Supporting Information

Synthesis, characterization, and in vitro evaluation of the selective $P2Y_2$ receptor antagonist AR-C118925

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Materials and methods

Radioligand receptor binding assays: CB_1 and CB_2

For the cannabinoid receptors CB_1 and CB_2, competition binding assays were performed as described before [1] using the CB agonist radioligand [^{3}H](−)-cis-3-[2-hydroxy-4-(1,1-dimethylheptyl)phenyl]-trans-4-(3-hydroxypropyl)cyclohexanol (CP55,940, final concentration = 0.1 nM). As a source for human CB_1 and CB_2 receptors, membrane preparations of Chinese hamster ovary cells stably expressing the respective receptor subtype were used (CB_1: 20 µg of protein/vial; CB_2: 2 µg of protein/vial). After addition of 15 µl of test compound in DMSO, 60 µl of [^{3}H]CP55,940 solution in assay buffer (50 mM TRIS, 3 mM $MgCl_2$, pH 7.4), and 60 µl of membrane preparation to 465 µl of assay buffer (50 mM TRIS, 3 mM $MgCl_2$, pH 7.4), the suspension was incubated for 2 h at room temperature. Total binding was determined by adding DMSO without test compound. Non-specific binding was determined in the presence of 10 µM of unlabeled CP55,940. Incubation was terminated by rapid filtration through GF/C glass fiber filters pre-soaked for 0.5 h with 0.3% aqueous polyethyleneimine solution. Filters were washed three times with ice-cold washing buffer (50 mM TRIS, 0.1% bovine serum albumin, pH 7.4) and then dried for 1.5 h at 50 °C. Radioactivity on the filters was determined in a Tri-Carb® 2810TR (PerkinElmer) liquid scintillation counter after 6 h of incubation with 3 ml of Lumasafe™ scintillation cocktail.

Radioligand receptor binding assays: adenosine receptors

For adenosine receptors, the procedure has been previously described in detail [2, 3]. Briefly, radioligand/Tris-buffer solution (100 µl) was given to 10 µl of compound diluted in DMSO and 790 µl of Tris-buffer (50 mM, pH 7.4), following which 100 µl of

membrane suspension containing the respective receptor was added and incubated for 1.25 h at room temperature. Following incubation, the radioligand bound to the receptor was separated from unbound radioligand by harvesting using a Brandel® MP48 harvester and GF/B glass fiber filters. Lumasafe™ scintillation cocktail (3 ml) was given to the filter and, after 9 h of incubation, radioactive counts were measured using a Tri-Carb® 2810TR (PerkinElmer) liquid scintillation counter. The radioligands used were [^{3}H]CCPA for the A_1 adenosine receptor, [^{3}H]MSX-2 for the A_{2A} adenosine receptor, [^{3}H]PSB-603 for the A_{2B} adenosine receptor, and [^{3}H]PSB-11 for the A_3 adenosine receptor.

β-Arrestin assays: GPR18, GPR55

The Chinese hamster ovary (CHO) cell lines expressing the human GPR18 and GPR55 receptors were purchased from DiscoverX (Fremont, CA, USA). The β-arrestin assays for GPR18 and GPR55 were done as described for the $P2Y_2$ receptor in the main text, with the exception that test compound dilutions were done in phosphate-buffered saline (PBS) containing 10% DMSO and 0.1 % bovine serum albumin. For GPR18, tetrahydrocannabinol (10 µM), and for GPR55 lysophosphatidylinositol (1 µM) was used as agonist.

NPP1-3 assays

Materials: 4-Aminoantipyrine, adenosine 5'-monophosphate (AMP), adenosine 5'-triphosphate (ATP), calcium chloride, choline oxidase, dimethyl sulfoxide (DMSO), magnesium chloride, 1-oleoyl-*sn*-glycero-3-phosphocholine (LPC (18:1)), peroxidase from horseradish, sodium hydroxide, and 3-(*N*-ethyl-3-methylanilino)-2-hydroxypropanesulfonic acid (TOOS) were supplied by Sigma (Steinheim, Germany).

2-(*N*-Cyclohexylamino)ethanesulfonic acid (CHES) and Tris(hydroxymethyl) aminomethane (Tris) were purchased from Applichem (Darmstadt, Germany). Disodium hydrogen phosphate was obtained from Carl Roth (Karlsruhe, Germany). Human recombinant soluble NPP1-3 stably expressed in Sf9 insect cells, were prepared in our laboratory [4].

NPP1 and NPP3 assays: For the assessments at human NPP1 and -3, we utilized a capillary electrophoresis (CE) assay based on the method described by Chang *et al.* [5] using adenosine 5'-triphosphate (ATP) as substrate. The test compounds were initially screened in a concentration of 100 µM. They were dissolved in reaction buffer (1 mM $MgCl_2$, 2 mM $CaCl_2$, 10 mM CHES (2-(*N*-cyclohexylamino)ethanesulfonic acid), pH 9.0) together with ATP as a substrate (400 µM). The reaction was initiated by adding 20 µl of human NPP1 (1.7 µg) or human NPP3 (43 µg), respectively. The mixture was then incubated for 30 min (NPP1) or 60 min (NPP3) and stopped by heating at 90 °C for 3 min. After cooling the reaction samples on ice, they were transferred into CE vials and injected into the CE instrument. The operation conditions in CE analyses were as follows: P/ACE MDQ capillary electrophoresis system (Beckman Instruments, Fullerton, CA, USA) equipped with a DAD detection system. Data collection and peak area analysis were performed by the P/ACE MDQ software 32 KARAT obtained also from Beckman Coulter (Fullerton, CA, USA). The electrophoretic separations were performed using a polyacrylamide-coated capillary (50 cm (eff.), x 50 µm (id) supplied by CS-Chromatography (Langerwehe, Germany)). Electrokinetic injections were performed using a voltage of –9 kV for 90 s and separations were carried out by a voltage of –20 kV. The amount of adenosine 5'-monophosphate (AMP) produced were detected at 260 nm. The running buffer

consisted of 50 mM phosphate buffer (pH 6.5). Between separations, the capillary was washed with water for 2 min (20 psi) and subsequently with running buffer for 2 min (20 psi) before each injection. For human NPP1, a concentration-inhibition curve was determined with different concentrations of AR-C118925 (**3**) and derivative **5** in a range of 0.02-200 µM. The IC_{50} value was then determined from three independent experiments using Prism 5.0 (Graphpad Software, San Diego, CA, USA).

NPP2 assay: Inhibitory activity at human NPP2 was determined using spectrophotometric assays with 1-oleoyl-*sn*-glycero-3-phosphocholine (LPC (18:1)) as a substrate [4, 5]. Lysophospholipase-D activities were measured at 37 °C in a final volume of 50 µl. The reaction mixture included 5 mM $MgCl_2$, 5 mM $CaCl_2$, 100 mM Tris, pH 9.0, together with 400 µM LPC (18:1). The reaction was started by the addition of 10 µl of human NPP2 (44 µg), and then incubated at 37 °C for 60 min. Subsequently, the released choline was quantified colorimetrically at 555 nm after incubation at 37 °C for 10 min with 50 µl of each, the peroxidase reagent (50 mM Tris at pH 9.0, 2 mM 3-(*N*-ethyl-3-methylanilino)-2-hydroxypropanesulfonic acid (TOOS), 5 U/ml peroxidase), and the choline oxidase reagent (50 mM Tris at pH 9.0, 2 mM 4-aminoantipyrine, 5 U/ml choline oxidase). All experiments were carried out twice in triplicates.

Human NTPDases 1-3, 8: malachite green assay

Substrate ATP (20 µl) in assay buffer (5 mM $CaCl_2$ and 80 mM Tris with the pH value adjusted to 7.4) was added to a well of a 96-well plate containing 10 µl 10% aq. DMSO or test compound in 10% aq. DMSO. The reaction was initiated by addition of 20 µl NTPDase protein extract in assay buffer to give a final concentration of 50 µM

(NTPDase1) or 100 μM (NTPDase2, 3, and 8) nucleotide and 4.0 ng/μl NTPDase1, 1.2 ng/μl ng/μl NTPDase2, 3.0 ng/μl NTPDase3, or 7.0 ng/μl NTPDase8 enzyme, respectively [4, 6, 7]. The mixture was incubated for 10 min at 37°C and terminated by the addition of 17.2 μl ammonium molybdate solution and 12.8 μl malachite green reagent [8]. After 20 min of incubation at room temperature, absorbance measurements were made at 623 nm on a BMG PheraStar FS plate reader (BMG Labtech GmbH, Ortenberg, Germany) and the percentage inhibition was calculated.

Ecto-5'-nucleotidase radiometric assay

The procedure for experiments on the ecto-5'-nucleotidase has been described in great detail before [9]. The substrate [^{3}H]AMP was separated from the product [^{3}H]adenosine by precipitation with lanthanum chloride and further filtration through glass fiber filters. The product was quantified using a Tri-Carb® 2810TR (PerkinElmer) liquid scintillation counter. The enzyme used was recombinant rat ecto-5'-nucleotidase expressed in Sf9 insect cells [10]. Three separate experiments were performed for determination of IC_{50} values from dose-response curves using 10 different concentrations of inhibitor.

Results and discussion

*Pharmacological evaluation of AR-C118925 (**3**) and its derivative **5** on targets not directly linked to purinergic signaling*

Complementing the testings on the P2Y, P2X and adenosine receptors, and on the ectonucleotidases presented in the main text, we also assessed AR-C118925 (**3**) and its derivative **5** for antagonism on a few targets that are not directly involved in purinergic signaling: the orphan receptors GRP18, and GPR55, as well as the cannabinoid receptors CB_1 and CB_2. For AR-C118925, moderate to weak antagonism was observed for both of the orphan receptors (Table 1). AR-C118925 is at least a hundred-fold selective for the $P2Y_2$ receptor over these other G protein-coupled receptors. The de-methylated oxo-analog **5** was less potent on GPR18, GPR55, and the CB_2 receptor but slightly more potent on the CB_1 receptor.

Table 1 Evaluation of AR-C118925 and its derivative **5** on other targets not directly related to purinergic signaling.

Receptor	**IC_{50} or K_i value (μM)**[a] (or % inhibition at indicated concentration)	
	AR-C118925 (3)	**Derivative 5**
Human GPR18 *β-arrestin assay*	**> 10**[b]	**> 10**[b] (36 % at 10 μM)
Human GPR55 *β-arrestin assay*	**> 10**[b]	**>> 10**[b] (6 % at 10 μM)
Human CB_1 *radioligand receptor binding assay*	***> 10*** *(34 % at 10 μM)*	***3.59*** *± 1.58*
Human CB_2 *radioligand receptor binding assay*	***> 10*** *(30 % at 10 μM)*	***> 10*** *(35 % at 10 μM)*

[a] n = 3-4

[b] receptor was activated with an agonist concentration that induced 80 % of maximal stimulation (EC_{80})

*Mode of antagonism of AR-C118925 (**3**) on the $P2Y_2$ receptor*

In order to determine whether AR-C118925 (**3**) acts as a competitive or an allosteric antagonist on the $P2Y_2$ receptor, we determined concentration-effect curves for the agonist UTP following pre-incubation with different, fixed concentrations of AR-C118925. Listed here are the EC_{50} values for UTP determined using the calcium mobilization assay (Table 2) and the β-arrestin assay (Table 3) – compare Fig. 5 in the main text.

Table 2 EC_{50} values and maximal receptor activation for the different dose-response curves of UTP following pre-incubation with different, fixed concentrations of AR-C118925, determined using the calcium mobilization assay

[AR-C118925 (3)] (μM)	EC_{50} of UTP (μM)[a]	Maximal receptor activation with 75 μM UTP (%)
0	**0.0838** ± 0.0223	103
0.15	**0.255** ± 0.019	114
0.3	**0.832** ± 0.246	114
1	**1.38** ± 0.14	98
3	**2.63** ± 0.14	102
30	n. d.[b]	5

[a] n = 3-6

[b] a full agonist curve could not be obtained at this concentration of antagonist

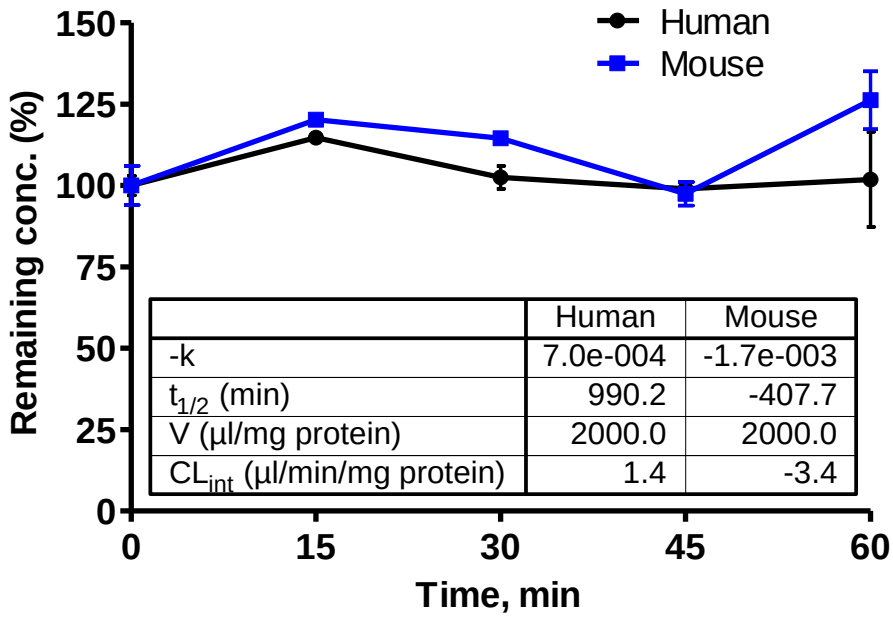
Table 3 EC_{50} values and maximal receptor activation for the different dose-response curves of UTP following pre-incubation with different, fixed concentrations of AR-C118925, determined using the β-arrestin assay

[AR-C118925 (3)] (μM)	EC_{50} of UTP (μM)[a]	Maximal receptor activation (%)
0	**0.858** ± 0.067	100 with 100 μM UTP
0.3	**4.48** ± 0.47	103 with 100 μM UTP
1	**10.6** ± 0. 4	93 with 100 μM UTP
3	**22.9** ± 4.2	91 with 1000 μM UTP
5	**38.6** ± 1.1	97 with 300 μM UTP

[a] n = 3

Physicochemical and pharmacokinetic properties of AR-C118925

The susceptibility to metabolism in the liver was tested by incubation of 1 µM of AR-C118925 (**3**) with human and mouse liver microsomes and measuring the concentration of the remaining compound at different time points. Over the entire course of 1 h, the concentration of AR-C118925 (**3**) remained at approx. 1 µM and the compound was, thus, not metabolized significantly (Fig. 1). These results show that AR-C118925 appears to have a very high metabolic stability, which is an important prerequisite for *in vivo* studies.



	Human	Mouse
-k	7.0e-004	-1.7e-003
$t_{1/2}$ (min)	990.2	-407.7
V (µl/mg protein)	2000.0	2000.0
CL_{int} (µl/min/mg protein)	1.4	-3.4

Fig. 1 Metabolic Stability of AR-C118925 (**3**) in human (black dots) and mouse (blue squares) liver microsomes. Data points represent mean values ± SEM of two separate experiments. Where no error bar is apparent, the SEM is too low to be seen.

The ability of AR-C118925 to permeate a monolayer of Caco2 cells was also determined to model the absorption properties of the human small intestine mucosa

following oral administration. We found that the permeability of AR-C118925 was moderate, similar to that of the hydrophilic reference compound atenolol (Table 4)

Table 4 Caco2 cell monolayer permeation of AR-C118925 in comparison to two reference compounds

Compound	**P_{app} x10^{-6} (cm/sec)**[a]	**SD x10^{-6}**
AR-C118925 (**3**)	1.1	0.2
Atenolol	0.7	0.6
Testosterone	8.5[b]	n.a.

[a] n = 2, unless stated otherwise
[b] n = 1

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