Profiling of a suramin-derived compound library at recombinant human P2Y receptors identifies NF272 as a competitive but non-selective P2Y₂ receptor antagonist

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Abstract

Extracellular nucleotides mediate multiple physiological effects such as proliferation, differentiation, or induction of apoptosis through G protein-coupled P2Y receptors or P2X ion channels. Evaluation of the complete physiological role of nucleotides has long been hampered by a lack of potent and selective ligands for all P2 subtypes. Meanwhile, for most of the P2 receptors, selective ligands are available, but only a few potent and selective P2Y₂ receptor antagonists are described. This limits the understanding of the role of P2Y₂ receptors. The purpose of this study was to search for P2Y₂ receptor antagonists by a combinatorial screening of a library of around 415 suramin-derived compounds. Calcium fluorescence measurements at P2Y₂ receptors recombinantly expressed in human 1321N1 astrocytoma cells identified NF272 [8-(4-methyl-3-(3-phenoxy-carbonylimino-benzamido)benzamido)-naphthalene-1,3,5-trisulfonic acid trisodium salt] as a competitive P2Y₂ receptor antagonist with a K_i of 19 μM which is 14-fold more potent than suramin at this receptor subtype. The SCHILD analysis of competitive inhibition resulted in a pA₂ value of 5.03 ± 0.22 (mean ± SEM) with a slope not significantly different from unity. Among uracil-nucleotide-preferring P2Y receptors, NF272 shows a moderate selectivity over P2Y₄ (3.6-fold) and P2Y₆ (5.7-fold). However, NF272 is equipotent at P2Y₁, and even more potent at P2Y₁₁ and P2Y₁₂ receptors. Up to 250 μM, NF272 showed no cytotoxicity in MTT cell viability assays in 1321N1, HEK293, and OVCAR-3 cells. Further, NF272 was able to inhibit the ATP-induced calcium signal in OVCAR-3 cells demonstrated to express P2Y₂ receptors. In conclusion, NF272 is a competitive but non-selective P2Y₂ receptor antagonist with 14-fold higher potency than suramin lacking cytotoxic effects. Therefore, NF272 may serve as a lead structure for further development of P2Y₂ receptor antagonists.

Keywords Purinergic receptors · P2Y₂ receptor · Calcium assay · Antagonist · Suramin

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Introduction

Nucleotides such as ATP (adenosine-5'-triphosphate) and UTP (uridine-5'-triphosphate) and their corresponding diphosphates ADP (adenosine-5'-diphosphate) and UDP (uridine-5'-diphosphate) are involved in cell signaling [1, 2]. They can be released into the extracellular space after apoptosis or necrosis [3, 4] and in response to various types of stress like hypoxia or pathogen invasion [1, 2], by exocytosis of secretory granules, through vesicular transport or through membrane channels (ABC transporters, pannexins, or connexins) [2, 5]. Upon release, nucleotides are able to interact with two distinct types of receptor families: ionotropic P2X receptors and metabotropic G protein-coupled P2Y receptors [1, 2]. The P2X receptors are trimeric ligand-gated ion channels with seven different subtypes numbered from P2X₁

to P2X₇. Both occurrences of heterotrimers and homotrimers are observed, distributed throughout the nervous system, vascular system, the pulmonary and digestive systems, skeletal muscle, bone, and hematopoietic cells [6, 7]. The P2Y family consists of eight receptor subtypes (P2Y₁, P2Y₂, P2Y₄, P2Y₆, P2Y₁₁, P2Y₁₂, P2Y₁₃, and P2Y₁₄) and can be subdivided based on their coupling to specific G proteins. P2Y₁, P2Y₂, P2Y₄, P2Y₆, and P2Y₁₁ receptors couple to G_q activating phospholipase C and eventually increasing intracellular calcium concentration. P2Y₁₂, P2Y₁₃, and P2Y₁₄ receptors couple to G_i inhibiting adenylyl cyclase [1]. P2Y receptors are widely expressed all over the human body, e.g., in epithelial and endothelial cells, immune cells, platelets, and therefore involved in diverse (patho)physiological functions [2, 7, 8]. However, for a long time, evaluation of their complete physiological role has been hampered due to the lack of selective and potent ligands [1, 7, 9–12]. Now, selective agonists and antagonists for many but not all of the P2Y receptors are available. P2Y₁₂ antagonists have long entered clinical use [10, 13]. Likewise for P2Y₁, P2Y₂, P2Y₄, P2Y₆, P2Y₁₁, and P2Y₁₄ receptors, selective agonists are reported as well as

selective antagonists for P2Y₁, P2Y₁₁, and P2Y₁₂ subtypes [11–13]. P2Y₂ receptors are sensitive for ATP and UTP [1, 2] and investigated as therapeutic targets in atherosclerosis [14–16], (lung) inflammation [3, 17–19], cystic fibrosis [20, 21], dry eye disease [22, 23], hypertension [24], pain [25, 26], cancer [27–31], or in neurodegenerative disorders [32]. In addition to the many known (patho)physiological functions, a cardiometabolic role of P2Y₂ receptors was recently described by Ali et al. [33, 34]. The dinucleoside tetraphosphates denufosal and diquafosal are clinically tested P2Y₂ receptor agonists [1, 7, 11]. Denufosal was investigated for treating patients with cystic fibrosis [20, 21]. However, clinical trials were stopped since no significant effects were observed in phase 3 [35]. In Japan and South Korea, diquafosal is now available for the treatment of dry eye disease [22, 23]. In contrast, only a few P2Y₂ receptor antagonists are described [1, 11–13]. Figure 1 summarizes the chemical structure of some currently known P2Y₂ antagonists. Reactive Blue 2 (RB-2) and suramin are competitive but non-selective antagonists of the P2Y₂ receptor [1, 9, 12, 36, 37]. AR-C 126313 and its analogue AR-C 118925 antagonize the P2Y₂ receptor

Fig. 1 Structures of P2Y₂ receptor antagonists

selectively in the low micromolar range, respectively, as published by Kemp et al. in 2004 [1, 38–40]. Recently, Rafehi and coworkers demonstrated that the thiouracil derivative AR-C118925 behaves as a competitive P2Y₂ antagonist with a pA₂ value of 7.4 and an IC₅₀ value of 57 nM [41]. PSB-716, a derivative of RB-2 (Fig. 1a) is described as a moderate and weak P2Y₂ receptor antagonist [37]. Suramin derivatives have been a rich source of lead compounds of potent and selective purinergic ligands [42–46]. In the present study, a suramin-derived compound library containing approximately 415 compounds was screened for P2Y₂ antagonism by fluorometric calcium measurement in human recombinant P2Y₂ receptor-expressing astrocytoma cells [47]. The library consisted of large (“suramin-type”) and small (“NF340-type”) symmetric ureas with variations of the cap region (aryl-sulfonic acid residues), variations of the aromatic substitution pattern (e.g., methyl, ethyl, isopropyl, phenyl, methoxymethyl, *tert*-butyl, fluorine, or chlorine substitution), symmetrical and asymmetrical ureas, and nitro and amino precursors of the ureas (Fig. 1b). Eventually, three compounds were identified as P2Y₂ antagonists: NF198 (8,8′-(carbonylbis(imino-3,1-phenylenecarbonylimino-3,1-(4-phenylphenylene) arbonylimino))bis(naphthalene-1,3,5-trisulfonic acid) hexasodium salt), NF272 (8-(4-methyl-3-(3-phenoxy carbonylimino-benzamido) benzamido)-naphthalene-1,3,5-trisulfonic acid trisodium salt), and NF292 3,3′-(Terephthaloyl-bis(imino-4,1-phenylene-carbonylimino)) bis (naphthalene-1,5-disulfonic acid) tetrasodium salt) (Fig. 1c). Due to its low cytotoxicity, NF272 was further investigated for potency, selectivity, and competitive antagonism.

Materials and methods

Materials

All nucleotides (ATP, UTP, ADP, and ADP), cisplatin, and other reagents were obtained from Sigma-Aldrich (Taufkirchen, Germany), unless otherwise stated. Suramin (Germanin®Bayer 205) was kindly provided by Bayer AG (Leverkusen, Germany). The suramin-derived library contained approximately 415 compounds synthesized in-house according to procedures formerly described [42, 43]. The general chemical structure is given in Fig. 1b. The library contained large and small ureas with different substituents in 4′-position (e.g., methyl, ethyl, isopropyl, phenyl, *tert*-butyl, methoxymethyl, fluorine, or chlorine), nitro and amino precursors of ureas, asymmetric and symmetric ureas and carbamates, and compounds with replacement of the central urea bridge by isophthalic and terephthalic acid bisamides. Structures of active compounds were confirmed as follows: structural confirmation of NF198 has been presented in

Ullmann et al. [43]. Structures of NF272 and NF292 were confirmed by ¹H-NMR, elemental analysis (carbon, hydrogen, and nitrogen) and mass spectrometry (see supplemental information). Purity was checked by high-performance liquid chromatography and was > 95% [48]. Molecular masses are as follows: NF198 1553.29 g/mol, NF272 821.68 g/mol, and NF292 1062.88 g/mol. The MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide) assay reagent was obtained from SERVA Electrophoresis GmbH, Heidelberg, Germany. Rabbit Anti-P2Y₂ (H-70) (sc-20124) and Mouse Anti-β-Actin (C4) (sc-47778) were obtained from Santa Cruz Biotechnology, Heidelberg. Goat Anti-Rabbit IgG HRP Affinity Purified PAb, Goat IgG (HAF008) and Goat Anti-Mouse IgG HRP Affinity Purified PAb, Goat IgG (HAF007) were purchased from R&D Systems, Wiesbaden. Alexa Fluor 488 goat polyclonal to rabbit (A-11008) was purchased from Invitrogen, Thermo Fischer Scientific, Rockford, USA.

Cell culture and stable transfection of cells

Human 1321N1 astrocytoma cells (European Collection of Cell Culture, ECACC, Salisbury, Wiltshire, England) were stably transfected with the expression vector pcDNA5/FRT (Invitrogen, Carlsbad, USA) containing the coding sequences of P2Y₁ (GenBank accession no. NM_002563), P2Y₂ (GenBank accession no. NM_002564), P2Y₄ (GenBank accession no. NM_002565), P2Y₆ (GenBank accession no. NM_176798), P2Y₁₁ (GenBank accession no. NM_002566), or P2Y₁₂ (GenBank accession no. NM_176876) respectively, by using the Invitrogen FlpIn™ System® for generating stable mammalian expression cell lines according to the manufacturer’s instructions. All cDNA-containing plasmids were purchased from the Missouri S&T cDNA Resource Center (Missouri, USA, www.cdna.org). Cloned cell lines were cultured in Dulbecco’s modified Eagle’s medium (DMEM, PAN Biotech GmbH, Aidenbach, Germany) supplemented with 10% fetal bovine serum (FBS), 100 U/ml penicillin G, 100 µg/ml streptomycin (PAN Biotech GmbH, Aidenbach, Germany), and 200 µg/ml Hygromycin B (Carl Roth GmbH & Co. KG, Karlsruhe, Germany). HEK293 (Deutsche Sammlung von Mikroorganismen und Zellen, Braunschweig, Germany) cells were cultured in DMEM supplemented with 10% FBS, 100 U/ml penicillin G, and 100 µg/ml streptomycin, and OVCAR-3 (ATCC/LGC Standards GmbH, Wesel, Germany) cells were cultured in Roswell Park Memorial Institute medium (RPMI, PAN Biotech GmbH, Aidenbach, Germany) supplemented with 10% FBS, 100 U/ml penicillin G, and 100 µg/ml streptomycin. All cell lines were incubated in a humidified atmosphere at 37 °C and 5% CO₂.

Measurements of intracellular calcium

Changes in intracellular calcium were measured by using a fluorescence microplate reader system with a built-in pipettor system (NOVOstar®, BMG LabTechnologies, Offenburg, Germany) according to the method described by Ullmann et al. [43] and by Kassack et al. [47]. The cells were seeded in 96-well plates at a density of 35,000 cells/well (1321N1 cells) or 45,000 cells/well (OVCAR-3 cells), respectively. After 24 h incubation at 37 °C and 5% CO₂, the medium was removed and the cells were washed with Krebs-HEPES buffer (KHB; 118.6 mM NaCl, 4.7 mM KCl, 1.2 mM KH₂PO₄, 4.2 mM NaHCO₃, 11.7 mM D-glucose, 10 mM HEPES (free acid), 1.3 mM CaCl₂, and 1.2 mM MgSO₄, pH 7.4) and loaded with 1.5 µM Oregon Green 488 BAPTA-1/AM (Molecular Probes) diluted in Krebs-HEPES buffer containing 0.03% Pluronic F-127 (Sigma-Aldrich, Taufkirchen, Germany) for 60 min (37 °C/5% CO₂). After removing the Oregon Green, the cells were washed twice and 180 µl (agonist-mode)/160 µl (antagonist-mode) of Krebs-HEPES buffer were added. Concentration-response curves of agonists were generated by injection of 20 µl of increasing concentrations of ATP or UTP, respectively. Concentration-inhibition curves of antagonists were obtained by preincubation of cells with 20 µl of the test compound for 30 min at 37 °C and subsequent injection of agonist (ATP or UTP, as indicated).

Combinatorial screening

Up to 16 different compounds were analyzed on one assay plate. Four different compounds were mixed and tested at two different concentrations (10 and 100 µM) by calcium assay. One millimolar suramin served as a control. 1321N1-P2Y₂ cells were preincubated with test compounds for 30 min at 37 °C followed by the injection of agonist (ATP 50 nM, UTP 20 nM) using the NOVOstar®. If one combination was able to inhibit the agonist-induced calcium signal, the four compounds were then analyzed separately.

Electrophysiological evaluation at recombinant P2X receptors

The selectivity of NF198 and NF272 was evaluated at *Xenopus laevis* oocytes recombinantly expressing rat (r) or human (h) P2X receptors (rP2X₁, hP2X₂, and S16V-rP2X₃) using previously described protocols [43, 44].

MTT cell viability assay

The MTT assay was used to determine the cytotoxicity of NF198 and NF272 as previously described [49, 50]. The cells were seeded in 96-well plates and incubated overnight at

37 °C and 5% CO₂. The seeding density depended on the growth characteristics of each cell line (1321N1, 3000 cells/well; OVCAR-3, 9000 cells/well; HEK293, 4000 cells/well). After the overnight incubation, the culture medium was replaced by 90 µl of fresh medium and the cells were treated with 10 µl of increasing concentrations of each test compound. After 72 h incubation, 25 µl of MTT solution (5 mg/ml) was added to each well. The incubation with MTT was terminated after 5 min by removing the supernatant and adding 75 µl of DMSO to dissolve the formazan crystals. The absorption at 544 nm (test wavelength) and at 690 nm (reference wavelength) was measured using the FLUOstar® (BMG LabTechnologies, Offenburg, Germany).

Immunoblot analysis

Standard protocols were used for protein extraction and immunoblotting as described in [51]. Diluted protein samples were loaded onto a 10% SDS-polyacrylamide gel and transferred to a polyvinylidene difluoride (PVDF) membrane by semi-dry blotting at 40 mA for 1 h. After blocking with 3% milk in TBST (10x TBST: 200 mM Tris, 9% NaCl, 1% Tween 20, pH 7.4) for 1 h, incubation with the specific primary antibody against P2Y₂ (dilution 1:200) was performed overnight at 4 °C. After washing twice with TBST and once with TBS (10x TBS: 200 mM Tris, 9% NaCl, pH 7.4), incubation with the corresponding HRP-conjugated secondary antibody was performed for 1 h at room temperature. After washing the membrane again twice with TBST and once with TBS, the proteins were visualized using the Western Blotting Luminol Reagent (Santa Cruz Biotechnologies) and the INTAS Science Imaging Instrument (Gel iX Imager, Göttingen, Germany). β-Actin was used as a loading control.

Fluorescence imaging

According to published protocols [52], the cells were grown on cover slides overnight. Then, the adherent cells were washed with PBS and fixed with 4% cold paraformaldehyde for 15 min. After washing with PBS, the non-specific binding sites were blocked with PBS 1x/3% bovine serum albumin for 1 h at room temperature, followed by incubation with primary antibody at 4 °C overnight. After the removal of primary antibodies, the cells were washed three times with PBS and the fluorescence-labeled secondary antibody (Alexa Fluor 488 goat polyclonal to rabbit, A-11008) was added (1:500) for another 2 h at room temperature in the dark. After washing three times with PBS and staining of nuclear DNA by diamidino-2-phenylindole-dihydrochloride (DAPI) using the VECTASHIELD Antifade Mounting Medium (VECTOR LABORATORIES, LTD, Peterborough, UK), microscopic slides were analyzed by fluorescence microscopy using the Olympus BX43 (Olympus Europa Holding GmbH,

Hamburg, Germany) or ArrayScan XTI Live High Content Platform (ThermoFisher Scientific Inc., USA).

Semi-quantitative real-time PCR

Total RNA was extracted from $0.5\text{--}1 \times 10^6$ cells using the *my*-Budget RNA Mini Kit (Bio-Budget Technologies GmbH, Krefeld, Germany) according to the manufacturer's instructions. A total of 2 µg of purified mRNA was taken to prepare cDNA using the High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Life Technologies GmbH, Darmstadt, Germany) and oligo(dT)23 anchored primers (Sigma-Aldrich, USA). The thermocycler program setting was initiated for 10 min at 25 °C then followed by 120 min at 37 °C. A total of 20 µl of cDNA was diluted in 100 µl TE buffer 1x (1 mM of Tris-Cl and 0.1 mM EDTA in distilled water) pH 7.5 and stored at –20 °C. The RT-PCR was performed by using 10 µl of the 2X QuantiTect-SYBR®Green-Master-Mix (Qiagen GmbH, Hilden, Germany), 1.5 µl of diluted cDNA, and 0.8 µl of forward and reverse primer (125 µM) filled up with water to a final volume of 20 µl, and was carried out with Mastercycler®personal (Eppendorf AG, Hamburg, Germany) programmed as follows: 95 °C for 120 s as first step ensued by 40 cycles at 94 °C for 20 s, 57 °C for 30 s, and 72 °C for 60 s. PCR products were separated by 2.0% agarose gel electrophoresis and after GelRed™ staining visualized by using the INTAS Gel iX Imager (INTAS Science Imaging, Goettingen, Germany). β-Actin was used as a control (housekeeping gene). Forward and reverse primer pairs are listed in Table S2.

Data analysis

To generate concentration-effect curves of agonists, effects of single doses of agonists (ATP or UTP) were expressed as the percentage of the maximum calcium fluorescence signal obtained in the experiment. Effects of combined or single doses of antagonists were expressed as percentage of agonist control response (50 nM ATP, 20 nM UTP, respectively). Apparent functional K_i and pK_i values were calculated according to the equation of Cheng and Prusoff [53]:

$$K_i = IC_{50} / (1 + L / EC_{50})$$

$$pK_i = -\log K_i$$

IC_{50} is the 50% inhibitory concentration of the antagonist, EC_{50} is the 50% effective concentration of the agonist used in the experiment, and L is the molar concentration of the used agonist. EC_{50} values for agonists and IC_{50} values for antagonists were derived from $-\log$ concentration-effect (inhibition) curves by fitting pooled normalized data to the nonlinear four-parameter logistic equation (Prism 4.00; GraphPad Software, San Diego, USA). The SCHILD analysis was performed as

previously described [54–56]. Additionally, analysis according to *Lew and Angus* was performed [57]. The MTT data were evaluated as follows: absorption at the reference wavelength was subtracted from the absorption at the test wavelength. Concentration-response curves were generated by nonlinear regression curve fitting using the four-parameter logistic equation as explained above. IC_{50} is the concentration of the tested compound leading to a decrease of 50% of the control signal. All experiments were performed in triplicates and repeated at least three times.

Results

Screening of a suramin-derived library at recombinant P2Y₂ receptors

P2Y₂ receptor-expressing 1321N1 cells (1321N1-P2Y₂) were stimulated by increasing the concentrations of ATP or UTP resulting in an increase in cytosolic Ca^{2+} concentration monitored by fluorescence calcium assay [43]. The concentration-effect curves are displayed in Fig. 2a. Kinetic records of the calcium assay (typical patterns of $[Ca^{2+}]_i$ responses to 1 µM ATP, 1 µM UTP, or KREBS-Hepes buffer as control) are shown in Figure S1A. As described in the literature, the human P2Y₂ receptor is activated by ATP and UTP with similar potency. In our test system, ATP has an EC_{50} of 25 nM ($pEC_{50} = 7.64 \pm 0.05$) and UTP of 10 nM ($pEC_{50} = 7.99 \pm 0.07$). These findings are in a similar range as published data [9]. The expression of P2Y₂ receptors in transfected 1321N1 cells was confirmed by western blot analysis and immunofluorescence microscopy (Fig. 2b). As expected, the wildtype 1321N1 cells did not express P2Y₂ receptor proteins either in western blot or in immunofluorescence experiments. On the other hand, these experiments demonstrated the successful expression of P2Y₂ receptor proteins in 1321N1-P2Y₂ cells after transfection. Notably, the P2Y₂ receptor seemed to be expressed by forming dimers or heteromers because of the five different bands detected in the western blot experiment. This fact is in line with recently published data by von Kügelgen et al. [12] and Abe et al. [58] describing the formation of dimers by P2Y₂ receptors or the formation of heteromers with P1A₁ [59] or with P2Y₁ receptors [60].

A total of 415 suramin-derived compounds were screened for antagonist effects at P2Y₂ receptors as described in the “Materials and methods” section “Combinatorial screening.” Three compounds, NF198, NF272, and NF292 (chemical structure, see Fig. 1), inhibited the response induced by 50 nM ATP (Fig. 2c). One micromolar suramin served as an inhibition control. Interestingly, only NF272 was able to fully inhibit the signal induced by 20 nM UTP. NF292 and NF198 were not able to inhibit the UTP-induced response in 1321N1-P2Y₂ cells to the same extent as the ATP-induced response.

Fig. 2 Pharmacological characterization of test compounds at 1321N1-P2Y₂ cells using the calcium assay

The concentration-effect curves of NF198, NF272, NF292, and suramin for inhibition of 50 nM ATP signal are displayed in Fig. 2d. IC₅₀, pIC₅₀, and apparent functional K_i values are summarized in Table 1. Thus, NF198 is 12-fold, NF272 14-fold, and NF292 is 8-fold more potent than suramin in our calcium studies. The concentration-inhibition curves of NF198, NF272, and NF292 show Hill coefficients not significantly different from unity. Since NF292 was approximately

2-fold less potent than NF198 and NF272, further experiments were undertaken only with NF198 and NF272.

Cytotoxicity of NF198 and NF272

To determine the antiproliferative properties of the most potent P2Y₂ antagonists NF198 and NF272, we tested both compounds in the MTT cell viability assay using the non-cancer cell line HEK293, the astrocytoma cell line 1321N1, and the ovarian cancer cell line OVCAR-3. Concentration-response curves are displayed in Fig. 3a, IC₅₀ values summarized in Table 2 (pIC₅₀ values ± SEM are listed in supplemental information Table S1). NF198 demonstrated significantly higher cytotoxicity than NF272. In all cell lines, NF198 is nearly 10-fold more cytotoxic than NF272 (Table 2). Figure 3b confirms a lack of cytotoxic effects of NF272 in all three cell lines up to a concentration of 250 μM, whereas 250 μM NF198 kills more than 50% of the cells. A total of 10 μM cisplatin was used as a positive control. Due to the unfavored cytotoxic

Table 1 Potency (IC₅₀, pIC₅₀, and apparent functional K_i) of NF198, NF272, NF292, and suramin at P2Y₂ receptors measured in 1321N1-P2Y₂ cells using the calcium assay. Data shown are average ± SEM (pIC₅₀) of *n* ≥ 3 independent experiments

	IC ₅₀ (μM)	pIC ₅₀ ± SEM	K _i (μM)
NF198	63	4.20 ± 0.05	21
NF272	58	4.23 ± 0.04	19
NF292	96	4.02 ± 0.06	32
Suramin	796	3.10 ± 0.08	265

Fig. 3 Cytotoxicity of NF198 and NF272 and functional activity of NF272 in OVCAR-3 cells natively expressing P2Y₂ receptors

properties of NF198 compared with NF272 with similar P2Y₂ antagonistic potencies, further studies were undertaken with NF272 only.

Effects of NF272 in the native P2Y₂ expression system OVCAR-3

Next, we studied the effect of NF272 in a cellular system with physiologically expressed P2Y₂ receptors. The human ovarian cancer cell line OVCAR-3 is reported to natively express P2Y₂ receptors [61–63]. First, the expression of P2Y₂ receptors was confirmed by semi-quantitative PCR analysis, western blotting, and immunofluorescence

Table 2 IC₅₀ values of NF198 and NF272 estimated at 1321N1, HEK293, and OVCAR-3 cells using the MTT assay. Data shown are the average of at least three independent experiments

IC ₅₀ (μ M)	1321N1	HEK293	OVCAR-3
NF198	124	106	246
NF272	1206	1020	2415

microscopy (Fig. 3c). The PCR experiments indicated that OVCAR-3 cells are expressing P2Y₆ besides P2Y₂ receptors. In western blot experiments, P2Y₂ receptors are shown to form dimers in this ovarian cancer cell line. Next, the concentration-effect curves of ATP and UTP were monitored using the fluorescence calcium assay. Kinetic records of the calcium assay (typical patterns of [Ca²⁺]_i responses to 1 μ M ATP, 1 μ M UTP, or KREBS-Hepes buffer) in OVCAR-3 cells are shown in Figure S1B. ATP gave an EC₅₀ of 86 nM, and UTP gave an EC₅₀ of 30 nM in OVCAR-3 cells (Fig. 3d), indicating an effect via P2Y₂ and not P2Y₆. EC₅₀ values were in the same range as data obtained in the recombinant cell line 1321N1-P2Y₂. Then, the inhibitory effect of NF272 on ATP-induced calcium release was studied. Indeed, NF272 inhibited the response of 200 nM ATP (approx. 2 $\times$ EC₅₀) in OVCAR-3 cells with similar potency as was found in recombinant 1321N1-P2Y₂ (Fig. 3e): IC₅₀ = 58 μ M (recombinant 1321N1-P2Y₂); IC₅₀ = 21 μ M (OVCAR 3). Suramin gave comparable results. IC₅₀, pIC₅₀, and K_i values are listed in Table 1 (1321N1-P2Y₂) and Table 3 (OVCAR-3). NF272 is thus a useful antagonist in recombinantly as well as natively P2Y₂ receptor-expressing cells.

Table 3 IC_{50} , pIC_{50} , and apparent functional pK_i values of NF272 and suramin, as estimated in OVCAR-3 cells using the calcium assay. Data shown are average $\pm$ SEM, respectively of $n \geq$ three independent experiments

	IC_{50} (μ M)	$pIC_{50} \pm$ SEM	K_i (μ M)
NF272	21	4.67 ± 0.07	6.3
Suramin	153	3.82 ± 0.10	46

Selectivity of NF272 for P2Y₂ over other P2 receptors

NF272 was tested for P2Y₂ selectivity over human P2Y₁, P2Y₄, P2Y₆, P2Y₁₁, and P2Y₁₂ receptors and hP2X₂, rP2X₁, and rP2X₃ receptors. For each P2Y receptor-expressing cell line, EC_{50} values of the native agonists were determined by calcium assay. EC_{50} values were as follows: P2Y₁ EC_{50} (ADP) = 400 nM; P2Y₄ EC_{50} (UTP) = 2 nM; P2Y₆ EC_{50} (UDP) = 5 nM; P2Y₁₁ EC_{50} (ATP) = 50 nM; P2Y₁₂ EC_{50} (ADP) = 150 nM. Resulting concentration-inhibition curves of NF272 are displayed in Fig. 4. IC_{50} and K_i values as well as fractional inhibition of 100 μ M NF272 are displayed in Table 4. The highest potency is found at P2Y₁₁ and P2Y₁₂, followed by P2Y₁, P2Y₂, P2Y₄, and P2Y₆. At P2Y₁ and P2Y₂ receptors, NF272 is equipotent: NF272 displays IC_{50} values of 55.2 μ M and 58.0 μ M, respectively (Table 4). NF272 is up

Table 4 Potency of NF272 at P2Y receptors recombinantly expressed in 1321N1 cells. IC_{50} values of pooled data and % inhibition caused by treatment with 100 μ M NF272 are shown. Data shown are the average of at least three independent experiments

Receptor	IC_{50} (μ M)	K_i (μ M)	% inhibition by 100 μ M NF272
P2Y ₁	55	18	81.2 ± 3.4
P2Y ₂	58	19	88.2 ± 1.6
P2Y ₄	210	70	34.2 ± 3.4
P2Y ₆	330	110	31.2 ± 2.6
P2Y ₁₁	2.6	0.9	98.7 ± 2.1
P2Y ₁₂	7.9	2.6	97.3 ± 3.8

to 21-fold more potent at P2Y₁₁ and P2Y₁₂ receptors than at P2Y₂: IC_{50} values are 2.6 μ M and 7.9 μ M, respectively (Table 4). Only among uracil-nucleotide activated receptors, NF272 shows moderate selectivity for ATP/UTP-activated P2Y₂ over uracil-nucleotide activated P2Y₄ (3.6-fold) and P2Y₆ (5.7-fold) receptors (Fig. 4, Table 4). Further, NF272 inhibits rP2X₁, hP2X₂, and rP2X₃ receptors in a similar range as found for P2Y receptors (IC_{50} values between 2 and 94.8 μ M; Table 5). Taken together, NF272 only displays a moderate preference for P2Y₂ among uracil-nucleotide-activated receptors P2Y₄, P2Y₆, and P2X₂ receptors, but not over other P2 receptors.

Fig. 4 Selectivity of NF272 over different P2Y receptor subtypes

Table 5 Potency of NF272 at P2X receptors recombinantly expressed in *Xenopus laevis* oocytes. IC₅₀ values of pooled data caused by treatment with 100 μ M NF272 are shown. Data shown are the average of at least three independent experiments

Receptor	IC ₅₀ (μ M)
rP2X ₁	2.0
P2X ₂	95
rP2X ₃	6.9

Mode of P2Y₂ receptor inhibition by NF272

To examine the mode of inhibition by NF272 of P2Y₂ receptors, concentration-response curves of ATP were monitored in the absence and presence of increasing concentrations of NF272 at recombinant 1321N1-P2Y₂. Figure 5a shows the rightward shift of ATP concentration-response curves. EC₅₀ values are summarized in Table 6. Analyses according to SCHILD [54–56] and *Lew and Angus* were performed [57]. Figure 5b displays the SCHILD plot with a pA₂ value of 5.03 $\pm$ 0.22. The pA₂ value is in a similar range as the pK_i derived from the inhibition curve in Fig. 2b (pK_i = 4.72) and the pK_i value calculated from the data of the OVCAR-3 cell line (pK_i = 5.20). The slope of the regression line is not significantly different from unity (95% confidence interval: slope 0.8522–1.676). Thus, a competitive inhibition of NF272 at P2Y₂ receptors can be assumed. Evaluation according to *Lew and Angus* gave the same result (Fig. 5c). pK_D (*Lew and Angus*) was estimated as 4.96 $\pm$ 0.11 (mean $\pm$ SEM) and slope was not significantly different from unity.

Discussion

P2Y₂ receptors are widely expressed all over the human body and involved in many (patho)physiological functions [2, 7, 8, 11]. The number of P2Y₂ receptor ligands is still manageable (Fig. 1a), and only one potent and selective P2Y₂ antagonist is available, namely AR-C118925 with a nanomolar IC₅₀ value of 57 nM [41]. Suramin has long been known to inhibit various P2X and P2Y receptors [7, 9]. However, suramin is neither potent nor selective for any P2 receptor subtype [1, 64], and suramin shows a broad range of side effects and a complex toxicological profile at concentrations inhibiting the P2Y₂ receptor limiting the use of suramin in in vitro and in vivo experiments [65]. In the past, our group has contributed a variety of purinergic ligands derived from suramin [42–46]. In this study, we aimed to screen for potent and selective P2Y₂ receptor ligands in a library containing approximately 415

Fig. 5 Functional characterization of NF272

suramin-derived compounds (Fig. 1b). Surprisingly, only three compounds (NF198, NF272, NF292) turned out as medium micromolar antagonists (Fig. 2c, d, Table 1). These suramin-derived compounds did by far not reach the potency of AR-C118925 (IC₅₀ 57 nM). However, the

Table 6 Potency of ATP at 1321N1-P2Y₂ cells in the absence or presence of increasing concentrations of NF272 derived from concentration-response curves in Fig. 5a. Data shown are average $\pm$ SEM of at least three independent experiments

NF272 (μ M)	EC ₅₀ (nM)	pEC ₅₀ $\pm$ SEM
0	65	7.18 $\pm$ 0.08
10	97	7.01 $\pm$ 0.08
31.6	197	6.71 $\pm$ 0.06
50	294	6.53 $\pm$ 0.08
100	1047	5.98 $\pm$ 0.07
316	2653	5.58 $\pm$ 0.07

potency of NF198, NF272, and NF292 is up to 14-fold higher than suramin (Table 1). NF292 is a terephthalic acid bisamide and showed the highest IC₅₀ among these three compounds (96 μ M). NF198 is a symmetrical suramin-type urea and equipotent to NF272 which contains only one-half of the suramin structure and a phenyl-carbamate moiety instead of urea. Notably, the amino or nitro precursors of suramin differing from NF272 only by lack of the phenyl-carbamate residue were totally inactive at P2Y₂ receptors up to a concentration of 100 μ M (data not shown). These results confirm that a central urea bridge and a symmetrical structure are not mandatory for interaction with the P2Y₂ receptor. Half of the suramin molecule as NF272 with only one cluster of anionic charges is sufficient for P2Y₂ antagonism and achieves the same potency as the large suramin-type urea NF198 differing

from suramin by replacement of two methyl by phenyl groups. The structure-activity relationships are summarized in Fig. 6.

Furthermore, although NF198 and NF272 are equipotent at P2Y₂ receptors (63 and 58 μ M, respectively, Table 1), NF272 is approximately 10-fold less cytotoxic at cancer and non-cancer cell lines, increasing the suitability of NF272 in cellular studies (Fig. 3a, b, Table 2). NF272 is a competitive antagonist (Fig. 5). The pA₂ (5.03, SCHILD analysis) or pK_D values (4.96, *Lew and Angus* analysis) of NF272 are in accordance with its pK_i from inhibition curves (4.72, Fig. 2d, Table 1). A drawback of NF272 is the lack of selectivity for P2Y₂ over purinergic receptors. Whereas most suramin-derived purinergic antagonists are potent and selective [42–46], NF272 preferentially inhibits ATP/ADP-preferring P2Y₁₁, rP2X₁, and P2Y₁₂ (Table 4). Only among uracil-nucleotide activated P2Y receptors, NF272 has a mild preference for P2Y₂ over P2Y₄ (3.6-fold) and P2Y₆ (5.7-fold) (Fig. 4, Table 4). In addition, NF272 inhibited ATP-induced calcium release in human ovarian cancer cells OVCAR-3 expressing natively P2Y₂ and P2Y₆ receptors (Fig. 3c) with similar potency as was found in recombinant 1321N1-P2Y₂ (Fig. 3e).

In conclusion, this study shows that suramin-derived ligands can serve as P2Y₂ antagonists with > 10-fold increased potency compared with suramin. Downsizing suramin to half (containing only one anionic cluster) yielded the competitive antagonist NF272 with 14-fold higher potency at P2Y₂ than suramin, moderate preference for P2Y₂ over P2Y₄ and P2Y₆, but no selectivity over other purinergic receptors, and no cytotoxic effects up to 250 μ M. NF272 can thus serve as a hit for the development of suramin-derived P2Y₂ antagonists.

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Compliance with ethical standards

Conflict of interest Nicole Brockmann declares that she has no conflict of interest.

Parichat Sureechatchaiyan declares that she has no conflict of interest.

David Müller declares that he has no conflict of interest.

Tatiana Hennicke declares that she has no conflict of interest.

Ralf Hausmann declares that he has no conflict of interest.

Gerhard Fritz declares that he has no conflict of interest.

Alexandra Hamacher declares that she has no conflict of interest.

Matthias U. Kassack declares that he has no conflict of interest.

Ethical approval This article does not contain any studies with human participants or animals performed by any of the authors.

K_i = 19 μ M: **NF272** R₁ = methyl, R₂ = phenoxy
 K_i = 21 μ M: **NF198** R₁ = phenyl, R₂ = R₃
 K_i = 265 μ M: **Suramin** R₁ = methyl, R₂ = R₃

Fig. 6 Structure-activity relationship

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