

The Role of P2Y₁₂ Receptor in Rodent Models of
Pain: in vivo Behavioural Pharmacology
Experiments and Explore Underlying Mechanisms

Doctoral thesis
Gergely Horváth

Semmelweis University
János Szentágothai Doctoral School of Neurosciences



Supervisor: Dr. Beáta Sperlách, PhD., professor

Official Reviewers: Dr. Gábor Pethő, PhD., professor

Dr. Pál Riba, PhD., professor

Chair of comprehensive exam: Dr. Éva Szökő, PhD., professor

Committee of comprehensive exam: Dr. Zsuzsanna Helyes, PhD., professor

Dr. Tibor Zelles, PhD., professor

Budapest
2017

1. Introduction

Pain is an unpleasant emotional and sensory experience what is fundamental for survival since it informs about actual and potential tissue damage. Pain medication is an ultimate activity and obligation of the physician therefore unsurprisingly analgesics are among the most frequently prescribed medicine. The different analgesics are varying in aspect of effectiveness and side effect, tolerance and dependency, and safety. That is why there is intensive research in finding new possible therapeutic targets for pain medication. The immune system and the nervous system can affect each other's action. The inflammatory mediators play an important role in pain transmission, not just locally in acute phase processing but also in subacute and chronic terms in the central nervous system. ATP is a universal compound of the living organism. It plays a central role in energy supplement of the cell and works as a neurotransmitter too. ATP and its metabolites -released due to neural activity, physical impact, inflammation or apoptosis- act on purinergic receptors. Since purinergic receptors show broad expression pattern they might play important role in different disease of the central nervous system (CNS) and in pain transmission as well. Human P2Y₁₂ receptor was cloned in 2001 for the first time. Since that time we know its exact amino acid sequence, 3D structure and the most important parameters. P2Y₁₂ receptor is the ADP receptor of the platelets but it is expressed by endothelial cells, smooth muscle cells and CNS microglial cells, respectively. The first papers searching for the role of P2Y₁₂ receptor in pain were published around 2006 in which the authors reported P2Y₁₂ receptor overexpression of spinal microglial cells in neuropathic pain model. P2Y₁₂ receptor antagonist and P2Y₁₂ receptor antisense oligonucleotide treatment alleviated allodynia and thermal hyperalgesia respectively.

2. Aims

Since P2Y₁₂ receptor was examined only in neuropathic pain and the used antagonists were not selective, the analgesic action of P2Y₁₂ receptor blockade was not so clear. Therefore our aim was to further investigate the role of P2Y₁₂ receptor in different pain models. Our experiments were focused on the main points listed below:

2.1. Effect of P2Y₁₂ receptor blockade in animal model of pain

P2Y₁₂ receptor antagonists were tested in the 3 pain models listed below:

- Acute pain
- CFA induced inflammatory pain
- Neuropathic pain (Seltzer model)

P2Y₁₂ receptor knockout mice were investigated in these models as a confirmation of our pharmacological data

2.2. In vitro activity of P2Y₁₂ receptor antagonists

These experiments were conducted in the laboratory of Prof. Ivar von Kügelgen, Department of Pharmacology and Toxicology, University of Bonn, Germany. The effect of P2Y₁₂ receptor antagonists on forskolin or isoproterenol induced cAMP level against the inhibitory effect of 2-MeSADP was measured.

2.3. Exploring the background mechanism P2Y₁₂ receptor blockade

In these experiments we were investigating the possible underlying mechanisms of P2Y₁₂ receptor blockade by using molecular biology tools. The efferent mechanisms were revealed by using in vivo behavioural pharmacology tests.

2.4. Survey of possible side effects of P2Y₁₂ receptor antagonists

The possible side effects of the selected compounds were examined by in vivo and ex vivo experiments.

3. Methods

3.1. Animals

Male wistar rats (5-7 weeks old, 200-250 g weight), P2Y₁₂ receptor deficient mice (70 days old, 25-30 g weight) and C57/Bl6 mice as controls for genetically modified ones (matching in age and weight) were used in our experiments. Animals were kept separately and under control at least 1 week prior to experiments. The experiments were conducted in a separate room isolated.

3.2. Treatment

The following antagonists were administered intraperitoneally (ip.) or intrathecally (it.), in 100-200 µL or 5 µL , 30 or 15 minutes before experiment respectively:

- MRS2395 (ip.)
- cangrelor (ip.)
- reactive blue 2 (ip.)
- clopidogrel (ip.)
- ticlopidine (ip.)
- PSB-0739 (it.)

3.3. Pain models

3.3.1. Acute pain model

Acute nociception was measured by an incremental hot plate test (IITC, Woodland Hills, CA, USA). Animals were put individually into a plexi glass observation chamber with 25 °C floor. After 10 minutes habituation the floors temperature was increased by a fix rate of 6 degrees/minute. Licking any of the paws was considered as thermal nociceptive threshold (PWT, Paw withdrawal Threshold) and experiment was stopped manually. Conditional measurement followed by two control measurement were performed for obtaining baseline values. Then animals were treated and the effect of the antagonists were measured.

3.3.2. CFA induced inflammatory pain model

Complete Freund Adjuvant emulsion (CFA, 50%) was injected into the plantar surface of the animals (100 μ L/rat, 30 μ L/mouse). Mechanical paw withdrawal threshold was measured before the CFA injection and 48/96 hours later by using Ugo Basile Dynamic Plantar aesthesiometer (von Frey, Stoelting, Woods Dale, IL, USA). Animals were placed into the observation chamber and after 15 minutes habituation mechanical stimuli were applied (0-50 g, increasing) on treated and contralateral hind paw. The volume of the inflamed hind paw was measured by using plethysmometer (Ugo Basile plethysmometer, Stoelting, Wood Dale, IL, USA).

3.3.3. Neuropathic pain model

Partial ligation of sciatic nerve was performed on anesthetized animals. Mechanical allodynia was measured before and 7 days after operation by using Ugo Basile electronic vonFrey device in the same manner as described in the inflammatory pain model. Antagonists were administered on the 7th day after surgery.

3.4. Molecular biology experiments

3.4.1. P2Y₁₂R mRNA expression by Quantitative Real-Time PCR

Inflamed peripheral tissue and lumbar spinal cord samples were collected 48/96 hours after animals received intraplantar CFA injection. Change of P2Y₁₂ receptor coding gene expression was measured by Real-Time PCR. Samples were homogenized in Trizol and total RNA was isolated by following the instruction of the manufacturer of RNeasy Lipid Tissue Mini Kit (Quiagen). Reverse transcription was performed by RevertAid First Strand cDNA Synthesis Kit (Fermentas, Vilnius, Lithuania). Gene expression was measured by using Real-Time PCR (Rotor-Gene 3000; Corbett Research, Sydney, Australia). The expression level of the target genes were normalized to the reference genes expression (18S rRNA)

3.4.2. Interleukin 1-beta expression measurement by ELISA

Inflamed peripheral tissue and lumbar spinal cord samples were collected 48/96 hours after animals received intraplantar CFA injection. Samples were homogenized and centrifuged then supernatant was collected in 500 μ L Tris-HCl buffer. IL-1 β production

was measured by using rat or mouse specific DuoSet IL-1 β (R&D System, Minneapolis, MN, USA), following the manufacturers instructions. The detection limit of the assay was 5 pg/mL. Absorbance was measured at 450 nm by Perkin-Elmer Victor3V 1420 Multilabel Counter. IL-1 β levels were expressed in pg/mL

3.4.3. Multiplex cytokin measurement by Luminex platform

Animals were injected by the selected P2Y₁₂ receptor antagonists 48/96 hours after intraplantar CFA/saline injection. Inflamed peripheral tissue and lumbar spinal cord samples were collected on dry ice and kept at -70⁰C until measurement. Inflammatory cytokine levels were determined by az R&D Systems microbead Fluorokine® MultiAnalyte Profiling kit, using Luminex-xMAP multiplex platform. Samples were homogenized and centrifuged then supernatant was collected in 500 μ L Tris-HCl buffer. Samples were measured in a 96 well filter microplate. Measurements were conducted in Semmelweis University Fluorescent Technics Core Facility. Cytokine production was expressed in pg/mL.

3.4.4. Cytokine measurement by FACS

Inflamed peripheral samples were prepared and kept on -80 $^{\circ}$ C until analysis. Samples were homogenized and centrifuged. IL-1 β concentration was measured by BD Cytometric Bead Array (CBA) Flex Sets (BD Biosciences). The experiment was performed on BD FACS Verse instrument and results were analyzed on FCAP Array v5 software (Soft Flow, USA). Cytokine concentrations were normalized to the whole protein levels of the tissue samples what was measured by photometry (Thermo Scientific Pierce BCA Protein Assay Kit; Rockford, USA). Absorbance was measured on 560 nm by Perkin Elmer Victor 3V 1420 Multilabel Counter (Perkin Elmer, USA) instrument.

3.4.5. HPLC analysis

Animals received systemic 6-OHDA (6-hydroxidopamine)/ saline treatment prior to intraplantar CFA/saline injection. HPLC analysis was performed in order to follow up the monoamine and metabolites of the inflamed peripheral tissue. The samples were homogenized and KOH solution was given to them prior to centrifugation. The liquid chromatographic system was controlled by 715 operation software (Gilson Medical

Electronics inc., Middletown, and WI USA). Eluent was connected to BAS 400 electrochemical detection and the oxidative potential was maintained by 0.75 V Eltron potentiostat. Separation was made after enrichment of the analytes. IP puffer was used for the analysis. The flow velocity was 0.98 mL/min.

3.5. Side effect studies

3.5.1. Rotarod test

Male wistar rats (140-190 g) were used for this experiment. Motor coordination was measured by using IITC (Woodland Hills, CA, USA) Rotarod instrument. The test was 300 s long, with incremental speed. Two days prior to experiment animals were trained and animals were excluded from further analysis if they could not reach at least 60 sec latency. Then animals received treatment and effect on latency of falling down was measured again.

3.5.2. *Ex vivo* platelet aggregation study

Animals received ip./it. antagonist/saline and 30/15 minutes later blood samples(3 mL) were drawn in anesthesia. Samples were centrifuged and platelet rich plasma (PRP) and platelet poor plasma (PPP) were collected separately. 450 μ L of both PRP and PPP were taken into cuvettes and aggregation of samples were induced. Aggregation was measured by using Carat TX4 optical aggregometer (Carat Diagnosztika Ltd., Budapest)

3.6. In vitro effectiveness of P2Y₁₂ receptor antagonists

1321N1 and Chinese Hamster Ovary (CHO) cells were used for in vitro experiments. Cells were stimulated by 10 nM isoproterenol or 10 μ M forskolin in the presence or absence of P2Y₁₂ receptor agonist 2-meSADP. Antagonists were administered 10 minutes before agonist treatment. cAMP levels in the supernatant were then quantified by incubation of an aliquot with cAMP binding protein and [3H]-cAMP (Perkin Elmer, Rodgau, Germany) and liquid scintillation counting after removal of the unbound [3H]-cAMP by charcoal. CHO cells cAMP response was measured by applying a Cre-dependent luciferase assay (the luciferase activity of the supernatant was measured by

luminometer: Bethold Technologies, Bad Wildbad, Germany). Concentration-response data were fitted by non-linear regression using GraphPad Prism (4.03) to estimate half-maximal concentrations. Apparent pKB-values were calculated according to: $pKB = \log(\text{dose ratio} - 1) - \log[B]$. pA2-values were determined by linear regression analysis.

3.7. Subdiaphragmatic vagotomy (VGX)

Vagotomy was performed in ketamin-xylozin anaesthesia. 3-4 cm midline incision was made on the ventral abdominal surface. The vagus nerve was explored and 1.5 cm long pieces of it were removed from both the anterior and posterior side by using a surgical microscope. The wound was closed and animals received 10 mL saline and were replaced in cages individually. Paw withdrawal threshold was measured before and 1 week after the surgical intervention. After the postoperative PWT measurement animals received intraplantar CFA treatment. Two days later the hyperalgesia and the effect of the P2Y₁₂ receptor antagonist were measured.

4. Results

4.1. Antinociceptive effect of P2Y₁₂ receptor antagonists in different pain models

Four of the tested P2Y₁₂ receptor antagonists had significant effect in acute pain model. MRS2395, ticlopidine and clopidogrel had dose dependent antinociceptive effect. reactive blue 2, cangrelor and intraperitoneally (ip.) administered PSB-0739 had no effect on nocifensive behaviour. Intrathecally (it.) administered PSB-0739 had dose independent significant effect in the acute pain model. Antagonists in clinical use (ticlopidine, clopidogrel) were effective in higher dose however MRS2395 had significant impact on nocifensive behaviour in lower dose. The most effective compound was found to be the potent and selective blocker of P2Y₁₂ receptor, PSB-0739.

All the tested compounds had dose dependent significant anti allodynic effect on CFA induced inflammatory pain. The clinically used drugs were effective in higher doses again. Ticlopidine was the only antagonist which could significantly decrease the volume of the inflamed hindpaw what suggests that in inflammatory pain the manifestation of the oedema and the decrease of its volume are independent on the change of PWT. Based on the rank order of the minimal effective dose the most effective compounds were it. administered PSB-0739 and cangrelor, respectively.

Five of the tested antagonists were effective in the neuropathic pain model. The dose-response curves of the antagonists were depressed compared to those in the inflammatory pain model. Ticlopidine was effective in higher dose again and in the highest tested dose (100 mg/kg) it brought the PWT value around the preoperative value. Reactive blue 2 and ip. administered PSB-0739 were ineffective in this model. The most effective compounds were it. administered PSB-0739 and cangrelor.

Table 1. Effect of P2Y₁₂ receptor antagonists in different pain models

Pain model	MRS2395	ticlopidine	clopidogrel	reactive blue 2	cangrelor	PSB-0739
Acute pain	Dose dependent	Dose dependent	Dose dependent	-	-	Dose independent
Inflammatory pain	Dose dependent	Dose dependent	Dose dependent	Dose dependent	Dose dependent	Dose dependent
Neuropathic pain	Dose dependent	Dose dependent	Dose dependent	-	Dose dependent	Dose dependent

Table 2. Rank order of P2Y₁₂ receptor antagonists (based on minimal effective dose)

Pain model	Rank order (mED)
Acute pain	PSB-0739 it. > MRS2395> clopidogrel> ticlopidine
Inflammatory pain	PSB-0739 it. = cangrelor> reactive blue2> MRS2395> ticlopidine = clopidogrel
Neuropathic pain	PSB-0739 it.= cangrelor> MRS2395> clopidogrel> ticlopidine

In order to confirm our pharmacological data we investigated the nocifensive behaviour of P2Y₁₂ receptor deficient animals in all three pain models. P2Y₁₂ receptor deficient mice tolerated higher temperature in the acute pain model than their wild-type littermates. PWT values decreased in both of the genotypes after intraplantar CFA treatment but P2Y₁₂ receptor knockout (KO) animals showed moderate decrease of PWT than the wild-type (WT) ones. The same effect of genetic deletion was found in the neuropathic pain model: after partial ligation of the sciatic nerve both P2Y₁₂ receptor KO and WT mice showed decreased PWT compared to the preoperative values but the nocifensive reaction of the P2Y₁₂ receptor deficient mice were slighter than showed by the wild-type mice.

4.2 Investigation of mechanism of action of P2Y₁₂ receptor blockers

We used molecular biology tools in order to find the possible mechanism of action of the blockade of P2Y₁₂ receptor.

P2Y₁₂ receptor mRNA and interleukin-1 beta expression showed time dependency in our inflammatory pain model. Both P2Y₁₂ receptor mRNA and interleukin-1 beta levels were increased in the inflamed hind paw 48 hours after

intraplantar CFA injection. The same increase in the spinal cord was found just in a later time point (96 hours after CFA treatment)

The effect of PSB-0739 and cangrelor on cytokine levels of the central nervous system (CNS) and the peripheral inflamed tissue was measured by Luminex Multiplex cytokine array-and FACS. PSB-0739 decreased the IL-1 β , TNF α , IL-10 and IL-6 levels of the inflamed peripheral tissue and the spinal IL-1 β level. Cangrelor affected the TNF α and IL-10 levels in the hind paw and IL-1 β in the spinal cord.

IL-1 β levels were measured in wild-type and knockout mice after CFA treatment. IL-1 β levels were increased in the inflamed hind paw regardless to genotype. In contrast spinal interleukin-1 beta levels were not elevated after intraplantar CFA injection in P2Y₁₂ receptor deficient mice.

Table 3. Effect of P2Y₁₂ receptor blockade on cytokine levels of the inflamed peripheral tissue and the spinal cord

	Inflamed hind paw		Spinal cord	
	Cangrelor	PSB-0739	Cangrelor	PSB-0739
IL-1 β	-	↓	↓	↓
TNF α	↓	↓	-	-
IL-10	↓	↓	-	-
IL-6	-	↓	-	-

Effect of intraplantar CFA injection

	Inflamed hind paw		Spinal cord	
	P2Y ₁₂ +/+	P2Y ₁₂ -/-	P2Y ₁₂ +/+	P2Y ₁₂ -/-
IL-1 β	↑	↑	↑	-

Centrally administered PSB-0739 affected not just nocifensive behaviour and spinal pro-inflammatory cytokine level but it modified on-site produced cytokine levels in the periphery suggesting efferent mechanism of the compound.

In order to explore the efferent mechanism of action we designed a new set of experiment in which a group of animals were treated by systemic 6-OHDA (6-hydroxidopamine) to block sympathetic fibers and another group of animals were

treated by MLA (Methyllycaconitine, $\alpha 7$ nicotinic acetylcholine receptor antagonist) to block cholinergic fibers. Treatments alone did not modify the PWT values. Intraplantar CFA treatment elicited mechanic allodynia. PSB-0739 had antinociceptive effect in 6-OHDA pretreated animals but remained ineffective in MLA treated ones. MLA seemed to suspend analgesic action of PSB-0739. To confirm our results we performed subdiaphragmatic vagotomy in a group of animals applying the same experimental paradigm. Vagotomy itself did not modify PWT values. The decrease in PWT was observed due to intraplantar CFA injection and PSB-0739 did not affect the lower PWT. This set of experiments revealed the suspected mechanism of action of PSB-0739 showing **Figure 1**. Inflammatory process starts in the hind paw after intraplantar CFA treatment. The primary afferent nociceptive fiber forward the signal to spinal interneuron and projection neuron towards higher brain structures. Intrathecally administered PSB-0739 has antinociceptive effect acting on neuron and microglial cells directly and able to modify peripheral cytokine response indirectly. The effect of PSB-0739 was inhibited by subdiaphragmatic vagotomy and systemic anti-cholinergic treatment (MLA) therefore we assume that the peripheral effect of PSB-0739 is mediated by parasympathetic, cholinergic fibers.

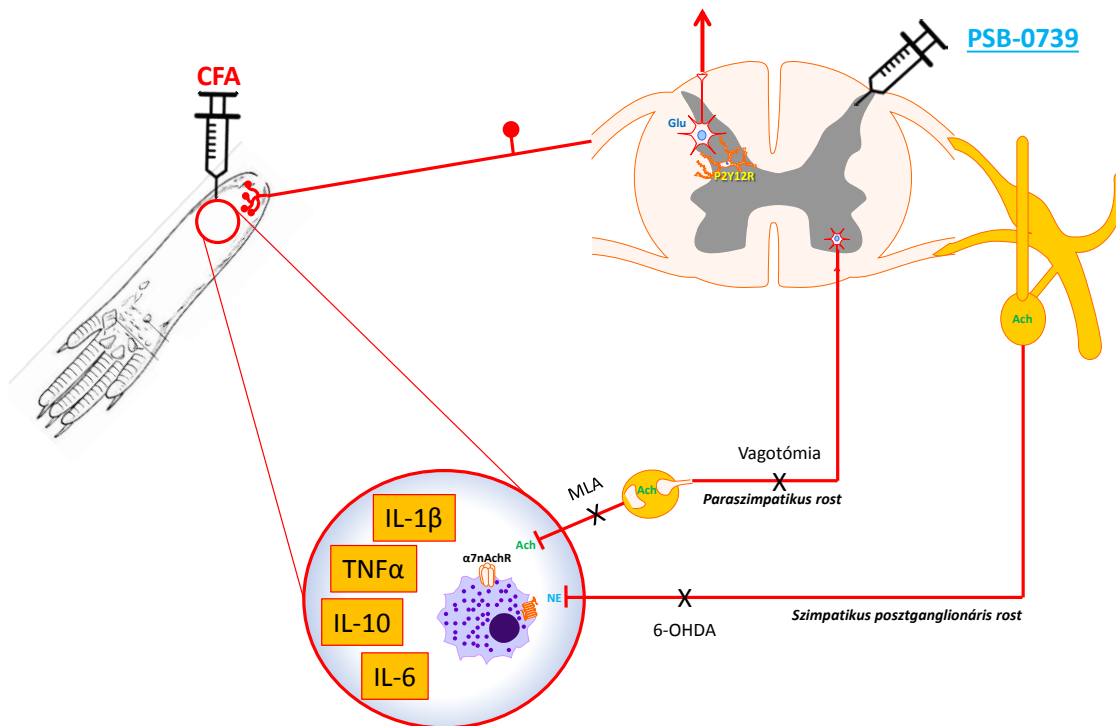


Figure 1. Suspected mechanism of action of PSB-0739

4.3 In vitro analysis of P2Y₁₂ receptor antagonists

PSB-0739 antagonized the effect of 2-MeSADP on cAMP levels (pA₂=9.8) and it was more potent on P2Y₁₂ receptor than on other P2Y receptors (P2Y₁, P2Y₂, P2Y₁₁ and P2Y₁₃). The effect of the antagonist was measured in the CHO model in which no effect was found on basal cAMP level. PSB-0739 shifted the concentration-response curve of 2-MeSADP to the right. PSB elicited no change in forskolin induced cAMP level. PSB-0739 is a potent and selective P2Y₁₂ receptor antagonist.

Suramin (3, 10, 30 μM) caused a rightward shift in the 2-MeSADP curve without changing the maximal effect of forskolin. Suramin was not a selective compound since it blocked P2Y₁, P2Y₁₃ and P2Y₁₂ receptors and with lower affinity P2Y₂, P2Y₄ and P2Y₆ receptors. Suramin is a non-selective, low affinity P2Y₁₂ receptor blocker.

Reactive blue 2 was found to be a competitive antagonist of human P2Y₁₂ receptor, showing higher efficacy than suramin but it had effect on various P2Y

receptors (pA2=6 on P2Y₆ and pA2 was around 5 on P2Y₁, P2Y₂ and P2Y₁₃ receptors). So it was a potent but non selective compound of P2Y₁₂ receptor.

Cangrelor showed similar potent effect as PSB-0739. It shifted the 2-MeSADP curve to the right. Cangrelor could antagonize P2Y₁₃ receptor. Cangrelor (100 nM) had no effect on basal cAMP level in the CHO model and it did not modify the forskolin induced cAMP level. Cangrelor is a potent antagonist of both P2Y₁₂ and P2Y₁₃ receptors.

MRS2395 did not change the basal cAMP production. 2-MeSADP dose-response curve on forskolin induced cAMP was not shifted to the right by MRS2395. In the presence of the antagonist cAMP levels were significantly higher than in the case of the solvent suggesting that MRS2395 can enhance cAMP level by itself. MRS2395 (20 μM) in the absence of P2Y₁₂ receptor agonist increased forskolin induced cAMP level. MRS2395 is not a genuine human P2Y₁₂ antagonist. The suspected P2Y₁₂ receptor antagonizing effect of MRS2395 was based on indirect data on native rat platelets. Our data show that the cAMP enhancing effect of the antagonist is independent of P2Y₁₂ receptor and possibly based on phosphodiesterase inhibition.

Prodrug clopidogrel and ticlopidine were unable to measure in this experiments therefore we assessed just in vivo effects of those compounds.

Table 4. P2Y receptor antagonists in vitro effects on human P2Y receptor subtypes

Antagonist	P2Y ₁	P2Y ₂	P2Y ₄	P2Y ₆	P2Y ₁₁	P2Y ₁₂	P2Y ₁₃	P2Y ₁₄
reactive blue 2	5.8 ^a	5.3 ^c	<4 ^{d,e}	6 ^{a,h}	<5 ^{d,f}	7.4 ^b	5.7 ^{c,g}	
suramin	5.5 ^a	4.5 ^c	<3.5 ^{d,i}	<4.5 ^{d,h}		5.7 ^b	5.6 ^{c,g}	
cangrelor						9.2 8.6 ^l 8.7	↓80% ^{g,i,k} (10 nM)	
MRS2395						non-competitive antagonist, increase cAMP level alone		
PSB-0739	- (>20 nM)	- (>20 nM)	- (>20 nM)	- (>20 nM)	- (>20 nM)	9.8 ^m 9.9	- (>20 nM)	- (>20 nM)

4.4 Side-effect studies

We have investigated the effect of cangrelor and PSB-0739 on motor coordination and platelet aggregation respectively. We chose the same dose and mode of administration of the compounds which were effective in the inflammatory pain model.

Rotarod test took 300 seconds each round. None of the tested antagonists had effect on motor coordination.

Platelet aggregation was induced by ADP and the extent of aggregation was described by maximal aggregation (A_{\max}) value. ADP induced platelet aggregation dose-dependently in naive animals. Cangrelor significantly decreased in contrast PSB-0739 significantly increased A_{\max} . Results of cangrelor treatment are similar to those found in literature showing P2Y₁₂R antagonists on anti-aggregate property what is more beneficial regarding cardiovascular risks.

5. Conclusion

Conclusion based on the summary of our results reflecting to the questions are collected below in points:

5.1. P2Y₁₂ receptor blockade in animal models of pain

Six compounds were measured in these experiments. Four of the antagonists were effective in acute pain, five compounds had analgesic effect on neuropathic pain and all of the tested drugs had anti-allodynic action in the inflammatory pain model. The potent and selective P2Y₁₂ receptor blocker PSB-0739 and the potent P2Y_{12/13} receptor blocker cangrelor were the most effective compounds in all of the pain models. PSB-0739 showed dose independent effect on acute nociception and dose dependent action in the other two pain models. Interestingly only the clinically used anti-aggregate ticlopidine had significant effect on the volume of oedema

P2Y₁₂ receptor deficient mice were investigated in the same models with their comparable control (wild-type mice). The KO animals showed nocifensive reaction on higher temperature than their WT littermates. After partial sciatic nerve ligation all the animals showed significant decrease in paw withdrawal thresholds regardless to genotype but mice lacking P2Y₁₂ receptor showed slighter PWT compared to wild-type animals. Similar results were obtained in the inflammatory pain model: genetically modified animals showed slighter PWT values two days after intraplantar CFA injection than the wild-type mice.

Interleukin-1 beta values changed parallel with the nocifensive behaviour: Pharmacological blockade of P2Y₁₂ receptor decreased the CFA induced elevated IL-1 β level. Spinal IL-1 β level remained unchanged in P2Y₁₂ receptor deficient mice after CFA treatment. Pharmacological and genetic blockade of P2Y₁₂ receptor alleviated pain in our in vivo models. The results showed correlation with in vitro data.

5.2. In vitro activity of P2Y₁₂ receptor antagonists

P2Y₁₂ blockers effect on 2-MeSADP inhibited cAMP levels were measured on transfected cell cultures. K_b, pA₂ and pK_B values were determined. PSB-0739 is a potent and selective antagonist of P2Y₁₂ receptor. Cangrelor can antagonize both P2Y_{12/13} receptors. The widely reported MRS2395 is not a genuine human P2Y₁₂R agonist.

Based on the in vivo behavioural pharmacology experiments, the potent and selective P2Y₁₂ receptor antagonist PSB-0739 was the most effective among the tested compounds what suggest that the selective blockade of P2Y₁₂ receptor leads to alleviation of pain. The other antagonists were less effective. The non-selective compounds may have their lower effectiveness due to their effect on other receptors. The antinociceptive effect of MRS2395 seems to be completely independent of P2Y₁₂ receptor.

5.3. Mechanism of action of P2Y₁₂ receptor blockade

We explored the mechanism of action of the in vivo anti-nociceptive effects of the compounds obtained in the first part of our study. Quantitative Real-Time PCR was used to investigate mRNA level of P2Y₁₂ receptor and ELISA and FACS were used to follow IL-1 β levels. Multiplex cytokine analysis was performed by luminex array in samples of inflamed peripheral hind paw and spinal cord, respectively.

Time dependent and delayed overexpression of P2Y₁₂ mRNA was found in the lumbar spinal cord (L4-6). Centrally administered PSB-0739 could affect both central and peripheral inflammatory cytokine levels.

Blockade of sympathetic postganglionic fibers by 6-OHDA did not suspend the analgesic effect of PSB-0739 but systemic nicotinic acetylcholine receptor α 7 subunit antagonist methyllycaconitine (MLA) affected PWT and inflammatory cytokine levels. Subdiaphragmatic vagotomy prevented the effect of PSB-0739. Based on these results we suspect that the potent and selective P2Y₁₂ receptor antagonist PSB-0739 has its efferent action on cholinergic fibers.

5.4. Side effect survey of the used P2Y₁₂ receptor antagonists

We have examined the effect of cangrelor and PSB-0739 on motor coordination and platelet aggregation.

Neither intraperitoneally administered cangrelor not intrathecally used PSB-0739 had effect on motor coordination in the rotarod test.

Cangrelor could inhibit platelet aggregation but PSB-0739 had contrary effect. Based on these results we suspect that the antagonists penetrating the blood-brain barrier can have stronger effect.

6. Publications

Publications in the field of the doctoral thesis

1, *Contribution of platelet P2Y12 receptors to chronic Complete Freund's Adjuvant-induced inflammatory pain.*

Bekő K, Koványi B, Göllöncsér F, Horváth G, Dénes Á, Környei Zs, Botz B, Helyes Zs, Mueller C, Sperlágh B

Journal of Thrombosis and Haemostasis. Accepted: 13th March, 2017

2, *Central P2Y12 receptor blockade alleviates inflammatory and neuropathic pain and cytokine production in rodents.*

Horváth G, Göllöncsér F, Csölle C, Király K, Andó RD, Baranyi M, Koványi B, Máté Z, Hoffmann K, Algaier I, Baqi Y, Müller CE, Von Kügelgen I, Sperlágh B.

Neurobiol Dis. 2014 Oct;70:162-78. doi: 10.1016/j.nbd.2014.06.011. Epub 2014 Jun 25.

Other publications

1, *A genetic study of 12 X-STR loci in the Hungarian population.*

Horváth G, Zalán A, Kis Z, Pamjav H.

Forensic Sci Int Genet. 2012 Jan;6(1):e46-7. doi: 10.1016/j.fsigen.2011.03.007. Epub 2011 Apr 3. No abstract available.

2, *Allele distribution of the new European Standard Set (ESS) loci in the Hungarian population.*

Molnár A, Zalán A, Horváth G, Pamjav H.

Forensic Sci Int Genet. 2011 Nov;5(5):555-6. doi: 10.1016/j.fsigen.2010.06.002. Epub 2010 Jul 4. No abstract available.