The Na^+/H^+ exchanger NHE3 inhibitor tenapanor prevents intestinal obstructions in CFTR-deleted mice

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Abstract

Background and purpose: Mutations in the CFTR chloride channel result in intestinal o-structive episodes in cystic fibrosis (CF) patients and in CF animal models. This study explores the possibility of reducing the frequency of obstructive episodes in the *Cftr*$^{-/-}$ mice by the oral application of a gut selective NHE3 inhibitor tenapanor and searches for the underlying mechanisms involved. Experimental approach: Sex and age-matched *Cftr*$^{+/+}$ and *Cftr*$^{-/-}$ mice were orally gavaged twice daily with 30mgkg^{-1} tenapanor or vehicle for a period of 21 days. Body weight and stool water content was assessed daily and gastrointestinal transit time (GTT) once weekly. The mice were sacrificed when an intestinal obstruction was suspected or after 21 days, and stool and tissues were collected for further analysis. Key results: 21 day tenapanor application resulted in a significant increase in stool water content, stool alkalinity, and a significant decrease in GTT in *Cftr*$^{+/+}$ and *Cftr*$^{-/-}$ mice. Tenapanor significantly reduced obstructive episodes to 8% compared to 46% in vehicle treated *Cftr*$^{-/-}$ mice and prevented mucosal inflammation. A decrease in cryptal hyperproliferation, mucus accumulation and mucosal mast cell number was also observed in tenapanor compared to vehicle treated unobstructed *Cftr*$^{-/-}$ mice. Conclusion and implications: Oral tenapanor application prevented obstructive episodes in CFTR deficient mice and was safe in *Cftr*$^{+/+}$ and *Cftr*$^{-/-}$ mice. These results suggest that tenapanor may be a safe and affordable adjunctive therapy in cystic fibrosis patients to alleviate constipation and prevent recurrent DIOS.

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Running title: Tenapanor prevents intestinal obstruction in CF mice

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Data availability: The original data are available from the authors upon reasonable request.

Bullet points:

What is already known :

NHE3 inhibitor tenapanor increases luminal fluidity and alkalinity in *Cftr* $^{-/-}$ mice during singlepass intestinal perfusion studies.

What this study adds:

3 week oral tenapanor application normalized intestinal transit time, reduced obstructive episodes, and increased survival.

It also reversed crypt-villus elongation, reduced mucus accumulation and prevented intestinal inflammation.

Clinical significance:

Tenapanor maybe a safe and affordable adjunctive therapy in cystic fibrosis patients to alleviate constipation and prevent recurrent DIOS

Abstract

Background and purpose : Mutations in the CFTR chloride channel results in intestinal obstructive episodes in cystic fibrosis (CF) patients and in CF animal models. This study explores the possibility of reducing the frequency of obstructive episodes in the *Cftr* $^{-/-}$ mice by the oral application of a gut selective NHE3 inhibitor tenapanor and searches for the underlying mechanisms involved.

Experimental approach: Sex and age-matched *Cftr* $^{+/+}$ and *Cftr* $^{-/-}$ mice were orally gavaged twice daily with 30mg kg^{-1} tenapanor or vehicle for a period of 21 days. Body weight and stool water content was assessed daily and gastrointestinal transit time (GTT) once weekly. The mice were sacrificed when an intestinal obstruction was suspected or after 21 days, and stool and tissues were collected for further analysis.

Key results: 21 day tenapanor application resulted in a significant increase in stool water content, stool alkalinity, and a significant decrease in GTT in *Cftr* $^{+/+}$ and *Cftr* $^{-/-}$ mice. Tenapanor significantly reduced obstructive episodes to 8% compared to 46% in vehicle treated *Cftr* $^{-/-}$ mice and prevented mucosal inflammation. A decrease in cryptal hyperproliferation, mucus accumulation and mucosal mast cell number was also observed in tenapanor compared to vehicle treated unobstructed *Cftr* $^{-/-}$ mice.

Conclusion and implications: Oral tenapanor application prevented obstructive episodes in CFTR deficient mice and was safe in *Cftr* $^{+/+}$ and *Cftr* $^{-/-}$ mice. These results suggest that tenapanor may be a safe and affordable adjunctive therapy in cystic fibrosis patients to alleviate constipation and prevent recurrent DIOS.

Key words: mucoviscidosis, DIOS, mucus, intestinal fluid absorption, sodium hydrogen exchange, chloride channel

Introduction

Cystic fibrosis (CF) patients develop intestinal obstructions, called "meconium ileus" in newborn and "distal intestinal obstructive syndrome" (DIOS) in juveniles and adults. Radiologic signs and clinical symptoms are that of mechanical ileus. Surgery is to be avoided, if at all possible, and conservative treatment consists of orally and rectally applied Gastrografin (meglumine diatrizoate), polyethylene glycol (PEG) lavage (20%), and oral laxatives and enemas (Houwen et al., 2010). Nevertheless, the need for surgery is high, the incidence of obstruction related segmental colectomies is increasing, with high morbidity and mortality (Hite et al., 2022; Sharma, Morton, Peckham & Jayne, 2012). Recent Cochrane analyses revealed a clear lack of evidence

for preventive as well as therapeutic treatment strategies, and suggested that randomised controlled trials need to be conducted (Carroll, Green & Gilchrist, 2021; Gilchrist, Green & Carroll, 2021).

A recent survey revealed a high rate of abdominal complaints with a likely origin in the lower gut in CF patients not suffering from recurrent DIOS episodes (Hayee et al., 2019). Other studies demonstrated a correlation between abdominal symptoms and an increase of inflammatory markers in the stool (Beaufils et al., 2020; Jaudszus et al., 2022), undermining the hypothesis derived from CF animal models that inflammation, secondary to bacterial overgrowth, predisposes to obstructive episodes, disease severity, and abdominal complications (De Lisle, 2007; Dorsey & Gonska, 2017; Talebi et al., 2022). However, this hypothesis has not been validated in animal models or CF patients.

It has also been shown that the treatment with CF modulators and correctors reduces CF-associated inflammation (Tétard et al., 2020). Nevertheless, many of the patients having undergone surgery for DIOS were already on CF corrector therapy, which is also not available for all CF mutations. This suggests that additional intestine targeted therapies that improve the sequelae of CFTR dysfunction, namely the reduced gut fluidity, high luminal acidity, mucus hyper-viscosity, increased transit time, microbial dysbiosis and mucosal inflammation and thereby hopefully reduce obstructive episodes in the intestine, are urgently needed. Because it is obviously difficult to perform double-blind placebo controlled studies in this highly compromised group of patients, it is important to carefully test pharmacological strategies to alleviate CF related intestinal disease in suitable animal models.

All currently available CF animal models display a very high incidence of intestinal obstruction, leading to death unless prevented by surgical, genetic or pharmacological rescue (Borowitz & Gelfond, 2013; Clarke, Gawenis, Franklin & Harline, 1996; Rogers et al., 2008; Stoltz et al., 2013). *Cftr* $^{-/-}$ mice do not display pancreatic or pulmonary disease, but consistently develop intestinal obstructive episodes. Hence, we considered this model ideal to test intestine-targeted drug therapies that have been approved for the treatment of constipation-prone irritable bowel disease and are believed to increase epithelial fluid secretion, decrease fluid absorption, or both.

In a previous report, we had examined the efficacy of the guanylate cyclase 2C ligand linaclotide, prostaglandin E1 analogue lubiprostone, and intestine-specific Na^+/H^+exchanger isoform 3 (NHE3) inhibitor tenapanor on jejunal and colonic fluid balance and alkaline output in anesthetized both *Cftr* $^{-/-}$ mice and in F508del mutant mice*in vivo* , titrating each drug to the minimal concentration that elicited a significant increase in gut fluidity and alkalinity (Tan et al., 2021). Although each of the drugs was able to increase jejunal alkaline output and reduce jejunal fluid absorption, linaclotide had no effect on the colon, and lubiprostone needed to be applied in much higher concentrations than were likely present in the gut lumen with the approved dosages for humans, suggesting that the anti-constipation effect may be due to an effect on motility rather than fluid balance. Tenapanor was able to decrease fluid absorption and increase alkaline output with similar efficacy in *Cftr* $^{-/-}$ and*Cftr* $^{+/+}$ mice, and did so in concentrations that were reasonably close to what can be expected in the gut lumen with the approved dosage for humans. We therefore chose tenapanor to test its efficacy to prevent obstructive episodes in*Cftr* $^{-/-}$ mice when given by oral gavage for 21 days. We also aimed to explore the effect of tenapanor on the hallmarks of CF intestinal dysfunction, namely the reduced gut fluidity and alkalinity, mucus accumulation, cryptal hyperproliferation and villus elongation, slowed gastrointestinal transit time, and mucosal inflammation.

MATERIALS AND METHODS

Animals and experimental protocol

All experiments were performed with wild type mice ($Cftr^{+/+}$) and CFTR null mice ($Cftr^{-/-}$) (FVB/N-CFTRtm1CAM) (Ratcliff et al., 1993). Both$Cftr^{+/+}$ and $Cftr^{-/-}$mice were bred and maintained at Hannover Medical School as previously described (Xiao et al., 2012), except for the use of an energy-rich diet for transgene animals (1414MOD141003, Altromin, Lage, Germany) instead the low-fiber diet (C1013, Altromin, Lage, Germany), after the mice had survived the first 2 months of age. *Cftr* $^{-/-}$and their respective wild type littermates were co-housed (unless the males need to be separated due to fighting), and received identical

diet (1414MOD141003, Altromin, Lage, Germany) and the osmotic laxative drinking solution (Oralav, 40 mmol $l^{-1}Na_2SO_4$, 75 mmol $l^{-1}NaHCO_3$, 10 mmol l^{-1} NaCl, 10 KCl, 23 g l^{-1} PEG 4000) to prevent obstruction and enhance survival post weaning.

The experimental protocol was performed in age-matched mice (9-13 weeks) with similar percentage of males and females. Post withdrawal of Oralav, *Cftr* $^{+/+}$ and *Cftr*$^{-/-}$mice were intragastrically gavaged twice daily (with a gap of 10-12 hours) with either the vehicle (PBS, pH 6.0) or with Tenapanor (30mg kg^{-1}) for 21 days. Mice were assigned into the vehicle or Tenapanor treated group randomly. The mice were monitored daily and sacrificed either at the end of the experiment or if obstruction was suspected. Mice were sacrificed when a suspicion of obstruction was present over two consecutive observation periods (1 observation period every 12 hours, lasting 12 hours). Suspicion of obstruction was present when the mice had a prominent abdomen, a hunched body composure, no passage of feces during one 12 hour observation period, or a score of 3 or higher in the mouse wellbeing score. The severity and scoring was assessed based on a published severity assessment system which is recommended by our institute for animal research (Bleich & Tolba, 2017). The abdomen was opened, the intestine was dissected, photographed and the site of obstruction was verified. Post sacrifice, the intestinal tissues were harvested for RT-PCR and histology.

The experimental protocol was approved by the Hannover Medical School Committee and an independent committee assembled by the local government. Animal studies are reported in accordance with the ARRIVE guidelines 2.0 and the guidelines from the British Journal of Pharmacology (Percie du Sert et al., 2020). The application and permission numbers are Az. 33.14-42502-04-14/1549 and Az. 33.12-42502-04-19/3197 for breeding "stressed strains" and Az. 33.9-42502-04-18/2829 for experimental procedures.

Whole GI transit time

The total GI transit time (GTT) using Carmine red stained food pellets was measured once per week as previously described with a few modifications (Kini et al., 2022). Briefly, the mice were fasted overnight (approximately 8 hours) and were placed in a clean transparent empty cage prior to the first gavage of the day. They were then fed the red pellets. The total GI transit time was measured as the interval between the first food intake until the appearance of the first red stool pellet.

Stool water content

Stool water content was measured every morning prior to gavage as previously described (Kini et al., 2020). Stool pellets were also collected every hour for 5 hours during the GI transit time measurement.

Stool pH measurement

Luminal stool pH was measured in *Cftr* $^{+/+}$ and*Cftr* $^{-/-}$ mice pre and 3 hours post gavage with Tenapanor. One to two fresh stool pellets were collected, placed in 50µl unbuffered distilled water and the pH determined with a pH-strip (Carl Roth, Karlsruhe, Germany). Following this, the pellets were homogenized and the pH was measured via a pre-calibrated TitraLab TIM 865 pH meter (Hach, Düsseldorf, Germany) with a Sension+ 5209 pH electrode (Hach, Düsseldorf, Germany).

Histology and immunohistochemistry

Dissected intestinal segments (jejunum, ileum,colon) were fixed in 4% paraformaldehyde and embedded in paraffin according to standard procedures. 3µm thick sections were stained with Hematoxylin and Eosin (HE) and basic histological parameters such as crypt-villus length, crypt depth and muscle layer thickness were assessed (Alshamy, Richardson, Hünigen, Hafez, Plendl & Al Masri, 2018). Crypt-villus length was defined as the distance from the crypt base to the apex of the villus in the jejunum and ileum. Crypt depth was measured from the basement of crypt to the surface epithelium in the proximal and mid-distal colon. Three to eight villi or crypts were measured and averaged for every section. Muscle layer thickness was measured from serosa to inner muscularis mucosae. Intestinal sections were also stained with Toluidine blue to detect mast cells numbers (Norkina, Kaur, Ziemer & De Lisle, 2004). Images were acquired under the

Leica DFC295 light microscope. Mast cells were then counted in five different sections per segment. The results are expressed as cells per mm^2 (image field area).

The dissected intestinal segments were processed for immunohistochemical (IHC) staining as previously described (Kini et al., 2020). The sections were incubated overnight at 4°C with primary antibodies to Muc2 (1:200, Santa Cruz Biotechnology, Heidelberg, Germany, Cat#sc-15334, RRID: AB_2146667) and MPO (1:250, Proteintech, Planegg-Matinsried, Germany, Cat# 22225-1-AP, RRID:AB_2879037). It was then incubated with the corresponding secondary antibody (Goat anti-rabbit Alexa 568; 1:500, Thermofisher, Waltham, MA, USA, Cat #A-11036, RRID AB_10563566) and DAPI to detect the nuclei. The slides were mounted with Mowiol 4-88 and images were acquired on the Olympus FluoView FV1000 confocal microscope. Muc2 was quantified as per previously described with a few modifications (Honda et al., 2017). Briefly, the intensity of green fluorescence indicating MUC2 stained mucins were quantified using Image J software. A region of interest (ROI) was determined (Supplementary Figure 1a) individually for intracellular and secreted mucins. Followed by normalizing the detected area with the background, the integrated density (I.D) ratio of Muc2 to Dapi was calculated for total, as well as for the intracellular and secreted mucins. The additive Muc2 to Dapi ratio of the intracellular and secreted mucins represents the total Muc2. MPO positive cells were counted by eye in image taken at 40X magnification in five different sections per segment. The results are expressed as cells per mm^2 (image field area). .

Quantitative PCR

Isolation of RNA, cDNA conversion and quantitative real-time PCR from the various intestinal segments (jejunum, ileum, proximal colon and mid-distal colon) were done as per manufacturer's instructions and as previously described (Tan et al., 2021). Ribosomal protein S9 (RPS9) was used as housekeeping gene. The list of primers used can be found in Supplementary Table 1. The total RNA was extracted with the RNeasy(r) Mini Kit (Qiagen GmbH, Hilden, Germany) based on the manufacturer's instructions. 1µg RNA was reverse transcribed using the QuantiTect® Reverse Transcription Kit (Qiagen GmbH, Hilden, Germany). cDNA was then diluted 1:20 with DNase free water, and 4µL of the dilution was used as a template for PCR. Each reaction contained 5µL qPCRBIO SyGreen Mix Lo-ROX (PCR Biosystems, Dueren, Germany), 4 µl template and an appropriate amount of primers.

Data and statistical analysis

The study design included a priori power analysis to determine the sample size for each experiment/subgroup. We used data from prior/similar experiments to determine the effect sizes and SDs. These were used in a power analysis with the software G*Power (Faul, Erdfelder, Lang & Buchner). To determine the necessary sample sizes, we assumed a type I error of $\alpha = 5\%$ and a statistical power of at least 80%. Mice were allocated to the experimental groups by a different person than the experimenter and this person also regenotyped the mice after killing and allocated the genotype to the mouse number. A fully blinded experiment is not possible, because the experienced experimenter has a high chance of correctly guessing the genotype based on the different phenotypes of knockout and wild-type mice. Sample sizes were calculated as biological replicates and were allocated equally to the experimental groups. Studies were designed to generate groups of equal size; however, accidental deaths (spontaneous or euthanasia due to disease) occurred during the preparatory period (transfer of the mice after group assignment to an animal experiment laboratory, one week obligatory acclimatization, removal of PEG laxative at day 1 of the experimental period), resulting in slightly uneven numbers in the *Cftr*$^{-/-}$ groups. The data were analyzed with GraphPad Prism Version 8.0.2, adhering to the British Journal of Pharmacology's recommendations for experimental design and analysis in pharmacology (Curtis et al., 2018). All results are presented as mean ± SEM. The body weight, stool water content and GI transit time are averaged for each time point. Unpaired Students't-test (for parametric data with normal distribution) or non-parametric Mann-Whitney U-test was used for the comparisons between vehicle and tenapanor treated groups and/or within genotype. Area under the curve was used to analyze the daily body weight and stool water content. Obstruction and survival curve was analysed by the Kaplan-Meier log-rank test. For multiple comparisons between the genotypes, the one-way ANOVA (for parametric data with normal distribution) was used with the Bonferroni post hoc analysis only if the F value of the ANOVA

reached significance. If the normality test failed with one or both groups, the non-parametric Kruskal-Wallis test was performed. Significant differences are indicated as: # and *p < 0.05.

Materials

Tenapanor was purchased from Adooq Bioscience (Irvine, CA, USA). Carmine red was from SERVA Electrophoresis (Heidelberg, Germany). Mayer's Hematoxylin Solution, Eosin Y Solution and Toluidine blue were all from Sigma-Aldrich (Deisenhofen, Germany).

RESULTS

Increased stool water content, more alkaline stool pH, but no difference in body weight between vehicle and tenapanor treated mice.

Cftr$^{+/+}$ and *Cftr* $^{-/-}$mice were intragastrically gavaged with either vehicle (1X PBS, pH 6.0) or tenapanor (30mg kg^{-1}) twice a day for 21 days in the absence of oral PEG-containing laxative (Figure 1a). Weight and stool water content was recorded daily. Body weight was slightly higher in the *Cftr*$^{+/+}$ group due to a higher number of males (Figure 1b). It was stable during the treatment period during the 3week period in the *Cftr*$^{+/+}$ group and slightly decreased in the *Cftr*$^{-/-}$ group (possibly due to the stress related to the gavage), but was not different between tenapanor- and vehicle-treated mice in either group (Figure 1c,d).

A significantly higher stool water content was observed in the tenapanor-treated *Cftr*$^{+/+}$ and *Cftr*$^{-/-}$ throughout the gavage period (Figure 1e,f). The vehicle treated *Cftr*$^{-/-}$ mice displayed a decrease of stool water content post removal of Oralav (Figure 1f). This was less prominent in the *Cftr*$^{+/+}$ mice (Figure1e). In a subset of mice, we also measured the stool pH before and 3 hours after gavage with tenapanor (Figure 1g), and found the pH to be significantly lower in *Cftr*$^{-/-}$ compared to *Cftr*$^{+/+}$ mice, and to be significantly increased after tenapanor gavage.

Accelerated GI transit time upon treatment with Tenapanor

Gastrointestinal transit time (GTT) is prolonged in CF patients and in CFTR deficient mice (De Lisle, 2007; Dellschaft et al., 2021; Malagelada et al., 2020; Vitko et al., 2016). We therefore measured GTT after oral gavage of 30 mgkg^{-1} tenapanor or vehicle once per week. Compared to *Cftr*$^{+/+}$, the *Cftr*$^{-/-}$ mice in the vehicle treated group showed a significantly longer transit time (Figure 2a,c). Upon treatment with Tenapanor, a significant decrease of transit time was observed from week 1 onwards in the *Cftr*$^{-/-}$ mice and in week 3 in the *Cftr*$^{+/+}$ mice (Figure 2a,c). This reduction of GTT in both vehicle treated and tenapanor treated *Cftr*$^{+/+}$ and *Cftr*$^{-/-}$mice from week 1 to week 3 was independent of the effect of tenapanor on the stool water content in the 5 hours during the determination of GTT which showed a significant difference between vehicle and tenapanor treated mice of both genotypes (Figure 2b,d). Interestingly, a difference in the pre-gavage (0h) stool water content (not significant until week 2, but significant in week 3) between the vehicle and tenapanor treated *Cftr*$^{+/+}$ mice but not in the *Cftr*$^{-/-}$ mice was observed (which would be a time point of approx. 11 hours after their last gavage). This may indicate a different durability of tenapanor action in *Cftr*$^{+/+}$ and *Cftr*$^{-/-}$intestine.

Significant reduction in obstructive episodes by Tenapanor

Treatment with Tenapanor resulted in a significantly lowered number of obstructive episodes in *cftr* $^{-/-}$ mice (8.3% vs. 46.2%, $p<0.05$) (Figure 3a), and a significantly better survival of 91.7% with tenapanor compared to 53.9% with vehicle treatment (because all mice with suspected obstruction were sacrificed). In the *Cftr* $^{+/+}$ group, survival was 100% both with tenapanor and with vehicle treatment. The site of obstruction was between the proximal and mid colon in most cases, and the segments in which the fecal material had accumulated was mostly restricted to the regions of the mid-distal ileum and the proximal colon (Figure 3b), because obstruction was suspected early. Table 1 shows the age and sex distribution of the obstructed and unobstructed *Cftr*$^{-/-}$ mice. While the total group of mice before gavage had near identical age and sex distributions in both the vehicle and the tenapanor treated groups, the mice that suffered an obstruction had a male and young age predominance.

Tenapanor treatment reverses cryptal hyperproliferation in the ileum and proximal colon

Assessment of the cryptal and villus length, the mucosal integrity and the muscle layer thickness was performed in all mice of the treatment groups. In the ileum, proximal and mid-distal colon, vehicle treated *Cftr*$^{-/-}$ mice displayed crypt villus/crypt elongation and increased muscle layer thickness compared to the *Cftr*$^{+/+}$ mice in the ileum and colon, but not the jejunum (Figure 4a-c). Treatment with tenapanor alleviated the crypt-villus/crypt elongation in the unobstructed ileum and proximal colon compared to the vehicle treated *Cftr*$^{-/-}$mice (Figure 4b, two middle panels). The increase in muscle layer thickness, seen in all segments except the jejunum in the *Cftr*$^{-/-}$ mice, was not significantly affected by tenapanor treatment. The jejunum did not display significant differences between the groups.

Obstructed segments display distorted architecture and loss of mucosal integrity

The obstructed group of mice displayed a highly distorted epithelial architecture in the ileum and proximal colon, which were the segments that were regularly affected by the accumulation of food particles and fecal bacteria rich material (Figure 4a). The mucosal barrier was clearly destroyed and a clear separation between intra- and extracellular mucus was not possible in many instances, and areas were identified where the epithelium was sloughed off the lamina propria. All these changes had occurred in the live mouse within a relatively short time span and demonstrates the severe ulcerative behaviour of the luminal content in conjunction with the altered physiological state of the intestinal segment.

Tenapanor treatment results in decreased mucus accumulation in *cftr*$^{-/-}$ intestine

Immunostaining against Muc2, the major component in intestinal secreted mucus, showed that tenapanor treatment reduced the total Muc2 mediated fluorescence intensity in relation to the Dapi mediated fluorescence intensity, compared to vehicle treatment (Figure 5a,b). In order to determine whether the decrease was due to intracellular Muc2 in the goblet cell thecae, or in the luminal (mucosa-attached plus secreted mucus), we optically separated the epithelium from the lumen in each section, and quantified the two components (Supplementary. Figure 1a). Because of the many different parameters that had to be studied in the intestine of the mice, including the mucosa adherent microbiome (the results of which will be published by the group of Soraya Shirazy-Beechey), it was technically not possible to perform a Carnoy fixation that preserves the firmly adherent mucus layer. Therefore the quantification of the extracellular mucus in the luminal compartment is only approximate. Nevertheless the data show a significantly increased accumulation of mucus in the *Cftr* $^{-/-}$ mice (Figure 5a-c). Tenapanor significantly reduced the mucus accumulation in the ileum and proximal colon of the *cftr* $^{-/-}$mice compared to vehicle treatment, while it did not affect these parameters in *cftr*$^{+/+}$ intestine (Figure 5a-d). In the segments proximal to the site of obstruction (ileum and proximal colon), mucus accumulation in the intracellular and luminal compartment was prominent, but the two components could often not be distinguished with certainty. Muc2 mRNA expression was not different in the small intestine in either genotype or in the two treatment groups, but it was non-significantly reduced compared to *Cftr*$^{+/+}$in the colon, similar to previous observations (Supplementary Figure 1b) (Kini et al. 2020). In contrast, mRNA expression of the membrane-resident Muc1 was increased in the *Cftr* $^{-/-}$ ileum and proximal colon, and tenapanor treatment reduced Muc1 expression relative to vehicle treatment (Figure 5e).

Mast cell number reduced in Tenapanor treated intestine

An increase in innate immune cells, particularly mast cells, has been shown in the small intestine of *Cftr*$^{-/-}$ mice (Norkina, Kaur, Ziemer & De Lisle, 2004). Importantly, a recent study highlighted the mast cell-dependent immune function to a worse survival in F50del mutant mice (Philp et al., 2018). We therefore studied mast cell numbers and the effect of tenapanor treatment. A significant increase of Toluidine Blue stained mast cells were seen in all examined intestinal segments of the *Cftr*$^{-/-}$ compared to *Cftr*$^{+/+}$ mice (Figure 6a, b). Treatment with tenapanor resulted in a significant reduction in the number of mast cells in the jejunum and mid-distal colon of *Cftr* $^{-/-}$ mice, which were also the segments with the highest mast cell numbers in the epithelium (outside of Peyer´s patches). In other segments, while there was a similar trend, it did not reach statistical significance. No differences in mast cell number were seen between vehicle and tenapanor treated *Cftr*$^{+/+}$ mice groups (Figure 6a,b).

Neutrophil infiltration in the segments proximal to the site of obstruction

Myeloperoxidase (MPO) staining was performed to assess neutrophil infiltration. The neutrophil count in the different segments of *Cftr*$^{+/+}$ and *Cftr*$^{-/-}$ intestine were very low, but there was a significant increase in the ileum and mid-distal colon between *Cftr*$^{+/+}$ and *Cftr*$^{-/-}$ mice, independent of treatment. Neutrophil infiltration was strongly increased in the segments immediately proximal to the site of obstructions, but not in the mid-distal colon, which was the segment that was always distal to the obstruction (Figure 7a,b). To verify this finding, we measured the mRNA expression of Ly6g, a marker for differentiated neutrophils and granulocytes (Figure 7c). *Ly6g* expression was much higher in the segments proximal to the obstruction.

Strong expression of proinflammatory cytokine expression in the intestinal segments proximal to the obstruction

Proinflammatory cytokine expression is more sensitive to detect low level inflammation than MPO staining. *Tνφα* , *Mcp1* , and*Ιλ1β* mRNA levels were assessed in the different intestinal segments of all gavaged mice (Figure 8a-c). The expression levels were low in *Cftr*$^{+/+}$ and *Cftr*$^{-/-}$ intestine, irrespective of treatment, except in the case of an obstruction having occurred. Proinflammatory cytokine expression levels were significantly increased in all segments proximal to the obstructed site, but not in the segment distal to the obstructed site (mid-distal colon). However, the relative increase was approx. 10 fold higher in the ileum and proximal colon, the segments that were in contact with the fecal matter, than in the jejunum, which is usually free of fecal content when the obstruction was clinically detected and the mice sacrificed. Please observe the different y-axis of the individual bargraphs.

Discussion

The primary goal of the study was to assess the potential of the oral intestine-specific NHE3 inhibitor tenapanor to prevent intestinal obstruction in CFTR null mice. Several major obstacles were noticed during the study preparation and had to be addressed: Tenapanor quickly precipitates from aqueous solutions at neutral or alkaline pH. Since microencapsulated tenapanor for use in mice is not available, it had to be applied by gavage, and dose finding studies were necessary. We aimed at finding a tenapanor dose and application frequency that resulted in a stable but mild increase in stool water, because diarrhea is quickly lethal for mice (Barone et al., 2009). 30 mgkg^{-1}tenapanor, applied twice daily by gavage, resulted in a mild increase in stool water, and it did not significantly influence the body weight of the mice. Surprisingly, the required dose was the same in *Cftr* $^{-/-}$ and *Cftr* $^{-/-}$mice, suggesting relative independence of the CFTR mediated route of fluid secretion and the NHE3 mediated route of fluid absorption. It is likely that the microencapsulation of tenapanor available for use in humans will permit lower doses in the CF population. An optimal dosing regimen to prevent obstructive episodes in CF patients without causing diarrhea or other unwanted side effects needs to be established.

The *Cftr* $^{-/-}$ mice experienced a mild loss of body weight in both the tenapanor and vehicle group, which may be related to an increased stress level due to the twice daily gavage. The lack of effect of tenapanor on body weight is an important finding, because it is known that NHE3 function is required for PEPT1-mediated dipeptide absorption (Thwaites & Anderson, 2007). It is in agreement with a lack of reduction of PEPT1-mediated substrate absorption during short term tenapanor ingestion in healthy volunteers (Johansson et al., 2017). However, NHE3 has also been shown to be involved in the absorption of amino acids (Anderson & Thwaites, 2005) and micronutrients (Shawki et al., 2016). Therefore, careful observation is necessary during long term studies in CF patients.

Replacing the drinking fluid containing the osmotic laxative with tap water may result in an initiation of an obstructive episode within the first day (which becomes evident only several days later); we therefore started the tenapanor gavage on the last day of the oral laxative administration. It is well known that the incidence of obstructive episodes (always lethal) in *Cftr* $^{-/-}$ mice is very high during weaning (Snouwaert et al., 1992), and 90% *Cftr* $^{-/-}$ mice are dead by day 30 unless preventive measures are taken (Clarke, Gawenis, Franklin & Harline, 1996), and decreases in adulthood. Previous studies that aimed at pharmacological prevention of obstructive episodes in *Cftr* $^{-/-}$ mice have therefore administered the pharmaceutical agent to mice aged

3-6 weeks (Walker, Simpson, Levitt, Boyle & Clarke, 2006), and 20 days, respectively (Lord et al., 2018). For our study, the mice had to tolerate twice daily gavage and preferably not loose body weight due to this procedure; we therefore chose the age range of 9-13 weeks. This explains why the percentage of obstructions is lower in our study compared to the studies mentioned above.

The primary goal of our study was the questions whether intestinal obstructive episodes can be prevented by tenapanor in a test group at high risk for obstructions, which is the CFTR null mouse (no CFTR protein present). Although our study was conducted in an age group in which the mice have a decreased risk compared to younger age, the results showed that the risk was still high (46% within the observation time) and was reduced to 8% with tenapanor treatment.

Apart from obtaining a positive result of the study, we wanted to understand how tenapanor conveyed this reduction in obstructive episodes at a cellular and molecular level. As previously studied in intestinally perfused mice, the direct effect of tenapanor will be a reduction of NHE3-mediated sodium and fluid absorption and an increase in the luminal alkalinity in both the small and large intestine (Tan et al., 2021), which was also seen in the stool of the mice in this study (Figure 1g). A normalization of the delayed GTT in the tenapanor-treated *Cftr* $^{-/-}$ mice is likely another key effect protecting the mice against obstructions.

Somewhat surprisingly, tenapanor treatment resulted in the reversal of other features of the intestinal abnormalities in *Cftr* $^{-/-}$ mice. Of particular interest to us was the finding that tenapanor treatment reversed the cryptal hyperproliferation in the ileum and proximal colon. A hyperproliferative response has been observed previously in *Cftr* $^{-/-}$ small (Gallagher & Gottlieb, 2001) and large intestine (Tan et al., 2021). The intracellular pH of *Cftr* $^{-/-}$ deficient enterocytes is more alkaline than that of corresponding *Cftr* $^{+/+}$ cells (Simpson, Gawenis, Walker, Boyle & Clarke, 2005). This feature is preserved in the crypts including the stem cells of intestinal organoids (Liu, Walker, Cook, Ootani & Clarke, 2012) and is causally related to the increased proliferative rate, which is preserved in *Cftr* $^{-/-}$ intestinal organoids (Strubberg et al., 2018). The presence of NHE3 has been reported both in undifferentiated and differentiated intestinal organoids (Foulke-Abel et al., 2016). Immunohistochemical staining of NHE3 overlaps with that of CFTR in the villus region and cryptal mouth region (Jakab, Collaco & Ameen, 2011). Therefore, it is possible that chronic tenapanor application reverses in part some of the pathological effects of the high intracellular pH on proliferative activity. A similar observation was made by Bradford *et al* . in their studies of mice which were homozygous negative for CFTR, for NHE3, or for both genes. While both *Cftr* $^{-/-}$ and *Nhe3* $^{-/-}$ mice displayed an increased cell proliferation in duodenal crypts, this increase was significantly reduced in the crypts of *Cftr* $^{-/-}$*/Nhe3* $^{-/-}$ mice (Bradford, Sartor, Gawenis, Clarke & Shull, 2009). Thus, long term tenapanor treatment, if proven safe in CF patients, may even have to potential to lower the risk for GI malignancy.

Another striking observation in tenapanor treated mice was a reduction in intestinal mucus accumulation. We previously reported an increase in the goblet cell counts, as well as enlarged goblet cell theca, in the mid-distal colon of *Cftr* $^{-/-}$ mice that did not display evidence of inflammation (Kini et al., 2020; Tan et al., 2021). Because of the well documented crypt-villus hyperproliferation in *Cftr* $^{-/-}$ intestine (Gallagher & Gottlieb, 2001; Tan et al., 2021), we used a fluorometric method of quantifying the Muc2 immunofluorescence in relation to that of the Dapi stained nuclei in this project. The total as well as the extracellular Muc2/Dapi immunofluorescence was significantly reduced in tenapanor vs vehicle-treated mice in the ileum and proximal colon, while it was very high when an obstruction was present.

We also noted a shift in the expression of the *Muc* genes, with a non-significantly lower expression of *Muc2* mRNA and a significantly higher *Muc1* mRNA. The 21 day treatment with tenapanor reduced *Muc1* mRNA expression compared to vehicle treatment. The somewhat lower *Muc2* mRNA expression was also seen in the present study, with no effect of tenapanor. It has been shown that *Muc1* deletion, although not a major component of intestinal mucin, reduces the thickness of the firmly adherent mucus layer (Malmberg et al., 2006). Muc1 deleted CF mice have reduced amounts of intestinal mucus and a better survival compared to Muc1-expressing CF mice (Parmley & Gendler, 1998). It is therefore likely that the observed tenapanor induced changes in Muc1 expression and mucus accumulation play a major role in reducing obstructive episodes.

The intestinal tract of patients with cystic fibrosis display an inflammatory phenotype in the absence of bacterial infection or enzyme induced fibrosing colitis. Multiple explanations have been provided, including bacterial overgrowth due to dysmotility, oxidative stress, intracellular accumulation of mutated CFTR protein, and lack of pancreatic and/or intestinal defensins (Bruzzese et al., 2004; Crites et al., 2015; Galli et al., 2012; Lisowska, Mdry, Pogorzelski, Szydłowski, Radzikowski & Walkowiak, 2010; Raia et al., 2000; Werlin et al., 2010). In mice, chronic exposure to PEG containing laxative in the drinking fluid both increased survival and reduced the inflammatory signature in the intestine of these mice (Bradford, Sartor, Gawenis, Clarke & Shull, 2009; Clarke, Gawenis, Franklin & Harline, 1996). We had previously reported no difference in proinflammatory cytokines in the different segments of *Cftr* $^{-/-}$ and *Cftr* $^{+/+}$ littermates while on the PEG containing laxative, yet several features that had been correlated with an absent CFTR function (in addition to the reduced anion and fluid secretory response) were present: increased enterocyte pH_i, an increased mucus content in the goblet cell theca, crypt-villus elongation, decreased surface pH with fluid hyper-absorption (Kini et al., 2020; Tan et al., 2021). In this study, a discreet but significant increase in the number of mast cells was observed in the lamina propria of the intestinal tract of the vehicle treated *Cftr* $^{-/-}$ mice compared to *Cftr* $^{+/+}$ mice, which was completely or partially prevented by tenapanor treatment. A very discreet increase in neutrophil infiltration was seen in *Cftr* $^{-/-}$ ileum and mid-distal colon, not accompanied by an increase in any of the proinflammatory cytokines. In contrast, a strong increase in neutrophil infiltration and in the expression of proinflammatory cytokines was only seen in mice with an obstruction, and was restricted to the intestinal segments proximal to the obstruction that were filled with fecal matter. These segments also exhibited severe alterations in the mucosal architecture. Thus, the intestinal inflammation was a sequelae of the obstruction, and not its cause.

A relative shortcoming in our study is that the application mode of the drug is relatively invasive, and therefore the study duration is fairly short. The number of mice permitted for the study by the animal committee was sufficient to reach the primary endpoint, but was not enough to determine significance for the reversal of every studied CF-associated intestinal abnormalities by tenapanor treatment in every intestinal segment. When we performed the GTT experiments, we noticed that the stool water content was still elevated in the *Cftr* $^{+/+}$ mice 11 hours after the last dosing, but not in the *Cftr* $^{-/-}$ mice, suggesting that more frequent dosing, not feasible with this form of application, may have been optimal. Obviously, this problem will not exist in CF patient trials. For a tenapanor trial in CF patients, it will be necessary to define the patient population at high risk for intestinal complications, and preferably test its prophylactic potential. Patients with meconium ileus at birth and prior DIOS episodes, and lung or liver transplanted patients are at particularly high risk, and CF diabetes, pancreatic insufficiency and female gender also carry a higher risk (Houwen et al., 2010; Lavie et al., 2015; Munck et al., 2016). While corrector and potentiator therapy is beneficial for CFTR-dependent functional outcome parameters (Graeber et al., 2022; Ooi et al., 2018), adjunctive therapy specifically targeting the constipation and obstructive episodes appears necessary in many CF patient subgroups.

Tenapanor treatment is considered safe for humans (Block et al., 2021; Sinagra et al., 2020). It has not been tested in children so far, and the longest observation time in a published trial is 52 weeks. Given the good safety profile, clinical trials testing the effect of tenapanor on the intestinal symptoms of CF patients seem warranted.

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Table 1:

	Gender Vehicle Male	**Gender Vehicle Female**	**Gender Tenapanor Male**	**Gender Tenapanor Female**	**Age (weeks) Vehicle**	**Age (weeks) Tenapanor**
Unobstructed	1	6	4	7	11.7 ± 0.522	11.5 ± 0.366
Obstructed	4	2	1	0	10.8 ± 0.477	9.0
Total	5	8	5	7	11.3 ± 0.365	11.3 ± 0.392

Table 1: Gender and age at the start of the experimental period of the mice that developed and obstruction and that did not.

FIGURE LEGENDS

FIGURE 1: Effect of 3 week tenapanor treatment on body weight, stool water content and stool pH in *Cftr*$^{+/+}$ and *Cftr*$^{-/-}$ mice: (a) Schematic description of experimental protocol. (b) Initial body weight of the male and female mice that did or did not develop obstructions. (c) The body weight remained stable during the 3 week gavage period in the *Cftr*$^{+/+}$ mice and (d) slightly decreased in the *Cftr*$^{-/-}$ mice, irrespective of treatment with tenapanor or vehicle. (e) A significantly higher stool water content was seen in the tenapanor treated treated *Cftr*$^{+/+}$and (f) *Cftr*$^{-/-}$ mice, in comparison to the vehicle treated groups. (g) Increased stool alkalinity 3 hours post gavage with tenapanor. Each dot represents one mouse. *Cftr*$^{+/+}$: n=10 in both tenapanor treated and vehicle treated groups; *Cftr*$^{-/-}$: n=12 in the tenapanor treated and n=13 in the vehicle treated group. Data is represented as mean ± SEM. # is used to represent the statistics performed between the two different genotypes and * is used to represent the statistics performed between the vehicle and tenapanor groups. # and * $p < 0.05$.

FIGURE 2: Faster GI transit time (GTT) in Tenapanor treated mice: (a,c) GTT was measured once per week, and it decreased in both *Cftr* $^{+/+}$ and *Cftr* $^{-/-}$mice from week 1 to week 3, and was decreased after gavage with tenapanor compared to vehicle. (b,d) The longitudinal assessment of the stool water content during the 5 hours post gavage showed a significant increase with tenapanor compared to vehicle treatment. 0h indicates stool samples collected pre gavage. Each dot in the GI transit measurement represents one mouse, with the same mouse then being simultaneously assessed for stool water content. Data is represented as mean ± SEM. *$p < 0.05$.

FIGURE 3: Significantly lower incidence of obstructions in Tenapanor treated *Cftr*$^{-/-}$ mice: (a) Treatment with tenapanor resulted in a significant reduction of the incidence of intestinal obstructions in *Cftr*$^{-/-}$ mice compared to the vehicle treated *Cftr*$^{-/-}$ mice. (b) A representative image of the obstruction

between ileum to proximal colon is shown. All mice but one were suspected to have suffered an obstruction while alive and were sacrificed prior to death. *p $<$ 0.05.

FIGURE 4: Reduced cryptal hyperproliferation in Tenapanor treated *Cftr*$^{-/-}$ mice: (a) Representative HE images of jejunum, ileum, proximal colon and mid-distal colon in *Cftr*$^{+/+}$ and *Cftr*$^{-/-}$mice treated either with vehicle or tenapanor. (b) Crypt-villus length or crypt depth measured showed reduced cryptal-villus prolongation in ileum and crypt prolongation in proximal colon of in the tenapanor treated *Cftr*$^{-/-}$ mice. (c) Muscle layer thickness displayed an increase in ileum, proximal colon and mid-distal colon of *Cftr*$^{-/-}$ mice, with no difference between vehicle and tenapanor treated group. Each dot represents one mouse. For each mouse, 5 different (not serial) sections per segment were analysed and averaged. Veh – vehicle, Ten – tenapanor, UO – unobstructed, O – obstructed. Data is represented as mean ± SEM. # is used to represent the statistics performed between the two different genotypes and * is used to represent the statistics performed between the vehicle and tenapanor groups. # and * p $<$ 0.05.

FIGURE 5: Decrease in mucus accumulation in Tenapanor treated *Cftr*$^{-/-}$ mice: (a) Representative images of Muc2 immunohistochemistry (major component of the secreted colonic mucin) of jejunum, ileum, proximal colon and mid-distal colon in *Cftr*$^{+/+}$ and *Cftr*$^{-/-}$mice treated with either vehicle or tenapanor. (b) Fluorescent intensity (I.D) ratio of Muc2 in relation to Dapi immunofluorescence (c) localized to the goblet cell thecae (intracellular mucus pool) and d) localized to the luminal compartment (secreted mucus). (e) mRNA expression of *Muc1* in these intestinal segments. Each dot represents one mouse. Veh – vehicle, Ten – tenapanor, UO – unobstructed, O – obstructed, I.D – Integrated density. Data is represented as mean ± SEM. # is used to represent the statistics performed between the two different genotypes and * is used to represent the statistics performed between the vehicle and tenapanor groups. # and * p $<$ 0.05.

FIGURE 6: Reduced numbers of mast cells in in Tenapanor treated *Cftr*$^{-/-}$ mice: (a) Representative images of toluidine blue stained mast cells (indicated by black arrows) in the jejunum, ileum, proximal colon and mid-distal colon in *Cftr*$^{+/+}$ and *Cftr*$^{-/-}$mice treated with either the vehicle or tenapanor. (b) Quantified mast cell counts within the selected intestinal segments. Each dot represents one mouse. Veh – vehicle, Ten – tenapanor, UO – unobstructed, O – obstructed. Data is represented as mean ± SEM. # is used to represent the statistics performed between the two different genotypes and * is used to represent the statistics performed between the vehicle and tenapanor groups. # and * p $<$ 0.05.

FIGURE 7: Neutrophil infiltration in obstructed *Cftr*$^{-/-}$ mice treated with vehicle: (a) Representative images of MPO stained neutrophils (indicated by white arrows) in the jejunum, ileum, proximal colon and mid-distal colon in *Cftr*$^{+/+}$ and *Cftr*$^{-/-}$mice treated with either the vehicle or tenapanor (n=5/genotype/group/intestinal segment). (b) Counting of MPO positive cells show an obvious neutrophils infiltration in the ileum and proximal colon of obstructed *Cftr*$^{-/-}$ mice treated with vehicle. (c) *Ly6g* mRNA expression further confirmed this observation. Each dot represents one mouse. Veh – vehicle, Ten – tenapanor, UO – unobstructed, O – obstructed. Data is represented as mean ± SEM. # is used to represent the statistics performed between the two different genotypes and * is used to represent the statistics performed between the vehicle and tenapanor groups. # and * p $<$ 0.05.

FIGURE 8: Inflammation in obstructed *Cftr*$^{-/-}$ mice treated with vehicle:The mRNA expression of pro-inflammatory cytokines (a) *Tνφα* , (b)*Mcp1* and (c) *Ιλ1β* are not significantly different between genotypes or between the tenapanaor vs vehicle treated mice without an obstruction, but show a dramatic increase in the ileum and proximal colon of obstructed *Cftr*$^{-/-}$ mice. Each dot represents one mouse. Veh – vehicle, Ten – tenapanor, UO – unobstructed, O – obstructed. Data is represented as mean ± SEM. # is used to represent the statistics performed between the two different genotypes and * is used to represent the statistics performed between the vehicle and tenapanor groups. # and * p $<$ 0.05.

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