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Supplementation by vitamin D compounds does not affect colonic tumor development in vitamin D sufficient murine models

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Abstract

Epidemiological studies indicate that sunlight exposure and vitamin D are each associated with a lower risk of colon cancer. The few controlled supplementation trials testing vitamin D in humans reported to date show conflicting results. We have used two genetic models of familial colon cancer, the *Apc*^{Pirc/+} (Pirc) rat and the *Apc*^{Min/+} (Min) mouse, to investigate the effect of 25-hydroxyvitamin D₃ [25(OH)D₃] and two analogs of vitamin D hormone on colonic tumors. Longitudinal endoscopic monitoring allowed us to test the efficacy of these compounds in preventing newly arising colonic tumors and in affecting established colonic tumors. 25(OH)D₃ and two analogs of vitamin D hormone each failed to reduce tumor multiplicities or alter the growth patterns of colonic tumors in the Pirc rat or the Min mouse.

Keywords

Chemoprevention; endoscopy; animal models

Introduction

Colorectal cancer is the third leading cause of cancer-related death in men and women in the US [1]. Colon cancer risk and mortality vary greatly around the world, indicating an environmental component to its pathogenesis. Epidemiological studies have shown that

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vitamin D is associated with a reduced risk of colon cancer. In particular, individuals with lower exposure to sunlight, resulting in reduced 25(OH)D₃ serum levels, have increased rates of colon cancer [2,3]. However, supplementation studies in human populations show conflicting results regarding the efficacy of vitamin D [4,5].

Three decades ago Garland and Garland recognized an association between lower exposure to sunlight and increased colon cancer mortality rates across the United States [2]. From this observation, they proposed a protective mechanism involving vitamin D. Five years later, Garland *et al* reported a 19-year prospective study in which increased vitamin D and calcium intake, as gathered from dietary histories, correlated with a decreased risk for developing colon cancer [6]. However, other studies based on dietary recall have failed to find a similar risk reduction [7,8]. Some association studies that measured serum 25(OH)D₃ levels and compared individuals in the lowest quartile with those in the highest quartile have shown a correlation with reduced colon cancer risk [9]. Similarly, the Nurse's Health Study, a nested case-control study where colorectal cancer incidence was the primary endpoint, identified 193 colorectal cancer cases diagnosed up to 11 years after a single blood collection [10]. Those findings indicated a significant association between high plasma 25(OH)D₃ levels and lower colorectal cancer incidence. Interestingly, the results were significant only among women older than 60 years of age, residing in areas with high levels of available sunlight, and for cancers of the distal colon and rectum. By contrast, other studies have failed to find an association between serum 25(OH)D₃ levels and colon cancer. In the Japan Public Health Centre-based Prospective Study, a nested case-control study, no association was seen between serum 25(OH)D₃ levels and the risk for developing colon cancer [11]. Looking further, some studies have found associations only with certain patient subpopulations or tumor sites within the colon [10,12].

Overall, epidemiological studies attempting to assess dietary and lifestyle factors for cancer susceptibility frequently encounter uncontrolled confounders. While many studies attempt to control for variables that may impact vitamin D status or colon cancer risk, other unappreciated confounding variables may exist. These variables, taken together, could lead to an apparent protective effect attributed to vitamin D status. In a prospective study, Freedman *et al* examined a list of lifestyle characteristics that varied by quantile of serum 25(OH)D₃ status [13]. They found an inverse association between colon cancer mortality and 25(OH)D₃ status, which was adjusted for sex, race/ethnicity and smoking status. However, trends are evident for confounding factors in individuals with higher 25(OH)D₃ status, including reduced total body fat and increased level of physical activity. A trend for lower body mass index and higher level of physical activity with higher circulating 25(OH)D₃ levels has also been observed in several other studies [12,14]. Plausibly, individuals with higher serum levels of vitamin D metabolites tend to be healthier overall and any combination of these lifestyle factors may play a role in protection from colon and other cancers. Thus, evidence for a *bona fide* link between circulating 25(OH)D₃ levels and colon cancer risk must take into account factors such as geographic location, diet, exercise, lifestyle and culture.

Although controlled prospective clinical trials are needed to assess causation, some reported studies have combined calcium with vitamin D supplementation, making it difficult to evaluate the effect of vitamin D itself [5,15]. Some studies have reported an inverse association between dietary calcium intake or serum calcium levels and colon cancer risk [8]. Similar effects have also been seen in animal models. One such study found that supplemental calcium alone was able to decrease tumor multiplicity and size but not tumor incidence [16]. Interestingly, studies have also shown an interaction between serum 25(OH)D₃ and calcium levels. In the Health Professionals Follow-up Study, a nested case-control study, the inverse association between a decreased risk for colon cancer and

increased serum 25(OH)D₃ levels was limited to patients who also had a high calcium intake [9]. Further, Jenab and colleagues showed that individuals with both low serum 25(OH)D₃ and low calcium levels had the highest risk for colon cancer (incidence rate 1.33), while those with both high serum 25(OH)D₃ and high calcium levels had the lowest risk (IR 0.72) [8].

As a result of the conflicting reports for the association between vitamin D and colon cancer in humans, we tested three vitamin D compounds in two rodent models of colon cancer. Both the Pirc rat and the Min mouse are heterozygous for mutations in *Apc*, a tumor suppressor gene that is mutated in approximately 80% of human colorectal cancers [17–19]. The Pirc rat spontaneously develops a high incidence and multiplicity of colonic tumors. Treatment of Pirc rats with dextran sodium sulfate (DSS), a polymer that further increases the multiplicity of colonic tumors, allows us to study tumors with a potentially different etiology. Therefore, both untreated and DSS-treated Pirc rats have been utilized to test these compounds. By contrast, the C57BL/6J Min mouse, the most commonly used genetic model of intestinal cancer, develops few colonic tumors. Thus, studies with the Min mouse may lack the power to detect an effect of agents on colonic tumors [20,21]. This limitation has been overcome by treating Min mice with DSS which increases the number of colonic tumors and by following the tumors longitudinally [22]. To complement terminal tumor counts, endoscopy was performed regularly to detect changes in colonic tumor emergence, growth rate, or regression [23].

Materials and Methods

Animal Breeding and Maintenance

Rats and mice were maintained under a protocol approved by the Animal Care and Use Committee of the University of Wisconsin School of Medicine and Public Health and in a facility in the McArdle Laboratory approved by the American Association of Laboratory Animal Care. Animals were housed in standard caging with free access to food and acidified water. F1 hybrid rats were generated by breeding ACI *Apc*^{+/+} females (Harlan, Indianapolis, IN) to F344/NTac *Apc*^{Pirc/+} males (developed in the laboratory of WFD and now commercially available through Taconic, Hudson, NY). The numbers of tumors in the small intestine versus the colon of Pirc rats is strain dependent in the F1 hybrids [17]. F1 generation rats were used in these studies to increase the number of colonic tumors and to shift the distribution of tumors even further in favor of the colon. F1 hybrid mice were generated by breeding SWR *Apc*^{+/+} females (Jackson Laboratory, Bar Harbor, ME) to C57BL/6J *Apc*^{Min/+} males (developed in the laboratory of WFD and now commercially available through Jackson Laboratory, Bar Harbor, ME). The distribution of tumors between the small intestine and colon also differs among the various inbred strains of Min mice [24]. F1 generation mice were used to decrease the number of tumors in the small intestine, thereby reducing blockage and increasing lifespan. In addition, using F1 generation animals creates the opportunity in future studies to examine any informative SNP sites for somatic changes in the genome or epigenome of tumors in response to these compounds.

Chemicals

Dextran sodium sulfate (500,000 kDa) was purchased from Fisher Scientific (Pittsburgh, PA). 25(OH)D₃ was purchased from Chemvion (Shanghai, China) and was determined to be 97% pure by HPLC [25]. The “high potency” vitamin D analog, 2 α -methyl-19-nor-(20S)-1 α ,25-dihydroxyvitamin D₃ (HP), and the relatively “non-calcemic” analog, 2-methylene-19-nor-(20S)-1 α -hydroxybismopregnacalciferol (NC), were developed in the laboratory of HFD [26,27]. These two analogs possess different tissue selective actions with NC having specific activity in the skin [28] and HP in the bone [27]. Another known

distinction between these two analogs is that the potency of HP is approximately one thousand-fold greater than that of NC. Both analogs efficiently suppress parathyroid hormone, suggesting a common pathway in the parathyroid gland [26 and unpublished work].

Diets

Control rats were fed either AIN-76A (Harlan, Madison, WI) or 5020 chow (Purina, St. Louis, MO); no difference between these diets was seen for tumor multiplicity, time to first tumor, or growth of tumors (data not shown). Therefore, control rat data from both diets were combined. Control mice were fed AIN-76A. All supplemented diets were formulated in the AIN-76A base diet. The vehicle used to incorporate the 25(OH)D₃ into the AIN-76A diet was 5% (w/w) Wesson soybean oil and 0.25% (v/w) ethanol (Pharmaco Aaper, Brookfield, CT). Daily doses of the test compounds were calculated based on a consumption rate of 20 grams of food per day for the rat and 4 grams of food per day for the mouse [29–32].

25(OH)D₃ was added to achieve a target dose of 1500 µg/kg body weight/day for the rat and 500 µg/kg body weight/day for the mouse. From pilot studies, these concentrations were sufficient to provide a 1–2 mg increase in calcium per 100 ml of serum, demonstrating that the 25(OH)D₃ was biologically effective. This increase in serum calcium did not affect the weight of the experimental animals versus the controls (male rats 338.5 ± 19.8 vs 351.8 ± 15.1 grams; female rats 218.8 ± 13.2 vs 224.2 ± 14.0 grams; male mice 28.4 ± 3.1 vs 26.2 ± 2.4 grams; female mice 22.8 ± 1.5 vs 23 ± 1.7 grams) as measured at sacrifice. This is an indicator that the increase in serum calcium did not affect the overall health of the animals during the study.

The HP analog diet was made up to reach a target dose of 0.02 µg/kg body weight/day in the rat and mouse; while the NC analog diet was made up to reach a target dose of 1200 µg/kg body weight/day in the rat and 600 µg/kg body weight/day in the mouse. These maximum tolerated doses each resulted in a 1–2 mg increase in calcium per 100 ml serum.

Rat Experimental Design

At 33 days of age, male and female (ACI x F344/NTac)F1 *Apc^{Pirc/+}* rats were assigned to either 25(OH)D₃-supplemented diet or vehicle diet. Assignments to protocols were random, except that sexes and littermates were evenly distributed (Fig 1). At 40 days of age, half of the animals were given 4% (w/v) DSS in the drinking water to study DSS-induced tumors (chemoprevention). The rat dosing regimen consisted of two rounds of DSS, each lasting seven days and separated by a seven-day period without DSS. The dose and molecular weight of DSS used in these experiments maintained normal crypt architecture, minimized inflammatory infiltrates, and did not affect body weight until the tumor burden became high (data not shown). Batches of DSS used in this study were consistent in the resultant multiplicity of rat colonic tumors and therefore one control group was used for all test diets. Another small set of male *Pirc* rats not treated with DSS were randomized to one of two analog diets: NC analog or HP analog. Animals underwent colonoscopy every other week starting before diet supplementation until sacrifice at 140 days of age or when they became moribund. Weights were measured during each colonoscopy procedure and at sacrifice. Tumors identified before supplementation, as well as those arising during supplementation, were used to test the effects of these compounds on existing tumors (treatment). Tumors that arose during supplementation provided a test of the effects of these compounds on newly arising tumors (chemoprevention). At sacrifice, the small intestine and colon were opened longitudinally, laid flat, washed with PBS and formalin-fixed; the small intestine was

divided into four equal sections before fixation. Blood was obtained at sacrifice by cardiac puncture.

Mouse Experimental Design

Starting at 25–40 days of age, all male and female (SWR x B6J)F1 *Apc*^{Min/+} mice were given 4% (w/v) DSS in the drinking water to increase the number of colonic tumors (Fig 1). The mouse dosing regimen consisted of two rounds of DSS, each lasting four days and separated by a 17-day period without DSS. As in the rat experiments, the dose and molecular weight of DSS used in these studies did not cause severe damage to the colonic epithelium (data not shown). In the chemoprevention mode (prior to the first DSS exposure) mice in one group were assigned to one of three diets: 25(OH)D₃, HP analog or vehicle alone. In the treatment mode (following completion of the last DSS exposure) mice in a second group were assigned to one of three diets: NC analog, HP analog, or vehicle alone. Assignments to protocols distributed sexes and littermates evenly across protocols, but were otherwise random. Batches of DSS used in this study differed in the resultant multiplicity of mouse colonic tumors, so each test diet in the mouse was analyzed with its own set of contemporaneous controls. Animals underwent colonoscopy every week until sacrifice at 130 days of age or when they became moribund. At sacrifice, the small intestine and colon were treated as described above. Blood was obtained at sacrifice by laceration of the abdominal aorta.

Endoscopy and Terminal Tumor Counts

Endoscopy was performed on rats and mice at predetermined intervals until sacrifice (Fig 1). Briefly, the animal was anesthetized with isoflurane and placed on a sterile surgical field, ventral side down. The colon was flushed with warm PBS to remove any fecal material and to provide lubrication. The anatomy of the rat allows the distal two-thirds of the colon to be visualized with endoscopy, while the anatomy of the mouse allows the distal half of the colon to be visualized. Video of the colonoscopy and still images of colonic tumors were obtained and analyzed after each colonoscopy procedure. For the Pirc rats, endoscopy began before dietary supplementation, which allowed us to examine the effects of the compounds both on existing tumors (treatment) and on newly arising tumors (chemoprevention). For the Min mice, endoscopy began after the second DSS treatment, because young mice were too small to allow insertion of the endoscope into the anus. The longitudinal fate of tumors was determined by three observers blinded to the diet of the animal. Tumors were examined during each procedure and any tumor that appeared at least twice during the study was given one of four scores: growing, static, regressing or unscorable; those judged to be unscorable were excluded from the longitudinal analysis. A score was generated based on agreement between at least two of the three blinded observers. After sacrifice, formalin-fixed tumors were counted on a dissecting microscope. Total tumor counts were obtained for each of the four small intestinal sections and for the entire colon.

Statistical Methods

For the biochemical assays, a two-sided Wilcoxon rank sum test was used; a p-value ≤ 0.05 was considered significant. Longitudinal analysis of endoscopic images utilized a chi-square test of growing versus non-growing tumors; no significant sex-specific differences in this ratio of tumor fates were observed for either the rat or the mouse, therefore sexes were combined for analysis. The means and standard deviations of tumor multiplicity data from Pirc rats and Min mice are not normally distributed, leading us to test our hypotheses using nonparametric statistics [17,33]. Tumor multiplicities in the Pirc rat show significant sex differences, as described previously [17]. Therefore, terminal multiplicity data of the Pirc rat were blocked based on Lehmann's extension to the Wilcoxon rank sum test and jointly tested for the effects of the test diets [34]. Since multiplicities of colonic tumors in the Min

mouse show no sex-specific differences, the data were combined to analyze the terminal multiplicity data in the mouse experiments, and a two-sided Wilcoxon rank sum test was employed. The Dunn-Sidak correction was utilized to correct for multiple testing in both the rat and the mouse studies. For the purpose of the Dunn-Sidak correction, experiments with and without DSS in the Pirc rat were considered to be two separate studies, as were the chemoprevention and treatment experiments in the Min mouse. Therefore, for the terminal multiplicity data in the rat, three comparisons were made without DSS and one comparison was made with DSS. A p -value ≤ 0.0167 for studies without DSS and a p -value ≤ 0.05 for studies with DSS was considered significant. For the terminal multiplicity data for the mouse, there were two comparisons in the prevention mode and two comparisons in the treatment mode. A p -value ≤ 0.025 was considered significant for each of these modes.

Biochemical Assays

Serum 25(OH)D₃ levels were measured using a ¹²⁵I-radioimmunological assay following manufacturer's instructions (DiaSorin; Stillwater, MN) [35]. Serum calcium was measured by atomic absorption spectrometry and the ABX Pentra clinical chemistry analyzer (Horiba ABX, France) [35].

Results

25(OH)D₃ did not reduce the multiplicity of colonic tumors in Pirc rats or Min mice

Groups of Pirc rats underwent endoscopy to identify existing tumors and were then placed on the 25(OH)D₃-supplemented diet. Although serum 25(OH)D₃ levels were significantly increased (Table 1A), there was no reduction in the multiplicity of tumors in the small intestine or colon (Fig 2A, Table 2A).

In an experiment specifically designed to test chemopreventive action against newly arising tumors, additional groups of Pirc rats underwent endoscopy to identify and exclude any existing tumors from analysis. They were then placed on the 25(OH)D₃-supplemented diet in advance of DSS treatment. Although serum 25(OH)D₃ levels were significantly increased (Table 1A), there was no reduction in the multiplicity of tumors in the small intestine or colon (Fig 2A, Table 2A).

Cohorts of Min mice were treated with DSS to increase the multiplicity of colonic tumors. To test chemopreventive action, they were placed on the 25(OH)D₃-supplemented diet in advance of DSS treatment. Again, although serum 25(OH)D₃ and calcium levels were significantly increased (Table 1B), there was no effect on the multiplicity of tumors in the small intestine or colon (Fig 2B, Table 2B).

Two analogs of vitamin D did not reduce the multiplicity of colonic tumors in Pirc rats or Min mice

To explore compounds related to vitamin D with different pharmacokinetic properties, we analyzed the effects of two vitamin D analogs on tumors of the small intestine and colon (Fig 1). Maximum tolerated dosages, even of the "non-calcemic" analog, were established on the basis of elevation of serum calcium compared to controls (Table 1B). The HP and NC analogs were tested in a small number of male Pirc rats not treated with DSS and no significant change in tumor multiplicity was seen (Fig 1B, Table 2B).

To test the chemopreventive action of the HP analog, a group of mice was placed on diet containing HP analog and then treated with DSS. To test the analogs in a treatment modality, other groups of mice were placed on diet containing HP analog or NC analog following the completion of the DSS administration. There was no effect of the HP analog

(chemoprevention or treatment) or NC analog (treatment) on the multiplicity of tumors in the small intestine or colon in the mice (Fig 2B, Table 2B).

Supplementation with 25(OH)D₃ or either of two vitamin D hormone analogs did not cause tumor regression in Pirc rats or Min mice

Both rats and mice routinely underwent endoscopy throughout the study. Thus, any substantial change in existing tumors or emergence of newly formed tumors in the distal colon would have been detected. Most of the colonic tumors were newly emerging during this study in both the rats and the mice. Supplementation of Pirc rats or Min mice with 25(OH)D₃ or either analog of vitamin D hormone did not appear to inhibit the emergence of these newly arising colonic tumors (Fig 3 and data not shown).

In the rat, only 1 of 101 scorable tumors appeared to regress; this tumor occurred in the control group. The tumor growth patterns were not significantly different in rats given the 25(OH)D₃-supplemented diet versus those in the control group (Table 3A).

In the mouse, only 1 of 111 scorable tumors appeared to regress; this tumor occurred in the HP prevention group. The growth patterns of tumors from the various control sets were indistinguishable and therefore were combined (data not shown). The tumor growth patterns were not significantly different in mice given either the HP analog diet or the NC analog diet versus those in the control group (Table 3B).

All rats not treated with DSS survived until the termination of the study (data not shown). However, about half of the DSS-treated rats had to be sacrificed before the scheduled 140 day time point, owing to the weight loss caused by their high tumor burden (data not shown). By contrast, no such weight loss or shortened time to morbidity was seen in any of the Min mice.

Discussion

These studies were designed to test the effects of 25(OH)D₃ and two analogs of vitamin D hormone both on existing tumors (treatment) and on newly arising tumors (chemoprevention) in two rodent models. Our studies are unique in two respects. First, to avoid issues of species-specificity in animal modeling of human disease, we used models for colon cancer in two distinct mammalian genera, the Pirc rat and the Min mouse. Second, to assess action by these agents in both chemopreventive and treatment modes, we have utilized longitudinal monitoring by endoscopy and tumor induction with DSS. Longitudinal monitoring provides the further advantages of detecting concurrent emergence and disappearance of tumors as well as changes in tumor size over time; such information would be lost with terminal tumor counting alone. Overall, these studies constitute a comprehensive investigation of whether supplementation by vitamin D or analogs of vitamin D hormone provides any protective effect against colon tumors in animal models.

We have found that 25(OH)D₃ and two analogs of vitamin D hormone each failed to reduce colonic tumor multiplicity in Pirc rats or Min mice. Further, we have also shown that these agents neither reduced the number of newly emerging tumors, nor changed the growth rate nor caused regression of existing tumors. Vitamin D supplementation should be evaluated carefully before routinely implemented in human populations [36].

Colon cancer is a disease with a long latency period [37]. Therefore, chemopreventive agents may need to be in place for a decade or more to show an effect in humans [37]. Thus, owing to their relatively short lifespan, rodent models may not provide an appropriate test of chemopreventive agents that require a long treatment period to exert a significant effect

[38]. In our study, supplemented diets were given for a significant portion of the animal's natural lifespan, which is approximately two years for rats and mice. Some agents have recently been shown to be chemopreventive in humans over a short period of time [39,40]. The negative results from our current study gain significance in contrast to positive results with other chemopreventive agents. A 40-day treatment with celecoxib was shown to significantly reduce tumor multiplicity in Pirc rats [17]. Additionally, 25–50 day studies of the combination of d,l- α -difluoromethylornithine (DFMO) and piroxicam in the Min mouse seeded the human trials of DFMO and sulindac [41,42]. Thus, rodent models can offer an important platform for short-term studies of chemoprevention. Clearly, short-term supplementation studies are the highest priority to explore, particularly for the individuals at risk of recurrent adenoma formation.

25(OH)D₃ may act at the initial stage of adenomagenesis and may need to be given for a long period, as speculated by Levine and Ahnen for aspirin [43]. Studies examining the efficacy of aspirin in humans as a colorectal chemopreventive agent have shown that significant effects can be observed only after five years of supplementation with follow-up performed at least ten years after starting the regimen [44]. This lag may represent a differential response to early stages of colon cancer. Reduced expression of the vitamin D receptor (VDR) may explain this lack of response. Many colon cancers lose VDR expression as they progress, possibly rendering useless any increase in vitamin D status at that time. We have shown that VDR gene expression decreases over time in Pirc rat tumors (data not shown). However, the test diet in this study was introduced before most of the tumors were visible by endoscopy and certainly before progression. It seems likely that VDR expression was intact in these early tumors. Using endoscopy, we demonstrated that 25(OH)D₃ and two analogs of vitamin D hormone each failed to prevent new tumor formation in two rodent models of familial colon cancer.

Prospective clinical supplementation trials of vitamin D are limited. In a randomized double-blind placebo-controlled trial of men and women between 65 and 85 years of age, Trivedi *et al* showed no significant change in colorectal cancer incidence after 5 years of vitamin D supplementation and follow-up [4], where development of cancer or cause of mortality were secondary endpoints. In addition, a double-blinded trial by the Women's Health Initiative, where participants were randomized to receive either placebo or 500 mg calcium plus 200 IU vitamin D twice daily, also showed no effect on colorectal cancer incidence after seven years [5]. Importantly, some studies have demonstrated that calcium supplementation alone appears to reduce colon adenoma occurrence [45]. Therefore, studies examining the combination of calcium and vitamin D make causation difficult to assess. Patients who have had a polyp removed are at enhanced risk of developing subsequent colonic tumors. Therefore, these individuals represent a focused group in which to study chemopreventive agents. To our knowledge, no clinical trials examining the efficacy of vitamin D supplementation on adenoma recurrence have been conducted in patients who have a history of colonic adenomas or cancer.

Evidence is accumulating to indicate that tumors develop through more than one pathway [46] and may respond differentially to various chemopreventive agents. In studies that do show an association between higher vitamin D levels and lower rates of colon cancer, the effect tends to be stronger on tumors in the distal colon and rectum than for those in the proximal region [10,11,47]. Similarly, Rothwell and colleagues have shown that aspirin protects against cancers of the proximal colon and rectum but not those in the distal colon [48]. Thus, the colon may not be uniform in response to chemopreventive agents across cancer sites. Accordingly, combination chemoprevention may be necessary to have an impact on all colon cancer risk. The combination of aspirin and 25(OH)D₃ may result in synergistic effects by complementary action between promotion of apoptosis by aspirin and

of terminal differentiation by vitamin D. To test this hypothesis, a pilot study involving cohorts of Pirc rats (with and without DSS) and Min mice (with DSS) were given a combination diet consisting of both 25(OH)D₃ and aspirin and followed longitudinally as in the above studies. We found no difference in small intestinal or colonic tumor multiplicities or in colonic tumor growth patterns (data not shown).

Current recommended daily intakes for vitamin D range from 400–800 IU, depending on age and gender [36]. Serum 25(OH)D₃ levels of at least 20 ng/ml are considered adequate for overall health in humans, and levels greater than 50 ng/ml are linked to potential adverse effects [36]. However, studies in humans have shown that doses of vitamin D much higher than recommended can be tolerated in some individuals [49]. Similarly, one study in rats has shown that serum 25(OH)D₃ levels up to 436 ng/ml were well tolerated; only when serum levels reached 688 ng/ml was severe hypercalcemia and weight loss observed [50]. The serum 25(OH)D₃ levels achieved in our studies were 552 ± 152 ng/ml in rats and 290 ± 11 ng/ml in mice. However, it remains unclear whether super-supplementation of vitamins above sufficiency is beneficial. Bashir and colleagues have reported that Min mice given a diet either low or high in multi-vitamin content had increased intestinal tumor multiplicities in both cases [51]. Future studies are needed to address whether a U-shaped curve exists for vitamin D in the area of cancer therapy, as for vitamin D protection against vascular calcification [52].

Several specific issues were not explored in our studies. The duration of treatment with the vitamin D compounds was between 11 and 16 weeks in our various studies. It is possible that in animal models with increased longevity, prolonged treatment may result in a different outcome. Our use of endoscopic methods allowed us to track tumors only in the distal portion of the colon, and thus limited our overall detection. Finally, the important issue whether 25(OH)D₃ deficiency would cause an increase in tumor formation remains to be addressed. Poor vitamin D status may be correlated with increased cancer risk, whereas supplementation above sufficiency may not. In a mouse xenograft model of colon cancer, mice with undetectable levels of serum 25(OH)D had 40% larger tumors than mice with sufficient serum levels [53]. This has also been shown to be true in carcinogen-induced rodent models of colon cancer. DMH-treated rats that were fed a high calcium and low vitamin D diet developed significantly larger colonic tumors than DMH-treated rats that were fed a high calcium diet [16].

We have found that 25(OH)D₃ failed to reduce tumor multiplicities in the small intestine or colon in both the Pirc rat and the Min mouse models. Longitudinal monitoring by endoscopy showed no change in the number of newly arising tumors or the growth patterns of existing tumors in rats and mice given the 25(OH)D₃ diet. Our study highlights the need for further investigation of these specific issues, and of the issue with which we began this report, the association between high sunlight exposure and low colon cancer incidence. Are there effects of sunlight beyond changes in serum vitamin D levels? A recent publication by Becklund *et al* showed that ultraviolet radiation exposure was able to suppress an experimental mouse model of autoimmune disease, independent of increases in serum 25(OH)D₃ [35].

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Highlights

1. The effect of vitamin D compounds on cancer in the *pirr* rat and *min* mouse has been studied.
2. The daily provision of increasing doses of 25(OH)D₃ neither prevents nor hinders the growth of tumors.
3. A highly potent analog of 1,25(OH)₂D₃ and a non-calcemic analog failed to affect initiation or growth of tumors.

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Figure 2.

Terminal total intestinal tumor counts for Pirc rats and Min mice. *A*, Supplementation with 25(OH)D₃, HP analog or NC analog failed to significantly reduce the multiplicity of intestinal tumors in rats. *B*, All Min mice were treated with DSS. Tumor multiplicities resulting from DSS treatment in the mouse vary with DSS batch; therefore, each experimental set is grouped with its own set of contemporaneous controls. Supplementation with 25(OH)D₃, HP analog or NC analog did not significantly reduce the multiplicity of intestinal tumors in mice.

Figure 3.

Longitudinal endoscopic tracking of colonic tumors in the Pirbright rat. The top row represents a single animal from the vehicle diet; the bottom row represents a single animal from the 25(OH)D₃-supplemented diet. Each column represents one time point when a still image of the tumor(s) was acquired. The 25(OH)D₃-supplemented diet did not prevent further growth of existing tumors (white arrow) or emergence of new tumors (black arrow). Qualitatively similar results were found for colonic tumors in the Min mice.

Table 1

Serum measurements for Pirc rats and Min mice

Species	Dietary supplement	Serum component measured	Level in serum, mean $\pm$ SD (n of animals)		P-value
			Control diet	Test diet	
A. Rat	25(OH)D ₃	25(OH)D ₃ (ng/ml)	28.0 $\pm$ 17.9 (12)	550 $\pm$ 169 (26)	< 0.00001
		Calcium (mg/dl)	11.8 $\pm$ 0.7 (14)	14.0 $\pm$ 1.1 (26)	< 0.00001
B. Mouse	25(OH)D ₃	25(OH)D ₃ (ng/ml)	26.2 $\pm$ 8.4 (7)	290 $\pm$ 11 (5)	0.004
		Calcium (mg/dl)	11.3 $\pm$ 1.1 (8)	13.2 $\pm$ 1.0 (5)	0.02
	HP analog	Calcium (mg/dl)	9.2 $\pm$ 0.3 (5)	10.4 $\pm$ 0.6 (16)	< 0.002
	NC analog	Calcium (mg/dl)	9.2 $\pm$ 0.3 (5)	11.7 $\pm$ 0.8 (9)	< 0.003

NOTE: Rat serum data combines male and female, as well as untreated and DSS-treated Pirc rats. Mouse serum data combines both male and female DSS-treated Min mice. P-values were determined using the Wilcoxon rank sum test for significance; a p-value $\leq$ 0.05 is considered significant for the biochemical assays.

Table 2

Terminal small intestinal and colonic tumor counts for Pirc rats and Min mice

Species	Treated with DSS	Test Diet	Supplementation mode	Small Intestinal Tumors		Colonic Tumors	
				Count, mean $\pm$ SD		Count, mean $\pm$ SD	
				Female	Male	Female	Male
A. Rat	No	Control		2 $\pm$ 2	6 $\pm$ 3	4 $\pm$ 2	23 $\pm$ 7
		25(OH)D ₃	Px/Tx	1 $\pm$ 1	9 $\pm$ 3	17 $\pm$ 3	42 $\pm$ 12
		NC Analog	Px/Tx	ND	12 $\pm$ 5	ND	34 $\pm$ 9
	Yes	HP Analog	Px/Tx	ND	5 $\pm$ 1	ND	39 $\pm$ 4
		Control		0.3 $\pm$ 0.6	10 $\pm$ 5	45 $\pm$ 11	58 $\pm$ 3
		25(OH)D ₃	Px/Tx	0 $\pm$ 0	4 $\pm$ 2	79 $\pm$ 11	80 $\pm$ 24
B. Mouse	Yes	25(OH)D ₃	Px	12 $\pm$ 4	17 $\pm$ 6	16 $\pm$ 8	16 $\pm$ 5
		HP Analog	Px	17 $\pm$ 9	23 $\pm$ 9	8 $\pm$ 5	9 $\pm$ 5
		NC Analog	Tx	23 $\pm$ 8	30 $\pm$ 10	2 $\pm$ 2	3 $\pm$ 3
		HP Analog	Tx		19 $\pm$ 7		2 $\pm$ 2
	No	Control					
		25(OH)D ₃					

NOTE: A. In chemoprevention/treatment mode (Px/Tx), rats underwent endoscopy prior to beginning the test diet; therefore, the effect of the diet was tested both on existing tumors (treatment) and on new tumors that arose during the study (chemoprevention). Rat p-values compare each test result to controls of the same DSS status with male and female values blocked and jointly tested. B. In chemoprevention mode (Px), mice were started on the test diet prior to the first DSS treatment. In treatment mode (Tx), mice were started on the test diet following completion of the last DSS treatment. Male and female data have been combined for the mice and corresponding controls are shown in the same row as test results. The same group of mice served as controls for both the HP and NC analog test diets in the treatment mode. Unadjusted p-values are reported; a p-value $\leq$ 0.0167 is considered significant for rat studies without DSS, a p-value $\leq$ 0.05 is considered significant for rat studies involving DSS, and a p-value $\leq$ 0.025 is considered significant for all mouse studies (see Materials and Methods). ND, not done.

Table 3

Longitudinal assessments of tumor growth for Pirc rats and Min mice

Species	Treated with DSS	Test Diet	Supplementation mode	Colonic tumors, % (n/total)		
				Grew	Static	Regressed
A. Rat	No	Control	None	71% (25/35)	26% (9/35)	3% (1/35)
		25(OH)D ₃	Px/Tx	85% (56/66)	15% (10/66)	0%
	Yes	Control	None	NS	NS	NS
		25(OH)D ₃	Px/Tx	NS	NS	NS
B. Mouse	Yes	Control	None	97% (35/36)	3% (1/36)	0%
		25(OH)D ₃	Px	NS	NS	NS
		HP Analog	Px	94% (32/34)	3% (1/34)	3% (1/34)
		HP Analog	Tx	95% (19/20)	5% (1/20)	0%
		NC Analog	Tx	90% (19/21)	10% (2/21)	0%

Note: No test diet in either the Pirc rats or the Min mice decreased the number of growing tumors as compared to control animals. All controls were combined in the Min mouse studies, as no significant difference in growth pattern was seen between the control groups. "Grew" includes existing or newly arising tumors that increased in size; "Static" includes tumors that showed no net change in size; "Regressed" includes tumors that decreased in size or disappeared during the study. NS, not scorable; individual tumors could not be reliably followed longitudinally owing to growth of existing tumors and abundance of emerging tumors. Px, chemoprevention; Tx, treatment.