

Single and combined supplementation of glutamine and *n*-3 polyunsaturated fatty acids on host tolerance and tumour response to 7-ethyl-10-[4-(1-piperidino)-1-piperidino]carbonyloxy-camptothecin (CPT-11)/5-fluorouracil chemotherapy in rats bearing Ward colon tumour

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Prior reports suggest that during irinotecan (7-ethyl-10-[4-(1-piperidino)-1-piperidino]carbonyloxy-camptothecin; CPT-11) chemotherapy in laboratory rats, the anti-tumour efficacy and diarrhoea toxicity could be modulated by *n*-3 PUFA and glutamine, respectively. We further examined how these two dietary elements, when provided individually and in combination, would affect the efficacy of a cyclical regimen of CPT-11/5-fluorouracil (5-FU), an accepted combination regimen for colorectal cancer. Prior to initiating chemotherapy, diets enriched either with glutamine (2%, w/w total diet) or *n*-3 PUFA (0.88%, w/w total diet) alone, inhibited Ward colon tumour growth ($P < 0.05$). These diets also completely or partially normalized the changes in peripheral leucocyte counts associated with the tumour-bearing state (e.g. neutrophil proportion/concentration and lymphocyte proportion). During chemotherapy, either glutamine- or *n*-3 PUFA-enriched diet enhanced tumour chemo-sensitivity, and reduced body weight loss, anorexia and muscle wasting (*v.* animals fed control diet, $P < 0.05$). Surprisingly, providing both glutamine and *n*-3 PUFA together did not confer a greater benefit on tumour inhibition either in the presence or absence of chemotherapy; individual benefits associated with single treatments, particularly in respect to host nutritional status (i.e. body weight, food intake and muscle weight) and immune (peripheral leucocyte counts) features were instead partially or completely lost when these two nutrients were combined. These results draw into question the common assumption that there are additive or synergistic benefits of combinations of nutrients, which are beneficial on an individual basis, and suggest that co-supplementation with glutamine and *n*-3 PUFA is not indicated during chemotherapy with CPT-11 and 5-FU.

Glutamine: *n*-3 PUFA: CPT-11: 5-Fluorouracil

Increasing evidence has emerged suggesting a promising role for certain nutritional factors in modulating efficacy and/or toxicity of cancer chemotherapy. Using nutritional adjuncts to chemotherapy is based on the premise that tumour and host responses to chemotherapy could potentially be favourably influenced (i.e. sensitization of the tumour to chemotherapy and protection of the host against chemotherapy-related toxicity). Glutamine and long-chain *n*-3 PUFA are two nutrients reported to modify efficacy or toxicity of cancer chemotherapy^(1–3). Glutamine mitigates chemotherapy toxicity by improving gastrointestinal-related symptoms, gut barrier function and whole-body nitrogen balance^(2–4). *n*-3 PUFA,

such as 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^(5,6). There is also some evidence that *n*-3 PUFA may alleviate chemotherapy-related toxicities^(1,5). Both glutamine and *n*-3 PUFA are recognized for their immunomodulatory roles by providing substrates for immune cells, regulating redox status and affecting the inflammatory cytokine/eicosanoid networks⁽⁷⁾. Evidence for their therapeutic utility in modifying immunity in the tumour-bearing state, with and without chemotherapy, is yet to be substantiated.

Abbreviations: CPT-11, 7-ethyl-10-[4-(1-piperidino)-1-piperidino]carbonyloxy-camptothecin; 5-FU, 5-fluorouracil; GLN + FO, glutamine + *n*-3 PUFA (fish oil) diet; GSSG, glutathione disulphide; rGSH, reduced glutathione.

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Interactions between some of the different nutrients that have been suggested to favourably affect host response to tumour and chemotherapy have not been explored, to the best of our knowledge. While there already exist some enteral 'immunonutrition' formulae featuring glutamine and *n*-3 PUFA combinations for applications in clinical nutrition^(8–10), most research on *n*-3 PUFA or glutamine nutrition has focused on the individual effects of these two nutrients^(11–16). It is commonly assumed that a greater benefit could be achieved by combining individually beneficial nutrients; however, evidence regarding the efficacy of nutrient combinations is lacking⁽¹⁷⁾.

We established a system to study interactions amongst tumour, chemotherapy and diet (rats bearing the Ward colon tumour), treated with irinotecan (7-ethyl-10-[4-(1-piperidino)-1-piperidino]carbonyloxy-camptothecin; CPT-11; Camptosar[®])⁽³⁾. Within our controlled dietary design, glutamine treatment mitigated late diarrhoea, the dose-limiting toxicity for CPT-11, whereas *n*-3 PUFA enhanced tumour response to CPT-11^(3,18). The current study aimed at comparing the effects of *n*-3 PUFA and glutamine, alone or in combination, on the response of the tumour and host to a combination chemotherapy similar to that used in the current treatment of colorectal cancer. The measured outcomes were mortality, food intake, weight loss, host leucocyte concentration, tumour growth and response to therapy. As oxidative stress is involved in anti-tumour activity as well as the pathophysiology of chemotherapy toxicity^(19–23), we further tested effects of the dietary interventions on redox status in host and tumour tissues.

Experimental methods

Animal treatments

Animal use was reviewed and approved by the Institutional Animal Care Committee and conducted in accordance with the Guidelines of the Canadian Council on Animal Care. Fisher 344 rats (150–180 g body weight and 11–12 weeks old) obtained from Charles River (St. Constant, QC, Canada) were used. Female animals were used for all studies, to avoid potential confounding effects of sex and to allow direct comparison with the body of prior work in the same tumour model⁽³⁾. Rats were housed two per cage in a temperature (22°C) and light (12 h light) controlled room; water and food were available for *ad libitum* consumption. Rats were separated into individual cages 1 week before chemotherapy. The Ward colorectal carcinoma was provided by Dr Y. Rustum, Roswell Park Institute⁽²⁴⁾. Tumour pieces (0.05 g) were transplanted subcutaneously on the flank of the rats via trocar under slight isoflurane anaesthesia. A major consideration in selecting this tumour site was to facilitate continuous evaluation of tumour growth and response to CPT-11 treatment. CPT-11 was provided by Pfizer as a ready-to-use clinical formulation. Atropine (0.6 mg/ml) was a clinical injectable formulation.

Diet

Tested nutrients were incorporated into a nutritionally complete diet (Table 1). The diets were based on an American Institute of Nutrition-76 (AIN-76) modified basal diet with

Table 1. Composition of the experimental diets*

Ingredient		Control diet (g/100 g diet)	<i>n</i> -3 PUFA (FO) diet (g/100 g diet)†	Glutamine (GLN) diet (g/100 g diet)	Glutamine + <i>n</i> -3 PUFA (GLN + FO) diet (g/100 g diet)†
Constant portion					
Modified AIN-76 basal mix (46 g/100 g)	Casein	25.2	25.2	25.2	25.2
	Methionine	0.25	0.25	0.25	0.25
	Glucose	13.95	13.95	13.95	13.95
	Vitamins AIN-76	1	1	1	1
	Minerals AIN-76	5	5	5	5
	Inositol	0.6	0.6	0.6	0.6
	Choline	0.3	0.3	0.3	0.3
Fibre (10 g/100 g)	Cellulose	10	10	10	10
Variable portion					
Lipids (20 g/100 g)	Soyabean 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 (24 g/100 g)	Control amino acid mixture‡	1.5	1.5	0	0
	Glutamine	0	0	2	2
	Maize starch	22.2	22.2	21.7	21.7
Total		100	100	100	100

AIN, American Institute of Nutrition.

* All diets contained 262 g protein and 15.48×10^3 kJ energy/kg. The constant portion consisted of the pre-mixed modified AIN-76 basal ingredients (Harlan Teklad, Madison, WI, USA); the variable portion was formulated to allow the addition of selected fat/fibre/amino acid elements. Other ingredients were supplied: soyabean stearine (ICN Bio-medicals Inc., Cleveland, OH, USA), safflower oil (Canadian Superstore, President's Choice, AB, Canada), linseed oil (Planet Organic, Gold Top, AB, Canada), fish oil (Ocean Nutrition Canada, Dartmouth, NS, Canada) and oligofructose-enriched inulin (Beneo Synergy 1[®]), kindly supplied by Orafiti, Tienen, Belgium.

† *n*-3 PUFA-enriched diets (FO and GLN + FO diets) contained 18:3(3), 20:5(3), 22:5(3) and 22:6(3), respectively, at 0.04, 0.64, 0.04, 0.16% of total diet (w/w, or 0.2, 3.2, 0.2, 0.8% of total fat, respectively), and had an *n*-6:*n*-3 ratio of 3.8. All other diets contained 18:3(3) as the only *n*-3 PUFA (0.2% of total diet, w/w) and had an *n*-6:*n*-3 ratio of 21.0.

‡ The control amino acid mixture contained an equimolar mixture of alanine, serine, glycine and histidine, and was isonitrogenous with glutamine-enriched diet.

40% of energy from fat. The modified fat component is formulated to be similar to typical North American dietary patterns in man (40% of energy; polyunsaturated to saturated fat ratio of 0.35) and have been described elsewhere⁽³⁾. Animals were initially fed Rodent Laboratory Chow (Harlan Teklad, Madison, WI, USA). During adaptation, this chow was mixed with our control diet (50:50, w/w) for 1 week, followed by full transition to experimental diets.

Experimental design

Two weeks prior to tumour implantation, rats were randomly assigned to one of four diets: (1) control diet (*n* 12); (2) glutamine diet (*n* 10); (3) *n*-3 PUFA (fish oil) diet (*n* 10); (4) glutamine + *n*-3 PUFA diet (GLN + FO; *n* 10).

When tumours reached approximately 2.3 cm³ (1.2% of body weight) a modified regimen of CPT-11/5-fluorouracil (5-FU) combination chemotherapy⁽²⁴⁾ was initiated. Drugs were administered intravenously 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 days 0 and 7, whereas 5-FU (50 mg/kg) was administered on days 1 and 8. Atropine (1 mg/kg, subcutaneously) was administered immediately before each CPT-11 injection to alleviate the early onset cholinergic symptoms⁽³⁾.

Another set of rats of the same age as the tumour-bearing rats were assigned to a reference group (*n* 7) for comparison. The REF group did not receive tumour implantation or chemotherapy and was on control diet throughout the study.

Outcome measures

Body weight and food intake were monitored every second day. Food intake and body weight at day 0 for each animal was considered the baseline value, and subsequent changes were expressed relative to that initial value. Tumour volume was measured at time-points indicated in the figures, in three dimensions with a caliper, the length (*L*), width (*W*) and height (*H*). Tumour volume was calculated: tumour volume (cm³) = 0.5 × *L* (cm) × *W* (cm) × *H* (cm)⁽³⁾. Tumour response was expressed as relative tumour volume, calculated relative to the volume at the start of chemotherapy, for each rat. Calculation of tumour growth inhibition was as described previously⁽³⁾.

Sample collection and assays

Animals were killed by CO₂ asphyxia followed immediately by exsanguination by cardiac puncture 13 d after completion of chemotherapy. The colonic mucosal tissue was scraped off from the first 6 cm section of the proximal colon and immediately frozen in liquid nitrogen for glutathione (GSH) assay. Tumour 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 d prior to chemotherapy (as baseline) and at the end of the study (13 d after completion of chemotherapy) and was used to measure leucocyte concentrations. The complete blood count and automated differential were performed on whole blood using a Hemavet instrument (CDC Technologies, Oxford, CT, USA).

Reduced glutathione (rGSH) and glutathione disulphide (GSSG) were determined with an Oxford rGSH/GSSG kit (product no. GT-30), which features 1-methyl-2-vinyl-pyridinium trifluoromethane sulphonate as a scavenger of rGSH⁽¹⁸⁾.

Statistical analysis

Data are expressed as means and their standard errors. Statistical analysis was performed using univariate or multivariate linear models of SPSS version 12.0 (SPSS Inc., Chicago, IL, USA). The models included glutamine, *n*-3 PUFA and time, and their interactions. Unless specified in the text, treatment differences in leucocyte counts, GSH, GSSG and muscle weight were analysed using one-way ANOVA followed by *post hoc* Tukey's test; whereas treatment differences in tumour growth, body weight and food intake changes were tested using two-way repeated-measures 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 analysed using the log-rank test. A probability of *P* < 0.05 was accepted as being statistically significant.

Results

Effects of dietary treatments during the pre-chemotherapy period

After tumour implantation but prior to chemotherapy, there was no significant effect of diet treatment on body weight and the food intake of tumour-bearing rats (data not shown). Although the total concentration of leucocytes did not differ between treatments, an effect of diet on the proportions of different leucocyte populations was observed. Tumour-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 2). Feeding *n*-3 PUFA diet alone partially corrected these alterations; feeding glutamine also lowered blood neutrophil concentration while increasing the proportion of lymphocytes in the tumour-bearing rats towards the values seen in the reference rats. Co-supplementation of glutamine and *n*-3 PUFA (GLN + FO) resulted in a lower proportion and concentration of monocytes as compared with reference rats (*P* < 0.05) and a lower lymphocyte concentration as compared with *n*-3 PUFA diet alone (*P* < 0.05).

Inhibition of tumour growth was observed with *n*-3 PUFA (−23.6 (SEM 5.9) %) or glutamine (−18.4 (SEM 5.8) %) supplemented individually, at 16 d after tumour implantation, compared with rats fed the control diet (*P* < 0.05; Fig. 1(A)). Co-supplementation of these two nutrients in the diet (GLN + FO) led to an identical tumour growth inhibition as seen with the single *n*-3 PUFA supplementation (Fig. 1(A)); tumour inhibition in this case was −23.9 (SEM 6.2) % (*P* < 0.05 *v.* control diet).

Effects of dietary treatments during the post-chemotherapy period

Tumour response to chemotherapy. CPT-11/5-FU therapy was initiated for each treatment group when the average

tumour burden in each diet group was identical (approximately 1.2% of body weight). Both glutamine and *n*-3 PUFA diets significantly enhanced anti-tumour activity of CPT-11/5-FU chemotherapy as compared with control diet ($P < 0.05$; Fig. 1(B)). However, these effects were not additive in the glutamine and *n*-3 PUFA co-enriched diet, which resulted in a similar potentiation of anti-tumour efficacy as

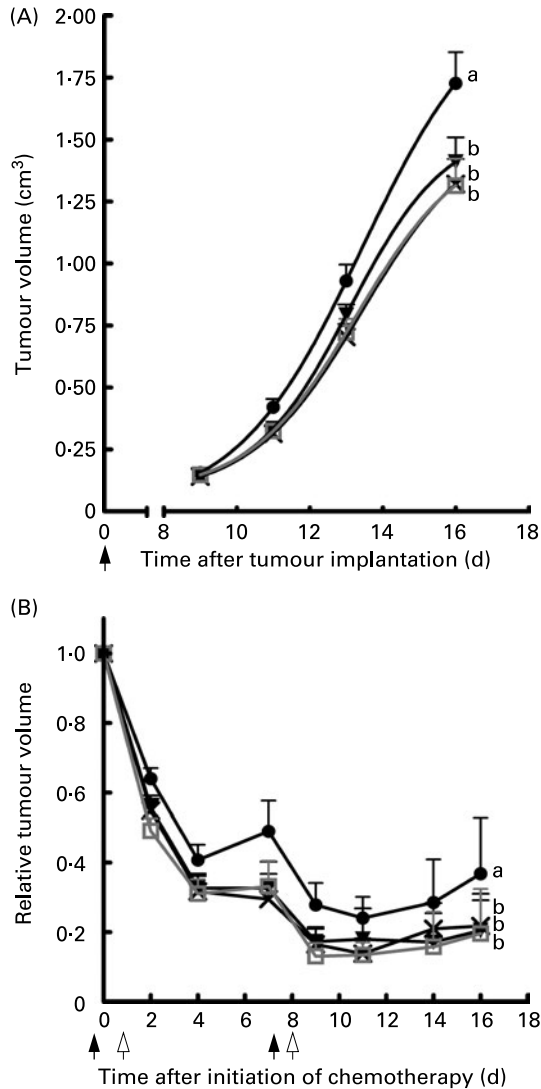


Fig. 1. Dietary effects on Ward colon tumour growth and anti-tumour efficacy of 7-ethyl-10-[4-(1-piperidino)-1-piperidino]carbonyloxy-camptothecin (CPT-11)/5-fluorouracil (5-FU) chemotherapy *in vivo*. Values are means with their standard errors depicted by vertical bars. (A), Effect of dietary treatment *per se* on Ward colon tumour growth *in vivo* (●, control diet (CON); ×, *n*-3 PUFA (fish oil) diet (FO); ▼, glutamine diet (GLN); □, glutamine + *n*-3 PUFA diet (GLN + FO)). Fisher rats were implanted with Ward colon tumour and changes in tumour volume were followed (day 0, tumour implantation). Co-supplementation of glutamine and *n*-3 PUFA in the diet (GLN + FO) led to an identical tumour growth inhibition as the single *n*-3 PUFA (FO) supplementation, and the tumour growth curves of these two groups overlap. (B), Dietary modification of anti-tumour efficacy of CPT-11/5-FU therapy. CPT-11/5-FU treatment was initiated when rats of all the dietary treatment groups had tumours of approximately 2.3 cm³ in volume. Relative tumour volume is compared to the baseline volume when chemotherapy was initiated (day 0). ▲, Single CPT-11 injection at 50 mg/kg; △, single 5-FU injection at 50 mg/kg. ^{a,b} Dietary treatments with unlike letters were significantly different ($P < 0.05$, *post hoc* Tukey's).

n-3 PUFA diet without the addition of glutamine (52.9 (SEM 16.7) % tumour inhibition by *n*-3 PUFA alone and 53.7 (SEM 13.0) % inhibition by GLN + FO as compared to the control diet on day 11; $P < 0.05$).

Host responses to chemotherapy. *Survival:* some mortality occurred during days 9–17 of the study (Fig. 2). As tumour burden was markedly reduced with CPT-11/5-FU treatment, the observed short-term mortality was considered to be attributable to the chemotherapy rather than cancer progression. The mortality was more prevalent in rats fed the control diet (five out of twelve, 41.7%). Mortality for glutamine, *n*-3 PUFA and GLN + FO groups were 20% (two out of ten), 30% (three out of ten) and 10% (one out of ten), respectively (Fig. 2); difference as compared to the control diet did not reach significance for any of these groups.

Blood cell counts: at 13 d following the last chemotherapy, there was a marked increase in the total leucocyte 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); lymphocyte concentration did not change significantly from the pre-chemotherapy baseline values (Table 2). These changes in the post-chemotherapy leucocyte concentrations were completely or partially mitigated in rats fed either *n*-3 PUFA or glutamine alone. However, the rats fed the combination diet had a similar post-chemotherapy increase in neutrophils, monocytes and total leucocytes as the rats fed the control diet.

Body weight: rat body weights during CPT-11/5-FU treatment displayed a loss–regain pattern concordant with the cyclic chemotherapy administration (Fig. 3(A)). Body weight loss was much greater upon administration of the second cycle of chemotherapy than after the first cycle for rats in all diet groups. For instance, rats fed control diet initially lost 5.5 (SEM 0.5) % of their weight following the

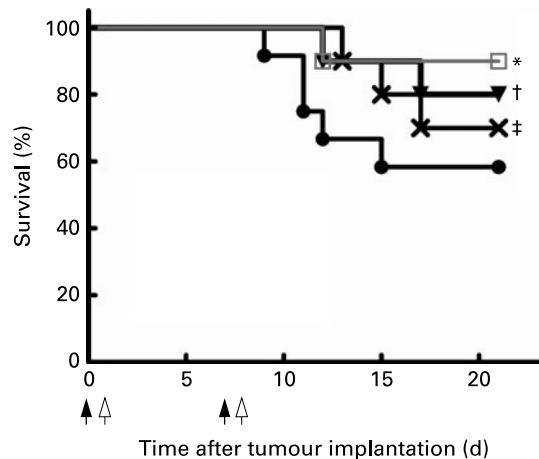


Fig. 2. Dietary effect on 7-ethyl-10-[4-(1-piperidino)-1-piperidino]carbonyloxy-camptothecin (CPT-11)/5-fluorouracil (5-FU)-induced mortality. Post-chemotherapy survival associated with different dietary treatments (●, control diet (CON); ×, *n*-3 PUFA (fish oil) diet (FO); ▼, glutamine diet (GLN); □, glutamine + *n*-3 PUFA diet (GLN + FO)) was analysed using the Kaplan–Meier method and overall survival between groups was analysed using the log-rank test. ▲, Single CPT-11 injection at 50 mg/kg; △, single 5-FU injection at 50 mg/kg. Differences as compared with the control diet did not reach significance for any of these groups: * $P = 0.43$, † $P = 0.23$, ‡ $P = 0.10$.

Table 2. Effects of dietary treatments on peripheral leucocyte counts before and after 7-ethyl-10-[4-(1-piperidino)-1-piperidino]carbonyloxy-camptothecin (CPT-11)/5-fluorouracil (5-FU) chemotherapy†

(Mean values with their standard errors)

Dietary treatment	Leucocyte ($\times 10^9/l$)		Neutrophils (% of leucocyte)		Neutrophils ($\times 10^9/l$)		Lymphocytes (% of leucocyte)		Lymphocytes ($\times 10^9/l$)		Monocytes (% of leucocyte)		Monocytes ($\times 10^9/l$)	
	Mean	SEM	Mean	SEM	Mean	SEM	Mean	SEM	Mean	SEM	Mean	SEM	Mean	SEM
Pre-chemotherapy														
REF (<i>n</i> 11)	8.81	1.06	19.84 ^a	1.91	1.56 ^a	0.18	73.02 ^b	1.46	6.36 ^{ab}	0.82	5.51 ^b	0.46	0.52 ^b	0.08
CON (<i>n</i> 12)	8.53	0.31	26.28 ^b	1.48	2.29 ^b	0.19	64.18 ^a	2.18	5.45 ^{ab}	0.21	4.18 ^{ab}	0.25	0.36 ^{ab}	0.03
GLN (<i>n</i> 10)	9.12	0.29	24.86 ^b	1.68	2.15 ^{ab}	0.16	69.44 ^{ab}	1.31	6.18 ^{ab}	0.16	3.98 ^a	0.20	0.36 ^{ab}	0.02
FO (<i>n</i> 10)	9.83	0.19	22.52 ^{ab}	0.91	2.22 ^{ab}	0.11	70.01 ^{ab}	1.13	7.01 ^b	0.12	4.69 ^{ab}	0.33	0.46 ^{ab}	0.04
GLN + FO (<i>n</i> 10)	7.56	0.66	23.96 ^b	1.54	1.64 ^{ab}	0.16	68.94 ^{ab}	1.13	5.18 ^a	0.41	3.93 ^a	0.48	0.31 ^a	0.06
Post-chemotherapy														
REF (<i>n</i> 11)	8.81 ^a	1.06	19.84 ^a	1.91	1.56 ^a	0.18	73.02 ^c	1.46	6.36	0.82	5.51	0.46	0.52 ^a	0.08
CON (<i>n</i> 7)	14.53 ^{ab}	2.76	41.43 ^{bc}	5.17	6.07 ^{ab}	1.53	47.00 ^{ab}	6.24	6.83	1.54	7.68	0.76	1.13 ^{ab}	0.25
GLN (<i>n</i> 8)	7.95 ^a	1.52	24.08 ^{ab}	1.60	1.95 ^a	1.53	67.25 ^{bc}	1.08	5.32	0.98	6.80	0.82	0.51 ^a	0.07
FO (<i>n</i> 7)	9.38 ^{ab}	2.46	30.53 ^b	2.16	2.99 ^{ab}	0.90	59.30 ^{ab}	1.20	5.50	1.40	7.75	1.05	0.66 ^a	0.10
GLN + FO (<i>n</i> 9)	16.68 ^{ab}	1.84	40.43 ^{bc}	2.76	6.59 ^{ab}	0.29	48.80 ^{ab}	3.22	8.30	1.53	8.30	0.84	1.34 ^{ab}	0.04

CON, control diet; FO, *n*-3 PUFA (fish oil) diet; GLN, glutamine diet; GLN + FO, *n*-3 PUFA + glutamine diet; REF, reference group.^{a,b,c} For both pre-chemotherapy and post-chemotherapy data, mean values within a column with unlike superscript letters were significantly different ($P < 0.05$).Mean values were significantly different from those prior to chemotherapy (paired *t* test): * $P < 0.05$.† For details of procedures and diets, see the Experimental methods section and Table 1. Whole blood was collected prior to initiation of CPT-11/5-FU chemotherapy when all groups had tumours of approximately 2.0 cm³ in volume (1.0% of body weight) and 13 d after completion of CPT-11/5-FU chemotherapy (at the end of the study).

first cycle at day 2 (nadir of the first cycle), and further suffered an additional 9.6 (SEM 1.8) % weight loss following the second cycle at day 11 (nadir of the second cycle) ($P < 0.05$, paired *t* test). Rats receiving either glutamine, *n*-3 PUFA treatment or both had significantly less body weight loss following two cycles of chemotherapy as compared to the rats fed the control diet ($P < 0.05$). Glutamine diet appeared to have a greater benefit in limiting post-chemotherapy body weight loss compared to *n*-3 PUFA and combination (GLN + FO) diets ($P < 0.05$). By the end of the study, rats fed the glutamine diet had entirely caught up and exceeded their baseline body weight by +2.1 (SEM 0.7) % ($P < 0.01$ v. control diet, Bonferroni post-test) and rats fed the *n*-3 PUFA diet also ended the study above baseline weight by +2.6 (SEM 1.4) % ($P < 0.05$ v. control diet, Bonferroni post-test) above their baseline body weight. However, rats fed GLN + FO had a slower post-chemotherapy body weight catch-up, ended the study with a -1.7 (SEM 1.1) % weight loss overall, which was significantly different from the glutamine and *n*-3 PUFA groups ($P < 0.05$, Bonferroni post-test) but not different from the control group.

Food intake: the time course of the food intake change during the chemotherapy is illustrated in Fig. 3(B). Both single glutamine and *n*-3 PUFA treatments significantly alleviated the anorexia that occurred following CPT-11/5-FU chemotherapy, as compared with the control diet ($P < 0.05$). However, food intake of rats fed GLN + FO were not different from rats on the control diet. At day 10, where rats from all groups had the lowest daily food intake, rats fed either glutamine or *n*-3 PUFA diet had a higher food intake level as compared with rats fed control diet ($P < 0.05$, Bonferroni post-test). However, feeding diet co-enriched with glutamine and *n*-3 PUFA was not associated with a higher food intake on day 10.

Muscle weight: cancer chemotherapy could be a potent catabolic stimulus for muscle wasting⁽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 (Fig. 3;

$P < 0.05$). Feeding either glutamine or *n*-3 PUFA diet completely prevented this muscle loss. However, feeding the GLN + FO diet 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 the reference group or rats with dietary glutamine or *n*-3 PUFA monotherapy ($P < 0.05$).

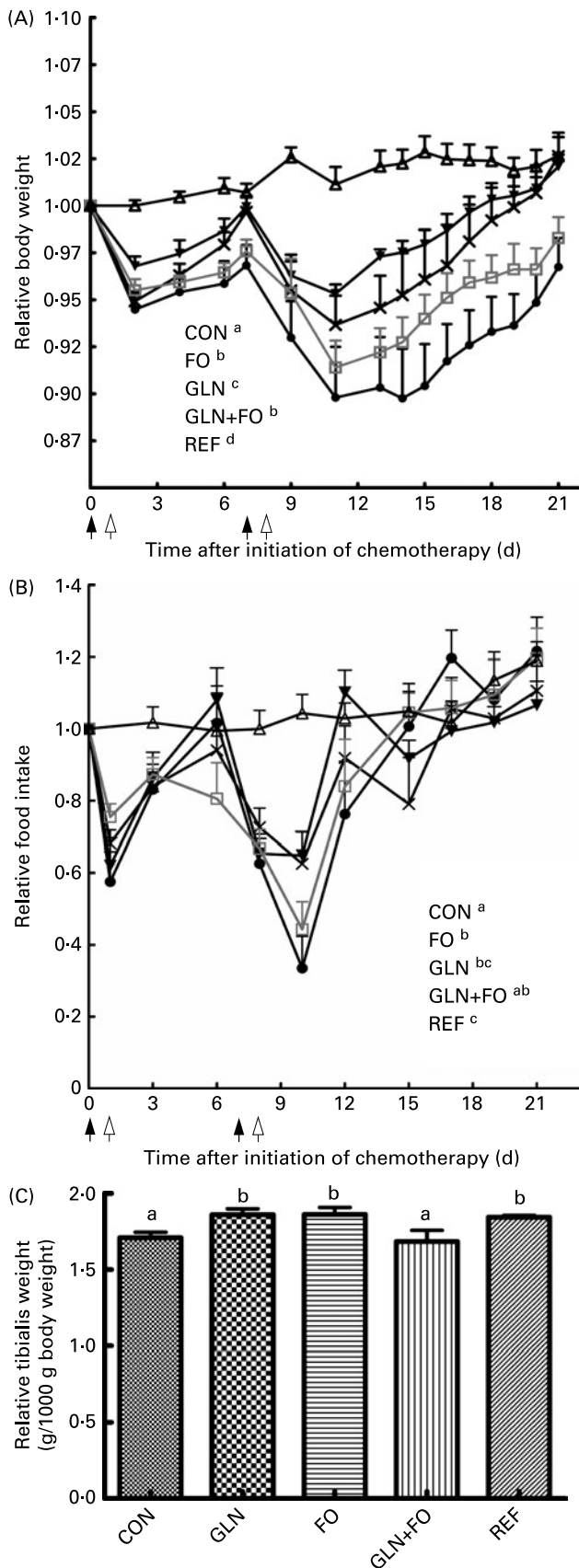
Effects of dietary treatments on glutathione stores in host and tumour tissues following 7-ethyl-10-[4-(1-piperidino)-1-piperidino]carbonyloxy-camptothecin/5-fluorouracil therapy

At 13 d after completing CPT-11/5-FU chemotherapy, rGSH/GSSG ratio was lowered by 50 % in colonic mucosa of control diet-fed rats (v. reference rats, $P < 0.05$; Table 3). This was mainly attributed to 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 glutamine and *n*-3 PUFA dietary treatments (but not for the combination treatment) as compared with rats on control diet ($P < 0.05$). In tumour tissue (Table 3), glutamine supplementation, either alone or in combination with *n*-3 PUFA, resulted in a lower rGSH/GSSG ratio, compared to the rats on control diet ($P < 0.05$).

Discussion

Individual effects of glutamine and n-3 PUFA monotherapy on the tumour and host

The Ward colon tumour caused an array of changes in peripheral leucocytes, i.e. elevation of neutrophil count and skewed relative abundance of neutrophils and lymphocytes, consistent with findings in patients with various malignant solid tumours^(26–28). These cancer-related abnormalities in peripheral blood leucocyte concentrations and proportions are suggested to negatively correlate with clinical prognosis in terms of response rate to chemotherapy, incidence of



post-treatment infectious complications and survival^(29–31). The present results show that these changes in the leucocyte concentration and populations that appear prior to chemotherapy could be at least partially normalized by diets with *n*-3 PUFA or glutamine. This may convert into advantage favouring *n*-3 PUFA or glutamine-fed rats during subsequent CPT-11/5-FU treatment. At 13 d after completion of the chemotherapy, we observed a marked neutrophilic and monocytic leucocytosis observed in rats on control and GLN + FO diets, but not on glutamine or *n*-3 PUFA diets. More detailed evaluations of peripheral leucocyte dynamics will be required to discern whether the neutrophilia and monocytosis associated with the control 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⁾.

Supplementing the diet with either *n*-3 PUFA or glutamine inhibited Ward tumour growth *in vivo*. A direct growth-inhibitory effect of dietary *n*-3 PUFA is abundantly documented in various tumour types^(34,35) including Ward colon tumour⁽³⁾. Incorporation of *n*-3 fatty acids in tumour membrane phospholipids affects a wide range of biological functions, such as biosynthesis of eicosanoids, membrane lipid peroxidation, signal transduction, membrane fluidity and cell interaction⁽⁵⁾. Compared to *n*-3 PUFA, which has been increasingly accepted as an anti-tumour nutrient, utility of glutamine supplementation is often challenged due to a prevalent notion that tumours are ‘glutamine traps’⁽³⁶⁾ and exogenous glutamine may promote tumour growth. This notion is based on findings in tissue culture with abundant oxygen supply^(37,38) and indirect evidence (i.e. tumour glutaminase activity⁽³⁹⁾ and lowered plasma glutamine concentration differences in tumour-bearing hosts⁽⁴⁰⁾). There is currently no direct evidence indicating glutamine can stimulate tumour growth *in vivo*, either in animals^(41,42) or clinical trials⁽⁴³⁾. Glutamine supplementation may conversely exert inhibitory effects on tumour growth^(44–46), possibly involving reduced production of PGE₂, enhanced natural kill cell activity, altered tumour GSH stores and reduced expression of apoptosis effectors^(45–47).

The Ward colon tumour model recapitulates the response of human colon cancer to the prevailing chemotherapy regimens based on CPT-11 alone or in combination with 5-FU^(3,24). Dietary supplementation with *n*-3 PUFA or glutamine has been shown to modulate the anti-tumour efficacy of various drugs including doxorubicin, cytosine arabinoside, mitomycin C, CPT-11 and methotrexate^(48,49). We additionally demonstrate

Fig. 3. Dietary effect on host nutritional features following 7-ethyl-10-[4-(1-piperidino)-1-piperidino]carbonyloxy-camptothecin (CPT-11)/5-fluorouracil (5-FU) therapy. Values are means with their standard errors depicted by vertical bars. (A), Body weight change. Body weight is relative to baseline value when the chemotherapy was initiated (day 0). (B), Food intake change. Daily food intake level is relative to that prior to chemotherapy initiation (day 0). ^{a–d} Dietary treatments with unlike superscript letters were significantly different ($P < 0.05$, *post hoc* Tukey's). (C), Muscle weight. At the end of the study, left tibialis anterior muscle was isolated from the killed rats and weighed. Muscle weight is relative to the whole body weight at kill. ^{a,b} Mean values with unlike letters were significantly different ($P < 0.05$, *post hoc* Tukey's). Δ , Reference group (REF); \bullet , control diet (CON); \times , *n*-3 PUFA (fish oil) diet (FO); ∇ , glutamine diet (GLN); \square , glutamine + *n*-3 PUFA diet (GLN + FO); \blacktriangle , single CPT-11 injection at 50 mg/kg; \blacktriangle , single 5-FU injection at 50 mg/kg.

Table 3. Dietary effects on glutathione (GSH) content in host colonic mucosa and tumour tissues following 7-ethyl-10-[4-(1-piperidino)-1-piperidino]carbonyloxy-camptothecin (CPT-11)/5-fluorouracil (5-FU) chemotherapy*

(Mean values with their standard errors)

Treatment group	tGSH ($\mu\text{mol/g}$ tissue)		GSSG ($\mu\text{mol/g}$ tissue)		rGSH ($\mu\text{mol/g}$ tissue)		rGSH/GSSG ratio	
	Mean	SEM	Mean	SEM	Mean	SEM	Mean	SEM
Colonic mucosa (<i>n</i> 6)								
REF	1.86	0.12	0.014 ^a	0.002	1.83	0.12	136 ^b	13
CON	1.65	0.06	0.033 ^b	0.010	1.58	0.05	68 ^a	18
GLN	1.57	0.10	0.012 ^a	0.001	1.55	0.10	136 ^b	13
FO	1.72	0.12	0.015 ^{ab}	0.003	1.69	0.11	124 ^b	13
GLN + FO	1.74	0.06	0.015 ^{ab}	0.002	1.71	0.11	118 ^{ab}	13
Tumour (<i>n</i> 5)								
CON	1.56	0.10	0.017	0.003	1.52	0.09	99 ^b	15
GLN	1.20	0.17	0.024	0.004	1.15	0.16	55 ^a	10
FO	1.26	0.14	0.023	0.004	1.22	0.14	59 ^{ab}	11
GLN + FO	1.10	0.12	0.028	0.006	1.04	0.12	47 ^a	13

CON, control diet; FO, *n*-3 PUFA (fish oil) diet; GLN, glutamine diet; GLN + FO, *n*-3 PUFA + glutamine diet; REF, reference group; rGSH, reduced GSH; tGSH, total GSH.^{a,b} For both colonic mucosa and tumour tissues, means within a column with unlike superscript letters were significantly different ($P < 0.05$).

* For details of procedures and diets, see the Experimental methods section and Table 1. GSH concentration was analysed in the host colonic mucosa and tumour tissue at the end of the study (13 d after completion of CPT-11/5-FU treatment).

that supplementation with either nutrient enhanced the responsiveness of Ward colon tumour to CPT-11/5-FU treatment. GSH is the major intracellular anti-oxidant that protects cells from injury caused by excessive oxidative stress, and is one possible effector of this altered tumour response. Reduced GSH/GSSG ratio is the most important regulator of intracellular redox status (the lower the ratio, the higher the oxidative burden)⁽⁵⁰⁾. Tumour GSH levels correlate with tumour cell proliferation and resistance to chemotherapy⁽⁵⁰⁾. Consistent with their chemo-sensitizing effects, feeding glutamine either alone or in combination with *n*-3 PUFA lowered GSH/GSSG ratio in tumour tissue. *n*-3 PUFA have been suggested to predispose tumour cells to oxidative injury caused by chemotherapy^(48,51). Although serving as a precursor for GSH synthesis, glutamine treatment paradoxically lowered the rGSH/GSSG ratio, consistent with some earlier reports^(18,52). Feeding *n*-3 PUFA alone also tended to lower rGSH/GSSG ratio in tumours, which could be ascribed to enhanced oxidative stress associated with *n*-3 PUFA incorporation in membranes^(1,53).

Adding either glutamine or *n*-3 PUFA to the diet improved clinical symptoms associated with CPT-11/5-FU therapy such as body weight loss, anorexia and muscle wasting. This work adds to previous findings on benefits associated with individual glutamine or *n*-3 PUFA supplementation in the setting of CPT-11 chemotherapy^(18,54). Improvement of these manifestations may contribute to the trend to increased survival after intensive CPT-11/5-FU treatment by animals in these diet treatment groups. Anorexia and accompanying weight loss could be a direct reflection of gastrointestinal toxicity of chemotherapy. In particular, a compromised gut barrier integrity has been suggested to serve as a pivotal mechanism by which chemotherapy triggers endotoxaemia, bacterial translocation and systemic inflammatory responses⁽⁵⁵⁾. Effects of dietary glutamine and *n*-3 PUFA on the endogenous intestinal GSH store have been suggested to play a role in preserving the structural and functional integrity of the gut barrier against exogenous insults^(52,56). Consistent with our previous finding with bolus

glutamine treatment, continuous glutamine feeding normalized colonic GSH stores and redox status, which was deteriorated by chemotherapy⁽¹⁸⁾. Feeding *n*-3 PUFA also normalized the GSH-related redox state following CPT-11/5-FU treatment. Both these nutrients regulated the host and tumour GSH-related redox status in a differential manner. In the tumour, glutamine and *n*-3 PUFA were pro-oxidative, whereas in the colon these two factors alleviated chemotherapy-related oxidative stress. This differential effect has been previously reported during glutamine feeding of tumour-bearing rats^(18,52). *n*-3 PUFA supplementation has been shown to reduce colonic oxidative burden in patients with inflammatory bowel disease or animal models of colitis^(57,58). This could be secondary to its anti-inflammatory effect by inhibiting the production of pro-inflammatory *n*-6 series eicosanoids, TNF- α , IL-6 and IL-1 β ^(59,60). Decreased generation of reactive oxygen species could result from reduction of the inflammation process, e.g. respiratory burst of immune cells⁽⁶¹⁾.

Interaction of glutamine and *n*-3 PUFA when combined

Despite the individual benefits of supplementing the diet with either glutamine or *n*-3 PUFA, additive benefits were notably lacking. When these two nutrients were combined there was no additive benefit on tumour inhibition either in the presence or absence of chemotherapy. Surprisingly, individual benefits associated with single supplementation of glutamine or *n*-3 PUFA, particularly in respect to host nutritional (i.e. body weight, food intake and muscle weight) and immune (peripheral leucocyte counts) features were instead partially or completely lost when these two nutrients were combined. The present results do not support supplementation of both glutamine and *n*-3 PUFA during CPT-11/5-FU chemotherapy. Taken together, the present study suggests the need to test any assumption that there are additive or synergistic benefits of nutrients that are beneficial on an individual basis. This has important ramifications for clinical nutrition. Enteral

food formulations containing both glutamine and *n*-3 PUFA have been marketed for various conditions such as sepsis, surgery, trauma and burns^(8–10). However, clear scientific evidence for combining these is lacking.

Mixtures of different nutrients may have antagonistic effects^(62–64) and the specific mechanisms underlying these are not well understood. In a rat model of head injury, feeding arginine alone reduced bacterial translocation more efficiently than the combination of arginine and *n*-3 PUFA⁽⁶⁵⁾. The same group also showed that combination of arginine and *n*-3 PUFA may exert some conflicting effects on gut barrier function and macrophage reactivity in a rat model of inflammation induced by turpentine. Co-supplementation resulted in increased bacterial translocation and impaired pro-inflammatory Th1 cytokine production compared with arginine alone (C Moineard, personal communication). Since enteral glutamine administration elevates arginine levels in tissue and plasma^(18,66) it is possible that arginine contributed to the negative interaction between glutamine and *n*-3 PUFA in the present study.

The current study reflects a single attempt to evaluate potential higher-order interactions between host physiology and dietary elements. Taking into account the large variety of dietary factors that are suggested to interact with tumour growth and response to therapy, as well as the potential for various nutrients to interact with one another, the number of relevant combinations of all of these elements is somewhat daunting. Experimental models, possibly involving expression of key proteins or genes, that could be useful in predicting the types of interactions shown here, would be a valuable asset for future work.

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