

Evidence of CFTR Function in Cystic Fibrosis after Systemic Administration of 4-Phenylbutyrate

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Most individuals with cystic fibrosis (CF) carry one or two mutations that result in a maturation defect of the full-length protein. One such mutation, $\Delta F508$, results in a mutant membrane glycoprotein that fails to progress to the apical membrane, where the wild-type protein normally functions as a cyclic AMP-regulated chloride channel. 4-Phenylbutyrate (Buphenyl), an orally bioavailable short chain fatty acid, modulates heat shock protein expression and restores maturation of the $\Delta F508$ protein *in vitro* and *in vivo*. We performed a randomized, double-blind, placebo-controlled, dose-escalation and safety study of Buphenyl in 19 adults with CF (homozygous $\Delta F508$) to test the hypothesis that Buphenyl would be safe, well-tolerated, and associated with an increase in chloride transport in nasal epithelia. Three dose levels (20, 30, or 40 g divided t.i.d.) of drug or placebo were given for 1 week. Serial measurements of chloride transport by nasal potential difference (NPD) testing and metabolic safety testing were performed. A maximum tolerated dose of 20 g was defined based on minimal adverse reactions, the safety profile, and a statistically significant induction of chloride transport that was maximal by day 3. This short-term phase I/II study demonstrates proof of principle that modulation of $\Delta F508$ CFTR biosynthesis and trafficking is a viable therapeutic approach for cystic fibrosis.

Key Words: butyrates, clinical trial, cystic fibrosis, mutation, chloride, sodium, sweating, CFTR, $\Delta F508$, Buphenyl

INTRODUCTION

The $\Delta F508$ CFTR mutation results in a mutant membrane glycoprotein that fails to progress to the apical membrane, where the wild-type protein normally functions as a cyclic AMP-regulated chloride channel [1,2]. Instead, the majority of nascent $\Delta F508$ CFTR molecules becomes ubiquitinated and rapidly degraded from the endoplasmic reticulum [3]. More efficient folding and maturation of $\Delta F508$ CFTR can be induced by high concentrations of glycerol [4] or by protein assembly at 27°C [5,6]. Both HSP70 and HSC70, distinct members of the 70 kDa heat shock protein family, interact with CFTR, and regulation of these heat shock protein-CFTR interactions can restore $\Delta F508$ CFTR trafficking [7,8]. We recently demonstrated that 4-phenylbutyrate (Buphenyl), an orally bioavailable short chain fatty acid, modulates heat shock protein function and restores $\Delta F508$ maturation *in vitro* and *in vivo* [7-9]. It is not known whether restoration of $\Delta F508$ CFTR to the

plasma membrane will be sufficient to reverse cystic fibrosis (CF) disease.

We performed a randomized, double-blind, placebo-controlled, dose-escalation, and safety study of Buphenyl in 19 adults with CF (homozygous $\Delta F508$). We hypothesized that Buphenyl would be safe, well-tolerated, and associated with a gain in chloride transport in nasal epithelia as quantified by nasal potential difference (NPD) testing. Drug or placebo was administered in three oral dose levels (20, 30, or 40 g) divided thrice daily (t.i.d.) for 1 week. Serial measurements of NPD, sweat electrolyte concentration, metabolic and hepatic function, pulmonary function, and sputum microbiology were performed during the study period and during a 1 month washout period. Pharmacokinetics were evaluated during the first 72 hours of study drug administration and will be reported separately.

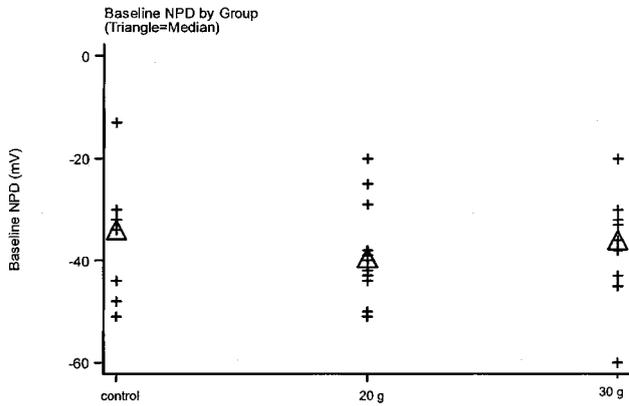


FIG. 1. Baseline NPD. Baseline NPD was recorded on day 0 during superfusion of Ringer's solution (triangle = median).

Nasal Epithelial Chloride Transport

NPD measurements were performed separately in the right and left nares. Several subjects presented with inflammation and tenderness on one side and NPD was not performed on that side if it was painful. Bilateral testing was resumed when the symptoms subsided. The baseline NPD did not differ between groups (Fig. 1). The means and standard deviations of the baseline sodium and chloride responses in each study group (Table 1) were comparable to data obtained in the Cystic Fibrosis Therapeutics Development Network (M.P.B. *et al.*, manuscript submitted), and there were no statistically significant differences in these baseline parameters between groups. In CF, the basal NPD is determined by the sodium potential which is unchecked (< -30 mV) in the absence of functional CFTR. Of the intended 38 measured basal NPDs, one naris in each of three subjects was too painful to allow measurement at the baseline. The baseline NPD was more positive than expected (-13 to -25) in 5 of 35 nares. This was due to technical difficulties related to patient reports of tenderness at the desired point of catheter placement. Although in those cases the catheter was repositioned for patient comfort and may not have represented the ideal site, these values were retained in the analysis. Average baseline NPD was similar across groups and indicative of CF.

The aggregate data for the measured low chloride/isoproterenol response over time of the placebo (Fig. 2A), 20 g (Fig. 2B), and 30 g (Fig. 2C) cohorts were assessed separately by group. No change was observed for the placebo group during the first 7 days. In contrast the low chloride/isoproterenol responses increased at days 2, 3, 4, and 7, respectively, for the 20 g (Fig. 2B) and 30 g (Fig. 2C) cohorts. The primary outcome variable, the change in the low chloride/isoproterenol response from baseline levels on each study day, was then compared across groups for days 2 (Fig. 3A), 3 (Fig. 3B), 4 (Fig. 3C), and 7 (Fig. 3D). A statistically significant induction of chloride transport potentials was observed in a dose-dependent relationship on day 3 (Fig. 3B), but this difference diminished by day 4 (Fig. 3C). The mean difference in low chloride/isoproterenol change between the 20 g cohort and the control group was -2.2 mV (95% confidence interval (CI): -10.1 ,

RESULTS

Demographics

There were 12 men and 7 women randomized in the study, and all 19 completed the final study visit. Mean age \pm SD was 28.5 years ± 7.1 , average weight was 62.6 kg ± 7.1 , and average FEV₁ (% predicted) was 63.7 ± 17.0 . There were no significant differences in gender, baseline age, weight, or FEV₁ among participants in the four groups (Kruskal-Wallis test, $P > 0.25$ for each comparison).

Adverse Events

As in our previous study, minor adverse events in the 20 g cohort included transient nausea, headache, and sleepiness after the initial dose, and body odor. The first three resolved with a dose of Tylenol, and hydration was encouraged. Body odor was an inconsistent complaint by family or friends of subjects. No dose adjustments were required. These complaints were also observed after the initial dose in the 30 g cohort. Several subjects reported visual disturbances that were transient after the first dose. One subject had severe headache that resolved with a reduction to 20 g daily. All three subjects in the 40 g cohort complained of nausea, headache, and visual disturbances, and one complained of cramps in the hands and fingers. One of these subjects tolerated 40 g of Buphenyl when it was divided into six daily doses. One tolerated a reduction to 30 g daily, and one subject found the symptoms to be so unpleasant that the drug had to be discontinued. The data and safety monitoring committee was convened to review the adverse event profile and recommended termination of the 40 g cohort. Although the maximum tolerated dose was 30 g daily, the number of tablets necessary and the side effect profile, as well as the physiologic outcome, suggest that the practical daily dose is 20 g daily divided t.i.d.

TABLE 1: Basal NPD parameters

Group	n	Amiloride inhibition (mV)	Low chloride response (mV)	Isoproterenol response (mV)	Low chloride/isoproterenol response (mV)
Control	7	22.3 (9.7)	3.9 (4.3)	0.1 (1.6)	4 (4.2)
20 g	11	23.9 (9.1)	4.4 (7.2)	3 (2.0)	7.5 (8.0)
30 g	11	25.4 (14.6)	4.5 (6.8)	1.4 (2.9)	5.9 (5.7)
40 g	6	27.2 (7.8)	-2 (4.1)	3.7 (4.6)	1.7 (5.5)

Data are expressed as mean (SD).

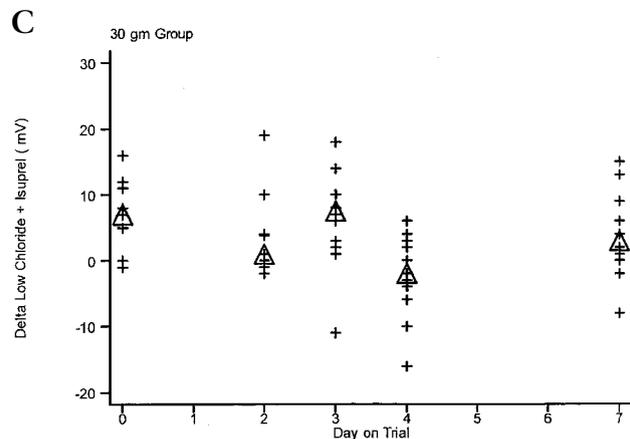
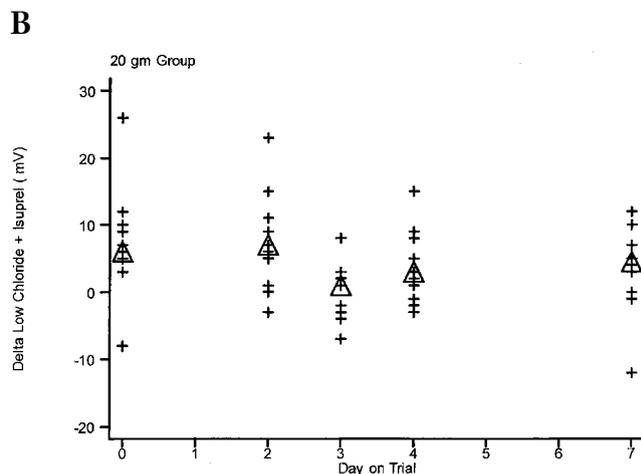
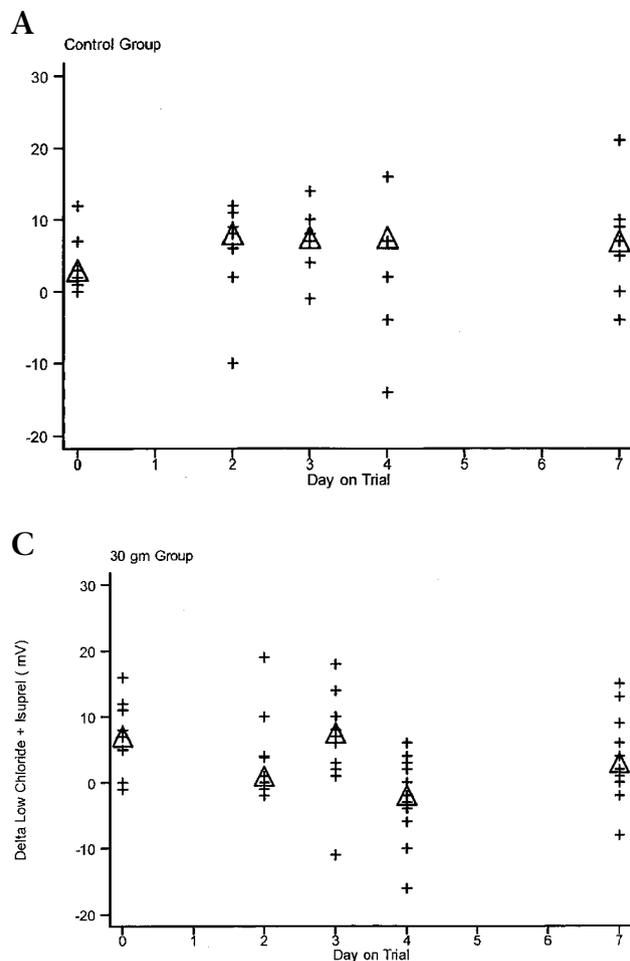


FIG. 2 Nasal epithelial chloride transport during treatment and washout periods. Individual data points representing the low chloride/isoproterenol responses at each study date during treatment are recorded by +. (A) Control group; (B) 20 g cohort; (C) 30 g cohort. Note that the induction of chloride transport in the phenylbutyrate treated cohorts is indicated by negative values crossing below the zero line.

5.8) and -11.9 mV (95% CI: $-18.9, -5.0$) on days 2 and 3, respectively. This difference decreased to -1.8 mV (95% CI: $-10.0, 6.3$) by day 4. A similar trend was observed in the comparison of the low chloride/isoproterenol change between the 30 g cohort and the control group from baseline to day 2 (-5.0 mV (95% CI: $-14.3, -4.2$)) and to day 3 (-6.3 mV (95% CI: $-16.7, 4.1$)).

Analysis of the isoproterenol response alone, without the previous low chloride maneuver, revealed the same patterns. A statistically significant, dose-dependent improvement of isoproterenol-stimulated chloride transport was observed on days 2 and 3, but this difference diminished by day 4. The mean difference in isoproterenol change between the 20 g cohort and the control group was -5.6 mV (95% CI: $-10.2, -0.9$) and -6.2 mV (95% CI: $-12.1, -0.3$) on days 2 and 3, respectively. This difference decreased to -2.8 mV (95% CI: $-10.3, 4.7$) by day 4. A similar trend was observed in the comparison of the difference in isoproterenol change on day 2 between the 30 g cohort and the control group (-5.6 mV (95% CI: $-9.4, -1.7$)) although the difference was not statistically significant by day 3 (-2.6 mV (95% CI: $-8.9, 3.7$)).

There was no statistically significant change from baseline in the amiloride-inhibited potential at any time point (data not shown). Thus, induction of chloride transport was not accompanied by inhibition of amiloride-regulated sodium transport as would be predicted for full correction of CFTR function. One example of a robust induction of chloride transport in an individual in the 30 g cohort on day 3 is shown (Fig. 4A). This NPD measurement tracing demonstrates the persistently high basal level of NPD in spite of normal levels of chloride transport, which again suggests that induction of chloride transport has no significant effect on amiloride-regulated sodium transport.

Urine and plasma collected on day 7 demonstrated the presence of study drug metabolite in all treated subjects and not in controls. Phenylbutyrate was efficiently converted to phenylacetate and excreted in the urine as phenacetylglutamine in the 20 g cohort. Increasing the dose by 33% from 20 g to 30 g daily was associated with a doubling in the AUC_{24} (11.9 ± 5.9 and 22.6 ± 5.0 mm \cdot h). Examples of the plasma phenylbutyrate (Fig. 4B) and phenylacetate (Fig. 4C) profiles for the same subject displayed in Fig. 4A are given. These graphs demonstrate the three expected daily peaks in phenylbutyrate concentration and more sustained levels of the first metabolite during the waking hours.

Accumulation of phenylacetate in the plasma was observed in one individual in the 30 g cohort. This suggests that in this subject, phenylbutyrate may have saturated the metabolic pathway to conversion to phenacetylglutamine, thus suggesting a maximum tolerated dose of 20 g daily.

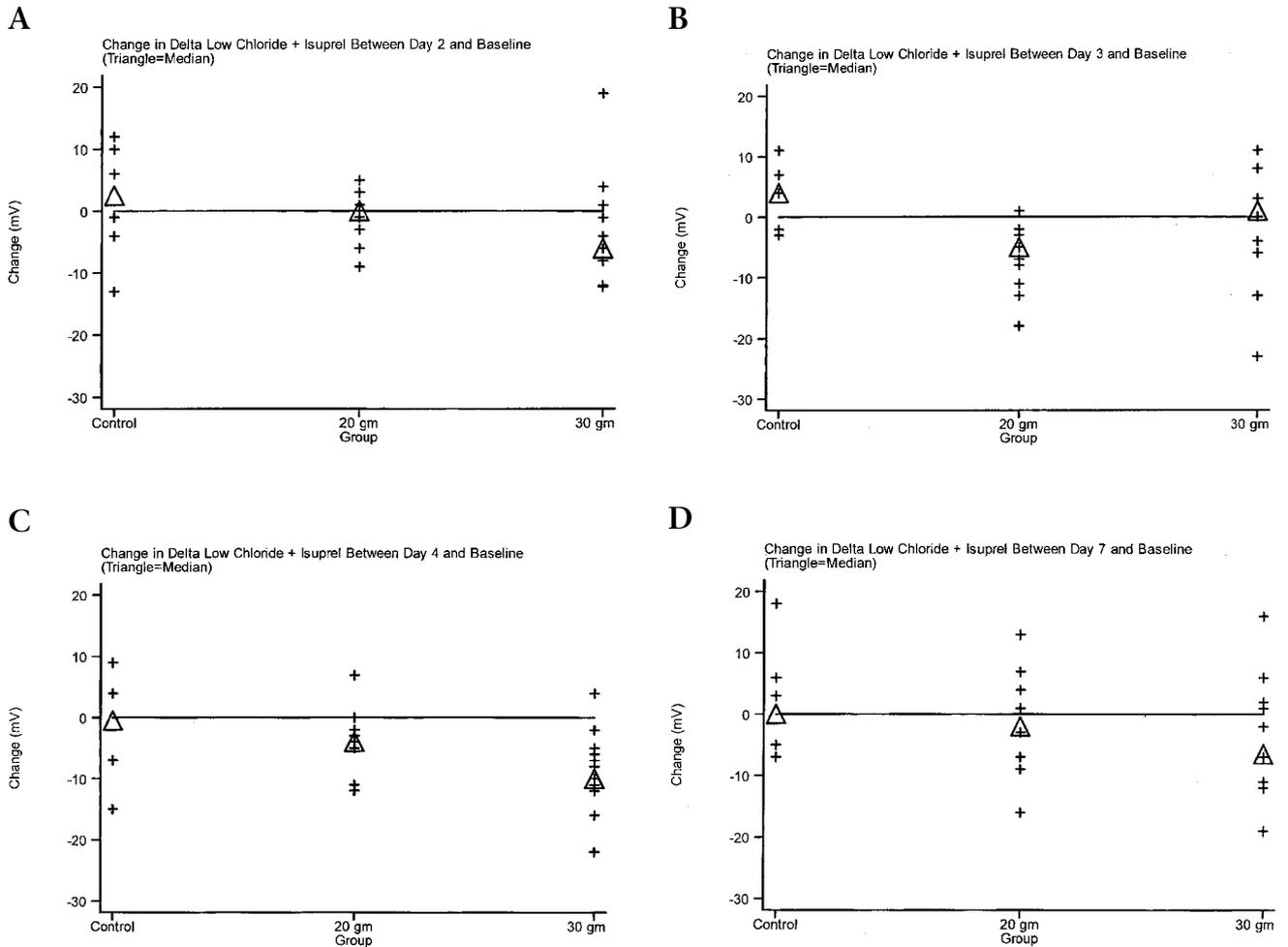


FIG. 3. Change from baseline in nasal epithelial chloride transport (triangle = median). Nasal epithelial chloride transport was measured on day 2 (A), day 3 (B), day 4 (C), and day 7 (D). Days 2 and 3 resulted in maximal inductions of chloride transport for 20 and 30 g cohorts.

Sweat Electrolytes

Although several individuals exhibited a decrease in sweat chloride while taking the study drug with restoration of pre-drug values during the washout, there was significant inter-subject variability and no statistically significant difference for the groups as a whole (Table 2).

Hepatic Enzymes

One subject had an isolated elevation in alkaline phosphatase at baseline which persisted. There was a trend toward reduction in alanine aminotransferase (ALT) and aspartate aminotransferase (AST) in the 20 g and 30 g cohorts and a statistically significant drop in total bilirubin by day 7 for the 30 g cohort (Table 2).

Uric Acid

Uric acids levels became mildly elevated while on study drug in the 30 g cohort and returned to baseline levels

during the washout period (Table 2). Phenacetylglutamine and urate may compete for the same transporter in the kidney.

Pulmonary Function Test

There was no statistically significant difference from baseline in FEV1 levels between groups, according to measurements made on day 0, 3, 4, or 7 within each group.

Microbiology

Pseudomonas aeruginosa and *Staphylococcus aureus* were scored semi-quantitatively at baseline and day 7 using a Likert scale from 0 (none) to 6 (heavy). Median score for *P. aeruginosa* at baseline was 4.5 in the controls, 2 in the 20 g cohort, and 5 in the 30 g cohort. There were no statistically significant differences in these scores at the baseline measurement between groups or between baseline and day 7 within each group.

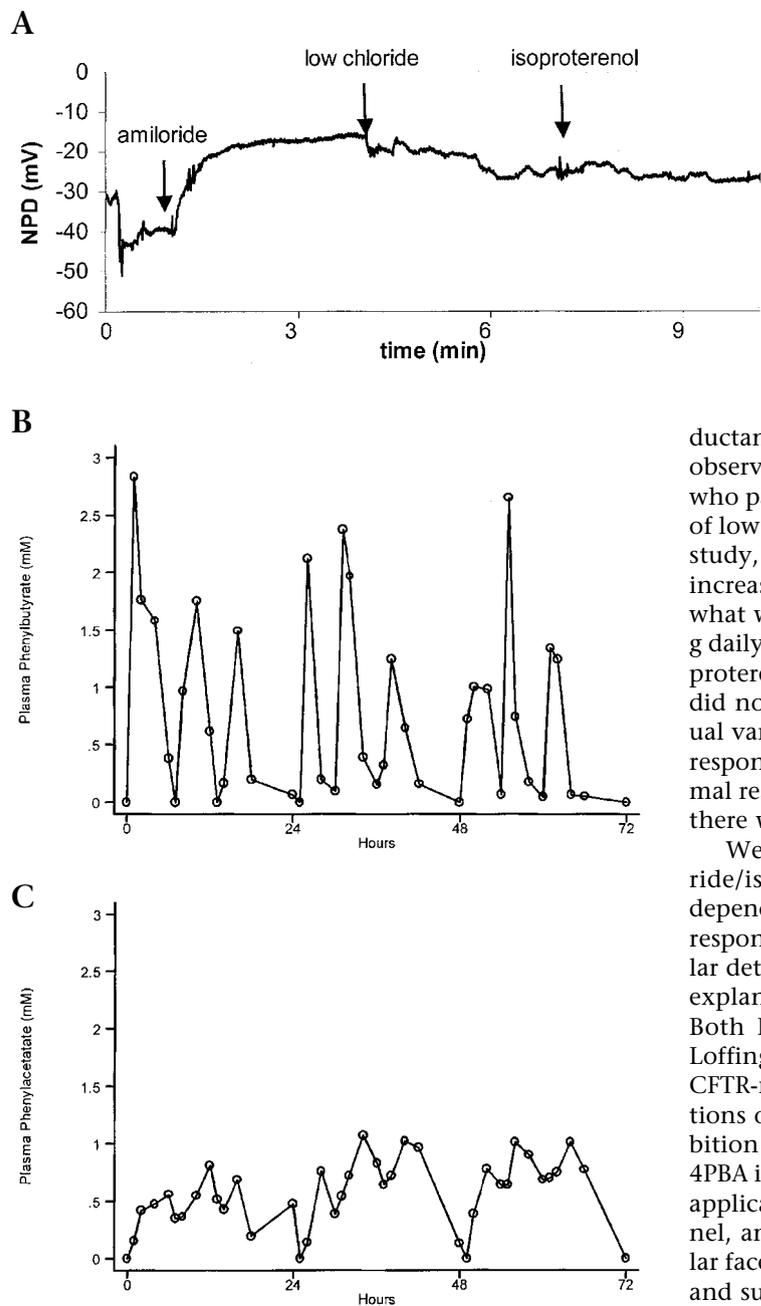


FIG. 4. Example of a NPD in a subject taking 30 g phenylbutyrate daily for 3 days. (A) NPD. (B) Plasma phenylbutyrate levels for study days 1–3. (C) Plasma phenylacetate levels for study days 1–3.

chloride/isoproterenol responses). CF can be associated with slightly higher or intermediate levels of low chloride/isoproterenol response, particularly in the setting of one or more pancreatic sufficient mutations [11–14]. In our study, restoration of the $\Delta F508$ CFTR to the plasma membrane may have been associated with a sub-maximal response because this mutation carries a shorter open time and lower conductance than wild-type CFTR [15].

We had previously observed, in subjects of this genotype taking 19 g daily who participated in a pilot study, a modest improvement of low chloride/isoproterenol response [8]. In the present study, a dose escalation was undertaken to attempt to increase the low chloride/isoproterenol response above what we observed in our first pilot clinical trial using 19 g daily. Whereas some subjects achieved low chloride/isoproterenol responses approaching normal values, others did not. Our results suggest additional genes or individual variation in pharmacokinetics may play a role in the response, but that on average, we have achieved a maximal response using 20 g daily. Our data also indicate that there was no additional advantage to taking 30 g daily.

We observed peak improvement of the low chloride/isoproterenol response between 3 and 4 days, depending on the dose, and a slight diminution in this response by day 7 in all dose groups. While the molecular detail of this observation is not known, two potential explanations are supported by published observations. Both Linsdell [16], in single channel recordings, and Loffing *et al.* [17], in Calu-3 cells, observed inhibition of CFTR-mediated Cl^- transport with millimolar concentrations of 4-phenylbutyrate (4PBA), suggesting direct inhibition of the channel by 4PBA. Inhibition of CFTR by 4PBA in single channel recordings was only observed with application of 4PBA to the cytoplasmic face of the channel, and not with application of 4PBA to the extracellular face. For this mechanism to apply *in vivo*, a significant and sustained intracellular accumulation of Buphenyl at millimolar concentrations would need to occur. Only transient plasma concentrations of Buphenyl greater than 1 mM (Fig. 4B) are observed. Rapid metabolism of Buphenyl is also observed in patients with urea cycle disorders, and our pharmacokinetic data (reported elsewhere), reporting quantitative conversion of Buphenyl to phenylacetate and phenacetylglutamine before excretion in the urine, argues against such a mechanism. Furthermore, patients with urea cycle disorders who have taken Buphenyl daily for many years have not developed lung disease, bronchiectasis, or phenotypic features of CF (Saul Brusilow, April 2002, pers. comm.).

DISCUSSION

Magnitude of Chloride Response in CF Nasal Epithelia

The combined low chloride/isoproterenol response in the NPD test has been recognized to be the most discriminatory measure of CFTR dysfunction and cystic fibrosis [10]. Homozygous $\Delta F508$ subjects (uniformly pancreatic insufficient) would be expected to have negligible, or at most, extremely low chloride responses (< -5 mV low

TABLE 2: Secondary outcome measures

	Control	20 g	30 g
Test	Mean (SD)	Mean (SD)	Mean (SD)
Sweat chloride – baseline	110.1 (16.6)	119.2 (12.7)	116.8 (16.8)
- Day 2	119.2 (7.4)	119.8 (10.4)	96.2 (45.3)
- Day 3	113.6 (13.6)	117.2 (9.0)	121.1 (13.5)
- Day 4	111.0 (8.7)	116.2 (9.9)	110.3 (16.2)
- Day 7	117.2 (10.3)	116.5 (10.7)	119.8 (4.4)
Uric acid – baseline	6.3 (1.0)	5.6 (1.8)	6.8 (0.9)
- Day 2	6.3 (2.0)	6.7 (1.9)	8.7 (1.7)*
- Day 3	6.0 (1.4)	6.6 (1.6)	9.4 (2.5)*
- Day 4	5.9 (1.4)	6.8 (1.6)	8.9 (2.6)
- Day 7	5.7 (1.6)	7.4 (2.0)	8.6 (2.1)
AST – baseline	45.0 (41.0)	23.5 (3.0)	28.3 (8.1)
- Day 2	27.5 (13.2)	20.2 (4.5)	27.2 (8.2)
- Day 3	29.2 (13.8)	19.2 (3.1)*	23.0 (8.5)
- Day 4	47.0 (34.0)	19.5 (3.7)*	38.0 (39.9)
- Day 7	59.0 (55.2)	22.4 (6.3)	33.7 (18.6)
ALT – baseline	49.5 (42.5)	28.5 (14.4)	35.0 (14.5)
- Day 2	40.0 (33.4)	23.7 (7.1)	32.6 (21.5)
- Day 3	41.2 (30.0)	21.0 (7.7)*	26.2 (18.5)*
- Day 4	56.2 (52.8)	19.2 (4.4)*	25.7 (15.1)*
- Day 7	81.5 (78.5)	19.4 (5.9)*	42.5 (74.8)
Alkaline phosphatase – baseline	238.2 (209.8)	98.2 (32.9)	162.8 (67.8)
- Day 2	228.0 (203.8)	102.8 (41.4)	149.6 (65.3)*
- Day 3	221.2 (194.4)	95.5 (35.2)	147.0 (63.3)*
- Day 4	216.0 (190.7)	92.0 (32.4)	120.8 (58.1)*
- Day 7	353.5 (277.9)	96.0 (15.0)	144.5 (74.8)
Total bilirubin – baseline	0.5 (0.3)	0.6 (0.4)	0.5 (0.2)
- Day 2	0.5 (0.2)	0.8 (0.7)*	0.5 (0.2)
- Day 3	0.4 (0.2)	0.7 (0.4)*	0.4 (0.2)
- Day 4	0.5 (0.1)	0.6 (0.3)	0.7 (0.7)
- Day 7	0.4 (0.1)	0.5 (0.2)	0.3 (0.1)*

*Significant change from baseline, $P < 0.05$.

We favor a second possible explanation for the diminution of the low chloride/isoproterenol response between 4 and 7 days, namely that the cell adapts to the perturbation of cellular homeostasis caused by Buphenyl that initially improved $\Delta F508$ trafficking. Such adapta-

tion returns the cell closer to its baseline condition in which $\Delta F508$ trafficking is aberrant. Such a mechanism is consistent with suggestions that Buphenyl alters molecular chaperone expression and interaction with $\Delta F508$ [7,18] and causes a more global cellular adaptation, one element of which is increased turnover of HSC70 mRNA [19]. Such global effects on the cell may result from Buphenyl's action as an inhibitor of histone deacetylase [20] and are also consistent with a long-term loss of Buphenyl's effect on increased hemoglobin F (HbF) expression in patients with β -hemoglobinopathies.

Upregulation of these intermediate levels of chloride transport was not associated with a reduction in the basal NPD nor with a reduction in the amiloride response, although the presence of functional $\Delta F508$ in the plasma membrane might have been expected to downregulate the epithelial sodium channel [21]. Because some members of the isoflavonoid family have been shown to activate chloride conductance through CFTR, it may be possible to increase $\Delta F508$ -mediated chloride transport by adding molecules that increase CFTR open time, decrease CFTR closed time, or prolong CFTR residence in the plasma membrane by inhibiting recycling. Perhaps full activation of $\Delta F508$ -mediated chloride transport would downregulate basal NPD and amiloride-inhibited sodium transport, as is suggested by Suaud *et al.* [22].

Systemic Administration

The ability to administer a butyrate through the gastrointestinal tract is a distinct advantage, because CF is a systemic disorder of exocrine glands and secretory epithelia. Sinusitis, pancreatitis, biliary tract disease, hepatitis, meconium ileus equivalent, and sweat losses leading to dehydration should theoretically respond to restoration of $\Delta F508$ CFTR trafficking. Our study was not suf-

ficiently powerful statistically to detect a difference in the secondary outcomes of sweat electrolytes, hepatic enzymes, or FEV₁ of subjects. Importantly, we did not observe an apparent worsening of any of these measures during phenylbutyrate treatment.

Tolerability

Phenylbutyrate is approved for infants, children, and adults with urea cycle disorders that lead to accumulation of nitrogen in the form of ammonia. Therapy must be daily and lifelong. In general, it is well-tolerated and associated with improvements in ammonia and liver function. Phenylbutyrate also has been tested in phase I and II clinical trials for a variety of solid tumors [23]. Some subjects have remained on extremely high doses of the drug for more than a year. Side effects are similar to the ones observed in our trial—mainly headache and nausea. Phenylbutyrate also has undergone testing in the hemoglobinopathies [24,25]. The therapy is pulsatile and lifelong. Identification of the appropriate dosage regimen for CF as well as potential synergy with channel activators should be explored.

Genetics

Upregulation of cellular $\Delta F508$ CFTR protein is desirable because the disease results from a deficiency of CFTR beyond the endoplasmic reticulum. Upregulation of other mutations which create a partially functional CFTR is also a desirable goal. Presumably a larger fraction of modestly impaired CFTR proteins in the plasma membrane would still improve salt and water balance across the epithelium overall. Phenylbutyrate has not been tested with any of the additional > 900 mutations seen in CF.

Applicability in Other Inherited Disorders

The $\Delta F508$ CFTR is prematurely degraded from the endoplasmic reticulum. Accumulation of undigested, misfolded CFTR in vacuoles retrieved from the endoplasmic reticulum is not a key feature of CF. The *zz* genotype of α_1 anti-trypsin (α_1 AT) deficiency produces a misfolded α_1 AT that is accumulated in the hepatocyte, the cell of origin, causing progressive cirrhosis in a subgroup of patients. The chaperones involved in α_1 AT biogenesis are similar to those involved in CFTR processing. We have implicated butyrate-mediated changes in HSC70–CFTR and HSP70–CFTR complexes as key events in the upregulation of CFTR and chloride transport. Similar mechanisms may account for *zz* α_1 AT secretion in the murine model [26]. Phenylbutyrate may therefore be helpful to the subset of CF subjects presenting with significant liver involvement.

METHODS

Human subjects. Patients of both genders with CF who were homozygous $\Delta F508$ genotype and ± 18 years of age were eligible to participate. Exclusion criteria included pregnancy or inability to practice birth control, breast-feeding, clinically significant liver disease, 24-hour oxygen therapy, acute upper respiratory infection, acute pulmonary exacerbation, and sinus surgery within 6 weeks or intravenous administration of antibiotics within 4 weeks of the start of the trial. All subjects signed informed consent forms. The study protocol and consent forms were

approved by the Joint Committee on Clinical Investigation of the Johns Hopkins School of Medicine (IRB).

Nineteen subjects entered the study and were randomized. The study drug was discontinued in one female subject from the 30 g cohort who experienced acute distal intestinal obstruction syndrome on day 2, and this subject was replaced by another subject. Due to unpleasant symptoms, three subjects required a dose reduction; two subjects from the 30 g cohort were reduced to 20 g and one from the 40 g cohort was reduced to 30 g daily. One subject in the 40 g cohort elected to discontinue use of the study drug due to intolerable symptoms. Randomization in the 40 g cohort was discontinued at the request of the data and safety monitoring committee after the third subject experienced unacceptable symptoms. This resulted in six subjects randomized to 20 g, six randomized to 30 g, three randomized to 40 g, and four randomized to placebo. All subjects completed the final visit.

Study design. A randomized, double-blind, dose-escalation design was used. Subjects were randomized in a 3:1 fashion to either the study drug or placebo. The designed study included eight subjects randomized at each of three sequential dose levels resulting in six subjects per dose cohort and six subjects in the placebo group. Escalation to the subsequent dose level proceeded unless unacceptable toxicity was observed. The primary outcome was the change in NPD between the baseline measurement and measurements taken on day X of study drug administration. A sample size of six subjects per group was planned with a power of 0.80 and a two-sided significance level of 0.05 to detect a difference in mean change in NPD of 5 mV between drug and placebo, assuming a standard deviation of the change of 1.1 mV in placebo and 2.9 mV in drug. These estimates were based on our previous investigation of the isoproterenol/low chloride response in homozygous $\Delta F508$ subjects [8]. Secondary outcomes included changes in metabolic and hepatic function, pulmonary function, sputum microbiology and pharmacokinetics.

Study drug and placebo. Buphenyl (sodium salt of 4-phenylbutyrate) in a 500 mg tablet was provided by Medicis Pharmaceutical Inc. A sodium lactate placebo tablet was formulated to maintain equivalent sodium salt load by Medicis Pharmaceutical Inc. A “no added salt” diet was prescribed for the duration of study drug administration. The 20 g daily dose was divided into 13 tablets to be taken at 8 AM and at 2 PM and 14 tablets to be taken at 8 PM. The 30 g daily dose was divided into 20 tablets to be taken at 8 AM, at 2PM, and at 8 PM. The 40 g daily dose was first prescribed as 27 tablets to be taken at 8 AM and at 2 PM and 26 tablets to be taken at 8 PM. This latter schedule proved unpleasant, and the doses were then divided into 14 tablets to be taken at 8 AM, 13 tablets to be taken at 11 AM, 14 tablets to be taken at 2 PM, and 13 tablets to be taken at 5 PM, at 8 PM, and at 11 PM. The dose was tolerated better when administered with food. Hydration, either by the oral route or through the intravenous line used for blood sampling, was associated with fewer symptoms of nausea and headache, which resolved in the first 24 hours.

Study protocol. Subjects were admitted to the Johns Hopkins Pediatric General Clinical Research Center (GCRC) for the first 4 days of study drug administration to monitor safety, to obtain frequent blood sampling and 24 hour urine collection to evaluate the pharmacokinetics, and to perform daily measurements of CFTR function. Routine safety tests consisted of measurement of hematologic indices, prothrombin time (PT) and partial thromboplastin time (PTT), hepatic function, urinalysis, spirometry, and electrolyte and uric acid levels. Blood sampling through a heparin lock or midline catheter was obtained at 0, 1, 2, 4, 6, and 8 hours post-dose on days 1, 2, and 3. Twenty-four hour urine collections were conducted on days 1, 2, and 3. Sputum was collected at baseline, on day 7, and weekly during the remainder of the protocol (1 month washout period). Sweat chlorides were measured in the Johns Hopkins Hospital chemistry lab on sweat collected by pilocarpine electrophoresis from equivalent sites on each forearm at baseline, and on days 2, 3, 4, and 7, and at weekly intervals during the washout period. NPD testing as described [8] (with minor modifications) was performed in both nares at baseline, on days 2, 3, 4, and 7, and weekly during the washout period. Testing was performed with room temperature solutions, Ringer's solution instead of an agar bridge for the subcutaneous electrode, and computer-based data acquisition for the last 5 patients.

Analysis of metabolites by HPLC. Plasma (0.1 ml) was treated with 0.3 ml methanol containing 0.1 mM benzoyl glycine methyl ester as an internal standard. After 3 minutes at room temperature, this sample was centrifuged in a microfuge at top speed for 5 minutes. The supernatant was analyzed for metabolites. Urine (0.25 ml) was prepared with 0.75 ml methanol containing the standard. A 1:25 dilution (volume:volume) of the supernatant was analyzed on a Waters Alliance system using a Waters C18 Bondapak column, 3.9 mm × 300 mm. The column temperature was maintained at 40°C, and the flow rate was 1.5 ml/min. The mobile phase consisted of (A) 1 mM phosphate, pH 3; (B) methanol; and (C) 1 mM phosphate, pH 4. Phenylbutyrate, phenylacetate, and phenacetylglutamine were resolved and quantified by A₂₂₀ measurement.

Statistical methods. Data were graphically displayed with exploratory techniques including dot plots and box and whisker plots. Differences in baseline characteristics between groups were assessed using the Kruskal–Wallis nonparametric analysis of variance method [27]. Changes in NPD, between baseline and treatment day within groups, were initially assessed using the Wilcoxon signed-rank test [27]. Changes in NPD over time were analyzed by a regression approach using a robust estimate of variance to adjust for the correlation between measures on different nares of the same individual [28].

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