Unexpected Antitumorigenic Effect of Fenbendazole when Combined with Supplementary Vitamins

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Abstract

Diet containing the anthelminthic fenbendazole is used often to treat rodent pinworm infections because it is easy to use and has few reported adverse effects on research. However, during fenbendazole treatment at our institution, an established human lymphoma xenograft model in C.B-17/Icr-*prkdc*^{scid}/Crl (SCID) mice failed to grow. Further investigation revealed that the fenbendazole had been incorporated into a sterilizable diet supplemented with additional vitamins to compensate for loss during autoclaving, but the diet had not been autoclaved. To assess the role of fenbendazole and supplementary vitamins on tumor suppression, 20 vendor-supplied 4-wk-old SCID mice were assigned to 4 treatment groups: standard diet, diet plus fenbendazole, diet plus vitamins, and diet plus both vitamins and fenbendazole. Diet treatment was initiated 2 wk before subcutaneous flank implantation with 3×10^7 lymphoma cells. Tumor size was measured by caliper at 4-d intervals until the largest tumors reached a calculated volume of 1500 mm^3. Neither diet supplemented with vitamins alone nor fenbendazole alone caused altered tumor growth as compared with that of controls. However, the group supplemented with both vitamins and fenbendazole exhibited significant inhibition of tumor growth. The mechanism for this synergy is unknown and

deserves further investigation. Fenbendazole should be used with caution during tumor studies because it may interact with other treatments and confound research results.

Abbreviation: HIF, hypoxia-inducible factor 1α

Pinworms are a common problem in rodent facilities[4,16] and typically are treated with anthelminthics.[17] Fenbendazole incorporated in the diet is used often because it is safe—the oral LD_{50} for rats and mice is in excess of 10,000 mg/kg[5]—and labor-efficient, and adverse effects in research rodents have rarely been reported.[20] More than 50% of ingested fenbendazole is absorbed and metabolized in the liver, primarily to the active form, fenbendazole sulfoxide.[19] Fenbendazole inhibits microtubule polymerization, and its efficacy as an anthelminthic results from its greater affinity for helminth tubulin than mammalian tubulin.[9]

During an 8-wk facility treatment for *Aspiculuris tetraptera* pinworms with fenbendazole diet at our institution, human lymphoma xenografts failed to grow in C.B-17/Icr-*Prkdc*^{scid}/Crl (SCID) mice. This well-established xenograft model is used to study the role of mitochondrial genes in tumorigenesis and usually results in 80% to 100% successful tumor growth within 21 d. However, during the facility treatment with fenbendazole, no tumors grew in 40 mice during the 30 d after injection. The mice in this study had not been diagnosed with pinworms but were part of a facility treatment. Rodents in this area customarily were fed a commercial irradiated diet (Global 2918, Harlan Teklad, Madison, WI). However the equivalent treatment diet containing 150 ppm fenbendazole was available only in a sterilizable form (2018S, Harlan Teklad) supplemented with vitamins A, D, E, K, and B (Table 1) to compensate for loss during sterilization. Because the animal facility was not configured for dietary sterilization, the sterilizable diet was fed unautoclaved, with the result that mice received higher-than-normal concentrations of vitamins. Therefore the observed antitumor effect could have resulted from either the additional vitamins or the fenbendazole. Therefore a controlled study was conducted to test whether fenbendazole, supplemented vitamins, or both in combination affected the growth of this human lymphoma cell line in SCID mice.

Materials and Methods

Mice were housed in an AAALAC-accredited facility under conditions compliant with the *Guide for the Care and Use of Laboratory Animals*.[12] Procedures were approved by the Johns Hopkins institutional animal care and use committee. The mice were housed in individually ventilated cages (Allentown Caging Equipment, Allentown, NJ) on autoclaved corncob bedding (Bed-O'Cobs, The Andersons, Maumee, OH). Mice received hyperchlorinated reverse-osmosis–treated water by means of an in-cage automated watering system (Edstrom Industries, Waterford, WI). Cages were changed by using chlorine-dioxide-based disinfectant (MB10 tabs, 100-ppm solution, Quip Laboratories, Wilmington, DE) in filtered-air change stations (Lab Products, Seaford, DE). The colony tested free of a wide range of viral and parasitic pathogens by sentinel surveillance; pathogens included Sendai virus, pneumonia virus of mice, mouse hepatitis virus, mouse minute virus, mouse parvovirus 1 and 2, Theiler mouse encephalomyelitis virus, reovirus, epizootic diarrhea of infant mice, lymphocytic choriomeningitis virus, ectromelia virus, murine adenovirus, murine cytomegalovirus, *Mycoplasma pulmonis*, fur mites, and pinworms.

Twenty 4-wk-old male SCID mice (Charles River Laboratories, Boston, MA) were assigned randomly to 4 groups housed 5 animals per cage: control diet (2018, Harlan Teklad), diet plus fenbendazole (2018 custom-formulated with 150 ppm fenbendazole, Harlan Teklad), diet plus supplemented vitamins (2018S, Harlan Teklad), and diet plus both fenbendazole and supplemented vitamins (2018S plus 150 ppm fenbendazole, Harlan Teklad). All diets had the same basic composition (18% protein, 5% fat; Table 1), and none of the diets was autoclaved. The mice were stabilized on their respective diets for 2 wk after arrival and before tumor cells were injected.

Table 1.

Vitamin levels in nonsterilizable (regular) and sterilizable (supplemented) diets

	Diet			
Vitamins	Regular	Supplemented	Units	% Increase
A	15.4	30.7	IU/g	100
Retinol	4.65	9.31	mg/kg	100

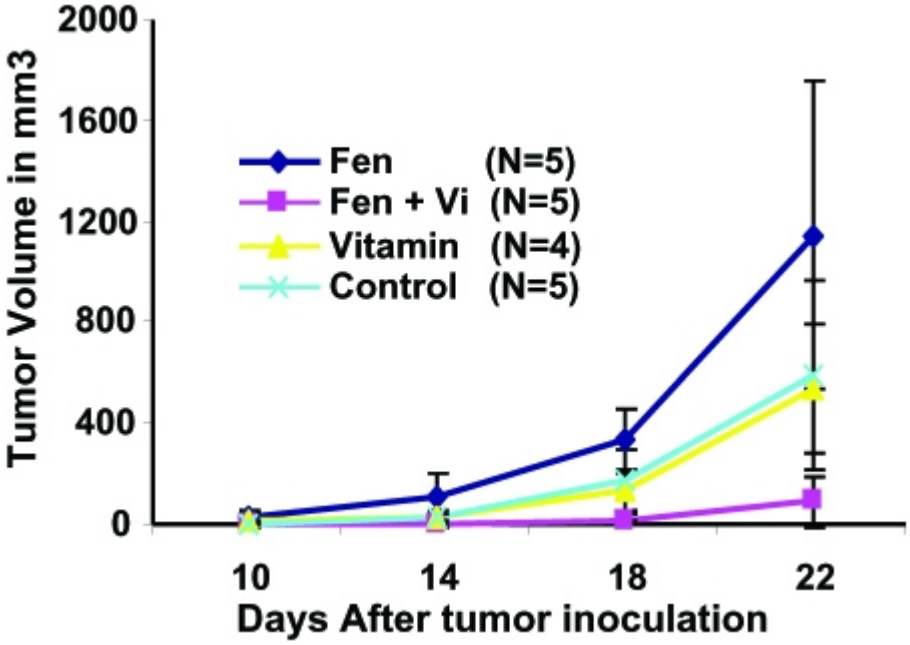
	Diet			
Vitamins	Regular	Supplemented	Units	% Increase
D3	1.54	2.05	IU/g	33
Cholecalciferol	38.39	51.18	g/kg	33
E	101	126	mg/kg	25
K3	51	102	mg/kg	100
B1	16.5	117.6	mg/kg	613
B2	14.9	27.2	mg/kg	83
Available niacin	41.2	87.3	mg/kg	112
B6	18.5	26.8	mg/kg	45
Pantothenic acid	33	141.6	mg/kg	329
B12	0.08	0.15	mg/kg	88
Available biotin	0.3	0.82	mg/kg	173
Folate	3.34	8.41	mg/kg	152

The day after arrival, blood was collected by puncture of the facial vein under manual restraint and processed by using an automated analyzer (Hemavet 950, Drew Scientific Group, Dallas, TX) for complete blood count. Human Burkitt lymphoma cells (P493-6 B cell line[8]) were cultured in RPM1 1640 plus 10% fetal calf serum containing 100 U/ml penicillin and 100 μg/ml streptomycin. The cells were washed, counted, and resuspended in PBS. Each mouse was restrained manually and received 3×10^7 lymphoma cells in 100 μL PBS injected subcutaneously in the flank. Growth of tumors was monitored every 4 d by using calipers, and tumor volume was calculated by using the formula length × width × width × 0.52 mm^3. Once the largest tumors reached a calculated volume of 1500 mm^3, the experiment was terminated. Before euthanasia of mice, blood was collected from the facial vein and analyzed for complete blood count. The size of tumors in each group at the endpoint was compared with that of the control group by using the Student *t* test. Total white cell, lymphocyte, and neutrophil counts were compared with those of controls at the beginning and end of the experiment.

Results

Tumor size.

Tumors in the fenbendazole plus vitamin group were significantly smaller ($P = 0.009$) and delayed in initial growth compared with those of the control group (Figure 1). Tumor growth did not differ between control and fenbendazole-only ($P = 0.12$) or vitamin-only ($P = 0.82$) groups. The apparent trend toward increased tumor size ($P = 0.12$) in the fenbendazole-only group was due to a single outlier.

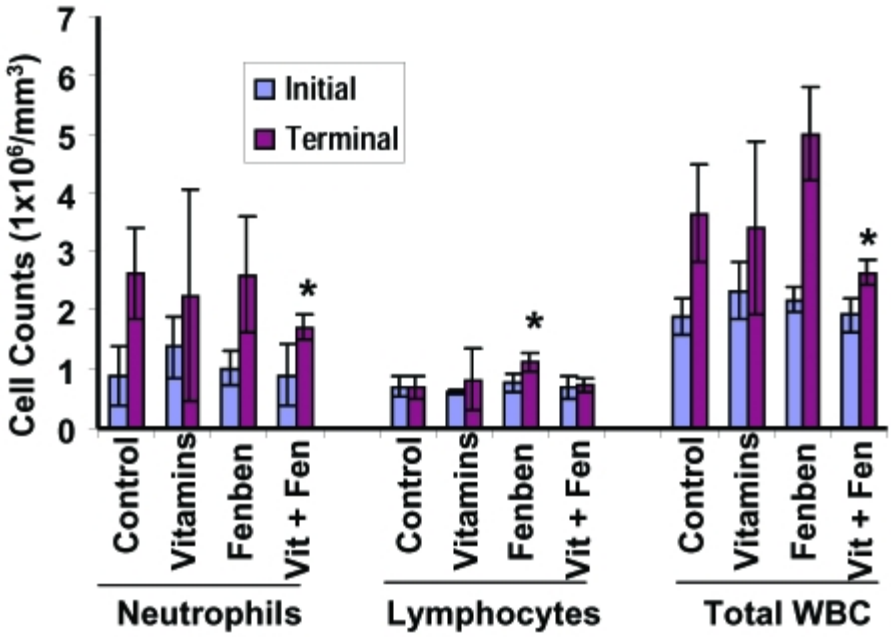


Growth in tumor volume (mean (SD) in mice on 4 different diets. After subcutaneous injection of lymphoma cells, tumor growth in mice receiving fenbendazole- or vitamin-supplemental diet did differ from that in controls. Tumor growth in mice on fenbendazole- plus vitamin-supplemented diet was significantly (P = 0.009) inhibited compared with that in controls.

White cell counts.

One mouse died during the initial blood collection, and 1 blood sample from the terminal group was lost. Initial complete blood counts (Figure 2) were typical of SCID mice, demonstrating a low white cell count with a paucity of lymphocytes. White cell counts did not differ significantly between test and control groups on arrival. At the end of the study (Figure 2), all groups demonstrated a leukocyte response consisting

primarily of neutrophils. The total white cell and neutrophil responses in the fenbendazole plus vitamin group were significantly smaller ($P = 0.001$ and $P = 0.04$, respectively) than in the control group. There was a trend ($P = 0.06$) toward increased total white cell count and a significantly increased lymphocyte count ($P = 0.009$) in the fenbendazole-only group compared with controls.



Initial and terminal white cell counts (mean ± SD) in mice on 4 different diet. Initial counts did not differ among the 4 groups. Terminal total white cell and neutrophil counts in mice receiving the (p=0.001 and p=0.04 respectively) lower than those in control animals, and terminal lymphocyte counts in the fenbendazole-only group were significantly (P = 0.009) higher than those in controls.

Discussion

This study demonstrated that a combination of supplemented vitamins and fenbendazole in the diet inhibited growth of a human lymphoma cell line in SCID mice, whereas fenbendazole or vitamins alone had no growth inhibitory effect. The mechanism for this synergy is as yet unknown. However, like other anticancer drugs such as taxanes,[14] quinolones,[3] and vinca alkaloids,[15] fenbendazole inhibits microtubule polymerization. In addition, fenbendazole is a member of a large group of related anthelminthics, the benzimidazoles, and another member of this group, mebendazole,

exerts antitumor effects by inhibition of tumor-induced neovascularization.[11] However, in the present case, fenbendazole likely contributes to the antitumorigenic effect through its antimicrotubule activity.

Supplemented vitamins included B, D, K, E, and A. Vitamins E and A both have antitumor properties by virtue of their antioxidant properties. Vitamin E causes antitumor and antimetastatic effects in several animal models of cancer; for example, it suppresses the nuclear transcription factor NFκB in prostate cell lines.[13] NFκB regulates proapoptotic and prometastatic proteins; thus suppression results in antitumor effects. Higher intake of dietary folate and vitamin B has been associated with lower incidence of colorectal cancer in women.[22] Recent work suggests that hypoxia-inducible factor 1α (HIF), which plays a key role in tumorigenesis by facilitating adaptation to hypoxia, is diminished by microtubule inhibitors,[7] and some antioxidants may exert their antitumor effects through reducing HIF rather than by reducing genetic instability.[8,10] We hypothesize that the combination of fenbendazole and supplemented vitamin antioxidants may have exerted a threshold effect, resulting in reduction of HIF and inhibition of tumorigenesis. Indeed, preliminary information from our laboratory confirms that fenbendazole inhibits HIF transcriptional activity in cell culture in an additive manner with other HIF inhibitors (data not shown).

This study demonstrated significant inhibition of tumor growth. Results were less dramatic than the total inhibition initially observed, perhaps due to inadvertent inclusion of lower vitamin concentrations during the study than during the initial observation. Vitamins in prepared diets deteriorate with time, and the study diet containing both vitamins and fenbendazole was within a week of its expiration date (6 mo after manufacture) at the time of this study. In contrast, diet used during the initial observation was newly ordered to treat the pinworm outbreak and so was likely more recently manufactured, with higher vitamin concentrations. Unfortunately expiration dates during the initial observation were not recorded and vitamin concentrations in the diet were not analyzed independently so this theory cannot be confirmed.

Figure 1 shows a trend ($P = 0.12$) toward *increased* tumor growth in the fenbendazole only treatment group. This trend was the result of 1 outlier. Nonetheless, fenbendazole may have tumor-promoting activity in rats at therapeutic dosages (that is, through

inhibition of connexin 32 and induction of cytochrome P450 enzymes 1A1 and 1A2).[21] In our experiment, the apparent increase in tumor size in the fenbendazole group was due to a single large-tumor outlier, and we believe that this trend is due to experimental noise. Further studies are needed to determine whether fenbendazole actually exhibits a tumor-promoting effect in this model.

Although the exact composition of T and B cells was not analyzed, complete blood counts confirmed that the numbers of white cells did not differ initially between the treatment and control groups, ruling out the possibility of a chance concentration of relatively immunocompetent 'leaky' SCID mice[1,2] in 1 group. At study termination, the fenbendazole plus vitamin group had significantly lower total white cell and neutrophil values ($P = 0.001$ and $P = 0.04$, respectively) than did the control group. This observation is consistent with significantly smaller tumors causing less compression and necrosis of adjacent tissues, although this supposition was not confirmed by histopathology. There was also a trend ($P = 0.06$) toward increased total numbers of white cells and a significantly larger ($P = 0.009$) lymphocyte response in the fenbendazole-only group. Fenbendazole has had immunomodulatory effects in sheep and mice[18] and stimulated proliferation of T and B cells in healthy mice,[6] but most studies have shown no effect on selected immune responses.[21] It is not possible to draw any conclusions regarding immunomodulation from the present study because additional analysis of cell types was not done.

Our study showed that fenbendazole alone did not significantly affect growth of the P493-6 human lymphoma cell line in SCID mice. Most importantly, our observation that fenbendazole in combination with supplemented vitamins significantly *inhibited* tumor growth has implications for its use during antitumor studies because it may cause unpredictable interactions with test substances and thus alter research results.

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