Bioorganic & Medicinal Chemistry 17 (2009) 5298–5311

Contents lists available at ScienceDirect

Bioorganic & Medicinal Chemistry

journal homepage: www.elsevier.com/locate/bmc

Molecular recognition in the $P2Y_{14}$ receptor: Probing the structurally permissive terminal sugar moiety of uridine-5′-diphosphoglucose

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ARTICLE INFO

Article history:
Received 11 March 2009
Revised 5 May 2009
Accepted 9 May 2009
Available online 15 May 2009

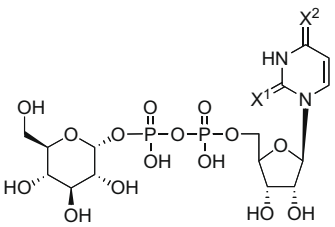
Keywords:
G protein-coupled receptor
Nucleotides
Pyrimidines
Phospholipase C
Carbohydrates
Uracil

ABSTRACT

The $P2Y_{14}$ receptor, a nucleotide signaling protein, is activated by uridine-5′-diphosphoglucose **1** and other uracil nucleotides. We have determined that the glucose moiety of **1** is the most structurally permissive region for designing analogues of this $P2Y_{14}$ agonist. For example, the carboxylate group of uridine-5′-diphosphoglucuronic acid proved to be suitable for flexible substitution by chain extension through an amide linkage. Functionalized congeners containing terminal 2-acylaminoethylamides prepared by this strategy retained $P2Y_{14}$ activity, and molecular modeling predicted close proximity of this chain to the second extracellular loop of the receptor. In addition, replacement of glucose with other sugars did not diminish $P2Y_{14}$ potency. For example, the [5″]ribose derivative had an EC_{50} of 0.24 μM. Selective monofluorination of the glucose moiety indicated a role for the 2″- and 6″-hydroxyl groups of **1** in receptor recognition. The β-glucoside was twofold less potent than the native α-isomer, but methylene replacement of the 1″-oxygen abolished activity. Replacement of the ribose ring system with cyclopentyl or rigid bicyclo[3.1.0]hexane groups abolished activity. Uridine-5′-diphosphoglucose also activates the $P2Y_2$ receptor, but the 2-thio analogue and several of the potent modified-glucose analogues were $P2Y_{14}$-selective.

1. Introduction

Purine and pyrimidine nucleotides, in addition to their well-known diverse intracellular functions, fulfill important roles as extracellular signaling molecules.[1] Receptors of the eight-membered P2Y family of metabotropic G protein-coupled receptors and the P2X family of ligand-gated ion channels detect these nucleotides and stimulate subsequent intracellular signaling pathways.[2–4] Two subfamilies comprise the P2Y receptors: a $P2Y_1$-like subgroup ($P2Y_1$, $P2Y_2$, $P2Y_4$, $P2Y_6$, $P2Y_{11}$) that preferentially couples to G_q to stimulate phospholipase C (PLC), and a $P2Y_{12}$-like subgroup ($P2Y_{12}$, $P2Y_{13}$, $P2Y_{14}$) that preferentially couples to G_i to inhibit adenylyl cyclase. P2Y receptors are distributed in a broad range of tissues and are the focus of therapeutic strategies for a wide range of targets, including antithrombotic therapy,[5] modulation of the immune system[6] and cardiovascular system,[7] and treatment of inflammation,[8] pain,[9] diabetes,[10] and cystic fibrosis and other pulmonary diseases.[11,12]



1 X^1, X^2 = O, UDP-glucose

2 X^1 = S, X^2 = O, MRS2690

3 X^1 = O, X^2 = S

Chart 1. Structures of a naturally occurring agonist of the $P2Y_{14}$ receptor, UDPG **1**, and two potent thio analogues, **2** and **3**.

Abbreviations: Boc, *t*-butyloxycarbonyl; CDI, 1,1′-carbonyldiimidazole; DCC, dicyclohexylcarbodiimide; DCM, methylene chloride; DMF, dimethylformamide; ED, ethylenediamine; EDC, *N*-ethyl-*N*′-dimethylaminopropylcarbodiimide; MCMM, Monte-Carlo Multiple Minimum; MCPBA, 3-chloroperbenzoic acid; PLC, phospholipase C; SAR, structure–activity relationship; TBAF, tetrabutylammonium fluoride; UDPG, uridine-5′-diphosphoglucose.

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doi:10.1016/j.bmc.2009.05.024

The $P2Y_{14}$ receptor is activated by uridine-5′-diphosphoglucose (UDPG, **1**, Chart 1) and other endogenous UDP-sugars.[13] It is distributed in various tissues including placenta, adipose, stomach, intestine, brain, spleen, thymus, lung, and heart.[1] Extracellular release of **1** upon trafficking of proteins to the plasma membrane has been demonstrated, suggesting its widespread role in signaling.[14] Uridine 5′-diphosphate (UDP) also has been reported to be a partial agonist/competitive antagonist at the human $P2Y_{14}$ receptor and a potent agonist at the rat $P2Y_{14}$ receptor.[15] Identification of the physiological functions of the $P2Y_{14}$ receptor has been difficult to establish,[16,17] in part due to a lack of selective high affinity agonists and antagonists for this receptor. The $P2Y_{14}$ receptor plays a role in the neuroimmune system, with expression occurring in T cells, dendritic cells, hematopoietic stem cells, and other tissues.

The structure–activity relationship (SAR) of synthetic nucleotides for activation of the human $P2Y_{14}$ receptor was recently probed.[13] Nearly all modifications of the uracil or ribose moieties abolished activity, suggesting that the binding pocket of the $P2Y_{14}$ receptor is among the least permissive for ligand modification among the P2Y receptors.[13] However, a 2-thiouracil modification in **2** (MRS2690) increased potency by sevenfold, and the corresponding 4-thio analogue **3** was equipotent to **1**. Molecular modeling of the human $P2Y_{14}$ receptor based on a rhodopsin template and ligand docking have aided in visualizing putative molecular interactions within the binding site.[13,16,18] Agonist ligand docking correctly predicted potent agonism of UDP-fructose, UDP-mannose, and UDP-inositol. The hexose moiety of **1** is proposed to interact with multiple H-bonding and charged resides and therefore may provide a fertile region for agonist modification.

In this study, we have further explored the receptor binding regions of the glucose, diphosphate, ribose, and uracil moieties of **1**. The glucose moiety was examined in greatest detail. Modifications included replacing glucose with other sugars, substitution of hydroxyl groups of glucose with fluorine, and chain extension. The pharmacological activities of analogues of **1** were characterized using a functional assay at the recombinant human $P2Y_{14}$ receptor. Activity of this receptor was conveniently followed through the stimulation of phosphoinositide hydrolysis, made possible by coexpression in COS-7 cells with a chimeric G protein that responds to G_i-coupled receptors.

2. Results and discussion

2.1. Chemical synthesis

Two major objectives of this study were to explore the SAR at the distal end of these uracil nucleotides, corresponding to the glucose region of the $P2Y_{14}$ receptor agonist **1**, and to identify the receptor-preferred conformation of the ribose moiety. Thus, analogues of **1** were synthesized with extensive modification in the sugar rings and limited modification in the nucleobase (Table 1).

The $P2Y_{14}$ receptor agonist activities of some glucose-modified and uracil-modified nucleotide analogues of UDPG have already been reported, and these results have been included in Table 1 for structural comparison.[13] For example, the 2-thio analogue **2** had enhanced potency while the 4-thio analogue **3** had potency comparable to **1**.[13] In this case, the modification of the uracil ring through replacement of the 2- or 4-oxygen atom by sulfur was compared to its replacement of uracil by N^4-methoxycytidine to give **4**.

In this study, replacement of glucose with various sugars produced sugar-modified derivatives of **1** ([1″]hexoses **5–9**, [6″]hexoses **18–20**, and pentoses **21–24**) that retain an unmodified uracil nucleobase. In many cases commercially available sugar monophosphates were employed. In another series, fluorine was substituted on the glucose moiety to produce compounds **11–14.** All of the nucleotide analogues were prepared according to the methods shown in Schemes 1–5 and isolated either as an ammonium or a triethylammonium (in cases where the final purification was by preparative HPLC) salt. The purified nucleotides were tested in functional assays for the $P2Y_{14}$ receptor and in selected cases, for the $P2Y_2$ receptor.

The nucleotide derivatives of **1** were obtained either by adaptation of previous methods (Schemes 1–3 and Scheme 5) or by using a new reaction sequence (Scheme 4). Compounds **11–14** were synthesized from the corresponding fluorinated sugar intermediates (**34–37**) which were prepared using known procedures (Scheme 1A).[19] The sodium or lithium salt of the appropriate sugar monophosphate was exposed to a cation-exchange resin (H^+) and neutralized with tributylamine. Addition of commercially available uridine 5′-monophosphate morpholidate (as the 4-morpholine-*N*,*N*-dicyclohexylcarboxamidine salt form) to a solution of the sugar monophosphate tributylammonium salt in DMF resulted in a condensation reaction to afford analogues **5**, **8**, **11–14**, **18–22**, and **24**, as shown in Scheme 1. The nucleotide analogues were characterized using HPLC, nuclear magnetic resonance (^{1}H NMR, ^{31}P NMR), and high resolution mass spectrometry.

N^4-Methoxycytidine-5′-diphosphoglucose (**4**) was synthesized as shown in Scheme 2. First, the reaction of uridine and *O*-methylhydroxylamine hydrochloride in pyridine afforded nucleoside **40**.[20] This was monophosphorylated with phosphorous oxychloride and then treated with cation-exchange resin and tributylamine, as above, to give the N^4-methoxycytidine-5′-monophosphate tributylammonium salt **41**. Compound **41** was activated with 1,1′-carbonyldiimidazole and then condensed with the tributylammonium salt of glucose 1-monophosphate to yield compound **4** (Scheme 2).

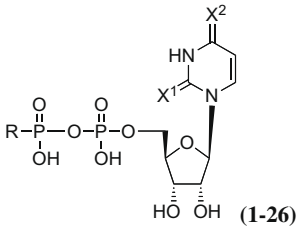
The uridine-5′-diphosphoglucuronic acid derivatives **16** (containing a 2-aminoethylamino moiety) and **17a** and **17b** (containing terminally acylated chains) were prepared by EDC coupling of **42** with the corresponding ethylenediamine derivative at pH 4.5–5.0 (Scheme 3).

A phosphonate derivative **26**, in which the oxygen bridge of **1** at the 1″ position of glucose was replaced with a methylene bridge, was prepared as described previously.[21]

In other modifications, the ribose ring of **1** was replaced with rigid bicyclo[3.1.0]hexane ring systems, that is, South (S) and North (N) methanocarba rings, to give **27** and **28**, respectively, and also with a simple carbocyclic ring to produce **29**. The methanocarba rings lock the ribose-like moiety into conformations that approximate the two conformations of freely twisting ribose that correspond to the most likely biologically active conformations of nucleosides and nucleotides.[22–24] These ring systems have been used extensively to probe the conformational requirements of the ribose moiety at other P2Y receptors and adenosine receptors. We recently reported the enantioselective synthesis of the (S)-methanocarba analogue of uridine **43**,[25] and we applied this to the preparation of **27**. We previously found the corresponding sterically constrained (S)-methanocarba analogue of 2′-deoxy-UDPG to be inactive at the $P2Y_{14}$ receptor.[13] Nonetheless, since 2′-deoxy-UDPG also was inactive,[13] we reasoned that the (S)-methanocarba analogue of **1** might be active, since **27** corresponds to the active riboside (UDPG) rather than the inactive deoxy-riboside (deoxy-UDPG).

For the synthesis of **27**, compound **43**[25] was monophosphorylated with phosphorous oxychloride to provide the 5′-monophosphate and this was treated as above with cation-exchange resin and tributylamine. The resulting (S)-methanocarba analogue of uridine 5′-monophosphate tributylammonium salt **44** was activated with 1,1′-carbonyldiimidazole at room temperature, and the intermediate containing a 2′,3′-cyclic carbonyl group was

Table 1
In vitro pharmacological data for UDPG, **1**, and its analogues in the stimulation of PLC at recombinant human $P2Y_{14}$ receptors expressed in COS-7 cells transiently transfected with $hP2Y_{14}$ and $G\alpha_{qi}$



(1-26)

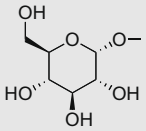
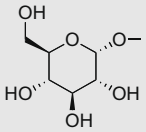
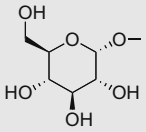
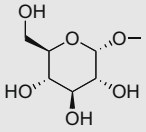
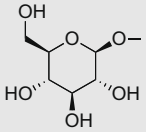
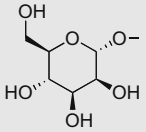
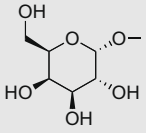
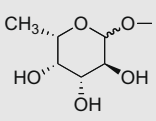
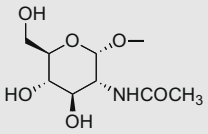
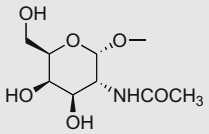
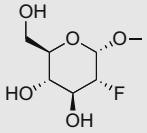
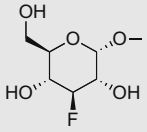
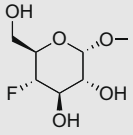
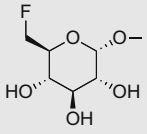
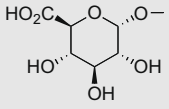
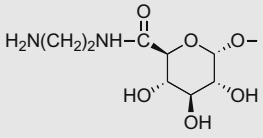
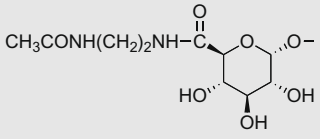
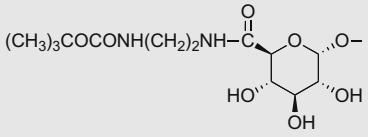
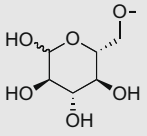
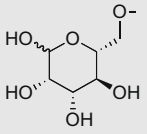
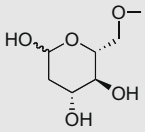
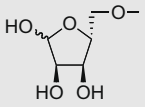
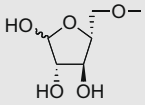
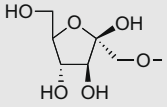
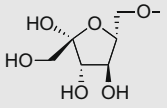
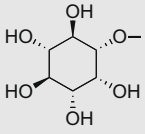
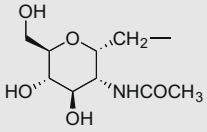
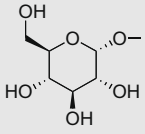
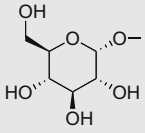
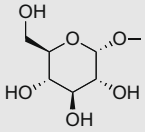
Compound	Modification	Structure R=	EC_{50} at $hP2Y_{14}$ receptor[a] (μM)
1	UDP-[1″]glucose		0.261 ± 0.053
2[b,c]	2-Thio-UDP-[1″]glucose,	 $X^1 = S$	0.049 ± 0.002
3[b,c]	4-Thio-UDP-[1″]glucose,	 $X^2 = S$	0.29 ± 0.16
4	N^4-Methoxy-CDP-[1″]glucose	 X^2 = N-OMe	<50% max at 10 μM
5	UDP-β-[1″]glucose		0.588 ± 0.130
6[b]	Up_2-[1″]mannose		0.910 ± 0.150
7	UDP-[1″]galactose		0.670 ± 0.090
8	Up_2-[1″]fucose		0.562 ± 0.173
9[b]	Up_2-[1″]*N*-Ac-glucosamine		4.38 ± 1.05

Table 1 (*continued*)

Compound	Modification	Structure R=	EC_{50} at hP2Y_{14} receptor[a] (μM)
10[b]	Up_2-[1″]*N*-Ac-galactosamine		0.810 ± 0.090
11	UDP-2″-F-[1″]2″-deoxy glucose		2.5 ± 0.9
12[c]	UDP-3″-F-[1″]3″-deoxy glucose		0.361 ± 0.094
13	UDP-4″-F-[1″]4″-deoxy glucose		0.567 ± 0.156
14	UDP-6″-F-[1″]6″-deoxy glucose		0.905 ± 0.429
15	UDP-[1″]glucuronic acid		0.370 ± 0.070
16	UDP-[1″]glucuronyl-ED		2.59 ± 1.59
17a[c]	UDP-[1″]glucuronyl-ED-Ac		0.496 ± 0.067
17b	UDP-[1″]glucuronyl-ED-Boc		0.951 ± 0.277
18	UDP-[6″]glucose		0.373 ± 0.073
19	Up_2-[6″]mannose		0.658 ± 0.022

(*continued on next page*)

Table 1 (*continued*)

Compound	Modification	Structure R=	EC_{50} at hP2Y_{14} receptor[a] (μM)
20	Up_2-[6″]2″-deoxyglucose		<50% max at 10 μM
21[c]	Up_2-[5″]ribose		0.238 ± 0.084
22	Up_2-[5″]arabinose		0.460 ± 0.057
23[b]	Up_2-[1″]fructose		0.880 ± 0.210
24	Up_2-[6″]fructose		0.323 ± 0.069
25	UDP-inositol		1.88 ± 1.10[b]
26	UDP-[2″-(acetylamino)-2″-deoxy α-D-glucopyranosyl] methyl phosphonyl uridine 5′yl phosphate		<50% max at 10 μM
27[d]	(S)mc-UDP-[1″]glucose (single enantiomer)		NE
28[d]	(N)mc-UDP-[1″]glucose (single enantiomer)		NE
29[d]	Carbocyclic-UDP-[1″]glucose		<50% max at 10 μM

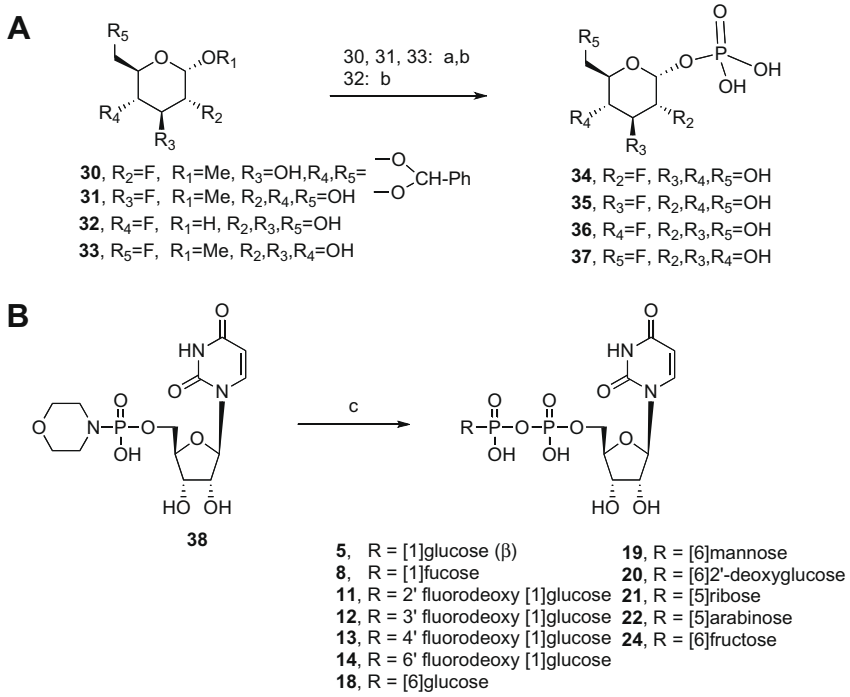
ED, ethylenediamine.
NE, no effect at 10 μM.
Unless noted: X^1, X^2 = O.

[a] Agonist potencies reflect stimulation of PLC, unless noted, and were calculated using a four-parameter logistic equation and the GraphPad software package (GraphPad, San Diego, CA). EC_{50} values (mean ± standard error) represent the concentration at which 50% of the maximal effect is achieved. Relative efficacies (%) were determined by comparison with the effect produced by a maximal effective concentration of reference agonist (uridine-5′-diphosphoglucose, **1**) in the same experiment. If no maximal effect is given, then 100% efficacy was achieved. $N = 3$, except for **1** ($n = 17$) and **8** ($n = 4$).

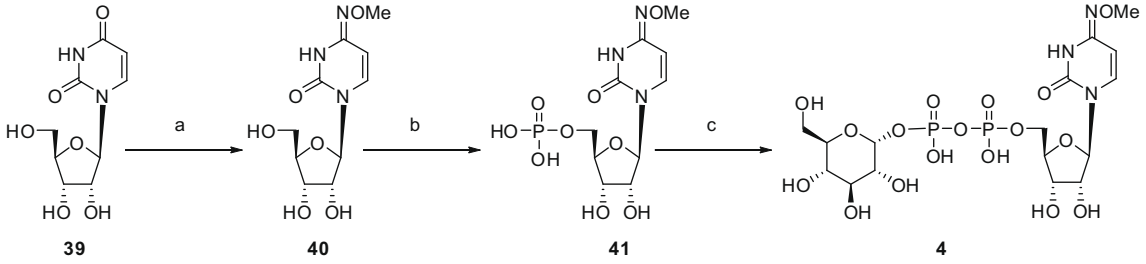
[b] Potency at P2Y_{14} receptor reported in Ko et al.[13] and Ivanov et al.[18]

[c] Compound **2**, MRS2690; **3**, MRS2670; **12**, MRS2825; **16**, MRS2820; **21**, MRS2738.

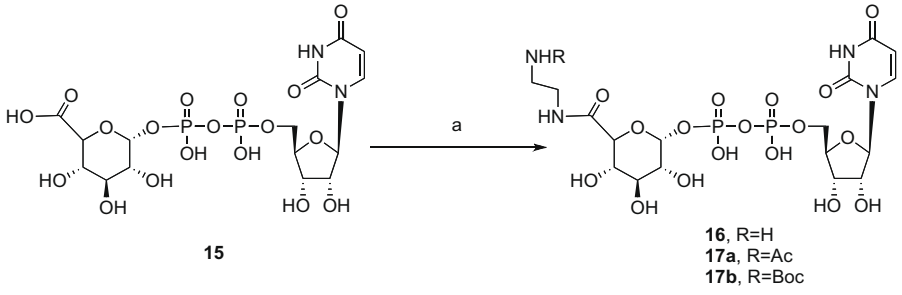
[d] Structures are given in Schemes 4 and 5.



Scheme 1. Preparation of monophosphorylated sugar intermediates (A) to be applied to the synthesis of analogues of **1**, as shown in (B). Reagents and conditions: (a) (i) 50% TFA, 100 °C, (ii) Ac_2O, sodium acetate, 110 °C; (b) (i) H_3PO_4, 50 °C, (ii) LiOH, rt; (c) $ROPO_3H_2$, DMF, rt.



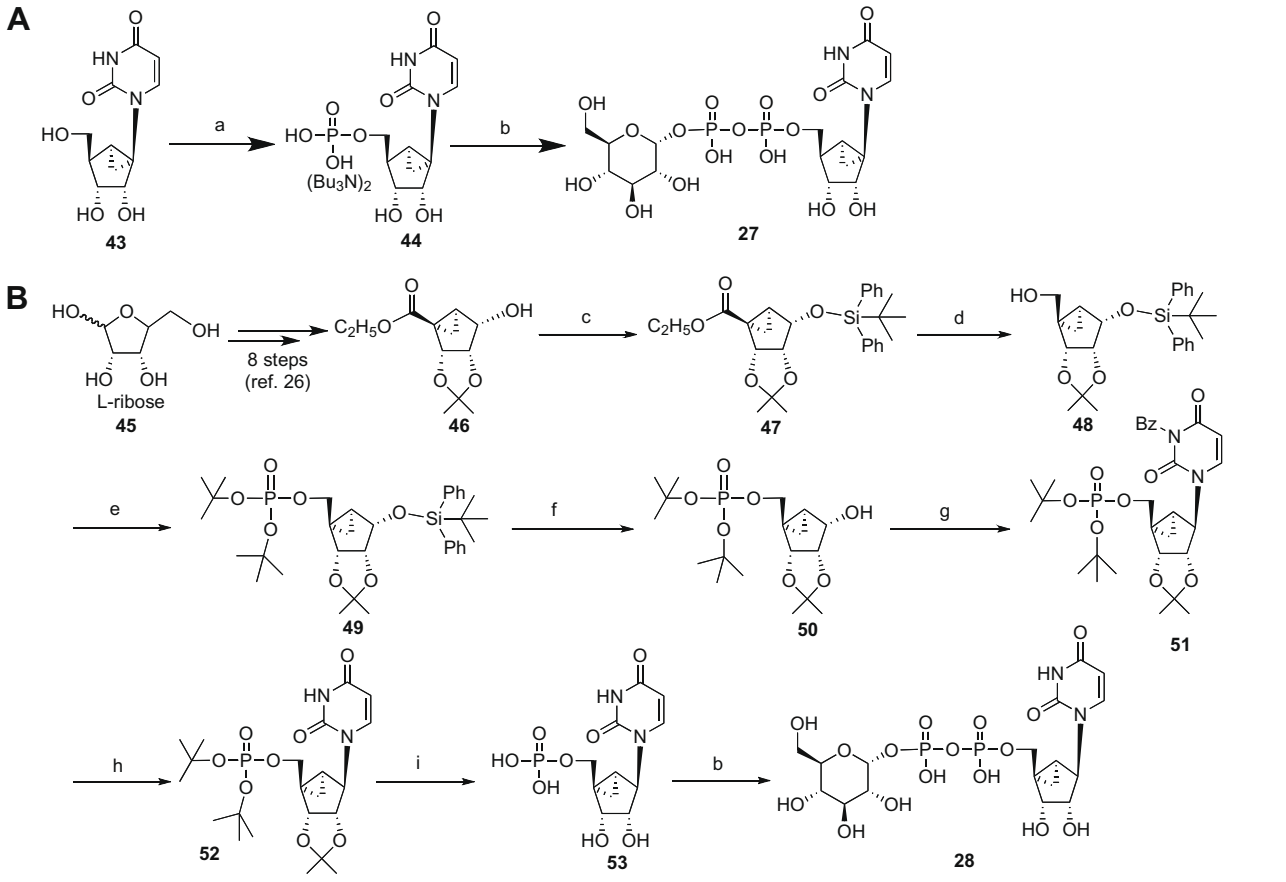
Scheme 2. Synthesis of an N^4-methoxycytidine derivative **4**. Reagents and conditions: (a) $MeONH_2$.HCl, Py, 100 °C; (b) (i) $POCl_3$, Proton Sponge, $PO(OMe)_3$, 0 °C, (ii) 0.2 M triethylammonium bicarbonate, rt; (c) (i) CDI, DMF, rt, (ii) 5% TEA in 1/1 MeOH/H_2O, (iii) glucose 1-monophosphate tributylammonium salt, DMF, rt.



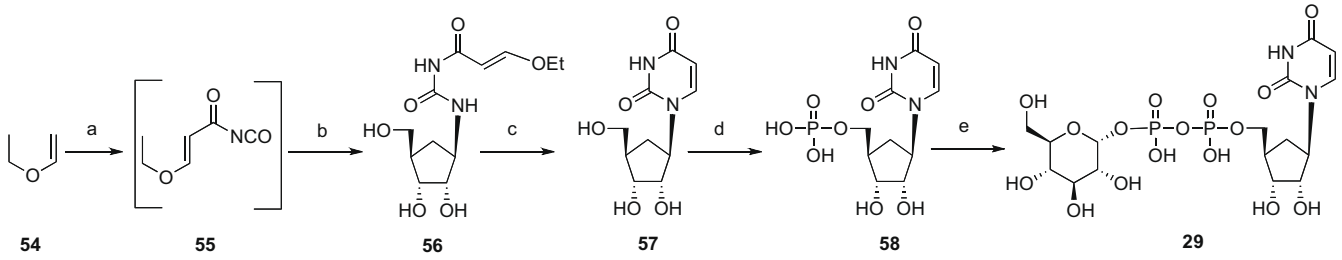
Scheme 3. Synthesis of extended chain amide derivates of uridine-5′-diphosphoglucuronic acid, compounds **16** and **17**. Reagents and conditions: (a) EDC–HCl, $RNHCH_2CH_2NH_2$ (**42**), pH 4.5–5.0, rt.

hydrolyzed with methanol and triethylamine. The activated intermediate was directly condensed with a tributylammonium salt of glucose 1-monophosphate in DMF to afford compound **27**, the (S)-methanocarba analogue of **1** (Scheme 4A).

The corresponding (N)-methanocarba analogue **28** was synthesized from L-ribose as shown in Scheme 4B. The multistep enantioselective synthesis of the key early intermediate **46** was performed as recently reported.[26] However, our synthetic route beyond compound **46** utilized a new approach to the (N)-methanocarba nucleotide analogues in the riboside series, in which the bicyclic moiety was pre-phosphorylated at the 5-position prior to the condensation with the nucleobase. Silylation of compound **46** with TBDPS-Cl followed by DIBAL-H reduction gave **48**, which was phosphorylated with di-*tert*-butyl-*N,N*-diethylphosphoramidite to



Scheme 4. Synthesis of (S)- and (N)-methanocarba analogues of **1**, compounds **27** (A) and **28** (B), respectively. Reagents and conditions: (a) (i) $POCl_3$, Proton Sponge, $PO(OMe)_3$, 0 °C, (ii) 0.2 M triethylammonium bicarbonate, rt; (b) (i) CDI, DMF, rt, (ii) 5% TEA in 1/1 MeOH/H_2O, (iii) glucose 1-monophosphate tributylammonium salt, DMF, rt; (c) imidazole, TBDPS–Cl; (d) DIBAL-H, DCM, −78 °C, 2 h, then rt 30 min; (e) di-*tert*-butyl-*N*,*N*-diethyl phosphoramidite, tetrazole, THF, rt, 1 h, then MCPBA, −78 °C; (f) TBAF, THF; (g) Ph_3P, 3-Bz-uracil, DIAD, THF; (h) NH_3, EtOH; (i) ion-exchange resin (H^+), MeOH, 80 °C; then 0.2 M TEAB, rt.



Scheme 5. Synthesis of the carbocyclic derivative **29** of UDPG **1**. Reagents and conditions: (a) *N*-(chlorocarbonyl)-isocyanate, THF, 0 °C then Et_3N, 0 °C; (b) (1*R*,2*S*,3*R*,4*R*)-2,3-dihydroxy-4-(hydroxymethyl)-1-aminocyclopentane hydrochloride, −40 °C; (c) 2 N H_2SO_4, 100 °C; (d) (i) $POCl_3$, Proton Sponge, $PO(OMe)_3$, 0 °C; (ii) 0.2 M triethylammonium bicarbonate, rt; (e) (i) 1,1′-carbonyldiimidazole, DMF, rt, (ii) 5% TEA in 1/1 MeOH/H_2O, (iii) glucose 1-monophosphate tributylammonium salt, $MgCl_2$, DMF, rt.

afford **49**. Deprotection of silylated compound **49** by TBAF followed by Mitsunobu condensation with a benzoyl-protected uracil gave compound **51**. This protected nucleotide was deprotected by sequential treatment with ammonia, Dowex 50 resin in the acid form, and tributylamine to afford the (N)-methanocarba analogue of uridine 5′-monophosphate **53** as the tributylammonium salt. The monophosphate **53** was then condensed with glucose 1-monophosphate using CDI in DMF to afford compound **28**, the (N)-methanocarba analogue of **1**.

Compound **29**, the carbocyclic analogue of **1**, was prepared from the cyclopentylamine derivative (1*R*,2*S*,3*R*,4*R*)-2,3-dihydroxy-4-(hydroxymethyl)-1-aminocyclopentane by the route shown in Scheme 5.[27,28] Reaction of *N*-(chlorocarbonyl) isocyanate with ethyl vinyl ether **54** afforded intermediate **55** which was treated with the carbocyclic amine to form intermediate **56**. Compound **56** was cyclized using sulfuric acid (2 N) to give the carbocyclic analogue of uridine, compound **57**. Compound **57** was monophosphorylated with phosphorous oxychloride and the product was treated with cation-exchange resin (H^+) and tributylamine to afford the carbocyclic 5′-monophosphate tributylammonium salt **58**. This 5′-monophosphate derivative was activated with 1,1′-carbonyldiimidazole, and the activated complex was treated with the tributylamine salt of glucose 1-monophosphate in DMF in the presence of magnesium chloride to give the carbocyclic analogue **29**.

2.2. Quantification of pharmacological activity

Activation of PLC was quantified in COS-7 cells transiently expressing the human $P2Y_{14}$ receptor and an engineered G protein

($G\alpha_{qi5}$) that allows the coupling of G_i-coupled receptors to activation of the phosphoinositide signaling pathway.[14] Thus, inositol lipid hydrolysis[29,30] served as a measure of agonist activity at the G_i-coupled $P2Y_{14}$ receptor.

The N^4-methoxycytidine derivative **4** was only weakly active at the $P2Y_{14}$ receptor. Inversion of the glycoside linkage in the β-[1″]glucose derivative **5** reduced the potency as a $P2Y_{14}$ receptor agonist by only twofold. As reported previously, uridine-5′-diphosphogalactose **7**, uridine-5′-diphospho-*N*-acetylglucosamine **9**, and uridine-5′-diphosphoglucuronic acid **15** were $P2Y_{14}$ receptor agonists with potencies similar to or less than that of **1**. Many modified sugar moieties, such as [5″]ribose **21**, [6′]fructose **24**, and [6″]glucose **18**, may be substituted for glucose with retention of activity. The most potent analogue among simple uridine-5′-diphosphosugar derivatives was the [5″]ribose derivative **21**, which exhibited an EC_{50} value of 0.24 μM. Inversion of the configuration of the 2″-hydroxyl group in **22** decreased potency twofold. Alternate attachment of the glucose moiety at the 6″ position in **18** maintained nearly the same potency as **1**. However, the corresponding [6″]2″-deoxyglucose analogue **20** was much less potent than **1** at the $P2Y_{14}$ receptor, achieving less than 50% of full receptor activation at 10 μM (Fig. 1A). Compound **20** differs from **18** and **19** only in the absence of one hydroxyl group. Thus, moderate sensitivity to structural changes exists in this region of the binding site.

Fluorodeoxy[1″]glucose isomers were synthesized to probe the importance of H-bonding in the recognition of **1** at the receptor. The 2″-deoxy-2″-fluoro analogue **11** and 6″-deoxy-6″-fluoro analogues **14** were 9.6-fold and 3.5-fold less active at the $P2Y_{14}$ receptor, respectively. In contrast, the 3″-deoxy-3″-fluoroanalogue **12** retained $P2Y_{14}$ receptor potency (Fig. 1B) 4″-deoxy-4″-fluoroanalogue **13** was twofold less potent. Thus, the potencies of fluorodeoxyglucose analogues of **1** were in the rank order of 3″-F > 4″-F > 6″-F > 2″-F. These results indicate a contribution to binding stabilization from H-bond donation by the 2″- and 6″-hydroxyl groups.

Replacement of the ribose moiety of **1** with a rigid methanocarba group, either in a North **28** or South **27** conformation, abolished agonist activity. The simple carbocyclic (cyclopentane) analogue **29** at 10 μM produced <20% of full activation of the receptor. Thus, the active, $P2Y_{14}$ receptor-preferred conformation of the ribose moiety could not be determined. In comparison to other P2Y subtypes, a clear preference for the North conformation at the $P2Y_1$ receptor and the South conformation at the $P2Y_6$ receptor was identified by similar structural modification of the respective ligands. A possible explanation for the inability of any of these carbocyclic analogues to activate the $P2Y_{14}$ receptor would be that the ring oxygen participates in recognition, but there may also be conformational factors.

Uridine-5′-diphosphoglucuronic acid **15** served as the basis for functionalized congeners **16** and **17**, in which an amide-linked chain was extended from the carboxylic acid. Functionalized congeners containing terminal 2-acylaminoethylamides **17a** and **17b** retained the ability to fully activate the $P2Y_{14}$ activity, with EC_{50} values of 496 and 951 nM, respectively. An amine functionalized congener **16** containing a terminal 2-aminoethylamino-[1″]glucuronic acid moiety was less potent, but still fully activated the receptor. The biological activities of **16**, **17a**, and **17b** further illustrate that this region in nucleotide sugar molecules is suitable for extensive structural modification and appears to occupy a relatively insensitive region of the putative binding site of the $P2Y_{14}$ receptor. Thus, the glucose moiety is the most structurally permissive region of the nucleotides for derivatization as $P2Y_{14}$ receptor agonists, although substantial variation in potency may be induced by structural modification in this region.

The P2Y receptor subtype selectivities of **1** and its various derivatives also were explored. Although **1** is the principal native agonist of the $P2Y_{14}$ receptor, it was also found to be a weak full agonist with an EC_{50} of 10 μM (data not shown) at the human $P2Y_2$ receptor stably expressed in 1321N1 cells. To probe the selectivity of the present nucleotide derivatives in activation of the $P2Y_{14}$ receptor, several of the most potent compounds were tested at the human $P2Y_2$ receptor expressed in 1321N astrocytoma cells for activation of PLC. They were found to be inactive at concentrations up to 10 μM. These $P2Y_2$ receptor-inactive analogues include ($P2Y_{14}$ receptor EC_{50} values in μM indicated): **2** (0.049), **12** (0.36), **17a** (0.50), and **24** (0.32). Therefore, the selectivity of these analogues for the $P2Y_{14}$ receptor was improved in comparison to **1**. This demonstrates an advantage for their use as pharmacological probes.

2.3. Molecular modeling

We constructed a new homology model of the human $P2Y_{14}$ receptor that is based on the high resolution X-ray crystallographic structure of the A_{2A} adenosine receptor, rather than rhodopsin.[31,32] The putative binding modes of the native agonist **1** and its long

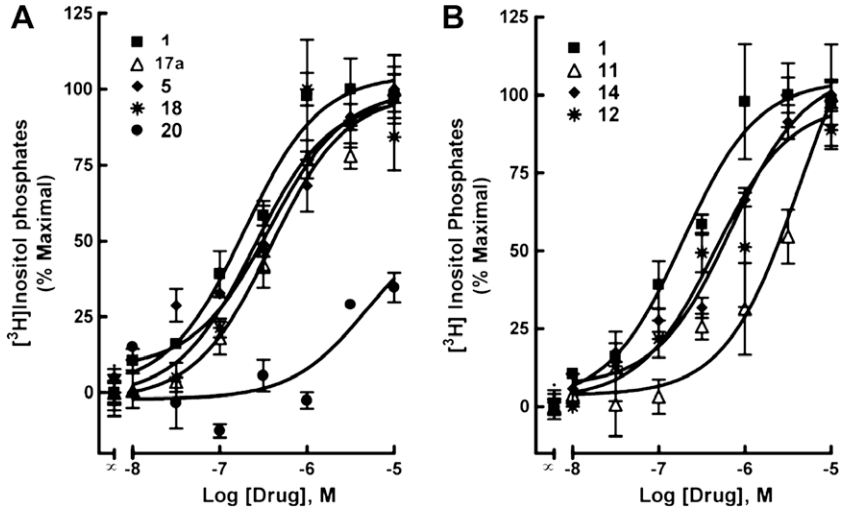


Figure 1. Activation of PLC by derivatives of UDPG **1** in COS-7 cells expressing both the human $P2Y_{14}$ receptor and an engineered Gα-q/i protein that allows the G_i-coupled receptor to stimulate inositol phosphate hydrolysis by PLC. (A) Effects on potency of attachment of an acetylaminoethyl amide-linked chain on a glucuronic acid moiety **17a**; a β-glucosyl linkage **5**; alternate attachment of the glucose moiety at the 6″ position **18** and its 2″-deoxy analogue **20**. (B) Variation of potency among fluorodeoxyglucose derivatives of UDPG (2″-F, **11**; 3″-F, **12**; 6″-F, **14**).

chain functionalized congener **17a** were studied in this model using molecular docking and Monte-Carlo Multiple Minimum (MCMM) calculations[33] as described in the Experimental Section. The UDP moieties of **1** and **17a** had the same position and orientation inside the receptor. The extended 2-acetylaminoethylamide chain of **17a** was favorably located in proximity to the second and third extracellular loops (EL2 and EL3), consistent with this being a structurally permissive region of the nucleotide.

In agreement with our previous studies using rhodopsin-based homology modeling,[13,18] the following ligand–receptor interactions were observed in the final models (Fig. 2). The uracil rings of **1** and **17a** were surrounded by Tyr29 (1.39), Met70 (2.53), Pro75 (2.58), Ile78 2.61), and Ala285 (7.43). The numbers in parentheses refer to the Ballesteros–Weinstein numbering system for residues in the TMs of GPCRs.[34] Also, the uracil ring of the compounds was involved in π–π interactions with the phenyl ring of Phe101 (3.32), which is conserved in the P2Y family.[35]

Differences between the two models were also observed. Previously, it was proposed that the 2′-and 3′-OH groups of the ribose moiety could interact with Asn104 (3.35) and Asn287 (7.45), respectively.[13] In the present model, these Asn residues were located far from the ribose OH-groups. However, both 2′-and 3′-OH groups of **1** and **17a** were found in close proximity to Tyr102 (3.33) and Ser284 (7.42). In the rhodopsin-based model of the $P2Y_{14}$ receptor,[13,18] the ribose ring was located deeper within the binding cavity, which might be related to a different conformation of EL2.[36] In contrast, in the present A_{2A} receptor-based model in which EL2 is more extended and less constraining, the ribose ring is higher and can interact with Tyr102 and Ser284.

Surprisingly, the ribose rings of **1** and **17a** showed preferences for different conformations. Molecular docking revealed that the South conformation is most favorable for the ribose ring of **1.** In contrast, the results obtained for **17a** indicated a preference for the North conformation of the ribose ring. In agreement with previous results of molecular modeling of P2Y receptors, the negatively charged phosphate chains of **1** and **17a** were located near the positively charged Arg253 (6.55) and Lys277 (7.35). The third conserved residue previously predicted to interact with the phosphate chain, Lys171 (EL2),[35] was located far from the ligand. In the present model, Lys171 is the last residue of the modeled part of EL2, and the modeling of preferred orientation of this residue as well as the modeling of the entire EL2 is problematic. Future experiments using site-directed mutagenesis of both positively charged residues and OH-containing residues of the $P2Y_{14}$ receptor could be used to explore the role of Lys171 (EL2) and H-bonding in interaction with the ligand.

The following interactions between the $P2Y_{14}$ receptor and functional groups of the glucose moiety of the ligands were observed. The 6″-OH-group of the glucose ring of **1** was H-bonded to Lys277 (7.35). The side chains of Arg274 (EL3) and Glu278 (7.36) formed H-bonds with the 4″-OH group. Also, the carboxylic oxygen atom of Glu278 was found at a distance of 3.6 Å from the 3″-OH group of the glucose ring of **1.** The 2″-OH group of the glucose ring of **1** formed an H-bond with Glu174. Similar interactions were observed between the $P2Y_{14}$ receptor and the glucose moiety of **17a**. In addition, in the model of $P2Y_{14}$—**17a**, the distal amido group of the functionalized chain of **17a** was located between Arg274 (EL3) and Lys277 (7.35). Lys277 was also found in proximity to the terminal acetyl oxygen atom of **17a** and thus could be involved in H-bonding with the ligand acetyl group (Fig. 3).

3. Conclusions

New ligands recently have become available for characterization of the P2Y receptors.[37] In the present study, we have further expanded the range of potent $P2Y_{14}$ receptor ligands through a systematic exploration of the SAR of analogues of **1** at this receptor, particularly with respect to the glucose moiety (Fig. 4). We have demonstrated that the glucose moiety is the most structurally permissive region of the ligand for derivatization. For example, replacement of glucose with other sugar moieties produced analogues that retained $P2Y_{14}$ potency, an example being the analogue **21** (EC_{50} 0.24 μM) that has the [5″]ribosyl group in place of glucose. This permissive nature of the sugar moiety will facilitate the development of new covalent chain attachment through a 5′-carboxylic group.

Other modifications of **1** presented in this study produced analogues that retained activity but with reduced potency at the $P2Y_{14}$ receptor. Many modified sugar moieties, such as ribose, fructose,

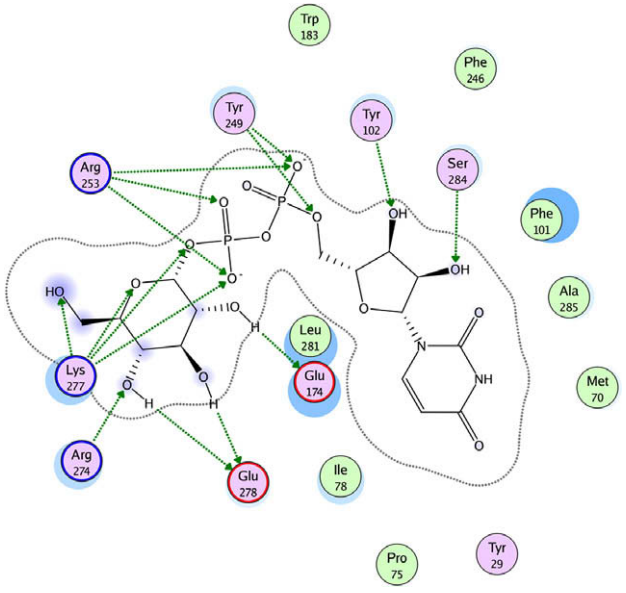


Figure 2. Schematic representation of the putative docking mode of UDPG **1** at the human $P2Y_{14}$ receptor. The figure was generated with MOE software.

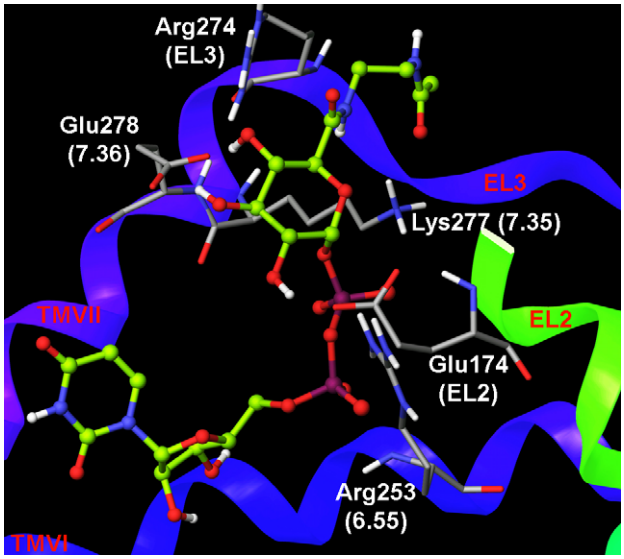


Figure 3. Compound **17a** docked to the $P2Y_{14}$ receptor. The glucose ring of the ligand is surrounded by several cationic and anionic residues. The terminal acetyl group of the functionalized chain of **17a** can be H-bonded to Lys277 (7.35). The numbers in the brackets correspond to the Ballesteros–Weinstein numbering system for GPCRs.[34] The carbon atoms of **17a** are colored in green.

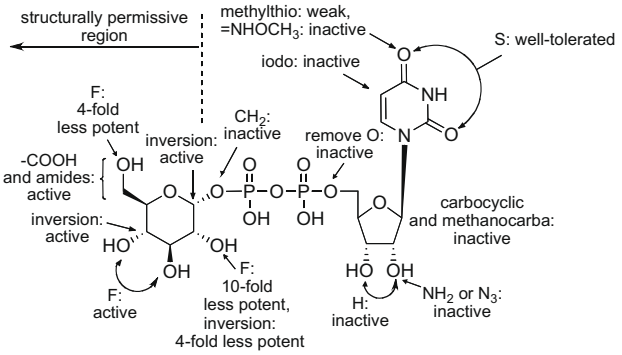


Figure 4. SAR patterns for analogues of **1** in activation of the human $P2Y_{14}$ receptor. Data are from present study and Refs. 13,18,38.

and various fluorinated glucose moieties, may be substituted for the glucose with retention of activity. In some cases, subtle changes in structure of the terminal sugar greatly influenced the potency, for example, the potent [6″]glucose **18** and [6″]mannose **19** analogues and their weakly active 2″-deoxy equivalent **20**. Evaluation of the monofluorinated glucose analogues allowed us to conclude that the 2″- and 6″-hydroxyl groups of **1** are more important for receptor recognition than other hydrogen bond donors of the glucose moiety. The β-glucoside of **1** was only twofold less potent than the native α-isomer, while replacement of the 1″-oxygen of the glucose moiety with CH_2 abolished activity.

Introduction of a rigid carbocyclic ring system that constrains the pseudoribose moiety in a North or South conformation or a simple cyclopentane ring abolished agonist activity. Therefore, we were unable to identify the active ribose conformation for the $P2Y_{14}$ receptor in contrast to our ability to do so for other P2Y subtypes using this strategy.

Compound **1** also activates the $P2Y_2$ receptor, but the previously reported potent 2-thio analogue **2** and several of the potent modified-glucose analogues were selective for the $P2Y_{14}$ receptor. Thus, we have discovered nucleotide analogues that have increased selectivity as agonists of the $P2Y_{14}$ receptor.

With the exception of 2- and 4-thio modifications, most modifications of the nucleobase or ribose moieties diminished the activity of **1** at the $P2Y_{14}$ receptor.[13] However, we have developed a strategy to preserve the potency and efficacy at the $P2Y_{14}$ receptor using a series of functionalized congeners containing a terminal 2-(acylamino)ethylamino-[1″]glucuronic acid moiety. Molecular modeling predicts the placement of a terminal acylaminoethylamino-[1″]glucuronic acid moiety to be close to EL2 of the $P2Y_{14}$ receptor. The flexibility of substitution at this position of the nucleotide series suggests that this site on the nucleotide is suitable for attachment to larger carrier moieties. Thus, analogues with chain extension at this site constitute functionalized congeners for probing the $P2Y_{14}$ receptor. Therefore this structural lead is potentially applicable to the design of a wide range of receptor probes, including fluorescent tracers and polymeric ligands. Work is in progress to explore these possibilities.

4. Experimental

4.1. Chemical synthesis

^1H NMR spectra were obtained with a Varian Gemini 300 spectrometer using D_2O 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 using a Varian XL 300 spectrometer (121.42 MHz) with orthophosphoric acid (85%) as an external standard.

The course of reaction and the purity of the final nucleotide derivatives were determined using a Hewlett–Packard 1100 HPLC equipped with a Zorbax Eclipse 5 mm XDB-C18 analytical column (250 × 4.6 mm; Agilent Technologies Inc, Palo Alto, CA), using a linear gradient solvent system: 5 mM TBAP (tetrabutylammonium dihydrogenphosphate)–CH_3CN from 80:20 to 40:60 in 20 min with a flow rate of 1 mL/min. Peaks were detected by UV absorption (254 nm) using a diode array detector. All derivatives tested for biological activity were shown to be at least 97% pure using this analytical HPLC system.

High-resolution mass measurements were performed on a Micromass/Waters LCT Premier Electrospray Time of Flight (TOF) mass spectrometer coupled with a Waters HPLC system. 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) ammonium bicarbonate as the mobile phase. This led to the isolation of the ammonium salt forms of the desired nucleotide derivatives. Some of the compounds were additionally purified, as needed, by HPLC leading to the isolation of the triethylammonium salt forms of the nucleotide derivatives. The semipreparative HPLC system consisted of a Luna 5 μ RP-C18(2) semipreparative column (250 × 10.0 mm; Phenomenex, Torrance, CA) using as mobile phase a linear gradient of 10 mM aqueous TEAA (triethylammonium acetate)–CH_3CN, from 100:0 to 95:5 (or up to 99:1–90:10) in 30 min, and a flow rate of 2 mL/min. Unless noted otherwise, reagents and solvents were purchased from Sigma–Aldrich (St. Louis, MO). The nucleotide analogues were stored at −20 °C.

4.2. General procedure for the preparation of non-fluorinated uridine 5′-diphosphosugar analogues

Uridine 5′-monophosphate morpholidate (4-morpholine-*N*,*N*-dicyclohexyl-carboxamidine salt) **38** (20 mg, 0.029 mmol) was dissolved in DMF (2 mL). The corresponding sugar monophosphate (0.035 mmol, tributylammonium salt form by treatment with Dowex 50WX2-200 ion-exchange resin (H) and tributylamine) was added to the solution. The reaction mixture was stirred at room temperature for 1–2 days (Scheme 1). Solvent was removed under reduced pressure and the resulting residue was purified by ion-exchange column chromatography using a Sephadex–DEAE A-25 resin and a mobile phase consisting of an increasing gradient of aqueous ammonium bicarbonate (0.01–0.5 M) to afford the corresponding nucleotides as their ammonium salts. Some of the compounds were additionally purified by HPLC as described above to afford the nucleotide triethylammonium salts. Data for compounds **5**, **8**, **18–22**, and **24** are provided in Supplementary data.

4.3. General procedure for the preparation of uridine-5′-(fluorodeoxyglucose-1″-diphosphate) triethylammonium salts (11–14)[19,39]

The appropriate protected fluorodeoxyglucose derivative (compounds **30**, **31**, and **33**, 0.76 mmol) was treated with 50% aq TFA (10 mL) for 8 h at 100 °C. The TFA was removed by co-evaporation with water using a rotary evaporator. The crude product or compound **32** was treated with a mixture of sodium acetate (50 mg) in acetic anhydride (5 mL) and the mixture was stirred for 5 h at 110 °C while the course of the reaction was followed by TLC. Excess Ac_2O was removed using a rotary evaporator, and the mixture was stirred with aq $NaHCO_3$ for 15 min. The product was extracted into DCM and washed with brine. The corresponding crude tetraacetyl derivative was purified by column chromatography. The monophosphate derivatives of the various fluorodeoxysugars (**34–37**) were prepared as the lithium salts using the MacDonald procedure.[39] The lithium salts (5.5 mg,

0.014 mmol) were converted to the tributylammonium salts by batchwise treatment with Dowex 50WX2–200 ion-exchange resin (H), and, after removal of the resin by filtration, neutralization with tributylamine. After removal of the water by lyophilization, the obtained tributylammonium salts were dried under high-vacuum overnight. The tributylammonium salt of the fluorinated sugar (10 mg, 0.04 mmol) **34–37**[19,39] dissolved in DMF (1 mL) was added to a stirred solution of uridine 5′-monophosphate morpholidate 4-morpholine-*N*,*N*-dicyclohexylcarboxamidine salt (20 mg, 0.029 mmol in DMF, 2 mL). The reaction mixture was stirred at room temperature for two days. Solvent was removed under the reduced pressure and the resulting residue was purified by ion-exchange column chromatography using a Sephadex–DEAE A-25 resin with a linear gradient (0.01–0.5 M) ammonium bicarbonate as the mobile phase to give the corresponding nucleotides as the ammonium salts. All of the uridine-5′-(fluorodeoxyglucose-1″-diphosphate) compounds (**11–14**) were additionally purified by semipreparative HPLC as described above to afford the triethylammonium salt forms. Data for these compounds are provided in Supplementary data.

4.4. Diphosphoric acid 1″-α-D-[1″]Glucopyranosyl Ester 2-((N^4-methoxy)cytidin-5′-yl)ester, triethylammonium salt (4)

A suspension of uridine (2 mmol, 0.48 g) (**39**) and *O*-methylhydroxylamine hydrochloride (4 mmol, 0.34 g) in pyridine (2 mL) was stirred at 100 °C for 4 h.[20] The reaction mixture was evaporated, and the residue was evaporated twice with toluene, triturated with chloroform, and filtered. The filtrate was evaporated, and the residue was purified by flash chromatography (chloroform–methanol, gradient of 3–10%) to afford N^4-methoxycytidine (215 mg, 0.92 mmol, 46%) (**40**). ^{1}H NMR (D_2O) δ 7.33 (d, J = 8.4 Hz, 1H), 5.95 (d, J = 5.1 Hz, 1H), 5.68 (d, J = 8.1 Hz, 1H), 4.23 (m, 3H), 3.89 (s, 3H), 3.85 (m, 2H).

A solution of N^4-methoxycytidine (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. Phosphorous oxychloride (0.008 mL, 0.08 mmol) was added in small portions. After 2 h at 0 °C, 0.2 M triethylammonium bicarbonate solution (1.5 mL) was added and the clear solution was stirred at room temperature for 1 h. After removal of solvents, the residue was purified by the method described above using Sephadex–DEAE A-25 resin. After lyophilizing the product-containing fractions, the resulting N^4-methoxycytidine-5′-monophosphate ammonium salt (5.5 mg, 0.014 mmol) was converted to the tributylammonium salt by treatment with Dowex 50WX2-200 ion-exchange resin (H) and tributylamine. After removal of water, N^4-methoxycytidine-5′-monophosphate tributylammonium salt (**41**) was dried under high-vacuum overnight.

N^4-Methoxycytidine 5′-monophosphate tributylammonium salt (**41**) and 1,1′-carbonyldiimidazole (6.0 mg, 0.036 mmol) dissolved in DMF (2 mL). The reaction mixture was stirred at room temperature for 5 h. A triethylamine solution (5%) in water/methanol (1:1, 1 mL) was added, and stirring was continued at room temperature for an additional 2 h. After removal of the solvent, the residue was dried in high vacuum and dissolved in DMF (2 mL). Glucose-1′-monophosphate tributylammonium salt (30 mg, 0.05 mmol) in DMF (0.2 mL) was added to this mixture. The reaction mixture was stirred at room temperature for two days. After removal of the solvent, the residue was purified as described in the general procedure using Sephadex–DEAE A-25 resin and semipreparative HPLC to obtain compound (**4**) (Scheme 2) (2.7 mg, 24%). ^{1}H NMR (D_2O) δ 7.25 (d, J = 8.4 Hz, 1H), 5.97 (d, J = 5.7 Hz, 1H), 5.84 (d, J = 8.1 Hz, 1H), 5.62 (dd, J = 3.3, 7.2 Hz, 1H), 4.38 (m, 2H), 4.26 (m, 1H), 4.19 (m, 2H), 3.94 (m, 1H), 3.89 (m, 1H), 3.83 (m, 1H), 3.82 (s, 3H), 3.79 (m, 1H), 3.55 (m, 1H), 3.48 (m, 1H); ^{31}P NMR (D_2O) δ −10.82 (d, J = 20.2 Hz), −12.49 (d, J = 19.5 Hz). HRMS-EI found 594.0740 $(M-H^+)^-$. $C_{16}H_{26}N_3O_{17}P_2$ requires 594.0737; purity >98% by HPLC (System A: 12.2 min).

4.5. General procedure for the preparation of uridine 5′-diphosphoglucuronic acid analogues (16, 17)

Uridine-5′-diphosphoglucuronic acid trisodium salt (**15**) (25 mg, 0.038 mmol) was dissolved in a minimum volume of water (1 mL). The mixture was treated with EDC–HCl (29 mg, 0.152 mmol) and was stirred under a nitrogen atmosphere, and then ethylenediamine, *N*-Boc-ethylenediamine, or *N*-Ac-ethylenediamine (15 equiv) was added. The pH of the reaction was adjusted to the range of 4.5–5.0 using dilute HCl. The mixture was stirred at room temperature for two days. After the removal of the solvents, the residue was purified as described in the general procedure using Sephadex–DEAE A-25 resin and HPLC to obtain compounds **16**, **17a**, and **17b**, respectively (Scheme 3).

4.5.1. Diphosphoric acid 1″-α-D-[1″](glucuronic acid-*N*-(2-amino-ethyl amide))pyranosyl ester 2-(uridin-5′-yl)ester, triethylammonium salt (16)

Compound **16** (6.5 mg, 21%) was obtained as a white solid following the general procedure, with final purification by semipreparative HPLC. ^{1}H NMR (D_2O) δ 8.07 (d, J = 8.1 Hz, 1H), 6.04 (d, J = 5.1 Hz, 1H), 6.04 (d, J = 7.8 Hz, 1H) 5.65 (dd, J = 3, 6.9 Hz, 1H), 4.43 (m, 2H), 4.38 (m, 1H), 4.26 (m, 2H), 4.01 (m, 3H), 3.17 (m, 3H), 3.59 (m, 2H); ^{31}P NMR (D_2O) δ −10.84 (dd, J = 20.7 Hz), −12.64 (d, J = 20.7 Hz); HRMS-EI found 621.0863 $(M-H^+)^-$. $C_{19}H_{29}N_4O_{18}P_2$ requires 621.0846; purity >98% by HPLC (5.0 min).

4.5.2. Diphosphoric acid 1″-α-D-[1″](glucuronic acid-*N*-(2-acetylethyl amide))pyranosyl ester 2-(uridin-5′-yl)ester, triethylammonium salt (17a)

Compound **17a** (5.9 mg, 18%) was obtained as a white solid following the general procedure, with final purification by semipreparative HPLC. ^{1}H NMR (D_2O) δ 7.96 (d, J = 8.1 Hz, 1H), 5.99 (m, 2H), 5.23 (dd, J = 3.6, 7.2 Hz, 1H), 4.34 (m, 5H), 4.01 (m, 1H), 3.82 (m, 1H), 3.59 (m, 1H) 3.34 (m, 5H), 1.98 (s, 3H); ^{31}P NMR (D_2O) δ −10.91 (dd, J = 20.2 Hz), −12.64 (d, J = 20.2 Hz); HRMS-EI found 663.0952 $(M-H^+)^-$. $C_{19}H_{29}N_4O_{18}P_2$ requires 663.0913; purity >98% by HPLC (12.9 min).

4.5.3. Diphosphoric acid 1″-α-D-[1″](glucuronic acid-*N*-(2-*t*-butyloxycarbonylethyl amide))pyranosyl ester 2-(uridin-5′-yl)ester, triethylammonium salt (17b)

Compound **17b** (3.5 mg, 10%) was obtained as a white solid following the general procedure, with final purification by semipreparative HPLC. ^{1}H NMR (D_2O) δ 7.93 (d, J = 8.4 Hz, 1H), 5.96 (m, 2H), 5.62 (dd, J = 3.6, 7.8 Hz, 1H), 4.36 (m, 5H), 3.99 (m, 1H), 3.80 (m, 1H), 3.55 (m, 1H), 3.33 (m, 2H), 3.20 (m, 3H), 1.42 (s, 9H); ^{31}P NMR (D_2O) δ −10.76 (dd, J = 19.18 Hz), −12.68 (d, J = 19.18 Hz); HRMS-EI found 721.1375 $(M+H^+)^-$. $C_{22}H_{35}N_4O_{19}P_2$ requires 721.1371; purity >98% by HPLC (14.0 min).

4.6. Diphosphoric acid 1″-α-D-[1″]glucopyranosyl Ester 2-((S)-Methanocarba-uridin-5′-yl)ester, triethylammonium salt (27)

Compound **27** was synthesized from (S)-methanocarba-uridine **43** as described below.[25a] (S)-Methanocarba-uridine (9.3 mg, 0.037 mmol) was stirred with a mixture of Proton Sponge (17 mg, 0.08 mmol) in trimethyl phosphate (1 mL) for 10 min at 0 °C. Phosphorous oxychloride (0.008 mL, 0.08 mmol) was added slowly to this reaction mixture, and the reaction was stirred for an additional 2 h at 0 °C. The reaction mixture then was treated with 0.2 M triethylammonium bicarbonate solution (1.5 mL) and stirring was continued for 1 h more at room temperature. The

solvent was removed and the residue was purified using Sephadex–DEAE A-25 resin and lyophilized. The resulting (S)-methanocarba-uridine-5′-monophosphate ammonium salt was treated with ion-exchange resin (Dowex 50WX2-200 (H)) and basified with tributylamine to form (S)-methanocarba-uridine 5′-monophosphate tributylammonium salt (5.01 mg, 0.015 mmol) **44**. The water was removed through lyophilization and the product was dried under high vacuum.

1,1′-Carbonyldiimidazole (1.0 mg, 0.006 mmol) was added to a solution of (S)-methanocarba-uridine-5′-monophosphate triethylammonium salt (1.40 mg, 0.002 mmol) (**44**) in DMF (0.5 mL) under a nitrogen atmosphere. The reaction mixture was stirred at room temperature for 5 h. Then 5% triethylamine solution in 1/1 water/methanol (1 mL) was added and stirring was continued at room temperature for an additional 2 h. After removal of the solvent, the residue was dried in high vacuum and dissolved in DMF (0.5 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 room temperature for two days. After removal of the solvent, the residue was purified as described in the general procedure using Sephadex–DEAE A-25 resin and semipreparative HPLC to obtain compound **27** (0.52 mg, 34%) as a white solid. ^{1}H NMR (D_2O) δ 7.73 (d, J = 8.1 Hz, 1H), 5.81 (d, J = 8.1 Hz, 1H), 5.58 (dd, J = 3.3, 7.2 Hz, 1H), 4.62 (m, 1H), 4.10 (t, J = 6.3 Hz, 2H), 3.80 (m, 4H), 3.47 (m, 3H), 2.34 (t, J = 6.3 Hz, 1H), 1.82 (dd, J = 4.8, 9.9 Hz, 1H), 1.67 (t, J = 5.7 Hz, 1H), 1.26 (m, 1H); ^{31}P NMR (D_2O) δ −10.53 (d, J = 21.4 Hz), −12.49 (d, J = 20.76 Hz); HRMS-EI found 575.0674 $(M-H^+)^-$. $C_{17}H_{25}N_2O_{16}P_2$ requires 575.0679; purity >98% by HPLC (11.0 min).

4.7. 5-(*tert*-Butyl-diphenyl-silanyloxy)-3,3-dimethyl-tetrahydro-2,4-dioxa-cyclopropa[α]pentalene-1α-carboxylic acid ethyl ester (47)

A solution of imidazole (0.54 g, 8 mmol) in dry acetonitrile (15 mL) was treated with *tert*-butyldiphenylchlorosilane (1.64 g, 6 mmol), and the resulting reaction mixture was stirred at room temperature for 15 min. The alcohol **46** (0.968 g, 4 mmol)[26] was added and the reaction mixture was stirred for an additional 6 h. Chloroform (100 mL) was added and the resulting organic layer was washed with 0.5 M HCl (50 mL), followed by brine. The organic layer was dried over $MgSO_4$ and concentrated. The final purification was effected by using silica-gel column chromatography eluting with $CHCl_3$/MeOH (95:5) to afford **47** as a colorless solid (1.38 g, 72%). ^{1}H NMR ($CDCl_3$) δ 7.77 (m, 4H), 7.39 (m, 6H), 5.12 (d, J = 8.8 Hz, 1H), 4.43 (t, J = 4.2, 2.8 Hz, 1H), 4.12 (m, 3H), 2.22(m, 1H), 1.91 (t, J = 7.6, 6.0 Hz, 1H), 1.48 (m, 1H), 1.15 (m, 18H); HRMS-EI found 481.2411 $(M+H^+)^-$. $C_{28}H_{37}O_5Si$ requires 481.2410.

4.8. [5-(*tert*-Butyl-diphenyl-silanyloxy)-3,3-dimethyl-tetrahydro-2,4-dioxa-cyclopropa[α]pentalen-1α-yl]-methanol (48)

A solution of ester **47** (0.96 g, 2 mmol) in dichloromethane (25 mL) stirred at −78 °C was treated with diisobutylaluminium hydride solution (4 mL, 1.0 M in toluene). After stirring the resulting reaction mixture at −78 °C for 2 h, the reaction was quenched with methanol (1 mL) and allowed to stir at room temperature for 30 min. The reaction mixture was then treated with 0.5 N sulfuric acid (5 mL) and dichloromethane (50 mL), and the organic layer was washed successively with water and brine. The resulting organic layer was concentrated and the residue was further purified using silica-gel column chromatography eluting with $CHCl_3$/MeOH (90:10) to afford alcohol **48** as a colorless solid (0.47 g, 54%). ^{1}H NMR ($CDCl_3$) δ 7.72 (m, 4H), 7.31 (m, 6H), 4.61 (m, 2H), 4.10 (t, J = 1.2, 8.8 Hz, 1H), 3.3 (m, 2H), 1.26 (m, 11H), 1.13 (s, 3H), 1.03 (s, 3H), 0.71 (m, 1H); HRMS-EI found 439.2307 $(M+H^+)^-$. $C_{26}H_{34}O_4Si$ requires 439.2305.

4.9. Phosphoric acid di-*tert*-butyl ester 5-(*tert*-butyl-diphenyl-silanyloxy)-3,3-dimethyl-tetrahydro-2,4-dioxa-cyclopropa[α]-pentalen-1α-ylmethyl ester (49)

A stirred solution of alcohol **48** (0.438 g, 1 mmol) in dry THF (10 mL) was treated successively with tetrazole (0.280 g, 4 mmol) and di-*tert*-butyl *N,N*-diethylphosphoramidite (0.74 g, 3 mmol). The resulting solution was stirred at room temperature for 1 h, and the reaction mixture was cooled to −78 °C. 3-Chloroperbenzoic acid (MCPBA, contains 50%, 0.516 g) was added, and the reaction mixture was allowed to stir at room temperature for 1 h. Triethylamine (2 mL) was added and the reaction mixture was stirred for 15 min and concentrated under reduced pressure. The resulting residue was subjected to silica-gel column chromatography eluting with $CHCl_3$/MeOH (90:5) to afford alcohol **49** as a colorless solid (0.113 g, 18%). ^{1}H NMR ($CDCl_3$) δ 7.73 (m, 4H), 7.29 (m, 6H), 4.78 (m, 1H), 4.5 (m, 1H), 4.15 (m, 1H), 3.81 (m, 2H), 1.53 (m, 19H), 1.41 (s, 3H), 1.20 (m, 4H), 1.1 (s, 9H), 0.68 (m, 1H); HRMS-EI found 631.3222 $(M+H^+)^-$. $C_{34}H_{51}O_7PSi$ requires 631.3220.

4.10. Phosphoric acid di-*tert*-butyl ester 5-hydroxy-3,3-dimethyl-tetrahydro-2,4-dioxa-cyclopropa[α]pentalen-1 α -ylmethyl ester (50)

The silyl ether **49** (0.063 g, 0.1 mmol) in THF (2 mL) was treated with tetrabutyl ammonium fluoride solution (1 M in THF, 1 mL) and the mixture was stirred for 12 h. The resulting reaction mixture was concentrated under reduced pressure, and the residue was subjected to careful silica-gel column chromatography eluting with $CHCl_3$/MeOH (90:5) to afford alcohol **50** as a colorless solid (0.021 g, 55%). ^{1}H NMR ($CDCl_3$) δ 5.0 (m, 1H), 4.55 (m, 2H), 4.5 (m, 1H), 4.30 (dd, J = 6.9, 11.1 Hz, 1H), 3.59 (dd, J = 6.6, 11.1 Hz, 1H), 1.48 (m, 19H), 1.42 (m, 4H), 1.25 (s, 3H), 0.72 (m, 1H); HRMS-EI found 393.2037 $(M+H^+)^-$. $C_{18}H_{33}O_7P$ requires 393.2042.

4.11. Phosphoric acid di-*tert*-butyl ester 5-(2,4-dioxo-3,4-dihydro-2*H*-pyrimidin-1-yl)-3,3-dimethyl-tetrahydro-2,4-dioxa-cyclopropa[α]pentalen-1-α-ylmethyl ester (52)

A stirred solution of diisopropyl azodicarboxylate (0.120 g 0.6 mmol) in dry THF (10 mL) was successively treated with triphenylphosphine (0.156 g, 0.6 mmol) and 3-benzoyl uracil (0.054 g, 0.25 mmol). The resulting reaction mixture was stirred at room temperature for 30 min. The alcohol **50** (0.078 g, 0.2 mmol) was added to the reaction mixture and the mixture stirred for an additional 6 h. The crude reaction mixture was concentrated and treated with 1 M ethanolic ammonia (2 mL) to effect debenzoylation. After 6 h of stirring at room temperature the reaction mixture was concentrated and subjected to careful silica-gel column chromatography using $CHCl_3$/MeOH (85:15) to afford alcohol **52** as a colorless solid (0.015 g, 16%). ^{1}H NMR ($CDCl_3$) δ 7.76 (d, J = 6.8 Hz, 1H), 6.13 (d, J = 6.7 Hz, 1H), 5.13 (m, 1H), 4.67 (m, 2H), 4.27 (dd, J = 6.5, 11.0 Hz, 1H), 3.79 (dd, J = 6.6, 11.1 Hz, 1H), 1.45 (m, 19H), 1.41 (m, 4H), 1.26 (s, 3H), 0.89 (m, 1H); HRMS-EI found 487.2208 $(M+H^+)^-$. $C_{22}H_{36}N_2O_8P$ requires 487.2209.

4.12. Phosphoric acid mono-[4-(2,4-dioxo-3,4-dihydro-2*H*-pyrimidin-1-yl)-2,3-dihydroxy-bicyclo[3.1.0]hex-1-ylmethyl] ester (53)

The isopropylidene derivative **52** (9.6 mg, 0.02 mmol) in a mixture of methanol (1 mL) and water (0.5 mL) was treated with ion-exchange resin (H^+-form, 0.050 g) and the resulting solution was

stirred at 80 °C for 4 h. The reaction mixture was treated with 0.2 M triethylammonium bicarbonate (0.3 mL) and the resulting mixture was lyophilized. The residue thus obtained was purified by an ion-exchange chromatography using a column of Sephadex–DEAE A-25 resin with a linear gradient (0.01–1.0 M) of 1.0 M ammonium bicarbonate as the mobile phase to give 3.0 mg of **53** as a white solid. The spectral properties of (N)-methanocarba nucleotide **53** were comparable with one reported in the literature.[25b]

4.13. Diphosphoric acid 1″-α-D-[1″]glucopyranosyl ester 2-((N)-methanocarba-uridin-5′-yl)ester, triethylammonium salt (28)

(N)-Methanocarba-uridine-5′-glucose-1′-diphosphate triethylammonium salt **28** was prepared from (N)-methanocarba-uridine-5′-monophosphate using the same procedure as described for the synthesis of compound **27** from **44**. Compound **28** (0.48 mg, 31%) was obtained as a white solid. ^{1}H NMR (D_2O) δ 7.85 (d, J = 7.8 Hz, 1H), 5.80 (d, J = 8.4 Hz, 1H), 5.44 (dd, J = 3.6, 7.2 Hz, 1H), 4.62–4.78 (m, 2H), 4.52 (m, 2H), 3.95 (m, 1H), 3.48–4.76 (m, 4H), 3.33–3.40 (m, 2H), 1.62 (m, 1H), 1.24 (m, 1H), 0.90 (m, 1H); ^{31}P NMR (D_2O) δ −10.68 (d, J = 20.8 Hz), −12.49 (d, J = 21.4 Hz); HRMS-EI found 575.0715 $(M-H^+)^-$. $C_{17}H_{25}N_2O_{16}P_2$ requires 575.0679; purity >98% by HPLC (10.9 min).

4.14. Diphosphoric acid 1″-α-D-[1″]glucopyranosyl ester 2-(carbocyclic uridin-5′-yl)ester, triethylammonium salt (29)[27,28]

A solution of ethylvinyl ether (**54**) (64 mg, 0.81 mmol) was added slowly to a stirred solution of *N*-(chlorocarbonyl) isocyanate (58 mg, 0.6 mmol) in THF at 0 °C. The stirring was continued for 30 min at the same temperature. Triethylamine (120 mg, 1.1 mmol) in THF was added to the reaction mixture, which was stirred for 10 min at 0 °C and then cooled to −40 °C. A solution of (1*R*,2*S*,3*R*,4*R*)-2,3-dihydroxy-4-(hydroxymethyl)-1-aminocyclopentane hydrochloride (100 mg, 0.54 mmol) in DMF was added, and the reaction mixture was allowed to warm to room temperature. The mixture was stirred overnight. The solvent was evaporated in a rotary evaporator, and the crude product (**56**) was dissolved in a mixture of 2 N H_2SO_4 (9 mL) and dioxane (6 mL). The mixture was refluxed at 100 °C for 4 h and then cooled and neutralized with 3 N ammonia (7 mL). The ammonium salt was precipitated with ethanol and the solid was filtered. The filtrate was evaporated under reduced pressure and was purified by column chromatography on silica gel eluting with MeOH/CH_2Cl_2 (90:10) to obtain compound (**57**) as a white solid (71 mg, 0.29 mmol). The spectral data was consistent with the assigned structure.[27,28]

Carbocyclic-uridine-5′-monophosphate (**58**) was prepared by first stirring compound (**57**) (9 mg, 0.037 mmol) and Proton Sponge (17 mg, 0.08 mmol) in trimethyl phosphate (1 mL) for 10 min at 0 °C. Phosphorous oxychloride (0.008 mL, 0.08 mmol) was added dropwise to this reaction mixture, and the mixture was stirred for 2 h at 0 °C. 0.2 M triethylammonium bicarbonate solution (1.5 mL) was added, and the reaction mixture was stirred at room temperature for 1 h. After the removal of solvents, the residue was purified using Sephadex–DEAE A-25 resin. The desired fractions were collected and lyophilized to obtain carbocyclic-uridine 5′-monophosphate ammonium salt **58**. Compound **58** (4.83 mg, 0.015 mmol) was converted to the tributylammonium salt by treatment with Dowex 50WX2–200 ion-exchange resin (H^+) and tributylamine. Water was removed through lyophilization, and the product was dried under high-vacuum overnight. Carbocyclic-uridine 5′-monophosphate tributylammonium salt **58** and 1,1′-carbonyldiimidazole (6.0 mg, 0.036 mmol) were dissolved in DMF (2 mL), and the mixture was stirred at room temperature for 5 h. 5% Triethylamine solution in water/methanol (1:1, 1 mL) was added, and stirring was continued at room temperature for an additional 2 h. After removal of the solvent, the residue was dried in high vacuum and redissolved in DMF (0.5 mL). Solutions in DMF (each 0.2 mL) of magnesium chloride (dissolved with warming) and glucose-1′-monophosphate tributylammonium salt (3 mg, 0.0045 mmol) were successively added. The reaction mixture was stirred at room temperature for two days. After removal of the solvent, the residue was purified as described in the general procedure using Sephadex–DEAE A-25 resin and semipreparative HPLC (1.71 mg, 15%) to obtain compound **29** as a white solid. ^{1}H NMR (D_2O) δ 7.82 (d, J = 8.4 Hz, 1H), 5.93 (d, J = 8.1 Hz, 1H), 5.62 (dd, J = 3.6, 7.8 Hz, 1H), 3.9 (m, 5H), 3.7 (m, 3H), 3.5 (m, 3H), 2.26 (m, 2H), 1.65 (m, 1H). ^{31}P NMR (D_2O) δ −10.35 (d, J = 20.63 Hz), −12.41 (d, J = 20.63 Hz); HRMS-EI found 563.0663 $(M-H^+)^-$. $C_{16}H_{25}N_2O_{16}P_2$ requires 563.0679; purity 97% by HPLC (11.3 min).

4.15. Assay of $P2Y_2$ receptor- and $P2Y_{14}$ receptor-stimulated PLC activity

COS-7 cells were transiently transfected with the human $P2Y_{14}$ receptor and $G\alpha_{qi5}$.[15] Twenty-four hours after transfection, the inositol lipid pool of the cells was radiolabeled by incubation in 200 μL of serum-free inositol-free Dulbecco's modified Eagle's medium, containing 0.4 μCi of *myo*-[^{3}H]inositol. No changes of medium were made subsequent to the addition of [^{3}H]inositol. Forty-eight hours after transfection, cells were challenged with 50 μL of the fivefold concentrated solution of receptor agonists in 200 mM *N*-(2-hydroxyethyl)-piperazine-*N*′-2-ethanesulfonic acid, pH 7.3, containing 50 mM LiCl for 20 min at 37 °C. Incubations were terminated by aspiration of the drug-containing medium and addition of 450 μL of ice-cold 50 mM formic acid. After 15 min at 4 °C, samples were neutralized with 150 μL of 150 mM NH_4OH. [^{3}H]Inositol phosphates were isolated by ion-exchange chromatography on Dowex AG 1-X8 columns as previously described.[30] Activity at the human $P2Y_2$ receptor stably expressed in 1321N1 astrocytoma cells was determined as described.[23,25,29,30]

5. Data analysis

Agonist potencies (EC_{50} values) were obtained from concentration–response curves by non-linear regression analysis using the GraphPad software package Prism (GraphPad, San Diego, CA). All experiments were performed in triplicate assays and repeated at least three times. The results are presented as mean ± 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.

6. Molecular modeling

The recently published crystal structure of the A_{2A} adenosine receptor was utilized as a template for homology modeling of $P2Y_{14}$.[32] All calculations in this study were performed with the Schrödinger suite.[33] To built a homology model of the $P2Y_{14}$ receptor the Prime program was utilized and its standard parameters were used. Since the prediction of the right configuration of the EL2 is problematic due to its high flexibility and low sequence identity between EL2 of the target and a template receptors only part of EL2 was modeled in the present study. Namely, all residues between Val164 and Lys171 were not included in the model. The model obtained was used for the docking studies of **1** and **17a**. The SITEMAP program of the Schrödinger suite was used to identify the potential ligand binding site of the $P2Y_{14}$ receptor. The binding site with the best value of the SiteScore function (0.9854) was selected as a site for molecular docking. Namely, the binding site was

formed by the following amino acid residues: Arg274, Lys277, Arg253, Glu278, Tyr249, Leu281, Gln22, Ile25, Pro26, Phe74, Lys77, Ile78, Leu79, Asp81, Ser82, Leu84, Ser97, Ala98, Leu100, Phe101, Cys172, Ile173, and Glu174. The InducedFit docking approach was utilized to initial docking of **1** to the $P2Y_{14}$ receptor. The receptor grid generation was performed for the box with automatically determined side and with a center in the centroid of the binding site identified with the SITEMAP. The default values were applied for all other parameters.

The model of the $P2Y_{14}$ receptor with **1** docked was subjected to Monte-Carlo Multiple Minimum (MCMM) calculations. The conformations of the side chains of residues located within 5 Å from **1** were refined by Mixed torsional/Low-mode sampling method, a shell of residues located within 2 Å was used. The following parameters were applied: MMFFs force field, water as an implicit solvent, a maximum of 500 iterations of the Polak-Ribier conjugate gradient minimization method was used with a convergence threshold of 0.05 kJ mol^{-1} $Å^{-1}$, the number of conformational search steps = 200, the energy window for saving structures = 100 kJ mol^{-1}.

The GLIDE program of Schrödinger suite and the $P2Y_{14}$–**1** complex obtained were utilized to dock **17a** to the $P2Y_{14}$ receptor. The extra precision (XP) mode was used. The grid generation was performed for the box with automatically determined side. The center of the box was defined as the centroid of UDPG, **1**. The ligand flexibility was allowed.

Acknowledgments

Mass spectral measurements were carried out by Dr. John Lloyd and Dr. Noel Whittaker and NMR by Wesley White (NIDDK). We thank Dr. Stefano Costanzi (NIDDK) for helpful discussion and Dr. Sonia de Castro, Dr. T. Santhosh Kumar, Nathaniel Kim, and Deepmala Kumar (all of NIDDK) and Lauren Burianek (UNC) for technical assistance. This research was supported in part by the Intramural Research Program of the NIH, National Institute of Diabetes and Digestive and Kidney Diseases. This work was supported by National Institutes of Health Grant GM38213 to T.K. Harden.

Supplementary data

Details of synthesis and characterization of analogues of **1**, including monofluorinated glucose derivatives and other sugar derivatives are available. Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bmc.2009.05.024.

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