Responses to Reviewers

Authors are thankful to reviewers for their prompt and constructive comments. The attached manuscript has been revised per their comments. Specific responses to reviewer's comments are as follows:

Reviewer 1

Q1. How concentrations of GC-C agonists were chosen.

Response: The most widely used bioassay to measure potency of GC-C agonists is a T84-cell based assay. T84 are human colon cancer cell line which overexpresses guanylate cyclase C (GC-C) receptors. This assay has been used for the last two decades because it is reliable and reproducible. The dose response curves for majority of GC-C agonists have been established and published using this bioassay. There is absolutely no correlation between the potencies of GC-C agonists in this assay with *in vivo* animal experiments. The dose needed in animal experiments to stimulate fluid secretion or to ameliorate GI inflammation is relatively much lower[1-3]. However, the dose response curves generated with GC-C agonists such as uroguanylin, plecanatide, dolcanatide, E.coli STa and linaclotide have been reproducible. Example of dose response curves for GC-C binding and cGMP production are shown below:

Fig. 1: Effect of pH on cGMP stimulation by GC-C agonists in T84 cells

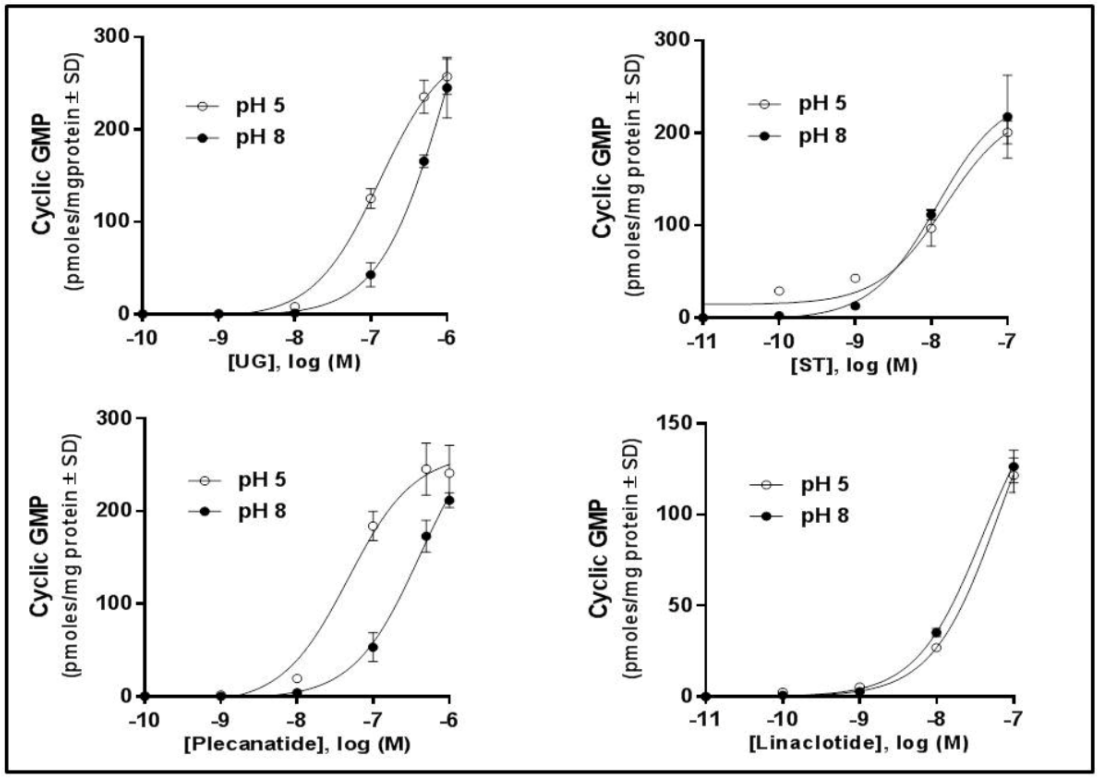
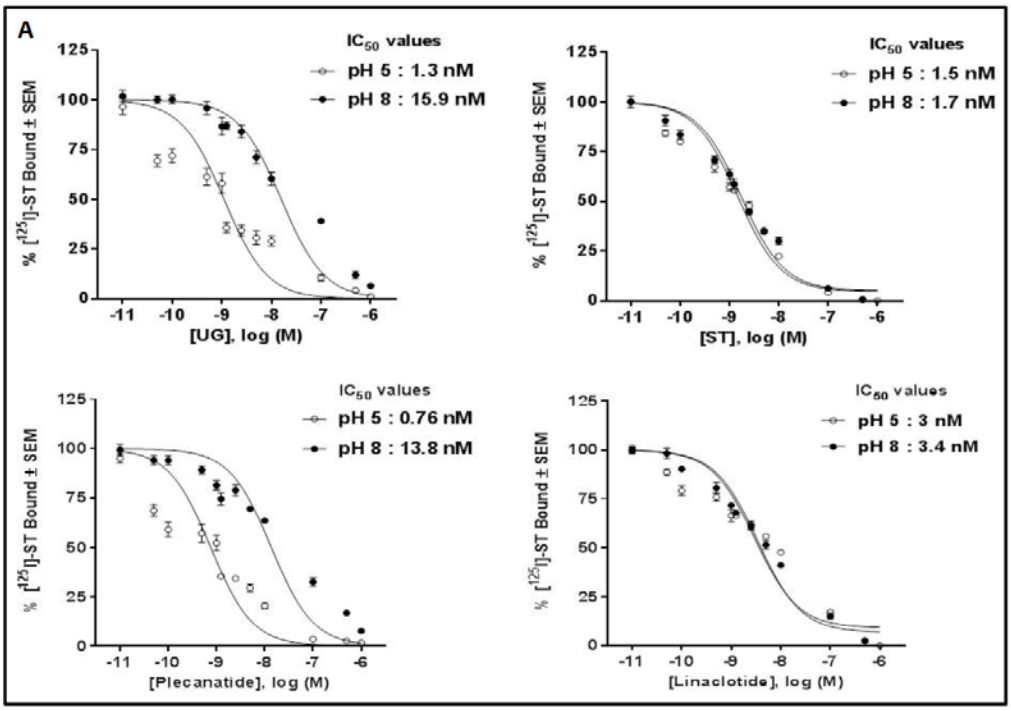


Fig. 2: Effect of pH on competitive binding of GC-C agonists in T84 cells



Data shown in Fig 1 and 2 on dose responses for cGMP production and GC-C binding are consistent with previously published reports. These data along with other experimental findings were presented at an oral presentation by Dr. Shailubhai at the annual meeting of Digestive Disease Week 2016 (Gastroenterology 150: S193-S194, 2016)[4]. The major finding of this study is that the binding and cGMP stimulatory activity of GC-C agonist is drastically regulated by the GI mucosal acidity. For example, uroguanylin and plecanatide are more active at the pH range 5 to 6, which corresponds to pH range of proximal small intestine and the site of fluid secretion in the gut. The activity of these two GC-C agonists is considerable reduced at pH 8, which is the pH range in the distal intestine (colon). The activity of E .coli STa and linaclotide in these assays was not altered by the pH. A manuscript reporting these findings along with results from a number of other *in vitro* and *in vivo* experiments is being prepared for full-length publication. The physiological implications of these results explains the underlying mechanism of travelers' diarrhea caused by E. coli STa and how the water secretory activity of uroguanylin is regulated by the GI mucosal acidity.

Q 2. Were any controls run with linaclotide?

We did not run controls with linaclotide in these experiments. However, other researchers have looked at the effect of linaclotide on cellular homeostasis and barrier function [5,6]. The primary objective of *in vitro* experiments conducted with cell lines (T84 and CaCo-2) and rat colon tissues was simply to examine if plecanatide and dolcanatide play role in integrity and localization of tight junction proteins and permeability. Initial experiments were conducted to

determine the optimum concentrations of each of the agonists, which was subsequently chosen for the final experiments.

Q 3. Gender of the animals: Genders of animal has been mentioned in the manuscript.

Cited references:

1. Shailubhai K, Yu HH, Karunanandaa K, Wang JY, Eber SL, Wang Y, Joo NS, Kim HD, Miedema BW, Abbas SZ, Boddupalli SS, Currie MG, Forte LR. Uroguanylin treatment suppresses polyp formation in the Apc(Min/+) mouse and induces apoptosis in human colon adenocarcinoma cells via cyclic GMP. *Cancer Res* 2000; **60**: 5151-5157 [PMID: 11016642]

2. Shailubhai K, Palejwala V, Arjunan KP, Saykhedkar S, Nefsky B, Foss JA, Comiskey S, Jacob GS, Plevy SE. Plecanatide and dolcanatide, novel guanylate cyclase-C agonists, ameliorate gastrointestinal inflammation in experimental models of murine colitis. *World J Gastrointest Pharmacol Ther* 2015; **6**: 213-222 [PMID: 26558155 PMCID: PMC4635161 DOI: 10.4292/wjgpt.v6.i4.213]

3. Chang WL, Masih S, Thadi A, Patwa V, Joshi A, Cooper HS, Palejwala VA, Clapper ML, Shailubhai K. Plecanatide-mediated activation of guanylate cyclase-C suppresses inflammation-induced colorectal carcinogenesis in Apc+/Min-FCCC mice. *World J Gastrointest Pharmacol Ther* 2017; **8**: 47-59 [PMID: 28217374 PMCID: PMC5292606 DOI: 10.4292/wjgpt.v8.i1.47]

4. Patwa, V., Joshi, A., Thadi, A., Eddy, P.E., Palejwala, V.A., Jacob, G. S., and Shailubhai, K. Plecanatide, like uroguanylin, activates guanylate cyclase-C signaling in a pH-dependent manner in T84 cells, murine intestinal cells and tissues. *Gastroenterology* 2016; **150:**S193-S194

5. Cuppoletti J, Blikslager AT, Chakrabarti J, Nighot PK, Malinowska DH. Contrasting effects of linaclotide and lubiprostone on restitution of epithelial cell barrier properties and cellular homeostasis after exposure to cell stressors. BMC Pharmacol. 2012 May 3;12:3. doi: 10.1186/1471-2210-12-3.

6. Kang SB, Marchelletta RR, Penrose H, Docherty MJ, McCole DF. A comparison of linaclotide and lubiprostone dosing regimens on ion transport responses in human colonic mucosa. Pharmacol Res Perspect. 2015 Mar;3(2):e00128. doi: 10.1002/prp2.128. Epub 2015 Mar 13.

Reviewer 2

Q1. There are too many cited references

Response: We agree with the reviewer and reduced the number of cited references where possible.

Q2. Abdominal withdrawl reflex method was not used.

Response: All animal experiments were conducted in laboratory of Dr. Lionel Bueno, who has contributed to this field immensely. Dr. Bueno's group also conducted a similar study with linaclotide[6]. However, the abdominal withdrawl method was not used.

7. Eutamene H, Bradesi S, Larauche M, Theodorou V, Beaufrand C, Ohning G, Fioramonti J, Cohen M, Bryant AP, Kurtz C, Currie MG, Mayer EA, Bueno L. Guanylate cyclase C-mediated antinociceptive effects of linaclotide in rodent models of visceral pain. *Neurogastroenterol Motil* 2010; **22**: 312-e384 [PMID: 19706070 DOI: 10.1111/j.1365-2982.2009.01385.x]