Talniflumate Increases Survival in a Cystic Fibrosis Mouse Model of Distal Intestinal Obstructive Syndrome

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ABSTRACT

Intestinal disease in cystic fibrosis (CF) mice closely mirrors aspects of obstructive syndromes in CF patients. The pathogenesis involves accumulation of mucoid debris in the crypts that fuse with intestinal content to form obstructing mucocellular impactions. Treatment involves modalities that increase the fluidity of the luminal content, such as osmotic laxatives and liquid diets. We investigated the effects of talniflumate (Lomucon, Genaera Corporation, Plymouth Meeting, PA), a compound that may be beneficial to treatment of CF intestinal disease based on three mechanisms of action: mucus synthesis inhibition by blockade of the murine calcium-activated chloride channel 3 (mCLCA3), nonsteroidal anti-inflammatory effects, and inhibition of Ci/HCO3 exchange(s) involved in intestinal NaCl absorption. Cohorts of CF mice were fed control diet or diets containing either talniflumate (0.4 mg/g chow) or ibuprofen (0.4 mg/g chow) for 21 days to assess survival. Talniflumate significantly increased CF mouse survival from 26 to 77%, whereas ibuprofen had no effect (22% survival). Oral talniflumate did not alter crypt goblet cell numbers or change intestinal expression of mCLCA3 but tended to decrease crypt mucoid impaction. Ussing chamber studies indicated that talniflumate slightly increased the basal short-circuit current of CF intestine, but the change was not sensitive to secretagogue stimulation or bumetanide inhibition. In contrast, intracellular pH measurements of intact intestinal villous epithelium indicated that talniflumate significantly inhibited apical membrane Cl/HCO3 exchange by >50%. We conclude that oral talniflumate increases the survival of CF mice, possibly by the beneficial effects of decreasing small intestinal NaCl absorption through the inhibition of apical membrane Cl/HCO3 exchange(s).

Cystic fibrosis (CF), a consequence of mutations in the cystic fibrosis transmembrane conductance regulator gene cftr and its protein product CFTR, is manifested by a number of disease entities including pancreatic enzyme insufficiency, meconium ileus, distal intestinal obstructive disease, and failure of mucociliary clearance in the airways (Welsh et al., 1995). Although respiratory disease is the most consequential aspect of CF, obstructive intestinal disease is a serious manifestation that includes the syndromes of meconium ileus, meconium ileus in the newborn and distal intestinal obstructive syndrome in older patients. Furthermore, a major cause of mortality in CF mice with severe mutations of the murine homolog of CFTR is intestinal obstructive disease (Grubb and Gabriel, 1997). Respiratory mucus alterations, however, are not manifestations in CF mice and do not contribute significantly to mortality (Grubb and Gabriel, 1997). The pathology of intestinal disease in CF mice closely mirrors intestinal obstruction syndromes in CF patients, and both are characterized by the development of mucoid casts within the intestinal crypts that fuse with luminal content to form mucocellular impactions. The pathogenesis of distal intestinal obstructive syndrome involves insufficient hydration of mucus and debris at mucosal surfaces because of abnormal transepithelial electrolyte and water transport in the absence of CFTR activity. CFTR is an epithelial, cyclic nucleotide-activated anion channel with known regulatory actions on a number of electrolyte transport processes including NaCl absorption (Anderson et al., 1991; Bear et al., 1992; Clarke et al., 1992; Gabriel et al., 1993; Stutts et al., 1995). In the CF small intestine (human and mouse), loss of CFTR function results in deficient anion secretion and dyregulation of electroneutral NaCl absorption, which together diminish hydration of the luminal content (Clarke and Harline, 1996; Gawenis et al., 2003). Osmotic laxatives are a palliative

ABBREVIATIONS: CF, cystic fibrosis; CFTR, cystic fibrosis transmembrane conductance regulator; PEG, polyethylene glycol; hCLCA1, human calcium-activated chloride channel 1; mCLCA3, murine calcium-activated chloride channel 3; WT, wild type; PCR, polymerase chain reaction; Isc, short-circuit current; BCECF-AM, 2’,7’-bis[2-carboxyethyl]-5-(and-6)-carboxyfluorescein acetoxyethyl ester; IBR, isethionate-bicarbonate Ringers; pH, intracellular pH; DMSO, dimethyl sulfoxide; CLD, congenital chloride diarrhea; IL, interleukin.
treatment for CF obstructive disease, and, in CF mice, the inclusion of polyethylene glycol (PEG) 3350-based laxatives in the drinking water can essentially eliminate mortality because of intestinal obstruction (Clarke et al., 1996).

Talniflumate (Lomucin, Genaera Corporation, Plymouth Meeting, PA) is a phthalidyl ester of niflumic acid. Previous studies have shown that niflumic acid is an inhibitor of the calcium-activated chloride channel hCLCA1 [and its murine homolog, murine calcium-activated chloride channel 3 (mCLCA3), alias gob-5] (Pauli et al., 2000; Zhou et al., 2002). Because the expression of hCLCA1 has been closely associated with increased mucin production by epithelia of CF and asthmatic patients (Toda et al., 2002; Hauber et al., 2003), the proposed mechanism of action for talniflumate or niflumic acid is inhibition of hCLCA function in mucus overproduction (Melton, 2002). This hypothesis has been strengthened by demonstrations that these compounds inhibit mucin synthesis and release in cell culture and animal model systems (Zhou et al., 2002; Bertrand et al., 2004) (M. McLane, K. J. Holroyd, and R. C. Levitt, unpublished data). However, drugs of this class possess other pharmacological properties, including nonsteroidal anti-inflammatory activity through an inhibitory action on cyclooxygenases (Insel, 1996) and inhibition of Slc26a Cl⁻/HCO₃⁻ exchange activity (i.e., Slc26a3, alas down-regulated in adenoma, and Slc26a6, alas putative anion transporter-1 or chloride formate exchanger), which play a major role in NaCl absorption across the intestine (Jacob et al., 2002; Wang et al., 2005).

The combination of these properties (mucus synthesis inhibitor, nonsteroidal anti-inflammatory drug, Cl⁻/HCO₃⁻ exchange inhibitor) in an orally tolerated drug may be useful in the treatment of CF intestinal disease. Therefore, we tested whether oral talniflumate treatment would reduce mortality resulting from obstructive disease in CF mice after acute withdrawal of PEG laxative treatment. These results were compared with the effect of oral ibuprofen, a cyclooxygenase inhibitor of the same class that does not inhibit mucin production (Zhou et al., 2002). In additional studies of CF mice maintained on PEG laxative, we evaluated the effect of talniflumate or control diet.

Materials and Methods

Animals. Mice (23–45 days old) homozygous for gene-targeted disruption of the murine homolog of cfr (S489X mutation, cfr¹⁰⁷¹¹¹¹¹¹¹¹¹¹¹¹¹¹¹¹¹¹¹¹¹¹¹¹¹¹¹¹¹¹¹¹¹¹¹¹¹¹¹¹¹¹¹¹¹¹¹¹¹¹¹¹¹¹¹¹¹¹¹¹¹¹¹¹¹¹¹¹¹¹¹¹¹¹¹¹¹¹¹¹¹¹¹¹¹¹¹¹¹¹¹¹¹¹¹¹¹¹¹¹¹¹¹¹¹¹¹¹¹¹¹¹¹¹¹¹¹¹¹¹¹¹¹¹¹¹¹¹¹¹¹¹¹¹¹¹¹¹¹¹¹¹¹¹¹¹¹¹¹¹¹¹¹¹¹¹¹¹¹¹¹¹¹¹¹¹¹¹¹¹¹¹¹¹¹¹¹¹¹¹¹¹¹¹¹¹¹¹¹¹¹¹¹¹¹¹¹¹¹¹¹¹¹¹¹¹¹¹¹¹¹¹¹¹¹¹¹¹¹¹¹¹¹¹¹¹¹¹¹¹¹¹¹¹¹¹¹¹¹¹¹¹¹¹¹¹¹¹¹¹¹¹¹¹¹¹¹¹¹¹¹¹¹¹¹¹¹¹¹¹¹¹¹¹¹¹¹¹¹¹¹¹¹¹¹¹¹¹¹¹¹¹¹¹¹¹¹¹¹¹¹¹¹¹¹¹¹¹¹¹¹¹¹¹¹¹¹¹¹¹¹¹¹¹¹¹¹¹¹¹¹¹¹¹¹¹¹¹¹¹¹¹¹¹¹¹¹¹¹¹¹¹¹¹¹¹¹¹¹¹¹¹¹¹¹¹¹¹¹¹¹¹¹¹¹¹¹¹¹¹¹¹¹¹¹¹¹¹¹¹¹¹¹¹¹¹¹¹¹¹¹¹¹¹¹¹¹¹¹¹¹¹¹¹¹¹¹¹¹¹¹¹¹¹¹¹¹¹¹¹¹¹¹¹¹¹¹¹¹¹¹¹¹¹¹¹¹¹¹¹¹¹¹¹¹¹¹¹¹¹¹¹¹¹¹¹¹¹¹¹¹¹¹¹¹¹¹¹¹¹¹¹¹¹¹¹¹¹¹¹¹¹¹¹¹¹¹¹¹¹¹¹¹¹¹¹¹¹¹¹¹¹¹¹¹¹¹¹¹¹¹¹¹¹¹¹¹¹¹¹¹¹¹¹¹¹¹¹¹¹¹¹¹¹¹¹¹¹¹¹¹¹¹¹¹¹¹¹¹¹¹¹¹¹¹¹¹¹¹¹¹¹¹¹¹¹¹¹¹¹¹¹¹¹¹¹¹¹¹¹¹¹¹¹¹¹¹¹¹¹¹¹¹¹¹¹¹¹¹¹¹¹¹¹¹¹¹¹¹¹¹¹¹¹¹¹¹¹¹¹¹¹¹¹¹¹¹¹¹¹¹¹¹¹¹¹¹¹¹¹¹¹¹¹¹¹¹¹¹¹¹¹¹¹¹¹¹¹¹¹¹¹¹¹¹¹¹¹¹¹¹¹¹¹¹¹¹¹¹¹¹¹¹¹¹¹¹¹¹¹¹¹¹¹¹¹¹¹¹¹¹¹¹¹¹¹¹¹¹¹¹¹¹¹¹¹¹¹¹¹¹¹¹¹¹¹¹¹¹¹¹¹¹¹¹¹¹¹¹¹¹¹¹¹¹¹¹¹¹¹¹¹¹¹¹¹¹¹¹¹¹¹¹¹¹¹¹¹¹¹¹¹¹¹¹¹¹¹¹¹¹¹¹¹¹¹¹¹¹¹¹¹¹¹¹¹¹¹¹¹¹¹¹¹¹¹¹¹¹¹¹¹¹¹¹¹¹¹¹¹¹¹¹¹¹¹¹¹¹¹¹¹¹¹¹¹¹¹¹¹¹¹¹¹¹¹¹¹¹¹¹¹¹¹¹¹¹¹¹¹¹¹¹¹¹¹¹¹¹¹¹¹¹¹¹¹¹¹¹¹¹¹¹¹¹¹¹¹¹¹¹¹¹¹¹¹¹¹¹¹¹¹¹¹¹¹¹¹¹¹¹¹¹¹¹¹¹¹¹¹¹¹¹¹¹¹¹¹¹¹¹¹¹¹¹¹¹¹¹¹¹¹¹¹¹¹¹¹¹¹¹¹¹¹¹¹¹¹¹¹¹¹¹¹¹¹¹¹¹¹¹¹¹¹¹¹¹¹¹¹¹¹¹¹¹¹¹¹¹¹¹¹¹¹¹¹¹¹¹¹¹¹¹¹¹¹¹¹¹¹¹¹¹¹¹¹¹¹¹¹¹¹¹¹¹¹¹¹¹¹¹¹¹¹¹¹¹¹¹¹¹¹¹¹¹¹¹¹¹¹¹¹¹¹¹¹¹¹¹¹¹¹¹¹¹¹¹¹¹¹¹¹¹¹¹¹¹¹¹¹¹¹¹¹¹¹¹¹¹¹¹¹¹¹¹¹¹¹¹ wParam on October 2, 2017 from jpet.aspetjournals.org Downloaded using license agreement
neutralization per hour and normalized to tissue surface area (microequivalents per centimeter squared per hour).

**Northern Blot Analysis.** Northern blot analysis of total mRNA was performed as previously described (Clarke et al., 2004). Total RNA from murine duodenum was extracted using TRI-Reagent (Molecular Research Center, Cincinnati, OH), according to manufacturer's instructions. RNA was mixed with Glyoxal sample buffer (Bio-Whittaker Molecular Applications, Rockland, ME), separated by 1% agarose gel electrophoresis, and transferred to a Hybond-N membrane (Amersham Biosciences, Piscataway, NJ). The blot was probed using a digoxigenin (DIG)-labeled cDNA PCR products for mCLCA3 and the L32 ribosomal protein. For the mCLCA3 probe, the RT-PCR product was obtained using the sense and antisense oligonucleotide primers with the following sequences: 5'-GAAAGCTGCAAGGTAGAT-GAATC-3' and 5'-GACTGTTGATTTCTGCTG-3'. For the L32 probe, the RT-PCR product was obtained using the sense and anti-sense oligonucleotide primers with the following sequences: 5'-CATCTGTTTTACCGCATCATG-3' and 5'-AGCTCCCATACCGATGTTGG-3'. Radiographic density of bands was measured using a Kodak Imaging Station 2000MR (Eastman Kodak, Rochester, NY), and the expression level of mCLCA3 was normalized to L32.

**Histology.** Jejunal or cecal sections were fixed in buffered 2.5% glutaraldehyde/2.0% paraformaldehyde, embedded in paraffin for microtoming. Sections extending from the base to crypt mouth (three crypts/section/mouse). Cryostat sections were stained with either H&E or Alcian Blue, pH 2.5, with a periodic acid Schiff counterstain. Using an upright microscope (BX50WI; Olympus, Tokyo, Japan), histological sections were scanned at low power for longitudinal crypt cross-sections extending from the base to crypt mouth (three crypts/section/mouse). Crypts were photographed using a SensoScan digital camera (Cooke, Auburn Heights, NY) at 100× magnification (40× objective plus 2.5× photo eyepiece), and morphological measurements of the crypt lumen diameter, crypt diameter, or number of goblet cells/crypt were obtained using ImagePro Plus (Media Cybernetics, Carlsbad, CA).

**Intracellular pH Measurement of Villous Epithelium.** The method used for imaging villous epithelial cells in intact murine intestine has been previously described (Simpson et al., 2005). Briefly, WT litter mates of CF mice, i.e., cfr+/+, were sacrificed, and proximal duodenum was removed, opened longitudinally, and the mucosa was stripped of the underlying muscle layers. The intestinal segment was mounted apical side up on a horizontal perfusion chamber in which mucosal and serosal surfaces were independently bathed. The intestinal preparations were treated with indomethacin (1 μM, mucosal and serosal baths) and tetrodotoxin (0.1 μM, serosal bath) to minimize the effect of endogenous prostaglandins and neuronal tone, respectively (Bukhave and Rask-Madsen, 1980; Sheldon et al., 1989). Villi were immobilized under a fine nylon mesh and washed for 5 min with a solution containing 100 mM DL-dithiothreitol. The duodenum was incubated in the presence of 16 mM 2, 7'-bis-(2-carboxyethyl)-5-(and-6)-carboxyfluorescein acetoxymethyl ester (BCECF-AM) for 10 min on the apical side in an isethionate-bicarbonate Ringer solution (IBR) containing 140.0 mM NaCl, 55.0 mM Cl⁻, 55.0 mM isethionate, 25.0 mM HCO₃⁻, 5.2 mM KCl, 5.0 mM Na⁻⁻⁻ N-Tris methyl-2-aminoethanesulfonic acid, 4.8 mM glucose, 2.8 mM PO₄⁻⁻, 1.2 mM Ca²⁺, 1.2 mM Mg²⁺, 10.0 mM glucose, and 6.8 mM mannitol that was gassed with 95% O₂/5% CO₂ at 37°C, pH 7.4. The basolateral superfuse consisted of a Cl⁻ free IBR (Cl⁻ replaced with isethionate) gassed with 95% O₂/5% CO₂ at 37°C, pH 7.4, and contained 1 mM 5-(N-ethyl-n-isopropyl)-amiloride to block the activity of Na⁺/H⁺ exchanger isoform 1. During BCECF-AM incubation, duodenal villi were viewed at 100× magnification (40× water immersion objective plus 2.5× photo eyepiece), and 10 epithelial cells from the midregion of a single villus were selected for ratiometric analysis. Changes in intracellular pH (pHᵢ) were measured by the dual-excitation wavelength technique (440 and 495 nm), and the villi were imaged at a 535-nm emission wavelength. Ratio-metric images were acquired at 20-s intervals with a SensoCam digital camera (Cooke), and images were processed using Axon Imaging Workbench 2.2 (Axon Instruments, Union City, CA). The 495:440-nm ratios were converted to pHᵢ using a standard curve generated by the K'/nigericin technique (Thomas et al., 1979; Boyarsky et al., 1998). Experiments to measure Cl⁻/HCO₃⁻ exchange consisted of initial pHᵢ alkalization induced by replacement of apical Cl⁻ with isethionate on an equimolar basis. After a stable pHᵢ was obtained (approximately 2 min), pHᵢ recovery was initiated by replacing isethionate with Cl⁻, as previously described (Simpson et al., 2005). Rates of anion exchange during alkalization and recovery (ΔpH/Δt) were calculated from a linear regression of the values from the first 90 s of the initial pHᵢ changes during Cl⁻ removal and replacement, respectively. For studies of talniflumate treatment, the intestinal preparation was exposed to either 100 μM talniflumate or vehicle (0.4% dimethyl sulfoxide (DMSO)) in the apical bath for 5 min prior to the measurements of Cl⁻/HCO₃⁻ exchange activity.

**Materials.** Talniflumate (Lomucin) and mouse chow diets containing talniflumate, ibuprofen, or vehicle were generously provided by Genaera Corporation. The fluorescent dye BCECF-AM was obtained from Molecular Probes (Eugene, OR). Tetrodotoxin was obtained from Biomol International L.P. (Plymouth Meeting, PA). All other materials were obtained from either Sigma Aldrich (St. Louis, MO) or Fisher Scientific (Springfield, NJ). For drug treatment of the in vitro preparations, talniflumate was added from a 100 mM stock solution in DMSO, forskolin was added from a 10 mM stock in DMSO, and carbachol was added from a 100 mM stock in IBR.

**Statistics.** A Kaplan-Meier log-rank analysis with a post hoc Holm-Sidak test for all pair-wise comparisons was used to compare survival curves between treatment groups. A Student’s t test assuming equal variances was used to compare data for two treatment groups, and a one-way analysis of variance with a post hoc Bonferroni’s test was used to compare data from more than two treatment groups. p < 0.05 was considered statistically significant. All data are expressed as mean ± S.E.M.

**Results**

**Talniflumate But Not Ibuprofen Increases the Survival of CF Mice.** Mucus and debris accumulating within the intestinal crypts can fuse with luminal contents, resulting in an obstructing impaction and the fatal sequela of peritonitis in untreated CF mice (Snowaert et al., 1992). To test the efficacy of talniflumate in preventing fatal intestinal disease, CF mice initially treated with a PEG laxative in the drinking water were divided into three groups with different diets (control, talniflumate, and ibuprofen). The ibuprofen group was included because compounds of this class have known nonstereoidal anti-inflammatory activity (Insel, 1996), and ibuprofen has been shown to have beneficial effects in the treatment of CF patients (Konstan et al., 1995). The CF mice in the three groups consumed similar amounts of the diets (control, 0.22 ± 0.01; talniflumate, 0.22 ± 0.04; and ibuprofen, 0.15 ± 0.06 g/g body weight/day, N.S.), resulting in an average daily dose of 88 mg talniflumate/kg body weight or 60 mg ibuprofen/kg body weight. As shown in Fig. 1A, 14 of 19 (73.7%) CF mice on the control diet died during the 21-day study period, with greatest loss occurring between days 4 and 10. Of the CF mice consuming the ibuprofen diet, seven of nine (77.8%) died during the survival study period. Most CF mice on the ibuprofen diet died within 4 days (66%), suggesting an additional untoward effect of the ibuprofen; however, the greatest loss in the control group also occurred early in the study (42.1% loss within 6 days), and differences between the two survival curves were not statistically significant. In contrast to the control and ibuprofen groups, only 3 of 13 (23.1%) CF mice consuming the talniflumate diet died.
during the 21-day period. The survival for CF mice consuming the talniflumate diet was significantly increased compared with that for CF mice consuming either the control or ibuprofen diets.

Withdrawal of the PEG laxative from the CF mice results in the highest mortality rates during the first few days (<10 days) after the switch to untreated drinking water. We questioned whether the beneficial effects of talniflumate would also be observed in CF mice that were subjected to a less severe disease insult. Preliminary studies indicated that dilution of PEG laxative to 70% of full-strength yields an approximately 50% mortality rate in untreated CF mice (Walker et al., 2004). Because the greatest mortality occurred within the first 2 weeks after PEG withdrawal (see Fig. 1A), the 14-day survival of CF mice maintained on 70% PEG laxative in the drinking water was compared between mice consuming either the control or talniflumate diets. As shown in Fig. 1B, all CF mice on the talniflumate diet survived, whereas 37.5% of the CF mice consuming the control diet died during the 14-day study period.

**Talniflumate Treatment Does Not Significantly Affect Goblet Cell Numbers or Mucus Impaction in the Intestinal Crypts of CF Mice.** It is well documented that the CF mouse intestine recapitulates the histopathological appearance of intestinal disease in CF patients, which is hallmarked by goblet cell hyperplasia and mucus impaction of the crypts (Grubb and Gabriel, 1997). Previous investigations in cell culture systems and animal disease models have shown that talniflumate treatment can significantly decrease rates of mucus production (Melton, 2002; Zhou et al., 2002). Therefore, we evaluated goblet cell numbers and the ratio of crypt lumen diameter to crypt diameter (as a measure of mucus impaction of the intestinal crypts) in CF mice treated with either the talniflumate or control diets. Because water consumption often decreases for 1 to 2 days after switching from PEG-containing drinking water to tap water (data not shown), the CF mice were maintained on 70% PEG laxative in the drinking water and consumed the test diets for a 7- to 10-day period. At the conclusion of the study, the CF mice were sacrificed, and intestinal samples were taken for histological examination. As shown by the cumulative data in Fig. 2A, goblet cell numbers in the crypts of either the small intestine (jejunum, left panel) or large intestine (cecum, right panel) were not significantly different between the CF mice consuming either the talniflumate or control diets. As a measure of small bowel crypt impaction, the ratio of jejunal crypt lumen diameter/crypt diameter in the two groups of CF mice was measured as shown diagrammatically in Fig. 2B, right panel. The cumulative data from these measurements (Fig. 2B, left panel) indicated that the mean crypt lumen/crypt diameter ratio for the talniflumate group was slightly, although nonsignificantly, reduced compared with that in CF mice on the control diet. Thus, we cannot rule out the possibility that talniflumate has a beneficial effect on reducing crypt impaction in CF mice.

**Talniflumate Treatment Does Not Alter the mRNA Expression of mCLCA3.** Previous studies of talniflumate and its parent compound niflumic acid have provided evidence that these agents inhibit the Cl̂ channel activity of mCLCA3, a reported Ca²⁺-activated channel, which has been associated with changes in mucin production in model systems (Melton, 2002; Toda et al., 2002). We asked whether daily treatment with talniflumate reduces the expression of mCLCA3, which might have a beneficial effect on mucus production in the CF mice. In these studies, the mice were treated with 70% PEG drinking water and either the control or talniflumate diet. After a 1-week treatment period, the CF mice were sacrificed, and intestinal samples were taken for Northern blot analysis. As shown by the Northern blot in Fig. 3A and the densitometry measurements in Fig. 3B, talniflumate treatment did not alter the mRNA expression of mCLCA3 in the intestine of the CF mice.

**Talniflumate Treatment Has Minimal Effects on Electrogenic Ion Transport Processes across the CF Mouse Small Intestine Ex Vivo.** Based on the known properties of talniflumate and niflumic acid as Cl̂ channel blockers (Bertrand et al., 2004), we investigated whether electronegative processes of ion transport across the CF intestine, as indexed by changes in the Isc, were altered by feeding the talniflumate diet. In these studies, the CF mice...
were maintained on 70% PEG laxative in the drinking water and provided either the control or talniflumate diet for 1 week. At the end of the treatment period, the CF mice were sacrificed, and intestinal sections were immediately removed for mounting in Ussing chambers. The only difference in bioelectrical parameters that was noted was a slight but statistically significant increase in the Isc of the talniflumate-treated group (Fig. 4, left panel). However, the magnitude of this current (\( \mu \text{A/cm}^2 \)) was not sufficient to increase the basal Isc in the CF intestine to a level equivalent with the basal Isc of WT intestine measured in this laboratory (dashed line, Fig. 4, left panel; from Gawenis et al., 2003). The basal Isc of the small intestine from both groups of CF mice was not affected by treatment with bumetanide, an inhibitor of Cl\(^-\) secretion that blocks the Na\(^+\)/K\(^+\)/2 Cl\(^-\) cotransporter at the basolateral membrane of the epithelium (data not shown). Investigation of second messenger regulation of the Isc did not indicate that the Isc of CF mice on either the control or talniflumate diets was stimulated by a cAMPi agonist (forskolin) or a Ca\(^{2+}\)-mobilizing agonist (carbachol) (see Fig. 4, left panel). To determine whether talniflumate treatment affects processes of Na\(^+\) absorption across the small intestine, we examined the Isc response induced by the addition of 10 mM glucose to the mucosal bath. Transepithelial resistances were not different between control and talniflumate intestine (data not shown). *, significantly different from control.
ex vivo studies (control G<sub>f</sub>, 25.1 ± 2.6 versus talniflumate
G<sub>f</sub>, 29.5 ± 3.7 mS/cm<sup>2</sup>, n = 18).

**Talniflumate Significantly Inhibits Cl<sup>-</sup>/HCO<sub>3</sub><sup>-</sup>
Activity in Murine Intestinal Epithelium in Vitro.** At least two
members of the Slc26a family of sulfate transporters (Slc26a3 and
Slc26a6) provide Cl<sup>-</sup>/HCO<sub>3</sub><sup>-</sup> exchange function
at the apical membrane of intestinal epithelia (Jacob et al.,
2002; Wang et al., 2005). These anion exchangers provide
important functions of bicarbonate secretion and Cl<sup>-</sup>
absorption across the intestine. For example, loss of function
mutations in Slc26a3 (alias down-regulated in adenoma) cause
the human disease congenital chloride diarrhea (CLD), which is
characterized by copious acidic diarrhea resulting
from reduced NaCl absorption across the intestine (Mount
and Romero, 2004). Studies of recombinant proteins have
shown that the activity of the Slc26a exchangers is signifi-
cantly inhibited by niflumic acid (Chernova et al., 2003,
2005), and, recently, we have shown that niflumic acid inhib-
its ~60% of the basal Cl<sup>-</sup>/HCO<sub>3</sub><sup>-</sup> exchange activity in native
intestinal epithelium from both WT and CF mice (Simpson et
al., 2005). To determine whether this effect of niflumic acid is
physiologically relevant, we performed ex vivo pH stat mea-
surements of net HCO<sub>3</sub><sup>-</sup> secretion across native murine
small intestine and found that 100 μM niflumic acid
decreased net HCO<sub>3</sub><sup>-</sup> secretion by 62.6% (net HCO<sub>3</sub><sup>-</sup>
secretory flux (in microequivulants per centimeter squared per hour):
control = 2.3 ± 0.2; niflumic acid = 0.9 ± 0.2, n = 11, p < 0.05).

Because niflumic acid significantly inhibits apical mem-
brane Cl<sup>-</sup>/HCO<sub>3</sub><sup>-</sup> exchange in both WT and CF mouse
intestinal epithelium (Simpson et al., 2005), we asked whether its phthalate
derivative talniflumate also inhibits Cl<sup>-</sup>/HCO<sub>3</sub><sup>-</sup> exchange
activity in the murine small intestine. Using WT villous
epithelium, microfluorometry studies show that robust Cl<sup>-</sup>/
HCO<sub>3</sub><sup>-</sup> exchange activity is present at the apical membrane
of the villous epithelium. As demonstrated in Fig. 5A, the
epithelial cells alkalize when Cl<sup>-</sup> is removed from the lumi-
nal bath because the Cl<sup>-</sup>/HCO<sub>3</sub><sup>-</sup> exchanger(s) operate in the
“reverse” mode, i.e., Cl<sup>-</sup><sub>out</sub>/HCO<sub>3</sub><sup>-</sup><sub>in</sub> exchange. After readdi-
tion of luminal Cl<sup>-</sup>, the cells acidify as the exchanger(s) operate in the “forward” mode, i.e., Cl<sup>-</sup><sub>in</sub>/HCO<sub>3</sub><sup>-</sup><sub>out</sub>, and the rate of Cl<sup>-</sup>/HCO<sub>3</sub><sup>-</sup> exchange is measured during the initial linear
phase of pH<sub>i</sub> changes. To evaluate the effect of talni-
flumate on this transport process, WT intestinal epithelium
was treated with either 100 μM talniflumate or vehicle (0.4%
DMSO) for approximately 5 min before measuring Cl<sup>-</sup>/
HCO<sub>3</sub><sup>-</sup> exchange activity by Cl<sup>-</sup> removal and replacement.
As shown in Fig. 5B, talniflumate significantly inhibited the
rate of Cl<sup>-</sup>/HCO<sub>3</sub><sup>-</sup> exchange by ~55% compared with vehicle
control. Thus, talniflumate, like niflumic acid, inhibits the
complement of apical membrane anion exchangers in the
murine intestinal epithelium.

**Discussion**

The major finding of the present study was that oral talni-
flumate treatment significantly increased survival of CF
mice by reducing the occurrence of obstructing intestinal
impactions after withdrawal of the PEG laxative. At the end
of the 21-day study period, 76.9% of the talniflumate-treated
group survived, whereas 26.3% of the control group survived
PEG withdrawal. All CF mice that died spontaneously or
that were sacrificed when moribund had intestinal impa-
tions in the distal small bowel and/or ileocecal region with
variable degrees of bowel necrosis and peritonitis. Overt
signs of toxicity were not observed in mice receiving oral
talniflumate either with or without simultaneous treatment
with PEG laxative. The survival rate of the untreated control
group was somewhat higher than reported in previous stud-
ies of CFTR knockout and ΔF508 CFTR mouse models (Snou-
waert et al., 1992; Davidson and Dorin, 2001). This difference
may be due to a beneficial effect of the pulverized diet be-
cause previous studies have shown high rates of survival for
CF mice consuming liquid diets, e.g., Peptamen (Eckman et
al., 1992). Another important control in the survival studies
was the group of CF mice consuming the ibuprofen diet.
Compounds of the class containing talniflumate and niflumic
acid have known nonsteroidal anti-inflammatory properties
(Insel, 1996). Ibuprofen, a member of this class of drugs, was
chosen because it is has been shown to have beneficial effects
in the treatment of CF (Konstan et al., 1995). However, the
survival rate of CF mice consuming the ibuprofen diet
(22.2%) was similar to that of CF mice on the control diet.
A modest (nonsignificant) trend toward lower daily consump-
tion of the ibuprofen diet was noted, suggesting a toxic effect
of the diet. However, all CF mice in the ibuprofen group that
died during the survival study had intestinal impactions, and
neither survivors nor impacted animals on the ibuprofen diet
were observed with mucosal ulceration or erosions in the upper gastrointestinal tract at necropsy. Taken together, the poor survival of the CF mice consuming the ibuprofen diet suggests that the beneficial effect of the talniflumate diet does not result from the nonsteroidal anti-inflammatory properties of talniflumate.

Survival studies of CF mice consuming either the talniflumate or control diets while simultaneously being treated with 70% PEG laxative in the drinking water were used to control for confounding factors associated with abrupt PEG withdrawal. For example, we had noted that water consumption often decreased for 1 to 2 days immediately after switching from PEG-containing drinking water to tap water (data not shown). Preliminary studies indicated that dilution of the PEG laxative to 70% of full strength resulted in an approximately 50% effective concentration with respect to CF mouse survival. Using 70% PEG laxative in the drinking water, survival of CF mice consuming the control diet was 62.5%, whereas 100% of CF mice consuming the talniflumate diet survived. Thus, talniflumate treatment appeared to have a beneficial effect even when disease severity was lessened by simultaneous laxative treatment.

Goblet cell metaplasia and mucus overproduction are fundamental to the pathogenesis of cystic fibrosis (Welsh et al., 1995). When tested in cell culture systems and animal models of airway disease, talniflumate and its parent compound niflumic acid have been shown to inhibit mucus production (Melton, 2002; Zhou et al., 2002; Bertrand et al., 2004). Investigations into the mechanism of action have largely focused on the ability of these compounds to block activity of the calcium-activated Cl− channels, hCLCA1 and mCLCA3 (Gandhi et al., 1998; Gruber et al., 1998). These proteins show a strong positive correlation with mucus overproduction in the lungs of interleukin (IL)-9 transgenic mice and in human primary lung cultures treated with IL-4, IL-9, and IL-13 (Toda et al., 2002; Hauber et al., 2003; M. McLane, K. J. Holroyd, and R. C. Levitt, unpublished data). Moreover, expression of hCLCA1 in NCI-H292 cells induces soluble gel-forming mucin production, which is inhibited by niflumic acid treatment (Zhou et al., 2002). Despite the beneficial effects of niflumic acid on these models of mucus production, the role of hCLCA1 Cl− channel activity in the process of mucus production remains unexplained. Recently, studies of calcium-stimulated granule exocytosis in mucin-secreting cells indicate that niflumic acid interferes with Ca2+ influx across the plasma membrane, a process that initiates mucin granule release (Bertrand et al., 2004). Based on evidence that talniflumate may reduce mucus production in the intestine, we examined goblet cell numbers, mCLCA3 expression, and the ratio of crypt lumen diameter/crypt diameter in mice consuming the talniflumate diet and treated with 70% PEG laxative in the drinking water (to avoid spurious effects of impending impaction). However, talniflumate treatment did not result in any additional effects beyond that provided by the 70% PEG laxative treatment. The only positive indicator was a trend toward a reduction of crypt lumen diameter, which is an indirect measure of mucus impaction of the crypt. Thus, the evidence from these basic measurements of intestinal mucus production was not consistent with a major effect of talniflumate on mucus overproduction in the CF intestine, but a beneficial effect of talniflumate cannot be ruled out because changes in mucus production may not be detectable by the methods used or the effect of talniflumate may overlap with the effects of simultaneous PEG laxative treatment.

The transepithelial bioelectric parameters of the intestine from CF mice consuming the talniflumate or control diets, in the presence of 70% PEG laxative, were compared ex vivo in Ussing chamber studies. Talniflumate treatment did not have an appreciable effect on the integrity of the intestine or the paracellular fluid loss (Kere et al., 1999). It has been shown in individuals affected with cystic fibrosis that intestinal Cl− absorption can result in severe diarrheal fluid loss (Kere et al., 1999). It has been shown in heterologous expression studies that SLC26A3 and SLC26A6 are inhibited by the action of niflumic acid (Chernova et al., 2003, 2005). Furthermore, it has been proposed that pharmacological inhibition of the SLC26A transporters in the intestine is a potential therapy for the clinical impaction states that characterize CF intestinal disease (Chernova et al., 2003). Using microspectrofluorometry, we have recently shown that niflumic acid inhibits Cl−/HCO3− exchange by 60 to 65% in intact intestinal epithelia from both WT and CFTR-null mice (Simpson et al., 2005). Therefore, using the same technique, we investigated the effect of luminal applied talniflumate on Cl−/HCO3− exchange across the apical membrane of villous epithelium in the WT murine duodenum, a site where both SLC26A3 and SLC26A6 are expressed (Jacob et al., 2002; Wang et al., 2002). Talniflumate resulted in >50% reduction in the rate of Cl−/HCO3− exchange across the apical membrane of the intestine. These results are consistent with the proposal that talniflumate has a beneficial effect on the survival of CF mice by reducing the incidence of intestinal impactions through pharmacological inhibition of intestinal Cl− absorption. A diagram depicting the beneficial effect of Cl−/HCO3− exchange inhibition in the CF intestine is shown in Fig. 6. In the absence of anion secretion in the CF
Reduced Luminal Fluidity in CF

A.

Net NaCl + Water Absorption

B.

Increased Luminal Fluidity

Fig. 6. Schematic diagram depicting the beneficial effects of Cl-/HCO₃⁻ exchange inhibition in the treatment of distal intestinal obstructive syndrome in CF patients. A, in CF patients, net NaCl and water secretion are deficient due to the absence of the CFTR anion channel activity and the process of NaCl absorption via coupled Na⁺/H⁺, Cl⁻/HCO₃⁻ exchangers (Na⁺/H⁺ exchanger isomorph 3 and AE, respectively) dominates in much of the intestine, thereby contributing to dehydration of the luminal content. B, inhibition of apical membrane Cl⁻/HCO₃⁻ exchanger(s) reduces intestinal Cl⁻ absorption and Na⁺ absorption (Kere et al., 1999), resulting in the retention of NaCl and water in the lumen that increases the fluidity of the luminal content.

References


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