Targeted delivery of vitamin D to the colon using β-glucuronides of vitamin D: therapeutic effects in a murine model of inflammatory bowel disease

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Running Head: Colon delivery of vitamin D reduces bowel inflammation.

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Abstract

1,25-Dihydroxyvitamin D₃ (1,25(OH)₂D) has been shown to inhibit development of Dextran Sodium Sulfate (DSS) induced colitis in mice, but can also cause hypercalcemia. The aim was to evaluate whether β-glucuronides of vitamin D could deliver 1,25(OH)₂D to the colon to ameliorate colitis while reducing the risk of hypercalcemia. Initial studies demonstrated bacteria residing in the lower intestinal tract were capable of liberating 1,25(OH)₂D from 1,25-dihydroxyvitamin D₃-25-β-glucuronide (β-gluc-1,25(OH)₂D). We also determined that treating mice with an oral dose of β-gluc-1,25(OH)₂D was able to induce a much greater up-regulation of the vitamin D-dependent 24-hydroxylase gene (Cyp24) in colon than 1,25(OH)₂D, demonstrating targeted delivery of 1,25(OH)₂D to the colon. We then tested β-glucuronides of vitamin D in the mouse DSS colitis model in two studies. Mice receiving DSS water and treated with either 1,25(OH)₂D, or β-gluc-1,25(OH)₂D had reduced severity of colitis in both studies. Combining β-gluc-1,25(OH)₂D with 25-hydroxyvitamin D₃-25-β-glucuronide (β-gluc 25-OH D) resulted in the greatest reduction of colitis lesions and symptoms in DSS treated mice. Mice treated with β-gluc-1,25(OH)₂D, alone or in combination with β-gluc 25-OH D, had lower plasma Ca concentrations than mice treated with 1,25(OH)₂D, which were hypercalcemic at time of sacrifice. β-glucuronides of vitamin D compounds can deliver 1,25(OH)₂D to the lower intestine and can reduce symptoms and lesions of acute colitis in this model.

Keywords: 1,25-Dihydroxyvitamin D₃, 1,25-dihydroxyvitamin D₃-25-β-glucuronide, 24-hydroxylase, Dextran sodium sulfate, inflammatory bowel
Introduction

Crohn’s disease and ulcerative colitis are forms of inflammatory bowel disease (IBD) affecting the lower ileum and colon. IBD, especially Crohn’s disease, is often considered an autoimmune disease, characterized by lower intestinal tract innate immune cells that inappropriately secrete pro-inflammatory cytokines in response to commensal bacteria residing in the lower intestinal tract. Lower intestinal tract Th1 lymphocytes react by secreting interleukins-2, 12, and 17, tumor necrosis factor-α (TNF-α), and interferon-γ (IFN-γ), inducing excessive inflammation in response to bacterial antigens.

Epidemiological evidence suggests geographical location, specifically distance from the equator, is associated with increased incidence of Crohn’s disease (17). One possible explanation for this epidemiological observation is that living closer to the equator increases exposure to the sun’s UV-B rays, which may promote cutaneous vitamin D synthesis (21). In support of this, less than 22% of patients recently diagnosed with Crohn’s disease had optimal serum 25-hydroxyvitamin D3 levels (25-OH)D), defined as 75 nmol 25-OH)D/L or greater (14). In a recent clinical trial treatment of Crohn’s patients with 1200 IU vitamin D3/day caused a small reduction in the risk of relapse (P<0.08). Plasma 25-OH D concentrations were increased from 31 to 96 nmol/L by this treatment (10).

Since dietary vitamin D absorption is often sub-optimal in Crohn’s patients, previous work in such patients often focused on the endocrine actions of vitamin D on calcium homeostasis and bone health. The endocrine effects of vitamin D are mediated by renal production of the hormone, 1,25-dihydroxyvitamin D$_3$ (1,25(OH)$_2$D), produced from the vitamin D metabolite, 25-(OH)D, which is synthesized by the liver. In addition to its endocrine actions, vitamin D plays an autocrine/paracrine role in the differentiation and regulation of cell function. Circulating 25-(OH)D is taken up by many cells of the body and converted to 1,25(OH)$_2$D by those cells that express the 25-hydroxyvitamin-D$_3$-1α-hydroxylase (1α-hydroxylase) enzyme. The 1,25(OH)$_2$D produced in this way can act directly within the cell, or diffuse out to neighboring cells, and bind to vitamin D receptors (VDR) to regulate transcription of genes. Vitamin D receptors are widely expressed in the epithelial cells and the immune cells found in the colon, including activated T lymphocytes, and antigen presenting macrophages and dendritic cells. The
1,25(OH)2D produced within the cell binds to the VDR and regulates transcription of a wide variety of genes in epithelial and innate immune cells that may be of benefit to the health of the lower intestine.

Several studies have demonstrated a direct therapeutic effect of vitamin D and 1,25(OH)2D in mouse models of IBD. Cantorna et al. (2) performed a series of elegant studies demonstrating that over 50% of vitamin D deficient interleukin-10 knockout mice (IL-10 KO) died of IBD by 8 wks of age. Vitamin D sufficient IL-10 KO mice showed no IBD symptoms through 9 wks of age. Treating vitamin D deficient IL-10 KO mice with as little as 12 pmol (5 ng) 1,25(OH)2D/day reversed vitamin D deficiency and prevented lesions associated with IBD from developing. Diet supplementation with 480 pmol (200 ng) 1,25(OH)2D/day was able to block further development of IBD in IL-10 mice once it started (2). Further studies demonstrated that 48 pmol (20 ng) 1,25(OH)2D/day treatment of vitamin D deficient IL-10 KO mice reduced expression of TNF-α, TNF-receptor superfamily 1A, and TNF-α induced protein2 in the colon while reducing or preventing the onset of IBD. Interestingly, this benefit was only observed in mice fed a calcium sufficient diet (29).

The absence of 1,25(OH)2D acting on its receptor in lower intestinal cells permits higher numbers of activated dendritic cells to exist in the intestine and may permit auto reactive T cells to develop (5, 27). Though administering 1,25(OH)2D can ameliorate induced IBD in mice, effective doses risk causing hypercalcemia. Another strategy that has been used is to use vitamin D receptor agonists with low calcemic index to treat IBD. Laverny et al., 2010 have administered one such compound, 1alpha,25(OH)(2)-16-ene-20-cyclopropyl-vitamin D to mice intra-rectally and demonstrated beneficial effects in the DSS mouse model, without causing hypercalcemia (13). A recent study by Miheller et al., 2009 treated Crohn’s patients with 2 x 0.25 µg 1,25(OH)2D/day without causing hypercalcemia and did improve the Crohn’s disease activity index in the patients six weeks after treatment (18).

Treatment with 1,25(OH)2D induces a 25-hydroxyvitamin-D-24-hydroxylase enzyme (Cyp24) in target tissues, which speeds the catabolism of 1,25(OH)2D. Over time, larger doses of 1,25(OH)2D must be given to get the same effect. IBD itself results in up-regulation of the Cyp24 enzyme in the proximal colon of mice induced to develop IBD with DSS (15). Froicu et al., demonstrated that administration of 120 pmol (50 ng) 1,25(OH)2D rectally, every other day, ameliorated symptoms of IBD in
the colons of DSS treated mice and did not cause hypercalcemia (5). While 1,25(OH)$_2$D suppositories may be possible it may be difficult to deliver 1,25(OH)$_2$D to the ileum, which is also commonly affected in Crohn’s disease.

We have conjugated glucuronic acid to the 25-carbon position of the 1,25(OH)$_2$D and 25-(OH)D molecules in a beta linkage to form 1,25-hydroxyvitamin D$_3$-25-$\beta$-glucuronide (β-gluc-1,25(OH)$_2$D) (Figure 1) and 25-hydroxyvitamin D$_3$-25-$\beta$-glucuronide (β-gluc-25-(OH)D). The addition of the glucuronide makes the compounds water-soluble. They are also essentially inactive until acted upon by a β-glucuronidase (20). Rambeck et al (1985) reported glycosidic forms of 1,25(OH)$_2$D had less than 1% of the intestinal VDR binding activity aglycone 1,25(OH)$_2$D (22). It is believed that little of the glycosidic forms of vitamin D compounds can be absorbed across the intestine (20). However, the possibility cannot be ruled out as we have no assay sensitive enough to detect the glycosidic forms in blood. When administered orally, the β-glucuronides of the vitamin D compounds are converted to the active vitamin D moiety upon hydrolysis of the β-glucuronide by β-glucuronidase produced by bacteria in the lower intestinal tract. This should allow targeted delivery of 1,25(OH)$_2$D to the ileum/colon cells affected by IBD. Cleaving the β-gluc-1,25(OH)$_2$D to free 1,25(OH)$_2$D in the colon would also reduce systemic absorption of the 1,25(OH)$_2$D compared to absorption of 1,25(OH)$_2$D from the duodenum. Rectally administered 1,25(OH)$_2$D is less hypercalcemic than orally administered 1,25(OH)$_2$D, perhaps because of reduced systemic absorption (5,13).

Destruction of 1,25(OH)$_2$D by up-regulation of the Cyp24 enzyme within IBD target cells (15) necessitates administration of very high doses of 1,25(OH)$_2$D to observe a benefit. In vitro, the effects of 1,25(OH)$_2$D can be enhanced if cells are also treated with a competitive inhibitor of Cyp24, such as 25(OH)D or 24,25-dihydroxyvitamin D$_3$ (24,25(OH)$_2$D) (24). Using these pro-drugs we postulated that it would be possible to deliver a therapeutic dose of 1,25(OH)$_2$D to the ileum/colon along with enough 25(OH)D (in the form of the glucuronide) to competitively inhibit 24-hydroxylation of 1,25(OH)$_2$D, thus potentiating 1,25(OH)$_2$D effectiveness. This could allow consistent effects on the colon with doses of vitamin D compounds that might otherwise be systemically toxic.
Materials and Methods

General

The β-25-monoglucuronides of 1,25(OH)₂D and 25(OH)D were synthesized from the corresponding provitamin D or its derivatives using the Koenigs-Knorr reaction (25). Their structure was confirmed by FT-IR and NMR. The β-gluc-1,25(OH)₂D (MW 592.76) was purified by HPLC to >97% purity and the β-gluc 25(OH)D (MW 576.7) was purified by HPLC to >90% purity. The 1,25(OH)₂D (MW 416.64), 25-(OH)D (MW 400.6) and 24,25(OH)₂D (MW 416.64) utilized were >98% pure (Sigma Aldrich, St Louis). Quantitation of vitamin D compounds in ethanol solutions utilized to prepare each diet treatment was based on absorbance at 265 nm (using a molar extinction coefficient of 18,300 absorbance units·mol⁻¹·L⁻¹).

Male C57BL/6 mice (Jackson Labs, Bar Harbor, ME) were fed Teklad 2018 rodent diet based on wheat, corn, and soybean meal and containing 1% Ca, 0.7% P and 1.5 IU vitamin D₃ / g (Harlan Labs, Madison, WI). Sprague-Dawley rats (Harlan Labs, Madison, WI) were fed Teklad 2018 diet prior to harvest of their intestinal tissue. Animals were housed in individual wire bottom cages to reduce coprophagy. The room was maintained at 24-26 °C with a 12 hour light on/off cycle. All procedures performed on the animals were submitted to and approved by the Iowa State University Institutional Animal Care and Use Committee (Protocols # 4-09-6378-M and 10-08-6651-R).

In vitro assessment of β-glucuronidase activity of intestine contents

Most mammals have relatively few bacteria in their duodenum and generally have bacterial species such as Bacteroides sp in their lower intestinal tract that are capable of cleaving β-glycosidic linkages (9). Rats were chosen to test the ability of the glucuronides of vitamin D to be cleaved in different sections of the intestinal tract. The microbes found in their intestine are similar to those of a mouse and the amount of intestinal lumen material that could be obtained from the rat intestines allowed for larger incubations of material to be carried out. The proximal 25 cm of duodenum/jejunum, the caudal 12 cm of ileum, and the cranial 12 cm of colon were removed from 18 Sprague-Dawley rats and the contents of the lumen from each section were flushed with 3 ml distilled water, collected, and pooled. Three-ml
 aliquots of intestinal contents were placed into tubes in duplicate. To some tubes, 3.53 pmol β-gluc-1,25(OH)₂D was added and incubated at 37°C for 0, 1, 3, or 6 hrs. To other tubes 0.36 pmol of 1,25(OH)₂D was added and incubated for 0 or 6 hrs (Table 1). The 1,25(OH)₂D tubes served as a control to confirm the ability to extract and detect 1,25(OH)₂D in this material and to determine if any degradation of 1,25(OH)₂D would occur. Tritiated 1,25(OH)₂D (2200 DPM of 156 Ci/mmol 1,25(OH)₂D , Amersham-GE Healthcare, Piscataway, NJ) was added to each tube to assess extraction efficiency. Acetonitrile (3 ml) was added to each tube after incubation to end all enzymatic activity, and was used to extract the 1,25(OH)₂D that was liberated. The preparations were cleaned using a 0.5g C₁₈OH SPE column followed by an analytical Zorbax Sil HPLC column (both purchased from Varian, Lexington, MA). β-gluc-1,25(OH)₂D , being more water-soluble than 1,25(OH)₂D , does not co-elute with 1,25(OH)₂D on either the C₁₈OH or Zorbax Sil column. Samples were analyzed for 1,25(OH)₂D content by RIA (Heartland Assays, Ames, IA) (8).

In vivo assessment of β-gluc 1,25-D vs. 1,25-D on Cyp24 gene expression in colon and duodenum

Our goal in this section of the study was to determine if the β-gluc-1,25(OH)₂D could be used to target delivery of 1,25(OH)₂D activity to the lower intestine. These acute studies allowed us to determine the site of action of a single dose. Two studies were conducted in mice to investigate the relative activity of β-gluc-1,25(OH)₂D and 1,25(OH)₂D on colon and duodenum, utilizing Cyp24 expression as an indicator of action of the secosteroid on the tissues. The first study was a dose titration study. Ten wk old male C57BL/6 mice fed ad libitum received a single oral dose (6, 12, 24, or 48 pmol) of either 1,25(OH)₂D or β-gluc-1,25(OH)₂D suspended in 50 µl peanut oil (4 mice/treatment). Mice were euthanized 6 hrs later, blood collected into heparinized tubes, and the plasma samples were analyzed for 1,25(OH)₂D content by RIA as described above. β-gluc-1,25(OH)₂D, being more water-soluble than 1,25(OH)₂D, elutes with the methanol wash of the 0.5g C₁₈OH SPE column (Varian, Lexington, MA) making it possible to measure only 1,25(OH)₂D in the samples. A 1 cm section of duodenum (between 2 and 3 cm from the pylorus) and a 1
cm section of colon (between 2 and 3 cm from the cecum) were obtained from each mouse for mRNA analysis. Tissue samples were flushed with ice-cold phosphate-buffered saline and immediately homogenized in 1 ml of TRIzol® reagent (Invitrogen Corp., Carlsbad, CA). Samples were then kept frozen at -86°C prior to processing for RNA.

The second study was a time course study. Similarly maintained mice were treated with a single oral 24 pmol dose of either 1,25(OH)₂D or β-gluc-1,25(OH)₂D suspended in 50 µl peanut oil (5 mice/treatment). Mice were then euthanized at 1, 3, 6 and 24 hrs after treatment and plasma and tissues were harvested as described above.

Each TRIzol® homogenate was thawed at room temperature and 500 ul placed in a clean microfuge tube, mixed thoroughly with 100 ul chloroform for 15 sec and then centrifuged at 12,000 X g for 15 min at 4°C. The upper aqueous phase was removed and mixed with 0.93 volumes of 75% ethanol. The mixture was then applied to an RNeasy spin column (Qiagen Inc., Germantown, MD) and processed as described by the manufacturer with the exception that an additional wash with 2M NaCl/2 mM EDTA (pH 4.0) was included (3). RNA was eluted in 50 ul of water and the concentration obtained by UV spectrometry. One microgram of RNA was then used as a template for production of cDNA in a 20 ul reaction volume using random hexamers and Superscript III as described by the manufacturer (Invitrogen, Carlsbad, CA). Afterwards, samples were diluted to 100 ul final volume with TE buffer and stored at -20°C prior to PCR analysis.

Quantitative real-time-PCR was performed using a Stratagene Mx3005p cycler (Stratagene, La Jolla, CA) and PerfeCTa SYBR Green FastMix, ROX reagent (Quanta Biosciences, Gaithersburg, MD). Amplification of target cDNAs was accomplished with the following primers (synthesized by Integrated DNA Technologies, Coralville, IA): Cyp24-For, 5’-CACACGCTGCGCTGGGACAC; Cyp24-Rev, 5’-GGAGCTCCGTAGCAGCAGCG; GAPDH-For, 5’-GAAGGTCGGTGTGAACGGATTTGGC; GAPDH-Rev, 5’-TTGATGTTAGTGGGGTCTCGCTCCTG. Aliquots (8.3 ng) of cDNA were amplified under the following conditions: 95°C for 30 sec, followed by 45 cycles of 95°C for 1 sec and 57°C for 30 sec. All reactions were performed in duplicate, with 4
or 5 animals/treatment and Cyp24 target gene expression was estimated using the ΔCT method relative to GAPDH expression as described previously (6).

**Testing the effect of vitamin D compounds on DSS induced colitis in mice**

Diet treatments were prepared so that the daily dose of each desired vitamin D compound was delivered in 3.5 g Teklad 2018 diet. Accordingly, the appropriate amount of each vitamin D compound dissolved in 40 ml ethanol was thoroughly mixed into the finely ground diet. The diets were then left uncovered at room temperature overnight with occasional stirring to allow the ethanol to evaporate. Thereafter the diets were stored at 4°C between feedings. The dietary treatments began 4 days prior to initiation of colitis with Dextran Sodium Sulfate (DSS) drinking water. The diets were fed at a rate of 3.5 g/day and any diet not consumed in 24 hrs was removed and weighed to determine feed refusal (data not shown). Mice were administered 2.5% DSS dissolved in distilled water ad libitum for seven days. DSS (MW 35-50,000) was obtained from MP Biomedicals (Solon, OH).

**IBD Study # 1.** Groups of ten-week old mice (9 mice/group) were randomly assigned to the different treatments. One group of mice was allowed to drink normal water (No DSS) while all other groups of mice received DSS in their drinking water. Of these latter groups of mice, one group received no vitamin D compounds added to the diet (DSS only). Other groups received treatments consisting of 1,25(OH)₂D incorporated into the diet to supply either 24 or 120 pmol/day. The β-gluc-1,25(OH)₂D was incorporated into the diet to supply 24, 120, or 600 pmol /day. The final three treatments consisted of the β-gluc 25(OH)D fed at 8.67 nmol/day by itself or combined with 24 or 120 pmol β-gluc-1,25(OH)₂D/day. The β-gluc 25(OH)D treatment was included to try to competitively inhibit the 24-hydroxylase in the colon and potentiate the effects of 1,25(OH)₂D in the colon.

**IBD Study # 2.** Six mice (14 weeks of age) were assigned to each treatment. Treatments consisted of No DSS, DSS only, DSS plus 171 pmol β-gluc-1,25(OH)₂D /day. This dose of β-gluc-1,25(OH)₂D was higher than that found to be effective in IBD Study #1. Animals were weighed daily and monitored clinically for bleeding from the rectum and general signs of morbidity. The blood loss from the rectum was scored as follows on the day of necropsy: No blood= 0; A single spot of blood in cage = 1; Less...
than 10 spots of blood within the cage = 2; More than 10 spots of blood in the cage = 3. One mouse in the treatment group receiving 600 pmol β-gluc-1,25(OH)2D/day in Experiment 1 failed to finish the trial as it developed severe bloody diarrhea and weight loss necessitating euthanasia on the 7th day of the DSS administration. Its tissues were collected and data from this mouse are retained in the study results. Following the 7th day of DSS water, the mice were offered regular tap water for 24 hrs and euthanized by guillotine while under isofluorane anesthesia.

Blood was collected from the cervical stump into heparinized tubes and each mouse’s plasma was harvested and frozen at -86°C until analyzed for calcium content by a colorimetric assay (Arsenazo III, Pointe Scientific, Canton, MI). Plasma 25(OH)D and 1,25(OH)2D concentrations were determined by radioimmunoassay on pooled samples of plasma collected from the mice (Heartland Assays, Ames, IA) (7, 8). In experiment 1, each pooled sample consisted of 50 ul plasma from either 4 or 5 mice, resulting in 2 samples/treatment. In experiment 2, each pooled sample consisted of 100 ul of plasma from 3 mice, resulting in 3 samples/treatment. Because of the small number of samples, the results of the vitamin D metabolite determinations were not subjected to statistical analysis.

The entire colon and rectum of each mouse was removed intact and the length recorded. The colon/rectum was cut in two and the caudal half discarded. The caudal 1 cm of the remaining half was fixed in formalin for histopathological analysis. The fixed colon section was stained with hematoxylin & eosin for microscopic histopathologic evaluation by a veterinary pathologist (JSH) blinded to the treatments. Each tissue section received a score from 0 to 4 from no lesion to severe extensive lesion for each of three criteria: (1) the degree of erosion/ulceration of colon mucosa; (2) the degree of infiltration of the tissues by inflammatory cells; and (3) the degree of submucosal edema. The sum of the score of each of these criteria constitutes the histopathological score for the tissue with a zero score representing normal tissue based on all criteria, and the worst possible outcome being a score of 12.

A second 1 cm piece of colon tissue cranial to the section used for histopathological analysis was obtained from each mouse for isolation of mRNA. In IBD Study #1 the tissues were flash frozen. Unfortunately the RNA isolated from these tissues was degraded. In IBD Study #2 the tissues were immediately placed into TRIzol® and homogenized as described earlier for the dose and time course studies.
on effects of a single dose of the vitamin D compounds. This method successfully preserved the RNA. Quantitative real-time PCR was performed and quantitated as described above. Amplification of target cDNAs for Cyp24, Tumor Necrosis Factor-α (TNFα), and Cadherin-1 (Cadh1) was accomplished with the following primers:

- TNFα-For, 5’-AGGGGCCACCACGCCTCTCT; TNFα-Rev, 5’-
- CACTCCAGCTGCTCCTCCACT; Cadh1-For, 5’-
- TGGAGGGATCCTCGCCCTGC; Cadh1-Rev, 5’-CATCCAGGCCCTGTGCAGC.

Statistical analysis

Data were analyzed by one way Analysis of Variance (SAS, Cary, NC). When ANOVA suggested a significant difference (P < 0.05), post hoc comparison of individual means was conducted using Fischer’s Test of Least Significant Difference. Results from studies of the mice treated with DSS to induce inflammation of the lower bowel are presented as treatment differences from either the Positive (No DSS) or Negative (DSS only) control means and are considered significant at the P < 0.05 level unless otherwise noted. Several of the treatments from IBD study #1 with no significant effects are not presented in the tables to improve clarity of presentation, but any significant effects attributable to these treatments are discussed.

Results

Ileal and colon, but not duodenal contents, contain high levels of β-glucuronidase activity

To determine the β-glucuronidase activity capable of liberating a vitamin D aglycone from the pro-drug form of the vitamin D compound in various parts of the small intestine, β-glucuronidase activity was tested in contents from intestinal subsections of rats. The assay system utilized recovered at least 60% of the added 1,25(OH)2D from intestinal content incubations at time zero and the level of 1,25(OH)2D did not change with time of incubation, suggesting the 1,25(OH)2D was not degraded during the course of the incubations and that the assay was reasonably able to detect 1,25(OH)2D from this type of sample (Table 1). When the β-gluc-1,25(OH)2D was incubated with duodenal contents, less than 5% of the compound was converted to the aglycone 1,25(OH)2D, even after 6 hrs incubation. When the β-gluc 1,25(OH)2D was incubated with ileal contents more than 85% was cleaved to free the aglycone 1,25(OH)2D within 1 hr of incubation. Colon contents liberated
65% of the 1,25(OH)\(_2\)D within one hour and the amount of 1,25(OH)\(_2\)D recovered increased slightly at the 3 and 6 hr time points for ileal and colon contents (data not shown). The results shown in Table 1 demonstrate the upper small intestine of rats is unlikely to contain sufficient β-glucuronidase activity to cause release of substantial amounts of 1,25(OH)\(_2\)D in the upper small intestine. On the other hand, the β-gluc-1,25(OH)\(_2\)D is likely to be rapidly cleaved upon entry to the ileum and colon where substantial numbers of bacteria reside that are capable of producing β-glucuronidase.

**β-gluc-1,25(OH)\(_2\)D vs. 1,25(OH)\(_2\)D effects on Cyp24 gene expression in colon and duodenum**

A prominent action of 1,25(OH)\(_2\)D on its target tissues is induction of the mRNA for the Cyp24 enzyme. The effect of increasing doses of β-gluc-1,25(OH)\(_2\)D and 1,25(OH)\(_2\)D on Cyp24 expression in the colon and duodenum relative to untreated control mice is presented in Fig 2A & B. At the highest dose of 1,25(OH)\(_2\)D utilized (48 pmol) there was approximately a 4.8 ± 4 fold increase in Cyp24 expression in the colon six hours after treatment. The equimolar dose of β-gluc-1,25(OH)\(_2\)D caused over a 400 fold increase in colon Cyp24 expression. Even at the 12 pmol dose, β-gluc-1,25(OH)\(_2\)D was able to cause a 60-fold increase in Cyp24 expression in the colon, which was about 20 times greater than the response from the equimolar dose of 1,25(OH)\(_2\)D. As expected, 1,25(OH)\(_2\)D was able to strongly induce Cyp24 gene expression in the duodenums of the same mice 6 hours after oral dosing, with maximal induction (>1000-fold) occurring at the highest dose (48 pmol) evaluated. Though induction of Cyp24 gene expression was also observed in the duodenums of mice treated with the β-gluc-1,25(OH)\(_2\)D, it was consistently less effective than the analogous dose of 1,25(OH)\(_2\)D. Plasma 1,25(OH)\(_2\)D concentration was not significantly increased at the 6 hr time point by 6 pmol of either 1,25(OH)\(_2\)D or β-gluc-1,25(OH)\(_2\)D (Fig. 2C). Higher doses of either compound resulted in higher levels of 1,25(OH)\(_2\)D in the blood. At the 48 pmol dose the β-gluc-1,25(OH)\(_2\)D resulted in higher blood 1,25(OH)\(_2\)D at the time of euthanasia, which was 6 hr after treatment in this study. Plasma calcium concentrations were similar to control mouse plasma calcium concentrations in all treatment groups, which likely reflects the short time duration of the experiment.
When 24 pmol of \( \beta \)-gluc-1,25(OH)\(_2\)D or 1,25(OH)\(_2\)D were administered orally and animals were sacrificed at intervals following treatment, the highest levels of expression of Cyp24 in both the colon and duodenum were observed at 3 or 6 hrs after treatment (Fig 3A & B). \( \beta \)-gluc-1,25(OH)\(_2\)D treatment caused Cyp24 in colon tissue to increase about 700-fold higher than in control mice at 6 hrs, whereas 1,25(OH)\(_2\)D was only able to increase colon Cyp24 about 5-fold. In the duodenum the relative effects of 1,25(OH)\(_2\)D and \( \beta \)-gluc-1,25(OH)\(_2\)D were reversed. Compared to control mice, 1,25(OH)\(_2\)D treatment steadily increased Cyp24 expression in the duodenum from the 1 hour time point (350-fold induction) to the 3 hour time point (1600-fold induction) to the 6 hour time point (more than 2500 fold). In contrast, the \( \beta \)-gluc-1,25(OH)\(_2\)D effects on Cyp24 peaked at 3 hrs in the duodenum with a 1300-fold increase in Cyp24 expression and had fallen to a 500-fold increase at 6 hours. The effects of both 1,25(OH)\(_2\)D and \( \beta \)-gluc-1,25(OH)\(_2\)D on Cyp24 gene expression in both tissues was similar to control mouse levels 24 hours after treatment.

Plasma concentrations of 1,25(OH)\(_2\)D peaked in 1 hr following the per os treatment with 24 pmol at 1280 pg/ml, approximately a 14-fold increase over control mouse plasma 1,25(OH)\(_2\)D (Fig. 3C). In contrast, the average plasma 1,25(OH)\(_2\)D concentration in mice treated with 24 pmol \( \beta \)-gluc-1,25(OH)\(_2\)D peaked approximately 3 hrs after treatment at 325 pg/ml, a level that was only 3.5-fold greater than control levels. By 24 hrs after treatment plasma 1,25(OH)\(_2\)D concentrations in both 1,25(OH)\(_2\)D and \( \beta \)-gluc-1,25(OH)\(_2\)D treated mice were slightly below the concentration observed in control animals. Plasma calcium concentrations were similar to control mouse plasma calcium concentrations at all time points of both treatment groups.

Taken together these two studies demonstrate the oral administration of \( \beta \)-gluc-1,25(OH)\(_2\)D has a greater effect on colon tissue and a lesser effect on duodenum than does the native hormone. Oral administration of \( \beta \)-gluc-1,25(OH)\(_2\)D also causes a much lower increase in plasma concentration of 1,25(OH)\(_2\)D than does the equimolar dose of 1,25(OH)\(_2\)D. However, the time for each drug to cause peak levels of 1,25(OH)\(_2\)D in the blood differs. As expected the highest plasma concentrations of 1,25(OH)\(_2\)D occur shortly after oral administration of 1,25(OH)\(_2\)D, and concentrations decline thereafter due to rapid metabolism of 1,25(OH)\(_2\)D in the mouse. However, there is a delay in the time to peak 1,25(OH)\(_2\)D concentrations in mice receiving \( \beta \)-
gluc-1,25(OH)\textsubscript{2}D. This likely represents the time it takes for the compound to reach the ileum and to be converted to 1,25(OH)\textsubscript{2}D and then be absorbed into the circulation to travel back to the duodenum. These tissues were also subjected to quantitative real-time-PCR to examine changes in expression of the vitamin D receptor (VDR) and calbindin-D9k, both of which are commonly reported to be upregulated by 1,25(OH)\textsubscript{2}D. We could not detect changes in expression of these genes in these tissues (data not shown). These animals were vitamin D replete- most studies demonstrating strong up-regulation of these proteins are done using vitamin D deficient animals. Our studies were completed in less than 24 hrs after treatment. Many of the studies demonstrating a VDR or calbindin-D9k response require repeated doses of 1,25(OH)\textsubscript{2}D over more than 24 hrs to see significant up-regulation in the animal’s tissues. And when VDR expression is upregulated it may be just a 3-4 fold increase (23).

**Effect of vitamin D compounds on DSS induced colitis**

**IBD Study # 1.**

Control mice that did not receive DSS gained 1.91 g body weight during the 8 days of the IBD induction period, had no blood in their feces, and their colon length was 6.89 ± 0.37 cm. Their plasma calcium was 9.54 ± 0.11 mg/dl. In the mice receiving DSS treatment alone, frank blood began to appear in the feces about 6 days after initiation of DSS water and at the time of sacrifice these animals had an average fecal blood score of 0.89 ± 0.34 and colon length of 5.22 ± 0.20 cm and had lost 1.03 g (4.7%) of initial body weight during the eight days following the start of DSS water feeding (Table 2 and 3). Their histopathology colon lesion score was 9.33 ± 0.62. The DSS treated mice were significantly hypocalcemic at the time of sacrifice with plasma calcium of 8.44 ± 0.36 mg/dl. Severe inflammatory processes often result in high levels of IL-1 and other cytokines which cause a decline in blood calcium level through mechanisms that may involve vitamin D, parathyroid hormone, or calcitonin (19, 28).

Mice receiving DSS water and treated with120 pmol / day of either 1,25(OH)\textsubscript{2}D or β-gluc-1,25(OH)\textsubscript{2}D had reduced fecal blood scores and reduced colon lesion scores. Colon length and weight loss in both groups was not statistically improved over the mice receiving DSS only. However, treating the mice with 120
pmol 1,25(OH)\textsubscript{2}D/day also resulted in severe hypercalcemia (11.58 \pm 0.18 mg Ca/dl), while feeding 120 pmol \(\beta\)-gluc-1,25(OH)\textsubscript{2}D/day did not (10.12 \pm 0.30 mg Ca/dl) (Tables 2 and 3).

Feeding 8.67 nmol/day of the \(\beta\)-gluc of 25(OH)D by itself was ineffective in preventing weight loss, improving fecal blood scores or colon length, or reducing histologic lesion score (data not shown). However, combining 120 pmol \(\beta\)-gluc-1,25(OH)\textsubscript{2}D with 8.67 nmol of \(\beta\)-gluc-25(OH)D significantly reduced fecal blood score, improved colon lesion score and prevented DSS-induced weight loss. This treatment was the only treatment that significantly improved colon length over that observed in mice receiving DSS only. Plasma calcium was not significantly elevated above that of control mice by the combination 120 pmol \(\beta\)-gluc-1,25(OH)\textsubscript{2}D and 8.67 nmol \(\beta\)-gluc-25(OH)D treatment (10.26 \pm 0.22 mg Ca/dl).

Lower doses (24 pmol) of either vitamin D compound were largely ineffective in preventing negative effects of DSS in the mice. In mice treated with 24 pmol 1,25-D/day, colon length, colon lesion score, and weight loss during DSS treatment was not significantly improved over mice receiving DSS only. Mice fed DSS water and treated with 24 pmol \(\beta\)-gluc-1,25(OH)\textsubscript{2}D/day, with or without concurrent treatment with 8.67 nmol/day of the \(\beta\)-gluc of 25(OH)D fared somewhat better. They had significantly lower colon lesion scores and lost less weight than DSS treatment alone, but had no improvement in colon length or fecal blood score (data not included in Tables 2 and 3 for clarity of presentation). Feeding 600 pmol \(\beta\)-gluc-1,25(OH)\textsubscript{2}D/day to mice receiving DSS water was of no benefit in reducing IBD symptoms or lesions in DSS treated mice and caused significant hypercalcemia (10.72 \pm 0.33 mg Ca/dl). Feed intake was significantly reduced in this group as early as the 4\textsuperscript{th} day of DSS treatment (8\textsuperscript{th} day of feeding the 600 pmol \(\beta\)-gluc-1,25(OH)\textsubscript{2}D/day) (data not shown). This group also had one mouse that needed to be euthanized on Day 7 of DSS treatment due to the severity of the IBD and weight loss it was experiencing.

Based on the pooled samples of plasma a qualitative assessment of plasma vitamin D metabolite concentrations is presented. Plasma 25(OH)D concentrations were decreased in animals receiving DSS water when compared to No DSS controls. The diarrhea in affected mice, though not severe, may have caused interference with the enterohepatic circulation of 25(OH)D. Mice receiving \(\beta\)-gluc-25(OH)D in their
diet had greatly increased plasma 25(OH)D concentrations. Plasma 1,25(OH)\(_2\)D concentrations were increased in animals receiving the 120 pmol 1,25(OH)\(_2\)D/ day and in mice receiving 600 pmol β-gluc-1,25(OH)\(_2\)D/day, but not in mice receiving 120 pmol β-gluc-1,25(OH)\(_2\)D/day. When combined with β-gluc-25(OH)D, plasma 1,25(OH)\(_2\)D concentrations increased in mice receiving 120 pmol β-gluc-
1,25(OH)\(_2\)D/day.

**IBD Study # 2**

All mice completed Experiment 2 and the degree of colitis, based on body weight loss, histopathologic colon lesion score and colon length, experienced by the DSS only group was milder than in Experiment 1. Compared to mice not receiving DSS water, the mice fed DSS only had significantly more blood in their feces, shorter colon length, and lost more body weight (Table 4). Fecal blood score was 1.33 ± 0.49 in DSS only mice. The treatment consisting of 171 pmol β-gluc-1,25(OH)\(_2\)D/day (a 42% increase in the dose found to be effective in IBD Study #1) significantly reduced fecal blood score (0.33 ± 0.33) (P< 0.05) and body weight loss (P<0.075) when compared to mice receiving DSS only. In addition, colon length of the mice receiving 171 pmol β-gluc-1,25(OH)\(_2\)D /day (7.40 ± 0.22 cm) was not statistically different from that of control mice receiving No DSS (7.57 ± 0.34 cm) and was statistically improved compared to DSS only mice (6.68 ± 0.19 cm) (P< 0.075). Plasma calcium of mice receiving DSS only was lower than that of no DSS mice (P<0.075), but none of the vitamin D treatments caused a significant increase in plasma calcium concentration (P > 0.075) (Table 5). Histopathology colon lesion score for the DSS only mice was much lower than in experiment 1 (3.67 in Expt. 1 vs. 9.33 in Expt. 2), indicating a milder inflammation was induced by DSS in this trial. Treatment with 171 pmol β-gluc-1,25(OH)\(_2\)D/day did not alter colon lesion score, though fecal blood score and colon length were improved over DSS only mice. Plasma 25(OH)D concentrations were decreased in animals receiving DSS water, similar to DSS trial 1. Plasma 1,25(OH)\(_2\)D concentrations were decreased in mice fed DSS water and increased in mice receiving 171 pmol β-gluc-
1,25(OH)\(_2\)D/day.

Gene expression data of colon tissue of No DSS, DSS only and DSS plus 171 pmol β-gluc-1,25(OH)\(_2\)D/day treatments are presented in Figure 4. There was a trend
toward increased Cyp24 expression in colon of DSS mice compared to No DSS controls, but this difference was not statistically significant. However, mice receiving 171 pmol β-gluc-1,25(OH)₂D/day exhibited about a 200 fold increase in Cyp24 expression. There was no statistically significant decrease in expression of the epithelial tight cell junction protein cadherin 1 in DSS only mice and treatment with β-gluc-1,25(OH)₂D did not increase cadherin 1 expression. Mice receiving DSS water had significantly increased expression of TNF-α, and administration of β-gluc-1,25(OH)₂D did not reduce TNF-α expression.

Discussion

Conjugating a glucuronide to the 1,25(OH)₂D or 25(OH)D molecules in a β conformation link makes the compounds water soluble but more importantly they are biologically inert until the glucuronide moiety is cleaved leaving the aglycone vitamin D compound. As these studies demonstrate, glucuronidase activity in the upper small intestine is unlikely to allow formation of significant amounts of the aglycone vitamin D compounds. However, the colon and ileum possess substantial numbers of bacteria, which are able to produce β-glucuronidase, and nearly all the glucuronide is cleaved from the vitamin D compounds very rapidly. Administering a single oral dose of 24 pmol 1,25(OH)₂D had a profound impact on duodenal Cyp24 expression that persisted at least 6 hrs, but was essentially gone by 24 hrs. The colon responded to the oral 24 pmol 1,25(OH)₂D with a five fold increase in colon Cyp24 expression. In contrast, the equimolar amount of β-gluc-1,25(OH)₂D administered per os was much less able to up-regulate duodenal Cyp24 expression than 1,25(OH)₂D. The β-gluc 1,25(OH)₂D was able to up-regulate colon Cyp24 expression about 700 fold by 6 hrs after treatment, more than 100 times as active on colon tissues as the native hormone. The effect of the vitamin D compounds at these doses on Cyp24 was short lived as Cyp24 was essentially back to basal levels by 24 hrs after treatment.

Not only did Cantorna et al. demonstrate that correction of vitamin D deficiency improved resistance to IBD in IL-10 KO mice (2), subsequent studies by Froicu and Cantorna also demonstrated that DSS-induced IBD could be ameliorated in vitamin D replete genetically normal animals by treatment with 1,25(OH)₂D whether administered orally or rectally (5). Our data corroborate these observations in that
feeding mice 120 pmol 1,25(OH)₂D/day reduced lesions and symptoms of IBD in the DSS treated mouse model. Unlike the mice in the study of Froicu and Cantorna, the mice fed 120 pmol 1,25(OH)₂D/day in our study developed rather severe hypercalcemia (11.58 mg Ca/dl). The lower dose of 24 pmol 1,25(OH)₂D used in our study did not cause hypercalcemia, but offered little amelioration of IBD.

Daily feeding of 24 pmol of β-gluc-1,25(OH)₂D improved a few aspects of IBD in the DSS treated mice but this effect was modest at best. Feeding 5 or 7 times this amount of the β-gluc-1,25(OH)₂D (120 or 171 pmol/day) resulted in a modest improvement in lesions of IBD over the lower dose. Feeding 600 pmol/day of the β-gluc-1,25(OH)₂D proved to be a toxic dose. It was associated with hypercalcemia (10.72 mg Ca/dl), the mice consumed less diet, lost more body weight than the other treatments, and had IBD that was nearly identical to the animals receiving DSS water only. Though less hypercalcemic than the native hormone 1,25(OH)₂D, β-gluc-1,25(OH)₂D can induce hypercalcemia.

One complication from the use of 1,25(OH)₂D and its analogs in treating disease is that the treatment induces CYP24 expression. Upon translation, this enzyme will speed the catabolism of 1,25(OH)₂D limiting the effectiveness of the 1,25(OH)₂D. Both 25(OH)D and 24,25(OH)₂D have been used as competitive inhibitors of 24-hydroxylase in vitro (24). When 8.67 nmol/day β-gluc-25(OH)D was administered along with 120 pmol β-gluc-1,25(OH)₂D/day to DSS treated mice, there was a substantial improvement in IBD over 120 pmol β-gluc-1,25(OH)₂D alone. Feeding the β-gluc-25(OH)D by itself was ineffective against IBD. We interpret this to mean that the β-gluc 25(OH)D was converted to 25(OH)D in the colon and competitively inhibited colon 24-hydroxylase, allowing a longer persistence of anti-inflammatory action by the 1,25(OH)₂D liberated from the β-gluc-1,25(OH)₂D in the colon.

Several attributes of 1,25(OH)₂D may help explain how administration of 1,25(OH)₂D could ameliorate IBD. A characteristic of Crohn’s disease is the inappropriate expression of the pro-inflammatory Th1 and Th17 cytokines by T cells in response to bacterial antigens. Vitamin D and its metabolites may influence this process. The active metabolite, 1,25(OH)₂D, causes T cell differentiation to shift away from Th1 and Th17 predominant phenotypes toward a Th2 phenotype (1) and down-regulates gut-homing receptors expressed by T cells that migrate selectively to
the intestinal tract (26). In support of this, vitamin D deficiency exacerbates symptoms of colitis in IL-10 knockout mice (2) and vitamin D receptor knockout mice are ultra-sensitive to DSS-induced IBD (5).

Some Crohn’s patients are known to have a genetic variant of the NOD2 gene, which is a pattern recognition receptor that recognizes bacterial peptidoglycans. It appears the innate immune cells such as monocytes and macrophages of these people fail to recognize certain bacterial cell wall antigens and thus the cells are unable to initiate a cascade of events leading to production of antimicrobial peptides that might keep “inflammation triggering” bacteria populations in check. NOD2 and Toll-like receptors (TLR) pathways also stimulate the innate immune cell’s ability to perform 1α–hydroxylation of 25(OH)D (16). Colon cells are also capable of producing 1,25(OH)2D. Though the role recognition of pathogen-associated molecular patterns plays on control of colon mucosa cell 1α–hydroxylase is unclear (12), one can speculate that people with defective NOD2 may not be producing adequate 1,25(OH)2D within their immune cells, even if adequately supplied with the 25(OH)D precursor. It has recently been demonstrated that exogenous treatment of normal human macrophages with 1,25(OH)2D can circumvent problems with endogenous production of 1,25(OH)2D due to inadequate 25(OH)D and results in production of antimicrobial peptides such as cathelicidin (16). Cathelicidin’s ability to kill Mycobacteria may be critical to preventing IBD, especially if a major factor precipitating IBD proves to be the presence of M. avium sp. paratuberculosis in the gut. Finally, the intestinal epithelial barrier, comprised of epithelial cells and intercellular tight junctions prevents microorganisms, toxins, and luminal antigens from entering the body. Impaired barrier function is a common finding in patients with inflammatory bowel disease (4). Studies in mice demonstrate that 1,25(OH)2D acts on enterocytes to increase expression of junction proteins to maintain the integrity of the intestinal mucosal barrier and allow rapid repair of damage to enterocyte tight junctions, which may facilitate recovery from a bout of IBD (11). In our study there appeared to be some loss of cadherin 1 as a result of DSS treatment, but this was not statistically significant and more importantly we could not demonstrate an increase in cadherin 1 expression following treatment with the β-gluc-

1,25(OH)2D.

Utilizing β-glucuronide forms of 1,25-D and 25-D allows delivery of
1,25(OH)$_2$D and 25(OH)D to the colon in amounts that stimulate colon gene expression to a much higher degree than is possible with the native hormone, 1,25(OH)$_2$D. These compounds, at the doses that proved effective in this mouse model, also avoid the development of severe hypercalcemia observed with native 1,25(OH)$_2$D. In this mouse model of IBD, the combination of $\beta$-gluc-1,25(OH)$_2$D with the $\beta$-gluc-25(OH)D provided superior amelioration of IBD to that achieved with $\beta$-gluc-1,25(OH)$_2$D alone. We believe this is due to competitive inhibition of the 24-hydroxylase enzyme, prolonging the action of 1,25(OH)$_2$D within the colon. It is also possible the provision of high amounts of 25(OH)D to the colon may also provide sufficient substrate to drive colon 1$\alpha$–hydroxylase and enhance 1,25(OH)$_2$D production within the epithelial and immune cells of the colon.

Acknowledgements
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Disclosures
Glycomyr, Inc. Ames, IA supplied the $\beta$-glucuronidated vitamin D compounds utilized in the studies. Authors Goff and Horst are president and vice president respectively of this company, which is working to develop commercial uses of the glycosides of vitamin D.
References


2. Cantorna MT, Munsick C, Bemiss C, and Mahon BD. 1,25-


29. Zhu Y, Mahon BD, Froicu M, and Cantorna MT. Calcium and 1 alpha,25-
dihydroxyvitamin D3 target the TNF-alpha pathway to suppress experimental
Figure Legends

Figure 1. Chemical structure of 1,25-dihydroxyvitamin D₃-25-β-glucuronide (β-gluc-1,25(OH)₂D)

Figure 2. Effect of increasing equimolar doses (6, 12, 24 or 48 pmol) of 1,25-dihydroxyvitamin D₃ (1,25(OH)₂D) or 1,25-dihydroxyvitamin D₃-25-β-glucuronide (β-gluc-1,25(OH)₂D) administered orally on colon and duodenum expression of Cyp24 and plasma concentrations of 1,25(OH)₂D. N= 4 mice/treatment time point. Mice were sacrificed 6 hrs after treatment. * denotes significantly different from control (P<0.05).

Figure 3. Colon and duodenum Cyp24 expression and plasma 1,25(OH)₂D concentrations in mice (N=5 mice/treatment time point) receiving 24 pmoles of either 1,25-dihydroxyvitamin D₃ (1,25(OH)₂D) or 1,25-dihydroxyvitamin D₃-25-β-glucuronide (β-gluc-1,25(OH)₂D) administered orally. Mice were sacrificed at 1, 3, 6, or 24 hrs after treatment. * denotes significantly different from control (P<0.05).

Figure 4. Colon expression of Cyp24, TNF-α, and Cadherin 1 (Cadh1) in mice from IBD study #2. Control mice received no Dextran sodium sulfate (DSS) in their drinking water and DSS mice received 2.5% DSS drinking water for seven days. DSS + β-gluc-1,25(OH)₂D mice received DSS drinking water and also had 171 pmole β-gluc-1,25(OH)₂D mixed with their diet each day. Mice were sacrificed 8 days after the initiation of DSS in the drinking water.
Fig. 1
Table 1. β-glucuronidase activity of duodenum and ileal contents of rats as assessed by cleavage of 1,25-dihydroxyvitamin D₃-25-β-glucuronide (β-gluc-1,25(OH)₂D) to the aglycone 1,25-dihydroxyvitamin D₃ (1,25(OH)₂D) following in vitro incubation of β-gluc-1,25(OH)₂D or 1,25(OH)₂D (as assay internal control) for varying lengths of time and determining the amount of 1,25(OH)₂D liberated. Mean ± SEM, N=2.

<table>
<thead>
<tr>
<th>Origin of Lumen Contents</th>
<th>Incubation Time (hrs.)</th>
<th>Vitamin D Form</th>
<th>Added Amount (pmol)</th>
<th>Measured 1,25(OH)₂D (pmol)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Duodenum</td>
<td>0</td>
<td>1,25(OH)₂D</td>
<td>0.36</td>
<td>0.23 ± 0.06</td>
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<td>Duodenum</td>
<td>6</td>
<td>1,25(OH)₂D</td>
<td>0.36</td>
<td>0.28 ± 0.02</td>
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<tr>
<td>Duodenum</td>
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<td>β-gluc-1,25(OH)₂D</td>
<td>3.54</td>
<td>0.07 ± 0.12</td>
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<td>Duodenum</td>
<td>6</td>
<td>β-gluc-1,25(OH)₂D</td>
<td>3.54</td>
<td>0.25 ± 0.02</td>
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<td>Ileum</td>
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<td>1,25(OH)₂D</td>
<td>0.36</td>
<td>0.31 ± 0.02</td>
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<tr>
<td>Ileum</td>
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<td>1,25(OH)₂D</td>
<td>0.36</td>
<td>0.31 ± 0.03</td>
</tr>
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<td>β-gluc-1,25(OH)₂D</td>
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<td>0.09 ± 0.01</td>
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<td>3.18 ± 0.67</td>
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<td>Colon</td>
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<td>1,25(OH)₂D</td>
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<td>0.17 ± 0.02</td>
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<td>Colon</td>
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<td>β-gluc-1,25(OH)₂D</td>
<td>3.54</td>
<td>0.91 ± 0.51</td>
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<tr>
<td>Colon</td>
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<td>β-gluc-1,25(OH)₂D</td>
<td>3.54</td>
<td>2.31 ± 0.41</td>
</tr>
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</table>

a Duplicate lost to analysis.
**Table 2.** Effect of vitamin D compounds on progression of colitis in mice with Dextran Sodium Sulfate (DSS)-induced colon IBD in IBD Study #1. Mice received diets containing treatments which included 1,25-dihydroxyvitamin D₃ (1,25(OH)₂D), 1,25-dihydroxyvitamin D₃-25-β-glucuronide (β-gluc-1,25(OH)₂D) and 25-hydroxyvitamin D₃-25-β-glucuronide (β-gluc-25-(OH)D). Diet treatments began 4 days prior to initiation of DSS water and continued until sacrifice. DSS water was administered for 7 days and mice were euthanized on Day 8. Mean ± SEM.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Fecal Blood Score</th>
<th>Colon Length (cm)</th>
<th>Colon Lesion score</th>
<th>Change in Body Weight (g)</th>
</tr>
</thead>
<tbody>
<tr>
<td>No DSS</td>
<td>0 ± 0 d</td>
<td>6.89 ± 0.37 d</td>
<td>0.55 ± 0.18 d</td>
<td>1.91 ± 0.31</td>
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<tr>
<td>DSS Only</td>
<td>0.89 ± 0.34</td>
<td>5.22 ± 0.20</td>
<td>9.33 ± 0.62</td>
<td>-1.03 ± 0.71 e</td>
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<tr>
<td>DSS + 120 pmole 1,25(OH)₂D</td>
<td>0.22 ± 0.12 d</td>
<td>5.74 ± 0.17</td>
<td>5.89 ± 0.54 d</td>
<td>-0.96 ± 0.51 e</td>
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<tr>
<td>DSS + 120 pmole β-gluc-1,25(OH)₂D</td>
<td>0.22 ± 0.08 d</td>
<td>5.41 ± 0.15</td>
<td>6.33 ± 0.74 d</td>
<td>-0.55 ± 0.38 e</td>
</tr>
<tr>
<td>DSS + 120 pmole β-gluc-1,25(OH)₂D + 8.67 nmole β-gluc-25(OH)D</td>
<td>0.11 ± 0.11 d</td>
<td>6.22 ± 0.19 d</td>
<td>6.78 ± 0.98 d</td>
<td>0.33 ± 0.44</td>
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a = Fecal Blood Score System: 0 = no blood to 3 = multiple blood spots in cage  
b = Histopathology colon lesion score: 0 = no lesions; 12 = severe erosion, hemorrhage, and submucosal edema  
c = Body wt on Day 8 of DSS period – average body wt over the 3 days prior to initiation of DSS treatment  
d = Significantly different from DSS Only mice (P< 0.05).  
e = Significantly different from No DSS mice (P< 0.05).
Table 3. Effect of vitamin D compounds on plasma calcium and plasma vitamin D metabolite concentrations in mice with Dextran Sodium Sulfate (DSS)-induced colon IBD in IBD Study #1. Mice received diets containing treatments which included 1,25-dihydroxyvitamin D3 (1,25(OH)2D), 1,25-dihydroxyvitamin D3-25-β-glucuronide (β-gluc-1,25(OH)2D) and 25-hydroxyvitamin D3-25-β-glucuronide (β-gluc-25-(OH)D). Diet treatments began 4 days prior to initiation of DSS water and continued until sacrifice. DSS water was administered for 7 days and mice were euthanized on Day 8. Mean ± SEM.

<table>
<thead>
<tr>
<th>Treatment (N=9 mice/treatment)</th>
<th>Calcium (mg/dl)</th>
<th>25-(OH)D (nmole/L)&lt;sup&gt;a&lt;/sup&gt;</th>
<th>1,25(OH)2D (pmole/L)&lt;sup&gt;a&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>No DSS</td>
<td>9.54 ± 0.11</td>
<td>77 ± 2.5</td>
<td>223 ± 26</td>
</tr>
<tr>
<td>DSS Only</td>
<td>8.44 ± 0.36&lt;sup&gt;b&lt;/sup&gt;</td>
<td>45 ± 2.5</td>
<td>182 ± 72</td>
</tr>
<tr>
<td>DSS + 120 pmole 1,25(OH)2D</td>
<td>11.58 ± 0.18&lt;sup&gt;b&lt;/sup&gt;</td>
<td>42 ± 2.5</td>
<td>389 ± 77</td>
</tr>
<tr>
<td>DSS + 120 pmole β-gluc-1,25(OH)2D</td>
<td>10.12 ± 0.30</td>
<td>40 ± 2.5</td>
<td>255 ± 60</td>
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<tr>
<td>DSS + 120 pmole β-gluc-1,25(OH)2D + 8.67 nmole β-gluc-25(OH)D</td>
<td>10.26 ± 0.22</td>
<td>188 ± 2.5</td>
<td>507 ± 137</td>
</tr>
</tbody>
</table>

<sup>a</sup> = Vitamin D metabolite concentrations of pooled plasma. Each pooled sample consisted of 50 ul plasma from either 4 or 5 mice, resulting in 2 samples/treatment. No statistical testing applied to these data.

<sup>b</sup> = significantly different from No DSS group, P < 0.05.
Table 4. Effect of vitamin D compounds on progression of colitis in mice with Dextran Sodium Sulfate (DSS)-induced colon IBD in IBD Study #2. Mice received diets containing treatments which included carrier or 171 pmole 1,25-dihydroxyvitamin D₃-25-β-glucuronide (β-gluc-1,25(OH)₂D). Diet treatments began 4 days prior to initiation of DSS water and continued until sacrifice. DSS water was administered for 7 days and mice were euthanized on Day 8. Mean ± SEM.

<table>
<thead>
<tr>
<th>Treatment (N=6 mice/treatment)</th>
<th>Body Wt changeᵃ (g)</th>
<th>Colon length (cm)</th>
<th>Fecal Blood Scoreᵇ</th>
<th>Colon Lesion Scoreᶜ</th>
</tr>
</thead>
<tbody>
<tr>
<td>No DSS</td>
<td>0.012 ± 0.011</td>
<td>7.56 ±0.33e</td>
<td>0 ± 0e</td>
<td>0.17 ± 0.17</td>
</tr>
<tr>
<td>DSS Only</td>
<td>-0.092 ± 0.016d</td>
<td>6.68 ±0.19d</td>
<td>1.33 ± 0.49d</td>
<td>3.67 ± 0.33d</td>
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<tr>
<td>DSS + 171 pmole β-gluc-1,25(OH)₂D</td>
<td>-0.052 ± 0.019d</td>
<td>7.40 ±0.21e</td>
<td>0.33 ± 0.33e</td>
<td>5.33 ± 0.80d</td>
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ᵃ = Body wt on Day 8 of DSS period – average body wt over the 3 days prior to initiation of DSS treatment;
ᵇ = Fecal Blood Score System: 0 = no blood to 3 = multiple blood spots in cage
ᶜ = Histopathology colon lesion score: 0 = no lesions; 12 = severe erosion, hemorrhage, and submucosal edema
ᵈ = significantly different from No DSS mice (P<0.05);
ᵉ = significantly different from DSS Only mice (P < 0.075)
Table 5. Effect of vitamin D compounds on plasma calcium and vitamin D metabolite concentrations in mice with Dextran Sodium Sulfate (DSS)-induced colon IBD in IBD Study # 2. Mice received diets containing treatments which included carrier or 171 pmoles of 1,25-dihydroxyvitamin D3-25-β-glucuronide (β-gluc-1,25(OH)2D). Diet treatments began 4 days prior to initiation of DSS water and continued until sacrifice. DSS water was administered for 7 days and mice were euthanized on Day 8. Mean ± SEM.

<table>
<thead>
<tr>
<th>Treatment (N=6 mice/treatment)</th>
<th>Plasma Calcium (mg/dl)</th>
<th>Plasma 25(OH)D (nmole/L)a</th>
<th>Plasma 1,25(OH)2D (pmole/L)a</th>
</tr>
</thead>
<tbody>
<tr>
<td>No DSS</td>
<td>9.46 ± 0.07</td>
<td>117 ± 2.5</td>
<td>259 ± 17</td>
</tr>
<tr>
<td>DSS Only</td>
<td>8.90 ± 0.27b</td>
<td>70 ± 2.5</td>
<td>120 ± 17</td>
</tr>
<tr>
<td>DSS + 171 pmoles β-gluc-1,25(OH)2D</td>
<td>9.84 ± 0.20</td>
<td>42 ± 12</td>
<td>504 ± 63</td>
</tr>
</tbody>
</table>

a = Vitamin D metabolite concentration of pooled plasma. Each pooled sample consisted of 100 ul of plasma from 2 mice, resulting in 3 samples/treatment. No statistical testing applied to these data.

b = significantly different from No DSS mice group (P< 0.075);