



Published in final edited form as:

J Med Chem. 2010 June 10; 53(11): 4488–4501. doi:10.1021/jm100287t.

Pyrimidine Ribonucleotides with Enhanced Selectivity as P2Y₆ Receptor Agonists: Novel 4-Alkyloxyimino, (S)-Methanocarba, and 5'-Triphosphate γ -Ester Modifications^a

Hiroshi Maruoka¹, Matthew O. Barrett², Hyojin Ko^{1,†}, Dilip K. Tosh¹, Artem Melman^{1,§}, Lauren E. Burianek², Ramachandran Balasubramanian¹, Barkin Berk^{3,+}, Stefano Costanzi³, T. Kendall Harden², and Kenneth A. Jacobson^{*,1}

¹Molecular Recognition Section, National Institutes of Diabetes and Digestive and Kidney Diseases, NIH, Bethesda, MD.

²Department of Pharmacology, University of North Carolina, School of Medicine, Chapel Hill, NC.

³Laboratory of Biological Modeling, National Institutes of Diabetes and Digestive and Kidney Diseases, NIH, Bethesda, MD.

Abstract

The P2Y₆ receptor is a cytoprotective G protein-coupled receptor (GPCR) activated by UDP (EC₅₀, 0.30 μ M). We compared and combined modifications to enhance P2Y₆ receptor agonist selectivity, including ribose ring constraint, 5-iodo and 4-alkyloxyimino modifications, and phosphate modifications such as α,β -methylene and extension of the terminal phosphate group into γ -esters of UTP analogues. The conformationally constrained (S)-methanocarba UDP is a full agonist (EC₅₀ 0.042 μ M). 4-Methoxyimino modification of pyrimidine enhanced P2Y₆, preserved P2Y₂ and P2Y₄, and abolished P2Y₁₄ receptor potency, in the appropriate nucleotide. *N*⁴-Benzyloxy-CDP (**15**, MRS2964) and *N*⁴-methoxy-Cp₃U (**23**, MRS2957) were potent, selective P2Y₆ receptor agonists (EC₅₀ 0.026 μ M and 0.012 μ M, respectively). A hydrophobic binding region near the nucleobase was explored with receptor modeling and docking. UTP- γ -aryl and cycloalkyl phosphoesters displayed only intermediate P2Y₆ receptor potency, but had enhanced stability in acid and cell membranes. UTP-glucose was inactive, but its (S)-methanocarba analogue and *N*⁴-methoxy-cytidine 5'-triphospho- γ -[1]glucose were active (EC₅₀ of 2.47 μ M and 0.18 μ M, respectively). Thus, the potency, selectivity, and stability of pyrimidine nucleotides as P2Y₆ receptor agonists may be enhanced by modest structural changes.

Keywords

G protein-coupled receptor; nucleotides; pyrimidines; phospholipase C; dinucleotide; uracil

^aPresented in part at the 238th ACS National Meeting, Aug. 2009, Washington, DC, MEDI187.

^{*}Corresponding author: Laboratory of Bioorganic Chemistry, National Institutes of Diabetes and Digestive and Kidney Diseases, NIH, Bldg. 8A, Rm.B1A-19, Bethesda, MD 20892-0810, USA kajacob@helix.nih.gov. Phone: 301-496-9024. Fax: 301-480-8422..

[†]present address: Gwangju Institute of Science and Technology 216 Cheomdan-gwagi-ro, Buk-gu, Gwangju 500-712, Republic of Korea.

[§]present address: Clarkson Univ., Potsdam, NY.

⁺Permanent address: Yeditepe University, Istanbul, Turkey.

Supporting Information Available. Time course of decomposition/hydrolysis of selected pyrimidine nucleotide derivatives in acidic solution and in the presence of cell membranes and stick representation of Fig. 2A. This material is available free of charge via the Internet at <http://pubs.acs.org>.

Introduction

The P2Y receptors are G protein-coupled receptors (GPCRs) that respond to naturally-occurring extracellular nucleotides including ATP, ADP, UTP, UDP, and UDP-glucose.¹ Five of the eight subtypes, P2Y₁, P2Y₂, P2Y₄, P2Y₆, and P2Y₁₁ receptors, are preferentially coupled through Gq to activate phospholipase C β (PLC- β) and represent a distinct structural subfamily.² The remaining P2Y₁₂, P2Y₁₃, and P2Y₁₄ receptors are coupled to inhibition of adenylate cyclase. Activation of the P2Y₆ receptor by its native ligand UDP **1** (Chart 1) is associated with vasoconstriction and cytoprotection.³⁻⁶ We reported the effects of P2Y₆ receptor agonists to counteract apoptosis induced by tumor necrosis factor- α in astrocytoma cells stably expressing the human P2Y₆ receptor and in mouse C2C12 skeletal muscle cells and MIN6 pancreatic cells that express an endogenous P2Y₆ receptor.⁴⁻⁶ Furthermore, *in vivo* administration of 5-iodo-UDP (MRS2693) **3**, which is a selective P2Y₆ receptor agonist, induced protection in a model of ischemia in hindleg skeletal muscle.⁶ The dinucleoside triphosphate INS48823 **4** activates the P2Y₆ receptor in bone and increased survival of osteoclasts.⁷ A role for the P2Y₆ receptor in inducing phagocytosis in microglial cells was recently discovered, suggesting the possible application of P2Y₆ receptor ligands for the treatment of neurodegenerative conditions.⁸ The P2Y₆ receptor is upregulated when neurons are damaged and in intestinal inflammation.^{8,9} UDP applied topically to the cornea reduced intraocular pressure, suggesting the use of P2Y₆ receptor agonists for the treatment of ocular hypertension and glaucoma.¹⁰ Studies of mice lacking the P2Y₆ receptor recently demonstrated a defective response to UDP in macrophages, endothelial cells, and vascular smooth muscle cells.¹¹ Thus, pharmacological modulation of the P2Y₆ receptor is of increasing interest for future therapeutics.¹²

Potent P2Y₆ receptor agonists, such as 3-phenacyl-UDP **5** with an EC₅₀ value of 70 nM, have been reported.¹³⁻¹⁶ Recently, we reported that UDP also activates the P2Y₁₄ receptor, and therefore, agonists that delineate between these two subtypes are needed.^{17,18} We have adopted a structure-based approach to developing P2Y₆ receptor agonist ligands with improved potency, selectivity, and stability. In the absence of an X-ray structure, rhodopsin-based homology modeling has predicted a putative binding site for nucleotides in the human P2Y₆ receptor.¹³ The receptor recognition of the 5'-diphosphate group is associated with three cationic residues in the transmembrane region, which serve as counterions and which are conserved in charge across the five Gq-coupled P2Y receptors.² A Monte Carlo analysis of conformations of receptor-docked UDP molecules, followed by molecular dynamics of the receptor-ligand complex in a lipid bilayer model, has predicted that the South (S) conformation of the ribose ring is preferred at the P2Y₆ receptor.¹³ This is the only Gq-coupled P2Y subtype at which the (S) conformation of the ribose or a ribose-like moiety of nucleotide ligands appears to be favored, and our comparison of the weak agonist 2'-deoxy-UDP **2** and the corresponding 2'-deoxy (S)-methanocarpa analogue **6**^{13,14} supported this idea.

We recently reported the first synthetic route to the enantiomerically pure (S)-methanocarba-uridine,¹⁹ and in this study we examined the hypothesis that the (S) conformation is preferred at the P2Y₆ receptor in the more potent 2'-hydroxynucleotide series. Modifications of the pyrimidine ring of UDP, including 5-iodo and 4-alkyloxyimino, and of the phosphate moiety, including α,β -methylene, and extension of the terminal phosphate group, were compared and combined in an effort to further enhance selectivity for the P2Y₆ receptor.^{14,16} Chemical and metabolic instability of mononucleotides is well-documented.²⁰ Mononucleotides are degraded enzymatically by ectonucleotidases into the lower nucleotides and eventually into the nucleoside. Chemical degradation of nucleotides is also evident at pH ranges less than 4 or greater than 10. The degradation products of nucleotides may interact with multiple P2Y receptors or with adenosine receptors and lead to side effects. Since various dinucleotides, such as diuridine triphosphates, show greater stability than analogues of mononucleotides,^{21,22} we

hypothesized that blockade of the terminal phosphate with an alkyl or aryl group may also increase its stability.

Results

Chemical synthesis

A series of known (**1-6**, **8**, **10**, and **11**) and newly synthesized derivatives of UDP (**7**, **9**) and CDP (**12-16**) was assembled and compared (Tables 1 and 2). Novel analogues of UTP and Up₃U (**18**, **20**, and **22-33**) also were included in this structure activity relationship (SAR) analysis (Table 3). The chemical synthesis of ribonucleoside 5'-di and triphosphate derivatives (Scheme 1) was performed by standard phosphorylation methods using the unprotected nucleoside precursors.^{23,24} Various 5'-diphosphate derivatives were then extended to form dinucleoside triphosphates or other 5'-triphosphate γ -esters (Scheme 2). (S)-Methanocarba nucleotides were synthesized as shown in Scheme 3. The synthesis of several of the pyrimidine nucleotides was reported in previous studies of the P2Y receptor (**3**, **5**, **6**, **8**, **10**, **19**, **21**, and **26**).¹⁴⁻¹⁶

5-Iodouridine **34** was available from commercial sources, while the 4-methoxyimino derivative **36** and (S)-methanocarba-uridine **41** were prepared as described,^{19,25} and compounds **37-39** were prepared by the same procedure as **36**. Compounds **34** and **36** were converted to the α,β -methylene diphosphonate analogues **11** and **16**, respectively, by a carbodiimide condensation. *N*⁴-Alkoxy-cytidines **36-39** were converted to the corresponding 5'-diphosphates **12-15** and *N*⁴-methoxy-cytidine **36** to the 5'-triphosphate **20** using phosphorous oxychloride followed by either phosphate or pyrophosphate.²⁴ Various 5'-diphosphates (**1**, **3**, **5**, **10**, and **12**) were converted to the corresponding 5'-triphosphate γ -esters by condensation with the appropriate monophosphate derivative in DMF.

Nucleotides **18** and **28** derived from (S)-methanocarba-uridine **40** were prepared by similar phosphorylation of the unprotected nucleoside (Scheme 3).²⁴ The 3',5'-cyclic diphosphate **9** was isolated as a side product in the synthesis of **28**. A similar cyclic diphosphate was isolated following phosphorylation of other uridine analogues.¹⁴

Pharmacological evaluation

The nucleotide analogues (Tables 1 – 3) were assayed for capacity to promote activation of phospholipase C (PLC) in 1321N1 human astrocytoma cells stably expressing the human P2Y₆ receptor.²⁶ Receptor selectivity of the novel analogues of CDP and UTP in Tables 2 and 3 was assessed by quantification of PLC activation in 1321N1 cells stably expressing either the P2Y₂ receptor or the P2Y₄ receptor. Previously reported data for several analogues (**2** – **6**, **8**, and **10**) were included for comparison.

The enantiomerically pure (S)-methanocarba-UDP **7** was more potent at the P2Y₆ receptor than either UDP **1** (7-fold) or the previously reported (S)-methanocarba-2'-deoxy-UDP **6** (5-fold). In contrast, the North (N)-methanocarba conformationally locked UDP analogue **8** was previously found to be inactive at the P2Y₆ receptor, confirming a strong conformational preference in P2Y₆ receptor recognition.^{13,14} The (S)-methanocarba-uridine-3',5'-cyclic diphosphate **9** was also inactive at the P2Y₆ receptor.

The carbon-bridged analogue α,β -methylene-UDP **10** was recently reported to be nearly as potent as UDP at the P2Y₆ receptor.¹⁶ Compound **11** contained the 5'- α,β -methylenediphosphonate modification, which would increase the stability of a nucleotide in biological systems, and the 5-iodo modification (as in **3**), both of which were previously noted to preserve potency at the P2Y₆ receptor.¹⁴ This combination of modifications resulted in a molecule **11** that potently activated the P2Y₆ receptor with an EC₅₀ value of 127 nM.

Introduction of the 4-methoxyimino modification of UDP, i.e., *N*⁴-methoxy-CDP **12**, resulted in high potency at the P2Y₆ receptor (EC₅₀ 70 nM), which represents a 4-fold gain of potency over UDP (Table 2, Figure 1A). Compound **12** was 47-fold selective for activation of PLC via the P2Y₆ receptor in comparison to inhibition of cyclic AMP accumulation in HEK-293 cells expressing the Gi-coupled human P2Y₁₄ receptor.¹⁸ The native agonist for P2Y₁₄, UDP-glucose, did not tolerate the 4-methoxyimino substitution.²⁷ However, the 4-methoxyimino modification of the pyrimidine ring in the 5'-triphosphate derivative **20** produced a gain of potency (EC₅₀ 80 nM) of >100-fold over UTP **17** at the P2Y₆ receptor (Table 3). The 4-methoxyimino modification of **20** was also well-tolerated at the P2Y₂ and P2Y₄ receptors, with EC₅₀ values of 50 nM. Thus, compound **20** was a nonselective agonist at these three P2Y receptor subtypes.

In the series of 4-alkoxyimino 5'-diphosphate analogues, the progression from MeO to larger *O*-alkyl substituents **12** – **15**, first decreased then increased the P2Y₆ receptor potency. The sterically bulky *O*-*t*-butyl group of **14** was not well tolerated. However, the *O*-benzyl analogue **15** was the most potent P2Y₆ receptor agonist in the series of 4-imino 5'-diphosphate analogues with 82- and 44-fold selectivity vs. P2Y₂ and P2Y₄ receptors, respectively. Curiously, the *O*-ethyl analogue **13** was potent at P2Y₂ and P2Y₄ receptors and was thus a non-selective hP2Y_{2/4/6} agonist. The potency-enhancing 4-methoxyimino modification was combined with α,β -methylene substitutions in compound **16**. However, there was no enhancement by the 4-methoxyimino modification, as **10** and **16** were equipotent at the P2Y₆ receptor.

Up₃U **21** slightly favored activation of the P2Y₆ receptor, in comparison to the P2Y₂ and P2Y₄ receptors, with an EC₅₀ value of 270 nM.¹⁶ Introduction of a carbon bridge in the corresponding α,β -methylene analogue **22** reduced P2Y₆ receptor potency 5-fold, but with increased selectivity. Given the P2Y₆ receptor potency of extended phosphate derivatives, such as dinucleoside triphosphates related to Up₃U **21** (including **4**), we tested other γ -phosphoesters of UTP for P2Y₆ receptor activity, including alkyl and aryl esters and Up₃-sugar derivatives. The analogue of Up₃U containing a single 4-methoxyimino modification **23** displayed 22-fold enhanced potency at the P2Y₆ receptor with an EC₅₀ value of 12 nM and selectivity versus the P2Y₂ and P2Y₄ receptors of 14- and 66-fold, respectively. The γ -cyclohexyl **25** and γ -phenyl **26** esters of UTP displayed moderate potency at the P2Y₆ receptor, with EC₅₀ values of 6.99 and 2.13 μ M, respectively. However, Up₃-[1]glucose **27** only weakly activated the P2Y₆ receptor, and an EC₅₀ value was not determined. The introduction of the potency enhancing (S)-methanocarpa modification in the corresponding ring-constrained analogue **28** increased potency, as predicted. Nevertheless, only a moderate potency of 2.47 μ M was attained.

Combinations of Up₃-glucose with other known modifications that are tolerated or favored at the P2Y₆ receptor were evaluated. Introduction of the 5-iodo modification in **29** only slightly enhanced potency in comparison to **27**. The corresponding 3-phenacyl **30** and α,β -methylene **31** analogues were only weakly active at the P2Y₆ receptor. However, the P2Y₆ receptor potency of the corresponding *N*⁴-methoxy-cytidine analogue **32** was greatly increased with an EC₅₀ value of 180 nM, which also displayed >100-fold selectivity versus P2Y₂ and P2Y₄ receptors. This finding suggested another analogue, *N*⁴-methoxy-cytidine-5'-triphosphate γ -phenyl ester **33**, but an additive effect on potency was absent, as it weakly activated the P2Y₆ receptor (EC₅₀ 3.49 nM).

Selected nucleotide analogues were also examined for the ability to raise levels of intracellular calcium ions in 1321N1 human astrocytoma cells expressing the human P2Y₆ receptor (Figure 1B). The rank order of potency was **23**, **3** > **21**, **24**, which was similar to the order of potency in PLC activation.

Stability of Nucleotide Derivatives

The chemical and enzymatic instability of nucleotide analogues in biological systems is a major limitation in their use as pharmacological probes. Therefore, to assess their potential for broad use, selected nucleotide derivatives were evaluated for stability during prolonged exposure to two different conditions. The derivatives were incubated at 37°C in aqueous medium at low pH or in the presence of 1321N1 astrocytoma membranes, which are known to contain ectonucleotidases, as a representative mammalian cell of the central nervous system. At regular intervals aliquots were taken for HPLC analysis (Table 4 and Supporting Information). The injection sample was pre-diluted with 5 mM tetrabutylammonium dihydrogenphosphate (TBAP) to allow complete equilibration with the mobile phase, in order to avoid the early HPLC elution at around one minute.

The stability in the presence of cell membranes was **25**, **22** > **12** > **21**, **26** > **17**, **27** > **15**, **23** > **31**. The potent P2Y₆ receptor agonist **12** was more stable than the related *N*⁴-benzyloxy cytidine 5'-diphosphate **15**. The γ -cyclohexyl ester **25** was more stable than either Up₃U **21**, which has a uridyl group at this terminal position, or the γ -phenyl ester **26**. The α,β -methylene analogue of Up₃U **22** was more stable than the parent Up₃U **21** in the presence of cell membranes, which may indicate that a α,β -methylene bridges blocks enzymatic breakdown, as has been observed for other P2 receptor agonists containing phosphonates.^{16,28,29} However, the introduction of a methylene bridge into Up₃U unfortunately also reduced potency at the P2Y₆ receptor 5-fold.

In acidic solution, the CDP derivatives **12** and **15** and the dinucleoside triphosphates were of enhanced chemical stability. Also, the γ -phenyl phosphoester **26** was relatively stable. However, the Up₃U analogue **22** containing a α,β -methylene bridge was significantly less stable than the parent Up₃U **21**. Likewise, the Up₃-[1]glucose analogue **31** containing a α,β -methylene bridge was less stable than the parent Up₃-[1]glucose **27**, which itself was very labile. Since both of the glucose derivatives **27** and **31** were rapidly degraded, the introduction of glucose was not a favored modification.

Molecular Modeling

A docking of the semi-rigid (S)-methanocarba analogue **7** in complex with our rhodopsin-based P2Y₆ receptor model¹³ (Figure 2A) suggested that **7** establishes with the receptor the same putative interactions characteristic of UDP. In particular, three cationic residues located in transmembrane domain 3 (TM3), TM6, and TM7 coordinate the diphosphate of **7**, namely R103(3.29), K259(6.55), and R287(7.39), while the base is coordinated by Y33(1.39) and S291(7.43), located in TM1 and TM7, respectively – see Experimental Section for residue indexing. The higher potency of **7** compared to UDP is induced by the rigid methanocarba scaffold, which constrains the ribose in the bioactive conformation, likely with a consequent gain in the entropic component of the binding affinity. A hydrogen bond is possibly formed between the ribose 2'-hydroxy group and the uracil 2-oxo group of **7** (Figure 2A). Furthermore, as shown in Figure 2A, the molecular dynamics simulation predicted the access of several water molecules to the receptor binding pocket from the extracellular milieu. These molecules surround the portion of the ligand exposed to the extracellular opening of the binding site, including the 5 position of the uracil ring. For entropic reasons, substituents capable of displacing these water molecules from the binding cavity to the bulk of the solvent are expected to enhance the affinity of the compounds. The increase in affinity noted upon the introduction of the iodo-substituent at the 5-position of the uracil ring (see compounds **1** and **3**) is consistent with this reasoning and supports the orientation of this moiety of the ligand towards the extracellular opening of the helical bundle of the receptor as suggested by our models.

A molecular model of *N*⁴-methoxy-CDP **12** docked in the P2Y₆ receptor showed similar coordination to that of UDP, i.e., S291(7.43) coordinates the base, and four cationic residues

coordinate the diphosphate moiety (Figure 2B). The methyl group at the 4 position seems to fit well in a sterically restricted hydrophobic pocket, which consisted of V32(1.38), Y33(1.39), I83(2.61) and P288(7.40). These hydrophobic interactions may be the reason for the increased activity of **12** (Table 2).

Discussion

The present set of analogues provides new insights into the structural requirements for activation of the P2Y₆ receptor at each of the three regions modified: ribose, nucleobase, and terminal phosphate. In addition to studying the SAR of pyrimidine nucleotides in receptor activation, we have also confirmed that blockade of the terminal phosphate with an alkyl or aryl group or other groups may increase its stability.

Using sterically constrained carbocyclics, we have extended to the ribo (2'-hydroxy) series the prediction that the (S) conformation of a ribose-like moiety is preferred for recognition at the human P2Y₆ receptor, consistent with previous activity of the 2'-deoxy analogues. The first synthesis of enantiomerically pure (S)-methanocarpa-uridine enabled the preparation of nucleotide derivatives in the 2'-hydroxy series (**7**, **18**, and **28**). These (S)-methanocarpa nucleotides exhibited enhanced potency over the corresponding natural ribose analogue in each case.

In homology-based modeling of the P2Y₆ receptor and ligand docking, an intramolecular hydrogen bond was detected between the 2'-hydroxy group and the uracil 2-oxo group of the conformationally constrained, potent analogue of UDP **7**. We suggest that this interaction might further restrain **7** in its bioactive conformation, and thus, contribute to the greater potency of **7** over the corresponding 2'-deoxy analogue **6**.

One of the striking findings of this study was that the 4-methoxyimino and 4-benzyloxyimino modifications of the pyrimidine ring in the appropriate nucleotide derivative served to enhance potency at the P2Y₆ receptor. This is one of the few modifications of the uracil ring that is tolerated for activation of the P2Y₆ receptor. CDP itself is nearly inactive at the human P2Y₆ receptor, and its EC₅₀ at the P2Y₁₄ receptor is >10 μM.^{17,30} The 4-methoxyimino 5'-diphosphate analogue **12** displayed a selectivity of 51- and 47-fold versus the P2Y₂ and P2Y₁₄ receptors, respectively, and a substituent as large as *O*-benzyl **15** was tolerated. In the 5'-triphosphate analogue **20**, the 4-methoxyimino modification also preserved potency at P2Y₂ and P2Y₄ receptors and abolished potency at the P2Y₁₄ receptor.²⁷ In Schemes 1 and 2, the imino group of *N*⁴-methoxycytidine is depicted as the imino-oxo tautomer, based on an early study of the poly C derivative after treatment with methoxyamine.³¹ This predominant form, which resembles the major lactam (keto) tautomer of UDP, was used in the molecular modeling. However, the amino tautomeric form, which is found in native cytosine in DNA, might also be present. The 4-methoxyimino group in a γ-[1]glucose triphosphate ester **32** also enhanced potency. However, the γ-[1]glucose triphosphate in another analogue **27** suffered from low stability, and therefore, **32** is not a likely candidate for generation of a potent and stable agonist.

The introduction of a phenyl or cyclohexyl ring at the terminal phosphate of UTP in compounds **25** and **26**, respectively, enhanced stability in both acidic solution and in the presence of membranes to at least the same extent as Up₃U. Both **25** and **26** containing terminal hydrophobic groups showed higher potency than UTP **17** at the P2Y₆ receptor, and the more hydrophilic Up₃-[1]glucose **27** was nearly inactive. Thus, the nature of the terminal group can greatly affect agonist activity. Since Up₄-[1]glucose is a potent agonist of the P2Y₂ receptor, and Up₂-[1]glucose is a native agonist of the P2Y₁₄ receptor, we speculated that the intermediate triphosphate homologue **27** might be an effective agonist of the P2Y₆ receptor.

However, this receptor is not as tolerant of a terminal glucose moiety as are the P2Y₂ and P2Y₁₄ receptors. Nevertheless, the introduction of a rigid (S)-methanocarba ring system in **18** and **28** resulted in agonists that exceeded the P2Y₆ potency of both UTP and Up₃-[1]glucose.

The combination of two modifications in the present set of compounds either produced an additive effect on P2Y₆ receptor potency, as in γ -[1]glucose triphosphate ester **32**, or failed to increase potency, as in the γ -phenyl ester **33** and the α,β -methylene analogue **16**. The inconsistent effects of combination of various structural modifications on receptor recognition suggested multiple modes of binding of these nucleotide series.

The homology modeling and docking, interpreted in light of the biological activity, indicate conformational cross-talk between the nucleobase and the terminal phosphate substituent. Although the molecular model of *N*⁴-methoxy-CDP **12** docked in the P2Y₆ receptor showed similar coordination to that of UDP, Up₃-O-phenyl **26** docked a different mode (not shown). The base of **26** did not coordinate with Y33(1.39) and S291(7.43), because the position of the base had shifted within the binding site. Also, the ribose ring showed a planar rather than a (S) conformation. The terminal phenyl group of **26** seemed to fit in a hydrophobic region in the model, which might be the reason for the dislocation of the nucleobase, i.e. by withdrawal of the entire molecule. On the other hand, the docked model of *N*⁴-methoxy-Cp₃-O-phenyl **33** in the receptor showed a position and coordination similar to *N*⁴-methoxy-CDP **12**, but the phenyl ring did not interact with the distal hydrophobic region occupied by **26**. The strong interaction of the methyl group of **33** in a hydrophobic pocket near the nucleobase may cause the phenyl group to exit its large hydrophobic binding region. This interpretation is consistent with the lack of enhanced activity of compound **33** in comparison to Up₃-O-phenyl **26**, despite the presence of methoxyimino moiety at the 4 position.

The modeling and receptor docking also predicted that various water molecules are located in proximity of the 5 position of the ligand. The enhanced affinity of the 5-iodo-substituted analogues could be a result of the fact that the halogen displaced some of the structured water molecules present in the binding site, with a consequent entropic gain.

Conclusions

We have identified novel nucleotides, such as *N*⁴-alkoxy-cytidine derivatives, that are more potent, selective, and potentially more stable *in vivo* than both the native agonist and previously reported synthetic agonists of the P2Y₆ receptor. Thus, *N*⁴-methoxy-cytidine nucleotides **12**, **15**, and **23** were relatively potent and selective agonists, and moreover, compound **23** displayed favorable stability. Thus, in future SAR studies (S)-methanocarba-(α,β -CH₂)*N*⁴-OMe-CDP could be a suitable combination to maximize the potency, while retaining selectivity and stability.

The P2Y₆ receptor is implicated in neurodegeneration, osteoporosis, ischemic effects in skeletal muscle, and possibly diabetes.^{4,32,33} Therefore, new potent and selective ligands are of interest in the search for new treatments for these conditions. Agonists of the P2Y₆ receptor are predicted to produce beneficial responses in for example muscle wasting and possibly neurodegeneration, and several compounds such as **23** from the present SAR analysis could provide useful pharmacological tools.

Experimental Section

Synthesis

¹H NMR spectra were obtained with a Varian Gemini 300 or Varian Mercury 400 spectrometer using D₂O, CDCl₃ or DMSO-*d*₆ as a solvent. The chemical shifts are expressed as relative

ppm from HOD (4.80 ppm). ^{31}P NMR spectra were recorded at room temperature (rt) by use of Varian XL 300 (121.42 MHz) or Varian Mercury 400 (162.10 MHz) spectrometers; orthophosphoric acid (85%) was used as an external standard. In several cases the signal of the terminal phosphate moiety was not visible due to high dilution.

Purity of compounds was checked using a Hewlett–Packard 1100 HPLC equipped with a Zorbax Eclipse 5 μm XDB-C18 analytical column (250 \times 4.6 mm; Agilent Technologies Inc, Palo Alto, CA). Mobile phase: linear gradient solvent system: 5 mM TBAP- CH_3CN from 80:20 to 40:60 in 20 min; the flow rate was 1 mL/min (System A) or Zorbax SB-Aq 5 μm analytical column (50 \times 4.6 mm; Agilent Technologies Inc, Palo Alto, CA). Mobile phase: linear gradient solvent system: 5 mM TBAP- CH_3CN from 80:20 to 40:60 in 13 min; the flow rate was 0.5 mL/min (System B). Peaks were detected by UV absorption with a diode array detector at 254, 275, and 280 nm. All derivatives tested for biological activity showed >99% purity by HPLC analysis (detection at 254 nm).

High-resolution mass measurements were performed on Micromass/Waters LCT Premier Electrospray Time of Flight (TOF) mass spectrometer coupled with a Waters HPLC system, unless noted. Purification of the nucleotide analogues for biological testing was carried out on (diethylamino)ethyl (DEAE)-A25 Sephadex columns with a linear gradient (0.01–0.5 M) of 0.5 M ammonium bicarbonate as the mobile phase. Compounds **7** were purified by Sephadex alone (and isolated in the ammonium salt form), and all other compounds were purified by HPLC with a Luna 5 μm RP-C18(2) semipreparative column (250 \times 10.0 mm; Phenomenex, Torrance, CA) and using the following conditions: flow rate of 2 mL/min; 10 mM triethylammonium acetate (TEAA)- CH_3CN from 100:0 to 95:5 (or up to 100:0 to 75:25) in 20–40 min (and isolated in the triethylammonium salt form).

All reagents were of analytical grade. 3-Phenacyl-UDP **5** was purchased from Tocris. 5-Iodouridine **34** was purchased from Berry Associates (Ann Arbor, MI). Compound **36** were synthesized as reported.^{19,25} Other reagents and solvents were purchased from Sigma-Aldrich (St. Louis, MO).

(S)-Methanocarpa-uridine-5'-diphosphate triethylammonium salt (**7**)

A solution of the (S)-methanocarpa-uridine **40** (1-[(1*S*,2*S*,5*S*,3*R*,4*R*)-2,3-dihydroxy-4-(hydroxymethyl) bicyclo[3.1.0]hexyl]-1,3-dihydropyrimidine-2,4-dione, 10 mg, 0.04 mmol, ref. 13) and Proton Sponge (17 mg, 0.08 mmol) in trimethyl phosphate (1 mL) was stirred for 10 min at 0 °C. Then phosphorous oxychloride (0.008 mL, 0.08 mmol) was added dropwise, and the reaction mixture was stirred for 2 h at 0 °C. A mixture of tributylamine (0.8 mL, 0.35 mmol) and a solution 0.35 M of the bis(tributylammonium) salt of phosphoric acid in DMF (0.7 mL) was added at once. After 10 min, 0.2 M triethylammonium bicarbonate solution (1.5 mL) was added, and the clear solution was stirred at rt for 1 h. After removal of solvents, the residue was purified using the same method as the general procedure using Sephadex-DEAE A-25 resin and HPLC to get compound **7** (1.3 mg, 45%) as a white solid. ^1H NMR (D_2O) δ 7.79 (d, J = 7.8 Hz, 1H), 5.86 (d, J = 8.1 Hz, 1H), 4.69 (m, 1H), 4.15 (m, 3H), 2.39 (t, J = 6.3 Hz, 1H), 1.87 (m, 1H), 1.71 (t, J = 6.0 Hz, 1H), 1.30 (ddd, J = 2.1, 6.3, 8.4 Hz, 1H); ^{31}P NMR (D_2O) δ -5.87 (d, J = 22.0 Hz), -10.10 (d, J = 23.2 Hz); HRMS-EI found 413.0170 ($\text{M} - \text{H}^+$). $\text{C}_{11}\text{H}_{15}\text{N}_2\text{O}_{11}\text{P}_2$ requires 413.01511; purity > 99% by HPLC (System A: 11.1 min).

5-Iodo-uridine-5'- α,β -methylenediphosphate triethylammonium salt (**11**)

Methylene diphosphonic acid (14 mg, 0.08 mmol) and DCC (25 mg, 0.121 mmol) were added to a solution of 5-iodo-uridine **34** (15 mg, 0.040 mmol) in DMF (1.0 mL). The mixture was stirred for 2 d at rt under a nitrogen atmosphere. Solvent was evaporated and the residue was purified on Sephadex-DEAE A-25 resin and HPLC using the same method as the general

procedure, to give the compound **11** (2 mg, 11%) as a white powder. $^1\text{H NMR}$ (D_2O) δ 8.26 (s, 1H), 5.98 (d, $J = 6.1$ Hz, 1H), 4.39-4.46 (m, 2H), 4.29-4.34 (m, 1H), 4.19-4.24 (m, 2H), 2.23 (t, $J = 19.1$ Hz, 2H); $^{31}\text{P NMR}$ (D_2O) δ 19.02 (d, $J = 8.34$ Hz), 15.04 (d, $J = 8.34$ Hz); HRMS-EI found 526.9108 (M-H), $\text{C}_{10}\text{H}_{15}\text{N}_2\text{O}_{11}\text{P}_2$ requires 527.9109.

***N*⁴-Methoxycytidine-5'-diphosphate ammonium salt (12)**

A solution of the *N*⁴-methoxycytidine **36** (10 mg, 0.037 mmol) and Proton Sponge (17 mg, 0.08 mmol) in trimethyl phosphate (1 mL) was stirred for 10 min at 0 °C. Then, phosphorous oxychloride (0.008 mL, 0.08 mmol) was added dropwise, and the reaction mixture was stirred for 2 h at 0 °C. A mixture of tributylamine (0.8 mL, 0.35 mmol) and a solution 0.35 M of the bis(tributylammonium) salt of phosphoric acid in DMF (0.7 mL) was added at once. After 10 min 0.2 M triethylammonium bicarbonate solution (1.5 mL) was added, and the clear solution was stirred at rt for 1 h. After removal of solvents, the residue was purified using the same method as the general procedure using Sephadex-DEAE A-25 resin and HPLC to get compound **12** (4.4 mg, 25%) as a white solid. $^1\text{H NMR}$ (D_2O) δ 7.23 (d, $J = 8.1$ Hz, 1H), 5.92 (d, $J = 5.7$ Hz, 1H), 5.79 (d, $J = 7.8$ Hz, 1H), 4.35 (m, 2H), 4.21 (m, 1H), 4.15 (m, 2H), 3.78 (s, 3H); $^{31}\text{P NMR}$ (D_2O) δ -7.74 (d, $J = 21.4$ Hz), -10.6 (d, $J = 21.4$ Hz); HRMS-EI found 432.0207 (M - H⁺). $\text{C}_{10}\text{H}_{16}\text{N}_3\text{O}_{12}\text{P}_2$ requires 432.0209; purity > 98% by HPLC (System A: 13.6 min).

***N*⁴-Ethoxycytidine-5'-diphosphate ammonium salt (13)**

A solution of the cytidine **35** (200 mg, 0.82 mmol) and *O*-ethylhydroxylamine hydrochloride (160 mg, 1.64 mmol) in pyridine (2 mL) was stirred overnight at 100 °C. The reaction mixture was concentrated, and the residue was purified on a silica gel column ($\text{CHCl}_3/\text{MeOH} = 10/1$) to obtain compound **37** (230 mg, 97%) as a yellow amorphous solid. $^1\text{H NMR}$ (acetone- d_6) δ 7.32 (d, $J = 8.2$ Hz, 1H), 5.86 (d, $J = 5.1$ Hz, 1H), 5.62 (d, $J = 8.2$ Hz, 1H), 4.27-4.21 (m, 2H), 4.07-3.95 (m, 3H), 3.79 (dd, $J = 12.0, 2.8$ Hz, 1H), 3.73 (dd, $J = 12.0, 2.8$ Hz, 1H), 1.22 (t, $J = 7.0$ Hz, 3H); HRMS-EI found 288.1196 (M + H⁺). $\text{C}_{11}\text{H}_{18}\text{N}_3\text{O}_6$ requires 288.1185

A solution of the *N*⁴-ethoxycytidine **37** (85 mg, 0.30 mmol) and Proton Sponge (95 mg, 0.44 mmol) in trimethyl phosphate (2.5 mL) was stirred for 10 min at 0 °C. Then, phosphorous oxychloride (0.054 mL, 0.59 mmol) was added dropwise, and the reaction mixture was stirred for 2 h at 0 °C. A mixture of tributylamine (0.56 mL, 2.4 mmol) and a 0.35 M solution of the bis(tributylammonium) salt of phosphoric acid in DMF (6.6 mL) was added at once. After 10 min 0.2 M triethylammonium bicarbonate solution (6.9 mL) was added, and the clear solution was stirred at rt for 1 h. After removal of solvents, the residue was purified using the same method as the general procedure using Sephadex-DEAE A-25 resin and HPLC to obtain compound **13** (21.3 mg, 14%) as a white amorphous solid. $^1\text{H NMR}$ (D_2O) δ 7.24 (d, $J = 8.2$ Hz, 1H), 5.96 (d, $J = 5.6$ Hz, 1H), 5.82 (d, $J = 8.2$ Hz, 1H), 4.42-4.33 (m, 2H), 4.26-4.14 (m, 3H), 4.05 (dd, $J = 14.1, 7.0$ Hz, 2H); HRMS-EI found 446.0366 (M - H⁺). $\text{C}_{10}\text{H}_{16}\text{N}_3\text{O}_{12}\text{P}_2$ requires 446.0373; purity > 98% by HPLC (System B: 7.54 min).

***N*⁴-*t*-Butyloxycytidine-5'-diphosphate ammonium salt (14)**

A solution of the cytidine **35** (200 mg, 0.82 mmol) and *O-t*-butylhydroxylamine hydrochloride (207 mg, 1.64 mmol) in pyridine (2 mL) was stirred overnight at 100 °C. The reaction mixture was concentrated, and the residue was purified on a silica gel column ($\text{CHCl}_3/\text{MeOH} = 10/1$) to obtain compound **38** (143 mg, 55%) as a white solid. $^1\text{H NMR}$ (acetone- d_6) δ 7.18 (d, $J = 8.2$ Hz, 1H), 5.85 (d, $J = 5.7$ Hz, 1H), 5.56 (d, $J = 8.2$ Hz, 1H), 4.27-4.17 (m, 2H), 3.95 (dd, 1H, $J = 6.1, 3.0$ Hz), 3.80-3.68 (m, 2H), 1.26 (s, 9H); HRMS-EI found 316.1509 (M + H⁺). $\text{C}_{13}\text{H}_{22}\text{N}_3\text{O}_6$ requires 316.1516.

A solution of the *N*⁴-*t*-butyloxycytidine **38** (39 mg, 0.12 mmol) and Proton Sponge (40 mg, 0.19 mmol) in trimethyl phosphate (1.2 mL) was stirred for 10 min at 0 °C. Then, phosphorous oxychloride (0.023 mL, 0.25 mmol) was added dropwise, and the reaction mixture was stirred for 2 h at 0 °C. A mixture of tributylamine (0.24 mL, 1.0 mmol) and a 0.35 M solution of the bis(tributylammonium) salt of phosphoric acid in DMF (2.8 mL) was added at once. After 10 min 0.2 M triethylammonium bicarbonate solution (3 mL) was added, and the clear solution was stirred at rt for 1 h. After removal of solvents, the residue was purified using the same method as the general procedure using Sephadex-DEAE A-25 resin and HPLC to obtain compound **14** (12.5mg, 21%) as a white amorphous solid. ¹H NMR (D₂O) δ 7.12 (d, *J* = 8.3 Hz, 1H), 5.84 (d, *J* = 6.0 Hz, 1H), 5.74 (d, *J* = 8.3 Hz, 1H), 4.32-4.23 (m, 2H), 4.13-4.03 (m, 2H), 3.08 (dd, *J* = 14.7, 7.4 Hz, 12H), 1.18 (s, 9H), 1.16 (t, *J* = 7.3 Hz, 18H); HRMS-EI found 474.0679 (M - H⁺)⁻. C₁₃H₂₂N₃O₁₂P₂ requires 474.0669; purity > 98% by HPLC (System B: 8.06 min).

***N*⁴-Benzyloxycytidine-5'-diphosphate ammonium salt (15)**

A solution of the cytidine **35** (200 mg, 0.82 mmol) and *O*-benzylhydroxylamine hydrochloride (263 mg, 1.64 mmol) in pyridine (2 mL) was stirred overnight at 100 °C. The reaction mixture was concentrated, and the residue was purified on a silica gel column (CHCl₃/MeOH = 10/1) to obtain compound **39** (288 mg, 100%) as pale yellow amorphous. ¹H NMR (acetone-d₆) δ 7.40-7.26 (m, 5H), 7.23 (d, *J* = 8.2 Hz, 1H), 5.85 (d, *J* = 5.6 Hz, 1H), 5.52 (d, *J* = 8.2 Hz, 1H), 5.00 (s, 2H), 4.27-4.18 (m, 2H), 3.96 (dd, *J* = 6.1, 3.0 Hz, 1H), 3.82-3.69 (m, 2H); HRMS-EI found 350.1352 (M + H⁺)⁺. C₁₆H₁₉N₃O₆ requires 350.1356.

A solution of the *N*⁴-benzyloxycytidine **39** (80 mg, 0.23 mmol) and Proton Sponge (74 mg, 0.34 mmol) in trimethyl phosphate (2.4 mL) was stirred for 10 min at 0 °C. Then, phosphorous oxychloride (0.042 mL, 0.46 mmol) was added dropwise, and the reaction mixture was stirred for 2 h at 0 °C. A mixture of tributylamine (0.43 mL, 1.83 mmol) and a 0.35 M solution of the bis(tributylammonium) salt of phosphoric acid in DMF (5.1 mL) was added at once. After 10 min 0.2 M triethylammonium bicarbonate solution (1.5 mL) was added, and the clear solution was stirred at rt for 1 h. After removal of solvents, the residue was purified using the same method as the general procedure using Sephadex-DEAE A-25 resin and HPLC to obtain compound **15** (54 mg, 47%) as a white amorphous solid. ¹H NMR (D₂O) δ 7.50-7.39 (m, 5H), 7.26 (d, *J* = 8.4 Hz, 1H), 5.95 (d, *J* = 5.9 Hz, 1H), 5.77 (d, *J* = 7.8 Hz, 1H), 5.07 (s, 2H), 4.46-4.43 (m, 1H), 4.37 (t, *J* = 5.7 Hz, 1H), 4.27-4.11 (m, 3H); ³¹P NMR (D₂O) δ -7.33 (s), -11.04 (s), -11.18(s); HRMS-EI found 508.0522 (M - H⁺)⁻. C₁₆H₂₀N₃O₁₂P₂ requires 508.0507; purity > 98% by HPLC (System B: 9.45 min).

***N*⁴-Methoxycytidine -5'-α,β-methylenediphosphate triethylammonium salt (16)**

Methylene diphosphonic acid (13 mg, 0.07 mmol) and DCC (23 mg, 0.11 mmol) were added to a solution of *N*⁴-Methoxycytidine **36** (10 mg, 0.037 mmol) in DMF (1.0 mL). The mixture was stirred for 2 d at rt under a nitrogen atmosphere. Solvent was evaporated and the residue was purified on Sephadex-DEAE A-25 resin and HPLC using the same method as the general procedure, to give the compound **16** (7.5 mg, 48%) as a white powder. ¹H NMR (D₂O) δ 7.30 (d, *J* = 8.1 Hz, 1H), 5.97 (br, 1H), 5.84 (d, *J* = 8.1 Hz, 1H), 4.42-4.39 (m, 2H), 4.28-4.23 (m, 1H), 4.17-4.12 (m, 2H), 3.83 (s, 3H), 2.20 (t, *J* = 19.5 Hz, 2H); ³¹P NMR (D₂O) δ 18.88 (br), 14.75 (br); HRMS-EI found 430.0425 (M-H⁺)⁻. C₁₁H₁₈N₃O₁₁P₂ requires 430.0417; purity > 98% by HPLC (System B: 6.93 min).

(S)-Methanocarba-uridine-5'-triphosphate triethylammonium salt (18)

A solution of (S)-methanocarba-uridine **41** (10 mg, 0.04 mmol) and Proton Sponge (17 mg, 0.08 mmol) in trimethyl phosphate (1 mL) was stirred for 10 min at 0 °C. Then phosphorous oxychloride (0.008 mL, 0.08 mmol) was added dropwise, and the reaction mixture was stirred

for 2 h at 0 °C. Tributylammonium pyrophosphate (1.6 moles C₁₂H₂₇N per mole H₄P₂O₇, 110 mg, 0.23 mmol) in DMF (0.3 mL) was added at once to the reaction mixture. After 10 min, 0.2 M triethylammonium bicarbonate solution (1.5 mL) was added, and the clear solution was stirred at rt for 1 h. After removal of solvents, the residue was purified using the same method as the general procedure using Sephadex-DEAE A-25 resin and HPLC to get compound **18** (2.0 mg, 56%) as a white solid. ¹H NMR (D₂O) δ 7.78 (d, *J* = 7.8 Hz, 1H), 5.85 (d, *J* = 8.1 Hz, 1H), 4.67 (m, 1H), 4.16 (m, 3H), 2.38 (t, *J* = 6.3 Hz, 1H), 1.86 (m, 1H), 1.71 (t, *J* = 6.0 Hz, 1H), 1.30 (ddd, *J* = 2.1, 6.3, 8.4 Hz, 1H); ³¹P NMR (D₂O) δ -6.76 (m), -10.7 (d, *J* = 20.2 Hz), -22.63 (app t); HRMS-EI found 492.9847 (M - H⁺). C₁₁H₁₆N₂O₁₄P₃ requires 492.9814; purity > 98% by HPLC (System A: 17.5 min).

N⁴-Methoxycytidine-5'-triphosphate ammonium salt (20)

A solution of N⁴-methoxycytidine **36** (10 mg, 0.037 mmol) and Proton Sponge (17 mg, 0.08 mmol) in trimethyl phosphate (1 mL) was stirred for 10 min at 0 °C. Then phosphorous oxychloride (0.008 mL, 0.08 mmol) was added dropwise, and the reaction mixture was stirred for 2 h at 0 °C. Tributylammonium pyrophosphate (1.6 moles C₁₂H₂₇N per mole H₄P₂O₇, 110 mg, 0.23 mmol) in DMF (0.3 mL) was added at once to the reaction mixture. After 10 min, 0.2 M triethylammonium bicarbonate solution (1.5 mL) was added, and the clear solution was stirred at rt for 1 h. After removal of solvents, the residue was purified using the same method as the general procedure using Sephadex-DEAE A-25 resin and HPLC to get compound **20** (3.9 mg, 31%) as a white solid. ¹H NMR (D₂O) δ 7.25 (d, *J* = 8.1 Hz, 1H), 5.97 (d, *J* = 6.3 Hz, 1H), 5.83 (d, *J* = 8.4 Hz, 1H), 4.40 (m, 2H), 4.22 (m, 3H), 3.81 (s, 3H); ³¹P NMR (D₂O) δ -8.82 (d, *J* = 20.2 Hz), -11.0 (d, *J* = 20.2 Hz), -22.40 (t, *J* = 19.5 Hz); HRMS-EI found 511.9873 (M - H⁺). C₁₀H₁₇N₃O₁₅P₃ requires 511.9857; purity > 98% by HPLC (System A: 17.2 min).

P¹,P³-Di(uridine-5'-)α,β-methylenetriphosphate triethylammonium salt (22)

Compound **10** (3 mg, 0.006 mmol) and UMP (1.6 mg, 0.006 mmol) were converted to the corresponding tributylammonium salts by ion exchange. A solution of compound **10**, tributylammonium salt, and *N,N'*-diisopropylcarbodiimide (DIC, 2.8 μL, 0.018 mmol) in DMF (0.06 mL) was stirred for 3 h at rt. Then a solution of the UMP tributylammonium salt in DMF (0.04 mL) and a solution of MgCl₂ (1.7 mg, 0.018 mmol) in DMF (0.02 μL) were added. After stirring the reaction mixture at rt for 16 h, the solvent was removed and water (0.6 mL) was added. The resulting solid was removed by filtration, and the filtrate was purified by semipreparative HPLC as described above to obtain **22** (1.2 mg, 27 %) as a white solid.

¹H NMR (D₂O) δ 8.03 (d, *J* = 8.1 Hz, 1H), 7.98 (d, *J* = 8.1 Hz, 1H), 6.02-5.94 (m, 4H), 4.43-4.35 (m, 2H), 4.32-4.17 (m, 3H), 2.37 (t, *J* = 20.1 Hz, 1H); ³¹P NMR (D₂O) δ 17.35 (s), 7.42 (d, *J* = 27.6 Hz), -10.93 (d, *J* = 27.6 Hz); HRMS-EI found 751.0009 (M + 2Na⁺ - 3H⁺). C₁₉H₂₄N₄Na₂O₁₉P₃ requires 751.0043; purity > 99% by HPLC (System B: 9.3 min).

P¹-Uridine-5'- P³-cyclohexyl-triphosphate triethylammonium salt (25)

Compound **1** (4 mg, 0.010 mmol) was exchanged to the tributylammonium salt form. A solution of compound **1**, tributylammonium salt, and DIC (4.6 μL, 0.030 mmol) in DMF (0.08 mL) was stirred for 3 h at rt. Then a solution of cyclohexyl phosphate tributylammonium salt in DMF (0.08 mL) and a solution of MgCl₂ (2.8 mg, 0.030 mmol) in DMF (0.04 mL) were added. After stirring the reaction mixture at rt for 16 h, the solvent was removed and water (0.4 mL) was added. The resulting solid was removed by filtration, and the filtrate was purified by semipreparative HPLC as described above to obtain **25** (1.35 mg, 18%) as a white solid.

¹H NMR (D₂O) δ 7.92 (d, *J* = 8.1 Hz, 1H), 7.39 (dd, *J* = 8.2, 8.7 Hz, 2H), 7.26 (d, *J* = 8.7 Hz, 2H), 7.19 (t, *J* = 8.2 Hz, 1H), 5.94 (d, *J* = 4.5 Hz, 1H), 5.92 (d, *J* = 8.7 Hz, 1H), 4.38-4.32 (m, 1H), 4.30-4.18 (m, 4H); ³¹P NMR (D₂O) δ -11.22 (d, *J* = 19.1 Hz), -15.71 (d, *J* = 19.9 Hz),

-23.07 (t, $J = 19.1$ Hz); HRMS-EI found 558.9928 ($M - H^+$). $C_{15}H_{18}N_2O_{15}P_3$ requires 558.9920; purity > 99% by HPLC (System B: 8.9 min).

P¹-Uridine-5'-P³-phenyl-triphosphate triethylammonium salt (26)

Compound **1** (4 mg, 0.010 mmol) and sodium phenyl phosphate dibasic dihydrate (5.0 mg, 0.020 mmol) were cation exchanged to the tributylammonium salts. A solution of compound **1**, tributylammonium salt, and DIC (4.6 μ L, 0.030 mmol) in DMF (0.08 mL) was stirred for 3 h at rt. Then a solution of phenyl phosphate tributylammonium salt in DMF (0.08 mL) and a solution of $MgCl_2$ (2.8 mg, 0.030 mmol) in DMF (0.04 mL) were added. After stirring the reaction mixture at rt for 16 h, the solvent was removed and water (0.4 mL) was added. The resulting solid was removed by filtration, and the filtrate was purified by semipreparative HPLC as described above to obtain **26** (1.24 mg, 16%) as a white solid.

1H NMR (D_2O) δ 8.02 (d, $J = 8.7$ Hz, 1H), 6.07-6.00 (m, 2H), 4.49-4.40 (m, 2H), 4.35-4.15 (m, 4H), 4.01-3.77 (m, 4H), 2.06-1.96 (m, 2H), 1.79-1.69 (m, 2H), 1.59-1.14 (m, 6H); ^{31}P NMR (D_2O) δ -11.5 (br, 2H), -23.1 (br, 1H); HRMS-EI found 565.0405 ($M - H^+$). $C_{15}H_{24}N_2O_{15}P_3$ requires 565.0390; purity > 99% by HPLC (System B: 9.8 min).

P¹-Uridine-5'-P³-[1]glucose-1'-triphosphate triethylammonium salt (27)

Compound **1** (10 mg, 0.025 mmol) and α -D-glucose-1-phosphate disodium salt hydrate (10 mg, 0.038 mmol) were exchanged to the tributylammonium salts. A solution of compound **1**, tributylammonium salt, and DIC (12 μ L, 0.075 mmol) in DMF (0.10 mL) was stirred for 3 h at rt. Then a solution of the α -D-glucose-1-phosphate tributylammonium salt in DMF (0.10 mL) and a solution of $MgCl_2$ (7 mg, 0.075 mmol) in DMF (0.05 μ L) were added. After stirring the reaction mixture at rt for 16 h, the solvent was removed and water (0.4 mL) was added. The resulting solid was removed by filtration, and the filtrate was purified by semipreparative HPLC as described above to obtain **27** (1.8 mg, 9%) as a white solid.

1H NMR (D_2O) δ 7.97 (d, $J = 7.8$ Hz, 1H), 6.01 (d, $J = 4.5$ Hz, 1H), 5.99 (d, $J = 7.8$ Hz, 1H), 5.63 (dd, $J = 7.2, 3.6$ Hz, 1H), 4.45-4.37 (m, 2H), 4.32-4.22 (m, 3H), 3.97-3.73 (m, 4H), 3.52 (dt, $J = 9.9, 3.3$ Hz, 1H), 3.45 (t, $J = 9.6$ Hz); ^{31}P NMR (D_2O) δ -11.27 (d, $J = 19.8$ Hz), -12.65 (d, $J = 19.8$ Hz), -22.70 (d, $J = 19.8$ Hz); HRMS-EI found 645.0100 ($M - H^+$). $C_{15}H_{24}N_2O_{20}P_3$ requires 645.0135; purity > 99% by HPLC (System B: 8.8 min).

(S)-Methanocarpa-uridine-3', 5'-cyclic diphosphate triethylammonium salt (9) and (S)-Methanocarpa-uridine-5'-glucose-1'-triphosphate triethylammonium salt (28)

To a solution of compound **7** (2.6 mg, 0.0036 mmol) in DMF (2 mL), 1,1'-carbonyldiimidazole (3.0 mg, 0.018 mmol) was added. The reaction mixture was stirred at rt for 5 h. Then, 5% triethylamine solution in 1/1 water/methanol (1 mL) was added and stirring was continued at rt for an additional 2 h. After removal of the solvent, the residue was dried under high vacuum and dissolved in DMF (2 mL). Glucose-1-monophosphate tributylammonium salt (4 mg, 0.006 mmol) in DMF (0.2 mL) was added to this mixture. The reaction mixture was stirred at rt for 2 d. After removal of the solvent, the residue was purified using the same method as the general procedure using Sephadex-DEAE A-25 resin and HPLC to get compound **28** (0.8 mg, 23%) and **9** (1.1 mg, 51%) additionally. Compound **28**: 1H NMR (D_2O) δ 7.79 (d, $J = 8.1$ Hz, 1H), 5.86 (d, $J = 8.1$ Hz, 1H), 5.64 (dd, $J = 3.3, 7.2$ Hz, 1H), 4.62 (m, 1H), 4.15 (t, $J = 6.3$ Hz, 2H), 3.86 (m, 4H), 3.50 (m, 3H), 2.39 (t, $J = 6.3$ Hz, 1H), 1.88 (dd, $J = 4.8, 9.9$ Hz, 1H), 1.72 (t, $J = 5.7$ Hz, 1H), 1.39 (m, 1H); ^{31}P NMR (D_2O) δ -10.87 (d, $J = 21.4$ Hz), -12.58 (d, $J = 20.76$ Hz); HRMS-EI found 655.0326 ($M - H^+$). $C_{17}H_{26}N_2O_{19}P_3$ requires 655.0343; purity > 98% by HPLC (System A: 16.9 min). Compound **9**: 1H NMR (D_2O) δ 7.81 (d, $J = 8.1$ Hz, 1H), 5.84 (d, $J = 8.1$ Hz, 1H), 5.30 (m, 1H), 4.08 (m, 3H), 2.63 (t, $J = 6.3$ Hz, 1H), 2.03 (m, 1H), 1.71 (t, $J = 6.0$ Hz, 1H), 1.50 (ddd, $J = 2.1, 6.3, 8.4$ Hz, 1H); ^{31}P NMR (D_2O) δ -9.55 (app d), -22.66

(app t); HRMS-EI found 395.0044 ($M - H^+$). $C_{11}H_{13}N_2O_{10}P_2$ requires 395.0045; purity > 98% by HPLC (System A: 12.0 min).

5-Iodouridine-5'-glucose-1'-triphosphate triethylammonium salt (29)

Compound **3** (2 mg, 0.003 mmol) and α -D-glucose-1-phosphate disodium salt hydrate (2 mg, 0.005 mmol) were exchanged to the tributylammonium salts. A solution of compound **3**, tributylammonium salt, and DIC (1.3 μ L, 0.008 mmol) in DMF (0.04 mL) was stirred for 3 h at rt. Then a solution of the α -D-glucose-1-phosphate tributylammonium salt in DMF (0.04 mL) and a solution of $MgCl_2$ (0.8 mg, 0.008 mmol) in DMF (0.02 μ L) were added. After stirring the reaction mixture at rt for 16 h, the solvent was removed and water (0.2 mL) was added. The resulting solid was removed by filtration, and the filtrate was purified by semipreparative HPLC as described above to obtain **29** (0.35 mg, 13%) as a white solid.

1H NMR (D_2O) δ 8.26 (s, 1H), 6.01 (d, $J = 4.8$ Hz, 1H), 5.68 (dd, $J = 3.0, 6.9$ Hz, 1H), 4.49-4.40 (m, 2H), 4.36-4.25 (m, 3H), 4.03-3.80 (m, 4H), 3.62-3.47 (m, 2H); ^{31}P NMR (D_2O) δ -11.46 (d, $J = 19.1$ Hz), -12.60 (d, $J = 19.1$ Hz), -22.60 (t, $J = 19.1$ Hz); HRMS-EI found 770.9131 ($M - H^+$). $C_{15}H_{23}IN_2O_{20}P_3$ requires 770.9102; purity > 99% by HPLC (System B: 9.7 min).

3-Phenacyluridine-5'-glucose-1'-triphosphate triethylammonium salt (30)

Compound **5** (2 mg, 0.005 mmol) and α -D-glucose-1-phosphate disodium salt hydrate (2 mg, 0.010 mmol) were exchanged to the tributylammonium salts. A solution of compound **5**, tributylammonium salt, and DIC (2 μ L, 0.015 mmol) in DMF (0.04 mL) was stirred for 3 h at rt. Then, a solution of the α -D-glucose-1-phosphate tributylammonium salt in DMF (0.04 mL) and a solution of $MgCl_2$ (1 mg, 0.015 mmol) in DMF (0.02 μ L) were added. After stirring the reaction mixture at rt for 16 h, the solvent was removed and water (0.2 mL) was added. The resulting solid was removed by filtration, and the filtrate was purified by semipreparative HPLC as described above to obtain **30** (1.0 mg, 29%) as a white solid.

1H NMR (D_2O) δ 8.08-8.04 (m, 3H), 7.80 (t, $J = 7.2$ Hz, 1H), 7.65 (t, $J = 7.2$ Hz, 2H), 6.17 (d, $J = 7.8$ Hz, 1H), 6.04 (d, $J = 4.5$ Hz, 1H), 5.66 (dd, $J = 3.6, 6.9$ Hz), 5.55 (s, 2H), 4.49-4.43 (br, 2H), 4.37-4.29 (br, 3H), 4.01-3.77 (m, 4H), 3.60-3.50 (br, 1H), 3.48 (t, $J = 9.1$ Hz, 1H); ^{31}P NMR (D_2O) δ -11.24 (d, $J = 25.4$ Hz), -12.63 (d, $J = 25.4$ Hz), -22.65 (d, $J = 25.8$ Hz); HRMS-EI found 763.0554 ($M - H^+$). $C_{23}H_{30}N_2O_{21}P_3$ requires 763.0589; purity > 99% by HPLC (System B: 9.9 min).

Uridine-5'-glucose-1'- α,β -methylenetriphosphate triethylammonium salt (31)

Compound **10** (2.4 mg, 0.004 mmol) and α -D-glucose-1-phosphate disodium salt hydrate (3.6 mg, 0.012 mmol) were exchanged to the tributylammonium salts. A solution of compound **10**, tributylammonium salt, and DIC (1.8 μ L, 0.012 mmol) in DMF (0.04 mL) was stirred for 3 h at rt. Then, a solution of the α -D-glucose-1-phosphate tributylammonium salt in DMF (0.04 mL) and a solution of $MgCl_2$ (2.6 mg, 0.028 mmol) in DMF (0.02 μ L) were added. After stirring the reaction mixture at rt for 16 h, the solvent was removed and water (0.4 mL) was added. The resulting solid was removed by filtration, and the filtrate was purified by semipreparative HPLC as described above to obtain **31** (2.7 mg, 80%) as a white solid.

1H NMR (D_2O) δ 8.07 (d, $J = 7.8$ Hz, 1H), 6.06-5.99 (m, 2H), 5.70-5.63 (m, 1H), 4.49-4.43 (m, 2H), 4.35-4.27 (m, 1H), 4.27-4.21 (m, 2H), 4.00-3.77 (m, 4H), 3.60-3.43 (m, 2H), 2.43 (t, $J = 19.8$ Hz, 2H); ^{31}P NMR (D_2O) δ 17.32 (d, $J = 9.1$ Hz), 8.27 (dd, $J = 9.1, 26.7$ Hz), -12.23 (d, $J = 26.7$ Hz); HRMS-EI found 643.0348 ($M - H^+$). $C_{16}H_{26}N_2O_{19}P_3$ requires 643.0343; purity > 99% by HPLC (System B: 9.2 min).

***N*⁴-Methoxycytidine-5'-glucose-1'-triphosphate triethylammonium salt (32)**

Compound **12** (4.9 mg, 0.009 mmol) and α -D-glucose-1-phosphate disodium salt hydrate (8.4 mg, 0.028 mmol) were exchanged to the tributylammonium salts. A solution of compound **12**, tributylammonium salt, and DIC (4.3 μ L, 0.028 mmol) in DMF (0.1 mL) was stirred for 3 h at rt. Then, a solution of the α -D-glucose-1-phosphate tributylammonium salt in DMF (0.1 mL) and a solution of MgCl₂ (2.6 mg, 0.028 mmol) in DMF (50 μ L) were added. After stirring the reaction mixture at rt for 16 h, the solvent was removed and water (1 mL) was added. The resulting solid was removed by filtration, and the filtrate was purified by semipreparative HPLC as described above to obtain **32** (0.62 mg, 10%) as a white solid.

¹H NMR (D₂O) δ 7.28 (d, *J* = 8.4 Hz, 1H), 5.99 (d, *J* = 5.4 Hz, 1H), 5.86 (d, *J* = 8.4 Hz, 1H), 5.65 (dd, *J* = 3.6, 7.2 Hz, 1H), 4.45-4.36 (m, 2H), 4.30-4.15 (m, 3H), 3.97-3.77 (m, 4H), 3.82 (s, 3H), 3.57-3.50 (m, 1H), 3.47 (t, *J* = 9.3 Hz, 1H); ³¹P NMR (D₂O) δ -11.27 (d, *J* = 19.1 Hz), -12.65 (d, *J* = 19.1 Hz), -22.71 (t, *J* = 19.1 Hz); HRMS-EI found 674.0387 (M - H⁺). C₁₆H₂₇N₃ O₂₀P₃ requires 674.0401; purity > 99% by HPLC (System B: 9.3 min).

***N*⁴-Methoxycytidine-5'-phenyl-triphosphate triethylammonium salt (33)**

Compound **12** (6.9 mg, 0.011 mmol) and sodium phenyl phosphate dibasic dihydrate (8.3 mg, 0.033 mmol) were exchanged to the tributylammonium salts. A solution of compound **12** tributylammonium salt and DIC (5.0 μ L, 0.033 mmol) in DMF (0.07 mL) was stirred for 3 h at rt. Then, a solution of the phenyl phosphate tributylammonium salt in DMF (0.07 mL) and a solution of MgCl₂ (3.1 mg, 0.033 mmol) in DMF (0.07 mL) were added. After stirring the reaction mixture at rt for 16 h, the solvent was removed and water (1 mL) was added. The resulting solid was removed by filtration, and the filtrate was purified by semipreparative HPLC as described above to obtain **33** (0.9 mg, 14%) as a white amorphous solid.

¹H NMR (D₂O) δ 7.44-7.36 (m, 2H), 7.31-7.17 (m, 4H), 5.92 (d, *J* = 6.0 Hz, 1H), 5.77 (d, *J* = 8.4 Hz, 1H), 4.38-4.28 (m, 2H), 4.24-4.15 (m, 3H); HRMS-EI found 588.0186 (M - H⁺). C₁₆H₂₂N₃ O₁₅P₃ requires 588.0199; purity > 99% by HPLC (System B: 10.3 min).

Pharmacological analyses

UDP was purchased from Sigma (St. Louis, MO). *Myo*-[³H]inositol (20 Ci/mmol) was obtained from American Radiolabeled Chemicals (St. Louis, MO). Measurement of intracellular calcium ion concentration in response to nucleotides was performed as described.⁴

Assay of PLC activity stimulated by P2Y₂, P2Y₄, and P2Y₆ receptors

Stable cell lines expressing the human P2Y₂, P2Y₄, or P2Y₆ receptor in 1321N1 human astrocytoma cells were generated as described.²⁶ Agonist-induced [³H]inositol phosphate production was measured in 1321N1 cells plated to 20,000 cells/well on 96-well plates two days prior to assay. Sixteen h before the assay, the inositol lipid pool of the cells was radiolabeled by incubation in 100 μ L of serum-free inositol-free Dulbecco's modified Eagle's medium, containing 1.0 μ Ci of *myo*-[³H]inositol. No changes of medium were made subsequent to the addition of [³H]inositol. On the day of the assay, the cells were challenged with 25 μ L of a fivefold concentrated solution of receptor agonists in 200 mM HEPES (N-(2-hydroxyethyl)-piperazine-N'-2-ethanesulfonic acid), pH 7.3 in HBSS, containing 50 mM LiCl for 30 min at 37°C. Incubations were terminated by aspiration of the drug-containing medium and addition of 450 μ L of ice-cold 50 mM formic acid. [³H]Inositol phosphate accumulation was quantified using scintillation proximity assay methodology as previously described in detail.²⁶

Data Analysis

Agonist potencies (EC_{50} values) were determined from concentration-response curves by non-linear regression analysis using the GraphPad software package Prism (GraphPad, San Diego, CA). All experiments examining the activity of newly synthesized molecules also included full concentration effect curves for the cognate agonist of the target receptor: UTP for the $P2Y_2$ receptor, UTP for the $P2Y_4$ receptor, and UDP for the $P2Y_6$ receptor. Each concentration of drug was tested in triplicate assays, and concentration effect curves for each test drug were repeated in at least three separate experiments with freshly diluted molecule. The results are presented as mean \pm SEM from multiple experiments or in the case of concentration effect curves from a single experiment carried out with triplicate assays that were representative of results from multiple experiments.

Stability of Nucleotide Derivatives

UTP was purchased from Sigma (St. Louis, MO). Up_3U **19** was synthesized by the procedure previously reported.²¹ HCl buffer (pH 1.2) solution was prepared as follows. Concentrated HCl (7 mL, 12 N) was added to 2.0 g of sodium chloride and diluted with water to a volume of 1000 mL. Membranes were prepared as follows. $P2Y_1$ receptor-expressing astrocytoma cells were grown to 80% confluence and then harvested. The cells were homogenized and the nuclear fraction was removed by centrifuging at $100 \times g$ for 5 min. The pellet was resuspended in 50 mM tris(hydroxymethyl)aminomethane (Tris) hydrochloride buffer (pH 7.4). The suspension was homogenized with a polytron homogenizer (Brinkmann) for 10 s and was ultracentrifuged at $20,000 \times g$ for 20 min at $4^\circ C$. The resultant pellets were resuspended in Tris buffer (pH 7.4), and the membrane was stored at $-80^\circ C$ until the experiments. The protein concentration of the membrane preparation was measured by Bradford assay.³⁴ The membrane preparation was diluted with 4 mL of Dulbecco's Phosphate-Buffered Saline (pH 7.4) and homogenized before use for the stability check. The final protein concentration was 14.9 $\mu g/mL$.

Ten μL of a 2 mg/mL aqueous solution of each nucleotide derivative was mixed with 90 μL of HCl buffer (pH 1.2) or the membrane suspension and incubated at $37^\circ C$. At regular intervals, a 6 μL aliquot of the above mixture was mixed with 54 μL of water or 5 mM TBAP. Then, 50 μL of the mixture was injected to the HPLC.

AUC of compounds were measured using a Hewlett-Packard 1100 HPLC equipped with a Zorbax Eclipse 5 μm XDB-C18 analytical column (250×4.6 mm; Agilent Technologies Inc, Palo Alto, CA). Mobile phase: linear gradient solvent system: 5 mM TBAP- CH_3CN from 80:20 to 40:60 in 6.5 min; the flow rate was 1 mL/min (System C) or System B. Peaks were detected by UV absorption with a diode array detector at 254, 275, and 280 nm and AUC were calculated based on the peak at 254 nm.

Molecular Modeling

$P2Y_6$ - Compound 7 complex—Molecular modeling was based on our previously published model of the complex between UDP and the rhodopsin-based $P2Y_6$ receptor, obtained through MonteCarlo conformational searches and molecular dynamics in a hydrated lipid bilayer.¹³

Here, compound **7** was sketched from the docked UDP, and subsequently subjected to a series of energy minimizations allowing flexibility to increasingly larger portions of the model, followed by a conformational search. In particular, the minimizations were performed on the cyclopropane moiety of the ligand, the ribose moiety of the ligand, and the entire ligand. The final minimization and the conformational search were performed on the entire ligand plus all the $P2Y_6$ residues and the water molecules located within a radius of 5 Å. All of the atoms

within an additional shell of a radius of 3 Å were also taken into account in the calculations as a frozen environment.

All the calculations were conducted using MacroModel, version 9.6, as implemented in the Schrödinger package, with the OPLS_2005 force field and water as implicit solvent (GB/SA model).³⁵ The minimizations were based on the Polak-Ribier conjugate gradient algorithm and were performed with a threshold on the gradient of 0.05 KJ/Mol. The conformational search was based on 1000 steps of extended Monte Carlo Multiple Minimum (MCMM) torsional sampling. The sampling regarded the torsional angles of residues and ligand, as well as the rotation and translation of the ligand.

P2Y₆ - Compound 12, 26 or 33 complex—Molecular modeling was based on the same structure described in the above section. Compounds **12**, **26** and **33** were sketched from the docked UDP, and subsequently subjected to a series of energy minimizations allowing flexibility to increasingly larger portions of the model, followed by a conformational search. All the conformational searches were performed on each entire ligand plus all the P2Y₆ residues located within a radius of 6 Å from the initial ligand, UDP. All of the atoms within an additional shell of a radius of 4 Å were also taken into account in the calculations as a frozen environment.

All the calculations were conducted using MacroModel, version 9.7.1, as implemented in the Schrödinger package, with the OPLS_2001 force field.³⁶ The minimizations were based on the Polak-Ribier conjugate gradient algorithm and were performed with a threshold on the gradient of 0.05 KJ/Mol. The conformational search was based on 100 steps of extended Monte Carlo Multiple Minimum (MCMM) torsional sampling. The sampling regarded the torsional angles of residues and ligand, as well as the rotation and translation of the ligand.

Residue indexing—To facilitate the comparison among receptors, residues are identified through the GPCR residue indexing system. In each TM, the index X.50, where X is the TM number, is assigned to the position hosting the most conserved residue among class A GPCRs. All other positions in the TM are numbered relatively to the X.50 position.³⁷

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

Mass spectral measurements were performed by Dr. Noel Whittaker and NMR by Wesley White and Dr. Herman Yeh (NIDDK). We thank Dr. Andrei A. Ivanov (NIDDK) for helpful discussions and Rhonda Carter for technical assistance. This research was supported in part by the Intramural Research Program of the NIH, NIDDK and by an NIH extramural grant GM38213 from the National Institute of General Medical Sciences.

Abbreviations

| | |
|-------|--|
| CDI | <i>N,N</i> -carbonyldiimidazole |
| DCC | <i>N,N'</i> -dicyclohexylcarbodiimide |
| DIC | <i>N,N'</i> -diisopropylcarbodiimide |
| DMF | dimethylformamide |
| HEK | human embryonic kidney |
| HBSS | Hank's balanced salt solution |
| HEPES | <i>N</i> -2-hydroxyethylpiperazine- <i>N'</i> -2-ethanesulfonic acid |

| | |
|------|--|
| HPLC | high performance liquid chromatography |
| PLC | phospholipase C |
| SAR | structure activity relationship |
| TBAP | tetrabutylammonium dihydrogenphosphate |
| TEAA | triethylammonium acetate |
| UDP | uridine-5'-diphosphate |
| UTP | uridine-5'-triphosphate |

Reference List

1. Abbracchio MP, Burnstock G, Boeynaems JM, Barnard EA, Boyer JL, Kennedy C, Knight GE, Fumagalli M, Gachet C, Jacobson KA, Weisman GA. International Union of Pharmacology LVIII: update on the P2Y G protein-coupled nucleotide receptors: from molecular mechanisms and pathophysiology to therapy. *Pharmacol. Rev* 2006;58:281–341. [PubMed: 16968944]
2. Costanzi S, Mamedova L, Gao ZG, Jacobson KA. Architecture of P2Y nucleotide receptors: structural comparison based on sequence analysis, mutagenesis, and homology modeling. *J. Med. Chem* 2004;47:5393–5404. [PubMed: 15481977]
3. Malmjö M, Hou M, Pendergast W, Erlinge D, Edvinsson L. Potent P2Y₆ receptor mediated contractions in human cerebral arteries. *BMC Pharmacol* 2003;3:4. [PubMed: 12737633]
4. Balasubramanian R, Ruiz de Azua I, Wess J, Jacobson KA. Activation of distinct P2Y receptor subtypes stimulates insulin secretion and cytoprotection in MIN6 mouse pancreatic β cells. *Biochem. Pharmacol* 2010;79:1317–132. [PubMed: 20067775]
5. Kim SG, Gao ZG, Soltysiak KA, Chang TS, Brodie C, Jacobson KA. P2Y₆ nucleotide receptor activates PKC to protect 1321N1 astrocytoma cells against tumor necrosis factor-induced apoptosis. *Cell. Mol. Neurobiol* 2003;23:401–418. [PubMed: 12825835]
6. Mamedova LK, Wang R, Besada P, Liang BT, Jacobson KA. Attenuation of apoptosis in vitro and ischemia/reperfusion injury in vivo in mouse skeletal muscle by P2Y₆ receptor activation. *Pharmacol. Res* 2008;58:232–239. [PubMed: 18805489]
7. Korcok J, Raimundo LN, Du X, Sims SM, Dixon SJ. P2Y₆ nucleotide receptors activate NF- κ B and increase survival of osteoclasts. *J. Biol. Chem* 2005;280:16909–16915. [PubMed: 15722352]
8. Koizumi S, Shigemoto-Mogam Y, Nasu-Tada K, Shinozaki Y, Ohsawa K, Tsuda M, Joshi BV, Jacobson KA, Kohsaka S, Inoue K. UDP acting at P2Y₆ receptors is a novel mediator of microglial phagocytosis. *Nature* 2007;446:1091–1095. [PubMed: 17410128]
9. Grbic DM, Degagné E, Langlois C, Dupuis AA, Gendron FP. Intestinal inflammation increases the expression of the P2Y₆ receptor on epithelial cells and the release of CXC chemokine ligand 8 by UDP. *J. Immunol* 2008;180:2659–2668. [PubMed: 18250478]
10. Markovskaya A, Crooke A, Guzmán-Aranguez AI, Peral A, Ziganshin AU, Pintor J. Hypotensive effect of UDP on intraocular pressure in rabbits. *Eur. J. Pharmacol* 2008;579:93–97. [PubMed: 18031728]
11. Bar I, Guns P-J, Metallo J, Wilkin F, Cammarata D, Boeynaems J-M, Bult H, Robaye B. Knock-out mice reveal a role for the P2Y₆ receptor in macrophages, endothelial cells and vascular smooth muscle cells. *Mol. Pharmacol* 2008;74:777–784. [PubMed: 18523137]
12. Jacobson KA, Boeynaems JM. P2Y nucleotide receptors: Promise of therapeutic applications. *Drug Disc. Today*. in press.
13. Costanzi S, Joshi BV, Maddileti S, Mamedova L, Gonzalez-Moa MJ, Marquez VE, Harden TK, Jacobson KA. Human P2Y₆ receptor: molecular modeling leads to the rational design of a novel agonist based on a unique conformational preference. *J. Med. Chem* 2005;48:8108–8111. [PubMed: 16366591]

14. Besada P, Shin DH, Costanzi S, Ko HJ, Mathé C, Gagneron J, Gosselin G, Maddileti S, Harden TK, Jacobson KA. Structure activity relationship of uridine 5'-diphosphate analogues at the human P2Y₆ receptor. *J. Med. Chem* 2006;49:5532–5543. [PubMed: 16942026]
15. El-Tayeb A, Qi A, Müller CE. Synthesis and structure-activity relationships of uracil nucleotide derivatives and analogues as agonists at human P2Y₂, P2Y₄, and P2Y₆ receptors. *J. Med. Chem* 2006;49:7076–7087. [PubMed: 17125260]
16. Ko H, Carter RL, Cosyn L, Petrelli R, de Castro S, Besada P, Zhou Y, Cappellacci L, Franchetti P, Grifantini M, Van Calenbergh S, Harden TK, Jacobson KA. Synthesis and potency of novel uracil nucleotide analogues as P2Y₂ and P2Y₆ receptor agonists. *Bioorg. Med. Chem* 2008;16:6319–6332. [PubMed: 18514530]
17. Carter RL, Fricks IP, Barrett MO, Burianek LE, Zhou Y, Ko H, Das A, Jacobson KA, Lazarowski ER, Harden TK. Quantification of Gi-mediated inhibition of adenylyl cyclase activity reveals that UDP is a potent agonist of the human P2Y₁₄ receptor. *Mol. Pharmacol* 2009;76:1341–1348. [PubMed: 19759354]
18. Das A, Ko H, Burianek LB, Barrett MO, Harden TK, Jacobson KA. Human P2Y₁₄ receptor agonists: Truncation of the hexose moiety of uridine-5'-diphosphoglucose and its replacement with alkyl and aryl groups. *J. Med. Chem* 2010;53:471–480. [PubMed: 19902968]
19. Melman A, Zhong M, Marquez VE, Jacobson KA. Synthesis of enantiomerically pure (S)-methanocarbaribo uracil nucleoside derivatives for use as antiviral agents and P2Y receptor ligands. *J. Org. Chem* 2008;73:8085–8088. [PubMed: 18811198]
20. Zimmerman H. Extracellular metabolism of ATP and other nucleotides. *Naunyn-Schmiedeberg's Arch Pharmacol* 2000;362:299–309. [PubMed: 11111825]
21. Shaver SR, Rideout JL, Pendergast W, Douglass JG, Brown EG, Boyer JL, Patal RI, Redick CC, Jones AC, Picher M, Yerxa BR. Structure activity relationships of dinucleotides: Potent and selective agonists of P2Y receptors. *Purinergic Signal* 2005;1:183–191. [PubMed: 18404503]
22. Yerxa BR, Sabater JR, Davis CW, Stutts MJ, Lang-Furr M, Jones AC, Cowlen M, Dougherty R, Boyer J, Abraham WM, Boucher RC. Pharmacology of INS37217 [P(1)-(uridine 5')-P(4)-(2'-deoxycytidine 5')]tetrphosphate, tetrasodium salt], a next-generation P2Y₂ receptor agonist for the treatment of cystic fibrosis. *J. Pharmacol. Exp. Ther* 2002;302:871–880. [PubMed: 12183642]
23. Kogo, S.; Yamada, K.; Iwai, Y.; Osawa, K.; Hayakawa, H. Process for producing di(pyrimidine nucleoside 5'-)polyphosphate.. 2008. WO 2008/012949 A1
24. Kim HS, Ravi RG, Marquez VE, Maddileti S, Wihlborg A-K, Erlinge D, Malmjö M, Boyer JL, Harden TK, Jacobson KA. Methanocarba modification of uracil and adenine nucleotides: High potency of Northern ring conformation at P2Y₁, P2Y₂, P2Y₄ and P2Y₁₁, but not P2Y₆ receptors. *J. Med. Chem* 2002;45:208–218. [PubMed: 11754592]
25. Ivanov MA, Antonova EV, Maksimov AV, Pigusova LK, Belanov EF, Aleksandrova LA. New N⁴-hydroxycytidine derivatives: synthesis and antiviral activity. *Coll. Czech. Chem. Comm* 2006;71:1099–1106.
26. Bourdon DM, Wing MR, Edwards EB, Sondek J, Harden TK. Quantification of Isozyme-Specific Activation of Phospholipase C-β2 by Rac GTPases and Phospholipase C-ε by Rho GTPases in an Intact Cell Assay System. *Methods in Enzymol* 2006;406:489–499. [PubMed: 16472681]
27. Ko H, Das A, Carter RL, Fricks IP, Zhou Y, Ivanov AA, Melman A, Joshi BV, Kováč P, Hajduch J, Kirk KL, Harden TK, Jacobson KA. Molecular recognition in the P2Y₁₄ nucleotide receptor: Probing the structurally permissive terminal sugar moiety of uridine-5'-diphosphoglucose. *Bioorg. Med. Chem* 2009;17:5298–5311. [PubMed: 19502066]
28. Chan SW, Gallo SJ, Kim BK, Guo MJ, Blackburn GM, Zamecnik PC. P1,P4-dithio-P2,P3-monochloromethylene diadenosine 5',5'''-P1,P4-tetrphosphate: a novel antiplatelet agent. *Proc. Natl. Acad. Sci. U. S. A* 1997;94:4034–4039. [PubMed: 9108100]
29. Eliahu SE, Camden J, Lecka J, Weisman GA, Sévigny J, Gélinas S, Fischer B. Identification of hydrolytically stable and selective P2Y₁ receptor agonists. *Eur. J. Med. Chem* 2009;44:1525–1536. [PubMed: 18760862]
30. Robaye B, Boeynaems JM, Communi D. Slow desensitization of the human P2Y₆ receptor. *Eur. J. Pharmacol* 1997;329:231–236. [PubMed: 9226417]

31. Phillips JH, Brown DM, Grossman L. The efficiency of induction of mutations by hydroxylamine. *J. Mol. Biol* 1966;21:405–419. [PubMed: 19768859]
32. Parandeh F, Abaraviciene SM, Amisten S, Erlinge D, Salehi A. Uridine diphosphate (UDP) stimulates insulin secretion by activation of P2Y₆ receptors. *Biochem. Biophys. Res. Commun* 2008;370:499–503. [PubMed: 18387359]
33. Ohtani M, Suzuki JI, Jacobson KA, Oka T. Evidence for the possible involvement of P2Y₆ receptor in Ca²⁺ mobilization and insulin secretion in mouse pancreatic islets. *Purinergic Signalling* 2008;4:365–375. [PubMed: 18784987]
34. Bradford MM. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein dye binding. *Anal. Biochem* 1976;76:248–254. [PubMed: 942051]
35. MacroModel, version 9.6. Schrödinger, LLC; New York, NY: 2008.
36. MacroModel, version 9.7. Schrödinger, LLC; New York, NY: 2009.
37. Ballesteros JA, Weinstein H. Integrated methods for the construction of three-dimensional models and computational probing of structure-function relations in G-protein coupled receptors. *Methods Neurosci* 1995;25:366–428.

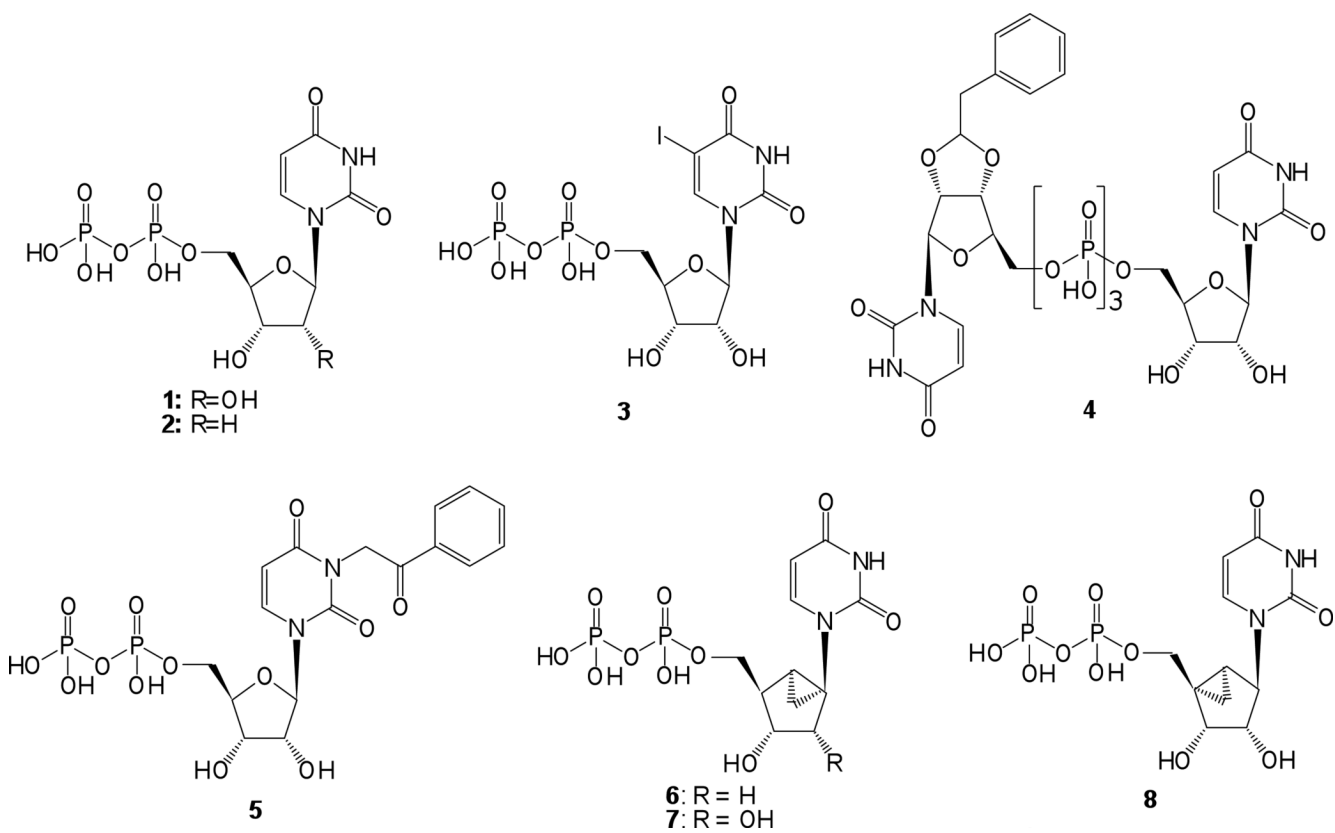
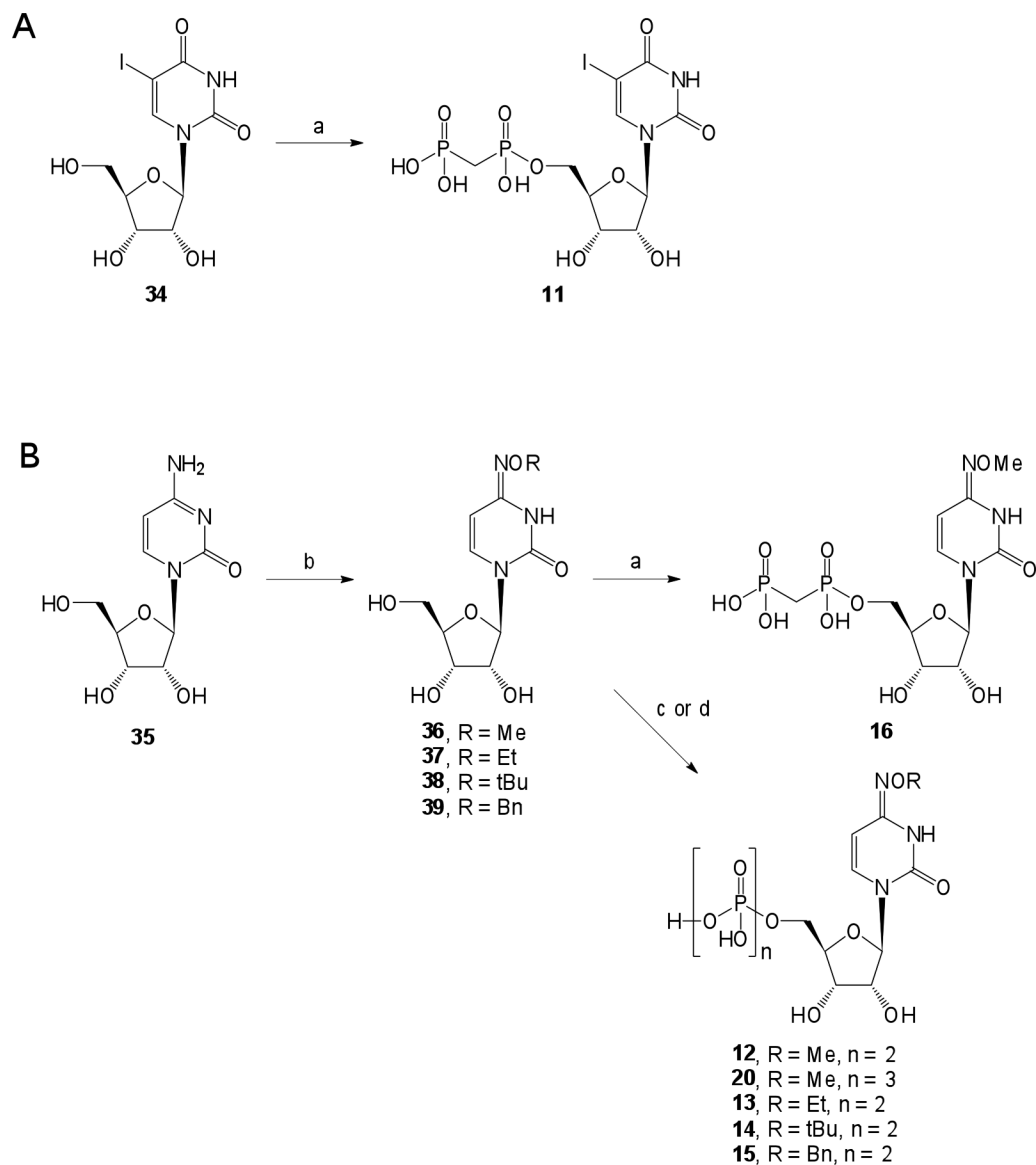
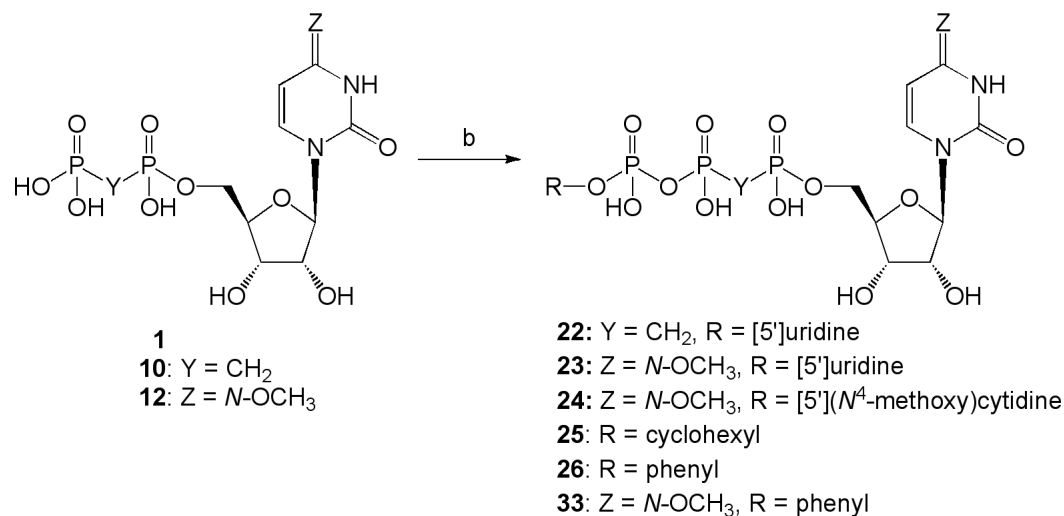
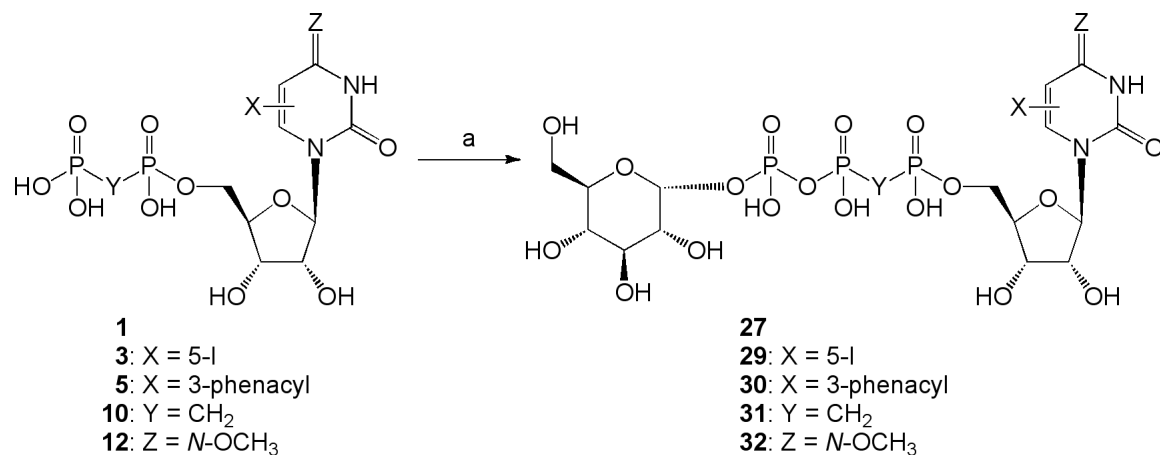


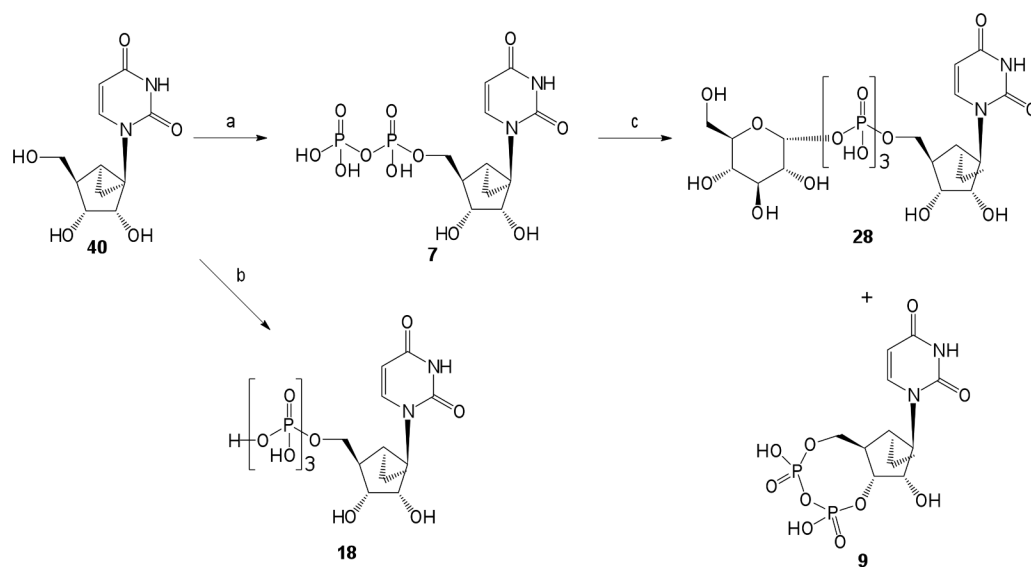
Chart 1.
Structures of prototypical agonist ligands for studying P2Y₆ receptors.

**Scheme 1.**

Synthesis of various pyrimidine ribonucleoside 5'-di and triphosphate derivatives. *Reagents and conditions*: (a) DCC, methylenebisphosphonate, rt, overnight, 11 % for **11**, 48 % for **16**; (b) RONH_2 , pyridine, 90°C , overnight, 55 – 100 %; (c) POCl_3 , Proton Sponge, $\text{PO}(\text{OMe})_3$, 0°C , 2 hr, followed by tributylammonium phosphate, rt, 0.5 hr (for **12 - 15**), 14 – 47 %; (d) POCl_3 , Proton Sponge, $\text{PO}(\text{OMe})_3$, 0°C , 2hr, followed by tributylammonium pyrophosphate, rt, 0.5 hr, 31 % (for **20**).

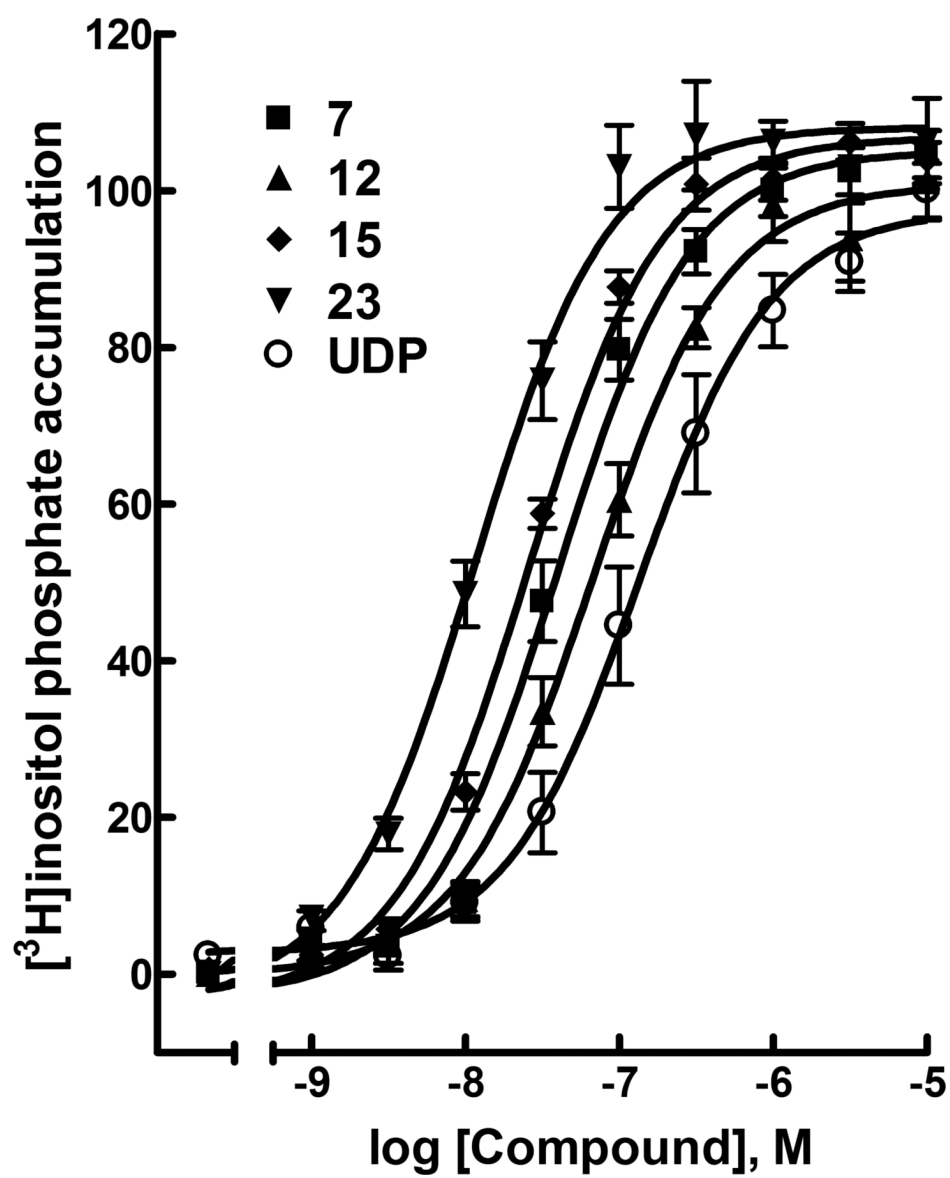
**Scheme 2.**

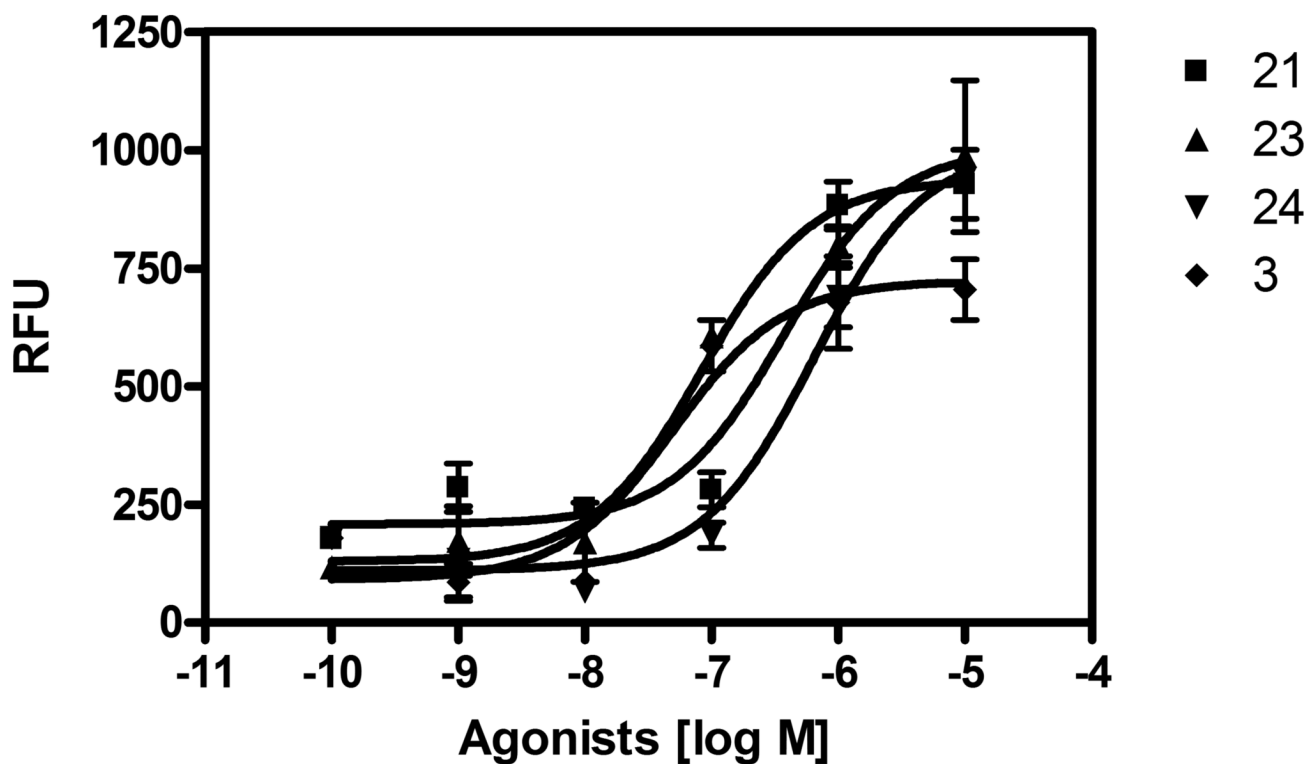
Synthesis of various pyrimidine ribonucleoside 5'-triphosphates and dinucleoside triphosphates. *Reagents and conditions:* (a) DIC, MgCl₂, glucose 1-monophosphate tributylammonium salt, DMF, rt, overnight, 9 - 80 %; (b) DIC, MgCl₂, respective monophosphate tributylammonium salt, e.g., uridine 5'-monophosphate, **35**, cyclohexyl monophosphate¹⁶ or phenyl monophosphate, in DMF, rt, overnight, 9-80%. Unless noted, X = H and Y, Z = O.

**Scheme 3.**

Synthesis of uracil ribonucleoside phosphate derivatives constrained with a (S)-methanocarba ring. *Reagents and conditions:* (a) POCl₃, Proton Sponge, PO(OMe)₃, 0°C, 2 hr; then tributylammonium phosphate, rt, 0.5 hr, 45 %; (b) POCl₃, Proton Sponge, PO(OMe)₃, 0°C, 2 hr; then tributylammonium pyrophosphate, rt, 0.5 hr, 56 %; (c) CDI, DMF, rt, 1 hr, then glucose 1-monophosphate tributylammonium salt, overnight, 23 % for **28**, 51 % for **9**.

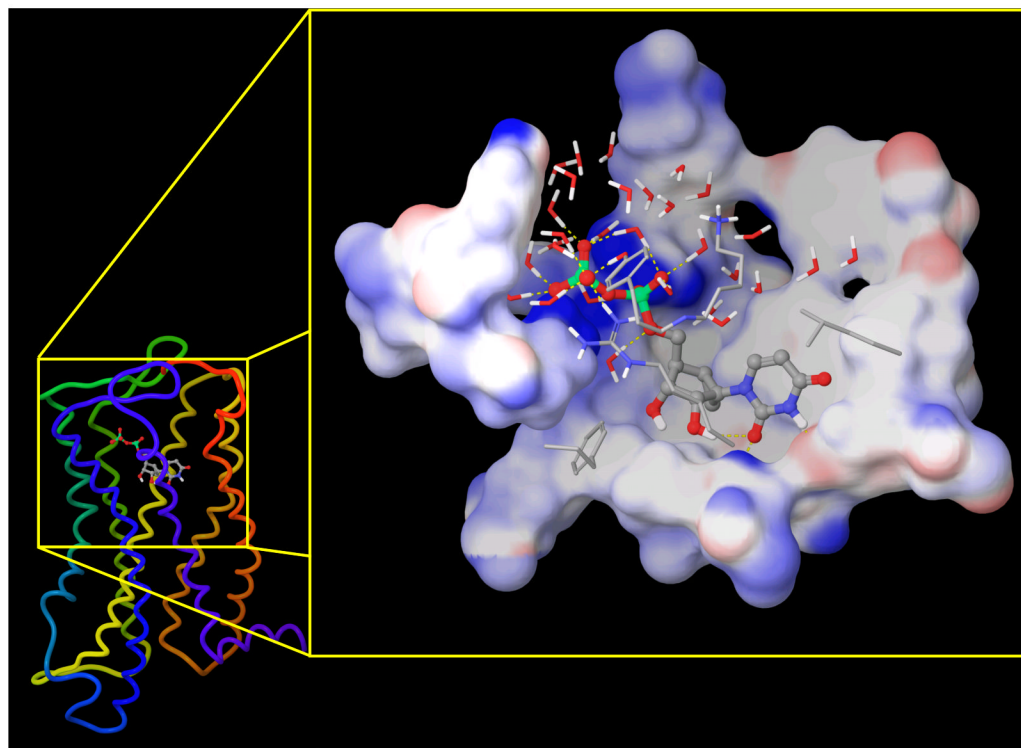
A



B**Figure 1.**

A) Activity of agonists **7**, **12**, **15**, and **23** at the human P2Y₆ receptor as indicated by activation of PLC in stably transfected 1321N1 human astrocytoma cells. The effect of UDP corresponds to 100%. B) Mobilization of intracellular calcium in astrocytoma cells expressing the human P2Y₆ receptor (EC₅₀ values shown in nM) by nucleotide agonists **3** (50), **21** (370), **23** (83), and **24** (630). The EC₅₀ value of **1** was 200 nM.

A



B

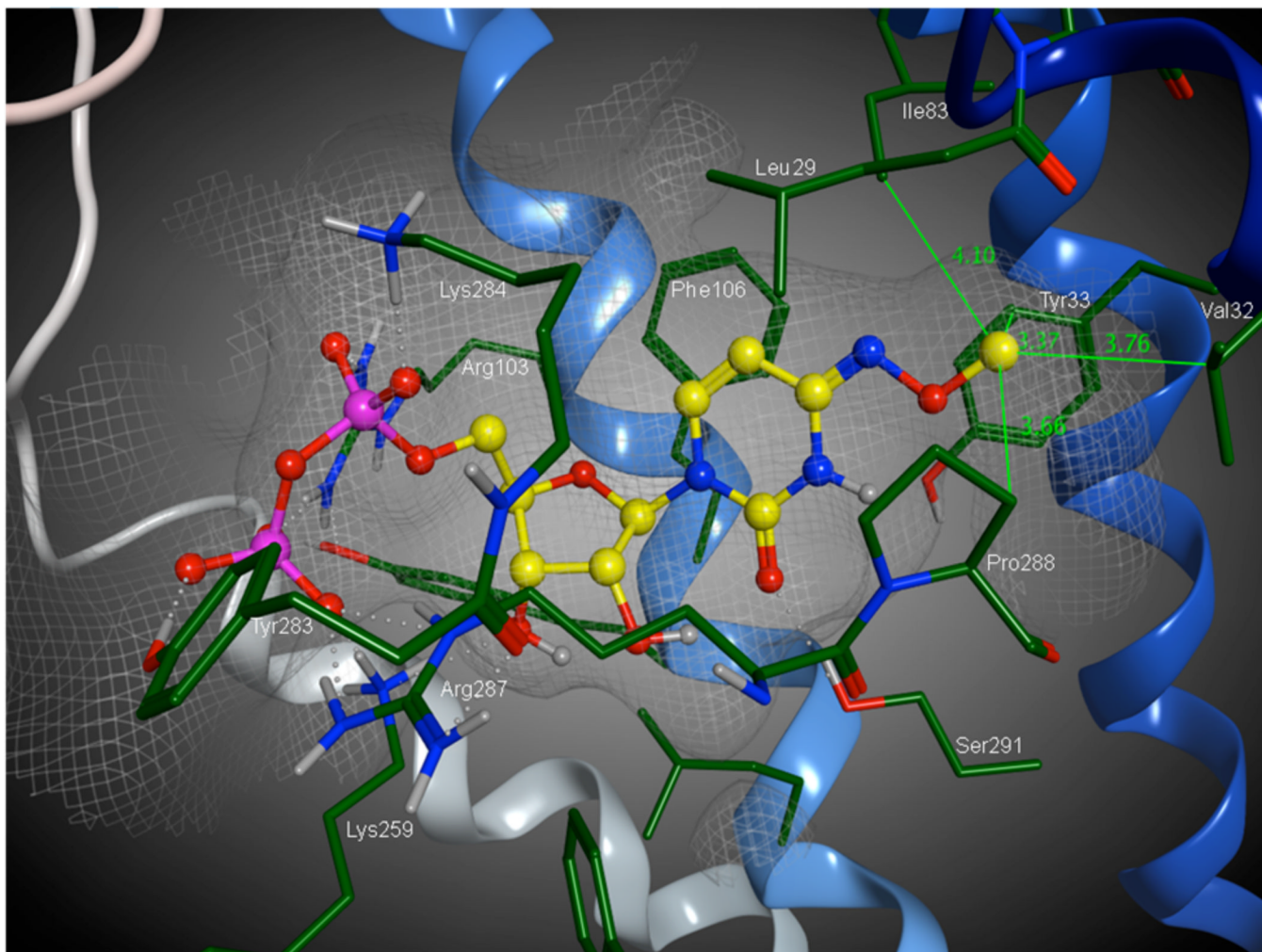


Figure 2. Molecular modeling of the human P2Y₆ receptor. A) Details of the nucleotide binding site of the receptor complexed with the agonist **7** as obtained after a fully flexible Monte Carlo conformational search. The ligand is represented as balls and sticks, colored by element. The binding pocket is represented as a van der Waals surface colored according to the electrostatic potential, with positive charges in blue and negative charges in red. For clarity, the residues located in front of the ligand are represented as sticks, colored by element. Labels indicate all of the residues represented as sticks (adjacent) and the key residues represented as van der Waals surfaces (through arrows). A schematic representation of the receptor-ligand complex is given in the lower left inset. In the tube representations the receptor is colored according to residue positions, with a spectrum of colors that ranges from red (N-terminus) to purple (C-terminus): TM1 is in orange, TM2 in ochre, TM3 in yellow, TM4 in green, TM5 in cyan, TM6 in blue, TM7 in purple. B) Docking of the potent agonist *N*⁴-methoxy-cytidine-5'-diphosphate **12**. A hydrophobic binding pocket for the *N*⁴-alkoxy substituent is defined in this docking model. Distances in Å from the methoxy carbon atom to neighboring amino acid residues are shown.

Table 1

Potencies of (S)-methanocarba analogues **6**, **7**, and **9**, in relation to known uridine 5'-diphosphate derivatives for activation of the human P2Y₆ receptor. Unless noted: R = uridine; and X = O.

X = H; Y = O, R = OH, unless noted.

| Compound | Modification | Structure | EC ₅₀ , μM ^a |
|------------------------|---|------------------------------|------------------------------------|
| | | | hP2Y ₆ |
| 1 | UDP | - | 0.30±0.06 |
| 2 | dUDP | R = H | 1.72±0.76 |
| 3^b | 5-I-UDP | X = 5-I | 0.015±0.002 |
| 5^b | phenacylUDP | X = 3-Ph-COCH ₂ | 0.070±0.006 |
| 6^b | (S)-methanocarba dUDP | R = H | 0.23±0.05 |
| 7 | (S)-methanocarba UDP | - | 0.042±0.008 |
| 8^{b,d} | (N)-methanocarba UDP | | NE |
| 9 | (S)-methanocarba U-cyclic 3',5'-diphosphate | | NE |
| 10^b | α,β-CH ₂ -UDP | Y = CH ₂ | 0.70±0.11 |
| 11 | α,β-CH ₂ -5-I-UDP | X = 5-I, Y = CH ₂ | 0.13±0.02 |

^c**6**, MRS2633; **7**, MRS2795.

^eEC₅₀ values³³ (μM) reported in activation of the hP2Y₁₄ receptor expressed in HEK-293 cells: **1**, 0.16±0.04; **10**, 0.011±0.005; **11**, 0.014±0.07.

NE – no effect at 10 μM.

^a Agonist potencies reflect stimulation of phospholipase C in 1321N1 human astrocytoma cells stably expressing the human P2Y₆ receptor. Potencies are presented in the form of EC₅₀ values, which represent the concentration of agonist at which 50% of the maximal effect is achieved. These values were determined using a four-parameter logistic equation and the GraphPad software package (GraphPad, San Diego, CA). The results are presented as mean ± standard error and are the average of three to six different experiments with each molecule.

^b Values reported in literature: **3**, Besada et al.¹⁴; **5**, El-Tayeb et al.⁸; **6** and **8**, Costanzi et al.¹³; **10**, Ko et al.¹⁶

^d Structure of **8** given in Chart 1.

Table 2

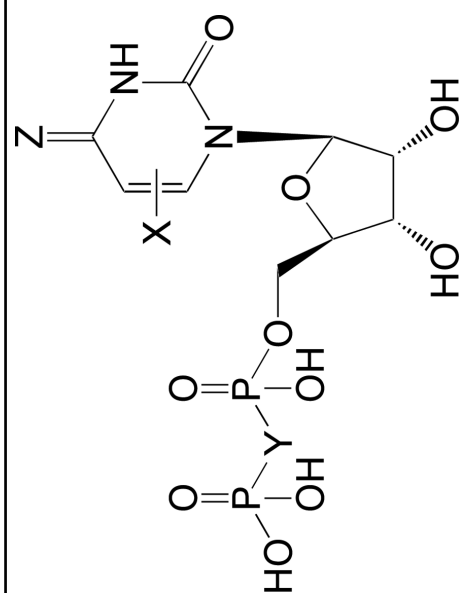
Potencies of *N*⁴-alkyloxy cytidine 5'-diphosphate derivatives for activation of the human P2Y₂, P2Y₄, and P2Y₆ receptors.

| Compound | Modification | Structure | EC ₅₀ , μM ^a | | |
|-----------------------|--|--|------------------------------------|-------------------|-------------------|
| | | | hP2Y ₂ | hP2Y ₄ | hP2Y ₆ |
| 12^b | <i>N</i> ⁴ -OMe-CDP | Z = <i>N</i> -OCH ₃ | 3.60±1.08 | 6.45±1.55 | 0.070±0.007 |
| 13 | <i>N</i> ⁴ -OEt-CDP | Z = <i>N</i> -OCH ₂ CH ₃ | 0.28±0.015 | 0.18±0.02 | 0.104±0.023 |
| 14 | <i>N</i> ⁴ -OtBu-CDP | Z = <i>N</i> -OC(CH ₃) ₃ | 3.51±1.52 | 6.23±1.88 | 2.55±0.70 |
| 15 | <i>N</i> ⁴ -OBn-CDP | Z = <i>N</i> -OCH ₂ Ph | 2.13±0.64 | 1.15±0.15 | 0.026±0.002 |
| 16 | α,β-CH ₂ - <i>N</i> ⁴ -OMe-CDP | Y = CH ₂ , Z = <i>N</i> -OCH ₃ | >10 ^c | NE | 0.678±0.018 |

ND – not determined. NE – no effect at 10 μM.

^a Agonist potencies reflect stimulation of phospholipase C in 1321N1 human astrocytoma cells stably expressing the human P2Y₂, P2Y₄, or P2Y₆ receptor. Potencies are presented in the form of EC₅₀ values, which represent the concentration of agonist at which 50% of the maximal effect is achieved. These values were determined using a four-parameter logistic equation and the GraphPad software package (GraphPad, San Diego, CA). The results are presented as mean ± standard error and are the average of three to six different experiments with each molecule.

^b EC₅₀ value³³ reported in activation of the hP2Y₁₄ receptor expressed in HEK-293 cells: **12**, 3.32±1.62 μM.



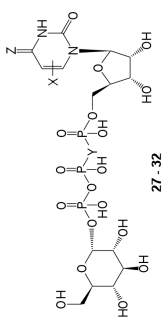
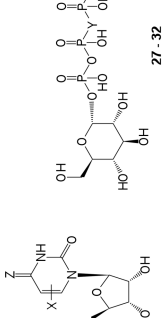
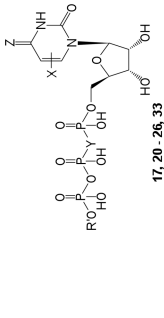
12 - 16

X = H; Y = O, unless noted.

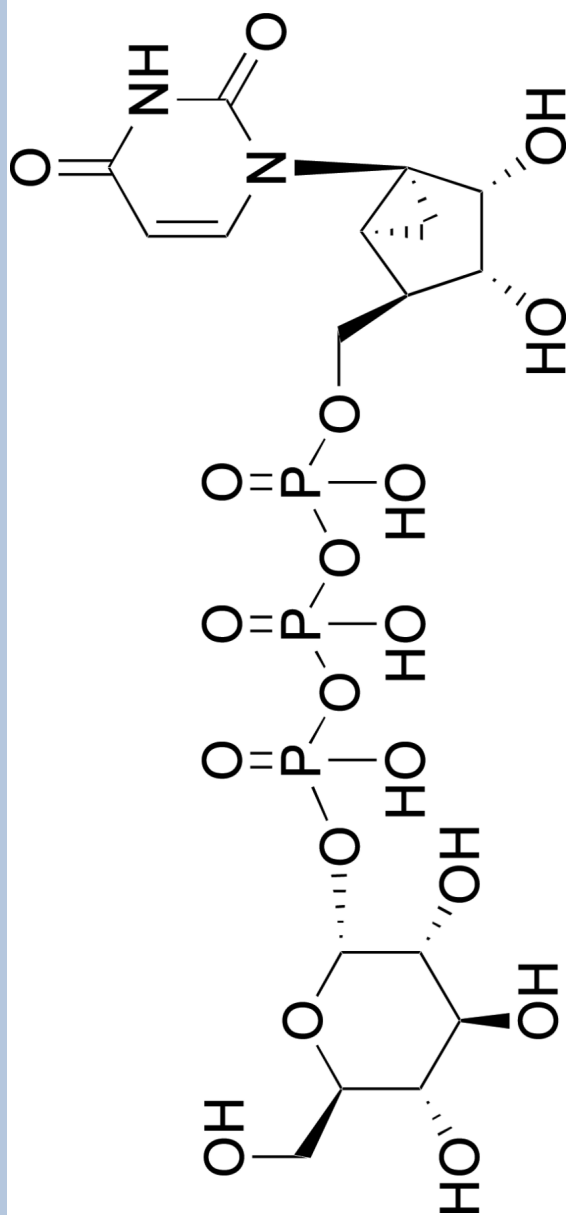
$c_{\leq 50\%}$ effect at 10 μM .

Table 3

Potencies of nucleotide (5'-triphosphate) derivatives for activation of the human P2Y₂, P2Y₄, and P2Y₆ receptors.

| Compound | Modification | Structure | EC ₅₀ , μM ^a | | |
|-----------------|--|---|------------------------------------|-------------------|-------------------|
| | | | hP2Y ₂ | hP2Y ₄ | hP2Y ₆ |
| 17 | UTP |  | 0.060±0.00 | 0.090±0.01 | >10 ^d |
| 18 | (S)-methanocarba UTP |  | 0.08±0.02 | 0.30±0.10 | 1.37±0.05 |
| 19 ^b | (N)-methanocarba UTP |  | 0.016±0.007 | 0.085±0.005 | NE |
| 20 | N ⁴ -OMe-CTP | Z = N-OCH ₃ | 0.05±0.00 | 0.05±0.02 | 0.08±0.02 |
| 21 ^b | Up ₃ U | R' = [5']uridine | 1.31±0.21 | 0.87±0.11 | 0.27±0.07 |
| 22 | α,β-CH ₂ -Up ₃ U | R' = [5']uridine, Y = CH ₂ | >10 ^d | NE | 1.30±0.18 |
| 23 | N ⁴ -OMe-Cp ₃ U | Z = N-OCH ₃ , R' = [5']uridine | 0.17±0.04 | 0.79±0.12 | 0.012±0.0024 |
| 24 | N ⁴ -OMe-Cp ₃ (N ⁴ -OMe)C | Z = N-OCH ₃ , R' = [5'](N ⁴ -methoxy)cytidine | 2.12±0.66 | 0.56±0.04 | 0.063±0.002 |
| 25 | Up ₃ -O-cyclohexyl | R' = cyclohexyl | 2.28±0.1 | NE | 6.99±0.18 |
| 26 | Up ₃ -O-phenyl | R' = phenyl | >10 ^d | NE | 2.13±0.18 |
| 27 | Up ₃ -gluc | | NE | NE | >10 ^d |
| 28 ^c | (S)-methanocarba-Up ₃ -gluc | | NE | NE | 2.47±0.39 |
| 29 | 5-I-Up ₃ -gluc | X = 5-I | NE | NE | 4.69±1.68 |
| 30 | 3-phenacyl-Up ₃ -gluc | X = 3-Ph-COCH ₂ | NE | NE | >10 ^d |
| 31 | α,β-CH ₂ -Up ₃ -gluc | Y = CH ₂ | NE | NE | >10 ^d |
| 32 | N ⁴ -OMe-Cp ₃ -gluc | Z = N-OCH ₃ | >10 ^d | NE | 0.18 ± 0.04 |
| 33 | N ⁴ -OMe-Cp ₃ -O-Ph | R' = phenyl, Z = N-OCH ₃ | >10 ^d | NE | 3.49 ± 1.63 |

X, R' = H, Y, Z = O, unless noted.



NE – no effect at 10 μ M.

ND – not determined.

^a Agonist potencies reflect stimulation of phospholipase C in 1321N1 human astrocytoma cells stably expressing the human P2Y₂, P2Y₄, or P2Y₆ receptor. Potencies are presented in the form of EC₅₀ values, which represent the concentration of agonist at which 50% of the maximal effect is achieved. These values were determined using a four-parameter logistic equation and the GraphPad software package (GraphPad, San Diego, CA). The results are presented as mean \pm standard error and are the average of three to six different experiments with each molecule.

^b Values reported in literature: **19**, Kim et al.²⁶; **21**, Ko et al.¹⁶

^c Structure of **28**:

^d \leq 50% effect at 10 μ M.

Table 4

Half-lives of nucleotide (5'-triphosphate) derivatives when incubated at 37°C in acidic aqueous solution or in the presence of mammalian (1321N1 human astrocytoma) cell membranes, determined by HPLC.

| Compound | t _{1/2} , h (pH 1.2) | t _{1/2} , h (membranes) | Pharmacological properties at hP2Y ₆ receptor |
|---|-------------------------------|----------------------------------|--|
| 12, N ⁴ -MeO-CDP | 116 | 55 | selective, potent agonist |
| 15, N ⁴ -BnO-CDP | 126 | 26 | selective, potent agonist |
| 17, UTP | 23 | 31 | ~inactive |
| 21, Up ₃ U | 218 | 42 | moderately potent agonist, only 4-5 fold selective |
| 22, α,β-me-Up ₃ U | 34 | 62 | weak agonist, selective |
| 23, N ⁴ -MeO-Cp ₃ U | 120 | 23 | selective, potent agonist |
| 25, Up ₃ -O-cyclohexyl | 193 | 62 | weak agonist, nonselective vs. P2Y ₂ receptor |
| 26, Up ₃ -O-Ph | 164 | 43 | weak agonist, selective |
| 27, Up ₃ -gluc | 5.1 | 29 | ~inactive |
| 31, α,β-me-Up ₃ -gluc | 2.8 | 19 | ~inactive |