No Detectable Improvements in Cystic Fibrosis Transmembrane Conductance Regulator by Nasal Aminoglycosides in Patients with Cystic Fibrosis with Stop Mutations

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Cystic fibrosis (CF) is an autosomal recessive disorder caused by many types of genetic defects, including premature stop codons. Gentamicin can suppress stop mutations in CF transmembrane conductance regulator (*CFTR*) *in vitro* and *in vivo*, leading to improvements in *CFTR*-dependent ion transport and protein localization to the apical surface of respiratory epithelial cells. The primary objective of this study was to test whether nasally administered gentamicin or tobramycin could suppress premature stop mutations in *CFTR*, resulting in full-length, functional protein. A secondary objective was to obtain data to aid in the design of multicenter trials using the nasal potential difference as a study endpoint. A multicenter study was conducted in two cohorts of patients with CF, those heterozygous for stop mutations in the *CFTR* gene and those without nonsense mutations, to investigate the effects of both gentamicin and tobramycin administered over a 28-d period on sequential nasal potential difference and airway cell immunofluorescence endpoints. Eleven patients with CF with stop mutations were enrolled in a randomized, double-blinded, crossover fashion to receive each drug, while 18 subjects with CF without stop mutations were randomized 1:1 in a parallel fashion to receive one drug. After demonstration of drug delivery, neither aminoglycoside produced detectable changes in nasal ion transport or *CFTR* localization in brushed cells from either study group. These results with first-generation suppressive agents suggest the need for improved drug delivery methods and/or more potent suppressors of nonsense mutations to confer *CFTR* correction in subjects with CF heterozygous for nonsense mutations. The study provides valuable information on parameters of the nasal potential difference measurements for use in future multicenter clinical trials.

Keywords: cystic fibrosis; nonsense mutations; chloride secretion; nasal potential difference

Nonsense mutations are the result of single base pair substitutions that produce in-frame stop codons (UGA, UAA, UAG)

CLINICAL RELEVANCE

This study demonstrates that not all premature stop mutations are equally sensitive to suppression *in vivo*, and will help researchers design clinical trials to detect *CFTR* activity.

within an exon and mRNA transcript, leading to premature truncation of protein translation (1). The resultant protein generally has reduced or absent function, and transcripts possessing premature stop mutations are inherently unstable (2–6). Together, these abnormalities elicit a profound defect in protein biosynthesis, and are frequently found in diseases with a direct (cystic fibrosis [CF], Hurler's syndrome, Duchenne's muscular dystrophy) or indirect contribution (mutations in the P53 gene or breast cancer susceptibility genes) from the affected gene (7–13).

Methods to improve the function of genes with premature termination codons have the potential for broad application to a variety of genetic disorders. In CF, premature stop codons are found in approximately 10% of the general population with CF (8–10). Affected individuals of certain ethnicities, such as those of Ashkenazi Jewish descent, have a much higher incidence of premature stop mutations in CF transmembrane conductance regulator (*CFTR*) (~85% harbor mutations of this class). These mutations in *CFTR* are generally predictive of a severe CF phenotype, particularly in regards to pancreatic insufficiency (8).

Aminoglycoside antibiotics have been shown to interact with the small ribosomal RNA subunit (18S) in eukaryotic cells, altering normal ribosomal proofreading during protein translation (11, 12, 14–18). Occasionally, this interaction leads to the binding of a near cognate amino-acyl tRNA at the premature stop codon, inserting an erroneous amino acid and allowing normal translation to progress to the end of the mRNA transcript. By this mechanism, suppression of premature stop mutations can lead to the production of full-length and functional protein. This effect has been demonstrated in a variety of models, from heterologous expression and cell-free systems, to cell lines and primary cells from patients possessing nonsense mutations, and recently in mouse models of muscular dystrophy and CF (11, 14, 15, 19–22). The specificity of the approach is attributable to greater stop codon fidelity at the authentic (3') end of mRNA (secondary to redundant termination signals). Gentamicin is a clinically approved antimicrobial that exhibits suppressive

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activity in certain models, and these preclinical findings have led to exploratory clinical trials evaluating both systemic and topical administration in subjects with stop mutations (12, 22–24). Recently, Wilschanski and colleagues in an Israeli trial provided compelling evidence that topical gentamicin, when applied directly in drop form to the nasal mucosa of CF patients possessing premature stop mutations, conferred improvements in CFTR-dependent ion transport (24). Immunocytochemical analysis confirmed localization of full-length CFTR protein to the airway cell surface specifically in treated subjects with nonsense alleles. The current study was designed to test if gentamicin or tobramycin (a potent antimicrobial with limited effects on stop codons *in vitro*), when administered as a nasal spray, could activate stop mutations in *CFTR* in patients with CF heterozygous for a wide array of premature termination mutations, as compared to control patients with CF without stop mutations. Another objective of this study was to gain longitudinal nasal potential difference (nasal PD) data to more accurately estimate the multicenter variability of the nasal PD to aid in the design of future CF clinical trials.

MATERIALS AND METHODS

Human Subjects

All human studies were approved by the institutional review boards and the general clinical research center scientific advisory committees within the participating centers. Informed consent was obtained from all participants before initiating the protocol. Inclusion criteria included: age greater than 7 yr, diagnosis of CF based on two positive sweat Cl⁻ tests and the identification of two causative mutations in *CFTR*, absence of systemic antibiotic use for four weeks prior to initiation of the protocol, and no use of topical nasal aminoglycosides. Study subjects treated with inhaled tobramycin therapy (TOBI) were instructed to wear nose clips during tobramycin nebulization. Subjects who used other topical nasal therapies (e.g., nasal steroids) were instructed to continue their use for the duration of the protocol to minimize nasal mucosal edema and inflammation. All subjects were in stable health as judged by their CF care physician. The participating study sites and number of subjects enrolled (in parentheses) included The University of Alabama at Birmingham (12), The University of North Carolina (3), The Johns' Hopkins University (4), The University of Cincinnati (3), the University of Washington (6), and Stanford University (1). Detailed demographic information is included in Table 1.

Before the protocol, limited studies were performed in six patients with CF to confirm study drug delivery by the standardized nasal spray device (Healthcare Logistics, Circleville, OH). For these studies, mean baseline PD measurements were tabulated from measurements at 1, 2, and 3 cm under the inferior turbinate (Ringer's perfusate, which was stopped upon contact with the nasal mucosa). The nasal catheter was removed, and subjects were then dosed with two sprays of Ringer's solution (100 μ l/spray, or 200 μ l total). The nasal PD measurements were repeated, and then the study patients were dosed with two sprays of Ringer's + amiloride (100 μ M). Basal PD measurements were repeated a third time, and the % inhibition of the initial mean PD was then determined for the mean PDs obtained after Ringer's and Ringer's + amiloride application. Paired *t* tests were used to compare the PD values in the presence and absence of amiloride. A *P* value of < 0.05 was considered statistically significant.

Subjects with CF possessing stop mutations were treated for 2 wk with nasally administered gentamicin sulfate and tobramycin sulfate (0.3 percent; Bausch and Lomb ophthalmic preparations, Rochester, NY) in a randomized, double-blind crossover format (Figure 1, top). Subjects received two sprays of study drug to each nostril three times daily for 2 wk. Study drug was administered with a standardized nasal spray device that provided 100 μ l per spray (200 μ l per application per nostril), yielding a total daily dose of 1,800 μ g of gentamicin or tobramycin (in 600 μ l) to each nostril per day. Subjects were instructed in use of the nasal spray device, and asked to lie with the treated side dependent for 3–5 min after administration to facilitate drug delivery under the inferior turbinate. One-week-supply bottles of study drug

TABLE 1. GENOTYPE AND DEMOGRAPHIC INFORMATION OF STUDY SUBJECTS

Age (yr)	Sex	Genotype
Premature stop mutation subjects		
16	Male	621+1G-T/E60X
16	Male	Δ F508/G542X
22	Male	Δ F508/G542X
12	Female	Δ F508/G542X
22	Female	Δ F508/G542X
11	Male	Δ F508/R553X
15	Female	621+1G-T/R553X
27	Female	Δ F508/R553X
32	Female	Δ F508/Y1092X
28	Male	Δ F508/R1162X
11	Female	Δ F508/W1282X
Mean yr (SD) 20.2 (8.9)	M:F 5:6	(six separate stop alleles represented)
Control subjects		
8	Male	Δ F508/ Δ F508
14	Male	Δ F508/ Δ F508
16	Female	Δ F508/ Δ F508
16	Female	Δ F508/ Δ F508
16	Male	Δ F508/ Δ F508
18	Female	Δ F508/ Δ F508
18	Male	Δ F508/ Δ F508
20	Male	Δ F508/ Δ F508
20	Female	Δ F508/ Δ F508
20	Male	Δ F508/ Δ F508
24	Female	Δ F508/ Δ F508
32	Female	Δ F508/ Δ F508
35	Male	Δ F508/ Δ F508
42	Female	Δ F508/ Δ F508
29	Male	Δ F508/G551D
59	Female	Δ F508/2789+5G-T
16	Male	Δ F508/3905InsT
15	Female	Δ F508/N1303K
Mean yr (SD) 23.2 (12.3)	M:F 9:9	Δ F508/ Δ F508: 14:18

were provided (with 25% overfill) at Days 0, 7, 42, and 49 for the premature stop group, and at Days 0 and 7 for the control group. Based on dispensed and returned bottle weights, the estimated compliance with study drug for the premature stop group was 72.44% (range, 62.08–108.83%) and 62.87% (range, 49.58–78.99%) for the control group. To assess for mucosal damage due to study drug application, the nasal mucosa was visually inspected at each study visit for mucosal integrity, erythema, swelling, bleeding, and friability (score range 0–4 for each parameter). The washout period between aminoglycosides was

Figure 1. Outline of study protocol. Each premature stop subject was treated with both study drugs (gentamicin, tobramycin) for 2 wk, with a 1-mo washout between administrations (random order of study drug treatment). Each control subject was treated with either gentamicin or tobramycin (random selection) for a single 2-wk period. Arrows: nasal PD measurements; * nasal curettage.

Figure 2. Effects of 100 μ M amiloride in Ringer's solution on baseline PD in patients with CF. (A) Percent inhibition of basal nasal PD after two sprays of Ringer's compared with Ringer's + amiloride (100 μ M) in six subjects with CF (mean of 1, 2, and 3 cm values for two nostrils each). * $P < 0.001$ for Ringer's compared with Ringer's + amiloride conditions. (B) Effects of Ringer's and Ringer's + amiloride spray on basal PD from one subject with CF (single nostril). *Left:* Basal PD at 1, 2, 3 cm as noted. *Middle:* Basal PD after two sprays of Ringer's. *Right:* Basal PD after two sprays of Ringer's + amiloride. Scale is as noted on tracing (mV), with upward deflections polarizing (more negative).

a minimum of 28 d. Nasal PD measurements were performed on Days 0, 7, 14, 28, 42, 49, 56, and 70 of the protocol. For each study drug period, the primary endpoint was an overall improvement in the nasal ion transport from baseline (Day 0) over the 14-d study drug administration period as well as the through the washout period at Day 28. Nasal curettage was performed after the nasal PD on Days 14, 28, 56, and 70. The secondary endpoint was detection of full-length CFTR in primary nasal cells by immunocytochemical staining over the 28-d study drug period. CF control subjects without stop mutations underwent a shortened protocol in which they were randomly assigned to receive 2 wk of either gentamicin or tobramycin (Figure 1, bottom). Nasal potential difference measurements were performed in control subjects on Days 0, 7, 14, and 28, and nasal curettage on Days 14 and 28. All safety data was reviewed by an independent external Data Safety Monitoring Board on an ongoing basis.

Nasal PD Measurements

The nasal PD is a bioelectric assay of CFTR-dependent ion transport that has been used in a variety of protocols designed to detect CFTR function. The assay is based on the description by Knowles and colleagues (25), and β -agonist-stimulated Cl^- conductance (in "0" $[\text{Cl}^-]$ + amiloride) is the most sensitive measure of CFTR activity within the nasal PD protocol. All Therapeutic Development Network (TDN) study sites were certified for the assay using standard operating procedures (SOPs) developed for multicenter studies (available on request from the CF-TDN Coordinating Center at the University of Washington). Solution #1 included Ringers solution, Solution #2 included Ringers + 100 μ M amiloride, Solution #3 included 0 $[\text{Cl}^-]$ + amiloride, Solution #4 included 0 $[\text{Cl}^-]$ + amiloride + isoproterenol (10 μ M), and Solution #5 was identical to solution #4 + 10 μ M ATP. Nasal PDs performed at the University of Alabama at Birmingham were based on a similar

protocol developed before availability of the SOP. Differences in the UAB protocol compared with the TDN protocol included (1) use of low chloride (6 mMol) solution rather than a 0-mMol chloride solution, (2) 2-min perfusion times with Ringer's solution plus amiloride (100 μ Mol) and low $[\text{Cl}^-]$ plus amiloride instead of 3-min perfusions for both conditions, and (3) absence of ATP perfusion (10 μ Mol) at the end of the measurement. All nasal PD tracings were scored independently at the University of North Carolina at Chapel Hill Nasal Potential Difference Interpretative Center in a blinded fashion.

Nasal Curettage and Immunocytochemistry

The nasal cell procurement, staining, and scoring methodology have previously been validated (24). Briefly, nasal cells were obtained from study subjects using the Rhinoprobe nasal curettage (Arlington Sciences, Springville, UT) after the completion of the nasal PD. Cells were gently scraped from the inferomedial region of the inferior turbinate (i.e., medial to the site of the nasal PD measurement) under direct visualization and spread directly onto glass slides (Fisher Scientific, Pittsburgh, PA). Slides were placed immediately in iced methanol (HPLC grade; Fisher Scientific) for 10 min to fix and dehydrate cells. The slides were then removed from methanol and air dried, placed in styrofoam mailers, and sealed within an airtight container with an accompanying humidity sponge (Control Company, Friendswood, TX). The containers were then sent to the University of Alabama at Birmingham Cystic Fibrosis Research Center for final processing and immunofluorescence scoring. A minimum of two slides per subject were obtained to allow for appropriate internal antibody controls.

Before testing, cells were rehydrated with PBS (5 min) and blocked with preimmune mouse serum for 20 min. The primary antibody (mouse monoclonal anti-CFTR 24-1, directed to the carboxy terminal four amino acids of full-length CFTR) was applied at a dilution of 1:100 for 2 h.

Figure 3. Examples of normal (*top*) and CF (*bottom*) nasal PD tracings. The x-axis is time (min) as described, and the y-axis is PD (mV, upward is polarizing). The normal phenotype includes a lower (less polarized) baseline PD in Solution #1, a small depolarizing change in PD after amiloride perfusion (Solution #2), and a repolarizing PD after perfusion with 0 [Cl⁻] and 0 [Cl⁻] + isoproterenol (Solutions #3 and #4—continued presence of amiloride). The CF phenotype includes a higher (more polarized) baseline PD, a large depolarizing change in PD after amiloride perfusion, and absent repolarization after perfusion with 0 [Cl⁻] and 0 [Cl⁻] + isoproterenol. ATP-stimulated PD (Solution #5, reflecting stimulation of Calcium Activated Cl⁻ Conductance) is retained in normal subjects and in subjects with CF.

Slides were then washed three times in PBS, and incubated with secondary antibody (Goat anti-mouse IgG, AlexaFluor 596; Molecular Probes, Portland, OR) for 1 h. Nuclei were identified by DAPI staining. Cells were imaged on a digital confocal fluorescent microscope, and images captured with IPlab Spectrum software (Scanalytics Inc., BD Biosciences Bioimaging, Rockville, MD) (24). All samples were analyzed in a blinded fashion by a single investigator. CFTR staining was classified as (1) absent, (2) perinuclear, or (3) cytoplasmic and surface. At least 25 cells in two separate areas of each slide were evaluated and scored (50 cells scored per slide).

Statistical Analysis

The parameters examined in this trial included (1) the baseline PD, Table 2, (2) the % change in PD after amiloride perfusion (in Ringer's, Solution #2, Table 3), and (3) the total CFTR-dependent Cl⁻ secretory response ("TCS," or change in PD from end of Solution #2 to end of Solution #4 perfusion, Table 4). Analyses were performed as follows: comparisons between groups (gentamicin-treated control subjects versus tobramycin-treated control subjects; premature stop group during gentamicin treatment versus all control subjects; premature stop group during tobramycin treatment versus all control subjects) and across the four time periods (before 0 d, 7 d, 14 d, and after 28 d) were performed using models containing group, time, and group by time interaction terms; in addition, comparisons across the four time periods were performed separately within each group (all control subjects, premature stop group during gentamicin, and premature stop group during tobramycin). These comparisons were performed using change from pretreatment values (Day 7 – Pre, Day 14 – Pre, and Post – Pre) in place of the actual values at each of the four time periods. All comparisons

were performed using mixed-models repeated measures analysis of variance, assuming a first-order autoregressive covariance matrix. Tukey's multiple comparisons test was then used to determine which specific pairs of means were significantly different. Results from the study are based on mean values of both nostrils per subject per nasal PD session. Additional analysis presented in this paper (examining inter- and intraindividual variability of the Cl⁻ secretory response, Tables 5–7) included (1) averaged responses (average of both nostrils per subject per session) and (2) "best" response (most polarizing response from either nostril during each nasal PD session). Aggregate data analysis of left right nostril comparisons and repeatability (between Days 0 and 28) comparing all sites, the TDN sites (without UAB), and the UAB site (without TDN sites) are also included (Tables 8 and 9). Analyses were performed using SAS software (version 9.1; SAS Institute Inc., Cary, NC). All statistical tests were two-sided and were performed at a 5% significance level (i.e., $\alpha = 0.05$).

RESULTS

Assessment of Drug Delivery

Figure 1 provides a summary of the clinical protocol (discussed in detail in the following section), and Figure 2 summarizes pre-study analyses of drug delivery. To ensure that topical application of drug reached the region of the nasal mucosa assayed during the nasal PD measurement, experiments to confirm drug delivery by the spray bottle were performed in subjects with CF before initiating the clinical trial. All measurements were performed at one site (UAB). Nasal PD measurements were

TABLE 2. RESTING OR BASELINE NASAL PD

Day of Dosing	Premature Stop Group: Gentamicin	Premature Stop Group: Tobramycin	Control Group: Gentamicin and Tobramycin
D01 "pre" values	–36.18 (17.16)	–37.73 (10.87)	–42.09 (10.12)
Day 07 (D07–D01)	2.43 (16.89)	3.48 (6.35)	6.40 (14.17)
Day 14 (D14–D01)	2.27 (16.09)	0.12 (9.44)	2.19 (18.21)
Post-values (D28–D01)	–4.30 (16.51)	1.45 (9.10)	3.88 (14.12)

Definition of abbreviation: PD, potential difference.

Change in PD from pretreatment in premature stop subjects during gentamicin and tobramycin treatment, and in control subjects during treatment (with either aminoglycoside). Values in the "Day 01 pretreatment" row are the actual mean resting PD ($\pm$ SD). Values below are changes from the Day 01 value (average values of both nostrils per subject). Day 07 and Day 14 are "on treatment." Positive changes in PD reflect improvements in CFTR function.

TABLE 3. PERCENT CHANGE IN NASAL PD AFTER AMILORIDE

Day of Study	Premature Stop Group: Gentamicin*	Premature Stop Group: Tobramycin†	Control Group: Gentamicin and Tobramycin
D01 "pre" values	40.20% (18.26)	45.26% (17.81)	42.06% (12.57)
Day 07 (D07–D01)	5.18 (15.82)	−0.31 (8.89)	−8.90 (13.71)
Day 14 (D14–D01)	−5.35 (18.62)	1.30 (13.70)	−5.33 (16.16)
Post-values (D28–D01)	1.24 (20.31)	4.01 (11.08)	−5.51 (15.61)

Definition of abbreviation: PD, potential difference.

Change in PD from pretreatment in premature stop subjects during gentamicin and tobramycin treatment, and in control subjects during treatment (with either aminoglycoside). Values in the "Day 01 pretreatment" row are the mean % change in PD after amiloride ($\pm$ SD). Values below are changes from the Day 01 value (average values of both nostrils per subject). Day 07 and Day 14 are "on treatment." Reduced changes in PD reflect improvements in CFTR function.

* There is a significant group $\times$ day interaction for the averaged nostril results ($P < 0.02$, groups = control subjects and premature stop subjects on gentamicin). None of the individual pair-wise comparisons are significant.

† There is significant difference between groups for the averaged nostril results ($P < 0.05$, groups = control subjects and premature stop subjects on tobramycin). None of the individual pair-wise comparisons are significant.

performed (mean of 1.0, 2.0, and 3.0 cm under the inferior turbinate) at baseline, after two sprays (200 μ l) of Ringer's solution, and after two sprays of 100 μ M amiloride in Ringer's solution as described in MATERIALS AND METHODS. The perfusate (Ringer's) was stopped after contact with the nasal mucosa to ensure that the dosed amiloride solution was not washed away by the PD measurement. The effects of each application on the mean resting potential were then compared with pretreatment values for each subject. Figure 2A summarizes data from six subjects with CF undergoing measurements. Amiloride + Ringer's solution depolarized the resting potential difference by $\sim 50\%$ compared with Ringer's solution alone ($P < 0.001$). Figure 2B shows a representative tracing from a single subject with CF before and after dosing with amiloride solution by spray bottle delivery. In general, measurements at multiple distances from the nasal vestibule were depolarized in a similar manner, suggesting even distribution of study drug after delivery. Together, these studies confirmed that the spray delivery system was able to effectively dose the region of the nasal mucosa accessed during nasal PD measurements, and provided confidence that the drug delivery system would provide reasonable distribution of study drug on the nasal mucosa.

Figure 3 provides examples of normal (*top*) and CF (*bottom*) nasal PD measurements. Upward deflections are polarizing. The nasal PD findings in normal subjects include a less polarized baseline PD in Ringer's (Solution #1), a small change (depolarizing) in PD after amiloride perfusion (Solution #2), and large repolarization after establishment of a Cl[−] secretory gradient and stimulation with isoproterenol (CFTR-dependent Cl[−] secretion; Solutions #3 and #4). The traditional CF phenotype includes a

high (polarizing) baseline PD, a large depolarizing response to amiloride, and absent repolarization after establishment of a Cl[−] secretory gradient and stimulation with isoproterenol. ATP-stimulated Cl[−] secretion is enhanced in CF relative to normal subjects (CFTR-independent Cl[−] secretion; Solution #5). The parameters measured in this study included (1) the mean baseline (or resting) PD, (2) the % change in PD after perfusion with Solution #2 (amiloride), and (3) the change in CFTR-dependent Cl[−] transport (change in PD from end of Solution #2 to the end of Solution #4 perfusion).

Clinical Trial

A total of 30 subjects enrolled in the protocol, and 29 were able to complete all components. One subject could not comply with the nasal PD measurement at baseline and withdrew from the protocol before initiating treatment. Eleven subjects with stop mutations in *CFTR* and 18 subjects without stop mutations were included in the final analysis. Table 1 summarizes CF genotypes. In the premature stop group, six different premature stop mutations in *CFTR* were represented (all heterozygous). The control group included 14 of 18 subjects who were homozygous for the $\Delta F508$ *CFTR* allele (and none of the mutations predicted retained CFTR activity).

In general, the study interventions were well tolerated, with infrequent adverse effects reported (mostly constitutional symptoms interpreted as unrelated to study drug or the nasal PD measurements). There were no differences in nasal mucosa visual scores during the study to suggest mucosal injury due to study drug application (either tobramycin or gentamicin). Tables 2–4 summarize the three parameters examined in the premature

TABLE 4. CHANGE IN PD AFTER PERFUSION WITH 0 [Cl[−]]/AMILORIDE AND ISOPROTERENOL (10 μ M) (SOLUTIONS #3 AND #4, OR TCS)

Day of study	Premature Stop Group: Gentamicin	Premature Stop Group: Tobramycin	Control Group: Gentamicin and Tobramycin
D01 "pre" values	3.97 (3.50)	3.89 (3.91)	2.99 (3.48)
Day 07 (D07–D01)	−0.42 (4.21)	−0.41 (4.42)	0.62 (3.81)
Day 14 (D14–D01)	2.88 (3.65)	1.59 (4.09)	0.11 (4.79)
Post-values (D28–D01)	1.50 (6.08)	1.43 (6.13)	0.26 (3.41)

Definition of abbreviations: PD, potential difference; TCS, total CFTR-dependent Cl[−] secretion.

Change in PD from pretreatment in premature stop subjects during gentamicin and tobramycin treatment, and controls during treatment (with either aminoglycoside). Values in the "Day 01 pretreatment" row are the mean change in PD after 0 [Cl[−]] and 10 μ M isoproterenol (Solutions #3 and #4 perfusion; $\pm$ SD). Values below are changes from the Day 01 value (average values of both nostrils per subject). Day 07 and Day 14 are "on treatment." Negative changes in PD (polarizing) reflect improvements in CFTR function.

Figure 4. Examples of CFTR staining in human nasal cells obtained by nasal curettage. Cells were derived from subjects not treated with aminoglycosides. (A) Three high-power fields (top panels and bottom left panel) of nasal cells from a normal subject that exhibit CFTR (red) and nuclear (blue) staining. Approximately 60% of cells stained positive for CFTR, with the majority demonstrating either a cytoplasmic or cytoplasmic/surface staining pattern (arrows). The bottom right panel is a negative antibody control (primary antibody omitted). (B) Low-power fields of nasal cells obtained from a normal subject, a patient with CF homozygous for the $\Delta F508$ CFTR mutation, and a subject with CF possessing a premature stop mutation (G542X/ $\Delta F508$). The first two panels demonstrate robust CFTR staining within the cytoplasm and at the cell surface (normal subject). The third panel shows perinuclear staining of CFTR ($\Delta F508/\Delta F508$ subject). The final panel demonstrates minimal CFTR staining (G542X/ $\Delta F508$ subject).

stop and control groups. Based on mixed model analysis, no interactions between groups over time were observed (baseline PD, % change in PD after amiloride, CFTR-dependent Cl^- secretion). No statistically significant differences within groups (during gentamicin or tobramycin treatment compared with off-treatment periods) were observed in the resting PD (Table 2), the % change in PD after amiloride perfusion (Table 3), or TCS (Table 4) within either the premature stop group or the control CF group. There were statistically significant differences in the % change in PD after amiloride perfusion (Table 3) between the control group and the premature stop group (discussed in Table 3). The clinical significance of these differences is unclear, given that they were not accompanied by improvements in other measures of CFTR function (baseline PD or Cl^- secretion, Tables 2 and

4, respectively), and no trends over treatment or time could be identified. No other significant differences in nasal PD parameters were seen comparing the premature stop group to the CF control group during treatment with either aminoglycoside. Repeat analysis of "best responses" revealed similar results (see detailed discussion in the following section).

Figure 4 provides examples of cytoplasmic and surface CFTR staining of nasal cells brushed from normal control subjects, perinuclear CFTR staining from a $\Delta F508/\Delta F508$ subject with CF, and sparse staining from a subject with CF heterozygous for a premature stop mutation (G542X/ $\Delta F508$; all staining shown was from cells isolated from subjects off of treatment). Approximately 50% of nasal curettage samples obtained during the protocol were adequate for subsequent analysis of CFTR localiza-

tion. No consistent changes in CFTR staining were demonstrated in premature stop subjects or control subjects with CF that could be attributed to aminoglycoside treatment.

Analysis of Aggregate Nasal PD Data

As CFTR-dependent Cl^- secretion has been shown to be the most sensitive marker of CFTR activity and this measurement displayed the least variability within our study (Tables 2–4), we limited subsequent data analysis to this nasal PD parameter. Tables 5–9 summarize nasal PD data obtained from subjects with CF off of study drug treatment (change in TCS between Day 0 and Day 28 for all subjects [premature stop and control groups analyzed together]). Table 5 summarizes descriptive statistics for Cl^- secretion, analyzing the data based on “average responses” (from two nostrils for each subject, providing one measurement/subject/d) and “best responses” (strongest Cl^- secretory response from either nostril, providing one measurement/subject/d). While the variability in the 28-d change is greater for the “best” nostril as compared to the average of the two nostril measures, the best nostril results may be more likely to show a treatment effect in the setting of an efficacious drug. Using estimates from Table 5, Table 6 summarizes power calculations to detect various differences in Cl^- secretion within a study group (change in TCS), and Table 7 summarizes similar calculations to detect differences in Cl^- secretion between two study groups (80% power, $\alpha = 0.05$). Thus, a sample size of 11 patients has 80% power to detect a 4-mV or greater change in PD over 28 d (within-group improvement in nasal Cl^- secretion based on averaging the nostrils), and a similar sample size would be able to detect a 5-mV or greater improvement based on the best nostril response. Based on the total number of subjects enrolled (premature stop group and controls), the current study had adequate power to detect a treatment effect of 4.72 mV for between-group comparisons (80% power, $\alpha = 0.05$). Sample sizes are approximately doubled for performing between-group comparisons. Importantly, the response based on averaging the nostrils is less variable for within-group comparisons, but the best nostril response elicits the greatest (most sensitive) treatment effect within a single group. This should be considered when choosing the primary analysis method to base future sample size calculations.

Additional analyses assessing variability of the aggregate data are included in Tables 8 and 9, and in Figures 5 and 6. Comparisons of left and right nostril responses (TCS) for the entire study group, the TDN sites only (UAB data omitted) and the UAB site (TDN data omitted) are included in Table 8. Left compared with right nostril values for each subject differed by approximately 1 mV in all analyses, and the least variability was seen for mean values of the left and right nostrils per subject. The TCS measurement from the TDN sites was generally less depolarized than the UAB data, likely reflecting the influence of the shorter amiloride exposure (Solution #2, 3 min for TDN sites versus 2

TABLE 6. REQUIRED SAMPLE SIZES (TOTAL NUMBER OF SUBJECTS) TO OBSERVE A WITHIN-GROUP EFFECT, 80% POWER, $\alpha = 0.05$, BY INCREASING TREATMENT EFFECT

Treatment Effect	Average of Left and Right Nostrils (per subject)	Best of Left and Right Nostrils (per subject)
–1 mV	144	230
–2 mV	38	59
–3 mV	18	28
–4 mV	11	17
–5 mV	8	12

min for the UAB site) or potentially differences in the content of the low $[\text{Cl}^-]$ solution (zero $[\text{Cl}^-]$ for the TDN sites, and 6 mMol for the UAB site). Figure 5 demonstrates modest correlation of left and right nostril responses within subjects with CF for the TCS measures ($r = 0.2433$). Table 9 summarizes repeatability of the TCS responses between Days 0 and 28 for all sites, the TDN sites only, and the UAB site only (mean of left and right nostrils per subject for each measurement); and Figure 6 summarizes repeatability for all study sites (mean TCS values per subject). Again, the TDN sites displayed less depolarization compared with the UAB (non-TDN) site, and the repeatability of TCS measurements off study drugs was modest ($r = 0.244$). These data provide support for the use of standardized procedures when performing the nasal PD as part of multicenter trials, as the mean values from the UAB site differed from the TDN sites, and the standard deviations of the UAB data was slightly higher than that of the cumulative data obtained from the TDN sites (using standardized procedures among all TDN study sites).

DISCUSSION

The goal of the current study was to examine whether treatment with nasally administered aminoglycosides was associated with improvement in nasal ion transport or full-length CFTR detection in airway cells of subjects with CF who were heterozygous for a variety of different CFTR stop mutations. Our results indicate that topical treatment with gentamicin or tobramycin was not associated with detectable improvements in airway Na^+ transport or Cl^- secretion. In addition, no differences were observed in CFTR immunocytochemical studies of nasal cells obtained from subjects before and during aminoglycoside treatment.

Our findings are in agreement with recent disparate reports from Phase II studies examining the effects of the oral stop codon suppressive agent PTC124 on nasal PD measures in American compared with Israeli populations with CF harboring premature stop mutations (26, 27). Our results also contrast with those recently published by Wilschanski and colleagues, which clearly demonstrated that topical gentamicin had significant positive effects on ion transport (both Cl^- secretion and Na^+ absorption)

TABLE 5. DESCRIPTIVE STATISTICS FOR ALL 29 SUBJECTS

	Average of Left and Right Nostrils (per subject)	Best of Left and Right Nostrils (per subject)
Mean	1.07	1.14
Median	1.00	1.00
SD	4.24	5.39
SEM	0.79	1.00

Difference between pretreatment (Day 0) and Day 28 measurements (total CFTR-dependent Cl^- secretion [end of Solution #2 through end of Solution #4], all obtained off study drug).

TABLE 7. REQUIRED SAMPLE SIZES (TOTAL NUMBER OF SUBJECTS TO BE RANDOMIZED 1:1 TO EACH GROUP) TO OBSERVE A BETWEEN-GROUP EFFECT, 80% POWER, $\alpha = 0.05$, BY INCREASING TREATMENT EFFECT

Treatment Effect	Average of Left and Right Nostrils (per subject)	Best of Left and Right Nostrils (per subject)
–1 mV	284	458
–2 mV	72	115
–3 mV	33	52
–4 mV	19	30
–5 mV	13	20

TABLE 8. COMPARISON OF TCS VALUES FROM LEFT, RIGHT, MEAN, AND BEST NOSTRIL RESPONSES (DAYS 0 AND 28 COMBINED) FOR ALL SITES, THE TDN SITES (UAB OMITTED, $n = 17$), AND THE UAB SITE (TDN SITES OMITTED, $n = 12$)

	Left	Right	Mean (Left and Right Nostrils)	Best of Left and Right Nostrils
Mean all sites	4.27 (4.43)	3.27 (4.57)	3.75 (3.54)	1.63 (3.92)
Mean TDN	2.99 (3.90)	2.18 (3.79)	2.58 (2.97)	0.73 (3.54)
Mean UAB	5.93 (4.56) [†]	4.64 (5.11)*	5.23 (3.68)*	2.72 (4.14) [†]

Definition of abbreviations: TDN, Therapeutic Development Network; UAB, University of Alabama at Birmingham.

Individual measurements were from the mean of left and right nostril per subject. The data are shown as the mean ($\pm$ SD).

* $P < 0.03$ compared with TDN sites.

[†] $P < 0.01$ compared with TDN sites.

as measured by the nasal PD, and accompanying improvements in CFTR localization in nasal mucosal cells (24). Important differences between the two trials likely account for the disparate results. First, the group reported by Wilschanski and colleagues was more homogenous. The majority of premature stop subjects were homozygous for stop mutations ($n = 11/19$), and all possessed at least one copy of the W1282X CFTR mutation. In contrast, no subject in our study had two copies of premature stop mutations, and only 1 of 11 subjects carried the W1282X CFTR mutation. Subjects possessing two different premature stop alleles might be predicted to recover more full-length, functional CFTR protein with a given dose of aminoglycosides than subjects carrying a single stop allele. Furthermore, *in vitro* studies completed in our laboratory have demonstrated that W1282X CFTR can retain partial function that is enhanced after stop codon suppression (28). Thus, increased activity of W1282X CFTR might be predicted in the context of higher expression levels. Consistent with these results, a recent report by Shushi and colleagues indicated that many of the CF subjects who failed to respond to gentamicin treatment in their previous trial had severely reduced levels of nonsense transcripts (29). Treatment with an inhibitor of nonsense-mediated decay (NMD) restored transcript levels and suppression by gentamicin *in vitro*. The authors hypothesized that NMD may act as a genetic modifier in this context. Thus, genetic differences in NMD between the Israeli and American populations with CF may account in part for differences in treatment effects (particularly in the context of the W1282X CFTR mutation).

Other systematic differences in the studies may account for the disparate results. The methods of drug delivery used in the two studies were different, and it is plausible that delivery of study drug by single drops produced higher local concentrations of aminoglycoside than the spray system used in this trial (total daily nasal dose of 900 μ g in Wilschanski's study compared with 1,800 μ g in the current study) (24). Finally, the study in Israel was conducted at a single center, a feature that might be expected

to limit the variability of the nasal PD measurement compared with the inclusion of multiple sites in this study. While standardized procedures were developed before initiating the study at the TDN sites, the variability of data for all sites (premature stop subjects: standard deviation for TCS = 4.27, $n = 22$ measurements—single nostril values) and for the TDN sites alone (standard deviation = 3.13, $n = 12$ measurements) was higher than those reported by Wilschanski and colleagues (23, 24). These findings highlight some of the difficulties in adapting the nasal PD assay for use at multiple different institutions (30).

The four most common stop mutations (G542X, R553X, R1162X, W1282X CFTR) all contain a UGA codon, and all have been shown to be suppressed by aminoglycoside treatment *in vitro* using heterologous expression systems. The less common stop mutations found in our study subjects (E60X, Y1092X) are produced by other codons (UAG or UAA, respectively) that are frequently less sensitive to suppression than the UGA codon (20). Thus, nucleotide differences seen in stop codons possessed by subjects within our study group may also have contributed to a reduced treatment effect.

Nasal cell immunocytochemistry did not demonstrate consistent improvements in CFTR localization in treated premature stop subjects compared with control subjects. This also differs from the previously published results (24). The similarity of the CFTR detection protocols suggest that differences in the two study populations (in terms of stop codons represented, number of patients heterozygous versus homozygous for stop mutations, or other population differences) or in the drug delivery method contributed to the different study results.

Several recent reports have used the nasal PD assay in multicenter studies, and have highlighted issues that can contribute to inter-site variability and the capacity to detect treatment effects (30–32). A recent report was able to demonstrate the positive effect of warming of solutions on isoproterenol-stimulated Cl[−] transport, including 32 normal subjects studied at four separate institutions (32). No studies, however, have demonstrated improvements in nasal PD measures in patients with CF using a multicenter study design. The nasal PD data obtained during this protocol provides a large dataset derived from multiple institutions in patients with CF with complete genotype information. Review of the data indicates that the number of study subjects needed for recruitment for future trials designed to improve nasal ion transport is dependent not only on the treatment effect size and variability, but by the method of data analysis. The choice between using the average of individual nostrils versus the “best” nostril should perhaps be made more on biological proof of concept than statistical grounds. If any nostril is shown to have significant response and this is thought to be clinically relevant, then an argument for using the best nostril for analysis could be made. It would be most thorough, however, to perform the analysis using both approaches, given their possible

TABLE 9. REPEATABILITY OF TCS MEASURES FROM ALL CF SUBJECTS, DAY 0 TO DAY 28 FOR ALL SITES, THE TDN SITES (UAB OMITTED, $n = 17$), AND THE UAB SITE (TDN SITES OMITTED, $n = 12$)

	Mean (Left and Right) Day 0	Mean (Left and Right) Day 28
Mean all sites	3.51 (3.54)	4.01 (3.56)
Mean TDN	2.45 (3.14)	2.71 (2.87)
Mean UAB	4.81 (3.66)*	5.68 (3.75) [†]

For definition of abbreviations, see Table 8.

Individual measurements were from the mean of left and right nostril per subject. The data are shown as the mean ($\pm$ SD).

Figure 5. Left versus right nostril TCS comparisons versus each study subject with CF ($n = 29$, Days 0 and 28 combined). The x-axis is left nostril values, and the y-axis is right nostril values (in mV) for each study subject.

disparity. A 5-mV change in PD has previously been shown to be highly specific to detect CFTR-dependent Cl^- transport in subjects with CF (22), and only 2 of 158 “averaged responses” (1.27%), 8 of 158 “best responses” (5.1%), and 8 of 318 (2.5%) from “all responses” were ≤ -5 mV (polarizing) throughout the entire trial. Based on these findings, analysis of results from multicenter trials should seek to reduce variability by providing single measurements per subject per nasal PD recording. Analysis of “averaged responses” are more specific and less likely to contribute to Type I errors, whereas “best responses” may improve sensitivity to detect low-level Cl^- secretion. Importantly, all three types of analysis of the current dataset failed to demonstrate consistent treatment effects within or between study groups.

The aggregate data shown in Figures 5 and 6 and Tables 8 and 9 compare left versus right nostril values and repeatability of mean TCS measurements (between Days 0 and 28) for all study subjects ($n = 29$), the subjects studied at TDN sites ($n = 17$), and the subjects studied at UAB ($n = 12$). The TCS measures from UAB differed from the TDN sites, likely representing subtle methodologic differences in nasal PD performance between the UAB and TDN study groups. These findings highlight the value of using standardized techniques and procedures when performing the nasal PD as an outcome measure in multicenter trials, and provide support for efforts to identify and assess factors that contribute to inter- and intrasite variability of the assay (such as nasal catheters, study subject interface, choice of electrodes, etc.).

In summary, the results of our studies indicate that nasally administered aminoglycosides did not produce detectable improvements in CFTR function or localization in subjects heterozygous for a variety of stop mutations within *CFTR*. Our results suggest the need to identify more efficacious agents with a broader spectrum of activity, improved systemic absorption, and reduced toxicity before this approach can be successfully used in a population with a diverse array of premature termination codons. The results of this study will provide valuable information to investigators planning multicenter trials of new therapeutics that use the nasal PD as a biological efficacy endpoint.

Conflict of Interest Statement: R.G. has been provided honoraria by the Cystic Fibrosis Foundation for being a member of their Clinical Research Committee and Participating in grant review twice per year. R.M. is a co-investigator on a clinical trial of an investigational drug for stop mutation disease funded by NIH/CFF and the company PTC. 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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Figure 6. Repeat TCS measurements in study subjects with CF ($n = 29$, Days 0 and 28). The x-axis is Day 0 values and the y-axis is Day 28 values (in mV, mean of left and right nostrils) for each study subject.

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