Pharmacology of INS37217 [P$^1$-(Uridine 5')-P$^4$-(2'-deoxyctydine 5')tetrAPHosphate, Tetrasodium Salt], a Next-Generation P2Y$_2$ Receptor Agonist for the Treatment of Cystic Fibrosis


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ABSTRACT

INS37217 [P$^1$-(uridine 5')-P$^4$-(2'-deoxyctydine 5')tetrAPHosphate, tetrasodium salt] is a deoxyctydine-uridine dinucleotide with agonist activity at the P2Y$_2$ receptor. In primate lung tissues, the P2Y$_2$ receptor mRNA was located by in situ hybridization predominantly in epithelial cells and not in smooth muscle or stromal tissue. The pharmacologic profile of INS37217 parallels that of UTP, leading to increased chloride and water secretion, increased cilia beat frequency, and increased mucin release. The combined effect of these actions was confirmed in an animal model of tracheal mucus velocity that showed that a single administration of INS37217 significantly enhanced mucus transport for at least 8 h after dosing. This extended duration of action is consistent with the ability of INS37217 to resist metabolism by airway cells and sputum enzymes. The enhanced metabolic stability and resultant increased duration of improved mucociliary clearance may confer significant advantages to INS37217 over other P2Y$_2$ agonists in the treatment of diseases such as cystic fibrosis.

Cystic fibrosis (CF) is a recessive genetic disease, characterized by pulmonary and reproductive tract dysfunctions, which affects more than 30,000 people in the United States (Davis et al., 1996). CF is caused by mutations in the CF transmembrane regulator (CFTR) gene, which encodes for an apical membrane epithelial protein that functions both as a cAMP-regulated chloride channel and a regulator of the epithelial sodium channel (Boucher, 1994). A defective CFTR leads to abnormal fluid and solute transport across epithelia, which contributes to the formation of viscous, dehydrated mucus in airways. The resulting mucostasis leads to progressive loss of ventilatory function and severe inflammatory responses to chronic bacterial infection (Mickle and Cutting, 1998). Most deaths of patients with CF occur as a consequence of pulmonary disease. Although improved treatment of lung disease has increased longevity, the median age for survival is still only 32 years, and patients have significant morbidity, including frequent hospitalizations, throughout their lives (Ramsey, 1996). Current therapies for CF include inhaled antibiotics, bronchodilators, mucolytics, and physiotherapy. Clearly, new therapeutic approaches are needed for the prevention and treatment of CF lung disease.

An emerging therapeutic paradigm for the treatment of cystic fibrosis is based on the identification and development of agents that act on the P2Y$_2$ receptor, which is involved in the regulation of cilia beat frequency and mucociliary clearance. The P2Y$_2$ receptor is expressed in airway epithelial cells, where it mediates the release of chloride and water, leading to increased ciliary beat frequency and mucin clearance. In this study, we evaluated the pharmacology of INS37217, a novel P2Y$_2$ agonist, in vitro and in vivo models of CF.

**ABSTRACT**

INS37217 [P$^1$-(uridine 5')-P$^4$-(2'-deoxyctydine 5')tetrAPHosphate, tetrasodium salt] is a deoxyctydine-uridine dinucleotide with agonist activity at the P2Y$_2$ receptor. In primate lung tissues, the P2Y$_2$ receptor mRNA was located by in situ hybridization predominantly in epithelial cells and not in smooth muscle or stromal tissue. The pharmacologic profile of INS37217 parallels that of UTP, leading to increased chloride and water secretion, increased cilia beat frequency, and increased mucin release. The combined effect of these actions was confirmed in an animal model of tracheal mucus velocity that showed that a single administration of INS37217 significantly enhanced mucus transport for at least 8 h after dosing. This extended duration of action is consistent with the ability of INS37217 to resist metabolism by airway cells and sputum enzymes. The enhanced metabolic stability and resultant increased duration of improved mucociliary clearance may confer significant advantages to INS37217 over other P2Y$_2$ agonists in the treatment of diseases such as cystic fibrosis.

**ABBREVIATIONS:** CF, cystic fibrosis; CFTR, CF transmembrane regulator; TMV, tracheal mucus velocity; INS365, P$^1$P$^4$-di(uridine 5')tetrAPHosphate, tetrasodium salt; INS37217, P$^1$-(uridine 5')-P$^4$-(2'-deoxyctydine-5')tetrAPHosphate, tetrasodium salt; HPLC, high-pressure liquid chromatography; DMEM, Dulbecco’s modified Eagle’s medium; ISH, in situ hybridization; PCR, polymerase chain reaction; IVT, in vitro transcription; DNase, deoxyribonuclease; I$_{sc}$, short circuit current; BEGM, bronchial epithelial growth medium; ALI, air/liquid interface; CBF, ciliary beat frequency; LHC, Laboratory of Carcinogenesis.
cystic fibrosis is to stimulate alternative modes of chloride secretion in the lung, thereby circumventing the genetic defect in the CFTR channel. Agents that correct the underlying ion transport defects in the airways may prove useful in normalizing airway secretions, leading to the improvement of mucociliary clearance and the prevention of chronic lung infections and progressive lung damage. One such method involves the use of inhaled nucleotides that activate P2Y₂ receptors on the airway epithelial surface. The P2Y₂ receptor is abundant on the luminal surface of polarized epithelial cells, especially those lining mucosal surfaces exposed to the external environment. The pharmacology of the P2Y₂ receptor has been established, and the effects of P2Y₂ agonists on epithelial cell functions are numerous, including stimulation of serosal to mucosal chloride and fluid transport (Knowles et al., 1991; Benali et al., 1994; Tarran et al., 2000), enhancement of mucin secretion from goblet cells (Lethem et al., 1993; Kim et al., 1996), increase in cilia beat frequency (Morse et al., 2001), and promotion of surfactant release from type II alveolar cells (Gobran et al., 1994). Additionally, tracheal mucus velocity (TMV), a measure of mucociliary clearance in a single large airway, has revealed the mucokinetic effects of nucleotides and various other agents in the lung in vivo (Sabater et al., 1996).

The discovery of diadenosine 5′-polyporphosphates (Ap₄A, in which “n” = 2–7) (Fig. 1) and their release from platelets has led to many studies on their biological activity and metabolism (Picher and Boucher, 1999; Pintor et al., 1999; Gurianowski, 2000; Hoyle et al., 2001). Diadenosine and diuridine polynucleophosphates have interesting pharmacological effects on nucleotide receptors, the latter class avoiding the liability of adenosine-containing metabolites with activities at adenosine receptors (Lazarowski et al., 1995; Pendergast et al., 2001). Recent advances in nucleotide biology indicate that mono- and dinucleotides are stored and released locally by epithelial cells in concentrations that effectively activate P2Y receptors (Donaldson et al., 2000; Pintor et al., 2002). In fact, shear forces caused by cough or osmotic stress may be sufficient to induce local nucleotide release in concentrations relevant for the activation of the P2Y₂ receptor. As with other extracellular signaling systems, P₂ purinergic signals are generally terminated when the nucleotides are hydrolyzed by ectonucleases. These ectonucleotidases quickly dephosphorylate mononucleotides and cleave the more stable dinucleotide tetraphosphates into nucleoside mono- and triphosphates (Zimmerman, 1996; Picher and Boucher, 2000).

The P₂Y₂ receptor agonist, Up₄U (INS365), has been evaluated in normal healthy volunteers and patients with CF. Clinical safety has been established for inhaled INS365 in normal healthy volunteers (nonsmokers and smokers) in single doses up to 400 mg in the nebulizer. In pediatric and adult CF patients (ages 5–12 and 18–63 years, respectively), single doses up to 40 mg were well tolerated (Shaffer et al., 1998). INS37217, a next-generation dinucleotide P₂Y₂ receptor agonist, is closely related to INS365 in terms of chemical structure (Fig. 1). However, we demonstrate that the deoxycytidine group that replaces one of the two uridine moieties imparts INS37217 with a significant resistance to enzymatic hydrolysis. The following in vitro and in vivo studies demonstrate how INS37217 is more suitable for treating CF lung disease in which an enhanced duration of action is believed to constitute a significant therapeutic advantage.

**Materials and Methods**

**Test Compounds and Reagents.** All compounds were obtained from Sigma-Aldrich (St. Louis, MO), unless otherwise stated. INS37217 was synthesized by Inspire Pharmaceuticals, Inc. (unpublished data). The purity of all nucleotide agonists was established by HPLC (95–99% purity). Fluor-3-AM was obtained from Molecular Probes (Eugene, OR). Dulbecco’s modified Eagle’s medium (DMEM), fetal bovine serum, genetin (G-418), and other cell culture reagents were obtained from the Tissue Culture Facility at the University of North Carolina or from Invitrogen (Carlsbad, CA). 1321N1 human astrocytoma cells stably expressing P₂Y₇, P₂Y₂, P₂Y₄, P₂Y₁₀, or P₂Y₁₁ receptors and the wild-type 1321N1 cell were obtained from the University of North Carolina at Chapel Hill.

**In Situ Hybridization.** Rhesus monkey lung specimens were obtained from Pathology Associates, A Charles River Company (Frederick, MD). Tissues were removed from healthy animals immediately following euthanasia and snap-frozen in embedding medium. Frozen tissues were stored at –80°C prior to cryosectioning. Tissues were cut in 5-μm sections and mounted on SuperFrost Plus microscope slides (Fisher Scientific, Pittsburgh, PA) for H&E staining and in situ hybridization (ISH). Sections stained by H&E were prepared to evaluate the quality and orientation of the tissues studied. Examination of H&E slides indicated that all tissues were suitable for ISH. Slide-mounted tissue sections were kept frozen until all sections were cut and then used immediately for ISH.
Nucleotides 272 to 627 of the P2Y<sub>1</sub> receptor gene were amplified with PCR primers designed to incorporate either an upstream T3 promoter or a downstream T7 promoter into the PCR product. The resulting PCR products were used to synthesize digoxigenin-labeled riboprobes by in vitro transcription (IVT). Antisense and sense riboprobes were synthesized using T7 and T3 RNA polymerases, respectively, in the presence of digoxigenin-11-UTP (Roche Diagnostics, Indianapolis, IN) using a MAXIscript IVT kit (Ambion, Austin, TX) according to the manufacturer’s instructions. Following IVT, template DNA was degraded with DNase-1, and unincorporated digoxigenin was removed by centrifugal ultrafiltration using Microcon columns (Millipore, Bedford, MA). Riboprobe integrity was assessed by electrophoresis through a denaturing polyacrylamide gel. Apparent molecular size was estimated by comparison with the electrophoretic mobility of a 100- to 1000-base pair RNA ladder (Ambion). Probe yield and labeling were evaluated by blot immunochemistry. Riboprobes were dispensed in 5-μl aliquots and stored at −80°C until used for ISH.

Frozen sections were fixed in 4% paraformaldehyde in phosphate-buffered saline, pH 7.4, for 15 min at room temperature. Tissue ribonucleases were further inactivated by treatment with 0.1% diethyl pyrocarbonate for 30 min. Sections were incubated overnight in hybridization buffer (50% formamide, 5× standard saline citrate, and 40 μg/ml sheared salmon sperm DNA) containing 0.5 μg/ml of either antisense or sense probe. Following hybridization, slides were subjected to a series of posthybridization stringency washes to reduce nonspecific hybridization. Hybridization was visualized by immunohistochemistry using an alkaline phosphatase-conjugated antidigoxigenin antibody and the alkaline phosphatase substrate nitroblue tetrazolium chloride/bromochloroindoly phosphate (Roche Diagnostics) according to the manufacturer’s protocol. Tissue sections were counterstained with nuclear fast red. Assay controls included omission of probe and omission of probe and anti-digoxigenin antibody. Cells were assessed for demonstration of hybridization with the antisense P2Y<sub>1</sub> receptor probe by visualizing dark cytoplasmic and/or perinuclear staining indicative of a positive ISH signal. Each cell type was compared with replicate sections that were hybridized with the negative control sense P2Y<sub>1</sub> receptor probe.

**Intracellular Calcium Mobilization.** 1321N1 human astrocytoma cells stably expressing the human P2Y<sub>2</sub>, P2Y<sub>4</sub>, P2Y<sub>6</sub>, P2Y<sub>11</sub>, and P2Y<sub>2/11</sub> receptors were grown in DMEM containing 4.5 g/l glucose, 5% fetal bovine serum, and 600 μg/ml G-418. For intracellular Ca<sup>2+</sup> measurements, cells were seeded in 96-well black-well/clear-bottom culture plates (3904; Corning Inc., Corning, NY), at a density of 35,000 cells/well, and assays were conducted 2 days later when the cells had reached confluence.

On the day of the assay, the growth medium in the culture plates was aspirated and replaced with 2.5 μM Fluo-3-AM in a final volume of 50 μl and incubated for 1 h at 25°C. Then, the dye was replaced with assay buffer (10 mM KCl, 118 mM NaCl, 2.5 mM CaCl<sub>2</sub>, 1 mM MgCl<sub>2</sub>, 10 mM glucose, and 20 mM HEPES, pH 7.4). For intracellular Ca<sup>2+</sup> measurements, cells were seeded in 96-well black-well/clear-bottom culture plates (3904; Corning Inc., Corning, NY), at a density of 35,000 cells/well, and assays were conducted 2 days later when the cells had reached confluence.

Chloride Secretion. The posterior membrane of fresh mongrel dog tracheas was excised and dissected free of trachealis muscle, and segments (1.5 cm wide) were cut, mounted in Ussing chambers, and bathed on the mucosal and serosal surfaces with Krebs-Ringer bicarbonate solution. The composition of the bath solution was 140 mM Na<sup>+</sup>, 120 mM Cl<sup>−</sup>, 5.2 mM K<sup>+</sup>, 25 mM HCO<sub>3</sub><sup>−</sup>, 2.4 mM HPO<sub>4</sub><sup>2−</sup>, 0.4 mM HPO<sub>4</sub><sup>2−</sup>, 1.1 mM Ca<sup>2+</sup>, 1.2 mM Mg<sup>2+</sup>, and 5.2 mM glucose, pH 7.4. Tissue baths were maintained at 37°C and gassed with humidified 95% O<sub>2</sub>:5% CO<sub>2</sub>.

Bioelectric properties, including short circuit current (I<sub>sc</sub>), transepithelial potential difference, and resistance, were monitored for each tissue. I<sub>sc</sub> was measured under voltage-clamp conditions. Data from individual clamps were collected and processed by Acquire & Analyze (Physiologic Instruments, San Diego, CA). Open circuit potential was recorded periodically. Resistance was monitored in the voltage-clamp mode by the current deflection in response to a 10-mV pulse. After a stable I<sub>sc</sub> was recorded, amiloride (100 μM) was added to the solution, bathing the apical surface to block sodium transport. The residual I<sub>sc</sub> measured under these conditions was a good approximation of chloride secretion (Boucher et al., 1981). After the establishment of a new steady-state I<sub>sc</sub>, indomethacin (10 μM) was added to the solution, bathing both the mucosal and serosal surfaces to block the generation of arachidonic acid metabolites that otherwise activate cAMP-stimulated chloride currents (e.g., CFTR). The residual I<sub>sc</sub> activity after the amiloride and indomethacin additions was considered the baseline I<sub>sc</sub> for each experiment. After recording a stable baseline, a solution of the test compound was added to the chamber, bathing the mucosal surface of the tracheal epithelium. The change in I<sub>sc</sub> was recorded. Concentration-response curves were obtained by the cumulative addition of higher concentrations of the test compound in 10-fold increments.

**Mucin Secretion.** Primary normal human tracheal/bronchial epithelial cells (donor-specific, nonsmoker), which had been shipped cryopreserved in the presence of retinoic acid, were obtained from Clonetics (East Rutherford, NJ; CC-2540). The cells were initially seeded on Transwell-Clear culture inserts (Corning-Costar 3460; Corning) and grown in bronchial epithelial growth medium (BEGM) (Clonetics; CC-3170 BEGM BulletKit media, plus supplements). After 2 to 3 days in culture, cells were switched to air/liquid interface (ALI) culture conditions as has been previously described by Gray et al. (1996). The 17Q2 mucin antibody was purified with a Protein G column (Pierce, Rockford, IL) from ascites fluid (University of California at Davis). Alkaline phosphatase was conjugated to 17Q2 antibody using the EZ-Link maleimide-activated alkaline phosphatase kit (Pierce).

Indicated concentrations of INS37217 were added to the apical surface of the cultures and incubated at 37°C for 2 h. At the end of incubation period, mucin-containing cell supernatants were removed from the apical compartment and stored at −70°C. Estimation of mucin production was carried out using an antigen/antibody enzyme-linked immunosassay as described previously (Wright et al., 1996).

**Ciliary Beat Frequency.** The effects of INS37217 on ciliary activity were determined on individual human ciliated nasal epithelial cells using techniques described previously (Geary et al., 1995; Morse et al., 2001). Briefly, epithelial cells were recovered from protease digests of human nasal turbinates procured through the Tissue Culture Core Facility of the Cystic Fibrosis/Pulmonary Research and Treatment Center at the University of North Carolina at Chapel Hill. The cells were seeded into 12-mm Costar Transwell-Col cell culture supports bearing epithelial explants at a density of 300,000 cells/cm<sup>2</sup> and incubated overnight in hormone-supplemented culture medium (Gray et al., 1996) at 37°C in an atmosphere of air (5% CO<sub>2</sub>, after which nonadherent cells were washed away to reveal small explants of the superficial epithelium as small clumps of ciliated cells that had attached to the substratum. These preparations were used within 4 days.

Transwell-Col cell culture supports bearing epithelial explants
were mounted in a chamber on the stage of an inverted microscope, superfused luminally, and warmed (35°C) as described previously (Morse et al., 2001). The control superfusion and the serosal bathing solution was Krebs-Ringer bicarbonate (KRB) with the following composition: 125 mM NaCl, 5.2 mM KCl, 1.2 mM MgCl₂, 1.2 mM CaCl₂, 25 mM NaHCO₃, 10 mM TES, 5 mM glucose (pH 7.4 when gassed with 5% CO₂). The explanted, native ciliated cells were viewed by phase contrast microscopy using a Zeiss IM microscope (Carl Zeiss Inc., Thornwood, NY) and 32× objective, and the image was monitored with a Dage 72 monochrome charge-coupled device video camera (Dage-MTI, Michigan City, IN). Ciliary beat frequency (CBF) was determined using a photosensor positioned over the image of an individual cell on the face of the video monitor to detect ciliary beating, as previously described (Morse et al., 2001). In all experiments, cultures were equilibrated with 1.5 h of superfusion with KRB. Each preparation was then subjected to two-10 min baseline and agonist stimulation periods, first, with a variable concentration of INS37217, then with 100 μM UTP as the agonist, with data recorded every minute for the determination of CBF. A 30-min KRB washout period separated the INS37217 challenge from the second baseline period. After fast Fourier transformation analyses for each experiment, the resulting CBF data were normalized to the respective mean baseline CBF (Fig. 6A). For the INS37217 concentration-response study shown in Fig. 5B, the peak CBF obtained with INS37217 was calculated relative to that obtained with 100 μM UTP. The data are reported as the mean ± S.E. of the peak response ratio (INS37217/100 μM UTP), relative to baseline, for n cultures. For each INS37217 concentration, cultures derived from the tissues of three or more patients were used.

Airway Surface and CF Sputum Metabolism. Well differentiated cultures from passage 1 human airway epithelial cells were grown as previously described (Gray et al., 1996). In brief, nasal epithelial cells were harvested from turbinates (Wu et al., 1995). Primary cultures were isolated by protein digestion and plated on a collagen-coated tissue culture dish (5–10 days) in LHC9 medium (Biosource International, Camarillo, CA) (Lechner and LaVeck, 1985) containing 25 ng/ml epidermal growth factor, 50 nM retinoic acid, 40 μg/ml gentamicin, 0.5 mg/ml bovine serum albumin, 0.8% bovine pituitary extract, 50 U/ml penicillin, 50 μg/ml streptomycin, and 0.125 mg/ml amphotericin termed BEGM. The cells were trypsinized and subpassaged on porous Transwell-Col filters (diameter, 7.5 cm in diameter (Mallinkrodt Medical Inc., St. Louis, MO), which had been shortened by 6 cm. The cuff of the tube was placed just below the vocal cords (verified by fluoroscopy) to allow for maximal exposure of the tracheal surface area. After intubation, the animals were allowed to acclimate for a period of 20 min before beginning measurements of TMV. During the course of the experiment, the inspired air was warmed and humidified using a Bennett humidifier (Puritan-Bennett, Lenexa, KS). To minimize possible impairment of TMV caused by inflation of the endotracheal tube cuff, the cuff was deflated throughout the study, except for the period of drug delivery. The sheep were periodically gavaged with tap water to avoid dehydration (Sabater et al., 1999).

TMV was measured in vivo by a roentgenographic technique as previously described (Sabater et al., 1996, 1999). Between 10 and 12 radiopaque Teflon/bismuth trioxide disks, which were 1 mm in diameter, 0.8 mm thick, and 1.8 mg in weight, were insufflated into the trachea. A modified suction catheter connected to a source of continuous compressed air at a flow of 3 to 4 l/min was used to introduce the particles via the endotracheal tube. The catheter remained within the endotracheal tube only, so that no contact with the tracheal surface was made. The cephalad-axial velocities of the individual disks were recorded on videotape from a portable image intensifier unit. Individual disk velocities were calculated by measuring the distance traveled by each disk during a 1-min observation period. For each run, the mean value of all individual disk velocities was calculated. A collar containing radiopaque reference markers of known length was worn by the sheep and used as a standard to correct for magnification effects inherent in the fluoroscopy unit. INS37217 at 40, 94, and 471 μmol (34, 80, and 400 mg, respectively) in sterile saline and sterile saline (placebo) were provided by Inspire Pharmaceuticals as individual 4-ml aliquots ready for immediate nebulization. After obtaining a baseline TMV, the sheep were administered a dose of INS37217 or placebo in a randomized fashion. The agents were delivered to the animals with a Pari LC Star nebulizer (Pari Respiratory, Richmond VA), via free breathing. The nebulizer was driven by wall air at a flow rate of 8 l/min, and the time
to reach dryness was approximately 10 to 12 min. TMV was measured immediately after drug administration (0 h) and remeasured at 0.25, 0.5, 1, 2, 4, 6, and 8 h after treatment.

Data were analyzed using Friedman’s analysis of variance followed by Wilcoxon’s paired test using SYSTAT for Windows, version 5 (Microsoft, Redmond, WA). Significance was accepted at $P < 0.05$. Values in the text and figures are mean $\pm$ S.E.

**Results**

**P2Y$_2$ Receptor Gene Expression in the Lung.** Nonradioisotopic ISH was used to determine the cellular localization of P2Y$_2$ receptor gene expression in cryosections of rhesus monkey lung (Table 1 and Fig. 2). Cytoplasmic ISH staining, indicative of P2Y$_2$ gene expression, was observed with the antisense probe in bronchial epithelium, including goblet cells, in bronchiolar and alveolar type I and II epithelium, and in submucosal gland epithelium, but not in submucosal gland ductal epithelium. In addition, vascular endothelial cells, intravascular white blood cells, and selected alveolar macrophages exhibited ISH staining consistent with expression of P2Y$_2$ mRNA. In contrast, no ISH staining was observed in peribronchial smooth muscle, vascular smooth muscle, mesothelium, or connective tissue stroma with the antisense probe. No staining was observed in any cell type other than P2Y$_2$ gene expression, was observed in situ hybridization using a labeled antisense probe. No staining was observed in any cell type with the antisense probe in bronchial epithelium, including goblet cells, in bronchiolar and alveolar type I and II epithelium, and in submucosal gland epithelium, but not in submucosal gland ductal epithelium. In addition, vascular endothelial cells, intravascular white blood cells, and selected alveolar macrophages exhibited ISH staining consistent with expression of P2Y$_2$ mRNA. In contrast, no ISH staining was observed in peribronchial smooth muscle, vascular smooth muscle, mesothelium, or connective tissue stroma with the antisense probe. No staining was observed in any cell type with the negative control P2Y$_2$ sense probe.

**INS37217 Activates P2Y$_2$ and P2Y$_4$ Receptors.** In a concentration-dependent manner, INS37217 induced the mobilization of intracellular calcium in 1321N1 astrocytoma cells stably expressing human P2Y$_2$ and P2Y$_4$ receptors with EC$_{50}$ values of 0.22 and 0.8 $\mu$M, respectively (Fig. 3). The efficacy of INS37217 was identical to that of UTP, indicating that INS37217 is a full agonist of P2Y$_2$ and P2Y$_4$ receptors. However, the potency of INS37217 at these two receptors is somewhat less than that for the native agonist, UTP. In contrast, INS37217 had little or no calcium-mobilizing activity in 1321N1 cells expressing the P2Y$_1$ receptor (Fig. 3A). INS37217 was a weak agonist of P2Y$_6$ receptors in which 100 $\mu$M INS37217 produced approximately 70% of the response observed with the natural agonist UDP. In conclusion, INS37217 is a full agonist specific for P2Y$_2$ and P2Y$_4$ receptors. Essentially, identical effects were observed in assays of phospholipase C-dependent accumulation of inositol phosphates (not shown).

**INS37217 Stimulates Chloride Secretion in Tracheal Epithelium.** Figure 4A shows a representative cumulative concentration-response curve for INS37217 on $I_{sc}$ in freshly isolated dog tracheal epithelial preparations mounted in Ussing chambers. Both INS37217 and UTP stimulated concentration-dependent increases in $I_{sc}$ activity over the concentration range of 0.1 to 100 $\mu$M (Fig. 4B). Maximal responses were observed with 100 $\mu$M INS37217 or 10 $\mu$M UTP. The maximal responses to INS37217 and UTP were similar, i.e., approximately 20 $\mu$A/cm$^2$ above baseline. The EC$_{50}$ values for stimulation of $I_{sc}$ activity by INS37217 and UTP were 1.9 and 0.3 $\mu$M, respectively. The kinetics of the responses differed somewhat for INS37217 and UTP. Both compounds stimulated immediate increases in $I_{sc}$ activity, which generally peaked within 7 s of compound addition. The response to INS37217 was sustained, whereas the response to UTP began a decline toward baseline soon after reaching its peak.

**INS37217 Stimulates Mucin Secretion from Human Airway Cell Culture.** To determine whether activation of P2Y receptors by INS37217 stimulated mucin production, fully differentiated cultures of human airway epithelium grown in an air/liquid interface were treated with the P2Y$_2$/$P2Y_4$ agonist. INS37217 stimulated in a concentration-dependent manner the production and release of mucin glycoproteins from these cultures (Fig. 5) with an EC$_{50}$ of 2.67 $\mu$M (average from two independent experiments). The efficacy of INS37217 to stimulate mucin production was identical to that observed with the endogenous agonist UTP (data not shown). The identification of specific mucin genes stimulated by INS37217 was not determined in these experiments. These results suggest that INS37217 is a potent and efficacious mucin secretagogue in human airway epithelium.

**INS37217 Stimulates Cilia Beat Frequency in Human Airway Explants.** As reported previously (Morse et al., 2001), purinergic challenge of human nasal ciliated cells stimulated ciliary activity above baseline rapidly, with peak responses occurring typically within 1 or 2 min (Fig. 6A). Because the agonist responses varied somewhat from explant to explant, the effects of INS37217 on ciliary activity were compared with a subsequent baseline using 100 $\mu$M UTP challenge as an internal control. The baseline frequencies recorded were 7.7 $\pm$ 0.2 Hz and 7.3 $\pm$ 0.2 Hz, respectively. Peak CBF responses to repeated 100 $\mu$M UTP challenges, in the earlier study, were similarly indistinguishable. INS37217 stimulated CBF significantly at concentrations above 1 $\mu$M, and these effects saturated at about 100 $\mu$M. To account for variability in explant responses to saturating concentrations of agonist, the effects of INS37217 were expressed relative to those of UTP in the same experiment (Fig. 6B). By this analysis, the EC$_{50}$ was 8.3 $\mu$M, and at 100 $\mu$M, the peak responses to UTP and INS37217 were 200 and 180% of baseline, respectively.

**INS37217 Is Resistant to Airway Surface Metabolism.** We compared the metabolic rates of 0.1 mM UTP, INS365, and INS37217 on the mucosal surface of human nasal epithelial cells in culture. The natural P2Y agonist UTP was rapidly hydrolyzed by the epithelium, with an average initial hydrolytic rate of 0.93 $\pm$ 0.12 nmol · min$^{-1}$ · cm$^{-2}$ and a half-life around 3 min (Fig. 7A). In contrast, dinucleotides displayed remarkable stability toward the membrane-bound enzymes. The concentration of INS365 was approximately 40 $\mu$M at the end of the 60-min incubation period, which corresponded to initial hydrolytic rates and

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**TABLE 1**

Cellular localization of P2Y$_2$ receptor gene expression as determined by in situ hybridization

<table>
<thead>
<tr>
<th>Cell Type</th>
<th>ISH Results</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bronchial epithelium</td>
<td>+</td>
</tr>
<tr>
<td>Goblet cells</td>
<td>+</td>
</tr>
<tr>
<td>Submucosal glands</td>
<td>+</td>
</tr>
<tr>
<td>Submucosal duct epithelium</td>
<td>−</td>
</tr>
<tr>
<td>Bronchiolar epithelium</td>
<td>+</td>
</tr>
<tr>
<td>Peribronchial smooth muscle</td>
<td>−</td>
</tr>
<tr>
<td>Alveolar I and II epithelium</td>
<td>+</td>
</tr>
<tr>
<td>Alveolar macrophages (selected)</td>
<td>+</td>
</tr>
<tr>
<td>Vascular endothelium (selected)</td>
<td>+</td>
</tr>
<tr>
<td>Vascular smooth muscle</td>
<td>−</td>
</tr>
<tr>
<td>Intravascular white blood cells</td>
<td>+</td>
</tr>
<tr>
<td>Connective tissue stroma</td>
<td>−</td>
</tr>
<tr>
<td>Mesothelium</td>
<td>−</td>
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</tbody>
</table>

+, ISH staining observed; −, no staining observed.

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half-lives of $0.15 \pm 0.02$ nmol $\cdot$ min$^{-1} \cdot$ cm$^{-2}$ and 50 min, respectively. Substitution of a uridine group by a deoxycytidine group increased the half-life of the dinucleotide to 3 h. The concentration of INS37217 on cell surfaces was approximately 90 $\mu$M after 60 min, for an average initial hydrolytic rate of $0.02 \pm 0.01$ nmol $\cdot$ min$^{-1} \cdot$ cm$^{-2}$. Therefore, INS37217...
was approximately 50 times and 6 times more stable than UTP and INS365, respectively, on the mucosal surface of human nasal epithelial cells.

Aerosolized dinucleotides are most likely to encounter a thick barrier of mucus before they reach their target receptors on airway epithelial surfaces. We therefore investigated the stability of UTP, INS365, and INS37217 in sputum samples obtained from CF patients. The rate of UTP hydrolysis obtained in human CF sputum (Fig. 7B) was comparable to those obtained on cultured airway cells (Fig. 7A), with a half-life of approximately 3 min. In contrast, the two dinucleotides were more stable in the sputum than on epithelial surfaces, with half-lives of about 3 h and 25 h for INS365 and INS37217, respectively (Fig. 7B). Thus, sputum would not be

![Graph A](image1.png)

**Fig. 4.** Effects of INS37217 and UTP on \( I_{sc} \) activity in excised dog tracheal epithelium. Dog tracheal epithelium was isolated and mounted in Ussing chambers as described. A, after a stable baseline of \( I_{sc} \) activity was achieved, amiloride (100 \( \mu \)M) and indomethacin (100 \( \mu \)M) were added to suppress epithelial sodium channel and cAMP-stimulated chloride channel activities, respectively. INS37217 was added as indicated. B, stimulants were added and peak changes in \( I_{sc} \) were recorded. Net changes in \( I_{sc} \) (stimulated \( I_{sc} \) minus baseline \( I_{sc} \)) were normalized to the maximal response elicited by each stimulant. Data represent the mean ± S.E.M. (\( n = 4 \)) of the normalized, net responses to INS37217 or UTP. EC\(_{50}\) values calculated from the fitted curve functions were 1.9 and 0.3 \( \mu \)M for INS37217 and UTP, respectively.
expected to significantly reduce the effective doses of aerosolized dinucleotides before reaching their target receptor(s) at the surface of human CF airway epithelial cells.

**INS37217 Increases Tracheal Mucus Velocity in Sheep.** Figure 8A illustrates the overall response of TMV to the three different doses of INS37217 compared with saline (placebo). The two highest doses of INS37217 significantly enhanced TMV over the 8-h period with respect to placebo (P < 0.05). In addition, there was a dose-dependent effect of the compound, with the 94-μmol dose showing the greatest stimulation within 0.25 h after treatment. Within 0.25 h of treatment with 94 μmol of INS37217, TMV increased to 160 ± 8% from a baseline value of 7.8 ± 0.4 mm/min. This increase is compared with 141 ± 3% after treatment with 471 μmol (from a baseline of 9.5 ± 0.4 mm/min) and 135 ± 5% (from a baseline of 8.2 ± 0.3 mm/min) after treatment with 40 μmol. Saline only produced a 119 ± 3% increase in TMV from a baseline of 8.2 ± 0.5 mm/min (Fig. 8B).

**Discussion**

The results from the present study indicate that P2Y₂ receptors are primarily expressed in bronchial, bronchiolar, alveolar, and submucosal gland epithelium, including mucous-secreting goblet cells. In vitro, INS37217 is a submicromolar, selective agonist for human P2Y₂ and P2Y₁ receptors, and stimulates chloride secretion and increases ciliary beat frequency. In vivo, INS37217 stimulates TMV, a marker of lung mucociliary clearance. Although earlier P2Y₂ agonists (INS365) have shown similar effects, the novel finding in this study is the duration of action of INS37217.

P2Y₂ receptor activation modulates several physiological activities that can increase mucociliary clearance. Receptor activation induces chloride secretion and water movement into the airway surface liquid, thereby hydrating mucus and optimizing periciliary fluid viscosity, mucin secretion, and increased ciliary beat frequency. The combined effects of these activities is predicted to enhance airway mucociliary clearance. In vivo studies with UTP and a first-generation P2Y₂ agonist confirmed this hypothesis (Sabater et al., 1999). Our current findings showing that INS37217 effectively improved TMV in sheep are consistent with these previous results. In the present study, INS37217 produced greater peak increases in TMV than did INS365, and the stimulatory effect was more prolonged than was seen with previous P2Y₂ agonists. Furthermore, these in vivo effects were achieved at micromolar concentrations of INS37217 compared with the millimolar concentrations used in the previous study. INS37217 showed a bell-shaped, dose-dependent increase in TMV. The reason(s) that the highest concentration used was not the most effective is not clear from these experiments. Possibilities include differences in baseline TMV values...
among the trials and the unknown effects of P2Y4 activation. Nevertheless, the major finding of the present study is that the increase in TMV after dosing was significant compared with placebo for up to 8 h for the two highest doses. This is the longest and largest increase in TMV observed for any nucleotide achieved in this model. Finally, it should be pointed out that TMV has been shown to be a reliable surrogate for whole lung mucociliary clearance when evaluating P2Y2 agonists.

Although all nucleotides examined in the present work effectively stimulate components of mucociliary clearance in airways, they present important differences in stability on human airway epithelia. The metabolism of extracellular nucleotides was recently reported at the surface of human airway epithelial cells (Picher and Boucher, 2001). These enzymes sequentially dephosphorylate mononucleotides such as UTP into UDP, UMP, and uridine. An alkaline phosphodiesterase activity was also identified on human nasal and bronchial epithelial cells in culture (Picher and Boucher, 2000). This enzyme activity catalyzes the asymmetrical cleavage of dinucleotides (NpN) into nucleoside 5'-monophosphate and NpN-1 ("N" = A, U, or G; "n" = 2–6). In the present study, we showed that INS365 (UpU) was approxi-
mately 10 times more stable than UTP on the mucosal surface of human nasal epithelial cells (Fig. 7). As a result, the half-life of the dinucleotide was 50 min compared with only 3 min for UTP. These findings were supported by the extended duration of INS365 on P2Y receptor-mediated responses. However, such improvement was not satisfactory for outpatient treatments of CF, in which less frequent dosing is desired. Interestingly, we have demonstrated that asymmetry significantly enhanced the metabolic stability of INS37217 (dCP, U) compared with the symmetrical dinucleotide, INS365 (Up, U). The half-life of the dinucleotide was extended from 50 min to 3 h by simple substitution of a uridine for a deoxycytidine. Accordingly, the longer duration of INS37217-derived, P2Y-mediated responses with respect to those obtained with INS365 could be explained by a higher metabolic stability in human airways.

Because aerosolized nucleotides and dinucleotides are most likely to interact with the mucus layer before they reach P2Y receptors, we explored the possibility that CF sputum could significantly diminish the effective drug concentration (Fig. 7B). Nucleotide and dinucleotide metabolism was also detected in sputum samples collected from human CF patients. Whereas the rate of UTP hydrolysis was comparable to the cell surface activity, the two dinucleotides were 5 times more stable in sputum. These enzymatic activities could originate from epithelial cell desquamation or as ubiquitous cytosolic enzymes released from lysed cells. These experiments indicated that sputum would not be expected to significantly reduce the effective doses of aerosolized mono- or dinucleotides before they reach their target receptor(s) at the surface of human CF airway epithelial cells.

P2Y2 agonist therapy represents an approach to the treatment of CF that attempts to bypass defective CFTR function in airway epithelia, taking advantage instead of the integrated actions of this class of agents on mucociliary clearance components not dependent on CFTR. The enhanced duration of action of this compound and its ability to resist metabolism on the airway surface may allow for prolonged activation of the alternative chloride channel, thus, providing more effective treatment of CF lung disease. It is anticipated that INS37217 inhalation solution, via activation of P2Y2 receptors in the airways, will replenish airway surface liquid volume, restore mucociliary clearance, and thereby promote removal of retained secretions in patients with cystic fibrosis. The potential clinical utility of INS37217 inhalation solution is currently being evaluated in patients with CF.

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References


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