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Synthesis and structure–activity relationships of carboxylic acid derivatives of pyridoxal as P2X receptor antagonists

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ABSTRACT

Carboxylic acid derivatives of pyridoxal were developed as potent $P2X_1$ and $P2X_3$ receptor antagonists with modifications of a lead compound, pyridoxal-5′-phosphate-6-azophenyl-2′,5′-disulfonate (**5b**, iso-PPADS). The designing strategies included the modifications of aldehyde, phosphate or sulfonate groups of **5b**, which may be interacted with lysine residues of the receptor binding pocket, to weak anionic carboxylic acid groups. The corresponding carboxylic acid analogs of pyridoxal-5′-phosphate (**1**), **13** and **14**, showed parallel antagonistic potencies. Also, most of 6-azophenyl derivatives (**24–28**) of compound **13** or **14** showed potent antagonistic activities similar to that of **5b** at human $P2X_3$ receptors with 100 nM range of IC_{50} values in two-electrode voltage clamp (TEVC) assay system on the *Xenopus* oocyte. The results indicated that aldehyde and phosphoric or sulfonic acids in **5b** could be changed to a carboxylic acid without affecting antagonistic potency at mouse $P2X_1$ and human $P2X_3$ receptors.

1. Introduction

The distinct activities of extracellular adenosine 5′-triphosphate (ATP), including the transmissions of pain and inflammatory signaling, are mediated through its activation of P2 receptors in various cells and organs, including the nervous, muscular, cardiovascular, renal and immune systems.[1,2] P2 nucleotide receptors have been classified into two families:[3] P2X receptors, which are ligand-gated cation channels; and P2Y receptors, which are G-protein coupled receptors. Activation of neuronal P2Y receptors[4] regulates both outwardly and inwardly rectifying K^+ channels, potentiates high-voltage activated Ca^{2+} channels, and triggers $InsP_3$-mediated release of Ca^{2+} from stores in the endoplasmic reticulum.[5] In contrast, P2X receptors as ion channels play significant roles in the functions of the peripheral and the central nervous system by impulse generation and synaptic modulation in primary afferents.[6,7] In past decades, genes encoding seven human P2X receptor subtypes ($P2X_{1-7}$) have been cloned.[8–14] Their encoded proteins are 30–50% identical and range in length from 379–595 amino acids. P2X receptor subunits have a similar structural topology, consisting of two transmembrane spanning domains (TM1 and TM2) connected by a long extracellular loop containing the ATP binding site, and intracellular N and C termini of various lengths.[15–19]

The gene encoding $P2X_1$ receptor was originally cloned from rat vas deferens,[14] and its protein was shown to be distributed within the smooth muscles that line a variety of hollow organs, including the urinary bladder, intestines, arteries, and vas deferens.[20–23] These channels mediate the purinergic component of sympathetic and parasympathetic nerve-mediated smooth muscle contraction in a variety of tissues including the urinary bladder,[24,25] vas deferens,[22,26] saphenous vein,[27] and renal microvasculature.[28] In a mouse model lacking the gene encoding $P2X_1$ receptor protein, nerve-mediated vasoconstriction and contraction of the urinary bladder and vas deferens were reduced by 50–70%.[22,24,29,30]

Homomeric $P2X_3$ and heteromeric $P2X_{2/3}$ receptors have been recognized as playing a major role in mediating the primary sensory effects of ATP.[31,32] These receptors are predominantly located on small-to-medium diameter C-fibers and Aδ sensory neurons within the dorsal root, trigeminal, and nodose sensory ganglia.[33,34] Electrophysiological studies on sensory neurons from $P2X_2$ and $P2X_3$ deficient mice have confirmed that $P2X_3$ and $P2X_{2/3}$ receptors account for nearly all responses to ATP in DRG sensory neurons,[35,36] while $P2X_2$ and $P2X_{2/3}$ receptors are predominant in nodose sensory neurons.[37,38] $P2X_3$ and $P2X_{2/3}$ receptors are present on both the peripheral and central terminals of primary sensory afferents projecting to a number of somatosensory and visceral organs including the skin, joints, bones, lungs, urinary bladder, ureter, and gastrointestinal tract.[39] Recent studies have demonstrated that epithelial tissues, including the bladder uroepithelium, airway epithelial cells, and pulmonary neuroepithelial bodies, express $P2X_3$ and $P2X_{2/3}$

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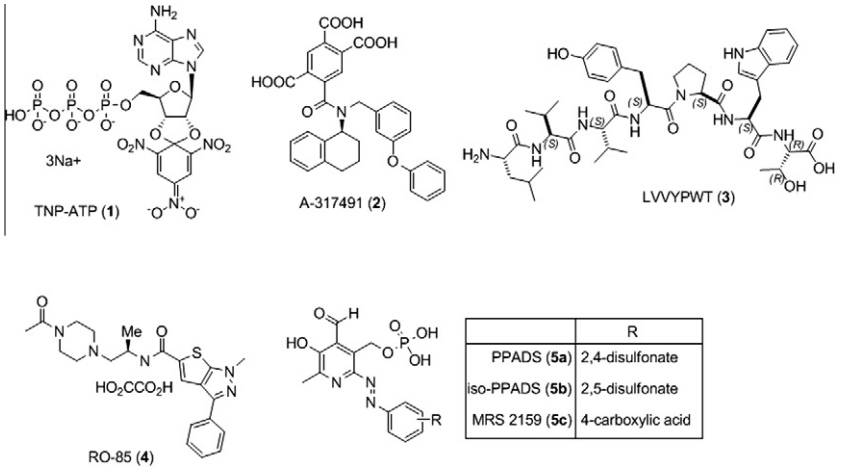
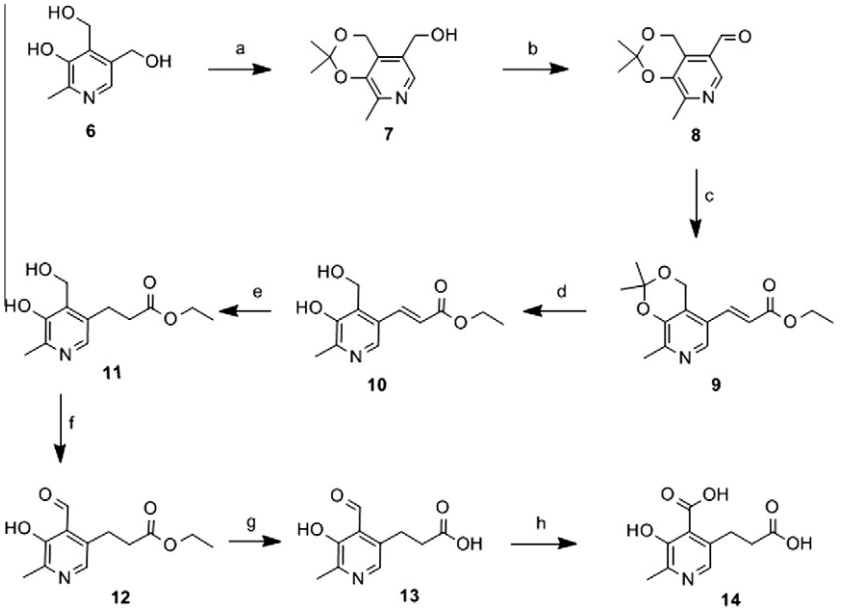


Figure 1. Structures of $P2X_3$ receptor antagonists (**1**–**4**) and pyridoxal-5-phosphate class P2X receptor antagonists (**5a**–**5c**).



Scheme 1. Synthesis of the pyridoxic-β^5-carboxylic acid. Reagents and conditions: (a) 2,2-dimethoxypropane, *p*-TsOH, acetone, 65.7%; (b) pyridinium dichromate, CH_2Cl_2, 67.5%; (c) triethylphosphoacetate, NaH, THF, 81.3%; (d) 10% formic acid in water, reflux, 3 h, 97.8%; (e) Pd/C, H_2, MeOH, 89.2%; (f) MnO_2, CH_2Cl_2, 71.1%; (g) 5% KOH, MeOH, 53.9%; (h) OXONE, 0.2 M DMF/CH_3CN, 47.7%.

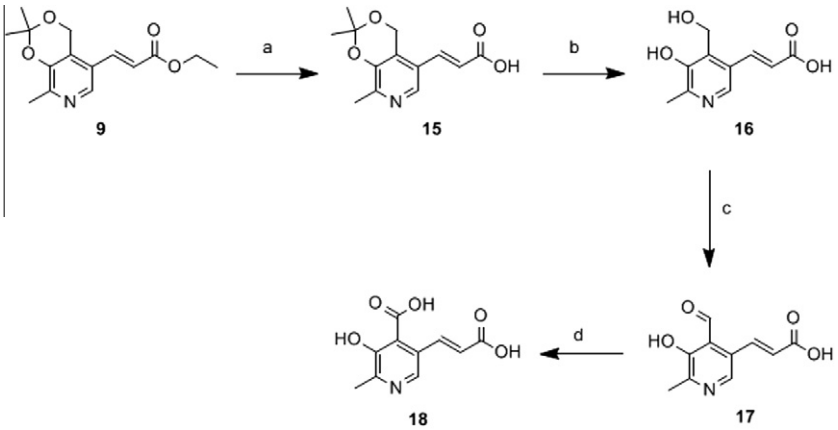
channels, which may modulate certain mechanosensory and chemosensory responses.[40,41] The therapeutic effects of blocking P2X receptors have been studied using potent and selective antagonists, such as TNP-ATP (**1**) at $P2X_1$, $P2X_3$, and $P2X_{2/3}$ receptors,[42] A-317491 (**2**) at $P2X_3$ and $P2X_{2/3}$ receptors,[43] Spinorphin (**3**) at $P2X_3$ receptors,[44] and RO-85 (**4**) at $P2X_3$, and $P2X_{2/3}$ receptors (Fig. 1).[45] These studies showed that peripheral and spinal $P2X_3$ and $P2X_{2/3}$ receptors are involved in transmitting persistent, chronic inflammatory and neuropathic pain signals. Another series of P2 receptor antagonists, pyridoxalphosphate-6-azophenyl-2′,4′-disulfonic acid (PPADS), and its derivatives (**5a–c**), were reported to be potent antagonists at $P2X_{1,3}$ receptors with IC_{50} values in the nanomolar range and selectivity over $P2Y_1$ receptors.[46,47]

Herein, we report the development of novel antagonists for $mP2X_1$ and $hP2X_3$ receptors by modification of the aldehyde, phosphate or sulfonate group of **5b**, which may interact with lysine residues in the ATP binding pocket of the receptors, to a weak anionic carboxylic acid group. The antagonistic effect of these analogs was evaluated using a two-electrode voltage clamp (TEVC) assay system on *Xenopus* oocytes expressing cloned $mP2X_1$ and $hP2X_3$ receptors.

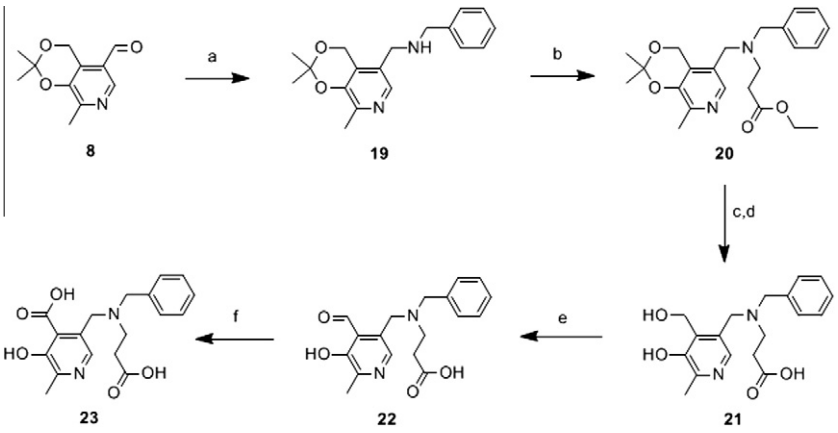
2. Results and discussion

2.1. Chemistry

A series of carboxylic acid derivatives of pyridoxal and pyridoxic acid derivatives of **5b** was synthesized using the routes shown in Schemes 1–3. Pyridoxine **6** was treated with 2,2-dimethoxypropane in the presence of *p*-toluenesulfonic acid in acetone, followed by the oxidation of the 3-hydroxyl group with pyridinium dichromate in dichloromethane to yield the 5-aldehyde derivative **8**. A Wittig–Horner reaction of **8** with triethylphosphoacetate produced compound **9** and subsequent deprotection of the acetonide with 10% formic acid yielded compound **10**. After reduction of the α,β-unsaturated double bond of compound **10** by catalytic hydrogena-



Scheme 2. Synthesis of the pyridoxic-β[5]-acrylic acid. Reagents and conditions: (a) 5% KOH in MeOH, rt, 3 h, 74.5%; (b) 10% formic acid in water, reflux, 3 h, 60.5%; (c) MnO_2, 0.3 M THF/Water, 52.4%; (d) OXONE, 0.2 M DMF/ACN, 71.3%.



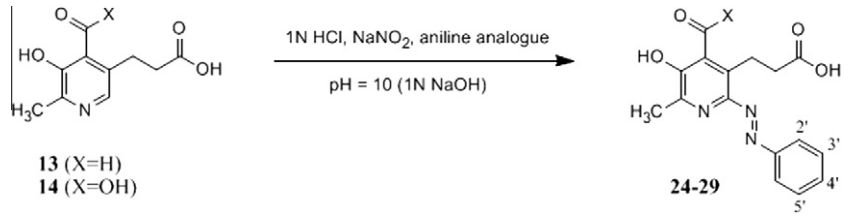
Scheme 3. Synthesis of the 5-((benzyl(2-carboxyetyl)amino)methyl pyridoxic acid. Reagents and conditions: (a) benzylamine, $NaBH_3CN$, 1% AcOH in DCM, 50.7%; (b) ethyl acrylate, EtOH, reflux, 24 h, 34.6%; (c) 10% formic acid in water, reflux, 3 h; (d) 5% KOH in MeOH, rt, 3 h, 46.9%; (e) MnO_2, 0.3 M THF/Water, 34.5%; (f) OXONE, 0.2 M DMF/ACN, 42.6%.

tion, the 4-hydroxymethyl group was selectively converted to an aldehyde using manganese dioxide. The ester group of **12** was hydrolyzed to produce the corresponding acid **13**, which was, in turn, converted to the 4-carboxylic acid analog, **14** by oxidation with OXONE (Scheme 1).

Compounds **17** and **18**, the α,β-unsaturated derivatives of **13** and **14**, respectively, were synthesized from compound **9** as shown in Scheme 2. Compound **23** was synthesized using a 6-step procedure (Scheme 3). The aldehyde group of compound **8** was reacted with benzylamine under conditions of reductive amination in the presence of $NaBH_3CN$ to produce **19**. A Michael type 1,4-addition reaction of the secondary amine of compound **19** with ethyl acrylate yielded compound **20**, followed by deprotection reactions with 10% formic acid for the acetonide and 5% KOH for the ethyl ester to produce compound **21**. Using oxidation reactions similar to those in Scheme 1 yielded the, 4-aldehyde analog, **22** and the 4-carboxylic acid analog **23**. Compounds **13** and **14** were transformed to compounds **24–29** by diazo coupling reactions with various aniline derivatives substituted with sulfonic or carboxylic acids (Scheme 4). However, the diazo coupling reactions with **17**, **18**, **22** and **23** failed to yield the 6-substituted derivatives.

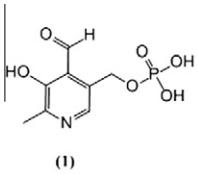
2.2. Biological evaluation

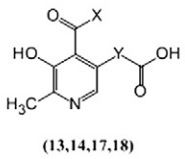
The antagonistic activity of the synthesized compounds at the $mP2X_1$ and $hP2X_3$ receptors was evaluated by performing two-electrode voltage clamp (TEVC) assays on *Xenopus* oocytes expressing cloned $mP2X_1$ and $hP2X_3$ receptors.[48] These

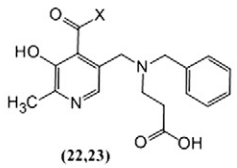


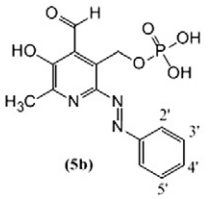
Scheme 4. General synthesis of pyridoxine-6-azophenyl-β[5]-carboxylic acid derivatives by azo coupling reactions.

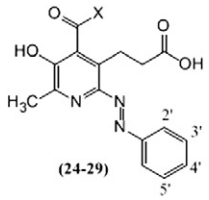
Table 1
Structures and antagonistic effects of pyridoxal carboxylic acid derivatives at $mP2X_1$ and $hP2X_3$ receptors











Compound	Position						% Inhibition	
	2′	3′	4′	5′	X	Y	$mP2X_1$[a]	$hP2X_3$[a]
1[b]							13.2 ± 3.1	20.1 ± 4.2
13[b]					H	CH_2CH_2	19.5 ± 5.1	22.3 ± 8.1
14[b]					OH	CH_2CH_2	14.8 ± 1.7	15.2 ± 7.7
17[b]					H	CH=CH	7.4 ± 1.9	9.3 ± 2.3
18[b]					OH	CH=CH	Inactive	Inactive
22[b]					H		Inactive	Inactive
23[b]					OH		Inactive	Inactive
5b[c] (iso-PPADS)	SO_3H	H	H	SO_3H			62.4 ± 3.1	48.3 ± 5.6
24[c]	SO_3H	H	H	SO_3H	H		58.6 ± 4.5	40.1 ± 8.4
25[c]	SO_3H	H	H	SO_3H	OH		60.2 ± 4.9	45.7 ± 10.8
26[c]	H	H	SO_3H	H	H		41.8 ± 9.8	46.5 ± 11.2
27[c]	H	H	SO_3H	H	OH		57.6 ± 8.8	37.6 ± 6.9
28[c]	H	H	CO_2H	H	H		48.5 ± 10.5	56.3 ± 8.8
29[c]	H	H	CO_2H	H	OH		51.7 ± 13.4	25.7 ± 6.9

[a] The ion current was induced by 2 μM ATP at the recombinant P2X receptors expressed in *Xenopus* oocytes, and the percentage inhibition of ion current by 10 μM and 100 nMof each compound was measured at $mP2X_1$ and $hP2X_3$ receptors (mean ± SEM, $n = 4$).
[b] At concentrations of 10 μM.
[c] At concentrations of 100 nM.

compounds were also evaluated for selectivity at $hP2X_7$ receptors using an ethidium bromide (EtBr) uptake assay.

Since pyridoxal-5′-phosphate, **1** has been reported to antagonize $P2X_1$ receptors with an IC_{50} value of roughly 10 μM (reference), the inhibitory activities of compound **1** and its carboxylic acid analogs at both receptors were investigated at concentrations of 10 μM. Compounds **13** and **14** showed similar antagonistic potencies as pyridoxal-5′-phosphate, **1** at $mP2X_1$ and $hP2X_3$ receptors, whereas compounds **17**, **18**, **22**, and **23** displayed weak or no activity (Table 1). The results obtained with compounds **22** and **23** suggest that there is no additional hydrophobic binding pocket around the 5-position carboxylic acid. Also, the comparison of compounds **13** and **17** indicated that a flexible propionic acid group may be associated with greater antagonistic activity than a more rigid propenic acid group. Therefore, **13** and **14** were selected as pyridine carboxylic acid analogs and subjected to diazo coupling reactions to prepare compounds **24–29**, 6-substituted derivatives with aniline-2,3-disulfonic acid, sulfanilic acid, and 4-aminobenzoic acid groups.

Most of these compounds showed antagonistic activities generally similar to that of the positive control, **5b** (iso-PPADS) with 100 nM concentrations showing 50–60% inhibition at $P2X_1$ receptors, indicating that pyridoxal-5′-phosphate could be replaced with pyridine carboxylic acid analogs. We found that the 6-substituted pyridine-4-carboxylic acid analogs **25**, **27** and **29** were more potent antagonists at $mP2X_1$ than at $hP2X_3$ receptors. In contrast, compounds **26** and **28,** with aldehyde groups at the 4-position, displayed greater antagonistic activities at $hP2X_3$ receptors than compounds **27** and **29** (Table 1). Therefore, substitutions at both the 4-carboxaldehyde and 4-acidic groups may enhance antagonism at $P2X_3$ relative to $P2X_1$ receptors.

Among the series of derivatives, compounds **24** and **28** were chosen as representative analogs for the evaluation of dose-dependent antagonisms. Compound **24** showed dose-dependent antagonism against ATP-induced ion currents at $mP2X_1$ receptors, with a complete reversal of antagonistic effects after wash-out (Fig. 2A). The full dose-response curves of **5b**, **24** and **28** at the $mP2X_1$ and $hP2X_3$ receptors are depicted in Figures 2B and C, respectively. The antagonistic potency of **24** and **28** at $P2X_1$ and $P2X_3$ receptors were maintained compared with **5b**, showing IC_{50} values in the range of 100 nM. However, the IC_{50} ratios for $P2X_1$ receptors relative to $P2X_3$ receptors were 0.3 for **5b**, 0.7 for **24** and 1.6 for **28**. Therefore, modifications of the pyridoxal-5′-phosphate and 6-phenylsulfonic acid moieties of **5b** by incorporation of carboxylic acids may shift selectivity profiles toward greater antagonisms toward $P2X_3$ receptors than toward $P2X_1$ receptors.

In the investigation of selectivity profiles of compounds 24–29 at $P2X_7$ receptor using ethidium bromide (EtBr) uptake assays in $hP2X_7$-expressing HEK293 cells, all the derivatives showed less than 50% antagonistic activity at a concentration of 10 μM (Supplementary Fig. S1). Thus, the selectivity of new pyridine carboxylic acid derivatives for $P2X_1$ and $P2X_3$ versus $P2X_7$ receptors was greatly improved, since the IC_{50} values of PPADS analogs, such as PPNDS, MRS 2159 have been reported to be in the range of 0.6–4.6 μM in various assay systems.[49]

3. Conclusion

We have described the successful modification of non-selective P2 receptor antagonists PPADS and iso-PPADS, by the replacement of strong anionic moieties such as phosphoric or sulfonic acids with weaker carboxylic acids. Overall, modification of the moiety at position 4 or 5 in the pyridine skeleton to a carboxylic acid group antagonized $mP2X_1$ and $hP2X_3$ receptors without loss of activity. In particular, a non-phosphate and non-sulfonate analog, **28**, showed similar antagonistic potencies as its parent compound, **5b**, with

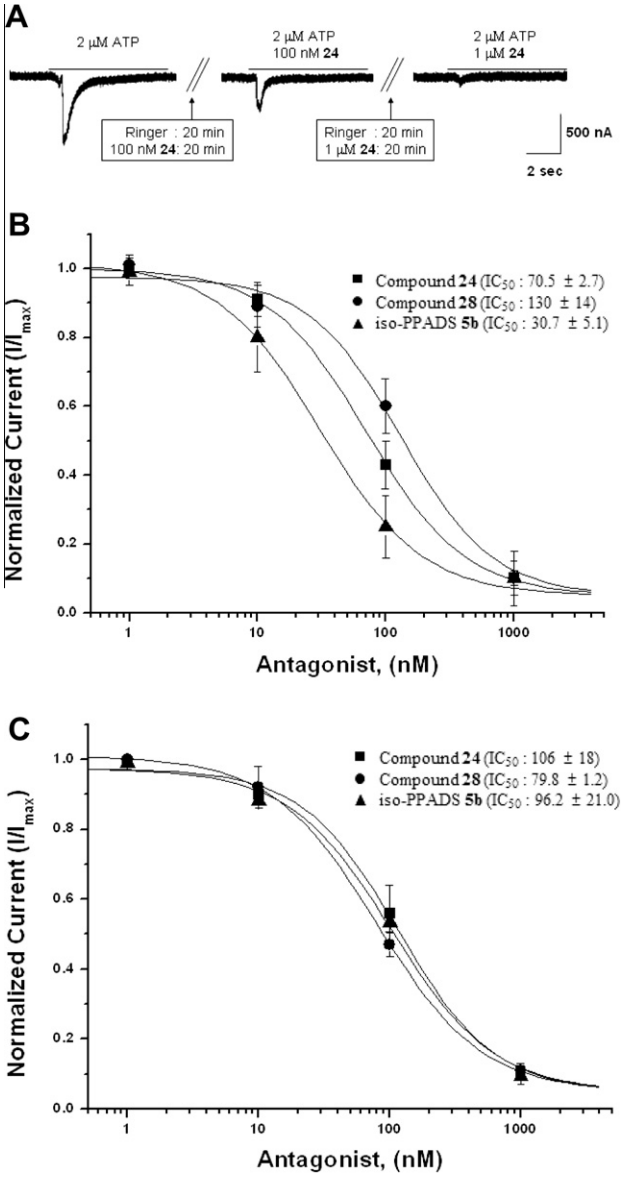


Figure 2. Inhibition of $mP2X_1$ and $hP2X_3$ receptor-mediated currents by compounds **24** and **28**. Inward currents were elicited by 2 μM ATP in the control and in the presence of two concentrations of compound **24** in *Xenopus* oocytes expressing $mP2X_1$ receptor subtypes (A). Concentration–inhibition curves of compounds **24** (■), **28** (●), and iso-PPADS **5b** (▲) in cells expressing (B) $mP2X_1$ and (C) $hP2X_3$ receptors. The continuous line for ATP was fit to the data using the equation $I = I_{max}/(1 + IC_{50}/L)^{nH}$, where I is the actual current for a ligand concentration (L), nH is the Hill coefficient, and I_{max} is the maximal current. In B, the IC_{50} values and Hill coefficients were: (■) 70.5 ± 2.7 nM, and −1.1 ± 0.4, respectively, for compound **24**; (●) 130 ± 14 nM, and −1.2 ± 0.5, respectively, for compound **28**; (▲) 30.7 ± 5.1 nM, and −1.1 ± 0.1, respectively, for compound **5b**. In C, the IC_{50} values and Hill coefficients were: (■) 106 ± 18 nM, and −1.2 ± 0.3, respectively, for compound **24**; (●) 79.8 ± 1.2 nM, and −1.1 ± 0.02, respectively, for compound **28**; (▲) 96.2 ± 21.0 nM, and −1.1 ± 0.3, respectively, for compound **5b**.

slightly improved selectivity for $P2X_3$ receptors. Moreover, all new pyridine carboxylic acid derivatives were highly selective for $mP2X_1$ and $hP2X_3$ receptors when compared with $hP2X_7$ receptors.

4. Experimental section

4.1. Chemistry

ATP (disodium salt), Pyridoxine, and the reagents for diazo coupling reactions were purchased from Aldrich (St. Louis, MO) and TCI (Tokyo). Aniline-2,5-disulfonic acid was obtained from K&K Laboratories, Inc. (Hollywood, CA). All other reagents and solvents were of analytical grade and purchased from Merck (Darmstadt, Germany). Solutions of agonists and antagonists were prepared daily from stock solutions (10 mM, stored frozen) made up in extracellular bathing solution. Analytical TLC was carried out on precoated silica glass plates (Merck silica gel 60, F254) and visualized with UV light or stained with sulfonic acid stain. Flash column chromatography was performed with silica (Merck, 70–230 mesh). Proton nuclear magnetic resonance spectroscopy was performed on a JEOL JNM-LA 300WB spectrometer, and spectra were taken in DMSO-d_6. Unless otherwise noted, chemical shifts are expressed as ppm downfield from internal tetramethylsilane, or relative ppm from DMSO (2.5 ppm). Data are reported as follows: chemical shift, multiplicity (s, singlet; d, doublet; t, triplet; m, multiplet; b, broad; app., apparent), coupling constants, and integration. Mass spectroscopy was carried out on MALDI-TOF and ESI instruments. The determination of purity was performed on a Shimadzu SCL-10A VP HPLC system using a Shimadzu Shim-pack C18 analytical column (250 mm × 4.6 mm, 5 μm, 100 Å) in linear gradient solvent systems. One solvent system (A) was 0.1 M triethylammonium acetate buffer/CH_3CN = 95:5 to 40:60 for 30 min with flow rate 1 mL/min. The other (B) was 5 mM tetrabutylammonium phosphate buffer/CH_3CN = 80:20 to 40:60 in 30 min with flow rate 1 mL/min. The cRNA of $hP2X_3$ receptor was obtained by reverse transcription of the cDNA of $hP2X_3$ receptor, which was generously provided by Dr. W. Stuhmer and Dr. F. Soto of the Max-Plank Institute. The EST clones containing full-length cDNAs of $mP2X_1$ (clone ID: 4189541) and $hP2X_7$ receptor (clone ID: 5286944) were purchased (Invitrogen, CA, U.S.A.), and their sequences were confirmed by DNA sequencing.

4.1.1. 2,2,8-Trimethyl-4*H*-[1,3]dioxino[4,5-c]pyridin-5-yl)methanol (7)

To a mixture of 20 g (0.1 mol) of pyridoxine and 244 mL (1.9 mol) of 2,2-dimethoxypropane was added 90 g (0.4 mol) of *para*-toluenesulfonic acid in flame-dried 1 L three-neck flask. This mixture was stirred for 30 h at room temperature and neutralized with $NaHCO_3$ and concentrated in evaporation. After extraction with dichloromethane, the combined organic layer was washed with brine, dried over $MgSO_4$, filtered, concentrated, and purified by column chromatography with dichloromethane/methanol = 20:1. Yield 65.7% ^{1}H NMR ($CDCl_3$) δ 1.52 (6H, s, 2 × CH_3), 2.34 (3H, s, CH_3), 4.52 (2H, s, CH_2), 4.91 (2H, s, CH_2), 7.78 (1H, s, H-6). MS (ESI): m/z = 210.2 (M^++1).

4.1.2. 2,2,8-Trimethyl-4*H*-[1,3]dioxino[4,5-c]pyridine-5-carbaldehyde (8)

To a stirred solution of 5 g (23.9 mmol) of **7** in 50 mL of anhydrous dichloromethane was added pyridinium dichromate with vigorous stirring. After overnight reaction, the mixture was filtered through celatum bed with methanol. The filtrate was evaporated under vacuum and purified by column chromagotraphy with *n*-hexanes/ethyl acetate = 3:1. Yield 67.5% ^{1}H NMR ($CDCl_3$) δ 1.57 (6H, s, 2 × CH_3), 2.51 (3H, s, CH_3), 5.18 (2H, s, CH_3), 8.48 (1H, s, H-6), 10.05 (1H, s, CHO). MS (ESI): m/z = 208.0 (M^++1).

4.1.3. Ethyl 3-(2,2,8-trimethyl-4*H*-[1,3]dioxino[4,5-*c*]pyridin-5-yl)acrylate (9)

To a suspension of 500 mg (16.9 mmol) of NaH in anhydrous tetrahydrofuran was added dropwise 3 mL (16.8 mmol) of triethylphosphoacetate. The mixture color was changed to clear yellow solution with H_2 gas release. After 5 min stirring, 3.2 g (15.3 mmol) of **8** in 40 mL tetrahydrofuran was added and the reaction solution was stirred for 1 h. The mixture extracted with chloroform and brine. The organic residue was combined and dried over $MgSO_4$, fil-

tered, concentrated, and purified by column chromatography with *n*-hexanes/ethyl acetate = 3:1. Yield 81.3% ^{1}H NMR ($CDCl_3$) δ 1.33 (3H, s, CH_3), 1.57 (6H, s, 2 × CH_3), 2.44 (3H, s, CH_3), 4.28 (2H, q, J = 8.8 Hz, CH_2), 4.93 (2H, s, CH_2), 6.37 (1H, d, J = 16.6 Hz, CH), 7.54 (1H, d, J = 16.6 Hz, CH), 8.26 (1H, s, H-6). MS (ESI): m/z = 278.3 (M^++1).

4.1.4. Ethyl 3-(5-hydroxy-4-(hydroxymethyl)-6-methylpyridin-3-yl)acrylate (10)

A solution of 3 g (10.8 mmol) of **9** in 20 mL solution of 10% formic acid was refluxed for 2 h. After cooling at room temperature, the mixture was extracted with chloroform and saturated $NaHCO_3$. The organic layer was combined and dried over $MgSO_4$, filtered, concentrated, and purified by column chromatography with dichloromethane/methanol = 10:1. Yield 97.8% ^{1}H NMR ($CDCl_3$) δ 1.35 (3H, s, CH_3), 2.48 (3H, s, CH_3), 4.28 (2H, q, J = 8.8 Hz, CH_2), 5.12 (2H, s, CH_2), 6.30 (1H, d, J = 16.6 Hz, CH), 7.67 (1H, d, J = 16.6 Hz, CH), 8.14 (1H, s, H-6). MS (ESI): m/z = 238.1 (M^++1).

4.1.5. Ethyl 3-(5-hydroxy-4-(hydroxymethyl)-6-methylpyridin-3-yl)propanoate (11)

The solution of 2 g (8.4 mmol) of **10** in dichloromethane was added 0.5 g of Pd/C and stirred for 4 h under H_2 atmosphere. The mixture was filtered through celatum bed. The filtrate was evaporated in vacuum and purified by column chromatography with chloroform/methanol = 10:1. Yield 89.2% ^{1}H NMR ($CDCl_3$) δ 1.23 (3H, t, J = 7.1 Hz, CH_3), 2.40 (3H, s, CH_3), 2.50 (2H, t, J = 7.6 Hz, CH_2), 2.80 (2H, t, J = 7.6 Hz, CH_2), 4.10 (2H, q, J = 7.1 Hz, CH_2), 4.99 (2H, s, CH_2), 7.73 (1H, s, H-6). MS (ESI): m/z = 240.1 (M^++1).

4.1.6. Ethyl 3-(4-formyl-5-hydroxy-6-methylpyridin-3-yl)propanoate (12)

To a solution of 1 g (4.2 mmol) of **11** in 50 mL of anhydrous dichloromethane was added activated manganese dioxide with vigorous stirring for 2 h at room temperature under N_2 atmosphere. The mixture was filtered through celatum bed and filtrated was evaporated in vacuum and purified by column chromatography with *n*-hexanes/ethyl acetate = 4:1. Yield 71.1% ^{1}H NMR ($CDCl_3$) δ 1.24 (3H, t, J = 7.1 Hz, CH_3), 2.51 (3H, s, CH_3), 2.69 (2H, t, J = 7.6 Hz, CH_2), 3.25 (2H, t, J = 7.6 Hz, CH_2), 4.12 (2H, q, J = 7.1 Hz, CH_2), 8.02 (1H, s, H-6), 10.46 (1H, s, CHO). MS (ESI): m/z = 238.1 (M^++1).

4.1.7. 3-(4-Formyl-5-hydroxy-6-methylpyridin-3-yl)propanoic acid (13)

To a solution of 5% KOH in methanol was added 330 mg (1.4 mmol) of **12** with vigorous stirring for 1 h. The reaction solvent was removed by evaporation and product was purified by ion-exchange column chromatography using Amberlite CG-50 resin. Yield 53.9% ^{1}H NMR (D_2O) δ 2.47 (3H, s, CH_3), 2.55 (2H, t, J = 7.5 Hz, CH_2), 3.15 (2H, t, J = 7.5 Hz, CH_2), 7.58 (1H, s, H-6), 10.44 (1H, s, CHO). MS (ESI): m/z = 210.2 (M^++1).

4.1.8. 5-(2-Carboxyethyl)-3-hydroxy-2-methylisonicotinic acid (14)

To a solution of 177 mg (0.8 mmol) in 0.2 M DMF in acetonitrile was added 520 mg (0.8 mmol) of oxone with vigorous stirring for 3 h. The reactant color was changed from yellow to colorless. Product was purified by ion-exchange column chromatography using Amberlite CG-50 resin and lyophilized. Yield 47.7% ^{1}H NMR (D_2O) δ 2.34 (3H, s, CH_3), 2.50 (2H, t, J = 7.5 Hz, CH_2), 2.74 (2H, t, J = 7.5 Hz, CH_2), 7.53 (1H, s, H-6). MS (ESI): m/z = 226.3 (M^++1).

4.1.9. 3-(2,2,8-Trimethyl-4*H*-[1,3]dioxino[4,5-c]pyridin-5-yl)acrylic acid (15)

To a solution of 50 mL of aqueous 5% KOH in 100 mL of methanol was added 3 g (12.1 mmol) of **9** with vigorous stirring for 3 h. The reaction solvent was evaporated in vacuum and purified by ion-exchange column chromatography suing Amberlite CG-50. Yield 74.5% ^{1}H NMR (D_2O) δ 1.57 (6H, s, 2 × CH_3), 2.38 (3H, s, CH_3), 5.08 (2H, s, CH_2), 6.43 (1H, d, J = 16.6 Hz, CH), 7.20 (1H, d, J = 16.6 Hz), 8.17 (1H, s, H-6). MS (ESI): m/z = 250.2 (M^++1).

4.1.10. 3-(5-Hydroxy-4-(hydroxymethyl)-6-methylpyridin-3-yl)acrylic acid (16)

To a solution of aqueous 4 N HCl in methanol was added 2 g (8.0 mmol) of **14** and stirred for 2 days. Reaction was monitored by analytical HPLC system and purified by ion-exchange column chromatography suing Amberlite CG-50. Yield 60.5% ^{1}H NMR (D_2O) δ 2.45 (3H, s, CH_3), 4.87 (2H, s, CH_2), 6.43 (1H, d, J = 16.6 Hz, CH), 7.26 (1H, d, J = 16.6 Hz, CH), 7.98 (1H, s, H-6). MS (ESI): m/z = 210.1 (M^++1).

4.1.11. 3-(4-Formyl-5-hydroxy-6-methylpyridin-3-yl)acrylic acid (17)

Following the compound **13** procedure. Yield 52.4% ^{1}H NMR (DMSO-d_6) δ 2.39 (3H, s, CH_3), 6.95 (1H, d, CH, J = 16.1 Hz), 7.58 (1H, d, CH, J = 16.1 Hz), 8.34 (1H, s, H-6). MS (ESI): m/z = 208.2 (M^++1).

4.1.12. 5-(2-Carboxyvinyl)-3-hydroxy-2-methylisonicotinic acid (18)

Following the compound **14** procedure. Yield 71.3% ^{1}H NMR (DMSO-d_6) δ 2.31 (3H, s, CH_3), 6.12(1H, d, CH, J = 16.8 Hz), 7.89 (1H, d, CH, J = 16.8 Hz), 8.76 (1H, s, H-6). MS (ESI): m/z = 224.2 (M^++1).

4.1.13. *N*-Benzyl-1-(2,2,8-trimethyl-4*H*-[1,3]dioxino[4,5-*c*]pyridin-5-yl) methanamine (19)

To a solution of 200 mg (1.0 mmol) of **8** in 1.0% acetic acid in dichloromethane was added 126 μL (1.2 mmol) of benzylamine and stirred for 30 min. 90 mg (1.4 mmol) of $NaBH_3CN$ was added reaction mixture and stirred for 4 h. The reaction mixture was extracted with chloroform and saturated $NaHCO_3$, dried over $MgSO_4$, filtered, concentrated, and purified by column chromatography with dichloromethane/methanol = 30:1.Yield 50.7% ^{1}H NMR ($CDCl_3$) δ 1.54 (6H, s, 2 × CH_3), 2.39 (3H, s, CH_3), 3.64 (2H, s, CH_2), 3.78 (2H, s, CH_2), 4.93 (2H, s, CH_2), 7.25–7.40 (5H, m, phenyl), 7.92 (1H, s, H-6). MS (ESI): m/z = 299.4 (M^++1).

4.1.14. Ethyl 3-(benzyl((2,2,8-trimethyl-4*H*-[1,3]dioxino[4,5-*c*]pyridin-5-yl)methyl) amino)propanoate (20)

To a solution of 50 mg (0.2 mmol) of **19** in ethanol was added 37 μL (0.3 mmol) of ethyl acrylate and stirred for overnight at room temperature. The mixture was concentrated in vacuum and purified by column chromatography with chloroform/methanol = 50:1. Yield 34.6% ^{1}H NMR ($CDCl_3$) δ 1.14 (3H, t, J = 7.2 Hz, CH_3), 1.45 (6H, s, 2 × CH_3), 2.31 (3H, s, CH_3), 2.39 (2H, t, J = 7.2 Hz, CH_2), 2.68 (2H, t, J = 7.2 Hz, CH_2), 3.36 (2H, s, CH_2), 3.46 (2H, s, CH_2), 4.01 (2H, q, J = 7.2 Hz, CH_2), 4.69 (2H, s, CH_2),, 7.17∼7.26 (5H, m, phenyl), 7.84 (1H, s, H-6). MS (ESI): m/z = 399.5 (M^++1).

4.1.15. 3-(Benzyl((5-hydroxy-4-(hydroxymethyl)-6-methylpyridin-3-yl)methyl) amino) propanoic acid (21)

Following the compound **13** procedure. Yield 46.9% ^{1}H NMR (D_2O) δ 2.42(3H, s, CH_3), 2.53 (2H, t, J = 7 .2 Hz, CH_2), 3.02 (2H, t, J = 7.2 Hz, CH_2), 3.95 (2H, s, CH_2), 4.62 (2H, s, CH_2), 7.35–7.45 (5H, m, phenyl), 7.62 (1H, s, H-6). MS (ESI): m/z = 331.4 (M^++1).

4.1.16. 3-(Benzyl((4-formyl-5-hydroxy-6-methylpyridin-3-yl)methyl) amino) propanoic acid (22)

Following the compound **12** procedure. Yield 34.5% ^{1}H NMR ($CDCl_3$) δ 2.47 (3H, s, CH_3), 2.51 (2H, t, J = 6.9 Hz, CH_2), 2.78 (2H, t, J = 6.9 Hz, CH_2), 3.61 (2H, s, CH_2), 3.76 (2H, s, CH_2), 7.24–7.33 (5H, m, phenyl), 8.01 (1H, s, H-6), 10.21 (1H, s, CHO). MS (ESI): m/z = 329.4 (M^++1).

4.1.17. 5-((Benzyl(2-carboxyethyl)amino)methyl)-3-hydroxy-2-methylisonicotinic acid (23)

Following the compound **14** procedure. Yield 42.6% ^{1}H NMR (D_2O) δ 2.58 (3H, s, CH_3), 2.55 (2H, t, J = 6.9 Hz, CH_2), 2.82 (2H, t, J = 6.9 Hz, CH_2), 3.71 (2H, s, CH_2), 3.82 (2H, s, CH_2), 7.24–7.33 (5H, m, phenyl), 8.01 (1H, s, H-6). MS (ESI): m/z = 345.4 (M^++1).

4.1.18. General procedure for the synthesis of azo group containing compounds (24–29)

8 mg (0.1 mmol) of $NaNO_2$ was added to a stirred solution of an aniline analogue (0.1 mmol) and 13 mg (0.1 mmol) of Na_2CO_3 in 1 mL of H_2O at 0 °C. 60 μL (0.3 mmol) of 6 N HCl was added dropwise, and the mixture was stirred for 5–10 min at 0 °C. A solution of carboxylic acid analogues of pyridoxal- or pyridoxic acid (0.1 mmol) in 1 mL of H_2O was added at once, and the pH of the mixture was adjusted to 10–11 by the addition of 1 N NaOH (~300 μL). The yellow color changed to red (sometimes gradually), indicating the reaction had occurred. After stirring the mixture for 30 min at 0 °C and 30 min at 25 °C, it was then purified by ion-exchange column chromatography using Amberlite CG-50 resin (H^+ form, weakly acidic) with the elution of water (flow rate 0.5 mL/min). The red fraction showing a single peak in HPLC was collected and lyophilized to give the desired compound with more than 95% purity.

4.1.19. 3-(2-((2,5-Disulfophenyl)diazenyl)-4-formyl-5-hydroxy-6-methylpyridin-3-yl) propanoic acid (24)

Following the general procedure for the synthesis of azo group containing compounds. Yield 28.5% ^{1}H NMR (D_2O) δ 2.39 (3H, s, CH_3), 2.65 (2H, t, J = 6.9 Hz, CH_2), 3.54 (2H, t, J = 6.9 Hz, CH_2), 8.34 (1H, d, J = 8.1 Hz, phenyl), 8.62 (1H, d, J = 8.1 Hz, phenyl), 9.06 (1H, s, phenyl), 10.20 (1H, s, CHO). ^{13}C NMR (DMSO-d_6, 400 MHz): δ 19.5, 21.3, 35.2, 126.6, 127.5, 129.9, 131.8, 138.0, 150.0, 150.9, 151.9, 173.7, 170.4, 179.5, 198.1. MS (MALDI-TOF): m/z = 474.4 (M^++1).

4.1.20. 3-(2-Carboxyethyl)-2-((2,5-disulfophenyl)diazenyl)-5-hydroxy-6-methylisonicotinic acid (25)

Following the general procedure for the synthesis of azo group containing compounds. Yield 32.4% ^{1}H NMR (D_2O) δ 2.74 (3H, s, CH_3), 2.85 (2H, t, J = 8.7 Hz, CH_2), 3.84 (2H, t, J = 8.7 Hz, CH_2), 8.03 (1H, d, J = 8.1 Hz, phenyl), 8.13 (1H, s, phenyl), 8.22 (1H, d, J = 8.1 Hz, phenyl). ^{13}C NMR (DMSO-d_6, 400 MHz): δ 19.0, 21.5, 34.5, 125.5, 126.8, 128.0, 129.7, 131.4, 149.7, 150.7, 159.2, 160.2, 167.4, 173.5, 174.2. MS (MALDI-TOF): m/z = 489.4 (M^++1).

4.1.21. 3-(4-Formyl-5-hydroxy-6-methyl-2-((4-sulfophenyl)diazenyl)pyridin-3-yl) propanoic acid (26)

Following the general procedure for the synthesis of azo group containing compounds. Yield 26.2% ^{1}H NMR (D_2O) δ 2.43 (3H, s, CH_3), 2.65 (2H, t, J = 6.9 Hz, CH_2), 3.50 (2H, t, J = 6.9 Hz, CH_2), 7.67 (2H, d, J = 8.4 Hz, phenyl), 7.82 (2H, d, J = 8.4 Hz, phenyl), 10.29 (1H, s, CHO). ^{13}C NMR (DMSO-d_6, 400 MHz): δ 19.5, 21.6, 35.9, 124.8, 127.3, 128.8, 130.2, 131.5, 148.1, 149.8, 156.0, 167.2, 174.0, 196.6. MS (MALDI-TOF): m/z = 394.3 (M^++1).

4.1.22. 3-(2-Carboxyethyl)-5-hydroxy-6-methyl-2-((4-sulfophenyl)diazenyl) isonicotinic acid (27)

Following the general procedure for the synthesis of azo group containing compounds. Yield 28.3% ^{1}H NMR (D_2O) δ 2.65 (3H, s, CH_3), 2.73 (2H, t, J = 7.2 Hz, CH_2), 3.68 (2H, t, J = 7.2 Hz, CH_2), 7.79 (2H, d, J = 7.2 Hz, phenyl), 7.98 (2H, d, J = 7.2 Hz, phenyl). ^{13}C NMR (DMSO-d_6, 400 MHz): δ 19.2, 23.9, 35.9, 121.6, 122.4, 127.3, 129.3, 131.5, 147.4, 148.4, 156.7, 168.3, 173.9, 175.3. MS (MALDI-TOF): m/z = 410.3 (M^++1).

4.1.23. 4-((3-(2-Carboxyethyl)-4-formyl-5-hydroxy-6-methylpyridin-2-yl) diazenyl) benzoic acid (28)

Following the general procedure for the synthesis of azo group containing compounds. Yield 32.5% ^{1}H NMR (D_2O) δ 2.53 (3H, S, CH_3), 2.62 (2H, t, J = 6.9 Hz, CH_2), 3.75 (2H, t, J = 6.9 Hz, CH_2), 7.94 (2H, d, J = 8.1 Hz, phenyl), 8.06 (2H, d, J = 8.1 Hz, phenyl), 10.52 (1H, s, CHO).). ^{13}C NMR (DMSO-d_6, 400 MHz): δ 18.3, 21.1, 32.9, 128.1, 128.6, 130.6, 131.9, 132.1, 149.6, 156.7, 168.4, 170.4, 176.9, 178.3, 199.2. MS (MALDI-TOF): m/z = 358.3 (M^++1).

4.1.24. 3-(2-Carboxyethyl)-2-((4-carboxyphenyl)diazenyl)-5-hydroxy-6-methy lisonicotinic acid (29)

Following the general procedure for the synthesis of azo group containing compounds. Yield 24.5% ^{1}H NMR (D_2O) δ 2.43 (3H, s, CH_3), 2.58 (2H, t, J = 7.5 Hz, CH_2), 3.52 (2H, t, J = 7.5 Hz, CH_2), 7.63 (2H, d, J = 8.1 Hz, phenyl), 7.98 (2H, d, J = 8.1 Hz, phenyl). ^{13}C NMR (DMSO-d_6, 400 MHz): δ 19.6, 22.4, 36.4, 120.7, 122.5, 130.9, 132.1, 139.3, 147.8, 153.9, 165.2, 167.7, 174.7, 175.5. MS (MALDI-TOF): m/z = 374.3 (M^++1).

4.2. Biology

4.2.1. Antagonistic activity at recombinant mP2X$_1$ and hP2X$_3$ receptors

Xenopus oocytes were harvested and prepared as previously described.[50] Defolliculated oocytes were injected cytosolically with mP2X$_1$ and hP2X$_3$ receptor cRNA (40 nL, 1 μg/mL), respectively, incubated for 24 h at 18 °C in Barth's solution and kept for up to 12 days at 4 °C until used in electrophysiological experiments. ATP-activated membrane currents (V_h = −70 mV) were recorded from cRNA-injected oocytes using the two-electrode voltage-clamp technique (Axoclamp 2B amplifier). Voltage recording (1–2 MΩ tip resistance) and current-recording microelectrodes (5 MΩ tip resistance) were filled with 3.0 M KCl. Oocytes were held in an electrophysiological chamber and superfused with Ringer's solution (5 mL/min, at 18 °C) containing (mM) NaCl, 110; KCl, 2.5; HEPES, 5; $BaCl_2$, 1.8, adjusted to pH 7.5. ATP was superfused over the oocytes for 60–120 s then washed out for a period of 20 min. For inhibition curves, data were normalized to the current evoked by ATP, at pH 7.5. Test substances were added for 20 min prior to ATP exposure; all peptides were tested for reversibility of their effects. The concentration required to inhibit the ATP response by 50% (IC_{50}) was taken from Hill plots constructed using the formula: $\log(I/I_{max} - I)$, where I is the current evoked by ATP in the presence of an antagonist. Data are presented as mean ± SEM (n = 4) for the data from different batches of oocytes.

4.2.2. Ethidium bromide (EtBr) accumulation assay at hP2X$_7$ receptors

hP2X$_7$-expressing HEK293 cells, a human embryonic kidney cells, were grown in DMEM supplemented with 10% fetal bovine serum as monolayer culture at 37 °C in a humidified atmosphere of 5% CO_2. Cells were harvested with treatment of Trypsin/EDTA solution, collected by centrifugation (200g for 5 min). The cells were resuspended at 2.5 × 10^6 cells/mL in assay buffers, consisting of (in mM) HEPES 10, *N*-methyl-D-glutamine 5, KCl 5.6, D-glucose

10, $CaCl_2$ 0.5 (pH 7.4), and then ethidium bromide (100 μM) was added. Cell suspensions were added to 96 well plates containing the $P2X_7$ receptor agonist, BzATP, at 2×10^5 cells/well. The plates were incubated at 37 °C for 120 min and cellular accumulation of ethidium ion was determined by measuring fluorescence with Bio-Tek FL600 fluorescent plate reader (excitation wavelength of 530 nm and emission wavelength of 590 nm).

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Supplementary data

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