

Efficacy of Dietary Antioxidants Combined with a Chemotherapeutic Agent on Human Colon Cancer Progression in a Fluorescent Orthotopic Mouse Model

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Abstract. We report here the efficacy of dietary antioxidants in combination with chemotherapy on tumor growth in the orthotopic COLO-205-green fluorescent protein (GFP) human colon cancer mouse model. The orthotopically-transplanted nude mice used for the study were randomly divided into 5 groups (A-E) after surgical orthotopic implantation (SOI) of tumor tissue. The following diets were given: Diet A, modified AIN-93M mature rodent diet with 4% fish oil; Diet B, modified AIN-93M which contains added antioxidants vitamin A, vitamin E, and selenium at levels present in the standard AIN-93M diet; Diet C, Diet A without added antioxidants vitamin A, vitamin E, or selenium; Diet D, Diet A with 5 times the amount of added antioxidants vitamin A, vitamin E, and selenium present in Diet B. Cisplatin, 7 mg/kg, was administered intraperitoneally on day 16 after SOI. Throughout the course of treatment, noninvasive whole-body imaging, based on the GFP expression of the tumor, permitted visualization of tumor progression. At sacrifice, the mean tumor weights showed significant statistical differences in all of the treated groups compared to the negative control (no cisplatin treatment) ($p \leq 0.001$). The mean tumor weight showed a significant statistical difference between the Diet D combined with the cisplatin group compared to Diet B combined with cisplatin ($p = 0.038$). Thus, we have demonstrated that Diet D is effective against tumor growth in combination with cisplatin in the fluorescent mouse model of colon cancer COLO-205 GFP. The results of the present study therefore indicate

enhancement of cisplatin efficacy by high-dose antioxidants in combination with fish oil for colon cancer progression and suggests the design of clinical trials for this regimen.

Results from animal studies to determine the effects of antioxidants on chemotherapy have been variable, perhaps due to differences in the timing of delivery, dosage and animal models used (1). Some studies have shown a positive effect of antioxidants on reducing chemotherapy-induced side-effects, or enhancing antitumor activity of drugs (2-5). The route of administration of these antioxidants also appears to be important since intraperitoneal or intravenous delivery of the antioxidants appear to be more effective, in many cases, as compared to oral delivery (6-10). On the other hand, some animal studies have not shown any additive effect of antioxidants use during chemotherapy (11, 12), and some studies revealed adverse effects of certain antioxidants on chemotherapy (4, 13).

The long-term effects of combining chemotherapy with oral antioxidants are still not known. Patients with cancer, especially those undergoing therapy, have a reduced food intake, which might translate to a reduction in the daily intake of nutrients leading to some deficiencies. However, no guidelines exist as to the quantities of nutrients (including antioxidants) that need to be supplied to cancer patients to preserve lean body mass without enhancing tumor growth. Thus, caution needs to be exercised with respect to clinical decisions to supplement the diet of cancer patients undergoing chemotherapy with antioxidants, until safety issues regarding long-term clinical use are resolved. It is imperative that the use of antioxidants does not in any way jeopardize the anti-neoplastic effects of the therapy over the course of treatment.

In this study, we report the efficacy of the treatment of dietary antioxidants during intraperitoneal (*i.p.*)-cisplatin administration in an orthotopic green fluorescent protein

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Table I. Study design.

Group	Diet	Diet schedule	Cisplatin dose (mg/kg)	Dose schedule	Route	Number of mice
Negative control, untreated + Diet B	Diet B	Every 24 hours	0	-	-	20
Positive control, Cisplatin + Diet B	Diet B	Every 24 hours	7	Once a week	<i>i.p.</i>	20
Cisplatin + Diet A	Diet A	Every 24 hours	7	Once a week	<i>i.p.</i>	20
Cisplatin + Diet C	Diet C	Every 24 hours	7	Once a week	<i>i.p.</i>	20
Cisplatin + Diet D	Diet D	Every 24 hours	7	Once a week	<i>i.p.</i>	20

Diet A (modified AIN-93M mature rodent diet with 4% fish oil); Diet B [modified AIN-93M with antioxidants vitamin A, vitamin E, and selenium at levels present in the standard AIN-93M diet (control)]; Diet C (Diet A without antioxidants vitamin A, vitamin E, or selenium); Diet D (Diet A with 5 times the amount of added antioxidants vitamin A, vitamin E, and selenium present in Diet B).

(GFP) orthotopic mouse model of human colon cancer. GFP expression allows for visualization and imaging of tumor growth and metastasis noninvasively throughout the course of treatment, permitting real-time analysis and comparison of therapeutic intervention with dietary antioxidants.

Materials and Methods

GFP expression vector. pLEIN was purchased from Clontech (Clontech Laboratories, Palo Alto, CA, USA). The vector expresses enhanced GFP and the neomycin resistance gene on the same bicistronic message that contains an internal ribosome entry site.

GFP packaging cell culture, vector production, transfection and subcloning. PT67, an NIH3T3-derived packaging cell line, expressing the 10 Al viral envelope, was purchased from Clontech. PT67 cells were cultured in DMEM supplemented with 10% fetal bovine serum. For vector production, packaging cells (PT67) at 70% confluence were incubated with a precipitated mixture of *N*-[1-(2,3-dioleoyloxy)propyl]-*N,N,N*-trimethylammoniummethyl sulfate reagent and saturating amounts of pLEIN plasmid for 18 h. Fresh medium was replenished at this time. The cells were examined by fluorescence microscopy 48 h post-transfection. For selection, the cells were cultured in the presence of 200-1000 µg/ml G418 for 7 days.

GFP gene transduction of COLO-205 cells. For GFP gene transduction, 20% confluent COLO-205 cells (CRL 1435; ATCC, Manassas, VA, USA) were incubated with a 1:1 precipitated mixture of retroviral supernatants of PT67 cells and Ham's F-12 K containing 7% fetal bovine serum for 72 h. Fresh medium was replenished at this time. COLO-205 cells were harvested by trypsin-EDTA 72 h post-transduction and subcultured at a ratio of 1:15 into selective medium that contained 200 µg/ml G418. The level of G418 was increased to 1000 µg/ml stepwise. The brightest COLO-205 cell clones expressing GFP were selected, combined, and then amplified and transferred by conventional culture methods.

Subcutaneous xenograft. Tumor stocks were made by subcutaneously (*s.c.*) injecting COLO-205 GFP cells at 5×10^6 cells/200 µl into the flank of nude mice. The strong GFP expression of tumors grown in the subcutis of mice was verified before harvest. The tumor tissues harvested from *s.c.* growth were inspected and

any grossly necrotic or suspected necrotic or non-GFP tumor tissues in mice were removed. Tumor tissues were subsequently cut into small fragments of approximately 2 mm³.

Animals. Fifty male and 50 female athymic NCr nude mice, between 5 and 6 weeks of age, were used in this study, with an average weight of 20-25 g. The animals were bred and maintained in a HEPA-filtered environment with cages, food and bedding sterilized by autoclaving. The breeding pairs were obtained from Taconic Quality Laboratory Animals and Services for Research (Germantown, NY, USA). The animal diets were obtained from Ross Laboratories (Columbus, OH, USA). Hydrochloric acid at 0.005 mg/ml (w/v) was added to the autoclaved drinking water. The animals were examined daily for signs of illness. A total of 100 animals were used for this study. The orthotopically-transplanted animals used for the study were divided into 5 groups 3 days before treatment. Groups for each of the cohort conditions were randomly chosen.

Surgical orthotopic implantation (SOI) (14). The animals were transplanted by surgical orthotopic implantation (SOI) using the COLO-205 GFP tumor fragments harvested from the stock animals described above. The animals were anesthetized with the ketamine mixture (10 µl ketamine HCl, 7.6 µl xylazine, 2.4 µl acepromazine maleate, and 10 µl H₂O) *via s.c.* injection. The surgical area was sterilized using iodine and alcohol. An incision approximately 1.0 cm long was made along the midline in the lower abdomen of the nude mouse using a pair of surgical scissors. The serosa of the cecum near the ascending colon was removed, and then three or four 1 mm³ fragments of COLO-205 GFP tumor were transplanted onto the wall of the colon with 8-0 surgical sutures (nylon). The colon was then returned to the abdominal cavity. The abdomen was closed with 6-0 surgical sutures (silk). All procedures of the operation described above were performed with the aid of a 7 × magnification microscope (Olympus Corp., Tokyo, Japan) under HEPA filtered laminar flow hoods.

Experimental design. The cohort study was grouped as follows for a total of 100 mice. Thirteen days after tumor tissue implantation (when SOI tumors reached an approximate mean caliper-measured size of 100-150 mm³), mice were grouped with five mice per cage, with four cages per group (10 males and 10 females). Each cage was provided with a feeding jar and a known quantity of food was supplied fresh each day. Cisplatin was administered intraperitoneally

(*i.p.*) 3 days after changing diets. The food was replaced every 24 hours and weighed to determined intake (see Table I).

Feeding regimen. Mice had free access to food and water at all times. Antibiotics (sulfamethoxazole and trimethoprim) were added to the drinking water to prevent outbreak of bacterial infections. The feeding regimen is indicated in Table I. In all groups, mice were fed regular chow diet (AIN-93M-Diet B) before and during the implantation of the tumor. Two or three days before the commencement of chemotherapy, the diets were switched to the experimental diets. Mice were maintained on their respective diets until the end of the study. The study continued until a large number of animals started to die. Each cage was provided with specialized mouse feeding jars and a known quantity of food was supplied daily. Twenty-four-hour food intake was determined by measuring the food left over from the previous day. Per mouse food consumption was determined by dividing the total amount consumed by the number of mice per cage.

Diets. Diet A (modified AIN-93M mature rodent diet with 4% fish oil); Diet B [modified AIN-93M with antioxidants vitamin A, vitamin E, and selenium at levels present in the standard AIN-93M diet (control)]; Diet C (Diet A without added antioxidants vitamin A, vitamin E, or selenium); Diet D (Diet A with 5 times the amount of added antioxidants vitamin A, vitamin E, and selenium present in Diet B). The modified AIN-93M diet has the same composition as standard AIN-93M diet except that soybean oil is replaced by corn oil, fat content is 11g % not 5g % and +BHQ is removed. All diets were prepared, weighed, and individually packaged at Abbott Laboratories-RPD (Columbus, Ohio, USA). The experimental diets were supplied as blinded diets. The individual diets were isocaloric and isonitrogenous. The pre-packaged diets were stored in the freezer until time of use.

Body weights. Thirteen days after tumor implantation, the body weight for each animal was followed on a weekly basis. An electronic balance was used to measure body weight.

Whole-body optical imaging of GFP-expressing tumors (15, 16). Seventeen days after implantation, whole-body optical imaging of GFP-expressing tumors was performed once a week. The time course of tumor progression was tracked by fluorescence imaging after transplantation. The time of tumor occurrence in different organs and the numbers of metastases were recorded in tumor-bearing animals. A Leica stereo fluorescence microscope model LZ2 equipped with a mercury lamp power supply was used. Selective excitation of GFP was produced through a D425/60 band-pass filter and 470 DCXR dichroic mirror. Emitted fluorescence was collected through a long-pass filter GG475 (Chroma Technology, Brattleboro, VT, USA) on a Hamamatsu C5810 3-chip cooled color CCD camera (Hamamatsu Photonics Systems, Bridgewater, NJ, USA). Experiments were controlled and images were processed for contrast and brightness and analyzed with the help of Image Pro Plus 3.1 software (Media Cybernetics, Silver Spring, MD, USA). High-resolution images were captured directly on the computer or continuously through video output on a high-resolution Sony VCR.

Direct open imaging of green fluorescent protein-expressing tumors (17). Mice were sacrificed, explored and images taken on day 44 after SOI when they appeared pre-morbid. GFP facilitated identification of tumors by fluorescence visualization.

Table II. *Statistical analyses of tumor weights.*

Group	No. of animals available	Mean final tumor weight (g)	<i>P</i> -value*	<i>P</i> -value**
Negative control (Diet B)	20	0.65±0.73	-	-
Positive control				
Cisplatin + Diet B	19	0.21±0.16	0.01	-
Cisplatin + Diet A	19	0.18±0.10	0.01	0.847
Cisplatin + Diet C	20	0.17±0.11	0.01	0.554
Cisplatin + Diet D	18	0.12±0.05	0.01	0.038

*All cisplatin-treated groups *versus* negative control. **All cisplatin-treated groups with Diet A, Diet C and Diet D *versus* positive control.

Termination. The final tumor weights and the GFP tumor images were acquired after animals were sacrificed at the termination of the study, which was on day 44 after SOI.

Statistical methods used in efficacy evaluation. Final tumor weights of all 5 groups were analyzed using the Student's *t*-test and ANOVA test (with the Dunnett two-sided test) with an $\alpha=0.05$.

Results

Effect of cisplatin + Diet D on primary tumor growth. At the end of the study, the primary tumor weights in the cisplatin-treated groups were compared to that of the negative control group without cisplatin with the Student's *t*-test with an $\alpha=0.05$. The tumor weights showed significant statistical differences in all of the treated groups compared to the negative control ($p\leq 0.001$) (Table II). The primary tumor weights in the cisplatin-treated groups with dietary antioxidant diets, which included Diet A, Diet C and Diet D, were compared to that of the positive control group (Diet B+cisplatin) using the Dunnett two-sided test with an $\alpha=0.05$. The tumor weight was significantly lower in mice or Diet D combined with cisplatin treatment group ($p=0.038$) compared to the positive control (Table II).

Average food intake of each mouse, body weight loss, and toxicity. In the cisplatin-treated mice, food intake abruptly decreased by day 16 while in the negative-control group, the food intake was stable (Figure 1).

The mean body-weight curve revealed significant loss in all cisplatin-treated groups within the duration of the experiment (Figure 2). At day 38 after SOI, the loss of mean body weight was 22.3% in the positive control group, 23.8% in the Diet A combined with cisplatin group, 20.6% in the Diet C combined with cisplatin group, and 18.0% in the Diet D combined with cisplatin group. In cisplatin-treated groups, toxicity-related death was observed. In addition, no decrease in mean body weight was noted in the negative-control group

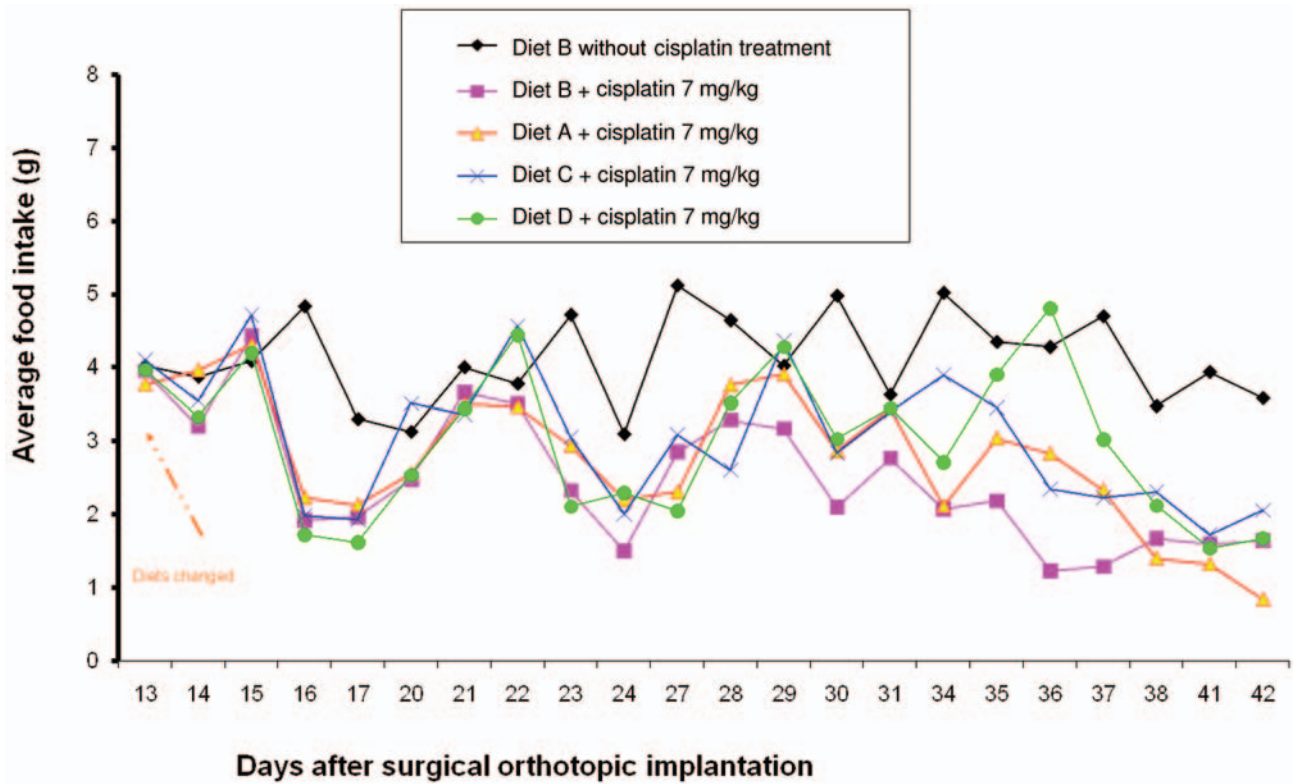


Figure 1. Average food intake of mice in study groups. See Materials and Methods for details.

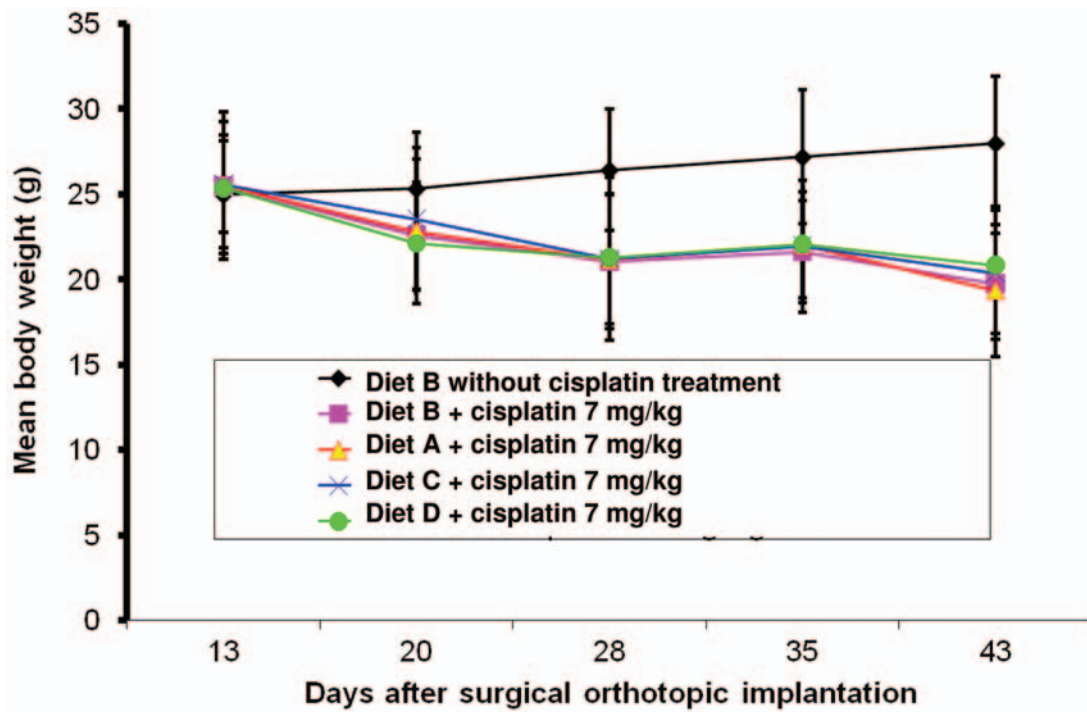


Figure 2. The body weight of each mouse was recorded once a week to determine systemic toxicity. In the cisplatin-treatment groups, the mean body weight declined >18%, which suggested that *i.p.* cisplatin administration had toxicity.



Figure 3. Whole-body images of one representative mouse from each group are shown. Real-time evaluation of therapeutic interventions on colon tumor growth and metastasis was facilitated by noninvasive fluorescence imaging. Treatment efficacy on orthotopically-implanted human colon tumors was visible in live mice by GFP fluorescence. The white arrows indicate primary tumor growth.

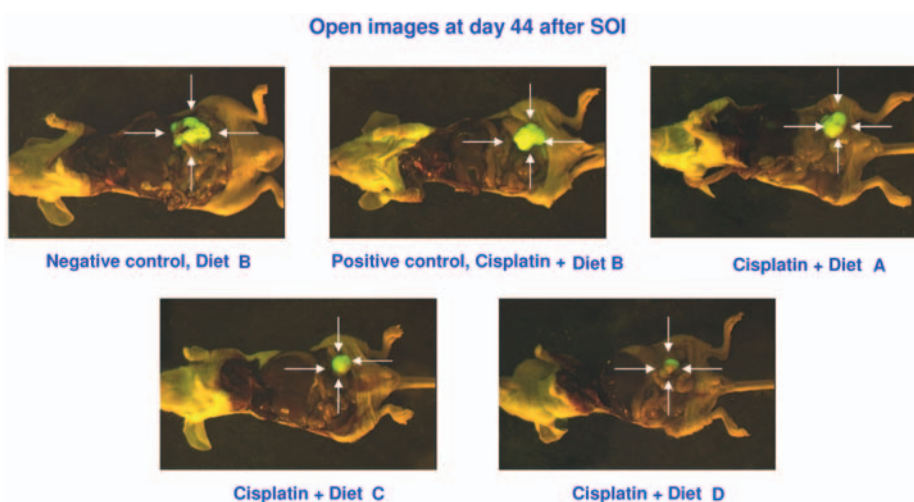


Figure 4. GFP images of primary tumor in the orthotopic COLO-205 GFP mouse model from a representative mouse from each group at the time of autopsy. White arrows show the primary colon tumor. Treatment efficacy on tumor growth of orthotopically-implanted human colon tumors in each group was visible by fluorescence. No metastasis was observed in the mice in any of the cisplatin-treated groups.

(Diet B not combined with cisplatin). However, the mean body weight of mice in the positive control group with Diet B combined with cisplatin treatment decreased by 22.3%. Thus, the results showed that the mean body-weight decrease was due to *i.p.* administered cisplatin.

Real-time imaging analysis of therapeutic efficacy. Real-time evaluation of therapeutic interventions on colon tumor growth and metastasis was carried out by fluorescence imaging. The primary tumor was sequentially imaged through the skin of the animal from days 31 to 43 after implantation. Figure 3 demonstrates that aggressive tumor growth was visible in the animals in the negative control group, beginning from days 31 to 38 after SOI. At days 38

and 44 after SOI, a significant increase in the fluorescent primary tumors was visible in the negative control group by both whole-body imaging and open imaging (see Figures 3 and 4). In contrast, no significant tumor growth was demonstrated in any of the cisplatin-treated groups given dietary antioxidants.

Discussion

A number of cancer therapeutics involve the generation of reactive oxygen species (ROS) which cause cellular damage and necrosis in malignant cells. These include the alkylating agents (*e.g.* cyclophosphamide, ifosamide) and the platinum compounds (*e.g.* cisplatin). In addition, there is evidence that

the anthracycline antitumor antibiotics (*e.g.* doxorubicin, bleomycin) generate ROS. However, the exact role of ROS in its antitumor efficacy remains to be determined (18). Ionizing radiation causes cell death through free radical formation. Thus there is concern that reduction in the levels of ROS generated in the tumor cell by the action of antioxidants might reduce the effectiveness of chemotherapy (19-21).

On the other hand, some studies suggest that the use of antioxidants during chemotherapy might actually be beneficial for the patient (22-24). Many antineoplastic agents produce severe side-effects due to the generation of excessive free radicals which have adverse effects on normal tissue cells. These include doxorubicin-induced cardiotoxicity, bleomycin-induced pulmonary toxicity, and cisplatin-induced nephrotoxicity (25). It has been suggested that treatment with antioxidants might actually protect normal cells from this free radical damage thus reducing the side-effects of chemotherapy.

The results of the present study therefore indicate enhancement of cisplatin efficacy by supplementation of high-dose antioxidants in combination with fish oil for colon cancer progression and suggests the design of clinical trials for this regimen.

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