Vasoactive Intestinal Peptide Nanomedicine for the Treatment of Inflammatory Bowel Disease

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THESIS

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DEDICATION

This dissertation is dedicated to my beloved parents Chrysantha and Mignonne Jayawardena for always being beside me through it all!

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Contribution of Authors

Chapter 1 provides background and basis for my dissertation question in the context of current knowledge witin the broad field and highlights the importance and significance of investigating the resreach question. **Chapter 2** includes all methods and materials used in answering the dissertation research question. **Chapter 3** consists of previoulsy published data from journal article (Previously published as Jayawardena, Dulari, Arivarasu N. Anbazhagan, Grace Guzman, Pradeep K. Dudeja, and Hayat Onyuksel. "Vasoactive Intestinal Peptide Nanomedicine for the Management of Inflammatory Bowel Disease." *Molecular pharmaceutics* 14, no. 11 (2017): 3698-3708.) which I was the primary author and major driver of reserch. Figures 21,23-29,39,40 and 42 were directly taken from the manuscript. Arivarasu Anbazhagan assisted with animal sacrifices, Dr. Guzman assisted with blinded pathological scoring reflected in Figure 28. Dr. Dudeja and Dr. Onyuksel my advisors assisted me in designing experiments and analysing data as well as correcting the manuscript after it was written by me. Additionally Figures 44-46 were directly taken from ther publication (Previoisly published as Jayawardena, Dulari, Grace Guzman, Ravinder K. Gill, Waddah A. Alrefai, Hayat Onyuksel, and Pradeep K. Dudeja. "Expression and localization of VPAC1, the major receptor of vasoactive intestinal peptide along the length of the intestine." American Journal of Physiology-Gastrointestinal and Liver Physiology 313, no. 1 (2017): G16-G25) where I was again the primary author, driver of research and person conducting experiments. Dr. Guzman assisted in analysing data, Dr. Alrefai and Dr. Gill revised written manuscript. Dr. Dudeja and Dr. Onyuksel my advisors assisted in analysing data and revised manuscript. **Chapter 4** consists of the discussion based on all the dissertation research

conducted and **Chapter 5** contains conclusions of the overall dissertation project. **Chapter 6** includes future directions and possibilities for novel revenues of the current research.

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LIST OF ABBREVIATIONS

ANOVA	Analysis of variance
AU	Arbitrary unit
CA	carbonic anhydrase
cAMP	Cyclic adenosine monophosphate
CD	Circular dichroism
CD	Crohn's disease
COPD	Chronic obstructive pulmonary disease
CMC	Critical micelle concentration
CFTR	Cystic fibrosis transmembrane regulator
COX	Cyclooxygenase
CXC	Chemokine (C-X-C motif) ligand
CCL	Chemokine (C-C motif) ligand
CDX2	Caudal related homeobox 2
DMEM	Dulbecco's modification of eagle's medium
DMSO	Dimethylsulfoxide
DSPE-PEG_{2000}	Distearoyl phosphatidylethanolamine-polyethylene $glycol_{2000}$
DRA	Down regulated in adenoma
DC	Dendritic cells
DAPI	2,4-Diamidino-2-phenylindole
DSS	Dextran sulfate sodium

LIST OF ABBREVIATIONS (Continued)

EMEM	Eagle's minimal essential medium
ELISA	Enzyme-linked immunosorbent assay
EPR	Enhanced permeability and retention effect
EIA	Enzyme immuno assay
FBS	Fetal bovine serum
FD	Freeze drying
FDA	Food and drug administration
FITC	Fluorescein isothiocyanate
GPCR	G-protein coupled receptors
GAPDH	Glyceraldehyde 3-phosphate dehydrogenase
GI	Gastrointestinal
GLP-1	Glucagon like peptide1
HED	Human equivalent dose
HPLC	High pressure liquid chromatography
HTAB	Hexadecyltrimethylammonium bromide
IBMX	3-isobutyl-1-methylxanthine
IBD	Inflammatory bowel disease
IL-1ß	Interleukin-1ß
IL-6	Interleukin-6
IL-10	Interleukin-10
IL-17	Interleukin- 17
IFNγ	Interferon gamma

LIST OF ABBREVIATIONS (Continued)

iNOS	Inducible nitric oxide synthase
MPO	Myeloperoxidase
MUC2	Mucin 2
mRNA	messenger ribonucleic acid
NF-κB	Nuclear factor-kappa B
NGS	Normal goat serum
NHE3	Sodium hydrogen exchanger isoform 3
NP-40	nonyl phenoxypolyethoxylethanol 40
OCT	Optimal cutting temperature
PAC1	Low affinity vasoactive intestinal peptide receptor 3
PAMP	Pathogen associated molecular patterns
PAS	Periodic acid-schiff
PFA	Paraformaldehyde
$PACAP_{1-38}$	Pituitary adenylate cyclase-activating $peptide_{1-38}$
PBST	Phosphate buffered saline tween
PBS	Phosphate buffered saline
PAMP	Pathogen associated molecular patterns
PEG	Polyethylene glycol
PLGA	Poly (lactic-co-glycolic acid)
QOL	Quality of life
qPCR	quantitative polymerase chain reaction
RPM	Revolutions per minute

LIST OF ABBREVIATIONS (Continued)

RT	Room temperature
SD	Standard deviation
SEM	Standard error of mean
SSM	Sterically stabilized phospholipid simple micelles
TNBS	2,4,6- trinitrobenzene sulfonic acid
TFF3	Trefoil factor 3
TNF-α	Tumor necrosis factor-α
TLR	Toll like receptor
TJ	Tight junctions
Th	T helper cells
T reg	T regulatory cells
USP	United States pharmacopoeia
UC	Ulcerative colitis
VIP	Vasoactive intestinal peptide
VPAC1	High affinity vasoactive intestinal peptide receptor 1
VPAC2	High affinity vasoactive intestinal peptide receptor 2
ZO-1	Zona occludens-1

SUMMARY

The overall goal of this dissertation project was to investigate the therapeutic potential of a biocompatible nanomedicine of Vasoactive Intestinal Peptide (VIP) as a treatment for Inflammatory Bowel Disease (IBD).

IBD is a global health burden currently affecting around 3 million people in the United States, and over 3 million people in Europe with increasing incidence worldwide. Development and pathogenesis of IBD is still unclear and is known to be multifactorial in nature making it difficult to manage. IBD is an umbrella term covering two main types of diseases; Ulcerative Colitis (UC) and Crohn's Disease (CD). These are distinct in certain characteristics and share some key features including inflammation of the intestine with the classic symptom of diarrhea.

Current pharmacological treatments for IBD are mainly symptomatic, with broad spectrum immune suppressants such as steroids and salicylates. In addition, with recent advancements, specific treatments such as anti- tumor necrosis factor-α antibodies have also emerged. However, all these drug classes have severe side effects and do not benefit all patients which results in acute flares of disease.

In this regard, VIP is an endogenous neuropeptide with a wide array of anti-inflammatory and immuno-modulatory effects. It is suggested to be an excellent therapeutic candidate against inflammation, due to being a native peptide present in the body. In addition, severe inflammation in the intestine is known to down regulate endogenous VIP. Furthermore, mice lacking VIP gene show profound abnormalities along the gastrointestinal (GI) tract. These may indicate

that the lack of VIP in severe inflammation, may lead to the exacerbation of IBD.

Although VIP possesses therapeutic activity in inflammatory conditions, it cannot be used in its native form due to its very short biological half-life (<5 minutes). Moreover, being a neuropeptide there are multiple off- target effects; one of the main being hypotension. To overcome these drawbacks, attempts have been made to synthesize VIP analogs. However, none of these analogs have reached clinical usage due to off-target side effects associated with them such as allergies and GI disturbances. Several nanoparticles of VIP have also been tested in pre-clinical models to manage airway inflammation, however, a suitable method to deliver active VIP is still far from achieved. In this regard, our laboratory has previously developed a nanomedicine of VIP in sterically stabilized micelles (VIP-SSM). This well characterized nanomedicine was studied previously, in a pre-clinical model of arthritis and demonstrated low toxicity, and higher therapeutic efficacy compared to the free peptide.

Based on these promising studies, we aimed to investigate the use of VIP-SSM nanomedicine as a novel treatment option for the management of IBD. Previous studies conducted in animal models have shown the benefit of free VIP in alleviating CD like colitis. However, its role in UC like colitis had not been investigated. Therefore, we aimed at determining the beneficial use of VIP in both forms of IBD by delivering the peptide in a more stable formulation (VIP-SSM). In addition, we tested the prospects of delivering the formulation directly to the site of disease (colon) via enteral route. To investigate these effects, the **Specific Aims**

of the project were to; **i)** Determine the effectiveness of VIP nanomedicine in alleviating inflammation in preclinical models of IBD after ip administration. **ii)** Delineate mechanisms of VIP involved in alleviating inflammation in colitis. **iii)** Determine the effectiveness of VIP-SSM when instilled directly to the lumen of the colon *in vivo*, and test the feasibility of VIP-SSM as an oral formulation.

Since IBD encompasses two disease sub-types, it was important to determine its pre-clinical efficacy in both UC and CD. Therefore, to represent these diseases, two separate mouse models of colitis were utilized. The widely used chemically induced colitis models, dextran sulfate sodium (DSS) colitis was used to represent human UC and 2,4,6- trinitrobenzene sulfonic acid (TNBS) colitis was used to represent human CD. The efficacy of the nanomedicine was tested both in a preventive (to manage low grade inflammation) and a therapeutic (to manage severe inflammation) manner. In the preventive studies, treatments were administered in multiple doses on alternate days from the beginning of the study, while colitis development progressed in mice. However, in the therapeutic studies, severe colitis was first established in mice, and then a single dose of the treatment was administered to determine the anti-inflammatory action of the nanomedicine.

Our data demonstrated that, ip doses of 0. 25 nmol VIP-SSM alleviated inflammation in both DSS and TNBS colitis. VIP-SSM ameliorated colitis, by improving the loss of body weight, dampening the robust increase in the pro-inflammatory cytokine mRNA levels, alleviating histopathology and the diarrheal phenotype However, the superiority of the nanomedicine was optimally

observed in a therapeutic model of DSS, where the effects of VIP-SSM were more pronounced compared to the free peptide.

Therefore, the therapeutic model of DSS, was employed to delineate mechanisms of VIP-SSM in alleviating inflammation. In this regard, effects of VIP-SSM on mucosal barrier and intestinal ion transporters were investigated. Mucosal barrier consists of the secreted mucus and the intact epithelial lining of the intestine. Mucus is a gel like material secreted by a specialized secretory cell type termed, goblet cells. These cells are significantly down-regulated in IBD and in mouse models of colitis. In the therapeutic model of DSS, VIP-SSM significantly recovered the loss of goblet cells seen with DSS to almost control levels. The next line of defense in the mucosal barrier is the intact intestinal epithelium. The integrity of the epithelium in maintained by specialized proteins present on the apical membrane, between cells termed as tight junction (TJ) proteins. These proteins are significantly depleted and redistributed from the apical membrane in human IBD and in mouse models of colitis. Therefore, in the therapeutic model of DSS colitis the levels of occludin, a key TJ protein was determined to test the action of VIP-SSM on epithelial barrier. Significantly down-regulated occludin mRNA and protein levels observed in DSS, were remarkably recovered with VIP-SSM. Additionally, the reduction and redistribution of occludin in the distal colons of colitis mice were also improved to almost control appearance with VIP-SSM. These data show other therapeutic benefits of administering VIP as a treatment for IBD.

Another key pathological hallmark of IBD is the severe diarrhea associated with the disease. In this regard, dysregulated electroneutral sodium chloride reabsorption mediated by intestinal ion transporters play a key role in the pathogenesis of diarrhea in IBD. Therefore, in the therapeutic model of DSS, since an overall recovery of diarrheal phenotype was observed, the levels of intestinal ion transporters were studied. In DSS colitis, inflammation is localized to the colon, and the chloride bicarbonate exchanger, SLC26A3 or Down Regulated in Adenoma (DRA) is the key ion transporter down-regulated. Additionally, in IBD patients, levels of DRA are also significantly reduced. Therefore, we aimed at determining the levels of DRA, to measure the effects of VIP-SSM in alleviating diarrheal phenotype. Protein and mRNA levels of DRA were significantly reduced in DSS but, treatment with VIP-SSM reversed this pattern. Next, using cell culture models we demonstrated that VIP had direct effects on the DRA protein expression which was mediated by VPAC1 receptors. Therefore, effects of VIP on DRA may be directly mediated by activation of receptors in the intestine and indirectly via dampening the local inflammation.

Finally, we tested the potential of our biocompatible nanomedicine VIP-SSM, to be used via enteral route to manage localized inflammation in IBD. Prior to conducting *in vivo* studies, there was a requirement to investigate the expression of VIP receptors in the GI tract, to understand if targeting could be possible via luminal delivery. We demonstrated that VIP receptors were expressed in the intestine, specifically on the luminal wall of the colon, which could be directly

accessed when the peptide is delivered to the lumen. Once we demonstrated the expression of VIP receptors at the right location, our next goal was to evaluate if VIP-SSM in solution administered via intrarectal route shows therapeutic effects. *In vivo* efficacy studies after direct colonic instillation of VIP-SSM in a therapeutic model of DSS colitis showed alleviation of inflammation. The nanomedicine was effective at the same dose of 0.25 nmol, which was used previously via ip route. Additionally, these effects were not mediated with the free peptide, demonstrating the benefit of using a nanoparticle to access inflamed tissue environment when administered directly to the lumen.

Therefore, we next aimed to test the feasibility of delivering the nanomedicine in a more stable and favorable oral formulation *in vitro*. In order to be delivered orally, our peptide nanomedicine needed to bypass the harsh conditions of the stomach from digestion and reach the site of action (colon) in the active nanomedicine form. Our previous findings had already demonstrated the freeze-drying capability of VIP-SSM without any lyo or cryo protectants. Furthermore, there are commercially available enteric coated capsules capable of withstanding acidity of the stomach and specifically dissolving at higher colonic pH. Thus, we used these capsules to overcome the stomach conditions and specifically release our nanomedicine at the target colonic environment. Next, the human equivalent dose was calculated based on the effective intra colonic dose in mice. The calculated human dose was prepared as a nanomedicine, then, this formulation was freeze-dried and filled in enteric coated capsules. The capsules

were evaluated for its release of active VIP at its required dose for humans and micelles at their original size after dissolving at colonic pH. Our data demonstrated that, capsules can be formed reproducibly with active VIP and is stable for at least 6 weeks when stored in dark at 4 ^{0}C.

In summary, we have demonstrated that VIP-SSM nanomedicine can alleviate inflammation associated with both UC and CD types of IBD. In addition to an overall anti-inflammatory action VIP-SSM also improves mucosal defense by improving the dysregulated goblet cell number and tight junction proteins. Moreover, the nanomedicine demonstrated anti-diarrheal effects by ameliorating diarrheal phenotype and reduced levels of DRA protein in the colon of DSS mice. Finally, due to the presence of luminal VIP receptors in the colon, direct instillation of VIP-SSM showed similar benefits to parenteral administration *in vivo*. Additionally, these data indicate, for the first time, the potential feasibility of delivering a peptide as a nanomedicine in a capsule via oral route.

1. INTRODUCTION

1.1 Inflammatory Bowel Disease

According to the center for disease control and prevention the prevalence of Inflammatory bowel disease (IBD) in the United states in 2018 is estimated to be around 600 cases per 100,000 persons, roughly accounting for 1% of the population (1). Although, many new insights into the pathogenesis of IBD have emerged in the past years, treatment options for its management have not been as effective. The pathogenesis of IBD is multifactorial in nature and factors which have been identified include; genetic susceptibility of the host, environmental factors, luminal microbial components of the intestine, immune response of the host and compromised barrier integrity (Figure 1) (2). The disease can affect individuals at any stage in life, however is more commonly diagnosed in middle aged (30-40 yrs) and elderly patients (>60 yrs.) (3, 4). One key pathological hallmark of IBD is diarrhea and severe abdominal pain which occurs in almost all patients, thus, debilitating them and reducing their quality of life (QOL) (5).

The disease is further classified into two subtypes, namely ulcerative colitis (UC) and Crohn's disease (CD) which has equal prevalence among patients (per 100,000 adults; CD 201, UC 238) and rarely occurs at the same time in a single patient. Although these share several similar clinical features, there are distinct differences which enables differential diagnosis of UC vs CD (TABLE I).

TABLE I. CLINICAL AND EPIDEMIOLOGICAL FEATURES OF THE TWO-MAJOR INFLAMMATORY BOWEL DISEASE SUBTYPES, CROHN'S DISEASE AND ULCERATIVE COLITIS

	Crohn's disease	**Ulcerative colitis**
Incidence patterns		
Sex	More common in women than men	Equal rates in men and women
Prevalence rates	CD is more prevalent than UC in developed countries	UC emerged before CD in developed countries, and is more prevalent in developing countries
Disease localization		
Affected areas	Entire gastrointestinal tract (from mouth to anus)	Colon, plus some potential backwash ileitis
Inflammation pattern	May occur as patchy, discontinuous inflammation	Continuous inflammation in the affected area (though sometimes a separate cecal patch)
Histopathology		
Penetrance	Transmural inflammation of the entire bowel wall	Inflammation restricted to the mucosal and submucosal layers (other than in fulminant colitis)
Appearance	Thickened colon wall with granulomas, deep fissures and a cobblestone appearance	Distorted crypt architecture, with shallow erosions and ulcers; granulomas, if present, only around crypts
Complications		
	Fistulas, abdominal mass (typically lower right quadrant), colonic and small-bowel obstructions, stomatitis	Haematochezia (rectal bleeding), passage of mucus or pus, fulminant colitis and toxic megacolon

Ulcerative colitis predominantly occurs in the distal intestine involving the colon and the rectum and has a continuous involvement of tissue (7). The extent of inflammation, when observed in a cross section of the whole intestine, is restricted to the superficial mucosa with ulceration and infiltration of neutrophils (8). Incidence of UC has equal occurrence among sexes, however, in some geographic locations, it is observed to be slightly higher in males (8, 9). In contrast, the occurrence of CD is slightly higher among women than males. CD can manifest anywhere in the intestine, most commonly in the distal small intestine (ileum) and colon, giving rise to transmural inflammation which involves deeper layers of the intestinal wall including the mucosa (10). In addition, CD presents as skip lesions where areas of tissue with inflammation is separated by healthy tissue (11).

Genome wide association studies (GWAS) have identified over 100 loci, which have been linked to IBD development (71 for CD and 47 for UC) out of which, one-third are common to both subtypes (12). A prominent contributing factor for the development of IBD is the dysregulated immune response to self-antigens and commensal microorganisms which leads to disease manifestation. Therefore, since IBD is a disease with autoimmune properties, its management would benefit if therapies can be targeted to the immune system. Thus, understanding the immunological basis of the disease could provide insights to discover new therapeutics.

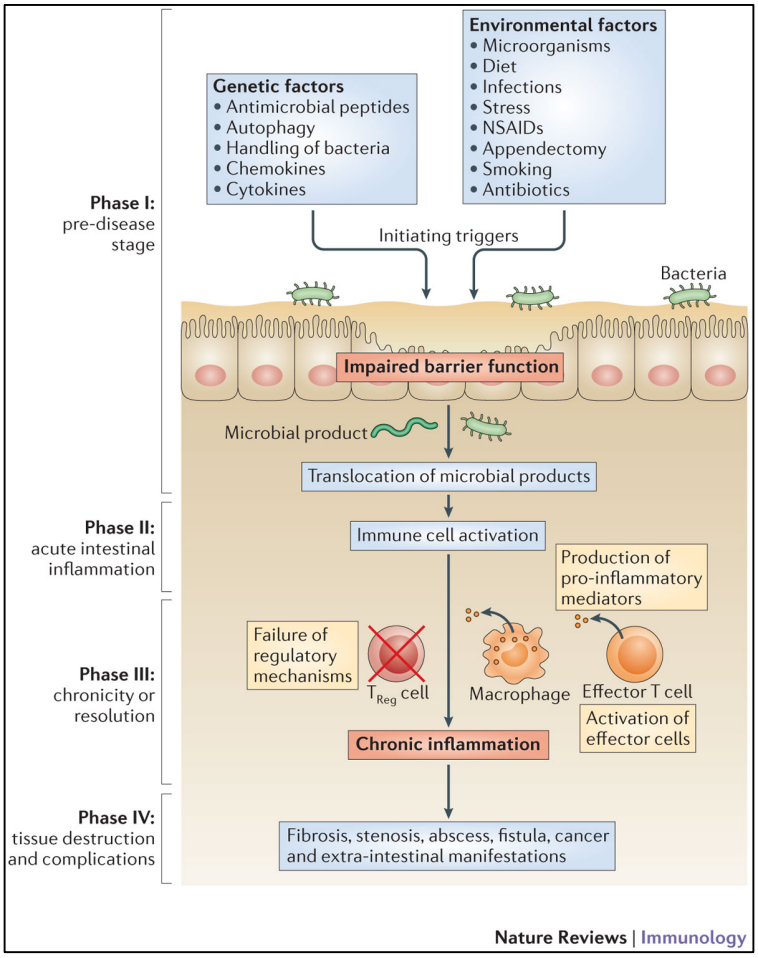

Figure 1: Progression of inflammatory bowel disease
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1.1.1 Immunological basis of inflammatory bowel disease

Pathogenesis of IBD is a complex process with high involvement of the immune system. As shown in Figure 1 above, a combination of genetic and environmental factors lead to the initiation of an immune response in susceptible individuals which in turn lead to disease (13). The innate and adaptive immune systems are both involved during this process. Key cytokines secreted by different immune cells and their regulation is highlighted in Figure 2. In a healthy individual, mucosal immunity of the gastrointestinal tract is maintained by tolerance mechanisms which prevent activation of mucosal dendridic cells (DC). However, in IBD patients, the homeostasis is disturbed and self-antigens and or commensal microbial and food components are processed by DC which trigger an immune response causing an expansion of effector T cells. In addition, the activated T cells resist suppression by regulatory T cells and thus the homeostasis is disturbed (Figure 3).

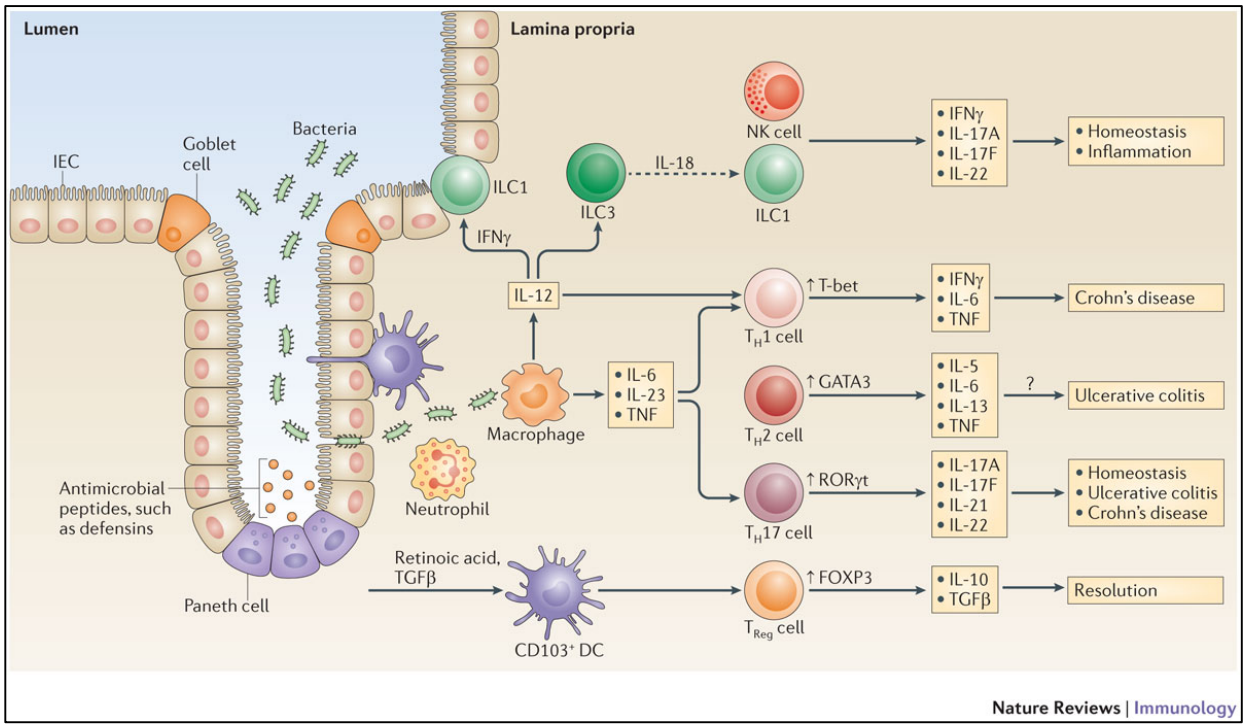

Figure 2: Involvement of immune cells and cytokines secreted during inflammation of the intestine in IBD Reprinted with permission from Ref (13). Springer: Nature, Nature reviews immunology, Copyright © 2014

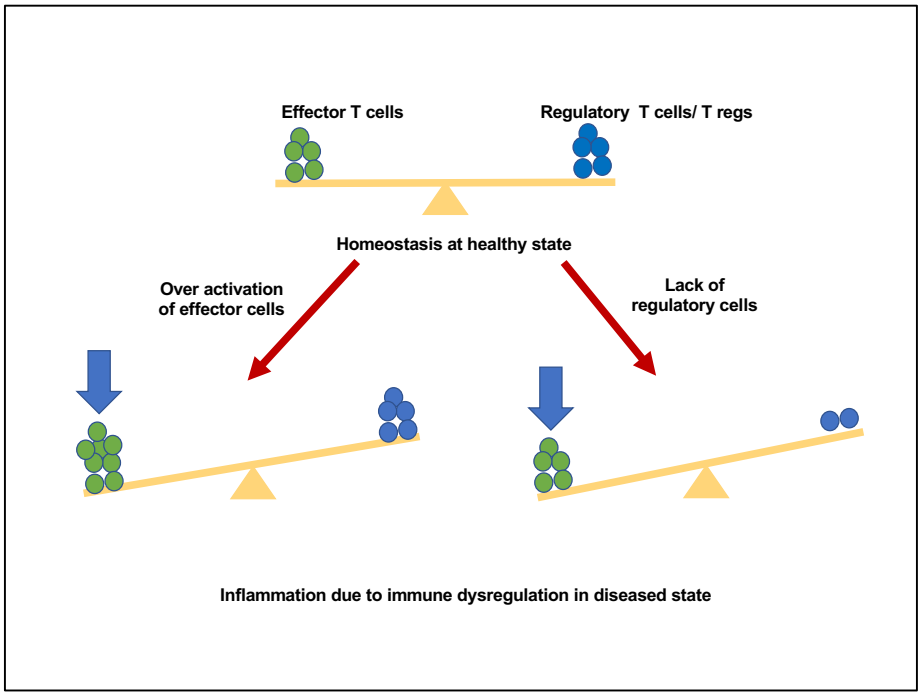

Figure 3: Dysregulation of the adaptive immune system in IBD

1.1.2 Current treatments available for managing inflammatory bowel disease

Main goal of treating IBD is to induce remission in patients and improve their QOL. Following the achievement of remission in these patients, treatments are tailored to maintain remission. Pharmacological agents are the mainstay of IBD management with surgical interventions used as needed (14). There are several commonly used drugs in the clinics today which are listed below.

1.1.2a Aminosalicylates

Aminosalicylates and salicylates have been widely used to manage multiple immunological disorders including rheumatoid arthritis (RA) and IBD (15, 16). Anti-inflammatory action of this drug class is mediated by inhibition of prostaglandins (PG's) and leukotriene biosynthesis, scavenging reactive oxygen metabolites, reducing macrophage chemotaxis and inhibition of cytokine production. Aminosalicylates have shown more benefit in managing UC over CD especially if targeted directly to the colon by oral or rectal administration (17). Mesalazine, 5-aminosalicylic acid (5-ASA) linked to sulfapyridine by a diazo bond is specifically cleaved by colonic bacteria and is the most widely used agent in managing newly diagnosed patients.

1.1.2b Corticosteroids

Corticosteroids are a mainstay treatment for many inflammatory diseases and mediate broad immunosuppressive action by dampening most functions of the

immune system. However, steroids are only initiated in patients who cannot be managed with aminosalicylates alone. It is very important that these drugs are tapered appropriately during treatment so that adverse effects such as hypertension, weight gain and diabetes are not experienced by patients. Systemic corticosteroids including prednisolone, hydrocortisone and methylprednisolone show significant benefit in both UC and CD patients (18). In addition, oral, enteric coated budesonide formulations are also beneficial in IBD patients and are also used to induce remission.

1.1.2c Immunosuppressive agents

Immunosuppressive drugs including thiopurine, azathioprine, 6-mercaptopurine and methotrexate are reserved for patients who cannot be managed by mesalazine and who are on chronic corticosteroids. These agents are used to reduce the dose of steroids and thus are termed steroid sparing agents. Due to their broad non-specific immune suppression and chemistry, thiopurines may give rise to serious side effects including pancreatitis, allergies and infections. However, a combination treatment of thiopurines with anti-tumor necrosis factor alfa (TNFα) therapy have shown potential benefit in some IBD patients. Furthermore, methotrexate has also shown promise in CD patients who failed other agents in maintenance of remission (19).

1.1.2d Antibiotics

Recent research has shown that the gut microbiome may play a role in the pathogenesis of IBD. In addition, high population of pathogenic bacteria in the

gastrointestinal (GI) tract of IBD patients have been managed by the administration of antibiotics (20). In this regard, mainstay antibiotics include ciprofloxacin, metronidazole specifically in patients who have developed complications such as perianal fistulas and pouchitis. Antibiotics are always used in combination with anti-inflammatory agents and have not shown benefit as a stand-alone treatment.

1.1.2e Anti tumor necrosis factor alfa therapy

Tumor necrosis factor alfa (TNF- α) also known as cachexin is a cell signaling protein involved in systemic inflammation. This protein is secreted by many immune cells including macrophages and mediates acute phase response which give rise to cell death. With the advent of novel therapeutics to manage inflammatory disorders, humanized monoclonal antibodies have made breakthrough progress. In this regard, anti-TNF α antibodies have been successful in managing both CD and UC patients by significant clinical improvement and mucosal healing. Currently marketed formulations include, infliximab (Remicade ®, Jassen), adalimumab (Humira ®, Abbvie) and certolizumab pegol (Cimzia ®, UCB) which are mainstay treatments for IBD in patients who do not respond to anti-inflammatory and immune modulating agents. Although TNF α therapy shows promise, in recent years, patients have shown treatment failure and relapse as well as severe side effects due to long term usage and possible development of an immune response (21). Due to these drawbacks, blockade of other cytokines has also been explored; such as, antibodies against Interferon gamma (INFγ) and Interleukin 17 (IL-17). However, use of these agents have not shown any therapeutic potential in managing IBD (22, 23).

1.1.2f Leucocyte adhesion inhibitors

Mucosal inflammation is a key hallmark of IBD where migration of leucocytes to the site of inflammation is imperative. Adhesion molecule alfa-4 beta-7 integrin is an important molecule present on the gut endothelium. Thus, blocking of these molecules by biologics are also used in clinics today. Natalizumab (Tysabri ®, Biogen) was used initially in IBD patients. However, due to the cross-reactivity of this biologic to another integrin molecule, alfa-4 beta-1, which is present on the skin, brain and bone marrow, progressive multifocal leukoencephalopathy (PML), a high-risk side effect was reported. Therefore, to overcome the non-specificity, a more specific antibody with higher affinity to the gut integrin, vedolizumab (Entyvio ®, Takeda) was introduced and is used clinically without any reported PML occurrences (24). These biologics are currently used in patients who relapse with anti TNF α treatment.

1.1.3 Novel treatment options for the management of inflammatory bowel disease

Although multiple pharmacological treatment options are currently available for the management of IBD, there is still a pressing need for developing novel therapeutics. In this regard, many advances have emerged and novel strategies identified. Listed in TABLE II are some potential treatment options which require further exploration. However, this list is not exclusive and due to the complexity of the disease and treatment failures observed, there is a constant need to identify novel therapeutics.

TABLE II: POTENTIAL FUTURE TREATMENTS FOR INFLAMMATORY BOWEL DISEASE

Pharmacological class	Mechanism of action	Route of administration	Agents
Small molecule inhibitors of RNA and intracellular cytokine pathways			
Antisense oligonucleotides	Inhibit specific messenger RNA (mRNA) or DNA sequences responsible for cytokines implicated in inflammation	Topical (alicaforsen enema), oral (mongersen)	Alicaforsen and mongersen
Janus Kinase inhibitors	Inhibitor of several enzymes responsible for signal transduction pathways for multiple cytokines, including proinflammatory cytokines in IBD	Oral	Tofacitinib, others
Biologics			
Interleukin-12 and -23 antagonist	Humanized monoclonal antibody that binds the p40 subunit used by IL-12 and IL-23	Subcutaneous injection	Ustekinimab
Sphingosine-1-phosphate receptor agonists	Agonist of sphingosine-1-phosphate receptor subtypes one and five that includes peripheral lymphocyte sequestration	Oral	Ozanimod
Anti-integrin monoclonal antibody	Humanized monoclonal antibody that binds the β7 subunit of both the α4β7 and αEβ7 integrin heterodimers	Subcutaneous injection	Etrolizumab
Therapies which modify the gut microbiome			
Fecal microbiota transplant	Promotes change/restoration of gut microbial composition and diversity	Topical (oral or via endoscopy)	Donor stool
Probiotics	Promotes change/restoration of gut microbial composition and diversity	Oral	Various

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1.2 Vasoactive intestinal peptide

Isolated first from the intestine by Said and Mutt, vasoactive intestinal peptide (VIP) is an endogenous 28 amino acid long neuropeptide with an array of biological functions (25). As depicted in Figure 4 below, in physiological buffers VIP is a cationic molecule with a net +3 charge, with two clusters of positively charged residues (Green), 2 well separated negatively charged residues (red) and a charged termini (26). This peptide although termed as intestinal, has a broader organ-wide distribution (27). It is known to be secreted by neuronal cells mainly in the central and peripheral nervous system (28), endocrine cells of the pituitary and pancreas (29-31) and immune cells including macrophages (32), lymphocytes (33) and mast cells (32, 34). Once its presence in the immune system was established, an increased attention was drawn towards the immunomodulatory properties of VIP (35).

Structurally, VIP has sequence homology to glucagon superfamily of peptides including glucagon like peptides (GLP) and secretin (36). In aqueous solutions, the peptide renders a random coil conformation and transfers to an alfa-helical form when associated with biological membranes.

N- terminal

(NH_3^+) His-Ser-Asp-Ala-Val-Phe-Thr-Asp-Asn-Tyr-Thr-Arg-Leu-Arg-Lys-Gln-Met-Ala-Val-Lys-Lys-Tyr-Leu-Asn-Ser-Ile-Leu-Asn-(NH_2)

C-terminal

Figure 4: Amino acid sequence of vasoactive intestinal peptide

1.2.2 Vasoactive intestinal peptide receptors

The following have already been published and is used with permission from reference (37).

The diverse physiological effects of VIP are mediated through binding of the peptide with seven transmembrane G-protein coupled receptors (GPCRs). VIP binds first, specifically to the N- terminus of its receptor and then to the other domains which require the entire 28 amino acid residues of the peptide (Figure 5) (38, 39). “The receptors for VIP are structurally related to the secretin/glucagon superfamily of receptors and are common to both VIP and PACAP (which shares almost 70% amino acid homology to VIP) (40). These receptors have been pharmacologically classified into two types, based on their affinity to VIP. The two high affinity receptors are termed VPAC1 and VPAC2, and the low affinity receptor termed PAC1 (38, 40). Since VIP receptors are stimulatory or type 2 GPCRs (Gs), the molecular pathways of VIP receptor activation and subsequent effects have been mainly attributed to increased intra-cellular cyclic adenosine mono phosphate (cAMP) through adenylate cyclase. Among the three receptors, VPAC1 has been identified as the predominant receptor in different organs such as the liver, lung, thyroid and reproductive organs (41)”.

“Currently, there is very limited information related to the expression of VIP receptors in the intestine. Early studies conducted by Laburthe *et al* with the aid of radiolabeled VIP binding showed high binding of VIP to the intestinal epithelial cells (42, 43). It should be noted that the discovery and characterization of the specific GPCR’s for VIP and its classification was only conducted later and was not defined

at the time these studies were undertaken (40, 44). In addition, the sub cellular localization of these receptors in the intestinal epithelial cells has also not been characterized. Thus, very limited knowledge is available regarding the expression of VIP receptors in the gastro intestinal mucosa."

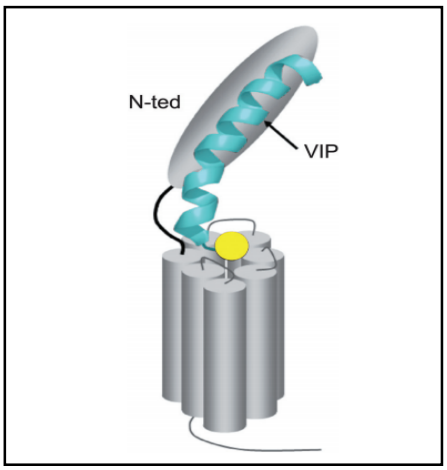


Figure 5: Schematic representation of VIP binding to its receptor

The receptor N-terminal ectodomain (N-ted) traps the central and C-terminal parts (6–28) of VIP (depicted in blue) and positions the N-terminal part (1–5) of VIP (yellow circle) in the receptor core for activation. Reprinted with permission from Ref. (38) Elsevier, peptides copyright © 2007.

1.2.3 Anti-inflammatory properties of vasoactive intestinal peptide

Vasoactive intestinal peptide is also termed as an anti-inflammatory cytokine as it is also released avidly from immune cells including macrophages, neutrophils, monocytes and lymphocytes. Broadly, the anti-inflammatory actions

of VIP can be classified into its effects on the innate and adaptive immune systems. The involvement of neuropeptides in modulating the immune system also known as neuro-immunomodulation was realized when mediators such as VIP were identified to be released from immune cells. This suggested a common biochemical language between the neuroendocrine and immune systems. In addition, receptors for these specific mediators are also present on the immune cells confirming the capacity of these agents to elicit biological functions in these cell types.

VIP has been shown to affect key players of the innate immune system specifically the macrophages. VIP is known as a deactivator of macrophages, microglia and dendritic cells. In general, activated macrophages are known to be inhibited by VIP by preventing their adherence, migration and the production of cytokines and reactive oxygen species (45). In addition, the second messenger responsible for these inhibitory functions has been identified to be cAMP. In this regard, VIP is also an important anti-inflammatory agent in its ability to further increase secretion of anti-inflammatory cytokine such as IL-10. In addition to inhibiting the pro-inflammatory cytokines, VIP also activates inhibitor of NO (iNOs) and inhibits cyclooxygenase 2 (COX2) which adds on to its anti-inflammatory mechanisms.

The accumulation of immune cells or chemotaxis at the site of inflammation is mainly mediated by chemokines such as C-X-C motif chemokine ligand (CXC); CXCL-1 or KC, CXCL2, C-C Motif chemokine ligand (CCL); CCL2, CCL3 and Interleukin (IL); IL8 in humans, VIP is also known to down regulate the expression

of these chemokines (46). Another key factor contributing to the development of an immune response by the innate immune system is through the identification of pathogen associated molecular patterns (PAMPS) with the aid of receptors such as Toll like receptors (TLR's); in this regard, VIP has also been shown to prevent the upregulation of TLR 2 and TLR 4 in models of inflammation (47-49). In the adaptive immune system, VIP plays a key modulatory role by affecting the T helper (Th) cell populations (Figure 6). VIP inhibits the production of Th1 sub population while switching the expansion of Th2 sub population by directly priming the DC (50). In addition, anti-inflammatory T regulatory (Treg) cells are expanded by directly affecting the transcription factor, forkhead box P3 (FOXP3) (50-52).

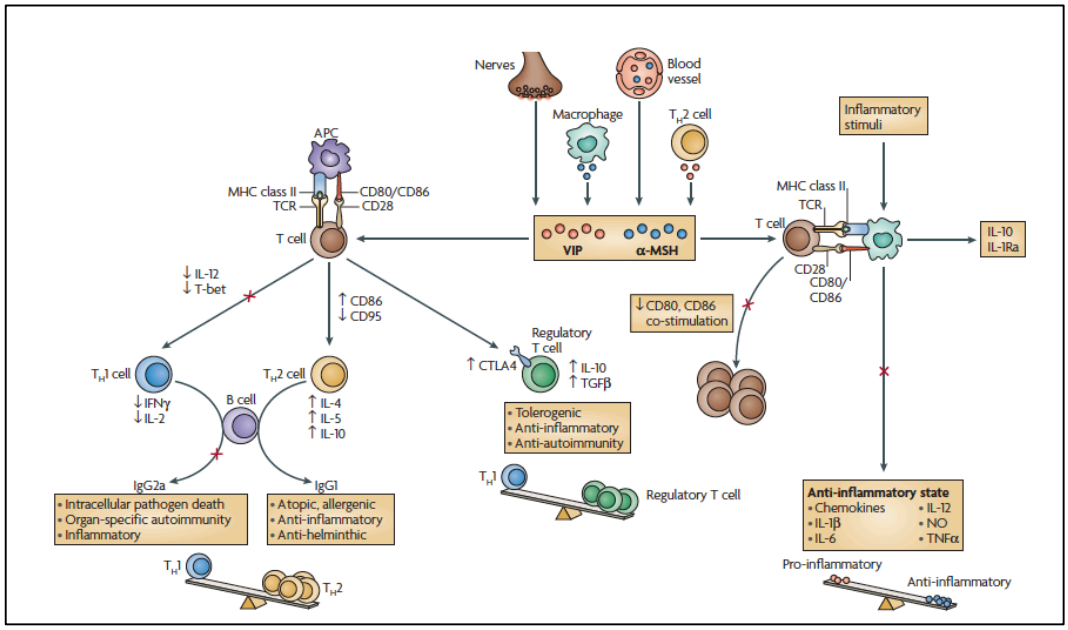


Figure 6: Immune tolerance mediated by vasoactive intestinal peptide

1.2.4 Effects of vasoactive intestinal peptide on physiology of the intestine

Isolated first from the porcine intestine, vasoactive intestinal peptide (VIP) is highly secreted in the intestinal tract, specifically by the enteric nervous system to mediate myriad of functions. In the gastrointestinal (GI) tract, VIP is known to regulate many physiological processes in different regions. For example, in the upper intestine it mainly mediates smooth muscle relaxation for peristaltic movement and sphincter functions (53, 54). VIP is also important in secretion of luminal ions and fluid in the pancreas and jejunum (55, 56) and is imperative as an enteric neuropeptide for tonic inhibitory control of the small intestinal circular muscle in the ileum (57, 58). In the colon, VIP has been shown to be involved in smooth muscle relaxation (59), ion transport (60, 61), mucus secretion (62). In colon carcinoma cell models, VIP has been shown to affect cellular biochemical processes such as glycogenolysis (63). Apart from these effects it also mediates epithelial regeneration (64) and tight junction barrier function (65, 66) in the intestinal epithelium. Furthermore, studies with VIP global knock out (KO) mice show severe abnormalities in the morphology and function of the GI tract, validating the significance of VIP as a key enteric hormone (67).

1.3 Vasoactive intestinal peptide as a therapeutic agent to manage inflammatory bowel disease

All the important properties that VIP possess, make it an ideal candidate for use in managing gastro intestinal disorders, specifically diseases with immunological disturbances such as IBD. Due to the limited success of the current

treatment strategies available for the management of IBD, it is of interest to pursue potential treatment options of endogenous origin which can be administered exogenously, so that minimal safety concerns are posed during clinical development. In this regard, several *in vivo* studies have been undertaken specifically in pre-clinical mouse models to study the potential use of VIP as a therapeutic agent in managing multiple immune disorders such as septic shock (68), multiple sclerosis (69), asthma (70), rheumatoid arthritis (RA) (71, 72) and CD (73, 74).

Several studies related to the use of VIP in IBD have also been conducted *in vivo*, to ascertain its therapeutic potential in alleviating colitis in mouse models as shown in TABLE III. In summary, these studies highlight the positive effects of VIP in combating inflammation associated with colitis by the broad anti-inflammatory and regenerative capacity of VIP. However, with the reported benefits observed with RA (75), studies with IBD models were more focused on CD like animal models due to the similarities in immune responses of these two diseases. Apart from the CD model, infectious models have been also explored to determine if VIP also has anti-infective properties and regenerative capacity (76).

TABLE III: USE OF VIP IN ALLEVIATING COLITIS IN MOUSE MODELS

Study	Colitis mouse model	VIP dose and route	Outcome
Abad et al. 2003 (73)	TNBS colitis	1 nmol every other day i.p	Reduced clinical and histopathological severity, reduced pro-inflammatory cytokines in blood and mucosa
Abad et al. 2005 (77)	TNBS colitis	1 nmol i.p on alternate days	VIP Treatment Down-regulated TNBS-induced expression of pro-inflammatory cytokines and chemokine receptors and TLR's
Newman et al. 2005 (78)	TNBS colitis	1 and 5 nmol VIP i.p or s.c infusion pumps	Prevented leucocyte migration but does not modify experimental colitis by clinical score and blood cytokines
Conlin et al. 2009 (76)	*Citrobacter rodentium*	0.5-5 nmol/mouse/day i.p	Ameliorated intestinal barrier disruption
Vijay et al. 2015 (79)	*Citrobacter rodentium*	Not used *in vivo*	Inhibited PKCε activation, attenuating pathogenic *E.coli* induced intestinal barrier disruption

With the availability of genetically modified animal models it was also of interest to determine the involvement of endogenous VIP during the pathogenesis of colitis in mouse models (TABLE IV). As indicated earlier, that genetically modified vasoactive intestinal peptide global knock out (VIP KO) mice demonstrated significant abnormalities in their intestine highlighting the potential involvement of the peptide during the development of the intestine and its physiology (67). In line with these findings, as shown in TABLE IV, in both studies

conducted by Azuma *et al* and Wu *et al*, mice showed a severe colitis development in VIP and PACAP (closely related peptide) KO animals. In contrast, in the studies conducted by Vu *et al* and Abad *et a*l it was discussed that, the VIP KO animals showed no abnormality in the intestines and that these mice showed resistance to colitis. It is however, unclear as to why these studies showed conflicting findings and how endogenous VIP may have promoted colitis while exogenous peptide was protective. The studies conducted with VIP receptor KO mice showed that VPAC2 but not VPAC1 on T cells were important for mediating the anti-inflammatory action of VIP. Overall, these studies provided further evidence of the important role VIP could play, as a therapeutic agent in managing IBD.

TABLE IV: STUDIES CONDUCTED TO DETERMINE THE INVOLVEMENT OF ENDOGENOUS VIP IN THE DEVELOPMENT OF COLITIS IN MICE

Year	**Colitis mouse model in genetically modified mice**	**Outcome**
Azuma et al. 2008 (80)	DSS colitis	PACAP KO mice showed severe colitis
Yadav et al. 2011 (81)	DSS colitis	Reduced pathology in VPAC1 KO mice vs severe pathology in VPAC2 KO mice
Vu et al. 2014 (82)	DSS colitis	Reduced pathology in VIP KO mice
Abad et al. 2015 (83)	TNBS colitis	Reduced pathology in VIP KO mice
Wu et al. 2015 (84)	DSS and DNBS colitis	Severe disease in VIP KO mice ameliorated with exogenous VIP

1.3 Vasoactive intestinal peptide in sterically stabilized micelles

The neuropeptide, VIP is secreted locally at required sites and rapidly degraded by enzymes present in biological fluids. Under physiological conditions, VIP is mainly cleaved by endopeptidases whereas during inflammation, it is readily cleaved by mast cell derived tryptase and chymase (85). Therefore, though VIP possesses favorable attributes as an anti-inflammatory agent, it cannot be used exogenously in its native form due to its very short biological half-life (86). If infused in high volumes as aqueous solutions, it can give rise to multiple off- target effects such as flushing, hypotension and tachycardia (87). In animal models, to achieve its effects, higher doses of free VIP were administered parenterally at increased frequency (78). Thus, due to the impractical nature of frequent administration and serious side effects, treatments with VIP lack clinical relevance.

Synthetic analogs of VIP have shown some potential after local administration in airway disorders such as asthma and COPD, however to date have not reached clinical development (88). Apart from that, nanomedicines of VIP have also been developed. Medical application of nanotechnology, also termed as nanomedicine was introduced in the early 2000's (89). Nanomedicines have a size range between 1-100 nm and are useful in transporting drug molecules with delivery challenges. The size of the particle allows accumulation at sites where inflammation persists; including in cancers and inflammatory conditions. This phenomenon is termed as enhanced permeation and retention (EPR) effect and allows passive targeting of nanoparticles to the site of disease (90). VIP containing nanomedicines have been developed by our group and a few others. Our

laboratory have extensive experience in delivering VIP in nanomedicine form either as an active targeting agent for imaging (91) or to deliver therapeutics into cancer cells (92). Additionally, VIP nanomedicine was also used as a treatment to manage RA (93). Other groups have also used nanomedicines of VIP, namely polymeric nanoparticles of VIP have been used to deliver VIP to the brain via intra nasal route (94). In addition, nano alumina particles of VIP and liposomes have been used for inhalation to manage asthma in animal models (95, 96). There have also been several efforts to formulate and characterize nanoparticles of VIP with silver (97) and with biodegradable protamine nanoparticles(98). However, these polymeric and metal based nanomedicines pose a risk of severe allergy development and have not yet been studied for any other indication.

In this regard, sterically stabilized micelles (SSM), composed of PEGylated phospholipid, DSPE-PEG_{2000} (component of a FDA approved pharmaceutical product (99) is a unique platform for improving stability of peptides (100). VIP can successfully self-associate with SSM when incubated above its optimum molar ratio to form VIP-SSM nanomedicine (26) (Figure 7). The preparation of VIP-SSM is simple due to the fact that the system is composed of only water soluble components. Empty micelles (SSM) are prepared and peptide solution are added to give a desired molar ratio of lipid:peptide (such as 1:25) and allowed to equilibrate. Self-association of the peptide with micelle results in a conformational change from random coil to an alfa helical form (Figure 7). Phospholipid micelles and VIP interact with coulombic coupling and VIP is believed to be present on the interface between the phospholipid head and the PEG corona of the micelle (26) .

VIP nanomedicine (VIP-SSM) was previously tested in our laboratory for its anti-inflammatory action in a different inflammatory disorder, RA where VIP was shown to have promising therapeutic outcomes. Collagen induced arthritis mouse model was employed to demonstrate the superior therapeutic benefit of VIP-SSM compared to free VIP in reducing inflammation associated with RA. Systemic administration of VIP-SSM showed increased stability, reduced toxicity and higher potency at a given dose in reversing inflammation and associated severity of disease (93).

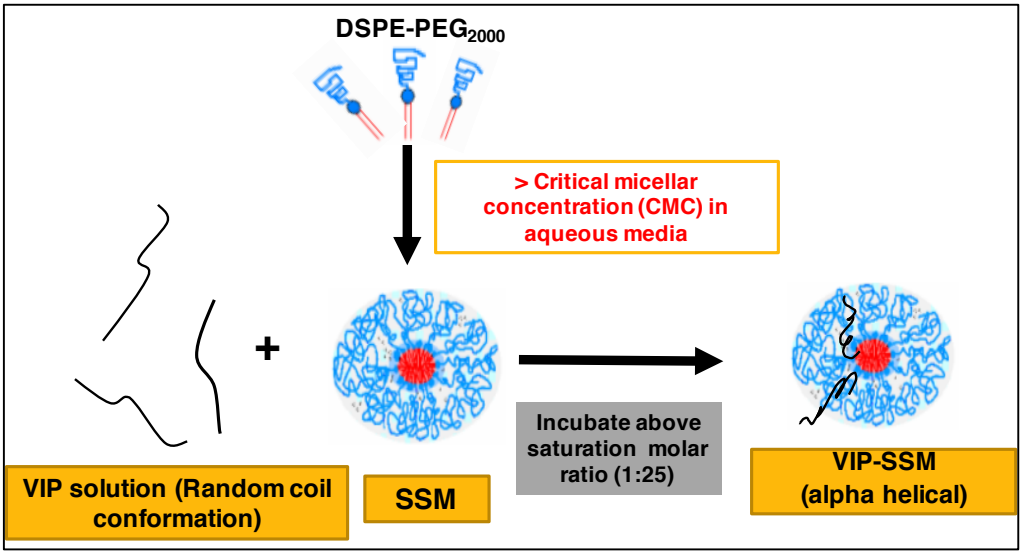


Figure 7: Formulation of VIP-SSM nanomedicine with simple dissolution method

One major side effect of systemic VIP administration is the drastic drop in blood pressure due to the well-known hypotensive properties of the peptide. Due to the small size of the peptide, it can readily extravasate and access smooth muscle cell receptors in healthy vasculature giving rise to hypotension. However, administration of VIP in SSM was capable of completely abrogating this toxic effect in mice after systemic administration (Figure 8). This is most likely due to the bigger size of VIP-SSM (~15 nm), which cannot extravasate out of vasculature to reach vascular smooth muscle cell VIP receptors. However, VIP-SSM efficiently accumulates at the diseased site by passage through leaky vasculature, and thus, only mediates anti-inflammatory action after binding to receptors on immune cells at the inflamed tissue.

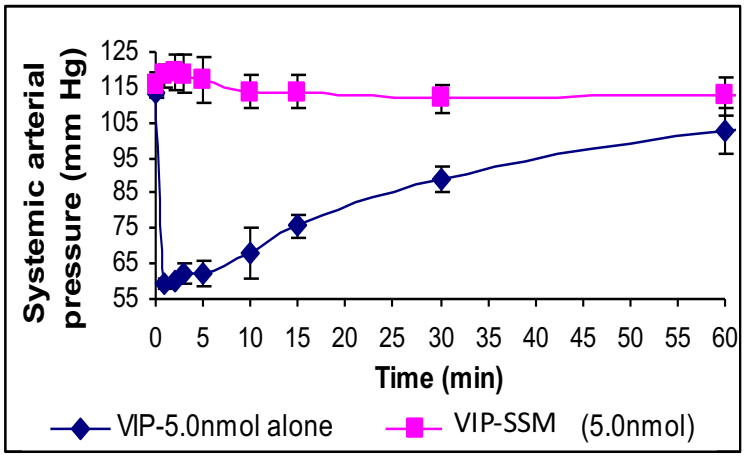


Figure 8: VIP-SSM abrogated the hypotensive toxicity of VIP after systemic administration .Adapted with permission from Ref. (93) Copyright © 2013 American Chemical Society

Another important aspect of SSM formulations, specifically for peptides, is its capability to be freeze-dried without adding additional ingredients such as lyo or cryo protectants, and have improved stability for desired shelf-life (101). Due to these favorable properties, VIP-SSM could be a potential drug candidate for other inflammatory disorders, such as IBD. The potential of VIP analogs or specific agonists were also explored in humans for therapeutic use in asthma and erectile dysfunction. However, these were hindered due to severe side effects such as allergies and GI disturbances associated with them (102). Therefore, use of VIP in a clinical setting is an unmet need and requires further investigations (103, 104). In this dissertation, we achieved the initial forward steps towards the clinical use of VIP nanomedicine for IBD.

1.4 Orally administered nanomedicines as potential drugs for treating inflammatory bowel disease

Being a disease of the GI tract, managing IBD with orally administered drugs which could be site specifically released, would be an ideal way of therapy. Oral route is the most popular and physiological route of administration. Therefore, patients prefer and show higher compliance to orally administered drugs over parenteral drugs. In this regard, a few studies have shown the potential of delivering nanomedicines specifically to inflamed mucosa in IBD. These studies have demonstrated specific accumulation of nanoparticles at the site of inflammation (105-107). Compromised epithelial integrity and lack of secreted mucus allows access of nanoparticles to the inner layers of the mucosa (Figure 9).

This phenomenon is termed, enhanced epithelial permeability and retention effect (108). Due to specific accumulation of nanoparticles at the site of inflammation they can interact with immune cells to mediate anti-inflammatory activity.

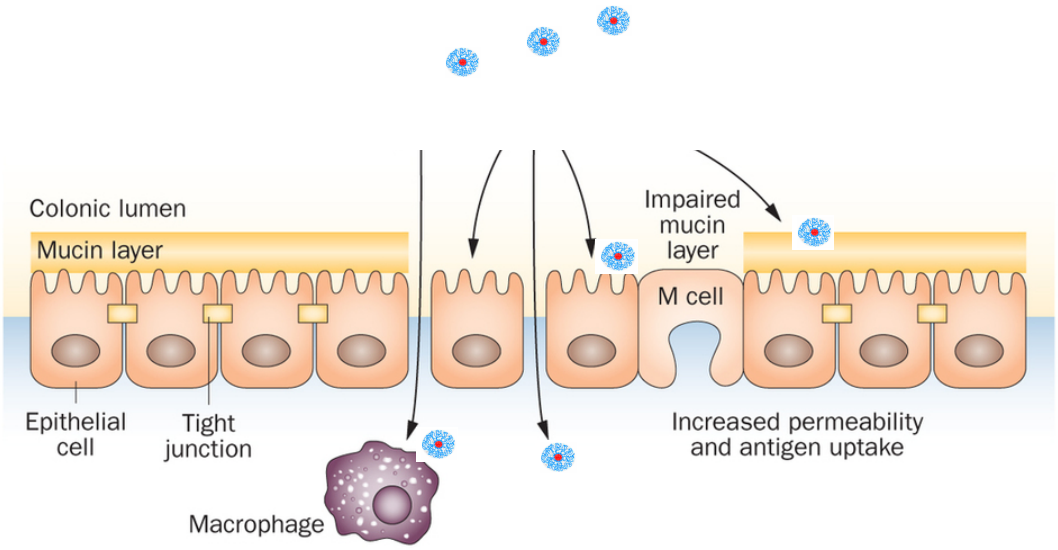


Figure 9: Advantages of using nanoparticles for oral delivery during inflammation in the intestine. Adapted with permission from Ref (105) Springer Nature: Macmillan, Nature reviews gastroenterology & hepatology Copyright © 2015

VIP-SSM nanomedicine has good potential for oral formulation if it can be protected from degradation in GI tract and specifically be targeted to the colon (site of inflammation). The size of the nanomedicine and the constituents of the formulation allows it to be a favorable targeted product for intestinal inflammation. Its nanosize should provide specific persorption at diseased site which facilitates retention in the mucosa in diarrheal conditions prevalent in IBD patients (106). In

addition to the size advantage, PEGylated phospholipid component of the nano-delivery system should allow muco-penetration due to the adherence and penetrance capacity of PEG into the secreted mucus of the intestine (109). Apart from this, PEG also has therapeutic benefit in alleviating inflammation in colitis by affecting barrier integrity of the intestine (110), thus, should add to the therapeutic effects of the active ingredient VIP. Therefore, VIP-SSM as an oral formulation to manage IBD, would provide a platform for delivering peptides in a biocompatible formulation with minimal additives. It would pave the way for other peptide drugs associated with SSM to be delivered by the same route for either local or potentially for systemic effects (100, 111-113).

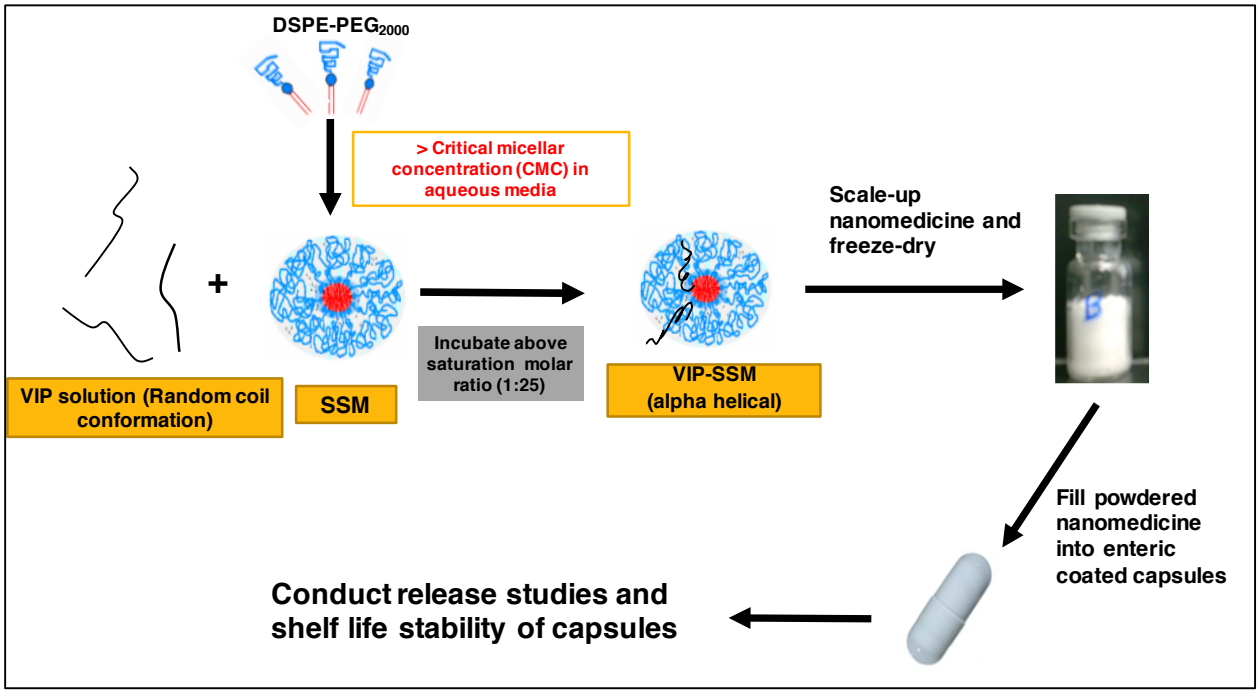


Figure 10: Schematic representation of freeze-dried VIP-SSM filled capsules

Conceptually, in this study we proposed freeze-drying VIP-SSM nanomedicine after scaling up the dose for human use and incorporating it in an enteric coated capsule which could specifically deliver the drug to be released in the colon. Once released, VIP should bind to its receptors, which are also present on colonocytes and on immune cells to mediate its therapeutic effects in IBD (Figure 10). The development of such a system would be a ground breaking achievement in the delivery of peptides in an oral formulation and could pave the way for other potential peptide nanomedicines or peptides to be delivered in this form. Previously, other groups have followed similar methods to deliver insulin as an oral formulation by incorporating it in a coated capsule (114). In addition, short peptide (lysine-proline-valine) have been delivered orally to target the colon in nanoparticles in a hydrogel system (115). Some other recent developments in oral delivery of peptides with the use of nanomedicines are presented in TABLE V below. However, none of these formulations have reached clinical trials and there is an urgent need for oral delivery of peptides.

TABLE V: RECENT EXAMPLES OF BIOACTIVE PEPTIDES INCORPORATED IN NANOPARTICLES FOR ORAL DELIVERY

Protein/ Peptides	**Nano delivery system**	**Therapeutic indication**	**Ref.**
Insulin	Nano hydroxyapatite functionalized with PEG	Diabetes	(116)
Lysine-Proline – Valine (KPV)	Poly lactic acid nanoparticles in Chitosan/Alginate hydrogel	colitis	(117)
GLP-1 and DPP-4 inhibitor	Chitosan modified porus silica nanoparticles coated with coated with HPMC succinate	Diabetes	(118)
Beta-lactoglobulin derived peptides	Ploy (lactic co- glycolic acid) nanoparticles	Prevention of cow's milk allergy	(119)
Exenatide	polyethylene glycol-poly(lactic-co-glycolic acid) (PEG-PLGA) NPs modified with Fc antibody fragment	Diabetes	(120)

Abrev. DPP-4: Dipeptidyl peptidase, PEG-ploy ethylene glycol, NP- nanoparticles

1.5 Mucosal defense barrier in inflammatory bowel disease pathogenesis

Mucosal defense barrier of the intestine consists of two important components; the epithelial cell barrier and the secreted mucus. Impaired intestinal epithelial barrier integrity has been shown to be an important factor contributing to the pathogenesis of IBD (8). Furthermore, VIP is known to affect the expression of tight junction proteins and mucus proteins under physiological conditions (65, 121-123). These two parameters will be elaborated in the sections below;

1.5.1 Mucus secreting goblet cells

The viscous gel overlying the mucosa of the intestine is formed largely by the secreted products of the goblet cell population. Among these, the high molecular weight mucin glycoproteins are the most recognized components of the mucus. In addition, mucus-producing cells have also been shown to secrete large amounts of trefoil peptides which together form a complex three-dimensional mesh to form a physical barrier. The amount of mucus and mucus secreting goblet cells are severely down regulated during intestinal inflammation (124).

1.5.2 Intestinal epithelial tight junction proteins

Epithelial barrier integrity is maintained by the proteins of the paracellular junction complex. Tight junctions located at the apical end of the paracellular junction complex perform a gate function controlling the passage of ions and solutes while maintaining a physical barrier to the luminal contents. Tight junctions consist of the transmembrane proteins (claudins and occludin) and cytoplasmic plaque proteins such as zonula occludens-1 (ZO-1). During inflammation, the levels of these proteins are known to be altered and redistributed leading to loss of barrier function (125).

1.6 Intestinal ion transporters and diarrhea associated with inflammatory bowel disease

Diarrhea could result from either a reduction in absorption of fluid and electrolytes from the intestine, an increase in its secretion or both. In this regard, intestinal ion transporters are the key mediators of fluid and electrolyte homeostasis in the intestine. Patients with IBD suffer from severe diarrhea due to

the mucosal damage observed in the intestine. In addition, the loss of key ion transporters have been shown to be associated with the pathogenesis of IBD. The key ion transporters responsible for maintaining this balance include, sodium hydrogen exchanger, isoform 3 (NHE3) and SLC26A3 also known as Down Regulated in Adenoma (DRA). DRA and NHE3 mediate electroneutral NaCl reabsorption in the intestine (Figure 11). In this regard, DRA the major Cl^-/HCO_3^- exchanger in the colon plays a key role in fluid and electrolyte absorption and is down regulated in colitis. Mutated human DRA gene is associated with a severe diarrheal disorder termed congenital chloride diarrhea (126).

Recent GWAS studies identified SLC26A3 as a gene positively associated with IBD patients in certain geographical locations further highlighting the importance of this ion transporters in IBD (127). In addition, DRA protein and mRNA levels are down regulated in mouse models of colitis and contribute to the associated diarrhea (128) Since chemically induced colitis only affects the distal colon, and DRA is the predominant ion transporter present in that segment of the intestine, studies were also undertaken specifically with regard to the expression of DRA in the diseased state.

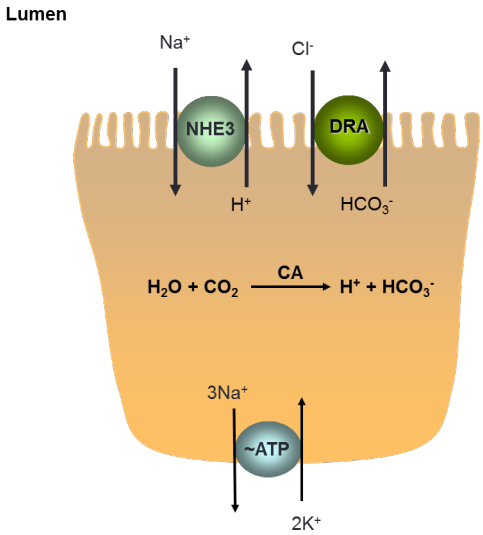


Figure 11: Schematic representation of key ion transporters mediating electroneutral sodium chloride absorption in the intestine. NHE3: sodium hydrogen exchanger isoform 3, DRA: SCL26A3, down regulated in adenoma chloride bicarbonate exchanger, CA: Carbonic anhydrase

1.7 Chemically induced models of colitis

Chemically induced colitis mouse models are the most commonly used systems to study intestinal inflammation. This is mainly due to the quick induction, ability to study mucosal injury, immune responses, relative cost and the ability to use both in acute and chronic settings. In the studies carried out herein, it was important to induce inflammation resembling both UC and CD forms of IBD in mice and thus, the two models described below were used in the acute phase.

1.7a Dextran Sulfate Sodium (DSS) induced colitis

Dextran sulfate sodium colitis is a widely used chemical injury model of colitis developed by Okayazu *et al* in 1980 (129). DSS is administered at doses

from 2% to 5% w/v in drinking water to mice and other animals. The model can be used both as an acute and chronic model based on the duration and dose of the DSS used to induce colitis. DSS specifically interacts with medium chain fatty acids in the distal colonic tissue and disrupts the epithelial barrier (130). This disruption provides an access point to bacteria in the colonic lumen to gain entrance to trans epithelial parts of the intestine and thus, give rise to an exaggerated immune response which resembles human ulcerative colitis (131). Mice start showing signs and symptoms of colitis including weight loss, rectal bleeding, shortening of colon, loose stools and watery diarrhea. Therefore, this model is a useful mean to study potential therapeutics which will be beneficial in managing human UC.

1.7b Two, four, six-trinitrobenzene sulfonic acid (TNBS) colitis

Trinitro benzene sulfonic acid colitis is another chemical injury model of colitis. However, the induction of disease requires priming of the immune system to the agent. Thus, a day or a week prior to the instillation of TNBS 3-5 % w/v in 50% ethanol, the dorsal skin of mice is exposed to a much larger dose of (~15%w/v) TNBS by application, to pre-sensitize mice to the agent for development of colitis (132, 133). It is shown in literature that C57BL/6 mice are relatively resistant to TNBS colitis (132), however, we and others have demonstrated the avid development of disease once pre-sensitization step is performed (134). TNBS is also termed as a haptenizing agent which modifies self-proteins into hapten-modified self-antigens. These antigens mediate a delayed hypersensitivity response mediated by T helper type 1 (Th1) cells (135-137). The

inflammation and associated signs and symptoms of colitis develop immediately after TNBS instillation and peaks at day 3, and persists until day 10 after instillation. Signs and symptoms of TNBS colitis resemble that of Crohn's colitis and include rectal bleeding, loss of body weight, transmural inflammation and shortening of the colon. This model is also used both in acute and chronic setting.

1.8. Rationale of the proposed studies

As described earlier, IBD is an umbrella term covering two main types of diseases; UC and CD. These two types are distinct in certain characteristics and share some key features including inflammation of the intestinal mucosa and the classic symptom of diarrhea (138). IBD associated diarrhea has been attributed to multiple factors where the loss of function and expression of intestinal ion transporters is considered to be a key event (139). Current pharmacological treatments for IBD is mainly symptomatic with broad spectrum immune suppressants such as steroids and salicylates. In addition, with recent advancements, specific agents such as anti-TNF-α antibodies have also emerged as a treatment. However, all these drug classes have severe side effects associated with them and do not benefit all patients. Therefore, better pharmacological agents are very much needed to manage IBD.

In this regard, VIP is an endogenous neuropeptide with a wide array of anti-inflammatory and immuno-modulatory effects (140). VIP is suggested to be an excellent therapeutic candidate against inflammation, partly due to being a native peptide present in the body. In addition, VIP amino acid sequence is identical in most mammals (141) and therefore, data derived from animal models have better

translation to the humans (142). Furthermore, VIP is known to have multiple beneficial effects in the intestine including improving the mucosal defense barrier by affecting both tight junction proteins (65) and mucus proteins (mucins and trefoil peptides) (122). Severe gut inflammation is known to down regulate endogenous VIP in the intestine. Furthermore, VIP knockout mice show profound abnormalities along the GI tract, indicating, that the lack of VIP in severe inflammation may contribute to the exacerbation of IBD.

Although VIP possesses therapeutic activity in inflammatory conditions, it cannot be used in its native form due to its very short biological half-life (86). Moreover, being a neuropeptide there are multiple off- target effects; one of the main being hypotension (143). To overcome these drawbacks previously our laboratory has successfully incorporated VIP into a nanomedicine VIP-SSM, and demonstrated its superiority over the free peptide (93). The nanomedicine VIP-SSM, works by protecting the peptide from degradation, preventing VIP interaction with off target receptors as well as delivering it to the site of inflammation by passive accumulation after parenteral administration. Additionally, we have recently demonstrated the therapeutic effects of a similar neuropeptide GLP-1 (glucagon like peptide-1) nanomedicine, in alleviating inflammation associated with DSS colitis(144). Based on all the afore mentioned, in this study, we evaluated the use of VIP-SSM peptide nanomedicine as a treatment for IBD.

1.9 Hypothesis and specific aims

Given all these beneficial effects of VIP in the GI tract, we hypothesized

that; “VIP-SSM nanomedicine after parenteral administration can effectively ameliorate inflammation and diarrhea associated with preclinical models of colitis and this nanomedicine has the potential to be developed as a solid oral dosage form”. Previous studies have shown VIP to be effective in alleviating inflammation associated with CD like disease (73). However, when other groups attempted to reproduce these studies, there were conflicting findings (78). We believe these discrepancies were partly due to the labile nature of the peptide and thus, inconsistencies in doses reaching the target site. Additionally, we also believe that since VIP is a broad spectrum anti-inflammatory agent, affecting multiple immune cells, it can benefit patients with UC. Therefore, to test our hypothesis under **Specific Aim 1**, two chemically induced colitis mouse models, DSS resembling UC and TNBS resembling CD were employed. VIP’s effect on inflammation and diarrhea was evaluated as nanomedicine or free form. Effect of VIP on parameters such as improving mucosal defense and expression of key ion transporters under the conditions of colitis has not been explored. Therefore, systematic investigation of these parameters was conducted under **Specific Aim 2**. As with most chronic diseases, IBD progresses with acute flares followed by remission which may require drugs to be tailored to the condition. One way of accomplishing this is by administering drugs by different routes i.e.; the same drug can be administered via parenteral route during acute state and while in remission could be switched to an oral formulation. Additionally, since IBD is a disease of the GI tract, oral route would be preferred by most patients. We have also shown recently, the presence of VIP receptors on the luminal surface of the enterocytes which may facilitate mediating

therapeutic activity of VIP if administered intra-luminally (145). Therefore, the potential of using VIP-SSM nanomedicine by luminal route was attempted for the first time in **Specific Aim 3**. VIP-SSM can be freeze dried and incorporated into enteric coated capsules as a powder, to be released at the target site to re-form micelles in liquid form. *In vitro* testing of this concept was performed after establishing the benefit of local administration of VIP-SSM in liquid form, in a pre-clinical model of colitis. We believe local delivery *in vivo* will mimic the action of the nanomedicine, once released at the site of inflammation after oral administration in a capsule. With the completion of these studies we believe that VIP-SSM shows proof of concept as a versatile nanomedicine to manage IBD where severe and low grade inflammation persists with either parenteral or local/oral routes of administration. Overall, results from this study can present VIP-SSM as a promising novel nanomedicine for effective management of IBD which can overcome challenges of stability, toxicity and potency associated with the free peptide.

The specific aims of this project are;

1. **To determine the effectiveness of VIP-SSM in ameliorating experimental colitis after intra peritoneal (ip) administration.**
    1.1. Determine effectiveness of VIP-SSM in preventive and therapeutic modes of DSS induced colitis after ip administration
    1.2. Determine effectiveness of VIP-SSM in preventive and therapeutic modes of TNBS induced colitis after ip administration

2. **To delineate mechanisms involved in alleviating inflammation in animal models of colitis by VIP-SSM**

    2.1 Determine the effect of VIP-SSM on epithelial permeability and mucosal defense barrier

        a. Determine the effect of VIP-SSM administration on mucus secreting goblet cells

        b. Determine the effect of VIP-SSM administration on epithelial tight junction protein expression and distribution

    2.2 Determine the effect of VIP-SSM on expression of intestinal chloride transporter: SLC26A3.

3. **To determine the feasibility of oral delivery of VIP-SSM**

    3.1. Determine the effectiveness of VIP-SSM administered by instillation locally (intra rectally) in an *in vivo* model of DSS colitis

        3.1.1. Determine the distribution of VIP receptors in the GI tract of mouse and humans

        3.1.2. Perform efficacy studies of local VIP-SSM delivery in a therapeutic model of colitis

    3.2. Perform *in vitro* release kinetic studies of freeze dried VIP-SSM in enteric coated capsules in simulated colonic fluids

        3.2.1. Prepare oral nanomedicine in capsules with a VIP dose scaled up to clinical use

3.2.2. Determine *in vitro* reformation of micelles and release of active VIP from freshly prepared capsules under simulated colonic pH

3.2.3. Determine the reformation of micelles and release of active VIP from capsules stored over time

1.10 Significance

Inflammatory bowel disease is a global health burden with growing incidence. IBD currently affects around 1.5 million people in the US(1), over 3 million people in Europe with increasing occurrence worldwide(146). In recent years, research on GI diseases has gained significant attention and this has resulted in an overall health improvement of individuals(147, 148). Therefore, the aim of this project was to investigate a better therapeutic modality for a key GI disorder, IBD, which is currently an unmet medical need. In this regard, endogenous VIP is known to be down regulated in IBD patients and cannot be administered exogenously due to its short half-life and side effects. However, the nanomedicine, VIP-SSM has superior attributes to the free peptide and in this project, we demonstrate its potential in pre-clinical models of colitis as an anti-inflammatory, mucosal healing and anti-diarrheal nanomedicine. Furthermore, the discrepancies observed in previous literature of VIP's therapeutic effects in colitis models were also partly resolved with the administration of the peptide in the nanomedicine form. Administering VIP as a nanomedicine at the same dose showed similar effects in two different models of colitis. Additionally, luminal administration of VIP-SSM to the colon also demonstrated the efficacy of the nanomedicine in alleviating inflammation in colitis. Finally, the feasibility of

delivering VIP-SSM as an oral formulation determined *in vitro*, widens prospects of delivering similar peptide nanomedicines by oral route to manage GI diseases.

Overall, this project demonstrated for the first time, evidence for the potential use of a peptide nanomedicine as a treatment option for IBD management, (both UC and CD) and show potential of its possible usage both as a parenteral and an orally/locally administered formulation.

2. MATERIALS AND METHODS

2.1 Materials

Sodium salt of 1,2-Distearoyl-*sn*-glycero-3-phosphoethanolamine-*N*-methoxy-poly (ethylene glycol 2000) (DSPE-PEG_{2000}) was purchased from Lipoid GmbH (Ludwigshafen, Germany). Synthetic human vasoactive intestinal peptide was custom synthesized using solid-phase synthesis by the Protein Research Laboratory at the Research Resource Center, University of Illinois at Chicago. All peptides were purified to a purity quality of >90 % by high performance liquid chromatography (HPLC) analysis. Caco-2 cells and Minimum Essential Medium (MEM), Dulbecco's Modification of Eagle's Medium (DMEM) and 0.25 % trypsin-EDTA were obtained from American Type Culture Collection (ATCC, Manassas, VA). Phosphate buffered saline (PBS), Cell culture plates and flasks were purchased from Corning inc. (Corning, NY). Fetal bovine serum (FBS) was purchased from Hyclone (Logan, UT). Penicillin (100 U/ml)/streptomycin (100 µg/ml) solution and PCR primers were purchased from Invitrogen (Carlsbad, CA), RNAeasy kits and Trizol reagent was purchased from Quiagen (Valencia, CA), Brilliant SYBR green qPCR master mix kit was purchased from Stratagene (La Jolla, CA). Commercially available enteric coated clear capsules were purchased from PCCA (Houston, TX). periodic acid-schiff staining kit was purchased from Sigma-Aldridge (St.Louis, MO), Hematoxylin and eosin staining kit was purchased from Scytek laboratories (Logan, UT). 2,4,6-trinitro benzene sulfonic acid was purchased from Sigma. Optimal cutting temperature (OCT) media and PAP pen for staining procedures was purchased from Fisher scientific (Hampton, NH),

Dextran sulfate sodium (DSS) was purchased from MP Biomedicals (Solon, OH), cAMP Enzyme immunoassay (EIA) kit was purchased from Cayman chemicals (Ann Arbor, MI), VIP ELISA kit was purchased from Ray biotech (Norcross, GA). All other chemicals unless specified were of analytical grade and were purchased from Sigma-Aldrich. Antibodies used are listed under the respective sections.

2.2 Mice

Male, 4-8 weeks old C57BL/6 mice were purchased from Jackson laboratories, (Bar Harbor, ME). All animal studies performed were approved by the animal care committee of the University of Illinois at Chicago and Jesse Brown Veterans Affairs Medical Center (JBVAMC) (Chicago, IL). Mice were fed with normal chow diet and drinking water ad libitum. Mice were housed 4 mice per cage at JBVAMC in 12-hour light/dark cycle and were acclimatized for 1 week prior to use.

2.3 Human specimens

De-identified formalin fixed, paraffin embedded human colon sections from healthy areas of cancer biopsy patients were kindly provided by the department of pathology, University of Illinois at Chicago. Human RNA was purchased commercially from BioChain (Newark, CA).

2.4 General methods

2.4.1 Preparation of nanomedicine

Vasoactive intestinal peptide nanomedicine was prepared as described earlier with minor modifications (93, 111). Briefly a stock solution of DSPE-PEG_{2000}

at a concentration of 1.12 mM, was prepared by dissolving the required amount of lipid in saline. The solution was mixed well using sonication for 2 minutes to allow complete dissolution of the lipid. This solution was then saturated with inert argon gas, incubated at room temperature (RT) for 1 hour for micelles to form. Next, a stock solution of VIP at a concentration of 25 μM was prepared by dissolving the peptide in saline with mild agitation. These two stock solutions were mixed at a volume ratio of lipid (9): peptide (1), by adding the peptide gradually to lipid and swirling the contents. The resulting solution was saturated with argon gas and kept at RT in the dark for 1 more hour for the nanomedicine to form (VIP-SSM). The final molarity of DSPE-PEG_{2000} was kept constant at 1 mM for all the nanomedicine formulations used *in vivo*. Control solution of 1 mM SSM was prepared by mixing the stock solution of DSPE-PEG_{2000} with saline, again at lipid (9): saline (1), volume ratio and incubated for 1 hr at RT. A peptide solution with free VIP was prepared freshly in a similar manner to the VIP-SSM preparation. For this purpose, a stock solution of 25 μM VIP was prepared and diluted 10 times in saline, swirled and kept aside for 15 minutes prior to injection. The final dose of VIP in each of these solutions (VIP-SSM and free VIP) were 0.25 nmol/100 μL.

2.4.2 Characterization of nanomedicine with dynamic light scattering (DLS)

Particle sizes of all solutions (SSM, VIP-SSM and VIP) were determined using dymamic light scattering (Agilent 7030 NICOMP DLS, Agilent Technologies, Santa Clara, CA). A sample size of 500 μL of each solution was aliquoted into a drop-in tube. The mean hydrodynamic particle diameter (d) in aqueous dispersions

were obtained from the Stokes-Einstein relation shown below using the measured diffusion of particles in solution; (d- hydrodynamic diameter, k- Boltzmann constant, T- temperature, η- solvent viscosity, D- diffusion coefficient).

$$d = \frac{\kappa T}{3\pi\eta D}$$

Based on the equation, diffusion coefficient (D), was calculated by the light scattered and is inversely correlated to the diameter of the particle in solution. Each reported experimental result was the average of at least three d values obtained from analysis of the autocorrelation function accumulated over at least 15 mins.

2.4.3 *In vitro* bioactivity of vasoactive intestinal peptide and its nanomedicine

2.4.3a Cyclic adenosine mono phosphate enzyme immuno assay

Prior to conducting *in vivo* efficacy studies, it was important to determine if VIP in free and nanomedicine form was bioactive. The bioactivity of the peptide was determined by its ability to specifically bind and activate the type II G-protein coupled receptors (GPCR's) to increase cAMP. To test this effect, HT29 cell line was employed. This cell line has high expression of VPAC1 receptor and thus serves as a good *in vitro* model to determine the bioactivity of the peptide (149, 150).

HT29 cells were maintained in T-75 tissue culture flasks in DMEM culture media supplemented with 10% FBS and 1% Penstrep. To perform the cAMP assay, cells were seeded in 24-well cell culture plates at $1X10^4$ cells/well for 3 days at 37 ^{0}C and 5% CO_2. The cells were then serum starved by replacing media with 1% FBS DMEM, for 2 hours. Next, cells were washed 3 times with PBS, incubated

the phosphodiesterase inhibitor isobutylmethylxanthine (IBMX) at a concentration of 1 mM (222.24 µg/mL), in DMEM for 15 min followed by the addition of saline, SSM, VIP in saline or VIP-SSM for an additional 10 min. Peptide concentration tested was 10 nM which has shown reasonable biological functions at cellular level (151). To prevent breaking up of micelles upon dilution in cell culture medium, 1 µM DSPE-PEG_{2000} solution in DMEM was first added to the respective wells in which SSM or VIP-SSM would be added later. At the end of study, the culture medium was removed and 300 µl of 0.1 M HCl was added into each well and incubated at RT over 20 min for cell lysis. The cell lysates were collected, centrifuged at 1000 g for 10 min and the resulting supernatants were assayed for cAMP concentration using commercial cAMP EIA kit per the manufacturer's protocol (Cayman Chemical).

2.4.3b Vasoactive intestinal peptide enzyme linked immuno sorbent assay (ELISA)

In addition to cAMP EIA assay used above, when *in vitro* studies were conducted, in order to determine the active VIP content directly, VIP ELISA (Ray biotech) was used. The samples were assayed directly as per the manufacturers protocol.

2.5 Methods for Specific Aim 1

The methods listed below were used to test *in vivo* efficacy of VIP nanomedicine on alleviating colitis as stated under specific aim 1.

2.5.1 *In vivo* efficacy studies

The following studies were conducted to determine the *in vivo* efficacy of

VIP nanomedicine in reducing colitis associated in two chemical injury mouse models. In addition, the superior effect of the nanomedicine over the free peptide was compared. As described in section 1.7, dextran sulfate sodium (DSS) colitis model was used to represent human UC and 2,4,6- trinitrobenzene sulfonic acid (TNBS) colitis was used to represent human CD.

In addition, these models were utilized in two treatment modes;

1. To test the preventive action of VIP-SSM in minimizing the insult caused during colitis development

2. To test the therapeutic action of VIP-SSM in alleviating severe colitis.

2.5.1.1 Dextran sulfate sodium colitis mouse model

Six to eight-week-old male C57Bl/6 mice were purchased from Jackson Laboratories (Bar Harbor, ME) and acclimated for 1 week at JBVAMC as mentioned earlier. DSS colitis was used to determine the benefit of VIP-SSM in treating UC like colitis.

2.5.1.1a Preventive Studies with dextran sulfate sodium colitis

For preventive studies 3% w/v of DSS was added in drinking water and given throughout the 8 days of the study. Treatments were administered intra peritoneally (ip) starting at day 1, and continued every alternate day (Figure 12). Treatment groups used with appropriate controls are listed in TABLE VI. Empty nanocarrier was used as a negative control and free VIP was used as a positive control. Mice were sacrificed at the end of the study (day 8) and distal colonic tissues were used for analyzing anti-inflammatory actions which will be described later.

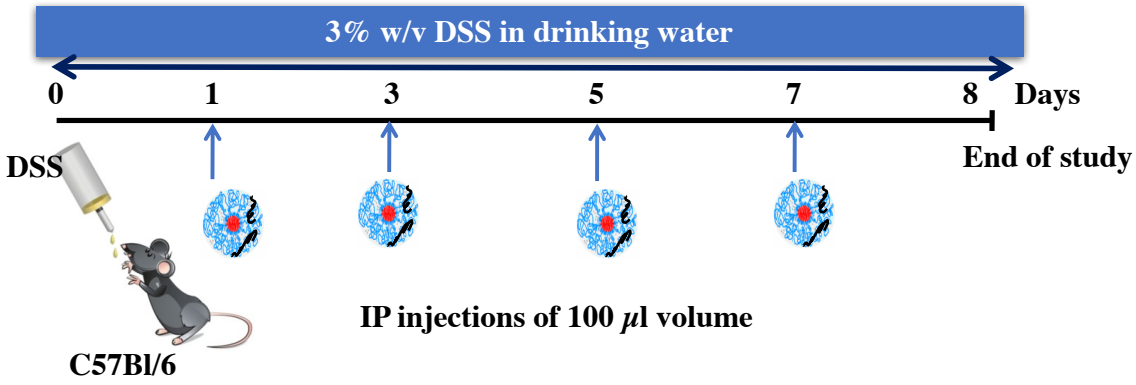


Figure 12: Preventive model of DSS colitis, administration of treatments is indicated by arrow heads. (Treatment groups are listed in TABLE VI)

TABLE VI: TREATMENT GROUPS USED IN DSS COLITIS STUDIES

Group	Treatment	Number of mice
Control	Healthy mice receiving ip injections of 1 mM SSM	5
VIP-SSM	Healthy mice receiving ip injections of 0.25 nmol of VIP in 1mM SSM	5
DSS	DSS mice receiving ip injections of 1 mM SSM	5
DSS + VIP-SSM	DSS mice receiving ip injections of 0.25 nmol of VIP in 1mM SSM	5
DSS + VIP	DSS mice receiving 0.25 nmol free VIP peptide	5

2.5.1.1b Therapeutic studies with dextran sulfate sodium colitis

For the therapeutic studies, 3.5 % w/v of DSS was used instead of 3% to assure development of severe inflammation at day 7 when mice were switched from DSS to tap water (Figure 13). Treatments were administered ip on day 8 as a single dose of 0.25 nmol VIP in free or nanomedicine form with appropriate controls as shown in TABLE VI. At the end of the study, mice were sacrificed and distal colonic tissues were used to analyze anti-inflammatory action.

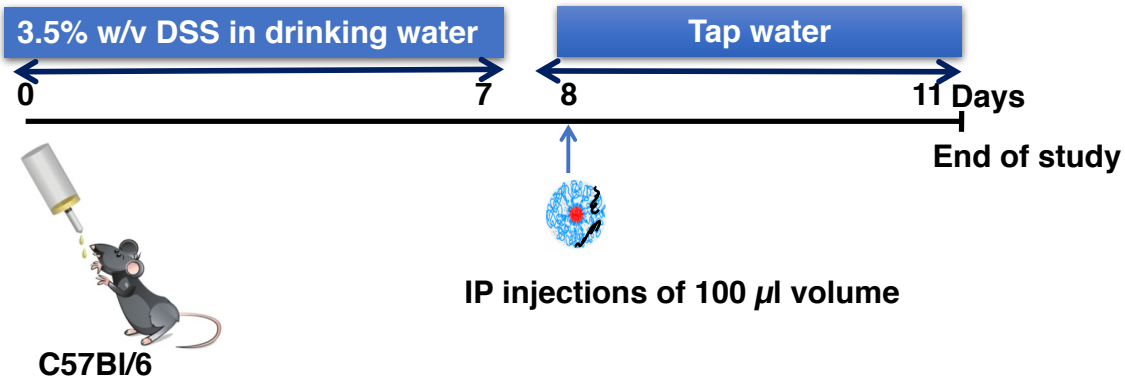


Figure 13: Therapeutic model of DSS colitis, arrow head indicates administration of treatments. (Treatment groups are listed in TABLE VI)

2.5.1.2 Trinitrobenzene sulfonic acid colitis mouse model

In the next set of studies, TNBS colitis was used to determine benefit of VIP-SSM in treating CD like colitis. The same strain of mice was used for TNBS. Therefore, the age of mice used in these studies were between 4-6 weeks due to the better development of colitis in younger mice (152).

2.5.1.2a Preventive studies with trinitrobenzene sulfonic acid colitis

Four to six-weeks-old male C57Bl/6 mice were purchased and acclimated for 1 week as described earlier. At the beginning of the study (day 0) mice were pre-sensitized with 3.75 mg of TNBS in 100 µL 50% ethanol by applying onto the shaved dorsal skin (133). On the next day, mice were anesthetized with ketamine/xylazine and a 3.5-Fr silicon catheter (Harvard Apparatus, Holliston, MA) was inserted 4 cm into the lumen of the colon with aid of a lubricant. Once introduced a syringe needle was inserted to the tubing and 100 µL of 3.5mg TNBS in 40 % ethanol was slowly instilled into the colonic lumen of mice. To ensure that the solution retains in the colonic lumen, mice were held from the tail in an inverted

position for 30 seconds and then kept back in cages to recover from anesthesia. Treatments listed in TABLE VII were administered with appropriate controls on alternate days throughout the study as indicated in Figure 14. At day 5, mice were sacrificed and distal colonic tissues were harvested for analysis.

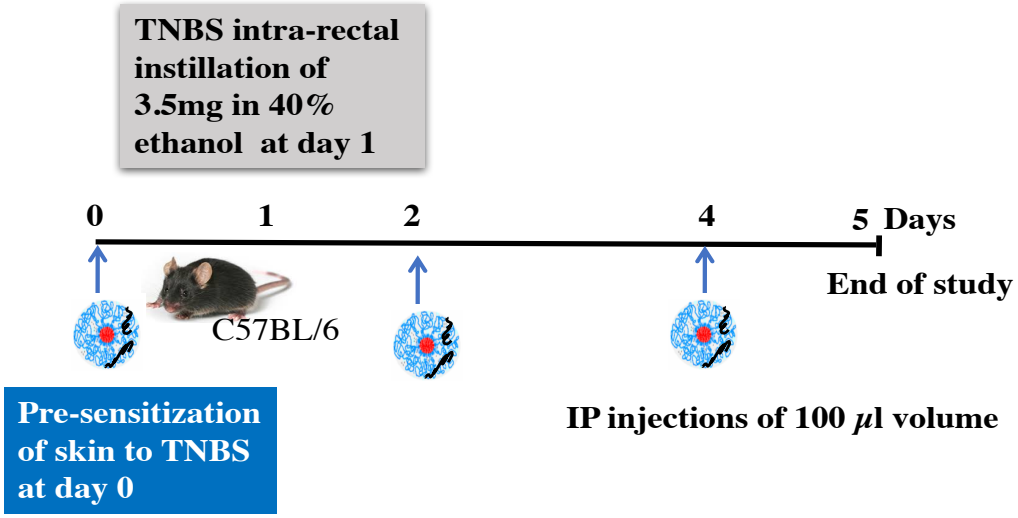


Figure 14: Preventive model of TNBS colitis, treatments were administered on days indicated by arrowheads. (Treatment groups listed in TABLE VII)

TABLE VII: TREATMENT GROUPS USED IN TNBS COLITIS STUDIES

Group	**Treatment**	**Number of mice**
Control	Healthy mice receiving ip injections of 1 mM SSM	5
VIP-SSM	Healthy mice receiving ip injections of 0.25 nmol of VIP in 1mM SSM	5
TNBS	TNBS mice receiving ip injections of 1 mM SSM	5
TNBS + VIP-SSM	TNBS mice receiving ip injections of 0.25 nmol of VIP in 1mM SSM	5
TNBS + VIP	TNBS mice receiving 0.25 nmol free VIP peptide	5

2.5.1.2b Therapeutic studies with trinitrobenzene sulfonic acid colitis

TNBS colitis was induced as described earlier in section 2.7.2a and a single dose of 0.25 nmol VIP either in the free form or nanomedicine form was administered on day 2 to determine the therapeutic potential of VIP in TNBS colitis. Study plan is shown in Figure 15 below and treatments were same as listed in TABLE VII. At the end of the study, mice distal colonic tissues were analyzed for anti-inflammatory action.

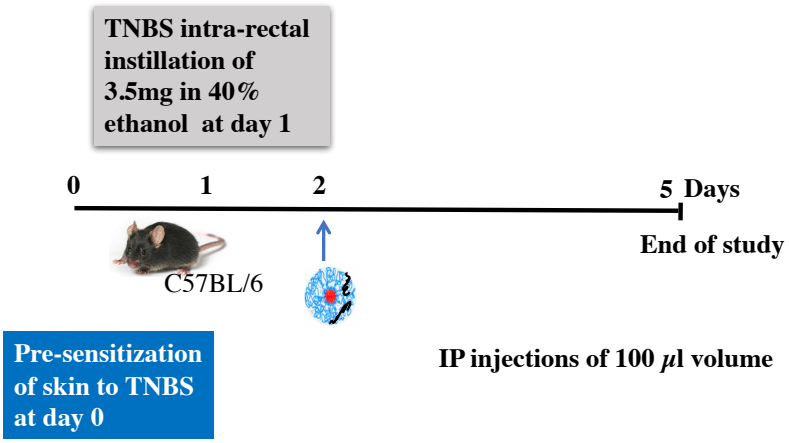


Figure 15: Therapeutic model of TNBS colitis, treatments were administered on the day indicated by arrowhead. (Treatment groups listed in TABLE VII)

2.5.1.3 Determination of the anti-inflammatory action of vasoactive intestinal peptide nanomedicine

The anti-inflammatory effect of VIP nanomedicine was assessed by processing mice tissues for the parameters briefly described below;

2.5.1.3a Body weight measurements

Mice body weights were recorded daily, during the duration of the studies and average weight per group was graphically represented and used for

comparison.

2.5.1.3b Myeloperoxidase assay for distal colonic tissues

Snap frozen distal colonic tissues were stored at -80^{0}C until use. Samples from each mouse in all groups was thawed on ice and individually weighed using a microbalance. These tissues were stored individually in eppendorf tubes and labeled accordingly. Hexadecyltrimethylammonium bromide (HTAB) at a concentration of 50 mg/mL was dissolved in pH 6 phosphate buffer (1 M). This lysis buffer was added to the tissues based on the weight i.e. 10 times the volume based on weight (Ex: 10 mg = 100 μl). These tissues were then homogenized for 4 minutes at 30 Hz with the aid of a tissue homogenizer (Bullet blender) (Homogenizers, Atkinsons, NH). Once homogenized, the samples were centrifuged for 6 minutes at 13000 X g at 4 ^{0}C. Tissue lysate supernatants were carefully collected and stored in -20 ^{0}C if not used immediately.

Colorimetric MPO assay was performed as described before (153). Seven micro liters of lysates prepared above, was pipetted in triplicates per sample into a clear 96-well-plate. Next, O- dianisidene dihydrochloride (Sigma) solution was prepared by combining 16.7 mg of o-dianisidine with 90 ml of deionized water and 10 ml of pH 6 phosphate buffer. To this solution, 4 μL of 30% hydrogen peroxide (H_2O_2) was added to prepare the reaction mixture. Two hundred μL of this reaction mixture was added to each well, and color development was observed over a known period. Usual time taken for color development in distal colons with colitis ranges from 1-5 minutes. Absorbance was measured using a microplate reader (Synergy 4, BioTek, Winooski, VT) at 450 nm after a detectable color development

had occurred. MPO activity was calculated based on the equation below;

$$\textbf{MPO units/mg tissue= (}\Delta\textbf{ Absorbance / }\Delta\textbf{Time) X }\frac{\boldsymbol{A}}{\boldsymbol{B}}$$

A- 1.13 X 10^{-2} nm/min (absorbance change of 1 µmol of H_2O_2)

B- Weight of tissue in 7 µL (0.35 mg)

2.5.1.3c Real time polymerase chain reaction for pro-inflammatory cytokines

Isolation of mRNA from mice distal colonic mucosa was carried out with Qiagen RNeasy kits (Valencia, CA). Mouse tissues were collected to Trizol reagent (Qiagen) and extracted to the aqueous phase using chloroform (Sigma) prior to isolation using the RNeasy kit. All distal colonic tissues were processed with lithium chloride as described before to avoid interaction with PCR reaction (154). Equal amounts of RNA were reverse transcribed and amplified using Brilliant SYBR green qPCR master mix kit (Stratagene). Glyceraldehyde 3- phosphate dehydrogenase (GAPDH) was amplified as an internal control for each sample. Relative expression of genes were calculated according to the ΔΔCt method (155). Average expression of each cytokine per group was graphed and compared across groups for changes. Cytokines assessed include interleukin 1 beta (IL-1β), C-X-C motif chemokine ligand-1and 2 (CXCL1, CXCL-2) and C-C Motif chemokine ligand-3 (CCL3) also known as macrophage inflammatory protein 1 (MIP-1) Primers used are listed in TABLE VIII.

TABLE VIII: GENE SPECIFIC PRIMER SEQUENCES

Gene	Sequence (5' → 3')
Human VPAC1	F-TCATCCGAATCCTGCTTCAGA R- AGGCGAACATGATGTAGTGTACT
Mouse VPAC1	F- GATGTGGGACAACCTCACCTG R- TAGCCGTGAATGGGGGAAAAC
Human VPAC2	F-CAGTGGCGTCTGGGACAAC R- CCGTCACTCGTACAGTTTTTGC
Mouse VPAC2	F- GGTGAGCAGCATCCATCCAG R- TCGCTAGTGCAGTTTTTGCTTA
Human PAC1	F-GTCGGAACCCTTCCCTCATTA R-GGCCTTCACTGACAGGTAGTA
Mouse PAC1	F-GGCTGTGCTGAGGCTCTACTTTG R-AGGATGATGATGATGCCGATGA
Human GAPDH	F-GAAATCCCATCACCATCTT R-AAATGAGCCCCAGCCTTCT
Mouse GAPDH	F-TGTGTCCGTCGTGGATCTGA R-CCTGCTTCACCACCTTCTTGAT
Mouse IL-1β	F- GCAACTGTTCCTGAACTCAACT R-ATCTTTTGGGGTCCGTCAACT
Mouse CXCL-1	F- AAAGATGCTAAAAGGTGTCCCCA R- AATTGTATAGTGTTGTCAGAAGCCA
Mouse CXCL-2	F-CCAACCACCAGGCTACAGG R-GCGTCACACTCAAGCTCTG
Mouse CCL3	F-TTCTCTGTACCATGACACTCTGC R- CGTGGAATCTTCCGGCTGTAG
Mouse Occludin	F- CCTCCAATGGCAAAGTGAAT R-CTCCCCACCTGTCGTGTAGT
Mouse DRA	F- TGGTGGGAGTTGTCGTTACA R-CCCAGGAGCAACTGAATGAT

2.5.1.3d Histopathology

2.5.1.3d.1 Hematoxylin and eosin staining

To study the histology of the distal colon and to understand the extent of inflammation, hematoxylin and eosin (H & E) staining was employed. Staining was performed on formalin fixed, paraffin embedded distal colonic tissue sections of 5 µm thickness. Staining was carried out after deparaffinization of tissue sections as follows: First slides were heated to 60 ^{0}C and immersed in Xylene (Fisher) for 20 minutes. These slides were then gradually immersed in solutions of ethanol of decreasing concentrations (100%, 90%, 70%,50% v/v ethanol) to rehydrate the tissues. Finally, slides were immersed in distilled water. Following the rehydration process, slides were stained with H & E staining kit (Scytek Laboratories) according to the manufacturers protocol.

2.5.1.3d.2 Histopathological scoring

All H & E stained slides were blinded by assigning random numbers and assessed by a pathologist, according to the following criterion; Scores ranging from 0-3 were given where 0 indicates no change and 3 indicates maximal change. Parameters scored for include; epithelial integrity, edema, chronic inflammatory infiltrate, crypt destruction and erosion and ulceration and goblet cell damage.

2.5.1.3e Diarrheal phenotype and colon length

Acute chemical injury colitis models cause diarrhea and inflammation in mice within a few days of inducing the disease. In DSS colitis, diarrhea appears within 5-6 days and with TNBS it manifests within 24 hours. Main reason for this difference in the models is the routes the chemicals are administered to mice. The

extent of inflammation and associated diarrhea in the colon is assessed by observing diarrheal phenotype. The consistency of the stool present in the colon and the colonic length at the day of tissue harvest is used for this purpose (156). As shown in Figure 16 below, colons with colitis appear shortened with loose stool. This is in contrast to healthy colons where solid formed pellets are observed. The lengths of the colons were also recorded and average of each group was used to compare across groups for changes.

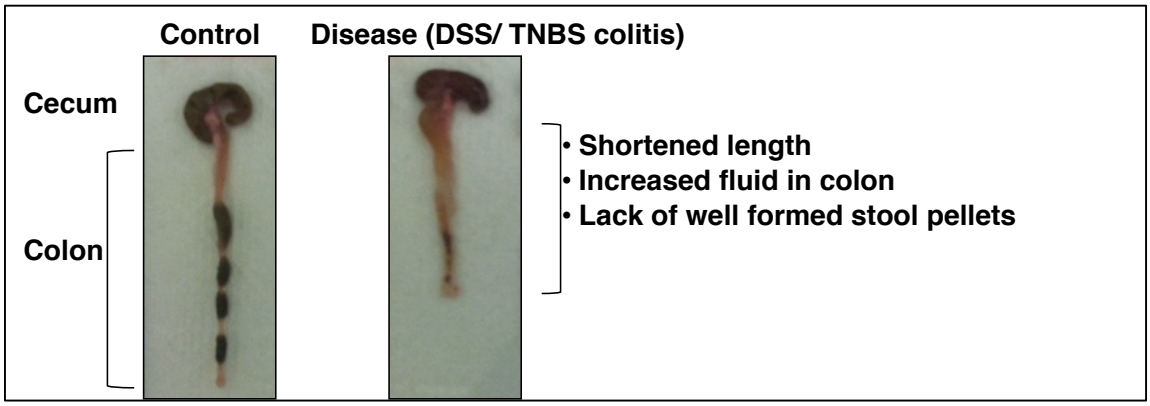


Figure 16: Features of diarrheal phenotype observed in mice with colitis

2.6 Methods for Specific Aim 2

Methods listed below were employed to determine effects of VIP nanomedicine in epithelial barrier and ion transporter expression as stated under **Specific Aim 2.**

2.6.1 Delineating mechanisms of VIP-SSM in alleviation of inflammation

The purpose of the following experiments was to define additional benefits of VIP-SSM in alleviating colitis. Tissue samples used for these studies were obtained from the therapeutic model of DSS colitis described in section 2.5.1.1b.

The parameters listed below were investigated for these effects.

2.6.1a Effect of VIP-SSM on goblet cell number in distal colonic tissue

As described earlier under section 1.5.1, mucus forms the first barrier in the intestine for luminal contents from accessing inner layers. To determine the effect of VIP on the loss of goblet cells observed in colitis, PAS staining was employed. Paraffin embedded distal colonic tissue sections were stained with PAS staining (Sigma) as per the manufacturers protocol after deparaffinization as described under 2.5.1.3d. PAS reagent binds to glycoproteins (neutral and acidic mucins) and lipids in the secreted mucus and stains goblet cells in magenta (157). The presence and number of goblet cells were assessed in each group to study effects of VIP nanomedicine on goblet cells during colitis as follows: The stained sections were visualized under a light microscope (20 X) and average count of goblet cells per crypt was assessed per mouse in at least 10 crypts per mouse. These values per group was averaged and plotted describe how goblet cells are counted

2.6.1b Effect of VIP-SSM on expression of tight junction proteins in the distal colon

Intestinal epithelial tight junction proteins play a key role in maintaining the epithelial integrity. These proteins are down regulated in colitis and thus result in a compromised barrier. To ascertain if VIP nanomedicine had effects on the expression of the TJ proteins the levels of these proteins were assessed in each group. Total mRNA expression of tight junction protein Occludin was also determined as described earlier under section 2.5.1.3c using qPCR analysis with mouse occludin primers listed in TABLE VIII. Additionally, the total protein levels

of occludin was determined by western blot analysis described below. Also, the immuno fluorescence studies were conducted in distal colonic tissue sections to determine the localization of TJ proteins, which will be described subsequently.

2.6.1b.1 Western blotting

Protein lysates from mouse intestinal mucosal scrapings from distal colons were extracted in RIPA lysis buffer (cell signaling, Danvers, MA) supplemented with protease inhibitor cocktail (Roche, Basel, Switzerland) and phosphatase inhibitor (Sigma). The tissues were homogenized and sonicated two times for 20 seconds and centrifuged. The supernatants were collected and 75 μg protein from each sample was used for western analysis as described earlier (158). Briefly, protein samples were prepared by with laemmli sample buffer (Bio-Rad, Hercules, CA) and boiled for 5 mins. Samples were then loaded to 7.5-10% Mini-Protean pre-cast gel (Biorad) and transferred to nitrocellulose membranes. The membranes were blocked with 5% milk in phosphate buffered saline (PBS) for 1 hour followed by incubation with primary antibody (occludin) at a ratio of 1:250 or anti-rabbit GAPDH (Sigma) antibody, at a ratio of 1: 10,000 in 1% milk in PBS overnight at 4^0C. The bound antibodies were detected by horseradish peroxidase-conjugated anti-rabbit Ig secondary antibody (Santa-Cruz, Santa Cruz, CA) followed by ECL detection system (Biorad) per manufacturer's instructions. Antibodies used are listed in TABLE IX.

TABLE IX: ANTIBODIES USED FOR IMMUNOBLOTTING AND IMMUNOFLUORESCENCE STUDIES

Antibody	Catalog no	Supplier
VPAC1	PA3-113	Thermo Fisher Scientific
Occludin	71-1500	Invitrogen
GAPDH	G9545	Sigma Aldridge
DRA	N/A	RRC Synthesized
Villin	Ab130751	Abcam
Zona Occludens-1	61-7300	Invtrogen

Commercially available antibodies were purchased from respective companies. DRA antibody was raised in house at protein research laboratory, University of Illinois at Chicago and validated extensively for authenticity (159, 160). VPAC1 antibody was commercially purchased and validated for its specificity and cross reactivity to mice (37).

2.6.1b.2 Immunofluorescence staining from optimal cutting temperature sections

Mouse distal colonic tissue segments were cryopreserved in optimal cutting temperature (OCT) media, (Fisher scientific) and frozen immediately at -80^{0}C. Afterwards, 5 µm sections were obtained with the aid of a cryostat microtome to glass slides and stored at -80^{0}C. These sections were stained for respective proteins (occludin and ZO-1) by following the common procedure described below;

Slides with tissue sections were taken out of the freezer, and left in dark in a humidified chamber until excess OCT media melted. The sections were washed two times with phosphate buffered saline (PBS), for 2 minutes to wash off all excess OCT and debris. Next, the sections were fixed in 4% v/v paraformaldehyde (PFA) in PBS (pH 8) for 20 minutes. The fixed sections were then washed again

two times in PBS as before and permeabilized with 0.3% NP 40 (Thermofisher) for 5 minutes. These slides were washed again in PBS and encircled with a water repellant pap pen (Termofisher) and blocked with 5% v/v normal goat serum (NGS) in PBS for 2 hours at RT, to prevent non-specific binding. The NGS solution was decanted and replaced with primary antibody of the respective proteins of interest (Occludin and ZO-1) in 1% v/v NGS at a ratio of 1:100 for 2 hours at RT. The slides were washed several times in PBS to remove excess primary antibody and replaced with fluorescently labelled secondary antibodies respective to the primary antibody tagged either with fluorophore Alexa fluor 488 (green) or 594 (red) (Invitrogen) at a ratio of 1:100 in 1% NGS for 1 hour at RT. The slides were then washed a few times in PBS and mounted with 4,6- Diamino-2-phenylindole or DAPI (Invitrogen) to stain nuclei. The coverslips were set in place by application of clear nail polish and stored at -80^{0}C until imaged. Images were acquired at 100 magnification with the aid of the fluorescent microscope Olympus BX51 or Zeiss Axiocam acc1 (Oberkochen, Germany).

2.6.1c Effect of VIP-SSM on epithelial chloride transporter: SLC26A3

Inflammation of the colon has been shown to significantly down regulate the major chloride bicarbonate exchanger SLC23A3. In addition, as described in section 1.5.2, the reduced expression of this transporter is directly linked to diarrheal disorders including inflammation associated diarrhea. Therefore, mRNA and protein expression of SLC26A3 or DRA was determined in the distal colonic tissues of the therapeutic DSS mice samples using the same methods listed qPCR, western blot and immuno fluorescence studies as stated under sections

2.5.1.3c and 2.6.1b.1-2

2.6.1c.1 Cell culture studies

To determine if VIP has a direct effect on DRA protein expression, fully differentiated human adenocarcinoma Caco2 (ATCC) cell monolayers were employed. The human Caco2 cells once differentiated are known to resemble the human small intestinal enterocytes (161, 162). Fully differentiated Caco2 cells have a high expression of the predominant receptor for VIP, VPAC1 (163, 164). Cells were cultured in minimum essential medium with 50 units/ml penicillin, 50 μg/mL streptomycin, 2 mg/L gentamycin and 20% fetal bovine serum. It has been shown that 20% serum enhances the expression of ion transporters such as SLC26A3 in Caco-2 cells (160, 165). Cells (passage 35-38) were seeded at 5000/well in a 12 well trans-well plate (corning inc.) pre-coated with 50 μl of collagen (Thermo fisher). The cells were allowed to grow at 37 ^{0}C and 5% CO_2 until fully confluent for up to 10-14 days. Cell media was changed every 2 days until treated. Prior to treatments, cells were serum starved overnight with sterile EMEM media. The following treatments were given from both the apical and basolateral chambers of the transwells and kept for 24 hrs prior to harvesting the cells. 300 nM VPAC1 receptor antagonist also known as, [Ala11,22,28] VIP (Tocris bioscience, Bristol, UK) was used to inhibit the receptor activity. The antagonist was pre-incubated with the cells in the respective groups treated with antagonist, 3 hours prior to the addition of VIP to assure inhibition (166). Treatments included saline, 10 nM VIP, 300 nM VPAC1 antagonist and 10 nM VIP + 300 nM VPAC1 antagonist. The treatments were prepared by dissolving all agents in normal saline

and adding them to EMEM media. Doses used were based on previous studies conducted by other groups and us, for optimal cellular effects (163). After 24 hrs, cells were harvested in cell lysis buffer (cell signaling, Danvers, MA) supplemented with protease inhibitor cocktail (Roche, Basel, Switzerland) and phosphatase inhibitor (Sigma). These lysates were processed for western blot analysis as stated under 2.8.2a.

2.7 Methods for Specific Aim 3

The methods listed in the following section were used to determine the feasibility of luminal delivery of liquid nanomedicine *in vivo* and for *in vitro* evaluation of the powdered nanomedicine as a potential oral formulation stated under **Specific Aim 3.**

2.7.1 Evaluation of VIP-SSM as a locally delivered treatment in colitis

As described under section 1.4, our long-term goal with VIP-SSM nanomedicine is to develop an oral dosage form that locally releases the drug at diseased site (colon). To this end, we first evaluated the effect of VIP-SSM when directly instilled into the colon by instillation in the liquid form. Then, we prepared freeze-dried powder of VIP-SSM, filled in enteric coated capsules that is known to dissolve at colonic pH, and tested the release of SSM with active VIP. The overall purpose of these studies was to demonstrate the potential use of VIP-SSM as an oral formulation.

Prior to conducting *in vivo* studies, it was important to determine the expression of VIP receptors in the mouse and human intestine. This was to determine the feasibility of targeting VIP receptors via luminal delivery to the colon.

2.7.1.1 Expression of vasoactive intestinal peptide receptors in the human and mouse intestine

To determine the expression of the receptors on intestinal mucosa; Real time PCR analysis (2.5.1.3c), was performed on human and mouse tissues (jejunum, ileum, ascending/proximal and sigmoid/distal colon). mRNA expression of all VIP receptors; VPAC1, VPAC2 and PAC1 was assessed in the intestine. Once the predominant receptors in these tissues were identified,

Protein expression was determined using western blot analysis in mouse tissues as described before in section 2.6.1b.1.

Finally, localization of the receptors in human and mouse paraffin embedded tissues were determined using immuno-localization studies as described below.

2.7.1.1a Immuno-fluorescence staining (paraffin embedded sections)

Formalin fixed, paraffin embedded 5 μm sections from mice proximal and distal colonic regions and the human colon were stained as described previously with some modifications (167). Briefly, slides were placed at 60 ^{0}C for 30 minutes. These slides were then immersed in xylene twice for 20 mins for deparaffinization. The slides were then placed in a series of ethanol solutions (100, 95, 90, 70 and 50%) in coupling jars for gradual rehydration. Afterwards the slides were immersed in distilled water for 5 mins. Antigen retrieval was performed by submerging the slides in a steam bath of 0.1M citrate buffer for 30 mins at 100 ^{0}C. The slides were subsequently allowed to cool to room temperature and rinsed in wash buffer (Tris-buffered saline containing 0.05% tween) for 5 mins followed by 5 min in permeabilization solution of tris- buffered saline containing 0.1% Triton X-100. The

tissue sections were encircled with a water repellant pen and the slides were then incubated for an hour in a moist, dark container with 10% NGS to block non-specific antibody binding. This was followed by incubation of the slides with the primary antibody at a ratio of 1:100, antibodies used; VPAC1 (Thermofisher) and monoclonal anti-mouse villin (abcam, Cambridge, MA) or monoclonal anti-mouse actin (Sigma-Aldrich) or monoclonal anti- mouse Na^+ / K^+ ATPase (Thermofisher) 4^0C overnight in wash buffer containing 1% NGS. Following several washes, the slides were incubated with anti-goat secondary antibodies conjugated to either anti-rabbit Alexa flour 568 (red) or anti-mouse Aexa flour 488 (green) (Invitrogen) for 1 hour at a ratio of 1:100 in wash buffer containing 1% NGS. After a few washes, the slides were mounted with prolong gold antifade/DAPI (Molecular probes) and sealed with clear nail polish. Slides were stored at -20^0C until imaged.

2.7.1.2 In vivo evaluation of VIP-SSM as a local treatment to the colon after instillation

These studies were conducted to determine effectiveness of liquid VIP-SSM nanomedicine, as a locally delivered agent to manage colitis. DSS colitis was used in a therapeutic setting with 3.5% w/v DSS as before, with treatments administered on day 5 intra luminally to the colon by intra rectal instillation (Figure 17). Day 5 was selected instead of day 8 to avoid possible rupture to colon due to local instillation conducted on highly inflamed tissues. Mice were anesthetized with ketamine/xylazine for luminal administration. Treatments of 100 μl volume, were administered directly to the colon by inserting a 3.5-Fr silicon catheter, 4 cm into the lumen (TABLE VI). However, each group in this study consisted of 9 mice.

The dose used was based on the therapeutic studies conducted under **Specific Aim 1** with DSS. Mice were held in an inverted position for 30 seconds and kept back in cages to recover from anesthesia. Tissues were harvested at the end of the study and anti-inflammatory as well as additional therapeutic effects of VIP were determined as described in earlier sections 2.7.3, 2.8.1 and 2.8.3. Parameters investigated included; body weight change, diarrheal phenotype and colon length, pro-inflammatory cytokine mRNA expression, histology, goblet cell numbers, the expression of SLC26A3 and expression of VPAC1 levels in colonic tissues.

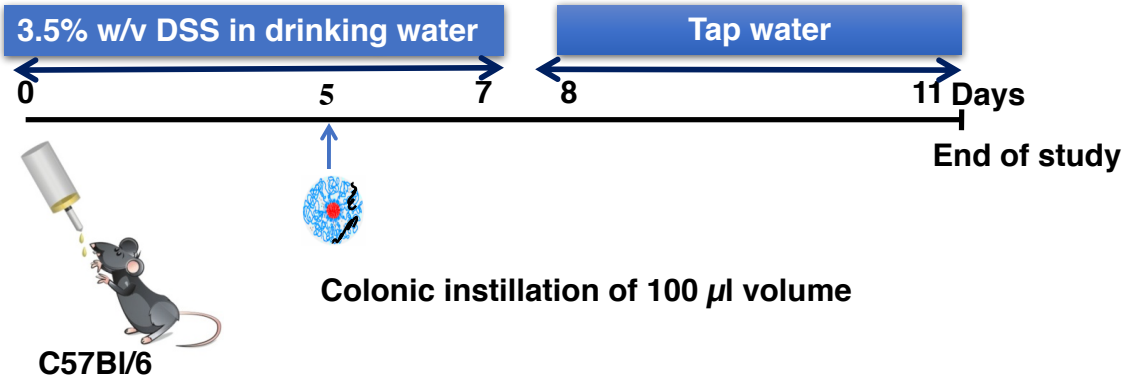


Figure 17: Therapeutic model of DSS colitis for intra colonic VIP-SSM treatment. (Treatment groups are listed in TABLE VI)

2.7.2 *In vitro* evaluation of VIP-SSM as an oral capsule

The following studies, were conducted to ascertain the potential use of VIP-SSM as a feasible oral capsule formulation. Capsules used were commercially available enteric coated capsules from PCCA (Houston, TX). These capsules were of size 1 per United States Pharmacopoeia (USP) standards, and resisted acidic

pH while dissolving at pH 6 and above this pH which resembles the colonic environment.

2.7.2.1 Determination of the optimal human equivalent dose of VIP-SSM

Prior to the preparation of the formulation, the dose determined from the animal studies needed to be converted to the equivalent human dose. For this purpose, a standard pharmacokinetic formula was utilized (168). This formula was based on allometric scaling, which is an empirical approach where the exchange of drug dose is based on normalization of dose to body surface area. This approach assumes that there are some unique characteristics on anatomical, physiological, and biochemical processes among species, and the possible difference in pharmacokinetics/physiological processes should be accounted for by the formula which is below.

$$\text{Mouse optimal dose (mg/kg)} = \frac{\text{Human dose (mg/kg)}}{12.3}$$

The effective dose of VIP-SSM after intra-colonic administration in mice was 0.25 nmol. Assuming the average weight of a mouse to be 20 g, this dose equivalent in milligrams per kilogram is; 41.76 X 10^{-3} mg/kg.

Therefore, based on the formula above, the corresponding dose for a 60 kg human would be;

$$\frac{0.042}{12.3} X\ 60 = 0.202\ mg\ (200\ \mu g)$$

Molar weight (MW) of VIP is 3326 g/mol therefore, this amount of VIP in mols;

(0.202/3326) = **60X 10^{-9} mol**

Available volume of fluid in the human colon is assumed to be between 10 -30 mL (169). Then the molarity of VIP in the lowest volume of fluid present in the colon (10 mL) would be;

(60 nmol/ 10 mL) X 1000 mL= **6 µM**

The lowest volume was used to calculate the drug to be incorporated into capsules in order to accommodate the highest VIP dose in the formulation. However, for practical feasibility, the capsule contents were dissolved in 30 mL of buffer. To detect particles with dynamic light scattering, molarity of SSM was kept at 1 mM after dilution (in 30 mL buffer).

Amounts were determined based on the calculated VIP dose as shown above, and the SSM to be 1 mM after dissolution in 30 mL buffer. Samples were prepared, sufficient for 4 capsules by simple dissolution as described previously in section 2.4.1 in the following manner;

2.7.2.1a Preparation of SSM 20 mM

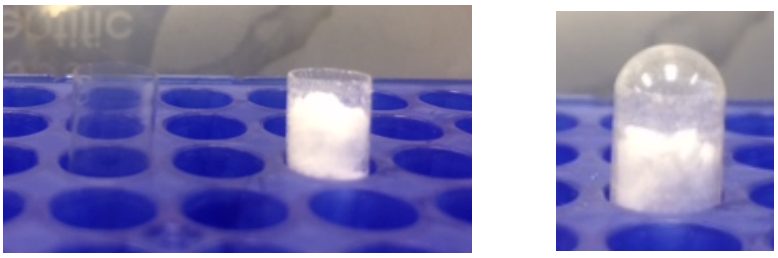
Approximately 404.6 mg of DSPE-PEG_{2000} was weighed and dissolved in 7.2 mL of saline. The solution was sonicated for 5 minutes carefully to prevent formation of bubbles and saturated with argon gas. This solution was incubated for 1 hour in the dark at RT.

2.7.2.1b Preparation of 1mg/mL VIP in SSM

Approximately 2.39 mg of VIP was weighed and dissolved immediately prior to use in approximately 2.4 mL of saline. The peptide solution was mixed with the pre-incubated micellar solution prepared above to obtain the VIP-SSM solution

(Lipid [15 mM]: peptide [0.075 mM] molar ratio; 1: 200) which assures all peptide molecules are associated with SSM (26). Next, the solution was saturated with argon gas and incubated for 2 hours in dark at RT.

After incubation, the solution of ~ 10 mL, was filled into a clean glass vial (total volume of 30 mL), frozen at – 80^0C and freeze dried overnight in Labconco freeze-drier (Kansas city, MO), to obtain lyophilized powder. Next, the powder was carefully broken-up with a clean spatula and mixed gently to obtain a uniform powder for filling. Each empty capsule, with cap and body was weighed. Amount of approximately 80 mg of VIP-SSM was filled manually per capsule as shown in Figure 18 below. Capsules were sealed tightly with cap until secondary lock sets in, to prevent powder leakage. Finally, the weight of each filled capsule was recorded to monitor fill weight. Capsules were stored in clean glass vials saturated with argon gas and kept in dark at RT until evaluation for drug release and SSM formation. For stability testing, filled capsules were stored at 4 ^{0}C in the dark until analysis.



Set-up for capsule filling with capsule bodies **Capsule filled and sealed**

Figure 18: Manual capsule filling set-up

2.7.2.2 Dissolution assay for capsules

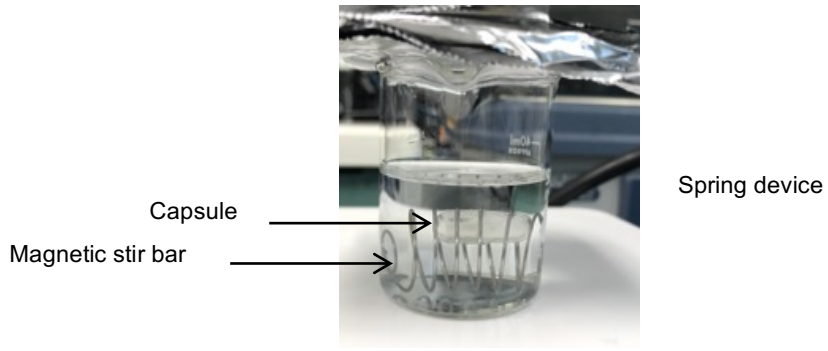


Figure 19: Dissolution assay apparatus modified for laboratory scale

Dissolution assay was performed using the set up shown in the Figure 19 above. 30 mL of pH 6.5 phosphate buffer was pre-incubated at 37 ^{0}C on a hot plate with constant stirring using a magnetic stirrer bar at 100 rpm. Capsules were taken one at a time and inserted into the spring device and immersed in the solution. One mL aliquots were sampled every 10 minutes up to 60 minutes from the same location in the beaker (upper left corner) to avoid sample variation. At each time point a sample was withdrawn, 1 mL of PBS was replaced to account for the loss in volume.

The reformation of micelles and presence of active VIP after capsule dissolution was determined by DLS analysis and VIP ELISA, respectively. 500 µL aliquots were used for NICOMP particle size analysis and another 500 µL aliquot was stored immediately in -20 ^{0}C in a clean glass vial to be used for ELISA at a later time. This dissolution assay was repeated for three separate capsules and

data was compared between capsules.

2.7.2.2a Determination of reformation of micelles after dissolution of freeze-dried cake

Particle size was analyzed using Agilent 7030 Nicomp DLS (Agilent 7030 NICOMP DLS, Agilent Technologies, Santa Clara, CA) as described earlier in section 2.5.

2.7.2.2b Determination of the presence of active vasoactive intestinal peptide after capsule dissolution

ELISA was performed at a later day with VIP EIA kit (Ray biotech) as per the manufacturers protocol and results were analyzed by plotting percentage VIP release Vs time. Since each capsule contains approximately 80 mg of VIP-SSM with approximately 300 μg of VIP, once dissolved each capsule should release approximately 9 μg/mL of VIP into the solution (Figure 20). The dilution of the beaker with 1 ml of fresh buffer at 6 time points results in sample dilution. However, since dilution factor at the final time point only accounted for 6% reduction in VIP concentration, this difference was not detectable in the ELISA and assumed to be insignificant.

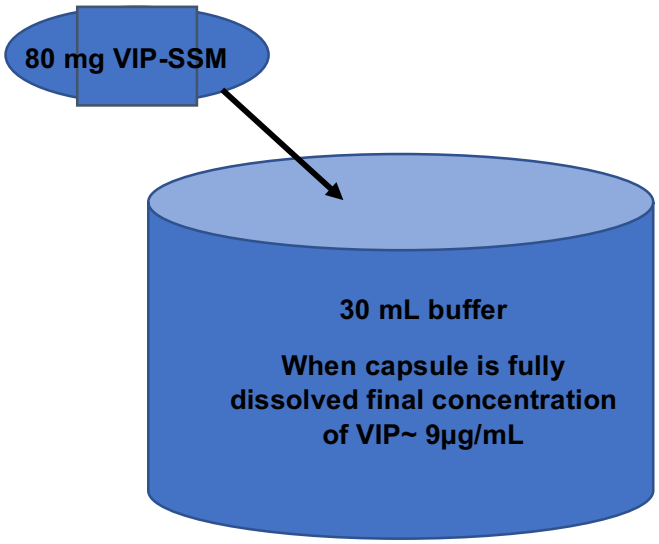


Figure 20: Dissolution of VIP-SSM in simulated colonic fluid

2.7.3 Detection of peptide association with SSM after released from capsules

Once capsules were dissolved at colonic pH, the released VIP levels were detected by ELISA. However, it was important to determine if the released peptide was in fact, associated with SSM and not in the free form. Literature supports evidence of the instability of free VIP in neutral buffer by autolysis when kept at 37 ^{0}C (170-172). *In vivo*, studies where VIP was administered directly to the colon demonstrated negligible biological activity of the free peptide. This shows the importance of the association of VIP with SSM for mediating its biological functions. Since VIP in SSM is more stable than the free peptide it allows adequate amounts of peptide to reach the target site for activity. Therefore, to determine the association of VIP with SSM a comparison of the VIP amount released from capsules containing the nanomedicine vs the free peptide was conducted, using

ELISA. As stated earlier under methods, free peptide in neutral solution is known to undergo autolysis. This breaks down the peptide at amino acids 17- 28 giving rise to fragmented peptide segments. The ELISA is known to recognize only the full-length peptide of VIP and thus, the degraded peptide should not interact with the kit and show no signal. SSM is known to protect VIP from enzymatic degradation and, therefore, would be also protective against autolysis.

To this end, capsules were filled with same fill weight (80 mg) of VIP-SSM or VIP in an inert diluent lactose (173, 174). Three capsules per formulation was prepared for comparison. The nanomedicine capsules were prepared as described under section 2.7.2. Lactose containing capsules were prepared by weighing out the same amount of VIP and lactose weighed as the same amount as the phospholipid to form the bulk of the powdered materials. VIP was homogenously mixed with lactose diluent using a mortar and pestle in geometric dilution method. The capsules were then subjected to dissolution assay as described under section 2.7.2.2. Aliquots of 200 μl samples were taken at time points 10,20,30,40,50,60 and 120 minutes and stored immediately at -20 ^{0}C for ELISA to detect differences in VIP amount. In addition, a freshly prepared VIP solution at a concentration of 10 ng/μl was used to to compare the stability between the two formulations.

2.7.4 Stability studies of VIP-SSM freeze dried capsules

The long-term stability of capsules stored at 4 ^{0}C in air tight glass containers for up to 6 weeks was determined. Seven capsules were prepared as stated under

section 2.7.2.1 and stored individually in tightly sealed glass containers saturated in argon gas. These glass containers were stored in a secondary light resistant container and stored in the mid-section of the refrigerator (4 ^{0}C). One capsule was used for dissolution assay as described in section 2.7.2.2 immediately and the rest was analyzed at each week up to 6 weeks. Data from ELISA were plotted and compared for release pattern of VIP at each week time point.

2.8 Statistical analysis

Each *in vivo* experiment was conducted with 5 or more mice per group and *in vitro* experiments were results from at least 3 independent samples. All data were statistically analyzed and a p value of 0.05 or less was considered statistically significant. Studies with 2 or more groups were subjected to one-way ANOVA and tukey's post hoc test and studies with two groups were compared with student's t-test (paired) statistical analysis.

3. RESULTS

Studies conducted with VIP nanomedidicne in DSS colitis was previously published as (Jayawardena, Dulari, Arivarasu N. Anbazhagan, Grace Guzman, Pradeep K. Dudeja, and Hayat Onyuksel. "Vasoactive Intestinal Peptide Nanomedicine for the Management of Inflammatory Bowel Disease." *Molecular pharmaceutics* 14, no. 11 (2017): 3698-3708.) and studies where the expression of VPAC1 receptor was analyzed was previously published as (Jayawardena, Dulari, Grace Guzman, Ravinder K. Gill, Waddah A. Alrefai, Hayat Onyuksel, and Pradeep K. Dudeja. "Expression and localization of VPAC1, the major receptor of vasoactive intestinal peptide along the length of the intestine." American Journal of Physiology-Gastrointestinal and Liver Physiology 313, no. 1 (2017): G16-G25.)

3.1 *In vitro* characterization of nanomedicine

Prepared nanomedicine was characterized for its size and biological action *in vitro* prior to being used *in vivo*.

3.1.1 Mean particle size and distribution determined by dynamic light scattering

These data were adapted from previously published work in (Jayawardena, Dulari, Arivarasu N. Anbazhagan, Grace Guzman, Pradeep K. Dudeja, and Hayat Onyuksel. "Vasoactive Intestinal Peptide Nanomedicine for the Management of Inflammatory Bowel Disease." *Molecular pharmaceutics* 14, no. 11 (2017): 3698-3708.)

All nanoparticle formulations were characterized for their mean size and distribution as described in previous studies, to confirm the formation of the nanomedicine (101). As shown in Figure 21, empty nanocarrier (SSM), and VIP

associated SSM (VIP-SSM), both showed a similar mean particle diameter (~15 nm) and a narrow size distribution. However, at the same concentration, VIP in saline showed particle populations with different sizes due to possible aggregation and decomposition.

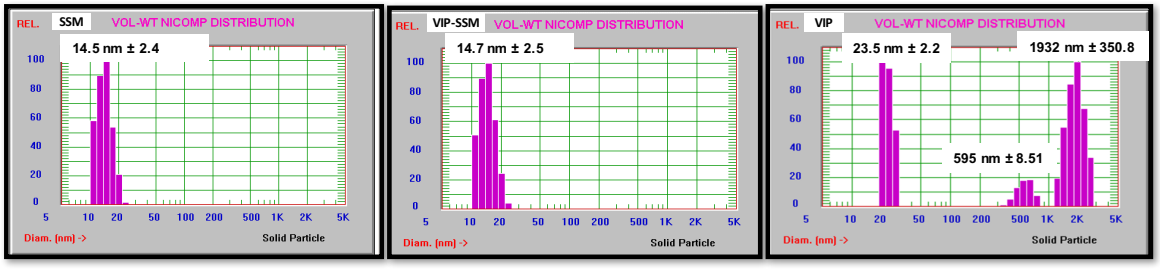


Figure 21: Characterization of formulations for their size using dynamic light scattering

3.1.2 *In vitro* bioactivity of VIP assessed by cyclic adenosine mono phosphate assay

Prior to conducting *in vivo* studies, it was important to determine the bioactivity of the peptide in SSM. The biological activity of VIP was determined by performing cAMP assay. Concentration of cAMP after addition of 10 nM of the peptide with or without SSM demonstrated equal amounts of intracellular cAMP concentrations of approximately 400 nM (Figure 22). These data provide confirmatory evidence of the synthesized peptide's capability to elicit an activation on specific receptors for VIP, in HT29 cells and therefore its biological activity.

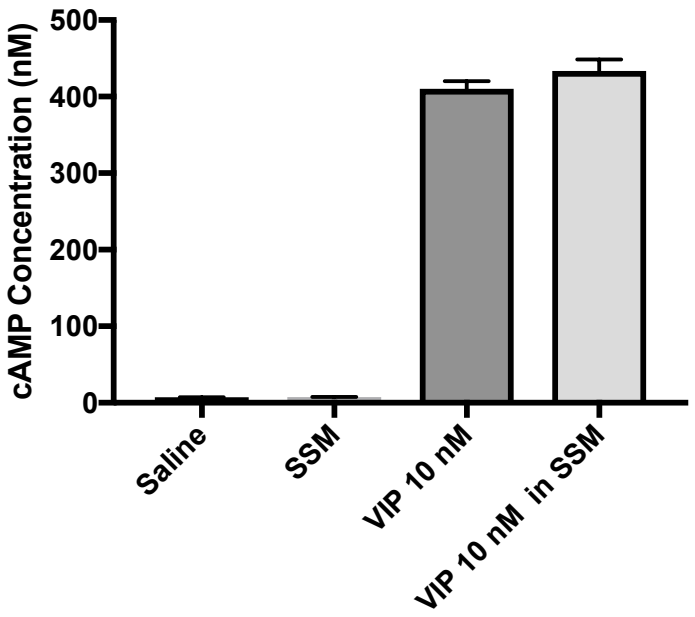


Figure 22: Intracellular cAMP concentration measured by cAMP assay in human HT29 cell line (passage 48-50). n=4

3.2 Results from Specific Aim 1

3.2.1 Effect of vasoactive intestinal peptide nanomedicine in a mouse model representing human ulcerative colitis

These data were adapted from previously published work in (Jayawardena, Dulari, Arivarasu N. Anbazhagan, Grace Guzman, Pradeep K. Dudeja, and Hayat Onyuksel. "Vasoactive Intestinal Peptide Nanomedicine for the Management of Inflammatory Bowel Disease." *Molecular pharmaceutics* 14, no. 11 (2017): 3698-3708.)

In vivo efficacy of VIP-SSM was studied in a mouse model of acute colitis induced by DSS which resemble human UC. DSS colitis animal model is well established in literature (131, 175) but there were no studies conducted using VIP.

However, other colitis mouse models listed in TABLE III, were tested to evaluate VIP effect on colitis. Therefore, we first performed some preliminary experiments to determine the right dose and time of administration as well as the route of injection. The doses tested included 0.05, 0.25, 0.5 and 1 nmol, based on literature (76, 77). The treatments were administered in two modes i) when the disease starts to develop (preventive) ii) after disease fully developed (therapeutic). With the use of 0.25 nmol and ip route selected based on our preliminary data, disease indicators including diarrheal phenotype, pro-inflammatory cytokine mRNA levels and histological damage were evaluated for both preventive and therapeutic studies.

3.2.1.1 Effect of vasoactive intestinal peptide on preventive studies with DSS colitis

As shown in Figure 12, colitis was induced with 3% DSS for 8 days and ip injections of the nanomedicine and free peptide at 0.25 nmol dose was administered on alternate days. Distal colonic tissues were harvested and analyzed for the following parameters.

3.2.1.1a Effect of vasoactive intestinal peptide on mRNA expression of pro inflammatory cytokines and histology of the distal colon in preventive model of DSS colitis

A hallmark of any inflammatory disease is the increase in pro inflammatory cytokines such as Interleukin-1 beta (IL-1β) and chemokines such as C-X-C motif chemokine ligand-1 (CXCL1). At the end of the study, mice receiving both free and nanomedicine form of VIP showed significant amelioration of the increased

cytokine mRNA expression observed with DSS colitis (Figure 23). Thus, these data demonstrate that in DSS colitis mouse model, VIP administration in a preventive manner significantly alleviated the local inflammation by reducing the mRNA expression of pro-inflammatory cytokines (Figure 23). This alleviation of inflammation was further confirmed by the reduced severity of histological damage that was observed in H & E stained sections shown in Figure 24. DSS colonic histology was severely disrupted with damage to colonic architecture and presence of swelling and inflammatory infiltrate. These effects were alleviated with both VIP and VIP-SSM treatments, indicating histologic recovery.

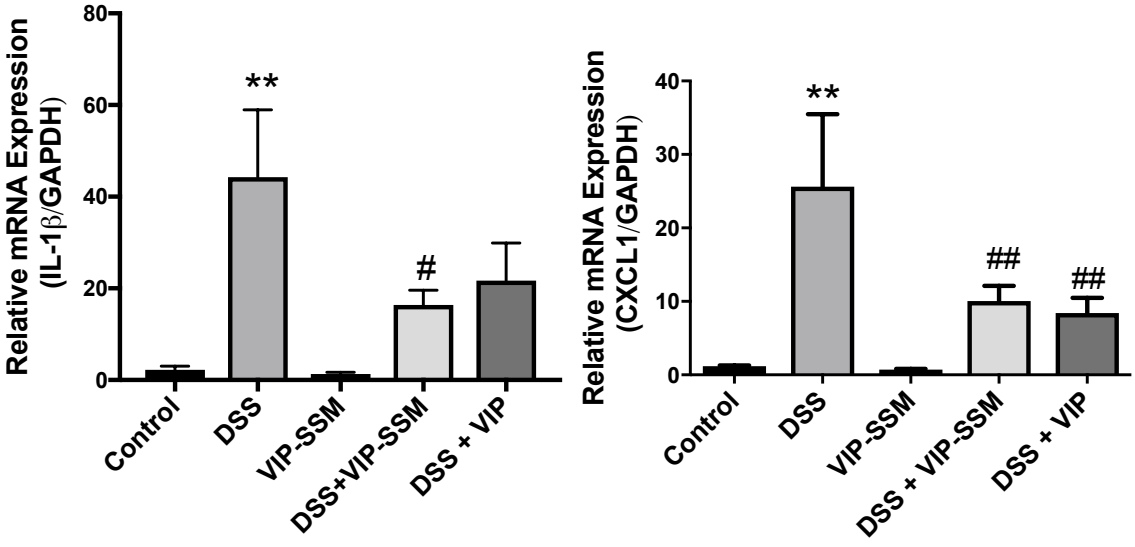


Figure 23: Effect of VIP on increased mRNA levels of pro-inflammatory cytokines in the distal colonic mucosa of DSS mice. n=5, **-p<0.005 Vs control, #-p<0.05, ##-p<0.005 Vs DSS. (Treatment groups are listed in TABLE VI) Modified and reused with permission from (176)

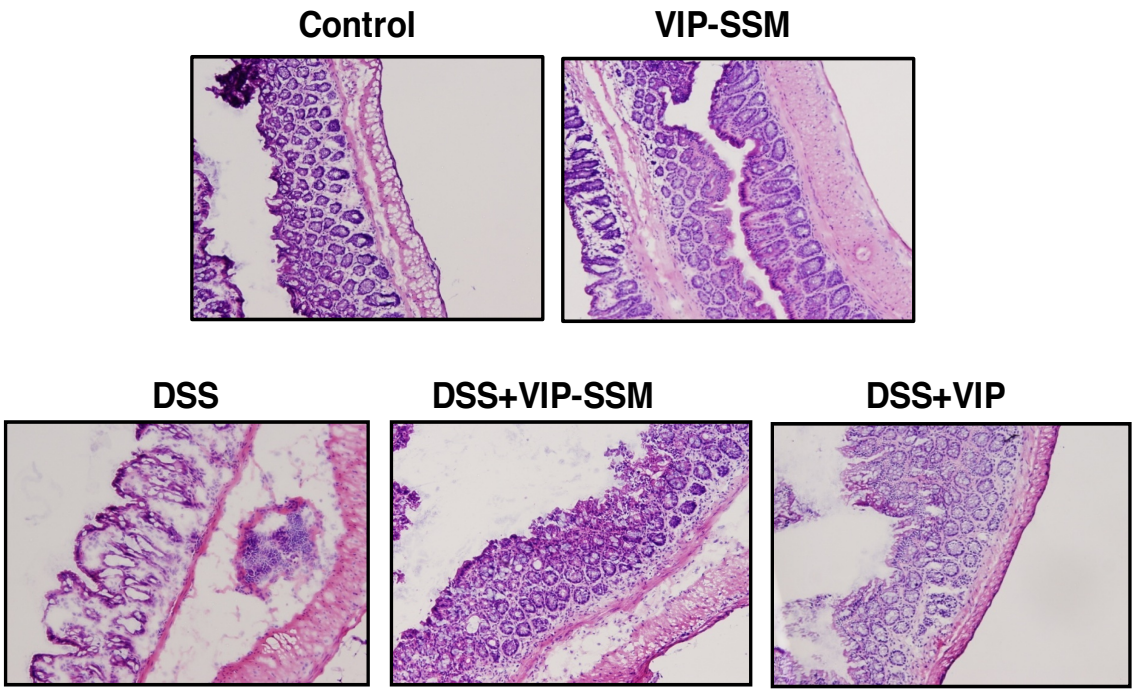


Figure 24: Effect of VIP on histological damage observed in DSS colitis (Treatment groups are listed in TABLE VI) Modified and reused with permission from (176)

3.2.1.1b Effect of VIP on diarrheal phenotype and colon length

When studying the effect of a therapeutic agent on diarrheal phenotype, the consistency of the stool in the colon as well as the total length are studied. Alleviation of inflammation was assessed by diarrheal phenotype as shown in Figure 25 below, DSS colitis causes severe mucosal damage which leads to swelling and edema and thus results in shortening of the colon. In addition, due to diarrhea, the stool pellets appeared loose and watery. Once treated, the colons appeared to have reformation of solid stool pellets and a significant improvement of the length of the colon as depicted in Figure 25B.

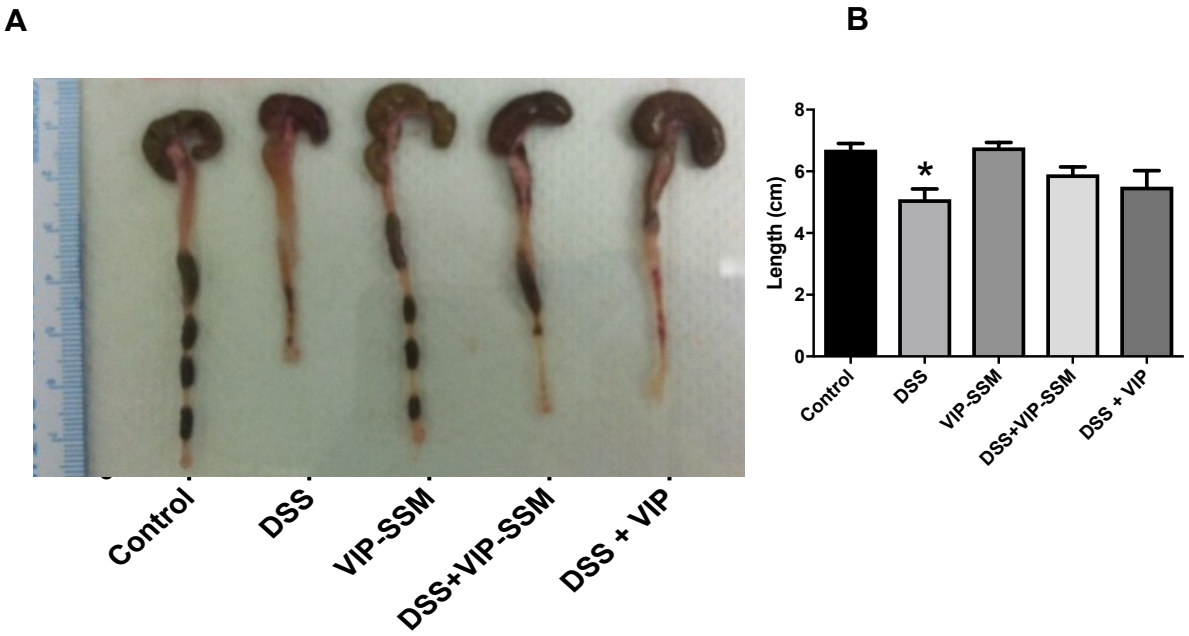


Figure 25: Effect of VIP on diarrheal phenotype and colon length in DSS colitis. A. representative images of whole colon of mice. B. graphical representation of average length of colon. n=5, *-p<0.05 Vs control (Treatment groups are listed in TABLE VI) Modified and reused with permission from (176)

After determining that VIP-SSM could alleviate inflammation in a preventive manner as effectively as the free peptide. We next explored the capability of the nanomedicine to lessen inflammation in a therapeutic model where severe inflammation was established.

During the preventive studies, because the treatments were administered frequently and before the onset of full inflammation, VIP and VIP-SSM showed similar benefits. Therefore, in our next studies, we used a fully established disease (therapeutic mode), and a single dose (0.25 nmol) of VIP to evaluate the beneficial

difference between free VIP and its nanomedicine. Same parameters in preventive studies were used to evaluate alleviation of inflammation.

3.2.1.2 Effect of vasoactive intestinal peptide on therapeutic studies with dextran sulfate sodium colitis

In this study, 3.5% DSS was administered to mice for 7 days and the mice were switched to tap water for the next 5 days. Treatments were administered at one time point, the day after removing DSS (Figure 13). VIP-SSM at a single intra peritoneal dose of 0.25 nmol alleviated inflammation and significantly reversed the parameters shown below.

3.2.1.2a Effect of VIP-SSM on body weight loss associated with therapeutic model of dextran sulfate sodium colitis

Colitis induced by DSS administration leads to loss of body weight inevitably, due to the severe damage to the colon. As shown in Figure 26 below, starting from day 6, mice on DSS lost significant amount of body weight (>10%). Additionally, mice with no treatment (DSS+SSM) or treated with the free peptide (DSS+VIP) did not show an improvement in body weight throughout the course of the study and demonstrated further deterioration. In contrast, mice receiving VIP-SSM as a single dose of 0.25 nmol, showed improvement in their body weight from the day after the treatment (day 9) and reached significant difference to DSS+SSM and DSS+VIP mice by day 11. This showed therapeutic potential of the administered dose of VIP-SSM in improving overall health of mice during acute flares of colitis.

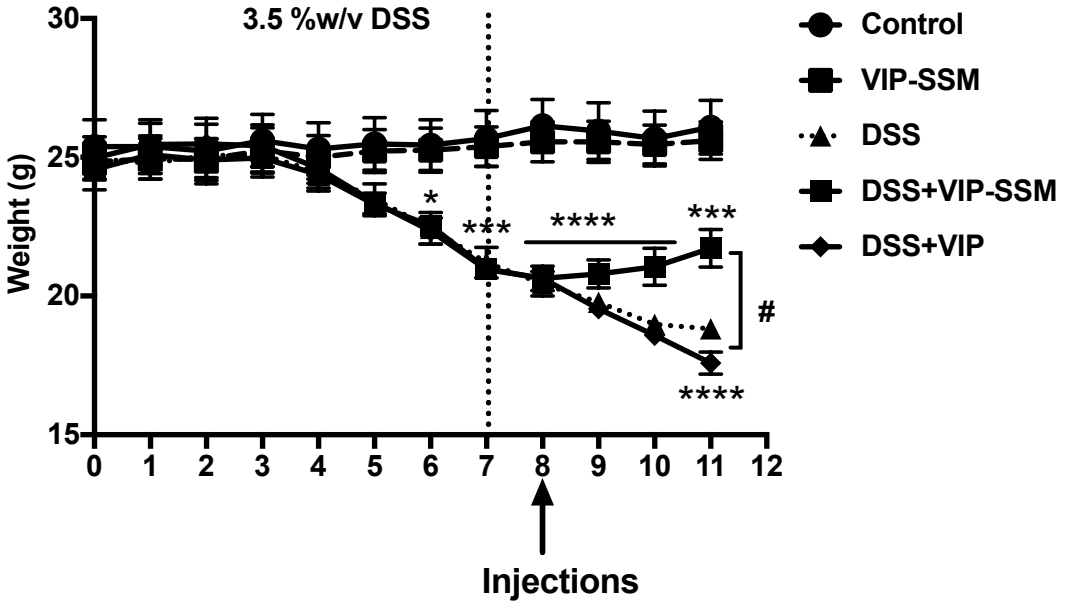


Figure 26: Effect of VIP-SSM on loss of body weight with DSS insult. Arrowhead indicated day of treatment. n=5, *p<0.05, **p<0.005, ***p<0.0005, ****p<0.0001 Vs control, # p<0.05 Vs DSS (Treatments groups listed in TABLE VI) Reused with permission from (176)

3.2.1.2b Effect of VIP-SSM on reducing severity of the local inflammatory response in therapeutic model of dextran sulfate sodium colitis

The local inflammatory reaction of the distal colon seen in DSS model was assessed by quantifying pro-inflammatory cytokine mRNA expression in the distal colonic mucosa. As shown in Figure 27, cytokines including IL-1β, CXCL-1, CXCL-2 and C-C Motif chemokine ligand-3 (CCL3) also known as macrophage inflammatory protein 1 (MIP-1), were greatly up regulated in DSS colitis. VIP-SSM treatment was capable of significantly reversing the increased cytokine expression to almost control levels at day 11 (Figure 27). This anti-inflammatory property of

VIP-SSM was further evidenced from the recovered distal colonic histology after H & E staining (Figure 28A). The extensively damaged colonic histology and avid accumulation of inflammatory infiltrate observed with DSS, was almost completely reversed to control histology with the nanomedicine. The results observed in histology were complemented with blinded histopathological scores. Data in Figure 28B demonstrated recovery of the histology with VIP-SSM administration. Mice treated with a single dose of VIP-SSM demonstrated significantly less chronic inflammatory infiltrate and edema supporting the results from cytokine mRNA expression. In addition, erosion and ulceration, crypt destruction and reduced epithelial integrity observed with DSS was also significantly abrogated with the nanomedicine indicating its healing properties. It should be noted that the free peptide did not have a significant effect on the expression of cytokines but appeared to have an intermediate improvement in histopathology as assessed by the scores. However, this effect was still negligible when comparing to the treatment with nanomedicine at the same dose.

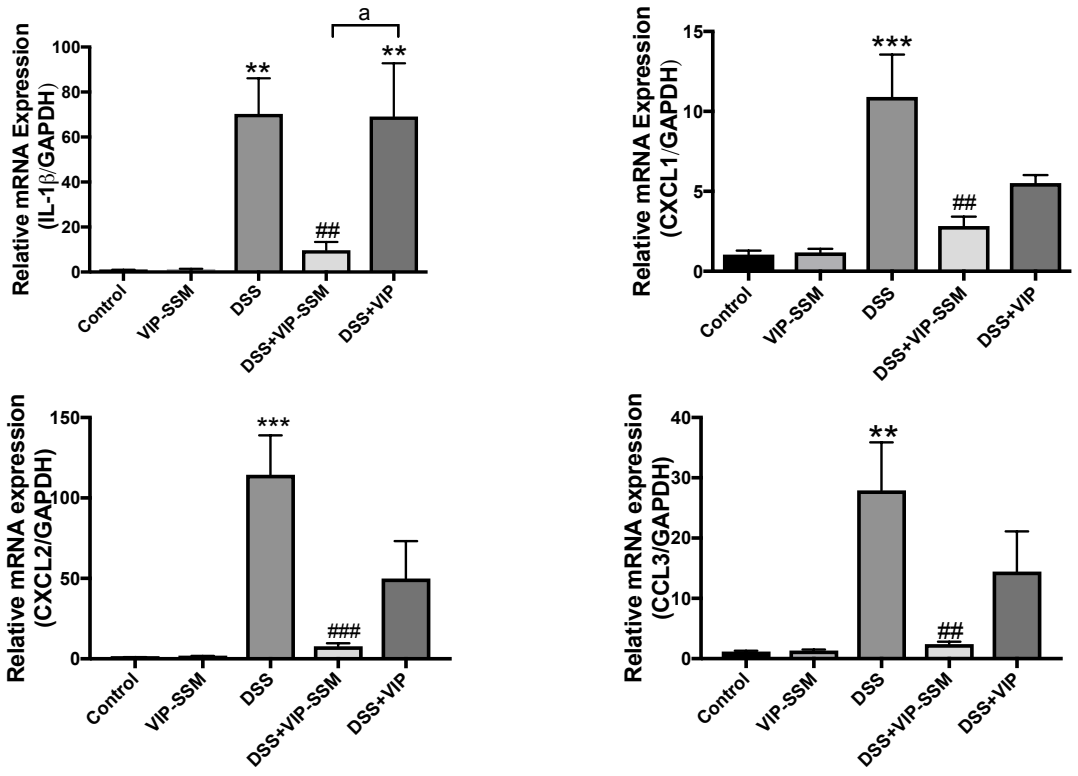
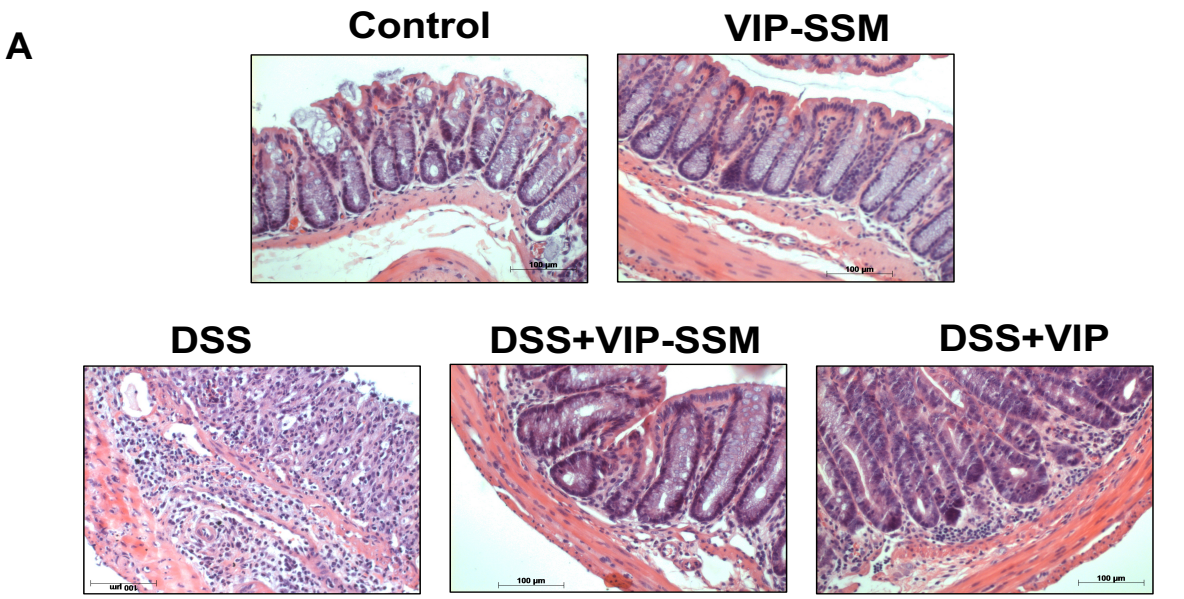


Figure 27: Effect of VIP-SSM on the increased pro-inflammatory cytokine mRNA levels in the distal colon of DSS colitis mice. n=5, **-p<0.005, ***-p<0.0005, ****-p<0.0001 Vs Control, ##-p<0.05 Vs DSS, a-p<0.05 (Treatments groups listed in TABLE VI) Reused with permission from (176)



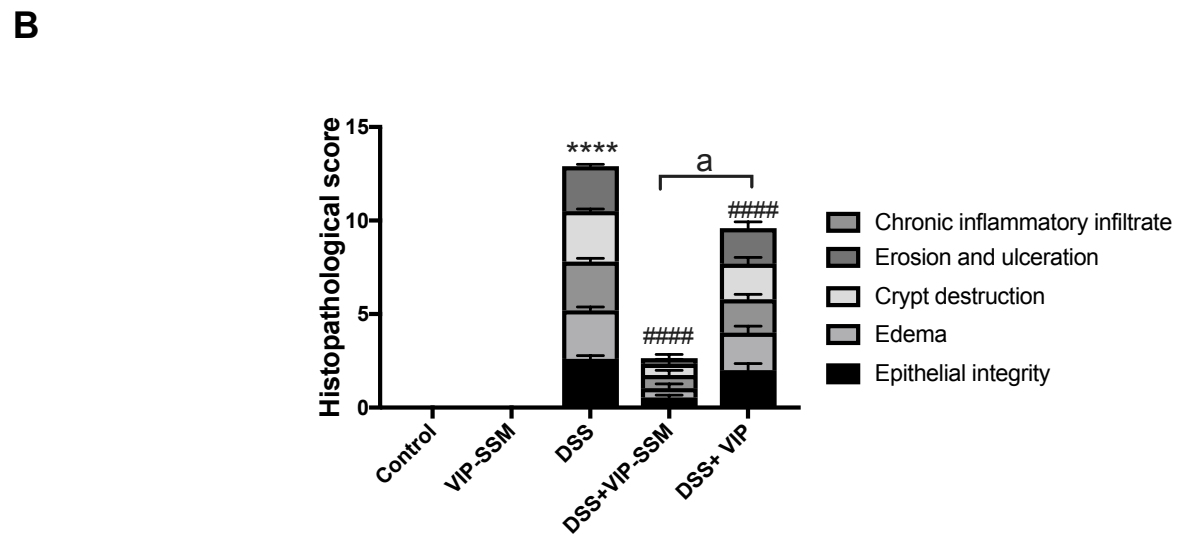


Figure 28: Effect of VIP-SSM on histopathology in distal colon of DSS colitis mice. A) Histology of the distal colon B) Blinded histopathological scores. n=5, ****-p<0.0001 Vs control, ####-p<0.0001 Vs DSS, a-p< 0.05 (Treatments groups listed in TABLE VI) Reused with permission from (176)

3.2.1.2c Effect of VIP-SSM on alleviating diarrheal phenotype observed with dextran sulfate sodium colitis

Dextran sulfate sodium colitis induces rectal bleeding and severe diarrhea in mice, the diarrheal phenotype associated with DSS shown in Figure 29A demonstrated the presence of loose stool. This pathological phenotype was significantly abrogated with the treatment of 0.25 nmol VIP-SSM but not with the free peptide at the same dose. This was also well evidenced by the shortening of colon which was only significantly recovered only with the nanomedicine treatment (Figure 29B).

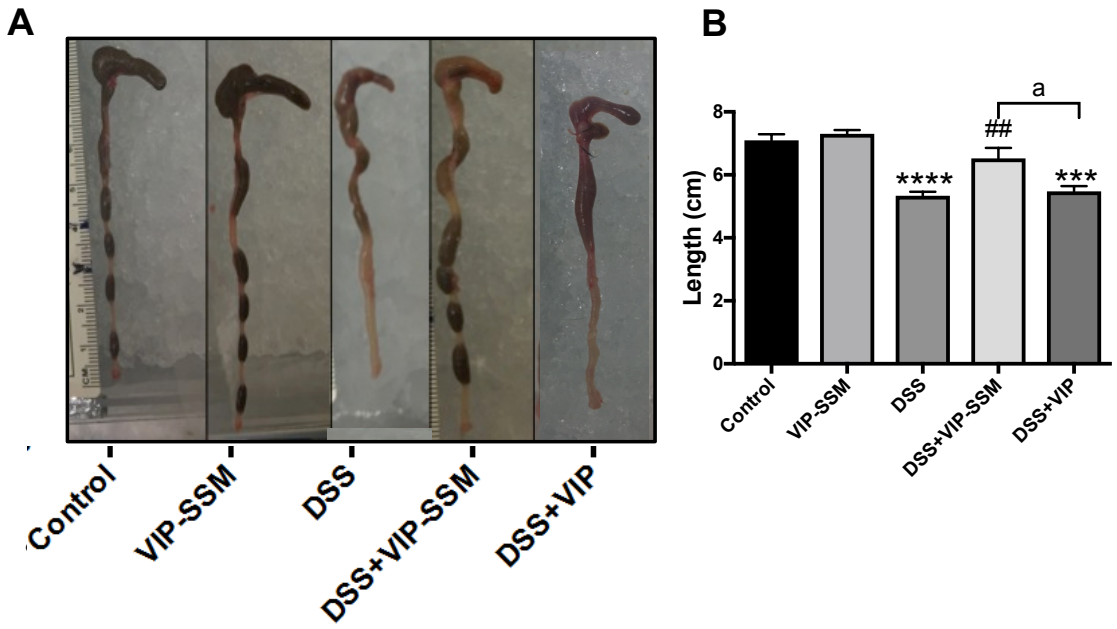


Figure 29: Effect of VIP-SSM in alleviating diarrheal phenotype associated with DSS colitis. A. representative images of whole colon of mice. B. graphical representation of average length of colon. n=5, ***-p<0.0005, ****-p<0.0001 Vs Control, ##-p<0.05 Vs DSS, a-p<0.05 (Treatments groups listed in TABLE VI) Reused with permission from (176)

Results from both preventive and therapeutic studies with DSS colitis mouse model demonstrated the anti-inflammatory and therapeutic benefit of VIP nanomedicine in improving signs and symptoms of the underlying local inflammation in mice. It is also important to highlight that once severe inflammation was established (therapeutic model) only the nanomedicine was effective, as a single dose of 0.25 nmol in ameliorating colitis.

3.2.2 Effect of vasoactive intestinal peptide nanomedicine in mouse model representing human Crohn's disease

The main goal of this project was to study the potential of VIP-SSM in treating IBD, which encompasses two disease sub types. Thus, similar studies were undertaken in an independent model resembling human CD with the same dose used in DSS in a preventive manner. TNBS colitis was selected as a model for human Crohn's colitis. Same strain of mice used in DSS model was used to determine the therapeutic potential of VIP nanomedicine in CD. VIP dose used was kept the same as in DSS colitis to compare the effects of preventive and therapeutic effects between the two models.

3.2.2.1 Effect of vasoactive intestinal peptide on preventive studies with trinitrobenzene sulfonic acid colitis

Previous studies in literature have shown the beneficial effects of VIP in mediating anti-inflammatory effects in a Th1 cytokine driven TNBS model of colitis (48, 73). Therefore, it was important to determine if these same benefits can be achieved with VIP-nanomedicine. Colitis was induced in mice after pre-

sensitization by directly instilling 3.5 mg of TNBS to the colon (Figure 14). The study was carried out for 5 days with treatments administered on alternate days starting from the day of pre-sensitization.

3.2.2.1a Effect of vasoactive intestinal peptide in relieving local inflammation in a preventive model of trinitrobenzene sulfonic acid colitis

In TNBS model of colitis, similar to DSS there is a robust inflammatory response in the distal colon of mice. The mRNA expression of pro inflammatory cytokines such as IL-1β, CXCL-1 and CCL3 were significantly upregulated with TNBS (Figure 30). These results showed an overall alleviation of the increased cytokine mRNA expression in both peptide and nanomedicine groups to a similar extent. When the histopathology of the colon was assessed, there was severe mucosal damage and inflammatory infiltrate observed in TNBS colitis colons (Figure 31).

In addition, appearance of red color distal colonic mucosa in TNBS shows evidence of necrosis. Administration of both VIP nanomedicine and the free peptide had beneficial effects, by improving overall histology in the distal colon. Histopathological score in mice treated with VIP nanomedicine showed a significant reversal in inflammation as indicated by the reduction in the overall score close to control levels. However, although the free peptide had significant changes in some cytokine mRNA expression, the histopathological score was alleviated to a significantly less extent as compared to the nanomedicine. Complementing these anti-inflammatory effects, the myeloperoxidase activity of the distal colon which represents the accumulation of neutrophils at the site of

disease was also significantly reversed by both nanomedicine and free peptide treatment (Figure 32).

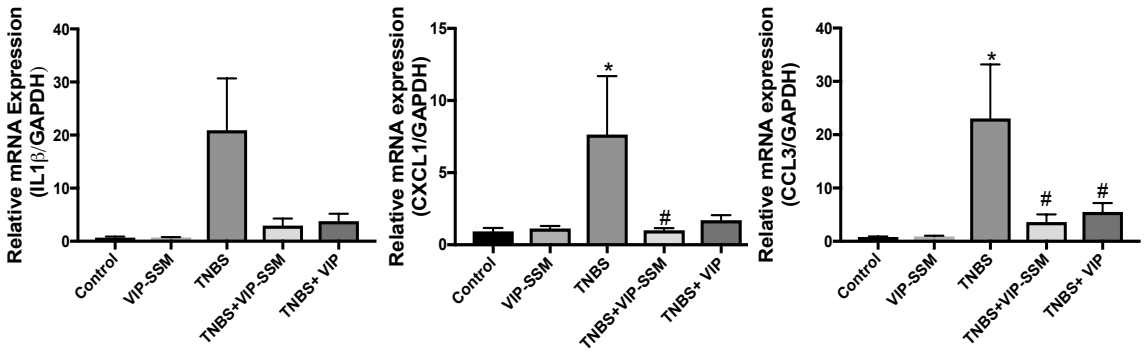


Figure 30: Effect of VIP on the increased pro-inflammatory cytokine mRNA expression in TNBS colitis mice distal colon. n=5, *-$p<0.05$ Vs control, #-$p<0.05$ Vs TNBS (Treatment groups listed in TABLE VII)

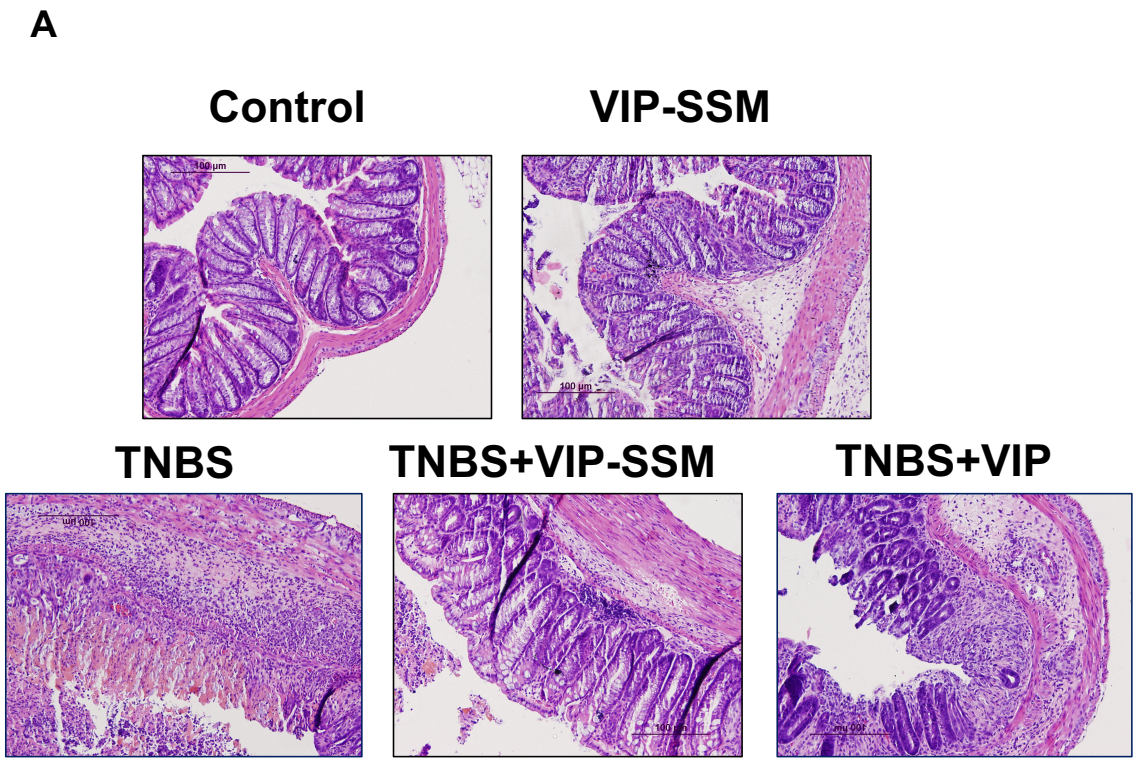


Figure 31: Effect of VIP in ameliorating the damaged distal colonic histology (A)and reduced histopathological score (B) in TNBS colitis. n=5, ****-p<0.0001 Vs control, ###-p<0.0005, ####-p<0.0001 Vs TNBS, a statistical significance of p<0.0001. (Treatment groups listed in TABLE VII)

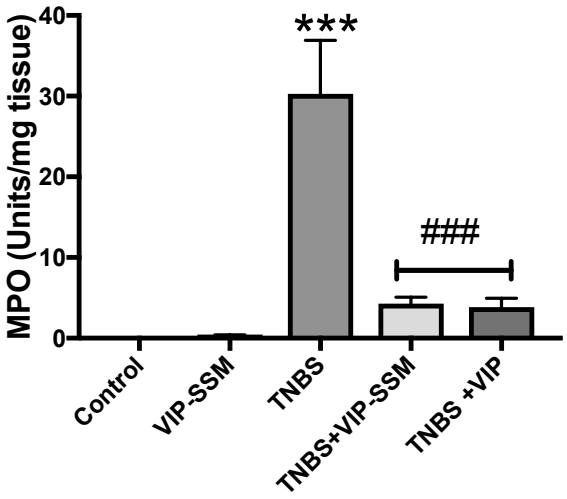


Figure 32: Effect of VIP on the increased myeloperoxidase activity at the distal colon in TNBS colitis. n=5, ***-p<0.0005 Vs control, ###-p<0.0005 Vs TNBS (Treatment groups listed in TABLE VII)

3.2.2.1b Effect of vasoactive intestinal peptide in alleviating diarrheal phenotype associated in a preventive model of trinitrobenzene sulfonic acid colitis

In TNBS colitis, inflammation persists in the colon and thus diarrhea is a key pathological feature. However, the overall appearance of the colons affected with TNBS (Figure 33) appeared uniquely distinct from those affected with DSS colitis. This was evident from the thicker colon that was observed in mice insulted with TNBS. The presence of loose stool was prominent even in TNBS colitis followed by a significant loss in colon length (Figure 33). These effects were alleviated with VIP treatment and appeared close to control animals.

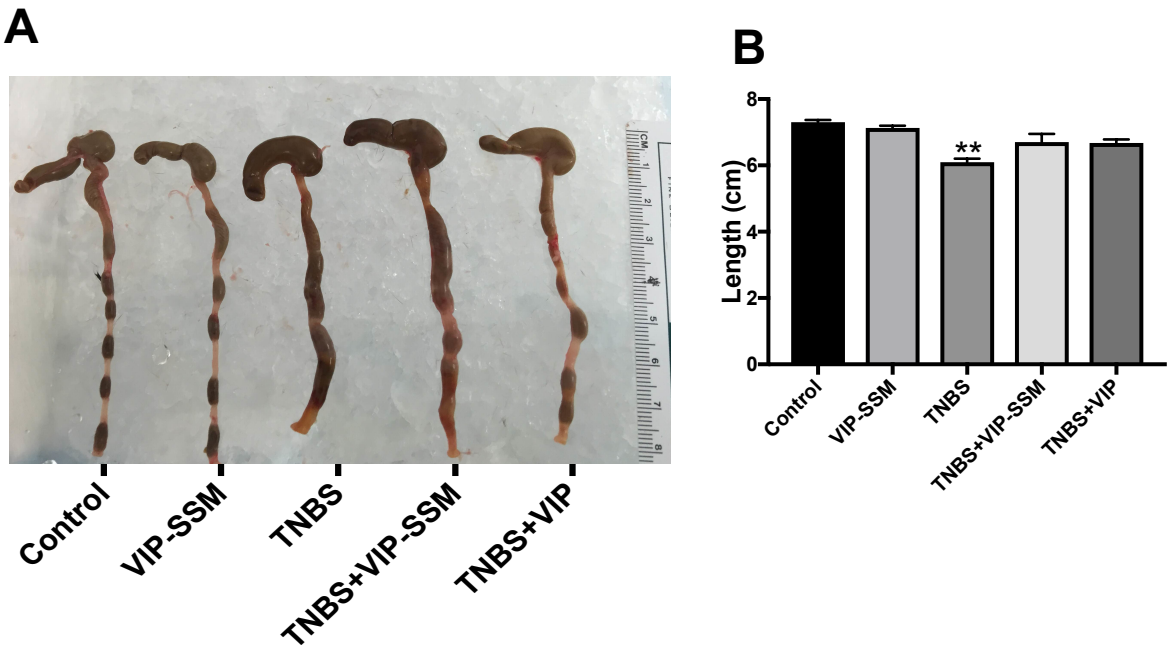


Figure 33: Effect of VIP on alleviating diarrheal phenotype in TNBS colitis. A. representative images of whole colon of mice. B. graphical representation of average length of colon. n=5, **-p<0.005 Vs control (Treatment groups listed in TABLE VII)

3.2.2.2 Effect of vasoactive intestinal peptide on therapeutic studies with trinitrobenzene sulfonic acid colitis

The purpose of the following studies, was to determine the therapeutic potential of a single dose of VIP nanomedicine in alleviating fully established inflammation in TNBS colitis. Since there was a significant improvement of DSS colitis with a single dose of VIP nanomedicine, it was of interest to determine if similar benefits could be achieved in TNBS colitis (Figure 15).

3.2.2.2a Effect of vasoactive intestinal peptide on improving overall health of mice by recovering the loss of body weight in a therapeutic model of trinitrobenzene sulfonic acid colitis

One important feature of any therapeutic agent beneficial in IBD is to be effective when acute flares of disease have occurred. In this regard, the therapeutic model of colitis provides a more clinically applicable model, resembling such instances. TNBS colitis was induced as described earlier, and on the day after colitis induction, a single dose of 0.25 nmol VIP in the nanomedicine form or free peptide was administered ip. As seen in DSS colitis, mice receiving treatments showed significant recovery in body weight almost resisting the weight drop observed in untreated TNBS mice (Figure 34). TNBS caused severe body weight drop starting from day 3, and continued to deteriorate until the end of the study. Mice treated with both free peptide and nanomedicine form, showed no significant loss in body weight indicating an overall improvement and recovery of inflammation in these groups.

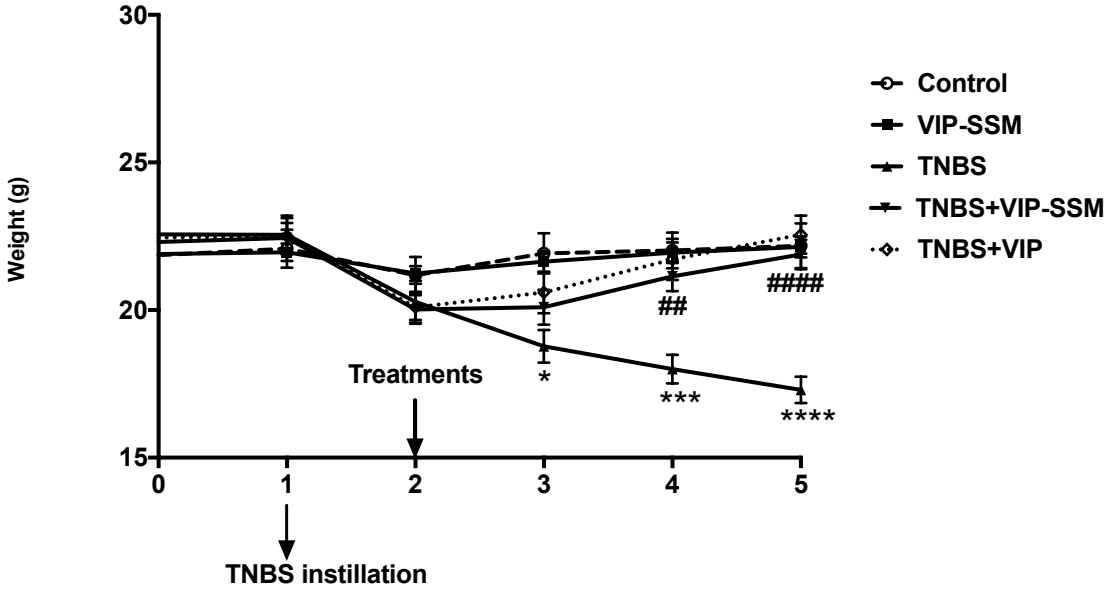


Figure 34: Effect of VIP on loss of body weight associated with TNBS colitis. n=5, -p<0.05, **-p<0.0005, ****-p<0.0001 Vs control, ##-p<0.005, ####-p<0.0001 Vs TNBS (Treatment groups listed in TABLE VII)

3.2.2.2b Effect of vasoactive intestinal peptide in reducing local inflammation in a therapeutic model of trinitrobenzene sulfonic acid colitis

A robust local inflammatory reaction occurs with the administration of TNBS at the distal colons of mice and persists over the next 10 days. Inflammation reaches its peak at day 3 and subsides within the next 10 days in C57BL/6 mice (134). Thus, at day 2, significant inflammation was established in mice as evidenced from the loss of body weight and diarrhea. Therefore, treatments were administered at day 2. A single dose of both free peptide and the nanomedicine form was able to down regulate the increased mRNA expression of key pro inflammatory cytokines upregulated in distal colonic mucosa (IL1β and CXCL2).

(Figure 35). In parallel to these findings it was also of interest to determine the histology of the distal colon. Representative micrographs of the distal colon in Figure 36 show clear recovery of the distal colonic histological damage with VIP nanomedicine and free peptide treatments. Therefore, these studies demonstrated that at a dose of 0.25 nmol, free peptide was capable of mediating its anti-inflammatory effects. Therefore, a lower dose of the nanomedicine form may still be able to effectively manage the disease in mice in TNBS colitis model.

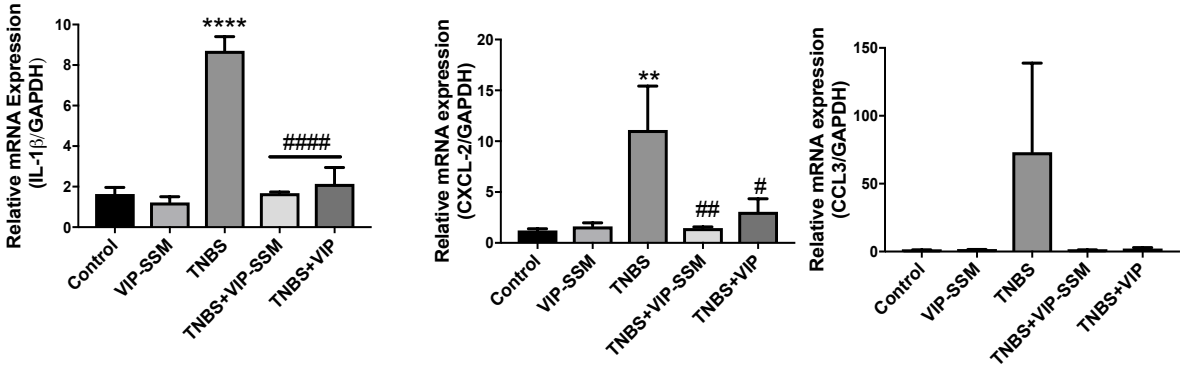


Figure 35: Effect of VIP on the increased pro-inflammatory cytokine mRNA expression in TNBS colitis mice distal colon. n=5, **-p<0.005, ****p<0.0001 Vs control, #-p<0.05, ##-p<0.005, ####-p<0.0001 Vs TNBS. (Treatment groups listed in TABLE VII)

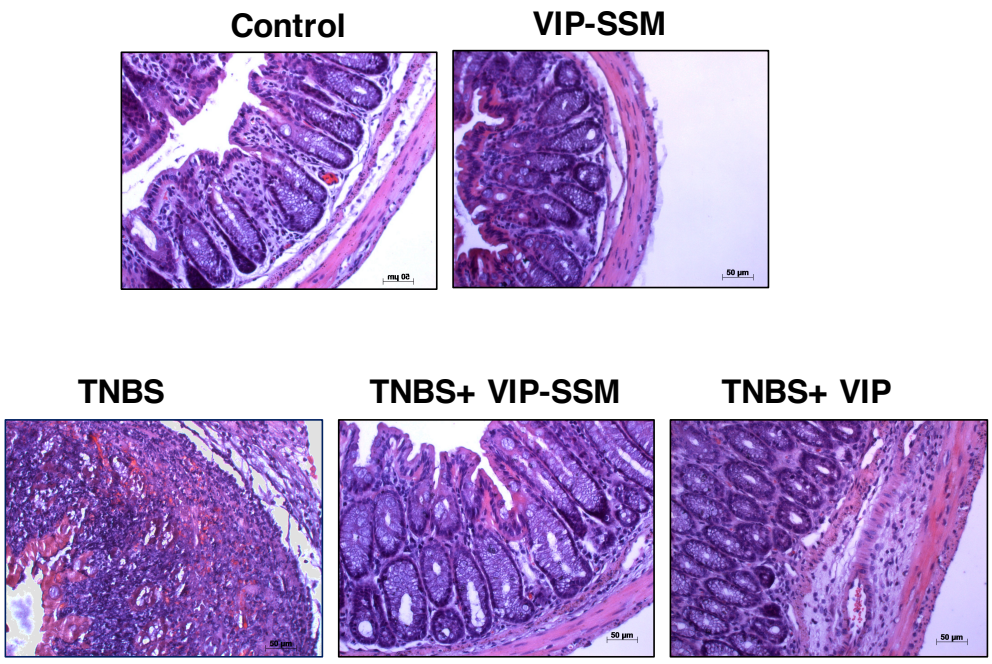


Figure 36: VIP alleviated the damaged distal colonic histology in TNBS colitis (Treatment groups listed in TABLE VII)

3.2.2.2c Effect of vasoactive intestinal peptide in alleviating diarrheal phenotype and associated reduction in colon length in a therapeutic model of trinitrobenzene sulfonic acid colitis

Therapeutic administration of a single dose of VIP significantly alleviated the diarrheal phenotype associated with TNBS (Figure 37). However, the accompanying reduction in colon length was only recovered with VIP nanomedicine and not with the free peptide (Figure 37). The free peptide showed only partial alleviation of reduced length compared to untreated TNBS.

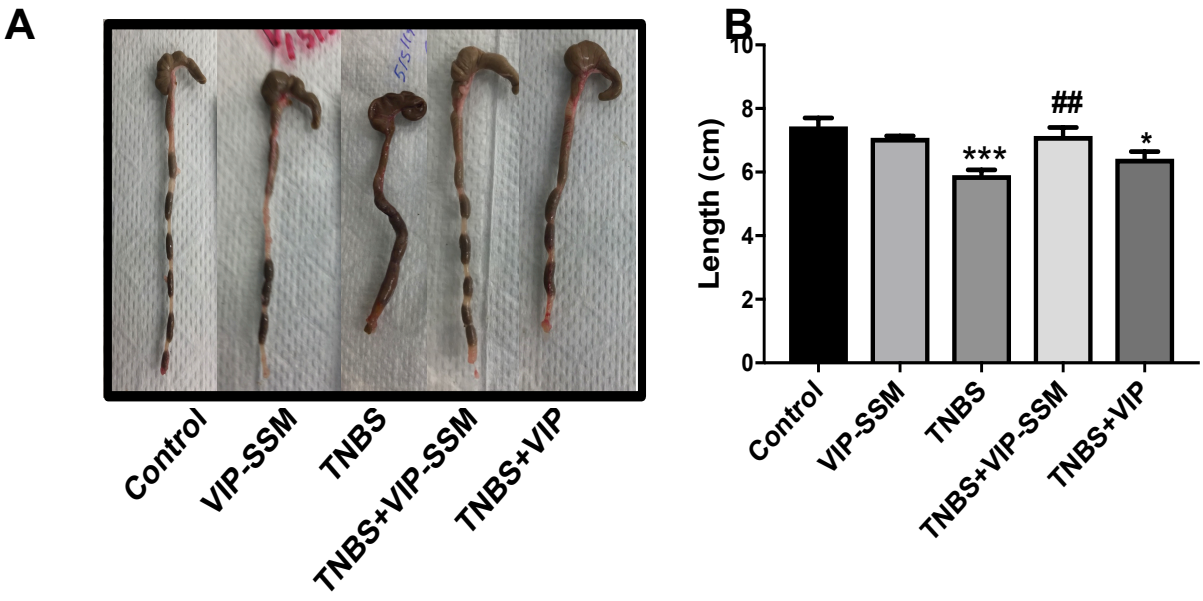


Figure 37: Effect of VIP on diarrheal phenotype associated with TNBS. n=5, *-p<0.05, ***-p<-0.0005 Vs control, ##-p<0.005 Vs TNBS (Treatment groups listed in TABLE VI)

3.3 Results from Specific Aim 2

The completion of specific aim 1 provided ample evidence in support of the anti-inflammatory action of VIP nanomedicine in colitis mouse models. Next, it was of interest to determine potential mechanisms underlying the beneficial effects of VIP nanomedicine in alleviating severe colitis in addition to its action on inflammatory cells. These beneficial effects were categorized as effects on mucosal barrier and on expression of ion transporter protein expression. These mechanisms were studied in the DSS model in a therapeutic setting. Treatment groups were as listed in TABLE VI via ip route of administration (Figure 13).

Therapeutic DSS model was selected because of the best beneficial effects of the nanomedicine vs the free peptide was observed in this model.

3.3.1 Effect of VIP-SSM on mucus secreting goblet cells in a therapeutic model of dextran sulfate sodium colitis

Mucus forms a physical barrier between the luminal contents and the underlying mucosa preventing access of luminal contents which could trigger a response in the host. In inflammatory conditions of the intestine, including IBD, the mucus barrier is known to be compromised (177). In addition, mouse models resembling human disease such as DSS and TNBS colitis, the loss of goblet cells has also been well defined (178, 179). Therefore, in the therapeutic model of DSS colitis the change in goblet cell numbers were determined by PAS staining.

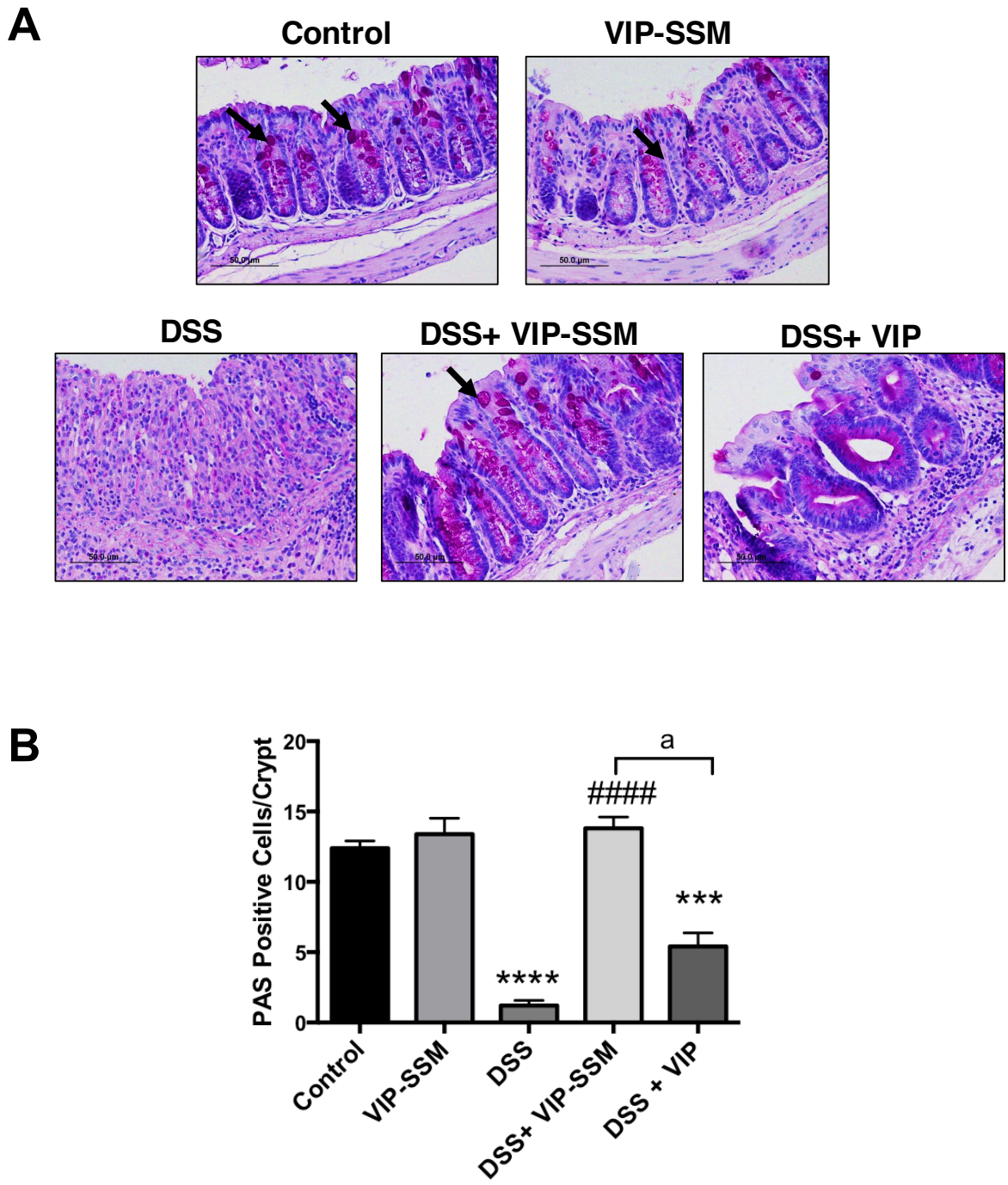


Figure 38: A) Representative micrographs of PAS stained paraffin embedded distal colonic tissue sections of DSS colitis mice. Arrow heads point at goblet cells stained in magenta. B) Quantification of PAS positive cells per crypt. n=5, ***-p<0.0005, ****-p<-0.0001 Vs control, ####-p<0.005 Vs DSS, a-p<0.0001 (Treatment groups listed in TABLE VI)

Goblet cells, stained in magenta (arrow heads) with PAS stain appear abundantly throughout the crypt-surface axis of the distal colonic cross sections in healthy mice. In DSS, there is a significant loss of the mucus secreting goblet cells due to the severe inflammation prevalent at the site. As shown in Figure 38 above, it is evident that VIP-SSM significantly improved the loss in goblet cell count during DSS colitis, improving it to almost control levels. Treatment with free peptide only improved the goblet cell numbers slightly and these results were parallel to the ones observed in histopathological scoring (Figure 28).

3.3.2 Effect of VIP-SSM on tight junction protein (TJ) expression in the distal colon in a therapeutic model of dextran sulfate sodium colitis

These data were adapted from previously published work in (Jayawardena, Dulari, Arivarasu N. Anbazhagan, Grace Guzman, Pradeep K. Dudeja, and Hayat Onyuksel. "Vasoactive Intestinal Peptide Nanomedicine for the Management of Inflammatory Bowel Disease." *Molecular pharmaceutics* 14, no. 11 (2017): 3698-3708.)

The epithelial lining of the GI tract forms the major first line of defense against potential pathogens from accessing the intestine. In this regard, TJ proteins play a pivotal role in maintaining the barrier integrity of the gut. Therefore, the expression and distribution of the TJ protein occludin was determined as a marker to study the beneficial effects of VIP-SSM in alleviating the compromised epithelial barrier in colitis.

Total mRNA and protein of the key TJ protein occludin in the distal colonic mucosa, was significantly down regulated in DSS colitis (Figure 39). There was

almost a 50% reduction in the total levels of occludin in the distal colonic mucosa of DSS group compared to control. When administered as a single dose of 0.25 nmol in a therapeutic manner, both VIP nanomedicine and the free peptide improved the mRNA and protein levels of occludin to control levels. mRNA levels were equally up regulated by both treatments, however, the free peptide showed only a positive trend, but the improvement at the protein level was not a statistically significant.

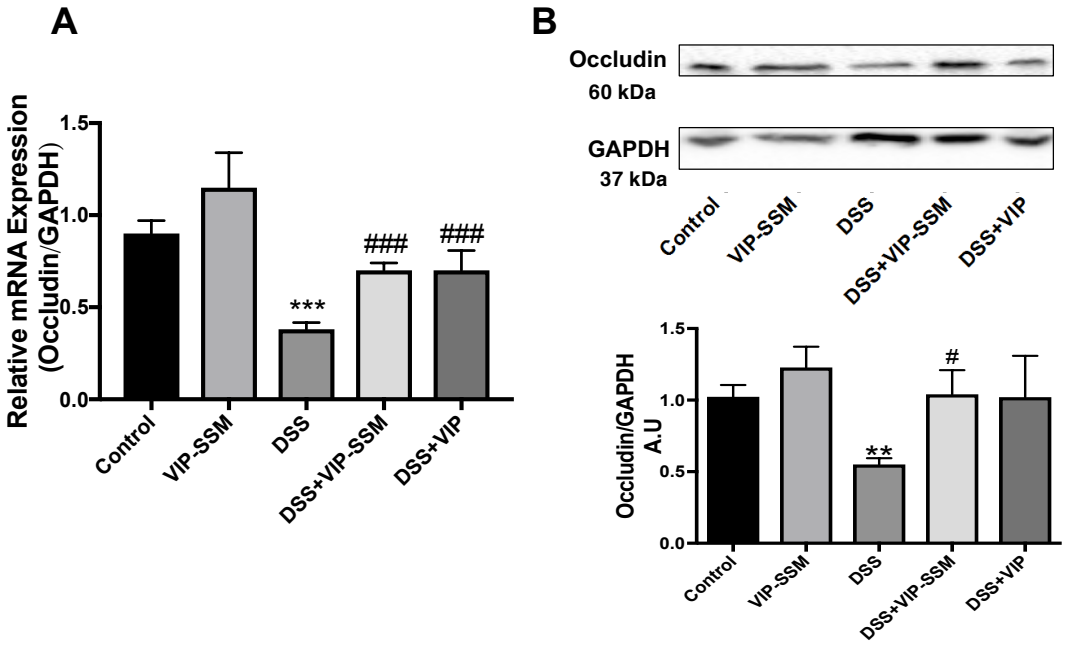


Figure 39: Effect of VIP-SSM on occludin mRNA and protein expression in mice distal colonic mucosa. A) mRNA B) Representative western blot with densitometric analysis. n=5, **-p<-0.005, ***-p<0.0005 vs control, #-p<0.05,###-p<0.0005 vs DSS (Treatment groups listed in TABLE VI) Modified and reused with permission from (176)

Although a significant alleviation of inflammatory parameters (cytokines, histology, diarrheal phenotype) was only observed with the nanomedicine administration (Therapeutic studies in DSS colitis), the free peptide seemed to have local effects in the mucosa. This is due to the improvement of the total mRNA and protein expression of occludin in the distal colon. Therefore, this was further investigated by studying the distribution of the TJ protein in the distal colonic mucosa. To determine the localization of the TJ protein occludin, in the distal colonic epithelium, immuno localization studies were conducted. Occludin (red) was stained along with the apical marker villin (green) (Figure 40). In healthy tissues, occludin has a beaded or punctate appearance in cross sections of the colons and co-localizes with the apical marker, villin (180). A Significant reduction and redistribution of the total occludin levels were seen with DSS. These levels were increased to almost control levels with both VIP-SSM and VIP, as depicted in Figure 40. However, in contrast to VIP-SSM, mice treated with free VIP showed aberrant, sub-apical localization of occludin in the epithelium (Figure 40). The punctate appearance of occludin indicating its presence at the junctional complex was only recovered with VIP-SSM treatment.

Additionally, the redistribution of another important cytoplasmic plaque protein in the TJ complex, zona occludens-1 (ZO-1) was determined in VIP-SSM treated mice. Total ZO-1 levels did not appear to reduce as drastically as occludin with DSS, however the redistribution to sub-apical compartment and the lack of beaded appearance was observed. This aberrant expression was recovered with VIP-SSM administration further confirming the positive effects of VIP

nanomedicine on epithelial integrity (Figure 41).

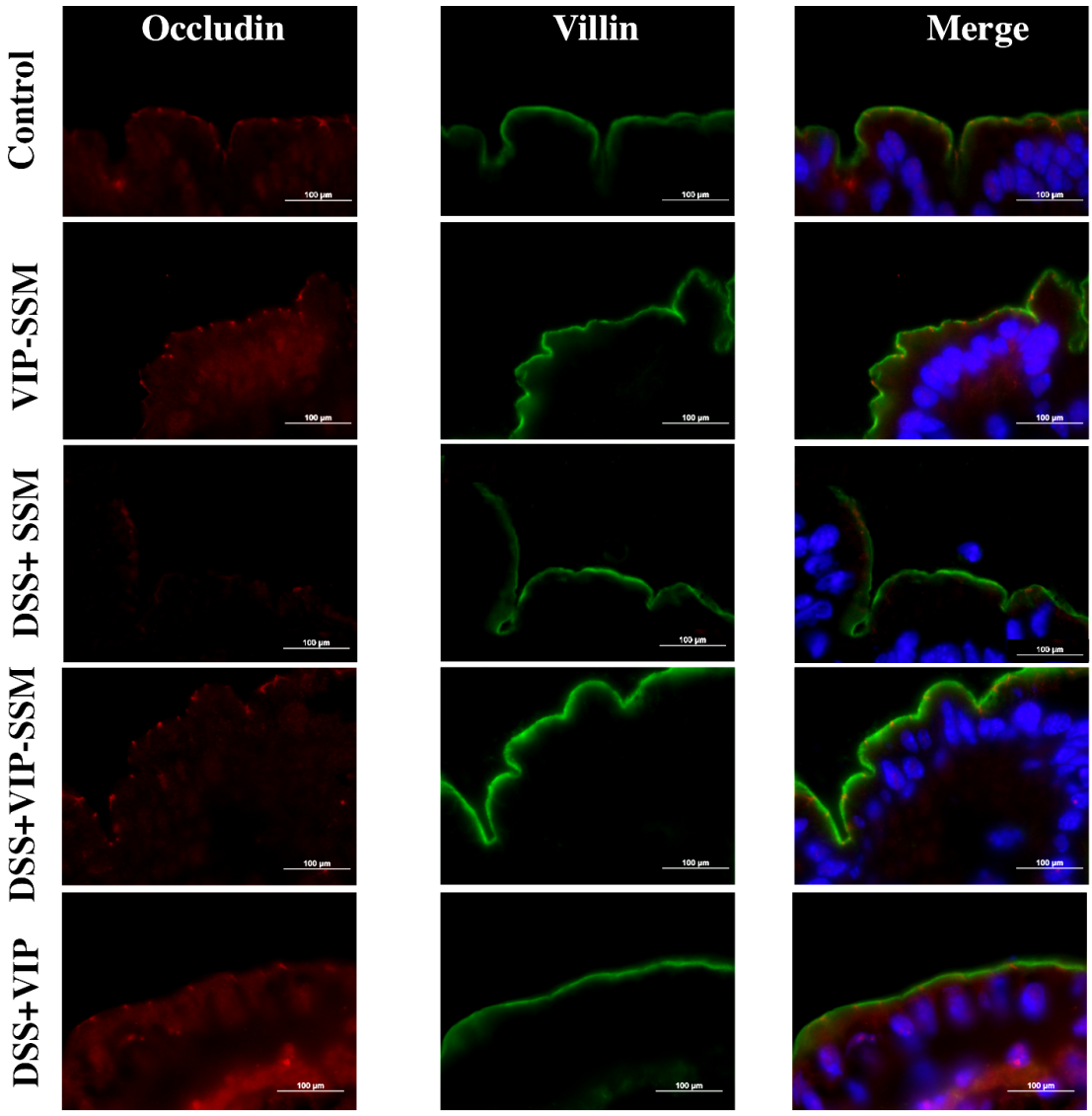


Figure 40: Effect of VIP-SSM on the localization of occludin in the distal colonic mucosa (Treatment groups listed in TABLE VI) Modified and reused with permission from (176)

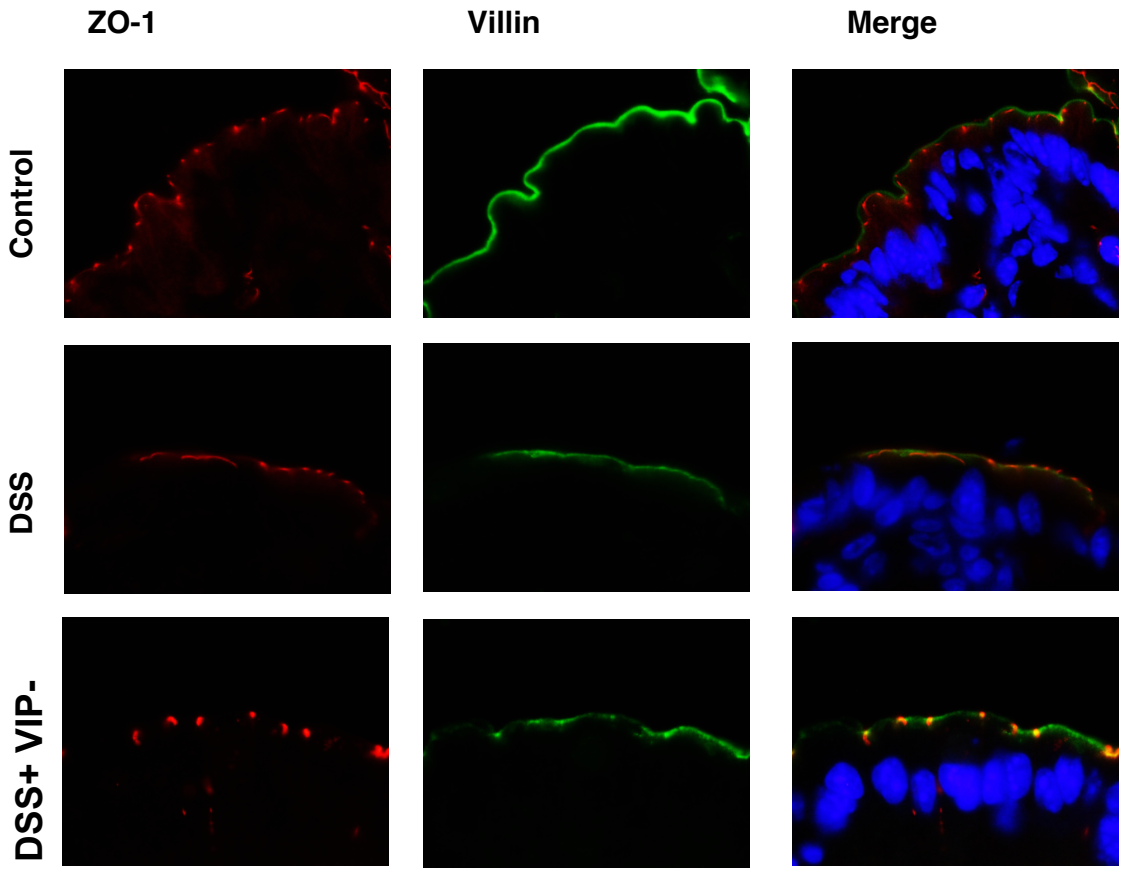


Figure 41: Effect of VIP-SSM on localization of cytoplasmic plaque protein ZO-1 in distal colons of DSS mice (Treatment groups listed in TABLE VI)

3.3.3 Effect of VIP-SSM on intestinal ion transporter (DRA) expression in the distal colon in therapeutic model of dextran sulfate sodium colitis

These data were adapted from previously published work in (Jayawardena, Dulari, Arivarasu N. Anbazhagan, Grace Guzman, Pradeep K. Dudeja, and Hayat Onyuksel. "Vasoactive Intestinal Peptide Nanomedicine for the Management of Inflammatory Bowel Disease." *Molecular pharmaceutics* 14, no. 11 (2017): 3698-3708.)

Intestinal ion transporters are key mediators of fluid and electrolyte homeostasis in the body. Patients with IBD suffer from severe diarrhea due to the mucosal damage observed in the disease. Recent GWAS studies identified SLC26A3 as a gene positively associated with IBD patients in certain geographical locations further highlighting the importance of this ion transporter in IBD (127). In DSS colitis, inflammation only persists in the distal colon of mice, and DRA is the major ion transporter expressed in these tissues (181). Therefore, the expression of DRA was studied to determine if VIP-SSM influenced the lost expression of this transporter in colitis.

Mice distal colonic mucosal scrapings were analyzed for total expression of DRA mRNA and protein. Figure 42 depicts, the drastic reduction in the expression of both mRNA (Figure 42 A) and protein (Figure 42 B) levels of DRA in DSS colitis. DSS treatment resulted in over 50% down regulation of DRA mRNA and over 80% down regulation of total protein. Thus, functionally, the intestine is incapacitated to balance the fluid intake which results in diarrhea (Figure 29). Treatment with VIP nanomedicine significantly improved both mRNA and protein expression of DRA.

However, treatment with the free peptide did not result in protein and mRNA recovery of the transporter. These findings are parallel to the diarrheal phenotype results (Figure 29) which were observed with the nanomedicine treatment. To supplement the protein data, immuno fluorescence studies were conducted to stain apically localized DRA (red) with villin (green) (Figure 42 C). These studies further confirmed the recovery of the down regulated DRA protein with VIP nanomedicine administration in DSS colitis.

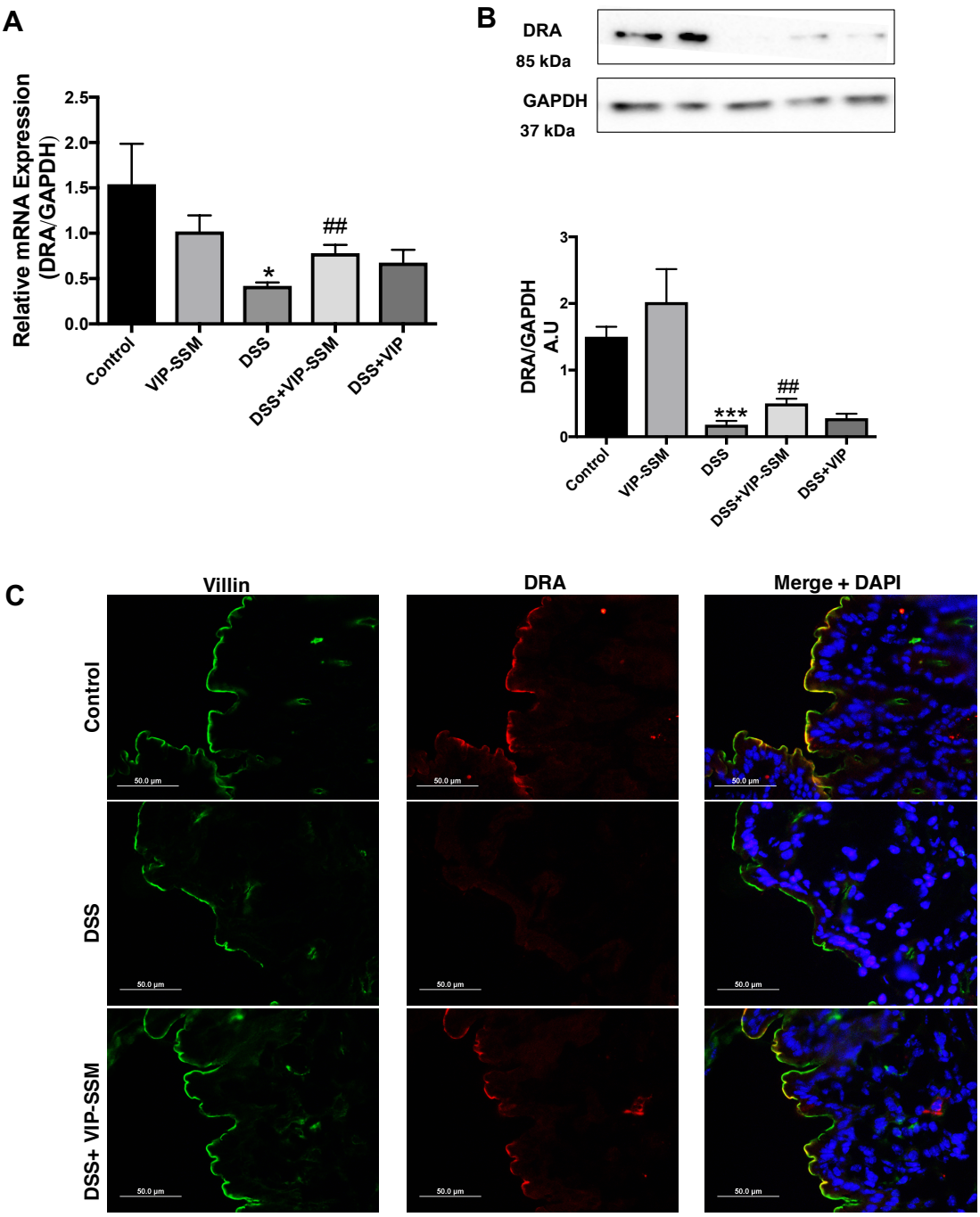


Figure 42: Effect of VIP-SSM on the down regulation of DRA mRNA and protein in DSS colitis. A) mRNA, B) representative western blot with densitometric analysis. C) Immuno-localization of DRA in distal colon. (Treatments groups listed in TABLE VI) Modified and reused with permission from (176)

Next, the specificity of VIP's effects on DRA protein expression was investigated in a cell culture model. This was to determine if the beneficial action on DRA was independent of its anti-inflammatory effects. Fully confluent Caco2 monolayers were treated with 10 nM VIP with or without the VPAC1 antagonist. The results demonstrated that, VIP had a direct effect on the protein expression of DRA by significantly increasing the levels from untreated controls up to 2 folds. This effect was mediated by the main VIP receptor VPAC1, since the specific antagonist [Ala 11,22,28] VIP was able to block these effects (Figure 43).

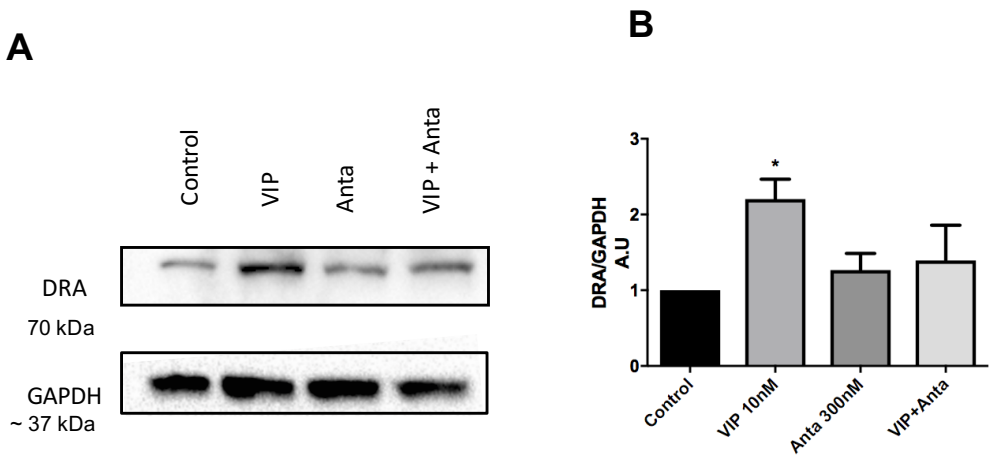


Figure 43: VIP directly upregulates DRA protein expression in Caco2 monolayers via VPAC1 activation. A) representative western blot, B) densitometric analysis. n=4, *-p<0.05 Vs control, Anta: VPAC1 receptor antagonist, [Ala 11,22,28] VIP

3.4 Results from Specific Aim 3

Under **Specific Aim 3** we explored the feasibility of luminal delivery of liquid nanomedicine *in vivo*. In addition, freeze-dried nanomedicine in powder form was evaluated as a potential oral formulation *in vitro*.

3.4.1 Expression of vasoactive intestinal peptide receptors in the gastrointestinal tract

These data were adapted from previously published work in (Jayawardena, Dulari, Grace Guzman, Ravinder K. Gill, Waddah A. Alrefai, Hayat Onyuksel, and Pradeep K. Dudeja. "Expression and localization of VPAC1, the major receptor of vasoactive intestinal peptide along the length of the intestine." American Journal of Physiology-Gastrointestinal and Liver Physiology 313, no. 1 (2017): G16-G25.)

The long-term goal of these studies is to treat IBD with an oral nanomedicine formulation to be released specifically at diseased tissue, the colon. However, there is a gap in knowledge regarding the expression of VIP receptors in the gastrointestinal mucosa. Therefore, studies were undertaken to determine the expression of VIP receptors in mice and human tissues to ascertain if targeting VIP to the lumen of the intestine could bring additional effects apart from its action on the inflammatory cells.

Mouse intestinal mucosa from jejunum, ileum proximal and distal colons were assessed for mRNA expression of the three receptors of VIP, VPAC1, VPAC2 and PAC1. As Shown in Figure 44A below, mRNA expression of VPAC1 was over 300-fold higher than VPAC2 and PAC1 along the length of the intestine. This demonstrated that VPAC1 to be the predominant receptor present in the

intestinal mucosa. Additionally, when the individual receptor VPAC1 mRNA expression was compared across the regions of the intestine, the expression showed significantly higher levels in the colon compared to the small intestine. These results were mirrored in the protein expression pattern (Figure 44 C).

Next, it was of interest, to determine if similar expression patterns were present in the human tissues so that data obtained from mice would provide insights into VIP's potential clinical use. As shown in Figure 45, human data suggests that VPAC1 mRNA levels are significantly higher in the distal part of the colon (sigmoid colon) as compared to other regions.

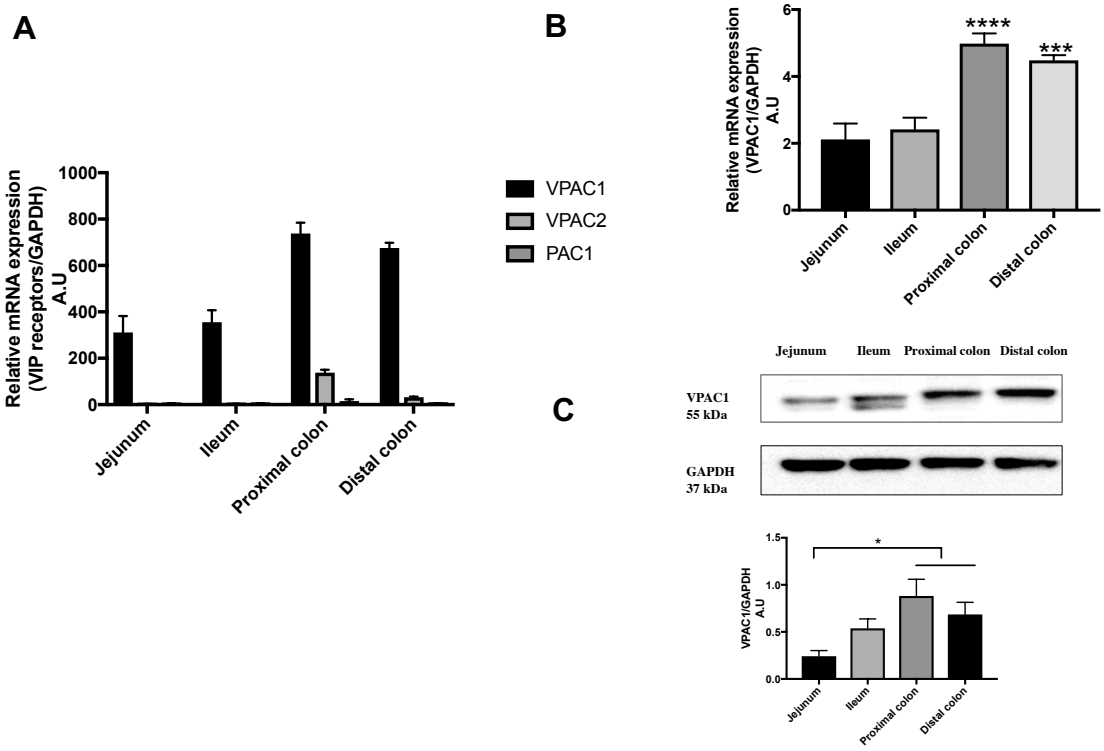


Figure 44: VIP receptor mRNA (A,B) and protein (C) expression along the length of the mouse intestine Values represent mean ± SEM, n=6, *p<0.05 Vs jejunum and ileum, ***p<0.0005 Vs jejunum and ileum.****p<0.0001 Vs jejunum and ileum. Reused with permission from Reference (37)

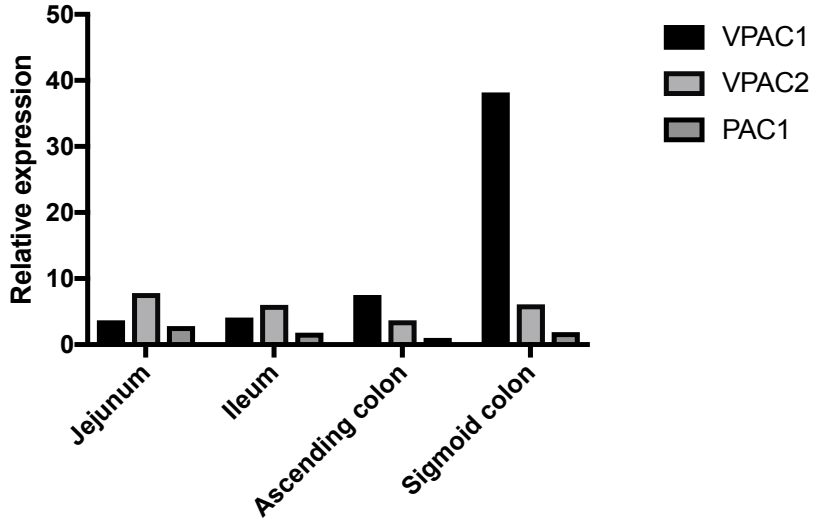


Figure 45: VIP receptor mRNA expression along the length of the human intestine. Modified and reused with permission from Reference (37)

Since higher expression was observed in the colons of both the human and mice tissues, immuno localization studies were conducted on paraffin embedded distal colonic tissues of mice and colonic biopsies from healthy areas of cancer patients. As shown in Figure 46, the localization of VPAC1 (red) was predominantly found on the apical membrane of the colon, which co-localized with the apical marker villin (green). This was similar in both mice and the human colon tissues.

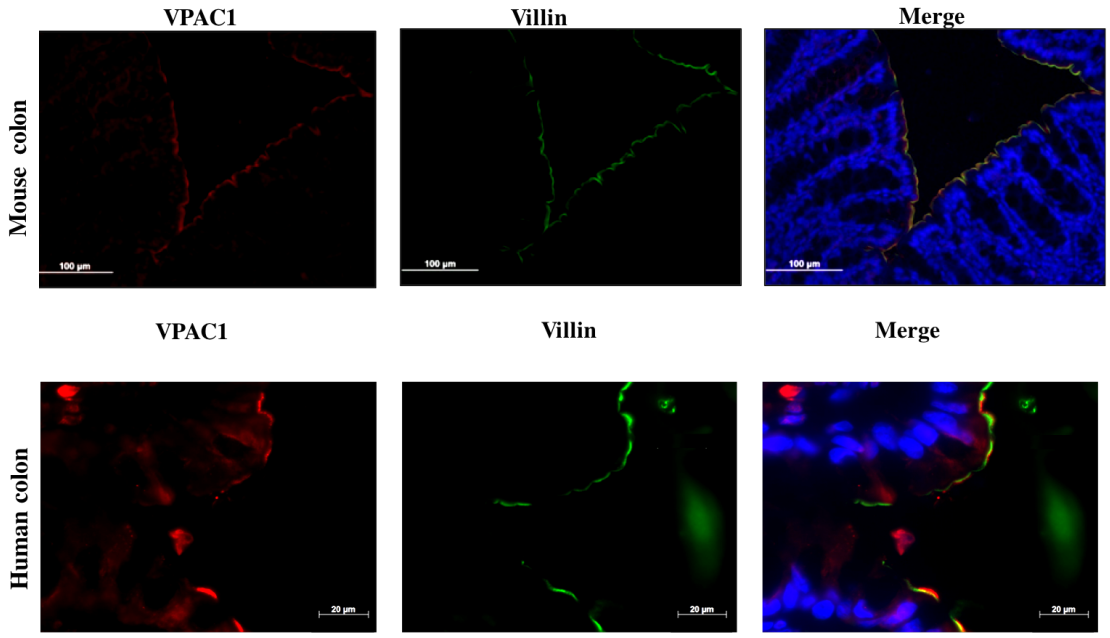


Figure 46: Co-localization of VAPC1(red) receptors with the apical marker villin (green) in the human and mouse colon. Modified and reused with permission from Reference (37)

3.4.2 *In vivo* efficacy of locally administered VIP-SSM nanomedicine in alleviating colitis

The favorable results obtained with the expression of VPAC1 receptors on the apical membrane in mice and humans colon facilitated the following studies to be conducted. Therefore, in order to determine the therapeutic benefit of VIP nanomedicine by colonic delivery in colitis, DSS model was used in a therapeutic setting (Figure 17). A single dose (0.25 nmol) of VIP nanomedicine or free peptide with respective controls were administered via intra rectal instillation at day 5 of the study (see Figure 47) by intra rectal instillation with similar treatment groups in TABLE VI. Administration of local VIP-SSM showed superior effects over the free

peptide in all parameters investigated. These beneficial effects are described below

3.4.2.1 Effect of Locally administered VIP-SSM via intra rectal route on weight loss associated in a therapeutic model of dextran sulfate sodium colitis

To complement previous findings, the therapeutic properties of VIP nanomedicine after a single local administration via intra rectal instillation, was determined by monitoring the body weight of mice throughout the duration of the study. As shown in Figure 47 below, mice started losing body weight from day 6 with DSS. Treatments were administered on day 5 and DSS was continued up to day 7. When mice were switched to tap water, nanomedicine treated mice started showing a recovery in average body weight compared to the untreated and free peptide treated groups. At the end of the study, mice in VIP-SSM treated group showed a significant improvement in body weight.

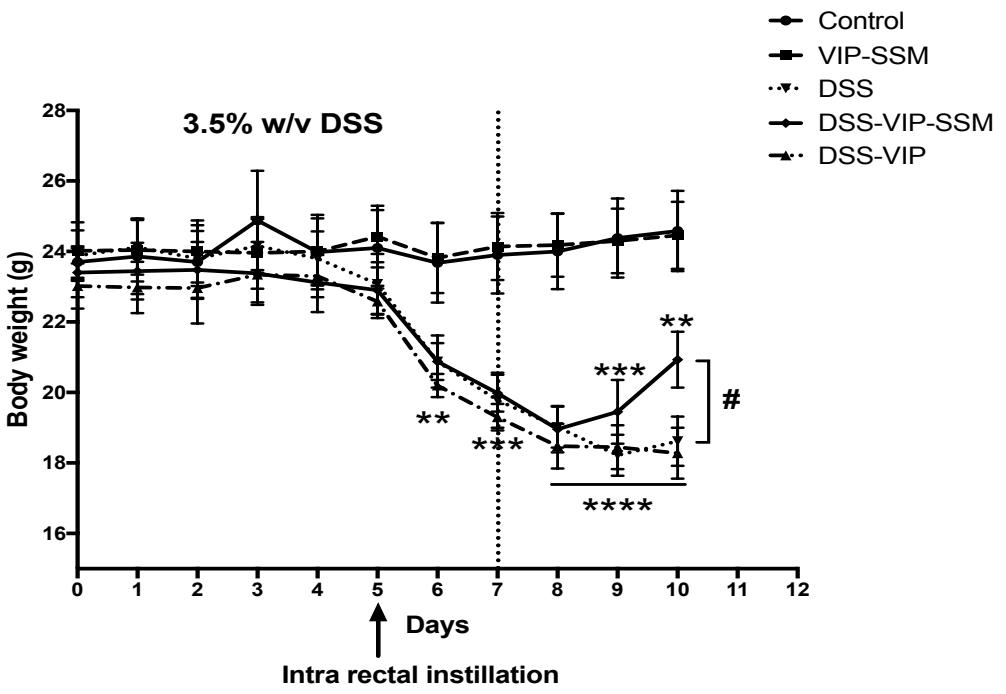


Figure 47: Effect of local VIP-SSM treatment on loss of body weight associated with DSS. n=9, **-p<0.005,***-p<0.0005,****-p<0.0001 Vs control, #-p<0.05 Vs DSS (Treatment groups listed in TABLE VI)

3.4.2.2 Effect of locally administered VIP-SSM to the colonic lumen in alleviating inflammation associated in a therapeutic model of dextran sulfate sodium colitis

The anti-inflammatory effects of VIP-SSM were determined by analyzing the distal colonic mucosa for mRNA expression of pro-inflammatory cytokines. Cytokines including IL-1β, CXCL-1 and CXCL-2 levels were significantly up regulated with DSS colitis (Figure 48). The administration of VIP-SSM nanomedicine as a single local dose of 0.25 nmol, significantly down regulated these cytokines to almost control levels. These effects were not present with the free peptide confirming the superior effects of the nanomedicine when

administered via intra rectally to the colonic lumen. These results were comparable to those obtained from systemic administration of VIP-SSM and further confirms the potential benefit of local administration of the nanomedicine to manage colitis.

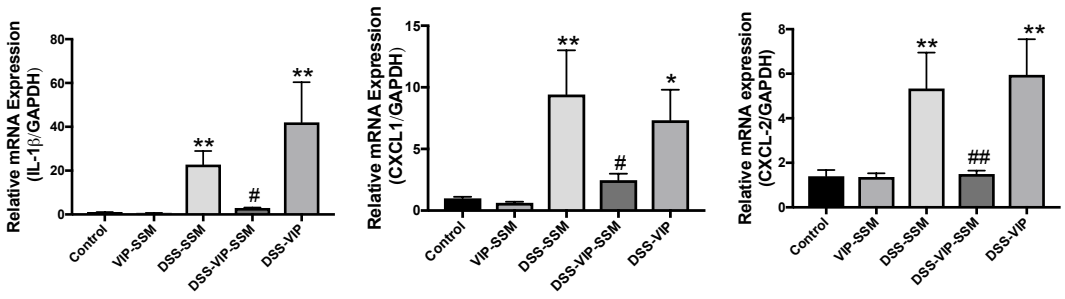


Figure 48: Effect of local delivery of VIP-SSM nanomedicine on the increased mRNA levels of pro-inflammatory cytokines in distal colon in DSS colitis. n=9, -p<0.05,*-p<0.005 vs control, #-p<0.05, ##-p<0.005 vs DSS (Treatment groups listed in TABLE VI)

In line with these findings, when the distal colonic histology was evaluated for pathological features of colitis including, epithelial damage, accumulation of inflammatory infiltrate and crypt destruction, VIP-SSM showed a remarkable recovery from diseased state (Figure 49). This was evident from the significantly low histopathological scores in mice treated with the nanomedicine. It should be noted that the free peptide had no such effects on the histopathological score and that the total values were similar to the untreated mice. In parallel, myeloperoxidase activity of the distal colon of the VIP nanomedicine group demonstrated significant amelioration of leucocyte infiltration compared to DSS

mice (Figure 50). Collectively, these results support the conclusion that VIP-SSM could successfully alleviate inflammation when administered locally to the colon.

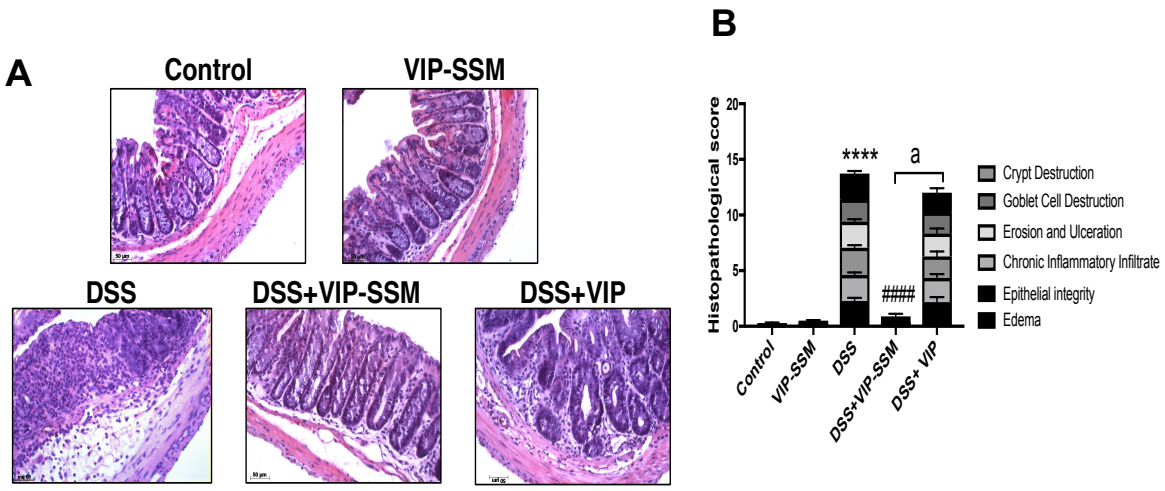


Figure 49: Effect of local delivery of VIP-SSM on alleviating the damaged distal colonic histology (A) as assessed by blinded histopathological score (B). n=9, ****-p<0.0001 vs control, ####-p<0.0001 vs DSS, a-p<0.0001 (Treatment groups listed in TABLE VI)

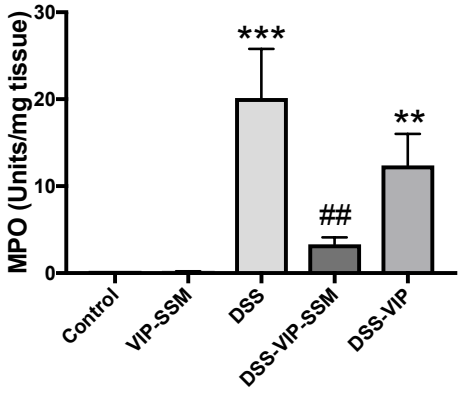


Figure 50: Effect of colonic delivery of VIP-SSM on the increased MPO activity in distal colon of DSS mice. n=9, **-p<0.005, ***-p<0.0005 vs control, ##-p<0.005 vs DSS (Treatment groups listed in TABLE VI)

In line with the above findings, at the end of the study when mice colons were harvested and analyzed for diarrheal phenotype only VIP-SSM mice showed improved stool consistency and recovered colonic length (Figure 51). These results show that local administration of the nanomedicine has beneficial effects at the same dose which showed alleviation of inflammation after systemic (ip) administration.

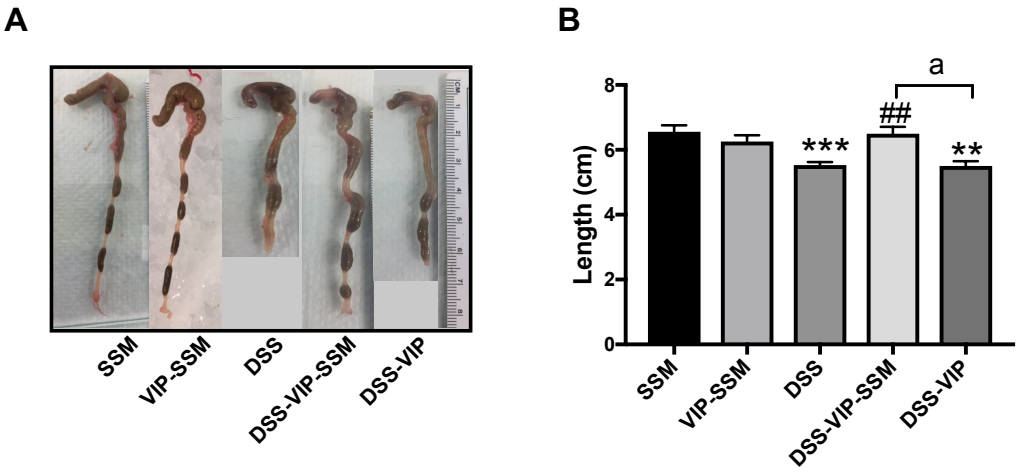


Figure 51: Effect of local VIP-SSM treatment on diarrheal phenotype.
A) representative images of whole colon of mice. B) graphical representation of average length of colon.n=9, **-p<0.005,***-p<0.0005 Vs control, ##-p<0.005 Vs DSS, a-p<0.05 (Treatment groups listed in TABLE VI)

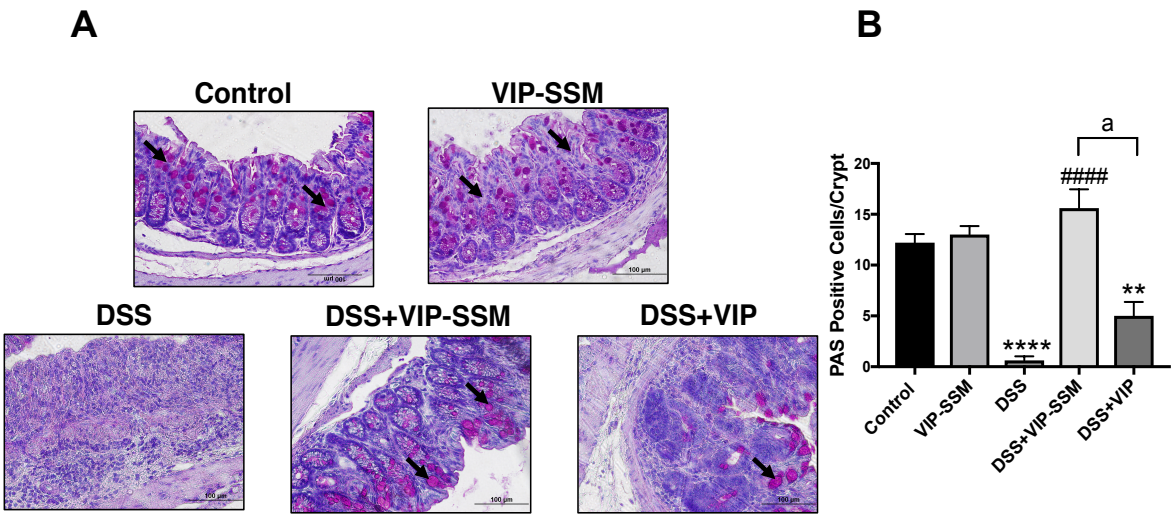


Figure 52: Effect of local delivery of VIP-SSM on the goblet cell count in DSS. A) Representative histologic micrographs of distal colon stained with PAS. Arrow heads indicate goblet cells stained in magenta. B) Quantification of goblet cell number per crypt. n=9, **-p<0.005,****-p<0.0001 Vs control, ####-p<0.0001 Vs DSS, a-p<0.0001 (Treatment groups listed in TABLE VI)

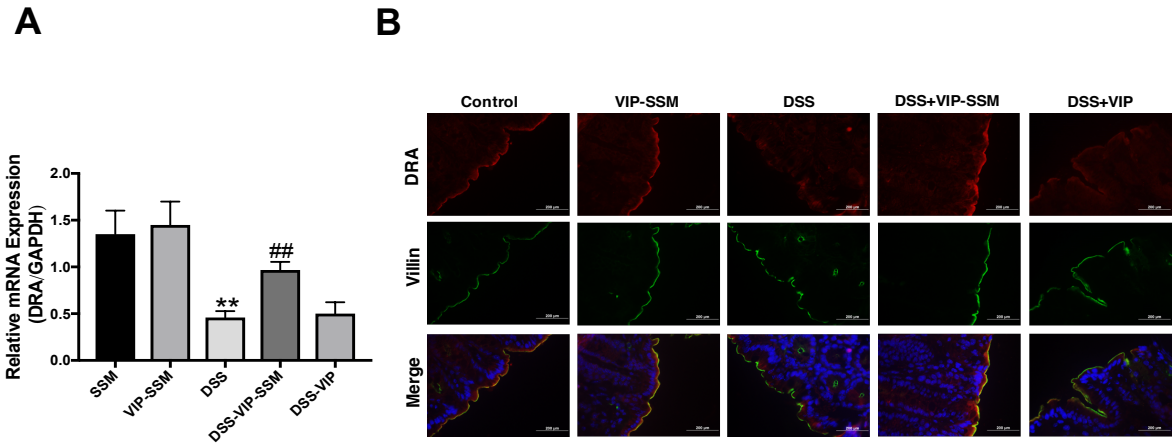


Figure 53: Effect of local VIP-SSM on down regulated, DRA expression in DSS colitis. A) mRNA B) protein levels by immuno-localization. n=9, **-p<0.005Vs control, ##-p<0.005 Vs DSS (Treatment groups listed in TABLE VI)

3.4.2.3 *Effect of therapeutic model of dextran sulfate sodium colitis with intra rectal VIP-SSM administration on expression of VPAC1 levels in the intestinal mucosa*

In our preliminary studies, we observed that the mRNA level of VPAC1 in a therapeutic model of colitis did not significantly down regulate with DSS. Therefore, in this study the expression of VPAC1 levels in the colonic mucosa was investigated in order to ascertain if DSS colitis could negatively affect its expression under diseased conditions. Thus, if the receptors are down- regulated luminal administration will not be successful in mediating these additional beneficial effects of the peptide.

VPAC1 mRNA, protein and immunofluorescence studies demonstrated no overall difference in its expression across groups in a therapeutic setting in DSS colitis (Figure 54, Figure 55). Thus, local targeting VIP-nanomedicine to the colon under inflammatory conditions could be potential treatment strategy to manage IBD.

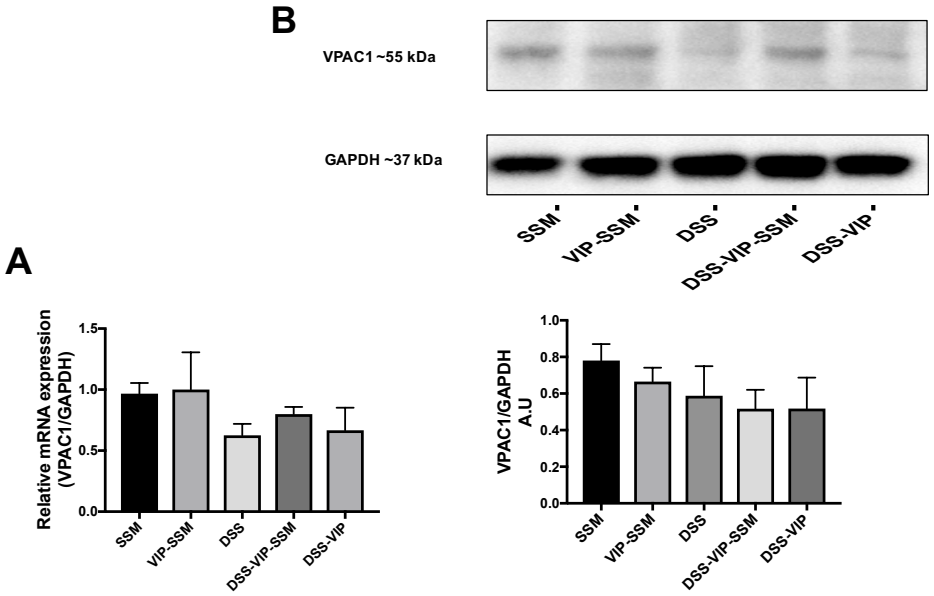


Figure 54: VPAC1 mRNA and protein expression in DSS colitis, A) mRNA, B) representative western blot and densitometric analysis. n=9 (Treatment groups are listed in TABLE VI)

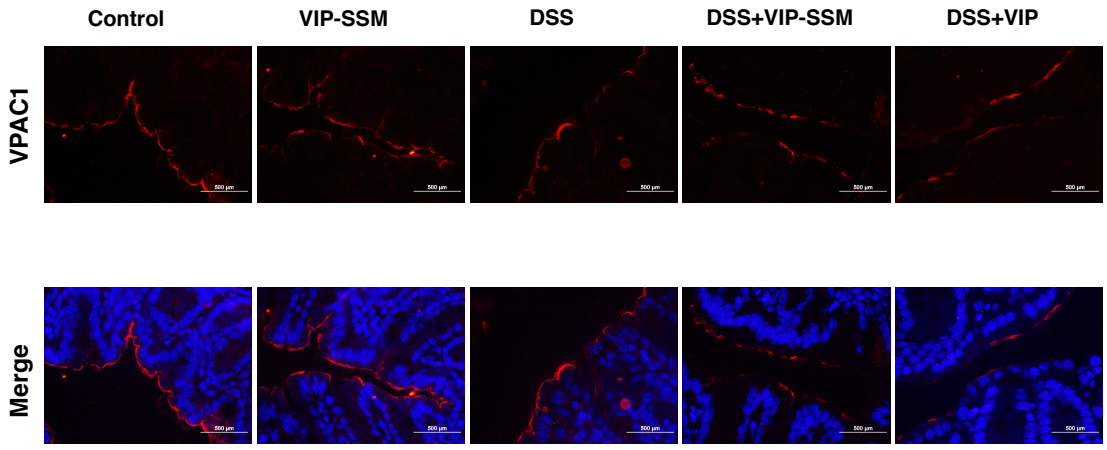


Figure 55: Immunofluorescence staining of VPAC1 (red) in the distal colonic tissues of DSS mice (Treatment groups are listed in TABLE VI)

With the completion of the *in vivo* studies after colonic luminal administration of the nanomedicine, we demonstrated that VIP nanomedicine alleviated inflammation and recovered mice from colonic damage. In the next stage, we explored the potential of this nanomedicine to be used as an oral formulation by *in vitro* studies.

3.4.3 *In vitro* evaluation of scaled up nanomedicine for human use

Studies conducted until this point showed potential of using VIP nanomedicine in animal models of colitis resembling both UC and CD. In addition to systemic delivery, local instillation of VIP-SSM in an animal model of DSS colitis further demonstrated potential for it to be used as a locally administered formulation. Due to the labile nature of the components of the nanomedicine (Phospholipid and native VIP), in order to be used as an oral formulation, it needs to by-pass the harsh conditions of the stomach. Fortunately, to circumvent this barrier there are available enteric coated capsules which can resist acidic conditions, and specifically release the drug at lower part of the GI tract. Using this concept, we planned to design an oral peptide nanomedicine for VIP nanomedicine, and test it by *in vitro* feasibility studies.

3.4.3.1 Formulation of freeze-dried VIP-SSM containing capsules

From the *in vivo* animal studies, a working dose effective for managing colitis was determined in mice. This dose was then converted to the human dose as described in methods section 2.11. This formulation was prepared by simple dissolution followed by freeze-drying to be incorporated into enteric coated capsules purchased from PCCA (Houston, TX). These capsules were then filled

with approximately 80 mg of VIP-SSM freeze-dried powder as shown in Figure 56 below, and was stored in air tight containers prior to performing the drug release test. Filled capsules were of uniform weight (TABLE X).

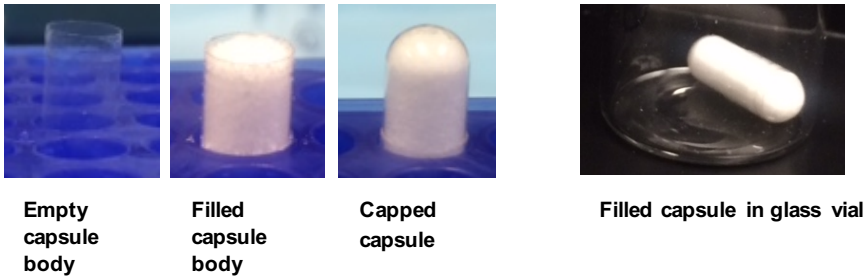


Figure 56: Manually filled VIP-SSM containing enteric coated capsules

TABLE X: FILL WEIGHTS OF MANUALLY FILLED CAPSULE

Capsule no	Weight after filling (mg)	Weight of empty capsule (mg)	Weight of VIP-SSM (mg)
1	163.8	83.8	80
2	164.9	84.8	80.1
3	163.2	82.5	80.3

3.4.3.2 Release of nanomedicine from capsules at colonic pH

To determine the release of capsule content (freeze dried VIP-SSM), dissolution assay was performed modified to laboratory scale, as shown in Figure 57 below. Capsules were immersed with the aid of a sinking device (spring) recommended by the Unites States Pharmacopoeia (USP), in simulated colonic pH of 6.5 at 37 ^{0}C.

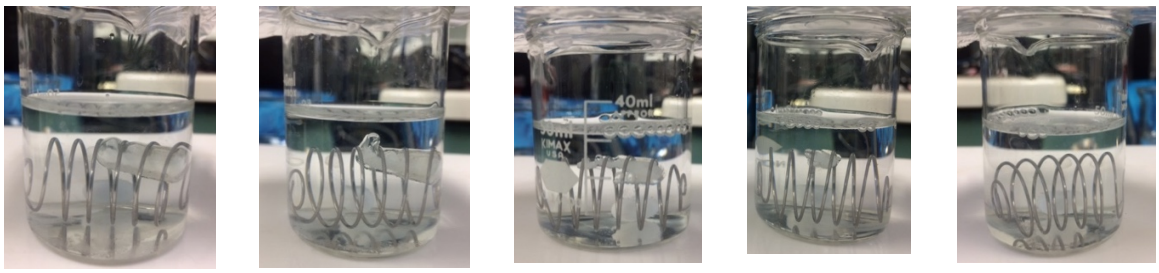


Figure 57: Representative photographs of freeze-dried VIP-SSM containing capsule dissolution

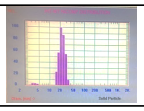
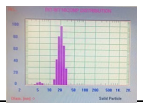
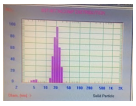
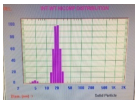
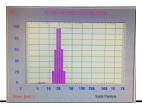
Capsules containing freeze-dried VIP-SSM started dissolving in the buffer from 10 minutes after immersion (Figure 57). The capsule contents gradually dissolved over the next 10 minutes showing release of contents from the edges of the capsule. Starting from 20 minutes there was rapid dissolution which resulted in a visibly clear solution at the end of 40 minutes. These observations indicated that VIP-SSM dissolved in colonic fluids and was completely released from the enteric coated capsules without forming clumps at the interfaces. The dissolution behavior of capsules resulted in identical outcomes for each capsule under study, including capsules prepared at different time points representing different batches or capsules stored for up to 6 weeks at 4 ^{0}C.

3.4.3.3 Reformation of micelles after capsule dissolution assessed by dynamic light scattering

Dissolution of capsules appeared to be successful with all contents being released from the capsules giving rise to a visually clear solution. However, for the

nanomedicine to be effective in managing human IBD, as shown *in vivo*, it is of utmost importance that once dissolved, the nanomedicine is intact with bio active VIP. In the following studies, the reformation of micelles after capsule dissolution was determined by dynamic light scattering. Presence of particles ranging in the size of approximately 15-20 nm was used to infer the presence of micelles in the dissolution contents (since micelles are ~ 15nm). TABLE XI below shows the population of particles corresponding to micelles in solution at each time point of analysis. Aliquots of 500 µL was used for dynamic light scattering analysis. Since the capsule contents did not dissolve well at 10 minutes and thus, very few micelles were be in solution, the aliquot used for DLS analysis was unable to initialize the NICOMP particle sizer. However, starting from 20 minutes, where the capsule showed reasonable swelling and dissolution, over 96% of the particles analyzed were in the size range of micelles (TABLE XI). This pattern was similar throughout the rest of the time aliquots were analyzed. At 60 minute time point, the majority of the particles were in micellar size range indicating successful reformation of micelles after freeze-dried nanomedicine was dissolved in simulated colonic fluid. As before, these results were similar between capsules prepared at different times and ones stored over a period of 6 weeks at 4 ^{0}C in air tight glass containers.

TABLE XI: REPRESENTATIVE READINGS FROM PARTICLE SIZE ANALYSIS SHOWING MICELLE FORMATION

Time point	Mean Diameter (nm)	Standard Deviation	Percentage	Data Output
20 min	22.4	4.3	96.51	
30 min	21	3.5	95.1	
40 min	20.9	3.5	94.5	
50 min	20.3	3.6	94.9	
60 min	20.4	3.6	100	

3.4.3.4 Presence of active vasoactive intestinal peptide in solution from dissolved capsules

The active ingredient in the capsules, VIP, should be present once capsules are dissolved, to mediate therapeutic benefits in IBD. Therefore, to understand if capsular contents, once dissolved, would have active VIP, ELISA was performed on aliquots taken at 10-minute time intervals. As shown in Figure 58 below, each capsule released VIP, starting from 20-minutes parallel to the findings from micelle reformation assessed by DLS. Beyond 20-minutes release of VIP from capsules showed an exponential increase fitting a 4-parametric sigmoidal curve (Figure 58).

Due to the complete dissolution of capsules after 40-minutes, the curve showed a plateau in release reaching complete release of VIP from capsules beyond 40 minutes. These data were comparable between capsules prepared at different times resembling capsules of different manufacturing batches.

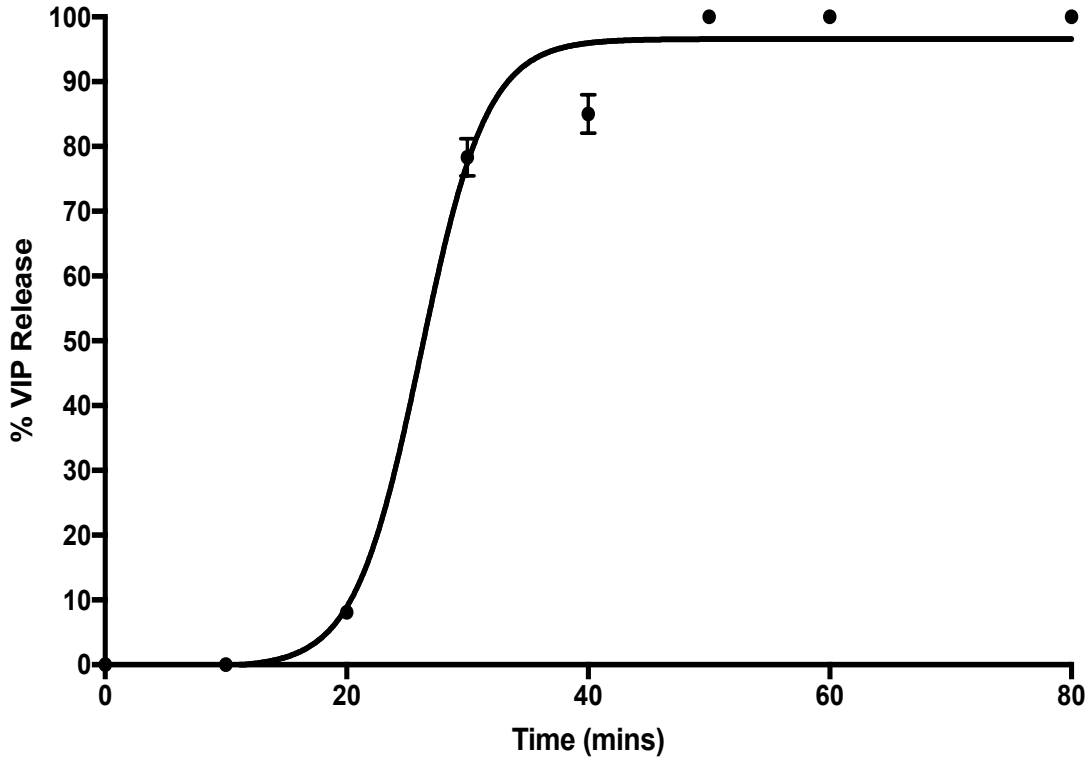


Figure 58: Percentage of VIP released from capsules dissolved in colonic pH as determined by ELISA. Results are from 5 individual capsules (3 prepared at the same time and 2 prepared at different times representing different batches of capsules). n=5, data points were fitted to a 4-parametric sigmoidal curve with a R^2 value of 0.99

3.4.3.5 Detection of peptide association with SSM after released from capsules

All prepared capsules had a uniform fill weight of 80 mg as shown in the TABLE XII below; In addition to the capsule dissolution fluids, a known concentration of VIP (10 ng/μl) was also assessed to compare the amounts of VIP.

TABLE XII: FILL WEIGHTS OF CAPSULES CONTAINING VIP-SSM OR FREE VIP WITH LACTOSE

Capsule	Weight before Filling	Weight after filling	Fill weight
1 SSM	85.1	164.4	80.3
2 SSM	84.4	164.6	80.2
3 SSM	84.9	165.4	80.5
1 Lactose	84.6	164.8	80.2
2 Lactose	85.0	165.3	80.3
3 Lactose	85.1	165.6	80.5

As shown in Figure 59 below, capsules containing VIP-SSM showed a dissolution pattern similar to which was observed previously, i.e. Releasing contents exponentially from 20 -40 minutes and then reaching a plateau after all contents were released (Figure 59). The capsules which had the same fill weight of VIP in lactose, released VIP faster initially but gave rise to lower total levels of VIP in solution at time points starting from 30 minutes compared to the capsules with VIP-SSM. In the initial phase of release, free VIP containing capsules released contents at a rapid rate compared to the nanomedicine powder containing

capsules, thus, this was reflected in the VIP release profile demonstrating higher VIP at the 20 minutes time point, compared to capsules with VIP-SSM (Figure 59). Most importantly, unlike capsules containing VIP-SSM, free peptide containing capsules started showing a relative drop in VIP concentrations once released, evidently from 20- 30 minutes and 40 minutes and beyond. A known concentration of free freshly prepared peptide was used to compare with the released peptide from capsules and is shown as a red triangle in Figure 59.

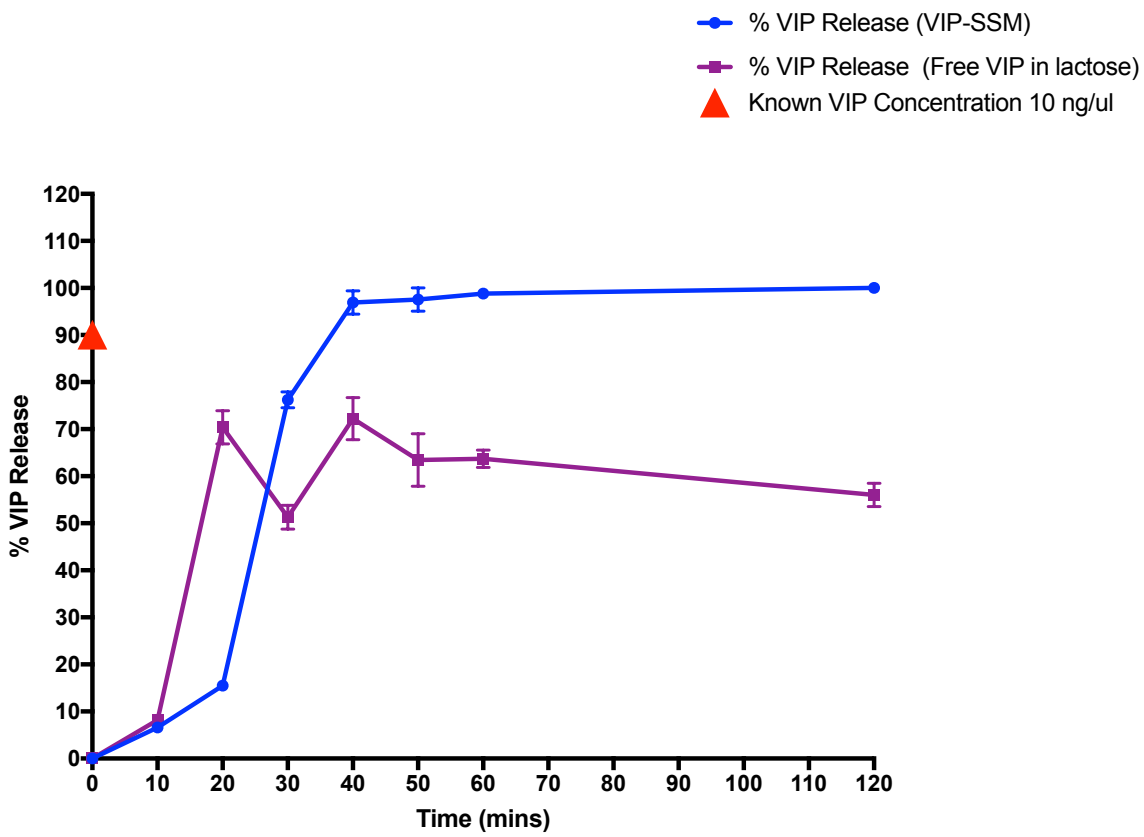


Figure 59: Association of VIP with SSM determined by ELISA

3.4.3.6 Stability of VIP-SSM containing capsules stored over a time

Pharmaceutical dosage forms used in clinics require storage life of appropriate duration. Therefore, for VIP-SSM in a capsule to be used in clinics, it was important to determine the stability of the formulation after storage. This was to determine if the reformation of micelles and release of VIP would show similar patterns to those observed as soon as the capsules were prepared.

As stated earlier, dissolution of the capsule and presence of micelles in solution after the capsules were stored for 6 weeks showed very similar outcomes to data presented earlier. Capsules dissolved completely after 40 minutes and from 20 minutes into dissolution, majority of particles present in the solution were in the size range of micelles.

The release of active VIP after storage at each week was determined and results are depicted in Figure 60 below, VIP release from capsules followed a similar pattern to the one observed earlier (Figure 6**0**) fitting a 4-parametric sigmoidal release. Data at each week of storage, did not show a significant difference in releasing active VIP. This shows reasonable evidence of the stability of the nanomedicine, with regard to the active ingredient VIP, in capsules in the freeze-dried form when stored in sealed containers at 4 ^{0}C for at least up to 6 weeks.

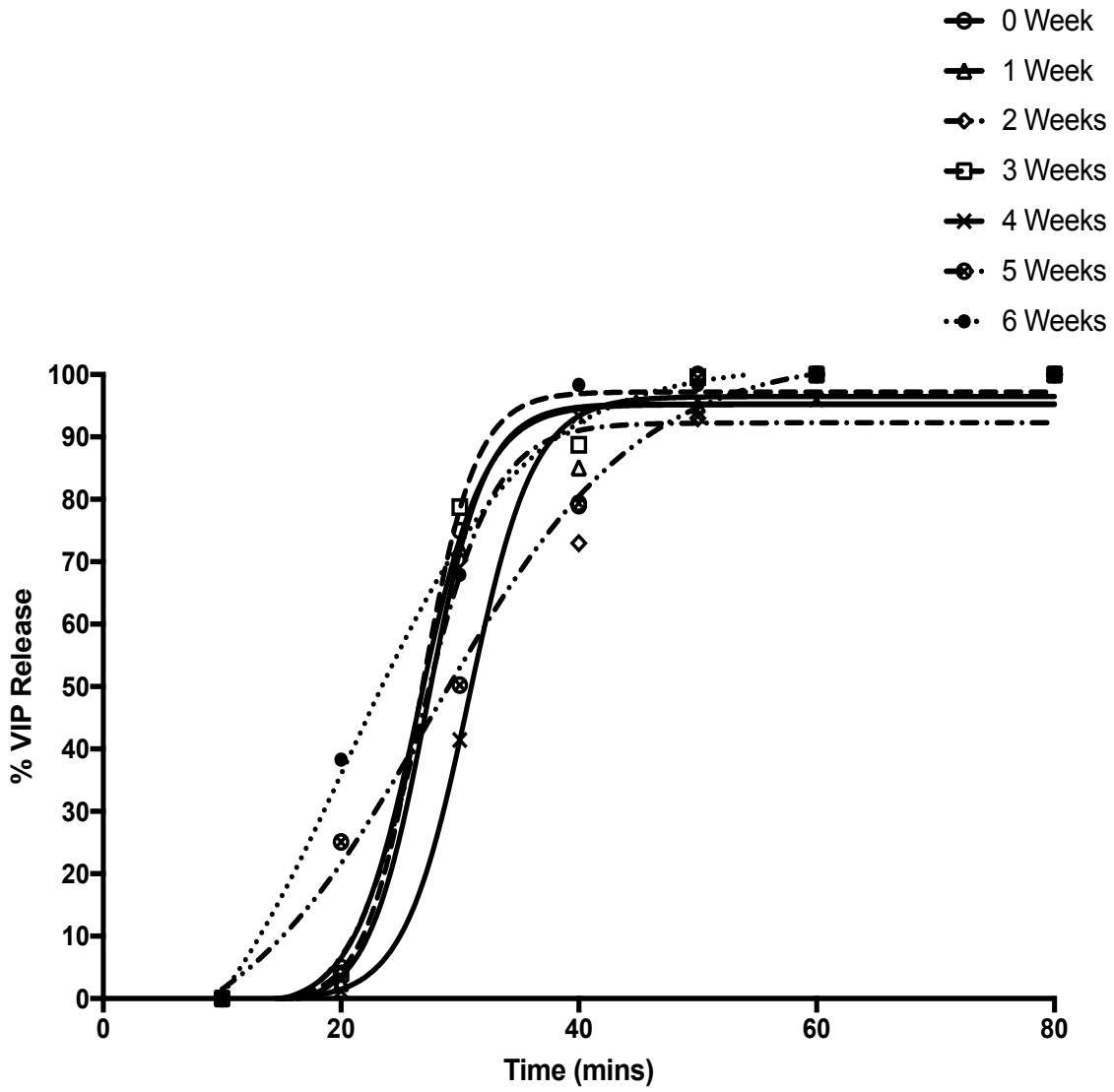


Figure 60: Percentage VIP release from capsules once dissolved after storage at 4 ^{0}C as assessed by ELISA. data points were fitted to a 4-parametric sigmoidal curve with a R^2 value of 0.99

4. DISCUSSION

The studies in this research project were undertaken to investigate the efficacy of a biocompatible peptide nanomedicine, and to test its potential as an oral formulation which can be delivered locally to the colon to manage colitis. The specific aims were to first; **i)** Identify the anti-inflammatory effects of VIP nanomedicine in alleviating colitis in animal models resembling human IBD. **ii)** Delineate mechanisms of VIP, involved in mediating therapeutic action. **iii)** Determining the potential of the nanomedicine as a locally delivered formulation.

4.1 In-vitro characterization of the nanomedicine

In vitro characterization of a nanomedicine is an important aspect prior to being used for any application. One key method of characterization of a nanoparticle is the determination of its particle size. The size is mainly assessed by dynamic light scattering, which is a sensitive method of detecting small particles which acquire Brownian motion in solution (182). The scattered light by the nanoparticle solution is used for calculating its size based on Stoke's Einstein relationship. The nanomedicine VIP-SSM developed in our laboratory has been widely characterized for its physicochemical and *in vitro* stability (26, 93, 101). Therefore, this project relied on these favorable attributes of the nanomedicine, to explore its usage to manage a novel therapeutic indication, IBD. *In vitro* characterization of the prepared nanomedicine was conducted at each preparation time to determine the particle size with DLS (Figure 21). Empty nanomedicine, SSM and VIP associated SSM (VIP-SSM) showed similar particle size distribution.

The similar size of VIP-SSM indicated that the peptide was associated with the nanocarrier.

In addition to size analysis, it was important to characterize the peptide nanomedicine for its bioactivity to confirm that association with the micelle does not interfere with VIP's biological functions. The bioactivity of VIP was determined by its ability to specifically bind and activate the type II GPCR's to increase intracellular cAMP. To test this effect, HT29 cell line was employed. The cell line has high expression of VPAC1 receptor and thus serves as a good *in vitro* model to determine the bioactivity of the peptide (149, 150). The results showed that, VIP-SSM had equal capacity to elicit an activation and thus increase in intra-cellular cAMP levels, similar to the free peptide *in vitro* (Figure 22). This confirmed that the association of the peptide with the nanocarrier does not affect its biological function. Once *in vitro* characterization was satisfactory, the peptide nanomedicine was tested *in vivo*, in mouse models of colitis for its anti-inflammatory action.

4.2 Determining the potential of VIP-SSM in alleviating inflammation in dextran sulfate sodium colitis

In these studies, effects of VIP in DSS colitis were explored in both preventive and therapeutic modes. DSS colitis is a widely used animal model and is well characterized in the literature (130). Disruption of the epithelial integrity of the colon by DSS, exposes the luminal contents and microbial antigens to the underlying dendritic cells to trigger an exaggerated immune response which recapitulates human UC like symptoms (183). DSS colitis is developed by

administering DSS in drinking water usually for 7 days. Mice develop colitis showing symptoms of rectal bleeding, body weight loss and diarrhea beginning from day 5 after DSS administration (137). This model can be used in various ways by changing the dose of DSS usually between 1% to 5% w/v, and by changing the duration of DSS administration. After 7 days of DSS acute colitis develops, however, if mice are switched to tap water they undergo self-recovery within two weeks of DSS removal (137). In a chronic setting DSS is usually administered at a lower dose (1% to 2.5%) for 7 days, followed by 7 days of water and DSS again in weekly cycles so that a low grade, chronic colitis is established. In our studies, we employed DSS model in an acute mode to determine preventive and therapeutic effects of VIP in this model.

Although, VIP was identified as a broad spectrum anti-inflammatory agent, capable of alleviating inflammatory responses, it is surprising as to why it had not been explored in DSS colitis model. One key reason for its lack of usage could be due to the focus of VIP treatment on Th1 type immune responses which shifted interests of VIP therapy to CD like colitis. In this regard, there were several earlier studies conducted in TNBS model where the effects of VIP have been studied extensively (48, 73, 77, 184).

Recently with the advent of KO animals, DSS model was used to study the importance of VIP in the development of colitis. In one such study, VIP KO animals demonstrated resistance to DSS colitis, which the authors described to be due to the involvement of endogenous VIP in mediating colitis development (82). However, these findings were completely negated in a more comprehensive study

conducted recently on VIP KO mice, which showed the therapeutic benefit of VIP in DSS (84). Therefore, in our studies DSS model was selected specifically for the following reasons; **i)** DSS model has been extensively studied and used as a pre-clinical model to evaluate current clinically used drugs in IBD (175, 185, 186). **ii)** Therapeutic use of VIP has never been studied in DSS colitis. **iii)** Our laboratory has used DSS model in multiple studies to determine therapeutic potential of other agents including a similar peptide nanomedicine (GLP-1-SSM) (144, 187).

In these studies, the VIP nanomedicine was tested for the first time as a treatment for colitis and therefore, the dose used needed to be determined. Preliminary studies were conducted with a dose of 1 nmol and 0.5 nmol which was used in previous studies (73, 84). In addition, lower doses of 0.25 and 0.05 nmol were also tested in a preventive model of DSS colitis. Based on these preliminary studies, 0.25 nmol showed the best effects in alleviating increased pro-inflammatory cytokine (IL-1β) levels, which are up regulated during DSS colitis. Finally, when 0.25 nmol was administered via different routes of iv, sc and ip, quite similar results were obtained, thus, ip route was chosen for all subsequent studies due to its ease of administration.

Initially, in the preventive studies, 3% w/v DSS was administered to mice throughout the study (8 days) with treatments administered on alternate days beginning at day 1 (Figure 12). The purpose of this study was to determine if administering VIP-SSM before severe inflammation sets in, could alleviate the development of colitis. In the preventive setting, VIP-SSM nanomedicine was as effective as the free peptide. This data demonstrated for the first time, that a

nanomedicine of VIP, was effective in preventing severe colitis in mice which resembles human UC. The pro-inflammatory cytokine mRNA levels, histology of the distal colon and the diarrheal phenotype of DSS were alleviated with both free and nanomedicine form of VIP (Figures 23-25). However, one interesting observation in these studies was the almost equal effectiveness of the free peptide at the same dose of 0.25 nmol as compared to the nanomedicine. The reasons for equal effectiveness of both these treatments (i.e nanomedicine vs free peptide) are not entirely clear, however, it is possible that intra peritoneal administration may have had the capacity to transport the drug directly back to the intestinal tissues avoiding first pass metabolism due to the proximity of the circulatory blood vessels between peritoneal cavity and the intestine. This may have allowed more free VIP to reach the site of inflammation (188, 189). In addition, since doses were administered every other day, degradation and instability of free VIP may have been compensated. Thus, the dose of the free VIP was possibly sufficient to alleviate the inflammation. Another factor could have been that since treatments were administered starting from day 1 of DSS, the extent of disease may have potentially been mild enough that a very small dose of VIP was sufficient to mediate anti-inflammatory effects. However, although a significant difference between nanomedicine and free peptide was not observed in a preventive setting, these studies provided substantial evidence of VIP nanomedicine to be effective in DSS colitis in dampening the inflammatory response.

In the next set of studies, it was important to determine if, VIP nanomedicine could be used to manage colitis in a therapeutic setting. In these studies, severe

inflammation was established prior to the administration of treatments. To create a more severe inflammation in the colon, DSS dose was increased from 3.0% to 3.5% w/v. Therefore, 3.5% DSS was administered to mice for 7 days and then mice were switched to tap water for the next 5 days. Symptoms of colitis developed in mice 5 days after DSS administration. These included diarrhea, body weight loss and rectal bleeding. All mice in this study exhibited a significant drop in body weight starting from day 6, which persisted throughout the duration of the study. It is known that after DSS removal, mice show acute inflammation which persists for the next 7 days and mucosal damage until 14 days after DSS removal (137, 190). However, after DSS removal, if left on drinking water, mice completely auto-recover from inflammation at the end of 2 weeks. Therefore, one day after switching to tap water, mice were treated with a single dose (0.25 nmol) of free or nanomedicine form of VIP, to test the therapeutic action.

Once treatments were administered, VIP-SSM treated mice significantly improved their body weight reaching a statistical significance when compared to untreated and free peptide treated mice by day 11 (end of study). These results demonstrated that VIP nanomedicine administration helped in recovering mice from the loss of body weight associated with colitis. The severe inflammation persisting at the end of 7 days of DSS treatment was mainly alleviated by a single dose (0.25 nmol) of VIP-SSM, but not with the free peptide. These results were in contrast to the results observed in preventive studies. Apart from the improvement in body weight, mice also demonstrated marked reduction in distal colonic inflammation, histological damage and diarrheal phenotype associated with DSS

(Figures 27-29).

The possible reasons for the differences in preventive vs therapeutic studies could have been partly be due to the single (therapeutic) vs multiple doses of VIP (preventive) utilized. Additionally, it is possible that the higher extent of inflammation associated in the therapeutic model allowed the nanomedicine to specifically accumulate at the site of inflammation due its size and protection of VIP from degradation. The ineffectiveness of the free peptide was possibly due to the low doses present at the site of action.

In our studies, we observed an overall anti-inflammatory action of VIP in alleviating DSS colitis. The involvement of the immune system and contribution of specific cell types in the disease were not studied. However, it is well-known that in DSS colitis model, there is an increase in activation of innate immune cells including neutrophils, macrophages and dendritic cells to mediate mucosal damage and inflammation in the colon (131, 137). In this regard, anti-inflammatory mechanisms of VIP could possibly be attributed to its action on these activated innate immune cells. The overall anti- inflammatory effects of VIP in DSS colitis in general should be mediated mainly by its broad spectrum anti-inflammatory action on activated dendritic cells and macrophages. VIP has shown to inactivate macrophages and inhibit cytokine and chemokine production in these cells (140, 191). In addition, it also promotes production of anti- inflammatory cytokines and mediates healing and immune tolerance (192, 193). Additionally, due to the reduction in neutrophil accumulation at the site of disease, the cascades of inflammatory events would be dampened, resulting in healing of the tissue and

recovery from colitis (194).

4.3 Determining the potential of VIP-SSM in alleviating trinitrobenzene sulfonic acid induced colitis

The studies conducted with DSS colitis provided sufficient evidence that VIP nanomedicine could be used to alleviate colonic inflammation associated with DSS colitis and is superior to the free peptide when administered as a single dose. However, earlier studies have demonstrated that VIP could impart therapeutic benefits in dampening the inflammation associated with TNBS colitis, a model resembling human CD. Therefore, it was of interest to determine if, this nanomedicine, at the same dose used in DSS colitis could have similar benefits in TNBS colitis.

These studies were conducted in 6-week old C57Bl6 male mice in both a preventive and therapeutic setting. It is known that younger C57Bl6 mice have a higher tendency to develop colitis compared to older mice and, therefore, mice from 4-6 weeks were used in these studies (152). TNBS colitis was induced by direct instillation of the chemical irritant, TNBS to the lumen of colon after pre-sensitization as described previously by Kremer *et.al (133).* Thus, inflammation develops rapidly starting from the day after instillation. Since C57Bl6 mice are relatively resistant to TNBS, pre-sensitization was conducted by applying 3.75 mg of TNBS on the shaved dorsal skin of the mice to prime the immune system for the development of an avid reaction which mediates colonic inflammation (132). The colonic inflammation peaks around 3 days after instillation and persists for 5 days

prior to self- recovery (134). Dose of TNBS, 3.5 mg (175 mg/kg) per mouse was established in our laboratory, for its capacity to elicit a robust inflammatory response and was selected for the current studies.

In the preventive studies, VIP was administered at 0.25 nmol on alternate days starting from the day of pre-sensitization. In TNBS colitis preventive studies, similar effects were observed with nanomedicine and free peptide treated mice (Figures 30-33). The anti-inflammatory effects were similar in both the free peptide and nanomedicine form, except for histological recovery (Figure 31). The histological damage may have been better recovered with the nanomedicine possibly due to more availability of the peptide at the site of inflammation. However, these findings were in parallel to the results observed in a preventive model of DSS colitis and, therefore, could be explained similarly; i.e. possibly due to the frequent administration of the peptide on alternate days. Therefore, the dose was sufficient to prevent severity of inflammation due to adequate quantities reaching the site of inflammation. Additionally, since the administration of treatments were started with the pre-sensitization step, VIP may have prevented the robust development of the immune response by interfering with disease development. Interestingly, previous studies conducted with the free peptide, in TNBS colitis in a different strain of mice (BALB/C) used a 4 times higher dose of VIP (free peptide, 1 nmol) for alleviating gut inflammation (73). The discrepancies in these findings could have been partly due to the differences in animal facilities of the two studies which could have affected the composition of gut microbiome in mice. Thus, the difference in gut microbiome may affect the extent of inflammation

in these mice at different facilities. Additionally, these discrepancies may also be due to the different mouse strains used to induce TNBS colitis. It is known that, C57Bl6 mice show resistance to TNBS colitis compared to other strains such as SJL and BALB/C mice (132). However, in our study setting we were able to optimize the TNBS model in C57Bl6 mice and this strain was, therefore, used for the ease of comparison between the two models of DSS vs TNBS colitis.

In the therapeutic setting with TNBS colitis model, which has not been studied before, we demonstrated that a single dose of 0.25 nmol, both the free peptide and nanomedicine form of VIP were still effective in alleviating inflammation. Both treatments were effective by improving the loss of body weight, dampening the robust increase in pro-inflammatory cytokine mRNA expression, histology and diarrheal phenotype (Figures 34-37). These effects showing lack of differences in efficacy between free and nanomedicine form of VIP were in contrast to the findings observed in a therapeutic model of DSS colitis (Figures 27-29). This discrepancy could be partly due to the extent of inflammation established in TNBS colitis with regards to the cytokine profile (Figures 30,35) which was much less robust compared to DSS colitis (Figure 23, 27). In addition, the immune response predominant in TNBS colitis model is more Th1 driven, and VIP has been shown to modulate therapeutic effects more favorably under these conditions (51). Furthermore, due to the priming of the immune system to the chemical, the inflammation developed from TNBS colitis may possibly give rise to a more systemic inflammation rather than a localized one as seen with DSS. It is also important to point out that 0.25 nmol dose of both nanomedicine and free peptide

was effective in TNBS model of colitis. However, the dose tested was same as for DSS and may not be optimal in this model to demonstrate superiority of the nanomedicine. Therefore, in CD like setting, a lower dose of the nanomedicine could be effective.

In this study we do not look at individual cellular components of the immune system in mediating alleviation of inflammation in TNBS colitis. However, previous studies have shown the anti-inflammatory action of VIP in TNBS colitis is mainly attributed to the modulation of pro-inflammatory Th1 cells. VIP mediates the balance between Th1 and Th2 effector cells and activates regulatory T cells (51). In addition, VIP has also been shown to reduce pro-inflammatory cytokines and chemokines as well as PAMP receptor TLR-2 and TLR-4 in the colonic mucosa to dampen the local inflammatory reaction (48, 77).

Overall, the studies conducted under **Specific Aim 1** demonstrated that VIP nanomedicine was effective in alleviating inflammation in colitis resembling both UC and CD forms of IBD and that 0.25 nmol dose was effective in both these settings. IBD occurs as acute flares and low grade inflammation and the main goal of treatment is to induce remission and maintain it in this stage. Therefore, we aimed to determine if VIP nanomedicine could benefit patients with severe and low grade colitis. Therapeutic models used in these studies resemble acute flares of active disease and administering VIP nanomedicine when severe damage and extensive inflammation was prevalent, demonstrated the maximal benefit. Therefore, it can be concluded that VIP nanomedicine may be more beneficial as a single dose to manage acute flares of UC. In addition, it appears that a lower

dose could be more effective in CD like colitis. Although free peptide showed therapeutic effects in mice, systemic administration of VIP in humans have shown severe hypotensive toxicity (195) and thus, precludes its clinical use and further bodes well for potential use of VIP nanomedicine as a superior therapeutic alternative.

4.4 Delineating the mechanisms of VIP-SSM nanomedicine in alleviating inflammation in colitis

The **Specific Aim 2** of this project was focused at delineating the mechanisms underlying the beneficial effects of VIP in mediating therapeutic effects in colitis. To study these effects, DSS model in a therapeutic setting was employed for the following reasons; **i)** This model demonstrated maximal beneficial effects and superiority of the nanomedicine vs the free peptide. **ii)** DSS model disrupts colonic epithelial mucosa and, therefore, assessing parameters associated with mucosal barrier and expression of epithelial proteins in this model is more favorable (196, 197)

In the current studies, the changes after VIP nanomedicine treatment in mucosal barrier (consisting of the secreted mucus and epithelial lining) and the improvement of diarrhea associated with DSS was investigated.

4.4.1 Effects of VIP-SSM on mucus secreting goblet cells and tight junction proteins

Mucosal barrier of the intestine consists of the secreted mucus and the intact epithelia lining. The secreted mucus forms a physical mesh like structure

and lines the GI epithelium. Mucus provides a barrier which prevents access of luminal microorganisms, including pathogens across the intestinal epithelium. Mucus is secreted by a specialized cell type termed as goblet cells. Goblet cells are present abundantly in the intestine. These cells have a swollen apical portion filled with mucus-laden granules which gives them a “goblet” like shape. Goblet cells synthesize and secrete mucin glycoproteins such as MUC2 and trefoil factor peptides (such as Tff3) and other molecules. The mucin, MUC2 forms polymers together with trefoil factors which result in cross-linking of the mucus to form a three-dimensional mesh like structure (124, 198).

In this regard, previous studies have reported that VIP increases mucus constituents including MUC2 and Tff3 (122, 199). In addition, under colitis conditions in VIPKO mice, VIP treatment was shown to recover the loss of goblet cell count and individual mucus proteins (84). In this regard, severe inflammation in the intestine associated with human UC and in mouse models of colitis including DSS, mucus barrier is also compromised and the number of goblet cells are severely reduced (200, 201). Therefore, studies were undertaken in a therapeutic model of DSS colitis, to determine VIP-SSM’s effects on mucus secretion by quantifying goblet cell numbers in distal colons with the use of PAS stain which specifically binds to mucus.

The Mucus secreting goblet cells were significantly down-regulated in DSS colitis (Figure 38) and treatment with VIP nanomedicine significantly alleviated the loss in goblet cells demonstrating these beneficial effects. The treatment with VIP-SSM significantly improved the number of goblet cells back to almost control levels

(Figure 38). The improvement in number of goblet cells by VIP treatment may partly be due to its anti-inflammatory effects in DSS colitis. Additionally, VIP has direct effects on increasing functional mucus secretion by transcriptionally activating MUC2 and secretion of TFF3 by molecular mechanisms secondary to intra cellular cAMP activation (122, 199). These effects may also contribute to improvement of the quality of mucus in the disease state. However, in our studies, we were unable to measure the thickness of the mucus in the colon. This is was mainly due to limitation in the processing of tissues after flushing luminal contents for other assays.

The next line of defense in the mucosal barrier, is the intact epithelial lining of the intestine. The TJ proteins, located at the apical part of the junctional complex, regulate epithelial integrity of the intestine. The TJ complex consists of several key proteins out of which, occludin is located in-between cells and forms a link between two adjacent cells in the junctional complex (202). Occludin also binds with the key cytoplasmic plaque protein zona occludens-1 (ZO-1) which in turn associates with the actin cytoskeleton of the cells (203). Therefore, both occludin and ZO-1 are key TJ proteins important in maintaining gate function of the epithelial lining. The neuropeptide VIP is known to regulate TJ proteins and improve barrier function under inflammatory conditions (76, 84). In addition, VIP has been shown to directly affect mRNA and protein expression of the TJ protein ZO-1 (65). Also, compromised barrier function has been associated with the pathogenesis of IBD, specifically with UC (204). In addition, patients with IBD and in DSS colitis model, important TJ proteins are known to be significantly down

regulated (205-207).

Therefore, DSS model in a therapeutic setting was used to explore the effects of VIP nanomedicine on TJ proteins. Occludin, a key TJ protein was selected as a model TJ protein in order to evaluate these effects (208). Total occludin mRNA and protein levels were significantly down regulated in DSS colitis (Figures 39). These effects were abrogated with both treatments of free and nanomedicine form of VIP in a therapeutic model of DSS colitis. These findings were in contrast to the observations in anti-inflammatory effects, where the free peptide showed no benefit in alleviating cytokine response in the therapeutic DSS model. It could be inferred that VIP has independent effects on the expression of TJ protein occludin, increasing the lost levels in the colonic mucosa during inflammation. Local presence of VIP at very low concentrations in the free form may have been sufficient to mediate up-regulation of occludin. However, the exact molecular mechanisms of this effect are unclear and may be similar to its up-regulation of ZO-1 via neural pathways.

It was also of interest to determine if the TJ proteins were optimally localized at the junctional complex, to determine if the integrity of the intestinal epithelium is compromised. However, when the distribution of occludin was investigated for its localization on the epithelium (Figure 40), it was evident that although the free peptide could increase the total levels, the correct localization could not be achieved. This could potentially be due to the lower dose of the free peptide present at the site of inflammation and consequently the higher grade of local inflammation present in mice treated with free VIP. The nanomedicine form

however, was able to restore correct localization of the TJ proteins both occludin (Figure 40) and ZO-1 (Figure 41) indicating the beneficial therapeutic effects and more availability of the peptide at the site, to mediate both anti-inflammatory and direct effects on TJ proteins.

4.4.2 Effect of vasoactive intestinal peptide on intestinal chloride transporter: SLC26A3

Diarrhea is a key hallmark symptom of IBD. Contributing factors to diarrhea may include, activation of secretion, reduction in absorption and a compromised barrier function of the epithelium (209). Another contributing factor to the pathogenesis of diarrhea in IBD, is the presence of high concentrations of inflammatory mediators such as pro inflammatory cytokines and chemokines in the lumen (210). In normal colonic mucosa, the absorption of salt is driven by active transporters coupled to passive movement of water. Majority of NaCl absorption is mediated by electroneutral pH coupled Na^+/H^+ and Cl^-/HCO^-_3 exchangers (211). The chloride bicarbonate exchanger SLC26A3 or DRA together with the sodium hydrogen exchanger isoform 3 (NHE3) mediate electroneutral NaCl reabsorption in the mamalian intestine.

In the colon, DRA is the predominant anion transporter and is more important under physiological and inflammatory conditions of the colon (212). The importance of DRA in normal function of the intestine is highlighted by the fact that, genetic mutations in its gene give rise to a rare genetic disorder termed as congenital chloride diarrhea (126). Similarly mice lacking DRA gene show severe

diarrhea similar to the human disorder (213). In relation to inflammatory conditions, in human IBD and in mouse models recapitulating the disease, DRA levels are significantly reduced and are associated with the accompanying diarrhea (139, 214). In this regard, previous studies have demonstrated that pro-inflammatory cytokines such as IL-1β, TNF-α and interferon (IFN)-γ have direct effects on the expression of DRA through transcriptional mechanisms (215-217). This transcriptional regulation of DRA was shown to be mediated via Signal Transducer and Activation of Transcription factor 1 (STAT-1) with IFN-γ and via nuclear factor (NF)-κB with TNF-α

Thus, it was of interest to determine if VIP had an effect on the expression of this colonic ion transporter in mediating the anti-diarrheal effects. The therapeutic model of DSS colitis was used to analyze the expression of this ion transporter across various treatment groups. The diarrheal phenotype (Figure 29) associated with DSS was directly correlated to the significant loss of both mRNA and protein levels of DRA (Figure 42). These findings were parallel to previous studies showing the direct influence of pro-inflammatory cytokines on DRA expression (215, 216). Treatment with VIP in the nanomedicine form significantly alleviated the loss of protein and mRNA levels of the transporter which may have been partly due to dampening of the robust inflammatory response at the site of inflammation. However, it was of interest to investigate if there was a direct effect of VIP (independent of its anti-inflammatory action) on the expression of this ion transporter.

To this end, cell culture models were used to delineate direct effects of VIP

on DRA. It was observed that VIP had direct effects on the protein expression of DRA (**Figure 43**). In addition, these effects seemed to be mediated by the key receptor VPAC1. Cell culture studies showed a direct effect of VIP on DRA protein expression, however, in mice receiving VIP alone, an increase in total DRA protein in the colonic mucosa was not observed. The reason for this discrepancy was not entirely clear, however it could have been possibly due to the following reasons; **i)** Since VIP-SSM nanomedicine is administered in healthy mice with no inflammation in the colon, accumulation of VIP in the colon may not have occurred as there would be no leaky vasculature. Thus, VIP may not have been able to reach the site at required dose. **ii)** In healthy mice, baseline expression level of DRA *in vivo* may not have been upregulated by VIP, as its expression is quite high in healthy colon. However, these effects were evident in cell lines since in differentiating cancer cells the baseline levels of DRA are quite low and thus up-regulation of DRA by VIP was possible. Under inflammatory conditions, since baseline levels *in vivo*, are down-regulated, the restoration of DRA protein was remarkable and could be partly attributed to the direct effects of VIP mediated by the luminal VPAC1 receptors.

Additionally, previous studies have demonstrated that VIP can upregulate the transcription factor caudal related homeobox (CDX)2 which is highly expressed in the intestine and is involved in differentiation of cells (84). In line with that, studies have shown upregulation of DRA expression by CDX2 (218). Therefore, it can be inferred that VIP may have had direct effects on CDX2 levels, which in turn may have upregulated the expression of DRA. However, further detailed studies

are required to identify exact mechanisms underlying these direct effects of VIP.

It should be noted that, VIP is a well-known pro-secretory agent in the small intestine, which is known to cause diarrhea, by increasing the chloride channel expression and function, namely Cystic Fibrosis Transmembrane Regulator (CFTR) (219-221). However, in our studies, we did not observe induction of diarrhea when VIP nanomedicine was administered to healthy animals. Additionally, in the colon, we observed an overall anti-diarrheal effect of VIP-SSM under colitis conditions. This may be partly due to the localized effects of VIP nanomedicine in the colon.

Overall, in **Specific Aim 2** we demonstrated that VIP nanomedicine could mediate therapeutic effects in DSS colitis at a single dose of 0.25 nmol. Apart from its overall anti-inflammatory action and effect on dampening the robust local inflammation, VIP also increased mucosal defense by increasing the lost goblet cell counts and TJ protein occludin expression. In addition, VIP reduced the diarrheal phenotype associated with DSS by partly alleviating the down-regulated ion transporter protein, DRA expression and thus, anti-diarrheal effects in colitis. Therefore, in acute flares of colitis, patients receiving VIP nanomedicine at a single dose, may have additional therapeutic benefits. Many of these effects maybe secondary to its anti-inflammatory effects, however, with the literature supporting direct effects of VIP on these individual parameters there could be direct effects as well.

4.5 Determining the potential usage of vasoactive intestinal peptide nanomedicine as an oral formulation

The purpose of the final aim of this project was to determine the feasibility of using VIP nanomedicine as an oral product. The oral route is the most physiological and patient-friendly due to its ease of administration (222). Out of the marketed dosage forms available in the clinics today, over 60% are orally administered (223). In the past few decades, peptide drugs have gained attention as therapeutic agents for their high specificity, potency and relatively low toxicity (224, 225). However, due to their high susceptibility to enzymatic degradation and low oral bioavailability, peptides are mostly administered by injection. Due to these limitations, there are only a very few peptide drugs available in the market as oral dosage forms. Some of the available drugs include, cyclic peptides such as Liaclotide (Linzess ®, Allergan), Plecanatide (Trulance ®, Synergy) and antibiotics such as Vancomycin (Vancocin ®) (226).

There have also been some attempts to deliver peptides as nanomedicines via oral route. In this regard, Insulin (227, 228), GLP-1 (229) and a few other peptides have been used as model peptide drugs to be delivered for systemic effects (TABLE V). In addition, notable efforts have also been taken to deliver peptide nanomedicines for local management of diseases in the GI tract (117, 230, 231). However, there is still an urgency to deliver peptides via oral route to improve their clinical use (232).

Therefore, in the current studies, we tested the potential of our biocompatible nanomedicine VIP-SSM, to be used as an oral formulation to

manage a local disease of the intestine. Our rationale was not only to develop an oral peptide product for ease of administration, but also for readily accessing the inflamed GI tissues in IBD, via enteral route. Therefore, prior to conducting *in vivo* studies there was a requirement to investigate the expression of VIP receptors in the GI tract, to understand if targeting could be possible via luminal delivery. We demonstrated that VIP receptors were expressed in the intestine, specifically on the luminal wall of the colon, which could be directly accessed when the peptide is delivered orally to the lumen (see results sections 3.4.1). Once we demonstrated the expression of VIP receptors at the right location, our next goal was to evaluate if VIP-SSM in solution when directly applied to the colon by instillation via intra rectal route shows therapeutic effects. *In vivo* efficacy studies showed therapeutic benefit of the nanomedicine and not the free peptide at the same dose when instilled directly to the colon in colitis (see results section 3.4.2.1).

Therefore, we next aimed to deliver the nanomedicine in a more stable and favorable oral formulation. In order to be delivered orally, our peptide nanomedicine needed to bypass the harsh conditions of the stomach, protected from digestion and reach the site of action (colon) in the active nanomedicine form. Our previous findings had already demonstrated the freeze-drying capability of VIP-SSM without any lyo or cryo protectants (101). Furthermore, there are commercially available enteric coated capsules capable of withstanding acidity of the stomach and specifically dissolving at higher colonic pH. Thus, we used these capsules to overcome the stomach conditions and specifically release our nanomedicine at the target colonic environment. In the next set of studies, we filled

the freeze-dried VIP-SSM in enteric coated capsules and evaluated its feasibility to release active VIP at its required dose for humans and micelles at their original size.

If an oral peptide product is introduced to the pharmaceutical market, based on our technology described above, it will have a great impact as the first of its kind. Our long-term goal for conducting these studies was not only to develop an oral peptide product for VIP, but also provide a platform for other peptides which can successfully associate with SSM to be delivered via the same means (111-113).

4.5.1 Expression of vasoactive intestinal peptide receptors in the human and mouse intestine

The following results have already been published and is used with permission from reference (37).

“The relative abundance of VIP receptors and its specific localization in the intestinal mucosa was not well understood. Our results show comparable results to previous studies on the existence of VIP receptors in the intestine (43, 233-236). In addition, it addresses the gap in knowledge of the specific receptor sub-types of VIP in the intestinal mucosa and opens-up investigation into the physiological relevance of these receptors.”

“The key findings were that the main receptor of VIP along the length of the mouse intestinal mucosa was VPAC1 and that the mRNA expression of VPAC1 was almost 300-times higher than the other receptors (Figure 44). In addition, the expression was higher in the colon as compared to jejunum and ileum. However,

in the human, VPAC1 was mostly expressed in the sigmoid colon (~4 fold) compared to other regions and it was similar in the jejunum, ileum and ascending colon (Figure 45). The dissimilarity in expression pattern could be due to the RNA extracted from mucosal scrapings in mice and whole tissues in the human, this was potentially due to other VIP receptors being present in layers of the intestine from mucosa to muscle layers as compared to the mucosa alone. It could also be due to the difference in species. Protein data in mice also showed similar results to mRNA with higher levels of VPAC1 in the colon (Figure 44). The most intriguing result was the atypical, apical localization of VPAC1 in the colonic epithelium (Figures 46). This was in contrast to previous studies, where radiolabeled VIP binding was indicated to be predominant in the basolateral membranes in the rat jejunum and rabbit ileum (237).” Additionally, in a therapeutic model of DSS colitis, we observed that VPAC1 receptors in the colon were not significantly changed due to colitis (Figure 54,55). This further demonstrated the feasibility of conducting studies with local VIP administration to manage colitis.

“The controversial finding of VPAC1 localization led us to undertake antibody authentication experiments, which would aid in validating our findings. This specific antibody raised in rabbit (PA3-113) have been used by other investigators in human and rodent models (238-240). However, to determine the specificity of the antibody in recognizing the protein of interest in human and mice tissues, we performed peptide competition assay. In addition, we used tissues from mice known to express less VPAC1 (kidney) as a negative control (241) and validated the results by performing qPCR on RNA and then confirmed this finding

by western analysis showing comparable results. ”

“Previous studies have provided valuable hints to the possible existence of VIP receptors in the epithelial cells and its potential presence on the apical membrane. Namely the radio-iodinated VIP binding studies in human colon sections demonstrated specific high binding of radiolabeled VIP to the colonic mucosa as compared to other parts in tissue cross sections, indicating existence of VIP binding through receptors to the mucosa (235). In addition, multiple studies performed by Laburthe *et al.* utilizing radiolabeled VIP indicated the existence of VIP receptors on epithelial cells isolated from intestinal mucosa from rats and humans (43, 236, 242). With the advent of specific receptor sub-type identification, another previous study utilized a radiolabeled analog of VIP with higher affinity for VPAC2 receptor (VPAC2 agonist) and showed minimal binding in the colonic epithelium (243). This was consistent with our data demonstrating a significantly less expression of VPAC2 as compared to VPAC1 in the colon.”

“The abundance and luminal localization of VPAC1 in colons of mice and humans may indicate that some of the key functions of VIP such as effects on epithelial regeneration (244), ion transport (60), fluid secretion (245), mucus secretion (62) and tight junction protein expression (65) can be directly mediated through these luminal receptors. The colonic epithelium undergoes constant regeneration and VIP affects epithelial regeneration and mucus secretion in mucosa including the intestine which could indicate that VIP may affect these parameters in the colon. The specific apical localization of VPAC1 also widens possibilities to target neuropeptide therapies to the colon, once administered

luminally. Previously, we and others have used VIP receptors as a targeting modality to various cancer cell populations (246-249). Being an immunomodulatory agent, VIP can also be successfully used to ameliorate colitis (73). If therapies with VIP can be administered via oral route, the apical receptors would directly participate in the mediation of the anti-inflammatory and regenerative effects of VIP for reducing the disease pathology. The abundance of VPAC1 receptors in the lumen of the colon can be utilized to deliver VIP in drug delivery systems which resists the acidity of the stomach, and is preferentially released at the colon." Therefore, as a next step *in vivo* local administration of VIP nanomedicine was performed in a therapeutic model of DSS colitis to determine the therapeutic effects of locally instilled VIP nanomedicine in alleviating colitis.

4.5.2 *In vivo* efficacy of locally administered VIP-SSM nanomedicine in alleviating colitis

In these studies, therapeutic DSS model was used to explore the potential benefit of locally administered VIP nanomedicine to alleviate inflammation. A dose of 3.5 % w/v DSS was administered in drinking water to mice for 7 days as before, then mice were switched to tap water and kept for the next 5 days **(**Figure 17). Treatments groups were kept similar to the therapeutic ip study protocol with the dose kept same at 0.25 nmol (TABLE VI). However, the intra colonic treatment via intra rectal instillation was performed at day 5 while DSS was still given to mice. This was different from the study design used in the therapeutic model of DSS colitis with ip administration where best therapeutic effects of VIP nanomedicine were observed. However, due to practical concerns, as instilling treatments to an

already damaged colon could pose a risk of potential rupture of the colonic wall, day 5 was used instead of day 8 in this study. The early instillation of treatments at day 5 could be a drawback of the study, due to severe inflammation not being established on the day of treatment instillation to the colon. However, comparative results to the ip study were observed with local instillation including, improved body weight, reduced local inflammation and improved distal colonic histology (Figures 47-50).

These positive effects of VIP nanomedicine could have been mediated mainly due to the capacity of the nanomedicine to specifically accumulate at the inflamed tissues and reach into the lamina propria to interact with immune cells. Additional benefits which could potentially be mediated by the luminal receptors such as improvement in goblet cell number (Figure 52), and ion transporter expression (Figure 53) were also apparent with the nanomedicine and not with the free peptide. These findings support our hypothesis that VIP nanomedicine could preferentially adhere and accumulate at the site of inflammation, bind to its receptors on the luminal membranes and immune cells and mediate therapeutic action.

There are several reasons why the nanomedicine has better effects than the free peptide after luminal delivery. **i)** Nanoparticles are known to adhere and retain at the site of inflammation better by persorption (106). **ii)** The size of the nanomedicine allows passive accumulation at the damaged intestinal mucosa by epithelial retention effect (108). **iii)** Finally, the PEG present on the surface of the nanomedicine may allow mucus penetration and interaction with VIP receptors in

the colonic mucosa (250). However, the retentive capacity of the free peptide is less due to potentially being flushed out by diarrhea and associated movements in the inflamed intestine, instability due to bacterial and enzymatic cleavage and therefore, would not reach the target receptors at the required dose. Although intra colonic instillation of a single dose of 0.25 nmol of VIP in nanomedicine form showed superior effects to free peptide we did not observe a significant difference to the studies conducted with ip treatment administration. Since a dose of 0.25 nmol is given directly to the site of inflammation, VIP receptor saturation may have occurred and theoretically, beneficial effects could be mediated even at a lower dose. We believe since there was a difference in the study designs (DSS therapeutic ip vs intra colonic) based on the day in which treatments were administered, direct comparison of the two studies with regards to the dose and effect is complicated. However, the results were quite similar to the ip study of therapeutic DSS model at the same dose of 0.25 nmol and may show the possible local effects that could be mediated by the ip route of administration, making it similar to the local instillation.

These studies provided comprehensive evidence of the potential usage of VIP-SSM nanomedicine as a locally administered nanomedicine to alleviate colitis. Ulcerative colitis is currently managed with enema solutions with active drugs. In this regard, these studies show effectiveness of using VIP nanomedicine solution by local instillation. However, to manage a GI disease with an oral formulation would be the best way to improve patient compliance. Therefore, in the next set of studies, it was of interest to show the feasibility of the use of VIP nanomedicine in

a clinically applicable dosage form for oral use.

4.5.3 *In vitro* evaluation of the scaled-up nanomedicine as an oral solid dosage form

Peptide nanomedicines with SSM have an additional advantage in that, they can be successfully freeze-dried without the addition of cryo and lyo protectants (101). Peptide associated nanomedicines withstand the freeze-drying process and reform micelles upon dissolution without significantly affecting the peptide-micellar interaction. Therefore, during this phase, it was of interest to determine if a formulation of VIP-SSM can be successfully freeze-dried and incorporated in colon specific release capsules. In addition, the formulated capsules could subsequently release the active peptide and form micelles once dissolved in the colon.

The required VIP dose to be filled in the capsule was calculated based on a formula on allometric scaling, which is an empirical approach where the conversion of drug dose is based on normalization of dose to body surface area. Because larger animals have lower metabolic rates and slower physiological processes, the dose required per kilogram is smaller. Although allometric scaling is used frequently for dose conversions among species, it may not be the best method, however, we employed this method due to its ease of usage and frequent usage in interspecies dose conversions. The correction factor for Human Equivalent Dose (HED) is calculated for each species by dividing the average body weight of the organism by its body surface area and for mice this value is 12.3 (168). Once the mouse to human dose was determined, the concentration of the

peptide in the colon was calculated based on the lowest volume which is known to be present in the colon (10 mL). This allowed incorporation of the highest VIP dose as mentioned in section 2.11.1. However, when the release studies were performed in the laboratory scale, the maximum volume in the colon of 30 mL was used to allow complete immersion of the capsule in the fluid. As shown in Figure 56,, capsules could be formulated and filled with uniformity of weight (TABLE X).

Once prepared the capsules were dissolved in simulated colonic pH to determine if micelles are formed and active VIP is released from capsules. Formation of micelles were tested with the aid of DLS to determine if the solution contained micelles and the amount of active VIP was determined by analyzing VIP content by ELISA. However, it would have been interesting to assess the presence of peptides in micellar form by determining its secondary structure in the solution as we did previously (101). We had demonstrated that VIP in SSM had higher alfa helicity compared to free peptide in solution (random coil). One way to analyze peptide association with micelles thus, increase in alfa helicity in this setting would be to conduct circular dichroism (CD) studies which could aid in determining the secondary structure of the peptide in the product. However, this method was not feasible to be performed to determine the peptide association with micelles due to the possible interference from the capsule polymer composition. Since, high purity of the sample is required for CD analysis (251). Therefore, indirect methods of size and activity was used to detect micelles in solution with active VIP.

The different batches of capsule formulations gave similar results. They all dissolved in colonic pH to give similar VIP release and particles in the size range

of micelles (TABLE XI). VIP was active as assessed by VIP ELISA (Figures 58). For stability studies, the capsules were stored in air tight glass containers saturated with an inert gas in dark to try and mimic the blister pack wrapping. Capsules stored over time at 4^0 C for 6 weeks, showed similar dissolution and release patters as determined by ELISA and, therefore, showed retention of VIP's activity, for up to at least 6 weeks (Figure 60). These studies showed promising findings and potential for developing VIP-SSM as an oral formulation with favorable activity. However, it is worthwhile to perform longer term storage studies of these capsules.

We believe the peptide was still associated with micelles due to the following possible reasons; **i)** The labile peptide is known to degrade rapidly in solution by autolysis at physiological pH following first order kinetics giving rise to multiple degradation products of inactive nature (170, 171). Therefore, during the dissolution process, carried out at 37^0 C under light, we believe that the free peptide would be degraded and the peptide associated with the micelles would resists autolysis to retain its activity. Therefore, detection of known concentrations of the peptide by ELISA indicated its presence in the micellar form. **ii)** The capsules used in these studies are composed of polymers, hypermellose phthalate (HPMCP) and hydroxyl propyl methyl cellulose (HPMC), these are considered FDA grade inactive ingredients. Additionally, these polymers are rapidly disintegrated at pH 6 and above, and has been shown not to interact with active ingredients, including peptides (252, 253). Therefore, the presence of polymers in solution may have minimal interference with peptide associating with micelles.

To demonstrate the presence of VIP in micellar form, a comparative study

was performed to detect if, loss of VIP by autolysis could be detected with ELISA, once released from capsules after dissolution. Figure 59 clearly indicates the degradation of the free peptide released from capsules compared to the peptide associated with SSM. The stability of the peptide in SSM confirms the association of VIP with SSM thus, resisting autolysis associated with the free peptide. The rate of autolysis is faster at dilute concentrations and can be attributed to the release profile of free VIP from 20-30 minutes showing a rapid drop in total levels. It could also be explained by the fact that VIP at lower concentrations, i.e. 0.4 μM, is in the monomeric form and not in its micellar form which is more stable (254). Therefore, the degradation observed becomes less pronounced at higher concentrations. Overall, the data above provide evidence of the association of VIP with SSM after dissolution from capsules. Since, the same dose of VIP released from capsules do not give rise to the correct amounts of VIP, the peptide in solution can only resist autolysis if present in the micellar form.

In summary, we have demonstrated the potential of a peptide nanomedicine with immunomodulatory properties to manage IBD in patients suffering from both severe and low grade inflammation. In addition, being an endogenous peptide which is depleted during the disease, VIP-SSM nanomedicine can be used to deliver active VIP to the target site with minimal side effects. Finally, the formulation is versatile in its capacity to be effective both as a systemic parenteral formulation and a locally delivered oral formulation. Furthermore, pediatric and geriatric patients with colitis are unable to administer drugs orally, the intra colonic instillation in these patients will be a feasible method for treatment. Additionally,

these data indicate the potential feasibility of delivering a peptide as nanomedicine in dry form, in a capsule by oral route, which is not achieved so far. This method has the potential for other peptide drugs to be delivered orally in a similar manner.

5. CONCLUSIONS

1. The nanomedicine, VIP-SSM can be prepared reproducibly by simple dissolution forming nanoparticles with a size of ~15 nm. The nanomedicine and free peptide elicit similar biological responses *in vitro* by increasing the intracellular cAMP levels in HT29 cell line. This shows that association of VIP with SSM nanocarrier does not change the biological activity of the peptide.

2. In the experiments conducted with DSS colitis, VIP and VIP-SSM nanomedicine were both effective in alleviating inflammation, when 0.25 nmol was administered on alternate days in a preventive manner. However, when severe inflammation was established, and a single dose of 0.25 nmol of the peptide or nanomedicine was administered to DSS mice, therapeutic effects were only mediated by VIP-SSM. This highlights the protective role of the nanocarrier (SSM), in avoiding degradation and instability of VIP and improving its accumulation at the site of inflammation. Therefore, VIP-SSM as a single dose of 0.25 nmol was most effective in alleviating severe inflammation associated with DSS.

3. Similar to studies with DSS colitis, VIP-SSM showed anti-inflammatory effects in TNBS colitis both in a preventive and therapeutic manner at a dose of 0.25 nmol. However, unlike DSS colitis, in TNBS colitis the therapeutic effects at 0.25 nmol dose was similar in both free VIP and VIP-

SSM. Therefore, due to the difference in the disease, in TNBS colitis 0.25 nmol dose is not optimal to demonstrate beneficial effects of the nanomedicine over the free peptide.

4. Neuropeptide hormone VIP is highly secreted from the intestine, and have demonstrated multiple beneficial effects on GI mucosa. In the therapeutic model of colitis associated with DSS, it was evident that VIP-SSM affected additional parameters such as improvement of goblet cell number and expression and distribution of tight junction protein occludin. These could improve epithelial barrier integrity and thus aid in recovery from colitis. In addition, VIP nanomedicine improved diarrheal phenotype and alleviated the loss in expression of the chloride transporter DRA in the colon. These effects could be mediated indirectly by VIP's anti-inflammatory action and directly by the activation of the luminal VIP receptor in the colon.

5. VPAC1 was the predominantly expressed receptor in the GI mucosa in mice. VPAC1 had atypical apical localization in the human and mouse colon and demonstrated high abundance in the colon compared to the small intestine. Additionally, in a therapeutic model of DSS colitis, the expression of VPAC1 was not down-regulated showing the potential of it being used as a target for VIP delivery to colon during colitis.

6. The presence of apical receptors facilitated local delivery of the VIP-SSM to the colon to treat inflammation. Similar to the findings with systemic ip administration, VIP-SSM alleviated inflammation and associated diarrhea with a dose of 0.25 nmol administered locally to the colon in a therapeutic model of DSS colitis. Additionally, beneficial effects such as recovering the loss of goblet cell number and DRA ion transporter levels were only observed with VIP-SSM and not the free peptide in DSS mice. Therefore, when administered locally, VIP-SSM nanomedicine was superior over the free peptide due to its capacity to resist flushing, adhere and penetrate mucus and access local tissues and lamina propria immune cells to mediate effects.

7. Locally delivered dose of VIP-SSM in mice could be successfully converted to equivalent human dose with the aid of a pharmacokinetic formula. This formulation of VIP-SSM was successfully freeze-dried and filled uniformly into commercially available enteric coated capsules. The capsules released active VIP and reformed micelles after dissolving in colonic pH. The stability of the capsules assessed by ELISA retained for at least 6 weeks when stored at 4 ^{0}C. Therefore, freeze-dried nanomedicine in enteric coated capsules show promise as a feasible oral preparation.

6. FUTURE DIRECTIONS

1. The nanomedicine VIP-SSM, demonstrated significant improvement of colitis associated signs and symptoms in DSS and TNBS colitis which resembles human UC and CD. Although these effects were studied in an acute model of both DSS and TNBS colitis it may be of interest to determine if therapy would be beneficial in managing chronic inflammation using chronic models of both DSS and TNBS colitis.

2. The optimal dose range of the nanomedicine can be determined by testing doses above and below the tested dose in the therapeutic models. To supplement these studies real time measurement of inflammation can be assessed by *in vivo* imaging using a luminescence probe or by fecal lipocalin-2 detection. This would aid in monitoring the level of local inflammation during the progression of the disease and effectiveness of the nanomedicine before the end-point of the study.

3. The effects mediated by VIP, apart from its anti-inflammatory action including effects on tight junction proteins and mucus secretion can further be explored by adding functional studies. The epithelial permeability can be measured with the aid of (Fluorescein isothiocyanate) FITC dextran assay and thesecretedmucus layer can be determined after fixing unflushed total colons in carnoy's fixative.

4. Intestinal inflammation is regulated by many factors, and in this regard, the gut microbiome has gained much attention. Therefore, it may be interesting to analyze the composition of the gut microbiome of mice with colitis and compare them to VIP treated mice. This may provide insights into the capability of the peptide to modulate the gut microbiome and thereby alleviate inflammation.

5. *In vitro* cell culture studies conducted with Caco2 cell monolayers demonstrated that VIP can directly increase the protein expression of the ion transporter DRA and that it was mediated by its specific receptor VPAC1. Further detailed studies can be conducted in cell lines and in intestinal organoids to delineate the exact molecular mechanisms of these effects.

6. The capsule formulation prepared, can be administered to larger animals capable of swallowing a capsule (dog or monkey), after tailoring the dose to test the *in vivo* release of the capsule contents to mediate therapeutic effects in colitis. Additionally, studies can be conducted to visually detect the accumulation of the nanomedicine in the inflamed colon in diseased state. After completion of these studies optimization of the solid oral nanomedicine can be conducted to determine long term stability of the capsules.

7. Since VIP-SSM can be a potential treatment option for managing IBD, it should be compared for its comparative efficacy with a current mainstay biological treatment such as anti-TNFα antibody to show its clinical potential. Once these studies show potential, this nanomedicine can be used as a novel therapeutic agent to manage IBD.

REFERENCES

1. Loftus Jr EV. Update on the incidence and prevalence of inflammatory bowel disease in the United States. Gastroenterology & hepatology. 2016;12(11):704.
2. Sartor RB. Mechanisms of disease: pathogenesis of Crohn's disease and ulcerative colitis. Nature clinical practice Gastroenterology & hepatology. 2006;3(7):390-407.
3. Al-Mofarreh MA, Al-Mofleh IA. Emerging inflammatory bowel disease in saudi outpatients: a report of 693 cases. Saudi journal of gastroenterology: official journal of the Saudi Gastroenterology Association. 2013;19(1):16.
4. Baars J, Kuipers E, van Haastert M, Nicolaï J, Poen A, van der Woude C. Age at diagnosis of inflammatory bowel disease influences early development of colorectal cancer in inflammatory bowel disease patients: a nationwide, long-term survey. Journal of gastroenterology. 2012;47(12):1308-22.
5. Bernstein CN, Fried M, Krabshuis J, Cohen H, Eliakim R, Fedail S, et al. World Gastroenterology Organization Practice Guidelines for the diagnosis and management of IBD in 2010. Inflammatory bowel diseases. 2010;16(1):112-24.
6. de Lange KM, Barrett JC. Understanding inflammatory bowel disease via immunogenetics. Journal of autoimmunity. 2015;64:91-100.
7. Hendrickson BA, Gokhale R, Cho JH. Clinical aspects and pathophysiology of inflammatory bowel disease. Clinical microbiology reviews. 2002;15(1):79-94.
8. Xavier R, Podolsky D. Unravelling the pathogenesis of inflammatory bowel disease. Nature. 2007;448(7152):427-34.
9. Kappelman MD, Rifas–Shiman SL, Kleinman K, Ollendorf D, Bousvaros A, Grand RJ, et al. The prevalence and geographic distribution of Crohn's disease and ulcerative colitis in the United States. Clinical Gastroenterology and Hepatology. 2007;5(12):1424-9.
10. Hart AL, Ng SC. Crohn's disease. Medicine. 2011;39(4):229-36.
11. Lennard-Jones J. Classification of inflammatory bowel disease. Scandinavian Journal of Gastroenterology. 1989;24(sup170):2-6.
12. Lees C, Barrett J, Parkes M, Satsangi J. New IBD genetics: common pathways with other diseases. Gut. 2011;60(12):1739-53.
13. Neurath MF. Cytokines in inflammatory bowel disease. Nature Reviews Immunology. 2014;14(5):329-42.
14. Love BL. Management of patients with inflammatory bowel disease: current and future treatments. Stroke. 2018;13:57.
15. Riley S, Turnberg L. Sulphasalazine and the aminosalicylates in the treatment of inflammatory bowel disease. QJM: An International Journal of Medicine. 1990;75(3):551-62.
16. Boardman P, Hart FD. Clinical measurement of the anti-inflammatory effects of salicylates in rheumatoid arthritis. British Medical Journal. 1967;4(5574):264.
17. Lim W-C, Hanauer S. Aminosalicylates for induction of remission or response in Crohn's disease. Cochrane Database Syst Rev. 2010;12.

18. Ford AC, Bernstein CN, Khan KJ, Abreu MT, Marshall JK, Talley NJ, et al. Glucocorticosteroid therapy in inflammatory bowel disease: systematic review and meta-analysis. The American journal of gastroenterology. 2011;106(4):590.
19. Seinen ML, Ponsioen CY, de Boer NK, Oldenburg B, Bouma G, Mulder CJ, et al. Sustained clinical benefit and tolerability of methotrexate monotherapy after thiopurine therapy in patients with Crohn's disease. Clinical Gastroenterology and Hepatology. 2013;11(6):667-72.
20. Khan KJ, Ullman TA, Ford AC, Abreu MT, Abadir A, Marshall JK, et al. Antibiotic therapy in inflammatory bowel disease: a systematic review and meta-analysis. The American journal of gastroenterology. 2011;106(4):661.
21. Fidder H, Schnitzler F, Ferrante M, Noman M, Katsanos K, Segaert S, et al. Long-term safety of infliximab for the treatment of inflammatory bowel disease: a single-centre cohort study. Gut. 2009;58(4):501-8.
22. Reinisch W, Hommes DW, Van Assche G, Colombel J-F, Gendre J-P, Oldenburg B, et al. A dose escalating, placebo controlled, double blind, single dose and multidose, safety and tolerability study of fontolizumab, a humanised anti-interferon γ antibody, in patients with moderate to severe Crohn's disease. Gut. 2006;55(8):1138-44.
23. Hueber W, Sands BE, Lewitzky S, Vandemeulebroecke M, Reinisch W, Higgins PD, et al. Secukinumab, a human anti-IL-17A monoclonal antibody, for moderate to severe Crohn's disease: unexpected results of a randomised, double-blind placebo-controlled trial. Gut. 2012:gutjnl-2011-301668.
24. Sandborn WJ, Feagan BG, Rutgeerts P, Hanauer S, Colombel J-F, Sands BE, et al. Vedolizumab as induction and maintenance therapy for Crohn's disease. New England Journal of Medicine. 2013;369(8):711-21.
25. Said SI. Vasoactive intestinal peptide. Journal of endocrinological investigation. 1986;9(2):191-200.
26. Vuković L, Madriaga A, Kuzmis A, Banerjee A, Tang A, Tao K, et al. Solubilization of therapeutic agents in micellar nanomedicines. Langmuir. 2013;29(51):15747-54.
27. Delgado M, Abad C, Martinez C, Juarranz M, Arranz A, Gomariz R, et al. Vasoactive intestinal peptide in the immune system: potential therapeutic role in inflammatory and autoimmune diseases. Journal of molecular medicine. 2002;80(1):16-24.
28. Fuxe K, Hökfelt T, Said S, Mutt V. Vasoactive intestinal polypeptide and the nervous system: immunohistochemical evidence for localization in central and peripheral neurons, particularly intracortical neurons of the cerebral cortex. Neuroscience Letters. 1977;5(5):241-6.
29. Konturek S, Pucher A, Radecki T. Comparison of vasoactive intestinal peptide and secretin in stimulation of pancreatic secretion. The Journal of physiology. 1976;255(2):497-509.
30. Davidson A, Moody TW, Gozes I. Regulation of VIP gene expression in general. Journal of Molecular Neuroscience. 1996;7(2):99-110.
31. JEFFREY MULCHAHEY GNJ, NEILL JD. Autocrine control of prolactin secretion by vasoactive intestinal peptide. Endocrinology. 1988;122(1):364-6.

32. Kawashima K, Yoshikawa K, Fujii YX, Moriwaki Y, Misawa H. Expression and function of genes encoding cholinergic components in murine immune cells. Life sciences. 2007;80(24):2314-9.
33. Martinez C, Delgado M, Abad C, Gomariz RP, Ganea D, Leceta J. Regulation of VIP production and secretion by murine lymphocytes. Journal of neuroimmunology. 1999;93(1):126-38.
34. Cutz E, Chan W, Track NS, GOTH A, SAID SI. Release of vasoactive intestinal polypeptide in mast cells by histamine liberators. Nature. 1978;275(5681):661-2.
35. Bellinger DL, Lorton D, Brouxhon S, Felten S, Felten DL. The significance of vasoactive intestinal polypeptide (VIP) in immunomodulation. Advances in neuroimmunology. 1996;6(1):5-27.
36. Bell GI. The glucagon superfamily: precursor structure and gene organization. Peptides. 1986;7:27-36.
37. Jayawardena D, Guzman G, Gill RK, Alrefai WA, Onyuksel H, Dudeja PK. Expression and localization of VPAC1, the major receptor of vasoactive intestinal peptide along the length of the intestine. American Journal of Physiology-Gastrointestinal and Liver Physiology. 2017;313(1):G16-G25.
38. Laburthe M, Couvineau A, Tan V. Class II G protein-coupled receptors for VIP and PACAP: structure, models of activation and pharmacology. Peptides. 2007;28(9):1631-9.
39. Solano RM, Langer I, Perret J, Vertongen P, Juarranz MG, Robberecht P, et al. Two basic residues of the h-VPAC1 receptor second transmembrane helix are essential for ligand binding and signal transduction. Journal of Biological Chemistry. 2001;276(2):1084-8.
40. Harmar AJ, Arimura A, Gozes I, Journot L, Laburthe M, Pisegna JR, et al. International Union of Pharmacology. XVIII. Nomenclature of receptors for vasoactive intestinal peptide and pituitary adenylate cyclase-activating polypeptide. Pharmacological reviews. 1998;50(2):265-70.
41. Reubi JC, Läderach U, Waser B, Gebbers J-O, Robberecht P, Laissue JA. Vasoactive intestinal peptide/pituitary adenylate cyclase-activating peptide receptor subtypes in human tumors and their tissues of origin1. Cancer Research. 2000;60(11):3105-12.
42. Couvineau A, Rouyerfessard C, Darmoul D, Maoret J-J, Carrero I, Ogierdenis E, et al. Human intestinal VIP receptor: cloning and functional expression of two cDNA encoding proteins with different N-terminal domains. Biochemical and biophysical research communications. 1994;200(2):769-76.
43. Salomon R, Couvineau A, Rouyer-Fessard C, Voisin T, Lavallée D, Blais A, et al. Characterization of a common VIP-PACAP receptor in human small intestinal epithelium. American Journal of Physiology-Endocrinology And Metabolism. 1993;264(2):E294-E300.
44. Laburthe M, Couvineau A. Molecular pharmacology and structure of VPAC receptors for VIP and PACAP. Regulatory peptides. 2002;108(2):165-73.
45. Gonzalez-Rey E, Chorny A, Delgado M. Regulation of immune tolerance by anti-inflammatory neuropeptides. Nature Reviews Immunology. 2007;7(1):52.

46. Delgado M, Ganea D. Inhibition of endotoxin-induced macrophage chemokine production by VIP and PACAP in vitro and in vivo. Archives of physiology and biochemistry. 2001;109(4):377-82.
47. Arranz A, Abad C, Juarranz Y, Torroba M, Rosignoli F, Leceta J, et al. Effect of VIP on TLR2 and TLR4 Expression in Lymph Node Immune Cells During TNBS-Induced Colitis. Annals of the New York Academy of Sciences. 2006;1070(1):129-34.
48. Gomariz RP, Arranz A, Abad C, Torroba M, Martinez C, Rosignoli F, et al. Time-course expression of Toll-like receptors 2 and 4 in inflammatory bowel disease and homeostatic effect of VIP. Journal of leukocyte biology. 2005;78(2):491-502.
49. Gomariz R, Arranz A, Juarranz Y, Gutierrez-Canas I, Garcia-Gomez M, Leceta J, et al. Regulation of TLR expression, a new perspective for the role of VIP in immunity. Peptides. 2007;28(9):1825-32.
50. Delgado M, Gonzalez-Rey E, Ganea D. VIP/PACAP preferentially attract Th2 effectors through differential regulation of chemokine production by dendritic cells. The FASEB journal. 2004;18(12):1453-5.
51. Delgado M. VIP: a very important peptide in T helper differentiation. Trends in immunology. 2003;24(5):221-4.
52. Jimeno R, Leceta J, Martínez C, Gutiérrez-Cañas I, Pérez-García S, Carrión M, et al. Effect of VIP on the balance between cytokines and master regulators of activated helper T cells. Immunology & Cell Biology. 2012;90(2):178-86.
53. Biancani P, Walsh J, Behar J. Vasoactive intestinal polypeptide. A neurotransmitter for lower esophageal sphincter relaxation. Journal of Clinical Investigation. 1984;73(4):963.
54. Wiley JW, O'dorisio T, Owyang C. Vasoactive intestinal polypeptide mediates cholecystokinin-induced relaxation of the sphincter of Oddi. Journal of Clinical Investigation. 1988;81(6):1920.
55. Krejs GJ, Fordtran JS, Fahrenkrug J, Schaffalitzky dMO, Fischer J, Humphrey C, et al. Effect of VIP infusion in water and ion transport in the human jejunum. Gastroenterology. 1980;78(4):722-7.
56. Coupar I. Stimulation of sodium and water secretion without inhibition of glucose absorption in the rat jejunum by vasoactive intestinal peptide (VIP). Clinical and Experimental Pharmacology and Physiology. 1976;3(6):615-8.
57. Crist J, He X, Goyal R. Both ATP and the peptide VIP are inhibitory neurotransmitters in guinea-pig ileum circular muscle. The Journal of Physiology. 1992;447:119.
58. Fox-Threlkeld J, Manaka H, Manaka Y, Cipris S, Daniel E. Stimulation of circular muscle motility of the isolated perfused canine ileum: Relationship to VIP output. Peptides. 1991;12(5):1039-45.
59. Grider J, Makhlouf G. Colonic peristaltic reflex: identification of vasoactive intestinal peptide as mediator of descending relaxation. American Journal of Physiology-Gastrointestinal and Liver Physiology. 1986;251(1):G40-G5.
60. Racusen LC, Binder H. Alteration of large intestinal electrolyte transport by vasoactive intestinal polypeptide in the rat. Gastroenterology. 1977;73(4 I):790-6.

61. Ballantyne GH, Goldenring JR, Fleming FX, Rush S, Flint JS, Fielding LP, et al. Inhibition of VIP-stimulated ion transport by a novel Y-receptor phenotype in rabbit distal colon. American Journal of Physiology-Gastrointestinal and Liver Physiology. 1993;264(5):G848-G54.
62. Plaisancié P, Barcelo A, Moro F, Claustre J, Chayvialle J-A, Cuber J-C. Effects of neurotransmitters, gut hormones, and inflammatory mediators on mucus discharge in rat colon. American Journal of Physiology-Gastrointestinal and Liver Physiology. 1998;275(5):G1073-G84.
63. Rousset M, Laburthe M, Chevalier G, Boissard C, Rosselin G, Zweibaum A. Vasoactive intestinal peptide (VIP) control of glycogenolysis in the human colon carcinoma cell line HT-29 in culture. FEBS letters. 1981;126(1):38-40.
64. Toumi F, Neunlist M, Cassagnau E, Parois S, Laboisse C, Galmiche JP, et al. Human submucosal neurones regulate intestinal epithelial cell proliferation: evidence from a novel co-culture model. Neurogastroenterology & Motility. 2003;15(3):239-42.
65. Neunlist M, Toumi F, Oreschkova T, Denis M, Leborgne J, Laboisse CL, et al. Human ENS regulates the intestinal epithelial barrier permeability and a tight junction-associated protein ZO-1 via VIPergic pathways. American Journal of Physiology-Gastrointestinal and Liver Physiology. 2003;285(5):G1028-G36.
66. Bakker R, Dekker K, De Jonge H, Groot J. VIP, serotonin, and epinephrine modulate the ion selectivity of tight junctions of goldfish intestine. American Journal of Physiology-Regulatory, Integrative and Comparative Physiology. 1993;264(2):R362-R8.
67. Lelievre V, Favrais G, Abad C, Adle-Biassette H, Lu Y, Germano P, et al. Gastrointestinal dysfunction in mice with a targeted mutation in the gene encoding vasoactive intestinal polypeptide: a model for the study of intestinal ileus and Hirschsprung's disease. Peptides. 2007;28(9):1688-99.
68. Delgado M, Martinez C, Pozo D, Calvo JR, Leceta J, Ganea D, et al. Vasoactive intestinal peptide (VIP) and pituitary adenylate cyclase-activation polypeptide (PACAP) protect mice from lethal endotoxemia through the inhibition of TNF-α and IL-6. The Journal of Immunology. 1999;162(2):1200-5.
69. FERNANDEZ-MARTIN A, GONZALEZ-REY E, Chorny A, Martin J, Pozo D, Ganea D, et al. VIP prevents experimental multiple sclerosis by downregulating both inflammatory and autoimmune components of the disease. Annals of the New York Academy of Sciences. 2006;1070(1):276-81.
70. Szema AM, Hamidi SA, Lyubsky S, Dickman KG, Mathew S, Abdel-Razek T, et al. Mice lacking the VIP gene show airway hyperresponsiveness and airway inflammation, partially reversible by VIP. American Journal of Physiology-Lung Cellular and Molecular Physiology. 2006;291(5):L880-L6.
71. Foey AD, Field S, Ahmed S, Jain A, Feldmann M, Brennan FM, et al. Impact of VIP and cAMP on the regulation of TNF-α and IL-10 production: implications for rheumatoid arthritis. Arthritis Res Ther. 2003;5(6):1.
72. Arranz A, Gutiérrez-Cañas I, Carrión M, Juarranz Y, Pablos JL, Martínez C, et al. VIP reverses the expression profiling of TLR4-stimulated signaling pathway in rheumatoid arthritis synovial fibroblasts. Molecular immunology. 2008;45(11):3065-73.

73. Abad C, Martinez C, Juarranz MG, Arranz A, Leceta J, Delgado M, et al. Therapeutic effects of vasoactive intestinal peptide in the trinitrobenzene sulfonic acid mice model of Crohn's disease. Gastroenterology. 2003;124(4):961-71.
74. Abad C, Juarranz Y, Martinez C, Arranz A, Rosignoli F, García-Gómez M, et al. cDNA array analysis of cytokines, chemokines, and receptors involved in the development of TNBS-induced colitis: homeostatic role of VIP. Inflammatory bowel diseases. 2005;11(7):674-84.
75. Delgado M, Abad C, Martinez C, Leceta J, Gomariz RP. Vasoactive intestinal peptide prevents experimental arthritis by downregulating both autoimmune and inflammatory components of the disease. Nature medicine. 2001;7(5):563.
76. Conlin VS, Wu X, Nguyen C, Dai C, Vallance BA, Buchan A, et al. Vasoactive intestinal peptide ameliorates intestinal barrier disruption associated with Citrobacter rodentium-induced colitis. American Journal of Physiology-Gastrointestinal and Liver Physiology. 2009;297(4):G735-G50.
77. Abad C, Juarranz Y, Martinez C, Arranz A, Rosignoli F, García-Gómez M, et al. cDNA array analysis of cytokines, chemokines, and receptors involved in the development of TNBS-induced colitis: homeostatic role of VIP. Inflammatory bowel diseases. 2005;11(7):674-84.
78. Newman R, Cuan N, Hampartzoumian T, Connor S, Lloyd A, Grimm M. Vasoactive intestinal peptide impairs leucocyte migration but fails to modify experimental murine colitis. Clinical & Experimental Immunology. 2005;139(3):411-20.
79. Morampudi V, Conlin VS, Dalwadi U, Wu X, Marshall KC, Nguyen C, et al. Vasoactive intestinal peptide prevents PKCε-induced intestinal epithelial barrier disruption during EPEC infection. American Journal of Physiology-Gastrointestinal and Liver Physiology. 2015;308(5):G389-G402.
80. Azuma YT, Hagi K, Shintani N, Kuwamura M, Nakajima H, Hashimoto H, et al. PACAP provides colonic protection against dextran sodium sulfate induced colitis. Journal of cellular physiology. 2008;216(1):111-9.
81. Yadav M, Huang M-C, Goetzl EJ. VPAC1 (vasoactive intestinal peptide (VIP) receptor type 1) G protein-coupled receptor mediation of VIP enhancement of murine experimental colitis. Cellular immunology. 2011;267(2):124-32.
82. Vu JP, Million M, Larauche M, Luong L, Norris J, Waschek JA, et al. Inhibition of vasoactive intestinal polypeptide (VIP) induces resistance to dextran sodium sulfate (DSS)-induced colitis in mice. Journal of Molecular Neuroscience. 2014;52(1):37-47.
83. Abad C, Cheung-Lau G, Coute-Monvoisin A-C, Waschek JA. Vasoactive intestinal peptide-deficient mice exhibit reduced pathology in trinitrobenzene sulfonic acid-induced colitis. Neuroimmunomodulation. 2015;22(3):203-12.
84. Wu X, Conlin VS, Morampudi V, Ryz NR, Nasser Y, Bhinder G, et al. Vasoactive intestinal polypeptide promotes intestinal barrier homeostasis and protection against colitis in mice. PloS one. 2015;10(5):e0125225.
85. Caughey GH, Leidig F, Viro NF, Nadel J. Substance P and vasoactive intestinal peptide degradation by mast cell tryptase and chymase. Journal of Pharmacology and Experimental Therapeutics. 1988;244(1):133-7.

86. Domschke S, Domschke W, Bloom S, Mitznegg P, Mitchell S, Lux G, et al. Vasoactive intestinal peptide in man: pharmacokinetics, metabolic and circulatory effects. Gut. 1978;19(11):1049-53.
87. Gozes I, Fridkin M, Hill J, Brenneman D. Pharmaceutical VIP: prospects and problems. Current medicinal chemistry. 1999;6:1019-34.
88. Onoue S, Yamada S, Yajima T. Bioactive analogues and drug delivery systems of vasoactive intestinal peptide (VIP) for the treatment of asthma/COPD. Peptides. 2007;28(9):1640-50.
89. Webster TJ. Nanomedicine: what's in a definition? : Dove Press; 2006.
90. Maeda H, Wu J, Sawa T, Matsumura Y, Hori K. Tumor vascular permeability and the EPR effect in macromolecular therapeutics: a review. Journal of controlled release. 2000;65(1-2):271-84.
91. Rubinstein I, Soos I, Onyuksel H. Intracellular delivery of VIP-grafted sterically stabilized phospholipid mixed nanomicelles in human breast cancer cells. Chemico-biological interactions. 2008;171(2):190-4.
92. Gülçür E, Thaqi M, Khaja F, Kuzmis A, Önyüksel H. Curcumin in VIP-targeted sterically stabilized phospholipid nanomicelles: a novel therapeutic approach for breast cancer and breast cancer stem cells. Drug delivery and translational research. 2013;3(6):562-74.
93. Sethi V, Rubinstein I, Kuzmis A, Kastrissios H, Artwohl J, Onyuksel H. Novel, biocompatible, and disease modifying VIP nanomedicine for rheumatoid arthritis. Molecular pharmaceutics. 2013;10(2):728-38.
94. Gao X, Wu B, Zhang Q, Chen J, Zhu J, Zhang W, et al. Brain delivery of vasoactive intestinal peptide enhanced with the nanoparticles conjugated with wheat germ agglutinin following intranasal administration. Journal of Controlled Release. 2007;121(3):156-67.
95. Athari SS, Pourpak Z, Folkerts G, Garssen J, Moin M, Adcock IM, et al. Conjugated Alpha-Alumina nanoparticle with vasoactive intestinal peptide as a Nano-drug in treatment of allergic asthma in mice. European journal of pharmacology. 2016;791:811-20.
96. Hajos F, Stark B, Hensler S, Prassl R, Mosgoeller W. Inhalable liposomal formulation for vasoactive intestinal peptide. International journal of pharmaceutics. 2008;357(1-2):286-94.
97. Fernandez-Montesinos R, Castillo PM, Klippstein R, Gonzalez-Rey E, Mejias JA, Zaderenko AP, et al. Chemical synthesis and characterization of silver-protected vasoactive intestinal peptide nanoparticles. Nanomedicine. 2009;4(8):919-30.
98. Wernig K, Griesbacher M, Andreae F, Hajos F, Wagner J, Mosgoeller W, et al. Depot formulation of vasoactive intestinal peptide by protamine-based biodegradable nanoparticles. Journal of Controlled Release. 2008;130(2):192-8.
99. Barenholz YC. Doxil®—the first FDA-approved nano-drug: lessons learned. Journal of controlled release. 2012;160(2):117-34.
100. Banerjee A, Onyuksel H. Peptide delivery using phospholipid micelles. Wiley Interdisciplinary Reviews: Nanomedicine and Nanobiotechnology. 2012;4(5):562-74.

101. Lim SB, Rubinstein I, Önyüksel H. Freeze drying of peptide drugs self-associated with long-circulating, biocompatible and biodegradable sterically stabilized phospholipid nanomicelles. International journal of pharmaceutics. 2008;356(1-2):345-50.
102. Klippstein R, Pozo D. Vasoactive intestinal peptide (VIP) nanoparticles for diagnostics and for controlled and targeted drug delivery. Advances in protein chemistry and structural biology. 98: Elsevier; 2015. p. 145-68.
103. Mathioudakis A, Chatzimavridou-Grigoriadou V, Evangelopoulou E, Mathioudakis G. Vasoactive intestinal peptide inhaled agonists: potential role in respiratory therapeutics. Hippokratia. 2013;17(1):12.
104. O'donnell M, Garippa R, Rinaldi N, Selig W, Simko B, Renzetti L, et al. Ro 25-1553: a novel, long-acting vasoactive intestinal peptide agonist. Part I: In vitro and in vivo bronchodilator studies. Journal of Pharmacology and Experimental Therapeutics. 1994;270(3):1282-8.
105. Lamprecht A. Nanomedicines in gastroenterology and hepatology. Nature Reviews Gastroenterology and Hepatology. 2015;12(4):195.
106. Hua S, Marks E, Schneider JJ, Keely S. Advances in oral nano-delivery systems for colon targeted drug delivery in inflammatory bowel disease: selective targeting to diseased versus healthy tissue. Nanomedicine: Nanotechnology, Biology and Medicine. 2015;11(5):1117-32.
107. Collnot E-M, Ali H, Lehr C-M. Nano-and microparticulate drug carriers for targeting of the inflamed intestinal mucosa. Journal of Controlled Release. 2012;161(2):235-46.
108. Xiao B, Merlin D. Oral colon-specific therapeutic approaches toward treatment of inflammatory bowel disease. Expert opinion on drug delivery. 2012;9(11):1393-407.
109. Lautenschläger C, Schmidt C, Lehr C-M, Fischer D, Stallmach A. PEG-functionalized microparticles selectively target inflamed mucosa in inflammatory bowel disease. European Journal of Pharmaceutics and Biopharmaceutics. 2013;85(3):578-86.
110. Videla S, Lugea A, Vilaseca J, Guarner F, Treserra F, Salas A, et al. Polyethylene glycol enhances colonic barrier function and ameliorates experimental colitis in rats. International journal of colorectal disease. 2007;22(6):571-80.
111. Lim SB, Rubinstein I, Sadikot RT, Artwohl JE, Önyüksel H. A novel peptide nanomedicine against acute lung injury: GLP-1 in phospholipid micelles. Pharmaceutical research. 2011;28(3):662-72.
112. Kuzmis A, Lim SB, Desai E, Jeon E, Lee B-S, Rubinstein I, et al. Micellar nanomedicine of human neuropeptide Y. Nanomedicine: Nanotechnology, Biology and Medicine. 2011;7(4):464-71.
113. Banerjee A, Onyuksel H. Human pancreatic polypeptide in a phospholipid-based micellar formulation. Pharmaceutical research. 2012;29(6):1698-711.
114. Sonaje K, Chen Y-J, Chen H-L, Wey S-P, Juang J-H, Nguyen H-N, et al. Enteric-coated capsules filled with freeze-dried chitosan/poly (γ-glutamic acid) nanoparticles for oral insulin delivery. Biomaterials. 2010;31(12):3384-94.

115. Xiao B, Xu Z, Viennois E, Zhang Y, Zhang Z, Zhang M, et al. Orally targeted delivery of tripeptide KPV via hyaluronic acid-functionalized nanoparticles efficiently alleviates ulcerative colitis. Molecular Therapy. 2017;25(7):1628-40.
116. Zhang Y, Zhang L, Ban Q, Li J, Li C-H, Guan Y-Q. Preparation and characterization of hydroxyapatite nanoparticles carrying insulin and gallic acid for insulin oral delivery. Nanomedicine: Nanotechnology, Biology and Medicine. 2018;14(2):353-64.
117. Laroui H, Dalmasso G, Nguyen HTT, Yan Y, Sitaraman SV, Merlin D. Drug-loaded nanoparticles targeted to the colon with polysaccharide hydrogel reduce colitis in a mouse model. Gastroenterology. 2010;138(3):843-53. e2.
118. Shrestha N, Araújo F, Shahbazi M-A, Mäkilä E, Gomes MJ, Airavaara M, et al. Oral hypoglycaemic effect of GLP-1 and DPP4 inhibitor based nanocomposites in a diabetic animal model. Journal of Controlled Release. 2016;232:113-9.
119. Kostadinova AI, Middelburg J, Ciulla M, Garssen J, Hennink WE, Knippels LM, et al. PLGA nanoparticles loaded with beta-lactoglobulin-derived peptides modulate mucosal immunity and may facilitate cow's milk allergy prevention. European journal of pharmacology. 2018;818:211-20.
120. Shi Y, Sun X, Zhang L, Sun K, Li K, Li Y, et al. Fc-modified exenatide-loaded nanoparticles for oral delivery to improve hypoglycemic effects in mice. Scientific reports. 2018;8(1):726.
121. Scuderi S, D'Amico AG, Castorina A, Imbesi R, Carnazza ML, D'Agata V. Ameliorative effect of PACAP and VIP against increased permeability in a model of outer blood retinal barrier dysfunction. Peptides. 2013;39:119-24.
122. Hokari R, Lee H, Crawley SC, Yang SC, Gum JR, Miura S, et al. Vasoactive intestinal peptide upregulates MUC2 intestinal mucin via CREB/ATF1. American Journal of Physiology-Gastrointestinal and Liver Physiology. 2005;289(5):G949-G59.
123. Ogata H, Podolsky DK. Trefoil peptide expression and secretion is regulated by neuropeptides and acetylcholine. American Journal of Physiology-Gastrointestinal and Liver Physiology. 1997;273(2):G348-G54.
124. Kim YS, Ho SB. Intestinal goblet cells and mucins in health and disease: recent insights and progress. Current gastroenterology reports. 2010;12(5):319-30.
125. Mennigen R, Nolte K, Rijcken E, Utech M, Loeffler B, Senninger N, et al. Probiotic mixture VSL# 3 protects the epithelial barrier by maintaining tight junction protein expression and preventing apoptosis in a murine model of colitis. American journal of physiology-Gastrointestinal and liver physiology. 2009;296(5):G1140-G9.
126. Höglund P, Haila S, Socha J, Tomaszewski L, Saarialho-Kere U, Karjalainen-Lindsberg M-L, et al. Mutations of the Down–regulated in adenoma (DRA) gene cause congenital chloride diarrhoea. Nature genetics. 1996;14(3):316.
127. Liu JZ, van Sommeren S, Huang H, Ng SC, Alberts R, Takahashi A, et al. Association analyses identify 38 susceptibility loci for inflammatory bowel disease

and highlight shared genetic risk across populations. Nature genetics. 2015;47(9):979.
128. Sullivan S, Alex P, Dassopoulos T, Zachos NC, Iacobuzio-Donahue C, Donowitz M, et al. Downregulation of sodium transporters and NHERF proteins in IBD patients and mouse colitis models: Potential contributors to IBD-associated diarrhea. Inflammatory bowel diseases. 2009;15(2):261-74.
129. Okayasu I, Hatakeyama S, Yamada M, Ohkusa T, Inagaki Y, Nakaya R. A novel method in the induction of reliable experimental acute and chronic ulcerative colitis in mice. Gastroenterology. 1990;98(3):694-702.
130. Laroui H, Ingersoll SA, Liu HC, Baker MT, Ayyadurai S, Charania MA, et al. Dextran sodium sulfate (DSS) induces colitis in mice by forming nano-lipocomplexes with medium-chain-length fatty acids in the colon. PloS one. 2012;7(3):e32084.
131. Chassaing B, Aitken JD, Malleshappa M, Vijay-Kumar M. Dextran sulfate sodium (DSS)-induced colitis in mice. Current protocols in immunology. 2014:15.25. 1-15.25. 14.
132. Wirtz S, Neufert C, Weigmann B, Neurath MF. Chemically induced mouse models of intestinal inflammation. Nature protocols. 2007;2(3):541.
133. Kremer B, Mariman R, van Erk M, Lagerweij T, Nagelkerken L. Temporal colonic gene expression profiling in the recurrent colitis model identifies early and chronic inflammatory processes. PloS one. 2012;7(11):e50388.
134. Keates AC, Castagliuolo I, Cruickshank WW, Qiu B, Arseneau KO, Brazer W, et al. Interleukin 16 is up-regulated in Crohn's disease and participates in TNBS colitis in mice. Gastroenterology. 2000;119(4):972-82.
135. Neurath M, Fuss I, Strober W. TNBS-colitis. International reviews of immunology. 2000;19(1):51-62.
136. Scheiffele F, Fuss IJ. Induction of TNBS colitis in mice. Current protocols in immunology. 2002:15.9. 1-.9. 4.
137. Alex P, Zachos NC, Nguyen T, Gonzales L, Chen T-E, Conklin LS, et al. Distinct cytokine patterns identified from multiplex profiles of murine DSS and TNBS-induced colitis. Inflammatory bowel diseases. 2008;15(3):341-52.
138. Greig E, Sandle GI. Diarrhea in ulcerative colitis: the role of altered colonic sodium transport. Annals of the New York Academy of Sciences. 2000;915(1):327-32.
139. Priyamvada S, Gomes R, Gill RK, Saksena S, Alrefai WA, Dudeja PK. Mechanisms Underlying Dysregulation of Electrolyte Absorption in Inflammatory Bowel Disease–Associated Diarrhea. Inflammatory bowel diseases. 2015;21(12):2926-35.
140. Pozo D, Delgado M, Martínez C, Guerrero JM, Leceta J, Gomariz RP, et al. Immunobiology of vasoactive intestinal peptide (VIP). Immunology today. 2000;21(1):7-11.
141. Du B-H, Eng J, Hulmes J, Chang M, Pan Y-C, Yalow R. Guinea pig has a unique mammalian VIP. Biochemical and biophysical research communications. 1985;128(3):1093-8.
142. Askar B, Ibrahim H, Barrow P, Foster N. Vasoactive intestinal peptide (VIP) differentially affects inflammatory immune responses in human monocytes

infected with viable Salmonella or stimulated with LPS. Peptides. 2015;71:188-95.
143. Said SI, Mutt V. Polypeptide with broad biological activity: isolation from small intestine. Science. 1970;169(3951):1217-8.
144. Anbazhagan AN, Thaqi M, Priyamvada S, Jayawardena D, Kumar A, Gujral T, et al. GLP-1 nanomedicine alleviates gut inflammation. Nanomedicine: Nanotechnology, Biology and Medicine. 2017;13(2):659-65.
145. Koch TR, Carney JA, Go LWV. Distribution and quantitation of gut neuropeptides in normal intestine and inflammatory bowel diseases. Digestive diseases and sciences. 1987;32(4):369-76.
146. Baumgart DC, Bernstein CN, Abbas Z, Colombel JF, Day AS, D'Haens G, et al. IBD Around the world: comparing the epidemiology, diagnosis, and treatment: proceedings of the World Digestive Health Day 2010–Inflammatory Bowel Disease Task Force meeting. Inflammatory bowel diseases. 2011;17(2):639-44.
147. Fung TC, Olson CA, Hsiao EY. Interactions between the microbiota, immune and nervous systems in health and disease. Nature neuroscience. 2017;20(2):145.
148. Aziz I, Palsson OS, Törnblom H, Sperber AD, Whitehead WE, Simrén M. The prevalence and impact of overlapping Rome IV-diagnosed functional gastrointestinal disorders on somatization, quality of life, and healthcare utilization: a cross-sectional general population study in three countries. The American journal of gastroenterology. 2018;113(1):86.
149. Alleaume C, Eychène A, Caigneaux E, Muller J-M, Philippe M. Vasoactive intestinal peptide stimulates proliferation in HT29 human colonic adenocarcinoma cells: concomitant activation of Ras/Rap1–B-Raf–ERK signalling pathway. Neuropeptides. 2003;37(2):98-104.
150. Lelièvre V, Meunier A-C, Caigneaux E, Falcon J, Muller J-M. Differential expression and function of PACAP and VIP receptors in four human colonic adenocarcinoma cell lines. Cellular signalling. 1998;10(1):13-26.
151. Couvineau A, Rousset M, Laburthe M. Molecular identification and structural requirement of vasoactive intestinal peptide (VIP) receptors in the human colon adenocarcinoma cell line, HT-29. Biochemical Journal. 1985;231(1):139-43.
152. Zhang Z, Zheng M, Bindas J, Schwarzenberger P, Kolls JK. Critical role of IL-17 receptor signaling in acute TNBS-induced colitis. Inflammatory bowel diseases. 2006;12(5):382-8.
153. Krawisz J, Sharon P, Stenson W. Quantitative assay for acute intestinal inflammation based on myeloperoxidase activity: assessment of inflammation in rat and hamster models. Gastroenterology. 1984;87(6):1344-50.
154. Viennois E, Chen F, Laroui H, Baker MT, Merlin D. Dextran sodium sulfate inhibits the activities of both polymerase and reverse transcriptase: lithium chloride purification, a rapid and efficient technique to purify RNA. BMC research notes. 2013;6(1):360.
155. Schmittgen TD, Livak KJ. Analyzing real-time PCR data by the comparative CT method. Nature protocols. 2008;3(6):1101-8.

156. Yan Y, Kolachala V, Dalmasso G, Nguyen H, Laroui H, Sitaraman SV, et al. Temporal and spatial analysis of clinical and molecular parameters in dextran sodium sulfate induced colitis. PloS one. 2009;4(6):e6073.
157. Lindén SK, Florin TH, McGuckin MA. Mucin dynamics in intestinal bacterial infection. PloS one. 2008;3(12):e3952.
158. Malhotra P, Soni V, Kumar A, Anbazhagan AN, Dudeja A, Saksena S, et al. Epigenetic modulation of intestinal cholesterol transporter Niemann-Pick C1-like 1 (NPC1L1) gene expression by DNA methylation. Journal of Biological Chemistry. 2014;289(33):23132-40.
159. Asghar MN, Priyamvada S, Nyström JH, Anbazhagan AN, Dudeja PK, Toivola DM. Keratin 8 knockdown leads to loss of the chloride transporter DRA in the colon. American Journal of Physiology-Gastrointestinal and Liver Physiology. 2016;310(11):G1147-G54.
160. Gill RK, Borthakur A, Hodges K, Turner JR, Clayburgh DR, Saksena S, et al. Mechanism underlying inhibition of intestinal apical Cl–/OH–exchange following infection with enteropathogenic E. coli. The Journal of clinical investigation. 2007;117(2):428-37.
161. Rousset M. The human colon carcinoma cell lines HT-29 and Caco-2: two in vitro models for the study of intestinal differentiation. Biochimie. 1986;68(9):1035-40.
162. Grasset E, Pinto M, Dussaulx E, Zweibaum A, Desjeux JF. Epithelial properties of human colonic carcinoma cell line Caco-2: electrical parameters. American Journal of Physiology-Cell Physiology. 1984;247(3):C260-C7.
163. Anderson CM, Mendoza ME, Kennedy DJ, Raldua D, Thwaites DT. Inhibition of intestinal dipeptide transport by the neuropeptide VIP is an anti-absorptive effect via the VPAC1 receptor in a human enterocyte-like cell line (Caco-2). British journal of pharmacology. 2003;138(4):564-73.
164. COUVINEAU A, MAORET J-J, ROUYER-FESSARD C, CARRERO I, LABURTHE M. The human vasoactive intestinal peptide/pituitary adenylate cyclase-activating peptide receptor 1 (VPAC1) promoter: characterization and role in receptor expression during enterocytic differentiation of the colon cancer cell line Caco-2Cl. 20. Biochemical Journal. 2000;347(3):623-32.
165. Raheja G, Singh V, Ma K, Boumendjel R, Borthakur A, Gill RK, et al. Lactobacillus acidophilus stimulates the expression of SLC26A3 via a transcriptional mechanism. American Journal of Physiology-Gastrointestinal and Liver Physiology. 2009;298(3):G395-G401.
166. Anderson CM, Thwaites DT. Indirect regulation of the intestinal H+-coupled amino acid transporter hPAT1 (SLC36A1). Journal of cellular physiology. 2005;204(2):604-13.
167. Gill RK, Pant N, Saksena S, Singla A, Nazir TM, Vohwinkel L, et al. Function, expression, and characterization of the serotonin transporter in the native human intestine. American Journal of Physiology-Gastrointestinal and Liver Physiology. 2008;294(1):G254-G62.
168. Nair AB, Jacob S. A simple practice guide for dose conversion between animals and human. Journal of basic and clinical pharmacy. 2016;7(2):27.

169. McConnell EL, Fadda HM, Basit AW. Gut instincts: explorations in intestinal physiology and drug delivery. International journal of pharmaceutics. 2008;364(2):213-26.
170. MODY R, TRAMONTANO A, PAUL S. Spontaneous hydrolysis of vasoactive intestinal peptide in neutral aqueous solution. Chemical Biology & Drug Design. 1994;44(5):441-7.
171. Cui X, Cao D, Qu C, Zhang X, Zheng A. A study of the chemical and biological stability of vasoactive intestinal peptide. Drug development and industrial pharmacy. 2013;39(12):1907-10.
172. Kahne D, Still WC. Hydrolysis of a peptide bond in neutral water. Journal of the American Chemical Society. 1988;110(22):7529-34.
173. Hoag S. Capsules Dosage Form: Formulation and Manufacturing Considerations. Developing Solid Oral Dosage Forms (Second Edition): Elsevier; 2017. p. 723-47.
174. Stegemann S, Bornem C. Hard gelatin capsules today-and tomorrow: Capsugel Library; 1999.
175. Elson CO, Sartor RB, Tennyson GS, Riddell RH. Experimental models of inflammatory bowel disease. Gastroenterology. 1995;109(4):1344-67.
176. Jayawardena D, Anbazhagan AN, Guzman G, Dudeja PK, Onyuksel H. Vasoactive Intestinal Peptide Nanomedicine for the Management of Inflammatory Bowel Disease. Molecular pharmaceutics. 2017;14(11):3698-708.
177. Bouma G, Strober W. The immunological and genetic basis of inflammatory bowel disease. Nature Reviews Immunology. 2003;3(7):521.
178. Steidler L, Hans W, Schotte L, Neirynck S, Obermeier F, Falk W, et al. Treatment of murine colitis by Lactococcus lactis secreting interleukin-10. Science. 2000;289(5483):1352-5.
179. Neurath MF, Fuss I, Kelsall BL, Presky DH, Waegell W, Strober W. Experimental granulomatous colitis in mice is abrogated by induction of TGF-beta-mediated oral tolerance. Journal of Experimental Medicine. 1996;183(6):2605-16.
180. Simonovic I, Rosenberg J, Koutsouris A, Hecht G. Enteropathogenic Escherichia coli dephosphorylates and dissociates occludin from intestinal epithelial tight junctions. Cellular microbiology. 2000;2(4):305-15.
181. Talbot C, Lytle C. Segregation of Na/H exchanger-3 and Cl/HCO3 exchanger SLC26A3 (DRA) in rodent cecum and colon. American Journal of Physiology-Gastrointestinal and Liver Physiology. 2010;299(2):G358-G67.
182. Pecora R. Dynamic light scattering measurement of nanometer particles in liquids. Journal of nanoparticle research. 2000;2(2):123-31.
183. Berndt BE, Zhang M, Chen G-H, Huffnagle GB, Kao JY. The role of dendritic cells in the development of acute dextran sulfate sodium colitis. The Journal of Immunology. 2007;179(9):6255-62.
184. Arranz A, Juarranz Y, Leceta J, Gomariz RP, Martínez C. VIP balances innate and adaptive immune responses induced by specific stimulation of TLR2 and TLR4. Peptides. 2008;29(6):948-56.

185. Sann H, von Erichsen J, Hessmann M, Pahl A, Hoffmeyer A. Efficacy of drugs used in the treatment of IBD and combinations thereof in acute DSS-induced colitis in mice. Life sciences. 2013;92(12):708-18.
186. Sartor R. How relevant to human inflammatory bowel disease are current animal models of intestinal inflammation? Alimentary pharmacology & therapeutics. 1997;11(s3):89-97.
187. Singh V, Kumar A, Raheja G, Anbazhagan AN, Priyamvada S, Saksena S, et al. Lactobacillus acidophilus attenuates downregulation of DRA function and expression in inflammatory models. American Journal of Physiology-Gastrointestinal and Liver Physiology. 2014;307(6):G623-G31.
188. Dedrick RL, Myers CE, Bungay PM, DeVita V. Pharmacokinetic rationale for peritoneal drug administration. Cancer Treat Rep. 1978;62:1-13.
189. Turner PV, Brabb T, Pekow C, Vasbinder MA. Administration of substances to laboratory animals: routes of administration and factors to consider. Journal of the American Association for Laboratory Animal Science. 2011;50(5):600-13.
190. Egger B, Bajaj-Elliott M, MacDonald TT, Inglin R, Eysselein VE, Büchler MW. Characterisation of acute murine dextran sodium sulphate colitis: cytokine profile and dose dependency. Digestion. 2000;62(4):240-8.
191. Delgado M, Ganea D. Inhibition of endotoxin-induced macrophage chemokine production by vasoactive intestinal peptide and pituitary adenylate cyclase-activating polypeptide in vitro and in vivo. The Journal of Immunology. 2001;167(2):966-75.
192. Delgado M, Munoz-Elias EJ, Gomariz RP, Ganea D. Vasoactive intestinal peptide and pituitary adenylate cyclase-activating polypeptide enhance IL-10 production by murine macrophages: in vitro and in vivo studies. The Journal of Immunology. 1999;162(3):1707-16.
193. Sun W, Tadmori I, Yang L, Delgado M, Ganea D. Vasoactive intestinal peptide (VIP) inhibits TGF-β1 production in murine macrophages. Journal of neuroimmunology. 2000;107(1):88-99.
194. De la Fuente M, Delgado M, Gomariz RP. VIP modulation of immune cell functions. Journal of Neuroimmunology. 1996;6(1):75-91.
195. Frase LL, Gaffney FA, Lane LD, Buckey JC, Said SI, Blomqvist CG, et al. Cardiovascular effects of vasoactive intestinal peptide in healthy subjects. American Journal of Cardiology. 1987;60(16):1356-61.
196. Chen Y, Chou K, Fuchs E, Havran WL, Boismenu R. Protection of the intestinal mucosa by intraepithelial γδ T cells. Proceedings of the National Academy of Sciences. 2002;99(22):14338-43.
197. Zhao H, Zhang H, Wu H, Li H, Liu L, Guo J, et al. Protective role of 1, 25 (OH) 2 vitamin D 3 in the mucosal injury and epithelial barrier disruption in DSS-induced acute colitis in mice. BMC gastroenterology. 2012;12(1):57.
198. Hansson GC. Role of mucus layers in gut infection and inflammation. Current opinion in microbiology. 2012;15(1):57-62.
199. Moro F, Levenez F, Durual S, Plaisancie P, Thim L, Giraud A, et al. Secretion of the trefoil factor TFF3 from the isolated vascularly perfused rat colon. Regulatory peptides. 2001;101(1-3):35-41.

200. Zheng X, Tsuchiya K, Okamoto R, Iwasaki M, Kano Y, Sakamoto N, et al. Suppression of hath1 gene expression directly regulated by hes1 via notch signaling is associated with goblet cell depletion in ulcerative colitis. Inflammatory bowel diseases. 2011;17(11):2251-60.
201. Gersemann M, Becker S, Kübler I, Koslowski M, Wang G, Herrlinger KR, et al. Differences in goblet cell differentiation between Crohn's disease and ulcerative colitis. Differentiation. 2009;77(1):84-94.
202. Gonzalez-Mariscal L, Betanzos A, Nava P, Jaramillo B. Tight junction proteins. Progress in biophysics and molecular biology. 2003;81(1):1-44.
203. Chiba H, Osanai M, Murata M, Kojima T, Sawada N. Transmembrane proteins of tight junctions. Biochimica et Biophysica Acta (BBA)-Biomembranes. 2008;1778(3):588-600.
204. Schmitz H, Barmeyer C, Fromm M, Runkel N, Foss H-D, Bentzel CJ, et al. Altered tight junction structure contributes to the impaired epithelial barrier function in ulcerative colitis. Gastroenterology. 1999;116(2):301-9.
205. Poritz LS, Garver KI, Green C, Fitzpatrick L, Ruggiero F, Koltun WA. Loss of the tight junction protein ZO-1 in dextran sulfate sodium induced colitis. Journal of Surgical Research. 2007;140(1):12-9.
206. Dai C, Zhao D-H, Jiang M. VSL# 3 probiotics regulate the intestinal epithelial barrier in vivo and in vitro via the p38 and ERK signaling pathways. International journal of molecular medicine. 2012;29(2):202-8.
207. Hering NA, Fromm M, Schulzke JD. Determinants of colonic barrier function in inflammatory bowel disease and potential therapeutics. The Journal of physiology. 2012;590(5):1035-44.
208. Oh-oka K, Kono H, Ishimaru K, Miyake K, Kubota T, Ogawa H, et al. Expressions of tight junction proteins Occludin and Claudin-1 are under the circadian control in the mouse large intestine: implications in intestinal permeability and susceptibility to colitis. PLoS One. 2014;9(5):e98016.
209. Lohi H, Makela S, Pulkkinen K, Hoglund P, Karjalainen-Lindsberg M-L, Puolakkainen P, et al. Upregulation of CFTR expression but not SLC26A3 and SLC9A3 in ulcerative colitis. American Journal of Physiology-Gastrointestinal and Liver Physiology. 2002;283(3):G567-G75.
210. Sartor RB. Cytokines in intestinal inflammation: pathophysiological and clinical considerations. Gastroenterology. 1994;106(2):533-9.
211. Lamprecht G, Gaco V, Turner JR, Natour D, Gregor M. Regulation of the intestinal anion exchanger DRA (downregulated in adenoma). Annals of the New York Academy of Sciences. 2009;1165(1):261-6.
212. Melvin JE, Park K, Richardson L, Schultheis PJ, Shull GE. Mouse down-regulated in adenoma (DRA) is an intestinal Cl−/HCO3− exchanger and is up-regulated in colon of mice lacking the NHE3 Na+/H+ exchanger. Journal of Biological Chemistry. 1999;274(32):22855-61.
213. Schweinfest CW, Spyropoulos DD, Henderson KW, Kim J-H, Chapman JM, Barone S, et al. slc26a3 (dra)-deficient mice display chloride-losing diarrhea, enhanced colonic proliferation, and distinct up-regulation of ion transporters in the colon. Journal of Biological Chemistry. 2006;281(49):37962-71.

214. Yang H, Jiang W, Furth EE, Wen X, Katz JP, Sellon RK, et al. Intestinal inflammation reduces expression of DRA, a transporter responsible for congenital chloride diarrhea. American Journal of Physiology-Gastrointestinal and Liver Physiology. 1998;275(6):G1445-G53.
215. Saksena S, Singla A, Goyal S, Katyal S, Bansal N, Gill RK, et al. Mechanisms of transcriptional modulation of the human anion exchanger SLC26A3 gene expression by IFN-γ. American Journal of Physiology-Gastrointestinal and Liver Physiology. 2009;298(2):G159-G66.
216. Ding X, Li D, Li M, Wang H, He Q, Wang Y, et al. SLC26A3 (DRA) prevents TNF-alpha-induced barrier dysfunction and dextran sulfate sodium-induced acute colitis. Laboratory Investigation. 2018:1.
217. Hodges K, Gill R. Infectious diarrhea: cellular and molecular mechanisms. Gut microbes. 2010;1(1):4-21.
218. Chatterjee I, Kumar A, Castilla-Madrigal RM, Pellon-Cardenas O, Gill RK, Alrefai WA, et al. CDX2 upregulates SLC26A3 gene expression in intestinal epithelial cells. American Journal of Physiology-Gastrointestinal and Liver Physiology. 2017;313(3):G256-G64.
219. Herkes SM, Smith CD, Sarr MG. Jejunal responses to absorptive and secretory stimuli in the neurally isolated jejunum in vivo. Surgery. 1994;116(3):576-86.
220. Ameen NA, Martensson B, Bourguinon L, Marino C, Isenberg J, McLaughlin GE. CFTR channel insertion to the apical surface in rat duodenal villus epithelial cells is upregulated by VIP in vivo. Journal of Cell Science. 1999;112(6):887-94.
221. Chao AC, Sauvage F, Dong Y, Wagner J, Goeddel D, Gardner P. Activation of intestinal CFTR Cl-channel by heat-stable enterotoxin and guanylin via cAMP-dependent protein kinase. The EMBO Journal. 1994;13(5):1065-72.
222. Morishita M, Peppas NA. Is the oral route possible for peptide and protein drug delivery? Drug discovery today. 2006;11(19-20):905-10.
223. DeVane C. Principles of pharmacokinetics and pharmacodynamics. The American Psychiatric Publishing Textbook of Psychopharmacology, 4th Edition: Am Neuropsych Assoc; 2009.
224. Frokjaer S, Otzen DE. Protein drug stability: a formulation challenge. Nature reviews drug discovery. 2005;4(4):298.
225. Lewis AL, Richard J. Challenges in the delivery of peptide drugs: an industry perspective. Therapeutic delivery. 2015;6(2):149-63.
226. Richard J. Challenges in oral peptide delivery: lessons learnt from the clinic and future prospects. Therapeutic delivery. 2017;8(8):663-84.
227. Sarmento B, Ribeiro A, Veiga F, Sampaio P, Neufeld R, Ferreira D. Alginate/chitosan nanoparticles are effective for oral insulin delivery. Pharmaceutical research. 2007;24(12):2198-206.
228. Sarmento B, Martins S, Ferreira D, Souto EB. Oral insulin delivery by means of solid lipid nanoparticles. International journal of nanomedicine. 2007;2(4):743.

229. Araújo F, Shrestha N, Shahbazi M-A, Fonte P, Mäkilä EM, Salonen JJ, et al. The impact of nanoparticles on the mucosal translocation and transport of GLP-1 across the intestinal epithelium. Biomaterials. 2014;35(33):9199-207.
230. Ensign LM, Cone R, Hanes J. Oral drug delivery with polymeric nanoparticles: the gastrointestinal mucus barriers. Advanced drug delivery reviews. 2012;64(6):557-70.
231. des Rieux A, Fievez V, Garinot M, Schneider Y-J, Préat V. Nanoparticles as potential oral delivery systems of proteins and vaccines: a mechanistic approach. Journal of controlled release. 2006;116(1):1-27.
232. Hamman JH, Enslin GM, Kotzé AF. Oral delivery of peptide drugs. BioDrugs. 2005;19(3):165-77.
233. Korman LY, Sayadi H, Bass B, Moody TW, Harmon JW. Distribution of vasoactive intestinal polypeptide and substance P receptors in human colon and small intestine. Digestive diseases and sciences. 1989;34(7):1100-8.
234. Laburthe M, Prieto J, Amiranoff B, Dupont C, Broyart J, Hoa DHB, et al. VIP receptors in intestinal epithelial cells: distribution throughout the intestinal tract. Hormone receptors in digestion and nutrition. 1979;1:241.
235. Zimmerman R, Gates T, Mantyh C, Vigna S, Passaro E, Mantyh PW. Vasoactive intestinal polypeptide receptor binding sites in the human gastrointestinal tract: localization by autoradiography. Neuroscience. 1989;31(3):771-83.
236. Prieto JC, Laburthe M, Hoa DHB, Rosselin G. Quantitative studies of vasoactive intestinal peptide (VIP) binding sites and VIP-induced adenosine 3′: 5′-monophosphate production in epithelial cells from duodenum, jejunum, ileum, coecum, colon and rectum in the rat. Acta endocrinologica. 1981;96(1):100-6.
237. Dharmsathaphorn K, Harms V, Yamashiro DJ, Hughes R, Binder H, Wright E. Preferential binding of vasoactive intestinal polypeptide to basolateral membrane of rat and rabbit enterocytes. Journal of Clinical Investigation. 1983;71(1):27.
238. Barbarin A, Séité P, Godet J, Bensalma S, Muller J-M, Chadéneau C. Atypical nuclear localization of VIP receptors in glioma cell lines and patients. Biochemical and biophysical research communications. 2014;454(4):524-30.
239. Fung C, Unterweger P, Parry LJ, Bornstein JC, Foong JP. VPAC1 receptors regulate intestinal secretion and muscle contractility by activating cholinergic neurons in guinea pig jejunum. American Journal of Physiology-Gastrointestinal and Liver Physiology. 2014;306(9):G748-G58.
240. Schulz S, Röcken C, Mawrin C, Weise W, Höllt V, Schulz S. Immunocytochemical identification of VPAC1, VPAC2, and PAC1 receptors in normal and neoplastic human tissues with subtype-specific antibodies. Clinical Cancer Research. 2004;10(24):8235-42.
241. Waschek JA, Richards ML, Bravo DT. Differential expression of VIP/PACAP receptor genes in breast, intestinal, and pancreatic cell lines. Cancer letters. 1995;92(2):143-9.
242. BROYART J-P, DUPONT C, LABURTHE M, ROSSELIN G. Characterization of Vasoactive Intestinal Peptide Receptors in Human Colonic

Epithelial Cells*. The Journal of Clinical Endocrinology & Metabolism. 1981;52(4):715-21.
243. Harmar AJ, Sheward WJ, Morrison CF, Waser B, Gugger M, Reubi JC. Distribution of the VPAC2 receptor in peripheral tissues of the mouse. Endocrinology. 2004;145(3):1203-10.
244. Guan C-X, Cui Y-R, Sun G-Y, Yu F, Tang C-Y, Li Y-C, et al. Role of CREB in vasoactive intestinal peptide-mediated wound healing in human bronchial epithelial cells. Regulatory peptides. 2009;153(1):64-9.
245. Holst JJ, Fahrenkrug J, Knuhtsen S, Jensen SL, Poulsen SS, Nielsen OV. Vasoactive intestinal polypeptide (VIP) in the pig pancreas: role of VIPergic nerves in control of fluid and bicarbonate secretion. Regulatory peptides. 1984;8(3):245-59.
246. Dagar S, Krishnadas A, Rubinstein I, Blend MJ, Önyüksel H. VIP grafted sterically stabilized liposomes for targeted imaging of breast cancer: in vivo studies. Journal of Controlled Release. 2003;91(1):123-33.
247. Dagar S, Sekosan M, Rubinstein I, Önyüksel H. Detection of VIP receptors in MNU-induced breast cancer in rats: implications for breast cancer targeting. Breast cancer research and treatment. 2001;65(1):49-54.
248. Reubi J, Gugger M, Waser B. Co-expressed peptide receptors in breast cancer as a molecular basis for in vivo multireceptor tumour targeting. European journal of nuclear medicine and molecular imaging. 2002;29(7):855-62.
249. Dagar A, Kuzmis A, Rubinstein I, Sekosan M, Onyuksel H. VIP-targeted cytotoxic nanomedicine for breast cancer. Drug delivery and translational research. 2012;2(6):454-62.
250. Taipaleenmäki EM, Mouritzen SA, Schattling PS, Zhang Y, Städler B. Mucopenetrating micelles with a PEG corona. Nanoscale. 2017;9(46):18438-48.
251. Greenfield NJ. Using circular dichroism spectra to estimate protein secondary structure. Nature protocols. 2006;1(6):2876.
252. Herrero EP, Alonso MJ, Csaba N. Polymer-based oral peptide nanomedicines. Therapeutic delivery. 2012;3(5):657-68.
253. Stern W, Gilligan JP. Oral peptide pharmaceutical products. Google Patents; 2000.
254. Önyüksel H, Bodalia B, Sethi V, Dagar S, Rubinsteina I. Surface-active properties of vasoactive intestinal peptide☆. Peptides. 2000;21(3):419-23.

APPENDICES

APPENDIX A

UIC UNIVERSITY OF ILLINOIS AT CHICAGO

Office of Animal Care and Institutional
Biosafety Committee (OACIB) (M/C 672)
Office of the Vice Chancellor for Research
206 Administrative Office Building
1737 West Polk Street
Chicago, Illinois 60612

10/18/2017

Pradeep K. Dudeja
Medicine/Gastroenterology and Hepatology
JAVAMC, R&D, MP-151
820 S. Damen, Suite 6215
Chicago, IL 60612

Dear Dr. Dudeja:

The protocol indicated below was reviewed in accordance with the Animal Care Policies and Procedures of the University of Illinois at Chicago and **renewed on 10/18/2017.**

Title of Application: Regulation of Ion Transport in Mouse Models of Colitis
ACC NO: 16-167
Original Protocol Approval: 11/17/2016 **(3 year approval with annual continuation required).**
Current Approval Period: 10/18/2017 **to** 10/18/2018

Funding: ***Portions of this protocol are supported by the funding sources indicated in the table below.***
Number of funding sources: 1

Funding Agency	**Funding Title**			**Portion of Funding Matched**
NIH	*Probiotics Potential Therapeutic Roles in Diahrrea (Institutional # 00019918)*			*All matched*
Funding Number	**Current Status**	**UIC PAF NO.**	**Performance Site**	**Funding PI**
RO1 DK818858 (years 5-9 original version)	*Funded*	*2013-02280*	*JBVAMC*	*Pradeep Dudeja*

This institution has Animal Welfare Assurance Number A3460.01 on file with the Office of Laboratory Animal Welfare, NIH. **This letter may only be provided as proof of IACUC approval for those specific funding sources listed above in which all portions of the grant are matched to this ACC protocol.**

Thank you for complying with the Animal Care Policies and Procedures of the UIC.

Sincerely,

John P. O'Bryan, PhD
Chair, Animal Care Committee
JPO/*kg*
cc: BRL, ACC File, Seema Saksena

Phone (312) 996-1972 • Fax (312) 996-9088

UIC UNIVERSITY OF ILLINOIS AT CHICAGO

Office of Animal Care and
Institutional Biosafety Committees (MC 672)
Office of the Vice Chancellor for Research
206 Administrative Office Building
1737 West Polk Street
Chicago, Illinois 60612-7227

November 18, 2016

Pradeep K. Dudeja
Medicine/Gastroenterology and Hepatology
JAVAMC, R&D, MP-151
820 S. Damen, Suite 6215
Chicago, IL 60612-

Dear Dr. Dudeja:

The protocol indicated below was reviewed at a convened ACC meeting in accordance with the Animal Care Policies of the University of Illinois at Chicago on **10/18/2016**. *The protocol was not initiated until final clarifications were reviewed and approved on* **11/17/2016**. *The protocol is approved for a period of 3* years with annual continuation.

Title of Application: Regulation of Ion Transport in Mouse Models of Colitis

ACC Number: 16-167

Initial Approval Period: 11/17/2016 to 10/18/2017

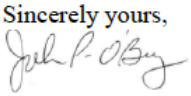
Current Funding: ***Portions of this protocol are supported by the funding sources indicated in the table below.***
Number of funding sources: 1

Funding Agency	**Funding Title**			**Portion of Proposal Matched**
NIH	*Probiotics Potential Therapeutic Roles in Diahrrea (Institutional # 00019918)*			*All matched*
Funding Number	**Current Status**	**UIC PAF NO.**	**Performance Site**	**Funding PI**
RO1 DK818858 (years 5-9 original version)	*Funded*	*201302280*	*JBVAMC*	*Pradeep Dudeja*

This institution has Animal Welfare Assurance Number A3460.01 on file with the Office of Laboratory Animal Welfare (OLAW), NIH. **This letter may only be provided as proof of IACUC approval for those specific funding sources listed above in which all portions of the funding proposal are matched to this ACC protocol.**

In addition, all investigators are responsible for ensuring compliance with all federal and institutional policies and regulations related to use of animals under this protocol and the funding sources listed on this protocol. Please use OLAW's "*What Investigators Need to Know about the Use of Animals*" (http://grants.nih.gov/grants/olaw/InvestigatorsNeed2Know.pdf) as a reference guide. Thank you for complying with the Animal Care Policies and Procedures of UIC.

Sincerely yours,



John P. O'Bryan, PhD
Chair, Animal Care Committee
JPO /*mbb*
cc: BRL, ACC File, Seema Saksena

DEPARTMENT OF VETERANS AFFAIRS
IACUC
Jesse Brown VA Medical Center
820 South Damen Ave, Room #6215
Chicago, IL. 60612

DATE:	February 28, 2017
TO:	Pradeep K. Dudeja, Ph.D
FROM:	Rhonda Kineman, Ph.D., Chair, JBVAMC IACUC
PROJECT TITLE:	1015140-1 Regulation of Ion Transport in Mouse Models of Colitis
SUBMISSION TYPE:	New Project
ACTION:	APPROVED
APPROVAL DATE:	February 28, 2017
EXPIRATION DATE:	February 28, 2020
REVIEW TYPE:	Full Committee Review

Thank you for your submission of IACUC #16-19 materials for the aboved named project. At the meeting held on February 8, 2017, the above named submission was brought before the JBVAMC IACUC. **The JBVAMC IACUC has APPROVED your submission.** This submission has received Full Committee Review based on applicable federal regulations. Notification of the JBVAMC IACUC decision will be submitted to the JBVAMC R&D Committee for their final approval. Note; your work is not to begin until the JBVAMC R&D Committee has approved your project and you receive an approval notice from the ACOS of Research. This approval is valid only for animals that are housed at the JBVAMC.

Please verify the information below. If corrections are required, notification must be sent to Robin McWherter, IACUC Administrator or Tyler Ridgeway, IACUC Coordinator at: CHS_IACUCSRS@va.gov immediately.

USDA CATEGORY:	B (120); C (6,290); D (2,076)
SPECIES:	Mice
# OF ANIMALS APPROVED:	8,486

Should it become necessary to make any additional changes to this protocol, you must submit a modification request for approval prior to initiating changes. The principal investigator is responsible for the modifications of their protocol when personnel are being added or removed. Failure to comply with these provisionss can result in a suspension and/or termination of your research.

If you have any questions or need further assistance, please do not hesitate to contact Robin McWherter, IACUC Administrator or Tyler Ridgeway, IACUC Coordinator at: CHS_IACUCSRS@va.gov. Please include your project title and reference number in all correspondence with this committee.

The JBVAMC IACUC wishes you success in your research endeavors.

Rhonda Kineman, Ph.D.
Chair, JBVAMC IACUC

cc: JBVAMC R&DC

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VITA

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- PhD in Biopharmaceutical Sciences (**GPA-3.97/4)**
  2018 University of Illinois at Chicago, Chicago, IL
- Bachelor of Pharmacy (1st Class Honors) (**GPA-4/4)**
  2011 University of Sri Jayewardenepura, Colombo, Sri Lanka
- Diploma in Information and Communication Technology
  2007 IDM Computer Studies, EDEXCEL, UK

Publications
Manuscripts

- Kumar, Anoop, Ishita Chatterjee, Arivarasu N. Anbazhagan, **Dulari Jayawardena**, Shubha Priyamvada, Waddah A. Alrefai, Jun Sun, Alip Borthakur, and Pradeep K. Dudeja. "Cryptosporidium parvum disrupts intestinal epithelial barrier function via altering expression of key tight junction and adherens junction proteins." *Cellular microbiology* (2018)
- **Jayawardena, Dulari,** Arivarasu N. Anbazhagan, Grace Guzman, Pradeep K. Dudeja, and Hayat Onyuksel. "Vasoactive Intestinal Peptide Nanomedicine for the Management of Inflammatory Bowel Disease." *Molecular Pharmaceutics* (2017)
- **Jayawardena, Dulari**, Grace Guzman, Ravinder K. Gill, Waddah A. Alrefai, Hayat Onyuksel, and Pradeep K. Dudeja. "Expression and localization of VPAC1, the major receptor of vasoactive intestinal peptide along the length of the intestine." *American Journal of Physiology-Gastrointestinal and Liver Physiology* (2017): ajpgi-00081
- Khaja, Fatima, **Dulari Jayawardena**, Antonina Kuzmis, and Hayat Onyuksel. "Targeted Sterically Stabilized Phospholipid siRNA Nanomedicine for Hepatic and Renal Fibrosis." Nanomaterials 6, no. 1 (2016): 8
- Anbazhagan, Arivarasu N., Mentor Thaqi, Shubha Priyamvada, **Dulari Jayawardena**, Anoop Kumar, Tarunmeet Gujral, Ishita Chatterjee et al.

"GLP-1 nanomedicine alleviates gut inflammation." *Nanomedicine: Nanotechnology, Biology and Medicine* 13, no. 2 (2017): 659-665.

Book chapters accepted

- Karina Espraza, Dulari Jayawardena and Hayat Onyuksel. “Phospholipid micelles for peptide drug delivery”. “Basic Protocols in Pharmaceutical Nanotechnology”, V. Weissig, T. Elbayoumi (Eds.).

Selected poster and podia presentations

- **Jayawardena D,** Anbazhagan A, Dudeja P and Onyuksel H. “Local Colonic Delivery of Vasoactive Intestinal Peptide (VIP) Nanomedicine Alleviates Colitis in Mice” Poster sessions, Experimental Biology, San Diego, CA, April 21-25 2018
- **Jayawardena D,** Anbazhagan A, Dudeja P and Onyuksel H. “Colonic luminal delivery of vasoactive intestinal peptide nanomedicine for the alleviation of colitis”. Poster sessions, COP Research day, Chicago,IL February 9, 2018
- **Jayawardena D,** Anbazhagan A, Dudeja P and Onyuksel H. “Colonic luminal delivery of vasoactive intestinal peptide nanomedicine for the alleviation of colitis”. Poster sessions, AAPS annual meeting and exposition, San Diego, CA, November 12-17 2017; T3018
- **Jayawardena D**, Guzman G, Gill R, Alrefai W, Onyuksel H and Dudeja P “Expression and localization of the major receptor of vasoactive intestinal peptide (VPAC1) along the length of the intestine”. **Poster sessions** Experimental Biology Meeting Chicago, IL, April 22-26 2017: F241
- **Jayawardena D,** Anbazhagan A, Priyamvada S, Kumar A, Dudeja P and Onyuksel H. “VIP and its nanomedicine in DSS induced colitis”. **Poster sessions,** AAPS annual meeting and exposition, Denver, CO, November 14-17 2016;17W1200
- **Jayawardena D**, Anbazhazhagan A, Dudeja P and Onyuksel H. Therapeutic Role of Vasoactive Intestinal Peptide (VIP) Nanomedicine in a Model of DSS Induced Colitis. UIC College of Pharmacy research day 2016

- **Jayawardena D**, Dudeja P, Önyüksel H. Vasoactive intestinal peptide nanomedicine against DSS induced colitis. **Special invited speaker** Pharmaceutics graduate student research forum, Kansas city, MO, June 2016
- **Jayawardena D**, Dudeja P, Onyuksel H. "Vasoactive intestinal peptide nanomedicine in inflammatory bowel disease", **Special invited speaker** French American Doctoral Exchange Program, Grenoble, France, March 2016

Leadership positions held in student societies (most recent)

- Student Co-moderator in Pharmacogenomics session at AAPS annual meeting 2017
- Vice president Controlled release society Illinois student chapter
- Departmental representative for UIC Graduate student council

Awards and Fellowships

- Van Doren Scholar, UIC College of Pharmacy (Jan 2018)
- Winner UIC College of Pharmacy Image of research completion (Nov 2017)
- Winner poster session, Pharmaceutics graduate student research meeting (PGSRM), University of Michigan (June 2017)
- **UIC Dean's Scholar Fellowship** (Apr 2017)
- Edward Benes Fellowship, UIC College of Pharmacy (Feb 2017)
- Winner for best poster at the UIC College of Pharmacy research day AbbVie Award for Excellence in Research, Chemistry Category (Feb 2017)
- UIC student presenter award (Feb 2017)
- UIC Graduate Student Council travel award (Jan 2017 and Dec 2015)
- **UIC Chancellors Graduate Research Fellowship**, (Dec 2016)
- Winner oral session, PGSRM, University of Missouri, Kansas (June 2016)
- **French-American Doctoral Exchange Program (FADEX) 2016:** Nanoparticles in Medicine: (Mar 2016)

- **UIC Center for Clinical and Translational Sciences Pre-Doctoral Education for Clinical and Translational Scientists (CCTS PECTS) Fellowship** (Dec 2015)
- First class honors and valedictorian, B. Pharm Degree, University of Sri Jayewardenepura, Colombo, Sri Lanka (February 2011)

Teaching experience

- Research Assistant — Aug 2015- Present
  Trained several graduate students, visiting scholars and undergraduate students
- Teaching Assistant — Aug 2013-Aug 2015
  University of Illinois at Chicago
  Involved in Pharm D courses, Drug delivery systems II and III, Principals of drug actions and therapeutics III, Drugs and disease and Physiology. Responsibilities included taking part in discussions, teaching labs, giving pre-lab talks and exam proctoring and grading
- Assistant lecturer — Feb 2012-May 2013
  B. Pharm unit, Faculty of Medical Sciences, University of Sri Jayewardenepura
  Actively involved in teaching pharmacy students and conducting laboratory sessions and exam procoring in pharmaceutical technology, clinical pharmacy, medicinal chemisty and Pharmacognosy

Professional experience

- Clinical Research Coordinator — Sep 2011- Feb 2012
  Clinical Trials Unit, University of Kelaniya, Sri Lanka
  Assigned to the National Hospital of Sri Lanka as the Clinical Research Co-ordinator for a pivotal muli-centre, global clinical trial in Rheumatoid Arthritis

Pharmacy Internships and Training — 2011-2012

- Trained in clinical pharmacy with Dr. Ian Coombs and group at the University of Queensland Australia, ward visits and patient consultations were conducted with the supervision of a specialized clinical pharmacist
- One year internship with 3 months per organization in the following government organizations of Sri Lanka: State Pharmaceutical Manufacturing Corporation, Drug Regulatory Authority, National Drug Quality Assurance Laboratory, Cancer Hospital, Medical Supplies Division, Medical Research Institute, State Pharmaceuticals Corporation

Memberships

- UIC Rho Chi society
- American Gastroenterologists society (**AGA**)
- Graduate Women in Science (**GWIS**) ETA chapter
- American Association of Pharmaceutical Scientists (**AAPS**)
- American Chemical Society (**ACS**)
- Pharmaceutical Society of Sri-Lanka and registered Pharmacist at the Sri Lanka Medical Council (No:7727).