Donald L. Trump
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Vitamin D and Cancer

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Preface

Over the past 30 years numerous provocative studies have provided clues suggesting that vitamin D may play an important role in cancer. In vitro studies have shown that cancer cells metabolize vitamin D and that vitamin D compounds can induce differentiation, inhibit cellular proliferation, and induce cell death. In addition, epidemiologic data suggest that vitamin D compounds may play a role in the prevention of cancer. In the past few years the understanding of the molecular effects of vitamin D has expanded substantially and investigators have begun to delineate the role of genetic factors that influence the response to vitamin D.

With this considerable history of development of vitamin D and cancer, it is timely and appropriate to summarize the current "state of the art" in the study of vitamin D and cancer. Scientists who have made many of the seminal contributions to this field of study have contributed to this volume. These collected data describe the foundation and current state for this important domain of cancer research – a domain that the coeditors of this book believe will yield important advances in cancer prevention and therapy.

Vitamin D Analogues as Antineoplastics: A Prologue Long Overdue?

Numerous investigators have drawn attention to the high prevalence of the vitamin D receptor (VDR) in human and murine cancer cells, the frequent evidence of intact vitamin D signaling pathways in such cells, and the ability of high concentrations of vitamin D analogues to inhibit the replication of cancer cells, induce apoptosis, and even inhibit angiogenesis. These data are cited in preceding and following chapters. Had such studies been completed with a new molecule – e.g., a new "targeted agent" – it is very likely that the following steps would have been undertaken promptly:

(a) Careful in vivo delineation of schedule and dose dependencies of these anticancer activities
(b) Careful determination of the maximum tolerated dose of analogues and exploration of optimal biologic dose

(c) *Direct* comparisons of toxicity and antitumor efficacy of analogues and parent compound (calcitriol) using the apparently most active drug schedules

Unfortunately, for vitamin D based studies in cancer, very little of this rudimentary work has been carried out. Studies with most analogues of vitamin D (paricalcitol, seocalcitol, inecalcitol) have employed continuous dosing schedules even though practically all in vitro and in vivo studies which have shown anticancer activity of vitamin D have exposed cells and tumors to intermittent, high-pulse doses. Many have been encouraged by the study of daily dosing of analogues and parent compound (calcitriol) and finding the analogue causes less hypercalcemia. Often and not surprisingly, the analogue binds less avidly to VDR. Such studies have led to small to medium sized studies using daily dosing algorithms which have shown no antitumor effects and been halted without any toxicity remotely resembling those defensible in patients with advanced cancer.

Further limiting work with calcitriol has been the absence of a formulation suitable for high-dose therapy. This limitation is due primarily to the lack of an economic motivation for the development of such formulations.

The following chapters provide excellent and comprehensive discussions of the potential role of vitamin D based therapies in breast, colorectal, prostate cancer, and leukemia and myelodysplastic syndromes. These chapters also point out that the focus on these diseases is largely determined by the interests and expertise of the outstanding scientists who have chosen to pursue vitamin D based cancer therapeutics. To our knowledge, every tumor type ever evaluated has shown some biochemical and antiproliferative response to vitamin D. Similarly, vitamin D analogues, especially calcitriol, potentiate almost every cytotoxic agent with which combination therapies have been tested. In our view the slow and halting development of vitamin D based cancer therapeutics could be greatly accelerated by following standard principles of anticancer drug development:

(a) Development of a standardized formula.
(b) Determination of MTD (current data indicate the MTD of calcitriol on an intermittent [weekly] schedule is ≥100 mcg). No reliable oral MTD have ever been determined and few data on the optimal biologic dose developed in the laboratory, much less in the clinic.
(c) Conduct of carefully designed clinical trials.

The field of vitamin D based cancer therapeutics has very few such data items available. Perhaps the extensive preclinical data on the antitumor effectiveness of high-dose vitamin D analogue therapy are misleading or in fact wrong. But until the agent is examined in the fashion one would follow for an antineoplastic – we will never know. The following chapters point out what is known and the direction that can be followed in clinical development of vitamin D as an anticancer agent.

Buffalo, NY — Donald L. Trump
Buffalo, NY — Candace S. Johnson

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Chapter 1
Vitamin D: Synthesis and Catabolism – Considerations for Cancer Causation and Therapy

Heide S. Cross

Abstract Protection from sporadic malignancies by vitamin D can be traced to the role of its hormonally active metabolite, 1,25-dihdroxyvitamin D_3 (1,25-$(OH)_2D_3$) which, by binding to the nuclear vitamin D receptor (VDR), can maintain cellular homeostasis. Human colonic, prostatic, and breast cells express the CYP27B1-encoded 25-(OH)D-1α-hydroxylase, the enzyme responsible for conversion of 25-$(OH)D_3$ to 1,25-$(OH)_2D_3$. In vitamin D insufficiency, availability of 25-$(OH)D_3$ is low, so that extrarenal CYP27B1 activity may not be high enough to achieve tissue concentrations of 1,25-$(OH)_2D_3$ necessary to control growth and prevent neoplastic transformation of colonocytes.

While adequate supply of the vitamin D precursor 25-$(OH)D_3$ is essential for prevention of tumor progression, activity of the extrarenal synthesizing CYP27B1 is of paramount importance especially in view of the fact that 1,25-$(OH)_2D_3$ catabolism is progressively elevated during tumor progression. To counteract catabolism, enhancement of 1,25-$(OH)_2D_3$ synthesis is discussed. Early during cancer progression growth factors and sex hormones may elevate CYP27B1 expression and suppress that of CYP24A1. Also, genetic variations and epigenetic regulation of vitamin D hydroxylases could determine actual accumulation of 1,25-$(OH)_2D_3$ in mammary, prostate, and colonic tissue and are considered both for prevention of progression as well as for potential therapy.

Primarily in the colon as part of the digestive system, the chemopreventive potential of vitamin D can also be augmented by nutrient factors that induce appropriate changes in CYP27B1 and/or CYP24A1 expression. Among these factors are calcium, the phytoestrogen genistein and potentially also folate. Adequate intake levels of these nutrients could augment effectiveness of 1,25-$(OH)_2D_3$ for prevention of cancers in humans. Especially folate, as a methyl donor, could affect epigenetic regulation of CYP27B1 and of CYP24A1, and could therefore play a central role in vitamin D-mediated inhibition of tumor progression.

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1.1 Introduction

The enzyme 25-hydroxyvitamin D3-1α-hydroxylase (CYP27B1) plays a central role in calcium homeostasis [1], but alternative physiological actions have been suspected for decades. The enzyme catalyzes the conversion of 25-hydroxyvitamin D_3 (25-(OH)D_3) to the hormone 1,25-dihydroxyvitamin D_3 (1α,25-$(OH)_2D_3$) that is known to regulate calcium and phosphate transport in intestine, bone, and kidney. While initially it was thought that only proximal tubule kidney cells express CYP27B1, it became evident in the mid-1980s that extrarenal cells, for instance, bone cells, macrophages, and keratinocytes (see, e.g., [2]) could also express CYP27B1 enzymatic activity in vitro. Mawer et al. [3] demonstrated that certain lung cells had measurable CYP27B1 activity. Apparently, this particular 25-hydroxyvitamin D3-1α-hydroxylase was not up-regulated by PTH and was not down-regulated by plasma calcium, a hallmark of the renal enzyme. In addition, while in renal cells sufficiency of serum 1,25-$(OH)_2D_3$ concentration leads to induction of the vitamin D-inactivating enzyme 1,25-$(OH)_2D_3$–24-hydroxylase (CYP24A1) [4], the extrarenal CYP27B1 is not necessarily inversely correlated with CYP24A1 expression, a fact that will be enlarged upon later in this chapter.

While extrarenal CYP27B1 activity in macrophages might be the reason for the hypercalcemia associated with sarcoidosis and lymphomas, there was also the possibility that it might be coded by a gene different from the renal one, and this could lead to alternative regulatory mechanisms. The renal CYP27B1 is a combination of three proteins: a cytochrome P450 as well as two other proteins, ferredoxin and ferredoxin reductase. Purified preparations of these proteins possess the CYP27B1 enzyme activity in vitro [5]. These enzyme complexes were cloned from rodents and human renal cells and response elements were found in promoter regions that allow up-regulation by PTH. Proof was provided that extrarenal CYP27B1 is a product of the same gene as the renal form. However, regulation of the newly discovered CYP27B1 suggested existence of a paracrine loop in extrarenal tissues for the modification of cellular proliferation and differentiation, though subsequent conversion of the active vitamin D metabolite into a C-24 oxidation product by CYP24A1 was similar to renal catabolism [6].

In the last few decades, there has been growing appreciation for the multitude of physiological roles that vitamin D has in many body tissues. As early as in 1979, Stumpf et al. demonstrated that cells from heart, stomach, pancreas, colon, brain, skin, gonads, etc., have the nuclear receptor for 1,25-$(OH)_2D_3$ [7], the so-called vitamin D receptor (VDR), and such tissues are potential targets for 1,25-$(OH)_2D_3$ activity. Many of these VDR-positive tissues are also positive for CYP27B1, i.e., the enzyme that can convert 25-(OH)D_3 to the active metabolite [8], and many of these tissues are known to be targets for development of malignancies.

As mentioned above, regulation of CYP27B1 in these non-renal tissues differs from that observed in the kidney and, importantly and in contrast to the renal enzyme, may be dependent on substrate concentration for activity. This led to the novel concept that maintenance of adequate serum 25-(OH)D_3 levels would be essential for providing the substrate for the synthesis of the active metabolite at extrarenal sites, which in turn would have physiological functions apart from those involved in bone mineral metabolism. This concept will be enlarged upon in the following. Evidence will be provided for the function and regulation of vitamin D synthesizing and catabolic hydroxylases, i.e., CYP27B1 and CYP24A1, respectively, in colorectal, prostate, and mammary gland-derived cells that are from organs particularly affected by sporadic malignancies during advancing age.

1.1.1 1,25-(OH)$_2$D$_3$ Synthesis

7-Dehydrocholesterol, the immediate precursor in the cholesterol biosynthetic pathway, is produced in rather large quantities in the skin of most vertebrates, also humans. Ageing decreases the capacity of skin to produce 7-dehydrocholesterol by as much as 75% [9] and this is of particular relevance when considering that sporadic cancers occur primarily in the elderly. When exposed to sunlight, skin cells absorb UVB radiation with wavelengths of 290–315 nm leading to a rearrangement of the molecular structure of 7-dehydrocholesterol to form the more thermodynamically stable previtamin D_3. Protection of the skin by topical sunscreens will reduce previtamin D_3 production by almost 100%. Persons that have greater amounts of melanin in their epidermis require much higher exposure to sunlight than whites to avoid vitamin D deficiency. Living at geographic latitudes above 35° will not provide enough UVB photons for sufficient production of vitamin D_3 in skin during winter time (for further reading see, e.g., [10]). Very few foods naturally contain vitamin D. Cod liver oil and oily fish are the best dietary source which, in some Scandinavian countries, can provide a positive balance to the lack of dermal vitamin D production.

Vitamin D_3 is first hydroxylated in the liver by CYP27A1, a cytochrome P450 25-hydroxylase, to the precursor 25-(OH)D_3. To be fully active, 25-(OH)D_3 must be converted to 1,25-(OH)$_2$D$_3$ by CYP27B1, a mitochondrial cytochrome P450 enzyme present primarily in proximal renal tubule cells but also in many extrarenal cells [11]. While the hormone regulates calcium and phosphate metabolism in intestine, bone, and kidney, at extrarenal sites it has a wide range of biological effects that are essentially noncalcemic in nature. The most surprising one is its ability to suppress hyperproliferative growth of cells and to support differentiation. In 1982, Tanaka et al. [12] provided the first evidence that 1,25-(OH)$_2$D$_3$ was able to promote differentiation of HL-60 leukemia cells. This, and a pronounced antimitotic effect, has subsequently been shown for many types of cancer cells in vitro (see, e.g., [13–18]), though only at nanomolar concentrations. However, serum 1,25-(OH)$_2$D$_3$ never exceeds picomolar concentrations, regardless of whether

sunlight exposure is increased or whether there is increased oral uptake of vitamin D [19], since its synthesis in renal cells is tightly regulated by PTH, calcium, and phosphate.

As early as 1980, Garland et al. raised the question whether sunlight and vitamin D can protect against colon cancer [20]. Strong support for this hypothesis was obtained when Garland et al. [21] in 1985 published the results of a 19-year prospective trial, showing that low dietary intakes of vitamin D and of calcium are associated with a significant risk of colorectal cancer. In the following decades, a compromised vitamin D status as indicated by low 25-$(OH)D_3$ serum levels has been associated with pathogenesis of diverse types of malignancy (for review see, e.g., [22, 23]). This, and the realization that there was vitamin D synthesis at extrarenal sites potentially enhancing 1,25-$(OH)_2D_3$ concentrations in certain tissues without contributing to serum levels of 1,25-$(OH)_2D_3$, suggested a hypothesis on how decreased sunlight exposure and low serum 25-$(OH)D_3$ could contribute to tumor pathogenesis.

1.2 Regulation of 1,25-$(OH)_2D_3$ Synthesis in Extrarenal Cells

Regulation of 1,25-$(OH)_2D_3$ production at multiple levels is a crucial determinant of nonclassical aspects of 1,25-$(OH)_2D_3$ function. When we showed that normal and neoplastic human colon epithelial cells are endowed with a functional 25-hydroxyvitamin D-1α-hydroxylase and can thus convert 25-$(OH)D_3$ to 1,25-$(OH)_2D_3$ [24–26], we hypothesized that adequate accumulation of the active metabolite could slow down or inhibit progression of malignant disease by promoting differentiation and apoptosis and by suppressing antimitotic activity locally. Renal CYP27B1 activity is tightly regulated by serum Ca^{++} and parathyroid hormone (PTH), as well as by feedback inhibition from 1,25-$(OH)_2D_3$. In contrast, CYP27B1 expression, at least in colonocytes and prostate cells, is relatively insensitive to modulation via the PTH/$[Ca^{++}]_o$ axis [27, 28]. Intracellular synthesis of 1,25-$(OH)_2D_3$ at extrarenal sites depends largely on ambient 25-$(OH)D_3$ levels and is not influenced by plasma levels of 1,25-$(OH)_2D_3$ [29]. This may explain why the incidence of vitamin D-dependent cancers, e.g., of the colorectum [30], breast [31], and prostate gland [32], is correlated with low serum 25-$(OH)D_3$ rather than with serum concentrations of 1,25-$(OH)D_3$. Strong support for the importance of intracellularly produced over circulating 1,25-$(OH)_2D_3$ for regulation of cell functions comes from a study by Rowling et al. [33] who have shown that in mammary gland cells VDR-mediated actions depended more on megalin-mediated endocytosis of 25-$(OH)D_3$ than on ambient 1,25-$(OH)_2D_3$. Also Lechner et al. [34] could induce the characteristic antimitogenic effect of 1,25-$(OH)_2D_3$ when human colon carcinoma cells were treated with 25-$(OH)D_3$, though only when they were CYP27B1-positive. Similar observations were made in prostate [35] and mammary cells [36].

However, at low serum levels of 25-(OH)D_3, CYP27B1 activity in extrarenal cells may be not high enough (in normal colonic mucosa without hyperproliferative signaling, positivity for CYP27B1 is extremely low [26]) to achieve those steady-state tissue concentrations of 1,25-$(OH)_2D_3$ necessary to maintain normal cellular growth and differentiation during hyperproliferation. In addition, 1,25-$(OH)_2D_3$ itself is an important regulator of CYP27B1 gene expression. Down-regulation of the CYP27B1 gene involves a negative vitamin D response element and cell specificity for this could be due to differential expression of protein complexes associated with the CYP27B1 promoter [37, 38].

1.2.1 Expression of CYP27B1 and of VDR During Hyperproliferation and Tumor Progression

The relevance of 1,25-$(OH)_2D_3$ to maintain normal epithelial cell turnover in the large intestine was demonstrated by studies with mice, which were genetically altered to block 1,25-$(OH)_2D_3$/VDR signaling: The colon mucosa of VDR null ($VDR^{-/-}$) mice show a pattern of increased DNA damage and cell division, the former probably due to formation of reactive oxygen species [39]. Interestingly, the large intestine reacts to inflammatory and hyperproliferative conditions with up-regulation of the VDR and of its ligand-synthesizing enzyme, CYP27B1: Liu et al. [40] reported that in a mouse model of ulcerative colitis, a disease considered to be a precursor lesion to colorectal cancer, expression of CYP27B1 was increased fourfold compared with controls. With respect to human colon cancer, we have shown that expression of CYP27B1 rises about fourfold in the course of progression from adenomas to well and moderately differentiated (G1 and G2) tumors, and then substantially declines during further progression [41]. Expression of the VDR showed the same dependence on tumor cell differentiation [41, 42]. However, cells from poorly differentiated (G3) colonic lesions, are frequently devoid of immunoreactivity for VDR and CYP27B1, while, at the same time, epidermal growth factor (EGF) receptor mRNA can be detected by in situ hybridization in almost any cancer cell [43]. Statistical evaluation actually showed an inverse expression of EGF receptor positivity compared to that of VDR. We suggested therefore that the 1,25-$(OH)_2D_3$/VDR system can be activated in colon epithelial cells in response to mitogenic stimulation, e.g., by EGF, respectively, TGF-α [43, 44]. A strong autocrine/paracrine antimitogenic action of 1,25-$(OH)_2D_3$ would retard further tumor growth as long as cancer cells retain a certain degree of differentiation and high levels of CYP27B1 activity and of VDR expression. However, during progression to high grade malignancy, signaling from the 1,25-$(OH)_2D_3$/VDR system would be too weak to effectively counteract proliferative effects from, e.g., EGF-R activation [43]. We confirmed these hypotheses by demonstrating that, in differentiated colon cell lines, EGF stimulates expression of VDR and CYP27B1, whereas in a primary culture derived from a G2 tumor, expression of VDR and of CYP27B1 was actually reduced [45]. Palmer et al. [46] demonstrated that induction of the adhesion protein

E-cadherin by vitamin D enhanced differentiation of colon cancer cells. This in turn opposed hyperproliferation and thus indicates the importance of vitamin D activity for normal maintenance of the wnt pathway. It is significant that repression of E-cadherin and of VDR, and parallel enhanced expression of the transcription factor SNAIL, was found in patients with aggressive tumor characteristics [47].

CYP27B1 and VDR expression is present also in some prostate and mammary gland-derived cells, since growth inhibition by 25-(OH)D_3 occurs with concomitant upregulation of CYP24A1. If mammary cells are negative for CYP27B1, there is no mitotic inhibition, and no induction of CYP24A1 expression [48]. When the antimitotic potencies of 25-(OH)D_3 and of 1,25-$(OH)_2D_3$, both in the nanomolar range, were studied in prostate cells, they were quite similar as long as cells expressed CYP27B1 [49]. However, it was suggested that during tumor progression, prostate cells no longer express CYP27B1 [35], though the biological grade of cells was not established in these studies. Quite similar to colon cells, EGF stimulated CYP27B1 promoter activity in prostate cell lines via involvement of the MAPK pathway, at least in those cancer cells that are still differentiated [50]. In normal human prostatic epithelial cells mitogen-activated protein kinase phosphatase 5 was induced by 1,25-$(OH)_2D_3$ leading to deactivation of protein kinase p38 [51]. Activation of p38 and downstream production of interleukin-6 are proinflammatory. Inflammation as well as interleukin-6 overproduction have been implicated in initiation and progression of prostate as well as of colon cancer [52]. Similar regulatory networks appear to exist in mammary gland cells (for review see [53]).

1.2.2 Expression of CYP24A1 During Hyperproliferation and Tumor Progression

It must be taken into account that the effective tissue concentration of 1,25-$(OH)_2D_3$ is determined not only by substrate availability but by additional regulatory factors that may govern also renal vitamin D synthesis: (i) in colonocytes, in prostate and mammary gland cells, 1,25-$(OH)_2D_3$ downregulates CYP27B1 and the VDR (see, e.g., [34]); (ii) 1,25-$(OH)_2D_3$ at the same time induces CYP24A1-encoded 25-(OH)D_3-24-hydroxylase, the enzyme that initiates stepwise degradation of the hormone; and (iii) at least in colon tumors, expression of CYP24A1 increases dramatically during progression to a poorly differentiated state (G3-G4) though CYP27B1 expression is diminished [54].

Therefore, one major mechanism for vitamin D resistance or reduced sensitivity in VDR-positive cancer cells is 1,25-$(OH)_2D_3$ catabolism via the C-24 hydroxylation pathway. An inverse relation between cellular metabolism of 1,25-$(OH)_2D_3$ via 24-hydroxylation and growth inhibition of prostate cancer cells by vitamin D has been suggested [55]. A 1,25-$(OH)_2D_3$ resistant prostate cell line was growth-inhibited when cultured with the active vitamin D metabolite combined with the CYP24A1 inhibitor liarozole [56]. Colon cells isolated from well-advanced (G3)

tumors express extremely high levels of CYP24A1, and cannot be growth-inhibited by 1,25-$(OH)_2D_3$. Actually, when these CYP27B1-negative cells are exposed to 16.6 nmol 25-$(OH)D_3$, they will efficiently use up the precursor within 12 h for 24,25-$(OH)_2D_3$ production and further degradation [34]. Androgen-independent prostate cell lines also tend to express high levels of CYP24A1, whereas CYP27B1 expression is negligible (see, e.g., [57]). These few examples clearly demonstrate an uncoupling of 1,25$(OH)_2D_3$ action from expression of CYP24A1 during advancing malignancy: whereas, in differentiated colon and prostate cancer cells, 1,25-$(OH)_2D_3$ will induce CYP24A1 expression, undifferentiated cells express basally extremely high levels of CYP24A1 that can no longer be enhanced by treatment with the active metabolite [38, 58]. Therefore, such basally high expression of CYP24A1 during advanced malignancy will not permit effective treatment of patients with vitamin D or vitamin D analogs that can be degraded via the C-24 pathway. However, this also clearly shows that inhibition of CYP24A1 activity in tumor cells could be of primary importance for cancer therapy. This aspect will be discussed further in the section on epigenetic regulation of CYP24A1 (see section 1.2.5.)

1.2.3 Regulation of CYP27B1 and CYP24A1 Expression by Sex Hormones

Although men and women suffer from similar rates of colorectal cancer deaths in their lifetime, the age-adjusted risk for colorectal cancer is less for women than for men [59]. This strongly indicates a protective role of female sex hormones, particularly of estrogens, against colorectal cancer (see, e.g., [60, 61]). Observational studies have further suggested that postmenopausal hormone therapy is associated with a lower risk for colorectal cancer and a lower death rate in women [62]. A meta-analysis of studies showed a 34% reduction in the incidence of this tumor in postmenopausal women receiving hormone replacement therapy [63]. A mechanism of action for estrogens in lowering colon cancer risk is not known yet. Since estrogen receptors are present in both normal intestinal epithelium and in colorectal cancers, the hormone is probably protective through these receptors and resultant post-receptor cellular activities.

While the colon cannot be considered an estrogen-dependent tissue, it must be defined as an estrogen-responsive organ. Expression of estrogen receptor (ER) subtypes α and β have been detected in cancer cell lines. Whereas human colon mucosa expresses primarily the ER-β type regardless of gender [64], ER-α is mainly expressed in the breast and the urogenital tract [65]. Both receptors bind estrogen, but they activate promoters in different modes. Studies of breast and prostate carcinogenesis suggested opposite roles for ER-α and ER-β in proliferation and differentiation [66]. Therefore, the ER-α/ER-β ratio has been suggested as a possible determinant of the susceptibility of a tissue to estrogen-induced carcinogenesis: in some cells, binding of estrogen to ER-α induces cancer-promoting effects, whereas binding to ER-β exerts a protective action. With respect to colon

cancer, the concept of a protective role of ER-β gained support recently: decreasing levels of the receptor were reported during colonic tumorigenesis compared with expression in the adjacent normal mucosa from the same patient [67].

Estrogens may indirectly oppose progression of malignancies by changing VDR expression or vitamin D metabolism in colonic epithelial cells. As early as in 1986, a study on the effect of endogenous estrogen fluctuation with respect to 25-$(OH)D_3$ metabolism was published [68]. This study in healthy premenopausal women suggested that 25-$(OH)D_3$ was metabolized predominantly to 24,25-$(OH)_2D_3$ at low estrogen, but to 1,25-$(OH)_2D_3$ at higher serum estrogen concentrations. While this, in 1986, primarily concerned renal synthesis of vitamin D metabolites, it was the first suggestion that estrogen elevates CYP27B1 expression.

Liel et al. [69] reported that estrogen increased VDR activity in epithelial cells of the gastrointestinal tract. In the colon adenocarcinoma-derived cell line Caco-2, which is ER-β positive but negative for ER-α, we demonstrated an increase of CYP27B1 mRNA expression and also of enzymatic activity after treatment with 17β-estradiol [70]. Based on these findings a clinical pilot trial was designed, in which postmenopausal women with a past history of rectal adenomas were given 17β-estradiol daily for 1 month to reach premenopausal serum levels. Rectal biopsies were obtained at the beginning and end of trial. A predominant result was the elevation of VDR mRNA [71]. We also observed significant induction of CYP27B1 mRNA in parallel to a decrease in COX-2 mRNA expression in those patients who had particularly high levels of the inflammatory marker at the beginning of the trial (Cross HS, The vitamin D system and colorectal cancer prevention. In: Vitamin D, 3rd edition. D. Feldman ed. Elsevier 2010).

To study modification of vitamin D hydroxylase activity by 17β-estradiol further, we used a mouse model to measure actual 1,25-$(OH)_2D_3$ synthesis and accumulation in colonic mucosa. In female compared with male mice, CYP27B1 mRNA was doubled and 1,25-$(OH)_2D_3$ concentration in the mucosa was increased by more than 50%. This occurred in the proximal colon only and suggested that there may be site-specific action of 17β-estradiol [127]. In this respect it is significant, that the estrogen receptor ESR1 is more methylated (inactivated) in the human distal than in the proximal colon [72].

There is equivocal evidence for the role of estrogen receptors (ER)-α and (ER)-β, and therefore for estrogenic activity, during mammary tumor progression. It has been suggested that higher ER-α expression in normal breast epithelium increases breast cancer risk. Since 1,25-$(OH)_2D_3$ synthesis, not only in colonocytes but also in mammary cells, may in part be regulated by 17β-estradiol [70], and since epidemiological evidence points to a correlation between breast cancer incidence and low levels of the precursor 25-$(OH)D_3$ [73], evaluation of the vitamin D system during progression of mammary carcinogenesis could be important. When normal breast tissue was compared with that derived from cancer patients, CYP27B1 mRNA was found in both tissues. In one study it was claimed, that expression was higher during early malignancy similar to colonic tissue [74]. Primary cultures established from human mammary tissue expressed CYP27B1 and were growth-inhibited by physiologically relevant concentrations of 25-$(OH)D_3$ [48], while

established breast cancer cell lines showed a wide range of vitamin D hydroxylase expression. In general, however, CYP27B1 mRNA expression is relatively low and that of CYP24A1 is rather high. For example, hydroxylation of 25-$(OH)D_3$ in MCF-7 cells occurred primarily on the C-24 pathway [38], though we were able to demonstrate that 17β-estradiol elevates CYP27B1 mRNA expression and activity in these cells as well [70]. Kemmis and Welsh [36] recently showed that oncogenic transformation of human mammary epithelial cells was associated with reduced 1,25-$(OH)_2D_3$ synthesis and decreased sensitivity to its antimitotic action. This suggests enhanced expression of the catabolic CYP24A1 during progression.

Growth and function of the prostate is dependent on androgens. Initial endocrine therapy in prostate cancer aims to eliminate androgenic activity from cells. However, cells invariably become refractory to this therapy and grow androgen-independently. During this progression, estrogen influence appears to increase and oxidative and reductive 17β-hydroxysteroid dehydrogenase activities are modified [75]. In another report, 17β-hydroxysteroid dehydrogenase subtypes 2, 4, and 5 were up-regulated in prostatic cell lines treated with 1,25-$(OH)_2D_3$ [76]. Interestingly, aromatase enzymatic activity was enhanced by 1,25-$(OH)_2D_3$ in prostate cancer cell lines suggesting synthesis of estradiol from testosterone, whereas 5α-reductase was not modified [77]. On the other hand, 1,25-$(OH)_2D_3$ apparently inhibited androgen glucuronidation and thus androgen inactivation [78], while it stimulated androgen receptor expression [79]. Quantification of CYP27B1 mRNA [80] and of enzymatic activity in prostate cancer compared with normal cells suggested deficiency during progression [35], which would result in reduced dependence on 25-$(OH)D_3$ for growth control.

1.2.4 Regulation of CYP27B1 and of CYP24A1 Expression by Splicing Mechanisms and Polymorphisms

Alternative gene splicing affects up to 70% of human genes and enhances genetic diversity by generating proteins with distinct new functions. In line with many cytochrome P450s, CYP27B1 is known to exhibit alternative splicing and, in kidney cells, this led to modified 1,25-$(OH)_2D_3$ synthesis [81]. There have been several reports on differential expression of splice variants for CYP27B1 also in cancerous cells derived from diverse tissues suggesting a role for gene splicing in tissue-specific regulation of 1,25-$(OH)_2D_3$ production [82–85]. In MCF-7 mammary cells, and several subclones of this cell line, at least six splice variants of CYP27B1 were detected resulting in at least six protein variants present in Western blots at varying band intensity [85]. It is yet unknown whether some of these splice variants present during breast tumor progression lack 1α-hydroxylation activity.

Splice variants of CYP24A1 could lead to abnormal vitamin D catabolism respectively reduced or enhanced 1,25-$(OH)_2D_3$ accumulation (see, e.g., [86, 128]). In prostate tumor-derived cell lines, constitutive CYP24A1 was expressed as a splice variant in some cells, whereas others had CYP24A1 splice variants after

treatment with 1,25-$(OH)_2D_3$ only [87]. In colon tumors, a CYP24A1 splice variant at 754 bp was much more prominent in differentiated (G1) tumors than in undifferentiated ones [25]. In colon cells derived from a G2 tumor, the normal CYP24A1 band as well as the variant were present with similar intensity, but the variant was not found in differentiated Caco-2 cells. This particular splice variant also disappeared after treatment with 1,25-$(OH)_2D_3$ [45].

Studies of genetic polymorphisms with respect to vitamin D hydroxylases are rare. In colon cancer patients, genetic variants of several markers, among them the VDR, were investigated to explore associations with microsatellite instability (MSI) or the CpG Island methylator phenotype (CIMP). Fok1 VDR polymorphism was associated with CIMP-positive tumors [88]. When investigating prostate tumors in a group of Caucasian and African American patients, several non-coding SNPs were identified in the CYP27B1 gene. However, these SNPs probably do not enhance susceptibility to tumors since they were found also in an unaffected control group [89]. Novel SNPs were detected in the human CYP24A1 promoter that did result in reduced expression of CYP24A1. This variant was found primarily in the African American population [90]. Since this population group is recognized to suffer from vitamin D insufficiency and to present with prostate tumors more frequently than Caucasian Americans, a relevance of this variant for protection against tumor incidence by the vitamin D system appears questionable.

1.2.5 Epigenetic Regulation of CYP27B1 and of CYP24A1 Expression

DNA methylation of cytosine residues of CpG islands in the promoter region of genes is associated with transcriptional silencing of gene expression in mammalian cells, while decreased methylation of CpG islands enhances gene activity. The CpG island methylator phenotype (CIMP) is a distinct phenotype in sporadic colorectal cancer. For instance, a CIMP-high status is significantly associated with tumors of the proximal colon. Also relative survival can be associated with methylation status [91]. While these studies certainly are not definitive yet, it seems unlikely that methylation/demethylation processes in general can be associated with colon tumor incidence; though CIMP status coupled with other information such as microsatellite instability could be used as a prognostic factor. However, methylation/demethylation processes concerning promoters of certain genes may predispose to, or protect against, sporadic malignancies.

In the normal colon, methylation is age- and also apparently site-related. When evaluating the promoter region of the estrogen receptor (ESR1), it was found to be more highly methylated (inactivated) in the human distal than in the proximal colon [72]. Since estrogen apparently enhances 1,25-$(OH)_2D_3$ synthesis in mucosal cells, this suggests that in women the distal colon is less protected by vitamin D against tumor incidence (see Sect. 1.2.3.).

Other genes modified by epigenetic events could be those coding for the vitamin D system. Kim et al. [92] demonstrated that the negative response element in the CYP27B1 promoter is regulated by the ligand-activated vitamin D receptor through recruitment of histone deacetylase, a critical step for chromatin structure remodeling in suppression of the CYP27B1 gene. In addition, this transrepression by VDR requires DNA methylation in the CYP27B1 gene promoter. However, this study was done in kidney cells and not in tumor-derived cells. Another study highlighted the relevance of different microenvironments (tumor versus normal) for the regulation of CYP24A1: CYP24A1 promoter hypermethylation was present in endothelial cells derived from tumors, but not from normal tissue [93].

In a mouse model of chemically induced colon cancer, protection against tumor incidence by estrogen was associated with decreased CpG island methylation of the VDR promoter and enhanced VDR expression [94]. When we tested colon cancer cell lines derived from moderately differentiated G2 tumors (COGA-1 cells) and from undifferentiated G3 tumors (COGA-13 cells) for expression of vitamin D hydroxylases and compared results with the differentiated colon cancer cell line Caco-2, it became evident that Caco-2 cells had high levels of CYP27B1 mRNA, while COGA-1 and COGA-13 had low expression or none. In contrast, constitutive CYP24A1 expression was extremely high in COGA-13, and not apparent in COGA-1 and Caco-2 cells (Fig. 1.1). Addition of the methyltransferase inhibitor 5-aza-2′-deoxycytidine induced CYP24A1 mRNA expression significantly in Caco-2 and also in COGA-1 cells. In COGA-13 cells, however, the methyltransferase inhibitor did not further raise the already high basal CYP24A1 expression. Interestingly, CYP27B1 appeared to be under epigenetic control as well, since COGA-1 and COGA-13 cells showed a distinct elevation of CYP27B1 mRNA after treatment with 5-aza-2′-deoxycytidine (Fig. 1.1) (Khorchide et al., manuscript in preparation).

Differences in expression of vitamin D hydroxylases in the course of tumor progression as observed in colon cancer patients [41, 54] could be caused by epigenetic regulation of gene activity via methylation/demethylation processes as well as histone acetylation/deacetylation. In low-grade cancerous lesions, CYP27B1 expression is exceedingly high compared to normal mucosa in non-cancer patients [26].

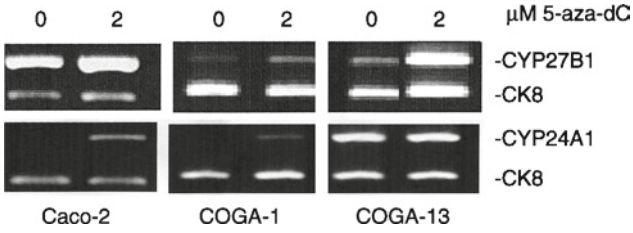

Fig. 1.1 Evaluation of CYP27B1 and CYP24A1 mRNA expression by RT-PCR in colon cancer cells. Cells were treated for 3 days with 2 μM 5-aza-deoxycytidine treatment. Caco-2, differentiated cells; COGA-1, established from a moderately differentiated tumor; COGA-13, established from an undifferentiated tumor. Reference mRNA was cytokeratin 8 (CK8)

Enhanced synthesis and accumulation of 1,25-$(OH)_2D_3$ in the colon mucosa would be responsible for up-regulation of transcriptional activity of CYP24A1 [34] and also for autocrine/paracrine inhibition of tumor cell growth. We suggest that this enhanced expression of CYP27B1 could be due, at least in part, to epigenetic regulation, i.e., demethylation, while raised CYP24A1 expression probably results from the normal regulatory loop following accumulation of 1,25-$(OH)_2D_3$ in colonic mucosa. However, in highly malignant tumors, an efficient antimitogenic effect by 1,25-$(OH)_2D_3$ is unlikely, because expression of the catabolic vitamin D hydroxylase by far exceeds that of CYP27B1. Our hypothesis, therefore, is that during cancer progression CYP27B1 would be inactivated by epigenetic mechanisms, whereas that of CYP24A1 would be activated. To test this, we studied expression of vitamin D hydroxylases in 105 colon tumor patients entering a Viennese hospital for tumor resection. Uncoupling of CYP24A1 expression from regulation by colonic 1,25-$(OH)_2D_3$ would lead to vitamin D hydroxylase expression in opposite directions during progression to a highly malignant state. This is actually the case: Transition from low- to high-grade cancers is associated with a further highly significant rise in CYP24A1 mRNA expression and a simultaneous decline of CYP27B1 activity (Fig. 1.2). Analysis of a selected (small) number of tumor biopsies

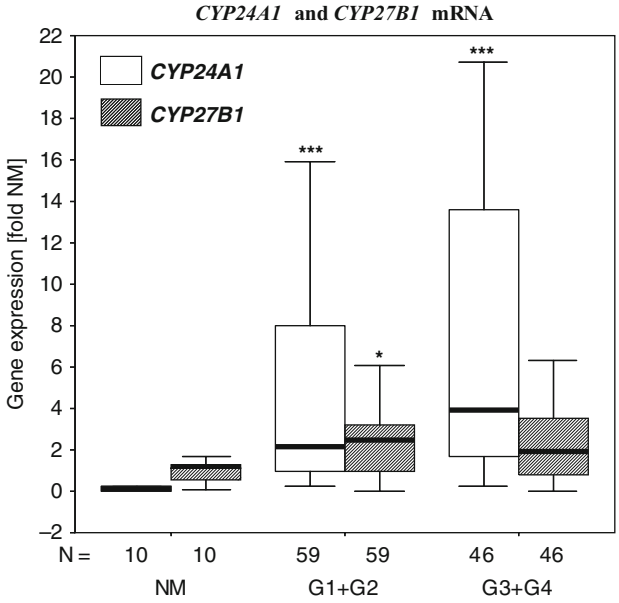

Fig. 1.2 CYP24A1 and CYP27B1 mRNA expression in 105 colon cancer patients. $n = 59$ patients with G1/G2 (highly to moderately differentiated) tumors; $n = 46$ patients with G3/G4 (low and undifferentiated) tumors. Cancer patient data were compared with those derived from non-cancer (NM) patients (tissue from stoma reoperations after diverticulitis surgery) $n = 10$. Densitometric data of tumor patients were expressed in fold increase compared to NM. Significant differences are expressed as: $*p \leq 0.05$; $***p \leq 0.001$

suggested that in poorly differentiated cancerous lesions, regions of the CYP24A1 promoter were demethylated and those of CYP27B1 were methylated (Khorchide et al., manuscript in preparation).

In prostate cells, Khorchide et al. [95] demonstrated that human normal prostate cells possess CYP27B1 expression, but are devoid of CYP24A1, whereas DU-145 prostate cancer cells display high CYP24A1 and very low CYP27B1 mRNA expression. Treatment with the methylation inhibitor 5-aza-2′-deoxycytidine together with the histone deacetylation (HDAC) inhibitor trichostatin A, elevated both CYP27B1 as well as CYP24A1 mRNA expression in the normal cell line. In DU-145 cells, 5-aza-2′-deoxycytidine plus trichostatin A elevated CYP27B1 mRNA and, importantly, also its activity as measured by HPLC [95]. Another HDAC inhibitor, SAHA, induced CYP27B1 mRNA expression in prostate cells as well, however at the high dose of 15 μM only [96]. In contrast, Banwell et al. were able to demonstrate that vitamin D-insensitive prostate and breast cells when treated with 1,25-$(OH)_2D_3$ together with nanomolar doses of HDAC inhibitors, were growth-inhibited synergistically. They suggest that insensitivity to vitamin D could be due to epigenetic mechanisms involving the VDR [97].

1.3 Regulation of CYP27B1 and of CYP24A1 Expression by Nutrition

The colorectum, as part of the digestive system, clearly is particularly affected by nutritional components. Therefore, this section will address nutrient regulation of vitamin D hydroxylases primarily in colorectal malignancies. However, there is some indication that also prostate as well as mammary cancer cells might be affected, though mechanistic evidence for this is more difficult to obtain.

It is clear that, for prevention of sporadic malignancies, average 25-$(OH)D_3$ levels at or above at least 40 nM need to be achieved in the general population, though there is still some discussion about the exact amount. However, optimization of extrarenal production of 1,25-$(OH)_2D_3$ is essential as well. Experimental proof is accumulating that nutrient factors such as calcium, phytoestrogens, and folate could regulate expression of vitamin D hydroxylases.

1.3.1 Regulation of Vitamin D Metabolism in the Gut Mucosa by Calcium

It is intriguing that vitamin D in combination with high intake of calcium from dietary sources or supplements, apparently is much more effective in reducing the risk of colorectal cancer than when given alone [98–100]. To investigate this further, we availed ourselves of a mouse model. Feeding male and female mice an AIN76 minimal diet containing 0.04% calcium led to enhanced positivity for

PCNA (proliferating cell nuclear antigen) and for cyclin D1, while that for p21, a cyclin-dependent kinase inhibitor, was diminished. Mice on a calcium-deficient diet also expressed CYP24A1 mRNA at a six- to eightfold higher level than their counterparts on a 0.9% calcium diet [27]. Interestingly, CYP27B1 mRNA was significantly up-regulated in animals on 0.04% compared to 0.9% calcium as well, though in female mice only [129]. Importantly, measurement of 1,25-(OH)D_3 concentrations in mucosal homogenates by a newly developed assay [127] indicated that up-regulation of CYP27B1 by low calcium is translated into increased CYP27B1 protein activity causing accumulation of 1,25-(OH)D_3 in colonic mucosal cells. In parallel, in these cells apoptotic pathways, i.e., expression of the downstream effector proteases, caspase-3 and of caspase 7, are stimulated. This strongly suggests that enhanced synthesis of 1,25-(OH)D_3 in females overrides the gender-independent stimulatory effect of low calcium on CYP24A1-mediated vitamin D catabolism, thereby providing protection against incipient hyperproliferation induced by inadequate calcium nutrition. This enhanced synthesizing activity occurred in the proximal colon only and suggests that there may be site-specific action of 17β-estradiol. As mentioned previously, the estrogen receptor ESR1 is more methylated (inactivated) in the human distal than in the proximal colon [72] (see also Sect. 1.2.3).

At present it is not clear whether signals from low luminal calcium are transduced by the calcium sensing receptor (CaR). Alternatively, a lack of calcium is known to increase concentrations of free bile acids in the gut lumen. Of these, lithocholic acid by binding to the VDR can induce expression of CYP24A1 [101]. Our results suggest that in humans also calcium supplementation could lower the risk of colorectal cancer because high dietary calcium suppresses vitamin D catabolism and this would favor accumulation of 1,25-(OH)D_3 in the colon mucosa. Furthermore, 1,25-(OH)D_3 would increase expression of the CaR by binding to a vitamin D responsive element in its promoter region [102].

1.3.2 Regulation of the Vitamin D System by Phytoestrogens

It can be inferred that in human colonocytes, estrogenic compounds have positive effects on endogenous synthesis of 1,25-$(OH)_2D_3$ and consequently on VDR-mediated anti-inflammatory and antimitogenic actions (see Sect. 1.2.3). In this context, it is of interest that in East Asian populations the risk of cancers of sex hormone-responsive organs, viz., breast and prostate gland, as well as of the colorectum is clearly lower than elsewhere. This has been traced to the typical diet in this part of the world, which is rich in soy products and therefore contains high amounts of phytoestrogens. Of these, genistein induced CYP27B1 and reduced CYP24A1 expression and activity in a mouse model and in human colon adenocarcinoma-derived cell lines [103], while daidzein, another phytoestrogen prominent in soy and, importantly, its metabolite equol, which is strongly active in other biological systems, did not affect any of the colonic vitamin D hydroxylases [70].

Genistein could also have anti-inflammatory properties in the colon: When mice were fed 0.04% dietary calcium, COX-2 mRNA and protein were increased two-fold in the female colon mucosa and to a lesser extent in males. Supplementation of genistein to the diet lowered COX-2 expression to control levels (0.5% dietary calcium) in both genders [104]. This suggests that genistein could have a beneficial effect on colonic inflammation similar to that seen with 17β-estradiol in the human pilot study described before (Sect. 1.2.3). Since genistein preferentially activates ER-β [105, 106], which is equally expressed in the colon of women and men, low rates of colorectal cancer incidence in both genders in soy-consuming populations could be due to appropriate modulation of the anti-inflammatory and anticancer potential of vitamin D by phytoestrogens.

Also the human prostate is frequently affected by inflammatory disease, which could predispose to development of malignancies. Since the inflammation-related prostaglandin pathway is negatively affected in prostate cancer cells by genistein [107], this suggests a potential mechanism of prostate cancer prevention in soy-consuming countries. Experimental data from Farhan et al. indicated that genistein very efficiently reduced the activity of CYP24A1 in human prostate cancer cells [57, 108], probably by direct binding to the CYP24A1 protein [58]. In contrast to the colon, genistein inhibited CYP27B1 mRNA expression in prostate cells, and this may involve histone deacetylation since trichostatin A rescues CYP27B1 from transcriptional inactivation [58] (see also [95]). Treatment of prostate cancer cells with 1,25-$(OH)_2D_3$ together with genistein potentiated the antimitotic activity of the active metabolite. This suggests an increased half-life of 1,25-$(OH)_2D_3$ due to inhibition of CYP24A1 activity [109], as already indicated in previous studies [58].

1.3.3 Effect of Folate on CYP24A1 Expression

Folate, a water-soluble vitamin of the B family, is essential for synthesis, repair, and methylation of DNA. As a methyl donor, folate could play an important role in epigenetic regulation of gene expression. While folic acid was supplemented to foods in the USA in the late 1990s to curb incidence of neural tube defects, and blood folate concentrations increased in the survey period shortly thereafter, there has been a decline since and its causes are unknown [110].

Sporadic cancers evolve over a lifetime and could therefore be at least equally affected by low folic acid intake as neural tube development. Older age and inadequate folate intake lead to altered methylation patterns [111]. Evidence is increasing that a low folate status predisposes to development of several common malignancies including colorectal cancer [112]. Giovannucci et al. [113] and others demonstrated that prolonged intake of folate above currently recommended levels significantly reduced the risk of colorectal cancer.

To investigate the relevance of folate for regulation of the vitamin D system, we used C57/BL6 mice on the semisynthetic AIN76A diet, which contained, among others, 5% fat, 0.025 μg/g vitamin D_3, 5 mg/g calcium, and 2 μg/g folic acid [114, 115].

When this basal diet was modified to contain high fat, low calcium, low vitamin D_3, and low folic acid, mice exhibited signs of hyperplasia and hyperproliferation in the colon mucosa [115], which were accompanied by a more than 2.5-fold elevated CYP24A1 mRNA expression [116]. When calcium and vitamin D_3 in the diet were optimized while fat was still high and folic acid low, CYP24A1 mRNA expression fell by 50%, but was still higher than in the colon mucosa of mice fed the basal (control) diet. Finally, when the diet contained high fat, low calcium, and low vitamin D, but folic acid content was optimized, only then any increment in colonic CYP24A1 due to dietary manipulations was completely abolished [116].

1.4 Can Regulation of Vitamin D Hydroxylases Be Implemented for Therapy?

The high levels of 1,25-$(OH)_2D_3$ respectively of its analogs initially used for cancer therapy invariably caused hypercalcemia. However, it was observed that doses of the active metabolite could be reduced without loss of activity when given as combination therapy.

1,25-$(OH)_2D_3$ and vitamin D analogs can enhance, either synergistically or additively, the antitumor activities of several classes of antineoplastic agents (see, e.g., [117–119]). This has led to several clinical studies with drugs such as docetaxel in combination with 1,25-$(OH)_2D_3$ in the treatment of androgen-independent prostate cancer, though mechanisms of action are poorly understood yet. It was observed that the antimitotic action of 1,25-$(OH)_2D_3$ associated with G0/G1 arrest, enhanced apoptosis, and differentiation could be achieved with lower concentrations of vitamin D substances when they were given to patients in combination therapy with cytotoxic agents such as carboplatin and taxanes. Even an intermittent 1,25-$(OH)_2D_3$ schedule was possible in this treatment regimen. It was also attempted to use ketoconazole, an unspecific cytochrome P450 inhibitor, for combination treatment. Very low doses of 1,25-$(OH)_2D_3$ could be used under such conditions since degradation of vitamin D was attenuated [120]. Recently it was demonstrated that antineoplastic agents themselves can target CYP24A1 for degradation by decreasing stability of CYP24A1 mRNA. When kidney cells positive for CYP27B1 were treated with 25-$(OH)D_3$, they synthesized 1,25-$(OH)_2D_3$ as expected. Treatment with daunorubicin, etoposide, and vincristine caused enhanced accumulation of 1,25-$(OH)_2D_3$. While CYP27B1 mRNA expression was not altered by cytotoxic drug treatment, that of CYP24A1 was reduced highly significantly [121]. Since mitogen-activated protein (MAP) kinases play an important role in mediating the stimulatory effect of 1,25-$(OH)_2D_3$ on CYP24A1 expression [122], and antineoplastic agents apparently stimulate activity of MAP kinases [123], this seems a likely mechanism of action.

Enhancing apoptotic activity of malignant cells could be another approach to cancer patient therapy. Pretreatment with a high dose of 1,25-$(OH)_2D_3$ augmented the antitumor activity of docetaxel, which manifested itself by an increased

population of apoptotic cells, raised Bax (a pro-apoptotic protein), and also reduced expression of a multidrug resistance-associated protein [124]. In an animal model for squamous cell carcinoma a combination of only 10 nM 1,25-$(OH)_2D_3$ together with cisplatin resulted in greater caspase-3 activation than either substance given alone. It was suggested that increased cytotoxicity resulting from a 1,25-$(OH)_2D_3$/cisplatin treatment could be due to raised 1,25-$(OH)_2D_3$-induced apoptotic signaling through the MEKK-1 pathway [118]. Also the anti-EGFR drug cetuximab applied together with 1,25-$(OH)_2D_3$ seems to provide increased cell cycle arrest and apoptosis in prostate cancer cell cultures [125].

Another valid approach to cancer therapy with 1,25-$(OH)_2D_3$ would be the use of vitamin D analogs to block CYP24A1 activity directly. A 24-phenylsulfone analog of vitamin D raised CYP24A1 mRNA expression in colon, prostate, and mammary cancer cells, but inhibited its activity very rapidly in a dose-dependent manner. This analog apparently binds to the VDR to stimulate transactivation, but also directly interacts with and inhibits CYP24A1 protein [126].

These few examples suggest that there are various options for the use of vitamin D for patient therapy. Most approaches are concerned with reducing activity of the catabolic hydroxylase CYP24A1. This is based on the hypothesis that reduced degradation of the active metabolite in combination therapy will allow the use of much lower concentrations of 1,25-$(OH)_2D_3$.

1.5 Conclusion

It is well-recognized that sporadic malignancies have a multifactorial etiology. While there is strong evidence that serum 25-$(OH)D_3$ levels are inversely related to tumor incidence, there are other factors equally important that will determine the optimal concentration of 1,25-$(OH)_2D_3$ synthesized from the precursor in extrarenal tissues. A person's genetic background with respect to VDR, CYP27B1 and CYP24A1 expression caused by specific splicing mechanisms and polymorphisms will determine production in kidney as well as in extrarenal cells. Growth factors and sex hormones regulate expression of vitamin D hydroxylases and of the VDR in several tissues known to be affected by sporadic cancers. Hyperproliferative cells early during tumor progression may express CYP27B1 strongly as a defense against progression, resulting in enhanced apoptosis and reduced mitosis. High concentrations of 1,25-$(OH)_2D_3$ in such tissues will invariably result in raised expression of the catabolic hydroxylase and this necessitates the use of potent CYP24A1 inhibitors to maintain tissue levels of the active metabolite. This highlights the need for reliable methods to measure tissue concentrations of 1,25-$(OH)_2D_3$. However, functional analysis of vitamin D metabolism in cancer is complicated by the heterogeneous composition of tumors, not only with respect to cell types but also to biological grade of cells. In at least 50% of G3 undifferentiated colon tumors, expression of CYP24A1 mRNA is extremely high whereas that of CYP27B1 is very low. This is probably because of epigenetic mechanisms and