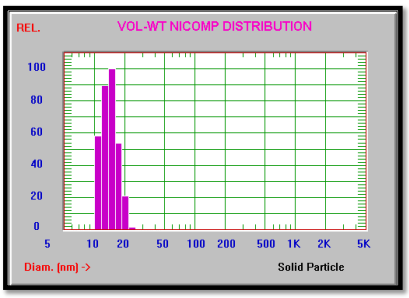
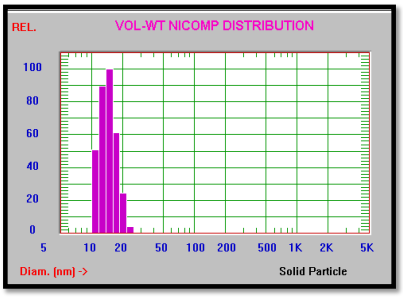
Supplementary materials

A **SSM**



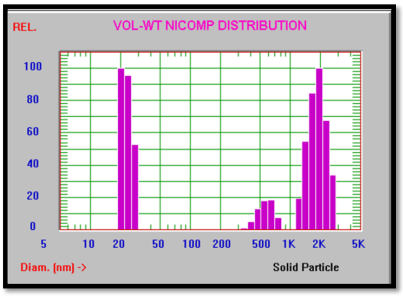
Peak	Mean Diameter	Standard deviation	Percent
1	14.8 nm	2.4	100

B **VIP-SSM**



Peak	Mean Diameter	Standard deviation	Percent
1	15.1 nm	2.5	100

C **VIP**



Peak	Mean Diameter	Standard deviation	Percent
1	24.3 nm	2.2	45.75
2	603.7 nm	99.7	8.51
3	2033.5 nm	348.8	45.73

Supplementary Figure 1: Characterization of various formulations for size.
Size of **A.** SSM, **B.** VIP-SSM, **C.** VIP peptide in Saline with dynamic light scattering. The various sizes of particles are defined in the table. VIP peptide solution undergoes decomposition and gives rise to multiple degradation and aggregation by products. SSM and VIP-SSM show similar particles size of ~ 15 nm.

Supplementary Method 1

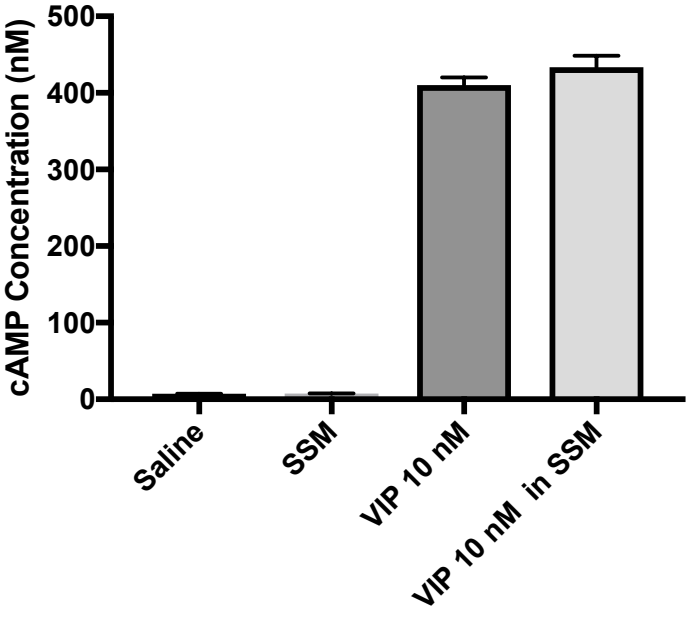
Determination of In vitro bioactivity of VIP and VIP-SSM uisng Cyclic adenosine mono phosphate enzyme immuno assay

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 [1, 2].

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 ^0C 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 [3]. 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).

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 (Supplementary Figure 2). 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.

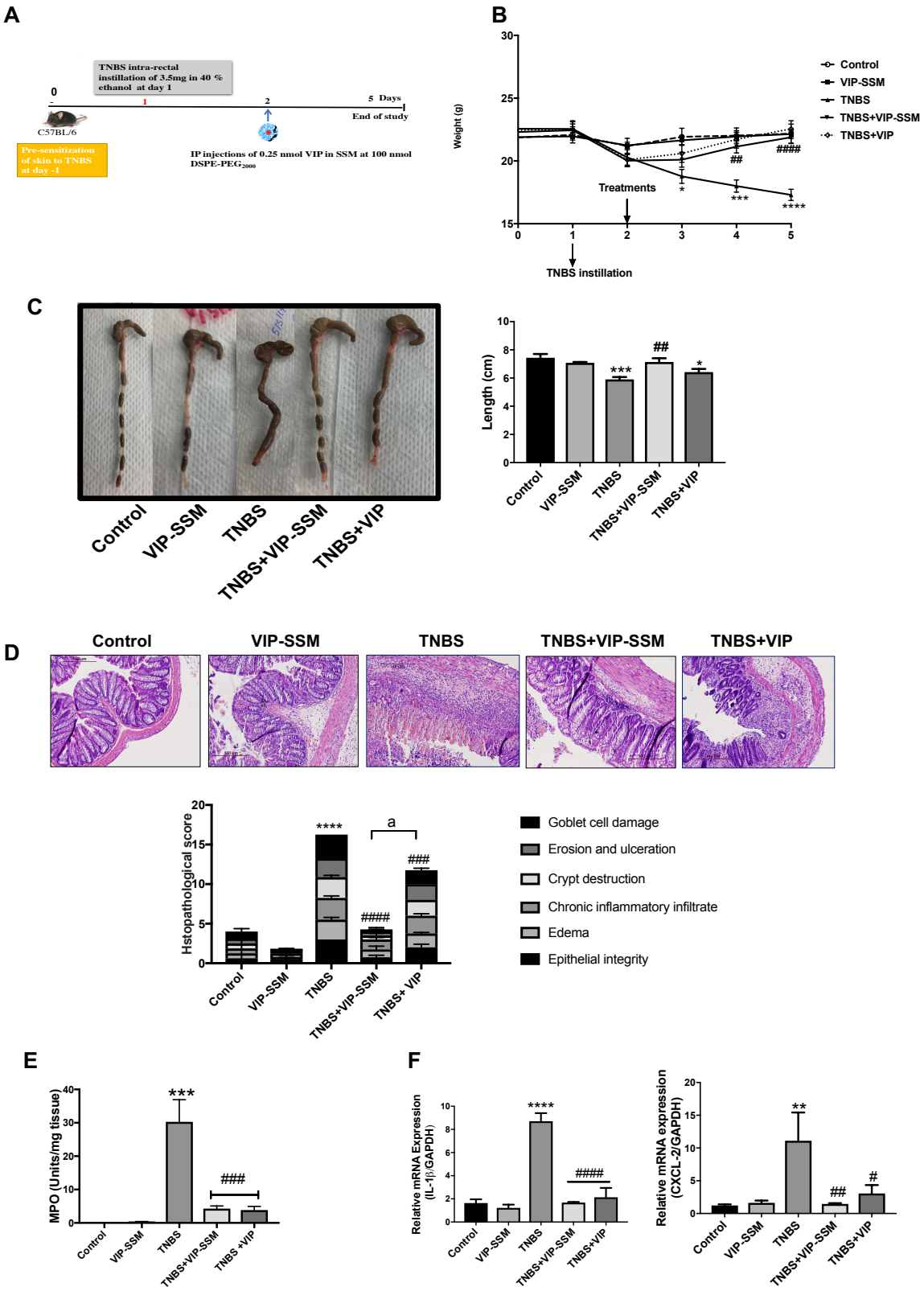


Supplementary Figure 2: Intracellular cAMP concentration measured by cAMP assay in human HT29 cell line (passage 48-50). n=4

Supplementary method 2.

TNBS study

Four to six-weeks-old male C57Bl/6 mice were purchased and acclimated for 1 week. 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 [4]. 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 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. At day 5, mice sacrificed and distal colonic tissues were harvested for analysis. At the end of the study, mice distal colonic tissues were analyzed for anti-inflammatory action.


A
TNBS intra-rectal instillation of 3.5mg in 40 % ethanol at day 1
0
1
2
5 Days
End of study
C57BL/6
Pre-sensitization of skin to TNBS at day -1
IP injections of 0.25 nmol VIP in SSM at 100 nmol DSPE-PEG2000
B
Weight (g)
Treatments
TNBS instillation
Control
VIP-SSM
TNBS
TNBS+VIP-SSM
TNBS+VIP
C
Control
VIP-SSM
TNBS
TNBS+VIP-SSM
TNBS+VIP
Length (cm)
D
Control
VIP-SSM
TNBS
TNBS+VIP-SSM
TNBS+VIP
Hstopathological score
Goblet cell damage
Erosion and ulceration
Crypt destruction
Chronic inflammatory infiltrate
Edema
Epithelial integrity
E
MPO (Units/mg tissue)
F
Relative mRNA Expression (IL-1β/GAPDH)
Relative mRNA expression (CXCL-2/GAPDH)

Supplementary Figure 3: VIP reversed inflammation associated with TNBS colitis after single dose

A. Schematic representation of animal study design. **B.** Body weight change of mice **C.** Representative photographs of whole excised colon mice in all treatment groups and graphical representation of colonic length. **D.** Representative histological micrographs of colons of mice and graphical representation of histopathological score. **E.** Graphical representation of myeloperoxidase activity in whole distal colonic tissues. **F.** Relative mRNA expression of proinflammatory cytokines. mRNA isolated from mouse intestinal mucosa from distal colon was subjected to qPCR with specific primers for interleukin 1 beta (IL-1β), C-X-C motif chemokine ligand- 2 (CXCL-2). Gene expression was normalized to internal control Glyceraldehyde-3-phosphate dehydrogenase (GAPDH).Data represented as mean $\pm$ SEM n=5 *=p<0.05,**-p<0.005, ***- p<0.0005 Vs control, ##-p<0.005, ###-p<0.0005, ####-p<0.0001 Vs DSS, a-p<0.0001 DSS-VIP-SSM Vs DSS-VIP.

Supplementary method 4.

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 [5]. This formula was based on allometric scaling, which is an empirical approach where the exchange of drug dose was based on normalization of dose to body surface area.

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

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 (60 nmol)**

Available volume of fluid in the human colon was assumed to be between 10-30 mL [6]. 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** (20 ng/µL)

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.

Supplementary method 5.

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.

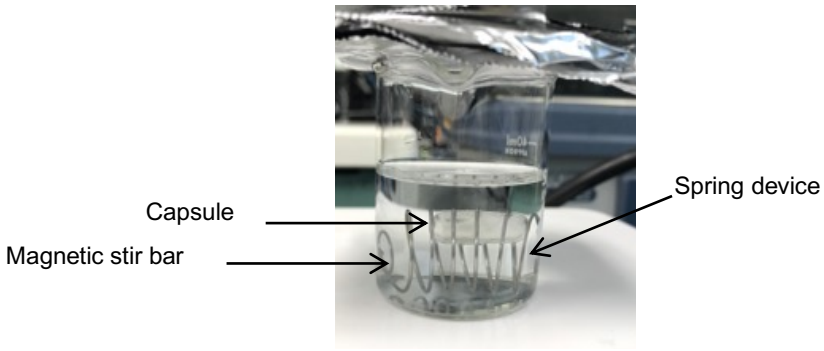
Preparation of 1mg/mL VIP in SSM

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 [7]. 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^{0}C 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. Capsules were sealed tightly with cap until secondary lock of the capsule 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.

Supplementary method 6.

Dissolution assay for capsules



Supplementary Figure 4: Dissolution assay apparatus modified for laboratory scale

Dissolution assay was performed using the set up shown in the Figure above. 30 mL of pH 6 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.

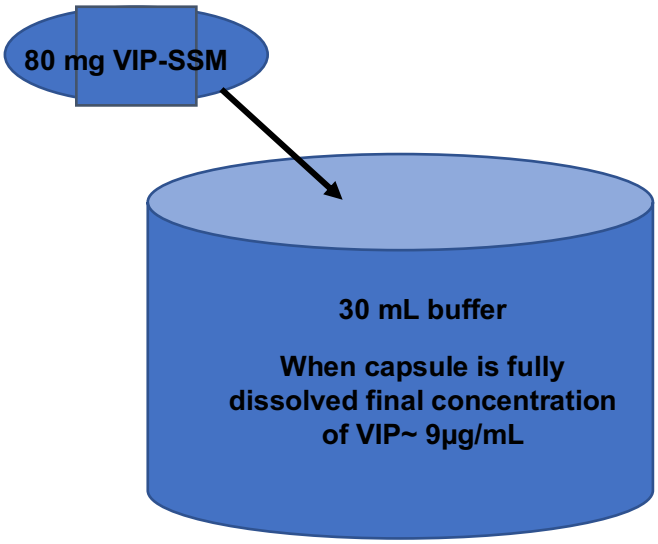
Supplementary method 7.

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 [8].

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

ELISA was performed at a later day with VIP EIA kit (RayBiotech, Peachtree Corners, GA) as per the manufacturer's 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 . The dilution of the beaker with 1 mL of fresh buffer at 6 time points resulted 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.



Supplementary Figure 5: Dissolution of VIP-SSM in simulated colonic fluid

Detection of peptide association with SSM after release 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 37^0C [9-11]. *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 with an inert filler diluent lactose [12, 13]. Three capsules per formulation were prepared for comparison. The nanomedicine capsules were prepared as described before. 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 before. 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 compare the stability between the two formulations.

Supplementary method 8.
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 before 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 earlier and the rest were 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.

Supplementary Table 1: Gene specific primer sequences

Gene	Sequence (5' → 3')
Mouse VPAC1	F- GATGTGGGACAACCTCACCTG R- TAGCCGTGAATGGGGGAAAAC
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 DRA	F- TGGTGGGAGTTGTCGTTACA R-CCCAGGAGCAACTGAATGAT

References:

1. 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* 37(2), 98-104 (2003).
2. 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* 10(1), 13-26 (1998).
3. 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* 231(1), 139-143 (1985).
4. 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* 7(11), e50388 (2012).

5. Nair AB, Jacob S. A simple practice guide for dose conversion between animals and human. *Journal of basic and clinical pharmacy* 7(2), 27 (2016).
6. Mcconnell EL, Fadda HM, Basit AW. Gut instincts: explorations in intestinal physiology and drug delivery. *International journal of pharmaceutics* 364(2), 213-226 (2008).
7. Vuković L, Madriaga A, Kuzmis A *et al*. Solubilization of therapeutic agents in micellar nanomedicines. *Langmuir* 29(51), 15747-15754 (2013).
8. Jayawardena D, Anbazhagan AN, Guzman G, Dudeja PK, Onyuksel H. Vasoactive intestinal peptide nanomedicine for the management of inflammatory bowel disease. *Molecular pharmaceutics* 14(11), 3698-3708 (2017).
9. Mody R, Tramontano A, Paul S. Spontaneous hydrolysis of vasoactive intestinal peptide in neutral aqueous solution. *Chemical Biology & Drug Design* 44(5), 441-447 (1994).
10. 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* 39(12), 1907-1910 (2013).
11. Kahne D, Still WC. Hydrolysis of a peptide bond in neutral water. *Journal of the American Chemical Society* 110(22), 7529-7534 (1988).
12. Hoag S. Capsules Dosage Form: Formulation and Manufacturing Considerations. In: *Developing Solid Oral Dosage Forms (Second Edition)*, (Ed.^(Eds).Elsevier 723-747 (2017).
13. Stegemann S, Bornem C. *Hard gelatin capsules today-and tomorrow*. Capsugel Library, (1999).