuropathic pain, neurological diseases, cancer and other conditions (see section 1.6). Therefore, P2X7R ion channels are targets for a range of therapeutic strategies. P2X7R channel activation can be inhibited by different pathways, including genetic modification by P2X7R KO mice or P2X7R SNPs and P2X7R antagonists. This thesis was undertaken to study P2X7R antagonists in more detail. Understanding how selective P2X7R antagonists act on the receptor is useful to facilitate drug design targeting P2X7R channel activation with various diseases.

The aim of this study was to identify the molecular basis of action for a range of selective P2X7R antagonists. This was done by generation of chimeras and mutations between the antagonist sensitive hP2X7R and the antagonist insensitive hP2X1R. My results have provided important additional information on antagonism at the P2X7R and contribute to understanding how selective P2X7R antagonists act at the receptor. My study has also identified residues that are responsible for species variations in antagonist sensitivity (see chapter 5) and identified the signature mutations that can decrease allosteric P2X7R antagonist sensitivity (see chapter 6).

7.1. P2X7 receptor channel activation blocked by allosteric inhibitor

The data presented in this thesis show that the five selective P2X7R antagonists AZ11645373, ZINC58368839, KN-62, Brilliant Blue G and Calmidazolium are allosteric inhibitors. All of these P2X7R antagonists bind at the allosteric pocket in the upper body domain. It is notable that the large molecule antagonists including KN-62, Brilliant Blue G and Calmidazolium extend into the central cavity. To develop a complete picture of large molecule P2X7R antagonists, further mutagenesis studies in the central cavity will need to be undertaken (e.g. mutations at R316, F317 and D318). The location, features and size of the P2X7R allosteric pocket will be discussed in more detail below.
(i) Location of the P2X7 receptor allosteric pocket

This study showed that the allosteric antagonist binding pocket of the P2X7R is located behind the orthosteric ATP binding site and surrounded by hydrophobic residues mainly from β-stands in the extracellular domain (figure 7.1), consistent to that published previously (Karasawa and Kawate, 2016). Interestingly, the binding site for eight allosteric inhibitors, five in this study and three from previous studies, shows direct interaction with residues F103 and V312. These findings represent a diagnostic test for antagonists that bind at the allosteric pocket. However, it is important to note that each of allosteric inhibitor has a distinct series of interactions required for high affinity binding. For instance, compared the allosteric pocket of AZ11645373 (this study) with AZ10606120 (Allsopp et al., 2017) showed that AZ11645373 interacts with residues F95, F108 and Q116 whereas AZ10606120 interacts with residues M105, Y295 and K297.

Figure 7.1. The Location of the P2X7 receptor allosteric pocket. Cartoon representation of the P2X7R shows the allosteric binding pocket as red spheres and the orthosteric ATP binding pocket as green spheres.
(ii) Features of the P2X7 receptor allosteric pocket

The current study suggests several features of the P2X7R allosteric pocket which are: (i) Residues at the base region of the allosteric pocket including F103 and V312 make direct interactions with all five P2X7R antagonists. Mutagenesis studies showed that both of these residues are essential for high affinity antagonist binding. (ii) Residues facing away the allosteric pocket do not interact directly with P2X7R antagonists but have potential effect on the shape/size of the allosteric pocket (e.g. residue aspartic acid at position 92 (D92)).

To gain insight how P2X7R allosteric antagonists act at the receptor, it is first necessary to understand the mechanism of P2X7R activation (see section 1.9.2). The pdP2X7R crystal structure suggested that allosteric antagonist pocket narrows during ATP binding and such of this conformational change is essential for P2X7R channel activation (Karasawa and Kawate, 2016). Therefore, the P2X7R allosteric antagonist can prevent narrowing of the allosteric pocket required for P2X7R channel activation. It seems likely that when P2X7R selective antagonist binds at the allosteric pocket surrounded by hydrophobic residues, the orthosteric ATP binding pocket is close including residues K64, K66 and L191 at one subunit and N292, F293 and K311 at neighbouring subunit.

(iii) Size of the P2X7 receptor allosteric pocket

Interestingly, the inter-subunit cavity of the allosteric pocket is larger in pdP2X7R than in zfP2X4R or in hP2X3R (Karasawa and Kawate, 2016). It is notable that there are two unique P2X7R residues (T90 and T94) and molecular dynamic stimulations of the P2X7R showed that both of these residues are essential for a dynamic effect on the short α-helix at the base of the allosteric pocket (Allsopp et al., 2017). Mutagenesis studies showed that a reduced dynamic effect of the short α-helix is associated with a reduction of the allosteric pocket. This may explain why the P2X7R has an unique large allosteric pocket. This fact might be important for P2X7R selectivity and provides an explanation as to why AZ11645373, ZINC58368839, KN-62, Brilliant Blue G and Calmidazoilum P2X7R antagonists are not be able to bind with high affinity at other P2XR subtypes. Furthermore, there is a negative charged aspartic acid residue at position 92 (D92) that is essential for stabilization of the β-stands that line the allosteric pocket. Mutagenesis
studies showed that reduced dynamic effect of the β-stands is associated with a reduction of the allosteric pocket (for details see sections 4.2.4 and 4.3.2).

To conclude, our data provide important information about the molecular basis of P2X7R antagonist action. This is useful to facilitate drug design targeting P2X7R channel activation associated with several pathological conditions. Modelling studies at residues T90, D92 and T94 provide more details about dynamic movements of the allosteric pocket. Therefore, further molecular dynamic studies at residues F103 and V312 are recommended.

7.2. Properties of the P2X7 receptor antagonist

P2X7R activation is associated with several neurological diseases such as Alzheimer’s disease, epilepsy, multiple sclerosis and others (see section 1.6). For CNS targets, it is necessary to use P2X7R antagonists that cross the blood brain barrier. Recently, several P2X7R antagonists with high CNS penetration have been described including JNJ-54175446, (4- Methyl- 6,7- dihydro- 4H- triazolo [4,5-c] pyridine). Autoradiography in rat brain showed that 10 mg/kg of JNJ-54175446 binding was sustained in the brain with occupancy of more than 80% at 24 hour (Letavic et al., 2017).

Positron emission tomography (PET) can be used to measure human brain target occupancy for drugs being developed for CNS indications. It was found that P2X7R PET ligand ([18F] JNJ-64413739) was surmountable by P2X7R brain penetrant P2X7R antagonist JNJ-54175446, the brain occupancy of P2X7R PET ligand decreased when the concentration of JNJ-54175446 was increased. This indicates that JNJ-54175446 has a mode of action similar to the P2X7R PET ligand (Bhattacharya, 2018). Taken together, these studies proved that P2X7R antagonist JNJ-54175446 has a high CNS penetration. Further research by using P2X7 PET radioligand is required to see whether our allosteric P2X7R inhibitors can cross blood brain barrier. This would be useful for pharmacokinetic profiling.

Interestingly, there are other approaches that can be used to inhibit P2X7R channel activation which are P2X7R antibodies. For example, a monoclonal antibody inhibited the BzATP induced current in HEK293 cells expressing the hP2X7R, with an IC$_{50}$ concentration of ~ 5 nM (Buell et al., 1998). Recently, it was published that BIL010t,
a topical P2X7R antibody, can be used for treatment of basal cell carcinoma. BIL010t contains sheep polyclonal antibodies and acts against of a non-pore functional P2X7R (nfP2X7R) form. This form is upregulated in response to high concentration of ATP that is associated with different types of cancer and is essential for cancer cell survival (Gilbert et al., 2017). It is important to note that P2X7R antibodies have low CNS penetration and low volume of distribution (Wulff et al., 2019). Therefore, it seems unlikely that P2X7R antibodies have therapeutic potential for treatment of neurological diseases. The site of action for BIL010t P2X7R antibody is unclear. It is possible that BIL010t P2X7R antibody blocks either ATP binding directly or the conformational change associated with P2X7R channel opening. Therefore, chimeras and mutations might be useful to identify the mode of binding for this P2X7R antibody. This would facilitate to develop other P2X7R antibodies.

According to properties of P2X7R antagonists, it seems likely that the brain penetrant P2X7R antagonist would be useful for treatment of several CNS diseases. It may also be used for treatment of different types of brain cancer. For instance, in the glioma brain tumour, brain penetrant P2X7R antagonist reduced tumour size by ~ 50% (Ryu et al., 2011). Recently, it was shown that P2X7R antagonist may be useful for the treatment of the skin cancer (basal cell carcinoma) (Gilbert et al., 2017). However, animal studies showed that progression of the melanoma is accelerated in P2X7R KO mice (Adinolfi et al., 2015). Therefore, P2X7R antagonist might be useful for certain types of skin cancer. It was also shown that cervical cancer was common in women with low levels of the P2X7R expression (Gorodeski, 2009). Therefore, P2X7R antagonist is not appropriate choice in this type of cancer. Furthermore, it was reported that P2X7R KO mice had reduced bone formation and increased bone resorption (Ke et al., 2003) and P2X7R SNP that result in loss function is associated with decreased bone density and increased fracture risk in postmenopausal women (Ohlendorff et al., 2007). This may suggest that osteoporosis is a potential side effect of using P2X7R antagonist. Further clinical studies are required to identify potential side effects of our allosteric P2X7R antagonists.

In conclusion, P2X7R activation is associated with inflammation, neuropathic pain and neurological diseases. Several approaches have been described to inhibit P2X7R channel activation; one of these methods is with P2X7R antagonists. In the last two years,
there have been major advances in understanding the molecular basis of P2X7R antagonists. This thesis provides insight about the molecular basis of action for five allosteric inhibitors and gives a template of P2X7R allosteric binding site. Understanding the molecular basis of antagonist action / binding sites can provide great opportunity for P2X7R drug design. The challenge now is forward these allosteric inhibitors into clinical studies.
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