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Development of Novel Therapies for the Treatment of Inflammatory Bowel Disease

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Development of Novel Therapies for the Treatment of Inflammatory Bowel Disease

By

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TABLE OF CONTENTS

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Shrey Kanvinde

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ABSTRACT

Development of novel oral therapies for the treatment of inflammatory bowel disease

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University of Nebraska Medical Center, 2018

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Inflammatory bowel disease (IBD) is a chronic and remittent inflammation of the gastrointestinal tract (GIT). Despite extensive research efforts, there is no cure nor a well-defined pathogenesis for IBD. Loss of epithelial barrier function, increased colonic immune cell infiltration and upregulation of pro-inflammatory cytokines are the hallmarks of IBD. Despite treatments like painkillers, aminosalicylates, steroids, and biologics, almost 70% patients require surgery at least once in their life time. The main limitation with most of the current treatments is they are either absorbed systemically or administered systemically resulting in adverse side effects. As a result, there is a huge unmet need for therapies that can be safely and locally delivered to treat inflammation.

Chloroquine (CQ) has been used as an anti-malarial for a long time and recently it has found anti-inflammatory applications. However, long term administration of CQ results in severe side effects like retinopathy because of systemic absorption. In this dissertation, we have re-designed CQ into a polymer (pCQ) and evaluated its potential as an orally administered IBD therapeutic. We found that pCQ showed preferential localization in the GIT which almost negligible systemic levels. We further evaluated the anti-inflammatory activity of pCQ in a mouse model of IBD and found reduction in colon

inflammation. We achieved this while reducing the systemic absorption almost 100 times which translates into an increased safety profile.

We then assessed the effect of local delivery of combination of TNFα siRNA and polymeric CXCR4 antagonist (PCXA) via chitosan (CS) nanoparticles (NPs) *in vivo*. We found that the particles not only demonstrated a desirable size but also protected the siRNA against biorelevant conditions which are usually encountered in the GIT. Our results also indicate uptake of these particles by macrophages which are target cells and infiltrated the inflamed colon tissue in IBD. We tested the particles *in vivo* in a mouse model of colitis. We observed the therapeutic effect due to CXCR4 inhibition as well as observed TNFα silencing in the colon.

Both these systems showed promise as local anti-inflammatory therapies. However, further development is needed to enhance their anti-IBD potential.

LIST OF FIGURES

LIST OF TABLES

LIST OF SCHEMES

LIST OF ABBREVIATIONS

GFP: Green fluorescent protein

CS: Chitosan

PCXA: Polymeric CXCR4 antagonist

siRNA: Small interfering ribonucleic acid

GIT: Gastrointestinal tract

IBD: Inflammatory bowel disease

NP: Nanoparticle

SGF: Simulated gastric fluid

SCF: Simulated colonic fluid with bile salts

AMD: AMD3100

GEN: Genistein

CPZ: Chlorpromazine

WRT: Wortmannin

6-FAM: 6-Carboxyfluorescein

DSS: Dextran sulfate sodium

C. rodentium: Citrobacter rodentium

PFA: Paraformaldehyde

pCQ: Polymeric Chloroquine

HCQ: Hydroxychloroquine

MPO: Myeloperoxidase

H&E: Hematoxylin and eosin

PFA: Paraformaldehyde

OD: Optical density

STAT3: Signal transducer and activator of transcription-3

TKNs: Thioketal nanoparticles

ROS: Reactive oxidative species

SOD: Superoxide dismutase

DHFR: Dihydrofolate reductase

5-ASA: 5- aminosalisylic acid

TNF: Tumor necrotic factor

FDA: Food and drug administration

EMA: European medicine agency

PML: Progressive multifocal leukoencephalopathy

JAK: Janus kinase

MGL: Macrophage galacotse-type lectin

E. coli: Escherichia coli

AIEC: Adherent-invasive *E. coli*

PDC: Polymer drug conjugates

TMC: Trimethyl chitosan

IVIS: *In vivo* imaging system

Chapter 1: Introduction

1.1 Inflammatory Bowel Disease

IBD is a collective term for a group of chronic inflammatory diseases affecting the GIT. It majorly manifests itself in two forms – ulcerative colitis (UC) and Crohn's disease (CD). UC and CD can share many clinical characteristics, although they are considered distinct inflammatory conditions. Chronic and relapsing mucosal inflammation is a major characteristic of both UC and CD. UC affects the colon which is the terminal part of the intestine. UC inflammation affects only the mucosa without affecting deeper tissue layers. In UC, the inflammation usually extends proximally from the colon and is continuous but confined to colon and rectum. Inflammation in CD can affect any region of the GIT and is usually discontinuous. CD inflammation in transmural, meaning it affects the deeper layers like muscularis and serosa as well. Other symptoms include intestinal swelling, abdominal pain, diarrhea, blood in stool and weight loss. IBD has an annual healthcare cost of over $1.7 billion in the United States. Despite extensive research efforts, the exact mechanism of how IBD occurs is still unknown. Current research points out in the direction that IBD is a result of a complex interplay between a variety of factors. Broadly the factors can be classified as follows –

1.1.1 Genetics

There have been recent technological developments in DNA analysis and sequences in conjunction with utilization of huge multinational databases [1]. This has led to many groups exploring genetic contributions to IBD development. All these studies have

identified 163 which are associated with IBD of which 30 are CD specific, 23 are UC specific and 110 are associated with both the conditions [2]. NOD2 which encodes for a protein recognizing MDP in bacteria was the first identified susceptibility gene for CD [3]. Stimulation of MDP induced autophagy and modulates the immune system. It also participates in MDP-independent pathways like T-cell regulation. A genome-wide meta-analysis has already shown an association between NOD2 and development of CD [4]. Numerous studies have reported the effect of autophagy in IBD immune response particularly two genes, ATG16L1 and IRGM [5]. Autophagy plays a role in intracellular homeostasis by degrading and recycling cytosolic contents as well as intracellular microbes. ATG16L1 mutations have been implicated in higher risk of IBD incidence [6]. IBD-associated polymorphisms in IRGM leads to reduced protein expression. Genetic screen studies have identified the involvement of IL23R, JAK2, STAT3 in both UC and CD [7]. Reported studies have proved that the expanding number of susceptibility gene loci described in IBD is an indication that genetic influences are critical components of the disease pathogenesis. However, explainable susceptibility loci discovered so far account for only 20%-25% of the heritability found in the above-mentioned studies. This is not only true for IBD, but also true for many other polygenetic diseases, and the phenomenon has been called “the mystery of missing heritability of common traits” or “genetic vacuum” [8]. New insights like gene-gene interactions, gene-pathway interactions and gene-environment interactions may lead to more valuable information in IBD pathogenesis than just focusing on finding new gene variants.

1.1.2 Environmental triggers

Environmental factors play an important role in the pathogenesis of IBD. The common environmental triggers include smoking, diet, drugs, geographical location, social stress, and psychological element. Amongst all these, smoking is the most studied and well-documented factor for IBD. In 1982, an inverse relationship between smoking and UC development was reported [9]. Subsequently few reports confirmed the protective effect of smoking on UC development where patients who were smokers had a lower rate of relapse of inflammation. However, reports on CD described that smoking increases the incidence of CD and correlates with higher rate of postoperative disease [10].

Many efforts have been directed at associating dietary excess or shortage of various foods as a factor in incidence of IBD [11]. Most commonly, higher intake of saturated fatty acids and monosaccharides, as well as low dietary fiber have been associated with a higher risk of CD development [12]. Many studies have emphasized the conflicting role of high intake of dietary monosaccharides on development of IBD. In retrospective studies, CD patients who felt sick had a higher percentage of monosaccharides in their diet [13, 14]. Russel and co-workers showed associated consumption of cola and chocolate with higher incidence of IBD [15]. Their observations were confirmed by Sakamoto and co-workers by demonstrating higher incidence of IBD in patients who consumed sweets and artificial sweeteners [16]. Contrary to these findings, Chan and co-workers conducted a large study having over 400000 volunteers that reported no association between the intake of monosaccharides and the incidence of either UC or CD [17]. Increased consumption of lactose was not reported to have any effect

on the development of IBD [14]. Few reports also demonstrated a slight increased risk of IBD development by excess consumption of animal protein. The same group also reported that a high fat diet, especially rich in cholesterol and animal fats may increase the rate of incidence of IBD [18]. These findings were corroborated by Ananthakrishnan and co-workers, who confirmed the effect of trans fatty acid consumption on UC development [19]. Consumption of linolenic acid has been linked to development of IBD. This fatty acid gives rise to arachidonic acid which is broken down into metabolites that exhibit pro-inflammatory properties [20]. John and co-workers carried out a study of over 25000 participants maintaining a 7-day food diary and demonstrated the protective effect of unsaturated omega-3 fatty acid intake on the occurrence of UC [21]. Many studies have reported that high fiber consumption reduces the risk of IBD up to 40% [22]. Vitamin D deficiency is commonly observed in IBD patients. As such, few researchers believe this deficiency to be a dietary factor that may increase the incidence of IBD. Increased intake of fruit whole or as fruit juice especially with Vitamin C has been implicated in reduced risk of IBD development [23]. No correlation between alcohol intake and IBD development has been observed [24]. Looking at the overall literature, dietary factors play an underestimated role in the development of IBD. Following a certain diet maybe of advantage in reducing the incidence of IBD, especially for individuals already affected by such conditions. This might help in achieving or maintaining stages of remission and improving the overall quality of life. However, there is no single specific diet for IBD patients, so dietary recommendations can be used in supplement to therapy [25].

According to ecological and epidemiological evidence, air pollution may play a role in the incidence of IBD as well. Countries with higher development of industrialization have shown an increase in the incidence of IBD [26]. Increase in pollution levels correlates with an increase in circulating polymorphonuclear leukocytes and plasma cytokines [27]. Overall, environmental factors represent a gray area in IBD pathogenesis. Multiple explanations have been reported describing the interactions of environmental factors with the components of immune system resulting in an abnormal inflammatory response, however, none of the explored factors have convincingly described the pathogenesis of IBD [28]. Thus, further studies are needed to explain the role of environmental determinants of IBD.

1.1.3 Microbial factors

The GI environment is rich in microorganisms. About 10^{11}-10^{14} microorganisms which contain 300-500 bacterial species are reported to be present in the gut [29]. In healthy individuals, bacteria play an important role in intestinal homeostasis. The role of gut bacteria in nutrition, immune development and energy metabolism has been elucidated [30]. Majority of the gut bacteria are gram-negative with minor population of gram-positive bacteria [31]. The diversity and the number of the gut microbiota are severely affected during presence of inflammation [32]. Almost 25% less genes were detected in the feces of IBD patients compared to healthy individuals [33]. There are multiple indications that bacteria may play a role in the development of IBD, as evidenced by the effectiveness of antibiotics in some IBD patients [34]. Secondly, IBD patients have increased antibody titers against bacteria which are present in gut under normal

conditions [35]. The genetic parameters associated with bacterial detection like NOD2 and T cell immunity are implicated in IBD [36]. Lastly, most of the animal models of IBD need luminal bacteria for initiation of inflammation [37]. To date many pathogens have been assessed as causative agents for IBD pathogenesis. Reduction of phyla *Firmicutes* is a well-defined change that has been reported by multiple metagenomic studies [32, 38]. Analysis of the fecal microbiome showed reduction in anti-inflammatory microbiota and an increase in the pro-inflammatory microbiota [39]. Most known pathogenic bacteria in the human GIT belong to phylum *proteobacteria* [40, 41]. Adherent-invasive *E. coli* has been reported in multiple studies. AIEC usually invades the epithelium and replicates in the macrophages [42]. AIEC has been detected more frequently in patients with IBD compared to healthy individuals [43]. Similar observations have also been made for *Campylobacter concisus, Clostridium difficile* and few other bacteria [44, 45]. Although, all this data suggests the potential role of bacterial imbalance might play a role in IBD development, no study has pointed out whether these changes are causative in IBD or just a consequence of the alterations in the GI environment during inflammation. Thus, further studies which point out the response of the gut microbiota to various other factors are needed to strengthen the role of the microbiota in the development of IBD.

1.1.4 Immunological factors

The role of mucosal immunity, especially T cell response has been studied for a considerable time in IBD pathogenesis. There is evidence that imbalance between the innate and the adaptive immune pathways plays a role in the erratic immune response observed in IBD. Adaptive immune response has been the focus of many pathogenesis

studies in IBD and lead to the conclusion that CD is driven by a Th1 response while UC has been associated with an unconventional Th2 response. Innate immune response has been recently studies and has been associated with epithelial barrier integrity, innate microbial sensing and unfolded protein response.

Innate immunity forms the first line of defense against pathogens which is often non-specific and allows the body to respond quickly to stimuli usually within minutes. It is mediated by epithelial cells as well as immune cells. This response is initiated by microbial pattern recognition receptors present on cell surface called TLRs and NOD-like receptors in the cytoplasm. Studies have found the behavior and expression of both these receptors to be significantly altered in IBD patients. Neutrophil accumulation, IL-1β and IL-8 production was reduced in IBD patients. Studies have revealed that mutations in NOD2 which are associated with CD affect the ability of the gut to response to LPS contributing to disease development. Although controversial, available reports point the role of NOD2 mutations in reduced activation of NF-κB as well as development of tolerance. IL-23 represents a key cytokine in immune responses against pathogens. Polymorphisms in IL-23 gene have been reported to play a role in both UC and CD. The epithelial barrier represents the second line of defense against the invasion by bacteria. Increase in the intestinal permeability and loss of the epithelial barrier has been observed in IBD. Mucus layer which covers the epithelium represents the first barrier against microbes. The cells which constitute the epithelium also secrete many anti-microbial peptides. The expression of these peptides has also been to be altered in IBD patients. The

role of the adaptive immune system in IBD development is still undergoing extensive research.

1.2 Current treatment strategies and challenges

Therapy options which are currently utilized include conservative measures as well as surgical procedures for patients who do not respond to treatment. Traditionally, the goal of IBD therapy has been to alleviate the symptoms of the disease and prolong remission. Many drug- as well as patient related factors influence the treatment of IBD. Drug-related factors include PK and PD, while patient-related factors include severity of disease, location of inflammation and responsiveness to treatment. Due to the absence of a permanent cure and the chronicity of the disease, patients essentially must be on treatment life-long [46]. Presently, a wide array of drugs both conventional and novel are available for treatment of IBD. Despite this, almost 80% of patients require surgery due to failure of therapy [47]. Current treatments for IBD can be divided into five main categories (Fig. 1) which are described as follows-

1.2.1 Anti-inflammatory drugs

Anti-inflammatory drugs are one of the earliest therapies which were used for the treatment of IBD. To date they represent one of the most frequently used therapy options in patients with ulcerative colitis. Aminosalicylates form a majority of this class of drugs. 5-ASA usually recommended as the first line of therapy for mild to moderate IBD. It is usually used as an oral or rectal formulation. Pro-drugs such as sulfasalazine, balsalazide are also available [48]. Aminosalicylates mainly function by inhibition of lipoxygenase

and cyclooxygenase pathways which are involved in metabolism of arachidonic acids which are the precursor of eicosanoids and downstream inflammatory cytokine signaling [49]. Patients who do not respond to aminosalicylates are usually treated with glucosteroids and corticosteroids [50]. Both these classes affect the immune and inflammatory responses, and function by reducing pro-inflammatory cytokines [51].

1.2.2 Immunosuppressants

Patients with severe IBD who are unresponsive to corticosteroid therapy are usually treated with immunosuppressive agents like cyclosporine or MTX. Most of the drugs of this class have been used in other diseases and cancers way before they were used for treatment of IBD. MTX is a folate analog and an inhibitor of DHFR [52]. It interferes with synthesis of DNA and exhibits anti-inflammatory effects including reduced production of pro-inflammatory cytokines and lymphocyte apoptosis [53]. Clinical trials for oral and IM therapies have been conducted. MTX showed significant improvement in IBD patients. MTX also maintained remission in patients [54]. However, MTX group also suffered more side effects which were nausea or liver test abnormalities [55]. Patients who are steroid dependent for remission are treated with azathioprine and mercaptopurine. Both these drugs are purine analogs and are converted to active metabolites that interfere with nucleic acid synthesis, lymphocyte proliferation. They have been studied in the treatment of IBD since 1960s, with multiple trials showing promising effects. However, most of the trials indicated that therapy with AZA or 6-MP would be usually for an indefinite amount of time for responding patients. Although effective in induction and maintenance of remission, long term usage of these drugs leads to

development of pancreatitis and leukopenia. As a result, the use of this medication is best coupled with another regimen like steroids.

1.2.3 Biologic agents

This class represents the newest and one of the most clinically used therapeutics which directly targets the main features of inflammation. Antibodies against upregulated pro-inflammatory cytokines form a major part in this group. TNFα is a potent mediator of inflammation which is commonly upregulated in the intestinal mucosa of IBD patients [56]. It is one of the late-stage cytokine of inflammatory cytokine cascade. It has been reported to play an important role in the development and progression of the disease. As a result, TNFα neutralization has been an extensively explored therapeutic strategy in various inflammatory disorders [57]. TNFα antibodies have been the most successful clinically translated biologic therapy for IBD.

Infliximab is a human chimeric monoclonal antibody which is a genetically constructed IgG1 murine antibody binding to both the soluble and the membrane-bound TNFα [58, 59]. It has been successful in the induction as well as remission maintenance of CD and UC [60, 61]. Adalimumab and Certolizumab are both TNFα antibodies which have been clinically approved for IBD therapy. Improvement in TNFα antibodies have also been made by modifying their structure. Certolizumab pegol is a pegylated, Fab' fragment of a humanized monoclonal antibody which binds TNFα [62]. The pegylation improves the stability and enhanced the circulation time in the body leading to reduction in dose [63]. It has shown better remission rates as compared to other antibodies on market

[64]. It does not contain Fc portion and consequently does not show cellular cytotoxicity [65].

Despite being successful, these therapies have face many limitations. Immunogenicity is observed in some patients as they develop antibodies against the monoclonal agents. The development of immunogenicity usually occurs through both thyroid-dependent and thyroid-independent mechanisms [66]. These anti-drug antibodies are usually IgG antibodies that hinder the binding of monoclonal therapeutic to its target cytokine or increase the rate of excretion via RES [67]. Almost 60% patients were reported to develop anti-drug antibodies against infliximab [68]. As a result, the dose of infliximab has been optimized which has lowered the prevalence of anti-infliximab antibodies to 10-20% in clinical trials [69]. However, in this patient population, loss of clinical response and rise of infusion reactions has been observed once they develop anti-infliximab antibodies [70]. Another limitation, majorly because of the systemic silencing of TNFα is the development of immunosuppression, which predisposes these patients to opportunistic infections and development of colon cancer [71]. Development of active TB with infliximab has been documented [72]. As a result, approaches other than targeting inflammation through cytokines have also been explored [73]. Such therapies can be used in conjunction with anti-TNFα therapies to improve the therapeutic outcomes in IBD therapy or for treating patients who are refractory to anti-TNFα therapies. One such strategy which has attracted significant attention is anti-adhesion therapy [74]. IBD involves extensive lymphocyte trafficking from the circulation which interact with the endothelial cells using adhesive interactions [75]. The proteins involved in these

interactions represent attractive targets for developing new therapeutic strategies for controlling inflammation in IBD. Antibodies against adhesion molecules and integrins have been developed. Natalizumab is developed against anti-α_4 integrin to reduce lymphocyte migration into the intestinal mucosa [76]. However, since this antibody was not specific to the intestine, there were concerns with development of PML leading to decline in its use [77]. This antibody became a building block for this concept and led to development of similar future drugs. First drug from the second-generation drug includes "gut selective" vedolizumab. It is an anti-$\alpha_4\beta_7$ antibody that shows gut specific immunosuppression. Clinical trials have not only indicated the efficacy but also reduced risk of PML with vedolizumab leading to approvals from FDA and EMA [78]. Similar gut selective antibodies like etrolizumab (anti-β_7 integrin) and PF-00547659 (anti-MadCAM-1) have been developed and undergoing clinical trials [79, 80]. AJM300 is a recently developed oral α_4 integrin antagonist [81]. It has concerns with development of PML, however, a discontinuous therapy with this agent is possible because of its short half-life (daily dosing of three times) providing a possible strategy if PML were to occur [82].

There are other newer generation biologic drugs in phase III clinical trials. Ustekinumab is an antibody against IL-12/23 which has been clinically approved and used for treatment of psoriasis and arthritis [83]. Its potential in treatment of IBD is being studied. Another class of upcoming biologic drug includes Tofacitinib which is a JAK1 and JAK3 inhibitor [84]. A novel aspect of this drug is that it has been developed for oral administration. It has been approved for rheumatoid arthritis and is under clinical trials for treatment of IBD.

1.2.4 Antibiotics

The GI ecosystem contains many bacteria, the composition of which changes with time, age, diet and geography [85]. Although a causative species has not been pointed out, there is plenty reports confirming the involvement of the GI microbiota in the development of inflammation in IBD [86]. No IBD development is observed in genetically engineered murine models that are raised in germ free environment but occurs when bacteria are introduced in the intestines. As a result, manipulation of the GI microbiome has been explored as an anti-IBD strategy [87]. Antibiotics can be used to control the inflammation by decreasing the bacterial population in the lumen and changing the composition of the intestinal microbiome to favor beneficial bacteria [88]. Antibiotics are usually used prophylactically in IBD treatment [73]. Few clinical trials have tested commonly used antibiotics like macrolides, fluoroquinolones, 5-nitroimidazoles and rifaximin either individually or in combination [34]. All the studies revealed significant benefits. Similar benefits were also observed in studies with ciprofloxacin and metronidazole in CD [89]. These studies even revealed that antibiotic treatments induced remission. However, the limitation with the use of antibiotics is the presence of many sources of variation. Other than patient-related factors, various antibiotics have different antibacterial spectrum. As a result, it becomes confusing to analyze data from trials which involve a combination of antibiotics. Additionally, antibiotics have side effects that they induce. Ciprofloxacin causes tendonitis and photosensitivity [90]. Metronidazole causes GI distress and peripheral neuropathy if used long term [91]. Antibiotics may also cause

a rebound effect in GI bacteria after the therapy is stopped leading to relapse [92]. Antibiotic resistance development is another limitation [93].

1.2.5 Symptomatic relief agents

Underlying mechanisms and pathophysiology of IBD have received significant attention. However, the absence of a solid causative mechanism makes treatment confusing. Surprisingly, there has been very few reports about the various symptoms that are experienced by IBD patients. One survey study reported that a significant number of patients experience diarrhea, fatigue, abdominal pain, joint ache and night sweats [94]. Presence of blood or mucus in stool was reported to be more prevalent in UC patients compared to CD patients. Even in phases where the patients were reported to have inactive disease, they still experienced the symptoms. Other than physical symptoms, almost half the patients experienced stress [95]. Most common reported stress was family stress followed by work and financial stress. However, it is difficult for clinicians to associate stress with the presence of symptoms as feeling stressed with the presence of symptoms is common. The reports on correlation between the pharmacological and psychological parameters in IBD is limited. As a result, this area needs better research.

The current symptomatic treatment focuses on management of pain and diarrhea. Acetaminophen is widely used in place of NSAIDs that have been considered as a potential cause of flares in IBD [96]. For more advanced pain relief, narcotics have been prescribed. However, their use is very minor as they are associated with increased risk of mortality [97]. Common anti-diarrheal drugs used in IBD include loperamide,

diphenoxylate, codeine and cholestyramine. Loperamide has been used most extensively in a postoperative setting. Clinical trials have proved the efficiency of this drug in managing diarrhea during IBD [98]. GI side effects as well as effects on normal physiology of the GIT were less as compared to placebo group [99]. Based on the no reduction in the defecation frequency and occurrence of side effects, many studies reported that diphenoxylate has no place in the management of IBD symptoms. Looking at the bigger picture, it is observed that a clinician's decision about the treatment is still influenced by the patient's symptoms [73]. Symptomatic relief therapies will always be needed even though a more pathophysiology based therapy is developed at some point of time. Thus, finding treatments that can manage abdominal pain and diarrhea with less side effects are needed. At the same time, assessing the efficacy of both psychological and pharmacological interventions in IBD is needed.

1.3 Need for oral therapies for IBD

As described in the previous section, majority of the current therapies in IBD are administered parenterally. Long-term dosing is needed in a chronic condition like IBD and these therapies, in the long run show severe side effects. As a result, the payoff for all these drugs comes at a huge risk. This has resulted in an unmet need therapies that act locally in the colon, at the site of inflammation. Oral route of delivery is an attractive option for delivering anti-inflammatory agents where the inflammation is local. In addition to its traditional advantages like ease of administration, it also offers a route to deliver the treatment directly to the site of action with minimal non-specific exposure. Rectal route is another interesting avenue. However, it is difficult to target discontinuous

inflammation or inflammation in the proximal part of the GIT with the rectal route. In case of oral administration, with appropriate modifications in the delivery system, a local effect as opposed to a systemic effect can be achieved. This will limit all the systemic side effects while allowing safe and prolonged dosing regimens.

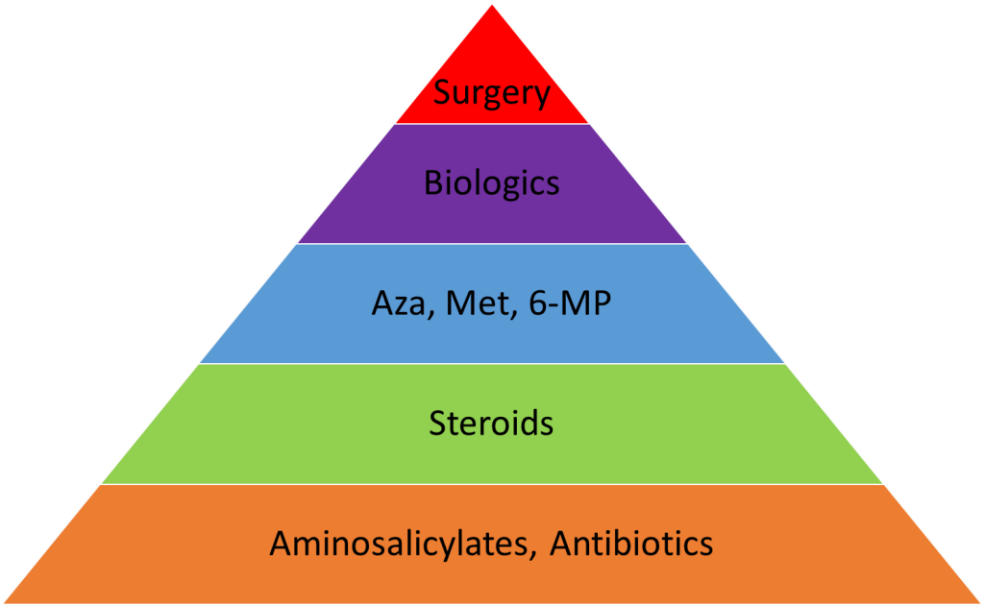


Figure 1. Options for treatment of IBD (Adapted from [52])

1.4 Challenges in oral delivery for IBD treatment

Despite being a promising route of administration, oral delivery to the colon presents its own challenges. The major challenge being that it relies on a number of physiological factors to achieve optimal drug concentration at the site of action, to make sure therapeutic efficacy is achieved. The physiological conditions in the colon of an IBD patient vary significantly from those in a healthy individual. The changes which occur in the GIT during inflammation are highlighted in this section (Fig. 2). They form an important consideration in the formulation of systems intended to deliver therapeutics to the colon.

1.4.1 Transit time

In humans, colonic transit time is usually 2 to 6 hours. However, gastric and colonic time in IBD patients vary significantly compared to healthy individuals. This is mainly due to the presence of diarrhea which is very common in IBD patients. Colonic transit times in IBD patients have been reported to range from 6 to 70 hours. This represents a significant hurdle in determining the time of dosing with respect to bowel movements. Changes in the transit time have been reported to significantly impact targeting specific regions of the colon using conventional formulations as the drugs show higher retention in the proximal GIT as compared to the colon.

1.4.2 pH

There are very few reports indicating any changes in the small intestinal pH in IBD patients. However, colonic pH is significantly acidic in both UC and CD patients. pH in

colon is influenced by many processes like microbial fermentation, metabolism of fatty acids by bile, various secretions, intestinal volume and transit times. Since all the parameters are disrupted during IBD, changes in the colonic pH are not unexpected. pH in the colon ranges from 6.8 to 7.2 from proximal to distal colon, respectively. These values can significantly differ in patients with IBD with the reported values being in the range of 5.5 to as low as 2.3, irrespective of the stage of the disease [100, 101]. These changes in the pH affect the composition of the colonic microbiome and well as the transit times. This can alter the behavior of formulations which are enzymatic or pH-dependent for release of drugs.

1.4.3 Intestinal volume

The composition of intestinal luminal contents varies significantly in IBD patients compared to healthy individuals. These changes directly impact the transit time as well as the pH conditions. Higher fluid secretion leads to dilution of the enzymes that play a vital role in absorption through the colon. This may also alter the composition of the intestinal flora which alters the digestion of carbohydrates leading to changes in transit times. These changes can affect the way conventional formulations are processed in the GIT and create hurdle to local delivery of drugs.

1.4.4 Histological changes

The intestinal epithelium represents one of the most selective barriers in the human body. It regulates the transport of the luminal contents into the tissue. Small molecules are restricted selectively while macromolecules are not transported. While this

barrier seems to be an attractive target that can be manipulated to achieve selective absorption of drugs, epithelial integrity is lost in IBD. Inflammation not only disrupts the barriers but also affects the individual cell types. Significant crypt damage as well as loss of the intestinal brush border affects the uptake and absorption properties of the epithelium. There is loss of goblet cells which are responsible for mucus production which results in thinning of the mucus layer which usually represents the first barrier to reach the tissue. This affects the permeability of lipophilic drugs and mucoadhesive systems.

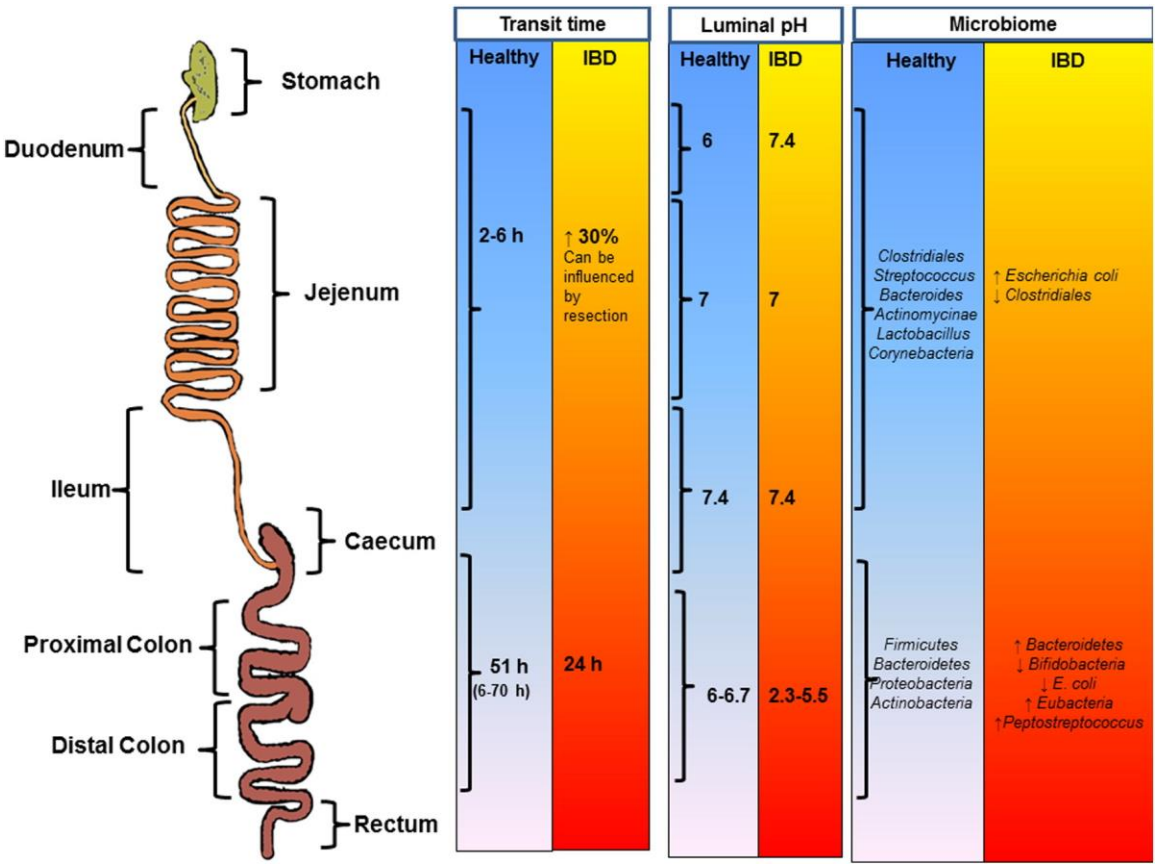


Figure 2. Physiological changes in the GIT in IBD. (adapted from [102])

1.5 Applicability of oral particulate delivery systems in colonic inflammation

As discussed previously, the current treatments for IBD are largely limited due to their systemic side-effects. With the biologic therapies like antibodies, development of resistance and immunosuppression are other concerns. With a view to overcome the limitations of traditional small molecule drugs for IBD, sustained-release drug delivery systems like capsules and enteric coating tablets have been developed for release drugs specifically in the colon. These delivery systems minimize the absorption of drugs in the upper GIT by releasing active agents influenced by colon specific conditions such as pH, enzymes, etc. However, as discussed in preceding section, the colonic conditions in the presence of active inflammation vary significantly compared to healthy colon. Inter-patient variability is another confounding factor. Consequently, current macroscopic delivery systems lack specificity to target only the inflamed parts [103]. Furthermore, about 90% of the IBD patients experience diarrhea which results in rapid intestinal clearance of these delivery systems, compromising their efficacy. To circumvent the limitations of the current macroscopic delivery systems for IBD, many groups have focused on utilizing particulate delivery systems. In 1998, Rein and co-workers laid down a hypothesis stating that the disruption of the intestinal barrier at the inflamed colonic regions could allow preferential accumulation of particles in those areas. Additionally, the accumulation of macrophages and dendritic cells at the inflammation site could result in higher uptake of particles. A size-dependent deposition study of fluorescent polystyrene microparticles and nanoparticles in an experimental model of colitis was carried out as a step in the direction towards this strategy. Higher adhesion of particles

was observed at the layers of inflamed tissues which had more mucus while in the ulcerated areas, a size dependency was observed. Targeting to inflamed areas became more effective with smaller particles. Particles in the submicron sizes showed higher uptake by macrophages and could evade clearance by diarrhea which is a common symptom of IBD. Encouraged by these studies, many groups started using particulate systems to encapsulate drugs or biologics to create more applicable and complex treatment strategies for IBD therapy which were either dependent on their size or harnessed a colon-specific target for accumulation. The following part reviews development of some of these systems according to their driving stimulus.

1.5.1 Size dependent systems

Particle size reduction to the nanometer scale has been shown to improve the accumulation of nanoparticles in the inflamed colonic areas thus a promising finding for IBD therapy. The size reduction allows for enhanced and specific delivery of active principles in inflamed tissue via an epithelial enhanced permeability and retention effect while allowing selective uptake by the immune cells that accumulate in these areas [104]. Accumulation in the inflamed tissue increases the local concentration of drugs which is a useful parameter for IBD therapeutics. Reduction in the size also helps particles escape quick clearance due to diarrhea [105]. NPs can undergo internalization by paracellular transport, transcytosis through M cells and through gaps in the intestinal villi [106, 107]. There have been numerous reports which look at particle deposition as well as uptake in inflamed colon based on their size. Highest binding to inflamed colon mucosa was observed with 100nm particles when polystyrene particles ranging from 0.1 to 10 μm were

orally administered for 3 days in TNBS colitis model in rats [108]. However, after washing off the mucus there was a significant reduction in the particles suggesting high proportion of particles getting entrapped in the mucus rather than being taken up by immune cells. This effect was observed independent of the particle material as a number of studies showed similar effect with various non-functionalized particles [109]. A comparative study between NP and microparticle uptake into rectal mucosa in human IBD patients showed that microparticles accumulated to a higher extent while only trace amounts of NPs were detected [110]. Although the microparticles showed enhanced bioadhesion and accumulation, there was negligible absorption across the epithelial barrier. On the contrary, NPs were found to be transported to the serosal compartment indicating microparticles might be a more suited drug delivery vehicle for local colonic application in humans. There is no explanation for the opposite observations for the effect of particle size on colon accumulation between animals and humans. While a glance at the reported study suggests that a reduction in particle size is essential for accumulation in the colon, there is no proof that that non-functionalized particles show specific accumulation in the inflamed versus the healthy parts of the colon *in vivo*. As a result, other strategies for enhancing the accumulation in inflamed regions including surface modification have been studied.

1.5.2 Surface charge dependent systems

Very few reports have been published about the effect of physicochemical properties other than particle size on the adhesion and accumulation of particles in the inflamed colon tissue [111]. The effect of surface charge on binding to inflamed tissue has

been reported mainly through *ex vivo* studies or *in vivo* studies with rectal administration with conflicting conclusions. Surface charge modification should theoretically improve selectivity towards inflamed tissue by influencing the electrostatic interactions between the particles and the tissues. Additionally, binding of surface charged modified particles to other components in GIT like bile acids during transit is another concern. Thus, additional pharmaceutical strategies are needed, in conjunction with surface charge modification to achieve localized drug delivery to the inflamed tissue.

Many studies have revealed improved deposition and therapeutic efficiency in IBD of particles having a positive surface charge. Cationic systems adhere to mucosal surfaces because of interaction between the nanocarrier and the negatively charged mucosa [112]. Mucins which form a major portion of the colonic mucosal surface possess a negative charge as most of their carbohydrates are substituted with sulfate and sialic acid residues [113, 114]. As a result of their charge interactions, most of the cationic materials possess mucoadhesive properties. Adhesion can be a desirable as it can promote more interaction between the mucosal surface resulting in higher particle uptake as well as reduced clearance in the presence of diarrhea, which is common in IBD. In CD, there is an increase in mucus production leading to a thicker mucus layer in the inflamed regions, making mucoadhesion an attractive strategy to enhance specificity and retention of delivery systems in those areas [115]. One of the most frequently used cationic polymer especially in oral delivery systems is CS. CS-functionalized PLGA NPs were reported to adhere onto human intestinal mucosa ex vivo [116]. However, very less uptake in the tissue was observed for both inflamed and healthy areas probably due to strong

association with the negative mucosal surface. This finding has also been supported by other reports which used TMC, a derivative of CS [112]. As a result, this approach might be effective in delivering drugs which act on extracellular domains [102].

Anionic systems have been developed with a goal to target positively charged proteins like eosinophil cationic protein and transferrin which are overexpressed in colons of IBD patients [117]. Such systems are able to diffuse through the mucus layer due to less association with the negative charge [118]. When compared ex vivo, anionic liposomes showed two-fold higher adhesion to inflamed tissue as compared to cationic and neutral liposomes [119]. The adherence was in direct proportion to the negative charge density. However, only limited conclusions can be drawn from such studies as the conditions *in vivo* might differ significantly.

Drawing from these studies, it is not very clear as to how the size or the charge affects the adhesion, uptake and absorption of the particles. As a result, an additional level of control and complexity has been added by synthesizing delivery systems that utilize physiological changes that occur during the course of GI transit or during inflammation.

1.5.3 pH- dependent delivery systems

These delivery systems make use of the difference in pH in different regions in the GIT. The pH in the GIT varies from highly acidic in the stomach to almost neutral in the ileum and the colon. As a result, a delivery system that disintegrates at colonic pH can provide colon-specific delivery. Traditional delivery systemic like tablets and capsules have been utilizing enteric coatings to create delayed release of drug in the colon to

achieve specific delivery [120]. One of the most used pH dependent polymers are methacrylic acid copolymers, commercially known as Eudragit. In addition to pH dependence, Eudragit polymers also show mucoadhesion. Innovating from these conventional dosage forms, many studies have been reported where such polymers have been adopted to form nano-delivery systems. Liposomes coated with Eudragit have shown superior mucoadhesion in pig intestine compared to other polymer coatings [121]. Although showing favorable release kinetics which were colon specific, this system was shown to degrade when incubated in the presence of bile salts which would hinder its application *in vivo* [122]. This led to development of pH dependent colon specific polymeric nanocarriers for IBD treatment. Budesonide loaded particles made up of PLGA and Eudragit S100 mixture showed superior anti-inflammatory effect than conventional enteric coated microparticles [123]. Similar system was applied and used for developing nanocapsules [124]. Nanocapsules showed a higher loading capacity for lipophilic drugs as they have a polymeric wall around a core [125]. Both these systems also showed colon specific release. These systems have also been tested and reported for their ability to reduce inflammation in various animal models. Although promising in preclinical testing, pH-dependent nanocarriers face hurdles in their clinical translation because of the inter as well as intra-patient variability in pH that is observed in IBD. As a result, a system which solely depends on pH difference in the GIT might not be reliable for IBD therapy.

1.5.4 ROS-responsive delivery systems

These delivery systems utilize high levels of ROS which are commonly observed in the inflamed tissue. Usually high concentrations of ROS are localized only to sites of

inflammation due to the accumulated phagocytes [126]. Although the concept of ROS-responsive delivery systems is relatively old, very few oral delivery systems have been developed which utilize it for treating inflammation, mainly because it necessitates the synthesis of novel materials which can be fabricated into nanocarriers. One such example are TKNs which were formulated from PPADT which degrades in presence of ROS [127]. This material was used to encapsulate TNFα siRNA complexed with a cationic lipid to form NPs. These particles showed selective accumulation in the inflamed colon areas *in vivo*. These particles were also shown to significantly reduce inflammation and inflammatory cytokines as compared to controls. Another system which utilized ROS was designed to mimic SOD/ catalase enzyme system which actively neutralizes ROS generated in the body [128]. This system degraded specifically in the presence of superoxide which is a major ROS in the body and reduced inflammation by neutralizing over produced ROS *in vivo* in the colon.

1.5.5 Actively targeted delivery systems

With the advent of systems that are colon specific, researchers have started looking into developing delivery systems that can deliver therapeutics specifically to the inflamed regions of the GIT. Such systems use ligands decorated on the surface of the particles to actively achieve specific accumulation in the inflamed areas to deliver therapeutics and get an enhanced anti-inflammatory effect [129]. The strategy is based on the concept that the ligand-receptor interaction expressed specifically at the inflammation sites will improve the accumulation of the formulation on those areas. The ligands are selected to harness various inflammation-induced changes like receptor or protein expression in the

tissue. This approach has been vastly used for developing parenteral delivery systems to target diseases like cancer and infections [130]. Encouraged by the success of these systems, many researchers have attempted this approach to develop orally administered particulate systems. Commonly used targeting moieties include monoclonal antibodies and peptides as they exhibit high targeting specificity [131]. However, development of formulations which utilize these targeting moieties is a challenge as they are labile to gastric acid as well as enzymes. Many targeting moieties have been tested for oral colon inflammation-specific drug delivery. The first proof of concept study utilized polystyrene nanoparticles coated with anti-ICAM-1 antibodies. The biodistribution and uptake of these particles, following oral administration was assessed *in vivo* using fluorescence and radiolabeling [132]. Almost 60% of the administered antibody dose was degraded, these particles showed targeting predominantly in the upper GIT. Although ICAM-1 is overexpressed on the surface of inflamed mucosal tissues, this study was not performed in an animal model of colitis [133]. Macrophages infiltrate the inflamed colon tissue in IBD. As a result, mannose receptors and MGL which are overexpressed by the macrophages under inflammatory conditions [134]. Bioreducible mannosylated cationic polymer which could complex siRNA by electrostatic interactions to form ~250nm NPs showed significant macrophage targeting ability [135]. The uptake was shown specifically by macrophages and not by epithelial cells showing that the targetability was because of the mannose. A similar system comprising of PLGA NPs grafted with mannose showed high accumulation *ex vivo* in inflamed areas of the colon. However, both these studies did not assess the accumulation in an *in vivo* model of colitis. A galactosylated trimethyl

chitosan-cysteine NP system complexed with Map4K4 siRNA was developed to target the MGL on the macrophages [136]. This system showed significantly higher uptake in macrophages compared to control NPs proving the effect of galactose receptor-mediated endocytosis. This system demonstrated significant reduction in inflammation in DSS-induced colitis mouse model. Based on the same concept various other systems which achieve targeting through Fab' portion of a macrophage specific ligand and other receptors like transferrin, CD98 have been developed and reported [137]. Not only polymeric nanoparticles but also liposomes and hydrogels have been actively targeted to inflamed areas in the colon.

1.6 Conclusion

In the following chapters, I will present the most important findings on the development of oral therapies for IBD treatment and the studies related to their physicochemical characterization and biological testing. The characterization and the validation studies we performed to assess the suitability of the animal models we have used in this dissertation are describe in chapter 2. Chapter 3 describes alteration of the PK of chloroquine using a PDC to make it more suitable for oral delivery for local treatment of inflammation. Chapter 4 explores the physicochemical testing and therapeutic effect of polydrug/ siRNA combination in IBD treatment.

Chapter 2: Characterization of animal models for suitability

2.1 Introduction

As discussed in the previously, IBD is a chronic and debilitating disorder. It is the result of complex interplay between various immunological, genetic and environmental factors. This has led to many groups have tried to elucidate the mechanism of development of IBD using a variety of *in vitro* studies because of the ability to control various causative factors independently. However, the complexity of the disease has negated all the advances in the study of IBD leading to a lot of fundamental gaps in our knowledge. A great deal of information about other human diseases as well as design of therapies has been carried out on the basis of studies carried out in animal models [138]. However, one of the problems with understanding the pathophysiology as well as designing therapies for IBD has been attributed to the lack of suitable animal models. An ideal model of IBD would be a disease in an animal that is identical in every factor to human IBD [139]. This would ensure that the animal model has similar pathological features as well as response to experimental treatments. In addition to this, the animal model also must be amenable to experimental manipulation to study disease progression and pathogenesis. Currently used IBD animal models are broadly categorized and their advantages and limitations are mentioned in the table below-

Table 1. Currently used IBD animal models (Adapted from [140]).

Category	Model	Advantages	Disadvantages
Gene knockout	IL-2 KO	Chance to identify the role of specific genes, target specific cells	Reduced pathologic relevance as human IBD rarely has single gene mutation
	IL-10 KO		
	T cell receptor mutant		
	TNF-3′ UTR KO		
Transgenic	IL-7 TG		
Spontaneous	C3H/HejBir	Disease with multifactorial causes which increases pathogenic relevance	Poor breeding ability, high cost for colony maintenance
	SAMP1/Yit		
Inducible	Acetic acid	Easy induction, inexpensive	Lack of reproducibility, varying protocols, overlooks pathogenic factors associated with the human disease
	Iodoacetic acid		
	TNBS		
	DSS		
	Oxazolone		

As described in the table, there have been many animal models of intestinal inflammation but the definition of an ideal model exposes many problems which spring from huge fundamental gaps in our knowledge on IBD. No single model captures all the complexities of human IBD, but takes into consideration only a particular major aspect of the disease. As a result, the animal models currently used are more of models of intestinal inflammation rather than IBD models. Another major challenge in testing novel oral drug delivery systems in IBD is mimicking the GI environment *in vitro*, particularly because of the diversity in the pH, enzymes and mucosal surfaces [101]. Despite having many limitations, the current animal models offer a lot of choices can give insight not only into how the GI environment changes during inflammation but also whether new therapeutic candidates may show promise in IBD-like disorders. As a result, from the perspective of developing and testing novel therapeutic candidates, it is not only essential to characterize the suitability of the animal model based on its pathophysiology but also for potential interactions between the inflammation inducing agent and the drug delivery system. In this chapter we discuss the characterization of DSS and the *C. rodentium* induced mouse models we performed to assess their suitability to our studies.

2.2 Materials and Methods

2.2.1 Materials

CaCO2 cells, CL antibodies and DAPI mounting medium were obtained from Dr. Amar Singh's lab, DSS (Mw 40,000) was obtained from TdB consultancy, DMEM, PBS and

methanol was purchased from Thermo Fisher Scientific Inc., NH_4Cl and Triton X-100 was bought from Sigma, MPO and CXCR4 antibodies were purchased from Abcam.

2.2.2 Immunofluorescent staining

24 h prior to the experiment, CaCO2 cells were seeded in 8 well glass chambers (Thermo Fisher Scientific Inc.) at 200,000 cells/ well in 500 µl media overnight. The cells were then treated with 4% DSS in complete media for 24 h. After the treatment, the media was removed and the cells were washed with ice PBS and fixed in 4 % PFA for 30 min. The cells were washed with PBS between each step. The cells were then incubated with 50 µM NH_4Cl for 10 min and washed. The cells were treated with chilled methanol for 10 min and washed before being treated with blocking buffer (0.01 % TritonX-100) for 1 h. Post blocking, primary antibodies for CL1, CL2, CL3 and CL7 were added and the cells were incubated on a shaker at 4°C overnight. On the following day, the cells were washed and treated with the appropriate fluorescently labeled secondary antibody for 1 h. The cells were then washed and then DAPI mounting media was added. The cells were visualized using Zeiss 710 confocal laser scanning microscope.

2.2.3 Induction of DSS colitis

Male Balb/c mice (6 weeks old, 18-20g) were obtained from Charles River Laboratories. All animal experiments were conducted according to the protocol approved by the University of Nebraska Medical Center Institutional Animal Care and Use Committee. Colonic inflammation was induced by substituting drinking water with 4 %

DSS solution. Animals were weighed before starting the DSS. The onset and progress of the disease was monitored by weighing the mice and presence of blood in the stool.

2.2.4 Induction of *C. rodentium* induced colitis

Male C57BL/6 mice (6 weeks old, 18-20g) were obtained from Charles River Laboratories and used for all *in vivo* studies. All animal experiments were conducted according to the protocol approved by the University of Nebraska Medical Center Institutional Animal Care and Use Committee. *C. rodentium* was used to induce colitis in mice as previously described [141]. Bacterial glycerol stock was streaked onto LB agar plates and the bacterial colonies were grown overnight at 37°C. A single colony was inoculated into 5 ml of 2% LB broth and incubated at 37°C, 280 rpm overnight to obtain a saturated primary culture. On the day of the experiment (Day 0), 2 ml of the primary culture was added into 300 ml of LB broth and the inoculum was placed on a shaker at 37°C at 280 rpm for 6 hours. Bacterial optical density was measured at 600 nm. Bacteria were diluted with LB broth and delivered to mice (n=3) via oral gavage in a 100 μl volume containing either 5×10^8 or 10×10^8 colony forming units (CFU). Healthy control group (n=3) was orally administered 100 μl of LB broth.

2.2.5 Tissue harvesting and processing

On the day 7 for DSS model and Day 14 for the bacterial model, the mice were sacrificed and the colons were harvested. For the DSS model, the fecal content was added to a pre-weighed tube and the tubes were placed in an oven at 50°C for 48 h. The weight of the dried feces was measured and the difference in the weights was used to calculate

the water content. For animals from both the models, the colons were opened longitudinally and excised into two parts along the length, which were stored accordingly for determination of cytokine mRNA levels by RT-PCR and histological analysis.

2.2.6 RT-PCR

Colon samples were stored in RNAlater™ (Thermo Fisher Scientific Inc.) at 4°C for 48 hours to allow sufficient time for tissue penetration followed by removal of excess solution. The tissues were then stored at -80°C until further processing. Stored frozen tissues were homogenized in TRIzol™ (Thermo Fisher Scientific Inc.) reagent using TissueLyser II (Qiagen) and mRNA was isolated from the homogenized tissues according to manufacturer's protocol. The extracted mRNA was quantified using Nanodrop One^{c} UV-Vis spectrophotometer (Thermo Fisher Scientific Inc.). The cDNA was synthesized from the mRNA using High-Capacity cDNA Reverse Transcription Kit with RNase Inhibitor per the manufacturer's protocol (Thermo Fisher Scientific Inc.). A volume corresponding to 1 μg of RNA as determined by UV spectrometer was used for cDNA synthesis. Synthesized cDNA was stored at -20°C until further use. RT-PCR was carried out using the synthesized cDNA from colon tissue samples to determine the levels of mRNA of the target genes. Healthy and untreated colons were used as controls. cDNA was mixed with 0.2 μM of primer pair of gene of interest and iTaq™ Universal SYBR® Green Supermix (Biorad) into an optical reaction tube (Qiagen). The RT-PCR reaction was carried out in Rotor-Gene Q 2plex thermal cycler (Qiagen) using the following cycle

program: 95°C for 3 minutes; 40 cycles 60°C for 30 seconds. Results obtained from the RT-PCR were analyzed by Ct method to determine the fold change in gene expression.

2.2.7 Histological studies

The part of the colon was rolled in a swiss roll fashion from distal to proximal end. The rolls were fixed for 24 h in 4% paraformaldehyde, embedded in paraffin, sectioned and stained with H&E. For other markers, the colon sections were stained with MPO (for DSS treated colon tissue) and CXCR4 antibodies.

2.2.8 Spleen bacterial load

For the bacterial model, the spleens were harvested, weighed and stored on ice. The spleens were homogenized in PBS and centrifuged at 15,000×g for 15 min at 4°C. The supernatant was streaked onto LB agar plates and the plates were incubated at 37 °C for 24 h. After 24 h, the plates were visually inspected and the number of colonies was counted.

2.2.9 Cytokine expression panel

Colitis was induced in male C57BL/6 mice using 5 x 10^8 CFU of *C. rodentium* as described in the previous sections (n=15). Five mice were sacrificed at each time-point (day 1, 10 and 14 after oral gavage of the *C. rodentium*). The colons were excised, cleaned, and their weight and length recorded. The entire colon was frozen and stored at -80°C. The colon was homogenized in 1.5 ml RIPA buffer using TissueLyserII (Qiagen) and the homogenates centrifuged at 15,000×g for 15 min at 4°C. The supernatant was analyzed for cytokine levels using ProcartaPlex™ Multiplex Immmunoassay (Invitrogen) following the

manufacturer's protocol. Briefly, magnetic beads coated with different cytokine antibodies were added at the required dilution to 96-well plate. Supernatants from the tissue homogenates and supplied fluorescent standards were added to the respective wells. The cytokines were detected by adding a detection antibody followed by addition of Streptavidin-PE. The beads were read on Luminex™ LX 200 Analyzer. The data obtained were analyzed using ProcartaPlex™ Analyst software to obtain cytokine concentrations.

2.3 Results and Discussion

2.3.1 *In vitro* effect of DSS on tight junction proteins

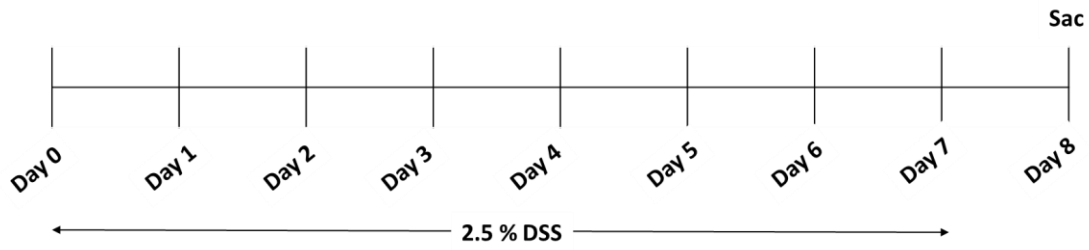
We assessed the effect of DSS on claudins which are the tight junction proteins in the colonic epithelium. The epithelial junctions in the colon represents one of the most effective barriers in the body which regulates the transport of nutrients, electrolytes as well as preventing luminal bacteria from infiltrating the colon tissue. Dysregulation of the epithelial barrier leading to leakage of the luminal contents into the tissue results in the immune response which causes inflammation. We tested the effect of DSS on tight junction proteins *in vitro* in CaCO2 cell line which is an epithelial cell line. We assessed the effect of DSS on expression of CL1, CL2, CL3 and CL7 in this cell line. We observed a decrease in the expression of CL1 and CL7 which were located at the cellular junctions. Post DSS treatment, these proteins appeared more punctate rather than at the tight junctions. Similar pattern was observed for CL2 which are seen both at the junctions as well as punctate. Post DSS treatment, CL2 showed a reduced expression overall. These experiments showed that DSS had an effect on the tested tight junction proteins and reduced their expression, thus affecting the integrity of the cell barrier.

We used two animal models for all the *in vivo* studies – DSS mouse model and *C. rodentium* mouse model of colitis. Before utilizing the models in our studies, it was important to confirm the suitability of the models to our experiments. It was essential to characterize the model to make sure that they exhibited the histological as well as the biochemical changes that commonly occur in human IBD. The following section will

discuss the studies and the analysis carried out to make sure that the models were suited to our studies.

2.3.2 DSS mouse model of colitis

One of the most commonly used animal models of colitis is a chemical injury model using dextran sodium sulfate (DSS) [142]. The mechanism by which DSS induces colitis is not entirely clear. However, it is postulated that the inflammation is likely due to the damage caused to the epithelial monolayer allowing dissemination of luminal colonic contents into the underlying tissue [143]. The DSS model is reproducible and the severity of the colitis can be controlled by adjusting the dose and frequency of DSS administration. One of the main advantages of the DSS-induced colitis is its histological similarity to human colitis.



Scheme 1. Timeline for the DSS model of colitis

2.3.2.1 Macroscopic observations

The results of mice weight measurements are shown in Fig 3a. The DSS treated group did not show any appreciable weight loss for the first five days of the experiment. On the sixth day all the mice in the DSS group lost almost 10 % of their original body weight. The weight loss continued the following date. On Day 7, the DSS group had lost close to 20 % of their body weight. No weight loss was observed in the healthy control group. Beginning day 3 most of the DSS treated mice began to show presence of blood in the stool (not shown). On the day of the sacrifice, the feces were collected and the weight before and after drying was determined (Fig. 3c). This allowed us to have an estimate of the diarrhea. Diarrhea is a very common symptom of IBD and usually persists throughout the course of the disease. We observed that the DSS group showed greater loss in fecal weight post drying indicating the presence of more water in the feces. This showed that the mice in the DSS group had diarrhea over the 7 days of DSS treatment. After sacrificing, we measured the length and the weight of the colons. We observed that the length of the DSS treated colon reduced significantly, however, the weight went up. As shown in Fig. 3b, the DSS group showed significantly higher weight by length ratio compared to the healthy group. In human as well as murine IBD, the weight of the colon increases because of the edema, immune cell infiltration and swelling which in turn also causes the colon to reduce in length. These observations proved that the DSS treated mice were showing symptoms as well as macroscopic parameters which are commonly observed in human IBD.

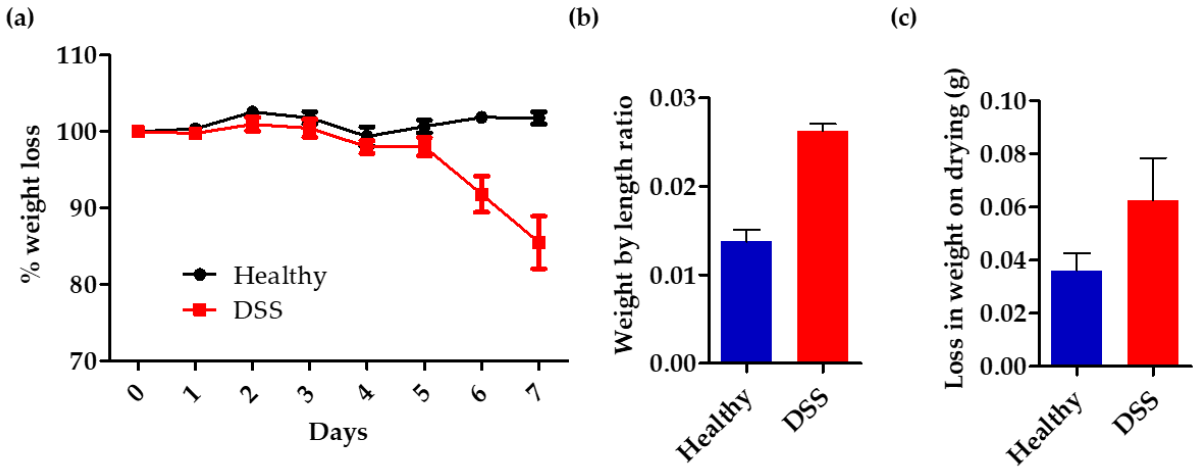


Figure 3. Macroscopic characteristics of DSS induced colitis. (a) Change in weight; (b) Change in the weight by length ratio of the colon; (c) Loss in fecal weight on drying. Results expressed as mean ± SD (n=5).

2.3.2.2Histology and immune cell markers

In the H&E stained colon sections, we observed major histological changes in the DSS treated group compared to the healthy controls (Fig. 4a). The colons of the DSS treated mice exhibited inflammation-associated evidenced by crypt fall out, presence of apoptotic cells and significant immune cell infiltrate. These changes correlated well with the changes which occur in the histology in human IBD.

We also stained the tissue sections for MPO (Fig. 4b). MPO is an enzyme which is specifically expressed by neutrophils which are the first immune cells in infiltrate the inflamed tissue. As a result, MPO is commonly used as a marker for neutrophil infiltration. We observed significantly higher MPO expression in the colons of the DSS treated group. This showed that DSS-induced inflammation was resulting in higher immune cell infiltration into the tissue compared to healthy control.

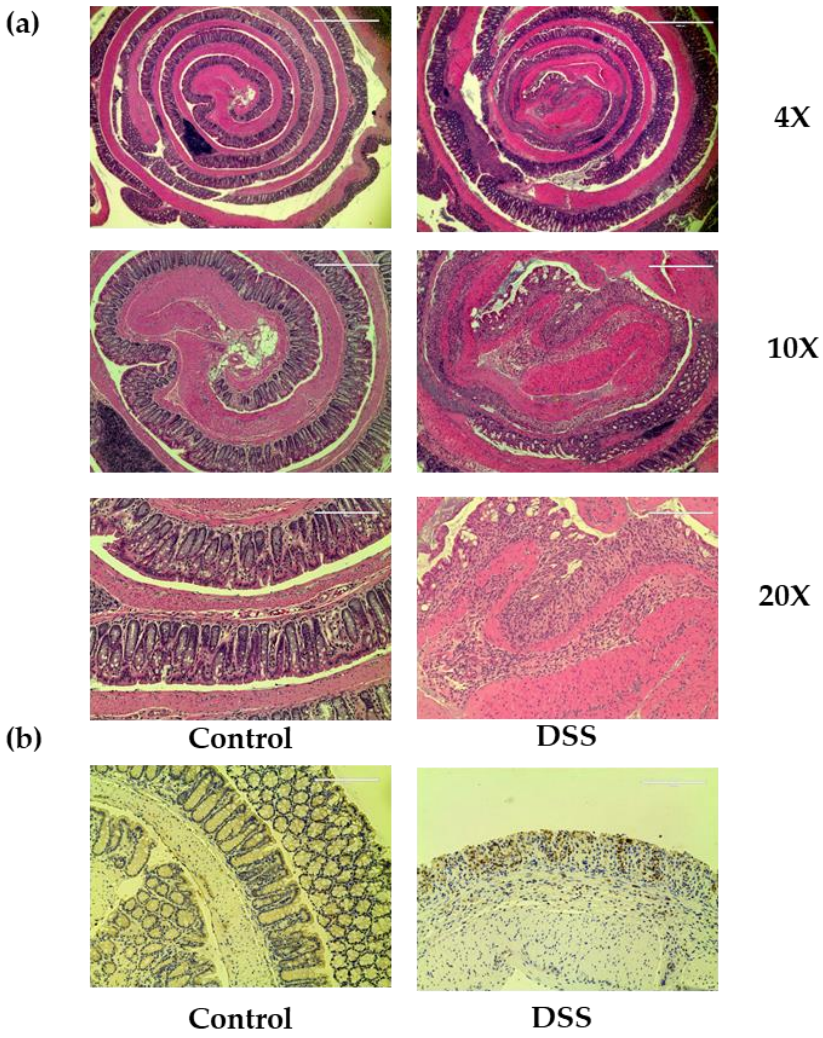


Figure 4. Histological features of DSS induced colitis. (a) Representative images of H&E stained control and DSS treated colon sections at various magnifications; (b) MPO stained control and DSS treated colon sections 20X magnification (n=3.)

2.3.2.3 CXCR4 expression

We also stained the colon sections for CXCR4 (Fig. 5). We observed higher expression of CXCR4 in the damaged crypts which were a part of the epithelium. CXCR4 plays an important role in inflammation, however, its exact role in the pathophysiology of IBD is unknown. CXCR4 is highly expressed on the peripheral T cells in patients with IBD [144]. Normal intestinal epithelial cells have been reported to express CXCR4. However, the expression of CXCR4 and CXCL12 is upregulated and more diffused in DSS induced colitis with the epithelial cells as well as the infiltrating immune cells expressing CXCR4 [145]. Our observations agree with previous studies. These studies point out that CXCR4 is responsible in migration of immune cells into the inflamed colonic tissue. Multiple reports have also pointed out the anti-inflammatory effect of CXCR4 blockade either by antibodies or AMD3100 in human IBD as well as animal model of colitis [146]. Our findings confirm the presence of CXCR4 expression in DSS model of colitis as well as draw confidence from reports that it can be a viable target to test our system discussed further.

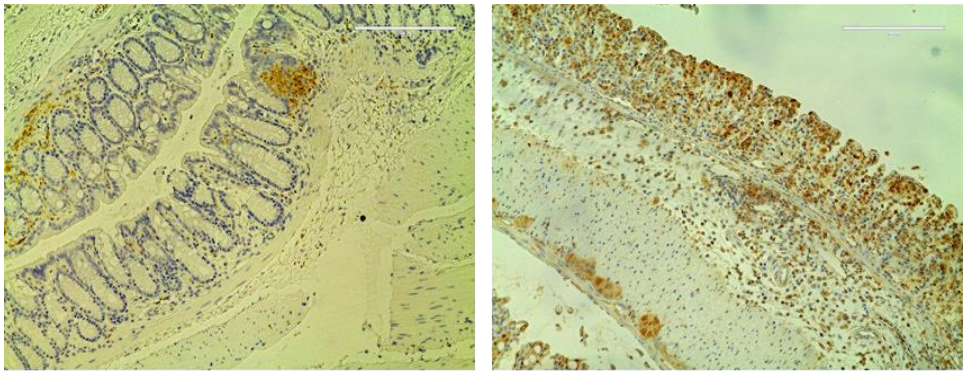
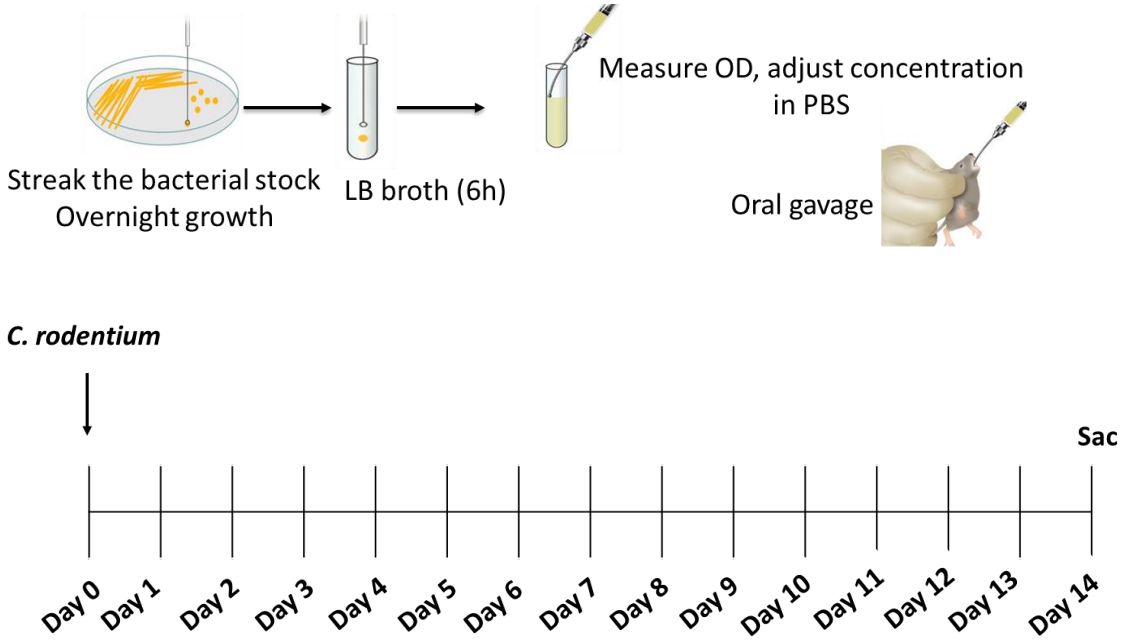


Figure 5. CXCR4 expression in DSS induced colitis. (a) Representative images of control and DSS treated colon sections stained for CXCR4 at 20X magnification (n=3).

2.3.3 *C. rodentium* model of colitis

As a second model of colitis, we decided to study and see whether *C. rodentium* induced colitis was suited for out studies. DSS model is a chemical induced injury model which gives a robust and replicable colonic inflammation in mice. However, the most characterized aspect of IBD pathophysiology involves immune response to bacterial infiltration from the lumen into the colonic mucosa. The DSS model fails to capture that aspect of the disease. *C. rodentium* is bacteria which is very similar to *E. coli* which is the major bacteria involved in the development of human IBD. It attaches to the apical side of the colon epithelium and invades the colon wall, thus eliciting an immune response [141]. As a result, *C. rodentium* colitis mimics the immune response to luminal bacteria which is one of the explanations in IBD pathogenesis, thus making it a biorelevant model. Unlike DSS which requires continuous administration at least for 7 days, *C. rodentium* model only necessitates single dose of bacteria which places relatively less stress on the animals. Other than the mentioned advantages, *C. rodentium* induced colitis also shows less interference with RT-PCR as well as testing of delivery systems which are commonly observed with DSS induced colitis [147]. We did a few characterization experiments to assess the suitability of this model to our studies.



Scheme 2. Timeline for *C. rodentium* model of colitis

2.3.3.1 Macroscopic observations

Over the 14 days of the induction, we weighed the mice every day. As opposed to the DSS model, weight reduction as well as presence of blood in stool are not significant characteristics of this model. Mice from both the groups which received bacteria did not exhibit any significant weight loss (~5% weight loss) (Fig. 6a).

Post sacrifice, we observed significant reduction in colon length for the mice which received the bacteria. In contrast, the weight of the colons from the bacteria group was significantly higher compared to healthy control (Fig. 6b). The increase in colon weight was the indication of inflammation in edema induced by *C. rodentium*. The increase in the colon weight was found to be bacterial dose dependent (Fig. 6c).

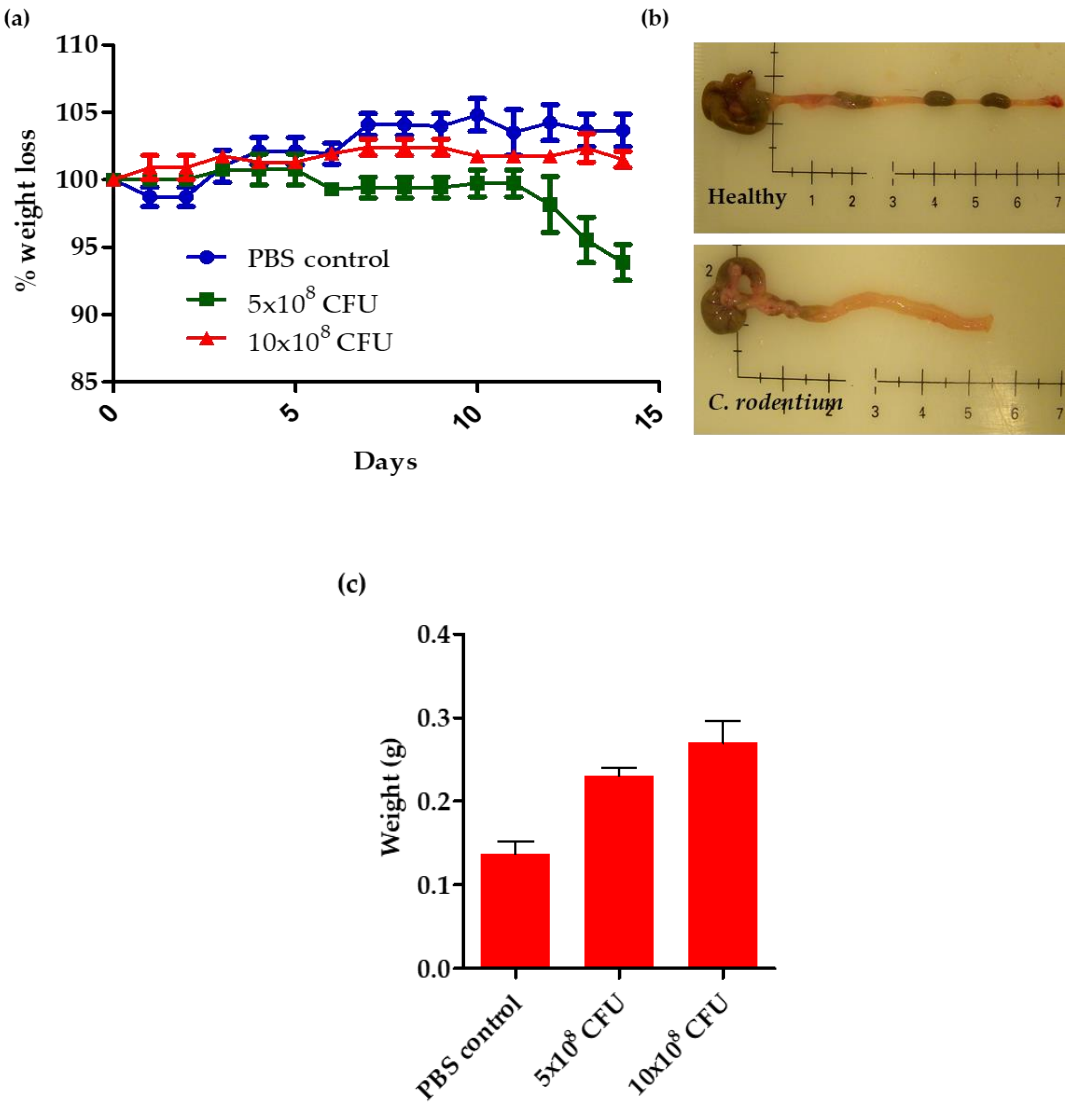


Figure 6. Macroscopic characteristics of *C. rodentium* induced colitis. (a) Change in weight; (b) Representative images of the colon; (c) Colon weight. Results expressed as mean ± SD (n=3).

2.3.3.2 Weight change and bacterial load in the spleen

We harvested the spleen and carried out further analysis. We observed a significant and dose dependent increase in the weight of the spleen (Fig. 7b). After infiltration into the colonic mucosa, the bacteria usually translocate to spleen which in the main organ involved in clearing of the infection. This results in the enlargement and subsequent increase in spleen weight (Fig 7a). We also measured the bacterial spleen load by plating the spleen homogenate on agar plates and counting the colonies that grew overnight (Fig. 7c). The healthy control spleens did not show any colonies after 24 h. However, spleens from both the bacteria treated groups showed bacterial growth confirming the translocation of the bacteria from the colon to the spleen.

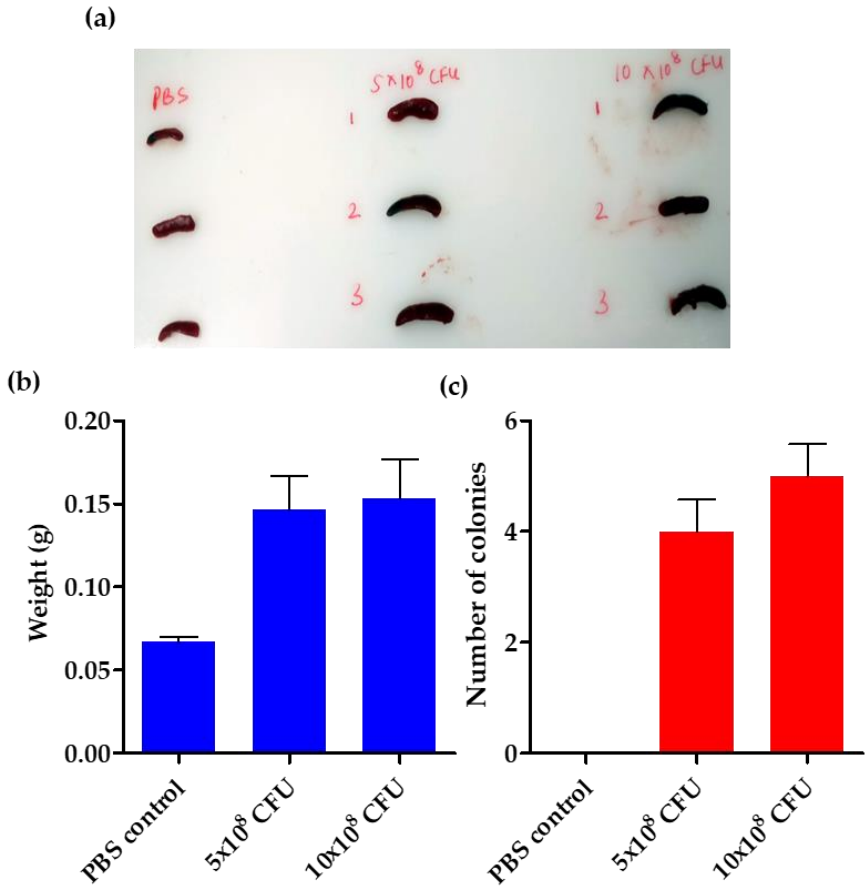


Figure 7. Changes in the spleen of *C. rodentium* infected mice. (a) Representative images of the spleens; (b) Weight of the spleen; (c) Bacterial load in the spleen. Results expressed as mean ± SD (n=3).

2.3.3.3 Histology

Histologically, colons of the infected mice exhibited inflammation-associated epithelial changes evidenced by crypt elongation, crypt fall out, presence of apoptotic cells, and significant inflammatory cell infiltration (Fig. 8). Distal colon showed more pronounced changes than the proximal colon, which was consistent with previous findings suggesting that *C. rodentium* preferentially colonizes the distal colon. The observed parameters correlate well with the inflammatory changes, which occur during the development of human IBD.

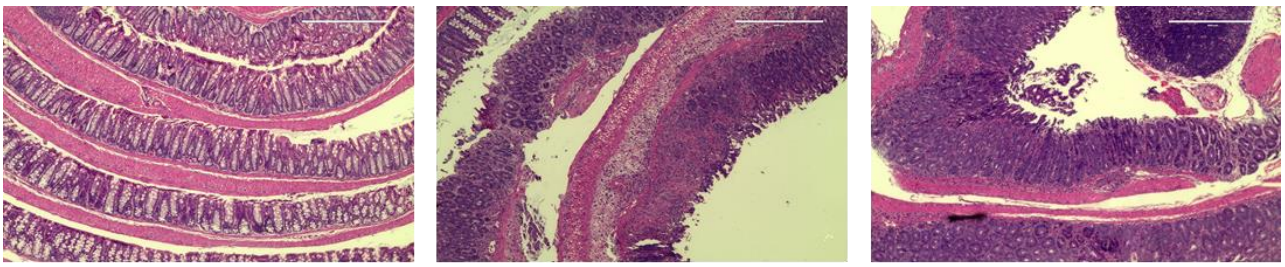


Figure 8. Histological changes in the colons of *C. rodentium* infected mice. Representative images of H&E stained colon sections.

2.3.3.4 Cytokine expression

It involves an erratic immune response to the gut microbiota and luminal contents which leak into the tissue when the epithelial barrier is broken. As a result, upregulation of pro-inflammatory cytokines is a hallmark of IBD. Cytokine dysregulation is a common aspect across all the animal models of IBD. The network of cytokines involved in IBD is which a variety of chemokines and growth factors are involved. However, most of the studies are restricted to individual cytokines. Many studies indicate the diagnostic value of cytokine profiling in IBD to design cytokine-based therapies [148]. As a result, we carried out a time dependent profiling analysis of local colonic cytokine levels in *C. rodentium* infected mice and observed many changes in the colon cytokine levels. Many cytokines of the IL family play a role in many autoimmune disorders [149]. We observed a time dependent increase in the colon levels of IL-1β, IL-6, IL-13 (Fig. 9). The main source of IL-1β in IBD are the monocyte and macrophages which release IL-1β into the colonic mucosa [150]. IL-6 was the cytokine which showed highest upregulation in colon levels. IL-6 signaling plays an important role in IBD pathogenesis via activation of STAT3 and has been commonly observed to be upregulated in IBD patients [151]. Not only mononuclear cells but also intestinal epithelial cells have been reported to be involved in producing IL-6 in lamia propria [152]. IL-13 has been reported to be an important cytokine in UC which is traditionally considered as a Th2 mediated disorder [153]. IL-13 mRNA levels are increased not only in UC affected mucosa, but also in lamia propria cells from patients cultured *ex vivo* [154]. We also observed a time dependent increase in the levels of TNFα. TNFα is heavily involved in the pathogenesis of IBD [155]. It has been reported

to exert pleiotropic effects as well as increase the production of IL-1β, IL-6 [156, 157]. The levels of TNFα have been shown to correlate with the disease activity index of IBD [157]. As a result, the most successful therapy for IBD are the TNFα antibodies [158]. The increase in the levels of all the cytokines results in over production of chemokines which involve majority of MCPs, MIPs and MMPs. These molecules play a major role in the recruitment and the migration of circulating immune cells [159]. We observed an upregulation in the levels of MCP-1, MCP-3, MIP-α, MIP-β in the colons of the infected mice (Fig. 10). Overall, the profiling of the cytokines in model revealed a time dependent increase in the levels over the 14 days of the model. The upregulation in these cytokines correlated with the histological changes we observed in this model.

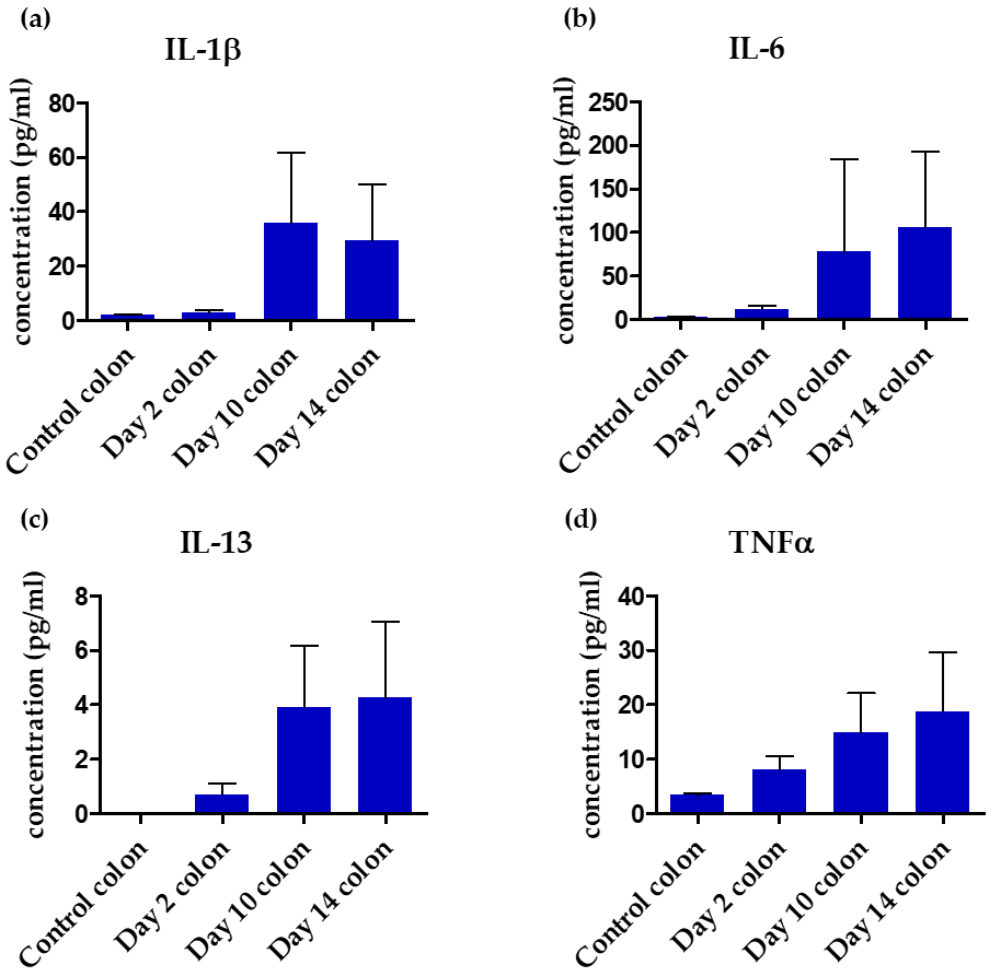


Figure 9. Changes in cytokine levels in *C. rodentium* infected mice. Colon cytokine levels were measured using Luminex cytokine panel (a) IL-1β; (b) IL-6; (c) IL-13; (d) TNFα; (e) MCP-4; (f) MCP-3; (g) MIP-α; (h) MIP-β. Results expressed as concentration in pg/ml (n=5).

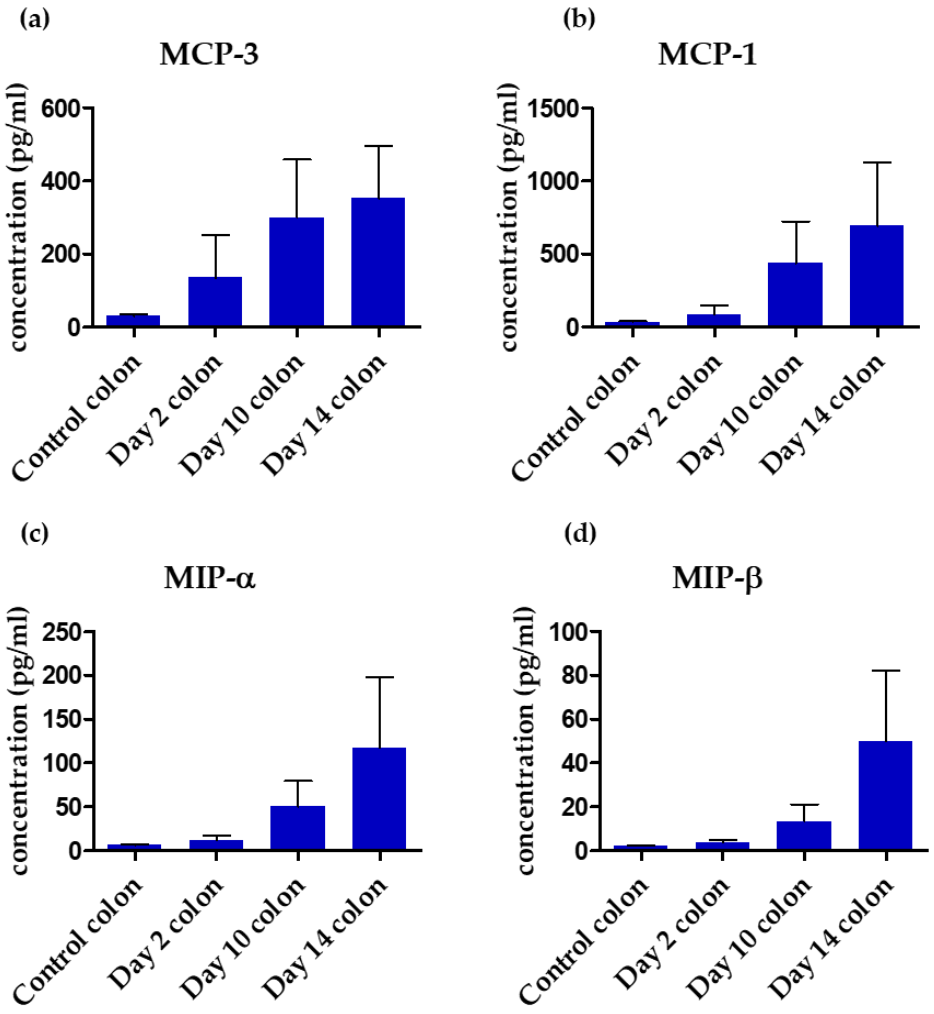


Figure 10. Changes in cytokine levels in *C. rodentium* infected mice. Colon cytokine levels were measured using Luminex cytokine panel (a) MCP-4; (b) MCP-3; (c) MIP-α; (d) MIP-β. Results expressed as concentration in pg/ml (n=5).

2.3.3.5 CXCR4 expression

We also assessed the mRNA levels of CXCR4 in the colon of *C. rodentium* infected mice (Fig. 11). We observed that CXCR4 mRNA levels were upregulated two-fold right from Day 2 post infection. Maximum fold increase in mRNA levels compared to healthy control was observed on Day 10, almost 5-fold. CCR4 mRNA levels decreased slightly on Day 14 compared to Day 10. A four-fold increase in CXCR4 mRNA levels was observed on Day 14 compared to healthy control.

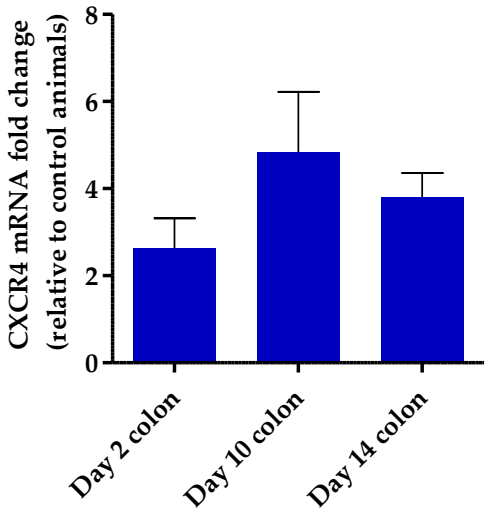


Figure 11. Changes in CXCR4 expression in *C. rodentium* infected mice. Colon CXCR4 mRNA levels over the course of the model measured using RT-PCR. Results are expressed as fold change in mRNA levels relative to healthy controls (n=3).

2.4 Conclusion

Our characterization of revealed the both the models gave a robust inflammation which was mainly localized in the colon. Both the models showed macroscopic characteristics, histological characteristics and the biochemical parameters similar to changes which occur in human IBD. All these factors can be used to compare the efficacy of our treatments. In conclusion, both the models are suitable for our planned studies.

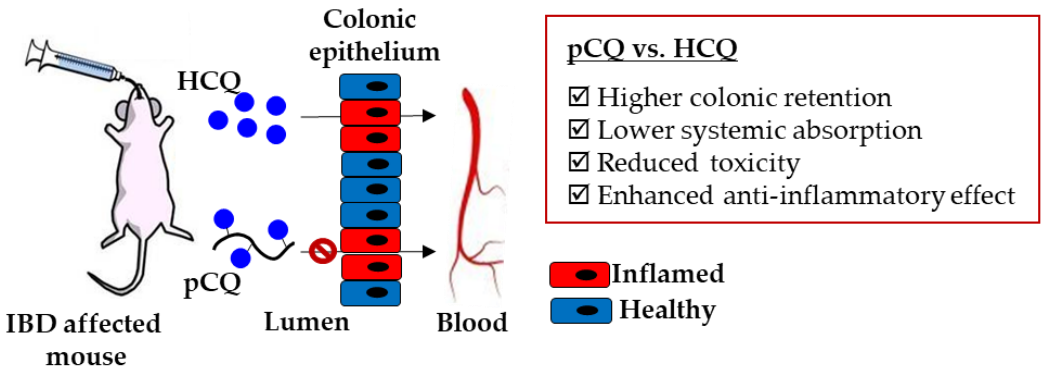
Chapter 3: Polymeric chloroquine as an oral therapeutic for treatment of IBD

3.1 Introduction

Polymers have been as therapies since a long time. PDC involve conjugating macromolecular polymers with drugs and have been applied as a promising drug delivery platform. In the last few years, the use of PDC in novel therapeutic applications is increasing. The first PDC reported conjugated mescaline with poly (vinyl pyrrolidine) in 1955. Following that, many PDCs have been synthesized and reported. Most common aim behind synthesizing a PDC is to change the characteristics of the free drug. Few common advantages a PDC may offer over a free drug involve increased water solubility, control of the release pattern of the drug, protection of the drug against degradation under physiological conditions, improved bioavailability in the target tissue. For a considerable time, only the physical properties of the drug were considered in designing a PDC. As a result, not all drugs were appropriate to be modified as PDCs. However, eventually the consideration of the route of administration became important as it has an impact on the accessibility of the drugs to their target site. With oral route of administration intended for drug delivery to the colon, it is important that the drug remains conjugated when during the transit as it encounters various pH and enzymatic conditions. There are numerous reports of PDCs synthesized with various goals have been synthesized for oral administration. Budesonide was conjugated with dextran for improving its aqueous solubility as well as reduce premature release in the upper GIT. A colon specific release of the drug obtained by degradation of the dextran backbone by dextranase, an enzyme present specifically in the colon. Another example was conjugation of Dexamethasone to

pDMAEMA to improve its mucoadhesive properties in the GIT and provide an enhanced anti-inflammatory effect. A commercial example of PDC is NKTR-118, a PEGylated form of naloxol which is currently under Phase III trials as an oral tablet for opioid-induced bowel disorders.

Chloroquine (CQ) is commonly used to prevent and treat malaria. In recent years, CQ has also found growing anti-inflammatory application in the treatment of autoimmune disorders like rheumatoid arthritis and lupus erythematosus [160]. Several reports indicate the promise of CQ in treating IBD [161]. However, the long-term use of CQ can result in severe ocular side effects including blurred vision and retinopathy [162]. We have previously synthesized a polymeric form of CQ (pCQ), which showed unexpected ability to safely inhibit cell migration. The epithelial tight junctions in the colon is one of the most regulated barriers in the body. It promotes the transport of nutrients and small molecules from the lumen into the systemic circulation, but macromolecules are virtually prevented from passing through. With pCQ being a macromolecule, we hypothesized that restricting it to the colon and minimizing systemic absorption could be a promising strategy to enhance its local anti-inflammatory effect in IBD (Scheme 3). We hypothesize that pCQ will have enhanced anti-inflammatory activity in IBD. In our current study, we analyzed the oral pharmacokinetics and evaluated therapeutic efficacy of pCQ using a widely used mouse model of IBD induced by oral administration of *C. rodentium*.



Scheme 3. Proposed mechanism of pCQ

3.2 Materials and methods

3.2.1 Materials. Hydroxychloroquine (HCQ) sulfate (98%), triethylamine, DMSO-d6 (99.8%) and chloroform-d (99.8%) were obtained from Acros Organics (Fisher Scientific). Methanol, acetonitrile (HPLC grade) were purchased from Fisher Scientific. pCQ (Fig. 1) with 16.7 mol % of CQ and weight average molecular weight 60 kDa was synthesized and characterized as previously reported [163].

3.2.2 Pharmacokinetics and biodistribution. The pharmacokinetics and biodistribution of single dose pCQ was evaluated in a mouse model of IBD. Colitis was induced using *C. rodentium* as described above and on day 14, the mice were given single dose of HCQ or pCQ in 200 μl of deionized water via oral gavage equivalent to 30 mg/kg HCQ. Blood was collected from the submandibular vein at 0.5, 1, 2, 4, 8, and 24 h post-administration. The animals were sacrificed and organs harvested at 0.5, 2, 8, and 24 h post-administration and stored at -80°C until further use. The mice were randomized to selected sampling times so that three blood samples and one terminal tissue collection were obtained from each. We have previously reported the single dose *intra venous* pharmacokinetics of HCQ in mice [164].

Blood, tissue and fecal samples were processed by two methods to determine free HCQ and total HCQ. For the first method, HCQ was isolated by a simple extraction from the organs as previously described [164]. The second method utilized base hydrolysis to release HCQ covalently bound to pCQ and subsequent HCQ extraction using solid phase extraction (SPE). Tissues and feces were homogenized in water prior to loading to the SPE

cartridge. The calibration and quality control samples were separately prepared for HCQ and pCQ by spiking 10 µl of appropriate calibration stock of HCQ and pCQ, in 100 µl of blank biomatrix, and 10 µl of internal standard solution (1.0 µg/ml) was added. For the study, 25 µl of blood and 100 µl of tissue homogenate were used. 400 µl of 1 M NaOH, 600 µl water and 100 µl methanol were added and the samples were incubated at 50 ºC for 1 h to hydrolyze HCQ from the pCQ. Subsequently, 400 µl 2% formic acid was added and the samples were vortexed for 30 s and centrifuged at 1300 x g for 10 min. The SPE was carried out using Oasis HLB 3cc, 60 mg extraction Cartridge (Waters). Cartridges were pre-conditioned with 1 ml acetonitrile followed by 1 ml water. Samples were loaded to the cartridges and washed with 2 ml of aqueous 15 % methanol and dried at high vacuum for 15 min. Analytes were eluted with 2 ml of acetonitrile. The eluents were collected in glass tubes and dried under nitrogen in water bath at 50°C. The dry residues were reconstituted in 400 µl 0.1% formic acid:methanol mixture (60:40) and centrifuged at 13000 x g and 10 µl supernatant was injected into the HPLC. The LC-MS/MS conditions used were according to our previous report [164]. The assay was linear over the range of 1 to 2000 ng/mL.

The pharmacokinetic parameters of HCQ and pCQ in blood and tissues were calculated using non-compartmental analysis with Phoenix WinNonlin 6.3 (Pharsight Corporation). The maximum concentration (C_{max}) and time to C_{max} (T_{max}) were obtained from visual inspection of the concentration-time plot. The area under the curve ($AUC_{0-\infty}$) was estimated using the linear trapezoidal method from 0-t_{last} and extrapolation from t_{last} to infinity based on the observed concentration at the last time point divided by the terminal

elimination rate constant (λ). The elimination half-life ($t_{1/2}$) was calculated as 0.693/k. Apparent clearance (Cl/F) and the apparent volume of distribution of the elimination phase (V_d/F) were calculated as dose/$AUC_{0-\infty}$ and dose/k*$AUC_{0-\infty}$, respectively. The mean residence time (MRT) was calculated as $AUMC_{0-\infty}$/ $AUC_{0-\infty}$. Mean tissue concentrations were calculated and expressed as ng/g tissues. The absolute bioavailability was calculated as:

$$F_{\mathrm{abs}} = 100 \cdot \frac{AUC_{\mathrm{po}} \cdot D_{\mathrm{iv}}}{AUC_{\mathrm{iv}} \cdot D_{\mathrm{po}}}$$

3.2.3 Therapeutic efficacy. Colitis was induced as stated above and mice were randomly assigned to healthy, HCQ, and pCQ treatment groups (n=5) and untreated group (n=8). Starting day 1, the mice received oral gavage of either HCQ or pCQ every other day (30 mg/kg HCQ equivalent in 200 µl sterile deionized water). Untreated controls were administered 200 µl sterile deionized water. On day 14, the mice were sacrificed and the colons were harvested. The colon was opened longitudinally, cleaned of fecal matter, and excised into two parts along the length, which were stored accordingly for determination of cytokine mRNA levels by RT-PCR and histological analysis.

3.2.4 Real-time PCR (RT-PCR). Colon samples from the therapeutic study were stored in RNAlater™ (Thermo Fisher Scientific Inc.) at 4°C for 48 hours to allow sufficient time for tissue penetration followed by removal of excess solution. The tissues were then stored at -80°C until further processing. Stored frozen tissues were homogenized in TRIzol™ (Thermo Fisher Scientific Inc.) reagent using TissueLyser II (Qiagen) and mRNA was

isolated from the homogenized tissues according to manufacturer's protocol. The extracted mRNA was quantified using Nanodrop One^{c} UV-Vis spectrophotometer (Thermo Fisher Scientific Inc.). The cDNA was synthesized from the mRNA using High-Capacity cDNA Reverse Transcription Kit with RNase Inhibitor per the manufacturer's protocol (Thermo Fisher Scientific Inc.). A volume corresponding to 1 μg of RNA as determined by UV spectrometer was used for cDNA synthesis. Synthesized cDNA was stored at -20°C until further use. RT-PCR was carried out using the synthesized cDNA from colon tissue samples to determine the levels of mRNA of the target genes. Healthy and untreated colons were used as controls. cDNA was mixed with 0.2 μM of primer pair of gene of interest (Table 1) and iTaq™ Universal SYBR® Green Supermix (Biorad) into an optical reaction tube (Qiagen). The RT-PCR reaction was carried out in Rotor-Gene Q 2plex thermal cycler (Qiagen) using the following cycle program: 95°C for 3 minutes; 40 cycles 60°C for 30 seconds. Results obtained from the RT-PCR were analyzed by Ct method to determine the fold change in gene expression.

Table 2. Primer sequences for RT-PCR

mRNA targets	Primer sequence (5′-3′)	
TNF-α	F	CATGAGCACAGAAAGCATGATC
	R	CCTTCTCCAGCTGGAAGACT
IL-6	F	ATGGATGCTACCAACTGGAT
	R	TGAAGGACTCTGGCTTTGTCT
IL-1β	F	CAACCAACAAGTGATATTCTCCATG
	R	GATCCACACTCTCCAGCTGCA
IL-2	F	TGAGCAGGATGGAGAATTACAGG
	R	GTCCAAGTTCATCTTCTAGGCAC

3.2.5 Histological evaluation. The longitudinally opened colons were rolled into a Swiss roll from distal to proximal end. The rolls were fixed for 24 h in 4% paraformaldehyde, embedded in paraffin, sectioned and stained with hematoxylin and eosin (H&E). The stained sections were evaluated by a pathologist without the knowledge of the identity of the samples using a light microscope. Histopathological scores were assigned based on criteria as previously described [165]. Scoring was performed based on severity of epithelial injury (graded 0-3, from absent to mild including superficial epithelial injury, moderate including focal erosions, and severe including multifocal erosions), the extent of inflammatory cell infiltrate (graded 0-3, from absent to transmural), and goblet cell depletion (0-1). For each tissue, a numerical score was assigned in a blinded manner to prevent bias. Scores from each tissue section group were averaged to obtain a mean histopathological score. Crypt heights were measured using ImageJ software, with 10 measurements taken in distal colons of each mouse. Only well-oriented and intact crypts were measured. Tissue sections were stained for cleaved caspase 3 (CC3) and macrophage infiltration (CD68). CC3 positive cells in the entire colon roll were counted at 20x magnification. Results were expressed as mean CC3 positive cells per entire colon roll. CD68 positive cells were counted in five randomly chosen areas in the colon roll at high power field (HPF). Results were expressed as mean of CD68 positive cells per HPF.

Statistics. Mann-Whitney test was used for statistical analysis of mean differences between treatment groups for biodistribution studies. One-way ANOVA followed by Tukey multiple comparison test was used for statistical analysis of mean differences among multiple groups. A value of $p < 0.05$ was considered statistically significant. All

statistical analysis was performed using Graphpad Prism v5 (ns = not significant, * = $p \leq 0.05$, ** = $p \leq 0.01$, *** = $p \leq 0.001$)

3.3 Results and discussion

The main hypothesis of this study was that the macromolecular nature of pCQ will significantly restrict oral bioavailability of chloroquine and thus allow us to test how local colon effects contribute to the overall anti-inflammatory activity of HCQ. Based on our prior studies, we further hypothesized that pCQ will act as a polymeric drug with pronounced ability to inhibit inflammatory cell infiltration in the colon. We have synthesized pCQ by copolymerization of N-(2-hydroxypropyl)methacrylamide (HPMA) with methacryloylated HCQ as reported in our previous studies (Fig. 12) [166].

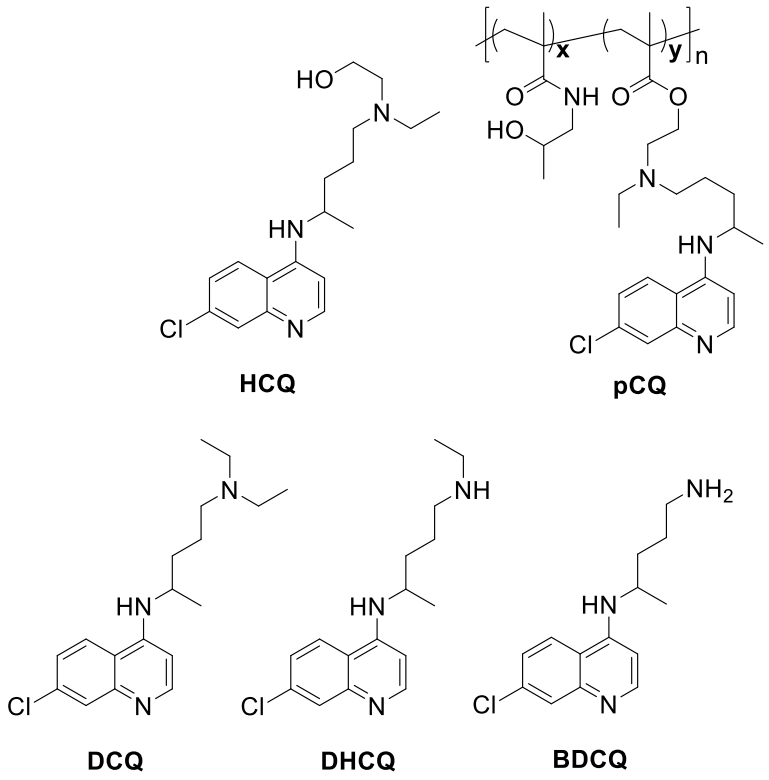


Figure 12. Structures of HCQ, pCQ and main HCQ metabolites

3.3.1 Pharmacokinetics

pCQ contains HCQ attached by a potentially hydrolyzable ester linker and it was thus important to be able to distinguish between polymer-conjugated HCQ and free HCQ released from pCQ (Fig. 12). We achieved this goal by using two LC-MS/MS analytical methods. First, we quantified HCQ using a simple extraction from blood and tissues to determine the amount of HCQ that was released from pCQ by hydrolysis in the GI tract, blood, or liver. In the second method, we included an alkaline hydrolysis step that was optimized to fully hydrolyze the ester linker between HCQ and the polymer, thus providing us with the information on the total (polymer bound + hydrolyzed) HCQ. The difference in the HCQ amount obtained from the two methods was used to calculate the percent of HCQ in the tissues that remained bound to the polymer. We have found that the HCQ amount quantified by both methods was similar in blood and tissues from animals treated with HCQ alone.

The blood concentration vs. time profile for the HCQ and pCQ after oral administration is shown in Fig. 13. The PK parameters of pCQ were determined from the total HCQ content in the blood and thus represent a combined PK of polymer-bound and hydrolyzed HCQ (Table 3). The drug reached a maximum concentration in blood (Cmax) of 2342.6 ± 46.3 and 12.2 ± 1.7 ng/mL for HCQ and pCQ treatment, respectively. The value of area under curve ($AUC_{0-\infty}$) were determined as 28182.4 ± 1475.8 and 231.3 ± 48.8 hr×ng/mL for HCQ and pCQ treatment, respectively. In comparison to HCQ, the pCQ formulation exhibited a significant reduction of Cmax (~192 fold) and $AUC_{0-\infty}$ (~122 fold) indicating that modifying HCQ into pCQ dramatically reduced its absorption. The

absolute bioavailability (oral to IV), was found to be 0.4% for pCQ compared to 80% for HCQ indicating that the pCQ formulation substantially reduced HCQ bioavailability and maintained drug in the gastrointestinal tract. One pharmacokinetic disadvantage of chloroquine and its metabolites is their exceptionally long residence time in the blood leading to severe side effects. In pCQ treatment group, HCQ levels were substantially lower in the systemic circulation, suggesting that prolonged exposure to HCQ and metabolites will not be a major systemic toxicant. This is an important finding for a small molecule drug like chloroquine which has high systemic bioavailability resulting in high non-specific tissue exposure. Reduction in systemic absorption and bioavailability is important for local therapy and reduction of systemic toxicities and our pCQ formulation resulted in very different blood PK profile compared to HCQ.

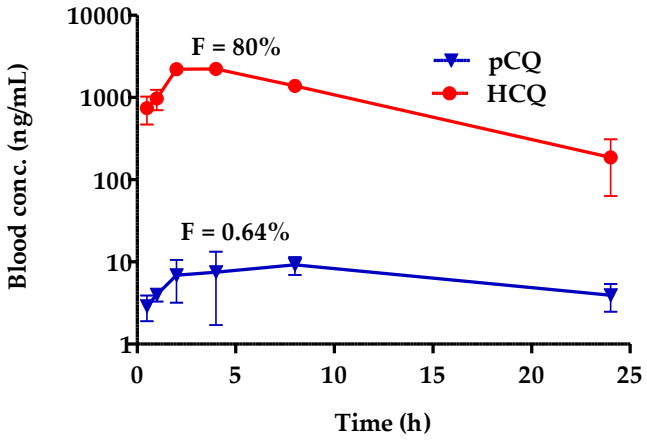


Figure 13. Blood concentration vs. time profile. Mice with *C. rodentium* induced colitis were orally administered with HCQ and pCQ equivalent to 30 mg/kg CQ dose, respectively. Results are expressed as HCQ blood concentration ± SD.

Table 3. Non-compartmental blood pharmacokinetic analysis in colitis mice

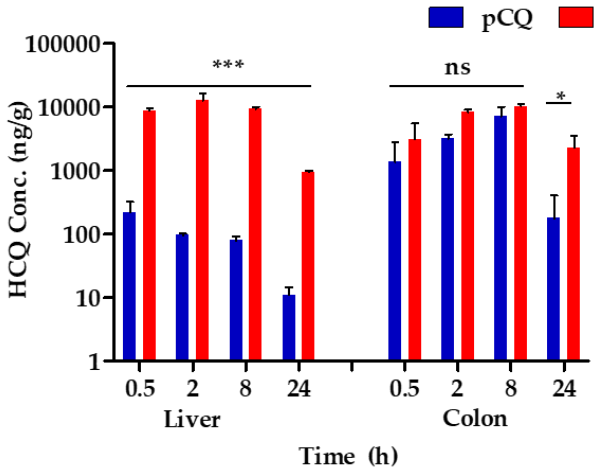
Pharmacokinetic parameter	**HCQ**	**pCQ**
C_{max} (ng/ml)	2,343 ± 46	12.2 ± 1.7
$t_{1/2}$ (h)	5.6 ± 1.9	11.7 ± 3.6
t_{max} (h)	3.3 ± 1.2	4.7 ± 3.1
$AUC_{0\text{-last}}$ (h×ng/ml)	26,446 ± 507	161 ± 8
$AUC_{0\text{-}\infty}$ (h×ng/ml)	28,182 ± 1476	231 ± 49
Cl/F (L/h/kg)	1.1 ± 0.1	134 ± 27
MRT (h)	6.8 ± 0.6	9.9 ± 0.7

3.3.2 Colon and liver distribution

There have been numerous reports indicating that conjugating small molecule drugs with polymers can change their PK and pre-dispose them to preferential accumulation in specific tissues. We next analyzed how the differences in blood PK affect relative distribution of pCQ and HCQ to the colon and liver following oral administration in the *C. rodentium* colitis model. The colon and liver PK and distribution results are shown in Table 3 and Fig. 14. HCQ and pCQ reached a Cmax of HCQ in colon of 10,304 ± 746 and 7,121 ± 2,984, respectively. The colon $AUC_{0-\infty}$was 208,917 ± 55,806 for HCQ and 94,515 ± 35,363 hr×ng/mL for pCQ treatment. HCQ appeared to show higher colon concentrations than pCQ probably due to faster transit time but the difference did not reach statistical significance. Both HCQ and pCQ showed increasing accumulation in the colon from the time of administration until at least 8 h, with subsequent decline by 24 h (Fig. 14). Both pCQ and HCQ showed similar colon PK behavior. The Tmax for HCQ and pCQ occurred at 8 h. However, major differences were observed in the hepatic PK parameters of pCQ and HCQ. As expected from the very low bioavailability, pCQ had much lower hepatic accumulation than HCQ with the liver Cmax for pCQ 58-times lower than the HCQ C_{max} and ~110-times lower AUC_{0-last} compared to HCQ. It was noteworthy, that pCQ concentrations in the liver declined from the first measured time point and were at all times lower than the liver levels of HCQ. These PK differences contributed to the preferential localization of pCQ in the colon as suggested by the calculated colon-to-liver ratios in Fig. 14. The pCQ colon:liver ratio was higher at all measure time points compared

to HCQ treatment. Fecal pCQ concentrations were higher than HCQ levels (data not shown). These observations reinforce the applicability of pCQ as a local colonic treatment.

Having established the local colon accumulation of pCQ, we then focused on the analysis of pCQ hydrolysis in the GI tract and the extent of release of free HCQ. We have analyzed the content of polymer-bound HCQ and the extent of pCQ hydrolysis using the two different sample preparation methods described above. As shown in Fig. 15, we have found that the vast majority (~97%) of the HCQ was polymer-bound until at least 24 h post-administration. The released (i.e. free) HCQ levels in the colon decreased, whereas the polymer-bound HCQ levels increased over time. While the released HCQ concentrations were highest in the colon at 1.5 h, the polymer-bound HCQ achieved maximum concentrations at 8 h. Calculating the hydrolyzed fraction at 8 h, only 1.2% of free HCQ was present in the colon tissue. This indicated that the hydrolyzed HCQ was being systemically absorbed while the polymer-bound HCQ had a higher transit time to localize in the colon before clearance at 24 h. Estimate of the colon $AUC_{0\text{-last}}$ showed about 37-fold difference between the polymer-bound HCQ and HCQ hydrolyzed from pCQ, suggesting that most of the therapeutic effects described below result from the activity of pCQ and not released HCQ.

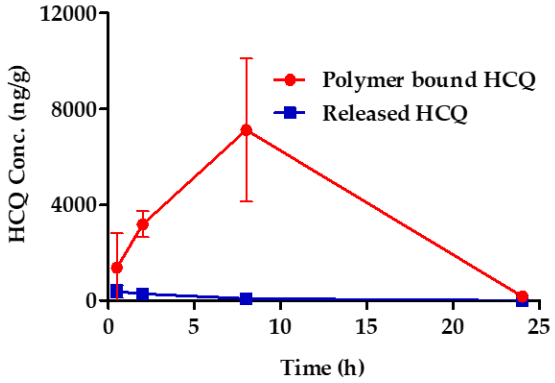


	Colon-to-Liver ratio	
Time (h)	pCQ	HCQ
2	33	0.7
8	89	1.1
24	16	2.4

Figure 14. Biodistribution of HCQ and pCQ. Animals were sacrificed at pre-determined timepoints and tissues were harvested and homogenized. HCQ levels were measured in liver and colon. Results are expressed as concentration of HCQ in tissues ± SD.

Table 4. Liver and colon pharmacokinetics in colitis mice.

Pharmacokinetic parameter	**Liver**		**Colon**	
	HCQ	**pCQ**	**HCQ**	**pCQ**
C_{max} (ng/ml)	12,807±3703	220.2±102.7	10,304 ± 746	7,121 ± 2,984
t_{max} (h)	2.0±0.0	0.5±0.0	8.0±0.0	8.0±0.0
$AUC_{0\text{-last}}$ (h×ng/ml)	167,944 ± 19,302	1,547 ± 100	166,377 ± 14,873	93,088 ± 34,403
$AUC_{0\text{-}\infty}$ (h×ng/ml)	175,852 ± 18,362	1,659 ± 78	208,917 ± 55,806	94,515 ± 35,363



$AUC_{0\text{-}last}$ (h*ng/ml)	
Polymer bound HCQ	93087.8
Released HCQ	2470.5

Figure 15. GI hydrolysis of pCQ. HCQ extracted with (polymer-bound) and without base hydrolysis (released) was measured in colon. Results are expressed as concentration of HCQ in colon ± SD.

3.3.3 HCQ and pCQ metabolism

To address tissue accumulation and subsequent metabolism of HCQ and pCQ, we measured the concentrations of HCQ metabolites in colon and liver at serial time points following oral administration. HCQ is metabolized in the liver by dealkylation into three major metabolites (Fig. 12): desethylchloroquine (DCQ), bisdesethylchloroquine (BDCQ) and desethylhydroxychloroquine (DHCQ) [167]. It was previously shown that DCQ has similar antimalarial activity as HCQ. All the N-dealkylated metabolites have been implicated in heart failure and retinopathy, with BDCQ being more cardiotoxic than HCQ [168]. Importantly for chronic use in IBD, HCQ and its metabolites have extremely long biological half-lives and thus their monitoring is important.

As expected, pCQ significantly decreased the extent and rate of HCQ metabolism due to the covalent bond formed between the polymer and the hydroxyl in HCQ. The metabolite concentration results in liver and colon are shown in Fig. 16. In the liver (Fig. 16a), which is the main organ for HCQ metabolism, both DCQ and BDCQ concentrations were 10-100-fold higher in the HCQ treated group as compared to the pCQ group. Both DCQ and BDCQ liver concentrations peaked at 8 h. DHCQ liver levels were undetectable in the pCQ group. Analysis of blood metabolite concentrations revealed similar trend as most metabolites were either undetectable or significantly lower in the pCQ group as a result of the very low bioavailability (data not shown).

Data in Fig. 16b suggest that HCQ metabolism occurs in the colon. In agreement with the liver metabolism findings, we observed that metabolite concentrations were

about 10-100-fold lower in the pCQ group than in the HCQ group. Calculating the percent of metabolites in colon at C_{max} (8 h), we observed that pCQ was metabolized to a lower extent than HCQ. While the major metabolite DCQ accounted for 16.5% in the HCQ group, only 4% of pCQ was metabolized to DCQ in the colon. BDCQ (4% of HCQ vs. 0.4% of pCQ) and DHCQ (8% of HCQ vs. 1% of pCQ) showed similar differences. This finding, coupled with the observation of elevated fecal pCQ concentrations, further support pCQ localization in the colon as opposed to systemic absorption. The covalent conjugation of HCQ in pCQ not only reduced its oral absorption due to its macromolecular nature but it also decreased accessibility to metabolic enzymes, thus preventing generation of toxic HCQ metabolites. Such a low systemic exposure to HCQ and its metabolites may result in reduction of adverse systemic side effects commonly observed with HCQ.

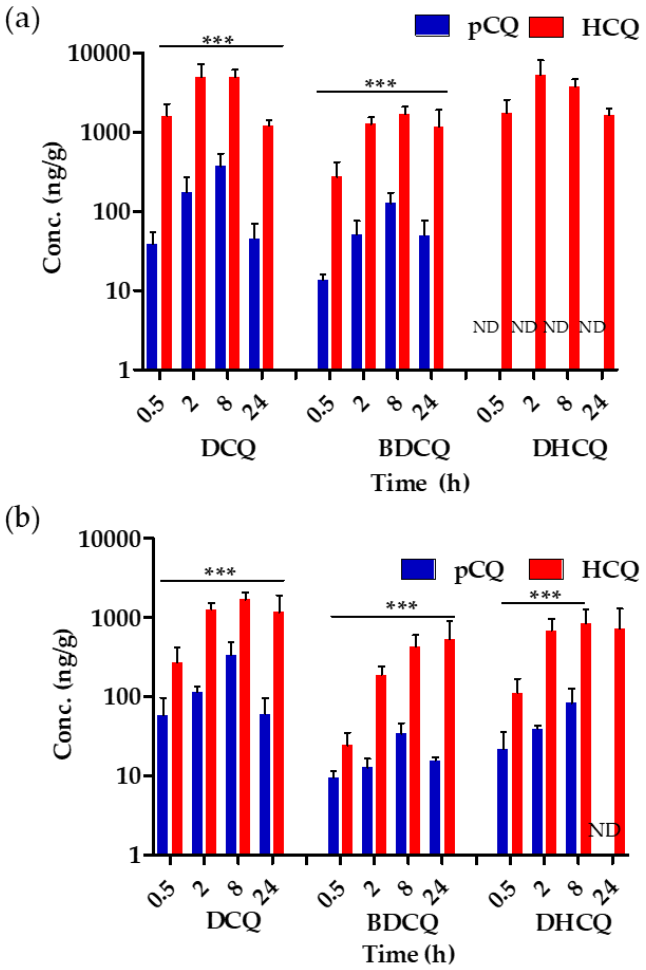


Figure 16. Metabolite levels of HCQ and pCQ in (a) liver and (b) colon. Tissue homogenates were analyzed for metabolites. Data are represented as mean metabolite concentration in ng/g of tissue.

3.3.4 Therapeutic efficacy

The local colon accumulation, limited systemic exposure, and low liver distribution of pCQ provided strong rationale for the testing of its anti-inflammatory activity in colitis. We conducted a therapeutic efficacy study designed to assess whether restricting the distribution of pCQ to the GI tract preserves the activity of HCQ. The mice with colitis were treated (every other day) with oral gavage of pCQ and HCQ. Histological changes in the colon were examined following animal sacrifice on day 14. As shown in Fig. 17, untreated animals showed superficial epithelial damage, marked reduction in the goblet cell population, mucin depletion, inflammatory cell infiltration and crypt hyperplasia [141]. Treatment with both, pCQ and HCQ, reduced the colon inflammation and eased the epithelial injury (Fig. 17a). Statistically significant reduction of the histological damage score was observed in animals treated with pCQ (Fig. 17c). Both treatments significantly reduced the colon crypt length when compared with the untreated group (Fig. 17b). Our findings support previous reports showing the efficacy of chloroquine in DSS-induced model of colitis and human patients [157, 161, 169].

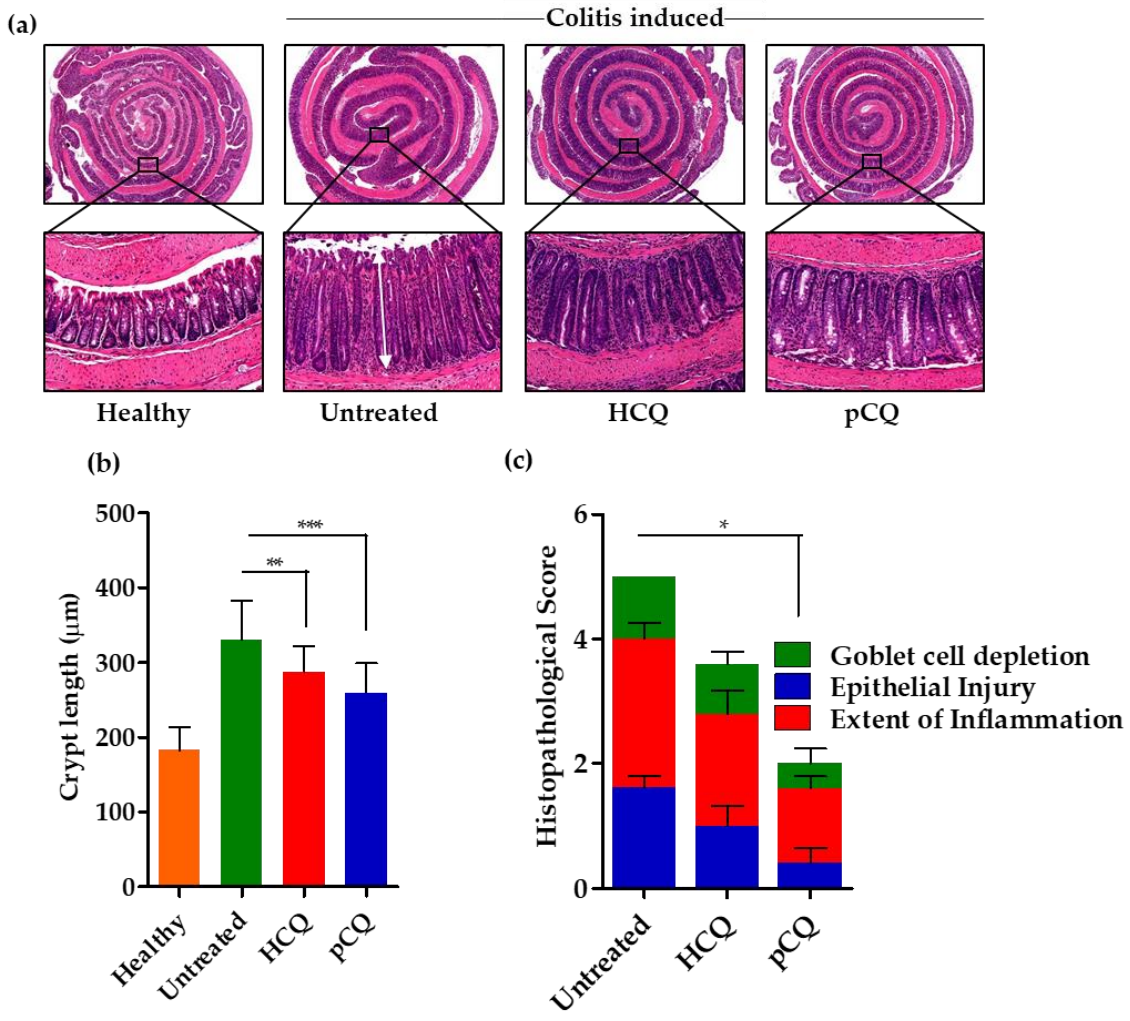


Figure 17. Effect of HCQ and pCQ on *C.rodentium*-associated colonic inflammation. Mice with *C.rodentium* induced colitis were orally administered with 7 doses at 30mg/kg CQ dose over 14 days. (a) Representative images of H&E stained colon tissue slides; (b) colon crypt length; (c) histopathological scores. Data are represented as mean ± SD.

To assess the immunohistochemical changes that occur during inflammation, we determined the effect of pCQ treatment on the expression of two markers (CD68, CC3) that are commonly measured in reported *in vivo* IBD studies. CD68 is a transmembrane glycoprotein specifically expressed by monocytes and macrophages. CD68 plays an important role in macrophage homing to tissues and is used to study macrophage infiltration in the inflamed colon. Colon sections from patients with active IBD have significantly higher macrophage infiltration than healthy subjects [170]. We observed elevated infiltration of $CD68^+$ macrophages in the colons of untreated mice with colitis (Fig. 18a). Treatment with pCQ and HCQ showed statistically significant reduction in the macrophage infiltration, with pCQ outperforming HCQ (Fig. 18b). $CD68^+$ macrophages have different roles in UC and CD, but they massively infiltrate throughout the inflamed colon [171] and targeting CD68 to reduce macrophage infiltration is a potential therapeutic strategy in IBD [172]. Based on our prior work which showed a broad ability of pCQ to inhibit migration and invasion of cells, we propose that reduction of macrophage infiltration could be a possible mechanism by which pCQ is exerting its anti-inflammatory activity. We next evaluated apoptosis as a marker for epithelial cell injury. CC3 immunostaining was used assess the apoptotic cells in the colon epithelium and we found that pCQ treatment showed statistically significant reduction in the number of apoptotic epithelial cells when compared with the untreated group (Fig. 18d). This observation combined with the decrease in the crypt length demonstrates the amelioration of epithelial cell injury by pCQ.

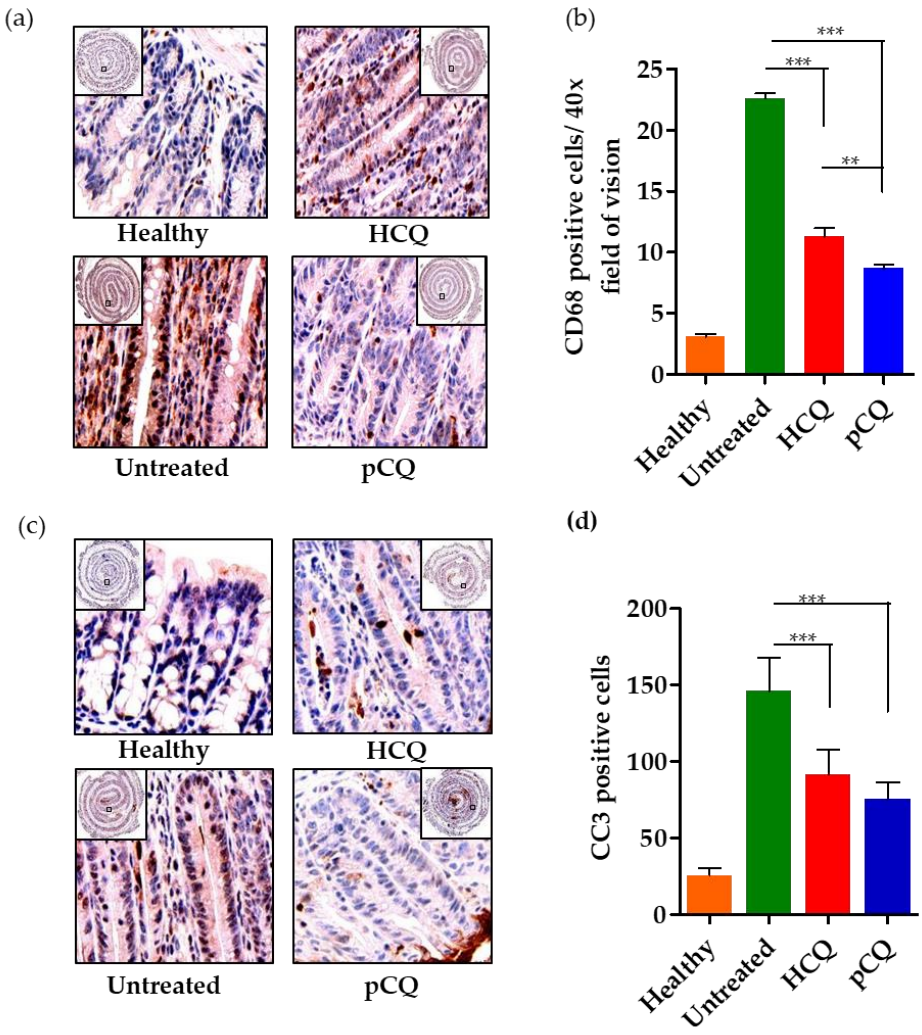


Figure 18. Effect of HCQ and pCQ on colonic macrophage infiltration and epithelial cell apoptosis. (a) Representative images of CD68 stained colon tissue slides; (b) quantitative results of CCD68 positive cells. Data are represented as mean CD68 positive cells per HPF ± SD. (c) Representative images of CC3 stained colon tissue slides; (d) quantitative results of CC3 positive cells. Data are represented as mean CC3 positive cells/ colon section ± SD.

3.3.5 Mechanism of action

Following histological evaluation, we sought to explore possible mechanisms for the anti-inflammatory activity of pCQ. Upregulation of pro-inflammatory cytokines is a hallmark of IBD and lowering local levels of cytokines has been shown to reduce colon inflammation. To investigate how pCQ treatment changes the cytokine expression profile, we measured the mRNA levels of selected pro-inflammatory cytokines in the colons after oral administration of seven doses of pCQ over 14 days. Although inhibition of TNFα is a well-established approach in the treatment of IBD and TNFα expression was 5-times higher in the untreated group, we observed no significant reduction in the colon TNFα expression after treatment with pCQ (Fig. 19a). We then measured expression levels of IL-6, IL-1β and IL-2 in the colon. Our initial studies indicated that IL-6 was highly upregulated in the *C. rodentium* model. Here, we have observed statistically significant reduction of IL-6 expression by both pCQ and HCQ (Fig. 19b). We have observed similar effect of pCQ and HCQ treatments on the expression of IL-1β (Fig. 19c).

In contrast to IL-6 and IL1β, both treatments resulted in upregulated IL-2 expression (Fig. 19d). Statistically significant difference was observed between IL-2 levels in untreated control and pCQ group. IL-2 knockout mice are an often-used animal model of IBD and there has been a reported clinical trial which investigated subcutaneously administered IL-2 as a way of enhancing regulatory T cells in IBD patients to reduce inflammation [173]. Based on these findings, upregulation of IL-2 may represent an interesting direction in the mechanistic studies of pCQ anti-inflammatory activity.

IL-6 has been the target of many mechanistic as well as clinical and preclinical studies [174]. The IL-6 downstream pathways promote immune response by increasing the $CD4^+$ T-cell migration into the inflamed colon, which consequently increases the migration of other immune cells to the inflamed areas [175]. Accumulated evidence suggests that activation of IL-6 is an important inflammatory event in the development of IBD. Hence, inhibiting IL-6 signaling pathways represents a possible mechanism for the observed anti-inflammatory activity of pCQ [176]. Overall, our observations pointed out that the therapeutic activity of pCQ seemed to be an effect of restoring the colonic immune imbalance that occurs in IBD.

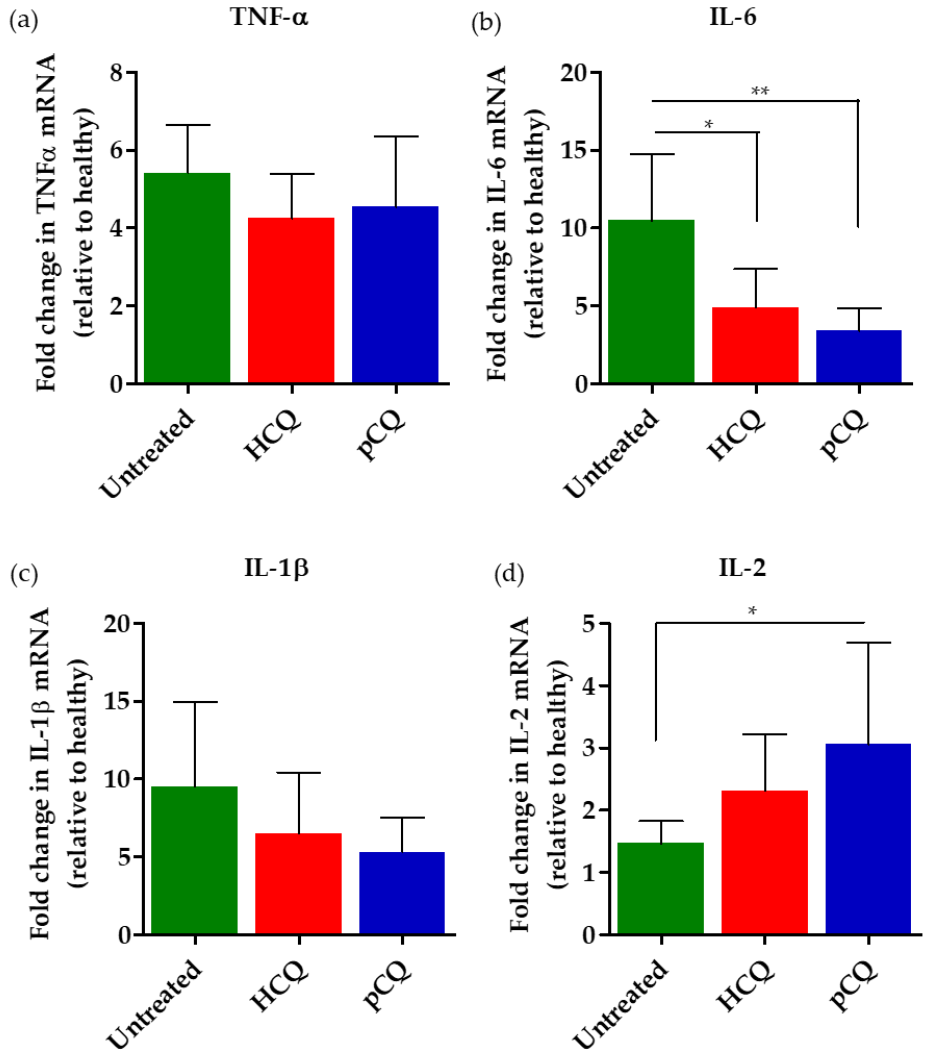


Figure 19. Effect of HCQ and pCQ on murine colonic cytokine levels. Fold change in colonic mRNA expression relative to healthy control (a) TNF-*α*; (b) IL-6;(c) IL-1β; (d) IL-2. Data are represented as mean ± SD.

3.4 Conclusions

This is the first report on the local anti-inflammatory effect of non-absorbable polymeric form of HCQ. We have demonstrated promising therapeutic efficacy of pCQ in murine model of colitis induced by infection with *C. rodentium*. Unlike epithelial injury models of IBD, this model captures the immunological and microbiological aspects of IBD, making it more relevant to human disease. Despite comparable colon accumulation as HCQ, pCQ showed significant reduction in colon inflammation. Further improvement of pCQ accumulation in the inflamed colon is likely to enhance the effect. We identified several putative mechanisms of action for pCQ as an anti-inflammatory agent. Imbalance of the immune system plays a major role in IBD and our investigation suggests that pCQ may restore the homeostasis between the pro-inflammatory and anti-inflammatory aspects of the immune system in the colon. The exact mechanism of how pCQ exerts its therapeutic activity is beyond the scope of this report and will be a topic for future investigations. Our findings are important for the development of safer local IBD therapies.

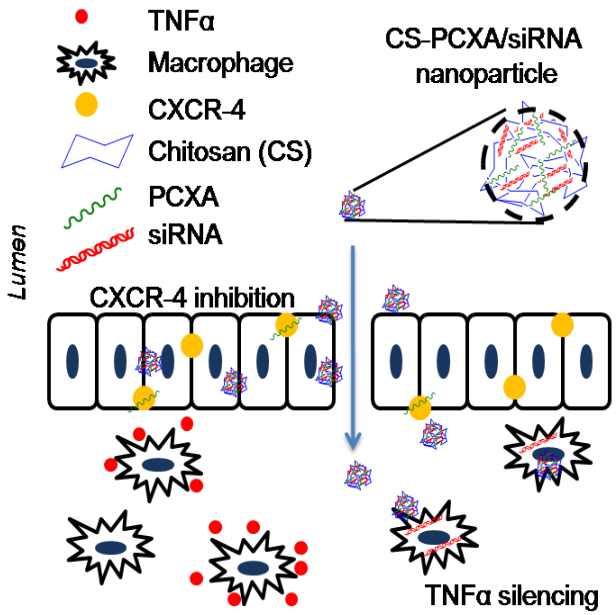
Chapter 4: Development of CXCR4 antagonist TNFα siRNA nanoparticles as an oral treatment for IBD

4.1 Introduction

Traditional IBD therapy includes palliative medication like systemically given anti-inflammatory and immunosuppressant drugs. Available evidence shows that IBD arises from aberrant immune response and concurrent upregulation of pro-inflammatory cytokines such as TNFα, IL-6 and IL-8. Success of treatments based on systemic administration of anti-TNFα antibodies confirms that TNFα plays a prominent role in the pathogenesis of IBD. However, TNFα inhibition exhibits a range of complications related to the cost of the treatment and systemic immune suppression leading to a risk of opportunistic infections and development of cancer. Chemokine receptor CXCR4 and its ligand CXCL12 have also been implicated in the IBD pathology. The CXCR4/CXCL12 axis is involved in regulating trafficking and invasion of inflammatory cells in the GI tract and its inhibition is known to exert therapeutic effect in experimental IBD animal models. Thus, both TNFα and CXCR4 are upregulated in IBD mucosa and represent an exciting combination target for local IBD therapy. There is a great need for novel local treatments of IBD as potentially safer alternatives to the current systemic treatments.

To better treat IBD, we sought to develop and test novel dual-function particles for combination oral treatment designed to safely reduce colonic inflammation. The particles are based on a recently developed polymeric CXCR4 antagonists (PCXA) designed to encapsulate, protect, and orally deliver small interfering RNA (siRNA). We believe is that

this combined approach will result in improved treatment of IBD as a result of decreased inflammation due to TNFα siRNA (siTNF) and inhibition of CXCR4 by PCXA.



Scheme 4. Proposed mechanism of CS-PCXA-siRNA NPs.

4.2 Materials and Methods

4.2.1 Materials

DSS (Mw 40,000) was obtained from TdB consultancy, DMEM, PBS, NaAc were purchased from Thermo Fisher Scientific Inc., Cy5.5 labeled siRNA was bought from Sigma Aldrich, Trizol reagent was purchased from Invitrogen, siTNFα and siSCR were obtained from Dharmacon, CXCR4 redistribution assay kit was purchased from Thermo Fisher Scientific Inc., CS was a kind gift from the lab of Dr. Kenneth Howard, Aarhus University, Denmark, RAW 264.7 cells were obtained from the lab of Dr. Yuri Lyubchenko.

4.2.2 Biodistribution of PCXA/siRNA particles in healthy mice

Mice were orally administered with PCXA/siRNA NPs. Cy5.5 labeled siRNA was used with every mouse receiving 4 μg siRNA. The mice were sacrificed 24 h post administration and the organs were harvested. Organs were visualized for fluorescence using IVIS.

4.2.3 Therapeutic activity of PCXA

Colitis was induced in mice using 5% DSS solution. Mice were divided into healthy controls, Untreated controls, PCXA i.p. and PCXA oral. After DSS treatment for 5 days, PCXA was administered either orally or i.p. three times a day for 3 days. The longitudinally opened colons were rolled into a Swiss roll from distal to proximal end. The rolls were fixed for 24 h in 4% paraformaldehyde, embedded in paraffin, sectioned and stained with hematoxylin and eosin (H&E). The stained sections were evaluated by a

pathologist without the knowledge of the identity of the samples using a light microscope. Histopathological scores were assigned based on criteria as previously described. The sections were graded based on ulceration, inflammation, crypt damage and edema. For each tissue, a numerical score was assigned in a blinded manner to prevent bias. Scores from each tissue section group were averaged to obtain a mean histopathological score.

4.2.4 Preparation and physical characterization of CS-PCXA-siRNA NPs

CS-PCXA-siRNA NPs were prepared by mixing CS, PCXA and siRNA solutions (40:4:1) in 200mM NaAc buffer, pH 5.5 and vortexing for 30 seconds. This was followed by incubation at room temperature for 20 minutes to stabilize the NPs before further use. siRNA complexation in the NPs was studied by agarose gel electrophoresis. The prepared NPs were loaded onto a 2% agarose gel containing 0.5 μg/ml ethidium bromide. Gels were run in an assembly at 75 V in 0.5x Tris/Borate/EDTA (TBE) buffer for 45 minutes and imaged under UV light using Kodak imager. The hydrodynamic radius of the NPs in 200mM NaAc buffer was measured using DLS. The results were expressed as mean ± SD of three measurements.

4.2.5 Resistance to simulated fluids

To study the resistance of CS-PCXA-siRNA NPs against RNase I, 20 μl of NP formulation containing a total amount of 0.2 μg siRNA were incubated with 2.5 units of RNase I at 37 °C for 30 minutes. The mixture was further incubated at 90 °C for 30 minutes to inactivate the enzyme. Heparin (200 μg/ml) was added to the samples and the mixtures

were incubated at room temperature for 30 minutes to dissociate the siRNA. Agarose gel electrophoresis was used to evaluate the siRNA integrity.

Resistance to SGF and SCF with bile salts was evaluated in a similar way. 20 μl of NP formulations were incubated with 20 μl of either SGF or SCF respectively. The mixtures were incubated at 37 °C for 30 minutes. Heparin was added and the mixtures were incubated at room temperature for 30 minutes to dissociate the siRNA. Agarose gel electrophoresis was used to evaluate the siRNA integrity.

4.2.6 CXCR4 inhibition assay

CXCR4 antagonism of the polycations and polyplexes was measured by CXCR4 redistribution assay using a high-contact fluorescence microscopy analysis. U2OS cells stably expressing functional EGFP-CXCR4 fusion protein were seeded at a density of 8,000 cells/well in 96-well black plates with optical bottom 24 h before the experiment. On the day of the assay, cells were washed twice with 100 μL assay buffer (DMEM supplemented with 2 mM L-glutamine, 1% FBS, 1% Pen-Strep, and 10 mM HEPES) and incubated with PCXA/siRNA, CS/siRNA, CS-PCXA/siRNA NPs or 300nM AMD3100 in triplicates in the assay buffer containing 0.25% DMSO at 37 °C for 30 min. Then, 10 nM SDF-1 was added to each well and the cells were incubated at 37 °C for 1 h. Cells were fixed with 4% paraformaldehyde at room temperature for 20 min, washed 4 times with PBS and stained in 1 μM Hoechst 33258 solution for 30 min before imaging (EVOS fl microscope).

4.2.7 Cellular uptake of CS-PCXA-siRNA NPs

Cellular uptake of NPs was studied in RAW 264.7 mouse macrophage cell line. Cells were seeded in 24-well plate at a density of 20,000 cells per well 24 h prior to the study. 6-FAM labeled fluorescent siRNA (Sigma-Aldrich) was used. On the day of the study, the cells were incubated with PCXA/siRNA, CS/siRNA and CS-PCXA/siRNA NPs in serum free media at an siRNA concentration of 100nM. After 4 h incubation, the media was removed. The cells were washed using cold PBS and scraped off using a cell scraper. The cells were resuspended in PBS containing 10% FBS and analyzed for fluorescence using flow cytometry. The results were processed using flow cytometry data analysis software Flowjo and expressed as % 6-FAM positive cells ± SD.

RAW 264.7 cells were seeded in a 23-mm glass-bottom dish (Nioptechs Inc.) at a density of 100,000 cells 24 h prior to study. 6-FAM labeled fluorescent siRNA was used. On the day of the study, the cells were incubated with PCXA/siRNA, CS/siRNA and CS-PCXA/siRNA NPs in serum free media at an siRNA concentration of 100nM. After 4 h incubation, the media was removed. washed twice with PBS, fixed with 4% paraformaldehyde, washed with PBS for additional 4 times and stained in 1 μM Hoechst 33258 solution. All the images were taken using Zeiss 710 confocal laser scanning microscope equipped with a 63x oil objective and 4 lasers (Blue Diode 405 nm, Argon 458/488/514 nm, DPSS 561 nm and He-Ne 633 nm).

4.2.8 Mechanism of cellular uptake of CS-PCXA-siRNA NPs

Cells were seeded in 24-well plate at a density of 20,000 cells per well 24 h prior to the study. On the day of experiment cells were pre-incubated for 30 minutes with various

endocytotic inhibitors including chlorpromazine (10 μg/ml), genistein (200 μg/ml) and wortmannin (50 nM). 6-FAM labeled fluorescent siRNA (Sigma-Aldrich) was used. On the day of the study, the cells were incubated with CS-PCXA/siRNA NPs in serum free media at an siRNA concentration of 100nM. After 4 h incubation, the media was removed. The cells were washed using cold PBS and scraped off using a cell scraper. The cells were resuspended in PBS containing 10% FBS and analyzed for fluorescence using flow cytometry. The results were processed using flow cytometry data analysis software Flowjo and expressed as % 6-FAM positive cells ± SD.

4.2.9 Selection of siRNA sequence

TNFα siRNA smartpool containing four different siRNA sequences from Dharmacon was used. RAW 264.7 cells were seeded on 24-well plates at 5×10^4 cells/well and cultured for 24 hours. Cell transfection was performed using Polyplyus INTERFERIN® transfection reagent. For each well, 0.6 pmol of siRNA was diluted and mixed in 100 μl serum-free DMEM. 2 μl of the transfection reagent was added to the diluted siRNA and the mixture was homogenized by vortexing for 10 seconds. Similar procedure was carried out to prepare complexes with scrambled siRNA. All the siRNA sequences were used individually to form the transfection complexes. The complexes were incubated at room temperature for 10 minutes to stabilize them. During this period, 500 μl of fresh DMEM containing 10 % FBS was added to the well having seeded cells. 100 μl transfection complexes were added to the wells and the plate was swirled gently to allow homogenization. The final volume in the well was 600 μl with the siRNA

concentration 1 nM. The plate was incubated at 37 °C for 24 hours before LPS stimulation (100 ng/ml) for 6 h. TNFα mRNA levels were measured using RT-PCR and the fold difference was calculated by $\Delta\Delta$Ct method.

4.2.10 Biodistribution of CS-PCXA-siRNA NPs in mice

Male C57Bl6 mice, 6 weeks old were obtained from Charles river laboratories. 20 mice were kept as healthy mice. Colonic inflammation was induced using DSS or *C. rodentium* as described below (20 mice in each group). NPs were prepared and 200 µl formulation containing 30 µg Cy 5.5 labeled siRNA was administered to the mice. At pre-determined timepoints, 5 mice from every group were sacrificed and the colon was harvested, cleaned of feces and snap frozen in liquid nitrogen. The colons were homogenized in RIPA buffer containing RNase inhibitor. The homogenate was centrifuged for 15 minutes and 17,000 x g. 100 µl supernatant was pipetted into a 96-well plate and fluorescence was measured using a 96-well plate reader comparing by standard curve generated by diluting a known amount of siRNA with colon homogenate obtained from control animals.

4.2.11 Effect of DSS on stability of CS-PCXA-siRNA NPs

CS-PCXA-siRNA NPs were prepared as previously described. The NPs were incubated with varying concentrations of DSS for 30 minutes. The samples were run using agarose gel electrophoresis and visualized under UV light to assess dissociation of siRNA from the particles.

4.2.12 Efficacy of CS-PCXA-siRNA NPs in *C. rodentium* mouse model of colitis

Inflammation was induced in male C57BL/6 mice, 6 weeks old using bacteria as described before. Mice were divided into healthy controls, untreated controls (n=8), CS-PCXA-siTNF and CS-PCXA-siSCR treated (n=5). Respective treatments were orally administered to mice every other day over the 14 days of the model. On day 14, the mice were sacrificed and the colons were harvested. On day 14, the mice were sacrificed and the colons were harvested. The colon was opened longitudinally, cleaned of fecal matter, and excised into two parts along the length, which were stored accordingly for determination of cytokine mRNA levels by RT-PCR and histological analysis.

4.2.13 RT-PCR

Colon samples from the therapeutic study were stored in RNAlater™ (Thermo Fisher Scientific Inc.) at 4°C for 48 hours to allow sufficient time for tissue penetration followed by removal of excess solution. The tissues were then stored at -80°C until further processing. Stored frozen tissues were homogenized in TRIzol™ (Thermo Fisher Scientific Inc.) reagent using TissueLyser II (Qiagen) and mRNA was isolated from the homogenized tissues according to manufacturer's protocol. The extracted mRNA was quantified using Nanodrop One^{c} UV-Vis spectrophotometer (Thermo Fisher Scientific Inc.). The cDNA was synthesized from the mRNA using High-Capacity cDNA Reverse Transcription Kit with RNase Inhibitor per the manufacturer's protocol (Thermo Fisher Scientific Inc.). A volume corresponding to 1 μg of RNA as determined by UV spectrometer was used for cDNA synthesis. Synthesized cDNA was stored at -20°C until further use. RT-PCR was carried out using the synthesized cDNA from colon tissue

samples to determine the levels of mRNA of the target genes. Healthy and untreated colons were used as controls. cDNA was mixed with 0.2 μM primer pair for TNFα and iTaq™ Universal SYBR® Green Supermix (Biorad) into an optical reaction tube (Qiagen). The RT-PCR reaction was carried out in Rotor-Gene Q 2plex thermal cycler (Qiagen) using the following cycle program: 95°C for 3 minutes; 40 cycles 60°C for 30 seconds. Results obtained from the RT-PCR were analyzed by Ct method to determine the fold change in gene expression.

4.2.14 Histological evaluation

The longitudinally opened colons were rolled into a Swiss roll from distal to proximal end. The rolls were fixed for 24 h in 4% paraformaldehyde, embedded in paraffin, sectioned and stained with hematoxylin and eosin (H&E). The stained sections were evaluated by a pathologist without the knowledge of the identity of the samples using a light microscope. Histopathological scores were assigned based on criteria as previously described [165]. Scoring was performed based on severity of epithelial injury (graded 0-3, from absent to mild including superficial epithelial injury, moderate including focal erosions, and severe including multifocal erosions), the extent of inflammatory cell infiltrate (graded 0-3, from absent to transmural), and goblet cell depletion (0-1). For each tissue, a numerical score was assigned in a blinded manner to prevent bias. Scores from each tissue section group were averaged to obtain a mean histopathological score.

4.3 Results and Discussion

The overall goal of this study was to develop NPs, made up of PCXA, CS and siRNA. The particles should exhibit stability in the GIT and have improved uptake in the colon when delivered orally.

4.3.1 Therapeutic activity of PCXA in DSS mouse model of colitis

Recently, the involvement of CXCR4 and its ligand CXCL12 in inflammation has attracted significant interest. CXCR4 and its ligand CXCL12 were thought to be exclusively involved in regulation of normal leukocyte recirculation and hematopoiesis. However in recent years their involvement in inflammation has been realized [177]. CXCR4 and CXCL12 are expressed by cells in the normal intestinal mucosa, contributing to cell migration. However, recently their presence has been extended to CXCR4+ lamina propria T cells and pathogenesis of IBD [178, 179]. CXCR4 is overexpressed in inflamed colons in humans as well as murine IBD models. Despite its important role in mediating homeostasis, CXCR4 blockade has been attributed to reduction in inflammation in the colon and intestinal tissue resurrection [180]. The ubiquitous expression of CXCR4 in the inflamed intestines makes the CXCR4/CXCL12 axis blockade a promising therapeutic strategy [144]. Unfortunately, chronic systemic administration of the only commercially available CXCR4 antagonist AMD3100 has been associated with significant cardiotoxicity thereby limiting its use as systemic IBD therapy [181, 182]. We have synthesized PCXA which is a polymer of AMD3100 and shown its benefits in as a dual activity polymer

which not only can inhibit CXCR4 but also complex nucleic acids. It was important to assess whether the synthesized PCXA shows any therapeutic promise in IBD. To test that, we conducted a preliminary study to investigate whether the anti-CXCR4 effect of PCXA reduced inflammation in the DSS mouse model of colitis. Higher dose of orally delivered PCXA was chosen to account for first pass metabolism. The results of the study are represented in figure 20. As expected, the mice which received no treatment showed significant inflammation and colon tissue damage elicited by the chemical injury due to DSS. There was significant crypt damage, edema and infiltration by immune cells. As opposed to the untreated controls, the groups which were administered PCXA showed an improvement in the disease severity. The reduction in inflammation was evidenced with higher number of intact crypts, presence of regular shaped goblet cells and significantly less immune cell infiltration into the colon tissue. The histopathological scores assigned to the H&E stained sections by a pathologist without the knowledge of identity of samples are shown in figure. All the groups which received DSS showed a significant increase in the inflammatory scores. However, the PCXA treated groups showed a reduction in the inflammatory scores compared to the untreated group. This indicated that the anti-CXCR4 activity of PCXA was reducing the colon inflammation. A significant reduction in the inflammatory score was observed with the group where PCXA was administered i.p. The group which received oral gavage of PCXA did not show a very appreciable reduction in the inflammation. We attribute this poor effect with less colon retention time for the orally administered PCXA. The PCXA administered i.p was delivered directly at the site of inflammation and thus, showed a better therapeutic effect.

In order to improve the GI retention time, we proposed the use of a mucoadhesive polysaccharide, CS in the PCXA-siRNA NP formulation.

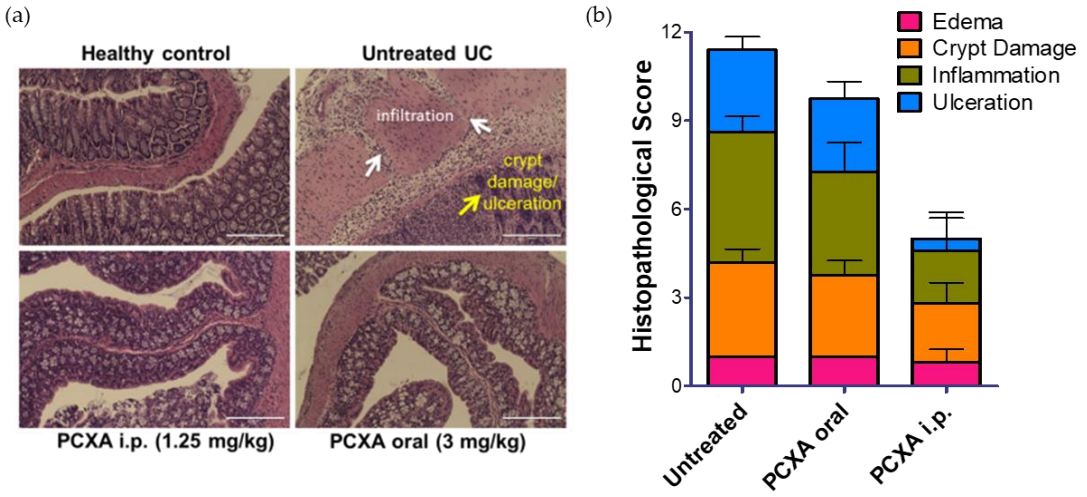


Figure 20. Treatment of IBD with PCXA. Acute IBD was induced by 5% DSS in drinking water and the mice were treated with i.p. or oral administration of PCXA (n=5). (a) Representative H&E stained images of colon sections; (b) Histopathological scores.

4.3.2 Biodistribution of PCXA/ siRNA particles in mice

After demonstrating the therapeutic promise of PCXA in a mouse model of IBD, we investigated whether PCXA/siRNA NPs could deliver siRNA to the colon when administered orally in healthy mice. Mice were gavaged with NPs and organs were visualized ex vivo post-sacrifice using IVIS for signal from fluorescently labeled siRNA (Fig. 21). Fluorescent signal for siRNA was observed in the colon 24 h post gavage. Systemic organs like liver and spleen did not show any fluorescent signal. These results gave us initial confidence that PCXA could deliver the siRNA to the colon via the oral route and there was less systemic absorption of siRNA.

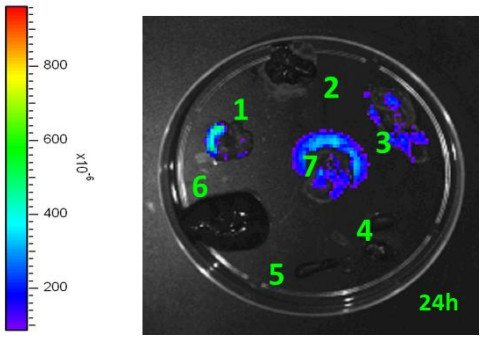


Figure 21. Biodistribution of orally administered PCXA/siRNA NPs in mice. (1 – stomach, 2 – small intestine, 3 – large intestine, 4 – liver, 5 – kidneys, 6 – spleen, 7 – lung).

4.3.3 Preparation of CS-PCXA-siRNA NPs

PCXA by itself can complex and condense siRNA into NPs. However, from our previous studies, PCXA-siRNA NPs did not show significant delivery of siRNA in the colon, probably due to stability issues as well as less retention time in the GIT. Additionally, oral administration of PCXA did not show significant therapeutic effect in a mouse model of colitis. These findings effected the need to modify the PCXA-siRNA NP system to make it more compatible with oral administration. Various cationic carriers have been investigated for the delivery of siRNA. Of all these CS has attracted considerable research interest, especially in the oral delivery of siRNA. CS can bind to the negatively charged siRNA to form NPs which can adhere to the negatively charged cell membrane and be taken up via endocytosis. This helps to restrict the action of siRNA to the colon which is desirable in a disease like IBD. Secondly, CS is available in a variety of molecular weights and degrees of deacetylation. Based on our preliminary studies and literature, we chose to use CS with molecular weight of 50 KDa and 80% degree of deacetylation. However, CS is insoluble at neutral and alkaline pH. Hence, CS was dissolved in 200 mM NaAc buffer, pH 4 at a concentration of 10 mg/ml and diluted further to required concentrations in 200 mM NaAc buffer, pH 5.5.

CS, PCXA were used to complex siRNA resulting in the formation of NPs via electrostatic interactions. This method prevents the use of heating or sonication, which avoids interaction of siRNA with toxic organic solvents and maintains its biological activity.

4.3.4 Physical characterization of CS-PCXA-siRNA NPs

Based on preliminary experiments, NPs were prepared from CS: PCXA: siRNA in the weight ratio of 40: 4: 1. Agarose gel electrophoresis was used to study the siRNA complexation of the NPs. As shown in the figure 22a, CS-PCXA could completely complex siRNA as evidenced by the absence of any free siRNA. This was expected at PCXA by itself could complex siRNA at the concentration used. CS by itself does not possess enough positive charge to completely complex siRNA. As a result, CS-siRNA showed presence of some amount of free siRNA as seen in the figure.

The particles sizes and the zeta potentials of various NPs prepared were measured using DLS and the results are expressed in figure 22b. All the NPs had diameters ranging from 150 to 200 nm. The zeta potentials for all the particles were positive which was expected because of all the cationic components used (not shown).

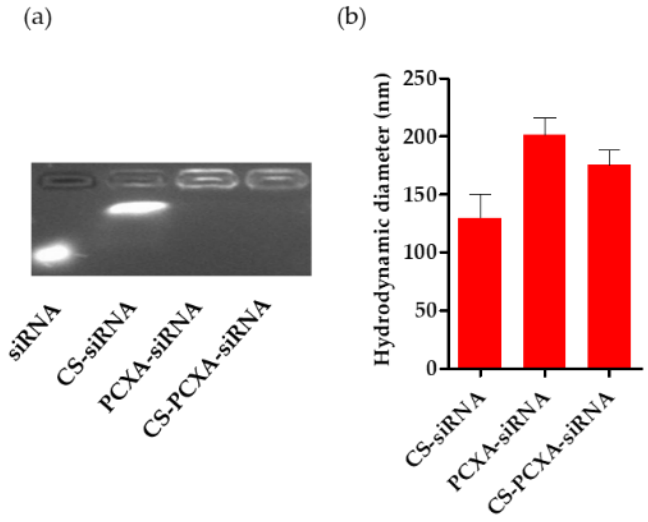


Figure 22. Physicochemical characterization of NPs. (a) siRNA complexation in CS-PCXA-siRNA NPs by agarose gel electrophoresis; (b) Particle sizes in nm

4.3.5 CXCR4 antagonistic activity

CXCL12 is the downstream signaling ligand for CXCR4. After binding it induces downstream processes through multiple pathways line RAS and PI3 kinase. CXCR4 antagonists not only blocks the CXCL12-induced downstream signaling but also inhibits endocytosis of the receptor [183]. To evaluate CXCR4 antagonism and to ensure that using CS as such high concentrations would not hamper the anti-CXCR4 activity of PCXA, CXCR4 redistribution assay was performed. The assay utilizes U2OS cells stably expressing human CXCR4 receptor tagged to the N-terminal of enhanced GFP. The assay monitors the cellular translocation of the GFP-CXCR4 receptors in response to stimulation with human CXCL12. The cells received appropriate treatments followed by stimulation with human CXCL12. As seen in figure 23, untreated cells exhibited the internalization of CXCR4 receptors into the endosomes, as suggested by the punctate distribution of the GFP fluorescence. On the contrary, treatment with AMD3100, a commercially available CXCR4 antagonist blocked the internalization of the receptor as evidenced by no punctate GFP fluorescence within the cells.

Next, we explored whether the NPs themselves exhibit CXCR4 antagonism. As expected PCXA-siRNA NPs exhibited CXCR4 antagonism (Figure 23). On the other hand, CS-siRNA NPs did not show any inhibition of CXCR4. This does not come as a surprise as CS is not reported to have any anti-CXCR4 activity. The CXCR4 antagonism of CS-PCXA-siRNA NPs was evaluated to make sure that CS which was at a concertation 10

times higher than PCXA, did not affect the anti-CXCR4 activity of PCXA in any way. It was observed that the CXCR4 inhibition for CS-PCXA-siRNA NPs was similar to the inhibition observed with PCXA-siRNA NPs.

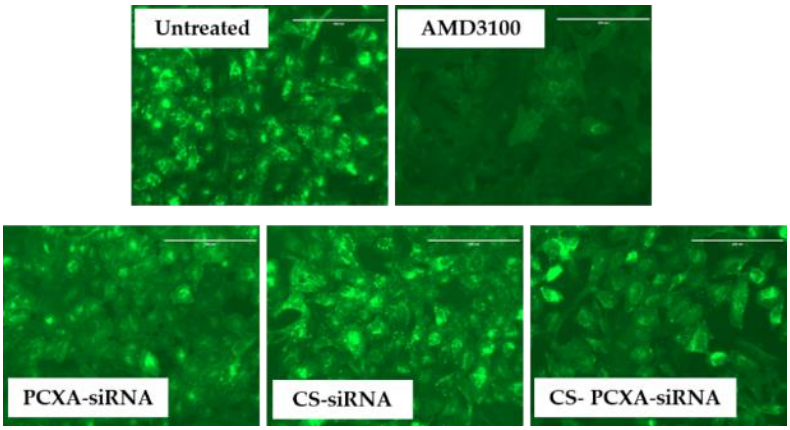


Figure 23. CXCR4 inhibition of NPs. U2OS cells having GFP-tagged CXCR4 receptor were treated with NPs followed by stimulation with SDF-1. Cells were observed under fluorescence microscope. Untreated cells (0% CXCR4 antagonism), cells treated with 300 nM AMD3100 (100% CXCR4 antagonism).

4.3.6 Resistance to biorelevant conditions

The structural integrity of NPs is important for the effective protection of their payload. Orally delivered delivery systems face a lot of hurdles before they can reach the colon. Few important factors include the volume of the digestive fluids, gastric juices, as well as the alterations in the pH and the ionic strength which can result in damage to the particle structure. Thus, assessing the stability of NPs against all these conditions forms an important consideration for orally delivered therapeutics. Hence, we investigated the effect of conditions which orally delivered systems usually encounter. We assessed the effect of SGF and SCF on particle integrity using agarose gel electrophoresis after incubating the NPs with SCF or SGF for 30 minutes. As seen from figure 24, siRNA showed degradation in the SGF which is strongly acidic in nature. No effect on free siRNA was observed in SCF which is close to a neutral pH. However, looking at all the NP formulations, no change in the particle stability was observed as evidence in almost no difference in the agarose gel bands compared to NP in formulation in buffer. No presence of free siRNA was observed when the NPs were treated with either SCF or SGF indicating no release of the siRNA cargo because of the conditions. This observation proved that the particles maintained their structural conditions under the diverse pH as well as the salt conditions commonly encountered in the GIT during transit.

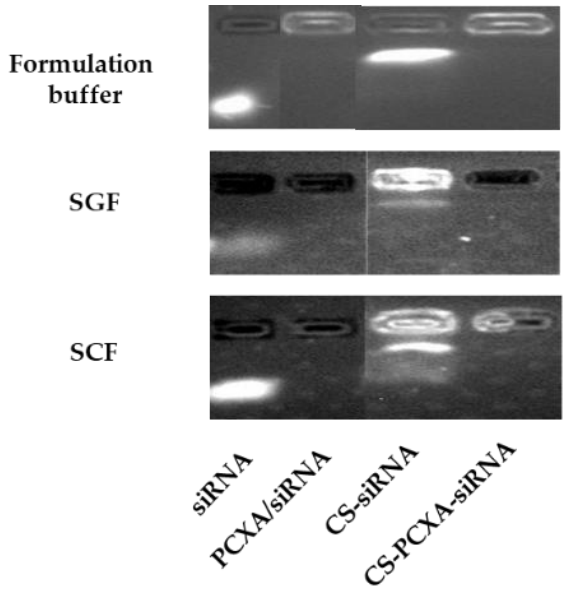


Figure 24. Stability of polyplexes to simulated gastric fluid (SGF) and simulated colonic fluid (SCF). Formulations were incubated for 30 minutes with 30 μl SGF or SCF at 37°C. Particle integrity was visualized using 2% agarose gel containing EtBr.

The GIT is not only a diverse environment in terms of pH and ionic gradients, but also in nucleases and other digestive enzymes. As a result, degradation of NP cargo is a common concern. Degradation of siRNA results in loss of activity and to ensure efficiency *in vivo* via oral delivery, siRNA must be protected from degradation either by nuclease or by the ionic and pH conditions in the GIT. Upon confirmation that the NPs maintained their structural integrity, we turned our attention to assessing the integrity of the complexed siRNA under RNase, SGF and SCF conditions (Fig. 25). Free siRNA and NPs were incubated with RNase, SGF and SCF, respectively for 1 hour. Post-incubation, heparin was to dissociate siRNA from the NPs and agarose gel electrophoresis was used to assess the siRNA integrity. As seen from figure, free siRNA treated with RNase and SGF was completely degraded, whereas clear siRNA band was observed after incubation with SCF. This suggested that siRNA was mainly degraded in the stomach due to the gastric juices and in presence of RNase while remaining comparatively intact in the colon. siRNA loaded into PCXA-siRNA NPs showed faint bands compared to free siRNA indicating partial degradation. On the contrary, CS-siRNA and CS-PCXA-siRNA NPs showed bright bands which were comparable in intensity to free siRNA bands suggesting that they provided good protection for their payload which correlated with the results of structural stability of NPs. No degradation of complexed siRNA was observed in the SCF which was expected from the fact that even free siRNA did not show any degradation under those conditions. These results might suggest that PCXA was not bulky compared to

chitosan which could prevent nucleases and other potential degradants from approaching siRNA via steric hindrance. This study proves the utility of both our cationic components where PCXA acts as a strong complexing agent as opposed to chitosan which provides complexation, in addition to making the NP system more amenable to oral delivery of siRNA by providing protection.

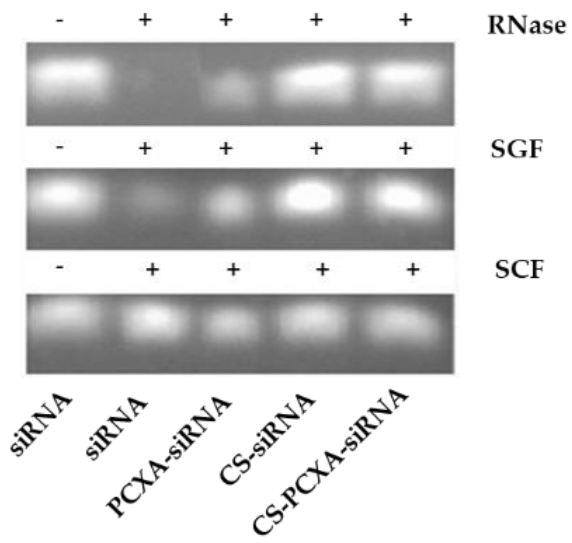


Figure 25. siRNA protection ability of NPs. Formulations were incubated for 30 minutes with 20 μg/ml RNase, 30 μl SGF or SCF at 37°C. siRNA was displaced by heparin and its integrity was visualized using 2% agarose gel containing EtBr.

4.3.7 Cellular uptake of NPs

Efficient cellular uptake is a pre-requisite for the therapeutic effect of siRNA. To determine the uptake of the NPs, RAW 264.7 cells were incubated with PCXA-siRNA, CS-siRNA and CS-PCXA-siRNA NPs containing fluorescent siRNA in serum free conditions, respectively. RAW 264.7 cells are a mouse derived macrophage cell line and are relevant to this study as our target cells for siRNA delivery are the immune cells which infiltrate the colonic epithelium. After 4 hours, the percentage of cells expressing fluorescence was quantified by flow cytometry. Binding of particles to the cell surface and giving a fluorescence signal is always a concern with NP uptake with flow cytometry. This results in a false positive fluorescence signal. To overcome this limitation, the cells were resuspended in flow buffer containing trypan blue to quench the fluorescence from the particles binding to the cell surface as well as eliminating any dead cell population from the uptake analysis (Fig. 26a). As seen from figure, cells incubated with free siRNA did not show a significant fluorescent signal. Similarly, PCXA-siRNA NPs did not show a significant increase in the uptake as compared to free siRNA. The inclusion of CS in the formulation significantly increased the uptake of NPs. Almost 40 % of the cells treated with CS-siRNA NPs showed a fluorescent signal. However, CS-PCXA-siRNA NPs, showed the highest signal with approximately 60 % of the cells showing fluorescence.

We visualized the uptake of CS-PCXA-siRNA NPs using confocal microscopy (Fig 26b). Our observations on the confocal microscope images correlated with the findings

with flow cytometry. The cellular uptake is shown in figure. We also took Z-stack images at various depths within the cells and observed that majority of the particles were internalized by the cells and not stuck to the cell surface (Fig. 27). This proved that our formulation not only possessed the physicochemical properties to complex and protect siRNA in the GIT on oral delivery, but also could deliver siRNA inside the macrophages which are the target cells for most of the anti-inflammatory IBD therapies.

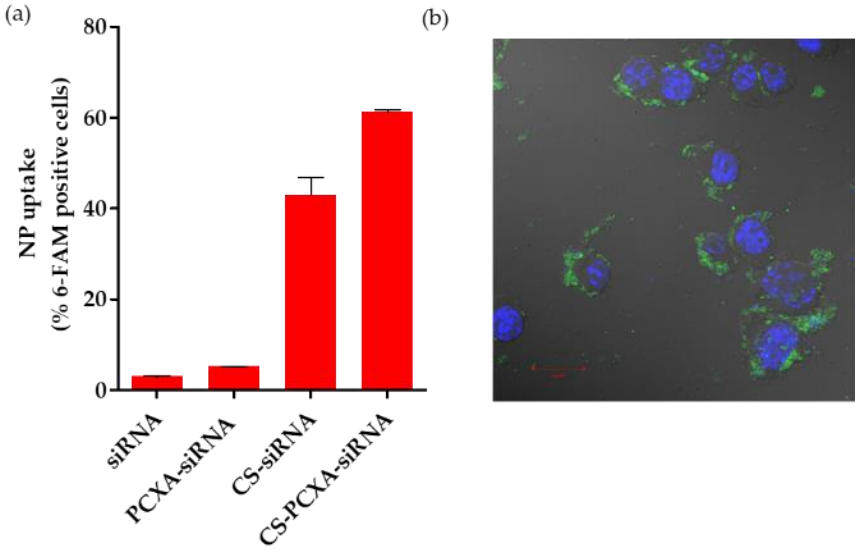


Figure 26. Cellular uptake of NPs. RAW 264.7 mouse macrophages were treated in serum-free media with polyplexes containing siRNA labeled with 6-FAM. Uptake was quantified by (a) flow cytometry and visualized by (b) confocal microscopy. Flow cytometry results expressed as percent 6-FAM positive cells ± SD (n=6).

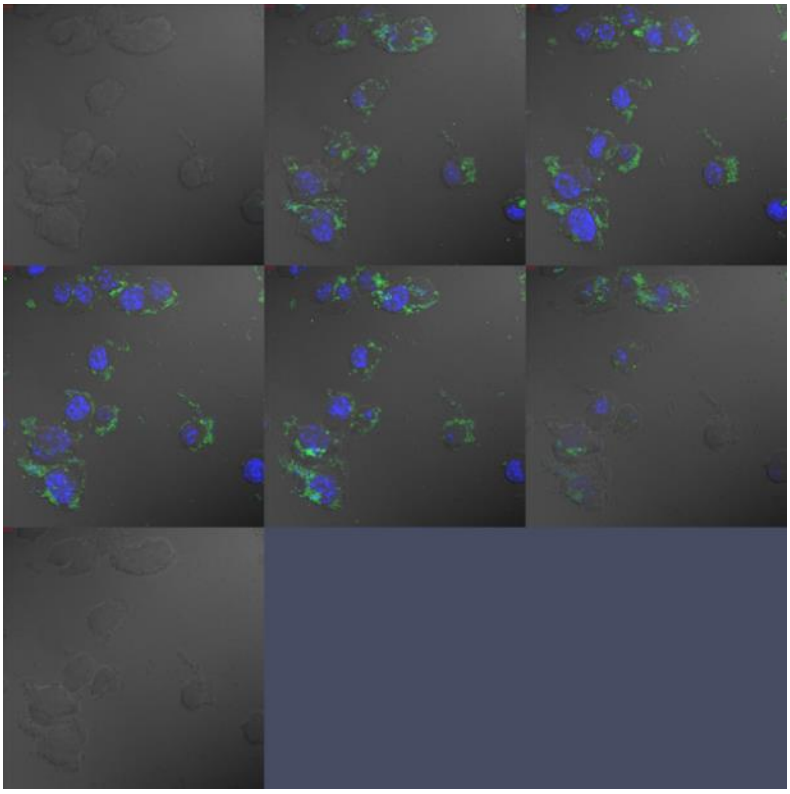


Figure 27. Z-stack images of cellular uptake of CS-PCXA-siRNA NPs. Image slices were taken at various depths in the cells to ensure internalization of particles.

4.3.8 Mechanism of cellular uptake of CS-PCXA-siRNA NPs

Upon confirmation of enhanced uptake of CS-PCXA-siRNA NPs by macrophages, we turned our attention to the mechanism for the uptake of the NPs. An efficient uptake of NPs is essential for the intracellular delivery of cargo [184]. RAW 264.7 cells are reported to express CXCR4 receptor [185]. Hence, the first goal was to identify if the uptake we observed was because of the ability to target CXCR4 receptor. We incubated RAW 264.7 cells with various concentrations of AMD as well as PCXA (not shown) for 30 minutes before quantifying for cellular uptake by flow cytometry. We observed no significant decrease in the uptake of the NPs after incubation with AMD (Fig. 28a). We also incubated cells with free PCXA, however, did not observe any reduction in the uptake of NPs. This proved that the uptake was not CXCR4 mediated and some other endocytosis mechanism was involved in the NP uptake by the cells.

To explore other mechanisms, we incubated RAW 264.7 cells with inhibitors of various endocytotic processes (Fig. 28b). It was made sure that the inhibitors were not toxic to the cells in the used concentrations (not shown). After incubation for 30 minutes, the cells were treated with CS-PCXA-siRNA NPs having 6-FAM labeled siRNA. The uptake was quantified using flow cytometry. We observed no decrease in the NP uptake for cells treated with CPZ and WRT. CPZ and WRT inhibit caveolin and clathrin-mediated endocytosis, respectively. Hence both these processed did not seem to be involved in the uptake of the NPs. However, a significant decrease in the uptake was observed for GEN treated cells. GEN is a specific inhibitor of micropinocytosis. Almost 30 % reduction in the

uptake was observed when micropinocytosis was inhibited which points out that it might be involved to a considerable degree in the uptake of these particles by macrophages.

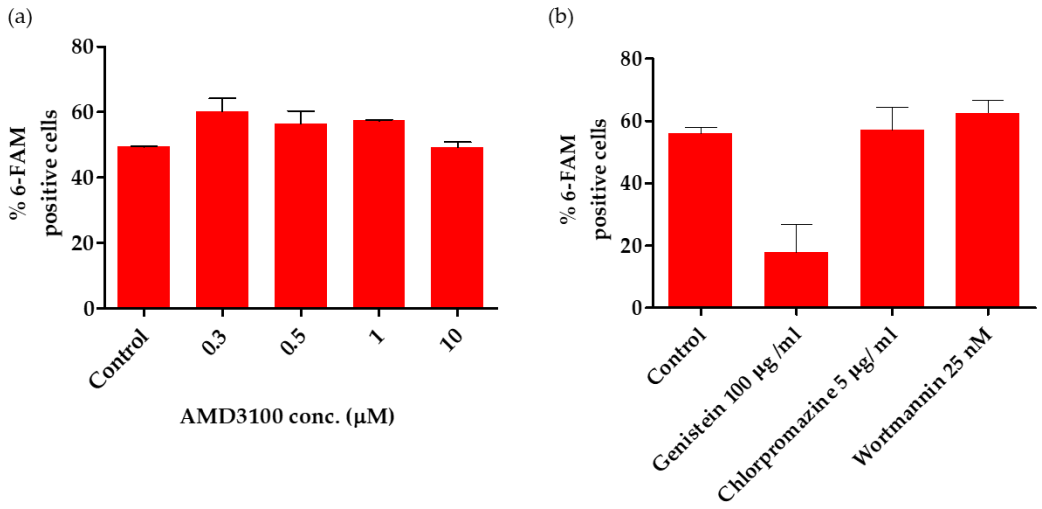


Figure 28. Mechanism of cellular uptake. Cells were treated for 30 minutes serum-free media with (a) AMD3100 and (b) various endocytic inhibitors and then with NPs containing 6-FAM-siRNA. Uptake was quantified by flow cytometry. Results expressed as percent 6-FAM positive cells (n=3).

4.3.9 Selection of siTNFα sequence

We used the smartpool siRNA commercially available from Dharmacon to choose a sequence which could efficiently silence TNFα. Four individual sequences and a mixture of the four siRNAs was used for the screening. We carried out our experiments in RAW 264.7 mouse macrophages which were treated with the respective siRNAs and then stimulated with LPS to induce TNFα 24 h later. Fold change in TNFα mRNA expression results determined by RT-PCR are shown in figure 29. As compared to the control cells, the cells treated with LPS showed almost 30-fold increase in TNFα expression. This proved that the LPS stimulation was working and upregulating the TNFα levels. The cells treated with the scrambled siRNA sequence showed TNFα mRNA levels similar to the untreated control cells indicating that the scrambled siRNA or the formulation components by itself did not affect the expression of TNFα in anyway. The cells treated with the siRNA mixture showed 20-fold increase in the expression as opposed to 30-fold upregulation seen in the untreated cells indicating that some sequence in the mixture was silencing TNFα. On testing individual sequences, we observed that sequences 1 and 2 did not show any downregulation of TNFα. However, sequences 3 and 4 showed significant TNFα silencing compared to untreated control. Sequence 3 was the best performing amongst the four siRNAs. Hence, we chose that sequence for our further experiments.

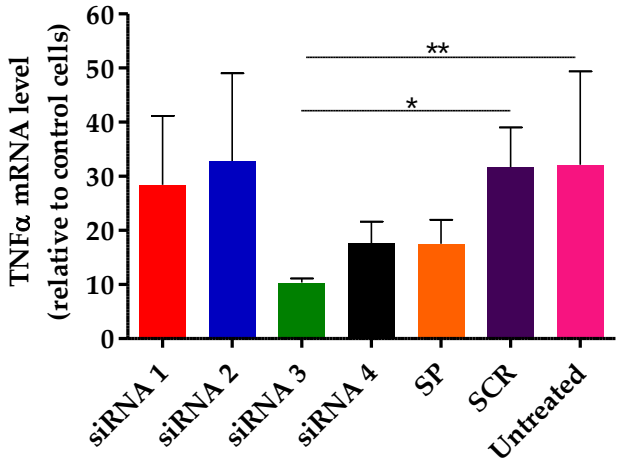


Figure 29. Selection of TNFα siRNA sequence. RAW 264.7 cells were treated with Polyplus transfection reagent-siRNA complexes. Different sequences of siRNA obtained from Dharmacon were used. 24h post treatment, cells were stimulated with LPS. TNFα mRNA levels were measured using RT-PCR. Results expressed as fold change in TNFα mRNA levels ± SD (n=6).

4.3.10 Biodistribution of CS-PCXA-siRNA NPs in mice

We assessed the biodistribution of our NPs to make sure that the particles were stable *in vivo* and were delivering the siRNA to the colon (Fig. 30). We evaluated the siRNA biodistribution in healthy as well as both the mouse models described in this dissertation. When assessed for biodistribution, the healthy mice showed fluorescence signal from 0.5 h post administration with about a 50 % reduction in signal at 12 h (Fig. 30a). In the *C. rodentium* model, the fluorescence signal was observed at 30 minutes (Fig. 30b). However, it diminished significantly within 2 h. This may probably be due to the presence of diarrhea and the formulation might be lost due to the less transit time. We were surprised to find that in the DSS model, no signal was observed. This finding might indicate that DSS which is a strong polyanion might be interacting with the NPs causing the dissociation and degradation of siRNA in the GIT. Secondly, it has been reported that DSS is deposited onto the mucosal surfaces resulting in formation of barrier that might interfere with the interaction of the delivery system with the tissue. Although, fluorescence is not a very quantitative method to quantify siRNA *in vivo*, this study gave us confidence that the NPs were delivering siRNA to the colon in sick animals. As a result, we proceeded to test the efficacy of these particles *in vivo* in *C. rodentium* mouse model of colitis.

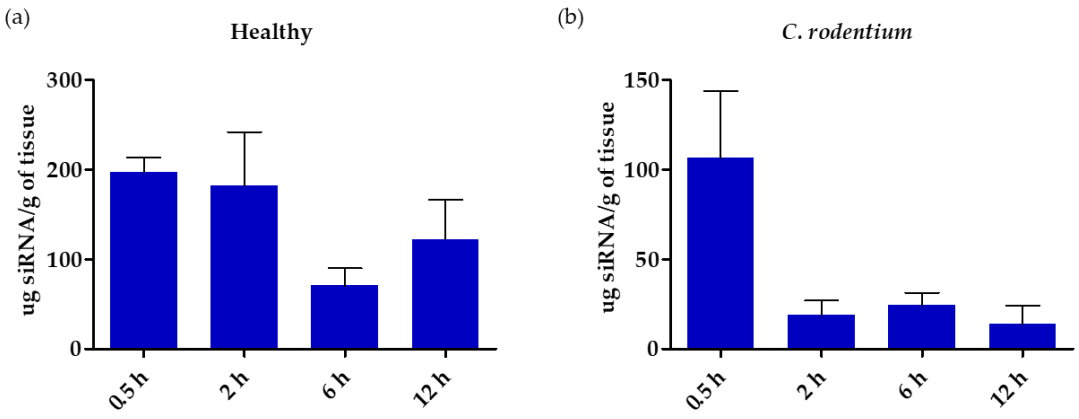


Figure 30. Biodistribution of siRNA *in vivo*. Mice were gavaged with CS-PCXA-siRNA NPs containing 30 μg Cy5.5 labeled siRNA and fluorescence signal in colon supernatant was measured at different timepoints in (a) Healthy mice; (b) Mice with *C. rodentium* induced colitis. Results are expressed as μg siRNA/ g of tissue ± SD (n=5).

4.3.11 Effect of DSS on stability of CS-PCXA-siRNA NPs

Looking at the mouse biodistribution data, we assessed the effect of DSS on the stability of NPs *in vitro* (Fig. 31). The results for the stability studies are represented in figure. We observed that with increasing concentrations of DSS, the particles began to lose their integrity. We observed displacement of siRNA from DSS concentration as low as 50 μg/ml. Complete dissociation of siRNA from the NPs was observed at a DSS concentration of 500 μg/ml. This correlated with our biodistribution studies *in vivo* where the particles did not show any signal in the colon of mice which were on DSS. The DSS concentrations at which the siRNA was being displaced were significantly lower than the concentrations that would be found in mice which were having DSS in their water. This confirmed our hypothesis that DSS because of its strong polyanionic nature was destabilizing the particles leading to premature release and degradation of siRNA in the GIT leading to no therapeutic effect. DSS is reported to exert its action by forming lipid vesicles with fatty acids in the GI lumen indicating its potential to interact electrostatically [143]. Also, DSS colon deposition studies have shown that DSS is deposited on the epithelium surface essentially forming a barrier between the delivery systems and the inflamed tissue [186]. All these factors make the use of DSS model of colitis an important consideration, especially when charge based delivery systems are involved.

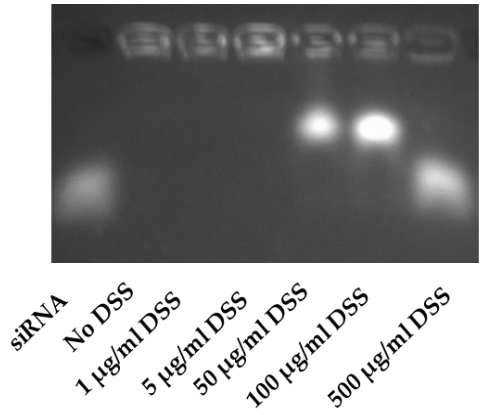


Figure 31. Effect of DSS on stability of NPs. CS-PCXA-siRNA NPs were incubated for 30 minutes with increasing concentrations of DSS. siRNA was visualized using agarose gel electrophoresis.

4.3.12 Efficacy of CS-PCXA- siTNFα NPs in *C. rodentium* mouse model of colitis

Encouraged by the colonic delivery of siRNA, we tested the efficacy of our NPs in colitis. We assessed the therapeutic efficacy of CS-PCXA-siTNF particles *in vivo*. The mice affected with colitis were treated with oral gavage of CS-PCXA-siTNF or CS-PCXA-siSCR NPs every other day. Histological changes in the colon were observed post animal sacrifice on day 14.

We also assessed the TNFα silencing in the colon using RT-PCR (Fig. 32). Upregulation of pro-inflammatory cytokines is a hallmark of IBD. TNFα plays a major role in inflammatory response and has been successfully explored as a major therapeutic target in IBD. TNFα plays a prominent role in IBD due to its contribution to the recruitment of immunocompetent cells that amplify the inflammatory response in T cells, macrophages, and mucosal cells [187, 188]. TNFα also has damaging effects on tight junctions, impairing barrier function, and enhancing the immune challenge by luminal antigens [189]. TNFα levels are increased in both serum and mucosa of IBD patients as well as in mouse models of IBD [190-192]. Consequently, blockade of TNFα can significantly improve or even prevent inflammation in both humans with IBD and animal IBD models. In addition to the successful use of systemic TNFα neutralization with antibodies [193], local TNFα gene silencing in the inflamed mucosa with antisense oligonucleotides and siRNA represents a promising alternative to attenuate IBD [194-199]. On day 14, we assessed the levels of TNFα mRNA in colon. Colons of untreated mice showed almost six-fold upregulation in TNFα as compared to healthy control mice. Not

much difference was observed between untreated group and the group which was treated with CS-PCXA-siSCR NPs. However, a significant difference was observed between the mRNA levels between the untreated and the CS-PCXA-siTNF treated group. The difference translated to almost a 40% TNFα silencing. However, the difference in mRNA levels between the CS-PCXA-siTNF and CS-PCXA-siSCR treated group was not found to be significant.

We assessed the histopathology of colons from the NP treated mice (Fig. 33). We found a significant reduction in the crypt length in the colons of mice who were treated with CS-PCXA-siTNF NPs compared to untreated as well as the mice treated with the NPs having the siSCR control (Fig. 33b). Significant reduction was observed in the histopathological scores for the NP treated mice compared to the sick control (Fig. 33c). However, no significant difference was observed in the scores between mice treated with NP containing siTNF versus those containing siSCR.

As a result, although we were able to deliver siRNA as well as saw reduction in the TNFα mRNA levels, the silencing was probably not significant enough to contribute to reduction in the inflammation. The observed reduction in the inflammatory scores seemed to be majorly due to CXCR4 inhibition by PCXA.

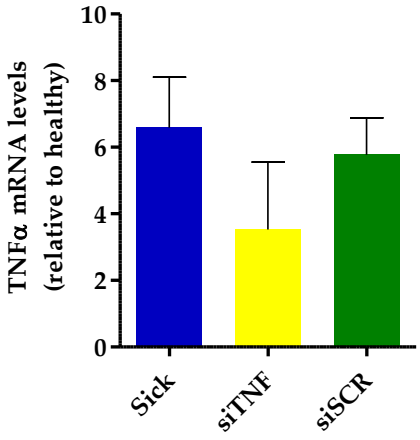


Figure 32. Effect of CS-PCXA-siTNF NPs on murine colonic TNFα mRNA levels. Fold change in colonic TNFα mRNA expression relative to healthy control. Data are represented as mean ± SD.

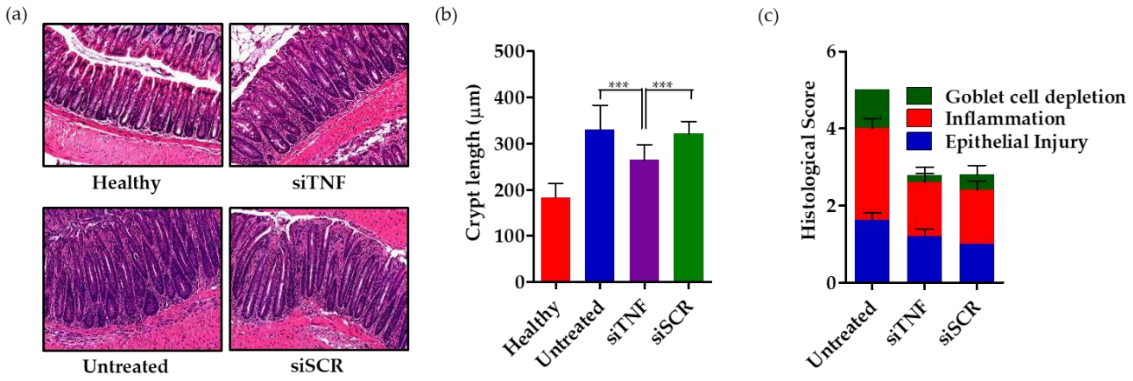


Figure 33. Therapeutic effect of CS-PCXA-siTNF NPs in *C. rodentium* model of colitis. (a) Representative H&E stained colon section images; (b) colon crypt length; (c) Histological scores.

4.4 Conclusion

We have demonstrated that CS-PCXA-siRNA particles are amenable to oral administration and subsequent delivery to the colon. We showed this by proving that these particles protect the siRNA from degradation in the GIT. Our biosdistribution studies point out the ability of these particles to deliver siRNA into the colon. We have shown *in vivo* that CXCR4 inhibition by PCXA has a therapeutic advantage in treating inflammation in the colon as well as shown gene silencing. Future efforts are needed to improve the formulation and enhance the anti-inflammatory effect.

CHAPTER 5: SUMMARY

IBD is a chronic inflammatory disorder which necessitates the patient to be on therapy almost life-long. Due to limitations like severe systemic side effects, current IBD therapy faces many hurdles. As the inflammation in IBD is local and confined to the GI tissue, therapies which can deliver drugs safely and locally are preferred. However, there is a lack of delivery systems that can achieve high local bioavailability of drugs. We tried to address this problem in this dissertation by developing delivery systems that can show preferential accumulation in the colon and improve therapeutic outcomes in IBD.

PDCs have been reported a lot in the treatment of many diseases. The advantage of a PDC is that it can change the properties of the drug to make it more suited to a particular physiological environment. Most of the reported PDCs for delivery to the colon have been synthesized with a goal of specific dissociation of drug under colonic pH. Although this strategy works for protecting the drug in the gastric environment, once released in the colonic environment, the drug still shows high systemic absorption which is not desirable from in treating local inflammation as well as from a safety perspective for some drugs. We selected CQ which is a widely used drug and has been in the clinics for a very long time. It has limited application as an anti-inflammatory agent and is an example of drug which shows high systemic absorption and long term side effects when administered orally. We synthesized a non-cleavable PDC of CQ to form pCQ. We showed that pCQ not only accumulates specifically in the GIT showing similar anti-inflammatory effects at HCQ, but also shows almost 1000-fold reduction in systemic absorption which is an indication of improved safety profile. We proved that by utilizing

this strategy we can make CQ amenable to multiple dosing which is difficult with the small molecule drug as it shows side effects like retinopathy after long term use. We proved all these findings in an animal model of colitis.

Secondly, we also investigated combination delivery of siRNA and a polymeric inhibitor of CXCR4 using mucoadhesive NPs. We found that although PCXA could deliver siRNA to the colon, it did not exert significant anti-inflammatory effect when administered orally. In order to increase the retention of PCXA-siRNA NPs, we included mucoadhesive polysaccharide CS to prepare CS-PCXA-siRNA NPs. We have shown that these nanoparticles were stable in simulated GI fluids as well as could protect siRNA against these fluids and RNase. Lastly, we have shown that we could achieve therapeutic effect through CXCR4 inhibition by PCXA and gene silencing by TNFα in a mouse model of colitis.

All these findings take a step in the direction of development of systems that can deliver therapeutics locally to the colon to treat inflammation. Our on-going efforts are focusing on improving both the above described formulations. We plan to formulate pCQ as particles and have performed preliminary testing which shows improved uptake into the colonic tissue for pCQ particles. Future efforts for CS-PCXA-siRNA particles will involve improving the inflamed tissue specificity as well as exploring novel targets.

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