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Review Article

$P2Y_2$ receptor agonists: structure, activity and therapeutic utility

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Summary

Molecular and cell biology research has highlighted the fundamental role of nucleotides and their derivatives in neurotransmission and metabotropic processes of a wide variety of cell types. This basic research has paved the way for using these compounds as potential therapeutics for treating many diseases, especially those that involve the mucosal epithelia. Molecular biology techniques have allowed for the discovery of two families of membrane bound receptors for these highly charged molecules. The P2X receptors are ligand-gated ion channels that are implicated in various neuromodulatory processes. The P2Y family of metabotropic receptors, on the other hand, is made up of 7-transmembrane G-protein-coupled receptors that bind to both purine and pyrimidine nucleotides.

The $P2Y_2$ receptor is found on the apical surface of airway epithelia and is believed to be the major coordinator of mucociliary clearance mechanisms in the lung. Nucleotide agonists of the $P2Y_2$ receptor, such as uridine 5'-triphosphate, have been shown to increase hydration of airway surface liquid and to mobilize these secretions by enhancing the cilia beat frequency of ciliated airway epithelial cells. Structure activity relationships of pyrimidine nucleotide analogs have provided new insights into the search for more potent and stable synthetic compounds that would lead to more treatment options for chronic diseases such as obstructive pulmonary disease and dry eye.

Introduction

The emphasis of this review is on the function of the $P2Y_2$ receptor and its utility as a therapeutic target for pulmonary and other diseases. Structure activity relationships (SAR) and medicinal chemistry of the $P2Y_2$ agonists will be included where appropriate. Since the nomenclature and classification of purinergic receptors is continuously evolving (1-3), only a brief discussion and update is provided. The signal transduction mechanisms of P2 receptors have been thoroughly reviewed (4, 5); thus, signal transduction discussions will be broad and focus only on cellular processes relevant to the therapeutic areas discussed. The mechanistic and therapeutic actions of uridine 5'-triphosphate (UTP) in the lung has not been reviewed since 1995 (6). Several reviews of P2 receptors have appeared in the last several years (7-10). It is the intention of this review to provide updated information on $P2Y_2$ agonists and their therapeutic utility, with emphasis on the SAR of pyrimidine nucleotides.

The concept of purinergic receptors was originally proposed by Burnstock (11, 12) to explain responses previously categorized as nonadrenergic and noncholinergic. Adenosine 5'-triphosphate (ATP) was proposed as the cognate ligand for these receptors. Further investigation in this area led Burnstock to propose the division of purinergic receptors into P1 receptors, responding to adenosine (Ado) and coupled to adenylate cyclase, and P2 receptors, responding to ATP and ADP (Fig. 1). Subsequent divisions have been made in the P2 receptor class based on the early observations of Burnstock and Kennedy: the P2X receptors are ligand-gated ion channels and the P2Y receptors are G-protein-coupled metabotropic receptors (GPCR) with 7 transmembrane domains (13).

Since the original classification was proposed, 5 receptors in the P2Y receptor family have been cloned (Table I). Some of these receptors respond to pyrimidine nucleotides as well as purines, and thus the family is now

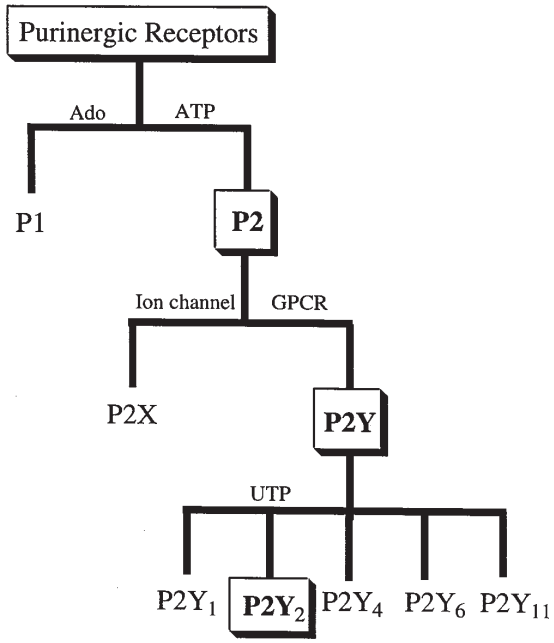

Fig. 1. Purinergic receptor superfamily tree. The pharmacologic lineage of the P2Y$_2$ receptor subtype is derived from the P2 receptors which respond to nucleotides.

termed P2Y receptors rather than P2Y purinergic receptors.

Signal transduction and mechanism of action

Activation of P2Y$_2$ receptors by the presumed endogenous agonist, UTP, has been associated with activation of phospholipase C, formation of inositol trisphosphate (IP_3) and increased intracellular calcium concentration (Fig. 2) (14-17). Activation of P2Y$_2$ receptors in respiratory epithelia has been related to increased mucociliary clearance (18) presumably through the combination of the following cellular actions: increased chloride and water transport across the luminal surface (19-22), increased cilia beat frequency (23), increased mucin release (24) and increased surfactant release (25).

P2Y$_2$ agonists

UTP and analogs

The first P2Y$_2$ agonists evaluated were the nucleoside triphosphates that were used to pharmacologically characterize the receptor (Fig. 3). These agonists, among

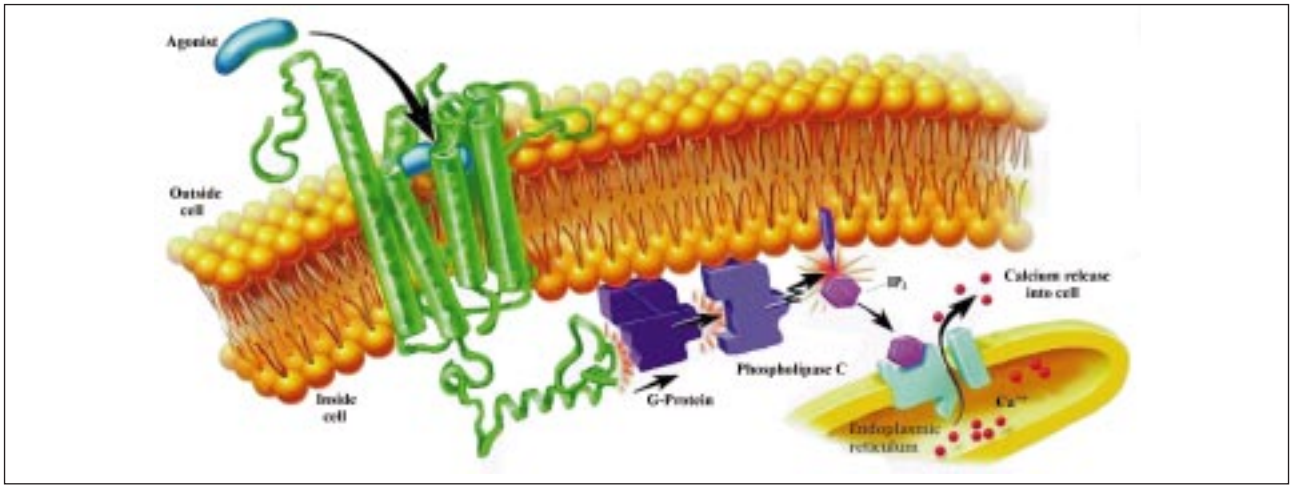

Fig. 2. P2Y$_2$ receptor in the cell membrane. Agonists bind to the P2Y$_2$ receptor, activating phospholipase C through a G-protein-coupled mechanism. The resultant increase in inositol (1,4,5)-trisphosphate (IP_3) levels causes release of Ca^{2+} from the endoplasmic reticulum. This stimulates cellular functions such as increased cilia beat frequency and Cl^- efflux, which improve mucociliary clearance.

Table I: Subtypes in the P2Y receptor family.

Receptor subtype	Pharmacology	Coupling
P2Y$_1$	2MeSATP > ATP (UTP inactive)	PLC/IP_3/Ca^{2+}
P2Y$_2$	**UTP = ATP >> 2 MeSATP**	**PLC/IP_3/Ca^{2+}**
P2Y$_4$	UTP > UDP > ATP	PLC/IP_3/Ca^{2+}
P2Y$_6$	UDP > UTP > ATP	PLC/IP_3/Ca^{2+}
P2Y$_{11}$	ATP > 2MeSATP >>> ADP	PLC/IP_3/Ca^{2+} AC/cAMP

2MeSATP = 2 methylthioATP; PLC = phospholipase C; IP_3 = inositol 1,4,5-trisphosphate.

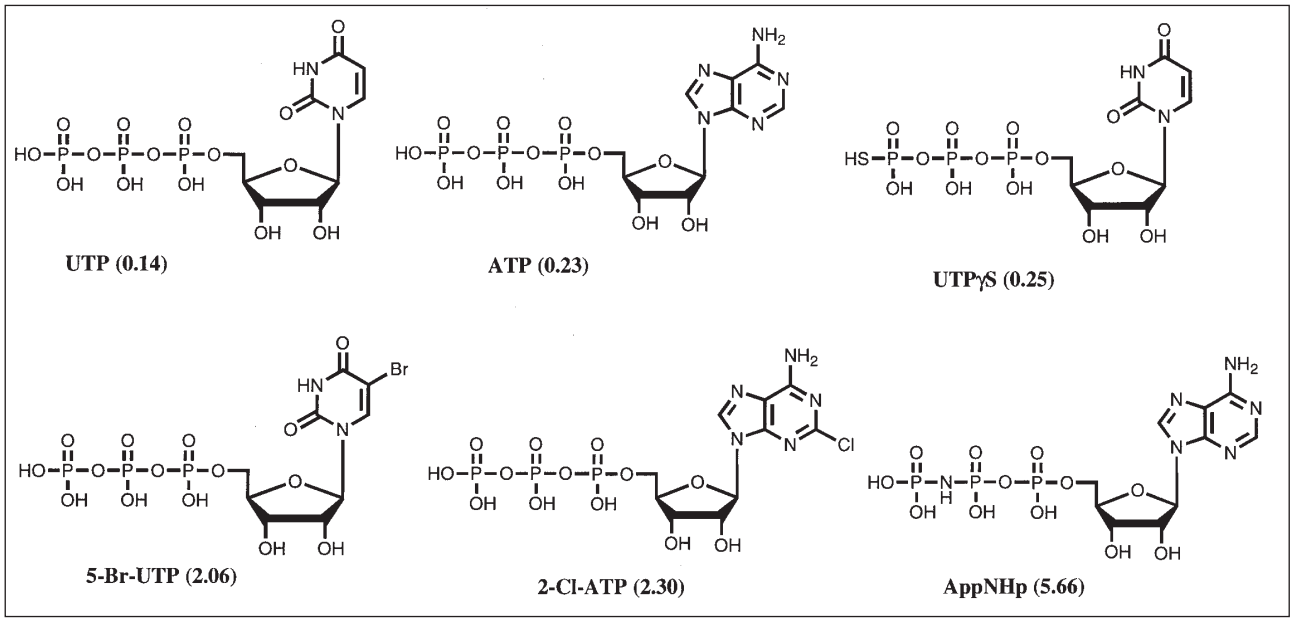

Fig. 3. Purine and pyrimidine nucleotide agonists of $P2Y_2$ receptor. Uridine and adenine nucleoside triphosphates are agonists of the $P2Y_2$ receptor. EC_{50} (μM) values are in parentheses.

others, included the commercially available purine and pyrimidine nucleoside triphosphates such as UTP and ATP and their halogenated derivatives (26). The terminal thiophosphate compound was also found to be a potent agonist with potential for increased biological stability. The triphosphate mimic, AppNHp, in which an imido group links the last two phosphate groups together, was a weak agonist of the $P2Y_2$ receptor, but may have increased stability over ATP. The diphosphates, UDP, ADP and GDP, are not potent agonists of the $P2Y_2$ receptor. At the $P2Y_1$ receptor, however, ADP is a potent and full agonist (27).

These agonists set the stage for the medicinal chemists and biologists to design, synthesize and test nucleoside triphosphates for $P2Y_2$ agonism. Since adenosine-containing nucleotides may have undesirable cardiovascular effects (28-30) via the action of its metabolite, adenosine, most of the SAR work has been focused around uridine nucleotides. Metabolism of UTP, for example, ultimately gives the inactive metabolite uridine. Although UTP itself is a drug candidate in human clinical trials (see later sections), it has two drawbacks: 1) it is chemically unstable and must be kept frozen and 2) it is rapidly metabolized by enzymes on the mucosal surface, leading to a relatively short duration of action.

Figure 4 outlines the general strategy used to make potent and stable analogs of UTP. The triphosphate moiety may be substituted with fraudulent linkers, including those that are nonhydrolyzable by enzymes. The pyrimidine base may be modified by substituting heteroatoms or adding R groups at various positions around the ring to explore space and to gain other favorable binding interactions. In addition, modifications of the sugar portion

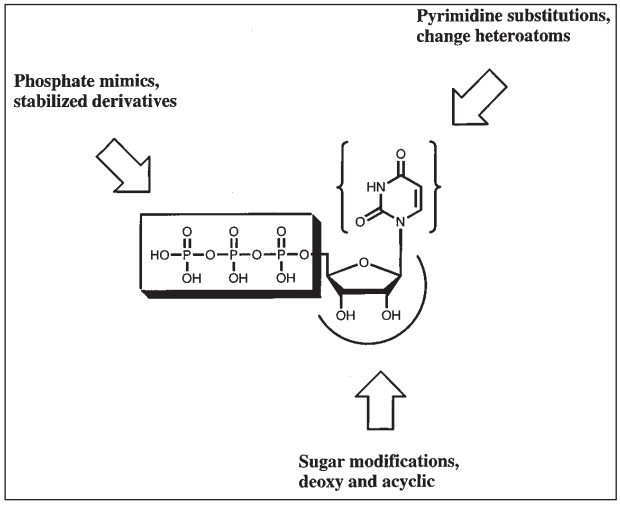

Fig. 4. Synthetic strategy for UTP analogs. Three approaches to the modification of UTP include phosphate mimics, changes to the pyrimidine base and modification of the ribose sugar.

may be explored. One can also imagine employing acyclic analogs which have been successful in the antiviral field. The following sections examine some of the synthetic chemistry and SAR of these types of pyrimidine nucleotide $P2Y_2$ agonists.

Phosphate mimics

As mentioned previously, the original $P2Y_2$ receptor pharmacology work included the agonist AppNHp which

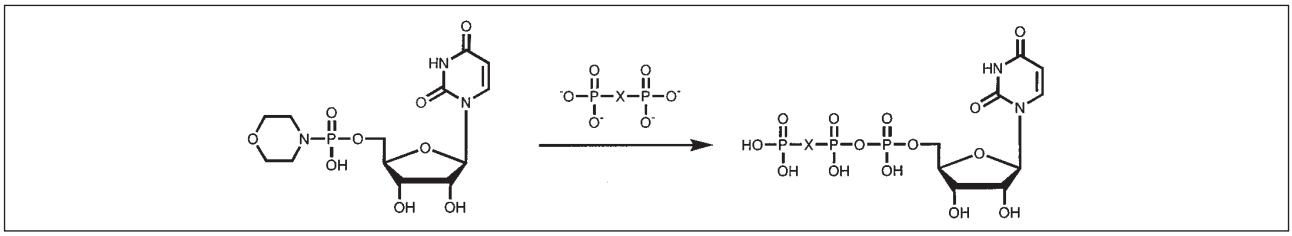

Fig. 5. Synthesis of fraudulent triphosphates. UTP analogs containing fraudulent triphosphate moieties are synthesized in one step from UMP-morpholidate.

Table II: Predicted and observed EC_{50} values for $P2Y_2$ agonists with fraudulent triphosphates.

x	Predicted EC_{50} (μm) QSAR*	COMFA#	Observed EC_{50} (μM) (mean ± SEM)
NH	0.80	1.20	1.45 ± 0.8
CH_2	63.5	80.9	73.3 ± 20.0
CF_2	1.90	7.60	8.92 ± 1.5

*QSAR+ module in Cerius[2] software from Molecular Simulations, Inc. #COMFA module in Sybyl software from Tripos, Inc.

served as a starting point for exploring the SAR of phosphate mimics with fraudulent linkers. Although early molecular modeling indicated that these molecules would not be as potent as the proposed native ligand, UTP, they were synthesized not only to evaluate their potency but also to test their ability to resist metabolism (31). The synthesis of a series of UTP analogs is shown in Figure 5 and the molecular modeling and biological assay results are presented in Table II. The most potent compounds in this series contain replacement atom(s) with an electronegativity closer to that of oxygen such as the imido and difluoromethylene groups.

Pyrimidine substitutions

UTP analogs substituted at the 4 position were not synthesized or tested for activity at the $P2Y_2$ receptor until 1997. These compounds can be made according to the synthetic scheme outlined in Figure 6. Uridine is per acetylated on the ribose moiety and then treated with phosphorous oxychloride and triazole to give the 4-triazolyl uridine derivative (32). This compound can be made on a large scale and stored in a dessicator. The triazole is then displaced with a variety of nucleophiles including

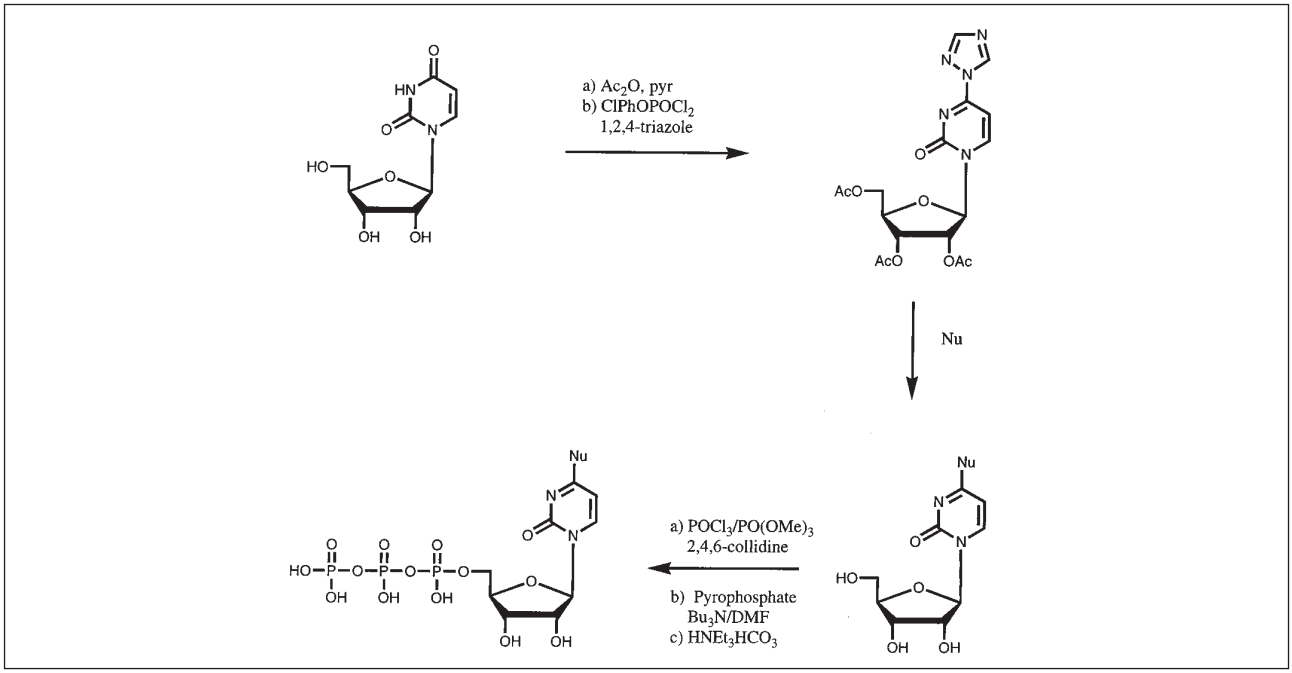

Fig. 6. 4-Substituted UTP analog synthesis. Pyrimidine nucleotides with various substituents at the 4-position can be made by attack of a nucleophile (Nu) on the triazole intermediate, followed by one-pot phosphorylation.

Table III: Structure activity relationship of 4-substituted UTP derivatives.

R	$P2Y_2$ EC_{50} (μm)*
O-Me	15.5
O-Hexyl	13.61
SH	0.03
S-Me	3.10
S-Hexyl	0.84
$N(Me)_2$	29.8
N-Hexyl	7.0
NH-cPentyl	Inactive
Morpholino	6.1

*Mean of at least 3 independent determinations.

amines and thiols to give the 4-substituted compounds. The corresponding 5'-triphosphates are then synthesized by the one-pot phosphorylating method using phosphorous oxychloride and trimethylphosphate followed by pyrophosphate (33). Table III shows the activity of selected 4-substituted UTP derivatives.

Sugar modifications

Modifications of the sugar backbone proved to be detrimental to $P2Y_2$ agonist activity (Fig. 7). The arabinose analog of UTP in which the 2'-hydroxyl is inverted, was several times less potent than UTP. Removal of a hydroxyl group as in the 2'- or 3'-deoxy UTP derivatives created less active compounds, and the 2',3'-dideoxy analog was inactive. Similarly, periodate oxidation of UTP to the dialdehyde destroyed activity. Recently, two series of acyclo-UTP derivatives were described although their activity at the $P2Y_2$ receptor was not disclosed (34). It appears thus far that the hydroxyls on the ribose sugar and an intact furanosyl moiety are important binding features for these $P2Y_2$ agonists.

Therapeutic utility

Mucociliary clearance

In the normal individual, about 10-20 ml of lower respiratory tract secretions reach the throat everyday, but this volume often exceeds 100 ml/day in certain disease states or may be abnormally low in others (35). The continuous, cephalad movement of lower respiratory material is necessary for the clearance of inhaled pathogenic organisms or injurious particles and is essential to maintain patent airways necessary for efficient gas exchange. The movement of airway secretions, along with accompanying lumenal cells and free foreign particles, is accomplished by the actions of several cell types within the respiratory tract. This process has been variously termed the mucociliary transport system, the mucociliary escalator or simply mucociliary clearance. Mucus is secreted by goblet cells and submucosal glands and forms a gel-like protective sheet within the lumen of the respiratory tract. This layer of mucus is propelled by the rhythmical, coordinated beat of the ciliated epithelial cells lining the airways from the terminal bronchi to the oropharynx and lining the nose. The viscous mucous sheet would be immovable except that it floats on a much

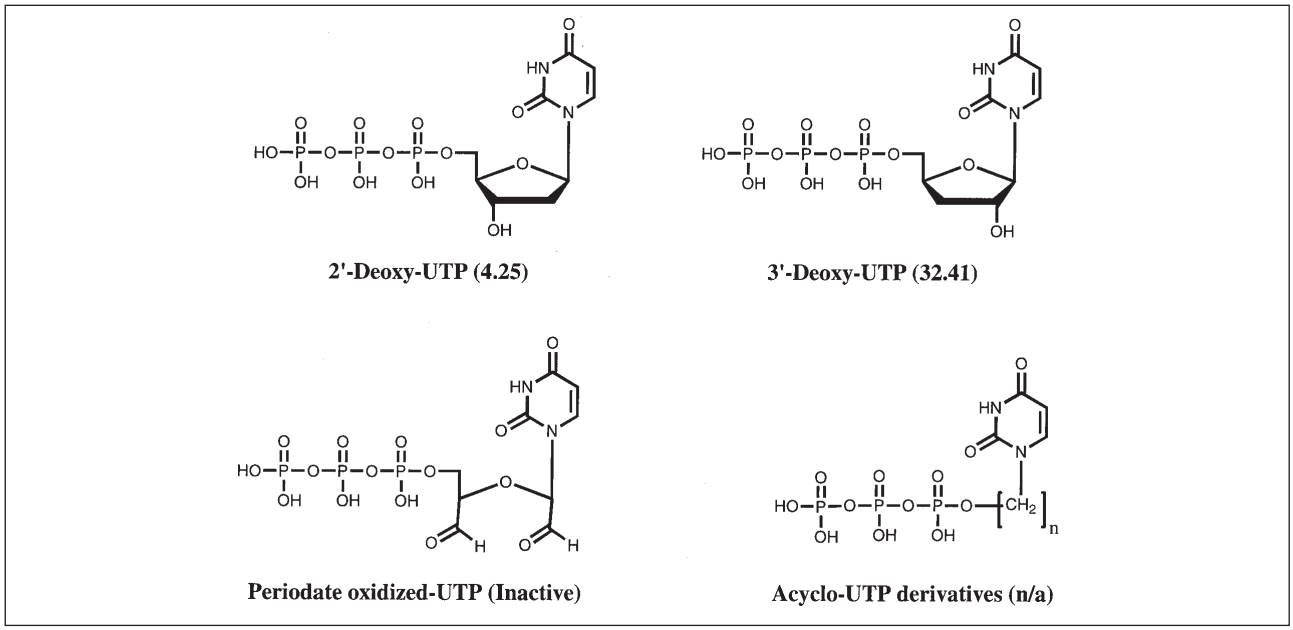

Fig. 7. UTP analogs with modified sugars. Changing the ribofuranosyl moiety of UTP is detrimental to agonist activity at the $P2Y_2$ receptor. EC_{50} (μM) values are in parentheses.

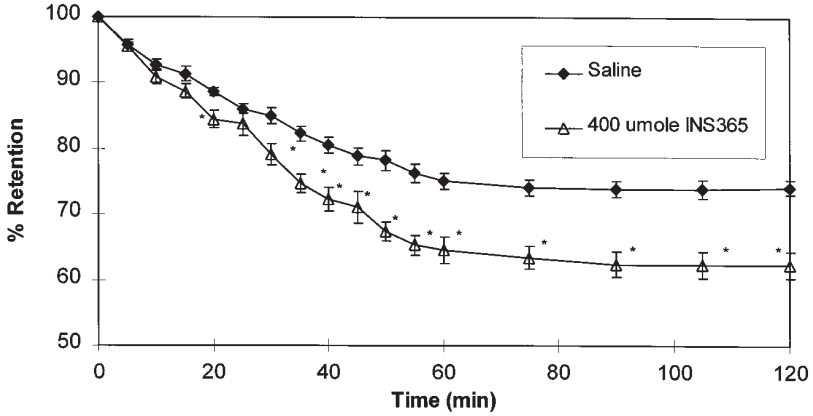

Fig. 8. Effects of INS-365 on mucociliary clearance (MCC) in sheep. This figure shows the effects of 400 μM INS365 on the retention of radiolabelled particles in the lungs of sheep (a measure of MCC). Data shown in this figure are the mean ± SEM for 7 sheep. *Statistical significance for differences between treatments (p <0.05).

less viscous layer of fluid above the beating cilia. This periciliary fluid layer is maintained by the transport of ions (chloride and sodium) across the epithelium onto the lumen of the airways followed by the passive movement of water.

INS365

The UTP SAR work led to the discovery of INS365, a new $P2Y_2$ receptor agonist that was shown to be equipotent to UTP in the inositol phosphate assay (36). Surprisingly, INS365 is very stable chemically, not requiring any refrigeration or special handling as with UTP. INS-365 was thus a breakthrough $P2Y_2$ receptor agonist, making it suitable for treating chronic indications.

UTP and INS365 have been shown to increase tracheal mucus velocity (TMV) in sheep (37). TMV is a surrogate marker for mucociliary clearance in a single large airway and serves as a good indication of effects on whole lung clearance. After demonstrating increased mucus transport in a single large airway, studies were designed to examine the effects of these agents on whole lung mucociliary clearance (MCC). Healthy adult ewes (n=7; 25-45 kg) received a solution containing 20 mCi of ^{99m}Tc-human serum albumin via inhalation over a 5-min period from which a baseline deposition image of the lungs was obtained. The spontaneously breathing, intubated animals then received by inhalation normal saline and 400 μM INS365 (4.0 ml over 10-12 min) in random order in separate treatments. The clearance of radioactivity was then measured following each treatment. Data from serial measurements of radioactivity remaining in the lung between 0 and 120 min after dosing were collected and stored for analysis. Figure 8 illustrates the stimulatory effect of INS365 on MCC in sheep. INS365 significantly accelerated clearance of radioactivity as compared with vehicle within 20 min of dosing (p <0.05) with peak effects observed 60 min after dosing (62% retention). Comparison of the clearance curves obtained following administration of UTP to the same sheep in earlier studies indicated that INS365 was equally potent as a stimulant of MCC (data not shown).

Normal ion transport, mucus secretion and coordinated, rapid beating of the cilia are all essential for the maintenance of normal mucociliary clearance of the respiratory tract. Several disease states in which specific elements of the mucociliary escalator are impaired or defective are associated with abnormal rates of mucociliary clearance, retention of respiratory secretions, impaired pulmonary function and high incidence of pulmonary infections. These diseases include cystic fibrosis and chronic bronchitis.

Cystic fibrosis

Cystic fibrosis (CF) is the most lethal genetic disease in Caucasians in the U.S., affecting approximately 1 in 2000 individuals (38-41). The median survival age for CF patients is 30 years, with the majority of deaths attributable to respiratory failure. Furthermore, the quality of life for patients afflicted with CF is significantly affected by this disease.

CF occurs due to mutations in the gene that codes for the CF transmembrane regulator (CFTR) protein (42-44). These mutations account for the ion conductance abnormalities that are characteristic of CF (45, 46). Abnormalities in sodium, chloride and water transport across epithelial cells result in dehydration and thickening of the mucus layer above the affected cells. The clinical expression of CF reflects the disease-related ion transport defects present in the gastrointestinal tract and the lung. Derangement of the ionic content of the airway surface liquid also may contribute to the susceptibility of CF patients to infection by inhibiting the normal bactericidal

Table IV: Effect of UTP versus placebo on mucociliary clearance in mild chronic bronchitis.

	Clearance rate (%/min)		
	Whole lung Mean (SD)	Central Mean (SD)	Peripheral Mean (SD)
Baseline	0.37 (0.09)	0.40 (0.12)	0.34 (0.07)
Placebo	0.56 (0.09)[a]	0.64 (0.12)[a]	0.49 (0.05)[c]
UTP - 20 mg	0.76 (0.07)[a,b]	0.90 (0.08)[a,b]	0.64 (0.07)[a,d]
UTP - 100 mg	0.78 (0.07)[a,b]	0.93 (0.07)[a,b]	0.66 (0.06)[a,d]

[a]$p < 0.001$, significantly different from baseline. [b]$p < 0.001$, significantly different from placebo. [c]$p < 0.01$, significantly different from baseline. [d]$p < 0.01$, significantly different from placebo.

activity of the airway surface liquid (47). The inability of CF patients to clear this dehydrated mucus and potential pathogens leads to chronic lung infection, progressive lung disease and impaired lung function. Lung infections account for approximately 90% of deaths from CF (38).

Additional therapeutic approaches clearly are needed for the prevention and treatment of CF lung disease. In particular, agents that correct the underlying ion transport defects in the airways may prove useful in normalizing airway secretions, leading to improved mucociliary clearance and preventing chronic lung infections and progressive lung damage. In this regard, evidence is accumulating indicating that $P2Y_2$ receptor agonists may enhance mucociliary clearance in CF patients. UTP has been demonstrated to stimulate chloride secretion via non-CFTR mechanisms in isolated normal and CF epithelial cells (19, 20). The stimulation of chloride secretion by UTP was accompanied by increased fluid transport across the apical surface (21, 22). In addition, UTP has also been shown to increase the beat frequency of cilia in isolated normal and CF epithelial cells (23). Because UTP is subject to rapid degradation in the lung *in vivo* (48), its activity is likely to be short-lived. Consequently, effective therapeutic agents in CF may require longer biological stability than that of UTP.

INS365 is a promising candidate as a therapeutic agent in CF. The results from an initial phase I clinical trial with INS365 indicate that it is safe and well-tolerated in normal nonsmokers and in smokers and that it produces a rapid increase in the quantity of sputum expectorated that is sustained for at least 1 h following a single dose (49). Clinical trials currently in progress in adult and pediatric CF patients will provide the first information on the potential of this compound to enhance mucociliary clearance in this disease.

Chronic bronchitis

Chronic bronchitis (CB) is commonly caused by smoking and environmental pollution. Cigarette smoke irritates airways and paralyzes the cilia lining the respiratory epithelia, resulting in retention of viscous mucus secretions and frequent respiratory infections. Smokers and patients with CB have impaired mucociliary clearance (50, 51). Currently, antibiotics, bronchodilators and antiinflammatory agents are used for the treatment of patients with CB. However, many physicians responsible for the management of these patients indicate that such patients would benefit from increased airway hydration and clearance of bronchial secretions. It is hypothesized that $P2Y_2$ receptor agonists would enhance the hydration of airways secretions, stimulate ciliary beat frequency, enhance cough clearance thereby facilitating airway mucus clearance, delay the onset of severe lung dysfunction and reduce symptoms associated with CB.

The potential for $P2Y_2$ receptor agonists to stimulate mucociliary clearance has been demonstrated in a clinical trial of UTP in normal subjects (18) and in a recently completed clinical proof of concept study of UTP in patients with mild CB. In this latter study, 15 subjects received by inhalation 20 mg UTP, 100 mg UTP and placebo (normal saline) on three separate occasions in a randomized order. Prior to dosing with drug, subjects also inhaled ^{99m}Tc-iron oxide, a radioactive marker that distributes throughout the lungs. Gamma scintigraphy of the lungs was performed at entry into the study and on the three occasions following dosing to determine the rate of clearance of radiolabel. Table IV shows the stimulatory effect of UTP on whole lung mucociliary clearance in these patients.

Sputum induction

Pulmonary cytology has long been used in the diagnosis of lung cancer. Because lung tumors and other preneoplastic lung lesions shed cells that can be identified in sputum specimens, analysis of these specimens has become one component of the diagnosis of lung cancer. Likewise, sputum samples are helpful in the diagnosis of certain pulmonary infections, including *Pneumocystis carinii* pneumonia and tuberculosis. However, obtaining evaluable, deep lung sputum specimens often proves difficult. Bronchoscopy is often performed to obtain evaluable specimens. However, it is a costly and invasive procedure which is not without risk, especially in certain subgroups of patients.

The reliability of the results of sputum evaluations depends highly upon the quality of the specimen obtained by the clinician. For the diagnosis of lung cancer, the ideal sputum specimen contains a significant number of cells

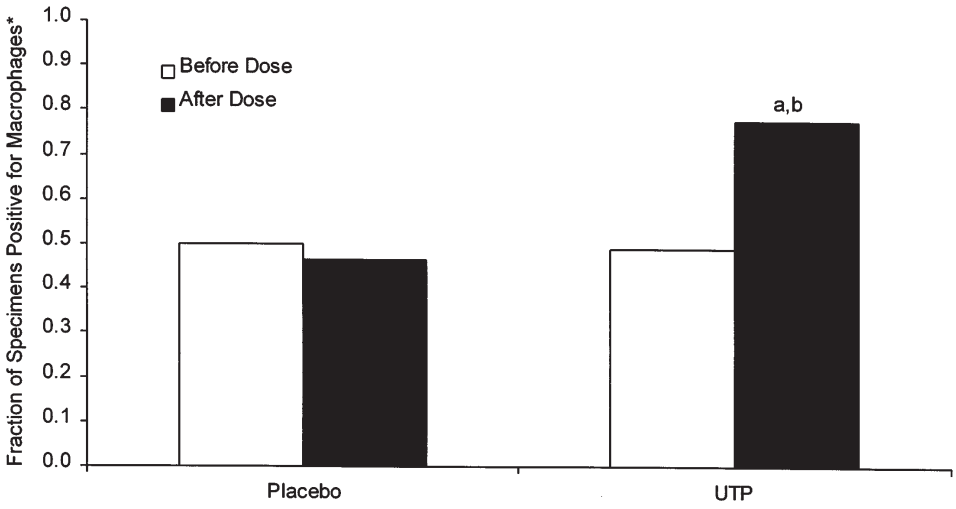

Fig. 9. Effect of UTP on sputum macrophage content in smokers with mild CB. UTP increased by approximately 2-fold the proportion of sputum specimens containing macrophages, indicative of the presence of deep lung material. *Positive specimens are those containing an average of ≥1 macrophage per high power field (400X). [a]$p \leq 0.05$, statistically greater than predose. [b]$p \leq 0.05$, statistically greater than placebo.

from deep within the lung. Such specimens are more likely to contain the cells of interest – exfoliated cells from dysplastic or neoplastic lesions. For the diagnosis of pulmonary infections, specimens containing predominantly white blood cells and/or deep lung cells in conjunction with few cells from the oropharynx are considered ideal. In most microbiology laboratories, sputum culture is attempted only on sputum specimens which contain significant numbers of macrophages (originating from the alveoli) and which meet adequacy criteria. Many patients have difficulty producing such evaluable sputum specimens spontaneously. Several techniques have been used to enhance the quantity and quality of expectorated sputum with variable success. Among these is inhalation of hypertonic saline (3-7% NaCl) for which there is only limited evidence of efficacy in normal subjects (52, 53), asthmatics (52-54) and HIV-infected patients with suspected *P. carinii* pneumonia (55). The safety of this method has not been rigorously assessed but has been reported to cause acute decreases in FEV_1 (52-54).

UTP, by virtue of its ability to hydrate airway secretions, stimulate mucin release and stimulate cilia beat frequency, may be useful in inducing evaluable sputum specimens from patients undergoing diagnostic evaluation for lung cancer or infectious disease. In a recent clinical trial, 15 healthy male smokers received solutions of placebo (normal saline) and 180 mg UTP by inhalation on 3 consecutive days in a randomized order. The quality of the sputum specimens obtained immediately before and after each dosing were evaluated using standard cytological methods. Figure 9 shows the ability of UTP to increase the number of sputum specimens obtained that result in an average of at least 1 macrophage per high power microscopic field in smokers. Figure 10 shows the ability of UTP to enrich sputum samples with macrophages relative to squamous epithelial cells in smokers.

As of July 1999, in clinical trials involving approximately 300 subjects including smokers and patients with mild CB, transient, mild to moderate coughing has been the most common side effect and is consistent with increased mobilization of airway fluids. Thus, UTP appears to have significant potential to shorten the time to diagnosis and improve the diagnostic value of sputum specimens in subjects being evaluated for possible lung cancer and serious infectious diseases.

Dry eye

Dry eye is characterized by a decrease in tear production or an improper mixture of tear film components often resulting in ocular surface disease (56-59). Although the main lacrimal glands produce the bulk of tear fluid, these glands are not easily activated by topical, nonirritating pharmacologic agents. As an alternative approach, compounds that stimulate the secretion of tear film components from the conjunctiva have been studied as potential pharmaceutical treatments of dry eye (60-63). The conjunctiva has received increased attention due to its large surface area, significant ion-transporting capabilities and numerous mucin-containing goblet cells.

It is now known that stimulation of $P2Y_2$ receptors with agonists such as UTP or INS365 causes secretion of chloride, fluid and mucin from the conjunctival epithelium (64-67). INS365 has been shown to safely increase tear volume upon instillation in rabbit eyes and is currently being developed as a treatment for chronic dry eye disease.

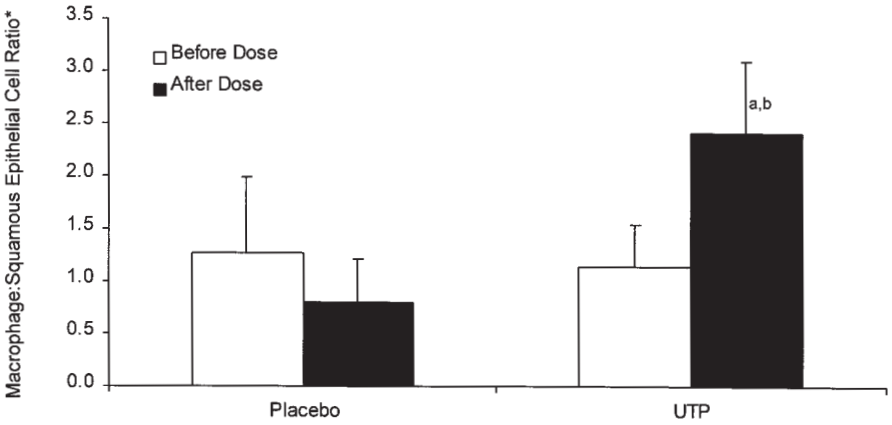

Fig. 10. Effect of UTP on sputum cytology in smokers with mild CB. UTP approximately doubled the average number of macrophages, indicative of the presence of deep lung material, in sputum specimens relative to squamous epithelial cells originating from the oropharynx. *Ratio is that of macrophages per high power field (400X) relative to squamous epithelial cells (SEC) per low power field (100X). To calculate a minimum ratio for specimens containing no SEC, the value of SEC was set to 1. [a]$p \leq 0.05$, statistically greater than placebo. [b]$p \leq 0.05$, statistically greater than predose.

Conclusions

This review of $P2Y_2$ receptor agonists is intended to give a perspective of how nucleotides have come to play a leading role in the field of mucociliary clearance and, in a broader sense, in the field of epithelial cell biology. The SAR of pyrimidine nucleotides has provided new insights into the design of potent and stable compounds, such as INS-365, which should be suitable for treating chronic diseases. The recent reports of the activity of $P2Y_2$ agonists in animal models of tracheal mucus velocity and whole lung mucociliary clearance confirm the activity of these compounds.

The ability of UTP to enhance expectoration of deep lung sputum opens up the possibility of improved diagnosis of lung cancer and infectious disease in patients via sputum cytology. In addition, the ability of $P2Y_2$ receptor agonists to stimulate mucociliary clearance in patients with mild CB demonstrates the potential for treating chronic obstructive pulmonary disease.

Finally, it should be emphasized that the $P2Y_2$ receptor is not only a target for treating lung disease but may also be useful for treating other indications such as dry eye.

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