CHARLES UNIVERSITY IN PRAGUE

FACULTY OF PHARMACY IN HRADEC KRÁLOVÉ

Department of Pharmaceutical Chemistry and Drug Control

DEVELOPMENT OF A RELIABLE TEST SYSTEM FOR PURINERGIC P2X3 RECEPTORS

Master thesis

Performed at:

University of Bonn

Pharmaceutical Institute

Department of Pharmaceutical Chemistry

Supervisors: Prof. Dr. Christa Elisabeth Müller

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Hradec Králové 2016

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Declaration

I declare that this master thesis has been developed and written independently by myself. Any thoughts from literature or other sources are clearly marked and properly listed in References. The master thesis was not used to achieve any academic grading.

Prohlašuji, že tato práce je mým původním autorským dílem. Veškerá literatura a další zdroje, z nichž jsem při zpracování čerpala, jsou uvedeny v seznamu použité literatury a v práci řádně citovány. Tato práce nebyla použita k získání jiného či stejného titulu.

Hradec Králové Šárka Tomanová

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Abstract

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Title of diploma thesis: Development of a reliable test system for purinergic P2X3 receptors

Purinergic P2X3 receptor is a ligand-gated ionotropic channel that occurs in all mammalian tissues. The highest occurrence has been observed in central and peripheral nervous system and smooth muscles, where P2X3 receptors participate in pathological disorders such as visceral and neurophatic pain, inflammatory reactions and psychiatric disorders. Compounds capable of blocking P2X3 receptor activity could be therefore used as potential drugs for treatment of these states. P2X3 receptor belongs to fast-desensitizing ionotropic channels, which makes the measurement of its activity very difficult. It was described that one point S15V mutation, in which the amino acid serine in a position 15 is replaced by amino acid valine, slows down the desensitization rate and the signal becomes easily measurable. This simple mutation may be used as an effective tool for characterization of insufficiently explored P2X3 receptor.

The P2X3 S15V receptor DNA was inserted into retrovirus and, subsequently, human 1321N1 astrocytoma cells were infected. Retroviruses carried not only receptor DNA but also a resistance to antibiotic G418, which allowed the selection of successfully transfected cells. Human cells expressing P2X3 S15V receptor were tested via fluorescence-based calcium assay, in which the intracellular calcium levels are indicated fluorescently. When the method was completely optimized the testing of known P2X3 agonist, antagonist and allosteric modulators plus yet untested potential P2X3 antagonists was performed. The measured data was analyzed in GraphPad Prism.

P2X3 S15V receptor mutant is a reliable surrogate for characterization of P2X3 ionotropic channel. The response to P2X3 agonists, antagonists and allosteric modulators was comparable to literature with one exception, compound A-317491. Moreover, blocking activity of one widely used drug and its metabolite was observed at P2X3 S15V receptor. Very interestingly, this could mean that the mechanism of action of this worldwide used compound was finally discovered.

Abstrakt

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Název diplomové práce: Vývoj spolehlivého testovacího systému pro purinergní P2X3 receptor

Purinergní P2X3 receptor je spojen s iontovým kanálem. Nalezen byl ve všech savčích tkáních, avšak nejhojnější výskyt byl pozorován v centrální i periferní nervové soustavě a v hladké svalovině, kde může být zodpovědný za patologické poruchy jako je viscerální a neuropatická bolest, zánětlivá onemocnění či psychiatrické stavy. Látky blokující aktivitu P2X3 receptoru by tedy mohly být využity jako potenciální léčiva těchto chorob. P2X3 receptor patří mezi iontové kanály s velmi rychlou desenzitizací, což značně ztěžuje měření jeho aktivity. Bylo zjištěno, že jednobodová S15V mutace, kdy je aminokyselina serin v poloze 15 zaměněna za aminokyselinu valin, prodlouží desenzitizaci a signál se stává měřitelným. Tato mutace by tedy mohla být účinným nástrojem pro farmakologickou charakterizaci dosud nedostatečně prozkoumaného P2X3 receptoru.

V naší práci byla DNA P2X3 S15V receptoru několika kroky vpravena do retroviru, kterým byly následně nakaženy astrocytomální buňky lidského mozku označované jako 1321N1 buňky. Spolu s DNA receptoru nesl virus i rezistenci k antibiotiku G418, což umožnilo snadnou selekci úspěšně transfekovaných buněk. Lidské buňky exprimující P2X3 S15V receptor byly podrobeny testování pomocí fluorescence-based calcium assay, kdy je změna hladiny intracelulárního vápníku měřena fluorescenčně. Po optimalizaci metody následovalo testování známých P2X3 agonistů, antagonistů, alosterických modulátorů ale i dosud netestovaných potenciálních P2X3 antagonistů. Naměřená data byla analyzována pomocí programu GraphPad Prism.

P2X3 S15V receptorový mutant je věrohodnou náhražkou pro charakterizaci P2X3 iontového kanálu. Reakce na přítomnost P2X3 agonistů, antagonistů a alosterických modulátorů byly až na sloučeninu A-317491 srovnatelné s literaturou. Navíc byla zjištěna blokace receptoru jedním hojně užívaným proléčivem a jeho metabolitem, což by mohlo znamenat objasnění dosud neznámého mechanismu účinku tohoto léčiva.

Content

1 Introduction

Purinergic signalling plays an important role in neurotransmission and neuromodulation. Recent studies showed that purinergic receptors, which are widely expressed throughout the central and peripheral nervous system, are involved in various pathological conditions such as neurological disorders, psychiatric states and inflammatory reactions.[5]

The superfamily of purinergic receptors is divided into two main families P1 and P2. P1 is subdivided into A_1, A_{2A}, A_{2B} and A_3 subtypes, while the P2 receptor family comprises P2X and P2Y receptors.[3] P2 receptors are activated by nucleotides such as adenosine triphosphate (ATP). ATP plays a role not only in cellular metabolism but also works as an extracellular messenger via activation of purinergic receptors. Together with adenosine diphosphate (ADP) and pyrimidine nucleotides uridine triphosphate (UTP) and uridine diphosphate (UDP) modulates cell functions.[8]

Purinergic P2X3 receptor is a ligand-gated ionotropic channel and occurs as a trimer especially in smooth muscles, central nervous system, nociceptive sensory and sympathetic neurons. P2X3 receptors participate in pathological disorders such as neuropatic pain, cough, visceral pain and urinary incontinence. Therefore, modulation of this receptor may be a strategy to influence these pathological states.[3,11]

However, P2X3 receptor is rapidly desensitizing ionotropic channel and that makes it difficult to measure its activity. It was observed that one-point mutation when serine (S) in position 15 was replaced by valine (V) slows the desensitization, which allows measurement of its activity. Other pharmacological properties remain unchanged. It follows that simple S15V mutation can be used as a useful tool for characterizing P2X3 receptor.

The purpose of this thesis is to develop a reliable test system for human P2X3 receptors which allows characterization of P2X3 receptor features. Therefore, our goal is to clone the human S15V-P2X3 receptor mutant into a retroviral vector and stably transfect it in 1321N1 human astrocytoma cells. Furthermore, the aim is to pharmacologically characterize the new cell line in fluorescence-based calcium assays using agonists and antagonists.

2 Theoretical background

2.1 Purinergic receptors

Extracellular actions of purine nucleotides and nucleosides were first observed and described by Drury and Szent-Györgyi in 1929.[1] Purinergic receptors also called purinoreceptors were then first defined in 1976 by Burnstock and soon after, in 1978, two types of purinergic receptors were distinguished.[2] Since then the superfamily of purinergic receptors is divided into two main families P1 and P2.

Sometimes the third purinergic receptor family called P0 (P zero) is distinguished. These G protein-coupled receptors are activated by nucleobase adenine and therefore named adenine receptors (AdeR). P0 receptors were firstly found in rat with high expression in dorsal root ganglia, brain cortex, hypothalamus, lungs, ovaries, small intestine and kidney.[16] Two adenine receptors Ade1R and Ade2R have been found in rodents.[26] Adenine plays a role in nociception and its neuroprotective effect was observed in cultures of rat Purkinje cells. Human adenine receptor has not been identified yet.[16] Scheme of purinergic receptor family is showed in Figure 1.

Conversely, P1 and P2 purinergic receptors were found in all mammalian organs and tissues,[3] especially in gastrointestinal tract and nervous system. It was observed that purinergic transmission is involved in gut reflexes regulation in animal models.[4] P1 and P2 receptors are also widely distributed in central nervous system and glial cells, including oligodendrocytes, astrocytes and microglia. Imbalance in purinergic transmission can, for instance, cause neurological diseases and psychiatric states.[5]

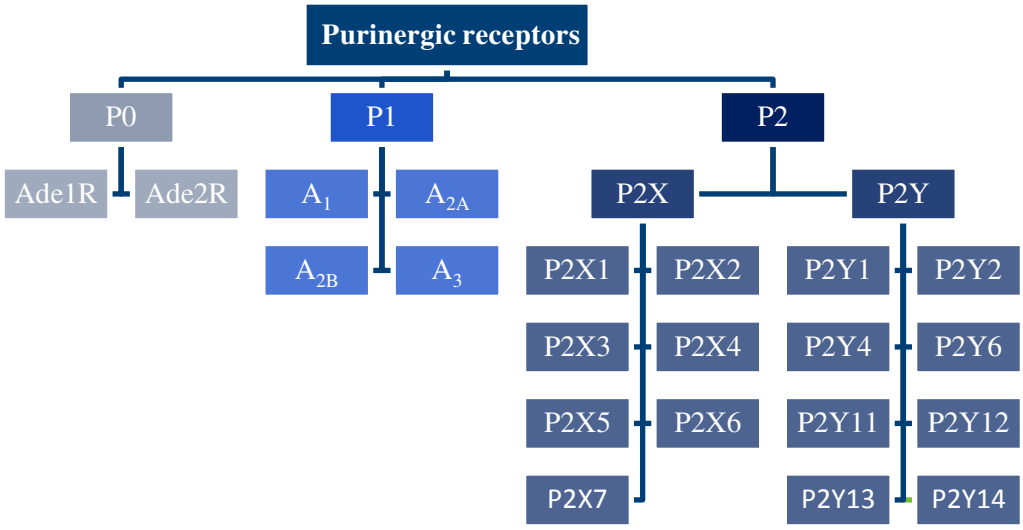


Figure 1: Purinergic receptor family.

2.1.1 P1 receptors

P1 purinoreceptors are broadly classified as receptors for nucleosides, for instance adenosine.[4] All P1 adenosine receptors are G protein-coupled and have seven putative transmembrane domains.[1] Binding of agonist to these G protein-coupled receptors (GPCRs) can provide wide variety of intracellular interactions via interactions with heterotrimeric guanine nucleotide binding proteins known as G-proteins. Activation of GPCRs causes changes in conformation state. GDP on its α-subunit is exchanged for GTP, which causes protein dissociation to Gα- and Gβγ- subunits (Figure 2). These subunits can then regulate both enzyme and ion channel activity.[7]

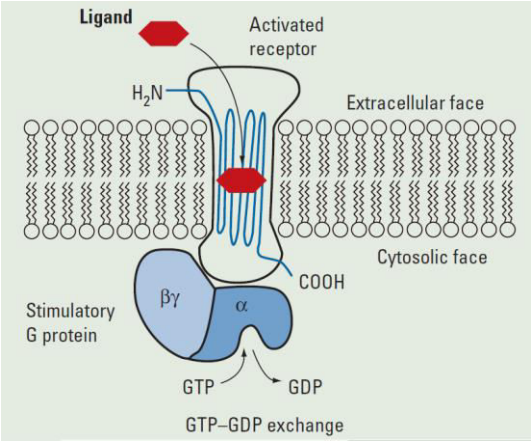


Figure 2: Scheme of G-protein coupled receptor.[18]

P1 receptors are subdivided into four subtypes A_1, A_{2A}, A_{2B} and A_3.[4] These subtypes differ in their main distribution and functions. $P1A_1$ receptors are located mainly in central nervous system and autonomic nerve terminals such as testis and heart and their main function comprises neuromodulation of neurotransmission and cardiac depression. $P1A_{2A}$ receptors occur in brain, lungs, spleen and heart and are responsible for neurotransmission and relaxation of smooth muscles. $P1A_{2B}$ receptors are located especially in bladder and large intestine and play a role in disorders of allergic and inflammatory origin. Similarly the $P1A_3$ receptors participate in allergic reaction and moreover they are cardioprotective and cytoprotective. Their main distribution was observed in lungs, brain, liver, testis as well as in heart.[5]

2.1.2 P2 receptors

P2 purinergic receptors can be activated by adenine nucleotides such as ATP and ADP as well as by pyrimidine nucleotides UTP and UDP.[6] These nucleotides play an important role in a diversity of tissue functions including nociception, fast excicatory neurotransmission, apoptotic cascade, platelet aggregation, pulmonary function and many others.[8]

P2 receptor group is a large family that comprises P2X ligand-gated ionotropic channel receptors and P2Y metabotropic G-protein coupled receptors.[3,4,8]

2.1.2.1 P2Y receptors

As mentioned above P2Y receptor family belongs to G-protein coupled receptors for extracellular nucleotides and hence they act slower than P2X ion channels.[9–11] Eight P2Y subtypes are known to exist in mammals ($P2Y_1$, $P2Y_2$, $P2Y_4$, $P2Y_6$, $P2Y_{11}$, $P2Y_{12}$, $P2Y_{13}$ and $P2Y_{14}$). Significant distinction in P2Y subtypes has been described in both pharmacological features and amino acid sequence. P2Y receptors can form homo- or heterodimers, which differ in their pharmacological properties when compared with monomers.[9]

The highest incidence of P2Y receptors was observed in endothelial cells, pancreatic β-cells, hepatocytes, platelets, kidney, bone marrow as well as in brain.[2] Therefore, P2Y forms possible targets for new therapeutic strategies such as modulation of microglial function, neuroprotective effect and control of bone metabolism.[9] New candidates for treatment of pulmonary diseases, dry eye disease and thrombosis has already been investigated.[10]

2.1.2.2 P2X receptors

The P2X receptors are ligand-gated ionotropic channels as opposed to P2Y receptors which are G protein-coupled.[10] P2X receptors are non-selective trimeric cation channels permeable for Na^+, K^+ and Ca^{2+}.[1,3] Activation of P2X receptors leads to cations influx which causes cell depolarization and activates cytosolic enzymes respectively.[10]

Seven different subunits of P2X (P2X1–7) have been found in mammals. Subunits can form homo- or heterotrimeric ion channels and that leads to different properties of these

transmembrane receptors.[12] P2X receptors are found exclusively in eukaryotes and are widely expressed in many tissues, especially in the central and peripheral nervous system, immune system as well as in smooth muscles.[3,27]

P2X receptors participate not only in neurotransmission from neuron to neuron but also in neuroeffector transmission from neuron to muscle. There is a strong evidence to suggest that P2X receptors are involved in various physiological and pathological processes including inflammatory and pain sensation, bone metabolism, taste, male fertility and they also participate in regulation of blood pressure.[10,13] Upregulated number of P2X receptors was observed after peripheral or central nerve injury, in brain ischemia and during inflammatory states.

P2X receptor structure still remains incompletely understood.[13] However, it is known that there are some unifying features.[12] All P2X subunites possesses two transmembrane segments named TM1 and TM2 and both N and C termini are located intracellular.[12,13] The scheme of P2X3 receptors is showed in Figure 3. ATP binds to large disulfide-rich extracellular domain.[27] When extracellular ATP binds, receptors switch from closed to open conformation state and cations can flow into the cell.[12,13] Besides ATP competitive antagonists and modulatory metal ions binds to extracellular domain, whereas transmembrane domains act as non-selective cation channel.[28]

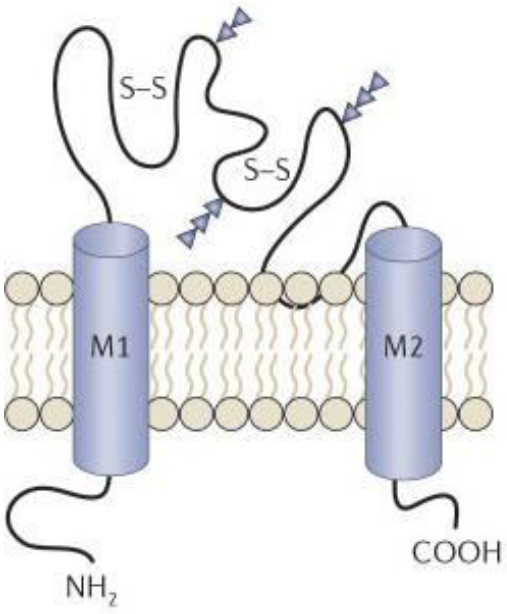


Figure 3: Scheme of P2X receptor (M1 and M2 are transmembrane domains, S-S marks disulfide bonds, NH_2 and COOH termini are located intracellular).[1]

The recent determination of the crystal structure of zebrafish P2X4 receptor confirmed the global architecture of P2X receptors and showed that each subunit is likened to shape of dolphin consisting of head, body, dorsal fin, both right and left flippers and tail (Figure 4).[12] The chalice-shaped P2X receptors are connected together by subunit-subunit contacts, which play an important role in receptor function. Binding of agonist may cause changes within and between subunits and thus enables ions to go through the channel.[12,17]

The zfP2X3 receptor consists of three subunits of the same conformation. Transmembrane helices 1 and 2 are antiparallel to one another and are angled approximately 45° from the membrane. The extracellular domain has a corrugated profile and is shaped like an equilateral triangle. ATP binds extracellularly to a putative ATP site which is shaped like an open jaw. It is suggested that antagonists occupy part or whole ATP site and thus block conformational rearrangement to an open state.[17]

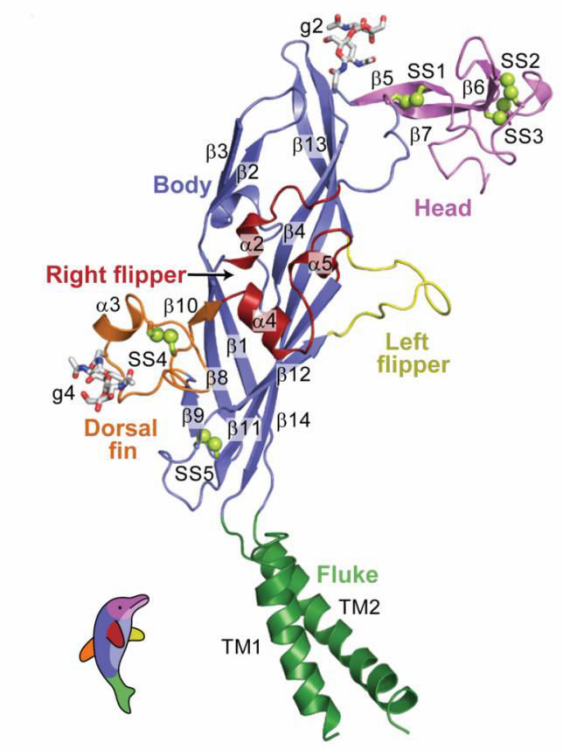


Figure 4: Dolphin-like subunit fold and intersubunit contacts of zebrafish P2X4 (β indicates beta strands, g marks glycans, SS marks disulfide bonds and TM are transmembrane helices).[12,17]

2.1.2.2.1 P2X3 receptors

High P2X3 receptor levels can be found in smooth muscles, central nervous system, nociceptive sensory and sympathetic neurons.[3] With regard to the nervous system they occur mainly in sensory neurons of the dorsal root ganglia, vagus and trigeminal nerve as well as in glossopharyngeal nerve. When present in sensory nerve endings they are responsible for temperature sensitivity and pain sensation.[11]

P2X3 receptors are also involved in patophysiology of visceral pain and other neurological diseases such as cough, urinary incontinence and neuropathic pain.[3,11] P2X3 knock-out mice demonstrate a reduced taste, temperature sensation and bladder reflexes as well as decreased pain behaviour.[3,43] When P2X3 subunit is deleted it affects not only homomeric P2X3 but also hetoremeric P2X3 receptors so a clear undestanding of the receptor effect is difficult to determine.[43] This fact complicates the assessment of the role of P2X3 *in vivo*.[45]

Therefore, P2X3 receptor modulation may be a strategy to reduce inflammatory and neuropathic pain as well as other neurological disorders.[3,11] Other potential indications comprise benign prostatic hyperplasia, bronchial hyperreactivity, asthma, chronic obstructive pulmonary disease, epilepsy and sleep disorders.[12]

P2X receptor subunits assemble together to form trimeric ion channels.[3,12] Homomeric P2X3 channels consist of three P2X3 subunits as opposed to heteromeric P2X2/3 ion channels which are built from both P2X2 and P2X3 subunits.[12] Non-desensitizing P2X2/X3 receptor chimera has been used as a reliable surrogate for analysis of P2X3 receptor, which desensitizes after agonist stimulation within one hundred milliseconds.[15]

Actually, there are two receptors from P2X family, P2X1 and P2X3, belonging to the group of rapidly desensitizing receptors and thus different from others, slow desensitizing receptors of the P2X receptor family. For this reason it is very difficult to measure their activity and functionally characterize them.[14,15] The desensitization rate of P2X receptors is not determined by ectodomain but by domains which flank the ectodomain. Not-ectodomains comprise both transmembrane domains TM1 and TM2, cytoplasmatic N- and C- domains.[15]

Strong delay in desensitization of P2X3 receptors was observed after single-point mutation of a rat P2X3 construct, in which the amino acid serine (S) at position 15 was replaced by a hydrophobic residue, especially by amino acid valine (V) which causes

the strongest delay in the desensitization. Amino acid sequence of P2X3 S15V receptor is shown in Figure 5, the desensitization delay in wild type P2X3 and S15V P2X3 receptor mutant is to be seen in Figure 6.

MNCISDFFTYETTKVVVVKSWTIGIINRVVQLLIISYFVGWVFLHEK
AYQVRDTAIESSVVTKVKGSGLYANRVMDVSDYVTPPQGTSVFVII
TKMIVTENQMQGFCPESEEKYRCVSDSQCGPERLPGGGILTGRCVN
YSSVLRTCEIQGWCPTEVDTVETPIMMEAENFTIFIKNSIRFPLFNFE
KGNLLPNLTARDMKTCRFHPDKDPFCPILRVGDVVKFAGQDFAKL
ARTGGVLGIKIGWVCDLDKAWDQCIPKYSFTRLDSVSEKSSVSPGY
NFRFAKYYKMENGSEYRTLLKAFGIRFDVLVYGNAGKFNIIPTIISS
VAAFTSVGVGTVLCDIILLNFLKGADQYKAKKFEEVNETTLKIAAL
TNPVYPSDQTTAEKQSTDSGAFSIGH

Figure 5: Amino acid sequence of P2X3 S15V receptor (provided by University of Bonn). Serine in position 15 was replaced by valine (in red).

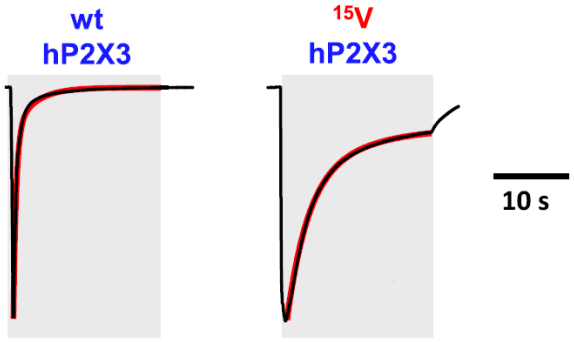


Figure 6: Desensitization rate of wild type hP2X3 (wt hP2X3) and S15V mutant (15V hP2X3).[15]

This fact strongly supports the theory that some of the N-terminal residues are crucial for receptor desensitization.[15] Chemical structures of serine and valine can be seen in Figure 7.

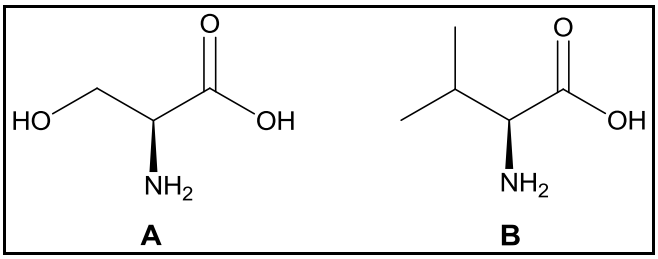


Figure 7: Chemical structure of serine (A) and valine (B).

Specific pharmacological properties after S15V mutation remained unchanged, but calcium imaging assays showed 10-fold higher calcium transients compared with those of the wild-type P2X3 receptor and the area under the curve of Ca^{2+} fluorescence signal was even 87-fold larger. Pharmacological properties were examined by comparing concentration-response analysis of mutated S15V-P2X3 and wild type P2X3 (wtP2X3), which showed similar EC_{50} values of ATP, A-317491 and α,β-me-ATP.[15]

Therefore, this slow-desensitizing phenotype of the P2X3 receptor offers the possibility to characterize its specific pharmacological features.[15] Moreover, the desensitization delay permits the reliable assessment of the activity of antagonists, that was formerly hampered by the rapid receptor desensitization.[14]

According to Bratt´s hypothesis P2X3 recovery consist of two steps. Firstly, the agonist slowly unbinds, which is recovery limitation step and rapid conformation follows in the second step. High affinity P2X3 receptor binding site is exposed during the process of desensitization and agonists that bind to this site can "trap" the receptor in desensitized state.[24]

Agonists and positive allosteric modulators at P2X3 receptor

Adenosine triphosphate is known mainly as a carrier of free energy but it is also crucial extracellular signalling molecule especially for receptors containing P2X3 subunits.[17,27,29] ATP is suggested to generate pain signals in sensory neurons. For instance, on nociceptive dorsal root ganglion neurons ATP operates via activity of P2X3 receptors.[30] When injected intradermally ATP causes intense pain.[44]

ATP is potent at all P2X receptors but activates them in different concentrations from low nanomolar to high micromolar.[10] EC_{50} value for human homotrimeric P2X3 receptor was determined as 1.6 micromolar.[24] P2X3 receptor is not activated only by

ATP but also by other nucleotides triphosphates such us CTP, UTP and nucleoside diphosphate ADP.[8,24,25]

ATP structural analogue α,β-methylene ATP (α,β-me-ATP) is a highly selective agonist for rapidly desensitizing receptors P2X1 and P2X3 with EC_{50} value of 54 nM and 350 nM, respectively.[10,21] P2X3 agonists group also includes other ATP structural analogues such as ADPβS, ATPγS, BzATP and 2-me-S-ATP.[8,24,25]

Adenosine-5´-O-(2-thiodiphosphate) abbreviated as ADPβS has considerably lower potency than ATP and α,β-me-ATP at P2X3 receptor. Contrariwise, ADPβS and other ADP structural analogues activate some P2Y receptors (P2Y1, P2Y12, P2Y13) with a higher potency than ATP itself.[31]

It was reported, that ATPγS, a less hydrolyzable ATP analog, activates P2X receptors expressed in rat dorsal horn neurons. In contrast, recent study claims that this effect was mediated by $P2Y_1$ receptors expressed in the same neurons.[32,33] This thesis was supported by several observations in presence of receptor-specific agonists and antagonists.[32] But it is still suggested, that ATPγS may also activate P2X4 receptor and poorly the P2X7 receptor.[33]

BzATP that was previously used as a selective P2X7 receptor agonist is, in fact, four to five orders of magnitude more potent at P2X3 and P2X1 receptors than at P2X7.[8,36] BzATP showed higher potency at zebra fish homomeric P2X3r than physiological ATP. No significant difference between its EC_{50} value for rat and zebrafish P2X3 was observed (8 μM and 5 μM, respectively).[35]

BzATP increases intracellular Ca^{2+} levels and stimulate nucleotide (ATP and ADP) release by activating P2X receptors. Nucleotide secretion is sensitive to intracellular calcium levels. BzATP does not act only through P2X receptors but is also known to activate P2Y receptors. Therefore, the mechanism of promoting nucleoside release still remains subject to debate.[34]

2-methylthioadenosine triphosphate (2-meS-ATP) produce equivalent maximum response as ATP but is more potent at P2X3r (EC_{50} value of 0.0203 μM).[25,37] Recovery of P2X3 receptor from desensitization was very slow after 2-meS-ATP stimulation compared to β,γ-meATP, α,β-me-ATP and ATP ($t_{1/2}$ = 3.22 min for 2-meS-ATP and 0.32 min in the case of β,γ-meATP).[37]

Symmetrical dinucleotide phosphate Ap4A evoked agonist response at rat P2X1, P2X2, P2X3 and P2X4 receptors.[25,38] Ap4A molecule was found endogenously and is present in neuroendocrine chromaffin cells in high concentration. Maximal response of 70 % of

the ATP response was observed after Ap4A stimulation at mammalian P2X3 receptors.[38] EC_{50} value reached 5 μM. It is suggested that Ap4A may have therapeutic potential for treatment of glaucoma.[39]

Cibacron Blue potentiates ATP responses at P2X3 receptor, 3- to 7-fold augmentation of Ca^{2+} influx was observed.[3,23] Cibacron Blue also known as Reactive Blue 2 is an anthraquinone derivative acting as P2X3 receptor positive allosteric modulator with EC_{50} value of 1.4 μM.[3] Another blue dye, Coomassie Blue, and cyclic pyridoxal phosphate derivative named MRS 2220 also enhance the effect of ATP at fast desensitizing P2X3 and P2X1 receptors.[20]

Also some metals, for instance zinc or copper, can modulate activity of some P2X receptors via allosteric mechanism.[12,20] Zink, the second most abundant trace metal in human body, modulates function of P2X2, P2X3, P2X4 and P2X5 receptors. Allosteric modulation has different effect depending on zinc concentration. At low (less than 100 μM) concentrations zinc has potentiating effect. On the contrary, at millimolar range zink inhibits some P2X receptors.[12] Chemical structures of selected P2X3 agonists are shown in Figure 8.

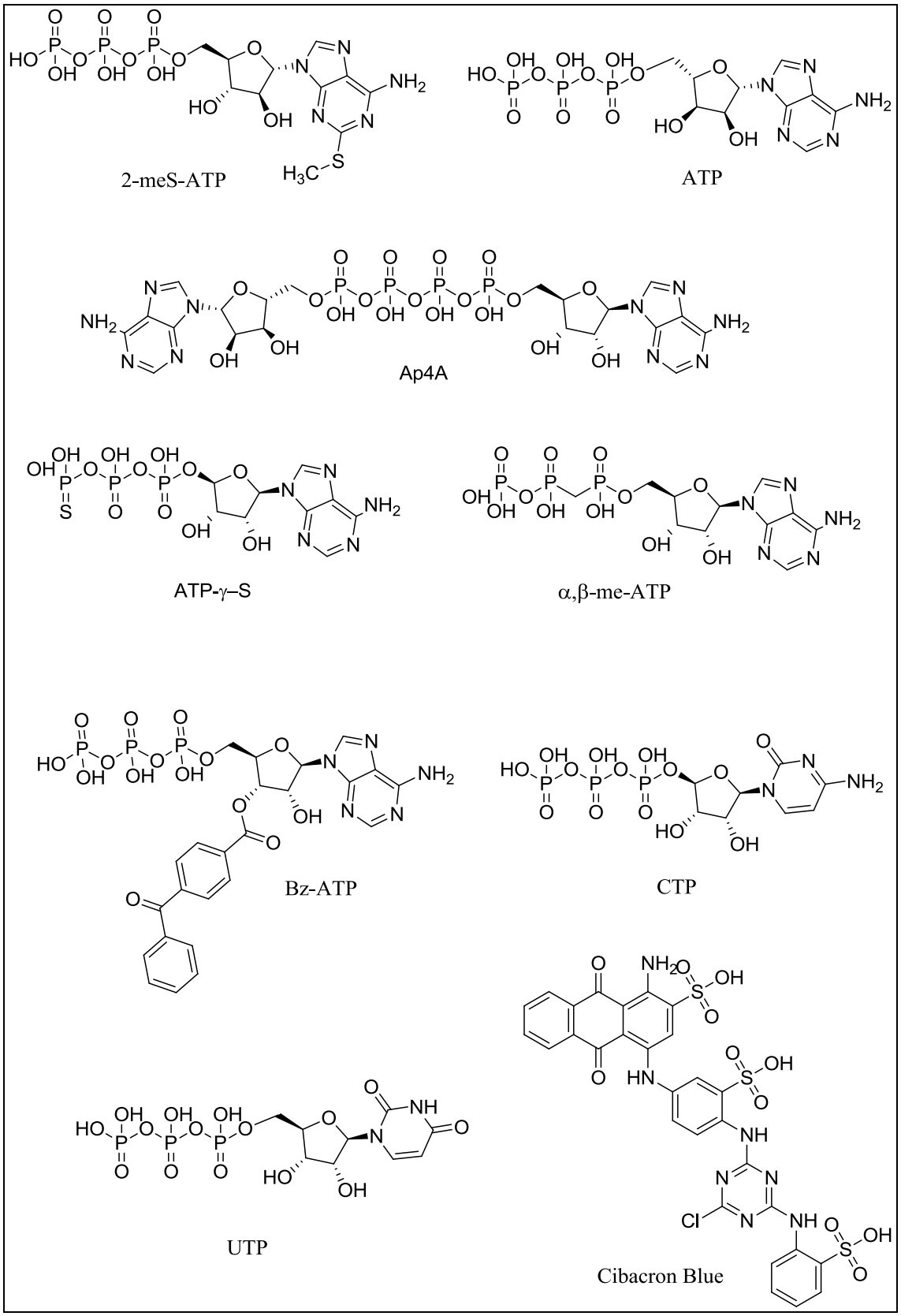


Figure 8: Chemical structures of selected P2X3 agonists.

Antagonists at P2X3 receptor

Large progress has been made in development of P2X3 antagonists in recent years.[20] Although there has been comprehensive literature describing the role of P2X3 receptors in various physiological and pathophysiological conditions the potential of P2X3 receptor antagonist as therapeutic agents has been limited by a lack of selective and potent drug-like molecules.[43]

However, an orally bioavailable antagonist of P2X3 receptors called AF-219 (diaminopyridine derivative with undisclosed structure) has successfully completed phase II clinical trials for chronic idiopathic cough, visceral pain and osteoarthritis of the knee.[3,14,15]

A-317491 is the first non-nucleotide antagonist with high selectivity and affinity for blocking homo- and heterotrimeric P2X3 channels. *R*-enantiomer (A-317344) showed significantly lower P2X3 blocking activity than *S*-enantiomer A-317491.[19] A-317491 binds to the extracellular part of P2X3 receptor as a competitive antagonist. However, high polarity of this compound disables its therapeutic usage because of low bioavailability and high plasma-protein binding.[3]

After systematic delivery of A-317491 either intrathecally or into the hindpaw in rats antinociceptive effect of chronic inflammatory and neuropathic pain was observed. Intrathecal administration was reported as more effective in reducing allodynia following peripheral nerve injury. These data proved that P2X3 and P2X2/3 receptors have significant effect on nociception.[40]

A-317491 as well as ATP structure analogue TNP-ATP interact with amino acids within the agonist binding pocket of P2X3 receptor, which is the place for binding agonists such as natural agonist ATP and its synthetic analogue α,β-methylene ATP.[21]

Trinitrophenyl ATP analogue TNP-ATP acts as an antagonist at P2X1, P2X3 and P2X2/3 receptors and is much less potent in cells expressing P2X2, P2X4 and P2X7 receptors.[8,22] TNP-ATP was previously judged to be a noncompetitive antagonist but re-examinations showed that TNP-ATP act as a competitive antagonist.[15]

Its IC_{50} value in cells expressing homomeric P2X3 receptor is close to 1 nM. TNP-ADP, TNP-AMP and trinitrophenyl-substituted guanosine triphosphate analogue TNP-GTP are of almost the same potency as TNP-ATP (1.3 nM, 2.9 nM, 0.4 nM, respectively).[22] However, TNP-ATP is in vivo rapidly metabolized, which limits its utilization as P2X3 antagonist.[20] Polyacidic functional groups present in TNP-ATP molecule limit bioavailability and potential as drug candidate.[43]

Trimethoprim derivative, AF-353 (formerly named as RO-4), is an antagonist of P2X3 receptors, both homomeric and heteromeric, human and rat.[3,20] Interestingly, antibacterial substance trimethoprim did not show any blocking activity at P2X3 receptor at high concentration of 30 μM. Conversely, IC_{50} value of AF-353 is 6 nM for human P2X3.

AF-353 has high potency at and high selectivity for P2X3 receptor and is likely to bind to allosteric site of ionotropic channel. Experiments proved his oral bioavailability in dogs and its penetration to brain.[3,41] AF-353 has equal affinity in nanomolar range to both human and rat P2X3 receptor and marginally lower affinity to heterotrimeric P2X2/3. Its attractive pharmacokinetic profile, which is shown in Figure 9, is suitable for *in vivo* studies.[41]

Parameter	*Result*	
	AF-353	*A-317491*[a]
Bioavailability (*F*)	32.9%	0%
Time to peak plasma concentration (T_{max})	0.5 h	NA
Plasma half-life ($T_{1/2}$)	1.63 h	NA
CNS penetration (brain/plasma ratio)	6	0
Plasma protein binding (bound)	98.2%	99.9%

[a]Estimates of bioavailability, CNS penetration and plasma protein binding were conducted at Roche Palo Alto using the same protocols as those used for AF-353 and employing powders synthesized in the research site's Department of Medicinal Chemistry.

Figure 9: Pharmacokinetic parameters of AF-353 and A-317491 P2X3 antagonists in rat.[41]

Diaminopyrimidine class of P2X3 antagonists comprises not only AF-353 but also RO-3 (IC_{50} value of 100 nM) and AF-353 close structural analogue, AF-010, that was previously called RO-10.[20,39,41] It is assumed that AF-010 similarly to AF-353 binds allosterically to ATP binding site of P2X3 receptor, because they did not behave in a simple, reversible competitive manner.[41]

Dual P2X3 and P2X2/3 antagonist AF-906, also known as RO-51, was created by a change in AF-353 molecule which led to a more polar molecule.[3,42] With its IC_{50} value

of 2 nM it is more active at hP2X3 than AF-353 itself.[3] At concentration up to 10 μM any antagonist activity of AF-906 was exhibited at P2X1, P2X2, P2X4, P2X5 and P2X7 receptors.[42]

Diinosine pentaphosphate (Ip_5I) is a potent antagonist at P2X1 and quite active at homomeric P2X3 but not on heteromeric P2X2/3 receptors, which could be a useful tool to clarify P2X receptor subunit composition of native P2X receptors. Micromalar concentration of Ip_5I caused inhibition of response to ATP with IC_{50} value of 2.8 μM in *Xenopus* oocytes.[44]

Another selective P2X3 receptor antagonist, thienoperazole derivative RO-85, showed 10-fold higher potency at rat P2X3 than at human P2X3, 89 % peroral bioavailability with 1.6 hour half-life was observed in rats.[3,43] RO-85 molecule showed high *in vitro* stability, 89 % protein binding and no CYP450 isoenzymes inhibition was detected at concentrations lower than 15 μM.[43]

AF-015 is well water-soluble P2X3 antagonist with appropriate pharmacological properties and IC_{50} value of 20-30 nM at human P2X3 receptor. The compound was efficient in reducing pain in rat model.[3] A related compound, MK3901, was reported as a selective P2X3 antagonist which IC_{50} reached 23 nM. It also penetrates into central nervous system and reduces neuropatic and inflammatory pain in several animal models.[3,29]

Pyridoxalphosphate-6-azophenyl-20,40-disulfonate (PPADS), a nonselective P2X antagonist, was extensively modified to achieve stable, potent and selective P2X3 antagonists.[46] This compound is (pseudo)irreversible P2X blocker with a chemical structure of large polyanions.[21,29] During its modification undesirable anionic groups were deleted or replaced by carboxylic acids. It was reported that PPADS successfully reduced the excitability of dorsal root ganglia neurons and neuropathic pain.[46]

Heptapeptide spinorphin showed potent antagonism at the human P2X3 receptor acting as allosteric modulator. The compound was reported to display an IC_{50} value of 8.3 pM in *Xenopus* oocytes.[47] Modification of spinorphin structure, for instance amino acid substitutions, always resulted in less potent antagonists.[3,47]

Suramine, the nonselective P2 antagonist, caused antinociceptive effect in rodents. The potential of suramin consist in modification of its structure. NF110 was reported as the first derivative which is significantly more potent antagonist at rat P2X3 but of almost the same potency at rat P2X1 receptors. NF 110 acting as competitive antagonist reached IC_{50} value of 90 nM.[48]

Other described P2X3 antagonists include Phenol Red (phenolsulphonephthalein sodium), which antagonise also P2X1 receptor.[49] Chemical structures of selected P2X3 antagonists are shown in Figure 10.

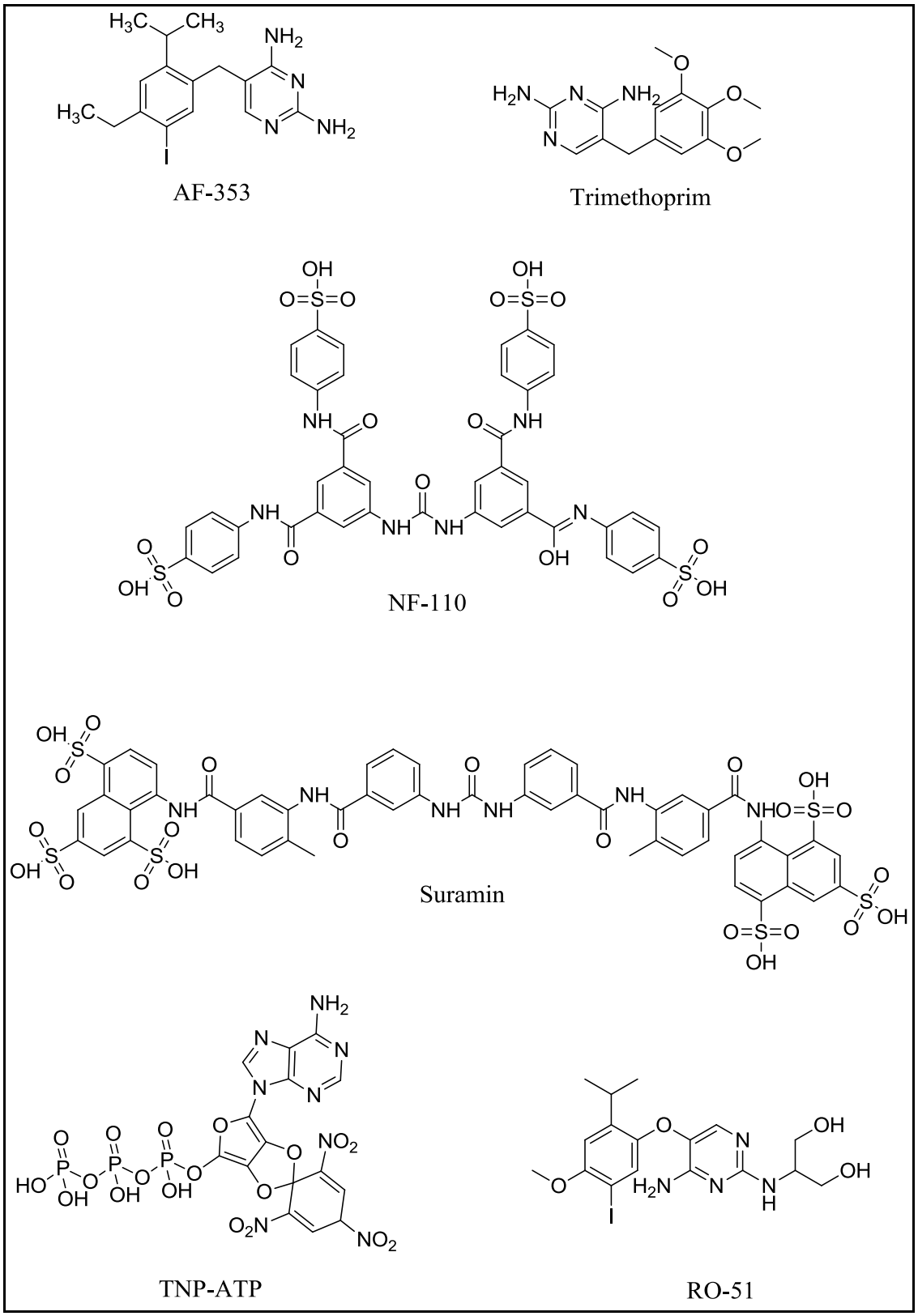


Figure 10: Chemical structures of selected P2X3 antagonists.

2.2 Methods to study P2X receptor ion channels activity via calcium measurement

Ion channels are necessary for many physiological processes throughout the body, they are pharmacologically accessible and thus they are a good drug targets. To screen the influence of different compounds on function of ion channel it is necessary to measure the channel function in a relevant biological context, for instance in a cell. Assays should be fast and sensitive and ideally compatible with automated high-throughput screening (HTS).[50]

Majority of the P2X receptor activity studies have been done using current measurements. Disadvantage of the current measurement comprises the possibility to measure one cell in one experiment and slow receptor recovery makes it difficult to perform repeated measurement.[53]

Two main approaches to measure P2X receptor activity are further described.

2.2.1 Calcium imaging assay

When P2X3 receptor is stimulated, ligand-gated ion channel opens and intracellular calcium level is increased.[15] Calcium release occurs within seconds after receptor activation. Fluorescence indicators with their high sensitivity are capable to determine changes in intracellular calcium level upon receptor activation.[50,52] Calcium assay is a widely applied technique and frequently used medium-throughput reverse pharmacology assay.[52]

The process starts with incubation of cells with calcium assay dye. During this period of time the indicator passes through the cell membrane into intracellular part where its acetoxymethyl (AM) group is cleaved off by esterases.[51] The AM ester group influences the properties of the molecule so that it enables the fluorescent dye to cross the membrane into intracellular part. Fluorophore is after esterification negatively charged which prevents it to diffuse out to extracellular matrix.[52]

After incubation period (usually approximately one hour) the cells are ready to be assayed. When ion channel opens and calcium enters the cell, Ca^{2+} sensitive dye binds to calcium and emits fluorescence.[50,51] Signal is detected upon receptor activation for every well in an assay plate by the Fluorescence Imaging Plate Reader (FLIPR), NOVOstar or FlexStation (Figure 11).[50,51,52]

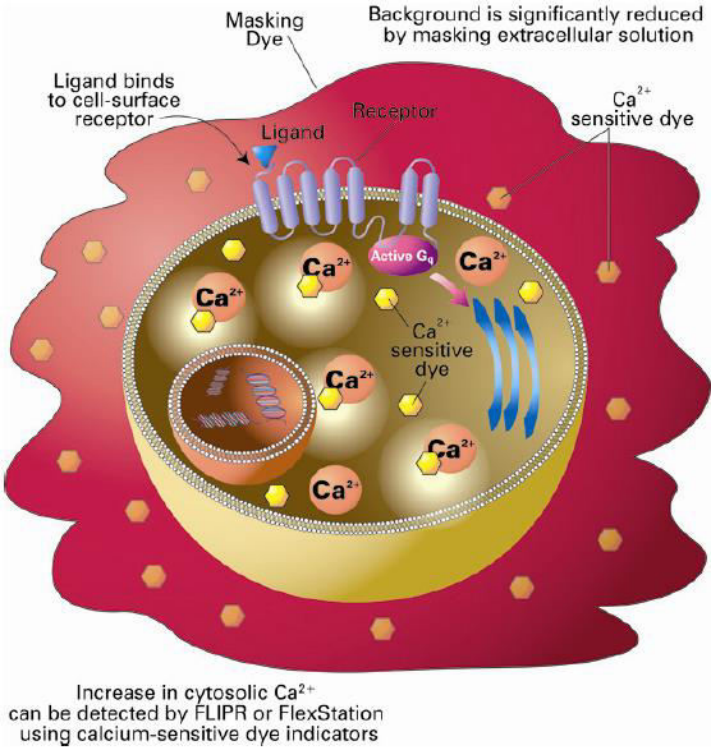


Figure 11: Principle of calcium assay. Once Ca^{2+} enters the intracellular part sensitive dye binds and emits fluorescence which is detected.[51]

The relative fluorescence unit (RFU) values are measured in each well of the plate separately starting before the addition of compounds (agonist) and then continuing. The RFU values can be analyzed and converted into concentration-response curves and EC_{50} value, IC_{50} value, calcium increase, percentage inhibition and many others values can be determined. Positive and negative control is highly recommended to be used.[52]

2.2.2 Patch-clamp electrophysiology

Since the patch clamp electrophysiology was discovered at the end on 1970s, there has been an increasing interest in its application to investigation of ion channels.[54] Patch-clamp recording is a gold standard for biophysiological characterization of compound action enabling a detailed characterization of ion-channel gating and the evaluation of permeability and drug interactions. The technique with its high sensitivity can measure activity of a single ion channel. The major disadvantage of patch-clamping is its difficulty to be automated for HTS.[50]

The procedure of patch clamping starts with an isolation of a patch of membrane and sealing of the pipettes (glass or quartz, containing electrolyte) with a blunt end onto the membrane. Suction is applied and thus isolates the membrane patch electrically. Currents fluxing through the channels hence flow into the pipette and are recorded by an electrode connected to a highly sensitive differential electronic amplifier (Figure 12).[55]

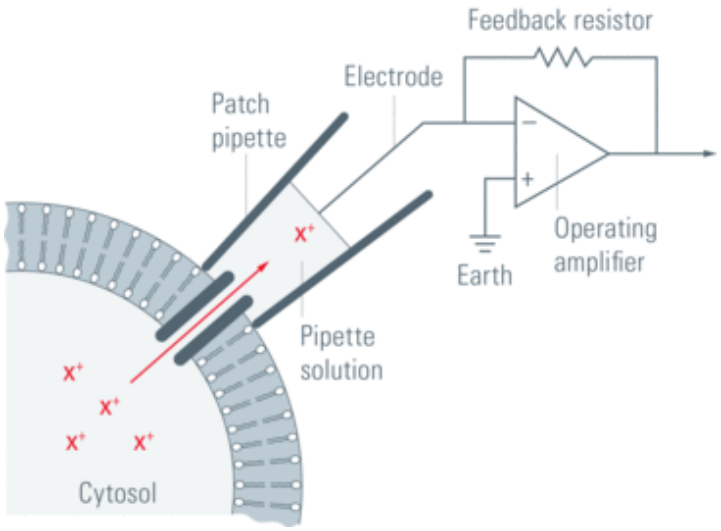


Figure 12: General principle of patch clamping.[55]

Successful combinations of patch clamping with other techniques such as calcium fluorescence or single-cell contraction are profitably used.[54]

3 Experimental part

3.1 Equipment

Autoclave	Systec VX-95
Balance	Sartorius CPA225D
Centrifuge	Allegra™ 21R Centrifuge, Beckman Coulter™
	Avanti™ J-20 I Centrifuge, Beckman
	Hettich zentrifugem Mikro 200R
Counting chamber	Merienfeld-Superior Germany, Neubauer
Electrophoresis machine	Power Pac 300, BioRad
Heated ultrasonic bath	Bandelin SONOREX™, RX52H
Heater	Heidolph MR 3001
Incubators	Memmert
	Binder, 15-11299
Laminar-air flow	Nalge NUNC International Bio-flow safety cabinet
Microplates	Corning® CellBIND Surface 3340 assay plate
	Rotilabo®- Mikrotest-Platten, V-Profil, Roth
Microscope	Axiovert 25, Zeiss
Microplate readers	NOVOstar, BMG Labtech 6mbH
	FlexStation3, Molecular Devices
Microwave	Severin Microwave 800
Multipette	Eppendorf Multipette® Plus
Pipettes	Eppendorf Research
Shaker-thermostat	Sky Line, ELMI
Sterile filter	Filtropur S 0.2
Thermocycler	Biometra® Tpersonal
Thermomixer	Eppendorf Thermomixer comfort
Vortexer	IKA®Vortexer Genius 3
	VWR VV3
Water bath	Memmert

3.2 Materials

3.2.1 Chemical substances

2-me-S-ATP	Tocris Bioscience, 1062
5X Q5 reaction buffer	BioLabs, B9028A
5X QS High GC Enhancer	BioLabs, B9028A
A-317491	Sigma, A2979
ADP	Sigma, A2754
AF-353	Afferent Pharmaceutials
Agarose	LONZA Sea Kem®LE
AMP	Acros, 61198
Ampicilin	Roth, K029.1
Ap4A	Sigma, A2979
ATP	Roth, HN35.2
BSA	BioLabs, B90019
BzATP	Sigma, B6396
Calcium assay dye	Calcium 5 assay Kit, Flipr, R8186
Calcium chloride dihydrate	Fluka, 21097
Cibacron Blue	Alexis Biochemicals, 550-293G005
CTP	Sigma, C1506
Cut Smart Buffer	BioLabs, B7204S
D-(+)-Glucose	Sigma, G-7021
Dimethylsulfoxid (DMSO)	Roth, 355228403
Disodium phosphate dihydrate	Roth, 4984
DMEM	Gibco®by life technologies™ 1722264
DNA Ligase Buffer	BioLabs, B02025
DNA Loading Dye	Thermo Science, R0611
dNTPs	MBI Fermentas, R0241
Drug X	Pharma Zentrum Bonn
EcoR1	Neb, R3101S
EDTA	Sigma E7889
Ethanoic acid	Merck, 1.000.631011
Ethanol	Merch, 64-17-5

Fetal Carf Serum (FCS)	Sigma-Aldrich, F7524
G418	Biomol, 24016-03-3
Gel Red™	Biotium, 41003
Glycerol	Acros
Hepes	Fluka, 54461
Isopropanol	ZVE Universität Bonn, 123903
LB agar	Roth, X9653
LB medium	Roth, X9618.1
Lipofectamine	Invitrogen, 11668-018
Magnesium sulphate	Sigma, M2643
NF110	Tocris Batch, 2A/115205
Not1HF	NEB, R3189L
Nuclease-free H_2O	Bio-Rad, 720001208
PBS	Sigma, D8537
Penicillin + Streptomycin	Gibco, 15140
Phi marker	BioLabs, N3026L
Polybrene	Aldrich, 10,768-9
Potassium chloride	Applichem, 2939
Potassium hydrogen phosphate	Roth, P749.1
Sodium bicarbonate	Sigma, S-5761
Sodium chloride	Roth, 3957.1
Suramin	Sigma, S2671
T4 DNA Ligase	Thermo Scientific, L0011
THV B01	Pharma Zentrum Bonn
THV B05-2	Pharma Zentrum Bonn
TNP-ATP	Tocris Bioscience, 2464
Trimethoprim	Pharma Zentrum Bonn, PZB20413133
Tris	Applichem, A2264
Trypsin	Pan Biotech™ for cell culture, 9330913
UTP	Sigma, 94370
YB 038	Pharma Zentrum Bonn
YB 087	Pharma Zentrum Bonn
YB 106	Pharma Zentrum Bonn
YB 107	Pharma Zentrum Bonn

YB 120	Pharma Zentrum Bonn
YB 149	Pharma Zentrum Bonn
YB 160	Pharma Zentrum Bonn
α,β-me-ATP	Tocris Bioscience, 3209
Λ marker	Thermo Scientific, SM0191

3.2.2 Buffer and culture media

50X Tris-acetate-EDTA (TAE) Buffer

242 g Tris was dissolved in distilled water, 57.1 ml pure acetic acid and 100 ml 0.5 M EDTA (pH 8.0) was added and filled up with distilled water to 1 litter. Afterwards, the pH was adjusted to 8.3.

1X TAE Buffer

40 ml of 50X TAE electrophoresis buffer was diluted with 1460 ml distilled water and mixed properly.

Hanks buffer solution (HBSS), pH 7.3

Hepec (4766 mg/l), NaCl (8000 mg/l), Glucose (1000 mg/l), $MgSO_4$ (92 mg/l), $CaCl_2.2H_2O$ (186 mg/l), $NaHCO_3$ (350 mg/l), KCl (400 mg/l), K_2HPO_4 (77 mg/l), $Na_2HPO_4.2H_2O$ (49 mg/l) were dissolved in autoclaved water and the pH was adjusted to 7.3 using 1N NaOH (40 g of NaOH dissolved in distilled water and make up to 1 liter gives 1 N NaOH).

HXM Medium

Penicillin (100 U/ml), streptomycin (100 µg/ml), hygromycin B (50 mg/ml), xanthine (10 mg/ml), hypoxanthin (10mg/ml) were added into 500 ml Dulbecco's Modified Eagle Medium (DMEM).

Medium for hP2X3 S15V 1321N1 cells

Penicillin (100 U/ml), streptomycin (100 µg/ml), G418 (800 µg/ml) and FCS (to final concentration of 10 %) were added into 500 ml DMEM media.

3.2.3 Cell lines

1321N1 human astrocytoma cells

GP+envAM12 packaging cells

3.2.4 Software

ChemDraw Ultra 12.0

GraphPad Prism 5.01

NovoSTAR 1.20.0

Quantity One 4.4.0

SoftMax Pro 5.4.1

3.3 Methodics

Experimental part of this master thesis was realized at the University of Bonn, Pharmaceutical Institute in the period of time from 1. 10. 2015 to 29. 2. 2016. Individual partial steps are simplified summarized in Figure 13.

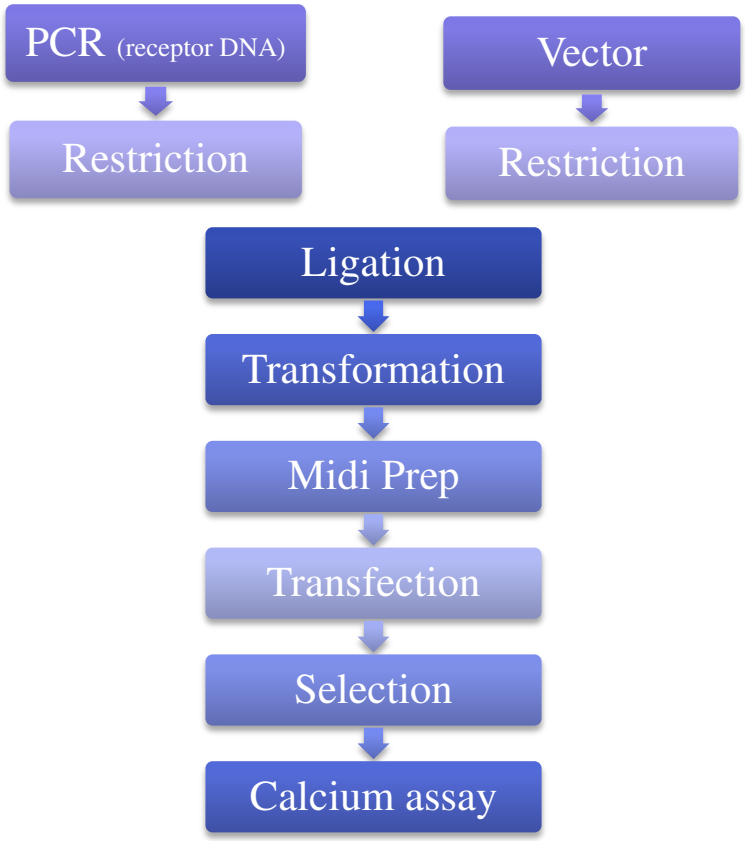


Figure 13: Simplified schematic representation of experimental part.

3.3.1 Preparation of stable P2X3 S15V 1321N1 cell line

3.3.1.1 PCR primers

Forward and reverse primers were designed as showed in Table 1. Melting temperature (T_m) of forward primer was 60 °C, T_m of reverse primer was 58 °C.

Primer	Sequence (5´-› 3´)
Forward	GTGACAGCGGCCGCATGAACTGCATATCCGACTT
Reverse	CTTACTAGAATTCCTAGTGGCCTATGGAGAAG

Table 1: DNA sequences of forward and reverse primers.

3.3.1.2 Polymerase Chain Reaction (PCR)

Polymerase chain reaction was performed to amplify template DNA of hP2X3 S15V receptor to produce thousands to billions of copies of this specific DNA sequence. At 98 °C the template DNA denaturates, forward and reverse primers start the multiplication of new strand which is elongated by DNA polymerase in the presence of nucleotide triphosphates.

Master mix was prepared:

5X Q5 reaction buffer	10 µl
dNTPs	5 µl
Brand (forward) primer (10 µmol/ml)	2.5 µl
Reverse primer (10 µmol/ml)	2.5 µl
5X QS High GC Enhancer	10 µl
DNA template	1.5 µl
QS Hot start high fidelity DNA polymerase	0.5 µl
Nuclease-free H_2O	18 µl

Three samples with DNA template and one with H_2O instead of DNA template were prepared. Final volume in each sample was 50 µl.

Polymerase chain reaction was carried out in thermometer with these requirements:

1. 98 °C for 4 minutes – initialization – heat activation
2. 98 °C for 1 minute – denaturation
3. 60 °C for 1 minute – annealing – primers anneal to the single stranded DNA
4. 72 °C for 2 minutes – elongation – DNA polymerase synthesizes complementary DNA strand in 5' to 3' direction
5. 72 °C for 10 minutes – final elongation – to ensure the single-stranded DNA is fully extended
6. 4 °C for the rest of the time – short time storage

Steps 2 to 4 were repeated thirty times.

3.3.1.3 Gel electrophoresis

For gel electrophoresis 1% agarose gel was used. Agarose in an amount of 250 mg was put into the conical flask and 25 ml of 1X TAE buffer was added. Mixture was heated in microwave oven until agarose was fully dissolved and the mixture completely transparent. Then 1.25 µl of Gel Red was added and mixture was poured into gel rack and the comb was inserted at one side of the gel.

When the gel became completely stiff, the comb was removed and the gel was placed into electrophoresis apparatus. TAE buffer was poured into the apparatus to cover the gel completely. DNA samples mixed with DNA Loading Dye were pipetted into the wells at one side made by comb. Apparatus was covered with the lid and the current was applied across the gel (100 A).

DNA is always negatively charged, therefore it is necessary to connect cathode, which has negative charge, closer to the sample so that DNA can migrate toward the anode which is positively charged. The smaller the DNA fragments are, the faster and farther they migrate through gel.

The visualization was made under UV light using BIO RAD machine and computer program Quantity One.

3.3.1.4 DNA cleaning

All samples except negative control were placed together to an Eppendorf tube and cleaned using DNA Clean Concentrator^{TM} – 5 following its protocol. Ultra-pure DNA was obtained.

3.3.1.5 Restriction of vector pQCXIP and PCR product

Two enzymes were used for restriction, namely EcoR1 HF and Not1 HF. Both of these enzymes produce DNA with “sticky” ends and were used to restrict PCR product as well as vector.

Restriction of PCR product:

BSA	2 µl
Cut Smart Buffer	2 µl
Nuclease-free H_2O	3 µl

PCR product	10 µl
Enzyme EcoR1 HF	1 µl
Enzyme Not1 HF	1 µl

Restriction of Vector pQCXIP:

BSA	1 µl
Cut Smart Buffer	1 µl
Nuclease-free H_2O	6 µl
Vector pQCXIP	1 µl
Enzyme EcoR1 HF	0.5 µl
Enzyme Not1 HF	0.5 µl

These mixtures were heated at 37 °C for one hour without shaking.

3.3.1.6 DNA recovery

To recover DNA fragments the extraction from an agarose gel was done. DNA fragments were visualized under UV light and desired DNA was cut out and transported into Eppendorf tubes. Recovery using Zymoclean™ Gel DNA Recovery Kit according to its protocol followed.

3.3.1.7 Ligation

Ligation was used to connect two nucleic acid fragments to create recombinant DNA molecules through the action of an enzyme T4 DNA Ligase.

PCR product	10 µl
Vector	4 µl
ATP	2 µl
Ligase Buffer	2 µl
T4 DNA Ligase	1 µl
Nuclease-free H_2O	1 µl

These compounds were mixed together and the reaction was performed at 16 °C overnight.

3.3.1.8 Transformation of ligation product and VSV-G DNA (glycoprotein G of the Vesicular stomatitis virus)

Whole amount of ligate product (or 1 µl of VSV-G DNA) was put to 100 µl of Top 10 recombinant bacteria, gently mixed and incubated in ice for 30 minutes. Heat shock followed after 30 minutes when the tube was put into water bath at 37 °C for 1 and half to 2 minutes and then transferred to ice for 2 minutes. Afterwards the LB medium without ampicillin in an amount of 300 µl was added. Mixture was incubated at 37°C for 1 hour with shaking (about 250 rpm).

The tube content was spread into agar plate with ampicillin using pipette. When the plate becomes dry it was placed upside down in the incubator at 37 °C with shaking overnight.

Few colonies (only one colony for VSV-G transformation product) were selected next day and put into a tube containing 5 ml of LB medium with ampicillin (100 µg/ml) and incubated at 37 °C with shaking overnight.

3.3.1.9 DNA preparation

Miniprep method allows extraction and purification of plasmid DNA. ZR-Plasmid Miniprep™ Kit – Classic was used according to its protocol. Acquired DNA was then restricted and sent for sequencing by GATC-biotech.

Restriction was performed mixing these compounds followed by incubation at 37 °C without shaking.

BSA	1 µl
Cut Smart Buffer	1 µl
Nuclease-freeH_2O	4 µl
DNA	3 µl
Enzyme EcoR1 HF	0.5 µl
Enzyme Not1 HF	0.5 µl

One millilitre of each bacteria suspension was stored in a fridge to allow later multiplication of desired colony. High pure plasmid DNA was obtained using PureLink™ HiPure Plasmid Filter Midiprep Kit following its protocol, purified DNA was stored at -20 °C.

3.3.1.10 Freezing of bacteria

Bacteria can be frozen to produce backups. 99% sterile glycerol in an amount of 200 µl is mixed with 800 µl of overnight bacteria suspension containing desired plasmid in an 1.5 ml Eppendorf tube. To homogenize this mixture the tube was gently inverted several times and then put into -20 °C freezer and after 24 hours moved to -80 °C freezer.

3.3.1.11 Counting chamber

To determine the number of cells in a certain volume of cell suspension the counting chamber was used.

$$\text{Cells per 1 µl} = \frac{\text{Number of cells}}{\text{Counted area (in mm²)x chamber depth (mm)x dilution}}$$

Counted area in our case was 1 mm^2, chamber depth 0.1 mm and dilution was not performed. The equation is thus simplified to

$$\text{Number of cells per 1 ml} = \text{Number of cells} \times 10^{4.56}$$

3.3.1.12 Retroviral gene transfection

Cultivation of packaging cells

GPenvAM12 packaging cells were quickly thawed and transferred into small flask with 5 ml of DMEM medium containing 10 % FCS and PS (penicillin 100 U/ml and streptomycin 100 µg/ml). When confluent, cells were transferred into a middle flask and later into large flask with HXM medium. When packaging cells become 70-80 % confluent, they were put into Falcon Tube and counted in a counting chamber.
For transfection 1.5 x 10^6 cells are required. Appropriate volume of cell suspension was put into small flask with medium (DMEM, FCS, PS).

In parallel, target cell line was thawed and cultivated in DMEM medium containing FCS and PS only.

Retrovirus production

In 1 ml tube 600 µl of DMEM was mixed with 25 µl of Lipofectamine 2 000 (587.5 µl DMEM and 37.5 µl Lipofectamine 2 000 for 15 µg DNA) and incubated at room temperature for twenty minutes. Lipofectamine 2 000 is cationic reagent which provides high transfection efficiency by forming complex with negatively charged DNA and thereby allowing DNA to overcome the electrostatic repulsion of negatively charged cell membrane and DNA can penetrate into intracellular space.[57]

For transfection 10 µg of plasmid DNA (6.25 µg of hP2X3 S15V receptor DNA and 3.75 µg of VSV-G protein DNA) is needed. Amount of DNA can be increased to 15 µg in case of insignificant expression rate. The tube with DNA was filled with DMEM without any additive to final volume of 625 µl.

After 20-minute incubation lipofectamine-DMEM mixture was together with DNA transferred into a small flask with adhered confluent packaging cells inside, old medium was previously removed.

After approximately 15 hours medium was changed for 3 ml of DMEM medium containing FCS and PS only and 30 µl of sodium butyrate was added. Sodium butyrate is used to increase basal expressions rates of stably transfected cell lines.[58] Afterwads, flask was placed into incubator at 32 °C for 48 hours. In this period of time, retroviruses leave the packaging cells and higher stability of retroviruses at 32 °C than at 37 °C was observed.[59]

One day before infection target cells were counted and split into flasks to achieve 5 x 10^5 cells per flask.

Target cell infection

Virus particles were separated via sterile filtration of liquid from packaging cells flask through 0.20 µm sterile non-pyrogenic filter. Into 3 ml of filtered liquid 6 µl of polybrene (4mg/ml in H_2O, sterile filtered) was added. Polybrene is a polycation that increases the transfection efficiency when glycoprotein of vesicular stomatitis virus (VSV-G) is used. VSV-G allows GPenvAM12 packaging cells to produce infectious viruses and is dependent on the presence of Polybrene.[60]

For successful transfection the ratio between DNA, target cells and viruses needs to be optimal. Therefore, we mixed viruses (produced using different amount of DNA, 10 µg and 15 µg) and target cells in three different ratios (Table 2).

DNA (µg)	Viruses solution (ml)	Target 1321 N1 cells (ml)
10	2.5	0.5
15	1.6	1
10	1	1

Table 2: Ratio between DNA used for viruses production, viruses and target cells used for transfection.

Culture medium was discarded from flasks with target cells and replaced by liquid containing virus particles. After 2.5 hour incubation at 32 °C medium was changed (DMEM, FCS, PS) and later, after 72 hours medium was replaced by medium containing antibiotic geneticin (G418) extra (DMEM, FCS 10%, penicillin (100 U/ml), streptomycin (100 µg/ml), G418 (800 µg/ml)). G418 performed the selection of cells that were successfully transfected[61] by DNA carrying the information not only of the hP2X3 S15V receptor but also the gen for G418 resistance. Incubation at 37 °C 10 % CO_2 followed.

The selection medium was changed several times after 24 or 48 hours to remove dead cells. G418 blocks the protein synthesis and therefore the selection is achieved within several days.[62]

3.3.2 Cell culture

3.3.2.1 Cells thawing

The cryovial containing frozen cells was put into water bath at 37 °C. When the suspension was completely melted, it was transferred into a middle flask with about 15 ml DMEM medium containing FCS and PS. Flask was put into an incubator at 37 °C, 95% humidity and 5 % CO_2 for few hours.

After few hours when the cells were attached the medium was changed for selection DMEM medium containing FCS, PS and G418. Dead cells did not attached and were removed with old medium.

3.3.2.2 Splitting cells

When cells in flask are approximately 70 to 80 % confluent there is a necessity to split them. After removing the culture medium with pipette the cells were washed with 10 ml of PBS to get rid of the remaining residual of old culture medium. To detach cells attached to the bottom of the flask 3 ml of trypsin was added. Incubation at 37 °C for around five minutes followed. These conditions increase enzymatic reactivity of trypsin and fasten the reaction.

To separate clusters of cells and to detach still attached cells it is possible to gently hit the flask from the side with hand. The reaction was stopped by adding about 7 ml of medium. The cell suspension needed to be mixed with pipette to stop the reaction in every part of the flask. The needed volume of cell suspension was transferred to new sterile flask with culture medium (approximately 20 – 25 ml) and mixed gently. The volume of cells suspension affects the speed of growing the cells to the confluent state.

3.3.2.3 Freezing cells

Freezing of cells is performed to create backups. After removing medium and washing with 10 ml of PBS reaction with trypsin was carried out in incubator at 37 °C for 5 minutes. Approximately 7 ml of medium was added to cell suspension to stop the reaction. This suspension was transferred to 50 ml Falcon Tube and refilled to a volume of 30 ml and then centrifuged at 200 x g for 5 minutes at 4 °C.

Supernatant was discarded and the cells adhered at the bottom of the tube were resuspended with 4 ml of freezing mixture. Freezing mixture consist of 90 % of FCS and 10 % of dimethylsulfoxid (DMSO). The solution was transferred to 2 ml cryovials making 1 ml aliquots and immediately frozen at -20 °C for one day and then relocated to -80 °C freezer.

3.3.3 Calcium imaging assay

Fluorescence based Ca^{2+} influx assay is a useful tool to identify and characterize endogenously expressed P2X receptors.[25] Calcium imaging assay showed the extent of P2X3 stimulation via intracellular calcium increase and therefore allows to pharmacologically characterize hP2X3 S15V receptor.

The plate had to be prepared one day before planned measurement. Cells were removed from flask as when splitting. Medium was taken away, cells were washed with 10 ml of PBS, 3 ml of trypsin was added and 5-minute incubation at 37 °C followed. Cells were checked under the microscope and clusters were separated by tapping the flask gently. The reaction with trypsin was stopped by adding 7 ml of culture medium.

Cells were counted in a counting chamber, diluted and placed into clear bottom 96-well microplate to achieve the cell density of 25 000 cells per well. One-hour incubation at room temperature and 24-hour incubation at 37 °C 5 % CO_2 followed.

Culture medium was removed and replaced by Calcium 5 assay dye (100 µl per well) and plate was incubated at 37 °C with shaking (125 rpm) for one hour. Dye was then substituted for 180 µl HBSS buffer (or 179 µl HBSS and 1 µl of antagonist dissolved in DMSO) and incubated at room temperature for 30 minutes. Into reagent V-profil plate 50-100 µl of tested agonist (or ATP for antagonist testing) dissolved in HBSS was added. Microplate reader (NovoSTAR, FlexStation) injected and transferred 20 µl into measurement plate and measured fluorescence in each well. Simplified flow diagram shows testing procedure in Figure 14. Settings of NOVOstar reader can be seen in Table 3. Acquired data was analyzed using GraphPad Prism 5.

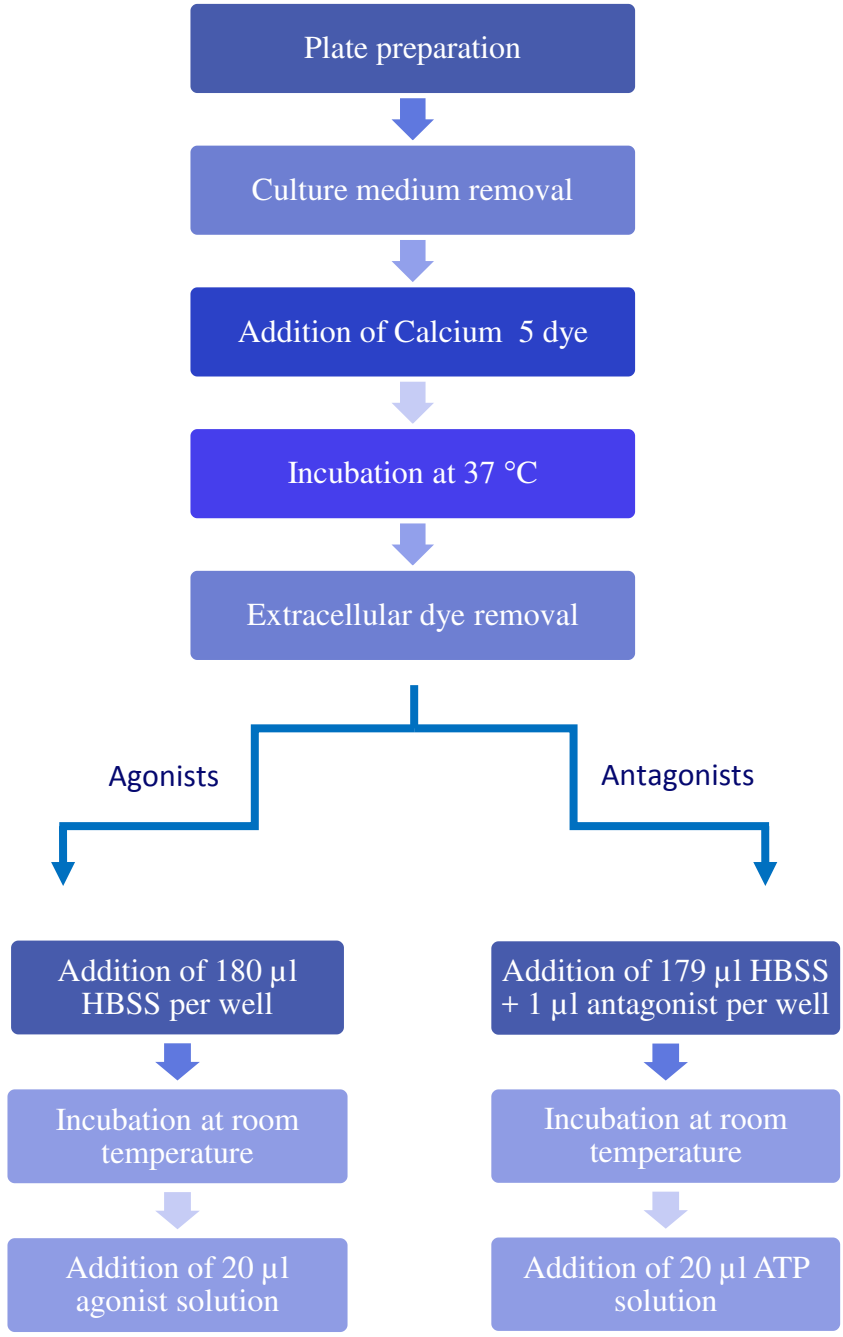


Figure 14: Simplified scheme of calcium-imaging assay.

Parameters	Settings
Gain	variable
Interval time	1.00 s
Number of flashes per well and interval	10
Injected volume	20 µl
Pump speed	65 µl/s
Shaking	no shaking
Injection start time	11.5 s

Table 3: Settings of NOVOstar microplate reader.

4 Results

4.1 Preparation of stable P2X3 S15V 1321N1 cell line

4.1.1 Polymerase Chain Reaction (PCR)

DNA for human P2X3 S15V receptor was multiplied by polymerase chain reaction. PCR was carried out in three Eppendorf tubes, fourth one was used as a negative control. Electrophoresis of PCR products and negative control is showed in Figure 15.

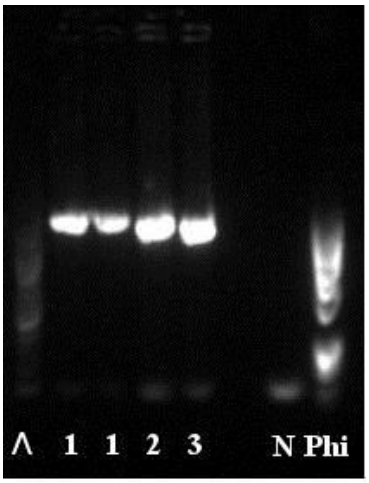


Figure 15: Electrophoresis of PCR products. (Numbers indicate number of sample, N indicates negative control, Λ marker and Phi marker were used as indicators.)

4.1.2 Restriction of vector pQCXIP and PCR product

PCR product and vector pQCXIP were both restricted by the same enzymes to enable their future ligation. EcoR1 HF and Not HF were used in Cut Smart Buffer which is compatible with both enzymes and does not negatively affect their activity. Electrophoresis of both restriction products can be seen in Figure 16.

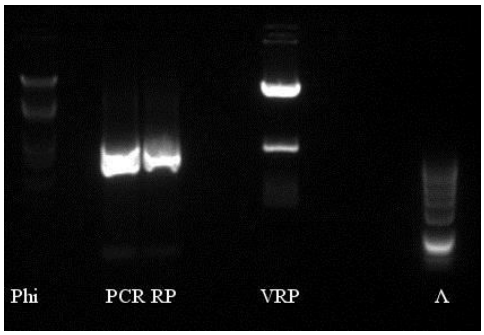


Figure 16: Electrophoresis of PCR restriction product (PCR RP) and vector pQCXIP restriction product (VRP). Phi and Λ markers were used as indicators.

4.1.3 DNA recovery

Desired restriction products were identified and exercised using scalpel from an agarose gel (Figure 17). Visualization of DNA fragments was performed under UV light. Both PCR and vector restriction products were recovered using Gel DNA recovery kit. Pure DNA segments were ligated straight away.

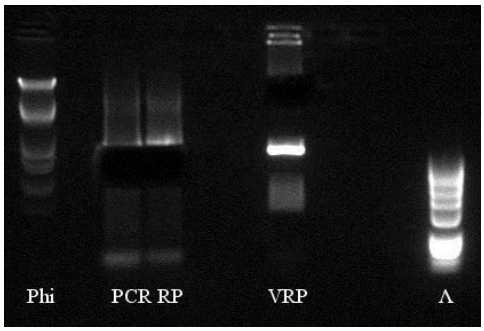


Figure 17: Electrophoresis gel after excision of restriction products.

4.1.4 Ligation and transformation

Ligation of restricted PCR product and vector pQCXIP was performed through activity of enzyme T4 DNA Ligase. Ligate product was then transformated into Top10 recombinant bacteria. Six colonies were chosen and grown in LB medium with ampicillin.

4.1.5 DNA preparation

When the transformated recombinant bacteria were grown plasmids were preparated using miniprep kit. Restriction followed, electrophoresis of restriction products was carried out (Figure 18) and clone 2 and 5 were sent for DNA sequencing by GATC-Biotech. Clone 2 and 5 were chosen because of their different DNA chain length.

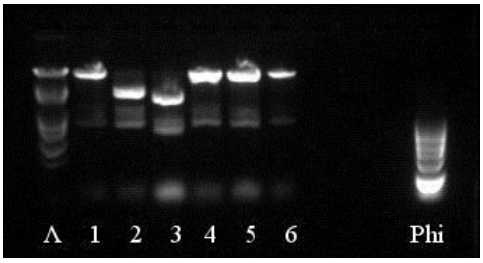


Figure 18: Electrophoresis of restricted ligation products. Numbers indicate number of bacterial colony. Λ and phi markers were used as indicators

Clone 5 was compliant with required DNA.

Bacteria suspension of clone 5 was grown in 100 ml of LB medium containing ampicillin (100 mg/ml) at 37 °C with shaking overnight. Ultra pure plasmid DNA was obtained via midiprep procedure in two concentrations. Electrophoresis of pure DNA is showed in Figure 19. Area that covers plasmid DNA was compared with area of Λ marker and DNA concentration was estimated (200 ng/µl for diluted DNA and 700 ng/µl for concentrated DNA). Plasmid DNA of clone 5 was frozen at – 20 °C and later used for transfection of target cells.

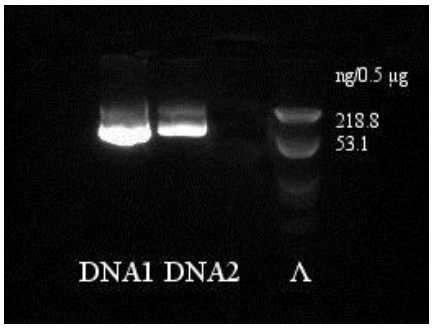


Figure 19: Electrophoresis of ultra pure DNA. DNA1 indicates diluted DNA, DNA2 is concentrated DNA, Λ marker was used as an indicator.

4.1.6 VSV-G transformation

DNA of VSV-G was transformated into Top10 recombinant bacteria. Bacteria were grown in agar plate and one colony was chosen and grown in LB medium containing ampicillin. Ultra pure DNA was obtained via midiprep method in two concentrations. To estimate concentration of VSV-G DNA electrophoresis was carried out (Figure 20).

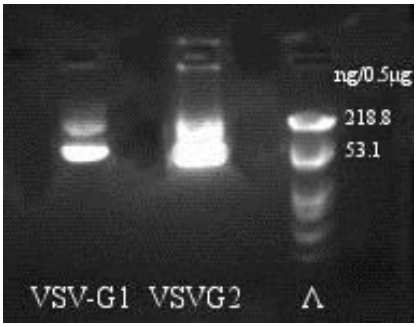


Figure 20: Electrophoresis of ultrapure VSV-G DNA. VSV-G1 marks diluted DNA, VSV-G2 indicates concentrated VSV-G DNA. Λ marker was used as an indicator.

4.1.7 Retroviral gene transfection

The DNA of human P2X3 S15V receptor was inserted into pQCXIP vector, which was together with VSV-G contransfected into GP+envAM12 packaging cells. VSV-G glycoprotein associates with non-infectious retrovirus particles and enables the packaging cells to produce infectious viruses and also enhances the efficiency of transfection when the naked plasmid vector is used.[60]

Human 1321N1 astrocytoma cells were then transfected by retroviruses produced by packaging cells. Transfected cells were selected using antibiotics G418 (800 µg/ml) to produce stable cell line expressing hP2X3 S15V receptor. Retrovirus carried not only hP2X3 S15V DNA but also resistance to G418.

4.2 Calcium imaging assay

4.2.1 Optimizing method

When the transfected cells were selected and become confluent calcium-imaging assay was performed for all three transfection products. The amount of 45 000 cells per one

well was pipette to Corning Cell Bind Surface plate 3340 and using Calcium 5 Assay Dye the effect of ATP agonist was tested.

Each transfection product showed positive stimulation after ATP application. The EC_{50} value of ATP at transfection product 1 (10 µg DNA, 2.5 ml of liquid containing viruses, 0.5 ml target cell suspension) was 0.03011 µM (n=1). See Figure 21.

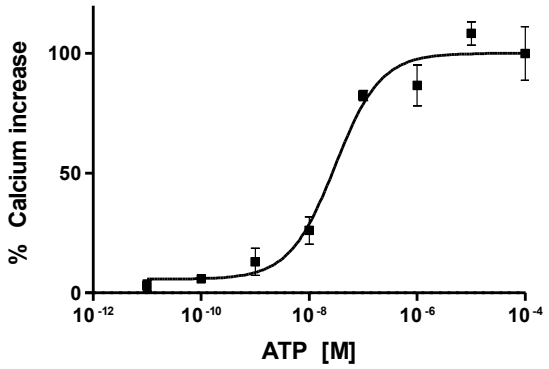


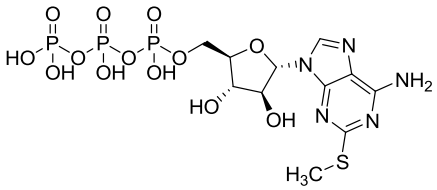
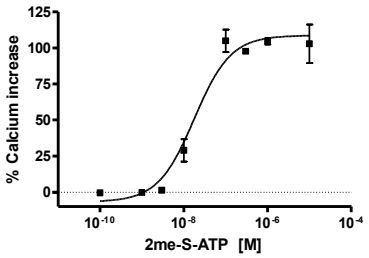
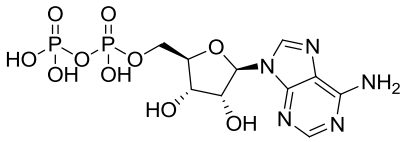
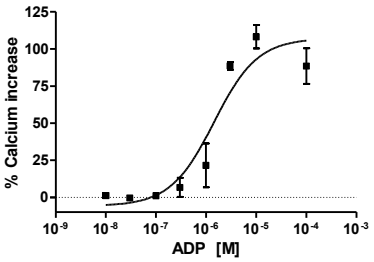
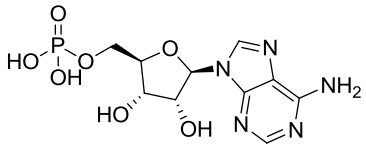
Figure 21: Concentration-response curve – hP2X3 S15V, transfection product one, ATP showed an EC50 value of 0.03011 µM (n=1).

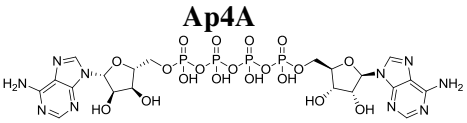
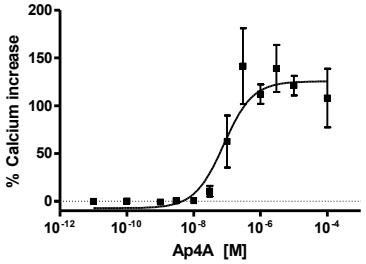
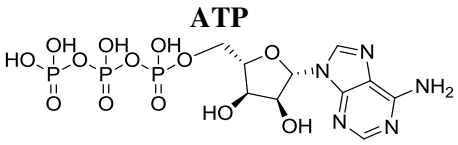
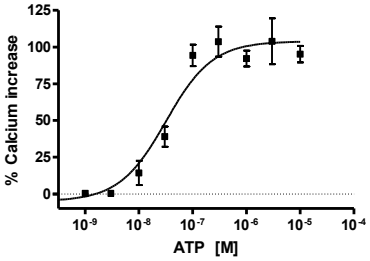
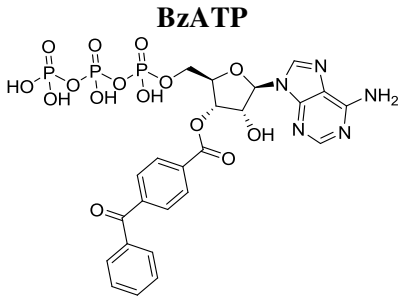
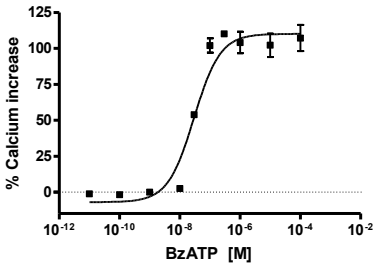
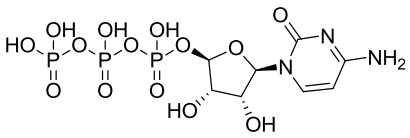
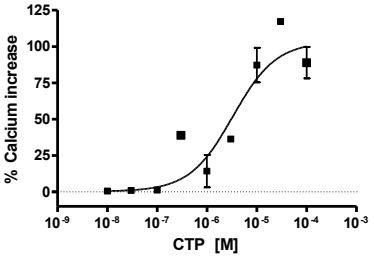
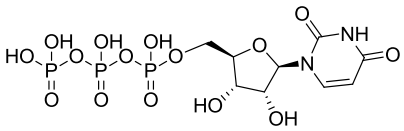
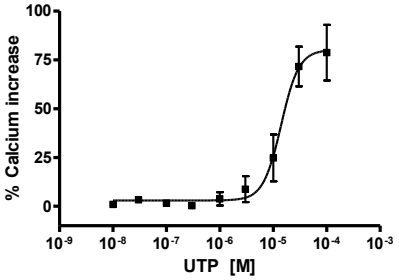
However, the signal of ATP-activated receptors of transfection product 2 and 3 was too high, that the measured values were not revealing. Therefore the calcium imaging assay was repeated with 20 000 and 25 000 cells per well to achieve lower signal that can be interpreted properly.

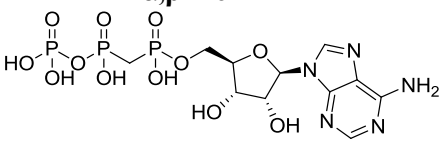
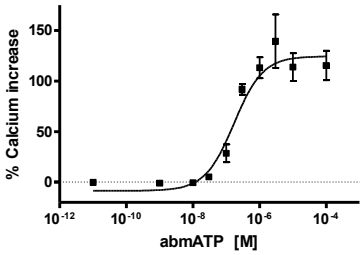
Transfection product 1 showed best results and hence used for characterization of human P2X3 S15V receptor via calcium imaging assay in an amount of 25 000 cells per well.

4.2.2 Functional characterization of hP2X3 S15V receptor agonists

Potential agonists of human P2X3 S15V receptor were found in literature and available compounds were tested via calcium imaging assay. Each compound was assayed at least three times (n=3) to achieve reliable results. Nine potential agonist were tested at hP2X3 S15V, namely 2-meS-ATP,[25] ADP,[25] AMP,[8] Ap4A,[8] ATP,[25] BzATP,[25,8] CTP,[24] UTP[25] and α,β-me-ATP.[20] Chemical structures of these compounds, their EC_{50} values, percentage stimulation and concentration-response curves can be seen in Table 4.

Compound	EC_{50} ± SEM in µM (% stimulation ± SEM at 100 µM compared to 100 µM ATP), n=3	Concentration-response curve
2-meS-ATP 	0.0203 ± 0.0054 (103 ± 13)	
ADP 	1.47 ± 0.19 (88 ± 12)	
AMP 	(7 ± 6)	Not active at 100 µM

Ap4A 	0.129 ± 0.042[b] (121 ± 10)[a]	
ATP 	0.0319 ± 0.0025[b] (94 ± 7)[a]	
BzATP 	0.0336 ± 0.0061 (103 ± 13)	
CTP 	4.01 ± 1.02 (89 ± 11)	
UTP 	29.3 ± 10.6 (79 ± 14)[c]	

α,β-me-ATP 	0.194 ± 0.036[b] (115 ± 15)	

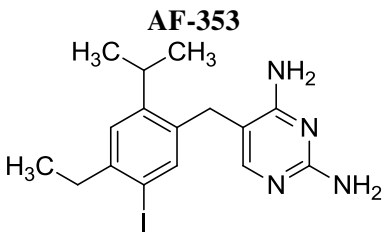
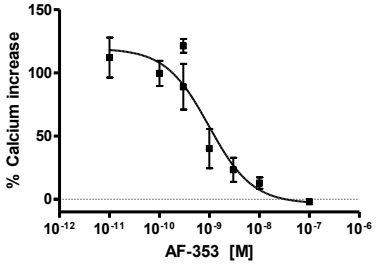
[a] at 10 µM; [b] n=4; [c] n=2

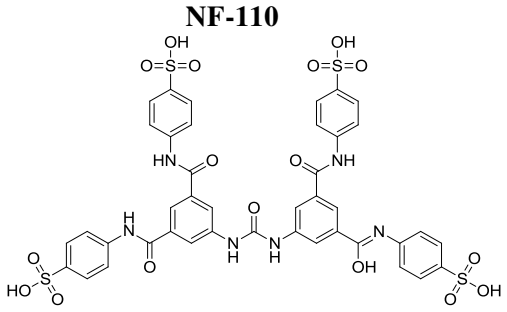
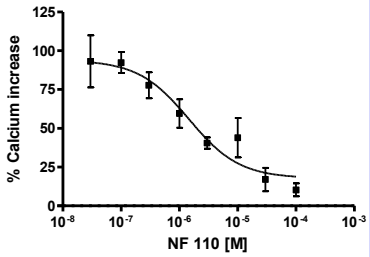
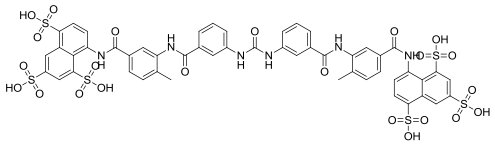
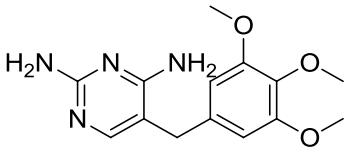
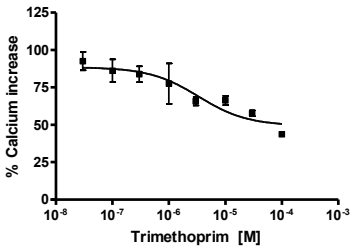
Table 4: EC_{50} value, % stimulation and concentration-response curve of compounds tested at human P2X3 S15V receptor expressed in 1321N1 astrocytoma cells as potential agonists.

4.2.3 Functional characterization of hP2X3 S15V receptor antagonists

Literature describes a large amount of P2X3 receptor antagonists, we tested four of them, namely AF-353,[3] NF 110[1] and suramin.[20] We also tested trimethoprim, which does not showed any P2X3 receptor inhibition at high concentration of 30 µM.[3] Chemical structures, IC_{50} values, percentage inhibition and concentration-response curves are shown in Table 5.

Surprisingly, A-317491,[3,20] described as high selective P2X3 antagonist, showed stimulation of human P2X3 S15V receptor. Therefore, information about A-317491 measurement is mentioned in chapter 4.2.4 Functional characterization of hP2X3 S15V receptor positive allosteric modulators.

Compound	IC_{50} ± SEM in µM (% inhibition ± SEM at 100 nM compared to 500 nM ATP), n=3	Concentration-response curve
AF-353 	0.00123 ± 0.00056[a] (102 ± 2)	

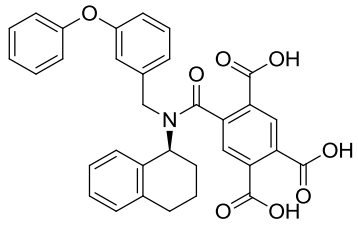
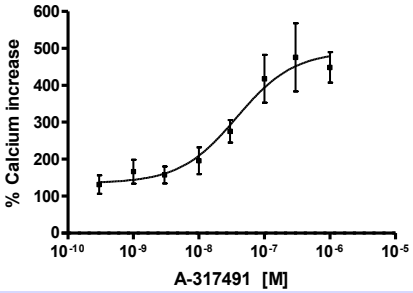
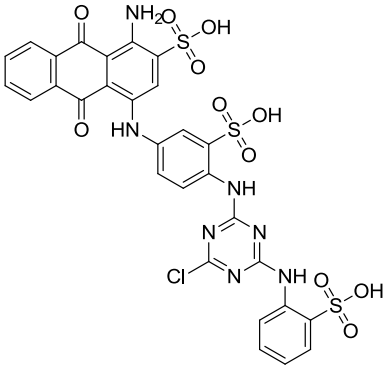
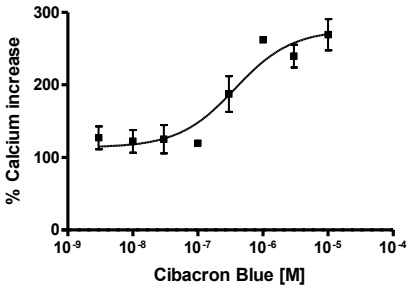
NF-110 	1.58 ± 4 (90 ± 4)[b]	
Suramin 	(10 ± 8)[b]	Not active at 100 µM
Trimethoprim 	3.52 ± 2.04 (56 ± 1)[b]	

[a] n=4, [b] at 100 µM

Table 5: IC_{50} value, % inhibition and concentration-response curve of compounds tested at human P2X3 S15V receptor expressed in 1321N1 astrocytoma cells as potential antagonists.

4.2.4 Functional characterization of hP2X3 S15V receptor positive allosteric modulators

Cibacron blue[3,8] was tested as a potential P2X3 allosteric modulator with positive results. A-317491 also act as a positive allosteric modulator at human P2X3 S15V receptor despite literature says the opposite.[20] Chemical structures, EC_{50} values, calcium increase and concentration-response curves can be seen in Table 6.

Compound	EC_{50} ± SEM in µM (Calcium increase ± SEM at 1 µM compared to 500 nM ATP), n=3	Concentration-response curve
A-317491 	0.0481 ± 0.0134 (448 ± 41)	
Cibacron Blue 	0.458 ± 0.190 (269 ± 22)[a]	

[a] at 10 µM

Table 6: EC_{50} value, % stimulation and concentration-response curve of positive allosteric modulators tested at human P2X3 S15V receptor expressed in 1321N1 astrocytoma cells.

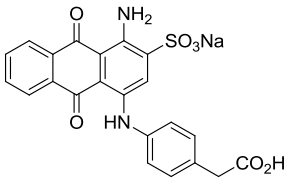
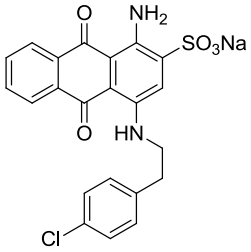
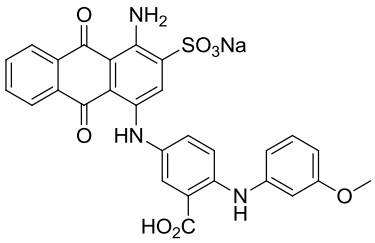
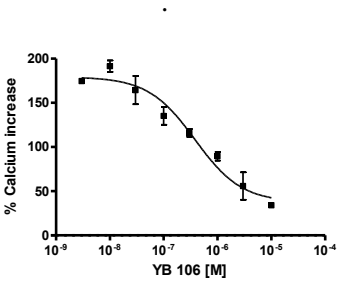
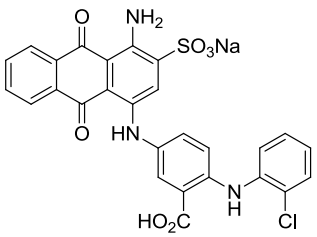
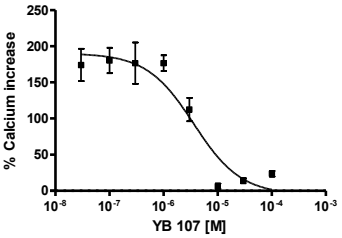
4.2.5 Testing compounds which were active at rat P2X3 S15V

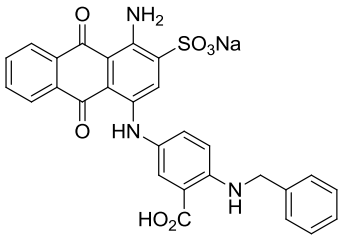
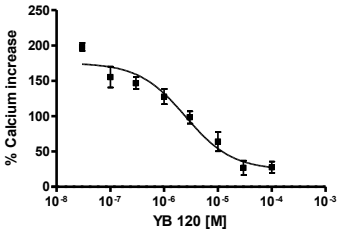
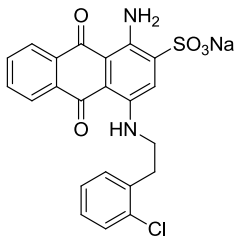
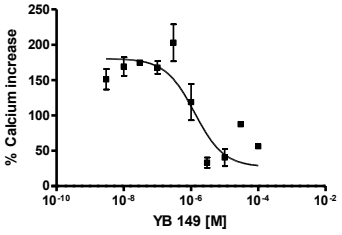
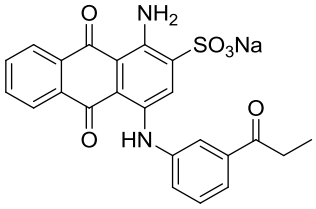
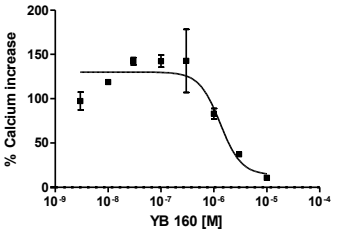
Screening of compound library has already been performed at rat P2X3 S15V receptor at the University of Bonn (data not published). Compounds, which showed interesting effect at rat P2X3 S15V receptor, were also tested at human P2X3 S15V receptor.

4.2.5.1 Anthraquinone derivatives

It was observed that some antraquinone derivatives influence rat P2X3 S15V receptor activity. Seven antharaquinone derivatives, synthetized by Younis Baqui (and therefore named YB), were tested at human P2X3 S15V receptor expressed at 1321N1 cells and

their influence on ATP response was monitored. Chemical structures, IC_{50} values, percentage inhibition and concentration-response curves are shown in Table 7.

Compound	IC_{50} ± SEM in µM (% inhibition ± SEM at 10 µM compared to 500 nM ATP), n=3	Concentration-response curve
YB 038 	(-57 ± 15 %) [a,b]	No effect at 100 µM Stimulatory effect at 1 µM
YB 087 	(-31 ± 16 %)[a,b]	No effect at 100 µM Stimulatory effect at 1 µM
YB 106 	0.387 ± 0.140 (66 ± 1) % inhibition at 10 nM (-92 ± 6 %)[c]	
YB 107 	3.61 ± 0.72 (94 ± 4) % inhibition at 30 nM (-74 ± 22 %)[d]	

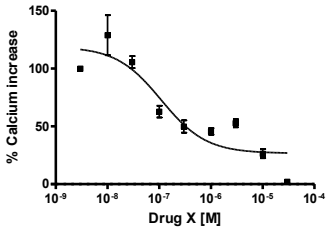
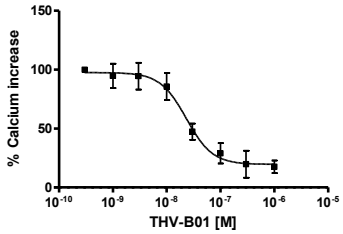
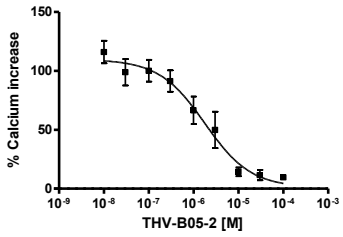
YB 120 	2.69 ± 0.63 (72 ± 8)[e] % inhibition at 30 nM (-98 ± 6 %)[d]		
YB 149 	2.29 ± 0.99 (59 ± 12) % inhibition at 300 nM (-103 ± 26 %)[b,f]		
YB 160 	1.25 ± 0.19 (89 ± 1) % inhibition at 300 nM (-43 ± 36 %)[f,g]		

[a] at 1 µM, [b] n=4, [c] at 10 nM, [d] at 30 nM, [e] at 100 µM, [f] at 300 nM, [g] n=2

Table 7: IC_{50} value, % inhibition and concentration-response curve of antharaquinone derivatives tested at human P2X3 S15V receptor expressed in 1321N1 astrocytoma cells as potential antagonists or allosteric modulators.

4.2.5.2 Commonly used drug and its derivatives

One drug, which is nowadays very commonly used, acts as very potent antagonist at rat P2X3 S15V. Name of this worldwide-used compound neither its chemical structure cannot be disclosed because this data has not been published yet. The chemical compound (named Drug X in this thesis) and its two derivatives THV B01 and THV B05-2, which were synthetized by The-Hung Vu (THV) at the University of Bonn, were tested at human P2X3 S15V receptor. All of them act as antagonists. The results of measurements can be seen in Table 8.

Compound	IC_{50} ± SEM in µM (% inhibition ± SEM at 30 µM compared to 500 nM ATP), n=3	Concentration-response curve
Drug X	0.186 ± 0.061 (98 ± 1)[a]	
THV B01	0.0270 ± 0.0083 82 ± 5[b]	
THV B05-2	2.09 ± 0.49 (88 ± 4)	

[a] n=4, [b] at 1 µM

Table 8: IC_{50} value, % inhibition and concentration-response curve of a Drug X and its derivatives tested at human P2X3 S15V receptor expressed in 1321N1 astrocytoma cells as potential antagonists.

5 Discussion

5.1 Preparation of stable cell line expressing P2X3 S15V receptor

The P2X3 S15V receptor DNA was multiplied via PCR method, inserted into retroviral vector and transfected into human 1321N1 astrocytoma cells. Antibiotic selection followed in order to select successfully transfected cells, in other words cells expressing P2X3 S15V receptor and also bearers of G418 antibiotic resistance. Measurement of receptor activity was performed via fluorescence-based calcium imaging assay. The method was optimized using ATP as an agonist in different conditions.

In conclusion, it can be said that cell line stably expressing P2X3 S15V receptor can be prepared via method described above in this thesis. 1321N1 cell lines expressing P2X3 S15V receptors responded very sensitively to ATP stimulation and intracellular signal of this slowly-desensitizing mutant was easily measured via fluorescence based calcium-imaging assay.

5.2 P2X3 S15V receptor mutant is a reliable surrogate for P2X3 receptor

Nine chemical compounds, that could possibly act as agonists at human P2X3 S15V were tested, namely 2-meS-ATP,[25] ADP,[25] AMP,[8] Ap4A,[8] ATP,[25] BzATP,[25,8] CTP,[24] UTP[25] and α,β-me-ATP.[20] The comparison of EC_{50} values measured at hP2X3 S15V receptor with EC_{50} values found in literature are shown in Table 9. All of them except AMP positively stimulated hP2X3 S15V receptor. AMP showed positive stimulation at some P2X receptor,[66] however, EC_{50} value of AMP at P2X3 receptor was not found.

Agonist	EC_{50} ± SEM in µM (n=3)	EC_{50} in µM according to literature
2-meS-ATP	0.0203 ± 0.0054	0.35[25]
ADP	1.47 ± 0.19	1.1[30]
AMP	–	
Ap4A	0.129 ± 0.042[a]	6.1[67]
ATP	0.0319 ± 0.0025[a]	1.6[24]
BzATP	0.0336 ± 0.0061	5[35]
CTP	4.01 ± 1.02	17.3[24]
UTP	29.3 ± 10.6	245[63]
α,β-me-ATP	0.194 ± 0.036[a]	2.4[24]

[a] n=4

Table 9: Comparison of EC_{50} values of agonists tested at human P2X3 S15V receptor expressed in 1321N1 astrocytoma cells to EC_{50} values found in literature.

Most of EC_{50} values we measured differ from EC_{50} values found in literature by one order of magnitude, sometimes by two orders of magnitude. But it is necessary to take into account the fact that measurement conditions may vary significantly.
For example, EC_{50} values of ATP, CTP and α,β-me-ATP were obtained using whole-cell electrophysiological recording at human P2X3. Effect of BzATP was also measured via electrophysiological study on zebra fish P2X3 receptor, ADP at rat P2X3 receptor. Conversely, activity of 2-meS-ATP, Ap4A and UTP was determined by intracellular calcium increase detection. Moreover, the results may differ due to the significantly slower desensitization of P2X3 S15V receptor.[15]

AF-353,[3] NF 110,[1] suramin[20] and trimethoprim were tested as potential antagonists at human P2X3 receptor. The comparison of IC_{50} values we measured with EC_{50} values found in literature are shown in Table 10.

Antagonist	IC_{50} ± SEM in µM (n=3)	IC_{50} in µM according to literature
AF-353	0.00123 ± 0.00056[a]	0.006[29]
NF110	1.58 ± 4	0.09[48]
Suramin	–	3.5[65]
Trimethoprim	3.52 ± 2.04	Not active at 30 µM[3]

[a] n=4

Table 10: Comparison of IC_{50} values of antagonists tested at human P2X3 S15V receptor expressed in 1321N1 astrocytoma cells to IC_{50} values found in literature.

Also in case of determination antagonists activity it is necessary to take into consideration measurement condition, for instance, method of measurement (patch-camp or fluorescence-based calcium-imaging assay), type of cells expressing P2X3 receptor (human, rat, zebrafish) but also chemical compound, which was used as an agonist during measurement of antagonist blocking activity (most often physiological ATP or synthetic BzATP or α,β-me-ATP).

IC_{50} values of AF-353 are very similar. Both of the measurements were carried out at human P2X3, ATP was used as an agonist and intracellular calcium influx was detected in both cases. Surprisingly, suramin was not active at hP2X3 S15V receptor at 100 µM and its derivative, NF110, was much less potent in comparison to literature. Both of the literature values were acquired via electrophysiology voltage-camp, ATP was used as an agonist and NF110 was tested at rat P2X3.

According to literature trimethoprim did not showed any blocking activity at P2X3 receptor at very high concentration of 30 µM.[3,29] In our hands trimethoprim act as antagonist at a concentration of 100 µM. Unfortunately, higher concentration could not be tested because of the high dilution factor and only 10 mM stock solution.

Study published in 1999 reported that Cibacron Blue potentiates ATP-responses of human P2X3 receptor responses. Three to seven fold potentiation of the maximal ATP response was observed. The receptor activity was detected via fluorescence imaging of intracellular Ca^{2+} increase. Similar EC_{50} value was achieved in our hands probably because of the very similar measurement conditions. Receptor response to ATP stimulation was almost three times higher.

A-317491 was expected to act as a very potent antagonist with IC_{50} value of 97 nM and high affinity and selectivity to P2X3 homomeric and P2X2/3 heteromeric receptors.[19] However, in our case A-317491 increased the stimulating effect of ATP and therefore worked as an allosteric modulator of hP2X3 S15V receptor. The EC_{50} value of A-317491 was 0.0481 ± 0.0134 µM (n=3). Calcium increase at 1 µM A-317491 was 448 ± 41 (n=3).

In Jarvis´s study, 3 µM α,β-meATP was used as an agonist at human P2X3 receptor. The receptor activity was measured using whole-cell patch-clamp recordings.[19] We had the advantage of the possibility to use physiological P2X3 receptor agonist, ATP. Unfortunately, the reason of this huge difference in response of P2X3 activity still remains a subject to debate. The comparison of EC_{50} values can be seen in Table 11.

Allosteric modulator	**EC_{50} ± SEM in µM (n=3)**	**EC_{50} in µM according to literature**
A-317491	0.0481 ± 0.0134	Antagonist 0.097[b,19]
Cibacron Blue	0.458 ± 0.190	1.4[64]

[a] at 10 µM, [b] IC_{50}

Table 11: Comparison of EC_{50} value of allosteric modulator tested at human P2X3 S15V receptor expressed in 1321N1 astrocytoma cells to EC_{50} or IC_{50} value found in literature.

In summary, this thesis demonstrated that P2X3 S15V receptor mutant is an appropriate surrogate to characterize human P2X3 receptor. The fluorescence-based calcium mobilization assay was successfully applied to confirm the functional characterization of P2X3 receptor.

5.3 Human P2X3 S15V receptor mutant facilitates reliable calcium-based high-throughput screening of compounds at P2X3

High throughput screening of compound library has already been carried out at rat P2X3 S15V receptor at University of Bonn. Chemical compounds that showed interesting effect at rP2X3 S15V receptor were also tested at human P2X3 S15V receptor.

Most of the antraquinone derivatives showed stimulatory effect at low nanomolar concentrations and inhibitory effect at high micromolar concentrations. Comparison of effect and IC_{50} values of antraquinone derivatives at rat and human P2X3 cannot be disclosed because this data has not been published yet.

Chemical compound named as Drug X showed lower potency at hP2X3 S15V than THV B01 but one fold higher potency than its second derivative THV B05-2. IC_{50} values are showed in Table 12.

	Drug X	**THV B01**	**THV B05-2**
IC_{50} in µM	0.186 ± 0.061	0.0270 ± 0.0083	2.09 ± 0.49

Table 12: IC_{50} values of Drug X and its two derivatives THV B01 and THV B05-2.

Drug X is a prodrug that is in living organism metabolized to metabolically more active THV B01. As shown in Table 12, THV B01 is one fold more potent than Drug X at hP2X3 S15V receptor. Drug X and its derivatives affected rat P2X3 S15V receptor very similarly. Concrete values cannot be published though.

Our results may be first step to finally elucidate mechanism of action of commonly used Drug X. Research on knock-out mice will follow and if this investigation proves our assumption, mechanism of action of this worldwide used drug could be completely clarified.

6 Conclusion

In conclusion, the human S15V-P2X3 receptor mutant was cloned into a retroviral vector and target 1321N1 human astrocytoma cells were infected. Retroviruses carried not only receptor DNA but also a resistance to antibiotic G418, which allowed simple and fast selection of successfully transfected target cells. The activity of P2X3 S15V receptor expressed in 1321N1 cells was tested via fluorescence-based calcium assay under different condition to optimize the method. For further measurements 25 000 cells per well were used, incubated at 37 °C and 5 % CO_2 for 24 hours and ATP was used as a positive control and agonist for testing of antagonists and allosteric modulators.

Pharmacological features of P2X3 S15V receptor mutant were characterized by analyzing the effect of nine known P2X3 agonists, six antagonists and one allosteric modulator. Measured data was compared to those found in literature. Most of the results showed comparable activity with known values. Very interesting effect showed non nucleotide compound, A-317491, which should act as a very potent P2X3 antagonist but in our hands acted as a strong positive allosteric modulator.

Moreover, the measurement of some compounds that have not been tested at P2X3 yet was carried out. Ten compounds which were formerly tested at rat P2X3 S15V receptor and showed interesting effect at rat receptor were also tested at human P2X3 S15V receptor. The most remarkable effect showed widely used drug called Drug X in this thesis and its more potent active metabolite. Their strong antagonistic effect may finally clarify the mechanism of action of this worldwide used drug. To confirm this hypothesis testing on knock-out mice will follow.

To conclude it, the reliable test system for P2X3 S15V receptor was developed. P2X3 S15V receptor is a credible surrogate for P2X3 receptor which allows simple and fast measurement of P2X3 agonists, antagonists and allosteric modulators. S15V receptor mutant and its simple activity measurement offer the opportunity to test large compound libraries which could, in future, be an important tool for discovery of new P2X3 antagonists that could be new candidates for treatment of many pathological states in which P2X3 receptor plays a key role.

7 List of abbreviations

2-me-S-ATP	2-Methylthioadenosine-5'-O-triphosphate
A-317491	5-[(3-phenoxyphenyl)methyl-[(1S)-1,2,3,4-tetrahydronaphthalen--1-yl]carbamoyl]benzene-1,2,4-tricarboxylic acid
AdeR	Adenine receptor
ADP	Adenosine diphosphate
AF-353	5-(5-iodo-4-methoxy-2-propan-2-ylphenoxy)pyrimidine-2,4-Diamine
AM	Acetoxymethyl
AMP	Adenosine monophosphate
Ap4A	P4-bis(5'-adenosyl)tetraphosphate
ATP	Adenosinetriphosphate
BSA	Bovine serum albumin
BzATP	3'-O-(4-Benzoyl)benzoyl adenosine triphosphate
CTP	Cytidine triphosphate
CYP 450	Cytochrome 450
DMEM	Dulbecco's Modified Eagle Medium
DMSO	Dimethylsulfoxid
DNA	Deoxiribonucleic acid
dNTP	Deoxinucleoside triphosphate
EC_{50}	Half maximal effective concentration
EDTA	ethylenediaminetetraacetic acid
FLIPR	Fluorescence Imaging Plate Reader
h	human
HBSS	Hanks buffer solution
Hepes	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
HF	High fidelity
dNTPs	Dinucleotides triphospate
FCS	Fetal calf serum
G	G-force (gravitional force)
G418	Geneticin
GDP	Guanosine diphosphate
GPCR	G-protein coupled receptor

GTP	Guanosine triphosphate
HBSS	Hanks buffer salt solution
HTS	High-throughput screening
IC_{50}	Half maximal inhibitory concentration
IP_5I	Diinosine pentaphosphate
LB medium	Lysogeny broth medium
n	number of measurements
NF-110	N-(4-sulfonatophenyl)-3-[(4-sulfonatophenyl)carbamoyl]-5-[[3-[(4-sulfonatophenyl)carbamoyl]-5-[(4-sulfophenyl)carbamoyl] phenyl]carbamoylamino]benzenecarboximidate
PBS	Phosphate buffered saline
PCR	Polymerase Chain Reaction
PS	Penicilin, Streptomycin
PPADS	4-[(2E)-2-[4-formyl-6-methyl-5-oxo-3-(phosphonooxymethyl) pyridin-2-ylidene]hydrazinyl]benzene-1,3-disulfonic acid
r	rat
RFU	Relative fluorescence unit
RO-85	N-[(2R)-1-(4-acetylpiperazin-1-yl)propan-2-yl]-1-methyl-3-phenylthieno[2,3-c]pyrazole-5-carboxamide
Rpm	Revolutions per minute
S	Serine
$t_{1/2}$	Half-life
TAE	Tris-acetate-EDTA
THV	The-Hung Vu
TM	Transmembrane segment
T_m	Primer melting temperature
TNP-ADP	2',3'-O-(2,4,6-Trinitrophenyl)adenosine-5'-diphosphate
TNP-AMP	2',3'-O-(2,4,6-Trinitrophenyl)adenosine-5'-monophosphate
TNP-ATP	2',3'-O-(2,4,6-Trinitrophenyl)adenosine-5'-triphosphate
TNP-GTP	2', 3'-O-(2, 4, 6-Trinitrophenyl)guanosine-5'-triphosphate
Tris	Tris(hydroxymethyl)aminomethane
UDP	Uridine diphosphate
UTP	Uridine triphosphate
U	Unit

UV	Ultraviolet
V	Valine
VSV-G	Glycoprotein G of the Vesicular stomatitis virus
YB	Younis Baqui
zf	zebrafish
α,β-me-ATP	α,β-methylene adenosine triphosphate

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