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The Effects Of 5-Ht4 Receptor Agonists On Interleukin-10 Knockout Mice

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THE EFFECTS OF 5-HT4 RECEPTOR AGONISTS ON INTERLEUKIN-10 KNOCKOUT MICE

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Abstract

Recent studies have demonstrated that activation of the 5-HT4 receptors in the colonic mucosa can have healing and protective actions in experimental models of colitis. These actions include increased mucus secretion, increased epithelial proliferation, and enhanced epithelial migration. Since these studies involved chemically induced models of colitis, the current investigation was conducted to test whether a protective action of 5-HT4 receptor stimulation could be detected in Interleukin-10 knockout (IL-10 KO), which develop colitis spontaneously due to the absence of the anti-inflammatory cytokine, interleukin-10.

Upon weaning, the IL-10 knockout mice were separated into two groups: an agonist group and a vehicle control group. The agonist group received 1 mg/kg tegaserod in a vehicle consisting of 0.9% saline each day by enema of dimethyl sulfoxide (DMSO) in saline each day, while the control group received daily enemas of vehicle over the course of 21 days. Several outcome measures were used to assess the effectiveness of the treatment. To evaluate the severity of colitis, disease activity index was monitored, and histologic damage was blindly scored.

Administration of tegaserod by enema to the IL-10 KO mice had a significant protective effect on the treated mice. The disease activity index (DAI) of agonist treated mice was significantly better than that of vehicle treated mice over time ($p<0.001$; 2-way ANOVA). Mice treated with vehicle had a more significant decline in health over time versus the agonists, with more blood present in feces and a looser/diarrhea-like consistency in stool. The histological damage score (HDS) was also improved by 5-HT4 agonist treatment ($p<0.05$, t-test). Sections of vehicle treated colons showed significantly greater damage, including epithelial erosions, the presence of polymorphonuclear cells, and abnormal crypt architecture (cryptitis), than those treated with the 5-HT4 receptor agonist tegaserod. During the 21-day course of the current investigation, there was no difference in the survival data between the two groups.

These data, when taken together, suggest that administration of the 5-HT4 receptor agonist tegaserod via enema to IL-10 KO mice has a greater healing and protective effect than seen in the IL-10 KO mice that received vehicle. We proposed to test the hypothesis that treatment of IL-10 knockout colitis with a 5-HT4 receptor agonist will attenuate the development of colitis and have healing and protective effects in the colons of the treated mice.

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CHAPTER 1: COMPREHENSIVE LITERATURE REVIEW

The Gastrointestinal Tract:

The gastrointestinal (GI) tract is an important organ system that extends from the mouth to the anus. It includes the esophagus, stomach, the large intestine, and the small intestine. Within the small intestine, there is also the duodenum, jejunum, and ileum. The GI tract is also associated with other organs such as the liver, pancreas and gall bladder. In addition, sphincters, such as the lower esophageal sphincter, play important physiologic roles, such as the regulation of passage of gut contents between areas of the GI tract. Next, the wall of the GI tract is organized into three layers, with each layer composed of different cell types. While the arrangement of the layers of the gut wall and the cellular components is similar along the length of the gut, there are differences. An example is the arrangement of the epithelium and smooth muscle thickness in different regions of the gut. Many of the gut's functions, like digestion, absorption of nutrients, movement, and defecation, occur due to coordinated actions of gut cells like smooth muscle cells, intrinsic neurons and different kinds of epithelial cells. These epithelial cells include absorptive enterocytes, mucus secreting goblet cells, and enteroendocrine cells (Saffrey 2014).

Next, the communication between the GI tract and brain by way of extrinsic autonomic and sensory nerves, as well as the hormones that are produced by endocrine and enteroendocrine cells is important. This communication occurs through the gut-brain axis and is bidirectional. While extrinsic signals regulate gut functions, information relayed from the gut by enteric neurons, extrinsic sensory neurons, and

enteroendocrine cells has influence over some central nervous system (CNS) activities which has impacts on behaviors, including the regulation of appetite.

The primary function of the GI tract is to break down food into nutrients to be used to generate energy for survival and reproduction (Sharkey and Savidge 2014). A secondary function is the defense of the host from ingested harmful food antigens, as well as bacteria, parasites, and toxins (Sharkey and Savidge 2014). The defensive function of the GI tract aids in the protection of not only the organ, but the host, from harmful effects of digestion, such as extreme pH, digestive enzymes, bile, and other potentially damaging chemicals (Fasano and Shea-Donohue 2005, McCole and Barrett 2007, Turner 2009). Digestion commonly produces antigenic peptides that can be dangerous to the host and lead to intense immune responses if not carefully regulated.

Important components of the GI tract are the small and large intestines, as disorders of the intestines is prevalent, mostly in the form of Inflammatory Bowel Disease (IBD) and Irritable Bowel Syndrome (IBS). IBD is a chronic inflammatory condition that causes many issues, like potential for obstruction, formation of ulcers, and damage to the epithelium. These issues can be best visualized histologically.

The small intestine, which measures about 20 feet in length, is the longest portion of the GI tract, and it serves as the primary site of digestion and absorbtion. The small intestine is divided into three segments, the first, the duodenum starts at the pyloris of the stomach, and is connected to the posterior abdominal wall, this is where absorption begins the jejunum is the next segment and is connected to the distal end of the duodenum. The jejunum is suspended from the posterior wall on the mesentery

and is where products of digestion are absorbed into the blood stream. Following this is the ileum, which makes up a large portion of the small intestine (three-fifths) and is also supported by mesentery. The ileum absorbs any remaining nutrients not absorbed by the jejunum, as well as bile acids. The small intestine is segmented in three parts because this allows for better breakdown and absorption of food and nutrients. The small intestine is organized primarily for the absorption of nutrients from food. Its efficacy in this matter is enhanced by many structural devices that increase the surface area. One such structural device is the length of the small intestine, at over 4 meters in length it is the longest portion of the GI tract. The surface area of the small intestine is further increased by transverse folds of the mucosa and submucosa called the plicae circulares, which project into the lumen. The plicae circulares are 8-10 mm in height, 3-4 mm thick, and are visible to the naked eye. The plicae circulares are enduring structures of the small intestine and are not flattened out by the distention of the intestine. They are most noticeable first in the first part of the jejunum, after which they diminish in number and height, and are not found in the ileum. The plicae circulares function to slow the passage of material through the intestine, and likely increase the surface area 3-fold. Small intestinal surface area is also increased by the intestinal villi. They are 0.1-0.5 mm in length, and are thin, leaf-like structures which project into the lumen. The intestinal villi are longest in the duodenum and proximal jejunum and decrease in length and become more cylindrical in the distal end of the jejunum and ileum. Villi are not present in the large intestine.

The large intestine, also called the colon, is shorter than the small intestine, measuring about the length of one's height. It is the primary site affected by the chronic inflammation that IBD is characterized by. There are seven regions within the

large intestine, in order as follows: the cecum, ascending colon, transverse colon, descending colon, sigmoid colon, rectum and anus. The function of the colon is to concentrate indigestible residues left over from food by absorbing water and electrolytes, and then moving these residues to the rectum to be eliminated. The mucosal surface is relatively smooth, containing no folds or villi. The surface epithelium, and the upper third of the glands, contains columnar absorptive cells and goblet cells. The lower two-thirds contain mostly goblet cells, and a small number of enteroendocrine cells and stem cells. The goblet cells are responsible for the secretion of mucus to coat the surface of the epithelium. This provides a protective, physical barrier between the epithelial surface and the microbes within the lumen of the colon, aids in the movement of content through the bowel. This secreted mucus also serves to protect the surface from damage that could be caused by increasingly concentrated contents. The lifespan of most epithelial cells is around 7 days. The stem cells divide often to generate new cells, which then move to a maturation area. At the maturation area the stem cells undergo structural and enzymatic maturation, and then provide the functional cell population of each region of the colon. The cells at the end of their lifespan are then shed in an area called the extrusion zone.

The layers of the colon wall include the mucosa, the submucosa, and the muscularis externa. Within these layers lie important histologic features of the colon that are affected by the characteristic chronic inflammation found in IBD. In the mucosa are crypts, which are test-tube like structures attached to the muscularis mucosae, a thin smooth muscle layer at the base of the mucosal layer. The crypts are covered by the colonic epithelium and contain a myriad of cell types like enteroendocrine cells, goblet cells, and enterocytes.

Histologic Features of Colonic Inflammation

Chronic inflammation, which is common in IBD, can damage many of these structures. In IBD, common histological changes include a disruption of the epithelium, an alteration in crypt architecture, such as branching crypts (cryptitis), the formation of abscesses, erosions, and ulcers. In addition, chronic inflammation can cause the infiltration of polymorphonuclear cells and mononuclear cells. One of the goals in the treatment of IBD is histological healing, or in other words, a return to a "healthy" appearance, as assessed by examination of histological sections of biopsy specimens (Marchal Bressenot, Riddell et al. 2015). In UC, this is the ultimate end goal, whereas in CD it is a minimum requirement for treatment. However, a generally accepted definition of histological healing does not currently exist. Though it should be noted that histological assessment, when used in drug trials, focuses primarily on improvement of inflammatory features and their regression. Histologic assessment of tissues is done with Hematoxylin and Eosin (H&E) stained sections of colon.

Examining the pathology of IBD relies mostly on two varieties of lesions which are architectural abnormalities, such as crypt branching or shortening, a decrease in the density of crypts, and an abnormal mucosal surface, and inflammatory features, such as an increase of mononuclear and polymorphonuclear cells in the lamina propria, and the presence of granulomas, ulcers and erosions (Jenkins, Balsitis et al. 1997, Geboes and Dalle 2002). It is important to note, however, that these histological features of IBD are variable over time as a result of the natural evolution of the disease, as well as the use of therapeutics. An example of the variable histologic features in IBD can be seen in UC, where, in the early stages, crypt distortion is not seen. Further, crypt architectural abnormalities can take up to 2

months to arise (Schumacher, Kollberg et al. 1994, Theodossi, Spiegelhalter et al. 1994). In addition, the decrease in the density of the crypts is generally not seen in the first week of UC and is present in nearly 75% of patients with UC in remission (Rubio, Johansson et al. 1982). Also, some histological features can help distinguish IBD patients from healthy subjects in nearly 90% of cases (Jenkins, Goodall et al. 1988).

In the realm of inflammatory features, increases in the cellularity of the lamina propria aids in discerning IBD afflicted patients from healthy subjects. Another histological feature used in this regard is an increase in plasma cells, where it was shown that there is an increase in IBD rectal samples as compared to controls (Scott, Goodall et al. 1983). While histological findings can aid in the identification of IBD, it should always be kept in mind that inflammatory features of IBD are not constant over time. This is evident in that granulomas, which are a distinct feature of CD, are not constantly present. They are generally seen in the early phases of the disease, and in children (Geboes and Dalle 2002, Rubio, Orrego et al. 2007).

In terms of disease activity, histological assessment is generally based on the combination of the presence of neutrophils and epithelial damage (Villanacci, Antonelli et al. 2013). Neutrophils can be recognized reliably, and they are known to release molecules that can damage tissue. It should be noted that the activity status of macrophages and lymphocytes cannot be assessed by H&E stained sections, and the influence of eosinophils remains unclear. However, elevated levels of eosinophils have been observed in colonic biopsy samples from patients with UC, and it has been noted that increased numbers of these eosinophils, as well as eosinophil-derived

granular proteins, correlate with morphological changes in the GI tract, disease severity, and GI dysfunction (Saitoh, Kojima et al. 1999, Carvalho, Elia et al. 2003).

Histologic assessment of mucosal healing is important. It is characterized by the resolution of abnormalities in crypt architecture and infiltration of inflammatory cells. Structurally, mucosal healing is associated with intact barrier function of the gut epithelium preventing the translocation of commensal bacteria and antigens in the lumen into the mucosa (Neurath 2012). The mucosa may, however, still show features of sustained damage, such as a decrease in crypt density, with cryptitis present (Price and Morson 1975, Rubio, Johansson et al. 1982). Though it should be noted that in some patients the mucosa appears to return to normal. Also, with mucosal healing, a reduction of epithelial regeneration generally reduces the depletion of mucin (Serafini, Kirk et al. 1981). The inflammatory cell infiltrate is variable in composition. It can be seen as a hypercellular lamina propria containing acute and chronic inflammatory cells, or a hypocellular lamina propria containing a reduction in the number of mononuclear cells. In remission, ultimately, the chronic inflammation decreases along with the basal plasmacytosis. With the persistence of a high number of eosinophils, and cellularity within the lamina propria gives a high risk of relapse (Schumacher, Kollberg et al. 1994, Bitton, Peppercorn et al. 2001, Azad, Sood et al. 2011). In terms of relapse predictions, there are certain histological features that may be indicative such as acute inflammatory cell infiltrate, the presence of crypt abscess, damaged surface epithelium, and the depletion of mucin (Riley, Mani et al. 1991).

Serotonin:

History:

Serotonin was first discovered in 1937 by Vittorio Erspamer. He first extracted it from rabbit gastric mucosa and called it “enteramine”. Nearly a decade later the team of Rapport, Page, and Green reported that they had isolated a compound found in bovine serum. They named it “serotonin” after its vasoconstrictive properties. A few years after the team of Rapport, Page, and Green reported their discovery, the structure was elucidated as the 5-Hydroxytryptamine (5-HT), and it was ultimately shown that enteramine and serotonin are one in the same. The name serotonin stuck simply because it was made available to researchers by the pharmaceutical company Upjohn Pharmaceuticals, referring to the compound as serotonin. In terms of function, Erspamer hypothesized that serotonin must be key in the gut, as he found it in the gastrointestinal tract of every vertebrate he studied. He also noted that the majority of serotonin is synthesized and contained within the gut.

Serotonin in the gut:

With a majority of our serotonin being found in the gut, it is important to understand serotonin’s functions there. Serotonin is present in the gut primarily in two sources. These are the enterochromaffin cells (EC cells) and myenteric neurons which project in descending pathways. The majority of the serotonin is contained in the EC cells (Mawe and Hoffman 2013). These two sources of serotonin are differentiated by their cellular location as well as their different synthetic pathways. In EC cells, located in the intestinal mucosa, serotonin is synthesized from L-tryptophan, and is mediated by the rate-limiting enzyme tryptophan hydroxylase 1 (TPH1). In neurons, however, serotonin is synthesis is mediated by tryptophan hydroxylase 2 (TPH2).

Neurons that contain serotonin, although small in amount (roughly 2% of myenteric neurons), have extensive projections, which suggests a role in modulating or initiating gut motility. Among these projections, those that could be considered most important in terms of gut motility would be those that project to other serotonergic neurons which would form a descending network of serotonergic neurons.

In terms of signaling, there are no intercellular degradative enzymes that degrade serotonin (Mawe and Hoffman 2013). This is because at physiological pH, serotonin is highly charged, and as such requires a transport mechanism to cross the plasma membrane where it can be degraded by intracellular enzymes like monoamine oxidase. In terms of reuptake, the serotonin selective reuptake transporter (SERT) is responsible for the reuptake of serotonin in not only the brain but in the gastrointestinal tract as well. As such, SERT is expressed by all of the epithelial cells in the intestinal mucosa (Mawe and Hoffman 2013). SERT internalizes serotonin through a sodium- and chloride-dependent mechanism. Due to the ubiquitous expression of SERT by the epithelial cells in the intestinal mucosa, SERT acts to remove serotonin from interstitial space, once released by the enterochromaffin cells. As such, SERT is critical in the role of local regulation of serotonin availability and action within the intestine.

Serotonin released from EC cells mediates many gastrointestinal functions such as motility, mucus secretion, perception of pain and nausea, and vasodilation (Mawe and Hoffman 2013). This is achieved through a diverse family of serotonin receptors, located on intrinsic and extrinsic nerve fibers that are located within the lamina propria. Serotonin interacts with many receptors that are present in the gut. There are 7 subtypes of serotonin, and within each subtype are many variants. Within

the 7 subtypes, the 5-HT3 and 5-HT4 families have been studied the most in terms of gut motility. In addition to these, the 5-HT7 family have also been hypothesized to have some hand in regulating gut motility, but their therapeutic usefulness and physiological role have yet to be elucidated. The two serotonin receptor subtypes that have been most extensively exploited as therapeutic targets in the gut are the 5-HT3 and 5-HT4 receptors.

5-HT3:

The 5-Hydroxytryptamine 3 (5-HT3) receptor is one of the subtypes of serotonin receptors mentioned above. It is part of the family of cys-loop ligand-gated ion channels. It is permeable to sodium, potassium, and calcium, and as such mediates rapid depolarizing responses in both pre and post synaptic neurons (Lummis 2012). In therapeutic use, 5-HT3 receptor antagonists block afferent and efferent synaptic transmission. In terms of a physiological role, the 5-HT3 receptor coordinates emesis and regulates motility and secretion within the gastrointestinal tract. As such, in therapeutic use the 5-HT3 receptor antagonist is used to combat nausea and vomiting common in cancer chemotherapy, radiation, and anesthesia. Also, the 5-HT3 receptor antagonist has found success in dealing with diarrhea predominant Irritable Bowel Syndrome. In the neurons where 5-HT3 is localized at resting membrane potential, the electrochemical gradient favors sodium and potassium. As such, 5-HT3 receptors depolarize neurons and mediate fast, excitatory synaptic transmission.

A large number of 5-HT3 receptors can be found in the gastrointestinal tract as well as the peripheral nervous system (Lummis 2012). The 5-HT3 receptors have their best documented effects in the gut, where they are said to regulate gastrointestinal motility and the vomiting reflex. They have also been indicated to

play a role in visceral pain and inflammation. In terms of the actual receptor, to date, 5 subunits have been cloned, so called A, B, C, D, and E. However, only the A and B subunits have been studied extensively. The 5-HT3 receptor has much in common with the cys-loop family to which it belongs: it is an integral membrane protein, pentameric in structure, containing a central ion channel pore. Mariq et al suggest the structure of the 5-HT3 receptor as a long extracellular N-terminus, four transmembrane spanning domains (TM1-TM4), and a short C-terminus. The N-Terminal domain would contain the recognition site for serotonin, the binding of which would occur through the interface of two subunits. With the homomeric A subunit of 5-HT3, there are five possible binding sites. However, only binding two molecules of agonist are necessary to open the channel and are sufficient to do so. Within these subunits are amino acid domains, “loops”, which participate in binding.

The 5-HT3 receptor has been studied in various animals (mouse, human, dog) and it has been noted that the receptors, in the animals studied, have similar ion permeabilities and current-voltage relationships (Machu 2011). Although the receptor orthologs have similar agonist potencies (including in serotonin) and affinities for numerous competitive antagonists, there are some differences in ligand recognition. In example, the human 5-HT3A receptor has a much lower affinity (1800 fold) for curare, a competitive antagonist of the 5-HT3 receptor than the 5-HT3A receptor found in mice (Machu 2011).

In terms of binding at the 5-HT3 receptor, when an agonist binds, it causes a conformational change in the receptor which results in channel opening. The site where the ligand binds is composed of loops from the principal face of one subunit and a series of β strands from the complementary face of an adjacent subunit. When

an agonist binds to the ligand binding domain, it is thought to initiate movement through bringing Loop C of the ligand binding domain from an uncapped and disengaged state to a capped and engaged position. Experimental evidence from several groups suggests that several “loops” are involved in the binding process. Loop 2, Loop 7 (the cys-loop), Loop 9 (from the opposing face of the ligand binding domain), and Loop 10 interact with transmembrane 2 – transmembrane 3 linker regions (Machu 2011). As these “loops” interacting with the transmembrane 2-3 linker region, they play key roles in transmitting agonist binding to channel opening. The transmission of agonist binding to channel opening is achieved by electrostatic and/or hydrophobic interactions, such that the movement of the transmembrane 2-3 linker moves the transmembrane-2, which is the channel lining element, resulting in channel opening.

Therapeutic Use of 5-HT3:

5-HT3 receptor antagonists are commonly used to treat various forms of nausea and vomiting. Vomiting is coordinated by neurons in the medulla oblongata. It is a complex process which requires stepwise gastric fundus relaxation, followed by intense contraction of abdominal, intercostal, and diaphragmatic muscles, then closing of the glottis, and relaxation of the upper esophageal sphincter prior to the expulsion of gastric contents (Machu 2011). The physiological responses leading to vomiting are caused by the activation of many efferent neurons, whose nuclei are spread through the medulla oblongata. Nausea and vomiting can be initiated by various factors. Harmful chemicals and toxins can act directly on the gastric and duodenal mucosa to stimulate abdominal vagal afferent nerves. These project to the dorsal vagal complex which contains the nucleus of the solitary tract, the dorsal motor

nucleus of the vagal nerve, and the area postrema. Circulating harmful chemicals act directly at the chemoreceptor trigger zone (CTZ) of the area postrema which is located on the floor of the fourth ventricle, an area of the CNS that is not bound by the blood-brain-barrier. Motion sickness and other vestibular disturbances stimulate nausea and vomiting. Changes in intracranial pressure, brain trauma, or lesions in/near the vomiting center can evoke vomiting, in addition, extreme emotion, bad odors, or hormonal changes associated with pregnancy can also elicit nausea and/or vomiting. Also, gastrointestinal dysfunction, such as gastroparesis, can produce nausea and vomiting.

In terms of the development of 5-HT3 receptor antagonists and the recognition that it plays a role in nausea and vomiting, these actions were discovered before the 5-HT3 receptor was identified as a ligand gated ion channel (Machu 2011). Two laboratories in the 1950's identified the 5-HT3 receptor through contraction of guinea pig ileum. Due to the ability of morphine to block the contractile response seen in this newly identified receptor, it was named "5-Hydrpxytryptamine M". In the mid-late 1970's Fozard and colleagues identified this 5-HT-M receptor in rabbit heart and set to investigating it. They observed that metoclopramide, a dopamine receptor antagonist, blocked the action of serotonin at the 5-HT-M receptor. This led to development of 5-HT-M selective agents. In the early 1980's, Florczyk and colleagues showed that a high dose of metoclopramide relieved cisplatin, a cancer chemotherapy drug, induced vomiting in ferrets. This then led two groups of investigators to show that an early 5-HT-M receptor antagonist, MDL 72222, was very effective in eliciting the same effect: reducing cisplatin-induced vomiting.

The mechanism, or mechanisms, by which 5-HT3 receptor activation causes nausea and vomiting could involve both a central, and peripheral, component. In the gastrointestinal tract, EC cells release serotonin in response to mucosal irritation, or cellular damage. The released serotonin binds to 5-HT3 receptors on vagal afferent nerves in the duodenal mucosa, which project to the dorsal vagal complex of the vomiting center. 5-HT3 receptors are on nerve terminals of the projecting vagal afferent nerves to the dorsal vagal complex. Post-synaptic 5-HT3 receptors could also be present in the dorsal vagal complex. Aside from the 5-HT3 receptor, there are three other classes of major neurotransmitter receptors that have been identified as targets for antiemetic drugs. These are the dopamine D2 receptor, the muscarinic M1 receptor, and the histamine H1 receptor. That there are so many drugs to combat nausea and vomiting (5-HT3 receptor antagonists, D2 receptor antagonists, antihistamines, and muscarinic M1 antagonists) elucidates that no single class of drugs can successfully stop the nausea and vomiting brought about by various causes. As such, the 5-HT3 receptor antagonists are primarily used to combat nausea and vomiting associated with chemotherapy, radiation treatment, and radiation surgery. The 5-HT3 receptor antagonists have little efficacy in treating other causes of emesis.

5-HT3 and Inflammatory Bowel Disease/Irritable Bowel Syndrome:

In addition to treating nausea and vomiting, 5-HT3 receptor antagonists have been used to combat diarrhea predominant Irritable Bowel Syndrome (IBS-D). IBS is a disorder characterized by periods of intense abdominal symptoms, followed by periods of no symptoms. Severe intestinal cramping or pain, in the absence of other disease, is the “calling card” of IBS. IBS can be accompanied by diarrhea, constipation, or an alternation of both. Over the period of 1 year, a patient must

experience at least 12 weeks of abdominal pain or discomfort, accompanied by at least 2 of the following 3 features: Abdominal distress relieved by defecation (1), abdominal distress accompanies a change in stool frequency (2), or there is an associated change in the appearance of the stool (3) (Machu 2011). IBS has sub-types, constipation predominant, diarrhea predominant, mixed, alternating, or unspecified, which is based upon the consistency of the stool of the patient. IBS is prevalent, with an occurrence of 10-20% worldwide.

Gastrointestinal function is regulated by intrinsic and extrinsic factors, which include endocrine and paracrine mediators (Machu 2011). In addition, the gastrointestinal tract is regulated by the sympathetic, parasympathetic, and enteric divisions of the autonomic nervous system. In the enteric nervous system, nerve plexuses form reflex arcs which coordinate activity within the gastrointestinal tract. The enteric nervous system can function independently of the parasympathetic and sympathetic nervous systems, however, the enteric nervous system modulated by their input. IBS has both altered motility (constipation, diarrhea), and increased visceral sensitivity. As there are a large number of hormones and neurotransmitters that are involved in gastrointestinal motility, as well as in transmitting sensory information within the gut, like pain, it should come as no surprise that there is a myriad of drug classes involved in the treatment of IBS, such as 5-HT4 agonists, 5-HT3 antagonists, anticholinergics, and probiotics. The underlying mechanisms surrounding IBS are not well understood. It is likely that they are multi-faceted, and likely differ based on the subtype of IBS a given patient has.

One of the roles of 5-HT3 receptors is to transmit sensory information to the central nervous system. This sensory information includes pain via spinal afferent

nerves, and non-painful sensory information such as the sensation of bloating done through parasympathetic afferent nerves (Gershon and Ratcliffe 2004). As such, the 5-HT3 receptor antagonist alosetron has been marketed as having the capability to reduce the visceral sensation that is associated with IBS-D.

5-HT4:

The 5-hydroxytryptamine 4 (5-HT4) receptor is G-protein coupled and promotes activation of the adenylate cyclase/ cyclic adenosine monophosphate (cAMP)/protein kinase A pathway (Hoffman, Tyler et al. 2012). It can affect a variety of cellular functions that include the facilitation of neurotransmitter release. In stimulating the presynaptic 5-HT4 receptors on the myenteric cholinergic nerve terminals, there is an enhanced fast excitatory synaptic input to neurons. There is also an increase in neurogenic muscle contractions in the intestines. Thus, presynaptic facilitation in the peristaltic reflex circuitry is thought to be responsible for the prokinetic actions seen in 5-HT4 receptor agonists.

In addition to the 5-HT3 receptor, the 5-HT4 receptor has been targeted for potential therapeutic effects. Stimulation of presynaptic 5-HT4 receptors through the intrinsic reflex circuitry of the gut potentiates the release of acetylcholine, substance P, and calcitonin gene related peptide. 5-HT4 receptor agonists, such as tegaserod, and cisapride, have been used in constipation predominant IBS (IBS-C), chronic idiopathic constipation, functional dyspepsia, and gastroparesis. However, both tegaserod and cisapride were pulled from the market for cardiovascular related side effects. Cisapride was pulled due to association with cardiac arrhythmias, such as ventricular tachycardia, ventricular fibrillation, and Torsades-de-pointes (Wong, Manabe et al. 2010). Tegaserod was pulled because of concern due to possible

ischemic cardiovascular events, but epidemiological studies performed failed to show such a correlation (Anderson, May et al. 2009, Loughlin, Quinn et al. 2010). It should be noted, however, that tegaserod and cisapride are not as selective for 5-HT4 receptors as the newer generation of agonists, such as velusetrag and prucalopride. Tegaserod, in addition to being an agonist at the 5-HT4 receptor, also interacts with the 5-HT 1B and 1D receptors as an agonist and the 5-HT2B receptor as an antagonist. Cisapride inhibits the human ether-àgo- go related gene (hERG) potassium channel at therapeutic concentrations (Wong, Manabe et al. 2010, Beattie, Armstrong et al. 2011). This inhibition of hERG can lead to QT prolongation and ultimately Torsades-de-pointes, as well as ventricular tachycardia, ventricular fibrillation. These off-target interactions are likely responsible for the cardiovascular events that resulted in the pulling of cisapride from the market and what raised concern about tegaserod.

With cisapride and tegaserod having been removed the market, a new generation of 5-HT4 receptor agonists was needed to combat IBS-C. Prucalopride is one such agent. As a part of the newer generation of 5-HT4 receptor agonists, prucalopride has a greater selectivity, and a higher affinity for, the 5-HT4 receptor. It promotes cholinergic and non-adrenergic, non-cholinergic neurotransmission via enteric neurons (Wong, Manabe et al. 2010). Prucalopride shows high affinity in binding to the 5-HT4 isoforms A and B. It also shows more than a 290-fold selectivity for the 5-HT4 receptor than other receptors to which it has shown ability to bind (namely the dopamine D4 receptor, 5-HT3 receptor, and alpha-1 receptor) (Wong, Manabe et al. 2010). As an agonist binding to the 5-HT4 receptor, prucalopride activates adenylate cyclase, as well as increasing intracellular cAMP levels. In addition, in the 5-HT4 receptors present in the gastrointestinal tract, there is a

promotion of increase motility and mucus secretion. As a result, in human studies prucalopride has shown an increase in stool frequency, as well as a loosening of stool. There was also an accelerated gastric emptying half-time, ascending colon emptying half-time, overall colonic transit, and whole gut transit. It should also be noted that, in contrast with cisapride and tegaserod, prucalopride has a high selectivity for the 5-HT4 receptor, which minimizes interactions with other receptors that would lead to serious adverse effects.

Also, prucalopride induces a smaller increase in the L-type calcium current as compared with serotonin (De Maeyer, Lefebvre et al. 2008). This would mean that it would be unlikely that prucalopride could bring forth atrial arrhythmias in patients being treated for constipation. It would instead block cardiac 5-HT4 receptors and could potentially prevent arrhythmogenic effects of serotonin. In addition, it is eliminated from the body without much metabolism, reducing potential drug-drug interactions with medications that affect hepatic or renal metabolism and clearance. There is also the possibility that 5-HT4 receptor agonists can act through a mucosal site of action (Hoffman, Tyler et al. 2012). Luminal administration of 5-HT4 receptor agonists promotes propulsive motility, as well as enhancing the ascending contractile and descending relaxatory limbs of the peristaltic reflex.

The 5-HT4 receptor, in addition to therapeutic use for IBS-C and gastroparesis, has been studied in some mouse models of colitis. Spohn and her colleagues in the Mawe laboratory showed the efficacy of 5-HT4 receptor agonists in two chemically induced models of colitis, namely Dextran sodium sulfate (DSS) and 2,4,6-Trinitrobenzene sulfonic acid (TNBS). She showed that in these models of colitis, the 5-HT4 receptor, when acted upon by an agonist, attenuated the

development of colitis, and accelerated healing from already established colitis (Spohn, Bianco et al. 2016). The 5-HT4 receptor agonist treatment was given by enema, which would limit its adverse effects, but as seen in Dr. Spohn's paper, did not limit its efficacy. Administration of a 5-HT4 agonist systemically by intraperitoneal injection failed to affect inflammation, and enema administration of the agonist was ineffective in 5-HT4 null mice.

Evidence collected to date indicates that the protective action of epithelial 5-HT4 receptor activation involves protection and restoration of the epithelial barrier. Animals given the 5-HT4 receptor agonist by enema showed a significant increase in epithelial proliferation, as measured by quantification of Ki67-immunoreactive cells. Furthermore, in experiments involving Caco-2 cells, a human colonic adenocarcinoma cell line, the 5-HT4 agonist increased the rate of cell migration and led to a resistance to oxidative stress-induced apoptosis (Spohn, Bianco et al. 2016). Together, along with the knowledge that 5-HT4 agonists activate mucus secretion from goblet cells (Hoffman, Tyler et al. 2012), these findings support the concept that 5-HT4 agonist promote healing by assisting with the restoration of the epithelial barrier.

In the body, the 5-HT4 receptor is widely expressed. Activation of the 5-HT4 receptor, in the periphery, has been shown to be involved in several responses in various organs like the gastrointestinal tract, the heart, and the urinary bladder (De Maeyer, Lefebvre et al. 2008). The 5-HT4 receptor is also present in the brain, where its highest expression can be found in the limbic structures, hippocampus, and the basal ganglia, which are linked to cognition. Thus the 5-HT4 receptor has been

implicated in various pathological disorders and represents a valuable target for designing new drugs.

That the 5-HT4 receptor is involved in peristalsis in humans, mice, and many other animals is well documented. Mucosal stimulation induces the release of serotonin from enterochromaffin cells, which activates intrinsic primary afferent neurons (IPANs) (De Maeyer, Lefebvre et al. 2008). This then releases acetylcholine (ACh) as well as calcitonin gene related peptide (CGRP). These neurotransmitters then connect through interneurons to ascending excitatory or descending inhibitory motor neurons, which results in ascending contraction and descending relaxation. Through functional studies and radioligand binding in the intestine, it is suggested that 5-HT4 receptors are present in all segments of the human gastrointestinal tract (De Maeyer, Lefebvre et al. 2008). They are expressed on enterochromaffin cells, IPANs, interneurons, and efferent neurons of the myenteric plexus as well as smooth muscle cells. Thus, their activation can affect all components of the peristaltic reflex.

In some species, such as humans, there are inhibitory 5-HT4 receptors that are expressed on enterochromaffin cells, which mediate autoregulation of serotonin release (De Maeyer, Lefebvre et al. 2008). In addition, the activation of 5-HT4 receptors on IPANs could either initiate or strengthen the peristaltic reflex. Of note, 5-HT4 receptors are present on the efferent limbs of the peristaltic reflex, activation of which, on efferent myenteric cholinergic excitatory neurons, leads to enhanced acetylcholine release, and thus an increase in contraction. This is likely the predominant mechanism through which 5-HT4 receptor agonists affect gastrointestinal motility. In therapeutic use, 5-HT4 receptors have the potential to be activated by a wide array of compounds from varying classes of drugs.

Metoclopramide, a prokinetic drug of the benzamide class, was found to be a ligand for both 5-HT3 and 5-HT4 receptors. It played a large role in the discovery of potent and selective 5-HT4 receptor drugs.

Briefly, there are 5-HT4 receptors in the heart as well. They are expressed in the atria and ventricles, though at low densities (De Maeyer, Lefebvre et al. 2008). Thus, many of the effects of 5-HT4 receptor agonists are lesser when compared with serotonin. Some of these effects include an increase in contractile force, an increase in the onset of muscle relaxation, and an increase in heart rate. That there is a lower efficacy for the 5-HT4 receptors in the heart as compared with serotonin is relevant because of the association between 5-HT4 receptor agonists and cardiovascular events. No evidence was found to substantiate these claims with the use of renzapride and prucalopride.

5-HT4 and Opioid receptors:

As stated above, 5-HT4 receptors seem to have a hand in motility in the gut. When acting in concert with delta-opioid receptor antagonists, there seems to be a synergistic effect in the stimulation of colonic propulsion. Endogenous opioid peptides, and selective opioid receptor agonists (d, k, and mu) decrease the velocity of fecal pellet propulsion in isolated segments of guinea pig colon, as reported by Foxx-Ornstein and colleagues (Foxx-Orenstein, Jin et al. 1998). They also reported that selective antagonists increased the velocity in a dose-dependent manner, where it was indicated that there was a preferential involvement of d-opioid receptors. 5-HT4 agonists, which are known to increase propulsion, acted synergistically with the opioid d-receptor antagonist naltrindole. Opioid receptor agonists have distinct actions, both neural and muscular, in the gastrointestinal tract (Foxx-Orenstein, Jin et

al. 1998). These effects include contraction of smooth muscle cells of the circular layer, as well as inhibition of neuronal activity, which is reflected by a decrease in the release of excitatory and inhibitory neurotransmitters.

The inhibition of opioid peptide release facilitates the descending phase of the peristaltic reflex, and thus enhances intestinal propulsion. Studies into peristaltic activity using the opioid antagonists naloxone and norbinaltorphimine showed an increase in peristaltic contractions. However, parenteral or oral administration of opioid agonists gives rise to rhythmic, uncoordinated, non-propulsive muscle contractions, which is likely mediated by the inhibition of the release of inhibitory neurotransmitters. Foxx-Ornstein and colleagues showed that selective 5-HT4 receptor agonists can initiate peristaltic activity through activation of intrinsic sensory calcitonin gene related peptide neurons, which are normally activated by the serotonin released by enterochromaffin cells (Foxx-Orenstein, Jin et al. 1998). In addition, they reported that studies in pellet propulsion in isolated segments of guinea pig colon showed that serotonin and 5-HT4 receptor agonists increased the velocity of propulsion in a dose-dependent manner.

Inflammatory Bowel Disease:

Inflammatory bowel disease (IBD) is a disorder marked by chronic intestinal inflammation. Its cause is unknown to date and is composed of 3 entities: Crohn's disease (CD), ulcerative colitis (UC), and Inflammatory bowel disease unclassified (IBDU). UC and CD are chronic relapsing inflammatory disorders which affect the gastrointestinal tract. They have a progressive and destructive nature and, therefore, can cause various complications including stenosis, abscesses, fistulas, extra-intestinal manifestations and colitis-associated neoplasias and cancer.

While the pathophysiology of IBD is not well understood, there are many hypotheses about its origin, such as an impairment in the mucosal barrier, commensal microbe dysbiosis, persistent pathogenic infection, and immune dysregulation. Individuals with a genetic susceptibility to IBD are exposed to a number of environmental factors, including diet and lifestyle, which can then induce an immune response that impairs the mucosal barrier and elicits chronic inflammation (Silva, Rodrigues et al. 2016). IBD occurs in the gastrointestinal tract, which houses the body's largest component of the immune system. The innate and adaptive immune systems are balanced in complex interactions with intestinal microbes under normal conditions. Also, gastrointestinal tract is in a critical position as it is the frontier of the innate immune system (Kim and Cheon 2017). The inner lining of the intestine works as a barrier to defend the host from harmful pathogens, as well as an area where interactions with commensal microorganisms can occur. These interactions are modulated carefully by the intestinal immune system, and they contribute to homeostasis. When intestinal homeostasis is disrupted, IBD can arise for a variety of reasons.

IBD is a multi-faceted immune-mediated disorder that is characterized by chronic inflammation of the intestine. The clinical characteristics of IBD include hemorrhagic diarrhea, abdominal pain, tenesmus, anorexia and weight loss (Silva, Rodrigues et al. 2016). The severity of symptoms can vary, from mild to severe, and patients that do not response to clinical management and have complications, generally require surgical intervention. Their differences lie in the location and the depth of the inflammation, as well as the complications and prevalence that can occur. It is currently believed that a disturbance of the immune system and/or an imbalance

in interactions with microbes leads to the rise of chronic intestinal inflammation upon environmental triggers of genetically susceptible hosts.

Ulcerative colitis and Crohn's Disease are chronic, relapsing, and remitting conditions for which no permanent cure exists, and from which long-term morbidity can result (M'Koma 2013). UC tends to affect the colon and is confined to the mucosal and submucosal compartments. CD, however, can involve any part of the gastrointestinal tract, from the oral cavity to the anus, and can involve all layers of the gut (M'Koma 2013). While the factors which contribute to the development of IBD remain largely unknown, IBD has long been considered a problem of Western societies, where the Western lifestyle contributes a great deal to their pathogenesis. IBD is most prevalent before age 20, between 20 and 30, as well as in the 60-70 age group (M'Koma 2013).

UC and CD are distinguished from each other as UC is centered in the distal colon, perineal disease, fistulas, histologic granulomas, and full-thickness as opposed to a largely mucosa-submucosa limited disease. In CD, granulomas are evident in up to 50% of patients, and fistulas are evident in up to 25%. While IBD susceptibility genes have been identified, such as *NOD2* gene variants, there also have been advances in defining environment risk factors that suggest smoking, oral contraceptives, diet, appendectomy, breast feeding, antibiotics, vaccination, infection, and childhood hygiene may be involved (Loftus , Jess, Riis et al. 2005, Gaya, Russell et al. 2006, Halfvarson, Jess et al. 2006, Molodecky and Kaplan 2010).

IBD is thought to arise from an interaction between a genetically susceptible host and environmental factors that influence the normal gut microbiota and trigger an

abnormal mucosal immune response. Recent data (Chouraki, Savoye et al. 2011, Jakobsen, Paerregaard et al. 2011) suggest that children and adolescents show a high incidence of IBD. About 25-30% of patients diagnosed with CD, and 20% of patients diagnosed with UC, present symptoms before age 20. In IBD, a dysfunctional interaction between the gut microbiome and the mucosal immune system occurs, which has the potential to cause the loss of intestinal immune tolerance through overreaction of effector T-cells which react against common microbial antigens. One of the myriad of mechanisms that affect host inflammatory reactions is associated with short-chain fatty acids (SCFAs), which are produced by specific types of colonic bacteria (Silva, Rodrigues et al. 2016). In IBD, the level of SCFA is significantly decreased, which may be a factor in compromising the intestinal and immune homeostasis.

In UC, goblet cell depletion and an associated reduction in the mucus layer are characteristic findings. Kim and colleagues noted evidence (Peltekova, Wintle et al. 2004, Jung, Park et al. 2017) showing that single nucleotide polymorphisms (SNPs) in the organic cation transporter (*OCTN*), which is responsible for the transport of organic cations across the cell membrane, were associated with susceptibility to CD. In addition, Intestinal Epithelial Cells (IECs) play a role as communicator between pathogens and the lamina propria. Generally, only a small number of bacteria can move into the intestinal epithelium. This is a method by which the intestinal mucosal immune system samples antigens and conducts immune surveillance essential for the host's immune homeostasis. Once the integrity of the intestinal epithelial layer is compromised, however, an influx of intestinal contents and/or a burden of microorganisms is thought to give rise to, and maintain, an inflammatory response

considered to be one mechanism underlying IBD (Sartor 2006). In example, Hermiston et al showed in an animal model where the barrier function of the intestinal epithelial layer is reduced, as is seen in mice with a dominant negative N-cadherin mutation, IBD-like enteritis is seen.

In IBD, large-bowel involvement is typical, and may result from UC, or IBDU, and possibly from the subset of non-complicated CD with an isolated colonic localization. In terms of classification of disease, however, the spread of the disease to other gastrointestinal segments leads, definitively, to the diagnosis of CD. It should be noted though, that one-third of patients with CD exhibit the disease in a purely colonic fashion, whereas two-thirds have a non-restrictive, non-penetrating disease behavior at the time of diagnosis, which shares many similarities with UC. In CD, disease localization appears to be a stable trait, with a spread to the small bowel in only a small number of cases. IBD restricted to the colon which cannot be classified as CD or UC is termed “inflammatory bowel disease unclassified” or IBDU. IBDU has been associated with a worse prognosis than UC due to the higher frequency of relapse, an increased risk of colon cancer, and less favorable outcomes following ileal pouch-anal anastomosis. There is, however, few data available on the prevalence of IBDU. A study done by Meucci et al of adults in European countries show the prevalence of IBDU varying from 3 to 7 per 100,000 inhabitants. The diagnosis of IBDU comprises 5-15% of new IBD cases. However, IBDU tends to represent a provisional diagnosis, as many studies (Meucci, Bortoli et al. 1999, Carvalho, Abadom et al. 2006, Tremaine 2012, Magro, Langner et al. 2013) show that 80% of those diagnosed with IBDU will be reclassified to CD or UC within 8 years. Though it should be noted that in a subset of cases, IBDU is the most accurate diagnosis.

The integrity of the epithelial layer allows the bacteria of the intestinal lumen to communicate with the immune system. The mucous layer is the first physical barrier on the mucosal surface. It is formed by inner and outer layers produced by polymerization of gel-forming mucins which are secreted by Goblet cells. The inner layer is sterile, while the outer layer is inhabited by commensal bacteria which consume nutrients such as mucin glycan. Next, the intestinal epithelium is the second barrier, and considered the second line of defense against bacterial invasion. It is composed of enterocytes and specialized epithelial cells called Goblet cells and Paneth cells. Intestinal epithelial cells (IECs)s also play a role in the mucosal barrier, as they prevent the influx of antigens, as well as invasion of both pathogens and commensal bacteria. The IEC is made up of many different cells, such as enterocytes, goblet cells, neuroendocrine cells, Paneth cells, M cells, and epithelial resident intestinal stem cells (Kim and Cheon 2017). IECs structurally constitute crypts and villi, with a columnar cell lining containing a tight junction. IECs also secrete mucus containing anti-microbial peptides. They separate intra-luminal pathogens from the sub-epithelial lamina propria.

A mucus layer covers the outer epithelial surface which serves as a protection for the mucosa. This mucus layer is comprised of glycosylated mucin from the goblet cells, and defensins from the Paneth cells and IECs. A critical component of mucin is encoded by *Muc2* and, when deleted, spontaneous colitis has been noted in mice. One study, conducted by Heazlewood et al, showed that aberrant mucin production was accompanied by endoplasmic reticulum stress. IECs are key in the maintenance of tolerance toward alimentary antigens and commensal bacteria. In addition, IECs activate both the innate and adaptive immune responses. IECs, in the protection of the

mucosal barrier, present tight junctions, and produce mucins and defensins. In addition, IECs express toll-like receptors (TLRs) and nucleotide oligomerization domain receptors (NOD), which are pathogen sensitive innate immune receptors (Silva, Rodrigues et al. 2016). IECs can also produce chemokines and cytokines to enlist immune cells. Thus, TLR signaling pathways can produce pro-inflammatory cytokines like IL-12 and 6 by way of IECs and can help to keep the epithelial barrier intact (Bamias and Cominelli 2007, Hisamatsu, Kanai et al. 2013). An impairment in the epithelial barrier can lead to an increased permeability within the intestine, which has been observed in CD and UC (Salim and Soderholm 2011). As noted by Silva et al, some Genome Wide Associative Studies (GWAS) suggest that this might represent a primary pathogenic mechanism in IBD (Tontini, Vecchi et al. 2015).

IBD is associated with intestinal dysbiosis, or the imbalance of functions of gut microorganisms which impair the host microbe and immune homeostasis (Miyoshi and Chang 2017). The human gut contains roughly 10^{11}-10^{12} microorganisms per gram of intestinal lumen content, which are called commensal bacteria. These commensal bacteria can be beneficial to the host under normal conditions, as they aid in the protection of the intestinal epithelium and digestion (Sartor 2006, Sung and Park 2013). Normally, many commensal bacteria are essential in the protection of intestinal homeostasis. These commensal bacteria affect nutrient provision, which is crucial, as well as the development of the immune system, and modulation of energy metabolism. Commensal bacteria consist mostly of gram-negative bacteria, like Bacteroidetes, and gram-positive bacteria, like Firmicutes. Other phyla consist of Proteobacteria, Actinobacteria, Fusobacteria, and Verrucomicrobia (Eckburg, Bik et al. 2005, Cho 2008). These mucosae associated

phyla show a reduction in number and diversity in individuals with IBD when compared to healthy individuals (Eckburg and Relman 2007, Frank, St Amand et al. 2007, Abraham and Cho 2009).

Individuals with genetic susceptibility are exposed to any number of environmental factors, like diet and lifestyle, which can then induce an immune response which alters the intestinal microbiota and can impair the mucosal barrier (Devkota, Wang et al. 2012, Hisamatsu, Kanai et al. 2013, Ward, Pierre et al. 2016). Devkota et al showed that a diet which does not change the intestinal microbiota is crucial in the prevention of IBD. They showed that there was an increase in prevalence of experimental UC when colitis-susceptible mice (see below) were fed with food containing high levels of saturated fat. This diet promoted the growth of a commensal bacterium called Bilophila wadsworthia (Silva, Rodrigues et al. 2016). The reported growth was likely due to changes in composition of bile acids caused by the high intake of saturated fat, which lead to dysbiosis. In addition, von Mutius et al suggested that exposure to commensal bacteria during childhood has potential protective effects against the development of IBD, as it is critical to stabilize immune tolerance (M'Koma 2013).

While these commensal bacteria are helpful, they can be harmful under certain conditions. Some evidence has been presented, which shows that antibiotic treatment is effective in some IBD patients (M'Koma 2013). In addition, IBD patients have been shown to have increased action against indigenous bacteria. Mononuclear phagocytes, like macrophages and DCs, are responsible for a lack of immunological response to the commensal bacteria, which is key in the maintenance of gut homeostasis. The microbiome is key in the production of pro-interleukin (IL)-1beta in

resident mononuclear phagocytes. When the epithelial barrier is intact, commensal bacteria cannot cause the maturation of pro-IL-1beta into its biologically active, mature counterpart. As a result, a state of low response is maintained (Strugnell and Wijburg 2010). The first defense against invading microorganisms and other potentially harmful agents is called innate immunity. Its response is activated mere minutes after invasion from microorganisms and can last a few hours. It has no classic immunological memory (Medzhitov and Janeway 2000).

Aside from innate immunity, adaptive immunity also plays an important role in disease. T-cells regulate the immune response from IBD and proliferate in the peripheral blood. They differentiate when stimulated by the presence of antigens. There is a subtype of T-cells, called T-helper cells (Th) that assist in the adaptive immune response. Some important Th cells are Th1, Th2, Treg, and Th17. Each subtype of Th cells has immune functions relevant to the adaptive immune response. Th1 cells aid in the elimination of pathogenic agents, and Th2 cells manage allergic reactions, as well as aiding in the protection from parasites. It is important to note that Th1 cells are important in the secretion of pro-inflammatory cytokines, like IL-1, IL-2, and IL-12. Next, Th17 cells aid in the removal of exogenous bacteria and fungi, and lastly, Treg cells promote the repair of tissues. While these cells aid in the adaptive immune response, alterations in their proliferation can have profound negative effects (Silva, Rodrigues et al. 2016) With alteration, these cells can cause a large increase in chemo- and cytokines, which would cause a worsening of the inflammatory process, or the maintenance of it. Once antigens have been identified in gut-associated lymphoid tissue (GALT), Th1 and Th2 cells are activated. In addition, B lymphocytes mature, and can produce antigen-specific immunoglobulins.

Experimental Animal Models of Colitis:

DSS:

Dextran sodium sulfate (DSS) induced colitis is one of the most commonly used models of colitis. DSS is a water soluble, negatively charged sulfated polysaccharide, with a varied molecular weight, which ranges from 5 to 1400 kilodaltons (kDa). The most severe colitis arising from this model results from the administration of between 40-50 kDa of DSS into the drinking water of the animal (Chassaing, Aitken et al. 2014). While the mechanism through which DSS induces intestinal inflammation characteristic of colitis is not clear, it is likely due to damage of the epithelial monolayer that lines the large intestine. This allows the dissemination of proinflammatory intestinal contents, such as bacteria and their products, into the underlying tissue (Chassaing, Aitken et al. 2014). The popularity of the model stems from its rapid onset, its simplicity, the reproducibility, and its controllability. Of note, in DSS colitis, unlike in human IBD, T and B cells are not required to develop the colitis this model produces (Chassaing, Aitken et al. 2014).

Once administration of DSS has begun, the animals used, usually mice, are typically monitored daily for changes in weight, and the presence of blood in their feces. In addition, any mice that lose 25-30% of their starting weight should be euthanized. Also, in this model the severity of disease and mortality can be variable, so a sufficient number of animals should be used (typically 5-10) (Chassaing, Aitken et al. 2014). Chassaing et al also reported that the colitis induced by DSS tends to occur gradually in younger mice as compared to older mice. This is likely due to younger mice having a lower demand for food and as such, water intake. As water intake is crucial for DSS, being that DSS is put into the water supply, this would make

sense that the younger mice develop the disease at a slower rate. The optimal age for best results in the DSS model range from 6-8 weeks. While both male and female mice develop the colitis which DSS induces, males develop more rapidly, more significantly, and more aggressively than their female counterparts (Chassaing, Aitken et al. 2014). It should also be noted that mice, regardless of sex, kept in a germ-free environment either do not develop colitis, or have a very mild onset.

DSS causes damage to the gastrointestinal tract because it carries has highly negative charged sulfate groups. This is toxic to colonic epithelia and induces erosions that ultimately compromise barrier integrity. This results in an increase in colonic epithelial permeability. Also, DSS has an anticoagulant property, which aggravates intestinal bleeding. DSS-induced pathology tends to be contained within the large intestine, more specifically the distal colon, for unknown reasons. How DSS passes through the mucosal epithelial cells has yet to be elucidated, but Laroui et al suggest that the colitis induced by DSS is formed by nano-lipocomplexes with medium-chain-length fatty acids (MCFAs) within the colon (Laroui, Ingersoll et al. 2012). In addition, the specificity of DSS for the colon could possibly be a function of the absorption of water and electrolytes in the presence of bacteria.

In terms of action, administration of DSS induces signs of colitis as quickly as 1 day after treatment, as shown by changes in expression of tight junction proteins, (Poritz, Garver et al. 2007) and through an increase in the expression of proinflammatory cytokines (Yan, Kolachala et al. 2009). The modest initial effects are then followed by an increase in more drastic symptoms such as an increase in intestinal permeability, severe bleeding, and, if severe enough., mortality.

Colitis induced by DSS appears to have similar clinical and histological features as human IBD, in particular UC. Acute changes in the histology of the colon can be induced through relatively short exposure to a high dose of DSS (4-7 days) (Chassaing, Aitken et al. 2014). Whereas chronic lesions can be brought forth by continuous treatment of a low dose of DSS, or through cyclical application. The acute histological changes are associated with symptoms like weight loss, diarrhea, blood in the feces, a hunched back, and in severe cases, death (Chassaing, Aitken et al. 2014). Common histological changes brought forth by DSS can include the depletion of mucin and goblet cells, the erosion of the epithelium, and ulceration. In addition, DSS induced colitis causes an influx of neutrophils into the lamina propria and submucosa. The trans-epithelial migration of neutrophils, also called cryptitis, and the extensive migration of neutrophils through mucosal epithelium and into the crypt lumen, called crypt abscess, are most commonly associated with human IBD. These effects are not as common in DSS colitis but have been reported. In chronic DSS colitis, there is an infiltration of mononuclear leukocytes, the disarray of crypt architecture, and increase in the gap between the base of the crypt and the muscularis.

TNBS:

In addition to the DSS model of colitis, the Trinitrobenzenesulfonic acid (TNBS) model of colitis is also commonly used. Its administration is combined with ethanol, at a dose of 100 mg/kg, intra-rectally. The use of ethanol is for the effective disruption of the intestinal barrier, and to enable the interaction of TNBS with colonic tissue proteins. TNBS is a classical contactant and acts as a hapten. Little et al in 1966 suggested that, when coupled with proteins with a high molecular weight, TNBS can bring forth significant immunologic responses, causing those proteins to elicit an

immune response from the host's immune system (Little and Eisen 1966). The combination of TNBS and ethanol requires only a single administration to lead to the development of excessive cell mediated immune response, which is characterized by Th1 inflammation (Antoniou, Margonis et al. 2016). Once colitis has been induced, the animals develop many manifestations of acute colitis, such as inconsistent formation of feces, and bloody diarrhea. Through intracolonic administration of the combination, a severe illness is induced characterized by bloody diarrhea, and a large decrease in weight within the first week. Following this, the weight increases but the diarrhea persists (for up to 2 weeks). Antoniou et al reported that, once TNBS was introduced to animals, there was a significant weight loss, the mean being 10%, as well as the development of liquid, bloody feces (Antoniou, Margonis et al. 2016). The control animals, which received saline, remained healthy, and gained weight.

The weight loss seen in this model of colitis is caused by the effects of TNBS itself within the gut, such as diarrhea and possibly a reduction in fluid absorption. In addition, a systemic inflammatory response could also have a role. Other signs of general deterioration, although non-specific, include piloerection of fur and a decrease in the movements of the TNBS treated animals. The onset of the symptoms and their severity, however, is variable, and can depend on the species of animal, the strain of animal, and the dose of ethanol and TNBS administered. Prior to induction of TNBS colitis, the animals are fasted, from 12-24 hours. Then, the mice are anesthetized. Once anesthetized, the TNBS, dissolved in ethanol, is administered intrarectally. Following administration, the animals are maintained in a head down position so as to prevent expulsion of the administered TNBS, as well as to achieve an even distribution. As is evident in the literature, the dosing of the TNBS and ethanol is

varied. Various researchers use a range of between 50 and 150 mg/kg body weight. Morris et al who established the TNBS model, report an ideal dosage of 100 mg/kg (Antoniou, Margonis et al. 2016). Mice that receive ethanol only show a normal appearance, with no histological abnormalities. A dose of 50mg/kg showed a low level of colitis, whereas a dose of 150 mg/kg had a mortality rate of between 20 and 30%, as associated with high to severe levels of colitis. Antoniou et al reported that death resulted primarily from excessive inflammation, and sometimes perforation, as shown in postmortem autopsy (Antoniou, Margonis et al. 2016). In the 100 mg/kg dosage, most of the mice (90% as reported by Antoniou et al) showed thickening of the colon, focal hyperemia, and intestinal ulcerations, in roughly 65% of the treated animals. It should be noted that TNBS colitis is somewhat variable, and the disease level can vary amongst the mice. Some mice show low levels of inflammation, whereas others may suffer lethal levels, or could die within 48 hours of TNBS treatment. Of note, during the course of disease, mucosal edema, distortion of the crypts, and formation of abscesses can occur over the course of the disease. In addition, there is the potential for hemorrhagic ulcerations. Through histological examination, Antoniou et al reported the infiltration of leukocytes and erythrocytes into the mucosa and submucosa was shown in the first week. Within the next two weeks, the mucosa, submucosa and muscularis propria were infiltrated by neutrophils, macrophages, and lymphocytes. In addition, they reported granuloma-like structures and fibroblasts.

TNBS induced colitis is a commonly used animal model which shares many properties with Crohn's disease in humans. Its advantages include its reproducibility, technical simplicity, and a low cost. This model can be used in rodents and guinea

pigs and can have significant strain-dependent differences. TNBS induced colitis is a strong tool with which to study immunopathogenesis, as well as possible treatments for the disease.

T-Cell Transfer:

Briefly, in addition to the chemically induced models mentioned, DSS and TNBS colitis, there is the T-cell Transfer model of colitis. This model of colitis builds on the foundation that T lymphocytes play a large role in the onset of colitis. T lymphocytes recognize antigens from antigen presenting cells and generate targeted immune responses to enteric bacteria or intestinal self-antigens (Bramhall, Flórez-Vargas et al. 2015). This model utilizes naïve T cells from wild-type mice and transfers them to mice who genetically lack T and B cells (such as mice with Severe Combined Immunodeficiency (SCID) or RAG 1/2). Colitis in this model occurs as a result of enteric antigen-drive activation, polarization, and homeostatic expansion of the naïve T-cells used (Ostanin, Bao et al. 2009). These helped produce cells that aid in the induction of colitis like Th1 and Th17. Colitis symptoms occur 2 weeks post transfer, and pancolitis is present at 4 weeks. Unlike in DSS and TNBS colitis, this model requires a more complex and labor-intensive protocol. This is due to extraction, isolation, purification, and injection of the T cells. Also, there are factors that affect the resulting colitis such as the strain of animal, the number and viability of the T-cells that are transferred and the presence, if any, of B cells in the animals to which the naïve cells are transferred.

IL-10 Knockout:

Interleukin-10 (IL-10) is an anti-inflammatory cytokine. It was initially described as a T helper derived cytokine, though it is now widely accepted that it was not restricted to certain subsets of T-cells; indeed, it is produced by almost all leukocytes (Iyer and Cheng 2012). The major sources of IL-10 include T helper cells, monocytes, macrophages, and dendritic cells. Also, a myriad of other immune effector cells has the capability of producing IL-10 in specific contexts. Such cells include B cells, cytotoxic T cells, NK cells, mast cells, and granulocytes like neutrophils and eosinophils (Iyer and Cheng 2012). As an anti-inflammatory cytokine, its immunosuppressive activity is mediated by a heterodimeric IL-10 receptor. While the IL-10 receptor is expressed to varying degrees in many cell types, it seems that monocytes and macrophages are the primary target. Binding of IL-10 to its receptor leads to large changes in the expression of immunomodulatory genes, which then causes the inhibition of proinflammatory mediators. IL-10 can directly or indirectly inhibit the development of some cells, as well as suppressing some cell and allergic responses. It can also positively enhance the activation and proliferation of certain immune cells types such as mast cells, CD8+ T cells, NK cells, and B cells.

Some initial studies showed that deficiencies in IL-10, by way of disruption of the IL-10 gene, or signaling by blockade of the receptor, most intracellular infections are better controlled, or cleared faster. Cessation of signaling leads to enhanced survival post infection and is associated with enhanced adaptive immune response. In addition, in the case of some parasitic infections, such as *Y. Pestis*, IL-10 is a biomarker for poor disease outcome (Iyer and Cheng 2012). The impairment of IL-10 signaling has effects in many experimental disease models, such as various infection

models, as well as inflammatory bowel disease, rheumatoid arthritis, multiple sclerosis, and others. Many pathogens have evolved mechanisms through which they selectively up-regulate IL-10 during infection, potentially create a more favorable environment. One such example is *Toxoplasma gondii,* which can shut down TLR4-mediated LPS signaling in such a way that blocks TNF-alpha expression but allows IL-10 to be produced (Iyer and Cheng 2012). However, it should be noted that while initially beneficial to the host, the absence of IL-10 in the long term is very detrimental. Such prolongation or enhancement of inflammatory cytokines, with no cessation, can lead to septic shock by viral, bacterial, or fungal infection. As inflammatory molecules have the potential to be potent activators of cell death, the increase of IL-10 levels can moderate the extent of apoptosis caused by the response to the infection. An example of this is in a model of Chlamydia pneumoniae. Bacterial clearance is enhanced by the absence of IL-10, and mice also develop severe inflammation, and elevated levels of apoptosis. Iyer et al reported that, in this model, blocking the action of CD8+ T-cell derived IL-10 causes enhanced pulmonary inflammation, and lethal injury. As such, this indicates that resolution of infection needs a coordinated response by which initial pro-inflammatory mechanisms clear the pathogen, and then are limited by IL-10 before negative effects can occur.

With the understanding that IL-10 is an important mediator of the inflammation response, the IL-10 knockout (IL-10 KO) model of colitis demonstrates the effect on mice of the loss of this important cytokine. This model of colitis was first generated in 1993 by Kuhn et al. Kuhn et al using a targeted mutation which disrupted the IL-10 gene by replacing a 500-base pair fragment of exon 1 with a termination codon, and a neo expression cassette, and by introducing a termination

codon into exon 3 (Keubler, Buettner et al. 2015). IL-10 deficient mice spontaneously develop colitis after weaning as IL-10 is a key gut mediator and essential for the maintenance of intestinal homeostasis. The colitis that is seen in this model arises from an aberrant response of CD4+ Th1-like T-cells in addition to an excessive secretion of IL-12, IL-17, and Interferon gamma (IFNg), which are all pro-inflammatory cytokines.

The colitis seen in this experimental model can be reversed by treatment with recombinant IL-10 (Keubler, Buettner et al. 2015). The IL-10 model of colitis is highly regarded because mice are found to have histological findings that are akin to those found in human IBD, and no chemical treatment is necessary. In this model, inflammatory cells infiltrate the lamina propria and submucosa, the epithelium is disrupted, mucin is depleted, the formation of crypt abscesses and cryptitis occurs, as well as the formation of ulcers and the thickening of the intestinal wall. The inflammation seen in this model is initially driven by a pro-inflammatory Th1 T-cell response, and thus can be ameliorated by the systemic administration of anti-IL-12p40 (Keubler, Buettner et al. 2015). Stimulation of the mucosal immune system by commensal microflora is necessary for the colitis seen in this model. As such, mice contained in a germ-free environment do not develop colitis, unless transferred from a germ-free environment to a pathogen-free environment. It should also be noted that in the IL-10 experimental model of colitis, the onset of colitis can be delayed using antibiotics, further suggesting that resident bacteria are necessary for the development of colitis in this model. In addition, Alan Sher and his colleagues showed that, in germ free IL-10 deficient mice, the bacteria *Helicobacter hepaticus* is responsible for the rise of colitis (Kullberg, Ward et al. 1998).

Importantly, while there has been efficacy shown in IL-10 recombinant therapy in preclinical models, recombinant IL-10, in practice, has shown weak therapeutic potential (Davidson, Leach et al. 1996, Duchmann, Schmitt et al. 1996, Tomoyose, Mitsuyama et al. 1998). There are several factors which contribute to the failure of recombinant IL-10 therapy in clinical trials. The first of these is that systemic administration might not be sufficient enough to deliver IL-10 to mucosal inflammatory sites upon which it can exert anti-inflammatory functions. Second, IL-10 impairment is essential for the development of colitis in the IL-10 deficient mouse. Specifically, it is the impairment of the IL-10 derived from CD4+, from T-regulatory cells, not B-cells, that is necessary (Murai, Turovskaya et al. 2009)

Specific Aim:

Approximately 1-1.3 million people in the United States have an Irritable Bowel Disease (IBD). IBDs are classified as Crohn's Disease and Ulcerative Colitis (UC). UC effects approximately 907,000 Americans today. It is characterized by inflammation of the colon, and the formation of ulcers. Unfortunately, treatment options are limited and ineffective in many individuals with IBD. Recent work performed in the Mawe laboratory has demonstrated that 5-HT4 agonists, acting in the colonic epithelium, could represent a novel and effective treatment for IBD. Spohn and colleagues demonstrated that 5-HT4 agonists, delivered by enema, attenuates the development of colitis, and accelerates the healing of, established colitis. These studies were performed using the trinitrobenzene sulfonic acid (TNBS) and dextran sulfate sodium (DSS) models of colitis, both of which involve chemical agents delivered to the lumen of the colon.

IBD is thought to develop because of an imbalance in the mucosal immune response. Another experimental model of colitis, which involves an imbalance of mucosal immunity, is the interleukin-10 (IL-10) knockout mouse. Since these mice lack the anti-inflammatory cytokine IL-10, colitis develops spontaneously without having to introduce exogenous chemicals to the lumen. In the proposed studies, we tested the hypothesis that intraluminal administration of a 5-HT4 agonist is protective in IL-10 KO mice.

Aim 1. Test the hypothesis that treating IL-10 knockout mice with 5-HT4 receptor agonist will attenuate the development of colitis. We treated IL-10 knockout mice immediately following weaning. We used the disease activity index, histologic damage score, and Ki67 stained slides to judge the effect of 5-HT4 agonist and vehicle control on IL-10 knockout mice. We used the disease activity index to check the mice used for weight loss, presence of fecal blood, and stool consistency. We also used the histologic damage score to test for epithelial damage, ulcers, erosions, to look at crypt architecture and whether it is altered, and to see if the lamina propria has been infiltrated by monocytes and polymorphonuclear cells.

This study tested the effect of 5-HT4 receptor agonists and their ability to attenuate the inflammatory actions of interleukin-10 knockout induced colitis. This study strengthened both previous data from the Mawe lab and our understanding on 5-HT4 receptor agonist treatment in experimental models of colitis.

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CHAPTER 2: THE EFFECTS OF 5-HT4 RECEPTOR AGONIGISTS ON INTERLEUKIN-10 KNOCKOUT MICE

Abstract:

Recent studies have demonstrated that activation of the 5-HT4 receptors in the colonic mucosa can exert healing and protective actions in experimental models of colitis. These actions include increased mucus secretion, increased epithelial proliferation, enhanced epithelial migration, and resistance to oxidative stress. Since these studies involved chemically induced models of colitis, the current investigation was conducted to test whether a protective action of 5-HT4 receptor stimulation could be detected in Interleukin-10 knockout (IL-10 KO) mice, which develop colitis spontaneously due to the absence of the anti-inflammatory cytokine, IL-10.

Upon weaning, the IL-10 knockout mice were separated into two groups: an agonist group and a vehicle control group. The agonist group received 1 mg/kg tegaserod in a vehicle consisting of 0.9% saline each day by enema of dimethyl sulfoxide (DMSO) in saline each day, while the control group received daily enemas of vehicle over the course of 21 days. The concentration of tegaserod utilized was the same as used in the study done by Spohn and her colleagues, so as to give a therapeutic dose of the drug but not to elicit potential adverse effects. Several outcome measures were used to assess the effectiveness of the treatment. To evaluate the severity of colitis, disease activity index (DAI) was monitored, and histologic damage was blindly scored. In addition, fecal lipocalin levels were evaluated by ELISA.

Administration of tegaserod by enema to the IL-10 KO mice had a significant protective effect on the treated mice. The DAI of agonist treated mice was significantly better than that of vehicle treated mice over time ($p<0.001$; 2-way ANOVA). Mice treated with vehicle had a more significant decline in health over time versus the agonists, with more blood present in feces and a looser/diarrhea-like consistency in stool. The histological damage score was also improved by 5-HT4 agonist treatment ($p<0.05$, t-test). Sections of vehicle treated colons showed significantly greater damage, including epithelial erosions, the presence of polymorphonuclear cells, and abnormal crypt architecture (cryptitis), than those treated with the 5-HT4 receptor agonist tegaserod. During the 21-day course of the current investigation, there was no difference in survival between the two groups.

Collectively, these data, suggest that administration of the 5-HT4 receptor agonist tegaserod via enema to IL-10 KO mice has a greater healing and protective effect compared with vehicle treatment. We tested the hypothesis that treatment of IL-10 knockout colitis with a 5-HT4 receptor agonist will attenuate the development of colitis and have healing and protective effects in the colons of the treated mice.

Introduction:

Serotonin (5-hydroxytryptamine, 5-HT) is classically thought of as a brain neurotransmitter. However, most of the body's serotonin is made and found in the gastrointestinal tract. Serotonin in the gut is responsible for many actions such as motility, mucus secretion, and chloride secretion from enterocytes. There are also a number of serotonin receptor subtypes found within the gastrointestinal tract, such as 5-HT3, 5-HT4, and 5-HT7. While there haven't been many studies conducted on how 5-HT7 might function, there have been numerous studies, and drugs, detailing the

effects of 5-HT3 and 5-HT4 on the gastrointestinal tract. The 5-HT3 receptor is ligand gated, and antagonists at this receptor constitute its therapeutic effects. Drugs like ondansetron are used to combat nausea and vomiting that are commonly associated with cancer chemotherapy, radiation therapy, and surgery. 5-HT4 receptors, however, are G-protein coupled, and therapeutic drugs that act on these receptors are agonists. 5-HT4 receptor agonists, like tegaserod and cisapride, were used to combat constipation-predominant Irritable Bowel Syndrome (IBS-C).

As 5-HT4 receptors have been shown to increase motility and have been proposed to have anti-inflammatory effects (Sung and Park 2013, Spohn, Bianco et al. 2016), we proposed to test the hypothesis that administration of the 5-HT4 receptor agonist tegaserod will attenuate the development of colitis and provide protective and healing effects in the interleukin-10 (IL-10) knockout experimental model of colitis. While Spohn and her colleagues used the DSS and TNBS models of colitis, which are chemically induced experimental models of colitis, we proposed to use the IL-10 knockout experimental model, which causes spontaneous colitis after weaning.

To assess the severity of the damage of colitis, and to determine the protective and healing effects of the tegaserod on the IL-10 KO model, we used a variety of tests. To determine the severity of the colitis, we used the disease activity index (DAI) and histologic damage score (HDS). The DAI measures the consistency of stool of each animal, and tests for fecal occult blood. The DAI was measured every day to obtain an accurate picture as to the progression of the disease. Typically weight is included in the DAI, but as the IL-10 deficient mice start off at lower weights, this metric was removed and utilized as a separate readout. The HDS was also used in the determination of the severity of the colitis and is a much more in-depth test. The HDS

uses hematoxylin and eosin (H&E) stained sections of colon to look at the structure changes of the crypts in the colonic epithelium, the presence of polymorphonuclear cells and mononuclear cells in the lamina propria, presence of granulomas, abscesses, erosions and ulcers, and epithelial damage. While the HDS gives a better picture of the colitis, it requires the euthanasia of the mice. In addition to these tests, immunostaining for the cell proliferation marker Ki67 was conducted. The Ki67 staining was used to assess the cell proliferation in the sectioned colons and would denote the healing effects seen in administration of tegaserod. Our findings here suggest that in the IL-10 experimental model of colitis, 5-HT4 receptor agonists attenuate the development of colitis and provide healing and protective effects against the inflammation which characterizes colitis.

Methods:

The mice used in this experiment were ordered from The Jackson Laboratory by Dr. Dimitry Krementsov. These mice, B6.129P2-IL10^{tm1Cgn}/J, are also known as IL-10 knockout mice. We then bred the mice used for the study from those that were ordered. This model of mouse was generated in 1993 by Kühn and colleagues.

Animal Preparation:

All experimental protocols were approved by the University of Vermont's Institutional Animal Care and Use Committee. The mice were euthanized by either carbon dioxide overdose and cervical dislocation, or isoflurane overdose and exsanguination.

Assessment of Inflammation:

Severity of colitis in the mice was measured using the disease activity index (DAI). The DAI used measures presence of fecal blood, and consistency of stool in determining the severity of colitis in the mouse and is used as a measure of the health of the animal. The determination of fecal blood was done through HemOccult Single Slide testing slides and their developing chemical. The developing chemical, per Beckman-Coulter, is "an alcoholic/aqueous stabilised solution of hydrogen peroxide". To utilize the HemOccult slide, a single fecal pellet was collected from each mouse. The fecal pellet was placed on one of the two testing windows on the card and smeared evenly. The developing chemical was applied to the feces, and if the developing chemical caused a blue color to show on the slide, the feces was HemOccult positive. The DAI uses a set of scores to denote the severity of the colitis seen in mouse as such: for the presence of fecal blood a score equivalent to 0 denotes no blood in the stool, a score of 2 for blood present through use of the developing chemical, and a score of 4 for gross bleeding. For the consistency of the stool a score of 0 denoted a normal stool sample. A score of 2 was softer than normal stool, and diarrhea received a score of 4. After 21 days, the mice were euthanized, and their colons were collected and fixed for immunohistochemistry.

Histological Assessment of Inflammation:

Tissues from the euthanized IL-10 knockout mice were fixed by immersion in 4% paraformaldehyde in 0.1M phosphate buffer overnight at 4°C. Tissue was then paraffin-embedded, sectioned at 10 μm, and stained with hematoxylin and eosin (H&E). The HDS is a scale that aids in the determination of changes in the crypt architecture, if any, infiltration of monocytes and polymorphonuclear cells into the

lamina propria, the presence of granulomas, erosions, and ulcers, as well as epithelial damage. The HDS used in the current study is the same as used in the work done by Spohn et al, which was co-created by her and a trained pathologist, Rebecca Wilcox. The slides for each mouse were scored blindly. Briefly, in determining the score for the HDS, we looked for epithelial damage, architectural changes, mononuclear and polymorphonuclear cells in the lamina propria, polymorphonuclear cells in the epithelium, and erosions and ulcers. In terms of epithelial damage, a score of 1 was noted by mild to moderate epithelial damage, and severe damage received a score of 2. In terms of architectural changes, a score of 1 was noted as less than 50% disturbed architecture, and a score of 2 (extensive damage) was greater than 50% disturbed architecture. Mononuclear cell infiltration into the lamina propria was judged their presence, where a moderate increase received a score of 1 and a severe increase a score of 2. Polymorphonuclear cells were judged by how many were seen in a given field of view. If there were less than 3, but more than 0, this section received a score of 1. If there were 3 or more in a field of view, the score was a 2. Polymorphonuclear cells in the epithelium were judged in a few ways. A score of 1 noted that polymorphonuclear cells were found in the surface epithelium. A score of 2 denoted the presence of cryptitis (branching crypts), and a score of 3 was noted as crypt abscess. Lastly, if any ulcers or erosions were found, a score of 1 was received.

Immunohistochemistry:

Unstained slides of sectioned colon from the agonist and vehicle treated mice were obtained. These slides were deparaffinized in xylene, and then 3 washes of ethanol at varying concentrations (100%, 95%, and 80%, respectively), and lastly a wash in water. Slides were blocked in 4% goat serum in 0.5% Triton-X in phosphate

buffered saline (PBS), slides were incubated in rat anti-mouse Ki-67 (1:100; eBioscience, San Diego, CA) in PBS with 0.5% Triton-X, rinsed, then incubated in goat anti-rat Cy3 antibody (1:1000; Jackson ImmunoResearch, West Grove, PA). Slides were counterstained with DAPI (1:10,000), a nucleic acid marker, to determine total number of cells. The slides were then covered and analyzed on a fluorescent filter microscope. First, the filter was set to so that the DAPI stained cells could be counted. This was noted as the total cell count per crypt. Then, the filter was switched so that the Ki67 stained cells were visible. These cells were counted and then calculated as a percent of the DAPI (the total number of cells). Ki-67 is a cell proliferation marker, and in this case is used to show wound healing effects of the 5-HT4R agonist treatment.

Results:

To test the effects of activation of the 5-HT4 receptor by agonist in colitis, the IL-10 experimental model of colitis was used. The IL-10 model was used because the colitis found in the IL-10 knockout mouse is characterized by histologic findings like that of human IBD (Keubler, Buettner et al. 2015). Specifically, these findings include crypt abnormalities, abscesses, erosions, and the infiltration of inflammatory cells into the lamina propria, which is consistent with the findings of the current study. In this experimental model of colitis, tegaserod was administered on the day of weaning, and each day after for 21 total days. The tegaserod was delivered intra-luminally via enema at a concentration of 1mg of tegaserod/ kg of body weight. The amount of enema given was 100 μL. At this time point, the mice exhibited signs of colitis, meaning that we were treating established colitis as opposed to waiting for the rise of

the disease. Colitis occurs in these mice was shown to occur as early as 4 weeks of age (Raza, Crothers et al. 2017).

The 5-HT4 receptor agonist tegaserod significantly decreased the DAI ($p \leq 0.001$, 2-way ANOVA, Figure 1) in the mice given agonist compared with those given vehicle. This finding suggests that the treatment was effective in improving the health of the mice over time, as compared to the vehicle treatment. The treatment also significantly improved the HDS ($p \leq 0.05$, t-test, Figure 2) in the mice treated with agonist. This shows that there was more protection in the mice given agonist than those given vehicle, as those given vehicle tended to have significantly higher HDS scores, and thus more damage to their colon. It also shows that there was attenuation of colitis in that there is less damage seen in the agonist treated mice in terms of epithelial damage, alteration in the crypt architecture, less infiltration of mononuclear cells and polymorphonuclear cells and no presence of erosion, which was seen in those treated with vehicle.

During the 3-week time course of the study, no significant difference in the survival pattern was detected between the two groups. Death was either as a result of the disease progression (before the treatment period was over) or at the time of euthanasia (21 days). Though this could likely be further elucidated with a longer end-point since most of the mice in both groups survived the full three weeks.

Over the course of the study, the mice were weighed each day for their treatment period of 21 days. Wild type and heterozygous (*IL-10+/+, IL-10 +/-, respectively*) untreated mice were also weighed for comparison as they do not develop spontaneous colitis (Raza, Crothers et al. 2017). When a 2-way ANOVA was used to

compare all three groups at all time points, Tukey's multiple comparison evaluation revealed differences between all three groups ($P \leq 0.0001$; Fig. 5). However, when the weights of the mice were compared on their last day of treatment by 1-way ANOVA, there were no differences between the vehicle and agonist treated mice ($P=0.42$), but there were significant differences between the wild type untreated and agonist ($P \leq 0.005$, 1-way ANOVA, Figure 6) and between the wild type untreated and vehicle ($P \leq 0.05$, 1-way ANOVA, Figure 6).

Additional sections of colons from these studies were stained via immunohistochemistry for the cell proliferation marker Ki67 and counter stained with the nucleic DAPI. Once the slides were stained, they were examined under a microscope with fluorescent filters. From each colon section on the slide, 5 whole crypts were selected and the amount of DAPI stained slides were counted to get a total number of cells per crypt. Then, the amount of Ki67 positive cells were counted, and this was calculated as a percent. This examination was not blinded. We found that the mice treated with agonist had a significantly higher average percent of Ki67 positive cells ($p \leq 0.05$; t-test, Figure 3) than did the vehicle. The agonist treated mice had roughly 30% of their total cells Ki67 positive, while the vehicle treated mice had just over 10%. The numbers seen from the agonist mice are similar to those reported by Spohn and her colleagues in their assessment of agonist treated mice in the DSS and TNBS experimental models of colitis. Due to Ki67 being seen in these mice, this shows that there is a wound healing effect, suggesting the protective effects of tegaserod are due to wound healing not immune suppression.

Discussion:

This study was conducted to test the hypothesis that 5-HT4 receptor agonist administration in the interleukin-10 knockout experimental model of colitis would attenuate the development of colitis and have protective and healing effects. Our findings suggest that treatment with the 5-HT4 receptor agonist tegaserod attenuates the development of colitis and protects against the damaging effects of colitis.

The current study advances previous work done in the Mawe lab, by Spohn and her colleagues, in that in shows the efficacy of tegaserod, a 5-HT4 receptor agonist, in another model of colitis. Spohn and her colleagues used chemically induced experimental models of colitis (DSS and TNBS). The current study used the IL-10 knockout experimental model of colitis. While there are differences between the models, such as TNBS having findings closer to human Crohn's colitis, each of these models have similarities to human IBD paradigms.

The current study further reinforces the significance of the data found by Spohn and her colleagues in that similar results are reported in a different model of colitis. The IL-10 model of colitis does not require the induction of disease as the mice are sick almost immediately after weaning. The data reported from the current study strengthens the results from the studies done by Spohn and her colleagues. In both cases, the DAI and HDS were significantly lower for the animals treated with 5-HT4 receptor agonist than those treated with vehicle. In addition, in both cases, sections of colon stained via immunohistochemistry for the cell proliferation marker Ki67 showed wound healing in the mice treated with tegaserod. This wound healing likely results in the anti-inflammatory actions that are a result of 5-HT4 receptor activation. The anti-inflammatory effect seen because of intra-luminal administration

is likely due to a wound healing/immune regulatory effect and not an immune suppressant effect. This is evidenced in the immunohistochemistry staining of sectioned colons with the cell proliferation marker Ki67. The agonist treated mice saw a significant increase in cells which showed Ki67 than the vehicle treated mice. This means that tegaserod has a healing effect. Spohn et al used Caco-2 cells to further explore 5-HT4 receptor activation. Caco-2 cells are a common epithelial colorectal adenocarcinoma cell line. Caco-2 cells are used for in vitro study. Specifically, Spohn et al studied scratches made in Caco-2 cell monolayers and their treatment with tegaserod versus vehicle cultures. Spohn et al showed that 5-HT4 receptor stimulation enhances cell migration and provides resistance to oxidative stress-induced apoptosis.

While this study shows significance in many areas, like the DAI, HDS, and Ki67 stained sections of colons, there are limits. This study used mice bred in-house, as a constraint of time only a small number of IL-10 knockout mice were able to be obtained. As a result, this study would need more time to be able to breed more mice to increase the n values and determine more strongly the significance of the data. In addition, larger n values would strengthen the results already shown, that there are anti-inflammatory and wound healing effects that are caused by the activation of 5-HT4 receptors by agonist.

There was no significant survival between the groups of mice, but this is likely due to the small n. It is likely that if the n value was increased the vehicle treated mice would have a lesser survival rate than the agonist treated mice due to the worsening of their disease, as shown by the results of the DAI and HDS tests, and in that they have less of a wound healing effect, shown by the data gathered from the Ki67 stained colons.

In addition, there is the need to perform further tests. Tegaserod is known to have other effects and so further trials with a 5-HT4 receptor antagonist would be necessary to elucidate the actions of tegaserod. While the trials performed by Spohn and her colleagues showed that antagonizing the 5-HT4 receptor does in fact inhibit the protective and attenuative actions seen in the activation of the 5-HT4 receptor by agonist in the DSS and TNBS experimental models of colitis, it would be prudent to perform similar agonist plus antagonist experiments in the IL-10 experimental model.

While this study reports low n values, it is prudent to note that significance, namely in DAI, HDS, and Ki67, were found. This significance can be further elucidated with more study and a higher n value. The advantage of increasing the n value of this study is to further elucidate the weight differences between the two treated groups, the survival in the treatment groups, and for further testing such as conducting an antagonist plus agonist group, and the use of a fecal lipocalin-2 ELISA.

The IL-10 experimental model of colitis was used because it has histologic features that are similar to those of human IBD (Chassaing, Aitken et al. 2014). IL-10, unlike DSS and TNBS colitis, causes colitis to occur spontaneously. IL-10 is an anti-inflammatory cytokine which is responsible for resolving inflammation responses. It is also a key gut mediator, which along with its potent anti-inflammatory capabilities, could explain why spontaneous colitis arises from its deficiency. It should be noted that some gut microbiome is necessary for the development of colitis in the IL-10 KO model (Iyer and Cheng 2012). It has been shown by many studies (Iyer and Cheng 2012, Bramhall, Flórez-Vargas et al. 2015, Kiesler, Fuss et al. 2015) where mice have IL-10 knocked out that being raised in a germ-free environment does not give rise to the development of colitis.

Potential Mechanisms of Protection through 5-HT4 Receptor Activation:

There are a few ways in which the activation of epithelial 5-HT4 receptors can elicit the protective effects that have been described above. The fact that 5-HT4 receptor agonists promote mucus secretion from goblet cells, and that 5-HT4 receptors are found in the colonic epithelium likely means that the effects seen here are protective, not anti-immune. As shown in the immunohistochemistry data from Spohn et al and from the current study, mice treated with agonist in all three models of colitis (DSS, TNBS, and IL-10 knockout) showed Ki67 positive cells in around 30% of their total cell count. This likely produces a wound healing effect. In addition, there is likely an anti-inflammatory effect as seen through the lower HDS and DAI scores seen in the mice given agonist. Histologically, healing from chronic inflammation is characterized by resolving abnormalities in crypt architecture, as well as resolving any infiltration of inflammatory cells (Neurath 2012). It should also be noted that after resolution of the chronic inflammation, there could still be features of sustained damage, such as a decrease in crypt density, and the presence of cryptitis (Price and Morson 1975, Rubio, Johansson et al. 1982). These criteria are met in the mice given agonist as they show less inflammatory cell infiltrate, they have less damage to the crypt architecture and to the colonic epithelium.

Figures:

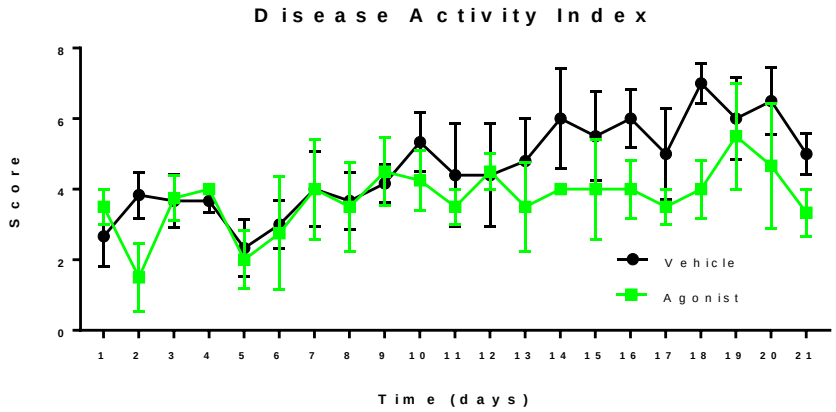

Figure 1: The disease activity index scores as they progressed over time. The agonist had significantly lower scores (n= 3 for agonist, n=4 for vehicle, P≤0.001, 2-way ANOVA).

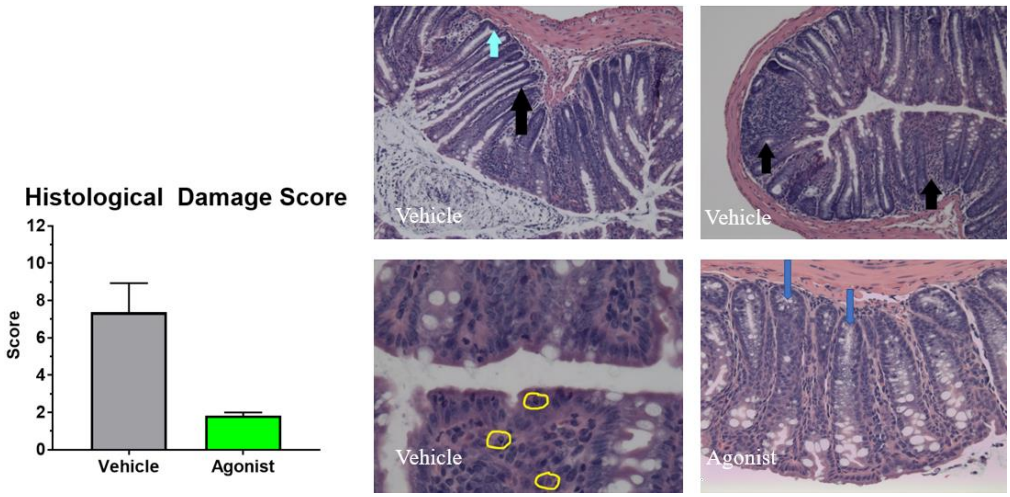

Figure 2: The histologic damage score, which looks at crypt architecture abnormalities, presence of abscesses, granulomas, erosions, ulcers, and infiltration of polymorphonuclear and mononuclear cells. The agonist had significantly lower scores than the vehicle (n=3 for agonist, n=4 for vehicle, P≤0.0304, unpaired t-test). To the side of the graph are micrographs of a vehicle animal which was closest to the mean

HDS (top left, top right, bottom left) and an agonist animal closest to the mean HDS (bottom right). The mean for the vehicle was 7.3 and the closest scored vehicle treated animal was a score of 6.5. The mean for the agonist was 1.8 and the closest scored animal was a 2. All micrographs are shown at 10X on a microscope. The vehicle treated micrographs show elongation of crypts and branching crypts (cryptitis; top left micrograph), infiltration of inflammatory cell (top right micrograph), and the presence of polymorphonuclear cells in the lamina propria (bottom left). The agonist treated micrograph (bottom right) shows more normal crypts (not elongated), and no evident infiltration of inflammatory cells.

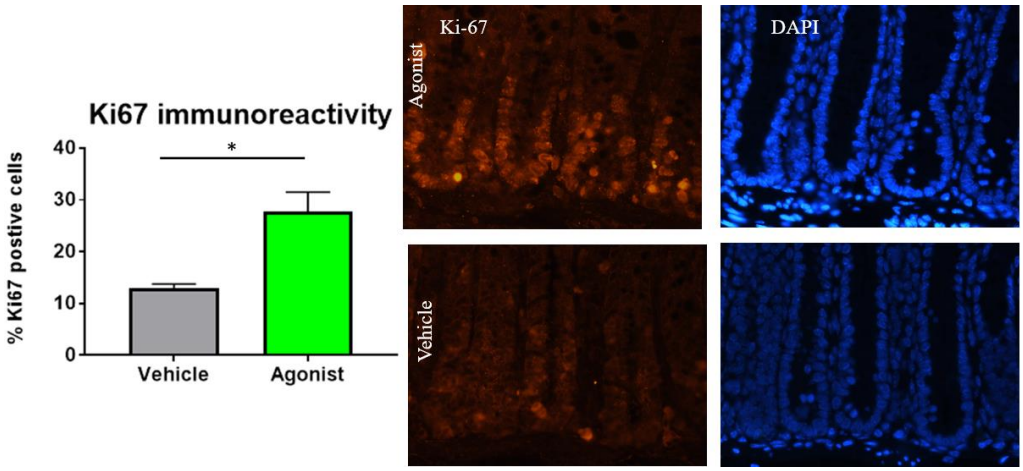

Figure 3: Sectioned colons stained by immunohistochemistry for Ki67 positive cells to show cell proliferation. Sectioned colons were counter stained with 4',6-diamidino-2-phenylindole (DAPI). A blue fluorescent filter was used to visualize DAPI. DAPI was counted in 5 crypts to obtain a total number of cells, and then the fluorescent filter was changed to red to visualize the Ki67 positive cells. The Ki67 positive cells were counted as a percent of the total cells (DAPI). The agonist had significantly more Ki67 positive cells than the vehicle (n=3 for agonist, n=4 for vehicle, $P \leq 0.05$, t-test). Ki-67 positive cells were determined by their fluorescence in comparison to the

rest of the slide. Ki-67 positive cells can be clearly seen by their fluorescence and are more abundant in the agonist treated mice. The vehicle treated has fewer Ki-67 positive cells.

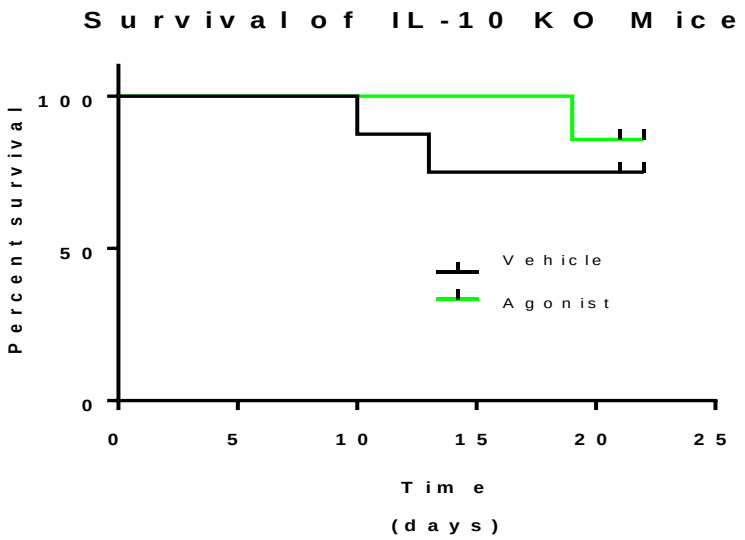

Figure 4: The survival of both treatment groups. There was no significant different between the treatment groups in terms of survival (n=8 for agonist, n=8 for vehicle, P=.5590, log-rank test).

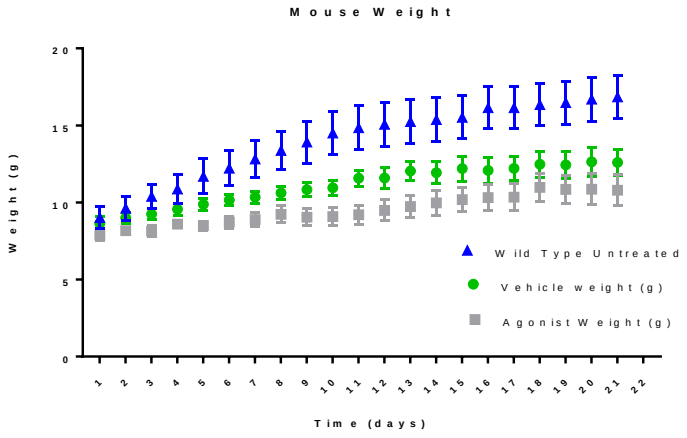

Figure 5: The weights of each mouse group over time. There was significant difference in weight over time, and the mice reacted to the treatment over time.

(P≤0.05, 2-way ANOVA)

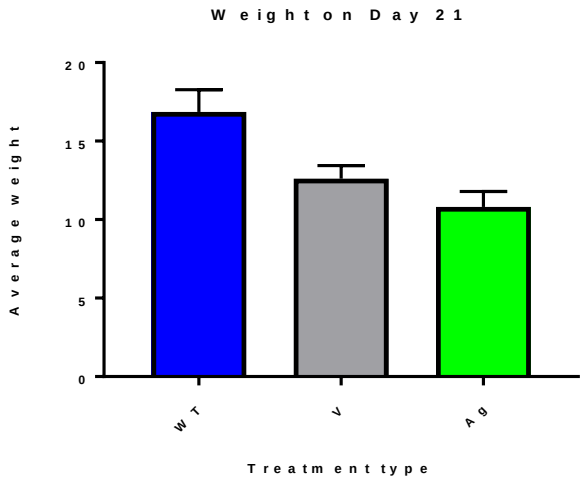

Figure 6: On the last day of treatment the weights between the vehicle and agonist showed no significant difference. However, there was significant difference between the agonist and wild type untreated and vehicle and wild type untreated mice (P=.4225, 1-way ANOVA).

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CHAPTER 3: FINAL CONCLUSIONS AND FUTURE DIRECTIONS

Summary and Conclusions:

Inflammatory Bowel Disease (IBD) is a chronic inflammatory disease of the gastrointestinal tract that exists in three forms: Crohn's Disease (CD), Ulcerative Colitis (UC), and Inflammatory Bowel Disease Unclassified (IBDU). IBD is characterized by its chronic inflammation, which can lead to obstruction and ulcers and is commonly accompanied by either severe diarrhea or constipation. Abdominal pain is also common, as is weight loss. Therapies for IBD include biologics, monoclonal antibodies, 5-aminosalicylates, and corticosteroids (Neurath, 2017). Steroids used like prednisone are not recommended for use long-term, and other therapies are either not very effective, or lose their efficacy over time. The goal of treatment, using drugs or by way of surgery, is to, according to the Mayo Clinic, reduce the inflammation that triggers the signs and symptoms. We continued the study of 5-HT4 receptor agonists, a novel therapeutic, which appears to serve an anti-inflammatory and wound healing role. Results from the current study show that the activation of the 5-HT4 receptors in the colonic epithelium attenuated the development of inflammation in the IL-10 knockout animal model of colitis. In addition, the mice treated with agonist increases cell proliferation and enhances wound healing.

Future Directions:

Potential future directions for this study include the addition of an antagonist plus agonist study. By using a 5-HT4 receptor antagonist combined with an agonist, we would be able to confirm the site of action of tegaserod. Tegaserod is known to have effect at other receptors, such as being an antagonist at the 5-HT2B receptor. If

an antagonist is given first, followed by the agonist treatment, the agonist should have no effect as its site of action is being blocked by the antagonist. This would then confirm the site of action of tegaserod. This is important because tegaserod has been reported to have off target effects leading to cardiovascular issues. While the evidence for this is weak, it would never the less be prudent to show that tegaserod mainly effects the 5-HT4 receptor.

It is also important to conduct further trials in which an alternate route of administration is used. Throughout the current study, the route of administration was by enema, directly into the lumen. This showed great efficacy in that we saw improved wound healing and anti-inflammatory effects. To expand on these results, it would be pertinent to study the effects of agonist activation of the 5-HT4 receptor by way of another route of administration, such as intraperitoneal administration. In the study conducted by Spohn and her colleagues, administration of tegaserod by intraperitoneal injection was less effective than administration by enema into the lumen (Spohn, Bianco et al. 2016). This would strengthen those results and show that enema is the most efficacious route of administering 5-HT4 receptor agonists in experimental animal models of colitis. It is important to note that oral administration would not be pertinent to study. It is known that there are potential adverse effects for oral administration of tegaserod that are a result of systemic absorption. The goal here is to determine therapeutic potential of tegaserod without systemic absorption i.e. administration of tegaserod intraluminally such that there is little-to-no systemic absorption.

It would also be important to increase the size of the study. The significance of the current study given the small sample size is promising, but to further elucidate the

results, more mice are needed. A larger “n” value would give a more accurate picture as to the wound healing and anti-inflammatory effects that are seen in the agonist treated mice. It would also clarify the weight loss and gain of the mice. Given a higher sample size, there would be further data as to which group, agonist or vehicle, maintains their weight, gains weight, or loses weight. Currently, there is no significant difference in the weights between either group. A possible explanation could be that the treatment is having a negative effect on the weight of the mice (they are losing weight as a result of treatment). The non-significance in weight could also be as a result of choosing mice for the agonist and vehicle groups at random and selecting smaller mice for the agonist treatment group. It would be prudent to continue treatment for this metric to ascertain a better picture as to what the treatment is doing to the weight of the mice.

Also, the use of a fecal lipocalin-2 ELISA would be prudent to run. The fecal lipocalin-2 ELISA is a measure of lipocalin-2 which is shown to be present in both the DSS and IL-10 knockout models of colitis (Chassaing, Srinivasan et al. 2012). The use of this readout would be to show a less severe colitis by way of lipocalin-2 level. This would show the effect of the 5-HT4R treatment in an objective manner. In the current study, a fecal-lipocalin-2 ELISA was run to determine the severity of colitis (data not shown). In the current study, the n values found by examination of fecal material were too low to be considered statistically viable. Future studies conducted should utilize the fecal lipocalin-2 ELISA and increase the n value to be statistically viable. The presence of fecal lipocalin-2 i of colitis in both the DSS and IL-10 knockout models of colitis (Chassaing, Srinivasan et al. 2012).

Lastly, it would be important to include longer studies for survival analysis. With the knowledge that the agonist treated mice have reduced inflammation and enhanced wound healing (Figures 1-3), it is likely that, given more time, the agonist would show a greater survival percent than the vehicle treated mice. The current study showed no difference in survival between groups, but the agonist group had later deaths (Figure 4) than the vehicle.

From the current study, further evidence has been reported for a novel treatment of colitis. The current study reports continuing evidence for the protective and anti-inflammatory actions of tegaserod reported in the work done by Spohn et al. Treatment of colitis in the IL-10 experimental model with the 5-HT4 receptor agonist tegaserod showed a decrease in inflammation in the animals treated, as well as wound healing effects. The effect of 5-HT4 receptors on the colonic epithelium is likely due to a variety of effects that include the promotion of mucus secretion from goblet cells, wound healing through increased proliferation, and anti-inflammation due to resolution of infiltration of inflammatory cells and less crypt abnormalities. This study provides further evidence that luminally restricted administration of 5-HT4 receptor agonists are safe and effective and would likely be efficacious in the treatment of colitis.

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