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Non digestible carbohydrates in the diet determine toxicity of irinotecan (CPT-11)/5-fluorouracil in rats independently of β-glucuronidase activity in intestinal lumen

by

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Abstract

Aim of this study was to assess effects of non-digestible carbohydrates on the pathobiology of mucosal injury after chemotherapy.

Rats bearing colon tumor (n=6/diet) were fed diets including 8% (w/w) as either cellulose, inulin, fructo-oligosaccharide (OligoF), 50:50 (w/w) inulin and oligoF (Synergy®), isomalto-oligosaccharide (IMO), type IV resistant starch (RS4) or cornstarch. Animals were treated with 2 cycles of CPT-11/5-fluorouracil.

Animals fed RS4 or Synergy® were least ill with the least weight loss and anorexia, highest levels of gut associated lymphoid tissue (GALT) CD3+ and CD3CD8+ cells, least acute phase responses (haptoglobin, α-1-acid glycoprotein), least GALT OX6+ cells, CD152+ and CD71+ expression on CD8+ cells. Animals fed IMO or cornstarch were the most ill by these same criteria ($p<0.05$); animals fed inulin, cellulose and OligoF showed intermediate sickness.

Dietary NDCHO determine chemotherapy toxicity in a controlled model of colon cancer treatment, this may be due to change in immune function.

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List of Abbreviations

5-FU: 5-fluorouracil

AGP: α-1 acid glycoprotein

AIN: American Institute of Nutrition

ANOVA: analysis of variance

AUC: area under curve

COX-2: cyclooxygenase-2

CPT-11: Irinotecan (7-ethyl-10-[4-(1-piperidino)-1-piperidino]carbonyloxy-camptothecin)

DSS: dextran sodium sulfate

FOLFIRI: combination chemotherapy regimen consisting of 5-FU, folinic acid and irinotecan

FOLFIRINOX: combination chemotherapy regimen consisting of oxaliplatin, irinotecan, fluorouracil, and leucovorin

GALT: gut associated lymphoid tissue

GI: gastrointestinal

IFN- γ: interferon-γ

IL: interleukin

IMO: Isomalto-oligosaccharides

KC: Keratinocyte Chemoattractant

LMW- β-glucan: low-molecular-weight β-glucan

LPS: lipopolysaccharide

MAPK: Mitogen-activated protein (MAP) kinases

MLN: mesenteric lymph node

L/M ratio: lactulose:mannitol ratio

LTB_4: leukotriene B4

NDCHO: non-digestible carbohydrates

OligoF: fructo-oligosaccharide

PCA: principal component analysis

PGE_2: prostaglandin E2

PUFA: polyunsaturated fatty acids

RS4: resistant starch type 4

SCFA: short chain fatty acids

SN-38: 7-ethyl-10-hydroxycamptothecin

SN38G: SN-38 (7-ethyl-10-hydroxycamptothecin) glucuronide

TJ: tight junction

TLR: toll-like receptor

TNBS: trinitro benzene sulfonic acid

TNFα: Tumor necrosis factor alpha

TXA_2: Thromboxane A2

TXB_2: Thromboxane B2

UGT: uridine diphosphate glucuronosyltransferases

Chapter 1: Introduction and literature review

1.1 Overview of CPT-11 chemotherapy and its toxicity

Chemotherapy is a major modality in current anti-cancer practice and for most cancer patients is inevitable at some point during the course of anti-cancer therapy. Toxicity cannot be avoided after chemotherapy and has influenced the way anticancer drugs are being used. In general, the greater the rate of mitosis and the more immature the cells are, the greater the sensitivity to chemotherapy is (1). Targets of chemotherapy agents are not only tumor cells, but also normal cells with high rate of mitosis from host tissues such as bone marrow, gastrointestinal mucosa, lymphoid tissues, hair follicles, and germinal epithelium (2).

Colorectal cancer is the second leading cause of death from cancer in Canada with 5-year survival rate of 63% (Canadian Cancer Society: http://www.cancer.ca/Canada-wide/About%20cancer/Cancer%20statistics/Stats%20at%20a%20glance/Colorectal%20cancer.aspx?sc_lang=en). Irinotecan (7-ethyl-10-[4-(1-piperidino)-1-piperidino]carbonyloxy-camptothecin, CPT-11, Camptosar®) is a semisynthetic derivative of camptothecin, a plant alkaloid isolated from *Camptotheca acuminate.* Since its approval in the United States in 1996; irinotecan-based combination chemotherapy regimens have been a cornerstone of the management of metastatic colorectal cancer (3-6). National Institute of Clinical Evaluation recommends cetuximab in combination with 5-flurauracil (5-FU), folinic acid and irinotecan (FOLFIRI) as first-line treatment of metastatic colorectal cancer when primary surgery has been carried out and metastatic disease is confined to the liver and is resectable. Treatment with CPT-11/5-FU was superior to a regimen of 5-FU for metastatic colorectal cancer in terms of progression-free survival and overall survival (5). Both sequences of 5-FU/CPT-11 followed by 5-FU/oxaliplatin or 5-FU/oxaliplatin followed by 5-FU/CPT-11 prolonged survival in

colorectal cancer patients (7). Irinotecan as second-line chemotherapy for patients with gastric cancer prolonged overall survival compared to best supportive care (8). Combination chemotherapy regimen consisting of oxaliplatin, irinotecan, fluorouracil, and leucovorin (FOLFIRINOX) was associated with a survival advantage compared with gemcitabine in treatment of metastatic pancreatic cancer patients (9). Irinotecan has been the backbone of chemotherapy for small cell lung cancer (10).

The main limitation of CPT-11 is its toxicity in gastrointestinal (GI) (especially diarrhea) and hematological systems (mainly neutropenia) (11-13). CPT-11 causes two types of diarrhea: Early (which occurs within 24 hours of CPT-11 injection) and late (which occurs > 24 hours after CPT-11 administration) (14, 15). Early-onset diarrhea can be managed by anticholinergics such as atropine or by opioid-receptor agonists such as loperamide inhibits smooth muscle tone leading to prolonged intestinal transit time and increased time for fluid reabsorption (14-16). Delayed diarrhea lasts for a long duration and doesn't respond well to loperamide (12). Reports indicate that delayed diarrhea occurs in 87% of patients, with 39% having severe (grade 3 or 4) diarrhea associated with severe dehydration and electrolyte abnormalities, with 16% of patients requiring hospitalization (14). Incidence of grade 4 (life-threatening) diarrhea is reported at incidence of 8% in patients (5). Proper management of gut toxicity, especially delayed diarrhea which is the hallmark toxicity of CPT-11 is essential to achieve optimal treatment results with regimens centered with CPT-11.

1.2 CPT-11: mechanism of action

CPT-11 is a prodrug and requires bioactivation by tissue carboxylesterase to form its active metabolite SN-38 (7-ethyl-10-hydroxycamptothecin). SN-38 interferes with the breakage-reunion reaction of cellular topoisomerase I, stabilizing the topoisomerase-DNA intermediate, and thus inhibits DNA religation. During S-phase, collision between the

drug-stabilizing DNA-topoisomerase complex and the advancing replication fork results in fork breakage, leading to irreversible double-strand DNA break and cell apoptosis (17, 18). In vitro, SN-38 is 250- to 1,000-fold more potent than CPT-11 in inhibition of topoisomerase-I activity (19). CPT-11 and its metabolites are subject to a pH-dependent reversible inter-conversion between the ring-closed lactone form and the ring-opened carboxylate form in aqueous environment. Only the closed ring (lactone) form cross cell membranes and are effective topoisomerase-I inhibitors, carboxylates are inactive. The liver excretes bile into the duodenum to neutralize the acidic conditions from the stomach, thus creating of neutral environment at small intestine and colon. At neutral pH, interconversion equilibrium between the two forms favors the inactive carboxylate form, while an acidic pH drives the equilibrium to the closed ring form (13). SN-38 is eventually deactivated by uridine diphosphate glucuronosyltransferases (UGT) to form its glucuronide, SN-38G prior to biliary excretion (11, 20).

1.3 CPT-11: mechanism of gut toxicity

SN-38 exposure in the bowel is a complex function of its rate of formation from CPT-11, its biliary excretion, the action of UGT to form its inactive glucronide (SN38G). Exposure to SN-38 in intestinal tissue is from both the basolateral and luminal sides. It has also been proposed that deconjugation of SN38G to SN-38 by β-glucuronidase produced by gastrointestinal microbiota reactivates the drug to its toxic form in the colon (21) . Much emphasis has been placed on the importance of local deconjugation of SN38G to SN-38 by enteric bacterial β–glucuronidase (Figure 1-1) as a crucial contributor to CPT-11-induced gut toxicity (22-24). SN38G has a longer half-life than SN-38, which favors localization of SN38G and subsequent accumulation of SN-38 in epithelial cells of the large intestine after deconjugation by microflora (23, 25). However, recent work has drawn into question whether deconjugation of SN38G to SN-38 by β-glucuronidase

produced by gastrointestinal microbiota is the primary determinant of SN-38 exposure (26).

1.3.1 Pathohistological alterations

Pathohistological alterations occurring in the large intestine are the most consistent findings associated with CPT-11 treatment, such as decrease in crypt cell proliferation and increase in villous cell apoptosis (27-30), disappearance of villi in ileum, shorter villi in the duodenum and jejunum (26, 31-33), severe degeneration of crypts in the ileum, caecum, and colon, mild degeneration in the duodenum and jejunum (28, 31), reduction of crypt length in the jejunum and colon (33), and destruction of healthy glandular structure (34), have been documented. Damage to integrity of intestinal structure has been associated with diarrhea symptoms (28-30, 35, 36). However, a direct link is still lacking for establishing the compromised structural integrity as the primary causative factor leading to diarrhea. Improvement of diarrhea outcomes independent of intestinal cell apoptosis has been observed using keratinocyte growth factors (35, 36) (Table 1-1).

1.3.2 Compromised barrier function

Treatment of rats with CPT-11 (250mg/kg for 2 days) compromised barrier function of small and large intestines by injuring tight junction proteins (reduction of claudin-1 protein expression).This treatment disrupted mucosal barrier function and increased intestinal permeability of the large intestine (reduction of electrical resistance), and caused systemic translocation of bacteria and / or lipopolysaccharide (LPS) (37) (Table 1-1).

1.3.3 Inflammation

CPT-11 treatment also causes inflammation in intestinal tissues. It induces cyclooxygenase (COX)-2 in colonic mucosa and enhances production of pro-inflammatory factors (e.g., PGE_2, TNFα, IL-1β, KC, and TXA_2) (31, 38-41). These could serve as pathogenic factor independent of structural destruction, as over-production of

eicosanoids (PGE_2 and TXA_2) may result in hypersecretion of Cl^- and thus excessive Na^+ and water secretion into the gut lumen (31). CPT-11 was shown to increase nuclear factor-*k*B translocation which plays a critical role in regulating gene expression of cellular mediators involved in inflammatory and oxidative stress-induced processes (32), increase inflammatory markers, as measured by macrophage inflammatory protein-2 content, myeloperoxidase activity of neutrophils in intestinal tissue (31, 32), and finally upregulate expression of transcription factors (Cebpb and Cebpd) and transcription activators (42) (Table 1-1). Myeloperoxidase activity of neutrophils in intestinal tissue is indicator of polymorphonuclear neutrophil primary granules and inflammatory granulocyte infiltration. Egr1, Fosl1 and Stat genes are known to be activated by inflammatory signals and act through MAPK signaling to induce apoptotic and inflammatory mediators in tissue injury.

1.3.4 Altered immunity

CPT-11 is associated with altered systemic and local immunity, suppression of systemic immunity, decrease in immune cells population (i.e lymphocytopenia and neutropenia), increased expression of activation markers, production of inflammatory cytokines, and reduction of regulatory cytokines. CPT-11- based therapy decreased CD8+CD28+ and CD19+ lymphocytes, increased expression of activation markers, and CD14+CD16+ monocytosis in patients with metastatic colorectal cancer (43). Patients with higher counts of CD3+CD4+ cells before CPT-11 treatment showed a trend for CD3+CD4+ lymphocytopenia (43). Three months after treatment with a CPT-11-based regimen, both CD4+ and CD8+ T cells in peripheral blood were significantly lower than pre-treatment (44). CPT-11 caused grade 4 neutropenia in 15.4% and grade 4 leukopenia in 5.1% of patients with nonsmall cell lung cancer (45). CPT-11 also caused grade 3–4 neutropenia in 66% of patients and 17.5% of cycles (46). During treatment with CPT-11-based therapy, IL-2, IFN-γ, IL-12 and IL-18 production, as well as autologous mixed lymphocyte reaction responses increased when peripheral blood mononuclear cells were stimulated with anti-CD3 monoclonal antibody, whereas IL-4 and IL-10 production decreased over baseline, yet their respective values never reached those of healthy donors (47). An

increase of serum soluble IL-2 receptor levels, CD4+CD25+ T-cells and the CD4/CD8 ratio was found in patients treated with CPT-11 who showed symptomatic adverse reactions. The mentioned parameters correlated with toxicity during chemotherapy and affected quality of life scores (48). After injection of CPT-11 to rats, splenocytes were unable to proliferate or produce proliferative and inflammatory cytokines (i.e., IL-2, IFN- γ, TNF- α, IL-1β, IL-6) with mitogen stimulation (suggesting suppression of systemic immunity), whereas mesenteric lymph node cells showed rebound-like recovery with hyper-proliferative response and a hyper-production of pro-inflammatory cytokines with mitogen stimulation (suggesting activated mucosal immunity) (49) (Table 1-1).

1.4 Dietary fibres and Non-digestible carbohydrates: introduction

It is more than 59 years since Hipsley (1953) first used the term dietary fibre for the non-digestible constituents of plant cell walls (50) and more than 40 years since Trowell (1972) adopted the term and suggested a definition for "dietary fibre" (51). Since this time there has been no accepted international regulatory definition until Codex Alimentarius Commission adopted a final definition in its 2009 meeting (52): "carbohydrate polymers with ten or more monomeric units which are not hydrolysed by the endogenous enzymes in the small intestine of humans and belong to the following categories:

- Edible carbohydrate polymers naturally occurring in the food as consumed
- Carbohydrate polymers, which have been obtained from food raw material by physiological, enzymic or chemical means and which have been shown to have a physiological effect of benefit to health as demonstrated by generally accepted scientific evidence to competent authorities
- Synthetic carbohydrate polymers which have been shown to have a physiological effect of benefit to health as demonstrated by generally accepted scientific evidence to competent authorities".

Dietary fibres have two main components: insoluble fibres and soluble fibres. Whole grain cereals and whole meal bread are good sources of insoluble fibres, while oats, fruits, vegetables and pulses (bean, lentils and chick peas) are good sources of soluble fibrs. Biological functions of dietary fibre vary depending on the type of fibre. Insoluble fiber act like sponges; they absorb and hold water and will mainly contribute to bulking of stool, improve bowel movements which decrease bowel transit time and reduce intestinal exposure to procarcinogens (53). As they pass through the colon, insoluble fibre alsobind to toxins and procarcinogens and clean them from intestinal lumen, but are not fermented by colonic microflora. Alternatively, soluble fibres are readily fermented in the colon into gases and physiologically active byproducts. Short chain fatty acids (SCFA), primarily acetate, propionate, and butyrate, are end products resulting from fermentation of fibres and are suggested to have beneficial properties in the host (53, 54).

Dietary intervention with fibres is done with whole plant extracts (i.e. oat bran) or purified, isolated oligo-saccharides (non-digestible carbohydrates, NDCHO). These oligo-saccharides are made up of fructose, glucose, isomaltose or other sugars, enjoined by indigestible bond structures (i.e. β-(1→2), α-(1→6) linkages) and are of varying length and degree of polymerization. Classification of these compounds is as follows:

- **High and low molecular weight fructans**:

  - *Inulin*: a β-(1→2) linked fructan with a degree of polymerization (DP) of 3 to about 60, with an average of 12 units

  - *Fructooligosaccharide* (*fructo-oligosaccharide, oligofructose, OligoF)*: is produced by the enzymatic hydrolysis of inulin to yield of oligosaccharides with a DP of 3 – 10; and a mixture (1:1w/w) of inulin and OligoF (Synergy®).

- **High and low molecular weight glucans**:

  - *Resistant starch Type 4 (RS4):* produced by enzymatic treatments of starch to produce a NDCHO polymer composed of predominantly α-(1→6) glucose moieties

  - *Isomalto-oligosaccharides (IMO):* α-(1→6) and α-(1→4) linked glucose

oligosaccharides also produced from starch to obtain a DP of 2 – 5.

-β-glucan: Insoluble β-glucan with greater biological activity is composed of β-(1→3) and β-(1→4) glucose moieties

- **Polymer of glucosamine and N-acetyl-glucosamine**

  -*Chitosan:* soluble polymer consisting of varying amounts of β-(1→4) linked residues of N-acetyl-2 amino-2-deoxy-D-glucose (glucosamine) and 2-amino-2-deoxy-D-glucose (N-acetyl-glucosamine) residues

  -*Chitin*: insoluble polymer of N-acetyl-glucosamine or glucosamine, or a mixture typically 10 - 30% acetylated).

1.5 NDCHO as a potential modulator of CPT-11 chemotherapy

There is a small amount of literature comprising just 3 papers where NDCHO have been fed to animals under different chemotherapy regimens and have been proposed to reduce chemotherapy toxicities (55-57). Oral administration of low-molecular-weight β-glucan (LMW β-glucan, 25, 50, 100 mg/kg twice daily, 14 consecutive days) delayed the onset of diarrhea after 1-(2-tetrahydrofuryl)-5-fluorouracil + uracil chemotherapy in mice bearing colon tumor. The damage to the small intestine villi was also prevented by LMW β-glucan (55). Oral administration of chitosan (150 and 750 mg/kg, 14 consecutive days) prevented reductions of body and spleen weights caused by cisplatin chemotherapy in sarcoma bearing mice (56). Oral administration of chitosan (150 and 750 mg/kg x 2/day, 8 consecutive days) in sarcoma bearing mice blocked the reduction of blood leukocyte counts caused by 5-FU administration, and prevented the injury of the small intestinal mucosa membrane and delayed the onset of diarrhea. The higher dose of chitosan (750 mg/kg) prevented the reduction of CD3+ and CD8+ T cell numbers (57). A methodological concern with these studies is that the tested NDCHO was added to the drinking water and there was no parallel control treatment. In our experiment we used a

controlled and well defined diet to exclude any confounding factor that would have originated from diet.

There is an intersection between mechanisms proposed for CPT-11 toxicity and beneficial actions of NDCHO (Table 1-1). However, the bulk of the evidence for NDCHO to alter the clinical severity and mechanisms of intestinal injury comes from non-chemotherapy related colitis in humans or experimental animals such as trinitro benzene sulfonic acid (TNBS) or dextran sodium sulfate (DSS) induced colitis or IL-10 deficient animals (which develop colitis spontaneously).

1.5.1 Preservation of mucosal structure integrity

NDCHO prevent loss of mucosal structural integrity. Oral *inulin* significantly reduced the total area of mucosa showing any degree of damage and crypt lesion and confined lesions to the distal colon in DSS-induced colitis in rats (58). Daily consumption of 24 g of *inulin* for three weeks, lowered total histological score (polymorphonuclear leukocyte infiltration score + ulceration per field score) in patients with an ileal pouch-anal anastomosis (59). *Fructo-oligosaccharide* treatment via orogastric gavage (1.5 g/mL, twice daily for 14 days) reduced colonic crypt loss and disease activity index and increased crypt depth in DSS-induced colitis in rats (60). Addition of *Inulin+ fructo-oligosaccharide* (5 g/day) to drinking water for 7 weeks decreased gross morphological scores (intestinal wall thickness, ranging from 0 to 4) and inflammatory histological scores (criterion includes: inflammatory cells and goblet cells counts, mucosa thickening, submucosa cell infiltration, and destruction of architecture) in the caecum and colon of HLA-B27 transgenic rats (which develop chronic intestinal inflammation) (61). Addition of this combination (50g/kg) to basal diet for 28 days resulted in higher villi and deeper crypts in distal jejunum of human flora-associated rats (62). Orally administered low-molecular-weight *β-glucan* (50 and 100 mg/kg twice daily, 14 days) inhibited damage to small-intestine villi after 1-(2-tetrahydrofuryl)-5-fluorouracil) + uracil chemotherapy (55).

Enzyme-treated rice fibre (3% w/w) effectively reduced mucosal damage score (criterion included: extent of inflammation, extent of crypt damage, cystic dilatation of crypts, and loss of crypt) in DSS-induced colitis in rats (63) (Table 1-1).

1.5.2 Reduction of intestinal permeability and bacterial translocation

NDCHO decrease intestinal permeability and bacterial translocation**.** *Inulin* + *fructo-oligosaccharide* (60 g/ day, 17 days) lowered bacterial translocation to mesenteric lymph nodes (MLN) and significantly reduced the portal endotoxin concentration in TNBS-induced colitis in rats (64). Bacterial translocation to MLN decreased significantly after treatment with *Inulin* + *fructo-oligosaccharide* via oro-gastric tube (0.5g/3ml, twice daily for 14 days) In DSS-induced colitis in rats (65). Dietary *Isomaltooligosaccharide* (20% w/w, 4 weeks) inhibited translocation of *Enterobacteriaceae* to liver and spleen in gamma-irradiated mice (66). Enteral feeding of g*alactooligosaccharide* (1 g/day) 7 days after severe acute pancreatitis induced by sodium taurocholate, increased transmembrane junction protein (occludin) mRNA level in colonic tissues (67). Xylooligosaccharide and galactooligosaccharid (10% w/w, 3 weeks) both increased resistance to oral infection with *L. monocytogenes* (decreased the quantification of bacteria strains in MLN, spleen and liver) in guinea pig (68). Supplementation of *Oat base* (intragastric infusion of oatmel soup, 4ml/day, 9 days) to elemental diet after reduced intestinal permeability and plasma endotoxin levels after methotrexate chemotherapy (69) (Table 1-1).

1.5.3 Reduction of inflammation

Feedning NDCHO reduce cytokines, eicosanoids, and inflammatory markers. *Inulin* (administrated either at 1% (wt/vol) in the drinking water, or at 400 mg/day in 2 ml tap water by oral gavage, 5 days after DSS- induced colitis) decreased intraluminal release of eicosanoids (TXB2, LTB4) and myeloperoxidase activity of neutrophils in colonic tissue

(58). Feeding *Fructo-oligosaccharide* (1 g/d, intragastric catheter for 7 or 14 d) reduced myeloperoxidase activity of neutrophils in colonic tissue in TNBS-induced colitis (70). Feeding *Inulin*+ *fructo-oligosaccharide* decreased caecal tissue pro-inflammatory cytokine (IL-1β) (61), reduced colonic tissue IL-1 β, malondialdehyde (index of lipid peroxidation, a measure of oxygen free radical damage), and myeloperoxidase activity of neutrophils (65). This combination (4 g, three times daily) decreased faecal calprotectin (a marker of intestinal inflammation) in ulcerative colitis patients (71). *Enzyme-treated rice fiber* decreased overexpression of inflammatory cytokines (IL-1β, IL-6, and IL-12p70) production in serum and mucosal tissue of colon (63) (Table 1-1).

1.5.4 Modulation of immunity

Feeding NDCHO modulate the immune system of the host, elevate different immune cell populations, enhance function of immune cells (i.e phagocytosis activity), increase secretion of immunoglobins, and attenuate over activation of immune cells. *Resistant starch* elevated CD4+ T cell population and the ratio of CD4+/CD8+ T cells in the mesenteric lymph nodes of healthy rats, as well as serum immunoglobulin A levels after 4 weeks of feeding (72). *Inulin* (11.3 g/100 g of bread, participants were instructed to consume 200 g of bread daily for 5 weeks) increased the percentage of blood B cells (defined as CD19+) in young smokers and non-smoker adults (73). *Iinulin* + *fructo-oligosaccharide* (10% (w/w) , 4 months feeding to rats) enhanced peritoneal macrophage phagocytic activity (superoxid anion production), increased T cell numbers in the spleen and thymus, and increased expression of major histocompatability complex (MHC) II molecule on the surface of an antigen-presenting cells of spleen, mesenteric lymph nodes, and thymus (74). *Iinulin* + *fructo-oligosaccharide* (10% (w/w), fed for 4 weeks) increased production of Il-10 in Peyer’s patches as well as the production of secretory IgA in the caecum, and increased natural killer cytotoxic function in the spleen of healthy rats (75). Feeding *Fructo-oligosaccharide* (10% (w/w), 16 days) increased B cell numbers in Peyer’s patches of healthy and endotoxaemic (LPS challenged) mice, and increased

the CD4+/CD8+ ratio only in endotoxaemic mice (76). It increased total faecal IgA, IgA secretion by Peyer's patches cells, and ileal IgA secretion rate of healthy mice (77, 78). Enhancement of T cell cytokine production has been reported in healthy mice fed *fructo-oligosaccharide* (7.5% (w/w), 4 weeks), with an increase in IL-10 and IFN-γ production from stimulated Peyer's patches CD4+ T cells, and high levels of IL-5 and IL-6 secretion from these cells (77). Fifteen geram of *fructo-oligosaccharide* for three weeks, increased colonic dendritic cells expressing IL-10, toll-like receptor (TLR)-2 receptor , and TLR-4 in Crohn's disease patients (79). Feeding *Isomaltooligosaccharide* (20% (w/w) for 4 weeks) increased levels of IgA in feces, upregulated the Th1 response and protected the mice from gamma-irradiation-induced lethality (80). *Galacto-oligosaccharides + fructo-oligosaccharide* increased the fecal secretory IgA in formula-fed infants (0.6g/100 ml of formula) (81). *Enzyme-treated rice fiber* (3% w/w) attenuated the T cell activation (CD4+CD69+) of spleen and MLN in DSS-induced colitis (63) (Table 1-1).

1.5.5 Alteration of intestinal microflora

The human large intestine can be described as a complex microbial ecosystem. NDCHO can stimulate the growth and/or activity of one or a limited number of bacteria in the colon that improve the host health. Fermentation of NDCHO can alter the colonic microflora towards a healthier composition of bacteria seen as health promoting, with indigenous *lactobacilli* and *bifidobacteria* currently being the preferred targets (82, 83). It may be speculated that this shift in bacterial population could result in alterations in β-glucuronidase producing bacteria and therefore β-glucuronidase activity and hence alter luminal SN-38 levels.

1.6 Limitation of current knowledge about effects of NDCHO on chemotherapy

There is some data on NDCHO and chemotherapy toxicity (55-57), but this is difficult to interpret as it is heterogenous with respect to selected chemotherapy (cisplatin, 5FU and

derivatives, CPT-11, methotrexate), NDCHO (chitosan, inulin, cellulose, hemicellulose, pectin, β-glucan), and other details. The aim of our study was to investigate the effects of several classes of NDCHO (with demonstrated efficacy in clinical or experimental colitis), in a standardized model of CPT-11 induced colitis in rats bearing a colon tumor.

1.7 Rational

There is an intersection between mechanisms proposed for CPT-11 toxicity and beneficial actions of NDCHO. Treatment with CPT-11, causes apoptosis of intestinal cells (3-6), local and systemic inflammation (7-9), and loss of gut barrier function (10). Systemic translocation of bacteria and bacterial lipopolysaccharide (LPS) (10) may occur after CPT-11 therapy (Table 1-1) with risks of sepsis during concurrent myelosupression. Feeding NDCHO significantly mitigate several forms of clinical and experimental colitis reported by investigations mostly carried out in non-chemotherapy related colitis conditions (Table 1-1). In these conditions dietary NDCHO prevents the loss of mucosal structural integrity (16, 19-21), decreases intestinal permeability and bacterial translocation (22-24), reduces inflammatory markers (19, 21, 22, 25), increases T cells in spleen and thymus and B cells in peripheral blood (26, 27), enhances macrophage phagocytosis (27) and natural killer cell activity (28) and increases luminal secretion of IgA (29) as well as T cell cytokine production (29). All of these would be beneficial to the damage caused by CPT-11 treatment. Our study will determine if feeding NDCHO improves the health of tumor bearing animals receiving CPT-11 treatment.

1.8 Hypothesis

The objective of this study is to determine the optimal dietary form of NDCHO that will improve the health after CPT-11 treatment in an animal model of colon cancer. More specifically we hypothesize that feeding highly fermentable NDCHO compared to the less fermentable will:

1) prevent or reduce weight loss and the decrease in food intake

2) reduce the intestinal injury and colitis
    a. preserve mucosal structure integrity ,
    b. reduce the increase in apoptosis of intestinal epithelium,
    c. lowering β-glucuronidase activity of caecal contents,
    d. change composition of caecal microbiota to healthier folra,
    e. increase SCFA in the caecum,
    f. increase tight junction proteins of caecal contents,
    g. reduce inflammatory mediators in the colonic tissue
3) inhibit bacterial translocation to MLN
    a. inhibit the acute phase responses in plasma,
4) improve the response of the MLN immune system

1.9 Research Approach

To test whether in an adopted model of diet, NDCHO can mitigate toxicity of two consecutive cycles of CPT-11/5-FU chemotherapy in colon tumor bearing rats. Controlled feeding of American Institute of Nutrition (AIN)-76 semi-purified diet included 8% (w/w) as either cellulose, inulin, fructo-oligosaccharide (OligoF), 50:50 (w/w) inulin and oligoF (Synergy®), isomalto-oligosaccharide (IMO), type IV resistant starch (RS4) or cornstarch. Blood, MLN, caecum and colon tissues and contents were collected. Primary outcomes to assess toxicity were to monitor body weight and food intake, intestinal tissues morphometry and apotosis, plasma acute phase proteins, and β-glucuronidase activity of caecal contents. Secondary outcomes for assessment of toxicity were MLN immune cell phenotypes, bacterial translocation to MLN, quantification of microflora, SCFA, and CPT-11 drug metabolites in caecum contents, and colonic tissue tight junction proteins and an inflammatory cytokine (IL-1 β).

Tables

Table 1-1. Parallel between the mechanisms of CPT-11 toxicity and mechanisms of the beneficial effects of NDCHO.

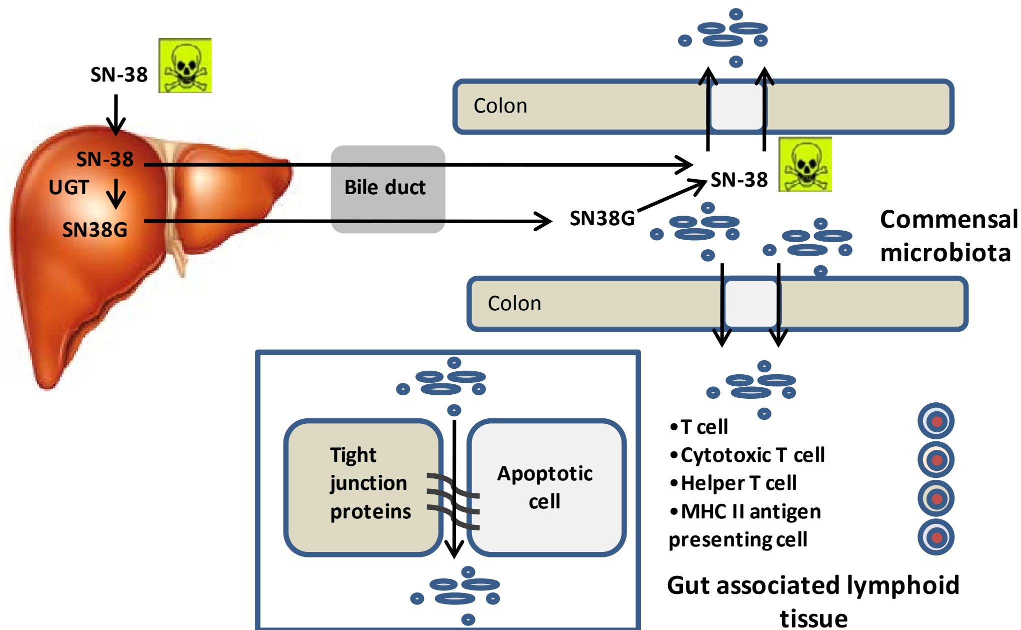
	Mechanisms of CPT-11 toxicity	Effects of NDCHO
Mucosal structure integrity loss	**Pathohistological alterations after CPT-11:** ***Hypoplasia and apoptosis**:* ↓ in crypt cell proliferation and ↑ in villous cell apoptosis (27-30). ***Villus structure**:* ↓ of villi in ileum, shorter villi in the duodenum and jejunum (26, 31, 32). ***Crypt structure**:* Severe degeneration of crypts in ileum, caecum, and colon, mild degeneration in the duodenum and jejunum (28, 31). ↓ of crypt length in the jejunum and colon (84). ***Colonic gland height:*** destruction of healthy glandular structure (34).	**Effects of NDCHO on intestinal mucosal structure:** ***Inulin*** ↓ extent of damage to mucosa and severity of colon crypt destruction in dextran sodium sulfate (DSS) induced colitis (58). ↓ total histological score in patients with an ileal pouch-anal anastomosis (59). ***Fructo-oligosaccharide*** ↓ colonic crypt loss and disease activity index, ↑ crypt depth in DSS-induced colitis (60). ***Inulin+ fructo-oligosaccharide*** ↓ gross morphological scores and inflammatory histological scores in the caecum and colon of HLA-B27 transgenic rats (61). Resulted in higher villi and deeper crypts in distal jejunum of human flora-associated rats (62). ***β-glucan*** ↓ damage to small-intestine villi by 1-(2-tetrahydrofuryl)-5-fluorouracil) + uracil chemotherapy (55). ***Enzyme-treated rice fiber*** ↓ mucosal damage score in DSS-induced colitis (63).
Functional changes	**Loss of barrier function:** ***Tight junction proteins and bacterial translocation:*** CPT-11 injured tight junction proteins and caused bacteria translocation to mesenteric lymph nodes or spleen (37).	**Effects of NDCHO on barrier function:** ***Inulin + fructo-oligosaccharide*** ↓ bacterial translocation to the mesenteric lymph nodes after TNBS and DSS-induced colitis (64, 65). ***Isomalto-oligosaccharide*** ↓ translocation of *Enterobacteriaceae* after irradiation (66). ***Galacto-oligosaccharide*** ↑ colonic tissue transmembrane junction protein (occludin) mRNA level in sodium taurocholate induced severe acute pancreatitis (67). ***Xylo-oligosaccharides and galacto-oligosaccharide*** *both* ↑ resistance to oral infection with *L. monocytogenes* (68). ***Oat base*** ↓ intestinal permeability, and plasma endotoxin after methotrexate chemotherapy (69).

	Mechanisms of CPT-11 toxicity	Effects of NDCHO
Functional changes	**Inflammation:** ***Eicosanoids and cytokines:*** ↑ production of PGE_2, TNFα, IL-1β, TXA2, ↑ COX-2 in colonic mucosa (31, 38-41). Over-production of PGE_2 and TXA2 may result in ↑ secretion of Cl^- and thus ↑ Na^+ and water secretion into the gut lumen (31). ***Inflammatory signal transduction****:* ↑ nuclear factor-kB translocation (32). ***Inflammatory markers****:* ↑ macrophage inflammatory protein-2 content, myeloperoxidase activity of neutrophils in intestinal tissue (31, 32). ***Genes involved in inflammation****:* ↑ expression of transcription factors (Cebpb and Cebpd) and transcription activators (Egr1, Fosl1 and Stat) genes and MAPK signaling to induce apoptotic and inflammatory mediators in tissue injury (42).	**Effect of NDCHO on inflammation:** ***Inulin*** ↓ intraluminal release of eicosanoids (TXB2, LTB4), and ↓ myeloperoxidase activity of neutrophils in colon tissue in DSS-induced colitis (58). ***Fructo-oligosaccharide*** ↓ myeloperoxidase activity of neutrophils in colonic tissue in TNBS-induced colitis (70). ***Inulin + fructo-oligosaccharide*** ↓ caecal tissue pro-inflammatory cytokine (IL-1β) in HLA-B27 transgenic rats (61). ↓ colonic tissue IL-1 β, malondialdehyde (index of lipid peroxidation), and myeloperoxidase activity of neutrophils in DSS-induced colitis (65). ↓ faecal calprotectin in ulcerative colitis patients (71). ***Enzyme-treated rice fiber*** ↓ overexpression of inflammatory cytokines (IL-1β, IL-6, and IL-12p70) production in serum and mucosal tissue of DSS-induced colitis (63).
Functional changes	**Immunity:** ***Systemic and local immunity:*** ↓ CD8+CD28+ and CD19+ lymphocytes, ↑ expression of activation markers, and CD14+CD16+ monocytosis (43). ↓ CD4+ and CD8+ T cells in peripheral blood compared to pre-treatment 3 months after treatment with CPT-11-based regimen (44). ↓ ability of splenocytes to proliferate and produce proliferative and inflammatory cytokines (i.e., IL-2, IFN-γ, TNFα, IL-1β, IL-6) on mitogen stimulation (suggesting anergy of systemic immunity) (49). ↑ mesenteric lymph node cells proliferative response and production of pro-inflammatory cytokines on mitogen stimulation (suggesting activated mucosal immunity) after CPT-11 (49). Caused grade 4 neutropenia in 15.4% and grade 4 leukopenia in 5.1% of patients with non-small cell lung cancer (45). Caused grade 3–4 neutropenia in 66% of patients and	**Effect of NDCHO on immune system:** ***Resistant starch*** ↑ CD4+ T cell and the ratio of CD4+/CD8+ T cells in the mesenteric lymph nodes of rats, ↑ serum immunoglobulin A levels (72). ↑ hemoglobin concentration of red blood cells, ↓ % of blood CD4+ cells in healthy growing pigs (85). ***Inulin*** ↑ % of blood B cells (CD19+) in young male adults (73). ***Iinulin + fructo-oligosaccharide*** ↑ peritoneal macrophage phagocytic activity (superoxid anion production), ↑ T cell numbers in the spleen and thymus, ↑ expression of major histocompatability complex (MHC) II molecule on the surface of an antigen-presenting cells of spleen, mesenteric lymph nodes, and thymus (74). ↑ production of Il-10 in Peyer's patches as well as the production of secretory IgA in the caecum, ↑ natural killer cytotoxic function in the spleen of healthy rats (77). ***Fructo-oligosaccharide*** ↑ B cell numbers in Peyer's patches of healthy and endotoxaemic mice, ↑ the CD4+/CD8+ ratio in endotoxaemic mice (76). ↑ total faecal IgA, IgA secretion by Peyer's patches cells, and ileal IgA secretion rate of healthy

	Mechanisms of CPT-11 toxicity	Effects of NDCHO
Functional changes	17.5% of cycles (46). ↑ IL-2, IFN-γ, IL-12 and IL-18 production, as well as autologous mixed lymphocyte reaction responses when peripheral blood mononuclear cells were stimulated with anti-CD3 mAb, ↓ IL-4 and IL-10 production over baseline (47). ↑ serum soluble IL-2 receptor levels, CD4+CD25+ T-cells and the CD4/8 ratio (48).	mice. ↑ in IL-10 and IFN-γ production from stimulated Peyer's patches CD4+ T cells, and high levels of IL-5 and IL-6 secretion from these cells in healthy mice (77). ↑ colonic dendritic cells expressing IL-10, TLR-2 receptor , and TLR-4 in Crohn's disease patients (79). ***Isomalto-oligosaccharide*** ↑ levels of IgA in feces, ↑ the Th1 response and protected the mice from gamma-irradiation-induced lethality (80). ***Galacto-oligosaccharides + fructo-oligosaccharide*** ↑ the fecal secretory IgA in formula-fed infants (81). ***Enzyme-treated rice fiber*** attenuated the T cell activation (CD4+CD69+) of spleen and MLN in DSS-induced colitis (63).

Figures

Figure 1-1. Mechanism of CPT-11 metabolism and intestinal toxicity. SN-38 (active metabolite of CPT-11) is deactivated by uridine diphosphate glucuronosyltransferases (UGT) in the liver to form its glucuronide (SN38G) prior to biliray excretion. Commensal bacteria are believed to reactivate SN38G to SN-38. Translocated bacteria and their endotoxins enter the intestinal tissues and blood circulation through impaired gut barrier function and stimulate the gut associated lymphoid tissues.



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Chapter 2: Non digestible carbohydrates in the diet determine toxicity of irinotecan (CPT-11)/5-fluorouracil in rats independently of intestinal β-glucuronidase activity

2.1 Introduction

The non-digestible portion of plants (fiber) escapes digestion in the small intestine, is selectively digested by colonic microbiota, which is proposed to alter intestinal mucosa physiology and response to injury. We hypothesized that non digestible carbohydrates (NDCHO) may modulate the severe late onset diarrhea (1) which is the main dose-limiting toxicity of irinotecan (7-ethyl-10-[4-(1-piperidino)-1-piperidino] carbonyloxy-camptothecin, CPT-11, Camptosar®) chemotherapy.

CPT-11 is used to treat colorectal cancer (2). SN-38, a metabolite generated by carboxylesterase activity from CPT-11, possesses antitumor activity, causes apoptosis of intestinal cells (3-6), local and systemic inflammation (7-9), and loss of gut barrier function (10). Systemic translocation of bacteria and bacterial lipopolysaccharide (LPS) (10) may occur after CPT-11 therapy (Table 1-1) with risks of sepsis during concurrent myelosupression (Figure 1-1). SN-38 exposure in the bowel is a complex function of its rate of formation from CPT-11, its biliary excretion, and actions of uridine diphosphate glucuronosyltransferases (UGT) to form its inactive glcronide (SN38G). Exposure to SN-38 in intestinal tissue occurs from both the basolateral and luminal sides. It has also been proposed that deconjugation of SN38G to SN-38 by β-glucuronidase produced by colonic microbiota reactivates the SN38G to SN-38 (11). Much emphasis has been placed on the importance of β-glucuronidase in the development of toxicity (1, 12, 13), although

recent work has drawn into question if this is the primary determinant of SN-38 exposure (14).

Dietary intervention with NDCHO is performed with whole plant extracts or purified, isolated oligo-saccharides consisting of fructose, glucose, isomaltose or other sugars. NDCHO are linked by indigestible bond structures (i.e. β-(1→2), α-(1→6) linkages) and are of varying length and degree of polymerization. Short chain fatty acids (SCFAs) are end products resulting from bacterial fermentation of dietary fibers in the colon and are suggested to have beneficial properties for the host (15).

Feeding NDCHO significantly mitigate several forms of clinical and experimental colitis (Table 1-1) and have been proposed to reduce chemotherapy-induced toxicity (16-18). There is an intersection between mechanisms proposed for CPT-11 toxicity and beneficial actions of NDCHO (Table 1-1). Dietary NDCHO alter the clinical severity and mechanisms of intestinal injury in non-chemotherapy-related colitis in humans and experimental animals (trinitro- benzene sulfonic acid or dextran sodium sulfate induced colitis or IL-10 deficient animals (which develop colitis spontaneously). NDCHO prevent loss of mucosal structural integrity (16, 19-21), decrease intestinal permeability and bacterial translocation (22-24), reduce inflammatory markers (19, 21, 22, 25), increase T cells in spleen and thymus and B cells in peripheral blood (26, 27), enhance macrophage phagocytosis (27) and natural killer cell activity (28) and increase luminal secretion of IgA (29) as well as T cell cytokine production (29) (Table 1-1). Dietary NDCHO modify the balance between bacterial species in intestinal microbiota in favor of protective microorganisms (30).

Some reports examined NDCHO and chemotherapy toxicity (16-18), but these are difficult to interpret as they are heterogeneous with respect to selected chemotherapy (cisplatin, 5-fluororuracil (5-FU), and derivatives, CPT-11, methotrexate), NDCHO

(chitosan, inulin, cellulose, hemicellulose, pectin, β-glucan), and other details. Our aim was to investigate effects of several NDCHO (with demonstrated efficacy in clinical or experimental colitis), in a standardized model of CPT-11 induced colitis in rats bearing a colon tumor. Results were analyzed per diet as well as integrated by modeling using Principal Component and *k*-means cluster analysis.

2.2 Experimental Methods

2.2.1 Animals

Animal use was approved by the Institutional Animal Care Committee and conducted in accordance with Guidelines of the Canadian Council on Animal Care. Fisher 344 rats (~180 g body weight and ~12 weeks of age) were from Charles River (QC, Canada). Female animals were used to enable comparison with prior work (31, 32). Rats were housed 2 per cage in a temperature (22°C) and light controlled (12h light) room; water and food were available for *ad libitum* consumption. One week before chemotherapy rats were separated into individual cages so they could be individually monitored.

2.2.2 Diet

NDCHO were incorporated into a nutritionally complete diet (Table 2-1) based on an American Institute of Nutrition (AIN)-76 formulation. The fat component was formulated to be similar to North American dietary patterns in humans (40% of calories, polyunsaturated: saturated fat ratio, 0.35) (31). Animals were initially fed Rodent Laboratory Chow (Harlan Teklad, Madison, WI). During adaptation, chow was mixed with study diet (50/50, w/w) for one week, followed by full transition to study diets. Rats were randomized to 7 diets (n=6/diet) (Table 2-1) composed of a constant fraction (92% of diet) and a variable NDCHO fraction (8%). We selected cellulose which is usually included in AIN-76 formulations and other oligosaccharides with different degrees of polymerization and digestibility (inulin, fructo-oligosaccharides (OligoF), 50:50 (w/w) inulin

and oligoF (Synergy®), isomalto-oligosaccharide (IMO), type IV resistant starch (RS4), cornstarch).

2.2.3 Experimental design

After habituation to experimental diets for 2 weeks, Ward colorectal carcinoma (Y Rustum, Roswell Park Institute) was implanted. Tumor pieces (0.05g) were transplanted subcutaneously (s.c) on the flank of the rats via trocar under slight isoflurane anesthesia. Subcutaneous tumor growth was continuously evaluated for 2 weeks, until ~2.3 cm^3 (1.2% body weight), then CPT-11/ 5-fluororuracil (5-FU) chemotherapy was initiated (31) by intraperitoneal administration. CPT-11 (Pfizer, USA) and atropine sulfate (Sandoz, Canada) were clinical injectable formulations. On day 0, CPT-11 (50 mg/kg) was administered followed by 5-FU (50 mg/kg) on Day 1. Atropine (1 mg/kg, s.c.) was administered immediately before each CPT-11 injection to alleviate early-onset cholinergic symptoms (31). A second cycle of CPT-11/5-FU was administered one week later at days 7 and 8 and rats were followed until day 9 which our prior studies (33) had shown to be the time of bacterial translocation, increased morbidity and mortality with this regimen (Figure 2-1). Body weight and food intake were monitored every 2nd day. Food intake and body weight for the 3 days preceding Day 0 for each rat was considered the baseline value.

2.2.4 Sample collection

Rats were killed by CO_2 asphyxiation. Blood was collected by cardiac puncture into heparinized tubes and centrifuged at 4˚C at 3,000 × g for 15 min. Plasma was removed and frozen at -70˚C. Mesenteric lymph nodes were collected aseptically, followed by dissection of the gastrointestinal tract. After sampling of caecal and colon digesta, tissues were washed in ice cold physiological saline. The first 6-cm of the proximal colon as well as the middle part of the caecum were collected and immediately frozen in liquid nitrogen.

The remaining colon was longitudinally cut in two halves, one of which was mounted on a wax strip and fixed in 10% (v/v) neutral buffered formalin.

2.2.5 Assays

2.2.5.1 Gut associated lymphoid tissue (GALT) cell phenotype: Mesenteric lymph node (MLN) cells were isolated (200,000 cells/well) and studied by 2 color direct immunofluorescence (34). Antibodies used were CD3, CD4, CD8, CD25, CD27, CD28, CD45, CD71, CD80, CD45RA (BD Bioscience, Mississauga ON, Canada) and OX6, OX12, OX62 (Cedarlane, Hornby ON, Canada); Streptavidin QR (Sigma, Oakville, ON, Canada) was added to all Biotin labeled antibodies. The percentage of cells expressing each antibody marker was determined using flow cytometry (FacScan, Becton Dickinson, Sunnyvale, CA, USA) (34).

2.2.5.2 Bacterial translocation: MLN were aseptically homogenized in 5 mL sterile water and 0.1 mL of these samples was inoculated on blood agar (for Gram+ bacteria) and McConkey agar (for Gram– bacteria). Cultures were incubated aerobically at 37°C for 48 h and then colony-forming units on each plate counted and corrected to the original tissue weight.

2.2.5.3 β-glucuronidase activity in caecal digesta was determined by a modified method of Freeman as previously described (31) and expressed as U (defined as 1.0 μg of phenolphthalein liberated from phenolphthalein glucuronide/h) /g protein.

2.2.5.4 Quantification of microflora by real time PCR: DNA of caecal digesta samples was extracted with Qiagen DNA extration kit (35). Group-specific primers used to quantify 16S rRNA genes were: total bacteria, *Bacteriodes* group, *Lactobacillus* group, *Bifidobacterium* spp., *Clostridial* clusters I, IV, XI, and XIVa, and *Enterobacteriaceae* (35).

Copy numbers of rDNA were quantified by fast real time PCR unit (Applied Biosystems, Streetsville, Canada) using absolute quantification method described previously (35).

2.2.5.5 Short Chain Fatty Acid (SCFA): Caecal digesta samples were diluted 3X with water and centrifuged (22,000g, 15min). Supernatants were then diluted with 7% perchloric acid (1:3 v/v) and incubated at 4 ˚C overnight to remove proteins. Samples were then centrifuged (22,000g, 5min) and supernatants used for high performance chromatography (HPLC). SCFA were separated using an Aminex 87H column (Bio-Rad, Mississauga, Ontario) at 70 ˚C, and the solvent was 5 mmol/L H_2SO_4, at a flow rate of 0.4 mL/min. SCFA were visualized using a UV detector (Agilent 1200 series variable wavelength detector G1314B, Agilent technologies Inc.) at 210 nm, and identified using external standards. SCFAs were analyzed in duplicate.

2.2.5.6. Intestinal morphometry: Fixed colon tissue samples were processed and embedded in paraffin. Sections (4µm) were dewaxed, rehydrated and stained in Mayer's hematoxylin. After differentiation in 1% v/v acid alcohol and bluing in Scott's tap water, sections were counterstained in eosin, dehydrated and mounted. Colon crypt height was calculated as the distance between basal and apical surfaces of each crypt. Colon crypts (25-35 per sample) were assessed and averaged for each sample using Axioskop 2 plus microscope and AxioVision LE software (Carl Ziess Imaging Resolution Inc.).

2.2.5.7 Apoptotic cells in colonic mucosa were identified with the DeadEnd Colorimetric terminal transferase-mediated dUTP nick end labeling (TUNEL) System according to the manufacturer's instruction (Promega, Wisconsin, USA). In each section, 20–25 areas of colonic mucosa, each of which contained at least 10 crypts, were randomly selected to count TUNEL-positive cells under 400x magnification using Axioskop 2 plus microscope and AxioVision LE software (Carl Ziess Imaging Resolution

Inc.). The apoptotic index was calculated by dividing the number of TUNEL-positive cells by the measured area.

2.2.5.8 Colonic tight junction protein claudin-1 expression: tissue samples were homogenized in lysis buffer (36). The homogenate was then separated on a 4%-12% Bis-Tris Gel (Invitrogen, Carlsbad, CA) and was transferred to a polyvinylidene difluoride (PVDF) membrane (Hybond-P; Amersham Biosciences, Piscataway, NJ). The membrane was incubated with a primary antibody against claudin-1 (1:200 dilution, Camarillo, CA) and then stained with horseradish peroxidase (HRP)-conjugated secondary antibody (Santa Cruz Biotechnology, Santa Cruz, CA). Claudin-1 was detected by enhanced chemiluminescence reagents (Pierce, Rockford, IL), and exposed with utilizing a UVP chemiluminescent darkroom system (UVP, Upland, CA). Quantification was done by scanning densitometry using LabWorks 4.0 Image Acquisition and Analysis Software (UVP, Inc., Upland, CA, USA). β-actin was used as loading control.

2.2.5.9 Colonic and caecum tissue IL-1β was quantified using sandwich enzyme-linked immunosorbent-assay (ELISA) technique using unlabelled capture antibodies and biotin-labeled detection antibodies followed HRP-labeled Streptavidin (37). Tissues were homogenized in PBS containing a cocktail of protease inhibitors, after which the homogenate was assayed in a 1:1 dilution for IL-1β according to the manufacturer's instructions (National Institute for Biological Standards and Controls, South Mimms, UK).

2.2.5.10 Acute phase proteins: Commercial ELISA kits for rat α-1-acid glycoprotein (AGP) and rat haptoglobin were purchased from Life Diagnostics (West Chester, Pennsylvania, USA) and conducted according to the manufacturer’s instructions.

2.2.5.11 CPT-11 metabolites (SN-38, and SN38G) were quantified using fast liquid chromatography–tandem mass spectroscopy method (38). A Shimadzu Nexera Ultra HPLC was coupled with a ABI MDS SCIEX 4000 Q TRAP™ LC/MS/MS. A Kinetex™ 2.6 µm C18 100 x 3 mm column was used. The flow rate was 0.4 mL/min. The acetonitrile gradient increased from 10% to 80 % from 0 to 18 min, maintained at 100% from 18.1 to 19.0 min, and returned to 10% from 19.4 to 25 min.

2.2.6 Statistics

Data were expressed as mean ± SEM. A probability 0.05 was considered significant. SPSS 18.0 (SPSS Inc) was used unless otherwise noted. Time effects on body weight following CPT-11 were analyzed using Generalized Estimating Equations which accounts for repeated measures data. Diet effects were analyzed using one-way ANOVA followed by *post hoc* least significant difference test; any values that were not normally distributed were log transformed before analysis. Comparison of β-glucuronidase and SN-38 subgroups was done by unpaired 2-tailed Student's t-test for independent samples. Principal Component Analysis (PCA) transforms all measured variables regardless of diet into sets of values called principal components. This transformation is defined in such a way that the 1st principal component has the largest possible variance (accounts for as much of the variability in the data as possible), and 2nd component in turn had the highest variance possible under the constraint that it was orthogonal to (uncorrelated with) the preceding component. All variables were log transformed, as PCA is sensitive to the relative scaling of original variables (SIMCA-P 11.0, Umetrics Inc.). K-means clustering analysis was used to partition variables into clusters in which each variable belonged to the cluster with the nearest mean. All variables were standardized to Z scores and clustering membership for each of them was evaluated. Bivariate correlations were done using two-tailed Pearson correlation.

2.3 Results

2.3.1 Effects of diet

Diet treatments resulted in variation of most measured constitutional, immune and intestinal variables. The following data presentation is organized in light of the observation of a distinct pattern of this variation: animals fed the IMO and cornstarch diets were most sick (based on weight loss, food intake, GALT cell phenotypes and acute phase response), while the animals fed RS4 and Synergy® were least sick (based on the same criteria). Animals fed cellulose, inulin and OligoF fell intermediate between these poles and were not significantly different from each other in most of the measured parameters (Figure 2-2). In this section, we use the term toxicity in a global sense to reflect variation of clinical, immune and biochemical state observed across the diets, and specifically to recognize that in the spectrum of responses animals were respectively more (or less) sick by multiple criteria.

2.3.1.1 Body weight loss occurred after the 1st cycle of chemotherapy, there was some recovery, followed by further loss after cycle 2 (Figure 2-3 A). Rats fed IMO and cornstarch had the largest weight loss and had persistently the lowest body weight at all time points. By contrast, animals fed RS4 and Synergy® maintained the highest body weights throughout the study. Cumulative *food intake* (area under the curve of food intake during chemotherapy, compared with the baseline value) paralleled body weight (Figure 2-3 B); rats fed RS4 and Synergy® had the highest food intake and animals fed IMO and cornstarch had the lowest. Total calorie intake after chemotherapy was significantly lower in animals fed IMO compared to animals fed RS4 and Synergy® (Figure 2-3 C).

2.3.1.2 GALT cell phenotypes: Animals fed IMO and cornstarch had the lowest proportions of T lymphocytes (CD3+) as a proportion of total cells (Figure 2-3 D). The

lower CD3+ proportion was due to a reduced proportion of CD3CD8+ (T cytotoxic cells, Figure 2-3 E) but not CD3CD4+ (T helper cells, data not illustrated). Rats fed IMO and cornstarch also showed the highest proportion of CD8+ cells expressing the transferrin receptor (CD71+) (Figure 2-3 F). CD71 is required for iron delivery from circulating transferrin to cells and is upregulated on lymphocytes during proliferative responses to antigens or infectious organisms. Animals fed IMO and cornstarch had the highest percentage of OX6+ cells (MHC II antigen presenting cells) (Figure 2-3 G). Animals fed IMO had higher proportion of OX12+ (B cells, 15.8±4.6) and OX62+ (dendritic cells, 0.6±0.1) compared to animals fed RS4 (11.0±2.4) and (0.4±0.1), respectively. However, these differences did not reach statistical significance; it is possible that the limited sample size used here (n=6/diet group) posed a limitation on the ability to detect some differences. IMO and cornstarch fed animals had a higher proportion of CD152+ (CTLA-4) cytotoxic T lymphocytes (Figure 2-3 H). There were no significant effects of diet on any of following single or combination of markers: IgA+, IgM+, and IgG+, CD25, CD27, CD28, CD45, CD80, CD45RA (data not shown).

2.3.1.3 Acute phase proteins: Rats fed IMO had the highest concentration of haptoglobin and AGP, whereas rats fed Synergy® had the lowest values of all diet treatments. Rats fed RS4 had lower haptoglobin and AGP compared to ones fed IMO ($p<0.05$) (Figure 2-3 I,J).

2.3.1.4 Microbiological findings: For majority of the bacterial strains evaluated as well as total bacteria, there was no significant variation by diet, with the exception that rats fed cornstarch had 1 log higher (10x) number of caecal *Enterobacteriaceae spp.* compared to any other diets (Figure 2-3 K). Positive bacterial cultures in MLN were observed in a 26 out of 42 animals (data not shown).

2.3.1.5 SCFA in caecal digesta: rats fed IMO had uniformly the lowest concentration (μmol/g) of butyrate, propionate and acetate of all treatments (3.4±1.2, 5.8± 0.5, 24.1±3.2 respectively). Butyrate levels were nearly 3-fold higher in rats fed inulin (10.7±4.0) versus IMO (3.4±1.2, p=0.015). The highest propionate levels were seen in rats fed RS4 (7.3±0.8 versus IMO 5.8±0.5, p=0.043), and highest levels of acetate were seen in rats fed OligoF (37.0±5.0 versus IMO 24.1±3.2, p=0.008).

2.3.1.6 Drug metabolites in caecal digesta: Animals fed cornstarch had the highest amount of SN-38 and this was significantly different than animals fed Synergy® or oligoF (Figure 2-3 L). Animals fed IMO and cornstarch had lower levels of β-glucuronidase activity (Figure 2-3 M) compared to animals fed Synergy® and RS4 (P<0.05). These findings are opposite to the generally accepted view of β-glucuronidase mediating CPT-11-induced toxicity. To further explore this, we grouped all 42 animals studied into tertiles based on β-glucuronidase activity (Figure 2-4). Animals in the highest tertile of enzyme activity (T3) had higher cumulative food intake, lost significantly less weight, had the lowest percentage of both CD8CD152+ and CD8CD71+ cells in MLN, less IL-1β in caecal tissue, less acute phase protein in plasma (haptoglobin), higher butyrate in caecal digesta, and finally less SN-38 in caecal digesta compared to animals in the lowest tertile of enzyme activity (T1).

2.3.2 Model for the CPT-11 toxicity

PCA and k-means clustering were used to explore associations in the data. PCA explained 37% of the variance in the data (R^2=0.37, Q^2=0.18). Figure 2-5 illustrates the PCA analysis, where the right hand side and especially the upper right quadrant includes indices associated with less toxicity (food intake, body weight, *clostridial* clusters I and XIV, SCFA, β-glucuronidase, claudin-1, CD3CD8+, CD8CD28+). Opposite to these, in the left and especially lower left quadrant are indices associated with more toxicity

(*Enterobacteriaceae*, *Clostridial* cluster XI, hapoglobin, AGP, CD8CD71+, CD8CD152+, CPT-11 and SN38).

K-means cluster analysis verified associations found by PCA. The distance between final 2 clustering centers was 7.8, reflecting robust separation. ANOVA table of K means clustering showed that the 2 clusters are different with respect to the following variables: body weight, β-glucuronidase activity, CD3+, CD3CD28+ (normal functioning T cells), CD8+,CD8CD28+ (normal functioning cytotoxic T cells), CD4+,CD4CD28+ (normal functioning helper T cells), CD8CD71+, CD8CD152+, OX6+, OX12+, OX62+, IgM+, IgA+, AGP, and CPT-11 ($p<0.05$). This analysis confirmed that SN-38 grouped with indices of toxicity and while β-glucuronidase activity clustered with indices of wellness.

Correlations also support patterns noted in PCA and k-means cluster analysis: cytotoxic T cell activation marker (CD8CD71+) correlated negatively with body weight ($r=-0.60$, $p<0.001$), food intake ($r=-0.36$, $p=0.02$), β-glucuronidase ($r=-0.32$, $p=0.03$), CD3+ cells ($r=-0.60$, $p<0.001$), CD8+ cells ($r=-0.69$, $p<0.001$) but correlated positively with CD8CD152+ cells ($r=0.69$, $p<0.001$), OX6+ cells ($r=0.52$, $p=0.001$), OX62+ cells ($r=0.59$, $p<0.001$), AGP ($r=0.48$,$p=0.002$), haptoglobin ($r=0.55$, $p<0.001$) and SN-38 concentrations ($r=0.42$, $p=0.009$).

SN-38 is the cytotoxic metabolite of CPT-11. The overall median SN-38 concentration in caecal digesta was 6.2 µg/g. A subset of rats (n=9) had SN-38 concentrations ≥12 µg/g (median 15.1 µg/g). Most animals in the high SN-38 group had been fed IMO or 2%cellulose (5 out of 9) and they lost more weight, had less cytotoxic T cells, possessed more activated cytotoxic T cells (CD8CD71+) and IgA+ cells in MLN, had higher haptoglobin plasma levels, more IL-1 β in caecal tissues and less butyrate in caecal digesta versus rats with low SN-38 levels (Figure 2-6 A).

Six rats that were amongst the most ill were selected on the basis of high weight loss, high acute phase protein levels and high levels of CD8CD71+; these animals had been

fed IMO or cornstarch diets. Six rats were identified as least ill based on the same criteria, from rats fed RS4 or cornstarch diets (Figure 2-6 B). Mean colon crypt length and apoptotic index were not significantly different between the 2 groups. Histological differences were unremarkable, but the least sick rats expressed twice higher amount of colon Claudin-1 protein compared to most sick group (1.1± 0.1 versus 0.5 ± 0.1, arbitrary units relative to β-actin housekeeping gene expression, $p<0.001$). These results suggest that the primary defect allowing translocation of bacteria and/or endotoxins may be tight junction failure.

2.4 Discussion

2.4.1 Dietary modulation of CPT-11 response

We conducted a controlled comparison of multiple NDCHO in an inbred rat strain with limited genetic variability using a standardized chemotherapy regimen and a strictly defined diet composition. All of the tested NDCHO had been reported to have some efficacy in mitigating intestinal injury in non-chemotherapy mediated models of colitis (19-23, 25-28, 39). Methodological concerns with prior studies (16-21) include adding tested NDCHO to the drinking water or administration of NDCHO by orogastric gavage and with no parallel control treatment. Under our controlled and well defined conditions, we document a broad spectrum of toxicity in the host animals, across the range of NDCHO chosen, with Synergy® and Type 4 resistant starch clearly emerging as the NDCHO associated with the least toxicity. One might speculate that higher amount of food intake in animals fed Synergy® and Type 4 resistant starch may have affected the less toxicity seen in these animals (Figure 2-3 B). Animals in these two diets ate on average 10-15% more food compared to animals in IMO and cornstarch diet. To best of our knowledge this difference is unlikely to affect outcomes of toxicity (i.e. boost immunity dramatically). Pair feeding of animals fed Synergy® and Type 4 resistant starch to the lower amount

taken by animals in IMO and cornstarch diets would help us to rule out the possibility of food intake as a confounding factor in overall toxicity.

There are some possible explanations for severe toxicity in rats fed IMO and cornstarch. It is suggested that highly digestible diets compromise gut barrier function, as enhanced bacterial translocation in animals fed an elemental diet (i.e. containing no non-digestible components) was reversed by addition of cellulose to the elemental feed (40). If NDCHO are partly digestible in the small intestine, this may lead to low NDCHO appearance in the large intestine for microbial fermentation and less production of SCFA. SCFA are anti-inflammatory and also a fuel for colonic epithelial cells (15). IMO is partially digestible in the small intestine (41) and here IMO-fed animals had the lowest mean levels of acetate, propionate and butyrate of any diet treatment. Rats fed cornstarch had the highest levels of SN-38 in caecal digesta of any group. An additional effect unique to the cornstarch diet was a 10-fold higher number of caecal *Enterobacteriaceae spp.* than those fed RS4 or Synergy®. *Enterobacteriaceae* is the main family of bacteria translocated to MLN, liver, spleen and peripheral blood (42) and this has been documented in our experimental model (43). We did not see variations in *Bifidobacterium* and *Lactobacillus* as had been reported in other models after feeding inulin, OligoF and Synergy® (21, 22, 25). The reasons for this are unknown.

2.4.2 Integrative model of biology of CPT-11 toxicity

Prior findings implicating β-glucuronidase in CPT-11 toxicity included evidence that incubation of bile from drug-treated animals with a caecal extract from an untreated animal increased the SN-38 content (11). Histological damage was greater in the caecum where microflora and β-glucuronidase were more abundant versus small intestine (13). Here, using a different approach, we observed that levels of β-glucuronidase activity did not explain variations in toxicity in CPT-11 treated rats. Furthermore β-glucuronidase activity levels were inversely associated with SN-38 levels in caecal contents. SN-38

exposure can be influenced by its rate of systemic formation from CPT-11, intestinal uptake from the blood, SN-38 biliary excretion and the action of UGT in liver and intestinal tissue (14). However the respective contribution of all of these to net appearance of SN-38 remains to be determined. Additionally the potential negative effect of higher levels of β-glucuronidase activity may have been offset by beneficial effects induced by other end products of bacterial fermentation such as SCFA.

Our integrative model suggests participation of 3 main elements contributing to CPT-11 mediated toxicity: SN-38 exposure, ability to maintain gut barrier function and immune competency. In the first instance, CPT-11 toxicity depends on the degree of **exposure to SN-38**, which in turn depends on its synthesis, excretion, glucuronidation and transport. The relative participation of these steps in toxicity is not well understood. It may be that the SN-38 arriving at the colon from the bile excretion is a more quantitatively important source than the generation of SN-38 locally in the colon by β-glucuronidase. As there are some evidence supporting that NDCHO shorten mouth-to-anus transit time significantly (44), future research is needed to elaborate if accelerated movement of bowel would clear SN-38 faster from colon lumen and therefore reduce toxicity; **Intestinal Barrier function is compromised after CPT-11 therapy**. Healthy control animals have no detectable bacterial translocation (32), so the presence of positive MLN bacterial cultures in 26 of 42 animals is direct evidence of barrier compromise. Translocation results have wide variation (45) and statistical differences are difficult to detect even with much larger samples than n=6 / diet, however considering all 42 rats in the PCA analysis, translocation grouped with other indices of toxicity. In the absence of gross histological damage or apoptosis, we speculate that bacterial translocation was associated with decreased claudin-1 (Figure 2-6). Strategies to maintain barrier function are needed to reduce toxicity. CPT-11 also induces **changes to the number and function of different subsets of immune cells involved in both innate and adaptive immunity.** Risk of infection has become a limiting factor in development of new intensive chemotherapy

treatment (46). Here, we show reduced levels of CD3+ cells, cytotoxic T lymphocytes (CD3CD8+) and notably higher CD152 expression on the latter cells. CTLA-4 (CD152) engagement results in down-regulation of T cell dependent immune activation and response. Taken together, reduced numbers of cytotoxic T cells with higher CD152 expression is suggestive of less effective defense against infections and tumor surveillance in IMO-fed and cornstarch-fed animals. Some of these immune changes may be potential targets for nutritional modulation.

Decreased proportions of CD3+ and CD8+ cells are potentially important. Metastatic colorectal cancer patients had depleted levels of CD3+, CD3+CD4+, CD3+CD8+, CD8+CD28+ and CD19+ cells compared to controls even before starting 1st line therapy (47). Three months after treatment with CPT-11-based regimen, both CD4+ and CD8+ T cells in peripheral blood were significantly lower than pre-treatment (48). CPT-11-based therapy caused grade 4 leukopenia in 5.1% and grade 4 neutropenia in 15.4% and of with nonsmall cell lung cancer (49). CD8+ cells lyse tumor cells and are important effector cells during tumor rejection and also play a crucial role in host defense against cancer immune surveillance (50). When CD3+ cells were removed from mice treated by means of anti-CD3 monoclonal antibody, increased tumor growth rate and reduced survival were observed (51).

2.5 Conclusion:

In our experiment, type of NDCHO in diet determined CPT-11 induced gut toxicity. Although all the tested NDCHO have been suggested to improve certain forms of gut injury mostly in conditions other than chemotherapy-induced colitis in literature, not all of them were equally effective to modulate CPT-11 chemotherapy toxicity. Our results suggest a possible benefit of NDCHO supplementation to reduce chemotherapy toxicity and might encourage researchers to design clinical trials to test effects of NDCHO in cancer patients treated with chemotherapy. We speculate that primary defect allowing

translocation of bacteria and/or endotoxins after CPT-11 chemotherapy is tight junction protein failure. Our findings are opposite to the generally accepted view of β-glucuronidase mediating CPT-11-induced toxicity.

Tables and figures

Table 2-1. Composition of experimental diets. The constant portion consisted of the pre-mixed modified AIN-76 basal ingredients (Harlan Teklad, Madison, WI); the variable portion was formulated to allow the addition of selected fiber elements. Other ingredients were supplied: Canola stearine (ICN Biomedicals Inc., Cleveland, OH), Flax seed oil (Canadian Superstore, President's Choice, AB), Sunflower oil (Planet Organic, Gold Top, AB), Resistant starch type IV, trans-glycosidated starch (National starch, NJ, US)

<table>
<tr><th colspan="3">Ingredient</th><th>Cellulose</th><th>Corn starch</th><th>IMO</th><th>OligoF</th><th>Inulin</th><th>Synergy®</th><th>RS4</th></tr>
<tr><td rowspan="7">Variable Portion 8%</td><td rowspan="7">Non digestible carbohydrate</td><td>Cellulose</td><td>8</td><td>0</td><td>0</td><td>0</td><td>0</td><td>0</td><td>0</td></tr>
<tr><td>Cornstarch</td><td>0</td><td>8</td><td>0</td><td>0</td><td>0</td><td>0</td><td>0</td></tr>
<tr><td>Isomalto-oligosacchride</td><td>0</td><td>0</td><td>8</td><td>0</td><td>0</td><td>0</td><td>0</td></tr>
<tr><td>Fructo-oligosaccharides</td><td>0</td><td>0</td><td>0</td><td>8</td><td>0</td><td>0</td><td>0</td></tr>
<tr><td>Inulin</td><td>0</td><td>0</td><td>0</td><td>0</td><td>8</td><td>0</td><td>0</td></tr>
<tr><td>Synergy®</td><td>0</td><td>0</td><td>0</td><td>0</td><td>0</td><td>8</td><td>0</td></tr>
<tr><td>Resistant Starch</td><td>0</td><td>0</td><td>0</td><td>0</td><td>0</td><td>0</td><td>8</td></tr>
<tr><td rowspan="2">Constant portion 92%</td><td>Modified AIN-76 basal mix</td><td>Casein (25.2g), Methionine (0.25g), Glucose (13.95g), Vitamins AIN 76 (1g), Minerals AIN 76 (5g), Inositol (0.6g), Maize starch (23.7g), cellulose 2 g</td><td>72</td><td>72</td><td>72</td><td>72</td><td>72</td><td>72</td><td>72</td></tr>
<tr><td>Lipids</td><td>Canola stearine (11.4g), Flax seed oil (0.8g), Sunflower oil (7.8g)</td><td>20</td><td>20</td><td>20</td><td>20</td><td>20</td><td>20</td><td>20</td></tr>
<tr><td>Total</td><td></td><td></td><td>100</td><td>100</td><td>100</td><td>100</td><td>100</td><td>100</td><td>100</td></tr>
</table>

Figure 2-1. Experimental design. After habituation to experimental diets for 2 weeks, colorectal carcinoma was implanted. After two more weeks of subcutaneous tumor growth, CPT-11/ 5-FU chemotherapy was initiated. A second cycle of CPT-11/5-FU was administered one week later.

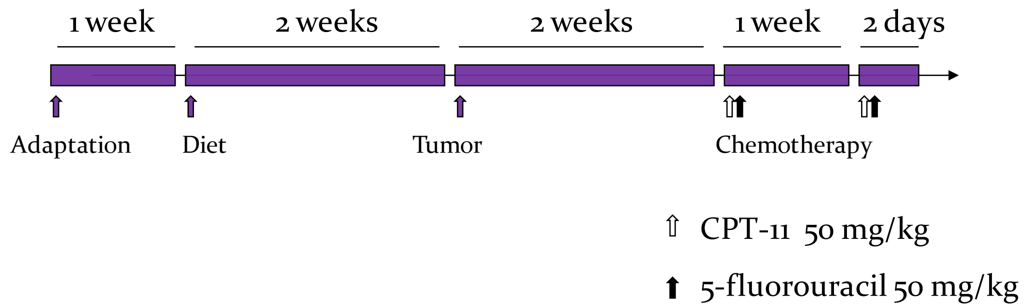


Figure 2-2. Diet effect stratification. Animals fed the IMO and cornstarch diets were most sick, while the animals fed RS4 and Synergy® were least sick. Animals fed cellulose, inulin and OligoF fell intermediate between these poles and were not significantly different from each other.

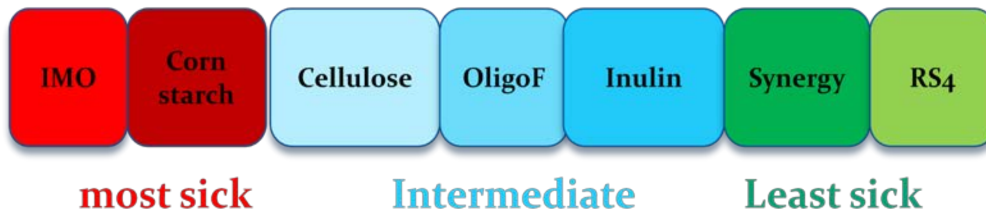
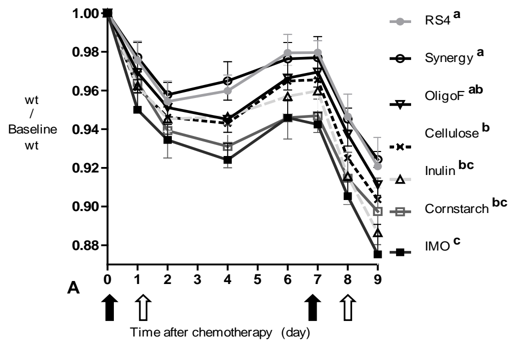
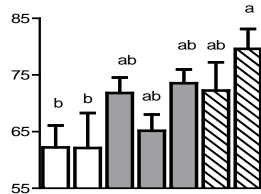
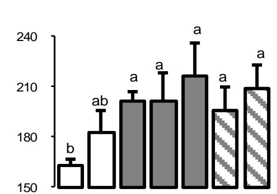
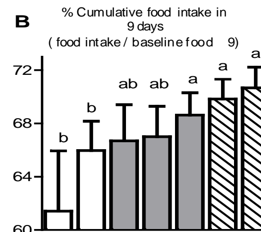


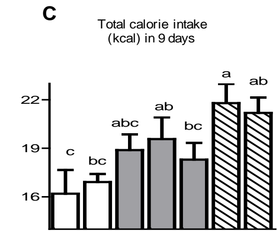
Figure 2-3. Dietary effect of NDCHO following CPT-11/5-fluorouracil (5-FU) therapy. (A) Relative body weight. (B) Cumulative food intake. (C, D) Total and cytotoxic T lymphocytes population in mesenteric lymph nodes (MLN). (E, F, G) Markers of activation and inhibition on immune cells in mesenteric lymph nodes. (H, I) acute phase proteins in plasma. (J, K, L) *Enterobacteriaceae* spp. population, SN-38 concentration and β-glucuronidase activity were measured in caecal digesta. [a–c] Dietary treatments with unlike superscript letters are significantly different ($P<0{\cdot}05$, Panel A: generalized estimating equation, Panels B to L: one-way ANOVA followed by *post hoc* least significant difference). Values are means with their standard errors depicted by vertical bars.

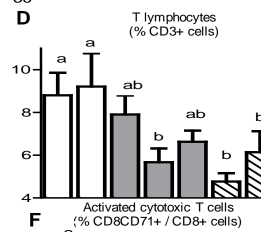


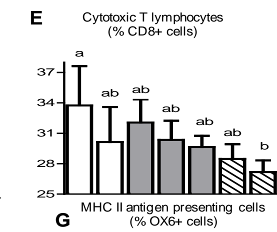


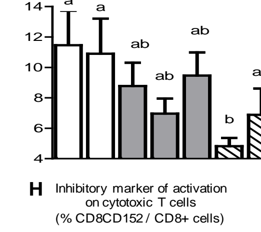


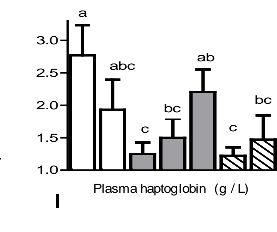


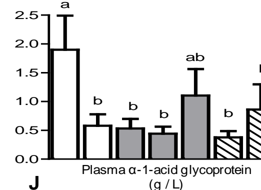


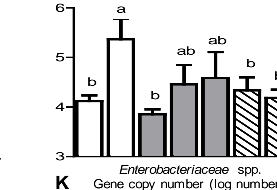


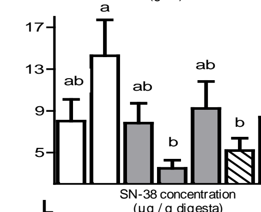


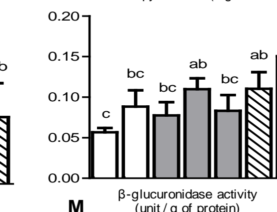


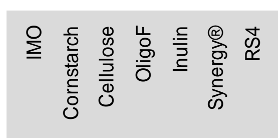












Diet

Figure 2-4. Association of β-glucuronidase and other measured variable. All 42 animals were divided into tertiles based on β-glucuronidase activity of caecal digesta; other data are reported for these tertiles.

[a–c] bars with unlike superscript letters are significantly different (P<0·05, one-way ANOVA followed by *post hoc* least significant difference).

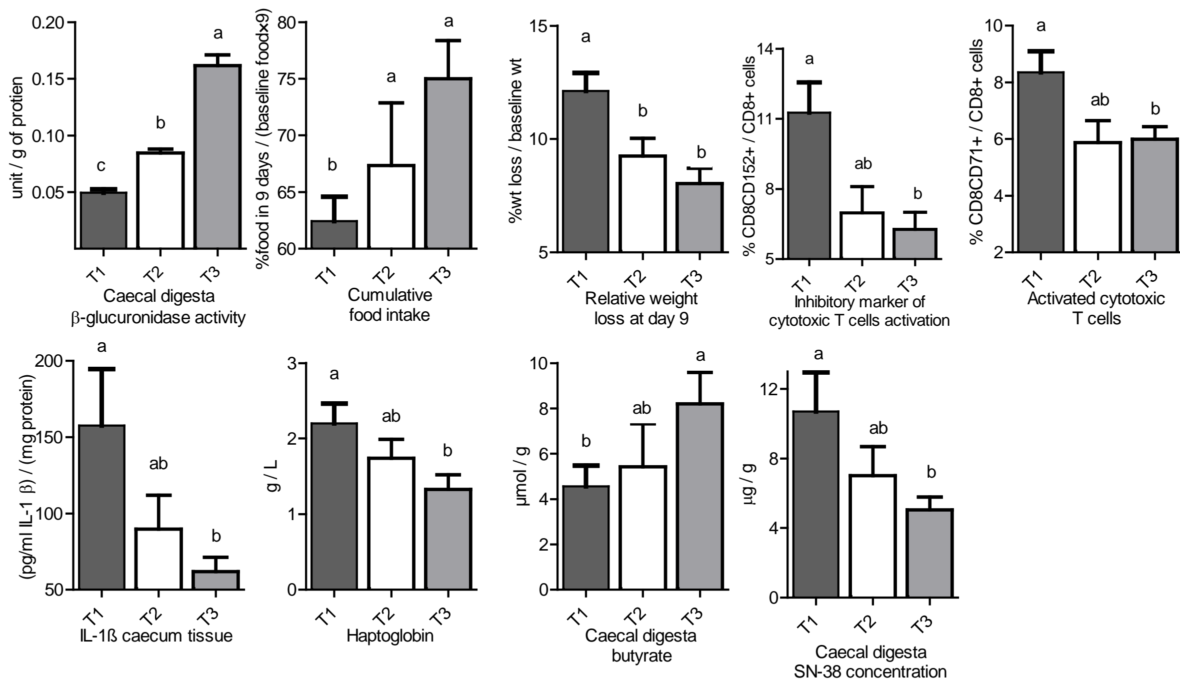


Figure 2-5. Principal Component Analysis (PCA) of CPT-11 metabolites, β-glucuronidase and other measured parameters of toxicity. PCA transforms data on all 42 animals into sets of values called principal components. Notably, SN-38 and CPT-11 grouped with multiple parameters of toxicity, distinct from β-glucuronidase activity which grouped with indices of wellness.

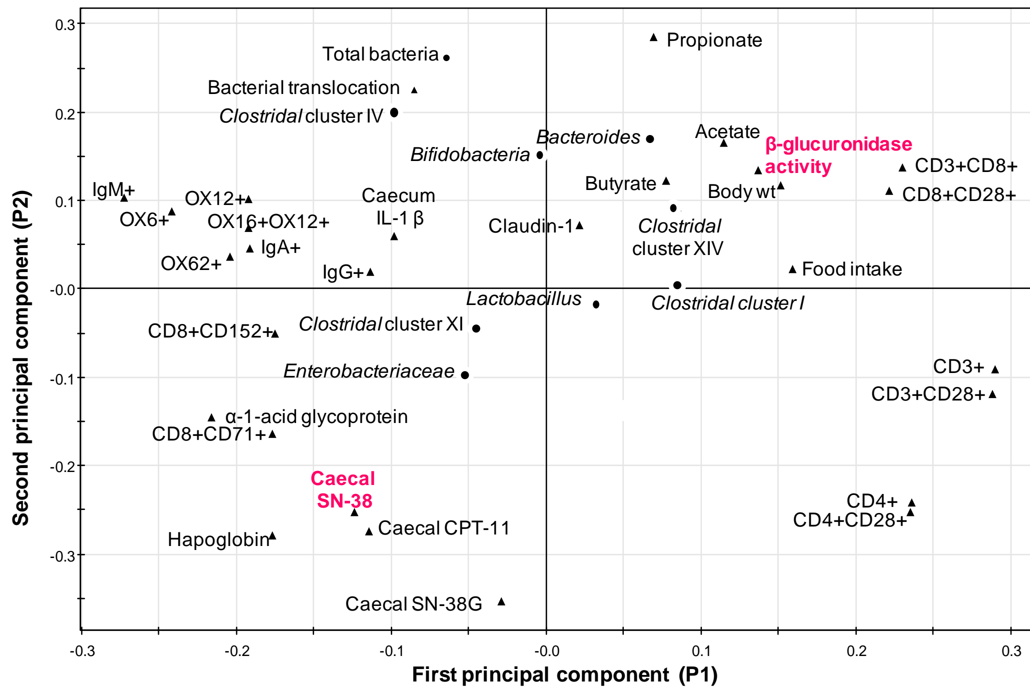
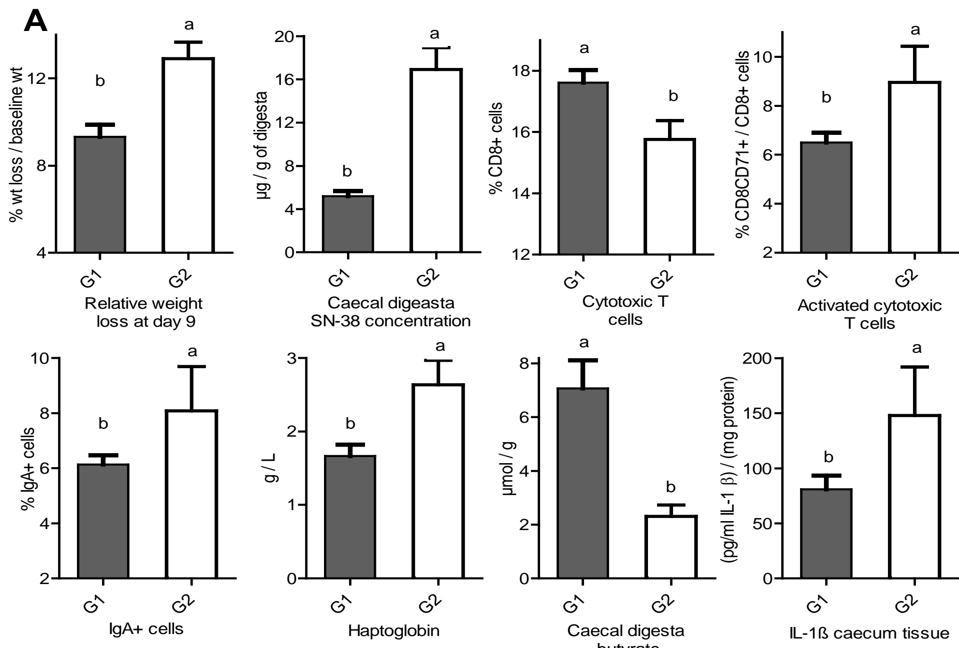
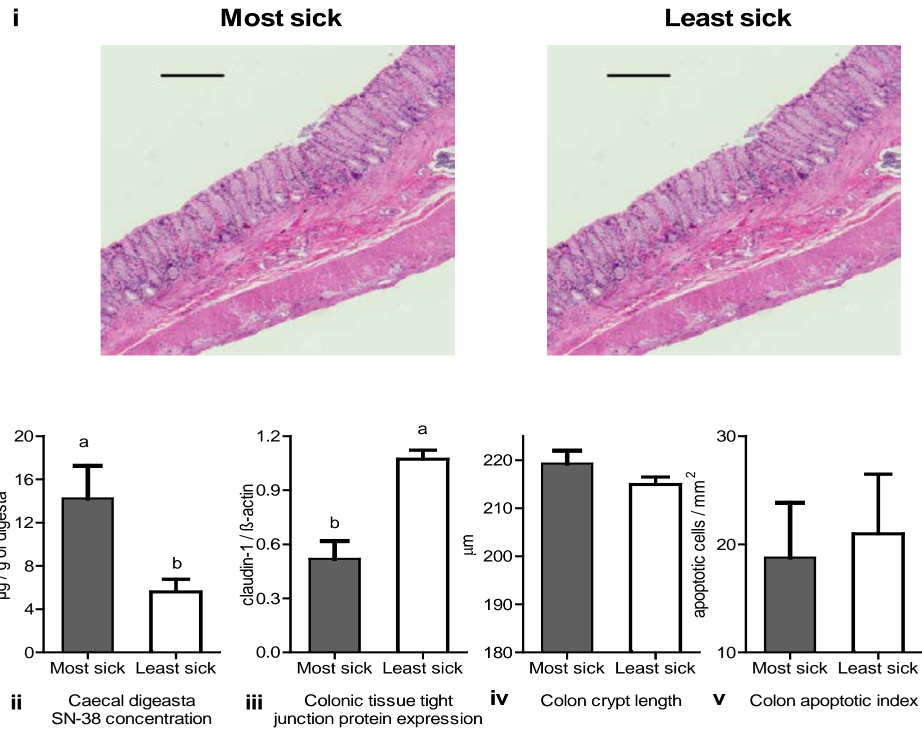


Figure 2-6. SN-38, apoptosis and tight junction. (A) All 42 animals were divided into two subgroups based on SN-38 concentration in caecal digesta: Group 1 (G1)<12 μg/g (n=30), Group 2 (G2)≥12 μg/g (n=9); other data are reported for these 2 groups. (B) Comparison of the most sick (n=6) and the least sick (n=6) rats, selected on the basis of weight loss and levels of acute phase proteins and CD8CD71+ cells. (i) Histological view of colon 48 hours after CPT-11 treatment, H&E staining, 100x magnification, scale bar represents 200 μm. (ii,iii) SN-38 concentration in caecal digesta and claudin-1 expression in caecal tissue were different between most and least sick animals ($p<0.05$). (iv,v) Colon crypt length and apoptotic index were invariable between most and least sick animals.


A
% wt loss / baseline wt
Relative weight loss at day 9
μg / g of digesta
Caecal digeasta SN-38 concentration
% CD8+ cells
Cytotoxic T cells
% CD8CD71+ / CD8+ cells
Activated cytotoxic T cells
% IgA+ cells
IgA+ cells
g / L
Haptoglobin
μmol / g
Caecal digesta butyrate
(pg/ml IL-1 β) / (mg protein)
IL-1ß caecum tissue
G1
G2
a
b


B
i
Most sick
Least sick
μg / g of digesta
claudin-1 / ß-actin
μm
apoptotic cells / mm2
Most sick
Least sick
ii Caecal digeasta SN-38 concentration
iii Colonic tissue tight junction protein expression
iv Colon crypt length
v Colon apoptotic index

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Chapter 3: General discussion and conclusion

Cancer chemotherapy disrupts normal cellular processes and is administered under the premise that cancer cells are more susceptible to these agents than normal tissues. Although cancer chemotherapy is effective against a wide array of tumor cells, toxicity and damage to normal cells is an inseparable part of chemotherapy which brings significant morbidity. In spite of increasing interest in NDCHO in different areas, evidence leading to rational advice for supplementation of NDCHO to cancer patients treated with chemotherapy remains weak and systematic research is needed to build up evidence-based strategies to supplement NDCHO for patients given different treatments. This thesis highlights limited evidence in this regard and generates evidence of translatable information for clinical strategy design. The thesis is built in a clinically relevant model system and a controlled dietary design; NDCHO are assessed in a comparative manner, and exhaustive investigations point to issues of chemotherapy toxicity from multiple points of view related to cancer chemotherapy.

Revolving around the main theme of dietary NDCHO during chemotherapy, this thesis work produces results primarily answering following questions: **what is the translational importance of the current findings; what we found about mechanisms of CPT-11 toxicity and our suggestions to modulate this toxicity.**

3.1 What is the translational importance of the current findings?

3.1.1 Clinical evidence on fibre supplementation*:*

3.1.1.1 Numerous applications of dietary supplementation with fibres: Nutritional interventions with fibres aiming to modulate disease process have been relatively well established in chronic bowel disease conditions (1-3). In epidemiological studies, dietary fibres have been also associated with lower incidence of total mortality, cardiovascular disease and mortality from these diseases, type II of diabetes,

development of cancers (especially colorectal cancer), promotion of satiety and weight loss and prevention of obesity, reducing prevalence and duration of diarrhea associated with infectious organisms and antibiotic-associated diarrhea, enhancing bioavailability and uptake of minerals (including calcium, magnesium, and possibly iron), and finally lowering inflammation and symptoms associated with inflammatory bowel diseases (4-7).

3.1.1.2 The most active agents of fibres: In the context of intestinal health, NDCHO are readily fermented in the colon into physiologically active byproducts. SCFA, primarily acetate, propionate, and butyrate, are end products resulting from fermentation of NDCHO and are suggested to have beneficial properties in the host (4, 8). The rate and amount of SCFA production depends on the substrate source, gut transit time and the number and species of microflora present in the colon (4, 8). SCFA are readily absorbed (9). Acetate enters the peripheral circulation and is metabolized by many tissues (8). Propionate is largely absorbed by the liver (8) and butyrate is the major energy source for colonocytes (10). Specific SCFA may reduce risk of developing gastrointestinal disorders, cancer, and cardiovascular diseases (4, 8). It has been suggested that butyrate, in particular, has functions at the level of gene expression, preventing malignant transformation by reducing cell proliferation and inducing differentiation and apoptosis (11-14). Butyrate was found to enhance expression of glutathione S-transferase, which leads to enhanced chemoprotection, reducing the impact of endogenous genotoxic risk factor 4-hydroxy-2-nonenal, and this is hypothesized to play a role in early and late stages of cancer prevention by reducing exposure to relevant risk factors (15).

3.1.1.3 Clinical evidence with negative and positive results for mitigation of inflammatory bowel diseases by NDCHO: There are a few clinical trials where NDCHO were supplemented to patients with inflammatory bowel conditions. One randomized, double-blind, crossover, placebo-controlled study of 20 patients with pouchitis demonstrated that, compared with placebo, 24 g/day of inulin for 3 week reduced both endoscopic and histological pouchitis inflammation scores, lowered fecal pH and concentration of

Bacteroides fragilis in fecal samples, and increased fecal butyrate concentration (3). It has been suggested that highly digestible diets compromise gut barrier function, as enhanced bacterial translocation in animals fed an elemental diet (i.e. containing no non-digestible components) was reversed by addition of cellulose to the elemental feed (16). However in our experiment, animals in highly digestible diets (IMO and cornstarch) were not different from animals in RS4 and Synergy® diets in assessed pathohistological damage after CPT-11 treatment. The reasons for this are unknown. In another randomized, double blind, placebo-controlled pilot study, participants (n=10) with active ulcerative colitis were administered 12 g/day mixture of OligoF and inulin for 2 weeks. Consumption of the mixture significantly decreased the concentration of calprotectin (an abundant neutrophil protein that is markedly elevated in patients with inflammatory bowel disease), and all participants in the test group achieved clinical remission (1). When10 patients with active ileocolonic Crohn's disease received 15 g/day of OligoF for three weeks, this treatment reduced disease activity index, and increased percentage of IL-10 positive dendritic cells, dendritic cells expressing TLR-2 and TLR4 (2). In our experiment, animals fed RS4 and Synergy® had lower immune activation markers and higher number of lymphocytes and cytotoxic Tcells. Our findings might be suggestive of immunity improvement by some NDCHO after chemotherapy. However in an adequately powered randomised double-blind placebo-controlled trial, when Benjamin *et al.* assessed the impact of oligoF in patients with active Crohn's disease they could not find a significant difference either in the number of patients achieving a clinical response or remission rates between the oligoF and placebo groups in the intention to treat analysis (17).

3.1.1.4 Is bowel injury of inflammatory bowel disease related to CPT-11 injury? Inflammatory bowel diseases are idiopathic inflammatory bowel disorders. Environmental factors (e.g. geography, cigarette smoking, sanitation and hygiene), microbes, ethnic origin, genetic susceptibility, and a dysregulated immune system can result in mucosal inflammation in these conditions (18). The potential benefits of NDCHO in inflammatory bowel diseases might have some value for interpretation in study of

NDCHO in CPT-11 bowel injury. Like inflammatory bowel diseases, intestinal microflora are suggested to play a key role in CPT-11 intestinal injury (by production of β-glucuronidase) (19-21), and shift in the intestinal microbes to more potential healthy population (e.g. *Bifidobacteria spp.* and *Lactobacillus spp.)* might benefit host in both inflammatory bowel diseases and CPT-11bowel injury. This similarity between both conditions of intestinal injury might encourage researchers to consider findings of clinical trials in inflammatory bowel conditions to design clinical trials for CPT-11 injury and NDCHO.

3.1.1.5 Clinical trial design to study NDCHO in cancer patients receiving CPT-11: Dietary fibres associate with reduced risk of colorectal cancer and low intake of dietary fibres has been suggested as an etiologic factor in cancer of colon in several large prospective observational studies and systemic reviews and meta-analysis studies (5, 22-24). Based on this evidence, one might assume that dietary fibre intake of many people (including cancer patients) might be lower than recommended. The American Dietetic association recommends daily use of 25 - 35 grams of fibre. One important consideration in the design of a clinical trial of NDCHO in colorectal cancer patients will be amount of fibres patients should consume on average during daily intake. Nutrition facts labels recommend intake of 25 g dietary fibre per day for 2000 kcal/day diet or 30 g/day for 2500 kcal/day diet as goals for healthy American individuals. The dietary reference intake table by Health Canada recommends 25-30 g/day of total dietary fibre as adequate intake amount (http://www.hc-sc.gc.ca/fn-an/nutrition/reference/table/index-eng.php). But there is no consensus agreement on recommended amount of dietary fibre intake for cancer patients treated with chemotherapy at this time. Dietary food intake records will help to assess the average amount of fibre taken by cancer patients. The total amount of 25-35 g/ day of fibre and NDCHO may be a suggested amount to achieve in order to prevent the gastrointestinal symptoms seen with high levels of fibres (e.g. flatulence, bloating, and abdominal discomfort), but further investigation is needed to decide about the optimum amount that will benefits cancer patients. Control arm of the proposed study would be patients treated with the same regimen of chemotherapy and same stage of cancer. Any unused NDCHO would

be returned by patients as measure of compliance to diet. The results of such a clinical study would probably help not only patients treated with CPT-11, but also other cancer patients dealing with burden of GI toxicities as results of variety of other chemotherapies.

3.1.2 Strengths and limitations of the animal models:

3.1.2.1 Current knowledge about NDCHO effects on chemotherapy toxicity: Although a few available preclinical findings support some palliative roles of NDCHO during cancer chemotherapy, a gap still remains preventing a translational value of these findings into a widely accepted and evidence-based rationale for clinical use (25-27). Most of the prior evidence on chemotherapy and dietary NDCHO had important limitations. NDCHO were given by gavage and not incorporated in the diet. Previous studies were generally been limited to a intervene with single NDCHO and lacked comparison with a control treatment or other NDCHO sources. The clinical relevance of the doses used was not justified (25-27). Thus, much of the evidence may not entirely represent what could be translatable to a clinical setting. This forms a major barrier preventing a meaningful comparison between these studies. The potential contribution of NDCHO intake level and type to colonic injury and toxicity associated with CPT-11- based regimens is completely unknown and there is no present clinical trial done with NDCHO in the context of chemotherapy toxicity. CPT-11 is not the only chemotherapeutic agent with GI toxicity; many other agents have cytotoxic side effects on GI. High-dose protocol of paclitaxel increased risk of *clostridium difficile*-associated diarrhea as high as 20% (28). 5-FU, capecitabine, oxaliplatin, docetaxel are examples of other agents associated with nausea, vomiting, diarrhea, and mucositis (29-31).

3.1.2.2 Our model of tumor, chemotherapy, and toxicity: Our preclinical experimental system is in the clinical range and mimics as exactly as possible, colon tumor, first line chemotherapy regimen, and diet (modeled on AIN-76 modified basal diet with 40% of calories from fat, the modified fat component is formulated to be similar to typical North American dietary patterns in humans) of patients with colorectal

cancer. The model recapitulates levels of morbidity and mortality seen clinically. Unlike most of the available studies, which are focused on a single NDCHO, this work made a back-to-back comparison of several NDCHO in an identical setting and makes possible the interpretation of their relative efficacy. The clinical ties inherent to our model system, dietary design and exhaustive end point selection also add to the translatability of this animal work.

3.1.2.3 Limitations of our experimental model: level of NDCHO: The dose of NDCHO used in current experiment (54.4 g for 2000kcal/day) is higher than current recommendations for healthy humans. This is because of the differences in gastrointestinal anatomy and physiology between humans and hindgut fermenters (rats) who have higher capability of fibre fermentation because of their relatively large caecum. There is no consensus agreement on recommended amount of dietary fibre intake for cancer patients treated with chemotherapy at this time.

3.1.2.4 Relative efficacy of NDCHO in our experiment and clinical value of this finding: Not all the tested NDCHO were equally effective to modulate toxicity related to CPT-11 chemotherapy, though all the NDCHO tested have been suggested to have improved certain forms of gut toxicity individually in different (non-chemotherapy related) conditions. Based on our findings, RS4 and Synergy® could potentially inhibit CPT-11-induced toxicity and feeding IMO can worsen CPT-11-induced toxicity. Our findings also suggest that low level of NDCHO (i.e. 2% vs 10%) in the diet may potentially worsen the chemotherapy toxicity of CPT-11. One important issue regarding the effects of NDCHO is individualized nature of response. This suggests that some individuals are less or more responsive to these nutrients. As it is not possible to predict which people will respond, the therapy could be wasted on these individuals. What differentiates these responders from non-responders is unknown, but one explanation may simply be due to the presence or absence of specific bacterial strains (*Bifidobacterium)* capable of using that NDCHO as a growth substrate (17, 32). We observed large variation in response to chemotherapy between six

animals that were in the same diet. What made RS4 and Synergy® diets decreasing the toxicity might have been simply the greater number of animals that benefited from NDCHO. Unlike IMO which is partly digestible in small intestine, any other of tested NDCHO (RS4, Synergy®, OligoF, inulin) can be used in the proposed clinical trial.

3.1.2.5 Prior findings in our model of CPT-11 toxicity; possible combination of different nutrients in a previous experiment in our model when glutamine, fish oil, and NDCHO (Inulin+oligoF) were directly compared: In rats treated with high dose of CPT-11 (125 mg/kg × 3 days), bolus glutamine was the only factor that demonstrated efficacy in preventing CPT-11-induced diarrhea (33). Glutamine's ameliorative effect on CPT-11-induced diarrhea was attributed to increasing accumulation of the stress-inducible heat shock proteins and oxidative stress regulation (increasing reduced glutathione:oxidized glutathione ratio) in colonic mucosa, and prevention of upregulated activity of a β-glucuronidase in caecal contents (34). At present, various immune-nutrition formulas are available featuring a mixing supply of glutamine and n-3 PUFAs and in most cases, combined with other substrates such as arginine, nucleotides and antioxidants (35-37). However, evidence about potential synergistic or antagonistic interactions between these nutrients when combined is still sparse. Given that cancer patients receiving chemotherapy are a targeted population for these combination formulas, further efforts are needed to understand potential interactions between nutrients and their role in the complex situations associated with cancer chemotherapy. Nutrients individually proven with therapeutic benefits during cancer chemotherapy do not necessarily produce a greater benefit when combined. One example is in an experiment in our model of chemotherapy, when glutamine and n-3 polyunsaturated fatty acids (n-3 PUFAs) were supplied in combination; an additive benefits were absent and combined nutrient appeared to be antagonist in many of the examined end points (38). Evidence-based rationale for mechanism of benefits of individual nutrients is needed as to understand how to maximize the therapeutic efficacy by optimally combining

different nutritional elements into a formulation. How these preparations are formulated in terms of component selection and dose optimization should be carefully justified.

3.1.2.6 How to supplement intelligently; is there any way to find the best diet to reduce chemotherapy toxicity, other than just by trial and error? Understanding of mechanisms of toxicity of individual chemotherapies, will lead us to design potentially advantageous dietary supplementation for cancer patients undergo chemotherapy. Researchers should understand the mechanisms of injury induced by chemotherapeutic agents and parallel these mechanisms with potential beneficial evidence of adjuvant nutrient supplements in order to design experimental dietary interventions. To better understand the importance of the mentioned parallel, CPT-11 can be used as an example. Structure integrity loss of intestinal mucosa is an important aspect of injury caused by CPT-11 (Table1-1) which can be a potential target for nutritional intervention with glutamine. Glutamine has an essential role in activation of the transcription factor Elk-1, which is pivotal for epithelial cell proliferation (39). Thus, dietary modulation that focuses on maximizing expression and activity of this transcription factor may be of interest.

3.1.2.7 Clinical validity of our suggested mechanisms; how to justify translational value of mechanisms of toxicity and NDCHO benefits in our experiment for clinical use? Late-onset diarrhea is a well recognized side-effect of CPT-11 chemotherapy; however a limited amount of research has been conducted to understand the underlying mechanisms of this type of diarrhea and to recognize other mechanisms of injury of CPT-11. Much of the information in the published literature about toxicity of CPT-11 is about clinical observations with limited basic studies existing. Immunological changes in terms of number and functional ability of immune cells have been documented in several human studies though (40-45), but most of the finding about pathohistological injuries, intestinal barrier dysfunction, and stimulatory effects of CPT-11 on inflammation in stemmed from animal experiments (46-54). Lack of comprehensive mechanistic investigations of humans is tangible and this gap makes it complicated to

target nutrients to improve toxicity profile of CPT-11. We applied exhaustive end point assessments to investigate CPT-11injury and NDCHO effects from several points of view which included measurements of clinical indices, bacterial and immunological analysis of changes, and evaluation of inflammation and drug metabolites. To confirm our suggested mechanisms of effect of NDCHO in CPT-11 toxicity or any other suggested favorable effects of nutritional intervention in experimental designs, clinical trials studies seem necessary. However, it is obvious that not all the mechanistic exploration done in animal studies would be feasible in human clinical trials. To test nutritional intervention effects on tight junction proteins injured by CPT-11, perhaps the widely trusted lactulose:mannitol ratio test (L/M ratio test) would be an appropriate candidate. L/M ratio has been used as a measure of permeability in clinical trials where the barrier function is compromised in variety of situations (55-58). The L/M ratio had a sensitivity of 85 % and a specificity of 46.2 % to detect severe mucosal damage and mucosal atrophy in follow-up visits in patients with celiac disease (59). Fecal calprotectin measurement has been proposed as a non-invasive biomarker of intestinal inflammation (60). Calprotectin is a calcium and zinc binding protein which accounts for 30 to 40% of neutrophils' cytosol. Neutrophils influx into the bowel lumen as a result of the inflammatory process. Measurement of faecal calprotectin has been shown to be strongly correlated with 111-indium-labelled leucocyte (which is considered the gold standard measurement of intestinal inflammation) (60). Fecal calprotectin can be a potential biomarker to evaluate nutrients and intestinal inflammation interaction; however one should consider that this marker has been mostly investigated in inflammatory bowel disease conditions (61). Fecal calprotectin was proposed as a potential biomarker of intestinal damage during radiotherapy in gynaecological and urological cancer patients during pelvic irradiation (62), but the accuracy of this test to detect intestinal inflammation in chemotherapy-induced intestinal inflammation is unknown.

3.2 What are we found about mechanisms of CPT-11 toxicity and our suggestions to modulate this toxicity?

3.2.1 What is the importance of β-glucuronidase in CPT-11 toxicity?

We observed that β-glucuronidase activity did not explain variations in toxicity in CPT-11 treated rats and furthermore the enzyme levels were inversely associated with SN-38 levels in caecal contents. Our findings are opposite to the generally accepted view of β-glucuronidase mediating CPT-11-induced toxicity. The evidence for role β-glucuronidase in causing toxicity was first proposed when the incubation of bile from drug-treated animals with a caecum extract from an untreated animal increased the SN-38 content. Therefore the metabolite SN-38G was suggested to be deconjugated by intestinal microflora to SN-38 (63). Takasuna *et al.* found a good correlation between β-glucuronidase activity in the intestinal lumen and the degree of intestinal damage and suggested that SN-38, derived from the hydrolysis of SN-38G by microflora β-glucuronidase, plays a key role in the intestinal toxicity induced by CPT-11 (20). They reported that antibiotics markedly reduced the area under curve ($AUC_{1\text{-}24h}$) of SN-38 (by about 85%) in the large intestine tissue and completely inhibited the deconjugation of SN-38G in the large intestine luminal contents (21). Gupta *et al.* found a correlation between the glucuronidation (biliary index) and diarrhea degree, again confirming the main role of β-glucuronidase in causing clinical toxicity of CPT-11 (diarrhea) (19). Our findings however, support that this enzyme is only one of the causes of CPT-11 toxicity but not the main culprit at least in our model. Our results does not negate the role of this enzyme in causing CPT-11 toxicity, but rather suggest that perhaps the SN-38 arriving at the colon from upstream small intestine billiary excretion is more quantitatively important than generation of SN-38 locally in the colon by β-glucuronidase. Also a potential negative effect of higher levels of β-glucuronidase activity is possibly offset by beneficial effects of SCFA. This possibility has not previously been considered. Our results are in accordance with Kurita *et al.* who found antibiotics alleviated diarrhoea in Gunn rats (which

have an inherited deficiency of UGT in the liver and therefore not able to excrete SN-38G in the bile (50). They proposed that antibiotics alleviate the diarrhea and decrease exposure of CPT-11 and SN-38 to the intestinal epithelium not by inhibition of β-glucuronidase in the lumen, but by inhibiting the absorption of CPT-11 from the intestinal lumen and the change of carboxylesterase and UGT activities in the intestinal epithelium (50).

3.2.2 Limiting SN-38 gut exposure; would be a primary therapy to reduce CPT-11 toxicity?

Strategies to limit intestinal toxicity of CPT-11 are possible by comprehensive understanding of the metabolism and elimination pathways of the drug and timing of these events, especially factors relating to the rate of appearance of SN-38 in the terminal ileum. As our findings point to the importance of SN-38 levels in large intestine to cause injury, limiting SN-38 appearance in the lumen would seem reasonably rational to reduce intestinal injury caused by CPT-11. Luminal SN-38 could be captured or inactivated in different ways. The *first approach could be to target β-glucuronidase by other means less harmful than antibiotics.* Inactivation of SN-38 by oral gavage of β-glucuronidase inhibitor (twice/day, 10µg) could effectively reduce toxicity and diarrhea without killing the commensal bacteria essential for host health (52). *Another approach can be to lower luminal appearance of SN-38 by substances that may bind it and block its action.* This has been suggested by some early studies with activated charcoal, which is used as an adsorbent to treat intoxications from ingestion of harmful drugs or substances (64). Timing of treatment with this substance is crucial for maximal efficacy of activated charcoal, and delays of even 30 minutes can decrease absorption substantially (64); which emphasize on significance of timing for this material to reach the large intestine when biliary excretion peaks. Charcoal acts as an insoluble carrier that non-specifically absorbs molecules (65), thereby this substance might absorb essential nutrients like vitamins and calcium and cause malnutrition if used regularly. Dietary fibres in guar gum were effective in binding to cholesterol and bile salts (66). NDCHO might be worth of investigation to assess their binding

properties to SN-38 and therefore reduced appearance of SN-38 in large intestine and ultimately decreasing CPT-11-induced intestinal toxicity. However, the idea of oral agents to trap or inactivate luminal SN-38 seems worthy of further consideration. Finally, as SN-38 is excreted in through the bile*, another possible approach to reduce SN-38 appearance inside large intestine is to minimize its rate of biliary excretion*. Limitation of intake during a window around the time of CPT-11 injection might serve the ultimate goal of minimal luminal exposure of SN-38. This idea again highlights the significance of fully comprehension of CPT-11metabolism in plasma to try to prevent the bile excretion when CPT-11 and SN-38 are at topmost levels in blood and bile. In a case series of 10 patients with cancer who voluntarily underwent short term fasting prior to or after an average 4 cycles of various chemotherapy drugs (or at both times), fatigue, weakness, and gastrointestinal side effects were reduced (67). Fasting might also potentiate the therapeutic efficacy of chemotherapy agents to destroy tumor cells. Normal cells and cancer cells differ in their ability to respond to fasting. In the absence of nutrients, normal cells switch their metabolism toward maintenance pathways, whereas tumor cells are unable to activate this protective response. The differences in metabolism between normal cells and cancer cells could be used to enhance anticancer therapy by selectively increasing the resistance of normal cells to chemotherapy (68).

3.2.3 Improving host resistance to CPT-11 chemotherapy by diet therapy to preserve intestinal barrier function and increase and enable immunity:

3.2.3.1 Preserving gut barrier function: CPT-11 reduced tissue expression of tight junction proteins, this break in the barrier function of intestine results in bacteria and toxins translocation (Table1-1). Tight junction (TJ) is an essential component of Intestinal epithelial barrier. Tight junctions are the most apical component of intercellular junctional complex. Dynamic regulation of TJ function is fundamental to many physiological processes, and disruption of TJ drastically alters paracellular permeability (69). TJ are composed of transmembrane proteins: claudins, occludin and cytoplasmic proteins, such as zona occludens-1 (69). Probiotics are one potentially interesting class of agent shown to

improve intestinal barrier function in variety of different challenges that cause barrier dysfunction (immature intestinal barrier, DDC-induced colitis, acute pancreatitis, and colorectomy). Probiotics are mono- or mixed culture of live microorganisms which applied to animal or man and benefits the host by improving the properties of the indigenous microflora (70). *Lactic acid* bacteria and *bifidobacteria* are the most common organisms used as probiotics; but certain yeasts and *bacilli* may also be used. Probiotics are commonly consumed as part of fermented foods with specially added active live cultures; such as in yogurt, soy yogurt, or as dietary supplements. Probiotics improve intestinal barrier function by regulating and maintaining intestinal tight junction protein expression in neonatal mice with immature intestinal epithelial barrier and DDS-induced mice (71, 72). Probiotic supplementation significantly lowered post-operative incidence of bacterial translocation and intestinal permeability test (assessed using the L/M ratio test) in colorectal cancer patients who underwent radical colorectomy (55).

3.2.3.2 Modulation of host immunity: Modulating host immune function may be assumed to contribute in prevention of CPT-11 toxicity. How the immunonutrients (e.g. glutamine and n-3 PUFAs), would potentially affect intestinal and systemic immunity, and how to direct their potential immunomodulatory actions towards systemic infection secondary to chemotherapy still needs further effort to be clarified. Chemotherapy results in complicated pathological changes in immunity which includes initial hematopoietic suppression and subsequent immunological reconstitution. These phasic changes may predict a temporally compromised immune responsiveness and vulnerability to certain opportunistic infections. At present, we don't know how the therapeutic window of these immunonutrients fit into the sequence of immunopatological changes of patients; so a challenging consideration regarding immunonutrients is timing of administration of these agents. Current evidences suggest that immunonutrients may not be equally effective for moderately immunosuppressed patients (at risk of infection) and the patients suffering from more serious illnesses (e.g sepsis, and organ failure) as result of cancer therapy (73, 74). This means that immunonutrients therapeutic window may also considerably

differ for the same chemotherapeutic regimen based on the facts that if the treated patient is only at the risk of infection or already has developed intensive systemic infection. This emphasizes the need for future systematical research on immunonutrients supplementation in cancer chemotherapy to categorize patients according to their immune status.

3.3 Conclusion:

Gastrointestinal injury is a major dose-limiting toxicity for many anticancer drugs. The gut is an attractive target for nutrition modulation, because of its direct exposure to the diet, participation in uptake and metabolism of nutrients, high rate of cell proliferation and responsiveness to nutrition stimuli. Optimal use of nutrients for specific injuries with cancer chemotherapies requires further study.

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