

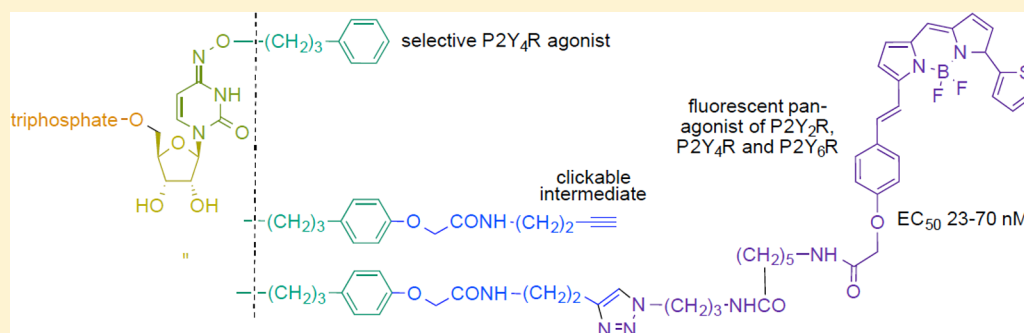
4-Alkyloxyimino Derivatives of Uridine-5'-triphosphate: Distal Modification of Potent Agonists as a Strategy for Molecular Probes of P2Y₂, P2Y₄, and P2Y₆ Receptors

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S Supporting Information



ABSTRACT: Extended N⁴-(3-arylpropyl)oxy derivatives of uridine-5'-triphosphate were synthesized and potently stimulated phospholipase C stimulation in astrocytoma cells expressing G protein-coupled human (h) P2Y receptors (P2YRs) activated by UTP (P2Y_{2/4}R) or UDP (P2Y₆R). The potent P2Y₄R-selective N⁴-(3-phenylpropyl)oxy agonist was phenyl ring-substituted or replaced with terminal heterocyclic or naphthyl rings with retention of P2YR potency. This broad tolerance for steric bulk in a distal region was not observed for dinucleoside tetraphosphate agonists with both nucleobases substituted. The potent N⁴-(3-(4-methoxyphenyl)-propyl)oxy analogue **19** (EC₅₀: P2Y₂R, 47 nM; P2Y₄R, 23 nM) was functionalized for chain extension using click tethering of fluorophores as prosthetic groups. The BODIPY 630/650 conjugate **28** (MRS4162) exhibited EC₅₀ values of 70, 66, and 23 nM at the hP2Y_{2/4/6}Rs, respectively, and specifically labeled cells expressing the P2Y₆R. Thus, an extended N⁴-(3-arylpropyl)oxy group accessed a structurally permissive region on three G_q-coupled P2YRs, and potency and selectivity were modulated by distal structural changes. This freedom of substitution was utilized to design of a pan-agonist fluorescent probe of a subset of uracil nucleotide-activated hP2YRs.

■ INTRODUCTION

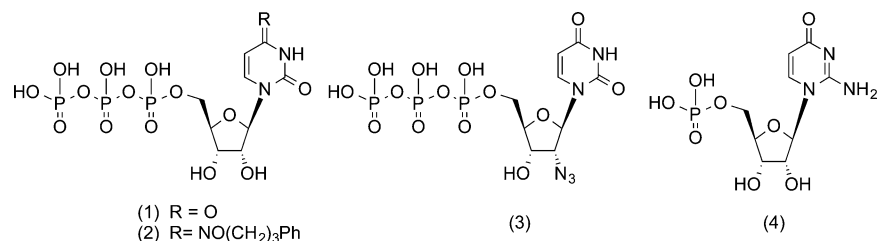
The P2Y receptors (P2YRs) are G protein-coupled receptors (GPCRs) that are activated by extracellular nucleotides.¹ The widely expressed² UTP-activated (P2Y₂R and P2Y₄R) and UDP-activated (P2Y₆R) receptors belong to the subfamily of P2Y₁-like receptors that promote inositol lipid signaling through G_q-mediated activation of phospholipase C-β (PLC-β). Few selective agonists and antagonists³ are available for pharmacological differentiation of these uracil nucleotide-regulated receptors, and P2Y₂R, P2Y₄R, and P2Y₆R exhibit much overlap in affinities for pyrimidine nucleotides. This study was initiated to synthesize nucleoside 5'-triphosphate derivatives as potent agonist probes of the P2Y₄R, including chain derivatization for incorporation of bulky reporter groups. The message for this P2YR subtype is expressed at high levels in intestine, pituitary, and brain, with lower levels observed in adipose tissue, prostate, spleen, lymphocytes, skeletal muscle,

and lung.^{2,3} The P2Y₄R has been shown to regulate cardiovascular development,⁴ and activation of this signaling protein results in intestinal chloride secretion,⁵ inhibition of contraction of the mouse ileum longitudinal muscle, and K⁺ secretion in the luminal membrane of the distal colon.^{6,7} Postsynaptic P2Y₄Rs are expressed on neurons of the rat hypothalamus that release neuropeptides.⁸ Thus, ligands of the P2Y₄R are of potential interest for pharmacotherapies directed at intestinal function, angiogenesis, cardiac remodeling, postischemic revascularization, CNS function, and inflammation.^{4,9}

Uridine-5'-triphosphate (UTP, **1**) is the endogenous P2Y₄R agonist (EC₅₀ = 80 nM),^{1,10} while adenosine-5'-triphosphate (ATP) antagonizes the human homologue of this receptor and

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Chart 1. Structures of Pyrimidine Nucleotides As Prototypical Agonist Ligands for Studying the P2Y₄R^a

^aEC₅₀ values for activation of PLC via the human P2Y₄R are: 2, 23 nM; 2'-azido-2'-deoxy-UTP 3, 1100 nM; iso-CMP 4, 4980 nM.^{11–13}

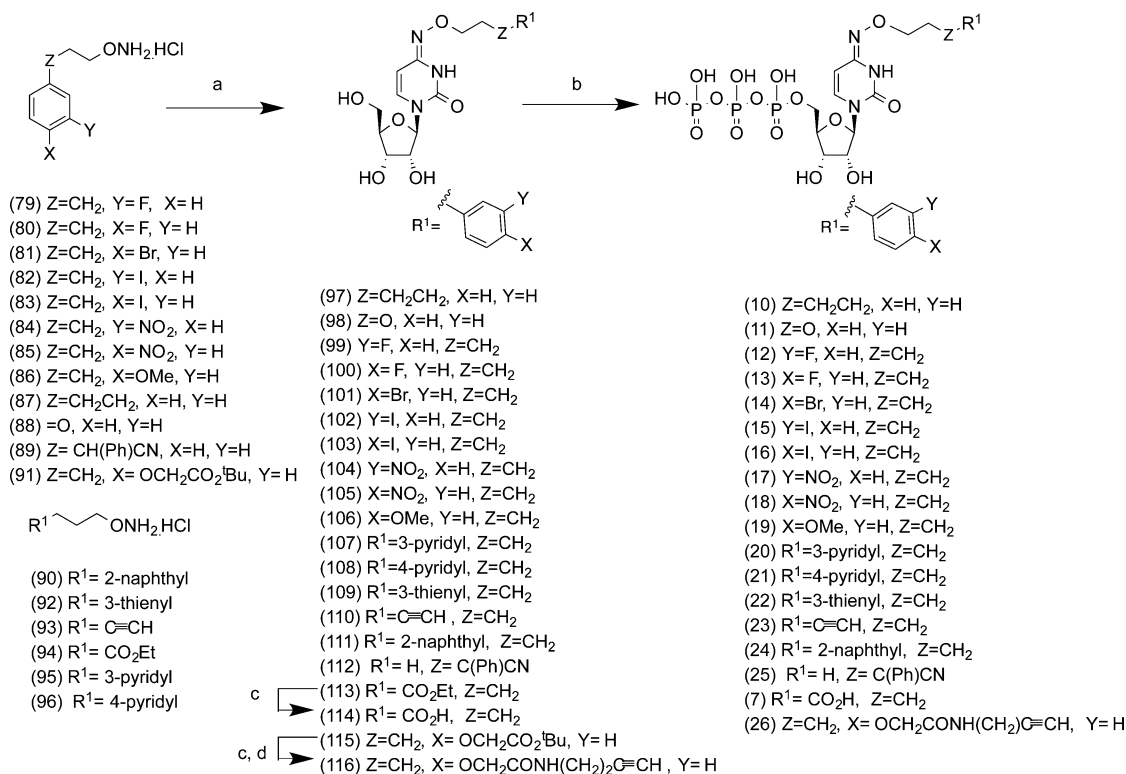
Table 1. Potency of a Series of Pyrimidine Nucleotide Derivatives at Three Subtypes of hP2YRs

no.	structure, R ¹ =	potency, EC ₅₀ (nM)			
		P2Y ₂ ^a	P2Y ₄ ^a	P2Y ₆ ^a	
S'-Triphosphates					
5 ^b	=N-O-CH ₃	28 ± 4	25 ± 3	130 ± 21	
6 ^b	=N-O-CH ₂ CH ₃	817 ± 93	210 ± 79	877 ± 50	
7	=N-O-(CH ₃) ₃ CO ₂ H	299 ± 56	470 ± 130	697 ± 167	
8 ^b	=N-O-CH ₂ Ph	620 ± 75	97 ± 14	230 ± 37	
9 ^b	=N-O-(CH ₂) ₂ Ph	1200 ± 270	73 ± 17	1210 ± 220	
2 ^b	=N-O-(CH ₂) ₃ Ph	640 ± 147	23 ± 4	740 ± 29	
10	=N-O-(CH ₂) ₄ Ph	214 ± 11	54 ± 14	251 ± 110	
11	=N-O-(CH ₂) ₂ O-Ph	3400 ± 880	430 ± 93	1780 ± 430	
12	=N-O-(CH ₂) ₃ -3-F-Ph	190 ± 28	100 ± 22	539 ± 28	
13	=N-O-(CH ₂) ₃ -4-F-Ph	547 ± 125	105 ± 27	1210 ± 290	
14	=N-O-(CH ₂) ₃ -4-Br-Ph	350 ± 70	141 ± 60	225 ± 39	
15	=N-O-(CH ₂) ₃ -3-I-Ph	325 ± 100	114 ± 13	299 ± 10	
16	=N-O-(CH ₂) ₃ -4-I-Ph	187 ± 61	91 ± 15	90 ± 13	
17	=N-O-(CH ₂) ₃ -3-NO ₂ -Ph	457 ± 11	95 ± 25	9700 ± 1500	
18	=N-O-(CH ₂) ₃ -4-NO ₂ -Ph	458 ± 145	84 ± 24	1330 ± 290	
19 ^c	=N-O-(CH ₂) ₃ -4-CH ₃ O-Ph	47 ± 2	23 ± 8	277 ± 75	
20	=N-O-(CH ₂) ₃ -3-pyridyl	327 ± 77	300 ± 230	2950 ± 1020	
21	=N-O-(CH ₂) ₃ -4-pyridyl	285 ± 20	250 ± 160	4280 ± 1330	
22	=N-O-(CH ₂) ₃ -3-thienyl	103 ± 7	20 ± 2	164 ± 48	
23	=N-O-(CH ₂) ₃ C≡CH	168 ± 30	410 ± 28	3000 ± 1500	
24	=N-O-(CH ₂) ₃ -(2-naphthyl)	139 ± 29	128 ± 26	90 ± 12	
25	=N-O-(CH ₂) ₂ -C(CN)(Ph) ₂	934 ± 92	47 ± 13	177 ± 21	
26	=N-O-(CH ₂) ₃ -Ph-(4-O-CH ₂ CONH-(CH ₂) ₂ C≡CH	109 ± 33	40 ± 14	183 ± 42	
27	Alexa Fluor 488 derivative	577 ± 146	200 ± 42	334 ± 36	
28	BODIPY 630/650 derivative	66 ± 17	70 ± 21	23 ± 7	
Dinucleoside Tetraphosphates					
	R ¹ =	R ² =	P2Y ₂ ^a	P2Y ₄ ^a	P2Y ₆ ^a
29 ^{b,c}	=O	=O	210 ± 30	130 ± 10	1160 ± 420
30	=N-O-(CH ₃) ₃ Ph	=O	389 ± 114	93 ± 19	332 ± 80
31	=N-O-(CH ₃) ₃ Ph	=N-O-(CH ₃) ₃ Ph	>10000	1330 ± 100	>10000

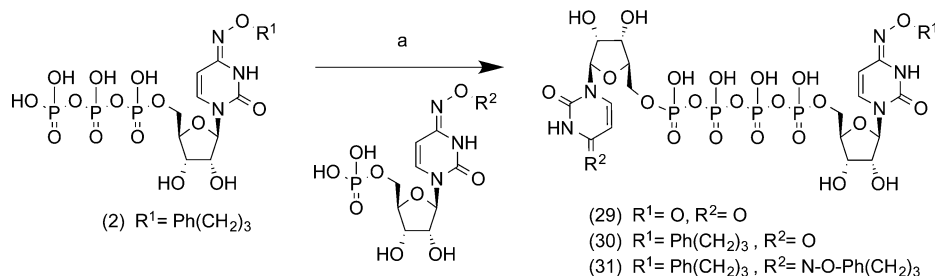
^aFunctional assays were conducted with 1321N1 astrocytoma cells expressing recombinant hP2Y₂, P2Y₄, or P2Y₆Rs. Values are expressed as the mean ± SEM. ^bData from Maruoka et al.¹⁰ ^c29, Up₄U.

activates the rat homologue.¹¹ Structure–activity relationship (SAR) studies of the P2Y₄R have been performed through

preparation of modified analogues in conjunction with molecular modeling. Two analogues of UTP, N⁴-(3-

Scheme 1. Synthesis of Various 4-Alkoxyiminopyrimidine Ribonucleoside 5'-Triphosphates^a

^aReagents and conditions: (a) cytidine, pyridine, 110 °C; (b) proton sponge, POCl₃, PO(OMe)₃, tributylammonium pyrophosphate, DMF; (c) 1 N NaOH, THF, 50 °C; (d) PyBOP, DIEA, but-4-yn-1-amine.

Scheme 2. Synthesis of 4-Alkoxyiminopyrimidine Dinucleoside Tetraphosphates^a

^aReagents: (a) DIC, MgCl₂, DMF.

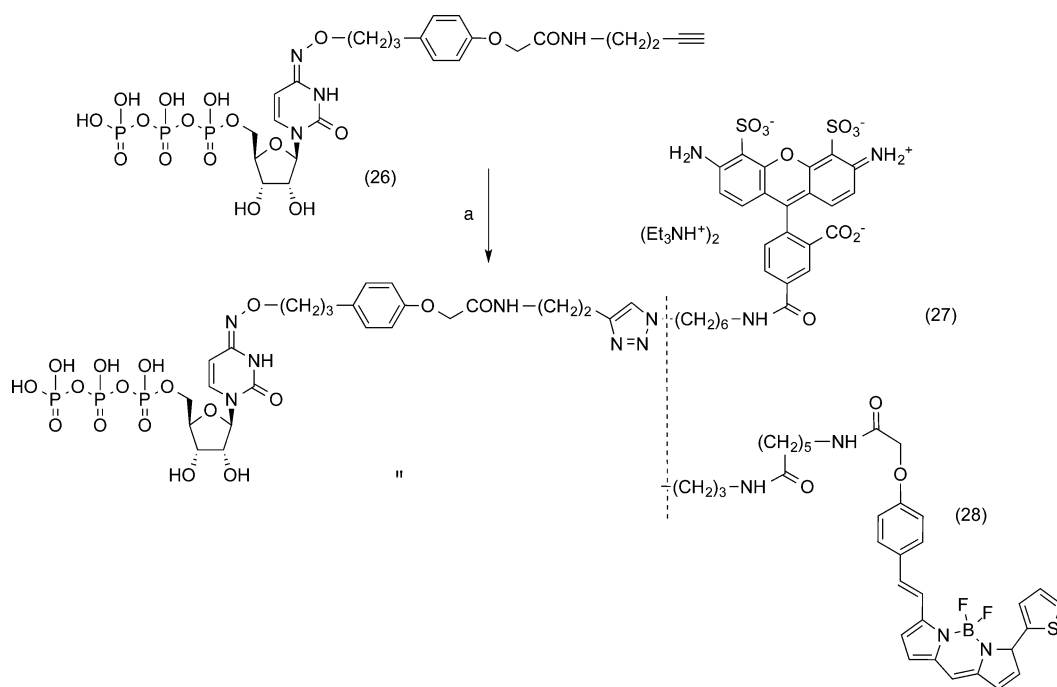
phenylpropyloxy)cytidine-5'-triphosphate, **2**, and 2'-azido-2'-deoxy-UTP, **3**, and one 5'-monophosphate derivative, iso-CMP **4**,^{10,12,13} have demonstrated (Chart 1) low to moderate selectivity for this subtype, but highly potent and selective P2Y₄R agonists have not been identified to date.^{10–16} The 4-benzyloxyiminopyrimidine group was identified as a structurally permissive site for synthesis of functionalized congeners,¹⁷ leading to high affinity molecular probes for the closely related UDP-activated P2Y₆R.¹⁴ Because of the structural similarity of the P2Y₄R and P2Y₆R, we focused on the same site for derivatization of analogues of **1** that included an elongated alkyl chain, i.e., a N⁴-phenylpropyloxy group, which we hypothesized would be more suitable for conferring P2Y₄R selectivity.¹⁰ One goal of these studies was to generate an analogue(s) with tolerance for steric bulk when bound to the receptor, potentially allowing attachment of a fluorophore or other reporter group to serve as a tracer for the receptor on live cells.

This approach has been validated for adenosine receptors and other GPCRs.^{18,19}

RESULTS

We considered a 4-arylalkoxyimino group in the pyrimidine nucleobase as an approach to expand the range of potent agonists of P2Y₄R and related P2YRs. In summary, we have modified the 4-(3-phenylpropyloxy)imino group of **2** with various ring substitutions and chain elongations and evaluated the new nucleotide derivatives in functional assays of the hP2Y₄R, P2Y₆R, and P2Y₂R. Because dinucleoside tetraphosphates are also known to activate the P2Y₄R,¹⁰ we included several derivatives in the dinucleotide series to test their compatibility with 4-alkoxyimino modifications.

Novel nucleotide derivatives **7**, **10–28**, and **30–31** (Table 1) were prepared and purified to homogeneity. Two series of nucleotide derivatives were chosen: 5'-triphosphates related to UTP and dinucleotides related to Up₄U **29**, which have

Scheme 3. Synthesis of Fluorescent 4-Alkoxyiminopyrimidine Ribonucleoside 5'-Triphosphates 27 and 28 by Click Reaction with Azido-Dyes^a

^aReagents and conditions: (a) azide (e.g., **124**, 1 equiv) and alkyne (1.4 equiv) precursors, ^tBuOH/water, TBTA, sodium ascorbate, cupric sulfate.

relatively high potency at both P2Y₂R and P2Y₄R. The synthetic routes to these groups of structurally modified pyrimidine nucleotides include: UTP analogues containing a N⁴-alkoxy group (Scheme 1), modified Up₄U dinucleotide analogues (Scheme 2), and UTP analogues containing Alexa Fluor 488 and BODIPY 630/650 fluorescent dyes^{19,27} (Scheme 3).

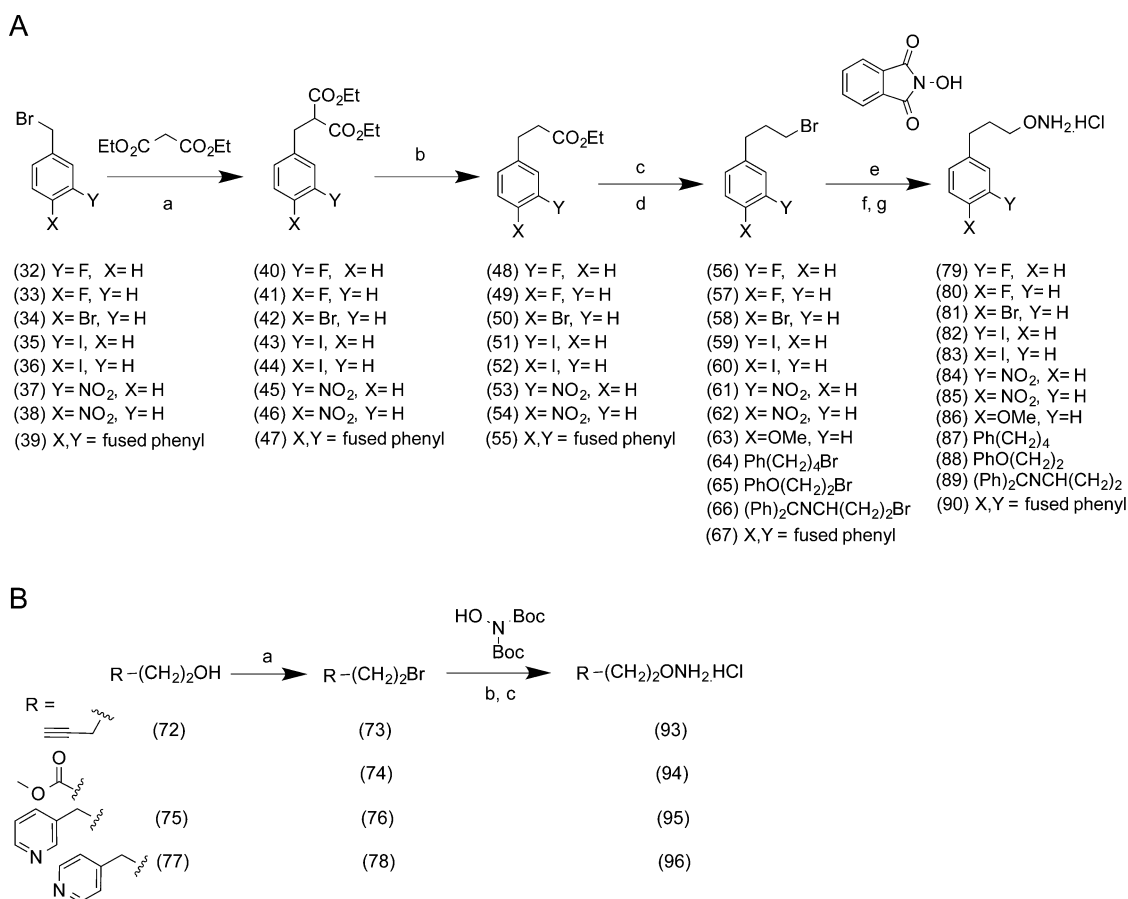
The synthesis of N⁴-alkoxy cytidines **10–26** from cytidine was performed using corresponding alkoxyamine hydrochlorides. Various alkoxy groups including phenyl, pyridyl, thienyl, naphthyl, and alkyl ester derivatives were chosen. These alkoxyamine derivatives were synthesized from the corresponding 3-arylpropyl bromides using Gabriel synthesis or a variation thereof using *N,N'*-di-*tert*-butoxycarbonylhydroxylamine as the source of the hydroxylamine moiety.^{22,25} The 3-alkylpropyl bromides **56–62** and **67** were synthesized from the corresponding benzyl bromides by homologation in four steps (Scheme 4A).²³ The 3-arylpropyl bromides **73**, **76**, and **78** were synthesized from the corresponding alcohols and converted to the alkoxyimino derivatives (Scheme 4B).²² (3-Thien-3-yl)propyl bromide **71** was synthesized from the corresponding α,β -unsaturated carboxylic acid (Scheme S1, Supporting Information). The resulting N⁴-alkoxy-cytidines were phosphorylated by standard methods¹⁰ to give the desired N⁴-alkoxy-cytidine 5'-triphosphates **10–26** (Scheme 1). In each case, the unprotected nucleoside was first treated with phosphorus oxychloride and the reaction mixture was treated immediately with bis(tri-*n*-butylammonium) pyrophosphate to produce the 5'-triphosphate. The synthesis of dinucleoside tetraphosphates, including an asymmetrically substituted analogue **30**, was performed using *N,N'*-diisopropylcarbodiimide (DIC) as a coupling reagent for condensing a 5'-monophosphate and 5'-triphosphate in the presence of magnesium chloride (Scheme 2), which dramatically increased

the reactivity compared to carbodiimide alone.²⁴ The fluorescent Alexa Fluor 488 5'-triphosphate **27** and BODIPY 5'-triphosphate **28** derivatives were synthesized from alkyne nucleotide precursor **26** and an azide derivative (e.g., a BODIPY 630/650 azide **124** for product **28**, Scheme S2, Supporting Information) using copper-catalyzed [2 + 3] cycloaddition click reactions with azides (Scheme 3).²⁰

The newly synthesized analogues were tested for potency against the P2Y₂R, P2Y₄R, and P2Y₆R.^{13–15} Similar N⁴-3-phenylalkoxy derivatives were shown to be only weakly active at the P2Y₁₄R;¹⁰ thus, this subtype was not part of the initial screen. The potencies of nine known reference compounds (**2**, **5–6**, **8–9**, and **29**) were included for comparison.^{10,14} Standard assays of potency for activation of PLC were performed as described²⁶ in 1321N1 human astrocytoma cell lines stably expressing either the P2Y₂R, P2Y₄R, or P2Y₆R (1321N1-P2YR cells).

Among substituted phenyl groups, 3-F **12**, 4-I **16**, and 4-MeO **19** provided relatively high potency at the three P2Y₂, P2Y₄, and P2Y₆Rs. Thus, we decided to elongate functionalized congeners based on *p*-substituted ethers related to **19**. Other aryl moieties that appeared to enhance potency were thien-3-yl **22** and naphth-2-yl **23**, indicating a tolerance of steric bulk in this region. The optimal chain length at the P2Y₄R was reached, with the *n*-propyl analogue **2** not substituted with an ether in the chain (cf. **11**). In contrast, the longer *n*-butyl homologue **10** displayed increased potency at the P2Y₂R and P2Y₆R. High potency at the P2Y₄R was achieved with a terminal alkyne at the N⁴-linked chain for tethering sterically bulky groups by click chemistry (**26**, EC₅₀ = 40 nM, Figure 1). However, potency at P2Y₂R and P2Y₆R also was largely retained in this ether- and amide-linked alkyne, with EC₅₀ values of 109 and 183 nM, respectively.

Scheme 4. Synthesis of *N*-Alkoxyamines: (A) 3-Phenylpropyl and 3-(naphth-2-yl)propyl Intermediates Prepared by the Gabriel Synthesis.²⁵ (B) *N*-Alkoxyamine Intermediates Prepared Using *N,N'*-di-*tert*-Butoxycarbonylhydroxylamine as Described^a



^aReagents and conditions: (A) (a) diethyl malonate, NaH, THF; (b) NaCl, DMSO; (c) DiB_{al}-H; (d) CBr₄, PPh₃; (e) Et₃N, DMF, 100 °C; (f) NH₂NH₂·H₂O; (g) 1 N HCl. Protected 3-(4-carboxymethoxy-phenyl)propyl (**91**) and 3-(thien-3-yl)propyl (**92**). *N*-alkoxyamine intermediates were prepared by analogous methods (Scheme S1, Supporting information): (a) K₂CO₃, CH₃CN, reflux; (b) CBr₄, PPh₃; (c) Et₃N, DMF, 100 °C; (d) NH₂NH₂·H₂O; (e) 10% Pd/C, H₂, MeOH; (f) LiAlH₄, THF; (g) Br₂, PPh₃, 2,6-lutidine.²² (B) (a) HBr in acetic acid, 50 °C; (b) DBU, CH₃CN, 50 °C; (c) 1 N HCl, CH₂Cl₂.

The fluorescent dye, Alexa Fluor 488, is a suitable fluorophore for flow cytometry (FCM) binding assays and live cell imaging.^{14,18} This fluorophore was incorporated in conjugate **27**, but only an intermediate potency was observed with this molecule at the three P2Y_Rs. An alternative BODIPY 630/650 fluorophore was incorporated in conjugate **28**. Curiously, in comparison to the precursor molecule **26**, the BODIPY conjugate exhibited enhanced potency at the P2Y₂R (EC₅₀ = 66 nM) and P2Y₆R (EC₅₀ = 23 nM) but slightly decreased potency at the P2Y₄R. Thus, **28** is a high affinity pan-agonist fluorescent probe with nearly equivalent potency at the hP2Y₂R, hP2Y₄R, and hP2Y₆R.

A preliminary feasibility study was carried out to determine if **28** could serve as a fluorescent P2Y_R probe. These experiments were with P2Y₆R-expressing 1321N1 cells because **28** is most potent at this P2Y_R subtype (Figure 2). Fluorescent binding of **28** was compared by FCM, using several known P2Y₆R ligands to compete for cell labeling. Incubation of cells with 100 nM **28** for 30 min at 37 °C in the presence of the ecto-nucleotidase inhibitor α,β -methylene-adenosine 5'-diphosphate **125** (100 μ M)^{33,34} resulted in significant cell labeling (Figure 2). The level of cell-associated fluorescence was not reduced by coinubation of **28** with 10 μ M *N*⁶-methyl-2-deoxyadenosine 3,5'-bisphosphate (**117**, MRS2179), a P2Y₁R-selective antago-

nist that is inactive at P2Y₂, P2Y₄, and P2Y₆R.²⁸ However, significant decreases in mean fluorescence intensity were observed after preincubation with P2Y₆R ligands, including the P2Y₆R-selective noncompetitive antagonist *N,N'*-1,4-butanediyl-bis[*N'*-(3-isothiocyanatophenyl)]thiourea (**118**, MRS2578, 10 μ M) and the competitive antagonist suramin (**119**, 10 μ M).^{29,30} Although **119** is a nonselective P2X and P2Y antagonist, we included it in this assay because it has been shown to effectively block the P2Y₆R.²⁶ The potent P2Y₆R-selective dinucleotide agonist P¹-(uridine 5'-)-P⁴-(*N*⁴-methoxycytidine 5'-)-triphosphate (**120**, MRS2957, 10 μ M)¹⁴ produced the largest decrease in cell-associated fluorescence. Fluorescence microscopy comparing binding to 1321N1-P2Y₆R cells in the presence and absence of **118** (Figure 3) indicated that 1 μ M **28** specifically labeled cells expressing the receptor.

DISCUSSION

We have identified a functionalization approach that provides versatile nucleotide analogues for further derivatization of high potency P2Y₂R, P2Y₄R, and P2Y₆R agonists. The initial approach was based on a P2Y₄R-selective agonist, and many of the new derivatives tend toward selectivity at this subtype. However, upon elongation of a functionalized chain at the *N*⁴

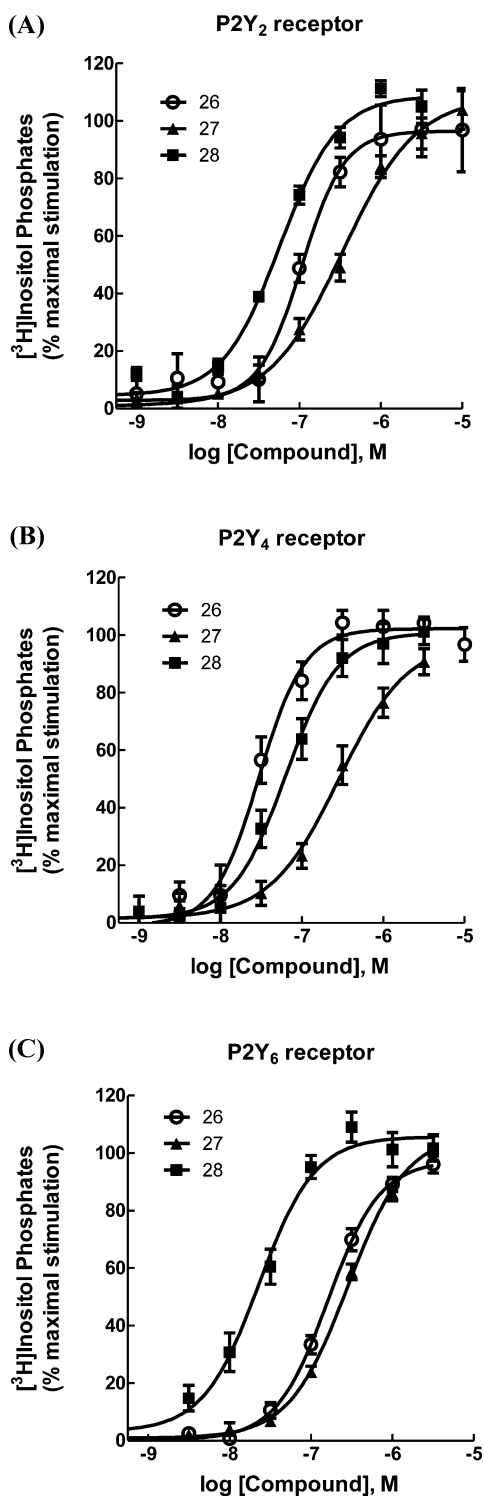


Figure 1. Comparison of potencies of alkyne 26 and fluorescent analogues 27 and 28 in activation of PLC via the human P2Y₂R (A), P2Y₄R (B), and P2Y₆R (C) in stably transfected 1321N1 human astrocytoma cells.

position, significant potency also was observed at the related P2Y₂R and P2Y₆R subtypes. For example, compound 19 was particularly potent at P2Y₂ and P2Y₄Rs.

The derivatization strategy was aimed to clarify where to modify the pyrimidine nucleotides to preserve or increase potency at any or all of the relevant P2YRs to allow the attachment of sterically large fluorophores²⁷ and other reporter

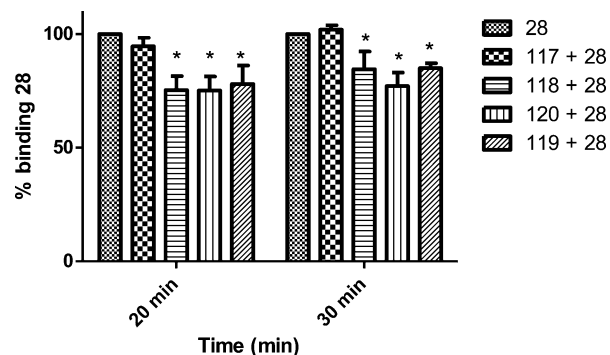


Figure 2. Fluorescence ligand binding experiments using FCM in stably transfected 1321N1-P2Y₆R cells with 28 after preincubation at 37 °C with known P2Y₆R agonist or P2Y₆R antagonists. Each column shows the brightness of each compound using 28 set at 100% and after correcting the MFI values for autofluorescence. Results are expressed as mean \pm SEM ($n = 4$). Binding of 28 at 20 and 30 min to the P2Y₆R was significantly blocked after preincubation with selective antagonist 118, selective agonist 120, and nonselective antagonist 119. No significant difference in MFI was observed for P2Y₁R antagonist 117. *, $p < 0.05$, when compared to cells treated with only 28. Average of MFI after 20 min incubation of 28: 107.0 ± 30.2 ; after 30 min incubation of 28: 222.8 ± 72.1 .

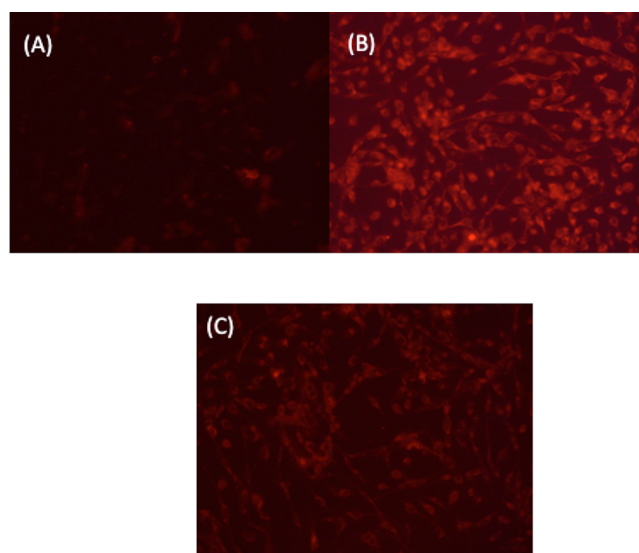


Figure 3. Fluorescent micrographs using a Keyence fluorescent microscope (BZ-9000) of P2Y₆R-expressing 1321N1 astrocytoma cells exposed to the fluorescent agonist 28 (1 μM , 30 min incubation at 37 °C). (A) Control cells in the absence of 14. (B) Incubation with 1 μM 14 at 37 °C for 30 min in medium. (C) Incubation with 1 μM 14 at 37 °C for 30 min in cells pretreated with noncompetitive antagonist 118 (10 μM).

groups. Questions to be answered were: If the pseudo-oxime chain of 2 proved to be a suitable attachment point, how long should the alkyl linker (i.e., propyl in 2) be for optimal potency (cf. 8, 9, 11, 25)? Were ring-substitutions (cf. 12–19) or replacement of the terminal phenyl moiety with heteroaryl or other group (cf. 7, 20–24) desirable for enhancing potency? What position for attachment of an elongated chain on the phenyl (cf. 12–19) or other aryl group was best suited for high potency? Thus, model compounds of various intermediate substructures were prepared and tested before the final target fluorescent conjugates were designed. Briefly, we chose the *p*-

position for extension of an alkyl ether chain on the 4-(3-phenylpropyloxy)imino group of **2**, which was click-linked²⁰ through a terminal alkyne, i.e., **26**, to an azide-bearing fluorophore. Other possible approaches could have been through chain elongation at the 2, 3, or 5 positions of a uracil ring.^{13,21}

Modifications of the distal phenyl ring of a 3-phenylpropyloxyimino group, shown previously to be optimal for P2Y₄R potency, allowed a bulky fluorescent reporter group to be introduced, but the P2Y₄R selectivity was compromised. Nevertheless, these molecules also provide high affinity leads for further modified agonists that could exhibit higher selectivity at one of these receptors. Possible directions for structural modifications of pyrimidine nucleotides conducive to P2Y₄R selectivity might include ribose and nucleobase modifications such as appear in compounds **3** and **4**.

This discovery provides an optimal new route for generation of fluorescent probes for facile pooled quantification of these three P2YRs in living cells. In cells expressing a single P2YR subtype, such as our astrocytoma cells, there is no ambiguity. Following the development of standard assay conditions, the potent fluorescent ligand **28** could potentially be used for high-throughput screening of drug libraries with P2Y₂R-, P2Y₄R-, or P2Y₆R-expressing cells. Radioligands are not available for any of these receptors. Our previously reported fluorescent nucleotide P2YR probe, a conjugate of Alexa Fluor 488, tethered from a cytosine nucleobase in the diphosphate series, was selective for the P2Y₆R.¹⁴ The current work adds a second viable fluorescent probes for studying the P2Y₆R. Although we did not directly examine its binding to P2Y₂R- and P2Y₄R-expressing cells, compound **28** adds the potential capability of fluorescent characterization of these subtypes for which no high affinity assay probes are available. Assay development for routine application of this fluorescent probe presents a separate experimental challenge that will be addressed in future work.

The high potency of **28** at the hP2Y₂R, hP2Y₄R, and hP2Y₆R suggests the existence of energetically favorable interactions with groups on the conformationally flexible extracellular regions of P2Y₂R and P2Y₆R. The modeling of these extracellular regions is difficult because of their flexibility and the lack of a close structural template. Further characterization of the location of the fluorescent label within the cells as well as delineation of the saturability, reversibility, kinetics, and other measurements will be required before this probe molecule can be used routinely for FCM labeling of cells expressing P2YRs.

CONCLUSIONS

A series of N⁴-(3-arylpropyloxy) derivatives of uridine-5'-triphosphate were synthesized and found to display high potency as agonists of the initial target G protein-coupled hP2Y₄R and also hP2Y₂R and hP2Y₆R. The phenyl group could be substituted with heterocyclic rings or a naphthyl ring with retention of P2YR affinity, indicating a broad tolerance for steric bulk in this distal region of the nucleotide. Thus, an extended N⁴-(3-arylpropyloxy) group accessed a structurally permissive region on various G_q-coupled P2YRs and was modulated by distal structural changes to alter selectivity. This freedom of substitution can be utilized for the design of pan-agonist affinity probes of the uracil nucleotide-activated, G_q-coupled P2Y_{2/4/6} subset of receptors.

EXPERIMENTAL SECTION

Chemical Synthesis. ¹H NMR spectra were obtained with a Varian Mercury 400 spectrometers using D₂O, MeOD, CDCl₃, or DMSO-*d*₆ as a solvent. The chemical shifts are expressed as relative ppm from HOD (4.80 ppm). ³¹P NMR spectra were recorded at room temperature (rt) on 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. High-resolution mass measurements were performed using a Micromass/Waters LCT Premier Electrospray time of flight (TOF) mass spectrometer coupled with a Waters HPLC system, unless noted. Alexa Fluor 488 azide was from Invitrogen (Carlsbad, CA). All other reagents were of analytical grade and were purchased from Sigma-Aldrich (St. Louis, MO).

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. Then these nucleotides were additionally purified by HPLC with a Luna 5 μ RP-C18(2) semipreparative column (250 mm × 10.0 mm; Phenomenex, Torrance, CA) and using the following conditions: flow rate of 2 mL/min; 10 mM triethylammonium acetate (TEAA)-CH₃CN from 100:0 to 95:5 (system B) (or up to 99:1 to 50:50 (system C)) in 30 min (and isolated in the triethylammonium salt form). All other compounds (cytidine derivatives) were purified from a silica column chromatography (CHCl₃:MeOH-gradient from 100:0 to 70:30). Purity of the compounds submitted for bioassay was checked using a Hewlett-Packard 1100 HPLC equipped with a Zorbax SB-Aq 5 μm analytical column (50 mm × 4.6 mm; Agilent Technologies Inc., Palo Alto, CA). Mobile phase: linear gradient solvent system; 5 mM TBAP (tetrabutylammonium dihydrogenphosphate)-CH₃CN from 80:20 to 40:60 in 13 min; the flow rate was 0.5 mL/min (system A). Peaks were detected by UV absorption with a diode array detector at 230, 254, and 280 nm. All nucleotide derivatives tested for biological activity showed >98% purity by HPLC analysis (detection at 254 nm).

General Procedure for the Preparation of Nucleoside Triphosphates (7, 10–26). A solution of the nucleoside 97–112, **114** (73.0 μmol), and 1,8-bis(dimethylamino)naphthalene (Proton Sponge, 24.0 mg, 0.11 mmol) in trimethyl phosphate (0.4 mL) was stirred for 10 min at 0 °C.¹⁵ Then, phosphorus oxychloride (13.0 μL, 0.13 mmol) was added dropwise, and the reaction mixture was stirred for 2 h at 0 °C. A solution of tributylammonium pyrophosphate (0.80 mL, 0.44 mmol) and tributylamine (69.0 μL, 0.29 mmol) in *N,N*-dimethylformamide (DMF, 1 mL) was added, and stirring was continued at 0 °C for additional 10 min. Then 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 by Sephadex-DEAE A-25 resin and preparative HPLC.

N⁴-(3-((4-But-3-yn-1-aminocarbonylmethyleneoxy)phenyl)propyloxy)cytidine-5'-triphosphate Triethylammonium Salt (26). Compound **26** (7.0 mg, 6.9 μmol, 10%) was obtained as a white solid using N⁴-(3-((4-but-3-yn-1-aminocarbonylmethyleneoxy)phenyl)propyloxy)cytidine **116** (35.5 mg, 70.1 μmol). ¹H NMR (D₂O) δ 7.27–7.19 (m, 3H), 6.94 (d, *J* = 8.6, 2H), 5.94 (d, *J* = 6.4 Hz, 1H), 5.77 (d, *J* = 8.2, 1H), 4.60 (s, 2H), 4.44–4.40 (m, 1H), 4.36 (t, *J* = 5.9, 1H), 4.26–4.14 (m, 3H), 4.06 (t, *J* = 6.0, 2H), 3.44 (t, *J* = 6.6, 2H), 2.75–2.63 (m, 2H), 2.44 (dt, *J* = 6.6, *J* = 2.7, 2H), 2.33 (t, *J* = 2.6, 1H), 2.07–1.91 (m, 2H). HRMS-EI found: 741.0974 (M - H⁺)⁻. C₂₄H₃₂N₄O₁₇P₃ requires 741.0970; analytical HPLC (system A: 6.54 min).

N⁴-(3-((4-(2-Ethylamino)carbonylmethyleneoxy-(Alexa Fluor 488-1H-1,2,3-Triazol-4-yl))phenyl)propyloxy)cytidine-5'-triphosphate Triethylammonium Salt (27). To a mixture of Alexa Fluor 488 azide (Alexa Fluor 488 5-carboxamido-(6-azido)hexanyl), bis(triethylammonium salt), 5-isomer, 0.5 mg, 0.8 μmol, 1 equiv), and alkyne **26** (0.8 mg, 0.8 μmol, 1.4 equiv) in a 0.20 mL of (1:1) mixture of ^tBuOH and water, were added tris[(1-benzyl-1H-1,2,3-triazol-4-yl)methyl] amine (TBTA, 0.1 mg) and freshly prepared 1 M aqueous sodium ascorbate solution (1.6 μmol, 1.6 μL, 2 equiv) followed by

7.5% aqueous copper sulfate pentahydrate solution (4.0 μL , 1.2 μmol , 1.5 equiv). The reaction mixture was stirred overnight at rt, solvent was evaporated, and the residue was purified by semipreparative HPLC as described above to obtain **27** (1.64 mg, 34% yield) as a yellow/orange solid. HRMS-EI found: 1399.2106 ($M - H^+$)⁻. $\text{C}_{51}\text{H}_{58}\text{N}_{10}\text{O}_{27}\text{S}_2\text{P}_3$ requires 1399.2104.

***N*⁴-(3-((4-*O*-CH₂CONH-(CH₂)₂(BODIPY-1*H*-1,2,3-Triazol-4-yl))-phenyl)propoxy)cytidine-5'-triphosphate Triethylammonium Salt (**28**)**. To a mixture of BODIPY 630/650 azide (**124**, Supporting Information, 1.0 mg, 1.0 μmol , 1 equiv) and alkyne **26** (2.0 mg, 1.8 μmol , 1.2 equiv) in a 200 μL of (1:1) mixture of ^tBuOH and water, and 20 μL of DMF, were added tris[(1-benzyl-1*H*-1,2,3-triazol-4-yl)methyl] amine (TBTA, 0.05 mg) and freshly prepared 1 M aqueous sodium ascorbate solution (1.1 μL , 1.6 μmol , 1 equiv) followed by 7.5% aqueous copper sulfate pentahydrate solution (2.5 μL , 0.8 μmol , 0.8 equiv). The bright-blue color reaction mixture was stirred overnight at rt, solvent was evaporated, and the residue was purified by semipreparative HPLC as described above to obtain desired triphosphate **28** as blue-colored solids. HRMS-EI m/z ($M + ES$ MS) for **28** found, 1388.3648 ($M + H^+$)⁺; calcd for $\text{C}_{64}\text{H}_{68}\text{N}_{11}\text{O}_{20}\text{BF}_2\text{P}_3\text{S}$, 1388.3631; analytical HPLC (system A: 7.91 min). Fluorescence absorption and emission spectra indicated max values at 634 and 650 nm, respectively (Supporting Information), as measured in solution in a cuvette using a SpectraMax M5 reader (Molecular Devices, Sunnyvale, CA).

General Synthesis of *P*¹-(*N*⁴-Phenylpropoxycytidine-5'-)*P*⁴-(uridine-5'-)-tetrphosphate Triethylammonium Salt (30**)**. Uridine 5'-monophosphate disodium salt (4.4 mg, 11.0 μmol , 2 equiv) and *N*⁴-(3-phenylpropoxy)cytidine-5'-triphosphate triethylammonium salt **2** (4.5 mg, 5.4 μmol , 1 equiv) were converted to the tributylammonium salts by treatment with ion-exchange resin (DOWEX 50WX2-200 (H)) and tributylamine. After removal of the water, the obtained uridine monophosphate dibutylammonium salt and the tributylammonium salt of *N*⁴-phenylpropoxycytidine-5'-triphosphate **2** (4.5 mg, 5.5 μmol , 1 equiv) were dried under high vacuum for 1 h in separate vials. DIC (1 μL , 5.5 μmol , 1 equiv) was added to a solution of *N*⁴-(3-phenylpropoxy)cytidine-5'-triphosphate tributylammonium salt **2** in DMF (200 μL). After stirring the reaction mixture at rt for 3 h, a solution of the uridine 5'-monophosphate tributylammonium salt (11.0 μmol) and MgCl_2 (1.0 mg, 11.0 μmol , 2 equiv) in DMF (100 μL) were added. The reaction mixture was stirred at rt overnight. After removal of the solvent, the MgCl_2 was removed by treatment with ion-exchange resin (DOWEX 50WX2-200 (H)) and ammonia bicarbonate or tri-*n*-butylamine, and the residue was purified by a semipreparative HPLC purification using system C. Compound **30** (1.0 mg, 0.8 μmol , 16%) was obtained as a white solid. ¹H NMR (D_2O) δ 7.93 (d, $J = 7.9$ Hz, 1H), 7.35–7.30 (m, $J = 8.3$ Hz, 4H), 7.25–7.21 (m, 2H), 6.02–5.91 (m, 2H), 5.78 (d, $J = 7.9$ Hz, 1H), 4.42–4.33 (m, 4H), 4.30–4.16 (m, 6H), 4.05 (t, $J = 4.8$ Hz, 2H), 2.74 (t, $J = 8.3$ Hz, 2H), 2.05–1.79 (m, 2H). ³¹P NMR (D_2O) δ -11.51 (br), -23.22 (br). HRMS-EI found: 922.0742 ($M - H^+$)⁻. $\text{C}_{27}\text{H}_{36}\text{N}_5\text{O}_{23}\text{P}_4$ requires 922.0752; analytical HPLC (system A: 7.92 min).

***P*¹-(*N*⁴-(3-Phenylpropoxy)cytidine-5'-)*P*⁴-(*N*⁴-(3-phenylpropoxy)cytidine-5'-)-tetrphosphate Triethylammonium Salt (**31**)**. Compound **31** (0.5 mg, 0.3 μmol , 10%) was obtained as a white solid using *N*⁴-(3-phenylpropoxy)cytidine-5'-triphosphate triethylammonium salt (3.0 mg, 3.0 μmol) and *N*⁴-(3-phenylpropoxy)cytidine-5'-monophosphate diethylammonium salt (3.0 mg, 6.1 μmol) from the previous procedure. ¹H NMR (D_2O) δ 7.45–7.10 (m, 12H), 5.95–5.87 (m, 2H), 5.79–5.63 (m, 2H), 4.39–4.29 (m, 4H), 4.27–4.16 (m, 6H), 4.06–4.00 (m, 4H), 2.80–2.65 (m, 4H), 2.05–1.93 (m, 4H). HRMS-EI found: 1055.1593 ($M - H^+$)⁻. $\text{C}_{36}\text{H}_{47}\text{N}_6\text{O}_{23}\text{P}_4$ requires 1055.1643; analytical HPLC (system A: 9.10 min).

Synthesis of compounds **40–96** is described in the Supporting Information.

General Procedure for the Synthesis of *N*⁴-Alkoxycytidine analogues (97–110**, **113**) *N*⁴-(4-Phenylbutyloxy)cytidine (**97**)**. A suspension of cytidine (360 mg, 1.5 mmol) and 4-phenylbutyloxy-

amine hydrochloride (**87**, 617 mg, 3.0 mmol, 2 equiv) in pyridine (4 mL) was stirred at 100 °C overnight. 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-(4-phenylbutyloxy)cytidine (**97**) (0.430 g, 1.10 mmol, 73%). ¹H NMR (400 MHz, MeOD): 7.52 (d, $J_1 = 8.20$ Hz, 1H), 7.20–7.17 (m, 2H), 7.15–7.05 (m, 3H), 5.83 (d, $J_1 = 4.84$ Hz, 1H), 5.63 (d, $J_1 = 8.12$ Hz, 1H), 4.13 (t, $J_1 = 5.20$ Hz, 1H), 4.09 (m, 1H), 4.00–3.92 (m, 3H), 3.78 (dd, $J_1 = 12.16$ Hz, $J_2 = 2.72$ Hz, 1H), 3.68 (dd, $J_1 = 12.20$ Hz, $J_2 = 3.16$ Hz, 1H), 2.60 (t, $J_1 = 6.88$ Hz, 2H), 1.70–1.60 (m, 4H). ¹³C NMR (400 MHz, MeOD): 149.2, 147.6, 142.0, 137.1, 135.0, 128.0, 127.9, 125.4, 95.0, 88.9, 84.8, 74.4, 73.7, 69.9, 60.9, 35.1, 27.7, 27.3. m/z ($M + ESI$ MS) found, 392.1813; calcd for $\text{C}_{19}\text{H}_{26}\text{N}_3\text{O}_6$, 392.1816.

***N*⁴-(3-((4-*tert*-Butyloxy-carbonylmethoxy)phenyl)propoxy)cytidine (**115**)**. Compound **115** (45 mg, 88.5 μmol , 65%) was obtained as a white solid from cytidine (29 mg, 0.12 mmol) and *N*⁴-(3-(4-*tert*-butyloxy-carbonylmethoxy)phenyl)propoxylamine hydrochloride (**91**) (79 mg, 0.25 mmol) using the above procedure. ¹H NMR (400 MHz, MeOD): 7.16 (d, $J_1 = 8.24$ Hz, 1H), 7.06 (d, $J_1 = 8.36$ Hz, 2H), 6.76 (d, $J_1 = 8.52$ Hz, 1H), 5.82 (d, $J_1 = 5.20$ Hz, 1H), 5.55 (d, $J_1 = 8.24$ Hz, 1H), 4.46 (s, 2H), 4.13–4.10 (m, 2H), 3.94–3.91 (m, 3H), 3.76 (d, $J_1 = 12.08$ Hz, 1H), 3.66 (dd, $J_1 = 12.12$ Hz, $J_2 = 3.00$ Hz, 1H), 2.58 (t, $J_1 = 7.08$ Hz, 2H), 2.01–1.86 (m, 2H), 1.42 (s, 9H). ¹³C NMR (400 MHz, MeOD): 169.0, 156.2, 150.1, 144.7, 134.7, 131.4, 129.1, 114.2, 97.6, 88.4, 84.6, 82.0, 73.4, 72.8, 70.3, 65.4, 61.3, 30.8, 30.4, 27.0. m/z ($M + ESI$ MS) found, 508.2294; calcd for $\text{C}_{24}\text{H}_{34}\text{N}_3\text{O}_9$, 508.2290.

***N*⁴-(3-((*tert*-Butyl-1-amino-4-methoxycarbonyl)phenyl)propoxy)cytidine (**116**)**. *N*⁴-(3-((4-*tert*-Butyloxy-carbonylmethoxy)phenyl)propoxy)cytidine **115** (44 mg, 0.12 mmol) was dissolved in 1.0 mL of THF and treated with 2.0 mL of 1 N NaOH. The reaction mixture was stirred at 50 °C for 2 h. The mixture was cooled to rt and neutralized by adding 1N HCl. After removal of the solvent, *N*⁴-(3-((4-methoxycarbonate)phenyl)propoxy)cytidine (31 mg, 68 μmol , 56%) was isolated after column chromatography. ¹H NMR (400 MHz, MeOD): 7.17 (d, $J_1 = 8.24$ Hz, 1H), 7.09 (d, $J_1 = 8.44$ Hz, 2H), 6.81 (d, $J_1 = 8.64$ Hz, 1H), 5.82 (d, $J_1 = 5.44$ Hz, 1H), 5.55 (d, $J_1 = 8.24$ Hz, 1H), 4.57 (s, 2H), 4.12 (t, $J_1 = 5.40$ Hz, 1H), 4.08 (m, 1H), 3.95–3.90 (m, 3H), 3.74 (dd, $J_1 = 12.08$ Hz, $J_2 = 2.88$ Hz, 1H), 3.66 (dd, $J_1 = 12.16$ Hz, $J_2 = 3.40$ Hz, 1H), 2.60 (t, $J_1 = 7.40$ Hz, 2H), 1.95–1.86 (m, 2H). ¹³C NMR (400 MHz, MeOD): 156.2, 150.1, 144.7, 134.7, 131.3, 129.0, 114.1, 97.4, 88.3, 84.7, 73.3, 72.7, 70.3, 64.6, 61.3, 30.8, 30.4, 27.0. m/z ($M + ESI$ MS) found, 442.1665; calcd for $\text{C}_{20}\text{H}_{26}\text{N}_3\text{O}_9$, 452.1664.

The above isolated acid (50 mg, 0.1 mmol), DIEA (0.128 mL, 0.93 mmol, 8.5 equiv), and 6.0 mL of DMF were added to round-bottom flask. Then 4-amino-1-butyne (13.5 μL , 0.16 mmol, 1.5 equiv) and 1.0 mL of DMF were added to the above mixture, which was then stirred for 10 min at rt. PyBOP (57 mg, 0.11 mmol, 1.1 equiv) was added, and the mixture was stirred overnight. Solvent was removed, and **116** (45.1 mg, 89.9 μmol , 80%) was isolated following column chromatography. ¹H NMR (400 MHz, MeOD): 7.17 (d, $J_1 = 8.28$ Hz, 1H), 7.11 (d, $J_1 = 8.56$ Hz, 2H), 6.86 (d, $J_1 = 8.64$ Hz, 2H), 5.82 (d, $J_1 = 5.44$ Hz, 1H), 5.53 (d, $J_1 = 8.24$ Hz, 1H), 4.43 (s, 2H), 4.15–4.00 (m, 2H), 3.95 (t, $J_1 = 6.36$ Hz, 2H), 3.91 (q, $J_1 = 3.32$ Hz, 1H), 3.75 (dd, $J_1 = 12.16$ Hz, $J_2 = 2.88$ Hz, 1H), 3.66 (dd, $J_1 = 12.12$ Hz, $J_2 = 3.36$ Hz, 1H), 3.38 (t, $J_1 = 7.08$ Hz, 2H), 2.62 (t, $J_1 = 7.40$ Hz, 2H), 2.37 (td, $J_1 = 7.08$ Hz, $J_2 = 2.64$ Hz, 2H), 2.25 (t, $J_1 = 2.64$ Hz, 1H), 2.01–1.85 (m, 2H). ¹³C NMR (400 MHz, MeOD): 170.11, 155.98, 150.03, 144.68, 135.27, 131.37, 129.5, 123.9, 114.7, 114.5, 112.1, 111.9, 97.4, 88.3, 84.7, 73.3, 72.5, 70.3, 61.3, 31.4, 30.1. m/z ($M + H$ ESI MS) found, 503.2139; calcd for $\text{C}_{24}\text{H}_{31}\text{N}_4\text{O}_8$, 503.2136.

Procedures for Phospholipase C Assay. Stable cell lines for study of the human (h) P2Y₂, P2Y₄, and P2Y₆Rs were produced by retroviral expression of the individual receptors in 1321N1 human astrocytoma cells, which do not natively express P2YRs.³¹ Agonist-stimulated [³H]inositol phosphate accumulation was quantified in cells plated at 20000 cells/well on 96-well plates two days prior to assay.

The inositol lipid pool of cells was radiolabeled 16 h prior to the assay by incubation with 100 μL of serum-free inositol-free Dulbecco's Modified Eagle's Medium, containing 1.0 μCi of [^3H]myo-inositol. No changes of medium were made subsequent to the addition of [^3H]inositol. Test drugs were added in 25 μL of 100 mM Hepes (*N*-(2-hydroxyethyl)-piperazine-*N'*-2-ethanesulfonic acid), pH 7.3, in HBSS containing 50 mM LiCl. Incubations were for 30 min at 37 $^{\circ}\text{C}$ and were terminated by aspiration of the drug-containing medium and addition of 90 μL of ice-cold 50 mM formic acid. The samples were neutralized with 30 μL of 150 mM NH_4OH and applied to Dowex AG1-X8 anion exchange columns. Total inositol phosphates were eluted, and radioactivity was measured using a liquid scintillation counter.²⁶

Data Analysis. Agonist potencies (EC_{50} values) were determined from concentration–response curves by nonlinear regression analysis using the GraphPad software package Prism (GraphPad, San Diego, CA). 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.

Cell Cultures for FCM. 1321N1 human astrocytoma cells overexpressing the $\text{P2Y}_6\text{R}$ were grown in DMEM with 5% FBS, 50 U/mL penicillin/streptomycin, and 2 mM L-glutamine. Cells were grown in 6-well plates (approximately 3×10^5 cells/well) and incubated at 37 $^{\circ}\text{C}$ and 5% CO_2 for 24 h. At 80% confluency, the medium was replaced with fresh preheated medium, in addition to α,β -methylene-adenosine 5'-diphosphate (**125**, AMP-CP, 100 μM) and a known agonist or antagonist of the $\text{P2Y}_6\text{R}$. After a 30 min incubation, **28** was added at different time intervals and the decrease in fluorescence intensity was measured by FCM.

Fluorescent Ligand Binding in 1321N1 Astrocytoma Cells Expressing $\text{P2Y}_6\text{R}$. Binding of **28** to the $\text{P2Y}_6\text{R}$ in overexpressing 1321N1 astrocytoma cells was blocked by using known $\text{P2Y}_6\text{R}$ ligands, such as antagonists **118** and **119** or the agonist **120** (all 10 μM). Ecto-nucleotidase inhibitor **125** (100 μM) was added to prevent the metabolism of 5'-di and triphosphate derivatives to the 5'-monophosphates.^{32–34} After a 30 min preincubation with the appropriate agonist or antagonist and **125** (100 μM), **28** (100 nM) was added at different time intervals from 20 to 30 min. At the end of incubation, the medium was removed, the cells were washed three times with ice-cold DPBS, and 1 mL of 0.2% EDTA was added to each well to detach the cells from the plate. Cells were then incubated at 37 $^{\circ}\text{C}$ for 5–10 min. After detaching, 1 mL of medium was added to neutralize the EDTA. The cell suspensions were transferred to 5 mL of polystyrene round-bottom BD Falcon tubes (BD, Franklin Lakes, NJ) and centrifuged for 5 min at 23 $^{\circ}\text{C}$ and 400g. After the supernatant was discarded, the cells were washed with 3 mL of DPBS and centrifuged again at 23 $^{\circ}\text{C}$ and 400g for 5 min. After centrifugation, the supernatant was discarded and the cells suspended in 0.5 mL of DPBS for analysis by FCM.

FCM Analysis. The intensity of fluorescence emission of each sample was measured by using FCM. Cell suspensions were vortexed briefly before analysis on a Becton and Dickinson FACSCalibur flow cytometer (BD, Franklin Lakes, NJ). Samples were maintained in the dark during the analysis to avoid photobleaching. Measured fluorescent intensities (MFIs) were obtained in the FL-4 channel in log mode. Ten thousand events were analyzed per sample. Data were collected and analyzed using Cell Quest Pro software (BD, Franklin Lakes, NJ).

Fluorescent Microscopy Studies. $\text{P2Y}_6\text{R}$ -expressing astrocytoma cells were grown on glass coverslips placed in a 6-well plate. When the cells reached 80% confluence, the medium was replaced with fresh medium, $\text{P2Y}_6\text{R}$ antagonist **118** (10 μM) was added to the cells, and incubation was continued for 30 min at 37 $^{\circ}\text{C}$. Then **16** was added to achieve a final concentration of 1 μM , and incubation continued for 30 min at 37 $^{\circ}\text{C}$. At the end of the incubation, the cells were washed with

ice-cold PBS and mounted on a glass slide. The cells were visualized using a Keyence BZ-9000 fluorescent microscope.

Data Analysis. Data analysis was performed with the Prism 5 (GraphPad, San Diego CA) software. The mean autofluorescence of 1321N1 astrocytoma cells was measured in the absence of the fluorescent ligand. The mean fluorescence intensity in the presence of fluorescent ligand was corrected by subtracting the autofluorescence.

■ ASSOCIATED CONTENT

Supporting Information

Chemical synthesis of intermediates and nucleotides, characterization data including selected NMR and MS spectra, HPLC purification and purity analysis procedures, cell culture, and membrane preparation. This material is available free of charge via the Internet at <http://pubs.acs.org>.

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Notes

The authors declare no competing financial interest.

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■ ABBREVIATIONS USED

1321N1- $\text{P2Y}_6\text{R}$ cells, 1321N1 human astrocytoma cells expressing a human P2Y_6 receptor; DCC, *N,N'*-dicyclohexylcarbodiimide; DIC, *N,N'*-diisopropylcarbodiimide; DIEA, *N,N*-diisopropylethylamine; DMF, *N,N*-dimethylformamide; DPBS, Dulbecco's phosphate buffered saline; EDC, *N*-(3-dimethylaminopropyl)-*N'*-ethylcarbodiimide; FCM, flow cytometry; GPCR, G protein-coupled receptor; HBSS, Hank's Balanced Salt Solution; HEPES, *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid; HPLC, high performance liquid chromatography; MFI, measured fluorescent intensity; PyBOP, (benzotriazol-1-yloxy)tripyrrolidinophosphonium hexafluorophosphate; $\text{P2Y}_6\text{R}$, P2Y_6 receptor; PLC, phospholipase C; SAR, structure–activity relationship; TBAP, tetrabutylammonium dihydrogenphosphate; TBTA, tris[(1-benzyl-1*H*-1,2,3-triazol-4-yl)methyl] amine; TEAA, triethylammonium acetate; THF, tetrahydrofuran; TLC, thin layer chromatography; UTP, uridine-5'-triphosphate

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