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Review

Natural Epigenetic Modulators of Vitamin D Receptor

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Featured Application: The article describes three natural and dietary agents that are epigenetic modulators of VDR, reporting the evidences of their activity as enhancers of VDR expression. The aim is to stimulate the search for other natural compounds active on VDR as epigenetic modulators, and to suggest that such dietary supplements could be exploited to revert the resistance to vitamin D in many diseases.

Abstract: Vitamin D plays an important role in every tissue due to its differentiating properties and the control of calcium homeostasis. The reversion of the epigenetic repression of the vitamin D receptor (VDR) could lead to an increased sensitivity of the cells to the beneficial activity of the hormone and could be exploited in many vitamin D-resistant diseases. In this study we analyzed the effects of three natural epigenetic modulators: sulforaphane, curcumin, and the products of the fermentative activity of probiotics. Sulforaphane and curcumin are inhibitors of the DNA methyltransferases (DNMT) and of the histone deacetylases (HDAC); it has been demonstrated that sulforaphane and curcumin increase VDR expression in intestinal epithelial cells and in a human liver cancer cell line, respectively. The anti-inflammatory properties associated with the probiotic administration in vivo can be linked to the increased activity of intestinal VDR. Butyrate, an inhibitor of HDAC and a known modulator of VDR expression, is the candidate byproduct of fermentation by gut microbiome that could mediate the enhanced expression of VDR triggered by probiotics in vivo. Many other natural compounds wait to be investigated and recognized as epigenetic modulators of VDR, thus opening promising therapeutic avenues for many diseases by natural means.

Keywords: vitamin D receptor; sulforaphane; curcumin; probiotics; butyrate; epigenetic modulation; nutraceuticals

1. Introduction

The scientific community has shown an ever growing interest towards natural epigenetic modulators; among them, natural molecules as curcumin, sulforaphane, resveratrol, genistein, and polyphenols have proved to be able to act as epigenetic modulators, influencing the activity of chromatin modifiers and transcriptional factors [1]. The effects of these molecules have been exploited for therapeutic purposes and these compounds have been also employed to increase the sensitivity of cancer cells to conventional chemotherapeutic agents [2]. The recently coined term "nutriepigenomics" is used to describe the study of the interactions between dietary compounds and gene expression modulated by epigenetics and it represents a research field that has been developing for the past decades with the advent of genome-wide expression profiling. These natural molecules that are introduced as part of the diet providing extra health benefits are therefore called "nutraceuticals". In the last years

numerous experiments and clinical trials had proven the efficacy of these compounds in the treatment of different pathologic conditions. In particular, several trials confirmed the beneficial anti-inflammatory and anti-oxidant properties of curcumin in the treatment of osteoarthritis, rheumatoid arthritis, and vascular endothelial function [3–5]. Some promising results of curcumin have been obtained also in oncology [6–8] in terms of safety and anti-cancer effects of curcumin as a complementary therapy in combination with gemcitabine in pancreatic cancer patients [9]. Moreover, curcumin has been found effective also on diabetes [10] since it reduces diabetes complications through decreasing triglyceride levels as well as indicators of inflammation; supplementation with curcumin improves also liver fat and transaminase levels in patients with non-alcoholic fatty liver disease (NAFLD) [11] and it is active in improving memory and attention [12] and in preventing cognitive decline in the elderly [13]. Clinical trials have demonstrated that sulforaphane has anti-oxidant [14,15] and tumor-protective [16,17] properties. Moreover, dietary supplementation of sulforaphane ameliorates liver function and insulin resistance in diabetes II patients [18,19]. Because of their high efficacy and safety, at the moment there are 64 ongoing clinical trials on curcumin and 19 on sulforaphane (https://clinicaltrials.gov/).

This review is aimed at illustrating the ability of three nutraceuticals to modulate the expression of vitamin D receptor and their beneficial effects on health due to the enhanced ubiquitous actions exerted by vitamin D; this collection of initial observations is meant to stimulate the search for other natural compounds with similar activity.

2. Epigenetic Modulation

Epigenetics can be identified as the ensemble of the genome modifications that are hereditable, reversible and that do not involve the alteration of the nucleotide sequence [20]. Epigenetic modifications affect gene expression and protein level, so that they are involved in normal cell growth and development, cell commitment, and differentiation [21]. The tridimensional organization of chromatin is a crucial epigenetic factor in the regulation of gene expression. Chromatin is essentially organized in nucleosomes; each nucleosome consists of DNA wrapped around an octamer of globular proteins named histones (H), more precisely one H3-H4 tetramer and two H2A-H2B-dimers. Chromatin is a complex and extremely dynamic structure that can be found either in a highly condensed inactive state, heterochromatin, in which genes are switched off and cannot be expressed, or in a less condensed state known as euchromatin, which is more accessible to transcriptional factors due to its open conformation that allows gene expression [22]. The main epigenetic modifications involve DNA methylation, histone modifications and microRNA regulation and expression (reviewed in [21–26]). These epigenetic mechanisms are indeed able to modify the chromatin organization by inducing or preventing gene expression [27]. Since DNA methylation has been found mostly in promoter regions of silenced genes, it has been associated with transcriptional repression, whereas CpG islands (sites of transcription initiation) are usually unmethylated in tissue-specific and in “housekeeping” genes, which are constitutively expressed in all tissue. The enzymes involved in the methylation process are DNA methyltransferases (DNMT) [28]. Post-translational histone modifications are involved in chromatin remodeling. The most well-known modifications are methylation and acetylation of lysine residues on histone H3 and H4 and acetylation of histone H2A and H2B (reviewed in [24]). Histone acetyltransferases (HATs) and histone deacetylases (HDACs) catalyze the acetylation and the deacetylation of histone tails residues, respectively. Active regions of chromatin are therefore characterized by high level of unmethylated DNA and acetylated histones; these reversible modifications ensure the expression of specific sets of genes depending on the developmental, metabolic, and environmental state of the cell [29–31].

Many epigenetic abnormalities are associated with the development of a great range of diseases, including neurological, autoimmune, cardiovascular, infectious, aging, and cancer. Cancer can be defined as a multistep process characterized by the accumulation of both genetic and epigenetic errors that transform a healthy cell into a tumor cell [27]. The global DNA methylation and histone

acetylation profiles of cancer cells show a targeted perturbation, characterized by a region-specific pattern, leading to a limited differentiation. The global hypomethylation occurs in gene bodies and within repetitive sequences whereas a specific hypermethylation can be found in the promoter region of tumor suppressor genes [32,33]. The epigenetic changes, when not consequent to a mutation on an epigenetic modulator, are dynamic, transient, and reversible. Therefore, epigenetic modulators have been considered new potential target for cancer treatment and an increasing number of small molecules acting upon epigenetic regulators have been approved by FDA in the last years [34–36].

3. Vitamin D and Its Receptor VDR

The active form of vitamin D3 is the 1α,25-dihydroxyvitamin D3, or calcitriol (also addressed as vitamin D in this review), which is obtained after the double hydroxylation of the precursor on C-25 and on C-1; these reactions take place in the liver and in the kidney, respectively, and are catalyzed by cytochrome P-450 enzymes (CYP). Kidney, as well as many tissues, is the site of both the final activation step and the degradation of calcitriol to the inactive 24,25-dihydroxyvitamin D and 1,24,25-trihydroxyvitamin D metabolites. Vitamin D acts through the binding to the vitamin D receptor (VDR) that drives the expression of VDR responsive genes. In addition to the tissues involved in calcium and phosphate homeostasis, the entire organism is affected by vitamin D activity (extensively reviewed in [37]). VDR is mostly expressed in colon and skin epithelial cells; in colon cells vitamin D-VDR induces the expression of transcription factors, immunomodulators, anti-inflammatory, and anti-oxidant effectors [37]. Vitamin D controls the expression of hundreds of different genes: At the same time it activates some key genes and represses others, inhibiting growth and promoting cell differentiation. VDR is a trans-acting transcription factor, a member of the nuclear receptor family. It is encoded by VDR gene which shares a high sequence similarity with steroid and thyroid hormone receptors. Vitamin D responsive genes contain multiple VDRE sites (vitamin D responsive elements) and usually just one of them is in the proximal promoter region, whereas the others are dispersed up to 100 kb downstream and upstream from the transcription initiation site. The individual VDREs seem to function synergistically in attracting co-modulators; all the docking sites need to be occupied in order to have the maximal induction of response. These distant regions are juxtaposed via chromatin looping, creating a single platform where the transcription machinery assembles (reviewed in [38,39]).

Genes responsive to vitamin D-VDR can be divided into several groups according to their biological function: They can be involved in bone metabolism, anabolism and resorption, mineral homeostasis, cell life (including proliferation, differentiation, migration, and death), immune modulation, and metabolism. For further details on the transcriptional activity of vitamin D, the reader is referred to very comprehensive reviews [37,40]. The transcription of these genes depends on the presence of the hormone, which binds VDR inducing a critical conformational change in the C terminal region from the closed conformation to the open one; the latter allows the binding to co-activators that stabilize VDR-RXR (retinoid X receptor) complex and the docking to VDREs. However, the chromatin condensation changes from tissue to tissue, so that in different tissues various VDREs can be bound by VDR-RXR active complex; the ensemble of all the genomic cis targets open to VDR docking is called VDR cistrome. ChIP-sequencing datasets analysis identified more than 23,000 unique VDR binding sites, 75% of which represent a cell type specific binding profile [41]. The ligand binding increases DNA docking of VDR by a factor of 2.5 [42]. These data demonstrate that the VDR cistrome can be modulated by the binding of calcitriol or other molecules. The expression of VDR and the presence of vitamin D are necessary but not sufficient to trigger the transcription of VDR target genes. In fact also the level of chromatin accessibility in the VDRE regions impacts on the transcription of vitamin D induced genes; if VDREs are within a hypermethylated region of heterochromatin they are not accessible and thus cannot be bound by the active form of VDR-RXR. This highly specific epigenetic regulation of VDR cistrome is one of the main principles that ensure tissue-specific gene expression. Basically, the transcriptional control of vitamin D responsive genes is exerted by three

components: Vitamin D availability, VDR expression levels, and VDREs accessibility; all steps are exquisitely modulated in physiological conditions.

VDR expression is shaped by environmental (i), genetic (ii), and epigenetic (iii) modulators according to Saccone and colleagues [43]. The main modulators are outlined in Supplementary Table S1. The environmental factors involved are diet, sun exposure, age, pollution, and infection, and the majority of these determinants act by modulating the plasmatic amount of vitamin D. Vitamin D affects VDR expression in different manners. The hormone is able to autoregulate the expression of its receptor through a positive feedback loop, since VDR is a target gene of the vitamin D-VDR-RXR complex; moreover the interaction with the ligand stabilizes VDR increasing its half-life [44].

As for the genetic modulation, the overall organization of VDR gene highlights the complex and meticulous genetic regulation that characterizes VDR expression in different tissues and under different conditions. VDR comprises many coding and non-coding regions, four distinct tissue-specific promoters, and four enhancer elements within and upstream VDR gene. The Genomatix database reports ten different experimentally verified and two annotated VDR transcripts, all expressed in a wide range of diverse tissues [43]. mRNA transcription and alternative splicing is tissue and disease-specific as demonstrated in multiple studies. Two genome-wide association studies identified the presence of polymorphisms and SNPs (single nucleotide polymorphisms) in VDR that seem to have a crucial effect on VDR expression and function [43]. Given the importance of VDR activity in all tissues, several studies have suggested a correlation between VDR polymorphisms and differential disease susceptibility, for example in osteoporosis, infectious diseases and diabetes (reviewed in [43]).

4. Vitamin D Resistance

The impact of a defective VDR, either as expression or activity, is crucial for the biological effects of vitamin D. Indeed, in many pathological conditions vitamin D resistance occurs, due to three distinct possible defects: (i) Lack of VDR expression, (ii) calcitriol deficiency or increased vitamin D catabolism, or (iii) repression of transcriptional activity of vitamin D-VDR-RXR complex. Vitamin D resistance can be inherited or de novo acquired; in the former case is caused by the mutation of VDR or RXR genes or the genes involved in calcitriol activation, catabolism, and signaling, in the latter case the resistance can be environment-dependent. Concerning the inherited resistance, most of the mutations observed where discovered either in VDR or RXR domains and are considered the main cause of the recessive form of rickets (type I) [45].

The prevalent mechanism of vitamin D acquired resistance present in pathological conditions, especially in cancer, involves the transcriptional repression of VDR. Although VDR is expressed in most cell types, it was shown that its expression is progressively reduced during the progression of different cancer types, such as skin, breast, and prostate cancer [46–48]. A decrease in the expression of VDR is associated with a late stage disease and a poor prognosis in urothelial bladder cancer, while a high VDR expression correlates with better survival in non-small cell lung cancer patients [46,49]. An impairment of VDR expression can be explained as a strategy used by tumor cells to counteract the tumor-suppressive effects of vitamin D. A first molecular mechanism that allows tumor cells to inhibit VDR expression consists in the over expression of the Snail family of transcriptional repressors. Indeed, in breast and colon cancer cells Snail1 and -2 can bind DNA near the promoter regions of VDR gene recruiting co-repressors that inhibit VDR transcription [50]. Furthermore, it was shown that the expression of K-Ras and H-Ras mutants, respectively in human and mouse colon cancer cells, suppresses VDR transcription [51]. The repression of VDR due to epigenetic mechanisms is discussed in the next section.

Another very common cause of resistance is the increase in vitamin D catabolism due to the up-regulation of the enzyme CYP24A1. In breast cancer CYP24A1 has been proposed as an oncogene because it was found amplified and it was correlated with poor prognosis and a decreased survival rate [52]. Colon, prostate, lung, ovarian, cervical, and many other types of cancer have shown an

up-regulation of this catabolic enzyme that positively correlates with advanced cancer stages and is associated with a poor outcome [53–55].

Cancer is not the only pathological condition characterized by the acquisition of vitamin D resistant phenotype. From infectious diseases such as tuberculosis or HIV (human immunodeficiency virus), to autoimmune disorders as systemic lupus erythematosus, rheumatoid arthritis, or Crohn's disease, many immunopathologies are often associated with a lack of VDR expression after a long activation of T-helper cells or in the presence of VDR polymorphisms [56]. Since vitamin D resistance plays a primary role in multiple pathological conditions, a great effort has been put into the research of possible treatments. One of the most promising approaches seems to be based on the epigenetic modulation.

5. Epigenetic Modulation of Vitamin D Effects

Several molecules can potentially influence the effects of vitamin D through epigenetic mechanisms. The epigenetic modulation acts principally on three levels: (i) VDR expression, (ii) modulation of VDR cistrome, and (iii) chromatin modification triggered by vitamin D-VDR activity.

5.1. VDR Expression Regulated by Epigenetics

Concerning methylation, VDR seems to follow the classical model, according to which normal gene expression is linked with promoter and enhancers hypomethylation and gene body hypermethylation. However, according to Smirnoff and colleagues, VDR expression is regulated by a non-classical methylation mechanism too. Indeed, the treatment of rat colonic mucosa with the established carcinogen DMH (dimethylhydrazinedihydrochloride) resulted in a gene-body hypermethylation and a consequent decreased expression of VDR, in conflict with the classical model [57].

In many pathological conditions, especially cancer, VDR expression is altered due to epigenetic aberration. Breast cancer cell lines treated with the DNMT inhibitor 5'deoxy-azacytidine showed a reduced aberrant VDR promoter hypermethylation, an enhanced responsiveness to vitamin D and an increased expression of target genes, supporting the classic model [58]. Besides cancer, infectious diseases as HIV and tuberculosis are characterized by an aberrant VDR methylation profile too. For instance, HIV infected T cells show an increase of 2.5 fold in VDR gene body methylation compared to controls [59].

Another mechanism involved in VDR transcriptional suppression in colon and breast cancer is the epigenetic silencing mediated by the methylation of CpG islands (CGI) within the VDR promoter region [58]. According to predictive model of CGIs made by Bock and colleagues, VDR contains three bona fide CGIs at promoter regions (CGI 1065, 1062, and 1060); in particular the presence of TaqI polymorphism influences the last CGI 1060 [60]. A change in methylation at 1060 CGI site results in a differential transcription of a long non-coding RNA present in this region that may potentially regulate VDR expression post-transcriptionally [43].

Although the impact of specific histone modifications on VDR transcription is not characterized in detail, it is quite clear that histone acetylation induced by vitamin D gives an important contribution to the regulation of VDR expression [61]. In fact, the treatment of osteosarcoma cells with vitamin D was reported to induce a strong increase in H4 acetylation in VDR enhancers, compatible with an increase in VDR expression [62]. Moreover, the treatment of resistant malignant melanoma cell lines with the HDAC inhibitor trichostatin-A significantly restored VDR expression. In general, several modifications of VDR histones have known effects; for instance H3K4me1 (monomethylation of lysine 4 on histone H3 protein) correlates with transcriptional elongation, while H3K4me3 (trimethylation) is associated with active transcription and it is found near the transcription start sites, H3K27ac (acetylation of lysine 27 of the histone H3 protein) correlates with active enhancers and H3K27me3 (trimethylation of lysine 27 on histone H3 protein) is associated with transcriptional inhibition if found in VDR gene-body [63–65].

Finally, also several microRNAs (miRNAs) are epigenetic modulators of VDR. miRNAs are a class of non-coding RNAs made of twenty two nucleotides, able to recognize and bind specific sequences

on mRNA. Usually mature miRNAs suppress gene expression by binding the 3′-UTR region of the target mRNA, although several studies have reported other interactions with 5′-UTR, coding sequence, and gene promoters [66]. miRNA are involved in the reversible silencing of VDR too, particularly in melanoma cells [67]. The mRNA of the receptor presents three experimentally verified MREs (miRNA response elements), located in the 3′-UTR of the transcript [68], which are recognized by miR-125b, miR-27b, and mmu-miR-298. Due to the characteristic reversibility of the epigenetic modifications, vitamin D resistance can be overcome with the administration of calcitriol and DNMT or miRNA inhibitors [58].

5.2. Epigenetic Modulation of VDR Cistrome and the Epigenetic Control Exerted by Vitamin D/VDR

VDR cistrome is modulated by ligand binding but not only. A complex system of epigenetic modulators is differentially expressed during embryogenesis and tissue differentiation, and they can change ligand-induced VDR/RXR docking at most sites across the genome responding to the requirements of the tissue. A comparison of the VDR cistromes in osteoblast lineage cells prior to and following differentiation [69,70] revealed striking changes in the genome after differentiating transition, which demonstrate that the cistrome for VDR can undergo significant remodeling affecting both the expression of the receptor and genomic distribution of its binding.

The studies of genome-wide analysis measured the consequences of VDR/RXR binding at target genes on the whole chromatin landscape. ChIP-chip and subsequent ChIP-seq analysis revealed that VDR/RXR binding across the genome results in the recruitment of coactivators such as SRC1, a histone acetyltransferase that increases histone acetylation and chromatin decondensation [71]. As consequence, the appearance of the VDR/RXR heterodimer evokes a striking increase in the levels of histone H3 and H4 lysine acetylation and changes in transcription machinery occupancy not only at promoters that are regulated by vitamin D but at other regions as well [72]. The ligand-dependent interference of VDR with chromatin modifiers such as lysine demethylase 6B explains how vitamin D can affect histone markers for active transcription start sites [73].

The epigenome-wide effects of vitamin D are also exerted on subsets of the cistromes of several transcription factors, such as the pioneer transcription factors PU.1 (purine-rich box 1), CEBPA (CCAAT/enhancer binding protein alpha), and the transcriptional repressor CTCF (CCCTC-binding factor) [74].

Based on these observations, it would be extremely useful to search for molecules, either synthetic or natural compounds, able to potentiate the activity of vitamin D/VDR through epigenetic mechanisms, which could influence both the expression of VDR and the actions of vitamin D on a genome-wide scale. Up to date there are no reports of natural compounds able to modulate VDR cistrome or VDR-dependent epigenetic landscape; further genome-wide studies are warranted. Instead, the existence of natural epigenetic modulators that act on VDR expression has been proven and some molecules have been investigated.

In this review we will deal with three natural modulators of VDR: Curcumin, sulforaphane, and butyrate, the latter as the product of the fermentative intestinal microbiome.

6. Sulforaphane

The sulforaphane (SF) is a thiocyanate naturally present in crucifers, especially in broccoli, which belong to the family of Brassicaceae. This natural compound is known for its reported antioxidant and anti-cancer properties. Due to its high lipophilicity and its low molecular weight, the SF is rapidly absorbed by the intestinal epithelium. It is conjugated to glutathione (GSH) by the enzyme glutathione S-transferase (GST) and it follows the mercapturic pathway with the excretion of N-acetyl cysteine in the urine. As a result, the SF metabolism and excretion depend on the GST activity and consequently they are influenced by the polymorphism of GST's subunits, especially GST-M1 and GST-T1 [75,76]. The likelihood of null genotype of these isoforms is relatively high in Caucasian and Asian population and it leads to a different SF biodistribution with an altered protective and anti-cancer activity [77].

The chemopreventive effect of SF has been intensively investigated both in vivo and in vitro, since it is associated to multiple mechanisms that synergize in reducing the risk of carcinogenesis [78]. As for the metabolic enzymes, SF inhibits the phase I cytochrome P450 (e.g., CYP1A1, CYP2B2, and CYP3A4) [79], whereas it induces enzymes of phase II (e.g., UGT1A1, GSTA1) [80]. In vitro studies demonstrate that SF is a potent inducer of the Nrf2-antioxidant response element signaling pathway [81], which controls the expression of genes involved in the expression of phase II enzymes. Many of the induced genes codify for xenobiotic-metabolizing enzymes that include GST and enzymes responsible for the GSH biosynthesis [82], therefore by increasing GSH tissue levels SF performs an antioxidant function [83]; moreover, SF modulates inflammation by downregulating genes involved in inflammation (e.g., NF-kB).

In addition to its anti-oxidant properties, SF triggers several epigenetic alterations by changing the histone modifications. In fact, recent in vitro and in vivo studies demonstrated the role of SF metabolites (e.g., SF-cysteine,) in the inhibition of the HDAC activity [84] and the increase of histone H3 and H4 acetylation [78]. In 2006 Myzak et al. showed the epigenetic mechanism of SF upon administration in APC^{min} mice (a mouse model of intestinal cancer) and demonstrated the consequent suppression of colon tumorigenesis [85]. Further clinical trials focused on the efficacy of SF assumption found that SF, given as daily dose of 150 μM for 12 weeks, is a safe phytochemical compound with a great potential in the treatment of irritability in children with autism spectrum disorder and to reduce hepatic glucose production and improve glucose control in obese patients with dysregulated type 2 diabetes [86,87].

Because VDR is regulated by epigenetic mechanisms, it would be interesting to carry out a clinical study testing the enhancement of vitamin D efficacy in patients treated with SF. The evidences supporting the hypothesis that SF could be beneficial in incrementing the response to vitamin D are provided by the study of Schwab et al. [88], investigating the anti-inflammatory effect of SF on three different human colorectal cancer cells. They demonstrated the role of SF in the production of the antimicrobial peptide human β-defensin-2 (hBD-2) and the involvement of the increased VDR expression in this mechanism. Defensins are small cationic peptides ubiquitously expressed throughout the gastrointestinal tract, including the colon. Here they exert their wide antimicrobial activity and in particular hBD-2 is induced in response to infection, proinflammatory mediators and probiotic bacteria. Moreover, hBD-2 seems to be involved in carcinogenesis and in several host immunity functions, such as the chemoattraction of different immune cells (e.g., T cells and neutrophils). Because of the relevant anti-inflammatory function of defensins, abnormalities in their expression have been observed in inflammatory bowel diseases (IBD) and Crohn's disease. Schwab et al. discovered a novel correlation among the SF-induced hBD-2 expression and the nuclear activity of VDR, a key regulator of immune response. The VDR signaling exerts a crucial role in regulating the innate immunity response, also by the indirect control over the production of hBD-2 mediated by NF-kB [89]. Interestingly, SF alone was able to increase the expression of VDR and the co-incubation of Caco2 cells with SF and the VDR antagonist ZK191732 drastically decreased the production of hBD-2 mRNA, revealing the crucial role of the receptor in the SF-induced hBD-2 expression.

In conclusion, the assumption of plants from the *Brassicaceae* family, especially Broccoli, is likely to improve health for several reasons. The SF is able to modulate and mitigate inflammation by transcriptional control, but also through epigenetic mechanisms. By the inhibition of HDAC and the increased acetylation of histones H3 and H4, SF can induce the expression of VDR, with the consequent regulation of the antimicrobial hBD-2 peptide. Further in vivo studies are required in order to investigate in depth the role of SF on VDR activity; these studies could be particularly interesting in the oncology field, considering the resistance to vitamin D and the decreased VDR expression often found in tumors.

7. Curcumin

In the last century curcumin was identified as a therapeutic agent for many diseases, affecting the skin, the pulmonary and the gastrointestinal system and also active on pain and liver disorders [90]. Extensive studies indeed describe curcumin as a potent anti-inflammatory and antioxidant agent acting through numerous mechanisms. For instance, it abates the activation of the pro-inflammatory transcription factor NF-kB [91], it downregulates the expression of many pro-inflammatory enzymes such as cyclooxygenase-2 (COX-2) [92], and it lowers the expression of chemokines and cytokines (e.g., TNF-α, IL-6, IL-8) [93]. Curcumin acts in a double way, since it modulates both antioxidant and pro-oxidant functions. It indirectly increases the production of reactive oxygen species (ROS) and through this mechanism it is able to kill cancer cells [94]. On the other hand, it modulates the redox state of cells also by playing an antioxidant function, for instance by increasing the intracellular concentration of GSH [95] and suppressing the lipid peroxidation [96]. In light of these data, curcumin is supposed to be a valid substitute of most steroidal and nonsteroidal anti-inflammatory drugs, due to their numerous side effects, such as the cardiovascular disease induced by the treatment with COXIBs, the selective inhibitors of COX-2 [90].

In addition to the antioxidant function, curcumin is active on numerous antitumoral pathways, which have been widely studied also in clinical trials. The results of the oncological studies revealed its safety, lack of toxicity and the antitumoral effect in several patients [97]. The poor bioavailability of curcumin is the main limitation for its use in clinical trials. For this reason, novel formulations suggest the use of theracurmin, a curcumin nanoparticle, which shows a remarkable improvement of bioavailability in healthy human volunteers [98].

Curcumin exerts its antitumoral function by modulating a wide range of targets, which mediate the downregulation and upregulation of many genes, most of them involved in the tumoral development or suppression, respectively. Overexpression of the P-glycoprotein (P-gp), which is a drug-efflux pump belonging to the ATP-binding cassette (ABC) transporters family, is one of the mechanisms involved in the development of the multidrug resistance (MDR). Notably, several in vitro and in vivo studies have demonstrated the role of curcumin in reversing MDR through not only the inhibition of P-gp activity, but also the modulation of its expression at both mRNA and protein level [99].

In the context of gene regulation, curcumin provokes numerous epigenetic changes, such as the histone acetylation and deacetylation. It is a powerful inhibitor of HDAC expression, especially of HDAC1, 3 and 8 [100], and an inducer of histone H4 acetylation [101]; moreover it restores oxidative stress-impaired HDAC2 activity and expression, thus exerting an anti-inflammatory role in case of chronic obstructive pulmonary disease [102]. At the same time, curcumin is also a potent HAT inhibitor, and through this mechanism it triggers several biological effects; for example by the inhibition of the acetylation of the viral HIV-tat protein it suppresses the proliferation of the virus [103]. Curcumin exerts its antitumoral activity also by inducing the chromatin demethylation, and this property has been recently evaluated both in vivo and in vitro. Liu et al. demonstrated the ability of curcumin (20–40 μM for 48 h) to induce the demethylation of the tumor suppressor Deleted in Liver Cancer 1 (DLC1), which is usually down-regulated or silenced in many cancers [104]. The consequent inhibition of the tumor growth in vitro was promising and the demethylating effect of curcumin was tested in vivo. When colitis-accelerated colon cancer mice were treated with curcumin, which produced a change of the CpGs methylation, the inflammation was reduced [105]. Curcumin is also able to directly bind the DNA [106], leading to epigenetic modifications. Moreover, curcumin controls many cellular signaling pathways (e.g., AP-1, NF-kB) by modulating an extensive range of miRNAs, as several studies have demonstrated in pancreatic, melanoma and lung cancer cells [107–109].

In 2018 for the first time Abdalla et al. investigated the correlation between the curcumin-induced hypomethylation and the VDR expression in hepatocellular carcinoma (HCC) [110]. Most cancers are characterized by a reduced sensitivity to vitamin D and its anti-inflammatory signaling pathways without inducing evident defects of its receptor, suggesting the involvement of epigenetic modifications. Studies on tumoral cells demonstrated that the hypomethylation of the VDR promoter region leads to

changes in gene transcription and the impairment of vitamin D functions [111]. Abdalla et al. proposed a novel approach able to contrast vitamin D resistance, by investigating the curcumin-dependent demethylation and the regulation of the VDR expression in tumoral cells [110]. In this study the analysis of the CpG methylation revealed that the higher levels of methylation found in the VDR promoter region of HCC samples are likely to be a good prognostic marker in patients, useful also for the discrimination between hepatocellular carcinoma and chronic liver disease (CLD) samples. Further analysis on the efficiency of VDR as biomarker, confirmed these observations [110]. The methylation of VDR gene promoter is also related to an inflammatory condition, since it was detected also in CLD samples. Most importantly, the treatment of hepatocarcinoma cells with different concentrations of curcumin (10-20 μM for 48 h) strongly inhibited the tumor growth by epigenetic mechanisms; in fact after the treatment with curcumin, the methylation of VDR gene promoter decreased, while the VDR relative expression increased.

In summary curcumin, a dietary phytochemical, exerts its anti-inflammatory and antioxidant activity by controlling both transcription and epigenetic events, therefore it is considered a promising antitumoral and chemo-preventive agent. Further studies are required in order to evaluate its efficacy in vivo and in clinical trials. In the same way, the effect of curcumin on the anti-inflammatory activity of vitamin D and the regulation exerted on VDR expression still remain to be fully explored, to unveil the details of the epigenetic modifications influencing VDR expression. Finally, as we mentioned the regulation exerted by curcumin on several miRNAs, future studies could also identify some miRNAs as the molecules linking curcumin activity and VDR expression.

8. Probiotics and Butyrate

The human body contains a huge amount (10^{14}) of microorganisms that all together constitute the microbiota. Above all the gastrointestinal tract is the most colonized, but every region of our body in contact with the external environment is actually rich of microorganisms. In humans the composition of microbiota strongly changes among individuals, depending on their health, age and local biogeography [112]. Independently on their composition, the importance of the intestinal microbiota resides in its numerous metabolic functions that lead to the production of nutrients, such as vitamins (e.g., folic acid, vitamin B12), amino acids and short-chain fatty acids (SCFA). Therefore, the intestinal microbiota is involved in physiological, metabolic, and nutritional processes with a further role in the development of the intestinal mucosal immunity [113]. As expected, the microbiota is sensitive to external and internal changes, whereby it undergoes relevant modifications (dysbiosis) in response to a high-fat diet [114], micronutrient deficiency [115], chronic inflammation such as the inflammatory bowel disease (IBD) [116], and in obesity [117].

Based on this background, the modulation of microbiota results an effective vehicle for improving and maintaining health. For this purpose, the use of probiotics and prebiotics, whose mixture constitutes the synbiotics, is suggested. According to the World Health Organization (WHO) criteria, a probiotic is "a live organism, which ensures a benefit to the host when provided in adequate quantities" [118]. The primary microorganisms classified as probiotics were various lactic acid-producing lactobacilli strains and a number of bifidobacteria strains; in addition, today several multistrain probiotic preparations are used. Over the last years the mechanisms of action of probiotics have been thoroughly investigated, whereby their roles have been defined, such as the regulation of the mucosal and systemic immune response of the host, the modification of the microbiota composition and the role in keeping the barrier functions of the intestinal epithelium. Moreover, probiotics are able to contrast the pathogen-induced epithelial damage, by reducing and preventing the adhesion of bacterial pathogen [119], and inducing the host to produce antimicrobial compounds [120]. In the last years the effect of probiotics against various diseases has been widely evaluated with promising results. In fact, in patients affected by obesity and hypertension the probiotic treatment reduced the blood pressure and the body mass index after only three weeks [121]; in addition, the probiotic mixture VSL#3 (*Bifidobacterium breve, Bifidobacterium infantis, Bifidobacterium longum, Lactobacillus acidophilus,*

Lactobacillus bulgaricus, *Lactobacillus casei*, *Lactobacillus planatarum*, and *Streptococcus salivarus subspecies thermophilus*) improved the health of overweight patients after a daily treatment for 6 weeks with 112.5×10^9 CFU of probiotic by enhancing the insulin sensitivity, changing the intestinal microbiota composition and limiting the concentration of lipids and inflammatory markers [122]. Several lines of evidence demonstrated the significant effect of probiotics on remission of patients after surgery due to Chron's disease and of patients with ulcerative colitis [123].

Apart from the promising direct effects of probiotics on intestinal microorganisms, additional effects can be found by analyzing the produced metabolites and their functions. In response to high fiber consumption, SCFA are naturally produced via fermentation in the colon, mainly as butyrate, propionate, and acetate. Among the SCFA, the butyrate has received a particular attention, due to its beneficial properties both in physiological and pathological conditions. Indeed, in the intestine it modulates cell proliferation and controls the intestinal barrier function, even protecting from the inflammatory disease [124]. The antitumoral activity of butyrate is well known, although its mechanisms of action have not been fully elucidated yet. In the oncologic field the literature identifies the butyrate as an epigenetic modulator, especially as an inducer of the HDAC inhibition [125]. It is indeed supposed to be a potential agent for both cancer therapy and prevention [126]. The main effect produced by butyrate through the epigenetic modulation is the anti-inflammatory one exerted through several mechanisms: The suppression of the proinflammatory cytokines such as IL-1β, TNF-α, and IL-6 [127], and the reduction of the pro-inflammatory NF-kB [128]. Besides its anti-inflammatory effect, butyrate has a direct effect on the tumoral development, by inhibiting the growth of colonic cancer cell lines [129]. Several lines of evidence show that the epigenetic modulation exerted by butyrate has a proliferative effect on noncancerous cells, whereas it inhibits the tumoral cell proliferation. Donohoe et al. assessed this duality in correlation with the cellular energy metabolism and the regulation of gene expression [130]. According to this study, the butyrate exerts its epigenetic mechanism in a cell-specific and dose-dependent manner. In normal colonocytes the butyrate is metabolized and transformed to acetyl-CoA, representing a fundamental oxidative energy source. In turn, the acetyl-CoA directly leads to the stimulation of HAT activity and consequently to the cell proliferation. On the contrary, in tumor cells the Warburg effect shifts the metabolic processes from oxidative to glycolytic, thus the butyrate is scarcely metabolized and it is accumulated in the nucleus, leading to an increase of HDAC inhibition. Although both mechanisms increase histone acetylation, different target genes are upregulated, and as a result, in malignant colonocytes butyrate inhibits cell proliferation and increases apoptosis [130]. Recently, the butyrate-induced chromatine remodeling has been corroborated by Sun et al., with the discovery of a further epigenetic role of SCFA in the demethylation processes [131]. In colorectal cancer cell lines it was demonstrated that butyrate up-regulates the isocitrate dehydrogenase 1 (IDH1), and that the consequent increase of α-ketoglutarate induces the DNA demethylation, up-regulating the promoter region of DNA mismatch repair genes (MMR) and inhibiting the colorectal carcinogenesis [131]. Based on these observations, it is clear that the butyrate is an epigenetic modulator with an antitumoral effect exerted through several mechanisms.

As for the differentiating properties of butyrate, it has been demonstrated that the butyrate potentiates the effects exerted by vitamin D, resulting in the increase of cell differentiation [132]. Indeed, the butyrate-induced cell differentiation in colon cancer cells is supposed to be mediated by VDR [133] through the TGFβ/SMAD3 pathway [134]. As demonstrated in recent studies, TGFβ controls the remodeling of VDR cistrome [135], induces VDR expression, and the receptor exerts a negative feedback regulation of TGFβ activities [136]. All together these observations lead to the conclusion that butyrate increases the activity of the TGFβ pathway, which upregulates VDR expression and activity by epigenetic mechanisms, creating an autoregulatory loop; the boosted VDR activity explains the differentiating properties of butyrate.

The accumulation of butyrate can lead to adverse effects. For example a clinical trial showed that in patients with advanced colorectal carcinoma the administration of high doses of butyrate

resulted in a high toxicity and liver insufficiency [137]. For this reason, the more physiological production of butyrate by the intestinal tissue is recommended, which can be incremented by probiotic administration. Probiotics can be orally administered, and they show no adverse effects both in patients and healthy adults. Appleyard et al. demonstrated in vivo the anti-inflammatory and antitumoral effect of the probiotic preparation VSL#3 in case of colitis-associated cancer [138], as a condition of chronic inflammation is commonly known to progress to dysplasia and colon cancer [139]. The modulation of the VDR expression plays a key role against inflammation, and vitamin D exerts a preventive effect against cancer by promoting apoptosis and cell differentiation. An elevated risk of colorectal cancer (CRC) is indeed related to low levels of vitamin D [140] as well as to decreased amounts of VDR [141]. Interestingly, the assumption of VSL#3 led to a remarkable increase of VDR levels in the proximal and distal colons of treated rats, affecting both chronic inflammation and cancer development [138]. Based on the previous considerations, it is conceivable that the probiotic administration (e.g., VLS#3) stimulates the microbiota population to produce more metabolites, such as the butyrate; the epigenetic activity of butyrate exerts many effects, among them the positive regulation of the VDR expression is crucial, possibly either through a direct epigenetic control or via TGFβ/SMAD3 signaling pathway. Similarly, another recent study found that the probiotic *Lactobacillus rhamnosus* strain GG and *Lactobacillus plantarum* increased VDR protein expression and transcriptional activity in both mouse and human intestinal epithelial cells and concluded that the VDR pathway is required for protection against inflammation exerted by probiotics in colitis [142].

In conclusion, a deep analysis of the microbiota composition and function reveals that its modulation can prevent and treat a wide range of diseases that include IBD, colon cancer, and obesity [121–123,143]. For this purpose, probiotics could be promising chemo-preventive and therapeutic agents, since they reinforce the microbiota and the immune system by the induction of numerous epigenetic mechanisms. Several studies reveal the safety and the tolerability of probiotic administration [123,143], in contrast to the single butyrate assumption [137,144,145]. However, further research is required to thoroughly investigate the effect of probiotic assumption in clinical trials and which mechanisms mediate its action. Moreover, the connection between probiotics and VDR levels, as well as the epigenetic mechanisms by which other metabolites could modulate the expression of this receptor, have to be explored in vivo.

9. Conclusions

Many natural dietary agents, consisting of bioactive compounds, have been shown to be effective nutraceuticals in inflammation and cancer prevention acting as epigenetic modulators.

Given the central role of VDR in healthy tissues and considering its frequent silencing, due to a faulty epigenetic remodeling occurring during differentiation, or to the epigenetic defects accumulated during neoplastic transformation, the ability to restore VDR expression and activity could be crucial in many conditions. A scarce sensibility to vitamin D often nullifies the dietary supplementation of the hormone, which is recommended by health authorities in the UK and US for older people. The resistance to vitamin D could be overcome by altering the epigenetic control of VDR. In our analysis we considered three molecules, sulforaphane, curcumin, and butyrate produced by the intestinal microbioma, which if added to the diet could enhance the expression of VDR through epigenetic mechanisms, facilitating the transcription of the VDR gene and the activity of this transcriptional factor.

The studies that have been described clearly indicate the following effects of the treatment with these natural molecules:

- The increased expression of VDR;
- the decrease of cancer cell proliferation and survival;
- the increased expression of antimicrobial and anti-inflammatory proteins; and
- the improvement of chronic inflammation and the reduction of neoplastic progression.

The analysis carried out in this review reveals two novel consequences of the epigenetic activity of these molecules, which are worth dwelling on:

1. The VDR is an epigenetic modulator itself; the studies of genome-wide profiling of accessible chromatin showed that nearly 9000 chromatin regions are changed in their accessibility when human monocytes are treated for 24 h with Vitamin D [42]. Therefore the nutraceutical compounds targeting VDR would trigger a double wave of chromatin remodeling, both directly and by inducing VDR activity. This would explain the broad and sometimes contrasting epigenetic effects described for some of these molecules. Figure 1 summarizes the influence of the considered nutraceuticals on epigenetics.
2. Most importantly, the resistance to Vitamin D could be reverted by the molecules described in our study and many natural substances could deserve further characterization. The enhanced expression of the receptor could be obtained by introducing some natural compounds in the diet, and could greatly influence the outcome of many diseases.

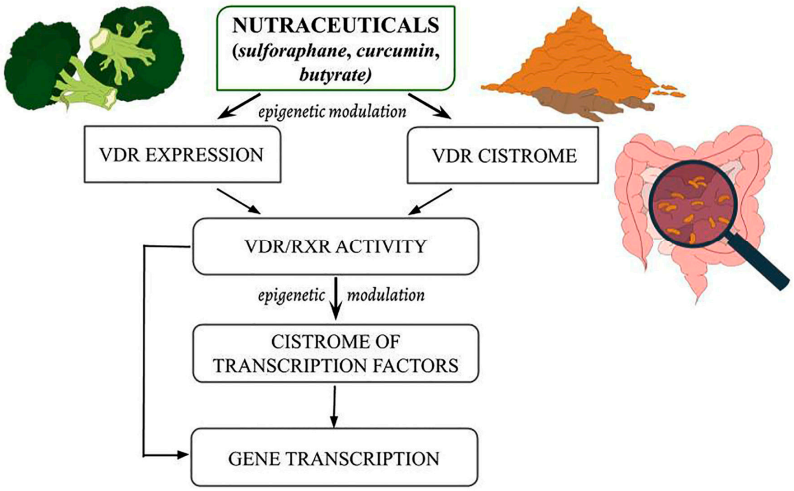

Figure 1. Schematic presentation of the possible effects of nutraceuticals on the epigenetic modulation exerted at three levels: vitamin D receptor (VDR) expression, VDR cistrome landscape, and chromatin remodeling triggered by vitamin D-VDR activity.

As many other natural molecules described as epigenetic modulators could be effective in upregulating the VDR, further studies on natural substances active as epigenetic agents is urgently needed to fully explore the potential of these nutraceuticals in the prevention and treatment of cancer and other diseases, either alone or in combination therapies. The most intriguing developments in the field could derive from the advent of the genome-wide cistrome analysis techniques, which could be exploited to investigate the changes in epigenetic landscape triggered by these natural molecules, and novel properties could be discovered.

Supplementary Materials: The following are available online at http://www.mdpi.com/2076-3417/10/12/4096/s1. Table S1: Factors affecting VDR regulation.

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