IF YOU HAVEN'T HAD AT LEAST A SLIGHT POETIC CRACK IN THE HEART,
YOU HAVE BEEN CHEATED BY NATURE.

~PHYLLIS BATTELLE

University of Alberta

Nutritional modulation of efficacy and toxicity related to irinotecan chemotherapy

by

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ABSTRACT

Dose-intensive systemic chemotherapy is a prevailing tactic used in medical oncology, but this is largely limited by inherent toxicities to various host systems including gastrointestinal, hematological and immune systems. The premise of this work is to modulate the treatment response to irinotecan (CPT-11), a first-line chemotherapy used to treat colorectal cancer, by adopting a nutritional perspective.

I used a rat model bearing the Ward colon tumor and a controlled dietary design to compare effects of three dietary elements on cancer progression, CPT-11-related efficacy and diarrhea toxicity. *N*-3 polyunsaturated fatty acids (PUFA) suppressed tumor growth and enhanced CPT-11's efficacy; whereas glutamine bolus limited CPT-11-induced delayed diarrhea. Prebiotic oligosachharides were ineffective with respect to diarrhea and did not affect CPT-11's efficacy. Diarrhea-ameliorative effects by bolus glutamine treatment was associated with a multiplicity of potentially beneficial and protective responses in the colon: enhanced heat shock response (HSR); increased reduced:oxidized glutathione (GSH) ratio; stablized β-glucuronidase activity; and increased proportions of cytotoxic T cells in mesenteric lymph nodes (MLN). By contrast, glutamine treatment did not alter CPT-11's anti-tumor activity, nor did activate cytoprotective mechanisms in the tumor. I further examined how glutamine and *n*-3 PUFAs, when provided individually and in combination, would affect the treatment response related to CPT-11/5-Fluorouracil (5-FU) combination chemotherapy. Combined

supply of glutamine and *n*-3 PUFAs didn't lead to a greater efficacy but rather abrogated some of benefits to the host associated with single supply of these two nutrients. This work shows the therapeutic promise of glutamine and *n*-3 PUFAs as potential adjuncts to CPT-11, supports the concept that glutamine may favorably alter the balance between the host and tumor via its selective protection, and shows the need to justify combined use of key nutrients in 'immunonutrition' formulae.

Infectious complications are a major cause of morbidity and mortality associated with systemic chemotherapy. Prophylactic use of a fluoroquinolone antibiotic, ciprofloxacin (Cipro), completely prevented CPT-11-related mortality and strikingly improved the overall toxicity profile, which was associated with boosted immune responsiveness in the systemic immune compartment(s) and alleviation of excessive proinflammatory responses mediating local gut injury.

TO MY PARENTS, FOR EVERYTHING THEY GIVE ME.

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LIST OF ABBREVIATIONS

5-FU	5-fluorouracil
AA	arachidonic acid
AIN	American Institute of Nutrition
Ara-C	arabinosylcytosine
BCA	bicinchoninic acid
bcl-2	B-cell leukemia/lymphoma 2
BMT	bone marrow transplantation
CBC	complete blood count
CDK	cyclin-dependent kinase
CFU	colony-forming unit
Cipro	Ciprofloxacin
Con A	concanavalin A
CPT-11	Irinotecan (7-ethyl-10-[4-(1-piperidino)-1-piperidino]carbonyloxy-camptothecin
COX	cyclooxygenase
DHA	docosahexaenoic acid
DPM	disintegrations per minute
DSS	dextran sodium sulphate
EDTA	ethylenediaminetetraacetic acid
EFA	essential fatty acid

EGFR	epidermal growth factor receptor
EPA	eicosapentaenoic acid
ERK	extracellular signal-related kinase
GBF	germinated barley foodstuff
GI	gastrointestinal
GLP	glucogan-like peptide
GSH	glutathione
GSSG	glutathione disulfide
GALT	gut-associated lymphoid tissue
H_2O_2	hydrogen peroxide
H&E	haematoxylin and eosin
HER2	human epidermal growth factor receptor 2
HPLC	high performance liquid chromatography
HRP	horseradish peroxidase
Hsc	heat shock cognate
HSE	heat shock element
HSF	heat shock factor
Hsp	heat shock protein
HSR	heat shock response
IFN	interferon
IL	interleukin

i.v. intravenous(ly)

JNK/SAPK Jun nuclear kinase

KPBS potassium phosphate buffer

LA linoleic acid [18:2(n-6)]

LNA α-linolenic acid [18:3(n-3)]

LOX lipoxygenase

LT leukotriene

M2VP 1-methyl-2-vinyl-pyridinium trifluoromethane sulfonate

MAPK mitogen-activated protein kinases

MLN mesenteric lymph nodes

MPA metaphosphoric acid

MTD maximal tolerated dose

n-3 PUFA n-3 polyunsaturated fatty acid

n-6 PUFA n-6 polyunsaturated fatty acid

NF-κB nuclear factor-κB

NP-40 Nonidet P-40

PBS phosphate buffered saline

PG prostaglandin

Pgp P-glycoprotein

PKC protein kinase C

PMA phorbol myristate acetate

PMSF phenylmethyl-sulphonyl-fluoride

PP Peyer's Patches

PVDF polyvinylidene difluoride

rGSH reduced glutathione

RIPA radioimmunoprecipitation assay

s.c. subcutaneous(ly)

SCFA short chain fatty acids

SDD selective decontamination of the digestive tract

SDS sodium dodecyl sulfate

SI stimulation index

SN-38 7-ethyl-10-hydroxycamptothecin

SN-38G SN-38 glucuronide, 7-ethyl-10-hydroxycamptothecin-glucuronide

TdT terminal deoxynucleotidyl transferase

tGSH total glutathione

TGF-β transforming growth factor-β

TMP/SMZ co-trimoxazole (Trimethoprim/sulfamethoxazole)

TNBS trinitrobenzene sulfonic acid

TNF tumor necrosis factor

TPN total parental nutrition

TUNEL terminal transferase-mediated dUTP nick end labeling

TX thromboxane

UGT	uridine diphosphate glucuronosyltransferases
VEGF	vascular endothelial growth factor
VRE	vancomycin-resistant enterococci
v:v	volume:volume
wt:v	weight:volume
wt:wt	weight:weight

CHAPTER 1 INTRODUCTION AND RATIONALE

1.1 Overview: Nutritional modulation of cancer chemotherapy

1.1.1 Chemotherapy-science of selective toxicity

Since the first anticancer agent, nitrogen mustard, was introduced into clinical trials (1), chemotherapy has rapidly developed as a major anti-cancer modality, with the number of drugs available for cancer chemotherapy growing exponentially. For most cancer patients, chemotherapy has to be used at a certain point during the whole trajectory of their anti-cancer treatment. Chemotherapy *per se* can cure some disseminated cancers and can be effective in decreasing tumor volume, alleviating symptoms and even prolonging life in many other types of metastatic cancer.

As implicated in the discovery of early anticancer drugs, which was ironically through toxic effects (1), toxicity is inherent to chemotherapy and has continued to have a major influence on the way anticancer drugs are used. All types of cells actively engaged in proliferation are among targets for cancer chemotherapy. These include tumor cells as well as normal cells from host tissues such as bone marrow, gastrointestinal (GI) mucosa, lymphoid tissues, hair follicles, and germinal epithelium (2). This fact makes chemotherapy a science of selective toxicity. The utility of chemotherapeutic drugs depends on their therapeutic index, the ratio between doses that are toxic to tumor tissue and to normal tissues.

Anticancer drugs are one of the few classes of therapeutic agents that are routinely given to patients at doses producing moderate to severe toxicity. For most of conventional anti-cancer drugs, the efficacy against sensitive tumors shows a clear dose dependency (2).

Unfortunately, so does the dose-response relationship for toxicity, and patient morbidity is almost invariably associated with attempts at curative therapies (2). To a large extent, dosages at which chemotherapeutic agents are administered to patients are not defined by their anti-tumor potential, the intended pharmacologic effect, but by their potential to induce toxicity. In the clinic, chemotherapy is commonly administered at a maximal tolerated dose (MTD), which reflects a deliberate effort in dose titration, aiming to maximize anti-tumor efficacy within the boundary of "acceptable" toxicities.

The toxicities found to occur with chemotherapy have been reported to affect almost every organ system and tissue. The major potentially lifethreatening organ toxicities of chemotherapy are GI, bone marrow, hepatic, renal, cardiovascular, neuromuscular, and respiratory (2-4). Other commonly occurring toxicities, such as nausea, vomiting and alopecia, although non-lifethreatening, can seriously affect quality of life of patients (5).

A persistently existing key issue for cancer chemotherapy is how to augment the therapeutic index of anti-cancer drugs, by enhancing a drug's anti-cancer potential or/and lowering its major dose-limiting toxicities, such that the therapeutic outcome could ultimately be improved. One strategy representing these efforts is through synthesis or discovery of new analogs or agents with higher tumor-targeting capacity; another strategy is to alter the drug's pharmacokinetics/pharmacodynamics by modifying its administration scheduling or delivery system. The third strategy is to modulate the therapeutic index of existing anti-cancer drugs by combing adjuvant factors/therapies, which, in many of the cases, turns out to be more reasonable via the 'cost-benefit analysis' (6).

1.1.2 Adjuvant modulators for cancer chemotherapy

All the adjuncts to cancer chemotherapy can be categorized into chemosensitizers or toxicity antagonists according to their mechanism of action. Chemosensitizers render cancer cells more susceptible to chemotherapy through its interaction with cancer cells and/or the anti-cancer drug; whereas toxicity antagonist mitigates chemotherapy's toxicity by directly interfering with the mechanism of toxicity or modulating normal tissue response(s) to injury (7). Both of them could directly or indirectly improve the net anti-tumor efficacy of the anti-cancer drug with equal or lesser toxicity.

Ever since the concept to combine modulators into chemotherapy regimens has emerged, efforts have been largely directed towards developing chemotherapeutic or pharmaceutical agents of potential for this combinatorial use. Chemosensitizers' action runs the gamut of overcoming drug resistance in biologically heterogeneous tumors to targeting specific molecules governing regulation of cell survival, apoptosis, cell cycle progression and angiogenesis (e.g., epidermal growth factor receptor (EGFR), human epidermal growth factor receptor 2 (HER2), cyclin-dependent kinases (CDK), protein kinase C (PKC), nuclear factor-κB (NF-κB), B-cell leukemia/lymphoma 2 (bcl-2) and vascular endothelial growth factor (VEGF)) (8-12). There has also been a rapid expansion in the number and diversity of toxicity antagonists or protectants, which are designed specifically to interrupt or block processes fundamental to tissue injury. Agents showing protective potential against acute or late toxicities include granulocyte colony stimulating factor (G-CSF), keratinocyte growth factor, transforming growth factor-β (TGF-β), misoprostol, lisofylline and amifostine (13-17).

One of the first nontraditional ideas about the relationship between food and health was the therapeutic use of certain nutrients in conditions with stress and potential injury. Not until three decades ago had such a conception been transformed into attempts to seek

therapeutic constituents naturally occurring in the diet, which could potentially modulate the host/tumor's response to cancer chemotherapy. These efforts provide an important complementary perspective in the discovery and development of chemotherapy modulators.

1.1.3 Dietary elements-potential chemotherapy modulators?

Ultimate outcome related to systemic cancer chemotherapy largely depends upon sophisticated interplays between cancer, host and the anti-cancer drug. Accumulating lines of evidence suggest that diet could extensively influence interactions between these three parameters and thus may hold a key to modulating the balance in a manner that favours increased anti-cancer efficacy or/and lesser injury to normal tissues. Dietary elements as diverse as certain amino acids, fatty acids, oligosaccharides, minerals, vitamins and antioxidant compounds have been suggested to affect processes involved in the progression of cancer and its responsiveness to the anti-cancer therapy, the host's integrity in the dual challenges presented by the cancer and anti-cancer chemotherapy. These effects may take place on multiple levels including modulating the pharmacokinetics of the anti-cancer drug, blocking/limiting key mechanisms for drug's toxicity, altering cytokine/hormone network, modifying cellular protective and repair machinery, and initiating/blocking signaling events involved in regulation of cell cycle and cell proliferation/death. Table 1-1 provides examples of dietary interventions tested in preclinical models, which have been shown to augment efficacy or reduce toxicity of cytotoxic chemotherapy in respective conditions.

A prerequisite for the utility of these nutritional interventions is that their action as chemosensitizer or toxicity antagonist has to be considerably differential in tumor and

host normal tissues. A toxicity antagonist confers protection to host tissues, but does not supposedly protect the tumor or renders the tumor more chemoresistant; whereas with the use of a chemosensitizer, the potentiated cytotoxic effect should be confined to tumor tissue, but does not extend to critical dose-limting host tissues. Mechanisms responsible for such a discriminative effect are not well studied. Experimental work available suggests that this could occur at various levels, depending on different cytotoxic drugs and nutrients:

1) The mechanism for the drug's anti-tumor activity is different from what is primarily responsible for the pathogenesis of toxicity, which forms the basis for the use of certain antioxidants to mitigate anthracycline-induced cardiotoxicity without affecting or even sensitizing tumor's response (18-20);

2) Intrinsic biochemical deficits in tumor cells could also be another important attributor for this discrimination. For instance, cancer cells may have different EFA levels on their plasma membrane (21,22) or an abnormal oxidation pathway for polyunsaturated fatty acids (PUFA) as compared to their normal counterpart (23), which could make them more susceptible to *n*-3 PUFA treatment. Glutamine supplementation has been suggested to modify host and tumor glutathione (GSH) stores in a differential manner. Blockade of GSH regeneration in the tumor is conjectured to be due to the acidic intratumoral environment and tumor's inability to upregulate essential enzymes for GSH regeneration upon glutamine induction (24).

1.1.4 Nutritional modulators for chemotherapy- Is the evidence sufficiently robust for clinical strategy-making?

When diagnosed with cancer, patients commonly become concerned about their

dietary patterns and are highly motivated to seek information about diet and dietary supplements. Currently oncologists face increasing demands about dietary advice such as what diet constituents have intrinsic anti-cancer activity; what dietary measure, if there is any, should be taken to favorably modulate response to antineoplastic treatment; what should best be avoided from the diet because they may potentially counteract treatment efficacy. As such, a clinically-driven thrust has been formed for systematic and comprehensive research which could provide robust evidence-based information and help formulate rational advice for patients and clinicians.

In spite of the interest which diet and dietary supplements continue to elicit among patients, clinicians and researchers, evidence permitting rational clinical advice in this regard remains weak. Despite the existence of almost 1000 current citations in the literature of work conducted in experimental animal models, an apparent gap still remains and hampers these preclinical findings being effectively translated into practical strategies for clinical use; questions regarding what nutrients, why these nutrients and how the nutrients could clinically work are still poorly answered. A recent analysis of potential benefit of nutritional intervention for outcome of patients with cancer or preinvasive lesion concludes that robust evidence is lacking for improvement of patient survival or disease prognosis by dietary modification (25).

The underlying reason for such a gap could be multifactorial: Reading of literature suggests 4 key problems (as summarized below) in the experimental nutrition approach to modulation of chemotherapy toxicity. In the following sections examining the literature specific to the 3 classes of nutrients studied in this thesis, a detailed analysis of issues related to those nutrients is given.

1) Lack of a systematic and comparative approach. Individual nutrients are rarely

compared with one another within a controlled dietary design, so their relative efficacy is unknown.

2) Enormously varied basal diet ranging from chemically defined elemental diets, semipurified diets to chow diets.

3) Inconsistency in the dose, schedule and route of a certain nutrient supply.

4) Model system lacking clinical translatability in drug treatment or measured toxicity outcome.

1.2 Nutritional modulation of irinotecan (CPT-11) chemotherapy

1.2.1 Overview: CPT-11 chemotherapy

Irinotecan (7-ethyl-10-[4-(1-piperidino)-1-piperidino]carbonyloxy-camptothecin, CPT-11, Camptosar®) is a water-soluble, semisynthetic derivative of camptothecin, a plant alkaloid isolated from *Camptotheca acuminata*. Since it was first discovered and synthesized in the early 1980s, CPT-11 has emerged as a cornerstone of the management of metastatic colorectal cancer (26-31). CPT-11 was recognized early in its development to be active in patients who had failed 5-fluorouracil (5-FU) (32). Subsequent studies have demonstrated in advanced colorectal cancer patients that treatment with 5-FU/CPT-11 was superior to 5-FU in terms of overall survival (30). The current standard of care for colorectal cancer patients is treatment with 5-FU/CPT-11 followed by 5-FU/oxaliplatin (33). CPT-11 has also shown potent activity against many other malignancies (34-39) and has been the backbone of chemotherapy for small cell lung cancer (36,40).

Unlike most other camptothecins, CPT-11 is a prodrug and requires bioactivation by

tissue carboxylesterase to form its active metabolite SN-38 (7-ethyl-10-hydroxycamptothecin). Antitumor activity of SN-38 is at least 100-fold greater than that of CPT-11, but its development as a clinical agent without the prodrug approach is limited by its short plasma half-life (41). SN-38 acts as a topoisomerase I inhibitor by 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 (42,43). CPT-11 and its metabolites undergo a pH-dependent reversible inter-conversion between the ring-closed lactone form and the ring-opened carboxylate form in aqueous environment. Only the lactone form is believed to be able to cross cell membranes and act as a topoisomerase I poison. At neutral pH, interconversion equilibrium between the two species favors the inactive carboxylate form (44). SN-38 is eventually deactivated by uridine diphosphate glucuronosyltransferases (UGT) to form its glucuronide, SN-38G (45,46).

1.2.2 Diarrhea- hallmark toxicity for CPT-11 chemotherapy

CPT-11 has a narrow therapeutic window. The dose of CPT-11 is mainly limited by its toxicity in GI and hematological systems (47). Gut toxicity, especially diarrhea is the hallmark toxicity related to CPT-11-based regimens (34,44,48). CPT-11 administration can induce two types of diarrhea. Early diarrhea, which occurs within 24 hours of CPT-11 administration, is a clinical component of the cholinergic syndrome that induces colonic hyperstimulation (49,50). It can often be managed by anticholinergics such as atropine (49,50). Delayed diarrhea (occurring more than 24 hours after CPT-11 injection), which is

usually of long duration and doesn't respond well to conventional symptomatic managements, usually turns out to be a serious problem in the clinic (34). Over 80% of patients receiving CPT-11 develop delayed diarrhea of varying severity. 31-39% of these patients experience grade 3/4 delayed diarrhea, which is associated with severe dehydration and electrolyte abnormalities (47,48). Delayed diarrhea can be life threatening for 8% of patients (30) and is one of the major contributors to the death of 2.4% patients with colorectal cancer treated with CPT-11/5-FU regimen (31). Proper management of gut toxicity, especially delayed diarrhea, is essential if optimal efficacy, safety, nutritional status as well as patient satisfaction are to be achieved with regimens centered with CPT-11.

1.2.3 Mechanisms for CPT-11-induced gut toxicity

SN-38 pharmacokinetics, the toxic metabolite of CPT-11, are believed to be directly related to induction of the gut toxicity (51). Duration of exposure to SN-38 is more critical for the severity of gut toxicity as compared to SN-38 concentration. A significant correlation was found between the extent of glucuronidation and severity of diarrhea, indicating that the rate of formation and elimination of the inactive metabolite SN-38G may be an important predictor of diarrhea (51).

Emerging evidence suggests that local deconjugation of SN-38G to SN-38 by enteric bacterial β–glucuronidase (Figure 1-1) may be a crucial contributor to CPT-11-induced gut toxicity (52,53). Reactivation of SN-38G by flora not only allows for more SN-38 to be absorbed into systemic circulation via enterohepatic recycling, but also intensifies the local intestinal exposure (52). SN-38G has a longer half-life than SN-38, which favors the

localization of SN-38G and subsequent accumulation of SN-38 in epithelia cells of the large intestine after deconjugation by microflora (52,54). A steady accumulation of SN-38 and SN-38G was observed in mucosal tissue of rat large intestine, irrespective of the rapid elimination of these two metabolites from plasma (55).

Nonetheless, pathogenic mechanisms of CPT-11-induced diarrhea are not fully understood. Damage integrity of intestinal structure has been reported to be associated with diarrhea symptoms (55-58). Pathohistological alterations occurring in the large intestine are the most consistent findings associated with CPT-11 treatment, such as crypt hypoplasia, crypt dilation and mucus over-secretion in the large intestine (55,56,59); villous atrophy and crypt hypoplasia were also observed in the small intestine (56,59). Reduced epithelial cell proliferation and increased cell apoptosis may precede these histological changes (56,60,61). 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 (57,58). CPT-11 was shown to induce cyclooxygenase (COX)-2 in colonic mucosa and enhance the production of prostaglandin (PG)E_2 and thromboxane (TX)A_2. This could serve as pathogenic factor independent of structural destruction, as over-production of these eicosanoids (PGE_2 and TXA_2) may result in hypersecretion of Cl^- and thus excessive Na^+ and water secretion into the gut lumen (62-64). Additionally, administration of CPT-11 may lead to disturbances to bacterial flora of the intestinal lumen, which may in turn alter drug pharmacokinetics in gut tissue by affecting bacterial β-glucuronidase activity (58). Moreover, impaired intestinal immunity resulting from CPT-11 administration makes intestinal mucosa more predisposed to infection of aerobic bacteria, which might be a direct but non-specific

cause for CPT-11-induced diarrhea (65).

1.2.4 Management of CPT-11-induced diarrhea: current clinical status and experimental antagonists on trial (Summarized in Table 1-2)

Currently, a reliable prophylactic therapy to effectively prevent CPT-11-induced delayed diarrhea is still lacking. Interventional symptomatic therapy is still the mainstay for managing CPT-11-induced delayed diarrhea. Non-specific anti-diarrheal agents such as loperamide, Lomotil® (diphenoxylate combined with atropine) can slow GI-transit time in order to promote colonic water re-absorption (34,48). These agents are however of no help in reducing the incidence of severe (grade 3 and 4) diarrhea (26). Some clinical studies report that conventional anti-diarrheal agents such as atropine and morphine can even exacerbate CPT-11-induced delayed diarrhea (50).

Trials on toxicity antagonists of CPT-11 have largely focused on pharmacological modulation of intestinal metabolism and disposition of CPT-11 and its metabolites. These approaches include inhibiting biliary excretion of CPT-11 and its metabolites by using agents such as cyclosporin (66,67) and probenecid (68); intestinal alkalization by using bicarbonate to favor conversion of the lactone forms of CPT-11 and SN-38 to the less toxic carboxylate forms (69); downregulating bacterial β-glucuronidase activity in the bowel and thus inhibiting formation of SN-38 from SN-38G, by using non-absorbable antibiotics (70) or natural β-glucuronidase inhibitors such as bacalin and TJ-14 (35,50).

Another approach on the experimental stage is to explore whether certain gut-trophic/protective agents could be used as gut-toxicity antagonists and confer selective protection to the gut following CPT-11 therapy. Examples of these biological or pharmaceutical agents are glucogan-like peptide (GLP)-2, interleukin (IL)-15 and JBT

3002 (59,60,71,72). However, the majority of these trials only show evidence that these agents provide benefits as to improve the structural integrity of the intestinal epithelium. It is hard to interpret whether these observed structural ameliorations suffice the symptomatic improvement of diarrhea, as few of these studies established a link between the gut pathology and symptomatology.

1.2.5 Rationale for nutritional modulation of CPT-11-induced gut toxicity

The idea that dietary elements can modulate gut physiology and response to stress and injury has been around for a long time. A wide variety of stresses and injuries to the gut have been studied (e.g., weaning, inflammatory bowel disease, short bowel syndrome, trauma, surgery, sepsis, hyperthermia) and a wide variety of different orally active compounds implicated (i.e., amino acids, EFAs, Pre/Probiotics, iron, zinc, nucleotides, vitamins). However, considering that CPT-11-induced gut toxicity, especially in the form of delayed diarrhea, has a high incidence and severe clinical consequences, there is a remarkable paucity of research directed towards attenuation of this form of intestinal injury through dietary intervention. Preventative dietary manipulation prior to the start of CPT-11 chemotherapy has not been tested in any preclinical studies.

Glutamine, *n*-3 PUFA and inulin/oligo-fructose prebiotics are three distinct nutrients arguably among those which are most widely studied in gut-stress/injury caused by various stressors/insults including anti-cancer drugs, and have demonstrated therapeutic benefits in settings such as inflammatory bowel disease, short bowel syndrome and certain types of refractory diarrhea. Each of these three elements is associated with suggested mechanisms, as summarized in Table 1-3. One of the major objectives of this study is to compare the relative therapeutic potential or efficacy of these three nutrients in

the identical setting of CPT-11-induced diarrhea.

1.2.5.1 Rationale for choosing glutamine as a potential modulator to CPT-11 chemotherapy

1.2.5.1.1 Overview: glutamine

Glutamine is a functionally versatile amino acid involved in a diverse range of physiological processes, i.e., interorgan nitrogen transport/exchange, pH homeostasis, regulation of protein synthesis and cell swelling (73-75). A preferential use of glutamine is characteristic of rapidly dividing cells such as enterocytes, fibroblasts, and lymphocytes (75,76), where glutamine is a vital source of ATP and an important precursor of proteins, as well as of GSH, amino sugars, purine and pyrimidine bases, and other amino acids, i.e., citrulline, arginine and proline (77). Prof. Hans Kreb's early assumptions that glutamine provided a source of respiratory fuel and nitrogen for biosynthetic reactions (78) is now firmly established and has recently been complemented by a realization that this amino acid plays diverse regulatory roles in relevant target cells (75).

1.2.5.1.2 Glutamine and cancer

Glutamine is normally considered a dietary dispensable amino acid in healthy individuals. Like several other amino acids of this category, glutamine can become essential during stress states such as cancer where demand for glutamine outstrips its synthesis from endogenous precursors. This situation is referred to as *conditional* essentiality (79). Tumors are said to be 'glutamine traps' based on several lines of evidence: 1) progressive tumor growth is associated with host glutamine depletion; 2)

tumor growth rate is correlated with tumor glutaminase activity; 3) low glutamine supply retards malignant cell growth *in vitro*. A prevalent concern arising from this notion is that exogenous glutamine supply may stimulate tumor growth, which has been documented in several *in vitro* conditions (80-82). For this reason and categorization of glutamine as non-essential amino acid, glutamine had been eliminated from total parental nutrition (TPN) formula, and with few exceptions, glutamine is present in oral and enteral diets only at relatively low levels that are characteristic of its concentration in most dietary proteins (83). In spite of such a concern, there is no evidence available suggesting glutamine supplementation would stimulate tumor growth *in vivo*. Size, protein synthetic rate, DNA content, GSH level and glutaminase activity of tumors were not affected by glutamine administration in *in vivo* tumor models (84-87). A few studies even observed an inhibition of tumor growth or enhanced tumor response to anti-cancer drugs in animals receiving glutamine treatment (24,88-92). Conversely, glutamine supplementation has been shown to correct host glutamine depletion, improve nitrogen retention and protein economy at both tissue and whole body levels, and reverse the impairment of intestinal functional and structural integrity associated with tumor-bearing state (84-86,93).

1.2.5.1.3 Glutamine and cancer chemotherapy-induced gut toxicity

Savarese *et al.* (94) and Ziegler (95) have recently reviewed the literature summarizing clinical findings with adjuvant glutamine treatment during chemotherapy and radiation. Glutamine supplementation has been extensively studied in patients receiving bone marrow transplantation (BMT) and high dose chemotherapy. High dose (defined as more than 0.2 g/(kg·day) by Melis *et al.*(96)) glutamine supplementation at 14-30 g/day, either orally or in parenteral nutrition, could increase plasma glutamine

levels, improve nitrogen balance (97), lower the incidence of intestinal infection and ameliorate GI symptoms, e.g., diarrhea and abdominal pain (98), preserve the histological and functional (intestinal permeability and absorptive) integrity of the gut and decrease the incidence of mucositis for patients receiving BMT (99-106). There has been just a little work exploring effects of glutamine supplementation in colorectal cancer patients receiving standard dose chemotherapy. Glutamine supplementation at 14-22 g/day was reported to reduce the incidence of 5-FU-induced diarrhea, improve intestinal absorption and permeability and reduce oral mucositis and ulcerations of gastric and duodenal mucosa with improvement of villous height/crypt depth ratio (100,107). Nonetheless, there is considerable variability and inconsistency in these clinical results, which may be largely attributed to widely varied doses, schedules, nature of the injury and end points investigated (108). Glutamine supplementation in CPT-11-related gut toxicity has only been documented in a small case study (109) as detailed in Chapter 2, and systematic studies on the potential role of glutamine supplementation in CPT-11-related gut toxicity and the possible mechanism are needed in this sense.

Many animal studies echo clinical findings on glutamine's protective role in chemotherapy-induced gut toxicity, although none of these have used CPT-11 so far. Most animal studies employed methotrexate or 5-FU induced enteritis models and were conducted under a chemically-defined elemental dietary background (110,111). Glutamine enriched elemental diets (1.3-2 g/(kg·day)) were shown to diminish methotrexate (or 5-FU)-induced gut injury, e.g., preventing mucosal hypoplasia and the decrease in mucosal content of protein and DNA, improving intestinal permeability and barrier function, ameliorating diarrhea and anorexia, and subsequently increasing survival rates (112-115). It should be noted that the use of elemental diet in these studies renders

them difficult to compare with their clinical counterparts as elemental diets *per se* may greatly exacerbate the basal level of chemotherapy toxicity (100,105).

1.2.5.1.4 Mechanisms of glutamine action

Precise mechanisms by which glutamine exerts its gut-protective effects in various stress conditions are not well defined. A substantial body of experimental evidence suggests glutamine's action may involve diverse regulatory processes within the cell.

Glutamine supplementation seems to affect both apoptotic and cell-proliferative signaling pathways in glutamine deprived conditions. Addition of glutamine to glutamine-starved cells can stimulate the activation of the extracellular signal-related kinases (ERK) and Jun nuclear kinase (the stress-activated protein kinase, JNK/SAPK). The activation of these two mitogen-activated protein kinases (MAPK) is followed by induction of immediate-early gene c-jun expression, which forms transcription factor AP-1 to upregulate expression of a series of target genes responsible for cell proliferation (116). Glutamine starvation enhanced JNK- or Fas-dependent apoptosis (116-118), and could lead to activation of caspase-2 and -3 (119). Therefore, glutamine supplementation may benefit cancer patients receiving major anti-cancer treatment, who are at the high risk of glutamine depletion, through the favorable modifications on the proliferation and apoptotic signaling pathways.

Another putative mechanism based on the findings in methotrexate-induced enteritis model, suggests that dietary glutamine can selectively modify the storage of the vital anti-oxidant, GSH, in tumor and normal tissues (24,120). Feeding with glutamine-enriched diet leads to lesser methotrexate-related gut injury and greater

anti-tumor efficacy. This was associated with a favorable increase in the GSH level of gut tissues and a decrease in that of tumor tissues. The increase in host GSH store could reduce the activity of redox-sensitive kinases and inhibit NF-κB-dependent inflammatory injury to the host tissue (40), and was associated with an enhanced NK cell-mediated tumor inhibition.

Recent studies suggest that potentiation of heat shock response (HSR) may be involved in glutamine's protective effects during endotoxin shock and hyperthermia, provided that it was administered in an oral or intravenous (*i.v.*) bolus manner (as extensively elaborated in Chapter 2 and 3, ref:(121,122)). Induction of heat shock proteins (Hsp), known as the hallmark of HSR, is an important innate mechanism employed by cells to protect themselves against various stressors (123,124). This finding suggests an additional mechanism underlying glutamine's function and may hint at that different glutamine-supplementation approaches seem to evoke different protective machinery. The benefits achieved by continuous feeding seems more dependent on glutamine's vital role in gut nutrition, e.g., important source of fuel, essential precursor for GSH synthesis; whereas effects exerted by the bolus approach seems more related to the up-regulation of the stress response (i.e., HSR).

Glutamine is known to modulate immune function both *in vitro* and *in vivo* via diverse effects on immune cell proliferation, antigen presentation, phagocytosis, production of cytokine, nitric oxide and superoxide (125). A requirement for glutamine was observed for expression of activation/maturation markers such as CD25, CD45RO, CD71, and for production of interferon (IFN)-γ and tumor necrosis factor (TNF)-α (77). Glutamine supplementation significantly increased *in vitro* proliferation of mitogen-stimulated blood lymphocytes from *E. coli*–infected or septicemic animals

(126,127). The superoxide anion (O_2^-) generated by NADPH oxidase initiates the cascade of reactive oxygen species production (128). These oxidants are used by neutrophils to kill invading microorganisms. Glutamine increases superoxide anion generation stimulated by phorbol myristate acetate (PMA) in rat neutrophils deprived of glutamine (129) partially due to regulation of expression of the components of NADPH oxidase (130). There are only a limited number of studies that have addressed the question of whether dietary glutamine modulates immune function in individuals receiving cancer chemotherapy. Available evidence suggests that high-dose glutamine supplementation promoted recovery of total blood lymphocyte, total CD4+ and total CD8+ numbers and enhanced the proliferative response of peripheral blood lymphocytes in BMT recipients or patients with esophageal cancer receiving radiochemotherapy (131,132). For dose-intensive chemotherapy, mucosal injury is a major contributor to the initiation and development of systemic infection by commensal organisms colonizing in the gut lumen (133). Gut-associated lymphoid tissue (GALT) forms the defense line of utmost importance in confronting gut-derived pathogens; and importantly, GALT is sensitive to alterations in luminal supply of nutritional substrates (134). Given this, research is warranted to explore the potential effect of oral/enteral glutamine supply on intestinal immunity in context of cancer chemotherapy.

1.2.5.2 Rationale for choosing *n*-3 PUFAs as a potential modulator to CPT-11 chemotherapy

1.2.5.2.1 Overview: *n*-3 PUFAs

Essential fatty acids (EFA), namely linoleic acid [18:2(*n*-6)] (LA) and α-linolenic acid [18:3(*n*-3)] (LNA), cannot be synthesized by mammalian cells and must be obtained from dietary sources (135). LA is found in most vegetable oils, especially corn, safflower and soybean oils, and meats, whereas LNA is found mainly in canola, soybean and flaxseed oils. Larger amounts of preformed long-chain *n*-3 PUFAs, i.e., eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA), can be found in fatty cold-water fish (135). In mammalian cells, the combined action of several enzymatic processes including desaturations and elongations results in production of numerous longer chain *n*-3 and *n*-6 PUFAs from the parent EFAs (135). *N*-6 and *n*-3 PUFAs have a number of vital functions in the human body (135,136). As structural phospholipids of cell membranes, they modulate membrane fluidity, cellular signaling and cellular interaction. Moreover, the 20-carbon fatty acids, EPA (*n*-3) and arachidonic acid (AA) (*n*-6) can be subsequently converted into PGs and TXs by COX; or into leukotrienes (LT) by lipoxygenase (LOX). All of these important immunoregulatory metabolites are collectively known as eicosanoids (135). Because cell membrane phospholipids normally contain much higher levels of AA than of the other 20-carbon PUFAs (137), AA is the most common eicosanoid precursor and gives rise to 2-series PGs and TXs and 4-series LTs. By contrast, EPA gives rise to 3-series PGs and TXs and 5-series LTs. *N*-6 series of eicosanoids tend to be more proinflammatory and proproliferative in most tissues than their *n*-3 counterparts.

N-3 and *n*-6 fatty acids cannot be interconverted, and they compete for the same enzymes for subsequent production of the respective *n*-3 and *n*-6 series of longer-chain PUFAs and eicosanoids as PGs, TXs and LTs. Enzymes such as desaturases and elongases

have a greater affinity for *n*-3 PUFAs (138). As a result, when dietary *n*-3 PUFA intake is high, they are preferentially metabolized and lead to a competitive inhibition of *n*-6 PUFA metabolism, resulting in decreased AA concentrations and reduced generation of *n*-6 series eicosanoids, and in the meanwhile, elevated generation of AA-derived eicosanoids (137,139-142).

1.2.5.2.2 Dietary *n*-3 PUFA supplementation in cancer

A variety of studies extensively explore the therapeutic potential and feasibility of *n*-3 PUFA (EPA and /or DHA or fish oil) supplementation to cancer-bearing individuals with regards to: 1) cancer chemoprevention (143-146); 2) anti-cancer treatment when used alone or in combination with chemotherapy (137,147-150); 3) palliative care to improve cancer cachexia (43,151-154).

N-3 PUFAs have emerged as an anti-carcinogenic nutrient and is shown to have independent inhibitory effect against the growth of certain actual tumors. They work through multiple actions to protect against cancer initiation, promotion and progression, including decreasing tumor cell proliferation, enhancing tumor cell apoptosis, promoting cell differentiation, and limiting angiogenesis, and modulating tumor-extracellular matrix interaction (155). Dietary *n*-3 PUFAs are recently reported to show therapeutic promise as adjunct to cancer chemotherapy. They can both enhance the toxicity of drugs to tumor cells at low doses and/or offer protection to non-target tissues and thus augment the therapeutic index of anti-cancer drugs (147,156,157). EPA and DHA alone or in combination from fish oil are reported to enhance the cytotoxicity of several widely used anti-neoplastic agents including anthracyclines, cisplatin, alkylating agents, tamoxifen, bleomycin, CPT-11 and 5-FU (158-160).

An association between *n*-3 PUFA supplementation and attenuation of chemotherapy-related toxicities has also been increasingly reported with anti-cancer drugs such as cyclophosphamide, arabinosylcytosine (Ara-C) and doxorubicin (156,157,161-164). Hardman *et al.* have shown some experimental evidence suggesting that fish oil may enhance tumor response to CPT-11 chemotherapy and in the meantime largely preserved the intestinal histological integrity following CPT-11 treatment (72,165). However, several issues (elaborated in Chapter 2) inherent to the work by Hardman *et al.*, i.e., endpoint selection, drug dose intensity, and control diet design (especially fat source and *n*-6/*n*-3 ratio), could hinder translation of their work into a practical clinical strategy design.

1.2.5.2.3 Mechanisms of *n*-3 PUFA action

There have been numerous reports that long-chain *n*-3 PUFA supplementation could change the fatty acid composition of tumors *in vitro* and *in vivo* (147-149,166-169), and especially decrease uptake of saturated fatty acids, monounsaturated fatty acids, and *n*-6 fatty acids (170,171). This alteration in fatty acid profiles of cell membranes forms the biochemical basis for modified chemosensitivity associated with *n*-3 fatty acid supplementation. Several inter-related mechanisms of *n*-3 PUFAs that interact with chemotherapy have been proposed: 1) facilitating cancer cells to overcome drug-resistance by increasing drug uptake (167,172-174), enhancing drug's intracellular accumulation/retention (168,174), reducing drug efflux by downregulating expression or activity of multidrug resistance related proteins such as P-glycoprotein (Pgp) (175,176); these benefits are proposed with the alterations of membrane properties associated with

n-3 fatty acid incorporation, as it has been suggested that drug transport in tumor cells is correlated with the unsaturation index in membrane phospholipids (177); 2) enhancing efficacy of prooxidant chemotherapy through oxidative stress. Enriching tumor cells with *n*-3 PUFAs significantly increases the degree of unsaturation of tumor lipids which would be expected to increase the susceptibility to lipid peroxidation and pro-oxidant therapy (147,163,177-179); 3) targeting cell signaling pathways to modulate chemo-sensitivity towards specific anti-cancer agents, such as inhibiting activity or expression of Akt or PKC to enhance chemosensitivity to tamoxifen and Ara-C respectively (180-182).

One crucial issue related to the utility of *n*-3 PUFAs in cancer chemotherapy is that its chemo-sensitizing effect has to be substantially differential between tumor and normal host cells. If sensitivity of host cells to the cytotoxic agent is also increased by altering the fatty acid composition to the same extent as malignant cells, the potential advantage of nutritional manipulation of fatty acid composition to enhance cancer chemotherapy would potentially be abolished. Mechanisms underlying the selectivity of *n*-3 PUFAs' action are poorly understood. A few lines of evidence may provide some hints on this: As cancer cells may lack in some essential enzymes, e.g., δ-6-desaturase, in forming long-chain PUFAs, they might have deficiency in long-chain *n*-3 PUFAs like EPA and therefore become vulnerable to the exogenous long-chain *n*-3 PUFA supplementation (183,184). A step wise decrease of EPA from normal mucosa, benign adenoma and cancer has been seen in the colonic mucosa (21,22). Besides, cancer cells might convert the parent *n*-3 PUFAs in a different manner from the host's normal tissue. An active NADPH-dependent ω-oxidation of PUFAs has been reported in colonic carcinoma tissue as opposed to its normal counterpart, resulting in conversion of DHA to ω-hydroxydocosahexaenoic acid

(ω-HDHA) (185). Another study suggests that the increased therapeutic index for mitomycin C in cells treated with EPA, is related to preferential incorporation of long-chain *n*-3 PUFAs into tumor cell membranes as compared to the non-malignant parent cell line (148). Additionally, greater sensitivity of the tumor to oxidative stress compared with healthy tissues can be explained by less effective antioxidative machinery in cancer cells. For instance, superoxide dismutase activity is weaker in cancer cells than in normal cells (186). Thus, accumulation of free radicals induces cell cycle arrest and apoptosis preferentially in the tumor cells (187). These facts may be attributable to the relatively high sensitivity of cancer cells to *n*-3 PUFAs.

Intriguingly, increasing experimental evidence shows that *n*-3 fatty acid treatment did not potentiate chemotherapy cytotoxicity to normal tissues, but instead, conferred considerable protection to the host against the drug toxicity (156,188). Although mechanisms of *n*-3 PUFAs' protection against chemotherapy-related injuries remain under-investigated, the ability of *n*-3 PUFAs to attenuate inflammatory injury to enterocytes has been well established in colitis models and proposed to be largely attributed to its immunomodulatory effect by altering the network of pro-inflammatory and anti-inflammatory mediators including cytokines and eicosanoids (189,190). The role of eicosanoids in the pathogenesis of CPT-11-induced diarrhea has been established by use of specific COX-2 inhibitors such as celecoxib (64), however less attention has been paid to the potential for dietary modulation as an avenue to influence chemotherapy-induced injury.

1.2.5.3 Rationale for choosing prebiotic oligosaccharides as a potential

modulator to CPT-11 chemotherapy

1.2.5.3.1 Overview: prebiotic oligosaccharides

It has been recognized that non-starch polysaccharides (i.e., the major chemically defined fraction of dietary fibre), resistant starch and miscellaneous low-digestible saccharides can escape hydrolysis by small intestine digestive enzymes and thus reach the distal bowel virtually intact and serve as substrates for flora of the large intestine (191). Prebiotics, defined as nondigestible but fermentable food ingredients that selectively stimulate the growth or activity of one or a limited number of bacterial species conferring benefits upon host well-being and health (191,192). Prebiotic fermentation is generally directed towards bacteria seen as health-promoting, with indigenous lactobacilli and bifidobacteria currently being the preferred targets (193). Examples of established prebiotics include lactosucrose, oligofructose, inulin, bran, chitosan, psyllium, and germinated barley foodstuff (GBF).

Central to the idea of prebiotic nutrition is the effect on the intestinal microflora. The role of intestinal bacteria in the pathogenesis of human inflammatory bowel diseases, particularly Crohn's disease, is well-recognized (194). The role of intestinal bacteria in the initiation and perpetuation of chronic intestinal inflammation is most convincingly demonstrated in rodent models of chronic intestinal inflammation in which genetically susceptible hosts develop spontaneous colitis in the presence of commensal intestinal organisms, whereas no disease occurs in the germ-free state (194). Furthermore, drug metabolism by intestinal microflora contributes to the pharmacological profile of various drugs. Hydrolysis of biliary drug conjugates is not only responsible for enterohepatic

circulation of a drug, which ultimately prolongs a drug's apparent half-life in systemic circulation, but is also responsible for localized accumulation of a drug into enterocytes (195,196). Examples of bacterial hydrolases which may profoundly modulate drugs' pharmacokinetics are β-glucuronidase, β-glucosidase, amide hydrolase, and arylsulfotransferase (195). Of special relevance to CPT-11 metabolism is β-glucuronidase produced by gut flora as elaborated earlier in this chapter.

Not all prebiotics are equally effective in preventing disease and their relative efficacy remains unclear (197). Inulin and oligofructose are the most studied and well-established prebiotics. In the many studies that investigated the effects of inulin and oligofructose on human gut microbiota both *in vitro* and *in vivo*, a selective stimulation of growth of beneficial flora, namely bifidobacteria, to lesser extent lactobacilli and possibly other species like *Clostridium coccoides–Eubacterium rectale* cluster known to be a butyrate producer has been reported (198,199). Feeding inulin plus oligofructose has been shown to prevent colitis in a genetically prone rat model (200). Oral inulin also decreased distal colonic lesions in rats after ingestion of dextran sodium sulphate (DSS) (201). In another induced model of colitis using trinitrobenzene sulfonic acid (TNBS) in rats intestinal inflammation was decreased by oral oligofructose (202). Dietary supplementation with inulin has also shown therapeutic promise in patients with relapsing pouchitis (203).

1.2.5.3.2 Prebiotic oligosaccharides and chemotherapy-related gut toxicity

Studies on the use of prebiotics in treating chronic inflammatory bowel diseases are relatively extensive (204,205), however research related to their therapeutic potential in

chemotherapy–induced toxicity is less known. Results from the few studies seem to be consistent with the notion that prebiotics offer protection. In methotrexate-induced entero-colitis rat model, both the severity of anorexia and the incidence of diarrhea were decreased when soybean fiber was added to a casein-based semi-purified diet (206). Supplementation of oatbase in the elemental diet decreases body weight loss, intestinal permeability, bacterial translocation and increased bowel mucosal mass (207). However, natural foodstuffs used in these studies are complex and heterogeneous in composition, comprised of several polysaccharides (e.g., soybean fiber) or even proteins (e.g., oatbase). Therefore a chemically-defined simple polysaccharide (e.g., oligofructose, inulin) is superior, in this sense, to be used for defining the therapeutic potential and mechanisms, without interference of other food components.

Prebiotics have been increasingly recognized for their anti-diarrhea effects mainly by reestablishing the gut micro-ecology, which could also be largely disrupted in the setting of chemotherapy treatment (58). In limited studies investigating prebiotic use in chemotherapy-related diarrhea, chitosan and GBF have been reported to prevent 5-FU or methotrexate-induced diarrhea (145,208). However, no study is yet to explore the potential use of prebiotics during CPT-11 chemotherapy whose gut toxicity largely depends on the action of gut flora, or to make a link between modulating bacterial enzyme activity and diarrhea severity with dietary prebiotics.

1.2.5.3.3 Mechanisms of prebiotic action

Mechanisms by which prebiotics may modulate chemotherapy-induced gut toxicity are just being unraveled and likely to be multifactorial (Table 1-3). In addition to the potential modulation of pharmacokinetics of anti-cancer drugs such as CPT-11 by altering

composition and metabolic activity of commensal flora, indeed, prebiotics may beneficially affect a series of GI functions by modulating both the structure/composition as well as miscellaneous activities of the mucosa and microflora.

1) Reversing the microbiota disruption and improve colonization resistance. Gut microflora themselves may act as barriers against invasion by potential pathogens. Bifidobacteria and lactobacilli can inhibit pathogens like *E. coli, Campylobacter* and *Salmonella spp.* (209). Lactic microflora of the human GI tract is thought to play a significant role in improved colonization resistance (193). However, chemotherapy may profoundly disturb floral balance and render potentially pathogenic bacteria to surmount colonization resistance afforded by indigenous gut microflora (210). Thus, an efficient prebiotic can favorably modify composition of microbiota with the predominance of health-promoting (probiotic) bacteria (i.e., bifidobacteria and lactobacilli), and thus inhibit the overgrowth of potential pathogens which cause secondary infectious diarrhea following chemotherapy.

2) Beneficially modulating the immune system. Prebiotics such as inulin and oligofructose could modulate function and phenotypic composition of immune cells in GALT. In mice infected with *Clostridium difficile*, oligofructose supplementation increased macrophage numbers in cecum and colon (211). Oligofructose consumption has also been shown to increase the size, number and cellularity of Peyer's Patches (PP) (145,212,213) in the upper intestinal tract. This also suggests that prebiotic fermentation in the large intestine induces changes in distant GALT compartments. Prebiotics may also directly (214-217) or, indirectly by stimulating growth of probiotic bacteria (218-220), modulate the inflammatory cytokine network and antibody production, such as inducing mucosal immunoregulatory cytokines as well as decreasing pro-inflammatory cytokine

production. For instance, oligofructose-enriched inulin supplementation significantly increased the production of IL-10 and IFN-γ in PP (221), augmented intestinal mRNA expression of IL-15 (222) and sIgA content in the cecum or feces (145,221). These prebiotics seem to exert a dual immunomodulatory effect, which confers protection against intestinal infection via boosting gut barrier (223-226) and in the meanwhile, mitigates inflammatory injury by rebalancing the pro/anti-inflammatory cytokine network (201-203,218-220).

3) Enhancing production of short chain fatty acids (SCFA) by bacterial fermentation in the large intestine. It is well established that fermentation of oligofructose and inulin increases production of SCFAs, primarily acetate, butyrate and propionate in the gut (191). SCFAs, the main energy source for colonic mucosal enterocytes, play a central metabolic role in upholding a dynamic epithelial cell barrier in the gut and to restore repair mechanisms which are likely to be essential for prevention or resolution of inflammation (227,228). SCFAs, especially butyrate, can serve to enhance post-resectional epithelial proliferation in both the small bowel and the colon (229,230). This suggests a systemic mediator might be involved in SCFA effects in the small intestine. Recent studies suggest that this mediator is probably an intestinotrophic peptide, glucagon-like peptide 2 (GLP-2) which is released upon systemic administration of SCFAs (231). Administration of GLP-2 promotes mucosal healing and improves intestinal adaptation after gut injury or resection (231-233). A recent study showed that exogenous administration of GLP-2 can markedly improve survival and attenuate intestinal injury in mice treated with lethal doses of CPT-11 (71).

Taken together, prebiotics may potentially modulate the metabolism and disposition of CPT-11 in the gut lumen, which remains uninvestigated; may beneficially modify

microbiota composition and thus limit growth and invasion of potentially harmful bacteria; favorably modulate immune and endocrine function enhancing production of gut-trophic factors such as SCFAs. Thus, their therapeutic potential as an adjuvant factor to CPT-11 chemotherapy warrants experimental exploration.

1.2.5.4 Potential interactions between different nutritional therapies

Proponents of dietary modulation of intestinal injury generally focus their interest on a single dietary element of interest in a relatively discrete manner. We lack a crucial understanding of how these different nutrients act or counteract and whether additivity or subtractivity can be achieved when they are provided in combination (234). However, it is a common and widespread assumption that greater benefits would be achieved by combining different nutrients that are beneficial on an individual basis. Compared to the considerably large and rapidly increasing number of nutrition formulae featuring combinations of various nutrients (235-237), there is disproportionate amount of rigorous and systematic experimental evidence to justify these combinations and to support the way in which these commercial products are formulated. The limited evidence available suggests these combinations may turn out to be synergistic or antagonistic in activity (238-240). For instance, EFAs may act as enhancers of beneficial actions of probiotics (241), whereas glutamine may antagonize GSH-enhancing effects of glycine supplied at the physiological level (240). As such, systematic research is warranted to evaluate the relative efficacy of nutrient combinations as compared to individual components, which could serve as a scientifically grounded rationale for maximizing therapeutic benefits through optimally combining nutrients with an individual health claim.

1.3 Cancer chemotherapy-related immunotoxicity and infection

1.3.1 Infectious complications associated with cancer chemotherapy

Cancer patients display varying degrees of immunosuppression at the time of presentation prior to initiating antineoplastic therapy (242). This immunosuppression is subsequently exacerbated by prolonged and intensive chemotherapy, which predictably suppresses the hematopoietic system, impairing host defense mechanisms against various potential pathogens (243). Patients receiving chemotherapy are at risk for a wide array of infectious diseases that cause significant morbidity and mortality (244,245). A variety of host and treatment-associated factors act together to predispose these patients to opportunistic infections.

Neutropenia is clearly the single most important risk factor for infection in cancer patients and is one of the most consistent dose-limiting factors for cancer chemotherapy including CPT-11 (2,246). Neutrophils are the first line of defense against infection as the first cellular component of the inflammatory response and a key component of innate immunity. Neutropenia blunts inflammatory responses to nascent infections, allowing bacterial multiplication and invasion (247). Quantitative and qualitative defects in innate phagocytosis usually predispose to bacterial, and secondarily, fungal infections. Cytotoxic chemotherapy can also profoundly compromise adaptive immunity via suppressing lymphopeoiesis occurring in the bone marrow, interfering with thymus-dependent T-cell development, affecting immunoglobulin production, disrupting the balance of other humoral factors such as cytokines (210,248). It is clear that deficiencies in T-cell or B-cell immune competence in cancer patients contribute to a susceptibility to infections with a wide array of bacterial, viral and parasitic pathogens (210,248). Nonetheless, given the fact 1) that more than one component of host immunity is usually impaired in the

individual patient; and 2) that innate and adaptive immune systems are highly inter-related and interactive, it is unrealistic to identify precise contribution of specific alterations in various components of host defense to specific infections caused by certain pathogens (210).

1.3.2 Intestinal mucosal injury- an important contributor to systemic infection following cancer chemotherapy

Mucosa of the GI tract serves as an important mechanical barrier that helps to prevent a local or systemic invasion of various microbes and absorption of microbial products that are normally present in the oral cavity and lumen of the gut (249). Derangement in the barrier function of the GI tract plays a central role in the pathophysiology of systemic infection, shock, and sepsis syndrome associated with cancer chemotherapy. The GI tract is a major source of bacteria in patients who develop systemic infection as a result of chemotherapy (250). Bacteremia from gut colonizing gram-negative bacilli has been the most problematic opportunistic infection in chemotherapy-induced neutropenia (133). In spite of the fact that the incidence of gram-negative bacterial infections in neutropenic patients has decreased over time possibly due to the empiric use of systemic broad-spectrum antibiotics in neutropenic patients (251), gram-negative rods such as *P. aeruginosa* continue to cause a disproportionate degree of morbidity and mortality in this patient population (252-254). Currently, bacteremia caused by gram-positive organisms is becoming more common (251,255). Substantial proportions of these gram-positive bacterial pathogens are viridans group streptococci (256). Mucosal lesions in the digestive tract form the most probable portal of entry for viridans streptococci that cause bacteremia (257-260). Infection caused by vancomycin-resistant enterococci (VRE) is

rapidly increasing in hospitalized patients and are associated with considerable morbidity (261). Mucositis has been implicated as a possible contributory factor associated with invasive VRE infection (262). Additionally, invasive infections caused by fungi such as *Candida* and *Aspergillus spp.* are frequent in patients undergoing cancer chemotherapy that results in prolonged neutropenia and after BMT (263). Pathogenesis of invasive fungal disease is also suggested to be linked to chemotherapy-related gut epithelial damage in the setting of fungal colonization of the gut (264,265).

The presumed mechanism for establishing systemic infection in cancer patients involves initial intestinal colonization of microorganism with subsequent translocation through viable mucosal barrier into mesenteric lymph nodes (MLN), and further to bloodstream (266-269). The combination of an intact intestinal mucosa and a normally functioning immune system provides adequate barrier function against translocation of the gut colonizing bacteria and their endotoxins. The intestinal immune system, also known as GALT which includes PP, lymphoid cells located within the intestinal lamina propria, intraepithelial lymphocytes, and aggregated lymphoid tissue within MLNs, is of utmost importance in protecting the host from invasion of gut-derived microorganisms and their pathogenic products. By contrast to the large body of studies directed towards understanding the immunosuppressive role of chemotherapy in systemic immunity represented by compartments such as peripheral blood (in human) and spleen (mostly in animals), how cancer chemotherapy impacts GALT, the first line of defense against gut-originated infection, is largely uninvestigated.

1.3.3 Prophylactic anti-bacterial treatment in cancer chemotherapy

Treatment of chemotherapy-related infection has improved significantly since the

1970s when empiric antibiotic therapy was introduced at the onset of febrile neutropenia (270). In recent years, the choice of antibiotics has changed a lot, but administration of parenteral broad spectrum antibiotics is still standard of care (271). Even though infection-related mortality has decreased substantially (approximately 4–6% in adult patients and 0.4–1.0% in pediatric patients (271-273)), prevention of infection remains extremely important in patients receiving chemotherapy.

The concept of chemoprophylaxis of bacterial infection in cancer patients was first developed by van der Waaij and colleagues (274), who used a strategy described as 'selective decontamination of the digestive tract' (SDD) to eliminate potentially pathogenic aerobic bacteria from the GI tract without affecting nonpathogenic anaerobic flora. Non-absorbable antibiotics were administered before the onset of neutropenia to achieve optimal eradication of potential pathogenic aerobic micro-organisms in the digestive tract. As the pattern of infectious microorganisms has changed significantly over time, the non-absorbable regimens have to a large extent been superseded by absorbable antibiotics alone or as a hybrid with non-absorbable ones to provide systemic antimicrobial concentrations while effecting local decontamination of the gut. Prophylaxis with co-trimoxazole (Trimethoprim/sulfamethoxazole; TMP/SMZ) has shown benefit in reducing rates of infection caused by enteric gram-negative rods and certain gram-positive pathogens in neutropenic patients (275-277). Despite reported successes of TMP/SMZ, several disadvantages were associated with its use, including prolonged duration of neutropenia (278,279), and occurrence of infections with resistant Gram-negative bacteria (280) and its lack of activity against certain bacteria such as *P. aeruginosa* (281).

In the 1980s, fluoroquinolones were considered more promising drugs as

prophylactic antibiotics since they had an increased activity against gram-negative bacteria. Moreover, compliance was better and they were not as myelosuppressive as TMP/SMZ (282). The activity spectrum of quinolones includes the most common causes of infection due to gram-negative bacilli, *S. aureus*, and many of the coagulase-negative *staphylococci* (283,284). Of all quinolones studied so far, only ciprofloxacin (Cipro) offers the most complete protection against gram-negative bacilli, including *P. aeruginosa* (285-287). Meta-analysis results showed a significant reduction in gram-negative bacteremia and a decrease in infection-related mortality was associated with the use of quinolone-based prophylactic regimens (282,288).

Despite the established therapeutic benefits of prophylactic quinolone regimens, an important concern against their prophylactic use is the development of antibiotic resistance (210,289). However, there is no robust evidence suggesting that patients treated with quinolones have a significant increase in colonization by quinolone-resistant bacteria (290). Risks associated with colonization and infections caused by quinolone-resistant organisms do not seem to outweigh the overall benefit in reducing infection rates, all-cause mortality and infection-related mortality (290).

1.3.4 Immunomodulatory effects of quinolones

Evidence is accumulating that, in addition to the intrinsic antibacterial activity, an important mechanism of quinolones' action *in vivo* is through interacting with host defense mechanisms and modulating interactions between bacteria and host immune cells. These immunomodulatory effects could take place at multiple levels including modulating phagocytosis, altering cytokine production and promoting hematopoiesis (291-296). Despite the fact that quinolones such as Cipro and rufloxacin are inactive

against *Bacteroides fragilis in vitro*, these agents could effectively eliminate this pathogen from infected animals associated with a modulation of TNF production, suggesting that their antibacterial activity *in vivo* largely relies on their immunomodulatory effect (297-300). Quinolones have been shown to protect mice from lethal or sublethal endotoxin shock, accompanied by a reduction of TNF and IL-12 circulating levels or production in response to lipopolysaccharhide (LPS) stimulation and enhancement of IL-10 circulating levels (301-304). However, these studies were performed in immunocompetent animals. No studies have thus far been conducted to relate quinolone's efficacy to their potential immunomodulatory effects in immunocompromised hosts. Cancer chemotherapy calamitously represses hematopoiesis and damages the integrity of host defense mechanisms, and thus puts the host at high risk of opportunistic infection by various pathogens. Given that quinolones are the most widely used oral antibiotics for treating or preventing chemotherapy-related infections, experimental studies are warranted to elucidate their immunomodulatory effects on hosts facing the dual immune challenges from intensive cancer chemotherapy and infectious pathogens/agents.

1.4 Premise, significance, scope and overall features of this thesis research

Systemic chemotherapy has long been a cornerstone for antineoplastic therapy. However, cancer chemotherapy toxicity is the single most important factor limiting the use of drugs that might more often be curative if higher doses could be used. Use of adjuvant factors including chemosensitizers and toxicity antagonists is an important approach to modify the therapeutic index of chemotherapeutic agents available. Exploration on the therapeutic adjuncts to cancer chemotherapy is traditionally recognized as a pharmaceutical area. Certain nutrients naturally occurring in the diet

could potentially be an important and promising complement to pharmaceutical and biological approaches for seeking chemotherapy modulators of therapeutic promise. The premise of this project is that modulation of therapeutic index of cancer chemotherapy, emerging as an important issue of clinical oncology and supportive care, is well suited for a systematic program of research adopting a nutritional perspective.

In overall, **this research was aimed to** explore whether certain nutritional elements, namely glutamine, *n*-3 PUFAs and prebiotic oligosaccharides, could potentially affect treatment response to CPT-11 chemotherapy in a favorable manner with enhanced anti-cancer efficacy or/and reduced gut toxicity and related systemic consequences.

This research used CPT-11-based chemotherapy as a drug model, which is characteristic of a common, predictable and substantially injurious assault on the GI system. Such a drug model not only provides a platform for focused investigations targeted on gut-related toxicity or injury, but also would typically reflect the complex crosstalks between different forms of toxicities (e.g., gut toxicity and immunotoxicity, which are closely interrelated and contributes altogether to the pathogenesis of systemic infection) and the potential critical consequences (i.e., sepsis and multiple organ failure) derived from these interactions at the systemic level.

Treatment response to CPT-11 chemotherapy was evaluated in a rat model with the transplantable Ward colon tumor, which was specifically developed with a view to clinical assessment of CPT-11-induced diarrhea and also allowed for evaluation of other clinical endpoints at different levels including tumor growth, immunity, infection and malnutrition. Mechanistic studies will be directed towards potential modulation of cytoprotective machinery (i.e., HSR and GSH), intestinal and systemic immune competence, and drug-nutrient metabolic interaction. Different from previous studies of

this kind which mostly focus on a single nutrient in a relatively discrete manner, this study adopts a longitudinal perspective rigorously comparing the relative efficacy of different nutrients, and moreover exploring the potential interaction between different nutrients (glutamine and *n*-3 PUFAs). This work will hopefully lend translatable information and compatible rationale for clinical strategy-design aimed to optimally incorporating dietary adjuncts to cancer chemotherapy regimens.

This thesis work is composed of **four** interrelated individual sub-studies which have their own discrete emphases on addressing specific hypotheses and objectives:

Chapter 2. Main theme: Horizontal comparison of different nutrients' therapeutic potential in modulating CPT-11 chemotherapy.

Dietary factors have been suggested to influence cancer progression, tumor response to anti-neoplastic treatments, and treatment-related toxicities to host tissues; however, work in this area to date has not rested upon a standardized dietary design. Controlled comparisons of different dietary elements in an identical setting of chemotherapy regimen and basal/background diet are not available. Here we proposed a controlled pre-clinical model with a tumor chemotherapy combination (Ward colon treated with CPT-11) established for the study of treatment–induced diarrhea and testing of new therapies for CPT-11-induced-injury, upon a platform of standardized basal diet with features comparable to typical North American diet. Dietary interventions were selected from dietary elements already established to modify other forms of intestinal injury and which have been hypothesized to mitigate chemotherapy-induced injury (glutamine, *n*-3 PUFAs and prebiotic oligosaccharides).

Hypotheses: 1) CPT-11 treatment induces substantial tumor growth inhibition as well as severe delayed diarrhea in Fisher rats bearing Ward colon tumor;

2) Glutamine, long-chain *n*-3 PUFAs and prebiotic oligosaccharides will provide therapeutic benefits to CPT-11 chemotherapy individually, by protecting against CPT-11-induced diarrhea or/and enhancing anti-tumor efficacy of CPT-11 chemotherapy;

3) Efficacy of glutamine in preventing CPT-11-induced diarrhea is dependent on the schedule or/and route of administration;

4) Prebiotic oligosaccharides can modulate CPT-11's metabolism in the gut lumen and thereby affect the gut toxicity related to the drug.

Objectives: 1) To characterize the profiles of both anti-tumor efficacy and diarrhea toxicity in a preclinical model which is clinically relevant and comparable in terms of chemotherapy's dose intensity (MTD) and anti-tumor efficacy as well as severity and kinetics of diarrhea toxicity.

2) To test whether treatments of glutamine, *n*-3 PUFAs and prebiotic oligosaccharides can individually alter the incidence and severity of CPT-11-induced diarrhea and change CPT-11's inhibitory effect on Ward tumor growth in the rats

3) To test whether different glutamine modalities (bolus feeding vs. continuous feeding by incorporating glutamine in the diet; oral bolus vs. *i.v.* bolus) can equally affect CPT-11-induced delayed diarrhea

4) To test whether activity of intestinal β-glucuronidase could be altered with prebiotic treatment and furthermore, whether this change is correlated its effect on CPT-11-induced diarrhea.

Chapter 3. Main theme: Mechanistic study of the action of bolus glutamine

This chapter focuses on the mechanistic investigations of bolus glutamine's gut-protective effect during CPT-11 chemotherapy. Incorporation of amino acids in diets is a conventional approach in experimental nutrition studies; however this feeding paradigm did not show a clear ability to alter severity of late diarrhea (Chapter 2). By contrast, bolus glutamine treatment was shown here, to substantially mitigate the incidence and severity of CPT-11-induced late diarrhea. One objective of this study was to relate effect of bolus glutamine to potentiation of innate cytoprotective HSR, which has been reported to be involved in the action of bolus glutamine during septic and hyperthermic stresses (121,122) but has not previously been investigated in the context of chemotherapy-induced injury. In addition to this, the ability of bolus glutamine to mitigate CPT-11-induced diarrhea was further related to other mechanisms occurring on various levels (i.e., GSH, intestinal immunity, apoptosis and bacterial β-glucuronidase activity), which have or have not yet been proposed for glutamine's action based on the literature.

As discussed earlier, the prerequisite for the utility of nutritional modulators to reduce treatment toxicity is that the nutrient should exert a substantially differential effect on the host and tumor. There is a highly prevalent concern that glutamine provided at high doses would 'feed the tumor' and support tumor cell proliferation. A major aim of this study was to investigate whether glutamine treatment, in the co-existence of its gut-protective effect, could also confer protection to the tumor against cytotoxic chemotherapy in association with activation of certain cytoprotective machinery such as GSH or HSR in the tumor tissue.

Hypotheses: 1) Protective effects of bolus glutamine treatment on CPT-11-induced diarrhea are associated with potentiation of HSR in the gut tissue;

2) Bolus glutamine treatment may also affect multiple other mechanisms such as GSH, apoptosis, gut immunity and drug metabolism in the gut lumen that would in overall favor its protection against CPT-11-related gut injury;

3) Bolus glutamine treatment does not affect efficacy of CPT-11 chemotherapy or modulate the tumor cytoprotective machinery in the same manner as in host tissues.

Objectives: 1) To test whether bolus glutamine can modulate expression of inducible Hsps (Hsp-27,-70 and -90α) and correlate this with its effects on CPT-11-induced diarrhea;

2) To test whether bolus glutamine could also affect intestinal GSH stores, epithelial apoptosis, phenotypic composition of MLNs and luminal β-glucuronidase activity, and correlate these with its therapeutic benefit on CPT-11-related gut toxicity;

3) To test whether Hsp expression and GSH stores in tumor tissue can also be affected by bolus glutamine treatment and correlate this to its effects on tumor response to CPT-11 chemotherapy.

Chapter 4. Main theme: Comparison of single and combined supply of glutamine and *n*-3 PUFAs

Chapters 1 and 2 have shown that anti-tumor efficacy and diarrhea toxicity related to CPT-11 mono-chemotherapy could be modulated by single supply of *n*-3 PUFAs and glutamine respectively. In this study, I will further examine how these two dietary elements, when provided individually and in combination, would affect the treatment response related to a cyclical regimen of CPT-11/5-FU, the 1st line combination chemotherapy for colorectal cancer management. Findings based on this combination regimen would provide a more comparable rationale for clinical strategy design of

adjuvant nutritional treatments for colorectal cancer chemotherapy.

As discussed earlier, one question that has rarely been addressed is the potential for additivity, synergy or subtractivity when two dietary factors, which are individually beneficial in certain settings, are used in combination. In the case of *n*-3 PUFAs or glutamine, most of the research available only focused on individual effects of these two factors. That a greater benefit could be achieved by combining them is a commonly held assumption, and notably, there already exist some enteral formulae featuring combinations of glutamine and *n*-3 PUFAs (235-237). However, evidence addressing the relative efficacy of such a combination is lacking. A major objective of this study is to assess the therapeutic potential of a combination *n*-3 PUFAs and glutamine and to investigate their potential interactions in the setting of CPT-11/5-FU chemotherapy.

Hypotheses: 1) Both *n*-3 PUFAs and glutamine could individually modulate treatment response to cyclical CPT-11/5-FU chemotherapy in a favorable manner, by which chemotherapy's anti-tumor efficacy or/and host's tolerance is augmented;

2) Single supply of *n*-3 PUFAs or glutamine per se could exert therapeutic benefits to tumor-bearing host by affecting tumor growth or/and ameliorate immunodefects inherent to tumor-bearing state;

3) When *n*-3 PUFAs and glutamine are provided in combination, greater therapeutic benefits could be achieved in terms of tumor or/and host's response to CPT-11/5-FU chemotherapy.

Objectives: 1) To test whether dietary supply of *n*-3 PUFAs and glutamine could individually alter Ward colon tumor growth kinetics and peripheral blood leukocyte counts prior to chemotherapy initiation;

2) To test whether supply of *n*-3 PUFAs and glutamine could individually modify

effects of CPT-11/5-FU chemotherapy on both tumor (i.e., growth kinetics) and host (i.e., survival, body weight loss, anorexia and muscle wasting), and to relate these clinical endpoints to changes in tumor and host GSH stores;

3) To test effects of combined supply of *n*-3 PUFAs and glutamine on the aforementioned endpoints in comparison with those associated with single supplies of these two nutrients and investigate possible interactions between them.

Chapter 5 Main theme: Effects of high-dose CPT-11 on systemic and intestinal immunity and its modulation by Cipro.

Infectious complications are a major contributor to the morbidity and mortality associated with dose-intensive cancer chemotherapy. Investigations on chemotherapy-related suppression and reconstitution of immune function have been largely confined to the systemic immune compartments such as peripheral blood. However, given that compromised gut mucosal integrity is key to systemic infection secondary to cancer chemotherapy and GALT serve as the first-line defense confronting insults from gut-derived pathogens, information regarding how systemic chemotherapy would potentially affect gut mucosal immunity is minimal.

Cipro, a quinolone antibiotic, is prevalently used in prophylaxis of opportunistic infection in high-risk neutropenic patients. In addition to its intrinsic antibacterial activity, potential immunomodulatory effects of Cipro on chemotherapy-treated immunodeficient hosts are poorly defined. This study aims to investigate effects of high-dose CPT-11 chemotherapy on gut and systemic immune competence. With the use of Cipro, the role of opportunistic bacterial infection in the overall CPT-11-related toxicity profile could be isolated. Furthermore, potential modulatory effects of Cipro on systemic and intestinal

immunity in hosts receiving dose-intensive CPT-11 chemotherapy are investigated.

Hypotheses: 1) In addition to severe diarrhea, systemic infection is a key contributor to the overall CPT-11-related toxicity profile;

2) Dose-intensive CPT-11 chemotherapy considerably affects both systemic and gut immune competence;

3) Prophylactic Cipro therapy will significantly improve host tolerance and ameliorate diarrhea toxicity and the overall toxicity profile related to dose-intensive CPT-11 treatment;

4) Cipro treatment will significantly modulate CPT-11's effects on systemic and gut immune function.

Objectives: 1) To compare CPT-11-related toxicity profiles (mortality, delayed diarrhea, weight loss, muscle wasting and bacterial translocation) in absence and presence of prophylactic Cipro treatment;

2) To test effects of Cipro treatment on β-glucuronidase activity and relate this to its effects on diarrhea;

3) To compare CPT-11-induced alterations in phenotypic composition, activation/maturation and functional integrity (proliferation and cytokine production upon mitogen stimulation *in vitro*) of immune cells in systemic and intestinal immune compartments (spleen vs. MLNs) in the absence and presence of Cipro treatment.

TABLES

Table 1-1. Examples of dietary modulators for cancer chemotherapy tested in experimental studies

Dietary elements		Increased efficacy	Decreased toxicity
glutamine		*methotrexate (chemically induced fibrosarcoma) (24,92)	gut toxicity related to methotrexate and 5-FU (92,100,112,113,115); doxorubicin-related cardiotoxicity (305); cisplatin-related renal toxicity (306)
n-3 PUFAs		doxorubicin (A-549 human lung carcinoma) (164), epirubicin (chemically induced rat mammary carcinoma) (150) cisplatin (3LL Lewis lung carcinoma) (307), mitomycin C (MX-1 human mammary carcinoma) (149), cyclophosphamide (MX-1 human mammary carcinoma) (183) CPT-11 (MCF-7 human breast carcinoma) (163) Ara-C (L-1210 murine leukemia) (162)	CPT-11-related gut toxicity (72) and myelosuppression (165), Ara C-related gut toxicity (156) , cyclophosphamide-related systemic toxicities (161)
Dietary fibres			methotrexate and 5-FU related gut toxicity (145,206-208)
vitamins	Vitamin C		cisplatin-related renal toxicity (308)
	Vitamin E		doxorubicin-related cardiotoxicity (309-312) cisplatin-related neurotoxicity (313)
	Co-enzyme Q10 (Ubiquinone)		doxorubicin-related cardiotoxicity (314-316)
minerals	zinc		methotrexate-induced gut toxicity (317)
	selenium	doxorubicin (doxorubicin-resistant human lung cancer cell line) (318), CPT-11 (human head and neck and colon carcinoma xenografts) (319,320)	doxorubicin-induced cardiotoxicity (321)
Flavonoids		cisplatin (human ovarian and breast cancer cell lines) (322), doxorubicin (doxorubicin-resistant P388 murine leukemia) (323)	doxorubicin-related cardiotoxicity (324)

* chemotherapy (the tumor system in which the combination of chemotherapy and dietary constituent was examined)

Table 1-2. Clinical and experimental approaches to manage CPT-11-related gut toxicity and the respective targeted mechanism

	Mechanisms	**Clinical/experimental managements**
Structural integrity loss	Decrease in crypt cell proliferation and increase in villous cell apoptosis (55,56,59-61)	Enterotrophic hormones, e.g., GLP-2 (71)
Functional changes	Up-regulated cholinergic activity through inhibition of acetylcholinesterase (53)	Loperamide, Atropine (26,34,48,50)
	Increase production of pro-inflammatory factors, e.g., PGE_2, TXA_2 (62-64)	COX-2 inhibitor (64)
	Increase in production of β-glucuronidase by colonic microflora resulting in conversion of SN-38G to SN-38 (52,58)	Non-absorbable antibiotics (53,70,325)
	Biliary excretion of CPT-11 (41,53)	Cyclosporin (67)
	Impaired intestinal immunity makes intestinal mucosa more predisposed to infection by aerobic bacteria, which might be a direct but non-specific cause for diarrhea (65)	Immunomodulator e.g., IL-15 (59)

Table 1-3. Suggested mechanisms of modulation of intestinal functions by dietary factors

Dietary factors	Mechanisms
Glutamine	1) Preferred energy fuel for enterocytes (75,76) 2) Precursor for GSH biosynthesis (24,120) 3) Hsp induction (121,122) 4) Regulate apoptotic/proliferative signaling (116-118) 5) Modulate intestinal and systemic immunity (112,125-127,131)
n-3 PUFAs	1) Attenuate intestinal inflammatory injury via affecting the pro-/anti-inflammatory cytokine/eicosanoid network (189,190) 2) Building block for tissue repair (as needed for membrane synthesis and cellular replication) (157)
Prebiotics (Inulin, Oligo-fructose)	1) Enhancing production of SCFAs and intestinotrophic hormones e.g., glucagon-like peptide 2 (GLP-2) (191,231) 2) Modulate intestinal anti-/pro-inflammatory cytokine network (214-217) 3) Boosting gut barrier function (223-226) 4) Growth inhibition of luminal as well as mucosa-associated pathogenic bacteria (191,193,209) 5) Potentially affecting β-glucuronidase activity (53,193,326)

FIGURE

Figure 1-1.

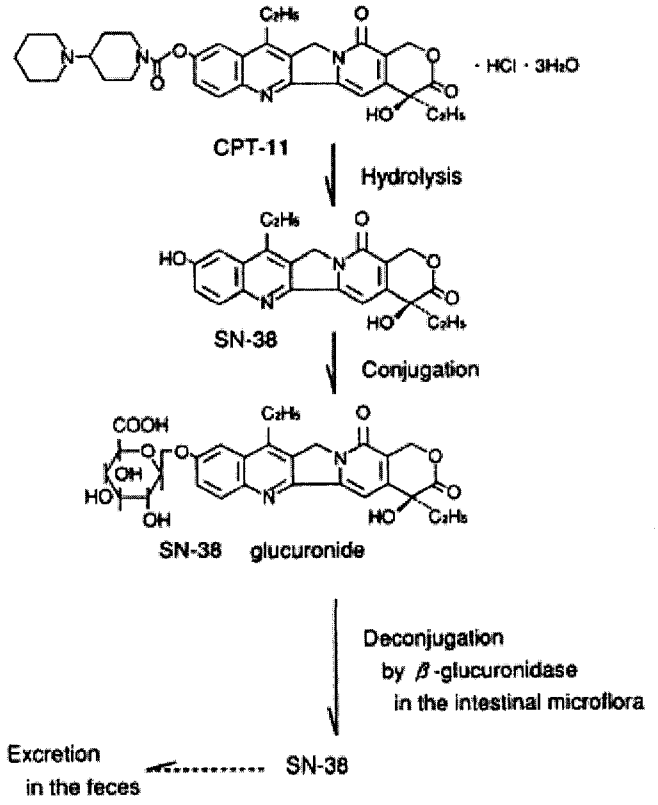

Figure 1-1. Metabolic pathway of CPT-11 (Reprinted from Takasuna *et al.* (52))

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CHAPTER 2 NUTRITIONAL MODULATION OF ANTI-TUMOR EFFICACY AND DIARRHEA TOXICITY RELATED TO CPT-11 CHEMOTHERAPY IN RATS BEARING THE WARD COLON TUMOR

2.1 Introduction

CPT-11 has emerged as a first-line drug for colorectal cancer and has also been shown to be effective in other malignancies (1,2). As elaborated in Chapter 1, use of CPT-11 is mainly limited by its GI toxicity with diarrhea as its dose-limiting toxicity; β-glucuronidase produced by intestinal microflora, which reconverts SN-38G to SN-38, is believed to play a critical role in mediating SN-38-related gut toxicity (3,4).

One approach to improve the therapeutic index of a chemotherapeutic agent is to combine adjuvant factors to enhance anti-tumor efficacy and/or to reduce toxicities. Specific dietary elements have been attracting attention in this context, including the amino acid glutamine, *n*-3 PUFAs such as EPA (C20:5*n*-3) and DHA (C22:6*n*-3), and prebiotic oligosaccharides. Prebiotic is the term used to define non-digestible fermentable food ingredients which stimulate growth of selected intestinal bacteria, (i.e., lactobacilli, bifidobacteria), that are important to the host's health (5). These nutrients have been clearly established to mitigate inflammation and tissue injury in chronic inflammatory bowel disease (6-8) and have been proposed to be potential modulators of gut-injury related to cancer chemotherapy (9-14). Various mechanisms of these effects have been proposed as dietary influences may be exerted on multiple levels including the physiology of cells and organs, signal transduction within cells and the endocrine, immune and GI systems. However, little is known about the potential capacity of these dietary factors to modulate CPT-11-induced diarrhea. Prebiotics have attracted attention for managing

diarrheas in other intestinal injuries that are related to disruption of intestinal flora balance. In the specific case of CPT-11, it is unknown how prebiotic oligosaccharides may alter intestinal flora composition and levels of β-glucuronidase produced by intestinal bacteria, thereby influencing SN-38G metabolism.

Effects of diet on tumors and host response to therapies remain poorly defined. The existing literature is hard to interpret due to a lack of a systematic approach. For any specific nutrient, the available literature encompasses diverse animal models, therapies, nutrient levels and basal (background) diets, making it quite daunting to perform a systematic interpretation that could be applied to humans. Almost all studies examined a single nutrient or nutrient class, so that the relative efficacy of different nutrients remains completely unknown. Application of clinically relevant anti-neoplastic therapy within a nutritionally relevant and controlled dietary design is required to move understanding forward in this area. The objective of our study was to comparatively assess the ability of glutamine, *n*-3 PUFAs and prebiotic oligosaccharides to alter CPT-11's efficacy and to mitigate its dose-limiting toxicity, diarrhea. Emerging debates have revolved around the scheduling and routes of glutamine administration (15,16) and we further compared glutamine incorporation in the diet, with oral and *i.v.* bolus administration.

2.2 Materials and methods

2.2.1 Features of the model system

The experimental system included an implanted tumor, CPT-11 chemotherapy (MTD as described by Cao *et al.* (17)), and specifically formulated basal diet and diet treatments. We selected CPT-11 chemotherapy in rats bearing the Ward colon tumor (17), which is particularly applicable for study of severe late-onset diarrhea, the clinically relevant dose-limiting toxicity. Kinetics of acute and delayed diarrhea is clearly observed in the rat and is consistent with those observed in patients treated with CPT-11 (18). Tumors were subcutaneously (*s.c.*) introduced to enable the assessment of rate of tumor growth.

Semi-purified diets permit definition of the lipid, protein and carbohydrate constituents of the diet. Our conception of dietary design in animal models is spelled out in detail in our recent publication (19). In brief, diets are formulated to meet or exceed nutrient requirements of laboratory rats and are based on the American Institute of Nutrition (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 (40% of calories, polyunsaturated: saturated fat ratio of 0.35) (Table 2-1) (19). CPT-11 toxicity in this dietary background is similar to what has been reported for chow-fed animals.

Savarese *et al.* (9) reviewed possible prevention of chemotherapy toxicity by glutamine. Since several recent studies demonstrated that glutamine bolus treatment rescued rats from lethal endotoxin shock or hyperthermic stress (20,21), we opted for the bolus dose used in those investigations (0.75 g/(kg·day)). Other experimental treatments were incorporated in the diet (Table 2-1). A glutamine-containing diet (2%, wt:wt) was initiated 7 days prior to the first dose of CPT-11 and continued thereafter; this was

calculated to provide an identical daily amount of glutamine as was provided in the bolus treatment. Control diets contained 1.1 % (wt:wt) of total fatty acids as *n*-3 and met EFA requirements of laboratory rats. Marine fish oil was used to formulate a diet containing 5% (wt:wt) total fatty acids as *n*-3; including 3.2% EPA and 0.8% DHA (Table 2-1). To allow for adequate incorporation of *n*-3 PUFAs into plasma membranes, diet treatment was started 14 days prior to tumor implantation and continued thereafter. We also studied a chickory-derived prebiotic mixture consisting of inulin and oligofructose (1:1 wt:wt) at doses that reduced chronic colitis in HLA-B27 transgenic rats (22). Prebiotic treatment was initiated 14 days prior to tumor implantation, which is sufficiently long to increase beneficial flora, such as bifidobacteria and lactobacilli (23,24), and was continued thereafter.

2.2.2 Rats, tumors and drugs

Rat use was reviewed and approved by the Institutional Animal Care Committee and conducted in accordance with Guidelines of the Canadian Council on Animal Care. Female Fisher 344 rats (body weight, 150–180 g), 11-12 weeks of age, were obtained from Charles River (QC, Canada). Rats were housed 2 per cage under aseptic conditions (positive-air-pressured room, cages, bedding and filter tops; handling under a laminar flow hood) in a temperature (22°C) and light controlled (12 h light) room; water and food were available for *ad libitum* consumption. One week before chemotherapy rats were separated into individual housing in wire-bottom cages. The Ward colorectal carcinoma was provided by Dr. Y Rustum, Roswell Park Institute (17). Tumor pieces (0.05 g) were transplanted *s.c.* into the left flank of rats via trocar under slight isoflurane anesthesia. A major consideration in selecting this tumor site was to facilitate continuous evaluation of

tumor growth and response to CPT-11 treatment.

CPT-11 was provided by Pfizer as a ready-to-use clinical formulation (20 g/L). Atropine (0.6 g/L), obtained from the hospital pharmacy, was in a clinical injectable formulation.

2.2.3 Experimental design

Acclimation to the semi-purified diets was initially with laboratory rodent chow blended with our control diet (50/50 wt:wt) for one week, followed by allocations to the test diets (control, *n*-3 PUFA, prebiotic, glutamine). When tumors reached ~2.0 cm^3 CPT-11 injections were initiated. The day when the first dose of CPT-11 was administered is designated Day 0.

2.2.3.1 Determination of CPT-11 MTD

Tumor-bearing rats fed the control diet were randomized into 4 groups, each of which received three consecutive daily *i.v.* CPT-11 injections at 75 (n=5), 100 (n=9), 125 (n=21) and 150 (n=7) mg/(kg·day). Seven days after the last dose of CPT-11 (Day 9), rats were killed.

2.2.3.2 Study on the dietary modulation of the anti-tumor efficacy and toxicity related to the 3-day CPT-11 treatment at MTD

Tumor-bearing rats were fed control (CON), *n*-3 PUFA enriched (FO), glutamine-enriched (GLN) and prebiotic-enriched (PRE) diets described in Table 2-1. In addition to the diet, each rat was given a bolus treatment (glutamine or sham). Bolus treatments were by oral gavage or *i.v.* infusion, and consisted of glutamine (Sigma-Aldrich, St. Louis, MO) or the same solution (sham) only. Rats treated with oral or *i.v.* bolus glutamine were fed the control diet throughout the study. Glutamine oral

bolus (0.75 g/kg) was administered by gavage 30 minutes before each daily CPT-11 injection, while rats from other groups received isovolemic water as sham. Oral glutamine 3% (wt:v) solution was made immediately before use, and was filtered with a 0.45-μm filter. Glutamine *i.v.* bolus (0.75 g/kg) was prepared immediately before use as a 3% (wt:v) solution in lactated Ringer solution and filtered with a 0.45-μm filter. Intravenous bolus glutamine was administered via the lateral tail vein of anesthetized rats at a rate of 0.5 ml/min, immediately before each daily dose of chemotherapy. All three modes of glutamine delivery (diet, *i.v.*, and gavage) provided a total daily dose of 0.75 g/(kg·day).

Eleven rats were allocated to each experimental treatment. At Day 9, rats were killed and tissues/organs were collected. A separate set of rats (5 from the control; 6 from oral bolus glutamine treatment) were killed 6 hours after the last injection of CPT-11, colonic mucosa, tumor and whole blood were harvested from these rats for assay of glutamine levels.

2.2.4 Outcome measures

Diarrhea was scored: 0 - normal, normal stool or absent; 1 - slight, slightly wet and soft stool with mild perianal staining; 2 - moderate, wet and unformed stool with moderate perianal staining of the coat; and 3 – severe, watery stool with severe perianal staining of the coat (25). Diarrhea was scored twice daily until Day 7 and daily thereafter. Data are presented as the incidence of diarrhea grade 3, total incidence of diarrhea grade 2 & 3 (25) and area under curve of diarrhea score. Incidence of delayed diarrhea was calculated for each rat by counting observations of a particular score(s) out of the total eight observations between Day 3 and Day 7 when diarrhea developed to its full severity (25). Area under curve of diarrhea score was calculated from the diarrhea score-time

graph of each individual rat between Day 3 and Day 7. All diarrhea severity assessments were conducted by one person who was blinded to experimental treatments.

Body weight was monitored daily. Tumors were measured at time points indicated in the figures, in three dimensions with a caliper, the length (*L*), width (*W*), and height (*H*). Tumor volume was calculated according to the following equation: tumor volume (cm^3) = 0.5 × *L*(cm) × *W*(cm) × *H*(cm) (26). Tumor volume doubling times were calculated as described (27). Relative tumor volume (*R*) for each tumor was calculated relative to its volume at the start of chemotherapy. Interactions between diet and CPT-11 was expressed as a ratio of relative tumor values between a given tumor (*R*) and the mean of all control tumors (RC_m), the formula of which is ($R/RC_m \times 100\%$). Tumor growth inhibition was expressed as 1- ($R/RC_m \times 100\%$). When assessing effects of diet alone on tumor growth, absolute volume value (*V*) was used in place of R because an equal amount of tumor tissue was assumed to be implanted into each rat.

Rats were killed by CO_2 asphyxia followed immediately by exsanguination by cardiac puncture. Aliquots of heparinized whole blood were centrifuged at 4°C at 3000g for 15 min. Plasma was removed and frozen at -70°C for later assessment of amino acid and fatty acid profiles. Tumor and tibialis anterior muscles were collected, weighed and then immediately frozen in liquid nitrogen. The full length of the colon (after the ceco-colonic junction) was longitudinally cut into 2 halves, one of which was mounted on a wax strip and fixed in 10% (v:v) neutral buffered formalin. Cecal contents, which were collected in an aseptic condition for β-glucuronidase assay, as well as mucosal tissue scraped off from the first 6-cm section of the other half of proximal colon, were collected and immediately frozen in liquid nitrogen.

Glutamine/glutamate concentrations were determined using high performance liquid

chromatography (HPLC). Amino acids were converted to their *o*-phthaldialdehyde derivatives using established methods (28). Calibration was done every 10 samples using a commercially prepared standard. All samples were run in duplicate and averaged.

Plasma triglycerides were separated and fatty acid methyl esters were prepared from the scraped silica band using 14 % (wt:v) BF_3/methanol reagent and separated by automated gas liquid chromatography (Varian CP 3800, Varian Instruments, Georgetown, ON) on a fused silica BP20 capillary column (25 m × 0.25 mm internal diameter, Varian Instruments), as previously described (29).

β-glucuronidase activity of cecal contents was determined by a modified method of Freeman (30). Briefly, samples were mechanically homogenized in 0.1 M potassium phosphate buffer (KPBS, pH 6.8) and centrifuged to obtain supernatant fractions. A reaction mixture containing 0.02 mol/L KPBS, 0.1 mmol/L ethylenediaminetetraacetic acid (EDTA), 1 mmol/L phenolphthalein-β-glucuronide and 100 mg total protein/L homogenate was incubated for 30 min at 37°C and then stopped with 3-volumes 0.2 mol/L glycine buffer (pH 10.4). The liberated phenolphthalein was measured at 540 nm. β-glucuronidase activity was expressed per g protein and 1 unit was defined as 1.0 g of phenolphthalein liberated from phenolphthalein glucuronide per hour at pH 6.8 at 37°C.

Formalin-fixed colon tissue was embedded in paraffin wax, sectioned, and stained with haematoxylin and eosin (H&E) for histopathological examination (31). The sections were viewed by the same individual who was blinded to the treatment. All images were acquired under 200× magnification with MetaMorph® 6.0 (Universal Imaging, West Chester, PA).

Data are expressed as mean ± SEM. Statistical analysis was performed using GraphPad Prism (GraphPad Inc., San Diego, CA). Differences among groups were tested using one-way ANOVA on the effect of CPT-11 or diet followed by *post-hoc* Tuckey's test. Effects of CPT-11's doses on tumor growth and effects of dietary treatments on tumor growth (before or after CPT-11 treatment) were tested using two-way ANOVA followed by *post hoc* Tukey's and Bonferroni posttest as specified in the text or figure captions. A probability $p<0.05$ was accepted as being statistically significant.

2.3 Results

2.3.1 Defining efficacy/toxicity profile of the CPT-11 regimen

We used diarrhea, the dose-limiting toxicity for CPT-11-based regimens, as the major endpoint. CPT-11 induced both early-onset diarrhea (within 12 h of each CPT-11 injection) as well as delayed diarrhea, which was of much greater intensity than early-onset diarrhea and which began 12 h after the 3rd dose (Figure 2-1A). Early-onset diarrhea was transitory and could be largely alleviated by atropine (1 mg/kg *s.c.* injection immediately before each CPT-11 injection). Atropine did not affect the time course or severity of late-onset diarrhea (data not shown) and was given as a standard prophylactic treatment in the main study.

Effects of CPT-11 were dose-dependent in respect to both anti-tumor efficacy (Figure 2-1B) and toxicity (Figure 2-1C). A reduction of tumor volume initially followed CPT-11 treatment with the maximal reduction at Day 4-5, and regrew thereafter for all the examined doses (Figure 2-1B). A dose of 125 mg/(kg·day) produced severe but self-limiting delayed diarrhea with a minimal mortality rate consistent with clinical data; 100 mg/(kg·day) did not consistently produce severe diarrhea, whereas 150 mg/(kg·day) was associated with 43% mortality (Figure 2-1C). Therefore, the maximum tolerated CPT-11 dose for the 3-day regimen was defined as 125 mg/kg. This dose provided the baseline profiles of anti-tumor efficacy and toxicity, on which effects of different dietary treatments were tested.

2.3.2 Effects of diet treatments

Prior to CPT-11 treatment, animals fed on different diets had identical feed intake. A reduction of feed intake initially followed CPT-11 treatment, and a rebound-like recovery

was observed thereafter; these changes were identical between all the dietary treatment groups (data not shown). CPT-11 treatment led to a small number of animal deaths, (1 from CON, 2 from PRE and 1 from FO), comparable with the mortality in the preliminary work.

Levels of glutamine and its immediate metabolite, glutamate were assessed and because these amino acids are relatively rapidly metabolized, these observations were made 6 h after glutamine administration. Oral bolus glutamine treatment significantly raised plasma glutamine levels (Table 2-2). Levels of glutamate were elevated in colonic mucosa, as would be expected due to high glutaminase activity in mucosal tissue (32). Oral bolus glutamine treatment did not affect levels of glutamine or glutamate in the tumor.

Plasma triglyceride levels generally reflect dietary fat intake. Plasma triglyceride composition of *n*-3 and *n*-6 fatty acids was not affected by chemotherapy (data not shown). At 7 days after the end of chemotherapy, in rats fed the control diet, neither EPA nor DHA were detectable in plasma triglyceride. *N*-3 PUFA feeding raised levels of EPA and DHA to 1.76 ± 0.64% and 1.76± 0.63% of total fatty acids, respectively ($p<0.05$). *N*-3 PUFA feeding also reduced plasma triglyceride levels of AA from 2.80± 0.60 % to 0.39 ± 0.28% ($p<0.005$) of total fatty acids in triglyceride.

We determined β-glucuronidase activity in cecal contents at Day 0 to study whether different diets affected activity of this enzyme produced by intestinal microflora (Figure 2-2). Feeding the prebiotic-enriched diet resulted in increased β-glucuronidase activity ($p<0.0001$) as compared to the control diet, whereas the *n*-3 PUFA and glutamine-enriched diet did not affect β-glucuronidase activity.

In the *n*-3 PUFA-fed rats there was a 33.0±6.5% ($p<0.05$) inhibition of tumor growth

16 days after tumor implantation compared with control diet rats. *N*-3 PUFAs lengthened tumor doubling time by 15.4% (3.87±0.15 vs. 3.35±0.14 days, $p<0.02$) (Figure 2-3A). Prebiotic-enriched and glutamine diets did not affect tumor growth (data not shown). CPT-11 therapy was initiated for each treatment group when the average tumor burden was of equal size (~1.3% of body weight). *N*-3 PUFAs combined with CPT-11 therapy retarded tumor regrowth after chemotherapy (two-way ANOVA, $p<0.05$) and caused a greater inhibition on tumor growth (26.7±9.1%, $p<0.05$) compared to the combination of control diet and CPT-11 treatment. Neither prebiotic enriched diet nor glutamine affected tumor response to CPT-11 compared to the control diet (Figure 2-3B). Tumor response to CPT-11 was identical with all three ways of glutamine administration (data not shown).

Rats fed the control diet developed late onset diarrhea of high incidence and cumulative severity following CPT-11 treatment (Figure 2-4A, B). Oral glutamine bolus significantly improved the diarrhea profile by decreasing the incidence of score 3 diarrhea and area under the curve of diarrhea score, and this was particularly prominent between Day 3 and Day 7 (Figure 2-4A, B). Rats receiving oral bolus glutamine treatment also showed less body weight loss after CPT-11 treatment (Figure 2-4B). The *i.v.* bolus glutamine improved the diarrhea profile (i.e., incidence of score 3 diarrhea and area under the curve of diarrhea score) to an identical extent as the same dose by oral gavage (Figure 2-4B). When the planned glutamine dose was incorporated in the diet, diarrhea profile (i.e., incidence and area under curve) for these rats were intermediate and were not significantly different from either control rats or those administered glutamine bolus (Figure 2-4B). Neither *n*-3 PUFA nor prebiotic treatment affected any dimension of the

diarrhea severity (Figure 2-4B).

At 6 h following completion of CPT-11 treatment, there was not yet any obvious disruption of the architecture of colonic mucosa except for mild shortening of crypt length (Figure 2-1A). No change in crypt number and goblet cell population was observed. At study completion, all rats had recovered from diarrhea symptoms, and rats treated with CPT-11 showed rebound crypt hyperplasia and goblet-cell hyperplasia in colonic mucosa (Figure 2-1A). No difference was found with different diet treatments at these two time points (data not shown).

2.4 Discussion

2.4.1 Modulation of disease and treatment response by dietary factors

Our experiments reveal that host×tumor×chemotherapy interactions can be modified by dietary modifications. Because feed intake during both pre- and post-chemotherapy phases was identical in all the dietary treatment groups, feed intake-related effects on the examined endpoints could be excluded. Feeding *n*-3 PUFAs slowed tumor growth (*per se)*, and also improved Ward colon tumor response to CPT-11 therapy. We also demonstrated the ability of glutamine therapy to mitigate CPT-11–induced diarrhea, when this amino acid was applied as a dose bolus immediately prior to chemotherapy. By contrast, *n*-3 PUFA and the prebiotic oligosaccharide mixture had no apparent efficacy in preventing CPT-11-induced diarrhea. Taken together, these results suggest that specific dietary modifications may increase the efficacy of therapy and mitigate its side effects. Future work employing the model system to assess for potential additivity or synergy of the effects of nutrient combinations will allow for the development of optimized dietary conditions that may eventually be conducive to escalation of chemotherapy doses within a specialized dietary background.

These systematic studies make it possible to study any beneficial (or deleterious) effects which may occur in response to specific dietary treatments over the progression of tumor and treatment. Our model allows a factorial approach incorporating host, tumor, and single or multiple dietary factors. Ours is the first study encompassing a controlled comparison of three classes of nutrients putatively able to alter tumor growth and host or tumor response to cytotoxic therapy. Prior work in this domain has generally been limited to subsets of the overall conception presented here. Usually, proponents of dietary modulation focus on a single dietary element. A variety of investigations of diet therapy

and chemotherapy have been conducted in healthy, rather than tumor bearing animals (13,33,34) or may not have been conducted in the range of MTD of a specific drug (4,31) and thus will not entirely represent clinical settings. Where interaction between diet and chemotherapy toxicity has been studied, the clinically relevant outcome (diarrhea) has often been replaced by surrogate measures such as histology, body weight loss, feed intake (17,31,35), which may or may not be related to diarrhea severity.

In our model, only **glutamine** bolus treatment significantly reduced severe diarrhea. There are no prior reports regarding the therapeutic potential of glutamine for CPT-11-related diarrhea in an animal model. This otherwise non-essential amino acid is hypothesized to become conditionally essential during stress states where demand for glutamine outstrips its synthesis from endogenous precursors (36). Savarese *et al.* (9) and Ziegler (10) reviewed clinical studies supporting prevention of chemotherapy toxicity by glutamine. There has been limited work exploring glutamine supplementation in colorectal cancer patients treated with 5-FU (37). Glutamine supplementation was reported in a small study series with 5 patients experiencing grade 4 CPT-11-induced diarrhea, which was significantly alleviated after receiving glutamine treatment and dose reescalation could be achieved (38).

We initially selected bolus glutamine treatment based on literature showing that this paradigm could substantially rescue rats from lethal endotoxemia, septic shock or hyperthermia. These conditions are associated with intestinal inflammation and compromised gut barrier function (20,21), as is CPT-11 treatment. While our initial approach of bolus administration was influenced by this thinking, there are several ways of glutamine administration that could be considered nutritionally relevant. The oral versus *i.v.* comparison is important, since glutamine is subject to considerable first-pass

metabolism in the small intestine and *i.v.* administration is the more direct means of raising systemic concentrations. Our results suggest that oral and *i.v.* administration are of equal efficacy, provided that glutamine was administered as a bolus. Incorporation of amino acids in diets is a conventional approach in experimental nutrition studies; however, here, this feeding paradigm did not show a clear ability to alter diarrhea severity. Mixing glutamine with the diet makes for a more continuous intake throughout the day and it may be that acutely and substantially raised tissue levels of glutamine achieved with the bolus treatment are required to evoke mechanisms essential for this protection. Wischmeyer *et al.* (20,21) related the bolus glutamine administration to potentiation of innate cytoprotective HSR during septic and hyperthermic stresses. It would be of interest to examine this link in context of CPT-11-related stress.

Data suggesting that high dietary intakes of *n*-6 PUFAs generally support tumor proliferation and that the corresponding ***n*-3 PUFAs** suppress tumor growth are becoming more abundant (11). This has been demonstrated for diverse tumor types, and is evident also for the Ward colon tumor. Dietary supplementation with *n*-3 PUFAs has been reported to both enhance cytotoxic effects of chemotherapy (e.g., doxorubicin, cisplatin, and bleomycin) and to offer protection to host tissues (11,12) in animal models. Hardman *et al.* (31) reported enhanced regression of MCF7 breast carcinoma xenografts by co-administration of *n*-3 PUFAs with CPT-11, and our results confirm their conclusions with respect to the ability of *n*-3 PUFAs to enhance anti-tumor activity of CPT-11. Here, *n*-3 PUFA treatment did not alter development of severe diarrhea in response to CPT-11. Hardman *et al.* (31) did not measure diarrhea, but showed some evidence for better preservation of intestinal epithelia in animals fed fish oil and treated with CPT-11. The planned time points in our study were respectively too early and too

late to coincide with the time point when glutamine's effect on diarrhea was observed, and new work will be needed for histological work. It should be noted, however, that well-preserved gut histology may not necessarily correlate with amelioration of diarrhea, which is in nature a functional disorder as the net result of intestinal secretion and fluid absorption. For example, oversecretion of Cl^- might be an important contributor to the pathogenesis of CPT-11-induced diarrhea, independent of intestinal epithelial destruction (25,39,40). There are other differences between our respective studies. Hardman *et al.* (31) used a comparatively long-term low-dose CPT-11 regimen; they used pure corn oil in their control diet; the very high *n*-6 fatty acid content of corn oil would be expected to increase inflammatory responses (41) and this may have made the response of the control animals to CPT-11 treatment worse than it would otherwise have been.

Prebiotic oligosaccharides are of interest for their anti-inflammatory effects in chronic bowel disease and in colon cancer prevention (8,42). Feeding oligofructose and/or inulin reduced genetically- or chemically-induced colitis in animal models (22,23) as well as in some emerging studies in human inflammatory bowel disease (IBD) (43). There are only a few reports on effects of dietary fibers on chemotherapy-induced intestinal toxicity, specifically with methotrexate and 5-fluoruracil (13,14). The case of CPT-11 is unique. Biliary excretion of CPT-11 as its inactive glucuronide, and reversal of this glucuronidation by enzymes produced by the intestinal flora, means that β-glucuronidase activity is influential in development of CPT-11 toxicity. Although it is an emerging nutritional concept to treat certain types of refractory diarrhea using prebiotics (44,45) and this would also appear relevant in methotrexate and 5-FU chemotherapy (14,34), our results suggested that caution may need to be exercised when employing prebiotic treatment in conjunction with CPT-11. Prebiotic oligosaccharides

doubled β-glucuronidase activity in cecal contents, which would be expected to increase formation of SN-38, the toxic metabolite of CPT-11, and thus to aggravate rather than ameliorate intestinal injury. Microbiological studies are warranted to determine which β-glucuronidase-producing intestinal bacterial strains are affected by diet components and which bacterial strains worsen CPT-11 induced intestinal injury.

Our observations raise some issues of potential clinical importance. We are currently conducting a study of oral high-dose glutamine therapy in colorectal cancer patients treated with CPT-11, in follow-up of these animal studies and the case series of Savarese *et al.* (9). Our experimental results demonstrate that specific prebiotics fed at nutritionally relevant levels doubled β-glucuronidase activity in cecal contents. It is also known from experimental studies that when intestinal bacterial loads are significantly reduced by antibiotic treatment, CPT-11 toxicity is greatly diminished, suggesting that intestinal bacteria mediate CPT-11 toxicity (46,47). It will thus be of interest to assess endogenous β-glucuronidase activity as a potential determinant of severe diarrhea, in patients treated with CPT-11. There are no published data relating to this point. Considering the great heterogeneity of dietary (prebiotic) fiber intake in humans, it seems plausible that fiber intake could be an innate variant determining CPT-11-induced gut toxicity by affecting formation of SN-38 in the distal GI tract. Dietary intake and physiological levels of *n*-3 PUFAs in cancer patients are also incompletely characterized and could vary considerably (48). The possibility that *n*-3 PUFAs could enhance tumor response to CPT-11 treatment remains to be tested in patients, and this would require a supplementation of a sufficient dose and duration to achieve effective levels.

2.4.2 A rationale for the adoption of defined dietary conditions in animal models of cancer

A clearly defined and nutritionally relevant dietary platform is needed for preclinical research on host×tumor×chemotherapy interactions. Within the body of literature using various animal models to investigate dietary effects on toxicity/efficacy of chemotherapy, diverse background diets have been used. These range from elemental diets and semi-purified diets to chow diets composed of natural ingredients (13,31,33). Elemental diets are made of purified triglycerides, free fatty acids, free amino acids, sugars, vitamins and minerals. Semi-synthetic diets are composed of purified proteins, starch, sugars and defined lipid components from specified fats and oils. Laboratory animal chows are not of standard macronutrient composition and are formulated from plant materials (e.g., corn, soybean meal), and from meat meal, fish meal, and fats which are frequently undefined. Application of elemental diets in human nutrition is limited to *i.v.* feeding and to a few isolated cases of specialized nutritional requirements, and they are not typically used in the nutritional support of patients receiving CPT-11 chemotherapy. While chow diets are composed of ingredients used in foods, their exact composition is incompletely characterized, variable between batches and brands, and more importantly, chows are delivered premixed and thus cannot be modified. For this reason, semi-purified diets are the most widely used in experimental research in clinical nutrition, as they are well–defined, permit systematic modulation of key nutrient classes, and are composed of whole proteins, complex carbohydrates and lipid sources commonly consumed by humans.

A careful consideration of basal diet is indicated. It should meet known nutrient requirements for the animal species under study, and its nutritional relevance must be considered. This is particularly important with respect to the lipid composition of the diet, since both levels and type of dietary fat (including relative and absolute amounts of

saturated, polyunsaturated, *n*-6 and *n*-3 fatty acids) have all been shown to affect tumor growth, response to therapy or toxicity. The amounts of *n*-3 PUFAs in our experimental diets can be defined as nutritionally relevant as it demonstrated efficacy in modulating intestinal and immune functions in studies using dosages that can realistically be achieved in clinical diets (49,50). The basal diet we used was selected to be similar in composition to typical diets of North American populations in terms of overall fat level, polyunsaturated/saturated fat ratio and *n*-6/*n*-3 ratio. Caution must be taken in interpreting studies which used diets with insufficient amounts of *n*-3 PUFAs or a highly unphysiological ratio of *n*-6 to *n*-3 PUFAs (31,51), as these are likely to induce *n*-3 EFA deficiency or are pro-inflammatory and/or promote tumor growth. There is a tendency to use a single oil or fat source, rather than to formulate a fat blend, as done here. For example, corn oil has been used as the sole fat source (31,51,52), and while a corn oil based diet is not likely to induce an *n*-3 EFA deficiency, in a short feeding study, the skewed ratio of *n*-6 to *n*-3 PUFAs in corn oil (i.e., polyunsaturated/saturated ratio of 40) may be supportive of enhanced tumor growth or increased inflammatory mediators (41).

TABLES

Table 2-1. Composition of experimental diets

Ingredient			Control	*N*-3 PUFA	Prebiotic	Glutamine
			g/100g of diet			
Constant portion	Modified AIN-76 basal mix 46g/100g	Casein	25.2			
		Methionine	0.25			
		Glucose	13.95			
		Vitamins AIN 76	1			
		Minerals AIN 76	5			
		Inositol	0.6			
		Choline	0.3			
Variable portion	Lipids 20g/100g	Soybean stearine	15.22	13.84	15.22	15.22
		Linseed oil	0.4	0.4	0.4	0.4
		Sunflower oil	0	3.46	0	0
		Safflower oil	4.38	0	4.38	4.38
		Fish oil*	0	2.3	0	0
	Fibers 10g/100g	Cellulose	10	10	2	10
		Inulin+ oligofructose	0	0	8	0
	Amino acids 24g/100g	Control amino acid mixture§	1.5	1.5	1.5	0
		Glutamine	0	0	0	2
		Corn starch	22.2	22.2	22.2	21.7
Total			100	100	100	100

All diets contained 262 g protein and 15.48 MJ of energy per kg. The constant portion consisted of the pre-mixed modified AIN-76 basal ingredients (Harlan Teklad, Madison, WI); the variable portion was formulated to allow addition of selected fat/fiber/amino acid elements. Other ingredients were supplied: soybean stearine (ICN Biomedicals Inc., Cleveland, OH), safflower oil (Canadian Superstore, President's Choice, AB), linseed oil (Planet Organic, Gold Top, AB), fish oil (Ocean Nutrition Canada, Dartmouth, NS), and Oligofructose-Enriched Inulin (Beneo Synergy I. ®, kindly supplied by Orafti, Tienen,

Belgium. § The control amino acid mixture contained an equimolar mixture of alanine, serine, glycine and histidine, and was isonitrogenous with glutamine-enriched diet. *N-3 PUFA containing diets contained C18:3(3), C20:5(3), C22:5(3) and C22:6(3), respectively, at 0.2, 3.2, 0.2 and 0.8% of total fatty acids, and had an n-6: n-3 ratio of 3.8. All other diets contained 1.1% of total fatty acids as C18:3(3) and had an n-6: n-3 ratio of 21.0.

Table 2-2. Effects of oral bolus glutamine treatment on levels of glutamine and glutamate in colonic mucosa and tumor tissue, and glutamine level in plasma 6 h after CPT-11 treatment (125 mg/(kg·day)×3 days)

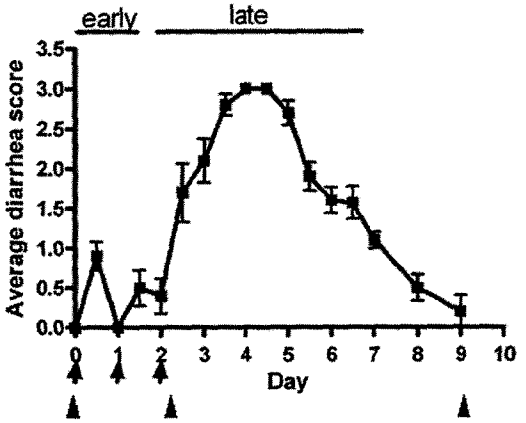
Treatment	Colonic mucosa			Plasma	Tumor		
	[glutamine]	[glutamate]	[glutamine+glutamate]	[glutamine]	[glutamine]	[glutamate]	[glutamine+glutamate]
	μmol/g	*μmol/g*	*μmol/g*	*μmol/L*	*μmol/g*	*μmol/g*	*μmol/g*
Glutamine (oral bolus)	3.48±0.53	2.81±0.34*	6.29±0.55*	664±43*	0.77±0.09	1.08±0.24	1.85±0.24
Control	2.60±0.52	1.70±0.20	4.30±0.69	562±22	0.75±0.07	1.06±0.17	1.82±0.17

Tumor-bearing rats fed on control diet received oral bolus glutamine or sham (Control) treatment 30 min before each CPT-11 injection. Six hours after the last CPT-11 injection, the rats were sacrificed, and blood, colonic mucosa and tumor tissues were harvested. Glutamate and glutamine were analyzed HPLC. *$p<0.05$ vs. CON.

FIGURES

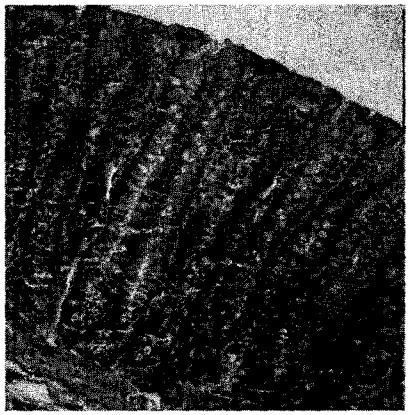
Figure 2-1.

A.



Before CPT-11

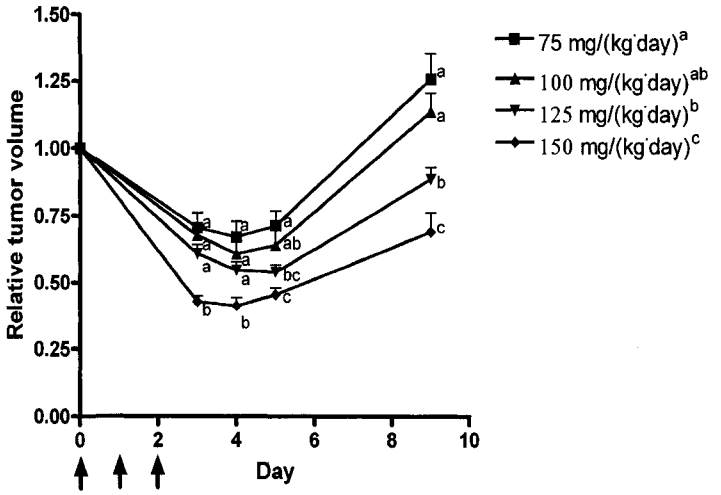
6 h after CPT-11



7 d after CPT-11

Figure 2-1 (Cont'd)

B.



C.

CPT-11 dose	*N	Incidence of delayed diarrhea		Area under curve of the diarrhea score	†Relative body weight at Day 6	‡Mortality
		Score 2 & 3	Score 3			
		%	%		%	%
75 mg/(kg·day)	5	0^{a}	0^{a}	2.6 ± 0.75^{a}	91.8 ± 0.82^{b}	0^{ab}
100 mg/(kg·day)	9	18.1 ± 4.7^{b}	0^{a}	9.0 ± 0.33^{b}	88.1 ± 0.80^{b}	0^{a}
125 mg/(kg·day)	21	86.2 ± 2.5^{c}	54.6 ± 3.8^{b}	18.9 ± 0.52^{c}	79.9 ± 0.48^{a}	4.8^{a}
150 mg/(kg·day)	7	85.7 ± 5.1^{c}	62.5 ± 6.1^{b}	19.7 ± 0.81^{c}	77.6 ± 1.73^{a}	43^{b}

Figure 2-1. Anti-tumor activity and toxicity profiles of CPT-11 treatment

A. Time course of CPT-11-induced diarrhea

Upper panel: tumor-bearing rats on the control diet received 3 daily *i.v.* injections of CPT-11 at 125 mg/(kg·day). Diarrhea was scored twice per day between Day 0 and Day 7 and once per day afterwards. Each black arrow indicates a single CPT-11 injection at 125

mg/kg. Triangles below indicate the time points when histological examination (lower) was performed, as corresponding to the diarrhea dynamics.

Lower panel: photomicrographs of H&E stained colonic sections (left to right) from rats killed before CPT-11, 6 h and 7 days after the last dose of CPT-11 (125 mg/(kg·day) × 3 days). Magnification, ×200.

B. Dose-dependent anti-tumor activity of the 3-day CPT-11 regimen Tumor-bearing rats on the control diet received 3 daily *i.v.* injections of CPT-11 at different dose levels. Each black arrow indicates a single injection of CPT-11 at the specified dose. Y axis represents the relative tumor volume as compared to the baseline volume when CPT-11 therapy was initiated.

In the legend, dietary treatments which do not share a common letter are different ($p<0.05$, *post hoc* Tukey's).

Mean tumor values were compared at the indicated time points between treatments at various doses, and means at a certain time point that do not share a common letter are significantly different ($p<0.05$, Bonferroni posttests).

C. Dose-dependent toxicity profiles associated with 3-day CPT-11 regimen Tumor-bearing rats on the control diet received 3 daily *i.v.* injections of CPT-11 at the indicated doses.

*N, total rat number of each treatment group

[†]Relative body weight at Day 6 was calculated by comparing the body weight at Day 6 to the weight at Day 0.

[‡]Mortality represents percentage of dead rats at the end of the study.

[§]Means within a column that do not share a common letter are significantly different ($p<0.05$).

All data are presented as mean ±SEM.

Figure 2-2.

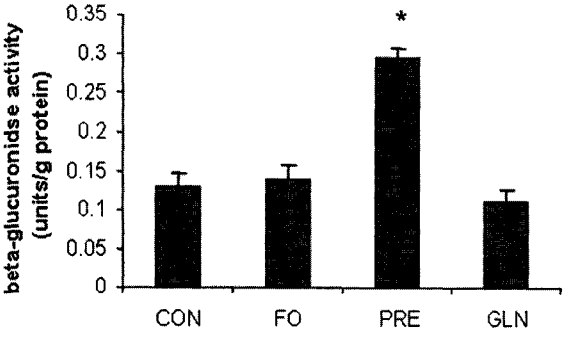
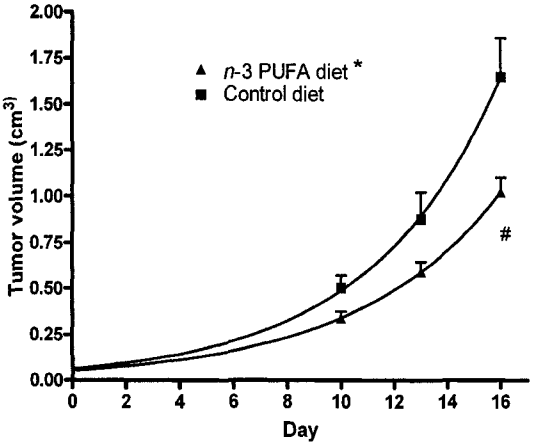


Figure 2-2. Prebiotic oligosaccharides increase β-glucuronidase activity in cecal contents. Rats fed on different diets (CON=control diet, FO= *n*-3 PUFA diet, PRE=prebiotic diet, GLN=glutamine-enriched diet) were killed at Day 0 and β-glucuronidase activity was determined in the collected cecal contents. Rats treated with bolus glutamine had been on the control diet and did not receive glutamine bolus until prior to CPT-11 administration, and the pre-CPT-11 enzyme activity in cecal contents should be identical to that of CON group. *$p<0.0001$ PRE vs. CON.

Figure 2-3.

A.



B.

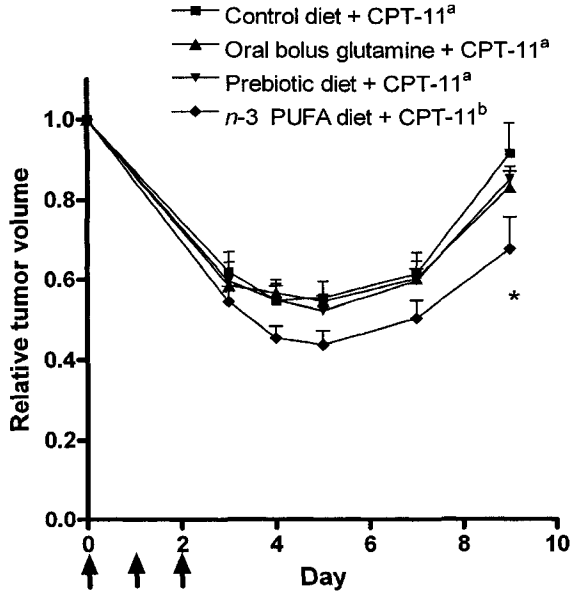


Figure 2-3. Effects of *n*-3 PUFA enriched diet on Ward colon tumor growth and anti-tumor efficacy of CPT-11 *in vivo*

A. *N*-3 PUFA diet inhibited Ward colon tumor growth *in vivo*. Fisher rats were

implanted with Ward colon tumor and changes in tumor volume were followed.

* Tumor growth was different between rats receiving control and *n*-3 PUFA diet treatments ($p<0.05$, two-way ANOVA)

# $p<0.05$ comparison for the tumor values at the indicated time point (16 days after tumor implantation) between control and *n*-3 PUFA diet treatments via Bonferroni posttests.

B. *N*-3 PUFA diet enhanced the anti-tumor efficacy of CPT-11 treatment. Three daily *i.v.* injections of CPT-11 at 125 mg/(kg·day) (as indicated by the black arrows) were initiated when rats of all dietary treatment groups had tumors of approximately 2.0 cm^3 in volume. Y axis represents the relative tumor volume as compared to the baseline volume when the chemotherapy was initiated.

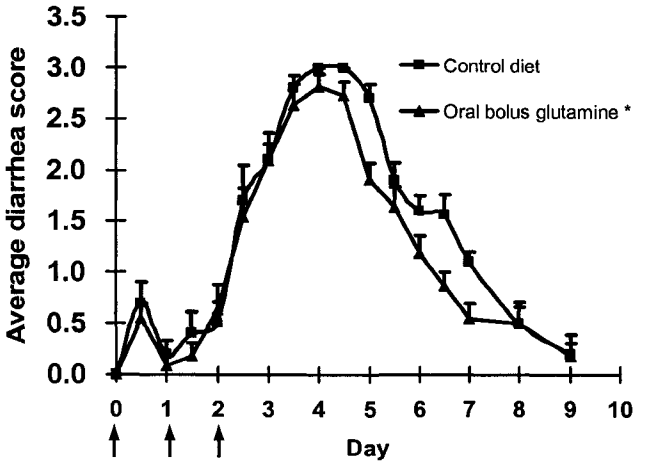
Data are presented as mean ±SEM. Significant difference was found in the tumor volume change between control and *n*-3 PUFA dietary treatments both before chemotherapy as *n*-3 PUFA diet treated alone (panel A) and following CPT-11 treatment (panel B) via two-way ANOVA comparison, $p<0.05$.

* $p<0.05$ comparison for the tumor values at the indicated time point (Day 9) between control and *n*-3 PUFA diet treatments via Bonferroni posttests.

In the legend, dietary treatments which do not share a common letter are different ($p<0.05$, *post hoc* Tukey's).

Figure 2-4.

A.



B.

Type of diet rats fed	Bolus Treatment	†N	Incidence of severe diarrhea	Area under curve of the diarrhea score	‡Relative body weight at Day 6	Tibialis muscle weight at Day 9
			%		*%*	*g/1000g body weight*
Control	Oral sham	10	53.8±4.2[b]	18.8±0.5[b]	79.7±0.5[a]	1.61±0.03
N-3 PUFA enriched	Oral sham	10	48.8±5.1[b]	18.7±0.6[b]	82.2±0.5[b]	1.60±0.06
Prebiotic enriched	Oral sham	9	55.6±6.9[b]	19.0±1.0[b]	80.1±0.9[a]	1.53±0.05
Glutamine enriched	Oral sham	9	43.1±8.0[ab]	18.0±1.5[a b]	80.7±1.3[ab]	1.62±0.05
Control	Oral glutamine	11	34.1±4.7[a]	16.5±1.0[a]	82.8±1.0[b]	1.62±0.05
Control	*i.v.* sham	5	55.5±5.0[b]	18.8±0.7[b]	76.8±1.0[a]	1.56±0.05
Control	*i.v.* glutamine	9	35.0±4.9[a]	16.6±0.8[a]	78.9±0.6[a]	1.54±0.04

Figure 2-4. Bolus glutamine mitigates CPT-11 induced diarrhea. Tumor-bearing rats on different dietary treatments received 3 daily *i.v.* injections of CPT-11 at 125 mg/(kg·day).

A. Effect of oral bolus glutamine on the time course of CPT-11-induced diarrhea

Diarrhea was scored twice per day between Day 0 and Day 7 and once per day afterwards. Each black arrow indicates a single CPT-11 injection at 125 mg/kg.

*Development of CPT-induced delayed diarrhea during Day 3 to Day 7 was different between rats on control diet and rats receiving oral bolus glutamine via two-way ANOVA analysis ($p<0.005$) .

B. Effect of different dietary treatments on the toxicity profiles associated with CPT-11 treatment

†N, total rat number of each treatment group

‡Relative body weight at Day 6 was calculated by comparing the body weight at Day 6 to the weight at Day 0.

All data are presented as mean ±SEM.

*Means within a column that do not share a common letter are significantly different ($p<0.05$).

ENDNOTES

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CHAPTER 3 BOLUS ORAL GLUTAMINE PROTECTS RATS AGAINST CPT-11–INDUCED DIARRHEA AND DIFFERENTIALLY ACTIVATES CYTOPROTECTIVE MECHANISMS IN HOST INTESTINE BUT NOT TUMOR

3.1 Introduction

Glutamine protects the gut during a variety of stress conditions including trauma, sepsis, burns and several cancer chemotherapies (5-FU, methotrexate, doxorubicin) (1-5). Several potential mechanisms for this protection are proposed. Glutamine preserves GSH in the gut during oxidative stress during methotrexate chemotherapy and endotoxin challenge (6,7). Glutamine may also preserve gut barrier integrity and gut–associated and systemic immune competence (8). Glutamine (0.3-2 g/(kg·day)) is usually provided continuously in the diet (in parenteral nutrition or mixed with enteral feed (3-5,7). Wischmeyer et al. (9,10) recently explored administration of glutamine as a single *i.v.* or oral bolus and a dose of 0.75 g/kg was optimally protective from lethal endotoxin shock or hyperthermia in rats (9,10). Wischmeyer suggests that additional protective mechanisms not seen with continuous feeding occur after bolus doses of glutamine, particularly the HSR, an inherent cellular cytoprotective response (9,10). Our recent work (Chapter 2, ref (11)) provides the first direct evidence that high dose oral bolus glutamine limits the development of severe diarrhea in rats treated with CPT-11.

There has been concern expressed that provision of glutamine may promote tumor growth, based on the prevalent notion that tumors have a propensity to use glutamine as a source of energy and of nitrogen for biosynthesis (12,13). The utility of nutritional treatments to reduce chemotherapy toxicity would depend upon a differential effect on the

host and tumor, such that host tissues would be protected from injury and there would be no protection (or even sensitization) of tumor tissues to chemotherapy. While such differential effects on host and tumor would be ideal, it could equally be predicted that nutritional support might invoke cellular protective mechanisms in the tumor, and may cause resistance to therapy. Based on these considerations, we designed a study to examine the effects of an oral bolus glutamine in tumor bearing rats treated with CPT-11. The specific objective of our work was to determine the effect of this treatment on specific cytoprotective effectors (including Hsp and GSH) in the host and tumor.

3.2 Materials and methods

Laboratory rat use, tumor implantation and drug formulation (CPT-11 and atropine) are detailed previously (Chapter 2). Our dietary design is spelled out in detail in our recent work (Chapter 2, ref:(11)). Rats were initially fed Rodent Laboratory Chow (Harlan. Teklad, Madison, WI). During the adaptation period, this chow was mixed with our control diet (50/50 wt:wt) for one week, followed by full transition to experimental diets throughout the whole study.

3.2.1 Experimental design

When tumor volume reached ~2 cm^3, three consecutive daily CPT-11 *i.v.* injections at 125 mg/(kg·day), the MTD determined from our prior study (11), were initiated. Atropine (1 mg/kg *s.c.*) was administered immediately before each CPT-11 injection to alleviate the early-onset cholinergic symptoms as described previously (Chapter 2, ref (11)).

Glutamine was administered by oral gavage (0.75 g/(kg·day)) 30 min before each daily CPT-11 injection. The sham treatment group was gavaged with isovolemic sterile water. Glutamine (Sigma) was made as a 3% (wt:v) solution immediately before use, and filtered with a 0.45-μm filter. The 1st day of CPT-11 administration was designated Day 0. Sham- and glutamine-treated rats (n=6/treatment) were killed on Day 3, 6 h after the last injection of CPT-11 (~6.5 h after last glutamine gavage). This time point was selected to capture early responses to glutamine treatment, especially Hsp expression. Additional sham- and glutamine–treated rats (n=12/treatment) were followed until Day 9 for tumor response to CPT-11 treatment and to evaluate diarrhea. β-glucuronidase activity was assayed in cecal contents collected from glutamine- and sham-treated rats killed at these

two time points (Day 3 and Day 9), and additionally prior to chemotherapy (Day 0) (n=6/treatment).

3.2.2 Outcome measures

Tumor volume was calculated as previously described (11). A clinically comparable 3-point scale was adopted in grading the severity of CPT-11-related **diarrhea** in rats (14). Incidence of grade 3 diarrhea and area under curve of diarrhea score were calculated as described previously (Chapter 2, ref:(11)). Rats were killed by CO_2 asphyxiation. Immediately after rats became unconscious, whole blood was collected via cardiac puncture into heparinized tubes. Cecal digesta content was collected under aseptic conditions. The full length of the colon was washed and longitudinally cut into halves, one of which was mounted on a wax strip and fixed in 10% (v:v) neutral buffered formalin, and the mucosa was gently scraped off the other half and immediately frozen in liquid nitrogen. Tumor was excised intact, blotted and weighed before the non-necrotic portions were collected and frozen in liquid nitrogen.

Apoptosis *in situ* via terminal transferase-mediated dUTP nick end labeling (TUNEL). Apoptotic cells in colonic mucosa were identified with the DeadEnd Colorimetric TUNEL System (Promega, Madison, WI). Prepared sections were treated with proteinase K (20 mg/L in phosphate buffered saline (PBS)) and endogenous peroxidase activity was quenched with 2% (v:v) hydrogen peroxide (H_2O_2) in PBS. Buffered terminal deoxynucleotidyl transferase (TdT) enzyme solution was applied, incubated at 37°C for 1 h and then slides were placed in stop/wash buffer. Antidigoxigenin peroxidase (55 µl) was added, and incubated for 30 min at room

temperature. Sections were again washed, and diaminobenzidine-H_2O_2 was used for color development. Sections were counterstained with 2% (wt:v) hematoxylin and mounted. Apoptotic cells were identified by brown-stained nuclei, or as apoptotic bodies, which are fragments of apoptotic cells engulfed by neighboring epithelial cells. 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 400×magnification. Apoptotic counts were calculated by dividing the number of positively stained cells by the area of the examined mucosa. The analysis was performed by a researcher who was blinded to the dietary treatment.

Hsp. Tissue samples were placed in radioimmunoprecipitation assay (RIPA) buffer (1% (v:v) Nonidet P-40 (NP-40), 0.5% (wt:v) sodiumdeoxycholate, 0.1% (wt:v) sodium dodecyl sulfate (SDS) in 1× PBS) with three fresh protease inhibitors (aprotinin (1:100), phenylmethyl-sulphonyl-fluoride (PMSF, 1:100) and pepstatin-A (1:1000), all from Sigma) and mechanically homogenized. The homogenate protein was determined using the Micro bicinchoninic acid (BCA) assay from Pierce (Rockford, IL). Lysates from heat shocked (45°C, 15 min) HeLa cells were used as positive controls for Hsp70, 90α and heat shock cognate (Hsc)70, while purified rat Hsp25 protein (Stressgen, Victoria, BC) was used as a positive control for Hsp25. Thirty micrograms of homogenate was separated on a 10% (wt:v) SDS-polyacrylamide gel and was transferred to a polyvinylidene difluoride (PVDF) membrane (Hybond-P; Amersham Biosciences, Piscataway, NJ). The membrane was blocked with 5% (wt:v) non-fat dry milk solution in Tris-buffered saline with 0.5% (v:v) Tween 20 (TBS-T) and incubated with the primary antibody (anti-Hsp70, 1:4000; anti-Hsp90α, 1:4000; anti-Hsp25, 1:12000) (Stressgen).

After washing and incubation with corresponding horseradish peroxidase (HRP) - conjugated secondary antibodies (Stressgen), all Hsps were detected by enhanced chemiluminescence plus (ECL plus, Amersham Pharmacia Biotech) and analyzed by autoradiography using a Typhoon 8600 Variable Mode Imager (Amersham Biosciences). The densitometry determination was performed by using ImageQuant 3.2 software (Molecular Dynamics, Sunnyvale, CA). Data shown are relative densitometric ratios against the value for Hsc70 (anti-Hsc70, 1:5000, Stressgen), the cognate constitutively expressed form of Hsp70 used as the protein loading control.

Reduced GSH (rGSH) and GSH disulfide (GSSG) were determined with a modified Tietze enzymatic recycling assay (15). Tissues were homogenized in 6 volumes of 5% (wt:v) metaphosphoric acid (MPA) with or without 33 mmol/L 1-methyl-2-vinyl-pyridinium trifluoromethane sulfonate (M2VP), a scavenger of rGSH. The homogenate was centrifuged (1,000*g*, 5 min). For GSH, the MPA extract was neutralized and diluted in buffer containing 100 mmol/L Na_3PO_4 and 5 mmol/L EDTA, pH 7.5. For GSSG, the homogenate with M2VP was neutralized and diluted in buffer containing 100 mmol/L Na_3PO_4 and 5 mmol/L EDTA, pH 10.05. Samples were mixed with 1.262 mmol/L 5,5-dithiobis-(2-nitrobenzoic acid) and 1,500 Units/L GSH reductase. The mixture was incubated (23°C, 5 min), and the absorbance was recorded at 412 nm, 3 min after addition of 3.8 μmol NADPH.

Free amino acid concentrations were determined using high performance liquid chromatography, after conversion to their *o*-phthaldialdehyde derivatives (16). Cysteine and proline are not detected by this method. ***β*-glucuronidase activity** was determined by

a modified method of Freeman (17) as described in Chapter 2. The β-glucuronidase activity is expressed as Units/g protein. One unit is defined as 1.0 μg of phenolphthalein liberated from phenolphthalein glucuronide per hour.

MLN cell phenotype. Immune cells were isolated from MLNs (18), and cells (200,000 cells/well) were used to determine cell phenotype using 2 color direct immunofluorescence (19). The following antibodies were used: CD3, CD4, CD8, CD25, CD45RA (BD Bioscience, Mississauga ON, Canada), and OX12 (Cedarlane, Hornby ON, Canada); Streptavidin QR (Sigma, Oakville, ON, Canada) was added to all Biotin labeled antibodies. The percentage of cells expressing each of the antibody markers was determined by flow cytometry (FacScan, Becton Dickinson, Sunnyvale, CA, USA) (19). We attempted to perform every phenotype assay on each rat, but this was not always possible due to limited lymph node size; the actual number of rats utilized for each assay is indicated in result tables.

Statistical Analysis. Data are expressed as mean ± SEM. The effect of glutamine on tumor growth following CPT-11 treatment was tested using two-way ANOVA followed by Bonferroni posttest (GraphPad Prism, GraphPad Inc., San Diego, CA). Treatment differences in apoptotic index and immune phenotype were analyzed using one-way ANOVA followed by *post hoc* Tukey's test (SPSS 12.0, SPSS Inc. Chicago, IL). For the other measures, the effects of glutamine were analyzed using the unpaired two-tailed Student's *t* test for independent samples. A probability 0.05 was accepted as being statistically significant.

3.3 Results

3.3.1 Bolus glutamine decreased diarrhea without altering anti-tumor efficacy of CPT-11

CPT-11 therapy resulted in a clinically comparable diarrhea profile and mortality of < 5% (11). Two sham-treated animals and no glutamine–treated animals died. Kinetics of tumor inhibition following CPT-11 treatment was identical between glutamine and sham-treated groups (Figure 3-1). CPT-11 treatment induced both early and delayed-onset diarrhea. Bolus glutamine treatment decreased the incidence of severe (grade 3) diarrhea (sham-treated 53.8 ± 4.2% vs. glutamine-treated 34.1 ± 4.7%; $p<0.005$) and area under curve of diarrhea score (sham-treated 18.8 ± 0.5 vs. glutamine-treated 16.5 ±1.0; $p<0.05$). CPT-11 caused a reduction of feed intake, and a rebound-like recovery thereafter; these changes were identical between groups. For the sham- and glutamine-treated groups respectively, the daily relative feed intake dropped to its nadir on Day 4 by 66±9% and 69±4% ($P=0.8$) and the daily relative feed intake overshot to its top value on Day 8 by 110±14% and 100±12% ($P=0.7$).

3.3.2 Bolus glutamine administration differentially induces cytoprotective mechanisms in host tissues following CPT-11 treatment

Amino acids were determined in samples taken ~ 6.5 h after the last glutamine bolus. This time point was selected for detection of the Hsp response and would likely be past the peak glutamine concentrations; however, at this time point, concentrations of glutamine, glutamate and aspartate were raised in plasma or/and colonic mucosa tissue from glutamine-treated rats ($p<0.05$) (Table 3-1). Glutamine treatment also raised concentrations of leucine, valine, tryptophan and phenylalanine, arginine, tyrosine,

glycine and asparagine in plasma or/and colon ($p<0.05$). Ornithine and citrulline were not detectable in colon or tumor, and in plasma their concentrations (ornithine 35.8±7.4 μmol/L; citrulline 16.5±0.8 μmol/L) were not affected by glutamine. Changes in amino acid levels in response to glutamine treatments of plasma and colon tissue were in general concordant with one another in both magnitude and direction. In spite of increased plasma concentrations of many individual amino acids, amino acid concentrations in tumor tissue were not altered by glutamine treatment.

In CPT-11–treated rats, bolus glutamine increased the accumulation of the stress-inducible Hsp, Hsp70, 25 and 90α, in the colonic mucosa by 3.1, 7.2 and 3.8 fold, respectively as compared to sham treatment (Figure 3-2A). However, the abundance of these inducible Hsp in tumor tissue was not affected by glutamine treatment (Figure 3-2B).

GSH concentration was analyzed in colonic mucosa and tumor at 6 h after the 3rd CPT-11 dose. Glutamine significantly enhanced the rGSH/GSSG ratio in colonic mucosa compared with sham-treated rats, mainly through an increasing trend in rGSH (Table 3-2). By contrast, glutamine treatment significantly decreased the reduced and total GSH (tGSH) concentrations in the tumor, resulting in a trend of decreased tumor rGSH/GSSG ratio.

Previous studies (20) suggested that apoptosis in colonic tissue peaked as early as 6 h after acute high-dose CPT-11 treatments, the time point at which we also opted to investigate whether glutamine treatment altered colonic apoptosis. While overall the apoptotic index of colonic mucosa 6 h following CPT-11 treatment was lower in

glutamine–treated animals, there was not a significant difference in this parameter (Figure 3-3).

We analyzed the β-glucuronidase activity in cecal contents at Day 0 (baseline); 6 h after the last dose of CPT-11, when the delayed diarrhea started; and 7 days after the last dose (Day 9) when late diarrhea resolved on its own. As shown in Figure 3-4, β-glucuronidase activity increased by 3.8-fold 6 h after completing CPT-11 treatment as compared to baseline levels, and returned to a level slightly lower than baseline levels at Day 9. Oral bolus glutamine treatment counteracted the immediate rise of cecal β-glucuronidase 6 h after treatment, but had no effects on enzyme activity on a longer time scale at Day 9.

Phenotypic composition of lymphocytes in MLNs was analyzed 7 days after completing CPT-11 chemotherapy. There were numerous effects of CPT-11 treatment compared with animals that had not received chemotherapy. CPT-11 decreased the proportion of CD3+ T cells (primarily CD3+CD4+ cells as suggested by the reduced CD3+CD4+/CD3+CD8+ ratio) and accordingly an increased proportion of B cells (OX12+) in MLNs, irrespective of glutamine treatment. Although only a small proportion of cells expressed IL-2 receptor, the proportion in CPT-11 treated rats was about 30% lower due to proportionately less CD8+CD25+ cells ($p<0.05$). CPT-11 also decreased proportions of cytotoxic T cells (CD3+CD8+ cells) and antigen mature CD8+ cells (CD45RA-) compared to healthy controls ($p<0.05$). Glutamine normalized the relative abundance of CD3+CD8+ cells and memory/antigen mature CD8+ cells (CD45RA-) to levels seen in healthy controls and did not affect the CD3+CD4+/CD3+CD8+ ratio or the proportion of CD8+CD25+ cells (Table 3-3).

3.4 Discussion

3.4.1 A dichotomy in tumor and host's response to glutamine treatment

We observed a striking dichotomy in the response of a tumor and of the tumor-bearing host to oral glutamine administration. In addition to symptomatic relief of severe diarrhea, several potentially beneficial protective mechanisms were invoked in host tissues: 1) increased proportion of rGSH, 2) HSR, 3) suppression of colonic β-glucuronidase activity, 4) increased proportions of cytotoxic T (CD3+CD8+) cells in MLNs, especially memory CD8+ T cells. By contrast, cytoprotective pathways were not induced in Ward colon tumor and tumor response to CPT-11 was unaffected by glutamine treatment. These data concur with the concept that a nutrient may alter the balance between the host and the tumor, in a manner that favors the host overall.

The potential for purified nutrients at high doses to 'feed the tumor' or to confer tumor protection against cytotoxic therapy remains a prevalent notion. Tumors are said to be 'glutamine traps', and glutaminolysis has been proposed as a major energy-producing process in tumor cells (12,13). In tissue culture tumor cells rely on glutamine for cell growth, as an essential energy source and biosynthetic precursor (21,22). In animal models, however, the size, protein synthetic rate and DNA content of tumors are not generally affected by supplemental glutamine (23,24). A majority of tested tumors indeed do not show net glutamine uptake from the arterial supply *in vivo* (25-27). Our findings principally agree with these studies and show that tumor amino acid pools remained strikingly unaffected by glutamine supplementation.

Overexpression of Hsp is thought to be involved in tumor chemoresistance by directly or indirectly permitting cancer cells to overcome chemotherapy-induced

apoptosis (28,29). Here, the failure of glutamine to modulate tumor Hsp expression and GSH may simply result from the lack of adequate and sustained alteration of the tumor glutamine pool. While only a single time point was studied, the concurrent alterations in amino acid concentrations in plasma and colon tissue would suggest that the selected time should have revealed alterations in tumor amino acid concentrations. Amino acid transport, metabolism, or both, could potentially contribute to the insensitivity of tumor tissue to amino acid supplementation.

3.4.2 A multiplicity of glutamine actions in the tumor-bearing host

Glutamine bolus significantly improved CPT-11-induced diarrhea, and we are currently pursuing a clinical investigation of this potential benefit of glutamine therapy. Because transient anorexia after CPT-11 chemotherapy was identical in sham and glutamine-treated rats, feed intake-related effects on the examined endpoints could be excluded.

There are not other published data on the effects of tumor and concurrent CPT-11 chemotherapy on plasma and tissue amino acids, and our results suggest notably low levels of arginine and citrulline compared with published results for normal rats. There are suggestions in the literature that hypocitrullinemia could be a potential quantitative marker for intestinal damage caused by intense chemo- and radiation therapy (30,31). Diminished plasma citrulline could limit arginine synthesis through the intestinal-renal axis, resulting in decreased arginine concentration (32,33).

Glutamine administered orally is subject to extensive first pass metabolism in the small intestine and liver (34), and its actions may be due as much to secondary

metabolites as to glutamine *per se*. The wide range of changes in plasma and colon amino acid concentrations (Table 1) reflects the sophisticated metabolic pathways of glutamine *in vivo*. Some products derived from glutamine metabolism, such as arginine, are important modulators of gut physiology and immunity (35). Consistent with previous findings, our glutamine bolus increased arginine concentration systemically and in the colon (36). T-cell depletion has been considered as the primary contributor to immunodeficiency related to high-dose chemotherapy (37) and both glutamine and arginine promote lymphocyte proliferation and activation (7). Our oral glutamine treatment attenuated the relative depletion of cytotoxic T cell, especially memory CD8+ cells in MLNs associated with CPT-11 treatment. This may add to the competence of gut-associated immunity against invasion of pathogens present in the gut lumen.

CPT-11 is associated with oxidative stress in normal tissues with diminished GSH store (38). GSH synthesis may play a pivotal role in protecting from CPT-11-related toxicity (39) . Glutamine (via glutamate) is a precursor for GSH synthesis and has been suggested to be limiting for GSH synthesis during stress (40). Glutamine treatment raised the ratio of reduced to oxidized GSH in the colon tissue but at the same time lowered the reduced and total GSH stores in tumor. Previous work in rats bearing tumors and treated with methotrexate suggest that glutamine decreased GSH content in tumor but increased it in generally in host organs including heart, kidney and intestine (6,41). These authors conjectured that the acidic tumor environment blocks GSH regeneration and ultimately depletes the GSH store in the tumor (6).

Glutamine increased Hsp25, 70 and 90α in colon mucosa, the three key inducible Hsp playing a central role in protecting cells by preserving the structure of normal

proteins and removing damaged ones. This response was somewhat variable between individual animals; however this variability remains largely unexplained. Wischmeyer et al. had also seen greater variability in the HSR of colon and ileum as compared to lung and heart (9). The action of glutamine to induce Hsp may be direct, as there is evidence that glutamine induces Hsp in cells in culture (42,43). Induction of Hsp preserves the functional integrity of epithelia, i.e., electrolyte absorption/secretion, intestinal myoelectric activity and mucosal barrier (44-46), and reduces production of pro-inflammatory mediators (45,47), which may in turn be involved in triggering diarrhea (14).

The protective ability of glutamine treatment may or may not have derived from alteration of apoptosis in colonic epithelium, and our results on this point were equivocal. The improvement of diarrhea outcomes independent of gut apoptosis has been observed by other authors using keratinocyte growth factors (48,49). Glutamine could exert its benefit on CPT-11-induced diarrhea largely through other mechanisms that may more predominantly correct intestinal absorptive/secretory function.

Local deconjugation of SN-38 glucuronide catalyzed by β-glucuronidase produced by colonic microflora, intensifies the epithelial exposure to SN-38 (50). We provide direct evidence that administration of CPT-11 affects colonic β -glucuronidase; a marked increase of the enzyme was detected 6 h after CPT-11 treatment. CPT-11 can increase levels of several β-glucuronidase-producing flora (*Escherichia*, *Clostridium* and *Staphylococcus* species) within 1-6 h following treatment (51). The post-CPT-11 increase of the enzyme activity was abolished with glutamine treatment, in a manner that might alleviate the exposure of intestinal epithelium to SN-38.

Glutamine induced Hsp and favorably altered GSH stores and β–glucuronidase activity in colon. The early induction of these effects suggests that they have the potential to contribute to the subsequent mitigation of severe diarrhea, which was most evident on Day 4-7. It may be that one of these effects is primary; however, it is also possible that their simultaneous occurrence may be important. As suggested by Wischmeyer *et al.* (9,10), bolus glutamine may represent a novel therapeutic paradigm for preventing chemotherapy-related injury via boosting the inherent stress response of tissues vulnerable to the drug. A future objective of research in this context will be to further clarify the dose, schedule of administration and additional dietary elements, to optimize the protective response.

TABLES

Table 3-1. Concentrations of free amino acids in rat plasma, colonic mucosa and tumor tissue after CPT-11 chemotherapy ± glutamine treatment

	Plasma		Colonic mucosa		Tumor	
	Sham	Glutamine	Sham	Glutamine	Sham	Glutamine
Amino acid	*μmol/L*		*μmol/g*		*μmol/g*	
Aspartate	8.9±1.1	11.5±1.6	0.428±0.054	0.892±0.125*	0.385±0.071	0.364±0.049
Glutamate	55.0±10.3	63.3±3.0	1.62±0.19	2.79±0.37*	1.07±0.15	1.03±0.09
Asparagine	36.9±2.7	40.9±4.2	0.136±0.019	0.237±0.025*	0.128±0.023	0.124±0.017
Serine	164±5	166±9	0.759±0.149	0.883±0.160	0.400±0.051	0.395±0.045
Glutamine	560±10	666±40*	2.59±0.50	3.53±0.62	0.773±0.094	0.765±0.073
Histidine	68.6±5.9	83.7±5.1	N.D.†	N.D.†	0.112±0.007	0.118±0.011
Glycine	148±3	168±9	0.491±0.111	1.372±0.272*	1.13±0.19	1.15±0.07
Threonine	204±14	196±6	0.506±0.053	0.652±0.091	0.552±0.078	0.563±0.069
Arginine	65.4±7.8	92.1±4.1*	0.075±0.014	0.170±0.021*	0.150±0.023	0.134±0.012
Tyrosine	49.2±2.7	69.1±1.8*	0.062±0.008	0.141±0.020*	0.142±0.012	0.148±0.021
Tryptophan	86±5	104±2*	0.076±0.012	0.168±0.042	0.062±0.005	0.054±0.003
Methionine	37.5±1.7	45.7±4.3	0.080±0.010	0.150±0.031	0.079±0.010	0.080±0.012

Valine	207±10	261±15*	0.295±0.041	0.403±0.047	0.296±0.019	0.309±0.036
Phenylalanine	74.2±3.8	92.2±1.6*	0.099±0.016	0.217±0.021*	0.141±0.014	0.140±0.016
Isoleucine	92±5	106±6	0.196±0.032	0.228±0.031	0.182±0.017	0.181±0.019
Leucine	157±10	194±12*	0.229±0.028	0.359±0.036*	0.311±0.024	0.325±0.038
Lysine	372±23	387±29	0.298±0.058	0.402±0.031	0.551±0.055	0.597±0.074
Alanine	-‡	-	-	-	0.95±0.14	1.15±0.12

Tumor-bearing rats received a bolus glutamine or isovolemic sham treatment 30 min before each CPT-11 injection and concentrations of free amino acids in plasma, colon and tumor tissues were analyzed 6 h after the last CPT-11 injection. All the data are reported as mean ± SEM; n=5 for Sham; n=6 for Glutamine.

†N.D. not detectable

‡For plasma and colonic tissue, alanine was not separated from taurine at the same run as the other amino acids.

*Different from Sham, $p<0.05$.

Table 3-2. GSH concentrations in rat colon and tumor after CPT-11 chemotherapy ± glutamine treatment

	tGSH	GSSG	rGSH	rGSH/GSSG ratio
	μmol/g tissue	*μmol/g tissue*	*μmol/g tissue*	
Colonic mucosa				
CPT-11+sham	1.04±0.16	0.027±0.003	0.99±0.16	41.9±11.8
CPT-11+glutamine	1.45±0.18	0.021±0.004	1.41±0.17	75.2±9.0*
Tumor				
CPT-11+sham	1.39±0.12	0.024±0.004	1.34±0.12	66.3±14.9
CPT-11+glutamine	1.02±0.10*	0.025±0.003	0.97±0.10*	40.6±5.9

Tumor-bearing rats received an oral bolus glutamine or sham treatment 30 min before each CPT-11 injection and GSH concentration was analyzed in the colonic mucosa and tumor tissue 6 h after the last CPT-11 injection. All the data are reported as mean ± SEM; n=5 for CPT-11+sham; n=6 for CPT-11+glutamine.

*Different from CPT-11+sham, $P < 0.05$

Table 3-3. Phenotype of immune cells in rat MLNs after CPT-11 chemotherapy ± glutamine treatment

subpopulations	Healthy control with sham treatments only	CPT-11+sham	CPT-11+glutamine
B cell + (OX12) (% of total cells)	19.1±0.6^{a} (4)	32.5±2.9^{b} (6)	31.2±2.5^{b} (7)
CD3+ (% of total cells)	73.0±2.1^{b} (5)	57.8±2.1^{a} (7)	59.6±1.4^{a} (7)
CD3+CD8+ (% of total cells)	22.2±1.2^{b} (5)	18.0±0.4^{a} (6)	21.1±1.1^{b} (7)
CD25+CD8+ (% of total cells)	1.8±0.1^{b} (5)	0.9±0.1^{a} (7)	0.9±0.1^{a} (7)
CD8+CD45RA- (% of total cells)	25.2±1.3^{b} (5)	20.3±0.6^{a} (6)	23.4±0.4^{b} (5)
CD3+CD4+ (% of total cells)	48.7±0.7^{b} (5)	38.0±2.8^{a} (7)	36.2±1.3^{a} (4)
CD25+CD4+ (% of total cells)	2.7±0.4 (4)	2.3±0.3 (7)	2.0±0.3 (7)
CD4+CD45RA- (% of total cells)	43.5±3.4 (5)	38.9±4.3 (5)	40.2±2.0 (7)
CD4+CD45RA+ (% of total cells)	1.8±1.1^{a} (5)	3.1±1.8^{ab} (5)	5.2±0.9^{b} (5)
CD3CD4/CD3CD8	2.2±0.6^{b} (5)	1.9±0.2^{a} (5)	1.8±0.2^{a} (4)
CD25+ (% of total cells)	3.5±0.2^{b} (5)	2.4±0.2^{a} (6)	2.4±0.2^{a} (7)

Tumor-bearing rats received an oral bolus glutamine or sham treatment 30 min before each CPT-11 injection and phenotype of MLN cells were determined 7 days after the last CPT-11 injection (Day 9). Values presented as cell population percentage mean ± SEM (number of rats); means within a row that do not share a common letter differ, $p<0.05$.

FIGURES

Figure 3-1.

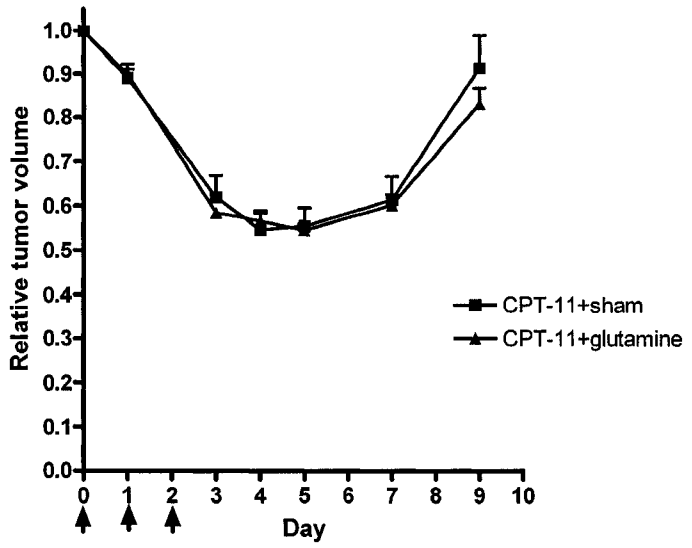
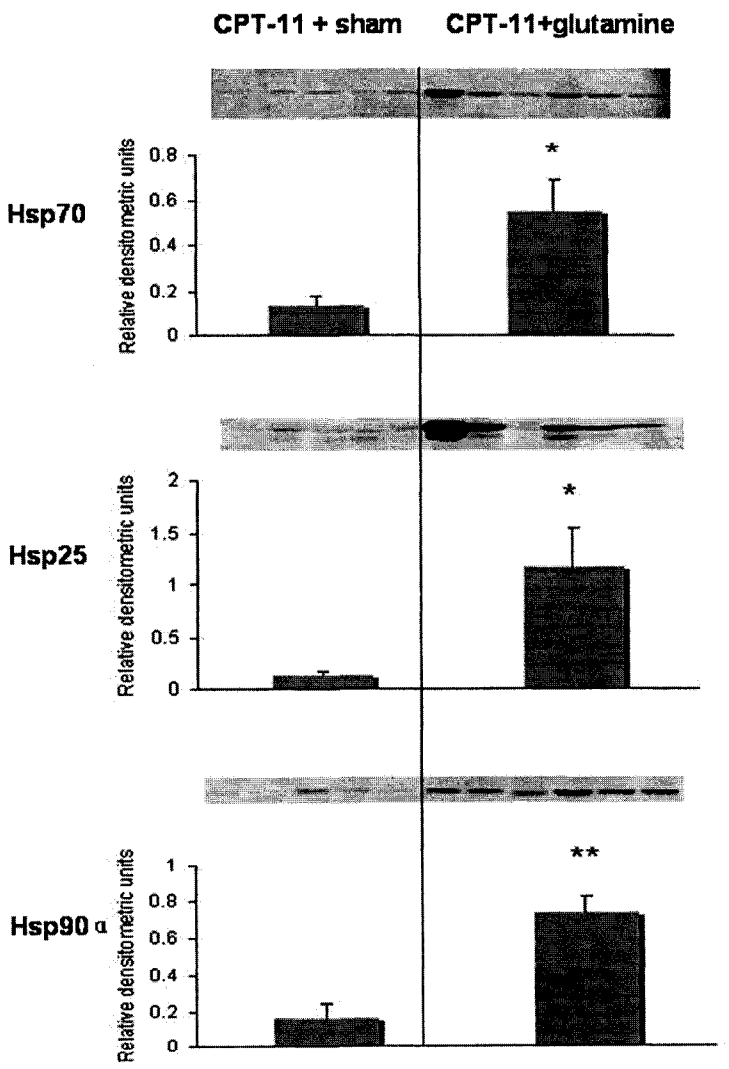


Figure 3-1. Oral bolus glutamine treatment did not alter CPT-11 anti-tumor efficacy. Three daily consecutive CPT-11 injections at 125 mg/(kg·day) (as indicated by the black arrows) were initiated when the rats had tumors of ~2 cm^3 in volume. Rats received a bolus of glutamine at 0.75 g/kg or isovolemic sham via oral gavaging 30 minutes before each CPT-11 injection. The Y axis represents the relative tumor volume as compared with the baseline volume when CPT-11 treatment was initiated. Data are presented as mean ±SEM.

Figure 3-2.

A.


CPT-11 + sham
CPT-11+glutamine
Hsp70
Relative densitometric units
0.8
0.6
0.4
0.2
0
*
Hsp25
Relative densitometric units
2
1.5
1
0.5
0
*
Hsp90 α
Relative densitometric units
1
0.8
0.6
0.4
0.2
0
**

B.

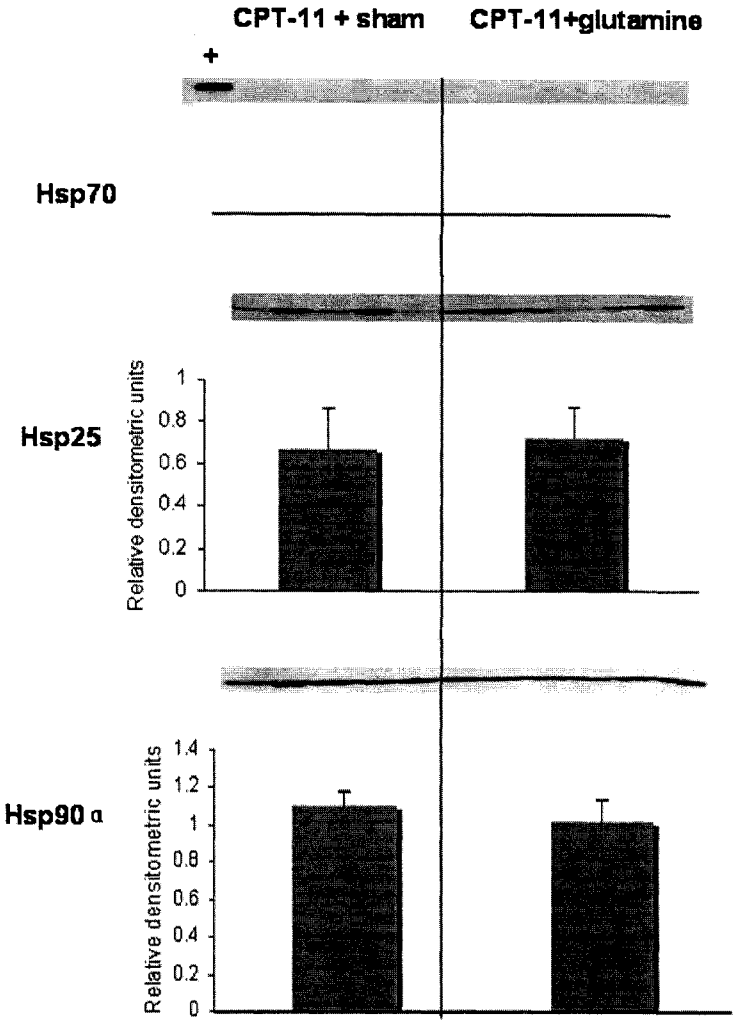


Figure 3-2. Oral bolus glutamine treatment differentially affects Hsp expression profile in colonic mucosa (A) and tumor tissue (B) 6 h following the 3rd dose of CPT-11.

Accumulation of stress-inducible Hsp (Hsp70, 25 and 90α) in colonic mucosa was examined by Western blot. Each lane represents the sample from an individual animal. Relative densitometry is expressed as mean ± SEM. Samples left of the central black line are from rats receiving CPT-11+sham treatment (n=5); samples on the right are from rats

receiving CPT-11+glutamine (n=6) .*$p<0.05$, **$p<0.001$, "+" indicates the positive control lane for tumor Hsp70 detection.

Figure 3-3.

A.

Healthy control sham treatments only | CPT-11+sham | CPT-11+glutamine

B.

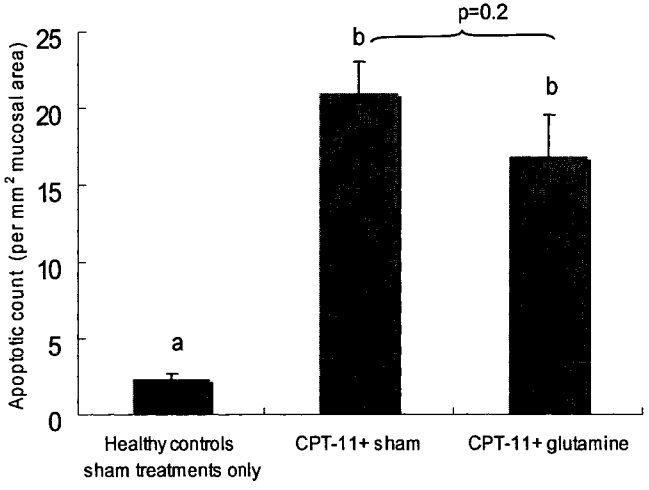


Figure 3-3. Effects of oral bolus glutamine treatment on CPT-11-induced apoptosis of colonocytes

Six hours after completion of CPT-11 treatment, colonic sections were harvested and apoptotic colonocytes in the crypt compartment were identified via TUNEL staining. For illustration of the level of apoptotic colonocytes in normal colonic mucosa, sections from 4 additional healthy animals receiving only sham treatments were also stained by the TUNEL method for comparison.

A. Photomicrographs of TUNEL-stained colonic sections (left to right) from the following treatment groups: healthy control with sham treatments only; CPT-11+sham; CPT-11+glutamine. Magnification × 400. Arrows indicate apoptotic colonocytes identified with TUNEL staining.

B. Mean apoptotic count per mm^2 mucosal area from the three groups: Healthy control with sham treatments only (n=4); CPT-11+sham (n=5); CPT-11+glutamine (n=6). Data are presented as mean ±SEM. Means that do not share a common letter differ, $p<0.05$.

Figure 3-4.

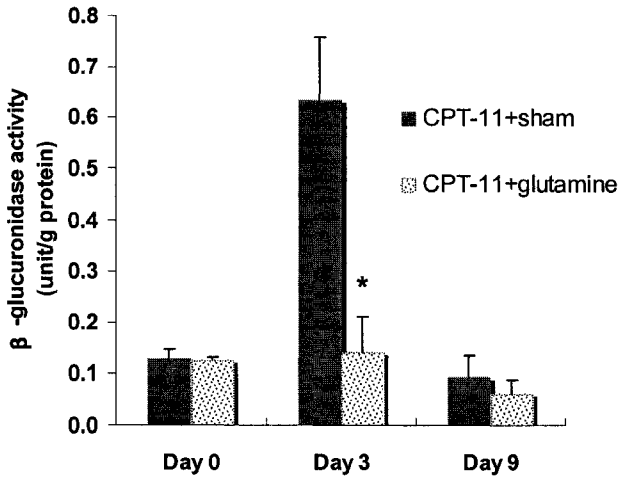


Figure 3-4. Oral bolus glutamine treatment countered the transiently up-regulated activity of β-glucuronidase in cecal contents following CPT-11 administration. Cecal contents were collected from glutamine- and sham-treated animals killed prior to chemotherapy (Day 0), 6 h after completion of CPT-11 therapy (Day 3), and 7 days after CPT-11 treatment (Day 9). Data represent average β-glucuronidase activity ± SEM (unit/g protein) calculated for each time point. *different from CPT-11+sham at Day 3, $p < 0.01$

ENDNOTES

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CHAPTER 4 EFFECTS OF SINGLE AND COMBINED SUPPLEMENTATION OF GLUTAMINE AND N-3 PUFAs ON HOST TOLERANCE AND TUMOR RESPONSE TO CPT-11/5-FU CHEMOTHERAPY IN RATS BEARING WARD COLON TUMOR

4.1 Introduction

Increasing evidence has emerged for the promising role of certain nutritional factors in modulating efficacy and/or toxicity related to cancer chemotherapy. This conception of using nutritional adjuncts to chemotherapy is based on the fact that tumor and host responses to the chemotherapeutic agents could potentially be affected in a differential manner, such that the overall outcome favors sensitization of tumor to the chemotherapy or/and protecting the host against injury related to the drug. Glutamine and long-chain *n*-3 PUFAs are two nutrients that have been demonstrated to modify efficacy/toxicity related to cancer chemotherapy. Glutamine has been demonstrated to mitigate the toxicity of chemotherapy by improving GI-related symptoms, gut barrier function and whole-body nitrogen-balance (1-7). *N*-3 PUFAs, EPA (20:5*n*-3) and DHA (22:6*n*-3) are reported to enhance the cytotoxicity of several widely used anti-neoplastic agents including anthracyclines, cisplatin and bleomycin via several different mechanisms (8,9). There is also some evidence suggesting that *n*-3 PUFAs may alleviate chemotherapy-related toxicities (8,10). Both glutamine and *n*-3 PUFAs have been increasingly recognized for their immunomodulatory roles by providing substrates for immune cells, regulating redox status and affecting inflammatory cytokine/eicosanoid network (11). Evidence-based linkage for their therapeutic utility in modifying immuno-defects/disruption related to both the tumor-bearing state and the tumor and host's response to major anti-cancer

treatment is yet to be substantiated.

The higher order interactions between some of the different nutrients that have been suggested to favorably affect host response to chemotherapy (and a variety of other diseases and insults) have rarely, if ever, been explored. While there already exist some enteral 'immunonutrition' formulae featuring the combination of glutamine and *n*-3 PUFAs (12-14) for applications in clinical nutrition (12-14), most of the research on *n*-3 fatty acid or glutamine nutrition only focused on the individual effects of these two nutrients when supplemented to nutritionally complete diets. It is commonly assumed that a greater benefit could be achieved by combining individually beneficial nutrients; however, evidence regarding the relative efficacy of nutrient combinations is lacking.

We earlier established a rat model to study the interactions amongst tumor, chemotherapy and diet (rats bearing the Ward colon tumor and treated with CPT-11 (15)). Within our controlled dietary design, glutamine treatment mitigated late diarrhea, the dose-limiting toxicity for CPT-11, whereas *n*-3 PUFAs could enhance the anti-tumor efficacy of CPT-11 therapy (15). The current study is an extension of these earlier studies aimed at comparing the effects of *n*-3 PUFAs, and glutamine alone or in combination on the response of the tumor and host to a combination chemotherapy similar to that used in the first line therapy of colorectal cancer (cycles of CPT-11 plus 5-FU). The measured outcomes of the study were mortality, feed intake, weight loss, tumor growth and response to therapy, and blood cell counts. As oxidative stress is involved in anti-tumor activity as well as the pathophysiology of toxicity related to these two drugs (16-20), we further related the potential effects of tested dietary interventions to possible redox status alterations in host and tumor tissues.

4.2 Materials and methods

Laboratory rat use, tumor implantation and drug formulation (CPT-11 and atropine) are detailed previously (Chapter 2). Our dietary design is spelled out in detail in our recent work (Chapter 2, ref:(15)) All tested nutrients were incorporated into a nutritionally complete diet (Table 4-1). Rats were initially fed Rodent Laboratory Chow (Harlan. Teklad, Madison, WI). During the adaptation period, this chow was mixed with our control diet (50/50 wt:wt) for one week, followed by full transition to experimental diets throughout the whole study.

4.2.1 Experimental design

Two weeks prior to tumor implantation, rats were randomly assigned to one of five experimental dietary treatments:

1) reference group (no tumor, no chemotherapy, control diet) (REF) (n=7)

2) control diet + chemotherapy (CON) (n=12)

3) glutamine diet +chemotherapy (GLN) (n=10)

4) *n*-3 PUFA diet + chemotherapy (FO) (n=10)

5) *n*-3 PUFA + glutamine diet + chemotherapy (GLN+FO) (n=10)

When rats had a tumor of ~ 2.3 cm^3, a modified regimen of CPT-11/5-FU combination chemotherapy (21) was initiated. Briefly, drugs were administrated *i.v.* once a week for 2 weeks. The day when chemotherapy was initiated was designated as Day 0. CPT-11 (50 mg/kg) was administered on Day 0 and Day 7, whereas 5-FU (50 mg/kg) was administered on Day 1 and Day 8. Atropine (1 mg/kg *s.c.*) was administered immediately before each CPT-11 injection to alleviate the early-onset cholinergic symptoms (15).

4.2.2 Outcome measures

After starting chemotherapy treatment, body weight and feed intake were monitored every second day. Feed intake and body weight at Day 0 for each rat was considered the baseline value, and the consumption and weight gain/loss were calculated relative to that initial weight. Tumor volume was calculated as described previously (15). Tumor response was expressed as relative tumor volume for each tumor, calculated relative to the volume at the start of chemotherapy. Calculation of tumor growth inhibition was as described previously (Chapter 2, ref: (15)).

Thirteen days after completion of chemotherapy, rats were killed by CO_2 asphyxia followed immediately by exsanguination by cardiac puncture. The colonic mucosal tissue was scraped off from the first 6-cm section of the proximal colon and immediately frozen in liquid nitrogen for GSH assay (15). Tumor and tibialis anterior muscles were collected, weighed and then immediately frozen in liquid nitrogen.

Blood was collected by jugular vein puncture into heparinized tubes 5 days prior to chemotherapy (as baseline) and at the end of the study (13 days after completion of chemotherapy) was used for a complete blood count (CBC) and automated differential count performed using a Hemavet instrument (CDC Technologies, Oxford, CT).

Tissue rGSH and GSSG concentrations were determined using a modified Tietze enzymatic recycling assay (22) as described previously (Chapter 3, ref: (23)).

Data are expressed as mean ± SEM. Unless specified in the text, treatment differences in leukocyte counts, GSH, and muscle weight were analyzed using one-way ANOVA followed by *post hoc* Tukey's test (SPSS, Inc. Chicago, IL), whereas treatment differences in tumor growth, body weight and feed intake were tested using two-way

ANOVA (dietary treatment ×time) followed by *post hoc* Tukey's test. Survival curves were obtained using the Kaplan–Meier method, and differences in survival between groups were analyzed using the log-rank test. A probability $p<0.05$ was accepted as being statistically significant.

4.3 Results

4.3.1 Effects of dietary treatments during the pre-chemotherapy period

During the initial phase of this study after tumor implantation but prior to chemotherapy, there was no significant effect of diet treatment on body weight and the feed intake of tumor-bearing rats (data not shown). Although the total concentration of white blood cells (WBC) did not differ between treatments, an effect of diet *per se* on the relative proportion of different lineages was observed when rats had a tumor burden about 1.2% of body weight. Tumor-bearing rats fed the control diet had a higher concentration and proportion of neutrophils and a lower proportion of lymphocytes in peripheral blood compared with the reference rats ($p<0.05$, Table 4-2A). *N*-3 PUFAs alone partially corrected such a skewed composition of WBCs (means of neutrophil proportion/concentration and lymphocyte proportion not significantly different from those of reference rats); glutamine also lowered neutrophil concentration while increasing lymphocyte proportion in the tumor-bearing rats towards the values seen in the reference rats (both means not significantly different from those of reference rats). Co-administration of *n*-3 PUFAs and glutamine lowered the proportion and concentration of monocytes as compared with reference rats ($p<0.05$) and led to a lower lymphocyte concentration as compared with single *n*-3 PUFA treatment ($p<0.05$).

Inhibition of tumor growth was observed with both *n*-3 PUFAs and glutamine provided individually within the pre-chemotherapy period as compared with rats fed the control diet ($p<0.05$, Figure 4-1A); there was a 23.6±5.9% (*n*-3 PUFA treatment) and 18.4±5.8% (glutamine treatment) inhibition of tumor growth 16 days after tumor implantation as compared with control diet rats ($p<0.05$). Not leading to a greater tumor-inhibitory effect, co-administration of these two nutrients led to an identical tumor

growth inhibition as the *n*-3 PUFA treatment (Figure 4-1A); tumor inhibition by the co-treatment of glutamine and *n*-3 PUFAs was 23.9±6.2% 16 days after tumor implantation ($p<0.05$ vs. control diet).

4.3.2 Effects of dietary treatments during the post-chemotherapy period

4.3.2.1 Tumor response to chemotherapy

CPT-11/5-FU therapy was initiated for each treatment group when the average tumor burden was of equal size (~1.2% of body weight). Both glutamine and *n*-3 PUFAs significantly enhanced anti-tumor activity of CPT-11/5-FU chemotherapy as compared with control diet ($p<0.05$, Figure 4-1B). However, these effects were not additive in the combination therapy with both nutrients, which resulted in a similar potentiation of chemotherapy's anti-tumor efficacy as *n*-3 PUFA treatment (52.9±16.7% more tumor inhibition by FO and 53.7±13.0% more inhibition by GLN+FO as compared to CON on Day 11, $p<0.05$)

4.3.2.2 Host responses to chemotherapy

4.3.2.2.1 Survival. There was a considerable mortality occurring during Day 9 - Day 17. As tumor burden was markedly reduced with CPT-11/5-FU treatment, the observed short-term mortality was considered to be attributed to the chemotherapy instead of cancer progression. The mortality was more prevalent in rats fed the control diet (5 out of 12, 41.7%). Mortality for GLN, FO and GLN+FO groups were 20% (2 out of 10), 30% (3 out of 10) and 10% (1 out of 10) respectively (Figure 4-2); difference as compared to CON does not reach a level of significance for any of these groups.

4.3.2.2.2 Blood cell counts. At 13 days following completion of

chemotherapy, there was a marked overshoot increase of total WBC count in rats fed the control diet, compared to the baseline level before chemotherapy. This was primarily attributable to expansion of the neutrophil population and to a lesser extent of the monocytes ($p<0.05$, paired *t-test)*, as the lymphocyte concentration did not change as compared to the pre-chemotherapy baseline values (Table 4-2B). These differences were not observed in the rats fed either the *n*-3 PUFAs or the glutamine diet. However the rats fed the combination diet also had an overshoot post-chemotherapy increase in neutrophils, monocytes and total WBCs to a comparable extent as the rats on control diet.

4.3.2.2.3 Body weight. Rat body weight during CPT-11/5-FU treatment displayed a loss-regain pattern concordant with the cyclic administration of chemotherapy (Figure 4-3A). Body weight loss was much greater upon administration of the 2nd cycle of chemotherapy than after the 1st cycle. For instance, rats fed control diet initially lost 5.5±0.5% of their weight following 1st cycle at day 2 (nadir of the first cycle), and further suffered an additional 9.6±1.8% weight loss following 2nd cycle at day 11 (nadir of the second cycle) ($p<0.05$, paired *t-test*). Rats receiving either glutamine or *n*-3 PUFA treatment or both had significantl less body weight loss following 2 cycles of chemotherapy as compared to the rats fed on control diet ($p<0.05$). Glutamine treatment led to a greater benefit in limiting post-chemotherapy body weight loss as compared to *n*-3 PUFA and combination (glutamine + *n*-3 PUFAs) treatments ($p<0.05$). By the end of the study, glutamine–treated rats had entirely caught up and exceeded their baseline body weight by +2.1±0.7% ($p<0.01$ vs. CON, Bonferroni posttest) and *n*-3 PUFA-treated rats also ended the study at a weight +2.6±1.4% ($p<0.05$ vs. CON, Bonferroni posttest) above their baseline body weight. However, rats receiving the combination treatment had a slower post-chemotherapy body weight catch-up, ended the study with a -1.7±1.1%

weight gain, which was significantly lower than GLN and FO groups ($p<0.05$, Bonferroni posttest) but not significantly different from the CON group.

4.3.2.2.4 Feed intake. Time course of the feed intake change during the chemotherapy was concordant with that of body weight change in overall (Figure 4-3B). Both single glutamine and *n*-3 PUFA treatments significantly alleviated anorexia following CPT-11/5-FU chemotherapy as compared with the control diet ($p<0.05$). However, co-treatment with glutamine and *n*-3 PUFAs failed to significantly limit the post-chemotherapy anorexia as compared with the control diet. At Day 10, where rats from all groups had the least daily feed intake, glutamine and *n*-3 PUFA-treated rats had lesser reduction in their feed intake level as compared with rats fed control diet ($p<0.05$, Bonferroni posttest), although the combination treatment was unable to significantly affect this nadir value at Day 10.

4.3.2.2.5 Muscle weight. Cancer chemotherapy could be a potent catabolic stimulus and causes muscle wasting (24). Muscle mass is thus an important indicator for chemotherapy-related cachexia/wasting and host overall nutritional state (25). At the end of the study, rats fed the control diet had a significantly lower relative weight of tibialis anterior muscle than reference rats ($p<0.05$). Either glutamine or *n*-3 PUFA treatment completely prevented such a muscle loss. However co-treatment with glutamine and *n*-3 PUFAs did not prevent the muscle loss and the relative tibialis weight for this group was comparable to that of the rats fed control diet and considerably lower than reference group and rats receiving single glutamine and *n*-3 PUFA treatments ($p<0.05$).

4.3.3 Effects of dietary treatments on GSH stores in host and tumor tissues following CPT-11/5-FU therapy

At 13 days after completing CPT-11/5-FU chemotherapy, rGSH/GSSG ratio was lowered by 50% in colonic mucosa of control diet-fed rats (vs. reference rats, $p<0.05$, Table 4-3). This was mainly through a higher GSSG level ($p<0.05$). By contrast, this ratio for the other dietary treatment groups was maintained at a comparable level as the reference rats and was significantly higher for single glutamine and *n*-3 PUFA treatments (but not for the combination treatment) as compared with rats on control diet ($p<0.05$). In tumor tissue (Table 4-3), glutamine supplementation, either in a single manner or in combination with *n*-3 PUFAs, resulted in a lower rGSH/GSSG ratio as compared with control diet ($p<0.05$).

4.4 Discussion

4.4.1 Individual effects of glutamine and *n*-3 PUFAs

Our data demonstrate that the presence of a tumor leads to an array of hematological changes, i.e., elevation of neutrophil count and skewed relative abundance of neutrophils and lymphocytes, consistent with findings in patients with various malignant solid tumors (26-28). These cancer-related abnormalities in peripheral leukocyte abundance are suggested to negatively correlate with clinical prognosis in terms of response rate to chemotherapy, incidence of post-treatment infectious complications and survival as reported (29-31). Our results show that these hematological disturbances appearing prior to chemotherapy could be at least partially normalized with *n*-3 PUFAs or glutamine treatment *per se*, which may as well convert into advantage favoring *n*-3 PUFA or glutamine-treated rats during the period of CPT-11/5-FU treatment. At 13 days after completion of the chemotherapy, there was a marked neutrophilic and monocytic leukocytosis observed in rats on CON and GLN+FO diets, but not on GLN or FO diets. More detailed hematological evaluation on peripheral leukocyte dynamics is required to discern whether the neutrophilia and monocytosis associated with CON and GLN+FO diets were merely a reflection of 'rebound-overshoot' immunological recovery after immunosuppressive chemotherapy (32), or myelopoiesis/neutrophil mobilization related to pathological conditions such as infection and inflammation (33).

Our results demonstrate that supplementing the diet with either *n*-3 PUFAs or glutamine *per se* inhibited Ward tumor growth *in vivo*. Evidence supporting a direct growth-inhibitory effect of dietary *n*-3 PUFAs is abundantly documented in various tumor types (34,35) including Ward colon tumor as reported by our group (15). Incorporation of *n*-3 PUFAs in tumor membrane phospholipids affects a wide range of

biological functions, such as biosynthesis of eicosanoids, membrane lipid peroxidation, signal transduction, membrane fluidity and cell interaction (8). As compared to *n*-3 PUFAs, which has been increasingly accepted as an 'anti-tumor' nutrient, utility of glutamine supplementation in cancer patients is often challenged due to a prevalent notion that tumor is a 'glutamine trap' (36,37) and exogenous glutamine may promote tumor growth. This notion is based on findings in tissue culture with abundant oxygen supply (38-40) and indirect in vivo evidence looking at enzyme analysis (41,42) and plasma glutamine concentration differences between tumor-bearing and normal hosts (43). However, there is no direct evidence indicating glutamine could stimulate tumor growth *in vivo* (44,45) and emerging evidence suggests that glutamine supplementation may conversely exert inhibitory effect on tumor growth (46-48). Proposed mechanisms for observed tumor-inhibitory effect may involve reduced production of PGE_2, enhanced natural kill cell activity, altered tumor GSH store and modulating expression of apoptosis effectors (47-49).

Ability of dietary supplementation with *n*-3 PUFAs and glutamine in modulating chemotherapy efficacy has been reported separately with various drugs including doxorubicin, AraC, mitomycin C, CPT-11 and methotrexate (50,51), but not previously with colorectal cancer treated with the combination regimen of CPT-11 and 5-FU. Our results demonstrate that supplementation with either *n*-3 PUFAs or glutamine enhanced responsiveness of Ward colon tumor to CPT-11/5-FU treatment. GSH is the major intracellular anti-oxidant protecting cells from injury caused by excessive oxidative stress. Tumor GSH level has been shown positively correlated with tumor cell proliferation and resistance to chemotherapy (52). Increment of *n*-3 PUFA content in plasma membrane may enhance cellular oxidative burden and make cells more predisposed to oxidative

injury caused by chemotherapy (50,53). Glutamine, as the precursor for biosynthesis of GSH, may as well modify cellular redox status indirectly. Therefore, we related effects of *n*-3 PUFAs and glutamine on tumor chemosensitivity to possible alterations of tumoral GSH store following chemotherapy. Both glutamine and *n*-3 PUFAs lowered rGSH/GSSG ratio, the most important regulator of intracellular redox status (the higher the ratio, the lower the oxidative burden) (52), correlated with their respective chemo-sensitizing effect. Altered rGSH/GSSG ratio could be ascribed to enhanced oxidative stress associated with *n*-3 PUFA incorporation in membrane (10,54). Although serving as a precursor for GSH synthesis, glutamine treatment paradoxically lowered the rGSH/GSSG ratio and intensified oxidative stress in tumor instead of alleviating that through boosting tumor GSH store. This phenomenon has been observed by our group (Chapter 3, ref:(23)) and others (55). However, question still remains on whether intensified tumoral oxidative stress associated with *n*-3 PUFAs or glutamine treatment is sufficient to explain the higher tumor response to chemotherapy in the current study. Bolus glutamine treatment as shown in our previous study also lowered rGSH level in tumor, but didn't result in enhanced tumor response to CPT-11 monotherapy (Chapter 3, ref:(23)).

Our study clearly demonstrated that adding either glutamine or *n*-3 PUFAs, improved constitutional symptoms associated with CPT-11/5-FU therapy including body weight loss, anorexia and muscle wasting. Improvement of these cachexia-related manifestations may largely contribute to the observed advantage in surviving the intensive CPT-11/5-FU treatment associated with glutamine and *n*-3 PUFA diets. Anorexia and accompanying weight loss could be a direct reflection of GI toxicity associated with CPT-11/5-FU chemotherapy. In particular, compromised gut barrier integrity caused by chemotherapy

has been suggested to serve as a pivotal mechanism triggering gut-derived infection, endotoxemia and concurring systemic inflammatory response (56). All of these are the major contributors to pathophysiology of chemotherapy-induced anorexia-wasting syndrome. We therefore investigated dietary effect of glutamine and *n*-3 PUFAs on endogenous intestinal GSH store, which plays a crucial role in preserving structural and functional integrity of gut barrier against exogenous insults such as chemotherapy (55,57). Consistent with our previous finding with bolus glutamine treatment, continuous feeding glutamine from diet could normalize the colonic GSH stores and redox status, which was deteriorated by chemotherapy (Chapter 3, ref:(23)). *N*-3 PUFAs also tended to exert positive effects on GSH-related redox state following CPT-11/5-FU treatment. We thus observed an interesting differential effect of diet on GSH stores in tumor and host tissues. In tumor, glutamine and *n*-3 PUFAs were pro-oxidative, correlated with enhanced tumor response to CPT-11/5-FU treatment; whereas in colonic tissue, these two factors alleviated chemotherapy-related oxidative stress, in concert with better host's tolerance to the chemotherapy. This striking dichotomy has been reported with glutamine treatment by others and us (45,55) (Chapter 3, ref:(23)). *N*-3 PUFA supplementation has been shown to mitigate colonic oxidative burden in patients with inflammatory bowel disease or animal models with colitis (58-60). This could be secondary to its anti-inflammatory effect by inhibiting the production of pro-inflammatory mediators such as *n*-6 series eicosanoids, TNF-α, IL-6 and IL-1β (61,62). Decreased generation of reactive oxygen species could result from reduction of inflammation process, e.g., respiratory burst of immune cells (63,64). Given this, more experimental work is entailed to address the potential differential effect of *n*-3 PUFAs on the redox states of host and tumor tissue in chemotherapy-induced colitis.

4.4.2 Interaction of glutamine and n-3 PUFAs when combined

Given the benefits associated with single glutamine and *n*-3 PUFA treatments, additive benefits were notably lacking when these two nutrients were combined. This combination treatment apparently did not confer a greater benefit on tumor inhibition either in the presence or absence of chemotherapy; individual benefits associated with single treatments, particularly in respect to host nutritional (i.e., body weight, feed intake and muscle weight) and immune (peripheral leukocyte counts) features, were instead partially or completely abrogated when these two nutrients were combined.

This raises an issue that is of importance to clinical nutrition but has not yet been addressed. Glutamine and *n*-3 PUFAs have been increasingly recognized as 'immunonutrients'; enteral feed formulations with both of these elements incorporated have been marketed for immuno-enhancement or modulation in various conditions such as sepsis, surgery, trauma and burns (12-14,65). However, the potential interaction between these two nutrients has not been fully evaluated under the context of different clinical conditions and thereby clear scientific evidence for combining them is lacking. A limited body of studies has just been emerging to focus on how different immunonutrients would act or counteract when provided in combination. Available data from these studies suggest immunomodulatory effects of combined immunonutrients are dose and end point-dependent, and may also depend on illness severity, timing and duration (66-68). In various situations associated with the aforementioned variables, mixtures of different immunonutrients may have synergistic but also antagonistic effects (66-68). For instance, arginine seemed to limit enterobacterial translocation in a head-injured rat model more efficiently alone than in an immunonutrition formula featuring combination of arginine

and *n*-3 PUFAs (69). The same group also demonstrated that arginine and *n*-3 PUFAs when combined may exert some conflicting effects on gut barrier function and macrophage reactivity in a rat model of inflammation and catabolism, and resulted in enhanced enterobacterial translocation and reduced Th1 cytokine production by stimulated macrophages (communication with Dr. Moinard).

Our results did not support the use of concurrent supplementation of glutamine and *n*-3 PUFAs in the enteral feeding during CPT-11/5-FU chemotherapy based on evaluation of overall effects on the host and tumor. The combination treatment may have a negative impact on the host redox state as suggested by the colonic rGSH/GSSG ratio showing a declining trend towards the value seen in rats on control diet. Our previous study and others have shown that enteral glutamine administration could effectively elevate arginine levels in tissue and plasma (Chapter 3, ref:(23,70). Arginine×*n*-3 PUFA interaction derived from enteral glutamine supplementation thus cannot be excluded. Taken together, our study underlines the importance of systematically evaluating and comparing the specific effects of component nutrients of immunonutrition formula, both in their single supply form and in the formulation form with coexistence of other nutrients. Future studies are warranted to be directed towards possible nutrient interaction, between but possibly not limited to, glutamine and *n*-3 PUFAs.

TABLES

Table 4-1. Composition of the experimental diets

Ingredient			Control (CON)	*N*-3 PUFA (FO)	Glutamine (GLN)	Glutamine + *n*-3 PUFA (GLN+FO)
			g/100g of diet			
Constant portion	Modified AIN-76 basal mix 46g/100g	Casein	25.2			
		Methionine	0.25			
		Glucose	13.95			
		Vitamins AIN 76	1			
		Minerals AIN 76	5			
		Inositol	0.6			
		Choline	0.3			
	Fiber 10g/100g	Cellulose	10			
Variable portion	Lipids 20g/100g	Soybean stearine	15.22	13.84	15.22	13.84
		Linseed oil	0.4	0.4	0.4	0.4
		Sunflower oil	0	3.46	0	3.46
		Safflower oil	4.38	0	4.38	0
		Fish oil	0	2.3	0	2.3
	Amino acids 24g/100g	Control amino acid mixture§	1.5	1.5	0	0
		Glutamine	0	0	2	2
		Corn starch	22.2	22.2	21.7	21.7
Total			100	100	100	100

Ingredient sources and fatty acid composition of each diets were detailed previously (Chapter 2).

Table 4-2. Effect of dietary treatments on peripheral WBC counts before and after CPT-11/5-FU chemotherapy

A. Effect of dietary treatments *per se* prior to CPT-11/5-FU chemotherapy

Dietary treatment	*WBC	Neutrophils	Neutrophils	Lymphocytes	Lymphocytes	Monocytes	Monocytes
	$\times 10^9$/L	*% of WBC*	*$\times 10^9$/L*	*% of WBC*	*$\times 10^9$/L*	*% of WBC*	*$\times 10^9$/L*
¶REF (n=11)	8.81±1.06	19.84±1.91^{a}	1.56±0.18^{a}	73.02±1.46^{b}	6.36±0.82^{ab}	5.51±0.46^{b}	0.52±0.08^{b}
CON (n=12)	8.53±0.31	26.28±1.48^{b}	2.29±0.19^{b}	64.18±2.18^{a}	5.45±0.21^{ab}	4.18±0.25^{ab}	0.36±0.03^{ab}
GLN (n=10)	9.12±0.29	24.86±1.68^{b}	2.15±0.16^{ab}	69.44±1.31^{ab}	6.18±0.16^{ab}	3.98±0.20^{a}	0.36±0.02^{ab}
FO (n=10)	9.83±0.19	22.52±0.91^{ab}	2.22±0.11^{ab}	70.01±1.13^{ab}	7.01±0.12^{b}	4.69±0.33^{ab}	0.46±0.04^{ab}
GLN+FO (n=10)	7.56±0.66	23.96±1.54^{b}	1.64±0.16^{ab}	68.94±2.24^{ab}	5.18±0.41^{a}	3.93±0.48^{a}	0.31±0.06^{a}

B. Effect of dietary treatments 13 days after completing of CPT-11/5-FU chemotherapy

Dietary treatment	*WBC	Neutrophils	Neutrophils	Lymphocytes	Lymphocytes	Monocytes	Monocytes
	$\times 10^9$/L	*% of WBC*	*$\times 10^9$/L*	*% of WBC*	*$\times 10^9$/L*	*% of WBC*	*$\times 10^9$/L*
REF (n=11)	8.81 ± 1.06^{a}	19.84 ± 1.91^{a}	1.56 ± 0.18^{a}	73.02 ± 1.46^{c}	6.36 ± 0.82	5.51 ± 0.46	0.52 ± 0.08^{a}
CON (n=7)	$14.53\pm2.76^{ab\dagger}$	$41.43\pm5.17^{c\dagger}$	$6.07\pm1.53^{b\dagger}$	$47\pm6.24^{a\dagger}$	6.83 ± 1.54	7.68 ± 0.76	$1.13\pm0.25^{b\dagger}$
GLN (n=8)	7.95 ± 1.52^{a}	24.08 ± 1.60^{ab}	1.95 ± 0.45^{a}	67.25 ± 1.08^{bc}	5.32 ± 0.98	6.8 ± 0.82	0.51 ± 0.067^{a}
FO (n=7)	9.38 ± 2.46^{ab}	30.53 ± 2.16^{b}	$2.99\pm0.90^{a\dagger}$	$59.3\pm1.2^{ab\dagger}$	5.5 ± 1.40	7.75 ± 1.046	0.66 ± 0.1^{a}
GLN+FO (n=9)	$16.68\pm1.84^{b\dagger}$	$40.43\pm2.76^{c\dagger}$	$6.59\pm0.29^{b\dagger}$	$48.8\pm3.22^{a\dagger}$	8.3 ± 1.53	8.3 ± 0.84	$1.34\pm0.037^{b\dagger}$

*Whole blood was collected prior to initiation of CPT-11/5-FU chemotherapy when all groups for had tumors of approximately 2.0 cm^3 in volume (1.2% of body weight) (Table 4-2A) and 13 days after completion of CPT-11/5-FU chemotherapy (at the end of the study) (Table 4-2B). Data are presented as mean ± SEM.

For both pre-chemotherapy and post-chemotherapy data, means within a column that do not share a common letter are different, $p<0.05$.

†$p<0.05$ vs. corresponding treatment prior to chemotherapy, paired *t-test.*

Table 4-3. Dietary effect on GSH content in host colonic mucosa and tumor tissues following CPT-11/5-FU chemotherapy

Treatment group	tGSH	GSSG	rGSH	rGSH/GSSG ratio
	μmol/g tissue	*μmol/g tissue*	*μmol/g tissue*	
Colonic mucosa				
REF	1.86±0.12	0.014±0.002[a]	1.83±0.12	136±13[b]
CON	1.65±0.06	0.033±0.010[b]	1.58±0.05	68±18[a]
GLN	1.57±0.10	0.012±0.001[a]	1.55±0.10	136±13[b]
FO	1.72±0.12	0.015±0.003[ab]	1.69±0.11	124±13[b]
GLN+FO	1.74±0.06	0.015±0.002[ab]	1.71±0.06	118±13[ab]
Tumor				
CON	1.56±0.10	0.017±0.003	1.52±0.09	99±15[b]
GLN	1.20±0.17	0.024±0.004	1.15±0.16	55±10[a]
FO	1.26±0.14	0.023±0.004	1.22±0.14	59±11[ab]
GLN+FO	1.10±0.12	0.028±0.006	1.04±0.12	47±13[a]

GSH concentration was analyzed in the host colonic mucosa and tumor tissue at the end of the study (13 days after completion of CPT-11/5-FU treatment).

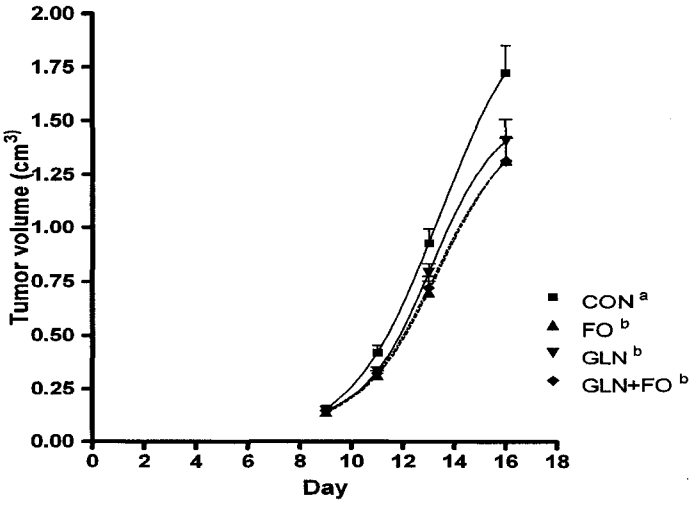
All the data are reported as mean ± SEM.

For both colonic mucosa and tumor tissues, means within a column that do not share a common letter are different, $p<0.05$.

FIGURES

Figure 4-1.

A.



B.

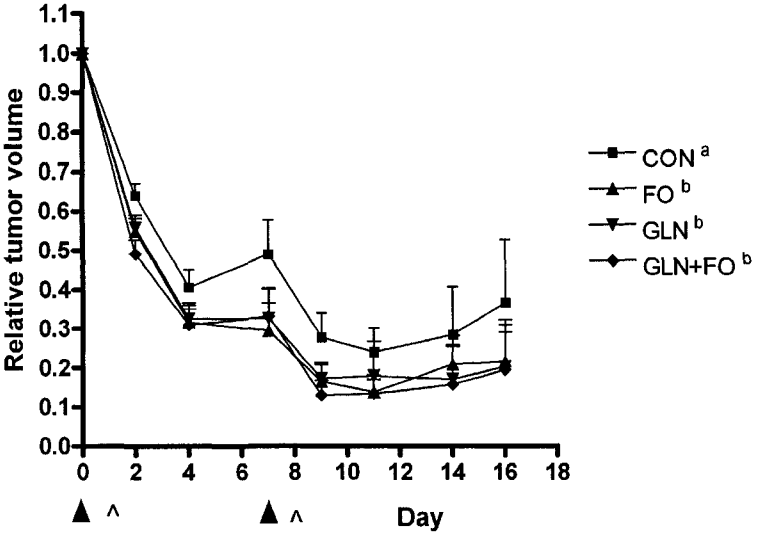


Figure 4-1. Dietary effects on Ward colon tumor growth and anti-tumor efficacy of CPT-11/5-FU chemotherapy *in vivo.*

A. Effect of dietary treatment per se on Ward colon tumor growth in vivo

Fisher rats were implanted with Ward colon tumor and changes in tumor volume were followed.

B. Dietary modification of anti-tumor efficacy of CPT-11/5-FU therapy

CPT-11/5-FU treatment was initiated when rats of all the dietary treatment groups had tumors of approximately 2.3 cm^3 in volume. Y axis represents the relative tumor volume as compared to the baseline volume when the chemotherapy was initiated. ▲indicates a single CPT-11 injection at 50 mg/kg; ^ indicates a single 5-FU injection at 50 mg/kg.

*In the legend of each figure, dietary treatments which do not share a common letter are different ($p<0.05$, *post hoc* Tukey's).

Data are presented as mean ±SEM.

Figure 4-2.

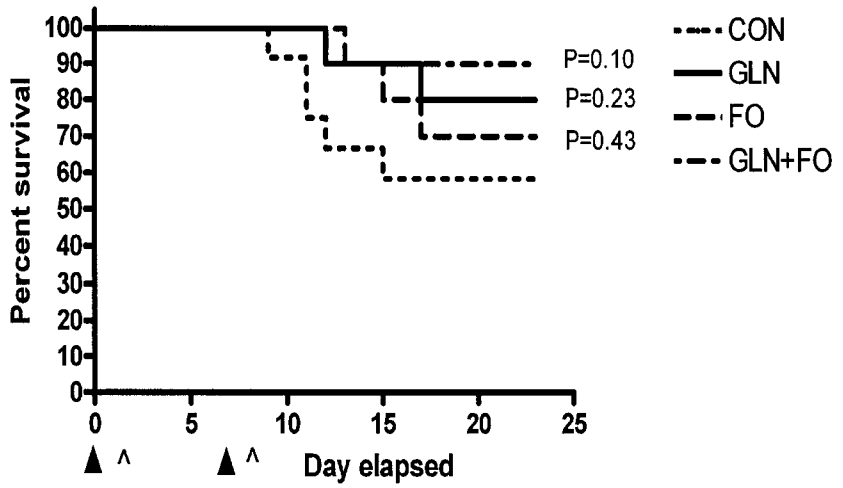
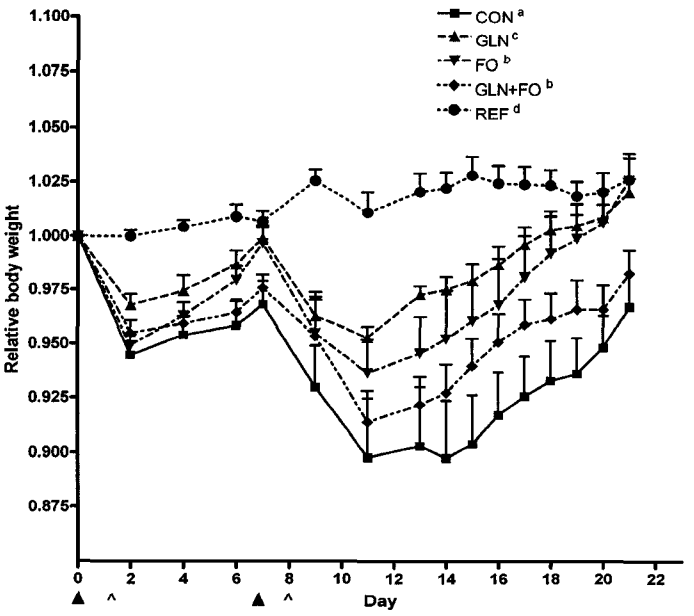


Figure 4-2. Dietary effect on CPT-11/5-FU-induced mortality

Post-chemotherapy survival associated with different dietary treatments was analyzed using Kaplan-Meier method and overall survival between groups were analyzed using the log-rank test. ▲indicates a single CPT-11 injection at 50 mg/kg; ^ indicates a single 5-FU injection at 50 mg/kg.

Figure 4-3.

A.


CON a
GLN c
FO b
GLN+FO b
REF d
Relative body weight
1.100
1.075
1.050
1.025
1.000
0.975
0.950
0.925
0.900
0.875
0 2 4 6 8 10 12 14 16 18 20 22
Day

B.

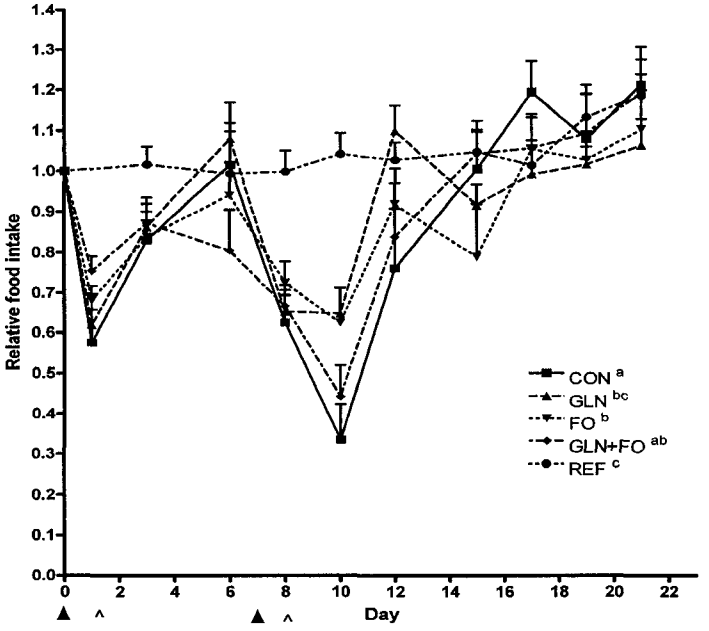

Relative food intake
1.4
1.3
1.2
1.1
1.0
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0.0
0 2 4 6 8 10 12 14 16 18 20 22
Day
CON a
GLN bc
FO b
GLN+FO ab
REF c

Figure 4-3. (Cont'd)

C.

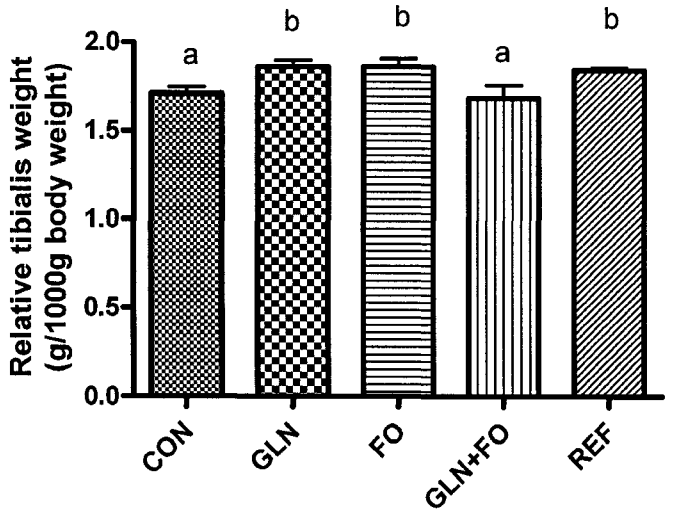


Figure 4-3. Dietary effect on host nutritional features following CPT-11/5-FU therapy

A. Body weight change. Y axis represents body weight relative to baseline value when the chemotherapy was initiated. ▲indicates a single CPT-11 injection at 50 mg/kg; ^ indicates a single 5-FU injection at 50 mg/kg.

B. Feed intake change. Y axis represents daily feed intake level relative to that prior to chemotherapy initiation. ▲indicates a single CPT-11 injection at 50 mg/kg; ^ indicates a single 5-FU injection at 50 mg/kg.

Data are presented as mean ±SEM.

C. Muscle weight. At the end of the study, left tibialis anterior muscle was isolated from the killed rats and weighed. Y axis represents the muscle weight relative to the whole body weight at kill.

Data are presented as mean ±SEM.

*In the legend of Figures 4-3A and 3B, dietary treatments which do not share a common letter are different ($p<0.05$, *post hoc* Tukey's).

In Figure 4-3C, means that don't share a common letter are significantly different ($p<0.05$, *post hoc* Tukey's).

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CHAPTER 5 PROPHYLACTIC CIPROFLOXACIN TREATMENT PREVENTED HIGH MORTALITY, AND MODIFIED SYSTEMIC AND INTESTINAL IMMUNE FUNCTION, IN TUMOR-BEARING RATS RECEIVING DOSE-INTENSIVE CPT-11 CHEMOTHERAPY

5.1 Introduction

Dose-intensive systemic chemotherapy is a prevailing tactic used in oncology. However, it presents a potentially fatal challenge to host defense systems. Compromised host immunity and infection is a major contributor to the morbidity and mortality associated with high-dose chemotherapy. CPT-11-based regimens have been consistently shown to compromise the integrity of intestinal epithelial lining (1-4), which could in turn create a nidus for infection. The intestinal epithelial surfaces in combination with the local specialized innate and adaptive mucosal immune system (GALT) form the foremost defense line against invasion by micro-organisms present in the gut lumen. Consistent with a high degree of compartmentalization, the GALT is populated by phenotypically and functionally distinct B cell, T cell and accessory cell subpopulations as compared with systemic lymphoid tissues (5,6). Investigations on chemotherapy-related suppression and reconstitution of immune function have been largely confined to the systemic immune compartments in peripheral blood (7-9). GALT, while less studied in response to chemotherapy, may be of greater interest in the case of agents with a dose-limiting intestinal toxicity such as CPT-11.

Prophylactic use of broad-spectrum antibiotics is a prevalent strategy of preventing systemic infection in high-risk neutropenic patients receiving chemotherapy, irrespective of some concerns over development of antibiotic resistance (10,11). An independent

review panel was struck by the National Cancer Institute of the United States following an excess number of deaths due to GI toxicities from CPT-11 treatment. The results of that review panel recommended that all patients treated with CPT-11 who have diarrhea persisting for more than 48 hours with loperamide treatment be treated with a fluoroquinolone antibiotic for seven days. Fluoroquinolone (e.g., Cipro)-based prophylactic regimens are highly effective against chemotherapy-induced bacteremia originating from gut colonizing bacteria (10-13). Increasing evidence suggests that antibiotics such as quinolones may exert an immunomodulating effect on the host by altering cytokine production of activated T lymphocytes, up-regulating colony-stimulating factor synthesis and thus enhancing hematopoiesis (14-18). However, it remains largely unknown how prophylactic use of antibiotics will potentially affect the functional competence of systemic and gut immunity in the immunosuppressive milieu of cancer chemotherapy.

We aimed to investigate effects of CPT-11 chemotherapy on gut and systemic immune competence. With the use of a quinolone antibiotic, Cipro, we sought to isolate the role of opportunistic bacterial infection in the overall CPT-11-related toxicity profile, and to study the potential modulation by Cipro on systemic and intestinal immunity.

5.2 Materials and methods

Laboratory rat use, tumor implantation and drug formulation (CPT-11 and atropine) are detailed previously (Chapter 2, ref:(19)). The diet used for all the treatment groups in this study was identical with the control diet used in Chapter 2 and 4 (Table 2-1 and 4-1).

5.2.1 Experimental design

All rats transplanted with Ward colon tumor were randomized to receive Cipro or not. Cipro treatment was continuous starting one week prior to initiation of CPT-11 therapy and continued throughout the whole study. Cipro was dissolved in the sterilized drinking water at 100 mg/L to provide ~10 mg/(kg·day) (n=11 rats). Antibiotic solutions were made every 2-3 days to ensure activity. Other rats (n=20) had *ad libitum* access to sterilized drinking water.

When rats had a tumor of ~ 2 cm^3, 3 daily CPT-11 *i.v.* injections at 125 mg/(kg·day) were initiated. Atropine (1mg/kg *s.c.*) was administered immediately before each CPT-11 injection to alleviate the early-onset cholinergic symptoms (19). The day when the first dose of CPT-11 was administered is designated Day 0. Seven days after the last dose of CPT-11 (Day 9), rats were killed. An additional group (healthy controls, n=8) of non-tumor-bearing rats not receiving CPT-11 or Cipro treatment were killed on Day 9.

5.2.2 Outcome measures

Diarrhea assessment and **tumor measurement** were detailed in Chapter 2 (ref:(19)). Rats were killed by CO_2 asphyxiation. Cecal content, spleen and MLNs were collected under aseptic conditions. Tumor, tibialis anterior and medial gastrocnemius muscles were collected and weighed. Whole blood collected respectively at Day 0 (as baseline), 3 and 9 was used for **CBC and differential WBC** count as detailed in Chapter 4. **β-glucuronidase activity** of cecal content was determined as previously described

(Chapter 2, ref:(19)).

5.2.2.1 Spleen and MLN cell phenotype. Immune cells were isolated from MLNs as previously described (20). Isolated cells (200,000 cells/well) were used to determine cell phenotype using 2 color direct immunofluorescence (21). The following antibodies were used: CD3, CD4, CD8, CD25, CD28, CD62L, CD71, CD80, CD45RA (BD Bioscience, Mississauga ON), and OX12 (Cedarlane, Hornby ON); Streptavidin QR (Sigma, Oakville, ON) was added to all Biotin labeled antibodies. The % of immune cells expressing each of the antibody markers was determined by flow cytometry (FacScan, Becton Dickinson, Sunnyvale, CA) (21). We attempted to perform every phenotype assay on each rat, but this was not always possible due to the total yield of tissue; the actual number of rats utilized for each assay is indicated in the result tables

5.2.2.2 Mitogen induced proliferation. Cells ($1.25x10^9$ cells/L) were incubated in a 96-well microtiter plate, in triplicate, in the presence or absence of 5 mg/L of the mitogen, concanavalin A (Con A) (ICN, Montreal, PQ) for 24 and 48 h. Eighteen hours prior to harvesting, cells were pulsed with 0.5 μCi of ^{3}H-thymidine (Amersham Life Sciences, Baie D'Urfe, PQ), harvested on glass-fiber paper filters using a multi-well harvester (Skatron, Lier, Norway) and counted in a β counter (LS-5801 Beckman Canada, Mississauga, ON). The ability to proliferate was defined as a stimulation index (SI), calculated as: the rate of ^{3}H-thymidine incorporated after incubation with Con A/the rate of ^{3}H-thymidine incorporated in the absence of Con A.

5.2.2.3 Mitogen-stimulated cytokine production. Splenocytes and cells from MLN (1.0×10^9 cells/L) were incubated (48 h) in the presence or absence of LPS (100 mg/L) in a humidified atmosphere at 37°C in the presence of 5% CO_2. Supernatants were

removed and stored at -70°C until all the samples were collected. The concentration of IL-1 and -6, IFN-γ and tumor necrosis factor-alpha (TNF-α) was determined using ELISA kits (BD Bioscience, Mississauga, ON) according to specifications using a standard curve. All plates were read at 450 nm in a microplate reader (SpectraMax 190, Molecular Device, Sunnyvale, CA). All cytokines were assayed in duplicate and variation (co-efficient of variance) between replicates determined. If the coefficient of variation between samples was greater than 15% samples were reanalyzed in duplicate. If a concentration of a cytokine was found to be less than the lower detection limit, the half value of lower detection limit was used for statistical analysis.

5.2.2.3 Bacterial translocation. MLNs were aseptically homogenized in 5 mL sterile water and 0.1 mL of these samples was inoculated with blood agar (for Gram+ bacteria) and McConkey agar (for Gram– bacteria). All cultures were incubated under aerobic conditions at 37°C for 48 h and then colony-forming units (CFU) on each plate counted and corrected to the weight of the original tissue.

All parameters were expressed as mean ± SEM. Effect of time on WBC counts following CPT-11 treatment was analyzed via one-way repeated measures ANOVA (SPSS 12.0, SPSS Inc. Chicago, IL). Treatment differences in the immune phenotype were analyzed using one-way ANOVA followed by *post hoc* Tukey's test (SPSS 12.0) unless specified in the table or figure. All immune parameters were tested for normal distribution. Values that were not normally distributed were log transformed prior to statistical analysis. A probability 0.05 was accepted as being statistically significant. We attempted to perform every immune assay on each rat, but this was not always possible; the actual number of rats utilized for each assay is indicated in the result tables.

5.3 Results

5.3.1 CPT-11-related toxicity

CPT-11 was associated with considerable mortality (9/20, 45%) in non-Cipro-treated rats; whereas rats given prophylactic Cipro all survived CPT-11 treatment. Moreover, Cipro treatment strikingly improved the host general nutritional status by reducing body weight loss and muscle wasting (Table 5-1). Rats receiving CPT-11 treatment developed a high incidence and cumulative severity of diarrhea (Table 5-1). Prophylactic Cipro treatment did not significantly alter the diarrhea profile (Table 5-1), nor affect cecal β-glucuronidase activity (Figure 5-1). CPT-11 treatment resulted in bacterial translocation into MLNs; however this was completely abolished with prophylactic Cipro treatment (Table 5-2).

5.3.2 Peripheral WBC counts and spleen weight

CPT-11 treatment alone led to a transient depletion of the peripheral WBC pool, with nadir counts of total WBCs, neutrophils and lymphocytes occurring 1-4 days after completing CPT-11 treatment (Figure 5-2A). There was a rebound-like recovery of WBC counts 7 days after completing CPT-11 treatment. However, this post-chemotherapy 'overshoot' recovery of WBC counts was abrogated with Cipro treatment (Figure 5-2B). Splenic hyperplasia also occurred 7 days after completing CPT-11 therapy; however, the splenic hyperplasia of rats receiving Cipro treatment was significantly smaller in magnitude (Figure 5-2C).

5.3.3 Phenotypic distribution of spleen and MLN cells

5.3.3.1 Non-Cipro-treated animals

In rats not receiving Cipro therapy, CPT-11 treatment resulted in a pronounced alteration in the phenotypic composition of immune cells in both MLNs and spleen (Table 5-3A). Changes in splenocytes largely paralleled with those observed in MLN cells.

CPT-11 treatment led to a relative depletion of CD3+ T cells in both spleen (due to a decrease in both the CD3+CD4+ and CD3+CD8+ population) and MLNs (due to a decrease in the CD3+CD4+ population) with an increased proportion of B cells (OX12+). The proportion of total cells expressing CD45RA+ (antigen naïve marker) was lower in both MLNs and spleen after CPT-11 therapy. More of B and CD8+ T cells, but not CD4+ T cells, were antigen-mature (CD45RA-) after CPT-11 treatment.

There was also a marked change in expression of activation markers by T cells after CPT-11 treatment. In both spleen and MLNs, there was a striking increase (4-13 fold) in helper and suppressor T populations that expressed the co-stimulatory molecule CD28 ($p<0.05$). Within the helper T population in spleen and MLNs, there were more cells expressing transferrin receptor (CD71+) and high abundance of IL-2 receptor (CD25+), but less cells expressing L-selectin (CD62L+).

5.3.3.2 Cipro-treated animals

Overall phenotypic changes following CPT-11 treatment were in the similar direction in rats receiving Cipro as compared to the non-Cipro-treated ones. Nevertheless, Cipro led to a further reduction of CD3+ proportion following CPT-11 in MLN cells, mainly ascribed to the reduction in the proportion of CD3+CD8+ cells. The relative percent of B cells was higher in the Cipro-treated MLNs. Cipro treatment restored the proportion of CD8+CD45RA+ in spleen to proportions not different from healthy control rats. In MLNs, the lower relative percent of CD3+CD8+ cells appeared to be at the expense of CD8+CD45RA- (antigen mature cells). As for other T cell activation markers, the most

consistent finding was that Cipro treatment resulted in strikingly higher number of helper and suppressor T cells expressing IL-2 receptors (CD25+) (as compared to both other two groups) in MLNs (but not spleen). The higher proportion of total CD28+ cells that occurred with CPT-11 treatment remained unaffected (or even further enhanced in suppressor T cells of MLNs) with Cipro therapy in the T populations of both MLNs and spleen. In MLNs and spleen, Cipro treatment raised the proportion of suppressor T cells expressing CD71, but lowered the proportion of CD71+ helper T cells following CPT-11 therapy as compared to non-Cipro-treated rats. Cipro resulted in a lower number of suppressor T cells expressing L-selectin (CD62L+) after CPT-11 therapy as compared to non-Cipro-treated ones.

5.3.4 Proliferative response to Con A.

In the CPT-11 treated group the basal (unstimulated) rates of ^{3}H-thymidine uptake by both MLN cells (24 and 48h) and splenocytes (24h) were significantly higher than healthy control rats (Table 5-3B). Providing Cipro resulted in an unstimulated rate of ^{3}H-thymidine uptake that was comparable or even below levels of healthy control rats. CPT-11 treatment resulted in a reduction in the SI in spleen but an increase in MLNs. The response by cells from Cipro-treated rats was not significantly different from healthy controls in MLNs, and although higher ($p<0.05$) than the CPT-11 cells in spleen, was still significantly lower than healthy controls.

5.3.5 LPS-stimulated cytokine production of splenocytes

In the absence of mitogen (unstimulated), all cytokines measured were below detection limits (data not shown). In the absence of Cipro, CPT-11 significantly suppressed production of IFN-γ and IL-1 in isolated splenocytes stimulated with the

bacterial mitogen, LPS (Table 5-3C). There was no effect of CPT-11 on the production of TGF-β, TNF-α and IL-6. Cipro treatment did not influence the chemotherapy-induced depression of IFN-γ and IL-1 production, but led to significantly higher production of TNF-α and IL-6 as compared to both healthy control rats and those receiving CPT-11 alone.

5.4 Discussion

Opportunistic infection is an important cause of morbidity and mortality in patients receiving dose-intensive cancer chemotherapy. GI tract structure and functional integrity is particularly vulnerable to chemotherapy treatment, and constitutes a major gateway for local and systemic invasion of various pathogens (22). This is particularly problematic when using CPT-11, which is preferentially cytotoxic to GI mucosal cells and results in prominent GI toxicities (1,19,23,24) frequently at the time of profound myelosuppression.

Prophylactic use of Cipro completely prevented CPT-11-induced mortality, and conferred significant protection from weight loss and muscle wasting. However, Cipro was unable to alter the severity or course of diarrhea, a hallmark and dose-limiting toxicity for CPT-11-based regimens, nor did it affect activity of bacterial β-glucuronidase, a key enzyme activating of the toxic CPT-11 metabolite, SN-38 (25,26). Therapeutic benefits associated with Cipro treatment were independent of the diarrhea toxicity. It is a plausible conjecture that bacteremia or septicemia secondary to CPT-11 treatment was the predominant contributor to mortality in the CPT-11 treated rats, and that Cipro was able to limit this by reducing total bacterial translocation and through the immunomodulatory activity of this agent.

5.4.1 Alterations of systemic and intestinal immune competence associated with CPT-11 treatment alone

At 7 days following CPT-11 chemotherapy, a quantitative rebound was observed in peripheral immune compartments as manifested with restored peripheral blood counts of various leukocyte lineages and splenic hyperplasia. Moreover, our results demonstrated a preponderance of activated T cells (increased percentages of CD45RA-, CD71, CD25 and

decreased percentage of CD62L in CD4+ and CD8+ T cells) following CPT-11 treatment. The antigen-naïve T cells can also be phenotypically characterized by expressions of the high-molecular-weight isoform of CD45 (CD45RA) and the peripheral lymph node homing receptor CD62L (L-selectin) (27,28). When naïve T cells are stimulated by specific antigens, their cell surface phenotype undergoes a number of changes. First, expression of CD45RA is lost and the low molecular weight isoform CD45RO is expressed; next to this, CD62L shed from the cell surface and various surface markers are upregulated at various stages of cell activation, such as transferrin receptor CD71 (early) and IL-2 receptor CD25 (late) (29-31). Such post-chemotherapy phenotypic activation has also been observed previously (32-35). Supported by the profound phenotypic activation, there was a concomitantly raised basal (unstimulated) rate of ^{3}H-thymidine uptake by both MLN cells and splenocytes. Apparently, a marked *in vivo* activation occurred in these different immune compartments after chemotherapy. Substantial translocation of pathogenic microorganisms and their products (i.e., endotoxin) could be a key attributable factor for the observed *in vivo* activation.

5.4.1.1 Hyporesponsive and anergic state of splenocytes

Quantitative changes of various subsets of immune cells have been a predominant focus of studies investigating effects of high-dose chemotherapy on immune system. However, alteration of functional competence following chemotherapy may or may not be reflected in these cell number changes. Our results show a striking 'discordance' in phenotypic and functional changes of splenocytes following CPT-11 therapy. Despite the overall quantitative recovery and phenotypic activation of immune cells in peripheral blood and spleen, splenocytes were unable to proliferate upon stimulation of ConA *in vitro*, and had a depressed ability of cytokine production upon LPS stimulation, especially

for the production of IFN-γ, a pivotal cytokine initiating Th1 response (36). The inability of splenocytes to mount an effective immune response upon *in vitro* mitogen stimulation suggests that the systemic immunity (rather than local intestinal immunity) was in a state of 'anergy' (37). Such functional incompetence observed *in vitro* may reflect the compromised capacity for response to antigen *in vivo* and as such an enhanced susceptibility to opportunistic infections.

Hakim *et al.* (33) found that *in vivo* phenotypic activation of T cells following chemotherapy was associated with a heightened susceptibility to activation-induced apoptosis upon mitogen stimulation *in vitro*. Our results showed that stimulation indices of splenocyte proliferation upon mitogen stimulation were below 1, which suggests that splenocytes may undergo cell death instead of being induced to proliferate by mitogens. The susceptibility to activation-induced apoptosis could be responsible for the observed depression of cytokine and proliferation response to mitogen stimulation *in vitro* as activated cells were eliminated, and could also result in the relative depletion of T cell abundance following chemotherapy. Furthermore, the potential contribution of severe systemic infection (i.e., septicemia), which occurred presumably in a high incidence among non-Cipro-treated rats, in the post-chemotherapy immune anergy should be taken into consideration. In septic patients, a hypoinflammatory state characterized by hyporesponsiveness and anergy of circulating leukocytes, a phase also named as compensatory anti-inflammatory response syndrome (CARS) (38), was regularly observed following the initial hyperinflammatory phase, also known as systemic inflammatory response syndrome (SIRS) (39,40). Taken together, the post-chemotherapy hyporeactivity of immune cells in the spleen (a compartment reflecting the systemic immunity) could be an integrated outcome reflecting both chemotherapy's direct

immunosuppression and the phasic progression of complicated sepsis.

5.4.1.2 Hyperresponsive state of MLN cells

Notably, despite the depressed responsiveness of splenocytes after CPT-11 treatment, immune cells from MLNs, a compartment of GALT, displayed a remarkably up-regulated proliferation response upon T cell mitogens *in vitro*. This dichotomy suggests that the effects of CPT-11 on the immunologic competence was compartmentalized with a primed local intestinal immunity and concomitantly suppressed systemic immunity. Our results were consistent with previous findings by de Koning *et al.* (41), who also demonstrated that innate and adaptive immune responses of GALT cells were intact or even primed following high-dose methotrexate treatment. CPT-11 treatment has been consistently shown to destroy intestinal barrier integrity, and this may render immune cells localized to various GALT compartments increasingly exposed to bacterial-derived immunogens such as antigens and LPS. Whether endogenous priming of GALT cells by gut-derived local stimuli contributes to the observed compartmentalized preservation of immune competence needs to be further defined in context of dose-intensive chemotherapy. The hyperresponsiveness of intestinal local immune cells may be required for the chemotherapy-induced gut injury (41), and presumably it may assume an important source for the antigen-driven peripheral T cell expansion to maintain the homeostasis of T cell pool after the depletion by chemotherapy (42). Moreover, this dichotomy observed in splenocytes and MLN cells may also be a reflection of the compartmentalizing effect derived from the complicated sepsis or SIRS following the dose-intensive chemotherapy. Numerous examples illustrate that the hyporeactivity subsequent to sepsis or SIRS is essentially observed in hematopoietic compartments (peripheral blood and spleen) (43-45), whereas lymphocytes derived from the inflamed tissues or infectious foci are

activated and primed and fully responsive to *in intro* mitogen stimulation (46,47). Localization of inflammatory response to the gut, where the infectious nidus and inflamed foci was developed with the destruction of mucosal integrity by chemotherapy, may serve as an important strategy the body employs to prevent systemic inflammation and igniting new inflammatory foci (48).

5.4.2 Effects of Cipro on the alterations of immune competence following CPT-11 therapy

Antibiotics do not act alone *in vivo* but in conjunction with host defenses through modulating innate or adaptive immune responses (14-18). Our work is the first to systematically investigate Cipro's immunomodulatory effect in the context of high-dose chemotherapy, in multiple dimensions including phenotypic distribution, functional competence and compartmentalized impacts (spleen vs. MLNs).

Interestingly, Cipro treatment seemed to exert a dual effect on immune response in different immune compartments. In the spleen, Cipro treatment abrogated the depressed mitogens response which might indicate that it prevented activation-induced cell death following CPT-11 therapy (SI of mitogen-stimulated proliferation was raised to 1). However the proliferative response was still depressed as compared to the healthy controls; whereas in MLN cells, the hyperresponsiveness following CPT-11 therapy was abolished and the proliferative reactivity was normalized to a comparable level as the healthy controls. Defense against infection of rapidly growing viruses and bacteria requires an immediate and adequate response to limit growth and dissemination of the pathogens (49). The partially improved proliferative response in spleen suggests an enhanced capacity to respond to immune challenges by blood-borne pathogens in

Cipro-treated rats. Activation and the hyperresponsiveness of GALT immune cells may be involved in the pathogenesis of high-dose chemotherapy-induced gut injury (41). It may reflect a favorable role of Cipro treatment in attenuating the excessive proinflammatory responses occurring locally in the gut upon CPT-11 insults. Further studies are needed to correlate the observed effect of Cipro on proliferative response with its potential modulatory effect on production of cytokines, i.e., TNF-α and Il-1, which are shown to mediate the gut injury (41,50) .

Accumulating evidence suggests that immunomodulatory actions of quinolone antibiotics, such as Cipro, also largely relies on their capacity to modify cytokine production (51). Inflammatory cytokines including IFN-γ, TNF-α, IL-1 and IL-6 are essential to evoke an effective immune response to invasive pathogens (52-54). Our results show the splenocytes were anergic after CPT-11 chemotherapy, with depressed capacity in producing IFN-γ and IL-1 upon LPS stimulation. This suggests suppressed cell-mediated immunity, which essentially depends on these Th1 cytokines (54), and could be attributable to enhanced susceptibility to secondary systemic infection as well as the high mortality caused by the post-chemotherapy sepsis (55,56). Despite the failure of influencing the production of IFN-γ and IL-1, Cipro treatment upregulated LPS-stimulated production of TNF-α and IL-6 by splenocytes. This was consistent with previous findings that therapeutic Cipro concentrations increase LPS-stimulated production of inflammatory cytokines including TNF-α and IL-6 by monocytes (57). Collectively, Cipro treatment appeared to correct the reduced mitogen-stimulated proliferative response and promoted inflammatory cytokine production in spleen, which may mitigate post-chemotherapy immunologic anergy and favor development of appropriate defences against pathogens disseminated systemically, meanwhile, it may

alleviate the activated proinflammatory response occurring in the local immune compartment (i.e., MLNs), which mediates chemotherapy-induced mucosal inflammatory injury.

TABLES

Table 5-1. Effects of Cipro treatment on the toxicity profile of CPT-11 treatment

Treatment		N	Mortality	Incidence of severe delayed diarrhea	Area under curve of the diarrhea score	Relative body weight at Day 6	Tibialis muscle weight at Day 9	Medial gastrocnemius muscle weight at Day 9
CPT-11	Cipro							
			%	%		%	*g/1000g body weight*	*g/1000g body weight*
None	None	8	-	-	-	98.5±0.6[c]	1.80±0.05[b]	2.05±0.02[b]
Yes	None	20	45	55.0±3.9	19.5±0.5	80.6±0.7[a]	1.60±0.03[a]	1.93±0.04[a]
Yes	Yes	11	0	47.7±5.5	18.6±0.6	90.5±0.5[b]	1.74±0.02[b]	2.04±0.04[b]

Means within a column that do not share a common letter are significantly different ($p<0.05$).

All data are presented as mean ± SEM.

Table 5-2. Effects of CPT-11 +/- Cipro on bacterial translocation into MLNs 7 days following completion of CPT-11chemotherapy (Day 9)

Treatment		Gram positive bacteria	Gram negative bacteria
CPT-11	Cipro	$\times 10^3$ CFU/g tissue*	$\times 10^3$ CFU/g tissue
None	None	0†	0
Yes	None	3.6±0.7‡	3.5±1.1‡
Yes	Yes	0	0

*CFU=colony-forming unit.

†Not detectable within the sensitivity of the method used.

‡$p<0.0001$ vs. Healthy controls and CPT-11+ Cipro

Table 5-3. Effects of CPT-11 +/- Cipro on systemic and intestinal immune function

A. Effects of CPT-11 treatment and Cipro on phenotypic distribution of immune cells in MLNs and spleen

	MLNs			Spleen		
Antibody	Healthy controls n=8	CPT-11 alone n=9	CPT-11 + Cipro n=6	Healthy controls n=8	CPT-11 alone n=9	CPT-11 + Cipro n=6
OX12+ (% of total cells)	13.0 ± 0.6^{a}	19.8 ± 1.8^{b}	27.3 ± 1.9^{c}	29.1 ± 0.4^{a}	36.4 ± 1.8^{b}	36.9 ± 1.1^{b}
% OX12+CD45RA-	1.5 ± 0.3^{a}	5.3 ± 0.6^{b}	5.0 ± 1.7^{b}	7.5 ± 0.8^{a}	28.7 ± 3.0^{b}	8.3 ± 0.9^{a}
% OX12+CD45RA+	98.5 ± 0.3^{b}	94.7 ± 0.6^{a}	95.0 ± 1.7^{a}	92.5 ± 0.8^{b}	71.3 ± 3.0^{a}	91.7 ± 0.9^{b}
% OX12+CD80+	0.7 ± 0.1^{a}	1.5 ± 0.5^{ab}	2.6 ± 1.1^{b}	2.8 ± 0.4	3.9 ± 0.7	2.5 ± 0.3
CD3+ (% of total cells)	70.8 ± 1.2^{c}	66.4 ± 1.4^{b}	57.5 ± 1.2^{a}	55.4 ± 0.6^{b}	46.4 ± 2.1^{a}	49.6 ± 0.8^{a}
CD3+CD8+ (% of total cells)	22.2 ± 0.8^{b}	23.0 ± 0.6^{b}	17.9 ± 0.5^{a}	31.9 ± 0.9^{b}	21.0 ± 0.9^{a}	23.9 ± 1.1^{a}
% CD8+CD71+	9.2 ± 1.2^{a}	10.5 ± 1.0^{a}	19.3 ± 2.6^{b}	25.6 ± 0.8^{a}	23.9 ± 1.2^{a}	28.8 ± 1.3^{b}
% CD8+CD28+	4.0 ± 0.6^{a}	50.8 ± 4.5^{b}	75.2 ± 2.9^{c}	10.0 ± 0.5^{a}	52.3 ± 6.3^{b}	44.5 ± 9.3^{b}
% CD8+CD25+	9.3 ± 0.3^{a}	9.4 ± 1.0^{a}	40.2 ± 9.1^{b}	8.6 ± 0.8^{a}	16.4 ± 3.7^{b}	14.0 ± 1.2^{b}
% CD8+CD62L+	38.0 ± 2.0^{b}	42.8 ± 3.1^{b}	30.1 ± 2.4^{a}	33.8 ± 0.9^{b}	34.7 ± 3.1^{b}	15.7 ± 0.6^{a}
% CD8+CD45RA-	62.4 ± 1.7^{b}	76.7 ± 6.5^{c}	38.4 ± 2.5^{a}	31.8 ± 1.2^{a}	45.9 ± 4.6^{b}	28.1 ± 1.5^{a}
% CD8+CD45RA+	37.6 ± 1.7^{b}	23.3 ± 6.5^{a}	61.6 ± 2.5^{c}	68.2 ± 1.2^{b}	54.1 ± 4.6^{a}	71.9 ± 1.5^{b}
CD3+CD4+ (% of total cells)	48.7 ± 0.7^{b}	43.3 ± 1.4^{a}	40.7 ± 1.2^{a}	32.4 ± 0.5^{b}	22.2 ± 1.0^{a}	20.4 ± 1.7^{a}
% CD4+CD71+	6.5 ± 0.7^{a}	9.2 ± 1.2^{b}	4.4 ± 0.9^{a}	22.1 ± 1.1^{a}	39.9 ± 3.8^{c}	26.7 ± 2.1^{b}
% CD4+CD28+	5.5 ± 0.3^{a}	73.9 ± 5.1^{b}	85.2 ± 2.2^{b}	15.2 ± 0.9^{a}	60.7 ± 10.4^{b}	77.6 ± 2.5^{b}

% CD4+CD25+	7.5 ± 0.4^{a}	9.8 ± 0.7^{b}	37.3 ± 11.9^{c}	9.4 ± 0.4^{a}	15.4 ± 2.2^{b}	10.8 ± 0.9^{ab}
% CD4+CD62L+	74.7 ± 4.2^{b}	53.3 ± 6.5^{a}	59.4 ± 4.4^{a}	67.8 ± 2.1^{b}	36.1 ± 4.7^{a}	28.4 ± 1.0^{a}
% CD4+CD45RA-	93.8 ± 0.7^{b}	91.9 ± 3.4^{ab}	90.6 ± 0.5^{a}	80.2 ± 0.5^{a}	79.9 ± 2.0^{ab}	83.4 ± 1.3^{b}
% CD4+CD45RA+	6.2 ± 0.7^{a}	8.1 ± 3.4^{ab}	9.4 ± 0.5^{b}	19.8 ± 0.5^{b}	20.1 ± 2.0^{ab}	16.6 ± 1.3^{a}
CD3+CD4+/CD3+CD8+	2.2 ± 0.1^{b}	1.9 ± 0.0^{a}	2.3 ± 0.1^{b}	1.0 ± 0.0^{b}	1.1 ± 0.0^{b}	0.9 ± 0.1^{a}
CD71 (% of total cells)	15.8 ± 0.7^{b}	12.8 ± 0.9^{a}	14.6 ± 0.6^{ab}	23.5 ± 0.8^{a}	33.5 ± 2.6^{b}	22.6 ± 2.1^{a}
CD25 (% of total cells)	4.3 ± 0.2^{a}	5.7 ± 0.4^{b}	27.1 ± 7.5^{c}	5.5 ± 0.5^{a}	10.2 ± 1.9^{b}	6.5 ± 0.6^{ab}
CD28 (% of total cells)	3.4 ± 0.2^{a}	44.8 ± 3.2^{b}	55.4 ± 1.9^{c}	7.4 ± 0.5^{a}	29.3 ± 2.9^{b}	38.5 ± 3.5^{b}
CD62L (% of total cells)	48.2 ± 2.8^{b}	46.7 ± 2.5^{b}	35.3 ± 1.1^{a}	39.3 ± 1.2^{c}	31.4 ± 1.4^{b}	15.3 ± 0.5^{a}
CD45RA+ (% of total cells)	36.3 ± 1.3^{b}	24.8 ± 1.7^{a}	35.0 ± 1.6^{b}	56.3 ± 0.5^{c}	36.4 ± 3.2^{a}	52.0 ± 1.1^{b}

Data presented as cell population percentage mean ± SEM; means within a row that do not share a common letter are significantly different ($p<0.05$).

B. Effects of CPT-11 +/- Cipro on *in vitro* proliferation in response to Con A by immune cells in MLNs and spleen

		MLNs			Spleen		
Mitogen	Unit	Healthy controls	CPT-11 alone	CPT-11 + Cipro	Healthy controls	CPT-11 alone	CPT-11+ Cipro
24 hours							
None	DPM	668±51 (8)^{b}	1359±143 (9)^{c}	482±65 (8)^{a}	2954±75 (8)^{a}	11204±1046 (9)^{b}	3498±348 (8)^{a}
48 hours after mitogen stimulation							
None	DPM	317±53 (8)^{a}	1023±128 (5)^{b}	251±57 (7)^{a}	5001±588 (8)^{a}	5603±840 (9)^{a}	3789±778 (8)^{a}
Con A	SI	35±10 (8)^{a}	104±16 (5)^{b}	29±11 (7)^{a}	8.70±1.50 (8)^{c}	0.10±0.02 (9)^{a}	1.05±0.33 (8)^{b}

Data are presented as mean ± SEM (number of rats), means within a row that do not share a common letter are significantly different ($p<0.05$).

C. Effects of CPT-11 treatment +/- Cipro on cytokine production by splenocytes stimulated by LPS

Cytokine	Healthy controls	CPT-11 alone	CPT-11+ Cipro
		$\times 10^{-9}$ g/L	
IL-1β	239±15 (8)[b]	186±19 (9)[a]	173±15 (7)[a]
IFN-γ	1891±90 (8)[b]	244±68 (8)[a]	127±34 (7)[a]
TNF-α	377±11 (8)[a]	437±48 (9)[a]	631±29 (6)[b]
IL-6	737±54 (8)[a]	868±75 (9)[a]	1247±114 (7)[b]

Data are presented as mean ± SEM (number of rats), means within a row that do not share a common letter are significantly different ($p<0.05$).

FIGURES

Figure 5-1.

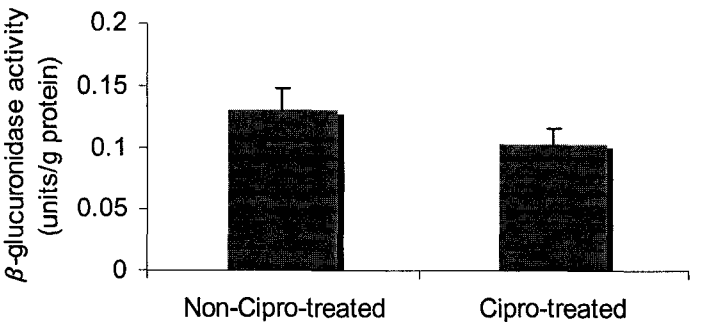
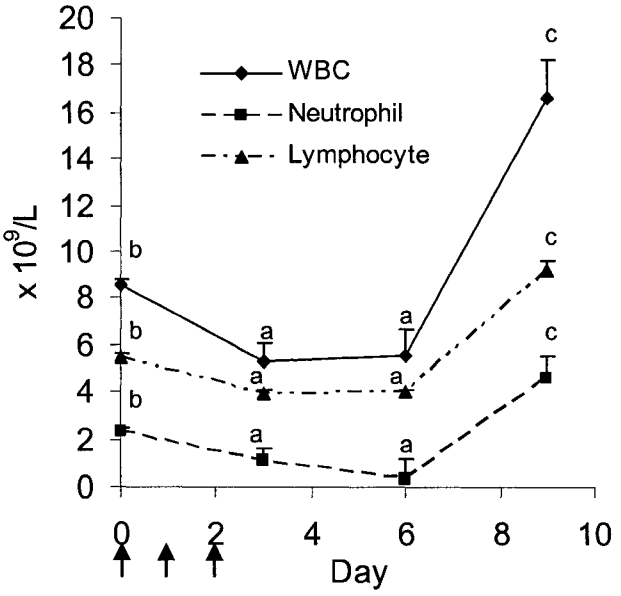


Figure 5-1. Effects of antibiotic treatment on β-glucuronidase activity in the cecal content

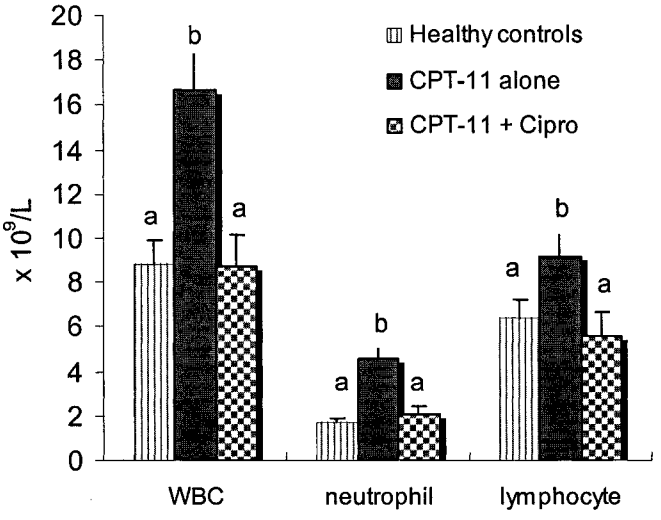
Rats receiving prophylactic antibiotic treatment were killed (without receiving CPT-11 treatment), and β-glucuronidase activity was determined in the collected cecal contents.

Figure 5-2.

A.


20
18
16
14
12
10
8
6
4
2
0
x 10^9/L
WBC
Neutrophil
Lymphocyte
b
a
a
c
b
a
a
c
b
a
a
c
0
2
4
6
8
10
Day

B.


20
18
16
14
12
10
8
6
4
2
0
x 10^9/L
Healthy controls
CPT-11 alone
CPT-11 + Cipro
a
b
a
a
b
a
a
b
a
WBC
neutrophil
lymphocyte

C.

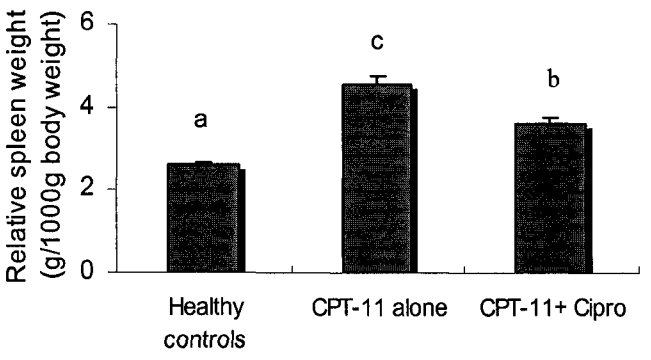


Figure 5-2. Effects of CPT-11 treatment +/- Cipro on peripheral WBC counts and splenic weight

A. Time course of peripheral WBC counts in rats receiving CPT-11 chemotherapy alone without Cipro. Whole blood was harvested from tumor-bearing rats at the indicated time points following CPT-11. Data (mean ± SEM) represents total WBC, neutrophil and lymphocyte counts at corresponding time points. Differences of total WBC, neutrophil and lymphocyte counts at different time points after chemotherapy were analysed by one-way repeated measures ANOVA followed by post hoc Tukey's. Means for a certain count (total WBC, neutrophil or lymphocyte) that do not share a common letter are different ($p<0.05$).

B. Differential WBC count in peripheral blood 7 days after CPT-11 chemotherapy. Data (mean ± SEM) represents total WBC, neutrophil and lymphocyte counts. Means that do not share a common letter are significantly different ($p<0.05$).

C. Effects of CPT-11 treatment +/- Cipro on spleen weights. Relative spleen weights (Y axis) are calculated by comparing with the total body weight on Day 9. Data are presented as mean ± SEM. Means that do not share a common letter are significantly different ($p<0.05$).

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CHAPTER 6 GENERAL DISCUSSION AND CONCLUSIONS

Cancer chemotherapy acts through disruption in normal cellular processes, with the premise that cancer cells are more susceptible to these agents than normal tissues, and thus provides a window of therapeutic efficacy. Although cancer chemotherapy succeeds in producing significant efficacy against a wide array of malignancies, toxicity is inherent to chemotherapy and has also brought with it significant morbidity or even mortality in patients being treated for various cancers. Diets or component bioactive substances in foods are garnering more attention and have been hypothesized to be a potentially important approach to modulate the therapeutic index of chemotherapy through multilateral influences on the interaction between host, cancer and the anti-cancer drug. In spite of increasing interest in diet and dietary supplements, evidence leading to rational advice to patients remains weak and systematic research is warranted to build up evidence-based rationales for integrating nutritional maneuvers into cancer chemotherapy regimens.

This thesis work, highlighted with the following major features, generates evidence of potential significance for lending translatable information for clinical strategy design:

The whole work is built in a clinically relevant model system and a controlled dietary design; nutritional therapies are assessed in a comparative manner; multi-leveled investigations point to issues resting on multiple dimensions related to cancer chemotherapy: role of diet in host×tumor×chemotherapy interaction; interaction between

different nutrients; interaction between different forms of toxicities (i.e., gut toxicity and immunotoxicity) in relation to systemic consequence caused by cancer chemotherapy (i.e., bacteremia and sepsis).

Revolving around the main theme on nutrition and chemotherapy, this thesis work produces results primarily answering questions resting on three levels: **what nutrients to choose; how do nutritional elements work; and how to administer/supplement these nutrients.**

6.1 What nutrients to choose

Within this domain, the following conclusions are derived from our observations:

- *Not all the three nutritional factors tested exhibited efficacy to modulate diarrhea toxicity or anti-tumor activity related to CPT-11 chemotherapy under identical diet/tumor/chemotherapy/toxicity settings. Glutamine could potentially be an antagonist for CPT-11-induced diarrhea; n-3 PUFAs are a potential chemosensitizing agent for CPT-11 chemotherapy; prebiotic oligosaccharides are not conducive to favorably affecting CPT-11's therapeutic index either by modulating diarrhea toxicity or the tumor's chemosensitivity.*

As mentioned in Chapter 1, although the preclinical findings which support a positive role of dietary manipulation during cancer chemotherapy are tantalizing, a gap still

remains preventing a straightforward translation of these preclinical findings into a consensus and evidence-based rationale for clinical use. The bulk of literatures attempting to support the supplementation of a certain nutrient are not set on the basis of a widely agreed basal (background) dietary design and controls. This forms a major barrier preventing a meaningful comparison between these studies. Furthermore, a wide variety of end points have been used, and a large number of studies do not assess clinically important outcomes or treat them as the primary end point, or use surrogates that have not been validated as predictive for clinical outcomes. For most of the nutrients studied, there is a lack of definitive and predictable physiologic end points identified from the preclinical findings and may render it difficult to define an appropriate end point for the clinical trials. In addition, the heterogeneous drug/tumor/toxicity models used and the wide variability of doses, timing, and duration of a certain dietary modality also hamper a meaningful interpretation and reconciliation of these preclinical results.

Not like most of the available studies, which are centered on a single factor of interest, this work made a back-to-back comparison of three nutrients in an identical setting, which makes it possible to interpret their relative efficacy on defined end points. The clinical ties inherent to our drug model system, dietary design and end point selection also add to the translatability of this animal work. Not all elements were equally effective in the same setting of drug, background diet and the selected end point (CPT-11-induced diarrhea), though all the three tested nutrients have been suggested to have benefits against certain forms of chemotherapy-induced gut toxicity individually in different

settings. A comparison like this could lend evidence-based information for choosing appropriate nutritional modality in the clinical trial.

Glutamine is the only factor amongst the three tested that demonstrated efficacy in preventing CPT-11-induced diarrhea. Histological damage caused by CPT-11 at a dose of low-intensity could be substantially mitigated by supply of *n*-3 PUFAs(1). But our results suggest supplying *n*-3 PUFAs in a nutritionally relevant design (dose/control/basal diet) did not confer a direct and observable benefit to the diarrhea toxicity related to maximal-tolerated-dosed CPT-11 treatment. Prebiotics have been suggested to be effective against some types of diarrhea including 5-FU-induced diarrhea (2-5), and there could be a predisposition in using prebiotics in managing CPT-11-induced diarrhea. Our results show that inulin/oligofructose prebiotics were assuredly of no help to prevent CPT-11-induced diarrhea, and actually doubled the activity of β-glucuronidase. As such, caution needs to be exercised when taking prebiotics during CPT-11 chemotherapy. Additionally, given that vast variation exists in the prebiotic fiber intake in human diets, the prebiotic fiber contents in the diet may also serve as an innate risk factor for CPT-11-induced diarrhea.

- *Both glutamine and n-3 PUFAs exhibited significant discrimination/selectivity in their actions towards the host vs. the tumor. Glutamine protected the host, but did not counteract chemotherapy's efficacy; n-3 PUFAs enhanced the tumor's sensitivity to chemotherapy, but did not potentiate chemotherapy's toxic effect on the host.*

There is a highly prevalent notion amongst both cancer patients and health care professionals that nutritional support "feeds the tumor" and supports tumor cell proliferation. It is especially a concern when purified nutrients are given at high doses. Our results demonstrated that high-dose glutamine treatment did not desensitize tumor's response to chemotherapy in the short term as demonstrated by the study with CPT-11 monotherapy, as well as in a relatively longer term study with CPT-11/5-FU combination chemotherapy, a situation in which glutamine treatment actually enhanced the tumor's response to chemotherapy. Long-term pretreatment of glutamine *per se* did indeed inhibit tumor growth instead of stimulating its growth in our study, which has also been reported with several other experimental tumor models (6-11). Similarly, a crucial challenge in seeking an appropriate chemosensitizer is how to target and confine its sensitizing effect to tumor tissues without affecting the host tissues' sensitivity to chemotherapy's cytotoxic effect (12-15). In both of our monotherapy and combination chemotherapy models, *n*-3 PUFAs consistently potentiated chemotherapy's efficacy in addition to their independent anti-tumor effects. Importantly, *n*-3 PUFAs did not seem to aggravate toxicities while increasing the drugs' anti-tumor activity. Conversely, supply of *n*-3 PUFAs was shown to mitigate the systemic toxicity associated with CPT-11/5-FU chemotherapy, though unable to show a clear ameliorative effect on diarrhea as a targeted endpoint for CPT-11-induced gut toxicity. Collectively, both glutamine and *n*-3 PUFAs have shown therapeutic promise to be further developed as nutritional adjuncts to chemotherapy regimens for treating

colorectal cancer.

- *Nutrients individually proven with therapeutic benefits during cancer chemotherapy do not necessarily produce a greater benefit when combined. Evidence-based rationale is needed as to how to maximize the therapeutic potential by optimally combining different nutritional elements into a formulation.*

At present, various enteral 'immunonutrition' 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 (16-22). Clinical benefits of these individual components are studied in a relatively extensive manner, yet information regarding potential synergistic or antagonistic interactions between these elements is meager. In this context, how these expensive enteral preparations are formulated in terms of component selection and dose optimization should be carefully justified. Our results demonstrate an absence of additive benefits when glutamine and *n*-3 PUFAs were supplied in combination; instead they appeared antagonistic in many of the end points examined. Given that cancer patients receiving chemotherapy are a targeted population for immunonutrition treatment, further efforts are surely warranted to understand potential nutrient×nutrient interactions and their role in the complex immunopathogical situations associated with cancer chemotherapy.

6.2 How dose glutamine work

On this level, the following conclusions are derived from our observations:

- *Glutamine's ameliorative effect on CPT-11-induced diarrhea could be a convergence of multifold mechanistic pathways occurring at various levels including innate cytoprotective response, redox regulation, intestinal immunity and drug metabolism modification.*

Precise mechanisms underlying glutamine's effects are hard to identify at this point because of the various intracellular metabolic pathways and diverse metabolites. Glutamine administered orally is subject to extensive 1st pass metabolism in the small intestine and liver, and its actions described here may be due as much to a variety of metabolites as to glutamine *per se*. Alike to glutamine, many of these metabolic by-products themselves, such as glutamate, arginine and proline, are important regulators for gut physiology and immunity. It is been debatable whether the benefits associated with glutamine supplementation are attributed to the intact molecule of glutamine or its various metabolites. Few studies have performed rigorous comparisons of these modulatory effects between glutamine and its metabolites, particularly with reference to how glutamine is handled intracellularly and which intermediate metabolites convey the potentiating effect on Hsp expression. Emerging evidence suggests glutamine's role in HSR may largely rely on its intracellular conversion into glutamate (23), but cannot be

reproduced with other metabolites such as arginine, proline and ammonium (23,24). Rhoads *et al.* have determined an essential role for glutamine in the activation of the transcription factor Elk-1, which is pivotal in epithelial cell proliferation (25,26). Additional evidence suggests that endogenously synthesized glutamine is essential for enterocyte survival irrespective of an adequate supply of extracellular glutamine (27,28). These intriguing findings suggest that glutamine may play a more subtle regulatory rather than merely a biochemical role in these cells and its regulatory role is at least, not completely and compellingly related to its intermediary metabolism.

Although diarrhea is a well recognized side-effect of CPT-11 chemotherapy, a disproportionate amount of research has been conducted leading to understanding the underlying mechanisms. Much of the information in the published literature is based on clinical observations with limited basic studies existing. This lack of mechanistic understanding makes hard to target treatment. Diarrhea is in nature caused by a misbalanced fluid absorption and secretion by intestinal epithelium. Although precise mechanism(s) responsible for CPT-11-induced diarrhea remains elusive, several factors have been proposed that may be contributable to a skewed absorption/secretion ratio: structural disruption of intestinal tract lining resulting in mismatched rates of absorption and secretion (29,30),; gut hypermotility leading to less absorption and osmotic diarrhea (31); biochemical disorders such as overproduction of proinflammatory and pro-secretory mediators (32-35); microflora disturbances and bacterial overgrowth which not only potentially change drug metabolism (36), but also increase the risk of opportunistic

infections (37,38) and in turn leading to a direct secretory effect on the intestinal mucosa through the actions of enterotoxins (37-39). It may be that one of these is the primary causative event; however, it is also possible that their simultaneous occurrence may be important. Glutamine enhanced mucosal HSR, alleviated its oxidative stress, modified immune composition of MLNs, and stabilized the production of bacterial β-glucuronidase, and thus its action seems to be multi-targeted. It is conceivable that these concurring effects may lead to an adequate compensation for the multi-faceted defects underlying the pathogenesis of CPT-11-induced diarrhea, and is ultimately reflected in an amelioration of the clinical symptomatology.

- *Glutamine selectively conferred protection to host tissues but not tumors against injury from chemotherapy. In line with this, glutamine exhibited a striking dichotomy in modulating stress response and redox potential in host and tumor tissues.*

It is an intriguing question as to why normal host tissues and tumor tissues behave differently in response to exogenous glutamine supply. As shown in Chapter 3, bolus glutamine elicited a wide array of changes in plasma and colonic amino acid pools 6 h after treatment, but did not lead to any detectable changes to tumor amino acid profiles. This observation may hint at the underlying reason for the differential actions of glutamine. The systemic circulation is the main source for glutamine supply to both tumor and colonic tissues. The unaffected tumor amino acid pools, irrespective of the elevated

glutamine level in plasma following glutamine administration, could involve one or more possibilities: limited glutamine uptake due to depressed glutamine oxidation in hypoxic tumors (40-43), insufficient blood supply or nutrient penetrance in tumor tissues (41), different glutamine transport efficiency of tumor cells (44), and a 'dissipative' consumption resulting in a failure of substantial accumulation of glutamine in tumor tissues(45). Induction of HSR may require a threshold for an inducer's intensity and duration (46) and such a threshold could be tissue-dependent (46). Failure to potentiate HSR in tumor tissues by glutamine treatment could be possibly due to lack of adequate and sustained elevation of glutamine levels or a different inherent threshold required for modulating HSR in tumors. Similarly, the differential effect of glutamine treatment on host and tumor GSH stores could be a reflection of the different free amino acid response in tumor and host tissues. Different preponderance in substrate utilization (45) and defects in GSH biosynthetic pathways inherent to tumor tissues (9) may also account for such a difference.

6.3 How to supplement

- *High dose glutamine bolus supply rather than continuous feeding from glutamine-enriched diet could significantly mitigate CPT-11-induced diarrhea; such a benefit of bolus glutamine was evident with either enteral or parenteral administration.*

Nutritional interventions aiming to modulate disease process have been extensively and relatively well established in chronic conditions such as diabetes, atherosclerosis, metabolic syndrome, chronic inflammatory bowel diseases, chronic or latent infection and cancer chemoprevention. In overall, nutritional management of acute disease conditions is not a traditional domain of the overarching 'nutrition and health' field, and investigations on how nutrition would affect an acute-onset and dose-concentrated disease process i.e., infection, inflammation and injury are much less substantialized. An important issue that remains unclear is, to achieve efficacy in these acute conditions, whether these nutrients should be given in the conventional manner as used in the chronic conditions, and if not, how to alter the way they are administered. Lack of such a crucial understanding is largely attributed to the infant state of nutritional pharmacology, a major aim of which is to understand the pharmacokinetics/pharmadynamics of a particular nutrient and relate this to the pathophysiology of a disease process. In our study on CPT-11-induced diarrhea, we opted for the high dose bolus glutamine administration which had shown efficacy in other acute lethal/sublethal conditions. Such a paradigm is not what is commonly used in the experimental nutritional studies, and actually blurs the lines between pharmacological and nutritional modes of administration. The high dose bolus administration resulted in an acute and substantial accumulation of glutamine in the targeted tissue, which could be essential for evoking required protective mechanism(s) and overcoming the threshold for establishing an efficacy on the diarrhea toxicity.

Compared to the oral route, *i.v.* administration of glutamine would presumably result

in a greater bioavailability to colonic tissue, as enteral glutamine supply is subject to a great deal of first-pass metabolism. However, these two routes were of equal efficacy in preventing CPT-11-induced diarrhea. Direct communication between gut mucosa and enteral glutamine may generate some benefits to the GI tract as a whole, such as modifying mucosal immunity, which could be unique to enteral administration (47,48). Future efforts are warranted to characterize the pharmacokinetic/pharmadynamics relationship and mechanistic features of these two routes.

- *Opportunistic infection after dose-intensive chemotherapy made a substantial contribution to the overall toxicity profile. Prophylactic Cipro treatment prevented mortality caused by CPT-11 chemotherapy, ameliorated systemic toxicity, and differentially corrected CPT-11-related disorders in systemic and intestinal immunity.*

Toxicity related to systemic cancer chemotherapy is not only confined to a single system in most cases, but rather implicates multiple systems. Interactions between defects or disorders occurring in different compartments together contribute to development of a systemic pathological condition subject to cancer chemotherapy, such as bacteremia, septicemia and shock. Compromised intestinal integrity constitutes the gateway for pathogen translocation, impaired immunity fails to localize and effectively eliminate translocated pathogens, which can enter the systemic circulation and may further elicit cascading events leading to septicemia (49). Despite being unable to affect diarrhea

toxicity, Cipro exerted a strikingly ameliorative effect on systemic toxicity. This suggests that gut toxicity, if isolated from the interaction with secondary infection by the use of Cipro, would be much less life-threatening. Moreover, we found a sepsis-like compartmentalized immune disorder after CPT-11 chemotherapy as revealed by the hyper-responsiveness of intestinal immune compartment independent of the paralyzed systemic immunity. Cipro treatment exerted a dual modulation on these two compartments by normalizing the hyperresponsiveness in MLN cells and meanwhile significantly mitigating systemic immune anergy. Therefore, in addition to their intrinsic antibacterial, therapeutic benefits of antibiotics may largely lie in their modulatory interaction with the host immunity. In the case of Cipro, it may favour development of appropriate defences against pathogens disseminated systemically, and also alleviate excessive proinflammatory response mediating local gut injury.

FUTURE DIRECTIONS

Findings in this work leave an array of important questions open for future explorations.

- *How different nutrients would potentially modulate immune function during dose-intensive chemotherapy?*

As discussed earlier, modulating host immune function may assume an important contributory role in antibiotics' action. How the tested nutrients, especially glutamine and *n*-3 PUFAs, which are known as 'immunonutrients', would potentially affect intestinal

and systemic immunity, and how to link their potential immunomodulatory actions to the overall therapeutic effects against systemic infection secondary to chemotherapy are amongst the major remaining questions entailing future efforts. Cancer chemotherapy results in a complex immuno-pathological situation featuring the initial hematopoietic suppression and subsequent immunological reconstitution. Such a phasic transition may herald a temporally-compartmentalized immune responsiveness and vulnerability to certain opportunistic infections. An intriguing question arising is the timing to administer these immunonutrients, specifically as to whether the therapeutic window of these nutrients only fit into a certain phase or the whole trajectory at the large. Immunonutrients' therapeutic window may also considerably differ for individuals only at the risk of infection and those who already develop fulminant systemic infection, the latter of which is just like our case. Accumulating evidence suggest that benefits from immunonutrients may not be necessarily equal for moderately immunosuppressed or infection-threatened patients and the critically ill suffering from sepsis, shock and organ failure (50-52). Pathophysiology of sepsis is at least biphasic, with an initial boost of cellular defence functions described as a 'hyperimmune' state followed by suppression of these functions (53), an effect described by the term 'immune paralysis' supported by our findings with splenocytes. Thus sepsis is paradoxically associated with an overwhelming proinflammatory milieu, as assessed by the imbalance of anti- and pro-inflammatory cytokine network, and a diminished ability of immune cells (especially from systemic compartments such as circulating leukocytes) to proliferate or produce cytokine upon *in*

vitro activation (53). Immunomodulatory effects of glutamine and *n*-3 PUFAs may or may not confer benefits for both of these phases with immune features at two extremities.

Therefore, future systematical research on immunonutrients supply in cancer chemotherapy may produce meaningful findings by stratifying chemotherapy patients according to their immune status, e.g., infection-threatened or septic, early post-chemotherapy immunosuppressed or substantially immuno-reconstituted at a much later point, hyperimmune or hypoimmune state with sepsis. Intestinal mucosal immunity is sensitive to alterations in luminal supply of nutritional substrates (48). Links between potential effects of glutamine and *n*-3 PUFAs on gut integrity, the GALT immune response, and gut-derived sepsis in the context of chemotherapy administration needs to be substantiated with future experimental work exploring the targeted actions of these two immunonutrients on (1) mucosal barrier function; (2) cellular defence function and (3) local or systemic inflammation.

- *Molecular mechanisms for glutamine's regulatory role*

As to modulation of HSR by glutamine, mechanisms behind such a finding remain elusive. Heat shock transcription factors (HSF) are the master regulator for heat shock gene expression. A coordinated series of activating events take place to enable HSFs to acquire their transactivation ability, including (1) oligomerization of HSF monomers, (2) acquisition of HSF DNA binding activity, (3) HSF trimer translocation to the nucleus, and (4) phosphorylation-dependent transcriptional activation at heat shock elements (HSE) located in heat shock gene promoters (54-56). It would be interesting to examine whether

glutamine's action may by mediated by modulation of any of these key events for HSF transactivation.

More work is warranted to explore mechanisms underlying the dichotomy in glutamine's action in tumor and host tissues in various dimensions: substrate availability which could be directly related to blood perfusion and amino acid transport efficiency (40,41,43); metabolic discrepancy as exemplified by key enzymes such as glutaminase and γ-glutamyl transferase (9,45); potentially different regulatory mechanisms governing tumor and host stress response (57-59).

- *Mechanisms responsible for enhancement of tumor chemosensitivity by nutritional interventions*

Dietary supply of *n*-3 PUFAs was consistently shown to potentiate the anti-tumor efficacy in both CPT-11 monotherapy and CPT-11/5-FU combination regimens; whereas long-term supply of glutamine was also shown with ability to enhance the tumor's response to the combination regimen. Capacities of these two nutrients in modulating chemotherapy's efficacy need to be followed and examined in different tumor/drug systems. More work is needed to define the underlying mechanisms leading to these observations. Our results show that such a chemosensitizing effect by both of these two nutrients was associated with a differential modulation on tumor and host redox potential. Immune competence is closely relevant to redox regulation. The link between glutamine/*n*-3 PUFAs supply, redox status and anti-tumor immunity needs to be

examined. Emerging evidence has shown that glutamine supply enhances host GSH stores, which may in turn augment NK cell anti-tumor activity (6,8). Apart from NK cell-mediated anti-tumor immunity, our bolus glutamine treatment reduced the relative depletion of total cytotoxic T cell and memory CD8+ cells in MLNs associated with CPT-11 chemotherapy. Accumulating evidence suggests a positive correlation between cytotoxic T cells, especially memory CD8+ cells within cancer cell nests and colon cancer prognosis (e.g., metastasis and survival) (60,61). It would be of interest to investigate whether glutamine treatment could raise CD8+ T subsets of tumor-infiltrating lymphocytes and relate this to glutamine's (either for a short-term or long-term supply) effect on tumor growth. In the case of *n*-3 PUFAs, in addition to modulating host anti-tumor immunity, other avenues of mechanisms potentially affecting the drug-tumor interaction need further investigations to pursue. For instance, how incorporation of *n*-3 PUFAs into cell membranes would potentially affect oxidative stress occurring in tumor cells and whether this is related to chemosensitizing effects exerted by *n*-3 PUFAs; how membrane enrichment of *n*-3 PUFAs would affect tumor's uptake and efflux of CPT-11 and the drug's intracellular retention; and how *n*-3 PUFAs would modulate CPT-11-induced pathways to apoptosis (62).

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