Donated Chemical Probe

P2X4 Inhibitor Probe BAY-1797

June, 2018

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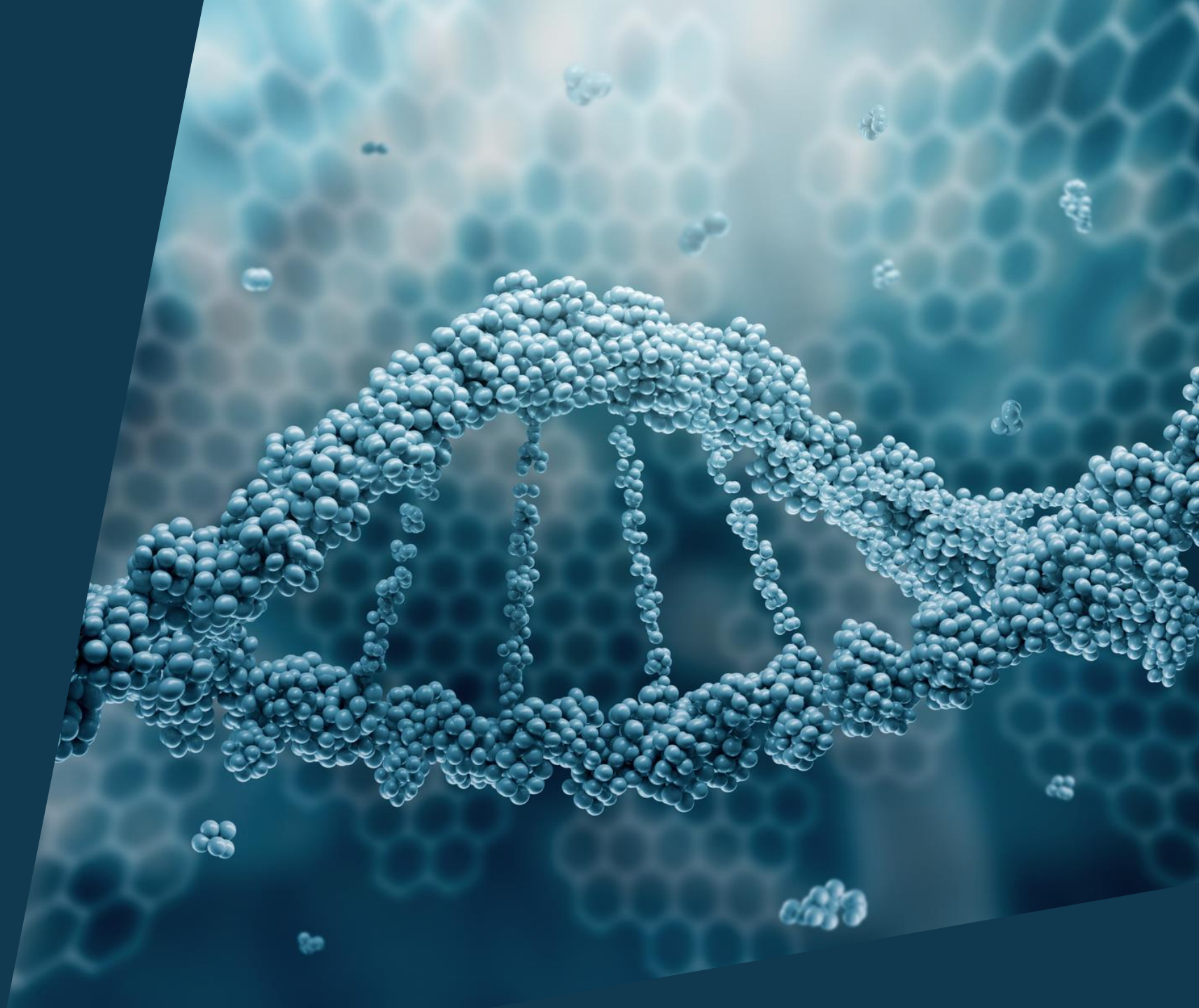
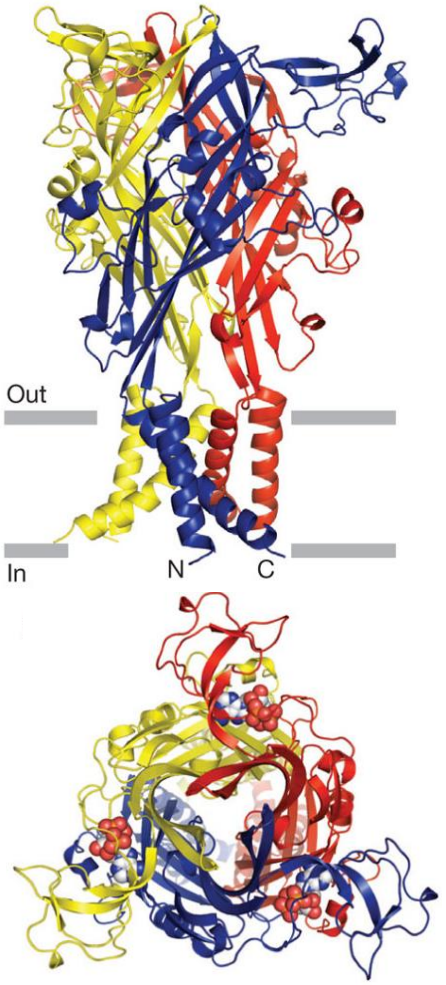


P2X4 Probe BAY-1797

Background P2X4

- Extracellular **nucleotides** have been recognized as important mediators, triggering multiple responses via plasma membrane receptors known as P2 receptors
- Among them, P2X4 receptors are **trimeric ATP-activated ion channels** permeable to Na^+, K^+ and Ca^{2+} leading to inflammasome, p38 MAPK and PLA2 activation
- P2X4 is widely expressed in central and **peripheral neurons**, in **microglia**, and also found in various **epithelial tissues** and **endothelial cells**
- P2X4 is described to be involved in different mechanisms including **pro-inflammatory responses**, **neuropathic and inflammatory pain** and **neuroinflammatory disorders**

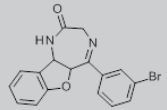
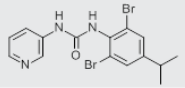
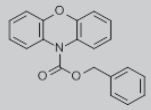
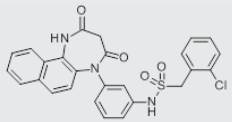
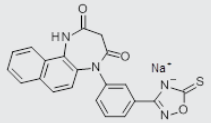
- P2X4 KO mice are **fertile** and show **no overt phenotype** when naïve
- P2X4 KO mice show **reduced inflammatory pain** and reduced PGE2 during inflammation
  (Ulmann et al., 2010; Tsuda et al., 2009)
- P2X4 KO mice show strongly reduced **mechanical allodynia in neuropathic pain models** (Ulmann et al., 2008; Tsuda et al., 2009)



Zebrafish P2X4 structure. Nature 2012

P2X4 Probe BAY-1797

Literature known P2X4 Inhibitors

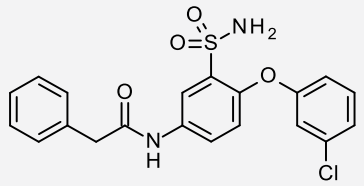
Structure	Code	Origin	Patent/Publication	hP2X4 IC_{50} (from Lit)	Comment
	5-BDBD	Bayer	WO 2004/085440 Balázs et al. (2013)	500 nM (CHO, Ca2+) 1300 nM (HEK, Ca2+)* 1600 nM (patch clamp)	
	BX 430	McGill University	Mol. Pharmacol. 2015, 87, 606	550 nM (patch clamp, HEK293)	human selective (mouse and rat IC_{50} >10µM)
	PSB-12054	Pharma Center Bonn	J. Med. Chem. 2012, 55, 9576	189 nM (Ca2+, 1321N1 astrocytoma) 2475 nM (HEK, Ca2+)*	allosteric, human selective (mP2X4 1.8 µM; rP2X4 2.1 µM)
	most active cmpd in this patent (Ex173)	Nippon Chemiphar	WO 2013/105608	64 nM (Ca2+, 1321N1 astrocytoma) 54 nM (HEK, Ca2+)*	high CL in rat Heps and high Efflux @Bayer assays
undisclosed	NCP-917	Nippon Chemiphar	EFIC Conference, Vienna 2015	300 nM (Ca2+) 28 nM (patch clamp, EZCells TT)	anti-allodynic effect in modified Chung model in rat after oral administration.
	NP-1815-PX	Nippon Chemiphar	Scientific Reports 2016, 6, 32461	260 nM (Ca2+, 1321N1 astrocytoma)	similar potency on rat and mouse P2X4 anti-allodynic effect in a mouse model of herpetic pain
undisclosed	NC-2600	Nippon Chemiphar & Kyushu University			Phase 1 (since June 2016) controlling pain through targeting glial cells; expected to attenuate neuropathic pain evoked in peripheral and central nervous system by oral administration

* same assay as used for BAY-1797 in this presentation

No potent/selective P2X4 inhibitors are commercially available

P2X4 Probe BAY-1797

Overall profile



BAY-1797

PhysChem

Lead-like properties

MW real / corr [g/mol]	417 / 401
TPSA [A²]	98
logD 7.5 (calc/exp)	3.5 / 2.8
Rotatable Bonds	6

Sol pH 2/4/7.4/8 [mg/L]	14-15
Plasmastab (4h) hum/rat [%]	94 / 103
Stability (24h) pH1/7/10 [%]	all 100

Pharmacology

Potent and selective P2X4 Inhibitor

P2X4 human FLIPR Ca^{2+} HEK IC_{50} [nM] (Efficacy in %)	211 (74)
P2X4 human / mouse / rat FLIPR Ca^{2+} 1321N1 astrocytoma IC_{50} [nM] (Efficacy in %)	108 (47) 112 (60) 233 (85)
P2X4 human Ephys Qpatch HEK IC_{50} [nM]	274
P2X4 human / mouse Ephys Qpatch 1321N1 astrocytoma IC_{50} [nM]	320 / 340

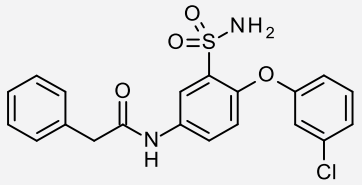
Assay description see backup

hP2X1 IC_{50} [µM]	>50
hP2X23 IC_{50} [µM]	>30
hP2X3 IC_{50} [µM]	8.3
hP2X7 IC_{50} [µM]	10.6
Carbonic Anhydrase II IC_{50} [µM]	>10
hCox-1 / hCox-2 IC_{50} [µM]	>6.7
h mPGES IC_{50} [µM]	>10
hERG IC_{50} [µM]	>10
Eurofins Lead Profiling Screen (n=67)	Na Channel site 2 57% at 10µM DAT IC_{50} 2.2µM
GPCR Profiler (n=25)	all>12.5 (agon) all>10µM (anatag)
Bayer Kinase Panel (n=22) IC_{50} [µM]	all >20
Ames / MNT / Cytotox	negative

BAY-1797 is a potent and selective P2X4 inhibitor

P2X4 Probe BAY-1797

Overall profile



BAY-1797

Pharmacokinetics

DMPK profile suitable for in vivo studies in rodents

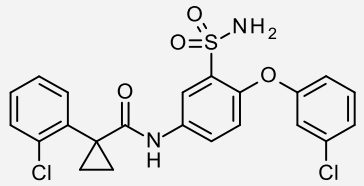
In vitro PK	CL_{bl} [L/h/kg]			F_{max} [%]		
LM hum / mouse / rat *	0.02	1.1	0.9	99	80	79
Heps hum / mouse / rat *			1.9			55
In vivo PK	AUC_{norm} [kg/h/L] iv/po	Vss [L/kg]	CL_{blood} [L/h/kg]	$t_{1/2}$ iv/po [h]	t_{max} po [h]	F po [%]
Rat male Wistar iv 1 mg/kg; po 2 mg/kg	1.1 / 0.47	2.8	0.48	2.6 / 2.6	4.0	43
	Hum	Mouse	Rat	Dog	Monkey	Williams E
Protein binding fu [%] *	1.2	2.9	2.2			41
	A-B [nm/s]		B-A [nm/s]		Efflux	
CaCo	132		84		0.6	
	1A2	2C8	2C9	2D6	3A4	TDI
CYP Inhibition [µM]	>20	3.4	6.1	4.6	>20	hint
	PXR		CYP1A2 [µg/L]		CYP3A4 [µg/L]	
CYP Induction $NOEL_{mRNA}$	red		>10000		41	

* Human mix Caucasian; Rat male Wistar; Mouse female NMRI; Dog female Beagle; Monkey female Cynomolgus

BAY-1797 shows a favorable pharmacokinetic profile, which makes the Chemical Probe suitable for in vivo studies in rodents

P2X4 Probe BAY-1797

Negative control BAY-207



BAY-207

Pharmacology

Inactive on human/mouse/rat P2X4

P2X4 human FLIPR Ca^{2+} HEK IC_{50} [nM] (Efficacy in %)	>25000 (20)
P2X4 human / mouse / rat FLIPR Ca^{2+} 1321N1 astrocytoma IC_{50} [nM] (Efficacy in %)	- >25000 (20) >25000 (29)
P2X4 human Ephys Qpatch HEK IC_{50} [nM]	
P2X4 human / mouse Ephys Qpatch 1321N1 astrocytoma IC_{50} [nM]	

hP2X1 IC50 [µM]	
hP2X23 IC50 [µM]	
hP2X3 IC50 [µM]	>50
hP2X7 IC50 [µM]	
Carbonic Anhydrase II IC50 [µM]	
hCox-1 / hCox-2 IC50 [µM]	
h mPGES IC50 [µM]	
hERG IC50 [µM]	
Eurofins Lead Profiling Screen (n=67)	
GPCR Profiler (N=25)	
Bayer Kinase Panel (n=12) IC50 [µM]	all >20 KDR 15

PhysChem

MW real / corr [g/mol]	477 / 427
TPSA [A²]	98
logD 7.5 (calc/exp)	3.1 / 3.4
Rotatable Bonds	6

Sol pH 2/4/7.4/8 [mg/L]	5-7 mg/L
Plasmastab (4h) hum/rat [%]	all 100
Stability (24h) pH1/7/10 [%]	all 100

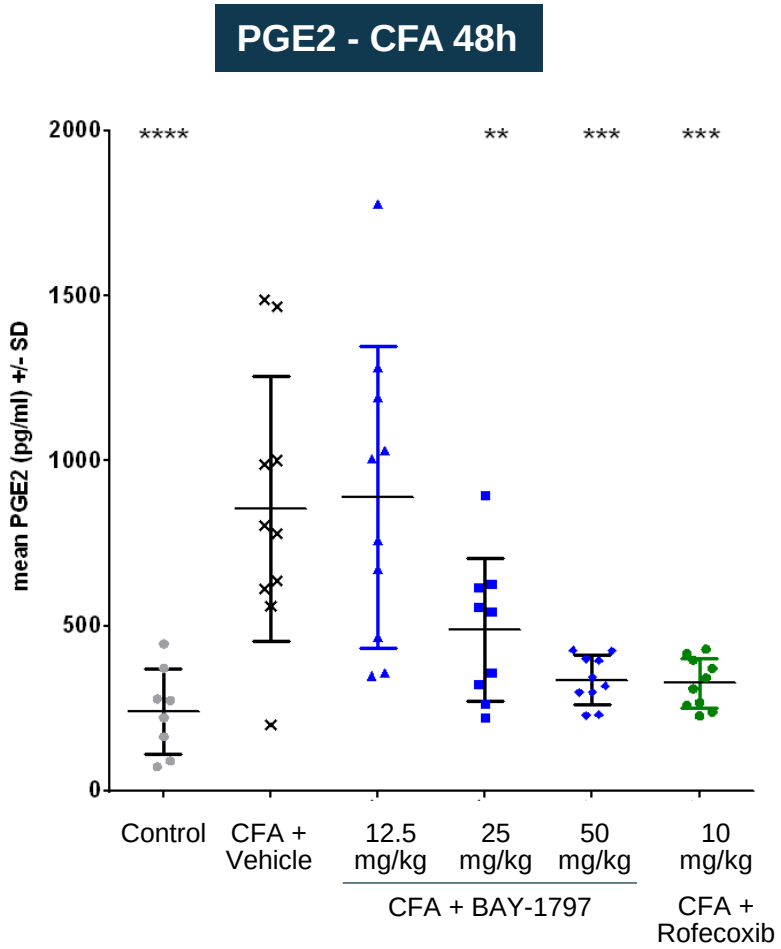
Pharmacokinetics

In vitro PK	**CL_{bl} [L/h/kg]**			**F_{max} [%]**		
LM hum / mouse / rat *	0.2	1.4	0.42	85	74	90
Heps hum / mouse / rat *			1.6			63
In vivo PK	AUC_{norm} [kg/h/L] iv/po	Vss [L/kg]	CL_{blood} [L/h/kg]	$t_{1/2}$ iv/po [h]	t_{max} po [h]	F po [%]
Rat male Wistar iv 1 mg/kg; po 2 mg/kg						
	Hum	Mouse	Rat	Dog	Monkey	Williams E
Protein binding fu [%] *						
	A-B [nm/s]		B-A [nm/s]		Efflux	
CaCo	95		39		0.4	
	1A2	2C8	2C9	2D6	3A4	TDI
CYP Inhibition [µM]	>20	3.9	6.8	>20	>20	hint
	PXR		CYP1A2 [µg/L]		CYP3A4 [µg/L]	
CYP Induction $NOEL_{mRNA}$	red					

* Human mix Caucasian; Rat male Wistar; Mouse female NMRI; Dog female Beagle; Monkey female Cynomolgus

P2X4 Probe BAY-1797

Initial Pharmacology of BAY-1797: Efficacy in mechanistic mouse model in vivo (CFA Model)



Dose (mg/kg)	unbound plasma conc. (60 min after dosing)
12.5	186 ± 43
25	374 ± 70
50	672 ± 154

Design

- CFA 48h, intraplantar inj. in C57Bl/6 mice
  - 30 µL, 1 mg/mL
  - Robinson et al., 2012
- BAY-1797
  - 0, 12.5, 25, 50 mg/kg, p.o.
  - 1 single dose at 1h before PGE2 sampling
- Vehicle: EtOH/Solutol/NaCl 1:5:94
- Read-out: PGE2 production in inflamed paw

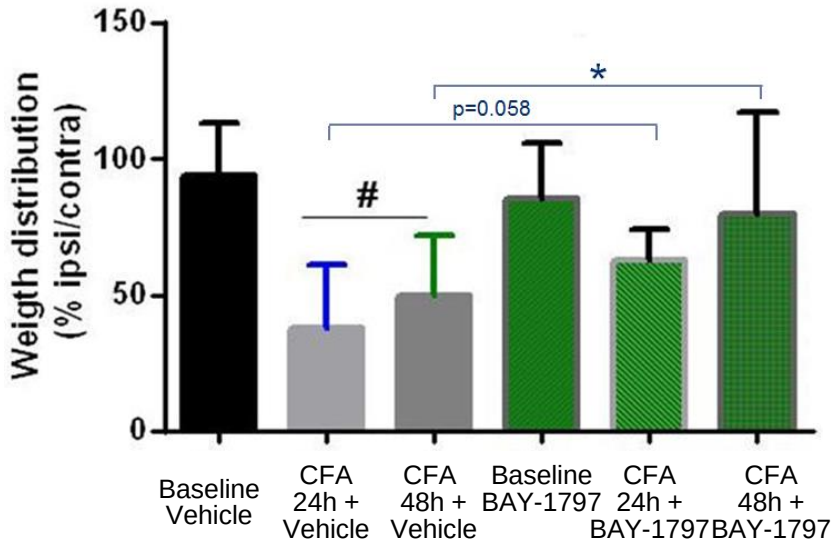
One-way analysis of variance, followed by Bonferroni's Multiple Comparison Test, n=9-10/group
**** p<0.0001 Control vs. CFA Veh at 48h. Data are mean ± SD
*** p<0.001 Treatment vs. CFA Veh at 48h. Data are mean ± SD
** p<0.01 Treatment vs. CFA Veh at 48h. Data are mean ± SD

- BAY-1797 dose-dependently reduced PGE2 levels in inflamed mouse paw
- Similar effects of BAY-1797 on PGE2 release was observed in rat CFA model

P2X4 Probe BAY-1797

Initial Pharmacology of BAY-1797: Efficacy in PD pain mouse model in vivo

Spontaneous pain behaviour - DWB



Dose (mg/kg)	unbound plasma conc. (80 min after dosing)
50	548 ± 32

Design

- CFA, intraplantar inj. in C57Bl/6 mice
  - 30 µl, 1 mg/ml
  - Robinson et al., 2012
- BAY-1797
  - 50 mg/kg, p.o.
  - 1 single dose each day at 1h before DWB
  - DWB testing at 24 and 48h after CFA
- Vehicle: EtOH/Solutol/NaCl 1:5:94
- Read-out: Dynamic Weight Bearing – DWB
  - Tétreault P. et al., 2011

One-way analysis of variance, followed by Bonferroni's Multiple Comparison Test, n=7-8/group
* p<0.05 Treatment vs. CFA Veh at 48h.
# p<0.01 CFA (24 and 48h) vs. Baseline Veh. Data are mean ± SD

- BAY-1797 significantly reduced pain behavior in CFA model
- At CFA 48h, weight bearing values in the treatment group were similar to baseline
- Effect correlates to markedly reduced PGE2 levels in inflamed paw

P2X4 Probe BAY-1797

Summary / Conclusion

Probe criteria	
Inhibitor/agonist potency: goal is < 100 nM (IC_{50}, Kd)	Potency as inhibitor of P2X4 ion channel demonstrated in FLIPR assay: Human P2X4 IC_{50} at 211 nM (HEK, Ca2+) and at 108 nM (1321N1 astrocytoma, Ca2+) Equipotent on human, rat and mouse P2X4
Selectivity within target family: goal is >30-fold	**Surpasses criteria**; Selectivity against family members was tested, all >30fold
Selectivity outside target family: describe the off-targets (which may include both binding and functional data)	**Surpasses criteria**; Selectivity in Eurofins Lead Profiling Screen, GPCR Profiling Screen and Bayer Kinase Panel was performed (only relevant IC_{50}: DAT 2.2µM)
On target cell activity for cell-based targets: goal is < 1 micromolar IC_{50}/EC_{50}	**Surpasses criteria**; Ephys human P2X4 IC_{50} at 274 nM (Qpatch, HEK) Efficacy in several *in vitro* and *in vivo* mechanistic models demonstrated; efficacy in PD pain inflammation model
On target cell activity for secreted targets: appropriate alternative such as mouse model or other mechanistic biological assay, e.g., explant culture	**Surpasses criteria**; Suitable pharmacokinetic profile for *in vivo* studies in rodents
Neg ctrl: *in vitro* potency – > 100 times less; Cell activity – >100 times less potent than the probe	**Surpasses criteria**; Structure related compound BAY-207 with no P2X4 activity (human P2X4 IC_{50} >25 µM (HEK, Ca2+)

We ask for acceptance of P2X4 inhibitor BAY-1797 as chemical probe, accompanied by BAY-207 as negative control

P2X4 Probe BAY-1797

Acknowledgement

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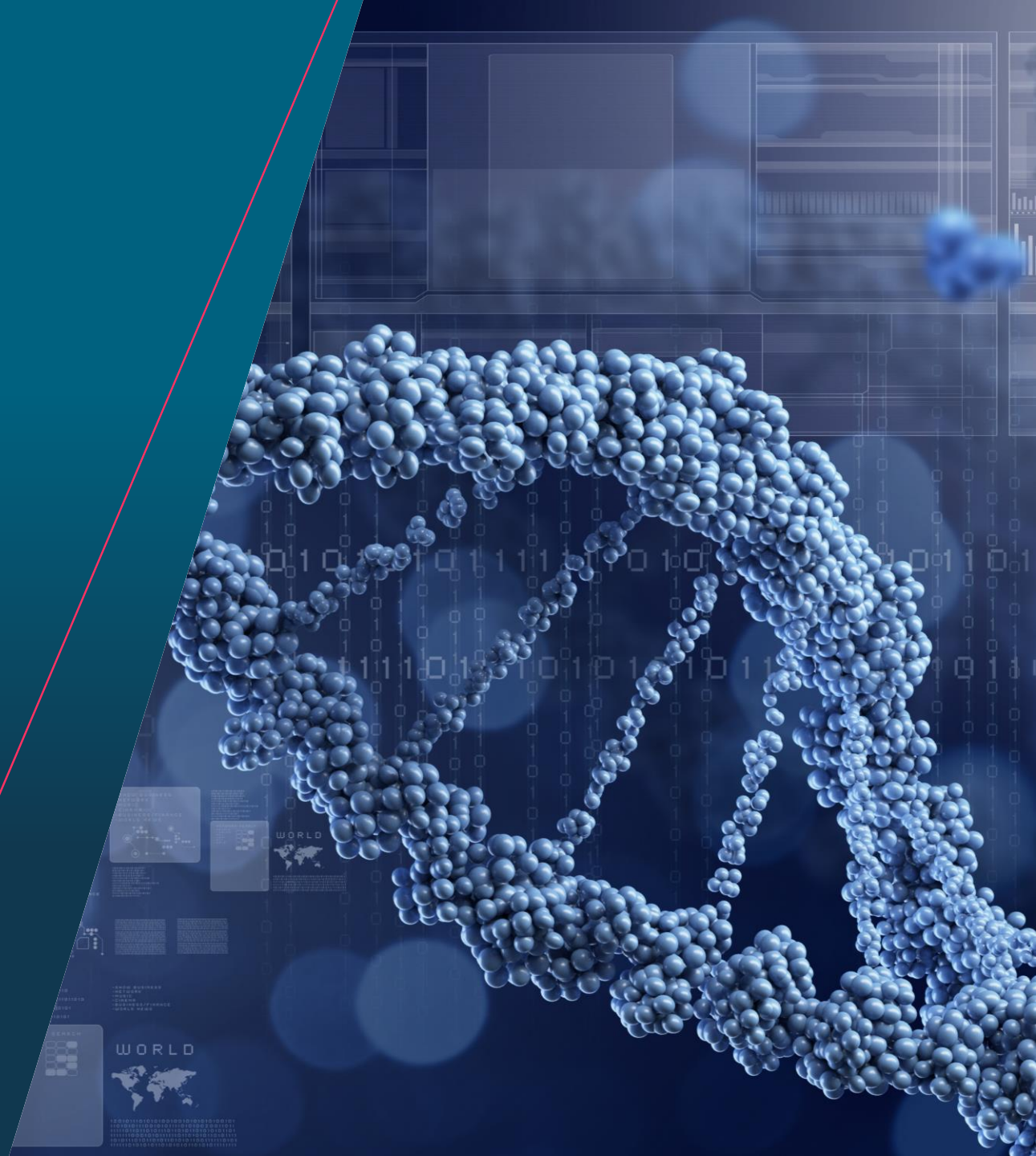
Thank You



P2X4 Probe BAY-1797

P2X Ion channel family

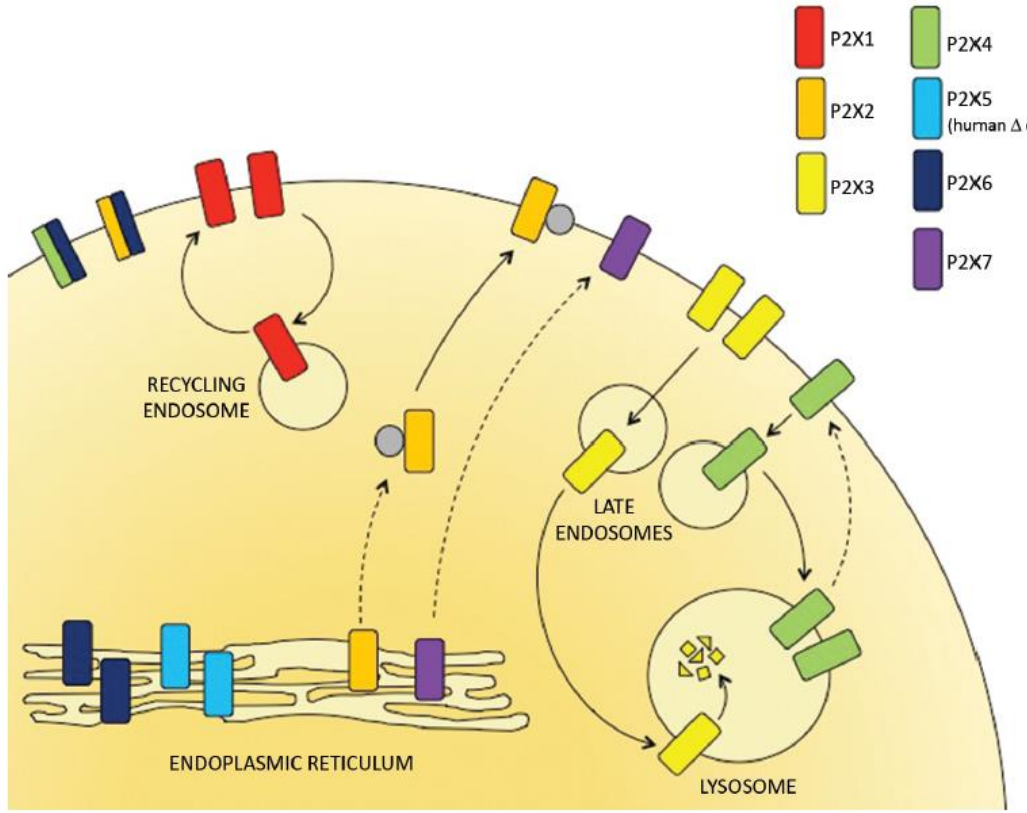


FIGURE 1 | The subcellular distribution of P2X receptors. P2X receptor subtypes differ in their trafficking properties and hence are localized to different subcellular compartments. P2X6 receptors are retained within the ER but can assemble with P2X4 and P2X6 subunits to form heterotrimers that traffic to the cell surface. The predominant human allele of P2X5 lacks exon 10 and is also retained in the ER. P2X2 and P2X7 receptors traffic relatively slowly through the secretory pathway but are stably expressed at the surface. P2X1 receptors are expressed at the cell surface but rapidly cycle to and from recycling endosomes. P2X3 and P2X4 receptors are consitutively internalized and delivered to late endosomes and lysosomes. Within the lysosomes, P2X3 receptors are rapidly degraded but P2X4 receptors resist degradation and can recycle back to the surface.

Murell-Lagnado, Front. Cell. Neurosci., 2013, Vol 3, Article 233

P2X4 Probe BAY-1797

Sequence Identity of P2X-Receptors

Whole Sequence \ allosteric Binding Site

		P2X4			P2X1			P2X2			P2X3			P2X5			P2X6			P2X7		
	Identity	h	r	m	h	r	m	h	r	m	h	r	m	h	r	m	h	r	m	h	r	m
P2X4	h	100	85	85	38	38	38	54	46	46	62	62	62	62	62	62	62	62	62	54	62	54
	r	80	100	77	38	38	38	54	46	46	62	62	62	62	62	62	54	54	54	46	54	46
	m	81	85	100	38	38	38	54	46	46	62	62	62	62	62	62	62	62	62	62	69	62
P2X1	h	26	25	25	100	92	85	69	62	62	62	62	62	62	54	62	54	62	62	54	46	54
	r	25	24	24	89	100	92	69	62	62	54	54	54	62	54	62	54	62	62	54	46	54
	m	25	24	24	89	97	100	62	54	54	54	54	54	54	46	54	54	62	62	54	46	54
P2X2	h	23	23	23	30	31	31	100	92	92	69	69	69	77	69	77	54	62	62	46	54	54
	r	23	23	23	30	31	31	77	100	100	62	62	62	69	62	69	46	54	54	38	46	46
	m	23	23	23	30	30	30	79	95	100	62	62	62	69	62	69	46	54	54	38	46	46
P2X3	h	24	23	23	41	40	40	39	39	38	100	100	100	77	69	77	62	69	69	54	62	54
	r	24	23	23	40	40	40	39	38	37	94	100	100	77	69	77	62	69	69	54	62	54
	m	24	23	23	40	40	40	38	38	37	94	99	100	77	69	77	62	69	69	54	62	54
P2X5	h	29	28	29	47	47	47	36	35	34	41	41	41	100	85	100	62	69	69	54	62	54
	r	28	28	29	46	46	46	36	35	34	42	42	42	87	100	85	62	62	62	46	54	46
	m	28	28	29	47	47	47	36	35	34	43	42	42	87	96	100	62	69	69	54	62	54
P2X6	h	22	22	21	35	34	34	32	34	33	35	35	36	42	42	41	100	92	92	54	62	54
	r	25	25	25	37	36	36	37	37	36	38	37	37	42	42	42	63	100	100	62	69	62
	m	24	24	24	37	36	36	36	36	36	37	36	37	42	42	42	62	95	100	62	69	62
P2X7	h	23	21	22	37	37	37	31	31	31	32	32	32	38	38	37	39	40	41	100	77	85
	r	23	22	22	40	40	40	30	29	29	34	34	34	42	41	42	37	39	39	70	100	85
	m	22	22	22	40	40	40	31	31	30	35	35	35	42	41	42	37	39	39	72	93	100

Allosteric binding site (top-right triangle) defined from published x-ray structures of P2X7, no similar P2X4 structure known

P2X4 Probe BAY-1797

Assay description FLIPR

Compound	BAY-1797
P2X4 human FLIPR Ca^{2+} HEK IC_{50} [nM] (Efficacy in %)	211 (74)
P2X4 human / mouse / rat FLIPR Ca^{2+} 1321N1 astrocytoma IC_{50} [nM] (Efficacy in %)	108 (47) 112 (60) 233 (85)

Human P2X4 HEK Cell FLIPR Assay (Assay 1)
HEK293 cells stably expressing human P2X4 were plated in poly-D-lysine-coated 384-well plates at a seeding density of 30000 cells/well and incubated overnight. P2X4 25 function was assessed by measuring intracellular calcium changes using the calciumchelating dye Fluo8-AM (Molecular Devices) on a fluorescent imaging plate reader (FLEX/FLIPR station; Molecular Devices). On the day of the assay, the medium was removed and the cells were incubated for 30 min at 37°C and 5% C02 in 30 µL of dye buffer (Hank's balanced salt solution, 10 mM HEPES, 1.8 mM CaCl2, 1 mM MgCl2, 2 mM 30 probenecid, 5mM D-glucose monohydrate, 5µM Fluo8-AM, pH=7.4). Compounds diluted in probenecid buffer (Hank's balanced salt solution, 10 mM HEPES, 1.8 mM CaCl2, 1 mM
MgCb, 2 mM probenecid, 5mM D-glucose monohydrate, pH=7.4) were added in a volume of 10 µL and allowed to incubate for 30 min at room temperature. The final assay DMSO concentration was 0.5%. The agonist, Bz-ATP (Tocris), was added in a volume of 10 µLat a concentration representing the ECao value. The ECao value of Bz-ATP was determined each assay day prior to compound profiling. The fluorescence was measured for an interval of 120 sec at 2 sec intervals. The excitation and emission wavelengths used to monitor fluorescence were 470-495 nm and 515-575 nm, respectively. The data s was analyzed based on the increase in peak relative fluorescence units (RFU) compared to the basal fluorescence and the data was normalized to the agonist control. The compounds were tested in triplicates per plate and mean values were plotted in Excel XLFit to determine IC50 values, percentage of maximal inhibition and the Hill coefficients.

Human/Mouse/rat P2X4 1321 N1 astrocytoma Cell FLIPR Assay (Assay 2)
5 1321 N1 Astrocytoma cells stably expressing human P2X4 or rat P2X4 or mouse P2X4 were plated in Collagen I TC-treated microplate at a seeding density of 10000 cells/well and incubated overnight. P2X4 function was assessed by measuring intracellular calcium changes using the calcium-chelating dye Fluo8-AM (Molecular Devices) on a fluorescent imaging plate reader (FLEX/FLIPR station; Molecular Devices). On the day of the assay, 10 the medium was removed and the cells were incubated for 30 min at 370C and 5% C02 in 30 µL of dye buffer (Hank's balanced salt solution, 10 mM HEPES, 1.8 mM CaCl2, 1 mM MgCb, 2 mM probenecid, 5 mM D-glucose monohydrate, 5 µM Fluo8-AM, pH=7.4). Compounds diluted in probenecid buffer (Hank's balanced salt solution, 10 mM HEPES,
1.8 mM CaCl2, 1 mM MgCl2, 2 mM probenecid, 5 mM D-glucose monohydrate, pH=7.4) 15 were added in a volume of 10 µL and allowed to incubate for 30 min at room temperature. The final assay DMSO concentration was 0.25%. The agonist, Mg-ATP (Sigma), was added in a volume of 10 µL at a concentration representing the ECao value. ECao was determined to be 0.5 µM for human and mouse P2X4 and 5 µM for rat P2X4. The fluorescence was measured for an interval of 120 sec at 2 sec intervals. The excitation 20 and emission wavelengths used to monitor fluorescence were 470-495 nm and 515-575 nm, respectively. The data was analyzed based on the increase in peak relative fluorescence units (RFU) compared to the basal fluorescence and the data was normalized to the agonist control. The compounds were tested in triplicates per plate and mean values were plotted in Excel XLFit to determine IC50 values, percentage of maximal 25 inhibition and the Hill coefficients.

P2X4 Probe BAY-1797

Assay description

Compound	BAY-1797
P2X4 human Ephys Qpatch HEK IC_{50} [nM]	274
P2X4 human / mouse Ephys Qpatch 1321N1 astrocytoma IC_{50} [nM]	320 / 340

Human P2X4 HEK Cell Qpatch Assay (Assay 3)

Cell culture conditions: HEK-293 mito-Photina pcDNA3(neo-)/pPURO N/pcDNA3_P2RX4, clone 2a/4 (HEK-293 mito-Photina/hP2RX4) cells were cultured in EMEM Minimum Essential Medium Eagle with Earl's salts Balanced Salt Solution (BioWhittaker cat. BE12-125F) supplemented with 5 ml of 200 mM Ultraglutamine1 (BioWhittaker cat. BE17-605E/U1), 5 ml of 100X Penicillin/Streptomycin (BioWhittaker cat. DE17-602E; final concentration 1%), 4 ml of 50 mg/ml G418 (Sigma cat. G8168-100ml; final concentration 400 µg/ml), 10 µL of 10 mg/ml Puromicin (InvivoGen cat. ant-pr-1; final concentration 0,2 µg/ml) and 50 ml of Fetal Bovine Serum (Sigma cat. F7524; final concentration 10%).

Experimental protocol: HEK-293 cell lines are seeded 72 or 96 hours before experiment, at a concentration of 5 or 2.5 million cells, respectively onto a T225 flask. Just before the experiments cells are washed twice with D-PBS w/o Ca2+/Mg2+ (Euroclone cat. ECB4004L) and detached from the flask with trypsin-EDTA (Sigma, cat. T4174 diluted 1/10). Cells are then re-suspended in the suspension solution: 25 ml EX-CELL ACF CHO medium (Sigma, cat. C5467); 0.625 ml HEP ES (BioWhittaker, cat. BE17-737E); 0.25 ml 25 of 100x Penicillin/Streptomycin (BioWhittaker, cat. DE17-602E), 0.1 ml of Soybean Trypsin Inhibitor 10 mg/ml (Sigma, cat. T6522) and placed on the QPatch 16X.

Compound preparation and storage: Compound stock solutions (10 mM; 100% DMSO; stored at -20°C) were used. Fresh solutions from stock (1 or 3 mM, 100% DMSO) were prepared just before the experiments (0.1 % final DMSO concentration). DMSO solution was obtained from SIGMA (cat.# D-5879) and stored at room temperature.

Patch clamp analysis with QPatch16X (Figure 1): Standard whole-cell voltage clamp experiments are performed at room temperature using the multihole technology. For the voltage clamp experiments on hP2X4, data are sampled at 2 KHz. After establishment of the seal and the passage in the whole cell configuration, the cells are held at -90 mV and the hP2X4 current is evoked by the agonist in the absence (vehicle period, i.e. 0.1 % DMSO) or in the presence of the compound under investigation at increasing concentrations; see the application protocol in Figure 1.

Output: the maximum inward current induced by the agonist (ATP 5 microM). The intracellular solution contained (mM) 135 CsF, 10 NaCl, 1 EGTA, 10 HEPES (pH 7.2 with CsOH) whereas the extracellular solution (mM) 145 NaCl, 4 KCl, 0.5 MgCl2, 1 CaCl2, 10 HEPES, 10 Glucose (pH 7.4 with NaOH).

For data collection, the Sophion software was used and the analysis was performed offline using Excel and GraphPad Prism.

When possible, i.e. when the % of inhibition with the highest concentration tested was more than 50 %, the dose-response curves data were fitted with the following equation:

Y=100/(1 +1 OA((LoglC50-X)*HillSlope))

[Xis log of concentration; Y is normalized response (100% down to 0%, decreasing as X increases); LoglC50 same log units as X; HillSlope is unitless slope factor or hill slope]

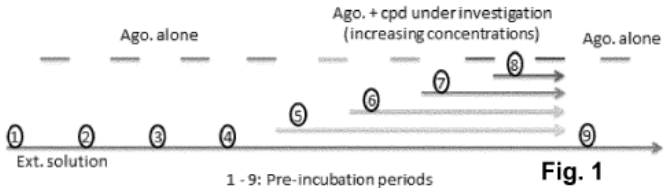


Fig. 1

QPatch methods for hP2X4 1321N1 cells (Assay 4)

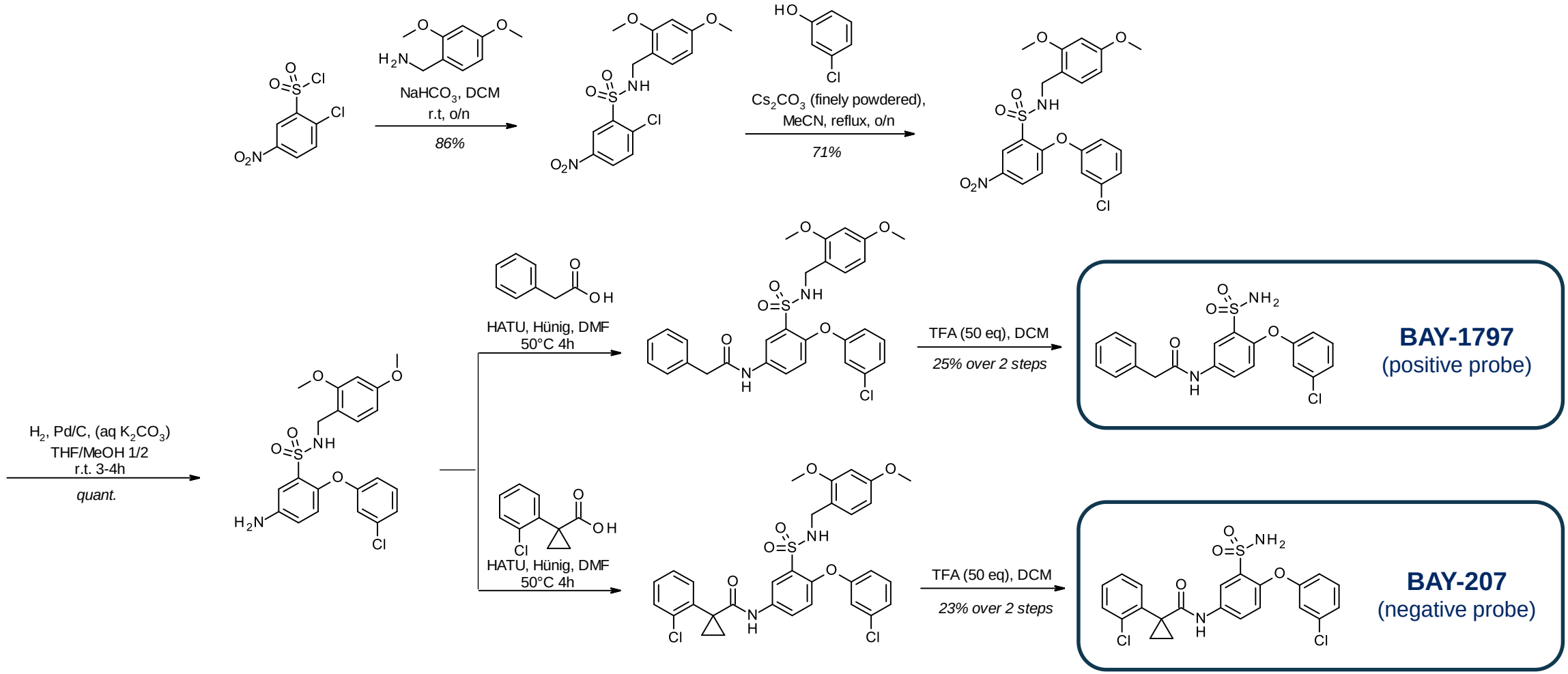
Human P2X4 stably expressed in 1321N1 cells were obtained from Prof. Müller (University of Bonn). Cells were grown for 2-4 days to a 70-90% confluency prior to the experiment and prior to the experiment, the cells were detached with Accutase and resuspended in 1 mL serum free medium.

P2X4 currents were measured with the automated patch clamp platform QPatch 48X (Sophion - Biolin scientific, Denmark) in multi-hole configuration (10x). Extracellular Buffer solution (in mM): NaCl 140, KCl 4, HEPES 10, CaCl2 2, MgCl2 1, D-glucose monohydrate 5, pH=7.4 and intracellular buffer (in mM): CsF 135, EGTA 1, HEPES 10, NaCl 10, pH 7.2 were used for the measurements. The membrane potential was held at -90 mV and the ligand agonist, adenosine 5'-trisphosphate (ATP, 5 µM) was added in a volume of 5 µl, directly washed off by extracellular buffer containing 30 U hexokinase/mL. Baseline was obtained by five consecutive additions of ATP, followed by increasing concentration of compound (0.3% DMSO) and ATP to assess the compound effect.

Data were sampled at 10 kHz and filtered using a 4-pole Bessel filter at 100 Hz. Series resistance was compensated by 70%. Data analysis was performed using Qpatch assay software version 5.0 (Sophion) and compound effect was assed as decrease in peak current amplitude. The IC50 values were obtained by nonlinear fit using the following equation: Y=Bottom + (Top - Bottom)/1+10^Log(IC50 – x)* HillSlope.

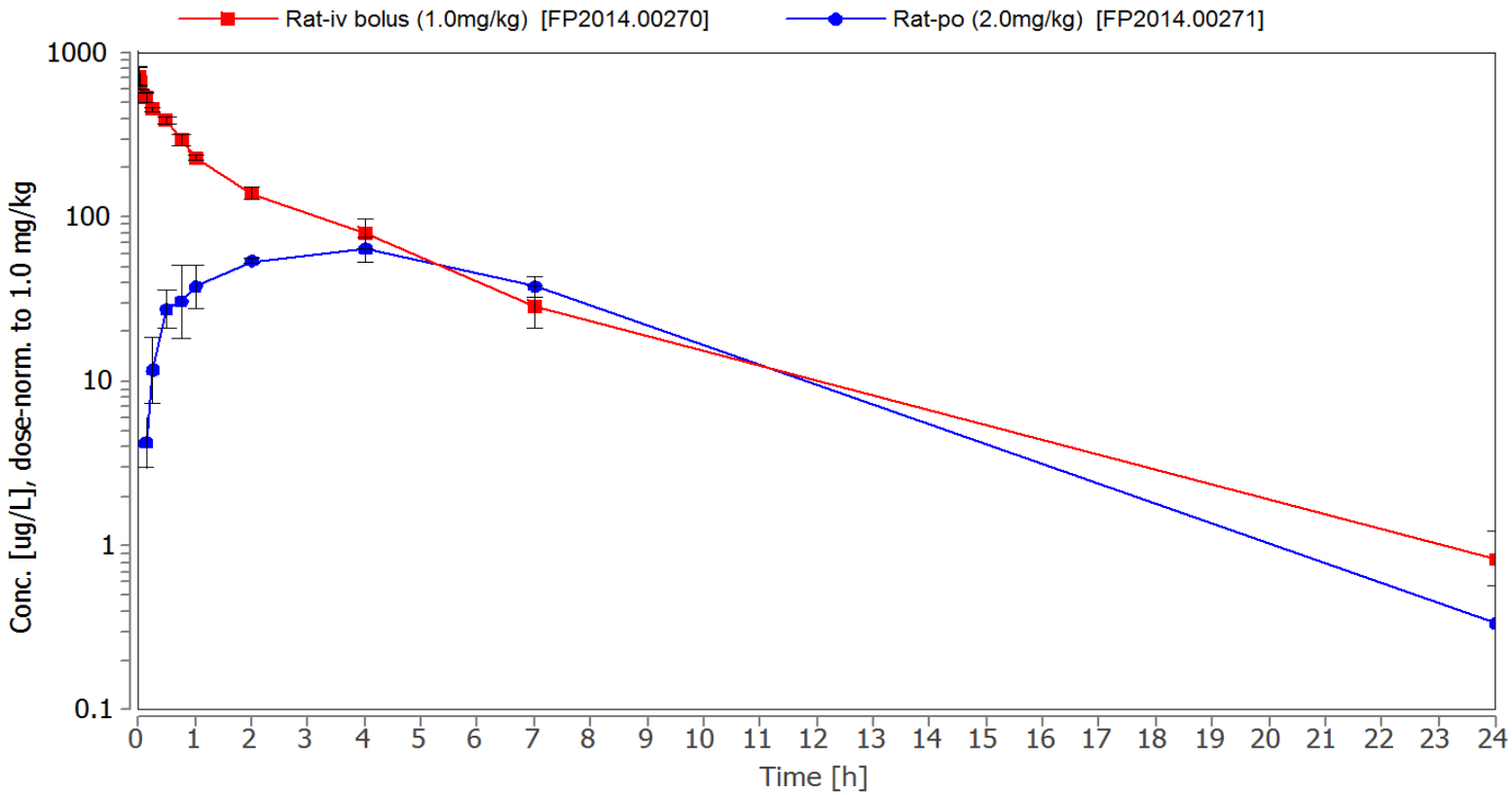
P2X4 Probe BAY-1797

Synthesis of BAY-1797 and BAY-207



P2X4 Probe BAY-1797

In vivo Pharmacokinetics (male Wistar Rat)



In vivo PK male Wistar Rat	AUC_{norm} [kg*h/L]	CL_{Plasma} [L/h/kg]	C_{Blood}/C_{Plasma}	CL_{Blood} [L/h/kg]	V_{SS} [L/kg]	$t_{1/2}$ [h]	t_{max} [h]	F [%]
i.v. PK (1 mg/kg)	1.1	0.94	1.9	0.48	2.8	2.6		
p.o. PK (2 mg/kg)	0.47					2.6	4.0	43

In vivo PK in male Wistar rat

- Low clearance
- High V_{SS}
- Moderate half-life time
- Late t_{max}
- Moderate bioavailibility

P2X4 Probe BAY-1797

Eurofins Lead Profiling Screen // GPCR Profiler

Cat #	Assay Name	Batch*	Spec.	Rep.	Conc.	% Inh.
Compound: CHH71-2013, PT #: 1172997						
200510	Adenosine A_1	336632	hum	2	10 µM	35
200610	Adenosine A_{2A}	336633	hum	2	10 µM	14
200720	Adenosine A_3	336655	hum	2	10 µM	47
203100	Adrenergic α_{1A}	336550	rat	2	10 µM	16
203200	Adrenergic α_{1B}	336551	rat	2	10 µM	9
203400	Adrenergic α_{1D}	336552	hum	2	10 µM	5
203630	Adrenergic α_{2A}	336627	hum	2	10 µM	26
204010	Adrenergic β_1	336578	hum	2	10 µM	3
204110	Adrenergic β_2	336580	hum	2	10 µM	2
285010	Androgen (Testosterone) AR	336531	rat	2	10 µM	17
212510	Bradykinin B_1	336499	hum	2	10 µM	11
212620	Bradykinin B_2	336500	hum	2	10 µM	10
214510	Calcium Channel L-Type, Benzothiazepine	336631	rat	2	10 µM	17
214600	Calcium Channel L-Type, Dihydropyridine	336508	rat	2	10 µM	21
216000	Calcium Channel N-Type	336654	rat	2	10 µM	6
217030	Cannabinoid CB_1	336582	hum	2	10 µM	44
219500	Dopamine D_1	336634	hum	2	10 µM	11
219700	Dopamine D_{2S}	336636	hum	2	10 µM	15
219800	Dopamine D_3	336637	hum	2	10 µM	28
219900	Dopamine $D_{4.2}$	336638	hum	2	10 µM	4
224010	Endothelin ET_A	336563	hum	2	10 µM	3
224110	Endothelin ET_B	336593	hum	2	10 µM	-1
225510	Epidermal Growth Factor (EGF)	336716	hum	2	10 µM	2
226010	Estrogen ERα	336509	hum	2	10 µM	5
226600	$GABA_A$, Flunitrazepam, Central	336594	rat	2	10 µM	0
226500	$GABA_A$, Muscimol, Central	336543	rat	2	10 µM	15
228610	$GABA_{B1A}$	336597	hum	2	10 µM	14
232030	Glucocorticoid	336566	hum	2	10 µM	20
232700	Glutamate, Kainate	336643	rat	2	10 µM	3
232810	Glutamate, NMDA, Agonism	336607	rat	2	10 µM	23
232910	Glutamate, NMDA, Glycine	336514	rat	2	10 µM	-9
233000	Glutamate, NMDA, Phencyclidine	336608	rat	2	10 µM	-1
239610	Histamine H_1	336518	hum	2	10 µM	15
239710	Histamine H_2	336626	hum	2	10 µM	-15

Cat #	Assay Name	Batch*	Spec.	Rep.	Conc.	% Inh.
239820	Histamine H_3	336498	hum	2	10 µM	0
241000	Imidazoline I_2, Central	336542	rat	2	10 µM	10
243520	Interleukin IL-1	336502	mouse	2	10 µM	24
250460	Leukotriene, Cysteinyl $CysLT_1$	336522	hum	2	10 µM	8
251600	Melatonin MT_1	336569	hum	2	10 µM	13
252610	Muscarinic M_1	336586	hum	2	10 µM	-6
252710	Muscarinic M_2	336587	hum	2	10 µM	4
252810	Muscarinic M_3	336588	hum	2	10 µM	-12
257010	Neuropeptide Y Y_1	336881	hum	2	10 µM	9
257110	Neuropeptide Y Y_2	336612	hum	2	10 µM	8
258590	Nicotinic Acetylcholine	336591	hum	2	10 µM	-11
258700	Nicotinic Acetylcholine α1, Bungarotoxin	336592	hum	2	10 µM	-4
260130	Opiate δ_1 (OP1, DOP)	336641	hum	2	10 µM	4
260210	Opiate κ(OP2, KOP)	336525	hum	2	10 µM	10
260410	Opiate μ(OP3, MOP)	336642	hum	2	10 µM	9
264500	Phorbol Ester	336656	mouse	2	10 µM	4
265010	Platelet Activating Factor (PAF)	336613	hum	2	10 µM	-2
265600	Potassium Channel [K_{ATP}]	336644	ham	2	10 µM	-9
265900	Potassium Channel hERG	336532	hum	2	10 µM	8
268420	Prostanoid EP_4	336568	hum	2	10 µM	21
268700	Purinergic P2X	336639	rabbit	2	10 µM	5
268810	Purinergic P2Y	336640	rat	2	10 µM	14
270000	Rolipram	336660	rat	2	10 µM	8
271110	Serotonin (5-Hydroxytryptamine) $5\text{-}HT_{1A}$	336528	hum	2	10 µM	7
271910	Serotonin (5-Hydroxytryptamine) $5\text{-}HT_3$	336544	hum	2	10 µM	3
278110	Sigma σ_1	336622	hum	2	10 µM	19
279510	Sodium Channel, Site 2	336630	rat	2	10 µM	57
255520	Tachykinin NK_1	336523	hum	2	10 µM	4
285900	Thyroid Hormone	336504	rat	2	10 µM	2
220320	Transporter, Dopamine (DAT)	336512	hum	2	10 µM	81
226400	Transporter, GABA	336510	rat	2	10 µM	-10
204410	Transporter, Norepinephrine (NET)	336511	hum	2	10 µM	47
274030	Transporter, Serotonin (5-Hydroxytryptamine) (SERT)	336574	hum	2	10 µM	4

Cat #	Assay Name	Species	Conc.	% Inh.	IC_{50}*	K_i	n_H
220320	Transporter, Dopamine (DAT)	hum	3 µM	60	2.17 µM	1.73 µM	1.19

In addition, BAY-1711 was tested in the GPCR profiler against 25 GPCRs

- 5-HT1A, 5-HT2B, 5-HT6, A2B, A3, ADRA1A, ADRA2A, ADRB1, ADRB2, CB1, D1, D2, EP3, H1, H2, H3, M1, M4, MC4, Motilin, NK1, OPRK1, OPRM1, P2RY2, SST4
- No agonistic activity @12.5 µM
- No antagonistic activity @10 µM