Investigation on 2',3'-*O*-substituted ATP derivatives and analogs as novel P2X3 receptor antagonists

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Supporting Material

Molecular modeling

All molecular modelling studies were performed on a Core i7 CPU (PIV 2.20 GHZ) PC workstation. The recently reported X-ray structure of the inactive state hP2X3 receptor in complex with TNP-ATP (PDB code: 5SVQ; 3.25-Å resolution[1]) was retrieved from the Protein Data Bank and refined and analyzed within Molecular Operating Environment (MOE, version 2016.08) suite, by filling missing loops and residues side chain. The structures of the design compound were docked into the binding site of the hP2X3 receptor using the genetic algorithm docking tool of CCDC Gold with default efficiency settings and by selecting GoldScore as scoring function. The top 30 poses for each ligand were outputted to a MOE database and analysed.[2,3]

Chemistry

Melting points were determined with a Büchi apparatus and are uncorrected. ^{1}H-NMR and ^{31}P-NMR spectra were obtained with Varian Mercury 400 MHz spectrometer; δ in ppm, J in Hz; all exchangeable protons were confirmed by addition of D_2O. Mass spectra were recorded on an HP 1100-MSD series instrument. All measurements were performed using electrospray ionization (ESI-MS) on a single quadrupole analyzer. TLC were carried out on pre-coated TLC plates with silica gel 60 F-254 (Merck). For column chromatography, silica gel 60 (Merck) was used. For ion exchange chromatography, Sephadex DEAE A-25 resin, HCO_3^- form, was used. Elemental analyses were determined on a Fisons model EA 1108 CHNS-O model analyzer and are within ± 0.4% of theoretical values.

5'-*O*-(4-methoxyphenyl)diphenylmethyl-N^6-(4-methoxyphenyl)diphenylmethyl-adenosine (2): To a solution of adenosine (**1**, 2.0 g, 7.48 mmol) in pyridine (15 mL) anhydrous 4-methoxytritylchloride (6.93 g, 22.44 mmol) was added and left under reaction at 60 °C for 1 h, then the reaction was cooled down to 0 °C and pH neutralized with a saturated solution of $NaHCO_3$. The aqueous solution was partitioned with CH_2Cl_2 (3 x 100 mL) and the organic extracts collected, anhydrified over Na_2SO_4, filtrate, and evaporated under vacuum. The crude residue was purified by flash silica gel column chromatography eluting with CH_2Cl_2/MeOH (100:0-97:3) to obtain compound **2** as white solid with 35%, yield. M.p.: 138-141 °C. ^{1}H-NMR (DMSO-d_6, 400 MHz) δ 3,17 (d, J = 4.8 Hz, 2H, H-5'), 3.69 (s, 3H, OCH_3), 3.70 (s, 3H, OCH_3), 4.03 (q, J = 4.4 Hz, 1H, H-4'), 6,08 (s, 1H, CH), 4.26 (q, J = 5.2 Hz, 1H, H-5'), 4.70 (q, J = 4.8 Hz, 1H, H-1'), 6.81-6.85 (m, 4H, H-Ph), 7.17-7.32 (m, 24H, H-Ph), 7.78 (s, 1H, H-8), 8.33 (s, 1H, H-2) ppm. Anal. calcd for $C_{50}H_{45}N_5O_6$: C, 73.96; H, 5.59; N, 8.63; found: C, 74.12; H, 5.79; N, 8.52. ESI-MS calcd for $C_{50}H_{45}N_5O_6$: 811.3; found, positive ion mode, *m/z*: 812.2 [M+H], 834.1 [M+Na].

2',3'-*O*-(methyliden)-5'-*O*-(4-methoxyphenyl)diphenylmethyl-N^6-(4-methoxyphenyl)diphenyl-methyl adenosine (3): To a solution of **2** (2.14 g, 2.63 mmol), CH_2Br_2 (5.54 mL, 78.90 mmol) and cetyltrimethylammonium bromide (87 mg, 0.24 mmol) in CH_2Cl_2 (21 mL) a solution of NaOH (7.89 g, 197.25 mmol) in H_2O (7.90 mL) was added dropwise. The reaction was heated at reflux for 24 h after which water was added (100 mL) and the mixture partitioned with CH_2Cl_2 (3 x 100 ml). Organic extracts were collected, anhydrified over Na_2SO_4, filtrate, and evaporated under vacuum. The crude residue was purified over flash silica gel column chromatography eluting with CH_2Cl_2/MeOH (100-0-99.5:0.5) to get **3** as white solid after crystallization with EtOH/EtOAc (1:4) with 82%, yield. M.p.: 131-133 °C. ^{1}H-NMR (DMSO-d_6, 400 MHz) δ 3.00-3.03 (m, 1H, H-5'), 3.18 – 3.22 (m, 1H, H-5'') 3.68 (s, 3H, OCH_3), 3.70 (s, 3H, OCH_3), 4.29-4.31 (m, 1H, H-4'), 4.88 (dd, *J* = 6.4 Hz, *J*=6.0 Hz 1H, H-3'), 5.10 (s, 1H, *H*-CH), 5.28 (dd, *J* = 6.0 Hz, *J* = 5.6 Hz 1H, H-2'), 6.22 (d, *J* = 2.4 Hz, 1H, H-1'), 6.79 (d, *J*=8.8 Hz, 2H, Ph), 6.83 (d, *J* = 8.8 Hz, 2H, H-Ph), 7.11-7.28 (m, 24H, H-Ph), 7.37 (s, 1H, NH) 7.73 (s, 1H, H-8), 8.39 (s, 1H, H-2) ppm. Anal. calcd for $C_{51}H_{45}N_5O_6$ C, 74.34; H, 5.51; N, 8.50; found: C, 74.62; H, 5.63; N, 8.26. ESI-MS calcd for $C_{51}H_{45}N_5O_6$: 823.3; found, positive ion mode, *m/z*: 824.5 [M+H]; 846.5 [M+Na].

2',3'-*O*-methylidenadenosine (4): To compound **3** (530 mg, 0.64 mmol) a 1:1 solution of HCl 0.2 M/CH3CN (6.4) has been added and the solution left under stirring at r.t. for 24 h. Then, the solution was neutralized by addition of Et_3N, H2O was added (20 mL) and the solution partitioned with EtOAc (3 x 50 ml). Organic extracts were collected, anhydrified over Na_2SO_4 anhydrous, filtrate, and evaporated under vacuum. The crude residue was purified by flash silica gel column chromatography eluting with CH_2Cl_2/MeOH (100:0-97:3) to obtain **4** as pure white solid with 70% yield after crystallization from CH_2Cl_2. M. p.: 212-214 °C. ^{1}H-NMR (DMSO-d_6, 400 MHz) δ 3.49-3.059 (m, 2H, H-5'), 4.14 (q, *J* = 3.6 Hz, 1H, H-4'), 4.88 (dd, *J* = 6.4 Hz, *J* = 6.4 Hz 1H, H-3'), 5.12 (s, 1H, *H*-CH), 5.15 (s, 1H, *H*-CH), 5.16 (t, *J* = 5.4 Hz, 1H, OH), 5.29 (t, *J* = 6.4 Hz, 1H, H-2'), 6.11 (d, *J* = 3.2 Hz, 1H, H-1'), 7.36 (br s, 2H, NH_2), 8.14 (s, 1H, H-8), 8.33 (s, 1H, H-2) ppm. Anal. calcd for $C_{11}H_{13}N_5O_4$: C, 47.31; H, 4.69; N, 25.08; found: C, 47.54; H, 4.79; N, 24.86. ESI-MS calcd for $C_{11}H_{13}N_5O_4$: 279.1; found, positive ion mode, *m/z*: 279.9 [M+H], 301.9 [M+Na].

General procedure for the synthesis of nucleotides 6 and 7: To a solution of the nucleoside **4** or **5** (1 mmol) in trimethylphosphate (5 mL) cooled at 0 °C in an ice bath, $POCl_3$ (372 µL, 4 mmol) was added drop by drop under nitrogen gas atmosphere. The reaction was left under stirring for 4 h and a 0.5 M solution of bis-(tri-n-butylammonium) pyrophosphate in dry DMF (20 mL) was added maintaining anhydrous conditions. The reaction was left under stirring at r.t. for 10 min and then cooled at 0 °C and quenched by addition of a cooled solution of TEAB 1M (13.8 mL) and stirred for 15 min at r.t. Solvent was extracted with di-tert-butyl ether (3 x 15 mL) and the water was removed from the aqueous solution under vacuum, the residue was co-evaporated with distilled water (3 x 10 mL) and the vitreous residue purified on ion exchange Sephadex DEAE A-25 (HCO_3^-) gel column chromatography eluting with a 0 – 0.4 M gradient of NH_4HCO_3 (1700 mL). Fractions containing desired compounds were collected, concentrated under vacuum and co-evaporated with distilled H_2O and lyophilized, giving nucleotides **6** and **7** as ammonium salts.

2',3'-*O*-methylidenadenosine-5'-triphosphate ammonium salt (6): Compound **6** has been obtained from **4** with 20% yield. ^{1}H-NMR (D_2O, 400 MHz) *δ* 4.07-4.13 (m, 2H, H-5'), 4.49-4.51 (m, 1H, H-4'), 4.97-5.00 (m, 1H, H-3'), 5.07 (s, 1H *H*CH), 5.15 (s, 1H *H*CH), 5.18-5.21 (m, 1H, H-2'), 6.12 (d, *J* = 3.6 Hz, 1H, H-1'), 8.10 (s, 1H, H-8), 8.30 ppm (s, 1H, H-2) ppm.^{31}P NMR (D_2O, 162 MHz) *δ* -8.30, -10.55, -21.43 ppm. Anal. calcd for $C_{11}H_{28}N_9O_{13}P_3$: C,

22.50; H, 4.81; N, 21.46; found: C, 22.70; H, 4.96; N, 21.35. ESI-MS calcd for $C_{11}H_{28}N_9O_{13}P_3$: 519.0; found, negative ion mode, *m/z*: 258.6 [(M-2H)/2], 517.8 [M-H].

2',3'-*O*-isopropylidenadenosine-5'-triphosphate ammonium salt (7): Compound **7** has been obtained from **5** with 29% yield. ^{1}H-NMR (D_2O, 400 MHz) δ 1.27 (s, 3H, CH_3), 1.49 (s, 3H, CH_3), 4.02 (m, 2H, H-5'), 4.51 (m, 1H, H-4'), 5.07 (m, 1H, H-3'), 5.21 (m, 1H, H-2'), 6.10 (d, *J*=3.2 Hz, 1H, H-1'), 8.06 (s, 1H, H-8), 8.27 (s, 1H, H-2) ppm. ^{31}P NMR (D_2O, 162 MHz) δ -5.16, -10.60, -21.17 ppm. Anal. calcd for $C_{13}H_{32}N_9O_{13}P_3$: C, 25.37; H, 5.24; N, 20.49; found: C, 25.63; H, 5.59; N, 20.19. ESI-MS calcd for $C_{13}H_{32}N_9O_{13}P_3$: 547.0; found, negative ion mode, *m/z*: 545.9 [M-H], 625.9 [M+Br].

2',3'-*O*-cyclohexylidenadenosine-5'-α,β-methylene-triphosphate ammonium salt (9): To a solution of the nucleoside **8**[3] (100 mg, 0.29 mmol) in dry trimethylphosphate (4.4 mL) cooled at 0 °C in an ice bath. (1 mmol) in trimethylphosphate (5 mL) cooled at 0 °C, methylenebis(phosphonic dichloride) (145 mg, 0.58 mmol) was added. The reaction was left to stir for 4.5 h at r. t. After cooling the mixture in an ice bath, n-tributylamine (1.6 mL) and a 0.5 M solution of n-tributylammonium phosphate in dry DMF (2.9 mL, 1.45 mmol) were added and stirred for 30 min. The reaction was quenched by slowly adding 5 mL of a cold 1M TEAB solution at 0 °C and then stirred for 15 minutes at room temperature. The mixture was directly charged into a Sephadex DEAE A-25 gel (HCO_3^- form) column eluting with a solvent gradient of 0 to 0.4 M NH_4HCO_3 buffer (1700 mL). The appropriate fractions were collected and concentrated under vacuum, co-evaporated several times with distilled H_2O, and freeze-dried to yield pure 9 as ammonium salts with yield = 14%. ^{1}H-NMR (D_2O, 400 MHz) δ 1.21-1.31 (m, 2H, *c*-Hex), 1.37-1.41 (m, 2H, *c*-Hex), 1.48-1.59 (m, 4H, *c*-Hex), 1.72-1.75 (m, 2H, *c*-Hex), 2.17 (app t, *J* = 20 Hz, 2H, CH_2), 3.99 (m, 2H, H-5'), 4.56 (m, 1H, H-4'), 5.08 (m, 1H, H-3'), 5.30 (m, 1H, H-2'), 6.15 (d, *J*=3.2 Hz, 1H, H-1'), 8.20 (s, 1H, H-8), 8.44 ppm (s, 1H, H-2) ppm. ^{31}P NMR (D_2O, 162 MHz) δ 18.26, 8.51, -9.62 ppm. Anal. calcd for $C_{17}H_{38}N_9O_{12}P_3$: C, 31.25; H, 5.86; N, 19.29; found: C, 31.43; H, 5.98; N, 19.01. ESI-MS calcd for $C_{17}H_{26}N_5O_{12}P_3$: 585.1; found, negative ion mode, *m/z*: 291.5 [(M-2H)/2], 584.0 [M-H].

2',3'-*O*-isopropylidenadenosine-5'-pyromellitic ester (10): A suspension of 2',3'-*O*-isopropylidenadenosine (**5**) in THF (6.5 mL) and Et_3N (0.91 mL) was slowly added dropwise to a solution of pyromellitic dianhydride (PMDA, 128 mg, 0.585 mmol) in THF (13 mL) at 0 °C in 30 min and then left to react for 4 h. Then, the reaction was taken to pH 5 by addition of HCl 1N and then evaporated to dryness under reduced pressure. The residue was purified over a reverse phase C18-silica gel column chromatography eluting with MeCN/H_2O (30:70) to afford the desired compound **13** as a white solid with 32% yield. ^{1}H-NMR: (D_2O) δ 1.42 (s, 3H, Ac), 1.59 (s, 3H, Ac), 4.30 (m, 1H, H-CH), 4.52 (m, 1H, H-CH), 4.75 (d, 1H, *J* = 2.5 Hz, H-4'), 5.22 (m, 1H, H-3'), 5.23 (m, 1H, H-2'), 6.16 (d, 1H, *J* = 2.5 Hz H-1'), 7.31 (s, 1H, Ph), 7.49 (s, 1H, Ph), 8.04 (s, 1H, H-2), 8.05 (s, 1H, H-8) ppm. Anal. calcd for $C_{23}H_{21}N_5O_{11}$: C, 50.83; H, 3.90; N, 12.89; found: C, 51.01; H, 3.98; N, 12.60. ESI-MS calcd for $C_{23}H_{21}N_5O_{11}$: 543.1; found, negative ion mode, *m/z*: 270.6 [(M-2H)/2], 542.2 [M-H].

2',3'-*O*-isopropylidenadenosine-4'-carboxilic acid (11): 2',3'-*O*-isopropylidenadenosine (**5**) was taken into a 1:1 mixture of MeCN/H_2O together with BAIB (1.156 g, 3.59 mmol) and TEMPO (52 mg, 0.33 mmol). The initial suspension became a solution and then a suspension again. After stirring at r.t. for 4 h, the mixture was filtered, and the residue was triturated sequentially with diethyl ether and then with acetone, affording the desired product **10** as a white powder (yield= 90%). ^{1}H-NMR: (DMSO- d*6*) δ 1.33 (s, 3H, CH_3), 1.49 (s, 3H, CH_3), 4.66 (d, 1H, *J* = 2.5 Hz,

H-4'), 5.45 (m, 1H, H-3'), 5.52 (m, 1H, H-2'), 6.30 (d, 1H, *J* = 2.5, Hz H-1'), 7.28 (s, 2H, NH_2), 8.06 (s, 1H, H-8), 8,23 (s, 1H, H-2), 12.78 (brs, 1H, OH-5') ppm. Anal. calculated for $C_{13}H_{15}N_5O_5$: C, 48.60; H, 4.71; N, 21.80; found: C, 48.79; H, 4.78; N, 21.55. ESI-MS calcd for $C_{13}H_{15}N_5O_5$: 321.1; found, negative ion mode, *m/z*: 320.1 [M-H], 641.1 [2M-H].

2',3'-*O*-isopropylidenadenosine-4'-(glutamic acid diethyl esther)carboxamide (12): To a solution of **11** in CH_2Cl_2 were added glutamic-L acid diethyl ester hydrochloride (177 mg, 0.74 mmol), DMAP (38 mg, 0.31 mmol), and HOBt (109 mg, 0.81 mmol) obtaining a suspension that, after the addiction of Et_3N **(**0.43 mL**)** became a yellow solution. The mixture was left to react for 24 h and then HOBt (30 mg) and of EDC (90 mg) were added to obtain the complete reaction after 6 h. Final product was purified by flash silica gel column chromatography eluting with CH_2Cl_2/MeOH (98:2) to obtain **12** as pure product after crystallyzation from CH_2Cl_2/diethyl ether (4:6) with 78% yield. ^{1}H-NMR: (DMSO- d*6*) δ 1.15 (m, 6H, 2 x CH_3, OCH_2CH_3), 1.34 (s, 3H, CH_3), 1.57 (s, 3H, CH_3), 1.66 (m, 1H, H-CH), 1.82 (m, 1H, H-CH), 2.11 (t, 2H, *J* = 7.5 Hz, CH_2), 4.05 (m, 4H, 2 x CH_2, OCH_2CH_3), 4.14 (m, 1H, CH), 4.61 (d, 1H, *J* = 2.5 Hz, H-4'), 5.26 (m, 1H, H-3'), 5.36 (m, 1H, H-2'), 6.32 (d, 1H, *J* = 2.5 Hz H-1'), 7.33 (s, 1H, NH_2), 8.10 (s, 1H, H-8), 8.25 (d, 1H, *J* = 5 Hz, NH), 8,31 (s, 1H, H-2) ppm. Anal. calcd for $C_{22}H_{30}N_6O_8$: C, 52.17; H, 5.97; N, 16.59; found: C, 52.47; H, 6.13; N, 16.49. ESI-MS calcd for $C_{22}H_{30}N_6O_8$: 506.2; found, positive ion mode, *m/z*: 507.2 [M+H], 530.3 [M+Na].

2',3'-*O*-isopropylidenadenosine-4'-(glutamic acid)carboxamide (13): To a solution of **12** in H_2O and MeOH (0.38 mL and 2.7 mL, respectively) K_2CO_3 (105 mg) was added and the mixture left under stirring 16 h. Then, the reaction was taken to pH 3 by addition of HCl 1N and then evaporated to dryness under vacuum. The crude residue was purified over a flash silica gel column chromatography eluting with CH_2Cl_2/MeOH (92:8–90:10) to afford the desired compound **13** as a white solid with 34% yield. ^{1}H-NMR: (DMSO- d*6*) δ 1.34 (s, 3H, CH_3), 1.57 (s, 3H, CH_3), 1.66 (m, 1H, H-CH), 1.82 (m, 1H, H-CH), 2.10 (t, 2H, J=7,5Hz, CH_2), 4.14 (m, 1H, CH), 4.61 (d, 1H, J=2,5Hz, H-4'), 5.26 (m, 1H, H-3'), 5.36 (m, 1H, H-2'), 6.32 (d, 1H, J=2,5Hz H-1'), 7.32 (s, 1H, NH_2), 8.09 (s, 1H, H-8), 8.05 (d, 1H, J=5Hz, NH), 8,32 (s, 1H, H-2), 12,5 (brs, 2H, OH) ppm. Anal. calcd for $C_{18}H_{22}N_6O_8$: C, 48.00; H, 4.92; N, 18.66; found: C, 48.35; H, 5.27; N, 18.35. ESI-MS calcd for $C_{18}H_{22}N_6O_8$: 450.2; found, negative ion mode, *m/z*: 449.2 [M-H].

Biological evaluation

Patch clamp recording assay

Full details about the isolation and culture of mouse TG sensory neurons were previously described.[4] Brains were removed to expose trigeminal ganglia of terminally-anesthetized (i.p. urethane, 10% solution) mice. All procedures were conducted in agreement with the Italian Welfare Act and the European Communities Council Directive (2010/63/EU), and were approved by the ethical committee of the Scuola Superiore di Studi Avanzati (SISSA). All the efforts were made to minimize animal suffering.

Currents were recorded from small/medium size mouse TG neurons (nociceptors that strongly express native P2X3 receptors) as previously reported[4-7] in whole cell voltage clamp configuration, at a holding potential of -65 mV after correction for liquid junction potential. Membrane currents were analysed in terms of their peak amplitude and rise time (10-90% of the peak). The receptor agonist α,β-meATP (Sigma) and test compounds were applied by rapid solution changer system (Rapid Solution Changer RSC-200; BioLogic Science Instruments, Claix, France). Test

compounds were kept at + 4 °C and dissolved to 10 mM concentration in distilled H_2O before experiments and finally diluted to the desired concentration in control salt solution. For routine tests, the P2X3 selective agonist α,β-meATP was applied at 10 μM concentration (2 s pulse) at least 3 times (at 5 min interval to prevent cumulative receptor desensitization) to obtain an average control response. Since activated P2X3 receptors desensitize rapidly in the sustained presence of agonist, the inward current transient is too short for the binding equilibrium to be reached when agonist and antagonist are co-applied. Thus, to assess potential receptor blocking activity, each test compound was continuously pre-applied for 5 min before the α,β-meATP application as previously reported.[4-7] Antagonist activity was quantified as percent inhibition of the α,β-meATP-induced current:

$$\% \text{ inhibition} = 100 * (1 - I_2/I_1), \qquad \text{(eq. 1)}$$

where I_1 is the control peak current, I_2 is the peak amplitude of the current after test compound. For the most potent compounds, antagonist dose-inhibition curves were constructed by applying for 5 min different concentrations of each test compound using the same maximal concentration (10 μM) of α,β-meATP agonist. Data were plotted and fitted with empirical Hill equation using Origin 6.0 (Microcal, Northampton, MA, USA):

$$\% \text{ inhibition} = 100/(1+ (IC_{50}/[Ant])^{nH}), \text{ (eq. 2)}$$

where [Ant] is the concentration of the antagonist, n_H is the Hill coefficient, IC_{50} is the concentration of antagonist required to block the maximal current by 50%.

Calcium influx assays at human P2X receptor subtypes

1321N1 astrocytoma cell lines stably expressing the respective P2X receptor subtype (P2X1, P2X2, P2X3, P2X4, or P2X7, respectively) were utilized as previously described.[8,9] The compounds were tested at a concentration of 100 μM for their potency to inhibit ATP-induced calcium influx with one of the human P2X receptor subtypes: P2X1, P2X2, P2X3, P2X4, respectively or Bz-ATP for P2X7 receptor. For potent compounds, a full concentration-response curves were determined, and IC_{50} values were calculated. An ATP or Bz-ATP concentration which caused ~80% of the maximal effect was used for receptor stimulation. Data are the means from 2 to 4 separate experiments, each performed in duplicate.

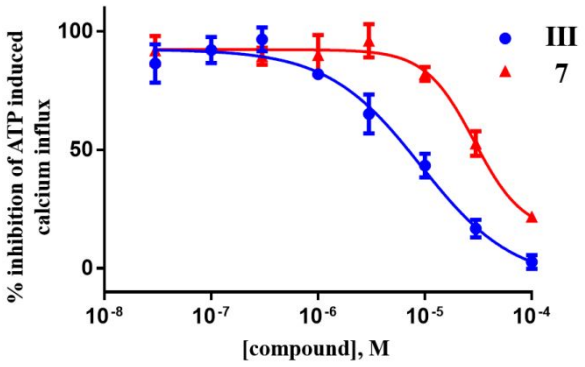

Figure 1. Concentration–response curves of selected antagonists at human P2X1 receptors expressed in 1321N1 astrocytoma cells. Cells were preincubated for 30 min with test compound and subsequently stimulated with ATP at a concentration approximately corresponding to its EC_{80} value (3 μM). For determined IC_{50} values see Table 1.

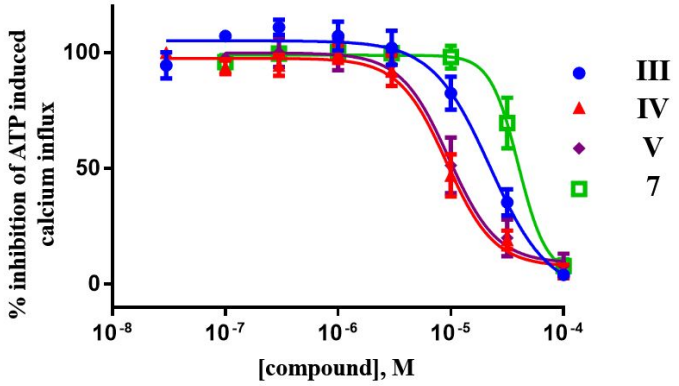

Figure 2. Concentration–response curves of selected antagonists at human P2X4 receptors expressed in 1321N1 astrocytoma cells. Cells were preincubated for 30 min with test compound and subsequently stimulated with ATP at a concentration approximately corresponding to its EC_{80} value (220 nM). For determined IC_{50} values see Table 1.

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