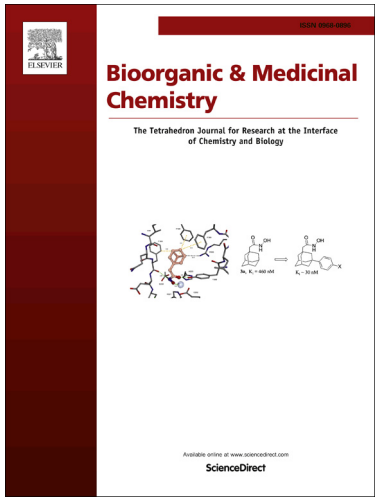

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Graphical Abstract

Synthesis and structure-activity relationship of uracil nucleotide derivatives leading to the identification of human $P2Y_6$ receptor antagonists

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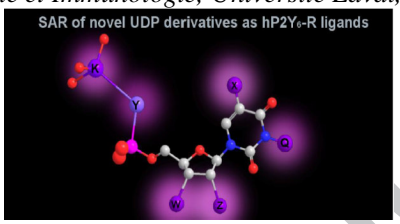

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ABSTRACT

$P2Y_6$ receptor ($P2Y_6$-R) is involved in various physiological and pathophysiological events. With a view to set rules for the design of UDP-based reversible $P2Y_6$-R antagonists as potential drugs, we established structure–activity relationship of UDP analogues, bearing modifications at the uracil ring, ribose moiety, and the phosphate chain. For instance, C5-phenyl- or 3-NMe-uridine-5'-α,β-methylene-diphosphonate, **16** and **23**, or lack of 2'-OH, in **12**-**15**, resulted in loss of both agonist and antagonist activity toward $hP2Y_6$-R. However, uridylyl phosphosulfate, **19**, selectively inhibited $hP2Y_6$-R (IC_{50} 112 μM) vs. $P2Y_{2/4}$-Rs. In summary, we have established a comprehensive SAR for $hP2Y_6$-R ligands towards the development of $hP2Y_6$-R antagonists.

1. Introduction

P2 receptors (P2-Rs) are membrane proteins that upon activation by nucleoside di- and tri-phosphate, and in some subtypes also by dinucleotides, lead to inhibitory and excitatory effects in many tissues and organs under both normal and pathophysiological conditions. The P2-Rs superfamily is divided into two families: the ATP-selective P2X receptor (P2X-R) family, which are ligand-gated ion channels, and the ATP (**1**; Fig. 1), ADP (**2**), UTP (**3**), UDP (**4**) and UPD-glucose sensitive GPCR P2Y receptor (P2Y-R) family.[1,2] P2-Rs are involved in the modulation of vasoconstriction[3], blood platelet aggregation,[4] regulation of intraocular pressure[5] and gastrointestinal disorders,[6] and hence represent important targets for novel drugs development.[7]

Among P2Y-Rs, $P2Y_6$-R is responsive to UDP, partially responsive to UTP and ADP, and not responsive to ATP.[8] $P2Y_6$-R is widely spread in the human body and has many physiological roles.[9-11]

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Pharmacological modulation of $P2Y_6$-R has been proposed to be useful for the treatment of various diseases, such as: osteoporosis, neurodegeneration, gout, ocular hypertension, glaucoma, inflammation, intestinal disorders, heart failure, and diabetes.[12-17]

While $P2Y_6$-R agonists are expected to be useful for the treatment of diseases such as glaucoma, neurodegeneration, and diabetes,[18-20] the treatment of inflammatory bowel diseases, for instance, requires the use of a $P2Y_6$-R antagonist.[21] Specifically, inflammation of the mucosa of the colon up-regulates the expression of $P2Y_6$-R,[6] and activation of the receptor by UDP results in increased damage of the colonic mucosal tissue. Blocking UDP activation of the over-expressed $P2Y_6$-R on epithelial cells, and consequently blocking the release of CXCL8, has been suggested for the treatment of inflammatory bowel diseases.[22-24] This hypothesis is corroborated by the finding that a $P2Y_6$-R (irreversible) antagonist, MRS2578, **5**, inhibited the induction levels of CXCL8 in Caco-2/15 cells.[21] Unlike $P2Y_6$-R agonists,[25-27] currently no UDP-based antagonists of this receptor are known.

Identification of potent and selective $P2Y_6$-R ligands has been a challenge for researchers over the past decade. This difficulty is exacerbated by the absence of a $P2Y_6$-R crystal structure. Hence, the structure–activity relationship (SAR) of pyrimidine nucleotide agonists at $P2Y_6$-R is only partially known,[28-30] and the mode of interaction between nucleotide

agonists and $P2Y_6$-R is not fully understood.[27] In this context, it is thus not surprising that only one class of $P2Y_6$-R antagonists has been reported.[7] The non-nucleotide di-isothiocyanate derivative MRS2578, **5**[6] (Fig. 1), is a potent and selective, yet irreversible, antagonist of $P2Y_6$-R. This isothiocyanate derivative which likely binds covalently to $P2Y_6$-R, is hydrophobic and has limited stability in aqueous solution.[7]

Hence, to allow controlled blocking of $hP2Y_6$-R, we targeted the identification of selective, reversible, stable and water-soluble $hP2Y_6$-R antagonists based on UDP scaffold.

Specifically, we describe here the synthesis of several series of UDP analogues, **6-23**, their SAR at $hP2Y_6$-R, as well as the metabolic stability of selected analogues.

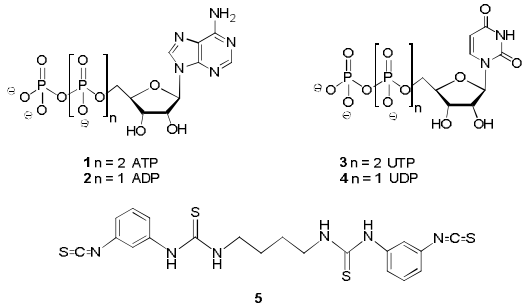


Figure 1. Natural nucleotides active at P2Y-Rs and MRS2578, **5**.

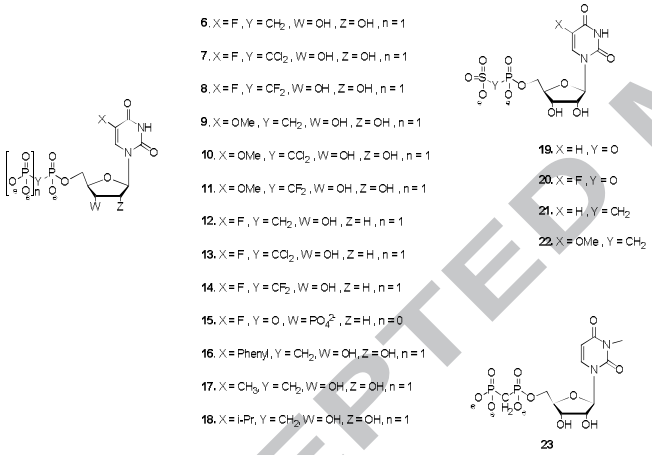


Figure 2. Uracil nucleotide analogues designed as potential $P2Y_6$-R antagonists.

2. Results

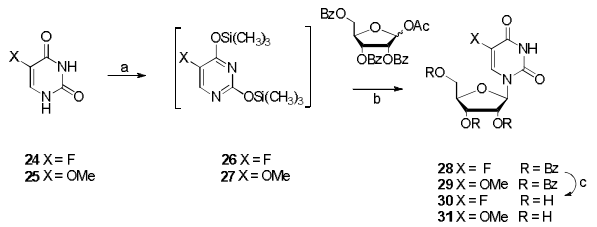
2.1. Synthesis of potential $P2Y_6$-R antagonists

We attempted the identification of potential $hP2Y_6R$ antagonists by modifying UDP-based $hP2Y_6R$ ligands, e.g. 5-OMe-UDP and 5-F-UDP scaffolds, which are a selective $P2Y_6$-R agonist / partial agonist, respectively,[8,31] or 5-alkyl/aryl-UDP scaffold, where C5-substituent is expected to improve the fit of the ligand to $P2Y_6$-R hydrophobic pocket.[8,32]

Specifically, UDP analogues **6-11** were obtained from nucleosides **32** and **33**. The latter were prepared by ribosylation of uracil derivatives **24** and **25** (Scheme 1).[33] Silylation of **24** and **25** was performed in HMDS in the presence of ammonium sulfate followed by ribosylation with 1-acetate 2,3,5-tribenzoate ribose. Compounds **30** and **31** were obtained by deprotection of the benzoate groups, using NH_3-MeOH for **30** and NaOMe for **31**, respectively. Next, 2'- and 3'-hydroxyl groups of **30** and **31**, were protected by an acetonide group upon reaction with dimethoxypropane and *p*-TsOH in acetone (Scheme 2).[34]

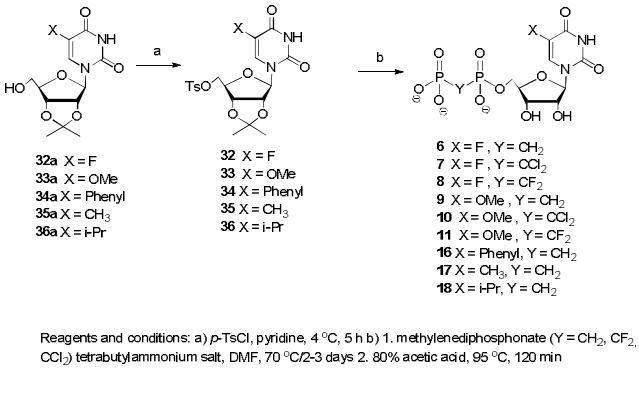
Compound **35a** was obtained by protection of commercially available 5-methyluridine. Compound **34a** was obtained by Suzuki coupling of acetonide protected 5-iodo uridine and phenyl boronic acid.[35] To prepare compound **36a**, first, Suzuki coupling was performed between acetonide protected 5-iodo uridine and isopropenylboronic acid pinacol ester,[35] followed by hydrogenation of the double bond over 10% Pd/C,[36] to get the desired isopropyl moiety (see Supplementary data for experimental details).

5'-Hydroxyl of 2',3'-acetonide-protected and 2'-deoxy derivatives, **32a-36a, 41a** and **37**, was activated with *p*-TsCl in pyridine. 5'-Tosyl-uridine analogues, **32-36, 38** and **41**, were used for the preparation of the corresponding diphosphate analogues (Schemes 2-5). First, the nucleosides were treated with the appropriate diphosphonate salt in DMF to produce the diphosphonate products **6-14, 16-18** and **23**. A high concentration of the nucleosides, 0.5-1 M, was required for the reaction, since 5'-tosyl-uridine is of limited reactivity. The progress of the reaction was monitored by ^{31}P-NMR and/or ^{19}F-NMR in 24 h time intervals. The integration of the products' signals in ^{31}P-NMR vs. that of diphosphonate reagent signal helped determining the completion of the reaction. The acetonide-protecting group was then removed using 80% acetic acid at 95°C. Finally, the diphosphonate products were purified by elution with 0.25-0.5 M ammonium bicarbonate on an ion-exchange chromatography (DEAE-A25). The yields of products **6-14, 16-18** and **23** were 6-77%. The purity of most products, determined on an analytical HPLC column in two solvents systems, was >97%. Compound **15** was obtained from 5-fluoro-2'-deoxyuridine, **37**, in 25% yield, according to literature.[37]



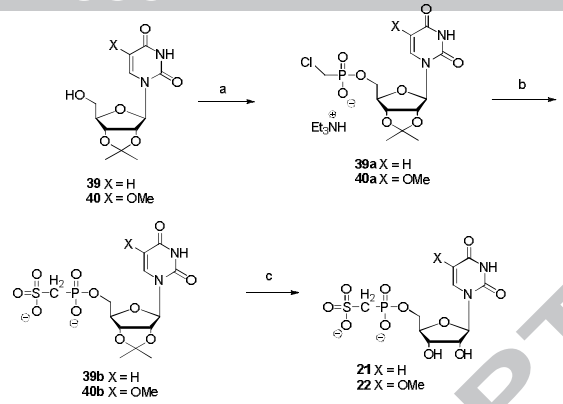
Reagents and conditions: a) HMDS, $(NH_4)_2SO_4$, reflux b) TMSOTf, $ClCH_2CH_2Cl$, RT c) NH_3-MeOH, RT

Scheme 1. Ribosylation of 5-F-uracil, **30**, and 5-OMe-uracil, **31**



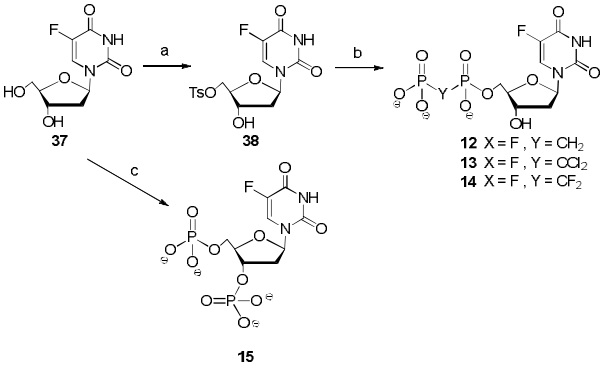
Reagents and conditions: a) *p*-TsCl, pyridine, 4 °C, 5 h b) 1. methylenediphosphonate (Y = CH_2, CF_2, CCl_2) tetrabutylammonium salt, DMF, 70 °C/2-3 days 2. 80% acetic acid, 95 °C, 120 min

Scheme 2. Synthesis of nucleotide analogues **6-11** and **16-18**.



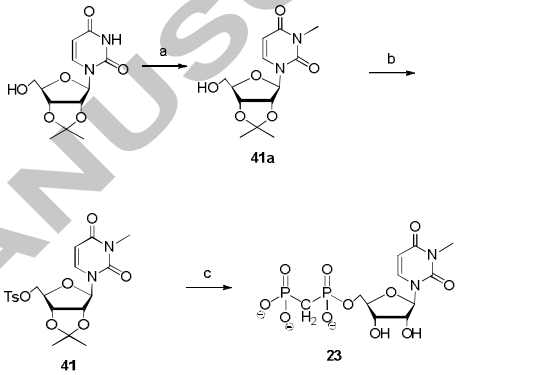
Reagents and conditions: a) chloromethylphosphonic acid, DCC, DMF, 60 °C, MW, 30 min b) sodium sulfite, water, pH 9-10, 130 °C, MW, 3.5 h c) 80% acetic acid, 95 °C, 120 min

Scheme 4. Synthesis of nucleotide analogues **21-22.**



Reagents and conditions: a) *p*-TsCl, pyridine, 4 °C, 5 h b) methylenediphosphonate (Y = CH_2, CF_2, CCl_2) tetrabutylammonium salt, DMF, 70 °C / 3 days c) $POCl_3$, TMP, 4 °C, 1 h

Scheme 3. Synthesis of nucleotide analogues **12-15.**



Reagents and conditions: a) Methyl iodide, K_2CO_3, acetone, DMF, RT, 4 h b) *p*-TsCl, pyridine, 4 °C, 5 h c) 1. methylenediphosphonate tetrabutyammonium salt, DMF, RT, 3 days 2. 80% acetic acid, 95 °C, 120 min.

Scheme 5. Synthesis of nucleotide analogue **23.**

Uridylyl phosphosulfate sodium salt, **19**, was also prepared according to literature.[38] 5-Fluoro-uridylyl phosphosulfate sodium salt, **20**, (see Supplementary data for experimental details) was prepared similarly from 5-fluoro-5'-monophosphate-uridine tri-n-octylammonium salt, tri-n-butyl-ammonium sulfate and diphenyl chloro-phosphate, and was obtained in 12% yield after HPLC separation. Analogues **21** and **22** were prepared according to literature.[39-42] Briefly, chloromethylphosphonic acid was coupled to the appropriate acetonide protected uridine using DCC in DMF. Then, sodium sulfite was added and after the removal of acetonide protecting group, products **21** and **22** were obtained. Compound **41** was obtained by methylation[43] of acetonide protected uridine followed by activation of 5'-OH with a tosyl group. Finally, compound **23** was obtained by treating **41** with methylenediphosphonate tetrabutyl ammonium salt, as described above.

2.2. Evaluation of uridine nucleotide analogues 6-23 as hP2Y$_6$-R ligands

Analogues **6-23** were first tested for their capacity to activate the human recombinant P2Y$_6$-R stably expressed in 1321N1 cells (Figs. 3-4). P2Y$_6$-R activation was determined by measuring variations in intracellular calcium concentration ($\Delta[Ca^{2+}]_i$) as previously described using UDP as control.[44,45]

Substitution of the 5-F-UDP and 5-OMe-UDP P_α, P_β- bridging oxygen atom by a CH_2 group, in compounds **6** and **9**, reduced agonist potency at hP2Y$_6$-R (Fig. 3A). The EC_{50} value for compound **6** could not be determined because of a yellowish background at concentrations above 10 µM. Although analogue **9** displayed some agonist activity, the elicited response at 10 µM was 27% of the UDP response at the same concentration (Fig. 3A). Likewise, CCl_2 and CF_2-substituted compounds **7**, **8**, **10** and **11,** were found to be poor P2Y$_6$-R agonists (Fig. 3A).

The absence of 2'-hydroxyl group, as in analogues **12** to **15**, inhibited the ability of these molecules to stimulate P2Y$_6$-R-dependent changes of $[Ca^{2+}]_i$ (Fig. 3B). Analogues **11**, **12** and **15** showed some residual P2Y$_6$-R activity at a concentration greater than 100 µM, whereas analogues **13** and **14** did not activate P2Y$_6$-R at all.

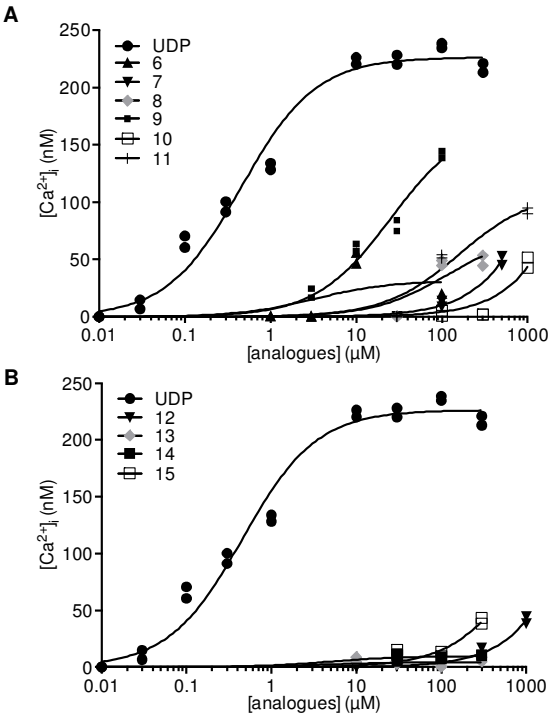


Figure 3. $P2Y_6$-R agonist activity of analogues **6-15** vs. UDP was determined by measuring intracellular calcium variation ($\Delta[Ca^{2+}]_i$) in 1321N1 astrocytoma cell line stably expressing recombinant human $P2Y_6$-R ($hP2Y_6$-R). A) Activity of analogues **6-11**. B) Activity of analogues **12-15**. The results of two experiments performed in duplicate are presented.

Among 5-alkyl/aryl-α,β-CH_2-UDP, **16**-**18,** analogues **16** and **18** could not activate the receptor (data not shown), however, compound **17** displayed significant $P2Y_6$-R activity with a response corresponding to 60% of the response induced by UDP at 100 μM (Fig. 4). 5-Fluoro-uridylyl phosphosulfate, **20**, also elicited a calcium response similar to the one produced by **17**, albeit less potent. However, the parent compound, uridylyl phosphosulfate, **19**, displayed only weak $P2Y_6$-R activation at concentration of 100 μM and higher. The related uridine-phosphono-methylene-sulfonate, **21**, and 5-OMe-uridine-phosphono-methylene-sulfonate, **22**, did not induce a calcium response even at 100 μM (data not shown). Finally, substitution of the hydrogen atom on N3 for a methyl group completely abolished the capacity of analogue **23** to induce $\Delta[Ca^{2+}]_i$ by $P2Y_6$-R (data not shown).

Since most of these analogues displayed only a partial agonist activity or even no activity at all, we tested these molecules for their ability to block changes of $[Ca^{2+}]_i$ induced by 1 μM UDP in 1321N1 cells stably expressing the recombinant $hP2Y_6$-R (Figs. 5-6). Compounds **9, 17** and **20** were not tested as antagonists due to their significant agonist activity as compared to UDP (Figs. 3A and 4). Compound **6**, tested at 10 μM, could not be analyzed due to the formation of an interfering yellowish color.

Analogues **7**, **8** and **10-15** tested at 30 μM failed to block UDP-induced $P2Y_6$-R activation, whereas at 100 μM analogues **8**, **10** and **11** significantly, but weakly, blocked $P2Y_6$-R stimulation by 1 μM UDP (Fig. 5).

Compounds **16**, **21, 22** and **23** were found to be neither $P2Y_6$-R agonists nor $P2Y_6$-R antagonists (Fig. 6). Compound **18** exhibited limited $P2Y_6$-R antagonist activity reducing UDP-induced calcium variation by 24% at 100 μM (the observed reduction is not statistically significant (p = 0.07)), and showing no agonist activity at $hP2Y_6$-R. On the other hand, analogue **19** significantly inhibited $hP2Y_6$-R at 100 μM (Fig. 6A). Concentration-response curve showed inhibition of $P2Y_6$-R activity with IC_{50} 112 μM (Fig. 6B). Hence, compound **19** may be a promising lead molecule for the design of UDP-based $P2Y_6$-R antagonists.

This result should be taken with care as it could be due to agonist-induced receptor desensitization and internalization,[46] and/or calcium pool depletion following the weak activation of the receptor by analogue **19** (Fig. 4).

Compound **19** was evaluated for its selectivity to $P2Y_6$-R vs. $hP2Y_2$- and $hP2Y_4$-Rs and showed no agonist activity at the latter receptors (data not shown).

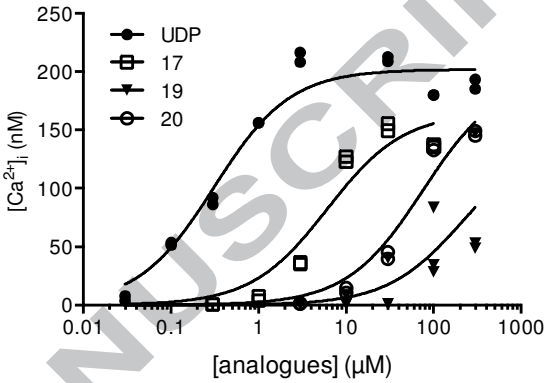


Figure 4. Activity of analogues **17, 19** and **20** vs. UDP at $hP2Y_6$-R. Potential $P2Y_6$-R agonist activity was determined by measuring intracellular calcium variation ($\Delta[Ca^{2+}]_i$) in 1321N1 astrocytoma cell line stably expressing recombinant $hP2Y_6$-R. The results of the two experiments performed in duplicate are presented.

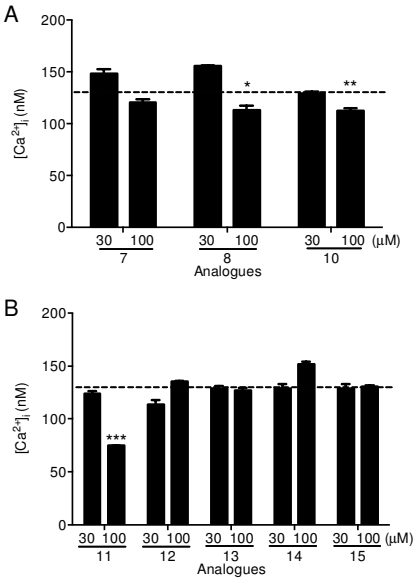


Figure 5. $hP2Y_6$-R antagonist activity of compounds **7-15**. The tested analogues were added prior to the addition of 1 μM UDP and $\Delta[Ca^{2+}]_i$ was measured. Horizontal bar showed the calcium response to 1 μM UDP. A) $P2Y_6$-R antagonist activity of compounds **7-10**. B) $P2Y_6$-R antagonist activity of compounds **11-15**. Results are presented as the mean ± SEM of two experiments performed in duplicate. Statistical significance was determined by one-way ANOVA with multiple comparisons post-test: $^*P < 0.05$, $^{**}P < 0.01$ and $^{***}P < 0.001$ compared to control vehicle (dashed line).

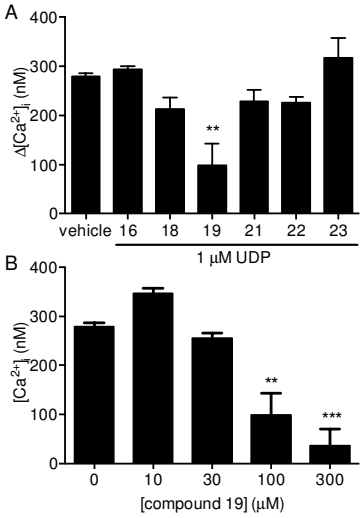


Figure 6. Antagonist activity of analogues **16**, **18**, **19** and **21-23** at human recombinant $P2Y_6$-R. **A)** Antagonists were tested at 100 μM upon 1 μM UDP stimulation of recombinant $hP2Y_6$-R stably expressed in 1321N1 cells. **B)** Concentration-dependent inhibitory effect of compound **19** on 1 μM UDP-dependent activation of $hP2Y_6$-R. Results are presented as the mean ± SEM of two experiments performed in duplicate. Statistical significance was determined by one-way ANOVA with multiple comparisons post-test: **P < 0.01 and ***P < 0.001 compared to control vehicle (A) or 0 μM (B).

2.3. Resistance of phosphosulfate and phosphono-methylene-sulfonate analogues 19-22 to hydrolysis by NPP1/3

To further evaluate the potential application of **19** as a $P2Y_6$-R antagonist, we studied its resistance to hydrolysis by nucleotide pyrophosphatase/phosphodiesterases (NPP1/3) vs. the hydrolysis of UDP and phosphosulfate and phosphonosulfonate analogues **20**-**22**. NPP1/3 are some of the major enzymes responsible for the hydrolysis of extracellular nucleotides.[47] The hydrolysis rate of analogues **19**-**22** by each enzyme was determined after incubation at 37 ºC in the appropriate buffer for 2 or 3 h (for NPP1 and NPP3, respectively), as compared to 100% hydrolysis of UDP. The enzymatic reaction was stopped by adding the reaction mixture into ice-cold perchloric acid. The stability of UDP and analogues **19-22** to hydrolysis by NPPs was determined by measuring the change in the integration of the HPLC peaks for each analogue vs. control, to take into consideration the degradation of the compounds due to the addition of acid to stop the enzymatic reaction. Percentages of hydrolysis of UDP and compounds **19-22** by NPP1 and 3 under the above conditions are shown in Table 1. Analogues **19** and **20** were partially metabolized by NPP1/3 vs. UDP. Surprisingly, analogues **21** and **22** were also hydrolyzed by NPP1/3, although to a lesser extent.

Compounds **21** and **22**, the enzymatic hydrolysis of which was relatively limited, were also tested as inhibitors of hNPP1/3 (Table 2). These compounds were found to be poor and non-selective hNPP1/3 inhibitors.

Table 1. Hydrolysis of analogues **19-22** by human ectonucleotidases, hNPP1/3.

	relative hydrolysis (% ± SD of UDP hydrolysis)[a]			
human ectonucleotidase	**19**	**20**	**21**	**22**
hNPP1	27.9 ± 1.0[b]	18.9 ± 1.0[b]	9.5 ± 2.5[b]	21.6 ± 3.8[b]
hNPP3	28.3 ± 0.1[b]	44.4 ± 1.9[b]	13.5 ±4.8[b]	11.0 ± 5.0[b]

[a] After incubation at 37 °C in buffer (1 mM $CaCl_2$, 200 mM NaCl, 10 mM KCl and 100 mM Tris, pH 8.5) for 2 h with NPP1/3.

[b] Values represent mean ± S.D. of three experiments.

Table 2. Inhibition of UDP hydrolysis by hNPP1/3 in the presence of analogues **21** and **22**.

	relative inhibition (% ± SD of UDP hydrolysis)[a]	
human ectonucleotidase	**21**	**22**
NPP1	26.7 ± 3.6[b]	48.4 ± 2.9[b]
NPP3	12.2 ±2.6[b]	10.5 ± 3.1[b]

[a] Analogue **21** or **22** was incubated at 37 ºC in buffer (1 mM $CaCl_2$, 140 mM NaCl, 5 mM KCl and 50 mM Tris, pH 8.5) for 2 h with hNPP1/3 and UDP.

[b] Values represent mean ± S.D. of three experiments.

3. Discussion

Currently, no reversible nucleotide-based $P2Y_6$-R antagonists are known. Likewise, structure-activity relationships (SAR) for $P2Y_6$-R antagonists are practically unknown. In the present study we have established SAR of several series of UDP derivatives at recombinant $hP2Y_6$-R stably expressed in 1321N1 cells. P_α,P_β–CH_2 modification[48] of $P2Y_6$-R agonist 5-OMe-UDP,[8] i.e. analogue **9**, resulted in reduced agonist activity.

CH_2 and CF_2 groups are considered as isosteres of the bridging oxygen atom in PPi.[49] Hence, in addition to P_α,P_β–CH_2 UDP analogues **6** and **9**, P_α,P_β–CF_2 and P_α,P_β–CCl_2 modified UDP analogues were synthesized, **10-11** and **13-14**, and found to be poor $hP2Y_6$-R agonists or no agonists at all (analogues **13** and **14**). Compound **11**, containing a bridging CF_2 group and 5-OMe substitution, reduced calcium release by 40% at 100 μM, but was not active at lower concentrations. At 100 μM analogue **10** weakly blocked $P2Y_6$-R stimulation by UDP. The minor or lack of $P2Y_6$-R agonist and antagonist activity of **10** and **7,** containing a bridging CCl_2 group (vs. **11** and **9**, Fig. 5) may be related to the large size of dichloro methylene bisphosphonate, vs. difluoro methylene bisphosphonate or methylene bisphosphonate anion,[50] which may not be compatible with the binding pocket of the receptor.

Reduced activation of $P2Y_6$-R by 2'-dUDP analogues has been described before.[26] 2'-Deoxy analogues **12-14**, showed neither agonist nor antagonist activity at $P2Y_6$-R. Likewise, 5-fluoro-2'-deoxyuridine 3',5'-bis-phosphate, **15**, was inactive as either an agonist or antagonist at $P2Y_6$-R.

However, C5-alkyl/aryl substitution in compounds **16-18** which was expected to improve the fit to the hydrophobic pocket of $P2Y_6$-R,[32] resulted in loss of any activity at $P2Y_6$-R or in poor agonist activity. These findings are in accordance with a report

on 5-substituted UTP derivatives as agonists of the related $P2Y_2$-R, showing that alkyl substituents at UTP C5-position are not tolerated by $P2Y_2$-R either.[51]

The loss of activity of compound **23**, bearing a methyl moiety on N3, at $hP2Y_6$-R may be due to a loss of a significant H-bond present in 98% of the complexes of uracil-nucleotides and proteins, between the uracil N3-H and a protein H-bond acceptor.[8,32]

At 100 μM, compounds **21** and **22**, bearing a phosphono-methylene-sulfonate moiety, neither activated $P2Y_6$-R nor inhibited its activity.

Analogue **19**, bearing a phosphosulfate moiety, although partially hydrolysable by NPP1/3, proved to be a very poor $P2Y_6$-R agonist and caused an apparent inhibition of $P2Y_6$-R with an IC_{50} value of 112 μM (Fig. 6). Furthermore it showed selectivity vs. $P2Y_2$-R and $P2Y_4$-R.

Hence, analogue **19** may be used as a scaffold to develop the next generation of potential UDP analogues displaying antagonist activity at human $P2Y_6$-R.

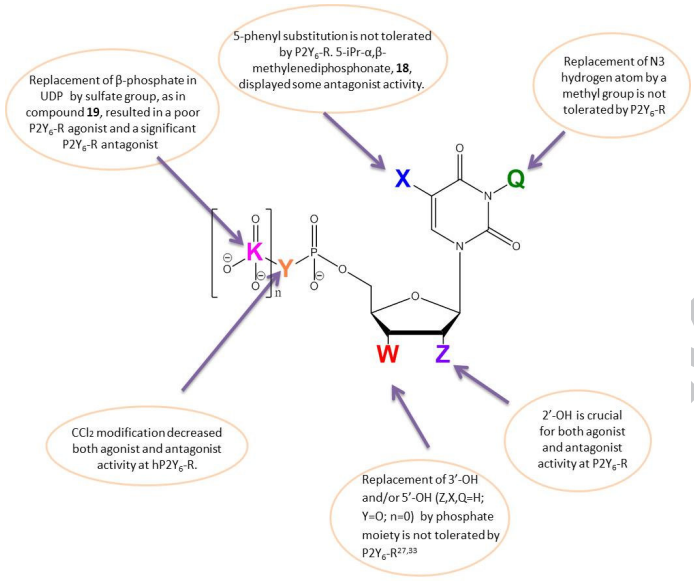


Figure 7. SAR of the new UDP derivatives presented herein.

4. Conclusions

Here, we synthesized several series of UDP analogues, and established their SAR as $hP2Y_6$-R ligands (Fig. 7). Analogues lacking 2'-OH (compounds **12-15**) had no agonist or antagonist activity at $hP2Y_6$-R, namely, 2'-OH is crucial for any activity at the receptor. Furthermore, 3-NMe and C5-alkyl/aryl modifications of P_α,P_β–CH_2-UDP, compounds **16-18** and **23**, were not tolerated by $hP2Y_6$-R, as well. They showed poor agonist activity towards $hP2Y_6$-R with the exception of compounds **16** and **23** that did not activate the receptor at all. Compounds **16** and **23** also did not inhibit $P2Y_6$-R. The replacement of β-phosphate in UDP by sulfate group, compounds **19-22**, reduced agonist activity, and even resulted in antagonist activity of compound **19**. P_α,P_β–CCl_2 modification of 5-F- and 5-OMe-UDP decreased agonist activity at $hP2Y_6$-R (compounds **7, 10**, and **13**), however, did not result in antagonist activity, while 5-OMe substitution of UDP-P_α,P_β–CF_2, **11**, resulted in 40% inhibition of $P2Y_6$-R at 100 μM. Yet, the corresponding 5-F-UDP analogues **6, 8**, and **14** proved to be poor $P2Y_6$-R ligands.

Analogue **19** is thus proposed as a lead structure for the development of $P2Y_6$-R antagonists. Furthermore, this compound was a $P2Y_6$-R selective ligand showing no activity at $hP2Y_2$- and $hP2Y_4$-Rs.

The SAR of the new UDP derivatives presented here and summarized in Fig. 7, may contribute to future design of selective ligands for $P2Y_6$-R and other uracil nucleotide-sensitive P2Y-Rs.

5. Experimental Section

5.1. Chemistry

5.1.1. General

All air and moisture sensitive reactions were carried out in flame-dried, argon-flushed, two-neck flasks sealed with rubber septa, and the reagents were introduced by syringe. The separation on the automatic column was carried out using an HPFC automated flash purification system (Biotage SP1 separation system (RP)). Compounds were characterized by NMR using Bruker AC-200, DPX-300, or DMX-600 spectrometers. ^{1}H NMR spectra were recorded at 200, 300, or 600 MHz. High resolution mass spectra were recorded on an AutoSpec Premier (Waters, UK) spectrometer by chemical ionization. Nucleotides were analyzed under ESI (electron spray ionization) conditions on a Q-TOF micro instrument (Waters, UK). Primary purification of the nucleotides was achieved on a LC (Isco UA-6) system using a Sephadex DEAE-A25 column, swollen in 1M $NaHCO_3$ at room temperature for 1 day. The resin was washed with deionized water before use. LC separation was monitored by UV detection at 280 nm. A buffer gradient of NH_4HCO_3 was applied as detailed below. Final purification of the nucleotides was achieved on an HPLC (Merck-Hitachi) system, using a semipreparative reverse-phase column (Gemini 5u C-18 110A, 250 X 10.00 mm, 5 μm, Phenomenex, Torrance, CA). The purity of the nucleotides was evaluated on an analytical reverse-phase column system (Gemini 5u, C-18, 110A, 150 X 4.60 mm, 5 μm, Phenomenex, Torrance, CA), in two solvent systems as described below. Aqueous solutions of the products were passed through a sodium form Dowex 50WX8-200 or CM Sephadex ion-exchange resin column and the products were eluted with deionized water to obtain the corresponding sodium salts after freeze-drying.

5.1.2. 5-Fluoro-uridine-5'-α,β-methylene-diphosphonate sodium salt, 6, was obtained from **32** (150 mg, 0.33 mmol) in a 14% yield (23 mg) after HPLC separation. Final separation was achieved by applying an isocratic TEAA/CH_3CN 97:3 in 13 min (5 mL/min): t_R 8.00 min. Purity data was obtained on an analytical column: t_R 2.58 min (100% purity) using solvent system I (98:2 TEAA/CH_3CN over 10 min, 1 mL/min); t_R 2.34 min (99.9% purity) using solvent system II (100:0 PBS/CH_3CN over 10 min, 1 mL/min). ^{1}H NMR (D_2O, 200 MHz): δ 8.15 (d, J = 6.2 Hz, H-6, 1H), 5.95 (d, J = 1.8 Hz, H-1', 1H), 4.43-4.32 (m, 2H), 4.31-4.23 (m, 1H), 4.23-4.08 (m, 2H), 2.19 (t, J = 20 Hz, PCH_2P, 2H) ppm. ^{31}P NMR (D_2O, 81 MHz): δ 19.03 (m, P_α), 15.19 (m, P_β) ppm. ^{19}F NMR (D_2O, 188 MHz): δ -165.28 (d, J = 6.25 Hz, F-5) ppm. HR MALDI (negative): calcd for $C_{10}H_{14}F_1N_2O_{11}P_2$, 419.0102; found, 419.0081.

5.1.3. 5-Fluoro-uridine-5'-α,β-dichloromethylene-diphosphonate sodium salt, 7, was obtained from **32** (150 mg, 0.33 mmol) in a 8% yield (14.3 mg) after HPLC separation. Final

separation was achieved by applying an isocratic TEAA/CH_3CN 97:3 in 13 min (5 mL/min): t_R 7.60 min. Purity data was obtained on an analytical column: t_R 8.66 min (99.9% purity) using solvent system I (98:2 TEAA/CH_3CN over 10 min, 1 mL/min); t_R 4.40 min (99.7% purity) using solvent system II (100:0 PBS/CH_3CN over 10 min, 1 mL/min). ^{1}H NMR (D_2O, 200 MHz): δ 8.14 (d, J = 6.2 Hz, H-6, 1H), 5.94 (d, J = 3.0 Hz, H-1', 1H), 4.40-4.28 (m, 4H), 4.27-4.18 (m, 1H) ppm. ^{31}P NMR (D_2O, 81 MHz): δ 8.90 (m, P_α), 8.20 (m, $P_ß$) ppm. ^{19}F NMR (D_2O, 188 MHz): δ -165.19 (d, J = 6.2 Hz, F-5) ppm. HR MALDI (negative): calcd for $C_{10}H_{12}Cl_2F_1N_2O_{11}P_2$, 486.9272; found, 486.9274.

5.1.4. 5-Fluoro-uridine-5'-α,β-difluoromethylene-diphosphonate sodium salt, 8, was obtained from **32** (45 mg, 0.10 mmol) in a 16% yield (8.5 mg) after HPLC separation. Final separation was achieved by applying an isocratic TEAA/CH_3CN 97:3 in 13 min (5 mL/min): t_R 9.00 min. Purity data was obtained on an analytical column: t_R 5.67 min (100% purity) using solvent system I (98:2 TEAA/CH_3CN over 10 min, 1 mL/min); t_R 2.36 min (99.8% purity) using solvent system II (100:0 PBS/CH_3CN over 10 min, 1 mL/min). ^{1}H NMR (D_2O, 200 MHz): δ 8.08 (d, J = 6.2 Hz, H-6, 1H), 5.94 (d, J = 3.0 Hz, H-1', 1H), 4.38-4.17 (m, 5H) ppm. ^{31}P NMR (D_2O, 81 MHz): δ 8.70-5.60 (m, P_α), 5.53-2.67 (m, $P_ß$) ppm. ^{19}F NMR (D_2O, 188 MHz): δ -116.03 to -117.10 (m, CF_2, 2F), -165.42 (d, J = 6.2 Hz, F-5) ppm. HR MALDI (negative): calcd for $C_{10}H_{12}F_3N_2O_{11}P_2$, 454.9902; found, 454.9861.

5.1.5. 5-OMe-uridine-5'-α,β-methylene-diphosphonate sodium salt, 9, was obtained from **33** (100 mg, 0.22 mmol) in a 30% yield (33.5 mg) after HPLC separation. Final separation was achieved by applying an isocratic TEAA/CH_3CN 97:3 in 13 min (5 mL/min): t_R 7.80 min. Purity data was obtained on an analytical column: t_R 2.77 min (99.5% purity) using solvent system I (96:4 TEAA/CH_3CN over 10 min, 1 mL/min); t_R 2.62 min (99.0% purity) using solvent system II (98:2 PBS/CH_3CN over 10 min, 1 mL/min). ^{1}H NMR (D_2O, 200 MHz): δ 7.30 (s, H-6, 1H), 5.95 (d, J = 5.3 Hz, H-1', 1H), 4.43-4.32 (m, 2H), 4.26-4.05 (m, 3H), 2.11 (t, J = 20 Hz, PCH_2P, 2H) ppm. ^{31}P NMR (D_2O, 81 MHz): δ 18.49 (m, P_α), 15.19 (m, $P_ß$) ppm. ^{13}C-NMR (D_2O, 100 MHz): δ 163.8, 152.2, 137.6, 120.3, 89.2, 84.1 (d, J = 7.9 Hz, C-4'), 73.9, 70.3, 64.1 (d, J = 4.6 Hz, C-5'), 58.3, 28.6 (t, J = 120.6 Hz, PCH_2P) ppm. HR MALDI (negative): calcd for $C_{11}H_{17}N_2O_{12}P_2$, 431.0260; found, 431.0252.

5.1.6. 5-OMe-uridine-5'-α,β-dichloromethylene-diphosphonate sodium salt, 10, was obtained from **33** (50 mg, 0.10 mmol) in a 28% yield (15.7 mg) after HPLC separation. Final separation was achieved by applying an isocratic TEAA/CH_3CN 97:3 in 13 min (5 mL/min): t_R 8.30 min. Purity data was obtained on an analytical column: t_R 5.31 min (99.9% purity) using solvent system I (96:4 TEAA/CH_3CN over 10 min, 1 mL/min); t_R 3.49 min (99.8% purity) using solvent system II (98:2 PBS/CH_3CN over 10 min, 1 mL/min). ^{1}H NMR (D_2O, 200 MHz): δ 7.32 (s, H-6, 1H), 5.97 (d, J = 5.1 Hz, H-1', 1H), 4.51-4.17 (m, 5H), 3.80 (s, OMe, 3H) ppm. ^{31}P NMR (D_2O, 81 MHz): δ 11.06 (d, J = 16.0 Hz, P_α), 8.62 (d, J = 16.0 Hz, $P_ß$) ppm. ^{13}C-NMR (D_2O, 151 MHz): δ 163.5, 151.9, 137.6, 120.8, 88.9, 84.3 (d, J = 6.8 Hz, C-4'), 80.9 (dd, J = 118.5 Hz, J = 135.7 Hz, PCH_2P), 73.8, 70.3, 67.1 (d, J = 5.3 Hz, C-5'), 58.8 ppm. HR MALDI (negative): calcd for $C_{11}H_{15}Cl_2N_2O_{12}P_2$, 498.9480; found, 498.9470.

5.1.7. 5-OMe-uridine-5'-α,β-difluoromethylene-diphosphonate sodium salt, 11, was obtained from **33** (46 mg, 0.10 mmol) in a 9% yield (4.6 mg) after HPLC separation. Final separation was achieved by applying an isocratic TEAA/CH_3CN 97:3 in 13 min (5 mL/min): t_R 7.70 min. Purity data was obtained on an analytical column: t_R 3.91 min (99.5% purity) using solvent system I (96:4 TEAA/CH_3CN over 10 min, 1 mL/min); t_R 2.31 min (97.8% purity) using solvent system II (98:2 PBS/CH_3CN over 10 min, 1 mL/min). ^{1}H NMR (D_2O, 200 MHz): δ 7.32 (s, H-6, 1H), 6.00 (d, J = 4.8 Hz, H-1', 1H), 4.48-4.18 (m, 5H), 3.79 (s, OMe, 3H) ppm. ^{31}P NMR (D_2O, 81 MHz): δ 8.70-5.60 (m, P_α), 5.53-2.67 (m, $P_ß$) ppm. ^{19}F NMR (D_2O, 188 MHz): δ -116.6 (m, CF_2, 2F) ppm. HR MALDI (negative): calcd for $C_{11}H_{15}F_2N_2O_{12}P_2$, 467.0071; found, 467.0062.

5.1.8. 5-Fluoro-uridine-2'-deoxy-5'-α,β-methylene-diphosphonate sodium salt, 12, was obtained from **38** (70 mg, 0.18 mmol) in a 17% yield (14 mg) after HPLC separation. Final separation was achieved by applying an isocratic TEAA/CH_3CN 97:3 in 13 min (5 mL/min): t_R 7.00 min. Purity data was obtained on an analytical column: t_R 3.11 min (99.5% purity) using solvent system I (98:2 TEAA/CH_3CN over 10 min, 1 mL/min); t_R 2.98 min (99.1% purity) using solvent system II (100:0 PBS/CH_3CN over 10 min, 1 mL/min). ^{1}H NMR (D_2O, 200 MHz): δ 8.08 (d, J = 6.3 Hz, H-6, 1H), 6.24 (t, J = 6.4 Hz, H-1', 1H), 4.60-4.50 (m, 1H), 4.19-4.00 (m, 3H), 2.33 (t, J = 5.7 Hz, 2H), 2.12 (t, J = 20.0 Hz, PCH_2P, 2H) ppm. ^{31}P NMR (D_2O, 81 MHz): δ 19.78 (d, J = 9.5 Hz, P_α), 14.71 (d, J = 9.5 Hz, $P_ß$) ppm. ^{19}F NMR (D_2O, 188 MHz): δ -165.26 (d, J = 6.3 Hz, F-5) ppm. HR MALDI (negative): calcd for $C_{10}H_{14}FN_2O_{10}P_2$, 403.0110; found, 403.0082.

5.1.9. 5-Fluoro-uridine-2'-deoxy-5'-α,β-dichloromethylene-diphosphonate sodium Salt, 13, was obtained from **38** (135 mg, 0.30 mmol) in a 6% yield (9.1 mg) after HPLC separation. Final separation was achieved by applying an isocratic TEAA/CH_3CN 97:3 in 13 min (5 mL/min): t_R 6.80 min. Purity data was obtained on an analytical column: t_R 3.63 min (98.7% purity) using solvent system I (98:2 TEAA/CH_3CN over 10 min, 1 mL/min); t_R 3.20 min (98.0% purity) using solvent system II (100:0 PBS over 10 min, 1 mL/min). ^{1}H NMR (D_2O, 200 MHz): δ 8.10 (d, J = 6.1 Hz, H-6, 1H), 6.30 (t, J = 6.6 Hz, H-1', 1H), 4.71-4.60 (m, 1H), 4.45-4.10 (m, 3H), 2.38 (t, J = 6.1 Hz, 2H) ppm. ^{31}P NMR (D_2O, 81 MHz): δ 11.02 (d, J = 16.0 Hz, P_α), 8.58 (d, J = 16.0 Hz, $P_ß$) ppm. ^{19}F NMR (D_2O, 188 MHz): δ -165.08 (d, J = 6.1 Hz, F-5) ppm. HR MALDI (negative): calcd for $C_{10}H_{12}Cl_2FN_2O_{10}P_2$, 470.9330; found, 470.9321.

5.1.10. 5-Fluoro-uridine-2'-deoxy-5'-α,β-difluoromethylene-diphosphonate sodium salt, 14, was obtained from **38** (71 mg, 0.18 mmol) in a 23% yield (21 mg) after HPLC separation. Final separation was achieved by applying an isocratic TEAA/CH_3CN 97:3 in 13 min (5 mL/min): t_R 7.20 min. Purity data was obtained on an analytical column: t_R 3.25 min (99.9% purity) using solvent system I (98:2 TEAA/CH_3CN over 10 min, 1 mL/min); t_R 2.56 min (99.3% purity) using solvent system II (100:0 PBS over 10 min, 1 mL/min). ^{1}H NMR (D_2O, 200 MHz): δ 8.07 (d, J = 6.5 Hz, H-6, 1H), 6.29 (t, J = 6.6 Hz, H-1', 1H), 4.68-4.54 (m, 1H), 4.38-4.06 (m, 3H), 2.44-2.30 (m, 2H) ppm. ^{31}P NMR (D_2O, 81 MHz): δ 8.70-5.60 (m, P_α), 5.53-2.67 (m, $P_ß$) ppm. ^{19}F NMR (D_2O, 188 MHz): δ -117.38 (dd, J = 88.0 Hz, 2F), -165.42 (d, J = 6.5 Hz, F-5) ppm. HR MALDI (negative): calcd for $C_{10}H_{12}F_3N_2O_{10}P_2$, 438.9921; found, 438.9901.

5.1.11. 5-Fluoro-2'-deoxyuridine 3',5'-bisphosphate disodium salt, 15. Starting from 40 mg (0.16 mmol) of 5-fluoro-2'-deoxyuridine, **37**, and following literature procedure,[37] 20 mg (25% yield) of **15** were obtained after HPLC separation. Final separation was achieved by applying an isocratic TEAA/CH_3CN

97:3 in 13 min (5 mL/min): t_R 6.20 min. Purity data was obtained on an analytical column: t_R 4.16 min (97.0% purity) using solvent system I (98:2 TEAA/CH_3CN over 10 min, 1 mL/min); t_R 3.81 min (96.5% purity) using solvent system II (100:0 PBS over 10 min, 1 mL/min). ^{1}H NMR (D_2O, 200 MHz) δ 2.38 (m, CH_2-2', 1H), 2.55 (m, CH_2-2', 1H), 4.00 (m, CH_2-5', 2H), 4.32 (m, H-4', 1H), 4.89 (m, H-3', 1H), 6.34 (t, J = 7.0 Hz, H-1', 1H), 8.20 (d, J = 6.5 Hz, H-5, 1H). ^{31}P NMR (D_2O, 81 MHz): δ 3.32 (s, 5'-P), 2.99 (s, 3'-P). ^{19}F NMR (D_2O, 188 MHz): δ -165.36 (d, J = 6.5 Hz, F-5). HR MALDI (negative): calcd for $C_9H_{12}FN_2O_{11}P_2$, 404.9922; found, 404.9902.

5.1.12. 5-Phenyl-uridine-5'-α,β-methylene-diphosphonate sodium salt, 16, was obtained from **34** (90 mg, 0.175 mmol) in a 20% yield (16 mg) after HPLC separation. Final separation was achieved by applying an isocratic TEAA/CH_3CN 90:10 in 10 min (4 mL/min): t_R 6.25 min. Purity data was obtained on an analytical column: t_R 5.31 min (99.5% purity) using solvent system I (92:8 TEAA/CH_3CN over 15 min, 1 mL/min); t_R 4.28 min (99.4% purity) using solvent system II (94:4 PBS/CH_3CN over 13 min, 1 mL/min). ^{1}H NMR (D_2O, 200 MHz): δ 7.87 (s, 1H, H-6), 7.42-7.52 (m, 5H, Ph), 6.01 (d, J = 5.4 Hz, 1H, H-1'), 4.48 (t, J = 5.4 Hz, 1H, H-2'), 4.37 (t, J = 4.6 Hz, 1H, H-3'), 4.2-4.3 (m, 1H, H-4'), 4.15-4.05 (m, 2H, H-5', H-5"), 1.98 (t, J = 19.8 Hz, 2H, PCH_2P) ppm. ^{31}P NMR (D_2O, 81 MHz): δ 19.10 (d, J = 8.9 Hz, P_α), 15.22 (d, J = 8.9 Hz, P_β) ppm. ^{13}C NMR (D_2O, 150 MHz): δ 164.8, 151.5, 138.7, 131.8, 128.8, 128.5, 116.2, 88.8, 83.6 (d, J = 7.8 Hz, C-4'), 73.4, 69.8, 63.4 (d, J = 5.0 Hz, C-5'), 27.5 (t, J = 123.2 Hz, PCH_2P). HR MALDI (negative): calcd for $C_{16}H_{19}N_2O_{11}P_2$, 477.0459; found, 477.0464.

5.1.13. 5-Methyl-uridine-5'-α,β-methylene-diphosphonate sodium salt, 17, was obtained from **35** (90 mg, 0.20 mmol) in a 33% yield (27 mg) after HPLC separation. Final separation was achieved by applying an isocratic TEAA/CH_3CN 98:2 in 11 min (4 mL/min): t_R 6.80 min. Purity data was obtained on an analytical column: t_R 4.56 min (99.9% purity) using solvent system I (99:1 TEAA/CH_3CN over 9 min, 1 mL/min); t_R 2.53 min (99.7% purity) using solvent system II (95.5:0.5 PBS/CH_3CN over 10 min, 1 mL/min). ^{1}H NMR (D_2O, 200 MHz): δ 7.77 (s, 1H, H-6), 5.97 (d, J = 4.2 Hz, 1H, H-1'), 4.43-4.32 (m, 2H, H-2', H-3'), 4.31-4.23 (m, 1H, H-4'), 4.23-4.08 (m, 2H, H-5', H-5"), 2.03 (t, J = 19.6 Hz, 2H, PCH_2P), 1.94 (s, 3H, CH_3) ppm. ^{31}P NMR (D_2O, 81 MHz): δ 21.07 (d, J = 8.4 Hz, P_α), 13.33 (d, J = 8.4 Hz, P_β) ppm. ^{13}C NMR (D_20, 150 MHz): δ 166.7, 152.1, 137.2, 111.7, 88.3, 83.3 (d, J = 7.3 Hz, C-4'), 73.5, 69.5, 63.3 (d, J = 4.6 Hz, C-5'), 28.1 (t, J = 120.8 Hz, PCH_2P), 11.66 ppm. HR MALDI (negative): calcd for $C_{11}H_{17}N_2O_{11}P_2$, 415.0313; found, 415.0302.

5.1.14. 5-Isopropyl-uridine-5'-α,β-methylene-diphosphonate sodium salt, 18, was obtained from **36** (100 mg, 0.2 mmol) in a 30% yield (27 mg) after HPLC separation. Final separation was achieved by applying an isocratic TEAA/CH_3CN 99:9 in 25 min (4 mL/min): t_R 18.40 min. Purity data was obtained on an analytical column: t_R 6.82 min (98.9% purity) using solvent system I (95:5 TEAA/CH_3CN over 22 min, 1 mL/min); t_R 3.61 min (99.9% purity) using solvent system II (98:2 PBS/CH_3CN over 10 min, 1 mL/min). ^{1}H NMR (D_2O, 600 MHz): δ 7.48 (s, 1H, H-6), 5.93 (d, J = 5.4 Hz, 1H, H-1'), 4.43 (t, J = 4.6 Hz, 1H, H-2'), 4.37 (dd, J = 5.4, 4.2 Hz, 1H, H-3'), 4.2-4.3 (m, 1H, H-4'), 4.10-4.18 (m, 2H, H-5', H-5"), 2.82 (septet, J = 7.2 Hz, 1H, CH), 2.12 (t, J = 19.2 Hz, 2H, PCH_2P), 1.16 (d, J = 1.8 Hz, 3H, CH_3), 1.15 (d, J = 1.8 Hz, 3H, CH'_3) ppm. ^{31}P NMR (D_2O, 81 MHz): δ 20.29 (d, J = 8.3 Hz, P_α), 14.08 (d, J = 8.3 Hz, P_β) ppm. ^{13}C NMR (D_2O, 150 MHz): δ 165.6, 151.6, 135.7, 121.7, 88.7, 83.3 (d, J = 7.8 Hz, C-4'), 73.1, 69.7, 63.5 (d, J = 4.8 Hz, C-5'), 27.8 (t, J = 124.1 Hz, PCH_2P), 25.8, 20.6, 20.4 ppm. HR MALDI (negative): calcd for $C_{13}H_{21}N_2O_{11}P_2$, 443.0626; found, 443.0615.

5.1.15. 5-Fluoro-uridylyl phosphosulfate sodium salt, 20, was synthesized according to literature,[38] and obtained in a 12% yield (22 mg) after HPLC separation. ^{1}H NMR (D_2O, 600 MHz): δ 8.02 (d, J = 6.4 Hz, 1H, H-6), 6.01 (dd, J = 4.9, 1.6 Hz, 1H, H-1'), 4.20-4.40 (m, 5H, H-2',H-3',H-4', H-5', H-5") ppm. ^{31}P NMR (D_2O, 81 MHz): δ -10.11 (s, 1P) ppm. ^{19}F NMR (D_2O, 188 MHz): -164.62 (d, J = 5.6 Hz) ppm. HR MALDI (negative): calcd for $C_9H_{11}FN_2O_{12}PS$, 420.9760; found, 420.9749. Purity data was obtained on an analytical column: t_R 7.65 min (88.0% purity) using solvent system I (99:1 TEAA/CH_3CN over 14 min, 1 mL/min); t_R 2.01 min (85.0% purity) using solvent system II (99:1 PBS/CH_3CN over 8 min, 1 mL/min).

5.1.16. Uridine-phosphono-methylene-sulfonate triethylammonium salt, 21, was synthesized according to literature,[42] and obtained in a 17% yield (20 mg) after LC separation. ^{1}H NMR (D_2O, 600 MHz): δ 8.02 (d, J = 8.4 Hz, 1H, H-6), 5.98 (d, J = 5.4 Hz, 1H, H-1'), 5.95 (d, J = 8.4 Hz, 1H, H-5), 4.36-4.40 (m, 2H, H-2',H-3'), 4.26-4.27 (m, 1H, H-4'), 4.17-4.20 (m, 2H, H-5', H-5"), 3.43 (d, J = 15.6 Hz, 2H, PCH_2S) ppm. ^{31}P NMR (D_2O, 162 MHz): δ 11.38 (s, 1P) ppm. ^{13}C NMR (D_2O, 150 MHz): δ 167.1, 152.7, 142.6, 103.3, 88.8, 84.3 (d, J = 8.0 Hz, C-4'), 74.4, 70.5, 64.6 (d, J = 5.3 Hz, C-5'), 49.6 (d, J 130.1 = Hz, PCH_2S) ppm. HR MALDI (negative): calcd for $C_{10}H_{14}N_2O_{11}PS$, 401.0050; found, 401.0030. Purity data was obtained on an analytical column: t_R 4.90 min (99.8% purity) using solvent system I (99:1 TEAA/CH_3CN over 15 min, 1 mL/min); t_R 2.51 min (99.9% purity) using solvent system II (99.8:0.2 PBS/CH_3CN over 10 min, 1 mL/min).

5.1.17. 5-OMe-uridine-phosphono-methylene-sulfonate triethylammonium salt, 22, was synthesized according to literature,[42] and obtained in a 40% yield (50 mg) after LC separation. ^{1}H NMR (D_2O, 600 MHz): δ 7.33 (s, 1H, H-5), 5.97 (d, J = 6.0 Hz, 1H, H-1'), 4.29-4.36 (m, 2H, H-2',H-3'), 4.17-4.19 (m, 1H, H-4'), 4.11-4.12 (m, 2H, H-5', H-5"), 3.69 (s, 3H, 5-OMe), 3.36 (d, J = 16.4 Hz, 2H, PCH_2S) ppm. ^{31}P NMR (D_2O, 162 MHz): δ 11.02 (s, 1P) ppm. ^{13}C NMR (D_2O, 150 MHz): δ 163.8, 152.4, 138.5, 121.1, 89.3, 85.4 (d, J = 7.8 Hz, C-4'), 74.6, 71.6, 65.9 (d, J = 5.1 Hz, C-5'), 49.6 (d, J 130.1 = Hz, PCH_2S) ppm. HR MALDI (negative): calcd for $C_{11}H_{16}N_2O_{12}PS$, 431.0156; found, 431.0170. Purity data was obtained on an analytical column: t_R 5.32 min (99.5% purity) using solvent system I (98:2 TEAA/CH_3CN over 15 min, 1 mL/min); t_R 3.16 min (99.4% purity) using solvent system II (99.5:0.5 PBS/CH_3CN over 10 min, 1 mL/min).

5.1.18. N3-Me-uridine-5'-α,β-methylene-diphosphonate sodium salt, 23, was obtained from **41** (100 mg, 0.22 mmol) in a 77% yield (75 mg) after HPLC separation. Final separation was achieved by applying a linear gradient of TEAA/CH_3CN 94:6 to 93:7 in 10 min (4 mL/min): t_R 5.5 min. Purity data was obtained on an analytical column: t_R 3.03 min (99.9% purity) using solvent system I (96:4 to 90:10 TEAA/CH_3CN over 10 min, 1 mL/min); t_R 1.90 min (100% purity) using solvent system II (99:1 to 93:17 of 0.01M KH_2PO_4 (pH=4.6)/ CH_3CN over 10 min, 1 mL/min). ^{1}H NMR (D_2O, 600 MHz): δ 7.99 (d, J = 8.4 Hz, 1H, H-6), 6.01 (d, J = 8.4 Hz, 1H, H-5), 5.96 (d, J = 3.6 Hz, 1H, H-1'), 4.35-4.40 (m, 2H, H-2', H-3'), 4.23-4.28 (m, 1H, H-4'), 4.12-4.21 (m, 2H, H-5', H-5"), 3.28 (s, 3H, N-CH_3), 2.12 (t, J = 21.0 Hz, 2H, PCH_2P) ppm. ^{31}P NMR (D_2O, 162 MHz): δ 20.66 (d, J = 9.1 Hz, P_α), 14.03 (d, J = 9.1 Hz, P_β) ppm. ^{13}C NMR (D_2O, 150 MHz): δ

165.5, 152.1, 139.5, 101.6, 89.6, 83.0 (d, J = 8.0 Hz, C-4'), 73.8, 69.0, 62.8 (d, J = 4.8 Hz, C-5'), 27.8 (t, J = 124.1 Hz, PCH_2P), 27.6 ppm. HRMS (ESI): calcd for $C_{11}H_{17}N_2O_{11}P_2$, 415.0312 found, 415.0311.

5.2. Evaluation of the resistance of analogues 19-22 to hydrolysis by hNPP1/3

100 µM analogues **19-22** was incubated with hNPP1 and hNPP3 at 37 °C in 487 µl of incubation buffer (1 mM $CaCl_2$, 200 mM NaCl, 10 mM KCl and 100 mM Tris, pH 8.5). After 2 h for hNPP1 and 3 h for hNPP3 the reaction was quenched by the addition of 350 µl of 1M perchloric acid. The samples were centrifuged for 1 min at 10,000 g and subsequently neutralized with 2M KOH until pH 7 and then centrifuged for 1 min at 10,000 g. Substance hydrolysis was analyzed by HPLC (on a Gemini analytical column (5u, C-18, 110A; 150 × 4.60 mm) using isocratic elution with TEAA (pH 7)/CH_3CN, at a flow rate of 1 mL/min). The percentage of degradation was calculated from the area under the curve of the nucleoside monophosphate peak, after subtraction of the control.

5.3. Evaluation of the inhibition of NPP1/3 mediated hydrolysis of UDP by analogues 21-22

Evaluation of analogues **21** and **22** on human NPP1/3 activity was carried out with UDP as the substrate. 21.44 µg of hNPP1 or 53.17µg of hNPP3 extract, was added to 0.38 or 0.37 mL, respectively, of incubation buffer (1 mM $CaCl_2$, 140 mM NaCl, 5 mM KCl and 50 mM Tris, pH 8.5) and pre-incubated at 37 ^{0}C for 3 min. The reaction was initiated by the addition of the analogues and UDP both at 100 µM final concentration. The reaction was stopped after 2 h, by adding 0.350 mL ice-cold 1 M perchloric acid and neutralized with 130 µL 2 M KOH in 4 ^{0}C and centrifuged for 1 min at 10,000 g. The reaction mixture was filtered and freeze-dried. The samples were analysed as described in before. Reference samples were prepared without the addition of the analogues so the native decomposition of UDP by the enzymes could be determined. The inhibition of UDP hydrolysis was determined by comparing these samples with the samples containing both UDP and the analogues.

5.4. Cytosolic [Ca^{2+}] measurement

132 1N1 cells (10×10^6 cells grown in 100-mm^2 dishes) were detached by a brief trypsin/EDTA treatment, re-suspended in complete culture medium, and washed by centrifugation for 3 min at 100 × *g* before being incubated with 1 µM Fluo-4/AM in 4.5 ml HBSS with Ca^{2+} and Mg^{2+} (Wisent, St-Bruno, QC) for 25 min at 37 °C. After a wash by centrifugation, cells were re-suspended in HBSS containing Ca^{2+} and Mg^{2+} and incubated for 25 min at 37°C to ensure complete hydrolysis of the Fluo-4/AM. Cells were then centrifuged again and re-suspended in 16 ml of HBSS with Ca^{2+} and Mg^{2+} and 2 ml of cells suspension was gently stirred in a quartz cuvette while $[Ca^{2+}]_i$ was monitored on a RF-5301 PC Shimadzu spectrofluorometer (Man-Tech, Guelph, ON) with excitation and emission wavelengths of 488 and 520 nm, respectively. Tested compounds were then added at the indicated concentrations to evaluate their agonist potential. For the determination of the antagonist potential, UDP analogues were added two minutes prior to the 1 µM UDP stimulation. Change in intracellular Fluo-4 fluorescence intensity (F) was acquired using the Panorama fluorescence 1.1 software. At the end of each recording, maximal (F_{max}) and minimal (F_{min}) fluorescence were determined by adding successively 0.1% Triton X-100 and 50 mM EDTA to cell suspensions. The following equation was used to relate the fluorescence intensity to Ca^{2+} levels: $[Ca^{2+}] = Kd(F - F_{min})/(F_{max} - F)$. Kd is the Ca^{2+} dissociation constant of Fluo-4 (345 nM).[52]

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Supplementary Material

Supplementary data associated with this article can be found, in the online version, at.