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Targeted delivery of 1,25-dihydroxyvitamin D₃ to colon tissue and identification of a major 1,25-dihydroxyvitamin D₃ glycoside from *Solanum glaucophyllum* plant leaves

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Abstract

Leaves of the *Solanum glaucophyllum* (*Sg*) plant, indigenous to South America, have long been known for their calcinogenic toxicity in ruminant animals. It was determined the leaves contained glycosidic derivatives of 1,25-dihydroxyvitamin D₃ (1,25D₃) and liberation of the free hormone by rumen bacterial populations elicited a hypercalcemic response. Our interest in the leaves is predicated on the concept that the glycoside forms of 1,25D₃ would target release of the active hormone in the lower gut of non-ruminant mammals. This would provide a means of delivering 1,25D₃ directly to the colon, where the hormone has been shown to have beneficial effects in models of inflammatory bowel disease (IBD) and colon cancer. We fed mice for 10 days with variable amounts of *Sg* leaf. Feeding 7–333 ug leaf/day produced no changes in plasma Ca²⁺ and 1,25D₃ concentrations, and only at > 1000 ug leaf/day did these values become significantly elevated compared to controls. Gene expression studies from colon tissue indicated a linear relationship between the amount of leaf consumed and expression of the Cyp24a1 gene. In contrast, Cyp24a1 gene expression in the duodenum and ileum of these mice was unchanged compared to controls. One of the major 1,25D₃-glycosides was isolated from leaves following extraction and purification by Sep-Pak cartridges and HPLC fractionation. Ultraviolet absorbance was consistent with modification of the 1-hydroxyl group, and positive ion ESI mass spectrometry indicated a diglycoside of 1,25D₃. 2-Dimensional NMR analyses were carried out and established the C1 proton of the A-ring was interacting with a C1' sugar proton, while the C3 proton of the A-ring was linked with a second C1' sugar proton. The structure of the isolated compound is therefore consistent with a beta-linked 1,3-diglycoside of 1,25D₃. Thus, *Sg* leaf administered to mice at up to 333 ug/day can elicit colon-specific enhancement of Cyp24a1 gene expression

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without inducing hypercalcemia, and the 1,3-diglycoside is one of the major forms of 1,25D₃ found in the leaf.

Keywords

1,25-dihydroxyvitamin D₃; colon; *Solanum glaucophyllum*; gene expression; hypercalcemia

Introduction

Vitamin D insufficiency has been linked to a variety of immune disorders, including inflammatory bowel disease (IBD) and colon cancer. Epidemiological studies now suggest poor vitamin D status may initiate development of IBD. [1, 2]. Animal models of IBD that are vitamin D-deficient develop the disease more quickly and exhibit greater inflammation than vitamin D-replete animals [3, 4]. Animal studies have also demonstrated the hormonal form of vitamin D, 1,25-dihydroxyvitamin D₃ (1,25D₃) has potent modulating effects on the immune system [5] and on intestinal epithelial barrier integrity [6, 7].

Epidemiologic evidence also suggests the prevalence of widespread vitamin D insufficiency may be linked to the development of several different types of cancer [8, 9], particularly colon cancer [10-16]. Treatment of a wide variety of cancer cell lines with 1,25D₃ reduces cell proliferation *in vitro*. Human colon cancer cell lines, such as HT-29 [17] and Caco-2 [18], and the mouse colon cancer cell line, MC-26 [19], all responded to the anti-proliferative and pro-differentiating effects of 1,25D₃ with reduced growth. Unfortunately, the doses of 1,25D₃ required to effectively treat tumors *in vivo* results in strong stimulation of the vitamin's classical effects on calcium homeostasis and the development of life-threatening hypercalcemia [20, 21]. A novel, alternative strategy is to develop a delivery system that would target 1,25D₃ to just the colon tissue. Smaller doses of 1,25D₃ could be administered and still achieve high therapeutic levels within the colon while reducing the risk of increasing systemic 1,25D₃ concentrations that lead to hypercalcemia.

The *Solanum glaucophyllum* plant (*Sg*; also referred to as *Solanum malacoxylon*), is widely distributed in southeastern South America. Ingestion of the leaf causes a "wasting away" syndrome in cattle due to hypercalcemia and nephrocalcinosis [22]. Investigations into the factor(s) responsible for the disease demonstrated the plant contains a water-soluble material that stimulated calcium metabolism much like 1,25D₃ [23]. Later reports indicated the leaves contained glycoside forms of 1,25D₃, which explained its water-soluble characteristics and lack of biological activity until the glycosides were cleaved by rumen bacteria to liberate 1,25D₃ [24-27]. Humans and monogastric animals also harbor bacteria with β -glycosidase activity, but only in their lower intestine. We reasoned, therefore, that 1,25D₃-glycosides present in the *Sg* leaf would provide a means of delivering 1,25D₃ specifically to the colon as a possible therapeutic in the treatment of colon disorders. In evaluating the potential therapeutic use of the *Sg* leaf we now report on the ability of the leaf to target colon-specific, vitamin D-dependent gene expression without raising blood Ca²⁺ concentrations, and describe the isolation and identification of one of the principal glycoside forms of 1,25D₃ found in the leaf.

Materials and Methods

General

The *Sg* leaf was obtained from Argentina as previously described [28]. The β -25-monoglucuronide of 1,25D₃ (β Gluc-1,25D₃) was synthesized as described previously [29], was purified by high performance liquid chromatography to >97% purity and the structure confirmed by FT-IR and NMR. The 1,25D₃ was purchased from Sigma-Aldrich (St Louis, MO) and was >98% pure. Quantitation of vitamin D compounds in ethanol solutions utilized to prepare each treatment was based on absorbance at 264 nm using a molar extinction coefficient of 18,200 absorbance units $\cdot$ mol⁻¹ $\cdot$ L⁻¹. Analytical high-performance liquid chromatography (HPLC) of extracts was performed on a Waters Associates modular system (Waters Associates, Milford, MA) equipped with two model 510 solvent pumps, a U6K manual injector, a model 440 detector and a model 996 photodiode array (PDA) detector. Ultraviolet (UV) spectra were obtained using a Beckman DU-600 scanning spectrophotometer. The ESI mass spectrum was obtained on a Finnigan 700 triple quadrupole mass spectrometer fitted with the API 1 Finnigan ESI interface. The NMR spectra were obtained on a Bruker Avance 500 MHz spectrophotometer in the indicated solvents.

Animal Experiments

Male C57BL/6 mice (The Jackson Laboratory, Bar Harbor, ME) were housed individually or in groups in solid bottom cages with contact bedding, in accordance with the accepted space requirements established by Laboratory Animal Resources at Iowa State University. Mice 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/gr (Harlan Labs, Madison, WI) and were assumed to have normal intestinal biomes. 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.

Oral doses were prepared by grinding Teklad 2018 pellets into a powder. This powder was then mixed with commercial low-fat peanut butter in a 2:1 ratio until a homogeneous consistency was obtained. One hundred grams of Teklad/peanut butter (Tpb) mixture was thoroughly mixed with 100 mg of dried, finely ground *Sg* leaf to create a stock of 1 mg leaf/gr Tpb mixture. Additional stock materials were prepared by removal of a weighed portion of leaf/Tpb mixture and thorough mixing with additional fresh Tpb of known weight, and this process repeated in a serial manner to obtain other leaf/Tpb stocks. Then multiple, approximate 1 gr portions were removed from each of the stock preparations and rolled into small balls that were stored at 4°C. In the dosing trials, mice (5 animals/treatment) were individually housed and fed the assigned 1 gr Tpb balls with varying amounts of *Sg* leaf each day, along with access to standard diet (Teklad 2018 pellets) and water. Control mice were fed 1 gr Tpb balls lacking *Sg* leaf. Mice were monitored daily to ensure that each round had been consumed. On the evening of day 9, access to regular food was denied and on the morning of day 10 the animals only received the leaf/Tpb rounds as a food source until the entire round was consumed. Mice were then euthanized ca. 6 hours later and plasma and tissues were harvested.

Other mice were treated orally with 24 pmoles of 1,25D₃ or βGluc-1,25D₃. Oral doses of 1,25D₃ or βGluc-1,25D₃ were prepared by suspending the vitamin D compounds in 50 μl of a peanut oil/ethanol mixture (90:10). Mice were returned to their cages and then euthanized 6 hrs after treatment and plasma and tissues were harvested as above.

Plasma analyses

Mice were euthanized by cervical dislocation while under isoflurane anesthesia. Blood was collected from the cervical stump into heparinized tubes, and plasma was collected and frozen at −86°C until analyzed. Plasma 1,25D₃ concentrations were determined by radioimmunoassay on individual samples (Heartland Assays, Ames, IA) [30, 31]. Calcium content was determined by colorimetric assay (Arsenazo III, Pointe Scientific, Canton, MI).

Tissue and RNA processing

A 1 cm section of duodenum (between 2 and 3 cm from the pylorus; wet tissue weight ca. 50-75 mg) and a 1 cm section of colon (between 2 and 3 cm from the cecum; wet tissue weight ca. 25-50 mg) were obtained from each animal for mRNA analysis. In a subset of animals a 1 cm section of ileum tissue (between 2 and 3 cm proximal from the ileocecal junction; wet tissue weight ca. 25-50 mg). Tissue samples were flushed with ice-cold 0.9% saline solution and immediately homogenized in 1 ml of TRIzol® reagent. Samples were then kept frozen at −86°C prior to processing for RNA.

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 × 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 [32]. RNA was eluted in 50 ul of water and the concentration obtained by UV spectrometry by microtiter plate. 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 (Invitrogen, Carlsbad, CA) as described by the manufacturer. 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 (qPCR)

qPCR was performed using a Stratagene Mx3005p cycler (Stratagene, La Jolla, CA) and GoTaq qPCR Master Mix (Promega Corp., Madison, WI). Amplification of murine target cDNAs was accomplished with the following primers (synthesized by Integrated DNA Technologies, Coralville, IA): mCyp24-For, 5'-CACACGCTGGCCTGGGACAC; mCyp24-Rev, 5'-GGAGCTCCGTGACAGCAGCG; mGapdh- For, 5'-GAAGGTCGGTGTGAACGGATTTGGC; mGapdh-Rev, 5'-TTGATGTTAGTGGGGTCTCGTCCTG. Aliquots (8.3 ng) of cDNA were amplified under the following conditions: 95°C for 2 min, followed by 45 cycles of 95°C for 3 sec and 55°C for 30 sec. All reactions were performed in duplicate, with 5 animals/treatment and Cyp24a1 gene expression was estimated using the C_t method normalized relative to Gapdh expression as described previously [29, 33].

Solanum extracts

The *Sg* leaf was extracted using a 10:1 ratio of purified H₂O/gr leaf. The *Sg* leaf suspension was incubated overnight at room temperature (ca. 16 hrs) with constant stirring. The suspension was then pelleted at 500×g for 10 min and the clarified aqueous solution was filtered (Whatman #1). The pelleted material was resuspended in fresh H₂O (1/2 vol) and re-extracted with vigorous agitation for several minutes, followed by centrifugation and filtration, and this process was repeated an additional 1×. The aqueous filtrates were combined and applied to C-18 Sep-Pak columns in 10 ml aliquots. The columns were washed with 4mL of 40:60 mix of MeOH:H₂O. The glycoside compound was eluted with 4mL of 80:20 MeOH:H₂O. The eluant was made basic with 10 ul of ammonium hydroxide and the mixture was then loaded onto Sep-Pak NH₂ columns. Samples were eluted with 1 ml more of the same basic MeOH:H₂O mixture and the solvent was removed prior to HPLC analysis.

The residue was resuspended in MeOH:H₂O (50:50) and loaded into a 6 ml loop of a U6K manual injector (Waters, Milford, MA) and applied to a C-18 preparative column (30 × 300 mm; 15µm particle size; 100A pore size). A gradient was run from 50:50 to 100% MeOH over 70 minutes (10 ml/min) and 2 minute fractions were collected. Fractions were evaporated to dryness in a vacuum centrifuge and re-suspended in MeOH:H₂O (92:8). The resuspension was diluted to 12% (v:v) with CHCl₃ for HPLC analysis on an Uporasil semi-preparative column (7.8 × 300 mm). This was run isocratic for 20 minutes (1:11:88; H₂O:MeOH:CHCl₃) and then stepped to 84% CHCl₃ (1.25:14:75:84; H₂O:MeOH:CHCl₃) for 15 minutes. The fraction collector was set to collect 1 minute fractions. All fractions with the proper UV signature (240 and 270 maximums) were evaporated to dryness in a vacuum centrifuge. Fractions were then resuspended in MeOH:H₂O (60:40) and run isocratically for 45 minutes on PKb100 column (4.6 × 250 mm) at 2 ml/minute. One minute fractions were collected, saved and combined across many individual runs to accumulate enough material for UV, mass spec and NMR analyses.

Glycosidase assay

The leaf samples were assayed by rumen fluid to quantify the 1,25D₃ content as described previously [28]. During the purification process collected fractions were assessed for 1,25D₃ content using a mixed glycosidase enzyme preparation (MP Biomedicals, Santa Ana, CA). Briefly, 100 ul of recovered sample is dried, resuspended in 100 ul of water and then mixed with 500 ul of glycosidase (5 mg/ml) in 0.1 M sodium phosphate /0.1 M citric acid buffer (pH 5.0). The sample is incubated at 37°C for 8 hrs, after which 1.0 ml of CH₃CN and 1.5 ml of water are added together with 1000 cpm of 1,25D₃ tracer for identification and recovery estimate. The mixture is briefly centrifuged and then applied to a C-18 Sep-Pak column as highlighted above. After washing and elution the samples are analyzed by HPLC (Zorbax/Silica; 4.6 × 250 mm) in comparison to a standard.

Statistical analysis

Statistical analysis was performed on untransformed Ct data and means compared by student's t-test using PSI-Plot (Pearl River, NY).

Results

Oral Dosing with *Sg* Leaf and Effects on Intestinal Gene Expression

Our initial interest in *Sg* leaf was to feed it to broiler chickens as an inexpensive means of supplementing 1,25D₃ to enhance leg bone strength [34]. Later work focused on developing a means to reliably determine 1,25D₃ concentrations in the leaf [28]. Our preliminary work with mice revealed feeding 2-4 mg leaf/day caused a substantial rise in plasma 1,25D₃ concentrations (data not shown), so subsequent studies focused on feeding <1 mg leaf/day. Mice were fed the indicated dose of dried, finely ground *Sg* leaf in a mixture of peanut butter and ground Teklad 2018 pellet for 10 consecutive days along with ad libitum access to Teklad 2018 pellets and water. Animals sometimes reject higher amounts of *Sg* leaf when distributed on food, thus incorporating the leaf in the combination of peanut butter and ground Teklad 2018 pellets ensured consumption at all of the test doses. All feed was withheld during the last night, and mice were then fed their final dose of leaf the next morning followed by euthanasia 6 hours later. As seen in Figure 1, when mice were fed < 333 µg of *Sg* leaf on a daily basis for 10 days the plasma Ca²⁺ and 1,25D₃ concentrations remained largely unchanged compared to controls. , Mice exhibited a significant increase in both plasma Ca²⁺ and 1,25D₃ concentrations when 1000 µg/day of *Sg* was fed.

RNA preparations from the colons of these mice demonstrated a dose-dependent increase in Cyp24a1 gene expression (Figure 2A). We also saw increases in Trpv6 gene expression at the higher amounts of *Sg* leaf (data not shown). Meanwhile, no statistically significant enhancement of the Cyp24a1 gene was observed in the corresponding duodenum preparations from the same mice (Figure 2B). However, a handful of individual mice at the 1000 µg dose of leaf exhibited enhanced expression of Cyp24a1 in the duodenum, but as a group this did not reach statistical significance.

Ileum samples were also collected from a subset of *Sg*-treated mice dosed at 1000 and 333 µg/day. In particular, we were interested in assessing the impact of the highest concentrations of *Sg* leaf on vitamin D-dependent gene expression in the ileum. We had previously reported that the majority of a synthetic glucuronide conjugate of 1,25D₃ (βGluc-1,25D₃) incubated with ileal contents recovered from rats could be broken down to free the hormone in as little as 1 hour of incubation, just as in the colon [29]. The βGluc-1,25D₃ also strongly enhances expression of vitamin D-dependent genes in the colon [29, 35]. Accordingly, we observed strong induction of Cyp24a1 gene expression in RNA recovered from ileum tissues when βGluc-1,25D₃ was orally administered to mice, while treatment with 1,25D₃ failed to impact ileal expression of the enzyme (Figure 3A). Surprisingly, no stimulation of ileal Cyp24a1 gene expression was observed in mice consuming *Sg* leaf at 1000 and 333 µg/day (Figure 3B) despite strong stimulation of this gene's expression in their corresponding colons (Figure 2A). Thus, it appears that, unlike the synthetic βGluc-1,25D₃, the 1,25D₃-glycosides present in the *Sg* leaf are resistant to breakdown in the ileum and the 1,25D₃ is liberated only in the colons of these animals.

Studies on Isolation and Purification of 1,25D₃-Glycosides from *Sg* leaf

We demonstrated the whole *Sg* leaf fed to mice in a standard rodent diet can activate vitamin D-dependent genes exclusively within the colon. However, the leaf reportedly contains multiple forms of 1,25D₃-glycosides [36], so we set about to begin identification of the vitamin D glycosides present in leaf extracts. Previous work in the field by Wassermann [37], and in our laboratory, revealed the 1,25D₃ activity in *Sg* leaf tissue consisted of water-extractable, neutral glycosides of 1,25D₃. Several possible forms of 1,25D₃-glycosides can occur. For example, glycosidic residues of various lengths and combinations could be attached through the C-1, C-3 or C-25 hydroxyls of 1,25D₃. In order to track the activity of these conjugates during the purification process the glycosidic residues are cleaved to yield free 1,25D₃, which can then be analyzed by routine methods.

Scanning UV analysis of the peak collected at 43 min (Fig. 4A) suggested that it was likely a C1-glycoside as evident by the unique UV signature peaks at 273 and 244 nm [38, 39] (Fig. 4B). The low resolution ESI mass spectrum indicated a molecular ion at 763 (M+Na) and a 2M+Na adduct at 1504, which is consistent with the addition of 2 sugar molecules to 1,25D₃ (Fig. 4C). The UV spectrum indicated a sugar substitution at the C1 hydroxyl of the vitamin D A-ring, meaning a second sugar molecule could be attached through either of the C3 or C25 alcohols, or a disaccharide could be linked at C1. The ¹H NMR revealed a complicated resonance pattern (Fig 4D). However, the C26/C27 methyl groups (ca. 1.2 ppm) appeared as a singlet, which is consistent with no glycoside modification of the C25 hydroxyl position [40]. This indicates the sugar modifications were localized to the A-ring. Accordingly, both the A-ring protons at C1 and C3 were shifted downfield (ca. 4.55 and 4.3 ppm), which is suggestive of modified hydroxyls at both positions. Distinct doublets were also evident at ca. 4.4 and 4.2 ppm corresponding to the C1' protons of the two sugar molecules, and the shifts and coupling constants (ca. 8 Hz) are consistent with β-linkages [41].

The material was then analyzed by 2-dimensional (2D) NMR spectroscopy techniques. As seen in Fig 5A, Heteronuclear Multiple Quantum Correlation (HMQC) analysis of C-H coupling confirmed the presence of distinct C1' signals at 102.5 ppm and 100.5 ppm from the 2 sugar molecules. A cluster of overlapping signals from ca. 70.5 to 77 ppm is consistent with C2'-C5' ring sugar carbons and protons, while the C6' sugar produced distinct carbon resonances at ca. 62.5 ppm in agreement with the distinct position of the hydrogen signals at ca. 3.65 and 3.85 ppm. Finally, Rotating frame nuclear Overhauser effect spectroscopy (ROESY) 2D NMR (DMSO-d₆) established the through-space interactions of the C1 proton of the A-ring at ca. 4.55 ppm with the C1' sugar proton at ca. 4.2 ppm, while the C3 proton of the A-ring (ca. 4.15 ppm) is linked with the C1' sugar proton at ca. 4.45 ppm (Fig 5B). In total, the data is consistent with the isolated *Sg* plant 1,25D₃-glycoside being singly glycosylated at the C1 and C3 hydroxyl positions of the vitamin D A-ring via β-C1' sugar beta linkages (Fig 5C).

Disucussion

The calcinogenic toxicity of *Sg* leaves to particularly ruminants is attributed to the presence of 1,25-glycosides that can be cleaved by rumen bacteria and release the free hormone [42].

Other glycoside forms of vitamin D have also been found in *Sg*, including 25D₃ and vitamin D₃ itself, but they are much less abundant, and are less important than glycosides of 1,25D₃ with regards to the hypercalcemic activity of the plant [43, 44]. Substantial evidence documents that the plant and/or plant extracts are active in monogastrics, and if given in high amounts can serve as a means of increasing plasma 1,25D₃ concentrations [34, 36, 45-50]. There is also an isolated report where the leaf was administered to a handful of human patients with renal failure as a source of 1,25D₃ to prevent secondary renal hyperparathyroidism [51]. This caused a rise in serum Ca²⁺ and an increase in intestinal Ca²⁺ absorption, indicating the leaf is active in humans as well. Bioassays reveal the dried *Sg* leaves can contain as much as 100 µg of 1,25D₃ activity/g material [52], although more recent sampling from a variety of locations in Argentina using a radioimmunoassay indicated an average of approximately 22 µg 1,25D₃ activity/gr dry leaf [28]. Weissenberg et al., estimated that under proper agronomic conditions *Sg* could yield as much as 3.3 tons of dried leaf material/hectare during two seasonal harvests [53], or about 300 gr of 1,25D₃/hectare.

We demonstrated *Sg* leaf dose-dependently enhanced Cyp24a1 gene expression in the colon, with as little as 21 µg leaf/day significantly stimulating this gene. Doses of leaf, up to 333 µg/day, did not stimulate duodenal Cyp24a1 gene expression or raise blood Ca²⁺ or 1,25D₃ concentrations, suggesting minimal risk of developing hypercalcemia with *Sg* fed at these concentrations (Figure 1 & 2). Furthermore, we observed differences in vitamin D-dependent gene enhancement in the ileums of mice treated with the synthetic βGluc-1,25D₃ versus the *Sg* leaf. The βGluc-1,25D₃ strongly stimulated Cyp24a1 gene expression in the ileum (Figure 3), suggesting breakdown by bacterial glucuronidases in this part of the digestive tract [29]. However, no enhancement of this gene was observed in the ileums when animals were fed *Sg* leaf, even though just a short distance away the colons of these same mice exhibited strong enhancement of Cyp24a1 gene expression (Figures 2 and 3). Glucuronide modification of the synthetic βGluc-1,25D₃ occurs at the C-25 alcohol group in the side chain, while in contrast, the purified glycoside from *Sg* leaf revealed modifications of the 1- and 3-position alcohols of the A-ring. Thus, the synthetic molecule with the modification in the side chain appears to be more readily targeted for removal by bacteria higher up the digestive tract. The *Sg* leaves, in contrast, may require further digestion by bacteria in the lower intestinal tract, the colon and perhaps cecum, before the 1,25D₃-glycosides are liberated from the plant tissue and available for cleavage by glycosidases, or the A-ring modifications may require the presence of specific bacterial populations with the requisite glycosidases that may be present only in the lower tract to effectively liberate the 1,25D₃ molecule. In either case the data support tissue differences in the breakdown of the simple, synthetic glucuronide relative to the 1,25D₃-glycoside moieties that exist in *Sg* leaf.

Accordingly, we have isolated and purified a major glycoside present in *Sg* leaf extracts and identified it as a 1,3-diglycoside of 1,25D₃ (Figure 5). The coupling constants seen in the ¹H NMR strongly suggest the sugar moieties are beta-linked to the 1,25D₃ molecule (Figure 4), which would be consistent with degradation of the molecule by β-glycosidases produced by bacterial populations. Subsequently, we have isolated other, less abundant, 1,25D₃-glycosides from the leaf and tentatively linked them by a combination of MS and HPLC

analyses to be sequential additions of a sugar molecule relative to the 1,25D₃-1,3-diglycoside (data not shown), of which the exact locations of the added sugars are currently unknown.

Several studies have demonstrated a direct therapeutic effect of vitamin D and 1,25D₃ in mouse models of IBD. A commonly utilized murine IBD model utilizes dextran sodium sulfate (DSS) administration in the drinking water to induce IBD-like lesions. Though administering 1,25D₃ in short-term studies can ameliorate IBD in mice, hypercalcemia is a common side effect that hinders use of 1,25D₃ in more chronic models of IBD. However, if the 1,25D₃ or its' analog is administered rectally the dose needed to ameliorate inflammation in the DSS mouse model is greatly reduced, as is the risk of developing hypercalcemia. Froicu et al. demonstrated rectal administration of 10 ng 1,25D₃ every other day for 10 days improved body weights and histopathology scores in colons of mice receiving DSS. This was more effective than feeding 50 ng 1,25D₃/day, and did not cause hypercalcemia [3]. Laverny et al. gave ~7.5 ng 1,25D₃ or 25 ng of a less hypercalcemic 1,25D₃ analog (BXL-62) daily per rectum to C57/Bl6 mice in the DSS model [54]. Both drugs reduced an index of disease activity in the mice without causing hypercalcemia. The data suggest 1,25D₃ delivered primarily or exclusively to the colon in relatively high doses can ameliorate IBD with decreased risk of developing hypercalcemia.

Our prior data indicate exogenously administered 1,25D₃ is largely unable to impact gene expression in the colon despite attaining greatly elevated circulating concentrations of the hormone [29, 35]. Thus, it has proved difficult to administer enough 1,25D₃ to achieve “therapeutic” effects in the lower gut without incurring systemic hypercalcemic side effects. Our approach is to unlock the potential of 1,25D₃ residing as glycosides in the *Sg* plant to specifically target 1,25D₃ release to the lower intestine. Our data indicate the *Sg* leaf offers a means of delivering high concentrations of 1,25D₃ locally to the colon lumen while the small total dose of 1,25D₃ in the glycoside limits the potential for hypercalcemia. By moving the 1,25D₃ through the duodenum in a form that will not interact with the duodenal VDR, it will limit active transport of Ca²⁺ in the upper intestine. The *Sg* leaf may therefore provide several advantages over a synthetic conjugate: it will be less expensive - the leaves are a readily accessible plant product that produces the 1,25D₃-glycosides in high amounts - and our preliminary data suggest the 1,25D₃ in the *Sg* leaf may not be released until it reaches the colon proper, thus it is highly tissue specific. Consequently, the *Sg* leaf, or purified extracts of 1,25D₃-glycosides derived from it, represents an attractive alternative means of delivering 1,25D₃ specifically to colon tissue, where the hormone has proven beneficial in a variety of disease states.

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References

- [1]. Garg M, Lubel JS, Sparrow MP, Holt SG, Gibson PR. Review article: vitamin D and inflammatory bowel disease--established concepts and future directions. *Alimentary pharmacology & therapeutics*. 2012; 36:324–344. [PubMed: 22686333]
- [2]. Ulitsky A, Ananthakrishnan AN, Naik A, Skaros S, Zadvornova Y, Binion DG, Issa M. Vitamin D deficiency in patients with inflammatory bowel disease: association with disease activity and quality of life. *JPEN. Journal of parenteral and enteral nutrition*. 2011; 35:308–316. [PubMed: 21527593]
- [3]. Froicu M, Cantorna MT. Vitamin D and the vitamin D receptor are critical for control of the innate immune response to colonic injury. *BMC immunology*. 2007; 8:5. [PubMed: 17397543]
- [4]. Strauch UG, Obermeier F, Grunwald N, Dunger N, Rath HC, Scholmerich J, Steinmeyer A, Zugel U, Herfarth HH. Calcitriol analog ZK191784 ameliorates acute and chronic dextran sodium sulfate-induced colitis by modulation of intestinal dendritic cell numbers and phenotype. *World journal of gastroenterology: WJG*. 2007; 13:6529–6537. [PubMed: 18161923]
- [5]. Ooi JH, Chen J, Cantorna MT. Vitamin D regulation of immune function in the gut: why do T cells have vitamin D receptors? *Molecular aspects of medicine*. 2012; 33:77–82. [PubMed: 22079836]
- [6]. Zhao H, Zhang H, Wu H, Li H, Liu L, Guo J, Li C, Shih DQ, Zhang X. Protective role of 1,25(OH)₂ vitamin D₃ in the mucosal injury and epithelial barrier disruption in DSS-induced acute colitis in mice. *BMC gastroenterology*. 2012; 12:57. [PubMed: 22647055]
- [7]. Kong J, Zhang Z, Musch MW, Ning G, Sun J, Hart J, Bissonnette M, Li YC. Novel role of the vitamin D receptor in maintaining the integrity of the intestinal mucosal barrier. *Am J Physiol Gastrointest Liver Physiol*. 2008; 294:G208–216. [PubMed: 17962355]
- [8]. Fleet JC, DeSmet M, Johnson R, Li Y. Vitamin D and cancer: a review of molecular mechanisms. *Biochem J*. 2012; 441:61–76. [PubMed: 22168439]
- [9]. Welsh J. Cellular and molecular effects of vitamin D on carcinogenesis. *Archives of biochemistry and biophysics*. 2011
- [10]. Ma Y, Zhang P, Wang F, Yang J, Liu Z, Qin H. Association between vitamin D and risk of colorectal cancer: a systematic review of prospective studies. *J Clin Oncol*. 2011; 29:3775–3782. [PubMed: 21876081]
- [11]. Giardina C, Madigan JP, Godman Tierney CA, Brenner BM, Rosenberg DW. Vitamin D resistance and colon cancer prevention. *Carcinogenesis*. 2012
- [12]. Garland CF, Garland FC. Do sunlight and vitamin D reduce the likelihood of colon cancer? *Int J Epidemiol*. 1980; 9:227–231. [PubMed: 7440046]
- [13]. Holick MF. Vitamin D and sunlight: strategies for cancer prevention and other health benefits. *Clin J Am Soc Nephrol*. 2008; 3:1548–1554. [PubMed: 18550652]
- [14]. Newmark HL, Yang K, Kurihara N, Fan K, Augenlicht LH, Lipkin M. Western-style diet-induced colonic tumors and their modulation by calcium and vitamin D in C57Bl/6 mice: a preclinical model for human sporadic colon cancer. *Carcinogenesis*. 2009; 30:88–92. [PubMed: 19017685]
- [15]. Jenab M, Bueno-de-Mesquita HB, Ferrari P, van Duijnhoven FJ, Norat T, Pischon T, Jansen EH, Slimani N, Byrnes G, Rinaldi S, Tjonneland A, Olsen A, Overvad K, Boutron-Ruault MC, Clavel-Chapelon F, Morois S, Kaaks R, Linseisen J, Boeing H, Bergmann MM, Trichopoulou A, Misirli G, Trichopoulos D, Berrino F, Vineis P, Panico S, Palli D, Tumino R, Ros MM, van Gils CH, Peeters PH, Brustad M, Lund E, Tormo MJ, Ardanaz E, Rodriguez L, Sanchez MJ, Dorransoro M, Gonzalez CA, Hallmans G, Palmqvist R, Roddam A, Key TJ, Khaw KT, Autier P, Hainaut P, Riboli E. Association between pre-diagnostic circulating vitamin D concentration and risk of colorectal cancer in European populations: a nested case-control study. *BMJ*. 2010; 340:b5500. [PubMed: 20093284]
- [16]. Fedirko V, Bostick RM, Goodman M, Flanders WD, Gross MD. Blood 25-hydroxyvitamin D₃ concentrations and incident sporadic colorectal adenoma risk: a pooled case-control study. *Am J Epidemiol*. 2010; 172:489–500. [PubMed: 20650953]

- [17]. Zhao X, Feldman D. Regulation of vitamin D receptor abundance and responsiveness during differentiation of HT-29 human colon cancer cells. *Endocrinology*. 1993; 132:1808–1814. [PubMed: 8384998]
- [18]. Chen A, Davis BH, Bissonnette M, Scaglione-Sewell B, Brasitus TA. 1,25-Dihydroxyvitamin D(3) stimulates activator protein-1-dependent Caco-2 cell differentiation. *The Journal of biological chemistry*. 1999; 274:35505–35513. [PubMed: 10585423]
- [19]. Kane KF, Langman MJ, Williams GR. Antiproliferative responses to two human colon cancer cell lines to vitamin D3 are differently modified by 9-cis-retinoic acid. *Cancer Res*. 1996; 56:623–632. [PubMed: 8564982]
- [20]. Harris DM, Go VL. Vitamin D and colon carcinogenesis. *J Nutr*. 2004; 134:3463S–3471S. [PubMed: 15570055]
- [21]. Smith DC, Johnson CS, Freeman CC, Muindi J, Wilson JW, Trump DL. A Phase I trial of calcitriol (1,25-dihydroxycholecalciferol) in patients with advanced malignancy. *Clin Cancer Res*. 1999; 5:1339–1345. [PubMed: 10389917]
- [22]. Worker NA, Carrillo BJ. “Enteque seco”, calcification and wasting in grazing animals in the Argentine. *Nature*. 1967; 215:72–74. [PubMed: 6053410]
- [23]. O'Donnell JM, Smith MW. Vitamin D-like action of *Solanum malacoxylon* on calcium transport by rat intestine. *Nature*. 1973; 244:357–358. [PubMed: 4355668]
- [24]. Wasserman RH. Active vitamin D-like substances in *Solanum malacoxylon* and other calcinogenic plants. *Nutrition reviews*. 1975; 33:1–5. [PubMed: 163014]
- [25]. Haussler MR, Wasserman RH, McCain TA, Peterlik M, Bursac KM, Hughes MR. 1, 25-dihydroxyvitamin D3-glycoside: identification of a calcinogenic principle of *Solanum malacoxylon*. *Life sciences*. 1976; 18:1049–1056. [PubMed: 933696]
- [26]. Haussler MR, Hughes MR, McCain TA, Zerwekh JE, Brumbaugh PF, Jubiz W, Wasserman RH. 1,25-Dihydroxyvitamin D3: mode of action in intestine and parathyroid glands, assay in humans and isolation of its glycoside from *Solanum malacoxylon*. *Calcified tissue research*. 1977; 22(Suppl):1–18. [PubMed: 912510]
- [27]. Peterlik M, Wasserman RH. 1,25-Dihydroxycholecalciferol-like activity in *Solanum malacoxylon*: purification and partial characterization. *FEBS letters*. 1975; 56:16–19. [PubMed: 169151]
- [28]. Gil S, Dallorso M, Horst R. Screening of Vitamin D activity (VDA) of *Solanum glaucophyllum* leaves measured by radioimmunoassay (RIA). *The Journal of steroid biochemistry and molecular biology*. 2007; 103:483–486. [PubMed: 17223548]
- [29]. Goff JP, Koszewski NJ, Haynes JS, Horst RL. Targeted delivery of vitamin D to the colon using beta-glucuronides of vitamin D: therapeutic effects in a murine model of inflammatory bowel disease. *American journal of physiology. Gastrointestinal and liver physiology*. 2012; 302:G460–469. [PubMed: 22114117]
- [30]. Hollis BW, Horst RL. The assessment of circulating 25(OH)D and 1,25(OH)2D: where we are and where we are going. *J Steroid Biochem Mol Biol*. 2007; 103:473–476. [PubMed: 17197173]
- [31]. Hollis BW. Quantitation of 25-hydroxyvitamin D and 1,25-dihydroxyvitamin D by radioimmunoassay using radioiodinated tracers. *Methods Enzymol*. 1997; 282:174–186. [PubMed: 9330287]
- [32]. Das A, Spackman E, Pantin-Jackwood MJ, Suarez DL. Removal of real-time reverse transcription polymerase chain reaction (RT-PCR) inhibitors associated with cloacal swab samples and tissues for improved diagnosis of Avian influenza virus by RT-PCR. *Journal of veterinary diagnostic investigation: official publication of the American Association of Veterinary Laboratory Diagnosticians, Inc*. 2009; 21:771–778.
- [33]. Giulietti A, Overbergh L, Valckx D, Decallonne B, Bouillon R, Mathieu C. An overview of real-time quantitative PCR: applications to quantify cytokine gene expression. *Methods*. 2001; 25:386–401. [PubMed: 11846608]
- [34]. Cheng YH, Goff JP, Sell JL, Dallorso ME, Gil S, Pawlak SE, Horst RL. Utilizing *Solanum glaucophyllum* alone or with phytase to improve phosphorus utilization in broilers. *Poultry science*. 2004; 83:406–413.

- [35]. Koszewski NJ, Horst RL, Goff JP. Importance of apical membrane delivery of 1,25-dihydroxyvitamin D3 to vitamin D-responsive gene expression in the colon. *American journal of physiology. Gastrointestinal and liver physiology*. 2012; 303:G870–878. [PubMed: 22837344]
- [36]. Bachmann H, Offord-Cavin E, Phothirath P, Horcajada MN, Romeis P, Mathis GA. 1,25-Dihydroxyvitamin D(3)-glycoside of herbal origin exhibits delayed release pharmacokinetics when compared to its synthetic counterpart. *The Journal of steroid biochemistry and molecular biology*. 2012
- [37]. Wasserman RH, Henion JD, Haussler MR, McCain TA. Calcinogenic factor in *Solanum malacoxylon*: evidence that it is 1,25-dihydroxyvitamin D3-glycoside. *Science*. 1976; 194:853–855. [PubMed: 982048]
- [38]. Furst A, Labler L, Meier W. Synthesis of Beta-D-Glucopyranosides of Some Hydroxylated Vitamin-D Compounds. *Helv Chim Acta*. 1983; 66:2093–2102.
- [39]. Londowski JM, Kost SB, Meier W, Labler L, Kumar R. Biological activity of the C-1, C-3, C-25, beta-D-glucopyranosides of 1,25-dihydroxyvitamin D3. *The Journal of pharmacology and experimental therapeutics*. 1986; 237:837–840. [PubMed: 3754894]
- [40]. Shimada K, Sugaya K, Kaji H, Nakatani I, Mitamura K, Tsutsumi N. Syntheses and Enzymatic-Hydrolysis of 25-Hydroxyvitamin-D Monoglucuronides. *Chem Pharm Bull*. 1995; 43:1379–1384.
- [41]. Roslund MU, Tahtinen P, Niemitz M, Sjöholm R. Complete assignments of the (1)H and (13)C chemical shifts and J(H,H) coupling constants in NMR spectra of D-glucopyranose and all D-glucopyranosyl-D-glucopyranosides. *Carbohydrate research*. 2008; 343:101–112. [PubMed: 17980865]
- [42]. Boland RL. *Solanum malacoxylon*: a toxic plant which affects animal calcium metabolism. *Biomedical and environmental sciences: BES*. 1988; 1:414–423. [PubMed: 3077267]
- [43]. Esparza MS, Vega M, Boland RL. Synthesis and composition of vitamin D-3 metabolites in *Solanum malacoxylon*. *Biochimica et biophysica acta*. 1982; 719:633–640. [PubMed: 6295509]
- [44]. Skliar MI, Boland RL, Mourino A, Tojo G. Isolation and identification of vitamin D3, 25-hydroxyvitamin D3, 1,25-dihydroxyvitamin D3 and 1,24,25-trihydroxyvitamin D3 in *Solanum malacoxylon* incubated with ruminal fluid. *The Journal of steroid biochemistry and molecular biology*. 1992; 43:677–682. [PubMed: 1335278]
- [45]. Napoli JL, Reeve LE, Eisman JA, Schnoes HK, DeLuca HF. *Solanum glaucophyllum* as source of 1,25-dihydroxyvitamin D3. *The Journal of biological chemistry*. 1977; 252:2580–2583. [PubMed: 856794]
- [46]. Zanuzzi CN, Nishida F, Portiansky EL, Fontana PA, Gimeno EJ, Barbeito CG. Effects of *Solanum glaucophyllum* toxicity on cell proliferation and apoptosis in the small and large intestine of rabbits. *Research in veterinary science*. 2012; 93:336–342. [PubMed: 21862088]
- [47]. von Rosenberg S, Wehr U, Bachmann H. Effect of vitamin D-containing plant extracts on osteoporotic bone. *The Journal of steroid biochemistry and molecular biology*. 2007; 103:596–600. [PubMed: 17368186]
- [48]. Done SH, Tokarina CH, Dammrich K, Dobereiner J. *Solanum malacoxylon* poisoning in pigs. *Research in veterinary science*. 1976; 20:217–219. [PubMed: 1265362]
- [49]. Rucksan BE, Wells GA, Lewis G. *Solanum malacoxylon* toxicity to pigs. *The Veterinary record*. 1978; 103:153–155. [PubMed: 695263]
- [50]. Mathis GA, Toggenburger A, Pokorny R, Autzen S, Ibanez R, Romeis P, Bachmann H. Human pharmacokinetic profile of 1,25-dihydroxyvitamin D-glycoside of herbal origin. *The Journal of steroid biochemistry and molecular biology*. 2013
- [51]. Mautalen CA, Ferraro C, Cabrejas M, Landi E, Gotlieb D. Effects of *Solanum malacoxylon* on calcium metabolism in patients with chronic renal failure. *Calcified tissue research*. 1977; 22(Suppl):534–537. [PubMed: 334348]
- [52]. Boland RL. Plants as a source of vitamin D3 metabolites. *Nutrition reviews*. 1986; 44:1–8. [PubMed: 3005932]
- [53]. Weissenberg M, Maoz A, Levy A, Wasserman RH. Radioimmunoassay for rapid estimation of vitamin D derivatives in calcinogenic plants. *Planta medica*. 1988; 54:63–65. [PubMed: 3375339]

- [54]. Laverny G, Penna G, Vetrano S, Correale C, Nebuloni M, Danese S, Adorini L. Efficacy of a potent and safe vitamin D receptor agonist for the treatment of inflammatory bowel disease. *Immunology letters*. 2010; 131:49–58. [PubMed: 20350569]

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Highlights

- Dose-dependent expression of vitamin D-regulated genes exclusively in the colons of mice fed Solanum leaf.
- No effects on gene expression in the duodenums and ileums of the mice.
- Plasma Ca and 1,25D₃ concentrations remained at control values, except at the highest dose of leaf.
- Isolation and identification of a 1,3-diglycoside of 1,25D₃ as a major isoform from leaf extracts

Figure 1.

Plasma 1,25D₃ and Ca²⁺ concentrations in mice fed *Sg* leaf. Mice (5/group) were fed the indicated amounts (μg) of *Sg* leaf/day for 10 days, after which plasma 1,25D₃ and Ca²⁺ concentrations were determined. a=plasma 1,25D₃ different from control (P<0.05); b=plasma Ca²⁺ different from control (P<0.05).

Figure 2.

Changes in Cyp24a1 gene expression in the intestinal tract. Mice (5/group) were fed different amounts of *Sg* leaf ($\mu\text{g}/\text{day}$) for 10 days. Expression of the Cyp24a1 gene (ddCt) was determined by qPCR from RNA isolated from the colon (A) and duodenum (B). * = different from control ($P < 0.05$). For comparison, one group of mice was treated orally with 24 pmol of 1,25D₃ and then sacrificed 6 hrs later (1,25D).

Figure 3. Cyp24a1 gene expression in ileums of mice. (A) Gel electrophoresis of Cyp24a1 gene expression by RT-PCR in ileums from mice (2/group) treated for 6 h with indicated compounds. (B) Gel electrophoresis of Cyp24a1 gene expression in ileums from mice (2/group) fed Sg leaf for 10 days and sacrificed 6 hours after consumption of final treatment. Arrow denotes unused primer band.

Figure 4.

Spectral analyses of the purified 1,25D₃-glycoside isolated from *Sg* leaves. (A) HPLC trace of the glycoside following its purification from *Sg* leaves. Elution time was at ca. 43 min and absorbance was monitored at 260 nm. (B) Scanning ultraviolet absorbance of the purified glycoside. (C) Low resolution ESI mass spectrum of the glycoside. (D) 500 MHz ¹H NMR of the glycoside obtained in MeOH-d₄.

Figure 5. 2-Dimensional NMR analyses of the purified 1,25D₃-glycoside isolated from *Sg* leaves. (A) HMQC spectral analysis of C-H coupling produced by the purified 1,25D₃-glycoside. (B) ROESY spectral analysis of through-space ¹H-¹H coupling exhibited by the purified 1,25D₃-glycoside. Boxed region highlights C1-C1' sugar and C3-C1' sugar interactions. (C) Proposed structure of the purified 1,25D₃-1,3-diglycoside isolated from *Sg* leaf extract.