Curcumin: a novel nutritionally derived ligand of the vitamin D receptor with implications for colon cancer chemoprevention


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Abstract

The nuclear vitamin D receptor (VDR) mediates the actions of 1,25-dihydroxyvitamin D₃ (1,25D) to regulate gene transcription. Recently, the secondary bile acid, lithocholate (LCA), was recognized as a novel VDR ligand. Using reporter gene and mammalian two-hybrid systems, immunoblotting, competitive ligand displacement and quantitative real-time PCR, we identified curcumin (CM), a turmeric-derived bioactive polyphenol, as a likely additional novel ligand for VDR. CM (10⁻⁵ M) activated transcription of a luciferase plasmid containing the distal vitamin D responsive element (VDRE) from the human CYP3A4 gene at levels comparable to 1,25D (10⁻⁸ M) in transfected human colon cancer cells (Caco-2). While CM also activated transcription via a retinoid X receptor (RXR) responsive element, activation of the glucocorticoid receptor (GR) by CM was negligible. Competition binding assays with radiolabeled 1,25D confirmed that CM binds directly to VDR. In mammalian two-hybrid assays employing transfected Caco-2 cells, CM (10⁻⁵ M) increased the ability of VDR to recruit its heterodimeric partner, RXR, and steroid receptor coactivator-1 (SRC-1). Real-time PCR studies revealed that CM-bound VDR can activate VDR target genes CYP3A4, CYP24, p21 and TRPV6 in Caco-2 cells. Numerous studies have shown chemoprotection by CM against intestinal cancers via a variety of mechanisms. Small intestine and colon are important VDR-expressing tissues where 1,25D has known anticancer properties that may, in part, be elicited by activation of CYP-mediated detoxification and/or up-regulation of the tumor suppressor p21. Our results suggest the novel hypothesis that nutritionally-derived CM facilitates chemoprevention via direct binding to, and activation of, VDR.

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1. Introduction

The active hormonal metabolite of vitamin D, 1α,25-dihydroxyvitamin D₃ (1,25D), has a broad spectrum of biological actions which have been extensively reviewed [1–3]. 1,25D stimulates intestinal calcium and phosphate absorption, bone calcium and phosphate resorption, and renal calcium and phosphate reabsorption. These actions of 1,25D effect calcium and phosphate homeostasis and ensure proper remodeling of the mineralized skeleton. An obligate mediator of 1,25D action is a transcription factor, the vitamin D receptor (VDR), a member of the nuclear and steroid receptor superfamily. Binding of 1,25D elicits conformation changes in VDR which lead to recruitment of its co-receptor, the retinoid X receptor (RXR). A liganded VDR–RXR heterocomplex then binds to short sequences of DNA, termed vitamin D responsive elements (VDREs), which are typically in the vicinity of 1,25D-regulated genes. Once bound to a VDRE, the VDR–RXR duplex induces transcription by recruiting coactivators with histone acetyl transferase activity, such as steroid receptor coactivator-1 (SRC-1), as well as helping assemble other components of the RNA polymerase promoter complex.

In addition to the classic target genes regulated by 1,25D-VDR that mediate bone and mineral homeostasis, of particular interest is the capacity of liganded VDR to regulate cell growth and division. The
ability of 1,25D analogs, such as calcipotriol, to prevent psoriasis [4–6]. Moreover, 1,25D and its analogs exhibit potential in chemoprevention of a variety of cancers, particularly those of the prostate and colon [7,8]. Mechanistically, it has been shown that 1,25D controls cell division by arresting cells in the G1/G0 phase of the cell cycle and by up-regulating powerful tumor suppressor genes such as p21 [9,10]. An important pathway for prevention of colon cancer by VDR may be detoxification of the carcinogenic secondary bile acid, lithocholate (LCA) [11]. LCA activates self-detoxification by inducing transcription of cytochrome P450-3A4 (CYP3A4) via direct binding to VDR. LCA acts as a low-affinity VDR ligand that is able to perform some of the same traditional functions of 1,25D in vitamin D-deficient rats [12]. The establishment of LCA as a bona fide VDR ligand led us to hypothesize that there may be additional novel ligands for VDR which may play a role in cancer chemoprevention. In this study, we evaluate curcumin (CM) as a potential VDR ligand.

Curcumin is a major biologically active component of turmeric, which is abundant in the traditional Indian diet. A number of studies reveal that CM inhibits tumor initiation by suppressing proinflammatory pathways and inducing Phase II conjugating enzymes, such as sulfotransferase and glutathione-S-transferase, that facilitate the excretion of carcinogens (reviewed in Ref. [13]). The conjugated nature of the CM molecule that leads to its ability to act as an antioxidant is another important factor for cancer chemoprevention [14]. CM exhibits particular promise as a therapeutic and preventative agent for gastrointestinal cancer, where it displays a modest bioavailability in the colon following oral administration [15]. Notably, there is a significant overlap among the molecular targets of 1,25D and CM; thus, both molecules prevent TNF-induced degradation of NF-κB, leading to attenuated activity of NF-κB, a well-known cancer promoter [16–18]. CM has also been shown to activate p21 in a p53-independent fashion in breast (MCF-7), prostate (PC-3) and colon (Colo-205) cancer cells [19–21]. The up-regulation of p21 by 1,25D and its analogs has also been documented in MCF-7 and PC-3 cells [22,23].

In the current study, we present the first evidence which supports a novel hypothesis that CM is a nutritionally-derived ligand of VDR. CM is able to bind VDR, induce recruitment of its co-receptor RXR and co-activator SRC-1, and activate transcription of a VDR-target gene, CYP3A4, in colon cancer cells. Moreover, we provide data that up-regulation of p21 by CM may be at least in part mediated by VDR, and we investigate the molecular mechanism that is perhaps responsible for the previously reported synergism between 1,25D and CM in promoting differentiation of HL-60 cells [24].

2. Methods and materials

2.1. Transfection of cultured mammalian cells and transcriptional activation assays

Cells were grown at 37°C under a humidified atmosphere of 5% carbon dioxide. All cell lines in this study originated from the ATCC (Manassas, VA, USA) and were transfected in Costar polystyrene 24-well plates from Corning, Inc. (Corning, NY, USA) using Lipofectamine Transfection Reagent in combination with Plus Reagent, both supplied by Invitrogen (Carlsbad, CA, USA). Human colorectal adenocarcinoma cells (Caco-2) were plated at a density of 40,000 cells/well approximately 24 h prior to transfection in minimum essential medium (MEM), supplemented with 20% fetal bovine serum (FBS), 1 mM sodium pyruvate, 0.1 mM nonessential amino acids, 100 U/ml penicillin and 100 μg/ml streptomycin. The transfection procedure was adapted from the manufacturer's protocol. Briefly, each well received 1 μl of Lipofectamine Reagent, 2 μl of Plus Reagent, 50 ng of pTZ18U carrier DNA plasmid and 20 ng of pRL-null (constitutively expressing low levels of Renilla reniformis luciferase) to monitor transfection efficiency. Each well also received 250 ng of plus-MCS plasmid (Stratagene, La Jolla, CA, USA) containing an oligonucleotide (cloned between the HindIII and BgII sites) with two copies of a nuclear receptor responsive element upstream of the firefly (Photinus pyralis) luciferase gene. The VDR-expressing XDR3, was the distal element from the human cytochrome P450 (CYP) 3A4 gene [25]. The entire sequence inserted into plus-MCS reporter vector was CAGAGCTGGTACACGTGTTACCT.
USA), and 100 μg of protein from each sample was run on a 5–15% gradient SDS-polyacrylamide gel, followed by electrotransfer to Immobilon P membrane (Millipore Corp., Bedford, MA, USA). The transfer was performed using a Transblot apparatus in 25 mM Tris-HEC, pH 7.4, 192 mM NaCl, 0.01% SDS and 20% methanol. The membrane was then blocked by incubation for 1 h with 3% dry milk, 10 mM Tris-HEC, pH 7.5 and 150 mM NaCl, followed by treatment for 3 h at room temperature with a 1:10,000 dilution of the anti-VDR monoclonal antibody, 9A7γ [30], or a 1:500 dilution of anti-CYP24 polyclonal antibody (H-87; Santa Cruz Biotechnology, Santa Cruz, CA, USA). A horseradish peroxidase conjugated anti-rat IgG (against 9A7γ) or anti-rabbit IgG (against H-87) was incubated with the membrane overnight at 4°C followed by washes and visualization using the Enhanced Chemiluminescence Detection System (Amer sham, Piscataway, NJ, USA).

2.5. Real-time PCR

Real-time PCR was performed on the human CYP3A4 gene using 5'-CCGATGTA-GAGATCTGGTTGAGC-3' and 5'-TCTTGGATGGTTGAGACAGTCG-3' primers (153 bp product); the human CYP24 gene using 5'-CAGCGACCTGACAAATGTGTCG-3' and 5'-TCTCCTCCTCATACACACCCAGGCG-3' primers (58 bp product); the human p21 gene using 5'-AGGAAGCATTGACCTGTCTAC-3' and 5'-GGCGGGTGTGGCTAGATCC-3' primers (147 bp product); the human TRPV6 gene using 5'-CCTCATACGACGAGGCTCTAAC-3' and 5'-TCTCAACCGAGCAGTTGAG-3' primers (156 bp product); and the rat VDR gene using 5'-CCAAAAGTGCTTCTACGCGGCGG-3' and 5'-TTGTCCTCGTGGCCGAAATTCTC-3' primers (80 bp product). Total RNA was isolated from 2×10^5 Caco-2 or ROS 17/2.8 cells (treated with ligands for 4 or 24 h in serum-free medium) using an Aurum Total RNA Mini Kit (Bio-Rad, Hercules, CA, USA). The RNA obtained was quantified using A260/A280 spectrophotometry. DNase-treated RNA (2 μg) was reverse transcribed using the Script cDNA Synthesis Kit (Bio-Rad). The obtained cDNA was used in 20-μl PCR reactions containing 10 μl iQ SYBR Green Supermix (Bio-Rad), 1 μl primers, 2 μl of cDNA template sample and 7 μl of molecular-grade water. Reactions were performed in 96-well PCR plates and read on a Bio-Rad iCycler iQ Real-Time PCR detection system or an ABI 7500 Fast instrument. Data were analyzed using the comparative Ct method as a means of relative quantitation, normalized to an endogenous reference (GAPDH cDNA) and relative to a calibrator (normalized Ct value obtained from vehicle-treated cells) and expressed as 2^-ΔΔCt according to Applied Biosystems’ User Bulletin 2: Rev B, “Relative Quantitation of Gene Expression.”

2.6. Epithelial cell migration assay

Cell migration assays were performed essentially as described previously [31]. Caco-2 cells were grown to 90% confluency in MEM, supplemented with 20% FBS, 1 mM sodium pyruvate, 0.1 mM nonessential amino acids, 100 U/ml penicillin and 100 μg/ml streptomycin in six-well plates. Cells were starved in serum-free media for 24 h, and a line was drawn on each plate. Detached cells were removed by gently washing in PBS. The cells were then incubated in MEM containing 1% FBS in the presence or absence of 10^{-5} M 1,25D or a range of CM concentrations (10^{-6} to 10^{-2} M) for 48 h. Cell migration across the lesion line was assessed under a microscope and recorded by color photography using Kodak UltraMAX 400 film.

3. Results

3.1. Curcumin activates VDR and stimulates VDRE-mediated transcription

We hypothesized that VDR may be one of the direct mediators of CM bioactivities. Several experiments were performed to test this hypothesis, including the use of transcription assays employing a VDRE-firefly luciferase reporter plasmid, mammalian two-hybrid assays, competition binding assays to evaluate the direct association of CM and VDR in vitro, and quantitative real-time PCR assessment of CM-mediated induction of VDR target genes, as well as a Caco-2 cell migration assay.

Fig. 1 shows the pooled results of three independent experiments in which Caco-2 human colon cancer cells were transfected with a reporter plasmid and treated with CM and other known VDR ligands. Treatment with 10^{-8} M 1,25D and 10^{-8} M LCA for 30 h in complete medium, including serum, boosted transcription of the reporter plasmid 4.2- and 3.3-fold, respectively. Cells treated with 6.7×10^{-6} M and 10^{-5} M CM also demonstrated a dose-dependent increase (2.1- and 5.0-fold, respectively) in the level of transcription of the VDRE-reporter plasmid.

![Figure 1](image-url)
ability of CM to induce transcription of reporter constructs containing a VDRE, RXRE or GRE was evaluated. Caco-2 cells were transfected with the indicated reporter constructs (described in the Methods and Materials) to compare the amount of transcription induced by CM in the same experiment. The results shown in Fig. 3 demonstrate that $10^{-5}$ M CM is 95% as efficient as $10^{-8}$ M 1,25D in inducing transcription of the reporter plasmid containing two distal VDREs from the human CYP3A4 gene. When cells transfected with the same reporter vector are treated with a mixture containing $10^{-5}$ M CM and $10^{-8}$ M LG101305 (Rex), a synthetic ligand for RXR, the level of transcription observed is 2.16-fold greater than in cells treated with $10^{-8}$ M 1,25D alone, and this difference is statistically significant ($P < 0.05$). Curcumin had a similar effect on the transcription of a reporter plasmid containing the RXRE (Fig. 3B). CM at $10^{-5}$ M was 89% as potent as $10^{-7}$ M LG101305 (Rex), a synthetic ligand for RXR, in stimulating the transcription of the reporter gene. When cells containing the same reporter plasmid were treated with both CM and Rex, 2.44 times more transcription was observed than in cells treated with Rex alone.

In contrast to the effect of CM on vitamin D- and retinoid X-responsive element reporter constructs, CM did not appear to significantly activate transcription of a reporter plasmid containing a GRE. Despite the observation that $10^{-5}$ M CM treatment displayed a modest-fold increase in transcriptional activity compared to the vehicle control, this increase was only 3% of the reporter transcription elicited by treatment with $10^{-8}$ M dexamethasone (Dex), a high affinity ligand of glucocorticoid receptor (Fig. 3C). In addition,
treatment with both CM and Dex produced less transcription than treatment with Dex alone. Thus, CM displays at least some level of nuclear receptor specificity when tested under the conditions of these assays.

3.4. Mechanism of CM-mediated potentiation of 1,25D action

In order to investigate the molecular basis for the large increase in the level of VDR-mediated transcription by the combined treatment of cells with CM and 1,25D, two routes were pursued. Employing real-time PCR, we explored the potential of 24-h treatment with 5 × 10^{-5} M CM to up-regulate expression of VDR mRNA in osteosarcoma (ROS 17/2.8) and Caco-2 cells. Increased levels of VDR mRNA could lead to a higher receptor concentration and more robust transcriptional initiation. However, the graph depicted in Fig. 4A indicates that CM alone did not increase ROS 17/2.8 VDR mRNA content, while dual treatment with 1,25D and CM was slightly less effective than 1,25D alone. Similar results were obtained with ligand treatment for 4 h in ROS 17/2.8 cells or for 4 or 24 h in Caco-2 cells (data not shown). Thus, CM is unlikely to potentiate the action of 1,25D-VDR by increasing the level of receptor mRNA.

An alternative mechanism for CM potentiation of VDR action is at the protein level (for example, by protecting VDR from degradation), rather than at the transcriptional level. We therefore performed Western blots to investigate directly the effect of CM on VDR content in ROS 17/2.8 cells. This cell line was used instead of Caco-2 because of abundant endogenous VDR levels that can be more readily detected by Western blotting [32]. Based on a representative experiment shown in Fig. 4B, 24-h treatment with CM (2 × 10^{-5} M) did not increase levels of VDR compared to the vehicle control. The cells treated with both 1,25D and CM had, in fact, a lower VDR content than cells treated with 1,25D alone. Thus, effects of CM on VDR mRNA or protein cannot explain the marked increase in transcriptional activity induced by the dual ligand treatment with 1,25D and CM observed in Fig. 3A.

3.5. Curcumin can compete with 1,25D for direct binding to VDR

To obviate the possibility that CM is not a VDR ligand, but rather affects VDRE-mediated transcription indirectly, perhaps through direct binding to only RXR, in vitro competition binding assays were performed [see Methods and Materials]. As illustrated in Fig. 5, which contains data from a single representative experiment from three independent tests, CM successfully competed with radiolabeled 1α,25-dihydroxy[26,27-methyl-^{3}H]cholecalciferol for binding to VDR in a transfected cell lysate containing both human VDR and RXR proteins. At 10^{-6} M, the CM ligand effectively competed more than 50% of the amount of radiolabeled 1,25D bound to VDR, compared to negligible competition by Dex, a lipophilic ligand without appreciable binding affinity for VDR. LCA, a recognized novel ligand of VDR, was included for comparison with CM and was only slightly more effective than CM in competing with 1α,25-dihydroxy[26,27-methyl-^{3}H]cholecalciferol for VDR binding [inhibition constant (K_i) = 2.1 and 2.9 µM, respectively, assuming a K_d for 1,25D-VDR = 10^{-10} M]. Overall, these findings not only indicate that CM directly binds to VDR, but also strongly suggest that CM activates transcription via a VDRE by recruiting co-receptor RXR and coactivator SRC-1.

3.6. Curcumin activates 1,25D target genes as assessed by RT-PCR

If CM is truly a biologically relevant VDR ligand, it must activate transcription of VDR target genes in their natural chromatin context. To test this hypothesis, we employed quantitative real-time PCR to measure the effect of CM treatment in Caco-2 cells on the mRNA levels of CYP3A4, CYP24, p21 and TRPV6, genes that are known to be regulated by 1,25D-VDR [1,2,25]. To test whether up-regulation of these genes is by a direct effect of CM on VDR or elicited by some other mechanism, the measurements were performed in cells containing

Fig. 4. Evaluation of VDR mRNA and protein levels in 1,25D- and CM-treated cells. (A) Real-time PCR. Rat osteoblast-like osteosarcoma cells (ROS 17/2.8) were treated with 10^{-7} M 1,25D, 5 × 10^{-5} M CM, or a combination of both compounds for 24 h in order to evaluate the potential regulation of VDR mRNA. Relative levels of VDR mRNA were measured using quantitative real-time PCR as described in Methods and Materials. (B) Western blots. ROS 17/2.8 cells were treated as in (A), followed by preparation of cell lysates in a solution of 2% SDS, 0.125 M Tris-HCl, pH 6.8 and 20% glycerol. The protein content of the lysates was determined using a BCA assay, and 100 µg of total protein from each sample was run on a 5–15% gradient SDS-polyacrylamide gel, followed by Western blotting with an antibody (9A7γ) directed against VDR (see Methods and Materials). This result is representative of three independent experiments.

Fig. 5. Ability of CM to compete with 1,25D for binding to VDR. Competition curves display the concentration range in which CM is able to compete for binding to VDR with 4.0 × 10^{-10} M [^{3}H]1,25D. Dexamethasone is a CR ligand and has no appreciable binding to VDR, thus serving as a noncompeting negative control. Lithocholic acid (LCA) was included as a positive (competing) control. This plot was generated in Prism4 (GraphPad Software, Inc.) and is representative of three independent experiments.
3.6. Curcumin stimulates Caco-2 cell migration in vitro

Intestinal epithelial repair is thought to be driven, in part, by 1,25D-activated, VDR-dependent pathways [31]. Thus, another readout of VDR activation in the context of a cellular assay employing Caco-2 cells is the ability of a putative VDR ligand to stimulate cell migration in a cell culture “scratch” assay. Migration is measured after cells are starved for 24 h in serum-free medium, then wounded by scratching, followed by growth in 1% serum-containing medium for 24–48 h. Treatment with 10⁻⁷ M 1,25D stimulated migration in a dose-dependent fashion. Table 1 summarizes the results obtained from four independent experiments expressed as fold induction of the mRNAs by 4-h treatment (in serum-free medium) with 10⁻⁷ M 1,25D or 5×10⁻⁵ M CM as compared to the vehicle control. CYP3A4 was up-regulated 1.6-fold in the presence of 1,25D, p21 was only induced 1.2-fold and TRPV6 was stimulated 2.4-fold under the same conditions. Providing exogenous VDR via cotransfection yielded a more potent induction of CYP3A4, p21 and TRPV6 mRNAs by 1,25D (3.1-, 2.5- and 3.3-fold, respectively). In untransfected cells, CM had a 1.4- and 2.2-fold effect on CYP3A4 and TRPV6, respectively. However, increased VDR content boosted its ability to activate these genes to new levels of 3.0- and 4.1-fold, respectively. Induction of p21 by CM is strong in the presence of endogenous VDR (7.9-fold), while CM treatment in the presence of additional co-transfected VDR leads to an even greater 11.6-fold increase in p21 mRNA. Taken together, these data indicate that CM treatment not only leads to up-regulation of these target genes, but also that this up-regulation is VDR dependent.

Given the VDR-augmented increase in mRNA from vitamin D target genes by treatment of cells with 1,25D or CM, we next sought to determine whether a classic vitamin D-regulated gene such as CYP24 could be induced by CM in a dose-dependent fashion. Fig. 6A illustrates an actual amplification plot from a real-time PCR experiment using Caco-2 cells that were treated with increasing amounts of CM. When these data are analyzed using the comparative Ct method as means of relative quantitation, and normalized to an endogenous reference (GAPDH), the results demonstrate a classic CM dose-dependent enhancement of both CYP24 (Fig. 6B) and p21 expression (data not shown), with the highest level of CM statistically comparable to 10⁻⁷ M 1,25D treatment (Fig. 6B, black bar). The CM dose-dependent transactivation effect is nearly perfectly reflected at the protein level as shown in the CYP24 immunoblot of extracts of Caco-2 cells (Fig. 6C) treated under the same conditions as in Fig. 6B. These results provide additional corroborative evidence that CM is a bona fide VDR ligand that can induce traditional vitamin D target genes at both the RNA and protein level.

Table 1

<table>
<thead>
<tr>
<th>Gene</th>
<th>Ligand</th>
<th>Average (±S.D.) relative fold increase in target gene mRNA (n=3)</th>
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<td></td>
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<td>Endogenous VDR</td>
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<tr>
<td>CYP3A4 (Xenobiotic detoxification)</td>
<td>+D (10⁻⁷ M)</td>
<td>1.55±0.29</td>
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<tr>
<td>p21 (Cell differentiation and division)</td>
<td>+CM (5×10⁻⁵ M)</td>
<td>1.41±0.19</td>
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<tr>
<td>TRPV6 (Intestinal calcium transport)</td>
<td>+D (10⁻⁷ M)</td>
<td>1.22±0.09</td>
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<td></td>
<td>+CM (5×10⁻⁵ M)</td>
<td>7.91±1.67</td>
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Fig. 6. Curcumin regulates CYP24 (25-hydroxyvitamin D 24-hydroxylase) in human Caco-2 cells in a dose-dependent fashion. (A) Results of a real-time PCR experiment using human CYP24 primers and cDNA prepared from Caco-2 cells incubated for 4 h with the indicated concentration of CM or ethanol vehicle. Results are plotted as ΔRn vs. cycle number, where Rn is the reporter dye signal normalized to the passive reference dye, and ΔRn is Rn from which the baseline dye signal has been subtracted. (B) Induction of CYP24 mRNA by the indicated ligands as compared to the ethanol control. Data were obtained using Ct values from real-time PCR as described in (A); error bars represent triplicate determinations±S.D. (C) Western blotting of CYP24 protein detected in cell lysates from Caco-2 cells incubated with the indicated ligand.
Cell migration was assessed under an inverted phase contrast microscope (magnification ×40). Where CM has shown promise in cancer chemoprevention, CM was administered intraperitoneally to mice, the concentration of CM in the intestine is greater levels of CM in colonic tissue leading to plasma concentrations greater than 10−6 M [33], and even greater levels of CM in colonic tissue [15]. When CM is administered intraperitoneally to mice, the concentration of CM in the intestine is about 200 times greater than in plasma [34]. Thus, in the intestinal tract, where CM has shown promise in cancer chemoprevention, CM concentrations may be sufficient to activate VDR, especially in individuals consuming a diet high in turmeric.

Current data suggest that CM is poorly absorbed from the GI tract and is also rapidly metabolized by glucuronidation [34]. Some human [35] and animal [36] trials have attempted to boost the bioavailability of CM by administering it along with piperine, which is known to inhibit glucuronidation of xenobiotics. Co-administration of piperine was shown to increase serum concentrations of CM as well as lengthen the half-life of CM in the body [35,36]. Also, absorption of CM in a topical skin application was enhanced by the use of a gel containing either eugenol or terpeniol [37]. These studies suggest that there may be mechanisms for enhancing the bioavailability of CM by either improving its uptake or by inhibiting its metabolism.

CM is also metabolized in mammals by sulfation [38] and reduction to tetra-, hexa- and octahydro derivatives [39]. The rapid and consistent metabolism of CM to other forms raises the possibility that a metabolite of CM may, in fact, be the biologically most active ligand for VDR. In such a case, using an agent to increase CM bioavailability might not be advisable if the action of that agent is to suppress CM metabolism.

Cancer chemoprevention by CM may be attributed, at least in part, to activation of tumor-suppressor genes like p21. Since CM has a wide range of molecular targets (reviewed in Ref. [40]), p21 induction could be caused by a secondary effect on the p21 promoter. Recently, C/EBPβ (a member of the CCAAT/enhancer binding protein family of transcription factors) has been shown to induce p21 in a p53-independent manner in the presence of antioxidants, such as vitamin E [41]. It has been reported that CM simultaneously induces C/EBPβ and p21 in a dose-dependent fashion, suggesting that C/EBPβ may be one of the mediators of the effect of CM on p21 expression [20]. However, our results demonstrate that overexpression of VDR leads to a greater up-regulation of p21 by CM. Thus it is possible that CM can stimulate p21 transcription not only via a secondary mechanism, but also directly through a VDRE in the p21 promoter by binding to VDR. Additionally, there is evidence that 1,25D-VDR induces C/EBPβ, hinting at another potential connection between VDR, 1,25D, CM and p21 [42].

Yet another novel mechanism by which CM might exert antitumor effects is via up-regulation of intestinal TRPV6, as shown in the current study. TRPV6 is a major calcium transporter in the small intestine [43], where its role in vitamin D-stimulated calcium transport has been well demonstrated [44]. Several studies have indicated that high dietary calcium protects against risk for colon cancer [45,46]. If CM is, in fact, a VDR ligand that up-regulates TRPV6 in vivo, then it is conceivable that CM may perhaps play a role similar to that of 1,25D in promoting calcium uptake as part of the protective effect against colon cancer.

Vitamin D and its analogs continue to be evaluated as colon cancer chemopreventive and treatment agents [47]. Of particular interest is identification of compounds that can be employed in combination with vitamin D analogs, allowing the application of lower doses to minimize undesired toxicity and side effects such as hypercalcemia, and providing a greater treatment efficiency. CM has been shown to act synergistically with 1,25D to elicit differentiation of human promyelocytic leukemia HL-60 cells [24]. In the current study, cells treated with 1,25D and CM had a higher level of transcription of a transfected VDRE-reporter construct than cells treated with 1,25D alone (Fig. 3A). Additive stimulation of the receptor is unlikely to explain this, because most of the ligand binding sites of VDR may be occupied by the high affinity 1,25D ligand. There are several other potential molecular mechanisms for this phenomenon. One of these is that our data have shown is the possible ability of CM to up-regulate VDR mRNA and VDR protein content since CM increased neither VDR mRNA nor protein levels. Another possibility is that CM could potentiate VDR transactivation via its demonstrated ability to bind
to RXR. Finally, the observation that CM evidently configures VDR as a dietary sensor of CM and perhaps other nutritionally derived beneficial lipids to effect tissue-specific chemoprevention, further challenging the notion that nuclear receptors solely bind to and mediate the activity of high affinity endocrine ligands.

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References


