Electronic Supplementary Material (ESI) for Medicinal Chemistry Communications

SUPPORTING INFORMATION

4-Alkyloxyimino-Cytosine Nucleotides: Tethering Approaches to Molecular Probes for the $P2Y_6$ Receptor

P. Suresh Jayasekara, Matthew O. Barrett, Christopher B. Ball, Kyle A. Brown, Eszter Kozma, Stefano Costanzi, Lucia Squarcialupi, Ramachandran Balasubramanian, Hiroshi Maruoka, and Kenneth A. Jacobson

Contents **Page**

Procedures for phospholipase C assay

Stable cell lines for study of the human (h) $P2Y_2$, $P2Y_4$, and $P2Y_6$Rs were generated by retroviral expression of the receptor in 1321N1 human astrocytoma cells, which do not natively express P2YRs.[1] Agonist-induced [^{3}H]inositol phosphate production was measured in cells plated at 20,000 cells/well on 96-well plates two days prior to assay. Sixteen h before the assay, the inositol lipid pool of the cells was radiolabeled by incubation in 100 μL of serum-free inositol-free Dulbecco's modified Eagle's medium, containing 1.0 μCi of [^{3}H]*myo*-inositol. No changes of medium were made subsequent to the addition of [^{3}H]inositol. On the day of the assay, cells were challenged with 25 μL of a five-fold concentrated solution of receptor agonists in 100 mM Hepes (N-(2-hydroxyethyl)-piperazine-N′-2-ethanesulfonic acid), pH 7.3 in HBSS, containing 50 mM LiCl for 30 min at 37°C. Incubations were terminated by aspiration of the drug-containing medium and addition of 90 μL of ice-cold 50 mM formic acid. After 30 min, supernatants 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.[2]

Data Analysis

Agonist potencies (EC_{50} values) were determined from concentration-response curves by non-linear regression analysis using the GraphPad software package Prism (GraphPad, San Diego, CA). 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 ± 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.

References

1. Nicholas, R. A.; Lazarowski, E. R.; Watt, W. C.; Li, Q.; Harden, T. K. Uridine nucleotide selectivity of three phospholipase C-activating P-2 receptors: Identification of a UDP-selective, a UTP-selective, and an ATP- and UTP-specific receptor. *Mol. Pharmacol.* **1996**, *50*, 224-229.

2. Bourdon, D. M.; Wing, M. R.; Edwards, E. B.; Sondek, J. and Harden, T. K. Quantification of isoenzyme-specific activation of phospholipase C-beta 2 by Rac GTPases and phospholipase C-epsilon by Rho GTPases in an intact cell assay system. *Methods Enzymol.* **2006**, *406*, 489-499.

Procedures and results of fluorescent assays

1. Materials and methods

1.1. Spectral characterization

Absorbance and fluorescence spectra of 5 µM **16** were measured in aqueous solution in cuvettes using a SpectraMax M5 microplate reader (Molecular Devices, Sunnyvale, CA).

1.2. Cell cultures for FCM

1321N1 astrocytoma cells expressing the $P2Y_1R$ or $P2Y_6R$ (1321N1-$P2Y_1R$ or 1321N1-$P2Y_6R$, respectively) were grown in DMEM with high glucose with 5% FBS, 2 mM L-glutamine, 50 U/ml penicillin/streptomycin and 800 µg/ml of G418. Cells were grown in 12-well plates (approximately $2*10^5$ cells/well) and incubated at 37 °C for 24 h (1321N1-$P2Y_1R$) or 36 h (1321N1-$P2Y_6R$) in the presence of 5% CO_2. When the confluency of the cells reached 80% (approximately $4*10^5$ cells/well), the medium was replaced with fresh medium. Compound **16** was added in the presence or absence of a competitor, and cells were processed for FCM.

1.3. Fluorescent ligand binding in intact 1321N1 astrocytoma cells expressing $P2Y_1$ or $P2Y_6$Rs

1321N1-$P2Y_1R$ and 1321N1-$P2Y_6R$ were incubated with different concentrations of fluorescent (Alexa Fluor 488) derivative **16** ranging from 10 nM to 1.5 µM for 1 h for saturation binding experiments. To study binding kinetics, we incubated 1321N1-$P2Y_6R$ with 400 nM **16** for different time intervals from 5 min to 3 h. Nonspecific binding was determined in the presence of 100 µM UDP (1321N1-$P2Y_6R$) or 10 µM MRS2500 (1321N1-$P2Y_1R$). Surface bound ligand was measured in media containing 0.4 M sucrose, after 15 min preincubation.[1,2]

At the end of each time interval, the plates were placed on ice, medium was removed and cells were washed 3 times with ice-cold DPBS. To study receptor internalization, after washing, cells were washed with 1 ml of ice-cold acid stripping buffer (DMEM with 0.2% BSA - bovine serum albumin - adjusted to pH 3.5 with HCl) 3 times for 5 min on shaking platform at 4 °C to remove surface bound ligand, and washed 3 times with ice-cold DPBS for 5 min on shaking platform [3]. After washing, 0.5 ml 0.2 % EDTA solution was added to each well, and cells were incubated at 37 °C for 3-5 min. Following cell detachment, 0.5 ml medium was added to each well to neutralize the EDTA. The cell suspensions were transferred to polystyrene round-bottom BD Falcon tubes (BD, Franklin Lakes, NJ) and centrifuged for 5 min at 23 °C and 400×*g*. After centrifugation, the supernatant was discarded, and cells were washed with 2 ml PBS and centrifuged again at 23 °C and 400×*g* for 5 min. After discarding the supernatant, cells were suspended in 0.3 ml PBS and analyzed by FCM.

1.4. 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) with 488 nm argon ion laser. Samples were maintained in the dark during the analysis to avoid photobleaching. MFIs were obtained in the FL-1 channel in log mode. Ten thousand events were analyzed per sample. Data were collected using Cell Quest Pro software (BD, Franklin Lakes, NJ) and analyzed by Cyflogic v. 1.2.1 software (CyFlo, Ltd., Turku, Finland). MESF calibration was done using Quantum Alexa Fluor-488 MESF beads (Bangs Laboratories, Inc., Fishers, IN) according to the instructions of the manufacturer.

1.5. Confocal microscopy studies

$P2Y_6R$-expressing astrocytoma cells were seeded into Nunc Lab-Tek 8-well chambered coverglass (Nalgene Nunc, Thermo Fischer Scientific, Rochester, NY) in a concentration confluent after 48 h. When 70% confluency was reached, the medium was refreshed with warm media, and cells were incubated in the presence or absence of **16** (2 μM) or **17** (100 nM-1 μM) for 60 min. After 60 min, cells were observed using Zeiss LSM 700 confocal laser scanning microscope and LSM Software ZEN 2009 (Carl Zeiss Microscopy GmbH, Germany). Results are shown in Figure 3.

1.6. Fluorescence microscopy studies using known P2Y6R ligands

$P2Y_6R$-expressing astrocytoma cells were grown on glass cover slips placed in a 6-well plate. When the cells reached 80% confluence the medium was replaced with fresh medium and competitive ligands were added to the cells and incubated for 30 min 37 ºC, followed by addition of **16** (final conc. 500 nM) and incubation for 60 min at 37 ºC. 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 equipped with filters for green fluorescence with excitation at 495 nm and emission at 519 nm. Results are shown in Figure 4.

1.6. Data analysis

All data were analyzed by non-linear least squares analysis (GraphPad Prism Software, San Diego, CA).

The MFIs were corrected with the subtraction of autofluorescence values of 1321N1 astrocytoma cells in the absence of any ligand and converted into MESF values using QuickCal program v. 2.3 (Bangs Laboratories, Inc., Fishers, IN)

2. Results

2.1. Spectral characterization of 16

The absorption spectrum of 5 μM **16** aqueous solution displays a maximum at 494 nm, so the fluorescence emission spectra was performed using an excitation wavelength of 494 nm and detecting emission from 470 to 570 nm, the maximum emission was measured at 518 nm (Figure S2).

2.2. Fluorescent ligand binding experiments with FCM

Saturation binding studies of the binding of **16** to the $P2Y_6R$ were performed by measuring the MFI with FCM. A saturation binding curve of **16** to 1321N1-$P2Y_6R$ was obtained using MESF values (Figure 1B). An apparent K_D value of 380 ± 10 nM was determined. We were not able to obtain saturation binding curves at $P2Y_1Rs$, the binding of the fluorescent compound was non-saturable, and could not be blocked by selective and potent antagonist MRS2500 (Figure S3). 1321N1-$P2Y_1R$ and 1321N1-$P2Y_6R$ in the absence of fluorescence ligand were used to measure autofluorescence, nonspecific binding was measured in the presence of 100 μM UDP or 10μM MRS2500 on $P2Y_6$ and $P2Y_1$, respectively. A FCM histogram of the total binding, nonspecific binding and the autofluorescence of 1321N1-$P2Y_6R$ using 400 nM **16** and 3h incubation is shown in Figure 1A.

Association kinetics of the fluorescent ligand **16** to the h$P2Y_6R$ was also determined using a FCM assay. Figure S5 shows the time-dependent binding of 400 nM **16** to 1321N1-$P2Y_6R$ cells. The $t_{1/2}$ was 37 min, and the association rate constant (K) was calculated to be 0.019 min^{-1}.

Incubation with inhibitors followed by brief exposure to **16**: 1321N1-$P2Y_6R$ cells were grown in 12-well plates overnight and on the day of the experiment, fresh media was added. Cells were pre-incubated with non-fluorescent $P2Y_6R$ ligands for 30 min, and then the cells were treated with fluorescent **16** for 2 min. At the end of the incubation, cells were washed twice with ice-cold PBS and the cells were detached from the plate using 0.2% EDTA. The cells were washed again with PBS and dissolved in 0.5 ml PBS and analyzed by FCM (Figure S6, FACSCalibur, BD Biosciences, San Jose, CA).

2.3. Internalization studies

It is well-known that acid stripping cells by washing with low pH solutions readily dissociates most ligands from their receptors.[3] It was also reported that hypertonic treatment (with 0.4 M sucrose) effectively blocks receptor endocytosis.[1,2] We used these two methods to determine the surface bound and the internalized amount of fluorescent ligand during the time course of incubation.

The internalization process of the agonist-occupied $P2Y_6R$ using fluorescent **16** was characterized using FCM during a 3-hour long incubation (Figure 2A, B). At each time point, the internalized amount of fluorescent ligand was determined by removal of the cell-surface bound ligand by washing in acidic aqueous medium (pH 3.5). The internalization occurred gradually, with a $t_{1/2}$ of 18 min (Figure 2A). After 60 min, the kinetic plot of receptor internalization reached its plateau, at which point 77% of the fluorescent compound was found in the intracellular compartment. Figure 2B shows the surface bound and internalized fractions of the fluorescent compound **16** after 60 min of incubation, in comparison with the nonspecific and the total binding. After reaching the plateau, only 17 % of the total binding was surface bound, 77 % was internalized and 23% of the total binding was nonspecifically bound.

A fluorescence micrograph also shows that after 60 min incubation, and fluorescence derived from exposure to 2 μM **16** mostly appears inside the 1321N1-$P2Y_6R$ cells rather than on

the cell surface (Figure 2C), which corresponds to the previous observations using FCM. In the absence of **16**, there was no significant cell-fluorescence.

References for pharmacology:

[1] Trincavelli ML, Tuscano D, Cecchetti P, Falleni A, Benzi L, Klotz KN, Gremigni V, Cattabeni F, Lucacchini A, Martini C. Agonist-induced internalization and recycling of the human A_3 adenosine receptors: role in receptor desensitization and resensitization. J Neurochem. 2000;75:1493-1501.
[2] Mundell SJ, Kelly E. The effect of inhibitors of receptor internalization on the desensitization and resensitization of three Gs-coupled receptor responses. British Journal of Pharmacology, 1998, 125:1594-1600.
[3] Li N, Hill KS, Elferink LA. Analysis of receptor tyrosine kinase internalization using flow cytometry. Methods Mol Biol. 2008;457:305-317.

Experimental procedures

Sequence alignment. The sequence of the hP2Y$_6$R was added to our published alignment of the CXCR4, P2Y$_1$, P2Y$_2$ and P2Y$_4$Rs and was aligned to the others using the sequence alignment algorithm implemented in MOE,[1] with the BLOSUM62 matrix and penalties for gap starts and extensions set to 7 and 4, respectively. The resulting sequence alignment is shown in **Figure S7**.

The template. The template for the homology modeling was prepared with MOE.[1] Our published CXCR4-based[2] model of the P2Y$_4$R was then used as the template for the construction of a CXCR4-based P2Y$_6$R model.[3] Prior to the construction of the model, the ligand docked into the P2Y$_4$R was transformed into UDP by deleting the γ-phosphate and substituting the alkoxyimino group at position 4 of the pyrimidine ring with a carbonyl oxygen atom. Moreover, we changed the puckering of the ribose moiety to the southern (S) conformation, which we demonstrated to be a requirement for binding of UDP to the P2Y$_6$R. This was done by subjecting the ribose to energy minimization, with the PFROSST force field, while restraining the dihedral angle between the C3', C2', C1' and O4' atoms to a value comprised between 30° and 40°. Before the energy minimization procedure, the partial charges of the ligands were calculated with the AM1-BCC semiempirical method. The energy minimization was terminated when the potential energy gradient reached the cutoff value of 0.05 kcal/(mol Å).

Construction of the homology model. The homology model was constructed with MOE.[1] Ten models were built and scored on the basis of electrostatic solvation energy (GB/VI). Intermediate and final models were subjected to energy minimizations with the PFROSST force field, with the refinement level set to "medium". We chose the "medium" refinement protocol, which is based on a brief cycle of energy minimizations intended to relief steric strain, to prevent from drifting away excessively from the template.

Optimization of the receptor model. The model was subsequently optimized with the "protein preparation wizard" workflow, as implemented in the Schrödinger package[4] to add hydrogen atoms and and calculate the protonation states of ionizable groups at pH 7. The workflow also optimized the orientation of hydroxyl groups, as well as Asn, Gln and His residues. Finally, the hydrogen atoms were minimized with the Impact molecular mechanics engine, allowing a maximum root mean square deviation (RMSD) of 0.30 Å from the original structure, whereas heavy atoms were held rigid.

Modeling of the receptor-ligand interactions. The study of the receptor-ligand interactions was conducted with the Schrodinger package and its MacroModel molecular mechanics engine.[4] Compound **16** was sketched from UDP through the Maestro interface of the Schrödinger package,[4, 5] by substituting the carbonyl group at position 4 of the pyrimidine ring with the large alkoxyimino group characteristic of the fluorescent ligand. The compound was then subjected to two rounds of energy minimization, conducted with the OPLS2005 force field and protracted until the potential energy gradient reached the cutoff value of 0.05 kJ/(mol Å), followed by 1000 steps of Monte Carlo conformational search. In the first round, flexibility was granted only to the large alkoxyimino substituent, while a shell of residues located within 5 Å from the flexible atoms where considered for the calculation of the potential energy but were held rigid. In the second minimization round and the subsequent Monte Carlo search, flexibility was granted only

to the entire ligand as well as all the residues located within 5 Å from. An additional shell of residues located within 5 Å from the flexible atoms where considered for the calculation of the potential energy but were held rigid. The lowest energy structure resulting from the MCMM calculation and all the structures within 20 kJ/mol from it were saved in the output of the conformational search.

References for modeling

1. The molecular operating environment (MOE), 2011.10, Chemical Computing Group, Inc., Montreal, Canada, http://www.chemcomp.com.
2. Wu, B.; Chien, E. Y.; Mol, C. D.; Fenalti, G.; Liu, W.; Katritch, V.; Abagyan, R.; Brooun, A.; Wells, P.; Bi, F. C.; Hamel, D. J.; Kuhn, P.; Handel, T. M.; Cherezov, V.; Stevens, R. C. *Science* **2010,** *330*, 1066.
3. Maruoka, H.; Jayasekara, M. P.; Barrett, M. O.; Franklin, D. A.; de Castro, S.; Kim, N.; Costanzi, S.; Harden, T. K.; Jacobson, K. A. *J Med Chem* **2011,** *54*, 4018.
4. Schrödinger Suite 2012, Schrödinger, LLC, New York, NY, USA, http://www.schrodinger.com.
5. MacroModel, version 9.9, Schrodinger, LLC, New York, NY, USA, http://www.schrodinger.com.
6. Costanzi, S.; Mamedova, L.; Gao, Z.; Jacobson, K. *J. Med. Chem.* **2004,** *47*, 5393.
7. Costanzi, S.; Joshi, B.; Maddileti, S.; Mamedova, L.; Gonzalez-Moa, M.; Marquez, V.; Harden, T.; Jacobson, K. *J. Med. Chem.* **2005,** *48*, 8108.
8. Besada, P.; Shin, D.; Costanzi, S.; Ko, H.; Mathé, C.; Gagneron, J.; Gosselin, G.; Maddileti, S.; Harden, T.; Jacobson, K. *J Med Chem* **2006,** *49*, 5532.
9. Jacobson, K.; Costanzi, S.; Ivanov, A.; Tchilibon, S.; Besada, P.; Gao, Z.; Maddileti, S.; Harden, T. *Biochem Pharmacol* **2006,** *71*, 540.
10. Hillmann, P.; Ko, G. Y.; Spinrath, A.; Raulf, A.; von Kügelgen, I.; Wolff, S. C.; Nicholas, R. A.; Kostenis, E.; Höltje, H. D.; Müller, C. E. *J Med Chem* **2009,** *52*, 2762.
11. Moro, S.; Guo, D.; Camaioni, E.; Boyer, J. L.; Harden, T. K.; Jacobson, K. A. *J Med Chem* **1998,** *41*, 1456.
12. Jiang, Q.; Guo, D.; Lee, B. X.; van Rhee, A. M.; Kim, Y. C.; Nicholas, R. A.; Schachter, J. B.; Harden, T. K.; Jacobson, K. A. *Mol Pharmacol* **1997,** *52*, 499.

Figure Legends

Figure S1. cAMP measurements in C6 glioma cells expressing the hP2Y$_{14}$R. Procedures were according to Carter et al.[3]

Figure S2. Absorption and fluorescence spectra of **16** (5 µM in H_2O). The maximum absorbance was measured at 494 nm; the fluorescence emission had a maximum at 518 nm. Both spectra were recorded using a cuvette and SpectraMax M5 microplate reader (Molecular Devices, Sunnyvale, CA).

Figure S3. FCM measurements of binding of the high affinity fluorescent P2Y$_6$R agonist (compound **16**) and a 5'-monophosphate control derivative (compound **42**) after 60 min incubation.

Figure S4. Fluorescent images using compound **17**. 1321N1-P2Y$_6$R cells were incubated with the fluorescent compound in different concentrations (100 nM-1µM) for 60 min at 37 °C, and fluorescent images were recorded using Zeiss LSM 700 confocal microscope. Although intense fluorescent labeling was observed with a 100 nM concentration of **17**, it could not be blocked using P2Y$_6$R agonist UDP (100 µM).

Figure S5. (A) Association binding kinetics of **16** to 1321N1-P2Y$_6$R cells determined using FCM. Cells were incubated with 400 nM **16** for different time intervals for 5 min to 3 h at 37 °C. Association rate constant (K) was determined to be 0.019 min^{-1}. Results are expressed as mean ± S.E. (n=3). (B) Saturation experiments for **16** binding to 1321N1-P2Y$_6$R determined using FCM. Comparison of 37˚C and 4˚C, binding for 90 min, with 100 µM UDP used to define nonspecific binding. sucrose and incubation at 4˚C were used to block internalization of the receptor. Results are expressed as mean ± S.E. (n=3).

Figure S6. Binding of MRS4129 **16** to the P2Y$_6$R was blocked by using known P2Y$_6$R ligands antagonist MRS2578 and agonists UDP and MRS2957. There was a 30 min pre-incubation with the receptor ligand followed by a brief treatment with the fluorescent ligand **16** (2 min). The decrease in fluorescent intensity was measured by FCM. A significant difference in mean fluorescent intensity was observed between MRS4129 alone treated cells and MRS4129+P2Y$_6$ ligands treated cells. *, $p<0.01$, when compared to MRS4129 alone treated cells.

Figure S7. Alignment of the sequences of the CXCR4, P2Y$_1$, P2Y$_2$, P2Y$_4$ and P2Y$_6$Rs. Transmembrane helices (TMs), extracellular loops (ELs) and intracellular loops (ILs) are indicated by labels. Blue dots indicate the conserved cationic residues putatively responsible for the coordination of the negatively charged phosphate groups of the ligands. Yellow boxes and connected by yellow lines indicate disulfide bridges. A red and a blue box, connected by a red and blue line indicate a salt bridge that putatively connects EL2 to the extracellular end of TM6 in P2Y receptors. Color-coded bars indicate the secondary structure of the template, with □-helices in red, β-strands in yellow, and turns in blue. Black dots indicate the TM.50 position for each helix, as defined in the GPCR indexing system. The figure was generated with the Molecular Operating Environment (MOE) software.

Figure S1.

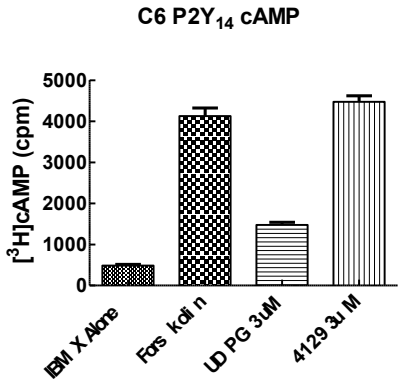


Figure S2.

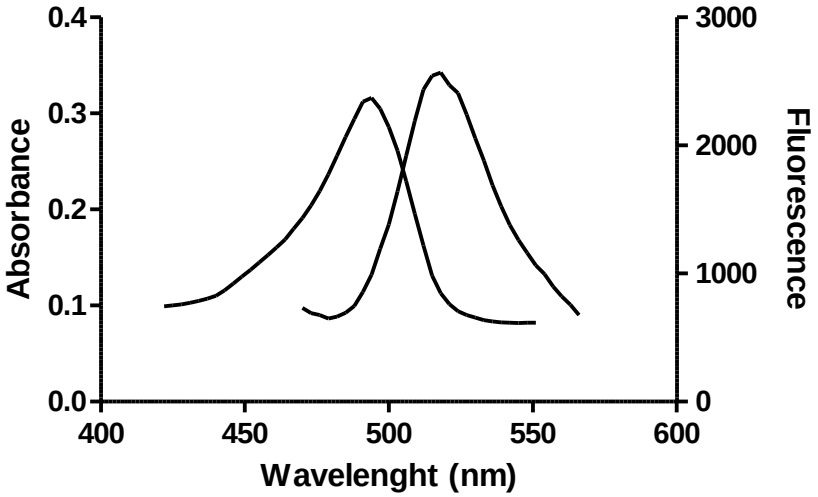
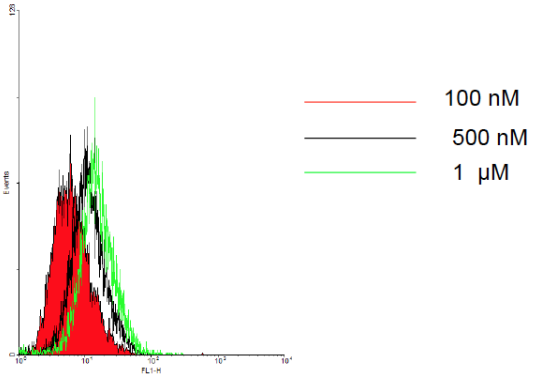


Figure S3.

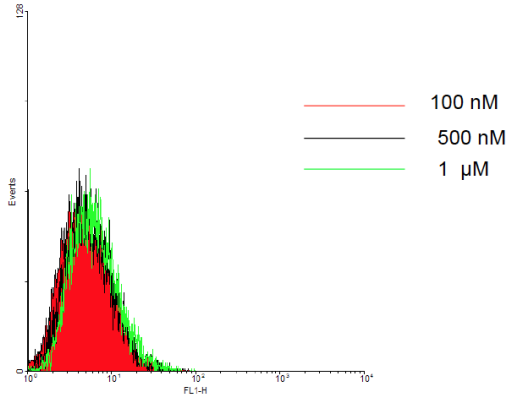
A

FCM Measurement of Binding of MRS4129 (Compound 16) in P2Y$_6$R-Expressing 1321N1 Astrocytoma Cells (90 min incubation)



B

FCM Measurement of Binding of MRS4158 (5'-Monophosphate 42) in P2Y$_6$R-Expressing 1321N1 Astrocytoma Cells (90 min incubation)



C

Comparison of Binding of Different Concentrations of MRS4129 and MRS4158 (5'-Monophosphate) in P2Y$_6$R-Expressing 1321N1 Astrocytoma Cells

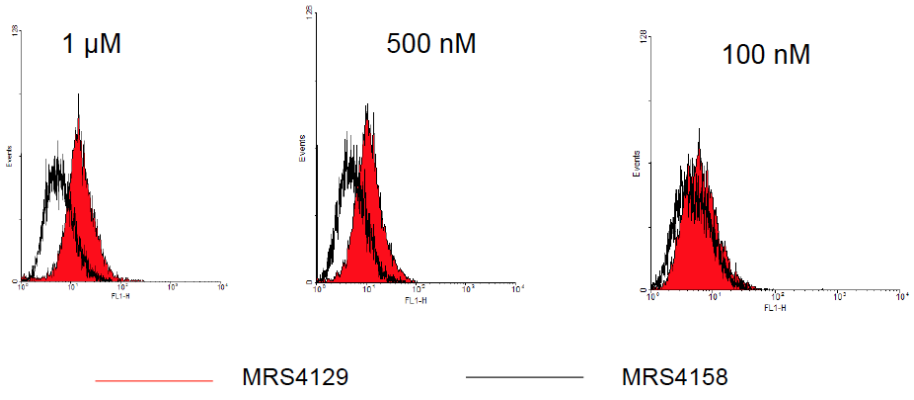


Figure S4.

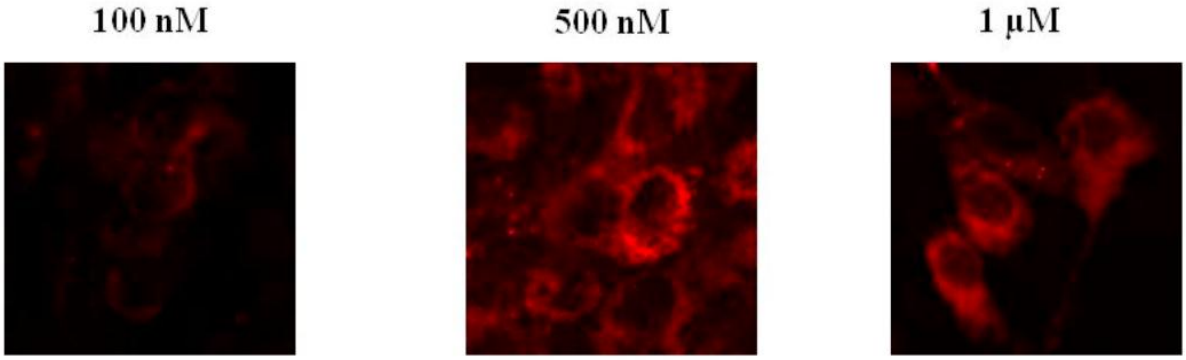
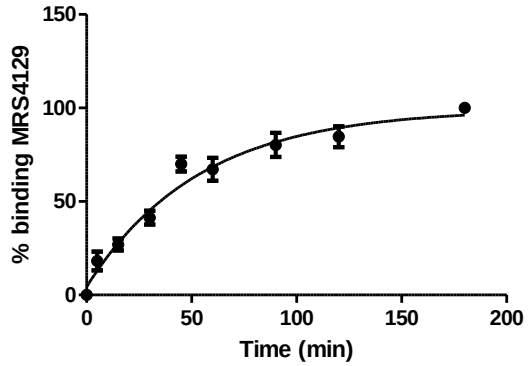


Figure S5.

A



B

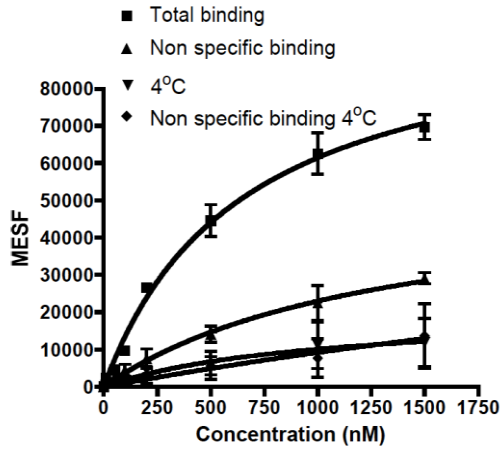


Figure S6.

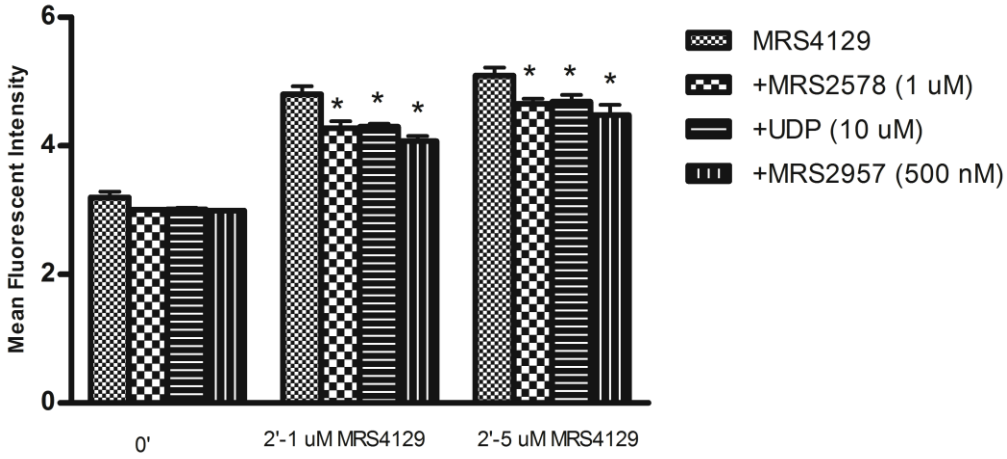
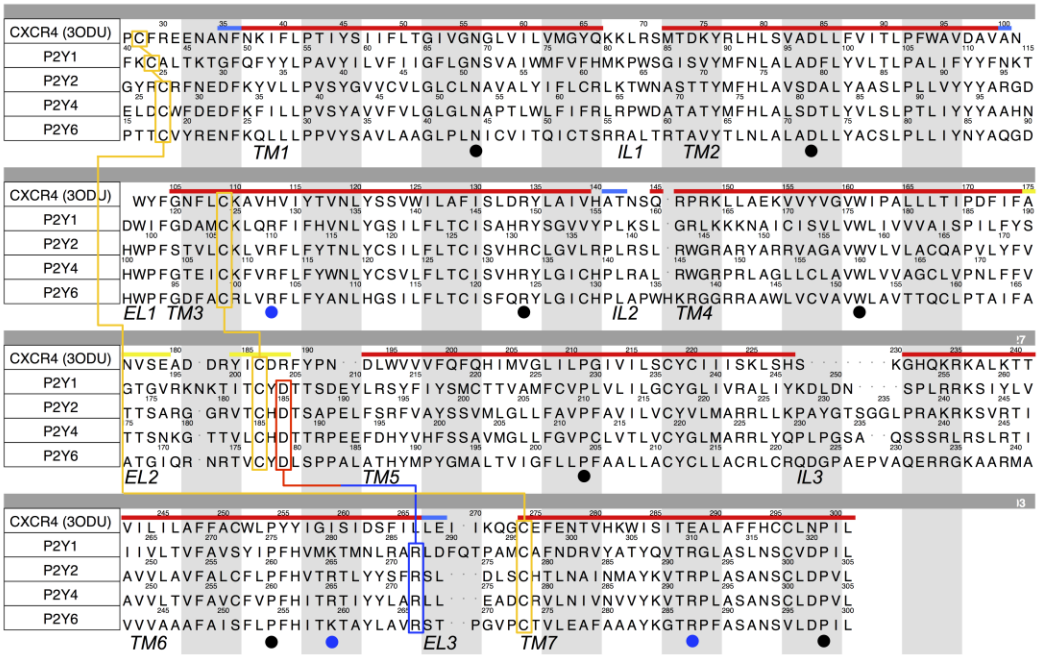


Figure S7.


CXCR4 (3ODU)
P2Y1
P2Y2
P2Y4
P2Y6
TM1
IL1
TM2
EL1
TM3
IL2
TM4
EL2
TM5
IL3
TM6
EL3
TM7

Chemical synthetic methods

Reagents and instrumentation

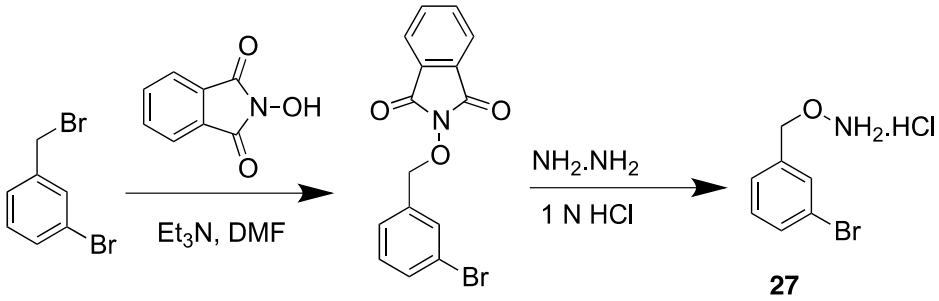
Alexafluor 488 azide and Cy5 azide were purchased from Invitrogen-Life Technologies (Grand Island, NY). Cytidine, and other reagents and solvents were purchased from Sigma-Aldrich (St. Louis, MO). ^{1}H NMR spectra were obtained with a Varian Gemini 300 or Varian Mercury 400 spectrometer using D_2O or $CDCl_3$ as a solvent. The chemical shifts are expressed as relative ppm from HOD (4.80 ppm). ^{31}P NMR spectra were recorded at room temperature (rt) by use of Varian XL 300 (121.42 MHz) or Varian Mercury 400 (162.10 MHz) spectrometers; orthophosphoric acid (85%) was used as an external standard. In several cases the signal of the terminal phosphate moiety was not visible because of high dilution.

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 unless otherwise noted. Purification of the nucleotide analogues for biological testing was carried out on (diethylamino)ethyl (DEAE)-A25 Sephadex columns with a linear gradient (0.01- 0.5 M) of 0.5 M ammonium bicarbonate as the mobile phase followed by HPLC purification with a Luna 5 μmRP-C18(2) semi-preparative column (250 mm X 10.0 mm; Phenomenex, Torrance, CA). Following mobile phase conditions were used for the purification: flow rate of 2 mL/min; 10 mM triethylammonium acetate (TEAA)-CH_3CN from 100:0 to 95:5 (or up to 100:0 to 75:25) in 20-40 min (and isolated in the triethylammonium salt form).

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

General procedure for the synthesis of *O*-benzylhydroxylamine hydrochloride analogues (27-31)

Synthesis of *O*-(3-bromobenzyl)-hydroxylamine hydrochloride (27)



27

***O*-(3-Bromobenzyl)-hydroxyphthalimide**

To a 25 mL round bottom flask was added *N*-hydroxyphthalimide (0.279 g, 1.69 mmol, 1.0 equiv.), DMF (2.25 mL) and Et_3N (0.571 mL, 4.11 mmol, 2.4 equiv.). To this dark brown solution was added 3-bromobenzyl bromide (0.5 g, 1.69 mmol, 1 equiv.), and the reaction mixture was heated (100 °C) for 4 h. The mixture was cooled, and 8 g of ice was added in portions with stirring. Resulted orange/brown semi solid was refrigerated for 1h and solids were filtered, washed with water and dried under high vacuum overnight. The crude material was used in subsequent experiment without further purification. ^{1}H NMR (400 MHz, $CDCl_3$): 7.79 (m, 2H), 7.72 (m, 2H), 7.67 (s, 1H), 7.47 (m, 2H), 7.24 (m, 1H), 5.14 (s, 2H). ^{13}C NMR (400 MHz, $CDCl_3$): 163.4, 135.9, 134.5, 132.5, 132.3, 130.1, 128.8, 128.2, 123.6, 122.5, 78.9.

***O*-(3-Iodobenzyl)-hydroxyphthalimide**

^{1}H NMR (400 MHz, $CDCl_3$): 7.86 (t, 1H, J_1=1.5 Hz), 7.80 (s, 1H), 7.43 (m, 2H), 7.72 (m, 2H), 7.68 (d, 1H, J_1 =7.68 Hz), 7.51 (d, 1H, J_1=7.68 Hz), 7.10 (m, 1H), 5.21 (s, 2H). m/z (M+ESI MS) found: 379.15; calc for $C_{15}H_{10}NO_3I$: 379.15.

***O*-(4-Bromobenzyl)-hydroxyphthalimide**

^{1}H NMR (400 MHz, $CDCl_3$): 7.78 (m, 2H), 7.72 (m, 2H), 7.47 (d, 2H, J_1 = 8.40 Hz), 7.38 (d, 2H, J_1 = 8.40 Hz), 5.13 (s, 2H). ^{13}C NMR (400 MHz, $CDCl_3$): 163.4, 134.5, 132.7, 131.7, 131.4, 128.8, 123.6, 123.5, 78.9.

***O*-(4-Iodobenzyl)-hydroxyphthalimide**

^{1}H NMR (400 MHz, $CDCl_3$): 7.78 (m, 2H), 7.70 (m, 4H), 7.25 (d, 2H, J_1=8.24 Hz), 5.12(s, 2H). ^{13}C NMR (400 MHz, $CDCl_3$): 163.63, 137.92, 134.73, 133.56, 131.70, 128.99, 123.77, 95.70, 79.27.

***O*-(4-Nitrobenzyl)-hydroxyphthalimide**

^{1}H NMR (400 MHz, $CDCl_3$): 8.20 (d, 2H, J_1 = 8.68 Hz), 7.81 (m, 2H), 7.74 (m, 2H), 7.72 (d, 2H, J_1 = 8.64 Hz), 5.27 (s, 2H). ^{13}C NMR (400 MHz, $CDCl_3$): 163.4, 148.0, 142.0, 134.7, 130.0, 128.7, 123.7, 123.7, 78.3.

***O*-(3-Iodobenzyl)-hydroxylamine hydrochloride (28)**

O-(3-Iodobenzyl)-hydroxyphthalimide according to the previous experiment was dissolved in 4 mL of ethanol and treated with hydrazine hydrate (0.146 mL,1.86 mmol, 1.1 equiv.). The reaction mixture was refluxed at 78 °C for 1.5 h. After completion, reaction mixture was cooled and 3% Na_2CO_3 (5 mL) was added and the mixture extracted with ether (20 mL x 3). Anhydrous sodium sulfate was added to the combined organic portion, filtered and dried. Then crude mixture was redissolved in 2 mL of CH_2Cl_2, and 1.8 mL of 4 M HCl in dioxane was added and the mixture stirred 3 h at room temperature. The resulting white colored salt was collected by filtration (1.2 mmol, 72% for 3 steps). ^{1}H NMR (400 MHz, $CDCl_3$) for ***O*-(3-Iodobenzyl)-hydroxylamine** : 7.70 (s, 1H), 7.61 (s, 1H, J_1 =7.84 Hz), 7.29 (d, 1H, J_1 =7.60 Hz), 7.07 (dd, 1H, J_1 =7.84 Hz, J_2 =7.60 Hz), 5.36 (Broad, 2H), 4.6 (s, 2H). m/z (M+ESI MS) found: 284.4; calc for C_7H_8NOI: 284.94.

***O*-(4-Iodobenzyl)-hydroxylamine**: ^{1}H NMR (400 MHz, $CDCl_3$): 11.12(br, 2H), 7.78 (d, 2H, J_1=7.57 Hz), 7.22 (d, 2H, J_1=7.60 Hz), 5.00(s, 2H). ^{13}C NMR (400 MHz, $CDCl_3$): 138.09, 134.23, 131.99, 96.35, 75.64.

General procedure for the synthesis of N^4-benzyloxycytidine analogues (34-38)

***N*^{4}-(*O*-(3-Bromobenzyloxy))cytidine (34)**

A suspension of cytidine (1.4 mmol, 0.38 g) and *O*-(3-bromobenzyl)-hydroxylamine hydrochloride (**27**, 2.79 mmol, 0.8 g) in pyridine (3.5 mL) was stirred at 100 °C for 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-(O-(3-bromobenzyloxy))cytidine (**34**) (548 mg, 1.28 mmol, 90%). ^{1}H NMR (400 MHz, $CDCl_3$): 7.50(s, 1H), 7.39 (d, 1H, J_1 = 7.89 Hz), 7.28 (d, 1H, J_1 =7.60 Hz), 7.21 (d, 1H, J_1 = 7.40 Hz), 7.16 (d, 1H, J_1 = 8.28 Hz), 5.82 (d, 1H, J_1 = 5.53 Hz), 5.52 (d, 1H, J_1 = 8.28 Hz), 4.94 (s, 2H), 4.11 (t, 1H, J_1 = 5.4 Hz), 4.07 (m, 1H), 3.90 (dt, 1H, J_1 = 3.44 Hz, J_2 = 3.28 Hz), 3.74 (dd, 1H, J_1 = 2.89 Hz, J_2 = 12.12 Hz), 3.65 (dd, 1H, J_1 = 12.12 Hz, J_2 = 3.40 Hz). ^{13}C NMR (400 MHz, $CDCl_3$):150.0, 145.4, 131.7, 130.4, 130.3, 129.7, 126.2, 121.9, 97.2, 88.4, 84.7, 74.2, 73.3, 70.2, 61.3. m/z (M+ESI MS) found: 428.0461; calc for $C_{16}H_{19}N_3O_6Br$: 428.0457.

***N*^{4}-(*O*-(3-Iodobenzyloxy))-cytidine (35)**

Following above procedure, N^4-(*O*-(3-iodobenzyloxy))cytidine (**35**) was prepared (298 mg, 0.63 mmol, 85%). ^{1}H NMR (400 MHz, $CDCl_3$): 8.14 (s, 1H), 7.66 (s, 1H), 7.62 (d, 1H, J_1 = 7.92 Hz), 7.27 (d, 1H, J_1 =7.48 Hz), 7.06 (m, 1H), 6.84 (d, 1H, J_1 = 8.2 Hz), 5.55 (m, 1H), 5.45 (d, 1H, J_1 = 5.00 Hz), 4.9 (s, 2H), 4.39 (s, 1H), 4.31 (s, 1H), 4.09 (m, 1H), 3.88 (m, 1H), 3.74 (m, 1H), 3.48 (s, 1H), 2.80 (br s, 1H), 2.46 (br s, 1H). m/z (M+ESI MS) found: 476.0314; calc for $C_{16}H_{19}N_3O_6I$: 476.0319.

***N*^{4}-(*O*-(4-Bromobenzyloxy))-cytidine (36)**

Following above procedure, N^4-(*O*-(4-bromobenzyloxy))cytidine was prepared (**36**) (442 mg, 0.93 mmol, 90%). ^{1}H NMR (400 MHz, $CDCl_3$): 7.41 (d, 2H, J_1 = 8.40 Hz), 7.23 (d, 2H, J_1 = 8.40 Hz), 7.15 (d, 1H, J_1 = 8.28 Hz), 5.81 (d, 1H, J_1 = 5.32 Hz), 5.51 (d, 1H, J_1 = 8.21 Hz), 4.92 (s, 2H), 4.11 (t, 1H, J_1 = 5.40 Hz), 4.07 (m, 1H), 3.91 (dt, 1H, J_1 = 3.44 Hz, J_2 = 3.32 Hz), 3.74 (dd, 1H, J_1 = 2.89 Hz, J_2 = 12.12 Hz), 3.65 (dd, 1H, J_1 = 12.12 Hz, J_2 = 3.40 Hz). ^{13}C NMR (400 MHz, $CDCl_3$):150.0, 145.3, 137.3, 131.6, 131.0, 129.5, 121.1, 97.3, 88.4, 84.7, 74.2, 73.3, 70.2, 61.3.m/z (M+ESI MS) found: 428.0464; calc for $C_{16}H_{19}N_3O_6Br$: 428.0457.

***N*^{4}-(*O*-(4-Iodobenzyloxy))cytidine (37)**

Following above procedure, N^4-4-iodobenzyloxycytidine (**37**) was isolated (544 mg, 1.11 mmol, 82%). ^{1}H NMR (400 MHz, $CDCl_3$): 7.63 (d, 2H, J_1 = 8.28 Hz), 7.05 (d, 2H, J_1 = 8.28 Hz), 6.96

(d, 1H, J_1 = 8.20 Hz), 5.64 (d, 1H, J_1 = 4.20 Hz), 5.53 (d, 2H, J_1 = 8.20 Hz), 4.90 (s, 2H), 4.25 (s, 2H), 4.00 (s, 1H), 3.75 (m, 2H), 3.44 (m, 4H), 2.84 (br, 1H). m/z (M+ESI MS) found: 476.0324; calc for $C_{16}H_{19}N_3O_6I$: 476.0319.

***N*^{4}-(*O*-(4-Nitrobenzyloxy))-cytidine (38)**

Following above procedure, *N*^{4}-(*O*-(4-nitrobenzyloxy))cytidine (**38**) was isolated (201mg, 0.422 mmol, 89%). ^{1}H NMR (400 MHz, $CDCl_3$): 8.12 (d, 2H, J_1 = 8.78 Hz), 7.52 (d, 2H, J_1 = 8.80 Hz), 7.17 (d, 1H, J_1 = 8.28 Hz), 5.82 (d, 1H, J_1 = 5.32 Hz), 5.53 (d, 1H, J_1 = 8.24 Hz), 5.08 (s, 2H), 4.11 (t, 1H, J_1 = 5.40 Hz), 4.07 (m, 1H), 3.91 (dt, 1H, J_1 = 3.44 Hz, J_2 = 3.32 Hz), 3.74 (dd, 1H, J_1 = 2.89 Hz, J_2 = 12.12 Hz), 3.65 (dd, 1H, J_1 = 12.12 Hz, J_2 = 3.40 Hz). ^{13}C NMR (400 MHz, $CDCl_3$):150.0, 147.3, 146.1, 145.6, 131.8, 127.9, 123.0, 97.2, 88.3, 84.7, 73.8, 73.4, 70.2, 61.3.m/z (M+ESI MS) found: 395.1189; calc for $C_{16}H_{19}N_4O_8$: 395.1203.

***N*^{4}-(*O*-(3-(1,5-Hexadiynyl))benzyloxy)-cytidine (39)**

N^4-3-Iodobenzyloxycytidine (**35**, 311 mg, 0.66 mmol), copper iodide (50 mg, 0.264 mmol, 0.4 equiv.), $Pd(PPh_3)_4$ (152 mg, 0.132 mmol, 0.2 equiv.) and DMF (13 mL) were added to a 50 mL Schlenk flask under N_2. The mixture was degassed 3 times and filled with N_2. 1,5-Hexadiyne (50% in pentane, 1.28 mL, 6.6 mmol, 10 equiv.) and Et_3N (0.183 mL, 1.32 mmol, 2 equiv.) were added to the above mixture and stirred for 2 h at room temperature. After completion, all the solvents were removed and the product *N*^{4}-(*O*-(3-(1,5-hexadiynyl))benzyloxy)cytidine (**39**) (90 mg, 2 mmol, 30%) was isolated after column chromatography (5%-20% methanol: CH_2Cl_2). 1H NMR (400 MHz, $CDCl_3$): 8.29 (Broad, 1H), 8.00 (s, 1H), 7.64 (m, 1H), 7.55 (m, 1H), 7.45 (m, 1H), 7.38 (m, 1H), 6.87 (d, 1H, J_1 = 8.16 Hz), 5.57 (d, 1H, J_1 = 4.80 Hz), 5.54 (d, 1H, J_1 = 8.20 Hz), 4.95 (s, 2H), 4.30 (m, 1H), 4.24 (m, 1H), 4.02 (s, 1H), 3.82 (m, 1H), 3.70 (m, 1H), 2.63 (t, 2H, J_1 = 6.80 Hz), 2.50 (m, 2H), 2.00 (s, 1H). m/z (M+ESI MS) found: 426.1670; calc for $C_{22}H_{24}N_3O_6$: 426.1656.

General procedure for the synthesis of analogues of *N*^{4}-(*O*-(Benzyloxy))cytidine-5′-diphosphate diethyl ammonium salts (10, 11, 12, 13, 14, 15)

***N*^{4}-(*O*-(3-Bromobenzyloxy))cytidine-5′-diphosphate diethyl ammonium salt (10)**

N^4-(*O*-(3-Bromobenzyloxy))cytidine (**34**, 30.1 mg, 0.07 mmol,) and trimethyl phosphate (0.7 ml) added to 25 mL round bottom flask and cooled to 0 °C. Proton sponge (22.6 mg, 0.11 mmol, 1.5 equiv.) was added to above mixture and stirred for 10 min at 0 °C. Then phosphorous

oxychloride (13 μL, 0.15 mmol) was added dropwise, and the reaction mixture was stirred for 2h at 0 °C. A mixture of tributylamine (0.138 mL) and a solution of 0.35 M of bis(tributylammonium) salt of phosphoric acid in DMF (0.344 mL) was added once. This salt was prepared by mixing tributylamine (0.2 ml, 0.83 mmol) and phosphoric acid (43 mg, 0.44 mmol) in 1.25 ml DMF. After 10 min, 1.5 mL of 0.2 M triethylammonium bicarbonate (TEAB) was added, and the reaction mixture was stirred for 30 min at room temperature. The mixture was lyophilized overnight and the residue was purified by Sephadex-DEAE resin ion-exchange column chromatography, followed by semipreparative HPLC as described above to obtain compound **10** (3 mg, 3.8 x 10^{-3} mmol, 8% yield). ^{1}H NMR (400 MHz, D_2O): δ 7.61(s, 1H), 7.54 (d, 1H, J_1 = 7.80 Hz), 7.38 (d, 1H, J_1 =7.76 Hz), 7.32 (d, 1H, J_1 = 7.80 Hz), 7.23 (d, 1H, J_1 = 8.28 Hz), 5.94 (d, 1H, J_1 = 6.04 Hz), 5.74 (d, 1H, J_1 = 8.20 Hz), 5.02 (s, 2H), 4.38 (dd, 1H, J_1 = 5.36 Hz, J_1 = 3.30 Hz), 4.34 (t, 1H, J_1 = 5.8 Hz), 4.22 (m, 1H), 4.15 (m, 2H. ^{31}P NMR (400 MHz, D_2O): δ -9.44, -11.31. m/z (M-ES MS) found: 585.9649; calc for $C_{16}H_{19}N_3O_{12}P_2Br$: 585.9627.

N^4-(*O*-(3-Iodobenzyloxy))-cytidine-5′-diphosphate diethyl ammonium salt (11)

Compound **11** was synthesized according to the previous procedure (3 mg, 3.5 x 10^{-3} mmol, 8% yield. ^{1}H NMR (400 MHz, D_2O): δ 7.82 (s, 1H), 7.75 (d, 1H, J_1 = 7.92 Hz), 7.41 (d, 1H, J_1 = 7.68 Hz), 7.18 (m, 2H), 5.93 (d, 1H, J_1 = 5.96 Hz), 5.74 (d, 1H, J_1 = 8.18 Hz), 4.98 (s, 2H), 4.36 (m, 2H), 4.32-4.14 (m, 2H). m/z (M-ES MS) found: 633.9476; calc for $C_{16}H_{19}N_3O_{12}P_2I$: 633.9489.

N^4-(*O*-(4-Bromobenzyloxy))-cytidine-5′-diphosphate diethyl ammonium salt (12)

Compound **12** was synthesized according to the previous procedure (1.2 mg, 1.5 x 10^{-5} mmol, 6% yield. ^{1}H NMR (400 MHz, $CDCl_3$): 7.59 (d, 2H, J_1 = 8.44 Hz), 7.34 (d, 2H, J_1 = 8.44 Hz), 7.24 (d, 1H, J_1 = 8.36 Hz), 5.93 (d, 1H, J_1 = 5.99 Hz), 5.75 (d, 1H, J_1 = 8.28 Hz), 5.01 (s, 2H), 4.41 (dd, 1H, J_1 = 5.35 Hz, J_2 = 3,52 Hz), 4.35 (t, 1H, J_1 = 5.7 Hz), 4.21 (m), 4.16 (m, 2H). m/z (M-ES MS) found: 585.9590; calc for $C_{16}H_{19}N_3O_{12}P_2Br$: 585.9627.

N^4-(*O*-(4-Iodobenzyloxy))-cytidine-5′-diphosphate diethyl ammonium salt (13)

Compound **13** was synthesized according to the previous procedure (3 mg, 3.5 x 10^{-3} mmol, 5% yield. ^{1}H NMR (400 MHz, D_2O): δ 7.75 (d, 2H, J_1 = 8.32 Hz), 7.24 (d, 1H, J_1 = 7.64 Hz), 7.20 (d, 2H, J_1 = 8.28Hz), 5.90 (d, 1H, J_1 = 5.96 Hz), 5.74 (d, 1H, J_1 = 8.40 Hz), 4.99 (s, 2H), 4.40 (m, 1H), 4.34 (m, 1H), 4.20 (m, 3H), 4.13 (m, 1H). m/z (M-ES MS) found: 633.9529; calc for $C_{16}H_{19}N_3O_{12}P_2I$: 633.9489.

***N*^{4}-(*O*-(4-Nitrobenzyloxy))-cytidine-5′-diphosphate diethyl ammonium salt (14)**

Compound **14** was synthesized according to the previous procedure (2.3 mg, 3.0 x 10^{-3} mmol, 6% yield). ^{1}H NMR (400 MHz, $CDCl_3$): 8.25 (d, 2H, J_1 = 8.80 Hz), 7.60 (d, 2H, J_1 = 8.68 Hz), 7.24 (d, 1H, J_1 = 8.20 Hz), 5.95 (d, 1H, J_1 = 5.96 Hz), 5.74 (d, 1H, J_1 = 8.12 Hz), 5.17 (s, 2H), 4.41 (m, 1H), 4.35 (t, 1H, J_1 = 5.7 Hz), 4.22 (m, 1H), 4.15 (m, 2H). ^{31}P NMR (400 MHz, $CDCl_3$): -8.44, -11.12. m/z (M-ES MS) found: 553.0353; calc for $C_{16}H_{19}N_4O_{14}P_2$: 553.0373

***N*^{4}-(*O*-(3-(1,5-Hexadiynyl))-benzyloxy)cytidine-5′-diphosphate diethyl ammonium salt (15)**

Compound **15** was synthesized according to the previous procedure. (8.90 x 10^{-3} mmol, 7 mg, 10% yield). ^{1}H NMR (400 MHz, D_2O): δ 7.5-7.4 (m, 4H), 7.24 (d, 1H, J = 7.6 Hz), 5.93 (d, 1H, J = 5.8 Hz), 5.76 (d, 1H, J = 8.0 Hz), 5.04 (s, 2H), 4.41-4.34 (m, 2H), 2.21 (m, 3H), 2.68 (dd, 2H, J_1= 7.2 Hz, J_2 = 6.40 Hz), 2.53 (m, 2H), 2.46 (m, 1H). m/z (M-ES MS) found: 584.0823; calc for $C_{22}H_{24}N_3O_{12}P_2$: 584.0835.

***N*^{4}-(*O*-(3-Methoxycytidine-5′-(β-naphthyl)-triphosphate triethylammonium salt (20)**

The aryl monophosphate and N^4-3-methoxycytidine-5′- diphosphate triethylammonium salt[2] were first converted to its tributylammonium salt form by treatment with ion-exchange resin (DOWEX 50WX2-200 (H)), which upon acidification of the supernatant was followed by addition of tributylamine until a basic pH was reached. The aqueous solvent was removed by lyophilization, and the residue was used without further purification. A solution of tributylammonium salt of **8** (3 mg, 0.0047 mmol), β-naphthyl-monophosphate triethylammonium salt (1.72 mg, 0.0054 mmol) and DIC (3.0 µL, 0.017 mmol) in DMF (0.01 mL) was stirred at rt for 16 h. The solvent was removed and the crude was purified by semipreparative HPLC to obtain **20** (0.5 mg, 13%) as a white amorphous solid. ^{1}H NMR (400 MHz, D_2O): δ 7.95-7.90 (m, 3H), 7.51 (s, 1H), 7.10-6.98 (m, 3H), 7.24 (d, 1H, J = 7.6 Hz), 5.93 (d, 1H, J = 5.8 Hz), 5.76 (d, 1H, J = 8.0 Hz), 4.39-4.34 (m, 5H), 3.71 (s, 3H). ^{31}P NMR (400 MHz, D_2O): δ -11.53, -16.25, -23.29. m/z (M-ES MS) found: 638.0337; calc for $C_{20}H_{23}N_3O_{15}P_3$: 638.0342.

General procedure for the preparation of nucleoside triphosphates (21).

A solution of the N^4-(*O*-(benzyloxy))cytidine (**33**, 0.025 g, 0.073 mmol) and Proton Sponge (24 mg, 0.11 mmol) in trimethyl phosphate (0.4 mL) was stirred for 10 min at 0 °C. Then, phosphorous oxychloride (0.013 mL, 0.13 mmol) was added dropwise, and the reaction mixture was stirred for 2 h at 0 °C. A solution of tributylammonium pyrophosphate (0.8 mL, 0.44 mmol) and tributylamine (0.069 mL, 0.29 mmol) in *N*,*N*-dimethylformamide (DMF, 1 mL) was added and stirring was continued at 0°C for additional 10 min. 0.2 M Triethylammonium bicarbonate solution (1.5 mL) was added, and the clear solution was stirred at rt for 1 h. After removal of solvents, the residue was purified by Sephadex-DEAE A-25 resin and preparative HPLC.

***N*^{4}-Benzyloxycytidine-5′-triphosphate triethylammonium salt (21).**

Compound **21** (16 mg, 0.02 mmol, 27% yield) was obtained as a white solid. ^{1}H NMR (D_2O) δ 7.42 (m, 6H), 7.22 (d, J = 8.4 Hz, 1H), 5.95 (d, J = 6.5 Hz, 1H), 5.76 (d, J = 8.4 Hz, 1H), 5.05 (s, 2H), 4.42-4.34 (m, 2H), 4.23-4.15 (m, 3H) ; ^{31}P NMR (D_2O) δ - 1.17, -11.39 (br), -23.04 (br); HRMS-EI found 588.0200 $(M - H^+)^-$. $C_{16}H_{21}N_3O_{15}P_3$ requires 588.0186; purity > 98% by HPLC.

General Procedure for the Preparation *N*^{4}-3-Benzyloxycytidine-5′-(3-butynyl) (22) and (4-Pentynyl) (23) triphosphate triethylammonium salt analogues.

The appropriate alkynyl[1] or aryl monophosphate and N^4-3-alkoxycytidine-5′- diphosphate triethylammonium salt[2] were first converted to its tributylammonium salt form by treatment with ion-exchange resin (DOWEX 50WX2-200 (H)), which upon acidification of the supernatant was followed by addition of tributylamine until a basic pH was reached. The aqueous solvent was removed by lyophilization, and the residue was used without further purification. A solution of tributylammonium salt of **18** (5 mg, 0.007 mmol) and DIC (3.36 µL, 0.021 mmol) in DMF (0.03 mL) was stirred for 3 h at rt. Then, a solution of 4-pentynylmonophosphate tributylammonium salt (5.0 mg, 0.0014 mmol) in DMF (0.03 mL) and a solution of $MgCl_2$ (1.96 mg, 0.021 mmol) in DMF (0.03 mL) were added. After stirring the reaction mixture at rt for 16 h, the solvent was removed and water (1 mL) was added. The resulting solid was removed by filtration, and the filtrate was purified by semipreparative HPLC as described above to obtain **23** (1.89 mg, 40%) as a white amorphous solid.

N^4-(*O*-(3-Benzyloxy))-cytidine-5′-(3-butynyl)-triphosphate triethylammonium salt (22)

Compound **22** was synthesized from the above procedure. (0.5 mg, 6 x10^{-4} mmol, 4.2% yield). ^{1}H NMR (400 MHz, D_2O): δ 7.44-7.40 (m, 5H), 7.23 (d, 1H, J = 8.20 Hz), 5.94 (d, 1H, J = 6.12 Hz), 5.77 (d, 1H, J = 8.16 Hz), 5.02 (s, 2H), 4.38-4.32 (m, 2H), 4.23 (m, 1H), 4.17 (m, 1H), 4.02 (dt, 2H, J_1= 7.12, Hz, J_2 = 6.64 Hz), 2.53 (td, 2H, J_1= 5.40 Hz, J_2 = 2.40 Hz), 2.31 (t, 1H, J_1= 2.36 Hz). m/z (M-ES MS) found: 640.0509; calc for $C_{20}H_{25}N_3O_{15}P_3$: 640.0499.

N^4-(*O*-(3-Benzyloxy))-cytidine-5′-(4-pentynyl)-triphosphate triethylammonium salt (23)

Compound **23** was synthesized from the above procedure. (1.89 mg, 40% yield). ^{1}H NMR (400 MHz, D_2O): δ 7.42 (m,5H), 7.23 (d, 1H, J_1 = 8.32 Hz), 5.94 (d, 1H, J_1 = 6.16 Hz), 5.77 (d, 1H, J_1 = 8.32 Hz), 5.00 (s, 2H), 4.36 (m, 2H), 4.17 (m, 3H), 3.19 (m, 2H), 2.28 (m, 3H), 1.81 (t, 2H, J_1 = 6.24Hz). m/z (M-ES MS) found: 645.0625; calc for $C_{21}H_{27}N_3O_{15}P_3$: 654.0655.

N^4-(*O*-(3-(3-AlexaFluor-1H-1,2,3-triazol-4-yl)hex-1-ynyl)benzyloxy)-cytidine-5′ diphosphate diethyl ammonium salt (16)

To a mixture of Alexafluor 488 azide (0.0016 mmol, 1.0 mg, 1 eq) and dialkyne **15** (0.0029 mmol, 2.0 mg, 2 eq) in a 0.40 mL of (1:1) mixture of ^{t}BuOH and water, was added tris[(1-benzyl-1H-1,2,3-triazol-4-yl)methyl] amine (TBTA, 0.05 mg) and freshly prepared 1 M aqueous sodium ascorbate solution (0.0016 mmol, 1.6 µL, 1 eq) followed by 7.5% aqueous copper sulfate pentahydrate solution (4.0 µL, 0.0012 mmol. 0.75 eq). The reaction mixture was stirred overnight at room temperature, solvent was evaporated, and the residue was purified by semipreparative HPLC as described above to obtain **16** (1.64 mg, 34% yield) as a yellow/orange solid. ^{1}H NMR (400 MHz, MeOD): δ 8.28 (s, 1H), 7.94 (s, 1H), 7.39 (d, 1H, J_1= 8.04 Hz), 7.30 (s, 1H), 7.20 (m, 6H), 6.95 (d, 2H, J_1= 9.28 Hz), 5.88 (d, 1H, J_1= 5.68 Hz), 5.72 (d, 1H, J_1= 8.32 Hz), 4.91 (s, 2H), 4.43 (t, 3H, J_1= 6.30 Hz), 4.38 (m, 1H), 4.31 (m, 1H), 4.18 (m, 3H), 2.97 (t, 2H, J_1= 6.80 Hz), 2.75 (m, 2H), 1.87 (m, 2H), 1.15 (m, 4H). m/z (M-ES MS) found: 1244.2157; calc for $C_{49}H_{52}N_9O_{22}S_2\ P_2$: 1244.2144.

N^4-(*O*-(3-(3-AlexaFluor-1H-1,2,3-triazol-4-yl)hex-1-ynyl)benzyloxy)-cytidine-5′ monophosphate diethyl ammonium salt (42)

Compound **42** (0.7 mg, 0.0004 mmol, 15% yield) was isolated from the HPLC purification as a byproduct of the synthesis of compound **16**. m/z (M-ES MS) found: 1162.2280; calc for $C_{49}H_{49}N_9O_{19}S_2P$: 1162.2324.

N^4-3-(3-Cy5-1H-1,2,3-triazol-4-yl)hex-1-ynyl)benzyloxy)-cytidine-5′-diphosphate diethyl ammonium salt (17)

To a mixture of Cy5 azide **43** (0.7 mg, 1.16 μmol, 1 eq) and dialkyne **15** (1.2 mg, 1.76 μmol, 1.5 eq) in a 0.40 mL of (1:1) mixture of ^{t}BuOH and water, was added tris[(1-benzyl-1H-1,2,3-triazol-4-yl)methyl] amine (TBTA, 0.05 mg) and freshly prepared 1 M aqueous sodium ascorbate solution (1.16 μL, 1.16 μmol, 1 eq) followed by 7.5% aqueous copper sulfate pentahydrate solution (2.5 μL, 0.87 μmol. 0.75 eq). The bright blue color reaction mixture was stirred overnight at room temperature, solvent was evaporated, and the residue was purified by semipreparative HPLC as described above to obtain desired diphosphate **17** and its monophosphate **44** as blue color solids. m/z (M-ES MS) for **17** found: 1148.4423; calc for $C_{57}H_{68}N_9O_{13}P_2$: 1148.4412 and for **44** found: 1068.4744; calc for $C_{57}H_{67}N_9O_{10}P$: 1068.4749.

***N^4*-(*O*-(3-Benzyloxy))cytidine-5′-((3-ethynyl-1H-1,2,3-triazol-4-yl)-(N-(2-ethyl)-acetamide)-triphosphate ammonium salt (24)**

To a mixture of *N*-(2-azidoethyl)acetamide **45** (1.26 mg, 9.90 μmol, 1.5 eq) and dialkyne **22** (4.8 mg, 6.60 μmol, 1.5 eq) in a 0.35 mL of (2:3:5) mixture of water, ^{t}BuOH and ethanol, was added freshly prepared 1 M aqueous sodium ascorbate solution (6.68 μL, 6.68 μmol, 1 eq) followed by 7.5% aqueous copper sulfate pentahydrate solution (11.15 μL, 3.33 μmol. 0.5 eq). The reaction mixture was stirred overnight at room temperature, solvent was evaporated, and the residue was purified by semipreparative HPLC as described above to obtain **24** (0.38 mg, 2.6 %) as a white solid. ^{1}H NMR (400 MHz, D_2O): δ 7.88 (s, 1H), 7.44 (m, 5H) 7.18 (d, 1H, J_1 = 8.40 Hz), 5.91 (d, 1H, J_1 = 6.68 Hz), 5.70 (d, 1H, J_1 = 8.16 Hz), 5.02 (s, 2H), 4.46 (m, 2H), 4.36-4.31 (m, 2H), 4.20 4.17 (m, 4H), 4.00 (m, 1H), 3.59 (m, 2H), 3.18 (dt, 2H, J_1 = 7.64 Hz, J_2 = 7.15 Hz), 3.0 (m, 2H), 2.25 (s, 3H). m/z (M-ES MS) found: 768.1180; calc for $C_{24}H_{33}N_7O_{116}P_3$: 768.1197.

Reference:

1. Das, A.; Ko, H; Burianek, L. E.; Barrett, M. O.; Harden, K. T.; Jacobson, K. A. *J. Med. Chem*. **2010**, *53*, 471–480.
2. Maruoka, H.; Barrett, M. O.; Ko, H.; Tosh, D. K.; Melman, A.; Burianek, L. E.; Balasubramanian, R.; Berk, B.; Costanzi, S.; Harden, K. T.; Jacobson, K. A. *J. Med. Chem*. **2010**, *53*, 4488–4501.
3. R. L. Carter, I. P. Fricks, M. O. Barrett, L. E. Burianek, Y. Zhou, H. Ko, A. Das, K. A. Jacobson, E. R. Lazarowski, and T. K. Harden, *Mol. Pharmacol.* 2009, **76**, 1341.