VITAMIN D AND BREAST CANCER

VITAMINE D EN BORSTKANKER

PROEFSCHRIFT

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LIST OF ABBREVIATIONS

$1,25\text{-}(OH)_2D_3$	1,25-dihydroxyvitamin D_3, calcitriol
$17\beta\text{-}E_2$	17ß-oestradiol
ATP	adenosine 5'-triphosphate
BSA	bovine serum albumin
BW	bodyweight
cDNA	copy-deoxyribonucleic acid
Ci	Curie
cpm	counts per minute
CT-FCS	charcoal-treated foetal calf serum
DMBA	7,12-dimethylbenz[a]anthracene
DNA	deoxyribonucleic acid
EC_{50}	median effective concentration
EGF	epidermal growth factor
ER	oestrogen receptor
FCS	foetal calf serum
GAPDH	glyceraldehyde-3-phosphate dehydrogenase
GTP	guanosine 5'-triphosphate
IGF-I, II	insulin-like growth factor-I, II
IGF-BP	insulin-like growth factor binding protein
i.m.	intramuscularly
i.p.	intraperitoneally
kb	kilo basepair
K_D	dissociation constant
kDa	kilo Dalton
MAP	mitogen activated protein
mRNA	messenger ribonucleic acid
NMU	N-nitroso-N-methylurea
PBS	phosphate-buffered saline
PDGF	platelet-derived growth factor
PTH	parathyroid hormone
Rb	retinoblastoma susceptibility gene
s.c.	subcutaneously
SD	standard deviation
SDS	sodium dodecyl sulphate
SEM	standard error of the mean
SSC	standard saline citrate
SFM	serum-free medium
TGF-α, ß	transforming growth factor α, ß
VDR	vitamin D receptor
VDRE	vitamin D response element

Chapter 1

GENERAL INTRODUCTION

GENERAL INTRODUCTION

Vitamin D regulates calcium and phosphorus blood levels and bone metabolism via effects on intestine, kidney, and bone. Vitamin D is formed from 7-dehydrocholesterol in the skin by ultraviolet irradiation or is taken up from dietary sources. Vitamin D must be metabolically altered successively in the liver and kidney to form the biologically most active compound, 1,25-dihydroxyvitamin D_3. This active compound acts through binding to a specific receptor, the vitamin D receptor, which is a member of the steroid hormone receptor family.

During the last fifteen years, it has become apparent that the active form of vitamin D exerts effects on a variety of tissues apparently unrelated to calcium homeostasis. 1,25-Dihydroxyvitamin D_3 has been shown to promote cellular differentiation and inhibit proliferation of haematopoietic cells, cancer cells, and keratinocytes. In addition, studies with animal models for cancer have shown that 1,25-dihydroxyvitamin D_3 administration can prolong the survival of leukaemic mice and suppress the growth of tumours of different origin, including breast, colon, skin, and lung.

These newly discovered properties suggested a possible role of the hormone in the treatment of cancer. However, a major drawback for a clinical application is that high doses are needed. These doses cause serum levels of 1,25-dihydroxyvitamin D_3 far above the physiological level, which may lead to a dangerous elevation of the blood calcium concentration. To circumvent this problem many investigators have tried to change the 1,25-dihydroxyvitamin D_3 molecule in a way that it retains its antiproliferative and differentiation inducing activity, but has a reduced effect on calcium and bone metabolism. This strategy has resulted in new synthetic vitamin D_3 analogues with clinical potential.

This thesis focuses on a possible role of vitamin D in the treatment of breast cancer. For the experimental work breast cancer cell lines were used, which are tumour cells originally derived from patients. These cells are cultured continuously in the laboratory and form a model system for breast cancer, in which under defined conditions the effect of individual test compounds can be studied. The studies in this thesis were undertaken to gain insight into the mechanism of the tumour suppressive action of 1,25-dihydroxyvitamin D_3 and vitamin D_3 analogues. Furthermore, it was studied whether a combination therapy with tamoxifen, the widely used hormonal drug for breast cancer treatment, may have clinical potential.

Chapter 2

AN INTRODUCTION ON VITAMIN D METABOLISM, MECHANISM OF ACTION, FUNCTION, AND VITAMIN D ANALOGUES

Chapter 2

2.1 VITAMIN D METABOLISM

2.1.1 Sources of vitamin D

Vitamin D_3 (cholecalciferol) is either synthesized in the skin from 7-dehydrocholesterol in a reaction catalyzed by ultraviolet light, or is obtained from the diet. Adequate amounts of vitamin D_3 are produced in the skin with casual exposure to sunlight and therefore, vitamin D_3 is not a vitamin in the sense of an essential nutritional substance. The major dietary sources of this fat-soluble vitamin are fortified dairy products (milk, milk products, butter), margarine, fatty fish, and fish liver oils. Fortified foods contain either vitamin D_3 or vitamin D_2 (ergocalciferol) which behaves metabolically like vitamin D_3 (1-3).

2.1.2 Biological activation of vitamin D

Vitamin D_3 itself is biologically inert. First, it is metabolized in the liver to 25-hydroxyvitamin D_3 and then in the kidney to 1,25-dihydroxyvitamin D_3 (calcitriol), which is the biologically most active form (Figs. 2.1 and 2.2).

The hydroxylation of vitamin D_3 in the liver is catalyzed by the enzyme 25-hydroxylase. This metabolic step is not strictly regulated and 25-hydroxyvitamin D_3 is stable with a half-life of approximately three weeks. Accordingly, the serum concentration of 25-hydroxyvitamin D_3 is a reflection of the vitamin D status of a person and summates the concentrations derived from both the diet and photo-formation.

The conversion of 25-hydroxyvitamin D_3 into the active hormone 1,25-dihydroxyvitamin D_3 in the kidney by the enzyme 1α-hydroxylase is under stringent control. The most important stimulatory factors of 1α-hydroxylase activity are parathyroid hormone, and reduced plasma calcium and phosphate levels, whereas the 1α-hydroxylase activity is inhibited by 1,25-dihydroxyvitamin D_3 via a negative feedback loop. Other factors that have been implicated in the regulation of renal 1,25-dihydroxyvitamin D_3 synthesis are oestrogen, prolactin, growth hormone, and insulin. The half-life of 1,25-dihydroxyvitamin D_3 is 4 to 6 hours. As a result normal circulating levels of 1,25-dihydroxyvitamin D_3 are precisely controlled and fluctuate in response to the mineral needs of a person.

The kidney can also convert 25-hydroxyvitamin D_3 to 24,25-dihydroxyvitamin D_3 by the enzyme 24-hydroxylase. Under circumstances of relative vitamin D_3 deprivation the kidney produces only 1,25-dihydroxyvitamin D_3. If the supply of vitamin D_3 is adequate both dihydroxylated metabolites are formed. Whether 24,25-dihydroxyvita-

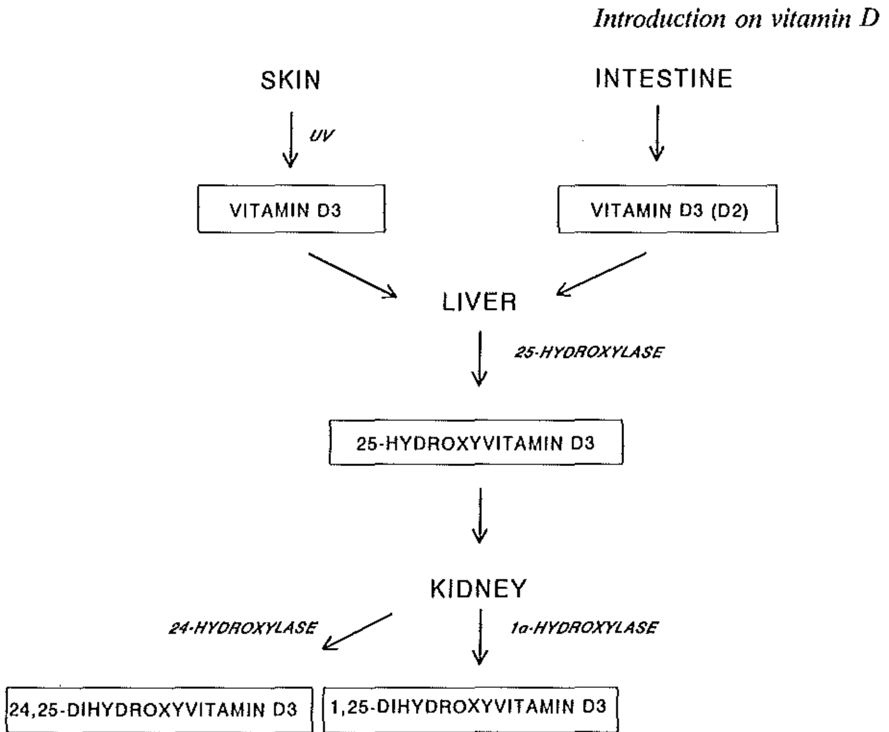

Figure 2.1. Metabolic activation of vitamin D.

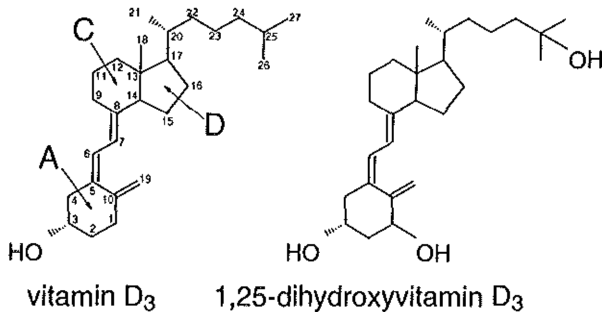

Figure 2.2. Chemical structures of vitamin D_3 and 1,25-dihydroxyvitamin D_3. The numbering of the carbon atoms and the ring nomenclature is shown for vitamin D_3. Vitamin D_3 has a side-chain of 8 carbon atoms (carbon 20-27). Note that the B ring is broken at the 9-10 carbon-carbon bond.

min D_3 has a biological role or whether its production initiates only a catabolic pathway is still under debate (1-3).

2.1.3 Inactivation of vitamin D metabolites

In addition to vitamin D_3 and its metabolites 25-hydroxyvitamin D_3, 1,25-dihydroxyvitamin D_3, and 24,25-dihydroxyvitamin D_3, a large number of other metabolites have been isolated from humans and animals. To date, these other metabolites have not been shown to possess any unique biological properties and appear to be breakdown products. The inactivation of vitamin D_3 metabolites takes place by sequential oxidations of the side-chain of the molecule (1,4).

2.1.4 Transport of vitamin D metabolites

Vitamin D_3 and its metabolites circulate in the blood primarily through binding to the vitamin D binding protein. This protein binds vitamin D_3 and its metabolites with a high affinity and is structurally homologous to two other blood proteins, albumin and α-fetoprotein. Besides binding to the vitamin D binding protein, vitamin D_3 metabolites bind with low affinity to albumin and low-density lipoproteins (5,6).

2.2 MECHANISM OF ACTION OF 1,25-DIHYDROXYVITAMIN D_3

2.2.1 The vitamin D receptor

The biological action of 1,25-dihydroxyvitamin D_3 is mediated through binding to a specific receptor, the vitamin D receptor. This receptor is an intracellular protein with a molecular weight of 50-60 kDa, which binds both 1,25-dihydroxyvitamin D_3 (K_D: 10^{-10}-10^{-11} M) and DNA. The vitamin D receptor is a member of the steroid receptor family, which are nuclear transcription factors. Other members of this family are the glucocorticoid, oestrogen, retinoic acid, and thyroid hormone receptor.

Comparison of the amino acid sequences of the vitamin D receptor and the other members of the steroid receptor family reveals a similarity in structural and functional organization. The proteins have six domains of evolutionarily conserved amino acids (region A to F in Fig. 2.3).

The region that exhibits the greatest degree of amino acid homology among the members of the receptor family is the DNA binding domain (region C). This region is required for DNA sequence recognition and receptor dimerization. The DNA binding domain consists of about 70 amino acids that folds in two so-called zinc-finger motifs.

Each finger binds a zinc ion that is tetrahedrally coordinated by four cysteines (Fig. 2.3).

The DNA binding domain is followed by a hinge region (region D) that links the DNA binding domain to the hormone binding domain (region E) located at the carboxy terminus of the receptor. The hormone binding domain, that encompasses about 210 amino acids, is moderately conserved and determines the steroid binding specifity of the receptor. It also participates in hormone-dependent transcriptional activation and receptor dimerization.

The amino-terminal domain (region A/B) is the most variable region and the length differs between the members of the family. The vitamin D receptor has a relatively short A/B domain of only 20 amino acids. The function of this region is less well-defined. For some receptors a transactivating function, which seems to be independent of ligand binding, has been assigned to this region (7-11).

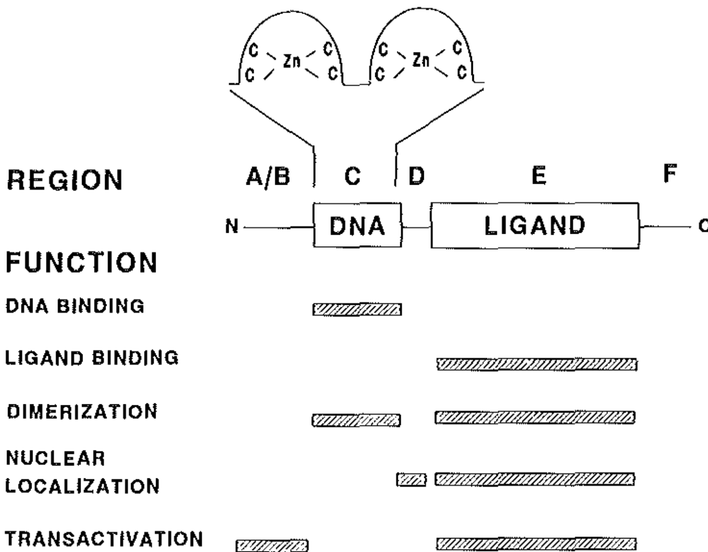

Figure 2.3. Structural and functional organization of steroid hormone receptors.

2.2.2 Phosphorylation of the vitamin D receptor

The vitamin D receptor, like other members of the steroid receptor family, can be phosphorylated (12). This phosphorylation is enhanced by the presence of 1,25-dihydroxyvitamin D_3. However, also ligand-independent phosphorylation has been observed. Certain kinases have been shown to phosphorylate the vitamin D receptor, including protein kinase C-ß (13,14), casein kinase II (15,16), and cAMP-dependent protein kinase (PK-A) (17). The phosphorylation occurs predominantly on serine residues. Specific phosphorylation sites have been located at serine residues 51 (13) and 208 (15) of the vitamin D receptor.

Although the exact function of the receptor phosphorylation is not clearly defined, several studies have shown that phosphorylation affects the transcriptional activation capacity of the vitamin D receptor complex (13,14,16-18).

2.2.3 Vitamin D response elements

A vitamin D response element is a nucleotide sequence located in the regulatory region of a gene, that is recognized by the DNA-binding domain of the vitamin D receptor. These sequences mediate the responsiveness of a gene to 1,25-dihydroxyvitamin D_3 through its receptor. The vitamin D response element is structurally similar to the general steroid hormone response elements.

Response elements are organized in two half-sites of 6 conserved nucleotides which are directly adjacent to each other or spaced by 1-5 unconserved nucleotides. The nucleotide sequence, orientation of the half-sites (direct repeat, palindromic, or inverted palindromic), and the spacing between the half-sites dictate selective recognition of the receptor. Vitamin D response elements have been identified in several genes that are induced by 1,25-dihydroxyvitamin D_3 (Table 2.1). Generally, the vitamin D response element is an imperfect direct repeat of 6 nucleotides separated by a 3-nucleotide spacer.

Receptors bind to their response elements as dimers, with each part of the dimer binding one half-site of the response element. The vitamin D receptor binds the vitamin D response element as a heterodimer with the retinoic acid receptor X-type (the retinoic X receptor). Alternatively, the vitamin D receptor may act as a homodimer or as a heterodimer with other, still unidentified nuclear accessory factors (8,11,19).

Table 2.1. Vitamin D response elements

Gene	Vitamin D response element
Rat osteocalcin	GGGTGA ATG AGGACA
Human osteocalcin	GGGTGA ACG GGGGCA
Mouse osteopontin	GGTTCA CGA GGTTCA
Rat calbindin D_{9K}	GGGTGT CGG AAGCCC
Rat 24-hydroxylase I	CGCACC CGC TGAACC
Rat 24-hydroxylase II	GCGGGA GTG AGTGGA
Mouse calbindin D_{28K}	GGGGGA TGTG AGGAGA
Human PTH	... TGAACC T...

Most vitamin D response elements (VDREs) involved in activation of gene expression are imperfect direct repeats of 6 nucleotides separated by a 3-nucleotide spacer. Exceptions are the mouse osteopontin VDRE that has two identical half-sites and the mouse calbindin D_{28K} VDRE that has a 4-nucleotide spacer. The human PTH VDRE is the first example of a VDRE that mediates suppression of gene transcription.

2.2.4 Modulation of gene expression by 1,25-dihydroxyvitamin D_3

The 1,25-dihydroxyvitamin D_3-receptor complex, once bound to the vitamin D response element, can either stimulate or repress the transcription rate of a target gene, thus resulting in an increased or decreased production of a mRNA species. This mRNA molecule is translated into a protein product, which after being processed, is ultimately responsible for the biological activity of 1,25-dihydroxyvitamin D_3 (Fig. 2.4).

It is not completely understood how the activated steroid hormone receptor, once positioned on the right promoter, activates gene transcription. Gene transcription involves the assembly of a stable preinitiation complex near the transcription start site. This complex is composed of multiple transcription initiation factors which interact with the DNA and/or with each other. When all factors are present RNA polymerase II can initiate the transcription of the target gene. Steroid receptors might stabilize the preinitiation complex, or stimulate the assembly of the complex. These interactions might involve co-activators that serve as a bridge between the receptor and the transcription initiation complex (7,9-11).

The mechanism behind negative regulation of transcription is also not well understood. For the human parathyroid hormone gene a distinct vitamin D response element (Table 2.1) has been identified, which suppresses gene expression in the presence of cell specific factors (20). In the osteocalcin promoter, the consensus sequence (AP-1 site) for the nuclear protooncogene encoded Fos and Jun proteins

overlaps the vitamin D receptor binding domain, and thereby one regulator (Fos-Jun dimer) may prevent the other regulator (1,25-dihydroxyvitamin D_3-receptor complex) from promoting transcription. Also, alternative mechanisms for hormonal repression have been proposed, e.g. a heterodimerization between the occupied receptor monomer and another nuclear factor, thereby preventing DNA binding or gene activation (9-11,21).

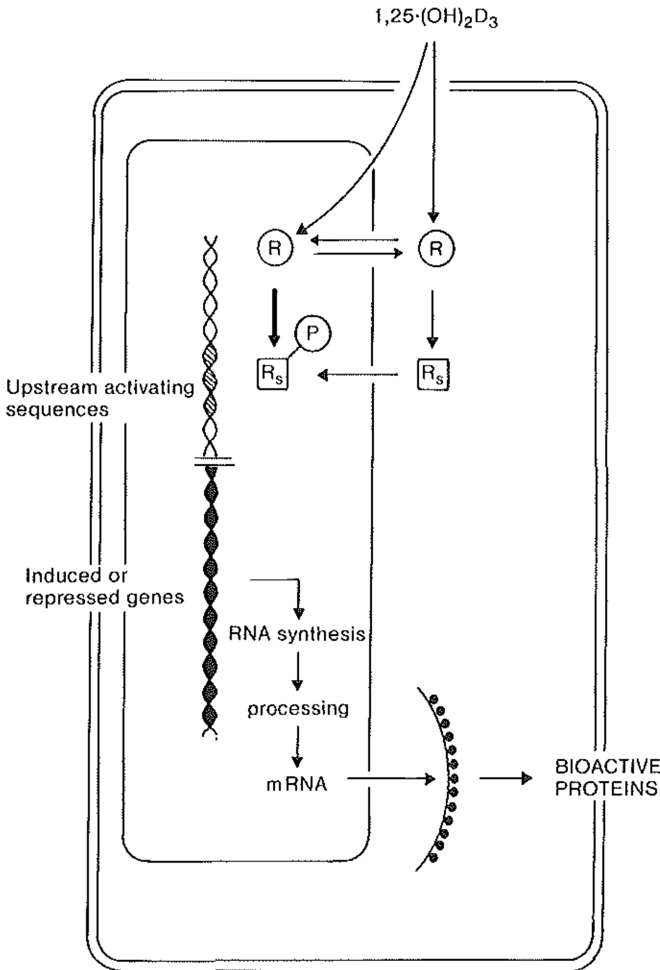

Figure 2.4. General mechanism of action of 1,25-dihydroxyvitamin D_3. R = unoccupied vitamin D receptor; R_S = occupied (phosphorylated) receptor

Table 2.2. A selection of vitamin D regulated genes

Gene or gene product	Regulation	VDRE	Gene or gene product	Regulation	VDRE
Mineral and bone metabolism			**Hormone and cytokine production**		
Calbindin D_{9K}	pos.	yes	Parathyroid hormone	neg.	yes
Calbindin D_{28K}	pos.	yes	Calcitonin	neg.	no
Collagen type I	neg.	yes	Interleukin II	neg.	no
24-Hydroxylase	pos.	yes	Tumour necrosis factor α	pos.	no
Osteocalcin	pos.	yes	**Proliferation and differentiation**		
Osteopontin	pos.	yes	fos	pos.	no
Vitamin D receptor	pos.	no	fms	pos.	no
Fibronectin	pos.	no	myb	neg.	no
			myc	neg.	no
			histone H4	neg.	no

An extensive number of genes are regulated by vitamin D. A selection of vitamin D regulated genes are indicated to illustrate the diversity of these genes. For references see Darwish and De Luca (11), Minghetti and Norman (22), and Walters (23). VDRE, vitamin D response element identified; pos./neg., positive/negative regulation of gene expression.

2.2.5 Vitamin D regulated genes

Several vitamin D regulated genes have thus far been identified in a number of different cell types and tissues from several species. These genes are associated with divergent functions, like mineral homeostasis, the metabolism of vitamin D, the secretion of peptide hormones, cellular proliferation and differentiation. A number of vitamin D regulated genes are shown in Table 2.2.

Despite the extensive number of genes and proteins that appear to be regulated by 1,25-dihydroxyvitamin D_3, evidence that the hormone directly modulates gene transcription exists only for some of these genes. Direct evidence achieved via the identification of a functional vitamin D response element has been obtained for the genes mentioned in Table 2.1.

2.2.6 Non-genomic mechanisms of action of vitamin D

1,25-Dihydroxyvitamin D_3 has been shown to regulate intracellular signalling processes, which have previously been associated with the signal transduction by polypeptide hormones through binding to their membrane-bound receptors. In a variety of tissues 1,25-dihydroxyvitamin D_3 has been shown to exert rapid effects

(within a few minutes) on intracellular calcium levels, phospholipid metabolism, and protein kinase C activity, which were insensitive to RNA and protein synthesis inhibitors (23-25). In the intestine, 1,25-dihydroxyvitamin D_3 induces a rapid stimulation of calcium transport, termed transcaltachia, which is also insensitive to RNA and protein synthesis inhibitors (26).

These data have indicated that the classical steroid hormone mechanism of action may not be the only mechanism by which 1,25-dihydroxyvitamin D_3 mediates cellular responses. To account for these apparently non-genomic actions a membrane-bound vitamin D receptor has been suggested, which may act in analogy to peptide hormone receptors (25). Recently, a 1,25-dihydroxyvitamin D_3 receptor has been isolated from the basal lateral membrane of chick intestinal epithelium, which is implicated in transcaltachia (27). However, in a number of other systems, genomic effects seemed to be involved in the 1,25-dihydroxyvitamin D_3-induced changes of intracellular signalling processes (23). In these cases the 1,25-dihydroxyvitamin D_3-induced calcium influx was slow and sustained, and abolished by RNA and protein synthesis inhibitors. Also, several reports have shown that 1,25-dihydroxyvitamin D_3-induced changes in phospholipid biosynthesis were sensitive to RNA and protein synthesis inhibitors, and a report of Obeid *et al.* (28) showed that 1,25-dihydroxyvitamin D_3 induces protein kinase C gene transcription.

2.3 1,25-DIHYDROXYVITAMIN D_3 REGULATES MINERAL AND BONE METABOLISM

2.3.1 Maintenance of mineral homeostasis

The principal physiological role of 1,25-dihydroxyvitamin D_3 is the regulation of plasma calcium and phosphate levels. This is achieved through actions on the intestine, kidney, and bone. The main action of 1,25-dihydroxyvitamin D_3 is to stimulate the absorption of dietary calcium and phosphate from the intestine. Further, 1,25-dihydroxyvitamin D_3 modulates calcium and phosphate reabsorption in the kidney, and when the dietary calcium intake is insufficient, 1,25-dihydroxyvitamin D_3 mobilizes calcium from bone.

1,25-dihydroxyvitamin D_3 works in concert with parathyroid hormone. A low serum calcium concentration stimulates the parathyroid glands to secrete parathyroid hormone. This hormone acts on bone and kidney to increase the serum calcium concentration. In addition, parathyroid hormone increases the serum calcium concentration indirectly via the stimulation of 1,25-dihydroxyvitamin D_3 synthesis in

the kidney (see Section 2.1.2). 1,25-Dihydroxyvitamin D_3 and an elevated serum calcium concentration suppress the production of parathyroid hormone in a negative feedback loop (1,3,29).

2.3.2 Bone metabolism

Bone tissue undergoes constant remodelling which is mediated by the bone resorbing cells, the osteoclasts, and the bone forming cells, the osteoblasts. Under normal conditions bone resorption and bone formation are in equilibrium. 1,25-Dihydroxyvitamin D_3, parathyroid hormone and a variety of other local and systemic factors regulate these dual processes.

1,25-Dihydroxyvitamin D_3 is an essential hormone for bone mineralization as well as a potent bone resorbing agent. 1,25-Dihydroxyvitamin D_3 promotes the mineralization of bone indirectly by providing calcium and phosphorus (see Section 2.3.1). On the other hand, 1,25-dihydroxyvitamin D_3 influences the action of the osteoblasts, which possess vitamin D receptors. A variety of effects of 1,25-dihydroxyvitamin D_3 on osteoblasts have been reported, e.g. regulation of osteoblast proliferation, regulation of the production of alkaline phosphatase, osteocalcin, osteopontin, and procollagen type I.

The osteoclasts are responsible for bone resorption. 1,25-Dihydroxyvitamin D_3 does not exert direct effects on osteoclasts, which lack vitamin D receptors, but influences the osteoblasts to secrete as yet uncharacterized factors that activate osteoclasts to resorb bone. Additionally, 1,25-dihydroxyvitamin D_3 plays a role in osteoclastgenesis (see Section 2.4.2) and thereby influences the bone resorption process on the long term (1,3,30,31).

2.4 1,25-DIHYDROXYVITAMIN D_3 MODULATES CELLULAR PROLIFERATION AND DIFFERENTIATION: THE BASIS OF NEW CLINICAL APPLICATIONS

2.4.1 Tissue distribution of the vitamin D receptor

The vitamin D receptor is not confined to tissues that play recognized roles in mineral metabolism, like intestine, kidney, bone, and parathyroid gland. Also a wide variety of tissues not primarily related to calcium and bone metabolism have been shown to express the vitamin D receptor. In addition to normal tissues and organs, the vitamin D receptor has been demonstrated in a number of malignant cell types.

Given the widespread tissue distribution of the vitamin D receptor, there are many putative target tissues for 1,25-dihydroxyvitamin D_3 (1,23).

2.4.2 Haematopoietic cells

A role of 1,25-dihydroxyvitamin D_3 in the differentiation of haematopoietic stem cells was suggested in 1981 by Abe and coworkers (32). They have demonstrated that immature mouse myeloid leukaemia cells differentiated towards more mature macrophage-like cells. This differentiation process was accompanied by a reduced growth rate. These observations were later extended to human leukaemic cell lines, and currently the cell lines HL60 (human promyelocytic leukaemia) and U937 (human histiocytic lymphoma) are frequently used to study the effects of vitamin D_3 compounds on haematopoietic differentiation and proliferation.

These *in vitro* studies suggested that 1,25-dihydroxyvitamin D_3 may be of clinical use in the treatment of leukaemia, since the hormone may induce the abnormal haematopoietic cells to differentiate into more mature, less aggressive cells. Honma and coworkers (33) suggested for the first time that a differentiation therapy with 1,25-dihydroxyvitamin D_3 may be operative *in vivo*: the survival of athymic mice that had been inoculated with mouse myeloid leukaemia cells was prolonged significantly by the administration of 1α-hydroxyvitamin D_3.

In addition to leukaemic cells, 1,25-dihydroxyvitamin D_3 induces differentiation of normal bone marrow cells. Immature bone marrow cells of the monocyte-macrophage lineage are believed to be precursors of the osteoclasts. 1,25-dihydroxyvitamin D_3 induces differentiation of immature myeloid cells towards monocytes-macrophages, and also activation and fusion of some macrophages. From these results is has been postulated that 1,25-dihydroxyvitamin D_3 stimulates differentiation and fusion of osteoclast progenitors into osteoclasts (31,34). Thus, the differentiation inducing capacity of 1,25-dihydroxyvitamin D_3 may also play an important role in the regulation of calcium and bone metabolism.

1,25-Dihydroxyvitamin D_3 has been shown to affect a wide range of specific functions of haematopoietic cells, e.g. it has been shown to inhibit the production of interleukin-2, interferon-gamma, and granulocyte-macrophage colony-stimulating factor by activated T cells, to inhibit the production of immunoglobulins by activated B cells, and to modulate the production of tumour necrosis factor-α and interleukin-1 by monocytes (35-38). Although dependent on the culture conditions and mode of activation, the *in vitro* effects of 1,25-dihydroxyvitamin D_3 are mainly immunosuppressive. By contrast, the effects of 1,25-dihydroxyvitamin D_3 on immune responses *in vivo* are more complex. In patients, a 1,25-dihydroxyvitamin D_3 deficient

or resistant state is associated with an impaired rather than enhanced immunity, indicating a role of vitamin D in sustaining normal functions of the immune system. On the other hand, an immunosuppressive effect of 1,25-dihydroxyvitamin D_3 administration has been observed in animal models for autoimmunity and transplantation (35-38).

2.4.3 Skin

The skin may be another tissue in which 1,25-dihydroxyvitamin D_3 exerts effects on cellular growth and differentiation. 1,25-Dihydroxyvitamin D_3 causes a decrease in proliferation and an increase in the morphological and biochemical differentiation of cultured keratinocytes (34,39).

In psoriasis the normal proliferation and maturation of epidermal cells is disturbed, resulting in a hyperproliferative state, and in addition, inflammatory processes are involved in this skin disease. Both processes represent potential targets for 1,25-dihydroxyvitamin D_3. The realization that 1,25-dihydroxyvitamin D_3 may have a role in the treatment of psoriasis, came from a case report of Morimoto *et al.* (40) in which a woman, treated with 1α-hydroxyvitamin D_3 for osteoporosis, had a dramatic remission of her psoriasis. This report was followed by a number of promising clinical studies with 1,25-dihydroxyvitamin D_3, 1,24-dihydroxyvitamin D_3, and 1α-hydroxyvitamin D_3, given orally or applied topically (38,41,42).

A problem of treatment with these vitamin D_3 metabolites is the risk of calcium-related side-effects. This applies both for oral and topical administration as vitamin D_3 is absorbed by the skin. Calcipotriol (MC903) is a synthetic 1,24-dihydroxyvitamin D_3 analogue containing a double bond and a cyclopropane ring in the side-chain (Fig. 2.5). These modifications result in a rapid conversion into inactive metabolites when given intravenously to rats (43). Therefore, calcipotriol is less suitable for systemic treatment. However, clinical studies have indicated that topical application of calcipotriol ointment is an effective and safe drug for the treatment of mild to moderate psoriasis (38,41,42). Calcipotriol ointment (Daivonex, LEO Pharmaceutical Products) is now registrated as treatment of psoriasis in many countries.

Table 2.3. *In vivo* effects of 1,25-dihydroxyvitamin D_3 and 1α-hydroxyvitamin D_3 in animal models for cancer

Tumour	Model	Administration[a]	Effect	Ref.
Breast	NMU[b]-induced breast cancer in rats	1α-(OH)D_3[f], i.p.	Tumour suppression	51
Breast	DMBA[c]-induced breast cancer in rats	1α-(OH)D_3, orally	Tumour suppression	58
Colon	Human colon cell line implanted into nude mice	1,25-$(OH)_2D_3$[g], i.p.	Tumour suppression	56,57
Colon	DMH[d]-induced colon cancer in rats	1,25-$(OH)_2D_3$, s.c.	Reduction of the incidence of colon adenocarcinomas	63
Leydig tumour	Leydig cell tumour implanted into rats	1,25-$(OH)_2D_3$, osmotic minipumps	Tumour suppression	60
Lung	Implantation of Lewis lung carcinoma into mice	1α-(OH)D_3, stomach tube	Reduction of the number of metastases (without suppression of primary tumour)	54
Lung	Implantation of lung carcinoma cells into mice	1,25-$(OH)_2D_3$, i.p.	Reduction of the number of metastases (without suppression of primary tumour)	59
Melanoma	Human melanoma cells implanted into nude mice	1,25-$(OH)_2D_3$, i.p.	Tumour suppression	56
Osteosarcoma	Human osteosarcoma cells implanted into nude mice	1α-(OH)D_3, i.p.	Tumour suppression	62
Retinoblastoma	Retinoblastoma cell line implanted into nude mice	1,25-$(OH)_2D_3$, i.p.	Tumour suppression	55
Retinoblastoma	Transgenic mice with retinoblastoma	1,25-$(OH)_2D_3$, i.p.	Tumour suppression	61
Skin	DMBA/TPA[e]-induced skin tumours in mice	1,25-$(OH)_2D_3$, topical	Inhibition of tumour formation	52
Skin	DMBA/TPA-induced skin tumours in mice	1,25-$(OH)_2D_3$, topical	Inhibition of tumour formation	53

[a]The dosage, duration of treatment, diet, and effects on serum/urinary calcium vary between the studies; [b]NMU, nitrosomethylurea; [c]DMBA, 7,12-dimethylbenz[a]anthracene; [d]DMH, 1,2-dimethylhydrazine dihydrochloride; [e]TPA, 12-O-tetradecanoylphorbol-13-acetate; [f]1α-(OH)D_3, 1α-hydroxyvitamin D_3; [g]1,25-$(OH)_2D_3$, 1,25-dihydroxyvitamin D_3.

2.4.4 Carcinoma cells

Receptors for 1,25-dihydroxyvitamin D_3 have been demonstrated in a variety of non-leukaemia cancer cell lines. Analogous to its effects on leukaemic cell lines, 1,25-dihydroxyvitamin D_3 induces differentiation and/or inhibits the proliferation of a wide variety of cell lines that have been established from solid tumours, including breast (44,45), colon (46,47), prostate (48,49), and melanoma (44,50). Several *in vivo* studies have confirmed the antiproliferative action observed *in vitro*. Table 2.3 summarizes the effects of 1,25-dihydroxyvitamin D_3 and 1α-hydroxyvitamin D_3 in animal models for cancer.

2.5 DEVELOPMENT OF NEW VITAMIN D_3 ANALOGUES

2.5.1 Rationale

As discussed in Section 2.4, there are new putative applications for 1,25-dihydroxyvitamin D_3 in the treatment of hyperproliferative disorders and immune-mediated diseases. However, the major problem is that higher than physiological doses of 1,25-dihydroxyvitamin D_3 are required to be effective. Thus, the use of 1,25-dihydroxyvitamin D_3 is restricted by a considerable risk on severe complications, like hypercalcaemia, calciuria, soft tissue calcification, and increased bone turnover.

To overcome these problems several pharmaceutical companies have developed new synthetic vitamin D_3 analogues in order to improve the selectivity of the hormone and to reduce the dosage needed. The clinical potential of the hormone will increase when vitamin D_3 analogues are available that have potent antiproliferative and differentiation inducing effects, or potent immunosuppressive effects, whereas the effects on calcium and bone metabolism are reduced (38,64).

2.5.2 *In vitro* and *in vivo* activity of new vitamin D_3 analogues

The majority of the new vitamin D_3 analogues synthesized have modifications in the side-chain of the 1,25-dihydroxyvitamin D_3 molecule (Fig. 2.5). Their ability to decrease proliferation and enhance differentiation is usually tested using the leukaemic cell lines U937 and HL60, which have well-defined differentiation markers. In Table 2.4 the *in vitro* growth inhibitory and differentiation inducing potential of some of the main analogues are compared to those of the native compound 1,25-dihydroxyvitamin D_3. Although the maximal inhibition of these analogues is not dramatically increased, the median effective concentrations (EC_{50}) of the analogues are sometimes several orders of magnitude lower.

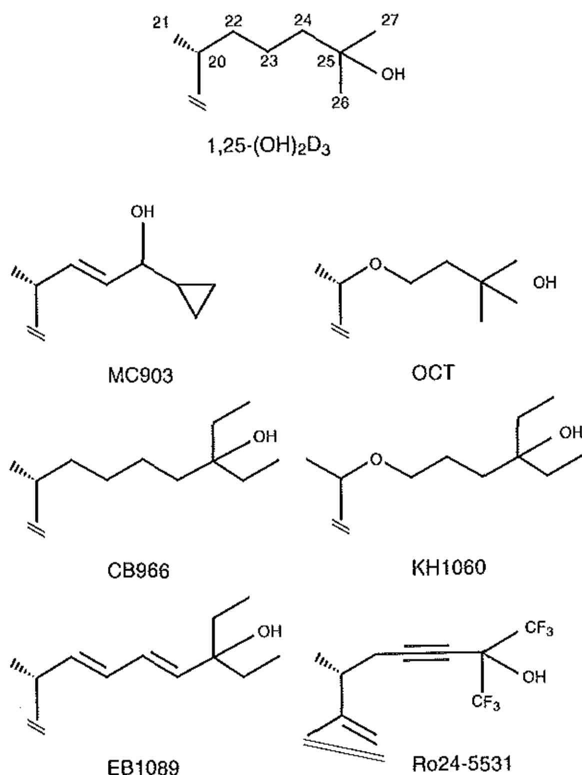

Figure 2.5. Side-chain modifications of 1,25-dihydroxyvitamin D_3. The analogues shown are discussed in the text. MC903 is also referred to as calcipotriol, OCT as 22-oxa-calcitriol, and Ro24-5531 as 1α,25-dihydroxy-16-ene-23-yne-26,27-hexafluoro-vitamin D_3. Note that Ro24-5531 has also a modification of the D ring.

The calcaemic activity of new analogues is tested by measuring serum calcium levels and/or urinary calcium excretion of rodents treated with an analogue or, alternatively, by measuring the intestinal calcium absorption in rachitic chickens. The analogues that have an increased growth inhibitory potential, but a similar or even reduced calcaemic activity compared to 1,25-dihydroxyvitamin D_3, offer promise for clinical applications (Table 2.4). Ultimately, these analogues have to be tested in animal models and in clinical studies to investigate whether the analogues inhibit tumour growth *in vivo*.

So far, only a few analogues have been tested in experimental animal models. The compound 1,25-dihydroxy-16-ene-23-yne-vitamin D_3 (Ro23-7553) increased the

Table 2.4. Cell regulating and calcaemic activity of vitamin D_3 analogues compared to 1,25-dihydroxyvitamin D_3

	RELATIVE TO 1,25-$(OH)_2D_3$			
Vitamin D_3 analogue	Inhibition of proliferation	Induction of differentiation	Calcaemic activity	Ref.
1,25-$(OH)_2D_3$	1	1	1	-
CB966	8	5	0.2	72
EB1089	68	67	0.4	73
KH1060	14,000	200,000	1.3	72
MC903	2	1	0.01	75
OCT	10	10	0.01	74,78
Ro23-7553	4	2	0.03	77
Ro24-5531	80	10	0.07	76

The effects on proliferation and differentiation were measured *in vitro* using U937 or HL60 leukaemic cell lines. Based on the EC_{50} values the relative potency compared to 1,25-dihydroxyvitamin D_3 was determined. The calcaemic effects were determined *in vivo* by measuring serum calcium levels or urinary calcium excretion in rats or mice, or by measuring the intestinal calcium absorption in chickens. The chemical structures of the analogues are shown in Figure 2.5, with the exception of Ro23-7553, which is 1,25-dihydroxy-16-ene-23-yne-vitamin D_3.

survival time of mice inoculated with leukaemic cells, whereas no measurable hypercalcaemia developed (65). The analogue EB1089 prolonged the survival time of rats implanted with Leydig tumours (60). Further, the analogues 22-oxa-calcitriol (OCT) (66,67), EB1089 (68,69), CB966 (68), and 1α,25-dihydroxy-16-ene-23-yne-26,27-hexafluoro-vitamin D_3 (Ro24-5531) (70) have shown promising results after systemic treatment of rats with mammary tumours. The mammary tumour models are further discussed in Section 3.5. In the only clinical study, which reported tumoursuppressive effects of vitamin D_3 analogues, it was shown that topical application of calcipotriol (MC903) on cutaneous deposits did prove effective in some patients with advanced breast cancer (71).

2.5.3 Possible mechanisms for the selective actions of vitamin D_3 analogues

In this section I will discuss a number of mechanisms to explain how the action of a vitamin D_3 analogue can differ from the action of 1,25-dihydroxyvitamin D_3 in 1) the activity, i.e. a specific biological response, like inhibition of cell growth, is achieved by lower concentrations of the analogue than of 1,25-dihydroxyvitamin D_3, and 2) the selectivity, i.e. suppression of tumour growth without raising serum calcium.

For a selective action on different target organs the pharmacological differences between the analogues and 1,25-dihydroxyvitamin D_3 are believed to play an important role. In serum the binding of the analogues to the vitamin D binding protein may affect the biological half-life of the analogues. A decreased binding may result in a rapid clearance of the analogue resulting in a decreased biological activity. On the other hand, low binding may result in a higher concentration of free, biologically available analogue compared to 1,25-dihydroxyvitamin D_3 (79,80). The transport of analogues may also proceed via other carrier proteins than the vitamin D binding protein. The analogue 22-oxa-calcitriol mainly circulates in the blood bound to lipoproteins, resulting in an altered pharmacokinetic profile compared to 1,25-dihydroxyvitamin D_3 (81). Thereby, an altered transport may account for a selective action on different target tissues.

Each action of the vitamin D_3 compounds has a distinct biological half-life. For example, Brown *et al.* (82) have shown that the rate of calcium transport returns to pretreatment levels within a few hours after 22-oxa-calcitriol disappears from the circulation. However, 22-oxa-calcitriol has much more prolonged effect on other processes such as induction of calbindin D_{9K} mRNA and 24-hydroxylase activity, and suppression of PTH secretion and 1α-hydroxylase activity. The short circulating half-life of 22-oxa-calcitriol might exploit the differences in the biological half-life between calcium transport and other processes to provide a selective action.

An altered metabolism at cellular level might contribute to the altered activity of vitamin D_3 analogues. Modifications in the side-chain influence the catabolism of the molecule, because the side-chain is sensitive to oxidation. An impaired cellular metabolism may lead to an accumulation of the analogue in the target cell. Alternatively, the analogue may be metabolized intracellularly into an active metabolite, as has been reported for 26,27-hexafluoro-1,25-dihydroxyvitamin D_3 (83). An additional factor involved in the selectivity of the analogues might be a differential intracellular metabolism in different target tissues. For example, the ability to degrade 22-oxa-calcitriol or 1,25-dihydroxyvitamin D_3 was shown to differ between normal monocytes and parathyroid cells (84).

The vitamin D receptor may play an important role in the selectivity and activity of the vitamin D compounds. An increased binding affinity to the vitamin D receptor could theoretically result in an increased activity. However, data obtained so far have shown no direct correlation between receptor binding and the biological activity of the analogues. Nevertheless, the presence of a vitamin D receptor seems to be essential for biological activity of 1,25-dihydroxyvitamin D_3 or vitamin D_3 analogues, since vitamin D receptor-negative cells are insensitive to the action of vitamin D_3 compounds (56,85). A selective action may be achieved via different types of the vitamin D receptor in the various target organs. However, it is currently accepted that vitamin D receptors are identical in all cell types.

Binding of an analogue instead of 1,25-dihydroxyvitamin D_3 to the vitamin D receptor may induce a different conformational change of the vitamin D receptor complex. This may lead to alterations in DNA binding, dimerization, phosphorylation, and intracellular clearance of the receptor complex, thus resulting in an altered transcriptional activation. All these changes may cause cell- and gene-specific actions of vitamin D_3 analogues and may alter the magnitude of the biological response.

Finally, there may be a difference between 1,25-dihydroxyvitamin D_3 and vitamin D_3 analogues in the activation of intracellular signalling processes via an apparently non-genomic mechanism of action (see Section 2.2.6). The contribution of these non-genomic processes to the ultimate biological response may vary between cell types. Thereby, a differential activation of non-genomic processes may result in a selective action (86,87).

A number of possibilities have been mentioned to explain the selectivity and altered activity of some vitamin D_3 analogues. However, the precise mechanism of action of the vitamin D_3 analogues has not yet been unravelled. Besides, it is conceivable that the explanation will differ for each of the analogues. Further research in this complex area will lead to a better understanding of the mechanism of action of vitamin D_3 analogues, which is important to enable investigators to optimize vitamin D_3 analogues for a specific application.

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Chapter 3

THE ROLE OF VITAMIN D IN THE TREATMENT OF BREAST CANCER

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3.1 BREAST CANCER

Breast cancer is the most frequent cause of cancer deaths in women in the western world. Breast cancer incidence and mortality rates for women in the USA are approximately 11 % and 3 %, respectively. A number of risk factors have been linked to the development of breast cancer, and many of these are related to oestrogens. Other factors are age, family history, and location of the country of residence (1-3).

Breast carcinomas derive from the epithelial lining of the milk ducts and the duct lobules. The tumour can either be confined within the ducts or lobules, or invades the surrounding tissue and gives rise to distant metastases. Many studies have tried to identify the causal factors responsible for the uncontrolled growth of the tumour cells. A variety of biochemical and genetic changes have been identified in breast carcinomas, and a wide range of factors have been shown to influence breast cancer growth. However, the exact mechanism of breast cancer development and progression has not been unravelled. The heterogeneity of the disease on the clinical, biological, and genetic levels further complicates this research.

3.2 SUNLIGHT AND BREAST CANCER INCIDENCE

Descriptive epidemiological studies have indicated that vitamin D might play a protective role against breast cancer. Incidence and mortality of breast cancer vary considerably world-wide and reveal a geographical pattern. The lowest rates of breast cancer generally occur in countries close to the equator. As the latitude increases, reported breast cancer incidence and mortality rates also increase. Studies in the USA and the former USSR have shown a negative correlation between available sunlight and breast cancer death rates. Sunlight exposure is a measure of vitamin D produced in the skin (see Section 2.1.1). Further, it is known that the primary source of vitamin D for adults in the USA is casual exposure to sunlight. Therefore, it has been hypothesized that vitamin D formed in the skin may reduce the risk of breast cancer (4,5).

3.3 THE VITAMIN D RECEPTOR IN BREAST CANCER

The presence of the vitamin D receptor in breast cancer was first demonstrated in the human breast cancer cell line MCF-7 (6). Later studies have extended this

finding to other breast cancer cell lines and to surgically obtained normal breast and breast tumour tissue (7).

The vitamin D receptor is present in approximately 80 % of human breast tumour specimens. The presence of the vitamin D receptor is not correlated to the presence of other steroid hormone receptors (oestrogen receptor, progesterone receptor) (8-11). Also, no relationship was observed between vitamin D receptor status and clinical indices (age, menopausal status, T-stage, histology, lymphnode involvement) and overall survival (9-12). However, two studies reported that the receptor status correlated positively with disease-free interval (11,12).

Regulation of the vitamin D receptor number may affect the cellular responsiveness to 1,25-dihydroxyvitamin D_3, the active form of vitamin D_3. In a number of different systems regulation of the vitamin D receptor by 1,25-dihydroxyvitamin D_3 itself (homologous upregulation), and by hormones and growth factors has been demonstrated (13-15). In breast cancer a homologous upregulation of the vitamin D receptor has been observed in MCF-7 cells (16), and a heterologous upregulation by serum, growth factors (EGF, insulin, IGF-I), and oestradiol was noticed in MCF-7 and T47-D cells (17,18).

3.4 VITAMIN D INHIBITS THE GROWTH OF CULTURED BREAST CANCER CELLS

Breast cancer cell lines have been established from primary breast tumours and pleural effusions of patients with advanced breast cancer. These cell lines have been proven to be very useful in studies of regulation of cell growth by steroid hormones and polypeptide growth factors. A number of cell lines have been widely used as models of oestrogen-responsive breast cancer, because they contain oestrogen receptors and their growth is stimulated by oestrogens. Oestrogen receptor-negative cell lines provide an *in vitro* model for oestrogen unresponsive breast cancer.

The first studies on the effect of 1,25-dihydroxyvitamin D_3 on breast cancer cells showed a biphasic growth response of the oestrogen receptor-positive T47-D human breast tumour cell line. At low concentrations (10^{-11} M) a stimulation of cell growth was observed, whereas at higher concentrations (10^{-8} M) an inhibition was observed (7,19,20). The presence of a biphasic effect and the extent of the growth inhibition was shown to be dependent on the culture conditions, i.e. the concentration and charcoal treatment of foetal calf serum supplemented to the culture medium (7,20,21).

Table 3.1. Inhibition of breast cancer cell growth by vitamin D_3 analogues

Vitamin D_3 analogue	Breast cancer cell line	Oestrogen receptor status	Relative potency compared to 1,25-$(OH)_2D_3$	Ref.
OCT	MCF-7	ER-pos.	approx. 10	22
	ZR-75-1	ER-pos.	"	
	T47-D	ER-pos.	"	
	MDA-MB-231	ER-neg.	"	
	BT-20	ER-neg.	"	
EB1089	MCF-7	ER-pos.	50	25
KH1060	T47-D	ER-pos.	800	27
MC903	MCF-7	ER-pos.	1	28
Ro24-5531	MCF-7	ER-pos.	10 - 100	26
	T47-D	ER-pos.	"	

Based on the EC_{50} values for the growth inhibition, the relative potency compared to 1,25-dihydroxyvitamin D_3 was measured. A relative potency of 1 means equally potent as 1,25-dihydroxyvitamin D_3. The chemical structures of the vitamin D_3 analogues are shown in Fig. 2.5; 1,25-$(OH)_2D_3$, 1,25-dihydroxyvitamin D_3; ER-pos./-neg., oestrogen receptor-positive/-negative.

The growth inhibitory effect of 1,25-dihydroxyvitamin D_3 was confirmed in other breast tumour cell lines and shown to be independent of the oestrogen receptor status (21,22). Treatment with 1,25-dihydroxyvitamin D_3 resulted in morphological changes, which may resemble a more differentiated status of the cells (20,23,24).

A few studies have demonstrated antiproliferative effects of synthetic vitamin D_3 analogues on breast cancer cells. These analogues have been shown to be more potent than 1,25-dihydroxyvitamin D_3 in the growth inhibition of leukaemic cells, whereas their *in vivo* calcaemic activity was similar or even reduced compared to 1,25-dihydroxyvitamin D_3 (see Section 2.5.2). The results are summarized in Table 3.1.

3.5 *IN VIVO* TUMOUR SUPPRESSION BY VITAMIN D

Several animal models for breast cancer are being used. Mammary tumours can be induced in rats by oral administration of the carcinogens N-nitroso-N-methylurea (NMU) or 7,12-dimethylbenz[a]anthracene (DMBA). NMU- and DMBA-induced tumours form a model for hormone-dependent tumours as they contain considerable amounts of oestrogen receptors and regress on ovariectomy and antioestrogen treatment (29,30). The presence of the vitamin D receptor has been demonstrated in

NMU tumours (31). In the nude mice model, immunedeficient athymic mice are inoculated subcutaneously with human breast cancer cells derived from continuous cell cultures. Alternatively, transplantable breast tumours can be propagated *in vivo* in the nude mice model. Both oestrogen-dependent and oestrogen-independent tumours can be studied in the nude mouse model (32).

Oral or intraperitoneal administration of 1,25-dihydroxyvitamin D_3 or 1α-hydroxyvitamin D_3, which is rapidly converted to 1,25-dihydroxyvitamin D_3 in the liver, resulted in an inhibition of the growth of NMU- (12,28) and DMBA-induced rat mammary tumours (33,34). By contrast, Noguchi *et al.* (35) did not find an effect of 1,25-dihydroxyvitamin D_3 on the incidence and growth of DMBA-induced rat mammary tumours. In the latter study, 1,25-dihydroxyvitamin D_3 was applied on shaved dorsal skin at doses that produced mild hypercalcaemia.

In order to achieve tumour suppression, high doses of 1,25-dihydroxyvitamin D_3 or 1α-hydroxyvitamin D_3 were needed. These high doses, approximately 0.5 μg/kg BW, resulted in the development of hypercalcaemia and subsequent weight loss. In an attempt to overcome this problem, synthetic vitamin D_3 analogues with low *in vivo* calcaemic activity have been developed (see Section 2.5.2). So far, only a few analogues have been evaluated for their potential use in the treatment of breast cancer. The data are summarized in Table 3.2, and show that with some analogues indeed an inhibition of tumour growth can be achieved, without raising serum calcium levels.

To date, only one clinical study on the effect of vitamin D_3 analogues on cancer growth in humans has been reported. Topical application of calcipotriol (MC903) in a small group of patients with locally advanced or cutaneous metastatic breast cancer showed a reduction of the size of treated lesions in 4 of 14 patients (36).

3.6 CURRENT THERAPIES FOR BREAST CANCER

Surgery and radiotherapy are used for treatment of the primary breast carcinoma and localized lesions. Endocrine and chemotherapy are given as systemic adjuvant therapy as part of the primary treatment, or for the treatment of advanced breast cancer, in order to manage distant (micro)metastases. Endocrine therapy is generally preferred, because in most cases it has lower toxicity and morbidity than chemotherapy. Chemotherapy is used for patients with aggressive or hormone receptor-negative tumours. In addition, patients failing to respond to, or relapsing after endocrine therapy are given chemotherapy (41,42).

Currently, a number of hormonal agents are used for endocrine treatment of breast cancer, namely antioestrogens, progestins, aromatase inhibitors, androgens, luteinizing hormone releasing hormone (LHRH) agonists, corticosteroids, and even high doses of oestrogens (41,43). The antioestrogen tamoxifen is the most widely used endocrine agent. Tamoxifen is effective in prolonging both disease-free interval and overall survival and has few side-effects. About one-third of all patients with breast cancer will respond to tamoxifen therapy. Patients with mammary carcinomas that are both oestrogen receptor- and progesterone receptor-positive display response rates of approximately 70 % compared to response rates of less than 20 % in patients with mammary carcinomas that are oestrogen receptor- and progesterone receptor-negative (41,43,44). A major problem of tamoxifen therapy is that in case of response, the tumour almost inevitably progresses to a tamoxifen-resistant state during prolonged therapy. Further, long-term tamoxifen therapy has been linked to an increased risk on endometrial cancer. Despite the efficacy of tamoxifen for breast cancer, alternative endocrine therapies are warranted, especially for tamoxifen-resistant and oestrogen receptor-negative tumours.

3.7 COMBINATION THERAPIES WITH VITAMIN D: *IN VITRO* AND *IN VIVO* LABORATORY RESULTS

The data obtained with 1,25-dihydroxyvitamin D_3 and synthetic vitamin D_3 analogues offer promise for the use of vitamin D in endocrine treatment of oestrogen receptor-positive and -negative breast cancer. Single agent treatment with a low calcaemic vitamin D_3 analogue could provide a new endocrine therapy, however, a combination therapy with established endocrine or cytotoxic agents may offer additional advantages, e.g. better response rates, lower dosages needed and thereby reducing the risk of negative side-effects.

Several *in vitro* and *in vivo* studies have focused on possible future combination therapies with vitamin D_3 compounds. Recently, Abe-Hashimoto *et al.* (39) observed a synergistic antiproliferative effect of submaximum dosages of 22-oxa-calcitriol and tamoxifen in MCF-7 and ZR-75-1 breast cancer cells. In addition, a synergistic action of 22-oxa-calcitriol and tamoxifen was observed *in vivo* in athymic mice implanted with MCF-7 cells. Also, Demirpence *et al.* (45) and James *et al.* (46) observed an augmented inhibition of MCF-7 cell growth by combined treatment of vitamin D_3 compounds (1,25-dihydroxyvitamin D_3 or the analogue EB1089) and antioestrogens

Table 3.2. Antitumour effects of vitamin D_3 analogues in animal models for breast cancer

Vitamin D_3 analogue	Model	Effective dose (administration)	Antitumour effect	Calcaemic effect	Ref.
OCT	Athymic mice implanted with ER-neg. MX-1 tumour	0.01-1 μg/kg BW (oral and intratumour)	Tumour suppression	No rise in serum Ca	22,39
OCT	DMBA-induced rat mammary tumour	0.1, 1 μg/kg BW (i.m.)	Tumour suppression	No rise in serum Ca	37
OCT	Athymic mice implanted with ER-pos. MCF-7 cells	0.001-1 μg/kg BW (oral)	Tumour suppression	No rise in serum Ca	39
MC903	NMU-induced rat mammary tumour	50 μg/kg BW (i.p.)	Tumour suppression	Small rise in serum Ca	28
EB1089	NMU-induced rat mammary tumour	0.5 μg/kg BW (oral)	Tumour suppression	No rise in serum Ca	38,40
CB966	NMU-induced rat mammary tumour	1 μg/kg BW (oral)	Tumour suppression	Small rise in serum Ca	40
Ro24-5531	NMU-induced rat mammary tumour	1.2 and 2.5 nmol/kg diet	Decreased tumour incidence	No rise in serum Ca	26

The chemical structures of the vitamin D_3 analogues are shown in Fig. 2.5.

(tamoxifen or ICI182,780), compared with the action of the compounds alone. Anzano *et al.* (26) have studied a possible role of 1,25-dihydroxy-16-ene-23-yne-26,27F_6-vitamin D_3 (Ro24-5531) in combination with low doses of tamoxifen for the prevention of breast cancer. They have observed that Ro24-5531 significantly enhanced the ability of tamoxifen to reduce the total tumour burden of rats treated with the carcinogen NMU.

Also, combinations with other endocrine therapies than antioestrogens have been studied. In a study of Iino *et al.* (33) 1α-hydroxyvitamin D_3 (0.5 μg/kg BW) was as effective as the progestin medroxyprogesterone acetate (MPA) (1 mg/kg BW) in the DMBA-induced rat mammary tumour model. Although a combination therapy did not result in an enhanced antitumour effect, MPA reduced the weight loss induced by 1α-hydroxyvitamin D_3. Vitamin A derivatives, like fenretinide, are currently tested in clinical trials as a preventive agent against recurrence of breast cancer, and animal studies point to a potential use of these compounds as therapeutic agents for breast cancer (47). A combination of retinoic acid and 1,25-dihydroxyvitamin D_3 was studied by Koga *et al.* (48) and they observed a synergistic growth inhibition of T47-D breast

cancer cells.

Furthermore, combinations with vitamin D_3 compounds and cytotoxic drugs have been studied. Abe *et al.* (22) have shown a more beneficial growth response by combined treatment with 22-oxa-calcitriol and adriamycin than by these agents alone, using athymic mice implanted with oestrogen receptor-negative MX-1 breast tumours. Iino *et al.* (33) have tested the effect of combined treatment with 1α-hydroxyvitamin D_3 and 5-fluorouracil in the DMBA-induced rat mammary tumour model. They have measured a similar tumour suppressive effect of 5-fluorouracil and 1α-hydroxyvitamin D_3 after 4 weeks. However, the combination therapy did not result in an enhanced antitumour effect. Cho *et al.* (49) observed that low concentrations of carboplatin and cisplatin interacted synergistically with 1,25-dihydroxyvitamin D_3 to inhibit MCF-7 cell growth.

Finally, Hassan *et al.* (50) have studied the effect of granulocyte-macrophage colony-stimulating factor (GM-CSF) on the antiproliferative effect of 1,25-dihydroxyvitamin D_3 in a MCF-7 clonogenic assay. They have observed that GM-CSF reduced the concentration of 1,25-dihydroxyvitamin D_3 required to induce the same antiproliferative effect as in the absence of GM-CSF.

3.8 MECHANISMS FOR THE ANTIPROLIFERATIVE ACTION OF VITAMIN D ON BREAST CANCER CELLS

3.8.1 Modulation of oncogene expression

Oncogenes affect cellular growth and differentiation through their protein products, which belong to various categories of the cell signalling machinery, such as growth factors, growth factor receptors, GTP-binding proteins, and nuclear proteins involved in transcription regulation (51,52). Oncogene expression at an inappropriate location or time period during cell cycle or maturation, overexpression of oncogenes, or mutations in oncogenes cause the transformation of cells in culture and induction of tumours in animals (51-53). A second class of cancer-related genes, the tumour suppressor genes, normally prevent tumour growth. Mutations or deletions in these genes cause their functional inactivation, which in turn contributes to cellular transformation (54,55).

A number of oncogenes and tumour suppressor genes have been implicated in the development of breast cancer (Table 3.3). A large number of reports have described amplification and/or overexpression of the erbB-2, int-2, and myc oncogenes in a high percentage of human breast cancer specimens. Deletions or

mutations in the DNA of human breast cancer specimens have been associated with the tumour suppressor genes Rb and p53. The "int" oncogenes have been found adjacent to the mouse mammary tumour virus (MMTV) integration sites in mouse breast cancer. Furthermore, studies with transgenic mice have indicated causal relationships between the expression of myc, erbB-2, and Ha-ras and the development of breast tumours. Finally, specific point mutations in the Ha-ras oncogene have been detected in chemical-induced breast cancers in rodents (56-60).

It has been proposed that oncogenes (and tumour suppressor genes) play a role in the tumour suppressive activity of vitamin D_3 compounds. The role of oncogenes in the cell regulating activity of 1,25-dihydroxyvitamin D_3 has been investigated most extensively in the leukaemic cell line HL60. In these cells 1,25-dihydroxyvitamin D_3 induced a progressive down-regulation of myc and a stimulation of fos and fms, which was initiated after approximately 4 hour of incubation and preceded the differentiation into monocyte-like cells and loss of proliferation capacity (61,62). In addition, 1,25-dihydroxyvitamin D_3 modulated the expression of myc, fos, and jun in other cell types than HL60 cells (63-67). However, very little data are available on the regulation of oncogenes by 1,25-dihydroxyvitamin D_3 in breast cancer cells. Only the report of Mathiasen *et al.* (25) describes a decreased myc expression after 3 hour, and a transient induction of fos expression with a maximum after 1 hour of 1,25-dihydroxyvitamin D_3 and EB1089 treatment of MCF-7 cells.

Table 3.3. Oncogenes and tumour suppressor genes associated with breast cancer

	Description of gene product
Oncogenes	
erbB-2 or HER-2	related to the epidermal growth factor receptor
int-2	related to the fibroblast growth factor family
myc	nuclear protein
Ha-ras	G-protein
Tumour suppressor genes	
Rb gene	nuclear phosphoprotein with DNA binding affinity
p53 gene	nuclear phosphoprotein

For reviews see Devilee and Cornelisse (56), Groner and Hynes (57), Mackay *et al.* (58), Van de Vijver and Nusse (59), and Groner (60).

3.8.2 Interaction with steroid hormones and polypeptide growth factors

Breast cancer cell growth is regulated by steroid hormones, acting via nuclear steroid hormone receptors, and polypeptide growth factors, acting via membrane receptors. Steroid hormones (oestrogens, progestins) may act directly on the tumour cell to stimulate growth, or indirectly via regulation of growth factor production and growth factor receptors. Breast cancer cells secrete a number of growth factors including TGFα, TGFß, IGF-I, IGF-II, and PDGF, which may act by autocrine loops, when the cells possess the adequate receptors. Alternatively, growth factors are derived from the circulation or are produced by stromal cells in the tumour and act by paracrine loops on breast cancer cells (68-71).

1,25-Dihydroxyvitamin D_3 may affect breast cancer cell growth by influencing these complex growth regulatory systems of steroid hormones and growth factors. 1,25-Dihydroxyvitamin D_3 may inhibit the secretion of stimulatory growth factors or stimulate the secretion of negative growth factors. Further, 1,25-dihydroxyvitamin D_3 may regulate the number of growth factor or steroid receptors. In addition, 1,25-dihydroxyvitamin D_3 may interfere with the action of steroid hormones on nuclear level, or with the intracellular signalling pathways of membrane bound growth factors.

Studies of Demirpence *et al.* (45) and James *et al.* (46) pointed to an interaction of 1,25-dihydroxyvitamin D_3 with oestradiol. Both studies showed that 1,25-dihydroxyvitamin D_3 (and the vitamin D_3 analogue EB1089) suppressed the mitogenic effect of oestradiol in MCF-7 cells. Further, it was shown that the oestrogen receptor concentration was decreased (46) and that oestrogen-induced gene transcription was inhibited (45) by treatment with 1,25-dihydroxyvitamin D_3.

There are some indications that 1,25-dihydroxyvitamin D_3 can interfere with paracrine or autocrine acting growth factors in breast cancer. A possible interaction with a paracrine loop was suggested by Saez *et al.* (34), who have observed that MCF-7 and BT-20 cells were growth stimulated by coculture with fibroblasts, whereas this process was reversed by 1,25-dihydroxyvitamin D_3 treatment. Furthermore, it has been shown that the number of EGF binding sites was decreased by 1,25-dihydroxyvitamin D_3 treatment in some breast cancer cell lines (MCF-7, T47-D), but increased in other cell lines (MDA-MB-231, BT-20) (34,72,73). As 1,25-dihydroxyvitamin D_3 reduced the growth of all these cell lines, the relation between EGF receptor regulation and growth inhibition by 1,25-dihydroxyvitamin D_3 is not clear.

TGF-ß is a negative growth factor that is produced by breast cancer cell lines (74-76). In several cell systems interactions with TGF-ß and 1,25-dihydroxyvitamin D_3 have been described. For example, human keratinocytes treated with 1,25-dihydroxyvitamin D_3 showed an increased expression of TGFß, and the

antiproliferative activity of 1,25-dihydroxyvitamin D_3 was partially blocked by neutralizing antibodies against TGF-ß (77). However, in breast cancer cells the effect of 1,25-dihydroxyvitamin D_3 on TGF-ß production has not been studied in relation with the antiproliferative effects. Yet, one report described a stimulation of TGF-ß secretion and TGF-ß mRNA expression by treatment with 1,25-dihydroxyvitamin D_3 and vitamin D_3 analogues in BT-20 breast cancer cells (78).

3.8.3 The role of calcium

Calcium (Ca^{2+}) is an important intracellular regulatory molecule and acts as an intracellular messenger for extracellular signalling molecules. Because of the well-known effects of 1,25-dihydroxyvitamin D_3 on calcium translocation in the intestine, it has been suggested that the antiproliferative and differentiation inducing effects of 1,25-dihydroxyvitamin D_3 are mediated via the regulation of calcium-related processes.

It has been demonstrated that 1,25-dihydroxyvitamin D_3 increases the calcium uptake in a number of different cell types. This phenomenon can either be a rapid mobilization of calcium, probably via a non-genomic mechanism, or a slow calcium influx, which may proceed via a genomic mechanism (e.g. via the production of vitamin D dependent calbindins) (14,79). An elevation of intracellular calcium appears to participate in monocytic differentiation of leukaemic cells (80,81), but in most other systems the physiological significance of the calcium uptake has not been identified.

The antiproliferative action of 1,25-dihydroxyvitamin D_3 on malignant cells was shown to be dependent on the extracellular calcium concentration. The growth inhibition by 1,25-dihydroxyvitamin D_3 was enhanced by a low extracellular calcium concentration in breast cancer cells (82), leukaemic cells (83,84), and colon cancer cells (85). However, the exact role of calcium in this enhanced growth inhibition remains to be determined.

3.9 VITAMIN D AND ANGIOGENESIS, INVASION, AND METASTASIS

The mechanisms of the antiproliferative action of vitamin D_3 compounds discussed in Section 3.8 are studied in cell culture systems. For the tumour suppressive activity of vitamin D_3 compounds *in vivo*, additional aspects may be involved. Vitamin D has been suggested to play a role in the processes of

angiogenesis, invasion, and metastasis as discussed below.

Angiogenesis, the formation of new capillary blood vessels, is an essential requirement for the growth of solid tumours. Inhibition of angiogenesis may contribute to the tumour suppressive activity of vitamin D compounds, as two groups have observed an anti-angiogenic effect of 1,25-dihydroxyvitamin D_3 and 22-oxa-calcitriol using different experimental model systems (86,87).

Invasion and metastasis of tumour cells are the primary causes for the fatal outcome of cancer diseases. A recent report of Mork Hansen *et al.* (88) indicated that 1,25-dihydroxyvitamin D_3 may be effective in reducing the invasiveness of breast cancer cells. They have shown that 1,25-dihydroxyvitamin D_3 inhibited the invasion and migration of a metastatic human breast cancer cell line (MDA-MB-231) using the Boyden chamber invasion assay.

Bone is the most frequent site of metastasis of advanced breast cancer (89). There are some indications from clinical studies that bone metastases develop preferentially in areas with high bone turnover (90,91). In contrast, agents that inhibit bone resorption have been reported to reduce the incidence of skeletal metastasis (92-94). As 1,25-dihydroxyvitamin D_3 is an important stimulator of bone resorption (see Section 2.3.2), treatment with 1,25-dihydroxyvitamin D_3 or vitamin D_3 analogues for breast cancer, might increase the risk of skeletal metastases. So far, no *in vivo* studies have been reported on this subject with respect to breast cancer. However, Krempien (95) has reported that following intra-arterial injection with Walker 256 tumour cells, rats treated with 1,25-dihydroxyvitamin D_3 developed significantly more bone metastases than untreated controls.

3.10 CONCLUDING REMARKS

The first reports on the antiproliferative action of 1,25-dihydroxyvitamin D_3 on breast cancer cells *in vitro*, 10 to 15 years ago, have since been confirmed *in vivo* in different animal models for breast cancer. It appears that the problem of the strong calcaemic activity of 1,25-dihydroxyvitamin D_3 can be circumvented by the use of new synthetic vitamin D_3 analogues, since it has been shown that a number of these analogues have more potent antiproliferative effects on breast cancer cells *in vitro*, and suppress breast cancer growth *in vivo* without marked calcaemic effects. However, it should be noticed that apart from calcaemic side-effects, other negative side-effects may arise, in particular immunosuppressive effects and an increased risk of bone metastases. But the development of new vitamin D_3 analogues continues, and in the

future analogues with even stronger antiproliferative action and better selectivity may be available.

The mechanism of the antiproliferative action of vitamin D remains largely unclear. There are some indications that vitamin D interferes with the action of oestrogen and polypeptide growth factors that influence breast cancer growth. More research is needed to define the role of oncogenes, tumour suppressor genes and intracellular calcium in the antiproliferative action of vitamin D.

A major advantage of an endocrine therapy with vitamin D may be that vitamin D suppresses breast tumour growth independent of the presence of the oestrogen receptor. Most established endocrine therapies are based on antioestrogenic action, and for oestrogen receptor-negative tumours therapeutic choices are limited. Vitamin D treatment could theoretically be beneficial for a large group of patients, since the vitamin D receptor is expressed in about 80 % of human breast cancers. Another promising aspect of vitamin D treatment might be its combination with other established endocrine (tamoxifen) or cytotoxic agents. In addition, epidemiological studies and laboratory results have suggested a role for vitamin D in the prevention of breast cancer. In the next years, clinical studies will hopefully confirm that vitamin D_3 analogues, either alone or in combination with other antitumour agents, can provide an effective treatment for breast cancer.

3.11 SCOPE OF THE THESIS

In vitro and *in vivo* studies have indicated that 1,25-dihydroxyvitamin D_3 and especially vitamin D_3 analogues with low calcaemic activity have potential for the treatment of breast cancer (Chapters 2 and 3). Presently, the antioestrogenic drug tamoxifen is the most widely used endocrine agent in the treatment of breast cancer (Section 3.6). The studies described in this thesis deal with the potential benefit of a combination therapy with tamoxifen and 1,25-dihydroxyvitamin D_3 or vitamin D_3 analogues. Furthermore, studies were undertaken in order to gain more insight into the antiproliferative action of vitamin D_3 compounds.

First of all, we have assessed the effect of combined treatment of 1,25-dihydroxyvitamin D_3 and tamoxifen on the growth of two oestrogen receptor-positive and vitamin D receptor-positive human breast cancer cell lines, MCF-7 and ZR-75-1 (Chapter 4). In Chapter 5 we have evaluated the antiproliferative action of four promising vitamin D_3 analogues (CB966, EB1089, KH1060, and 22-oxa-calcitriol) alone, and in combination with tamoxifen on MCF-7 and ZR-75-1 cells. The effect of

the vitamin D_3 compounds on tamoxifen-resistant cells was studied in Chapter 5. Furthermore, we have addressed the question whether 1,25-dihydroxyvitamin D_3 and analogues can interfere with the growth stimulation by oestradiol (Chapters 4 and 5), and evaluated a possible role of the myc oncogene in the growth inhibition by the vitamin D_3 compounds (Chapter 5).

In Chapter 6 we have extended the investigations of the combination of 1,25-dihydroxyvitamin D_3/analogues and tamoxifen to the regulation of bone resorption. As discussed in Section 3.9, treatment of breast cancer with 1,25-dihydroxyvitamin D_3 or vitamin D_3 analogues might have an unfavourable effect on the incidence of bone metastases, due to the bone resorbing activity of vitamin D_3 compounds. Tamoxifen, in contrast, has been shown to have protective effects on the skeleton (96,97). Therefore, it was studied in an *in vitro* model whether tamoxifen inhibits the bone resorption induced by 1,25-dihydroxyvitamin D_3 and two vitamin D_3 analogues (EB1089 and KH1060), and thereby potentially reduces the risk of skeletal metastasis.

In Chapters 7 and 8, it was investigated whether the tumour suppressive activity of 1,25-dihydroxyvitamin D_3 and analogues could be explained by an interference with polypeptide growth factors. First, we have studied the effect of 1,25-dihydroxyvitamin D_3 and the analogue EB1089 on insulin- and IGF-I-stimulated growth of MCF-7 cells. Subsequently, we have investigated the effect of 1,25-dihydroxyvitamin D_3 and EB1089 on the number of insulin receptors and IGF-I receptors, and studied the role of the fos oncogene (Chapter 7). In Chapter 8 we have further evaluated a possible interaction of the vitamin D_3 compounds with the IGF regulatory system, and studied the regulation of the expression of IGF-binding proteins and of the secretion of IGF-I by MCF-7 cells.

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Chapter 4

COMBINED EFFECTS OF 1,25-DIHYDROXY-VITAMIN D_3 AND TAMOXIFEN ON THE GROWTH OF MCF-7 AND ZR-75-1 HUMAN BREAST CANCER CELLS

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SUMMARY

In the present study we assessed the effect of combined treatment with 1,25-dihydroxyvitamin D_3 (1,25-$(OH)_2D_3$) and tamoxifen (TAM) on the growth of oestrogen-responsive (MCF-7) and oestrogen-dependent (ZR-75-1) human breast cancer cells. Both basal and 17ß-oestradiol (17ß-E_2)-stimulated growth were studied. 1,25-$(OH)_2D_3$ (10^{-10} - 10^{-7} M) time- and dose-dependently inhibited basal growth of MCF-7 cells, with growth arrest at 10^{-7} M. Also, 17ß-E_2-stimulated growth of MCF-7 and ZR-75-1 cells was inhibited by 1,25-$(OH)_2D_3$ in a time- and dose-dependent manner. TAM inhibited 17ß-E_2-stimulated growth of both cell lines and at high concentration (10^{-6} M) it also inhibited basal growth of MCF-7 cells. 10^{-6} M TAM together with 1,25-$(OH)_2D_3$ resulted in a further inhibition of basal (MCF-7 cells) as well as 17ß-E_2-stimulated proliferation (MCF-7 and ZR-75-1 cells) compared to the inhibition by these agents alone. TAM in combination with 10^{-7} M 1,25-$(OH)_2D_3$ resulted in growth arrest of 17ß-E_2-stimulated growth of MCF-7 cells. The inhibition of basal and 17ß-E_2-stimulated growth of MCF-7 cells was additive at early time points (4 days), but less than additive at later time points (8-10 days). It was demonstrated that with co-treatment of MCF-7 cells an equipotent inhibition of basal growth could be reached with lower concentrations of 1,25-$(OH)_2D_3$, compared to treatment with 1,25-$(OH)_2D_3$ alone. Studies with low concentrations ($< 10^{-7}$ M) of TAM revealed a partial oestrogenic effect, i.e. stimulation of MCF-7 proliferation in the absence of 17ß-E_2. This effect, which may resemble TAM-induced tumour flare, was completely prevented by co-treatment with a low concentration of 1,25-$(OH)_2D_3$ (10^{-9} M). Together, these results demonstrate the potent inhibition of breast cancer cell proliferation by 1,25-$(OH)_2D_3$ combined with TAM and indicate a potential benefit of combining these agents for the treatment of breast cancer.

INTRODUCTION

The seco-steroid hormone 1,25-dihydroxyvitamin D_3 (1,25-$(OH)_2D_3$) is the biologically most active form of vitamin D_3 and plays an important role in the regulation of calcium homeostasis and bone metabolism. The effects of 1,25-$(OH)_2D_3$ are mediated via the vitamin D receptor (VDR) in target tissues such as bone, intestine and kidney (1). VDRs are not confined to the classical target tissues, but have also been demonstrated in a variety of cells and tissues not directly related to calcium homeostasis. Several studies have indicated that 1,25-$(OH)_2D_3$ induces

differentiation and inhibits proliferation of haemopoietic, epidermal, and many cancer cells (1-3).

The VDR is present in most breast cancer cell lines and tumours (2, 4-7) and 1,25-$(OH)_2D_3$ has been shown to inhibit proliferation of breast cancer cells *in vitro* irrespective of their oestrogen dependence (2, 7-11). Studies *in vivo* have shown that 1α-hydroxyvitamin D_3, which is converted to 1,25-$(OH)_2D_3$ in the liver, suppressed the growth of carcinogen-induced rat mammary tumours (7, 12). These findings implicate a potential use of 1,25-$(OH)_2D_3$ for the treatment of breast cancer. However, high doses of the sterol are needed and it remains to be established whether 1,25-$(OH)_2D_3$ can produce long-term antitumour effects without unacceptable side-effects, like the development of hypercalcaemia.

Until now the most effective endocrine therapy for oestrogen receptor (ER)-positive breast tumours is the treatment with synthetic antioestrogens e.g. tamoxifen (TAM) (13). The effect of TAM on breast cancer cells is believed to be predominantly mediated through competition with oestrogen for the ER thereby attenuating the proliferative effect of oestrogen (14, 15). Although antioestrogens are very effective in ER-positive tumours, not all ER-positive tumours respond favourably and during prolonged antioestrogen therapy even patients with responsive tumours can be expected to become eventually resistant (16). For ER-negative tumours therapeutic choices are limited and therefore treatment with 1,25-$(OH)_2D_3$ may offer a new approach.

In the present study we assessed the effects of combined treatment with 1,25-$(OH)_2D_3$ and TAM on the growth of the ER-positive and VDR-positive human breast cancer cell lines MCF-7 and ZR-75-1. The cell lines have different growth characteristics. MCF-7 cells have partially escaped from hormonal regulation and are called oestrogen-responsive. These cells are able to grow in steroid-free culture medium without further additions and are growth stimulated by 17ß-E_2. The proliferation of ZR-75-1 cells is dependent on the presence of oestrogens. We have studied the effects of co-treatment on basal as well as 17ß-E_2-stimulated proliferation.

MATERIALS AND METHODS

Materials

17ß-E_2, TAM, RPMI-1640 culture medium, ethidium bromide, DNA (type I, highly polymerized), and Ribonuclease A were purchased from Sigma Chemical Co., St. Louis, MO. 1,25-$(OH)_2D_3$ was generously provided by LEO Pharmaceutical Products BV, Weesp, The Netherlands. Glutamine, penicillin, streptomycin, and foetal calf serum (FCS) were obtained from Life Technologies, Breda, The Netherlands. Trypsin was from Boehringer, Mannheim, Germany, Hank's balanced salts solution was

from Imperial Laboratories, Andover, UK, and heparin solution (5000 IU/ml) was from Organon, Boxtel, The Netherlands.

Cell culture and growth-experiments

MCF-7 and ZR-75-1 cells were generously provided by Dr. JA Foekens (Department of Endocrine Oncology, Dr. Daniel den Hoed Cancer Center, Rotterdam, The Netherlands). For proliferation studies cells were seeded in six-well dishes at a density of 16,000 cells/cm^2 for MCF-7 and 32,000 cells/cm^2 for ZR-75-1 cells in phenol red-free RPMI-1640 medium supplemented with 2 mM glutamine, 100 units/ml penicillin, 100 μg/ml streptomycin, 24 mM sodium bicarbonate, and 10% FCS. The cells were allowed to attach for 6-7 h. Next, medium was changed to medium with 2% charcoal-treated FCS (CT-FCS) and the agents to be tested or vehicle (0.1% ethanol) were added. For MCF-7 cells medium and agents were replaced every 24 h. For ZR-75-1 cells medium and agents were in initial experiments replaced every 24 h, in later experiments every 3 days. Similar results were obtained with both incubation procedures. At the end of the incubation medium was aspirated and DNA content was measured according to the ethidium bromide method of Karsten and Wollenberger (17). Cells were scraped in 200 μl trypsin solution (0.5 mg/ml in Hank's balanced salts solution) and suspended in 1.5 ml PBS containing 0.1% Triton X-100 (PBS-Triton). Cells were sonicated during 2 x 5 sec using a Soniprep 150 (Sanyo Gallenkamp PLC). Aliquots of the DNA samples were adjusted to 0.5 ml with PBS-Triton and incubated with 1 ml heparin solution (8.33 IU/ml in PBS) and 0.5 ml RNAse A solution (0.05 mg/ml in PBS) for 30 min at 37 °C. Next, 0.5 ml ethidium bromide solution (0.025 mg/ml in PBS) was added and the samples were measured using a Perkin-Elmer LS-2B filterfluorimeter. Excitation and emission wavelength were 340 and 590 nm, respectively. A DNA stock solution (25 μg/ml in PBS-Triton) was used for a standard curve.

VDR levels of MCF-7 and ZR-75-1 cells, 28 $\pm$ 12 and 40 $\pm$ 9 fmol/mg protein respectively, were determined as described previously (18).

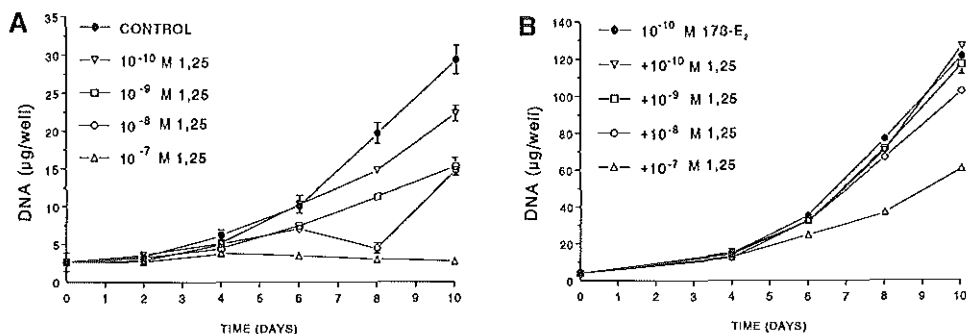
Data presented are representative for at least 2 independent experiments. All values are presented as mean $\pm$ SD of duplicate wells. Where no error bar appears the error is smaller than the symbol.

RESULTS

Effect of 1,25-$(OH)_2D_3$ on basal and 17ß-E_2-stimulated growth of MCF-7 cells

As shown in Fig. 4.1A, 1,25-$(OH)_2D_3$ inhibited proliferation of MCF-7 cells in a time- and dose-dependent manner. The first significant effects were observed with 10^{-7} and 10^{-8} M 1,25-$(OH)_2D_3$ after 4 days. After 6 days 10^{-9} M, and after 8 days 10^{-10} M 1,25-$(OH)_2D_3$ also significantly inhibited cell growth. 10^{-7} M 1,25-$(OH)_2D_3$ arrested cell growth, but after 4 and 10 days of incubation cell growth could be regained by adding fresh medium supplemented with 10% FCS (data not shown).

After evaluating the effect of 1,25-$(OH)_2D_3$ on basal cell growth, we investigated whether 1,25-$(OH)_2D_3$ was able to inhibit 17ß-E_2-stimulated proliferation. 17ß-E_2 stimulated cell growth very potently and maximal stimulation was already reached at 10^{-11} M. Comparison of Figs. 4.1A and 4.1B demonstrates that 1,25-$(OH)_2D_3$ inhibited basal growth more potently than 17ß-E_2-stimulated growth. With 10^{-7} M 1,25-$(OH)_2D_3$ basal cell growth was arrested, whereas on day 10 this concentration inhibited 17ß-E_2-stimulated cells by only 50%. Also, with a lower concentration



Figures 4.1A and B. Effect of 1,25-(OH)$_2$D$_3$ on basal and 17ß-E$_2$-stimulated proliferation of MCF-7 cells. MCF-7 cells were cultured in 2% CT-FCS containing medium without (A) or with 10^{-10} M 17ß-E$_2$ (B), and a dose-range of 1,25-(OH)$_2$D$_3$ (1,25). DNA was measured at the indicated times.

of 1,25-(OH)$_2$D$_3$ (10^{-10} M) an inhibition of 23% of basal proliferation was observed, whereas it had no effect on 17ß-E$_2$-stimulated proliferation.

Effect of TAM on basal and 17ß-E$_2$-stimulated growth of MCF-7 cells

TAM had a biphasic effect on the proliferation of MCF-7 cells. 10^{-8} M TAM stimulated proliferation, whereas with 10^{-6} M an inhibition was observed. (Figs. 4.2, 4.3, 4.6). In contrast to the biphasic effect on basal growth, both concentrations of TAM inhibited 17ß-E$_2$-stimulated (10^{-11} M) growth, with 10^{-6} M being more potent than 10^{-8} M (Fig. 4.2). With a higher dose of 17ß-E$_2$ (10^{-9} M) the effect of 10^{-6} M TAM could partially be reversed (data not shown). With ZR-75-1 cells a similar phenomenon was observed (Fig. 4.7).

Combined effects of 1,25-(OH)$_2$D$_3$ and TAM on MCF-7 proliferation

Subsequently, we investigated a possible interaction between 1,25-(OH)$_2$D$_3$ and TAM. First, we assessed the effect of co-treatment with 1,25-(OH)$_2$D$_3$ and a growth inhibitory dose of TAM (10^{-6} M). Fig. 4.3 shows that 10^{-6} M TAM alone resulted in an inhibition of 68% on day 10. A further inhibition up to 100% could be achieved by co-treatment with 1,25-(OH)$_2$D$_3$ (10^{-10} - 10^{-7} M). At early time points (4 days) an additive effect could be observed i.e. the reduction in DNA content, expressed in µg/well, by TAM and 1,25-(OH)$_2$D$_3$ alone adds up in the combined treatment. At later time points (8-10 days) the effect of combined treatment was, although not addi-

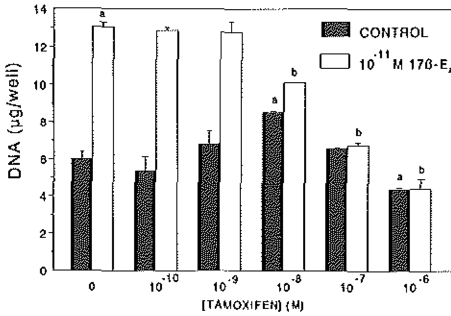


Figure 4.2. Dose-response of TAM in the presence and absence of 17ß-E_2. MCF-7 cells were cultured in 2% CT-FCS containing medium with the indicated concentrations of TAM in the presence or absence of 10^{-11} M 17ß-E_2. After 4 days DNA content was measured. a, $p<0.05$ versus control (no TAM); b, $p<0.001$ versus 10^{-11} M 17ß-E_2 (no TAM) as calculated with the Student's t-test.

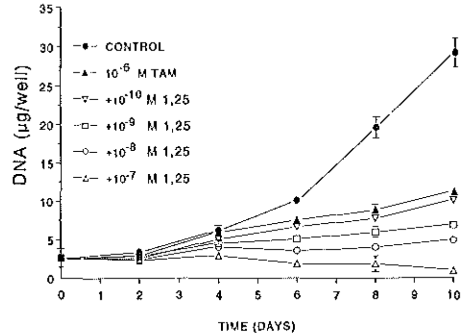


Figure 4.3. Combined treatment with 1,25-$(OH)_2D_3$ and a growth-inhibitory dose of TAM. MCF-7 cells were cultured in 2% CT-FCS containing medium with 10^{-6} M TAM plus the indicated concentrations of 1,25-$(OH)_2D_3$ (1,25). Control cultures received vehicle only. DNA content was measured every two days.

tive, stronger than the effect of either compound alone. The co-treatment was cytostatic rather than cytotoxic, since cell growth could be regained by adding fresh medium supplemented with 10% FCS after 4 and 10 days of incubation with 10^{-7} M 1,25-$(OH)_2D_3$ together with 10^{-6} M TAM (data not shown).

Fig. 4.4 shows the percentage inhibition by treatment with 1,25-$(OH)_2D_3$ alone and in combination with 10^{-6} M TAM on days 6 and 10. This figure illustrates for example that on day 6 an inhibition of 70% was achieved with 5.10^{-8} M 1,25-$(OH)_2D_3$, whereas a similar inhibition was achieved with a 50 times lower 1,25-$(OH)_2D_3$ concentration (10^{-9} M) when combined with TAM (Fig. 4.4A). In addition, on day 10, 80% inhibition was achieved with 3.10^{-8} M 1,25-$(OH)_2D_3$ alone and with 4.10^{-10} M 1,25-$(OH)_2D_3$ when combined with TAM (Fig. 4.4B). In this situation a 75 times lower concentration of 1,25-$(OH)_2D_3$ resulted in a similar inhibition when used in combination with TAM. The effect of co-treatment with 1,25-$(OH)_2D_3$ and TAM (10^{-6} M) on 17ß-E_2-stimulated proliferation is shown in Figure 4.5. Although TAM was a very potent inhibitor of 17ß-E_2-stimulated proliferation (Fig. 4.2), 10^{-6} M TAM did not completely inhibit the growth of MCF-7 cells. Co-treatment with 1,25-$(OH)_2D_3$ resulted in a further dose-dependent inhibition and growth arrest at 10^{-7} M

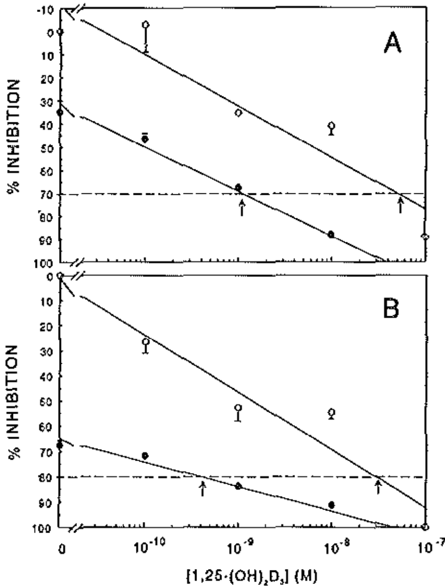


Figure 4.4. Comparison of the effect of 1,25-$(OH)_2D_3$ alone and in combination with TAM on basal growth. MCF-7 cells were treated for 6 days (A) and 10 days (B) with 1,25-$(OH)_2D_3$ (1,25) alone (open circles) or with 1,25-$(OH)_2D_3$ plus 10^{-6} M TAM (solid circles). DNA values were corrected for DNA values on day 0 and expressed as percentage inhibition relative to control (vehicle only).

1,25-$(OH)_2D_3$.

Next we investigated the effect of co-treatment with 1,25-$(OH)_2D_3$ and agrowth-stimulatory dose of TAM (10^{-8} M). As shown in Fig. 4.6, 1,25-$(OH)_2D_3$ inhibited TAM-stimulated growth in a time- and dose-dependent manner. 10^{-10} M 1,25-$(OH)_2D_3$ caused a small reduction of TAM-stimulated growth whereas 10^{-9} M1,25-$(OH)_2D_3$ resulted in an inhibition to control level (no TAM), thereby completely preventing TAM-induced growth stimulation. 10^{-8} and 10^{-7} M 1,25-$(OH)_2D_3$ even suppressed TAM-stimulated growth to below control level.

Effects of 1,25-$(OH)_2D_3$ and TAM on ZR-75-1 cells

Besides MCF-7 cells we tested the effects of 1,25-$(OH)_2D_3$ and TAM on the proliferation of another ER-positive breast cancer cell line. ZR-75-1 cells did not grow in the steroid-free culture medium we used for the proliferation experiments with MCF-7 cells. Addition of 17ß-E_2 to the culture medium resulted in a dose- dependent stimulation of proliferation. TAM (10^{-6} M) caused a complete inhibition of 10^{-10} M 17ß-E_2-stimulated growth whereas the effect of 10^{-9} M 17ß-E_2 was partially inhibited (Fig. 4.7).

1,25-$(OH)_2D_3$ inhibited 17ß-E_2-stimulated growth of ZR-75-1 cells in a dose-dependent manner (data not shown). 10^{-7} M 1,25-$(OH)_2D_3$ completely inhibited 10^{-10} M 17ß-E_2-stimulated growth similar to TAM (10^{-6} M). Also, an almost complete inhibition of 10^{-9} M 17ß-E_2-stimulated growth was observed using 10^{-7} M 1,25-$(OH)_2D_3$, whereas 10^{-6} M TAM was less potent (Fig. 4.7). Further, the inhibition of 17ß-E_2-stimulated growth by 1,25-$(OH)_2D_3$ was more effective in ZR-75-1 cells compared to MCF-7 cells. Comparison of Figs. 4.1B and 4.7 shows a partial inhibition

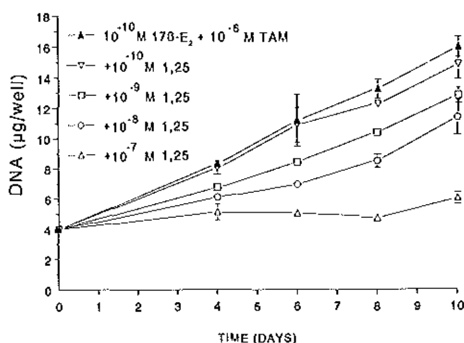


Figure 4.5. Combined treatment with 1,25-$(OH)_2D_3$ and TAM of cells stimulated with 17ß-E_2. MCF-7 cells were cultured in 2% CT-FCS containing medium with 10^{-10} M 17ß-E_2 and 10^{-6} M TAM (solid triangles) and were co-treated with a dose range of 1,25-$(OH)_2D_3$ (1,25; open symbols). DNA was measured at the indicated times.

of 10^{-10} M 17ß-E_2-stimulated growth of MCF-7 cells by 10^{-7} M 1,25$(OH)_2D_3$ and a complete inhibition of ZR-75-1 cells after 9 days. This may be directly related to the difference in response to 17ß-E_2. However, in several experiments it was observed that an equipotent growth stimulation by 17ß-E_2 of MCF-7 and ZR-75-1 cells was also inhibited more potently by 10^{-7} M 1,25-$(OH)_2D_3$ in ZR-75-1 cells (data not shown).

Although treatment with 1,25-$(OH)_2D_3$ (10^{-7} M) and TAM (10^{-6} M) alone resulted in a complete inhibition of 10^{-10} M 17ß-E_2 stimulated growth, still a further inhibition to below control level was observed when used in combination. Also, at higher concentration 17ß-E_2 (10^{-9} M) the effect of co-treatment was more potent than the effect of either compound alone (Fig. 4.7).

DISCUSSION

The present study describes for the first time effects of combined treatment with 1,25-$(OH)_2D_3$ and TAM on the growth of ER-positive and VDR-positive human breast cancer cells *in vitro*. As circulating levels of oestrogens are believed to play an important role in promoting the growth of ER-positive breast tumours, we have studied the effects of both compounds on basal as well as 17ß-E_2-stimulated proliferation. The current data show that basal growth of MCF-7 cells is inhibited by 1,25-$(OH)_2D_3$. ZR-75-1 cells did not grow in the absence of 17ß-E_2 and therefore no effects on basal growth could be assessed. Our data are consistent with several reports describing the inhibitory effect of 1,25-$(OH)_2D_3$ on human breast cancer cells (2, 7-11). In these studies the inhibition of cell proliferation was investigated using culture media supplemented with FCS or CT-FCS but to our knowledge the effect of 1,25-$(OH)_2D_3$ on a specific growth stimulus like 17ß-E_2 was not studied. Our data demonstrate that 1,25-$(OH)_2D_3$ inhibits 17ß-E_2-stimulated proliferation of both MCF7

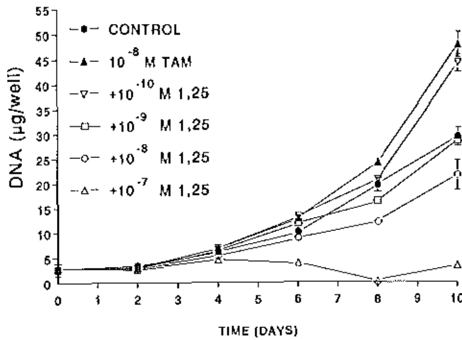


Figure 4.6. Combined treatment with 1,25-(OH)$_2$D$_3$ and a growth-stimulatory dose of TAM. MCF-7 cells were cultured in 2% CT-FCS containing medium with 10^{-8} M TAM plus the indicated concentrations of 1,25-(OH)$_2$D$_3$ (1,25). Control cultures received vehicle only. DNA content was measured every two days.

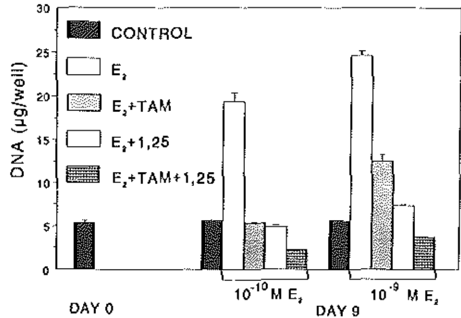


Figure 4.7. Combined treatment with 1,25-(OH)$_2$D$_3$ and TAM of ZR-75-1 cells. ZR-75-1 cells were cultured in 2% CT-FCS containing medium with vehicle, 10^{-10} or 10^{-9} M 17ß-E$_2$ (E$_2$) for 9 days. Cells were treated with 10^{-6} M TAM or 10^{-7} M 1,25-(OH)$_2$D$_3$ (1,25) or 10^{-6} M TAM together with 10^{-7} M 1,25-(OH)$_2$D$_3$. Medium and test agents were replaced every three days. DNA content was measured on days 0 and 9.

and ZR-75-1 cells. Although the VDR levels were comparable, 17ß-E$_2$ stimulated growth of ZR-75-1 cells is more sensitive to 1,25-(OH)$_2$D$_3$ than 17ß-E$_2$-stimulated growth of MCF-7 cells. These results are suggestive for a difference in stimulation by 17ß-E$_2$. It has been reported (19, 20) that 17ß-E$_2$ acts synergistically with insulin and possibly insulin-like growth factors on MCF-7 cells, whereas others (21) did not find this synergistic action on ZR-75-1 cells. Therefore, the 17ß-E$_2$-stimulated growth we observed in MCF-7 cells could be the result of a synergistic action of 17ß-E$_2$ with serum-derived insulin-like growth factors, whereas the growth stimulation by 17ß-E$_2$ in ZR-75-1 cells could be less sensitive to serum factors.

TAM antagonizes 17ß-E$_2$-stimulated growth by competition with 17ß-E$_2$ for the ER (14, 15). Consistent with these findings TAM dose-dependently inhibited 17ß-E$_2$-stimulated growth of MCF-7 and ZR-75-1 cells. It was also observed that 10^{-6} M TAM inhibited the growth of MCF-7 cells in steroid-free culture medium. This might be explained by antigrowth factor activity of TAM which has been demonstrated in several reports (20, 22, 23).

Both basal proliferation (MCF-7 cells) and 17ß-E$_2$-stimulated proliferation (MCF-7 and ZR-75-1 cells) were inhibited more potently by the combination of 1,25-

$(OH)_2D_3$ and TAM than by either compound alone. We have shown that an equipotent inhibition of basal growth of MCF-7 cells could be achieved with lower concentrations of 1,25-$(OH)_2D_3$ when combined with TAM compared to treatment with 1,25-$(OH)_2D_3$ alone. This is an interesting observation since it is of clinical importance to use 1,25-$(OH)_2D_3$ as an antiproliferative compound at the lowest possible doses in order to prevent the development of hypercalcaemia.

In MCF-7 cells we observed a growth stimulation by TAM at low concentrations ($< 10^{-7}$ M) in the absence of oestrogens. This effect is consistent with previous work (20, 24) and is thought to be due to a partial oestrogen agonistic action mediated via the ER. It is hypothesized that TAM-induced tumour flare, which is often observed in patients, is the result of this oestrogenic effect of TAM (25). We observed that growth stimulation by TAM was already completely prevented by a low concentration (10^{-9} M) of 1,25-$(OH)_2D_3$. Thereby these data are pointing to a possible role of 1,25-$(OH)_2D_3$ in the prevention of tumour flare. A further interesting observation was that a growth-stimulatory concentration of TAM (10^{-8} M) was nevertheless able to inhibit 17ß-E_2-stimulated growth. This phenomenon agrees with previous studies (20, 24), however, the underlying mechanism is not yet clear.

The observation that at early time points the inhibitory effects of 1,25-$(OH)_2D_3$ and TAM on the proliferation of MCF-7 cells are additive suggests that they inhibit cell growth via different mechanisms. This could indicate that 1,25-$(OH)_2D_3$ acts independent of the 17ß-E_2-induced pathway leading to proliferation. Further support for this 17ß-E_2-independent action comes from the observations in previous reports that both ER-positive and ER-negative breast cancer cell lines are growth-inhibited by 1,25-$(OH)_2D_3$ (2, 7-11). Further, in MCF-7 cells 1,25-$(OH)_2D_3$ inhibited basal growth more potently than 17ß-E_2-stimulated growth. However, several observations do point to an interference of 1,25-$(OH)_2D_3$ with the 17ß-E_2-induced pathway. Firstly, in ZR-75-1 cells 1,25-$(OH)_2D_3$ caused a complete blockade of 17ß-E_2-induced growth. Secondly, TAM-stimulated growth of MCF-7 cells, which is probably mediated via the ER, was potently inhibited by 1,25-$(OH)_2D_3$. Moreover, a recent report about a synergistic growth inhibition of MCF-7 and ZR-75-1 cells by a vitamin D_3 analog and TAM (26) is suggestive for an interaction. More experiments are needed to define precisely whether 1,25-$(OH)_2D_3$ interferes with the 17ß-E_2-mediated pathway and presently we are investigating the role of 1,25-$(OH)_2D_3$ in other 17ß-E_2-mediated responses.

In conclusion, the current results demonstrate a potent inhibition of breast cancer cell proliferation by combined treatment with 1,25-$(OH)_2D_3$ and TAM. The combined treatment may provide the advantages that a) tumours positive for both ER

and VDR have a more beneficial response, b) lower doses of 1,25-$(OH)_2D_3$ can be used which do not cause hypercalcaemia, and c) in tumours heterogenous for the ER both ER-positive and ER-negative cells can be inhibited. In addition, TAM may diminish the stimulatory side-effect of 1,25-$(OH)_2D_3$ on bone resorption since several reports have indicated that TAM exerts positive oestrogenic effects on bone and protects against steroid-induced bone loss (27, 28). An important drawback for the clinical use of 1,25-$(OH)_2D_3$ as an antiproliferative compound is the development of hypercalcaemia at high doses. At the moment, numerous attempts are being made to develop vitamin D_3 analogues with potent growth inhibitory, and reduced calcaemic activity. In the future co-treatment with these vitamin D_3 analogues and TAM may provide an even greater benefit and studies on this subject are currently in progress.

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Chapter 5

INHIBITION OF BREAST CANCER CELL GROWTH BY COMBINED TREATMENT WITH VITAMIN D_3 ANALOGUES AND TAMOXIFEN

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ABSTRACT

The steroid hormone 1,25-dihydroxyvitamin D_3 (1,25-$(OH)_2D_3$) has potential to be used as an antitumour agent, but its clinical application is restricted by the strong calcaemic activity. Therefore, new vitamin D_3 analogues are developed with increased growth inhibitory and reduced calcaemic activity. In the present study, we have examined the antiproliferative effects of four novel vitamin D_3 analogues (CB966, EB1089, KH1060, and 22-oxa-calcitriol (OCT)) on breast cancer cells, either alone or in combination with the antioestrogen tamoxifen. The oestrogen-dependent ZR-75-1 and oestrogen-responsive MCF-7 cell lines were used as a model. It was shown that, with EB1089 and KH1060 the same growth inhibitory effect as 1,25-$(OH)_2D_3$ could be reached at up to 100-fold lower concentrations, whereas CB966 and OCT were nearly equipotent with 1,25-$(OH)_2D_3$. The growth inhibition by the vitamin D_3 compounds could be augmented by combined treatment with tamoxifen. At the maximal effective concentrations of the vitamin D_3 compounds, the effect of combined treatment was additive (MCF-7 cells) or less than additive (ZR-75-1 cells). Tamoxifen increased the sensitivity of the cells to the vitamin D_3 compounds 2- to 4000-fold, which was expressed by a shift to lower median effective concentration values. Thereby, the vitamin D_3 compounds may be used at even lower dosages in combination therapy with tamoxifen. A major problem of tamoxifen therapy is the development of tamoxifen-resistance. We have observed that tamoxifen-resistant clones of ZR-75-1 cells retain their response to the vitamin D_3 compounds. Regulation of the growth-related oncogene c-myc (mRNA level) and the oestrogen receptor (protein level) were studied but appeared not to be related to the antiproliferative action of the vitamin D_3 compounds. Together, our data point to a potential benefit of combination therapy with 1,25-$(OH)_2D_3$ or vitamin D_3 analogues and tamoxifen for the treatment of breast cancer.

INTRODUCTION

The seco-steroid hormone 1,25-dihydroxyvitamin D_3 (1,25-$(OH)_2D_3$), the biologically most active metabolite of vitamin D_3, is a well-known regulator of calcium homeostasis and bone metabolism (1). Over the last decade, evidence has accumulated that 1,25-$(OH)_2D_3$ is also involved in the regulation of proliferation and differentiation of cells and tissues not primarily related to mineral metabolism (2). Also in breast cancer cells and tumours, an antiproliferative effect of 1,25-$(OH)_2D_3$

has been demonstrated (3-5). These nonclassical effects offer promise for the use of 1,25-$(OH)_2D_3$ as an antiproliferative drug (6). A major drawback for its clinical application is that high doses are needed for tumour suppression, which may result in negative side-effects like the development of hypercalcaemia. Therefore, vitamin D_3 analogues were developed in an attempt to dissociate effects on growth and differentiation from effects on intestinal calcium absorption and bone resorption.

Most analogues synthesized have modifications in the side-chain of the 1,25-$(OH)_2D_3$ molecule. The vitamin D_3 side-chain analogues used in this study, CB966, EB1089, KH1060, and 22-oxa-calcitriol (OCT), have been shown to be more potent than 1,25-$(OH)_2D_3$ in the inhibition of proliferation of leukaemic cells, whereas the calcaemic activity *in vivo* was similar or even weaker (7-10). A few studies have also demonstrated antiproliferative effects of OCT, EB1089, and KH1060 on breast cancer cells in culture (8,11-13). In addition, in animal models for breast cancer, OCT and EB1089 suppressed tumour growth without development of hypercalcaemia (11,12). Thereby these data, together with the high incidence of vitamin D receptors in human breast tumours (14,15), demonstrate the potential role of vitamin D_3 analogues in the treatment of breast cancer. Presently, the mechanism of the suppression of tumour growth by 1,25-$(OH)_2D_3$ and analogues is still unclear.

The growth of normal and many breast carcinoma cells is regulated by oestrogens. Antioestrogens are effective in controlling the growth of oestrogen-responsive tumours, and the antioestrogen tamoxifen is currently widely used in endocrine therapy for breast cancer (16). During prolonged treatment, however, most tumours become eventually resistant to tamoxifen (17). For ER-negative tumours and tamoxifen-resistant tumours, therapeutic choices are limited. Because 1,25-$(OH)_2D_3$ and analogues have been shown to inhibit breast cancer growth irrespective of the oestrogen dependence (4,11), treatment with vitamin D_3 analogues may be effective for ER-positive, ER-negative, and tamoxifen-resistant breast tumours. Moreover, combined treatment with tamoxifen and vitamin D_3 analogues may provide a more beneficial effect on breast cancer.

In an earlier report we established a complementary action of tamoxifen and 1,25-$(OH)_2D_3$ on the growth of MCF-7 and ZR-75-1 oestrogen-responsive breast cancer cells (18). In view of the promising effects of low calcaemic vitamin D_3 analogues (7-9,11-13), we examined in the present study whether these compounds, in combination with tamoxifen, resulted in an even better inhibition of breast cancer cell growth. In addition, we have studied ER regulation and evaluated a possible role of the growth-related oncogene c-myc in the growth inhibition by 1,25-$(OH)_2D_3$ and analogues.

Chapter 5

MATERIALS AND METHODS

Chemicals

1,25-$(OH)_2D_3$, CB966, EB1089, and KH1060 were kindly donated by Dr. L. Binderup of LEO Pharmaceutical Products (Ballerup, Denmark), and OCT by Dr. N. Kubodera of Chugai Pharmaceutical Co. (Shizuoka, Japan). The chemical structures of the vitamin D_3 compounds are depicted in Fig. 5.1. [23,24-^3H]-1,25-$(OH)_2D_3$ (120 Ci/mmol) was purchased from Amersham International (Aylesbury, United Kingdom). 17ß-oestradiol, tamoxifen, RPMI-1640, human transferrin, and sodium selenite were purchased from Sigma Chemical Co. (St. Louis, MO). Bovine serum albumin fraction V was from ICN Biomedicals Inc. (Costa Mesa, CA). Glutamine, penicillin, streptomycin, and FCS were obtained from Life Technologies (Breda, The Netherlands). Insulin (Actrapid) was from Novo Nordisk A/S (Bagsvaerd, Denmark).

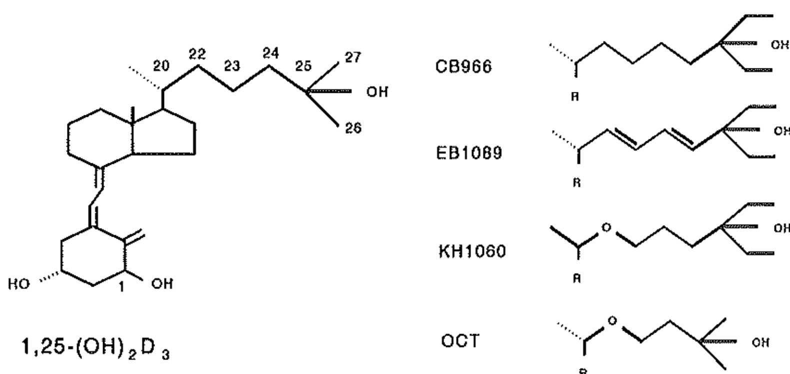

Figure 5.1. Chemical structures of 1,25-$(OH)_2D_3$ and vitamin D_3 side-chain analogs. The analogs have different types of side-chain modifications: elongation (CB966, EB1089, and KH1060), introduction of an oxygen atom at the C22 position (OCT and KH1060), unsaturation (EB1089), and altered conformation at the C20 position (KH1060).

Cell culture

MCF-7 and ZR-75-1 cells were generously provided by Dr. J.A. Foekens (Department of Endocrine Oncology, Dr. Daniel den Hoed Cancer Center, Rotterdam, The Netherlands). MCF-7 cells were maintained in RPMI-1640 supplemented with 2 mM glutamine, 100 units/ml penicillin, 100 μg/ml streptomycin, 24 mM sodium bicarbonate (basal RPMI medium), 10% FCS, and 10 μg/ml insulin. ZR-75-1 cells were maintained in basal RPMI medium supplemented with 10% FCS and 1 nM 17ß-E_2. Both cell lines were passaged weekly. Tamoxifen-resistant derivatives of ZR-75-1 cells were isolated and cultured as described previously (19). ZR/HERc cells, which are EGF receptor-positive derivatives of ZR-75-1 cells, were isolated and cultured as described by Van Agthoven *et al.* (20).

Growth experiments

Cells were seeded into six-well dishes at a density of 16,000 cells/cm^2 for MCF-7 and 32,000 cells/cm^2 for ZR-75-1 cells in phenol red-free basal RPMI medium supplemented with 2% charcoal-treated FCS. Cells were allowed to attach for 24 h. Next, medium was changed to SFM (basal RPMI medium supplemented with 30 nM sodium selenite, 10 μg/ml transferrin, and 0.2% bovine serum albumin). After another 24 h the medium was refreshed and the agents to be tested or vehicle (0.2%

ethanol) was added. Medium and test agents were replaced after 2 and 5 days of incubation, and after 8 days of incubation, DNA content was measured using the ethidium bromide method as described previously (18). Proliferation experiments with tamoxifen-resistant ZR-75-1-derived cells and ZR/HERc cells were performed using crystal violet absorbance (21). Parental ZR-75-1 cells were seeded 2000 cells/well; clones XI 13 and VIII 24 (19) 5000 cells/well; and ZR/Herc cells (20) 2200 cells/well into 96 well microplates in basal RPMI medium supplemented with 10% FCS. 10^{-10} M 17ß-E_2 was added to parental ZR-75-1 cells and 10 ng/ml EGF to ZR/Herc cells. Cells were incubated 5 to 7 days after a single addition of the vitamin D_3 compounds.

RNA isolation and hybridization

To study the effects of 17ß-E_2 and vitamin D_3 compounds on c-myc mRNA expression we have used a similar incubation procedure as in the growth experiments, i.e., 24 h after seeding (1.5×10^6 cells in 25 cm^2 culture flasks) in basal RPMI medium with 2% charcoal-treated-FCS, medium was changed to SFM, and after another 24 h test agents were added. Total RNA was prepared by acid guanidinium thiocyanate-phenol-chloroform extraction (22). Samples of 10-20 μg RNA were fractionated on a 1% agarose-2% formaldehyde gel and transferred onto a Hybond N+ nylon membrane (Amersham; Ref. 23). The membrane was prehybridized for at least 2 h at 42°C in hybridization buffer (50% formamide, 6% dextran sulphate, 1x Denhardt's solution, 1 μg/ml denatured herring sperm DNA, 5x SSC (1x SSC = 150 mM sodium chloride, 15 mM sodium citrate), 0.2% SDS, and 0.02 M NaH_2PO_4). Hybridization was carried out at 42°C for 16-24 h. The probes, a 1.4 kilobase *Cla*I-*Eco*RI fragment of the human c-myc gene (24) and a 0.8 kilobase *Eco*RI-*Hin*dIII fragment of the human GAPDH gene (25), were labelled with [^{32}P]ATP using random primers. After hybridization, membranes were washed twice in 2x SSC, 0.1% SDS for 5 min at room temperature, twice in 2x SSC, 0.1% SDS for 20 min at 42°C, and twice in 0.5x SSC, 0.1% SDS for 20 min at 42°C. Membranes were exposed to medical X-ray films (Fuji Photo Film Co., Tokyo, Japan), and autoradiographs were quantified using a Bio-Rad Videodensitometer (Richmond, CA). Before rehybridization, membranes were washed at least 2 h at 65°C in 5 mM Tris-HCl (pH 8.0), 0.2 mM EDTA (pH 8.0), 0.05% sodium pyrophosphate, and 0.1x Denhardt's solution.

Determination of oestrogen receptor content

Cells were grown to subconfluence in 175 cm^2 culture flasks. Next, cells were washed 2 times in SFM during 24 h to remove steroids. Cells were incubated with the vitamin D_3 compounds or vehicle (0.1% ethanol) in SFM for 24 or 48 h prior to harvesting with 3 mM EDTA in phosphate-buffered saline. Cell pellets were quickly frozen in liquid nitrogen and homogenized using a microdismembrator as described by Van Agthoven *et al.* (20). Cytosolic extracts were prepared by high-speed centrifugation at 100,000 x g for 20 min and ER content was measured with an enzyme immunoassay (Abbott ER-EIA; Abbott Laboratories, Chicago, IL).

Vitamin D receptor binding assay

Cells were grown to subconfluence and washed for 24 h in SFM to remove steroids. A VDR binding assay was performed as described previously (26). Briefly, cells were harvested by trypsinization, and the cell pellets were extracted on ice in a hypertonic buffer consisting of 300 mM KCl, 10 mM Tris-HCl, 1 mM EDTA, 5 mM dithiotreitol, 10 mM sodium molybdate, and 0.1% Triton X-100 (pH 7.4). High-speed supernatants were obtained by centrifugation at 100,000 x g for 1 h at 4°C. Aliquots of cytosolic extracts (containing approximately 1 mg protein) were incubated for 3 h at 0°C with 0.25 nM [^{3}H]1,25-$(OH)_2D_3$ and increasing concentrations (5×10^{-11} M - 5×10^{-7} M) of unlabelled 1,25-$(OH)_2D_3$ or vitamin D_3 analog. Receptor-bound and free [^{3}H]1,25-$(OH)_2D_3$ were separated by charcoal adsorption. The 1,25-$(OH)_2D_3$/analog concentrations resulting in 50% displacement of bound [^{3}H]1,25-$(OH)_2D_3$ were calculated.

RESULTS

Effect of vitamin D_3 compounds on the growth of MCF-7 cells

MCF-7 cells were able to grow exponentially in SFM without further additions (= autonomous growth). After an 8-day incubation period, DNA content of control cultures increased from 6 μg/well to about 50 μg/well. As shown in Fig. 5.2, 1,25-$(OH)_2D_3$ and the synthetic analogues OCT, CB966, EB1089, and KH1060 inhibited autonomous growth. The chemical structures of the analogues are depicted in Fig. 5.1. The maximum effect (25% inhibition) was similar for 1,25-$(OH)_2D_3$ and analogues, and was reached at approximately 10^{-8} M EB1089 and KH1060, 10^{-7} M 1,25-$(OH)_2D_3$ and CB966, and 10^{-6} M OCT. The concentrations of the analogues needed to achieve the half-maximal effect of 1,25-$(OH)_2D_3$ (designated as EC_{50}) were assessed, and based on these concentrations, the relative potencies with respect to 1,25-$(OH)_2D_3$ were calculated.

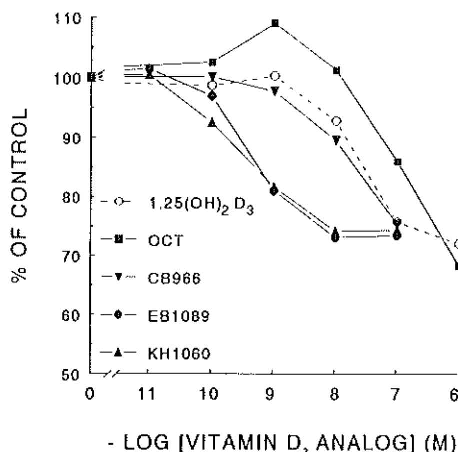

Figure 5.2. Inhibition of autonomous growth of MCF-7 cells by 1,25-$(OH)_2D_3$ and vitamin D_3 analogs. MCF-7 cells were cultured for 8 days in SFM as described in "Materials and Methods" in the absence or presence of various concentrations of the vitamin D_3 compounds. DNA content of the control cultures was set at 100% (49 μg DNA/well). Data represent the mean of three separate experiments each consisting of duplicate wells.

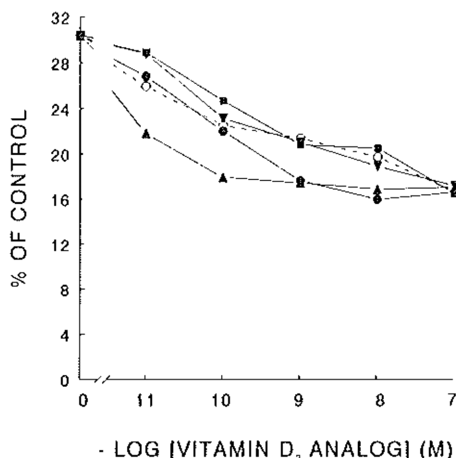

Figure 5.3. Inhibition of 17β-E_2-stimulated growth of MCF-7 cells by combined treatment with tamoxifen and vitamin D_3 compounds. Cells were cultured for 8 days in SFM supplemented with 10^{-10} M 17β-E_2 and 10^{-6} M tamoxifen, in the absence or presence of various concentrations of the vitamin D_3 compounds. Tamoxifen alone reduced 17β-E_2-stimulated proliferation to 30% (30 μg DNA/well), which is indicated as the starting value on the vertical axis. Data represent the mean of three separate experiments each consisting of duplicate wells. Symbols, see Fig. 5.2.

Table 5.1. Growth inhibition of MCF-7 cells by 1,25-$(OH)_2D_3$ and vitamin D_3 analogs and their affinity for the VDR

	GROWTH INHIBITION							
			17ß-E_2-stimulated growth					
	Autonomous growth		Without tamoxifen		With tamoxifen		Ratio of EC_{50} without and with tamoxifen[b]	VDR binding relative to 1,25-$(OH)_2D_3$
Analogue	EC_{50} (M)[a]	Relative to 1,25-$(OH)_2D_3$	EC_{50} (M)	Relative to 1,25-$(OH)_2D_3$	EC_{50} (M)	Relative to 1,25-$(OH)_2D_3$		
1,25-$(OH)_2D_3$	2×10^{-8}	1	1×10^{-8}	1	5×10^{-11}	1	200	1
OCT	8×10^{-8}	0.25	2×10^{-8}	0.50	2×10^{-10}	0.25	100	0.1
CB966	1×10^{-8}	2	1×10^{-8}	1	8×10^{-11}	0.63	125	0.7
EB1089	3×10^{-10}	67	1×10^{-10}	100	5×10^{-11}	1	2	0.7
KH1060	2×10^{-10}	100	1×10^{-10}	100	7×10^{-12}	7	14	0.7

[a]The concentrations of the analogs needed to achieve the half-maximal effect of 1,25-$(OH)_2D_3$ (designated as EC_{50}) were assessed on basis of the data in Figs. 5.2 and 5.3. The maximum inhibition of autonomous growth was 25%, and of 17ß-E_2-stimulated growth 12%. Tamoxifen inhibited 17ß-E_2-stimulated growth by 70% and tamoxifen combined with the vitamin D_3 compounds resulted in a maximum inhibition of 83%.
[b]The ratio expresses the magnitude of the shift of the EC_{50} by cotreatment with tamoxifen and was calculated by division of the EC_{50} in the absence of tamoxifen by the EC_{50} in the presence of tamoxifen. Binding of 1,25-$(OH)_2D_3$ and analogs to the VDR was measured in cytosolic extracts by displacement of $[^3H]$1,25-$(OH)_2D_3$.

OCT displayed a somewhat decreased potency and CB966 a small increased potency, whereas EB1089 and KH1060 were clearly more potent than 1,25-$(OH)_2D_3$, 67 and 100 times, respectively (Table 5.1).

Since 17ß-E_2 plays an important role in breast cancer development and growth, we have investigated the effect of the vitamin D_3 analogues on 17ß-E_2-stimulated proliferation of MCF-7 cells. 17ß-E_2 (10^{-10} M) resulted in a 2-fold stimulation of control cultures. Inhibition of 17ß-E_2-stimulated growth by the vitamin D_3 compounds was relatively small (approximately 12%; data not shown). The absolute inhibition of 17ß-E_2-stimulated growth expressed in μg DNA/well was similar to the absolute inhibition of the autonomous growth, indicating that there was no specific inhibition of the 17ß-E_2 effect. Also, the EC_{50}s of 1,25-$(OH)_2D_3$ and analogues were in the same order of magnitude as for autonomous growth (Table 5.1).

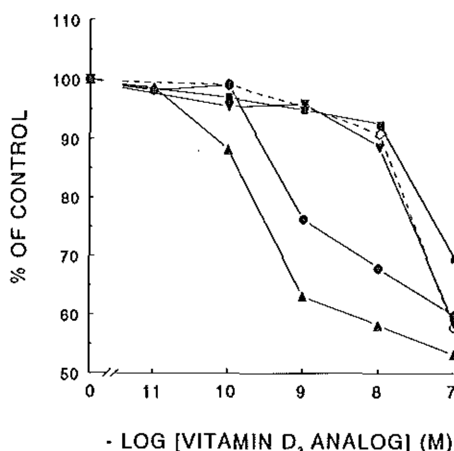

Figure 5.4. Growth inhibition of ZR-75-1 cells by 1,25-$(OH)_2D_3$ and vitamin D_3 analogs. ZR-75-1 cells were grown for 8 days in SFM supplemented with 10^{-10} M 17ß-E_2 in the presence or absence of various concentrations of the vitamin D_3 compounds. DNA values of control cultures (no vitamin D added) increased from 7 to 21 μg DNA/well in 8 days. The absolute increase in DNA content (14 μg DNA/well) was set at 100%. Data represent the mean of three separate experiments each consisting of duplicate wells. Symbols, see Fig. 5.2.

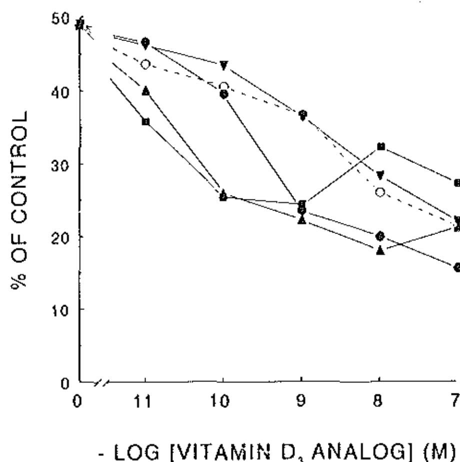

Figure 5.5. Growth inhibition of ZR-75-1 cells by combined treatment with tamoxifen and vitamin D_3 compounds. Cells were cultured for 8 days in SFM supplemented with 10^{-10} M 17ß-E_2 and 10^{-7} M tamoxifen, in the absence or presence of various concentrations of the vitamin D_3 compounds. DNA content was measured and corrected for DNA content at the start of the experiment. Tamoxifen alone reduced 17ß-E_2-induced proliferation to 49%, which is indicated as the starting value on the vertical axis. Data represent the mean of three separate experiments each consisting of duplicate wells. Symbols, see Fig. 5.2.

Table 5.2. Growth inhibition of ZR-75-1 cells by 1,25-$(OH)_2D_3$ and vitamin D_3 analogs

Analogue	17ß-E_2-stimulated growth: Without tamoxifen: EC_{50} (M)[a]	Without tamoxifen: Relative to 1,25-$(OH)_2D_3$	With tamoxifen: EC_{50} (M)	With tamoxifen: Relative to 1,25-$(OH)_2D_3$	Ratio of EC_{50} without and with tamoxifen[b]
1,25-$(OH)_2D_3$	2×10^{-8}	1	1×10^{-9}	1	20
OCT	4×10^{-8}	0.5	1×10^{-11}	100	4000
CB966	2×10^{-8}	1	1×10^{-9}	1	20
EB1089	7×10^{-10}	29	2×10^{-10}	5	4
KH1060	2×10^{-10}	100	2×10^{-11}	50	10

[a]The concentrations of the analogs needed to achieve the half-maximal effect of 1,25-$(OH)_2D_3$ (designated as EC_{50}) were assessed on basis of the data in Figs. 5.4 and 5.5. The maximal inhibition of the 17ß-E_2-stimulated growth was 40%. Tamoxifen inhibited the 17ß-E_2-stimulated growth 51% and tamoxifen combined with the vitamin D_3 compounds resulted in a maximum inhibition of 79%.
[b]The ratio expresses the magnitude of the shift of the EC_{50} by cotreatment with tamoxifen and was calculated by division of the EC_{50} in the absence of tamoxifen by the EC_{50} in the presence of tamoxifen.

Inhibition of 17ß-E_2-stimulated growth of breast cancer cells by the antioestrogen tamoxifen is well documented. In MCF-7 cells, tamoxifen not onlycompletely blocked 17ß-E_2-stimulated growth but also inhibited part of the autonomous growth of MCF-7 cells, i.e., DNA values of cultures treated with 10^{-10} M 17ß-E_2 and 10^{-6} M tamoxifen (30 μg/well) were lower than DNA values of control cultures (49 μg/well). Subsequently, we examined the effect of combined treatment with 1,25-$(OH)_2D_3$/analogues and tamoxifen on 17ß-E_2-stimulated growth. Fig. 5.3 shows that, although tamoxifen alone causes a potent growth inhibition (reduction to 30% of 17ß-E_2-stimulated growth), addition of 1,25-$(OH)_2D_3$ or analogues resulted in a still further inhibition (from 30% down to 17% of 17ß-E_2-stimulated growth). In combination with tamoxifen, the EC_{50}s of 1,25-$(OH)_2D_3$ and analogues shifted lower concentrations. The EC_{50}s of 1,25-$(OH)_2D_3$, CB966, and OCT were 200-, 125- and 100-fold lower, respectively, in the presence of tamoxifen, whereas the EC_{50}s of KH1060 and EB1089 were only 14- and 2-fold lower, respectively (Table 5.1). Consequently, the differences between 1,25-$(OH)_2D_3$ and the analogues are smaller in the presence of tamoxifen. Although the sensitivity to 1,25-$(OH)_2D_3$/analogues was increased by combined treatment with tamoxifen the inhibitory effect at the maximal effective concentrations of 1,25-$(OH)_2D_3$/analogues was additive.

Table 5.3. Growth inhibition of tamoxifen-resistant and EGF receptor-positive derivatives of ZR-75-1 cells by 1,25-$(OH)_2D_3$ and KH1060

	% of control			
Condition	ZR-75-1	XI 13	VIII 24	ZR/HERc
Control	100 ± 6	100 ± 3	100 ± 3	100 ± 9
10^{-9} M 1,25-$(OH)_2D_3$	107 ± 9	113 ± 3	88 ± 4	98 ± 6
10^{-7} M 1,25-$(OH)_2D_3$	55 ±8	78 ± 3	35 ± 1	30 ± 7
10^{-9} M KH1060	59 ± 6	80 ± 4	37 ± 3	29 ± 7
10^{-7} M KH1060	49 ± 4	79 ± 3	35 ± 3	28 ± 7

Cells were cultured in basal RPMI medium supplemented with 10% FCS in the absence or presence of 1,25-$(OH)_2D_3$ or KH1060. 17ß-E_2 (10^{-10} M) was added in cultures of parental ZR-75-1 cells and EGF (10 ng/ml) in cultures of EGF receptor-positive ZR/HERc cells to induce the growth of these cells. The tamoxifen-resistant XI 13 and VIII 24 cells are able to grow autonomously. Proliferation of XI 13 cells was measured after 5 days, and proliferation of ZR-75-1, VIII 24, and ZR/HERc cells after 7 days by crystal violet absorbance. Data are expressed as the percentage of control and represent mean ± SD of nine wells.

Effect of vitamin D_3 compounds on the growth of ZR-75-1 cells

Next, we studied the effect of the vitamin D_3 compounds on another oestrogen-responsive breast cancer cell line. As opposed to MCF-7 cells, ZR-75-1 cells are dependent on 17ß-E_2 for growth. 17ß-E_2 (10^{-10} M) induced an increase in DNA from 7 to 21 μg DNA/well in 8 days. Fig. 5.4 shows that, in contrast to 17ß-E_2-stimulated growth of MCF-7 cells, 17ß-E_2-stimulated growth of ZR-75-1 cells was potently inhibited by the vitamin D_3 compounds. The EC_{50}s for the growth inhibition of ZR-75-1 cells were similar to those of MCF-7 cells with the exception of EB1089, which had a somewhat reduced potency in ZR-75-1 cells (Table 5.2).

Analogous to MCF-7 cells, we have analyzed the combined effects of vitamin D_3 analogues and tamoxifen on the growth of ZR-75-1 cells. Tamoxifen (10^{-6} M) completely blocked 17ß-E_2-stimulated growth of ZR-75-1 cells and, thereby, the entire proliferation. Therefore, we have used a suboptimal concentration of tamoxifen (10^{-7} M) to test whether there is an interaction between tamoxifen and vitamin D_3 compounds. Fig. 5.5 demonstrates that the inhibition by tamoxifen (reduction to 49% of 17ß-E_2-stimulated growth) can be augmented by addition of 1,25-$(OH)_2D_3$ or analogues (further reduction from 49% down to an average of 21% of 17ß-E_2-stimulated growth). Similar to MCF-7 cells, the EC_{50}s for the inhibition by the vitamin D_3 compounds were shifted to the left in the presence of tamoxifen. The EC_{50} of OCT was even 4000 times lower; the EC_{50}s of 1,25-$(OH)_2D_3$ and CB966, 20

times; and the EC_{50}s of EB1089 and KH1060, 4 and 10 times; respectively (Table 5.2). Despite the increased sensitivity of 1,25-$(OH)_2D_3$ and analogues, the effect of combined treatment with tamoxifen was less than additive at the maximal effective concentrations of the vitamin D_3 compounds.

To assess whether treatment with 1,25-$(OH)_2D_3$/analogues could be useful in an antiestrogen-resistant situation, we have tested the effect of 1,25-$(OH)_2D_3$ and KH1060 on tamoxifen-resistant derivatives of ZR-75-1 cells. These resistant cells were cloned from oestrogen-dependent ZR-75-1 cells subjected to tamoxifen selection after retrovirus infection (19). Table 5.3 demonstrates that the growth of the resistant XI 13 and VIII 24 cells was indeed inhibited by 1,25-$(OH)_2D_3$ and KH1060, with KH1060 being more potent. Furthermore, EGF-dependent proliferation of EGF receptor-positive derivatives of ZR-75-1 cells (20) was strongly inhibited by 1,25-$(OH)_2D_3$ and KH1060 (Table 5.3).

Regulation of oestrogen receptor content

To study whether vitamin D_3 compounds exert their effects on proliferation via regulation of ER level, we have measured ER content of both cell lines using an enzyme immunoassay. The vitamin D_3 compounds 1,25-$(OH)_2D_3$, CB966, EB1089, KH1060 and OCT (10^{-8} M) had no effect on the ER level of MCF-7 (750 fmol/mg protein) and ZR-75-1 cells (180 fmol/mg protein) after 24 and 48 h of incubation (data not shown).

Regulation of c-myc mRNA expression

To study a possible involvement of c-myc in the growth inhibition by vitamin D_3 compounds, we have studied c-myc mRNA expression in MCF-7 and ZR-75-1 cells. In an attempt to relate growth inhibition to effects on c-myc mRNA expression, we have measured c-myc under the same conditions as the proliferation experiments. c-myc mRNA levels of autonomously growing MCF-7 cells incubated with 10^{-7} M 1,25-$(OH)_2D_3$ for 0.5 up to 48 h did not change significantly with respect to the control incubation (vehicle added; data not shown). 17ß-E_2-stimulated growth of MCF-7 and ZR-75-1 cells was studied using 10^{-10} M 17ß-E_2. At this concentration, c-myc was rapidly induced in MCF-7 cells (25-fold) and ZR-75-1 cells (2-fold) with a peak at 1 h and a gradual return towards prestimulation level in 4 h. In MCF-7 cells 10^{-10} M 17ß-E_2 was the maximal effective concentration, whereas in ZR-75-1 cells a maximal 6-fold stimulation was achieved at 10^{-9} M 17ß-E_2. Fig. 5.6 demonstrates that neither 1,25-$(OH)_2D_3$ nor vitamin D_3 analogues modulated the 17ß-E_2-induced c-myc mRNA expression in MCF-7 cells (Fig. 5.6, lanes 2-17). Tamoxifen (10^{-6} M) almost

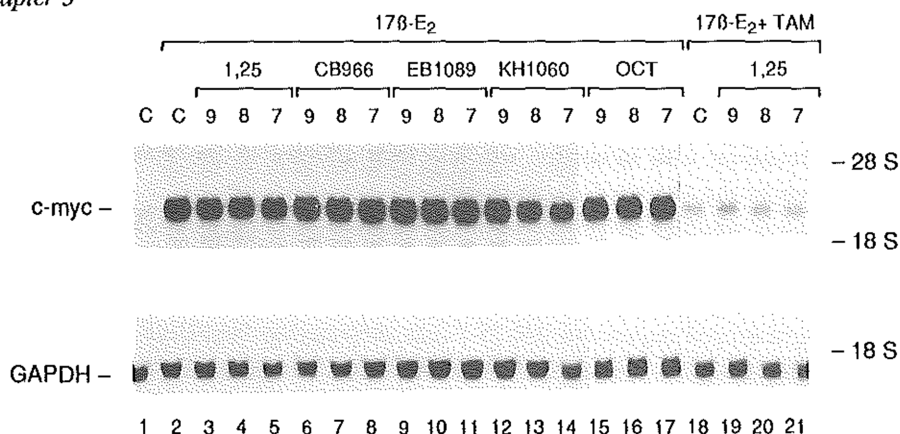

Figure 5.6. Northern analysis of c-myc in MCF-7 cells. Autoradiograph of RNA hybridized with ^{32}P-labeled c-myc and GAPDH probes. GAPDH was used to check equal loading of the lanes and ribosomal RNA bands (28 S, 18 S) served as molecular weight markers. Cells were cultured as described in Materials and Methods and incubated for 1 h with vehicle (lane 1 and 18), 10^{-10} M 17ß-E_2 (lanes 2-21), or 10^{-6} M tamoxifen (lanes 18-21). 1,25-$(OH)_2D_3$ (10^{-9}, 10^{-8}, and 10^{-7} M) was added in lanes 3-5 and 19-21, CB966 in lanes 6-8, EB1089 in lanes 9-11, KH1060 in lanes 12-14, and OCT in lanes 15-17.

completely inhibited c-myc induction by 17ß-E_2 (Fig. 5.6, lane 18). With ZR-75-1 cells similar results were obtained (data not shown). Subsequently, we have tested in MCF-7 cells the combined effects of 1,25-$(OH)_2D_3$/analogues and tamoxifen. The inhibition of 17ß-E_2-induced c-myc expression by tamoxifen was neither augmented by combined treatment with 1,25-$(OH)_2D_3$ (Fig. 5.6, lanes 19-21) nor by the vitamin D_3 analogues (data not shown).

Vitamin D receptor binding

VDR binding of the analogues was measured to study whether the differences between the vitamin D_3 analogues in their ability to inhibit breast cancer cell growth were related to their affinity for the VDR. It is demonstrated in Table 5.1 that the analogues had a lower affinity for the VDR compared to 1,25-$(OH)_2D_3$. The reduced growth inhibitory potential of OCT corresponded with a lower VDR affinity compared to 1,25-$(OH)_2D_3$, but the increased growth inhibitory potential of EB1089 and KH1060 did not correspond with their lower VDR affinity.

To study whether the increased sensitivity to the vitamin D_3 compounds by cotreatment with tamoxifen was caused by increased VDR binding, we have measured VDR concentration in MCF-7 cells after incubation for 24 h with 10^{-6} M tamoxifen. The VDR concentration of 15 fmol/mg protein was not changed by treatment with tamoxifen. Also, 17ß-E_2 had not effect on VDR levels.

DISCUSSION

The present data show that the growth inhibitory action of vitamin D_3 compounds and tamoxifen are complementary. In MCF-7 cells, which have partially escaped from oestrogenic control, combined treatment resulted in a stronger inhibition, than treatment with either compound alone. In ZR-75-1 cells, which are fully oestrogen-dependent, the entire proliferation could be blocked by a high concentration of tamoxifen, whereas at lower tamoxifen concentration, vitamin D_3 compounds were able to augment the inhibitory effect of tamoxifen, similar as in MCF-7 cells. The clinical use of lower dosages of tamoxifen may be beneficial, considering the relationship between tamoxifen and an increased risk on endometrial cancer (16). Resistance to tamoxifen therapy frequently occurs (17), and in this situation, treatment with vitamin D_3 analogues could also be useful. This is supported by our data that derivatives of ZR-75-1 cells, which had acquired resistance to tamoxifen as a result of retroviral insertional mutagenesis (19), had not lost their response to the growth-inhibitory action of the vitamin D_3 compounds.

Despite the promising antiproliferative effects *in vitro*, the calcaemic effects of vitamin D_3 compounds may result in the development of hypercalcaemia when applied *in vivo*. The present data show that the analogues EB1089 and KH1060 have the same growth-inhibitory action as 1,25-$(OH)_2D_3$ at up to 100-fold lower concentrations, whereas *in vivo* studies have shown that the calcaemic activity of these compounds was equal or even lower (8,9). These results support the idea that the antiproliferative effects can be (partly) dissociated from the calcaemic effects. Our data on the potent growth inhibitory effects of EB1089 and KH1060 are in line with other reports (8,12,13). Additionally, we show for the first time that EB1089 and KH1060 can also inhibit the growth of a fully 17ß-E_2-dependent cell line (ZR-75-1) with increased potency compared to 1,25-$(OH)_2D_3$. In various ER-positive and ER-negative human breast cancer cell lines OCT was 10 times more potent than 1,25-$(OH)_2D_3$ (11). In our hands the potency of OCT was somewhat diminished compared to 1,25-$(OH)_2D_3$ in both cell lines studied. The reason for this discrepancy is not yet known. CB966 and 1,25-$(OH)_2D_3$ were equipotent, and to our knowledge there are no other reports on the effect of CB966 on breast cancer cells.

For translation of the antiproliferative action of vitamin D_3 analogues on breast cancer cells in culture to the *in vivo* situation, the pharmacokinetic properties of the analogues play an important role (27). Further, negative side-effects have to be monitored. Apart from the development of hypercalcaemia other effects may arise, for instance the oxa-compounds OCT and KH1060 have been shown to exert strong

immunosuppressive activity (8,28), and stimulation of bone resorption might increase the incidence of skeletal metastases (29)

In view of the clinical importance of using low doses, the observation that, in combination with tamoxifen, the EC_{50}s of 1,25-$(OH)_2D_3$ and analogues shifted to lower concentrations might be of interest. This shift was more pronounced for 1,25-$(OH)_2D_3$ and the compounds with an EC_{50} close to 1,25-$(OH)_2D_3$ (OCT and CB966) than for the compounds with a more favourable EC_{50} (EB1089 and KH1060) in the absence of tamoxifen. Thereby the differences in potencies between the analogues were smaller in the presence of tamoxifen. The shift of the EC_{50}s of the vitamin D_3 compounds in the presence of tamoxifen points to an interaction between both types of growth inhibitors. This is in agreement with a report from Abe-Hashimoto *et al.* (30), who have observed synergism between OCT and tamoxifen in MCF-7 and ZR-75-1 cells *in vitro* as well as in MCF-7 tumour *in vivo*. The mechanism of the interaction between tamoxifen and vitamin D_3 compounds is yet unclear, but the present data show that it was not achieved via a change in VDR level. Notwithstanding the interaction between both growth inhibitors, at the maximal effective concentrations of the vitamin D_3 compounds the effect of combined treatment with tamoxifen was additive (MCF-7 cells) or less than additive (ZR-75-1 cells).

Our data on the effect of combined treatment with tamoxifen and vitamin D_3 compounds point to several potential advantages when applied *in vivo*: (a) a more beneficial response can be achieved than by either agent alone; (b) lower concentrations of 1,25-$(OH)_2D_3$/analogues and tamoxifen can be used with reduced risk of negative side-effects (hypercalcaemia, increased bone turnover, endometrial cancer); (c) since tumours are believed to be heterogenous with respect to ER status (31), combination therapy may have the advantage that both ER-positive and ER-negative cells are inhibited; (d) tumour flare in response to tamoxifen may be prevented since 1,25-$(OH)_2D_3$ has been shown to inhibit the oestrogenic effect of tamoxifen (18); and (e) tamoxifen may attenuate the negative effects of vitamin D_3 compounds on bone metabolism by its positive oestrogenic effect on bone (32).

Since 17ß-E_2 is an important regulator of breast cancer growth, we have addressed the question whether vitamin D_3 compounds can interfere directly with the growth stimulation by 17ß-E_2. The data obtained with MCF-7 cells clearly indicate an oestrogen-independent mechanism of action: (a) the inhibition of 17ß-E_2-stimulated growth, expressed in μg DNA/well, by the vitamin D_3 compounds was similar to the inhibition of autonomous growth (maximum and EC_{50}s); (b) the ER level was not down-regulated; (c) the rapid induction of c-myc mRNA, which is thought to mediate

the growth effect of 17ß-E_2 (33) was not affected; and (d) the expression of the 17ß-E_2-regulated pS2 gene was not changed[1]. Moreover, an oestrogen independent mechanism of action is consistent with the fact that 1,25-$(OH)_2D_3$ or analogues inhibit breast cancer cell growth irrespective of the presence of the ER (4,11). The inhibition of 17ß-E_2-induced growth of ZR-75-1 cells seems to contradict an entirely 17ß-E_2-independent mechanism of action of the vitamin D_3 compounds. However, as in MCF-7 cells, the ER and c-myc levels were not affected. Furthermore, EGF-dependent proliferation of ZR/HERc cells (derived from ZR-75-1) was also inhibited, indicating that vitamin D_3 compounds affect ZR-75-1 cell growth at a late stage of the signal cascades used by both the ER and EGF receptor. This may be achieved by induction of a negative growth factor or the inhibition of an autocrine loop. Also, the observed interaction between vitamin D_3 compounds and tamoxifen may occur at this level, since tamoxifen has been shown to exert antioestrogenic as well as antigrowth factor activity mediated via the ER (34).

In MCF-7 cells and several other cell types it has been demonstrated 1,25-$(OH)_2D_3$-induced differentiation and growth inhibition was preceded by a decrease in mRNA of the protooncogene c-myc (13,35-37), indicating that c-myc gene regulation could be involved in the pathway for 1,25-$(OH)_2D_3$-mediated control of cell proliferation. However, we did not find changes in c-myc mRNA by 1,25-$(OH)_2D_3$ neither in autonomously growing MCF-7 cells nor in 17ß-E_2-stimulated MCF-7 and ZR-75-1 cells, suggesting that downregulation of c-myc mRNA is not a prerequisite for the growth inhibition by vitamin D_3 compounds in breast cancer cells.

The differences between the growth inhibitory potential of the analogues are difficult to explain. The present data show that the maximal inhibition by 1,25-$(OH)_2D_3$ and analogues was the same, suggesting that they all act via the same pathway. Measurements of VDR affinity showed that the increased growth inhibitory potential of EB1089 and KH1060 was not related to a higher VDR affinity. Also, VDR-independent or nongenomic mechanisms of action have been attributed to 1,25-$(OH)_2D_3$ and analogues (2). However, it has been reported that the antiproliferative action of 1,25-$(OH)_2D_3$/analogues is restricted to VDR-positive cells (38). Other explanations could be differences in cellular metabolism of the analogues, receptor phosphorylation or conformational changes of the receptor-ligand complex.

In conclusion, a more beneficial growth response of ER-positive breast cancer cells was observed by combined treatment with vitamin D_3 analogues and tamoxifen than by treatment with these compounds alone. The vitamin D_3 analogues were active

[1]T. Vink-van Wijngaarden, H.A.P. Pols, J.C. Birkenhäger, and J.P.T.M. van Leeuwen, unpublished data.

at up to 100-fold lower concentrations than the native compound 1,25-$(OH)_2D_3$, which is important to reduce the risk on negative side-effects. Tamoxifen increased the sensitivity to 1,25-$(OH)_2D_3$ and analogues, which could implicate that in combination therapy even lower concentrations of the vitamin D_3 compounds can be used. Further, it was shown that also the growth of antioestrogen-resistant cells could be inhibited by 1,25-$(OH)_2D_3$ and analogues. Thereby, vitamin D_3 analogues are interesting candidates for breast cancer therapy.

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Chapter 6

ANTIOESTROGENS INHIBIT IN VITRO BONE RESORPTION STIMULATED BY 1,25-DIHYDROXYVITAMIN D_3 AND THE VITAMIN D_3 ANALOGUES EB1089 AND KH1060

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ABSTRACT

1,25-Dihydroxyvitamin D_3 (1,25-$(OH)_2D_3$) has been shown to inhibit breast cancer cell growth both *in vitro* and *in vivo*. A major drawback is that high doses of 1,25-$(OH)_2D_3$ are needed which may result in undesirable side effects like the development of hypercalcaemia and an increased risk of bone metastases due to the stimulation of bone resorption by 1,25-$(OH)_2D_3$. Several newly developed 1,25-$(OH)_2D_3$ analogues have a reduced calcaemic activity, but their direct effects on bone resorption have not yet been examined. Presently, the antioestrogen tamoxifen is the most important endocrine therapy for breast cancer. Recent studies have demonstrated the benefit of the combination tamoxifen and 1,25-$(OH)_2D_3$/analogues for the inhibition of breast cancer cell growth. Besides inhibition of breast cancer growth tamoxifen appeared to have beneficial effects on bone. The purpose of the present study was to investigate the effect of tamoxifen on 1,25-$(OH)_2D_3$- and analogues (EB1089 and KH1060)-stimulated bone resorption in an *in vitro* model. Bone resorption was stimulated by 1,25-$(OH)_2D_3$ and analogues in a dose-dependent manner with KH1060 and EB1089 being more potent than 1,25-$(OH)_2D_3$. Tamoxifen caused a strong dose-dependent inhibition (70 % at 10 μM) of 1,25-$(OH)_2D_3$- and EB1089-stimulated bone resorption. KH1060-stimulated bone resorption was also inhibited by tamoxifen but to a lesser extent (36 %). Also the pure antioestrogen ICI164,384 but not 17ß-oestradiol inhibited 1,25-$(OH)_2D_3$-stimulated bone resorption. Together, this study demonstrates that tamoxifen considerably reduces 1,25-$(OH)_2D_3$/analogues-stimulated bone resorption and therefore may be useful to reduce the risk of bone metastases. This together with the observed beneficial effects on breast cancer cell growth indicates that tamoxifen together with 1,25-$(OH)_2D_3$ /analogues is an interesting combination for the treatment of breast cancer. The mechanism of the bone resorption inhibitory action is not yet known but seems to be independent of the oestrogen pathway.

INTRODUCTION

Besides regulation of calcium and bone metabolism, both *in vivo* and *in vitro* studies have shown that 1,25-dihydroxyvitamin D_3 (1,25-$(OH)_2D_3$) inhibits the growth of breast cancer cells (1,2). These findings suggest a potential use of 1,25-$(OH)_2D_3$ for the treatment of breast cancer. However high doses of the sterol are needed. Therefore it remains to be established whether 1,25-$(OH)_2D_3$ can produce long term antitumour effects without unacceptable side effects, like the development of hypercalcaemia. Also,

the relation between increased bone resorption, which is stimulated by 1,25-$(OH)_2D_3$, and the development of bone metastases has been described (3-6). Rats treated with 1,25-$(OH)_2D_3$ had significantly more skeletal metastases than untreated controls when intra-arterially injected with Walker 256 tumour cells (5). A number of vitamin D_3 analogues have been developed which inhibit breast cancer cell growth at lower concentrations compared to 1,25-$(OH)_2D_3$ (7,8). The analogues EB1089 and 22-oxa-calcitriol inhibited tumour growth without marked hypercalcaemic effects in animal models for breast cancer (9,10). So far, the effects on bone resorption have not been examined.

Tamoxifen is currently the most widely used endocrine therapy for breast cancer. Recently, interactions between 1,25-$(OH)_2D_3$/analogues and tamoxifen with respect to growth inhibition of breast cancer cells have been observed (8,11). We have shown that in combination with tamoxifen significantly lower concentrations could be used which would increase the therapeutic potential of 1,25-$(OH)_2D_3$/analogues (8). Finally, in breast cancer patients it was shown that tamoxifen has protective effects on the skeleton (12,13).

In the present study we extended the investigations on the combination of 1,25-$(OH)_2D_3$/ analogues and tamoxifen to the regulation of bone resorption. Thereby these data may provide evidence whether tamoxifen combined with 1,25-$(OH)_2D_3$/analogues not only enhances the inhibition of breast cancer cell growth but that tamoxifen also potentially reduces the risk of skeletal metastases by inhibiting 1,25$(OH)_2D_3$/analogues-stimulated bone resorption.

MATERIALS AND METHODS

Chemicals

1,25-$(OH)_2D_3$, EB1089 (7), and KH1060 (16) were kindly donated by Dr. L. Binderup, LEO Pharm. Products, Ballerup, Denmark. Tamoxifen, 17ß-oestradiol, and MTT were purchased from Sigma Chemical Co. St. Louis, MO, USA, and $^{45}CaCl_2$ from Amersham, Aylesbury, UK. ICI164,384 was a generous gift of Dr. A.E. Wakeling, ICI Pharm., UK.

In vitro bone resorption

The *in vitro* bone resorption assay is based on the release of ^{45}Ca from prelabelled fetal mouse long bones, radii and ulnae, during two successive culture periods of three days and performed as described previously (14). The ^{45}Ca content of the medium at 3 and 6 days and of 5 % formic acid extracts of the bones were measured by liquid scintillation counting and used to calculate the cumulative percentage ^{45}Ca released.

Toxicity assay

In order to test the toxicity of the antioestrogens in the long bone cultures we have measured at the end of each resorption experiment the conversion of MTT into formazan by viable cells by measuring the absorbance at 540 nm (14,15).

Data analysis

Interactions between 1,25-$(OH)_2D_3$/analogues and the antioestrogens and 17ß-oestradiol were evaluated using analysis of variance for two-way design.

RESULTS

1,25-$(OH)_2D_3$, EB1089, and KH1060 stimulated *in vitro* bone resorption in a dose-dependent manner (Figs. 6.2A-C). Based on ED_{50}, KH1060 and EB1089 were about 100 and 10 times more potent than 1,25-$(OH)_2D_3$, respectively. As shown in Fig. 6.1, 1,25-$(OH)_2D_3$-stimulated bone resorption was inhibited by 1 μM tamoxifen both after 3 (47 % inhibition) and after 6 days (50 % inhibition).

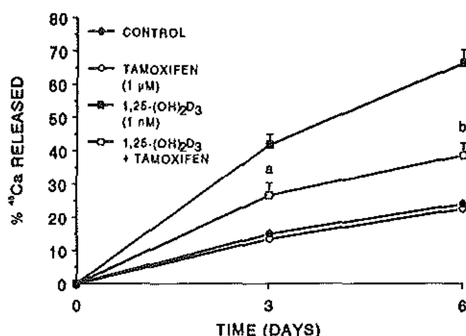
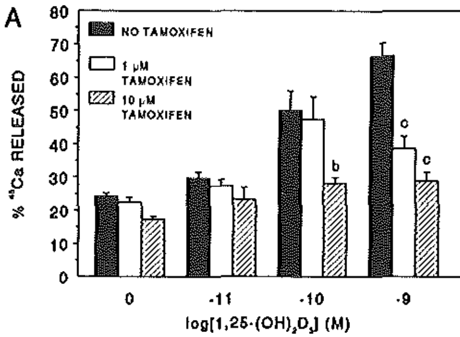
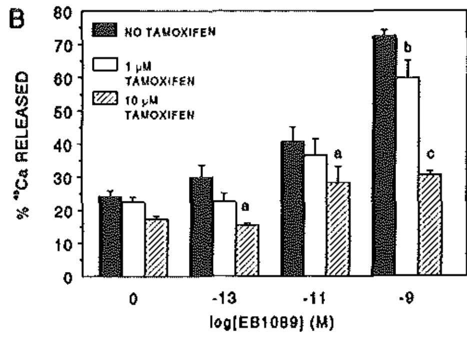
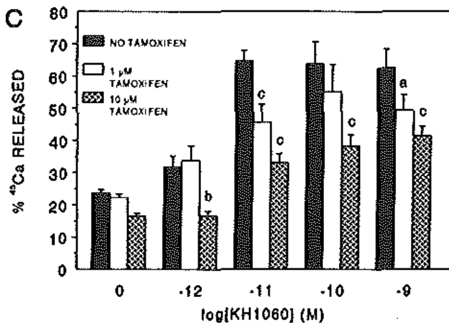


Figure 6.1. Inhibition of 10^{-9} M 1,25-$(OH)_2D_3$-stimulated bone resorption by 1 μM tamoxifen after 3 and 6 days of culture. Data are expressed as mean ± SEM of 3 independent experiments, each consisting of 4 bones per group. a: $p<0.005$, b: $p<0.0001$ calculated as the significance of interaction between 1,25-$(OH)_2D_3$ and tamoxifen.

EB1089- and KH1060-stimulated bone resorption was also inhibited after 3 as well as 6 days of culture (data not shown). Tamoxifen inhibited the 1,25-$(OH)_2D_3$- and EB1089-stimulated bone resorption in a dose-dependent manner with 10 μM tamoxifen being the most potent. In all experiments performed 10 μM but not 1 μM tamoxifen caused a small but significant reduction ($p<0.001$) of basal bone resorption (Figs. 6.2A-C). Maximally stimulated bone resorption by 10^{-9} M 1,25-$(OH)_2D_3$ and EB1089 was almost completely reduced to control level by 10 μM tamoxifen (Figs. 6.2A and B). KH1060-stimulated bone resorption was also inhibited by tamoxifen. However, in contrast to 70 % inhibition of the 1,25-$(OH)_2D_3$ and EB1089 effect, 10^{-9} M KH1060-stimulated bone resorption was only reduced by 36 % by 10 μM tamoxifen (Fig. 6.2C). Concentrations up to 10 μM tamoxifen were not toxic, whereas with higher concentrations (25 and 50 μM) cell death was observed (data not shown). 1,25-$(OH)_2D_3$-stimulated bone resorption was also inhibited by the 4-hydroxy metabolite of tamoxifen (data not shown).







Figures 6.2A, B, and C. Inhibition of A) 1,25-$(OH)_2D_3$-, B) EB1089-, and C) KH1060-stimulated bone resorption by tamoxifen after 6 days of culture. Data are expressed as mean ± SEM of 2-4 independent experiments, each consisting of 4 bones per group. a: $p<0.05$, b: $p<0.025$, c: $p<0.0005$ calculated as the significance of interaction between 1,25-$(OH)_2D_3$/ analogues and tamoxifen.

As tamoxifen has been reported to have oestrogen agonistic and antagonistic activity both the effects of a pure antioestrogen, ICI164,384, and that of 17ß-oestradiol on 1,25-$(OH)_2D_3$-stimulated bone resorption were examined. 17ß-oestradiol alone did not change *in vitro* bone resorption nor inhibited 10^{-9} and 10^{-8} M 17ß-oestradiol 1,25-$(OH)_2D_3$-stimulated bone resorption (data not shown). Like tamoxifen, the pure antioestrogen ICI164,384 (10 μM) inhibited 10^{-9} M 1,25-$(OH)_2D_3$-stimulated bone resorption after 3 and 6 days of culture by 47 and 61 %, respectively (Fig. 6.3). With 5 μM ICI164,384 a small but not significant inhibition was observed whereas 1 μM was not effective (data not shown).

In order to investigate whether the inhibitory effect of tamoxifen was specific for 1,25-$(OH)_2D_3$/analogues the effect on PTH-stimulated bone resorption was studied. Tamoxifen (10 μM) caused a small but not statistically significant reduction of 10^{-9} M PTH-stimulated bone resorption.

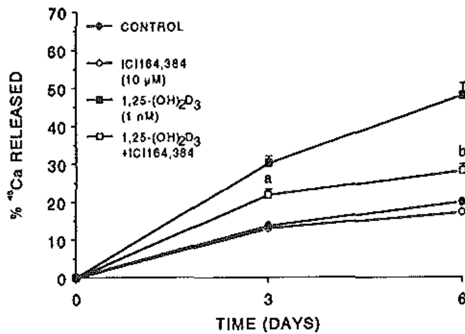


Figure 6.3. Inhibition of 10^{-9} M 1,25-$(OH)_2D_3$-stimulated bone resorption by 10 μM ICI164,384 after 3 and 6 days of culture. Data are expressed as mean ± SEM of 3 independent experiments, each consisting of 4 bones per group. a: $p<0.005$, b: $p<0.0005$ calculated as the significance of interaction between 1,25-$(OH)_2D_3$ and ICI164,384.

DISCUSSION

A potential side-effect of treatment of breast cancer by 1,25-$(OH)_2D_3$/analogues is the increased risk of skeletal metastases due to the stimulation of bone resorption by 1,25-$(OH)_2D_3$ and analogues (5). Several studies have described the relation between bone resorption and metastasis to bone (3,4,6). In contrast to the development of hypercalcaemia during treatment with 1,25-$(OH)_2D_3$/analogues, in the *in vivo* studies performed so far with 1,25-$(OH)_2D_3$ and analogues in relation to tumour growth inhibition this aspect has not been addressed (9,10). The present study demonstrates for the first time that bone resorption stimulated by 1,25-$(OH)_2D_3$ and 1,25-$(OH)_2D_3$-analogues can be inhibited by the antioestrogen tamoxifen. Although we have not directly measured bone metastases, this study suggests that a tamoxifen-1,25-$(OH)_2D_3$/ analogues combination therapy may result in a reduced risk of skeletal metastases due to 1,25-$(OH)_2D_3$/analogues-stimulated bone resorption.

The analogues EB1089 and KH1060 are potent inhibitors of breast cancer cell growth (8). However, these analogues are also more potent stimulators of *in vitro* bone resorption compared to 1,25-$(OH)_2D_3$. Irrespective of their difference in bone resorbing potency tamoxifen caused a similar inhibition of 1,25-$(OH)_2D_3$- and EB1089-stimulated bone resorption. KH1060-stimulated bone resorption was also reduced by tamoxifen, but to a much lesser extent. It is unlikely that KH1060 will be a possible candidate for treatment of breast cancer or other malignancies as it has a profound immunosuppressive effect (16), whereas with EB1089 already promising animal studies have been performed

(10). An explanation for the observed discrepancy between 1,25-$(OH)_2D_3$/ EB1089 and KH1060 is unknown and studies on the precise inhibitory mechanism of tamoxifen and the mechanism of action of KH1060 are needed.

Tamoxifen is a widely used antioestrogen in the treatment of breast cancer in postmenopausal women (17). However, it exhibits antioestrogenic as well as oestrogenic properties, depending on the organ or cellular response studied. On bone tamoxifen acts as a partial oestrogen agonist (18,19), moreover it has protective effects on the skeleton in breast cancer patients (12,13). The present data question whether the observed inhibition of bone resorption is due to an oestrogenic effect because with 17ß-oestradiol no inhibition of stimulated bone resorption was observed. Moreover, the pure antioestrogen ICI164,384, which lacks oestrogen agonistic activity (20), caused a comparable inhibition of 1,25-$(OH)_2D_3$-stimulated bone resorption as tamoxifen. The mechanism by which tamoxifen and ICI164,384 inhibit stimulation of bone resorption is yet unknown. Protein kinase C is a possible target as tamoxifen has been described to inhibit protein kinase C (21) and we have shown that protein kinase C is involved in the stimulation of bone resorption by 1,25-$(OH)_2D_3$ (14). The inhibitory effect of tamoxifen (up to 10 μM) seems to be specific for 1,25-$(OH)_2D_3$/analogues as PTH-stimulated bone resorption was not affected. This is in accordance with data of Stewart and Stern (22). In contrast, they observed inhibition of the PTH action with higher concentrations (40 - 100 μM) of tamoxifen. However, these concentrations appeared to be toxic in our studies.

Finally, the current study adds important data to the observed beneficial effects of combined treatment of breast cancer cells with tamoxifen and 1,25-$(OH)_2D_3$/analogues (8,11,23). These latter studies showed that co-treatment with 1,25-$(OH)_2D_3$/analogues and tamoxifen results in a stronger growth inhibition of breast cancer cells and that lower concentrations of 1,25-$(OH)_2D_3$ and analogues can be used to achieve a similar effect. Together these data underscore the potential benefit of the combination of tamoxifen and 1,25-$(OH)_2D_3$/analogues in the treatment of breast cancer.

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Chapter 7

INHIBITION OF INSULIN- AND INSULIN-LIKE GROWTH FACTOR-I-STIMULATED GROWTH OF HUMAN BREAST CANCER CELLS BY 1,25-DIHYDROXYVITAMIN D_3 AND THE VITAMIN D_3 ANALOGUE EB1089

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Submitted for publication

ABSTRACT

1,25 Dihydroxyvitamin D_3 (1,25-$(OH)_2D_3$) and a number of synthetic vitamin D_3 analogues with low calcemic activity, have been shown to inhibit breast cancer cell growth *in vitro* as well as *in vivo*. The purpose of the present study was to investigate a possible interaction of 1,25-$(OH)_2D_3$ and the vitamin D_3 analogue EB1089 with the insulin/IGF-I regulatory system. The oestrogen receptor-positive MCF-7 human breast cancer cells used in this study are able to grow autonomously and their growth is stimulated by insulin. In order to avoid interference of IGF-binding proteins (IGF-BPs) we have used an analogue of IGF-I, long R^3 IGF-I, which stimulated MCF-7 cell growth similar to insulin. The growth stimulation by insulin and by long R^3 IGF-I was completely inhibited by 1,25-$(OH)_2D_3$ and EB1089. Also the autonomous growth was inhibited by 1,25-$(OH)_2D_3$ and EB1089. The analogue EB1089 was active at 50 times lower concentrations than 1,25-$(OH)_2D_3$. It was shown that the growth inhibition was not achieved via a down-regulation of the insulin and IGF-I binding after 48 h. Paradoxically, after prolonged treatment (8 days) an upregulation of the insulin and IGF-I binding was observed. Two possible intracellular mediators of the insulin/IGF mitogenic signal are c-fos and mitogen activated protein (MAP) kinase. Insulin-induced c-fos mRNA was inhibited by 1,25-$(OH)_2D_3$, suggesting that c-fos could be involved in the growth inhibition by 1,25-$(OH)_2D_3$. MAP kinase activation appeared not to be involved in the growth stimulation by both insulin and IGF-I. Together, the present study demonstrates that vitamin D_3 compounds can block the mitogenic activity of insulin and IGF-I, which may contribute to their tumour suppressive activity observed *in vivo*.

INTRODUCTION

Besides regulation of calcium and bone metabolism the most active metabolite of vitamin D, 1,25-dihydroxyvitamin D_3 (1,25-$(OH)_2D_3$) regulates growth and differentiation of a wide variety of cells and tissues (1,2). Both *in vitro* and *in vivo* studies have shown that 1,25-$(OH)_2D_3$ inhibits the growth of breast cancer cells (3-7) and these findings suggest a potential use of 1,25-$(OH)_2D_3$ for the treatment of breast cancer. A major drawback is that high doses are needed for tumour suppression. This may result in undesirable side-effects, like the development of hypercalcemia. To overcome these problems, a number of promising synthetic vitamin D_3 analogues have been developed, with more potent effects on growth and differentiation and a

reduced *in vivo* calcemic activity (8,9).

Breast cancer growth is regulated by numerous factors, including steroid hormones and polypeptide growth factors (10). Insulin, insulin-like growth factor I (IGF-I) and insulin-like growth factor II (IGF-II) are structurally related molecules which are believed to play an important role in promoting breast tumour growth (11-14). The IGFs and insulin are derived from the circulation. The IGFs are also produced by breast tumour cells and by neighbouring stromal cells in the breast tumour and can act in an autocrine or paracrine manner (11,13,15). These three polypeptide hormones exert their biological activity by binding to distinct transmembrane receptors: the closely related insulin and type I IGF receptor, and the type II IGF receptor or mannose-6-phosphate receptor. Considerable cross-reactivity exists between the various ligands and receptors (16). In addition to the three receptors, the IGFs bind to other binding proteins which are found in serum and are produced by a wide variety of different cell types. To date six IGF binding proteins (IGF-BPs) have been cloned (17). Breast tumours and breast cancer cell lines express IGF-BP2 to IGF-BP6 in a heterogenous pattern, while little or no expression of IGF-BP1 is seen (18-20). At the cellular level IGF-BPs can either potentiate or suppress the activity of the IGFs (17,18).

In earlier reports we have studied the interference of 1,25-$(OH)_2D_3$ with the growth stimulation of human breast cancer cell lines by oestradiol. It was shown in MCF-7 cells that the inhibition by 1,25-$(OH)_2D_3$ and several vitamin D_3 analogues was independent of oestradiol (7,21). So far, no data are available on the interaction of vitamin D_3 compounds with the insulin and IGF-I regulatory system of breast cancer cells. In the present study, we have investigated the effects of 1,25-$(OH)_2D_3$ and a promising vitamin D_3 analogue, EB1089, on insulin- and IGF-I-stimulated growth of MCF-7 cells. As IGF-BPs are important, but often complex regulators of IGF actions, we have avoided an interference with IGF-BPs produced by MCF-7 cells by using insulin and analogues of IGF-I, long R^3 IGF-I and des(1-3)IGF-I, which have a reduced binding to IGF-BPs. Furthermore, we have studied the effects of 1,25-$(OH)_2D_3$ and EB1089 on insulin and IGF-I binding, and intracellular mediators of the insulin/IGF signal: c-fos mRNA expression (22,23) and mitogen-activated protein (MAP) kinase activity (24).

MATERIALS AND METHODS

Chemicals

1,25-$(OH)_2D_3$ and EB1089 (25) were kindly donated by Dr. L. Binderup of LEO Pharmaceutical Products (Ballerup, Denmark). The chemical structure of EB1089 is depicted in Figure 7.1. Insulin, RPMI-1640 cell culture medium, human transferrin, sodium selenite, and bacitracin were purchased from Sigma Chemical Co. (St. Louis, MO, U.S.A.). IGF-I, long R^3 IGF-I, and des(1-3)IGF-I were from GroPep Pty. Ltd. (Adelaide, Australia). Long R^3 IGF-I is an analogue of human IGF-I synthesized in *E.coli* and has a substitution of an ARG for GLU at position 3 and an extension of 13 amino acids at the N-terminus (26). Des(1-3) is a truncated form of IGF-I which lacks three N-terminal amino acids (27). $[^{125}I]$-insulin labeled at tyrosine-A14 (2000 Ci/mmol) and $[^{125}I]$-IGF-I (2000 Ci/mmol) were from Amersham International (Buckinghamshire, U.K.). The αIR3 antibody against the type I IGF receptor was purchased from Oncogene Science Inc. (Uniondale, NY, U.S.A.). Bovine serum albumin fraction V (BSA) was purchased from ICN Biomedicals Inc. (Costa Mesa, CA, U.S.A.). Glutamine, penicillin, and streptomycin were obtained from Life Technologies (Paisley, U.K.), and fetal calf serum (FCS) was from Sera-Tech Zellbiologische Produkte GmbH (St. Salvator, Germany).

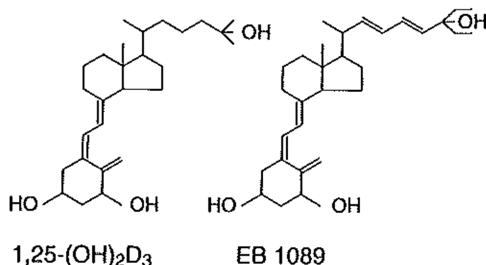

Figure 7.1. Chemical structures of 1,25-$(OH)_2D_3$ and EB1089.

Cell culture

MCF-7 cells were generously provided by Dr. J.A. Foekens, Dr. Daniel den Hoed Cancer Center (Rotterdam, The Netherlands). MCF-7 cells were routinely maintained in phenol red-free RPMI-1640 medium supplemented with 2 mM glutamine, 100 units/ml penicillin, 100 μg/ml streptomycin, 24 mM sodium bicarbonate (= basal RPMI medium) plus 10% FCS and 10 μg/ml insulin. Prior to the start of the experiments the cells were deprived from insulin and serum as follows: the cells were trypsinized and seeded in phenol red-free basal RPMI medium supplemented with 2% charcoal-treated FCS (25,000 cells/cm^2). The FCS was added for the attachement of the cells. After 24 h the medium was changed to serum-free basal RPMI medium supplemented with 2 mg/ml BSA, 10 μg/ml transferrin, and 30 nM sodium selenite (= SFM). Next, the medium was refreshed every two or three days. After one week the cells had grown to subconfluence and the procedure was repeated. For the experiments we have used cells that were cultured in SFM for several weeks. The experiments were started by seeding the cells in basal RPMI medium supplemented with 2% charcoal-treated FCS. After 24 h the medium was changed to SFM and after another 24 h the SFM was refreshed and the agents to be tested were added. For long-term experiments the medium and test agents were replaced every 3 days.

Proliferation experiments

MCF-7 cells were seeded into 24-well dishes at a density of 4000 cells/cm^2 and incubated with the compounds to be tested as described in the section "Cell Culture". After 8 days of incubation DNA content was measured using the ethidium bromide method as described previously (7).

Insulin and IGF-I binding

The insulin and IGF-I binding assay was performed as described by Maassen *et al.* (28). For the short-term incubations MCF-7 cells were grown to subconfluence in SFM in six-well dishes and were subsequently exposed to the vitamin D_3 compounds for 24 or 48 h. For the long-term incubations (8 days) the cells were exposed to the vitamin D_3 compounds from the start of the experiment as described in the section "Cell Culture". At the end of both the short- and long-term incubations, the cells were

washed twice in PBS (pH 7.8) plus 10 mg/ml BSA. Subsequently the cells were incubated with 3 x 10^{-11} M [^{125}I]-insulin in PBS (pH 7.8) plus 10 mg/ml BSA for 2.5 h at room temperature or with 3 x 10^{-11} M [^{125}I]-IGF-I in PBS (pH 7.8) plus 10 mg/ml BSA and 2 mg/ml bacitracin for 3.5 h at 4 C. Non-specific binding was assessed by incubating the cells with labeled insulin in the presence of 1 μM unlabeled insulin or with labeled IGF-I in the presence of 0.1 μM unlabeled IGF-I. After the incubation the cells were washed 5 times with ice-cold PBS plus 1 mg/ml BSA and solubilized in 0.1 M NaOH, 0.1% SDS. The amount of bound [^{125}I]-insulin or [^{125}I]-IGF-I was determined in a gamma counter (NE1600, NE Technology Ltd., Reading, U.K.) and corrected for the absorption at 260 nm, which was used as a measure for the cell number.

Assay of MAP kinase activity

MCF-7 cells were seeded (2 x 10^4 cells/cm^2) and incubated with the compounds to be tested for 5 to 30 min as described in the section "Cell Culture". Upon activation MAP kinase is phosphorylated. Activation of MAP kinase was detected by immunoblotting of cell homogenates as described by Van den Berghe *et al.* (29). The method is based on a shift in gelelectrophoretic migration due to phosphorylation of MAP kinase.

RNA isolation and hybridization

Cells were seeded (1.5 x 10^6 cells/25 cm^2 flask), and incubated with the compounds to be tested as described in the section "Cell Culture". Total RNA was isolated by guanidinium thiocyanate-phenol-chloroform extraction and Northern analysis with 10-20 μg RNA was performed as described previously (21). Northern blots were hybridized with [^{32}P]-labeled human cDNA fragments specific for c-fos (30) and GAPDH (31). After hybridization the Northern blots were washed twice in 2x SSC, 0.1% SDS for 5 min at room temperature, twice in 2x SSC, 0.1% SDS for 20 min at 60 C, and twice in 0.5x SSC, 0.1% SDS for 20 min at 60 C. Membranes were exposed to medical X-ray films (Fuji Photo Film Co., Tokyo, Japan) with intensifying screens at -80 C, and the autoradiographs were quantified using a ScanJet IIcx scanner (Hewlett-Packard Co., Minneapolis, U.S.A.). Before rehybridization with GAPDH, Northern blots were washed in 5 mM Tris-HCl (pH 8.0), 0.2 mM EDTA (pH 8.0), 0.05 % sodium pyrophosphate, and 0.1x Denhardt's solution for at least 2 h at 65 C.

RESULTS

Inhibition of insulin- and IGF-I-stimulated growth by vitamin D_3 compounds

The MCF-7 cells in our laboratory are able to grow autonomously in SFM without further additions. The autonomous growth was stimulated by treatment with insulin. After 8 days of treatment the DNA content of the wells containing SFM supplemented with insulin was 1.5- to 2-fold higher than the DNA content of the control wells without insulin. The insulin dose-response curve was steep with the maximum growth stimulation reached at 5 μg/ml and a median effective concentration of 1 μg/ml insulin (Fig. 7.2).

The growth stimulation by 10 μg/ml insulin could be dose-dependently inhibited both by 1,25-$(OH)_2D_3$ and the vitamin D_3 analogue EB1089 (Fig. 7.3). Based on the median effective concentration, EB1089 was approximately 50 times more potent than 1,25-$(OH)_2D_3$ in the inhibition of insulin-stimulated growth. Both

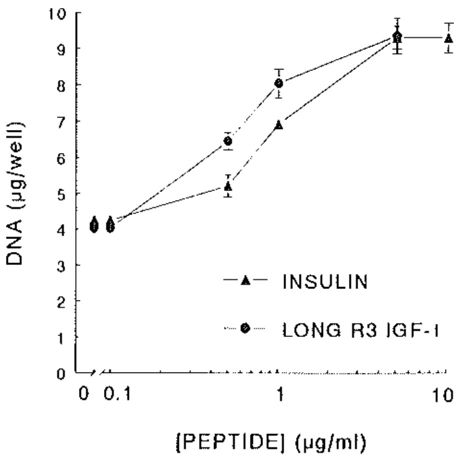

Figure 7.2. Growth stimulation of MCF-7 cells by insulin and long R^3 IGF-I. Cells were cultured for 8 days in SFM as described in the section "Cell Culture" with various concentrations of insulin or long R^3 IGF-I. Subsequently DNA content was measured. Each point represents mean ± SD of duplicate wells.

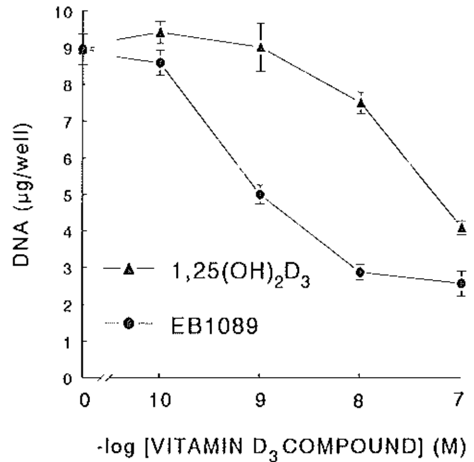

Figure 7.3. Inhibition of insulin-stimulated growth of MCF-7 cells by 1,25-$(OH)_2D_3$ and EB1089. Cells were cultured for 8 days in SFM supplemented with 10 μg/ml insulin and the indicated concentrations of the vitamin D_3 compounds or vehicle (0.1% ethanol) as described in the section "Cell Culture". Each point represents mean ± SD of duplicate wells.

vitaminD_3 compounds not only reversed the growth stimulation by insulin but also inhibited part of the autonomous growth of MCF-7 cells.

Since insulin binds not only the insulin receptor but also the IGF type I receptor with low affinity (16), it is possible that the observed stimulation by supraphysiological concentrations of insulin is mediated via the type I IGF receptor. To test this possibility we have studied whether the insulin-stimulated growth could be inhibited by the type I IGF receptor blocking antibody αIR3. The antibody inhibited the insulin-stimulated growth by 24%. A small inhibition of the autonomous growth (9%) was observed (Fig. 7.4).

Next, we have examined the mitogenic effect of IGF-I on MCF-7 cells. To avoid an interference with IGF-BPs secreted by MCF-7 cells, we have tested two analogues of IGF-I, long R^3 IGF-I and des(1-3)IGF-I, which have a reduced binding to IGF-BPs (26,27). Fig. 7.2 demonstrates that MCF-7 cells respond to long R^3 IGF-I with a similar increase of DNA content as obtained with insulin. The median effective concentration was 0.5 μg/ml long R^3 IGF-I. Also for des(1-3)IGF-I relatively high concentrations were needed to elicit a proliferative response. Des(1-3)IGF-I was not

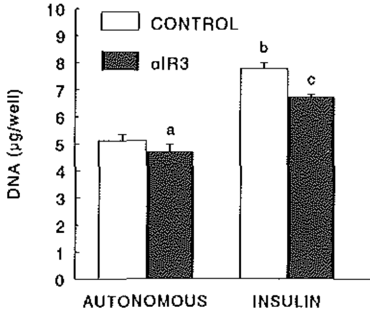

Figure 7.4. Effect of αIR3 on insulin-stimulated growth of MCF-7 cells. Cells were cultured for 8 days in SFM (autonomous growth), or SFM supplemented with 10 µg/ml insulin. The cells were treated with or without 10 µg/ml αIR3. Data are presented as mean ± SD of duplicate wells. a: $p<0.025$, b: $p<0.001$ vs. autonomous control, and c: $p<0.001$ vs. insulin-stimulated control.

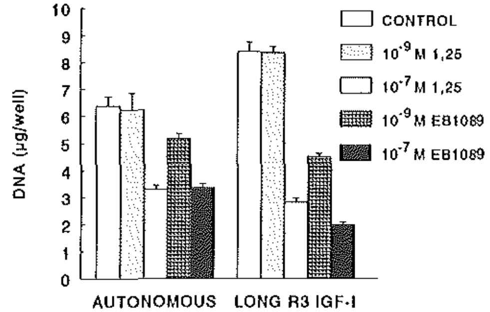

Figure 7.5. Inhibition of autonomous and long R^3 IGF-I-stimulated growth of MCF-7 cells by 1,25-$(OH)_2D_3$ and EB1089. Cells were cultured for 8 days in SFM (autonomous growth), or SFM supplemented with 1 µg/ml long R^3 IGF-I. The cells were treated with vehicle (0.1% ethanol), 1,25-$(OH)_2D_3$ (1,25) or EB1089 as indicated in the legend. Data are presented as mean ± SD of duplicate wells.

active at concentrations below 1 µg/ml (data not shown). Several studies have shown a synergistic action of insulin/IGF-I and oestradiol (32-34). However, in our culture system the mitogenic effects of insulin and the IGF-I analogues could not be sensitized by co-treatment with oestradiol (data not shown).

Similar to the inhibition of insulin-stimulated growth, the growth stimulation by long R^3 IGF-I could be completely reversed by 1,25-$(OH)_2D_3$ and the analogue EB1089 (Fig. 7.5). At 10^{-9} M the differences in potency between 1,25-$(OH)_2D_3$ and EB1089 are clearly demonstrated. In addition, Fig. 7.5 demonstrates that the autonomous growth was also potently inhibited by 1,25-$(OH)_2D_3$ and EB1089.

Insulin and IGF-I binding

Insulin binding was maximal after 2 h and non-specific binding was 9% of the total binding. For IGF-I the non-specific binding was 22% of the total binding. Competitive binding studies of $[^{125}I]$-insulin showed that insulin competed at approximately 300 times lower concentrations than IGF-I (Fig. 7.6).

Next, we have studied whether the vitamin D_3 compounds could exert their

antiproliferative action on insulin- and IGF-I-stimulated growth via regulation of insulin or IGF-I binding. Incubations for 48 h with 1,25-$(OH)_2D_3$ and EB1089 (10^{-9}, 10^{-7} M) had no effect on the specific insulin or IGF-I binding (Table 7.1). In addition we have studied the effect of long-term incubations with 1,25-$(OH)_2D_3$ and EB1089 at time-points where the growth inhibition by the vitamin D_3 compounds was observed. As shown in Table 7.1, treatment with 1,25-$(OH)_2D_3$ and EB1089 resulted in a stimulation of approximately 2-fold the control insulin binding and 3-fold the control IGF-I binding.

Table 7.1. Insulin and IGF-I binding of MCF-7 cells treated with 1,25-$(OH)_2D_3$ and EB1089.

	Short-term incubation (48 h)			
Condition	Insulin binding		IGF-I binding	
	exp. 1	exp. 2	exp. 1	exp. 2
Control	100 ± 13	100 ± 5	100 ± 2	100 ± 7
10^{-9} M 1,25-$(OH)_2D_3$	103 ± 9	99 ± 1	N.D.	N.D.
10^{-7} M 1,25-$(OH)_2D_3$	122 ± 11	125 ± 33	135 ± 3	106 ± 5
10^{-9} M EB1089	129 ± 23	98 ± 2	N.D.	N.D.
10^{-7} M EB1089	112 ± 0	85 ± 10	120 ± 8	101 ± 8
	Long-term incubation (8 days)			
Condition	Insulin binding		IGF-I binding	
	exp. 1	exp. 2	exp. 1	exp. 2
Control	100 ± 20	100 ± 9	100 ± 2	100 ± 4
10^{-9} M 1,25-$(OH)_2D_3$	98 ± 2	125 ± 26	N.D.	N.D.
10^{-7} M 1,25-$(OH)_2D_3$	153 ± 18	165 ± 11	268 ± 10	392 ± 1
10^{-9} M EB1089	135 ± 13	239 ± 46	N.D.	N.D.
10^{-7} M EB1089	198 ± 49	230 ± 45	274 ± 1	263 ± 35

MCF-7 cells were incubated during 48 h or 8 days with or without 1,25-$(OH)_2D_3$ and EB1089 and subsequently the specific [^{125}I]-insulin and [^{125}I]-IGF-I binding was measured as described under "Materials and Methods". The specific binding was corrected for the cell number and expressed of % of control ± SD of duplicate wells. N.D., not determined.

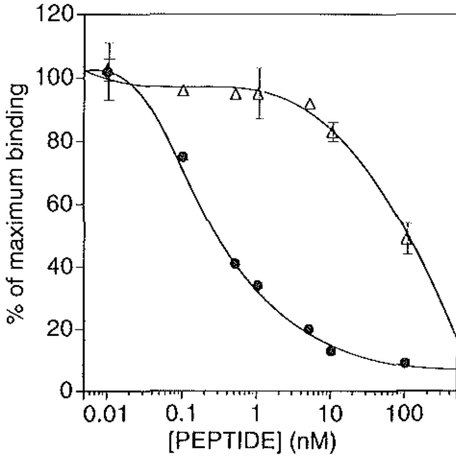

Figure 7.6. Binding of insulin to monolayers of MCF-7 cells. MCF-7 cells were grown to confluence in SFM and incubated with 3 x 10^{-11} M [^{125}I]-insulin for 2.5 h in the presence of the indicated concentrations of unlabeled insulin (solid circles) or IGF-I (triangles). Bound [^{125}I]-insulin was measured as described under "Materials and Methods". Data are expressed as mean ± SD of duplicate wells.

Effect of the vitamin D_3 compounds on intracellular mediators of the insulin/IGF-I signal: c-fos mRNA expression and MAP kinase

Induction of c-fos expression is believed to play a central role in the transduction of mitogenic signals in the nucleus (22,23). c-fos mRNA expression was rapidly induced by insulin with a peak at 30 to 60 min and a gradual return towards prestimulation level in 4 h (Fig. 7.7). Figure 7.8 shows that the rapid induction of c-fos mRNA by insulin was inhibited by treatment with 1,25-$(OH)_2D_3$. Basal c-fos mRNA expression was not affected by 1,25-$(OH)_2D_3$ (not shown).

Activation of MAP kinase is another intracellular mechanism involved in the signal transduction of growth factors (24). We wanted to study whether the vitamin D_3 compounds could affect MAP kinase activity as a mechanism for the observed inhibition of insulin-/long R^3 IGF-I-stimulated growth. However, in MCF-7 cells there was no detectable activation of MAP kinase by insulin, nor by long R^3 IGF-I in a time course from 5 to 30 min. As a positive control of the assay 10% FCS was used, which caused activation of MAP kinase after 10 min of incubation (not shown).

DISCUSSION

In the present study we show that 1,25-$(OH)_2D_3$ and the vitamin D_3 analogue EB1089 are able to reverse the mitogenic effect of insulin and an IGF-I analogue (long R^3 IGF-I) on MCF-7 breast cancer cells. The vitamin D_3 analogue EB1089 was active at 50-fold lower concentrations than the native compound 1,25-$(OH)_2D_3$. This is in line with other studies on the antiproliferative action of EB1089 on breast cancer

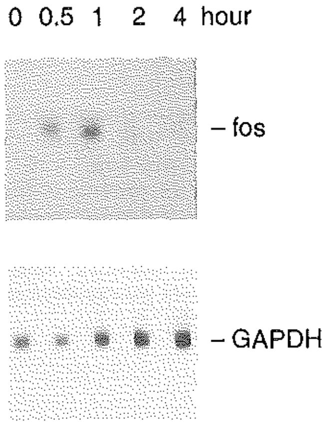

Figure 7.7. Time curve of insulin-induced c-fos mRNA expression. MCF-7 cells were incubated with 10 μg/ml insulin for 0, 0.5, 1, 2, or 4 h. RNA was isolated and Northern blots were prepared as described under "Materials and Methods" and the blots were hybridized with [^{32}P]-labeled fos and GAPDH probes.

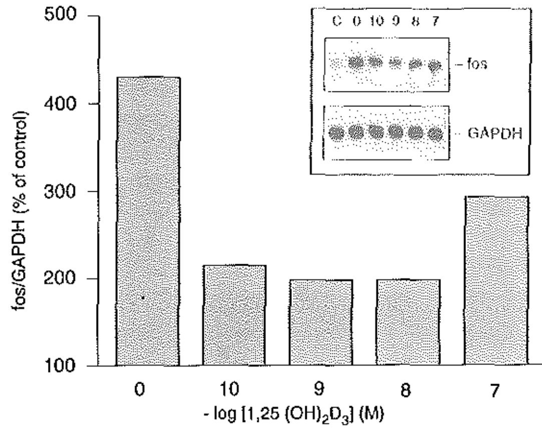

Figure 7.8. Inhibition of insulin-induced c-fos mRNA by 1,25-(OH)$_2$D$_3$. MCF-7 cells were treated without insulin (C) or with 10 μg/ml insulin and 10^{-10} - 10^{-7} M 1,25-(OH)$_2$D$_3$ (10 - 7) or vehicle (0) for 30 min. Subsequently RNA was isolated, Northern blots were prepared and hybridized [^{32}P]-labeled probes specific for GAPDH (1.6 kb mRNA) and c-fos (2.2 kb mRNA). The autoradiograph shown was quantitated densitometrically. The c-fos signal was divided by the GAPDH signal to correct for equal loading of the lanes.

cells (21,35,36). *In vivo* studies with rats have shown that EB1089 has a decreased ability to affect calcium metabolism compared to 1,25-(OH)$_2$D$_3$ (36). Moreover, Colston *et al.* showed that EB1089 suppressed tumour growth in rats with chemical-induced breast cancer (35). Thereby, EB1089 appears to be a promising analogue for the treatment of breast cancer.

IGF-I as well as insulin are believed to play an important role in the development and growth of breast tumours (11-14). In oestrogen-responsive cells insulin and the IGFs can contribute to oestrogen-induced growth (32-34). Thereby, interruption of insulin/IGF-induced growth by the vitamin D$_3$ compounds may affect both oestrogen-independent and oestrogen-dependent breast tumours. This may provide an explanation for the observation that vitamin D$_3$ compounds inhibit the growth of breast cancer cells and tumours irrespective of the presence of the oestrogen receptor (4,37).

Although most attention has been focused on the tumour promoting role of the IGFs, also insulin may play a role in breast tumour growth and development

acting through its own receptor (12). The high doses of insulin needed for growth stimulation of MCF-7 cells (EC_{50} = 1 μg/ml) suggested that insulin was acting through the type I IGF receptor, which has a low affinity for insulin, and mimicked the effect of IGF-I. However, the small inhibition of insulin-stimulated growth by αIR3 (24% inhibition) suggested that insulin acted predominantly via the insulin receptor. The antibody was tested in a concentration 100-fold higher than the K_D of αIR3 for binding to the type I IGF receptor in MCF-7 cells (38). Also the studies of Milazzo *et al.* (12) and Cullen *et al.* (14) with αIR3 suggested an involvement of the insulin receptor in the transmission of the mitogenic effect of insulin in breast cancer cells. The presence of specific insulin binding sites on MCF-7 cells was demonstrated by binding studies with [^{125}I]-insulin. Half-maximal displacement of [^{125}I]-insulin was achieved with insulin at 300 times lower concentrations than with IGF-I. Also other reports have demonstrated the presence of functional insulin receptors on several breast cancer cell lines, including MCF-7 (12). In addition, a role of the insulin receptor in breast cancer initiation and/or progression is supported by the observations that the insulin receptor expression is increased in human breast cancer specimens and that the insulin receptor content is positively correlated with tumour grade and size (39). Lastly, an insulin analogue with enhanced mitogenic activity increased the incidence of mammary tumours in female rats (40).

Besides insulin, also the IGF-I analogues long R^3 IGF-I and des(1-3)IGF-I were needed in high concentrations (0.5 μg/ml or more) to stimulate the growth of MCF-7 cells, whereas previous studies have shown that IGF-I and the IGF analogues can elicit a proliferative response at concentrations ranging from 1 to 50 ng/ml (13,14,26). There are several possible explanations for the low sensitivity of the MCF-7 cells to insulin and IGF-I, regardless of the receptor activation pathway. Firstly, we have used serum-free culture conditions, and possibly additional serum-derived factors play a synergistic role in the growth stimulation by insulin and IGF-I. Secondly, the cells could be less responsive because of the high basal growth rate. The independence of supplemented growth factors for normal growth suggests that MCF-7 cells produce autocrine acting growth factors (10). Possibly IGF-I is involved, as we[1] and others (15,41) have measured considerable amounts of immunoreactive IGF-I in the conditioned medium of MCF-7 cells.

To gain insight into the mechanism of the antigrowth factor activity of 1,25-$(OH)_2D_3$ and EB1089 we have studied several aspects of the insulin and IGF-I signal transduction pathway. Firstly, it was shown that 1,25-$(OH)_2D_3$ and EB1089 did not suppress the insulin and IGF-I binding. The vitamin D_3 compounds had no direct effect on the insulin and IGF-I binding after 48 h of treatment. At time points where the growth inhibition by the vitamin D_3 compounds was observed (8 days), an

increase of insulin and IGF-I binding was observed by treatment with 1,25-$(OH)_2D_3$ and EB1089. The physiological implications of these findings are not clear, but a similar inverse relation between receptor regulation and growth inhibition by 1,25-$(OH)_2D_3$ was observed for the epidermal growth factor receptor in BT-20 breast cancer cells (42). Possibly the stimulation of the insulin and IGF-I binding is a secondary effect of 1,25-$(OH)_2D_3$ and EB1089 treatment, which is not linked to the inhibitory effect on cell growth. Also, a role of IGF-BPs in the growth inhibition by 1,25-$(OH)_2D_3$ and EB1089 was unlikely because of the use of insulin and analogues of IGF-I which have a reduced binding to IGF-BPs.

We have also studied two intracellular responses that can be activated by insulin and IGF-I: c-fos mRNA expression (22,23) and MAP kinase activity (24). The rapid induction of c-fos mRNA expression by insulin was inhibited by 1,25-$(OH)_2D_3$, indicating that c-fos mRNA expression is a possible target for 1,25-$(OH)_2D_3$ and analogues in the growth inhibition of these breast cancer cells. It should be noted, however, that the inhibition of insulin-induced c-fos mRNA expression could already be observed at lower concentrations than the inhibition of proliferation, indicating that solely inhibition c-fos expression is not sufficient to inhibit insulin-induced cell growth. Furthermore, a stimulation of c-fos mRNA levels by 1,25-$(OH)_2D_3$ was associated with an inhibition of cell proliferation in the MCF-7 cell line (36) and in the HL-60 leukemic cell line (43). Finally, we have studied whether MAP kinase is involved in the growth inhibition by 1,25-$(OH)_2D_3$ and EB1089. MAP kinase has been detected in a wide variety of cells and tissues and is activated by a number of growth factors (insulin, IGF-I, and epidermal growth factor), hormones, and other extracellular signals. Activated MAP kinase on its turn phosphorylates other proteins, including the proto-oncogene products of myc and jun, which act as transcription factors (24). It appeared however that MAP kinase phosphorylation was not detected, which may imply that MAP kinase is not involved in the growth stimulation by insulin and IGF-I in the MCF-7 breast cancer cells.

In conclusion, it was shown that 1,25-$(OH)_2D_3$ and the more potent analogue EB1089 block the mitogenic effect of insulin and IGF-I on MCF-7 breast cancer cells. The observed interactions with the insulin/IGF regulatory system could contribute to the tumour suppressive activity of vitamin D_3 compounds.

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Chapter 8

1,25-DIHYDROXYVITAMIN D_3 AND THE VITAMIN D_3 ANALOGUE EB1089 REGULATE INSULIN-LIKE GROWTH FACTOR (IGF) BINDING PROTEIN EXPRESSION AND IGF-I SECRETION BY HUMAN BREAST CANCER CELLS

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Submitted for publication

SUMMARY

Insulin-like growth factor-I (IGF-I) is believed to play an important role in the growth of breast cancer cells by paracrine or autocrine effects. In a previous study, we showed that 1,25-dihydroxyvitamin D_3 (1,25-$(OH)_2D_3$) and its more potent analog EB1089 inhibit insulin- and IGF-I-stimulated growth of MCF-7 breast cancer cells. In the present study, we have examined whether 1,25-$(OH)_2D_3$ and EB1089 regulate the expression of IGF binding proteins (IGF-BPs) and the secretion of IGF-I by MCF-7 human breast cancer cells. In our serum-free MCF-7 cell cultures, the overall IGF-BPs produced by the cells appeared to have an inhibitory effect on IGF-I action, since IGF-I had no effect on the growth of MCF-7 cells, whereas IGF-I analogs with a reduced binding to IGF-BPs, long R^3 IGF-I and des(1-3)IGF-I, as well as insulin stimulated the cell growth. Western ligand blots showed that IGF-BP2 was predominantly produced in the conditioned medium of MCF-7 cells. Further we have detected bands of 38-40 kDa and 25 kDa, which were indicative for IGF-BP3 and IGF-BP4, respectively. The MCF-7 cells expressed IGF-BP2, IGF-BP4, and IGF-BP5 mRNA. IGF-BP2 was abundantly expressed, followed by IGF-BP4, whereas the expression of IGF-BP5 was relatively low. 1,25-$(OH)_2D_3$ and EB1089 (0.1 nM) stimulated the expression of IGF-BP5 mRNA after 24 h of incubation, which persisted up to 48 h. The expression of IGF-BP2 and IGF-BP4 mRNA was unaffected. Besides IGF-BPs, MCF-7 cells secrete considerable amounts of immunoreactive IGF-I (iIGF-I) into the medium. Strikingly, the growth inhibitors 1,25-$(OH)_2D_3$ and EB1089 stimulated the secretion of iIGF-I in a time- and dose-dependent manner. Already after 48 h a 3-fold stimulation was measured, whereas at this time point no effect on cell growth was observed. The stimulation of IGF-I secretion was specific for the vitamin D_3 compounds as another growth inhibitor, tamoxifen, was ineffective. Together, we show for the first time that 1,25-$(OH)_2D_3$ and its analog EB1089 change the pattern of expression of IGF-BPs and modulate the secretion of immunoreactive IGF-I in breast cancer cells. These data suggest that 1,25-$(OH)_2D_3$ and EB1089 can influence IGF action on breast cancer cells, but at this point it is unclear how these effects directly relate to the growth inhibitory effects of vitamin D_3 compounds.

INTRODUCTION

In addition to its role in calcium and bone metabolism, 1,25-dihydroxyvitamin D_3 (1,25-$(OH)_2D_3$) regulates growth and differentiation of a wide variety of cells and tissues, both normal and malignant (1,2). 1,25-$(OH)_2D_3$ mediates its effects via binding to the intracellular vitamin D receptor (VDR), which is a member of the steroid hormone receptor family (3). The VDR is expressed in most human breast tumors and breast cancer cell lines (4,5). 1,25-$(OH)_2D_3$ has been shown to inhibit the growth of breast cancer cell lines and in animal models for breast cancer 1,25-$(OH)_2D_3$ suppressed tumor growth (6-9). To improve the clinical application of 1,25-$(OH)_2D_3$ as a tumor suppressive drug, new synthetic vitamin D_3 analogs have been developed with increased antiproliferative and reduced *in vivo* calcemic activity (10,11). The analog EB1089 has been shown to inhibit breast cancer cell growth at 50- to 100-fold lower concentrations than 1,25-$(OH)_2D_3$ (12-14), whereas *in vivo* studies with rats have shown that EB1089 has a decreased ability to affect calcium metabolism (13). Moreover, Colston et al. showed that EB1089 suppressed tumor growth in rats with chemical-induced breast cancer (12).

Breast cancer growth is regulated by numerous factors, including steroid hormones and polypeptide growth factors (15). Insulin-like growth factor-I (IGF-I) is a potent stimulator of breast cancer cell growth *in vitro* and in estrogen receptor positive cells IGF-I can contribute to the growth stimulation by estrogen (16,17). IGF-I can be derived from the circulation, or is produced locally by breast tumor cells and/or neighbouring stromal cells. The activity of IGF-I is influenced by specific binding proteins (IGF-BPs). These proteins are found in serum, but can also be produced by a wide variety of cell types. At present six IGF-BPs have been cloned. They can either enhance or inhibit the effect of IGF-I depending on the type of IGF-BP or the experimental conditions employed (18-20). Breast tumors and breast cancer cell lines express IGF-BP2 to IGF-BP6 in a heterogenous pattern, while little or no expression of IGF-BP1 is seen (19-21).

In a previous study[1], we showed that 1,25-$(OH)_2D_3$ and the vitamin D_3 analog EB1089 inhibit the growth stimulation induced by IGF-I in the estrogen receptor- and vitamin D receptor-positive MCF-7 human breast cancer cell line. In the present study, we have further examined the interactions of the vitamin D_3 compounds with the IGF regulatory system. Therefore, we have studied in MCF-7 cells a possible regulation of IGF-BP expression and IGF-I secretion by 1,25-$(OH)_2D_3$ and EB1089.

[1]T. Vink-van Wijngaarden, H.A.P. Pols, C.J. Buurman, J.C. Birkenhäger, and J.P.T.M. van Leeuwen, manuscript submitted for publication.

MATERIALS AND METHODS

Chemicals

1,25-$(OH)_2D_3$ and the side-chain analog EB1089 (22) were kindly donated by Dr. L. Binderup of LEO Pharmaceutical Products (Ballerup, Denmark). Insulin, 17ß-estradiol, RPMI-1640 cell culture medium, human transferrin, and sodium selenite were purchased from Sigma Chemical Co. (St. Louis, MO, U.S.A.). IGF-I, long R^3 IGF-I, and des(1-3)IGF-I were from GroPep Pty. Ltd. (Adelaide, Australia). Long R^3 IGF-I is an analog of human IGF-I synthesized in *E.coli* and has a substitution of an ARG for GLU at position 3 and an extension of 13 amino acids at the N-terminus (23). Des(1-3) is a truncated form of IGF-I which lacks three N-terminal amino acids (24). [^{125}I]-IGF-II was kindly donated by Dr. S. van Buul-Offers (Wilhelmina Childrens Hospital, Utrecht, The Netherlands). Bovine serum albumin fraction V (BSA) was purchased from ICN Biomedicals Inc. (Costa Mesa, CA, U.S.A.). Glutamine, penicillin, and streptomycin were obtained from Life Technologies (Paisley, U.K.), and fetal calf serum was from Sera-Tech Zellbiologische Produkte GmbH (St. Salvator, Germany).

Cell culture

MCF-7 cells were generously provided by Dr. J.A. Foekens (Dr. Daniel den Hoed Cancer Center, Rotterdam, The Netherlands). MCF-7 cells were routinely maintained in phenol red-free RPMI-1640 medium supplemented with 2 mM glutamine, 100 units/ml penicillin, 100 μg/ml streptomycin, 24 mM sodium bicarbonate (= basal RPMI medium), 10% fetal calf serum, and 10 μg/ml insulin. All experiments were performed in serum-free basal RPMI medium, supplemented with 2 mg/ml B.S.A., 10 μg/ml transferrin, and 30 nM sodium selenite (= SFM). Prior to the start of an experiment, the cells were seeded in basal RPMI medium supplemented with 2% charcoal-treated fetal calf serum to allow the attachment of the cells. This medium was changed to SFM after 24 h.

Proliferation experiments

MCF-7 cells were seeded into 24-well dishes at a density of 4000 cells/cm^2. After 24 h the medium was changed to SFM and after another 24 h medium was refreshed and the test agents were added. Medium and test agents were replaced every two or three days. After 8 days of incubation DNA content was measured using the ethidium bromide method as described previously (9).

RNA isolation and hybridization

Cells were seeded in 25 cm^2 flasks (60,000 cells/cm^2), after 24 h the medium was changed to SFM, and after another 24 h medium was refreshed and the test agents were added. Total RNA was isolated by guanidinium thiocyanate-phenol-chloroform extraction and Northern analysis with 10-20 μg RNA was performed as described previously (14). Northern blots were hybridized with [^{32}P]-labeled cDNA fragments specific for IGF-BP2 (25), IGF-BP3 (26), IGF-BP4 (27), IGF-BP5 (28), IGF-BP6 (27), and GAPDH (29). After hybridization the Northern blots were washed twice in 2x SSC, 0.1% SDS for 5 min at room temperature, twice in 2x SSC, 0.1% SDS for 20 min at 60 C, and twice in 0.5x SSC, 0.1% SDS for 20 min at 60 C. Membranes were exposed to medical X-ray films (Fuji Photo Film Co., Tokyo, Japan) with intensifying screens at -80 C, and the autoradiographs were quantified using a ScanJet IIcx scanner (Hewlett-Packard Co., Minneapolis, U.S.A.). The exposure time was several hours to overnight for the IGF-BP2 and GAPDH probes, overnight to 2 days for the IGF-BP4 probe and 3-7 days for the IGF-BP5 probe. Before rehybridization, Northern blots were washed in 5 mM Tris-HCl (pH 8.0), 0.2 mM EDTA (pH 8.0), 0.05 % sodium pyrophosphate, and 0.1x Denhardt's solution for at least 2 h at 65 C.

Western ligand blotting and immunoblotting

Subconfluent cultures of MCF-7 cells grown in SFM in 25 cm^2 flasks were incubated with the vitamin D_3 compounds for 48 h in basal RPMI medium supplemented with 1 mg/ml BSA. Proteins were precipitated from 100 μl samples of conditioned medium by an overnight incubation with 7% trichloroacetic acid at 4 C, followed by a short centrifugation step. The proteins were dissolved in non-

reducing loading mix, and heated at 95 C until the trichloroacetic acid was evaporated. Western ligand blots were prepared essentially as described by Hossenlopp et al. (30). Proteins were separated on a 12% SDS gel under non-reducing conditions, and transferred onto nitrocellulose filters by electroblotting. The filters were washed first in 100 mM Tris-HCl (pH 7.5), 0.9% NaCl (= 1xTBS) containing 3% Nonidet P-40 for 30 min, then in TBS, 3% BSA for 2 h, and subsequently incubated overnight with [^{125}I]-IGF-II in TBS, 1% BSA, 0.1% Tween-20 at 4 C. The filters were washed twice in TBS, 0.1% Tween-20 for 15 min, 3 times in TBS for 15 min, and subsequently exposed to medical X-ray films with intensifying screens at -80 C for up to 14 days. The position of IGF-BP2 was verified by immunostaining using a polyclonal antibody against IGF-BP2. The filters were blocked in a TBS, 3% BSA solution and then incubated with the antibody. The filters were washed with TBS, 0.1% Nonidet P-40 and incubated with an alkaline phophatase-conjugated second antibody. Next the filters were washed in TBS, 0.1% Nonidet P-40 and binding was detected by 4-nitro blue tetrazolium chloride (NBT) and 5-bromo-4-chloro-3-indolyl-phosphate (BCIP) substrate solutions (Boehringer, Mannheim, Germany). As an antibody against IGF-BP5 was not available, this binding protein could not identified by immunodetection.

Determination of IGF-I in conditioned medium

For the short-term incubations (48 h) the cells were grown to subconfluence in SFM in 25 cm^2 flasks. Subsequently, the medium was refreshed and the cells were exposed to the vitamin D_3 compounds. For the long-term incubations (up to 8 days) the cells were seeded in six-well dishes (4000 cells/cm^2) and exposed to the test compounds from the start of the experiments as described for the proliferation experiments. Immunoreactive IGF-I secreted into the medium was measured using the radioimmunoassay kit for somatomedin-C of Medgenix Diagnostics SA (Fleurus, Belgium). To remove IGF-BPs the samples were pre-treated by an acid-ethanol extraction according to the protocol of the manufacturer.

RESULTS

Mitogenic action of IGF-I and IGF-I analogs on MCF-7 cells

MCF-7 cells in our laboratory are able to grow autonomously in serum-free medium. When the cells were treated with IGF-I (1 to 1000 ng/ml) we did not observe a stimulation of cell growth (data not shown). Next, we have tested two analogs of IGF-I, long R^3 IGF-I (23) and des(1-3)IGF-I (24), which have a reduced binding to IGF-BPs. As shown in Figure 8.1, long R^3 IGF-I and insulin treatment resulted in a growth stimulation of MCF-7 cells. Also, with des(1-3)IGF-I (1 μg/ml) a growth stimulation was observed.

Regulation of IGF-binding proteins by vitamin D_3 compounds

We have performed Western ligand blots on conditioned medium of MCF-7 cells. The ligand blots showed a predominant band migrating just above the position of the 30 kDa molecular weight marker, which was identified as IGF-BP2 (34 kDa) by immunoblotting (data not shown). Also two faint bands of higher molecular weight were detected. These bands are most likely to be IGF-BP3 (38-40 kDa) which migrates as a doublet (20,21). In some experiments we have also observed an IGF-BP

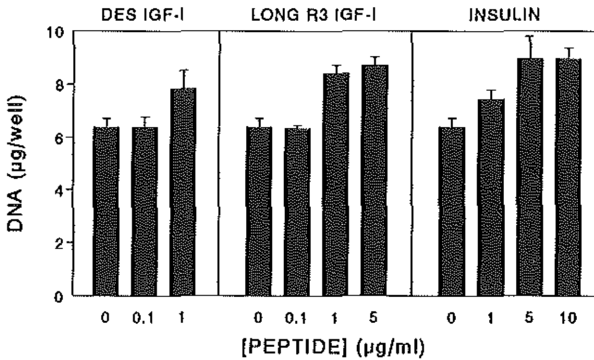

Figure 8.1. Mitogenic effect of long R^3 IGF-I and insulin on MCF-7 cells. Cells were grown in serum-free medium with the indicated concentrations of long R^3 IGF-I and insulin during 8 days as described under "Materials and Methods". DNA content was determined. Data are expressed of mean ± SD of duplicate wells.

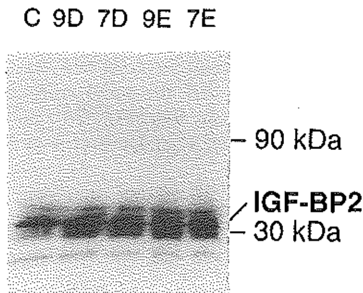

Figure 8.2. Western ligand blot of $[^{125}I]$-IGF-II detected IGF-BPs in 48 h conditioned medium of MCF-7 cells. Subconfluent cultures of MCF-7 cells were incubated with vehicle (C), 10^{-9} M 1,25-$(OH)_2D_3$ (9D), 10^{-7} M 1,25-$(OH)_2D_3$ (7D), 10^{-9} M EB1089 (9E), or 10^{-7} M EB1089 (7E) for 48 h. 100 µl of concentrated conditioned medium was separated on a SDS-polyacrylamide gel under non-reducing conditions. The proteins were transferred onto nitrocellulose and the IGF-BPs were detected by incubation with $[^{125}I]$-IGF-II and subsequent autoradiography. The exposure time was 17 days. IGF-BP2 was identified by immunoblotting and its migration position is marked.

species of lower molecular weight, which is probably IGF-BP4 (25 kDa). Treatment with 1,25-$(OH)_2D_3$ and EB1089 for 48 h did not markedly change this pattern of IGF-BPs (Fig. 8.2).

At mRNA level we have detected IGF-BP2 (1.6 kb), IGF-BP4 (2.6 kb), and IGF-BP5 (6 kb). IGF-BP2 was abundantly expressed, followed by IGF-BP4, whereas the expression of IGF-BP5 was much lower. IGF-BP3 and IGF-BP6 mRNA transcripts were not detected. MCF-7 cells were treated during 1 to 48 h with 1,25-$(OH)_2D_3$ or EB1089 (10^{-8} M). As shown in Fig. 8.3, the expression of IGF-BP5 mRNA was increased by treatment with 1,25-$(OH)_2D_3$ and EB1089 in a time-dependent manner, while the expression of IGF-BP2 and IGF-BP4 was not affected. The maximal stimulation of IGF-BP5, 2- to 3-fold the control level, was reached after 24 h of treatment with the vitamin D_3 compounds and after 48 h the IGF-BP5 mRNA level was still elevated. Dose-response studies with 1,25-$(OH)_2D_3$ and EB1089 revealed that already at 10^{-10} M a maximal stimulation of IGF-BP5 could be observed (Fig. 8.4).

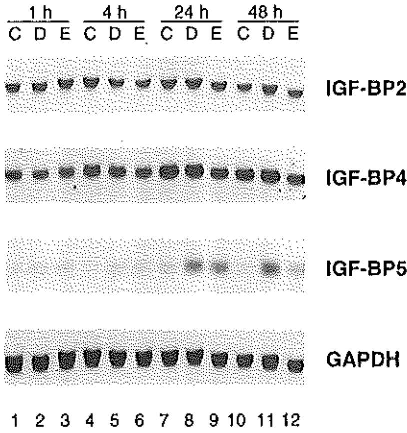

Figure 8.3. Time course of IGF-BP mRNA expression during treatment with 1,25-$(OH)_2D_3$ and EB1089 in MCF-7 cells. Cells were treated with vehicle (C), 10^{-8} M 1,25-$(OH)_2D_3$ (D) or 10^{-8} M EB1089 (E) for the indicated time periods and RNA was isolated as described in the section "Materials and Methods". The Northern blot was hybridized successively with IGF-BP5, IGF-BP4, IGF-BP2, and GAPDH probes.

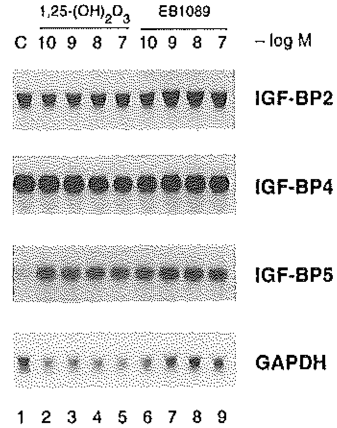

Figure 8.4. Dose response of the effect of 1,25-$(OH)_2D_3$ and EB1089 on IGF-BP mRNA expression in MCF-7 cells. Cells were treated with the indicated concentrations of 1,25-$(OH)_2D_3$ and EB1089 for 24 h. Subsequently RNA was isolated and hybridized successively with IGF-BP5, IGF-BP4, IGF-BP2, and GAPDH probes as described in the section "Materials and Methods".

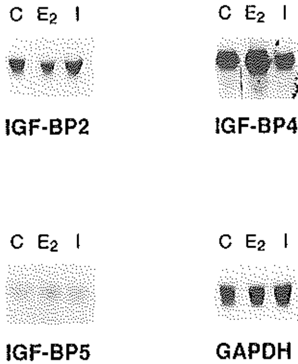

Figure 8.5. Effect of estradiol and IGF-I on IGF-BP mRNA expression. Cells were treated with vehicle (C), 10^{-10} M 17ß-estradiol (E_2), or 50 ng/ml IGF-I (I) during 24 h. RNA was isolated and hybridized successively with IGF-BP5, IGF-BP4, IGF-BP2, and GAPDH probes as described in the section "Materials and Methods".

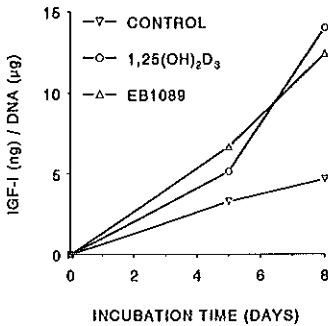

Figure 8.6. Time course of the 1,25-$(OH)_2D_3$- and EB1089-induced increase of the IGF-I secretion of MCF-7 cells. MCF-7 cells were cultured in SFM and incubated with vehicle, 100 nM 1,25-$(OH)_2D_3$ or 100 nM EB1089. 72 h conditioned medium was collected after 5 and 8 days of incubation and the amount of IGF-I in the medium was determined using a radioimmunoassay. The samples were acid-ethanol pre-treated to remove IGF-BPs.

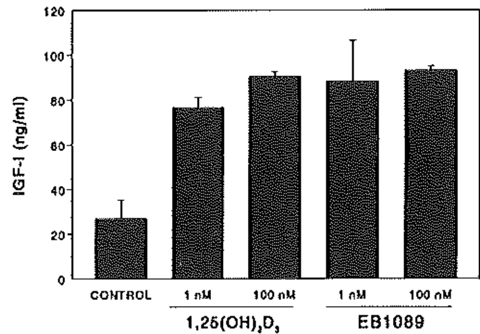

Figure 8.7. IGF-I secretion after 48 h of incubation with 1,25-$(OH)_2D_3$ and EB1089. MCF-7 cells were cultured to subconfluence in SFM and were subsequently incubated with vehicle, 1,25-$(OH)_2D_3$ or EB1089 for 48 h. The 48 h conditioned medium was pre-treated to remove IGF-BPs and the concentration of IGF-I was determined using a radioimmunoassay. The 48 h treatment with 1,25-$(OH)_2D_3$ and EB1089 had no effect on DNA content. Data are presented as mean ± SD of two separate experiments.

Since IGF-BPs have previously been shown to be regulated by estrogens, IGF-I, and insulin in MCF-7 cells (19,31), we have also examined the regulation by these compounds in our culture system. It was observed that IGF-BP4 mRNA expression was markedly increased by estradiol treatment, whereas estradiol had no clear effects on IGF-BP2 and IGF-BP5 expression. Insulin and IGF-I had no clear effect on the mRNA expression of IGF-BP2, IGF-BP4 and IGF-BP5 (Fig. 8.5).

Regulation of IGF-I secretion by MCF-7 cells

Immunoreactive IGF-I (iIGF-I) was detected in conditioned medium of MCF-7 cells, and the concentration increased during the cell culture from 3.3 ng iIGF-I/µg DNA on day 5 to 4.7 and 6.4 ng iIGF-I/µg DNA on days 8 and 11, respectively. Fig. 8.6 shows that incubation with 1,25-$(OH)_2D_3$ and EB1089 (100 nM) resulted in a time-dependent increase of the secretion of iIGF-I into the medium. Lower concentrations of 1,25-$(OH)_2D_3$ and EB1089 (1 nM) stimulated iIGF-I secretion on day 8 from 4.7 to 9.6 and 18.4 ng IGF-I/µg DNA, respectively.

To study whether the increased secretion was related to the growth inhibition and specific for the vitamin D_3 compounds, we have studied the effect of another growth inhibitor. We have previously shown that tamoxifen (10^{-6} M) as well as 1,25-$(OH)_2D_3$ and EB1089 inhibit the autonomous growth of MCF-7 cells (9,14). It appeared, however, that tamoxifen had no effect on the iIGF-I secretion after 8 days of incubation (control cultures 4.7, and tamoxifen-treated cultures 5.0 ng iIGF-I/μg DNA). Further, we have studied whether the vitamin D_3 compounds were able to regulate the iIGF-I secretion independent of the growth inhibitory effects. As shown in Figure 8.7, 1,25-$(OH)_2D_3$ and EB1089 (1 and 100 nM) resulted in a 3- to 4-fold increase of the iIGF-I concentration after 48 h of treatment, whereas these short-term incubations did not yet result in growth inhibition.

DISCUSSION

IGF-I is regarded as an important growth factor for breast cancer (16,17). The cellular activity of IGF-I is modulated by IGF-BPs. They can either enhance or inhibit IGF-I action depending of the type of IGF-BP, cell type, or culture conditions (18,19). In our MCF-7 culture system, IGF-I had no effect on cell growth, whereas IGF-I analogs with reduced binding affinity for IGF-BPs, long R^3 IGF-I (23) and des(1-3)IGF-I (24), were able to stimulate the cell growth, indicating that IGF-BPs secreted by MCF-7 cells into the medium might block the mitogenic activity of IGF-I. Regulation of the pattern of expression of IGF-BPs secreted by breast tumor cells, may be an important mechanism to modulate IGF action and thereby tumor growth. Previous studies have shown a different regulation of IGF-BP expression by positive (estrogen, IGF-I) and negative (retinoic acid, tamoxifen) regulators of MCF-7 cell growth (19,32-36), which suggests that the changes in expression of IGF-BPs are related to their effects on cell growth.

The regulation of IGF-BP expression by 1,25-$(OH)_2D_3$ or analogs has not yet been documented in breast cancer cells. In line with other studies with MCF-7 cells (19,20) we have detected IGF-BP2, IGF-BP4, and IGF-BP5 mRNA species. IGF-BP3 was not detected by Northern blot analysis, but a band indicative for IGF-BP3 was detected on Western ligand blots. Possibly the expression of IGF-BP3 mRNA was too low for detection under the conditions used. The present study shows that treatment with 1,25-$(OH)_2D_3$ and EB1089 resulted in a strong stimulation of IGF-BP5 mRNA expression after 24 h of treatment. The effect of the vitamin D_3 compounds was specific for IGF-BP5 because no effect on IGF-BP2 and IGF-BP4 expression was

observed. The stimulation of IGF-BP5 was also specific for the vitamin D_3 compounds because estradiol had no effect on IGF-BP5 expression, but stimulated IGF-BP4 expression. It is not clear whether the increased mRNA level resulted in an increased secretion of IGF-BP5 protein into the medium. IGF-BP5 could not be visualised on Western ligand blots because its expression is relatively low and IGF-BP5 (28-30 kDa) migrates closely with the predominantly produced IGF-BP2 (34 kDa). As no specific IGF-BP5 antibody was available, also subsequent immunodetection was not possible.

Limited data have been published about the function and regulation of IGF-BP5. The expression and/or production of IGF-BP5 in breast cancer cells has been shown to be stimulated by IGF-I, and estradiol in some (19,20,34,36), but not all studies (31,36), including ours. Similar to the other IGF-BPs, both inhibitory (37) and enhancing (38,39) activity of IGF-BP5 on IGF-action has been described. Because a functional role of IGF-BP5 for breast cancer cells has not yet been reported, the physiological significance for the observed upregulation remains to be determined. Regulation of IGF-BPs by 1,25-$(OH)_2D_3$ occurs also in other cell types than breast cancer cells. In osteoblast-like cells 1,25-$(OH)_2D_3$ has been shown to stimulate the expression of IGF-BP2 (40), IGF-BP3 (41), and IGF-BP4 (42). However, to our knowledge there are no reports on the regulation of IGF-BP5 mRNA by 1,25-$(OH)_2D_3$.

We have measured considerable amounts of immunoreactive IGF-I in the conditioned medium of MCF-7 cells. So far, conflicting results have been reported with respect to the production of IGF-I by breast tumor cells. IGF-I mRNA was not detected in human breast cancer cell lines using a sensitive RNAse protection assay (43), and the immunoreactive IGF-I in the conditioned medium of MCF-7 cells was shown to be due predominantly to the presence of cross reacting IGF-BPs (44). However, we and others (45,46) have measured immunoreactive IGF-I after removal of the binding proteins.

The MCF-7 cells we have used are able to grow logarithmically in serum-free medium. Possibly, IGF-I supports the autonomous growth via an autocrine action. We have previously shown that 1,25-$(OH)_2D_3$ and EB1089 block the mitogenic effect of long R^3 IGF-I on MCF-7 cells[2]. In addition, we have shown that the autonomous growth of MCF-7 cells is inhibited by 1,25-$(OH)_2D_3$ and analogs (9,14). In the present study we have investigated whether the growth inhibitory effects of the vitamin D_3 compounds could be related to an inhibition of IGF-I secretion. However, we have observed that the growth inhibition by 1,25-$(OH)_2D_3$ and EB1089 was accompanied by a stimulation of the iIGF-I secretion. This stimulation was probably a direct effect

of the vitamin D_3 compounds and not an aspecific compensatory mechanism for the growth inhibition, because the growth inhibition by tamoxifen was not accompanied by an increased iIGF-I secretion. This is further supported by the observation that the iIGF-I secretion increased already after 48 h of treatment with 1,25-$(OH)_2D_3$ and EB1089, whereas a growth inhibition could not yet be measured. An explanation for this inverse relationship is yet unclear, but these data indicate that the site of the growth inhibition by 1,25-$(OH)_2D_3$ and EB1089 may be located distal from autocrine production of IGF-I or IGF-I-like activity.

In conclusion, we show for the first time that 1,25-$(OH)_2D_3$ and its analog EB1089 regulate the expression of IGF-BP5 and the secretion of iIGF-I in human breast cancer cells. These data indicate that vitamin D_3 compounds interfere with the complex IGF regulatory system, and thereby possibly influence IGF action. However, presently it is unclear how the regulation of IGF-BP expression and IGF production directly relate to the antiproliferative effects of vitamin D_3 compounds.

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Chapter 9

GENERAL DISCUSSION

9.1 INTRODUCTION

1,25-Dihydroxyvitamin D_3 has been shown to inhibit the growth of cultured breast cancer cells and to suppress the growth of breast tumours in experimental animals, which suggested a role of the hormone in the treatment of breast cancer. However, a major drawback for the clinical use of 1,25-dihydroxyvitamin D_3 is that high dosages are needed. When given to patients, these high dosages may cause serious complications resulting from a disturbed calcium and bone metabolism. In order to make 1,25-dihydroxyvitamin D_3 suitable as an anticancer drug, vitamin D_3 analogues have been synthesized with the aim to dissociate the antiproliferative from the calcaemic activity.

The studies described in this thesis were undertaken to investigate the antiproliferative action of 1,25-dihydroxyvitamin D_3 and several novel vitamin D_3 analogues on the growth of human breast cancer cells in culture (discussed in Section 9.2). The antioestrogen tamoxifen is currently the most widely used endocrine agent for the treatment of breast cancer. Therefore, studies were undertaken to investigate the potential benefits of a combination therapy of 1,25-dihydroxyvitamin D_3/analogues and tamoxifen for breast cancer (discussed in Section 9.3). First, the effects of combined treatment were studied on breast cancer cell growth and subsequently, the investigations were extended to the regulation of bone resorption (discussed in Section 9.4). Several studies have been performed in order to gain insight into the mechanism of the antiproliferative action of the vitamin D_3 compounds and the results are discussed in Section 9.5. Finally, in Section 9.6 suggestions for further *in vitro* and *in vivo* research are given.

9.2 ANTIPROLIFERATIVE ACTION OF 1,25-DIHYDROXYVITAMIN D_3 AND VITAMIN D_3 ANALOGUES ON HUMAN BREAST CANCER CELLS

1,25-Dihydroxyvitamin D_3 has been shown to inhibit the growth of both oestrogen receptor-positive and oestrogen receptor-negative breast cancer cells (1,2). Furthermore, it has been shown that the vitamin D receptor is present in approximately 80 % of human breast tumours and that its presence is not related to the oestrogen receptor status (3). These data have suggested a role for 1,25-dihydroxyvitamin D_3, and especially vitamin D_3 analogues with a low calcaemic activity, in the treatment of both oestrogen receptor-positive and -negative breast

tumours. Most endocrine therapies that are currently used for breast cancer are based on antioestrogenic activity, and therefore treatment with vitamin D_3 compounds may provide an interesting alternative.

The studies in this thesis regard the antiproliferative action of 1,25-dihydroxyvitamin D_3 and four novel synthetic vitamin D_3 analogues on the oestrogen receptor- and vitamin D receptor-positive MCF-7 and ZR-75-1 human breast cancer cell lines. The cell lines have different growth characteristics: MCF-7 cells are able to grow autonomously and are growth stimulated by oestradiol, whereas ZR-75-1 cells are dependent on oestradiol for their growth.

The study described in Chapter 4 shows that basal growth of MCF-7 cells and oestradiol-induced growth of ZR-75-1 cells is arrested by 10^{-7} M 1,25-dihydroxyvitamin D_3. By contrast, in the study described in Chapter 5 a partial growth inhibition was observed and the cells were less sensitive to the antiproliferative action of 1,25-dihydroxyvitamin D_3 (higher EC_{50}). These discrepancies can be explained by differences in culture conditions, i.e. the presence of serum in the culture medium and the frequency of addition of the hormone. In the earlier studies (Chapter 4) we have used medium supplemented with 2 % charcoal-treated foetal calf serum, whereas in later studies (Chapter 5) we have used serum-free medium. In an additional experiment it was demonstrated that the growth inhibition by 1,25-dihydroxyvitamin D_3 was increased by the presence of charcoal-treated serum in the medium (Fig. 9.1). Therefore, it can be concluded that serum contains factors which synergize with 1,25-dihydroxyvitamin D_3 to enhance its antiproliferative action. The second difference between the two studies is that medium and 1,25-dihydroxyvitamin D_3 were refreshed daily in the earlier studies and every 3 days in the later studies. A stronger growth inhibition as a result of more frequent refreshments of hormone and medium can be explained as follows: 1) the hormone accumulates by adhesion to the cells and culture wells resulting in a higher effective concentration, 2) the influence of degradation of the hormone is lower, resulting in a higher effective concentration, and 3) autocrine acting growth factors produced by the cells cannot accumulate and thereby the cells may become more sensitive to the antiproliferative action of 1,25-dihydroxyvitamin D_3.

Chapter 5 describes a study of the effects of four novel vitamin D_3 analogues on breast cancer cell growth. The chemical structures of these side-chain analogues, CB966, EB1089, KH1060, and 22-oxa-calcitriol, are shown in Figure 2.5. Previous studies have shown that these analogues have an increased potency to inhibit the growth of leukaemic cell lines. The *in vivo* calcaemic activity of these analogues was similar or even reduced compared to that of 1,25-dihydroxyvitamin D_3 (Table 2.4).

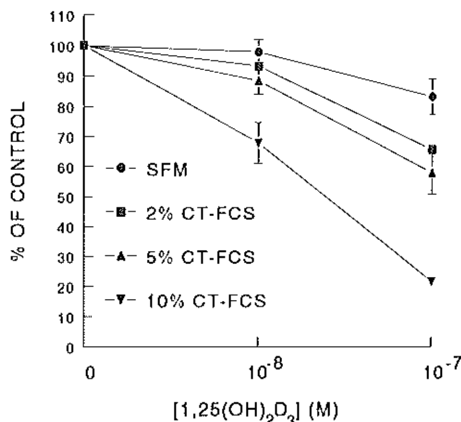

Figure 9.1. Effect of charcoal-treated serum on the growth inhibition of MCF-7 cells by 1,25-dihydroxyvitamin D_3. MCF-7 cells were cultured for 8 days with or without 1,25-dihydroxyvitamin D_3 (1,25-$(OH)_2D_3$) in serum-free medium (SFM) or in medium containing various concentrations charcoal-treated foetal calf serum (CT-FCS). Data are presented as mean ± SD of duplicate wells. See Chapter 5 for details of culture conditions and determination of DNA content.

These data supported the idea that the antiproliferative action of 1,25-dihydroxyvitamin D_3 can indeed be (partly) dissociated from the calcaemic activity.

At the time of our studies little data was available on the anti-proliferative action of these analogues on breast cancer cells. It was shown in Chapter 5 that 1,25-dihydroxyvitamin D_3 and the analogues 22-oxa-calcitriol and CB966 were almost equally potent in inhibiting the growth of MCF-7 and ZR-75-1 cells, whereas the analogues EB1089 and KH1060 were clearly more potent. Although the maximal inhibition was similar for 1,25-dihydroxyvitamin D_3 and the analogues, the EC_{50}'s of EB1089 and KH1060 were 50- and 100-fold lower, respectively. These data are largely in line with other reports of leukaemic and breast cancer cell lines (see Table 2.4 and Table 3.1). The variations in the absolute figures can be attributed to differences in culture conditions, e.g. presence of serum in the culture medium, frequency of medium refreshment and hormone addition (see above), and differences in sensitivity between cells.

The strong antiproliferative action on breast cancer cell lines, combined with the low *in vivo* calcaemic activity of vitamin D_3 analogues offer promise for a clinical use of these compounds in the treatment of breast cancer. However, one has to be careful in the translation of *in vitro* data to the *in vivo* situation. In the animal or human body the pharmacokinetic properties of the analogues play an important role, e.g. an analogue with potent *in vitro* effects may be ineffective *in vivo* because it is rapidly degraded in the body (see also Section 2.5.3). So far, only a few *in vivo* studies with vitamin D_3 analogues have been reported. Yet, the data are very promising. It was shown that the analogues 22-oxa-calcitriol and EB1089 caused tumour suppression without raising serum calcium in animal models for breast cancer (2,4). However, apart from hypercalcaemia other negative side-effect may arise. For

instance, some vitamin D_3 analogues (22-oxa-calcitriol, KH1060) have been shown to exert strong immunosuppressive activity (5). Further, the bone resorbing activity of vitamin D_3 compounds might increase the risk of bone metastases. The latter subject is further discussed in Section 9.4. It is clear that more *in vivo* studies and clinical trials are necessary to determine the effectiveness of vitamin D_3 compounds in the treatment of breast cancer.

9.3 COMBINATION THERAPY OF VITAMIN D AND TAMOXIFEN

Not only a single-agent treatment with vitamin D_3 analogues may have potential, but also a combination therapy with established endocrine or cytotoxic agents may be of clinical interest. We have studied whether the antioestrogen tamoxifen is a good candidate for a combination therapy with a vitamin D_3 analogue, as this is currently the most frequently used endocrine agent in the treatment of breast cancer.

The antiproliferative action of 1,25-dihydroxyvitamin D_3 and vitamin D_3 analogues in combination with tamoxifen has been studied in breast cancer cells in culture. Combined treatment of 1,25-dihydroxyvitamin D_3 and tamoxifen resulted in a stronger growth inhibition of MCF-7 and ZR-75-1 cells than treatment with either compound alone (Chapter 4). With the vitamin D_3 analogues a similar effect was observed (Chapter 5). An interesting observation was that in combination with tamoxifen the cells were more sensitive to the antiproliferative action of 1,25-dihydroxyvitamin D_3 and the analogues, i.e. the EC_{50}'s of the vitamin D_3 compounds in the presence of tamoxifen were lower (2-fold to 4000-fold) than in the absence of tamoxifen (Chapter 5). This shift in EC_{50} values was more pronounced for 1,25-dihydroxyvitamin D_3 and the analogues with an EC_{50} close to 1,25-dihydroxyvitamin D_3 (22-oxa-calcitriol and CB966) than for the compounds with a more favourable EC_{50} (EB1089 and KH1060). As a result the differences in potency between the vitamin D_3 compounds were smaller in the presence of tamoxifen, than in its absence. In MCF-7 cells, the EC_{50} of 1,25-dihydroxyvitamin D_3 shifted from 2×10^{-8} M in the absence of tamoxifen to 5×10^{-11} M in the presence of tamoxifen, which is a decrease by 200-fold. It is intriguing that the EC_{50} in the presence of tamoxifen is within the normal range of serum concentrations of 1,25-dihydroxyvitamin D_3. This raises the question whether physiological 1,25-dihydroxyvitamin D_3 concentrations may play a role in the response to tamoxifen therapy.

Recent studies of Demirpence *et al.* (6) and James *et al.* (7) have supported our

observations that a stronger growth inhibition can be achieved by combined treatment of 1,25-dihydroxyvitamin D_3/analogues and tamoxifen than by treatment with each compound alone. These investigators have observed similar effects using either 1,25-dihydroxyvitamin D_3 and tamoxifen, or EB1089 and the antioestrogen ICI182,780. In addition, our data on the interaction between vitamin D_3 compounds and tamoxifen are consistent with the report of Abe-Hashimoto *et al.* (8), that was published at the time of our studies. They have observed a synergistic antiproliferative effect of 22-oxa-calcitriol and tamoxifen in MCF-7 and ZR-75-1 cells in culture. Moreover, they have observed a synergistic inhibition of MCF-7 tumours grown *in vivo* in athymic mice. The synergism between 1,25-dihydroxyvitamin D_3/analogues and tamoxifen could be very beneficial for a possible clinical application as the use of lower dosages of the vitamin D_3 compounds will reduce the risk of negative side-effects.

The complementary growth inhibition by vitamin D_3 compounds and tamoxifen indicates that also lower dosages of tamoxifen may be used in a combination therapy, which may be beneficial considering the relationship between tamoxifen treatment and an increased risk of endometrial and liver cancer (9,10). In ZR-75-1 cells, which are fully oestrogen dependent, the entire proliferation could be blocked by a high concentration of tamoxifen. Therefore, a lower concentration of tamoxifen was used, which resulted in a partial growth inhibition. The growth inhibition by this suboptimal tamoxifen concentration was augmented by combined treatment with vitamin D_3 compounds (Chapter 5). Thus, the partial growth inhibition by suboptimal concentrations of tamoxifen can be compensated by combined treatment with vitamin D_3 compounds. However, a complete inhibition of cell growth could not be achieved in this way.

Tamoxifen is believed to act via the oestrogen receptor to suppress the growth of breast tumours. Breast tumours are heterogeneous with respect to the steroid receptor content, and consist of both oestrogen receptor-positive and -negative cells, and thus of putative tamoxifen-responsive and -unresponsive cells. Also, distant metastases may loose the oestrogen responsiveness of the primary tumour (11). A major advantage of 1,25-dihydroxyvitamin D_3 is that it inhibits the growth of breast tumours irrespective of the oestrogen receptor status (1,2). Therefore, a tamoxifen-vitamin D combination therapy may offer better response rates than treatment with tamoxifen alone.

A major problem of tamoxifen therapy is that after prolonged treatment, resistance to tamoxifen frequently occurs. A variety of mechanisms have been proposed for the development of tamoxifen-resistance that arises in patients, animals and cultured breast cancer cells, e.g. mutant oestrogen receptors (12), other dominant

Table 9.1. Possible advantages of a vitamin D-tamoxifen combination therapy for breast cancer

- Better response rate
  - Stronger growth inhibition by combined treatment
  - Both ER-positive and ER-negative cells are growth inhibited
- Lower dosages of vitamin D may be used with a reduced risk of negative side-effects
  - EC_{50}'s for the growth inhibition are lower in the presence of tamoxifen
- Lower dosages of tamoxifen may be used
  - Complementary growth inhibition
- Delay of tamoxifen resistance?
  - Antioestrogen-resistant cells are growth inhibited by vitamin D
  - Tamoxifen-induced growth stimulation is inhibited by vitamin D
- Prevention of tumour flare?
  - Tamoxifen-induced growth stimulation is inhibited by vitamin D
- Reduced risk of bone metastases?
  - Tamoxifen attenuates the bone resorption induced by vitamin D

Vitamin D, 1,25-dihydroxyvitamin D_3 or synthetic vitamin D_3 analogues; ER, oestrogen receptor. See Sections 9.3 and 9.4 for further details.

genetic alterations (13), or the induction of enzyme systems which metabolize tamoxifen (14). It has been proposed that tumour progression to the tamoxifen-resistant state includes not only the selection and expansion of subpopulations that have become resistant to tamoxifen, but also of subpopulations that are growth stimulated by tamoxifen (14,15). Growth stimulation by tamoxifen, has been attributed to a partial oestrogen agonistic activity. This agonistic activity of tamoxifen has been shown to be dependent on the tissue, cellular response studied, and concentration used (16). Tamoxifen-induced tumour flare, which is sometimes observed in patients within the first weeks of therapy, has been attributed to an agonistic activity of low concentrations of tamoxifen (17,18).

We have demonstrated that derivatives of ZR-75-1 cells, which had acquired resistance to tamoxifen as a result of retroviral insertional mutagenesis, had not lost their responsiveness to the growth-inhibitory action of 1,25-dihydroxyvitamin D_3 and the analogue KH1060 (Chapter 5). Further, in MCF-7 cells a growth stimulatory effect of low concentrations (10^{-8} M) of tamoxifen in the absence of oestradiol was observed (Chapter 4). This growth stimulation by tamoxifen was completely prevented by 1,25-dihydroxyvitamin D_3 (Chapter 4). These data suggest that treatment with vitamin D_3 compounds could be useful for both tamoxifen-resistant cells and cells that are growth stimulated by tamoxifen. Thereby, it can be hypothesised that a combination therapy of tamoxifen and vitamin D_3 compounds delays the development of tamoxifen-resistance of the tumour. Furthermore, the data indicate that the

combined treatment may play a role in the prevention of tumour flare.

Table 9.1 summarizes the potential benefits of an endocrine therapy for breast cancer with 1,25-dihydroxyvitamin D_3/analogues combined with tamoxifen. It should be stressed that these potential benefits are mostly based on single *in vitro* model systems for the very complex phenomena of tumour growth and tamoxifen-resistance. Of course studies with experimental animals and clinical trials are needed to determine whether all these possible advantages can be achieved in the *in vivo* situation.

9.4 BONE RESORBING ACTIVITY OF VITAMIN D_3 ANALOGUES: THE EFFECT OF TAMOXIFEN

Several studies have described a positive relation between bone resorption and metastasis to bone (see Section 3.9). As 1,25-dihydroxyvitamin D_3 is a potent stimulator of bone resorption, treatment of breast cancer patients with this compound might increase the risk of bone metastases. This aspect may have severe consequences for the potential use of vitamin D_3 compounds in the treatment of breast cancer, the more so as bone is the most frequent site of metastasis of breast cancer.

The vitamin D_3 analogues we have used have previously been tested for their ability to increase serum or urinary calcium concentrations of rats. However, their effects on bone turnover have not been examined. We have demonstrated that 1,25-dihydroxyvitamin D_3 and the analogues EB1089 and KH1060 stimulated the resorption of foetal mouse long bones, using an *in vitro* organ culture model (Chapter 6). The analogues EB1089 and KH1060 were more potent than 1,25-dihydroxyvitamin D_3 (10 to 100-fold) and thereby not only the antiproliferative action of these analogues is increased, but also the bone resorbing activity. By contrast, the reported *in vivo* relative calcaemic activities of EB1089 and KH1060 were only 0.4 and 1.3, respectively, compared to 1,25-dihydroxyvitamin D_3 (19,20). These differences in relative potencies between bone resorbing and calcaemic activity might be explained by organ specific effects of the analogues, i.e. the sensitivity of bone tissue for the analogues is higher than that of the intestine. Alternatively, the pharmacokinetic properties of the analogues may result in different relative potencies *in vivo* and *in vitro* (see also Section 2.5.3). *In vivo* animal studies are needed to determine whether EB1089 and KH1060 can exert the same potent effects on bone resorption *in vivo* as was observed *in vitro*.

Tamoxifen has anti-oestrogenic as well as oestrogenic activity depending on the

organ or cellular response studied. On bone tamoxifen acts as a partial oestrogen agonist. It has been shown that tamoxifen prevented skeletal changes after ovariectomy in rats (21). Further, tamoxifen had protective effects on the skeleton of breast cancer patients, whereas bone loss associated with a decreased oestrogenic activity was expected (22).

In Chapter 6 it was studied whether the bone preserving effects of tamoxifen offer protection against the bone resorbing activity induced by the vitamin D_3 compounds. It was shown that the bone resorption induced by 1,25-dihydroxyvitamin D_3, EB1089, and KH1060 was potently inhibited by tamoxifen. Although bone metastases were not directly measured, this study suggested that an increased risk of bone metastases due to an increased bone resorption induced by vitamin D_3 compounds, might be prevented by combined treatment with tamoxifen. However, *in vivo* studies are necessary for a direct examination of the relation between vitamin D-induced bone resorption and the risk of metastasis of breast tumours to bone, and the effect of tamoxifen on this process.

The mechanism by which tamoxifen inhibits vitamin D-induced bone resorption is not clear. In this study an oestrogenic effect of tamoxifen is unlikely, as oestradiol was ineffective, and the antioestrogen ICI164,384, which lacks oestrogen agonistic activity, caused a similar inhibition as tamoxifen. An alternative explanation could be the involvement of protein kinase C, as it has been shown that tamoxifen inhibits protein kinase C (23) and protein kinase C inhibitors suppress 1,25-dihydroxyvitamin D_3-induced bone resorption (24).

9.5 MECHANISM OF THE ANTIPROLIFERATIVE ACTION OF VITAMIN D

9.5.1 Interaction with oestradiol

Oestradiol plays an important role in the development and progression of breast tumours. Oestradiol acts through the oestrogen receptor, which belongs to the same family of steroid hormone receptors as the vitamin D receptor. As part of the investigations directed to unravelling the mechanisms responsible for the tumour suppressive activity of vitamin D_3 compounds, we have addressed the question whether 1,25-dihydroxyvitamin D_3 and several vitamin D_3 analogues interfere with the action of oestradiol (Chapters 4 and 5). It was observed that 1,25-dihydroxyvitamin D_3 and the analogues CB966, EB1089, KH1060 and 22-oxa-calcitriol caused only a small inhibition of the oestradiol-stimulated growth of MCF-7 cells, and when expressed in

μg DNA/well, this inhibition was similar to the inhibition of the autonomous growth. By contrast, the oestradiol-induced growth of ZR-75-1 cells was potently inhibited by the vitamin D_3 compounds. Therefore, the inhibition of oestradiol-stimulated growth by vitamin D_3 compounds seemed to be cell line specific and might be related to the oestradiol dependency of the cell line.

To gain further knowledge of the interactions with oestradiol, it was studied whether the vitamin D_3 compounds can interfere with oestradiol-induced gene transcription and with the oestrogen receptor level. The myc and pS2 genes are induced by oestradiol, but the oestradiol-induced mRNA levels of these genes were not affected by simultaneous addition of one of the vitamin D_3 compounds. Also, the oestrogen receptor content of both cell lines was not influenced by the vitamin D_3 compounds. On the contrary, a report of Demirpence *et al.* (6) showed that 1,25-dihydroxyvitamin D_3 inhibited oestradiol-induced gene expression of an endogenous gene (pS2), as well as various exogenous transfected genes in MCF-7 cells. Furthermore, James *et al.* (7) observed a decrease in the oestrogen receptor content by 1,25-dihydroxyvitamin D_3 and EB1089. An explanation for these differences is presently unclear. The presence of foetal calf serum may influence the results as we have used serum-free medium and James *et al.* and Demirpence *et al.* have used medium supplemented with foetal calf serum. As discussed earlier (Section 9.1) factors present in serum influence the growth inhibitory effect of 1,25-dihydroxyvitamin D_3 and possibly also the interaction with oestradiol. A second explanation could be that the MCF-7 cell line responds differently in the various laboratories, due to the many passages of the cells.

Together, it seems that vitamin D can interact with the action of oestradiol in some cases, either directly or indirectly. However, the interaction with oestradiol is probably not the main mechanism behind the tumour suppressive activity of vitamin D, because 1,25-dihydroxyvitamin D_3 and 22-oxa-calcitriol have been shown to inhibit breast cancer growth *in vitro* and *in vivo* irrespective of the presence of the oestrogen receptor (1,2).

9.5.2 Interaction with insulin and IGF-I

In addition to oestradiol, polypeptide growth factors are believed to play an important role in promoting the growth of breast tumours. It has been hypothesized that the autonomous, oestrogen-independent growth of breast cancer cells in culture is supported by autocrine acting growth factors (25). Therefore, the observed inhibition of the autonomous growth of MCF-7 cells by 1,25-dihydroxyvitamin D_3 and analogues might be explained by an interaction with autocrine acting growth factors.

In Chapters 7 and 8 we have studied the interaction of 1,25-dihydroxyvitamin D_3 and EB1089 with specific growth factors, namely insulin and IGF-I. Insulin, IGF-I, and also IGF-II are structurally related molecules. They act via three receptors, namely the insulin, IGF type I, and the IGF type II receptor, however considerable cross-reactivity exists between the various ligands and receptors. In addition to the three membrane-bound receptors, the IGFs bind to other soluble proteins, the IGF-binding proteins (IGF-BPs). To date six of these IGF-BPs have been cloned. At cellular level IGF-BPs can either potentiate or suppress the activity of the IGFs, depending on the type of IGF-BP, cell line, and culture conditions.

Several lines of evidence support a biological role for IGF-I in the proliferation of human breast cancer cells: 1) IGF-I is mitogenic for cultured breast cancer cells, 2) IGF-I is produced in the tumour by stromal cells, 3) exogenous IGF-I supported the growth of oestrogen-dependent MCF-7 breast cancer cells in nude mice, in the absence of oestrogen supplementation, and 4) a blocking antibody against the IGF type I receptor inhibited MDA-MB-231 tumour growth in nude mice (for review see ref. 26).

The mitogenic effect of IGF-I is believed to be mediated via the type I IGF receptor. This receptor has a high affinity for IGF-I and a low affinity for insulin. As IGF-I is an expensive peptide, researchers sometimes use high doses of insulin, which is much cheaper, to mimic the mitogenic effect of IGF-I. However, the work presented in this thesis and data from the literature demonstrate that insulin may also stimulate breast cancer growth via its own receptor: 1) insulin receptors have been demonstrated on several breast cancer cell lines (Chapter 5 and ref. 27), 2) a blocking antibody against the type I IGF receptor did not (completely) block the mitogenic action of insulin (Chapter 5, refs. 27 and 28) and 3) the insulin receptor content is increased in human breast tumour specimens and positively correlated to tumour size and grade (29). Finally, Dideriksen *et al.* (30) made the interesting observation that an insulin analogue with enhanced mitogenic activity increased the incidence of rat mammary tumours. However, it has not been investigated via which receptor this insulin analogue exerts its mitogenic activity.

Both insulin and IGF-I stimulated the growth of MCF-7 cells. It was observed that 1,25-dihydroxyvitamin D_3 and EB1089 reversed these mitogenic effects, suggesting that interruption of the insulin and/or IGF-I mitogenic pathway is involved in the antiproliferative action of vitamin D_3 compounds. Subsequently, it was tried to gain more insight into the mechanism of this anti-growth factor activity. It was studied whether the inhibition was achieved via downregulation of the insulin and IGF-I receptors. 1,25-Dihydroxyvitamin D_3 and EB1089 had no direct effect on the insulin

and IGF-I binding after 48 hour of incubation. On the contrary, an increased insulin and IGF-I binding was measured at time points on which the growth inhibition was measured (8 days) (Chapter 7). The physiological meaning of this observation is not clear, but a similar inverse relationship between receptor regulation and growth inhibition by 1,25-dihydroxyvitamin D_3 was observed for the EGF receptor in MDA-MB-231 and BT-20 cells (31,32). It seems that the stimulation of growth factor receptors by vitamin D_3 compounds is a secondary effect, which is not directly responsible for the growth inhibition.

Inhibition of IGF-I-stimulated growth, but not insulin-stimulated growth, may also involve regulation of IGF-BPs. It was shown in Chapter 8 that 1,25-dihydroxyvitamin D_3 and EB1089 stimulated the mRNA expression of IGF-BP5 in MCF-7 cells. The physiological role of IGF-BP5 for breast cancer cells is yet unknown, and hence the function of IGF-BP5 regulation by vitamin D_3 compounds. It is unlikely that the regulation of IGF-BP5 by 1,25-dihydroxyvitamin D_3 and EB1089 plays a dominant role in the inhibition of IGF-I stimulated growth. The inhibition of IGF-I-stimulated growth described in Chapter 7 was independent of IGF-BPs, since analogues of IGF-I were used, which have a strongly reduced binding to IGF-BPs.

MCF-7 cells secrete immunoreactive IGF-I into the medium. A possible mechanism for the inhibition of the autonomous growth by vitamin D_3 compounds is an inhibition of the secretion of autocrine acting IGF-I. However, it appeared that the secretion of immunoreactive IGF-I by MCF-7 cells was increased rather than decreased by treatment with 1,25-dihydroxyvitamin D_3 and EB1089 (Chapter 8). This stimulation of IGF-I secretion was already observed after 48 h of treatment and thus seemed to be a direct effect of the vitamin D_3 compounds. The inhibition of the autonomous growth of MCF-7 cells by the vitamin D_3 compounds probably takes place at a site located distal from IGF-I secretion.

In summary, it has been shown that 1,25-dihydroxyvitamin D_3 and EB1089 inhibit the mitogenic effect of insulin and IGF-I in MCF-7 cells. In addition, it has been shown that 1,25-dihydroxyvitamin D_3 and KH1060 inhibit the mitogenic action of EGF in ZR/HERc cells (Chapter 5). Furthermore, the vitamin D_3 compounds have been shown to regulate the number of growth factor receptors (insulin, IGF-I, and EGF), growth factor production (IGF-I), and IGF-BP expression. Therefore, it can be concluded that the vitamin D_3 compounds interfere with the action of growth factors at different levels. However, it remains to be established how precisely these effects relate to the antiproliferative action of vitamin D_3 compounds in breast cancer cells.

9.5.3 The role of the oncogenes myc and fos

The gene products of myc and fos are nuclear proteins which act as transcription factors. The expression of both genes is rapidly and transiently induced by multiple external signals, including steroid hormones, polypeptide growth factors, and serum. The myc and fos protein products are believed to play important roles in cellular proliferation and differentiation. 1,25-Dihydroxyvitamin D_3 has been shown to regulate the mRNA expression of myc and fos in several cell types, which suggested that these oncogenes play a role in the antiproliferative and/or differentiation inducing activity of vitamin D_3 compounds.

In breast cancer there is a strong positive correlation between myc expression and cell growth (33). Correspondingly, suppression of myc mRNA has been associated with growth inhibition by 1,25-dihydroxyvitamin D_3 in HL60 leukaemic cells (34) and in MCF-7 cells (35). However, we have not observed an inhibition of myc mRNA expression in MCF-7 and ZR-75-1 cells, neither under basal growth conditions, nor in the oestradiol-stimulated situation (Chapter 5).

Stimulation of fos mRNA expression by 1,25-dihydroxyvitamin D_3 has been associated with growth inhibition of HL60 leukaemic cells (36) and MCF-7 cells (35). We have not observed an effect of 1,25-dihydroxyvitamin D_3 on basal fos mRNA expression of MCF-7 cells, but observed that insulin-induced fos mRNA expression was inhibited by 1,25-dihydroxyvitamin D_3 (Chapter 7). However, because fos mRNA is induced by both growth inhibitory and growth stimulatory agents, fos mRNA expression and the proliferative status of breast cancer cells cannot be linked.

It can be concluded that, in order to define the role of myc, fos and other oncogenes or tumour suppressor genes in the antiproliferative action of vitamin D_3 compounds, the study of mRNA regulation is not sufficient. A better answer may be obtained from transfection experiments, in which it is possible to overexpress or inactivate genes of interest.

9.6 SUGGESTIONS FOR FURTHER RESEARCH

9.6.1 *In vitro* studies

More studies are needed to clarify the mechanism of the antiproliferative action of vitamin D_3 compounds. The fact that 1,25-dihydroxyvitamin D_3 inhibits the growth of cells of many different origins suggests a common mechanism of action. A possible site of interaction is the regulation of genes that are directly involved with the cell division machinery, e.g. certain oncogenes and tumour suppressor genes.

Studies in this thesis and elsewhere (37) have demonstrated an anti-growth factor activity of vitamin D_3 compounds. This might indicate an interaction at a site common in the signal transducing pathway for different polypeptide growth factors. Also, the intracellular messenger calcium may play a role, especially as calcium has been shown to influence the growth inhibition by vitamin D_3 compounds (see Section 3.8.3).

Studies on the regulation of differentiation of breast cancer cells by 1,25-dihydroxyvitamin D_3 have been hampered by the lack of good differentiation markers. When good markers are available, it can be established whether inhibition of proliferation by vitamin D_3 compounds is accompanied by induction of differentiation.

The development of tamoxifen-resistance is a major problem of tamoxifen therapy. Tamoxifen is an unnatural pharmaceutical compound, but it acts via the oestrogen receptor and changes in this receptor may contribute to tamoxifen resistance (12). For a future clinical application of vitamin D_3 compounds it is important to investigate whether breast cancer cells develop vitamin D resistance after prolonged treatment, analogous to tamoxifen resistance.

The mechanism behind the synergistic inhibition of breast cancer cell growth by 1,25-dihydroxyvitamin D_3/analogues and tamoxifen has not been clarified and needs further study. A possible site of interaction is the regulation of the vitamin D receptor number. Tamoxifen had no effect on the vitamin D receptor level of MCF-7 cells after 24 h of treatment (Chapter 5). However, Escaleira *et al.* (38) have shown that tamoxifen stimulated the vitamin D receptor level of T47-D cells after 72 h of treatment. Therefore, this should also be investigated in MCF-7 cells at later time-points. Tamoxifen and 1,25-dihydroxyvitamin D_3 have in common that they both inhibit the mitogenic action of certain growth factors, and this may be an other clue for the observed interaction between both types of compounds. Further, tamoxifen may inhibit the metabolism of the vitamin D_3 compounds. As the effect on metabolism may differ between the vitamin D_3 analogues, this may also explain the different extent of synergism of tamoxifen with the various vitamin D_3 analogues. However, it is very difficult to define the site of interaction when the mechanism of the growth inhibitory action of 1,25-dihydroxyvitamin D_3 itself is still largely unknown.

9.6.2 *In vivo* studies

In animal models for breast cancer the antiproliferative action of vitamin D_3 compounds has been studied by measuring the size of the tumours. However, much more valuable information can be obtained from the tumours. Analysis of parameters that have been linked to response to therapy and prognosis, e.g. cell proliferation

indices, oncogene expression, steroid receptor content, and growth factor receptor content (39), will give a better insight into the activity of vitamin D_3 compounds. Also, an investigation on vitamin D receptor status in relation to response to vitamin D therapy may provide helpful information in predicting the responsiveness of a tumour to vitamin D therapy.

The promising *in vitro* results of a vitamin D-tamoxifen combination therapy have to be confirmed by more *in vivo* studies. Several vitamin D_3 analogue-tamoxifen combinations should be tested in the chemical-induced breast cancer model, or in the nude mice model, using different breast cancer xenografts. To examine the synergistic interaction different concentrations of both compounds may be analyzed.

Little attention has been given to the effects of vitamin D_3 analogues on bone in relation with tumour suppression. The bone resorbing activity induced by vitamin D_3 compounds may increase the risk of skeletal metastases (see Section 3.9), and/or may weaken the skeleton after prolonged treatment. These undesired side-effects may have consequences for the clinical application of these compounds as tumour suppressive agents. Therefore, it is important to monitor serum markers of bone turnover and/or to perform histomorphometrical analysis of bone. These studies could be done both in the absence and presence of tamoxifen, as tamoxifen has been shown to have protective effects on vitamin D-induced bone resorption in an *in vitro* model (Chapter 6). If these data can be confirmed in an *in vivo* model, this would mean an additional advantage of a tamoxifen-vitamin D therapy.

A direct determination of bone metastases in response to vitamin D therapy, requires an *in vivo* model of highly metastatic breast cancer cells, analogous to the model described by Krempien *et al.* (40). The advantage of the nude mice model described by Brünner *et al.* (41) is that metastases and suppression of the primary tumour can be measured in the same model. These models will make it possible to determine whether vitamin D_3 compounds increase the risk of bone metastases, and whether tamoxifen has a protective effect on this process.

9.7 REFERENCES

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Chapter 10

SUMMARY

SUMMARY

The biologically most active form of vitamin D, 1,25-dihydroxyvitamin D_3 is mainly known for its important role in calcium and bone metabolism. More recently it has been shown that 1,25-dihydroxyvitamin D_3 inhibits the growth of breast cancer cells in culture and suppresses the growth of breast tumours in experimental animals. These findings have suggested a potential use of 1,25-dihydroxyvitamin D_3 in the treatment of breast cancer. However, high doses are needed for tumour suppression, which could result in a dangerous elevation of blood calcium levels in patients. Furthermore, an increased bone turnover induced by 1,25-dihydroxyvitamin D_3 might increase the risk of skeletal metastases. To overcome these problems new vitamin D_3 analogues have been synthesized, which have a strong antiproliferative, but a reduced calcaemic activity.

The studies in this thesis describe the effects of 1,25-dihydroxyvitamin D_3 and several novel vitamin D_3 analogues on the growth of the MCF-7 and ZR-75-1 human breast cancer cell lines. Both cell lines are vitamin D receptor- and oestrogen receptor-positive. In addition, the bone resorption induced by the vitamin D_3 compounds was measured using an organ culture system of ^{45}Ca labelled foetal mouse long bones. The effects of the vitamin D_3 compounds on cell growth and bone resorption were studied both in the absence and presence of the antioestrogen tamoxifen, in order to investigate a potential benefit of a combination therapy of vitamin D_3 compounds and tamoxifen. Tamoxifen is currently the most widely used endocrine agent used in the treatment of breast cancer. Although good results have been obtained with this agent, alternative or additional endocrine therapies are still warranted.

The vitamin D_3 analogues that have been tested are CB966, EB1089, KH1060, and 22-oxa-calcitriol. Only the analogues EB1089 and KH1060 were clearly more potent than 1,25-dihydroxyvitamin D_3 in the inhibition of MCF-7 and ZR-75-1 cell growth. These analogues were active at 30- to 100-fold lower concentrations than 1,25-dihydroxyvitamin D_3. Combined treatment of the vitamin D_3 compounds and tamoxifen resulted in a stronger inhibition than the inhibition by each of these agents alone. Further, it was observed that tamoxifen increased the sensitivity of the cells to the vitamin D_3 compounds. In the presence of tamoxifen, the vitamin D_3 compounds were active at 2- to 4000-fold lower concentrations than in its absence. These data demonstrate that a combination therapy of 1,25-dihydroxyvitamin D_3 or a vitamin D_3 analogue and tamoxifen may have the advantage that 1) a stronger growth inhibition can be achieved, and 2) lower dosages of the vitamin D_3 compounds can be used with

a reduced risk on negative side-effects. In addition, it was observed that the growth stimulatory effect of low concentrations of tamoxifen was reversed by co-addition of 1,25-dihydroxyvitamin D_3 to MCF-7 cells, and that 1,25-dihydroxyvitamin D_3 and KH1060 inhibited the growth of tamoxifen-resistant derivatives of the ZR-75-1 cell line. These data suggested a role of vitamin D_3 compounds in the treatment of tamoxifen-resistant tumours and in the prevention of tamoxifen-induced tumour flare. Finally, it was shown that tamoxifen considerably suppressed the bone resorption induced by 1,25-dihydroxyvitamin D_3, EB1089, and KH1060. These findings suggested that an increased risk on bone metastases due to an increased bone resorption induced by vitamin D_3 compounds, might be prevented by combined treatment with tamoxifen.

Oestradiol and a number of polypeptide growth factors play an important role in the development and growth of breast tumours. Therefore, we have studied whether the vitamin D_3 compounds can interfere with the actions of these mitogens. The inhibition of oestradiol-stimulated growth was cell line specific: 1,25-dihydroxyvitamin D_3 and the analogues inhibited the oestradiol-stimulated growth of ZR-75-1 cells, but not of MCF-7 cells. Oestradiol-induced mRNA expression of the myc and pS2 genes was not affected, neither was the oestrogen receptor level changed by treatment with the vitamin D_3 compounds. Therefore, in our culture system, the vitamin D_3 compounds did not seem to interfere directly with the action of oestradiol. By contrast, the vitamin D_3 compounds clearly interfered with the action of the polypeptide growth factors insulin and IGF-I at different levels. It was observed in MCF-7 cells that besides inhibition of the mitogenic effect of insulin and IGF-I, 1,25-dihydroxyvitamin D_3 and EB1089 increased the number of insulin and IGF-I binding sites, stimulated the secretion of immunoreactive IGF-I, and stimulated the expression of IGF-BP5. Further, 1,25-dihydroxyvitamin D_3 inhibited insulin-induced fos mRNA expression. However, additional research is needed to assess the direct relation between these effects and the antiproliferative action of vitamin D_3 compounds.

In conclusion, 1,25-dihydroxyvitamin D_3 and especially the vitamin D_3 analogues EB1089 and KH1060 are potent inhibitors of breast cancer cell growth. As EB1089 and KH1060 have been shown to have a low *in vivo* calcaemic activity, these analogues may have potential for treatment of breast cancer. A major advantage of a vitamin D therapy in comparison with most established endocrine therapies is that vitamin D_3 compounds inhibit breast cancer growth irrespective of the oestrogen receptor. Studies in this thesis have indicated that a vitamin D-tamoxifen combination therapy may offer additional advantages compared to treatment with these agents alone. However, in order to establish whether these promising laboratory data have

clinical potential, further research with an animal model for breast cancer is an essential next step.

SAMENVATTING

SAMENVATTING

De biologisch meest actieve vorm van vitamine D, 1,25-dihydroxyvitamine D_3 is vooral bekend door zijn belangrijke rol in de calciumhuishouding en in botmetabolisme. Meer recent is aangetoond dat 1,25-dihydroxyvitamine D_3 de groei remt van gekweekte borsttumorcellen en van borsttumoren in proefdieren. Deze bevindingen wijzen erop dat 1,25-dihydroxyvitamine D_3 mogelijkheden zou kunnen bieden voor de behandeling van borstkanker. Er zijn echter hoge doseringen nodig voor de tumorremmende werking en dit zou bij patiënten kunnen resulteren in een gevaarlijke verhoging van de calcium concentratie in het bloed. Verder zou een verhoogde botombouw geïnduceerd door 1,25-dihydroxyvitamine D_3 het risico op botmetastases kunnen doen toenemen. Om deze problemen het hoofd te bieden zijn nieuwe vitamine D_3 analogen gesynthetiseerd die een sterke tumor-remmende werking hebben, maar minder de calciumhuishouding verstoren.

In dit proefschrift worden de resultaten beschreven van de effecten van 1,25-dihydroxyvitamine D_3 en een aantal nieuwe vitamine D_3 analogen op de groei van de humane borsttumorcellijnen MCF-7 en ZR-75-1. Beide cellijnen zijn vitamine D receptor- en oestrogeen receptor-positief. Ook werd de botresorptie geïnduceerd door de vitamine D_3 verbindingen gemeten. Hiervoor werd een orgaan-kweeksysteem van ^{45}Ca gelabelde foetale muizebotjes gebruikt. De effecten van de vitamine D_3 verbindingen op tumorcelgroei en botresorptie werden bestudeerd in de aan- en afwezigheid van het anti-oestrogeen tamoxifen, dit om na te gaan of een combinatie-behandeling van vitamine D_3 verbindingen en tamoxifen betere mogelijkheden zou kunnen bieden voor de behandeling van borstkanker. Tamoxifen is momenteel het meest gebruikte endocriene middel voor de behandeling van borstkanker. Ondanks de goede resultaten die verkregen zijn met dit middel, zijn andere of additionele endocriene behandelingen nog steeds gewenst.

De vitamine D_3 analogen die werden getest zijn: CB966, EB1089, KH1060 en 22-oxa-calcitriol. Alleen de analogen EB1089 en KH1060 waren duidelijk potenter dan 1,25-dihydroxyvitamine D_3 in de remming van de groei van MCF-7 en ZR-75-1 cellen. Deze analogen werkten bij een 30 tot 100 maal lagere concentratie dan 1,25-dihydroxyvitamine D_3. Gecombineerde behandeling met de vitamine D_3 verbindingen en tamoxifen resulteerde in een sterkere remming dan de remming door deze stoffen afzonderlijk. Verder werd waargenomen dat tamoxifen de gevoeligheid van de cellen voor de vitamine D_3 verbindingen verhoogt. In de aanwezigheid van tamoxifen waren de vitamine D_3 verbindingen werkzaam bij 2 tot 4000 maal lagere concentraties dan in de afwezigheid van tamoxifen. Deze gegevens wijzen erop dat een gecombineerde

behandeling met 1,25-dihydroxyvitamine D_3 of een vitamine D_3 analoog en tamoxifen het voordeel zou kunnen bieden dat 1) een sterkere groeiremming bereikt kan worden, en 2) lagere doseringen van de vitamine D_3 verbindingen gebruikt kunnen worden met een verminderd risico op bijwerkingen. Verder werd gevonden dat de groeistimulerende werking van lage concentraties tamoxifen tegengegaan werd door gelijktijdig 1,25-dihydroxyvitamine D_3 toe te dienen aan MCF-7 cellen, en dat 1,25-dihydroxyvitamine D_3 en KH1060 de groei remden van tamoxifen-resistente cellen, die afgeleid zijn van de ZR-75-1 cellijn. Deze gegevens suggereerden een rol voor vitamine D_3 verbindingen in de behandeling van tamoxifen-resistente tumoren en in de preventie van een door tamoxifen geïnduceerd "tumour flare" effect. Tenslotte werd aangetoond dat tamoxifen de botresorberende activiteit van 1,25-dihydroxyvitamine D_3, EB1089 en KH1060 aanzienlijk remt. Deze bevindingen suggereerden dat een verhoogd risico op botmetastases als gevolg van een verhoogde botresorptie geïnduceerd door vitamine D_3 verbindingen, voorkomen zou kunnen worden door een gecombineerde behandeling met tamoxifen.

Oestradiol en een aantal polypeptide groeifactoren spelen een belangrijke rol in de ontwikkeling en groei van borsttumoren. Daarom is bestudeerd of de vitamine D_3 verbindingen invloed kunnen uitoefenen op de werking van deze mitogene stoffen. 1,25-Dihydroxyvitamine D_3 en de analogen remden de oestradiol-gestimuleerde groei van ZR-75-1 cellen, maar niet die van MCF-7 cellen. Verder werden noch de mRNA expressie van de myc en pS2 genen geïnduceerd door oestradiol, noch het aantal oestrogeen receptoren beïnvloed door de vitamine D_3 verbindingen. Daarom lijkt het erop dat in ons celkweeksysteem de vitamine D_3 verbindingen geen directe invloed kunnen uitoefenen op de werking van oestradiol. De vitamine D_3 verbindingen kunnen echter wel interfereren met de werking van de polypeptide groeifactoren insuline en IGF-I op verschillende niveaus. In MCF-7 cellen werd waargenomen dat 1,25-dihydroxyvitamine D_3 en EB1089 het groeistimulerende effect van insuline en IGF-I remden. Daarnaast werd een toename gevonden van het aantal bindingsplaatsen voor insuline en IGF-I, een stimulatie van de secretie van immunoreactief IGF-I en een stimulatie van de expressie van het IGF-bindend eiwit 5. Verder werd de inductie door insuline van het fos mRNA geremd door 1,25-dihydroxyvitamine D_3. Er is echter meer onderzoek nodig om de directe relatie tussen al deze effecten en de groeiremmende werking van de vitamine D_3 verbindingen vast te stellen.

Geconcludeerd kan worden dat 1,25-dihydroxyvitamine D_3 en vooral de vitamine D_3 analogen EB1089 en KH1060 potente remmers zijn van de groei van borsttumorcellen. Aangezien aangetoond is dat EB1089 en KH1060 een lage *in vivo*

calcaemische activiteit hebben, zouden deze analogen mogelijkheden kunnen bieden voor de behandeling van borstkanker. Een belangrijk voordeel van een vitamine D therapie, in vergelijking met de meeste bestaande endocriene therapieën, is dat vitamine D_3 verbindingen borstkankergroei remmen onafhankelijk van de aanwezigheid van de oestrogeen receptor. Het onderzoek beschreven in dit proefschrift geeft aan dat een vitamine D-tamoxifen combinatietherapie additionele voordelen zou kunnen bieden vergeleken met een behandeling met deze middelen afzonderlijk. Maar om te weten of deze veelbelovende laboratoriumresultaten ook klinisch toepasbaar zijn is onderzoek in een diermodel voor borstkanker een essentiële volgende stap.

CURRICULUM VITAE

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1 juni 1995	Aanstelling als "medical affairs officer", Pharmachemie BV, Haarlem

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