Author's response to reviews

Title: Lubiprostone ameliorates the cystic fibrosis mouse intestinal phenotype

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Author's response to reviews: see over
We thank the reviewers for their insightful and constructive comments. The manuscript has been revised in accord with these comments, and we believe the paper has been improved in the process. We hope that our manuscript is now acceptable for BMC Gastroenterology.

Referee 1: Dr Christina Haston

Major compulsory revisions

1. Introduction. References are needed to substantiate statements in the first paragraph, and the assertion that "Various novel approaches that correct mutant CFTR function, or activate alternate electrolyte secretory mechanisms are under investigation." The references for the CF phenotype being similar in patients and mice are all to mice. References to clinical data are needed here.

   **Appropriate references have been added.**

2. The effect of lubiprostone on CLC2 is hypothesized but not evaluated. The hypothesis actually tested should be indicated or the effect on CLC2 function in the intestines of treated mice assessed.

   We have modified the sentence stating our hypothesis to "Despite these controversial issues, the encouraging results using lubiprostone in CF patients [18] and with CF cells and tissues [2,17], made it reasonable to test the hypothesis that lubiprostone would ameliorate the intestinal phenotype in the Cftr knockout mouse."

3. Preliminary experiments showed that a dose of 10 µg/kg-day was maximally effective (data not shown). What effect was maximal at this dose?

   Our initial analysis was on motility, and gastric emptying was found to be maximally affected at this dose. This is now stated in the manuscript

4. In figure 1 the comparison tested is not clear. What is the weight of CF mice on Peptamen alone, for comparison? Why is CF mouse weight gain compared to WT?

   As stated in the Methods, "To increase the statistical power of these comparisons, weights of CF mice were calculated as percent of WT mice of the same age, gender, and treatment group". Also, as stated in the Results, 'Lubiprostone did not affect weight gain in WT mice as compared to control WT'. Thus, there is no drug effect on weight gain in WT mice, which is an important control. Basically, body weights of WT mice of a given age and gender were set to be 100% and CF mice of the same ages and genders were then compared to this. This allowed pooling of mice of both genders with the same genotype into a single group which increased statistical power of the analysis.

5. The effect of the intervention on mucous accumulation is modest, at best, within the CF mice. What is the p value comparing the distribution of lubiprostone treated CF mice to that of CF controls in fig 3b? The analysis presented in fig 3C should be statistically justified or removed.

   The data in panel B include all the data from 200-300 crypts per mouse and
thus it is not appropriate to perform a statistical analysis of such data. The data in Fig. 3C present the average values per animal and these were analyzed by ANOVA with a post-hoc test giving the resultant p-values as presented in the figure legend. This is now stated more explicitly in the Methods section.

6. What statistical test was applied to show the effect of lubiprostone treatment on bacterial counts in CF mice? Is there literature support for this change as physiologically relevant? Would such a change alter the intestinal distress in the CF mouse?

The Mann Whitney U test was used, as these data are not normally distributed. To our knowledge, there is no information in the literature regarding an association of bacterial load (quantity of bacteria) and extent of physiological effect/dysfunction. The standard definition of small intestinal bacterial overgrowth (>10^5 cfu/ml) is arbitrary and not based on systematic analysis. I do not believe enough is known about small intestinal bacterial overgrowth to indicate whether or not these changes in bacterial load will result in physiological changes. However, the lack of altered gene expression in lubiprostone treated WT mice suggests that the observed change in bacterial load does not have substantial effects on gut function.

7. Gene regulation was not assessed in this study so references to up/down regulation in the Tables and text should be corrected. How were functions listed in table determined? The table notes should indicate n=1.

The term ‘gene regulation’ is used in a descriptive sense. Any change in gene expression, deductively, involves altered gene regulation, either pre- or post-transcriptionally. Functions of genes were from the OMIM database (www.ncbi.nlm.nih.gov/omim), and from the scientific literature. We have now included the following statement in the table legends "Equal amounts of total RNA were pooled from 7-10 mice in each group and were used to interrogate the Affymetrix Mouse 430 2.0 GeneChip (n=1 per group)."

8. A more complete description of the RT experiment is needed. Are the data technical replicates of the chip result? If yes, the chip result should be removed as the more valid data are from the RT experiment.

More detail on the qRT-PCR approach is now stated in the Methods section. While the microarray and qRT-PCR share the same purpose, to discover differences in gene expression levels, they are radically different approaches, each with its strengths and drawbacks. The microarray, a relatively expensive procedure, allows interrogation of thousands of genes which is not practical using qRT-PCR. qRT-PCR is considered more reliable than microarray data, is used to confirm array results, and is much less expensive to perform for a limited number of genes. Thus, the two approaches are complementary even when the same RNA samples are used. For these reasons, we believe both datasets are useful and appropriate to include in the manuscript.

9. That Mcpt2 is increased in CF and decreased by treatment is not enough data to support an effect on the innate immune response. This conclusion should be justified with more data or removed.

As shown in the microarray data (Table 1) as presented in the Results
section, several genes associated with innate immunity are upregulated in the control CF intestine as compared to control WT, and downregulated in lubiprostone-treated CF mice as compared to control CF.

Minor:

1. Mouse strain C57BL/6J is stated in abstract, not methods.
   
   corrected

2. The effects of lubiprostone on body weight gain was assessed. Grammatical change needed.
   
   corrected

3. page 14, drug name misspelled.
   
   corrected

Referee 2: Jonathan Kaunitz

Comment:

1. Although bacterial overgrowth has been reported in CF (e.g. Fridge J Ped. Gastroenterol Nutr 2007 44:212, 2007), the most commonly reported GI problem is recurrent obstruction and constipation, which has been treated successfully with lubiprostone (O’Brien Ann Pharmacotherapy 2010 44:577). The manuscript should include a bit more background information.

   The O’Brien paper became available after our manuscript was submitted. A discussion of it is now included in the revised manuscript.

2. Although there is controversy surrounding the mechanism of lubiprostone, the authors have omitted perhaps the most compelling publication, Bijvelds (Gastroenterol 2009 137:976). In the paper, the authors showed that the effects of lubiprostone were entirely inhibited by prostaglandin EP4 antagonists whereas a CLC-2 channel inhibitor was without effect in three model systems. Furthermore, almost all in situ data supports a basolateral localization of the CLC-2 channel in the enterocyte. In light of these data, the predominance of the data favors lubiprostone acting as a prostaglandin.

   We cite the Bijvelds et al. paper in the Background and Discussion sections our manuscript covering the issues on which the Reviewer commented. We have also included reference to a recent review that discusses in more detail the controversy involving the mechanism of action of lubiprostone (Wood 2010).

3. The authors use an indirect means to assess mucus secretion, measurement of intervillous (crypt) width. Since they hypothesize that lubiprostone increases the rate of mucus turnover, they should measure it directly, perhaps by the methods described in ref. 21.

   We agree that a more direct measurement of mucus production is required before a definitive conclusion can be reached. We are in the process of establishing in our lab the methods used in (Garcia, Yang, et al. 2009) to test
the potential role of lubiprostone as a goblet cell mucus secretagogue, presumably via a prostaglandin-like pathway. However, it is beyond the scope of the present study to wait until such experiments are complete.

4. Lubiprostone substantially increased bacterial overgrowth in normal mice. Is this effect clinically important? Given that lubiprostone is approved to treat IBS, and bacterial overgrowth may worsen IBS, it would seem that this effect of lubiprostone would impair its therapeutic effects in the IBS population.

While there was a statistically significant increase in bacterial load in lubiprostone treated WT mice, the magnitude of change was small compared to bacterial overgrowth in control CF mice. The microarray analysis of the treated WT mice did not reveal any evidence of inflammation or other informative changes in gene expression. Such changes would be expected if the degree of bacterial load had a physiological effect in the treated WT mice. It is therefore unlikely that this increase in bacterial load is clinically important.

5. The authors stated on p. 15 that lubiprostone "decreased the innate immune response". This seems like too strong a statement given the data presented, since it is difficult to extrapolate changes in RNA expression with an immune response. In general, the gene chip analysis does not seem to add much to the authors' main argument and should be deleted.

An innate immune response in the gut can include recruitment of cells such as mast cells. The strong significant decrease in mast cell markers on the array shows that this recruitment is prevented by lubiprostone treatment of CF mice. Another reason we believe the genechip data are important is that these data show that lubiprostone has little effect on gene expression in WT mice, which is an important (negative) result.

6. If lubiprostone is a CLC-2 channel activator, why does it affect motility? Would it not be more likely that it acts as a PG, which has well known effects on motility? Although the author has previously measured altered motility in CF mice, is this also true in humans? In one article (Tonelli, J Cyst Fibros. 2009 May;8(3):193-7), gastroparesis was unusual and is thought the be due to lung disease or diabetes in most cases. In general, the motility data adds little to testing the stated hypothesis and could be deleted.

Dysmotility is one aspect of CF gut function with important consequences. There is a complex relationship between the intestinal microbiota and gut motility, and the normal healthy balance is lost in CF. We previously showed that oral osmotic laxative, which is believed to act by restoring sufficient hydration to the gut lumen, eradicated bacterial overgrowth and normalized intestinal transit in CF mice (De Lisle, Roach et al. 2007). We had hypothesized that if lubiprostone were as effective at correcting the fluid deficit of the CF gut as laxative, then similar improvements would be observed. Also, fluid in the lumen can have a bulk effect that stimulates GI motility. The fact that lubiprostone did not improve small intestinal transit in CF mice is an important observation, even though it provides evidence against our starting hypothesis. Therefore, we feel these data should be in the manuscript.

7. The Discussion could be reduced by 30% by deleting much of the speculation and focusing on the core findings.
We have carefully edited the Discussion to reduce its length. Because of the controversies about the mechanism of action of lubiprostone, it was not possible to reduce the Discussion as much as the Reviewer suggested without ignoring reasonable possibilities and while still presenting the analysis in a unbiased manner.

8. Fig. 4. It seems that the n is much lower for controls than for the lubi groups. Is this the case? Could this bias the interpretation?

   Our lab has been working with this CF mouse model for a number of years and the values obtained from the control mice in this study are consistent with our previous work. Therefore, we are confident that a smaller number of controls did not result in a misleading bias in the current study.

9. How valid is the method described for the quantitation of intestinal flora in the absence of standard culturing techniques?

   PCR amplification of the bacterial 16S rRNA gene has distinct advantages over culture-based techniques. It has been found that the majority of bacteria that can inhabit the intestines have not been cultured and thus culture could significantly underestimate bacterial load. For example, in a previous study we found that almost 40% of 16S sequences from the WT small intestine were from uncultured strains (Norkina, Burnett, et al. 2004). There are also disadvantages, as discussed in that paper, but the advantages far outweigh the potential problems.

Minor:
1. Please use the term "anion channel" rather than the confusing "Cl-/HCO3- channel", especially since the nature of the ionic species transported by CFTR remains controversial.

   Changed as requested.

2. Also, please use the term "anion exchangers" rather than "anion transporters that exchange".

   Changed as requested.

Referee 3: Dr Hugo De Jonge

Major Compulsory Revisions:

1. All Cftr-/- mice in this study are maintained on a liquid diet (Peptamen) rather than solid diet. Unfortunately, this choice did not allow the authors to investigate potential beneficial effects of lubiprostone on intestinal obstruction and survival, i.e. the most prominent intestinal phenotype of CF mice. If lubiprostone would be able to activate intestinal chloride and fluid secretion through a non-CFTR mediated pathway, possibly involving ClC2, one would expect to see a reduced obstruction and increased survival in lubiprostone-treated CF mice.

   As the reviewer is aware, the Cftr<sup>tm1UNC</sup> Cftr knockout mouse has a severe intestinal phenotype, in contrast to other Cftr knockout mice. This necessitates either using a liquid diet or administration of oral osmotic laxative to prevent lethal intestinal obstruction. It was part of our original
intent to test the possibility that lubiprostone would allow CF mice to survive on chow without laxative treatment. However, the lubiprostone treated CF mice on the liquid diet failed to show a dramatic benefit and we did not have a reasonable expectation that lubiprostone could spare the mice from lethal intestinal obstruction. Therefore, we could not ethically justify performing this experiment.

2. A very recent study performed too in Cftr-/- mice (Harmon GS et al 2010 Nature Med 16: 313-318) shows that PPARγ signaling is defective in CF colon and that PPARγ agonists (e.g. rosiglitazone) can induce bicarbonate secretion, reduce mucus retention, and promote survival of CF mice in a Cftr-independent fashion. In the present manuscript, stimulation of bicarbonate and mucus solubility and secretion rather than chloride secretion by lubiprostone, possibly triggered through EP4 receptors, is suggested as the most plausible mechanism by which lubiprostone may exert its anti-inflammatory and multiple other actions in the CF intestine. Both studies together raise the question whether lubiprostone, similar to 15-keto-PGE2, might additionally act as a PPARγ agonist and could partially rescue the intestinal CF phenotype through this pathway. This possibility should be explored, or at least discussed in the manuscript.

This paper was not yet published when our manuscript was submitted. The Reviewer raises an interesting point but we feel that a comparison of lubiprostone to 15-keto-PGE2 is too speculative to include in the Discussion of the current study.

3. Even if lubiprostone targets CFTR rather than apical ClC2 channels in the enterocyte (if they exist), it remains possible that the compound promotes luminal hydration in Cftr-/- mice through an EP4/cAMP/PKA signaling pathway that is known to result in inhibition of NHE3 and NaCl/fluid absorption. Such a potential anti-absorptive action is ignored in the present manuscript.

We have not included this possibility in the Discussion because we are not electrophysiologists and we did not perform such analysis.

4. Most experiments were apparently performed using total mouse intestine, despite the fact that intestinal functions may vary considerably along the length of the intestine. For example, the bacterial overgrowth and mucus retention from goblet cells is expected to be more prominent in the ileum as compared with jejunum. Were the measurements of crypt width (as an estimate of mucus accumulation) performed in ileum, jejunum, or in both segments? Idem for the microarrays?

Although obstruction occurs in the distal small intestine or proximal large intestine, the pathological changes are present all along the CF mouse small intestine and some are actually more pronounced in the proximal regions (Norkina et al. 2004). Also, previous work from our lab has shown that bacterial overgrowth is actually greater in the proximal small intestine in the Cftr<sup>tm1UNC</sup> mouse [see fig.3 (De Lisle 2007)]. Mucus accumulation is pronounced in all regions of the CF mouse small intestine. We routinely use tissue from the middle portion of the small intestine, and this is now stated in the Methods section.

Minor Essential Revisions:
1. p. 14, l. 10: The finding that lubiprostone can activate PGE2 receptors in the intestine is confirmed in ref. 10, showing inhibition of lubiprostone-induced anion secretion by a specific
EP4 antagonist. Therefore this reference should be mentioned in addition to ref. 23.

*The data from (Bijvelds, Bot, et al. 2009) with respect to the EP4 antagonist results are now cited.*

2. p. 17, l. 13-14: A crucial finding reported in ref. 10 is that lubiprostone not only failed to induce intestinal chloride secretion in Cftr^-/- mice but also in the ileal and rectal epithelium from human CF patients. This finding should be quoted in the Discussion.

*The data from (Bijvelds, Bot, et al. 2009) using human control and CF tissues have now been cited in the Discussion.*