STUDIES ON THE MECHANISM OF ACTION OF SYNTHETIC LIGANDS OF THE VITAMIN D AND ESTROGEN RECEPTOR

I read the news today, oh boy: "Four thousand holes in Blackburn, Lancashire".
And though the holes were rather small, they had to count them all.
Now they know how many holes it takes to fill the Albert Hall.
A day in the life, J. Lennon & P. McCartney

STUDIES ON THE MECHANISM OF ACTION OF SYNTHETIC LIGANDS OF THE VITAMIN D AND ESTROGEN RECEPTOR

ONDERZOEK NAAR DE WERKINGSMECHANISMEN VAN SYNTHETISCHE LIGANDEN VAN DE VITAMINE D EN OESTROGEEN RECEPTOR

Proefschrift

ter verkrijging van de graad van doctor aan de

Erasmus Universiteit Rotterdam op gezag van de

rector magnificus Prof. dr. P.W.C. Akkermans M.A.

en volgens besluit van het College van Promoties.

De openbare verdediging zal plaatsvinden op

woensdag 21 juni 2000 om 11.45 uur.

door

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geboren te Breda

Promotiecommissie

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Druk: Haveka B.V., Alblasserdam

Cover: White Rabbit, Breda

Het onderzoek beschreven in dit proefschrift werd verricht op de Afdeling Inwendige Geneeskunde van de faculteit der Geneeskunde en Gezondheidswetenschappen van de Erasmus Universiteit Rotterdam/Erasmus Medisch Centrum Rotterdam

The investigations described in this thesis were performed at the Department of Internal Medicine of the Erasmus University/Erasmus Medical Center Rotterdam, The Netherlands

CONTENTS:

List of abbreviations

$1,25\text{-}(OH)_2D_3$	1,25-Dihydroxyvitamin D_3
$1,25\text{-}(OH)_2\text{-}F_6\text{-}D_3$	1,25-Dihydroxy-26,26,26,27,27,27-hexafluoro-vitamin D_3
AF-1/2	Activation function 1/2
AP-1	Activating protein-1
ATP	Adenosine 5'-triphosphate
cAMP	cyclic-3',5'Adenosine monophosphate
CB966	24a,26a,27a-Trihomo-1,25-dihydroxyvitamin D_3
DBP	Vitamin D binding protein
DNA	Deoxyribonucleic acid
DR3/4/6	Direct repeat with 3/4/6 nucleotide spacing
DRIPs	Vitamin D receptor interacting proteins
E_2	17ß-Estradiol
EB1089	24a,26a,27a,-Trihomo-22,24-diene-1,25-dihydroxyvitamin D_3
ED_{50}	Median effective dose
ED-71	2ß-(3-Hydroxypropoxy)-1,25-dihydroxyvitamin D_3
ERα/ß	Estrogen receptor alpha/beta
ERE	Estrogen response element
α/βERKO	Estrogen receptor alpha/beta knock out
GRIP1	Glucocorticoid receptor interacting protein 1
HAT	Histone acetyltransferase
ICI 164,384	N-n-butyl-11-(3,17β-dihydroxyoestra-1,3,5(10)-trien-7α-yl)n-methylundecanamide
ICI 182,780	7-α-[9-(4,4,5,5,5-Pentafluoro-pentylsulphinyl)nonyl]oestra-1,3,5(10)-triene-3,17 beta-diol
IP9	Inverted palindrome with 9 nucleotide spacing
kDa	kilo Dalton
KH1049	20-Epi-22-oxa-24a,25a-di-homo-1,25-dihydroxyvitamin D_3
KH1060	20-Epi-22-oxa-24a,26a,27a-tri-homo-1,25-dihydroxyvitamin D_3
MC1288	20-Epi-1,25-dihydroxyvitamin D_3
MC903	1,24S-Dihydroxy-22-ene-25,26,27-cyclopropylvitamin D_3
αMEM	alpha Minimal essential medium
OCT	22-Oxo-1,25-dihydroxyvitamin D_3
OD	Optical density
PTH	Parathyroid hormone
(m)RNA	(messenger) Ribonucleic acid
RO 23-7553	1,25-Dihydroxy-16-ene-23-yne-vitamin D_3
RO 24-2637	1,25-Dihydroxy-16-ene-vitamin D_3
RO 24-5531	1,25-Dihydroxy-16-ene-23-yne-26,26,26,27,27,27-hexafluoro-vitamin D_3
RXR	Retinoid X receptor
SDS-PAGE	Sodium dodecyl sulfate-polyacrylamide gel electrophoresis
SERM	Selective estrogen receptor modulator

SRC-1	Steroid receptor coactivator-1
SUG1	Suppressor of gal1
TF	Transcription factor
TGFα/ß	Transforming growth factor alpha/beta
TNT lysate assay	Coupled *in vitro* transcription and translation rabbit reticulocyte lysate system
VDR	Vitamin D receptor
VDRE	Vitamin D response element

Chapter 1

INTRODUCTION

In preparation for submission:

Van den Bemd GJCM, Pols HAP, Van Leeuwen JPTM. Mechanistic aspects of vitamin D analog action.

1. INTRODUCTION

Nuclear hormones play a key role in embryonic development, growth and differentiation of cells and tissues, and in maintenance of homeostasis. They exert their action via a large group of nuclear transcription factors gathered in the nuclear receptor superfamily (Evans 1988, Beato 1989, O'Malley 1990). This superfamily consists of receptors for estrogen, glucocorticoid, mineralocorticoid, androgen, progesterone, but also contains receptors for vitamin D, retinoids, fatty acids, and thyroid hormone. Furthermore, a large number of so-called orphan receptors are included for which ligands and functions are at the moment unknown (Kliewer 1999).

A. VITAMIN D RECEPTOR-MEDIATED GENE REGULATION

The vitamin D receptor (VDR) (Baker 1988, McDonnell 1988, Haussler 1998) is a 50-60 kDa receptor protein that becomes active after binding of its ligand: the secosteroid 1,25-dihydroxyvitamin D_3 (1,25-$(OH)_2D_3$) (For details on 1,25-$(OH)_2D_3$ formation see Section A.4.1.). In its activated form, the VDR regulates transcription of various genes involved in bone metabolism and in maintenance of calcium and phosphate homeostasis (Reichel 1989, DeLuca 1997). In addition to these classic functions, binding of 1,25-$(OH)_2D_3$ to the VDR initiates a complex of biochemical processes that modulates proliferation and differentiation of cells (Pols 1990, Walters 1992, Bikle 1992, Van den Bemd 2000a).

A.1. VITAMIN D RECEPTOR EXPRESSION

The VDR is expressed in various tissues and cells (see Table 1.1). VDRs are both present in the cytoplasm and in the nucleus (Walters 1986). However, it is still controversial whether unliganded VDRs reside in the cytoplasm or in the nucleus (Barsony 1999).

Table 1.1. Cells, tissues, and organs containing vitamin D receptors
Adapted from: Bikle 1992, Walters 1992, Bouillon 1995, Norman 1997

target tissues related to classic vitamin D function
Bone (bone marrow, cartilage, osteoblasts, osteoclast presursors)
Intestine
Kidneys
Parathyroid glands

target tissues related to nonclassic vitamin D function

Adipose	Muscle
Adrenals	Ovary
Brain	Pancreas ß-cells
Breast	Parotids
Colon	Pituitary
Endothelium	Placenta
Epididymis	Prostate
Ganglion	Retina
Hair follicle	Skin (keratinocytes, melanocytes, fibroblasts)
Liver	Stomach
Lung	Testis
Lymphocytes (activated)	Thyroid
Monocytes	Uterus

Malignant cells

Breast carcinoma	Lung carcinoma
Cervical carcinoma	Osteosarcoma
Gall bladder carcinoma	Prostate carcinoma

A.2. VITAMIN D RECEPTOR STRUCTURE

The VDR and other members of the nuclear receptor superfamily share a basic structure consisting of a variable N-terminal region, a short and well conserved cysteine-rich central domain and a relatively well-conserved C-terminal region (Figure 1.1).

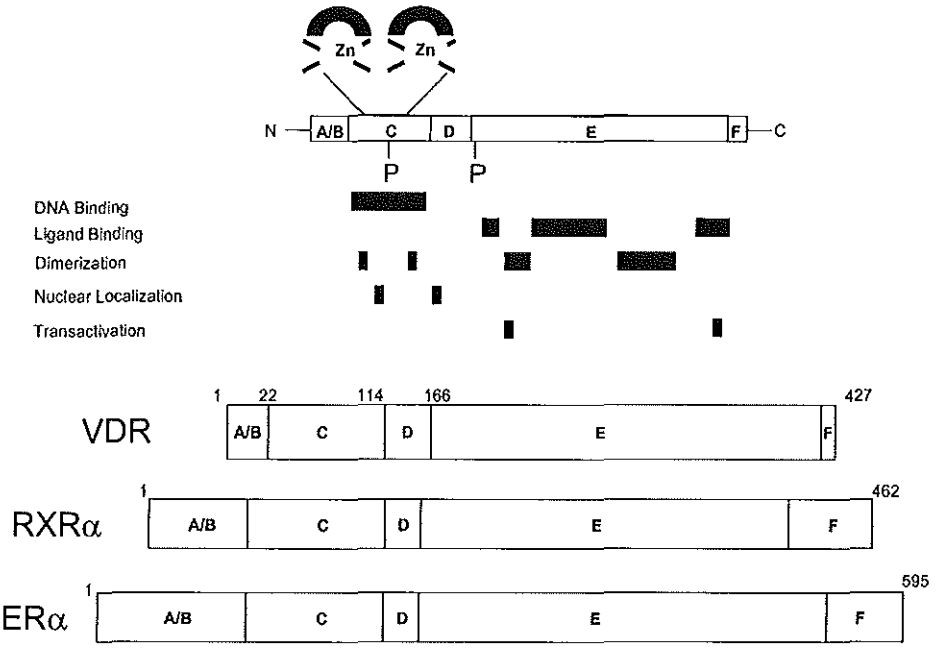

Figure 1.1. Schematic structure of VDR, retinoid X receptor α (RXRα), and estrogen receptor α (ERα). *The specific functions of the domains A through F are indicated. P indicates a phosphorylation site.*

The cysteine-rich domain (domain C in Figure 1.1) contains two characteristic zinc-binding clusters of amino acid residues that are responsible for DNA binding of the receptor. C-terminal of the DNA-binding domain lies the hinge region (domain D) that gives the VDR its flexibility. The ligand binding domain of the VDR (designated E) consists of 12 α helixes containing regions involved in nuclear translocation, receptor dimerization, and ligand-dependent transcriptional activation (AF-2). The AF-2 domain of the VDR is located on helix 12 between amino acids 417 and 420 (Jurutka 1997), but also tyrosine 236, lysine 246, and lysine 264 within helix 3 play an important role in cofactor binding and ligand-dependent transactivation (Nakajima 1998, Jiménez-Lara 1999, Kraichely 1999). Another region important for transactivation (i.e. AF-1) lies in the N-terminal A/B domain. The AF-1 domain functions in a ligand-independent manner and has been identified in most nuclear hormone receptors, although the VDR probably lacks AF-1 function. Compared to estrogen receptor (ER), retinoid X receptor (RXR), and retinoic acid receptor, the VDR A/B domain is short and its removal does not affect VDR function (Sone 1991).

However, elements between amino acids 14 and 23 in the N-terminal region of the VDR are important for optimal transcriptional activity and binding to the basal transcription factor IIB (TFIIB) [Jurutka 1998]. The function of the C-terminal F domain, which is not present in all receptors (e.g. the progesterone receptor), has not yet been elucidated.

A.3. FUNCTION OF 1,25-DIHYDROXYVITAMIN D_3

Classic function of 1,25-dihydroxyvitamin D_3

The hormone 1,25-$(OH)_2D_3$ is the natural ligand for the VDR. The classic action of 1,25-$(OH)_2D_3$ involves regulation of calcium and phosphate homeostasis and bone metabolism. Increased 1,25-$(OH)_2D_3$ levels induce elevation of the calcium concentration in the blood by increasing calcium absorption from the intestine by stimulating transcaltachia and the production of calbindin D-9k, a protein involved in calcium transport from the intestinal lumen through the enterocytes into the blood stream [Strom 1992]. Furthermore, 1,25-$(OH)_2D_3$ increases calcium reabsorption in the kidney.

For optimal regulation of calcium and bone metabolism interaction of 1,25-$(OH)_2D_3$ with parathyroid hormone (PTH) is crucial. PTH takes care of the rapid (within minutes) response to changes in serum calcium levels, whereas longer term control of homeostasis is maintained by 1,25-$(OH)_2D_3$. PTH not only regulates calcium homeostasis indirectly via 1,25-$(OH)_2D_3$ (Section A.4.1) but also directly increases calcium reabsorption in the kidney.

When uptake of dietary calcium via the intestine does not suffice, 1,25-$(OH)_2D_3$ can stimulate the release of calcium from bone, which is the major store of calcium in the body. Calcium release from bone is also increased by PTH, by a stimulatory effect on the number, activity, and life span of osteoclasts, the bone resorbing cells (more details on bone are discussed below).

A third hormone, calcitonin, secreted by C-cells in the thyroid gland, is also involved in regulation of the serum calcium concentration. Calcitonin stimulates the 1,25-$(OH)_2D_3$ concentration and decreases 24-hydroxylase activity. In addition, the number and activity of osteoclasts is reduced by calcitonin [Finkelman 1985]. The expression of receptors for calcitonin in newly formed osteoclasts is regulated by 1,25-$(OH)_2D_3$ [Minkin 1991].

1,25-$(OH)_2D_3$ plays a major role in bone metabolism [Huffer 1988]. The bone forming cells, the osteoblasts, and the bone resorbing cells, the osteoclasts, are constantly remodelling bone [Nijweide 1986]. 1,25-$(OH)_2D_3$ is acting on osteoblasts via their VDRs and regulates their growth and differentiation. The synthesis of several bone matrix proteins by osteoblasts (e.g. osteocalcin, osteopontin, osteonectin, type I procollagen, alkaline phosphatase) is under control of 1,25-$(OH)_2D_3$. The role of

most of these proteins is not completely clear and ranges from cell matrix attachment to control of mineralization. The synthesis of certain cytokines and growth factors (e.g. transforming growth factor β (TGFß), insulin-like growth factor I) by osteoblasts is also regulated by 1,25-$(OH)_2D_3$ [Petkovich 1987, Scharla 1991]. Mature osteoclasts lack the VDR and are indirectly regulated by 1,25-$(OH)_2D_3$ via stimulated secretion of specific factors (osteoclast differentiation factor) by the osteoblasts [McSheehy 1987, Greenfield 1999]. 1,25-$(OH)_2D_3$ regulates differentiation of osteoclast progenitors into mature osteoclasts [Suda 1992]. Studies using cocultures of osteoblasts from VDR knockout mice and wild-type spleen cells (as a source of osteoclast progenitors) showed that 1,25-$(OH)_2D_3$-mediated osteoclast formation was abolished, indicating that the VDR in osteoblasts is essential for 1,25-$(OH)_2D_3$-stimulated osteoclast formation. The VDR was not required for osteoclast formation stimulated by PTH and interleukin 1α [Takeda 1999]. A key role of the VDR in regulating calcium and phosphate homeostasis by 1,25-$(OH)_2D_3$ was further demonstrated by findings of hypocalcaemia, hypophosphatemia, hyperparathyroidism, and severely impaired bone formation in the VDR knock out mice [Yoshizawa 1997].

Nonclassic function of 1,25-dihydroxyvitamin D_3

As shown in Table 1.1, the VDR is also expressed in cells and tissues not directly involved in calcium and bone metabolism – so-called nonclassic target tissues. Also in various malignant cell types the VDR is expressed. 1,25-$(OH)_2D_3$ exerts growth regulatory and differentiation inducing effects on these cells and tissues [Reichel 1989, Pols 1990, Walters 1992, Van den Bemd 2000a]. In the section below these nonclassic effects of 1,25-$(OH)_2D_3$ will be discussed briefly.

The growth of various normal cells (e.g. keratinocytes, epithelium and stromal cells) is reduced by 1,25-$(OH)_2D_3$ while their differentiation is stimulated [Smith 1986, Regnier 1991, Pechl 1994]. In addition, 1,25-$(OH)_2D_3$ regulates growth and differentiation of various malignant cell types. Abe *et al.* and Colston *et al.* demonstrated that 1,25-$(OH)_2D_3$ inhibited proliferation and stimulated differentiation of mouse myeloid leukemia cells and human melanoma cells *in vivo* [Abe 1981, Colston 1981]. Furthermore, 1,25-$(OH)_2D_3$ prolonged the survival time of mice inoculated with myeloid leukemia cells [Honma 1983], suppressed the *in vivo* growth of human melanoma, colon cancer and breast cancer solid tumor xenografts [Eisman 1987, Eisman 1989], and inhibited the number of lung metastases after implantation of lung carcinoma cells into mice [Sato 1982]. These cell growth inhibitory effects of 1,25-$(OH)_2D_3$ were also observed *in vitro* in hematopoietic tumor cells, primary breast cancers, and breast cancer cell lines [Olsson 1983, Koeffler 1984, Mangelsdorf 1984, Chouvet 1986, Eisman 1986, Vink-Van Wijngaarden 1994]. More detailed information on 1,25-$(OH)_2D_3$ effects on tumor cell growth and differentiation can be found elsewhere [Pols 1994, Van Leeuwen 1997, Van den Bemd 2000a].

The discovery that VDRs are expressed in monocytes and activated lymphocytes (Bhalla 1983) has led to the search for an immunomodulatory role of 1,25-$(OH)_2D_3$ (Lemire 1992, Hewison 1992). These studies revealed that 1,25-$(OH)_2D_3$ stimulates differentiation of monocytes into mature macrophages and inhibits the proliferation of T and B lymphocytes (Koeffler 1984). Monocytes and macrophages activate lymphocytes by secretion of cytokines; e.g. interleukin 1α, interleukin 1β, interleukin 6, and tumor necrosis factor α. Activated T cells produce interleukin 2, interferon γ, lymphotoxin, and granulocyte-macrophage colony-stimulating factor that in return further activate the macrophages. The growth and differentiation of T cells into T helper cells and cytotoxic T cells and of B cells into immunoglobulin producing plasma cells depends on the production of these cytokines and the expression of their receptors. 1,25-$(OH)_2D_3$ down-regulates the production of certain cytokines by monocytes/macrophages and lymphocytes (Bhalla 1984, Bhalla 1986), probably by shortening the half-life of their encoding mRNA (Tobler 1988, Müller 1992), but also by blocking the transcription of genes encoding for these cytokines (Alroy 1995, Towers 1999). Furthermore, 1,25-$(OH)_2D_3$ decreases the production of immunoglobulin by B lymfocytes *in vitro*, probably by an inhibitory effect on the T cell and monocytes/macrophage helper functions (Müller 1991). Also *in vivo*, 1,25-$(OH)_2D_3$ has immunoregulatory activity. In mice, 1,25-$(OH)_2D_3$ suppressed graft rejection of transplanted skin allografts (Chiocchia 1991) and blocked development of experimental autoimmune encephalomyelitis (Lemire 1991). The cell proliferation inhibitory property of 1,25-$(OH)_2D_3$ together with its immunomodulatory effects are of interest in the treatment of psoriasis, a skin disease characterized by abnormal growth and differentiation of epidermal cells and local inflammation with infiltration of neutrophils and T lymphocytes (Holick 1989). Also treatment of autoimmune diabetes with 1,25-$(OH)_2D_3$ was reported (Mathieu 1994).

A.4. VITAMIN D RECEPTOR: TRANSCRIPTIONAL ACTIVATION

Interaction of a nuclear hormone receptor with its ligand triggers a cascade of events resulting in transcriptional activation or suppression of specific target genes.

Events that modulate gene transcription:

- Synthesis, transport, and metabolism of receptor ligand
- Binding of ligand to the receptor
- Dissociation of heat shock proteins
- Phosphorylation of the receptor

- Dimerization of the receptor
- Translocation of the receptor to the nucleus
- Interaction with DNA
- Complex formation with basal transcription factors and cofactors

↓

Enhanced or decreased target gene transcription

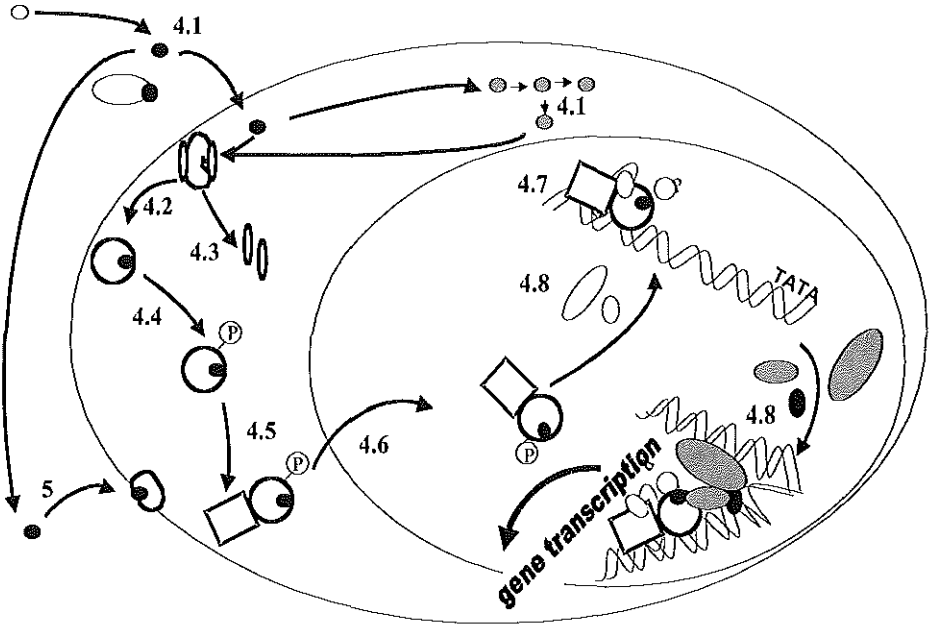

Figure 1.2. Schematic presentation of the cascade of events resulting in the regulation of gene transcription. *Major steps in the cascade are indicated by numbers that refer to the Sections A.4.1.-A.4.8. and A.5. for the VDR, and B.4.1-B.4.8. and B.5. for the ER.*

A.4.1. SYNTHESIS, TRANSPORT, AND METABOLISM OF 1,25-DIHYDROXYVITAMIN D_3

The hormone 1,25-$(OH)_2D_3$ is the active form of vitamin D_3 and the natural ligand for the VDR. Its inactive precursor vitamin D_3 is produced in the skin out of 7-dehydrocholesterol under influence of ultraviolet B photons (290-315 nm). Furthermore, dietary uptake (fatty fish, fish oil, vitamin D_3 fortified food) is a source of vitamin D_3. Subsequently, vitamin D_3 is first hydroxylated in the liver at the C-25 position to form 25-hydroxyvitamin D_3 (25-$(OH)D_3$) and secondly by renal 1α-hydroxylase at the C-1 position to form biologically active 1,25-$(OH)_2D_3$ (Figure 1.3).

PTH, but also other factors (calcitonin, estrogens; see Sections A.3. and B.3.) influence 1,25-$(OH)_2D_3$ synthesis in the kidney [Kawashima 1981]. The production of 1,25-$(OH)_2D_3$ and PTH is stimulated by low calcium levels in the blood, but 1,25-$(OH)_2D_3$ and PTH also directly regulate each others synthesis via a negative feedback loop. PTH stimulates 1α-hydroxylase in the kidney while 1,25-$(OH)_2D_3$ has a direct inhibitory effect on the production and secretion of PTH by the parathyroid glands [Garabedian 1972, Fraser 1973, DeMay 1992a]. In addition, 1,25-$(OH)_2D_3$ down-regulates 1α-hydroxylase activity [Reichel 1989].

1,25-$(OH)_2D_3$ and 25-$(OH)D_3$ are transported in the blood bound to vitamin D binding protein (DBP), that also functions as a storage for vitamin D compounds [Haddad 1987]. In plasma, only 0.4% of 1,25-$(OH)_2D_3$ and 0.04% of 25-$(OH)D_3$ is present in an unbound form. The largest fraction (>85%) is bound to DBP. The rest is bound to albumin (>10%) and low density lipoproteins [Haddad 1999]. Also other vitamin D_3 metabolites are bound by DBP. DBP has the highest binding affinity for 25$(OH)D_3$ (Kd 10^{-9} M), followed by 24,25-$(OH)_2D_3$ and 25,26-$(OH)_2D_3$ (Kd 10^{-8} M) and vitamin D_3 and 1,25-$(OH)_2D_3$ (Kd 10^{-7} M). Upon dissociation from DBP the hormones are available for entry and biological action in target cells. In this way DBP determines bioavailability and activity of vitamin D compounds, since only free vitamin D compounds are considered to be biologically active [Bikle 1989].

It is not likely that DBP plays a role in protecting the organism against toxic levels of vitamin D compounds since it was shown that the DBP knock out mouse was less sensitive for vitamin toxicity than the wild type mouse [Safadi 1999]. On the other hand, vitamin D depletion resulted in hyperparathyroidism and bone abnormalities associated with vitamin D deficiency in the DBP knock out mouse, whereas wild type mice were not affected. This indicates that the major role of DBP is protection of the organism against vitamin D deficiency by providing a pool of vitamin D compounds with prolonged serum half-lifes.

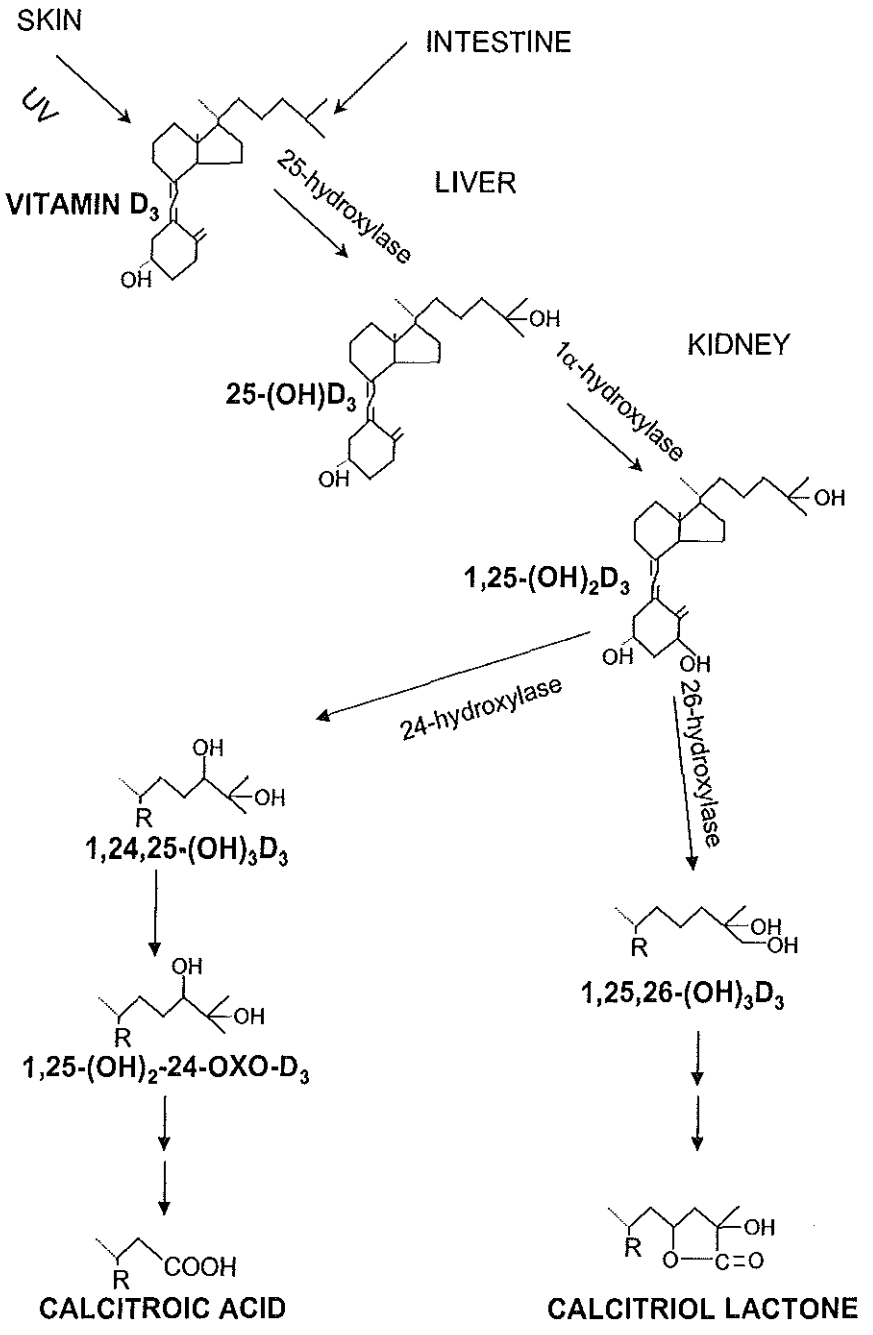

Figure 1.3. Formation and degradation of $1,25\text{-}(OH)_2D_3$

In order to protect the organism against toxic levels of vitamin D_3 (and its metabolites) further hydroxylation takes place, eventually leading to formation of inactive products that can be excreted from the body. The first step is hydroxylation at the C-24 position. Both 25-(OH)D_3 as well as 1,25-$(OH)_2D_3$ become 24-hydroxylated by the same enzyme (CYP24), although 1,25-$(OH)_2D_3$ is 10 times more efficiently hydroxylated than 25-(OH)D_3 [Jones 1999]. CYP24 is expressed at high levels in the kidney, but also in most other target tissues (e.g. bone, intestine, skin) 24-hydroxylase activity has been found [Jones 1987]. 1,25-$(OH)_2D_3$ is an inducer of its own 24-hydroxylation and PTH acts synergistically on this process in osteoblast-like cells [Van Leeuwen 1996, Ambrecht 1998]. In addition, hydroxylation at C-26 plays an important role in the metabolism of vitamin D_3 metabolites. The reason for the existence of two metabolic pathways is unclear. The 26-hydroxylation pathway might be a backup pathway for the 24-hydroxylase pathway [Jones 1999]. It is also not clear whether metabolites formed via 24- or 26-hydroxylation are active or just intermediate products in the catabolic cascade of 25-(OH)D_3 and 1,25-$(OH)_2D_3$. Fact is that additional hydroxylation at C-24 or C-26 leads to a decrease in the affinity for the VDR [Van Ham 1988], but it was also found that 24,25-$(OH)_2D_3$ might play a role in mineralization of bone [Ornoy 1978, Kanis 1978, Sebert 1982] and in regulation of intestinal calbindin D-9k production [Hemmingsen 1996]. Furthermore, 24,25-$(OH)_2D_3$ exerts nongenomic regulatory effects on protein kinase C activity [Sylvia 1996]. Recently, the possible role of 24,25-$(OH)_2D_3$ in bone metabolism was reviewed by Van Leeuwen *et al.* [Van Leeuwen 2000]. The 1,25-$(OH)_2D_3$ metabolite 1,24,25-$(OH)_3D_3$ has stimulatory effects on intestinal calcium absorption [Wang 1993]. 25,26-$(OH)_2D_3$ has intestinal calcium transport activity [DeLuca 1970], and the end product of the C-26 hydroxylation pathway, 1α,25R$(OH)_2$-26,23S-lactone-D_3 probably has specific effects on bone cells and cartilage [Ishizuka 1984, Stern 1984, Arai 1997, Ishizuka 1997].

Recently, a third metabolic pathway was identified in human keratinocytes, human colon carcinoma cells, rat osteoblastic cells and bovine parathyroid cells that metabolizes 1,25-$(OH)_2D_3$ into 1,25-$(OH)_2$-3-epi-D_3 [Reddy 1994, Bischof 1998, Brown 1999, Siu-Caldera 1999]. Interestingly, the activity of the different metabolic pathways might depend on the stage of cell differentiation [Bischof 1998].

A.4.2. VITAMIN D RECEPTOR LIGAND BINDING

A key event in receptor activation is the ligand-induced change in receptor conformation [Allan 1992] which has been described for several members of the nuclear receptor superfamily [Simons 1989, Leng 1993, Beekman 1993, Toney 1993, Keidel 1994, Kuil 1994, Nayeri 1995, Berger 1999]. The ligand-binding domain of nuclear hormone receptors consists of 12 α helixes. Upon ligand binding the position of the AF-2-containing helix 12 changes in such a way that it covers the ligand-binding pocket, closing the so-called mouse trap.

The closed ligand-binding pocket exposes sites for interaction with specific cofactors, and triggers the cascade of events leading to transcriptional activation.

Possibly directly linked to the conformation of the receptor is its stability: the folding of the protein will determine its susceptibility to proteases in the cell and this will influence intracellular clearance of the receptor. In general, binding of ligand to the receptor will lead to a receptor conformation with decreased protease sensitivity. This will result in an increased stabilization of the receptor in comparison to the unliganded state [Kemppainen 1992, Wiese 1992, Arbour 1993]. In Chapter 3, 1,25-$(OH)_2D_3$- and 1,25-$(OH)_2D_3$ analog-induced VDR stability will be discussed in more detail. However, ligand interaction does not always lead to increased receptor stability. Several steroids and antihormones bind to the androgen receptor and cause nuclear translocation but fail to induce increased receptor stability [Kemppainen 1992], and in the human breast tumor cell line T-47D ligand binding to the VDR results in decreased receptor half-life compared to the unliganded situation [Sher 1985]. Another example of ligand-induced decrease in receptor stability will be discussed in Chapter 6.

Another mechanism by which the biological activity of nuclear hormones is mediated is by regulation of their receptor levels in target tissues. For several nuclear hormones a direct correlation between their receptor level and some biological responses was observed [Vanderbilt 1987]. (For ER see Section B.4.2.). 1,25-$(OH)_2D_3$ is able to up-regulate its own receptor level, both *in vivo* [Costa 1986, Merke 1989, Strom 1989, Sandgren 1990, Li 1999], and *in vitro* [Costa 1985, Pols 1988a, Toaka 1993, Van Leeuwen 1996, Solvsten 1997, Li 1999]. This homologous receptor up-regulation has been reported to result from an increased transcription rate of the VDR gene [Solvsten 1997], an increased mRNA stability and/or an increased receptor half-life [Wiese 1992, Arbour 1993, Santiso-Mere 1993, Davoodi 1995]. *In vivo* and *in vitro* studies with human skin and cultured human keratinocytes suggested that 1,25-$(OH)_2D_3$ has no effect on VDR gene transcription but increases VDR levels by blocking ubiquitin/proteasome-mediated degradation of the VDR [Li 1999].

Also heterologous regulation of VDR by a wide variety of factors has been shown, for instance PTH [Pols 1988, Van Leeuwen 1992, Klaus 1994, Krishnan 1995, Van Leeuwen 1996, Armbrecht 1998], PTH-related peptide [Pols 1988, Van Leeuwen 1992], growth hormone [Chen 1997], glucocorticoids [Chen 1986, Davoodi 1995], epidermal growth factor [Van Leeuwen 1991], TGFβ [Staal 1994], phorbol esters [Reinhardt 1994], forskolin [Van Leeuwen 1992], prostaglandin E_2 [Van Leeuwen 1992], retinoic acid [Chen 1985, Davoodi 1995], ER ligands [Liel 1992, Escaleira 1993, Ishibe 1995, Chen 1997, Liel 1999], androgen receptor ligands [Escaleira 1993], progesterone receptor ligands [Davoodi 1995], and phosphorus [Sriussadaporn 1995].

The biological relevance of this change in VDR number needs to be established, since the number of 1,25-$(OH)_2D_3$-responses that parallel the VDR content in the target cells is only limited. *In vivo* studies with ovariectomized rats showed that estrogen- and growth hormone-induced rise in intestinal VDR levels was accompanied by an increase in intestinal calcium absorption, transcription of the calbindin 9k gene, and alkaline phosphatase activity in response to 1,25-$(OH)_2D_3$

(Chen 1997, Liel 1999). In addition, the increase of VDR levels in intestine and bone might explain hypercalciuria, increased intestinal calcium absorption and enhanced bone resorption as observed in idiopathic hypercalciuric rats (Li 1993a, Krieger 1996). The observation in rats that homologous up-regulation of the VDR is observed in kidney and intestine but not in tissues not directly involved in regulating plasma calcium homeostasis (testis, heart, lung) is speculative for a functional role of homologous VDR up-regulation (Gensure 1998). Also *in vitro* studies a relationship between VDR levels and biological response was found. For instance, correlation exists between VDR levels and the extent of inhibition of collagen synthesis by 1,25-$(OH)_2D_3$ in osteoblast-like cells from mice and rats (Chen 1986, Chen 1986a). Also in osteoblasts a direct relationship between 1,25-$(OH)_2D_3$-, TGFβ-, epidermal growth factor-, and PTH-induced changes in VDR level and 24-hydroxylase activity was found (Chen 1985, Chen 1986, Krishnan 1992, Armbrecht 1998, Staal 1997). Enhancement by 1,25-$(OH)_2D_3$ of glucocorticoid-induced expression of aromatase cytochrome P_{450} in primary cultured human osteoblasts was found to depend on their VDR level (Tanaka 1996). The antiproliferative activity of 1,25-$(OH)_2D_3$ was associated with VDR expression in skin lesions of patients with psoriasis (Chen 1996), and the sensitivity of three osteoblast-like osteosarcoma cell lines for the growth regulating potency of 1,25-$(OH)_2D_3$ was correlated with the number of VDRs in each cell line (Dokoh 1984). However, this is not a general rule, since no relation was found between VDR content of pancreatic cancer cells or prostate tumor cells and the antiproliferative effect of 1,25-$(OH)_2D_3$ (Kawa 1996, Zhuang 1997). A dissociation between VDR levels and 1,25-$(OH)_2D_3$ action was also observed by Staal *et al.* showing that the TGFβ-induced rise in VDR content of osteoblast-like cells *in vitro* was accompanied by decreased induction of osteocalcin and osteopontin production by 1,25-$(OH)_2D_3$ (Staal 1994).

As already mentioned, 1,25-$(OH)_2D_3$-induced VDR up-regulation might be due to inhibition of proteasome-mediated VDR degradation (Li 1999). However, the proteasome is probably also involved in 1,25-$(OH)_2D_3$-induced down-regulation of the VDR. It was shown that 1,25-$(OH)_2D_3$ dose-dependently stimulated interaction of VDR with cofactor SUG1, a component of the 26S proteasome, and that 1,25-$(OH)_2D_3$-induced binding of SUG1 to VDR enhanced VDR degradation by proteasomes. A similar process might also play a role in E_2-mediated turnover of the ER (Masuyama 1998).

A.4.3. DISSOCIATION OF HEAT SHOCK PROTEINS

In the cytoplasm, some of the members of the nuclear receptor superfamily (androgen receptors, ERs, glucocorticoid receptors, mineralocorticoid receptors, progesterone receptors and peroxisome proliferator-activated receptors) are associated with heat shock proteins (Huang 1994). The heat shock proteins might have a regulatory role in the action of the receptors, and they might be involved in

protection of the receptor during synthesis and transport to the nucleus. Until recently, it was thought that the VDR was not associated with heat shock proteins. However, Swamy *et al.* and Craig *et al.* showed that specific residues in the hinge region and ligand-binding domain of the VDR might form interaction sites of heat shock proteins 70 and DnaK and that an inhibitor of heat shock protein 90 reduced 1,25-$(OH)_2D_3$-mediated gene activation in osteoblasts [Swamy 1999, Craig 1999]. Upon ligand binding, heat shock proteins dissociate from the receptor, allowing the receptors to bind to specific sites in DNA and interact with cofactors, where they can transactivate target genes. However, removal of heat shock proteins alone is not sufficient to obtain transcriptionally active receptors [O'Malley 1990, Picard 1990, Bagchi 1991].

A.4.4. VITAMIN D RECEPTOR PHOSPHORYLATION

An additional step in the cascade leading to receptor activation is phosphorylation of the receptor [Auricchio 1989, Orti 1992]. Functional consequences of nuclear hormone receptor phosphorylation are not completely clear. Hormone binding activity, translocation, transcription factor binding and interaction with DNA might be influenced by phosphorylation. In addition, dimerization and receptor half-life might also be affected [Kuiper 1994]. The VDR is a phosphoprotein that is further phosphorylated under influence of 1,25-$(OH)_2D_3$. The VDR can be phosphorylated at multiple sites by several kinases (Figure 1.1) [Brown 1991]. For instance, human VDR is selectively phosphorylated by protein kinase C at serine 51 between the two zinc-binding amino acid clusters crucial to its transactivation function and by casein kinase II at serine 208 near the ligand-binding domain and probably involved in positive modulation of transactivation [Hsieh 1991, Jurutka 1993a, Jurutka 1996]. A cAMP-dependent protein kinase also plays a role in VDR phosphorylation and enhances 1,25-$(OH)_2D_3$-dependent transcriptional activation [Darwish 1993, Jurutka 1993].

A.4.5. VITAMIN D RECEPTOR DIMERIZATION

The 'classic' nuclear hormone receptors (ERs, glucocorticoid receptors, progesterone receptors) form homodimers and in this form they regulate gene transcription. In contrast, the VDR and several other members of the nuclear receptor superfamily (e.g retinoic acid receptors, thyroid hormone receptors, peroxisome proliferator-activated receptors) predominantly operate as heterodimers by binding to RXR [Kliewer 1992, Rosen 1993, Schulman 1998], although VDR homodimers were also described [Carlberg 1993, Freedman 1994, Kahlen 1994, Polly 1996]. The biological relevance of these VDR homodimers is uncertain [MacDonald 1995, Lemon 1996, Thompson 1998a]. Some investigators claim existence of heterodimers of VDR and retinoic acid receptor [Schräder 1993] or VDR and thyroid hormone receptor [Schräder 1994], although this was questioned recently [Raval-Pandya 1998, Thompson 1998].

1,25-$(OH)_2D_3$ is a strong enhancer of VDR/RXR dimerization [MacDonald 1995, Zhao 1997], whereas the ligand of RXR, 9-*cis* retinoic acid, negatively modulates this action of 1,25-$(OH)_2D_3$: it suppresses heterodimerization of VDR and RXR and stimulates the formation of RXR homodimers [MacDonald 1993, Cheskis 1994, Cheskis 1995, Thompson 1998a]. Another RXR-specific ligand (CD2809) also has an inhibitory effect on 1,25-$(OH)_2D_3$ action. This RXR ligand decreases the 1,25-$(OH)_2D_3$-induced increase of VDR levels in human keratinocytes and inhibits *in vitro* VDR/RXR binding [Jensen 1998]. Furthermore, *in vivo* 9-*cis* retinoic acid and the RXR-specific ligand LG153 attenuate the transcriptional induction by 1,25-$(OH)_2D_3$ [MacDonald 1993, Lemon 1996]. In contrast, others have reported no effect [Ferrara 1994, Zhao 1997] or a synergistic effect of 9-*cis* retinoic acid on 1,25-$(OH)_2D_3$ action [Carlberg 1993, Sasaki 1995].

A.4.6. VITAMIN D RECEPTOR TRANSLOCATION

It was shown that in cells cultured in the absence of ligand, the VDR resides mainly in the cytoplasm. Upon exposure to 1,25-$(OH)_2D_3$, a rapid (within a few minutes) translocation of VDRs to the nucleus takes place [Barsony 1990]. These observations were later confirmed in living cells using 1,25-$(OH)_2D_3$ attached to a fluorescent dye [Barsony 1997]. In addition, trafficking of VDR both into and out of the nucleus - a process called nucleocytoplasmic shuttling - is observed. The role of nucleocytoplasmic shuttling is not known, but it might be important for receptor modification and certain biological activities of the nuclear hormone receptor in the cytoplasm. Trafficking across the nuclear envelope is not a specifc VDR feature, since the same phenomenon can be observed for the ER, progesterone receptor, and glucocorticoid receptor [Guiochon-Mantel 1996].

A.4.7. VITAMIN D RECEPTOR-DNA BINDING

For the VDR and several other nuclear hormone receptors (e.g. progesterone receptors, glucocorticoid receptors) ligand binding is a prerequisite for *in vivo* DNA binding [Groyer 1987, Meyer 1990]. The dimerized receptor interacts with DNA at specific sites, the so-called hormone response element in the promoter region of a target gene. In general, the hormone response element consists of two palindromic, inverted palindromic or direct repeat hexameric nucleotides spaced by 0 through 5 base pairs. The hormone response elements of the receptors for androgen, progesterone, glucocorticoid, and mineralocorticoid consists of the consensus sequence TGTTCT, whereas the consensus binding motif for VDR, ER, retinoic acid receptor and thyroid hormone receptor is AGGTCA. Although the receptors show substantial resemblance in structure and bind to related DNA structures, there is a complex mechanism to specify gene activation and to diminish the risk

of false activation of gene transcription. The spacing between half-sites as well as minor differences in nucleotide sequence of half-sites and flanking sites play a crucial role in achieving this selective hormonal response (Umesono 1991, Towers 1993, Mader 1993, Glass 1994). The importance of nucleotide half-site sequences in determining the binding efficiency of the VDR will be further examined and discussed in Chapter 4.

For several VDR target genes a functional vitamin D response element (VDRE) has been defined (Table 1.2). However, for numerous other target genes, specific VDR binding sites have not yet been identified (Hannah 1994). VDREs generally consist of two imperfect direct repeat hexameric nucleotides spaced by 3 base pairs (DR3 type VDRE) and although the consensus sequence AGGTCA is still recognizable considerable heterogeneity in half-site sequences exists (Table 1.2). In most cases, the proximal (3'-) half-site is the binding site for the VDR, whereas the distal (5'-) half-site is the binding site for its dimer partner RXR (Lemon 1996, Jin 1996, Staal 1996). However, for the chicken carbonic anhydrase II VDRE the prefered polarity of binding of VDR and RXR for the proximal and distal half-sites seems reversed (Quélo 1994). Besides DR3-type VDREs, DR4-type VDREs and inverted palindromic motifs spaced by 9 nucleotides (IP9) have been characterized as functional VDREs. Furthermore, VDR binding to direct repeats with a spacing of 6 nucleotides, a palindrome without spacing and an inverted palindrome with a 12-nucleotide spacing has been reported (Carlberg 1993).

Most of the VDREs listed in Table 1.2 are positive regulatory sites: binding of a 1,25-$(OH)_2D_3$-VDR/RXR complex to such a VDRE will lead to increased transcription of the corresponding gene. However, also negative regulatory sites have been characterized: binding of the VDR complex to e.g. the human and avian PTH VDRE, mouse osteocalcin VDRE, and rat bone sialoprotein VDRE leads to suppression of gene transcription (DeMay 1992a, Li 1993, Liu SM 1996, Lian 1997). How the VDR negatively modulates gene transcription is still unclear and probably not similar for all 1,25-$(OH)_2D_3$ suppressed genes. The rat bone sialoprotein gene for instance, contains a VDRE that overlaps a general transcription regulation site (TATA box). As a consequence, VDR/RXR binding to this VDRE will prevent the TATA box binding protein from binding and thereby blocks gene transcription (Li 1993, Kim 1996).

Another possible mechanism is that the VDRE sequence of the suppressed gene is in such an orientation that the VDR occupies the distal 5' half-site instead of the proximal 3' half-site as seen with VDREs of positively modulated target genes, leading to inactive gene transcription. The cellular context (e.g. presence or absence of certain cofactors) might also interfere in the repression of gene transcription. The human PTH gene mediated transcriptional repression in response to 1,25-$(OH)_2D_3$ in pituitary GH4CI cells, whereas no transcriptional repression was observed when the human PTH gene was transfected into osteoblast-like ROS 17/2.8 cells (Demay 1992a).

Table 1.2.

1,25-Dihydroxyvitamin D_3-regulated genes and nucleotide sequences of vitamin D response elements identified in these genes

GENE	NUCLEOTIDE SEQUENCE	(Ref)
Positive VDREs:		
DR3 elements		
Chicken $ß_3$ integrin	5'-GAGGCA gaa GGGAGA-3'	(Cao 1993)
Chicken carbonic anhydrase II	5'-GGGGGA aaa AGTCCA-3'	(Quélo 1994)
Human 24-hydroxylase (proximal)	5'-AGGTGA gcg AGGGCG-3'	(Chen 1995)
Human 24-hydroxylase (distal)	5'-GAGTTC acc GGGTGT-3'	(Chen 1995)
Human 5'-lipoxygenase	5'-AGGGCA aag GGTGGA-3'	(Carlberg 1996)
Human Na^+/phosphate co-transporter	5'-GGGGCA gca AGGGCA-3'	(Taketani 1997)
Human osteocalcin	5'-GGGTGA acg GGGGCA-3'	(Morrison 1989)
Human cdk inhibitor $p21^{WAF1}$	5'-AGGGAG att GGTTCA-3'	(Liu M 1996)
Human placental lactogen	5'-AGCTGA ctc AGGTGG-3'	(Stephanou 1994)
Human TGFβ2 (proximal)	5'-AATGAA gtt GGTGGA-3'	(Wu 1999)
Human TGFβ2 (distal)	5'-TGTAGA aca AGTAGA-3'	(Wu 1999)
Mouse osteopontin	5'-GGTTCA cga GGTTCA-3'	(Noda 1990)
Pig osteopontin	5'-GGGTCA tat GGTTCA-3'	(Zhang 1992)
Rat atrial natriuretic factor	5'-AGGTCA tga AGGACA-3'	(Kahlen 1996)
Rat calbindin D-9k	5'-GGGTGT cgg AAGCCC-3'	(Darwish 1992)
Rat 24-hydroxylase (proximal)	5'-AGGTGA gtg AGGGCG-3'	(Ohyama 1994)
Rat 24-hydroxylase (distal)	5'-GGTTCA gcg GGTGCG-3'	(Jurutka 1994)
Rat osteocalcin	5'-GGGTGA atg AGGACA-3'	(DeMay 1992)
Rat osteopontin (proximal)	5'-AGGTCA cac AGGGCA-3'	(Ridall 1995)
Rat osteopontin (distal)	5'-AGGTCA tat GGTTCA-3'	(Ridall 1995)
DR4 elements		
Mouse calbindin D-28k	5'-GGGGGA tgtg AGGAGA-3'	(Gill 1993)
Mouse Pit-1	5'-AGTTCA tgag AGTTCA-3'	(Rhodes 1993)
DR6 elements		
Human osteocalcin	5'-TGGTGA ctcacc GGGTGA-3'	(Kerner 1989)
Human fibronectin	5'-GGGTGA cgtcac GGGGGA-3'	(Polly 1996)
Human phospholipase C-γ1	5'-AGGTCA gaccac TGGACA-3'	(Xie 1997)
Mouse fibronectin	5'-GGGTGA cgtcac GGGGTA-3'	(Polly 1996)
Rat 24-hydroxylase	5'-GGTCGA gcccag GGTTCA-3'	(Kahlen 1994)
IP9 elements		
Human calbindin D-9k	5'-TGCCCT tccttatgg GGTTCA-3'	(Schräder 1995)
Mouse c-fos	5'-TGACCC tgggaaccg GGTCCA-3'	(Schräder 1997)
Rat osteocalcin	5'-TGCACT gggtgaatg AGGACA-3'	(Schräder 1995)
Negative VDREs:		
Chicken PTH	5'-GGGTCA gga GGGTGT-3'	(Liu SM 1996)
Chicken protein kinase A inhibitor	5'-ATGTTG ctg AGGTCA-3'	(Rowland-Goldsmith 1997)
Human interferon-γ	5'-TGGGCA taa TGGGTC-3'	(Cippitelli 1998)
Human PTH	5'-GGTTCA aag CAGACA-3'	(Demay 1992a)
Mouse osteocalcin	5'-GGGCAA atg AGGACA-3'	(Lian 1997)
Rat bone sialoprotein	5'-AGGGTT tat AGGTCA-3'	(Kim 1996)
Rat PTH related peptide (proximal)	5'-AGGTTA ctc AGTGAA-3'	(Falzon 1996)
Rat PTH related peptide (distal)	5'-GGGTGG aga GGGGTG-3'	(Kremer 1996)

The nucleotide sequence of the VDRE is also of major importance in regulating gene transcription. The nucleotide sequence of the VDREs clearly influences the conformation of the VDR/RXR complex [Staal 1996]. Obviously, this conformational change can influence subsequent steps (binding of transcription factors and cofactors) leading to gene transcription. The importance of nucleotide sequences was also illustrated by the work of Koszewski *et al.* demonstrating that only minor differences in nucleotide sequence within half-sites determined the direction of transcriptional modulation. They showed that introduction of selective mutations of only two nucleotides within the proximal half-site of the avian PTH VDRE changed the negatively modulated gene into a positively modulated gene [Koszewski 1999].

Besides hormone response elements, other DNA elements like the activating protein-1 (AP-1) element might play a role in nuclear hormone receptor-mediated gene transcription. The AP-1 element is the DNA binding site for the proto-oncogene protein products fos and jun [Rauscher 1988]. Fos and jun are transcription factors that play an important role in promoting cell growth. There is evidence that nuclear hormone receptors interact with the AP-1 element or its binding proteins. For the ER [Paech 1997], glucocorticoid receptor [Uht 1997], retinoic acid receptor [Pfahl 1993], and also for the VDR interaction with the fos/jun pathway has been described. In the human osteocalcin gene, the distal region of the VDRE is closely juxtaposed to an AP-1 binding site [Ozono 1990] and the rat osteocalcin VDRE contains an internal AP-1 site [Owen 1990]. In the VDRE/AP-1 domain both cell differentiation and cell proliferation regulatory elements are integrated: On the one hand, binding of fos and jun to the AP-1 element might inhibit binding of VDR/RXR to the VDRE and consequently blocks transcription of the VDR responsive gene, on the other hand interaction of VDR with fos and jun at an AP-1 site might suppress proto-oncogene activities like stimulation of cell growth. Freedman and co-workers showed that interaction of VDR with fos/jun also has an impact on immune responses. They showed that VDR suppressed formation of a complex between AP-1 binding elements fos and jun and T cell specific transcription factor NFATp and suggested that this blockade might underly the immunosuppressive activity of $1,25\text{-}(OH)_2D_3$ [Alroy 1995, Towers 1999]. In addition, synergism between fos, jun, and the VDR has been described. It was found that the VDR and AP-1 binding factors synergistically induced transcription of a reporter construct containing an osteopontin VDRE and an AP-1 binding site. Also transcriptional synergism was observed for VDR and the transcription factors SP1, nuclear factor-1, and octamer transcription factor-1 [Liu 1994].

A.4.8. VITAMIN D RECEPTOR-COFACTOR BINDING

Binding of a receptor to a hormone response element results in a specific conformational change of the receptor [El-Ashry 1989, Toney 1993, Ikeda 1996, Staal 1996, Wood 1998] and

in bending of DNA [Nardulli 1993, Nardulli 1993a, Nardulli 1995, Scott 1995, Kimmel-Jehan 1996, Robinson 1998, Kimmel-Jehan 1999], providing a structure suitable for gene transcription regulation. After binding of the receptor dimer to a hormone response element other transcription factors (TFs) can bind and interaction with the basic TATA transcriptional machinery will occur. Transcriptional activation requires assembly of TFIID, TFIIA, TFIIB, TFIIF, TFIIE, TFIIH, and RNA polymerase II that form the preinitiation complex. A nuclear hormone receptor dimer bound to a hormone response element can interfere in formation and/or stability of the preinitiation complex and thereby interferes in regulation of gene transcription [Klein-Hitpass 1998]. As already described, both stimulation and repression of gene transcription can be the result.

The ligand-binding domain of the VDR interacts with the general transcription factor TFIIB and this interaction leads to cooperative activated transcription after stimulation with 1,25-$(OH)_2D_3$ [MacDonald 1995, Blanco 1995]. In addition to basic transcription factors, other cofactors (coactivators and corepressors) interfere in the transactivation process [Jenster 1998]. For example, steroid receptor coactivator-1 (SRC-1) and glucocorticoid receptor interacting protein 1 (GRIP1) can bind to the VDR and other nuclear hormone receptors (thyroid hormone receptor, retinoic acid receptor, RXR, mineralocorticoid receptor, glucocorticoid receptor, ER) and thereby enhances transcriptional activation in a receptor ligand-dependent manner [Hong 1997, Masuyama 1997]. Other cofactors (transcription associated factor (II) 135, VDR interacting proteins (DRIPs), RAC-3, suppressor of gal1 (SUG1), receptor interacting protein 140 (RIP140)) interact with the VDR at the AF-2 domain [Vom Bauer 1996, Masuyama 1997, Mengus 1997, Rachez 1998, Rachez 1999]. The AF-2 domain is crucial for transcriptional activation by cofactors. Specific mutations in the AF-2 domain did not affect ligand binding, heterodimerization with RXR, and VDRE binding, but gene transcription and interaction with cofactors (SUG1, SRC-1 and RIP140) were abolished [Masuyama 1997, Jurutka 1997, Gill 1998]. However, how cofactors exactly interfere in gene transcriptional regulation remains to be elucidated. Some of these cofactors might be involved in chromatin remodelling. DNA is stored in the nucleus as nucleosomes: DNA wrapped around a complex of the histones H2A, H2B, H3, and H4. The amino acid tail domain of the histones sticks out of the nucleosomes and can interact with cofactors that possess histone acetyltransferase (HAT) activity causing acetylation of lysine-rich domains of the histones. As a result DNA relaxes and the nucleosomes get less tight. In this way, the receptor complex gains access to transcription regulatory sites in the DNA [Grunstein 1997]. DRIPs and SRC-1 possess HAT activity [Spencer 1997, Rachez 1998] and interaction of these factors with the AF-2 domain of the VDR results in increased transcriptional activation activity of the VDR [Gill 1998, Rachez 1998].

A.5. NONGENOMIC EFFECTS OF 1,25-DIHYDROXYVITAMIN D_3

1,25-$(OH)_2D_3$ also exerts effects that seem to be not mediated via an interaction of the VDR with the genome [Cancela 1988, Baran 1994]. These nongenomic processes include: the rapid changes in intracellular calcium [Lieberherr 1987, Caffrey 1989, Civitelli 1990, Baran 1991, Farach-Carson 1991, Nemere 1991] and the rapid stimulation of Ca^{2+} transport in the intestine (transcaltachia) [De Boland 1990]. Furthermore, 1,25-$(OH)_2D_3$ can rapidly stimulate phosphoinositide metabolism [Lieberherr 1989, Civitelli 1990], leading to activation of protein kinase C [Wali 1990]; an important regulator of cell proliferation and differentiation [Farago 1990]. Recently, in chondrocytes a membrane-associated VDR has been identified that might play a role in the activation of protein kinase C by 1,25-$(OH)_2D_3$ [Nemere 1998]. In addition, also for 24,25-$(OH)_2D_3$ a specific membrane receptor seems to exist [Pedrozo 1999]. Although discovery of the membrane receptors is challenging their importance in other nongenomic effects of vitamin D metabolites and the way these membrane receptors might interact with the nuclear VDR-mediated pathway remains to be established [Fleet 1999].

A.6. 1,25-DIHYDROXYVITAMIN D_3 ANALOGS

As discussed in Section A.3., besides its classic action on bone, intestine, kidney, and parathyroids, 1,25-$(OH)_2D_3$ exerts effects on cells and tissues not directly related to bone and mineral homeostasis. These growth and differentiation regulatory effects are potentially of clinical interest. However, high doses of 1,25-$(OH)_2D_3$ necessary to achieve these effects may result in hypercalcemia and hypercalciuria [Koeffler 1985, Vieth 1990]. This has stimulated the search for synthetic 1,25-$(OH)_2D_3$ analogs with selective biological activity. The main goal was to develop analogs with an activity profile in favor of the effects on tumor cell growth, differentiation, and/or immunosuppression compared to the calcemic effects [Bikle 1992, Bindenip 1992, Pols 1994, Bouillon 1995, Van Leeuwen 1997, Van den Bemd 2000a]. In addition, analogs might also be useful in the treatment of metabolic bone diseases (e.g. due to an increased anabolic effect on bone or the potency to lower PTH levels without affecting the serum calcium concentration) [Nishii 1993].

A.6.1. BIOLOGICAL EFFECTS AND CLINICAL APPLICATIONS

In the past 15 years numerous 1,25-$(OH)_2D_3$ analogs have been developed. Most changes to the 1,25-$(OH)_2D_3$ structure (80% of the 820 modifications tabulated in the review by Bouillon *et al.*) are side chain modifications. Around 10% are A ring

modifications, 2% are B ring modifications and 10% are C/D ring modifications (Bouillon 1995). In general, 1,25-$(OH)_2D_3$ is still recognizable in the analog structure. However, recently it was shown that also nonsecosteroidal compounds (bis-phenyl derivatives and analogs lacking C and D rings) exert VDR modulating activities and mimic 1,25-$(OH)_2D_3$ effects on cell growth and differentiation (Verstuyf 1998, Boehm 1999). Although these compounds may be promising as therapeutics for cancer and psoriasis, this thesis will focus on VDR ligands with a chemical structure based on the structure of 1,25-$(OH)_2D_3$, the side chain modified-analogs in particular.

The changes in the 1,25-$(OH)_2D_3$ side chain structure include:

(See Figure 1.4 for chemical structures)

- Shortening of the side chain

  e.g. 26,27-dinor-1,25-$(OH)_2D_3$

- Elongation of the side chain

  e.g. 20-epi-22-oxa-24a,26a,27a-tri-homo-1,25-dihydroxyvitamin D_3 (KH1060),

  24a,26a,27a-tri-homo-1,25-dihydroxyvitamin D_3 (CB966)

- Substitution of a carbon atom with an oxygen atom

  e.g. 22-oxo-1,25-$(OH)_2D_3$ (OCT),

  20-epi-22-oxa-24a,25a-di-homo-1,25-dihydroxyvitamin D_3 (KH1049), KH1060

- Substitution of a hydrogen atom with a fluorine

  e.g. 1,25-$(OH)_2$-26,26,26,27,27,27-hexafluoro-D_3 (1,25-$(OH)_2$-F_6-D_3),

  1,25-$(OH)_2$-16-ene-23-yne-26,26,26,27,27,27-hexafluoro-D_3 (RO 24-5531)

- Addition of hydroxyl groups at carbon atom 22, 23, 24 or 26

  e.g. 1,23,25-trihydroxyvitamin D_3 (1,23,25-$(OH)_3D_3$)

- Moving the 25 hydroxyl group to another position in the side chain

  e.g. 1,24S-dihydroxy-22-ene-25,26,27-cyclopropylvitamin D_3 (MC903)

- Introduction of double or triple bonds between carbon atoms

  e.g. 1,25-$(OH)_2$-16-ene-23-yne-D_3 (RO 23-7553), RO 24-5531,

  24a,26a,27a,-tri-homo-22,24-diene-1,25$(OH)_2D_3$ (EB1089)

- Altered stereochemistry (epimerization) at carbon atom 20

  e.g. 20-epi-1,25-$(OH)_2D_3$ (MC1288), KH1060

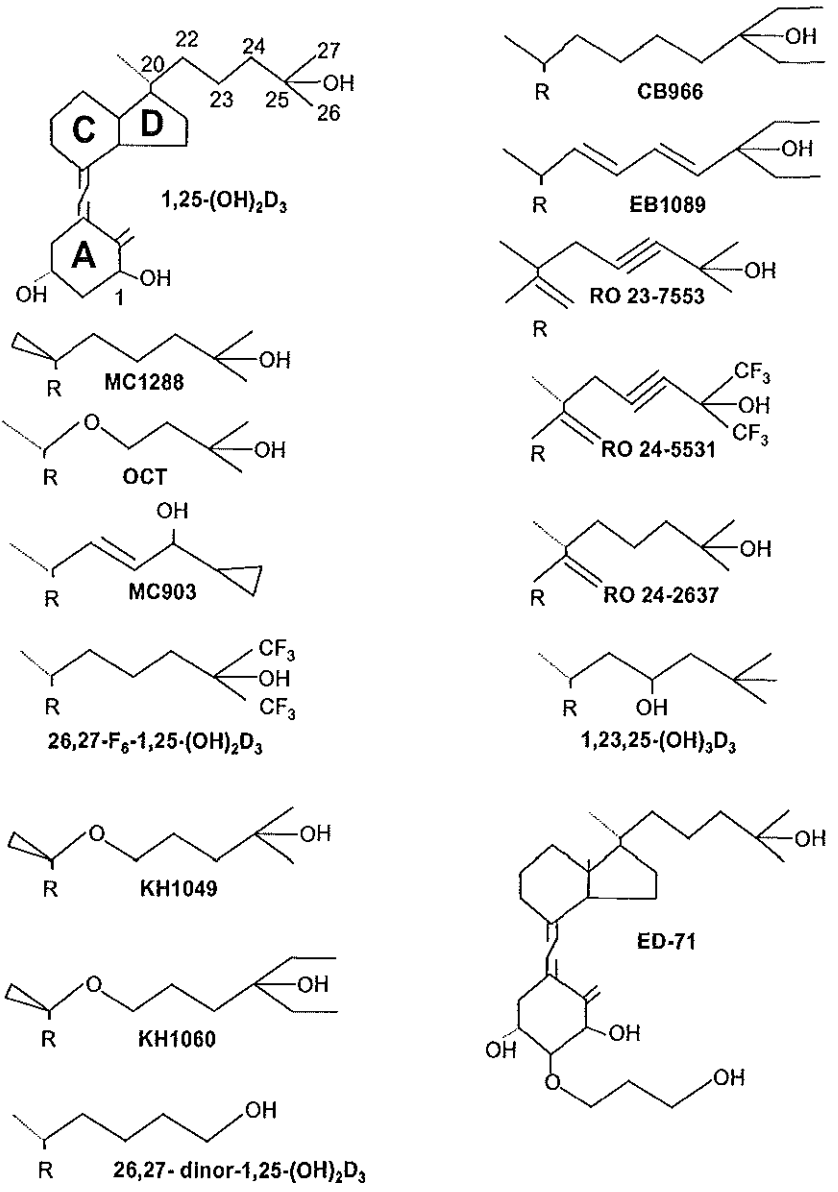

Figure 1.4. Examples of 1,25-(OH)$_2$D$_3$ analogs. *Side chain modifications can be introduced separately (e.g. OCT, MC1288), or in combination (e.g. KH1060, EB1089). Furthermore, side chain modifications can be combined with alterations at other sites of the 1,25-(OH)$_2$D$_3$ molecule (e.g. RO 23-7553). 2β-(3-Hydroxypropoxy)-1,25-(OH)$_2$D$_3$ (ED-71) and 1,25-(OH)$_2$-16-ene-D$_3$ (RO 24-2637) are shown as examples of analogs with modifications only at the A and D ring, respectively.*

The newly synthesized 1,25-$(OH)_2D_3$ analogs were extensively studied *in vitro* with respect to their effect on:

- Cell proliferation (Abe 1987, Binderup 1988, Norman 1990, Binderup 1991, Jones 1994, Elstner 1994, Vink-Van Wijngaarden 1994, Elstner 1995, Puthier 1996, Kawa 1996)
- Cell differentiation (Binderup 1991, Brown 1994, Mathiasen 1993, Elstner 1995, Munker 1996, Moore 1996, Mork Hansen 1996)
- Immunomodulation (Binderup 1991, Puthier 1996)
- PTH/PTH-related peptide synthesis (Brown 1989, Falzon 1998)
- Bone resorption (Abe 1987, Pols 1991, Wiberg 1995)
- Osteoclast recruitment (Wiberg 1995)
- Intestinal calcium transport (Wang 1993)
- Bone matrix protein synthesis (Pernalete 1991, Evans 1991, Ryhänen 1996)
- 24-Hydroxylase activity (Pols 1991)

In Table 1.3 the therapeutic potential of some of the 1,25-$(OH)_2D_3$ analogs in the treatment of immunological disorders and/or hyperproliferative diseases based on *in vivo* experiments are summarized. Furthermore, Table 1.3 summarizes analogs with properties that might be beneficial in the treatment of hyper- and hypoparathyroidism and osteoporosis.

Table 1.3. *In vivo* effects of 1,25-$(OH)_2D_3$ analogs and their potential clinical applications

ANALOG	*IN VIVO* EFFECT	THERAPEUTIC ROLE	(Ref)
CB966	tumor growth inhibition	breast cancer	(Colston 1991)
	Immunosuppression	organ transplantation	(Veyron 1993)
EB1089	tumor growth inhibition	breast cancer	(Colston 1991, Colston 1992, James 1998, VanWeelden 1998, Koshizuka 1999)
	tumor growth inhibition	colon cancer	(Akhter 1997)
	tumor growth inhibition	epithelial cancer	(El Abdalmi 1999)
	tumor growth inhibition	leydig cell carcinoma	(Haq 1993)
	tumor growth inhibition	pancreas cancer	(Colston 1997)
	tumor metastasis inhibition	prostate cancer	(Lokeshwar 1999)
KH1060	immunosuppression	immunological disorders	(Lillevang 1992)
	immunosuppression	type I diabetes	(Mathieu 1995, Casteels 1998)
	immunosuppression	organ transplantation	(Veyron 1993)

MC903	immunosuppression	psoriasis	(Kragballe 1991)
	tumor growth inhibition	breast cancer	(Bower 1991, Colston 1992a)
MC1288	immunosuppression	organ transplantation	(Johnsson 1994, Johnsson1995)
	immunosuppression	arthritis	(Larsson 1997)
	immunosuppression	type I diabetes	(Casteels 1998a)
OCT	immunosuppression	immunological disorders	(Abe 1989)
	tumor growth inhibition	breast cancer	(Abe 1991, Oikawa 1991, Abe-Hashimoto 1993, Matsumoto 1999)
	tumor growth inhibition	pancreatic cancer	(Kawa 1996, Endo 1998)
	tumor growth inhibition	lung cancer	(Endo 1998)
	tumor growth inhibition	pharynx carcinoma	(Endo 1998)
	decreased tumorigenesis	colon cancer	(Otoshi 1995)
	decreased tumorigenesis	intestinal carcinoma	(Otoshi 1995)
RO 23-7553	tumor growth inhibition	leukemia	(Zhou 1990, Shtemfeld 1996)
	tumor growth inhibition	prostate cancer	(Schwartz 1995)
	tumor growth inhibition	retinoblastoma	(Sabet 1999)
RO 24-2637	immunosuppression	organ transplantation	(Lemire 1992a, Lemire 1994)
RO 24-5531	decreased tumorigenesis	breast cancer	(Anzano 1994)
	decreased tumorigenesis	colon cancer	(Wali 1995)
	decreased tumorigenesis	seminal vesicle carcinoma	(Lucia 1995)
	decreased tumorigenesis	prostate cancer	(Lucia 1995)
1,25-$(OH)_2$-F_6-D_3	correcting hypocalcemia	hypoparathyroidism	(Nakatsuka 1992)
ED-71	increased bone mineralization	osteoporosis	(Okano 1991, Ikeda1992, Nishii 1993, Tsurukami 1994)
OCT	suppression of PTH production	hyperparathyroidism	(Slatopolsky 1992, Monier-Faugere 1992)

Although large quantitative differences in potency between 1,25-$(OH)_2D_3$ and certain analogs are observed, all the analogs discussed above exert activities qualitatively comparable to 1,25-$(OH)_2D_3$, i.e. they decrease cell proliferation and stimulate cell differentiation. This is in fact a general finding, although recently antagonistic activities were subscribed to 1,25-$(OH)_2$-26,23-lactone D_3 and its analogs. The lactone analogs inhibited 1,25-$(OH)_2D_3$-induced osteoclast formation (Arai 1997), and stimulated proteoglycan and collagen synthesis in chondrocytes from rabbits, whereas 1,25-$(OH)_2D_3$ had an inhibitory effect (Ishizuka 1997).

A.6.2. STRUCTURE-FUNCTION ANALYSIS

General characteristics with respect to structure-function relationship will be summarized. More detailed information on this topic can be found elsewhere (Norman 1990, Bikle 1992, Bouillon 1995, Jones 1997, Binderup 1997, Uskokovic 1997; and references therein).

Shortening and lengthening of the side chain

Shortening of the side chain by one carbon eliminates its potency *in vivo* and decreases its potency *in vitro* ten fold. In contrast, lengthening of the side chain results in an increased *in vitro* and *in vivo* activity. Elongation of the side chain with one carbon results in an increased potency of the analog to inhibit cell proliferation, whereas its calcemic effect is diminished. The dissociation between calcemic effect and the cell growth inhibitory effect is further increased after elongation with two carbon atoms. Lengthening by three carbon atoms results in a complete loss of *in vivo* activity, whereas some *in vitro* effects are only modestly reduced. Elongation by four or more carbons results in complete loss of biological activity.

Substitution of an oxygen atom

Substitution of an oxygen atom at carbon 22 in the side chain (OCT), leads to a 10 times increased cell differentiating activity compared to 1,25-$(OH)_2D_3$, whereas the calcemic activity is 50-100 times reduced. In general, hydroxylation at the carbon atom adjacent to the oxagen atom results in formation of metabolic unstable products. Indeed, OCT is rapidly degraded into the unstable hexanor-1α,20-dihydroxyvitamin D_3 and hexanor-20-oxo-1α-hydroxyvitamin D_3. KH1139, a combination of elongation (as in CB966) and substitution of an oxygen atom (as in OCT) is 150 times more potent than 1,25-$(OH)_2D_3$ in inhibiting cell growth, whereas its calcemic activity is three times lower.

Substitution of fluorine

Substitution of one fluorine at carbon 24 results in an analog with 3 times lower cell differentiating and calcemic activity compared to 1,25-$(OH)_2D_3$. In contrast, substitution of two fluorines at carbon 24 increases the capacity to induce cell differentiation 10 times without affecting the calcemic activity. Introduction of 6 fluorine groups at carbon 26 and 27 further increases cell differentiating activity and increases calcemic activity 10-fold compared to 1,25-$(OH)_2D_3$. In general, substitution of fluorine for hydrogen at carbon 23, 24, 25, 26 or 27 might diminish susceptibility to hydroxylases, resulting in longer half-life of the analog.

Introduction of double or triple bonds

Introduction of a double or triple bond in the side chain results in a moderately increased effect on cell growth inhibition and a diminished calcemic activity compared to 1,25-$(OH)_2D_3$. Introducing two double bonds (at carbon 22 and 24; EB1089) further increases the potency of the analog to inhibit cell proliferation, whereas its calcemic effect is 3 times lower than 1,25-$(OH)_2D_3$. In general, introduction of double or triple bonds might affect 24-hydroxylase activity, either directly (by blocking the carbon 24 position) or indirectly (because of a

conformational change of the side chain that diminishes susceptibility to hydroxylases). The double bond at carbon 16 (RO 24-2637) increases the cell growth inhibitory activity 100 times, while the calcemic activity is only moderately increased. Combining this modification with a triple bond in the side chain (RO 23-7553) lowers the calcemic activity 6 times.

20-Epi configuration

Analogs with 20-epi configurations have an increased biological potency compared to corresponding compounds with a normal 20*S* configuration. For instance, MC1288 which only difference with 1,25-$(OH)_2D_3$ is its 20-epi configuration, is 100 times more potent in inhibiting cell proliferation, but only 2 times more calcemic than 1,25-$(OH)_2D_3$. The analog KH1139, except for its 20*S* configuration identical to KH1060, is 45 times more potent than 1,25-$(OH)_2D_3$ in inhibiting proliferation, whereas KH1060 is 31,000 times more potent than 1,25-$(OH)_2D_3$. The potency of these analogs is even more evident in immunosuppresion tests, where the activity of 1,25-$(OH)_2D_3$ is overridden several 1,000 times. However, the therapeutic potential of these 20-epi-analogs is limited by an increased calcemic activity. A combination of double bonds (RO 24-2637) and 20-epimerization (MC1288) results in formation of an analog (1,25-$(OH)_2$-16-ene-20-epi-D_3) with a cell differentiation activity similar to that of MC1288, but a 80 times lower calcemic activity than 1,25-$(OH)_2D_3$. The calcemic activity of the 20-epi form of RO 23-7553 is even further diminished (200 times lower than 1,25-$(OH)_2D_3$).

A.6.3. POSSIBLE MECHANISMS FOR THE SELECTIVE ACTION OF 1,25-DIHYDROXYVITAMIN D_3 ANALOGS

It is not possible to provide a general mechanism of action to explain the changes in biological profile of every 1,25-$(OH)_2D_3$ analog. In fact, any modification in the 1,25-$(OH)_2D_3$ molecule will lead to formation of a ligand with a changed mechanism of action, i.e. the ligand may differently interact with one or more of the steps leading to transcriptional activation depicted in Figure 1.2. In the section below, possible steps that could determine the selective biological activity of a 1,25-$(OH)_2D_3$ analog are summarized. It is very likely that not just one of these sites of modulation is differently affected by an analog, but that subsequent steps in the cascade are affected as well.

I. RECEPTOR NUMBER, AFFINITY, CONFORMATION, AND HALF-LIFE

Presence of a functional VDR seems to be essential to observe effects of 1,25-$(OH)_2D_3$ and its analogs on bone metabolism and cell proliferation [Dokoh 1984, Yamaoka 1986, Eisman 1987, Elstner 1994, Hedlund 1996, Hedlund 1996a, Yoshizawa 1997, Zhuang 1997, Takeda 1999]. As already mentioned in Section A.4.2., 1,25-$(OH)_2D_3$ up-regulates expression of the VDR. In addition, 1,25-$(OH)_2D_3$ analog-induced VDR up-regulation was also reported [Pols 1991, Pemalete 1991]. However, the biological relevance of this increase in VDR content is not completely resolved.

Receptor affinity, as measured by classic ligand displacement, is of little value in predicting the biological potency of an analog (See also Chapter 2, [Vink-Van Wijngaarden 1994]). Analogs with extremely low affinity are usually weak activators but for analogs with moderate to high VDR affinity there is no correlation between receptor affinity of an analog and its biological activity [Ferrara 1994, Imai 1995, Munker 1996, Peleg 1996, Zhao 1997, Okano 1998]; i.e. analogs with low VDR affinity may have high biological activity, and *vice versa*. The fact that compared to 1,25-$(OH)_2D_3$ only very few analogs have an increased affinity for the VDR, supports the notion that VDR affinity is not the only factor to explain the biological activity of an analog [Bouillon 1995, Mork Hansen 1996].

Ligand-induced changes in VDR conformation are believed to be crucial for transactivation of the receptor [Peleg 1995]. 1,25-$(OH)_2D_3$ binding results in repositioning of helix 12 and closure of the mouse trap formed by the ligand-binding pocket, causing a change in the three dimensional structure of the VDR. Modifications of the 1,25-$(OH)_2D_3$ molecule will lead to different contact sites within the ligand-binding pocket of the VDR and induces a different folding of the VDR. This is nicely illustrated by a report of Liu *et al.* showing that deletion of helix 11 and 12 of the VDR resulted in loss of 1,25-$(OH)_2D_3$ binding, whereas binding of MC1288 or KH1060 was not affected. These 20-epi analogs of 1,25-$(OH)_2D_3$ interact with helix 5 of the ligand-binding pocket, instead of helix 11 and 12 which contact the side chain of 1,25-$(OH)_2D_3$ [Liu 1997]. Since it was shown that the conformation of unliganded VDR possibly prevents binding of SRC-1 and GRIP-1 and blocks DNA interaction [Hong 1997, Masuyama 1997], it is hypothesized that ligand-induced conformational changes can result in exposure of specific sites of the receptor (e.g. the AF-2 domain) important for interaction with cofactors and DNA binding [Nayeri 1996a]. Liu *et al.* speculated that the increased potency of 20-epi analogs of 1,25-$(OH)_2D_3$ might be due to different interactions with AF-2 residues in helix 12: 20-epi analogs do not bind directly to the AF-2 residues and allow optimal folding and exposure of coactivator binding sites, while 1,25-$(OH)_2D_3$ and analogs with a normal side chain orientation actually make contact with the AF-2 region and as a consequence probably induce VDR folding and exposure of coactivator binding sites that results in relatively lower VDR transactivation [Liu 1997].

Ligand-induced conformational changes of the VDR will affect its stability. Similar to the enzymes used in limited protease digestion analysis to study receptor conformational changes, the efficiency of enzymes in the cell involved in receptor processing will be affected by ligand-induced folding of the receptor (i.e. the accessibility of cleavage sites). The VDR conformation induced by MC1288 or KH1060 is more stable and exerts increased transcriptional activity [Liu 1997]. In Chapter 3, the effect of the 20-epi-analog KH1060 on VDR conformation and stability is studied in more detail.

II. RECEPTOR PHOSPHORYLATION

Since phosphorylation might be important for VDR activation [Hsieh 1991, Darwish 1993, Jurutka 1993, Jurutka 1993a, Kuiper 1994, Jurutka 1996], it is plausible that the increased biological activity of some 1,25-$(OH)_2D_3$ analogs could be attributed to their capability to induce VDR phosphorylation. Although it was reported that 1,25-$(OH)_2D_3$ analogs can induce VDR phosphorylation, a relationship with their specific biological activity could not yet be established [Jurutka 1993a].

III. RECEPTOR DIMERIZATION

Most of the 1,25-$(OH)_2D_3$ target genes with identified VDREs are regulated by VDR/RXR dimers, although also functional VDR homodimers have been described [Carlberg 1994]. Formation of VDR/RXR and VDR/VDR dimers is regulated by 1,25-$(OH)_2D_3$. It is obvious that an increased (or decreased) formation of dimers will influence the rate of gene transcription. The increased potency of the 20-epi 1,25-$(OH)_2D_3$ analog MC1288 for instance might be explained by its increased stimulatory effect on dimerization of VDR and RXR [Peleg 1995]. This might be a factor of general importance since Zhao *et al.* demonstrated, using the two-hybrid system, that the heterodimerization potency of most analogs correlated with their transcriptional activity [Zhao 1997]. One of the exceptions is ED-71, showing strong dimerization potency but relatively low tranactivation activity [Zhao 1997].

It was reported by Carlberg *et al.* that VDR homodimer-mediated gene transcription via a DR6-type VDRE showed higher ligand sensitivity than VDR/RXR heterodimer-mediated gene transcription via a DR3-type VDRE [Carlberg 1994]. It is possible that certain 1,25-$(OH)_2D_3$ analogs specifically stimulate formation of either dimer type and in this way selectively regulate gene transcription.

IV. TRANSLOCATION OF THE RECEPTOR TO THE NUCLEUS

Recently it was shown that the dose-response curve of an analog to activate gene transcription is similar to the dose-response curve to stimulate translocation of VDR to the nucleus (Racz 1999). The AF-2 domain seems to be an essential element in this process and it is therefore feasible that specific interactions with an analog will influence VDR translocation to the nucleus and subsequent activation of the VDR. Therefore, analogs could be developed that block VDR translocation, and in this way these analogs could be used to antagonize the effect of $1,25\text{-}(OH)_2D_3$.

V. DNA INTERACTION

The VDR interacts with specific responsive sites in the DNA, and by doing so it induces remodelling of chromatin to fascilitate interaction with the preinitiation complex. Vitamin D analog-selective effects on this DNA bending will affect subsequent activation steps (e.g. cofactor interaction) and this will likely influence the biological potency of this analog.

The increased biological activity of an analog might be explained by induction of an increased VDR affinity for the VDRE. For instance, the increased DNA binding affinity of $1,25\text{-}(OH)_2\text{-}F_6\text{-}D_3$-VDR/RXR compared to $1,25\text{-}(OH)_2D_3$-VDR/RXR is put forward as a possible explanation for its increased potency to inhibit the growth of leukemic cells (Inaba 1989, Sasaki 1995). It was also suggested that this feature could play a role in the increased biological potency of 20-epi analogs like GS1500, MC1288, and KH1060 (Mathiasen 1998, Peleg 1995).

In most cases, the VDRE is of the DR3 type, but also other VDRE types have been described (see Section A.4.7.). The selective action of certain $1,25\text{-}(OH)_2D_3$ analogs might be the result of their increased effect on gene transcription via VDREs of a specific type. For instance, the increased inhibitory effect on cell proliferation by the $1,25\text{-}(OH)_2D_3$ analog EB1089 might be explained by its increased stimulatory effect on gene transcription from IP9 elements. Based on these findings Carlberg and coworkers proposed that transcription of genes involved in cell growth regulation might be under control of IP9 VDREs (Nayeri 1995, Schräder 1997).

VI. COFACTOR INTERACTION AND CELLULAR CONTEXT

Selective modulation by $1,25\text{-}(OH)_2D_3$ analogs of VDR interaction with basic transcription factors that influence formation/stability of the preinitiation complex or with cofactors that modulate DNA relaxation, rate of gene transcription, or VDR stability (e.g. SUG1) might be involved in the selective biological activity of $1,25\text{-}(OH)_2D_3$ analogs. OCT induces such selective interaction of VDR with coactivators: Unlike $1,25\text{-}(OH)_2D_3$ and the analogs ED-71 and $1,25\text{-}(OH)_2\text{-}F_6\text{-}D_3$ which induce interaction of VDR with SRC-1, AIB, and TIF2, OCT only

potentiates interaction with TIF2 (Takeyama 1999). The increased potency of the 20-epi analogs MC1627 and MC1288 is possibly linked to the ability of these compounds to recruite and bind DRIP205, a subunit of the DRIP coactivator complex (Yang 1999). In addition to this, quantitative and qualitative differences in distribution of cofactors might provide an unique environment leading to cell- or tissue-specific effects of 1,25-$(OH)_2D_3$ analogs.

Recently, tissue-specific variant transcripts of the VDR gene were identified and it was suggested that these different transcripts encode for different VDR isoforms that might contribute to tissue-specific effects of 1,25-$(OH)_2D_3$ (Crofts 1998).

VII. LIGAND METABOLISM

Ligand metabolism is another important factor that might interfere with selective effects of the 1,25-$(OH)_2D_3$ analogs. The rate at which the 1,25-$(OH)_2D_3$ analog is inactivated depends on:

- Vitamin D binding protein binding and cellular uptake

DBP binding can work two ways: low DBP binding can result in rapid clearance, while unbound ligand can lead to greater accessibility to target cells. The low affinity for DBP might play a role in the increased biological potency of MC1288 (Dilworth 1994). The 1,25-$(OH)_2D_3$ analog OCT is as potent as 1,25-$(OH)_2D_3$ in many biological responses. However, its decreased DBP binding results in reduced serum half-life and might explain why OCT is less calcemic than 1,25-$(OH)_2D_3$ (Brown 1993). Cellular uptake of an analog is probably cell- or tissue-specific, and might therefore underly tissue-specific effects of analogs. The low bone mobilizing activity of OCT *in vivo* for instance might be explained by reduced uptake of OCT in comparison to 1,25-$(OH)_2D_3$ by bone, while uptake of OCT in other organs (small intestine, parathyroid glands, kidney, and liver) is increased compared to 1,25-$(OH)_2D_3$ (Kobayashi 1994). On the other hand, the high affinity of ED-71 for DBP might explain its increased half-life (Okano 1989).

- Catabolic susceptibility of the analog

Modifications in the structure of 1,25-$(OH)_2D_3$ might result in a decreased or increased susceptibility of the analog for catabolic pathways, leading to an increased or decreased half-life of the analog, respectively. The fluor groups in 1,25-$(OH)_2$-F_6-D_3 as well as double or triple bonds in the side chain (e.g. RO 23-7553) might inhibit hydroxylase activity, resulting in increased half-life of the analog (Tanaka 1984, Inaba 1993). In contrast, the modifications in MC903 lead to rapid disappearance from the circulation (Pols 1991). Analogs with an increased resistance against metabolic degradation could be used in the treatment of hypocalcemia (e.g. dihydrotachysterol), whereas the rapidly metabolized analogs might be useful for short term, local effects (e.g. MC903) (Jones 1997).

- Intrinsic metabolic capacity of the target cell

Tissue-specific rates of catabolic activity might also underly the selective activity of analogs. Certain tissues might have a more effective mechanism to degrade the analog into nonactive compounds, whereas in other tissues the analog accumulates and exerts its activity for a longer period of time. In keratinocytes OCT is degraded more rapidly than $1,25\text{-}(OH)_2D_3$ (Bikle 1995), while in monocytes OCT is more stable than $1,25\text{-}(OH)_2D_3$ (Kamimura 1993), possibly explaining the decreased and increased potency of OCT in keratinocytes and monocytes, respectively.

- Activity of metabolites of analogs

The metabolites of the analogs formed may also be active and can contribute to the biological activity of the mother compound (Watanabe 1996, Dilworth 1997). The $1,25\text{-}(OH)_2D_3$ analog RO 24-2637 for instance, is metabolized into $1,25\text{-}(OH)_2$-16-ene-24-oxo-D_3. This metabolite is stable and its effect on growth and differentiation of a human myeloid leukemic cell line is comparable to its parent compound. In contrast, the equivalent metabolite of $1,25\text{-}(OH)_2D_3$, $1,25\text{-}(OH)_2$-24-oxo D_3, is rapidly degraded to inactive calcitroic acid (Ferrara 1994, Siu-Caldera 1996). More aspects on the biological activity of metabolites of $1,25\text{-}(OH)_2D_3$ analogs will be discussed in Chapter 5.

VIII. NONGENOMIC EFFECTS

The selective action of $1,25\text{-}(OH)_2D_3$ analogs might also be the result of their changed capability to exert nongenomic effects. For instance, the transient transcaltachia activity of OCT might explain its decreased calcemic activity compared to $1,25\text{-}(OH)_2D_3$ (Farach-Carson 1993). Also other analogs with low calcemic activity (MC903, RO 23-7553) have low transcaltachia activity (Zhou 1992). Conversely, the $25\text{-}(OH)D_3$ analog 25-(OH)-16-ene-23-yne-D_3 is a strong stimulator of transmembrane calcium influx, whereas it only binds weakly to the nuclear VDR and has little transcriptional activity (Farach-Carson 1991). In this respect, the findings obtained with $1\beta,25\text{-}(OH)_2D_3$ are also of interest. This analog lacks any transcriptional activity and binds to the VDR with very low affinity. However, $1\beta,25\text{-}(OH)_2D_3$ is a potent antagonist of a $1,25\text{-}(OH)_2D_3$-mediated nongenomic effect (opening of Ca^{2+} channels in osteoblast-like cells) (Norman 1993). Therefore, studying the properties that determine the preference of an analog for binding the nuclear or the membrane-associated form of the VDR (Nemere 1998) might help to develop analogs with selective genomic or nongenomic potencies (Kabat 1998).

B. ESTROGEN RECEPTOR-MEDIATED GENE REGULATION

The nuclear hormone 17β-estradiol (E_2) is a major regulator of growth, differentiation, and function of numerous tissues including tissues of the female as well as the male reproductive system. Also in bone and the cardiovascular and central nervous system E_2 plays an important role. The effects of E_2 are primarily mediated by the ER; a member of the nuclear receptor superfamily that exists as two subtypes: ERα (molecular weight 60-66 kDa) [Green 1986] and the more recently cloned ERß (molecular weight 51-61 kDa) [Kuiper 1996, Mosselman 1996, Tremblay 1997].

B.1. ESTROGEN RECEPTOR EXPRESSION

ERα and ERβ are expressed in a wide variety of cells and tissues [Ciocca 1995, Kuiper 1999]. Although in many tissues both subtypes are expressed there are also differences in tissue distribution and cellular localization within certain tissues [Kuiper 1999]. For instance, within the rat ovary ERβ is observed in granulosa cells and not in interstitium and thecal cells. In contrast, ERα is expressed in theca and interstitial cells, and not or only weakly in granulosa cells [Kuiper 1996, Sar 1999, Fitzpatrick 1999]. Also, certain regions of the rat brain mainly express ERβ and virtually no ERα and *vice versa* [Shughrue 1997]. In the rat bone, prostate and in Sertoli cells and spermatocytes in the testis, ERβ is more abundant than ERα, whereas the opposite is found in kidney, pituitary, adrenal, and in stromal and epithelial cells of the endometrium [Onoe 1997, Kuiper 1997, Shughrue 1998, Saunders 1998, Van Pelt 1999]. The tissue-specific distribution of ERα and ERβ is species-specific, e.g. ERβ is detected in pituitaries from rats [Kuiper 1997] and humans [Brandenberger 1997], but not in mice [Couse 1997]. In addition, expression of ER subtypes is cell differentiation-dependent, as observed in *in vitro* cell culture models using human, rat, and mouse osteoblasts [Arts 1997, Onoe 1997, Ishibashi 1998].

Furthermore, of both ER subtypes several isoforms are detected. In mouse bone marrow cultures, normal rat pituitary cells and human pituitary tumors, as well as in human testis different ER isoforms are expressed [Shupnik 1998, Bhat 1998, Gruber 1999, Pasqualini 1999]. Among others, a rat ERβ isoform (ERβ2) with a much lower affinity for E_2 than the initially reported ERβ was found which is expressed in many rat tissues [Petersen 1998, Hanstein 1999]. In the review by Kuiper *et al.* this and other ERβ isoforms are summarized and discussed in more detail [Kuiper 1999].

B.2. ESTROGEN RECEPTOR STRUCTURE

Figure 1.5 shows the basic structure of ERα and ERβ, which consists of six major domains; A through F. ERα and ERβ share this structure with the VDR and other members of the nuclear receptor superfamily. Domain C is important for receptor dimerization and contains two zinc-binding clusters of amino acid residues that bind to DNA sequences in the vicinity of the promoter of target genes, so-called estrogen response elements (EREs). The C-terminal zinc-binding amino acid cluster is involved in non-specific DNA binding, whereas the N-terminal zinc cluster seems important in discriminating and specifying ER binding to EREs [Green 1988, Mader 1989]. The ligand binding domain (E) contains sites involved in nuclear translocation and dimerization. In addition, AF-2 is located in the ligand binding domain. AF-2 acts as a docking site for coactivator proteins and transcription factors that form the preinitiation complex [McKenna 1999] (See also Section B.4.8.). Unlike the VDR, the ER can also be activated in a ligand-independent fashion by AF-1 located in the N-terminal A/B domain, [Lees 1989, Aronica 1993, Ignar-Trowbridge 1993, Bunone 1996] (see also Section B.6.2., part VI). However, one must not consider these AFs as two independently operating sites of receptor activation but rather as cooperative activating elements [Pham 1992]. The ligand binding domain (E) and the DNA binding domain (C) is separated by a hinge region (D). The function of the F domain is not completely clear. It was demonstrated that deletion of the F domain had no influence on transcriptional activation by E_2 [Kumar 1987, Pakdel 1993], ER stability [Pakdel 1993], and phosphorylation state of the ER [Pakdel 1993]. However, it was suggested that the F domain is involved in agonistic/antagonistic action of certain antiestrogens [Montano 1995, Nichols 1998] and in interaction with specific cofactors [Peters 1999].

ERα and ERβ are encoded by different genes located on different chromosomes (human chromosome 6 and 14, respectively), but show considerable homology: 96% in the DNA binding domain and about 60% in the ligand binding domain [Mosselman 1996]. However, the amino terminal A/B domain containing AF-1, hinge region and F domain show little homology (Figure 1.5.).

B.3. FUNCTION OF 17β-ESTRADIOL

Reproductive organs, bone, cardiovascular system, and cognition

The function of estrogens (E_2, estriol and estrone) is very diverse and includes effects on female as well as male reproductive organs (uterus, vagina, ovary, testis, and prostate), and is also important for development of female secondary sex characteristics. In addition, development and maintenance of the skeleton, and function of the cardiovascular system, brain, and nervous system are importantly

influenced by estrogens. The synthesis of growth factors and their receptors (e.g. insulin-like growth factors I and II, colony stimulating factor I, TGF α and ß, insulin-like growth factor I receptor, epidermal growth factor receptor), peptide hormones (prolactin, luteinizing hormone, follicle stimulating hormone), protooncogenes (e.g. c-myc, c-fos, c-jun, ras), heat shock proteins (heat shock protein 27 and 90), and steroid hormone receptors (e.g. glucocorticoid receptors, ERs, VDRs, progesterone receptors) is influenced by estrogens [Liel 1992, Korach 1994, Ciocca 1995].

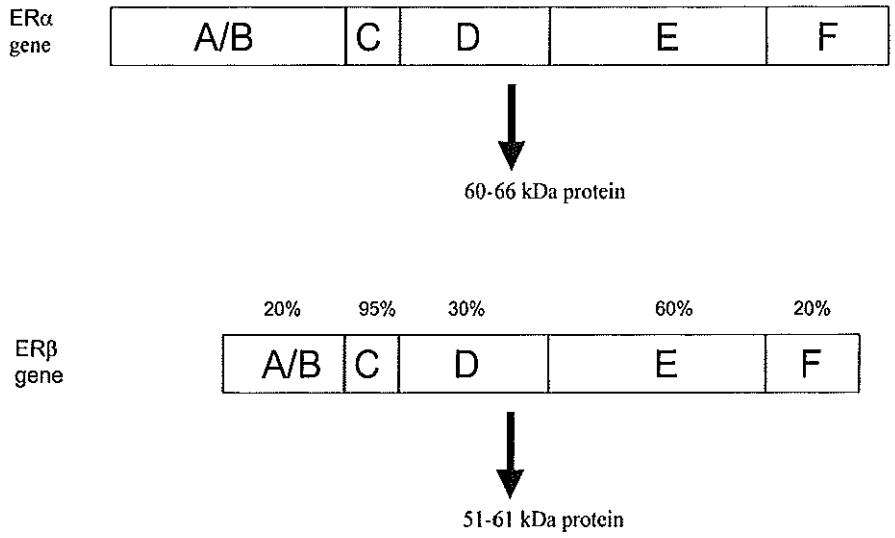

Figure 1.5. Schematic structure and homology of ERα and ERß. *The percentages in amino acid homology are depicted. The functions of domains A through F are described in Section B.2.*

The importance of E_2 for bone becomes most apparent after menopause, when the dramatic fall in E_2 and estrone levels lead to an imbalanced ratio between bone formation and bone resorption, resulting in an increased net bone loss. Furthermore, increased malabsorption of calcium could underly postmenopausal bone loss. Also in males estrogens play an important role in bone metabolism. The enzyme aromatase cytochrome P_{450} converts androgens into estrogens (Figure 1.6). It was shown that

men lacking this enzyme due to a gene mutation had low bone density and delayed bone maturation (Morishima 1995), and that treatment of these patients with E_2 improved bone mineral density (Carani 1997).

Bone is an important target tisssue of E_2 and in osteoblasts and osteocytes ERα and ERβ has been demonstrated (Hoyland 1997, Arts 1997, Onoe 1997, Vidal 1999). The presence of ERs in osteoclasts remains controversial. A preosteoclastic cell line expressed ERα, but no ER expression was observed in mature osteoclasts (Oreffo 1999). Administration of estrogens (estrogen replacement therapy) or a combination of estrogens and progestins (hormone replacement therapy) can reduce the risk of developing postmenopausal osteoporosis (Lindsay 1976, Munk-Jensen 1988). E_2 treatment results in improved calcium absorption in postmenopausal women. As an explanation for this effect E_2-stimulated production of 1,25-$(OH)_2D_3$ was proposed (Constillo 1977). However, in rats others observed increased absorption of calcium and phosphorus in the intestine without an alteration in serum levels of 25-$(OH)D_3$, 1,25-$(OH)_2D_3$, and PTH (Arjmandi 1994, Liel 1999), and our own laboratory and Chen *et al.* reported that the E_2-mediated increase in calcium absorption was accompanied by a decrease in 1,25-$(OH)_2D_3$ serum levels (Colin 1999, Chen 1997). Interestingly, in the study by Chen *et al.* and in the study of Liel *et al.* E_2 treatment resulted in a significant increase in intestinal VDR expression compared to ovariectomized or sham operated rats (Chen 1997, Liel 1999). Therefore, both a direct effect of E_2 and/or an E_2-stimulated responsiveness to 1,25-$(OH)_2D_3$ might explain the stimulating effect of E_2 on calcium absorption.

Furthermore, interaction of E_2 and calcitonin could be involved in the reduction in bone mass after menopause. Calcitonin is an inhibitor of osteoclastic bone resorption. Postmenopausal women treated with E_2 demonstrated increased blood levels of calcitonin and an increased vertebral bone mass compared to placebo treated women (Agnusdei 1990). The antiresorptive action of E_2 might also be mediated by the induction of osteoclast apoptosis (Kameda 1997), or by a decreasing effect on the synthesis of bone resorption-inducing cytokines like interleukin 1, interleukin 6, and tumor necrosis factor α (Kassem 1996, Jilka 1998).

In addition, a direct anabolic effect of E_2 on bone forming cells (osteoblasts) might play a role in the beneficial effect of E_2 on bone mass. A stimulatory effect of E_2 on proliferation and differentiation of osteoblast-like cells derived from male and female adult trabecular bone was reported (Scheven 1992). In femoral bone tissue from elderly female rats E_2 and the isoflavonoids daidzein and genistein increased DNA and calcium content, and stimulated alkaline phosphatase activity (Gao 1999).

It has been suggested that estrogens might have a beneficial effect on the cardiovascular system. After menopause, low density lipoproteins rise, which may result in an increased risk for cardiovascular diseases (myocardial infarction and

cerebrovascular accidents). Administration of estrogens may decrease low density lipoprotein levels, may increase high density lipoproteins (Lobo 1991), and thereby may reduce the rate of cardiovascular diseases (Barrett-Connor 1991, Stampfer 1991). In addition, direct effects of estrogens on vessel wall might play an important role (Lafrati 1997).

Estrogens might also play a role in cognition. It was found that hormone replacement therapy improved cognition and was associated with a decreased incidence of Alzheimer's disease (Sherwin 1996, Paganini-Hill 1996).

The central role of E_2 and its receptors in the functions mentioned above became evident studying mice that lack either a functional ERα or ERβ. The ERα knock out (αERKO) mouse clearly demonstrated the importance of E_2 in fertility. Both male and female αERKO mice are infertile and their reproductive organs are reduced in size (Lubahn 1993, Hess 1997). The fertility of the female ERβ knock out (βERKO) mouse is reduced because of a diminished ovarian efficiency, whereas the male βERKO mouse only shows abnormalities (hyperplasia of the bladder and prostate) at older age (Krege 1998). The male βERKO mouse displays no bone abnormalities compared with wild type mice. However, the cortical bone mineral content of adult female βERKO mice is increased due to increased radial bone growth. It was suggested that ERβ might have repressive function in regulating bone growth during female adolescence (Windahl 1999).

Also in humans insight into the function of E_2 and ER was gained with observations obtained with an ERα deficient male. The ERα gene of this patient contains a single base pair mutation in exon 2 and codes for a truncated ERα protein lacking the DNA and ligand binding domain. The patient had low bone mineral density, immature epiphyses and tall stature. The bone mineral density did not improve and the growth plates did not close after E_2 therapy. Interestingly, the patient developed normally until puberty. This suggests that other pathways can compensate the lack of a functional ERα or that E_2 is not a key regulator in prepubertal development. However, the ERα-mediated pathway seems elementary for bone metabolism during and after puberty (Smith 1994).

The importance of estrogens in male bone homeostasis is also illustrated by observations of severe osteoporosis and continued linear growth in men with mutations in the aromatase gene. The aromatase enzyme complex converts androgens (testosterone) to estrogens. Treatment of these aromatase deficient men with estrogens increases bone mineral density and closure of the epiphyses.

Cell growth

The stimulatory effect on growth of the uterus is a classic effect of E_2. Growth of other cells, both normal and malign is directly influenced by E_2. It was for instance found that estrogens have a mitogenic effect on the ER-positive breast cancer cell line MCF-7 (Lippman 1976). Development of certain breast and endometrial tumors is stimulated by estrogens (Beresford 1997). One possible explanation for these observations might be that certain estrogen metabolites show carcinogenic activity. Estrogens can be metabolized into 4-hydroxyestradiol and it was shown that this metabolite is a source of free radicals. These free radicals might damage DNA and tumors could arise (Zhu 1996). Due to metabolic stability of 4-hydroxyestradiol high concentrations in certain tissues could develop.

B.4. ESTROGEN RECEPTOR: TRANSCRIPTIONAL ACTIVATION

The transcriptional activation activity of the ER is initiated by interaction with ligand (See Figure 1.2 and the cascade of events mentioned in Section A.4.). Kuiper *et al.* reported that both receptor subtypes bind a large variety of compounds (estrogens, antiestrogens, some androgens, phytoestrogens, and environmental estrogens) (Kuiper 1997). However, the natural ligand for both receptor subtypes is E_2.

B.4.1. SYNTHESIS, TRANSPORT, AND METABOLISM OF 17ß-ESTRADIOL

Like 1,25-$(OH)_2D_3$, E_2 is synthesized out of cholesterol (see Figure 1.6). E_2 is mainly produced by granulosa cells in the follicles, but also in adrenal glands, liver, and kidneys. After a first conversion by a cholesterol side chain cleavage enzyme complex, pregnenolone is formed. Pregnenolone is converted into progesterone by 3ß-hydroxysteroid dehydrogenase or into 17α-hydroxypregnenolone by a 17α-hydroxylase. Both compounds can be converted into 17α-hydroxyprogesterone, that is further metabolized to androstenedione. Androstenedione can be converted into estrone by an aromatase or by 17ß-hydroxysteroid dehydrogenase into testosterone. In addition to the tissues mentioned above, aromatase activity is detected in placenta, fat tissue, fibroblasts, and brain tissue. Also in osteoblast and breast tumor cell lines aromatase activity is found (Purohit 1992, Pasqualini 1996, Tanaka 1996, Janssen 1999).

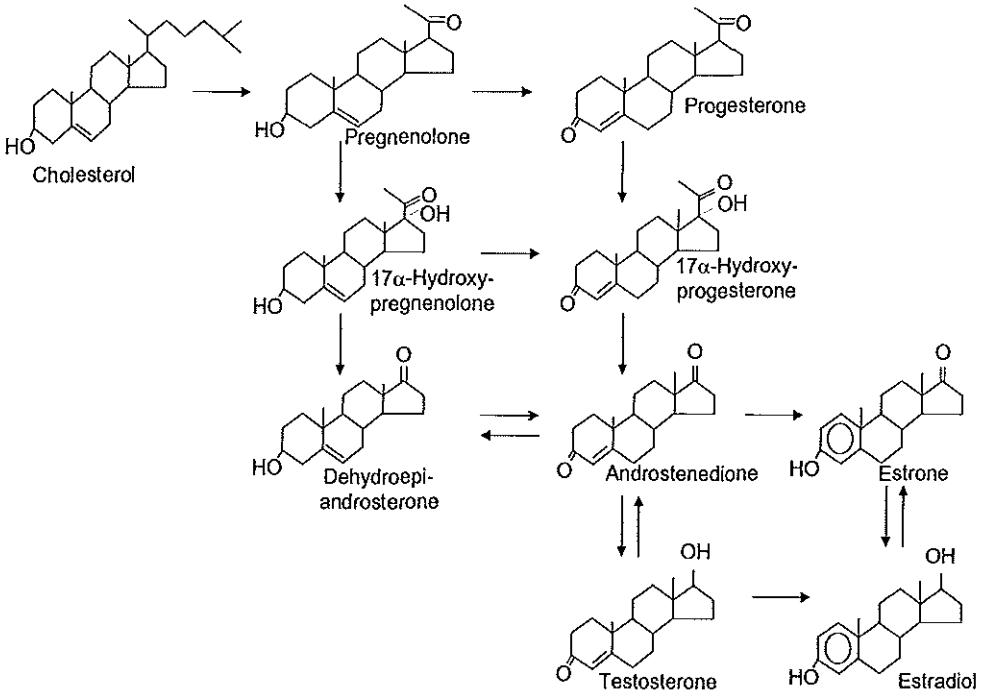

Figure 1.6. Formation of E_2. *Both testosterone and estrone are substrates for E_2 synthesis. Testosterone is converted by an aromatase, whereas estrone is metabolized by 17ß-hydroxysteroid dehydrogenase.*

More than 70% of E_2 secreted in the blood by the ovary is bound to albumin or sex hormone-binding globulin (Baker 1998). Metabolism of estrogens occurs mainly in the liver, but also in various other target tissues estrogen metabolism has been observed. Conjugated with glucuronic or sulfuric acid, the metabolites are secreted in the bile, reabsorbed into the blood and excreted in the urine as ß-glucuronides or sulfate esters.

B.4.2. ESTROGEN RECEPTOR LIGAND BINDING

An initial step in receptor activation is the ligand-induced conformational change of the ER (Beckman 1993). Ligand binding induces repositioning of the AF-2-containing helix 12 of the ligand binding domain, resulting in closure of the ligand binding pocket like

a mouse trap. Because of the more compact folding of the receptor its sensitivity for proteases is lowered, which was confirmed by limited proteolytic digestion analysis (Beekman 1993), and Chapter 6.

The cell sensitivity to estrogens is importantly influenced by ER levels in the cell. In a variety of cell lines a correlation was found between ER numbers and cellular responsiveness to estrogen (for references: see below). Modulation of ER levels is therefore an important mechanism to control target gene transcription. Like VDR and other nuclear receptors (See Section A.4.2., (Read 1988, Bellingham 1992, Wolf DA 1993), expression and/or stability of ER is under control of its ligand. During estrous cycle up-regulation of uterine ER mRNA and protein level is observed in an E_2 plasma concentration-dependent manner (Bergman 1992). E_2 increased ER expression in endometrium of ovariectomized ewes by increasing the stability of ER mRNA (Ing 1996, Zou 1998, Robertson 1998, Ing 1999). On the other hand, in a pituitary lactotrope cell line ER levels decreased after incubation with E_2 (Alarid 1999), and in liver of ovariectomized ewes single and chronic administration of E_2 resulted in ER down-regulation (Zou 1998). Bodine *et al.* demonstrated that the effect of E_2 on ER expression in rat calvarial-derived osteoblasts depended on cell differentiation stage. During the nodule-forming stage E_2 decreased ER mRNA, whereas during late mineralization stage E_2 up-regulated ER mRNA. As suggested by the investigators the developmental expression of ER mRNA might play a role in the observed osteoblast differentiation stage-dependent expression of bone cell genes like alkaline phosphatase, osteocalcin, and osteonectin (Bodine 1998). In the human breast cancer cell line T47D E_2 caused an increase in ER mRNA (Read 1989), whereas in MCF-7 cells ER mRNA and protein levels were down-regulated by E_2 (Read 1989, Saceda 1998, Alarid 1999, El Khissiin 1999). The SERM LY 117,018 itself was without effect but completely prevented E_2-induced down-regulation of ER in MCF-7 cells (Read 1989). The synthetic ER ligand tamoxifen induced ER up-regulation in MCF-7 cells, (Legros 1997) and mimicked the effect of E_2 on ER mRNA and protein levels in endometrium of ewes (Robertson 1998). In women a single dose of tamoxifen induced an increase in ER levels in endometrium (Gorodeski 1992), whereas chronic administration of tamoxifen caused down-regulation of ER levels (Perez-Lopez 1993). In breast cancer biopsies of postmenopausal women treated with tamoxifen increased ER levels were measured (Noguchi 1993).

Also heterologous regulation of ER has been observed. It was found that 1,25-$(OH)_2D_3$ (Bellido 1993), triiodothyronine (Fujimoto 1997), and the antiprogestin RU486 (Savoldi 1995), all increased ER mRNA stability and/or ER concentration in murine bone marrow-derived stromal cells, rat pituitary cells, and human breast cancer cell lines, respectively, whereas TGFβ (Read 1989), the androgen 5α-dihydrotestosterone (Poulin 1989), the synthetic progestin R5020 (Read 1989, Savoldi 1995), and 1,25-$(OH)_2D_3$ (Stoica 1999) had a decreasing effect on ER protein and mRNA levels in human breast cancer cell lines. A functional relevance for progestin-induced ER down-regulation in MCF-7 and

T47D breast cancer cells was found, as the decrease in ER levels was shown to correlate with decreased pS2 protein synthesis in response to E_2 (Savoldi 1995). Other studies also suggested that ER expression regulation has a functional role. For instance, ERα up-regulation in rabbit heart and aorta after cardiac-aorta allograft transplantation might be related to the cardiovascular protective effect of E_2 (Lou 1998), and the inverse relationship between circulating E_2 levels and ER mRNA expression in the anteroventral periventricular nucleus of the preoptic region of the rat is suggestive for a functional role of ER levels in the responsiveness of neurons of the anteroventral periventricular nucleus to E_2 (Simerly 1996). However, the change in ER levels is not always related to the biological response of the compound that induces the change in ER levels. For instance, tamoxifen-induced up-regulation of ER in MCF-7 breast cancer cells was not related to the growth inhibitory effect of tamoxifen on these cells (Legros 1997). All together these data indicate that ER level regulation is not an unequivocal process, but probably depends on the cell/tissue and its differentiation stage, the compound that induces the change in ER levels, and the way the compound is administered.

Regarding receptor degradation, like the VDR, the ER is probably ubiquitinated and subsequently degraded by the proteosome in a ligand-dependent manner (Nirmala 1995, Masuyama 1997, Alarid 1999, El Khissiin 1999, Nawaz 1999). SUG1, a cofactor involved in VDR turnover also binds to ER (Vom Bauer 1996) and is probably also playing a role in ER degradation by the proteasome (Masuyama 1997).

B.4.3. DISSOCIATION OF HEAT SHOCK PROTEINS

When present in the cytoplasm ERs are associated with heat shock proteins. Heat shock proteins probably play a role in stabilizing the receptor during synthesis and transport to the nucleus. Furthermore, the complex of receptor and heat shock proteins probably keeps the ER in a conformation with high affinity for ligand and low affinity for DNA. Ligand binding induces dissociation of heat shock proteins (Smith 1993). However, removal of heat shock proteins is not sufficient to obtain a transcriptional active ER (Picard 1990).

B.4.4. ESTROGEN RECEPTOR PHOSPHORYLATION

Like the VDR, the ER is phosphorylated under influence of its ligand (Auricchio 1987). Also other factors like epidermal growth factor, TGFα, and insulin-like growth factor I can activate ERs possibly by stimulating their phosphorylation at certain sites (Aronica 1993, Ignar-Trowbridge 1993, Bunone 1996). It was shown that phosphorylation of tyrosine within or near

the ligand binding domain increases ligand binding capacity (Migliaccio 1989), ER dimerization, and ERE binding (Arnold 1995). Phosphorylation by protein kinase A of serine 236 within the DNA binding domain of ERα also regulates ER dimerization (Chen 1999). Furthermore, serine 118 in the N-terminal A/B region is a specific phosphorylation site linked to AF-1 activity (Ali 1993, Joel 1995, Endoh 1999). Mitogen-activated protein kinase that is activated by E_2 and certain growth factors (e.g. epidermal growth factor) is involved in ER phosphorylation at serine 118 (Kato 1995, Bunone 1996, Endoh 1997).

B.4.5. ESTROGEN RECEPTOR DIMERIZATION

After E_2 binding ER dimerization is stimulated (Wang 1995) and DNA binding is facilitated (Kuntz 1997). Both ERα and ERß homodimers as well as ERα/ERß heterodimers have been described *in vitro* (Pace 1997, Cowley 1997, Pettersson 1997).

B.4.6. ESTROGEN RECEPTOR TRANSLOCATION

It was found that in the absence of ligand ERs are mainly located in the cytoplasm and that administration of E_2 leads to an increase in nuclear ER levels (Ferreira Mendes 1996). ER continuously shuttles between nucleus and cytoplasm (Guiochon-Mantel 1996).

B.4.7. ESTROGEN RECEPTOR-DNA BINDING

When looking at the numerous ER-regulated genes (Ciocca 1995), relatively few ERE-containing target genes have been identified (Table 1.4). It is conceivable that the ER regulates gene transcription of many genes not containing an ERE by modulating the activity of transcription factors like AP-1, NF kappa B, and Sp1 (Weisz 1993, Hyder 1995, Webb 1995, Porter 1997, Paech 1997, Duan 1998, Kleinert 1998, Cerillo 1998, Kuiper 1999, Xie 1999, Boffelli 1999).

Like the VDRE, ERE half-sites are (A)GGTCA or related motifs. The fact that the binding sites display almost perfect rotational symmetry suggests that ERs bind as dimers to these EREs (Kumar 1988, Klein-Hitpass 1989). However, exceptional ERE sequences have also been reported. For instance, the ERE found in the ovalbumine gene consists of four synergistically acting TGACC motifs separated from each other by more than 100 bp (Kato 1992) and EREs found in the rat prolactin gene have nonpalindromic sites (Mundoch 1995). Similar to the VDRE, most EREs are located in the 5'-region upstream of

their promoter, although EREs were also identified in the 3'-untranslated region (mouse c-fos) and within the coding sequence of the target gene (rat c-jun, Xenopus laevis ER, Oreochromis aureus vitellogenin). Note that several genes contain multiple ERE-like sequences. These sequences are only slightly active as isolated EREs but activate gene transcription in a cooperative way.

The presence of EREs within E_2-responsive genes can be species-specific. For instance, in the rat calbindin D-9k gene an imperfect palindromic ERE sequence was revealed (Darwish 1991), whereas no such sequence was found in the human and porcine calbindin D-9k gene (Jeung 1994, Krisinger 1995). Similar species-dependent differences in ER binding were observed between the rat luteinizing hormone β gene that contains an ERE (Shupnik 1991), and the bovine gene that lacks an ERE (Keri 1994).

The EREs reported to this date were characterized using ERα as the DNA binding protein. Based on the large homology in DNA binding domains between the two ER subtypes, it could be expected that ERα and ERß bind to the same EREs. In more detailed *in vitro* studies was found that the affinity of ERα for the vitellogenin ERE was higher than that of ERβ (Tremblay 1997, Cowley 1997), although others could not confirm this (Pettersson 1997). Also, gel mobility shift experiments performed with the choline acetyltransferase ERE and the cathepsin D ERE demonstrated that ERβ required higher DNA concentrations than ERα to form stable complexes *in vitro*, indicating that the affinity of ERα for these EREs might be higher than that of ERβ (Hyder 1999). Cowley *et al.* showed that ERα homodimers, ERß homodimers, as well as ERα/ERß heterodimers bound to the same ERE and that the affinity for the ERE depended on dimer composition: ERß homodimer exhibited the lowest affinity and ERα homodimers the highest, while heterodimers demonstrated intermediate affinity. For the transcriptional activation potency the same pattern was observed (Cowley 1997). In addition, it is still possible that specific EREs for the ER subtypes exist.

Some *in vivo* studies indicated that ligand seems a requirement to observe ER binding to DNA (Pham 1991, Gilbert 1992), although other studies show the opposite (Tzukerman 1990). Also *in vitro* the role of ligand in ER-ERE binding is not clear. In most gel mobility shift assays it was shown that ligand is not essential to observe ER binding to an ERE (Fawell 1990, Murdoch 1990, Sabbah 1991, Curtis 1991, Murdoch 1991, Inano 1994, Metzger 1995, Pace 1997, Tremblay 1998, Hyder 1999). However, some *in vitro* studies show ligand-dependent ER binding to DNA (Kumar 1988, Brown 1990, Beekman 1993, Hanstein 1999), possibly due to stringent

conditions in the gel mobility shift assay (e.g. magnesium concentration and temperature) (Brown 1990).

Table 1.4 Estrogen receptor-regulated genes and nucleotide sequences of estrogen response elements identified in those genes

GENE	NUCLEOTIDE SEQUENCE	(Refs)
Chicken vitellogenin A2	5'-GGTCA gcg TGACC-3'	(Burch 1988)
Chicken vitellogenin A2	5'-GGTCA aca TAACC-3'	(Burch 1988)
Chicken apo VLDLII	5'-GGGCT cag TGACC-3'	(Wijnholds 1988)
Chicken apo VLDLII	5'-GGTCA gac TGACC-3'	(Wijnholds 1988)
Human cathepsin D	5'-GGCCG ggc TGACC-3'	(Wang 1997)
Human c-fos	5'-CGGCA gcg TGACC-3'	(Weisz 1990)
Human lactoferrin	5'-GGTCA agg CGATC-3'	(Teng 1992)
Human oxytocin	5'-GGTGA cct TGACC-3'	(Richard 1990)
Human prolactin	5'-TGTCA cct TGGCC-3'	(Berwaer 1991)
Human pS2	5'-GGTCA cgg TGGCC-3'	(Berry 1989)
Mouse c-fos	5'-GGTCT agg AGACC-3'	(Hyder 1992)
Mouse choline acetyltransferase	5'-GGCCA cga TGACA-3'	(Hyder 1999)
Mouse lactoferrin	5'-GGTCA agg TAACC-3'	(Teng 1992)
Oreochromis aureus vitellogenin	5'-GGGCA cac TGACA-3'	(Teo 1998)
Oreochromis aureus vitellogenin	5'-CCAGT cca ACTTG-3'	(Teo 1998)
Rabbit uteroglobin	5'-GGTCA cca TGCCC-3'	(López de Haro 1990)
Rat calbindin D-9k	5'-GGTCA ggg TGATC-3'	(Darwish 1991)
Rat creatine kinase B	5'-GGTCA gaa CACCC-3'	(Wu-Peng 1992)
Rat c-jun	5'-GCAGA gca TGACC-3'	(Hyder 1995a)
Rat luteinizing hormone β	5'-GACAG atg GTGTC-3'	(Shupnik 1991)
Rat progesterone receptor	5'-GTTCA gtg TGAAC-3'	(Kraus 1994)
Rat progesterone receptor	5'-TGTCA aga TGTCC-3'	(Kraus 1994)
Rat progesterone receptor	5'-GGTCG tca TGACT-3'	(Kraus 1994)
Rat progesterone receptor	5'-GGACA gcc TGCCC-3'	(Kraus 1994)
Rat progesterone receptor	5'-GGACA gcc TGCCC-3'	(Kraus 1994)
Rat prolactin	5'-GGTCA caa GCTGC-3'	(Murdoch 1995)
Rat prolactin	5'-TGTCA cta TGTCC-3'	(Murdoch 1995)
Salmon gonadotropin II beta	5'-TGTCA atc TGACC-3'	(Liu 1995)
Xenopus laevis ER	5'-GGTCA gtt TGACG-3'	(Lee 1995)
Xenopus laevis vitellogenin A1	5'-TGCCA cac TGAGC-3'	(Walker 1984)
Xenopus laevis vitellogenin A1	5'-AGTCA ctt TGACT-3'	(Walker 1984)
Xenopus laevis vitellogenin A1	5'-GGTCA caa TGACC-3'	(Walker 1984)
Xenopus laevis vitellogenin A2	5'-GCCCA gag TGCCC-3'	(Walker 1984)
Xenopus laevis vitellogenin A2	5'-GGTCA cag TGACC-3'	(Klein-Hitpass 1986)
Xenopus laevis vitellogenin B1	5'-GGCCA cag TGAAC-3'	(Martinez 1987)
Xenopus laevis vitellogenin B1	5'-AGTCA ctg TGACC-3'	(Martinez 1987)
Xenopus laevis vitellogenin B1	5'-AGTTA tca TGACC-3'	(Martinez 1987)
Xenopus laevis vitellogenin B2	5'-AGTCA ctt TGACC-3'	(Walker 1984)
Xenopus laevis vitellogenin B2	5'-AGTTA tca TGACC-3'	(Walker 1984)

Despite these differences most researchers agree that addition of ligand changes the conformation of the ER-ERE complex. This might explain why in gel mobility shift assays the mobility of liganded ER-ERE complex is different from the mobility of unliganded ER-ERE complex (Lees 1989, Fawell 1990, Reese 1991, Sabbah 1991, Tremblay 1998). The ligand-induced conformation is a prerequisite for interaction with basal transcription factors and cofactors and essential for full transcriptional activation. Furthermore, presence of ligand seems to increase the stability of the ER-ERE complex (Dauvois 1992, Metzger 1995, Pace 1997).

As mentioned above, besides via EREs, ERs regulate gene transcription via AP-1 elements, Sp1 binding sites, and NF kappa B response elements (Weisz 1993, Hyder 1995, Webb 1995, Porter 1997, Paech 1997, Duan 1998, Kleinert 1998, Cerillo 1998, Kuiper 1999, Xie 1999, Boffelli 1999). For instance, synthesis of chicken insulin-like growth factor I is controlled by E_2 via an AP-1 motif located in its promoter region (Umayahara 1994). Also transcription of the chicken ovalbumin gene is activated by interaction of fos, jun (AP-1) and the ER (Gaub 1990). Instead of DNA binding, protein-protein interactions seem to be involved in ER activation at AP-1 sites since the DNA binding domain of the ER is not absolutely required (Webb 1995). Interestingly, besides E_2 also (pure) antiestrogens are able to activate the AP-1-mediated response by ER in the context of the collagenase promoter (Paech 1997).

B.4.8. ESTROGEN RECEPTOR-COFACTOR BINDING

ER binding to an ERE results in DNA bending (Sabbah 1992, Gilbert 1992, Nardulli 1993, Nardulli 1993a, Nardulli 1995). *In vivo* ER-induced alterations in chromatin structure are dependent upon the presence of E_2 (Gilbert 1992). However, *in vitro* no difference in DNA bending was observed with liganded and unliganded ER, indicating that ER-induced DNA bending alone is not sufficient to activate gene transcription. However, it is still believed that DNA bending is essential for gene transcription: it might facilitate interaction between proteins bound to separate sites on DNA as was indicated by the finding of interaction between the AF-2 domain with certain factors (TFIIB, and TBP, a subunit of TFIID) of the preinitiation complex at the TATA box) (Ing 1992).

In addition to basal transcription factors also (preformed complexes of) cofactors are essential for optimal regulation of gene transcription (Jenster 1998, McKenna 1999). The ligand-induced repositioning of the AF-2-containing helix 12 exposes specific amino acids critical for cofactor binding (See Sections A.2, A.4.8., and B.2.) (Tzukerman 1994). Binding of the nuclear protein RIP140, SRC-1, TIF 1, TIF2/GRIP1, and estrogen receptor-associated protein ERAP160 has been observed (Halachmi 1994, Cavaillès 1995, Onate 1995, McInerney 1996, Le Douarin 1996, Voegel 1996, Hong 1997, Cowley 1997, Tremblay 1997). E_2, other steroids, estrogen

receptor agonists and phytoestrogens are able to stimulate interaction of ER with these cofactors, and may thereby determine their estrogenic activity [Nishikawa 1999].

B.5. NONGENOMIC EFFECTS OF ESTROGENS

Estrogens also exert nongenomic effects [Pietras 1977, Weiss 1988, Brubaker 1999]. It was for instance shown that E_2, estrone and estriol rapidly (i.e. within 5 seconds) increased the calcium ion concentration of chicken and pig granulosa cells. The transcription inhibitor actinomycin D and the protein synthesis inhibitor cycloheximide were not effective in blocking this rapid response. However, inhibitors of inositol phospholipid hydrolysis could abolish the effect of the estrogens, indicating that estrogens trigger the release of calcium from intracellular stores through induction of phosphoinositide breakdown [Morley 1992]. Furthermore, it was shown that E_2-induced activation of mitogen-activated protein kinase is preceded by mobilization of intracellular calcium, suggesting that nongenomic regulation of calcium homeostasis and mitogen-activated protein kinase signaling pathway are connected by the action of E_2 [Improta-Brears 1999]. The rapid stimulation of alkaline phosphatase in chondrocytes by E_2 appeares to be mediated by nongenomic stimulation of protein kinase C [Sylvia 1998]. Furthermore, Alarid *et al.* attributed rapid E_2-induced degradation of ER in pituitary lactotrope cells and MCF-7 breast cancer cells to a nongenomic activity of estrogens [Alarid 1999].

Similar to the VDR, a membrane version of the ER that could mediate these rapid responses of E_2 was detected in a pituitary tumor cell line and in MCF-7 human breast cancer cells [Watson 1999, Norfleet 1999]. Also in rabbit uterus membrane estrogen binding proteins structurally related to intracellular ER were detected that could be important in mediating nongenomic effects of E_2 [Monje 1999]. Razandi *et al.* demonstrated that both nuclear and membrane ERs are derived from the same cDNA transcript. They also showed that membrane versions of both ERα and ERβ exist [Razandi 1999]. However, the biological relevance of these membrane receptors and the mechanism of action by which these receptors might act are still to be resolved.

B.6. SELECTIVE ESTROGEN RECEPTOR MODULATORS

The side effects of estrogen replacement therapy and hormone replacement therapy: resumption of menses, breast tenderness, abdominal bloating [Upton 1982] and possible increased relative risk for breast and uterine cancer, have led to an intensified search for synthetic ER ligands with the beneficial effects of E_2 (the effects on bone mass and the cardiovascular system), but with a diminished risk for development of breast

and uterus cancer. Because of their antagonistic effect on estrogen-dependent breast cancer growth, these compounds were originally named antiestrogens. The fact that these compounds exert their action via the ER and based on their tissue selective agonistic or antagonistic activity, makes the designation 'selective estrogen receptor modulators' (SERMs) more appropriate. Table 1.5 summarizes the agonistic/antagonistic action of E_2 and several synthetic ER ligands in various target tissues. Between the two extremes- E_2, as complete agonist, and N-n-butyl-11-(3,17β-dihydroxyoestra-1,3,5(10)-trien-α-yl)n-methyl-undecanamide (ICI 164,384) and 7-α-[9-(4,4,5,5,5-pentafluoro-pentylsulphinyl)nonyl]oestra-1,3,5(10)-triene-3,17 beta-diol (ICI 182,780) known as pure antagonists- there is a range of SERMs that exert tissue-specific agonistic or antagonistic activity (Bryant 1998).

Table 1.5. Agonistic/antagonistic activities of E_2 and several synthetic ER ligands in various target tissues

	Uterus	Bone	Breast	Cardiovascular
E_2	Agonist	Agonist	Agonist	Agonist
Diethylstilbestrol	Agonist	Agonist	Agonist	Agonist
Raloxifene	Antagonist	Agonist	Antagonist	Agonist
Tamoxifen	Partial agonist	Agonist	Antagonist	Agonist
4-Hydroxytamoxifen	Partial agonist	Agonist	Antagonist	Agonist
ICI 164,384	Antagonist	Antagonist	Antagonist	Antagonist
ICI 182,780	Antagonist	Antagonist	Antagonist	Antagonist

The activities of E_2 and SERMs in the various target tissues are discussed in more detail in Section B.6.1. Partial agonists mimic the action of E_2, albeit to a lesser extent, whereas an antagonist counteracts the action of E_2. In Figure 1.7 the chemical structures of these compounds are presented.

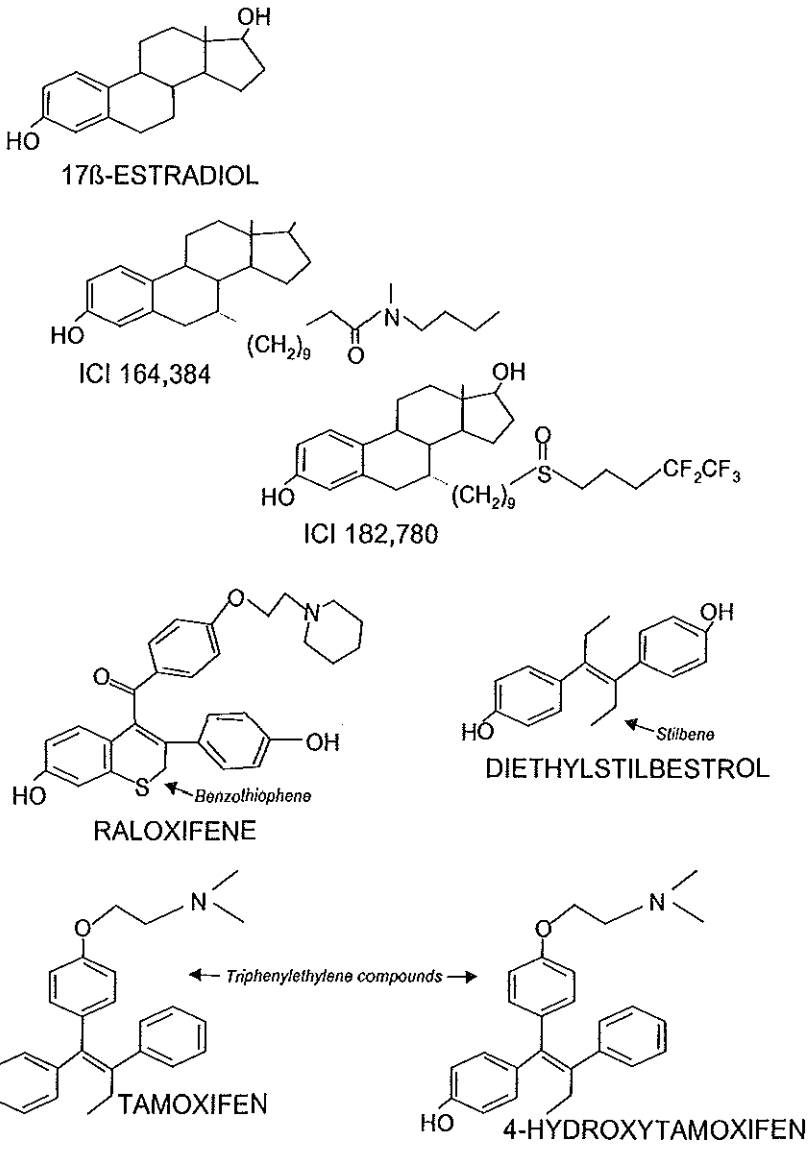

Figure 1.7. Chemical structures of E_2 and a selection of synthetic ER ligands. *These synthetic compounds were developed by successive chemical modifications at the triphenylethylene nucleus, formed by addition of an extra phenyl ring to the stilbene nucleus as present in for instance diethylstilbestrol, the most potent synthetic estrogen. The ICI compounds contain extensions at C7 of E_2* (Grainger 1996, Wakeling 1992).

B.6.1. BIOLOGICAL EFFECTS AND CLINICAL APPLICATIONS

Uterus

In the uterus tamoxifen can be classified as an agonist of E_2: It mimics the effect of estrogen on uterine weight [Kedar 1994]. The risk of this stimulatory effect on the uterus by tamoxifen is illustrated by the report of an increased number of patients with uterine carcinomas after breast cancer treatment with tamoxifen [Fisher 1994]. In most tissues raloxifene and tamoxifen have a similar profile. However, raloxifene is an antagonist in the uterus: Treatment of postmenopausal women with raloxifene did not result in uterine stimulation [Draper 1996]. In most studies, ICI 182,780 and ICI 164,384 lack any agonistic activity. However some studies demonstrated agonistic activity in uterine cells [Chetrite 1991], and endometrial cancer cells [Jamil 1991, Castro-Rivera 1998]. Therefore, although their selective effect is limited, the ICI compounds should perhaps also be considered as SERMs. The possible mechanism underlying the agonistic potency of the ICI compounds will be discussed in more detail in Chapter 7.

Bone

In vitro experiments demonstrated that high concentrations (100 μM) of SERMs like tamoxifen and clomiphene inhibit bone resorption induced by PTH, prostaglandin E_2, and 1,25-$(OH)_2D_3$ [Stewart 1986]. We showed that E_2 did not inhibit 1,25-$(OH)_2D_3$-stimulated *in vitro* bone resorption, whereas ICI 164,384 and tamoxifen did [Vink-Van Wijngaarden 1995]. *In vivo* E_2 and tamoxifen decreased bone resorption in ovariectomized rats [Turner 1988]. In addition, breast cancer patients treated with tamoxifen were protected against increased bone loss [Love 1992]. Raloxifene exerts also E_2-like effects on bone. Both E_2 and raloxifene inhibited interleukin 6-induced bone resorption and stimulated the TGFβ-inhibitory effect on osteoclast differentiation and activity [Bryant 1999]. Postmenopausal osteoporotic women treated with raloxifene or E_2 had increased bone mineral density values, whereas women receiving placebo had decreased bone mineral density values compared to values at base-line. Furthermore, raloxifene reduced fracture incidence [Draper 1996, Delmas 1997, Lufkin 1998].

Cardiovascular system

Beneficial effects of estrogens and SERMs on the cardiovascular system (e.g. on vascular smooth muscle proliferation, low density lipoproteins oxidation and effects on endothelial factors) are known. Administration of tamoxifen and raloxifene led to a decrease in cholesterol levels in ovariectomized rats and postmenopausal women [Draper 1996, Walsh 1998]. The number of cardiovascular events in breast cancer patients treated with tamoxifen was decreased [Rutqvist 1993].

Breast tumors

E_2 is an important regulator of normal and malignant cell growth. After estrogen replacement therapy increased risk for development of breast cancer is still a cause for concern and as mentioned above was a stimulus to find new ER ligands with a more beneficial biological profile. Tamoxifen was one of the first synthetic ER ligands evaluated for this purpose. *In vitro*, in the absence of E_2, tamoxifen and the tamoxifen metabolite 4-hydroxytamoxifen stimulate the growth of the human breast cancer cell line MCF-7 [Bignon 1988, Vink-Van Wijngaarden 1993]. However, in co-treatment experiments these synthetic ER ligands can be classified as antagonists: they inhibit E_2-induced cell proliferation [Vink-Van Wijngaarden 1993]. In clinical studies, tamoxifen was very successful in the treatment of breast cancer. More than 50% of human breast cancers contain ERs and 70% of these ER-positive tumors are inhibited in their growth by tamoxifen [Jordan 1992]. Nowadays tamoxifen is the most widely used endocrine therapy for breast cancer. However, during prolonged therapy tamoxifen resistance and relapse of breast tumor growth is observed. As underlying mechanism of tamoxifen resistance several possibilities were proposed: e.g. the occurrence of ER mutations and the accumulation of tamoxifen metabolites with less antagonistic or even agonistic activity [Wiebe 1992], although others showed that these aspects are probably not playing a major role [Wolf DM 1993, Katzenellenbogen 1997]. Alternatively, alternate ER activation pathways (alterations in ER phosphorylation, changed interactions with AP-1-mediated gene transcription) might be involved. See also B.6.2., part V.

B.6.2. POSSIBLE MECHANISMS FOR THE SELECTIVE ACTION OF SELECTIVE ESTROGEN RECEPTOR MODULATORS

The mechanism of action of a pure E_2 antagonist could, in theory, be very simple: a ligand that lacks any estrogenic effect will compete with E_2 for binding to the ER and in this way it prevents transcriptional activation. However, SERMs exert partial agonistic/antagonistic activity. The fact that agonistic/antagonistic activities are species-, tissue-, cell-, and target gene-specific makes the matter even more complicated. Fact is that the mechanism of action starts with competition of the SERM with the natural ligand E_2 for the ER ligand binding site. After that, any defective interaction with an event in the cascade leading to activation of the ER (see Section A.4.) will result in full or partial reduction of the potency of the SERM-ER complex to modulate target gene transcription. In the section below several possible mechanisms of action to explain selective antagonistic/agonistic activity of SERMs are presented.

I. RECEPTOR NUMBER, AFFINITY, CONFORMATION AND HALF-LIFE

As mentioned in Section B.4.2. the biological response of ER ligands is importantly influenced by the level of ERs in target cells. It was reported that tamoxifen mimics E_2 in increasing ER mRNA levels in endometrium (Gorodeski 1992, Robertson 1998). In contrast, ICI 164,384 and ICI 182,780 induce rapid loss of ER in uterus and other cells, a feature that might underly the antagonistic property of this compound (Gibson 1991, Reese 1991, Dauvois 1992, Jensen 1999). In addition, SERMs might have an ER subtype-selective up-regulatory effect. In Chapter 6 we will show that the effect of ICI on ER stability is ER subtype-dependent. Also for RU 58668 strongly decreased ER levels in the human breast cancer cell line MCF7 was reported, indicating that this pure antagonist of E_2 might act via a similar mechanism as the ICI compounds (Jin 1995).

ERα and ERβ show around 60% homology in their ligand binding domain and, in general, both ER subtypes demonstrate similar binding affinity for a large number of ligands. However, some phytoestrogens (coumestrol, genistein) show increased binding affinity for ERß (Kuiper 1997). Therefore, it might be feasible that ER subtype-selective ligands exists or can be developed. However, one should note that, similar to the situation with the VDR, the binding affinity of a ligand for the ER probably does not reflect its biological potency (Yang 1996).

Differences in effect on ER conformation probably forms the basis of the selective action of SERMs. X-ray crystal structure studies (Brzozowski 1997, Shiau 1998), partial digestion analysis (Beekman 1993, McDonnell 1995), antibody detection (Martin 1988), and phage ELISA (Paige 1999) revealed that E_2 and SERMs induce distinct conformational changes of ER. Ligand-induced changes in ER conformation will also interfere with subsequent steps in the transactivation cascade: ER dimerization, DNA binding and cofactor interaction. Furthermore, a change in ER conformation can also result in a change in ER half-life, since the accessibility of protease cleavage sites of enzymes which take care of receptor processing can also be affected.

E_2 and synthetic agonists like diethylstilbestrol (see Figure 1.7 for chemical structure) induce a conformational change of the ER characterized by refolding of the ligand binding domain. In this process described in the so-called mouse trap model, helix 12 is positioned over the ligand binding pocket formed by helices 3, 5/6, and 11. Raloxifene contacts the hydrofobic groove formed by helices 3 and 5, but in addition the alkylaminoethoxy side chain of raloxifene interacts with amino acid 351 in helix 3 (Brzozowski 1997). This specific interaction prevents helix 12 from rotating and might affect interaction of the receptor with cofactors essential for full receptor activation. Interestingly, studying the effect of raloxifene on TGFα mRNA induction in MDA-MB 231 human breast cancer cells this SERM is an antagonist with wild type ER, whereas raloxifene acquires agonistic potency when the cells were transfected with ERs with a mutation at position 351 (Levenson 1998). Likewise, the side chain of 4-hydroxytamoxifen prevents helix 12 from folding over the ligand binding pocket (Shiau

[1998]. Changed folding of helix 12 will influence presentation of certain residues (AF-2) important for cofactor interaction. This will affect AF-2 function and might explain why raloxifene and 4-hydroxytamoxifen exert tissue-dependent partial agonistic/antagonistic activity.

The conformational change of ERα induced by ICI 164,384 and ICI 182,780 might be an explanation for their antagonistic potency in many responses studied. ICI 164,384 increases turnover of ERα, both *in vitro* [Dauvois 1992] and *in vitro* [Gibson 1991] and it is hypothesized that this is a direct consequence of the induced conformational change (see Chapter 6).

II. RECEPTOR PHOSPHORYLATION

Although little is known concerning SERM-specific effects on ER phosphorylation, some data indicate that this aspect might be involved in the selective effect of SERMs. For instance it was shown that tamoxifen inhibited E_2-stimulated ER phosphorylation [Auricchio 1987], and that in the absence of E_2, ICI 164,384 and 4-hydroxytamoxifen stimulated ER phosphorylation although not as effectively as the natural ligand [Ali 1993].

III. RECEPTOR DIMERIZATION

Although ligand seems to be no prerequisite to observe ER dimerization *in vitro* [Sabbah 1989, Chen 1999], it was shown that E_2 and 4-hydroxytamoxifen promoted ER dimerization [Dauvois 1993]. In contrast, it was proposed that the antagonistic activity of ICI 164,384 is based on its inhibitory effect on ER dimerization, resulting in decreased ERE binding and diminished transcriptional activation [Fawell 1990, Arbuckle 1992]. Furthermore, impaired dimerization might result in a shorter half-life of the receptor, as was observed in mouse uterus, mouse testes cells, human breast cancer cells, and transfected COS-1 cells [Gibson 1991, Reese 1991, Dauvois 1992]. However, Metzger *et al.* and Lees *et al.* reported that ICI 164,384 did not impair ER dimerization [Lees 1989, Metzger 1995]. Also, Wang *et al.* demonstrated, using the yeast two-hybrid system, that not only the partial agonist/antagonist tamoxifen but also the pure antagonist ICI 182,780 was able to induce ER dimerization, although to a lesser extent than E_2. However, when E_2 and the antiestrogens were added simultaneously, dimerization was perturbed [Wang 1995].

IV. TRANSLOCATION OF THE RECEPTOR TO THE NUCLEUS

As already mentioned, E_2 induces translocation of ER to the nucleus [Guiochon-Mantel 1996]. SERMS also interfere with migration of ER to the nucleus. In addition, export from the nucleus, nucleocytoplasmic shuttling, also takes place. The observed disruption of nucleocytoplasmic shuttling of ERs observed after treatment with ICI could also be a consequence of impaired ER dimerization. It was found that ICI 182,780 and ICI

164,384 were able to block nucleocytoplasmic shuttling of ERs and caused accumulation of ERs in the cytoplasm (Dauvois 1993, Ferreira Mendes 1996). Interestingly, treatment with tamoxifen also led to decreased nucleocytoplasmic shuttling, but instead of accumulation in the cytoplasm nuclear retention of ERs was observed (Ferreira Mendes 1996).

V. DNA INTERACTION

Partial agonistic/antagonistic effect of SERMs is probably not a result of blocking ER-ERE binding. In most studies it was shown that binding of *in vitro* synthesized ER to an ERE did not require ligand and that ER-ERE binding was not affected quantitatively by E_2 or SERMs (Lees 1989, Reese 1991, Metzger 1995). Also *in vivo* ER binding to DNA was not prevented by SERMs (Reese 1991, Metzger 1995). However, for pure antagonists like ICI 164,384 and ICI 182,790 this matter seems more complicated. Some reports showed an impaired *in vitro* DNA binding of ERs after treatment with the ICI compounds (Fawell 1990, Arbuckle 1992). In contrast, others demonstrated ER binding to DNA when the receptor is occupied by ICI (Lees 1989, Martinez 1989, Berry 1990, Reese 1991, Sabbah 1991, Tremblay 1998). Also *in vivo* data are conflicting. Reese and Katzenellenbogen showed that ERs exposed to ICI 164,384 *in vivo* did not bind to DNA (Reese 1991). However, a report by Pham *et al.* demonstrated that ICI 164,384 was able to induce ERE binding *in vivo* (Pham 1991). Also, more recently ICI-induced *in vivo* ER-ERE binding was reported (Metzger 1995).

Binding of the ER dimer to an ERE induces folding of DNA (Sabbah 1992, Nardulli 1993, Nardulli 1993a, Nardulli 1995). It is plausible that interaction of ERs with a SERM-induced conformation will result in folding of DNA distinct from the situation where the ER conformation is induced by E_2. However, data from Pham *et al.* showed similar alterations of chromatin structure when ER is bound to E_2 or the antiestrogen nafoxidene, although the DNAse I sensitivity assay used is not very sensitive (Pham 1991).

Differences in DNA folding/conformation can be visualized to some extent in gel mobility shift assays. Several studies showed that an E_2-ER-ERE complex had increased gel mobility compared to antiestrogen-ER-ERE complexes, indicating that distinct conformational differences between the agonistic- and antagonistic-ER-ERE complexes exist (Kumar 1988, Lees 1989, Fawell 1990, Curtis 1991, Reese 1991, Sabbah 1991, Beekman 1993, Pace 1997). Furthermore, work from Klinge *et al.* provided immunological data indicating that conformations of E_2-ER-ERE, tamoxifen-ER-ERE, and 4-hydroxytamoxifen-ER-ERE are clearly distinct (Klinge 1996, Klinge 1998).

In addition it was shown that stability and kinetics of ER-ERE complexes formed differed between ER ligands, (Cheskis 1997) and also depended on flanking sequences of the ERE, as well as on cellular context (e.g. cell specific cofactors) (Anolik 1993, Anolik 1996). The stability of the ligand-induced ER-ERE complex may also differ among ER

subtypes. For instance, in gel mobility shift assays ICI 182,780 could not prevent loss of ERα binding to an ERE after temperature elevation, whereas ERβ-DNA binding was not affected [Pace 1997]. Furthermore, it was demonstrated that the agonistic activity of tamoxifen and nafoxidene depended on the ERE sequence itself [Dana 1994] and on the promoter context in which the ERE was studied [Watanabe 1997].

Homodimers of ERα or ERß, as well as ERα/ERß heterodimers have been described and it might be possible that each dimer interacts preferentially with a specific ERE. Differences in affinity between the three dimer forms (ERα/ERα, ERα/ERβ, ERβ/ERβ) for the same ERE is in this respect suggestive [Cowley 1997]. Experiments with transfected COS-1 cells showed that ERα has greater gene transcriptional activity via the luteinizing hormone ß ERE, whereas ERß preferentially activates vitellogenin ERE transcription [Pennie 1998]. Therefore, possible SERM-specific induction of a certain ER dimer form may lead to differential activation of ER target genes.

Besides via EREs, ERs regulate gene transcription via protein-protein interaction at AP-1 elements [Webb 1995, Paech 1997]. Webb *et al.* speculated that activation of AP-1-mediated gene transcription by tamoxifen might underly tissue-specific agonistic effects of this SERM. This feature of tamoxifen might also underly tamoxifen-stimulated growth of ER positive tumor cells (discussed in Section B.6.1.). In this respect, the observation of Johnston *et al.* is of interest. They observed increased AP-1 binding and c-jun NH_2-terminal kinase activity (an AP-1 activating enzyme) in tamoxifen-treated ER positive breast tumors with acquired tamoxifen resistance compared to untreated tumors or tumors that were arrested in their growth by tamoxifen [Johnston 1999]. Paech *et al.* reported differential activation of ERα and ERß by E_2 and SERMs at an AP-1 element: With ERα, both E_2 and SERMs stimulated gene transcription, whereas with ERß, E_2 inhibited and the SERMs stimulated gene transcription [Paech 1997]. Interestingly, the ICI compounds were also able to activate AP-1 responsive genes via ERα and ERβ [Webb 1995, Paech 1997].

Another example of ligand-specific interaction with regulatory elements in DNA is the strong *in vitro* activation of the bone matrix protein TGFß3 gene by raloxifene, tamoxifen, and ICI 164,384, but only weak activation by E_2 via a sequence in the promoter region of the TGFß3 gene, called the raloxifene response element [Yang 1996]. Interestingly, binding of the ER to this response element was not dependent on the DNA binding domain of the ER, indicating that an adaptor protein might interact [Yang 1996].

VI. COFACTOR INTERACTION AND CELLULAR CONTEXT

In vitro studies revealed that identical reporter constructs in different cell types gave contrasting results. In some cells SERMs acted as agonists, whereas in other cell types antagonistic properties were observed [Berry 1990, Watanabe 1997]. The ER contains two AFs (see Section B.2. and Figure 1.5) [Tora 1989] and in some tissues full agonistic activity is only achieved when both AF-1 and AF-2 are active, whereas in other tissues AF-1 is sufficient to obtain maximal transcriptional activity of the ER [Kuiper 1999]. SERMs like tamoxifen and its metabolite 4-hydroxytamoxifen block AF-2 activity, whereas AF-1 activity is only partially decreased [Webster 1988, Berry 1990]. This might explain the partial agonistic/antagonistic activity of these compounds in uterus where the AF-1 activity is high. In breast tissue, both AF-1 and AF-2 are required for maximal transcriptional activity of the ER and here tamoxifen acts antagonistic [Berry 1990]. However, cell- or tissue-specific differences in AF-1 activity do not provide a complete explanation for the tissue-specific agonistic/antagonistic activity of tamoxifen, since tamoxifen also exerts agonistic activity in cells with low AF-1 activity (e.g. endometrial and cervical cells). As already mentioned in Section V on DNA interaction, the activation of AP-1-mediated genes as suggested by Webb *et al.* [Webb 1995] as well as other transcription factor-mediated gene transcription processes might be involved.

The cell-, and tissue-dependent activity of AF-1 and AF-2 indicates that the cellular and nuclear context is an important determinator in this respect. Therefore, presence and relative distribution of cofactors (both activators and suppressors), combined with the potency of the ER ligand to induce their interaction with the ER are likely important factors in deciding whether a SERM acts agonistic or antagonistic in a specific cell type. Here, prevention of interaction of ERα and ERß with the coactivators SRC-1, GRIP1, TIF2, ERAP160 and RIP140 by synthetic ER ligands like ICI 164,384, ICI 182,780, 4-hydroxytamoxifen and EM-800 is of interest [Cavaillès 1995, Halachmi 1994, Voegel 1996, Schwartz 1998, Tremblay 1998]. Work of Shiau *et al.* demonstrated clear differences in ER folding and subsequent cofactor binding when an estrogen (diethylstilbestrol) or a SERM (4-hydroxytamoxifen) is bound. Binding of diethylstilbestrol to the ligand binding domain of ERα results in exposition of a hydrofobic groove formed by residues from helix 3, 4, 5, and 12, and the turn between helixes 3 and 4. Within this groove interaction sites for cofactor GRIP1 are located. In contrast, with 4-hydroxytamoxifen these sites are occluded by helix 12 and, as a consequence, GRIP1 binding is prevented [Shiau 1998]. Also work of Norris *et al.* using phage display indicated that E_2- and SERM-mediated interaction with cofactors is distinct [Norris 1999].

A different distribution of ER subtypes in target tissues might be involved in tissue-selective agonistic or antagonistic activities of tamoxifen and other SERMs. Although both ERs are highly homologous (see Section B.2.), the A/B domain

containing the AF-1 function is poorly conserved (about 20%). It was shown that AF-1 of ERß was not sufficient to activate ERß bound to tamoxifen [Watanabe 1997]. However, tamoxifen regained agonistic activity with ERß when its A/B domain was replaced with the A/B domain of ERα [McInerney 1998]. Observations by Kobori *et al.* indicate that the cell-, and tissue-specific distribution of ER subtypes and cofactors might explain cell type-specific effect of ER ligands. They found that transactivation by E_2 of ERE-containing reporter gene constructs was much higher with ERß than with ERα in osteoblast cell lines, whereas in non-osteoblast cell lines the transactivation activity of both receptors was reversed [Kobori 1998].

In a rat prostate cDNA library an ERβ splice variant (ERβ2) was found with a 18 amino acids insertion within the hormone binding domain [Hanstein 1999]. In contrast to the original ERβ and ERα, ERβ2 did not interact with the cofactor SRC-1 and its sensitivity for E_2 was 1000-fold lower [Hanstein 1999]. Recently, ER subtype-selective interaction with cofactors was also described by Endoh *et al.* [Endoh 1999]. They found that coactivator p68 specifically interacted with the AF-1 region of ERα and not with ERβ. In addition, Tremblay *et al.* showed that SRC-1 and Ras enhanced the transcriptional activity of ERα only in the presence of E_2, while the activity of ERβ was enhanced by these cofactors both in the absence and presence of E_2 [Tremblay 1998]. Furthermore, certain ligands and distinct DNA interaction sites (ERE, AP-1, SP1) might have a preference to bind one of the two ER subtypes [Kuiper 1997, Kuiper 1998, Hyder 1999]. Therefore, more abundant expression of either ERα or ERβ might influence the tissue- or cell-specific response. In this respect the report of Pujol *et al.* of a shift in the ratio of ERα/ERß during carcinogenesis of the ovary in favor of ERα is of interest [Pujol 1998].

VII. LIGAND METABOLISM

The efficacy of SERM delivery via the blood stream and entrance of the SERM into the target cell will determine its local concentration in the target cell. Binding of the SERM to albumin, sex hormone-binding globulin, or other carrier proteins are important aspects in this respect. In addition, SERMs might have a regulatory effect on the production of these serum binding proteins. It was for instance shown that tamoxifen or droloxifene therapy of postmenopausal breast cancer patients increased the serum sex hormone-binding globulin concentration [Rose 1992, Geisler 1995], whereas ICI 182,780 had no effect [Howell 1996]. Furthermore, SERMs might interfere in the percentage free and protein bound E_2. Tamoxifen therapy reduced the percentage of non-protein bound E_2 and albumin-bound E_2, whereas it increased sex hormone-binding globulin-bound E_2 [Rose 1992]. Also, metabolic stability of the SERM, as well as the biological activity of the metabolites formed might determine the eventual potency. These processes could be cell- or tissue-specific. However, for two major

metabolites of raloxifene (raloxifene-4'-ß-glucuronide and raloxifene-6-ß-glucuronide) no target tissue-differential conversion to raloxifene was observed, indicating that the tissue-specific effect of raloxifene (agonistic in bone and antagonistic in uterus), does not result from tissue-selective deconjugation of these metabolites (Dodge 1997).

VIII. NONGENOMIC EFFECTS

Although little data on this topic are reported, it is known that SERMs also exert nongenomic effects (Weiss 1988). It was found that tamoxifen had an inhibitory effect on protein kinase activity (O'Brian 1985) and possibly induces apoptosis of a hypothalamic neuronal cell line via a nongenomic pathway (Hashimoto 1997).

C. SCOPE OF THE THESIS

Several of the synthetic ligands of VDR (1,25-$(OH)_2D_3$ analogs) and ER (SERMs) have improved therapeutic characteristics compared to the natural ligands. For 1,25-$(OH)_2D_3$ analogs, these include the increased divergence between effects on cell proliferation/differentiation and calcemic effects. For the SERMs, clinical relevance lies in an enlarged separation between beneficial effects on bone and the cardiovascular system on the one hand and effects on development and growth of breast and uterus cancer on the other hand.

The mechanism(s) of action by which the synthetic ligands of VDR and ER determine the rate of gene transcription, often in a gene-, cell-, or tissue-dependent manner are largely unknown. Gene transcription is regulated by a complex cascade of events including ligand-receptor binding, receptor phosphorylation, and receptor-DNA interaction. The aim of the studies presented in this thesis is to provide more insight into the processes playing a role in gene transcription regulation and to investigate the relevance of these processes to the altered biological profiles of synthetic VDR and ER ligands.

Several aspects of interaction of 1,25-$(OH)_2D_3$ analogs and SERMs with their receptors were studied. In Chapter 2, the effects of 1,25-$(OH)_2D_3$ analogs on the growth and differentiation of osteoblast-like cells and the effect on *in vitro* bone resorption were evaluated. A possible explanation for the observed increased biological activity of one of the most potent 1,25-$(OH)_2D_3$ analogs, KH1060 was further investigated. We have studied its effect on the conformational change and stabilization of the VDR (Chapter 3). The effect of nucleotide sequences of VDREs on 1,25-$(OH)_2D_3$- and KH1060-induced VDR/RXR binding and transactivation was examined in Chapter 4. The contribution of metabolites to the biological potency of

the parent compound KH1060 is described in Chapter 5. In Chapter 6 we have studied the effect on ERα and ERß conformation by the SERMs tamoxifen, ICI 164,384, and ICI 182,780. Finally, in Chapter 7, the results are discussed and summarized and suggestions for further research are made.

CHAPTER 2

DIFFERENTIAL EFFECTS OF 1,25-DIHYDROXYVITAMIN D_3 ANALOGS ON OSTEOBLAST-LIKE CELLS AND ON *IN VITRO* BONE RESORPTION

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J Steroid Biochem Molec Biol 55, 337-346, 1995

2.1. ABSTRACT

Although numerous studies have shown potent antiproliferative and differentiation-inducing effects of 1,25-$(OH)_2D_3$ and its analogs on cells not directly related to bone metabolism, only few reports focussed on the effects of these analogs on bone. We compared the action of several recently developed analogs with that of 1,25-$(OH)_2D_3$ on human (MG-63) and rat (ROS 17/2.8) osteoblast-like cells and on *in vitro* bone resorption. In MG-63 cells the analogs EB1089 and KH1060 were about 166,000 and 14,000 times more potent than 1,25-$(OH)_2D_3$ in stimulating type I procollagen and 100 and 6,000 times more potent in stimulating osteocalcin production, respectively. Also in ROS 17/2.8 cells EB1089 and KH1060 were most potent in inducing osteocalcin synthesis. *In vitro* bone resorption was 2.3 and 17.5 times more potently stimulated by EB1089 and KH1060, respectively. In MG-63 cells, 1,25-$(OH)_2D_3$ and the analogs inhibited cell proliferation, whereas both 1,25-$(OH)_2D_3$ and the analogs stimulated the growth of ROS 17/2.8 cells. Differences in potency could neither be explained by affinity for the VDR nor by a differential involvement of protein kinase C in the action of the analogs. Together, these data show that also in bone the analogs EB1089 and KH1060 are more potent than 1,25-$(OH)_2D_3$, but that the potency of the analogs compared to 1,25-$(OH)_2D_3$ is dependent on the biological response. On basis of these observations it can be concluded that the reported reduced calcemic effect *in vivo* is not the result of a decreased responsiveness of bone to these analogs. Lastly, in view of eventual clinical application of 1,25-$(OH)_2D_3$ analogs, the observed stimulation of *in vitro* bone resorption and growth of an osteosarcoma cell line warrant *in vivo* studies to further examine these effects.

2.2. INTRODUCTION

The central role of vitamin D in the regulation of calcium and bone metabolism is well established (Reichel 1989). In addition, in 1981, Abe *et al.* (Abe 1981) demonstrated that 1,25-$(OH)_2D_3$ inhibited the proliferation and stimulated the differentiation of mouse myeloid leukemia cells. This finding was promising for the use of 1,25-$(OH)_2D_3$ in the treatment of hyperproliferative diseases and immunological disorders (Walters 1992). However, the high doses of 1,25-$(OH)_2D_3$ needed to achieve these nonclassic effects may lead to undesirable side effects on calcium metabolism (hypercalcemia, hypercalciuria). This has prompted the development of 1,25-$(OH)_2D_3$ analogs with potent cell growth regulating properties but relative low calcemic activity (Colston 1992, Mathiasen 1993, Binderup 1988, Abe 1989, Binderup 1991). Hitherto the *in vitro* effects of these analogs were mostly studied in cells not directly related to bone and calcium metabolism. Therefore, knowledge on their effects on bone

and bone cells is limited and mainly restricted to the analogs MC903 and OCT (Marie 1990, Valaja 1990, Evans 1991, Pemalete 1991, Pols 1991). In the present study, we have analyzed the effects of 1,25-$(OH)_2D_3$ and several of its analogs with side chain modifications on osteoblast-like cells and on *in vitro* bone resorption and compared their potencies. These analysis may provide insights whether the *in vivo* observed reduced calcemic activity reflects a decreased responsiveness of the skeleton to these analogs.

2.3. MATERIALS AND METHODS

Reagents

Non-radioactive 1,25-$(OH)_2D_3$ and the 1,25-$(OH)_2D_3$ analogs MC903, CB966, EB1089, KH1049 and KH1060 were provided by dr. L. Binderup, Leo Pharmaceuticals, Ballerup, Denmark. OCT was a gift from dr. N. Kubodera, Chugai Pharmaceutical Co., Ltd, Tokyo, Japan. Chemical structures are depicted in Figure 1.3 The sterols were dissolved in absolute ethanol and stored at -20 °C at a concentration of 10^{-4} M. [23,24-^{3}H]-1,25-$(OH)_2D_3$ (120 Ci/mmol) and ^{45}Ca were obtained from Amersham International, Amersham, U.K.. Alpha minimal essential medium (αMEM) and neutral red were from Sigma Chemical Co., St. Louis, MO, USA. The rat osteocalcin antiserum was a gift of Prof. R. Bouillon, Katholieke Universiteit Leuven, Leuven, Belgium. Penicillin, streptomycin, and L-glutamine were from Gibco Life Technologies Ltd., Paisley, Scotland. Fetal bovine serum was from Sera-Tech, St. Salvator, Germany. Biggers Gwatkin-Judah medium was made using reagents from Merck, Darmstadt, Germany. 1-O-hexadecyl-2-O-methyl-rac-glycerol was purchased from Bachem AG, Bubendorf, Switzerland. All other reagents were of the best grade commercially available.

Cells

The human osteoblast-like MG-63 cells were provided by Prof. R. Bouillon, Katholieke Universiteit Leuven, Leuven, Belgium. The rat osteoblast-like osteosarcoma cell line ROS 17/2.8 and the non-osteoblast-like osteosarcoma cell line ROS 25.1 were a gift from dr. S.B. Rodan, Merck, Sharp & Dohme Research Laboratories, West Point, USA. The cells were cultured in αMEM supplemented with 2 mM L-glutamine, 100 units/ml penicillin, 100 μg/ml streptomycin, 0.1% D-glucose plus the indicated concentration fetal bovine serum or charcoal-treated fetal bovine serum.

Osteocalcin measurements

The cells were seeded in 24 wells culture plates at a density of 40,000 cells/cm^2

and cultured for 24 hours (MG-63) or 48 hours (ROS 17/2.8) in αMEM plus 10% fetal bovine serum. Next, the medium was replaced by αMEM with 2% charcoal-treated fetal bovine serum and the cells were cultured in the absence or presence of various concentrations of 1,25-$(OH)_2D_3$ or its analogs. After 24 hours medium was collected and osteocalcin content was determined. Osteocalcin measurements in medium of ROS 17/2.8 cells were performed according to the method described by Verhaeghe *et al.* [Verhaeghe 1989]. Osteocalcin measurements in the medium of MG-63 cells were performed by radioimmunoassay (Incstar, Stillwater, MN, USA).

Type I procollagen measurements

MG-63 cells in αMEM plus 10% fetal bovine serum were seeded in 24 wells culture plates at a density of 40,000 cells/cm^2. After 24 hours medium was replaced by αMEM with 2% charcoal-treated fetal bovine serum and the various concentrations of 1,25-$(OH)_2D_3$ or its analogs. After 24 and 48 hours media were collected and analyzed for type I procollagen by radioimmunoassay (Orion Diagnostica, Espoo, Finland).

Proliferation assay

Cell proliferation was studied using the neutral red assay described by Löwik *et al.* [Löwik 1993]. The absorbance of neutral red is proportional to the number of viable cells. In short, 1,000 MG-63 cells/cm^2 and 3,000 ROS 17/2.8 or ROS 25.1 cells/cm^2 were seeded in a 96 wells plate and cultured for 24 hours in αMEM plus 10% fetal bovine serum. After 24 hours the medium was replaced by αMEM with 2% charcoal-treated fetal bovine serum with or without various concentrations of 1,25-$(OH)_2D_3$ or 1,25-$(OH)_2D_3$ analogs. After 3 days medium was replaced by new medium with or without the adition of the various analogs. After 6 days 50 µl of a neutral red solution (0.5 mg/ml in 0.9% NaCl) was added to each well for a 90 minutes incubation. Next, the medium was removed, the wells were washed twice with 100 µl phosphate-buffered saline and the neutral red was extracted from the cells with 100 µl 0.05 M NaH_2PO_4 in 50% ethanol. The absorbtion was measured at 540 nm (630 nm reference filter) in a microplate reader (Bio-Rad 450). All measurements were performed in quadruplicate and expressed as the percentage of control optical density (OD) values.

Bone resorption assay

The *in vitro* bone resorption assay was performed with 17-day-old fetal mouse radii/ulnae, using a method based on the fetal rat limb explant assay described by Raisz [Raisz 1965]. By injecting the mother on day 16 of gestation with 30 µCi of ^{45}Ca, the fetal radii/ulnae were labeled *in utero*. After explantation on day 17 the

radii/ulnae were precultured for 24 hours in 400 µl of Biggers Gwatkin-Judah medium with 5% charcoal-treated fetal bovine serum at 37 °C, to reduce free exchangeable calcium. Next, the medium was changed to Biggers Gwatkin-Judah medium supplemented with 5% charcoal-treated fetal bovine serum with or without various concentrations of 1,25-$(OH)_2D_3$ or analogs. After 3 days the medium was replaced by fresh medium, i.e. with or without 1,25-$(OH)_2D_3$ or the analogs. The ^{45}Ca content of the medium at 3 and 6 days of culture and of the 5% formic acid extracts of the bones was measured by liquid scintillation counting and used to calculate the total ^{45}Ca and the cumulative percentage ^{45}Ca released.

VDR binding studies

The competitive receptor binding assays were performed as previously described (Pols 1988). Receptor protein was prepared by cytosolic extraction on ice of MG-63 and ROS 17/2.8 cells with a hypertonic buffer consisting of 10 mM Tris-HCl, 300 mM KCl, 1 mM EDTA, 5 mM dithiothreitol, 10 mM sodiummolybdate and 0.1% Triton X-100 (pH 7.4). After centrifugation at 100,000 g for 1 hour at 4 °C, the cytosolic extracts (250 µl, ± 1 mg protein/ml) were incubated at 0 °C with 0.5 nM [23,24-^3H]-1,25-$(OH)_2D_3$ and increasing concentrations of non-radioactive 1,25-$(OH)_2D_3$ or 1,25-$(OH)_2D_3$ analogs. After 3 hours the bound and free [23,24-^3H]-1,25-$(OH)_2D_3$ were separated by charcoal adsorption. Concentrations of 1,25-$(OH)_2D_3$ or the analogs at which half-maximal displacement of [23,24-^3H]-1,25-$(OH)_2D_3$ was achieved were calculated.

DNA measurements

DNA measurements were performed according to the method of Karsten and Wollenberger (Karsten 1977).

Statistical analysis

The data shown are the mean of at least two independent experiments each consisting of two-four separate cultures. For clarity purposes standard deviations were not depicted in the figures. In all cases standard deviations were smaller than 10%.

2.4. RESULTS

Effect of 1,25-$(OH)_2D_3$ and 1,25-$(OH)_2D_3$ analogs on osteocalcin production by MG-63 and ROS 17/2.8 cells

In both cell lines 1,25-$(OH)_2D_3$ and the analogs stimulated osteocalcin production in a dose-dependent manner (Figure 2.1). Besides differences in

median effective dose (ED_{50}), in MG-63 cells but not in ROS 17/2.8 cells, also a difference in maximal response was detected.

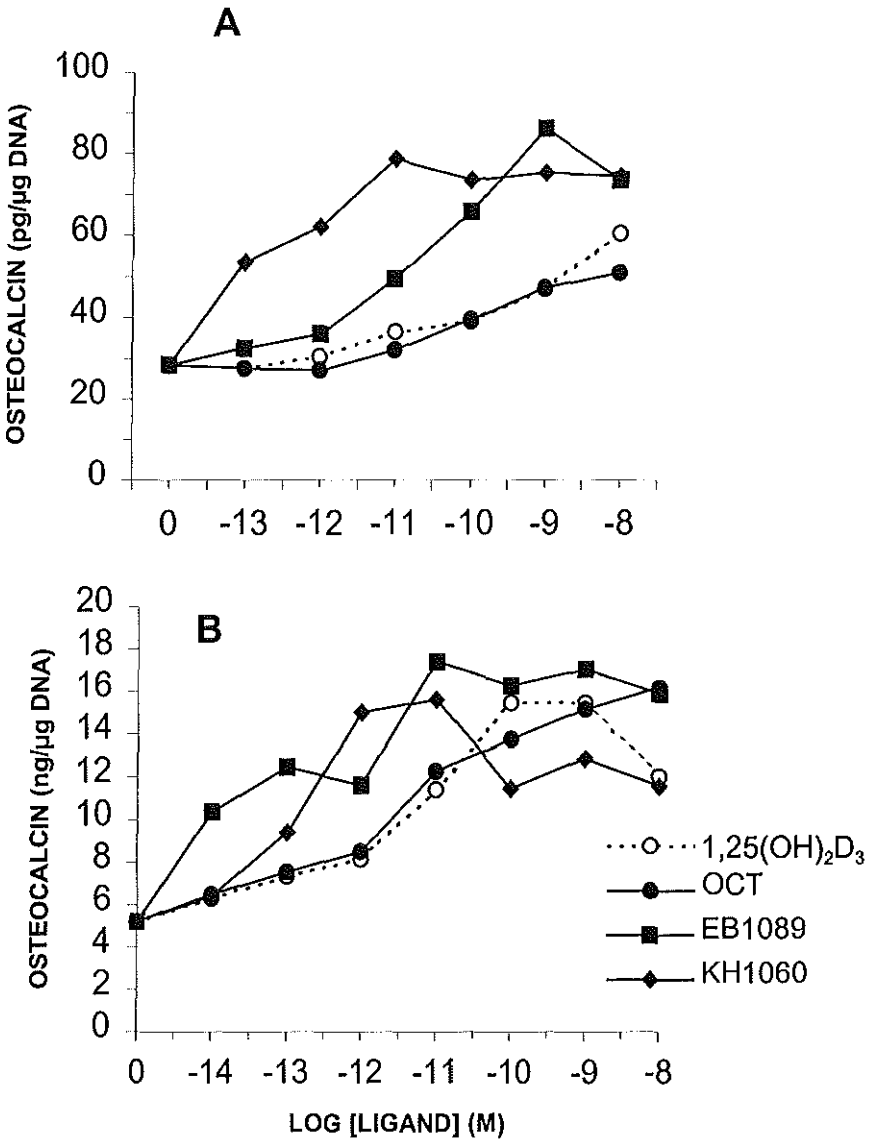


Figure 2.1. Effect of $1,25\text{-}(OH)_2D_3$ and $1,25\text{-}(OH)_2D_3$ analogs on osteocalcin production in MG-63 (A) and ROS 17/2.8 cells (B). *The cells were stimulated with $1,25\text{-}(OH)_2D_3$ or the $1,25\text{-}(OH)_2D_3$ analogs for 24 hours and osteocalcin was measured as described in Materials and Methods, Section 2.3.*

The maximal stimulation of osteocalcin production by the compounds with the lowest ED_{50} (KH1060, EB1089, KH1049) was about twice that of 1,25-$(OH)_2D_3$, OCT, and CB966 (Figure 2.1A: 1,25-$(OH)_2D_3$, OCT, EB1089, KH1060). Except for KH1060 and KH1049, the ROS 17/2.8 cells appeared to be more sensitive for 1,25-$(OH)_2D_3$ and the analogs than the MG-63 cells (Table 2.1).

Effect of 1,25-$(OH)_2D_3$ and 1,25-$(OH)_2D_3$ analogs on type I procollagen production by MG-63 cells

The effect on type I collagen synthesis was assessed by measuring the concentration of carboxyterminal propeptide of type I procollagen (Melkko 1990) in the medium of MG-63 cells after 24 and 48 hours treatment with 1,25-$(OH)_2D_3$ or its analogs. Figure 2.2 shows the dose-response curves after 48 hours of incubation with 1,25-$(OH)_2D_3$ and the most potent analogs.

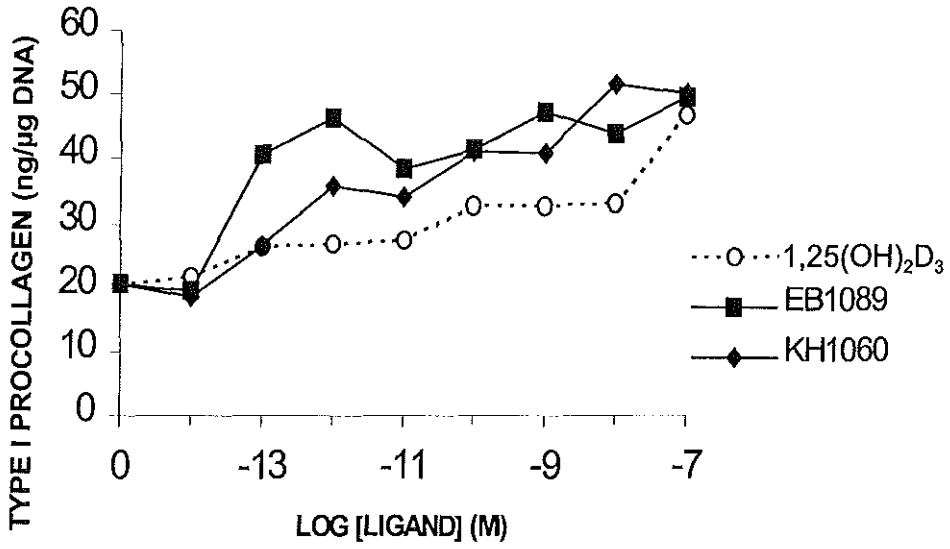


Figure 2.2. Effect of 1,25-$(OH)_2D_3$ and 1,25-$(OH)_2D_3$ analogs on the production of type I procollagen by MG-63 cells after 48 hours of incubation. *Medium was evaluated for type I procollagen content by radioimmunoassay as described in Materials and Methods, Section 2.3.*

KH1060 and EB1089 were about 14,000 and 166,000 times respectively more potent than 1,25-$(OH)_2D_3$. The ED_{50}'s were $7x10^{-13}$ M and $6x10^{-14}$ M for KH1060

and EB1089, respectively, and $1x10^{-8}$ M for 1,25-$(OH)_2D_3$. Only a slight effect of the sterols could be detected after 24 hours of culture (data not shown).

TABLE 2.1. Effects of 1,25-$(OH)_2D_3$ and 1,25-$(OH)_2D_3$ analogs on osteocalcin production and cell proliferation and their affinity for the VDR

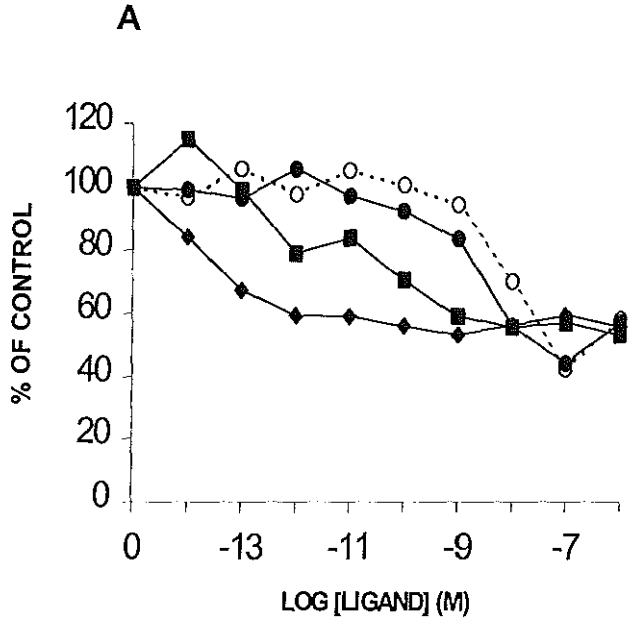
	MG-63 Cells		
Compound	Osteocalcin production ED_{50} (M)[a]	Growth inhibition ED_{50} (M)	Relative affinity for the VDR
1,25-$(OH)_2D_3$	$6x10^{-10}$ (1)[b]	$1x10^{-8}$ (1)	1[c]
OCT	$6x10^{-10}$ (1)	$3x10^{-9}$ (3)	0.15
MC903	ND	$1x10^{-8}$ (1)	0.21
CB966	$2x10^{-10}$ (3)	$2x10^{-9}$ (5)	0.70
EB1089	$6x10^{-12}$ (100)	$5x10^{-11}$ (200)	0.71
KH1049	$2x10^{-12}$ (300)	$6x10^{-12}$ (1,660)	0.22
KH1060	$1x10^{-13}$ (6,000)	$4x10^{-14}$ (250,000)	0.29
	ROS 17/2.8 Cells		
Compound	Osteocalcin production ED_{50} (M)[a]	Growth inhibition ED_{50} (M)	Relative affinity for the VDR
1,25-$(OH)_2D_3$	$4x10^{-12}$ (1)	$2x10^{-10}$ (1)	1
OCT	$5x10^{-12}$ (0.8)	$6x10^{-10}$ (0.3)	0.08
MC903	$4x10^{-12}$ (1)	ND	0.33
CB966	$2x10^{-12}$ (2)	ND	0.80
EB1089	$1x10^{-14}$ (400)	$1x10^{-14}$ (20,000)	0.80
KH1049	$1x10^{-12}$ (4)	ND	0.19
KH1060	$1x10^{-13}$ (40)	$1x10^{-12}$ (200)	0.20

The effect of 1,25-$(OH)_2D_3$ and the analogs on the production of osteocalcin was measured by radioimmunoassay. Cell proliferation was determined by the

neutral red assay after 6 days of culture in the presence of 1,25-$(OH)_2D_3$ or the analogs. Binding to the VDR was measured in cytosolic extracts by displacement of [23,24-^{3}H]-1,25-$(OH)_2D_3$. [(a)]The concentration needed to achieve the half-maximal effect of 1,25-$(OH)_2D_3$ is designated as ED_{50}. [(b)]The potency of the analogs (at ED_{50}) was calculated in relation to that of 1,25-$(OH)_2D_3$. [(c)]Results are expressed as relative affinity (at half-maximal displacement) in comparison with 1,25-$(OH)_2D_3$. The data are the mean of three independent experiments. ND: Not determined.

Effect on the proliferation of MG-63 cells and ROS 17/2.8 cells

The proliferation of the human osteoblast-like cell line MG-63 was inhibited by 1,25-$(OH)_2D_3$ and the analogs tested. No significant difference in maximum inhibitory effect (approximately 45%) between 1,25-$(OH)_2D_3$ and the analogs was found. Figure 2.3A shows dose-response curves of 1,25-$(OH)_2D_3$, OCT, EB1089, and KH1060 after 7 days of culture. The ED_{50}'s and the potencies relative to 1,25-$(OH)_2D_3$ (at ED_{50}) for these and the other analogs are summarized in Table 2.1.



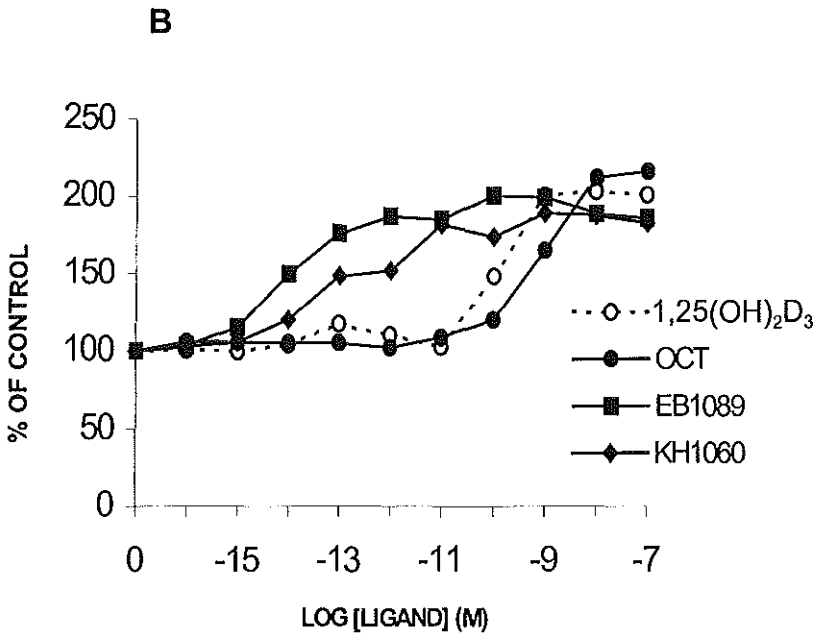


Figure 2.3. Effect of 1,25-$(OH)_2D_3$ and 1,25-$(OH)_2D_3$ analogs on the proliferation of MG-63 (A) and ROS 17/2.8 cells (B). *The effects on cell proliferation were studied using the neutral red assay. See Materials and Methods, Section 2.3. The data represent the mean percentage of control OD values.*

KH1060 was the most effective analog (ED_{50} was $4x10^{-14}$ M compared to $1x10^{-8}$ M for 1,25-$(OH)_2D_3$). KH1049 and EB1089 also were more potent than 1,25-$(OH)_2D_3$; 25 and 200 times respectively, whereas the other analogs were equipotent or only slightly more potent (Table 2.1).

In contrast to MG-63 cells, the growth of the rat osteoblast-like cells ROS 17/2.8 was not inhibited but stimulated by 1,25-$(OH)_2D_3$ and the analogs tested. The proliferation was stimulated in a dose-dependent manner and no significant difference in maximum effect between 1,25-$(OH)_2D_3$ and the analogs was observed (Figure 2.3B). EB1089 and KH1060 were most potent, reaching 50% growth stimulation at $1x10^{-14}$ M and $1x10^{-12}$ M, respectively, compared to $2x10^{-10}$ M for 1,25-$(OH)_2D_3$ (Table 2.1). Changing culture conditions (serum free and serum containing medium) and seeding density did not change the effect of 1,25-$(OH)_2D_3$ and the analogs (data not shown). The observed stimulation of the proliferation by 1,25-$(OH)_2D_3$ and the analogs was confirmed by cell counts

using a Coulter counter (Sysmex-Toa, model F-300, Kobe, Japan).

Effect of 1,25-$(OH)_2D_3$ and 1,25-$(OH)_2D_3$ analogs on in vitro bone resorption
1,25-$(OH)_2D_3$ and all analogs tested stimulated *in vitro* bone resorption in a dose-dependent manner, both after 3 and 6 days of culture. Figure 2.4 shows the *in vitro* bone resorption activity of 1,25-$(OH)_2D_3$ and the analogs OCT, EB1089, KH1049 and KH1060 after 6 days of culture. In Table 2.2 the concentrations of the sterols leading to a half-maximal ^{45}Ca release after 6 days of culture and the relative potency compared to the ED_{50} of 1,25-$(OH)_2D_3$ are presented. There was no difference in ED_{50}-values between 3 and 6 days of culture (data not shown). KH1049, KH1060, and EB1089, analogs with a far more potent effect on extracellular matrix synthesis and cell proliferation, were also more potent than 1,25-$(OH)_2D_3$ in inducing *in vitro* bone resorption (35, 17.5, and 2.3 times more potent, respectively), whereas CB966 and OCT were as potent as 1,25-$(OH)_2D_3$.

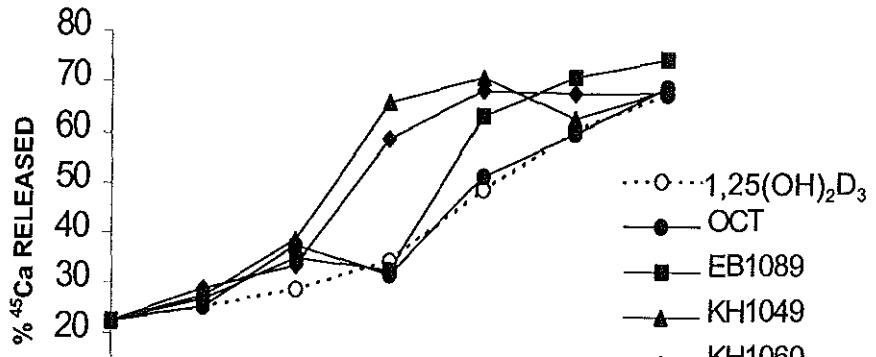


Figure 2.4. Effect of 1,25-$(OH)_2D_3$ and 1,25-$(OH)_2D_3$ analogs on ^{45}Ca release from fetal mouse radii and ulnae after 6 days of culture. *For details see Materials and Methods, Section 2.3.*

TABLE 2.2. Stimulation of *in vitro* bone resorption by 1,25-$(OH)_2D_3$ and the analogs after 6 days of culture

COMPOUND	ED_{50} (M)[a]	Potency relative to 1,25-$(OH)_2D_3$
1,25-$(OH)_2D_3$	$7x10^{-11}$	1
CB966	$7x10^{-11}$	1
OCT	$6x10^{-11}$	1.2
EB1089	$3x10^{-11}$	2.3
KH1060	$4x10^{-12}$	17.5
KH1049	$2x10^{-12}$	35

The *in vitro* bone resorptive activity of 1,25-$(OH)_2D_3$ and the analogs was determined by measuring ^{45}Ca release from pre-labeled radii/ulnae from fetal mice. [a]The concentration needed to achieve the half-maximal effect of 1,25-$(OH)_2D_3$ is designated as ED_{50}. The ED_{50} values are the mean of three independent experiments.

Ability of the 1,25-$(OH)_2D_3$ analogs to compete with 1,25-$(OH)_2D_3$ for binding to the VDR

In view of the potent effects of some analogs the ability to bind to the VDR from MG-63 and ROS 17/2.8 cells was examined. Displacement studies performed with cytosolic extracts demonstrated that all analogs exhibited lower affinity for the VDR compared to 1,25-$(OH)_2D_3$ (Figure 2.5). In both cell types similar results were obtained (Table 2.1).

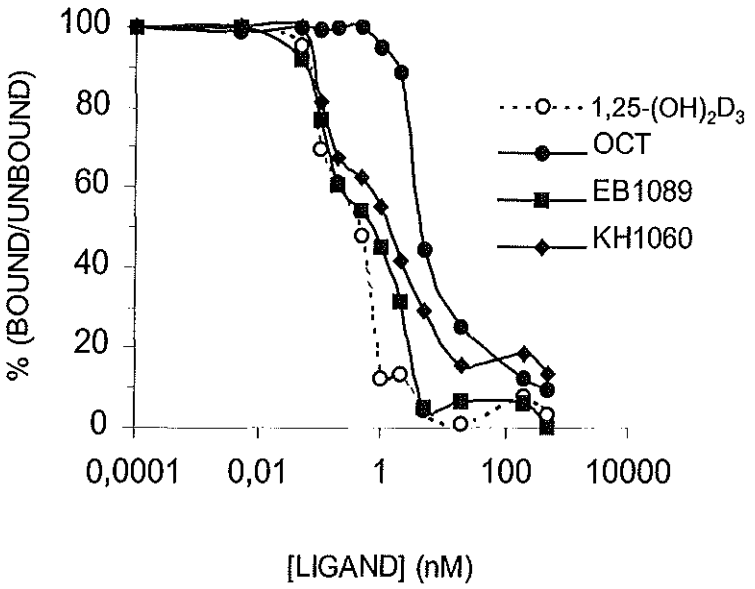


Figure 2.5. VDR affinity of 1,25-$(OH)_2D_3$ and the 1,25-$(OH)_2D_3$ analogs. *High salt cytosolic extracts of MG-63 cells were incubated with 0.5 nM ^{3}H-1,25-$(OH)_2D_3$ and increasing concentrations of 1,25-$(OH)_2D_3$ or 1,25-$(OH)_2D_3$ analogs as described in Materials and Methods, Section 2.3.*

Effect of 1,25-$(OH)_2D_3$ and KH1060 on the proliferation of ROS 25.1 cells

Regarding the very potent effects of some analogs and their reduced affinity for the VDR we further examined the role of the VDR. The ROS 25.1 cell line is a non-osteoblast-like rat osteosarcoma cell line with no detectable VDR expression. The growth of ROS 25.1 cells was not affected by neither 1,25-$(OH)_2D_3$ nor by the potent regulator of proliferation in both MG-63 and ROS 17/2.8 cells, KH1060. In addition, as expected, also no effects on osteocalcin production by 1,25-$(OH)_2D_3$ or the analogs OCT, KH1049, EB1089, and KH1060 were observed (data not shown).

Role of protein kinase C in 1,25-$(OH)_2D_3$ and 1,25-$(OH)_2D_3$ analogs stimulated osteocalcin production

Considering the dissociation between VDR binding and biological activity and the fact that protein kinase C has been shown to play a role in 1,25-$(OH)_2D_3$ action in bone (Van Leeuwen 1992a) an attempt was made to investigate a differential involvement of protein kinase C in the action of 1,25-$(OH)_2D_3$ and the analogs. To examine the role of protein kinase C in the stimulation of osteocalcin production we cultured the ROS 17/2.8 cells with various concentrations of 1,25-$(OH)_2D_3$ or analogs, in combination with 1-O-hexadecyl-2-O-methyl-rac-glycerol (25 μM), a specific inhibitor of protein kinase C. In both the 1,25-$(OH)_2D_3$- and analogs-incubated cells 1-O-hexadecyl-2-O-methyl-rac-glycerol decreased the maximal osteocalcin production to similar extent (± 40% inhibition). 1-O-hexadecyl-2-O-methyl-rac-glycerol did not cause a shift in ED_{50} values (data not shown).

2.5. DISCUSSION

The present study demonstrates that in bone the same analogs are more potent compared to 1,25-$(OH)_2D_3$ than, as has been reported before, in cells not directly related to bone and calcium metabolism. However, the potency of the analogs compared to 1,25-$(OH)_2D_3$ is dependent on the biological response. For example, the analogs KH1060 and EB1089 are far more potent stimulators of type I procollagen and osteocalcin synthesis than 1,25-$(OH)_2D_3$, whereas the differences in ED_{50} for the stimulation of bone resorption are less marked. This observation indicates that, at least in an *in vitro* situation, for these analogs the balance between stimulation of bone matrix proteins synthesis and bone resorption is in favor of bone formation (Table 2.3). One may hypothesize that both analogs may direct the osteoblast to a more mature phenotype with higher bone formation capabilities without affecting bone resorption in a similar way. The shift in the balance between bone resorption and bone formation in favor of bone formation is an interesting characteristic for a possible application in metabolic bone diseases. Although the use of 1,25-$(OH)_2D_3$/derivatives is still controversial (Parfitt 1988, Bikle 1994, Fujita 1992), several studies have shown positive effects of vitamin D metabolites, e.g. 1,25-$(OH)_2D_3$, in the treatment of metabolic bone diseases (Gallagher 1982, Riggs 1985). Treatment with vitamin D compounds has been shown to reduce bone mineral loss (Aloia 1988, Gallagher 1990) and to reduce vertebral fracture rates (Tilyard 1992).

In vivo studies have demonstrated that some of these analogs have a reduced calcemic activity (Colston 1992, Mathiasen 1993, Binderup 1988, Abe 1989, Binderup 1991, Veyron 1993). However, despite a favorable balance between bone resorption and bone formation, both

EB1089 and KH1060 still exert an increased *in vitro* bone resorptive activity compared to 1,25-$(OH)_2D_3$. Therefore, the present data show that the reduced calcemic activity of the analogs *in vivo* is not the result of a decreased responsiveness of bone to these analogs. Considering *in vivo* application of 1,25-$(OH)_2D_3$ analogs to inhibit tumor cell growth the present observations are important in view of the relationship between stimulated bone resorption and increased risk for bone metastases [Orr 1993]. Also, in patients with malignant tumors and active Paget's disease the first metastases were found in the pagetic bone lesions with high bone resorption and formation [Agha 1976, Powell 1983].

In relation to the use of 1,25-$(OH)_2D_3$ analogs as antitumor agents the observed stimulation of ROS 17/2.8 cell growth by 1,25-$(OH)_2D_3$ and the analogs is also significant. The more so, since Yamaoka *et al.* [Yamaoka 1986] reported that 1,25-$(OH)_2D_3$ promoted the growth of tumors arising from intracutaneous inoculations of athymic nude mice with ROS 17/2.8 cells. Furthermore, 1,25-$(OH)_2D_3$ also stimulated the formation of skin tumors in mice treated chronically with 7,12-dimethylbenz[a]anthracene [Wood 1985].

TABLE 2.3. Ratio of the ED_{50}'s for stimulation of *in vitro* bone resorption and stimulation of extracellular matrix synthesis

Compound	Bone resorption / Osteocalcin MG-63 Cells	Bone resorption / Osteocalcin ROS 17/2.8 Cells	Bone resorption / Type I procollagen MG-63 Cells
1,25-$(OH)_2D_3$	0.1	17.5	0.007
OCT	0.1	12	ND
EB1089	5	3,000	500
KH1060	40	40	5.7

A ratio >1 indicates stimulation of the synthesis of the extracellular matrix proteins at lower concentrations than stimulation of bone resorption whereas a ratio <1 indicates the opposite situation. ND: Not determined.

With low concentrations of 1,25-$(OH)_2D_3$ stimulation of *in vitro* cell proliferation has been described before (Majeska 1982, Freake 1981, Mangelsdorf 1984, Dokoh 1984). Although at higher 1,25-$(OH)_2D_3$ concentrations usually a growth inhibition is observed some studies reported also at these concentrations a stimulation of cell proliferation (Marie 1990, Van Auken 1994, Ishida 1993). It has been argued that the observation of growth stimulation is due to culture conditions or cell density (Rodan 1984, Eisman 1984). However, in our hands, experiments performed in serum free conditions and in 2% charcoal-treated fetal bovine serum containing medium resulted in similar effects and no relationship between seeding density and the 1,25-$(OH)_2D_3$ effects on proliferation could be demonstrated. Another explanation for the differential actions of 1,25-$(OH)_2D_3$ on cell proliferation might be the large heterogeneity within the ROS 17/2.8 cell line (Van Auken 1994, Grigoriadis 1985, Spiess 1986, Majeska 1982). In contrast to ROS 17/2.8 cells, with MG-63 cells a growth inhibition was observed. Interestingly, in both cells the same analogs were, although opposite, the most potent growth regulators. This different growth regulation does not reflect a general difference between these cell lines because in both cell lines 1,25-$(OH)_2D_3$ and the analogs stimulated the osteocalcin production.

All analogs examined have a lower affinity for the VDR in comparison with the natural ligand. Despite a decreased VDR affinity, the analogs were equipotent or far more potent in their biological responses. Other studies also reported a dissociation between receptor affinity and biological activity (Binderup 1991, Pemalete 1991, Calverley 1994, Posner 1992, Vink-Van Wijngaarden 1994, Abe 1987). Therefore, it can be concluded that VDR affinity is not predictive for the biological activity of the 1,25-$(OH)_2D_3$ analogs. Posner *et al.* (Posner 1992) suggested that the biological action of the analogs they tested is not regulated via binding to the VDR. However, the findings of others (Yamaoka 1986, Dokoh 1984, Eisman 1987) and our own results obtained with VDR deficient ROS 25.1 cells underline that presence of the VDR is essential for biological activity of 1,25-$(OH)_2D_3$ and the analogs. In addition, Carlberg *et al.* (Carlberg 1994) recently demonstrated that the analogs studied in the present paper are able to activate gene reporter systems both via the VDR homodimer and the VDR/RXR heterodimer pathway. In this respect it is tempting to speculate about a 1,25-$(OH)_2D_3$- or analog-specific induction of homo- or heterodimer formation. A possible higher sensitivity of the VDR target genes for one of these signalling pathways might then explain the observed differences in potency between 1,25-$(OH)_2D_3$ and the analogs.

Although the exact role of DBP is not clear, its presence can affect 1,25-$(OH)_2D_3$ action. On the one hand it is known that DBP decreases the free concentration of 1,25-$(OH)_2D_3$ in the circulation. In both *in vivo* (Brown 1993, Dusso 1991) and *in vitro* studies (Vanham 1988, Bikle 1989) DBP was shown to limit the access of 1,25-$(OH)_2D_3$ to the target cells. Therefore, analogs with low affinity for DBP, and thus a high free concentration, may have more potent cellular effects. On the

other hand, the unbound sterol is less protected against degradation and cleared more rapidly. For OCT the low DBP affinity has been put forward as an explanation for the decreased calcemic effect *in vivo* (Okano 1989a). However, the differences in potency between 1,25-$(OH)_2D_3$ and the analogs we observed cannot solely be explained by a lower affinity for DBP, since both equipotent (OCT) and much more potent analogs (EB1089 and KH1060) exhibit diminished affinity for DBP compared to 1,25-$(OH)_2D_3$ (Carlberg 1994, Okano 1989a). Also, studies performed with different cellular conditions, e.g. in the absence or presence of serum, resulted in similar differences in potency between 1,25-$(OH)_2D_3$ and the analogs (our unpublished observations).

Since the observed increased potency in biological responses could not be explained by a stronger affinity for the VDR and probably not by a lower affinity for DBP, other nonclassic mechanisms of action might play a role. As we have reported earlier (Van Leeuwen 1992a), protein kinase C is involved in the action of 1,25-$(OH)_2D_3$ in bone and bone cells. 1-O-hexadecyl-2-O-methyl-rac-glycerol, a specific inhibitor of protein kinase C, inhibited the 1,25-$(OH)_2D_3$ stimulated osteocalcin production in ROS 17/2.8 cells and in non-transformed isolated fetal rat osteoblasts. In the current study we investigated the role of protein kinase C in the action of 1,25-$(OH)_2D_3$ analogs on osteocalcin production in ROS 17/2.8 cells. The finding that 1-O-hexadecyl-2-O-methyl-rac-glycerol inhibited the 1,25-$(OH)_2D_3$ and 1,25-$(OH)_2D_3$ analogs stimulated osteocalcin production to the same extent without a shift in ED_{50}, suggests that the protein kinase C signalling pathway is not differently regulated by 1,25-$(OH)_2D_3$ and the analogs. In other words, activation of protein kinase C by the 1,25-$(OH)_2D_3$ analogs can not account for the large difference in potency compared to 1,25-$(OH)_2D_3$ in the stimulation of osteocalcin production.

Although the mechanism(s) underlying the differential changes in biological potencies remains unknown, the present data show that modifications in the side chain of the 1,25-$(OH)_2D_3$ molecule can lead to analogs with enhanced potential in osteoblast-like cells and on *in vitro* bone resorption. Lengthening of the side chain, especially in combination with the introduction of double bonds (EB1089) or an altered stereochemistry at position C-20 and substitution of an oxygen atom at C-22 (KH1060, KH1049) results in 1,25-$(OH)_2D_3$ analogs with enhanced biological activity within osteoblast-like cells. Furthermore, the analogs EB1089 and KH1060 exhibit characteristics that might be promising for the application of 1,25-$(OH)_2D_3$ analogs in case of metabolic bone diseases. The increase in bone resorption induced by these analogs and the observed stimulation of the proliferation of an osteosarcoma cell line by 1,25-$(OH)_2D_3$ and the analogs urges caution in the systemic application of these compounds in the treatment of hyperproliferative disorders like cancer and points out that further investigations on longterm *in vivo* and *in vitro* responses are required.

CHAPTER 3

CONFORMATIONAL CHANGE AND ENHANCED STABILIZATION OF THE VITAMIN D RECEPTOR BY THE 1,25-DIHYDROXYVITAMIN D_3 ANALOG KH1060

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Proc Natl Acad Sci USA 93, 10685-10690, 1996

3.1. ABSTRACT

The 1,25-$(OH)_2D_3$ analog KH1060 exerts very potent effects on cell proliferation and cell differentiation via the VDR. However, the activities of KH1060 are not associated with an increased affinity for the VDR. We now show that increased stabilization of the VDR-KH1060 complex could be an explanation for its high potencies. VDR half-life studies performed with cycloheximide-translational blocked rat osteoblast-like ROS 17/2.8 cells demonstrated that, in the absence of ligand, VDR levels rapidly decreased. After 2 hours less than 10% of the initial VDR level could be measured. In the presence of 1,25-$(OH)_2D_3$, the VDR half-life was 15 hours. After 24 hours less than 20% of the initial VDR content was detectable, whereas, at this time-point, still 80% of the VDR was present when the cells were incubated with KH1060. Differences in 1,25-$(OH)_2D_3$- and KH1060-induced conformational changes of the VDR could underlie the increased VDR stability. As assessed by limited proteolytic digestion analysis, both 1,25-$(OH)_2D_3$ and KH1060 caused a specific conformational change of the VDR. Compared to 1,25-$(OH)_2D_3$, KH1060 induced a conformational change which led to a far more dramatic protection of the VDR against proteolytic degradation. In conclusion, the altered VDR stability and the possibly underlying change in VDR conformation caused by KH1060 could be an explanation for its enhanced bioactivity.

3.2. INTRODUCTION

Besides the traditional effects on calcium and phosphate metabolism (Reichel 1989), the effects of 1,25-$(OH)_2D_3$ on cellular differentiation and proliferation and on immunological processes (Bikle 1992) might have relevance for the treatment of hyperproliferative and autoimmune diseases. However, the side effects (hypercalcemia and hypercalciuria) induced by the high doses needed to achieve these effects limit the use of 1,25-$(OH)_2D_3$ in clinical practice. This has prompted the development of new 1,25-$(OH)_2D_3$ analogs with reduced calcemic activity.

Previous studies have shown that modifications in the side chain of the 1,25-$(OH)_2D_3$ molecule can lead to far more potent analogs. One of the most potent analogs until now, KH1060 (Bouillon 1995), is a strong inhibitor of tumor cell growth (Binderup 1991, Vink-Van Wijngaarden 1994) and an inducer of cell differentiation (Binderup 1991, Brown 1994, Van den Bemd 1995). KH1060 also exhibits strong immunosuppressive activity in both *in vitro* and *in vivo* studies (Binderup 1991, Veyron 1993, Mathieu 1995). Furthermore, KH1060 is more potent than 1,25-$(OH)_2D_3$ in stimulating *in vivo* and *in vitro* bone resorption and osteoclast recruitment in murine bone marrow cultures (Binderup 1991, Van den Bemd 1995, Wiberg 1995).

The differences in biological activity between 1,25-$(OH)_2D_3$ and KH1060 could not be explained by an increased affinity for the VDR (Binderup 1991, Vink-Van wijngaarden

1994, Van den Bemd 1995). However, the presence of a (functional) VDR is essential for the biological responses of 1,25-$(OH)_2D_3$ and KH1060 (Van den Bemd 1995, Eisman 1987, Elstner 1994). It is known that, through binding to the VDR, 1,25-$(OH)_2D_3$ stabilizes the receptor (Costa 1986, Wiese 1992, Arbour 1993). Since VDR stabilization might influence biological activity of the ligand, we studied the effect of KH1060 on the VDR half-life in ROS 17/2.8 osteoblastic cells. We examined the effect of KH1060 on the conformation of the VDR. A 1,25-$(OH)_2D_3$ analog with normal stereochemistry at C-20 but, like KH1060, with an oxygen atom at the C-22 position, OCT was used as a reference compound in this study.

3.3. MATERIALS AND METHODS

Reagents

1,25-$(OH)_2D_3$ and the analog KH1060 were generously provided by dr L. Binderup (Leo Pharmaceutical Products, Ballerup, Denmark). OCT was a gift from dr N. Kubodera, Chugai Pharmaceutical Co., Ltd., Tokyo, Japan. The chemical structures of 1,25-$(OH)_2D_3$, OCT, and KH1060 are depicted in Figure 1.3. rRNasin ribonuclease inhibitor, luciferase DNA, brome mosaic virus RNA, and the coupled *in vitro* transcription and translation rabbit reticulocyte lysate system (TNT lysate assay) were purchased from Promega, Madison, WI, USA. [23,24-^{3}H]-1,25-$(OH)_2D_3$ (120 Ci/mmol), L-[^{35}S]methionine (>1,000 Ci/mmol), and Enhanced Chemiluminescence Western blotting reagents were obtained from Amersham International, Buckinghamshire, UK. αMEM, cycloheximide, trypsin, and chymotrypsin were from Sigma Chemical Co., St. Louis, MO, USA. Proteinase K was obtained from Boehringer Mannheim, Germany. N,N,N',N'-tetramethylethylenediamine and ammonium persulfate were obtained from BioRad Laboratories, Richmond, CA, USA. Acrylamide and methylenebisacrylamide were purchased from Pharmacia Biotech, Uppsala, Sweden. The human VDR cDNA was a generous gift from dr M.R. Haussler (University of Arizona, Tucson, AZ, USA). Nitrocellulose was from Schleicher & Schuell, Dassel, Germany. Penicillin, streptomycin, and L-glutamine were from Gibco Life Technologies Ltd., Paisley, Scotland. Fetal bovine serum was purchased from Sera-Tech, St. Salvator, Germany.

Cells

The rat osteoblast-like osteosarcoma cell line ROS 17/2.8 (kindly provided by dr S.B. Rodan, Merck, Sharp & Dohme Research Laboratories, West Point, PA, USA) was cultured in αMEM supplemented with 2mM L-glutamine, 100 units/ml penicillin, 100 μg/ml streptomycin, 0.1% D-glucose, and 10% fetal bovine serum. Incubations with 1,25-$(OH)_2D_3$, OCT, KH1060, or vehicle were performed in the presence of 2% charcoal-treated fetal bovine serum.

VDR stability study

ROS 17/2.8 cells ($2.0x10^6$) were seeded in 100x15 mm tissue culture dishes and cultured for 2 days. Then, at 80% confluency, medium was changed to 2% charcoal-treated fetal bovine serum αMEM containing 10 μM cycloheximide to block translational activity and thereby new synthesis of VDR. Next, vehicle, $1,25\text{-}(OH)_2D_3$, OCT, or KH1060 ($1x10^{-8}$ M) was added and, after varying incubation periods, cell extracts were prepared by Dounce homogenization in 500 μl TED buffer (50 mM Tris-HCl, pH 7.4, 1.5 mM EDTA, 5mM dithiothreitol) and an equal volume of TED buffer with 600 mM NaCl according to the method described by Wiese *et al.* (Wiese 1992). The supernatants were frozen in liquid N_2 and stored at -80°C until they were assayed for VDR content using the immunoradiometric assay described by Sandgren and DeLuca (Sandgren 1990).

In vitro transcription and translation

Human VDR cDNA (1 μg) inserted into the *Eco*RI site of pGem 4 (Baker 1988) was *in vitro* transcribed with Sp6 RNA polymerase and translated in the presence of [^{35}S]methionine and ribonuclease inhibitor rRNasin, using Promega's TNT lysate assay according to the manufacturer's instructions. Brome mosaic virus RNA and luciferase DNA were used as templates in the TNT lysate assay for the production of molecular mass markers.

Limited proteolytic digestion of in vitro synthesized VDR protein

The *in vitro* synthesized VDR protein was treated for 20 minutes at room temperature with various concentrations of $1,25\text{-}(OH)_2D_3$, OCT, KH1060, the noncognate ligands all-*trans* retinoic acid, 9-*cis* retinoic acid, E_2, thyroid hormone, progesterone, or with vehicle (0.01% ethanol). Protease solution (2 μl) was added to hormone/vehicle-treated VDR (2 μl). Trypsin, chymotrypsin, and proteinase K were used at concentrations of 5 to 500 μg/ml. After a digestion period of 10 minutes at room temperature, 20 μl of denaturing SDS-sample buffer was added and the samples were heated for 5 minutes at 95 °C. The samples were loaded on a 12.5% (w/v) SDS polyacrylamide gel for electrophoresis (SDS-PAGE) and the labeled fragments were visualized by overnight exposure to Fuji RX medical X-ray film at -80°C.

Immunoblotting

The *in vitro* synthesized VDR proteins were incubated for 20 minutes at room temperature with vehicle, $1,25\text{-}(OH)_2D_3$, OCT, or KH1060 and subsequently treated for 10 minutes at room temperature with vehicle or trypsin (25 μg/ml). After SDS-PAGE (12.5% gel), the protein fragments were transferred electrophoretically to nitrocellulose. Western blotting was performed using the IVG8C11 monoclonal antibody to the porcine intestinal VDR (Dame 1986).

Immunoreactive proteins were visualized using Enhanced Chemiluminescence Western blotting detection reagents.

3.4. RESULTS

Differences in VDR affinity and in vitro effects between 1,25-$(OH)_2D_3$, OCT, and KH1060

To emphasize the differences in biological potencies between 1,25-$(OH)_2D_3$ and the analogs a summary of two biological responses is presented in Table 3.1. In addition, the relative affinity of the analogs for the VDR is shown. The effects of OCT, KH1060, and 1,25-$(OH)_2D_3$ on the production of the bone matrix protein osteocalcin by the ROS 17/2.8 osteoblastic cells and on *in vitro* bone resorption are shown as examples, but KH1060 was also more potent than 1,25-$(OH)_2D_3$ in other responses, whereas OCT was equipotent (Van den Bemd 1995). Despite these increased potencies, KH1060 displayed reduced affinity for the VDR extracted from ROS 17/2.8 cells.

Table 3.1. VDR affinity and *in vitro* biological effects of 1,25-$(OH)_2D_3$, OCT, and KH1060*

Ligand	Osteocalcin production ED_{50} (M)†	*In vitro* bone resorption ED_{50} (M)	Relative affinity for the VDR
1,25-$(OH)_2D_3$	$4x10^{-12}$ (1)‡	$7x10^{-11}$ (1)	1
OCT	$5x10^{-12}$ (0.8)	$6x10^{-11}$ (1.2)	0.08
KH1060	$1x10^{-13}$ (40)	$4x10^{-12}$ (17.5)	0.20

These data are presented in extended form in Chapter 2. In short, osteocalcin production by ROS 17/2.8 cells was measured by radioimmunoassay. *In vitro* bone resorption was assayed by measuring ^{45}Ca release from pre-labeled radii/ulnae from fetal mice. Data after 6 days of culture are presented. VDR affinity was determined in the cytosolic extract of ROS 17/2.8 cells by competitive receptor binding assays with [23,24-^{3}H]-1,25-$(OH)_2D_3$. †The concentration needed to achieve the half-maximal effect of 1,25-$(OH)_2D_3$ is designated as ED_{50}. ‡The potency of OCT and KH1060 (at ED_{50}) was calculated in relation to that of 1,25-$(OH)_2D_3$.

In other osteoblastic cells (human osteosarcoma MG-63 cells) (Van den Bemd 1995) and in

cells not directly related to bone metabolism (e.g. the human breast cancer cell line MCF-7) (Vink-Van wijngaarden 1994), a reduced VDR affinity of KH1060 compared with 1,25-$(OH)_2D_3$ was observed.

VDR Half-life in rat osteoblast-like cells

When no ligand was present, the VDR in ROS 17/2.8 cells was almost completely degraded within 4 hours (half-life = 1.5 hours) (Figure 3.1). However, when the cells were incubated with 1,25-$(OH)_2D_3$, the VDR half-life was prolonged by 13.5 hours. Less than 20% of the initial VDR level could be detected after 24 hours. A comparable result was obtained when the cells were treated with OCT. KH1060 had a much stronger stabilizing effect on the VDR. After 24 hours, still 80% of the initial VDR level was detectable.

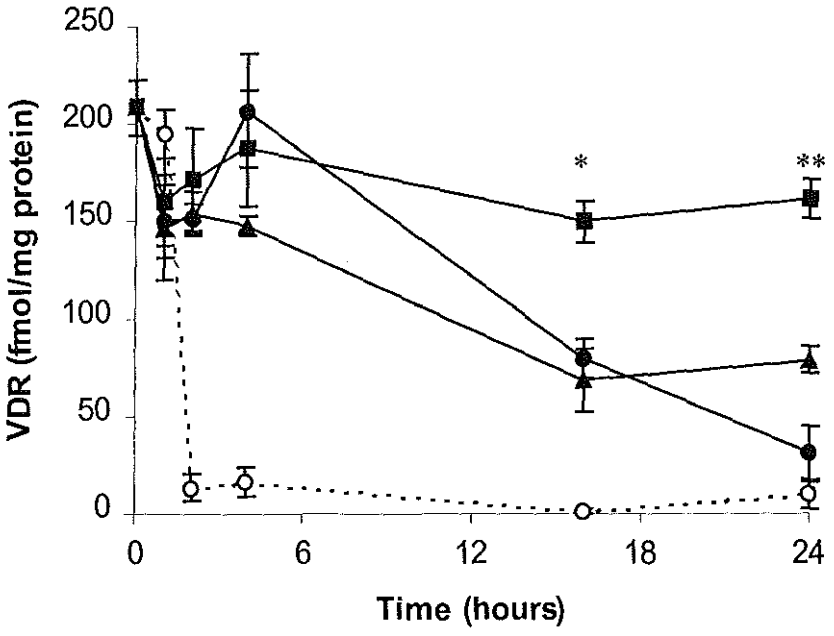
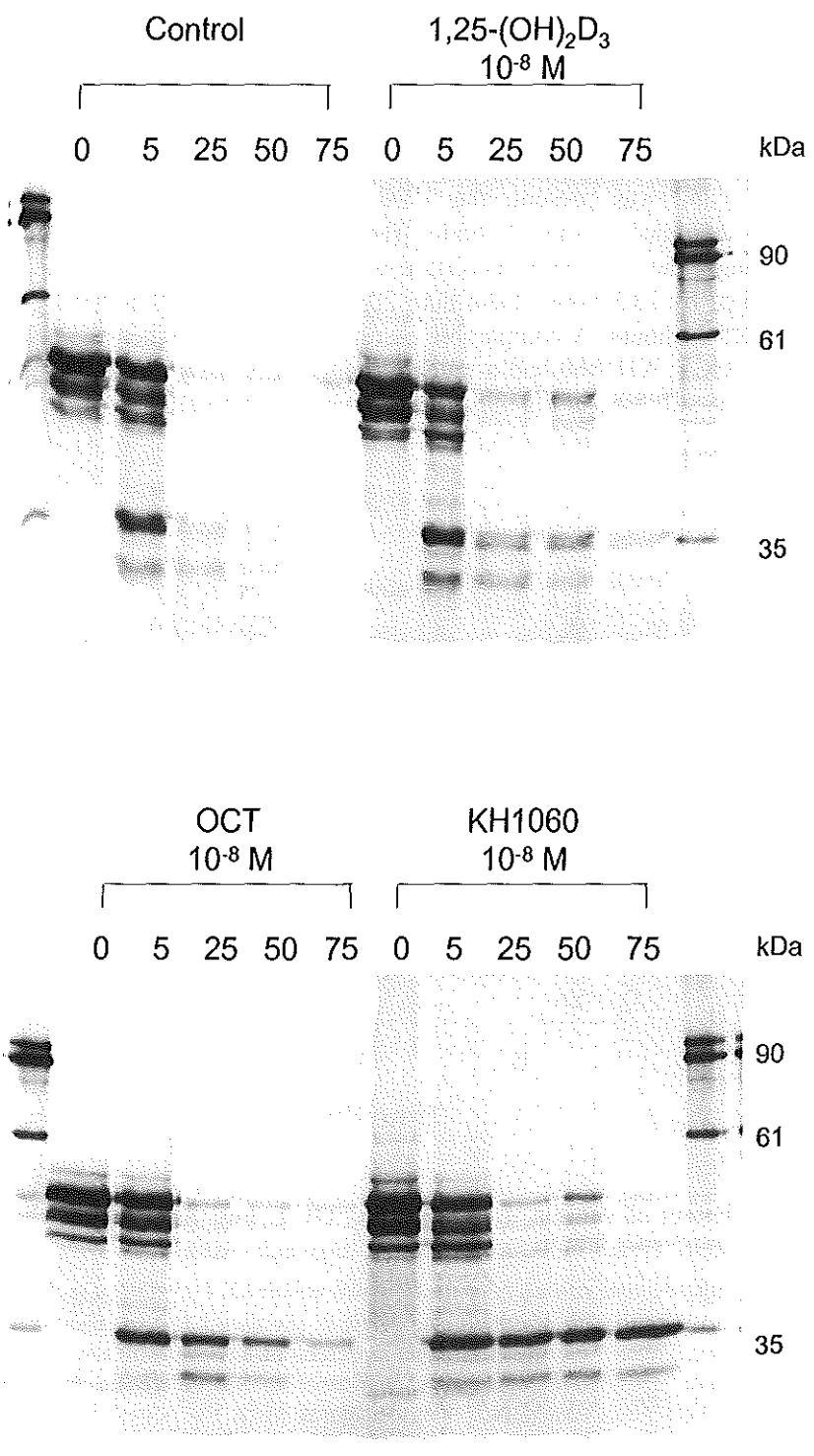


Figure 3.1. Effect of vehicle, 1,25-$(OH)_2D_3$, OCT, and KH1060 on the VDR half-life in ROS 17/2.8 cells. *Extracts of cycloheximide-treated ROS 17/2.8 cells were prepared after different time periods of incubation with vehicle ○, 1,25-$(OH)_2D_3$ ●, OCT ▲, or KH1060 ■ as described in Materials and Methods (Section 3.3), and assayed for VDR content by immunoradiometric assay. Each point represents mean ± standard error of duplicate cultures that were measured in triplicate. Significance of the differences between 1,25-$(OH)_2D_3$- and KH1060-incubated cells was calculated using the paired Student's t test. $^*P<0.005$, $^{**}P<0.0005$.*

A


Control
1,25-(OH)2D3
10-8 M
0 5 25 50 75 0 5 25 50 75
kDa
90
61
35
OCT
10-8 M
KH1060
10-8 M
0 5 25 50 75 0 5 25 50 75
kDa
90
61
35

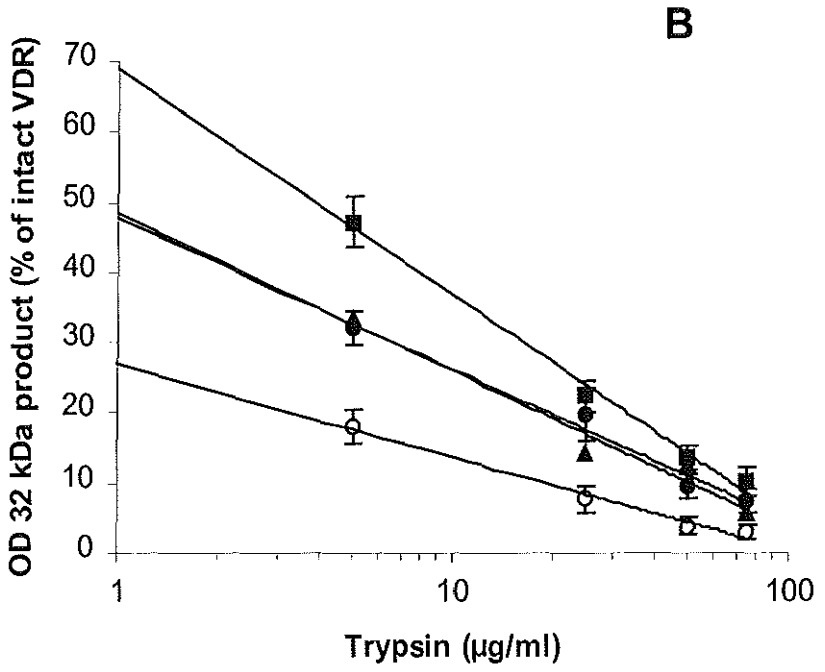


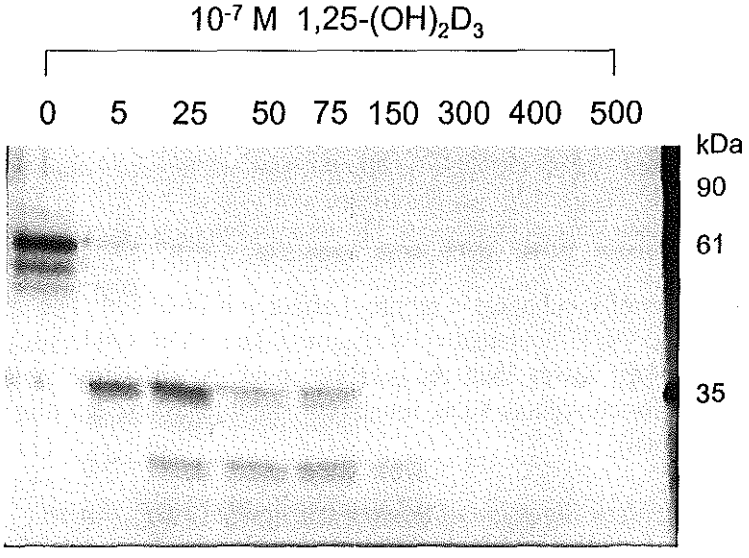
Figure 3.2. Effect of 1,25-$(OH)_2D_3$, OCT, and KH1060 on VDR conformation. *(A) In vitro synthesized human VDR was incubated with vehicle, 10 nM 1,25-$(OH)_2D_3$, 10 nM OCT, or 10 nM KH1060 and subsequently treated with increasing concentrations of trypsin. The numbers above the lanes indicate the amount of protease added (0-75 µg/ml). The fragments were analyzed by SDS-PAGE. The sizes of the molecular weight markers are indicated. (B) Computerized scan of the 32 kDa resistant fragment at increasing trypsin concentrations. X-rays of experiments similar to the experiment presented in Figure 3.2A were scanned, and the OD of the 32 kDa fragment was expressed as percentage of intact VDR OD values. Figure 3.2B was constructed with data of 6 (OCT) to 13 (vehicle, 1,25-$(OH)_2D_3$, KH1060) different experiments. The same symbols as in Figure 3.1 were used.*

After 48 hours of incubation, a rebound effect was observed: The control-, 1,25-$(OH)_2D_3$-, and OCT-incubated cells regained their VDR synthesis, but the VDR content was still lower than the starting level. At this time point, KH1060-incubated cells contained VDR levels comparable to the starting point levels (data not shown).

Limited proteolytic digestion of in vitro synthesized VDR protein

The increased VDR half-life could be the result of a conformational change of the VDR. We used partial enzymatic digestion of *in vitro* synthesized human

VDR as a means to gain insight into ligand-induced conformational changes of the VDR. The rationale behind this technique is that a changed VDR conformation will lead to a changed accessibility of cleavage sites within the VDR molecule. So, changes in VDR conformation will lead to an altered VDR digestion. *In vitro* synthesized [^{35}S]methionine-labeled VDR protein was separated by SDS-PAGE into three bands of 51, 48, and 45 kDa (Figure 3.2A), probably representing different forms (e.g. phosphorylated) of the VDR. Immunoblot analysis revealed that all three proteins were immunoreactive with the IVG8C11 antibody to the VDR (Figure 3.6). When trypsin was added, two distinct fragments of 32 and 27 kDa appeared (Figure 3.2A). Increasing the trypsin concentration to 50 µg/ml led to a complete degradation of the VDR. When VDR was incubated with 1,25-$(OH)_2D_3$ before trypsin treatment, degradation was retarded, resulting in more persistent fragments (Figure 3.2A). Compared with 1,25-$(OH)_2D_3$, VDR treatment with OCT resulted in a similar protection against proteolysis (Figures 3.2A and B), whereas incubation of VDR with KH1060 resulted in a dramatic increase in the resistance against protease activity (Figures 3.2A and B). To further illustrate the large difference in potency to protect the VDR against protease activity between 1,25-$(OH)_2D_3$ and KH1060, we extended the trypsin concentration range and found that, at a trypsin concentration of 500 µg/ml, fragments were still detectable when the VDR was incubated with KH1060, whereas 1,25-$(OH)_2D_3$-incubated VDR was already completely degraded at a trypsin concentration of 150 µg/ml (Figure 3.3).



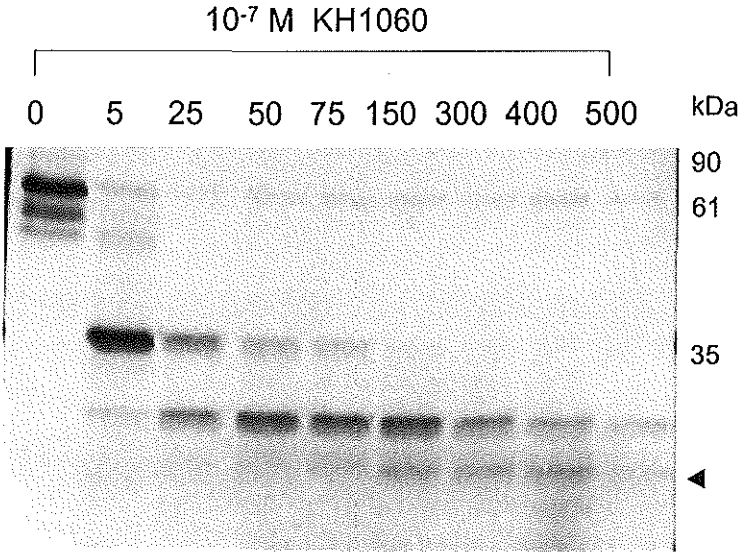


Figure 3.3. Comparison of the effects of 1,25-$(OH)_2D_3$ (*Upper*) and KH1060 (*Lower*) on the conformational change of the VDR. *In vitro synthesized human VDR was incubated with 100 nM of ligand and then treated for 10 minutes with 0-500 μg/ml of trypsin. The numbers above the lanes indicate the amount of protease added. The degradation fragments were separated by SDS-PAGE and visualized by autoradiography. The arrowhead marks the 24 kDa fragment.*

Notice that in the KH1060-incubated VDR, a third fragment of approximately 24 kDa is present that could not be detected when VDR was incubated with 1,25-$(OH)_2D_3$ or OCT (Figures 3.2A and 3.3). This 24 kDa fragment could be the result of processing of the larger fragments.

The observed protection was not due to direct blockade of specific cleavage sites of trypsin, since with chymotrypsin (Figure 3.4) or with the broadband protease proteinase K (data not shown) virtually the same observations were made: 1,25-$(OH)_2D_3$, OCT, and KH1060 protected the VDR against proteolytic breakdown, resulting in more preserved fragments with KH1060 being far more effective than 1,25-$(OH)_2D_3$ or OCT.

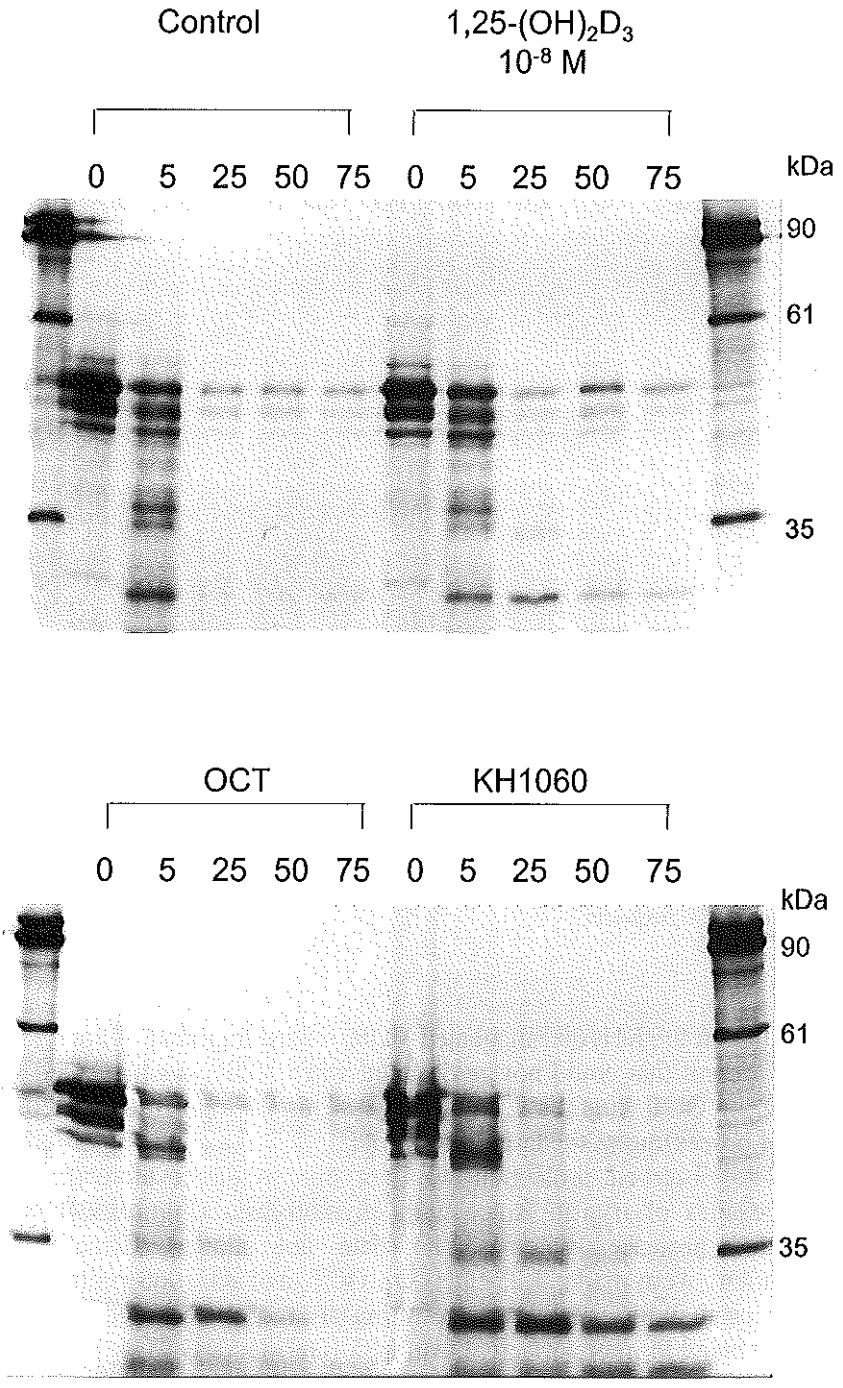


Figure 3.4. 1,25-$(OH)_2D_3$-, OCT-, and KH1060-induced conformational changes of the VDR. *In vitro synthesized human VDR was incubated with vehicle, 10 nM 1,25-$(OH)_2D_3$, 10 nM OCT, or 10 nM KH1060 and subsequently treated with increasing concentrations of chymotrypsin. The numbers above the lanes indicate the amount of protease*

added (0-75 μg/ml). The VDR fragments were separated by SDS-PAGE. The sizes of the molecular weight markers are indicated.

The ligand-induced conformational change of the VDR was ligand-concentration dependent (Figure 3.5). Furthermore, the conformational change was ligand-specific. Incubating the VDR with the noncognate ligands all-*trans* retinoic acid, progesterone, E_2, thyroid hormone, and 9-*cis* retinoic acid did not result in an increased preservation of distinct VDR fragments (data not shown).

To exclude the possibility that the observed protection was due to direct inactivation of the protease by 1,25-$(OH)_2D_3$ or the analogs we performed time course studies. Adding KH1060 and trypsin simultaneously did not result in an increased protection against proteolytic degradation, whereas a 10 minutes preincubation with KH1060 was effective (data not shown).

The observed differences in protective effect between 1,25-$(OH)_2D_3$, OCT, and KH1060 was not specific for the incubation temperature routinely used in this study (room temperature). When the temperature during the ligand and the trypsin incubation period was raised to 37°C, KH1060 was still much more potent than 1,25-$(OH)_2D_3$ in protecting the VDR against proteolytic breakdown (data not shown).

Immunoblot analysis of proteolytic VDR fragments

To gain insight whether the proteolytic VDR fragments and the 32 kDa product in particular contain at least part of the DNA binding site, we performed immunoblot analysis with the IVG8C11 monoclonal antibody directed to the DNA binding site of the porcine intestinal VDR. Incubation with 1,25-$(OH)_2D_3$, OCT, or KH1060 did not affect the immunological signal of the three main bands around 50 kDa. Incubation with trypsin (25 μg/ml) resulted in a loss of immunoreactivity, both in the presence and absence of 1,25-$(OH)_2D_3$, OCT, or KH1060. With all three ligands, none of the proteolytic fragments were immunoreactive (Figure 3.6).

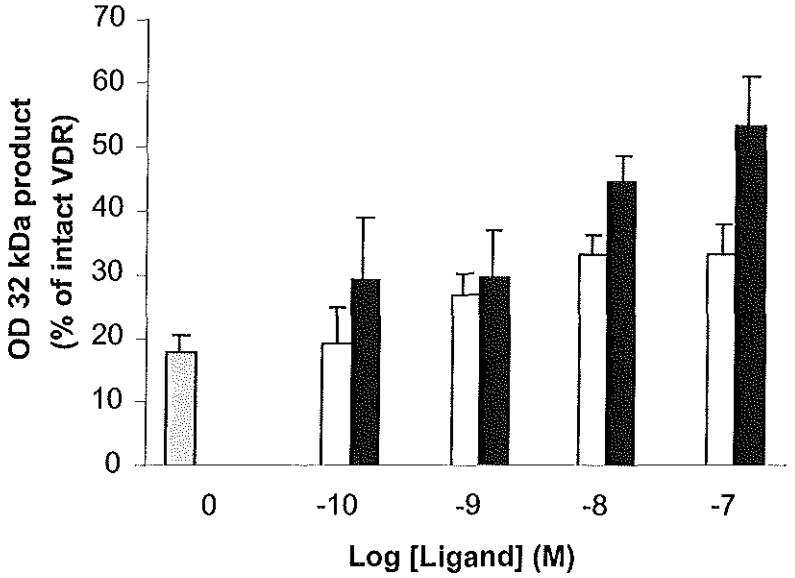


Figure 3.5. Effect of ligand concentration on the presence of the 32 kDa VDR fragment. *In vitro synthesized human VDR was incubated with vehicle (shaded bar) or increasing concentrations of 1,25-$(OH)_2D_3$ (open bars) or KH1060 (solid bars) (10^{-10}-10^{-7} M) and subsequently treated with 5 μg/ml of trypsin. After SDS-PAGE, the fragments were scanned from the autoradiograph and OD were expressed as percentage of the intact VDR OD values.*

3.5. DISCUSSION

It is known that modifications in the side chain of the 1,25-$(OH)_2D_3$ molecule can result in analogs with increased biological activity. One of these interesting 1,25-$(OH)_2D_3$ analogs, KH1060, is a much more potent regulator of cell proliferation and differentiation (Binderup 1991, Vink-Van Wijngaarden 1994, Van den Bemd 1995). However, the mechanism(s) underlying the differences in potency remain unclear. The increased potency of KH1060 is not associated with an increased affinity for the VDR (Vink-Van Wijngaarden 1994, Van den Bemd 1995), although the presence of the receptor is essential for the effects on cell proliferation and differentiation (Van den Bemd 1995).

The present study demonstrates that, compared with 1,25-$(OH)_2D_3$, KH1060 increases the half-life of the VDR, which could provide an explanation for the increased biological potencies of KH1060. Binding of KH1060 to the VDR causes an almost complete stabilization of the VDR, whereas with 1,25-$(OH)_2D_3$ after 24 hours only 20% of the initial amount of VDR was present.

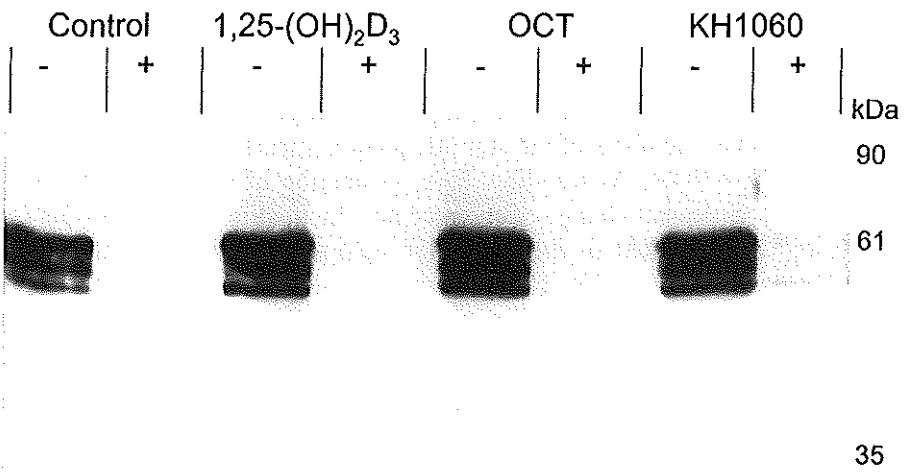


Figure 3.6. Immunoblot analysis of *in vitro* synthesized human VDR and protease degradation products. *Human VDR was incubated with vehicle, 100 nM of 1,25-(OH)2D3, 100 nM OCT, or 100 nM KH1060 and subsequently treated with vehicle (-) or with 25 μg/ml of trypsin (+). The samples were separated by SDS-PAGE and then transferred to a nitrocellulose membrane. Immunological detection was performed with the 1VG8C11 monoclonal antibody and Enhanced Chemiluminescence Western blotting detection reagents.*

Earlier studies showed that 1,25-$(OH)_2D_3$ or naturally occurring 1,25-$(OH)_2D_3$ metabolites stabilized the VDR in LLC-PK1 pig kidney cells (Costa 1986), mouse fibroblasts, rat intestinal epithelial cells (Wiese 1992), and ROS 17/2.8 cells (Arbour 1993). Our data are consistent with the VDR half-life of 2 hours in the absence of 1,25-$(OH)_2D_3$ reported by Arbour *et al.* (Arbour 1993). They also observed a VDR stabilization when the ROS 17/2.8 cells were treated with 1,25-$(OH)_2D_3$. However, they were not able to determine a VDR half-life, since the VDR content was unchanged within the 6 hours studied. The present paper is, to our knowledge, the first report concerning the effect of 1,25-$(OH)_2D_3$ analogs on VDR stability.

Besides VDR stabilization, the effect of 1,25-$(OH)_2D_3$, OCT, and KH1060 on the VDR conformation was examined. As suggested by Allan *et al.* (Allan 1992), hormone-induced conformational changes might be crucial for DNA binding, dimerization, and transcriptional activation, but in addition it could also play a role in receptor stability. We used partial enzymatic digestion of *in vitro* synthesized human VDR protein as a means to gain insight into ligand-induced conformational changes of the VDR. Partial enzymatic digestion has proven to be a useful method for studying conformational changes of the VDR (Nayeri 1995, Peleg 1995, Keidel 1994) and other members of the steroid receptor superfamily (Allan 1992, Keidel 1994, Leng 1993, Simons 1989, Kuil 1994, Beekman 1993). As we show here, incubation of the VDR with ligand leads to a conformational change of the receptor. Incubation with 1,25-$(OH)_2D_3$, KH1060, or OCT resulted in the protection against proteolytic breakdown of specific fragments of the VDR. This protection was not due to a direct effect of the ligand on the enzymes. In contrast to a 10 or 20 minutes preincubation with ligand, simultaneous addition of ligand and protease did not cause enhanced protection against degradation of the VDR. Also, it was not the result of blockade of specific cleavage sites by the ligands because with three different proteases similar results were obtained. In this respect, it is noteworthy that the ligand binding domain of the VDR contains over 20 evenly distributed cleavage sites for trypsin. Therefore, it seems unlikely that a relatively small molecule like 1,25-$(OH)_2D_3$ is able to protect a 32 kDa fragment from proteolytic degradation via direct blockade of these cleavage sites. The protection against protease activity could only be achieved when the receptor was incubated with its cognate ligand: VDR was not protected by E_2, progesterone, all-*trans* retinoic acid, thyroid hormone, and 9-*cis* retinoic acid.

In an attempt to identify the VDR fragments that are protected by ligand incubation, we performed immunoblot analysis. None of the fragments were immunoreactive with the IVG8C11 antibody which epitope has been mapped to amino acids 57 to 164 (Brown 1991), encompassing the second Zinc finger of the DNA binding domain (amino acids 21-87) and most of the hinge region (amino acids 88-188) (Figure 3.6). Furthermore, the theoretically calculated molecular weight of the ligand binding domain of the VDR is about 30 kDa, which is indeed comparable to the size of the major VDR degradation product. This suggests that the protected fragments are predominantly part of the ligand binding domain. As we do not possess specific antibodies against this region of the VDR, we cannot provide proof of this. For the progesterone receptor, a progesterone-induced 30 kDa fragment and for the estrogen receptor an estrogen-induced 32 kDa, both corresponding to their ligand binding domains, were reported (Allan 1992).

The observation that 1,25-$(OH)_2D_3$, OCT, and KH1060 induce a conformational change of *in vitro* synthesized VDR, leading to enhanced resistance against protease activity, makes it tempting to speculate about the role of VDR conformation in its stabilization. As shown in Figures 3.2A, 3.3, and 3.4,

KH1060 is much more potent than 1,25-$(OH)_2D_3$ and OCT in the protection against proteolytic degradation. This is further illustrated when the major degradation product, a 32 kDa fragment, was quantified and plotted against the protease concentration (Figure 3.2B). At high protease concentrations with KH1060, a 24 kDa VDR fragment was found that was not observed after incubation with 1,25-$(OH)_2D_3$ or OCT (Figure 3.3). Most of the conformational analysis were performed at room temperature, but, at 37°C, the difference between KH1060 and 1,25-$(OH)_2D_3$ was present, indicating that the observed phenomenon also holds at a physiological more relevant temperature. Together these data indicate a different VDR conformation after binding KH1060, which can be involved in the increased VDR stabilization observed in cells. This is even more likely when the background of the receptor conformational analysis is taken into account: protection against proteolytic breakdown due to reduced accessibility of cleavage sites in the receptor by ligand binding. Moreover, in the VDR conformational studies 1,25-$(OH)_2D_3$ and OCT showed similar effects and induced a comparable increase in VDR half-life (Figures 3.1, 3.2A, 3.2B, and 3.4).

Based on the observed different effects between OCT and KH1060 on VDR half-life and VDR conformation, we conclude that only specific alterations in the side chain structure of the 1,25-$(OH)_2D_3$ molecule result in more potent analogs. The reported *in vitro* (Table 3.1) and *in vivo* biological effects of OCT (Van den Bemd 1995, Abe 1989, Dusso 1991, Pernalete 1991, Brown 1993) and the data presented here with respect to its effect on VDR half-life and VDR conformation (Figures 3.1, 3.2, and 3.4) show that substitution of the C-22 by an oxygen atom results in an analog that mimics most of the activities of 1,25-$(OH)_2D_3$. Therefore, we conclude that the potent effect of KH1060 is not the result of the substituted oxygen atom at C-22, but is probably due to (combinations with) other modifications in the side chain.

In view of the fact that stabilization and conformational change of the VDR could be responsible for increased receptor-mediated responses in target cells (Arbour 1993, Allan 1992), the present study provides a means by which KH1060 can exert biological responses more potently than 1,25-$(OH)_2D_3$. In this respect, the report by Sasaki *et al.* (Sasaki 1995) is of interest. They speculated that the higher biological activity of the analog 1,25-$(OH)_2$-F_6-D_3 could (partly) be due to a structural change of the VDR induced by this analog, leading to enhanced binding affinity of the VDR for the VDRE and subsequently to enhanced transcriptional activity. It should, however, be taken into account that in addition also pharmacokinetic aspects (e.g. in relation to DBP affinity or altered metabolism) may contribute to the increased bioactivity of KH1060 (Kissmeyer 1995) and other 20-epi-analogs (Dilworth 1994).

CHAPTER 4

VITAMIN D RECEPTOR-DNA BINDING AND TRANSACTIVATION IS DIRECTED BY SPECIFIC NUCLEOTIDES IN VITAMIN D RESPONSE ELEMENTS AND BY CELLULAR CONTEXT

Van den Bemd GJCM, Jhamai P, Staal A, Van Wijnen AJ, Lian JB, Stein GS, Pols HAP, Van Leeuwen JPTM. In preparation.

Part of the data presented in this paper was already published in:

Van den Bemd GJCM, Pols HAP, Staal A, Van Wijnen AJ, Lian JB, Stein GS, Van Leeuwen JPTM. Specific nucleotides in the proximal half-site of DR3-type vitamin D response elements direct vitamin D receptor-retinoid X receptor binding. In: Vitamin D, Chemistry, Biology and Clinical Applications of the Steroid Hormone. Norman AW, Bouillon R, Thomasset M (Eds.).Walter de Gruyter, Berlin, p 284-285, 1997

4.1. ABSTRACT

The 1,25-$(OH)_2D_3$ 20-epi-analog KH1060 exerts its strong biological activity via the VDR. As we reported earlier, changes in VDR conformation and enhanced VDR stability induced by KH1060 and some of its metabolites are part of the mechanism that underlies the increased potency. In the present study we elaborated further on the mechanism of action and investigated 1,25-$(OH)_2D_3$- and KH1060-induced VDR binding to VDREs of three different target genes: rat osteocalcin, human osteocalcin, and mouse osteopontin.

To study VDR-VDRE binding, gel mobility shift assays were performed with *in vitro* synthesized VDR and its dimer partner RXRα, or with nuclear extracts from rat osteoblast-like ROS 17/2.8 cells. ^{32}P-labeled oligonucleotides encoding the rat osteocalcin, human osteocalcin, or mouse osteopontin VDRE were used as probes. To study the impact of differences in nucleotide sequences in more detail, hybrid VDREs were used in which mouse osteopontin VDRE half-sites or single nucleotides were substituted in the rat osteocalcin VDRE context. Transcriptional activity via these VDREs was tested by transient transfection of a luciferase reporter construct into ROS 17/2.8 cells.

We found that both 1,25-$(OH)_2D_3$ and KH1060 dose-dependently increased binding of *in vitro* synthesized VDR/RXRα to the VDREs. KH1060 was only slightly more potent than 1,25-$(OH)_2D_3$ in stimulating VDR/RXRα binding to the mouse osteopontin VDRE. Surprisingly, despite of its increased biological potency, KH1060-stimulated VDR/RXRα binding to human osteocalcin VDRE equally effective as 1,25-$(OH)_2D_3$ and to the rat osteocalcin VDRE even less active than 1,25-$(OH)_2D_3$. When gel mobility shift assays were performed with nuclear extracts of ROS 17/2.8 cells, KH1060 was clearly more potent than 1,25-$(OH)_2D_3$ with all three VDREs. Furthermore, we observed for both 1,25-$(OH)_2D_3$ and KH1060 considerable differences between the three VDRE types in intensity of the ligand-induced retarded bands. Binding to the rat osteocalcin VDRE was less compared to binding to the human osteocalcin VDRE and binding was even stronger when mouse osteopontin VDRE was used as probe. Studies with a series of substitution mutations demonstrated that the third and/or fourth nucleotide (both thymidines) in the proximal half-site of the mouse osteopontin VDRE determined this stronger VDR binding. Transfection studies with a VDRE-luciferase reporter construct in ROS 17/2.8 cells showed that introduction of these thymidines into the proximal half-site of the rat osteocalcin VDRE resulted in a two-fold enhancement of 1,25-$(OH)_2D_3$-induced transcriptional activation compared to the native rat osteocalcin VDRE.

Based on these findings we conclude that the extent of both 1,25-$(OH)_2D_3$- and KH1060-induced VDR/RXR-VDRE binding and transactivation activity depends on specific nucleotide sequences of VDREs and that the nuclear context is a prerequisite to obtain an analog-enhanced magnitude of

VDR/RXR-VDRE binding that reflects the increased biological activity of that analog.

4.2. INTRODUCTION

The classic role of 1,25-$(OH)_2D_3$ includes regulation of calcium and bone metabolism [DeLuca 1997]. In addition, the hormone is an important mediator of cell growth and differentiation [Walters 1992, Van den Bemd 2000a]. The 20-epi-analog KH1060 mimics these biological activities of 1,25-$(OH)_2D_3$, albeit with considerably increased potency [Binderup 1991, Van den Bemd 1995, Peleg 1995]. KH1060 is more potent than 1,25-$(OH)_2D_3$ in stimulating genes encoding for bone matrix proteins (e.g. human collagen type I, rat osteocalcin, human osteocalcin, and mouse osteopontin), both at the level of transcription and translation [Van den Bemd 1995, Ryhänen 1996, Dilworth 1997].

The search for an underlying mechanism to explain the increased biological activity still continues and focuses on studying the cascade of events that leads to modulation of gene transcription (See Chapter 1, Section A.4.). According to this classic model, ligand (1,25-$(OH)_2D_3$ or an analog) binds to the VDR and induces a conformational change [Van den Bemd 1996, Peleg 1995]. As a consequence, the VDR can form dimers with another nuclear receptor, usually RXR [Yu 1991], although homodimerization has been described as well [Carlberg 1993, Freedman 1994, Kahlen 1994, Polly 1996]. Next, the VDR/RXR dimer binds to specific DNA sequences, so-called VDREs in the promoter region of 1,25-$(OH)_2D_3$ target genes after which the preinitiation complex is formed and gene transcription is initialized or suppressed.

Earlier we showed that KH1060 changed the three-dimensional folding of the VDR in such a way that its sensitivity for proteases was diminished [Van den Bemd 1996]. We also found that the KH1060-induced increase in protease resistance was reflected by a KH1060-induced increased VDR half-life in cells [Van den Bemd 1996]. In addition, specific metabolites of KH1060 were found to be biologically active and shared the VDR stabilizing property of their parent compound [Van den Bemd 2000]. In the present study we elaborated on the potential mechanism of action and investigated whether the KH1060-induced change in VDR conformation affects binding of the VDR/RXR dimer to a selection of natural occurring VDREs. Using gel mobility shift assays we studied the binding of *in vitro* synthesized VDR and RXRα, as well as the binding of nuclear extracts (containing VDR and RXR [Staal 1996]) of ROS 17/2.8 cells to the rat osteocalcin (5'-GGGTGA atg AGGACA-3') [DeMay 1992], mouse osteopontin (5'-GGTTCA cga GGTTCA-3') [Noda 1990], and human osteocalcin VDRE (5'-GGGTGA acg GGGGCA-3') [Morrison 1989]. These VDREs are of the DR3-type [Umesono 1991] to which the VDR/RXR complex binds with a defined polarity: the distal half-site is occupied by RXR, while VDR binds to the proximal half-site [Lemon 1996, Jin 1996, Staal 1996]. The human osteocalcin VDRE and mouse osteopontin VDRE differ within their half-sites by the underlined nucleotides from the rat osteocalcin VDRE. The impact of differences in nucleotide

sequences was further unraveled using hybrid VDREs in which the rat osteocalcin VDRE half-sites were replaced by mouse osteopontin VDRE half-sites and by introducing a series of substitution mutations in the rat osteocalcin VDRE half-site. Finally, we transfected ROS 17/2.8 cells with VDRE-driven luciferase reporter constructs to study the influence of distinct nucleotides in the VDRE half-sites on the transcriptional activation by 1,25-$(OH)_2D_3$ and KH1060.

4.3. MATERIALS AND METHODS

Reagents
1,25-$(OH)_2D_3$ and KH1060 were a gift from dr. L. Binderup, Leo Pharmaceuticals, Ballerup, Denmark. Chemical structures are depicted in Figure 1.3. αMEM was from Sigma Chemical Co., St. Louis, MO, USA. VDRE-encoding oligonucleotides, fetal bovine serum, penicillin, streptomycin, and L-glutamine were purchased from Gibco Life Technologies Ltd., Paisley, Scotland. The TNT lysate assay, *Nhe*I, *Bgl*II, Tfx-50, the pGL3 control and reporter plasmids, and luciferase assay reagent were from Promega, Madison, WI, USA.

Cells
The rat osteoblast-like osteosarcoma cell line ROS 17/2.8 was cultured in αMEM supplemented with 2 mM L-glutamine, 100 units/ml penicillin, 100 μg/ml streptomycin, 0.1% D-glucose plus 10% fetal bovine serum. When the cells reached subconfluence medium was replaced by αMEM containing 2% charcoal-treated fetal bovine serum. After 24 hours of culture ligand incubations or transfections were performed for the indicated period of time.

In vitro synthesis of VDR and RXR$_\alpha$ and preparation of nuclear extracts
Human recombinant VDR and RXRα were *in vitro* synthesized with Promega' s TNT lysate assay according the instructions of the manufacturer using cDNA encoding for human VDR (in pGem4; a gift from dr M.R. Haussler, University of Arizona, Tucson, AZ, USA) and RXRα (in pSG5; a gift from dr P. Chambon, INSERM, Strasbourg, France). For preparation of nuclear extracts, rat osteoblastic ROS 17/2.8 cells were incubated for 1 hour with 1,25-$(OH)_2D_3$ or KH1060. Next, nuclear extracts were prepared in 20 mM HEPES, pH 7.5, 420 mM KCl, 25% glycerol and 0.2 mM EDTA, according to the method described previously [Staal 1996].

Gel mobility shift assay
Gel mobility shift assays were performed as described earlier [Staal 1996]. In brief: 10 μl

of a mixture of *in vitro* synthesized VDR and RXRα treated for 15 minutes at 37 °C with ligand ($1,25\text{-}(OH)_2D_3$ or KH1060); or 10 µl of diluted nuclear extract (5 µg protein) was incubated for 15 minutes at room temperature with 10 µl of ^{32}P-labeled oligonucleotides (10 fmol). The oligonucleotides used in this study are presented in Table 4.1. The protein/DNA complexes formed were separated on a 5% polyacrylamide gel (acrylamide:bisacrylamide = 80:1) in 0.5 x TBE buffer. In the competition experiments, ^{32}P-labeled oligonucleotides and different concentrations of unlabeled oligonucleotides were mixed and subsequently incubated with nuclear extract. The VDR ligand-responsive complex was visualized by exposure to Fuji RX medical X-ray film. All gel mobility shift experiments were performed with excess probe, but for reasons of clarity only the shifted bands are shown.

Table 4.1. DR3-type VDREs used in this study

ROC	5'-ctgcact	GGGTGA atg AGGACA	ttactga-3'
MOP	5'-acaa	GGTTCA cga GGTTCA	cgtct-3'
HOC	5'-gactcacc	GGGTGA acg GGGGCA	ttgcga-3'
OP/OC	5'-ctgcact	GGTTCA atg AGGACA	ttactga-3'
OC/OP	5'-ctgcact	GGGTGA atg GGTTCA	ttactga-3'
OP/OP	5'-ctgcact	GGTTCA atg GGTTCA	ttactga-3'
PM 3T	5'-ctgcact	GGGTGA atg AGTACA	ttactga-3'
PM 4T	5'-ctgcact	GGGTGA atg AGGTCA	ttactga-3'
PM 3T4T	5'-ctgcact	GGGTGA atg AGTTCA	ttactga-3'
PM 1G3T	5'-ctgcact	GGGTGA atg GGTACA	ttactga-3'
PM 1G4T	5'-ctgcact	GGGTGA atg GGGTCA	ttactga-3'

ROC = rat osteocalcin, MOP = mouse osteopontin, HOC = human osteocalcin. The oligonucleotides OP/OC, OC/OP, OP/OP, PM 3T, PM 4T, PM 3T4T, PM 1G3T, and PM 1G4T contain substitution mutations within the rat osteocalcin VDRE context. The underlined nucleotides are distinct from the corresponding nucleotides within the rat osteocalcin VDRE half-sites.

Transactivation assays

Oligonucleotides with *Bgl*II- and *Nhe*I-compatible ends containing the rat osteocalcin: 5'-CTAGCTGCACT**GGGTGAatgAGGACA**TTACTGA-3', or OC/OP VDRE: 5'-CTAGCTGCACT**GGGTGAatgGGTTCA**TTACTGA-3' (VDREs are shown in bold. The underlined nucleotides are distinct from the

corresponding nucleotides within the proximal rat osteocalcin VDRE half-site) were cloned into the multiple cloning site of the pGL3 vector containing luciferase cDNA as the reporter gene. Sequences were confirmed by dideoxysequencing using a 310 genetic analyzer (Perkin Elmer). ROS 17/2.8 cells cultured in 10 cm^2 dishes were transfected with 5 μg of reporter plasmid/well using Tfx-50 reagent (1:3). The pGL3 control plasmid was used as the normalization vector. Cells recovered for 4 hours, and were then treated with vehicle (0.1% ethanol) or 10^{-8} M 1,25-$(OH)_2D_3$. Luciferase activity was measured after 24 hours of ligand incubation using luciferase assay reagent and the Lumac Biocounter M2500.

4.4. RESULTS

Gel mobility shift assays revealed that ligand-induced binding of *in vitro* synthesized VDR/RXRα to the VDREs was ligand-dependent. In the absence of 1,25-$(OH)_2D_3$, only minor VDR/RXRα binding was observed. 1,25-$(OH)_2D_3$ and KH1060 dose-dependently enhanced VDR/RXRα binding (Figure 4.1). With the mouse osteopontin VDRE KH1060 was only slightly more potent than 1,25-$(OH)_2D_3$.

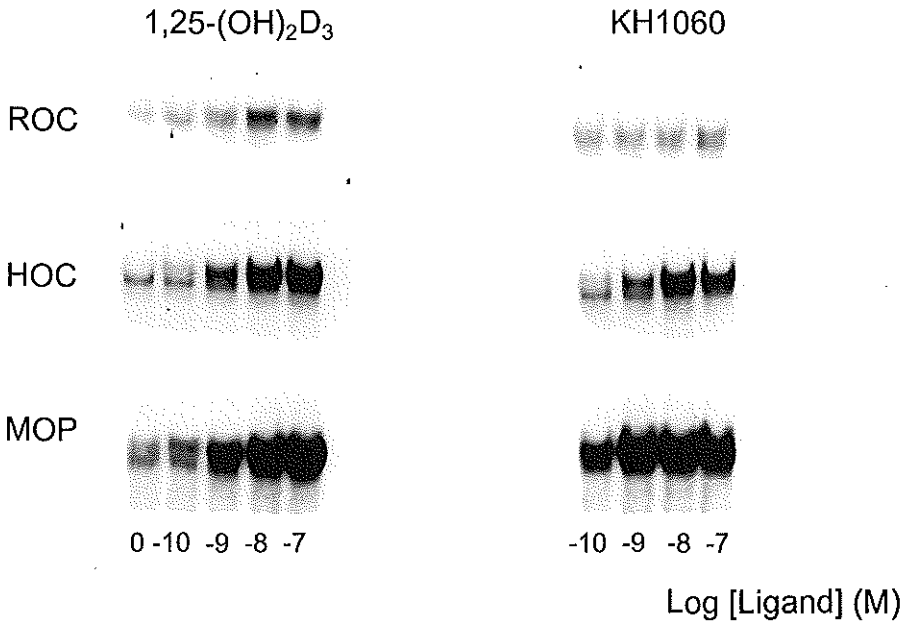


Figure 4.1. Binding of *in vitro* synthesized VDR and RXRα to rat

osteocalcin, human osteocalcin, or mouse osteopontin VDREs. *In vitro synthesized VDR and RXR$_\alpha$ were incubated for 10 minutes with vehicle (0.1% ethanol) or increasing amounts of 1,25-(OH)$_2$D$_3$ or KH1060 (10^{-10}-10^{-7} M) and subsequently incubated with 10 fmol of ^{32}P-labeled rat osteocalcin, human osteocalcin, or mouse osteopontin VDREs and protein/DNA complexes were separated by gel electrophoresis.*

However, unexpectedly in view of its potent induction of osteocalcin production (Van den Bemd 1995), KH1060 and 1,25-(OH)$_2$D$_3$ had a comparable stimulatory effect on VDR/RXRα binding to the human osteocalcin VDRE and the potency of KH1060 to induce VDR/RXRα binding to the rat osteocalcin VDRE was even lower than that of 1,25-(OH)$_2$D$_3$ (Figure 4.1).

In the present study we also performed gel mobility shift assays using nuclear extracts of ROS 17/2.8 cells, in which we already demonstrated the presence of VDR and RXR using monoclonal antibodies (Staal 1996). When nuclear extracts of 1,25-(OH)$_2$D$_3$- or KH1060-treated ROS 17/2.8 cells were used, ligand dose-dependent binding of nuclear proteins to all three VDREs was observed (Figure 4.2). In contrast to the studies performed with *in vitro* synthesized VDR and RXRα, the experiments with nuclear extracts demonstrated a stronger effect of KH1060 than of 1,25-(OH)$_2$D$_3$, not only on the binding to the mouse osteopontin and human osteocalcin VDRE, but also to the rat osteocalcin VDRE (compare Figures 4.1 and 4.2).

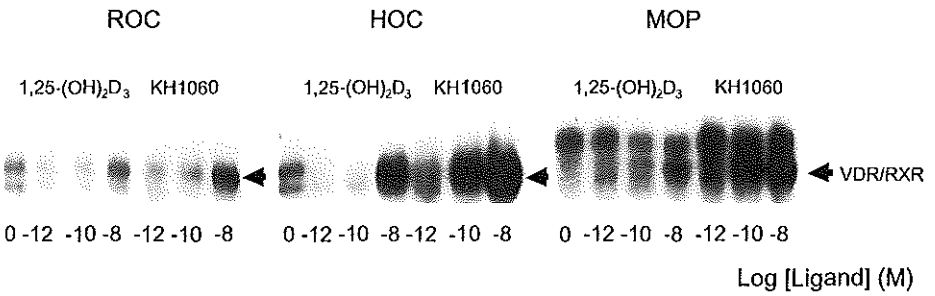


Figure 4.2. Binding of nuclear proteins of ROS 17/2.8 cells to rat osteocalcin, human osteocalcin, and mouse osteopontin VDREs. *Gel mobility shift assays were performed with nuclear extracts from vehicle-, 1,25-(OH)$_2$D$_3$-, or KH1060-treated (10^{-12}-10^{-10}-10^{-8} M, 1 hour) ROS 17/2.8 cells. Extracts were incubated with 10 fmol of ^{32}P-labeled rat osteocalcin, human osteocalcin and mouse osteopontin VDREs and protein/DNA*

complexes were separated by gel electrophoresis. Ligand-responsive complexes for the various VDREs are indicated by the arrowhead.

Figure 4.1 also shows that, when compared with the rat osteocalcin VDRE the liganded (either 1,25-$(OH)_2D_3$ or KH1060) VDR/RXRα complex exhibits a preference for binding the mouse osteopontin VDRE, while with the human osteocalcin VDRE intermediate VDR/RXRα-VDRE binding was observed. Likewise, the intensity of the shifted complex with nuclear extracts was strongest for the mouse osteopontin VDRE, followed by the human osteocalcin and rat osteocalcin VDRE (Figure 4.2).

To study the contribution of the various hexamer motifs in this differential preference for DNA we introduced substitution mutations, replacing rat osteocalcin VDRE half-sites by mouse osteopontin VDRE half-sites (Table 4.1).

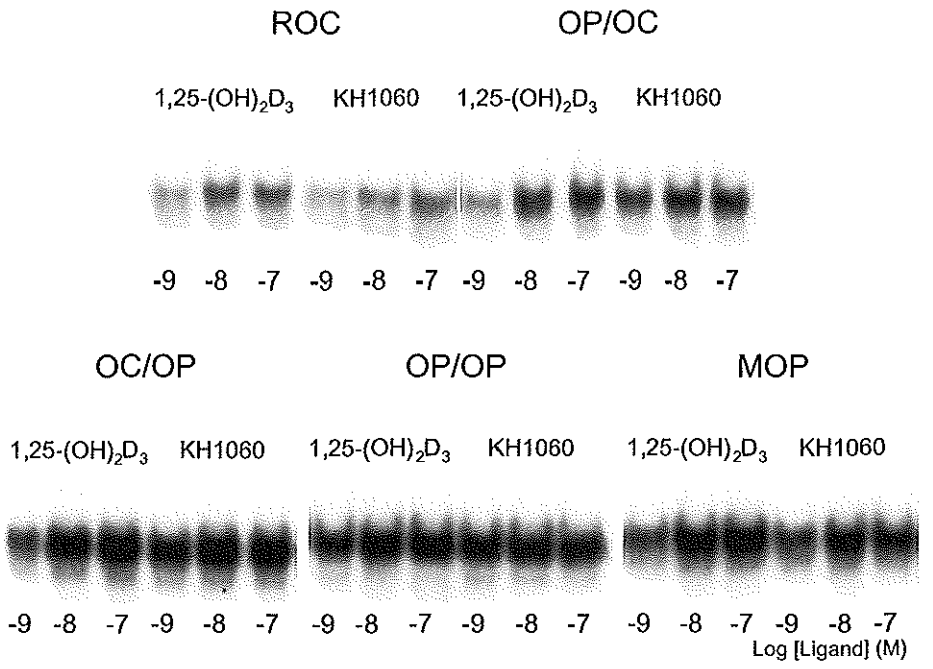


Figure 4.3. Binding of *in vitro* synthesized VDR and RXRα to hybrid rat osteocalcin and mouse osteopontin VDREs. *In vitro synthesized VDR and RXR$_\alpha$ were incubated for 10 minutes with vehicle (0.1% ethanol) or increasing amounts of 1,25-$(OH)_2D_3$ or KH1060 (10^{-9}-10^{-8}-10^{-7} M) and subsequently incubated with 10 fmol of ^{32}P-labeled oligonucleotides encoding for wild-type and substitution mutation VDREs as presented in Table 4.1. Protein/DNA complexes were separated by gel electrophoresis.*

With both *in vitro* synthesized VDR and RXRα (Figure 4.3) and nuclear extracts (Figure 4.4) replacement of the distal rat osteocalcin VDRE half-element by the corresponding mouse osteopontin VDRE half-site (OP/OC) only slightly increased the intensity of the 1,25-$(OH)_2D_3$- and KH1060-induced shifted band. Substitution of the proximal half-site alone (OC/OP), or in combination with the distal half-site (OP/OP), led to further increased DNA binding to levels comparable to that of the intact mouse osteopontin VDRE (MOP).

This was observed with both *in vitro* synthesized VDR/RXRα and nuclear extracts and with 1,25-$(OH)_2D_3$ as well as KH1060 (Figures 4.3 and 4.4). So far these findings clearly indicate that the proximal VDRE half-site, i.e. the VDR binding-site, has the largest impact on the extent of VDR/RXR binding to DNA.

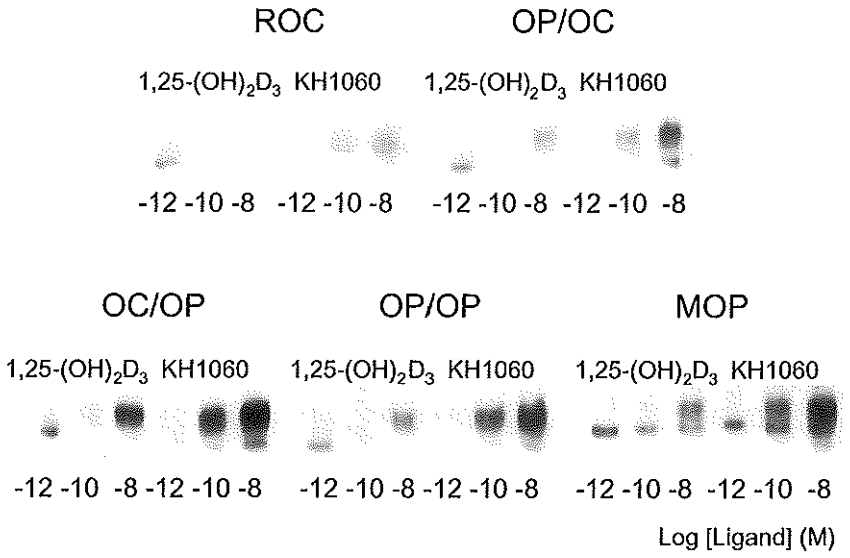


Figure 4.4. Binding of nuclear proteins of ROS 17/2.8 cells to hybrid rat osteocalcin and mouse osteopontin VDREs. *Gel mobility shift assays were performed with nuclear extracts from vehicle-, 1,25-$(OH)_2D_3$-, or KH1060-treated (10^{-12}-10^{-10}-10^{-8} M, 1 hour) ROS 17/2.8 cells. Nuclear extracts were incubated with 10 fmol of ^{32}P-labeled oligonucleotides encoding for wild-type and substitution mutation VDREs (Table 4.1). Protein/DNA complexes were separated by gel electrophoresis.*

Using single substitution mutations we further investigated which nucleotide(s) in

the proximal half-site is/are significant in determining the observed differences in VDRE binding. The significance of the 3T and 4T nucleotides in the proximal half-site of the mouse osteopontin VDRE was already notified (Freedman 1994, Staal 1996). Here, we show that introduction of one (PM 3T, PM 4T) or both of these nucleotides (PM 3T4T) strongly enhanced 1,25-$(OH)_2D_3$-induced VDR/RXR-VDRE-binding. Additional substitution of 1G (PM 1G3T and PM 1G4T) had no clear supplementary effect (Figure 4.5).

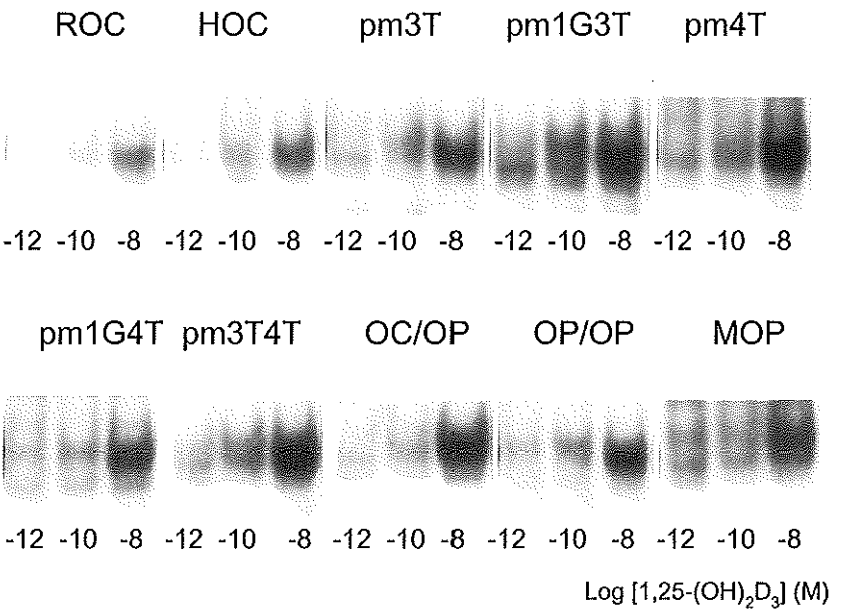


Figure 4.5. Binding of nuclear proteins of ROS 17/2.8 cells to rat osteocalcin VDREs containing specific point mutations. *Gel mobility shift assays were performed with nuclear extracts from 1,25-$(OH)_2D_3$-treated (10^{-12}-10^{-10}-10^{-8} M, 1 hour) ROS 17/2.8 cells and point mutated VDREs as presented in Table 4.1.*

We also studied whether differences in binding to VDREs are reflected by differences in affinity for these VDREs. Competition analysis using ^{32}P-labeled rat osteocalcin VDRE and increasing amounts (10-1,000 fmol) of unlabeled competitor oligonuceotides (rat osteocalcin or mouse osteopontin VDRE) showed that *in vitro* synthesized VDR/RXRα displayed increased affinity for mouse osteopontin VDRE (Figure 4.6).

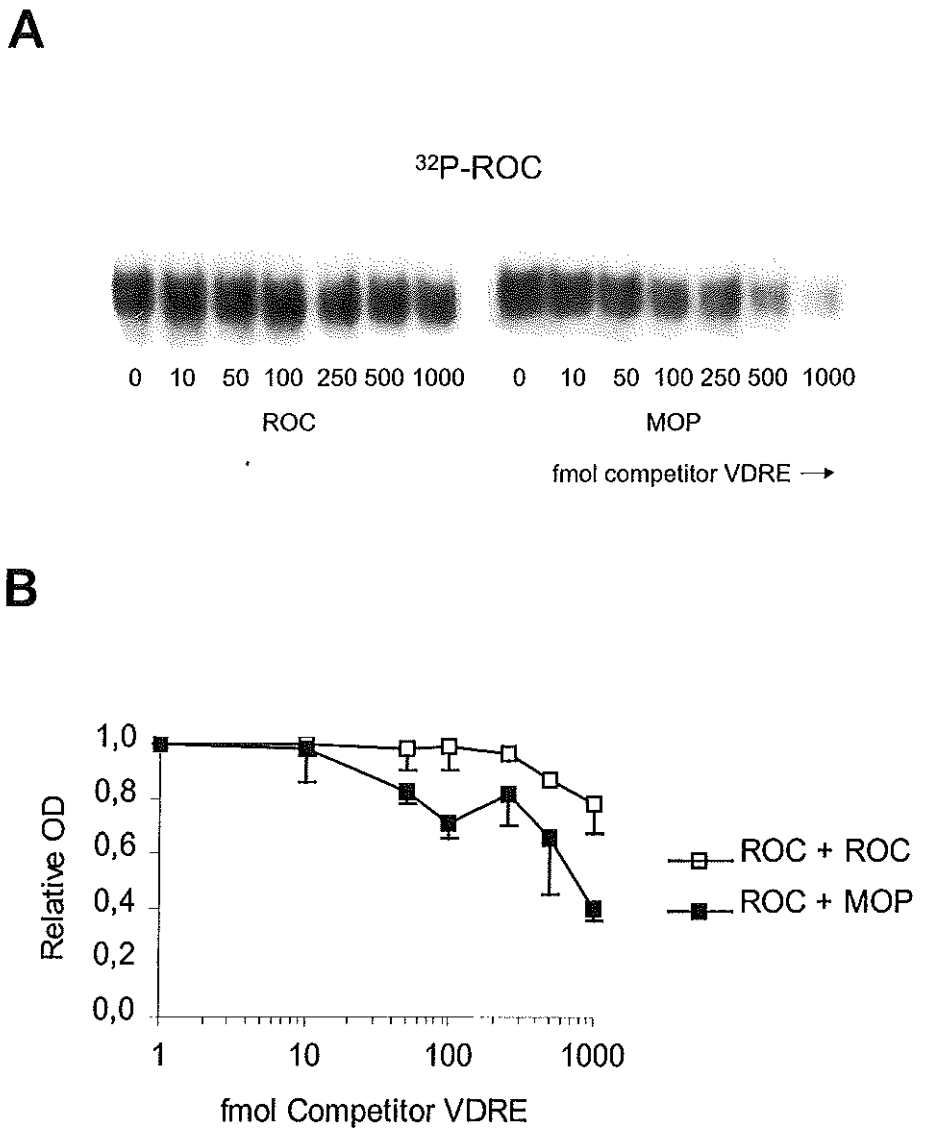
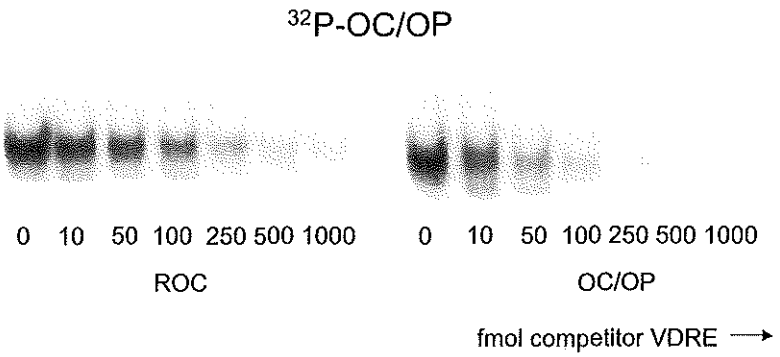


Figure 4.6. Competition gel mobility shift analysis with *in vitro* synthesized VDR/RXRα and rat osteocalcin VDRE. *In panel A, in vitro synthesized VDR/RXR$_\alpha$ incubated with 1,25-(OH)$_2$D$_3$ (10^{-8} M, 10 minutes) was incubated with a mixture of 10 fmol of ^{32}P-labeled rat osteocalcin VDRE-containing oligonucleotides and increasing amounts of unlabeled rat osteocalcin (ROC) or mouse osteopontin (MOP) competitor oligonucleotides (10-1000 fmol). In panel B, a computerized optical density scan of the shifted complex is shown. Data represent the means of two independent experiments +/- standard deviations. The OD value of the shifted complex in the absence of competitor was set to 1.*

A



B

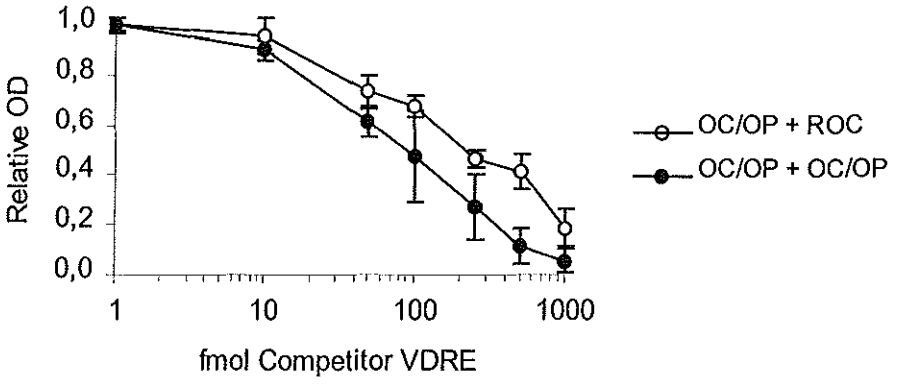


Figure 4.7. Competition gel mobility shift analysis with nuclear extracts of ROS 17/2.8 cells and OC/OP VDRE. *In panel A, nuclear extract of ROS 17/2.8 cells incubated with 1,25-$(OH)_2D_3$ (10^{-8} M, 1 hour) was incubated with a mixture of 10 fmol of ^{32}P-labeled OC/OP VDRE-containing oligonucleotides and increasing amounts of unlabeled competitor rat osteocalcin (ROC) or OC/OP oligonucleotides (10-1000 fmol). In panel B, a computerized optical density scan of the shifted complex is shown. Data represent the means of three independent experiments +/- standard deviations. The OD value of the shifted complex in the absence of competitor was set to 1.*

We further investigated the impact of differences in nucleotide sequences on the affinity of nuclear extracts for the wild type rat osteocalcin VDRE and the OC/OP VDRE. Competition assays revealed that nuclear proteins of ROS 17/2.8 cells displayed an increased binding affinity for OC/OP VDRE (Figure 4.7).

Finally, we investigated whether the differences in VDR/RXR binding to DNA observed in the gel mobility shift assays are reflected by differences in transactivation activity. ROS 17/2.8 cells were transfected with a pGL3 luciferase reporter vector containing rat osteocalcin or OC/OP VDRE sequences. As can be seen in Table 4.1 these VDREs differ only by 3 nucleotides in the proximal half-site. We found that the transactivation activity of 1,25-$(OH)_2D_3$ was enhanced two-fold when the first, third, and fourth nucleotide of the proximal half-site of the rat osteocalcin VDRE were replaced by corresponding nucleotides of the mouse osteopontin VDRE (Figure 4.8).

4.5. DISCUSSION

The present paper shows that the increased biological potency of KH1060 is not reflected by an increased binding of *in vitro* synthesized VDR and RXRα to various VDREs. KH1060-induced binding of *in vitro* synthesized VDR/RXRα to the mouse osteopontin VDRE was slightly increased, while binding to human osteocalcin and rat osteocalcin VDREs was comparable to or even less than with 1,25-$(OH)_2D_3$. Studies by Imai *et al.* with RO 24-2637 and RO 23-7553 also demonstrated a lack of parallelism between the potency of these analogs to induce binding of recombinant human VDR and RXRα to the human osteocalcin VDRE and the capacity of these analogs to activate a human osteocalcin VDRE-driven reporter gene [Imai 1995]. However, when we performed the gel mobility shift assays with nuclear extracts of osteoblast-like cells, the biological potency of KH1060 was paralleled by an increased binding of nuclear factors to the different VDREs studied. These observations underline the absolute importance of the cellular context, i.e. absence or presence of (nuclear) cofactors for the interaction between VDR/RXR and VDRE and to observe KH1060-induced VDR/RXR-DNA binding that reflects its biological potency. Also Zhao *et al.* showed that the ability of KH1060 and other 1,25-$(OH)_2D_3$ analogs (RO 24-5531, MC903, ED-71) to enhance binding of VDR expressed in COS-7 cells and RXRα from yeast extract to the human osteocalcin VDRE correlated with their potency to transactivate a human osteocalcin VDRE-driven reporter gene [Zhao 1997]. The importance of a nuclear/cellular context is also illustrated by cell type-dependent repression of gene transcription via the human PTH VDRE. In bovine parathyroids and in rat pituitary GH4C1 cells, but not in ROS 17/2.8 cells the human PTH VDRE mediates transcriptional repression [MacKey 1996].

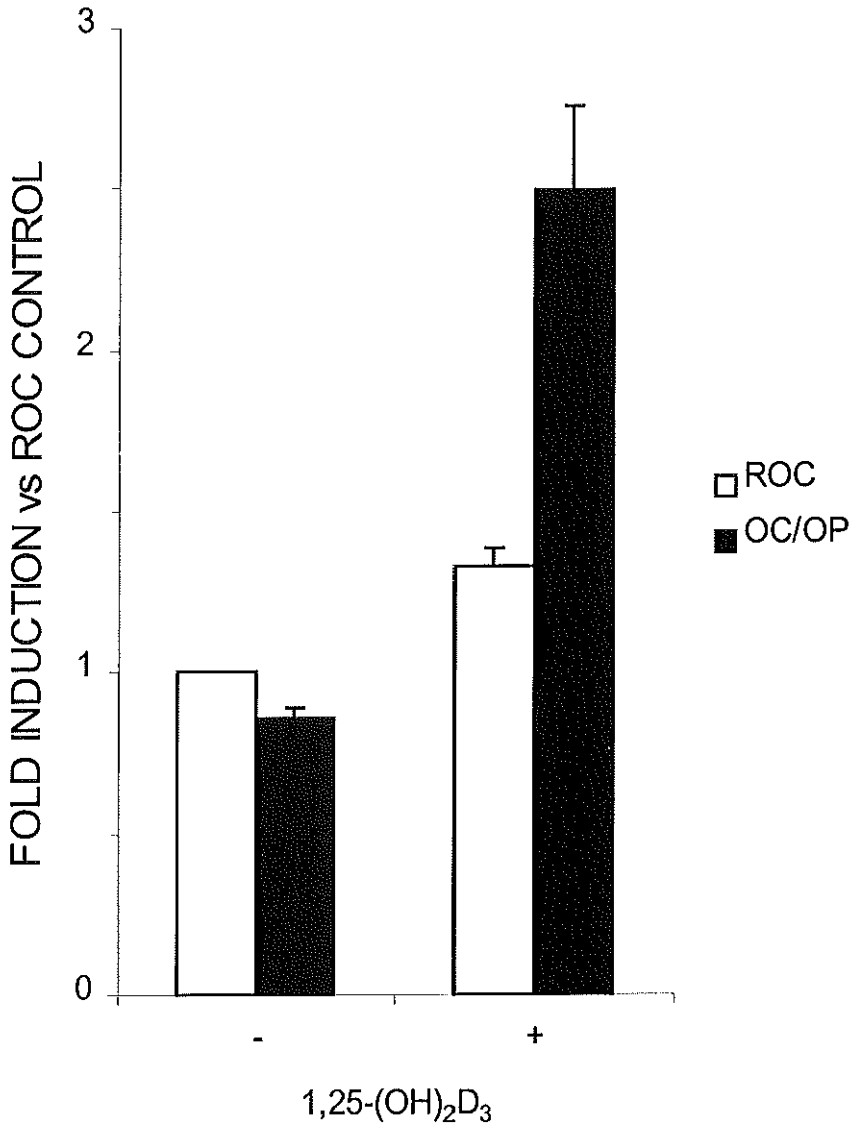


Figure 4.8. Transactivation assay with ROS 17/2.8 cells transiently transfected with a luciferase reporter gene driven by either the rat osteocalcin VDRE or OC/OP hybrid VDRE. *The rat osteocalcin (ROC) VDRE and OC/OP VDRE were transiently transfected into ROS 17/2.8 cells as described in Materials and Methods, Section 4.3. Luciferase activity was measured after 24 hours of ligand incubation. The luciferase activity measured in the vehicle-treated ROS 17/2.8 cells transfected with the wild type rat osteocalcin (ROC) VDRE was set to 1.*

This cell type-specific difference was paralleled by distinct protein-DNA binding: in the extracts of bovine parathyroids and GH4C1 cells VDR homodimer binding to the human PTH VDRE was observed, whereas the complex formed with extracts of ROS 17/2.8 cells contained VDR/RXR heterodimers. Transcriptional repression of the human PTH gene seems, therefore, dependent on the ability of the cell (i.e. dependent on the presence or absence of certain cofactors) to induce VDR-VDRE binding without interference of RXR (Mackey 1996).

Several processes might be involved in the increased potency of KH1060 to induce VDR/RXR-DNA binding. Incubation of ROS 17/2.8 cells with KH1060 might have a different effect than 1,25-$(OH)_2D_3$ on the amount and/or distribution of VDR, RXR, and/or cofactors in the nucleus leading to changed formation and/or affinity of DNA-binding complexes. Although the ligand incubation period used was relatively short (1 hour) it is not unlikely that *de novo* synthesis of receptors or cofactors has taken place (Candeliere 1991). In addition, during the incubation period migration of receptors/cofactors from the cytoplasm to the nucleus might occur. It was for instance shown that the VDR migrates from the cytoplasm to the nucleus within several minutes after addition of 1,25-$(OH)_2D_3$ (Barsony 1997). In addition, compared to 1,25-$(OH)_2D_3$ KH1060 might induce the formation of a more stable VDR/RXR-DNA complex. Cheskis *et al.* demonstrated, by using surface plasmon resonance, that certain analogs with stronger transcriptional activation activity than 1,25-$(OH)_2D_3$ induced increased stability of the VDR/RXR-mouse osteopontin VDRE complex (Cheskis 1995).

Another important observation in the present study is the difference in intensity in the DNA-bound complexes between the three VDRE types tested. Over 30 different genes have been identified that contain a responsive element for 1,25-$(OH)_2D_3$ (See Table 1.2). The various VDREs show higher or lower resemblance to the consensus sequence G/AGGTCA ttg AGTTCA (Nishikawa 1994). Variations in flanking and half-site sequences may be important discriminators involved in specific gene regulation. Earlier, our laboratory showed the impact of differences in nucleotide sequences within VDREs on the immunoreactivity of the VDR/RXR complex (Staal 1996), indicating that specific conformational changes occur upon VDR/RXR-VDRE binding. These conformational changes will affect subsequent interaction with cofactors and basal transcription factors and eventually will result in altered gene transcription. Here we show that ligand-induced binding of VDR/RXR complexes to rat osteocalcin VDRE was less pronounced compared to the binding to human osteocalcin and mouse osteopontin VDREs. This confirms our previous work (Staal 1996a), and is supported by work from others (Kuno 1994, Thompson 1998a). Our observation that VDREs containing the mouse osteopontin proximal half-site have a higher affinity for VDR/RXR and osteoblast nuclear extracts than wild type rat osteocalcin VDREs is consistent with findings

of Mackey *et al.* showing that VDR/RXR complexes from GH4C1 cells bound with higher affinity to the mouse osteopontin VDRE than to the rat osteocalcin VDRE (Mackey 1996).

In addition, we show that the intensity of ligand-induced VDR/RXR binding to functional VDREs can mainly be attributed to the VDR half-site, the 3 and/or 4 T in particular. The impact on the extent of VDR/RXR-DNA binding by minor changes in nucleotide sequences is also illustrated by work of others (Jääskeläinen 1995, Koszewski 1999, Ozono 1998). Ozono *et al.* showed that a non-VDR binding accessory element within the rat 24-hydroxylase gene was converted to a VDR-binding site when the fourth nucleotide within its proximal half-site was substituted by adenine or thymidine (Ozono 1998). Koszewski *et al.* demonstrated that two mutations in the proximal half-site of the avian PTH VDRE converted the negative activity of this VDRE into a positive one (Koszewski 1999). The large impact of only small changes in nucleotide sequences on receptor-DNA binding is not restricted to VDR-VDRE interaction. For instance, within EREs a change of one base pair in the proximal half-site (the vitellogenin A2 ERE versus the human pS2 ERE; see Table 1.4) resulted in a three-fold lower ER affinity (Curtis 1991), and introduction of two mutations converted the vitellogenin A2 ERE into a glucocorticoid responsive element (Martinez 1987).

In summary, the present data demonstrate that for the 1,25-$(OH)_2D_3$ analog KH1060 a cellular/nuclear context (i.e. absence or presence of nuclear cofactors) is crucial to observe ligand-induced VDR-VDRE binding that reflects its increased biological potency (Van den Bemd 1995, Ryhänen 1996, Dilworth 1997). Thereby, this study implicates the significance of these nuclear cofactors for determining the extent of transcriptional activity. Finally, the present data emphasize that VDR/RXR binding is directed by specific nucleotides in VDRE sequences. This might form part of a mechanism to achieve response selectivity in the action of 1,25-$(OH)_2D_3$ and 1,25-$(OH)_2D_3$ analogs.

CHAPTER 5

CONTRIBUTION OF SEVERAL METABOLITES OF THE VITAMIN D ANALOG KH1060 TO THE OVERALL BIOLOGICAL ACTIVITY OF KH1060 BY A SHARED MECHANISM OF ACTION

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Biochem Pharmacol, 59, 621-627, 2000

5.1. ABSTRACT

The synthetic 1,25-$(OH)_2D_3$ analog KH1060 is considerably more potent than its cognate hormone. The mechanism of action of KH1060 includes interaction with the VDR. We previously showed that KH1060 increases VDR stability in ROS 17/2.8 osteoblastic cells by inducing a specific conformational change in the VDR. KH1060 is metabolized, both *in vivo* and *in vitro*, into several stable products. In the present study, we investigated whether these metabolites might contribute to the increased biological activity of KH1060. We found that the potencies of two of these metabolites, 24a-OH-KH1060 and 26-OH-KH1060 were similar to that of 1,25-$(OH)_2D_3$ in inducing osteocalcin production by the osteoblast cell line ROS 17/2.8. This report further showed that these metabolites had the same effect as KH1060 on VDR: they increased VDR stability in ROS 17/2.8 cells, while limited proteolytic analysis revealed that they caused a conformational change in the VDR, resulting in an increased resistance against proteolytic cleavage. Furthermore, as shown in gel mobility shift assays, both compounds clearly induced VDR binding to VDREs. Together, these results show that the potent *in vitro* activity of KH1060 is not only directed by the effects on the VDR conformation/stabilization of the analog itself, but also by certain of its long-lived metabolites, and emphasizes the importance of detailed knowledge of the metabolism of synthetic hormonal analogs.

5.2. INTRODUCTION

The clinical usefulness of 1,25-$(OH)_2D_3$ in the treatment of cancer and immunological disorders is limited by its calcemic activity (Vieth 1990). In an attempt to obtain agents with a more favorable therapeutic profile, numerous 1,25-$(OH)_2D_3$ analogs have been developed (Bouillon 1995). Some of these analogs exert increased *in vivo* and *in vitro* activity compared to 1,25-$(OH)_2D_3$. One of the most potent, KH1060, has very strong effects on *in vitro* cell growth and differentiation and has high immunosuppressive activity (Binderup 1991, Binderup 1992, Vink-Van Wijngaarden 1994, Van den Bemd 1995). The mechanism(s) underlying the increased potency of KH1060 are not completely clear. Interaction with the VDR is crucial for the action of KH1060 (Van den Bemd 1995). In a previous study, we showed that, compared to 1,25-$(OH)_2D_3$, KH1060 potently increased VDR stability in ROS 17/2.8 osteoblastic cells. KH1060 also induced a different conformation of the VDR, resulting in an increased protease resistance which is in line with the VDR stability data (Van den Bemd 1996). Besides these VDR localized mechanisms, the metabolic characteristics of the analogs might also be important.

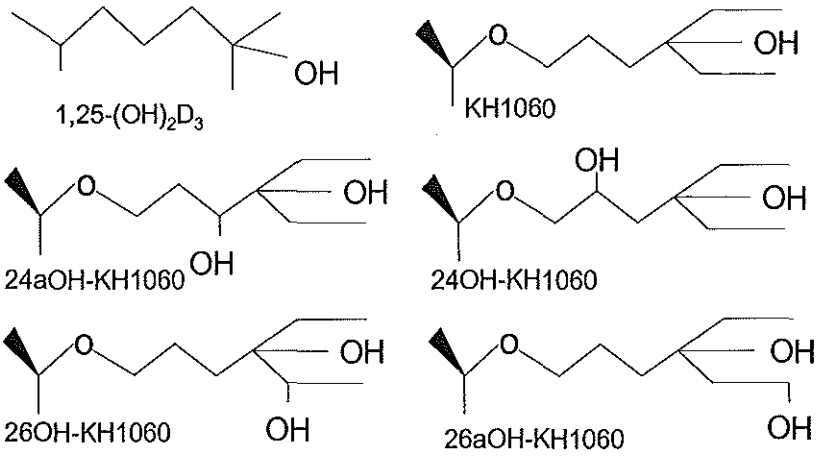


Figure 5.1. Chemical structure of the side chain of 1,25-$(OH)_2D_3$ and the modifications in the side chain of the synthetic analog KH1060 and its metabolites 24a-OH-KH1060, 24-OH-KH1060, 26-OH-KH1060, and 26a-OH-KH1060

In vitro KH1060 is metabolized into at least 22 different compounds, including several stable and biologically active ones (Dilworth 1997). The formation of these metabolites might contribute to the increased biological activity of KH1060. In the present study, the 4 most abundant metabolites (24a-OH-KH1060, 24-OH-KH1060, 26a-OH-KH1060, and 26-OH-KH1060) were examined as to their capability to stimulate osteocalcin synthesis in the osteoblast cell line ROS 17/2.8. Furthermore, the effects of these metabolites on VDR stability, VDR conformation, and VDR binding to VDREs were investigated.

5.3. MATERIALS AND METHODS

Reagents

αMEM and cycloheximide were from Sigma Chemical Co. L-Glutamine, penicillin, and streptomycin were from Gibco Life Technologies Inc. Fetal bovine serum was purchased from BioWhittaker. ^{32}P-ATP and [^{35}S]methionine

were from Amersham. Ribonuclease inhibitor recombinant RNasin and the TNT lysate assay were from Promega. Trypsin was from Boehringer Mannheim. Poly[dI-dC] was purchased from Pharmacia.

Generation, Extraction and Purification of Metabolites of KH1060

HPK1A-*ras* cells (a gift from R. Kremer, Royal Victoria Hospital, McGill University, Montreal, Canada) were incubated with KH1060 (provided by L. Binderup and A.-M. Kissmeyer, Leo Pharmaceutical Products) to generate KH1060 metabolites as described earlier (Dilworth 1997). The most abundant metabolites, i.e. 24a-OH-KH1060, 24-OH-KH1060, 26-OH-KH1060, and 26a-OH-KH1060, were further examined in this study. Their identities were confirmed by GC-MS and their chemical structures are depicted in Figure 5.1.

Osteocalcin Production Measurements

The rat osteoblast-like cell line ROS 17/2.8 (provided by S.B. Rodan, Merck, Sharp & Dohme) was cultured for 24 hours with vehicle, or with 1,25-$(OH)_2D_3$, KH1060, or the metabolites (10^{-14}-10^{-8} M) as described earlier (Van den Bemd 1995). Osteocalcin production was measured by radioimmunoassay (Verhaeghe 1989).

VDR Stability Study

As described earlier (Van den Bemd 1996), the ROS 17/2.8 cells were seeded in 100x15 mm tissue culture dishes and cultured for 2 days in αMEM supplemented with 2 mM L-glutamine, 100 units/ml penicillin, 100 µg/ml streptomycin, 0.1% D-glucose, and 10% fetal bovine serum. At 80% confluency, the medium was changed to αMEM containing 2% charcoal-treated fetal bovine serum and 10 µM cycloheximide to block translation and thereby new synthesis of VDR. Next, vehicle, 1,25-$(OH)_2D_3$, KH1060, or its metabolites (1 nM) were added and after 4 and 24 hours cell extracts were prepared (Wiese 1992). In the extracts, VDR content was measured using an enzyme-linked immunoassay (Uhland-Smith 1996).

In vitro Transcription and Translation

Human VDR cDNA (Baker 1988), a gift from M. R. Haussler of the University of Arizona, was *in vitro* transcribed and translated in the presence of [^{35}S]methionine (specific activity 1000 Ci/mmol) and ribonuclease inhibitor recombinant RNasin, using the TNT lysate assay according to the manufacturer's instructions.

Limited Proteolytic Digestion of in vitro Synthesized VDR

In vitro synthesized VDR was incubated with 1,25-$(OH)_2D_3$, KH1060, or the metabolites (10^{-12}-10^{-9} M, 20 minutes, room temperature) and subsequently treated with trypsin (25 µg/ml) for 10 minutes at room temperature. Trypsin concentration-dependent (0-500 µg/ml) resistance was tested at 10 nM ligand. The labeled fragments were separated on a 12.5% (w/v) polyacrylamide gel and visualized by exposure to Fuji RX medical x-ray film.

Gel Mobility Shift Assay

Gel mobility shift assays with nuclear extracts from ROS 17/2.8 cells treated for 1 hour with vehicle, 1,25-$(OH)_2D_3$, KH1060, or the metabolites (1 nM) were performed as described earlier (Staal 1996). The ^{32}P-labeled rat osteocalcin (5'-CTGCACTGGGTGAATGAGGACATTACTGA-3') and rat cytochrome P_{450} (CYP24) VDRE oligo (5'-CGCGAGGTGAGTGAGGGCGCCGC-3') were incubated with 5 µg of nuclear protein in a final KCl concentration of 50 mM and in the presence of 0.1 µg/µl of poly[dI-dC] non-specific competitor DNA. The protein-DNA complexes were electrophoretically separated on a 5% non-denaturing polyacrylamide gel in 0.5 x TBE (0.045 M Tris-borate; 0.001 M EDTA) buffer and visualized by autoradiography. The shifted probe was scanned from the autoradiograph, and OD values were expressed relative to the OD of the shifted probe after vehicle treatment. For reasons of clarity standard deviations (always smaller than 10%) were not depicted in most of the figures.

5.4. RESULTS

Effect of 1,25-$(OH)_2D_3$, KH1060, and the Metabolites on Osteocalcin Production by ROS 17/2.8 Cells

Figure 5.2 shows that 1,25-$(OH)_2D_3$, KH1060, and the KH1060 metabolites induced osteocalcin production in a dose-dependent manner. On the basis of ED_{50}, KH1060 was the most potent analog ($4.5x10^{-13}$ M) followed by 24a-OH-KH1060 ($1.3x10^{-11}$ M), 1,25-$(OH)_2D_3$ ($7.2x10^{-11}$ M), 26a-OH-KH1060 ($1.2x10^{-10}$ M), 26-OH-KH1060 ($2.5x10^{-10}$ M), and 24-OH-KH1060 ($1.4x10^{-9}$ M). All metabolites and 1,25-$(OH)_2D_3$ induced osteocalcin production with a similar maximum, whereas KH1060 had a somewhat lower maximal response.

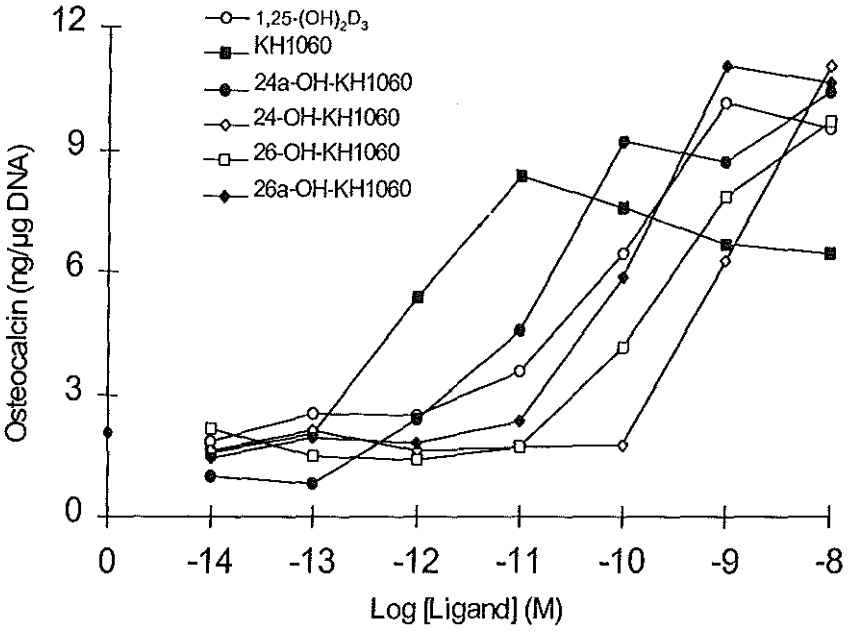


Figure 5.2. Effect of 1,25-$(OH)_2D_3$, KH1060, and its metabolites 24a-OH-KH1060, 24-OH-KH1060, 26-OH-KH1060, and 26a-OH-KH1060 on the synthesis of osteocalcin by ROS 17/2.8 cells. *Cells were treated for 24 hours with vehicle or with 1,25-$(OH)_2D_3$, KH1060, or its metabolites 24a-OH-KH1060, 24-OH-KH1060, 26-OH-KH1060, and 26a-OH-KH1060 (10^{-14}-10^{-8} M) and osteocalcin secreted into the medium was determined as described in Materials and Methods (Section 5.3). Each point represents the mean of two independent cultures in duplicate.*

VDR Half-Life in ROS 17/2.8 Cells

Figure 5.3 shows the ligand-induced stabilization of the VDR in ROS 17/2.8 cells. In the absence of ligand, VDR was rapidly degraded. At 1 nM, 1,25-$(OH)_2D_3$, KH1060, and its metabolites increased the VDR half-life in ROS 17/2.8 cells, although there was a marked difference in potency. Incubation with KH1060 and 24a-OH-KH1060 resulted in the most potent stabilization of the VDR. After 24 hours incubation, still about 60% (KH1060) and 45% (24a-OH-KH1060) of the initial VDR content was present. The other metabolites stabilized the VDR comparably to 1,25-$(OH)_2D_3$, while only 24-OH-KH1060 seemed less effective.

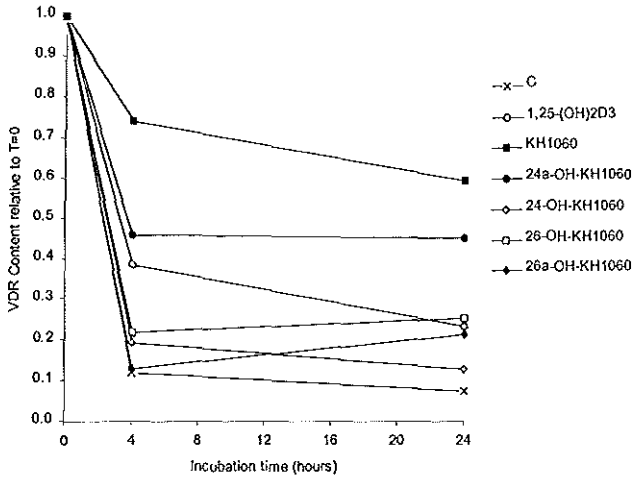
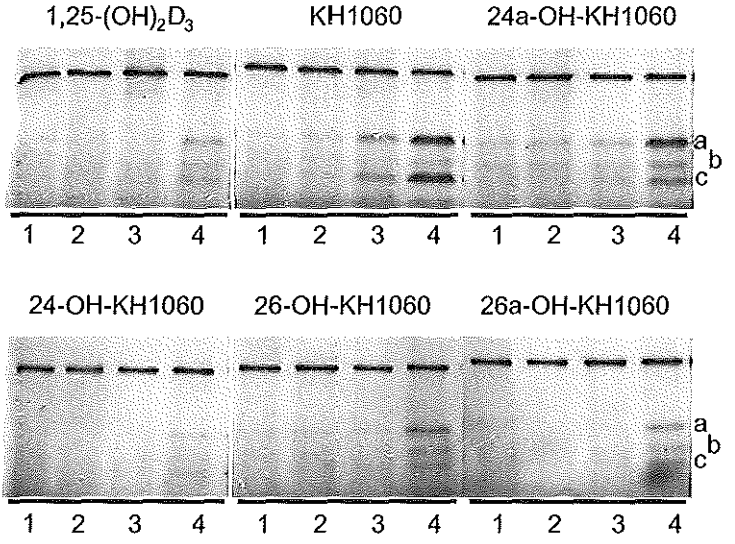


Figure 5.3. Effect of 1,25-$(OH)_2D_3$, KH1060, and its metabolites 24a-OH-KH1060, 24-OH-KH1060, 26-OH-KH1060, and 26a-OH-KH1060 on VDR half-life in ROS 17/2.8 cells. *Cycloheximide-treated ROS 17/2.8 cells were incubated for 4 or 24 hours with vehicle or with 1,25-$(OH)_2D_3$, KH1060, or its metabolites 24a-OH-KH1060, 24-OH-KH1060, 26-OH-KH1060, and 26a-OH-KH1060 (1 nM). Then, extracts were prepared and assayed for VDR content by enzyme-linked immunoassay. Data represent the means of three independent experiments and were expressed as VDR content relative to T=0.*

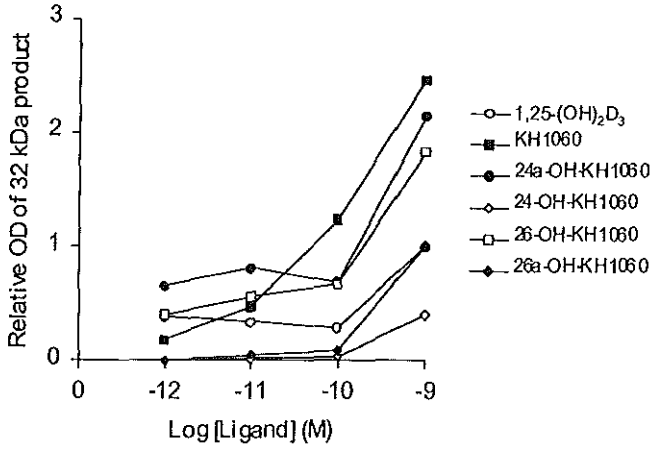
Limited Proteolytic Digestion of in Vitro Synthesized VDR

As shown in Figures 5.4A and B, 1,25-$(OH)_2D_3$, KH1060, and its metabolites 24a-OH-KH1060, 26-OH-KH1060, and 26a-OH-KH1060 all protected, in a dose-dependent manner, *in vitro* synthesized VDR against trypsin activity. There was a conservation of distinct fragments of 32, 30, and 27 kDa (Figures 5.4A and C). We found that VDR incubated with KH1060 or its metabolites 24a-OH-KH1060 and 26-OH-KH1060 was less sensitive to trypsin than VDR incubated with 1,25-$(OH)_2D_3$ or 26a-OH-KH1060. The metabolite 24-OH-KH1060 was virtually ineffective in protecting VDR against protease action. The effects on VDR conformation were further studied by taking a fixed ligand concentration (10 nM) and a dose range of trypsin (Figures 5.4C and D). In this set-up, the same order of potency of the ligands to protect the 32 kDa product was observed.

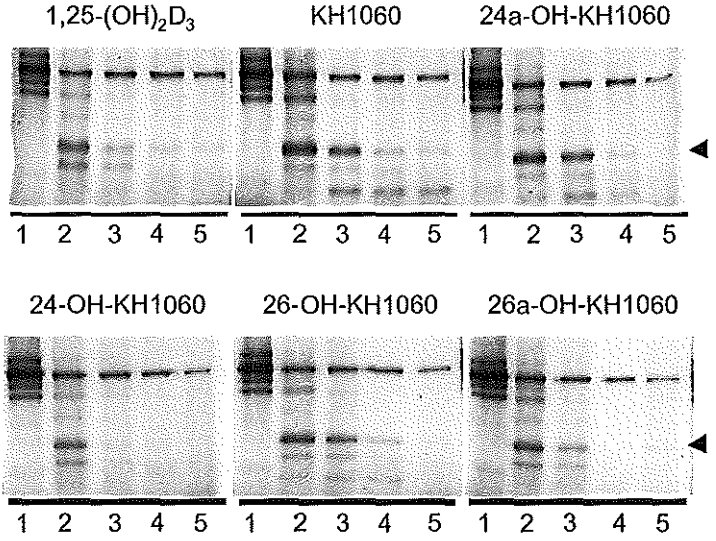
A


1,25-(OH)2D3
KH1060
24a-OH-KH1060
a
b
c
1 2 3 4 1 2 3 4 1 2 3 4
24-OH-KH1060
26-OH-KH1060
26a-OH-KH1060
a
b
c
1 2 3 4 1 2 3 4 1 2 3 4

B


Relative OD of 32 kDa product
3
2
1
0
0
-12
-11
-10
-9
Log [Ligand] (M)
1,25-(OH)2D3
KH1060
24a-OH-KH1060
24-OH-KH1060
26-OH-KH1060
26a-OH-KH1060

C


1,25-(OH)2D3
KH1060
24a-OH-KH1060
1 2 3 4 5 1 2 3 4 5 1 2 3 4 5
24-OH-KH1060
26-OH-KH1060
26a-OH-KH1060
1 2 3 4 5 1 2 3 4 5 1 2 3 4 5

D

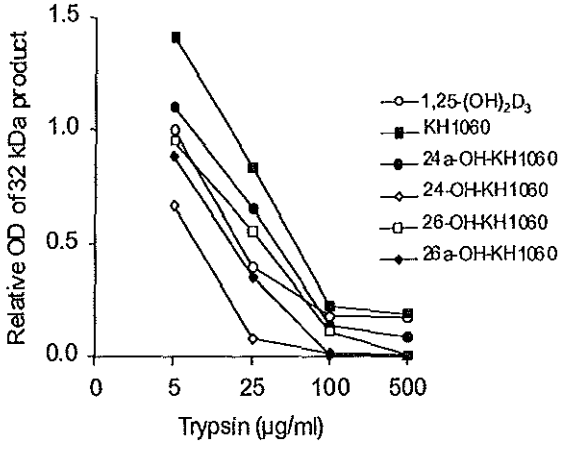

Relative OD of 32 kDa product
1.5
1.0
0.5
0.0
0
5
25
100
500
Trypsin (μg/ml)
1,25-(OH)2D3
KH1060
24a-OH-KH1060
24-OH-KH1060
26-OH-KH1060
26a-OH-KH1060

Figure 5.4. Effect of 1,25-$(OH)_2D_3$, KH1060, and its metabolites 24a-OH-KH1060, 24-OH-KH1060, 26-OH-KH1060, and 26a-OH-KH1060 on VDR conformation. *In panel A, in vitro synthesized human VDR was incubated with increasing concentrations of 1,25-$(OH)_2D_3$, KH1060, or its metabolites 24a-OH-KH1060, 24-OH-KH1060, 26-OH-KH1060, and 26a-OH-KH1060. Then, VDR protein was treated with trypsin (25 µg/ml), and the protease-resistant fragments (32, 30, and 27 kDa, marked a, b and c, respectively) were analyzed by SDS-PAGE. The ligand concentrations tested, 10^{-12}-10^{-9} M, are indicated by 1-4. In panel B, a computerized OD scan (mean of two experiments) of the 32 kDa fragment (marked 'a' in panel A) at increasing ligand concentrations is shown. The ODs of the 32 kDa product were normalized to the effect of 1 nM 1,25-$(OH)_2D_3$. A representative gel of a trypsin concentration-dependent degradation of in vitro synthesized VDR treated with 10 nM 1,25-$(OH)_2D_3$, 10 nM KH1060, or 10 nM of its metabolites is shown in panel C. The trypsin concentrations tested, 0-5-25-100-500 µg/ml, are indicated by 1-5. Panel D represents a computerized OD scan (mean of two experiments) of the 32 kDa product (marked by the arrowheads in panel C) normalized to the 1,25-$(OH)_2D_3$ effect at 5 µg/ml trypsin.*

Gel Mobility Shift Assays

In order to assess whether the KH1060 metabolites induce binding of the VDR to DNA, gel mobility shift analysis were performed. As shown in Figure 5.5, 1,25-$(OH)_2D_3$, KH1060, and its metabolites induced binding of the VDR to the rat osteocalcin VDRE, with KH1060 and 24a-OH-KH1060 the most active. 26-OH-KH1060 and 26a-OH-KH1060 were also more active than 1,25-$(OH)_2D_3$ in stimulating VDR binding to the osteocalcin VDRE, while 24-OH-KH1060 was least effective. With the CYP24 VDRE, KH1060 metabolite-induced VDR binding was also observed, and again 24-OH-KH1060 was least effective (data not shown). The shifted band (marked by the arrowhead) could be disrupted by adding the anti-VDR monoclonal antibody IVG8C11 (Uhland-Smith 1996) (data not shown).

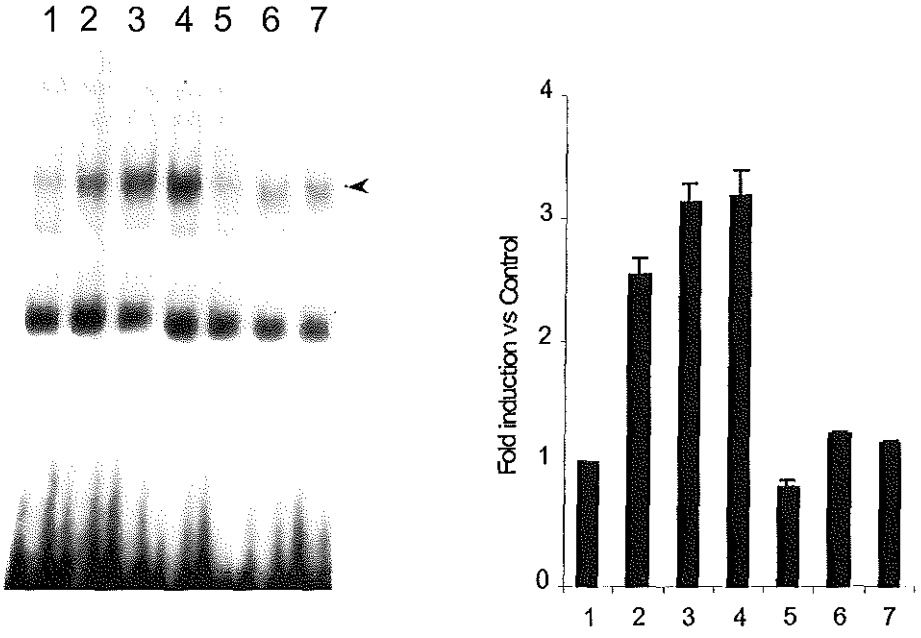


Figure 5.5. Gel mobility shift assay with the rat osteocalcin VDRE probe and nuclear extracts of ROS 17/2.8 cells. *Results from a gel mobility shift assay with nuclear extracts from ROS 17/2.8 cells treated for 1 hour with vehicle (lane 1), 1 nM 1,25-(OH)$_2$D$_3$ (lane 2), 1 nM KH1060 (lane 3), or 1 nM of its metabolites 24a-OH-KH1060 (lane 4), 24-OH-KH1060 (lane 5), 26-OH-KH1060 (lane 6), or 26a-OH-KH1060 (lane 7) are presented in panel A. A computerized OD scan (mean +/- standard deviations of two experiments) of the shifted probe is presented in panel B. The OD of the shifted probe after vehicle treatment was set to 1.*

5.5. DISCUSSION

The present study addresses the important issue of the metabolism of synthetic hormone analogs and the biological activity and mechanism of action of the metabolites formed. Previously, we showed that part of the increased biological potency of the 1,25-(OH)$_2$D$_3$ analog KH1060 probably lies in a specific interaction with the VDR. KH1060 enhanced the VDR half-life in ROS 17/2.8

cells (Van den Bemd 1996) and induced an altered conformational change in the VDR (Van den Bemd 1996, Peleg 1995, Nayeri 1996). Besides these VDR-based mechanisms, metabolic aspects (e.g. increased metabolic stability or formation of biologically active metabolites) might also contribute to the increased potency of KH1060. For other potent analogs of $1,25\text{-}(OH)_2D_3$, such as EB1089, MC1288, and $1,25\text{-}(OH)_2\text{-}F_6\text{-}D_3$, *in vivo* and *in vitro* metabolic stability could play a role in their increased potency (Inaba 1993, Dilworth 1994, Kissmeyer 1995, Shankar 1997). However, for KH1060, the stability of the compound itself is not likely to be an important factor, since both *in vivo* and *in vitro* KH1060 is metabolized at a rate comparable to or faster than that of $1,25\text{-}(OH)_2D_3$ (Dilworth 1997, Kissmeyer 1995).

In addition to the metabolic stability of the analogs, the metabolites generated can also contribute to the biological potency of an analog. Both biological activity and stability determine the impact of metabolites on the eventual biological potency of a parent compound. The biological activity of a metabolite does not necessarily have to surpass the activity of the parent compound to contribute to the overall effect. A metabolite with modest activity but increased stability will also add to the eventual effect (Jones 1997, Jones 1998). An example of generation of metabolites with increased stability and significant biological activity is $1,23,25\text{-}(OH)_3\text{-}F_6\text{-}D_3$, the major metabolite of $1,25\text{-}(OH)_2\text{-}F_6\text{-}D_3$, which demonstrated distinct transcriptional activity in a reporter gene expression system (Sasaki 1995). Another example is the target tissue specific 3ß-hydroxy epimerization of $1,25\text{-}(OH)_2D_3$ (Reddy 1997) and potentially its analogs. However, for the metabolites of the $1,25\text{-}(OH)_2D_3$ analogs EB1089 (Shankar 1997, Kissmeyer 1997), OCT (Watanabe 1996), and MC903 (Kissmeyer 1991), no contribution of the metabolites to the biological potency of the parent compound could be assessed.

The supposition that metabolites of KH1060 might also add to the biological potency of the parent compound is underscored by the finding that several of its *in vitro* generated metabolites (e.g. 24a-OH-KH1060 and 26-OH-KH1060) retained *trans*-activating and mRNA inducing activity (Dilworth 1997). In addition, the metabolites of KH1060 might contribute to the biological activity of the analog *in vivo*: in serum and in liver tissue taken from rats injected with KH1060, both 24a-OH-KH1060 and 26-OH-KH1060 could be identified (Dilworth 1997), and 24a-OH-KH1060 was detected in pig liver incubations (Rastrup-Andersen 1992). Here, we extend the observation that KH1060 metabolites are biologically active and elucidate mechanisms involved in their action. Some of the metabolites were as potent as $1,25\text{-}(OH)_2D_3$, while others were somewhat less potent but still able to induce osteocalcin production by ROS 17/2.8 cells. An interesting aspect of these metabolites is that they are stable. Even after 72 hours, they could be detected in cells treated with KH1060 (Dilworth 1997). This is in marked contrast to the metabolites of $1,25\text{-}(OH)_2D_3$, which disappear very rapidly (Masuda 1994).

Analysis of the possible mechanism(s) involved in the action of these

metabolites demonstrated that, like the parent compound (Van den Bemd 1996), they affect VDR stability. The metabolites 24a-OH-KH1060, 26-OH-KH1060, and 26a-OH-KH1060 enhance VDR stability comparably to or more than 1,25-$(OH)_2D_3$. The metabolites 24a-OH-KH1060 and 26-OH-KH1060 also induce conformational changes in the VDR that result in an increased resistance to proteolytic cleavage compared to 1,25-$(OH)_2D_3$, while 26a-OH-KH1060 exerts similar effects as 1,25-$(OH)_2D_3$. Gel mobility shift analysis with nuclear extracts from ROS 17/2.8 cells revealed that KH1060 metabolites also induce binding of the VDR to VDREs of vitamin D responsive genes. The low activity of 24-OH-KH1060 on the biological processes presented here underscores the significance of hydroxylation at C-24 in the 1,25-$(OH)_2D_3$ side chain in the inactivation of the hormone. In contrast, hydroxylation at C-24a only leads to marginal reduction in biological activity.

From a conceptual point of view the present data are interesting. Not only the parent molecule itself but also its (long-lived) metabolites may exert effects and act via similar receptor-mediated mechanisms. In so doing metabolites can significantly contribute to the eventual biological effect of an analog. Therefore, knowledge of both the metabolism of synthetic analogs and of the mechanism of action of the metabolites formed is of utmost importance. It is conceivable that this is not only applicable to vitamin D analogs, but also to other hormone analogs such as estrogen-like compounds, which have been the object of much recent attention (Langan-Fahey 1990, Osborne 1994, Dodge 1997). An additional aspect in the concept of the metabolism of synthetic analogs is target tissue-specific metabolism. Not only may synthetic analogs be metabolized to different, more active metabolites than the natural occurring counterpart, but this metabolism may also be target cell-/tissue-specific (Reddy 1997). This latter aspect may also be part of the clinically interesting target tissue-specific effects of vitamin D analogs as well as estrogen antagonists.

CHAPTER 6

DISTINCT EFFECTS ON THE CONFORMATION OF ESTROGEN RECEPTOR α AND β BY BOTH THE ANTIESTROGENS ICI 164,384 AND ICI 182,780 LEADING TO OPPOSITE EFFECTS ON RECEPTOR STABILITY

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Biochem Biophys Res Commun 261, 1-5, 1999

6.1. ABSTRACT

Tissue-specific effects of E_2 and synthetic ER ligands on target gene regulation might at least partly be explained by a selective ligand-induced conformational change of their receptors (ERα and ERß). In this study, the effects of E_2 and the synthetic ER ligands tamoxifen, ICI 164,384 and ICI 182,780 on the conformation of ERα and ERß were examined using limited proteolytic digestion analysis. We found that E_2 induced a conformational change of ERα resulting in the protection of a 30 kDa product whereas tamoxifen protected a 28 kDa fragment. Strikingly, the ERα conformational change induced by both ICI 164,384 and ICI 182,780 did not result in protection but rather seems to induce a ligand concentration-dependent increase in proteolytic degradation of the 30 and 28 kDa products. Incubation of ERβ with E_2 resulted in an increased protection of a 30 kDa fragment whereas with tamoxifen protection of a 29 kDa fragment was observed. In contrast to the situation with ERα, ICI 164,384 and ICI 182,780 incubation induced the protection of a similar 30 kDa fragment as E_2. In addition the ICI compounds also induced in a dose-dependent manner the preservation of a 32 kDa fragment. Our observations demonstrate that ICI 164,384 and ICI 182,780 have distinct effects on the conformation of ERα and ERβ, resulting in receptor subtype-selective opposite effects on receptor stability *in vitro*.

6.2. INTRODUCTION

The ER is expressed in two distinct forms, ERα (Green 1986) and ERß (Kuiper 1996, Mosselman 1996). Both ER subtypes bind estrogens and SERMs (Kuiper 1996, Mosselman 1996, Cowley 1997), form dimers (Cowley 1997, Pace 1997), interact with basal transcription factors and coactivators (Cowley 1997, Tremblay 1998), and bind to EREs in the DNA (Cowley 1997, Pace 1997) eventually leading to modulation of target gene transcription (Mosselman 1996, Cowley 1997, Paech 1997). The distribution of both ER types varies among cells and tissues. Some tissues express predominantly one type of ER, while others express ERα and ERß at more equal levels (Kuiper 1997). Furthermore, the ER subtypes can be differentially expressed during cell differentiation (Arts 1997). It is tempting to speculate that the tissue-specific effect of estrogens and SERMs is at least partly based on the differential distribution of ER subtypes and selective interactions of the various ligands with these receptor subtypes.

In view of the fact that the cascade of events resulting in target gene modulation is initialized by a conformational change of the receptor after ligand binding (Brzozowski 1997), we investigated whether the conformation of ERα and ERβ is changed differently by the estrogen E_2 and the synthetic ER ligands tamoxifen, ICI 164,384 and ICI 182,780. Tamoxifen is a partial estrogen agonist/antagonist

and considered a SERM, whereas the ICI compounds are generally denoted as pure antiestrogens, although some agonistic effects have been reported (Paech 1997, Jamil 1991, Chetrite 1991, Barkhem 1998, Sibonga 1998, Castro-Rivera 1998).

6.3. MATERIALS AND METHODS

Chemicals

E_2 and tamoxifen were purchased from Sigma Chemical Co. St.Louis, MO, USA. ICI 164,384 and ICI 182,780 were supplied by Zeneca Pharmaceuticals, Macclesfield, UK. Chemical structures are depicted in Figure 1.7.

In vitro Transcription and Translation

Human ERα cDNA (Tora 1989a) and ERß cDNA (Paech 1997) were *in vitro* transcribed and translated for 2 hours at 30°C using the TNT lysate assay (Promega). Translation was performed in the presence of [^{35}S]methionine (Amersham) to produce radioactive receptor protein.

Conformational studies

Conformational studies were performed as described by Beekman *et al.* (Beekman 1993). Shortly, *in vitro* synthesized ERs were incubated with ligand (E_2, tamoxifen, ICI 164,384, or ICI 182,780) for up to 30 minutes at 37°C in the presence of 10 mM Mg^{2+}. The co-incubation studies were performed with 10^{-8} M E_2 and increasing concentrations of either ICI 164,384 or ICI 182,780 added simultaneously to the ERs. After treatment with trypsin for 10 minutes at room temperature loading buffer was added. The samples were stored at -20°C or were directly analysed by SDS-PAGE (12.5% w/v). The gel was dried and after radiography the bands were quantified by densitometry.

6.4. RESULTS

In general, ERß was less sensitive to trypsin than ERα (Figure 6.1A). Detailed analysis of the digestion products showed that trypsin treatment (5 µg/ml) of ERα resulted in the formation of a distinct 28 kDa fragment and a faint 30 kDa fragment. Increasing the trypsin concentration resulted in complete degradation of these fragments. Both E_2 and tamoxifen enhanced the trypsin resistance of ERα. However, the size and intensity of the preserved fragments varied among the ligands studied. Incubation of ERα with E_2 had a preserving effect on the 30 kDa fragment, whereas incubation with tamoxifen resulted in an increased protection of the 28 kDa fragment (Figure 6.1).

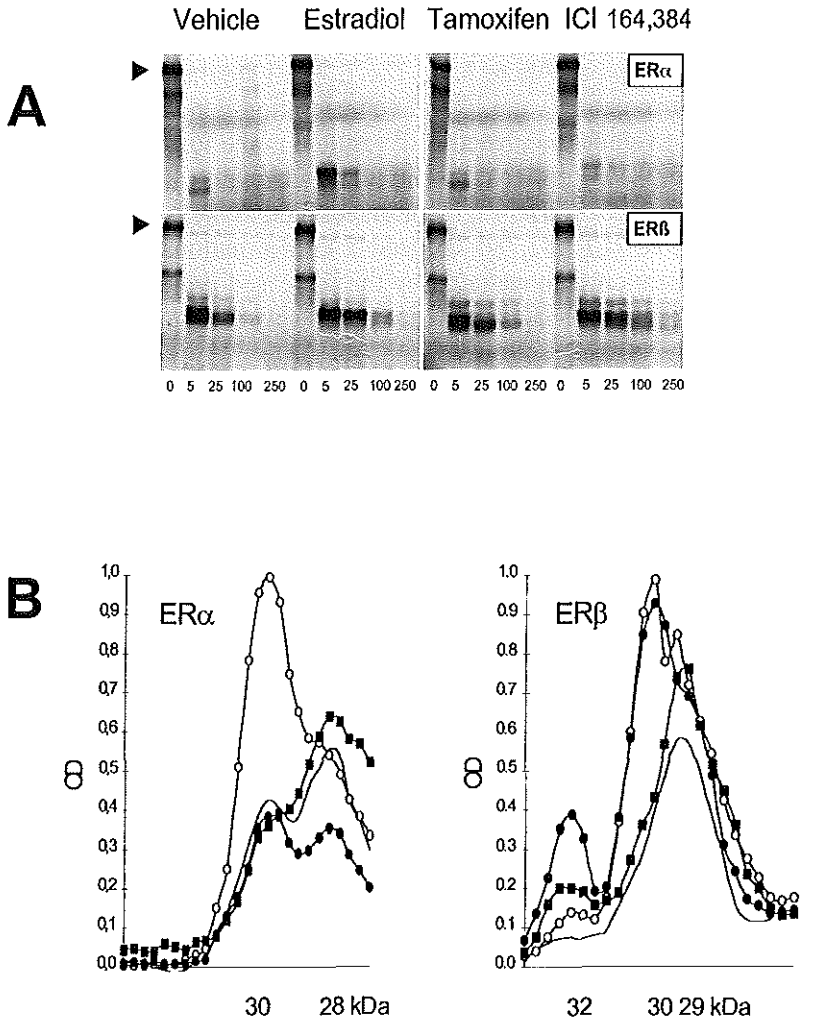

Figure 6.1. Trypsin resistance of ERα and ERß after incubation with vehicle, E_2, tamoxifen, or ICI 164,384. *In vitro synthesized ER was incubated with vehicle (-) (0.01% ethanol) or ligand: E_2 (○), ICI 164,384 (•), or tamoxifen (■) (10^{-6} M), and subsequently treated with increasing trypsin concentrations (0-250 µg/ml). The arrowhead indicates the intact ERs (Panel A). Computerized OD scans of the degradation products of ER_α (at 5 µg/ml trypsin) and ERß (at 25 µg/ml trypsin) are presented in panel B. The maximal OD value measured in the E_2 scans was set at one and was used as a reference for the other OD values. Each scan represents the mean of five independent experiments.*

In contrast, incubation of ERα with both ICI compounds did not result in increased protection of distinct fragments, but rather led to a slightly enhanced degradation of the receptor (Figure 6.2). The E_2- and synthetic ligands-induced effects on ERα conformation were ligand concentration-dependent (Figure 6.2). Trypsin treatment of vehicle-incubated ERß resulted in the formation of a 29 kDa fragment (Figure 6.1). E_2 protected a 30 kDa fragment, whereas tamoxifen mainly had an effect on a 29 kDa fragment. In contrast to the situation with ERα, both ICI 164,384 and ICI 182,780 induced a conformational change of ERß resulting in a stabilization demonstrated by the increased protection of a similar 30 kDa fragment as seen with E_2. Again these effects were ligand dose-dependent (Figure 6.2).

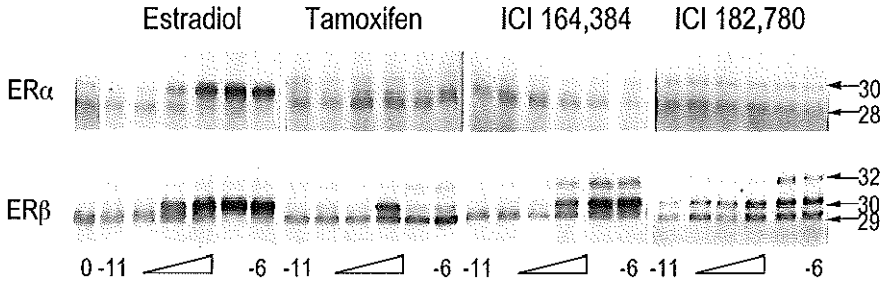

Figure 6.2. E_2- and SERM-induced conformational changes of ERα and ERß. *In vitro synthesized ER_α and ERß were incubated with increasing concentrations of E_2, tamoxifen, ICI 164,384 or ICI 182,780 (10^{-11}-10^{-6} M) as described in Materials and Methods (Section 6.3) and subsequently treated with 5 (ER_α) or 25 µg/ml (ERß) of trypsin.*

Furthermore, the ICI compounds had a marked protective effect on an additional 32 kDa fragment. At higher tamoxifen concentrations the 32 kDa fragment is also observed, whereas E_2 had hardly an effect on this fragment (Figure 6.2). Co-incubation studies in which a fixed concentration of E_2 and increasing concentrations of the ICI compounds were added simultaneously to the receptors

demonstrated that the E_2 effect on ER conformation can be overruled by the ICI compounds (Figure 6.3).

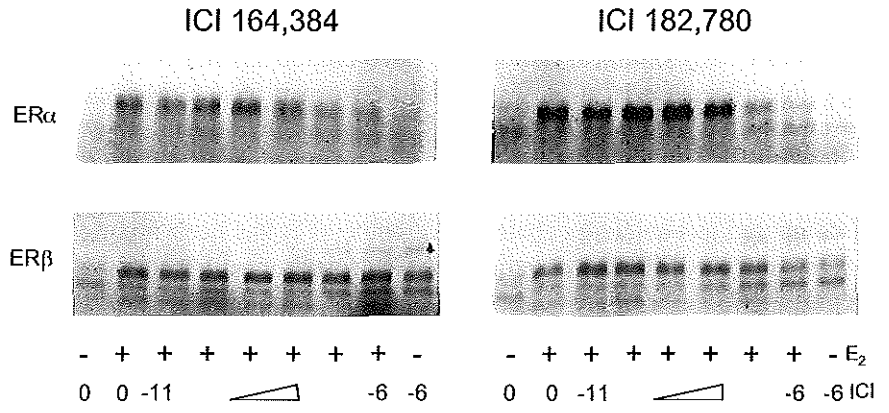

Figure 6.3. The effect of co-incubation of E_2 (10^{-8} M) and increasing concentrations of ICI 164,384 or ICI 182,780 (10^{-11}-10^{-6} M) added simultaneously to *in vitro* synthesized ERα or ERß. *After treatment with 5 (ER_α) or 25 µg/ml (ERß) of trypsin, the protease resistant fragments were separated on a SDS-polyacrylamide gel and visualised by radiography.*

6.5. DISCUSSION

Ligand-induced changes in receptor conformation are a common feature in steroid hormone action (Beekman 1993, Allan 1992, Kuil 1994, Van den Bemd 1996, McDonnell 1995) and it is believed that this event initiates a cascade of processes, eventually resulting in modulation of target gene transcription. Therefore, ligand-specific-induced changes in receptor conformation might underly the cell- and tissue-specific effects of estrogens and SERMs (Webb 1995, Grese 1997, Bryant 1998). In addition, another aspect that could contribute to this was the identification of a second ER and the finding of a cell differentiation-dependent (Arts 1997) and tissue-specific distribution of ER subtypes (Kuiper 1997). The current study shows distinct effects of the antiestrogens ICI 164,384 and ICI 182,780 on the conformation of the ER subtypes, resulting in

opposite effects on the stability of ERα and ERβ.

In general, we found that ERß is more stable than ERα as exemplified by the increased protease resistance. This is not due to a diminished number of potential trypsin cleavage sites within the entire receptor molecule (about 60 in both receptor types) or ligand binding domain (about 20 in both receptor types), but is probably a direct consequence of its different conformation and resulting accessibility of trypsin cleavage sites. Whether the increased protease resistance of ERß can also be observed in cells and tissues in terms of an increased receptor stability needs to be examined, but our previous conformational studies with the vitamin D receptor showed a close parallel between protease resistance of the receptor and its half-life in cells (Van den Bemd 1996). This is further supported by our data obtained with ERα and the ICI compounds. The ligand-dose experiments showed that ICI 164,384- and ICI 182,780-incubated ERα was slightly more sensitive to trypsin compared to vehicle-incubated ERα while as it has been reported earlier ICI 164,384 impairs ERα dimerization (Fawell 1990), resulting in reduced half-life of the receptor (Dauvois 1992). Also, the observed loss of ER in uterine tissue *in vivo* after ICI 164,384 treatment in mice was ascribed to an ICI 164,384-induced conformational change of ER, resulting in a reduced stability and proteolysis of the receptor (Gibson 1991). The effect of both ICI compounds on ERß is in marked contrast with the effect on ERα. With ERβ we observed a clear increased protease resistance. Interesting in this respect is the finding by Pace *et al.* (Pace 1997) showing that ICI 182,780 was not effective in preventing loss of ERα binding to an ERE when the incubation temperature was increased from 4°C to 37°C whereas elevating the temperature did not affect ERß-ERE binding. Loss of a specific ERα conformation suitable for DNA binding after incubation with this ICI compound was put forward as an explanation for the decreased ERα-ERE binding. Their observation that ICI 182,780 did protect ERß from heat inactivation might result from a distinct effect on the conformation of ERβ in line with our findings presented in this study.

Both ICI 164,384 and ICI 182,780 are denoted as pure antagonists (Wakeling 1992, Nicholson 1995), although some studies report an agonistic potency that could be based on an ER subtype-selective interaction (Paech 1997, Jamil 1991, Chetrite 1991, Barkhem 1998, Sibonga 1998, Castro-Rivera 1998). However, it is yet not possible to directly translate the present data into a receptor subtype-selective agonistic or antagonistic potency of the ICI compounds. In this respect one should take into account that the agonistic effects of SERMs may be cell type specific, dependent on the presence or absence of certain transcription factors (Webb 1995). Also, studies performed by Watanabe *et al.* (Watanabe 1997) showed that the agonistic effect of tamoxifen was dependent on ER subtype, in combination with cell type and ERE promoter context. Therefore, conformational studies performed in a cellular context of different target cells might provide more insight.

Finally, our protease digestion assays clearly demonstrated different ER

conformations induced by E_2 and the synthetic ER ligands with both ERα and ERβ. Furthermore, there was a marked difference between the ER conformations induced by ICI 164,384 and ICI 182,780 and the ER conformations induced by tamoxifen (Figures 6.1 and 6.2) and other synthetic ER ligands with partial agonistic/antagonistic properties (4-hydroxytamoxifen, idoxifen and LY 117,018-HCl; data not shown). Also in line with observations by McDonnell *et al.* [McDonnell 1995] we could not discriminate between tamoxifen and other ER ligands with partial agonistic/antagonistic activities (data not shown).

In conclusion, the present study shows that ERα and ERß respond to both ICI 164,384 and ICI 182,780 with a distinct conformational change: ERα conformation changes into a less stable, more protease sensitive form, whereas ERß conformation is changed into a more stable, less protease sensitive form. On basis of these clear opposite effects of the ICI compounds which were not observed with E_2 and tamoxifen it is tempting to speculate that the ICI compounds are ER subtype selective ligands. The observed ligand- and receptor-specific effect on receptor stability, together with the tissue specific distribution of ERα and ERß may be part of the mechanism that determines tissue specific agonistic/antagonistic properties of ER ligands.

CHAPTER 7

SUMMARY AND GENERAL DISCUSSION

7.1. INTRODUCTION

Both ER and VDR belong to the same superfamily of nuclear receptors and both receptors are activated by interaction with their ligands. Via a complex cascade of events already discussed in Chapter 1 this interaction will eventually lead to regulation of target gene transcription. Conformational changes and phosphorylation of the receptor, as well as interaction with dimer-partners, cofactors, basal transcription factors, and specific sites in the DNA are major events in this cascade.

1,25-$(OH)_2D_3$ and E_2, the natural ligands for VDR and ER, respectively, have potential therapeutic properties. However, their use as therapeutic agents is limited because of certain side effects (Chapter 1, Sections 1.3.1 and 1.6.1). The search for compounds with a more beneficial therapeutic profile initialized the development of synthetic ligands for VDR (1,25-$(OH)_2D_3$ analogs) and ER (SERMs). In case of VDR ligands this means that the dissociation between calcemic effects and effects on cell proliferation and differentiation is enlarged, in case of ER ligands this means that the beneficial agonistic effects of E_2 (e.g. maintenance of bone mass, improved cognitive function) are mimicked, while undesirable effects (e.g. increased risk for breast and uterine cancer) are antagonized. Indeed, several of the newly synthesized compounds have improved characteristics compared to the natural ligands. An explanation for the changed biological profile is not completely clear and obviously not similar for all synthetic ligands. It probably lies in specific interactions with one or more of the events in the cascade mentioned above (see also Sections 1.3.3. and 1.6.3.).

The aim of the studies presented in this thesis was to extend the knowledge on the mechanism of action of the VDR and ER, and their modified ligands in particular. Therefore we evaluated the differences in effect of 1,25-$(OH)_2D_3$ and a selection of its analogs on differentiation and growth of osteoblast-like cells and on *in vitro* bone resorption (Chapter 2), and investigated the role of VDR affinity (Chapter 2), conformational change of the VDR (Chapter 3), VDR stability (Chapter 3), VDRE binding (Chapter 4), and metabolism of 1,25-$(OH)_2D_3$ analogs (Chapter 5). In Chapter 6 the effect of a selection of SERMs on ER conformation was studied. In the Sections below the results are discussed and suggestions for further research are given.

7.2. THE VITAMIN D RECEPTOR, 1,25-DIHYDROXYVITAMIN D_3, AND 1,25-DIHYDROXVITAMIN D_3 ANALOGS

The first part of this thesis focusses on ligand interaction with the VDR.

Strikingly, most investigations studying 1,25-$(OH)_2D_3$ analogs do not use cells of bone origin - a classic target tissue - but rather nonclassic target cells like breast cancer cells and cells of the immune system. However, in our view also the effects on bone should be evaluated to establish an accurate biological profile of the analogs. Therefore, the rat osteoblast-like cell line ROS 17/2.8 and the human osteoblast-like cell line MG-63 was used as a model to evaluate the effect of several side chain-modified 1,25-$(OH)_2D_3$ analogs on growth and differentiation of these cells and to compare their potency with that of 1,25-$(OH)_2D_3$. Furthermore, isolated long bones from fetal mice were used to study the capacity of 1,25-$(OH)_2D_3$ and the analogs to release calcium from bone.

EFFECTS ON CELL GROWTH AND DIFFERENTIATION AND ON *IN VITRO* BONE RESORPTION

All 1,25-$(OH)_2D_3$ analogs examined in this thesis exhibited agonistic activity in all responses tested: like 1,25-$(OH)_2D_3$ the analogs stimulated the synthesis of the bone matrix proteins osteocalcin and type I procollagen. Furthermore, the analogs mimicked the natural ligand in their effect on osteoblast-like cell growth and in their stimulatory effect on *in vitro* bone resorption (Chapter 2). Some analogs (OCT, MC903, CB966) exhibited an *in vitro* biological potency comparable to that of 1,25-$(OH)_2D_3$, whereas others (KH1049, KH1060, EB1089) were much more potent than 1,25-$(OH)_2D_3$. This observation is supported by almost every *in vivo* and *in vitro* study published so far: 1,25-$(OH)_2D_3$ analogs are super, full, or partial agonists of 1,25-$(OH)_2D_3$. However, recent data from Arai *et al.*, Ishizuka *et al.*, and Miura *et al.* indicate that a metabolite of 1,25-$(OH)_2D_3$; 1,25-$(OH)_2$-26,23-lactone D_3, and its analogs might have antagonistic properties (Arai 1997, Ishizuka 1997, Miura 1997). Although these data are preliminary, they indicate that VDR ligands with specific antagonistic properties can be developed.

We found that the potency of the 1,25-$(OH)_2D_3$ analogs was related to the biological response. In some responses their potency (compared to 1,25-$(OH)_2D_3$) was only moderately increased, while in other responses the increase in potency was much more striking. In Table 2.3 this differential effect on gene regulation is expressed as the ED_{50} ratio for stimulation of *in vitro* bone resorption and stimulation of extracellular bone matrix protein synthesis. It clearly demonstrates that for EB1089 and KH1060 the ED_{50} ratio is increased compared to the ED_{50} ratio for 1,25-$(OH)_2D_3$. The finding that for some analogs the ED_{50} ratio between bone formation parameters (stimulation of osteocalcin and type I collagen synthesis) and bone resorption parameters (stimulation of calcium release) is increased compared to the ED_{50} ratio for 1,25-$(OH)_2D_3$ might support a therapeutic potential of these analogs in the treatment of metabolic bone diseases, i.e. osteoporosis. A relationship between increased bone turnover and the risk for metastasis of cancer to bone has been reported

[Agha 1976, Orr 1993]. The *in vivo* potency of these analogs to induce bone resorption must therefore be examined with great care.
The studies presented in Chapter 2 show cell type-specific differences in sensitivity for 1,25-$(OH)_2D_3$ and some of the analogs. For instance, in MG-63 cells maximal stimulation of osteocalcin production by EB1089, KH1049, and KH1060 was almost two-fold increased compared to 1,25-$(OH)_2D_3$ and the analogs with moderate activity (OCT, MC903, CB966), while no difference in maximal stimulation was observed in ROS 17/2.8 cells. However, in general, MG-63 cells were considerably less sensitive than ROS 17/2.8 cells. For instance, in MG-63 cells the ED_{50} for osteocalcin synthesis induced by 1,25-$(OH)_2D_3$, OCT, CB966, or EB1089 was reached at 100-600 times higher concentrations than in ROS 17/2.8 cells. Cell type-specific differences in VDR content and/or function might underly this phenomenon. We found that MG-63 cells have a much lower VDR content compared to ROS 17/2.8 cells and do not show homologous VDR up-regulation (data not shown). In addition, cell type-specific differences in presence and/or distribution of cofactors might play a role. We also observed that the ED_{50}'s for both KH1049 and KH1060 (both 20-epi analogs) were comparable between the two cell lines, indicating that for these analogs cell type-specific differences might be of less importance. Another possibility is that KH1049 and KH1060 are metabolized more efficiently by ROS 17/2.8 cells, (or less efficiently by MG-63 cells) in relation to 1,25-$(OH)_2D_3$ and the other analogs. This could lead to relatively high concentrations of KH analogs in MG-63 cells or relatively low concentrations of these compounds in ROS 17/2.8 cells.

With most cell types 1,25-$(OH)_2D_3$ has an inhibitory effect on growth; a feature that gave rise to the thought to use 1,25-$(OH)_2D_3$ or its analogs for the treatment of hyperproliferative diseases [Colston 1981, Abe 1981]. Likewise, with the human osteosarcoma cell line MG-63 and the human breast cancer cell lines MCF-7 and ZR75-1 our laboratory observed dose-dependent inhibition of proliferation by 1,25-$(OH)_2D_3$ and its analogs (Chapter 2). [Vink-Van wijngaarden 1994] However, the rat osteblast-like ROS 17/2.8 cells were stimulated in their growth. Interestingly, the analogs exhibited a similar order of potency in their growth inhibitory activity (in MG-63 cells) and in their growth stimulatory activity (in ROS 17/2.8 cells). The observed 1,25-$(OH)_2D_3$- and analog-induced growth stimulation of ROS 17/2.8 cells is in line with other reports [Majeska 1982, Van Auken 1996]. 1,25-$(OH)_2D_3$ also caused an increased growth of ROS 17/2.8 cells inoculated into athymic nude mice [Yamaoka 1986]. However, others have shown an inhibitory effect of 1,25-$(OH)_2D_3$ on the growth of these cells, although they also observed a statistically nonsignificant stimulation of cell growth at a lower 1,25-$(OH)_2D_3$ concentration (0.1 nM) [Dokoh 1984]. An explanation for this discrepancy could be the subclone heterogeneity within this cell line [Grigoriadis 1985], while also experimental conditions (serum

composition, differences in cell densities or differentiation state of the cells) could play a role. Also in several other studies with other cells 1,25-$(OH)_2D_3$ and 1,25-$(OH)_2D_3$ analogs have shown to exert growth stimulatory effects [Grillier 1997, Freake 1981, Grauer 1995, Wood 1985, Garach-Jehoshua 1999, Ishida 1993, Delissalde 1998, Gache 1999, Gniadecki 1995].

An explanation for the induced cell growth is still not revealed. Yamaoka *et al.* showed that presence of the VDR seems essential to observe growth stimulation by 1,25-$(OH)_2D_3$ since tumor weight in mice inoculated with VDR deficient ROS 24/1 cells or with G-361 melanoma cells with low VDR levels was not affected by 1,25-$(OH)_2D_3$ [Yamaoka 1986]. A 1,25-$(OH)_2D_3$-induced increase of ErbB1, ErbB2, and ErbB3 -proteins that mediate signal transduction by EGF receptor ligands- was proposed as a possible mechanism [Garach-Jehoshua 1999]. Also, the 1,25-$(OH)_2D_3$-induced rise in c-myc mRNA expression was put forward as an explanation for the stimulatory effect on cell growth [Grauer 1995]. Nevertheless, our data and the reports mentioned above indicate that caution is needed in the use of 1,25-$(OH)_2D_3$ or its analogs in the treatment of cancer.

VDR CONFORMATION AND VDR STABILITY

One of the key events in the cascade leading to regulation of gene transcription is the ligand-induced conformational change of the VDR. To study the effects of 1,25-$(OH)_2D_3$ and KH1060 on VDR conformation partial proteolytic digestion analysis was used. The method is based on the idea that accessibility of potential protease cleavage sites within a receptor molecule alters as a result of ligand-induced changes in receptor conformation. Consequently, changes in receptor conformation will lead to changes in the protease digestion pattern of the receptors. We found that in the absence of ligand *in vitro* synthesized VDR is rapidly degraded by proteases. In contrast, 1,25-$(OH)_2D_3$ and the analogs changed the VDR conformation, resulting in enhanced protease resistance of distinct VDR fragments. In general 1,25-$(OH)_2D_3$ analogs can be regarded as agonists [Bouillon 1995], although the potency by which they mimic the natural ligand differs between tissues, cells, and responses studied. This probably explains why we could not establish qualitative differences in the VDR protease digestion profiles of 1,25-$(OH)_2D_3$ and the analogs: the sizes of the protease resistant VDR fragments were not distinct, only the intensity of the preserved fragments was different. Also other investigators could not discriminate in a qualitative way between 1,25-$(OH)_2D_3$ and 1,25-$(OH)_2D_3$ analogs using this technique [Väisänen 1997, Koszewski 1999a].

Interestingly, we and others found that treatment of unliganded *in vitro* synthesized VDR with low trypsin concentrations resulted in preservation of VDR fragments with a similar size as the fragments observed with liganded VDR (Chapter 3) [Nayeri 1996, Nayeri 1997]. When protease digestion was intensified (by

increasing the trypsin concentration or the trypsin incubation time) these fragments disappeared, whereas the fragments of liganded VDR were still protected. Nayeri *et al.* suggested that this might indicate that ligand interaction has more impact on VDR stability (by preserving the conformation) than on the conformation itself (Nayeri 1997). However, partial protease digestion analysis is a rather insensitive method and based on findings obtained with other techniques it is clear that ligand-induced changes in VDR conformation do occur (Liu 1997). Therefore, we think that both stabilization as well as induction of a conformational change are import in the interaction of analog and receptor.

Nayeri *et al.* found a correlation between the transcriptional activity of a defined series of 20-epi-analogs with closely related structures and their potency to change/stabilize the VDR conformation (i.e. the functional receptor affinity) (Nayeri 1996). Our observation that 1,25-$(OH)_2D_3$ analogs and metabolites with strong biological activity are also strong inducers of a VDR conformation with increased protease resistance and *vice versa* is in line with their finding (Chapters 2, 3, and 4). However, one should be cautious in overestimating the quantitative differences obtained by limited protease digestion analysis. For instance, the strong biological effect of EB1089 (an analog with normal configuration at C-20) is not reflected by a strong increase in the intensity of protease resistant VDR fragments (data not shown) (Quack 1998, Koszewski 1999a). One can not exclude the possibility that some potent 1,25-$(OH)_2D_3$ analogs might induce a conformational change of the VDR that does not lead to increased protease resistance but even to increased protease sensitivity.

Crystal structure analysis revealed that liganded nuclear receptors adopt a more compact structure, obviously less sensitive to protease activity (Renaud 1995). In cells this property could lead to increased VDR stability. Indeed, our studies presented in Chapters 3 and 5 show that 1,25-$(OH)_2D_3$, analogs and specific metabolites of the analog KH1060 prolong VDR half-life in ROS 17/2.8 cells. These observations are consistent with findings by others (Wiese 1992, Arbour 1993, Liu 1997, Masuyama 1998). Recently it was reported that protesomes are involved in VDR degradation (Masuyama 1998), and that chymotrypsin-, and trypsin-like proteasome activities are observed in human osteoblast-like cells (Brochmann Murray 1998), indicating that our observations obtained with trypsin and chymotrypsin in the partial digestion analysis also might play a role in intact cells. Recently it was reported that 1,25-$(OH)_2D_3$-induced up-regulation of the VDR might be due to inhibition of proteasome-mediated VDR degradation (Li 1999). In proteasome-mediated receptor degradation the cofactor SUG1 is probably important. Interestingly, it was found that SUG1 binds the VDR in a 1,25-$(OH)_2D_3$-dependent manner and enhances its proteosome-mediated degradation (Masuyama 1998). So, on the one hand ligand interaction leads to a conformational change of the VDR by which the receptor becomes less vulnerable for proteasome-mediated degradation, whereas on the other hand ligand interaction seems to stimulate proteosome-mediated

degradation of the receptor. Although this seems contradictory, it is thought that just because of its increased stability, the liganded VDR requires a more specialized mechanism to control its transcriptional activity [Masuyama 1998]. Therefore, besides ligand metabolism also receptor degradation might be a mechanism to control the biological activity of VDR ligands.

DNA BINDING

The ligand-induced conformational change of the VDR probably fascilitates dimerization with RXR [Zhao 1997] and enhances binding to specific regulatory elements (VDREs) in the vicinity of 1,25-$(OH)_2D_3$ target genes. In Chapter 4 the potency of KH1060 was compared with the potency of 1,25-$(OH)_2D_3$ to induce binding of the VDR to VDREs. Both *in vitro* synthesized VDR/RXR as well as nuclear extracts of ROS 17/2.8 cells were used to investigate VDR binding to VDREs from three different target genes: rat osteocalcin, human osteocalcin and mouse osteopontin. Both 1,25-$(OH)_2D_3$ and KH1060 induced VDR binding to these VDREs in a ligand dose-dependent manner, independent of the source of VDRs (*in vitro* synthesized or nuclear extracts). All three VDREs are of the DR3 type: two hexameric half-sites separated by three nucleotides, and despite of minor differences in nucleotide sequence (see Table 4.1) large differences in VDR-VDRE affinity were observed. The VDR bound with lowest affinity to the rat osteocalcin VDRE, and with highest affinity to the mouse osteopontin VDRE. We further elaborated on this interesting finding and produced hybrid VDREs in which the rat osteocalcin VDRE half-sites were replaced by one or both mouse osteopontin VDRE half-sites. Gel mobility shift assays performed with these VDREs clearly showed that the proximal half-site of the mouse osteopontin VDRE (i.e. the OC/OP VDRE) was mainly responsible for the increased affinity. Transfection studies in ROS 17/2.8 cells revealed that 1,25-$(OH)_2D_3$-induced transcription of the OC/OP VDRE-driven reporter gene was more effective than via the rat osteocalcin VDRE-driven reporter. Further substitution studies revealed that in particular the third and/or fourth nucleotides (both thymidine) determined the difference in VDR-VDRE affinity.

We found that KH1060 was a stronger inducer of VDR/RXR binding to the mouse osteopontin VDRE than 1,25-$(OH)_2D_3$. However, surprisingly, when we studied binding of *in vitro* synthesized VDR/RXR to the human and rat osteocalcin VDRE KH1060 exhibited no increased potency compared to 1,25-$(OH)_2D_3$. In contrast, when VDR protein was extracted from ligand-treated cells KH1060 was a stronger inducer of VDR/RXR-VDRE binding than 1,25-$(OH)_2D_3$ with all VDRE types tested. So, this indicates that the KH1060-induced conformational change of the VDR does not necessarily result in enhanced VDRE binding, but in addition depends on the VDRE nucleotide sequence and furthermore underlines the importance of a cellular context (e.g. presence of

cofactors) to obtain optimal VDR-VDRE binding. Therefore, it seems justified to speculate that KH1060 changes the VDR conformation in such a way that, compared to the 1,25-$(OH)_2D_3$-induced VDR conformation, cofactor binding is fascilitated or that other KH1060-specific cofactors are involved and that this will lead to increased VDR-VDRE binding.

ANALOG METABOLITES

Formation of biologically active metabolites might be a cause for concern. Obviously, from a therapeutic point of view rapidly acting analogs with inactive metabolites are much better to control than analogs that are metabolized into one or more active metabolites. For each of these metabolites the mechanism of action and possible side effects should be established before safe clinical use can be guaranteed. Chapter 5 focusses on the potential contribution of metabolites of KH1060 to the biological potency of the parent compound. Earlier was shown that *in vitro* KH1060 is metabolized into at least 22 different compounds (Dilworth 1997). The metabolites 24a-OH-KH1060, 26-OH-KH1060, and 26a-OH-KH1060 were potent inducers of osteopontin mRNA expression, rat osteocalcin VDRE-driven reporter gene transcription (Dilworth 1997), and active stimulators of osteocalcin synthesis (Chapter 5). The same metabolites were also active inducers of VDR binding to rat osteocalcin and rat cytochrome P450 (CYP24) VDREs (Chapter 5). Next, we investigated whether the biological potency of these metabolites was correlated with their potency to induce a conformational change of the VDR with enhanced protease resistance. Indeed, partial protease digestion analysis showed that the biological active metabolites changed the VDR conformation resulting in enhanced protease resistance, whereas a metabolite with weak biological activity was inactive in the protease digestion analysis. A corresponding pattern was observed studying the effect of the metabolites on VDR stability in ROS 17/2.8 cells.

KH1060: THE MECHANISM OF ACTION RESOLVED?

KH1060, one of the most potent analogs was studied in more detail. The studies enclosed in this thesis present several indications of differential interactions of KH1060 with the cascade of events leading to gene transcription. Based on these investigations and the studies of others we constructed a scheme depicting potential explanations for the increased biological activity of KH1060 (Figure 7.1).

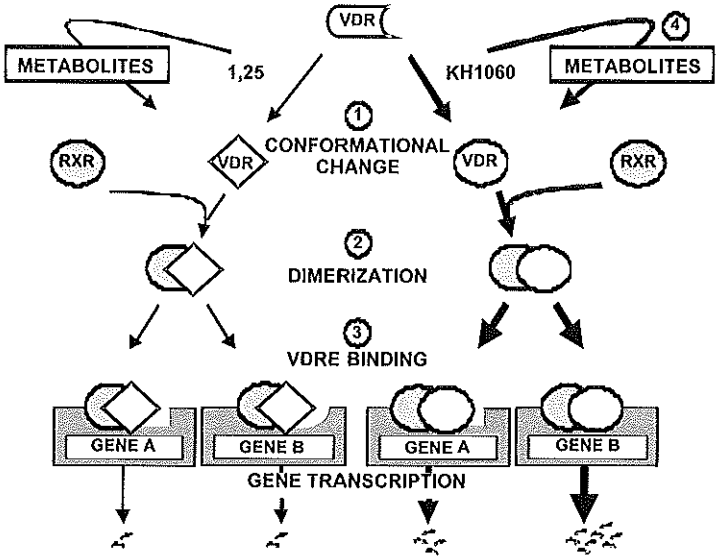

Figure 7.1. Possible explanations for the increased biological potency of KH1060. *The numbers refer to the section below. Arrows in bold indicate that a process takes place with increased efficiency compared to the 1,25-(OH)$_2$D$_3$ situation.*

1. The effect of KH1060 on the conformational change and stability of the VDR

In Chapter 3, using partial digestion analysis, we showed that the KH1060-induced VDR conformation is more resistant against protease activity than the 1,25-(OH)$_2$D$_3$-induced VDR conformation. Similar observations were obtained by others [Peleg 1995, Nayeri 1996]. The conformational change is probably the trigger of the changes in all subsequent steps. The KH1060-induced increase in receptor stability in cells is in parallel with the KH1060-induced increase in protease resistance in the partial digestion analysis. These and recent findings by Brochmann Murray *et al.* indicate that resistance for enzymes like trypsin and chymotrypsin might reflect the resistance for enzymes involved in receptor processing in cells [Brochmann Murray 1998].

2. **The effect of KH1060 on dimerization**

Peleg *et al.* showed that KH1060 was more potent than 1,25-$(OH)_2D_3$ in stimulating VDR-RXR dimerization [Peleg 1995].

3. **The effect of KH1060 on VDR/RXR binding to VDREs**

KH1060 was more potent than 1,25-$(OH)_2D_3$ in stimulating the binding of VDR/RXR to different VDREs. The variations in nucleotide sequences between these VDREs had an impact on the affinity of the VDR/RXR complex, but also on the relative potency of KH1060 compared to 1,25-$(OH)_2D_3$ to induce this binding. This might underly the differential effects of KH1060. As illustrated in Figure 7.1, at genes of the 'A' type, KH1060 is only moderately more potent than 1,25-$(OH)_2D_3$, while at 'B' genes KH1060 has a much stronger activity. The importance of a cellular context to VDR-VDRE binding was also demonstrated in these studies (Chapter 4).

4. **The contribution of KH1060 metabolites**

Some of the metabolites exert biological activities comparable to or stronger than those of 1,25-$(OH)_2D_3$ (Chapter 5) [Dilworth 1997]. Like the parent compound, these KH1060 metabolites decreased the protease sensitivity of the VDR, increased the VDR stability in cells, and enhanced VDR/RXR binding to VDREs. Together these data indicate that certain metabolites might contribute to the overall biological activity of KH1060.

Although other mechanisms are not excluded we think that these aspects are of major importance in determining the increased biological activity of KH1060.

7.3. THE ESTROGEN RECEPTOR, 17β-ESTRADIOL, AND SELECTIVE ESTROGEN RECEPTOR MODULATORS

The second part of the thesis focusses on ligand interaction with the ER. We studied the effect of E_2 and several synthetic ER ligands on the conformation of both ER subtypes: ERα and ERß. One of the tested ER ligands, tamoxifen, is a SERM with tissue-dependent partial agonistic/antagonistic activities: it mimics E_2 with anabolic effects on bone, protective effects on the cardiovascular system, and beneficial effects on cognitive function, whereas it counteracts the

stimulatory effect of E_2 on breast tumors (See Section B.5.2.). An explanation for the tissue-dependent partial agonistic/antagonistic activition of SERMs is still unknown.

Using partial protease digestion analysis we showed that tamoxifen changed the conformation of both ERα and ERβ differently from that induced by E_2. Also other partial agonists/antagonists (4-hydroxytamoxifen, idoxifen, and the raloxifene derivative LY 117,018-HCl) had a similar effect as tamoxifen. Like other investigators we could not discriminate between different SERMs based on the protease digestion profiles (McDonnell 1995). Other techniques, like phage enzyme-linked immunoassays have shown to be more suitable for this purpose (Paige 1999). Furtermore, it is difficult to translate the intensity of the preserved fragments obtained with the partial protease digestion analysis into a functional receptor affinity as was done for VDR analogs (Nayeri 1996). Therefore, protease resistance (as a tool to study ligand-induced conformational changes of a receptor) can only be used to calculate a functional receptor affinity as was done by Nayeri *et al.* when the investigated ligands exert agonistic activities. However, for nuclear hormone receptors like the progesterone receptor and ER (partial) agonistic/antagonistic ligands are known. Interaction with these ligands lead to a conformational change of the receptor that is clearly distinguishable by partial digestion analysis from the conformational change induced by ligands with pure agonistic action (Allan 1992, McDonnell 1995).

ICI 164,384/ICI 182,780: THE MECHANISM OF ACTION RESOLVED?

ICI 164,384 and ICI 182,780 are generally considered as pure antagonists of E_2 (Wakeling 1992) and, therefore, no real SERMs. However, data are accumulating that show some agonistic effects of ICI compounds. In Table 7.1 the agonistic effects of the ICI compounds both *in vivo* and *in vitro* are summarized.

Table 7.1. *In vivo* and *in vitro* agonistic effects of ICI 164,384 and ICI 182,780

GENE / RESPONSE	CELL / TISSUE / SPECIES	(REF)
AP-1 reporter gene	different cell types	(Paech 1997)
Collagenase reporter gene	different cell types	(Webb 1995)
Creatine kinase B reporter gene	human endometrial cancer cells	(Castro-Rivera 1998)
Progesterone receptor level	human endometrial cancer cells	(Jamil 1991)
Progesterone receptor level	guinea pig vagina, uterus cells	(Chetrite 1991)

Quinone reductase	human breast cancer cells	(Montano 1997)
Retinoic acid receptor α-1	human breast cancer cells, liver cells	(Elgort 1996, Zou 1999)
TGFβ3 reporter gene	human osteosarcoma cells	(Yang 1996)
ERE reporter gene	human kidney epithelial cells	(Barkhem 1998)
ERE reporter gene	chinese hamster ovary cells	(Montano 1995)
ERE reporter gene	yeast	(Lyttle 1992, McDonnell 1992, Kohno 1994)
Oxytocin / prostaglandin F2α	blood; ewes	(Al-Matubsi 1998)
Cancellous bone turnover	bone; ovariectomized rats	(Sibonga 1998)
Bone maturation	bone; immature mice	(Gunther 1999)

An explanation for the agonistic properties of the ICI compounds is not known. Webb *et al.* suggested that the low number of reports showing agonistic effects of ICI compounds is due to the fact that most studies focus on classic ERE-controlled genes while agonistic properties of ICI can be observed with AP-1 responsive genes (Webb 1995). However, others reported also with ERE-controlled genes agonistic activities of the ICI compounds (Lyttle 1992, McDonnell 1992, Kohno 1994, Montano 1995, Barkhem 1998). Montano *et al.* only observed agonistic activity of ICI 164,384 at concentrations lower than 10^{-8} M, whereas higher concentrations were not active probably because of the observed ICI-induced ER degradation (Montano 1995). In line with this, Webb *et al.* found that induction of the collagenase promoter by ICI required supraphysiological ER levels, and concluded that because of this requirement *in vivo* agonistic activity of ICI compounds will be rare (Webb 1995).

In the partial protease digestion analysis presented in Chapter 6 we found a receptor subtype-dependent effect of the ICI compounds: the ERα conformation was changed in such a way that increased protease sensitivity was observed, whereas with ERβ increased protease resistance was induced. It is tempting to speculate that the agonistic effect of the ICI compounds might be exerted via ERβ and not ERα. However, Barkhem *et al.* showed an agonistic effect of ICI 164,384 through ERα and not ERβ (Barkhem 1998). An explanation for this discrepancy is not known, but might be due to differences in cellular context (e.g. cofactors) in which these and our studies were performed.

Our observation of ICI-induced enhanced protease sensitivity of ERα fits nicely with the findings of a decreased ER half-life in cells and tissues after treatment with the ICI compounds (Gibson 1991, Reese 1991, Dauvois 1992). As an explanation for the decreased ER half-life ICI-induced impaired ER dimerization was suggested (Dauvois 1993), although others could not confirm this (Wang 1995, Metzger 1995). Assuming that ER binding to EREs only takes place as dimers, we also find no indication for impaired ER dimerization by the ICI compounds, since we did not find that the ICI compounds decreased ERE binding of *in vitro* synthesized ERα and ERβ (data not shown). It was suggested by Dauvois *et al.* that the reduced ER half-life induced by the ICI compounds also might result from disruption of

nucleocytoplasmic shuttling [Dauvois 1993]. Unpublished data of Alarid *et al.* indicate that in pituitary lactotrope cells the rapid degradation of ER by the ICI compounds occurs through a proteasome-mediated mechanism (referred to in [Alarid 1999]). Although this might be a relevant factor in intact cells, our data obtained in a cell free system indicate that reduction in half-life might be a direct consequence of increased protease sensitivity due to the ICI-induced conformational change of the ER.

Based on these findings we conclude that the ICI compounds can be considered as SERMs, although their agonistic activity is only limited. In certain conditions, i.e. in certain cells, via certain DNA interaction sites, and possibly via ERβ the ICI compounds might exert agonistic effects. However, in most tissues and most responses, the ICI compounds lack any estrogenic activity and counteract E_2 and, therefore, these compounds remain useful in the treatment of patients with advanced breast cancer resistant to tamoxifen [Howell 1996].

7.4. SUGGESTIONS FOR FURTHER RESEARCH

In contrast to the ER and other steroid hormone receptors, for the VDR no ligands devoid of biological activity were discovered for a long time [Bouillon 1995]. However, recent data showed that 1,25-$(OH)_2D_3$-26,23-lactone analogs exert antagonistic activities [Miura 1997, Arai 1997, Ishizuka 1997]. These analogs bind the VDR with moderate affinity (10 times less than 1,25-$(OH)_2D_3$) and counteract in a dose-dependent manner induction of cell differentiation by 1,25-$(OH)_2D_3$. In the absence of 1,25-$(OH)_2D_3$ these analogs did not affect cell differentiation. With respect to the search for VDR ligands with an enlarged dissociation between calcemic activity and cell proliferation-/differentiation-inducing activity, the finding that antagonistic VDR ligands exist or can be developed, is promising. The underlying mechanism by which these compounds act antagonistic is presently unknown. Therefore, it would be very interesting to study the effect of these 1,25-$(OH)_2D_3$ analogs with antagonistic activities on VDR conformation, half-life and DNA binding properties as performed in this thesis. Similar studies performed with the ER (as presented in Chapter 6) and other steroid hormone receptors demonstrated that ligands with (partial) antagonistic activity also change the receptor conformation and that this also results in increased protease resistance of receptor fragments, albeit of sizes distinct from those protected by agonists. However, our studies with the pure antagonists ICI 164,384 and ICI 182,780 show that increased protease sensitivity of the receptor is also possible (Chapter 6).

The importance of the cofactor SUG1 in 1,25-$(OH)_2D_3$-mediated VDR degradation has already been mentioned [Masuyama 1998]. Based on the observed stabilizing effect on the VDR by KH1060 it would be interesting to investigate

the role of this and other cofactors in the action of 1,25-$(OH)_2D_3$ analogs on VDR stability.

A specific role of the ER subtypes (if there is any) should be resolved. The observed ER subtype-specific tissue distribution [Kuiper 1997, Kuiper 1999], together with our findings with the partial protease digestion studies (Chapter 6) and studies of others [Tremblay 1997, Paech 1997, Watanabe 1997, McInerney 1998, Kobori 1998, Barkhem 1998, Zou 1999, Sun 1999, Gaido 1999] indicate that specific functions are not unlikely. Evaluation of data obtained by knockout models (both naturally occurring and generated) might provide more insight. In addition, investigations should focus on the search for ER subtype-specific target genes and regulatory elements (ERβ-specific EREs?). Finally, ligands with a preferential affinity for either one of the receptors are known [Kuiper 1997, Kuiper 1998] and points out that ER subtype-selective ligands can be developed.

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SAMENVATTING

1,25-Dihydroxyvitamin D_3 (1,25-$(OH)_2D_3$) en 17ß-oestradiol (E_2) zijn door het lichaam uit cholesterol gevormde steroidhormonen die, na binding aan een specifiek receptoreiwit, respectievelijk de vitamine D receptor (VDR) en de oestrogeen receptor (ER), een breed scala van processen aansturen en controleren. De bekendste functie van 1,25-$(OH)_2D_3$ is de regulatie van de calcium- en fosfaat-huishouding en het botmetabolisme. Van E_2 zijn vooral de effecten op de vrouwelijke geslachtsorganen bekend. Echter, beide hormonen spelen ook een belangrijke rol bij de groei, differentiatie en functie van diverse andere cellen, weefsels en organen. 1,25-$(OH)_2D_3$ heeft bijvoorbeeld een groeiremmend en differentiatie-inducerend effect op cellen; een eigenschap die mogelijkheden suggereert voor de klinische toepassing van 1,25-$(OH)_2D_3$ bij de behandeling van (hyper)proliferatieve aandoeningen (bijvoorbeeld kanker en psoriasis). E_2 heeft een gunstig effect op het functioneren van het hart en vaatstelsel, het centraal zenuwstelsel en het skelet. Het belang van E_2 voor het skelet wordt het duidelijkst geïllustreerd met het dalen van de botmassa en het toenemen van het risico op botbreuken bij vrouwen na de menopauze (postmenopauzale osteoporose). Echter, de toepassing van zowel 1,25-$(OH)_2D_3$ als E_2 wordt belemmerd door het optreden van bijwerkingen. Door de hoge dosis 1,25-$(OH)_2D_3$, noodzakelijk om de effecten op celgroei en differentiatie te induceren, ontstaat het risico op te hoge calciumspiegels in het bloed (hypercalcemie). Bij de behandeling van bijvoorbeeld postmenopauzale osteoporose patiënten met E_2 is er verhoogd risico op het optreden van borst- en baarmoederkanker. Er zijn dan ook meerdere onderzoeken die zich richten op de ontwikkeling van synthetische VDR en ER liganden die wel de gunstige, maar niet de ongunstige eigenschappen van de natuurlijke hormonen bezitten. Inmiddels zijn er vele synthetische VDR liganden (1,25-$(OH)_2D_3$ analoga) en synthetische ER liganden (Selective Estrogen Receptor Modulators; SERMs) ontwikkeld, waarvan verschillende een veelbelovend werkingsprofiel hebben en al daadwerkelijk worden toegepast als therapeuticum. Echter, een mechanistische verklaring voor de veranderde eigenschappen van deze synthetische hormonen is over het algemeen niet bekend. Onderzoek naar de werkingsmechanismen geeft inzicht in de mogelijke oorzaken van steroid hormoon-gereguleerde fysiologische en pathologische processen en zal uiteindelijk kunnen leiden tot de ontwikkeling van synthetische receptor liganden met sterk verbeterde klinische toepassingsmogelijkheden. In dit proefschrift worden een aantal onderzoeken beschreven die de mogelijke werkingsmechanismen van 1,25-$(OH)_2D_3$ analoga en SERMs nader bestudeert. De Hoofdstukken 2 tot en met 5 handelen over 1,25-$(OH)_2D_3$ analoga, Hoofdstuk 6 beschrijft een onderzoek met SERMs.

In het onderzoek beschreven in Hoofdstuk 2 werden een aantal 1,25-$(OH)_2D_3$ analoga onderzocht op hun effecten op de groei en differentiatie van botcellijnen: de humane osteoblast-achtige cellijn MG-63 en de ratten osteoblast-achtige cellijn ROS 17/2.8. De richting van de effecten van al de onderzochte analoga was gelijk aan die van 1,25-$(OH)_2D_3$: In beide cellijnen werd de productie van het botmatrixeiwit osteocalcine gestimuleerd, evenals de synthese van procollageen type I door de MG-63 cellen. De groei van de MG-63 cellen werd door 1,25-$(OH)_2D_3$ en de analoga geremd en van de ROS 17/2.8 cellen gestimuleerd. Verder stimuleerden zowel 1,25-$(OH)_2D_3$ als de analoga de afgifte van calcium (een maat voor de botresorptie activiteit) uit vrijgeprepareerde pijpbeentjes van foetale muizen. Echter, er was een aanzienlijk verschil in de sterkte van de effecten: enkele analoga waren al bij veel lagere concentraties dan 1,25-$(OH)_2D_3$ actief. Het mogelijk werkingsmechanisme van één van de meest actieve analoga uit dit onderzoek, KH1060, werd nader bestudeerd. Een mogelijke verhoogde affiniteit voor de VDR bleek geen rol te spelen: KH1060 bindt de VDR met een lagere affiniteit dan 1,25-$(OH)_2D_3$.

Receptor binding van een steroidhormoon leidt tot verandering in de vorm van de receptor. Door deze zogenaamde receptor conformatieverandering, wordt een reeks van processen in gang gezet, die tot veranderde expressie van specifieke genen en uiteindelijk tot veranderde celfunctie leidt. In Hoofdstuk 3 werd het effect van 1,25-$(OH)_2D_3$ en KH1060 op de conformatieverandering van de VDR bestudeerd. De partiële protease assay die hierbij werd toegepast is gebaseerd op de veronderstelling dat de bereikbaarheid van bepaalde knipplaatsen voor een protease (= een eiwit afbrekend enzym, bijvoorbeeld trypsine) in de VDR zal veranderen ten gevolge van zo'n conformatieverandering. Omdat wordt uitgegaan van radioactief-gelabeld VDR zullen de fragmenten die ontstaan door het knippen met een protease ook radioactief-gelabeld zijn. De receptor fragmenten worden met behulp van een gel op grootte gescheiden en door middel van röntgenfilm zichtbaar gemaakt. Deze experimenten toonden aan dat VDR onder invloed van KH1060 een conformatieverandering had ondergaan die, in vergelijking met 1,25-$(OH)_2D_3$ gebonden VDR tot verhoogde bescherming tegen de activiteit van de proteases leidde. Vervolgens werd onderzocht of de verhoogde bescherming tegen proteolytische afbraak ook in intacte cellen kon worden waargenomen. In de ROS 17/2.8 cellen werd de nieuwe vorming van eiwitten (waaronder VDR) geblokkeerd, waarna de cellen voor 1 uur werden behandeld met 1,25-$(OH)_2D_3$ of KH1060. Vervolgens werd met behulp van een immunologische bepaling op diverse tijdstippen het VDR gehalte in de cellen gemeten. Het bleek dat het VDR gehalte in de cellen behandeld met KH1060 veel langer hoog bleef dan het VDR gehalte in de cellen behandeld met 1,25-$(OH)_2D_3$. Met andere woorden: de stabiliteit van de VDR neemt toe wanneer het KH1060 heeft gebonden.

Een volgende stap in het proces dat leidt tot stimulatie of remming van transcriptie van bepaalde genen is de binding van de VDR aan vitamine D respons elementen (VDREs); een serie specifieke nucleotiden (DNA bouwstenen) in de aanstuureenheid (de promoter) van het gen. Binding van de VDR aan een VDRE wordt gestimuleerd door 1,25-$(OH)_2D_3$ en vindt in de meeste gevallen plaats als een complex met een andere steroid hormoon receptor: de retinoic X receptor (RXR). Of KH1060 ook in staat is om VDR/RXR binding aan VDREs te stimuleren werd onderzocht in Hoofdstuk 4. De binding van VDR/RXR (zowel verkregen uit *in vitro* synthese als uit celextracten) aan de VDREs van een drietal natuurlijk voorkomende genen: ratten osteocalcine, humaan osteocalcine en muizen osteopontine, werd bestudeerd. Er werd hierbij gebruik gemaakt van een bepaling waarbij radioactief gelabelde VDREs worden behandeld met VDR/RXR en ligand (1,25-$(OH)_2D_3$ of KH1060). Vervolgens wordt het mengsel op een gel gescheiden, waarbij het aan de VDRE gebonden VDR/RXR een tragere migratie van de VDRE in de gel veroorzaakt. Ondanks de grote mate van overeenkomst in de nucleotide volgorde van deze VDREs bleek er toch een aanzienlijk verschil te bestaan in de bindingsaffiniteit van VDR/RXR. Zowel 1,25-$(OH)_2D_3$ als KH1060 stimuleerden de VDR/RXR binding aan alledrie de VDREs, echter de affiniteit voor de muizen osteopontine VDRE was hoger en die voor de ratten osteocalcine VDRE lager dan die voor de humane osteocalcine VDRE. Door gebruik te maken van synthetische VDREs met variaties in de nucleotide volgorde kon het belang van twee nucleotides voor het waargenomen verschil in VDR/RXR binding grotendeels worden verklaard. Bovendien toonden deze onderzoeken aan dat het stimulerend effect van 1,25-$(OH)_2D_3$ en KH1060 op de binding van *in vitro* gesynthetiseerd VDR/RXR aan de VDREs van het humaan osteocalcine gen en het muizen osteopontine gen vergelijkbaar is. Interessant was de waarneming dat, wanneer de binding van *in vitro* gesynthetiseerd VDR/RXR aan de ratten osteocalcine VDRE werd bestudeerd, KH1060 minder effectief was dan 1,25-$(OH)_2D_3$. Echter, met VDR/RXR uit celextracten was KH1060 niet alleen via de humane osteocalcine VDRE en de muizen osteopontine VDRE, maar ook via de ratten osteocalcine VDRE een sterkere stimulator dan 1,25-$(OH)_2D_3$. Dit duidt op het belang van een cellulaire context (de aan- of afwezigheid van bepaalde cofactoren) voor de VDR/RXR-VDRE binding.

Voor 1,25-$(OH)_2D_3$ is een complex afbraakmechanisme bekend dat zorg draagt voor het controleren van de concentratie en daarmee van de effecten van dit hormoon. Ook 1,25-$(OH)_2D_3$ analoga worden enzymatisch afgebroken. Sommige van die afbraakproducten (metabolieten) zijn mogelijk zelf ook biologisch actief. In Hoofdstuk 5 werd onderzocht of bepaalde metabolieten bijdragen aan de sterke biologische activiteit van KH1060. Hiertoe werden door huidcellen gevormde metabolieten van KH1060 onderzocht op hun vermogen

om de conformatie van de VDR te veranderen, zoals dat reeds in Hoofstuk 3 is beschreven voor 1,25-$(OH)_2D_3$ en KH1060. Het bleek dat bepaalde metabolieten het vermogen hadden om de VDR dusdanig van conformatie te doen veranderen dat de resistentie voor proteases toenam. Dezelfde metabolieten bleken ook in staat om de stabiliteit van de VDR in ROS 17/2.8 cellen te verhogen. De biologische activiteit van deze metabolieten kwam ook tot uiting in het stimulerend effect op de binding van VDR/RXR complexen aan VDREs en uit de toegenomen productie van osteocalcine door ROS 17/2.8 cellen. Al deze waarnemingen hebben geleid tot de conclusie dat ook metabolieten van KH1060 bijdragen tot de uiteindelijk hoge biologische activiteit van dit analoog.

In Hoofdstuk 6 werden de effecten van een aantal SERMs op de conformatieverandering van de ER onderzocht. De ER komt voor als twee subtypen: ERα en ERβ. De receptoren vertonen grote overeenkomsten in structuur en functie, maar het is niet onwaarschijnlijk, of zelfs te verwachten dat de subtypen naast een gemeenschappelijke functie, ook een eigen, specifieke functie bezitten. De SERMs die in het onderzoek werden gebruikt zijn: tamoxifen, dat in bepaalde weefsels en op bepaalde genen het effect van E_2 nabootst (de agonistische werking) en in andere weefsels of andere genen de effecten van E_2 tegenwerkt (de antagonistische werking), en ICI 164,384 en ICI 182,780, die over het algemeen worden beschouwd als pure antagonisten van E_2, hoewel een aantal agonistische effecten zijn beschreven. In de protease assay, zoals die ook voor de VDR werd gebruikt, bleek dat bij behandeling van beide ER subtypen met tamoxifen een protease patroon werd verkregen dat duidelijk afweek van dat verkregen met E_2. De partiële agonist/antagonist tamoxifen veroorzaakte bij zowel ERα als ERβ een andere conformatieverandering dan de pure agonist E_2. Echter, opvallender was de waarneming met de ICI componenten. Met ERα veroorzaakte zowel ICI 164,384 als ICI 182,780 een dusdanige conformatieverandering dat de receptor een verhoogde gevoeligheid voor de proteases verwierf, terwijl met ERβ een toegenomen resistentie tegen proteolytische afbraak werd waargenomen. Hoewel er geen direct biologische activiteit aan dit ER-subtype-specifieke verschijnsel kan worden verbonden, is een mogelijke betrokkenheid van ERβ in de (zeldzame) agonistische effecten van de ICI componenten niet uitgesloten.

De onderzoeken met 1,25-$(OH)_2D_3$ analoga en SERMs beschreven in dit proefschrift tonen aan dat veranderingen in chemische structuur van een ligand kunnen leiden tot veranderingen in de reeks van processen die de uiteindelijke mate van gentranscriptie bepalen. Onderzoeken met de 1,25-$(OH)_2D_3$ superagonist KH1060 of de "pure" E_2 antagonisten, ICI 164,384 en ICI 182,780, tonen aan dat deze en andere synthetische liganden niet alleen een mogelijke klinische relevantie hebben, maar ook van grote waarde zijn voor het ophelderen van de werkingsmechanismen van natuurlijke en synthetische receptor liganden.

CURRICULUM VITAE

Gert-Jan van den Bemd werd op 28 januari 1964 te Breda geboren. Hij behaalde het HAVO diploma in 1981 (Maris Stella te Dongen). In 1986 voltooide hij de Hogere Laboratorium Opleiding, klinisch chemische differentiatie aan het Dr. Struycken Instituut te Etten-Leur. Het bijbehorende stage-onderzoek naar de glycosylering van transferrine werd onder leiding van dr. J.M. Pekelharing uitgevoerd op de afdelingen Klinische Chemie (dr. W.G. Haije) en Biochemie (dr. J.A. Foekens) van de Dr. Daniël den Hoed Kliniek te Rotterdam. Na een diensttijd van 16 maanden als Wachtmeester-Instructeur der Cavalerie (Bernhardkazerne te Amersfoort), werd hij als research analist aangesteld op een onderzoek naar de expressie van cytokines tijdens ontstekingsreacties bij peritoneaal dialyse patiënten (Afdeling Inwendige Geneeskunde I (prof. dr. M.A.D.H. Schalekamp) / Insituut Farmacologie (prof. dr. I.L. Bonta) van de Erasmus Universiteit Rotterdam, onder leiding van dr. M.W.J.A. Fieren). In 1989 behaalde hij het diploma Algemene Milieukunde aan de Open Universiteit Heerlen. In 1990 volgde een aanstelling als research analist op het Calcium- en Botstofwisselings Onderzoeks-laboratorium van de Afdeling Inwendige Geneeskunde III van de Erasmus Universiteit / Academisch Ziekenhuis Rotterdam (prof. dr. J.C. Birkenhäger en later prof. dr. S.W.J. Lamberts en prof. dr. H.A.P. Pols). In 1992 werd bij de stichting Posthoger Onderwijs Laboratoriummedewerkers Amsterdam het certificaat Endocrinologie behaald. In datzelfde jaar werd onder leiding van prof. dr. H.A.P. Pols en dr. J.P.T.M. van Leeuwen aangevangen met het onderzoek naar de werkingsmechanismen van synthetische liganden van de vitamine D en oestrogeen receptor, waarvan de resultaten in dit proefschrift zijn beschreven. In 1995 werd het COGEM certificaat en het diploma Veilig werken met recombinant DNA technieken (VMT) behaald (Academisch Ziekenhuis Utrecht). Momenteel werkt hij aan een onderzoek naar de expressie van bekende en onbekende genen in humane osteoblasten onder invloed van (synthetische) liganden van de vitamine D en oestrogeen receptor gedurende verschillende stadia van groei en differentiatie.

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DANKWOORD / ACKNOWLEDGMENTS

Allereerst wil ik mijn promotor professor Pols bedanken. Beste Huib, bedankt voor het geboden vertrouwen en de vrijheid die je me gaf om aan een 'eigen' stuk onderzoek te gaan werken. Het was een lange weg en het kostte heel wat 'effort', maar mede dankzij jouw steun is het toch volbracht. Mijn copromotor dr. Hans van Leeuwen wil ik bedanken voor zijn grote inzet bij de begeleiding van het onderzoek en de hulp bij het voltooien van de manuscripten. Ook wil ik hier stilstaan bij de interesse die wijlen professor Birkenhäger in mijn werk heeft gehad. Zijn betrokkenheid was voor mij een belangrijke stimulans.

Van de mensen van het vitamine D lab wil ik in het bijzonder Cok Buurman bedanken. Cok, vanaf het begin bood je een helpende hand bij de experimenten en een luisterend oor wanneer het weer eens tegenzat. Ada Staal (Department of Cell Biology, University of Massachusetts Medical Center, Worchester, MA, USA) wil ik bedanken voor de steun, eerst van nabij, later op afstand. Verder wil ik ook de andere mensen van 'het eerste uur': Edgar Colin, Wendy Geertsma-Kleinekoort, Trudy Vink-van Wijngaarden en Angelique Weel bedanken voor de gezelligheid en prettige samenwerking. André Uitterlinden, 'beste vriend André', bedank ik voor de leerzame discussies en de gezelligheid tijdens congresreizen. Mila Jhamai bedank ik voor haar grote inzet bij de kloneringsexperimenten, die de acceptatie van Hoofdstuk 4 van dit proefschrift hopelijk zullen bespoedigen. Marien Fieren (Inwendige Geneeskunde I) wil ik bedanken voor de geboden mogelijkheid om vanaf het allereerste moment zelfstandig onderzoek uit te voeren. Deze eerste kennismaking met de wetenschap was voor mij de basis. George Kuiper dank ik voor zijn opbouwende kritiek op de Inleiding over de oestrogeen receptor en Hoofdstuk 6. Buurman Joop Janssen bedank ik voor de leerzame discussies en zijn kritische blik op de Inleiding. Roel Docter wil ik bedanken voor het aanpassen van de computerprogramma's waardoor het analyseren van de densitometrische data een stuk eenvoudiger werd. Martijn, bedankt voor je geweldige hulp met de computer! Ronald van der Wal, bedankt voor de hulp bij het sequencen van de VDRE-constructen. Pascal Arp, Arjan Bergink, Marjolein van Driel, Yu Fang, Marjolein van der Klift, Joyce van Meurs, Saskia Postma, Franci Weyts, Carola Zillekens en alle andere niet met naam genoemde Labvrienden van Interne (III) wil ik bedanken voor de goede werksfeer en collegialiteit.

I am very grateful to dr. L. Binderup (Leo Pharmaceuticals, Ballerup, Denmark) for the vitamin D analogs, dr. N. Kubodera (Chugai Pharmaceutical Co., Ltd, Tokyo, Japan) for OCT, dr. A.E. Wakeling (Zeneca Pharmaceuticals, Macclesfield, Cheshire, England) for the gift of ICI 164,384 and ICI 182,780, and to dr. P. Chambon (INSERM, Strasbourg, France) for the supply of the human ERα expression vector. I would like to thank prof. R. Bouillon

(Katholieke Universiteit Leuven, Leuven, Belgium) for the antiserum used in the osteocalcin radioimmunoassay and for the MG-63 cells, dr. S.B. Rodan (Merck, Sharp & Dohme Research Laboratories, West Point, USA) for the ROS 17/2.8 and ROS 25.1 cells, and prof. H.F. DeLuca and Ms. J.M. Prahl (Department of Biochemistry, University of Wisconsin-Madison, Wisconsin, USA) for the VDR antibodies and the protocol for the VDR immunoradiometric assay. Ms. V. Byford (Department of Biochemistry, Queen's University, Kingston, Ontario, Canada) is acknowledged for the generation of KH1060 metabolites.

Vanzelfsprekend wil ik ook het 'thuisfront' hier noemen. Vrienden van leutorkest De Korte Keu, Magnus-vrienden, en overige vrienden en kennissen wil ik bedanken voor hun interesse en hun begrip voor het feit dat ik ze de laatste maanden wat heb verwaarloosd. Mijn ouders, schoonouders en familieleden bedank ik voor hun betrokkenheid en steun. Frank en Willem, bedankt dat jullie als paranimf willen optreden. Tenslotte, lieve Martijn en Lucas, liefste Minette, zonder jullie had ik dit dankwoord nooit hoeven of kunnen schrijven.