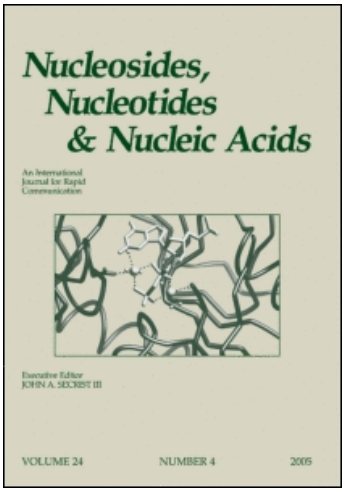

Nucleosides, Nucleotides and Nucleic Acids
Publication details, including instructions for authors and subscription information:
http://www.informaworld.com/smpp/title~content=t713597286

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To cite this Article Shaver, Sammy R. , Pendergast, William , Siddiqi, Suhaib M. , Yerxa, Benjamin R. , Croom, Dallas K. , Dougherty, Robert W. , James, Michael K. , Jones, A. Neil and Rideout, Janet L.(1997) '4-Substituted Uridine 5'-Triphosphates as Agonists of the P_{2Y2} Purinergic Receptor', Nucleosides, Nucleotides and Nucleic Acids, 16: 7, 1099 — 1102
To link to this Article: DOI: 10.1080/07328319708006141
URL: http://dx.doi.org/10.1080/07328319708006141

NUCLEOSIDES & NUCLEOTIDES, 16(7-9), 1099-1102 (1997)

4-SUBSTITUTED URIDINE 5'-TRIPHOSPHATES AS AGONISTS OF THE P_{2Y2} PURINERGIC RECEPTOR

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Abstract Uridine 5'-O-triphosphate (UTP) is a potent agonist of the purinergic receptor designated P_{2Y2}. UTP is rapidly metabolized in human tissue. To find a compound with similar activity that may be more slowly metabolized, a series of 4-substituted uridine 5'-triphosphates were prepared and evaluated in a P_{2Y2} receptor second messenger assay.

A class of purinergic receptors known as P_2 receptors are involved in platelet aggregation and wound healing, insulin secretion, mitogenesis, vasodilation, and transepithelial ion transport[1]. The family of P_2 receptors consists of many subtypes; P_{2X} (excitatory ion channel), P_{2Y} (G-protein coupled), P_{2Z} (membrane pore in mast cells, now thought to be P_{2X7}), and P_{2T} (ADP receptor on platelets).

The P_{2Y2} receptor is widely distributed in human tissue, including the heart, liver, kidney, and lung. The airway epithelial cells have a high density of P_{2Y2} receptors. An agonist acting at these P_{2Y2} receptors activates phospholipase C via coupling to the G-protein Gq. When this occurs, there is an increase in cilia beat frequency through an increase in internal Ca^{2+} concentration, an increase of chloride efflux that promotes hydration of airway secretions, and an increase of mucin release from goblet cells which all result in improved lung clearance[2,3]. This is a desirable result in individuals with a decreased lung function and mucociliary clearance that could result from disease states such as cystic fibrosis (CF) or primary ciliary dyskinesia (PCD). Therefore, an agonist of the P_{2Y2} receptor could serve as a therapeutic agent against these disease manifestations.

Uridine 5'-triphosphate (UTP) is a selective agonist at the P_{2Y2} receptor with an average EC50 of 0.12 μM in an inositol phosphate second messenger assay. UTP is currently in Phase I/II clinical trials and preliminary data suggest that UTP may have utility in the treatment of cystic fibrosis[4]. Studies seem to indicate that UTP has a very short half-life on the lung mucosal surface. A compound with a similar pharmacologic profile that would be

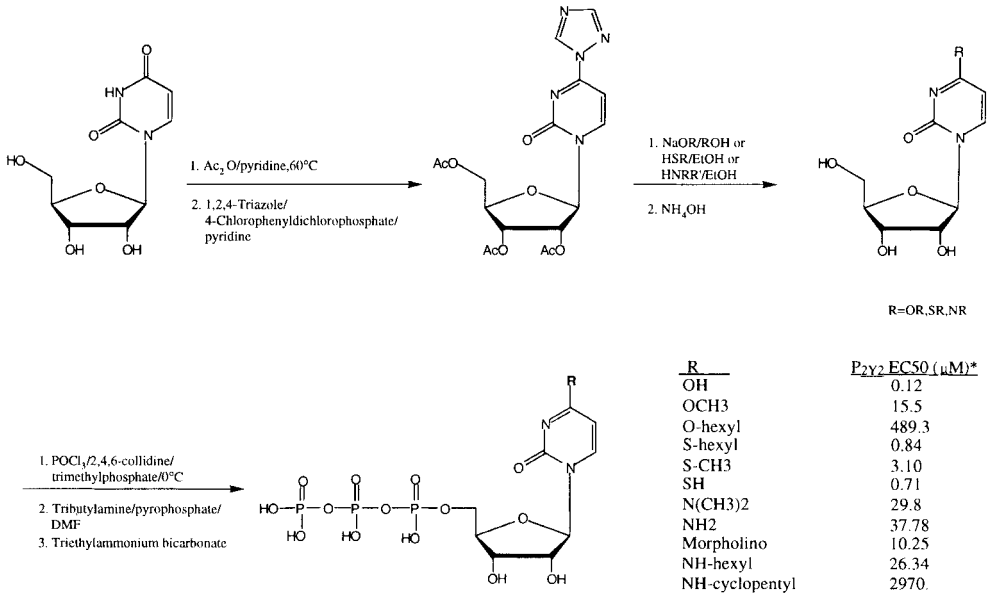
metabolized more slowly by endogenous enzymes (ectonucleotidases, etc.) may have a therapeutic advantage. Modifications of the pyrimidine base could result in compounds which will be poorer substrates than UTP for these catabolic enzymes. A series of substituted uridine and cytidine analogs were prepared and tested in the MUCOSA™ second messenger assay to identify possible candidates for further *in vivo* study.

Uridine was dissolved in pyridine and acetic anhydride added slowly over ten minutes. The solution was heated at 60°C for 3 hours then quenched by addition of ice. The reaction was partitioned between equal volumes of $CHCl_3$ and H_2O, the organic layer washed with cold 1N HCl, H_2O, brine, then dried over Na_2SO_4. The solvent was removed under vacuum to yield a gummy solid that was stirred with Et_2O to give 2',3',5'-tri-O-acetyluridine (92%) as a powder.

This material was dissolved in anhydrous pyridine at 0°C followed by slow addition of 4-chlorophenyldichlorophosphate[5]. After 10 minutes 1,2,4-triazole was added and the reaction stirred for 18 hours at ambient temperature. The reaction was quenched by addition of ice and partitioned with an equal volume of chloroform. The organic layer was dried, reduced in volume, and applied to a silica column and eluted with ethyl acetate/hexane (4:1 v/v). The product fractions were collected and evaporated to dryness to yield 4-(1,2,4-triazol-1-yl)-1-(2,3,5-tri-O-acetyl-β-D-ribofuranosyl) pyrimidin-2(1H)-one in 65% yield.

This material was suspended in EtOH and treated with cyclopentylamine at ambient temperature for 48 hours. The solvent was removed and the residue dissolved in MeOH. Treatment with concentrated NH_4OH for 48 hours yielded the desired nucleoside in 78% yield. The nucleoside was stirred with 2,4,6-collidine in trimethylphosphate for 10 minutes then treated with $POCl_3$ at 0°C for 2 hours[6]. Tributylamine and a DMF solution of tributylammonium pyrophosphate were added to the reaction and stirred for 10 minutes then poured into an 0.2M aqueous solution of triethylammonium bicarbonate. After stirring for 45 minutes, the solvent was removed under vacuum keeping the bath temperature below 30°C. The residue was dissolved in a minimum amount of H_2O, applied to a Sephadex DEAE A-25 column and eluted with a linear 0 to 0.5 M ammonium bicarbonate gradient. The triphosphate fractions were collected (by UV detection at 280nm) and repeatedly coevaporated with H_2O to yield 4-cyclopentylamino-1-(β-D-ribofuranose-5-triphosphatyl) pyrimidin-2(1H)-one. ^1H NMR (D_2O) δ 7.62 (d,J=7.7HZ,1H,H6), 5.84-5.80 (m,2H,H5,H1'), 4.21-4.05 (m,5H,H2',H3',H4',H5'), 1.85-1.30 (m,9H,cyclopentyl); ^{31}P NMR (D_2O) δ -0.375 (d), -10.74 (d), -22.04 (t); HPLC: Synchropak AX-300 (4.6 x 250mm) 75%H_2O/25%CH_3CN to 75% 0.5M KH_2PO_4 /25% CH_3CN 2mL/min. linear gradient for 15min. then final conditions for 10 min.; retention=18.5 min. (>98% pure).

Displacement of the triazolyl functionality with appropriate nucleophiles gave the other nucleosides in good yields. They were isolated, purified, and characterized in a manner analogous to that described above.



R	P_{2Y2} EC50 (μM)*
OH	0.12
OCH3	15.5
O-hexyl	489.3
S-hexyl	0.84
S-CH3	3.10
SH	0.71
N(CH3)2	29.8
NH2	37.78
Morpholino	10.25
NH-hexyl	26.34
NH-cyclopentyl	2970.

*Average of three values

The functional activities of test compounds were assessed (MUCOSA™ assay) measuring the stimulated accumulations of [^{3}H]-inositol phosphates in 1321N astrocytoma cells stably overexpressing human P_{2Y2} receptor[7] by modifications of published methods[8]. Briefly, confluent cultures of cells in 96-well format were incubated for 20-24 hours in inositol-free DMEM-H containing 0.1 μCi [^{3}H]-inositol/well to radiolabel inositol phospholipid pools to high specific activities. Prior to assay, labeled cells were incubated with 10 mM LiCl for 15 minutes prior to a 90 minute challenge with test compounds. Reactions were terminated by aspirating the reaction mixture followed by the addition of 150 μL boiling 1.0 mM EDTA. [^{3}H]-inositol phosphates were resolved by anion exchange chromatography as described.[9]

All of the compounds were assayed in the inositol triphosphate second messenger assay (MUCOSA™). The EC50's of 4-thiouridine 5'-triphosphate and 4-thiohexyluridine 5'-triphosphate (0.71 and 0.84 μM, respectively) were within an order of magnitude of UTP (0.12 μM). The remaining compounds were much less active than UTP in this assay. This apparent loss of agonist activity at the P_{2Y2} receptor is disappointing and likely due to unfavorable binding interactions. Any delayed metabolism of the phosphate moieties in these compounds becomes unimportant given the marginal activities shown.

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