Responses to Reviewer Comments:

Authors are thankful to reviewers for their constructive suggestions and comments. Their comments were very helpful to improve the manuscript. We believe the manuscript is now significantly improved. Our responses to reviewer's comments are given point-by-point below.

1. **Format and writing requirements:** We diligently followed the guidelines for manuscript revision. The revised manuscript is formatted as per the **Writing Requirements** of the World Journal of Pharmacology and Gastroenterology (WJGP) as described in sections 2.1 to 2.31.

2. **Language Editing:** Majority of authors are English speaking. The manuscript has been checked carefully for English and grammar. In addition, the revised manuscript was also checked using the Grammarly software version 6.4.104.5108 for grammar and plagiarism.

3. **Plagiarism detection software:** We contacted the Cross Check company and we were advised that the CrossRef software is only for institutional subscription and it is not for individual subscription. Although we assure you that the manuscript was carefully checked for plagiarism by using Grammarly software, but we request you to pass the manuscript through the CrossRef software, if you have access to the software.

4. **Copyright Transfer Agreement**: The copyright agreement is attached. The copyright agreement was slightly revised to state that Shailubhai K, Foss JA, Comiskey S, Palejwala V and Jacob G are employees of Synergy Pharmaceuticals Inc., and Plevy SE received compensation as a consultant from Synergy Pharmaceuticals, Inc. The conflict of interest statement is given on page 2 of the manuscript. The Copyright Agreement is signed by all authors except Dr. S.E. Plevy. He has now moved to Johnson and Johnson Pharmaceuticals and we are trying to get in touch with him to sign the agreement. In the mean time, we are submitting the agreement pending his signature to save time and to meet the due date for submission of the revised manuscript.

5. **Audio Clip:** A digitally recorded audio clip emphasizing the importance of the findings reported in the manuscript is attached

Detailed responses to scientific comments/questions are described in the next few pages.

Major Points

1. *The rationale for performing prophylactic studies?* Chemically induced colitis develops very rapidly in mice, and the total duration of these murine models is only 7 days. Hence, these models, typically used for prophylactic intervention, might not be suitable for therapeutic intervention with test agents due to the short duration. DSS and TNBS induced colitis animal studies presented here represent proof-of-concept evaluating whether administration of GC-C agonists could prevent or delay the onset of the GI inflammation. Therefore, we chose to administer plecanatide (SP-304) and dolcanatide (SP-333) at the time of DSS or TNBS treatment to evaluate their effect on amelioration of colitis. We agree with the reviewer that our results might be more representative of the prophylactic intervention and may not truly be relevant to the therapeutic intervention in clinical situation. Nevertheless, these models are widely used for evaluation of drug candidates for the treatment of ulcerative colitis. In addition, we also conducted experiments in the chronic model of murine colitis using TCR$\alpha^{-/-}$ mice that spontaneously develop colitis. In both chemical as well as spontaneously induced colitis models, plecanatide and dolcanatide effectively ameliorated colitis. Our future studies are directed for therapeutic evaluation of dolcanatide in the adoptive T-cell transfer model. Taken together, results from these animal studies were sufficiently convincing for Synergy Pharmaceuticals, Inc., to advance dolcanatide for clinical studies, and the drug is currently under Phase II clinical evaluation in patients with ulcerative colitis.

2. *Evidence for the ability of uroguanylin analogs to stimulate cGMP production in mice?* It is very well established in the literature that uroguanylin and other agonists of GC-C stimulate cyclic GMP *via* activation of GC-C in T84 cells as well as in rodent models. During the initial studies leading to the discovery of plecanatide and dolcanatide, we conducted several studies involving intestinal loop assay in mice and rats to establish that the pharmacological mechanism of action of these compounds is *via* stimulation of cyclic GMP in the GI tract. Nevertheless, we conducted a study to find out if orally administered plecanatide stimulates cGMP production in colon tissues. The primary objective of this study was to examine the potential of orally administered plecanatide to delay the onset of colitis in to the development of colon tumors in $Apc^{+/min\text{-}FCCC}$ mice (cited ref 44 in

the manuscript). Results from this study indicated that orally administered plecanatide delayed the onset of colitis into colon carcinogenesis in $Apc^{+/min\text{-}FCCC}$ mice *via* enhancing cGMP production in colon tissues. The poster describing these outcomes was chosen for the ACG Presidential Award and is attached here for your review only. Also, the results showing stimulation of cGMP in colon tissues are described later in this document for your review only. Recent clinical results showed that plecanatide was efficacious in Phase III trials in patients with chronic constipation. Similarly, oral treatment with dolcanatide was found to be efficacious in Phase II clinical evaluation in patients with opioid-induced constipation. Importantly, these recent clinical studies confirm that orally administered GC-C agonists are minimally absorbed into systemic circulation. Taken together, these pre-clinical and clinical studies confirm that the pharmacological actions of orally administered plecanatide and dolcanatide are primarily through activation of GC-C receptors in the gut lumen. We have included a paragraph in the discussion section to explain the mechanism of action of GC-C agonists based on the pre-clinical and clinical studies. This paragraph is highlighted in Yellow in the manuscript.

3. *Evaluation of mucosal expression of GC-C in colitis model?* Yes, we have conducted an independent study in $Apc^{+/Min\text{-}FCCC}$ (C57BL/6J) mice designed to evaluate the effect of plecanatide on cGMP levels and on expression of GC-C transcripts in colonic tissues during the acute phase of colonic inflammation. These results are described later in this document.

Minor Points

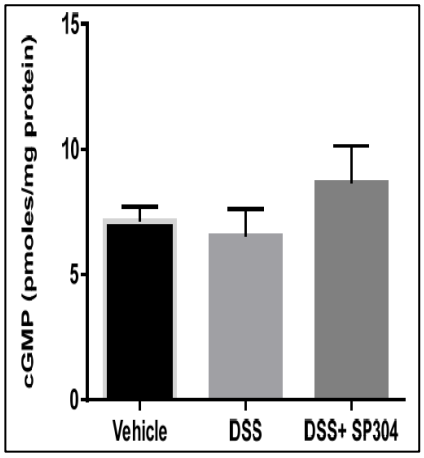
1. *Is SP-304 same as plecanatide?* Yes, legend has been updated.
2. *Missing p-values from figures 3*-5? All figures updated to reflect statistical significance

Amendment: We have now received name Dolcanatide for SP-333 from USAN agency. Hence, SP-333 is replaced with dolcanatide throughout the manuscript. We have included a statement in the core tip section indicating that plecanatide is SP-304, and dolcanatide is SP-333.

Supporting data: Only for review and not to be included in the manuscript

The data given below are to further support our responses described in sections 2 and 3 above. These data are from a study conducted to evaluate the potential of GC-C agonists to delay the onset of DSS-induced colitis into the development of colon tumors in $Apc^{+/min\text{-}FCCC}$ mice. These two figures are part of another manuscript that is being prepared for communication. This study was conducted in collaboration with researchers at the Fox Chase Cancer Center, Philadelphia. Thus, we cannot include the data described below in this manuscript. Data presented here support that the orally administered plecanatide activates GC-C receptors in the colon to stimulate cGMP production in DSS-induced colitis $Apc^{+/min\text{-}FCCC}$ mice.

1. ***Orally administered plecanatide stimulates cGMP production in the colon***: Mice were randomly divided into three cohorts of 6 mice each: 1) vehicle-treated, 2) DSS + vehicle and 3) DSS + plecanatide (SP-304) treated. $Apc^{+/min\text{-}FCCC}$ mice were administered DSS in the drinking water for 4 days followed by 3 days of untreated water. Starting on day 1 mice received an oral gavage of vehicle (cohorts 1 and 2) or 2.5 mg/kg of plecanatide (cohort 3). The cGMP-stimulatory activity of GC-C agonists are known to be transient and the optimal time for measuring cGMP in colon tissues is typically between 45-60 mins. Therefore, on day 7, mice were given oral gavage with vehicle (cohorts 1 and 2) or plecanatide (cohort 3) and subsequently euthanized within 45 mins. Colon tissues were excised immediately, cut in pieces of ~ 1 cm length, snap frozen and stored at -80^{o}C. For cGMP analysis, 6 pieces (1 piece from each mouse) were combined from each of the cohorts. Colon tissues were homogenized in 6% TCA followed by neutralization with 5N NaOH and levels of cGMP levels were determined using T84 cell-based bioassay. The cGMP levels normalized to the amount of protein in the lysate are expressed as an average of triplicates ± SD. As shown in the figure, the levels of cGMP in colon tissues from DSS + plecanatide treated mice were considerably higher than those in colon tissues from vehicle-treated or Vehicle + DSS treated mice. These results suggest that the orally administered plecanatide stimulates cGMP production in the colon tissues of $Apc^{+/min\text{-}FCCC}$ mice treated with DSS.



2. ***GC-C expression is not altered in DSS-colitic mice***: $Apc^{+/Min\text{-}FCCC}$ mice (n= 6/group) randomized to 4 treatment groups: DSS alone (no plecanatide in diet; vehicle control) or DSS plus diet supplemented with 2, 10 or 20 ppm of plecanatide. All animals were given 2% DSS in the drinking water for 4 days, followed by water *ad libitum*. Mice were euthanized after 7 weeks, colon excised, cut in pieces, snap frozen and stored at -80^{0}C for later analyses. Quantitative RT-PCR was used to determine the relative levels of GC-C transcript compared to GAPDH in the same sample. Results expressed as fold change (mean ± SEM) by comparing levels in plecanatide-treated animals with those of animals treated with DSS alone (control). The GC-C transcript levels in various fragments of colon were comparable. However, levels in proximal colon tissue from plecanatide (10 ppm)-treated mice were lower. It should be noted that the colon tissues used for analyses were half of the vertically cut pieces of proximal, middle and distal colon segments. Hence, these tissues representing different segments may or may not have colon tumors. It is believed that the expression of GC-C might be altered in colon tumors but its expression in the adjoining normal tissue is robust. It was difficult to dissect out the colon tissues with tumors only. Thus, levels of GC-C transcripts are representative of the transcript levels of the whole segment.