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September 16, 2022

BY ELECTRONIC SUBMISSION

Division of Dockets Management (HFA-305)
Food and Drug Administration
Department of Health and Human Services
5630 Fishers Lane, Rm. 1061
Rockville, MD 20852

Re: Docket No. FDA-2007-D-0369
Comments by Salix Pharmaceuticals, Inc. on the FDA's Product-Specific Draft Guidance on Plecanatide (Recommended Nov. 2018; Revised Sept. 2019)

Dear Sir or Madam:

On behalf of Salix Pharmaceuticals, Inc. ("Salix"), we respectfully submit these comments on the product-specific guidance issued by the United States Food and Drug Administration ("FDA"), entitled "Draft Guidance on Plecanatide" ("Plecanatide PSG").[1] These Comments address testing needed to ensure that the active ingredient sameness requirement[2] is met by any abbreviated new drug application ("ANDA") referencing Trulance® (plecanatide) tablets (the "Trulance® Product") as the Reference Listed Drug (RLD).

Should you have any questions regarding these comments, please do not hesitate to contact the undersigned.

[1] U.S. FOOD & DRUG ADMIN., DRAFT GUIDANCE ON PLECANATIDE (Recommended Nov. 2018; Revised Sept. 2019) [hereinafter PLECANATIDE PSG].
[2] 21 U.S.C. § 355(j)(4)(C)(i).

Sincerely,

Lance L. Shea
Partner

Comments by Salix Pharmaceuticals, Inc. on the FDA's Product-Specific Draft Guidance on Plecanatide (Recommended Nov. 2018, Revised Sept. 2019)
Docket No. FDA-2007-D-0369

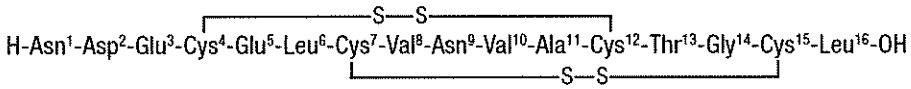
Contents

I. Description of the Trulance® Product

The Trulance® Product was approved for the treatment of chronic idiopathic constipation ("CIC") on January 19, 2017, and for the treatment of irritable bowel syndrome with constipation ("IBS-C") on January 24, 2018.

The active ingredient in the Trulance® Product is plecanatide. As explained in the Trulance® Product label:

> TRULANCE (plecanatide) is a guanylate cyclase-C (GC-C) agonist. Plecanatide is a 16 amino acid peptide with the following chemical name: L-Leucine, L-asparaginyl-L-α-aspartyl-L-α-glutamyl-L-cysteinyl-L-α-glutamyl-L-leucyl-Lcysteinyl-L-valyl-L-asparaginyl-L-valyl-L-alanyl-L-cysteinyl-L-threonylglycyl-L-cysteinyl-, cyclic (4→12),(7→15)bis(disulfide).
>
> The molecular formula of plecanatide is C65H104N18O26S4 and the molecular weight is 1682 Daltons. The amino acid sequence for plecanatide is shown below:



The solid lines linking cysteines illustrate disulfide bridges.

> Plecanatide is an amorphous, white to off-white powder. It is soluble in water. TRULANCE tablets are supplied as 3 mg tablets for oral administration. The inactive ingredients are magnesium stearate and microcrystalline cellulose.[3]

The Trulance® Product's route of administration is oral; its route of delivery is topical: Plecanatide delivered by the Trulance® Product acts topically in the gastrointestinal ("GI") lumen. The sites of drug action are guanylyl cyclase 2C receptors ("GUCY2C receptors")[4] located on the gastric epithelium in the lower pH environment of the proximal small bowel.[5] As explained in the Trulance® Product label:

> Activation of [GUCY2C Receptor] results in an increase in both intracellular and extracellular concentrations of cyclic guanosine monophosphate (cGMP). Elevation of extracellular cGMP has been associated with a decrease in the activity of pain-sensing nerves in animal models of visceral pain. Elevation of intracellular cGMP stimulates secretion of chloride and bicarbonate into the intestinal lumen, mainly through activation of the cystic fibrosis transmembrane conductance regulator (CFTR) ion channel, resulting in increased intestinal fluid and accelerated transit. In animal models, plecanatide has been shown to increase fluid secretion into the gastrointestinal (GI) tract, accelerate intestinal transit, and cause changes in stool consistency.[6]

Following Trulance® Product administration, systemic availability of plecanatide is negligible.[7]

II. The Trulance® Product is a complex product.

As discussed above, the Trulance® Product's active ingredient is plecanatide, a 16 amino acid peptide. Although no signals of immunogenicity related adverse events were seen in Trulance® Product clinical trials, the FDA Summary Review noted that plecanatide presented potential immunogenicity concerns.[8] Such active ingredients are classified as complex active ingredients by the FDA.[9] Further, the Trulance® Product is classified as having a complex route

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[3] SALIX PHARM. INC., TRULANCE® FULL PRESCRIBING INFORMATION § 11 (Rev. 2021) [hereinafter Trulance PI].
[4] Names for this receptor vary in the literature, as will be seen in quotations *infra*.
[5] Trulance PI § 12.1.
[6] *Id.*
[7] *Id.* § 12.3.
[8] U.S. FOOD & DRUG ADMIN., DIVISION DIRECTOR SUMMARY REVIEW FOR REGULATOR ACTION – APPLICATION NUMBER 208745ORIG1S000 at 4 (Jan. 19, 2017) ("Due to structural similarity between plecanatide and the endogenous peptides uroguanylin and guanylin, there is a theoretical immunogenicity risk for deficiency if patients develop cross-reacting anti-plecanatide antibodies. No signals of deficiency-related adverse events (e.g., hypertension, edema, pulmonary edema, hypernatremia, weight gain) were seen in the clinical trials database for plecanatide.").
[9] U.S. FOOD & DRUG ADMIN., MANUAL OF POLICY AND PROCEDURES – CLASSIFYING APPROVED NEW DRUG PRODUCTS AND DRUG-DEVICE COMBINATION PRODUCTS AS COMPLEX PRODUCTS FOR GENERIC DRUG DEVELOPMENT PURPOSES 3 (MAPP 5240.10 Apr. 13, 2022) ("Peptides with no greater than 40 amino acids (AAs) having complex higher-order structure and/or potential immunogenicity concerns are also considered as complex APIs (e.g., liraglutide, calcitonin).").

of delivery, because it delivers plecanatide topically in the GI lumen.[10] Plecanatide delivered by the Trulance® Product is negligibly absorbed into the systemic circulation. Further, due to its primary structure, plecanatide's binding affinity to GUCY2C receptors decreases once it leaves the lower pH environment of the proximal small intestine. Having a complex active ingredient and complex route of delivery, the Trulance® Product is classified as a complex product.

III. Trulance® Product indications and mechanisms of action

This section reviews the diseases for which the Trulance® Product is indicated and discusses the paracrine signaling process that comprises the Trulance® Product's mechanism of action. As discussed above, the Trulance® Product is indicated for treatment of CIC and IBS-C. Biological markers have not been identified for the diagnosis of either IBS-C or CIC. Thus, both diseases are defined by their symptom-based diagnostic criteria.

Clearly, the symptom common to IBS-C and CIC is constipation. Constipation describes not only infrequency of bowel movements, but also sensation of incomplete bowel movement, straining, bloating and stool consistency that is hard to pass (hard, lumpy, etc.), and need for manual stool removal.[Cash 2018; Rao 2018] The symptom of incomplete bowel movement refers to the inability to fully evacuate stool from the rectum, resulting in continuing sensations of urgency, pain and discomfort.

One symptom differentiates IBS-C from CIC: abdominal pain. In IBS-C patients, improvement in constipation symptoms is not ensured to relieve abdominal pain.[Lacy et al. 2016 at 1395]

The following is an overview of IBS-C and CIC diagnostic criteria.

A. Overview of IBS-C and CIC diagnostic criteria

The diagnostic criteria for each disease are set by the Rome Foundation and referred to as the "Rome Criteria." The Rome Criteria are standard of care in U.S. medical practice. The current edition is the fourth, "Rome IV."

1. Diagnostic criteria for IBS-C

Under Rome IV, IBS-C is diagnosed in two steps. In the first step, the patient is diagnosed with IBS. The diagnostic criteria for IBS are as follows:

> Diagnostic Criteria[a] for Irritable Bowel Syndrome
>
> Recurrent abdominal pain, on average, at least 1 day per week in the last 3 months, associated with 2 or more of the following criteria:
>
> 1. Related to defecation
> 2. Associated with a change in frequency of stool
> 3. Associated with a change in form (appearance) of stool

[10] *Id.* at 4.

[a] Criteria fulfilled for the last 3 months with symptom onset at least 6 months before diagnosis.[Lacy et al. 2016]

As made clear by Rome IV, recurrent abdominal pain is the symptom that defines IBS – a diagnosis of IBS is invalid unless it is based on that symptom.[Lacy et al. 2016 at 1395] Thus, bowel movement frequency, alone, is not a criterion sufficient for diagnosis for IBS-C. Notably, "a large subset of IBS patients do not have an improvement in abdominal pain with defecation, but instead report a worsening."[Lacy et al. 2016 at 1395]

In the second step, the IBS subtype is diagnosed. Rome IV lists 3 subtypes, which are IBS with predominant:

- Constipation (IBS-C);
- Diarrhea (IBS-D); or,
- Irregular bowel habits (IBS "mixed" / IBS-M).[Lacy et al. 2016 at 1395]

The criteria for the IBS subtypes are based on the Bristol Stool Form Scale.[Lewis & Heaton 1997]

The criteria for IBS-C are: "[m]ore than one-fourth (25%) of bowel movements with Bristol stool form types 1 or 2 and less than one-fourth (25%) of bowel movements with Bristol stool form types 6 or 7."[Lacy et al. 2016 at 1395]

2. Diagnostic criteria for CIC

Rome IV calls CIC "functional constipation" or "FC." Diagnosis of CIC excludes the symptom predominant abdominal pain: "FC is a functional bowel disorder in which symptoms of difficult, infrequent, or incomplete defecation predominate. Patients with FC should not meet IBS criteria, although abdominal pain and/or bloating may be present but are not predominant symptoms."[Lacy et al. 2016 at 1399] Rome IV sets forth the CIC diagnostic criteria as follows:

Diagnostic Criteria[a] for Functional Constipation

1. Must include 2 or more of the following:
    a. Straining during more than one-fourth (25%) of defecations
    b. Lumpy or hard stools (BSFS 1-2) more than one-fourth (25%) of defecations
    c. Sensation of incomplete evacuation more than one-fourth (25%) of defecations
    d. Sensation of anorectal obstruction/blockage more than one-fourth (25%) of defecations
    e. Manual maneuvers to facilitate more than one fourth (25%) of defecations (eg, digital evacuation, support of the pelvic floor)
    f. Fewer than 3 spontaneous bowel movements per week
2. Loose stools are rarely present without the use of laxatives

3. Insufficient criteria for irritable bowel syndrome
   [a]Criteria fulfilled for the last 3 months with symptom onset at least 6 months prior to diagnosis.[Lacy et al. 2016 at 1399-1400]

Rome IV makes clear that a patient diagnosed with IBS-C has a condition distinct from CIC.

B. Trulance® Product mechanism of action

Plecanatide is an agonist of the GUCY2C receptors. GUCY2C receptors in the GI tract are part of the signaling mechanism that regulates fluid and electrolyte transport in the GI lumen.

Guanylate cyclase C (GUCY2C) is an epithelial cell receptor expressed along the rostral-caudal axis of the intestine from proximal of the duodenum to the distal rectum. It was first identified as the receptor for heat-stable enterotoxins (STs) produced by enterotoxigenic bacteria that causes diarrheal diseases.[Waldman et al. 2019 at 2]

In humans, GUCY2C receptors are activated by the endogenous peptide uroguanylin:

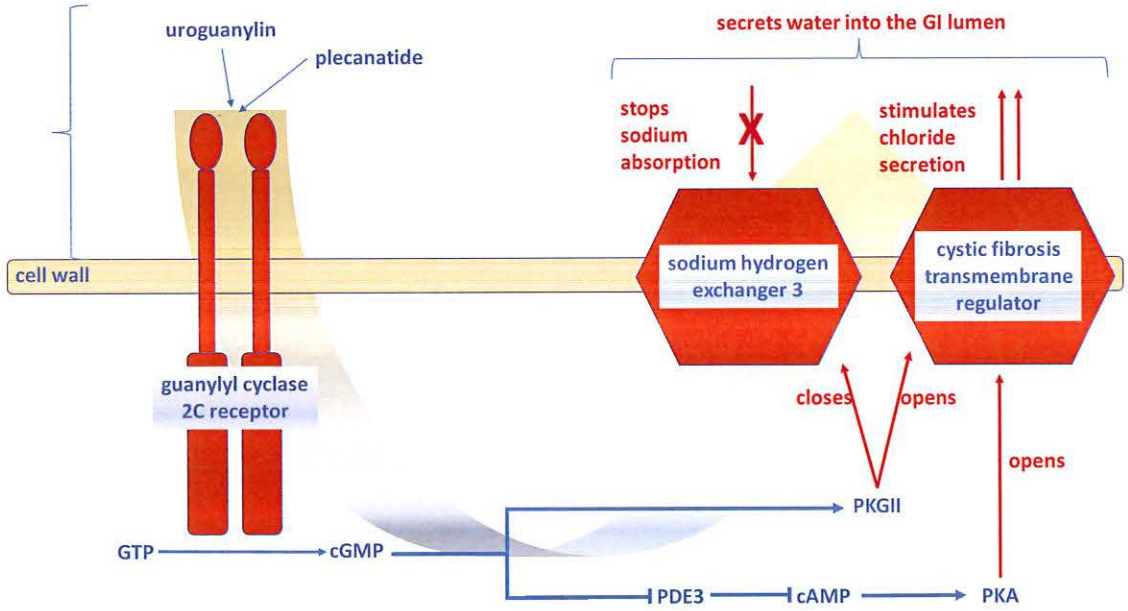
> [U]roguanylin (UGN) is a 16-amino acid peptide that activates GUCY2C with maximum potency in the slightly acidic (pH 5–6) environments of the duodenum and proximal jejunum. UGN is expressed by tuft-like epithelial cells, primarily in the proximal small intestine, with lower levels in the stomach, the distal small intestine, and the colorectum. [Waldman et al. 2019 at 2]

Generally, the signaling mechanism may be described as follows:

> The GC-C receptor is activated by its endogenous peptides uroguanylin and guanylin that differentially bind to the receptor in the varying pH environments found along the GI tract. Uroguanylin is primarily expressed and preferentially binds GC-C receptors in the slightly acidic (pH 5-6) regions of the duodenum and jejunum. . . . Binding of uroguanylin or guanylin to the GC-C receptor initiates a signaling cascade leading to accumulation of intracellular cyclic guanosine monophosphate (cGMP), which helps maintain fluid and electrolyte balance, promotes visceral analgesia, and reduces inflammation in the GI tract.[Brancale et al. 2017 at 2]

More specifically as shown in Figure 1, when an agonist binds to the GUCY2C receptor, cGMP is formed from guanosine triphosphate ("GTP"). This results in activation of protein kinase G II ("PKGII") which opens the cystic fibrosis transmembrane regulator ("CFTR") to release fluid into the GI lumen, and closes the sodium hydrogen exchanger 3 ("NHE3") to prevent counterflow from the lumen. Concurrently, activation of the GUCY2C receptor results in the accumulation of cyclic adenosine monophosphate ("cAMP") from adenosine triphosphate ("ATP"). This results in activation of protein kinase A ("PKA") that also opens the CFTR. When plecanatide enters the proximal small bowel and binds to GUCY2C receptors, fluid is released through the columnar epithelium into the lumen.

Figure 1. GUCY2C signaling from uroguanylin or plecanatide agonism



To have binding activity with the GUCY2C receptor, a peptide agonist must have a certain conformation. Human uroguanylin adopts "two topological isoforms (A and B) of which only the A form is biologically active."[Brancale et al. 2017 at 2] Isoform A binds to the GUCY2C receptor, while B does not.[Chino et al. 1998]

Plecanatide shares the same amino acid sequence as uroguanylin with the exception of a single amino acid residue, a glutamic acid rather than aspartic acid at position 3. Like uroguanylin, plecanatide contains 4 cysteine residues that form 2 intramolecular disulfide bonds, between cysteine 4 and 12, and cysteine 7 and 15.

The number and orientation of those bonds are necessary to generate the peptide conformation necessary for binding to the GUCY2C receptor. Although similar in structure to uroguanylin, plecanatide binds to the GUCY2C receptor with potency greater than that of uroguanylin[Brancale et al. 2017 at 7-8] when present in the lower pH conditions of the proximal small bowel.[Shailubhai 2008] The dominant conformation at low pH is maximally active in binding with the GUCY2C receptor.[Shailubhai 2008 at 10]

Human uroguanylin's binding activity is not pH-dependent.[Shailubhai 2008 at 11] The substitution of glutamic acid for aspartic acid results in plecanatide's pH-dependent activity. That feature provides a plecanatide conformation that is active in the lower pH conditions of the proximal small bowel but also results in a different conformation that is less active in the relatively higher pH regions of the distal small bowel and colon. This is supported by computational modeling of plecanatide binding activity at pH values of 2.0, 5.0 and 7.0:

> [Molecular dynamics] simulations of plecanatide in this study revealed plecanatide to be a flexible structure that is . . . able to adopt numerous conformations in response to a range of simulated pH environments. Optimally active conformations were observed in pH 5.0 simulations which approximates the pH values of the duodenum and proximal jejunum. . . . In contrast, at simulated pH values of 2.0 and 7.0, plecanatide adopted conformations that would make it less likely to bind GC-C receptors.[Brancale et al. 2017 at 7]

More specifically:

> Computational models in this study revealed that at pH 5.0, the charged end of the Glu3 residue interacts with the Asn9 residue, potentially serving to stabilize the structure when [plecanatide] forms its most active conformation. Interestingly, the corresponding interaction between Asp3 and Asn9 is not present in uroguanylin, probably because the side chain of Asp3 is not long enough to reach Asn9. Stabilizing the molecule in this way allows plecanatide to remain in its active conformer for a longer period of time at this pH and explain the superior binding profile of plecanatide versus uroguanylin witnessed in earlier studies.
> Brancale et al. 2017 at 8

Studies of pH dependency of plecanatide cGMP production in T84 colon carcinoma cells had similar results. Testing the range of pH values 5.5, 6.0, 6.5 and 7.0 indicated the following:

> [Plecanatide's] greatest effect on cGMP production occurred at pH 6.0-6.5, peaking at pH 6.0. Uroguanylin, on the other hand showed very little pH dependency, showing similar activity to [Plecanatide] only at pH 7.0 where [plecanatide's] was lowest. . . . [plecanatide's] general ability to stimulate cGMP synthesis in cells incubated at pH values ranging from 6.0-6.5 was superior to that of uroguanylin and two other similar peptides used in the study, SP-302 and SP-303.[Shailubhai 2008 at 11]

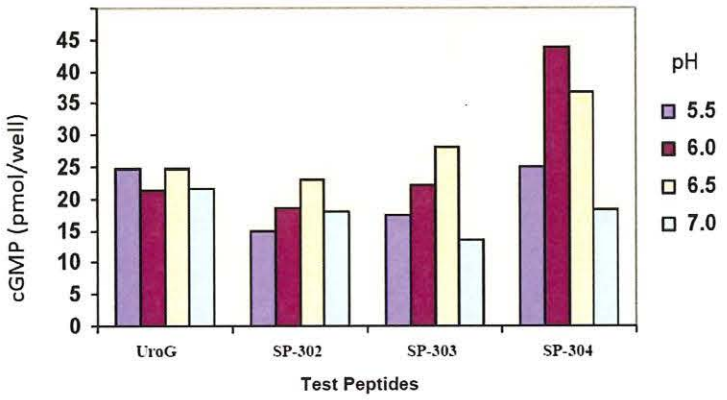
This area-specific activity provides a key safety feature: mitigation of diarrhea that can be caused by peptides that are active in a broader pH range, hence along a greater length of the GI tract. As indicated by the preceding quotation, this safety feature can be altered by small differences in plecanatide's amino acid sequence. The structures are shown in Figure 2. Comparative cGMP production is shown in Figure 3.

Figure 2: Amino acid sequences of peptides tested in Study SP-PH-004, differences in sequence highlighted.[Shailubhai 2008 at 8, Fig. 1]

Test Peptide	Mol. Weight	Amino Acid Sequence *
Uroguanylin	1668	Asn^{1} Asp^{2} Asp^{3} Cys^{4} Glu^{5} Leu^{6} Cys^{7} Val^{8} Asn^{9} Val^{10} Ala^{11} Cys^{12} Thr^{13} Gly^{14} Cys^{15} Leu^{16}
SP-302	1696	Asn^{1} Glu^{2} Glu^{3} Cys^{4} Glu^{5} Leu^{6} Cys^{7} Val^{8} Asn^{9} Val^{10} Ala^{11} Cys^{12} Thr^{13} Gly^{14} Cys^{15} Leu^{16}
SP-303	1682	Asn^{1} Glu^{2} Asp^{3} Cys^{4} Glu^{5} Leu^{6} Cys^{7} Val^{8} Asn^{9} Val^{10} Ala^{11} Cys^{12} Thr^{13} Gly^{14} Cys^{15} Leu^{16}
SP-304	1682	Asn^{1} Asp^{2} Glu^{3} Cys^{4} Glu^{5} Leu^{6} Cys^{7} Val^{8} Asn^{9} Val^{10} Ala^{11} Cys^{12} Thr^{13} Gly^{14} Cys^{15} Leu^{16}

* Note: brackets indicate the location of disulfide bonds

Figure 3: Effect of 30-minute incubations of 0.1 μM concentrations of plecanatide (SP-304), uroguanylin, SP-302 and SP-303 on cGMP production at various physiological pH values in T84 cells.[Shailubhai 2008 at 11, Fig. 2]



Differences in peptide structure result in differences in thermostability. After incubation at 95° C for 90 minutes, plecanatide retained virtually 100% activity, whereas uroguanylin lost about 15% of its activity, and SP-302 and SP-303 lost about 10% of their activities.[Shailubhai 2008 at 11]

As discussed more fully below, a Proposed Generic active ingredient containing these or other potential molecular and physicochemical differences would not meet the active ingredient sameness requirement.

IV. The active ingredient sameness requirement

To be approved under FDCA Section 505(j), a Proposed Generic must be proven therapeutically equivalent to the Trulance® Product.

Section 505(j) of the Federal Food, Drug and Cosmetic Act ("FDCA" or "Act") requires, *inter alia*, that a generic drug must satisfy two distinct statutory criteria: (1) "sameness" of active ingredients[11] and (2) bioequivalence.[12]

In this statutory construct, generic drugs are considered to be safe and effective therapeutic equivalents ("TE")[13] of (and hence, interchangeable with) the RLD if they are *both* pharmaceutically equivalent ("PE")[14] and bioequivalent ("BE")[15] to the RLD.[Alex Yu 2014] This can be expressed simply: **TE = PE + BE**.[Chen 2014; Conner 2014]

The pharmaceutical equivalence (active ingredient sameness) requirement must be satisfied for approval of Proposed Generics. FDA regulations define "active ingredients" as follows:

> Active ingredient means any component that is intended to furnish pharmacological activity or other direct effect in the diagnosis, cure, mitigation, treatment, or prevention of disease, or to affect the structure or any function of the body of man or other animals. The term includes those components that may undergo chemical change in the manufacture of the drug product and be present in the drug product in a modified form intended to furnish the specified activity or effect.[16]

Also, FDA regulations define "pharmaceutical equivalents" as follows: "*Pharmaceutical equivalents* are drug products in identical dosage forms and route(s) of administration that contain identical amounts of the **identical active drug ingredient**, i.e., the same salt or ester of the same therapeutic moiety . . . ."[17] Additionally, the FDA regulations define the "same as" requirement as follows: "Active ingredients. . . For a combination drug product, information to show that the active ingredients are the same as those of the reference listed drug except for any different active ingredient that has been the subject of an approved petition . . . ."[18] Finally, FDA regulations state the following about determining whether the "same as" requirement is met:

> For determining the suitability of an abbreviated new drug application, the term "same as" means **identical in active ingredient(s), dosage form, strength, route**

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[11] 21 U.S.C. § 355(j)(4)(C)(i).
[12] 21 U.S.C. § 355(j)(4)(F).
[13] 21 C.F.R. § 314.3(b) ("*Therapeutic equivalents* are approved drug products that are pharmaceutical equivalents for which bioequivalence has been demonstrated, and that can be expected to have the same clinical effect and safety profile when administered to patients under the conditions specified in the labeling.").
[14] In this petition, Bausch Health uses "pharmaceutically equivalent" to describe products that are "pharmaceutical equivalents."
[15] 21 C.F.R. § 314.3(b).
[16] 21 C.F.R. § 314.3(b).
[17] 21 C.F.R. § 314.3 (emphasis added).
[18] 21 C.F.R. § 314.94(a)(5)(ii).

> **of administration, and conditions of use**, except that conditions of use for which approval cannot be granted because of exclusivity or an existing patent may be omitted.[19]

Thus, the pharmaceutical equivalence / active ingredient sameness requirement is met by demonstrating that generics are formulated with the **identical active ingredients and dosage form** as the RLD, in the same strengths, and where needed, other properties.[20]

V. Active ingredient sameness testing recommended by the Plecanatide PSG

The Plecanatide PSG recommends the following categories of testing to demonstrate that a Proposed Generic's plecanatide active ingredient is the same as the Trulance® Product active ingredient:

> Sameness of synthetic plecanatide can be demonstrated based on comparative physicochemical and biological characterizations. The characterizations should include the following categories to support API sameness:
>
> 1. Primary peptide sequence and related molecular properties such as molecular formula, specific optical rotation, and spectroscopic properties
> 2. Configuration of the two disulfide bonds
> 3. In vitro biological activity (e.g., binding, functional assays)[21]

VI. Testing necessary to ensure active ingredient sameness between Proposed Generics and the Trulance® Product

Generally, Salix concurs with the categories of active ingredient sameness testing recommended by the Plecanatide PSG; however, the PSG's discussion of those categories does not identify testing that is sufficient to demonstrate active ingredient sameness. The following sections set forth the specific additional testing that is needed to ensure molecular and physicochemical sameness of a generic's plecanatide active ingredient to the Trulance® Product active ingredient.

A. Physicochemical structure and disulfide analyses

This section addresses points 1 and 2 of the Plecanatide PSG's sameness testing recommendations. Plecanatide was designed based on uroguanylin, the endogenous agonist of the GUCY2C receptor. Plecanatide's primary peptide sequence is comprised of 16 amino acids. It is identical to uroguanylin except for a single amino acid substitution: glutamic acid for aspartic acid at position 3.[Shailubhai et al. 2013] As recommended by the Plecanatide PSG, analyses to determine the primary peptide sequence are necessary to ensure physicochemical sameness of the generic and Trulance® Product active ingredients; however, the PSG's recommendations are inadequate for that purpose.

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[19] 21 C.F.R. § 314.92(a)(1) (emphasis added).
[20] That is, absent an approved petition as allowed by 21 C.F.R. § 314.93.
[21] PLECANATIDE PSG at 1.

To bind with the GUCY2C receptor, the Trulance® Product's active ingredient has a specific physicochemical form, with two disulfide bridges between cysteine residues, and exhibits pH-dependent conformational preferences both of which are crucial to its receptor binding and mode of action.[Brancale et al. 2017] Thus, to satisfy the active ingredient sameness requirement, the generic sponsor must demonstrate that the structure of the generic active ingredient is identical to the structure of the Trulance® Product active ingredient. The demonstration should be made through the following methods and procedures:

1. Peptide mapping analysis

Peptide mapping of the generic active ingredient must be performed in reduced and non-reduced forms of those ingredients.

2. Amino acid analysis

Amino acid analysis must be performed on the reduced form of the generic active ingredient. It is suggested to perform an automated Edman degradation-HPLC analysis using a peptide sequencer. The methods for those analyses are well established.

3. Intact mass analysis

Intact and reduced liquid chromatography-mass spectrometry (LC-MS) molecular analysis of the generic active ingredient and comparison with the Trulance® Product active ingredient must be performed to confirm identical intact mass. The active ingredients should be desalted, separated by reverse-phase high performance liquid chromatography and identified using electrospray ionization mass spectrometry. High-resolution mass spectrometry will confirm the molecular formula of the generic active ingredient and allow comparison with the Trulance® Product active ingredient.[Li et al. 2020] Alternatively, matrix assisted laser desorption ionization – time of flight (MALDI-TOF) mass spectrometry may be used. Further, tandem mass spectrometry – LC-MS/MS – should be performed to provide a fingerprint comparison of peptide fragments to confirm sequence identity of the generic active ingredient and the Trulance® Product active ingredient.

4. Peptide concentration analysis

Peptide content of the generic active ingredient will determine the dose delivered by the generic formulation. Thus, for the active ingredient sameness requirement to be met, the peptide concentration of the generic active ingredient must be the same as the peptide content of the Trulance® Product active ingredient. The determination of peptide concentration of each active ingredient should be determined through UV-vis spectroscopy by measurement of the absorbance when irradiated with UV light at a wavelength of 280nm, coupled with quantitative HPLC or LC-MS to give a direct measure of the quantity of active agent present.

5. Secondary and tertiary structure analysis

The binding of plecanatide to the GUCY2C receptor and agonism of that receptor are critically dependent on the three-dimensional structure of the drug. This includes secondary structure (local conformational preferences) and tertiary structure (global conformational preference). For instance, only the 1-3, 2-4 disulfide regioisomer shows biological activity, whereas all other permutations are not biologically active.[Brancale et al. 2017] Similarly, in common with the natural ligand uroguanylin topological isomers even of the 1-3, 2-4 disulfide show differences in biological activity.[Marx et al. 1998] Thus, for the active ingredient sameness requirement to be met, the generic sponsor must demonstrate that the secondary and tertiary structures of the generic active ingredient are the same as the secondary and tertiary structures of the Trulance® Product active ingredient.

Secondary structure can be compared with plecanatide by three main methods: circular dichroism, infrared spectroscopy and NMR spectroscopy. UV circular dichroism may be used to assess distinctive optical rotation properties of local peptide conformers when irradiated with polarized light. Fourier-transformed infrared spectroscopy (FT-IR) may be used to assess conformational sameness, in particular analysis of nitrogen-hydrogen (N-H) bond stretches which are distinctive in hydrogen-bonded conformers and also the region between 1500 and 400 cm^{-1} (fingerprint region) which can be directly compared between the generic and Trulance® Product active ingredients. ^{1}H NMR spectroscopy techniques may also be used to compare secondary structure of the generic active ingredient to the plecanatide active ingredient – particularly analysis of the chemical shift of N-H protons which are characteristic of secondary structure motifs.

Tertiary structure can be compared with plecanatide by two main methods: NMR spectroscopy[Chino et al. 1998] and differential scanning calorimetry/fluorimetry.[Johnson 2013] 2D NMR techniques such as Nuclear Overhauser Effect Spectroscopy (NOESY) and Total Correlation Spectroscopy (TOCSY) are recommended to determine tertiary structure (and also confirm secondary structure) through a measurement of close interactions formed between amino acid residues within the peptide, formed as a result of conformational preferences. A direct comparison can be made between spectra from a generic active ingredient and the Trulance® Product active ingredient. The use of differential scanning calorimetry (DSC) measures the thermal stability of a peptide towards unfolding. The temperature at which unfolding is observed will depend on the tertiary structure of the peptide. Thus, a comparison of DSC thermal stability profiles will demonstrate whether a generic active ingredient is identical in its tertiary structure to the Trulance® Product active ingredient. Differential scanning fluorimetry could be used as an alternative technique. It is a similar technique to DSC but relies on the use of a fluorescent dye to detect unfolded protein.

6. Disulfide regioisomer identification

As described above, plecanatide is only active in an oxidized form where two disulfide bonds are formed between four cysteine amino acid residues. Of all the possible permutations, only the 1-3, 2-4 disulfide regioisomer shows biological activity: other regioisomers have been shown to be inactive.[Marx et al. 1998] Thus, it is necessary to unambiguously establish which disulfide isomer is

present, and whether any inactive disulfide isomers are present as contaminants. Disulfide regioisomers may be distinguished by HPLC-low energy collision induced dissociation tandem mass spectrometry, a technique which allows the separation of disulfide regioisomers and preserves the disulfide linkages in the mass spectrometer.[Bradock 1998]

7. pH-specific conformation analysis

Sameness testing must demonstrate that the generic active ingredient's active conformations at pH 5 and pH 8 are the same as the Trulance® Product active ingredient's active conformations at those pH values. This is because plecanatide from the Trulance® Product maximally binds to the GUCY2C receptor in the duodenum's pH 5 environment, but binds with lower affinity to that receptor in the lower GI tract's pH 8 environment.[Brancale et al. 2017] This can be performed using two techniques: NMR analysis to quantify relative proportions of active and inactive conformers,[Chino et al. 1998] and HPLC –ESI-mass spectrometry.[Moss et al. 2009] This pH-dependent conformational shift can be also monitored by NMR and HPLC-mass spectrometry as discussed above.

8. Peptide size and purity analysis

The final step in plecanatide synthesis is the oxidation of the cysteine residues to produce the 1-3, and 2-4 disulfide linkages. This step can produce not only the inactive disulfide regioisomers as biproducts, but also intermolecular disulfide bonds leading to oligomers or polymers which are also inactive. The identity and quantity of these potential contaminant impurities can therefore influence the drug's efficacy.[Liu 2019] With respect to the efficacy issue – if there is a smaller contribution of appropriately disulfided peptide, then by simple proportionality there are going to be lower concentrations of active agent per weight of material (per "dose") making it, by definition, of lower efficacy. Sameness of peptide size can be determined by techniques including reduced and non-reduced capillary electrophoresis, and size exclusion chromatography coupled to UV, dynamic light scattering and mass spectrometry detection. This latter technique also allows a quantification of purity as it will provide the proportion of aggregates, oligomers formed through intermolecular disulfide formation and also fragments all of which are inactive. Overall charge can be determined by techniques including capillary isoelectric focusing, which is a high resolution technique that distinguishes peptides based on their isoelectric point (the pH at which they bear a net charge of 0), and HPLC ion exchange chromatography, a technique which separates peptides based on charge.

B. In vitro biological activity analyses

This section addresses point 3 of the Plecanatide PSG's sameness testing recommendations. As introduced above, the GUCY2C receptor agonists such as plecanatide induce fluid and electrolyte secretion into the GI lumen through a multi-step mechanism of action, in which the steps are discrete, yet interdependent.[Kuhn 2016; Lucas et al. 2000] In the first step, plecanatide binds to the extracellular domain of the GUCY2C receptor (Step 1 – ligand binding).[Cohen et al. 1993; Cohen et al. 1987; Crane et al. 1992; Deshmane et al. 1995; Deshmane et al. 1997; Guarino et al. 1987; Hugues et al. 1991; Ivens et al. 1990; Mezoff et al. 1992] In the second step, plecanatide-GUCY2C interaction is coupled to activation of cyclic cGMP production (Step 2 – cGMP production).[Carr et al. 1989; Cohen et al. 1993; Crane et al. 1992; Field 1979a, 1979b; Field et al. 1978; Gazzano et al. 1991; Hakki et al. 1993; Hugues et al. 1992; Mezoff et al. 1992; Parkinson et al. 1997; Parkinson et al. 1994; Parkinson

& Waldman 1996; Pattison et al. 2016; Rao et al. 1980; Rao et al. 1981; Vaandrager, van der Wiel, et al. 1993; Waldman et al. 1986; Zhang et al. 1999 In the third step, cGMP signals inside the cell through cGMP-dependent protein kinase II to activate the CFTR to stimulate chloride secretion, and block the NHE3 to stop sodium absorption (Step 3 – ion flux modulation). Those steps are necessary to creating the osmotic drive needed to secrete water into the GI lumen (Secretion Result – luminal secretion). Chen et al. 2019; Das et al. 2018; Field 1979a, 1979b; Field et al. 1978; Guandalini et al. 1982; Mezoff et al. 1992; Parkinson et al. 1997; Pattison et al. 2016; Rao et al. 1981; Vaandrager et al. 1997; Vaandrager et al. 2000; Waldman et al. 1986 In order for a plecanatide active ingredient to relieve constipation, all of the Steps must be operating and the Secretion Result must be achieved.

In order to demonstrate active ingredient sameness, a generic sponsor must demonstrate that the Proposed Generic matches the Trulance® Product's performance at Steps 1 and 2, as well as in the magnitude of the Secretion Result. The metrics to be tested are: binding affinity, cGMP production and luminal secretion (the "Sameness Metrics"). Testing of the three metrics are necessary for at least four reasons.

First, plecanatide active ingredients, particularly in their dosage forms, are not necessarily molecularly or physicochemically identical. This is especially true when they are produced by different manufacturing methods and in different facilities. Even if physicochemical sameness of the active ingredient of the Proposed Generic to the active ingredient of the Trulance® Product is demonstrated using the methods described in Section 1 above, this alone is not sufficient to support pharmacological sameness (or biological sameness). Pharmacological action of a drug is also very well known to be dependent on other factors such as formulation, drug polymorphism etc. It is very well known that differences in formulation can impact properties such as speed of dissolution, onset of action and pharmacokinetic profile, all of which in turn can impact bioactivity. Levy & Nelson 1961 Even when the excipients and formulation of a drug are identical, differences in polymorphism (i.e. the solid state structure of the drug) are also known to impact activity through altered dissolution behavior, pharmacokinetic and pharmacodynamic behaviors. Raza 2014; Zhou et al. 2018

Second, the Steps are inter-dependent, each relying on the activity of the others; however, equivalent performance of a Proposed Generic at one step does not ensure equivalent performance at subsequent steps.

Third, Step 1 and Step 2 are dependent on the molecular structure and physicochemical conformation of the plecanatide active ingredient. A demonstration of a Proposed Generic's binding to the GUCY2C receptor alone is not sufficient, as it is well known in pharmacology that a drug binding to a receptor can lead to different functional responses (agonism, antagonism or neutral). In that context, GUCY2C receptors can be prepared from rodent intestine or human colon cells that bind to agonists, but remain silent with respect to the catalytic production of cGMP (SA Waldman, personal communication). To demonstrate pharmacological sameness it is therefore crucial that a Proposed Generic is shown to also act as a functional agonist at GUCY2C through stimulating cGMP production.

Fourth, the exact mechanisms linking the subsequent Steps to the Secretion Result remain undefined. Thus, the magnitude of the Proposed Generic's Secretion Result must be empirically assessed.

The Proposed Generic's performance must match the Sameness Metrics at all pH ranges present in the GI lumen, in vivo. Plecanatide is a structural analog of uroguanylin, in which the glutamic acid in the fourth position has been replaced with an aspartic acid residue.[Shailubhai et al. 2013; Waldman & Camilleri 2018] As discussed above, this substitution creates an "acidic conformational switch" at GUCY2C receptor sites of action: Exposure of the peptide to the acidic pH environment of the proximal duodenum induces a conformational change in the peptide that is particularly favorable for GUCY2C receptor binding and stimulation.[Brancale et al. 2017; Hamra et al. 1997; Shailubhai et al. 2013; Shailubhai et al. 2015; Waldman & Camilleri 2018] This acidic switch is what makes plecanatide especially fit for purpose.[Brancale et al. 2017; Hamra et al. 1997; Shailubhai et al. 2013; Shailubhai et al. 2015; Waldman & Camilleri 2018] Plecanatide is highly active in the acidic duodenal environment, inducing the Secretion Result that is associated with constipation relief, but losing activity further down the length of the bowel where the pH environment is more neutral.[DeMicco et al. 2017; Quigley & Neshatian 2016; Shailubhai et al. 2013; Waldman & Camilleri 2018] Thus, to meet the sameness requirement, Proposed Generics must match the Trulance® Product's performance on the Sameness Metrics at all pH ranges encountered in the GI lumen.

1. **Step 1 – Ligand binding analysis: To satisfy the active ingredient sameness requirement, the Proposed Generic active ingredient must have binding affinity for duodenal GUCY2C receptors equal to that of the Trulance® Product active ingredient.**

Activation of cGMP production and fluid and electrolyte secretion are directly related, and quantitatively proportional, to ligand receptor occupancy.[Bryant et al. 2010; Busby et al. 2010; Carr et al. 1989; Cohen et al. 1993; Cohen et al. 1989; Crane et al. 1992; Field et al. 1978; Hakki et al. 1993; Mezoff et al. 1992; Pattison et al. 2016; Vaandrager, van der Wiel, et al. 1993] Essentially, the more GUCY2C receptors that are occupied by a ligand such as plecanatide, the more cGMP is produced. GUCY2C ligands bind to the receptor with an affinity that is defined by the equilibrium dissociation constant (K_D).[Bryant et al. 2010; Busby et al. 2010; Carr et al. 1989; Cohen et al. 1993; Cohen et al. 1989; Cohen et al. 1987; Crane et al. 1992; Deshmane et al. 1995; Deshmane et al. 1997; Field et al. 1978; Guarino et al. 1987; Hakki et al. 1993; Hugues et al. 1991; Hugues et al. 1992; Mezoff et al. 1992; Pattison et al. 2016; Vaandrager, van der Wiel, et al. 1993; Waldman & O'Hanley 1989] This is the concentration of ligand resulting in 50% receptor occupancy at equilibrium and it is a unique characteristic of a ligand-receptor pair.[Waldman 2009] The lower the K_D, the higher the receptor occupancy and activation, and the higher the cGMP production.[Bryant et al. 2010; Busby et al. 2010; Carr et al. 1989; Cohen et al. 1993; Cohen et al. 1989; Cohen et al. 1987; Crane et al. 1992; Deshmane et al. 1995; Deshmane et al. 1997; Field et al. 1978; Guarino et al. 1987; Hakki et al. 1993; Hugues et al. 1991; Hugues et al. 1992; Mezoff et al. 1992; Pattison et al. 2016; Vaandrager, van der Wiel, et al. 1993; Waldman & O'Hanley 1989]

K_D values translate directly to the potency and efficacy of the ligand: if two different ligands for the same receptor have different K_D's, they will have different potencies.[Waldman 2009] Because plecanatide's K_D depends on its molecular structure, conformation and orientation at the receptor site, a Proposed Generic active ingredient with a K_D different than that of the Trulance® Product active ingredient cannot be verified as having the Trulance® Product active ingredient's

molecular structure or conformational preference. Thus, the Proposed Generic must be demonstrated to have the same K_D as the Trulance® Product in order for the sameness requirement to be met.

As noted previously, plecanatide is highly active in the acidic duodenal environment, inducing water secretion that is associated with constipation relief, but losing activity further down the length of the bowel where luminal pH is more neutral. DeMicco et al. 2017; Quigley & Neshatian 2016; Shailubhai et al. 2013; Waldman & Camilleri 2018 Thus, to meet the sameness requirement, Proposed Generics must have the same K_D values in all of the GI luminal pH environments encountered in vivo.

To compare K_Ds of Proposed Generics to the Trulance® Product, assessments should be performed in cell membranes prepared from human small intestinal epithelial cells (the target for fluid secretion by uroguanylin and plecanatide),Brancale et al. 2017; Forte 1999; Hamra et al. 1997; Shailubhai et al. 2013 rather than from mice or from human colorectal cancer cells in culture. This is because it remains unknown whether the precise structure and function of GUCY2C receptors from these sources are identical to the structure and function of GUCY2C receptors in human small intestinal cells.Cohen et al. 1989; Crane et al. 1992; Deshmane et al. 1995; Hugues et al. 1991; Ivens et al. 1990; Vaandrager, Schulz, et al. 1993; Vaandrager et al. 1994 For example, the heterogeneous glycosylated isoforms of the GUCY2C receptorsIvens et al. 1990; Vaandrager, Schulz, et al. 1993 in small intestine are not identical to those expressed in the colorectum (Scott A. Waldman, unpublished data shown in Figure 4).

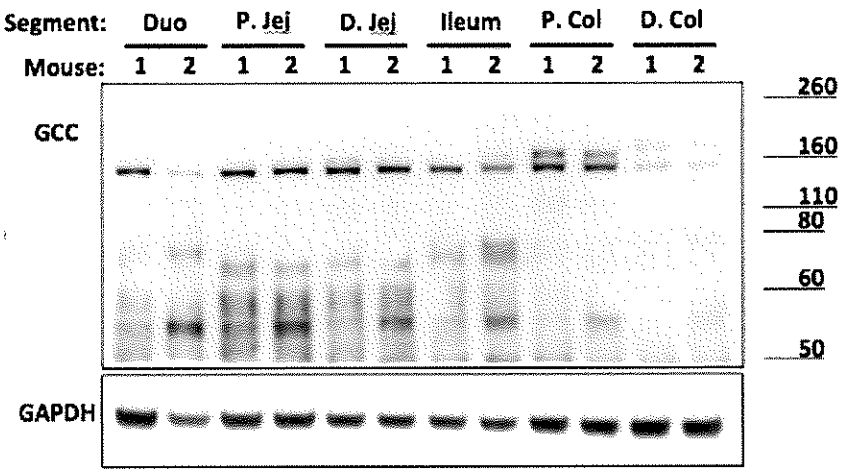

Figure 4. Immunoblot of GUCY2C glycoforms along the rostral-caudal axis of the intestine. Duo, duodenum; P. Jej, proximal jejunum; D. Jej, distal jejunum; P. Col, proximal colon; D. Col, distal colon. Note there is a single glycoform in the small intestine, but two glycoforms in the colon.

Finally, the assessments must be performed using small intestinal cells from multiple different patients to insure that results are generalizable to the user population. The following equilibrium binding analyses should be required:Crane et al. 1992

a. Membrane and ligand preparation

Intestinal epithelial cell membranes are prepared, resuspended at a protein concentration of 8-12 mg/ml, and stored at -70°C, in aliquots, until use. Peptide ligands (e.g., plecanatide) are iodinated to a specific activity of 1000 Ci/mmol and purified by HPLC. Radioiodinated ligands are tested for full efficacy and potency in assays of guanylyl cyclase activation.[Field et al. 1978; Ivens et al. 1990]

b. Binding assays

Binding assays are performed in buffer containing 50 mM Tris - HC1, pH 7.6 or pH 5.0, 0.67 mM, cystamine, 150 mM NaCl, 0.1% bacitracin, and 1 mM EDTA (standard binding buffer).

c. Kinetic binding assays

Association (on-rate) and dissociation (off-rate) kinetics of ligand binding are measured at 37°C unless stated otherwise. Association kinetics are initiated by addition of membranes, and bound ligand is measured at different times by filtration of an aliquot of incubation mixture (containing about 250,000 cpm) on Whatman GF/B filters that have been presoaked in 0.3% polyethylenimine. Filters are washed three times with 5 ml of ice-cold 20 mM phosphate buffer, pH 7.2, containing 150 mM NaCl and 1 mM EDTA, and bound radioactivity is quantified using a gamma counter. After equilibrium is achieved, dissociation is initiated by the addition of excess unlabeled toxin (0.25 μM). Part of the binding mixture does not receive unlabeled ligand, to assess the stability of binding, which should remain stable throughout the course of dissociation. Dissociation of labeled ligand from receptors is followed by measurement of the decrease in bound labeled ligand, by the filtration method described above. In some experiments, reaction mixtures containing membranes are incubated for up to 2 h at 37°C before initiation of association by addition of labeled ligand. Experiments utilizing multiple ligand concentrations are performed essentially as described above, except that ligand concentrations are varied from 1.0 to 6.8 nM. Specific binding is obtained by subtracting nonspecific binding, determined in parallel experiments conducted in the presence of a 100-fold excess of unlabeled ligand, from total binding. Nonspecific binding should remain constant throughout the course of the experiment.

d. Equilibrium binding analysis

Intestinal membranes (0.67 mg of protein/ml) are incubated in the presence of increasing concentrations of labeled ligand, from 100 pM to 100 nM. Reactions are performed in duplicate, incubated at 37°C for 3 h to reach equilibrium, and terminated by the filtration technique described above. Nonspecific binding is determined by the addition of excess unlabeled ST (0.25 μM) to parallel incubations.

e. Miscellaneous

Protein is measured using bovine serum albumin as standard. Binding curves are fitted and kinetic constants determined using GraphPad Prism Mean values ± standard deviations are reported unless stated otherwise.

2. Step 2 – cGMP production analysis: To satisfy the active ingredient sameness requirement, the Proposed Generic active ingredient must induce cGMP production equal to that of the Trulance® Product active ingredient.

Once plecanatide binds to the GUCY2C receptor, ligand-receptor interaction is transduced to activation of the GUCY2C catalytic domain and cGMP production, in proportion to the fractional occupancy of receptors. Bryant et al. 2010; Busby et al. 2010; Carr et al. 1989; Cohen et al. 1993; Cohen et al. 1989; Cohen et al. 1987; Crane et al. 1992; Deshmane et al. 1995; Deshmane et al. 1997; Field et al. 1978; Guarino et al. 1987; Hakki et al. 1993; Hugues et al. 1991; Hugues et al. 1992; Mezoff et al. 1992; Pattison et al. 2016; Vaandrager, van der Wiel, et al. 1993; Waldman & O'Hanley 1989 Production of cGMP is dependent on receptor binding affinity, hence the ligand's molecular structure and physicochemical conformation. Thus, a Proposed generic cannot meet the active ingredient sameness requirement unless its cGMP production is demonstrated to be the same as that from the Trulance® Product.

Similarity of cGMP production is assessed by comparing the active ingredients' EC_{50}, the concentration producing 50% maximum cGMP response and the active ingredients' E_{MAX}, the concentration producing maximum production of cGMP. Carr et al. 1989; Crane et al. 1992; Field et al. 1978; Gazzano et al. 1991; Hakki et al. 1993; Hugues et al. 1991; Pattison et al. 2016; Waldman et al. 1986 Given plecanatide's acidic conformation switch, EC_{50} and E_{MAX} values must be assessed for similarity at all relevant GI luminal pH values. Hamra et al. 1997

These assessments should be performed in organoids Pattison et al. 2016 prepared from human small intestinal epithelial cells (the target for fluid secretion by uroguanylin and plecanatide), Brancale et al. 2017; Forte 1999; Hamra et al. 1997; Shailubhai et al. 2013 rather than from mice or from human colorectal cancer cells in culture. This is because it remains unknown whether the precise structure and function of GUCY2C receptors from these sources are identical to GUCY2C receptors in human small intestinal cells. Cohen et al. 1989; Crane et al. 1992; Deshmane et al. 1995; Hugues et al. 1991; Ivens et al. 1990; Vaandrager, Schulz, et al. 1993; Vaandrager et al. 1994 As discussed above, the heterogeneous glycosylated isoforms of GUCY2C receptors in small intestine are not identical to those expressed in the colorectum. Of course, the assessments may be conducted in small intestinal cells from multiple patients to insure generalizability to the user population.

The following cGMP production analyses should be required:

a. Crypt isolation and enteroid culture from human small intestine Pattison et al. 2016

Enteroids from fresh small intestine segments from surgical waste. Tissue samples are minced, vortexed in phosphate-buffered saline (PBS) to remove debris, transferred to 30 ml of ice-cold Dulbecco's PBS (DPBS) supplemented with 8 mM EDTA and 1 mM dithiothreitol, and incubated on a rocker at 4°C for 30 min. Supernatants are discarded and replaced with 30 ml ice-cold DPBS and vortexed vigorously 10 times at 3 s per pulse, and supernatants were collected. This procedure is repeated six times, yielding 6 crypt-containing fractions. Fractions are centrifuged at 100 × g for 5 min and resuspended in 2 ml of DPBS supplemented with 10% fetal bovine serum to remove contaminating villi. Fractions are combined and centrifuged for 2 min at 100 × g, and supernatants are removed and immediately centrifuged at 120 × g for 2 min. Supernatants are removed and pellets were pooled in 5 ml of resuspension medium (advanced

Dulbecco's modified Eagle's medium [DMEM]/Ham's F-12, 2 mM GlutaMAX, 10 mM HEPES, 1% penicillin-streptomycin). Pelleted crypts are resuspended in GFR matrigel matrix (Corning), and 250 crypts in 50 µl of matrigel are seeded per well in a 24-well plate and incubated at 37°C for 10 to 15 min. Cultures are then overlaid with 500 µl of complete medium (Advanced DMEM/Ham's F-12, 2 mM GlutaMAX, 10 mM HEPES, 1% penicillin-streptomycin) supplemented with 1× N-2 (Life Technologies, Carlsbad, CA), 1× B-27 (Life Technologies), 1 mM N-acetyl-l-cysteine, Wnt3a-conditioned medium (1:1; from CRL-2647 cells [ATCC]), 50 ng/ml mouse recombinant epidermal growth factor (catalog number 315-09; PeproTech, Rocky Hill, NJ), 100 ng/ml mouse recombinant Noggin (catalog number 250-38; PeproTech), 1 µg/ml human recombinant R-spondin 1 (catalog number 4645-RS-025/CF; R&D Systems), 500 nM A83-01 (Sigma), 10 µM SB202190 (catalog number S7067; Sigma), 10 nM gastrin (catalog number G9145; Sigma), and 10 mM nicotinamide (catalog number NO636; Sigma). Human enteroids are maintained at 37°C in a 5% CO2 atmosphere for at least 7 days before passaging or peptide stimulation. Enteroids from passages 1 to 3 are used for peptide stimulation assays.

b. <u>Competitive cGMP ELISA</u>

To improve assay sensitivity and prevent cGMP degradation, enteroids are pretreated with a phosphodiesterase inhibitor (3-isobutyl-1-methylxanthine [IBMX]; 1 mM) for 1 h at 37°C. Since most of the cGMP generated by enteroids in response to GUCY2C activation is secreted into the medium, medium samples are tested for all cGMP quantification determinations. Following pretreatment, medium samples are collected and replaced with fresh DMEM/IBMX mixture, either at pH 7.6 or pH 5.0, containing increasing concentrations of ligands (100 pM-1 µM) and incubated for an additional 2 h at 37°C. Medium samples from treated enteroids and cGMP standards are subjected to acetylation prior to analysis to improve assay sensitivity. Cyclic GMP is quantified by ELISA. EC_{50} and E_{MAX} are quantified using GraphPad Prism.

3. <u>Secretion Result – Luminal secretion analysis: To satisfy the active ingredient sameness requirement, the Proposed Generic active ingredient must produce luminal secretion equal to that of the Trulance® Product active ingredient.</u>

The integrated response to GUCY2C agonist binding, cGMP production, and ion flux production is the secretion of fluid.[Busby et al. 2010; Das et al. 2018; DeMicco et al. 2017; Guandalini et al. 1982; Kuhn 2016; Lucas et al. 2000; Mezoff et al. 1992; Pattison et al. 2016; Quigley & Neshatian 2016; Shailubhai et al. 2013; Waldman & Camilleri 2018; Waldman & O'Hanley 1989; Waldman et al. 1994; Zhang et al. 1999] It is this fluid secretion that mediates the relief of constipation in patients.[Busby et al. 2010; DeMicco et al. 2017; Guandalini et al. 1982; Kuhn 2016; Lucas et al. 2000; Quigley & Neshatian 2016; Shailubhai et al. 2013; Waldman & Camilleri 2018]

Quantitative metrics include the EC_{50}, the concentration of agent producing 50% maximum secretion, and , E_{MAX}, the concentration producing maximum secretion.[Busby et al. 2010; Cohen et al. 1989; Mezoff et al. 1992; Pattison et al. 2016; Waldman et al. 1994; Zhang et al. 1999] Given plecanatide's acidic conformation switch, ion flux EC_{50} and E_{MAX} values must be assessed for similarity at all relevant GI luminal pH values.

One analytical approach is to compare the pharmacological characteristics of generic and reference compounds in pre-clinical animal models of intestinal secretion. [Busby et al. 2010; Cohen et al. 1989; Mezoff et al. 1992; Pattison et al. 2016; Sharman et al. 2017; Waldman et al. 1994; Zhang et al. 1999] Models could include the suckling mouse model, the rodent ileal loop model, and the mouse intestinal transit model. [Busby et al. 2010; Cohen et al. 1989; Mezoff et al. 1992; Pattison et al. 2016; Sharman et al. 2017; Waldman et al. 1994; Zhang et al. 1999] All of these models assess the clinical properties of GUCY2C peptides to induce secretion and are amenable to quantification of EC_{50} and E_{MAX} for each peptide.

Regardless, it would be more relevant to measure these pharmacological characteristics of fluid secretion in human intestinal cells ex vivo, which are amenable to interrogating these characteristics at the relevant pH ranges. Recent work revealed that 3-dimensional ex vivo cultures of human intestinal epithelial cells (organoids) recapitulate the heterogeneity of intestinal epithelia cell diversity and anatomical structure.[Dekkers et al. 2013; Foulke-Abel et al. 2016; In et al. 2016; Kovbasnjuk et al. 2013; Sato & Clevers 2013; Zachos et al. 2016] Indeed, these organoids ("mini-guts") form intestinal lumen structures that mediate vectoral water and electrolyte transport into that lumen, closely mimicking the structure and function of intestinal epithelium in vivo.[Dekkers et al. 2013; Foulke-Abel et al. 2016; In et al. 2016; Kovbasnjuk et al. 2013; Pattison et al. 2016; Sato & Clevers 2013; Zachos et al. 2016] Also, recent work revealed the utility of these organoids to quantify fluid secretion of GUCY2C receptor agonists.[Pattison et al. 2016]

Thus, the assessments should be performed in human small intestinal epithelial organoids. Quantification of the EC_{50} and E_{MAX} of generic and reference GUCY2C receptor agonists for stimulating fluid secretion should be determined in small intestinal epithelial organoids from different patients to ensure generalizability to the user population. As above, the analyses should be performed in all relevant GI luminal pH ranges.

More specifically, the following analyses should be performed:

a. <u>Crypt isolation and enteroid culture from human small intestine[Pattison et al. 2016]</u>

Enteroids from fresh small intestine segments from surgical waste. Tissue samples are minced, vortexed in phosphate-buffered saline (PBS) to remove debris, transferred to 30 ml of ice-cold Dulbecco's PBS (DPBS) supplemented with 8 mM EDTA and 1 mM dithiothreitol, and incubated on a rocker at 4°C for 30 min. Supernatants are discarded and replaced with 30 ml ice-cold DPBS and vortexed vigorously 10 times at 3 s per pulse, and supernatants were collected. This procedure is repeated six times, yielding 6 crypt-containing fractions. Fractions are centrifuged at 100 × g for 5 min and resuspended in 2 ml of DPBS supplemented with 10% fetal bovine serum to remove contaminating villi. Fractions are combined and centrifuged for 2 min at 100 × g, and supernatants are removed and immediately centrifuged at 120 × g for 2 min. Supernatants are removed and pellets were pooled in 5 ml of resuspension medium (advanced Dulbecco's modified Eagle's medium [DMEM]/Ham's F-12, 2 mM GlutaMAX, 10 mM HEPES, 1% penicillin-streptomycin). Pelleted crypts are resuspended in GFR matrigel matrix (Corning), and 250 crypts in 50 µl of matrigel are seeded per well in a 24-well plate and incubated at 37°C for 10 to 15 min. Cultures are then overlaid with 500 µl of complete medium (Advanced DMEM/Ham's F-12, 2 mM GlutaMAX, 10 mM HEPES, 1% penicillin-streptomycin)

supplemented with 1× N-2 (Life Technologies, Carlsbad, CA), 1× B-27 (Life Technologies), 1 mM N-acetyl-l-cysteine, Wnt3a-conditioned medium (1:1; from CRL-2647 cells [ATCC]), 50 ng/ml mouse recombinant epidermal growth factor (catalog number 315-09; PeproTech, Rocky Hill, NJ), 100 ng/ml mouse recombinant Noggin (catalog number 250-38; PeproTech), 1 µg/ml human recombinant R-spondin 1 (catalog number 4645-RS-025/CF; R&D Systems), 500 nM A83-01 (Sigma), 10 µM SB202190 (catalog number S7067; Sigma), 10 nM gastrin (catalog number G9145; Sigma), and 10 mM nicotinamide (catalog number NO636; Sigma). Human enteroids are maintained at 37°C in a 5% CO2 atmosphere for at least 7 days before passaging or peptide stimulation. Enteroids from passages 1 to 3 are used for peptide stimulation assays.

b. Secretion Assay[Pattison et al. 2016]

Established enteroids from a 5- to 7-day-old culture are seeded into a 48-well plate in 30 µl matrigel and 300 µl of IntestiCult medium, at a concentration of 250 to 500 crypts per well. One week after seeding, enteroids are incubated for 60 min with various concentrations of GUCY2C-stimulating ligands or the cell-permeable cGMP homolog, 8-Br-cGMP (Sigma; positive control) in DMEM at pH 7.6 or pH 5.0. For CFTR inhibition experiments, mouse and human enteroids are preincubated for 2 to 3 h with 50 µM CFTRinh-172 (Sigma), followed by addition of increasing concentrations of GUCY2C-stimulating ligands to human enteroid cultures (should inhibit fluid secretion). Control and stimulated enteroids are imaged using an Evos FL microscope (Life Technologies) and analyzed using ImageJ. Enteroid area (measure of fluid secretion) is calculated using ImageJ and converted to volume $[4/3\pi[\sqrt{(area/\pi)}]^3]$. The relative increase in volume of individual enteroids is compared to time zero measurements for each enteroid.

VII. Application to the Trulance® Product indications

The testing presented in these Comments is necessary to ensure that Proposed Generics will have pharmaceutical equivalence sufficient to ensure therapeutic equivalence to the Trulance® Product in the treatment of CIC symptoms.

In addition to treatment of CIC, the Trulance® Product is approved for treatment of IBS-C. The Plecanatide PSG does not address the Trulance® Product's IBS-C indication. As discussed above:

- Abdominal pain is the symptom that differentiates IBS-C from CIC.
- IBS-C patients experience abdominal pain regardless of whether they achieve relief of other constipation symptoms.
- The nature and extent of GUCY2C signaling is determined by the molecular and physicochemical structure of the plecanatide active ingredient.

Additionally, GUCY2C signaling attenuates the sensation of abdominal pain through several mechanisms, in addition to enhanced secretion:

> The [GUCY2C] signaling axis modulates afferent pathways involved in GI pain sensation. . . . The intracellular cGMP that is produced on ligand binding to GC-C on epithelial cells can be transported into the extracellular space through MRP4 cyclic nucleotide efflux pumps located on the basolateral membrane, decreasing conduction of submucosal afferent nociceptive neurons and attenuating the sensation of visceral pain. Also, GC-C signaling may contribute to analgesia indirectly by reducing inflammation through maintenance of the intestinal barrier, restricting luminal factors from gaining access to nociceptive or immune mechanisms in the lamina propria and beyond. Waldman & Camilleri 2018 at 1548

Further, abdominal pain relief cannot be assessed by in vitro testing methods. Thus, for approval under the Plecanatide PSG's in vitro option,[22] Proposed Generics must match the Trulance® Product's performance on the Sameness Metrics by the methods discussed in these Comments. Failure to do so will raise the risk of therapeutic **in**equivalence in the treatment of abdominal pain (as well as the other constipation symptoms) from pharmaceutical **in**equivalence of Proposed Generic and Trulance® Product active ingredients.

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