INO-4995 Therapeutic Efficacy Is Enhanced with Repeat Dosing in Cystic Fibrosis Knockout Mice and Human Epithelia

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Progressive lung damage in cystic fibrosis (CF) has been linked to inadequate airway mucosal hydration. We previously demonstrated that an inositol tetrakisphosphate analog, 1-*O*-octyl-2-*O*-butyryl-*myo*-inositol 3,4,5,6-tetrakisphosphate octakis(propionoxymethyl)ester (INO-4995), regulates airway secretory and absorptive processes, affecting mucosal hydration by prolonged (24 h) inhibition of Na^+ and fluid absorption in CF human nasal epithelia (CFHNE). The objectives of this study were to further assess clinical potential of INO-4995 in CF through ascertaining *in vivo* activity in mice with CF, determining the effects of repeated administration on potency and determining cytoplasmic half-life. Uptake and metabolism of [^{3}H]INO-4995 was monitored with HPLC to calculate intracellular half-life. INO-4995 was administered *in vitro* repeatedly over 4 to 8 days to CFHNE. Fluid absorption was assessed by blue dextran exclusion, and basal short-circuit current was measured in Ussing chambers. INO-4995 (1–100 μg/kg) was dosed intranasally either as a single dose or once per day over 4 days to gut-corrected CF mice. [^{3}H]INO-4995 was rapidly taken up by epithelial cultures and converted to the active drug, which had a half-life of 40 hours. Repeated daily application of INO-4995 to CFHNE lowered the effective concentration for inhibition of fluid absorption and amiloride-sensitive short-circuit current in cultured CFHNE, and reduced nasal potential difference to nearly control levels in gut-corrected CF mice. Ca^{2+}-activated Cl^- channel activity was also boosted in cultures. Mouse nasal levels fell from abnormal levels to within 2 μA of normal with repeated exposure to 0.8 μg/kg over 4 days. These data support further development of INO-4995 for the treatment of CF.

Keywords: cystic fibrosis; nasal potential difference; inositol polyphosphate

Premature mortality in patients with cystic fibrosis (CF) has been attributed to inadequate hydration of airway epithelia, leading to reduced mucociliary clearance and repeated infections (1, 2), especially in the lungs, where chronic inflammation results in irreversible lung damage (3). Therefore, we have sought a therapeutic approach to restore normal mucus hydration by regulating fluid secretion and absorption. In previous studies, we have demonstrated that an inositol polyphosphate analog and prodrug, 1-*O*-octyl-2-*O*-butyryl-*myo*-inositol 3,4,5,6-tetrakisphosphate octakis(propionoxymethyl)ester (INO-4995), reduces elevated basal short-circuit current (I_{sc}) and inhibits Na^+ and fluid absorption in CF airway epithelia. These actions can be explained at least in part by inhibition of the epithelial Na^+ channel (ENaC), which is overactive in CF due to absence of regulation by means of the CF transmembrane regulator (CFTR) (4–6). The effect of INO-4995 on basal I_{sc} and fluid absorption was much more prominent in tissue from patients with CF than tissue from donors without CF (4).

CLINICAL RELEVANCE

This study demonstrates the clinical potential for a compound for the treatment of cystic fibrosis. Its effectiveness serves to validate the use of inositol-based therapeutics in drug development, and demonstrates the role of inositol tetrakisphosphates in airway physiology.

The structure of INO-4995 and possible metabolites are depicted in Figure 1 and reproduced for convenience in Figure E1 of the online supplement. The de-esterified form of INO-4995 most active in patch clamp studies (7), 1-*O*-octyl-2-*O*-butyryl-*myo* inositol 3,4,5,6-tetrakisphosphate (INO-4913), is delivered to the intracellular compartment via exposure of cells to the prodrug, INO-4995. However, the precise kinetics of the uptake of INO-4995, its conversion to INO-4913 and other possible metabolites, and the half-life of intracellular metabolites after exposure of the cells to INO-4995 is not known.

Based on uptake studies with other membrane-permeant inositol polyphosphates with the same or similar protecting groups, INO-4995 cell entry is facilitated by its hydrophobic propionoxy(methyl)ester protecting groups, which can be hydrolyzed by intracellular carboxyesterases (8) after the prodrug enters the cell. Bioassay studies of methylester derivatives of inositol polyphosphates suggest that the methylester protecting groups are removed very rapidly (9). Once the propionoxy protecting groups are removed, the metabolites with ether-linked octyl groups are expected to be more slowly metabolized.

In a clinical setting, we anticipate dosing INO-4995 on a daily basis. Therefore, in the current study, we tested whether repeated dosing increases efficacy of INO-4995. We studied the effects of repeated daily dosing on the potency of INO-4995 effects on basal I_{sc}, resistance, open-circuit potential difference (PD), and response to P2Y2 receptor stimulation with ATP. We also tested whether the physiological effects of INO-4995 in human CF tissue culture *in vitro* translated into significant reduction in nasal PD (NPD) *in vivo* in the CF gut-corrected fatty acid–binding protein (FABP)/δF508CFTR$^{-/-}$ mice (10). In the FABP/δF508 CFTR$^{-/-}$ mice, the gastrointestinal defect is corrected with a gut-specific promoter, whereas the nasal electrophysiology is similar to that of the noncorrected δF508

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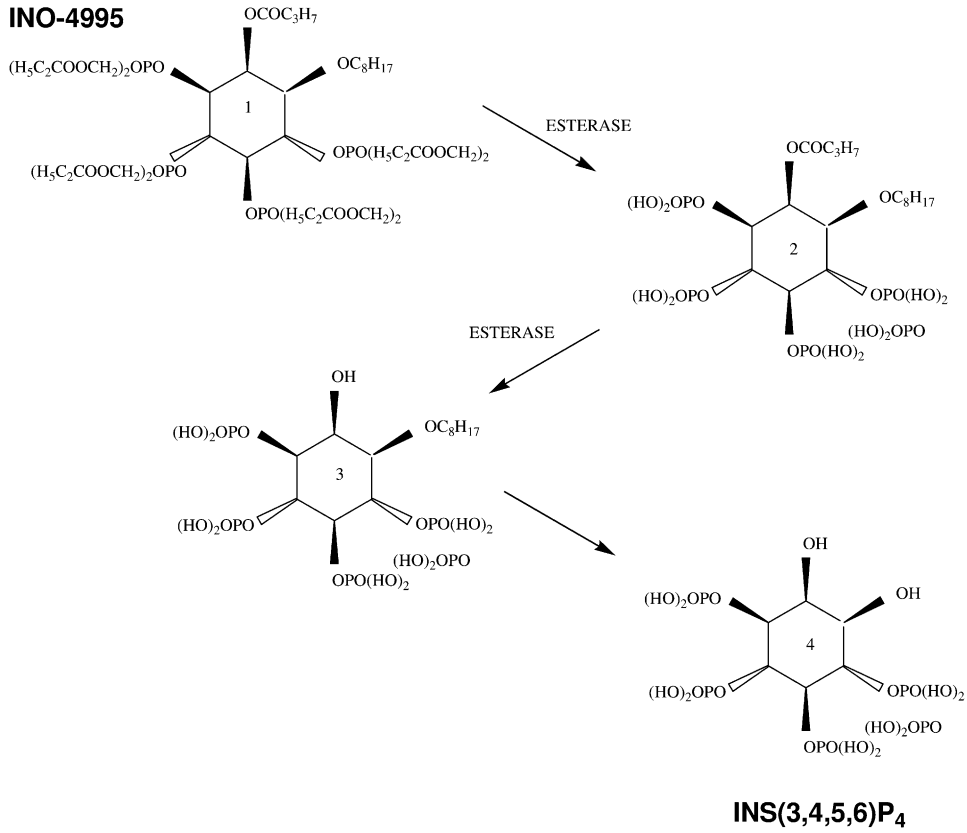

Figure 1. Pathway depicting 1-*O*-octyl-2-*O*-butyryl-*myo*-inositol 3,4,5,6-tetraki sphosphate octakis(propionoxymethyl)ester (INO-4995) and de-esterification products, 1-*O*-octyl-2-*O*-butyryl-*myo* inositol 3,4,5,6-tetrakisphosphate (INO-4913), and 1-*O*-octyl-2-*O*-butyryl-*myo* inositol 3,4,5,6-tetrakisphosphate (INO-4949). INO-4995 entry into cells is facilitated by the propionoxymethylester groups (PMs), which conceal the charges on the phosphate groups. Once inside the cell, endogenous carboxyesterases cleave the protecting PMs, resulting in an inositol tetrakisphosphate analog, INO-4913 (*2*). Further hydrolysis of the 2-butyrate yields another inositol polyphosphate analog, INO-4949 (*3*). Although it is not known whether the 1-octyl group would be cleaved by plasmologenases, which would yield the endogenous inositol polyphosphate, Ins(3,4,5,6)P_4, both INO-4913 and INO-4949 are subject to dephosphorylation by way of phosphatases, yielding, successively, InsP_3, InsP_2, InsP_1 analogs, and 1-octyl-inositol. In the current study, only products labeled with the [^{3}H]octyl group would be detectable by radio-HPLC.

$CFTR^{-/-}$ mice (10). A portion of the studies reported here have been published in abstract form (11, 12).

MATERIALS AND METHODS

Reagents

INO-4995 was obtained from SiChem GmbH (Bremen, Germany) and Industrial Research Ltd, (Lower Hutt, New Zealand). [^{3}H]INO-4995 (octyl*) was obtained from Amersham (GE Healthcare, Piscataway, NJ). Tritiated, de-esterified derivatives of [^{3}H]INO-4995, [^{3}H]INO-4913, and [^{3}H] 1-*O*-octyl-2-*O*-butyryl-*myo* inositol 3,4,5,6-tetrakisphosphate (INO-4949) were used as HPLC standards, and prepared from [^{3}H]INO-4995, as described *below* (*see also* Figure 1). [^{32}P]Inositol hexakisphosphate(InsP_6) prepared from mung beans and [^{32}P]inositol polyphosphate standards were produced as previously described (13, 14). All other reagents were provided by Sigma-Aldrich (St. Louis, MO) unless otherwise indicated.

Tissue Culture

Surgically excised nasal polyps were obtained from volunteers in collaboration with Sharon McNamara and Dr. Bonnie Ramsey at Children's Hospital, Seattle, Washington. Non-CF tissue was obtained from Dr. Ludwig Allegra at the Northwest Nasal Sinus Center, Seattle. Informed consent was obtained before receiving tissue specimens. All protocols were in compliance with institutional guidelines and approved by the Institutional Review Board at Children's Hospital in Seattle.

CF tissue was homozygous for the ΔF508 mutation. The tissue samples were processed as described previously (4).

Fluid Absorption: Multiple Exposure of CF Airway Epithelia (8 d)

INO-4995 was prepared in positive-pressure, sterile, filtered Ringer's buffer with the vehicles listed subsequently here. Ringer's buffer with INO-4995 (1 or 0.1 μM) or control vehicle were placed on the apical surface of monolayers of CF human nasal epithelia (CFHNE) that had been cultured to air–liquid interface for 2 hours (100 μl each) and incubated at 37°C. After incubation, the buffer was aspirated, and the apical surface was rinsed with 0.5 ml "differentiation media" (*see below*) and then aspirated. The monolayers were then incubated normally until the next exposure. This protocol was repeated daily for 8 days. On the ninth day, the monolayers were analyzed using a modified blue dextran (BD) assay, as previously described (4). Briefly, 100 μl buffer containing 1 μM BD was added to the apical surface of the cultures, which were then incubated in a high-humidity incubator for 18 hours, after which time 60-μl aliquots of the apical solution was removed, and the concentration of BD ascertained by spectrophotometry using 660 filter in a Packard Spectracount (Perkin Elmer, Waltham, MA). BD will not cross monolayers with tight junctions. Therefore, the change in BD concentration over time is proportional to the volume of fluid absorbed, which was calculated as previously described (4). After removal of the buffer for BD measurement in one experiment, the monolayers were mounted in Ussing chambers and I_{sc} was measured, as described elsewhere (4), to determine whether the changes in absorption corresponded to I_{sc}. Differentiation medium was based on a protocol described previously (15), and consisted of a 1:1 mixture of bronchial epithelial basal medium/Dulbecco's modified Eagles medium supplemented with 0.5 μg/ml hydrocortisone, 5 μg/ml insulin, 10 μg/ml transferrin, 0.5 μg/ml epinephrine, 6.5 ng/ml triiodothyronine, 52 μg/ml bovine pituitary extract, 0.05 ng/ml epidermal growth factor (EGF), 5 ml/500 ml nonessential amino acids (Sigma), penicillin (100 U/ml)/streptomycin (0.2 mg/ml) plus L-glutamine (2 mM) (Sigma), 3 μg/ml BSA (Sigma), and 50 nM all trans retinoic acid. Bronchial epithelial basal medium and bronchial epithelial growth medium kits were obtained from Clonetics Corp. (San Diego, CA).

Airway Surface Liquid Measurement

Primary bronchial epithelial cells from patients with CF were incubated in Transwells (Corning-Costar, Lowell, MA) with INO-4995 or vehicle (four incubations with 5 μM for 2 h/d for 4 d). Airway

surface liquid (ASL) height was measured using confocal microscopy 48 hours after the final dose, as described previously (16).

Ussing Studies: Multiple Exposure of CF Airway Epithelia (4 d)

I_{sc}, resistance, and conductance were measured in monolayers mounted in Ussing chambers, as described previously (4). Resistance was measured periodically with a bipolar voltage pulse and calculated with Ohm's law. Only monolayers with resistance values above 500 Ohms cm^2 were used in these experiments. Either INO-4995 or vehicle (vehicle control consisted of a 1:1 mixture of DMSO and DMSO plus 5% [wt/vol] pluronic-F127 diluted to a 0.1% final concentration) was supplied as a basolateral tissue culture medium and replenished daily, for a total of 96 hours, and the monolayers were mounted in Ussing chambers. No vehicle or compound was present during the recordings. INO-4995 levels in serum-free media over CFHNE decreased to 10% of initial concentration after 2 hours in the presence of CF airway epithelial monolayers using reversed-phase HPLC with extracted ion current chromatogram detection $[M+Na]^+$.

Uptake of [^{3}H]INO-4995 and Half-Life of Derivatives in HeLa and T_{84} Cell Extracts

HeLa cells were grown in 10-cm dishes in Dulbecco's modified Eagle's medium supplemented with 10% FBS at 37°C in a 5% CO_2 incubator. T_{84} cells were grown as described previously (17). Before labeling, cells were trypsinized in trypsin/EDTA, washed with serum-containing media, and plated in 6-well plates or 10-cm dishes.

Uptake experiments. Cells that were 90 to 100% confluent were incubated for varying periods of time with [^{3}H]INO-4995 in the presence of 5 μM unlabeled INO-4995 in serum-free media. HeLa cells grown on 2 × 60 mm dishes were incubated for varying periods of time with 1 ml/well of serum-free media containing 2×10^6 cpm/ml [^{3}H]INO-4995 and 5 μM cold INO-4995. T_{84} cells required labeling with higher levels of radioactivity to obtain sufficient levels for analysis. Therefore, T_{84} cells on 10-cm tissue culture dishes were incubated with 3 ml of serum-free media containing [^{3}H]INO-4995 at 4×10^6 cpm/ml and 5 μM cold carrier. Cells were incubated with [^{3}H]INO-4995 for 5–180 minutes, and were subsequently lysed with 2 ml MeOH:0.5 N HCl. Plates were washed twice with MeOH:0.5 N HCl containing 1 mM $InsP_6$ to neutralize nonspecific binding. The cell pellets were extracted three times with 3 ml chloroform, vortexed, and centrifuged at 3,000 rpm at 4°C for 10 minutes. Two phases were formed, and the lower chloroform phase containing the prodrug [^{3}H]INO-4995 and any lipophilic products was collected; the lower phases from successive extractions were combined. The combined lower phases were dried, and the extract resuspended in DMSO before reverse-phase HPLC to assess [^{3}H]INO-4995 levels, as described subsequently here. The interface and lower phase was washed with 1 ml MeOH:HCl. The upper, aqueous phases containing the hydrophilic, de-esterified products were combined, and levels of tritiated products, including [^{3}H]INO-4913, were assessed by strong anion exchange (SAX)-HPLC, as described in the online supplement.

Pulse-chase experiment. For the pulse-chase experiment, HeLa cells in 60-mm plates were labeled for 2 hours with 2.67×10^6 cpm/ml/plate; T_{84} cells were labeled in one 10-cm plate/time point with 3 ml of 4×10^6/ml. The media containing the radiolabeled compound was removed after 2 hours, and the plates were washed twice with PBS. Media containing serum was added back, radioactivity was chased for varying intervals, from 6 to 96 hours, and inositol phosphates extracted as described previously here.

Uptake of [^{3}H]INO-4995 into Primary Cultures of Human Tracheal Epithelial Cells

Primary cultures of human tracheal epithelial cells were grown on collagen-coated Transwell inserts and allowed to form tight junctions and differentiate. The cell sheets developed a transepithelial resistance of over 1,000 W cm^2 within 5 days of plating. The cell sheets also developed a visible mucus layer 14 days after plating. At this point, basal media were changed, and the apical liquid was aspirated. The mucus layer of half the filters was removed by repeated washing in cold PBS, and 100 ml of PBS containing ^{3}H-INO-4913 and ^{14}C-mannitol was added to the apical surface of each filter. One pair of filters was analyzed at each of the following time points: 15, 30, 60, and 120 minutes. Two 20-ml aliquots were taken from the apical liquid, and the basolateral volume (1 ml) was analyzed in its entirety; the cell sheets, along with the filter, were washed (15 s) in ice-cold PBS and lysed in 500 ml of 0.1 N NaOH, which was neutralized with 0.1 N HCl before analysis. Radioactive counts in solution samples and cell lysates were determined using a Packard liquid scintillation analyzer TR 2500.

Mouse NPD

Female, CF, gut-corrected FABP/δF508CFTR$^{-/-}$ mice between the ages of 12 and 31 weeks were used (10). NPD was measured 1–3 days before administration of drug, as described previously (3). Mouse nasal passages were perfused with 20 μl Ringer's solution containing INO-4995 or vehicle at a rate of 7 μl/min, and the PD across the mouse nasal epithelial membrane was measured as described previously (10) at various intervals after drug administration. The protocol was approved by the Case Western Reserve University Institutional Animal Care and Use Committee.

RESULTS

Uptake and Metabolism of [^{3}H] INO-4995 in Cultured Cells

Cultures (95% confluent) of HeLa or T_{84} cells were incubated for varying periods of time ranging from 10 minutes to 3 hours with [^{3}H]INO-4995 and 5 μM INO-4995 cold carrier, followed by extraction to separate the aqueous and lipid-soluble products. The lipid-soluble products were analyzed by reversed-phase (RP)–HPLC to confirm INO-4995 de-esterification, whereas the aqueous products were analyzed by SAX-HPLC, as described in online supplement. HPLC resolution of radiolabeled standards and cell extracts are depicted in Figures E2 and E3.

The time course of the uptake in T_{84} cultures is plotted in Figure 2A and shows that levels of INO-4913 become maximal after 1 hour. The time course of the uptake in HeLa cells was very similar (data not shown). The rate of uptake is consistent with the estimate that the intracellular concentration of [^{3}H]INO-4913 reaches 0.1–0.5 μM after a 60-minute incubation with 5 μM INO-4995. The timing of the rise in intracellular [^{3}H]INO-4913 levels corresponds to the timing of physiological efficacy (18).

Intracellular Half-Life of De-Esterified Derivatives of [^{3}H]-INO-4995

We further investigated the levels of radiolabeled metabolites of [^{3}H]INO-4995 in HeLa and T_{84} cultures over time with pulse-chase experiments to ascertain half-life and route of degradation. HeLa or T_{84} cells were incubated with [^{3}H]INO-4995 for 2 hours in media without serum supplemented with 5 μM INO-4995 as cold carrier, followed by removal of the media containing the radioactive compound, washing the cells with buffer, and chasing for 6 to 120 hours with cold media. Aqueous and organic cell extracts were subjected to SAX and reverse-phase HPLC, respectively. With chasing, the counts in the [^{3}H]INO-4913 region in T_{84} diminished exponentially, with a half-life of 40 hours (Figure 2B). The half-life in HeLa cells was similar (48 h [data not shown]).

The appearance and timing of peaks eluting with retention times corresponding to $InsP_3$, $InsP_2$, and $InsP_1$ suggests that INO-4913 is metabolized, at least in part, by progressive dephosphorylation. This is consistent with the conclusion that the primary mode of metabolism of Ins(3,4,5,6)P_4 phosphorylation to $InsP_5$ (19, 20) is not available to [^{3}H]INO-4913 due to the [^{3}H]octyl group interference on the 1 position. The kinetics of [^{3}H]INO-4913 intracellular appearance and persistence correspond to the time course of physiological efficacy of INO-4995(4), which remains fairly constant for 2–24 hours after a 2-hour exposure. Removal of the [^{3}H]octyl group at any point in the degradative

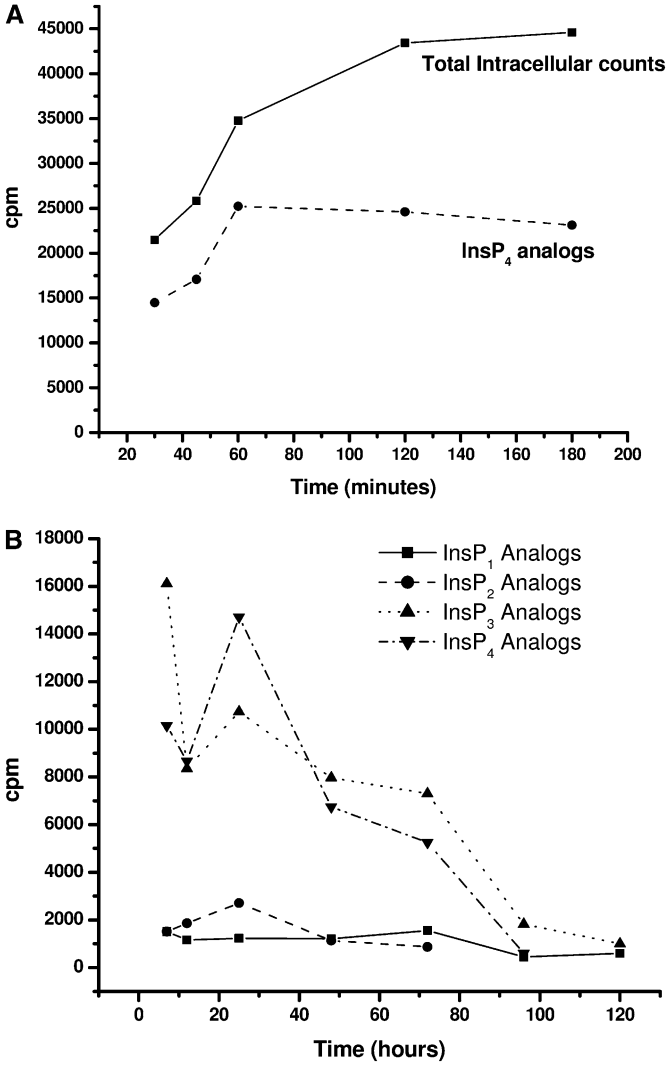

Figure 2. HPLC of de-esterified products of [^{3}H]INO-4995 extracted at various time points from T_{84} cells. (*A*) Effect of duration of exposure to [^{3}H]INO-4995 on levels of $InsP_4$ analogs in T_{84} cells. *Squares*, total intracellular counts; *circles*, $InsP_4$ analogs. (*B*) Comparison of levels of tritiated inositol polyphosphate derivatives during pulse-chase analysis of [^{3}H]INO-4995 metabolism in T_{84} cells. *Squares*, $InsP_1$ analogs; *circles*, $InsP_2$ analogs; *triangles*, $InsP_3$ analogs; *inverted triangles*, $InsP_4$ analogs.

metabolism would result in the formation of unlabeled inositol polyphosphates indistinguishable from native compounds, and would not be detected using the current protocol.

Effect of Repeated Exposure to INO-4995 on CFHNE

To better assess the optimal dose and regimen for *in vivo* studies, we tested the effects of repeated exposure to INO-4995 on fluid absorption measured with a blue dextran concentration dilution assay described previously. Here, we demonstrate that 1 μM INO-4995 administered apically 2 hours per day over 8 days inhibited flux rate by approximately 15% ($P = 0.0015$, Student's unpaired t test; $n = 6$), which was comparable to the inhibition after a single exposure to 50 μM (Figure 3). In one experimental set, I_{sc} was also measured in the same monolayers used to test the effect of repeated exposure on absorption. In this case, amiloride-inhibitable I_{sc} was also significantly reduced 23% ($P = 0.0321$).

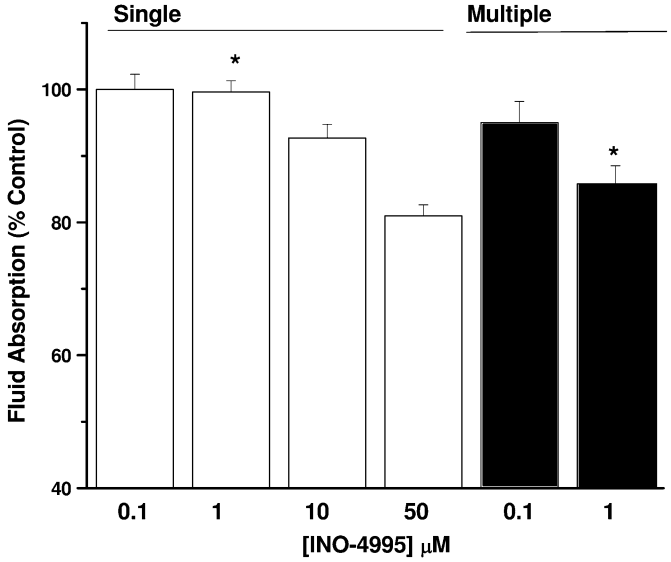

Figure 3. Repeat dosing effects on fluid absorption. The effect of repeated exposure to INO-4995 (2 h/day for 8 d) on fluid absorption was measured over 18 hours with blue dextran, as previously described (4). A portion of the control data has been described elsewhere (10 and 50 μM), and are reproduced here for comparison only (4). Comparison of effect of single (*open bars*) versus multiple doses (*closed bars*, 4 d) of INO-4995 on fluid absorption expressed as mean percent control ± SEM. *$P = 0.0015$, Student's unpaired t test comparing 1 μM single dose versus 1 μM multiple dose ($n = 6$).

We next tested whether basal I_{sc} was affected after a 4-day exposure. In these experiments to circumvent compromising the air–liquid interface by addition of compound to the apical surface, the medium in the basolateral compartment was exchanged every 24 hours with fresh medium containing 0.5, 1, or 2.5 μM INO-4995. On the fifth day, 24 hours after the final exposure, the monolayers were mounted in Ussing chambers, and open-circuit PD, basal I_{sc}, conductance, resistance, and response to ATP after amiloride were measured. After the monolayers mounted in the Ussing chambers reached a stable baseline, amiloride was added to determine the amiloride-inhibitable I_{sc}. ATP was then added to the apical compartment to assess the responsiveness to ATP and as a further test of the viability of the monolayers. The effects of repeated administration of INO-4995 on amiloride-inhibitable I_{sc} in CFHNE compared with a one-time exposure is depicted in Figure 4. I_{sc} inhibition after repeated daily dosing with 1 μM basolaterally for 4 days is indistinguishable from the inhibition observed after daily apical dosing with 1 μM (^) for 8 days or a single exposure to 20 μM. Slight increases in resistance after repeated exposure to 2.5 μM INO-4995 (data not shown) were not significantly different than with single exposures to 2 μM (4).

Repeated daily exposure to INO-4995 causes an elevation in ASL that is observable 48 hours after the cessation of treatment. Primary bronchial epithelial cells from patients with CF were incubated in Transwells (Costar) with INO-4995 or vehicle (four incubations with 5 μM for 2 h/d for 4 d). At 48 hours after the final dose, ASL height was measured using confocal microscopy, as previously described (16). The mean (±SD) ASL height for the control group was 4.16 (±0.19) ($n = 10$) versus 5.65 (±0.28) ($n = 13$) for the INO-4995 treated. Differences between treated and control values were determined to be statistically significant by a two-tailed Student's t test ($P < 0.0001$).

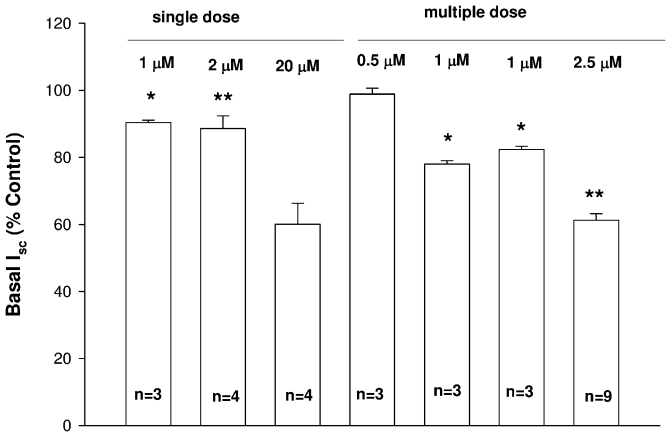

Figure 4. The effect of repeated exposure to INO-4995 on basal short-circuit current (I_{sc}) in cystic fibrosis (CF) human nasal epithelia (CFHNE). Comparison of amiloride-inhibitable I_{sc} in single versus multiple dose (4 d basolateral or 8 d repeated apical exposure, the latter denoted with ^) measured 20 minutes after mounting in Ussing chamber. Data (I_{sc}, % control) are expressed as means (±SEM). Control values were calculated from averaging basal I_{sc} from coincubated monolayers 20 minutes after mounting in Ussing chambers. *Multiple dose exposure to 1 μM was statistically different from a single dose exposure by Student's unpaired *t* test (1 μM, $P = 0.0032$; 1 μM^, $P = 0.0006$); **similarly, 2.5 μM multiple exposure was statistically different from single exposure to 2 μM ($P = 0.0001$).

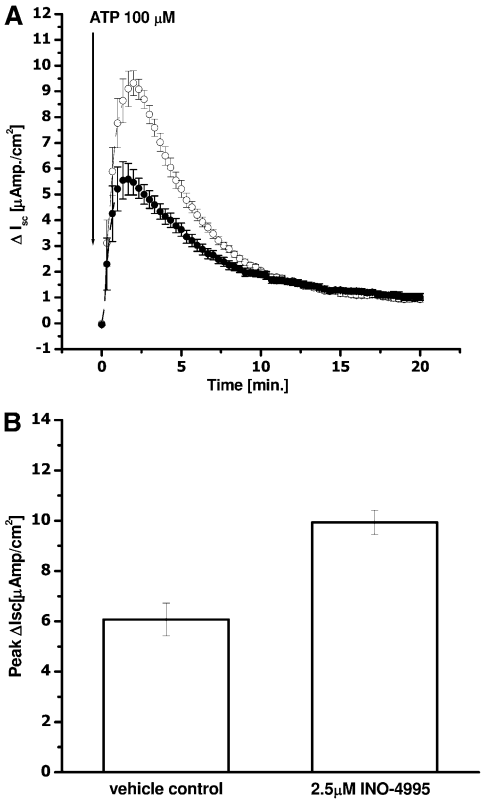

Figure 5. Response to ATP is enhanced after a 4-day exposure to 2.5 μM INO-4995. Comparison of the peak and duration of ATP-stimulated I_{sc} in the presence of amiloride after repeated 4-day exposure of CFHNE cells to 2.5 μM INO-4995 versus controls treated with vehicle. (*A*) Traces represent the average of I_{sc} over time from nine individual chambers, three separate experiments in triplicate (mean ± SEM; $n = 9$). The data were normalized to baseline just before the point of addition of 100 μM ATP. *Closed circles*, control; *open circles*, 2.5 μM INO-4995. (*B*) Peak ΔI_{sc} from the experiment depicted in *A* showing the difference between CFHNE subjected to repeated exposure to INO-4995 versus vehicle ($P < 0.0002$, Student's two-tailed, unpaired *t* test).

ENaC-Independent Effects of INO-4995 Repeat Dosing

Enhanced calcium-activated chloride secretion. When ENaC is blocked with amiloride, it is possible to observe a transient increase in I_{sc} after addition of 100 μM ATP to the apical compartment of the Ussing chamber. This rise is attributable to a transient increase in intracellular Ca^{2+} and ensuing Ca^{2+}-activated Cl^- secretion. We tested the effects of pretreatment with INO-4995 on the response to ATP in amiloride-treated monolayers in Ussing chambers. Although no difference in the response to ATP was observed after a single exposure to any dose of INO-4995 up to 200 μM (data not shown), a statistically significant increase in the peak and duration of ATP-stimulated ΔI_{sc} ($P < 0.0002$, Student's two-tailed, unpaired *t* test) was observed in the presence of amiloride in monolayers that had been exposed repeatedly to INO-4995 (Figure 5).

Mouse NPD

Like CFHNE (21), CF mouse nasal epithelia exhibit increased Na^+ absorption, which can be quantified by measuring the basal NPD (22, 23). However, studies with $CFTR^{-/-}$ mice have been hampered by the low survival rate due to digestive and absorptive deficiencies resulting from CF defects. In the FABP/$CFTR^{-/-}$ mice, the gastrointestinal defect is corrected with a gut-specific promoter, whereas the nasal electrophysiology is similar to that of the noncorrected $CFTR^{-/-}$ mice averaging between −23 and −30 mV. Therefore, these mice have a higher survival rate, but remain a good model for CF nasal airway disease. We compared the effect of single versus repetitive administration of INO-4995 on NPD in FABP/$CFTR^{-/-}$ mice. The average NPD for FABP/$CFTR^{-/-}$ mice used in these studies was 25 (±3) mV (mean [SD]). In contrast, NPD in wild-type mice average 11.1 (±0.4) mV (mean [SEM]; $n = 19$) (10). Mouse NPD was tested 3 days before the addition of various doses of INO-4995 or vehicle (−3 d) to measure baseline NPD. The NPD was measured again 1 day after the last dose with drug (+1 d). In some cases, NPD was again tested 10 days after the last dose of INO-4995. INO-4995 was tested at a dose range of 0.001–1.00 mg/ml (20 μl administered resulting in a dose of 0.02–20.00 μg) given as a single dose (acute). The vehicle or extremely low dose of 0.00001 mg/ml had no effect on the NPD values (data not shown). Data for the 0.001 and 0.01 mg/ml single doses are presented in Figure 6, revealing a statistically significant drop in the baseline NPD of 9 mV for 0.01 mg/ml ($P < 0.05$, paired *t* test). The single dose of 0.001 mg/ml resulted in an average NPD decrease of 4.2 mV that was not statistically significant compared with values before treatment.

Next, we tested whether repetitive dosing enhances responses in mouse basal NPD. Mice received the lowest dose used in the previous set of studies (0.001 mg/ml or 0.067 μg/cm² or ~1 μg/kg), which was administered daily over 4 days. The data (Figure 7) are from an experiment in which 16 animals were dosed over 4 days, and the NPD measurements were taken 24 hours after the last dose. The values for the individual mice before and after treatment are depicted in Figure 7A. In this experiment, all but one of the mice (no. 5) responded with decreased NPD after INO-4995 treatment. In Figure 7B, it can be seen that the average NPD was brought to wild-type levels on Day 5, 24 hours after animals were treated with the final dose of INO-4995. This decrease was statistically significant ($P < 0.005$). In addition, the effect of daily repeated exposure to 0.001 mg/ml INO-4995 over 4 days was

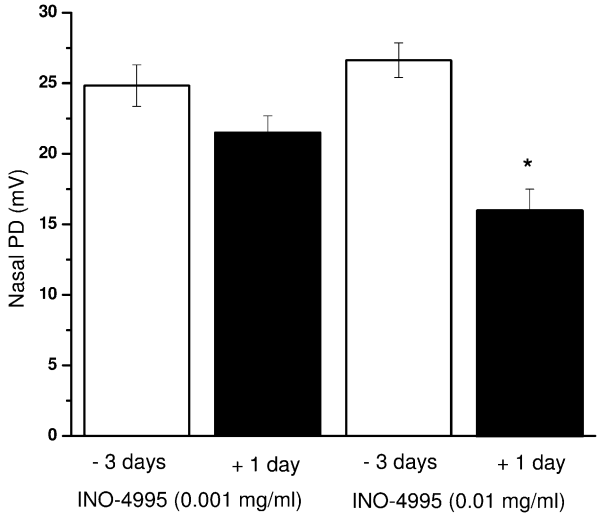

Figure 6. Effect of a single dosing of INO-4995 on nasal potential difference (NPD) in gut-corrected fatty acid–binding protein/CF transmembrane regulator knockout mice measured 24 hours after exposure compared with values in the same mice 3 days before dosing. INO-4995 was dripped into mouse nasal passages. NPD measurements were taken 3 days before drug exposure (−3 d), and 24 hours after drug delivery (+1 d). Comparison of NPD 3–5 days before addition of INO-4995 or 24 hours after addition of 20 μl of INO-4995 at either 0.001 mg/ml (0.8 μg/kg; $n = 4$) or 0.01 mg/ml (8 μg/kg; $n = 3$). The effect at 24 hours after addition of 0.01 mg/ml was statistically significant compared with the PD in the same mice 3 days before dosing (Student's paired t test, $P < 0.05$).

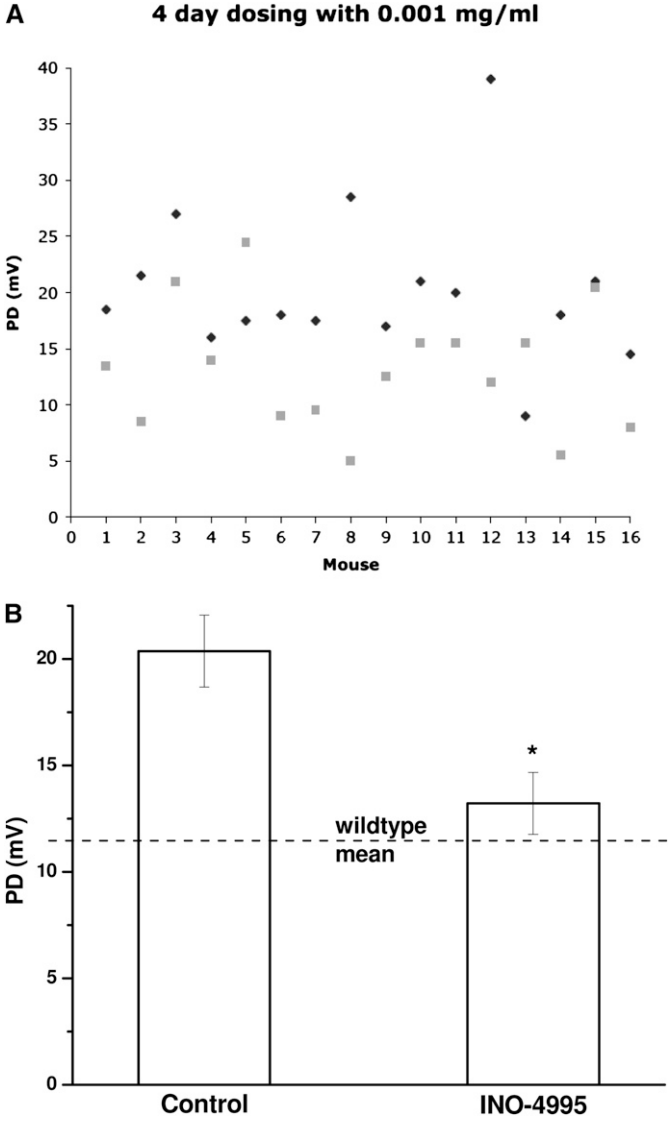

Figure 7. Effect of repeat dosing with INO-4995 (20 μl of 0.001 mg/ml or 0.8 μg/kg was delivered to mouse nasal passages once per day for 4 d). On the fifth day, NPD was measured. (*A*) *Scatter graph* depicting control (*diamonds*) and treated (*squares*) values in individual mice. The control represents NPD for the same mice 3–5 days before the administration of INO-4995. (*B*) *Bar graph* showing difference in NPD between mice before and after 4-day treatment with INO-4995. *Dotted line* shows average wild-type NPD. Data are means (±SEM) ($n = 16$). Significance was evaluated with a Student's paired t test ($P < 0.005$). The values for the group treated with 20 μl of 0.001 mg/ml INO-4995 for 4 days was also significantly different from the values for the group treated with the same dose once and displayed in Figure 5 (Student's unpaired t test, $P = 0.013$).

statistically different from a single exposure to the same dose (PD for a single dose, 21.5 ±1.19 versus PD for repeat dose, 13.2 ± 1.45 [means ± SEM]; $P = 0.013$, Student's unpaired, two-tailed t test).

We estimated the dose reaching the target tissue by taking the area of mouse nasal epithelia to be 0.3 cm^2, and calculated that the effective doses ranged from 0.067 μg/cm^2 for repeat dosing with 20 μl of 0.001 mg/ml (0.73 μM) to 0.67 μg/cm^2 for an individual dose of 20 μl of 0.01 mg/ml (7.3 μM). This is the same dose range as the lowest effective concentration in cultures of human CF nasal epithelium, where 100 μl of 1 μM INO-4995 added to cells growing on a 1-cm^2 filter is equivalent to 0.137 μg/cm^2.

DISCUSSION

We have previously demonstrated that INO-4995 has potential for treating pulmonary symptoms of CF, due to remediating actions on fluid secretion, ion movements, and electrophysiological parameters in cultured epithelia from patients with CF. The current study aimed to extend these studies to provide information to guide dosing and regimen parameters for subsequent studies. We investigated the cellular uptake, metabolism, and the effects of repeated administration in primary CF airway epithelia in comparison with *in vivo* effects in a CF mouse model. We demonstrated that repeated exposure to the prodrug, INO-4995, enhances physiological responses therapeutically relevant to CF compared with the same doses given once.

Here, we show that repeated administration of INO-4995 to human CF airway epithelia increased the potency of the compound's actions on fluid absorption and inhibition of ENaC measured as basal I_{sc}. Repeated dosing also resulted in new effects on parameters that were not affected by single doses. For example, ATP triggers a transient increase in Ca^{2+}-activated Cl^- secretion in amiloride-treated monolayers of CFHNE, measured as a transient increase in I_{sc}. The amplitude of this effect was not altered by a single exposure to INO-4995 up to 200 μM (data not shown). In contrast, repeated exposure of CFHNE (but not non-CF HNE) to 1 μM or 2.5 μM INO-4995 resulted in enhanced responses to ATP consistent with the up-regulation of Ca^{2+} activated Cl^- channel activity. These data suggest that INO-4995 could be complimentary, with CF therapeutics acting through the PY2Y receptor, such as Denufosol (24). The enhanced potency with repeated dosing was also evident in *in vivo* studies with gut-corrected $CFTR^{-/-}$ mice, where repeated exposure to 8 μg/kg

INO-4995 for 4 days resulted in greater inhibition of NPD than a single dose of a 10-fold higher concentration.

These findings demonstrate that repetitive dosing with INO-4995 results in enhanced benefits for CF treatment, such as increased potency on reversing the pathologically elevated basal I_{sc}, enhancing fluid absorption, and up-regulation of Ca^{2+}-activated Cl^- channel (CaCC) responses. No evidence of toxicity, such as deterioration of tight junctions, was observed. In fact, a small increase in basal resistance values and a small decrease in resistance in the presence of amiloride was observed, which is opposite of what would be expected if tight junction integrity were compromised. Safety studies in mice indicated that an acute intraperitoneal or intravenous dose of INO-4995 of 0.15 to 150.00 mg/kg had no adverse effect on survival, weight gain, or blood chemistries when measured immediately after or on Day 14 after dosing (25). Tarran and colleagues (16) have demonstrated that, although up-regulation of CaCC in CF airway epithelia does not result in increased basal I_{sc}, it does augment acute responses to calcium-elevating agents, such as PY2Y agonists. The success of Denufosol in augmenting mucociliary clearance in CF further demonstrates that increased activity of CaCC is clinically relevant (26). Therefore, the observation that responses to ATP, a PY2Y Ca^{2+}-elevating agonist, were enhanced in the INO-4995–treated human CF epithelia suggests that INO-4995 could also serve as an adjunctive treatment that would synergize with Ca^{2+}-elevating drugs, such as the PY2Y receptor agonist, Denufosol (24), in the treatment of CF airway disease.

One possible explanation for the enhanced effects with repeat administration is the long half-life of the active drug in cells. Membrane-permeant inositol polyphosphate analogs, such as INO-4995, penetrate the cell and become trapped inside once the labile protecting groups are removed by intracellular esterases. For instance, INO-4995 is a prodrug with its de-esterified form, INO-4913, being the active drug. [^{3}H]INO-4995 appears to enter the cells rapidly and become quickly de-esterified. We hypothesized that the enhanced responses with repeated dosing could be due to increased intracellular drug concentrations, stemming from its slow metabolism inside the cell. This conclusion is supported by our findings that the radiolabeled prodrug, [^{3}H]INO-4995, was taken up into cells and rapidly converted to the active species, [^{3}H]INO-4913, as well as the less active species, [^{3}H]INO-4949. Radioactivity in the $InsP_3$ region, and the delayed appearance of radioactivity in the $InsP_2$ region, indicate that $InsP_4$ analogs were dephosphorylated. The time course and duration of [^{3}H]INO-4913 and [^{3}H]INO-4949 elevation in cells corresponded to the time course of the physiological effects of INO-4995 in human CF airway epithelia (4). The protracted half-life of [^{3}H]INO-4913 may account for the observation that CF airway epithelia exposed repeatedly to INO-4995 exhibited quantitatively different responses to INO-4995 than epithelia exposed just once.

During the uptake experiments, [^{3}H]INO-4913 accounted for 50–75% of the total intracellular counts. This proportion shifted to 35–50% of the intracellular counts during the pulse-chase experiments, and even though the total cellular counts decreased, the proportion associated with $InsP_4$ analogs remained above 35% for 96 hours. The half-life of $InsP_4$ analogs, [^{3}H]INO-4913 and [^{3}H]INO-4949, in T_{84} cell studies was roughly 40 hours. A similar half-life in airway epithelia *in vivo* would indicate that daily dosing could result in an accumulation of drug inside the cells, and may account for our observation reported here of enhanced efficacy in mouse NPD experiments with repeated dosing versus single dosing.

We also considered the possibility that logistical difficulties in dosing mice could contribute to the advantage of repetitive administration. For instance, it is estimated that a highly variable amount of fluid, ranging from 5 to 50% of the 20 μl administered liquid, remains in the mouse nasal passages. However, variability inherent in the difficulties in delivering consistent doses in mice by itself cannot account for the magnitude of the difference in responses of mice, as the effect of a single dose of 10 μg/kg was not as great as that of four doses of 1 μg/kg.

The potency of INO-4995 on NPD responses in CF knockout mice measured *in vivo* corresponded to effects on fluid secretion and I_{sc} responses in human CF nasal epithelia measured *in vitro*. Thus, repeated exposure to INO-4995 enhances physiological actions that have therapeutic potential in the treatment of CF. This enhanced potency with repetitive treatment should be taken into consideration when developing dosing strategies for subsequent studies and clinical trials.

Conflict of Interest Statement: A.E.T.-K. is a founder, director, and employee of the company Inologic, Inc., (dba ISM Therapeutics), which sponsored the current study. B.L.-W. received reimbursement, salary, and stock options as an employee of the commercial entity, Inologic/ISM Therapeutics for from May 2002 to January 2008, and has served as a director for Inologic/ISM Therapeutics from November 2007 to the present, but receives no compensation. Some of the research activities of B.L.-W. in 2003–2005 were supported in part by a $1.3 million grant from the Cystic Fibrosis Foundation and by a $750,000 SBIR phase II grant from the National Institutes of Health. None of the other authors has a financial relationship with a commercial entity that has an interest in the subject of this manuscript.

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